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(54) Titre : PEPTIDES LIANT DES RECEPTEURS KDR ET VEGF/KDR ET LEURS UTILISATIONS EN DIAGNOSTIC ET THERAPIE

(54) Title: KDR AND VEGF/KDR BINDING PEPTIDES AND THEIR USE IN DIAGNOSIS AND THERAPY

(57) Abrégé/Abstract:
The present invention provides binding polypetides for KDR or VEGF/KDR complex, which have a variety of uses wherever treating, Detecting, isolating or localizing angiogenesis is advantageous. Particularly disclosed are synthetic, isolated polypeptides capable of binding KDR or VEGF/KDR complex with high affinity (e.g., having a K_D<1 μM).

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(54) Title: KDR AND VEGF/KDR BINDING PEPTIDES AND THEIR USE IN DIAGNOSIS AND THERAPY

(57) Abstract: The present invention provides binding polypetides for KDR or VEGF/KDR complex, which have a variety of uses wherever treating, Detecting, isolating or localizing angiogenesis is advantageous. Particularly disclosed are synthetic, isolated polypeptides capable of binding KDR or VEGF/KDR complex with high affinity (e.g., having a K_D<1 μM).

KDR AND VEGF/KDR BINDING PEPTIDES AND THEIR USE IN DIAGNOSIS AND THERAPY

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/360,851, filed March 1, 2002, and U.S. Provisional Application No. 60/440,411, filed January 15, 2003.

BACKGROUND OF THE INVENTION

In the developing embryo, the primary vascular network is established by *in situ* differentiation of meso-dermal cells in a process called vasculogenesis. After embryonic vasculogenesis however, it is believed that all subsequent generation of new blood vessels, in the embryo or in adults, is governed by the sprouting or splitting of new capillaries from the pre-existing vasculature in a process called angiogenesis (Pepper, M. *et al.*, 1996. *Enzyme Protein*, 49:138-162; Risau, W., 1997. *Nature*, 386:671-674). Angiogenesis is not only involved in embryonic development and normal tissue growth and repair, it is also involved in the female reproductive cycle, establishment and maintenance of pregnancy, and in repair of wounds and fractures. In addition to angiogenesis that takes place in the normal individual, angiogenic events are involved in a number of pathological processes, notably tumor growth and metastasis, and other conditions in which blood vessel proliferation is increased, such as diabetic retinopathy, psoriasis and arthropathies. Angiogenesis is so important in the transition of a tumor from hyperplastic to neoplastic growth, that inhibition of angiogenesis has become an active cancer therapy (Kim, K. *et al.*, 1993. *Nature*, 362:841-844).

Tumor-induced angiogenesis is thought to depend on the production of pro-angiogenic growth factors by the tumor cells, which overcome other forces that tend to keep existing vessels quiescent and stable (Hanahan, D. and Folkman, J., 1996. *Cell*, 86:353-364). The best characterized of these pro-angiogenic agents is vascular endothelial growth factor (VEGF) (Neufeld, G. *et al.*, 1999. *FASEB J.*, 13:9-22).

VEGF is produced naturally by a variety of cell types in response to hypoxia and some other stimuli. Many tumors also produce large amounts of VEGF, and/or induce nearby stromal cells to make VEGF (Fukumura, D. *et al.*, 1998. *Cell*, 94:715-725). VEGF, also referred to as VEGF-A, is synthesized as five different splice isoforms of 121, 145, 165, 189, and 206 amino acids. $VEGF_{121}$ and $VEGF_{165}$ are the

main forms produced, particularly in tumors (see, Neufeld, G. *et al.* 1999, *supra*). $VEGF_{121}$ lacks a basic domain encoded by exons 6 and 7 of the VEGF gene and does not bind to heparin or extracellular matrix, unlike $VEGF_{165}$.

VEGF family members act primarily by binding to receptor tyrosine kinases. In general, receptor tyrosine kinases are glycoproteins having an extracellular domain capable of binding one or more specific growth factors, a transmembrane domain (usually an alpha helix), a juxtamembrane domain (where the receptor may be regulated, *e.g.*, by phosphorylation), a tyrosine kinase domain (the catalytic component of the receptor), and a carboxy-terminal tail, which in many receptors is involved in recognition and binding of the substrates for the tyrosine kinase. There are three endothelial cell-specific receptor tyrosine kinases known to bind VEGF: VEGFR-1 (Flt-1), VEGFR-2 (KDR or Flk-1), and VEGFR-3 (Flt4). Flt-1 and KDR have been identified as the primary high affinity VEGF receptors. While Flt-1 has higher affinity for VEGF, KDR displays more abundant endothelial cell expression (Bikfalvi, A. *et al.*, 1991. *J. Cell. Physiol.*, 149:50-59). Moreover, KDR is thought to dominate the angiogenic response and is therefore of greater therapeutic and diagnostic interest (see, Neufeld, G. *et al.* 1999, *supra*). Expression of KDR is highly upregulated in angiogenic vessels, especially in tumors that induce a strong angiogenic response (Veikkola, T. *et al.*, 2000. *Cancer Res.*, 60:203-212).

KDR is made up of 1336 amino acids in its mature form. Because of glycosylation, it migrates on an SDS-PAGE gel with an apparent molecular weight of about 205 kDa. KDR contains seven immunoglobulin-like domains in its extracellular domain, of which the first three are the most important in VEGF binding (Neufeld, G. *et al.* 1999, *supra*). VEGF itself is a homodimer capable of binding to two KDR molecules simultaneously. The result is that two KDR molecules become dimerized upon binding and autophosphorylate, becoming much more active. The increased kinase activity in turn initiates a signaling pathway that mediates the KDR-specific biological effects of VEGF.

From the foregoing, it can be seen that not only is the VEGF binding activity of KDR *in vivo* critical to angiogenesis, but the ability to detect KDR upregulation on endothelial cells or to detect VEGF/KDR binding complexes would be extremely beneficial in detecting or monitoring angiogenesis, with particular diagnostic applications such as detecting malignant tumor growth. It would also be beneficial in therapeutic applications such as targeting tumorcidal agents or angiogenesis

inhibitors to a tumor site or targeting KDR, VEGF/KDR, or angiogenesis agonists to a desired site.

SUMMARY OF THE INVENTION

The present invention relates to polypeptides and compositions useful for detecting and targeting primary receptors on endothelial cells for vascular endothelial growth factor (VEGF), *i.e.*, vascular endothelial growth factor receptor-2 (VEGFR-2, also known as kinase domain region (KDR) and fetal liver kinase-1 (Flk-1)), and for imaging and targeting complexes formed by VEGF and KDR. The involvement of VEGF and KDR in angiogenesis makes the VEGF/KDR and KDR binding polypeptides of the present invention particularly useful for imaging important sites of angiogenesis, *e.g.*, neoplastic tumors, for targeting substances, *e.g.*, therapeutics, including radiotherapeutics, to such sites, and for treating certain disease states, including those associated with inappropriate angiogenesis.

A group of polypeptides has been discovered that bind to KDR or VEGF/KDR complex (referred to herein as "KDR binding polypeptides" or "KDR binding moieties" and homologues thereof). Such KDR and VEGF/KDR binding polypeptides will concentrate at the sites of angiogenesis, thus providing a means for detecting and imaging sites of active angiogenesis, which may include sites of neoplastic tumor growth. Such KDR and VEGF/KDR binding polypeptides provide novel therapeutics to inhibit or promote, *e.g.*, angiogenesis. The preparation, use and screening of such polypeptides, for example as imaging agents or as fusion partners for KDR or VEGF/KDR-homing therapeutics, is described in detail herein.

In answer to the need for improved materials and methods for detecting, localizing, measuring and possibly inhibiting affecting, *e.g.*, angiogenesis, we have now surprisingly discovered seven families of non-naturally occurring polypeptides that bind specifically to KDR or VEGF/KDR complex. Appropriate labeling of such polypeptides provides detectable imaging agents that can bind, *e.g.*, at high concentration, to KDR-expressing endothelial cells or cells exhibiting VEGF/KDR complexes, providing angiogenesis specific imaging agents. The KDR and VEGF/KDR binding polypeptides of the instant invention can thus be used in the detection and diagnosis of such angiogenesis-related disorders. Conjugation or fusion of such polypeptides with effective agents such as VEGF inhibitors or tumorcidal agents can also be used to treat pathogenic tumors, *e.g.*, by causing the

conjugate or fusion to "home" to the site of active angiogenesis, thereby providing an effective means for treating pathogenic conditions associated with angiogenesis.

This invention pertains to KDR and VEGF/KDR binding polypeptides, and includes use of a single binding polypeptide as a monomer or in a multimeric or polymeric construct as well as use of more than one binding polypeptide of the invention in multimeric or polymeric constructs. Binding polypeptides according to this invention are useful in any application where binding, detecting or isolating KDR or VEGF/KDR complex, or fragments thereof retaining the polypeptide binding site, is advantageous. A particularly advantageous use of the binding polypeptides disclosed herein is in a method of imaging angiogenesis *in vivo*. The method entails the use of specific binding polypeptides according to the invention for detecting a site of angiogenesis, where the binding polypeptides have been detectably labeled for use as imaging agents, including magnetic resonance imaging (MRI) contrast agents, x-ray imaging agents, radiopharmaceutical imaging agents, ultrasound imaging agents, and optical imaging agents.

Another advantageous use of the KDR and VEGF/KDR complex binding polypeptides disclosed herein is to target therapeutic agents, (including compounds capable of providing a therapeutic, radiotherapeutic or cytotoxic effect.) or delivery vehicles for therapeutics (including drugs, genetic material, etc.) to sites of angiogenesis or other tissue expressing KDR.

Constructs comprising two or more KDR or KDR/VEGF binding polypeptides show improved ability to bind the target molecule compared to the corresponding monomeric binding polypeptides. For example, as shown in Experiment 5, tetrameric constructs of KDR binding polypeptides provided herein showed improved ability to bind KDR-transfected 293H cells. Combining two or more binding polypeptides in a single molecular construct appears to improve the avidity of the construct over the monomeric binding polypeptides as shown by a decrease in K_D.

In addition, as demonstrated herein, constructs comprising two or more binding polypeptides specific for different epitopes of KDR and/or KDR/VEGF (*e.g.*, "heteromeric" or "heteromultimeric" constructs, see U.S. application number 60/440,201, and the application, filed concurrently herewith, having attorney's docket number 50203/010004, the contents of each is incorporated herein) were made. Constructs comprising two or more binding polypeptides provided herein are

expected to block multiple sites on KDR or VEGF/KDR. The heteromeric constructs show superior binding ability over both the corresponding monomers, as well as multimeric constructs comprising multiple copies of the same binding polypeptide. Furthermore, heteromeric constructs comprising two or more binding peptides specific for different epitopes, together with a control peptide were also able to efficiently bind KDR-transfected 293H cells. Thus, inclusion of two or more binding polypeptides that recognize different epitopes further improves the avidity of the construct for the target molecule, as demonstrated by a decrease in K_D.

Heteromeric constructs of the binding polypeptides provided herein show improved ability to inhibit receptor tyrosine kinase function. Based on experiments described herein, dimeric and other multimeric constructs of the present invention comprising at least two binding polypeptides specific for different epitopes of KDR and/or KDR/VEGF are expected to inhibit the function of receptor tyrosine kinases. In particular, such constructs are expected to inhibit the function of VEGF-2/KDR, VEGF-1/Flt-1 and VEGF-3/Flt-4.

For the purposes of the present invention, receptor tyrosine kinase function can include any one of: oligomerization of the receptor, receptor phosphorylation, kinase activity of the receptor, recruitment of downstream signaling molecules, induction of genes, induction of cell proliferation, induction of cell migration, or combination thereof. For example, heteromeric constructs of binding polypeptides provided herein inhibit VEGF-induced KDR receptor activation in human endothelial cells, demonstrated by the inhibition of VEGF-induced phosphorylation of the KDR receptor. In addition, heteromeric constructs of binding peptides provided herein inhibit VEGF-stimulated endothelial cell migration. As shown herein, targeting two or more distinct epitopes on KDR with a single binding construct greatly improves the ability of the construct to inhibit receptor function. Even binding peptides with weak ability to block receptor activity can be used to generate heteromeric constructs having improved ability to block VEGF-induced receptor function.

Therefore, the present invention is drawn to constructs comprising two or more binding polypeptides. In one embodiment, the multimeric constructs comprise two or more copies of a single binding polypeptide. In another embodiment, the multimeric constructs of the present invention comprise two or more binding polypeptides, such that at least two of the binding polypeptides in the construct are

specific for different epitopes of KDR and/or KDR/VEGF. These constructs are also referred to herein as "heteromeric constructs," "heteromultimers," etc. The constructs of the present invention can also include unrelated, or control peptide. The constructs can include two or more, three or more, or four or more binding polypeptides. Based on the teachings provided herein, one of ordinary skill in the art is able to assemble the binding polypeptides provided herein into multimeric constructs and to select multimeric constructs having improved properties, such as improved ability to bind the target molecule, or improved ability to inhibit receptor tyrosine kinase function. Such multimeric constructs having improved properties are included in the present invention.

Consensus sequences 1-14) have been determined based on the specific KDR and VEGF/KDR binding polypeptides shown in Tables 1-7. In specific embodiments, KDR and VEGF/KDR binding polypeptides of the invention comprise one or more of these sequences. Such preferred KDR or VEGF/KDR complex binding polypeptides include polypeptides with the potential to form a cyclic or loop structure between invariant cysteine residues comprising, or alternatively consisting of, an amino acid sequence selected from Consensus Sequences 1-5 below:

Consensus Sequence 1: X_1–X_2–X_3–Cys–X_5–X_6–X_7–X_8–X_9–X_{10}–Cys–X_{12}–X_{13}–X_{14} (TN8), wherein

X_1 is Ala, Arg, Asp, Gly, His, Leu, Lys, Pro, Ser, Thr, Trp, Tyr or Val;

X_2 is Asn, Asp, Glu, Gly, Ile, Leu, Lys, Phe, Ser, Thr, Trp, Tyr or Val;

X_3 is Asn, Asp, Gln, Glu, Ile, Leu, Met, Thr, Trp or Val;

X_5 is Ala, Arg, Asn, Asp, Gln, Glu, His, Ile, Lys, Phe, Pro, Ser, Trp or Tyr;

X_6 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X_7 is Ala, Asn, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X_8 is Ala, Asp, Glu, Gly, Leu, Phe, Pro, Ser, Thr, Trp or Tyr;

X_9 is Arg, Gln, Glu, Gly, Ile, Leu, Met, Pro, Thr, Trp, Tyr or Val;

X_{10} is Ala, Arg, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Trp or Tyr;

X_{12} is Arg, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

X_{13} is Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp or Tyr; and

X_{14} is Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp or Tyr;

and wherein the polypeptide binds KDR or a VEGF/KDR complex; or

Consensus Sequence 2: X_1–X_2–X_3–Cys–X_5–X_6–X_7–X_8–X_9–X_{10}–X_{11}–X_{12}–X_{13}–X_{14}–Cys–X_{16}–X_{17}–X_{18} (TN12), wherein

X_1 is Ala, Asn, Asp, Gly, Leu, Pro, Ser, Trp or Tyr (preferably Asn, Asp, Pro or Tyr);

X_2 is Ala, Arg, Asn, Asp, Gly, His, Phe, Pro, Ser, Trp or Tyr (preferably Asp, Gly, Pro, Ser or Trp);

X_3 is Ala, Asn, Asp, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr or Val (preferably Trp);

X_5 is Arg, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Thr, Trp, Tyr or Val (preferably Glu, Ile or Tyr);

X_6 is Ala, Arg, Asn, Cys, Glu, Ile, Leu, Met, Phe, Ser, Trp or Tyr (preferably Glu, Phe or Tyr);

X_7 is Arg, Asn, Asp, Gln, Glu, His, Ile, Leu, Pro, Ser, Thr, Trp, Tyr or Val (preferably Glu);

X_8 is Ala, Asn, Asp, Gln, Glu, Gly, His, Met, Phe, Pro, Ser, Trp, Tyr or Val (preferably Gln or Ser);

X_9 is Asp, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp or Tyr (preferably Asp);

X_{10} is Ala, Arg, Asn, Asp, Gln, Glu, Gly, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val (preferably Lys or Ser);

X_{11} is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Lys, Trp, Tyr or Val (preferably Gly or Tyr);

X_{12} is Ala, Arg, Gln, Gly, His, Ile, Lys, Met, Phe, Ser, Thr, Trp, Tyr or Val (preferably Trp or Thr);

X_{13} is Arg, Gln, Glu, His, Leu, Lys, Met, Phe, Pro, Thr, Trp or Val (preferably Glu or Trp);

X_{14} is Arg, Asn, Asp, Glu, His, Ile, Leu, Met, Phe, Pro, Thr, Trp, Tyr or Val (preferably Phe);

X_{16} is Ala, Asn, Asp, Gln, Glu, Gly, Lys, Met, Phe, Ser, Thr, Trp, Tyr or Val (preferably Asp);

X_{17} is Arg, Asn, Asp, Cys, Gly, His, Phe, Pro, Ser, Trp or Tyr (preferably Pro or Tyr); and

X_{18} is Ala, Asn, Asp, Gly, His, Leu, Phe, Pro, Ser, Trp or Tyr (preferably Asn, Pro or Trp),

and wherein the polypeptide binds KDR or a VEGF/KDR complex; or

Consensus Sequence 3: X_1–X_2–X_3–Cys–X_5–X_6–X_7–Gly–X_9–Cys–X_{11}–X_{12}–X_{13} (TN7), wherein

X_1 is Gly or Trp;

X_2 is Ile, Tyr or Val;

X_3 is Gln, Glu Thr or Trp;

X_5 is Asn, Asp or Glu;

X_6 is Glu, His, Lys or Phe;

X_7 is Asp, Gln, Leu, Lys Met or Tyr;

X_9 is Arg, Gln, Leu, Lys or Val;

X_{11} is Arg, Phe, Ser, Trp or Val;

X_{12} is Glu, His or Ser; and

X_{13} is Glu, Gly, Trp or Tyr,

and wherein the polypeptide binds KDR or a VEGF/KDR complex; or

Consensus Sequence 4: X_1–X_2–X_3–Cys–X_5–X_6–X_7–X_8–X_9–X_{10}–X_{11}–Cys–X_{13}–X_{14}–X_{15} (TN9), wherein

X_1 is Arg, Asp, Gly, Ile, Met, Pro or Tyr (preferably Tyr);

X_2 is Asp, Gly, His, Pro or Trp (preferably Gly or Trp);

X_3 is Gly, Pro, Phe, Thr or Trp (preferably Pro);

X_5 is Ala, Asp, Lys, Ser, Trp or Val (preferably Lys);

X_6 is Asn, Glu, Gly, His or Leu;

X_7 is Gln, Glu, Gly, Met, Lys, Phe, Tyr or Val (preferably Met);

X_8 is Ala, Asn, Asp, Gly, Leu, Met, Pro, Ser or Thr;

X_9 is His, Pro or Trp (preferably Pro);

X_{10} is Ala, Gly, His, Leu, Trp or Tyr (preferably His or Trp);

X_{11} is Ala, Asp, Gln, Leu, Met, Thr or Trp;

X_{13} is Ala, Lys, Ser, Trp or Tyr (preferably Trp);

X_{14} is Asp, Gly, Leu, His, Met, Thr, Trp or Tyr (preferably His, Trp, or Tyr); and

X_{15} is Asn, Gln, Glu, Leu, Met, Pro or Trp (preferably Glu, Met or Trp),

and wherein the polypeptide binds KDR or a VEGF/KDR complex; or

Consensus Sequence 5: X_1–X_2–X_3–Cys–X_5–X_6–X_7–X_8–Ser–Gly–Pro–X_{12}–X_{13}–X_{14}–X_{15}–Cys–X_{17}–X_{18}–X_{19} (MTN13; SEQ ID NO:1), wherein

X_1 is Arg, Glu, His, Ser or Trp;

X_2 is Asn, Asp, Leu, Phe, Thr or Val;

X_3 is Arg, Asp, Glu, His, Lys or Thr;

X_5 is Asp, Glu, His or Thr;

X_6 is Arg, His, Lys or Phe;

X_7 is Gln, Ile, Lys, Tyr or Val;

X_8 is Gln, Ile, Leu, Met or Phe;

X_{12} is Asn, Asp, Gly, His or Tyr;

X_{13} is Gln, Gly, Ser or Thr;

X_{14} is Glu, Lys, Phe or Ser;

X_{15} is Glu, Ile, Ser or Val;

X_{17} is Glu, Gly, Lys, Phe, Ser or Val;

X_{18} is Arg, Asn, Ser or Tyr; and

X_{19} is Asp, Gln, Glu, Gly, Met or Tyr,

and wherein the polypeptide binds KDR or a VEGF/KDR complex.

Further analysis of the polypeptides isolated from the TN8 library (see Consensus Sequence 1) revealed sub-families of preferred binding polypeptides, which are described by the Consensus Sequences 6, 7 and 8 as follows:

Consensus Sequence 6: X_1–X_2–X_3–Cys–X_5–X_6–X_7–X_8–X_9–Tyr–Cys–X_{12}–X_{13}–X_{14}, wherein

X_1 is Ala, Arg, Asp, Leu, Lys, Pro, Ser or Val;

X_2 is Asn, Asp, Glu, Lys, Thr or Ser (preferably Asn, Asp, Glu or Lys);

X_3 is Ile, Leu or Trp;

X_5 is Ala, Arg, Glu, Lys or Ser (preferably Glu);

X_6 is Ala, Asp, Gln, Glu, Thr or Val (preferably Asp or Glu);

X_7 is Asp or Glu;

X_8 is Trp or Tyr;

X_9 is Thr or Tyr (preferably Tyr);

X_{12} is Glu, Met, Phe, Trp or Tyr (preferably Trp, Phe, Met, or Tyr);

X_{13} is Ile, Leu or Met; and

X_{14} is Ile, Leu, Met, Phe or Thr (preferably Thr or Leu),

and wherein the polypeptide binds KDR or a VEGF/KDR complex; or

Consensus Sequence 7: Trp–Tyr–Trp–Cys–X_5–X_6–X_7–Gly–X_9–X_{10}–Cys–X_{12}–X_{13}–X_{14} (SEQ ID NO:2), wherein

X_5 is Asp, Gln or His;

X_6 is His or Tyr (preferably Tyr);

X_7 is Ile, His or Tyr;

X_9 is Ile, Met or Val;

X_{10} is Gly or Tyr;

X_{12} is Asp, Lys or Pro;

X_{13} is Gln, Gly or Trp; and

X_{14} is Phe, Ser or Thr,

and wherein the polypeptide binds KDR or a VEGF/KDR complex; or

Consensus Sequence 8: X_1–X_2–X_3–Cys–X_5–X_6–X_7–X_8–Gly–X_{10}–Cys–X_{12}–X_{13}–X_{14}, wherein

X_1 is Gly, Leu, His, Thr, Trp or Tyr (preferably Trp, Tyr, Leu or His);

X_2 is Ile, Leu, Thr, Trp or Val (preferably Val, Ile or Leu);

X_3 is Asp, Glu, Gln, Trp or Thr, (preferably Glu, Asp or Gln);

X_5 is Ala, Arg, Asn, Asp, His, Phe, Trp or Tyr (preferably Tyr, Trp or Phe);

X_6 is Ala, Asp, Gln, His, Lys, Met, Ser, Thr, Trp, Tyr or Val;

X_7 is Ala, Asn, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr or Val;

X_8 is Asp, Phe, Ser, Thr, Trp or Tyr (preferably Thr, Ser or Asp);

X_{10} is Ala, Arg, Gln, His, Ile, Leu, Lys, Met, Phe, Trp or Tyr (preferably Arg or Lys);

X_{12} is Arg, Gln, His, Ile, Lys, Met, Phe, Thr, Trp, Tyr or Val (preferably Tyr, Trp, Phe, Ile or Val);

X_{13} is Arg, Asn, Asp, Glu, His, Met, Pro, Ser or Thr; and

X_{14} is Arg, Gln, Glu, Gly, Phe, Ser, Trp or Tyr,

and wherein the polypeptide binds KDR or a VEGF/KDR complex.

Further analysis of the polypeptides isolated from the TN12 library (see Consensus Sequence 2) revealed sub-families of preferred binding polypeptides, which are described by Consensus Sequences 9-12 as follows:

Consensus Sequence 9: X_1–X_2–X_3–Cys–X_5–X_6–X_7–X_8–Trp–Gly–Gly–X_{12}–X_{13}–Cys–X_{15}–X_{16}–X_{17} (TN11, *i.e.*, 11-mer binders isolated from the TN12 library; SEQ ID NO:3), wherein

X_1 is Ser, Phe, Trp, Tyr or Gly (preferably Ser);

X_2 is Arg, Gly, Ser or Trp (preferably Arg);

X_3 is Ala, Glu, Ile or Val (preferably Val or Ile);

X_5 is Ala, Phe or Trp (preferably Trp or Phe);

X_6 is Glu or Lys (preferably Glu);

X_7 is Asp, Ser, Trp or Tyr (preferably Asp, Trp or Tyr);

X_8 is Phe, Pro or Ser (preferably Ser);

X_{12} is Gln or Glu (preferably Glu);

X_{13} is Ile, Phe or Val;

X_{15} is Gln, Ile, Leu, Phe or Tyr (preferably Phe, Tyr or Leu);

X_{16} is Arg, Gly or Pro (preferably Arg); and

X_{17} is Gln, His, Phe, Ser, Tyr or Val (preferably Tyr, Phe, His or Val),

and wherein the polypeptide binds KDR or a VEGF/KDR complex; or

Consensus Sequence 10: Tyr–Pro–X_3–Cys–X_5–Glu–X_7–Ser–X_9–Ser–X_{11}–X_{12}–X_{13}–Phe–Cys–X_{16}–X_{17}–X_{18} (TN12; SEQ ID NO:4), wherein

X_3 is Gly or Trp (preferably Trp);

X_5 is His or Tyr (preferably His, or Tyr);

X_7 is His, Leu or Thr;

X_9 is Asp or Leu (preferably Asp);

X_{11} is Gly or Val (preferably Val);

X_{12} is Thr or Val (preferably Thr);

X_{13} is Arg or Trp (preferably Arg);

X_{16} is Ala or Val (preferably Val);

X_{17} is Asp or Pro (preferably Pro); and

X_{18} is Gly or Trp (preferably Trp),

and wherein the polypeptide binds KDR or a VEGF/KDR complex; or

Consensus Sequence 11: X_1–X_2–X_3–Cys–X_5–X_6–X_7–X_8–X_9–X_{10}–Gly–X_{12}–Trp–X_{14}–Cys–X_{16}–X_{17}–X_{18} (TN12; SEQ ID NO:5), wherein

X_1 is Asp, Gly, Pro or Ser (preferably Asp);

X_2 is Arg, Asn, Asp, Gly or Ser (preferably Asp, Asn, or Ser);

X_3 is Gly, Thr, Trp or Tyr (preferably Trp or Tyr);

X_5 is Glu, Met or Thr (preferably Glu);

X_6 is Ile, Leu, Met or Phe (preferably Met, Leu, or Phe);

X_7 is Arg, Asp, Glu, Met, Trp or Val;

X_8 is Asn, Gln, Gly, Ser or Val;

X_9 is Asp or Glu;

X_{10} is Lys, Ser, Thr or Val (preferably Lys);

X_{12} is Arg, Gln, Lys or Trp (preferably Trp, Arg, or Lys);

X_{14} is Asn, Leu, Phe or Tyr (preferably Tyr, Phe, or Asn);

X_{16} is Gly, Phe, Ser or Tyr (preferably Tyr or Phe);

X_{17} is Gly, Leu, Pro or Ser (preferably Pro or Ser); and

X_{18} is Ala, Asp, Pro, Ser, Trp or Tyr,

and wherein the polypeptide binds KDR or a VEGF/KDR complex; or

Consensus Sequence 12: Asn–Trp–X_3–Cys–X_5–X_6–X_7–X_8–X_9–X_{10}–X_{11}–X_{12}–X_{13}–X_{14}–Cys–X_{16}–X_{17}–X_{18} (TN12; SEQ ID NO:6), wherein

X_3 is Glu or Lys;

X_5 is Glu or Gly;

X_6 is Trp or Tyr;

X_7 is Ser or Thr;

X_8 is Asn or Gln;

X_9 is Gly or Met;

X_{10} is Phe or Tyr;

X_{11} is Asp or Gln;

X_{12} is Lys or Tyr;

X_{13} is Glu or Thr;

X_{14} is Glu or Phe;

X_{16} is Ala or Val;

X_{17} is Arg or Tyr; and

X_{18} is Leu or Pro,

and wherein the polypeptide binds KDR or a VEGF/KDR complex.

Analysis of the binding polypeptides isolated from a linear display library (Lin20) defined two families of preferred embodiments including the amino acid sequences of Consensus Sequences 13 and 14 as follows:

Consensus Sequence 13: Z_1–X_1–X_2–X_3–X_4–X_5–Z_2 (Lin20), wherein,

Z_1 is a polypeptide of at least one amino acid or is absent;

X_1 is Ala, Asp, Gln or Glu (preferably Gln or Glu);

X_2 is Ala, Asp, Gln, Glu Pro (preferably Asp, Glu or Gln);

X_3 is Ala, Leu, Lys, Phe, Pro, Trp or Tyr (preferably Trp, Tyr, Phe or Leu);

X_4 is Asp, Leu, Ser, Trp, Tyr or Val (preferably Tyr, Trp, Leu or Val);

X_5 is Ala, Arg, Asp, Glu, Gly, Leu, Trp or Tyr (preferably Trp, Tyr or Leu); and

Z_2 is a polypeptide of at least one amino acid or is absent,

and wherein the polypeptide binds KDR or a VEGF/KDR complex; or

Consensus Sequence 14: X_1–X_2–X_3–Tyr–Trp–Glu–X_7–X_8–X_9–Leu (Lin20; SEQ ID NO:7), wherein, the sequence can optionally have a N-terminal polypeptide, C-terminal polypeptide, or a polypeptide at both termini of at least one amino acid; and wherein

X_1 is Asp, Gly or Ser (preferably Gly);

X_2 is Ile, Phe or Tyr;

X_3 is Ala, Ser or Val;

X_7 is Gln, Glu, Ile or Val;

X_8 is Ala, Ile or Val (preferably Ile or Val);

X_9 is Ala, Glu, Val or Thr;

and wherein the polypeptide binds KDR or a VEGF/KDR complex.

Preferred embodiments comprising the Consensus Sequence 1 above, include polypeptides in which X_3 is Trp and the amino acid sequence of X_7-X_{10} is Asp-Trp-Tyr-Tyr (SEQ ID NO:8). More preferred structures include polypeptides comprising Consensus Sequence 1, wherein X_3 is Trp and the amino acid sequence of X_5-X_{10} is Glu-Glu-Asp-Trp-Tyr-Tyr (SEQ ID NO:9). Additional preferred polypeptides comprising Consensus Sequence 1 include polypeptides in which: X_3 is Trp and the amino acid sequence of X_5-X_{10} is Glu-Glu-Asp-Trp-Tyr-Tyr (SEQ ID NO:9), and the peptide X_{13}-X_{14} is Ile-Thr. Of these preferred polypeptides, it is additionally preferred that X_1 will be Pro and X_{12} will be one of Phe, Trp or Tyr.

Particular embodiments of the cyclic polypeptide families described above are disclosed in Tables 1, 2, 4, 5, and 7, *infra*.

Additional cyclic polypeptides found to bind a KDR or VEGF/KDR target have a cyclic portion (or loop), formed by a disulfide bond between the two cysteine residues, consisting of ten amino acids, for example, as follows:

Asn–Asn–Ser–Cys–Trp–Leu–Ser–Thr–Thr–Leu–Gly–Ser–Cys–Phe–Phe–Asp (SEQ ID NO:10), Asp–His–His–Cys–Tyr–Leu–His–Asn–Gly–Gln–Trp–Ile–Cys–Tyr–Pro–Phe (SEQ ID NO:11),

Asn–Ser–His–Cys–Tyr–Ile–Trp–Asp–Gly–Met–Trp–Leu–Cys–Phe–Pro–Asp (SEQ ID NO:12).

Additional preferred embodiments include linear polypeptides capable of binding a KDR or VEGF/KDR target comprising, or alternatively consisting of, a

polypeptide having an amino acid sequence selected from the group of amino acid sequences set forth in Table 3, *infra*.

The polypeptides of the invention can optionally have additional amino acids attached at either or both of the N- and C-terminal ends. In preferred embodiments, binding polypeptides according to the invention can be prepared having N-terminal and/or C-terminal flanking peptides of one or more, preferably two, amino acids corresponding to the flanking peptides of the display construct of the phage selectant from which the binding polypeptides were isolated. Preferred N-terminal flanking peptides include Ala–Gly– (most preferably for TN7, TN8, TN9 sequences), Gly–Ser– (most preferably for TN10 sequences), Gly–Asp– (most preferably for TN12 sequences), Ala–Gln– (most preferably for linear sequences), and Ser–Gly– (most preferably for MTN13 sequences). Preferred C-terminal flanking peptides include –Gly–Thr (most preferably for TN7, TN8, TN9 sequences), –Ala–Pro (most preferably for TN10 sequences), –Asp–Pro (most preferably for TN12 sequences), –Gly–Gly (most preferably for linear sequences), and –Gly–Ser (most preferably for MTN13 sequences). Single terminal amino acids may also be added to the binding polypeptides of the invention, and preferred terminal amino acids will correspond to the parental phage display construct, *e.g.*, most preferably, N-terminal amino acids will be selected from Gly– (most preferably for TN7, TN8, TN9, MTN13 sequences), Ser– (most preferably for TN10 sequences), Asp– (most preferably for TN12 sequences), and Gln– (most preferably for linear sequences), and most preferably C-terminal amino acids will be selected from –Gly (most preferably for TN7, TN8, TN9, MTN13 and linear sequences), –Ala (most preferably for TN10 sequences), and –Asp (most preferably for TN12 sequences). Conservative substitutions (*i.e.*, substitute amino acids selected within the following groups: {Arg, His, Lys}, {Glu, Asp}, {Asn, Cys, Glu, Gly, Ser, Thr, Tyr}, {Ala, Ile, Leu, Met, Phe, Pro, Trp, Val}) for such flanking amino acids are also contemplated.

Examination of the sequence information and binding data from the isolates of libraries containing polypeptides with the potential to form loop structures (*e.g.*, libraries designated TN7, TN8, TN9, TN10, TN12 and MTN13) identifies a series of KDR or VEGF/KDR complex binding polypeptides that may form loop structures. In specific embodiments, cyclic KDR or VEGF/KDR binding polypeptides of the invention comprise, or alternatively consist of, an amino acid sequence selected from Loop Consensus Sequences 15-20 as follows:

Loop Consensus Sequence 15: Cys–X_2–X_3–X_4–X_5–X_6–X_7–Cys (TN8),

wherein

X_2 is Ala, Arg, Asn, Asp, Gln, Glu, His, Ile, Lys, Phe, Pro, Ser, Trp or Tyr (preferably Asp, Glu or Tyr);

X_3 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val (preferably Glu, Met or Tyr);

X_4 is Ala, Asn, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val (preferably Asp);

X_5 is Ala, Asp, Glu, Gly, Leu, Phe, Pro, Ser, Thr, Trp or Tyr (preferably Trp or Thr);

X_6 is Arg, Gln, Glu, Gly, Ile, Leu, Met, Pro, Thr, Trp, Tyr or Val (preferably Gly or Tyr); and

X_7 is Ala, Arg, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Trp or Tyr (preferably Lys or Tyr),

and wherein the polypeptide binds KDR or a VEGF/KDR complex; or

Loop Consensus Sequence 16: Cys–X_2–X_3–X_4–X_5–X_6–X_7–X_8–X_9–X_{10}–X_{11}–Cys (TN12), wherein

X_2 is Arg, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Thr, Trp, Tyr or Val (preferably Glu, Ile or Tyr);

X_3 is Ala, Arg, Asn, Cys, Glu, Ile, Leu, Met, Phe, Ser, Trp or Tyr (preferably Glu, Phe or Tyr);

X_4 is Arg, Asn, Asp, Gln, Glu, His, Ile, Leu, Pro, Ser, Thr, Trp, Tyr or Val (preferably Glu);

X_5 is Ala, Asn, Asp, Gln, Glu, Gly, His, Met, Phe, Pro, Ser, Trp, Tyr or Val (preferably Gln or Ser);

X_6 is Asp, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp or Tyr (preferably Asp);

X_7 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val (preferably Lys or Ser);

X_8 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Lys, Trp, Tyr or Val (preferably Gly or Tyr);

X_9 is Ala, Arg, Gln, Gly, His, Ile, Lys, Met, Phe, Ser, Thr, Trp, Tyr or Val (preferably Trp or Thr);

X_{10} is Arg, Gln, Glu, His, Leu, Lys, Met, Phe, Pro, Thr, Trp or Val (preferably Glu

or Trp); and

X_{11} is Arg, Asn, Asp, Glu, His, Ile, Leu, Met, Phe, Pro, Thr, Trp, Tyr or Val (preferably Phe),

and wherein the polypeptide binds KDR or a VEGF/KDR complex; or

Loop Consensus Sequence 17: Cys–X_2–X_3–X_4–Gly–X_6–Cys (TN7), wherein

X_2 is Asn, Asp or Glu;

X_3 is Glu, His, Lys or Phe;

X_4 is Asp, Gln, Leu, Lys, Met or Tyr; and

X_6 is Arg, Gln, Leu, Lys or Val,

and wherein the polypeptide binds KDR or a VEGF/KDR complex; or

Loop Consensus Sequence 18: Cys–X_2–X_3–X_4–X_5–X_6–X_7–X_8–Cys (TN9), wherein

X_2 is Ala, Asp, Lys, Ser, Trp or Val (preferably Lys);

X_3 is Asn, Glu, Gly, His or Leu;

X_4 is Gln, Glu, Gly, Met, Lys, Phe, Tyr or Val (preferably Met);

X_5 is Ala, Asn, Asp, Gly, Leu, Met, Pro, Ser or Thr;

X_6 is His, Pro or Trp (preferably Pro or Trp);

X_7 is Ala, Gly, His, Leu, Trp or Tyr (preferably Trp); and

X_8 is Ala, Asp, Gln, Leu, Met, Thr or Trp,

and wherein the polypeptide binds KDR or a VEGF/KDR complex; or

Loop Consensus Sequence 19: Cys–X_2–X_3–X_4–X_5–Ser–Gly–Pro–X_9–X_{10}–X_{11}–X_{12}–Cys (MTN13; SEQ ID NO:13), wherein

X_2 is Asp, Glu, His or Thr;

X_3 is Arg, His, Lys or Phe;

X_4 is Gln, Ile, Lys, Tyr or Val;

X_5 is Gln, Ile, Leu, Met or Phe;

X_9 is Asn, Asp, Gly, His or Tyr;

X_{10} is Gln, Gly, Ser or Thr;

X_{11} is Glu, Lys, Phe or Ser; and

X_{12} is Glu, Ile, Ser or Val,

and wherein the polypeptide binds KDR or a VEGF/KDR complex.

Preferred embodiments of the cyclic peptides of Loop Consensus Sequence 15 include KDR and/or VEGF/KDR complex binding polypeptides comprising Loop Consensus Sequences 20-22 as follows:

Loop Consensus Sequence 20: Cys–X_2–X_3–X_4–X_5–X_6–Tyr–Cys (TN8),

wherein

X_2 is Ala, Arg, Glu, Lys or Ser (preferably Glu);

X_3 is Ala, Asp, Gln, Glu, Thr or Val (preferably Asp or Glu);

X_4 is Asp or Glu;

X_5 is Trp or Tyr; and

X_6 is Thr or Tyr (preferably Tyr); or

Loop Consensus Sequence 21: Cys–X_2–X_3–X_4–Gly–X_6–X_7–Cys (TN8),

wherein

X_2 is Asp, Gln or His;

X_3 is His or Tyr (preferably Tyr);

X_4 is His, Ile or Tyr;

X_6 is Ile, Met or Val; and

X_7 is Gly or Tyr; or

Loop Consensus Sequence 22: Cys–X_2–X_3–X_4–X_5–Gly–X_7–Cys (TN8),

wherein

X_2 is Ala, Arg, Asn, Asp, His, Phe, Trp or Tyr (preferably Tyr, Trp or Phe);

X_3 is Ala, Asp, Gln, His, Lys, Met, Ser, Thr, Trp, Tyr or Val;

X_4 is Ala, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Pro, Ser, Thr or Val;

X_5 is Asp, Phe, Ser, Thr, Trp or Tyr (preferably Thr, Ser or Asp); and

X_7 is Ala, Arg, Gln, His, Ile, Leu, Lys, Met, Phe, Trp or Tyr (preferably Arg or Lys).

Preferred embodiments of the cyclic peptides of Loop Consensus Sequence 16 include KDR and/or VEGF/KDR complex binding polypeptides comprising sequences of Loop Consensus Sequences 23-26 as follows:

Loop Consensus Sequence 23: Cys–X_2–X_3–X_4–X_5–Trp–Gly–Gly–X_9–X_{10}–Cys (TN11, *i.e.*, 11-mers based on isolates of the TN12 library; SEQ ID NO:14), wherein

X_2 is Ala, Phe or Trp (preferably Trp or Phe);

X_3 is Glu or Lys (preferably Glu);

X_4 is Asp, Ser, Trp or Tyr (preferably Asp, Trp or Tyr);

X_5 is Phe, Pro or Ser (preferably Ser);

X_9 is Gln or Glu (preferably Glu); and

X_{10} is Ile, Phe or Val; or

Loop Consensus Sequence 24: Cys–X_2–Glu–X_4–Ser–X_6–Ser–X_8–X_9–X_{10}–

Phe–Cys (TN12; SEQ ID NO:15), wherein

X_2 is His or Tyr;

X_4 is Leu, His or Thr;

X_6 is Asp or Leu (preferably Asp);

X_8 is Gly or Val (preferably Val);

X_9 is Thr or Val (preferably Thr); and

X_{10} is Arg or Trp (preferably Arg); or

Loop Consensus Sequence 25: Cys–X_2–X_3–X_4–X_5–X_6–X_7–Gly–X_9–Trp–X_{11}–Cys (TN12; SEQ ID NO:16), wherein

X_2 is Glu, Met or Thr (preferably Glu);

X_3 is Ile, Leu, Met or Phe (preferably Met, Leu or Phe);

X_4 is Arg, Asp, Glu, Met, Trp or Val;

X_5 is Asn, Gln, Gly, Ser or Val;

X_6 is Glu or Asp;

X_7 is Lys, Ser, Thr or Val (preferably Lys);

X_9 is Arg, Gln, Lys or Trp (preferably Trp, Arg or Lys); and

X_{11} is Asn, Leu, Phe or Tyr (preferably Tyr, Phe or Asn); or

Loop Consensus Sequence 26: Cys–X_2–X_3–X_4–X_5–X_6–X_7–X_8–X_9–X_{10}–X_{11}–Cys (TN12), wherein

X_2 is Glu or Gly;

X_3 is Trp or Tyr;

X_4 is Ser or Thr;

X_5 is Asn or Gln;

X_6 is Gly or Met;

X_7 is Phe or Tyr;

X_8 is Asp or Gln;

X_9 is Lys or Tyr;

X_{10} is Glu or Thr; and

X_{11} is Glu or Phe.

Preferred embodiments of the cyclic peptides of Loop Consensus Sequence 17 include KDR and/or VEGF/KDR complex binding polypeptides comprising sequences of Loop Consensus Sequence 27 as follows:

Loop Consensus Sequence 27: Cys–X_2–X_3–X_4–Gly–X_6–Cys (TN7), wherein

X_2 is Asn, Asp or Glu;

X_3 is Glu, His, Lys or Phe;

X_4 is Asp, Gln, Leu, Lys, Met or Tyr; and

X_6 is Arg, Gln, Leu, Lys or Val.

Preferred embodiments of the cyclic peptides of Loop Consensus Sequence 18 include KDR and/or VEGF/KDR complex binding polypeptides comprising sequences of Loop Consensus Sequence 28 as follows:

Loop Consensus Sequence 28: Cys–X_2–X_3–X_4–X_5–X_6–X_7–X_8–Cys (TN9), wherein

X_2 is Ala, Lys, Ser, Trp or Val (preferably Lys);

X_3 is Asn, Glu, Gly, His or Leu;

X_4 is Glu, Gly, Lys, Met or Tyr (preferably Met);

X_5 is Ala, Asn, Asp, Leu, Met, Pro or Ser;

X_6 is His, Pro or Trp (preferably Pro);

X_7 is His, Leu, Trp or Tyr (preferably Trp or His); and

X_8 is Ala, Asp, Gln, Leu, Met, Thr or Trp.

Preferred embodiments of the cyclic peptides of Loop Consensus Sequence 19 include KDR and/or VEGF/KDR complex binding polypeptides comprising sequences of Loop Consensus Sequence 29 as follows:

Loop Consensus Sequence 29: Cys–X_2–X_3–X_4–X_5–Ser–Gly–Pro–X_9–X_{10}–X_{11}–X_{12}–Cys (MTN13; SEQ ID NO:17), wherein

X_2 is Asp, Glu, His or Thr;

X_3 is Arg, His, Lys or Phe;

X_4 is Gln, Ile, Lys, Tyr or Val;

X_5 is Gln, Ile, Leu, Met or Phe;

X_9 is Asn, Asp, Gly, His or Tyr;

X_{10} is Gln, Gly, Ser or Thr;

X_{11} is Glu, Lys, Phe or Ser; and

X_{12} is Glu, Ile, Ser or Val.

The modifications, chemical or physical, as well as any sequence modifications described herein are encompassed to be used for any of the specific sequences disclosed herein and/or any specific sequences that conform to any of the consensus sequences described herein.

The KDR and VEGF/KDR binding polypeptides described above can

optionally have additional amino acids attached at either or both of the N- and C-terminal ends and can be modified, optimized or employed in multimeric constructs. Further, the invention includes homologues of the KDR and VEGF/KDR complex binding peptides as defined herein.

Another aspect of the present invention relates to modifications of the foregoing polypeptides to provide specific angiogenesis imaging agents by detectably labeling a polypeptide according to the present invention. Such detectable labeling can involve radiolabeling, enzymatic labeling, or labeling with MR paramagnetic chelates or microparticles; incorporation into ultrasound bubbles, microparticles, microspheres, emulsions, or liposomes; or conjugation with optical dyes.

In another aspect of the present invention, methods for isolating KDR or KDR-expressing cells using the present binding polypeptides are provided.

Additionally, the KDR and VEGF/KDR complex binding polypeptides of the invention can be used as therapeutic agents, either as the sole bioactive agent in a pharmaceutically acceptable composition or conjugated to (or in combination with) other therapeutic agents to treat diseases or conditions involving angiogenesis or diseases associated with a number of pathogens, including, for example, malaria, HIV, SIV, Simian hemorrhagic fever, etc.

When used as therapeutic agents, it may be advantageous to enhance the serum residence time of the peptides. This can be accomplished by: a) conjugating to the peptide a moiety, such as maleimide, that reacts with free sulfhydryl groups on serum proteins, such as serum albumin, b) conjugating to the peptide a moiety, such as a fatty acid, that binds non-covalently to serum proteins, especially serum albumin, c) conjugating to the peptide a polymer, such as PEG, that is known to enhance serum residence time, and d) fusing DNA that encodes the KDR-binding peptide to DNA that encodes a serum protein such as human serum albumin or an antibody and expressing the encoded fusion protein.

These and other aspects of the present invention will become apparent with reference to the following detailed description.

In another aspect of the invention, methods of screening polypeptides identified by phage display for their ability to bind to cells expressing the target are provided. These methods permit rapid screening of the binding ability of polypeptides, including polypeptides with monomeric affinities that are too low for

evaluation in standard cell-binding assays. Additionally, these methods may be used to rapidly assess the stability of the peptides in the presence of serum.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 (panels A and B) are graphs illustrating the saturation binding curves of binding peptide/neutravidin-HRP complexes. FIG. 1A illustrates the saturation binding curve for SEQ ID NO:264 and SEQ ID NO:294. FIG. 1B illustrates the saturation binding curve for SEQ ID NO:277 and SEQ ID NO:356. All peptides had a C-terminal biotin and JJ spacer.

FIG. 2 is a graph illustrating the binding of peptide/neutravidin-HRP complexes: control (biotinylated with spacer, and SEQ ID NOS:264, 294, 277 and 356) to KDR-transfected and Mock-transfected 293H cells at a single concentration (5.55 nM). All peptides had a C-terminal biotin and JJ spacer.

FIG. 3 is illustrates peptide structures, with and without both spacer (di(8-amino-3,6-dioxaoctanoic acid) "JJ") and biotin tested in Example 5((a) biotinylated SEQ ID NO:264 with a JJ spacer; (b) SEQ ID NO:264 with an N-terminal biotin; (c) biotinylated SEQ ID NO:294 with the JJ spacer (d) biotinylated SEQ ID NO:294).

FIG. 4 is a bar graph illustrating binding of peptide/neutravidin HRP complexes to KDR-transfected and mock-transfected 293H cells at single a concentration (2.78 nM); peptides include (a) control (with spacer); (b) control; (c) biotinylated SEQ ID NO:264 with a JJ spacer; (d) SEQ ID NO:264 with an N-terminal biotin; and (e) biotinylated SEQ ID NO:294 with the JJ spacer; and biotinylated SEQ ID NO:294.

FIG. 5 is a bar graph illustrating specific binding (binding to KDR transfected cells minus binding to Mock transfected cells) of peptide/neutravidin-HRP complexes with and without 40% rat serum. (a) SEQ ID NO:294; (b) SEQ ID NO:264; (c) SEQ ID NO:277; (d) SEQ ID NO:356. Concentration of peptide/avidin HRP solutions was 6.66 nM for (a) and (b), 3.33 nM (c), and 2.22 nM for (d).). All peptides had a C-terminal biotin and JJ spacer.

FIG. 6 is a bar graph illustrating binding of polypeptide/avidin-HRP solutions (SEQ ID NO:294 and/or SEQ ID NO:264) to mock- and KDR-transfected cells plotted as absorbance at 450 nm. The proportions of control and KDR binding peptides used to form each tetrameric complex are indicated in the legend for each tested multimer.

FIG. 7 is a bar graph illustrating specific binding of a biotinylated SEQ ID NO:264 with a JJ spacer/avidin-HRP complex to KDR transfected cells (background binding to mock-transfected cells subtracted), plotted as absorbance at 450 nm. Increasing concentrations (as indicated in the X axis) of uncomplexed peptides were added to the assay as indicated in the legend. Only free SEQ ID NO:264 was able to decrease the binding of the SEQ ID NO:264 complex to KDR-transfected cells.

FIG. 8 illustrates structures of binding polypeptide sequences tested in Example 6: SEQ ID NOS:294 and 368-372.

FIG. 9 is a bar graph illustrating the binding of fluorescent beads to KDR-transfected and mock-transfected cells. Neutravidin-coated beads with the indicated biotinylated ligands attached were tested for binding to KDR-expressing and non-expressing 293H cells.

FIG. 10 is a bar graph illustrating percent inhibition of ^{125}I-labeled VEGF binding by binding polypeptides (a) acetylated SEQ ID NO:294 (without the modified C-terminus, "P6", GDSRVCWEDSWGGEVCFRYDP; SEQ ID NO:374); (b) SEQ ID NO:263 (without the modified C-terminus, "P4", AGDSWCSTEYTYCEMIGT; SEQ ID NO:375); (c) biotinylated SEQ ID NO:264 with a JJ spacer; and (d) SEQ ID NO:277 (biotinylated with the JJ spacer), at two concentrations (30 μM and 0.3 μM), to KDR-expressing 293H transfectants.

FIG. 11 depicts chemiluminescent detection on film demonstrating that activated (phosphorylated) KDR was not detected in immunoprecipitates from unstimulated (-V) HUVECs, but was abundant in immunoprecipitates from VEGF-stimulated (+V) HUVECs (upper panel). Reprobing the blot with anti-KDR demonstrated that comparable amounts of total KDR were present in both immunoprecipitates (lower panel).

FIG. 12 depicts chemiluminescent detection on film demonstrating the ability of an anti-KDR antibody (1 μg/mL) to partially block VEGF-mediated phosphorylation.

FIG. 13 depicts chemiluminescent detection on film demonstrating the ability of a KDR-binding polypeptide SEQ ID NO:306 (10 μM) to block VEGF-mediated KDR phosphorylation.

FIG. 14 is a bar graph showing binding of a Tc-labeled polypeptide (SEQ ID NO:339) to KDR-transfected 293H cells.

FIG. 15 is a graph showing the percentage inhibition of ^{125}I-labeled VEGF

binding by peptides P12-XB (SEQ ID NO:277) D2, D1, D3, and P13-D (AQDWYYDEILSMADQLRHAFLSGG; SEQ ID NO:376) at three different concentrations (10 μM, 0.3 μM, and 0.03 μM) to KDR-transfected 293H cells. The results are from one experiment carried out in triplicate +/- S.D.

FIG. 16 is a photograph showing the ability of D1 to completely block the VEGF-induced phosphorylation of KDR in HUVECs at 10 nM and the majority of phosphorylation at 1 nM. Reprobing the blot for total KDR (lower panel) demonstrated that the effects of the tested compounds was not due to reduced sample loading. Homodimers composed of the two binding sequences contained in D1 did not interfere with the phosphorylation at up to 100 nM.

FIG. 17 is a graph showing that D1 potently blocks the migration/invasion of endothelial cells induced by VEGF. Migrating cells were quantitated by fluorescence measurement after staining the migrated cells with a fluorescent dye.

FIG. 18 is a graph showing the binding of ^{125}I-labeled D5 to mock and KDR transfected 293H cells in the absence and presence of 40% mouse serum.

FIG. 19 is a graph showing the specific binding (KDR-MOCK) of ^{125}I-labeled D5 to KDR-transfected 293H cells in the absence and presence of 40% mouse serum.

FIG. 20 is a graph of plasma clearance as percent injected dose per mL versus time.

FIG. 21 shows SE-HPLC profiles of plasma from the Superdex peptide column. Top panel, sample injected; followed by 0min, 30min, and 90min. The insert within each panel shows time point, animal number and volume injected for HPLC analysis.

FIG. 22 is a graph showing the results of testing of KDR peptides in HUVEC proliferation assay. A: D6; B: SEQ ID NO:277; C: SEQ ID NO:377 (AEGTGDLHCYFPWVCSLDPGPEGGGK; negative control); F: SEQ ID NO:377; negative control.

FIG. 23 shows the kinetic analysis of D1 (see FIG. 36), binding to murine KDR-Fc. All sensograms are fit to the bivalent analyte model.

FIG. 24 shows the kinetic analysis of D7, a heterodimer of SEQ ID NO:264 and SEQ ID NO:294. All sensograms are fit to the bivalent analyte model.

FIG. 25 shows Kinetic analysis of fluorescein labeled SEQ ID NO:277 binding to murine KDR-Fc. All sensograms are fit to the 1:1 Langmuir model.

FIG. 26 depicts examples of alpha, beta, gamma or delta dipeptide or turn mimics (such as α, β, γ, or δ turn mimics), shown in panels **1**, **2** and **3**

FIG. 27 shows an oxime linker. The amino acids containing an aminoalcohol function (**4**), and containing an alkoxyamino function (**5**), are incorporated into the peptide chain, not necessarily at the end of the peptide chain.

FIG. 28 shows an Example of cyclization of cysteine with a pendant bromoacetamide function.

FIG. 29 is a schematic showing the formation of cyclic peptides with a thiazolidine linkage via intramolecular reaction of peptide aldehydes with cysteine moieties. FIG. 30 is a schematic showing lactam surrogate for the disulfide bond via quasiorthogonal deprotection of Lys and Asp followed by on-resin cyclization and cleavage from resin. FIG. 31 is a schematic showing lactam surrogate for the disulfide bond via quasiorthogonal deprotection of Lys and Asp using allyl-based protecting groups followed by on-resin cyclization and cleavage from resin. FIG. 32 is a schematic depicting Grubbs Olefin Metathesis Cyclization.

FIG. 33 shows phospholipid structures.

FIGS. 34A-F depict preferred structures of chelators.

FIG. 35 shows the structure of a chelating agent.

FIG. 36 shows dimer 1 (D1; Ac-AGPTWCEDDWYYCWLFGTGGGK(SEQ ID NO:277)[(Biotin-JJK-(O=)C(CH_2)$_3$C(=O)-JJ-NH(CH_2)$_4$-(S)-CH((Ac-VCWEDSWGGEVCFRYDPGGG(SEQ ID NO:370))-NH)$CONH_2$]-NH_2).

FIG. 37 shows dimer 2 (D2; Ac-AGPTWCEDDWYYCWLFGTGGGK(SEQ ID NO:277) [(Biotin-JJK-(O=)C(CH_2)$_3$C(=O)-JJ-NH(CH_2)$_4$-(S)-CH((Ac-AGPTWCEDDWYYCWLFGTJ(SEQ ID NO:338))-NH)$CONH_2$]-NH_2).

FIG. 38 shows dimer 3 (D3; Ac-VCWEDSWGGEVCFRYDPGGGK(SEQ ID NO:337)[(Biotin-JJK-(O=)C(CH_2)$_3$C(=O)-JJ-NH(CH_2)$_4$-(S)-CH((Ac-VCWEDSWGGEVCFRYDPGGG(SEQ ID NO:370))-NH)$CONH_2$]-NH_2).

FIG. 39 shows dimer 4 (D4; Ac-AGPTWCEDDWYYCWLFGTJK(SEQ ID NO:338)[DOTA-JJK-(O=)C(CH_2)$_3$C(=O)-JJ-NH(CH_2)$_4$-(S)-CH((Ac-VCWEDSWGGEVCFRYDPGGG(SEQ ID NO:370))-NH)$CONH_2$]-NH_2).

FIG. 40 shows dimer 5 (D5; Ac-VCWEDSWGGEVCFRYDPGGGK(SEQ ID NO:337) (JJ-C(=O)(CH_2)$_3$C(=O)-K-NH(CH_2)$_4$-(S)-CH((Ac-

AGPTWCEDDWYYCWLFGTGGG(SEQ ID NO:378))-NH)CONH$_2$)-NH$_2$).

FIG. 41 shows dimer 8 (D8; Ac-AQDWYYDEILSMADQLRHAFLSGGGGGK(SEQ ID NO:356){Ac-AQDWYYDEILSMADQLRHAFLSGGGGGK(SEQ ID NO:356)(J-Glut-)-NH$_2$}K(Biotin-JJ)-NH$_2$).

FIG. 42 shows dimer 9 (D9; Ac-AQDWYYDEILSMADQLRHAFLSGGGGGK(SEQ ID NO:356){[Ac-GDSRVCWEDSWGGEVCFRYDPGGGK(SEQ ID NO:294)(JJ-Glut-)]-NH$_2$}K-NH$_2$).

FIG. 43 shows dimer 10 (D10Ac-AGPTWCEDDWYYCWLFGTGGGK(SEQ ID NO:277){[Ac-GDSRVCWEDSWGGEVCFRYDPGGGK(SEQ ID NO:294) (JJ-Glut-NH(CH$_2$)$_4$-(S)-CH(PnAO6-Glut-NH)(C=O-)]-NH$_2$}-NH$_2$).

FIG. 44 shows dimer 11 (D11; Ac-AGPTWCEDDWYYCWLFGTGGGK(SEQ ID NO:277){Ac-VCWEDSWEDSWGGEVCFRYDPGGGK(SEQ ID NO:475)[JJ-Glut-NH(CH$_2$)$_4$-(S)-CH(DOTA-JJ-NH-)(C=O)-]-NH$_2$}-NH$_2$).

FIG. 45 shows dimer 12 (D12; Ac-AGPTWCEDDYCWLFGTGGGK(SEQ ID NO:476){[PnAO6-Glut-K(Ac-VCWEDSWGGEVCFRYDPGGGK(SEQ ID NO:337)(-C(=O)CH$_2$(OCH$_2$CH$_2$)$_2$OCH$_2$C(=O)-)-NH$_2$]}-NH$_2$).

FIG. 46 shows dimer 13 (D13; Ac-AGPTWCEDDWYYCWLFGTGGGK(SEQ ID NO:277){Ac-VCWEDSWGGEVCFRYDPGGGK(SEQ ID NO:337)[JJ-Glut-K(BOA)]-NH$_2$}-NH$_2$).

FIG. 47 shows dimer 14 (D14; Ac-AQDWYYDEILSMADQLRHAFLSGGGGGK(SEQ ID NO:356){PnAO6-Glut-K[Ac-GSDRVCWEDSWGGEVCFRYDPGGGK(SEQ ID NO:477) (JJ-Glut)-NH$_2$]}-NH$_2$).

FIG. 48 shows dimer 15 (D15; Ac-AGPTWCEDDWYYCWLFGTGGGK(SEQ ID NO:277){[[Ac-GDSRVCWEDSWGGEVCFRYDPGGGKJJ(SEQ ID NO:294)-Glut]-NH$_2$]-K(PnAO6-Glut)}-NH$_2$).

FIG. 49 shows dimer 16 (D16; Ac-AGPTWCEDDWYYCWLFGTGGGGK(SEQ ID NO:277){PnAO6-Glut-K [Ac-

GDSRVCWEDSWGGEVCFRYDPGGGK(SEQ ID NO:294)[-C(=O)$CH_2O(CH_2CH_2O)_2CH_2C(=O)NH(CH_2)_3O(CH_2CH_2O)_2(CH_2)_3NH$C(=O)$CH_2O(CH_2CH_2O)_2CH_2C(=O)$-]-$NH_2$]}-$NH_2$).

FIG. 50 shows dimer 17 (D17; Ac-AQDWYYYDEILJGRGGRGGRGGK(SEQ ID NO:478){K[Ac-VCWEDSWGGEVCFRYDPGGGK(SEQ ID NO:294)(JJ-Glut)-NH_2]}-NH_2).

FIG. 51 shows dimer 18 (D18; Ac-AGPTWCDYDWEYCWLGTFGGGK(SEQ ID NO:479){PnAO6-Glut-K[Ac-GVDFRCEWSDWGEVGCRSPDYGGGK (SEQ ID NO:489)(JJ-Glut)-NH_2]}-NH_2).

FIG. 52 shows dimer 19 (D19; Ac-AGPTWCEDDWYYCWLFGTGGGK(SEQ ID NO:294){Biotin-K[Ac-VCWEDSWGGEVCFRYDPGGGK(JJ-Glut)-NH_2]}-NH_2).

FIG. 53 shows dimer 20 (D20; (-JJAGPTWCEDDWYYCWLFGTGGGGK(SEQ ID NO:480)-NH_2)-Glut-VCWEDSWGGEVCFRYDPGGG(SEQ ID NO:370)-NH_2).

FIG. 54 shows dimer 21 (D21; [-JJAGPTWCEDDWYYCWLFGTGGGGK(SEQ ID NO:480)(PnAO6-Glut)-NH_2]-Glut-VCWEDSWGGEVCFRYDPGGG(SEQ ID NO:370)-NH_2).

FIG. 55 shows dimer 22 (D22; Ac-GDSRVCWEDSWGGEVCFRYDPGGGK(SEQ ID NO:294){JJ-Glut-JJ-AGPTWCEDDWYYCWLFTGGGK(SEQ ID NO:481)-NH_2}-NH_2).

FIG. 56 shows dimer 23 (D23; Ac-AGPTWCEDDWYYCWLFGTGGGK(SEQ ID NO:277){Ac-VCWEDSWGGEVCFRYDPGGGK(SEQ ID NO:337) [JJ-Glut-K(SATA)]-NH_2}-NH_2. D23 is also D5 functionalized with the SATA (S-Acetylthioacetyl) group).

FIG. 57 shows dimer 24 (D24; Ac-AGPTWCEDDWYYCWLFGTGGGK(SEQ ID NO:277){SATA-JJK[Ac-VCWEDSWGGEVCFRYDPGGGK(SEQ ID NO:337)(JJ-Glut)-NH_2]}-NH_2).

FIG. 58 shows dimer 25 (D25; Ac-AGPTWCEDDWYYCWLFGTGGGK(SEQ ID NO:277){Ac-GDSRVCWEDSWGGEVCFRYDPGGGK(SEQ ID NO:294)[JJ-Glut-NH(CH_2)4-(S)-$CH(NH_2)C(=O)$-]-NH_2}-NH_2).

FIG. 59 shows dimer 26 (D26;

AGPTWCEDDWYYCWLFGTGGGGK(SEQ ID NO:277){(-Glut-JJ-VCWEDSWGGEVCFRYDPGGG(SEQ ID NO:370)-NH_2)-K}-NH_2).

FIG. 60 shows dimer 27 (D27; Ac-AGPTWCEDDWYYCWLFGTGGGGK(SEQ ID NO:277){Ac-VCWEDSWGGEVCFRYDPGGGK(SEQ ID NO:337)[S(GalNAc(Ac)$_3$-alpha-D)-G-S(GalNAc(Ac)$_3$-alpha-D)-Glut-S(GalNAc(Ac)$_3$-alpha-D)-G-S(GalNAc(Ac)$_3$-alpha-D)-NH$(CH_2)_4$-(S)-CH(Biotin-JJNH-)C(=O)-]-NH_2}-NH_2).

FIG. 61 shows a dimeric binding peptide of the invention.

FIG. 62 shows a dimeric binding peptide of the invention.

FIG. 63 shows a dimeric binding peptide of the invention.

FIG. 64 shows a dimeric binding peptide of the invention.

FIG. 65 is a graph showing the inhibition of tumor growth by D6 as a function of D6 concentration.

FIG. 66 shows that D26 (squares) with its glycosylation and modified spacer is able to block the effects of VEGF in the migration assay to block VEGF-stimulated migration even more potently than D24 (diamonds), which lacks those chemical modifications.

FIG. 67 shows that Adjunct A enhances the potency of D6 in blocking the biological effects of VEGF in a migration assay with cultured HUVECs. Diamonds: D6 alone at the indicated concentrations. Squares: D6 at the indicated concentrations plus 100nM Adjunct A (constant).

FIG. 68 is a schematic showing Scheme 1 (synthesis of Peptide 2).

FIG. 69 is a schematic showing Scheme 2 (synthesis of Peptide 4).

FIG. 70 is a schematic showing Scheme 3 (synthesis of D26).

FIG. 71 depicts % inhibition ± s.d. of specific ^{125}I-VEGF binding to KDR-transfected cells by SEQ ID NO:504 (squares) and D1 (diamonds).

FIG. 72 depicts % maximum VEGF-stimulated migration ± s.d. of HUVEC cells in the presence of the indicated concentrations of SEQ ID NO:504 (diamonds) D1 (squares).

FIG. 73 is a graphical representation showing total binding of complexes of control peptide and the test peptides (SEQ ID NOS:321, 320 and 323) with ^{125}I-streptavidin (in the presence of VEGF) to mock-transfected and KDR-transfected cells. Only the complex containing SEQ ID NO:321 showed specific binding (KDR-mock).

FIG. 74 is a graphical representation showing specific binding of complexes of peptide (SEQ ID NO:321) and ^{125}I-streptavidin (in the absence and presence of VEGF) to KDR-transfected cells at various conc. (0-13.33 nM) of peptide-^{125}I-streptavidin complex.

FIG. 75 shows that homodimeric D8 (squares) does not block the effects of VEGF in the migration assay as carried out in Example 28 as well the heterodimeric D17 (diamonds).

FIG. 76 is a schematic showing the synthesis of cyclic lactam peptides (sample procedure).

FIG. 77 is a graphical representation showing binding of SEQ ID NO:482 derivatives with different spacer length and biotin. Derivatives have none, one J and two J spacers respectively in between the SEQ ID NO:482 targeting sequence and biotin.

FIG. 78 depicts the binding of Tc-labeled D10 to KDR-transfected 293H cells as described in Example 32. Panel B depicts the lack of binding of Tc-labeled D18 to KDR-transfected 293H cells as described in Example 32. Mock = mock-transfected. Trans = KDR-transfected. MS = mouse serum.

FIGS. 79A-G show derivatives of binding peptides of the invention.

FIG. 80 Summarizes the results of a radiotherapy study with D13 conducted in nude mice implanted with PC3 tumors. Each plotted line represents the growth over time for an individual tumor in a treated mouse, except for the heavy dashed line, which represents the average tumor growth in a set of untreated mice, as described in Example 34.

FIG. 83 shows uptake and retention of bubble contrast in the matrigel or tumor up to 30 minutes post injection for suspensions of microbubbles conjugated to KDR peptides of the invention. In contrast, the same bubbles showed only transient (no more than 10 minutes) visualization/bubble contrast in the AOI situated outside the matrigel or tumor site.

FIG. 84 shows uptake and retention of bubble contrast in the matrigel or tumor up to 30 minutes post injection for suspensions of microbubbles conjugated to KDR peptides of the invention. In contrast, the same bubbles showed only transient (no more than 10 minutes) visualization/bubble contrast in the AOI situated outside the matrigel or tumor site.

FIG. 85 shows a typical example of peptide-conjugated ultrasound contrast

agents bound to KDR-or mock-transfected cells in presence of 10% human serum. (magnification:100x)

DEFINITIONS

In the following sections, the term "recombinant" is used to describe non-naturally altered or manipulated nucleic acids, host cells transfected with exogenous nucleic acids, or polypeptides expressed non-naturally, through manipulation of isolated DNA and transformation of host cells. Recombinant is a term that specifically encompasses DNA molecules which have been constructed *in vitro* using genetic engineering techniques, and use of the term "recombinant" as an adjective to describe a molecule, construct, vector, cell, polypeptide or polynucleotide specifically excludes naturally occurring such molecules, constructs, vectors, cells, polypeptides or polynucleotides.

The term "bacteriophage" is defined as a bacterial virus containing a DNA core and a protective shell built up by the aggregation of a number of different protein molecules. The terms "bacteriophage" and "phage" are used herein interchangeably.

The term "polypeptide" is used to refer to a compound of two or more amino acids joined through the main chain (as opposed to side chain) by a peptide amide bond (–C(:O)NH–). The term "peptide" is used interchangeably herein with "polypeptide" but is generally used to refer to polypeptides having fewer than 40, and preferably fewer than 25 amino acids.

The term "binding polypeptide" as used herein refers to any polypeptide capable of forming a binding complex with another molecule. An equivalent term sometimes used herein is "binding moiety". "KDR binding polypeptide" is a polypeptide that forms a complex *in vitro* or *in vivo* with vascular endothelial growth factor receptor-2 (or KDR, Flk-1); "VEGF/KDR complex binding polypeptide" is a polypeptide that forms a complex *in vitro* or *in vivo* with a binding complex formed between vascular endothelial growth factor (VEGF) and KDR, in particular the complex of homodimeric VEGF and one or two KDR molecules that is believed to form at the surface of endothelial cells during angiogenesis. Specific examples of KDR and VEGF/KDR binding polypeptides include but are not limited to the peptides presented in Tables 1-7, *infra*, and include hybrid and chimeric polypeptides incorporating such peptides. Also included within the definition of KDR and

VEGF/KDR complex binding polypeptides are polypeptides which are modified or optimized as disclosed herein.

Specific examples of such modifications are discussed in detail *infra*, but include substitution of amino acids for those in the parent polypeptide sequence to optimize properties, obliterate an enzyme cleavage site, etc.; C- or N-terminal amino acid substitutions or elongations, *e.g.*, for the purpose of linking the binding polypeptide to a detectable imaging label or other substrate, examples of which include, *e.g.*, addition of a polyhistidine "tail" in order to assist in purification; truncations; amide bond changes; translocations; retroinverso peptides; peptoids; retroinversopeptoids; the use of N-terminal or C-terminal modifications or linkers, such as polyglycine or polylysine segments; alterations to include functional groups, notably hydrazide (-NH-NH_2) functionalities or the C-terminal linker -Gly-Gly-Gly-Lys (SEQ ID NO:18), to assist in immobilization of binding peptides according to this invention on solid supports or for attachment of fluorescent dyes; pharmacokinetic modifications, structural modifications to retain structural features, formation of salts to increase water solubility or ease of formulation, and the like.

In addition to the detectable labels described further herein, other suitable substrates for the binding polypeptides include a tumorcidal agent or enzyme, a liposome (*e.g.*, loaded with a therapeutic agent, an ultrasound appropriate gas, or both), or a solid support, well, plate, bead, tube, slide, filter, or dish. Moreover, dimers or multimers of one or more KDR or VEGF/KDR binding polypeptides may be formed. Such constructs may, for example, exhibit increased ability to bind to KDR. All such modified binding polypeptides are also considered KDR or VEGF/KDR complex binding polypeptides so long as they retain the ability to bind the KDR or VEGF/KDR targets.

"Homologues" of the binding polypeptides described herein may be produced using any of the modification or optimization techniques described herein or known to those skilled in the art. Such homologous polypeptides will be understood to fall within the scope of the present invention and the definition of KDR and VEGF/KDR complex binding polypeptides so long as the substitution, addition, or deletion of amino acids or other such modification does not eliminate its ability to bind either KDR or VEGF/KDR complex. The term "homologous", as used herein, refers to the degree of sequence similarity between two polymers (*i.e.*, polypeptide molecules or nucleic acid molecules). When the same nucleotide or amino acid residue or one

with substantially similar properties (*i.e.*, a conservative substitution) occupies a sequence position in the two polymers under comparison, then the polymers are homologous at that position. For example, if the amino acid residues at 60 of 100 amino acid positions in two polypeptide sequences match or are homologous then the two sequences are 60% homologous. The homology percentage figures referred to herein reflect the maximal homology possible between the two polymers, *i.e.*, the percent homology when the two polymers are so aligned as to have the greatest number of matched (homologous) positions. Polypeptide homologues within the scope of the present invention will be at least 70% and preferably greater than 80% homologous to at least one of the KDR or VEGF/KDR binding sequences disclosed herein.

The term "binding" refers to the determination by standard assays, including those described herein, that a binding polypeptide recognizes and binds reversibly to a given target. Such standard assays include, but are not limited to equilibrium dialysis, gel filtration, and the monitoring of spectroscopic changes that result from binding.

The term "specificity" refers to a binding polypeptide having a higher binding affinity for one target over another. The term "KDR specificity" refers to a KDR binding moiety having a higher affinity for KDR over an irrelevant target. The term "VEGF/KDR specificity" refers to a VEGF/KDR complex binding moiety having a higher affinity for a VEGF/KDR complex over an a given target. Binding specificity may be characterized by a dissociation equilibrium constant (K_D) or an association equilibrium constant (K_a) for the two tested target materials, or can be any measure of relative binding strength. The binding polypeptides according to the present invention are specific for KDR or VEGF/KDR complex and preferably have a K_D for KDR or VEGF/KDR complex that is lower than 10μM, more preferably less than 1.0μM, most preferably less than 0.5μM or even lower.

The term "patient" as used herein refers to any mammal, especially humans.

The term "pharmaceutically acceptable" carrier or excipient refers to a non-toxic carrier or excipient that may be administered to a patient, together with a compound of this invention, and which does not destroy the biological or pharmacological activity thereof.

The following common abbreviations are used throughout this specification: 9-fluorenylmethyloxycarbonyl (fmoc or Fmoc), 1-hydroxybenzotriazole (HOBt),

N,N'-diisopropylcarbodiimide (DIC), acetic anhydride (Ac_2O), (4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde), trifluoroacetic acid (TFA), Reagent B (TFA:H_2O:phenol:triisopropylsilane, 88:5:5:2), N,N-diisopropylethylamine (DIEA), O-(1H-benzotriazole-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU),O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorphosphate (HATU), N-hydroxysuccinimide (NHS), solid phase peptide synthesis (SPPS), dimethyl sulfoxide (DMSO), dichloromethane (DCM), dimethylformamide (DMF), and N-methylpyrrolidinone (NMP).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel binding moieties that bind KDR or a complex of VEGF and KDR. Such binding moieties make possible the efficient detection, imaging and localization of activated endothelial cells exhibiting upregulated KDR expression and binding to VEGF. Such endothelial cells are characteristic of active angiogenesis, and therefore the polypeptides described herein provide a means of detecting, monitoring and localizing sites of angiogenesis. In particular, the binding polypeptides of this invention, when appropriately labeled, are useful for detecting, imaging and localizing tumor-induced angiogenesis. Thus, the binding polypeptides can be used to form a variety of diagnostic and therapeutic agents for diagnosing and treating neoplastic tumor growth or other pathogenic instances of angiogenesis. In addition, the binding polypeptides can themselves be used as therapeutic agents.

Specific KDR and VEGF/KDR complex binding polypeptides according to the present invention were isolated initially by screening of phage display libraries, that is, populations of recombinant bacteriophage transformed to express an exogenous peptide on their surface. In order to isolate new polypeptide binding moieties for a particular target, such as KDR or VEGF/KDR, screening of large peptide libraries, for example using phage display techniques, is especially advantageous, in that very large numbers (*e.g.*, 5×10^9) of potential binders can be tested and successful binders isolated in a short period of time.

In order to prepare a phage library of displaying polypeptides to screen for binding polypeptides such as KDR or VEGF/KDR complex binding polypeptides, a candidate binding domain is selected to serve as a structural template for the

peptides to be displayed in the library. The phage library is made up of a multiplicity of analogues of the parental domain or template. The binding domain template may be a naturally occurring or synthetic protein, or a region or domain of a protein. The binding domain template may be selected based on knowledge of a known interaction between the binding domain template and the binding target, but this is not critical. In fact, it is not essential that the domain selected to act as a template for the library have any affinity for the target at all: Its purpose is to provide a structure from which a multiplicity (library) of similarly structured polypeptides (analogues) can be generated, which multiplicity of analogues will hopefully include one or more analogues that exhibit the desired binding properties (and any other properties screened for).

In selecting the parental binding domain or template on which to base the variegated amino acid sequences of the library, the most important consideration is how the variegated peptide domains will be presented to the target, *i.e.*, in what conformation the peptide analogues will come into contact with the target. In phage display methodologies, for example, the analogues will be generated by insertion of synthetic DNA encoding the analogues into phage, resulting in display of the analogue on the surfaces of the phage. Such libraries of phage, such as M13 phage, displaying a wide variety of different polypeptides, can be prepared using techniques as described, *e.g.*, in Kay *et al.*, *Phage Display of Peptides and Proteins: A Laboratory Manual* (Academic Press, Inc., San Diego, 1996) and US 5,223,409 (Ladner *et al.*), incorporated herein by reference.

In isolating the specific polypeptides according to this invention, seven cyclic peptide (or "loop") libraries, designated TN6/VI, TN7/IV, TN8/IX, TN9/IV, TN10/IX, TN12/I, and MTN13/I, and a linear library, designated Lin20, were used. Each library was constructed for expression of diversified polypeptides on M13 phage. The seven libraries having a "TN" designation were designed to display a short, variegated exogenous peptide loop of 6, 7, 8, 9, 10, 12 or 13 amino acids, respectively, on the surface of M13 phage, at the amino terminus of protein III. The libraries are designated TN6/VI (having a potential 3.3×10^{12} amino acid sequence diversity), TN7/IV (having a potential 1.2×10^{14} amino acid sequence diversity), TN8/IX (having a potential 2.2×10^{15} amino acid sequence diversity), TN9/IV (having a potential 4.2×10^{16} amino acid sequence diversity, TN10/IX (having a potential 3.0×10^{16} amino acid sequence diversity), TN12/I (having a sequence

diversity of 4.6×10^{19}), MTN13/I (having a potential 8.0×10^{17} amino acid sequence diversity), and Lin20 (having a potential 3.8×10^{25} amino acid sequence diversity).

The TN6/VI library was constructed to display a single microprotein binding loop contained in a 12-amino acid template. The TN6/VI library utilized a template sequence of Xaa_1–Xaa_2–Xaa_3–Cys–Xaa_5–Xaa_6–Xaa_7–Xaa_8–Cys–Xaa_{10}–Xaa_{11}–Xaa_{12}. The amino acids at positions 2, 3, 5, 6, 7, 8, 10, and 11 of the template were varied to permit any amino acid except cysteine (Cys). The amino acids at positions 1 and 12 of the template were varied to permit any amino acid except cysteine (Cys), glutamic acid (Glu), isoleucine (Ile), Lysine (Lys), methionine (Met), and threonine (Thr).

The TN7/IV library was constructed to display a single microprotein binding loop contained in a 13-amino acid template. The TN7/IV library utilized a template sequence of Xaa_1–Xaa_2–Xaa_3–Cys–Xaa_5–Xaa_6–Xaa_7–Xaa_8–Xaa_9–Cys–Xaa_{11}–Xaa_{12}–Xaa_{13}. The amino acids at amino acid positions 1, 2, 3, 5, 6, 7, 8, 9, 11, 12, and 13 of the template were varied to permit any amino acid except cysteine (Cys).

The TN8/IX library was constructed to display a single microprotein binding loop contained in a 14-amino acid template. The TN8/IX library utilized a template sequence of Xaa_1–Xaa_2–Xaa_3–Cys–Xaa_5– Xaa_6–Xaa_7–Xaa_8–Xaa_9–Xaa_{10}–Cys–Xaa_{12}–Xaa_{13}–Xaa_{14}. The amino acids at position 1, 2, 3, 5, 6, 7, 8, 9, 10, 12, 13, and 14 in the template were varied to permit any amino acid except cysteine (Cys).

The TN9/IV library was constructed to display a single microprotein binding loop contained in a 15-amino acid template. The TN9/IV library utilized a template sequence Xaa_1–Xaa_2–Xaa_3–Cys–Xaa_5–Xaa_6–Xaa_7–Xaa_8–Xaa_9–Xaa_{10}–Xaa_{11}–Cys–Xaa_{13}–Xaa_{14}–Xaa_{15}. The amino acids at position 1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 13, 14 and 15 in the template were varied to permit any amino acid except cysteine (Cys).

The TN10/IX library was constructed to display a single microprotein binding loop contained in a 16-amino acid template. The TN10/IX library utilized a template sequence Xaa_1–Xaa_2–Xaa_3–Cys–Xaa_5–Xaa_6–Xaa_7–Xaa_8–Xaa_9–Xaa_{10}–Xaa_{11}–Xaa_{12}–Cys–Xaa_{14}–Xaa_{15}–Xaa_{16}. The amino acids at positions 1, 2, 15, and 16 in the template were varied to permit any amino acid selected from a group of 10 amino acids: D, F, H, L, N, P, R, S, W, or Y). The amino acids at positions 3 and 14 in the template were varied to permit any amino acid selected from a group of 14 amino acids: A, D, F, G, H, L, N, P, Q, R, S, V, W, or Y). The amino acids at positions 5, 6, 7, 8, 9, 10, 11, and 12 in the template were varied to permit any amino

acid except cysteine (Cys).

The TN12/I library was constructed to display a single microprotein binding loop contained in an 18-amino acid template. The TN12/I library utilized a template sequence Xaa_1–Xaa_2–Xaa_3–Cys–Xaa_5–Xaa_6–Xaa_7–Xaa_8–Xaa_9–Xaa_{10}–Xaa_{11}–Xaa_{12}–Xaa_{13}–Xaa_{14}–Cys–Xaa_{16}–Xaa_{17}–Xaa_{18}. The amino acids at position 1, 2, 17, and 18 in the template were varied to permit any amino acid selected from a group of 12 amino acids: A, D, F, G, H, L, N, P, R, S, W, or Y). The amino acids at positions 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 16 were varied to permit any amino acid except cysteine (Cys).

The MTN13/I library was constructed to display a single microprotein binding loop contained in a 19-amino acid template featuring two variable regions of equal size (*i.e.*, eight amino acids) separated by a constant region of three amino acids (Ser–Gly–Pro). The MTN13/I library utilized a template sequence Xaa_1–Xaa_2–Xaa_3–Cys–Xaa_5–Xaa_6–Xaa_7–Xaa_8–Ser–Gly–Pro–Xaa_{12}–Xaa_{13}–Xaa_{14}–Xaa_{15}–Cys–Xaa_{17}–Xaa_{18}–Xaa_{19} (SEQ ID NO:19). The amino acids at position 1, 2, 3, 5, 6, 7, 8, 12, 13, 14, 15, 17, 18, and 19 in the template were varied to permit any amino acid except cysteine (Cys).

The Lin20 library was constructed to display a single linear peptide in a 20-amino acid template. The amino acids at each position in the template were varied to permit any amino acid except cysteine (Cys).

The binding polypeptides provided herein can include additions or truncations in the N- and/or C- termini. Such modified binding polypeptides are expected to bind KDR or VEGF/KDR complex. For example, the -GGGK linker present at the N-terminus of some of the binding polypeptides provided herein is an optional linker. Therefore, polypeptides having the same sequence, except without the terminal -GGGK sequences are also encompassed by the present invention. In addition, binding polypeptides comprising the loop portion of the templates and sequences provided herein are expected to bind KDR and/or VEGF/KDR complex and are also encompassed by the present invention. The loop portion of the templates and sequences includes the sequences between and including the two cysteine residues that are expected to form a disulfide bond, thereby generating a peptide loop structure. Furthermore, the binding polypeptides of the present invention can include additional amino acid residues at the N- and/or C-termini.

The phage display libraries were created by making a designed series of mutations or variations within a coding sequence for the polypeptide template, each mutant sequence encoding a peptide analogue corresponding in overall structure to the template except having one or more amino acid variations in the sequence of the template. The novel variegated (mutated) DNA provides sequence diversity, and each transformant phage displays one variant of the initial template amino acid sequence encoded by the DNA, leading to a phage population (library) displaying a vast number of different but structurally related amino acid sequences. The amino acid variations are expected to alter the binding properties of the binding peptide or domain without significantly altering its structure, at least for most substitutions. It is preferred that the amino acid positions that are selected for variation (variable amino acid positions) will be surface amino acid positions, that is, positions in the amino acid sequence of the domains which, when the domain is in its most stable conformation, appear on the outer surface of the domain (*i.e.*, the surface exposed to solution). Most preferably the amino acid positions to be varied will be adjacent or close together, so as to maximize the effect of substitutions.

As indicated previously, the techniques discussed in Kay *et al.*, *Phage Display of Peptides and Proteins: A Laboratory Manual* (Academic Press, Inc., San Diego, 1996) and US 5,223,409 are particularly useful in preparing a library of potential binders corresponding to the selected parental template. The seven libraries discussed above were prepared according to such techniques, and they were screened for KDR or VEGF/KDR complex binding polypeptides against an immobilized target, as explained in the examples to follow.

In a typical screen, a phage library is contacted with and allowed to bind the target, or a particular subcomponent thereof. To facilitate separation of binders and non-binders, it is convenient to immobilize the target on a solid support. Phage bearing a target-binding moiety form a complex with the target on the solid support whereas non-binding phage remain in solution and may be washed away with excess buffer. Bound phage are then liberated from the target by changing the buffer to an extreme pH (pH 2 or pH 10), changing the ionic strength of the buffer, adding denaturants, or other known means. To isolate the binding phage exhibiting the polypeptides of the present invention, a protein elution was performed, *i.e.*, some phage were eluted from target using VEGF in solution (competitive elution); and also, very high affinity binding phage that could not be competed off incubating with

VEGF overnight were captured by using the phage still bound to substrate for infection of *E.coli* cells.

The recovered phage may then be amplified through infection of bacterial cells and the screening process repeated with the new pool that is now depleted in non-binders and enriched in binders. The recovery of even a few binding phage is sufficient to carry the process to completion. After a few rounds of selection, the gene sequences encoding the binding moieties derived from selected phage clones in the binding pool are determined by conventional methods, described below, revealing the peptide sequence that imparts binding affinity of the phage to the target. When the selection process works, the sequence diversity of the population falls with each round of selection until desirable binders remain. The sequences converge on a small number of related binders, typically 10-50 out of the more than 10 million original candidates from each library. An increase in the number of phage recovered at each round of selection, and of course, the recovery of closely related sequences are good indications that convergence of the library has occurred in a screen. After a set of binding polypeptides is identified, the sequence information may be used to design other secondary phage libraries, biased for members having additional desired properties.

Formation of the disulfide binding loop is advantageous because it leads to increased affinity and specificity for such peptides. However, in serum, the disulfide bond might be opened by free cysteines or other thiol-containing molecules. Thus, it may be useful to modify the cysteine residues to replace the disulfide cross-link with another less reactive linkage. The $-CH_2-S-S-CH_2-$ cross-link has a preferred geometry in which the dihedral bond between sulfurs is close to 90 degrees, but the exact geometry is determined by the context of other side groups and the binding state of the molecule. Preferred modifications of the closing cross-link of the binding loop will preserve the overall bond lengths and angles as much as possible. Suitable such alternative cross-links include thioether linkages such as $-CH_2-S-CH_2-CH_2-$, $-CH_2-CH_2-S-CH_2-$, $-CH_2-CH_2-S-CH_2-CH_2-$; lactam linkages such as $-CH_2-NH-CO-CH_2-$ and $-CH_2-CO-NH-CH_2-$; ether linkages such as $-CH_2-CH_2-O-CH_2-CH_2-$; alkylene bridges such as $-(CH_2)_n-$ (where n = 4, 5, or 6); the linkage $-CH_2-NH-CO-NH-CH_2-$, and similar groups known in the art.

Although polypeptides containing a stable disulfide-linked binding loop are most preferred, linear polypeptides derived from the foregoing sequences may be

readily prepared, *e.g.*, by substitution of one or both cysteine residues, which may retain at least some of the KDR or VEGF/KDR binding activity of the original polypeptide containing the disulfide linkage. In making such substitutions for Cys, the amino acids Gly, Ser, and Ala are preferred, and it is also preferred to substitute both Cys residues, so as not to leave a single Cys that may cause the polypeptide to dimerize or react with other free thiol groups in a solution. All such linearized derivatives that retain KDR or VEGF/KDR binding properties are within the scope of this invention.

Direct synthesis of the polypeptides of the invention may be accomplished using conventional techniques, including solid-phase peptide synthesis, solution-phase synthesis, etc. Solid-phase synthesis is preferred. See Stewart *et al.*, *Solid-Phase Peptide Synthesis* (W. H. Freeman Co., San Francisco, 1989); Merrifield, *J. Am. Chem. Soc.*, 85:2149-2154 (1963); Bodanszky and Bodanszky, *The Practice of Peptide Synthesis* (Springer-Verlag, New York, 1984), incorporated herein by reference.

Polypeptides according to the invention may also be prepared commercially by companies providing peptide synthesis as a service (*e.g.*, BACHEM Bioscience, Inc., King of Prussia, PA; Quality Controlled Biochemicals, Inc., Hopkinton, MA). Automated peptide synthesis machines, such as manufactured by Perkin-Elmer Applied Biosystems, also are available.

The polypeptide compound is preferably purified once it has been isolated or synthesized by either chemical or recombinant techniques. For purification purposes, there are many standard methods that may be employed, including reversed-phase high-pressure liquid chromatography (RP-HPLC) using an alkylated silica column such as C_4-, C_8- or C_{18}-silica. A gradient mobile phase of increasing organic content is generally used to achieve purification, for example, acetonitrile in an aqueous buffer, usually containing a small amount of trifluoroacetic acid. Ion-exchange chromatography can also be used to separate peptides based on their charge. The degree of purity of the polypeptide may be determined by various methods, including identification of a major large peak on HPLC. A polypeptide that produces a single peak that is at least 95% of the input material on an HPLC column is preferred. Even more preferable is a polypeptide that produces a single peak that is at least 97%, at least 98%, at least 99% or even 99.5% or more of the input material on an HPLC column.

In order to ensure that the peptide obtained using any of the techniques described above is the desired peptide for use in compositions of the present invention, analysis of the peptide composition may be carried out. Such composition analysis may be conducted using high resolution mass spectrometry to determine the molecular weight of the peptide. Alternatively, the amino acid content of the peptide can be confirmed by hydrolyzing the peptide in aqueous acid, and separating, identifying and quantifying the components of the mixture using HPLC, or an amino acid analyzer. Protein sequenators, which sequentially degrade the peptide and identify the amino acids in order, may also be used to determine the sequence of the peptide.

KDR or VEGF/KDR complex binding polypeptides according to the present invention also may be produced using recombinant DNA techniques, utilizing nucleic acids (polynucleotides) encoding the polypeptides according to this invention and then expressing them recombinantly, *i.e.*, by manipulating host cells by introduction of exogenous nucleic acid molecules in known ways to cause such host cells to produce the desired KDR or VEGF/KDR complex binding polypeptides. Such procedures are within the capability of those skilled in the art (see Davis *et al.*, *Basic Methods in Molecular Biology*, (1986)), incorporated by reference. Recombinant production of short peptides such as those described herein may not be practical in comparison to direct synthesis, however recombinant means of production may be very advantageous where a KDR or VEGF/KDR complex binding moiety of this invention is incorporated in a hybrid polypeptide or fusion protein.

In the practice of the present invention, a determination of the affinity of the KDR or VEGF/KDR complex binding moiety for KDR or VEGF/KDR complex relative to another protein or target is a useful measure, and is referred to as specificity for KDR or VEGF/KDR complex. Standard assays for quantitating binding and determining affinity include equilibrium dialysis, equilibrium binding, gel filtration, or the monitoring of numerous spectroscopic changes (such as a change in fluorescence polarization) that may result from the interaction of the binding moiety and its target. These techniques measure the concentration of bound and free ligand as a function of ligand (or protein) concentration. The concentration of bound polypeptide ([Bound]) is related to the concentration of free polypeptide

([Free]) and the concentration of binding sites for the polypeptide, *i.e.*, on KDR or VEGF/KDR complex, (N), as described in the following equation:

$$[\text{Bound}] = N \times [\text{Free}]/((1/K_a)+[\text{Free}]).$$

A solution of the data to this equation yields the association constant, K_a, a quantitative measure of the binding affinity. The association constant, K_a is the reciprocal of the dissociation constant, K_D. The K_D is more frequently reported in measurements of affinity. Preferred KDR or VEGF/KDR complex binding polypeptides have a K_D for KDR or VEGF/KDR complex in the range of 1 nanomolar (nM) to 100 micromolar (μM), which includes K_D values of less than 10 nM, less than 20 nM, less than 40 nM, less than 60 nM, less than 80 nM, less than 1 μM, less than 5 μM, less than 10 μM, less than 20 μM, less than 40 μM, less than 60 μM, and less than 80 μM.

Where KDR or VEGF/KDR complex binding moieties are employed as imaging agents, other aspects of binding specificity may become more important: Imaging agents operate in a dynamic system in that binding of the imaging agent to the target (KDR or VEGF/KDR complex, *e.g.*, on activated endothelium) may not be in a stable equilibrium state throughout the imaging procedure. For example, when the imaging agent is initially injected, the concentration of imaging agent and of agent-target complex rapidly increases. Shortly after injection, however, the circulating (free) imaging agent starts to clear through the kidneys or liver, and the plasma concentration of imaging agent begins to drop. This drop in the concentration of free imaging agent in the plasma eventually causes the agent-target complex to dissociate. The usefulness of an imaging agent depends on the difference in rate of agent-target dissociation relative to the clearing rate of the agent. Ideally, the dissociation rate will be slow compared to the clearing rate, resulting in a long imaging time during which there is a high concentration of agent-target complex and a low concentration of free imaging agent (background signal) in the plasma.

Quantitative measurement of dissociation rates may be easily performed using several methods known in the art, such as fiber optic fluorimetry (see, *e.g.*, Anderson & Miller, *Clin. Chem.*, 34(7):1417-21 (1988)), surface plasmon resonance (see, Malmborg *et al.*, *J. Immunol. Methods*, 198(1):51-7 (1996) and Schuck,

Current Opinion in Biotechnology, 8:498-502 (1997)), resonant mirror, and grating coupled planar waveguiding (see, *e.g.*, Hutchinson, *Molec. Biotechnology*, 3:47-54 (1995)). Automated biosensors are commercially available for measuring binding kinetics: BIAcore surface plasmon resonance sensor (Biacore AB, Uppsala SE), IAsys resonant mirror sensor (Fisons Applied Sensor Technology, Cambridge GB), BIOS-1 grated coupled planar waveguiding sensor (Artificial Sensor Instruments, Zurich CH).

Methods of Screening Polypeptides Identified by Phage Display For Their Ability To Bind To Cells Expressing The Target:

In another aspect of the invention, methods of screening binding polypeptides identified by phage display for their ability to bind to cells expressing the target (and not to cells which do not express the target) are provided. These methods address a significant problem associated with screening peptides identified by phage display: frequently the peptides so identified do not have sufficient affinity for the target to be screened against target-expressing cells in conventional assays. However, ascertaining that a particular phage-identified peptide binds to cells that express the target (and does not bind to cells that do not) is a critical piece of information in identifying binding peptides which are potential *in vivo* targeting moieties. The method takes advantage of the increase in affinity and avidity associated with multivalent binding and permit screening of polypeptides with low affinities against target-expressing cells.

The method generally consists of preparation and screening of multimeric constructs including one or more binding polypeptides. For example, polypeptides identified by phage display as binding to a target are biotinylated and complexed with avidin, streptavidin or neutravidin to form tetrameric constructs. These tetrameric constructs are then incubated with cells that express the desired target and cells that do not, and binding of the tetrameric construct is detected. Binding may be detected using any method of detection known in the art. For example, to detect binding the avidin, streptavidin, or neutravidin may be conjugated to a detectable marker (*e.g.*, a radioactive label, a fluorescent label, or an enzymatic label which undergoes a color change, such as HRP (horse radish peroxidase), TMB (tetramethyl benzidine) or alkaline phosphatase).

The biotinylated peptides are preferably complexed with neutravidin-HRP.

Neutravidin exhibits lower non-specific binding to molecules than the other alternatives due to the absence of lectin binding carbohydrate moieties and cell adhesion receptor-binding RYD domain in neutravidin. See, Hiller *et al.*, *Biochem. J.*, 248:167-171 (1987); Alon *et al.*, *Biochem. Biophys. Res. Commun.*, 170:1236-41 (1990).

The tetrameric constructs may be screened against cells which naturally express the target or cells which have been engineered via recombinant DNA technologies to express the target (*e.g.*, transfectants, transformants, etc.). If cells which have been transfected to express the target are used, mock transfected cells (*i.e.*, cells transfected without the genetic material encoding the target) may be used as a control.

The tetrameric complexes may optionally be screened in the presence of serum. Thus, the assay may also be used to rapidly evaluate the effect of serum on the binding of peptides to the target.

The methods disclosed herein are particularly useful in preparing and evaluating combinations of distinct binding polypeptides for use in dimeric or multimeric targeting contructs which contain two or more binding polypeptides. Use of biotin/avidin complexes allows for relatively easy preparation of tetrameric constructs containing one to four different binding peptides. Furthermore, it has now been found that affinity and avidity of a targeting construct may be increased by inclusion of two or more targeting moieties which bind to different epitopes on the same target. The screening methods described herein are useful in identifying combinations of binding polypeptides which may have increased affinity when included in such multimeric constructs.

In a preferred embodiment, the screening methods described herein may be used to screen KDR and VEGF/KDR complex binding polypeptides identified by phage display, such as those described herein. As described in more detail in Example 5 *infra*, these methods may be used to assess the specific binding of KDR binding polypeptides to cells which express KDR or have been engineered to express KDR. Tetrameric complexes of biotinylated KDR binding polypeptides of the invention and neutravidin-HRP may be prepared and screened against cells transfected to express KDR as well as mock transfected cells (without any KDR).

As shown in Example 5, the assay may be used to identify KDR binding polypeptides which bind specifically to KDR-expressing cells (and do not bind to

cells that do not express KDR) even when the monodentate K_D of the polypeptide is on the order of 200nM-300nM. The assay may be used to screen homotetrameric constructs containing four copies of a single KDR binding polypeptide of the invention as well as heterotetrameric (constructs containing two or more different KDR binding polypeptides. The methods described herein are particularly useful for assessing combinations of KDR binding polypeptides for use in multimeric constructs, particularly constructs containing two or more KDR binding polypeptides which bind to different epitopes of KDR.

The assay may also be used to assess the effect of serum on the KDR binding polypeptides. Indeed, using the screening methods disclosed herein, KDR binding polypeptides, such as SEQ ID NOS:264, 294, and 356, were identified whose binding is not significantly affected by serum.

Modification or Optimization of KDR and VEGF/KDR Complex Binding Polypeptides:

As discussed, modification or optimization of KDR and VEGF/KDR complex binding polypeptides is within the scope of the invention and the modified or optimized polypeptides are included within the definition of "KDR and VEGF/KDR complex binding polypeptides". Specifically, a polypeptide sequence identified by phage display can be modified to optimize its potency, pharmacokinetic behavior, stability and/or other biological, physical and chemical properties.

Substitution of Amino Acid Residues

For example, one can make the following isosteric and/or conservative amino acid changes in the parent polypeptide sequence with the expectation that the resulting polypeptides would have a similar or improved profile of the properties described above:

Substitution of alkyl-substituted hydrophobic amino acids: Including alanine, leucine, isoleucine, valine, norleucine, S-2-aminobutyric acid, S-cyclohexylalanine or other simple alpha-amino acids substituted by an aliphatic side chain from C1-10 carbons including branched, cyclic and straight chain alkyl, alkenyl or alkynyl substitutions.

Substitution of aromatic-substituted hydrophobic amino acids: Including

phenylalanine, tryptophan, tyrosine, biphenylalanine, 1-naphthylalanine, 2-naphthylalanine, 2-benzothienylalanine, 3-benzothienylalanine, histidine, amino, alkylamino, dialkylamino, aza, halogenated (fluoro, chloro, bromo, or iodo) or alkoxy (from C1-C4)-substituted forms of the previous listed aromatic amino acids, illustrative examples of which are: 2-,3- or 4-aminophenylalanine, 2-,3- or 4-chlorophenylalanine, 2-,3- or 4-methylphenylalanine, 2-,3- or 4-methoxyphenylalanine, 5-amino-, 5-chloro-, 5-methyl- or 5-methoxytryptophan, 2'-, 3'-, or 4'-amino-, 2'-, 3'-, or 4'-chloro-, 2,3,or 4-biphenylalanine, 2',-3',-or 4'- methyl-2,3 or 4-biphenylalanine, and 2- or 3-pyridylalanine.

Substitution of amino acids containing basic functions: Including arginine, lysine, histidine, ornithine, 2,3-diaminopropionic acid, homoarginine, alkyl, alkenyl, or aryl-substituted (from C1-C10 branched, linear, or cyclic) derivatives of the previous amino acids, whether the substituent is on the heteroatoms (such as the alpha nitrogen, or the distal nitrogen or nitrogens, or on the alpha carbon, in the pro-R position for example. Compounds that serve as illustrative examples include: N-epsilon-isopropyl-lysine, 3-(4-tetrahydropyridyl)-glycine, 3-(4-tetrahydropyridyl)-alanine, N,N-gamma, gamma'-diethyl-homoarginine. Included also are compounds such as alpha methyl arginine, alpha methyl 2,3-diaminopropionic acid, alpha methyl histidine, alpha methyl ornithine where alkyl group occupies the pro-R position of the alpha carbon. Also included are the amides formed from alkyl, aromatic, heteroaromatic (where the heteroaromatic group has one or more nitrogens, oxygens or sulfur atoms singly or in combination) carboxylic acids or any of the many well-known activated derivatives such as acid chlorides, active esters, active azolides and related derivatives) and lysine, ornithine, or 2,3-diaminopropionic acid.

Substitution of acidic amino acids: Including aspartic acid, glutamic acid, homoglutamic acid, tyrosine, alkyl, aryl, arylalkyl, and heteroaryl sulfonamides of 2,4-diaminopriopionic acid, ornithine or lysine and tetrazole-substituted alkyl amino acids.

Substitution of side chain amide residues: Including asparagine, glutamine, and alkyl or aromatic substituted derivatives of asparagine or glutamine.

Substitution of hydroxyl containing amino acids: Including serine, threonine,

homoserine, 2,3-diaminopropionic acid, and alkyl or aromatic substituted derivatives of serine or threonine. It is also understood that the amino acids within each of the categories listed above may be substituted for another of the same group.

Substitution of Amide Bonds

Another type of modification within the scope of the patent is to substitute the amide bonds within the backbone of the polypeptide. For example, to reduce or eliminate undesired proteolysis, or other degradation pathways which diminish serum stability, resulting in reduced or abolished bioactivity, or to restrict or increase conformational flexibility, it is common to substitute amide bonds within the backbone of the peptides with functionality that mimics the existing conformation or alters the conformation in the manner desired. Such modifications may produce increased binding affinity or improved pharmacokinetic behavior. It is understood that those knowledgeable in the art of peptide synthesis can make the following amide bond-changes for any amide bond connecting two amino acids with the expectation that the resulting peptides could have the same or improved activity: insertion of alpha-N-methylamides or peptide amide backbone thioamides, removal of the carbonyl to produce the cognate secondary amines, replacement of one amino acid with an aza-aminoacid to produce semicarbazone derivatives, and use of E-olefins and substituted E-olefins as amide bond surrogates.

Introduction of D-Amino Acids

Another approach within the scope of the patent is the introduction of D-alanine, or another D-amino acid, distal or proximal to the labile peptide bond. In this case it is also understood to those skilled in the art that such D-amino acid substitutions can, and at times, must be made, with D-amino acids whose side chains are not conservative replacements for those of the L-amino acid being replaced. This is because of the difference in chirality and hence side-chain orientation, which may result in the accessing of a previously unexplored region of the binding site of the target which has moieties of different charge, hydrophobicity, steric requirements etc. than that serviced by the side chain of the replaced L-amino acid.

Modifications To Improve Pharmacokinetic or Pharmacodynamic Properties

It is also understood that use of the KDR or VEGF/KDR complex binding

polypeptide in a particular application may necessitate modifications of the peptide or formulations of the peptide to improve pharmacokinetic and pharmacodynamic behavior. It is expected that the properties of the peptide may be changed by attachment of moieties anticipated to bring about the desired physical or chemical properties. Such moieties may be appended to the peptide using acids or amines, via amide bonds or urea bonds, respectively, to the N- or C-terminus of the peptide, or to the pendant amino group of a suitably located lysine or lysine derivative, 2, 3-diaminopropionic acid, ornithine, or other amino acid in the peptide that possesses a pendant amine group or a pendant alkoxyamine or hydrazine group. The moieties introduced may be groups that are hydrophilic, basic, or nonpolar alkyl or aromatic groups depending on the peptide of interest and the extant requirements for modification of its properties.

Glycosylation of Amino Acid Residues

Yet another modification within the scope of the invention is to employ glycosylated amino acid residues (*e.g.* serine, threonine or asparagine residues), singly or in combination in the either the binding moiety (or moieties) or the linker moiety or both. Glycosylation, which may be carried out using standard conditions, can be used to enhance solubility, alter pharmacokinetics and pharmacodynamics or to enhance binding via a specific or non-specific interaction involving the glycosidic moiety. In another approach glycosylated amino acids such as O-(2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranosyl) serine or the analogous threonine derivative (either the D- or L- amino acids) can be incorporated into the peptide during manual or automated solid phase peptide synthesis, or in manual or automated solution phase peptide synthesis. Similarly D- or L-N^{γ}-(2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranosyl)-asparagine can be employed. The use of amino acids glycosylated on a pendant oxygen, nitrogen or sulfur function by the agency of suitably functionalized and activated carbohydrate moieties that can be employed in glycosylation is anticipated. Such carbohydrate functions could be monosaccharides, disaccharides or even larger assemblies of oligosaccharides (Kihlberg, Jan. (2000) Glycopeptide synthesis. In: Fmoc Solid Phase Peptide Synthesis – A Practical Approach (Chan, W.C. and White, P.D. Eds) Oxford University Press, New York, NY Chap. 8, pp195-213).

Also anticipated is the appendage of carbohydrate functions to amino acids

by means other than glycosylation via activation of a leaving group at the anomeric carbon. Linkage of the amino acid to the glycoside is not limited to the formation of a bond to the anomeric carbon of the carbohydrate function. Instead, linkage of the carbohydrate moiety to the amino acid could be through any suitable, sufficiently reactive oxygen atom, nitrogen atom, carbon atom or other pendant atom of the carbohydrate function via methods employed for formation of C-heteroatom, C-C or heteroatom-heteroatom (examples are S-S, O-N, N-N, P-O, P-N) bonds known in the art.

Formation of Salts

It is also within the scope of the invention to form different salts that may increase the water solubility or the ease of formulation of these peptides. These may include, but are not restricted to, N-methylglucamine (meglumine), acetate, oxalates, ascorbates, etc.

Structural Modifications which Retain Structural Features

Yet another modification within the scope of the invention is truncation of cyclic polypeptides. The cyclic nature of many polypeptides of the invention limits the conformational space available to the peptide sequence, particularly within the cycle. Therefore truncation of the peptide by one or more residues distal or even proximal to the cycle, at either the N-terminal or C-terminal region may provide truncated peptides with similar or improved biological activity. A unique sequence of amino acids, even as small as three amino acids, which is responsible for the binding activity, may be identified, as noted for RGD peptides (see, *e.g.*, Plow *et al.*, *Blood*, 70(1): 110-5 (1987); Oldberg *et al.*, *Journal of Biological Chemistry*, 263(36):19433-19436 (1988); Taub *et al.*, *Journal of Biological Chemistry*, 264(1):259-65 (1989); Andrieux *et al.*, *Journal of Biological Chemistry*, 264(16):9258-65 (1989); and US 5,773,412 and US 5,759,996, each of which is incorporated herein by reference.

It has also been shown in the literature that large peptide cycles can be substantially shortened, eliminating extraneous amino acids, but substantially including the critical binding residues. See, US 5,556,939, incorporated by reference herein.

The shortened cyclic peptides can be formed using disulfide bonds or amide

bonds of suitably located carboxylic acid groups and amino groups.

Furthermore, D-amino acids can be added to the peptide sequence to stabilize turn features (especially in the case of glycine). In another approach alpha, beta, gamma or delta dipeptide or turn mimics (such as α, β, γ, or δ turn mimics), some of which are shown in schematics 1, 2 and 3 as shown in FIG. 26, can be employed to mimic structural motifs and turn features in a peptide and simultaneously provide stability from proteolysis and enhance other properties such as, for example, conformational stability and solubility (structure 1: Hart *et al.*, *J. Org. Chem.*, 64, 2998-2999(1999); structure 2: Hanessian *et al.*, "Synthesis of a Versatile Peptidomimetic Scaffold" in *Methods in Molecular Medicine, Vol. 23: Peptidomimetics Protocols*, W.M. Kazmierski, Ed. (Humana Press Inc., Totowa, N.J., 1999), Chapter 10, pp. 161-174; structure 3: WO 01/16135.

Substitution of Disulfide Mimetics

Also within the scope of the invention is the substitution of disulfide mimetics for disulfide bonds within the KDR or VEGF/KDR complex binding peptides of the invention.

When disulfide-containing peptides are employed in generating ^{99m}Tc-based radiopharmaceuticals, a significant problem is the presence of the disulfide bond. The integrity of the disulfide bond is difficult to maintain during procedures designed to incorporate ^{99m}Tc via routes that are reliant upon the reduction of pertechnetate ion and subsequent incorporation of the reduced Tc species into substances bearing Tc-specific chelating groups. This is because the disulfide bond is rather easily reduced by the reducing agents commonly used in kits devised for one-step preparation of radiopharmaceuticals. Therefore, the ease with which the disulfide bond can be reduced during Tc chelation may require substitution with mimetics of the disulfide bonds. Accordingly, another modification within the scope of the invention is to substitute the disulfide moiety with mimetics utilizing the methods disclosed herein or known to those skilled in the art, while retaining the activity and other desired properties of the KDR-binding polypeptides of the invention:

1.) Oxime Linker

The oxime moiety has been employed as a linker by investigators in a

number of contexts. Of the most interest is the work by Mutter et. al. (Wahl and Mutter, *Tetrahedron Lett.*, 37:6861-6864 (1996)). The amino acids **4**, containing an aminoalcohol function, and **5**, containing an alkoxyamino function, are incorporated into the peptide chain, not necessarily at the end of the peptide chain (FIG. 27). After formation of the peptide the sidechain protecting groups are removed. The aldehyde group is unmasked and an oxime linkage is formed.

2.) Lanthionine Linker

Lanthionines are cyclic sulfides, wherein the disulfide linkage (S-S) is replaced by a carbon-sulfur (C-S) linkage. Thus, the lability to reduction is far lower. Lanthionines have been prepared by a number of methods since 1971.

Preparation of Lanthionines using Bromoacetylated Peptides

Lanthionines are readily prepared using known methods. See, for example, Robey *et al.*, *Anal. Biochem.*, 177:373-377 (1989); Inman *et al.*, *Bioconjugate Chem.*, 2:458-463 (1991); Ploinsky *et al.*, *Med. Chem.*, 35:4185-4194 (1992); Mayer *et al.*, "Peptides, Frontiers of Peptide Science", in Proceedings of the 15th American Peptide Symposium, Tam & Kaumaya (Eds.), June 14-19, 1995, Nashville, Tenn. (Klumer Academic Pub., Boston), pp. 291-292; Wakao *et al.*, Jpn. Kokai Tokyo Koho, JP 07300452 A2 (1995). Preparation of peptides using Boc automated peptide synthesis followed by coupling the peptide terminus with bromoacetic acid gives bromoacetylated peptides in good yield. Cleavage and deprotection of the peptides is accomplished using HF/anisole. If the peptide contains a cysteine group its reactivity can be controlled with low pH. If the pH of the medium is raised to 6-7 then either polymerization or cyclization of the peptide takes place. Polymerization is favored at high (100 mg/mL) concentration whereas cyclization is favored at lower concentrations (1 mg/mL), *e.g.*, **6** cyclizes to 7 (Scheme 1; FIG. 28).

Inman *et al.* demonstrated the use of N^{α}-(Boc)-N^{ε}-[N-(bromoacetyl)-β-alanyl]-L-lysine as a carrier of the bromoacetyl group that could be employed in Boc peptide synthesis thus allowing placement of a bromoacetyl bearing moiety anywhere in a sequence. In preliminary experiments they found that peptides with 4-6 amino acids separating the bromoacetyl-lysine derivative from a cysteine tend to cyclize, indicating the potential utility of this strategy.

Preparation of Lanthionines via Cysteine Thiol Addition to Acrylamides

Several variants of this strategy may be implemented. Resin-bound serine can be employed to prepare the lanthionine ring on resin either using a bromination-dehydrobromination-thiol addition sequence or by dehydration with disuccinimidyl carbonate followed by thiol addition. Ploinsky *et al.*, *M. J. Med. Chem.*, 35:4185-4194 (1992); Mayer *et al.*, "Peptides, Frontiers of Peptide Science", in Proceedings of the 15th American Peptide Symposium, Tam & Kaumaya (Eds.), June 14-19, 1995, Nashville, Tenn. (Klumer Academic Pub., Boston), pp. 291-292. Conjugate addition of thiols to acrylamides has also been amply demonstrated and a reference to the addition of 2-mercaptoethanol to acrylamide is provided. Wakao *et al.*, Jpn. Kokai Tokyo Koho, JP 07300452 A2 (1995).

3.) Diaryl Ether or Diarylamine Linkage: Diaryl Ether Linkage From Intramolecular Cyclization of Aryl Boronic Acids and Tyrosine

Recently the reaction of arylboronic acids with phenols, amines and heterocyclic amines in the presence of cupric acetate, in air, at ambient temperature, in dichloromethane using either pyridine or triethylamine as a base to provide unsymmetrical diaryl ethers and the related amines in good yields (as high as 98%) has been reported. See, Evans *et al.*, *Tetrahedron Lett.*, 39:2937-2940 (1998); Chan *et al.*, *Tetrahedron Lett.*, 39:2933-2936 (1998); Lam *et al.*, *Tetrahedron Lett.*, 39:2941-2944 (1998). In the case of N-protected tyrosine derivatives as the phenol component the yields were also as high as 98%. This demonstrates that amino acid amides (peptides) are expected to be stable to the transformation and that yields are high. Precedent for an intramolecular reaction exists in view of the facile intramolecular cyclizations of peptides to lactams, intramolecular biaryl ether formation based on the S_NAr reaction and the generality of intramolecular cyclization reactions under high dilution conditions or on resin, wherein the pseudo-dilution effect mimics high dilution conditions.

4.) Formation of Cyclic Peptides with a Thiazolidine Linkage via Intramolecular Reaction of Peptide Aldehydes with Cysteine Moieties

Another approach that may be employed involves intramolecular cyclization of suitably located vicinal amino mercaptan functions (usually derived from placement of a cysteine at a terminus of the linear sequence or tethered to the

sequence via a side-chain nitrogen of a lysine, for example) and aldehyde functions to provide thiazolidines which result in the formation of a bicyclic peptide, one ring of which is that formed by the residues in the main chain, and the second ring being the thiazolidine ring. Scheme 2 (FIG. 29) provides an example. The required aldehyde function can be generated by sodium metaperiodate cleavage of a suitably located vicinal aminoalcohol function, which can be present as an unprotected serine tethered to the chain by appendage to a side chain amino group of a lysine moiety. In some cases the required aldehyde function is generated by unmasking of a protected aldehyde derivative at the C-terminus or the N-terminus of the chain. An example of this strategy is found in: Botti *et al.*, *J. Am. Chem. Soc.*, *118*:10018-10034 (1996).

5.) Lactams Based on Intramolecular Cyclization of Pendant Amino Groups with Carboxyl Groups on Resin.

Macrocyclic peptides have been prepared by lactam formation by either head to tail or by pendant group cyclization. The basic strategy is to prepare a fully protected peptide wherein it is possible to remove selectively an amine protecting group and a carboxy protecting group. Orthogonal protecting schemes have been developed. Of those that have been developed the allyl, trityl and Dde methods have been employed most. See, Mellor *et al.*, "Synthesis of Modified Peptides", in Fmoc Solid Phase Synthesis: A Practical Approach, White and Chan (eds) (Oxford University Press, New York, 2000), Chapt. 6, pp. 169-178. The Dde approach is of interest because it utilizes similar protecting groups for both the carboxylic acid function (Dmab ester) and the amino group (Dde group). Both are removed with 2-10% hydrazine in DMF at ambient temperature. Alternatively, the Dde can be used for the amino group and the allyl group can be used for the carboxyl.

A lactam function, available by intramolecular coupling via standard peptide coupling reagents (such as HATU, PyBOP etc), could act as a surrogate for the disulfide bond. The Dde/Dmab approach is shown in Scheme 3a (FIG. 30).

Thus, a linear sequence containing, for example, the Dde-protected lysine and Dmab ester can be prepared on a Tentagel-based Rink amide resin at low load (~0.1-0.2 mmol/g). Deprotection of both functions with hydrazine is then followed by on-resin cyclization to give the desired products.

In the allyl approach, shown in Scheme 3b (FIG. 31), the pendant carboxyl

which is to undergo cyclization is protected as an allyl ester and the pendant amino group is protected as an alloc group. On resin, both are selectively unmasked by treatment with palladium tris-triphenylphosphine in the presence of N-methylmorpholine and acetic acid in DMF. Residual palladium salts are removed using sodium diethyldithiocarbamate in the presence of DIEA in DMF, followed by subsequent washings with DMF. The lactam ring is then formed employing HATU/HOAt in the presence of N-methylmorpholine. Other coupling agents can be employed as described above. The processing of the peptide is then carried out as described above to provide the desired peptide lactam.

Subsequently cleavage from resin and purification can also be carried out. For functionalization of the N-terminus of the peptide, it is understood that amino acids, such as trans-4-(iV-Dde)methylaminocyclohexane carboxylic acid, trans-4-(iV-Dde)methylaminobenzoic acid, or their alloc congeners can be employed. Yet another approach is to employ the safety catch method to intramolecular lactam formation during cleavage from the resin.

Thus, a linear sequence containing, for example, the Dde-protected lysine and Dmab ester may be prepared on a Tentagel-based Rink amide resin at low load (~0.1-0.2 mmol/g). Deprotection of both functions with hydrazine is then followed by on-resin cyclization to give the desired products. Subsequently cleavage from resin and purification may also be carried out. For functionalization of the N-terminus of the peptide it is understood that diamino acids such as trans-4-(iv-Dde)methylaminocyclohexane carboxylic acid or trans-4-(iv-Dde)methylamino benzoic acid would be required. An alternative scenario is to employ the safety catch method to intramolecular lactam formation during cleavage from the resin.

6.) Cyclic Peptides Based on Olefin Metathesis

The Grubbs reaction (Scheme 4, FIG. 32) involves the metathesis/cyclization of olefin bonds and is illustrated as shown below. See, Schuster *et al.*, *Angewandte. Chem. Int. Edn Engl.*, 36:2036-2056 (1997); Miller *et al.*, *J. Am. Chem. Soc.*, 118:9606-9614 (1996).

It is readily seen (FIG. 32) that if the starting material is a diolefin (**16**) that the resulting product will be cyclic compound **17**. The reaction has in fact been applied to creation of cycles from olefin-functionalized peptides. See, *e.g.*, Pernerstorfer *et al.*, *Chem. Commun.*, 20:1949-50 (1997); see, also, Covalent capture

and stabilization of cylindrical β-sheet peptide assemblies, Clark *et al.*, *Chem.Eur. J.*, 5(2):782-792 (1999); Highly efficient synthesis of covalently cross-linked peptide helices by ring-closing metathesis, Blackwell *et al.*, *Angew. Chem., Int. Ed.*, 37(23):3281-3284 (1998); Synthesis of novel cyclic protease inhibitors using Grubbs olefin metathesis, Ripka *et al.*, *Med. Chem. Lett.*, 8(4):357-360 (1998); Application of Ring-Closing Metathesis to the Synthesis of Rigidified Amino Acids and Peptides, Miller *et al.*, *J. Am. Chem. Soc.*, 118(40):9606-9614 (1996); Supramolecular Design by Covalent Capture, Design of a Peptide Cylinder via Hydrogen-Bond-Promoted Intermolecular Olefin Metathesis, Clark *et al.*, *J. Am. Chem. Soc.*, 117(49):12364-12365 (1995); Synthesis of Conformationally Restricted Amino Acids and Peptides Employing Olefin Metathesis, Miller *et al.*, *J. Am. Chem. Soc.*, 117(21):5855-5856 (1995). One can prepare either C-allylated amino acids or possibly N-allylated amino acids and employ them in this reaction in order to prepare carba-bridged cyclic peptides as surrogates for disulfide bond containing peptides.

One may also prepare novel compounds with olefinic groups. Functionalization of the tyrosine hydroxyl with an olefin-containing tether is one option. The lysine ε-amino group is another option with appendage of the olefin-containing unit as part of an acylating moiety, for example. If instead the lysine side chain amino group is alkylated with an olefin containing tether, it can still function as a point of attachment for a reporter as well. The use of 5-pentenoic acid as an acylating agent for the lysine, ornithine, or diaminopropionic side chain amino groups is another possibility. The length of the olefin-containing tether can also be varied in order to explore structure activity relationships.

Manipulation of Peptide Sequences

Other modifications within the scope of the invention include common manipulations of peptide sequences, which can be expected to yield peptides with similar or improved biological properties. These include amino acid translocations (swapping amino acids in the sequence), use of retroinverso peptides in place of the original sequence or a modified original sequence, peptoids and retro-inverso peptoid sequences. Structures wherein specific residues are peptoid instead of peptidic, which result in hybrid molecules, neither completely peptidic nor completely peptoid, are anticipated as well.

Linkers

Additional modifications within the scope of the invention include introduction of linkers or spacers between the targeting sequence of the KDR or VEGF/KDR complex binding peptide and the detectable label or therapeutic agent. Use of such linkers/spacers may improve the relevant properties of the binding peptide (*e.g.*, increase serum stability, etc.). These linkers may include, but are not restricted to, substituted or unsubstituted alkyl chains, polyethylene glycol derivatives, amino acid spacers, sugars, or aliphatic or aromatic spacers common in the art. Furthermore, linkers which are combinations of the moieties described above, can also be employed to confer special advantage to the properties of the peptide. Lipid molecules with linkers may be attached to allow formulation of ultrasound bubbles, liposomes or other aggregation based constructs. Such constructs could be employed as agents for targeting and delivery of a diagnostic reporter, a therapeutic agent (*e.g.*, a chemical "warhead" for therapy) or a combination of these.

Multimeric Constructs of KDR and VEGF/KDR Complex Binding Polypeptides

Constructs employing dimers, multimers or polymers of one or more VEGF or VEGF/KDR complex binding polypeptides of the invention are also contemplated. Indeed, there is ample literature evidence that the binding of low potency peptides or small molecules can be substantially increased by the formation of dimers and multimers. Thus, dimeric and multimeric constructs (both homogeneous and heterogeneous) are within the scope of the instant invention. Indeed, as discussed in more detail in the Examples, it is within the scope of the present invention to include multiple KDR or VEGF/KDR complex binding polypeptide sequences in a dimeric or multimeric construct. Moreover, as shown in Example 4 *infra*, these constructs may exhibit improved binding compared to a monomeric construct. The polypeptide sequences in the dimeric constructs may be attached at their N- or C- terminus or the N-epsilon nitrogen of a suitably placed lysine moiety (or another function bearing a selectively derivatizable group such as a pendant oxyamino or other nucleophilic group), or may be joined together via one or more linkers employing the appropriate attachment chemistry. This coupling

chemistry may include amide, urea, thiourea, oxime, or aminoacetylamide (from chloro- or bromoacetamide derivatives, but is not so limited. For example, any of the following methods may be utilized to prepare dimeric or multimeric constructs of KDR or VEGF/KDR complex binding polypeptides of the invention.

Method A

Fully protected KDR-binding peptides can be built up on Ellman-type safety catch resin using automated or manual Fmoc peptide synthesis protocols. Backes et al, *J. Am. Chem. Soc.*, 118(12):3055-56 (1996). Separately, using standard methods known in the art of peptide synthesis, a di-lysine derivative can be constructed on 2-chlorotrityl resin. See, for example, Fields et al, "Principles and Practice of Solid Phase Synthesis" in *Synthetic Peptides, A Users Guide*, Grant, Ed. (W.H. Freeman Co., New York, 1992), Chapt. 3, pp. 77-183; Barlos *et al.*, "Convergent Peptide Synthesis" in *Fmoc Solid Phase Peptide Synthesis*, Chan, W.C. and White, P.D., Eds. (Oxford University Press, New York, 2000), Chapt. 9, pp. 215-228. Liberation of this from the 2-chlorotrityl resin without removal of the side-chain protecting groups, activation of the carboxyl group and coupling to any amine-functionalized labeling group provides a di-lysine derivative whose protected pendant nitrogen atoms may be unmasked to give two free amino groups. The prior-mentioned safety-catch resin is activated and the desired N-deprotected labeling group-functionalized di-lysine derivative is added to the activated safety-catch resin. The pendant amino groups are acylated by the carboxy-terminus of the safety-catch resin-bound peptide which is now detached from the resin and an integral part of the di-lysine structure. An excess of the safety-catch resin-bound peptide can be employed to insure complete reaction of the amino groups of the di-lysine construct. Optimization of the ratio of the reacting partners in this scheme optimizes the yield. The protecting groups on the KDR-binding peptides are removed employing trifluoroacetic acid based cleavage protocols.

The synthesis of dimeric and multimeric constructs wherein two or more KDR-binding peptides are present in one construct is easily accomplished. Orthogonal protection schemes (such as an allyloxycarbonyl group on one nitrogen and an Fmoc group on the other, or employing the Fmoc group in conjunction with the iV-Dde protecting group on the other, for example) can be employed to distinguish the pendant nitrogen atoms of the di-lysine derivatives described above.

Unmasking of one of the amino groups, followed by reaction of the resulting product with an activated safety-catch resin-bound KDR-binding peptide as described above, provides a di-lysine construct having a single KDR-binding peptide attached. Removal of the second protecting group unmasks the remaining nitrogen. See, also, Mellor *et al.*, "Synthesis of Modified Peptides" in *Fmoc Solid Phase Peptide Synthesis*, Chan, W.C. and White, P.D., Eds. (Oxford University Press, New York, 2000), Chapt. 6, pp. 169-176. The resulting product may be reacted with a second safety-catch resin bearing another KDR-binding peptide to provide a fully-protected homodimeric construct, which after removal of protecting groups with trifluoroacetic acid, provides the desired material.

Method B

A KDR-binding peptide is assembled on a Rink-amide resin by automated or manual peptide coupling methods, usually employing Fmoc peptide synthesis protocols. The peptide may possess a C-terminus or N-terminus functionalized with a linker or a linker-labeling group construct that may possess an additional nucleophilic group such as the ε-amino group of a lysine moiety, for example. Cleavage of the protecting groups is accomplished employing trifluoroacetic acid with appropriate modifiers depending on the nature of the peptide. The fully deprotected peptide is then reacted with a large excess of a bifunctional electrophile such as the commercially available glutaric acid bis-N-hydroxysuccinimide ester (Tyger Scientific, Inc.). The resulting monoamidated, mono-N-hydroxysuccinimidyl ester of glutaric acid is then treated with an additional equivalent of the same peptide, or an equivalent of a different KDR-binding peptide. Purification of the resulting material by HPLC affords the desired homo-dimeric construct bearing a suitable labeling group.

Method C

A modular scheme can be employed to prepare dimeric or higher multimeric constructs bearing suitable labeling groups as defined above. In a simple illustration, fmoc-lysine(iV-Dde) Rink amide resin is treated with piperidine to remove the fmoc moiety. Then a labeling function, such as biotin, 5-carboxyfluorescein or N,N-Dimethyl-Gly-Ser(O-t-Bu)-Cys(Acm)-Gly-OH is coupled to the nitrogen atom. The resin is next treated with hydrazine to remove the iV-Dde group. After thorough washing, the resin is treated with cyanuric chloride and a hindered base such as diisopropylethylamine in a suitable solvent such as DMF, NMP or dichloromethane

to provide a monofunctionalized dichlorotriazine bound to the resin. Subsequent successive displacement of the remaining chlorine atoms by two equivalents of a KDR-binding peptide provides a resin-bound homo-dimeric labeling group-functionalized construct. Falorni *et al.*, *Tetrahedron Lett.*, 39(41):7607-7610 (1998); Johnson *et al.*, *Tetrahedron Lett.*, 54(16):4097-4106 (1998); Stankova *et al.*, *Mol. Diversity*, 2(1/2):75-80 (1996). The incoming peptides may be protected or unprotected as the situation warrants. Cleavage of protecting groups is accomplished employing trifluoroacetic acid-based deprotection reagents as described above, and the desired materials are purified by high performance liquid chromatography.

It is understood that in each of these methods lysine derivatives may be serially employed to increase the multiplicity of the multimers. The use of related, more rigid molecules bearing the requisite number of masked, or orthogonally protected nitrogen atoms to act as scaffolds to vary the distance between the KDR-binding peptides, to increase the rigidity of the construct (by constraining the motion and relative positions of the KDR-binding peptides relative to each other and the reporter) is entirely within the scope of methods A-C and all other methods described herein. The references cited above are incorporated by reference herein in their entirety.

Uses for KDR or VEGF/KDR Complex Binding Polypeptides

The KDR or VEGF/KDR complex binding moieties according to this invention will be extremely useful for detection and/or imaging of KDR or VEGF/KDR complex *in vitro* or *in vivo*, and particularly for detection and/or imaging of sites of angiogenesis, in which VEGF and KDR are intimately involved, as explained above. Any suitable method of assaying or imaging KDR or VEGF/KDR complex may be employed. The KDR and VEGF/KDR complex binding moieties of the invention also have utility in the treatment of a variety of disease states, including those associated with angiogenesis or those associated with a number of pathogens. The KDR and VEGF/KDR complex binding moieties of the invention may themselves be used as therapeutics or may be used to localize one or more therapeutic agents (*e.g.*, a chemotherapeutic, a radiotherapeutic, genetic material, etc.) to KDR expressing cells, including sites of angiogenesis.

In vitro:

For detection of KDR or VEGF/KDR complex in solution, a binding polypeptide according to the invention can be detectably labeled, *e.g.*, fluorescently labeled, enzymatically labeled, or labeled with a radioactive or paramagnetic metal, then contacted with the solution, and thereafter formation of a complex between the binding polypeptide and the KDR or VEGF/KDR complex target can be detected. As an example, a fluorescently labeled KDR or VEGF/KDR complex binding peptide may be used for *in vitro* KDR or VEGF/KDR complex detection assays, wherein the peptide is added to a solution to be tested for KDR or VEGF/KDR complex under conditions allowing binding to occur. The complex between the fluorescently labeled KDR or VEGF/KDR complex binding peptide and KDR or VEGF/KDR complex target can be detected and quantified by measuring the increased fluorescence polarization arising from the KDR or VEGF/KDR complex-bound peptide relative to that of the free peptide.

Alternatively, a sandwich-type "ELISA" assay may be used, wherein a KDR or VEGF/KDR complex binding polypeptide is immobilized on a solid support such as a plastic tube or well, then the solution suspected of containing KDR or VEGF/KDR complex target is contacted with the immobilized binding moiety, non-binding materials are washed away, and complexed polypeptide is detected using a suitable detection reagent, such as a monoclonal antibody recognizing KDR or VEGF/KDR complex. The monoclonal antibody is detectable by conventional means known in the art, including being detectably labeled, *e.g.*, radiolabeled, conjugated with an enzyme such as horseradish peroxidase and the like, or fluorescently labeled, etc.

For detection or purification of soluble KDR or VEGF/KDR complex in or from a solution, binding polypeptides of the invention can be immobilized on a solid substrate such as a chromatographic support or other matrix material, then the immobilized binder can be loaded or contacted with the solution under conditions suitable for formation of a binding polypeptide:KDR complex or binding polypeptide:VEGF/KDR complex. The non-binding portion of the solution can be removed and the complex may be detected, *e.g.*, using an anti-KDR or anti-VEGF/KDR complex antibody, or an anti-binding polypeptide antibody, or the KDR or VEGF/KDR complex target may be released from the binding moiety at appropriate elution conditions.

The biology of angiogenesis and the roles of VEGF and KDR in initiating and maintaining it have been investigated by many researchers and continues to be an active field for research and development. In furtherance of such research and development, a method of purifying bulk amounts of KDR or VEGF/KDR complex in pure form is desirable, and the binding polypeptides according to this invention are especially useful for that purpose, using the general purification methodology described above.

In vivo:

Diagnostic Imaging

A particularly preferred use for the polypeptides according to the present invention is for creating visually readable images of KDR expressing tissue, such as, for example, neoplastic tumors, which require angiogenesis for survival and metastasis, or other sites of angiogenic activity. The KDR and VEGF/KDR complex binding polypeptides disclosed herein may be converted to imaging reagents by conjugating the polypeptides with a label appropriate for diagnostic detection, optionally via a linker. Preferably, a peptide exhibiting much greater specificity for KDR or VEGF/KDR complex than for other serum proteins is conjugated or linked to a label appropriate for the detection methodology to be employed. For example, the KDR or VEGF/KDR complex binding polypeptide may be conjugated with or without a linker to a paramagnetic chelate suitable for magnetic resonance imaging (MRI), with a radiolabel suitable for x-ray, PET or scintigrapic imaging (including a chelator for a radioactive metal), with an ultrasound contrast agent (*e.g.*, a stabilized microbubble, a ultrasound contrast agent, a microsphere or what has been referred to as a gas filled "liposome") suitable for ultrasound detection, or with an optical imaging dye.

Suitable linkers can be substituted or unsubstituted alkyl chains, amino acid chains (*e.g.*, polyglycine), polyethylene glycols, polyamides, and other simple polymeric linkers known in the art.

In general, the technique of using a detectably labeled KDR or VEGF/KDR complex binding moiety is based on the premise that the label generates a signal that is detectable outside the patient's body. For example, when the detectably labeled KDR or VEGF/KDR complex binding moiety is administered to the patient in which it is desirable to detect, *e.g.*, angiogenesis, the high affinity of the KDR or VEGF/KDR complex binding moiety for KDR or VEGF/KDR complex causes the

binding moiety to bind to the site of angiogenesis and accumulate label at the site of angiogenesis. Sufficient time is allowed for the labeled binding moiety to localize at the site of angiogenesis. The signal generated by the labeled peptide is detected by a scanning device which will vary according to the type of label used, and the signal is then converted to an image of the site of angiogenesis.

In another embodiment, rather than directly labeling a KDR or VEGF/KDR complex binding polypeptide with a detectable label or radiotherapeutic construct, the peptide(s) of the invention can be conjugated with for example, avidin, biotin, or an antibody or antibody fragment that will bind the detectable label or radiotherapeutic. For example, one or more KDR-binding peptides can be conjugated to streptavidin (potentially generating multivalent binding) for *in vivo* binding to KDR-expressing cells. After the unbound targeting construct has cleared from the body, a biotinylated detectable label or radiotherapeutic construct (*e.g.*, a chelate molecule complexed with a radioactive metal) can be infused which will rapidly concentrate at the site where the targeting construct is bound. This approach in some situations can reduce the time required after administering the detectable label until imaging can take place. It can also increase signal to noise ratio in the target site, and decrease the dose of the detectable label or radiotherapeutic construct required. This is particularly useful when a radioactive label or radiotherapeutic is used as the dose of radiation that is delivered to normal but radiation-sensitive sites in the body, such as bone-marrow, kidneys, and liver is decreased. This approach, sometimes referred to as pre-targeting or two-step, or three-step approaches was reviewed by S.F. Rosebrough in *Q. J. Nucl. Med.*, 40:234-251 (1996), which is incorporated by reference herein.

A. Magnetic Resonance Imaging

The KDR or VEGF/KDR complex binding moieties of the present invention can advantageously be conjugated with one or more paramagnetic metal chelates in order to form a contrast agent for use in MRI. Preferred paramagnetic metal ions have atomic numbers 21-29, 42, 44, or 57-83. This includes ions of the transition metal or lanthanide series which have one, and more preferably five or more, unpaired electrons and a magnetic moment of at least 1.7 Bohr magneton. Preferred paramagnetic metals include, but are not limited to, chromium (III), manganese (II), manganese (III), iron (II), iron (III), cobalt (II), nickel (II), copper (II), praseodymium (III), neodymium (III), samarium (III), gadolinium (III), terbium (III), dysprosium

(III), holmium (III), erbium (III), europium (III) and ytterbium (III), chromium (III), iron (III), and gadolinium (III). The trivalent cation, Gd^{3+}, is particularly preferred for MRI contrast agents, due to its high relaxivity and low toxicity, with the further advantage that it exists in only one biologically accessible oxidation state, which minimizes undesired metabolysis of the metal by a patient. Another useful metal is Cr^{3+}, which is relatively inexpensive. Gd(III) chelates have been used for clinical and radiologic MR applications since 1988, and approximately 30% of MR exams currently employ a gadolinium-based contrast agent. Additionally, heteromultimers of the present invention also can be conjugated with one or more superparamagnetic particles.

The practitioner will select a metal according to dose required to detect angiogenesis and considering other factors such as toxicity of the metal to the subject (Tweedle *et al.*, *Magnetic Resonance Imaging (2nd ed.)*, vol. 1, Partain *et al.*, Eds. (W.B. Saunders Co. 1988), pp. 796-797). Generally, the desired dose for an individual metal will be proportional to its relaxivity, modified by the biodistribution, pharmacokinetics and metabolism of the metal.

The paramagnetic metal chelator(s) is a molecule having one or more polar groups that act as a ligand for, and complex with, a paramagnetic metal. Suitable chelators are known in the art and include acids with methylene phosphonic acid groups, methylene carbohydroxamine acid groups, carboxyethylidene groups, or carboxymethylene groups. Examples of chelators include, but are not limited to, diethylenetriaminepentaacetic acid (DTPA), 1,4,7,10-tetraazacyclo-tetradecane-1,4,7,10-tetraacetic acid (DOTA), 1-substituted 1,4,7,-tricarboxymethyl-1,4,7,10-teraazacyclododecane (DO3A), ethylenediaminetetraacetic acid (EDTA), and 1,4,8,11-tetra-azacyclotetradecane-1,4,8,11-tetraacetic acid (TETA). Additional chelating ligands are ethylene bis-(2-hydroxy-phenylglycine) (EHPG), and derivatives thereof, including 5-Cl-EHPG, 5Br-EHPG, 5-Me-EHPG, 5t-Bu-EHPG, and 5sec-Bu-EHPG; benzodiethylenetriamine pentaacetic acid (benzo-DTPA) and derivatives thereof, including dibenzo-DTPA, phenyl-DTPA, diphenyl-DTPA, benzyl-DTPA, and dibenzyl DTPA; bis-2 (hydroxybenzyl)-ethylene-diaminediacetic acid (HBED) and derivatives thereof; the class of macrocyclic compounds which contain at least 3 carbon atoms, more preferably at least 6, and at least two heteroatoms (O and/or N), which macrocyclic compounds can consist of one ring, or two or three rings joined

together at the hetero ring elements, *e.g.*, benzo-DOTA, dibenzo-DOTA, and benzo-NOTA, where NOTA is 1,4,7-triazacyclononane N,N',N''-triacetic acid, benzo-TETA, benzo-DOTMA, where DOTMA is 1,4,7,10-tetraazacyclotetradecane-1,4,7, 10-tetra(methyl tetraacetic acid), and benzo-TETMA, where TETMA is 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-(methyl tetraacetic acid); derivatives of 1,3-propylene-diaminetetraacetic acid (PDTA) and triethylenetetraaminehexaacetic acid (TTHA); derivatives of 1,5,10-N,N',N''-tris(2,3-dihydroxybenzoyl)-tricatecholate (LICAM); and 1,3,5-N,N',N''-tris(2,3-dihydroxybenzoyl) aminomethylbenzene (MECAM). A preferred chelator for use in the present invention is DTPA, and the use of DO3A is particularly preferred. Examples of representative chelators and chelating groups contemplated by the present invention are described in WO 98/18496, WO 86/06605, WO 91/03200, WO 95/28179, WO 96/23526, WO 97/36619, PCT/US98/01473, PCT/US98/20182, and US 4,899,755, US 5,474,756, US 5,846,519 and US 6,143,274, all of which are hereby incorporated by reference.

In accordance with the present invention, the chelator of the MRI contrast agent is coupled to the KDR or VEGF/KDR complex binding polypeptide. The positioning of the chelate(s) should be selected so as not to interfere with the binding affinity or specificity of the KDR or VEGF/KDR complex binding polypeptide. Preferably, the chelate(s) will be appended either to the N-terminus or the C-terminus, however the chelate(s) may also be attached anywhere within the sequence. In preferred embodiments, a chelator having a free central carboxylic acid group (*e.g.*, DTPA-Asp(β-COOH)-)OtBu) makes it easy to attach at the N-terminus of the peptide by formation of an amide bond. The chelate(s) could also be attached at the C-terminus with the aid of a linker. Alternatively, isothiocyanate conjugation chemistry could be employed as a way of linking the appropriate isothiocyanate group bearing DTPA to a free amino group anywhere within the peptide sequence.

In general, the KDR or VEGF/KDR complex binding moiety can be bound directly or covalently to the metal chelator (or other detectable label), or it may be coupled or conjugated to the metal chelator using a linker, which may be, without limitation, amide, urea, acetal, ketal, double ester, carbonyl, carbamate, thiourea, sulfone, thioester, ester, ether, disulfide, lactone, imine, phosphoryl, or phosphodiester linkages; substituted or unsubstituted saturated or unsaturated alkyl

chains; linear, branched, or cyclic amino acid chains or a single amino acid or different amino acids (*e.g.*, extensions of the N- or C- terminus of the KDR or VEGF/KDR complex binding moiety); derivatized or underivatized polyethylene glycol, polyoxyethylene, or polyvinylpyridine chains; substituted or unsubstituted polyamide chains; derivatized or underivatized polyamine, polyester, polyethylenimine, polyacrylate, poly(vinyl alcohol), polyglycerol, or oligosaccharide (*e.g.*, dextran) chains; alternating block copolymers; malonic, succinic, glutaric, adipic and pimelic acids; caproic acid; simple diamines and dialcohols; any of the other linkers disclosed herein; or any other simple polymeric linkers known in the art (see, *e.g.*, WO 98/18497, WO 98/18496). Preferably the molecular weight of the linker can be tightly controlled. The molecular weights can range in size from less than 100 to greater than 1000. Preferably the molecular weight of the linker is less than 100. In addition, it may be desirable to utilize a linker that is biodegradable *in vivo* to provide efficient routes of excretion for the imaging reagents of the present invention. Depending on their location within the linker, such biodegradable functionalities can include ester, double ester, amide, phosphoester, ether, acetal, and ketal functionalities.

In general, known methods can be used to couple the metal chelate(s) and the KDR or VEGF/KDR complex binding moiety using such linkers. See, *e.g.*, WO 95/28967, WO 98/18496, WO 98/18497 and discussion therein. The KDR or VEGF/KDR complex binding moiety can be linked through its N- or C-terminus via an amide bond, for example, to a metal coordinating backbone nitrogen of a metal chelate or to an acetate arm of the metal chelate itself. The present invention contemplates linking of the chelate on any position, provided the metal chelate retains the ability to bind the metal tightly in order to minimize toxicity. Similarly, the KDR or VEGF/KDR complex binding moiety may be modified or elongated in order to generate a locus for attachment to a metal chelate, provided such modification or elongation does not eliminate its ability to bind KDR or VEGF/KDR complex.

MRI contrast reagents prepared according to the disclosures herein may be used in the same manner as conventional MRI contrast reagents. When imaging a site of angiogenesis, certain MR techniques and pulse sequences may be preferred to enhance the contrast of the site to the background blood and tissues. These techniques include (but are not limited to), for example, black blood angiography

sequences that seek to make blood dark, such as fast spin echo sequences (see, *e.g.*, Alexander *et al.*, *Magnetic Resonance in Medicine*, 40(2): 298-310 (1998)) and flow-spoiled gradient echo sequences (see, *e.g.*, Edelman *et al.*, *Radiology*, 177(1): 45-50 (1990)). These methods also include flow independent techniques that enhance the difference in contrast, such as inversion-recovery prepared or saturation-recovery prepared sequences that will increase the contrast between angiogenic tumor and background tissues. Finally, magnetization transfer preparations may also improve contrast with these agents (see, *e.g.*, Goodrich *et al.*, *Investigative Radiology*, 31(6): 323-32 (1996)).

The labeled reagent is administered to the patient in the form of an injectable composition. The method of administering the MRI contrast agent is preferably parenterally, meaning intravenously, intraarterially, intrathecally, interstitially, or intracavitarilly. For imaging active angiogenesis, intravenous or intraarterial administration is preferred. For MRI, it is contemplated that the subject will receive a dosage of contrast agent sufficient to enhance the MR signal at the site of angiogenesis at least 10%. After injection with the KDR or VEGF/KDR complex binding moiety-containing MRI reagent, the patient is scanned in the MRI machine to determine the location of any sites of angiogenesis. In therapeutic settings, upon angiogenesis (*e.g.*, tumor) localization, a tumorcidal agent or anti-angiogenic agent (*e.g.*, inhibitors of VEGF) can be immediately administered, if necessary, and the patient can be subsequently scanned to visualize tumor regression or arrest of angiogenesis.

B. Ultrasound Imaging

When ultrasound is transmitted through a substance, the acoustic properties of the substance will depend upon the velocity of the transmissions and the density of the substance. Changes in the acoustic properties will be most prominent at the interface of different substances (solids, liquids, gases). Ultrasound contrast agents are intense sound wave reflectors because of the acoustic differences between the agent and the surrounding tissue. Gas containing or gas generating ultrasound contrast agents are particularly useful because of the acoustic difference between liquid (*e.g.*, blood) and the gas-containing or gas generating ultrasound contrast agent. Because of their size, ultrasound contrast agents comprising microbubbles, ultrasound contrast agents, and the like may remain for a longer time in the blood stream after injection than other detectable moieties; a targeted KDR or VEGF/KDR

complex-specific ultrasound agent therefore may demonstrate superior imaging of sites of angiogenesis.

In this aspect of the invention, the KDR or VEGF/KDR complex binding moiety may be linked to a material which is useful for ultrasound imaging. For example, the KDR or VEGF/KDR complex binding polypeptides may be linked to materials employed to form vesicles (*e.g.*, microbubbles, ultrasound contrast agents, microspheres, etc.), or emulsions containing a liquid or gas which functions as the detectable label (*e.g.*, an echogenic gas or material capable of generating an echogenic gas). Materials for the preparation of such vesicles include surfactants, lipids, sphingolipids, oligolipids, phospholipids, proteins, polypeptides, carbohydrates, and synthetic or natural polymeric materials. See, *e.g.*, WO 98/53857, WO 98/18498, WO 98/18495, WO 98/18497, WO 98/18496, and WO 98/18501, incorporated herein by reference in their entirety.

For contrast agents comprising suspensions of stabilized microbubbles (a preferred embodiment), phospholipids, and particularly saturated phospholipids are preferred. The preferred gas-filled microbubbles of the invention can be prepared by means known in the art, such as, for example, by a method described in any one of the following patents: EP 554213, US 5,413,774, US 5,578,292, EP 744962, EP 682530, US 5,556,610, US 5,846,518, US 6,183,725, EP 474833, US 5,271,928, US 5,380,519, US 5,531,980, US 5,567,414, US 5,658,551, US 5,643,553, US 5,911,972, US 6,110,443, US 6,136,293, EP 619743, US 5,445,813, US 5,597,549, US 5,686,060, US 6,187,288, and US 5,908,610, which are incorporated by reference herein in their entirety. In a preferred embodiment, at least one of the phospholipid moieties has the structure 18 or 19 (FIG. 33) and described in US 5,686,060, which is herein incorporated by reference.

Examples of suitable phospholipids include esters of glycerol with one or two molecules of fatty acids (the same or different)and phosphoric acid, wherein the phosphoric acid residue is in turn bonded to a hydrophilic group, such as choline, serine, inositol, glycerol, ethanolamine, and the like groups. Fatty acids present in the phospholipids are in general long chain aliphatic acids, typically containing from 12 to 24 carbon atoms, preferably from 14 to 22, that may be saturated or may contain one or more unsaturations. Examples of suitable fatty acids are lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, oleic acid,

linoleic acid, and linolenic acid. Mono esters of phospholipid are also known in the art as the "lyso" forms of the phospholipids.

Further examples of phospholipids are phosphatidic acids, i.e. the diesters of glycerol-phosphoric acid with fatty acids, sphingomyelins, i.e. those phosphatidylcholine analogs where the residue of glycerol diester with fatty acids is replaced by a ceramide chain, cardiolipins, i.e. the esters of 1,3-diphosphatidylglycerol with a fatty acid, gangliosides, cerebrosides, etc.

As used herein, the term phospholipids includes either naturally occurring, semisynthetic or synthetically prepared products that can be employed either singularly or as mixtures.

Examples of naturally occurring phospholipids are natural lecithins (phosphatidylcholine (PC) derivatives) such as, typically, soya bean or egg yolk lecithins.

Examples of semisynthetic phospholipids are the partially or fully hydrogenated derivatives of the naturally occurring lecithins.

Examples of synthetic phospholipids are e.g., dilauryloyl-phosphatidylcholine ("DLPC"), dimyristoylphosphatidylcholine ("DMPC"), dipalmitoyl-phosphatidylcholine ("DPPC"), diarachidoylphosphatidylcholine ("DAPC"), distearoyl-phosphatidylcholine ("DSPC"), 1-myristoyl-2-palmitoylphosphatidylcholine ("MPPC"), 1-palmitoyl-2-myristoylphosphatidylcholine ("PMPC"), 1-palmitoyl-2-stearoylphosphatid-ylcholine ("PSPC"), 1-stearoyl-2-palmitoyl-phosphatidylcholine ("SPPC"), dioleoylphosphatidylycholine ("DOPC"), 1,2 Distearoyl-sn-glycero-3-Ethylphosphocholine (Ethyl-DSPC), dilauryloyl-phosphatidylglycerol ("DLPG") and its alkali metal salts, diarachidoylphosphatidylglycerol ("DAPG") and its alkali metal salts, dimyristoylphosphatidylglycerol ("DMPG") and its alkali metal salts, dipalmitoyl-phosphatidylglycerol ("DPPG") and its alkali metal salts, distearolyphosphatidylglycerol ("DSPG") and its alkali metal salts, dioleoylphosphatidylglycerol ("DOPG") and its alkali metal salts, dimyristoyl phosphatidic acid ("DMPA") and its alkali metal salts, dipalmitoyl phosphatidic acid ("DPPA") and its alkali metal salts, distearoyl phosphatidic acid ("DSPA"), diarachidoyl phosphatidic acid ("DAPA") and its alkali metal salts, dimyristoyl phosphatidyl-ethanolamine ("DMPE"), dipalmitoyl phosphatidylethanolamine ("DPPE"), distearoyl phosphatidyl-ethanolamine ("DSPE"), dimyristoyl

phosphatidylserine ("DMPS"), diarachidoyl phosphatidylserine ("DAPS"), dipalmitoyl phosphatidylserine ("DPPS"), distearoylphosphatidylserine ("DSPS"), dioleoylphosphatidylserine ("DOPS"), dipalmitoyl sphingomyelin ("DPSP"), and distearoyl sphingomyelin ("DSSP").

Other preferred phospholipids include dipalmitoylphosphatidylcholine, dipalmitoylphosphatidic acid and dipalmitoylphosphatidylserine. The compositions also may contain PEG-4000 and/or palmitic acid. Any of the gases disclosed herein or known to the skilled artisan may be employed; however, inert gases, such as SF_6 or fluorocarbons like CF_4, C_3F_8 and C_4F_{10}, are preferred.

The preferred microbubble suspensions of the present invention may be prepared from phospholipids using known processes such as a freeze-drying or spray-drying solutions of the crude phospholipids in a suitable solvent or using the processes set forth in EP 554213; US 5,413,774; US 5,578,292; EP 744962; EP 682530; US 5,556,610; US 5,846,518; US 6,183,725; EP 474833; US 5,271,928; US 5,380,519; US 5,531,980; US 5,567,414; US 5,658,551; US 5,643,553; US 5,911,972; US 6,110,443; US 6,136,293; EP 619743; US 5,445,813; US 5,597,549; US 5,686,060; US 6,187,288; and US 5,908,610, which are incorporated by reference herein in their entirety. Most preferably, the phospholipids are dissolved in an organic solvent and the solution is dried without going through a liposome formation stage. This can be done by dissolving the phospholipids in a suitable organic solvent together with a hydrophilic stabilizer substance or a compound soluble both in the organic solvent and water and freeze-drying or spray-drying the solution. In this embodiment the criteria used for selection of the hydrophilic stabilizer is its solubility in the organic solvent of choice. Examples of hydrophilic stabilizer compounds soluble in water and the organic solvent are, *e.g.*, a polymer, like polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), polyethylene glycol (PEG), etc., malic acid, glycolic acid, maltol, and the like. Such hydrophilic compounds also aid in homogenizing the microbubbles size distribution and enhance stability under storage. Any suitable organic solvent may be used as long as its boiling point is sufficiently low and its melting point is sufficiently high to facilitate subsequent drying. Typical organic solvents include, for example, dioxane, cyclohexanol, tertiary butanol, tetrachlorodifluoro ethylene ($C_2Cl_4F_2$) or 2-methyl-2-butanol. 2-methyl-2-butanol and $C_2Cl_4F_2$ are preferred.

Prior to formation of the suspension of microbubbles by dispersion in an

aqueous carrier, the freeze dried or spray dried phospholipid powders are contacted with air or another gas. When contacted with the aqueous carrier the powdered phospholipids whose structure has been disrupted will form lamellarized or laminarized segments that will stabilize the microbubbles of the gas dispersed therein. This method permits production of suspensions of microbubbles which are stable even when stored for prolonged periods and are obtained by simple dissolution of the dried laminarized phospholipids (which have been stored under a desired gas) without shaking or any violent agitation.

Alternatively, microbubbles can be prepared by suspending a gas into an aqueous solution at high agitation speed, as disclosed e.g. in WO 97/29783. A further process for preparing microbubbles is disclosed in co-pending European patent application no. 03002373, herein incorporated by reference, which comprises preparing an emulsion of an organic solvent in an aqueous medium in the presence of a phospholipid and subsequently lyophilizing said emulsion, after optional washing and/or filtration steps.

Additives known to those of ordinary skill in the art can be included in the suspensions of stabilized microbubbles. For instance, non-film forming surfactants, including polyoxypropylene glycol and polyoxyethylene glycol and similar compounds, as well as various copolymers thereof; fatty acids such as myristic acid, palmitic acid, stearic acid, arachidic acid or their derivatives, ergosterol, phytosterol, sitosterol, lanosterol, tocopherol, propyl gallate, ascorbyl palmitate and butylated hydroxytoluene may be added. The amount of these non-film forming surfactants is usually up to 50% by weight of the total amount of surfactants but preferably between 0 and 30%.

Other gas containing suspensions include those disclosed in, for example, US 5,798,091, WO 97/29783, also EP 881 915, incorporated herein by reference in their entirety. These agents may be prepared as described in US 5,798,091 or WO97/29783.

Another preferred ultrasound contrast agent comprises ultrasound contrast agents. The term "microballoon" refers to gas filled bodies with a material boundary or envelope. More on microballoon formulations and methods of preparation may be found in EP 324 938 (US 4,844,882); US 5,711,933; US 5,840,275; US 5,863,520; US 6,123,922; US 6,200,548; US 4,900,540; US 5,123,414; US 5,230,882; US 5,469,854; US 5,585,112; US 4,718,433; US 4,774,958; WO

95/01187; US 5,529,766; US 5,536,490; and US 5,990,263, the contents of which are incorporated herein by reference.

The preferred microballoons have an envelope including a biodegradable physiologically compatible polymer or, a biodegradable solid lipid. The polymers useful for the preparation of the microballoons of the present invention can be selected from the biodegradable physiologically compatible polymers, such as any of those described in any of the following patents: EP 458745, US 5,711,933, US 5,840,275, EP 554213, US 5,413,774 and US 5,578,292, the entire contents of which are incorporated herein by reference. In particular, the polymer can be selected from biodegradable physiologically compatible polymers, such as polysaccharides of low water solubility, polylactides and polyglycolides and their copolymers, copolymers of lactides and lactones such as ε-caprolactone, γ-valerolactone and polypeptides. Other suitable polymers include poly(ortho)esters (*see e.g.*, US 4,093,709; US 4,131,648; US 4,138,344; US 4,180,646); polylactic and polyglycolic acid and their copolymers, for instance DEXON (see J. Heller, Biomaterials 1 (1980), 51; poly(DL-lactide-co- ε-caprolactone), poly(DL-lactide-co- γ -valerolactone), poly(DL-lactide-co- γ-butyrolactone), polyalkylcyanoacrylates; polyamides, polyhydroxybutyrate; polydioxanone; poly-ß-aminoketones (A. S. Angeloni, P. Ferruti, M. Tramontini and M. Casolaro, The Mannich bases in polymer synthesis: 3. Reduction of poly(beta-aminoketone)s to poly(gamma-aminoalcohol)s and their N-alkylation to poly(gamma-hydroxyquaternary ammonium salt)s, Polymer 23, pp 1693-1697, 1982.); polyphosphazenes (Allcock, Harry R. Polyphosphazenes: new polymers with inorganic backbone atoms (Science 193(4259), 1214-19 (1976)) and polyanhydrides. The microballoons of the present invention can also be prepared according to the methods of WO-A-96/15815, incorporated herein by reference, where the microballoons are made from a biodegradable membrane comprising biodegradable lipids, preferably selected from mono- di-, tri-glycerides, fatty acids, sterols, waxes and mixtures thereof. Preferred lipids are di- or tri-glycerides, *e.g.*, di- or tri-myristin, -palmityn or -stearin, in particular tripalmitin or tristearin. The microballoons may employ any of the gases disclosed herein of known to the skilled artisan; however, inert gases such as fluorinated gases are preferred. The microballoons may be suspended in a pharmaceutically acceptable liquid carrier with optional additives known to those of ordinary skill in the art and stabilizers.

Other gas-containing contrast agent formulations include microparticles

(especially aggregates of microparticles) having gas contained therein or otherwise associated therewith (for example being adsorbed on the surface thereof and/or contained within voids, cavities or pores therein). Methods for the preparation of these agents are as described in EP 0122624; EP 0123235; EP 0365467; US 5,558,857; US 5,607,661; US 5,637,289; US 5,558,856; US 5,137,928; WO 95/21631 or WO 93/13809, incorporated herein by reference in their entirety.

Any of these ultrasound compositions should also be, as far as possible, isotonic with blood. Hence, before injection, small amounts of isotonic agents may be added to any of above ultrasound contrast agent suspensions. The isotonic agents are physiological solutions commonly used in medicine and they comprise aqueous saline solution (0.9% NaCl), 2.6% glycerol solution, 5% dextrose solution, etc. Additionally, the ultrasound compositions may include standard pharmaceutically acceptable additives, including, for example, emulsifying agents, viscosity modifiers, cryoprotectants, lyoprotectants, bulking agents etc.

Any biocompatible gas may be used in the ultrasound contrast agents useful in the invention. The term "gas" as used herein includes any substances (including mixtures) substantially in gaseous form at the normal human body temperature. The gas may thus include, for example, air, nitrogen, oxygen, CO_2, argon, xenon or krypton, fluorinated gases (including for example, perfluorocarbons, SF_6, SeF_6) a low molecular weight hydrocarbon (*e.g.*, containing from 1 to 7 carbon atoms), for example, an alkane such as methane, ethane, a propane, a butane or a pentane, a cycloalkane such as cyclopropane, cyclobutane or cyclopentene, an alkene such as ethylene, propene, propadiene or a butene, or an alkyne such as acetylene or propyne and/or mixtures thereof. However, fluorinated gases are preferred. Fluorinated gases include materials which contain at least one fluorine atom such as SF_6, freons (organic compounds containing one or more carbon atoms and fluorine, *i.e.*, CF_4, C_2F_6, C_3F_8, C_4F_8,C_4F_{10},$CBrF_3$, CCI_2F_2,C_2CIF_5, and $CBrClF_2$) and perfluorocarbons. The term perfluorocarbon refers to compounds containing only carbon and fluorine atoms and includes, in particular, saturated, unsaturated, and cyclic perfluorocarbons. The saturated perfluorocarbons, which are usually preferred, have the formula C_nF_{n+2}, where n is from 1 to 12, preferably from 2 to 10, most preferably from 3 to 8 and even more preferably from 3 to 6. Suitable perfluorocarbons include, for example, CF_4, C_2F_6, C_3F_8 C_4F_8, C_4F_{10}, C_5F_{12}, C_6F_{12}, C_7F_{14}, C_8F_{18}, and C_9F_{20}. Most preferably the gas or gas mixture comprises SF_6 or a perfluorocarbon

selected from the group consisting of C_3F_8 C_4F_8, C_4F_{10}, C_5F_{12}, C_6F_{12}, C_7F_{14}, C_8F_{18}, with C_4F_{10} being particularly preferred. See also WO 97/29783, WO 98/53857, WO 98/18498, WO 98/18495, WO 98/18496, WO 98/18497, WO 98/18501, WO 98/05364, WO 98/17324.

In certain circumstances it may be desirable to include a precursor to a gaseous substance (*e.g.*, a material that is capable of being converted to a gas *in vivo*, often referred to as a "gas precursor"). Preferably the gas precursor and the gas it produces are physiologically acceptable. The gas precursor may be pH-activated, photo-activated, temperature activated, etc. For example, certain perfluorocarbons may be used as temperature activated gas precursors. These perfluorocarbons, such as perfluoropentane, have a liquid/gas phase transition temperature above room temperature (or the temperature at which the agents are produced and/or stored) but below body temperature; thus they undergo a phase shift and are converted to a gas within the human body.

As discussed, the gas can comprise a mixture of gases. The following combinations are particularly preferred gas mixtures: a mixture of gases (A) and (B) in which, at least one of the gases (B), present in an amount of between 0.5 - 41% by vol., has a molecular weight greater than 80 daltons and is a fluorinated gas and (A) is selected from the group consisting of air, oxygen, nitrogen, carbon dioxide and mixtures thereof, the balance of the mixture being gas A.

Since ultrasound vesicles may be larger than the other detectable labels described herein, they may be linked or conjugated to a plurality of KDR or VEGF/KDR complex binding polypeptides in order to increase the targeting efficiency of the agent. Attachment to the ultrasound contrast agents described above (or known to those skilled in the art) may be via direct covalent bond between the KDR or VEGF/KDR complex binding polypeptide and the material used to make the vesicle or via a linker, as described previously. For example, see WO 98/53857 generally for a description of the attachment of a peptide to a bifunctional PEG linker, which is then reacted with a liposome composition. See also, Lanza *et al.*, *Ultrasound in Med. & Bio.*, 23(6):863-870 (1997).

A number of methods may be used to prepare suspensions of microbubbles conjugated to KDR or VEGF/KDR complex binding polypeptides. For example, one may prepare maleimide-derivatized microbubbles by incorporating 5 % (w/w) of N-MPB-PE (1, 2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-4-(p-maleimido-

phenyl butyramide), (Avanti Polar-Lipids, Inc) in the phospholipid formulation. Then, solutions of mercaptoacetylated KDR-binding peptides (10 mg/ml in DMF), which have been incubated in deacetylation solution (50 mM sodium phosphate, 25 mM EDTA, 0.5 M hydroxylamine.HCl, pH 7.5) are added to the maleimide-activated microbubble suspension. After incubation in the dark, under gentle agitation, the peptide conjugated microbubbles may be purified by centrifugation.

Compounds that can be used for derivatization of microbubbles typically include the following components: (a) a hydrophobic portion, compatible with the material forming the envelope of the microbubble or of the microballoon, in order to allow an effective incorporation of the compound in the envelope of the vesicel; said portion is represented typically by a lipid moiety (dipalmitin, distearoyl); and (b) a spacer (typically PEGs of different molecular weights), which may be optional in some cases (for example, microbubbles may for instance present difficulties to be freeze dried if the spacer is too long) or preferred in some others (*e.g.*, peptides may be less active when conjugated to a microballoon with short spacers); and (c) a reactive group capable of reacting with a corresponding reacting moiety on the peptide to be conjugated (*e.g.*, maleimido with the –SH group of cysteine).

Alternatively, KDR-binding polypeptide conjugated microbubbles may be prepared using biotin/avidin. For example, avidin–conjugated microbubbles may be prepared using a maleimide-activated phospholipid microbubble suspension, prepared as described above, which is added to mercaptoacetylated-avidin (which has been incubated with deacetylation solution). Biotinylated KDR or VEGF/KDR complex-binding peptides (prepared as described herein) are then added to the suspension of avidin-conjugated microbubbles, yielding a suspension of microbubbles conjugated to KDR or VEGF/KDR complex-binding peptides.

Unless it contains a hyperpolarized gas, known to require special storage conditions, the lyophilized residue may be stored and transported without need of temperature control of its environment and in particular it may be supplied to hospitals and physicians for on site formulation into a ready-to-use administrable suspension without requiring such users to have special storage facilities. Preferably in such a case it can be supplied in the form of a two-component kit, which can include two separate containers or a dual-chamber container. In the former case preferably the container is a conventional septum-sealed vial, wherein the vial containing the lyophilized residue of step b) is sealed with a septum through which

the carrier liquid may be injected using an optionally prefilled syringe. In such a case the syringe used as the container of the second component is also used then for injecting the contrast agent. In the latter case, preferably the dual-chamber container is a dual-chamber syringe and once the lyophilizate has been reconstituted and then suitably mixed or gently shaken, the container can be used directly for injecting the contrast agent. In both cases means for directing or permitting application of sufficient bubble forming energy into the contents of the container are provided. However, as noted above, in the stabilised contrast agents according to the invention the size of the gas microbubbles is substantially independent of the amount of agitation energy applied to the reconstituted dried product. Accordingly, no more than gentle hand shaking is generally required to give reproducible products with consistent microbubble size.

It can be appreciated by one ordinary skilled in the art that other two-chamber reconstitution systems capable of combining the dried powder with the aqueous solution in a sterile manner are also within the scope of the present invention. In such systems, it is particularly advantageous if the aqueous phase can be interposed between the water-insoluble gas and the environment, to increase shelf life of the product. Where a material necessary for forming the contrast agent is not already present in the container (e.g. a targeting ligand to be linked to the phospholipid during reconstitution), it can be packaged with the other components of the kit, preferably in a form or container adapted to facilitate ready combination with the other components of the kit.

No specific containers, vial or connection systems are required; the present invention may use conventional containers, vials and adapters. The only requirement is a good seal between the stopper and the container. The quality of the seal, therefore, becomes a matter of primary concern; any degradation of seal integrity could allow undesirable substances to enter the vial. In addition to assuring sterility, vacuum retention is essential for products stoppered at ambient or reduced pressures to assure safe and proper reconstitution. As to the stopper, it may be a compound or multicomponent formulation based on an elastomer, such as poly(isobutylene) or butyl rubber.

Ultrasound imaging techniques which may be used in accordance with the present invention include known techniques, such as color Doppler, power Doppler, Doppler amplitude, stimulated acoustic imaging, and two- or three-dimensional

imaging techniques. Imaging may be done in harmonic (resonant frequency) or fundamental modes, with the second harmonic preferred.

In ultrasound applications the contrast agents formed by phospholipid stabilized microbubbles may, for example, be administered in doses such that the amount of phospholipid injected is in the range 0.1 to 200 μg/kg body weight, preferably from about 0.1 to 30 μg/kg. Microballoons-containing contrast agents are typically administered in doses such that the amount of wall-forming polymer or lipid is from about 10 μg/kg to about 20 mg/kg of body weight.

C. Optical Imaging, Sonoluminescence or Photoacoustic Imaging

In accordance with the present invention, a number of optical parameters may be employed to determine the location of KDR or VEGF/KDR complex with *in vivo* light imaging after injection of the subject with an optically-labeled KDR or VEGF/KDR complex binding polypeptide. Optical parameters to be detected in the preparation of an image may include transmitted radiation, absorption, fluorescent or phosphorescent emission, light reflection, changes in absorbance amplitude or maxima, and elastically scattered radiation. For example, biological tissue is relatively translucent to light in the near infrared (NIR) wavelength range of 650-1000 nm. NIR radiation can penetrate tissue up to several centimeters, permitting the use of the KDR or VEGF/KDR complex binding polypeptides of the present invention for optical imaging of KDR or VEGF/KDR complex *in vivo*.

The KDR or VEGF/KDR complex binding polypeptides may be conjugated with photolabels, such as optical dyes, including organic chromophores or fluorophores, having extensive delocalized ring systems and having absorption or emission maxima in the range of 400-1500 nm. The KDR or VEGF/KDR complex binding polypeptide may alternatively be derivatized with a bioluminescent molecule. The preferred range of absorption maxima for photolabels is between 600 and 1000 nm to minimize interference with the signal from hemoglobin. Preferably, photoabsorption labels have large molar absorptivities, *e.g.*, $> 10^5$ $cm^{-1}M^{-1}$, while fluorescent optical dyes will have high quantum yields. Examples of optical dyes include, but are not limited to those described in WO 98/18497, WO 98/18496, WO 98/18495, WO 98/18498, WO 98/53857, WO 96/17628, WO 97/18841, WO 96/23524, WO 98/47538, and references cited therein. The photolabels may be covalently linked directly to the KDR or VEGF/KDR complex binding peptide or

linked to the KDR or VEGF/KDR complex binding peptide via a linker, as described previously.

After injection of the optically-labeled KDR or VEGF/KDR complex binding moiety, the patient is scanned with one or more light sources (*e.g.*, a laser) in the wavelength range appropriate for the photolabel employed in the agent. The light used may be monochromatic or polychromatic and continuous or pulsed. Transmitted, scattered, or reflected light is detected via a photodetector tuned to one or multiple wavelengths to determine the location of KDR or VEGF/KDR complex in the subject. Changes in the optical parameter may be monitored over time to detect accumulation of the optically-labeled reagent at the site of angiogenesis. Standard image processing and detecting devices may be used in conjunction with the optical imaging reagents of the present invention.

The optical imaging reagents described above may also be used for acousto-optical or sonoluminescent imaging performed with optically-labeled imaging agents (see, US 5,171,298, WO 98/57666, and references cited therein). In acousto-optical imaging, ultrasound radiation is applied to the subject and affects the optical parameters of the transmitted, emitted, or reflected light. In sonoluminescent imaging, the applied ultrasound actually generates the light detected. Suitable imaging methods using such techniques are described in WO 98/57666.

D. Nuclear Imaging (Radionuclide Imaging) and Radiotherapy.

The KDR or VEGF/KDR complex binding moieties may be conjugated with a radionuclide reporter appropriate for scintigraphy, SPECT, or PET imaging and/or with a radionuclide appropriate for radiotherapy. Constructs in which the KDR or VEGF/KDR complex binding moieties are conjugated with both a chelator for a radionuclide useful for diagnostic imaging and a chelator useful for radiotherapy are within the scope of the invention.

For use as a PET agent a peptide is complexed with one of the various positron emitting metal ions, such as ^{51}Mn, ^{52}Fe, ^{60}Cu, ^{68}Ga, ^{72}As, ^{94m}Tc, or ^{110}In. The binding moieties of the invention can also be labeled by halogenation using radionuclides such as ^{18}F, ^{124}I, ^{125}I, ^{131}I, ^{123}I, ^{77}Br , and ^{76}Br. Preferred metal radionuclides for scintigraphy or radiotherapy include ^{99m}Tc, ^{51}Cr, ^{67}Ga, ^{68}Ga, ^{47}Sc, ^{51}Cr, ^{167}Tm, ^{141}Ce ,^{111}In, ^{168}Yb, ^{175}Yb, ^{140}La, ^{90}Y, ^{88}Y, ^{153}Sm, ^{166}Ho, ^{165}Dy, ^{166}Dy, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{97}Ru, ^{103}Ru, ^{186}Re, ^{188}Re, ^{203}Pb, ^{211}Bi, ^{212}Bi, ^{213}Bi, ^{214}Bi, ^{105}Rh, ^{109}Pd, ^{117m}Sn, ^{149}Pm, ^{161}Tb, ^{177}Lu, ^{198}Au and ^{199}Au. The choice of metal will be

determined based on the desired therapeutic or diagnostic application. For example, for diagnostic purposes the preferred radionuclides include ^{64}Cu, ^{67}Ga, ^{68}Ga, ^{99m}Tc, and ^{111}In. For therapeutic purposes, the preferred radionuclides include ^{64}Cu, ^{90}Y, ^{105}Rh, ^{111}In, ^{117m}Sn, ^{149}Pm, ^{153}Sm, ^{161}Tb, ^{166}Dy, ^{166}Ho, ^{175}Yb, ^{177}Lu, $^{186/188}Re$, and ^{199}Au. ^{99m}Tc is particularly preferred for diagnostic applications because of its low cost, availability, imaging properties, and high specific activity. The nuclear and radioactive properties of Tc-99m make this isotope an ideal scintigraphic imaging agent. This isotope has a single photon energy of 140 keV and a radioactive half-life of about 6 hours, and is readily available from a ^{99}Mo-^{99m}Tc generator.

The metal radionuclides may be chelated by, for example, linear, macrocyclic, terpyridine, and N_3S, N_2S_2, or N_4 chelants (see also, US 5,367,080, US 5,364,613, US 5,021,556, US 5,075,099, US 5,886,142), and other chelators known in the art including, but not limited to, HYNIC, DTPA, EDTA, DOTA, DO3A, TETA, and bisamino bisthiol (BAT) chelators (see also US 5,720,934). For example, N_4 chelators are described in US 6,143,274; US 6,093,382; US 5,608,110; US 5,665,329; US 5,656,254; and US 5,688,487. Certain N_3S chelators are described in PCT/CA94/00395, PCT/CA94/00479, PCT/CA95/00249 and in US5,662,885; US 5,976,495; and US 5,780,006. The chelator may also include derivatives of the chelating ligand mercapto-acetyl-acetyl-glycyl-glycine (MAG3), which contains an N_3S, and N_2S_2 systems such as MAMA (monoamidemonoaminedithiols), DADS (N_2S diaminedithiols), CODADS and the like. These ligand systems and a variety of others are described in Liu and Edwards, *Chem Rev.*, 99:2235-2268 (1999) and references therein.

The chelator may also include complexes containing ligand atoms that are not donated to the metal in a tetradentate array. These include the boronic acid adducts of technetium and rhenium dioximes, such as are described in US 5,183,653; US 5,387,409; and US 5,118,797, the disclosures of which are incorporated by reference herein, in their entirety.

In another embodiment, disulfide bonds of a KDR or VEGF/KDR complex binding polypeptide of the invention are used as two ligands for chelation of a radionuclide such as ^{99m}Tc. In this way the peptide loop is expanded by the introduction of Tc (peptide-S-S-peptide changed to peptide-S-Tc-S-peptide). This has also been used in other disulfide containing peptides in the literature (Chen *et al.*, *J. Nucl. Med.*, 42:1847-1855(2001)) while maintaining biological activity. The

other chelating groups for Tc can be supplied by amide nitrogens of the backbone, another cystine amino acid or other modifications of amino acids.

Particularly preferred metal chelators include those of Formula 20, 21, 22, 23a, 23b, 24a, 24b and 25 (FIGS. 34A-F). Formulas 20-22 (FIGS. 34A-C) are particularly useful for lanthanides such as paramagnetic Gd^{3+} and radioactive lanthanides such as ^{177}Lu, ^{90}Y, ^{153}Sm, ^{111}In, or ^{166}Ho. Formulas 23a-24b (FIG. 34D and F) are particularly useful for radionuclides ^{99m}Tc, ^{186}Re, or ^{188}Re. Forumula 25 (FIG. 34F) is particularly useful for ^{99m}Tc. These and other metal chelating groups are described in US 6,093,382 and US 5,608,110, which are incorporated by reference herein in their entirety. Additionally, the chelating group of formula 22 (FIG. 34C) is described in, for example, US 6,143,274; the chelating group of formula 24 is described in, for example, US 5,627,286 and US 6,093,382, and the chelating group of formula 25 is described in, for example, US 5,662,885; US 5,780,006; and US 5,976,495.

In the above Formulas 24a and 24b (FIG. 34E), X is either CH_2 or O; Y is C_1-C_{10} branched or unbranched alky, aryl, aryloxy, arylamino, arylaminoacyl, or arylalkyl comprising C_1-C_{10} branched or unbranched alkyl groups, hydroxy or C_1-C_{10} branched or unbranched polyhydroxyalkyl groups, C_1-C_{10} branched or unbranched hydroxy or polyalkoxyalkyl or polyhydroxy-polyalkoxyalkyl groups; J is C(=O)–, OC(=O)–, SO_2–, NC(=O)–, NC(=S)–, N(Y), NC(=NCH_3)–, NC(=NH)–, N=N–, homopolyamides or heteropolyamines derived from synthetic or naturally occurring amino acids; and n is 1-100. Other variants of these structures are described, for example, in US 6,093,382. The disclosures of each of the foregoing patents, applications and references are incorporated by reference herein, in their entirety.

The chelators may be covalently linked directly to the KDR or VEGF/KDR complex binding moiety or linked to the KDR or VEGF/KDR complex binding polypeptide via a linker, as described previously, and then directly labeled with the radioactive metal of choice (see, WO 98/52618, US 5,879,658, and US 5,849,261).

Complexes of radioactive technetium are particularly useful for diagnostic imaging and complexes of radioactive rhenium are particularly useful for radiotherapy. In forming a complex of radioactive technetium with the reagents of this invention, the technetium complex, preferably a salt of Tc-99m pertechnetate, is reacted with the reagent in the presence of a reducing agent. Preferred reducing agents are dithionite, stannous and ferrous ions; the most preferred reducing agent is

stannous chloride. Means for preparing such complexes are conveniently provided in a kit form comprising a sealed vial containing a predetermined quantity of a reagent of the invention to be labeled and a sufficient amount of reducing agent to label the reagent with Tc-99m. Alternatively, the complex may be formed by reacting a peptide of this invention conjugated with an appropriate chelator with a pre-formed labile complex of technetium and another compound known as a transfer ligand. This process is known as ligand exchange and is well known to those skilled in the art. The labile complex may be formed using such transfer ligands as tartrate, citrate, gluconate or mannitol, for example. Among the Tc-99m pertechnetate salts useful with the present invention are included the alkali metal salts such as the sodium salt, or ammonium salts or lower alkyl ammonium salts.

Preparation of the complexes of the present invention where the metal is radioactive rhenium may be accomplished using rhenium starting materials in the +5 or +7 oxidation state. Examples of compounds in which rhenium is in the Re(VII) state are NH_4ReO_4 or $KReO_4$. Re(V) is available as, for example, $[ReOCl_4](NBu_4)$, $[ReOCl_4](AsPh_4)$, $ReOCl_3(PPh_3)_2$ and as $ReO_2(pyridine)_4^+$, where Ph is phenyl and Bu is n-butyl. Other rhenium reagents capable of forming a rhenium complex may also be used.

Radioactively-labeled scintigraphic imaging agents provided by the present invention are provided having a suitable amount of radioactivity. In forming Tc-99m radioactive complexes, it is generally preferred to form radioactive complexes in solutions containing radioactivity at concentrations of from about 0.01 mCi to 100 mCi per mL.

Generally, the unit dose to be administered has a radioactivity of about 0.01 mCi to about 100 mCi, preferably 1 mCi to 20 mCi. The solution to be injected at unit dosage is from about 0.01 ml to about 10 mL.

Typical doses of a radionuclide-labeled KDR or VEGF/KDR complex binding imaging agents according to the invention provide 10-20 mCi. After injection of the KDR or VEGF/KDR complex-specific radionuclide imaging agent into the patient, a gamma camera calibrated for the gamma ray energy of the nuclide incorporated in the imaging agent is used to image areas of uptake of the agent and quantify the amount of radioactivity present in the site. Imaging of the site *in vivo* can take place in a matter of a few minutes. However, imaging can take place, if desired, in hours or even longer, after the radiolabeled peptide is injected into a

patient. In most instances, a sufficient amount of the administered dose will accumulate in the area to be imaged within about 0.1 of an hour to permit the taking of scintiphotos.

Proper dose schedules for the radiotherapeutic compounds of the present invention are known to those skilled in the art. The compounds can be administered using many methods which include, but are not limited to, a single or multiple IV or IP injections, using a quantity of radioactivity that is sufficient to cause damage or ablation of the targeted KDR-expressing tissue, but not so much that substantive damage is caused to non-target (normal tissue). The quantity and dose required is different for different constructs, depending on the energy and half-life of the isotope used, the degree of uptake and clearance of the agent from the body and the mass of the tumor. In general, doses can range from a single dose of about 30-50 mCi to a cumulative dose of up to about 3 Curies.

The radiotherapeutic compositions of the invention can include physiologically acceptable buffers, and can require radiation stabilizers to prevent radiolytic damage to the compound prior to injection. Radiation stabilizers are known to those skilled in the art, and may include, for example, para-aminobenzoic acid, ascorbic acid, gentistic acid and the like.

A single, or multi-vial kit that contains all of the components needed to prepare the complexes of this invention, other than the radionuclide, is an integral part of this invention.

A single-vial kit preferably contains a chelating ligand, a source of stannous salt, or other pharmaceutically acceptable reducing agent, and is appropriately buffered with pharmaceutically acceptable acid or base to adjust the pH to a value of about 3 to about 9. The quantity and type of reducing agent used would depend highly on the nature of the exchange complex to be formed. The proper conditions are well known to those that are skilled in the art. It is preferred that the kit contents be in lyophilized form. Such a single vial kit may optionally contain labile or exchange ligands such as glucoheptonate, gluconate, mannitol, malate, citric or tartaric acid and can also contain reaction modifiers such as diethylenetriamine-pentaacetic acid (DPTA), ethylenediamine tetraacetic acid (EDTA), or α,β,or γ cyclodextrin that serve to improve the radiochemical purity and stability of the final product. The kit may also contain stabilizers, bulking agents such as mannitol, that are designed to aid in the freeze-drying process, and other additives known to those

skilled in the art.

A multi-vial kit preferably contains the same general components but employs more than one vial in reconstituting the radiopharmaceutical. For example, one vial may contain all of the ingredients that are required to form a labile Tc(V) complex on addition of pertechnetate (*e.g.*, the stannous source or other reducing agent). Pertechnetate is added to this vial, and after waiting an appropriate period of time, the contents of this vial are added to a second vial that contains the ligand, as well as buffers appropriate to adjust the pH to its optimal value. After a reaction time of about 5 to 60 minutes, the complexes of the present invention are formed. It is advantageous that the contents of both vials of this multi-vial kit be lyophilized. As above, reaction modifiers, exchange ligands, stabilizers, bulking agents, etc. may be present in either or both vials.

Other Therapeutic Applications

The KDR or VEGF/KDR complex binding polypeptides of the present invention can be used to improve the activity of therapeutic agents such as anti-angiogenic or tumorcidal agents against undesired angiogenesis such as occurs in neoplastic tumors, by providing or improving their affinity for KDR or VEGF/KDR complex and their residence time at a KDR or VEGF/KDR complex on endothelium undergoing angiogenesis. In this aspect of the invention, hybrid agents are provided by conjugating a KDR or VEGF/KDR complex binding polypeptide according to the invention with a therapeutic agent. The therapeutic agent may be a radiotherapeutic, discussed above, a drug, chemotherapeutic or tumorcidal agent, genetic material or a gene delivery vehicle, etc. The KDR or VEGF/KDR complex binding polypeptide portion of the conjugate causes the therapeutic to "home" to the sites of KDR or VEGF/KDR complex (*i.e.*, activated endothelium), and to improve the affinity of the conjugate for the endothelium, so that the therapeutic activity of the conjugate is more localized and concentrated at the sites of angiogenesis. Such conjugates will be useful in treating angiogenesis associated diseases, especially neoplastic tumor growth and metastasis, in mammals, including humans, which method comprises administering to a mammal in need thereof an effective amount of a KDR or VEGF/KDR complex binding polypeptide according to the invention conjugated with a therapeutic agent. The invention also provides the use of such conjugates in the manufacture of a medicament for the treatment of angiogenesis associated diseases in mammals, including humans.

Suitable therapeutic agents for use in this aspect of the invention include, but are not limited to: antineoplastic agents, such as platinum compounds (*e.g.*, spiroplatin, cisplatin, and carboplatin), methotrexate, adriamycin, mitomycin, ansamitocin, bleomycin, cytosine, arabinoside, arabinosyl adenine, mercaptopolylysine, vincristine, busulfan, chlorambucil, melphalan (*e.g.*, PAM, L–PAM, or phenylalanine mustard), mercaptopurine, mitotane, procarbazine hydrochloride, dactinomycin (actinomycin D), daunorubcin hydrochloride, doxorubicin hydrochloride, taxol, mitomycin, plicamycin (mithramycin), aminoglutethimide, estramustine phosphate sodium, flutamide, leuprolide acetate, megestrol acetate, tamoxifen citrate, testoiactone, trilostane, amsacrine (m-AMSA), aparaginase (L-aparaginase), *Erwina* aparaginase, etoposide (VP-16), interferon *cx*-2a, Interferon *cx*-2b, teniposide (VM-26, vinblastine sulfate (VLB), vincristine sulfate, bleomycin sulfate, adriamycin, and arabinosyl; anti-angiogenic agents such as tyrosine kinase inhibitors with activity toward signaling molecules important in angiogenesis and/or tumor growth such as SU5416 and SU6668 (Sugen/Pharmacia & Upjohn), endostatin (EntreMed), angiostatin (EntreMed), Combrestatin (Oxigene), cyclosporine, 5-fluorouracil, vinblastine, doxorubicin, paclitaxel, daunorubcin, immunotoxins; coagulation factors; antivirals such as acyclovir, amantadine azidothymidine (AZT or Zidovudine), ribavirin and vidarabine monohydrate (adenine arahinoside, ara-A); antibiotics, antimalarials, antiprotozoans such as chloroquine, hydroxychloroquine, metroidazole, quinine and meglumine antimonate; anti-inflammatories such as diflunisal, ibuprofen, indomethacin, meclofenamate, mefenamic acid, naproxen, oxyphenbutazone, phenylbutazone, piroxicam, sulindac, tolmetin, aspirin and salicylates.

The KDR or VEGF/KDR complex binding polypeptides of the present invention may also be used to target genetic material to KDR-expressing cells. Thus, they may be useful in gene therapy, particularly for treatment of diseases associated with angiogenesis. In this embodiment, genetic material or one or more delivery vehicles containing genetic material useful in treating an angiogenesis-related disease may be conjugated to one or more KDR binding moieties of the invention and administered to a patient. The genetic material may include nucleic acids, such as RNA or DNA, of either natural or synthetic origin, including recombinant RNA and DNA and antisense RNA and DNA. Types of genetic material that may be used include, for example, genes carried on expression vectors

such as plasmids, phagemids, cosmids, yeast artificial chromosomes (YAC's) and defective or "helper" viruses, antigene nucleic acids, both single and double stranded RNA and DNA and analogs thereof, such as phosphorothioate and phosphorodithioate oligodeoxynucleotides. Additionally, the genetic material may be combined, for example, with lipids, proteins or other polymers. Delivery vehicles for genetic material may include, for example, a virus particle, a retroviral or other gene therapy vector, a liposome, a complex of lipids (especially cationic lipids) and genetic material, a complex of dextran derivatives and genetic material, etc.

In a preferred embodiment the constructs of the invention are utilized in gene therapy for treatment of diseases associated with angiogenesis. In this embodiment, genetic material, or one or more delivery vehicles containing genetic material, *e.g.*, useful in treating an angiogenesis-related disease, can be conjugated to one or more KDR or VEGF/KDR complex binding polypeptides or heteromultimers of the invention and administered to a patient.

Constructs including genetic material and the KDR-binding polypeptides of the invention may be used, in particular, to selectively introduce genes into angiogenic endothelial cells, which may be useful not only to treat cancer, but also after angioplasty, where inhibition of angiogenesis may inhibit restenosis.

Therapeutic agents and the KDR or VEGF/KDR complex binding moieties of the invention can be linked or fused in known ways, using the same type of linkers discussed elsewhere in this application. Preferred linkers will be substituted or unsubstituted alkyl chains, amino acid chains, polyethylene glycol chains, and other simple polymeric linkers known in the art. More preferably, if the therapeutic agent is itself a protein, for which the encoding DNA sequence is known, the therapeutic protein and KDR or VEGF/KDR complex binding polypeptide may be coexpressed from the same synthetic gene, created using recombinant DNA techniques, as described above. The coding sequence for the KDR or VEGF/KDR complex binding polypeptide may be fused in frame with that of the therapeutic protein, such that the peptide is expressed at the amino- or carboxy-terminus of the therapeutic protein, or at a place between the termini, if it is determined that such placement would not destroy the required biological function of either the therapeutic protein or the KDR or VEGF/KDR complex binding polypeptide. A particular advantage of this general approach is that concatamerization of multiple, tandemly arranged KDR or VEGF/KDR complex binding polypeptides is possible,

thereby increasing the number and concentration of KDR or VEGF/KDR complex binding sites associated with each therapeutic protein. In this manner KDR or VEGF/KDR complex binding avidity is increased which would be expected to improve the efficacy of the recombinant therapeutic fusion protein.

Similar recombinant proteins containing one or more coding sequences for a KDR and VEGF/KDR complex binding polypeptide may be useful in imaging or therapeutic applications. For example, in a variation of the pre-targeting applications discussed infra, the coding sequence for a KDR or VEGF/KDR complex binding peptide may be fused in frame to a sequence encoding an antibody (or an antibody fragment or recombinant DNA construct including an antibody, etc.) which, for example, binds to a chelator for a radionuclide (or another detectable label). The antibody expressing the KDR or VEGF/KDR complex binding polypeptide is then administered to a patient and allowed to localize and bind to KDR-expressing tissue. After the non-binding antibodies have been allowed to clear, the chelator-radionuclide complex (or other detectable label), which the antibody recognizes is administered, permitting imaging of or radiotherapy to the KDR-expressing tissues. Additionally, the coding sequence for a KDR or VEGF/KDR complex binding peptide may be fused in frame to a sequence encoding, for example, serum proteins or other proteins that produce biological effects (such as apoptosis, coagulation, internalization, differentiation, cellular stasis, immune system stimulation or suppression, or combinations thereof). The resulting recombinant proteins are useful in imaging, radiotherapy, and therapies directed against cancer and other diseases that involve angiogenesis or diseases associated with the pathogens discussed herein.

Additionally, constructs including KDR or KDR/VEGF complex binding polypeptides of the present invention can themselves be used as therapeutics to treat a number of diseases. For example, where binding of a protein or other molecule (*e.g.*, a growth factor, hormone etc.) is necessary for or contributes to a disease process and a binding moiety inhibits such binding, constructs including such binding moieties could be useful as therapeutics. Similarly, where binding of a binding moiety itself inhibits a disease process, constructs containing such binding moieties could also be useful as therapeutics.

As binding of VEGF and activation of KDR is necessary for angiogenic activity, in one embodiment constructs including KDR complex binding

polypeptides that inhibit the binding of VEGF to KDR (or otherwise inhibit activation of KDR) may be used as anti-angiogenic agents. Some peptides of the invention that inhibit activation of KDR are discussed in Example 9 *infra*. Certain constructs of the invention including multimers and heteromultimers that inhibit activation of KDR are also discussed in the Examples. A particularly preferred heteromultimer is the heterodimer-containing construct D1 (structures provided by the examples). Other preferred heterodimer constructs include D4, D5, and D6 (structures provided in Examples 12 and 18 below). The binding polypeptides and constructs thereof of the present invention are useful as therapeutic agents for treating conditions that involve endothelial cells. Because an important function of endothelial cells is angiogenesis, or the formation of blood vessels, the polypeptides and constructs thereof are particularly useful for treating conditions that involve angiogenesis. Conditions that involve angiogenesis include, for example, solid tumors, tumor metastases and benign tumors. Such tumors and related disorders are well known in the art and include, for example, melanoma, central nervous system tumors, neuroendocrine tumors, sarcoma, multiple myeloma as wells as cancer of the breast, lung, prostate, colon, head & neck, and ovaries. Additional tumors and related disorders are listed in Table I of U.S. Patent No. 6,025,331, issued February 15, 2000 to Moses, *et al.,* the teachings of which are incorporated herein by reference. Benign tumors include, for example, hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas. Other relevant diseases that involve angiogenesis include for example, rheumatoid arthritis, psoriasis, and ocular diseases, such as diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rebeosis, Osler-Webber Syndrome, myocardial angiogenesis, plaque neovascularization, telangiectasia, hemophiliac joints, angiofibroma and wound granulation. Other relevant diseases or conditions that involve blood vessel growth include intestinal adhesions, atherosclerosis, scleroderma, and hypertropic scars, and ulcers. Furthermore, the binding polypeptides and constructs thereof of the present invention can be used to reduce or prevent uterine neovascularization required for embryo implantation, for example, as a birth control agent. Heteromultimers of this invention can also be useful for treating vascular permeability events that can result when VEGF binds KDR. In renal failure, for example, it has been shown that anti-VEGF antibodies can reverse damage. In a similar way, the compounds of the

present invention can reverse renal permeability pathogenesis in, for example, diabetes.

Furthermore, the KDR or VEGF/KDR complex binding polypeptides of the present invention may be useful in treating diseases associated with certain pathogens, including, for example, malaria, HIV, SIV, Simian hemorrhagic fever virus, etc. Sequence homology searches of KDR-binding peptides identified by phage display using the BLAST program at NCBI has identified a number of homologous proteins known or expected to be present on the surface of pathogenic organisms. Homologies were noted between the polypeptides of the invention and proteins from various malaria strains, HIV, SIV, simian hemorrhagic fever virus, and an enterohemorrhagic *E. coli* strain. Some of the homologous proteins, such as PfEMP1 and EBL-1, are hypermutable adhesion proteins known to play roles in virulence. These proteins possess multiple binding sites that are capable of binding to more than one target molecule on the host's surface. Their high mutation and recombination rates allow them to quickly develop new binding sites to promote survival and/or invasion. Similarly, proteins such as gp120 of HIV (which also has homology to some of the KDR-binding peptides disclosed herein) play critical roles in the adhesion of pathogens to their hosts. Although not reported previously, it is possible that many of the pathogen proteins with homology to the KDR-binding peptides disclosed herein also bind to KDR. Comparison of the pathogen protein sequences with the corresponding peptide sequences may suggest changes in the peptide sequence or other modifications that will enhance its binding properties. Additionally, the KDR-binding peptide sequences disclosed herein may have usefulness in blocking infection with the pathogen species that possesses the homology. Indeed, a similar strategy is being employed to block HIV infection by trying to prevent virus envelope proteins from binding to their known cellular surface targets such as CD4. See, Howie *et al.*, "Synthetic peptides representing discontinuous CD4 binding epitopes of HIV-1 gp120 that induce T cell apoptosis and block cell death induced by gp120", *FASEB J*, 12(11):991-998 (1998). Thus, KDR may represent a previously unknown target for a number of pathogens, and the KDR binding peptides of the invention may be useful in treating the diseases associated with those pathogens.

The binding polypeptides and constructs thereof can be administered to an individual over a suitable time course depending on the nature of the condition and

the desired outcome. The binding polypeptides and constructs thereof can be administered prophylactically, *e.g.*, before the condition is diagnosed or to an individual predisposed to a condition. The binding polypeptides and constructs thereof can be administered while the individual exhibits symptoms of the condition or after the symptoms have passed or otherwise been relieved (such as after removal of a tumor). In addition, the binding polypeptides and constructs thereof of the present invention can be administered a part of a maintenance regimen, for example to prevent or lessen the recurrence or the symptoms or condition. As described below, the binding polypeptides and constructs thereof of the present invention can be administered systemically or locally.

The quantity of material administered will depend on the seriousness of the condition. For example, for treatment of an angiogenic condition, *e.g.*, in the case of neoplastic tumor growth, the position and size of the tumor will affect the quantity of material to be administered. The precise dose to be employed and mode of administration must per force in view of the nature of the complaint be decided according to the circumstances by the physician supervising treatment. In general, dosages of the agent conjugate of the present invention will follow the dosages that are routine for the therapeutic agent alone, although the improved affinity of a binding polypeptide or heteromultimer of the invention for its target may allow a decrease in the standard dosage.

Such conjugate pharmaceutical compositions are preferably formulated for parenteral administration, and most preferably for intravenous or intra-arterial administration. Generally, and particularly when administration is intravenous or intra-arterial, pharmaceutical compositions may be given as a bolus, as two or more doses separated in time, or as a constant or non-linear flow infusion.

As used herein the term "therapeutic" includes at least partial alleviation of symptoms of a given condition. The binding polypeptides and constructs thereof of the present invention do not have to produce a complete alleviation of symptoms to be useful. For example, treatment of an individual can result in a decrease in the size of a tumor or diseased area, or prevention of an increase in size of the tumor or diseased area. Treatment can result in reduction in the number of blood vessels in an area of interest or can prevent an increase in the number of blood vessels in an area of interest. Treatment can also prevent or lessen the number or size of metastatic outgrowths of the main tumor(s).

Symptoms that can be alleviated include physiological characteristics such as VEGF receptor activity and migration ability of endothelial cells. The binding polypeptides and constructs thereof of the present invention can inhibit activity of VEGF receptors, including VEGF-2/KDR, VEGF-1/Flt-1 and VEGF-3/Flt-4. Such inhibition can be detected, for example, by measuring the phosphorylation state of the receptor in the presence of or after treatment with the binding polypeptides or constructs thereof. Such inhibition can also be detected by measuring the ability of endothelial cells to migrate in the presence of or after treatment with the binding polypeptides or constructs thereof. Based on the teachings provided herein, one of ordinary skill in the art would know how and be able to administer a suitable dose of binding polypeptide or construct thereof as provided herein, and measure the effect of treatment on the parameter of interest. For example, the size of the area of interest (*e.g.,* the tumor or lesion) can be measured before and after treatment. In another embodiment, the phosphorylation state of the relevant receptor, or the migration ability of endothelial in an area of interest can be measured in samples taken from the individual. The VEGF receptors or endothelial cells can be isolated from the sample and used in assays described herein.

The dosage of the polypeptides and constructs thereof may depend on the age, sex, health, and weight of the individual, as well as the nature of the condition and overall treatment regimen. The biological effects of the polypeptides and constructs thereof are described herein. Therefore, based on the biological effects of the binding polypeptides and constructs provided herein, and the desired outcome of treatment, the preferred dosage is determinable by one of ordinary skill in the art through routine optimization procedures. Typically, the daily regimen is in the range of about 0.1 μg/kg to about 1 mg/kg.

The binding polypeptides and constructs thereof provided herein can be administered as the sole active ingredient together with a pharmaceutically acceptable excipient, or can be administered together with other binding polypeptides and constructs thereof, other therapeutic agents, or combination thereof. In addition, the binding polypeptides and constructs thereof can be conjugated to therapeutic agents, for example, to improve specificity, residence time in the body, or therapeutic effect. Such other therapeutic agents include, for example, other anti-angiogenic compounds, and tumoricidal compounds. The therapeutic agent can also include antibodies.

Furthermore, the binding polypeptide or constructs thereof of the present invention can be used as an endothelial cell homing device. Therefore, the binding polypeptide or constructs thereof can be conjugated to nucleic acid encoding, for example, a therapeutic polypeptide, in order to target the nucleic acid to endothelial cells. Once exposed to the nucleic acid conjugated binding polypeptide, the endothelial can internalize and express the conjugated nucleic acid, thereby delivering the therapeutic peptide to the target cells.

In another embodiment of the invention, the therapeutic agent can be associated with an ultrasound contrast agent composition, said ultrasound contrast agent including the KDR or VEGF complex binding peptides of the invention linked to the material employed to form the vesicles (particularly microbubbles or microballoons) comprised in the contrast agent, as previously described. For instance, said contrast agent/therapeutic agent association can be carried out as described in US 6,258,378, herein incorporated by reference. Thus, after administration of the ultrasound contrast agent and the optional imaging of the contrast agent bound to the pathogenic site expressing the KDR or VEGF/KDR complex, the pathogenic site can be irradiated with an energy beam (preferably ultrasonic, e.g. with a frequency of from 0.3 to 3 MHz), to cause the bursting of microvesicles, as disclosed for instance in the above cited U.S. Patent No. 6,258,378. The therapeutic effect of the therapeutic agent can thus be advantageously enhanced by the energy released by the burst of the microvesicles, in particular causing an effective delivery of the therapeutic agent to the targeted pathogenic site.

The binding polypeptides and constructs thereof can be administered by any suitable route. Suitable routes of administration include, but are not limited to, topical application, transdermal, parenteral, gastrointestinal, intravaginal, and transalveolar. Compositions for the desired route of administration can be prepared by any of the methods well known in the pharmaceutical arts, for example, as described in *Remington: The Science and Practice of Pharmacy*, 20th ed., Lippincott, Williams and Wilkins, 2000.

For topical application, the binding polypeptides can be suspended, for example, in a cream, gel or rinse which allows the polypeptides or constructs to penetrate the skin and enter the blood stream, for systemic delivery, or contact the area of interest, for localized delivery. Compositions suitable for topical application

include any pharmaceutically acceptable base in which the polypeptides are at least minimally soluble.

For transdermal administration, the polypeptides can be applied in pharmaceutically acceptable suspension together with a suitable transdermal device or "patch." Examples of suitable transdermal devices for administration of the polypeptides of the present invention are described, for example, in U.S. Patent No. 6,165,458, issued December 26, 2000 to Foldvari, *et al.*, and U.S. Patent No. 6,274,166B1, issued August 4, 2001 to Sintov, *et al.*, the teachings of which are incorporated herein by reference.

For parenteral administration, the polypeptides can be injected intravenously, intramuscularly, intraperitoneally, or subcutaneously. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Other pharmaceutically acceptable carriers include, but are not limited to, sterile water, saline solution, and buffered saline (including buffers like phosphate or acetate), alcohol, vegetable oils, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, paraffin, etc. Where necessary, the composition may also include a solubilizing agent and a local anaesthetic such as lidocaine to ease pain at the site of the injection, preservatives, stabilizers, wetting agents, emulsifiers, salts, lubricants, *etc.* as long as they do not react deleteriously with the active compounds. Similarly, the composition may comprise conventional excipients, *i.e.* pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral, enteral or intranasal application which do not deleteriously react with the active compounds. Generally, the ingredients will be supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent in activity units. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade "water for injection" or saline. Where the composition is to be administered by injection, an ampoule of sterile water for injection or saline may be provided so that the ingredients may be mixed prior to administration.

For gastrointestinal and intravaginal administration, the polypeptides can be incorporated into pharmaceutically acceptable powders, pills or liquids for ingestion, and suppositories for rectal or vaginal administration.

For transalveolar, buccal or pulmonary administration, the polypeptides can be suspended in a pharmaceutically acceptable excipient suitable for aerosolization and inhalation or as a mouthwash. Devices suitable for transalveolar administration such as atomizers and vaporizers are also included within the scope of the invention. Suitable formulations for aerosol delivery of polypeptides using buccal or pulmonary routes can be found, for example in U.S. Patent No. 6,312,665B1, issued November 6, 2001 to Pankaj Modi, the teachings of which are incorporated herein by reference.

In addition, the polypeptides of the present invention can be administered nasally or ocularly, where the polypeptide is suspended in a liquid pharmaceutically acceptable agent suitable for drop wise dosing.

The polypeptides of the present invention can be administered such that the polypeptide is released in the individual over an extended period of time (sustained or controlled release). For example, the polypeptide can be formulated into a composition such that a single administration provides delivery of the polypeptide for at least one week, or over the period of a year or more. Controlled release systems include monolithic or reservoir-type microcapsules, depot implants, osmotic pumps, vesicles, micelles, liposomes, transdermal patches and iontophoretic devices. In one embodiment, the polypeptides of the present invention are encapsulated or admixed in a slowly degrading, non-toxic polymer. Additional formulations suitable for controlled release of the polypeptides provided herein are described in U.S. Patent No. 4,391,797, issued July 5, 1983, to Folkman, *et al.*, the teachings of which are incorporated herein by reference.

Another suitable method for delivering the polypeptides of the present to an individual is via *in vivo* production of the polypeptide. A gene encoding the polypeptide can be administered to the individual such that the encoded polypeptide is expressed. The gene can be transiently expressed. In a particular embodiment, the gene encoding the polypeptide is transfected into cells that have been obtained from the patient, a method referred to as *ex vivo* gene therapy. Cells expressing the polypeptide are then returned to the patient's body. Methods of *ex vivo* gene therapy are well known in the art and are described, for example, in U.S. Patent No. 4,391,797, issued March 21, 1998 to Anderson, *et al.*, the teachings of which are incorporated herein by reference.

Isolation of KDR or VEGF/KDR complex binding moieties in accordance with this invention will be further illustrated in the following examples. The

specific parameters included in the following examples are intended to illustrate the practice of the invention, and they are not presented to in any way limit the scope of the invention.

EXAMPLES

Example 1: Library Screening Against KDR and KDR/VEGF Complex Targets

Chimeric fusions of Ig Fc region with human KDR (#357-KD-050), murine KDR (#443-KD-050), human VEGFR-1 (#321-FL-050), human VEGFR-3 (#349-F4-050), and human Trail R4 (#633-TR-100) were purchased in carrier-free form (no BSA) from R & D Systems (Minneapolis, MN). Trail R4 Fc is an irrelevant Fc fusion protein with the same Fc fusion region as the target Fc fusion (KDR Fc) and is used to deplete the libraries of Fc binders. $VEGF_{165}$ (#100-20) was purchased in carrier-free form from Peprotech (Rocky Hill, NJ). Protein A Magnetic Beads (#100.02) were purchased from Dynal (Oslo, Norway). Heparin (#H-3393) was purchased from Sigma Chemical Company (St. Louis, MO). A 2-component tetramethyl benzidine (TMB) system was purchased from KPL (Gaithersburg, MD).

In the following procedures, microtiter plates were washed with a Bio-Tek 404 plate washer (Winooski, VT). ELISA signals were read with a Bio-Tek plate reader (Winooski, VT). Agitation of 96-well plates was on a LabQuake shaker (Labindustries, Berkeley, CA).

Eight M13 phage display libraries were prepared for screening against immobilized KDR and VEGF/KDR targets: Cyclic peptide display libraries TN6/VI, TN7/IV, TN8/IX, TN9/IV, TN10/IX, TN12/I, and MTN13/I, and a linear display library, Lin20. The design of these libraries has been described, *supra*.

The DNA encoding the library was synthesized with constant DNA on either side so that the DNA can be PCR amplified using *Taq* DNA polymerase (Perkin-Elmer, Wellesley, MA), cleaved with *Nco*I and *Pst*I, and ligated to similarly cleaved phage display vector. XL1-Blue MFR' *E. coli* cells were transformed with the ligated DNA. All of the libraries were constructed in same manner.

KDR Selection Protocol in the Presence of Heparin

Protein A Magnetic Beads were blocked once with 1X PBS (pH 7.5), 0.01% Tween-20, 0.1% HSA (Blocking Buffer) for 30 minutes at room temperature and then washed five times with 1X PBS (pH 7.5), 0.01% Tween-20, 5 μg/ml heparin

(PBSTH Buffer).

The cyclic peptide, or "constrained loop", libraries were pooled for the initial screening into two pools: TN6/VI, TN7/IV and TN8/IX were in one pool; TN9/IV, TN10/IX and TN12/I were in the second pool. The two pooled libraries and the linear library (Lin20) were depleted against Trail R4 Fc fusion (an irrelevant Fc fusion) and then selected against KDR Fc fusion. 10^{11} plaque forming units (pfu) from each library per 100 µl PBSTH were pooled together, *e.g.*, 3 pooled libraries would result in a total volume of ~350 µl in PBSTH.

To prepare the irrelevant Fc fusion beads, 500 µl of Trail R4-Fc fusion (0.1µg/µl stock in PBST (no heparin)) were added to 1000 µl of washed, blocked protein A magnetic beads. The fusion was allowed to bind to the beads overnight with agitation at 4°C. The next day, the magnetic beads were washed 5 times with PBSTH. Each phage pool was incubated with 50 µl of Trail R4 Fc fusion beads on a Labquake shaker for 1 hour at room temperature (RT). After incubation, the phage supernatant was removed and incubated with another 50 µl of Trail R4 beads. This was repeated for a total of 5 rounds of depletion, to remove non-specific Fc fusion and bead binding phage from the libraries.

To prepare the KDR target beads, 500 µl of KDR-Fc fusion (0.1 µg/µl stock in PBST (no heparin)) were added to 500 µl of washed, blocked beads. The KDR-Fc fusion was allowed to bind overnight with agitation at 4°C. The next day, the beads were washed 5 times with PBSTH. Each depleted library pool was added to 100 µl of KDR-Fc beads and allowed to incubate on a LabQuake shaker for 1 hour at RT. Beads were then washed as rapidly as possible with 5 X 1 ml PBSTH using a magnetic stand (Promega) to separate the beads from the wash buffer. Phage still bound to beads after the washing were eluted once with 250 µl of VEGF (50 µg/ml, ~1 µM) in PBSTH for 1 hour at RT on a LabQuake shaker. The 1-hour elution was removed and saved. After the first elution, the beads were incubated again with 250 µl of VEGF (50µg/ml, ~1µM) overnight at RT on a LabQuake shaker. The two VEGF elutions were kept separate and a small aliquot taken from each for titering. Each elution was mixed with an aliquot of XL1-Blue MRF' (or other F' cell line) *E. coli* cells which had been chilled on ice after having been grown to mid-logarithmic phase. The remaining beads after VEGF elution were also mixed with cells to amplify the phage still bound to the beads, *i.e.*, KDR-binding phage that had not

been competed off by the two VEGF incubations (1-hour and overnight (O/N) elutions). After approximately 15 minutes at room temperature, the phage/cell mixtures were spread onto Bio-Assay Dishes (243 X 243 X 18 mm, Nalge Nunc) containing 250 ml of NZCYM agar with 50 μg/ml of ampicillin. The plate was incubated overnight at 37°C. The next day, each amplified phage culture was harvested from its respective plate. Over the next day, the input, output and amplified phage cultures were titered for FOI (*i.e.*, Fraction of Input = phage output divided by phage input).

In the first round, each pool yielded three amplified eluates. These eluates were panned for 2-3 more additional rounds of selection using ~10^{10} input phage/round according to the same protocol as described above. For each additional round, the KDR-Fc beads were prepared the night before the round was initiated. For the elution step in subsequent rounds, the amplified elution re-screen on KDR-Fc beads was always eluted in the same manner and all other elutions were treated as washes. For example, for the amplified elution recovered by using the still-bound beads to infect *E. coli*, the 1-hour and overnight VEGF elutions were performed and then discarded as washes. Then the beads were used to again infect *E. coli* and produce the next round amplified elution. Using this procedure, each library pool only yielded three final elutions at the end of the selection. Two pools and one linear library, therefore, yielded a total of 9 final elutions at the end of the selection.

This selection procedure was repeated for all libraries in the absence of heparin in all binding buffers, *i.e.*, substituting PBST (PBS (pH 7.5), 0.01% Tween-20) for PBSTH in all steps.

KDR:VEGF Complex Selection Protocol in the Presence of Heparin

Protein A magnetic beads were blocked once with Blocking Buffer for 30 minutes at room temperature and then washed five times with PBSTH.

Two pools of constrained loop libraries and a linear library (Lin20) were prepared as before and then depleted against KDR Fc fusion alone, instead of Trail-R4 Fc fusion, to remove binders to the receptor without bound VEGF. Once depleted, the libraries were selected against the KDR:$VEGF_{165}$ complex.

To prepare KDR-Fc fusion depletion beads, 1 mL of KDR-Fc fusion (0.1 μg/μl stock in PBST (no heparin)) was added to 1 mL of washed, blocked beads. The fusion was allowed to bind overnight with agitation at 4°C. The next day, the

beads were washed 5 times with PBSTH. Each phage pool was incubated with 50 µl of KDR-Fc fusion beads on a LabQuake shaker for 1 hour at RT. After incubation, the phage supernatant was removed and incubated with another 50µl of KDR-Fc beads. This was repeated for a total of 5 rounds of depletion.

To prepare the KDR:VEGF complex beads, 300 µl of KDR-Fc fusion beads from above were incubated with 15 µl of VEGF (1 mg/ml). VEGF was allowed to bind for 1 hour at RT. The beads were washed 5 times with PBSTH. Each depleted library pool was added to 100 µl of KDR:VEGF complex beads and allowed to incubate on a LabQuake shaker for 1 hour at RT. Beads were then washed as rapidly as possible with 5 × 1 mL PBSTH using a magnetic stand (Promega) to separate the beads from the wash buffer. To elute the phage still bound after washing, the beads were mixed with cells to amplify the phage still bound to the beads. After approximately 15 minutes at room temperature, the phage/cell mixtures were spread onto Bio-Assay Dishes (243 × 243 × 18 mm, Nalge Nunc) containing 250 ml of NZCYM agar with 50 µg/ml of ampicillin. The plate was incubated overnight at 37°C. The next day, each amplified phage culture was harvested from its respective plate. Over the next day, the input, output and amplified phage cultures were titered for FOI. This selection protocol was repeated for two additional rounds using 10^{10} input phage from each amplified elution.

KDR and KDR/VEGF Screening Assay

100 µl of KDR-Fc fusion or Trail R4-Fc fusion (1µg/ml) were added to duplicate Immulon II plates, to every well, and allowed to incubate at 4°C overnight. Each plate was washed twice with PBST (PBS, 0.05% Tween-20). The wells were filled to the top with 1X PBS, 1% BSA and allowed to incubate at RT for 2 hours. Each plate was washed once with PBST (PBS, 0.05% Tween-20).

To assess binding to KDR:VEGF complex, another set of KDR plates was prepared as above and then 100 µl of VEGF (1µg/ml) in PBST was added to each KDR well and allowed to incubate at RT for 30 minutes. Each plate was then washed with PBST (PBS, 0.05% Tween-20).

Once the plates were prepared, each overnight phage culture was diluted 1:1 (or to 10^{10} pfu if using purified phage stock) with PBS, 0.05% Tween-20, 1% BSA. 100 µl of each diluted culture was added and allowed to incubate at RT for 2-3

hours. Each plate was washed 5 times with PBST. The binding phage were visualized by adding 100 µl of a 1:10,000 dilution of HRP-anti-M13 antibody conjugate (Pharmacia), diluted in PBST, to each well, then incubating at room temperature for 1 hr. Each plate was washed 7 times with PBST (PBS, 0.05% Tween-20), then the plates were developed with HRP substrate (~10 minutes) and the absorbance signal (630 nm) detected with plate reader.

KDR and VEGF/KDR complex binding phage were recovered, amplified, and the sequences of the display peptides responsible for the binding were determined by standard DNA sequencing methods. The binding peptides of the phage isolates are set forth in Tables 1-7, *infra*.

After isolation of KDR and VEGF/KDR complex isolates in initial selection rounds, certain isolates were selected to act as templates for the construction of secondary libraries, from which additional high affinity binding polypeptides were isolated. In a secondary TN8 library, the phage isolate sequence PKWCEEDWYYCMIT (SEQ ID NO:21) was used as a template to construct a library that allowed one-, two-, and three-base mutations to the parent sequence at each variable codon. In a secondary TN12 library, the phage isolate sequence SRVCWEDSWGGEVCFRY (SEQ ID NO:88) was used as a template to construct a library that allowed one-, two-, and three-base mutations to the parent sequence at each variable codon. In a another TN8 secondary library, a recurrent motif from the initial TN8 sequences was kept constant (WVEC---TG-C---; SEQ ID NO:260) and all of the other codon positions (*i.e.*, at "-") were allowed to vary (all possible 20 amino acids) using NNK codon substitution, where N stands for any nucleotide and K stands for any keto nucleotide (G or T).

Using a method of peptide optimization by soft randomization as described by Fairbrother *et al.*, *Biochemistry*, 37(51):17754-17764 (1998), two libraries were prepared based on the SEQ ID NO:21 and SEQ ID NO:88 sequences. At each residue position, each nucleotide within a particular codon was allowed to evolve by adding fixed amounts of the other three nucleotides that did not correspond to the nucleotide of the parent codon. This nucleotide mixing is accomplished in the synthesis of the template DNA used to make the library. For these libraries, the parent nucleotide within each codon was maintained at 64% for SEQ ID NO:21 and 67% for SEQ ID NO:88, whereas the other nucleotides were added at the remainder frequency divided by three. Since the parent nucleotides are in the majority, the

overall consensus sequence for the whole library should still contain the parental sequence. Inspection of individual isolates, however, shows that multiple mutations are possible, thus allowing selection of peptides with improved binding ability compared to the parent sequence.

For the third library, the TN8 motif described above was kept constant and all of the other positions in were allowed to vary with NNK substitution in the template oligonucleotide. To extend the substitution, NNK diversity was also permitted in the two flanking amino acid positions, thus adding variable amino acid positions N-terminal and C-terminal to the display peptide. The secondary library template, therefore, encoded a display peptide of the following sequence: Xaa–Xaa–Trp–Val–Glu–Cys–Xaa–Xaa–Xaa–Thr–Gly–Xaa–Cys–Xaa–Xaa–Xaa–Xaa–Xaa (SEQ ID NO:261), where Xaa can be any amino acid. Unlike the previous two libraries, where the consensus sequence remains the parental sequence, this library was quite diverse in all allowed positions and only resembled the parent motif in the residues that were held constant.

A total of 2×10^{11} pfu from each library was used as before, except the elution strategy was changed. Competition elution of bound phage was performed using the parental peptide (50 μM) that was used to make the particular secondary library (*i.e.*, peptides of SEQ ID NOS:21, 88, and 40, respectively). Binding phage were eluted through three steps: (1) elution for 1 hour at room temperature, the eluted phage being used to infect cells for amplification, (2) elution overnight, wherein fresh competition elution peptide was added to the bound phage and incubated at 4°C overnight with mixing, the eluted phage being then used to infect cells for amplification, and (3) the remaining beads (bearing uneluted binding phage) were used to infect cells directly. Three rounds of selections were performed. Plaques were picked from rounds 2 and 3 and analyzed by ELISA and sequencing. KDR positive isolates were assayed further for competition with 50 μM free parent peptide. Those peptides that showed minimal competition with the parent peptide were deemed higher affinity binders and were synthesized. These sequences are listed in the following table as SEQ ID NOS:22-33 for the TN8 secondary library and SEQ ID NOS:89-95 for the TN12 secondary library.

Table 1: TN8/IXLibrary Isolates

Sequence	SEQ ID NO:	Elution	Class

DSWCSTEYTYCEMI	20	1 HR	NA
PKWCEEDWYYCMIT	21	1 HR	(III)
SDWCRVDWYYCWLM	22	O/N	III
ANWCEEDWYYCFIT	23	O/N	III
ANWCEEDWYYCWIT	24	O/N	III
PDWCEEDWYYCWIT	25	O/N	III
SNWCEEDWYYCYIT	26	O/N	III
PDWCAADWYYCYIT	27	O/N	III
PEWCEVDWYYCWLL	28	CELL	III
PTWCEDDWYYCWLF	29	O/N	III
SKWCEQDWYYCWLL	30	CELL	III
RNWCEEDWYYCFIT	31	O/N	III
VNWCEEDWYYCWIT	32	O/N	III
ANWCEEDWYYCYIT	33	O/N	III
VWECAKTFPFCHWF	34	1 HR	I
VTVCYEGTRICEWH	35	1 HR	NA
WVECRYSTGLCINY	36	O/N	NA
WYWCDYYGIGCKWT	37	1 HR	NA
WVECWWKSGQCYEF	38	1 HR, CELL	(II)
WIQCDMETGLCTHG	39	1 HR, CELL	II
WVECFMDTGACYTF	40	CELL, O/N	II
WLECYAEFGHCYNF	41	CELL, O/N	II
WIECDMLTGMCKHG	42	CELL	NA
SVECFMDTGACYTF	43	CELL	I
WIQCNSITGHCTSG	44	CELL	II
WIECYHPDGICYHF	45	CELL	(III)
QAWVECYAETGYCWPRSW	46	NA	NA
VGWVECYQSTGFCYHSRD	47	NA	NA
FTWVECHQATGRCVEWTT	48	NA	NA
DWWVECRVGTGLCYRYDT	49	NA	NA
DSWVECDAQTGFCYSFLY	50	NA	NA
GGWVECYWATGRCIEFAG	51	NA	NA
ERWVECRAETGFCYTWVS	52	NA	NA
GGWVECRAETGHCQEYRL	53	NA	NA
VAWVECYQTTGKCYTFRG	54	NA	NA
EGWVECFANTGACFTYPR	55	NA	NA
GVECYKHSGMCRSW	56	O/N	II
GVWCDMVTGWCYHG	57	CELL	II
WIECHYKTGHCIHS	58	CELL	II
DFNCKMIDGFCLLK	59	1 HR	II
WIQCDRKAGRCSRG	60	CELL	II
TITCWMDTGHCMHE	61	CELL	II
GINCYPATGKCQMG	62	CELL	II
WTECHYATGKCHSF	63	CELL	II
LNICKEDWYYCFLL	64	1 HR	I/III
GITCYSATGKCQMW	65	CELL	II
WVQCASDTGKCIMG	66	CELL	II
TGNCQEDWYYCWYF	67	CELL	II
KELCEDDWYYCYLM	68	1 HR	I/III
HWECYSDTGKCWFF	69	O/N	II
GITCYSDTGKCFSF	70	CELL	II

AVTCWALTGHCVEE	71	O/N	II
YVDCYYDTGRCYHQ	72	CELL	II
WYWCQYHGVCPQS*	73	1 HR	I/III
LVMCISPEGYCYEI	74	O/N	II
LIECYAHTGLCFDF	75	O/N	II
HWWCAFQPQECEYW	76	1 HR	III
HYECWYPEGKCYFY	77	CELL	II
WYWCHHIGMYCDGF	78	1 HR	III
WEWCPIDAWECIML	79	1 HR	II
WLECYTEFGHCYNF	80	1 HR	II
WVECWWKYGQCYEF	81	1 HR	II
PNTCETFDLYCWWI	82	1 HR	II
WIICDGNLGWCWEG	83	O/N	II
GEQCSNLAVACCST	84	O/N	II
WVECYDPWGWCWEW	85	CELL	NA
WYWCMHYGLGCPYR	86	CELL	NA

Table 2: TN12/I Library Isolates*

Sequence	SEQ ID NO:	Elution	Class
YPWCHELSDSVTRFCVPW	87	1 HR	(III)
SRVCWEDSWGGEVCFRY	88	1 HR	(III)
SRVCWEYSWGGEVCYRV	89	O/N	III
FGECWEYFWGGEFCLRV	90	CELL	III
WRICWESSWGGEVCIGH	91	CELL	III
YGVCWEYSWGGEVCLRF	92	CELL	III
SSVCFEYSWGGEVCFRY	93	CELL	III
SRVCWEYSWGGQICLGY	94	CELL	III
FSVCWEYSWGGEVCLRQ	95	CELL	III
DHMCRSPDYQDHVFCMYW	96	CELL	(II)
PPLCYFVGTQEWHHCNPF	97	CELL	(II)
WWECKREEYRNTTWCAWA	98	CELL	II
DSYCMMNEKGWWNCYLY	99	CELL	NA
PAQCWESNYQGIFFCDNP	100	CELL	II?
GSWCEMRQDVGKWNCFSD	101	CELL	II
GWACAKWPWGGEICQPS	102	CELL	(II)
ASTCVFHDHPYFPMCQDN	103	CELL	I/III
PDTCTMWGDSGRWYCFPA	104	CELL	(II)
NWKCEYTQGYDYTECVYL	105	O/N	II
NWECGWSNMFQKEFCARP	106	1 HR	(III)
SGYCEFESDTGRWFCSSW	107	O/N	II
GGWCQLVDHSWWWCGDS	108	O/N	II
DNWCEIVVEKGQWFCYGS	109	O/N	II
YPGCYETSLSGVWFCADG	110	CELL	II
GWCQMDAQGIWSCWAD	111	1 HR	II
DRWCMLDQEKGWWLCGPP	112	CELL	II
NSECGCPNMLHKEFCARH	113	1 HR	I/III
PFWCKFQQSKAMFPCSWF	114	1 HR	II
YPWCHEHSDSVTRFCVPW	115	1 HR	III
SDLCYNQSGWWELCYFD	116	O/N	I/II?
LGYCMYDYENRGWTCYPP	117	O/N	II
YYQCQRYWDGKTWWCEYN	118	1 HR	I/III

DSWCELEHQSGIWRCDFW	119	CELL	II
DWACDEYWSAYSVLCKHP	120	CELL	II
LSLCYNDMHGWWEHCQWY	121	CELL	II
YSHCIETSMENIWFCDFD	122	CELL	II
PPFCIYQEPSGQWWCYDH	123	CELL	II
PGWCDFSPQLGQWMCDWF	124	CELL	II
LDNCIWNVWKGVQDCEYS	125	O/N	II
AGWCEYVAPQGAWRCFHN	126	CELL	II
WDDCIWHMWLKKKDCNSG	127	O/N	II
PGHCEYIWIDEQPWCVRL	128	CELL	III
YSDCLFQLWKGSVCPPS	129	CELL	II
YFFCSFADVAYESCHPL	130	CELL	NA
NYMCESEDHTYMFPCWWY	131	CELL	NA
DAVCYNPWFKYWETCEYN	132	CELL	NA
NYMCEYEDHTYMLTCECN	133	CELL	NA
WDDCIYSMWMVHTVCDR	134	CELL	NA
NWKCDAHQEGRIHICWGY	135	CELL	NA
NGSCWYDFGWETEICFHN	136	CELL	II

Table 3: Lin20 Library Isolates*

Sequence	SEQ ID NO:	Elution	Class
QVQYQFFLGTPRYEQWDLDK	137	CELL	II
EPEGYAYWEVITLYHEEDGD	138	CELL	(II)
WYYDWFHNQRKPPSDWIDNL	139	1 HR	III
AFPRFGGDDYWIQQYLRYTD	140	1 HR	(III)
GDYVYWEIIELTGATDHTPP	141	O/N	(III)
RGDYQEQYWHQQLVEQLKLL	142	1 HR	(III)
RSWYLGPPYYEEWDPIPN	143	CELL	II
PSNSWAAVWEDDMQRLMRQH	144	CELL	II
PRLGDDFEEAPPLEWWWAHF	145	CELL	II
MPPGFSYWEQVVLHDDAQVL	146	CELL	II
KKEDAQQWYWTDYVPSYLYR	147	1 HR	III?
WVTKQQFIDTYGRKEWTILF	148	CELL	II
WLYDYWDRQQKSEEFKFWSQ	149	1 HR	III
PVTDWTPHHPKAPDVWLFYT	150	1 HR	III?
EWYWTEHVGMKHGFFV	151	1 HR	I/III
DALEAPKRDWYYDWFLNHSP	152	1 HR	III
PDNWKEFYESGWKYPSLYKPL	153	1 HR	NA
EWDAQYWHDLRQQYMLDYIQ	154	1 HR	I/III
AFEIEYWDSVRNKIWQHFPD	155	1 HR	I/III
AFPRFGGDDYWIQQYLRYTF	156	1 HR	I/III
AHMPPWRPVAVDALFDWVE	157	CELL	NA
AHMPPWWPLAVDAQEDWFE	158	CELL	NA
AQMPPWWPLAVDALFDWFE	159	CELL	II
ARMGDDWEEAPPHEWGWADG	160	CELL	II
DWYWQRERDKLREHYDDAFW	161	1 HR	I/III
DWYWREWMPMHAQFLADDW	162	1 HR	I/III
DWYYDEILSMADQLRHAFLS	163	1 HR	III
EEQQALYPGCEPAEHWVYAG	164	1 HR	III
FDVVNWGDGIWYAYPS	165	CELL	II
FPSQMWQQKVSHHFFQHKGY	166	CELL	II

GSDHVRVDNYWWNGMAWEIF	167	1 HR	II
ISPWREMSGWGMPWITAVPH	168	1 HR	I/III
LEEVFEDFQDFWYTEHIIVDR	169	1 HR	II
MPPGFSYWEQAALHDDAQDL	170	CELL	II
PEDSEAWYWLNYRPTMFHQL	171	1 HR	I/III?
QIEYVNDKWYWTGGYWNVPF	172	1 HR	II
QVQYQFILGTPRYEQWDPDK	173	CELL	II
RDEWGWTGVPYEGEMGYQIS	174	1 HR	II
STNGDSFVYWEEVELVDHPY	175	O/N	II
SYEQWLPQYWAQYKSNYFL	176	1 HR	I/III?
TKWGPNPEHWQYWYSHYASS	177	1 HR	I/III?
VSKGSIDVGEGISYWEIIEL	178	1 HR	III
WESDYWDQMRQQLKTAYMKV	179	1 HR	I/III
WYHDGLHNERKPPSHWIDNV	180	1 HR	III
APAWTFGTNWRSIQRVDSLT	181	CELL	NA
EGWFRNPQEIMGFGDSWDKP	182	CELL	NA
GWDLSVNRDKRWFWPWSSRE	183	CELL	NA
KSGVDAVGWHIPVWLKKYWF	184	CELL	NA
GMDLYQYWASDDYWGRHQEL	185	CELL	NA
GVDIWHYWKSSTRYFHQ	186	CELL	NA

Table 4: TN7/IV Library Isolates

Sequence	SEQ ID NO:	Elution	Class
GVECNHMGLCVSW	187	CELL	II
GITCDELGRCVHW	188	CELL	II
WIQCNHQGQCFHG	189	CELL	II
WIECNKDGKCWHY	190	CELL	II
WVECNHKGLCREY	191	CELL	II
WYWCEFYGVCSEE	192	1 HR	I/III

Table 5: TN9/IV Library Isolates

Sequence	SEQ ID NO:	Elution	Class
IDFCKGMAPWLCADM	193	1 HR	(III)
PWTCWLEDHLACAML	194	CELL	II
DWGCSLGNWYWCSTE	195	CELL	NA
MPWCSEVTWGWCKLN	196	CELL	II
RGPCSGQPWHLCYYQ	197	O/N	II
PWGCDHFGWAWCKGM	198	O/N	NA
MPWCVEKDHWDCWWW	199	CELL	NA
PGPCKGYMPHQCWYM	200	CELL	NA
YGPCAEMSPWLCWYP	201	CELL	NA
YGPCKNMPPWMCWHE	202	CELL	NA
GHPCKGMLPHTCWYE	203	CELL	NA

Table 6: TN10/IX Library Isolates

Sequence	SEQ ID NO:	Elution	Class
NNSCWLSTTLGSCFFD	204	O/N	NA
DHHCYLHNGQWICYPF	205	CELL	(III)
NSHCYIWDGMWLCFPD	206	CELL	(II)

Table 7: MTN13/I Library Isolates

Sequence	SEQ ID NO:	Elution	Class
SNKCDHYQSGPHGKICVNY	207	CELL	NA
SNKCDHYQSGPYGEVCFNY	208	CELL	NA
RLDCDKVFSGPYGKVCVSY	209	CELL	NA
RLDCDKVFSGPDTSCGSQ	210	CELL	NA
RLDCDKVFSGPHGKICVRY	211	CELL	NA
RLDCDKVFSGPHGKICVNY	212	CELL	NA
RVDCDKVISGPHGKICVNY	213	CELL	NA
RTTCHHQISGPHGKICVNY	214	CELL	NA
EFHCHHIMSGPHGKICVNY	215	CELL	NA
HNRCDFKMSGPHGKICVNY	216	CELL	NA
WQECTKVLSGPGTFECSYE	217	CELL	NA
WQECTKVLSGPGQFSCVYG	218	CELL	NA
WQECTKVLSGPGQFECEYM	219	CELL	NA
WQECTKVLSGPNSFECKYD	220	CELL	NA
WDRCERQISGPGQFSCVYG	221	CELL	NA
WQECTKVLSGPGQFLCSYG	222	CELL	NA
RLDCDMVFSGPHGKICVNY	223	CELL	NA
KRCDTTHSGPHGIVCVVY	224	CELL	NA
SNKCDHYQSGPYGAVCLHY	225	CELL	NA
SPHCQYKISGPFGPVCVNY	226	CELL	NA
AHQCHHWTSGPYGEVCFNY	227	CELL	NA
YDKCSSRFSGPFGEICVNY	228	CELL	NA
MGGCDFSFSGPFGQICGRY	229	CELL	NA
RTTCHHQISGPFGDVCVSY	230	CELL	NA
WYRCDFNMSGPDFTECLYP	231	CELL	NA
WMQCNMSASGPKDMYCEYD	232	CELL	NA
GISCKWIWSGPDRWKCHHF	233	CELL	NA
WQVCKPYVSGPAAFSCKYE	234	CELL	NA
GWWCYRNDSGPKPFHCRIK	235	CELL	NA
EGWCWFIDSGPWKTWCEKQ	236	CELL	NA
FPKCKFDFSGPPWYQCNTK	237	CELL	NA
RLDCDKVFSGPYGRVCVKY	238	CELL	NA
RLDCDKVFSGPYGNVCVNY	239	CELL	NA
RLDCDKVFSGPSMGTCKLQ	240	CELL	NA
RTTCHHHISGPHGKICVNY	241	CELL	NA
QFGCEHIMSGPHGKICVNY	242	CELL	NA
PVHCSHTISGPHGKICVNY	243	CELL	NA
SVTCHFQMSGPHGKICVNY	244	CELL	NA
PRGCQHMISGPHGKICVNY	245	CELL	NA
RTTCHHQISGPHGQICVNY	246	CELL	NA
WTICHMELSGPHGKICVNY	247	CELL	NA
FITCALWLSGPHGKICVNY	248	CELL	NA
MGGCDFSFSGPHGKICVNY	249	CELL	NA
KDWCHTTFSGPHGKICVNY	250	CELL	NA
AWGCDNMMSGPHGKICVNY	251	CELL	NA
SNKCDHIMSGPHGKICVNY	252	CELL	NA
SNKCDHYQSGPFGDICVMY	253	CELL	NA
SNKCDHYQSGPFGDVCVSY	254	CELL	NA
SNKCDHYQSGPFGDICVSY	255	CELL	NA
RTTCHHQISGPFGPVCVNY	256	CELL	NA

RTTCHHQISGPYGDICVKY	257	CELL	NA
PHGKICVNYGSESADPSYIE	258	CELL	NA
RYKCPRDLSGPPYGPCSPQ	259	CELL	NA

* During the course of DNA synthesis, there is always a small percentage of incomplete couplings at each cycle. Since the libraries used for these experiments were constructed using TRIM technology to couple trinucleotides (codons) instead of nucleotides, the library template DNA often has a small percentage of deleted codons. In the case of the TN12 library, for instance, it has been observed that approximately 5.3% of the total library is phage expressing a cyclic 11-mer, rather than a 12-mer, and indeed some phage expressing 11-mers were isolated in the selections described above (see Table 2).

In the foregoing tables, Class I peptides only bind KDR in the absence of heparin, and therefore presumably target the heparin binding domain of KDR; Class II peptides bind in the presence or absence of heparin or VEGF, and therefore presumably bind at a non-involved site on KDR; Class III peptides exhibit binding characteristics that are not affected by heparin but are perturbed in the presence of VEGF, and therefore presumably these bind either to VEGF or the VEGF binding domain of KDR. NA signifies data not available. In the elution column, 1 HR, O/N, and Cell stand for 1 hour VEGF, overnight VEGF, and bead infection elutions, respectively. In some cases, a particular isolate sequence was observed in two different elutions. For the isolates identified by second generation library, VEGF elutions were substituted with peptide elutions (see below).

Example 2: Peptide Synthesis and Fluorescein Labeling

Selected KDR or VEGF/KDR complex binding peptides corresponding to positive phage isolates were synthesized on solid phase using 9-fluorenylmethoxycarbonyl protocols and purified by reverse phase chromatography. Peptide masses were confirmed by electrospray mass spectrometry, and peptides were quantified by absorbance at 280 nm. For synthesis, two N-terminal and two C-terminal amino acids from the phage vector sequence from which the peptide was excised were retained and a –Gly-Gly-Gly-Lys-NH_2 linker (SEQ ID NO:262) was added to the C-terminus of each peptide. Each peptide was N-terminally acetylated. For peptides with selected lysine residues, these were protected with 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde), which allows

selective coupling to the C-terminal lysine, is not removed during peptide cleavage, and can be removed after coupling with 2% hydrazine in DMF or 0.5 M hydroxylamine, pH 8, in water.

Each peptide was labeled with fluorescein on the C-terminal lysine using fluorescein (N-hydroxysuccinimide ester derivative) or fluorescein isothiocyanate (FITC) in DMF, 2% diisopropylethylamine (DIPEA). If the peptide contained an ivDde protected lysine, the reaction was quenched by the addition of 2% hydrazine, which reacts with all free NHS-fluorescein and removes the internal protecting group. For all other peptides, the reaction was quenched by the addition of an equal volume of 0.5 M hydroxylamine, pH 8. The quenched reactions were then diluted with water to less than 10% DMF and then purified using C18 reverse phase chromatography. The peptides were characterized for purity and correct mass on an LC-MS system (HP1100 HPLC with in-line SCIEX AP150 single quadrapole mass spectrometer).

Example 3: Fluorescence Anisotropy Measurements and BiaCore Assays

Fluorescence anisotropy measurements were performed in 384-well microplates in a volume of 10 µl in binding buffer (PBS, 0.01% Tween-20, pH 7.5) using a Tecan Polarion fluorescence polarization plate reader. In some cases, heparin (0.5 µg/ml) or 10% human serum was added to the binding buffer (data not shown). The concentration of fluorescein labeled peptide was held constant (20 nM) and the concentration of KDR-Fc (or similar target) was varied. Binding mixtures were equilibrated for 10 minutes in the microplate at 30°C before measurement. The observed change in anisotropy was fit to the equation below via nonlinear regression to obtain the apparent K_D. This equation (1) assumes that the synthetic peptide and KDR form a reversible complex in solution with 1:1 stoichiometry.

$$r_{obs} = r_{free} + \left(r_{bound} - r_{free}\right)\frac{\left(K_D + KDR + P\right) - \sqrt{\left(K_D + KDR + P\right)^2 - 4 \cdot KDR \cdot P}}{2 \cdot P}$$

(1),

where r_{obs} is the observed anisotropy, r_{free} is the anisotropy of the free peptide, r_{bound} is the anisotropy of the bound peptide, K_D is the apparent dissociation constant, KDR is the total KDR concentration, and P is the total fluorescein-labeled peptide

concentration. K_D was calculated in a direct binding assay ($K_{D,B}$) (see Table 8), and therefore these values represent KDR binding to the fluorescein labeled peptide.

For BiaCore determinations of K_D, KDR-Fc(or other protein targets) was cross-linked to the dextran surface of a CM5 sensor chip by the standard amine coupling procedure (0.5 mg/ml solutions diluted 1:20 with 50 mM acetate, pH 6.0, R_L KDR-Fc = 12859). Experiments were performed in HBS-P buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% polysorbate 20 (v/v)). Peptide solutions quantitated by extinction coefficient were diluted to 400 nM in HBS-P. Serial dilutions were performed to produce 200, 100, 50, and 25 nM solutions. For association, peptides were injected at 20 µl/min. for 1 minute using the kinject program. Following a 1-minute dissociation, any remaining peptide was stripped from the target surface with a quick injection of 1M NaCl for 25 sec. at 50 µl/min. All samples were injected in duplicate. Between each peptide series a buffer injection and a non-target binding peptide injection served as additional controls. Sensorgrams were analyzed using the simultaneous k_a/k_d fitting program in the BIAevaluation software 3.1. Apparent K_D by this method is set forth as BiaK_D in Table 8. Unlike the fluorescence anisotropy experiments above, the unlabeled peptide was used for all testing using this assay and therefore, these values represent KDR binding to the unlabeled peptide. Binding affinities determined for the synthesized polypeptides are set forth in Table 8, below. The putative disulfide-constrained cyclic peptide moieties of the polypeptides are underlined.

Table 8: Binding Affinities for Synthesized Peptides

Sequence	$K_{D,B}$ (µM)	BiaK_D (µM)	SEQ ID NO:
TN8			
AGDSWCSTEYTYCEMIGTGGGK	>2		263
AGPKWCEEDWYYCMITGTGGGK	0.28	0.027	264
AGVWECAKTFPFCHWFGTGGGK	2.60		265
AGWVECWWKSGQCYEFGTGGGK	1.3		266
AGWLECYAEFGHCYNFGTGGGK	>10		267
AGWIQCNSITGHCTSGGTGGGK	0.24		268
AGWIECYHPDGICYHFGTGGGK	0.32	0.32	269
AGSDWCRVDWYYCWLMGTGGGK	0.064		270
AGANWCEEDWYYCFITGTGGGK	0.310		271
AGANWCEEDWYYCWITGTGGGK	0.097		272
AGPDWCEEDWYYCWITGTGGGK	0.075		273
AGSNWCEEDWYYCYITGTGGGK	0.046		274

AGPDWCAADWYYCYITGTGGGK	0.057		275
AGPEWCEVDWYYCWLLGTGGGK	0.075		276
AGPTWCEDDWYYCWLFGTGGGK	0.0032	0.079	277
AGSKWCEQDWYYCWLLGTGGGK	0.400		278
AGRNWCEEDWYYCFITGTGGGK	0.190		279
AGVNWCEEDWYYCWITGTGGGK	0.260		280
AGANWCEEDWYYCYITGTGGGK	0.180		281
AGQAWVECYAETGYCWPRSWGTGGGK	0.71		282
AGQAWIECYAEDGYCWPRSWGTGGGK	1.40		283
AGVGWVECYQSTGFCYHSRDGTGGGK	1.30		284
AGFTWVECHqATGRCVEWTTGTGGGK	2.00		285
AGDWWVECRVGTGLCYRYDTGTGGGK	0.93		286
AGDSWVECDAQTGFCYSFLYGTGGGK	2.30		287
AGGGWVECYWATGRCIEFAGGTGGGK	NB		288
AGERWVECRAETGFCYTWVSGTGGGK	2.10		289
AGGGWVECRAETGHCQEYRLGTGGGK	1.60		290
AGVAWVECYQTTGKCYTFRGGTGGGK	~2		291
AGEGWVECFANTGACFTYPRGTGGGK	2.10		292
TN12			
GDYPWCHELSDSVTRFCVPWDPGGGK	0.98	0.18	293
GDSRVCWEDSWGGEVCFRYDPGGGK	0.069	0.12	294
GDDHMCRSPDYQDHVFCMYWDPGGGK	0.48	0.14	295
GDPPLCYFVGTQEWHHCNPFDPGGGK	0.60		296
GDDSYCMMNEKGWWNCYLYDPGGGK	1.3		297
GDPAQCWESNYQGIFFCDNPDPGGGK	2.3		298
GDGSWCEMRQDVGKWNCFSDDPGGGK	0.62	0.18	299
GDGWACAKWPWGGEICQPSDPGGGK	1.0	1.5	300
GDPDTCTMWGDSGRWYCFPADPGGGK	0.49	0.26	301
GDNWKCEYTQGYDYTECVYLDPGGGK	0.82		302
GDNWECGWSNMFQKEFCARPDPGGGK	0.21	0.99	303
GDWWECKREEYRNTTWCAWADPGGGK			486
GDSSVCFEYSWGGEVCFRYDPGGGK	0.058		487
GDSRVCWEYSWGGQICLGYDPGGGK	0.32		488
Lin20			
AQQVQYQFFLGTPRYEQWDLDKGGK	1.7		304
AQEPEGYAYWEVITLYHEEDGDGGK	0.27	0.73	305
AQAFPRFGGDDYWIQQYLRYTDGGK	0.53	0.25	306
AQGDYVYWEIIELTGATDHTPPGGK	0.18		307
AQRGDYQEQYWHQQLVEQLKLLGGK	0.31	5.3	308
AQRSWYLGPPYYEEWDPIPNGGK	1.8		309
AQDWYYDEILSMADQLRHAFLSGGGK		0.05	310
TN9			
AGIDFCKGMAPWLCADMGTGGGK	0.73	0.18	311
AGPWTCWLEDHLACAMLGTGGGK	3.9		312
AGDWGCSLGNWYWCSTEGTGGGK	2.0		313
TN10			
GSDHHCYLHNGQWICYPFAPGGGK	0.26	0.15	314
GSNSHCYIWDGMWLCFPDAPGGGK	0.74		315

MTN13			
SGRLDCDKVFSGPYGKVCVSYGSGGGK	1.05		316
SGRLDCDKVFSGPHGKICVNYGSGGGK	~2		317
SGRTTCHHQISGPHGKICVNYGSGGGK	0.65		318
SGAHQCHHWTSGPYGEVCFNYGSGGGK	~2		319

For the analysis of those peptides that bind specifically to KDR/VEGF complex, each peptide was tested for binding to the complex in both assays (fluorescence anisotropy/Biacore) as above. In the anisotropy assay, KDR-VEGF complex was formed by mixing together a two fold molar excess of VEGF with KDR-Fc. This mixture was then used in the direct binding titration using a fluorescein labeled peptide as done previously. As a control, each peptide was also tested for binding to KDR and VEGF alone to assess their specificity for complex. Since none of the peptides bound VEGF to any extent, the presence of excess VEGF in the assay should not affect the K_D determination. As shown in Table 9, below, all of the peptides showed a dramatic binding preference, binding for KDR/VEGF complex over VEGF. Some of them, however, did show some residual binding to free KDR. To confirm the anisotropy results, the unlabeled peptides were tested in Biacore as before, except the chip was saturated with VEGF to form KDR/VEGF complex prior to the injection of the peptides. In the peptides tested, the BiaK_D was within at least 2-fold of the anisotropy measurement.

Table 9: KDR/VEGF Complex Specific Peptides

SEQ ID NO:	Sequence	K_D,B (KDR)	K_D,B (VEGF)	K_D,B (KDR/VEGF)	BiaK_D (KDR/VEGF)
320	AGMPWCVEKDHWDCWWWGTGGGK	NB	10	0.14	
321	AGPGPCKGYMPHQCWYMGTGGGK	0.4	NB	0.06	0.08
322	AGYGPCAEMSPWLCWYPGTGGGK	3.7	NB	0.13	
323	AGYGPCKNMPPWMCWHEGTGGGK	1.8	NB	0.18	0.42
324	AGGHPCKGMLPHTCWYEGTGGGK	>10	NB	3.3	
325	AQAPAWTFGTNWRSIQRVDSLTGGGGGK	NB	NB	0.84	
326	AQEGWFRNPQEIMGFGDSWDKPGGGGGK	NB	NB	1.4	

The putative disulfide-constrained cyclic peptide moiety is underscored.

METHODS FOR EXAMPLES 4-10

The following methods were employed in Examples 4-10. The following common abbreviations are used: 9-fluorenylmethyloxycarbonyl (Fmoc), 1-hydroxybenzotriazole (HOBt), N,N'-diisopropylcarbodiimide (DIC), N-methylpyrrolidinone (NMP), acetic anhydride (Ac_2O), (4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde), trifluoroacetic acid (TFA), Reagent B (TFA: H_2O: phenol: triisopropylsilane 88:5:5:2), diisopropylethylamine (DIEA), O-(1H-benzotriazole-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), N-hydroxysuccinimide (NHS), solid phase peptide synthesis (SPPS), dimethyl sulfoxide (DMSO), dichloromethane (DCM), dimethylformamide (DMF), human serum albumin (HSA), and radiochemical purity (RCP).

Method 1 for the ACT 357 MPS and ACT 496 MOS Synthesizers

The peptides were synthesized on NovaSyn TGR (Rink amide) resin (0.2 mmol/g) using the Advanced ChemTech ACT 357 or ACT 496 Synthesizers employing Fmoc peptide synthesis protocols, specifically using, HOBt/DIC as the coupling reagents and NMP as the solvent. The Fmoc was removed by treating the Nova-Syn TGR (Rink amide-available from NovaBiochem, San Diego CA) resin-bound peptide with 25% piperidine in DMF twice (4 min and 10 min). All amino acids were dissolved in NMP (DMF was added when the amino acid was not soluble in pure NMP). The concentration of the amino acid was 0.25M, and the concentrations for HOBt and DIC respectively were 0.5 M.

For a 0.04 mmol scale synthesis:

A typical amino acid coupling cycle (not including wash steps) was to dispense piperidine solution (2.4 mL) to each well and mix for 4 min, then empty all wells. NMP (320 µL), HOBt solution (320µL, 4 eq), amino acid (640µL, 4 eq) and DIC (320 µL, 4 eq) solutions were dispensed to each well. The coupling time was 3h; then the resin was washed. The cycle was repeated for each amino acid. After the last amino acid coupling, the resin-bound peptide was treated with 25% piperidine to remove the Fmoc protecting group. After washing, the resin bound peptide was capped with 1.0M Ac_2O (1.2 ml per well) and diisopropylethylamine in DMF, optionally including varying amounts of HOBt in the mixture for 30 min. The resin was washed with methanol and then dichloromethane and dried. Cleavage of

the peptides from the resin and side-chain deprotection was accomplished using Reagent B for 4.5 h. The cleavage solutions were collected and the resins were washed with an additional aliquot of Reagent B. The combined solutions were concentrated to dryness. Ether was added to the residue with swirling or stirring to precipitate the peptides. The ether was decanted, and solid was collected. This procedure was repeated 2-3 times to remove impurities. The crude linear peptides were dissolved in DMSO and water mixtures, and purified by HPLC (column: Waters Associates Xterra C18, 19 × 50 mm; solvents: H_2O with 0.1% TFA and CH_3CN with 0.1% TFA; UV 220 μm; Flow rate: 50-60 ml/min). The solutions containing the peptide were lyophilized to give the desired peptides as white fluffy lyophilizates (> 90% purity). The purified linear di-cysteine containing peptides were dissolved in water, mixtures of water-acetonitrile, or mixtures of water-DMSO at concentrations between 0.1 mg/ml and 2.0 mg/ml. The choice of solvent was a function of the solubility of the crude peptide in the solvent. The pH of the solution was adjusted to pH 7.5-8.5 with aqueous ammonia, aqueous ammonium carbonate or aqueous ammonium bicarbonate. The mixture was stirred vigorously in air for 24-48 hrs. In the case of non-DMSO containing solvent systems, the pH of the solution was adjusted to pH 2 with aqueous trifluoroacetic acid. The mixture was lyophilized to provide the crude cyclic disulfide containing peptide. The cyclic disulfide peptide was then dissolved to a volume of 1-2 ml in aqueous (0.1% TFA) containing a minimum of acetonitrile (0.1% TFA). The resulting solution was loaded onto a reverse phase column and the desired compound obtained by a gradient elution of acetonitrile into water, employing a C18, or C8 reverse phase semipreparative or preparative HPLC column. In the case of the DMSO-containing solutions, the solution was diluted until the DMSO concentration was minimal without precipitation of the peptide. The resulting mixture was quickly acidified to pH 2 with dilute trifluoroacetic acid and loaded onto the reverse phase HPLC system and purified as described. Fractions containing the desired materials were pooled and the peptides isolated by lyophilization.

Method 2 for the ACT 357 MPS and ACT 496 MOS Synthesizers

The peptides were synthesized as in Method 1 with the following changes. HBTU/HOBt/DIEA were used as the coupling reagent and NMP as the solvent. A low load (~0.2 mmol/g) Fmoc-GGGK(Boc)-NovSyn-TGR-resin-prepared from the

above-described Nova-Syn TGR resin was employed for peptide synthesis on 0.01 mmol scale.

For a 0.01mmol scale synthesis:

After the Fmoc group was removed, a standard coupling procedure used a solution of HOBt (720 µl, 6 eq), amino acid (804µl, 6.6 eq), HBTU (720µl, 6 eq) and DIEA (798 µl, 13.3 eq). The mixture was agitated for 15 min., emptied and the resin washed. After all couplings and after cleavage and purification as above, the solutions containing desired linear peptides were lyophilized to give the peptides (> 90% purity) as white fluffy solids. The crude ether-precipitated linear di-cysteine containing peptides were cyclized by dissolution in water, mixtures of aqueous acetonitrile (0.1% TFA), or aqueous DMSO and adjustment of the pH of the solution to pH 7.5 – 8.5 by addition of aqueous ammonia, aqueous ammonium carbonate, or aqueous ammonium bicarbonate solution. The peptide concentration was between 0.1 and 2.0 mg/ml. The mixture was stirred in air for 24-48 hrs., acidified to a pH 2 with aqueous trifluoroacetic acid, and then purified by preparative reverse phase HPLC employing a gradient of acetonitrile into water. Fractions containing the desired material were pooled and the peptides were isolated by lyophilization.

Method 3 for the ACT 496 MOS Synthesizer

The peptides were synthesized by using an Advanced ChemTech ACT 496 MOS Synthesizer as in method 1. The low load (~0.2 mmol/g) GGGK(Boc)-NovaSyn-TGR resin was employed for peptide synthesis. The coupling solvent was NMP/DMSO 8:2. The synthesis was performed at a 0.02 mmol scale using a coupling time of 3h. The crude linear peptides were further processed as described for Method 1.

Method 4 for the ACT 496 MOS Synthesizer

The peptides were synthesized using method 3 on the ACT 496 with HBTU/DIEA as the coupling reagents, and NMP as the solvent. 2,4,6-collidine as a 1 M solution was used as the base. The low load Fmoc-GGGK(ivDde)-Novsyn-TGR resin (~0.2 mmol/g) was used for peptide synthesis. The coupling time was 30 minutes. The crude linear peptides were further processed as described for Method 1.

Method 5 for the ABI 433A Synthesizer

Synthesis of peptides was carried out on a 0.25 mmol scale using the FastMoc protocol (Applied Biosystems Inc). In each cycle of this protocol, 1.0 mmol of a dry protected amino acid in a cartridge was dissolved in a solution of 0.9 mmol of HBTU, 2 mmol of DIEA, and 0.9 mmol of HOBt in DMF with additional NMP added. The peptides were made using 0.1 mmol of NovaSyn TGR (Rink amide) resin (resin substitution 0.2 mmol/g). The coupling time in this protocol was 21 min. Fmoc deprotection was carried out with 20% piperidine in NMP. At the end of the last cycle, the synthesized peptide was acetylated using acetic anhydride/DIEA/HOBt/NMP. The peptide resin was washed and dried for further manipulations or cleaved from the resin (using reagent B). Generally, the cleaved peptides were cyclized as in Method 1 before purification.

Method 6: Biotinylation of Resin-Bound Peptides

The peptides were prepared using Method 5. The ivDde protecting group on the C-terminal lysine was selectively removed by treatment with 10% hydrazine in DMF. The resin was then treated with a solution of Biotin-N-hydroxysuccinimidyl ester in DMF in the presence of DIEA. After washing, the resin was dried and cleavage was performed using with Reagent B. The resin was filtered off and the filtrate concentrated to dryness. The biotinylated peptide was dissolved in neat DMSO and treated with DIEA and stirred for 4-6 hours to effect disulfide cyclization. The crude mixture was purified by preparative HPLC.

In a typical experiment, 200 mg of the resin -bound peptide was treated with 10% hydrazine in DMF (2 × 20 mL) and washed with DMF (2 × 20 mL) and then with dichloromethane (1 × 20 mL). The resin was resuspended in DMF (10 mL) and treated with a solution of Biotin-NHS ester (0.2 mmol, 5 equivalents) and DIEA (0.2 mmol) and the resin was mixed with the reagents for 4 h. The completion of the reaction was checked by the ninhydrin test. The peptide was then released from the resin by treatment with Reagent B (10 mL) for 4 h. The resin was filtered off, Reagent B was removed *in vacuo* and the peptide was precipitated by addition of anhydrous ether. The solid formed was collected, washed with ether and dried. The solid was dissolved in anhydrous DMSO and the mixture was adjusted to pH 7.5 with DIEA and stirred for 4-6 h to effect disulfide cyclization. The disulfide cyclization reaction was monitored by analytical HPLC. After completion of the

cyclization, the mixture solution was diluted with 25% acetonitrile in water and directly purified by HPLC on a reverse phase C18 column using a gradient of acetonitrile into water (both containing 0.1 % TFA). Fractions were analyzed by analytical HPLC and those containing the pure product were collected and lyophilized to obtain the required biotinylated peptide.

Method 7: Biotinylation of Purified Peptides

The purified peptide (10 mg, prepared by methods 1-5) containing a free amino group was dissolved in anhydrous DMF or DMSO (1 ml) and Biotin-NHS ester (5 equivalents) and DIEA (5 equivalents) were added. The reaction was monitored by HPLC and after the completion of the reaction (1-2 h.), the crude reaction mixture was directly purified by preparative HPLC. Fractions were analyzed by analytical HPLC and those containing the pure product were collected and lyophilized to obtain the required biotinylated peptide.

Method 8: Biotinylation of Resin-Bound Peptides Containing Linkers

In a typical experiment, 400 mg of the resin- containing peptide (made using the ABI 433A Synthesizer and bearing an ivDde-protected lysine) was treated with 10% hydrazine in DMF (2 × 20 ml). The resin was washed with DMF (2 × 20 ml) and DCM (1 × 20 ml). The resin was resuspended in DMF (10 ml) and treated with Fmoc-aminodioxaoctanoic acid (0.4 mmol), HOBt (0.4 mmol), DIC (0.4 mmol), DIEA (0.8 mmol) with mixing for 4 h. After the reaction, the resin was washed with DMF (2 × 10 ml) and with DCM (1 × 10 ml). The resin was then treated with 20% piperidine in DMF (2 × 15 ml) for 10 min. each time. The resin was washed and the coupling with Fmoc-diaminodioxaoctanoic acid and removal of the Fmoc protecting group were repeated once more. The resulting resin, containing a peptide with a free amino group, was treated with a solution of Biotin-NHS ester (0.4 mmol, 5 equivalents) and DIEA (0.4 mmol, 5 equivalents) in DMF for 2 hours. The peptide-resin was washed and dried as described previously and then treated with reagent B (20 mL) for 4h. The mixture was filtered, and the filtrate concentrated to dryness. The residue was stirred with ether to produce a solid that was collected, washed with ether and dried. The solid was dissolved in anhydrous DMSO and the pH adjusted to 7.5 with DIEA. The mixture was stirred for 4-6 hr to effect the disulfide cyclization reaction, which was monitored by analytical HPLC. After the

completion of the cyclization, the DMSO solution was diluted with 25% acetonitrile in water and applied directly to a reverse phase C-18 column. Purification was effected using a gradient of acetonitrile into water (both containing 0.1 % TFA). Fractions were analyzed by analytical HPLC and those containing the pure product were collected and lyophilized to provide the required biotinylated peptide.

Method 9: Formation of 5-Carboxyfluorescein-Labeled Peptides

Peptide-resin obtained via Method 5, containing an ivDde protecting group on the epsilon nitrogen of lysine, was mixed with a solution of hydrazine in DMF (10% hydrazine/DMF, 2 × 10 ml, 10 min) to remove the ivDde group. The epsilon nitrogen of the lysine was labeled with fluorescein-5-isothiocyanate (0.12 mmol) and diisopropylethylamine (0.12 mmol) in DMF. The mixture was agitated for 12 h (fluorescein-containing compounds were protected from light). The resin was then washed with DMF (3 × 10 mL) and twice with CH_2Cl_2 (10 mL) and dried under nitrogen for 1h. The peptide was cleaved from the resin using reagent B for 4h and the solution collected by filtration. The volatiles were removed under reduced pressure and the residue was dried under vacuum. The peptide was precipitated with ether, collected and the precipitate was dried under a stream of nitrogen. The precipitate was added to water (1 mg/ml) and the pH of the mixture was adjusted to 8 with 10% aqueous meglumine. Cyclization of the peptide was carried out for 48 h and the solution was freeze-dried. The crude cyclic peptide was dissolved in water and purified by RP-HPLC on a C_{18} column with a linear gradient of acetonitrile into water (both phases contained 0.1%TFA). Fractions containing the pure product were collected and freeze-dried. The peptides were characterized by ES-MS and the purity was determined by RP-HPLC (linear gradient of acetonitrile into water/0.1% TFA).

Method 10: Preparation of Peptidic Chelate for Binding to Tc
by Coupling of Single Amino Acids

Peptides were synthesized starting with 0.1 mmol of NovaSyn-TGR resin (0.2 mmol/g substitution). Deprotected (ivDde) resin was then treated according to the protocol A for the incorporation of Fmoc-Gly-OH, Fmoc-Cys(Acm)-OH and Fmoc-Ser(tBu)-OH.

Protocol A for manual coupling of single amino acid:

1. Treat with 4 equivalents of corresponding Fmoc-amino acid and 4.1 equivalents of HOBt and 4.1 equivalents of DIC for 5 h.
2. Wash with DMF (3 × 10 mL)
3. Treat with 20% piperidine in DMF (2 × 10 mL, 10 min.)
4. Wash with DMF (3 × 10 mL)

The Fmoc-protected peptide loaded resin was then treated with 20% piperidine in DMF (2 × 10 mL, 10 min.) and washed with DMF (3 × 10 mL). A solution of N,N-dimethylglycine (0.11 mmol), HATU (1 mmol), and DIEA (0.11 mmol) in DMF (10 mL) was then added to the peptide loaded resin and the manual coupling was continued for 5 h. After the reaction the resin was washed with DMF (3 × 10 mL) and CH_2Cl_2 (3 × 10 mL) and dried under vacuum.

Method 11: Formation of Mercaptoacetylated Peptides Using S-Acetylthioglycolic acid N-Hydoxysuccinimide Ester

S-acetylthioglycolic acid N-hydroxysuccinimide ester (SATA) (0.0055mmol) was added to a solution of a peptide (0.005 mmol, obtained from Methods 1-5 with a free amine) in DMF (0.25 mL) and the reaction mixture was stirred at ambient temperature for 6 h. The volatiles were removed under vacuum and the residue was purified by preparative HPLC using acetonitrile-water containing 0.1%TFA. Fractions containing the pure product were collected and freeze-dried to yield the mercaptoacetylated peptide. The mercaptoacetylated peptide was characterized by ESI-MS and the purity was determined by reverse phase HPLC analysis employing a linear gradient of acetonitrile into water (both containing 0.1% TFA).

Method 12: Formation of Mercaptoacetylated Peptides using S-Acetylthioglycolic acid

Purified peptides from method 5, after disulfide cyclization, was coupled with S-acetylthioglycolic acid(1.5-10 eq.)/HOBt (1.5-10 eq.)/DIC (1.5-10 eq.) in NMP for 2-16 hours at room temperature. The mixture was then purified by preparative HPLC; the fractions containing pure peptide were combined and lyophilized. In the case of compounds with another lysine protected by an ivDde group, the deprotection reaction employed 2% hydrazine in DMSO for 3h at room temperature. Purification of the reaction mixture afforded pure peptide.

In the case when a preparing a compound with S-acetylthioglycolic acid

coupled to two aminodioxaoctanoic acid groups and the peptide, the purified peptide from method 5 (having a free amino group, was coupled to $AcSCH_2CO$-$(NH$-CH_2-CH_2-O-CH_2-CH_2-O-CH_2-$CO)_2$-OH (30 eq.)/HOBt (30 eq.)/DIC (30 eq.) in NMP for 40 hours at room temperature. The mixture was purified and the ivDde group was removed. A second purification gave the final product as a white lyophilizate.

Alternatively Fmoc aminodioxaoctanoic acid was coupled twice successively to the peptide (produced by method 5) followed by Fmoc removal and coupling to S-acetylthioglycolic acid.

Method 13: Preparation of Homo and Heterodimers

The required purified peptides were prepared by SPPS using Method 5. To prepare homodimers, half of the peptide needed to prepare the dimer was dissolved in DMF and treated with 10 equivalents of glutaric acid bis N-hydoxysuccinimidyl ester. The progress of the reaction was monitored by HPLC analysis and mass spectroscopy. At completion of the reaction, The volatiles were removed *in vacuo* and the residue was washed with ethyl acetate to remove unreacted bis-NHS ester. The residue was dried, re-dissolved in anhydrous DMF and treated with another half portion of the peptide in the presence of 2 equivalents of DIEA. The reaction was allowed to proceed for 24 h. This mixture was applied directly to a Waters Associates C-18 XTerra reverse phase HPLC column and purified by elution with a linear gradient of acetonitrile into water (both containing 0.1% TFA).

In the case of heterodimers, one of the monomers was reacted with the bis NHS ester of glutaric acid and after washing off the excess of bis NHS ester, the second peptide was added in the presence of DIEA. After the reaction, the mixture was purified by preparative HPLC.

Example 4: Preparation of KDR and VEGF/KDR Complex Binding Polypeptides

Utilizing the methods set forth above, biotinylated versions the KDR and VEGF/KDR complex binding polypeptides set forth in Table 10 were prepared. The letter "J" in the peptide sequences refers to a spacer or linker group, 8-amino-3,6-dioxaoctanoyl.

The ability of the biotinylated polypeptides (with the JJ spacer) to bind to KDR was assessed using the assay set forth in Example 5, following the procedures disclosed therein. Several biotinylated peptides bound well to the KDR-expressing cells: SEQ ID NO:356 (K_D 1.81 nM +/- 0.27), SEQ ID NO:264 (K_D 14.87+/- 5.0

nM, four experiment average), SEQ ID NO:294 + spacer (K_D 10.00+/-2.36 nM, four experiment average), SEQ ID NO:301 (K_D 4.03+/-0.86 nM, three experiment average), SEQ ID NO:337 (K_D 6.94+/-1.94 nM, one experiment), and SEQ ID NO:338 (K_D 3.02+/-0.75 nM, one experiment).

Table 10: KDR, VEGF/KDR Complex Binding Polypeptides

SEQ ID NO:	Structure (or) Sequence	Mol. Wt.	MS
294	Ac-GDSRVCWEDSWGGEVCFRYDPGGGK-NH_2	2801.98	1399.6 $[M-H]^-$
329	Ac-AGMPWCVEKDHWDCWWGTGGGK-NH_2	2730.14	-
311	Ac-AGIDFCKGMAPWLCADMGTGGGK-NH_2	2324.02	-
264	Ac-AGPKWCEEDWYYCMITGTGGGK-NH_2	2361	-
266	Ac-AGWVECWWKSGQCYEFGTGGGK-NH_2	2474.06	-
330	Ac-AQEGWFRNPQEIMGFGDSWDKPGGGK-NH_2	2934.35	-
299	Ac-GDGSWCEMRQDVGK(iv-Dde)WNCFSDDP-GGGK-NH_2	3075.29	1537.5 $[M^{2-}]$
299	Ac-GDGSWCEMRQDVGKWNCFSDDPGGGK-NH_2	2869.16	-
303	Ac-GDNWECGWSNMFQK(iv-Dde)EFCARPDP-GGGK-NH_2	3160.36	1579.6 $[M^{2-}]$
303	Ac-GDNWECGWSNMFQKEFCARPDPGGGK-NH_2	2954.23	-
294	Ac-GDSRVCWEDSWGGEVCFRYDPGGGK(Biotin)-NH_2	3030.29	1512.4 $[M^{2-}]$
331	Ac-AQRGDYQEQYWHQQLVEQLK(iv-Dde)LLGGGK-NH_2	3318.71	1659.1 $[M^{2-}]$
331	Ac-AQRGDYQEQYWHQQLVEQLKLLGGGK-NH_2	3112.58	-
332	Ac-AGWYWCDYYGIGCK(iv-Dde)WTGGGK-NH_2	2673.18	
333	Ac-AGWYWCDYYGIGCKWTGTGGGK-NH_2	2467.05	
334	Ac-AQWYYDWFHNQRKPPSDWIDNLGGGK-NH_2	3218.51	-
323	Ac-AGYGPCKNMPPWMCWHEGTGGGK-NH_2	2502.05	-
335	Ac-AGPKWCEEDWYYCMITGTGGGK(N,N-Dimethyl-Gly-Ser-Cys(Acm)-Gly)-NH_2	2836.204	2833.4 $[M-H]^-$
264	Ac-AGPK(iv-Dde)WCEEDWYYCMITGTGGGK-NH_2	2698.11	2695.7 $[M-H]^-$; 1347.8 $[M-2H]^{2-}/2$
336	Ac-WQPCPWESWTFCWDPGGGK($AcSCH_2C(=O)$-)-NH_2	2422.71	2420.7 $[M-H]^-$, 1209.9 [M-2H]/2
264	Ac-AGPKWCEEDWYYCMITGTGGGK(Biotin)-NH_2	2718.13	2833.4 $(M-H)^-$
264	Ac-AGPKWCEEDWYYCMITGTGGGK(Biotin-JJ-)-NH_2	3008.44	1502.6.4 $(M-2H)^{2-}/2$
264	Ac-AGPKWCEEDWYYCMITGTGGGK ($AcSCH_2C(=O)$-)-NH_2	2608.96	1304, $[M-2H]^{2-}/2$
294	Ac-GDSRVCWEDSWGGEVCFRYDPGGGK(Biotin-JJ-)-NH_2	3316.4	1657.8, $[M-2H]^{2-}/2$
294	Ac-GDSRVCWEDSWGGEVCFRYDPGGGK ($AcSCH_2C(=O)$-)-NH_2	2917.15	1457.4, $[M-2H]^{2-}/2$
294	Biotin-JJGDSRVCWEDSWGGEVCFRYDPGGGK-NH_2	3272.34	1636.7, $[M-2H]^{2-}/2$
264	Ac-AGPKWCEEDWYYCMITGT-GGGK($AcSCH_2C(=O)$-JJ-)-NH_2	2899.28	1449.2, $[M-2H]^{2-}/2$
277	Ac-AGPTWCEDDWYYCWLFGTGGGK(Biotin-JJ-)-NH_2	3066.27	1532.8, $[M-2H]^{2-}/2$
337	Ac-VCWEDSWGGEVCFRYDPGGGK(Biotin-JJ-)-NH_2	2903.24	1449.3, $(M-2H)^{2-}/2$; 965.8, $(M-3H)^{3-}/3$
338	Ac-AGPTWCEDDWYYCWLFGTJK(Biotin-JJ-)-NH_2	3042.44	1519.7, $(M-2H)^{2-}/2$-; 1012.8 $(M-3H)^{3-}/3$
294	Ac-GDSRVCWEDSWGGEVCFRYDPGGGK ($AcSCH_2C(=O)$-JJ-)-NH_2	3208.48	1602.6, $[M-2H]^{2-}/2$

339	Ac-AGPTWCEDDWYYCWLFGTGGGK(N,N-Dimethyl-Gly-Ser-Cys(Acm)-Gly-JJ-)-NH_2	3242.33	1621.5, $[M-2H]^{2-}/2$
277	Ac-AGPTWCEDDWYYCWLFGTGGGK($AcSCH_2C(=O)$-JJ-)-NH_2	2907.29	1453.1, $[M-2H]^{2-}/2$
340	Ac-AQAHMPPWRPVAVDALFDWVEGG-GGGK(Biotin-JJ-)-NH_2	3404.64	1701.6, $[M-2H]^{2-}/2$
341	Ac-AQAHMPPWWPLAVDAQEDWFEGG-GGGK(Biotin-JJ-)-NH_2	3493.59	1746.2, $[M-2H]^{2-}/2$
342	Ac-AQAQMPPWWPLAVDALFDWFEGG-GGGK(Biotin-JJ-)-NH_2	3487.64	1743.2, $[M-2H]^{2-}/2$
343	Ac-AQDWYWREWMPMHAQFLADDWGG-GGGK(Biotin-JJ-)-NH_2	3751.64	1874.3, $[M-2H]^{2-}/2$
344	Ac-AQK(ivDde)K(iv-Dde)EDAQQWYWTDYVPSY-LYRGGGGGK(Biotin-JJ-)-NH_2	4220.06	2108.9, $[M-2H]^{2-}/2$
345	Ac-AQPVTDWTPHHPK(iv-Dde)APDVWLFYT-GGGGGK(Biotin-JJ-)-NH_2	3781.86	1890.4, $[M-2H]^{2-}/2$
346	Ac-AQDALEAPK(iv-Dde)RDWYYDWFLNHSP-GGGGGK(Biotin-JJ-)-NH_2	3897.85	1948.0, $[M-2H]^{2-}/2$
347	Ac-KWCEEDWYYCMITGTGGGK(Biotin-JJ-)-NH_2	2781.2	1390.0, $[M-2H]^{2-}/2$
348	Ac-AGPKWCEEDWYYCMIGGGK(Biotin-JJ-)-NH_2	2747.15	1373.5, $[M-2H]^{2-}/2$
349	Ac-KWCEEDWYYCMIGGGK(Biotin-JJ-)-NH_2	2522.04	1260.8, $[M-2H]^{2-}/2$
350	Ac-AQPDNWK(iv-Dde)EFYESGWK(iv-Dde)-YPSLYK(iv-Dde)PLGGGGGK(Biotin-JJ-)-NH_2	4377.2	2188.4, $[M-2H]^{2-}/2$
351	Ac-AQMPPGFSYWEQVVLHDDAQVLGG-GGGK(Biotin-JJ-)-NH_2	3499.7	1749.2, $[M-2H]^{2-}/2$
352	Ac-AQARMGDDWEEAPPHEWGWADGG-GGGK(Biotin-JJ-)-NH_2	3480.5	1740.2, $[M-2H]^{2-}/2$
353	Ac-AQPEDSEAWYWLNYRPTMFHQLGG-GGGK(Biotin-JJ-)-NH_2	3751.7	1875.8, $[M-2H]^{2-}/2$
354	Ac-AQSTNGDSFVYWEEVELVDHPGG-GGGK(Biotin-JJ-)-NH_2	3554.6	1776.4, $[M-2H]^{2-}/2$
355	Ac-AQWESDYWDQMRQQLK(iv-Dde)TAYMK(iv-Dde)VGGGGGK(Biotin-JJ-)-NH_2	4187.02	2093.0, $[M-2H]^{2-}/2$
356	Ac-AQDWYYDEILSMADQLRHAFLSGGGGGK(Biotin-JJ-)-NH_2	3641.69	1820.9, $[M-2H]^{2-}/2$

The putative disulfide constrained cyclic peptide is indicated by underlining.

Example 5: Binding of KDR binding peptides/avidin HRP complex to KDR transfected 293H cells

To determine the binding of peptides identified by phage display to KDR expressed in transiently-transfected 293H cells, a novel assay that measures the binding of biotinylated peptides complexed with neutravidin HRP to KDR on the surface of the transfected cells was developed. This assay was used to screen the biotinylated peptides set forth in Example 4. Neutravidin HRP was used instead of streptavidin or avidin because it has lower non-specific binding to molecules other than biotin due to the absence of lectin binding carbohydrate moieties and also due to the absence of the cell adhesion receptor-binding RYD domain in neutravidin.

In the experiments described herein, tetrameric complexes of KDR-binding peptides SEQ ID NO:294, SEQ ID NO:264, SEQ ID NO:277 and SEQ ID NO:356 and a control peptide, which does not bind to KDR, were prepared and tested for

their ability to bind 293H cells that were transiently-transfected with KDR. All four tetrameric complexes of KDR-binding peptides were niotinylated and contained the JJ spacer, and bound to the KDR-expressing cells; however, SEQ ID NO:356 exhibited the best K_D (1.81nM). The tetrameric complexes of KDR-binding peptides SEQ ID NO:294, SEQ ID NO:264 exhibited improved binding over monomers of the same peptides. Moreover, inclusion of a spacer between the KDR-binding peptide and the biotin was shown to improve binding in Experiment B.

In Experiment C, it was shown that this assay can be used to assess the effect of serum on binding of peptides of the invention to KDR and VEGF/KDR complex. The binding of SEQ ID NO:264, SEQ ID NO:294, and SEQ ID NO:356 was not significantly affected by the presence of serum, while the binding of SEQ ID NO:277 was reduced more than 50% in the presence of serum.

In Experiment D, it was shown that this assay is useful in evaluating distinct combinations of KDR and VEGF/KDR complex binding polypeptides for use in multimeric targeting constructs which contain more than one KDR and VEGF/KDR complex binding polypeptide. Moreover, Experiments D and E establish that tetrameric constructs including two or more KDR binding peptides which bind to different epitopes exhibited superior binding to "pure" tetrameric constructs of the targeting peptides alone.

Experiment A

Preparation of m-RNA & 5' RACE ready cDNA library

HUVEC cells were grown to almost 80% confluence in 175 cm^2 tissue culture flasks (Becton Dickinson, Biocoat, cat # 6478) and then 10 ng/ml of bFGF (Oncogene, cat # PF003) was added for 24 h to induce expression of KDR. mRNA was isolated using the micro-fast track 2.0 kit from Invitrogen (cat. # K1520-02). 12 μg of mRNA (measured by absorbance at 260 nM) was obtained from two flasks (about 30 million cells) following the kit instructions. Reverse transcription to generate cDNA was performed with 2 μg of mRNA, oligo dT primer (5'-$(T)_{25}$GC-3') and/or smart II oligo (5'AAGCAGTGGTAACAACGCAGAGTACGCGGG-3') (SEQ ID NO:357) using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase. The reaction was performed in a total volume of 20 μl and the reaction mix contained 2 μl of RNA, 1 μl smart II oligo, 1 μl of oligo dT primer, 4 μl of 5X first-strand buffer (250 mM Tris HCl pH 8.3, 375 mM KCl, 30 mM $MgCl_2$)

1 µl DTT (20 mM, also supplied with reverse transcriptase), 1 µl dNTP mix (10 mM each of dATP, dCTP, dGTP, and dTTP in ddH_2O, Stratagene, cat. # 200415), 9 µl ddH_2O and 1 µl MMLV reverse transcriptase (Clonetech, cat #8460-1). The reverse transcription reaction was performed for 90 minutes at 42°C, and the reaction was stopped by adding 250 µl of tricine-EDTA buffer (10 mM tricine, 1.0 mM EDTA). The reverse transcription product, a 5' RACE ready cDNA library, can be stored for 3 months at –20°C. Note that all water used for DNA and RNA application was DNAse and RNAse free from USB (cat. # 70783).

Cloning of s-KDR into TOPOII Vector

In order to clone s-KDR, a 5' oligo (G ATG GAG AGC AAG GTG CTG CTG G) (SEQ ID NO:358) and a 3' oligo (C CAA GTT CGT CTT TTC CTG GGC A) (SEQ ID NO:359) were used. These were designed to amplify the complete extracellular domain of KDR (~2.2 kbps) from the 5' RACE ready cDNA library (prepared above) using polymerase chain reaction (PCR) with pfu polymerase (Stratagene, cat. # 600135). The PCR reaction was done in total volume of 50 µl and the reaction mix contained 2 µl 5' RACE ready cDNA library, 1 µl 5' oligo (10 µM), 1 µl 3' oligo (10 µM), 5 µl 10X PCR buffer [PCR buffer (200 mM Tris-HCl pH 8.8, 20 mM $MgSO_4$, 100 mM KCl, 100 mM $(NH_4)_2SO_4$) supplied with pfu enzyme plus 1% DMSO and 8% glycerol], 1 µl dNTP mix (10 mM) and 40 µl ddH_20. The PCR reaction was performed by using a program set for 40 cycles of 1 minute at 94C, 1 minute at 68C and 4 minutes at 72C. The PCR product was purified by extraction with 1 volume of phenol, followed by extraction with 1 volume of chloroform and precipitated using 3 volume of ethanol and 1/10 volume of 3M sodium acetate. The PCR product was resuspended in 17 µl of ddH_2O, the 2 µl of 10X Taq polymerase buffer (100 mM Tris-HCl pH 8.8, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin) and 1 µl of *Taq* polymerase (Stratagene, cat. # 600131) was added to generate an A overhang to each end of the product. After incubating for 1 hour at 72C the modified product was cloned directly into a TOPOII vector (InVitrogen, Carlsbad, CA) following the manufacturer's protocol to give TOPO-sKDR. The TOPO vector allows easy cloning of PCR products because of the A-overhang in *Taq* (PCR enzyme)-treated PCR products.

Cloning the transmembrane and cytoplasmic domains of KDR into TOPO II Vector

In order to clone the transmembrane and cytoplasmic domains of KDR, a 5' oligo (TCC CCC GGG ATC ATT ATT CTA GTA GGC ACG GCG GTG) (SEQ ID NO:360) and a 3' oligo (C AGG AGG AGA GCT CAG TGT GGT C) (SEQ ID NO:361) were used. These were designed to amplify the complete transmembrane and cytoplasmic domains of KDR (~1.8 kbps) from the 5' RACE ready cDNA library (described above) using polymerase chain reaction (PCR) with pfu polymerase. PCR reaction conditions and the program were exactly the same as described above for s-KDR. Just as with the s-KDR sequence, the PCR product was purified using phenol chloroform extraction, treated with Taq polymerase and cloned into TOPOII vector from Invitrogen to give TOPO-CYTO.

Cloning of full-length KDR into pcDNA6 Vector

To create the full-length receptor, the extra-cellular domain and the cytoplasmic domain (with trans-membrane domain) were amplified by PCR separately from TOPO-sKDR and TOPO-CYTO respectively and ligated later to create the full-length receptor. An oligo with a Not1 site at the 5' end of the extracellular domain (A TAA GAA TGC GGC CGC AGG ATG GAG AGC AAG GTG CTG CTG G) (SEQ ID NO:362) and an oligo complimentary to the 3' end of the extracellular domain (TTC CAA GTT CGT CTT TTC CTG GGC ACC) (SEQ ID NO:363) were used to amplify by PCR the extracellular domain from TOPO-sKDR. Similarly, the 5' oligo (ATC ATT ATT CTA GTA GGC ACG GCG GTG) (SEQ ID NO:364) and the 3' oligo, with a *Not*1 site (A TAA GAA TGC GGC CGC AAC AGG AGG AGA GCT CAG TGT GGT C) (SEQ ID NO:365), were used to amplify by PCR the cytoplasmic domain of KDR (with transmembrane domain) from TOPO-CYTO. Both PCR products were digested with Not1 and ligated together to create the full-length receptor. The cDNA encoding the full-length receptor was purified on an agarose gel and ligated into the Not I site of the pcDNA6/V5-HisC vector. Purification of DNA and ligation was done as described earlier for psKDR. The ligation reaction was used to transform a culture of DH5α bacteria and a number of individual clones were analyzed for the presence and orientation of insert by restriction analysis of purified plasmid from each clone with EcoRI enzyme.

Cell Culture

293H cells were obtained from Invitrogen (cat. # 11631) and grown as monolayer culture in their recommended media plus 1ml/L pen/strep (Invitrogen, cat. # 15140-148). All the cells were grown in presence of antibiotic for everyday culture but were split into antibiotic free media for 16-20 hours prior to transfection.

Preparation of DNA for Transfection

E. coli bacteria DH5α containing pf-KDR was streaked onto LB with 50 μg/ml ampicillin (LB agar from US biologicals, cat. # 75851 and ampicillin from Sigma, cat. #A2804) plates from a glycerol stock and plates were left in a 37°C incubator to grow overnight. Next morning, a single colony was picked from the plate and grown in 3 ml of LB/ampicillin media (LB from US biologicals, cat. # US75852) at 37°C. After 8 hours, 100 μl of bacterial culture from the 3 ml tube was transferred to 250 ml of LB/ampicillin media for overnight incubation at 37°C. Bacteria were grown up with circular agitation in a 500 ml bottle (Beckman, cat. # 355605) at 220 rpm in a Lab-Line incubator shaker. The next day, the bacterial culture was processed using maxi-prep kit (QIAGEN, cat. # 12163). Generally, about 1mg of plasmid DNA (as quantitated by absorbance at 260 nm) was obtained from 250 ml of bacterial culture.

Transfection of 293H cells in 96 well plate

Transfection was done as recommended in the lipofectamine 2000 protocol (Invitrogen, cat# 11668-019) using a poly-D-lysine-coated 96 well plate. 320 ng of KDR DNA (pc-DNA6-fKDR)/per well in 0.1 ml was used for 96 well transfection. Transfection was done in serum-containing media, the transfection reagent mix was removed from cells after 6-8 hours and replaced with regular serum-containing medium. Transfection was done in black/clear 96-well plates (Becton Dickinson, cat. # 354640). The left half of the plate (48 wells) were mock-transfected (with no DNA) and the right half of the plate was transfected with KDR cDNA. The cells were 80-90% confluent at the time of transfection and completely confluent next day, at the time of the assay, otherwise the assay was aborted.

Preparation of M199 media

In order to prepare M199 media for the assay, one M199 medium packet (GIBCO, cat. # 31100-035), 20 ml of 1 mM HEPES (GIBCO, cat. #15630-080) and

2 gm of DIFCO Gelatin (DIFCO, cat. # 0143-15-1) were added to 950 ml of ddH_2O and the pH of the solution was adjusted to 7.4 by adding approximately 4 ml of 1N NaOH. After pH adjustment, the M199 media was warmed to 37°C in a water bath for 2 hours to dissolve the gelatin, then filter sterilized using 0.2 μm filters (Corning, cat. # 43109), and stored at 4°C to be used later in the assay.

Preparation of SoftLink soft release avidin-sepharose

SoftLink soft release avidin-sepharose was prepared by centrifuging the sepharose obtained from Promega (cat. # V2011) at 12,000 rpm for 2 minutes, washing twice with ice cold water (centrifuging in-between the washes) and resuspending the pellet in ice cold water to make a 50% slurry in ddH_2O. A fresh 50% slurry of avidin-sepharose was prepared for each experiment.

Preparation of peptide/neutravidin HRP solution

Biotinylated peptides SEQ ID NOS:294, 264, 277, 356, and the non-binding biotinylated control peptide were used to prepare 250 μM stock solutions in 50% DMSO and a 33 μM stock solution of neutravidin-HRP was prepared by dissolving 2 mg of neutravidin-HRP (Pierce, cat. # 31001) in 1 mL of ddH_2O (all polypeptides contained the JJ spacer). Peptide stock solutions were stored at -20°C, whereas the Neutravidin HRP stock solution was stored at -80°C. To prepare peptide/neutravidin-HRP complexes, 10 μl of 250 μM biotinylated peptide stock solution and 10 μl of 33 μM neutravidin-HRP were added to 1 ml of M199 medium. This mixture was incubated on a rotator at 4°C for 60 minutes, followed by addition of 50 μl of soft release avidin-sepharose (50% slurry in ddH_20) to remove excess peptides and another incubation for 30 minutes on a rotator at 4°C. Finally, the soft release avidin-sepharose was pelleted by centrifuging at 12,000 rpm for 5 minutes at room temperature, and the resulting supernatant was used for the assays. Fresh peptide/neutravidin-HRP complexes were prepared for each experiment.

Preparation of peptide/neutravidin HRP dilutions for the assay

For saturation binding experiments, 120 μl, 60 μl, 20 μl, 10 μl, 8 μl, 6 μl, 4 μl, and 1 μl of peptide/neutravidin HRP complex were added to 1.2 ml aliquots of M199 medium to create dilutions with final concentrations of 33.33 nM, 16.65 nM, 5.55 nM, 2.78 nM, 1.67 nM, 1.11 nM and 0.28 nM complex, respectively.

Preparation of blocking solution for transfected 293H cells

Blocking solution was prepared by adding 20 ml of M199 medium to 10 mg of lyophilized unlabeled neutravidin (Pierce, cat. # 31000). Fresh blocking solution was used for each experiment.

Assay to detect the binding of peptide/neutravidin-HRP

24 hours after transfection, each well of the 293H cells was washed once with 100 µl of M199 medium and incubated with 80 µl of blocking solution at 37°C. After one hour, cells were washed twice with 100 µl of M199 media and incubated with 70 µl of peptide/neutravidin-HRP dilutions of control peptide, SEQ ID NO:264, SEQ ID NO:294, SEQ ID NO:277, and SEQ ID NO:356 for two and half hours at room temperature. Each dilution was added to three separate wells of mock as well as KDR-transfected 293H cells (two plates were used for each saturation binding experiment). After incubation at room temperature, plates were transferred to 4°C for another half-hour incubation. Subsequently, cells were washed 5 times with ice-cold M199 media and once with ice-cold PBS (in that order). After the final wash, 100 µl of ice cold TMB solution (KPL, cat. # 50-76-00) was added to each well and each plate was incubated for 30 minutes at 37°C in an air incubator. Finally, the HRP enzyme reaction was stopped by adding 50 µl of 1N phosphoric acid to each well, and binding was quantitated by measuring absorbance at 450 nm using a microplate reader (BioRad Model 3550).

Binding of peptide/neutravidin HRP to KDR-transfected cells

In this assay, complexes of control peptide, SEQ ID NO:264, SEQ ID NO:294, SEQ ID NO:277, and SEQ ID NO:356 peptides, each biotinylated with the JJ spacer and conjugated with neutravidin-HRP, were prepared as described above and tested for their ability to bind 293H cells that were transiently-transfected with KDR. During the peptide/neutravidin complex preparation, a 7.5-fold excess of biotinylated peptides over neutravidin-HRP was used to make sure that all four biotin binding sites on neutravidin were occupied. After complex formation, the excess of free biotinylated peptides was removed using soft release avidin-sepharose to avoid any competition between free biotinylated peptides and neutravidin HRP-complexed biotinylated peptides. The experiment was performed at several different

concentrations of peptide/neutravidin-HRP, from 0.28 nM to 33.33 nM, to generate saturation binding curves for SEQ ID NO:264 and SEQ ID NO:294 (FIG. 1A) and 0.28 to 5.55 nM to generate saturation binding curve for SEQ ID NO:277 and SEQ ID NO:256 (FIG. 1B). In order to draw the saturation binding curve, the background binding to mock-transfected cells was subtracted from the binding to KDR-transfected cells for each distinct peptide/neutravidin HRP complex at each concentration tested. Therefore, absorbance on the Y-axis of FIG. 1 (below) is differential absorbance (KDR minus mock) and not the absolute absorbance. Analysis of the saturation binding data in FIG. 1 using Graph Pad Prism software (version 3.0) yielded a K_D of 10.00 nM (+/-2.36) for the tetrameric SEQ ID NO:294, 14.87 nM (+/- 5.066) for the tetrameric SEQ ID NO:264, 4.031 nM (+/- 0.86) for the tetrameric SEQ ID NO:277, and 1.814 nM (+/- 0.27) for the tetrameric SEQ ID NO:356 peptide complexes. These binding constants are, as expected, lower than those measured by FP against the KDRFc construct for the related monodentate peptides SEQ ID NO:294 (69 nM), SEQ ID NO:264 (280 nM), SEQ ID NO:310 (51 nM), but similar to monodentate peptide SEQ ID NO:277 (3 nM). As expected, no saturation of binding for the control (non-binder) peptide/neutravidin HRP-complex was observed. The binding of peptide/neutravidin HRP complexes (FIG. 2) at a single concentration (5.55 nM) was plotted to demonstrate that a single concentration experiment can be used to differentiate between a KDR binding peptide (SEQ ID NOS:264, 295 and 277) from a non-binding peptide.

Experiment B

Experiment B was designed to look at the requirement of spacer (JJ, Table 10) between the KDR binding sequences (SEQ ID NOS:294 and 264) and biotin. In this experiment, biotinylated peptides with and without spacer JJ were tested (*e.g.*, biotinylated SEQ ID NO:264 with the JJ spacer, biotinylated SEQ ID NO:264 without the JJ spacer, SEQ ID NO:294 with a spacer, and biotinylated SEQ ID NO:294 without the spacer), and a non-KDR binding, biotinylated control peptide (with and without spacer, prepared as set forth above) was used as a control. The peptide structure of all the KDR-binding sequences tested in this experiment is shown in FIG. 3.

This experiment was performed as set forth in Experiment A described above, except that it was only done at a single concentration of 2.78 nM.

Results: It is evident from the results shown in the FIG. 4 that the spacer is enhances binding of SEQ ID NO:294 and SEQ ID NO:264. The spacer between the binding sequence and biotin can be helpful in enhancing binding to target molecule by multiple mechanisms. First, it could help reduce the steric hindrance between four biotinylated peptide after their binding to single avidin molecule. Second, it could provide extra length necessary to reach multiple binding sites available on a single cell.

Experiment C

Experiment C was designed to look at the serum effect on the binding of SEQ ID NOS: 294, 264, 277 and 356. In this procedure, biotinylated peptide/avidin HRP complexes of SEQ ID NOS:294, 264, 277 and 356 were tested in M199 media (as described above in Experiment A) with and without 40% rat serum. This experiment was performed as described for Experiment A except that it was only done at single concentration of 6.66 nM for SEQ ID NOS: 294 and 264, 3.33 nM for SEQ ID NO:277 and 2.22 nM for SEQ ID NO:356. Each of the polypeptides were biotinylated and had the JJ spacer.

Results: Results in FIG. 5 indicate that binding of SEQ ID NO:264, SEQ ID NO:294, and SEQ ID NO:356 was not significantly affected by 40% rat serum, whereas binding of SEQ ID NO:277 was more than 50% lower in presence of 40% rat serum. More than an 80% drop in the binding of Tc-labeled SEQ ID NO:277 with Tc-chelate was observed in the presence of 40% rat serum (FIG. 27). Since the serum effect on the binding of Tc-labeled SEQ ID NO:277 is mimicked in the avidin HRP assay disclosed herein, this assay may be used to rapidly evaluate the serum effect on the binding of peptide(s) to KDR.

Experiment D

Experiment D was designed to evaluate the binding of tetrameric complexes of KDR and VEGF/KDR complex-binding polypeptides SEQ ID NO:294 and SEQ ID NO:264, particularly where the constructs included at least two KDR binding polypeptides. The KDR binding peptides and control binding peptide were prepared as described above. This experiment was performed using the protocol set forth for

Experiment A, except the procedures set forth below were unique to this experiment.

Preparation of Peptide/Neutravidin Solutions: 250 μM stock solutions of biotinylated peptides SEQ ID NOs:264, 294 and control peptide were prepared in 50% DMSO and a 33 μM stock solution of Neutravidin HRP was prepared by dissolving 2 mg of Neutravidin HRP (Pierce, cat. # 31001) in 1 mL of ddH_2O. Peptide stock solutions were stored at -20C, whereas the Neutravidin HRP stock solution was stored at –80C. The sequences of the biotinylated peptides are shown above. To prepare peptide/neutravidin HRP complexes, a total 5.36 μL of 250 μM biotinylated peptide stock solution (or a mixture of peptide solutions, to give peptide molecules four times the number of avidin HRP molecules) and 10 μL of 33 μM Neutravidin HRP were added to 1 mL of M199 medium. This mixture was incubated on a rotator at 4C for 60 minutes, followed by addition of 50 μL of soft release avidin-sepharose (50% slurry in ddH_20) to remove excess peptides and another incubation for 30 minutes on a rotator at 4C. Finally, the soft release avidin-sepharose was pelleted by centrifuging at 12,000 rpm for 5 minutes at room temperature, and the resulting supernatant was used for the assays. Fresh peptide/neutravidin HRP complexes were prepared for each experiment.

Assay to Detect the Binding of Peptide/Neutravidin HRP: 24 hours after transfection, each well of the 293H cells was washed once with 100 μL of M199 medium and incubated with 80 μL of blocking solution at 37C. After one hour, cells were washed twice with 100 μL of M199 media and incubated with 70 μL of 3.33 nM peptide (or peptide mix)/neutravidin HRP solutions (prepared by adding 10 μL of stock prepared earlier to 1 mL of M199 media) for two and half hours at room temperature. Each dilution was added to three separate wells of mock as well as KDR-transfected 293H cells. After incubation at room temperature, plates were transferred to 4C for another half-hour incubation. Subsequently, cells were washed five times with ice-cold M199 media and once with ice-cold PBS (in that order). After the final wash, 100 μL of ice cold TMB solution (KPL, Gaithersburg, MD) was added to each well and each plate was incubated for 30 minutes at 37C in an air incubator. Finally, the HRP enzyme reaction was stopped by adding 50 μL of 1N phosphoric acid to each well, and binding was quantitated by measuring absorbance at 450 nm using a microplate reader (BioRad Model 3550).

Results: This experiment establishes that SEQ ID NO:294 and SEQ ID NO:264 bind to KDR in multimeric fashion, and cooperate with each other for binding to KDR in 293H transfected cells. A biotinylated control peptide that does not bind to KDR was used. As expected, a tetrameric complex of the control peptide with avidin-HRP did not show enhanced binding to KDR-transfected cells. Tetrameric complexes of SEQ ID NO:294 and SEQ ID NO:264 bound to KDR-transfected cells significantly better than to mock-transfected cells (see FIG. 6). SEQ ID NO:294 tetramers, however, bound much better than SEQ ID NO:264 tetramers. If the control peptide was added to the peptide mixture used to form the tetrameric complexes, the binding to the KDR-transfected cells decreased. The ratio of specific binding of tetramer to monomer, dimer and trimer was calculated by dividing the specific binding (obtained by subtracting the binding to mock transfected cells from KDR transfected cells) of tetramer, trimer and dimer with that of monomer. Results indicate that there is co-operative effect of multimerization of SEQ ID NOS:264, 294 and 356 on the binding to KDR-transfected cells.

	Tetramer	Trimer	Dimer
SEQ ID NO:264	45.4	5	4.3
SEQ ID NO:294*	38.6	7.1	2.7
SEQ ID NO:277	1	1.1	1.1
SEQ ID NO:356	16	5.7	2.3

*monomeric peptide binding at 2.22 nM was zero, therefore ratios were calculated using binding at 5.55 nM.

A mixture of 25% non-binding control peptide with 75% SEQ ID NO:264 did not bind significantly over background to KDR-transfected cells, indicating that multivalent binding is critical for the SEQ ID NO:264/avidn-HRP complex to remain bound to KDR throughout the assay. This phenomenon also held true for SEQ ID NO:294, where substituting 50% of the peptide with control peptide in the tetrameric complex abolished almost all binding to KDR on the transfected cells.

Surprisingly, a peptide mixture composed of 50% control peptide with 25% SEQ ID NO:294 and 25% SEQ ID NO:264 bound quite well to KDR-transfected cells relative to mock-transfected cells, indicating that there is a great advantage to

targeting two sites or epitopes on the same target molecule. Furthermore, it was noted that tetrameric complexes containing different ratios of SEQ ID NO:294 and SEQ ID NO:264 (3:1, 2:2, and 1:3) all bound much better to KDR-transfected cells than pure tetramers of either peptide, in agreement with the idea that targeting two distinct sites on a single target molecule is superior to multimeric binding to a single site. This may be because multimeric binding to a single target requires that the multimeric binding entity span two or more separate target molecules which are close enough together for it to bind them simultaneously, whereas a multimeric binder which can bind two or more distinct sites on a single target molecule does not depend on finding another target molecule within its reach to achieve multimeric binding.

Experiment E

Experiment E was designed to confirm that SEQ ID NO:294 and SEQ ID NO:264 bind to distinct sites (epitopes) on KDR. If these peptides bind to the same site on KDR, then they should be able to compete with each other; however, if they bind to different sites they should not compete. This experiment was performed using a single concentration of SEQ ID NO:264/avidin HRP (3.33 nM) solution in each well and adding a varying concentration (0-2.5 μM) of biotinylated control peptide with spacer, SEQ ID NO:264 and SEQ ID NO:294, none of which were complexed with avidin.

Results: It is evident from FIG. 7 that SEQ ID NO:264 does compete with SEQ ID NO:264/avidin HRP solution for binding to KDR transfected cells whereas control peptide and SEQ ID NO:294 do not compete with SEQ ID NO:264/avidin HRP solution for binding to KDR transfected cells. Thus, SEQ ID NO:264 and SEQ ID NO:294 bind to distinct and complementary sites on KDR.

Example 6: Binding of Analogs of a KDR-binding Peptide to KDR-expressing Cells

N-terminal and C-terminal truncations of a KDR binding polypeptide were made and the truncated polypeptides tested for binding to KDR-expressing cells. The synthesized polypeptides are shown in FIG. 8. Binding of the polypeptides to KDR-expressing cells was determined following the procedures of Example 3.

All of the peptides were N-terminally acetylated and fluoresceinated for

determining apparent K_D according to the method described above (Example 3). The results indicate that, for the SEQ ID NO:294 (FIG. 8) polypeptide, the C-terminal residues outside the disulfide-constrained loop contribute to KDR binding.

Example 7: Bead-binding Assay to Confirm Ability of Peptides Identified by Phage Display to Bind KDR-expressing Cells

The following procedures were performed to assess the ability of KDR-binding peptides to bind to KDR-expressing cells. In this procedure, KDR-binding peptides containing SEQ ID NOS:264, 337, 363, and 373 were conjugated to fluorescent beads and their ability to bind to KDR-expressing 293H cells was assessed. The experiments show these peptides can be used to bind particles such as beads to KDR-expressing sites. The results indicate that the binding of both KDR binding sequences improved with the addition of a spacer.

Protocol

Biotinylation of an anti-KDR antibody: Anti-KDR from Sigma (V-9134), as ascites fluid, was biotinylated using a kit from Molecular Probes (F-6347) according to the manufacturer's instructions.

Preparation of peptide-conjugated fluorescent beads: 0.1 mL of a 0.2 mM stock solution of each biotinylated peptide (prepared as set forth above, in 50% DMSO) was incubated with 0.1 ml of Neutravidin-coated red fluorescent microspheres (2 micron diameter, custom-ordered from Molecular Probes) and 0.2 ml of 50mM MES (Sigma M-8250) buffer, pH 6.0 for 1 hour at room temperature on a rotator. As a positive control, biotinylated anti-KDR antibody was incubated with the Neutravidin-coated beads as above, except that 0.03 mg of the biotinylated antibody preparation in PBS (Gibco #14190-136) was used instead of peptide solution. Beads can be stored at 4°C until needed for up to 1 week.

Binding Assay: From the above bead preparations, 0.12 mL was spun for 10 minutes at 2000 rpm in a microcentrifuge at room temperature. The supernatant was removed and 0.06 ml of MES pH 6.0 was added. Each bead solution was then vortexed and sonicated in a water bath 15 min. To 1.47 ml of DMEM, high glucose (GIBCO #11965-084) with 1X MEM Non-Essential Amino Acids Solution (NEAA)

(GIBCO 11140-050) and 40% FBS (Hyclone SH30070.02) 0.03 ml of the sonicated bead preparations was added. 96-well plates seeded with 293H cells which have been mock-transfected in columns 1 to 6, and KDR-transfected in columns 7 to 12 (as in Example 5), were drained and washed once with DMEM, high glucose with 1X NEAA and 40% FBS. To each well 0.1 ml of bead solution was added, six wells per bead preparation. After incubating at room temperature for 30 minutes, the wells were drained by inverting the plates and washed four times with 0.1 ml PBS with $Ca^{++}Mg^{++}$ (GIBCO #14040-117) with shaking at room temperature for 5 minutes each wash. After draining, 0.1 ml of PBS was added per well. The plates were then read on a Packard FluoroCount fluorometer at excitation 550nm/emission 620nm. Unconjugated neutravidin beads were used as a negative control while beads conjugated with a biotinylated anti-KDR antibody were used as the positive control for the assay.

To calculate the number of beads bound per well, a standard curve with increasing numbers of the same fluorescent beads was included in each assay plate. The standard curve was used to calculate the number of beads bound per well based on the fluorescence intensity of each well.

Results: The positive control beads with anti-KDR attached clearly bound preferentially to the KDR-expressing cells while avidin beads with nothing attached did not bind to either cell type (FIG. 9). Biotinylated SEQ ID NO:264 beads did not bind to the KDR-transfected cells significantly more than to mock-transfected cells, but adding a hydrophilic spacer between the peptide moiety and the biotin group (biotinylated SEQ ID NO:264 with a JJ spacer beads) enhanced binding to KDR cells without increasing the binding to mock-transfected cells. Biotinylated SEQ ID NO:294 beads showed greater binding to KDR-transfected cells, and adding a hydrophilic spacer between the peptide portion and the biotin of the molecule (biotinylated SEQ ID NO:294 with the JJ spacer) significantly improved the specific binding to KDR in the transfected cells. Thus, the peptide sequences of both SEQ ID NO:264 and SEQ ID NO:294 can be used to bind particles such as beads to KDR expressing sites. Addition of a hydrophilic spacer between the peptide and the group used for attachment to the particle should routinely be tested with new targeting molecules as it improved the binding for both of the peptides evaluated here.

Example 8: Competition of KDR binding peptides and ^{125}I-labeled VEGF for binding to KDR-transfected 293H cells

KDR-binding polypeptides were next assessed for their ability to compete with ^{125}I-labeled VEGF for binding to KDR expressed by transfected 293H cells. The results indicate that KDR-binding polypeptide SEQ ID NO:263 (Ac-AGDSWCSTEYTYCEMIGTGGGK-NH_2) did not compete significantly with ^{125}I-labeled VEGF, and SEQ ID NOS:294, 264, and SEQ ID NO:277 competed very well with ^{125}I-labeled VEGF, inhibiting 96.29±2.97% and 104.48±2.074% of ^{125}I-labeled VEGF binding.

Transfection of 293H cells: 293H cells were transfected using the protocol described in Example 5. Transfection was done in black/clear 96-well plates (Becton Dickinson, cat. # 354640). The left half of the plates (48 wells) were mock-transfected (with no DNA) and the right half of the plates were transfected with KDR cDNA. The cells were 80-90% confluent at the time of transfection and completely confluent the next day, at the time of the assay; otherwise the assay was aborted.

Preparation of M199 media: M199 medium was prepared as described in Example 5.

Preparation of peptide solutions: 3 mM stock solutions of peptides SEQ ID NO:294, SEQ ID NO:263, SEQ ID NO:264 and SEQ ID NO:277 were prepared as described above in 50% DMSO.

Preparation of ^{125}I-labeled VEGF solution for the assay: 25 µCi of lyophilized ^{125}I-labeled VEGF (Amersham, cat. # IM274) was reconstituted with 250 µl of ddH_2O to create a stock solution, which was stored at -80C for later use. For each assay, a 300 pM solution of ^{125}I-labeled VEGF was made fresh by diluting the above stock solution in M199 medium. The concentration of ^{125}I-labeled VEGF was calculated daily based on the specific activity of the material on that day.

Preparation of 30 µM and 0.3 µM peptide solution in 300 pM ^{125}I-labeled VEGF: For each 96 well plate, 10 ml of 300 pM ^{125}I-labeled VEGF in M199 medium was

prepared at 4°C. Each peptide solution (3 mM, prepared as described above) was diluted 1:100 and 1:10000 in 300 µl of M199 media with 300 pM ^{125}I-labeled VEGF to prepare 30 µM and 0.3 µM peptide solutions containing 300 pM of ^{125}I-labeled VEGF. Once prepared, the solutions were kept on ice until ready to use. The dilution of peptides in M199 media containing 300 pM ^{125}I-labeled VEGF was done freshly for each experiment.

Assay to detect competition with ^{125}I-labeled VEGF in 293H cells: Cells were used 24 hours after transfection, and to prepare the cells for the assay, they were washed 3 times with room temperature M199 medium and placed in the refrigerator. After 15 minutes, the M199 medium was removed from the plate and replaced with 75 µl of 300 pM ^{125}I-labeled VEGF in M199 medium (prepared as above) with the polypeptides. Each dilution was added to three separate wells of mock and KDR transfected cells. After incubating at 4°C for 2 hours, the plates were washed 5 times with cold binding buffer, gently blotted dry and checked under a microscope for cell loss. 100 µl of solubilizing solution (2% Triton X-100, 10% Glycerol, 0.1% BSA) was added to each well and the plates were incubated at room temperature for 30 minutes. The solubilizing solution in each well was mixed by pipeting up and down, and transferred to 1.2 ml tubes. Each well was washed twice with 100 µl of solubilizing solution and the washes were added to the corresponding 1.2 ml tube. Each 1.2 ml tube was then transferred to a 15.7 × 100 cm tube to be counted in an LKB Gamma Counter using program 54 (^{125}I window for 1 minute).

Competition of peptides with ^{125}I-labeled VEGF in 293H cells: The ability of KDR-binding peptides SEQ ID NO:294, SEQ ID NO:263, SEQ ID NO:264 and SEQ ID NO:277 to specifically block ^{125}I-labeled VEGF binding to KDR was assessed in mock-transfected and KDR-transfected cells. SEQ ID NO:263 was used in the assay as a negative control, as it exhibited poor binding to KDR in the FP assays described herein and would therefore not be expected to displace or compete with VEGF. To calculate the specific binding to KDR, the binding of ^{125}I-labeled VEGF to mock-transfected cells was subtracted from KDR-transfected cells. Therefore, the binding of ^{125}I- labeled VEGF to sites other than KDR (which may or may not be present in 293H cells) is not included when calculating the inhibition of ^{125}I-labeled VEGF binding to 293H cells by KDR-binding peptides. Percentage inhibition was

calculated using formula [(Y1-Y2)*100/Y1], where Y1 is specific binding to KDR-transfected 293H cells in the absence peptides, and Y2 is specific binding to KDR-transfected 293H cells in the presence of peptides or DMSO. Specific binding to KDR-transfected 293H cells was calculated by subtracting binding to mock-transfected 293H cells from binding to KDR-transfected 293H cells.

As shown in FIG. 10, in 293 cells, SEQ ID NO:263, which due to its relatively high K_d (>2 μM) was used as a negative control, did not compete significantly with ^{125}I-labeled VEGF, 12.69±7.18% at 30 μM and -5.45±9.37% at 0.3 μM (FIG. 10). At the same time, SEQ ID NOS:294 and 277 competed very well with ^{125}I-labeled VEGF, inhibiting 96.29±2.97% and 104.48±2.074% of ^{125}I-labeled VEGF binding at 30 μM and 52.27±3.78% and 80.96±3.8% at 0.3 μM (FIG. 10) respectively. The percentage inhibition with SEQ ID NO:264 was 47.95±5.09% of ^{125}I-labeled VEGF binding at 30μM and 24.41±8.43% at 0.3 μM (FIG. 10). Thus, the three strongly KDR-binding polypeptides did compete with VEGF, and their potency increased with their binding affinity. This assay will be useful for identifying peptides that bind tightly to KDR but do not compete with VEGF, a feature that may be useful for imaging KDR in tumors, where there is frequently a high local concentration of VEGF that would otherwise block the binding of KDR-targeting molecules.

Example 9: Inhibition of VEGF-induced KDR receptor activation by peptides identified by phage display

The ability of KDR-binding peptides identified by phage display to inhibit VEGF induced activation (phosphorylation) of KDR was assessed using the following assay. A number of peptides of the invention were shown to inhibit activation of KDR in monomeric and/or tetrameric constructs. As discussed *supra*, peptides that inhibit activation of KDR may be useful as anti-angiogenic agents.

Protocol

Human umbilical vein endothelial cells (HUVECs) (Biowhittaker Catalog #CC-2519) were obtained frozen on dry ice and stored in liquid nitrogen until thawing. These cells were thawed, passaged, and maintained as described by the manufacturer in EGM-MV medium (Biowhittaker Catalog #CC-3125). Cells seeded into 100 mm dishes were allowed to become confluent, then cultured overnight in basal EBM medium lacking serum (Biowhittaker Catalog #CC-3121). The next

morning, the medium in the dishes was replaced with 10 ml fresh EBM medium at 37C containing either no additives (negative control), 5 ng/ml VEGF (Calbiochem Catalog #676472 or Peprotech Catalog #100-20) (positive control), or 5 ng/ml VEGF plus the indicated concentration of the KDR-binding peptide (prepared as described above). In some cases, a neutralizing anti-KDR antibody (Catalog #AF357, R&D Systems) was used as a positive control inhibitor of activation. In such cases, the antibody was pre-incubated with the test cells for 30 min at 37°C prior to the addition of fresh medium containing both VEGF and the antibody. After incubating the dishes 5 min. in a 37°C tissue culture incubator they were washed three times with ice-cold D-PBS containing calcium and magnesium and placed on ice without removing the last 10 ml of Delbecco's phosphate buffered saline (D-PBS). The first dish of a set was drained and 0.5 ml of Triton lysis buffer was added (20 mM Tris base pH 8.0, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA (ethylenediaminetetraacetic acid), 1 mM PMSF(phenylmethylsulfonylfluoride), 1 mM sodium orthovanadate, 100 mM NaF, 50 mM sodium pyrophosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin). The cells were quickly scraped into the lysis buffer using a cell scraper (Falcon, Cat No. #353087), dispersed by pipeting up and down briefly, and the resulting lysate was transferred to the second drained dish of the pair. Another 0.5 ml of lysis buffer was used to rinse out the first dish then transferred to the second dish, which was then also scraped and dispersed. The pooled lysate from the two dishes was transferred to a 1.5 ml Eppindorf tube. The above procedure was repeated for each of the controls and test samples (KDR-binding peptides), one at a time. The lysates were stored on ice until all the samples had been processed. At this point samples were either stored at -70° C or processed to the end of the assay without interruption.

The lysates, freshly prepared or frozen and thawed, were precleared by adding 20 μl of protein A-sepharose beads (Sigma 3391, preswollen in D-PBS, washed three times with a large excess of D-PBS, and reconstituted with 6 ml D-PBS to generate a 50% slurry) and rocking at 4° C for 30 min. The beads were pelleted by centrifugation for 2 min. in a Picofuge (Stratgene, Catalog #400550) at 2000 ×g and the supernatants transferred to new 1.5 ml tubes. Twenty μg of anti-Flk-1 antibody (Santa Cruz Biotechnology, Catalog #sc-504) was added to each tube, and the tubes were incubated overnight (16-18 hr.) at 4C on a rotator to

immunoprecipitate KDR. The next day 40 µl of protein A-sepharose beads were added to the tubes which were then incubated 4C for 1 hr. on a rotator. The beads in each tube were subsequently washed three times by centrifuging for 2 min. in a Picofuge, discarding the supernatant, and dispersing the beads in 1 ml freshly added TBST buffer (20 mM Tris base pH 7.5, 137 mM NaCl, and 0.1% Tween 20). After centrifuging and removing the liquid from the last wash, 40 µl of Laemmli SDS-PAGE sample buffer (Bio-Rad, Catalog #161-0737) was added to each tube and the tubes were capped and boiled for 5 min. After cooling, the beads in each tube were pelleted by centrifuging and the supernatants containing the immunoprecipitated KDR were transferred to new tubes and used immediately or frozen and stored at -70C for later analysis.

Detection of phosphorylated KDR as well as total KDR in the immunoprecipitates was carried out by immunoblot analysis. Half (20 µL) of each immunoprecipitate was resolved on a 7.5% precast Ready Gel (Bio-Rad, Catalog #161-1154) by SDS-PAGE according to the method of Laemmli (*Nature*, 227:680-685 (1970)).

Using a Bio-Rad mini-Protean 3 apparatus (Catalog #165-3302). The resolved proteins in each gel were electroblotted to a PVDF membrane (Bio-Rad, Cat. No. 162-0174) in a Bio-Rad mini Trans-Blot cell (Catalog #170-3930) in CAPS buffer (10 mM CAPS, Sigma Catalog #C-6070, 1% ACS grade methanol, pH 11.0) for 2 hr. at 140 mA according to the method of Matsudaira (*J. Biol. Chem.*, 262:10035-10038 (1987)). Blots were blocked at room temperature in 5% Blotto-TBS (Pierce Catalog #37530) pre-warmed to 37° C for 2 hr. The blots were first probed with an anti-phosphotyrosine antibody (Transduction Labs, Catalog #P11120), diluted 1:200 in 5% Blotto-TBS with 0.1% Tween 20 added for 2 hr. at room temp. The unbound antibody was removed by washing the blots four times with D-PBS containing 0.1% Tween 20 (D-PBST), 5 min. per wash. Subsequently, blots were probed with an HRP-conjugated sheep anti-mouse antibody (Amersham Biosciences Catalog #NA931) diluted 1:25,000 in 5% Blotto-TBS with 0.1% Tween 20 added for 1 hr. at room temp., and washed four times with D-PBST. Finally, the blots were incubated with 2 ml of a chemiluminescent substrate (ECL Plus, Amersham Catalog #RPN2132) spread on top for 2 min., drip-drained well, placed in plastic sheet protector (C-Line Products, Catalog #62038), and exposed to X-ray film (Kodak BioMax ML, Cat No. 1139435) for varying lengths of time to achieve

optimal contrast.

To confirm that similar amounts of KDR were compared in the assay, the blots were stripped by incubating for 30 min. at 37° C in TBST with its pH adjusted to 2.4 with HCl, blocked for 1 hr. at room temp. with 5% Blotto-TBS with 0.1% Tween 20 (Blotto-TBST), and reprobed with an anti-Flk-1 polyclonal antibody (Catalog #sc-315 from Santa Cruz Biotech), 1:200 in 5% Blotto-TBST with 1% normal goat serum (Life Tech Catalog #16210064) for 2 hr. at room temp. The unbound antibody was removed by washing the blots four times with D-PBST, 5 min. per wash. Subsequently, the blots were probed with an HRP-conjugated donkey anti-rabbit secondary antibody (Amersham Biosciences Catalog #NA934) diluted 1:10,000 in 5% Blotto-TBST for 1 hr. at room temp., and washed four times with D-PBST. Finally, the blots were incubated with 2 ml of chemiluminescent substrate and exposed to X-ray film as described above.

Results: Immunoblots of KDR immunoprecipitates prepared from HUVECs with and without prior VEGF stimulation demonstrated that activated (phosphorylated) KDR could be detected when the HUVECs were stimulated with VEGF. An anti-phosphotyrosine antibody (PY-20) detected no phosphorylated proteins close to the migration position of KDR from unstimulated HUVECs on the blots, but after five minutes of VEGF stimulation, an intense band was consistently observed at the expected location (FIG. 11, upper panel). When the blots were stripped of bound antibodies by incubation in acidic solution then reprobed with an anti-KDR antibody (sc-315), the identity of the phosphorylated protein band was confirmed to be KDR. Moreover, it was observed that immunoprecipitates from unstimulated HUVECs contained about as much total KDR as immunoprecipitates from VEGF-stimulation HUVECs (FIG. 11, lower panel).

The foregoing results indicate that the phosphorylated KDR detected was formed from pre-existing KDR through autophosphorylation of KDR dimers resulting from VEGF binding, as five minutes is not enough time to synthesize and process a large glycosylated cell-surface receptor such as KDR.

The ability of this assay to detect agents capable of blocking the VEGF activation of KDR was assessed by adding a series of compounds to HUVECs in combination with VEGF and measuring KDR phosphorylation with the immunoblot assay described above. As negative and positive controls, immunoprecipitates from

unstimulated HUVECs and from HUVECs stimulated with VEGF in the absence of any test compounds were also tested in every assay. When a neutralizing anti-KDR antibody (Catalog #AF-357 from R&D Systems) was combined with the VEGF, the extent of KDR phosphorylation was greatly reduced (FIG. 12, upper panel), indicating that the antibody was able to interfere with the ability of VEGF to bind to and activate KDR. This result was expected since the ability of the antibody to block VEGF-induced DNA synthesis is part of the manufacturer's quality control testing of the antibody. Re-probing the blot with an anti-KDR antibody (FIG. 12, lower panel) indicated that slightly less total KDR was present in the VEGF+antibody-treated lane (+V+α-KDR) relative to the VEGF-only-treated lane (+V), but the difference was not great enough to account for the much lower abundance of phosphorylated KDR in the antibody-treated lane.

To assess the potency of a linear KDR-binding peptide (AFPRFGGDDYWIQQYLRYTD, SEQ ID NO:140) identified by phage display, the assay was repeated with a synthetic peptide containing the KDR-binding sequence, Ac-AQAFPRFGGDDYWIQQYLRYTDGGK-NH_2 (SEQ ID NO:306) in the presence of VEGF. SEQ ID NO:306 was able to inhibit the VEGF-induced phosphorylation of KDR. Re-probing the blot for total KDR showed that there is even more total KDR in the VEGF+SEQ ID NO:306-treated cells (+V+SEQ ID NO:306) than in the VEGF only-treated cells (+V) (FIG. 13, lower panel). Thus, it is clear that the decreased phosphorylation of KDR in the presence of SEQ ID NO:306 is not due to differential sample loading, but rather the ability of the polypeptide to inhibit VEGF-activation of KDR.

Repeating the foregoing assay, the following polypeptides demonstrated at least a 50% inhibition of VEGF-induced KDR phosphorylation at 10 μM:

Ac–AGWIECYHPDGICYHFGTGGGK–NH_2 (SEQ ID NO:269)

Ac–AGWLECYAEFGHCYNFGTGGGK–NH_2 (SEQ ID NO:267)

Ac–GDSRVCWEDSWGGEVCFRYDPGGGK–NH_2 (SEQ ID NO:294)

Ac–GDWWECK(ivDde)REEYRNTTWCAWADPGGGK–NH_2 (SEQ ID NO:366 having a blocked K)

Ac–GDPDTCTMWGDSGRWYCFPADPGGGK–NH_2 (SEQ ID NO:301)

Ac–AQEPEGYAYWEVITLYHEEDGDGGK–NH_2 (SEQ ID NO:305)

Ac–AQAFPRFGGDDYWIQQYLRYTDGGK–NH_2 (SEQ ID NO:306)

Ac–AQGDYVYWEIIELTGATDHTPPGGK–NH_2 (SEQ ID NO:307)

SEQ ID NOS: 269 and 294 were the most potent compounds in the assay, producing at least a 50% inhibition of VEGF-induced KDR phosphorylation at 1 μM.

The following peptides were tested in the assay and did not produce significant inhibition of KDR activation at 10 μM:

Ac–AGPK(ivDde)WCEEDWYYCMITGTGGGK–NH_2 (SEQ ID NO:264)

Ac–GSDHHCYLHNGQWICYPFAPGGGK–NH_2 (SEQ ID NO:314)

Ac–GDYPWCHELSDSVTRFCVPWDPGGGK–NH_2 (SEQ ID NO:293)

Ac–GDDHMCRSPDYQDHVFCMYWDPGGGK–NH_2 (SEQ ID NO:295)

Ac–GDPPLCYFVGTQEWHHCNPFDPGGGK–NH_2 (SEQ ID NO:296)

Ac–GDGSWCEMRQDVGK(ivDde)WNCFSDDPGGGK–NH_2 (SEQ ID NO:299)

Ac–AQRGDYQEQYWHQQLVEQLK(ivDde)LLGGGK–NH_2 (SEQ ID NO:331)

Ac–GDNWECGWSNMFQK(ivDde)EFCARPDPGGGK–NH_2 (SEQ ID NO:303)

Ac–AGPGPCK(ivDde)GYMPHQCWYMGTGGGK–NH_2 (SEQ ID NO:367)

Ac–AGYGPCAEMSPWLCWYPGTGGGK–NH_2 (SEQ ID NO:322)

In addition, tetrameric complexes of biotinylated derivatives of SEQ ID NOS:294 and 277 (prepared as described above) produced at least a 50% inhibition of VEGF-induced KDR phosphorylation at 10 nM.

Example 10: Binding of Tc-labeled SEQ ID NO:339 to KDR-transfected 293H cells

The ability of Tc-labeled SEQ ID NO:339 to bind KDR was assessed using KDR-transfected 293H cells. Tc-labeled SEQ ID NO:277 (*i.e.*, Ac-AGPTWCEDDWYYCWLFGT-GGGK(N,N–dimethyl–Gly-Ser-Cys–Gly–di(aminodioxaocta–))–NH_2) bound significantly better to KDR transfected 293H cells than to mock transfected 293H cells and binding increased with concentration of Tc-labeled SEQ ID NO:339 in a linear manner.

Preparation of Peptidic chelate for binding to Tc by SPPS (FIG. 35)

To a 250 mL of SPPS reaction vessel was added 6.64 mmol of H-Gly-2-Cl-trityl resin (0.84 mmol/g, Novabiochem). It was swelled in 80 mL of DMF for 1h.

For each coupling cycle the resin was added 26.6 mmol of DIEA, 26.6 mmol of a Fmoc-amino acid in DMF (EM Science), 26.6 mmol of HOBT (Novabiochem) in DMF, and 26.6 mmol of DIC. The total volume of DMF was 80 ml. The reaction mixture was shaken for 4h. The resin then was filtered and washed with DMF (3 x 80 ml). A solution of 20% piperidine in DMF (80 mL) was added to the resin and it was shaken for 10 min. The resin was filtered and this piperidine treatment was repeated. The resin finally was washed with DMF (3 x 80 mL) and ready for next coupling cycle. At the last coupling cycle, N,N-dimethyl glycine (Aldrich) was coupled using HATU/DIEA activation. Thus, to a suspension of N,N-dimethyl glycine (26.6 mmol) in DMF was added a solution of 26.6 mmol of HATU (Perseptive Biosystems) in DMF and 53.1 mmol of DIEA. The clear solution was added to the resin and shaken for 16 h. Following the synthesis, the resin was filtered and washed with DMF (3 x 80 ml), CH_2Cl_2 (3 x 80 ml) and dried. The resin was mixed with 80 mL of $AcOH/CF_3CH_2OH/DCM$ (1/1/8, v/v/v) and shaken for 45 min. The resin was filtered and the filtrate was evaporated to a paste. Purification of the crude material by silica gel chromatography using 25% MeOH/DCM afforded 2.0 g of the final product.

Coupling of the peptidic chelate to the peptide (*Fragment coupling*)

Diisopropylcarbodiimide (0.0055 mmol) was added to a mixture of purified Me_2N-Gly-Cys-(Trt)-Ser(tBu)-Gly-OH and hydroxybenzotriazole (0.0055mmol) in DMF (0.25 mL), and the mixture was stirred at RT for 6 h. The peptide (0.005 mmol) in DMF (0.25 mL) was then added to the reaction mixture, and stirring was continued for an additional 6 h. DMF was removed under vacuum and the residue was treated with reagent B and stirred for 3h. TFA was removed under reduced pressure and the residue was purified by preparative HPLC using acetonitrile-water containing 0.1%TFA. Fractions containing the pure product were collected and freeze dried to yield the peptide. The peptide was characterized by ES-MS and the purity was determined by RP-HPLC (acetonitrile-water/0.1% TFA) gradient.

Synthesis of ^{99m}Tc Labeled SEQ ID NO:339

A stannous gluconate solution was prepared by adding 2 ml of a 20 µg/ml $SnCl_2 \cdot 2H_2O$ solution in nitrogen-purged 1N HCl to 1.0 ml of nitrogen-purged water containing 13 mg of sodium glucoheptonate. To a 4 ml autosampler vial was added

20-40 µl (20 - 40 µg) of SEQ ID NO:339 ligand dissolved in 50/50 ethanol/H_2O, 6-12 mCi of $^{99m}TcO_4^-$ in saline and 100 µl of stannous glucoheptonate solution. The mixture was heated at 100°C for 22 min. The resulting radiochemical purity (RCP) was 10 - 47% when analyzed using a Vydac C18 Peptide and Protein column that was eluted at a flow rate of 1 ml/min. with 66% H_2O (0.1% TFA)/34% ACN(0.085% TFA). The reaction mixture was purified by HPLC on a Vydac C18 column (4.6 mm × 250 mm) at a flow rate of 1 ml/min., using 0.1% TFA in water as aqueous phase and 0.085% TFA in acetonitrile as the organic phase. The following gradient was used: 29.5% org. for 35 min., ramp to 85% over 5 min., hold for 10 min. The fraction containing ^{99m}Tc SEQ ID NO:339 was collected into 500 µl of a stabilizing buffer containing 5 mg/ml ascorbic acid and 16 mg/ml hydroxypropyl-α-cyclodextrin in 50 mM phosphate buffer. The mixture was concentrated using a speed vacuum apparatus to remove acetonitrile, and 200 µl of 0.1% HSA in 50 mM pH 5 citrate buffer was added. The resulting product had an RCP of 100%. Prior to injection into animals, the compound was diluted to the desired radioconcentration with normal saline.

Transfection of 293H cells

293H cells were transfected using the protocol described above. Transfection was done in black/clear 96-well plates (Becton Dickinson, cat. # 354640). The left half of the plates (48 wells) were mock-transfected (with no DNA) and the right half of the plate was transfected with KDR cDNA. The cells were 80-90% confluent at the time of transfection and completely confluent the next day, at the time of the assay; otherwise the assay was aborted.

Preparation of opti-MEMI media with 0.1% HSA

Opti-MEMI was obtained from Invitrogen (cat. # 11058-021) and human serum albumin (HSA) was obtained from Sigma (cat. # A-3782). To prepare opti-MEMI media with 0.1% HSA, 0.1% w/v HSA was added to opti-MEMI, stirred at room temperature for 20 min. and then filter sterilized using 0.2 µm filter.

Preparation of Tc-labeled SEQ ID NO:339 dilutions for the assay

Stock solution of Tc-labeled SEQ ID NO:339 (117 µCi/ml) was diluted 1:100, 1:50, 1:25 and 1:10 in opti-MEMI with 0.1% HSA to provide solutions with

final concentration of 1.17, 2.34, 4.68 and 11.7 μCi/ml of Tc-labeled SEQ ID NO:339.

Assay to detect the binding of Tc-labeled SEQ ID NO:339

Cells were used 24 hours after transfection, and to prepare the cells for the assay, they were washed once with 100 μl of room temperature opti-MEMI with 0.1% HSA. After washing, the opti-MEMI with 0.1% HSA was removed from the plate and replaced with 70 μl of 1.17, 2.34, 4.68 and 11.7 μCi/ml of Tc-labeled SEQ ID NO:339 (prepared as above). Each dilution was added to three separate wells of mock- and KDR-transfected cells. After incubating at room temperature for 1 hour, the plates were transferred to 4°C for 15 minutes and washed 5 times with 100 μl of cold binding buffer (opti-MEMI with 0.1% HSA), gently blotted dry and checked under a microscope for cell loss. 100 μl of solubilizing solution (2% Triton X-100, 10% Glycerol, 0.1% BSA) was added to each well and the plates were incubated at 37°C for 10 minutes. The solubilizing solution in each well was mixed by pipeting up and down, and transferred to 1.2 ml tubes. Each well was washed once with 100 μl of solubilizing solution and the washes were added to the corresponding 1.2 ml tube. Each 1.2 ml tube was then transferred to a 15.7 × 100 cm tube to be counted in an LKB Gamma Counter using program 12 (Tc-window for 20 sec).

Binding of Tc-labeled SEQ ID NO:339 to KDR transfected cells

The ability of Tc-labeled SEQ ID NO:339 to specifically bind to KDR was assessed using transiently transfected 293H cells.

As shown in FIG. 14, Tc-labeled SEQ ID NO:339 bound significantly better to KDR transfected 293H cells as compared to mock transfected 293H cells. To calculate specific binding to KDR, the binding of Tc-labeled SEQ ID NO:339 polypeptide to mock-transfected cells was subtracted from the binding to KDR-transfected cells. A linear increase in the specific binding of Tc-labeled SEQ ID NO:339 to KDR was observed with increasing concentration of Tc-labeled SEQ ID NO:339 (FIG. 26). Linear binding was not surprising because concentration of Tc-labeled SEQ ID NO:339 was only ~100 pM (even at the highest concentration, 11.7 μCi/ml, tested in the assay), which is far below the K_D value of ~3-4 nM of SEQ ID NO:277 (as calculated using avidin HRP assay), so no saturation of binding would be expected.

Example 11: Preparation of peptides and dimeric peptide construction

The following methods were used for the preparation of individual peptides and dimeric peptide constructs described in the following Examples (11-15).

Automated Peptide Synthesis

Peptide synthesis was carried out on a ABI-433A Synthesizer (Applied Biosystems Inc., Foster City, CA) on a 0.25 mmol scale using the FastMoc protocol. In each cycle of this protocol preactivation was accomplished by dissolution of 1.0 mmol of the requisite dry N^{α}-Fmoc side-chain protected amino acid in a cartridge with a solution of 0.9 mmol of HBTU, 2 mmol of DIEA, and 0.9 mmol of HOBt in a DMF-NMP mixture. The peptides were assembled on NovaSyn TGR (Rink amide) resin (substitution level 0.2 mmol/g). Coupling was conducted for 21 min. Fmoc deprotection was carried out with 20% piperidine in NMP. At the end of the last cycle, the N-terminal Fmoc group was removed and the fully protected resin-bound peptide was acetylated using acetic anhydride / DIEA / HOBt / NMP.

Cleavage, Side-chain Deprotection and Isolation of Crude Peptides

Cleavage of the peptides from the resin and side-chain deprotection was accomplished using Reagent B for 4.5h at ambient temperature. The cleavage solutions were collected and the resins were washed with an additional aliquot of Reagant B. The combined solutions were concentrated to dryness. Diethyl ether was added to the residue with swirling or stirring to precipitate the peptides. The liquid phase was decanted, and solid was collected. This procedure was repeated 2-3 times to remove impurities and residual cleavage cocktail components.

Cyclization of Di-cysteine Peptides

The crude ether-precipitated linear di-cysteine containing peptides were cyclized by dissolution in water, mixtures of aqueous acetonitrile (0.1% TFA), aqueous DMSO or 100% DMSO and adjustment of the pH of the solution to 7.5 – 8.5 by addition of aqueous ammonia, aqueous ammonium carbonate, aqueous ammonium bicarbonate solution or DIEA. The mixture was stirred in air for 16-48 h, acidified to pH 2 with aqueous trifluoroacetic acid and then purified by preparative reverse phase HPLC employing a gradient of acetonitrile into water.

Fractions containing the desired material were pooled and the purified peptides were isolated by lyophilization.

Preparation of Peptides Containing Linkers

In a typical experiment, 400 mg of the resin-bound peptide bearing an ivDde-protected lysine) was treated with 10% hydrazine in DMF (2 x 20 mL). The resin was washed with DMF (2 x 20 mL) and DCM (1 x 20 mL). The resin was resuspended in DMF (10 mL) and treated with Fmoc-8-amino-3,6-dioxaoctanoic acid (0.4 mmol), HOBt (0.4 mmol), DIC (0.4 mmol) and DIEA (0.8 mmol) with mixing for 4 h. After the reaction, the resin was washed with DMF (2 × 10 mL) and with DCM (1 × 10 mL). The resin was then treated with 20% piperidine in DMF (2 × 15 mL) for 10 min each time. The resin was washed and the coupling with Fmoc-8-amino-3,6-dioxaoctanoic acid and Fmoc protecting group removal were repeated once more.

The resulting resin-bound peptide with a free amino group was washed and dried and then treated with reagent B (20 mL) for 4 h. The mixture was filtered and the filtrate concentrated to dryness. The residue was stirred with ether to produce a solid, which was washed with ether and dried. The solid was dissolved in anhydrous DMSO and the pH adjusted to 7.5 with DIEA. The mixture was stirred for 16h to effect the disulfide cyclization and the reaction was monitored by analytical HPLC. After completion of the cyclization, the reaction mixture was diluted with 25% acetonitrile in water and applied directly to a reverse phase C-18 column. Purification was effected using a gradient of acetonitrile into water (both containing 0.1% TFA). Fractions were analyzed by HPLC and those containing the pure product were combined and lyophilized to provide the required peptide.

Preparation of Biotinylated Peptides Containing Linkers

In a typical experiment, 400 mg of the resin-bound peptide bearing an ivDde-protected lysine, was treated with 10% hydrazine in DMF (2 x 20 mL). The resin was washed with DMF (2 x 20 mL) and DCM (1 x 20 mL). The resin was resuspended in DMF (10 mL) and treated with Fmoc-8-amino-3,6-dioxaoctanoic acid (0.4 mmol), HOBt (0.4 mmol), DIC (0.4 mmol) and DIEA (0.8 mmol) with mixing for 4 h. After the reaction, the resin was washed with DMF (2 x 10 mL) and with DCM (1x 10 mL). The resin was then treated with 20% piperidine in DMF (2 x

15 mL) for 10 min each time. The resin was washed and the coupling with Fmoc-8-amino-3,6-dioxaoctanoic acid and removal of the Fmoc protecting group were repeated once more.

The resulting resin-bound peptide with a free amino group was treated with a solution of Biotin-NHS ester (0.4 mmol, 5 equiv.) and DIEA (0.4 mmol, 5 equiv.) in DMF for 2 h. The resin was washed and dried as described previously and then treated with Reagent B (20 mL) for 4 h. The mixture was filtered and the filtrate concentrated to dryness. The residue was stirred with ether to produce a solid that was collected, washed with ether, and dried. The solid was dissolved in anhydrous DMSO and the pH adjusted to 7.5 with DIEA. The mixture was stirred for 4-6 h to effect the disulfide cyclization which was monitored by HPLC. Upon completion of the cyclization, the reaction mixture was diluted with 25% acetonitrile in water and applied directly to a reverse phase C-18 column. Purification was effected using a gradient of acetonitrile into water (both containing 0.1% TFA). Fractions were analyzed by HPLC and those containing the pure product were collected and lyophilized to provide the required biotinylated peptide.

Preparation of DOTA-Conjugated Peptides for Labeling with Selected Gadolinium or Indium Isotopes

In a typical experiment, 400 mg of the resin-bound peptide bearing an N^{ε}-ivDde-protected lysine moiety was treated with 10% hydrazine in DMF (2 × 20 mL). The resin was washed with DMF (2 × 20 mL) and DCM (1 × 20 mL). The resin was resuspended in DMF (10 mL) and treated with Fmoc-8-amino-3,6-dioxaoctanoic acid (0.4 mmol), HOBt (0.4 mmol), DIC (0.4 mmol), DIEA (0.8 mmol) with mixing for 4 h. After the reaction, the resin was washed with DMF (2 × 10 mL) and with DCM (1 × 10 mL). The resin was then treated with 20% piperidine in DMF (2 × 15 mL) for 10 min each time. The resin was washed and the coupling with Fmoc-8-amino-3,6-dioxaoctanoic acid and removal of the Fmoc protecting group were repeated once. The resulting resin-bound peptide with a free amino group was resuspended in DMF (10 mL) and treated with a solution of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid,-1,4,7-tris-t-butyl ester (DOTA-tris-t-butyl ester, 0.4 mmol, 5 equiv.), HOBt (0.4 mmol), DIC (0.4 mmol) and DIEA (0.8 mmol) in DMF (10 mL) with mixing for 4 h. Upon completion of the reaction, the resin was washed with DMF (2 × 10 mL) and with DCM (1 x 10 mL) and treated

with Reagent B (20 mL) for 4 h. The mixture was filtered and the filtrate concentrated to dryness. The residue was stirred in ether to produce a solid that was collected, washed with ether, and dried. The solid was dissolved in anhydrous DMSO and the pH adjusted to 7.5 with DIEA. The mixture was stirred for 16 h to effect the disulfide cyclization, which was monitored by HPLC. Upon completion of the cyclization, the mixture was diluted with 25% acetonitrile in water and applied directly to a reverse phase C-18 HPLC column. Purification was effected using a gradient of acetonitrile into water (both containing 0.1% TFA). Fractions were analyzed by HPLC and those containing the pure product were combined and lyophilized to provide the required biotinylated peptide.

The following monomeric peptides of Table 11 were prepared by the above methods, "PnAO6", as used herein, refers to 3-(2-amino-3-(2-hydroxyimino-1,1-dimethyl-propylamino)-propylamino)-3-methyl-butan-2-one oxime.

Table 11. Sequence or Structure of Monomeric Peptides and Peptide Derivatives

Structure or Sequence	SEQ. ID NO: or dimer
Ac-AGPTWCEDDWYYCWLFGTGGGK(BiotinJJ-K)-NH_2	277
(Ac-AGPTWCEDDWYYCWLFGTGGGKK(BiotinJJ-)-NH_2)	373
Ac-AGPTWCEDDWYYCWLFGTJK(DOTAJJ-K)-NH_2	493
Ac-AGPTWCEDDWYYCWLFGTJK(JJ)-NH_2	493
Ac-AGPTWCEDDWYYCWLFGTGGGK[K(ivDde)]-NH_2	373
Ac-VCWEDSWGGEVCFRYDPGGGK(Biotin-JJK)-NH_2	337
(Ac-VCWEDSWGGEVCFRYDPGGGKK(Biotin-JJ)-NH_2)	494
Ac-VCWEDSWGGEVCFRYDPGGGK(JJ)-NH_2	337
Ac-AQDWYYDEILSMADQLRHAFLSGGGGGK(J)-NH_2 Seq 12 derivative	356
Ac-AQDWYYDEILSMADQLRHAFLSGGGGGKK(ivDde) Application seq 12 derivative	495
Ac-GDSRVCWEDSWGGEVCFRYDPGGGK(JJ)-NH_2 Seq 5 derivative	294
Ac-GDSRVCWEDSWGGEVCFRYDPGGGK(JJ)-NH_2 Seq 5 deriv	294/D10
Ac-AGPTWCEDDWYYCWLFGTGGGK[(PnAO6-C(=O)$(CH_2)_3$C(=O)-K]-NH_2 A Seq 11 derivative	277/D10
Ac-AGPTWCEDDWYYCWLFGTGGGK[(DOTA-JJK(iV-Dde)]-NH_2 A Seq 11 derivative	277/D11
Ac-AGPTWCEDDWYYCWLFGTGGGK[(PnAO6-C(=O)$(CH_2)_3$C(=O))K]-NH_2 A Seq 11 derivative	476/D12
Ac-VCWEDSWGGEVCFRYDPGGGK-NH_2 A Seq 5 derivative	337/D12

specifically: Seq 5 residues 5-25	
Ac-AGPTWCEDDWYYCWLFGTGGGK[K(BOA)]-NH_2 Seq 11 derivative	277/D13
Ac-AQDWYYDEILSMADQLRHAFLSGGGGGK[PnAO6-C(=O)$(CH_2)_3$C(=O)-K(iV-Dde)]-NH_2 Application seq 12 derivative	356/D14
Ac-GDSRVCWEDSWGGEVCFRYDPGGGK(JJ)-NH_2 Seq 5 deriv linker = Glut	294/D15
Ac-AGPTWCEDDWYYCWLFGTGGGK-[PnAO6-C(=O)$(CH_2)_3$C(=O)-K]-NH_2 A Seq 11 derivative, new sequence	277/D16
Ac-AQDWYYEILJGRGGRGGRGGK[K(ivDde)]-NH_2 A Seq 12 (1-9) derivative	496/D17
Ac-APGTWCDYDWEYCWLGTFGGGK[(6PnAO-C(=O)$(CH_2)_3$C(=O)-K]-NH_2 A scrambled Seq 11 derivative used as a control. A new sequence.	497/D18
Ac-GVDFRCEWSDWGEVGCRSPDYGGGK(JJ)-NH_2 A scrambled Seq 5 derivative. New Sequence.	489/D18
Ac-AGPTWCEDDWYYCWLFGTGGGK(Biotin-K)-NH_2 , A Seq 11 derivative	294/D19
JJAGPTWCEDDWYYCWLFGTGGGK(iV-Dde)-NH_2 (SEQ ID NO:277)	277/D20
JJVCWEDSWGGEVCFRYDPGGG-NH_2	370/D20
JJAGPTWCEDDWYYCWLFGTGGGK(iV-Dde)-NH_2	277/D21
Ac-AGPTWCEDDWYYCWLFGTGGGK[K(SATA)]-NH_2	373/D22
Ac-AGPTWCEDDWYYCWLFGTGGGK[SATA-JJ-K]-NH_2	339/D23
Ac-GDSRVCWEDSWGGEVCFRYDPGGGK(JJ)-NH_2	294/D24
H_2N-AGPTWCEDDWYYCWLFGTGGGK[K(iV-Dde)]-NH_2	373/D25
Ac-AGPTWCEDDWYYCWLFGTGGGK{Biotin-JJK[NH_2-Ser(GalNAc$(Ac)_3$-alpha-D)-Gly-Ser(GalNAc$(Ac)_3$-alpha-D]}-NH_2	339/D26
Ac-VCWEDSWGGEVCFRYDPGGGK(NH_2-Ser(GalNAc$(Ac)_3$-alpha-D)-Gly-Ser(GalNAc$(Ac)_3$-alpha-D)-NH_2	337/D26
Ac-GSPEMCMMFPFLYPCNHHAPGGGK[(PnAO6)-C(=O)$(CH_2)_3$C(=O)-K]}-NH_2 A modified cMet Binding Sequence	482/D27

Example 12: Preparation of homodimeric and heterodimeric constructs

The purified peptide monomers mentioned above in Example 8 were used in the preparation of various homodimeric and heterodimeric constructs.

Preparation of Homodimer-Containing Constructs

To prepare homodimeric compounds, half of the peptide needed to prepare the dimer was dissolved in DMF and treated with 10 equivalents of glutaric acid bis-N-hydoxysuccinimidyl ester. The progress of the reaction was monitored by HPLC

analysis and mass spectroscopy. At completion of the reaction, the volatiles were removed *in vacuo* and the residue was washed with ethyl acetate to remove the unreacted bis-NHS ester. The residue was dried, re-dissolved in anhydrous DMF and treated with another half portion of the peptide in the presence of 2 equivalents of DIEA. The reaction was allowed to proceed for 24 h. This mixture was applied directly to a YMC reverse phase HPLC column and purified by elution with a linear gradient of acetonitrile into water (both containing 0.1% TFA).

Preparation of Heterodimer-Containing Constructs

In the case of heterodimers, one of the monomers ("A") was reacted with the bis-NHS ester of glutaric acid and after washing off the excess of bis-NHS ester (as described for the homodimeric compounds), the second monomer ("B") was added in the presence of DIEA. After the reaction the mixture was purified by preparative HPLC. Typically, to a solution of glutaric acid bis N-hydoxysuccinimidyl ester (0.02 mmol, 10 equivalents) in DMF (0.3 mL) was added a solution of peptide "A" and DIEA (2 equiv) in DMF (0.5mL) and the mixture was stirred for 2 h. The progress of the reaction was monitored by HPLC analysis and mass spectroscopy. At completion of the reaction, the volatiles were removed *in vacuo* and the residue was washed with ethyl acetate (3 x 1.0 mL) to remove the unreacted bis-NHS ester. The residue was dried, re-dissolved in anhydrous DMF (0.5 mL) and treated with a solution of peptide "B" and DIEA (2 equiv) in DMF (0.5 mL) for 24 h. The mixture was diluted with water (1:1,v/v) and applied directly to a YMC C-18 reverse phase HPLC column and purified by elution with a linear gradient of acetonitrile into water (both containing 0.1% TFA). Fractions were analyzed by analytical HPLC and those containing the pure product were combined and lyophilized to obtain the required dimer. The dimmers depicted in FIGS. 36-63 were prepared by this method (structure, name, compound reference number as described in the "Brief Description of the Drawings").

For the preparation of the dimer D5, after the coupling reaction of the individual peptides, 50 μL of hydrazine was added to the reaction mixture (to expose the lysine N^{ε}-amino group) and the solution was stirred for 2 min. The reaction mixture was diluted with water (1.0 mL) and the pH was adjusted to 2 with TFA. This was then purified by the method described above.

The HPLC analysis data and mass spectral data for the dimeric peptides are

given in Table 12 below.

Table 12. Analytical Data for Homodimeric and Heterodimeric Peptide Constructs

HPLC Analysis System

	Retention Time (System)	**Mass Spectral data (API-ES, - ion)**
D1	8.98 min. (A)	1987.7 (M-3H)/3, 1490.6 (M-4H)/4, 1192.3 (M-5H)/5
D2	16.17 min (B)	2035.3 (M-3H)/3, 1526.1 (M-4H)/4, 1220.7 (M-5H)/5
D3	8.74 min (C)	1933.6 (M-3H)/3, 1449.9 (M-4H)/4, 1159.4 (M-5H)/5
D4	10.96 min (D)	2032.8 (M-3H)/3
D5	6.57 min (E)	1816.2 (M-3H)/3, 1361.8 (M-4H)/4, 1089.4 (M-5H)/5, 907.7 (M-6H)/6
D8	4.96 min; (F)	2379.3 [M-3H]/3
D9	5.49 min; (G)	2146.4 [M-3H]/3
D10	5.44 min; (H)	2082.7 [M-3H]/3, 1561.7 [M-4H]/4, 1249.1 [M-5H]/5, 1040.7 [M-6H]/6
D11	7.23 min; (E)	2041.8 [M-3H]/3, 1531.1 [M-4H]/4, 1224.6 [M-5H]/5
D12	5.84 min; (H)	1877.1 [M-3H]/3, 1407.6 [M-4H]/4, 1125.9 [M-5H]/5, 938.1 [M-6H]/6.
D13	5.367 min; (E)	1965.3 [M-3H]/3, 1473.8 [M-4H]/4, 1178.8 [M-5H]/5, 982.2 [M-6H]/6
D14	4.78 min; (I)	2275.0 [M-3H]/3, 1362.8 [M-5H]/5
D15	5.41 min; (H)	1561.3 [M-4H]/4, 1249.1 [M-5H]/5, 1040.8 [M-6H]/6, 891.8 [M-7H]/7.
D16	5.44 min; (J)	2150.8 [M-3H]/3, 1613.1 [M-4H]/4, 1289.9 [M-5H]/5, 1074.8 [M-6H]/6, 920.9 [M-7H]/7.
D17	4.78 min; (K)	1789.4 [M-3H]/3, 1347.7 [M-4H]/4.
D18	4.74 min; (L)	2083.1 [M-3H]/3, 1562.7 [M-4H]/4, 1249.5 [M-5H]/5.
D19	7.13 min; (O)	1891.9 [M-3H]/3, 1418.4 [M-4H]/4, 1134.8 [M-5H]/5, 945.5 [M-6H]/6.
D20	9.7 min; (P)	2700.4 [M-2H]/2, 1799.3[M-3H]/3
D21	6.1 min; (P)	2891.3 [M-2H]/2, 1927.2[M-3H]/3, 1445.1 [M-4H]/4, 1155.8 [M-5H]/5.
D22	6.23 min; (Q)	1994.4 [M-3H]/3, 1495.7 [M-4H]/4, 1196.3 [M-5H]/5
D23	7.58 min; (J)	1854.4 [M-3H]/3, 1390.8 [M-4H]/4, 1112.7 [M-5H]/5, 927 [M-6H]/6
D24	8.913 min; (R)	1952.1 [M-3H]/3, 1463.4 [M-4H]/4, 1171.1 [M-5H]/5, 975.3 [M-6H]/6
D25	5.95 min; (E)	1954.9 [M-3H]/3, 1466.1 [M-4H]/4, 1172.4 [M-5H]/5, 976.8 [M-6H]/6.
D26	6.957 min; (S)	1759.1 [M-3H]/3, 1319.6 [M-4H]/4, 1055.1 [M-5H]/5

D27	5.5 min; (M)	2317.6 [M-3H]/3, 1737.2[M-4H]/4, 1389.3[M-5H]/5, 1157.7 [M-6H]/6.

Table 13: Dimer sequences and linkers

Dimer #	Sequence
D8	Ac-AQDWYYDEILSMADQLRHAFLSGGGGGK{Ac-AQDWYYDEILSMADQLRHAFLSGGGGGK(J-Glut-)-NH_2}K(Biotin-JJ)-NH_2
D9	Ac-AQDWYYDEILSMADQLRHAFLSGGGGGK{[Ac-GDSRVCWEDSWGGEVCFRYDPGGGK(JJ-Glut-)]-NH_2}K-NH_2
D10	Ac-AGPTWCEDDWYYCWLFGTGGGK{[Ac-GDSRVCWEDSWGGEVCFRYDPGGGK(JJ-Glut-$NH(CH_2)_4$-(S)-CH(PnAO6-Glut-NH)(C=O-)]-NH_2}-NH_2
D11	Ac-AGPTWCEDDWYYCWLFGTGGGK{Ac-VCWEDSWEDSWGGEVCFRYDPGGGK[JJ-Glut-$NH(CH_2)_4$-(S)-CH(DOTA-JJ-NH-)(C=O)-]-NH_2}-NH_2
D12	Ac-AGPTWCEDDYCWLFGTGGGK{[PnAO6-Glut-K(Ac-VCWEDSWGGEVCFRYDPGGGK(-C(=O)$CH_2(OCH_2CH_2)_2OCH_2C$(=O)-)-NH_2]}-NH_2
D13	Ac-AGPTWCEDDWYYCWLFGTGGGK{Ac-VCWEDSWGGEVCFRYDPGGGK[JJ-Glut-K(BOA)]-NH_2}-NH_2: Dimer 13 (D13)
D14	Ac-AQDWYYDEILSMADQLRHAFLSGGGGGK{PnAO6-Glut-K[Ac-GSDRVCWEDSWGGEVCFRYDPGGGK(JJ-Glut)-NH_2]}-NH_2
D15	Ac-AGPTWCEDDWYYCWLFGTGGGK{[[Ac-GDSRVCWEDSWGGEVCFRYDPGGGKJJ-Glut]-NH_2]-K(PnAO6-Glut)}-NH_2
D16	Ac-AGPTWCEDDWYYCWLFGTGGGGK{PnAO6-Glut-K[Ac-GDSRVCWEDSWGGEVCFRYDPGGGK[-C(=O)$CH_2O(CH_2CH_2O)_2CH_2C$(=O)NH$(CH_2)_3O(CH_2CH_2O)_2(CH_2)_3$NH C(=O)$CH_2O(CH_2CH_2O)_2CH_2C$(=O)-]-$NH_2$]}-$NH_2$
D17	Ac-AQDWYYDEILJGRGGRGGRGGK{K[Ac-VCWEDSWGGEVCFRYDPGGGK(JJ-Glut)-NH_2]}-NH_2
D18	Ac-AGPTWCDYDWEYCWLGTFGGGK{PnAO6-Glut-K[Ac-GVDFRCEWSDWGEVGCRSPDYGGGK(JJ-Glut)-NH_2]}-NH_2
D19	Ac-AGPTWCEDDWYYCWLFGTGGGK{Biotin-K[Ac-VCWEDSWGGEVCFRYDPGGGK(JJ-Glut)-NH_2]}-NH_2
D20	(-JJAGPTWCEDDWYYCWLFGTGGGGK-NH_2)-Glut-VCWEDSWGGEVCFRYDPGGG-NH_2
D21	[-JJAGPTWCEDDWYYCWLFGTGGGGK(PnAO6-Glut)-NH_2]-Glut-VCWEDSWGGEVCFRYDPGGG-NH_2
D22	Ac-GDSRVCWEDSWGGEVCFRYDPGGGK{JJ-Glut-JJ-AGPTWCEDDWYYCWLFTGGGK-NH_2}-NH_2
D23	Ac-AGPTWCEDDWYYCWLFGTGGGK{Ac-VCWEDSWGGEVCFRYDPGGGK[JJ-Glut-K(SATA)]-NH_2}-NH_2
D24	Ac-AGPTWCEDDWYYCWLFGTGGGK{SATA-JJK[Ac-VCWEDSWGGEVCFRYDPGGGK(JJ-Glut)-NH_2]}-NH_2
D25	Ac-AGPTWCEDDWYYCWLFGTGGGK{Ac-GDSRVCWEDSWGGEVCFRYDPGGGK[JJ-Glut-$NH(CH_2)$4-(S)-$CH(NH_2)C$(=O)-]-NH_2}-NH_2
D26	AGPTWCEDDWYYCWLFGTGGGGK{(-Glut-JJ-VCWEDSWGGEVCFRYDPGGG-NH_2)-K}-NH_2
D27	Ac-AGPTWCEDDWYYCWLFGTGGGGK{Ac-VCWEDSWGGEVCFRYDPGGGK[S(GalNAc$(Ac)_3$-alpha-D)-G-S(GalNAc$(Ac)_3$-alpha-D)-Glut-S(GalNAc$(Ac)_3$-alpha-D)-G-S(GalNAc$(Ac)_3$-alpha-D)-$NH(CH_2)_4$-(S)-CH(Biotin-JJNH-)C(=O)-]-NH_2}-NH_2

HPLC Analysis Systems

System A: Column: YMC C-4 (4.6 x 250 mm); Eluents: A: Water (0.1%

TFA), B: ACN (0.1% TFA); Elution: initial condition, 25% B, linear gradient 25-60% B in 10 min; flow rate: 2.0 ml/ min; detection: UV @ 220 nm.

System B: Column: YMC C-4 (4.6 x 250 mm); Eluents: A: water (0.1% TFA), B: ACN (0.1% TFA); Elution: initial condition, 25 % B, linear gradient 25-60% B in 20 min; flow rate: 2.0 mL/min; detection: UV @ 220 nm.

System C: Column: YMC C-4 (4.6 x 250 mm); Eluents: A: water (0.1% TFA), B: ACN (0.1% TFA); Elution: initial condition, 30% B, linear gradient 30-60% B in 10 min; flow rate: 2. 0 mL/ min; detection: UV @ 220 nm.

System D: Column: YMC C-4 (4.6 x 250 mm); Eluents: A: water (0.1% TFA), B: ACN (0.1% TFA); Elution: initial condition, 20% B, linear gradient 20-60% B in 10 min; flow rate: 2. 0 mL/ min; Detection: UV @ 220 nm.

System E: Column: Waters XTerra, 4.6 x 50 mm; Eluents:A: water (0.1%TFA), B: ACN (0.1%TFA); Elution: initial condition, 10 % B, linear gradient 10-60 % B in 10 min; flow rate: 3.0 mL/min; detection: UV @ 220 nm.

System F: Column: Waters XTerra, 4.6 x 50 mm; Eluents:A: water (0.1%TFA), B: Acetonitrile (0.1%TFA); Elution: Initial condition, 30 % B, Linear Gradient 30-70 % B in 10 min; Flow rate: 3.0 mL/min; Detection: UV @ 220 nm.

System G: Column: Waters XTerra, 4.6 x 50 mm; Eluents:A: water (0.1%TFA), B: ACN (0.1%TFA); Elution: initial condition, 30 % B, linear gradient 30-75 % B in 10 min; flow rate: 3.0 mL/min; detection: UV @ 220 nm.

System H: Column: Waters XTerra, 4.6 x 50 mm; Eluents:A: water (0.1%TFA), B: ACN (0.1%TFA); Elution: initial condition, 20 % B, linear gradient 20-52 % B in 10 min; flow rate: 3.0 mL/min; detection: UV @ 220 nm.

System I: Column: Waters XTerra, 4.6 x 50 mm; Eluents:A: water (0.1%TFA), B: ACN (0.1%TFA); Elution: initial condition, 10 % B, linear gradient 10-65 % B in 10 min; flow rate: 3.0 mL/min; detection: UV @ 220 nm.

System J: Column: Waters XTerra, 4.6 x 50 mm; Eluents:A: water (0.1%TFA), B: ACN (0.1%TFA); Elution: initial condition, 20 % B, linear gradient 20-60 % B in 10 min; flow rate: 3.0 mL/min; detection: UV @ 220 nm.

System K: Column: Waters XTerra, 4.6 x 50 mm; Eluents:A: water (0.1%TFA), B: ACN (0.1%TFA); Elution: initial condition, 5 % B, linear gradient 5-60 % B in 10 min; flow rate: 3.0 mL/min; detection: UV @ 220 nm.

System L: Column: Waters XTerra, 4.6 x 50 mm; Eluents:A: water (0.1%TFA), B: ACN (0.1%TFA); Elution: initial condition, 5 % B, linear gradient

5-65 % B in 10 min; flow rate: 3.0 mL/min; detection: UV @ 220 nm.

System M: Column: Waters XTerra, 4.6 x 50 mm; Eluents:A: water (0.1%TFA), B: ACN (0.1%TFA); Elution: initial condition, 15 % B, linear gradient 15-50 % B in 10 min; flow rate: 3.0 mL/min; detection: UV @ 220 nm.

System N: Column: Waters XTerra, 4.6 x 50 mm; Eluents:A: water (0.1%TFA), B: ACN (0.1%TFA); Elution: initial condition, 10 % B, linear gradient 20-80 % B in 10 min; flow rate: 3.0 mL/min; detection: UV @ 220 nm.

System O: Column: YMC-C18, 4.6 x 250 mm; Eluents:A: water (0.1%TFA), B: ACN (0.1%TFA); Elution: initial condition, 30 % B, linear gradient 30-60 % B in 10 min; flow rate: 2.0 mL/min; detection: UV @ 220 nm.

System P: Column: YMC-C18, 4.6 x 250 mm; Eluents:A: water (0.1%TFA), B: ACN (0.1%TFA); Elution: initial condition, 20 % B, linear gradient 20-80 % B in 20 min; flow rate: 2.0 mL/min; detection: UV @ 220 nm.

System Q: Column: YMC-C18, 4.6 x 250 mm; Eluents:A: water (0.1%TFA), B: ACN (0.1%TFA); Elution: initial condition, 20 % B, linear gradient 20-60 % B in 6 min; flow rate: 2.0 mL/min; detection: UV @ 220 nm.

System R: Column: YMC-C18, 4.6 x 250 mm; Eluents:A: water (0.1%TFA), B: ACN (0.1%TFA); Elution: initial condition, 25 % B, linear gradient 25-60 % B in 10 min; flow rate: 2.0 mL/min; detection: UV @ 220 nm.

System S: Column: YMC-C18, 4.6 x 100 mm; Eluents:A: water (0.1%TFA), B: ACN (0.1%TFA); Elution: initial condition, 10 % B, linear gradient 10-60 % B in 10 min; flow rate: 3.0 mL/min; detection: UV @ 220 nm.

Example 13: Competition with ^{125}I-VEGF for binding to KDR on HUVECs and KDR-transfected cells

The following experiment assessed the ability of KDR-binding peptides to compete with ^{125}I-labeled VEGF for binding to KDR expressed by transfected 293H cells.

Protocol:

293H cells were transfected with the KDR cDNA or mock-transfected by standard techniques. The cells were incubated with ^{125}I-VEGF in the presence or absence of competing compounds (at 10 μM, 0.3 μM, and 0.03 μM). After washing the cells, the bound radioactivity was quantitated

on a gamma counter. The percentage inhibition of VEGF binding was calculated using the formula [(Y1 - Y2) × 100/Y1], where Y1 is specific binding to KDR-transfected 293H cells in the absence peptides, and Y2 is specific binding to KDR-transfected 293H cells in the presence of peptide competitors. Specific binding to KDR-transfected 293H cells was calculated by subtracting the binding to mock-transfected 293H cells from the binding to KDR-transfected 293H cells.

Results

As shown in FIG. 15, all of the KDR-binding peptides assayed were able to compete with ^{125}I-VEGF for binding to KDR-transfected cells. The heterodimer (D1) was clearly the most effective at competing with ^{125}I-VEGF, even over the two homodimers (D2 and D3), confirming the superior binding of D1.

Example 14: Receptor Activation Assay

The ability of KDR-binding peptides to inhibit VEGF induced activation (phosphorylation) of KDR was assessed using the following assay.

Protocol

Dishes of nearly confluent HUVECs were placed in basal medium lacking serum or growth factors overnight. The dishes in group (c), below were then pretreated for 15 min in basal medium with a KDR-binding peptide, and then the cells in the dishes in groups (a), (b), and (c) were placed in fresh basal medium containing:

(a) no additives (negative control),

(b) 5 ng/ mL VEGF (positive control), or

(c) 5 ng/ mL VEGF plus the putative competing/inhibiting peptide.

After 5 min of treatment, lysates were prepared from each set of dishes. KDR was immunoprecipitated from the lysates was analyzed sequentially by immunoblotting for phosphorylation with an anti-phosphotyrosine antibody, and for total KDR with an anti-KDR antibody (to control for sample loading).

Results

As shown in FIG. 16, D1 was able to completely block the VEGF-induced

phosphorylation of KDR in HUVECs at 10 nM. More than half of the phosphorylation was inhibited by the compound at 1 nM. Homodimers D2 and D3, made up of the two individual binding moieties that are contained in D1, had no effect on phosphorylation at up to 100 nM, demonstrating the benefit of heterodimer constructs in blocking a receptor-ligand interaction. In multiple experiments, the IC_{50} for D1 in this assay varied between 0.5 and 1 nM. A different heterodimer containing unrelated binding sequences, a tail-to-tail heterodimer comprising the polypeptides of SEQ ID NO:305 and SEQ ID NO:306 (FIG. 64), had no effect on phosphorylation at 100 nM in spite of it's high binding affinity (11 nM for KDR by SPR), suggesting that the choice of KDR-binding moieties is important when constructing a multimer to compete with VEGF for binding to KDR. One of ordinary skill in the art would be able to construct suitable heteromultimers using the binding polypeptides provided herein and routine screening assays.

Even though the affinity of D1 for KDR is 10-fold higher than that of D2 (by SPR analysis), the IC_{50} of D1 in the activation assay is at least 100-fold lower. Without wishing to be bound by theory, this suggests that targeting two distinct epitopes on KDR with a single binding molecule can generate greater steric hindrance than a molecule with similar affinity that only binds to a single epitope on KDR and, therefore, improve the ability to inhibit VEGF induced KDR activity. Similarly, it should be pointed out that the two KDR-binding moieties within D1, when tested as monomeric free peptides (SEQ ID NO:277 and SEQ ID NO:337 in the receptor activation assay, had IC_{50}s of 0.1 and 1 micromolar, respectively. The IC_{50} for the monomeric free peptides were 100 to 1000-fold higher than the IC_{50} for D1 in the assay and 14 to 30-fold higher than the K_Ds for the fluoresceinated derivatives of the monomeric peptides. Thus, creating a dimer containing two peptides with weak VEGF-blocking activity has resulted in a molecule with very potent VEGF-blocking activity that goes well beyond the increased binding affinity of D1.

Example 15: Migration Assay

The following experiment assessed the ability of D1 to block the VEGF-induced migration of HUVECs in culture.

Protocol

Serum-starved HUVECs were placed, 100,000 cells per well, into the upper chambers of BD Matrigel-coated FluoroBlok 24-well insert plates (#354141). Basal medium, containing either nothing or different attractants such as VEGF (10 ng/mL) or serum (5% FBS) in the presence or absence of potential VEGF-blocking/inhibiting compounds, was added to the lower chamber of the wells. After 22 hours, quantitation of cell migration/invasion was achieved by post-labeling cells in the insert plates with a fluorescent dye and measuring the fluorescence of the invading/migrating cells in a fluorescent plate reader. The VEGF-induced migration was calculated by subtracting the migration that occurred when only basal medium was placed in the lower chamber of the wells.

Results:

VEGF induced a large increase in endothelial cell migration in the assay, which was potently blocked by D1. At 5 nM D1, the VEGF-stimulated endothelial cell migration was 84% blocked (see FIG. 17). At 25 nM D1, this migration was almost completely blocked. In other experiments, a known KDR inhibitor, SU-1498 (Strawn, L. *et al.*, 1996, *Cancer Res.*, 56:3540-3545) was tested in the assay. SU-1498 at 3 micromolar did not block the VEGF-induced migration as well as D1 (47% blocked at 3 micromolar). D6 (structure shown below in Example 18), at 50 nM, also produced essentially complete inhibition of the migration stimulated by VEGF. Serum was a very powerful attractant in the assay when used in place of VEGF, but its effect was not significantly diminished by D1, indicating that D1 specifically inhibits endothelial migration induced by VEGF.

Example 16: Preparation of labeled compounds

The following experiments describe methods used to prepare Tc, In, and I-labeled compounds.

Preparation of ^{99m}Tc-378 (Ac-AGPTWC*EDDWYYC*WLFGTGGGK(PnAO$_6$-NH-(O=)C(CH$_2$)$_3$C(=O)-JJ)-NH$_2$; SEQ ID NO:378).

$SnCl_2 \cdot 2H_2O$ (20 mg) was dissolved in 1 mL of 1 N HCl, and 10 µL of this solution was added to 1 mL of a DTPA solution that was prepared by dissolving 10 mg of Ca Na_2 DTPA·2.5 H_2O (Fluka) in 1 mL of water. The pH of the stannous DTPA solution was adjusted to pH 6-8 using 1N NaOH. SEQ ID NO:378 (50 µg in

50 μL of 10% DMF) was mixed with 20 μL of $^{99m}TcO_4^-$ (2.4 to 4 mCi, Syncor), followed by 100 μL of the stannous Sn-DTPA solution. After 30 minutes at RT, the radiochemical purity (RCP) was 93%. The product was purified on a Supelco Discovery C16 amide column (4 x 250 mm, 5 um pore size) eluted at a flow rate of 0.5 mL/min using an aqueous/organic gradient of 1g/L ammonium acetate in water (A) and acetonitrile (B). The following gradient was used: 30.5% B to 35% B in 30 minutes, ramp up to 70% B in 10 min. The compound, which eluted at a retention time of 21.2 minutes was collected into 500 μL of 50 mM citrate buffer (pH 5.2) containing 1% ascorbic acid and 0.1% HSA, and acetonitrile was removed using a Speed Vacuum (Savant). After purification, the compound had an RCP of >98%.

Preparation of ^{111}In-Ac-AGPTWCEDDWYYCWLFGTJK(JJ-DOTA)-NH_2 (SEQ ID NO:338).

SEQ ID NO:338 (50 μg in 50 μL of 10% DMF) was mixed with $^{111}InCl_3$ (50 μL, 400 μCi, Mallinckrodt) and 100 μL of 0.2M ammonium acetate or citrate buffer at a pH of 5.3. After being heated at 85°C for 45 minutes, the radiochemical purity (RCP) ranged from 44% to 52.2% as determined using HPLC. The ^{111}In-labeled compound was separated from unlabeled ligand using a Vydac C18 column (4.6 x 25 cm, 5 micron pore size) under following conditions: aqueous phase, 1g/L ammonium acetate (pH 6.8); organic phase, acetonitrile. Gradient: 23% org. to 25% org. in 30 minutes, up to 30% org. in 2 minutes, hold for 10 minutes. The compound, which eluted at a retention time of 20.8 min, was collected into 200 μL of 50 mM citrate buffer (pH 5.2) containing 1% ascorbic acid and 0.1% HSA, and the acetonitrile was removed using a Speed Vacuum (Savant). After purification the compound had an RCP of >93%.

Preparation of ^{111}In-D4

A histidine buffer was prepared by adjusting a 0.1M solution of histidine (Sigma) to pH 6.25 with concentrated ammonium hydroxide. Ammonium acetate buffer was prepared by adjusting a 0.2 M solution of ammonium acetate (99.99%, Aldrich) to pH 5.5 using concentrated HCl (J. T. Baker, Ultra Pure). High purity $^{111}InCl_3$ (100 μL, 1.2 mCi, Malinckrodt, Hazelwood, MO) was added to D4 (200 μg in 200 of 50% DMF, 10% DMSO, 20% acetonitrile and 20% water), followed by addition of 300 μL of histidine buffer. The final pH was 5.5. After incubation of the

reaction mixture at 85°C for 45 minutes, the RCP was 20%.

Alternatively, $^{111}InCl_3$ provided with a commercially available OctreoScan™ Kit (134 μL, 0.6 mCi, Mallinkrodt) was added to D4 (135 μg) in 162 μL of 0.2M ammonium acetate buffer. The final pH was 5.5. After incubation of the reaction mixture at 85°C for 45 min. the RCP was 20%.

Preparation of ^{125}I-D5

D5 (200 μg), in 30 μL of DMF that had been previously adjusted to pH 8.5-9.0 using diisopropyl amine, was added to 1 mCi of mono-iodinated ^{125}I Bolton-Hunter Reagent (NEX-120, Perkin-Elmer) that had been evaporated to dryness. The vial was shaken and then incubated on ice for 30 minutes with occasional shaking. After this time, the RCP was 23%. ^{125}I-D5 was purified by HPLC at a flow rate of 1 mL/min using a Vydac C18 column (4.6 x 250 mm, 5 micron pore size) under the following conditions. Aqueous phase: 0.1% TFA in water; organic phase: 0.085% TFA in acetonitrile. Gradient: 30% org. to 36% org. in 30 minutes, up to 60% org. in 5 minutes, hold for 5 minutes. The compound was collected into 200 μL of 50 mM citrate buffer (pH 5.2) containing 1% ascorbic acid and 0.1% HSA. Acetonitrile was removed using Speed Vacuum (Savant). The resulting compound had an RCP of 97% (see FIG. 65).

Example 17: Binding to KDR-Transfected Cells -

An experiment was performed to test the ability of ^{125}I-labeled D5 to bind to KDR-transfected 293H cells. In this experiment, different amounts of ^{125}I-labeled D5 (1-4 μCi/ml, labeled with ^{125}I-Bolton-Hunter reagent and HPLC-purified) were incubated with mock and KDR-transfected 293H cells in 96-well plates for 1 hr at room temperature. Binding was performed with and without 40% mouse serum to evaluate the serum effect on binding to KDR-transfected cells. After washing away the unbound compound, the cells in each well were lysed with 0.5 N NaOH and the lysates were counted with a gamma counter.

The results of this experiment are summarized in FIG. 18 and FIG. 19. ^{125}I-labeled D5 is able to specifically bind to KDR-transfected cells and its binding is not affected by the presence of 40% mouse serum. Somewhat more binding to KDR-transfected cells was observed in the absence of serum as compared to binding in the presence of 40% mouse serum. However, the binding of ^{125}I-D5 to mock-transfected

cells was also increased by about the same extent when serum was omitted during the assay, indicating that the increased binding in the absence of serum was non-specific (FIG. 18). Specific binding to KDR-transfected cells (after subtracting binding to mock-transfected cells) looked almost identical with or without mouse serum (as shown in FIG. 19). In this experiment, 10-14% of the total CPM added were specifically bound to KDR-transfected cells (data not shown).

Example 18: Biacore analysis of heterodimer binding to KDR-Fc and determination of affinity constant

A peptide heterodimer (FIG. 66) composed of SEQ ID NO:277 and SEQ ID NO:294 was prepared as previously described in Example 12 using glutaric acid bis N-hydoxysuccinimidyl ester. The heterodimer was tested for binding to KDR-Fc using Biacore and an affinity constant was determined as follows.

Three densities of KDR-Fc were cross-linked to the dextran surface of a CM5 sensor chip by the standard amine coupling procedure (0.5 mg/mL solution diluted 1:100 or 1:50 with 50 mM acetate, pH 6.0). Flow cell 1 was activated and then blocked to serve as a reference subtraction. Final immobilization levels achieved:

R_L Fc 2 KDR-Fc = 1607

R_L Fc 3 KDR-Fc = 3001

R_L Fc 4 KDR-Fc = 6319

Experiments were performed in PBS (5.5 mM phosphate, pH 7.65, 0.15 M NaCl) + 0.005% P-20 (v/v)). D6 was diluted to 250 nM in PBS and serial dilutions were performed to produce 125, 62.5, 31.3 15.6, 7.8, and 3.9 nM solutions. All samples were injected in duplicate. For association, peptides were injected at 20 µL/min for 12.5 minutes using the kinject program. Following a 10 minute dissociation, any remaining peptide was stripped from the KDR surface with a quickinject of 50 mM NaOH +1 M NaCl for 12 s at 75 µL/min. Sensorgrams were analyzed using BIAevaluation software 3.1 and a hyperbolic double rectangular regression equation in SigmaPlot 6.0. Heterodimer steady state binding affinities (K_{DAV}) were determined at all three KDR immobilization densities (Table 14).

Table 14. Summary of Parameters

		K_{D1} (nM)	R_{max1}	K_{DAV} (nM)	R_{maxAV}	R^{2*}
D6	Vs. 1600RU	46	13.1	1.5	12.6	0.995
	Vs. 3000RU	25.5	21.2	0.665	22.7	0.991
	Vs. 6000RU	17	61.3	0.662	62.2	0.993

From this data, it appears that at the higher immobilization densities, the heterodimer binds KDR with a sub-nanomolar affinity (~0.6 nM).

To assess the *in vivo* clearance of this peptide heterodimer, a small amount of material was iodinated using iodogen and $Na^{125}I$ according to standard protocols (Pierce). One tube coated with the iodogen reagent was pre-wet with 1 mL of 25 mM Tris, 0.4M NaCl, pH 7.5. This was discarded and 100 µl of the same buffer added. Using a Hamilton syringe 11 µL of the ^{125}I-NaI was transferred to the reaction tube. Based on original estimates of the $Na^{125}I$ concentration of 143.555 mCi/ml, the 11 µL should contain about 1.5 mCi. After addition, the sample was swirled and set in a lead pig to incubate for 6min with a swirl every 30 sec. After 6 min, the entire sample was transferred to the protein that was in an Eppendorf tube. The sample was swirled and set to incubate for 8 min, with a swirl every 30 sec. After 8 min the reaction was quenched (terminated) with tyrosine (10mg/mL, a saturated solution), allowed to sit for 5 min, and then 2 µL was removed for a standard.

For purification a 10 mL column of the D-salt polyacrylamide 1800 was used to separate the labeled peptide from labeled tyrosine. The column was first washed with 10 mL saline, then 5 mL of 25 mM Tris, 0.4M NaCl, pH 7.5 containing 2.5% HSA to block non-specific sites. After the HSA buffer wash, the column was eluted with 60mL of the 25 mM Tris, 0.4 M NaCl buffer, and the column was stored overnight at 4°C. The labeled sample contained 1.355 mCi, as determined by the dose calibrator. The 2 µl sample that was removed as a standard contained 8.8 µCi. The peptide sample was applied to the D-salt 1800 column and eluted with the

Tris/NaCl buffer, pH 7.5. The flow was controlled by applying single 0.5ml aliquots for each fraction, #1-14, and then 1.0 mL for fractions 25-43. FIG. 20 shows the elution profile of activity versus fraction number. The peak of activity in fractions # 9, 10, and 11, was assumed to be the peptide. The radioactivity in 24 through ~40 is likely the labeled tyrosine. From this purification, fractions #9-12 were pooled together and used for the subsequent clearance study (concentration of ^{125}I-D6 in pool is 7.023 μg/mL; 100 μL = 0.702μg with 8.6μCi).

A total of 15 mice were injected with 100 μL ^{125}I-D6 and mice (in sets of 3) were sacrificed at the following time points: 0, 7, 15, 30, 90 minutes. After injection more than 2 μCi was found remaining in the syringe, so actual activity injected was about 6 μCi. With 6 μCi injected, the corresponding protein administered was ~ 0.5 μg per animal. Once sacrificed, the counts were determined in a 50 μL plasma sample from each animal. For each set of three animals at each time point, the counts were averaged, converted to % injected dose/ml plasma (ID%/mL), and then plotted to assess the rate of clearance (FIG. 20). This data was fit to either a 4 or 5 parameter equation to determine the biphasic half life of this molecule. The 4 parameter fit resulted in a $T_{1/2\alpha}$ of 2.55 minutes and a $T_{1/2\beta}$ of 64.66 minutes. The 5 parameter fit resulted in a $T_{1/2\alpha}$ of 2.13 minutes and a $T_{1/2\beta}$ of 23.26 minutes.

Larger volumes of plasma were also taken from mice sacrificed at the 0, 30, and 90 minute time points. These samples were injected onto a Superdex peptide column (Pharmacia)-coupled to a radioactivity detector to assess the association of the peptide with serum proteins (FIG. 21). As shown, the labeled peptide does associate with higher MW proteins, which could explain its biphasic half life clearance behavior.

To help assess the potency of the peptide as an anti-angiogenesis inhibitor, D6 was tested in an endothelial cell proliferation assay using HUVECs and BrdU detection. Briefly, freshly isolated HUVECs (between p3 – 6) were cultured in RPMI + 10% FCS +1% antibiotics + 1% L-glutamine + 0.4% BBE (bovine brain extract) and seeded per well, 5000-10000/well in100 μL. The cells were allowed to recover for 24 hrs prior to use. Then the cells were washed with PBS twice and treated for 48 hrs with anti-VEGF antibody (positive control) or peptides A, B and C (0.1 and 10 ug/mL) in RPMI + 0.1% BSA + 1% L-glutamine. The following 6 variables were tested in 2 series (n=4):

Series I: w/o VEGF

Series II: w/ VEGF (30 ng/mL)

1. Standard medium: RPMI + 10% FCS +1% antibiotics + 1% L-glutamine + 0.4% BBE
2. Negative control 1: RPMI (true starvation)
3. Negative control 2: RPMI + 0.1% BSA + 1% L-glutamine
4. Positive control: anti-VEGF 10 μg/ml in RPMI + 0.1% BSA + 1% L-glutamine
5. 0.1 μg/ml KDR peptides in RPMI + 0.1% BSA + 1% L-glutamine
6. 10 μg/ml KDR peptides in RPMI + 0.1% BSA + 1% L-glutamine

Protocol:

1) cells are incubated for 48 hours under various conditions
2) 10μL BrdU dilution (1:100 in EBM) is added to each well at 24 hours
3) incubate for another 24 hours (total 48 hrs)
4) aspirate the culture medium
5) add 100μL FixDenat (Roche Applied Science, Indianapolis, IN) to each well, incubate at room temperature for 30 min.
6) Discard FixDenat solution
7) 100 μL antibody-solution (PBS 1% BSA and anti-BrdU PO) added to each well.
8) incubate at RT for 90 minutes.
9) wash 3 times with PBS, 200 μL/well, 5 min.
10) add substrate solution (TMB), incubate for 10-30 minutes
11) transfer all to a flexible plate
12) stop the reaction by adding 2 M H_2SO_4, 25 μL/well
13) read absorbance at 450 nm within 5 minutes after stopping the reaction.

Background binding was determined by omitting the anti-BrdU antibody in 4 wells with control cells (cultured in complete medium; EBM + BulletKit (Clonetics, BioWhittaker, Inc., MD) and by complete labeling of cells that was not exposed to BrdU.

Of the two KDR binding peptide tested (D6 and SEQ ID NO:277) as shown in FIG. 22, D6 completely inhibits HUVEC proliferation at 10 μg/mL in the presence of VEGF, similar to an anti-VEGF antibody (positive control). On the other hand, SEQ ID NO:277 (one of the peptides that make up the heterodimer) did not inhibit proliferation in this assay at the highest concentration tested (10 μg/mL). As a result, the heterodimer shows an enhanced ability to compete with VEGF in comparison with SEQ ID NO:277 alone.

Example 19: BIAcore Analysis—murine KDR-Fc Binding of Peptide Dimers D1 and D7

Using BIAcore, the binding constants of peptide dimers D1 (a heterodimer of SEQ ID NO:277 and SEQ ID NO:294 and D7 (a heterodimer of SEQ ID NO:264 and SEQ ID NO 294; see FIG. 67) for murine KDR-Fc were determined.

Procedure

Three densities of recombinant murine KDR-Fc were cross-linked to the dextran surface of a CM5 sensor chip by the standard amine coupling procedure (0.5 mg/mL solution diluted 1:100 or 1:40 with 50 mM acetate, pH 6.0). Flow cell 1 was activated and then blocked to serve as a reference subtraction. Final immobilization levels achieved:

R_L Fc 2 KDR-Fc = 2770

R_L Fc 3 KDR-Fc = 5085

R_L Fc 4 KDR-Fc = 9265

Experiments were performed in PBS buffer (5.5 mM phosphate, pH 7.65, 0.15 M NaCl) + 0.005% P-20 (v/v)). SEQ ID NO:277, run as a control, was diluted to 125 nM in PBS. Serial dilutions were performed to produce 62.5, 31.3, 15.6, 7.8, and 3.9 nM solutions. D1 and D6 were diluted to 50 nM in PBS and serial dilutions were performed to produce 25, 12.5, 6.25, 3.13, 1.56, 0.78, and 0.39 nM solutions. All samples were injected in duplicate. For association, peptides were injected at 30 µL/min for 3 minutes using the kinject program. Following a 10 minute dissociation, any remaining peptide was stripped from the rmKDR-Fc surface with a quickinject of 50 mM NaOH + 1 M NaCl for 12s at 75 µL/min.

Sensorgrams were analyzed using the simultaneous k_a/k_d fitting program in the BIAevaluation software 3.1. The Results are shown in Table 15 and FIGS. 23-25. The fact that about the same K_{D2} constant was achieved for both heterodimers even when the density of receptor on the sensor chip was reduced by half is consistent with multimeric binding of the heterodimers to individual receptors rather than cross-link-type binding between receptors.

Table 15. Summary of Kinetic Parameters.

		ka1 (1/Ms)	Kd1 (1/s)	ka2 (1/RUs)	kd2 (1/s)	KD1[#] (nM)	KD2[‡] (nM)	Chi[2*]
D1	vs. 2700RU	7.94E+05	0.0139	3.31E-04	5.96E-04	17.5	0.751	0.077
	vs. 5000RU	5.54E+05	8.88E-03	1.17E-04	4.57E-04	16.0	0.825	0.323

D7	vs. 2700RU	7.59E+05	0.011	3.36E-04	6.44E-04	14.5	0.848	0.082
	vs. 5000RU	5.21E+05	7.39E-03	1.17E-04	4.68E-04	14.2	0.898	0.278
Fluorescein SEQ ID NO:277	vs. 2700RU	1.02E+06	0.037	-	-	36.4	-	0.073
	vs. 5000RU	5.18E+05	0.0174	-	-	33.6	-	0.167

K_{D1} is a calculated K_D based on kd_1/ka_1

‡ K_{D2} is a calculated K_D based on kd_2/ka_1 (*i.e.*, avidity factor)

* The chi2 value is a standard statistical measure of the closeness of the fit. For good fitting to ideal data, chi2 is of the same order of magnitude as the instrument noise in RU (typically < 2).

Example 20. In Vivo inhibition of tumor growth.

Conditions are described providing methods for determining efficacy of three (3) concentrations for Test Article (binding peptide, D6) suspected of having anti-angiogenic activity on SW-480 human colon carcinoma cells using an *in vivo* xenograft tumor model.

Athymic nude mice are acceptable hosts for the growth of allogenic and heterogenic cells. Nude mice are required in *Points to Consider in the Characterizatioñ of Cell Lines used to Produce Biologicals* (*Points to Consider in the Characterization of Cell Lines used to Produce Biologicals*, FDA 1993).

D6 is a synthetic heterodimeric peptide suspected of having anti-angiogenic activity. This peptide binds to the human VEGF receptor 2 (KDR) with high affinity and competes with VEGF binding.

SW-480 Human Carcinoma Cells

Colon carcinoma, SW-480, cells (ATCC) were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 4 mM L-glutamine, 0.1 mM non-essential amino acids, 50 mg/mL Gentamicin, 250 mg/mL Fungizone and 10% heat inactivated fetal bovine serum at 37°C in 95% air and 5% CO_2.

Exponentially growing cells were harvested, washed twice in phosphate buffered saline (PBS) to remove any traces of trypsin or serum. Cells were suspended in Hanks Balanced Salt Solution (HBSS) for injections.

Sterile phosphate buffered saline (BioWhittaker) was manufactured in accordance with cGMP regulations and was cell culture tested to assure compatibility; having a pH of 7.3-7.7 and an osmolarity of 271-287 mOsm/kg. PBS was the vehicle used to reconstitute Test Articles and for vehicle control injections.

Cisplatin (American Pharmaceutical Partners, Inc.; Los Angeles, CA) was

prepared according to manufacture's specifications. Cisplatin was prepared in an aseptic fashion using a BL2 BioChem guard hood.

TEST SYSTEM

A. Species/Strain: *Mus musculus*, Crl:NU/NU-nuBR mice (nude mice)

B. Sex: Female

C. Age: 6-8 weeks at initiation of treatment

D. Weight Range: No weight requirement

E. Source: Animals were received from the Gnottobiotic Department at Charles River Laboratories, Wilmington, MA.

F. Number: A total of 115 animals were received and injected for this study, with 90 mice used on study.

G. Method of Identification:

Mice were uniquely numbered using an ear tag system. Additionally, cages were marked with cage cards minimally identifying group number, animal number, study number and IACUC protocol number.

H. Randomization:

Animals were randomly assigned to treatment groups using Microsoft® Excel 97 SR-1 program.

I. Humane Care of Animals:

Treatment and care of the animals were in accordance with the standard operating procedures of Charles River Laboratories, which adheres to the regulations outlined in the USDA Animal Welfare Act (9 CFR, Parts 1, 2, and 3) and the *Guide for the Care and Use of Laboratory Animals*.

This study protocol was covered under the Charles River Laboratories Institutional Animal Care and Use Committee (IACUC Protocol Number: P07182001I).

ANIMAL CARE

A. Diet and Drinking Water:

Mice were fed gamma-irradiated rodent chow *ad libitum*. Tap water was sterilized and supplied via bottle and sipper tube *ad libitum*.

B. Animal Environment:

Animals were housed by groups in semi-rigid isolators. Mice were housed in flat bottom caging containing five to ten animals. Cages contained gamma-irradiated contact bedding. The number of mice in each cage may have been altered due to the behavior of the mice, changes were noted in the isolator inventory. The housing conforms to the recommendations set forth in the *Guide for the Care and Use of Laboratory Animals*, National Academy Press, Washington, D.C., 1996 and all subsequent revisions.

Environmental controls were set to maintain a temperature of 16-26°C (70 ± 8°F) with a relative humidity of 30-70. A 12:12 hour light: dark cycle was maintained.

C. Acclimation:

Once animals were received, they were allowed to acclimate to the laboratory environment for 24-hours prior to the study start. Mice were observed for signs of disease, unusual food and/or water consumption or other general signs of poor condition. At the time of animal receipt, animals were clinically observed and appeared to be healthy.

EXPERIMENTAL DESIGN

A. General Description:

Female athymic nude mice (Crl:NU/NU-*nu*BR) at 6-8 weeks of age were used in this study. A total of 115 mice were injected subcutaneously into the right lateral thorax with 5×10^6 SW-480, human colon carcinoma cells. When tumors reached a target window size of approximately 150 ± 75 mg, 90 tumor-bearing mice were randomly selected and distributed into one of nine groups. Test Articles and vehicle were administered intraperitoneally (IP), Cisplatin was administered intravenously (IV). Tumor measurements were recorded twice weekly using hand-held calipers. Mice were monitored daily for signs of toxicity and morbidity. At study termination, animals were euthanized by carbon dioxide overdose and necropsied for tissue collection.

B. Group Assignments:

A total of nine (9) groups were used in this study. Each group contained ten (10) tumor-bearing mice. Groups 1 and 2 contained untreated and vehicle treated negative control mice, respectively. Groups 3, 4, and 5 contained mice that received

one of three different concentrations of the D6 anti-angiogenic peptide. Group 9 contained mice that received cisplatin, a standard chemotherapeutic compound as a positive control.

C. Dosing Levels and Regiment:

Dose levels for each group are provided in Table 16. Dosing began the same day that animals were randomly sorted into groups (Study Day 7). Each dose was removed from the dose vial using aseptic technique for each animal and the injection site was wiped with an alcohol swab prior to dose administration. Doses were administered with a 1.0 mL syringe and a 27-gauge x ½" needle for each mouse

The Test Article- and vehicle-treated mice received daily intraperitoneal (IP) injections for 15 days. Cisplatin was administered every other workday for a total of five (5) doses via an intravenous route.

Table 16. Study Treatment Groups

Group	Test Article	Concentration mg/kg	Number of Animals
1	Untreated	-	10
2	Vehicle	0	10
3	D6	0.05	10
4	D6	0.5	10
5	D6	5.0	10
9	Cisplatin	6.0	10

D. Clinical Observations of Animals:

Clinical Observations of each animal were performed and recorded at least once daily for toxicity, morbidity and mortality. Morbidity included signs of illness such as, but not limited to, emaciation, dehydration, lethargy, hunched posture, unkempt appearance, dyspnea and urine or fecal staining.

E. Tumor Measurements:

In accordance with the protocol tumor measurements were taken twice weekly throughout the study by measuring the length and width of tumors with calibrated calipers. Measurements occurred a minimum of 3-4 days apart, except when animals were euthanized and measurements were taken; this sometimes resulted in an interval of less than 3 days. Tumor weights were calculated using the

following formula: mg = (L × W^2)/2. Animals were euthanized either when mean tumor weight was ≥ 1000 mg per group over two (2) consecutive measurements, or if tumors became ulcerated, impaired the animal's ability to ambulate or obtain food and water.

F. Unscheduled Euthanasia and Unexpected Deaths:

1. Unscheduled Euthanasia:

None of the animals required unscheduled euthanasia while on study.

2. Unexpected Deaths:

None of the animals died while on study.

G. Necropsy:

1. Euthanasia and Necropsy Order:

All mice in groups 1, 2, 3, 4, and 5 (50 total) were submitted for necropsy when tumors reached a group mean target size of ≥ 1000 mg over two (2) consecutive measurements within a group. Animals were submitted for necropsy to the Charles River Laboratories Health Monitoring Laboratory (HM), Wilmington, MA. All animals were euthanized on Study Day 22, short of received the full 28 day treatment regiment with Test Articles because mean tumor size was ≥ 1000 mg in Test Article Treated Groups 3-8.

All animals were humanely euthanized by carbon dioxide (CO_2) inhalation.

2. Tissue Collection:

Tumors were dissected free of surrounding tissue and overlying skin. Additionally the kidneys were collected. Any abnormalities noted on the renal surfaces were noted.

Frozen blocks were made of tumors and kidneys for each animal. A representative section of the tissue (tumor, kidneys) was taken. Kidney sections included the cortex and medulla. Tissue sections were placed in the bottom of a labeled plastic-freezing mold. Tissue was embedded with OCT medium. Blocks were submerged into isopentane chilled with dry ice until frozen. Blocks were briefly examined for quality, and stored on dry ice.

Blocks were labeled with the animal number and a letter code corresponding to tissue (A = left kidney; B = right kidney; C = mass). Blocks from one animal were placed into a labeled bag.

RESULTS

A. In-Life Measurements and Observations:

1. Clinical Observations, Morbidity and Mortality Summary Statement:

All animals appeared healthy and were within normal limits throughout the study. D6 showed no signs of toxicity at the doses used in this study.

Animals were euthanized on Study Day 22. All mice, except Group 9 mice, were euthanized prior to completing Test Article administration, because mean tumor size was $\geq$ 1000 mg in Groups 1-8. Group 9, Cisplatin-treated animals were euthanized on Study Day 22 when mean tumor weight was 995 mg. No animals died while on study.

2. Mass Palpation Summary Statement:

Throughout the study palpable masses were detected in all mice, with tumors progressively growing for the duration of the study. As expected tumors in untreated and vehicle treated negative control mice (Groups 1 and 2) grew the fastest, reaching a mean tumor size of 1000 mg on or before Study Day 20. In addition, animals treated with Cisplatin (Group 9) developed tumors that grew the slowest reaching a mean tumor size of 995 mg at study termination (Day 22).

In general, except for Group 3 mice, all animals treated with Test Article resulted in slower tumor growth (FIG. 68). Animals in Group 3, which were treated with the low dose of D6 (0.05 mg/kg) had tumors that grew at approximately the same rate as the tumors in untreated and vehicle treated animals in Groups 1 and 2. Animals treated with either higher doses of D6 (Groups 4 and 5) had tumors that grew slower; reaching a mean tumor size of 1000 mg on Study Day 21. When compared to control Groups 1 and 2 mice, Test Article treatment resulted in a delay of tumor growth of approximately 1 day.

B. Conclusions:

Data from this study validate the model used because tumor-bearing mice in negative control Groups 1 and 2 and positive control Group 9 responded as expected.

Throughout the study palpable masses were observed in all groups. In addition, all animals were healthy and within normal limits throughout the study. Furthermore, D6 did not adversely affect the animals. Therefore, these data would suggest that animals treated with D6 Test Article had tumors that grew slowly

(approximately 1 day slower over the 22 day test period than controls). Also, since the Test Article did not show any significant toxic effects, higher concentrations of Test Article could also be used with potentially better tumor regression.

Table 17.

		Test Article					
		Untreated	Vehicle	D6			Cisplatnin
		Control	Control	0.005 mg/kg	0.05 mg/kg	0.5 mg/kg	6 mg/kg
Days After Cell Injection	4	48	49	43	51	50	34
	7	164	156	157	163	154	160
	8	180	164	156	133	168	173
	11	340	388	333	298	310	407
	14	684	648	726	596	577	675
	20	1064	986	973	857	978	635
	21	1412	1571	1468	983	1056	839
	22	1967	1863	2026	1474	1526	995
Tumor Weights (mg)							

Example 21: In vitro cell proliferation assay

Microvascular endothelial cells (MVECs, Cascade Biologics, Portland, OR) were used to assess the *in vitro* efficacy of D6 and related analogues for their ability to inhibit VEGF-stimulated proliferation. MVECs (passage 2) were grown to 90% confluency, trypsinized and plated in gelatin-coated 96-well microtiter plates at a density of 4-8 x 10^3 cells/well. Sixteen to 24 hours after plating, the cells were washed one time (200μL/well) with media devoid of fetal bovine serum but containing 0.1% bovine serum albumin (BSA). Fresh BSA-containing media was added to each well and the cells were incubated for an additional 24 hours. After this 24 hour period of starvation, fresh BSA-containing media (containing 25 ng/mL VEGF) with or without D6 was added and the cells were incubated for an additional 48 hours at 37C. To assess the dose reponse in this assay, multiple D6 concentrations were tested in duplicate wells. The media was removed and fresh BSA-containing media was added with or without BrdU and the cells were incubated for an additional 24 hours prior to determining the level of incorporation exactly as described by the manufacturer. Results are shown in FIG. 84.

Example 22.

The following experiment assessed the ability of D25 and D27 to block the VEGF-induced migration of HUVECs in culture and demonstrated that the added glycosylation and/or distinct spacer structure used in D27 enhanced its potency.

Protocol: Serum-starved HUVECs were placed, 100,000 cells per well, into the upper chambers of BD fibronectin-coated FluoroBlok 24-well insert plates. Basal medium, with or without VEGF (10 ng/mL) in the presence or absence of D25 or D27, was added to the lower chamber of the wells. After 22 hours, quantitation of cell migration/invasion was achieved by post-labeling cells in the insert plates with a fluorescent dye and measuring the fluorescence of the invading/migrating cells in a fluorescent plate reader. The VEGF-induced migration was calculated for each experimental condition by subtracting the amount of migration that occurred when only basal medium was added to the lower chamber of the wells.

Results: VEGF induced a large increase in endothelial cell migration in the assay, which was potently blocked by both D25 and D27 (FIG. 69). D27 was ten-fold more potent than D25 (IC_{50} 0.5 nM and 5 nM respectively), indicating that the glycosylation of D27 and/or its distinct spacer properties has enhanced its ability to bind KDR and block the effects of VEGF.

Example 23.

The following experiment assessed the ability of "Adjunct A" multimeric construct of TKPPR peptide (SEQ ID NO:503; binds to NP-1, a VEGF receptor that enhances the effects of VEGF mediated by KDR), to enhance the inhibition of the VEGF-induced migration of HUVECs in culture produced by D6. Adjunct A = 5CF-Gly-N{[$CH_2CH_2C(=O)$-Gly-N($CH_2CH_2C(=O)$-Adoa-Thr-Lys-Pro-Pro-Arg-OH]$_2$}$_2$ where Adoa = 3,6-dioxa-8-aminooctanoyl, 5CF = 5-carboxyfluoresceinyl.

Protocol: Serum-starved HUVECs were placed, 100,000 cells per well, into the upper chambers of BD fibronectin-coated FluoroBlok 24-well insert plates. Basal medium, containing with or without VEGF (10 ng/mL) in the presence or absence of varying concentrations of D6 , or varying concentrations of D6 in combination with a constant 100 nM Adjunct A (synthesized as described in WO 01/91805 A2), was added to the lower chamber of the wells. After 22 hours, quantitation of cell

migration/invasion was achieved by post-labeling cells in the insert plates with a fluorescent dye and measuring the fluorescence of the invading/migrating cells in a fluorescent plate reader. VEGF-induced migration was calculated for each experimental condition by subtracting the amount of migration observed in the absence of VEGF.

Results: VEGF induced a large increase in endothelial cell migration in the assay, which was potently blocked by D6 (IC_{50} about 12.5 nM), but not by 100 nM Adjunct A alone (FIG. 70). Surprisingly however, Adjunct Awas able to enhance the potency of D6 by about ten-fold when used in the assay simultaneously with D6 (IC_{50} about 2.5 nM). This indicates that compounds containing the TKPPR sequence (or similar) found in Adjunct A can be used to enhance the potency of certain compounds such as D6, which compete with VEGF for binding to KDR. In addition, a heteromultimer containing the peptide sequences found in D6 or similar) as well as the TKPPR sequence (or similar), in one or more repetitions, would likely possess enhanced activity in this assay.

Example 24: Synthesis of D27

Synthesis of **1** and **3** (see FIGS. 71 and 72)

Synthesis of the monomers were carried out as described in Method 5 on a 0.25 mmol scale employing as the starting resin Fmoc-GGGK(iV-Dde)NH-PAL-PEG-PS resin. The peptide resin was washed and dried before cleavage or further derivatization by automated or manual methods.

Procedure Synthesis of Peptide **2** and Peptide **4** (see FIGS. 71 and 72)

Appendage of Biotin-JJ, Lysyl, Glycyl and Serinyl(GalNAc$(Ac)_3$-α-D moieties onto **1** and **3** was done by manual SPPS such as described in Method 6 and Method 8. The coupling of amino acids was carried out in DMF using HOBt/DIC activation (except for Ser(GalNAc$(Ac)_3$-α-D). Fmoc removal was carried out with 20% piperidine in DMF. All couplings were 5-16 hours duration. After each coupling, the completion was confirmed by the Kaiser test. In the case of Ser(GalNAc$(Ac)_3$-α-D, the coupling was performed in DMF employing HATU/DIEA as the coupling agent. In cases where the Kaiser test indicated unreacted amino groups the coupling was repeated. Removal of the N-terminal

Fmoc group and cleavage from resin was performed. The crude peptide was precipitated in ether and washed twice by ether and dried under vacuum. The linear crude peptide was directly cyclized by dissolving the peptide in DMSO (40 mg/mL). The pH of the solyution was adjusted to 8 by addition of aqueous N-methylglucamine.and the solution was was stirred in air for 48h at room temperature. The peptides were then purified employing gradient HPLC as described in Method 1 employing a Waters-YMC C-18 ODS preparative column (250 mm x 4.6 mm i.d.). The pure product-containing fractions were combined and lyophilized to provide the needed peptides.

Procedure: Synthesis of D27 – Compound **6** (see FIG. 73)

To a solution of glutaric acid bis-NHS ester (0.122 mmol, Pierce Scientific Co.) in anhydrous DMF was added dropwise a solution of **4** in DMF (40 mg, 0.0122 mmol, DIEA was added to neutralize the trifluoroacetic acid bound to the peptide and N-hydroxysuccinimide formed during the reaction). This 0.7 mL solution was stirred for 4h. The reaction was monitored by HPLC and mass spectroscopy. DMF was removed under vacuum. The excess diester was removed by addition of ethyl acetate which precipitated the peptide-monoester **5** while dissolving glutaric acid bis-NHS ester. The mixture was centrifuged and the liquid portion decanted. This was repeated twice. The residue was kept under vacuum for 10 min. The residue was dissolved in DMF and mixed with a solution of **2** (37 mg, 0.009 mmol) in DMF (pH 7). It was stirred at ambient temperature for 16 h. The volatiles were removed under high vacuum and the acetate functions were removed by treatment of the residue with 1 mL of hydrazine/MeOH (15/85, v/v) solution with stirring for 2.5 h at ambient temperature. Acetone was added to quench the excess of hydrazine and the volatiles were removed under vacuum. The resulting residue was dissolved in DMSO and purified by preparative HPLC as described above to provide 9 mg of the pure material.

Sequence and Analytical Data for Peptides **2**, **4** and **6**

Compound identifier	**Sequence**	**HPLC Ret.**	**Mass Spectrum (ESI, neg. ion)**

		time (System)	
Peptide 2 : New Seq, a Seq 11 derivative	Ac-AGPTWCEDDWYYCWLFGTGGGK{Biotin-JJK[NH_2-Ser(GalNAc(Ac)$_3$-α-D)-Gly-Ser(GalNAc(Ac)$_3$-α-D)-]}-NH_2	7.4 min (T)	2041.3 [M - 2H]/2
Peptide 4: New Seq, a Seq 5 derivative	Ac-VCWEDSWGGEVCFRYDPGGGK(NH_2 Ser(GalNAc(Ac)$_3$-α-D)-Gly-Ser(GalNAc(Ac)$_3$-α-D)-NH_2	8.0 min (T)	1636.3 [M - 2H]/2
D27	Ac-AGPTWCEDDWYYCWLFGTGGGGK{Ac-VCWEDSWGGEVCFRYDPGGGK[S(GalNAc-α-D)-G-S(GalNAc-α-D)-Glut-S(GalNAc-α-D)-G-S(GalNAc-α-D)-NH(CH_2)$_4$-(S)-CH(Biotin-JJNH-)C(=O)-]-NH_2}-NH_2	5.50 min (M)	1737.2 (M - 4H)/4; 1389.3 (M – 5H)/5; 1157.7 [M – 6H]/6

System T: Column: Waters XTerra, 4.6 x 50 mm; Eluents:A: Water (0.1%TFA), B: Acetonitrile (0.1%TFA) : Elution: Initial condition, 15 % B, Linear Gradient 15-50 % B in 8 min; Flow rate: 3.0 mL/min; Detection: UV @ 220 nm.

Example 25: Demonstration of the distinction between binding affinity and biological potency through in vitro assays

The following experiments showed that heteromultimeric peptides can display much greater biological potency than a monomeric peptide with similar binding affinity to the same target.

Protocol experiment 1: 293H cells were transfected with the KDR cDNA or mock-transfected by standard techniques described in Example 5. The cells were incubated with ^{125}I-VEGF in the presence or absence of SEQ ID NO:504 or D1 (at

300, 30, 3, and 0.3 nM). After washing the cells, the bound radioactivity was quantitated on a gamma counter. The percentage inhibition of VEGF binding was calculated using the formula [(Y1-Y2)x100/Y1], where Y1 is specific binding to KDR-transfected 293H cells in the absence peptides, and Y2 is specific binding to KDR-transfected 293H cells in the presence of peptide competitors. Specific binding to KDR-transfected 293H cells was calculated by subtracting the binding to mock-transfected 293H cells from the binding to KDR-transfected 293H cells.

Protocol experiment 2: Serum-starved HUVECs were placed, 100,000 cells per well, into the upper chambers of BD fibronectin-coated FluoroBlok 24-well insert plates. Basal medium, with or wihout VEGF (10 ng/mL) in the presence or absence of increasing concentrations of SEQ ID NO:504 or D1, was added to the lower chamber of the wells. After 22 hours, quantitation of cell migration/invasion was achieved by post-labeling cells in the insert plates with a fluorescent dye and measuring the fluorescence of the invading/migrating cells in a fluorescent plate reader. VEGF-stimulated migration was derived by subtracting the basal migration measured in the absence of VEGF.

Results experiment 1: As shown in FIG. 74, SEQ ID NO:504 AND D1 competed about equally well with ^{125}I-VEGF for binding to KDR-transfected cells, indicating that they possess comparable binding affinities as well as a comparable ability to inhibit VEGF from binding to KDR.

Results experiment 2: Despite the fact that both SEQ ID NO:504 and D1 potently block ^{125}I-VEGF binding to KDR-expressing cells to the same degree (FIG. 75), the heterodimeric D1 was much more potent in blocking the biological effects of VEGF as demonstrated in an endothelial cell migration assay (FIG. 75) than the monomeric SEQ ID NO:504. At up to 62.5 nM, SEQ ID NO:504had no effect on VEGF-stimulated migration whereas D1 completely blocked VEGF-stimulated migration at 50 nM.

Example 26: Identification of fragments of SEQ ID NO:356 with KDR binding activity

The following experiment showed that fragments of SEQ ID NO:356 can

maintain significant KDR binding activity.

Protocol: 293H cells were transfected with the KDR cDNA or mock-transfected by standard techniques described in Example 6. Binding of the streptavidin-HRP complexes to the cells was carried out as in Example 6 with a complex concentration of 5.5 nM in the presence of 0 to 250 nM or 0 to 1000 nM of the following competing peptides: SEQ ID NOS:356, 462, 463, and 465. After determining the specific binding under each experimental condition, the IC_{50} for each peptide was determined (where possible).

Results: As shown in Table 18, SEQ ID NO:462, composed of just the Asp-Trp-Tyr-Tyr (SEQ ID NO:490) binding motif that is also shared with SEQ ID NO:286 along with the non-targeted Gly-Gly-Gly-Lys (SEQ ID NO:286) sequence that was added to most monomeric peptides synthesized based on phage display data, was the smallest fragment able to block peptide/streptavidin-HRP complex binding with an IC_{50} below one micromolar. Surprisingly, a larger fragment derived from SEQ ID NO:356, failed to significantly inhibit complex binding at one micromolar. However, when a solubilising motif, $(Gly\text{-}Arg\text{-}Gly)_3$ was added to the latter peptide to make SEQ ID NO:465, it was able to compete with the complex for binding with an IC_{50} of 175 nM, confirming that certain fragments of SEQ ID NO:356 containing the Asp-Trp-Tyr-Tyr (SEQ ID NO:490) motif retain KDR-binding activity.

Table 18. Fragments of SEQ ID NO:356 in a displacement assay competing with a complex composed of binding peptide and streptavidin-HRP for binding to KDR-expressing cells.

Fragment (SEQ ID NO)	IC_{50}, nM
356	93
462	850
463	>1000
465	175

Example 27: Cell based assay for binding of KDR/VEGF complex binders

The ability of a KDR/VEGF complex-binding peptide to selectively bind to the KDR/VEGF complex was demonstrated.

Reagent preparation

The reagents for this assay were prepared as described in Example 5 except where noted.

Preparation of peptide-^{125}I-neutravidin solution

Biotinylated peptides SEQ ID NOS:321, 320 and 323, and a biotinylated non-binding control peptide were used to prepare 1.25 μM stock solutions in 50% DMSO. A 33.33 nM stock solution of ^{125}I-streptavidin was purchased from Amersham (Buckinghamshire, UK). A stock solution of 13.33 nM ^{125}I-streptavidin/100 nM VEGF was prepared by mixing 850 mL of ^{125}I-streptavidin with 22 μL of 10 μM VEGF and 1275 μL of M199 media. Another stock solution was prepared in the same manner, but lacking VEGF. To prepare 13.33 nM peptide-^{125}I-streptavidin complex solutions ± VEGF, 500 μL of the ^{125}I-streptavidin (with and without VEGF) stock solutions (prepared in last step) were mixed with 24 μL of 1.25 μM peptide solution of SEQ ID NOS:321, 320 and 323, or control peptide. The mixtures were incubated on a rotator at 4C for 60 minutes, followed by addition of 50 μL of soft release avidin-sepharose (50% slurry in ddH_20) to remove excess peptides and another incubation for 30 minutes on a rotator at 4C. Finally, the soft release avidin-sepharose was pelleted by centrifuging at 12,000 rpm for 5 minutes at room temperature, and the resulting supernatants were used for the assays.

Binding of peptide/neutravidin HRP to KDR-transfected cells

Complexes of control peptide and the test peptides (SEQ ID NOS:321, 320 and 323) with ^{125}I-streptavidin in the presence or absence of VEGF (prepared as above) were tested for their ability to bind 293H cells that were transiently-transfected with KDR. The complex of SEQ ID NO:321 with ^{125}I-streptavidin specifically bound to KDR-transfected 293H cells as compared to mock transfected cells in the presence of VEGF (FIG. 76), but not where VEGF was omitted (FIG. 77). SEQ ID NO:321, was also the best KDR/VEGF complex binder among the peptides tested using fluorescence polarization and SPR (BIAcore) assays (Table 9). This example shows that peptide (SEQ ID NO:321) can specifically bind to the KDR/VEGF complex present on the cell surface. This establishes a utility for the assay as useful for targeting the KDR/VEGF complex *in vitro* and *in vivo* for diagnostic or therapeutic purposes. Since the KDR/VEGF binding peptide only detects the functional and active KDR receptor and not all the KDR present on cell

surface, it will be useful in detecting and/or treating active angiogenesis in tumors, metastasis, diabetic retinopathy, psoriasis, and arthropathies.

Example 28.

More evidence that heterodimeric peptides targeting two epitopes on a single target molecule can be superior to a homodimeric peptide that binds one of the two epitopes on the target molecule.

The following experiment provides further evidence that heterodimeric constructs are superior to homodimeric peptides in their ability to block the biological effects of a peptide growth factor or cytokine.

Protocol: Serum-starved HUVECs were placed, 100,000 cells per well, into the upper chambers of BD fibronectin-coated FluoroBlok 24-well insert plates. Basal medium, containing either nothing or VEGF in the presence or absence of increasing concentrations of homodimericD8 or heterodimeric D17, was added to the lower chamber of the wells. After 22 hours, quantitation of cell migration/invasion was achieved by post-labeling cells in the insert plates with a fluorescent dye and measuring the fluorescence of the invading/migrating cells in a fluorescent plate reader.

Results: VEGF induced a large increase in endothelial cell migration in the assay, which was potently blocked by D17 but not D8. D17 blocked VEGF-induced migration with an IC_{50} of about 250 nM while D8 had no significant effect on migration even at 800 nM. This is in spite of the fact that D8 used the full targeting sequence found in SEQ ID NO:356 while D17 contained a truncated version of the SEQ ID NO:356 sequence (as seen in SEQ ID NO:465) with a lower affinity for KDR (as demonstrated in Example 26).

Example 29.

Disulfide bond substitution analogs of SEQ ID NO:301, where the Cys residues at position 6 and 13 are replaced by a pair of amino acids, one with a carboxy-bearing side-chain (either Glu or Asp) and the other with an amino-bearing side chain [(Lys or Dpr (2,3-diaminopropanoic acid)] were prepared. The cycle, encompassing the same sequence positions as those included in SEQ ID NO:301

(made by formation of the disulfide bond) was made by condensation of the side-chain amino and side-chain acid moieties, resulting in a lactam ring which bridges the residues 6-13 as does the disulfide bond of SEQ ID NO:301.

Table 19 below displays some examples of the substitutions made for Cys^6 and Cys^{13} of SEQ ID NO:301 in lactam analogs.

Table 19.

Lactam Analogs of SEQ ID NO:301			
Sequence	**Position 6**	**Position 13**	**Difference in Ring Size vs SEQ ID NO:301**
Seq 301 (parent seq)	Cys	Cys	-
453	Glu	Lys	4
454	Lys	Glu	4
455	Dpr	Asp	0
456	Asp	Dpr	0
457	Asp	Lys	3

Synthesis of Resin bound peptide 1

Synthesis of **1** was carried out using Method 5 on a 0.25 mmol scale. The peptide resin **1** was washed and dried for further derivatization manually (see FIG. 78).

Synthesis of 4 (SEQ ID NO:453)

To **1** (240 mg, 0.06 mmol) was added NMM (N-methyl morpholine)/HOAc/DMF 1/2/10 (v/v/v) (65 mL). Palladium tris-triphenylphosphine [$Pd(PPh_3)_4$, 554.4 mg, 0.48 mmol] was added and the resin was shaken for 20h shielded from light. The resin was filtered and washed with a solution of sodium diethyldithiocarbamate (0.5 g)/DIEA (0.5 ml)/DMF (100 mL), and finally with DMF (3 x 70 mL). This treatment served to expose only the carboxy and amino groups of Glu6 and Lys13 which are required for the lactam forming reaction. The on-resin cyclization of **2** was carried out using HATU (114 mg, 0.3 mmol), NMM (66 μL, 0.6

mmol) and DMF (10 mL) for 3 h. The completion of the cyclization was monitored by Kaiser test. The peptide was cleaved from the peptide resin 3 using reagent B for 4 h. The resin was filtered and the filtrate was evaporated to a paste. The crude peptide was precipitated in ether and washed twice with ether. The cyclic peptide was purified by preparative reverse phase linear gradient HPLC using a Waters-YMC C-18 column (250 mm x 30 mm i.d.) with CH_3CN into H_2O (both with 0.1% TFA) as the eluent. Lyophilization of the product-containing fractions afforded 8 mg of (SEQ ID NO:453). SEQ ID NOS:454, 455, 456 and 457 were prepared similarly.

Example 30: Replacement of a disulfide bridge of while retaining KDR-binding activity

The following experiment demonstrated that the lactam SEQ ID NO:454 replaced a chemically reactive disulfide bridge to maintain significant KDR binding activity.

Protocol: 293H cells were transfected with the KDR cDNA or mock-transfected by standard techniques described in Example 6. Streptavidin-HRP complexes were prepared as in Example 6. Binding of the streptavidin-HRP complexes to the cells was carried out as in Example 6 with a complex concentration of 5.5 nM in the presence of 0 to 250 nM SEQ ID NO:454. After determining the specific binding under each experimental condition, the IC_{50} for each peptide was determined.

Results: As shown in Table 20, SEQ ID NO:454, containing a lactam disulfide bridge replacement, was still able to compete with peptide-streptavidin-HRP complexes for binding to KDR although some affinity was lost (IC_{50} 108 nM versus 13 nM).

Table 20. A binding peptide and SEQ ID NO:454 (disulfide bridge replacement analog) in a displacement assay competing with a streptavidin-HRP/binding peptide complex for binding to KDR-expressing cells.

Fragment (Ref Number)	IC_{50}, nM
Binding peptide	13
454	108

Example 31: Binding of cMet binding peptides/avidin HRP complex to MDA-MB-231 cells

This example demonstrates the advantage of making homodimers with optimized linkers and/or spacers.

The spacer length requirements for the binding of a biotinylated derivative of a cMet binding peptide, SEQ ID NO:482, to c-Met expressing MDA-MB-231 cells were determined. In order to decide the spacer length to be placed in between peptide and biotin, SEQ ID NO:482 was synthesized with no spacer, one J spacer or a JJ spacer. These three different derivatives of cMet-binding peptide SEQ ID NO:482 and a control peptide that does not bind to c-Met, were tested as tetrameric complexes with neutravidin HRP for their ability to bind c-Met expressing MB-231 cells. All three tetrameric complexes of c-Met-binding peptides bound to the MB231 cells as compared to control peptide; however, the derivative with two spacers exhibited the best K_D (12.62 nM). This suggests that inclusion of two JJ-spacers between the c-Met-binding peptide and the biotin is better than one or no spacer.

Cell Culture: MDA-MB231 cells were obtained from ATCC and grown as monolayer culture in their recommended media plus 1ml/L pen/strep (InVitrogen, Carlsbad, CA). Cells were split the day before the assay, 35000 cells were added to each well of a 96 well plate. The rest of the experiment was conducted as in Example 6, except as noted below.

Binding of peptide/neutravidin HRP to MDA-MB-231 cells

Complexes of control peptide, and SEQ ID NO:482 derivatives with 0, 1 or 2 J spacers with neutravidin-HRP were prepared as described above and tested for their ability to bind MDA-MB-231 cells. During the peptide/neutravidin-HRP complex preparation, a 7.5-fold excess of biotinylated peptides over neutravidin-HRP was used to make sure that all four biotin binding sites on neutravidin were occupied. After complex formation, the excess of free biotinylated peptides was removed using soft release avidin-sepharose to avoid any competition between free biotinylated peptides and neutravidin HRP-complexed biotinylated peptides. The

experiment was performed at several different concentrations of peptide/neutravidin-HRP, from 0.28 nM to 33.33 nM, to generate saturation binding curves for derivatives with no or one spacer (FIG. 80) and 0.28 to 16.65 nM to generate a saturation binding curve for the derivative with two spacers (FIG. 80). In order to draw the saturation binding curve, the background binding of the control peptide/neutravidin HRP complex was subtracted from the binding of the binding derivative peptide/neutravidin-HRP complexes for each concentration tested. Therefore, absorbance on the Y-axis of FIG. 80 is differential absorbance (c-Met-binding peptide minus control peptide) and not the absolute absorbance. Analysis of the saturation binding data in FIG. 80 using Graph Pad Prism software (version 3.0) yielded a K_D of 12.62 nM (+/-3.16) for the tetrameric derivative with two spacers, 155.4 nM (+/- 86.56) for the tetrameric derivative with one spacer and 123.8 nM (+/-37.71) for the tetrameric derivative without a spacer. These binding constants are, as expected, lower than that measured by FP for the related monodentate peptide SEQ ID NO:482 (880 nM).

Results: It is evident from FIG. 80 that the SEQ ID NO:482 derivative with a JJ spacer showed much better binding to c-Met on MDA-MB-231 cells than derivatives with a J spacer or without a spacer, with a K_D of 12.62 nM after subtracting binding of control peptide as background binding (n = 1). This suggests that a certain minimum spacer length may be required to be able to reach multiple different binding sites on cells and thus achieve multimeric binding. This minimum spacer length could depend on the spacing between different target molecules on cells. As was the case where the binding target was KDR, the neutravidin-HRP assay with biotinylated peptides identified with phage display was useful for identifying peptides capable of binding to an immobilized target even when the affinity of the monomeric binding sequence is too low for an ELISA-type assay (with washing steps after binding) to work well.

Example 32: Binding of Tc-labeled heterodimeric polypeptides to KDR-transfected 293H cells

The ability of Tc-labeled D10 to bind KDR was assessed using KDR-transfected 293H cells. The results show that Tc-labeled D10 binds significantly better to KDR transfected 293H cells than to mock transfected 293H cells, and good binding was maintained in the presence of 40% mouse serum. In addition, a derivative of Tc-labeled D10 with its amino acid sequence scrambled, D18, was shown to possess no affinity for KDR-expressing cells, confirming the specificity of the D10 binding to those cells.

Synthesis of ^{99m}Tc-labeled peptides

Preparation of ^{99m}Tc-D10 and ^{99m}Tc-D18

See Example 37.

Transfection of 293H cells

293H cells were transfected using the protocol described in Example 5. Transfection was done in black/clear 96-well plates (Becton Dickinson, cat. # 354640). The cells in one half of the plate (48 wells) were mock-transfected (without DNA) and the cells in the other half of the plate were transfected with KDR cDNA. The cells were 80-90% confluent at the time of transfection and completely confluent the next day, at the time of the assay (the assay was aborted if these conditions were not satisfied).

Preparation of opti-MEMI media with 0.1% HSA

Opti-MEMI was obtained from InVitrogen (Carlsbad, CA) and human serum albumin (HSA) was obtained from Sigma (St. Louis, MO). opti-MEMI media was prepared by adding 0.1% HSA, 0.1% w/v HSA to opti-MEMI, followed by stirring at room temperature for 20 minutes. The media was filter sterilized using 0.2 μM filter.

Preparation of Tc-labeled peptide dilutions for the assay Stock solutions of Tc-labeled

D10 and D18 were diluted in opti-MEMI with 0.1% HSA to provide solutions with final concentrations of 1.25, 2.5, 5.0, and 10 μCi/mL of each Tc-

labeled heterodimer. A second set of dilutions was also prepared using a mixture of 40% mouse serum/60% opti-MEMI with 0.1% HSA as the diluent.

Assay to detect the binding of the Tc-labeled heterodimers

Cells were used 24 h after transfection, and to prepare the cells for the assay, they were washed once with 100 μL of room temperature opti-MEMI with 0.1% HSA. After washing, the opti-MEMI with 0.1% HSA was removed from the plate and replaced with 70 μL of 1.25, 2.5, 5.0, and 10 μCi/mL of Tc-labeled D10 or D18 (prepared as above with both diluent solutions). Each dilution was added to three separate wells of mock- and KDR-transfected cells. After incubating at room temperature for 1 h, the plates were washed 5 times with 100 μL of cold binding buffer (opti-MEMI with 0.1% HSA). 100 μL of solubilizing solution (0.5 N NaOH) was added to each well and the plates were incubated at 37C for 10 minutes. The solubilizing solution in each well was mixed by pipeting up and down, and transferred to 1.2 mL tubes. Each well was washed once with 100 μL of solubilizing solution and the washes were added to the corresponding 1.2 mL tube. Each 1.2 mL tube was then transferred to a 15.7 mm x 100 cm tube to be counted in an LKB Gamma Counter.

Binding of Tc-labeled peptide to KDR transfected cells

The ability of Tc-labeled D10 and D18 to bind specifically to KDR was demonstrated using transiently transfected 293H cells. As shown in FIG. 81, Tc-labeled D10 bound better to KDR transfected 293H cells, as compared to mock-transfected 293H cells in both the presence and absence of 40% mouse serum, although there was some inhibition in the presence of serum. The total specific binding of this Tc-labeled heterodimer to KDR-expressing cells was greater than that observed previously with a Tc-labeled monomeric peptide (Example 10). Tc-labeled D18, on the other hand, displayed no affinity for either mock-transfected or KDR-transfected 293H cells (FIG. 81), confirming the specificity of D10 binding.

Example 33: Binding of a Lu-labeled heterodimeric polypeptide to KDR-transfected 293H cells

The ability of Lu-labeled D13 to bind KDR was assessed using KDR-transfected 293H cells. The results show that Lu-labeled D13 binds better to KDR transfected 293H cells than to mock transfected 293H cells, and significant binding was maintained in the presence of 40% mouse serum.

Preparation of ^{177}Lu-D13

See Example 37.

Transfection of 293H cells

293H cells were transfected using the protocol described in Example 5. Transfection was performed in black/clear 96-well plates (Becton Dickinson, San Jose, CA). The cells in one half of the plate (48 wells) were mock-transfected (without DNA) and the cells in the other half of the plate were transfected with KDR cDNA. The cells were 80-90% confluent at the time of transfection and completely confluent the next day, at the time of assay (the assay was aborted if these conditions were not satisfied).

Preparation of opti-MEMI media with 0.1% HSA

Opti-MEMI was prepared as in Example 32.

Preparation of Lu-labeled peptide dilutions for the assay

A stock solutions of Lu-labeled D13 was diluted in opti-MEMI with 0.1% HSA to provide solutions with final concentrations of 1.25, 2.5, 5.0, and 10 μCi/mL of labeled heterodimer. A second set of dilutions was also prepared using a mixture of 40% mouse serum/60% opti-MEMI with 0.1% HSA as the diluent.

Assay to detect the binding of the Lu-labeled heterodimers

Detection of binding was measured as detailed in Example 32 except that Lu-labeled D13 was used in place of the Tc-labeled heterodimers.

Binding of Lu-labeled peptide to KDR transfected cells

The ability of Lu-labeled D13 to bind specifically to KDR was demonstrated using transiently-transfected 293H cells. Lu-labeled D13 bound significantly better to KDR transfected 293H cells, as compared to mock-transfected 293H cells in both

the presence and absence of 40% mouse serum, although there was some binding inhibition in the presence of serum.

Example 34: Radiotherapy with a Lu-labeled heterodimeric peptide in tumor-bearing mice

In this example, the ability of Lu-labeled D13 to inhibit the growth of PC3 cell tumors implanted in nude mice is demonstrated.

Synthesis of ^{177}Lu-labeled D13

See Examle 37.

Animal model

PC3 cells from ATCC, grown as recommended by the supplier, were injected subcutaneously between the shoulder blades of nude mice. When their tumors reached 100-400 mm^3, twelve mice were injected i.v. with 500 microcuries of Lu-labeledD13 and their growth monitored for an additional 18 days. Mice were sacrificed if they lost 20% or more of their body weight or their tumors exceeded 2000 mm^3. Tumor growth in the treated mice was compared with the average tumor growth in 37 untreated nude mice implanted with PC3 tumors.

Results

In 6 of the 12 treated mice in the study, the tumors experienced a significant or complete growth delay (FIG. 83) relative to untreated tumor mice, indicating that D13 was effective in slowing PC3 tumor growth under the conditions employed.

Example 35.

Rat tumor model

Cell line: a rat mammary adenocarcinoma, designated 13762 Mat B III, was obtained from ATCC (CRL-1666) and grown in McCoy's 5a medium + 10% FCS. 1% glutamine and 1% pen/strep (InVitrogen, Carlsbad, CA). Cells in suspension were collected whereas slightly adherent cells were detached with EDTA. Cells were washed in growth medium, counted, centrifuged and resuspended in PBS or growth medium at 1 x 10^7 cell per mL.

Induction of tumor: 1 x 10^6 cells in 0.1 mL were injected into the mammary

fat pad of anesthetized female Fisher 344 rat weighing 120 to 160 g. Tumors usually grow to a diameter of 5-8 mm within 8 days.

Rat and mouse sponge model

Material: knitted alphaLite polyester swabs with long handle were obtained from Texwipe (Saddle River, NJ).

Swab insertions: Sterile mini spongy polyester fiber swab was subcutaneously implanted on the dorsal flank of the animals. Animals (mice and rats) were sacrified by overdose of anesthetic at day 15 (rat and mice) or at day 18 (mice). Swabs were removed for immunohistological examination.

Immunohistochemistry on cryosections

Material: Rabbit anti mouse flk-1 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Rat anti mouse flk-1 monoclonal antibody (Chemicon, Temecula, CA). HRP-conjugated goat anti rabbit IgG (H+L) antibody (KPL, Gaithersburg, MD). HRP conjugated rabbit anti rat IgG (H+L) antibody, and reagent grade rabbit IgG (Sigma, St. Louis, MO). Rat IgG, (Serotec, Raleigh, NC). AEC: aminoethyl carbazole substrate kit: bottles with substrate buffer, with chromogen solution, hydrogen peroxide solution.(Zymed, San Francisco, CA). Substrate for horseradish peroxidase. Hematoxylin counterstain reagent (Zymed). Glycerol vinyl alcohol aqueous mounting solution (Zymed). Superfrost Plus glass slides (Menzel-glaser, Germany).

Immunohistochemistry: Swabs and tumors were excised, frozen in isopentan and cut into 10 µm sections using a cryostat. Sections were mounted onto Superfrost Plus glass slide then fixed in cold acetone for 20 minutes. After two washes in PBS for 5 minutes, endogenous peroxidase activity was quenched by incubation with 0.5% of H_2O_2 during 30 minutes and then washed again in PBS. Sections were first treated with 0.2% BSA in PBS for 1 hour before being incubated overnight at RT with the anti-VEGF-R_2 antibody (1/50) or the biotinylated-peptides (2 µM) or non specific IgG (1/50) in PBS or PBS only. Sections were washed 3 times in PBS during 5 minutes and then incubated with goat anti-rabbit HRP antibody at the dilution 1/200 or streptavidin-HRP (for biotinylated peptides) at the dilution 1/250 during 1 hour at room temperature. Sections were washed again 3 times in PBS during 5 minutes, stained with AEC, rinsed with H_2O and counterstained with hematoxylin for 3

minutes. Tissue sections were mounted for light microscopy.

Swabs and Tumors	Antibodies		Peptides	
	Flk-1 (rabbit serum)	Flk-1 (rat mAb)	BiotinylatedSEQ ID NO:264	BIOTINYLATED seq id no:502
Swab, rat (15 days)	+++	-	++	-
Swab, mouse (15-18 days)	-	++	++ (periphery)	-
Tumor, rat (5-8 days)	Endoth. cel. +++ Tumor cel. ++	nd	Endoth. cel. +++ (periphery) Tumor cel. -	Endoth. cel. - Tumor cel. -
Tumor, mouse (14 days)	nd	Endoth. cel. + Tumor cel. +++	Endoth. cel. +++ Tumor cel. ++	Endoth. cel. - Tumor cel. -

In vivo ultrasound imaging

Material: Ultrasound imaging system: ATL HDI 5000, equipped with a linear array probe (L7-4).

Imaging: Peptide-conjugated microbubbles described in Example 36 were injected intravenously in mice with implanted swabs. Intermittent pulse inversion B-mode imaging was used to monitor the accumulation of the targeted microbubbles in the neovessels of the swab. Control experiments were performed with unconjugated microbubbles or non-specific peptide coupled to the microbubbles. Echogenic area corresponding to regions expressing the VEGF receptor2 were observed when KDR-specific microbubbles were used.

Example 36.

Suspensions of phospholipid stabilized microbubbles conjugated to KDR-binding peptides of the invention were prepared. These suspensions are useful as ultrasound contrast agents. As described in more detail above, the microbubbles

conjugated to KDR-binding peptides of the invention can be administered to an animal (including a human) and used to generate an ultrasound image of regions of the animal expressing KDR (including angiogenic areas such as tumnors).

Preparation of composition for ultrasonic echography conjugated to KDR peptide binders

Gas bubbles prepared from lipid suspensions

A series of phospholipid aqueous suspensions were prepared with the following compositions:

A) 40mg of DSPC, 10mg of DPPA, 2.5mg of N-MPB-PE (1, 2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-4-(p-maleimido-phenyl butyramide, (Avanti Polar-Lipids, Inc, Alabaster, AL) and 3g of lactose

B) 50mg of DPPS, 2.5mg N-MPB-PE and 1.5g of glycerol and 5g of propylene glycol

The components of the each composition were dispersed in 30mL of saline solution (0.9%-NaCl) by heating at 70C and then extruded 3 times through 0.2μm polycarbonate membranes (Nuclepore®). The resulting suspensions were then treated according to the following process to generate gas microbubbles: Suspension A: was frozen at -45C and lyophilised under a reduced pressure of 20mbar; the obtained dried sample was exposed to C4F10 in a vial (100mg of the lyophilisate/vial) and then reconstituted with 10mL of water; Suspension B: was homogenized under high speed mechanical agitation using Polytron® (12' 000 rpm and 2min.) under C4F10 gas.

The suspensions became milky and opaque after reconstitution or agitation. The resulting gas microbubbles were then counted using Coulter Multisizer. Gas microbubbles were observed with a size varying from 1 to 15μm and a number varying from 10^8 to 10^9 according to the type of suspension and the method of activation.

Gas bubbles prepared from dried formulations containing phospholipids

An equal amount of DSPC and of DPPG were mixed to N-MPB-PE 5% (w/w) and 1g of Macrogol-4000 (Clarian, Germany) then dissolved in tert-butanol at 60C to obtain a clear solution. The solution was aliquoted into glass vials and rapidly frozen at -45C and lyophilised. The resulting lyophilisates were exposed to

C4F10 by replacing air and sealed with stopper within the freeze-dryer (Christ®). The lyophilisates samples were reconstituted with 10mL saline solution (0.9% NaCl) per vial. After reconstitution, the bubble formation (a milky suspension), the echogenicity (backscatter coefficient at 7 MHz; see Schneider, M., 1999. *Echocardiography*, 16(7 pt 2):743-746), the resistance to pressure and the concentration (see Schneider *et al.* EP 0 554 213 B1) were determined.

Preparation of Conjugated Microbubbles Using Maleimide

Solutions of the mercaptoacetylated peptides (SEQ ID NOS:294, 264 and 286, prepared as set forth above) were prepared at 10mg/mL in DMF. To 9 mL of PBS-EDTA 10 mM, pH 7.5 was added 20µL of the peptide solution and 1mL of deacetylation solution (50 mM sodium phosphate, 25 mM EDTA, 0.5 M hydroxylamine.HCl, pH 7.5). The mixture was incubated for 30 minutes at room temperature before the addition of the maleimide-activated microbubble suspension. After two hours of incubation in the dark, under gentle agitation, the conjugated microbubbles were purified by centrifugation.

Thioacetylation of avidin: The cross-linker reagent SATA (Pierce) was used to introduce protected sulfhydryl groups into avidin (Fluka) according to the manufacturer instructions and the protein was purified by dialysis.

Avidin-conjugated microbubbles: To a solution of mercaptoacetylated-avidin was added 1/10 in volume of deacetylation solution (50 mM sodium phosphate, 25 mM EDTA, 0.5 M hydroxylamine.HCl, pH 7.5). The mixture was incubated for 30 minutes at room temperature before the addition of the maleimide-activated microbubble suspension. After two hours of incubation in the dark, under gentle agitation, the conjugated microbubbles were separated from the unconjugated protein by centrifugation. The amount of conjugated avidin was determined spectrophotometrically by using the dye HABA.

Formation of Peptide Conjugated Microbubble Suspensions

Biotinylated peptide (SEQ ID NOS:294 and 264, prepared as set forth above) was added to the suspension of avidin-conjugated microbubbles in PBS at a ratio of 10 mole of peptide per mole of avidin as determined above and incubated for 30 minutes at RT under gentle agitation. The excess of peptide was remove by

centrifugation.

Example 37.

Table

Code	SEQ ID NO: (isotope)	Sequence
P12-C (Example 5)		Ac-AGPTWCEDDWYYCWLFGTGGGK(nSbGJJ)-NH2; Ac-Ala-Gly-Pro-Thr-Trp-Cys-Glu-Asp-Asp-Trp-Tyr-Tyr-Cys-Trp-Leu-Phe-Gly-Thr-Gly-Gly-Gly-Lys(DMG-Ser-Cys(Acm)-Gly-Adoa-Adoa)-NH2; Ne22-DMG-Ser-Cys(Acm)-Gly-Adoa-Adoa
P12-XDT (Example 13)	In-887	Ac-AGPTWCEDDWYYCWLFGTJK(JJ-DOTA)-NH2 ---- from Chemistry Database
P12-P (Example 13)	Tc-378	Ac-Ala-Gly-Pro-Thr-Trp-Cys-Glu-Asp-Asp-Trp-Tyr-Tyr-Cys-Trp-Leu-Phe-Gly-Thr-Gly-Gly-Gly-Lys(PnAO-NH-(=O)C(CH2)3C(=O)-J-J)-NH2
D4 (Example 13)		Ac-AGPTWCEDDWYYCWLFGTJK(CONH2)K(JJDOTA-GLUT-[Ac-VCWEDSWGGEVCFRYDPGGGK(CONH2)JJ]
preparation protocol below	Tc-D10	Ac-AGPTWCEDDWYYCWLFGTGGGK[(6-PnAO)-C(=O)(CH2)3-C(=O)-K(-(O=)C(CH2)3C(=O)-JJ-NH(CH2)4-(S)-CH(Ac-GDSRVCWEDSWGGEVCFRYDPGGG-NH)-CONH2)]NH2; Ac-Ala-Gly-Pro-Thr-Trp-Cys-Glu-Asp-Asp-Trp-Tyr-Tyr-Cys-Trp-Leu-Phe-Gly-Thr-Gly-Gly-Gly-Lys[(6-PnAO)-C(=O)(CH2)3-C(=O)-K(-(O=)C(CH2)3C(=O)-ADOA-ADOA-NH(CH2)4-(S)-CH(Ac-Gly-Asp-Ser-Arg-Val-Cys-Trp-Glu-Asp-Ser-Trp-Gly-Gly-Glu-Val-Cys-Phe-Arg-Tyr-Asp-Pro-Gly-Gly-Gly-NH)-CONH2)]NH2;Angiogenesis Agent/KDR Reporter 6-PnAO ---- from Chemistry Database
preparation protocol below	Lu-D11	Ac-AGPTWCEDDWYYCWLFGTGGGK[DOTA-JJK(C(=O)(CH2)3C(=O)-JJ-NH(CH2)4-(S)-CH(Ac-VCWEDSWGGEVCFRYDPGGG-NH)-CONH2)]NH2; Ac-Ala-Gly-Pro-Thr-Trp-Cys-Glu-Asp-Asp-Trp-Tyr-Tyr-Cys-Trp-Leu-Phe-Gly-Thr-Gly-Gly-Gly-Lys[DOTA-ADOA-ADOA-Lys(C(=O)(CH2)3C(=O)-ADOA-ADOA-NH(CH2)4-(S)-CH(Ac-Val-Cys-Trp-Glu-Asp-Ser-Trp-Gly-Gly-Glu-Val-Cys-Phe-Arg-Tyr-Asp-Pro-Gly-Gly-Gly-NH)-CONH2)]NH2 ---- from Chemistry Database
preparation protocol below	Lu-D12	Ac-AGPTWCEDDWYYCWLFGTGGGK[(6-PnAO)-C(=O)(CH2)3-C(=O)-K(C(=O)CH2O(CH2)2O(CH2)2OCH2C(=O)-3C(=O)-NH(CH2)4-(S)-CH(Ac-VCWEDSWGGEVCFRYDPGGG-NH)-CONH2)]NH2; Ac-Ala-Gly-Pro-Thr-Trp-Cys-Glu-Asp-Asp-Trp-Tyr-Tyr-Cys-Trp-Leu-Phe-Gly-Thr-Gly-Gly-Gly-Lys[(6-PnAO)-C(=O)(CH2)3C(=O)-Lys(-C(=O)CH2O(CH2)2O(CH2)2OCH2C(=O)-3C(=O)-NH(CH2)4-(S)-CH(Ac-Val-Cys-Trp-Glu-Asp-Ser-Trp-Gly-Gly-Glu-Val-Cys-Phe-Arg-Tyr-Asp-Pro-Gly-Gly-Gly-NH)-CONH2)]NH2 ---- from Chemistry Database
preparation protocol below	Lu-D13 (DTPA)	Ac-AGPTWCEDDWYYCWLFGTGGGK(Ac-VCWEDSWGGEVCFRYDPGGGK(Adoa-Adoa-Glut-K(BOA))-NH2)-NH2; Ac-Ala-Gly-Pro-Thr-Trp-Cys-Glu-Asp-Asp-Trp-Tyr-Tyr-Cys-Trp-Leu-Phe-Gly-Thr-Gly-Gly-Gly-Lys(Ac-Val-Cys-Trp-Glu-Asp-Ser-Trp-Gly-Gly-Glu-Val-Cys-Phe-Arg-Tyr-Asp-Pro-Gly-Gly-Gly-Lys-(Adoa-Adoa-Glut-Lys(BOA))-NH2)-NH2 ---- from Chemistry Database
preparation	Tc-D14	Ac-Ala-Gln-Asp-Trp-Tyr-Tyr-Asp-Glu-Ile-Leu-Ser-Met-Ala-Asp-

protocol below		Gln-Leu-Arg-His-Ala-Phe-Leu-Ser-Gly-Gly-Gly-Gly-Gly-Lys((6-PnAO)-C(=O)(CH2)3C(=O)-Lys(C(=O)(CH2)3CO-ADOA-ADOA-NH(CH2)4-(S)-CH(Ac-Gly-Asp-Ser-Arg-Val-Cys-Trp-Glu-Asp-Ser-Trp-Gly-Gly-Glu-Val-Cys-Phe-Arg-Tyr-Asp-Pro-Gly-Gly-Gly-NH)CONH2)NH2 ---- from Chemistry Database
preparation protocol below	Tc-D18	Ac-Ala-Pro-Gly-Thr-Trp-Cys-Asp-Tyr-Asp-Trp-Glu-Tyr-Cys-Trp-Leu-Gly-Thr-Phe-Gly-Gly-Gly-Lys(PnAO6-Glut-K(Glut-ADOA-ADOA-NH(CH3)4-(S)-CH(Ac-Gly-Val-Asp-Phe-Arg-Cys-Glu-Trp-Ser-Asp-Trp-Gly-Glu-Val-Gly-Cys-Arg-Ser-Pro-Asp-Tyr-Gly-Gly-Gly-NH)CONH2))-NH2 ---- from Chemistry Database (Scrambled peptide)

Preparation of ^{99m}Tc-D10

$SnCl_2$ $2H_2O$ (20 mg) was dissolved in 1 mL of 1 N HCl, and 10 μL of this solution was added to 1 mL of a DTPA solution that was prepared by dissolving 10 mg of Ca Na_2 DTPA·2.5 H_2O (Fluka) in 1 mL of water. D10 (100 μg in 100 μL of 50% DMF) was mixed with 75 μL of 0.1 M, pH 9 phosphate buffer and 50 μL of $^{99m}TcO_4^-$ (2.4 to 5 mCi, Syncor), followed by 100 μL of the stannous Sn-DTPA solution. After 15 min at RT, the radiochemical purity (RCP) was 72%. The product was purified on a Supelco Discovery C16 amide column (4 x 250 mm, 5 um pore size) eluted at a flow rate of 0.7 mL/min using an aqueous/organic gradient of 0.1% TFA in water (A) and 0.085% TFA in acetonitrile (B; "ACN"). The following gradient was used: 30% B to 42% B in 36 min, ramp up to 70% B in 10 min. The compound, which eluted at a retention time of 32 min., was collected into 500 μL of 50 mM citrate buffer (pH 5.2) containing 0.2% HSA, and acetonitrile was removed using a Speed Vacuum (Savant). After purification, the compound had an RCP of >90%.

Preparation of ^{177}Lu-D11

D11 (5 μL of a ~1 μg/μL solution in 0.05N NH_4OH/10% EtOH) was added to a glass insert microvial containing 80 μL of 0.2M NaOAc buffer, pH 5.6. ^{177}Lu was added to bring the ligand:Lu ratio to 2:1 (1-5 mCi). The vial was crimp-sealed and heated at 100C for 15-20 minutes, cooled for 5 minutes, and treated with 3 μL of 1% Na_2EDTA $2H_2O$ in H_2O. The entire reaction mixture was injected onto a Supelco Discovery RP Amide C16 column (4 mm x 250 mm x 5 μm). The following HPLC conditions were used: column temperature = 50C, Solvent A = H_2O with 0.1% TFA, Solvent B = ACN with 0.085% TFA, gradient 0.6/0.25 mL/min A/B at t = 0 minutes to 0.5/0.4 mL/min A/B at t = 60 minutes. The retention time

for D11 was ~40 minutes; that of ^{177}Lu-D11 was ~42 minutes. The radioactive peak was collected into 0.7 mL of 0.05M citrate buffer, pH 5.3 containing 0.1% Human Serum Albumin Fraction V and 1.0% Ascorbic Acid, and the mixture was spun down in a Savant Speed Vac to remove organic solvents. Radiochemical purities of greater than 80% were obtained.

Preparation of ^{99m}Tc-D12

$SnCl_2$ $2H_2O$ (20 mg) was dissolved in 1 mL of 1 N HCl, and 10 μL of this solution was added to 1 mL of a DTPA solution that was prepared by dissolving 10 mg of Ca Na_2 DTPA·2.5 H_2O (Fluka) in 1 mL of water. D12 (100 μg in 100 μL of 50% DMF) was mixed with 75 μL of 0.1 M, pH 9 phosphate buffer and 60 μL of $^{99m}TcO_4^-$ (2.4 to 4 mCi, Syncor), followed by 100 μL of the stannous Sn-DTPA solution. After 10 min at 40C, the radiochemical purity (RCP) was 16%. The product was purified on a Supelco Discovery C16 amide column (4 x 250 mm, 5 um pore size) eluted at a flow rate of 0.7 mL/min using an aqueous/organic gradient of 0.1% TFA in water (A) and 0.085% TFA in ACN (B). The following gradient was used: 30% B to 42% B in 36 min, ramp up to 70% B in 10 min. The compound, which eluted at a retention time of 37.1 min., was collected into 500 μL of 50 mM citrate buffer (pH 5.2) containing 0.2% HSA, and ACN was removed using a Speed Vacuum (Savant). After purification, the compound had an RCP of >90%.

Preparation of ^{177}Lu-D13

D13 (306 μg) was added to a 2-mL autosampler vial with a ~450 μL conical insert and dissolved in 0.01N NH_4OH (50 μL). To this was added 300 μL of 0.5M Ammonium Acetate containing Sodium Ascorbate, Sodium Gentisate, L-Methionine and L-Tryptophan each at 10 mg/mL, plus Human Serum Albumin Fraction V at 2 mg/mL, final pH = 7.6 adjusted with NaOH. A 6.8 μL aliquot of $^{177}LuCl_3$ in 0.05N HCl (39.3 mCi) was added, the vial was crimp-sealed, warmed for 15 min at 37C, cooled for ~5 minutes, and 10 μL of 1% Na_2EDTA $2H_2O$ in H_2O was added. A 350 μL aliquot of the reaction mixture was injected onto a Supelco Discovery RP Amide C16 column (4 mm x 250 mm x 5 μm). The following HPLC conditions were used: column temperature = 37C, Solvent A = H_2O containing 2 g/L NH_4OAc buffer, pH 7.0, Solvent B = 80% ACN/20% H_2O, gradient 0.56/0.24 mL/min A/B at t = 0 minutes to 0.47/0.33 mL/min A/B at t = 30 minutes. The retention time for D13 was

~28 minutes; the retention time for ^{177}Lu-D13 was ~29 minutes. The radioactive peak was collected into 1 mL of a buffer containing Sodium Ascorbate, Sodium Gentisate, L-Methionine and L-Tryptophan each at 10 mg/mL, plus Human Serum Albumin Fraction V at 2 mg/mL, final pH = 7.6 adjusted with NaOH). It was then spun down ~40 minutes using a Speed Vacuum (Savant) to remove ACN. The RCP of the isolated product was 86%.

Preparation of ^{99m}Tc-D14

$SnCl_2$ $2H_2O$ (20 mg) was dissolved in 1 mL of 1 N HCl, and 10 μL of this solution was added to 1 mL of a DTPA solution that was prepared by dissolving 10 mg of Ca Na_2 DTPA·2.5 H_2O (Fluka) in 1 mL of water. D14 (100 μg in 100 μL of 50% DMF) was mixed with 50 μL of $^{99m}TcO_4^-$ (6 mCi, Syncor) and 125 μL of 0.1M phosphate buffer, pH 9 followed by 100 μL of the stannous Sn-DTPA solution. After 15 min at 40C, the radiochemical purity (RCP) was 21%. The product was purified on a Vydac peptide C18 column (4.6 x 250 mm) eluted at a flow rate of 1 mL/min using an aqueous/organic gradient of 0.1% TFA in water (A) and 0.085% TFA in acetonitrile (B). The following gradient was used: 30% B to 45% B in 40 min. The compound, which eluted at a retention time of 34.9 min., was collected into 500 μL of 50 mM citrate buffer (pH 5.3) containing 0.2% HSA, and ACN was removed using a Speed Vacuum (Savant). After purification, the compound had an RCP of 92.5 %.

Preparation of ^{99m}Tc-D18

$SnCl_2$ 2H2O (20 mg) was dissolved in 1 mL of 1 N HCl, and 10 μL of this solution was added to 1 mL of a DTPA solution that was prepared by dissolving 10 mg of Ca Na_2 DTPA 2.5 H_2O (Fluka) in 1 mL of water. D18 (100 μg in 100 μL of 50% DMF) was mixed with 50 μL of 0.1 M, pH 9 phosphate buffer and 90 μL of $^{99m}TcO_4^-$ (14 mCi, Syncor), followed by 100 μL of the stannous Sn-DTPA solution. The reaction was warmed for 20 minutes at 37C. The entire reaction was injected on a Vydac 218TP54 C18 column (4.6 x 250 mm, 5 um silica) and eluted at a flow rate of 1.5 mL/min using an aqueous/organic gradient of 0.1% TFA in water (A) and 0.085% TFA in ACN (B). The following gradient was used: 32% to 39% B in 30 minutes, ramp up to 80% B in 2 min. The free ligand eluted at a retention time of 19

minutes. The complex, which eluted at 24 minutes, was collected into 500 µL of 50 mM citrate buffer (pH 5.3) containing 0.1% HSA and 1% Ascorbic Acid. ACN and excess TFA were removed using a Speed Vacuum (Savant) for 40 minutes. After purification, the compound had an RCP of 93%.

Example 38: Preparation of derivatized microbubbles for peptide conjugation

200 mg of DSPC (distearoylphosphatidylcholine), 275 mg of DPPG· Na (distearoylphosphatidylglycerol sodium salt), 25 mg of N-MPB-PE were solubilized at 60C in 50 mL of Hexan/isopropanol (42/8). The solvent was evaporated under vacuum, and then PEG-4000 (35.046 g) was added to the lipids and the mixture was solubilized in 106.92 g of t-butyl alcohol at 60C, in a water bath. The solution was filled in vials with 1.5 mL of solution. The samples were rapidly frozen at -45C and lyophilized. The air in the headspace was replaced with a mixture of C_4F_{10}/Air (50/50) and vials capped and crimped. The lyophilized samples were reconstituted with 10 mL saline solution (0.9%-NaCl) per vial.

Peptide conjugation

Peptides, *e.g.*, SEQ ID NO:374 and SEQ ID NO:277, were conjugated to a preparation of microbubbles as above described, according to the following methodology.

The thioacetylated peptide (200µg) was dissolved in 20µL DMSO and then diluted in 1 mL of Phosphate Buffer Saline (PBS). This solution was mixed to the N-MPB-functionalized microbubbles dispersed in 18 mL of PBS-EDTA 10 mM, pH 7.5 and 2 mL of deacetylation solution (50 mM sodium phosphate, 25 mM EDTA, 0.5 M hydroxylamine.HCl, pH 7.5) was added. The headspace was filled with C_4F_{10}/Air (35/65) and the mixture was incubated for 2.5 hours at room temperature under gentle agitation (rotating wheel), in the dark. Conjugated bubbles were washed by centrifugation.

Example 39: Preparation of derivatized microbubbles for peptide conjugation

Distilled water (30 mL) containing 6 mg of dipalmitoylphosphatidylserine (DPPS, Genzyme), 24 mg of distearoylphosphatidylcholine (DSPC, Genzyme) and 3g of mannitol was heated to 65C in 15 minutes then cooled to room temperature. N-MPB-DPPE (1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-

maleimidophenyl) butyramide] Na salt – Avanti Polar Lipids) was added (5% molar – 1.9mg). This derivatized phospholipid was dispersed in the aqueous phase using an ultrasonic bath (Branson 1210 – 3 minutes).

Perfluoroheptane (2.4 ml from Fluka) was emulsified in this aqueous phase using a high speed homogenizer (Polytron® , 10000rpm, 1 minute).

The emulsion was washed once by centrifugation (200 g/10 min) then resuspended in 30 mL of a 10% solution of mannitol in distilled water. The washed emulsion was frozen (-45C, 5 minutes) then freeze dried (under 0.2 mBar, for 24 hours).

Atmospheric pressure was restored by introducing a mixture of C_4F_{10} and air. The lyophilizate was dissolved in distilled water (30 mL). Microbubbles were washed once by centrifugation and redispersed in 10 mL of Phosphate Buffer Saline.

Peptide conjugation

Thioacetylated peptide (200µg) was dissolved in 20µL DMSO and then diluted in 1 mL of Phosphate Buffer Saline (PBS). This solution was mixed to 5 mL of the N-MPB-functionalized microbubbles. 0.6 mL of deacetylation solution (50 mM sodium phosphate, 25 mM EDTA, 0.5 M hydroxylamine.HCl, pH 7.5) was added and the suspensions were stirred by inversion for 2h30.

Microbubbles were washed twice with a solution of maltose 5% and Pluronic F68 0.05% in distilled water, by centrifugation (200 g/10 minutes). The final volume was fixed to 5mL.

Example 40: Preparation of derivatized microballoons for peptide conjugation

Distilled water (30 mL) containing 40 mg of distearoylphosphatidylglycerol (DSPG, Genzyme) was heated to 65C during 15 minutes then cooled to 40C.

DPPE-PEG2000-Maleimide(3.5mg - Avanti Polar Lipids) and tripalmitine (60mg – Fluka) were dissolved in cyclohexane (0.6 ml) at 40C in a ultrasound bath for 2min.

This organic phase was emulsified in the aqueous phase using a high speed homogenizer (Polytron® , 10000 rpm, 1 minute).

Polyvinylalcohol (200 mg) dissolved in distilled water (5 mL) was added to the emulsion. The mixture was cooled to 5C, then frozen (-45C, 10 minutes) and finally freeze dried (under 0.2 mBar, for 24 hours).

The lyophilisate was dispersed in distilled water (15 mL). The mixture was stirred for 30 min to obtain a homogenous suspension of microballoons.

Peptide conjugation

The thioacetylated peptide (200µg) was dissolved in 20µL DMSO then diluted with PBS (1mL).

7.5 mL of the suspension of microballoons obtained as above described were centrifuged (500rpm for 5min). The infranatant was discarded and microballoons were redispersed in Phosphate Buffer Saline (2mL).

The microcapsule suspension was mixed with the solution of peptide. Three hundred microliters of a hydroxylamine solution (10.4 mg in PBS 50 mM, pH: 7.5) was added to the suspension to deprotect the thiol. The suspension was stirred by inversion for two and a half hours.

The microballoons were washed twice by centrifugation (500g/5min) with distilled water containing 5% maltose and 0.05% Pluronic F68 and finally redispersed in 3mL of this solution.

In vitro assay on transfected cells

The ability of microbubbles conjugated to peptides of the invention to bind to KDR-expressing cells was assessed using 293H cells transfected to expresss KDR.

Transfection of 293H cells on Thermanox® coverslips

293H cells were transfected with KDR DNA. The transfected cells were incubated with a suspension of peptide-conjugated microbubbles or with a control peptide (a scrambled version of the conjugated peptide having no affinity for KDR).

For the incubation with the transfected cells a small plastic cap is filled with a suspension containing 1 to 3 x 10^8 peptide-conjugated microbubbles and the cap covered with an inverted Thermanox® coverslip as to put the transfected cells in contact with the conjugated microbubbles. After about 20 min at RT, the coverslip is lifted with tweezers, rinsed three times in PBS and examined under a microscope to assess binding of the conjugated microbubbles.

FIG. 85 indicates that microballoons conjugated to peptides of the invention bind specifically to KDR-expressing cells. Indeed, microballoons conjugated to KDR-binding peptide bound to KDR-expressing cells while they did not bind appreciably to mock transfected cells and microballoons bearing a scrambled control peptide showed no appreciable binding.

Determination of the % of surface covered by microvesicles

Images were acquired with a digital camera DC300F (Leica) and the percent of surface covered by bound microbubbles or microballoons in the imaged area was determined using the software QWin (Leica Microsystem AG, Basel, Switzerland).

The following table shows the results of the binding affinity (expressed as coverage % of the imaged surface) of targeted microvesicles of the invention towards KDR transfected cells, as compared to the binding of the same targeted microvesicles towards Mock-transfected cells or (only in the case of the peptide) to the binding of microvesicles targeted with a scrambled peptide towards the same KDR transfected cells.

As shown in Table 21, targeted microvesicles show increased binding affinity for KDR.

Table 21.

	Coverage %		
	KDR	Mock	Scrambled pept.
Example 1 Binding peptide	6.7	0.2	0.1
SEQ ID NO:374	3.5	0.9	n.a.
SEQ ID NO:277	16.8	1.0	n.a.
Example 2	14.2	1.4	2.1
Example 3	15.7	0.3	1.0

n.a.: not available

In Vivo animal models

Known models of angiogenic tissue (rat matrigel model and rat Mat B III model) were used to examine the ability of the peptide conjugated ultrasound conjugates to localize to and provide an image of angiogenic tissue.

Animals: Female Fisher 344 rat (Charles River Laboratories, France) weighing 120 to 160g were used for the MATBIII tumor implantation. Male OFA rats (Charles River Laboratories, France) weighing 100 to 150g were used for Matrigel injection.

Anesthesia: Rats were anesthetized with an intramuscular injection (1mL/kg) of Ketaminol/xylazine (Veterinaria AG/Sigma) (50/10mg/mL) mixture before implantation of Matrigel or MatBIII cells. For imaging experiments, animals were anesthetized with the same mixture, plus subcutaneous injection of 50% urethane (1g/kg).

Rat MATBIII tumor model: A rat mammary adenocarcinoma, designated 13762 Mat B III, was obtained from ATCC (CRL-1666) and grown in McCoy's 5a medium + 10% FCS. 1% glutamine and 1% pen/strep (Invitrogen cat# 15290-018). Cells in suspension were collected and washed in growth medium, counted, centrifuged and resuspended in PBS or growth medium at 1.10^7 cells per mL. For tumor induction:

1×10^6 cells in 0.1 mL were injected into the mammary fat pad of anesthetized female Fisher 344 rat. Tumors usually grow to a diameter of 5-8 mm within 8 days.

Rat matrigel model: Matrigel (400 µL) (ECM, Sigma, St Louis, MO) containing human bFGF (600ng/mL) (Chemicon: ref: GF003) was subcutaneously injected in the dorsal flank of each rat.

Matrigel solution was kept liquid at 4C until injection. Immediately after matrigel injection, the injection site was maintained closed for a few seconds with the hand in order to avoid leaking of the matrigel. At the body temperature, matrigel becomes gelatinous. Ten days post-injection, neoangiogenesis was observed in matrigel plug of rat and imaging experiment were performed.

In vivo ultrasound imaging: Mat B III tumor or matrigel imaging was performed using an ultrasound imaging system ATL HDI 5000 apparatus equipped with a L7-4 linear probe. B-mode pulse inversion at low acoustic power (MI=0.05) was used to follow accumulation of peptide conjugated-microbubbles on the KDR receptor expressed on the endothelium of neovessels. For the control experiments, an intravenous bolus of unconjugated microbubbles or microbubbles conjugated to non-specific peptide was injected. The linear probe was fixed on the skin directly on line with the implanted tumors or matrigel plug and accumulation of targeted bubbles was followed during thirty minutes.

In both models, a perfusion of SonoVue® was administrated before injecting the test bubble suspension. This allows for the evaluation of the vascularization status; the video intensity obtained after SonoVue® injection is taken as an internal reference.

A baseline frame was recorded and then insonation was stopped during the bubble injection. At various time points after injection (1, 2, 5, 10, 15, 20, 25, 30 minutes) insonation was reactivated and 2 frames of one second were recorded on a videotape.

Video frames from matrigel or Mat B III tumor imaging experiments were captured and analysed with the video-capture and Image-Pro Plus 2.0 software respectively. The same rectangular Area of Interest (AOI) including the whole sectional area of the tumor or matrigel was selected on images at different time

points (1, 2, 5, 10, 15, 20, 25, 30 minutes). At each time point, the sum of the video pixel inside the AOI was calculated after the substraction of the AOI baseline. Results are expressed as the percentage of the signal obtained with SonoVue, which is taken as 100%. Similarly, a second AOI situated outside from matrigel or tumor, and representing the freely circulating contrast agent, is also analysed.

The results indicate that ultrasound contrast agents bearing KDR binding moieties of the invention localize to angiogenic (and thus KDR expressing) tissue in animal models. Specifically, FIG. 84 shows uptake and retention of bubble contrast in the tumor up to 30 minutes post injection for suspensions of phospholipids stabilized microbubbles conjugated to KDR peptides of the invention prepared according to Example 38. In contrast, the same bubbles showed only transient (no more than 10 minutes) visualization/bubble contrast in the AOI situated outside the tumor site. Similarly, FIG. 85 and FIG. 86 show uptake and retention of bubble contrast in the matrigel at up to 30 minutes post injection for suspensions of phospholipids stabilized microbubbles conjugated to KDR peptides of the invention prepared according to Example 38 (SEQ ID NO:374). In contrast, the same bubbles showed only transient (no more than 10 minutes) visualization/bubble contrast in the AOI situated outside the matrigel site.

Example 41: Enhancing the serum residence of KDR-binding peptides

It is known in the art that compounds that contain maleimide and other groups that can react with thiols react with thiols on serum proteins, especially serum albumin, when the compounds are injected. The adducts have serum life times similar to serum albumin, more than 14 days in humans for example.

Conjugation to maleimide

Methods are available that allow for the direct synthesis of maleimide-labeled linear peptides encompassed by the present invention (Holmes, D. *et al.*, 2000. *Bioconjug. Chem.*, 11:439-444).

Peptides that include disulfides can be derivatized with maleimide in one of several ways. For example, a third cysteine can be added at the carboxy terminus. The added cysteine is protected with protecting group that is orthogonal to the type of groups used for the cysteines that are to form the disulfide. The disulfide is formed by selectively deprotecting the intended cysteines and oxidizing the peptide.

The final cysteine is then deprotected and the peptide reacted with a large molar excess of a bismaleimide. The resulting compound has one of the maleimides free to react with serum albumin or other thiol-containing serum proteins.

Alternatively, a cyclic peptide of the present invention is synthesized with a lysine-containing C-terminal extention, such as -GGGK (SEQ ID NO:262). Lysines of the KDR-binding motif are protected with ivDde and the C-terminal lysine is deprotected. This lysine is reacted with a maleimide-contining compound, such as N-[e-maleimidocaproyloxy]succinimide ester (Pierce Biotechnology, Rockford, IL) or N-(a-Maleimidoacetoxy)succinimide ester (Pierce Biotechnology).

Conjugation to a moiety that binds serum albumin non-covelently

Polypeptides having a molecular weight less than 50-60 kDa are rapidly excreted. Many small molecules, such as fatty acids, bind to serum albumin. Fatty acids containing 10 to 20 carbon atoms have substantial affinity for serum albumin. Linear and branched fatty acids can be used. This binding in serum can reduce the rate of excretion. Using methods known in the art, serum-albumin-binding moieties can be conjugated to any one of the peptides herein disclosed. The serum-ablumin-binding moiety can be joined to the KDR-binding peptide through a linker. The linker can be peptidic or otherwise, such as PEG. Linkers of zero to about thirty atoms are preferred. It is preferred that the linker be hydrophilic. The serum-albumin-binding moiety can be conjugated to the KDR-binding peptide at either end or though a side group of an appended amino acid. Suitable side groups include lysine and cysteine. Such compounds can also comprise chelators for radionuclides, as discussed herein. A KDR-binding peptide joined to a serum-ablumin-binding moiety will bind KDR.

Conjugation to PEG

As is well known in the art, attachment of poly(ethyleneglycol) (PEG) to proteins and peptides enhances the serum residence of these molecules. Attachment of PEG (linear or branched) to a KDR-binding peptide is expected give substantial enhancement of serum residence time. The molecular weight of the PEG should be at least 10 kDA, more preferably at least 20 kDa, and most preferably 30 kDa or more. The PEG could be attached at the N- or C-terminus. Methods of attaching PEG to peptides are well known in the art (Roberts M. *et al.*, 2002. *Adv. Drug.*

Deliv. Rev., 54:459-476). PEG can be attached to reactive side groups such as lysine or cysteine.

Fusion to serum protein

It is known in the art that proteins comprising serum albumin (SA) and other proteins have enhanced serum residence times. The amino-acid sequence of human SA (hSA) is shown in Table 22. Table 23 shows a fusion protein comprising AGDWWVECRVGTGLCYRYDTGTGGGK(SEQ ID NO:286)::
PGGSGGEGGSGGEGGRPGGSEGGTGG::mature hSA::
GGSGGEGGSGGEGGSGPGEGGEGSGGRP ::
GDSRVCWEDSWGGEVCFRYDPGGGK(SEQ ID NO:294). The KDR-binding peptides are separated from mature hSA by linkers that are rich in glycine to allow flexible spacing. It is known in the art that one need not use all of hSA to obtain an injectable protein that will have an enhanced serum residence time. It is also known in the art that chemical groups, such as maleimide and alpha bromo carboxylates, react with the unpaired cysteine (residue 34) to form stable adducts. Thus, one could attach a single chelator to hSA fusion proteins so that the adduct will bind a radionuclide. One could prepare a chelator with a maleimide group and couple that to hSA or an hSA derivative. Alternatively, hSA or an hSA derivative could be reacted with a bismaleimide and a chelator carrying a reactive thiol could be reacted with the bismaleimide-derivatized hSA.

Construction of genes that encode a given amino-acid sequence are known in the art. Expression of HSA fusions in *Saccharomyces cerevisiae* is known in the art (Sleep, D *et al.*, 1991. *Biotechnology (NY)*, 9:183-187).

Pretargeting radioactivity or toxins to KDR expressing tumors

Conventional radioimmune cancer therapy is plagued by two problems. The generally attainable targeting ratio (ratio of administered dose localizing to tumor versus administered dose circulating in blood or ratio of administered dose localizing to tumor versus administered dose migrating to bone marrow) is low. Also, the absolute dose of radiation or therapeutic agent delivered to the tumor is insufficient in many cases to elicit a significant tumor response. Improvement in targeting ratio or absolute dose to tumor would be of great importance for cancer therapy.

The present invention provides methods of increasing active agent localization at a target cell site of a mammalian recipient. The methods include, for example, a) administering to a recipient a fusion protein comprising a targeting moiety and a member of a ligand-anti-ligand binding pair; b) thereafter administering to the recipient a clearing agent capable of directing the clearance of circulating fusion protein via hepatocyte receptors of the recipient, wherein the clearing agent incorporates a member of the ligand-anti-ligand binding pair; and c) subsequently administering to the recipient an active agent comprising a ligand/anti-ligand binding pair member.

It is known in the art that hexoses, particularly the hexoses galactose, glucose, mannose, mannose-6-phosphate, N-acetylglucosamine, pentamannosyl phosphate, N-acetylgalactosamine, thioglycosides of galactose, and mixtures thereof are effective in causing hepatic clearance. Binding of sugars to hepatic receptors is not, however, the only means of directing a molecule to the liver.)

> "Clearance of carcinoembryonic antigen (CEA) from the circulation is by binding to Kupffer cells in the liver. We have shown that CEA binding to Kupffer cells occurs via a peptide sequence YPELPK representing amino acids 107-112 of the CEA sequence. This peptide sequence is located in the region between the N-terminal and the first immunoglobulin like loop domain. Using native CEA and peptides containing this sequence complexed with a heterobifunctional crosslinking agent and ligand blotting with biotinylated CEA and NCA we have shown binding to an 80kD protein on the Kupffer cell surface. This binding protein may be important in the development of hepatic metastases." (Thomas, P. *et al.*, 1992. *Biochem. Biophys. Res. Commun.*, 188:671-677

To use YPELPK (SEQ ID NO:498) as a clearance agent, one fuses this sequence via a linker to a moiety that binds the fusion protein (Ab). For example, if the Ab has affinity for DOTA/Re, one would make a derivative having YPELPK attached to DOTA/Re; for example, rvYPELPKpsGGG-DOTA. 'rvYPELPKps' is a fragment of CEA which includes the YPELPK sequence identified by Thomas et al. Any convenient point on DOTA can be use for attachment. RVYPELPKPSGGG-DOTA/cold Re (SEQ ID NO:499) would then be used as a clearing agent. The Fab corresponding to the fusion Ab would have affinity for the clearing agent of Kd <

100 nM, preferably Kd < 10 nM, and most preferably Kd < 1 nM.

The therapeutic agent would contain DOTA/^{185}Re. In a preferred embodiment, the therapeutic agent would contain two or more DOTA moieties so that the Ab immobilized on the tumor would bind the bis-DOTA compound with high avidity. The two DOTA moieties would preferably be connected with a hydrophilic linker of ten to thirty units of PEG. PEG is a preferred linker because it is not degraded, promotes solubility. Ten to thirty units of PEG is not sufficient to give the bis DOTA compound a very long serum residence time. A half life of 30 minutes to 10 hours is acceptable. The serum half life should be longer than the radioactive half life of the radionuclide used so that most of the radiation is delivered to the tumor or to the external environment.

In one embodiment, a "fusion protein" of the present invention comprises at least one KDR-binding peptide fused to the amino terminus or the carboxy terminus of either the light chain (LC) or the heavy chain (HC) of a human antibody. Optionally and preferably, two or more KDR-binding peptides are fused to the antibody. The antibody is picked to have high affinity for a small molecule that can be made radioactive or have a toxin attached. Preferably, the affinity of the Fab corresponding to the Ab has affinity for the small molecule with Kd less than 100 nM, more preferably less than 10 nM, and most preferably less than 1 nM. The small molecule could be a chelator capable of binding a useful radioactive atom, many of which are listed herein. The small molecule could be a peptide having one or more tyrosines to which radioactive iodine can be attached without greatly affecting the binding property of the peptide.

Any KDR-binding peptide (KDR-BP) of the present invention can be fused to either end of either chain of an antibody that is capable of binding a small radioactive compound. Useful embodiments include:

1) KDR-BP#1::link::LC / HC,

2) LC::link::KDR-BP#1 / HC,

3) LC / KDR-BP#1::link::HC,

4) LC / HC::link::KDR-BP#1,

5) KDR-BP#1::link1::LC::link2::KDR-BP#2 / HC,

6) LC / KDR-BP#1::link1::HC::link2::KDR-BP#2,

7) KDR-BP#1::link1::LC / KDR-BP#2::link2::HC,

8) KDR-BP#1::link1::LC / HC::link2:: KDR-BP#2,

9) LC::link1::KDR-BP#1 / KDR-BP#2::link2::HC,

10) LC::link1::KDR-BP#1 / HC::link2:: KDR-BP#2,

11) KDR-BP#1::link1::LC::link2::KDR-BP#2 / KDR-BP#3::link3::HC,

12) KDR-BP#1::link1::LC::link2::KDR-BP#2 / HC::link3::KDR-BP#3,

13) KDR-BP#3::link3::LC / KDR-BP#1::link1::HC::link2::KDR-BP#2,

14) LC::link3::KDR-BP#3 / KDR-BP#1::link1::HC::link2::KDR-BP#2, and

15) KDR-BP#1::link1::LC::link2::KDR-BP#2 / KDR-BP#3::link3::HC::link4::KDR-BP#4.

In cases (5)-(15), the linkers (shown as "link1", "link2", "link3", and "link4") can be the same or different or be absent. These linkers, if present, are preferably hydrophilic, protease resistant, non-toxic, non-immunogenic, and flexible. Preferably, the linkers do not contain glycosylation sites or sequences known to cause hepatic clearance. A length of zero to fifteen amino acids is preferred. The KDR-binding peptides (KDR-BP#1, #2, #3, and #4) could be the same or different. If the encoded amino-acid sequences are the same, it is preferred that the DNA encoding these sequences is different.

Since antibodies are dimeric, each fusion protein will present two copies of each of the fused peptides. In case (15), there will be eight KDR-BPs present and binding to KDR-displaying cells should be highly avid. It is possible that tumor penetration will be aided by moderate KDR affinity in each of the KDR-BPs rather than maximal affinity.

One group of preferred embodiments have SEQ ID NO:294 as one of the KDR-BPs and SEQ ID NO:286 as the other. For example, in case (7) (KDR-BP#1::link1::LC / KDR-BP#2::link2::HC), KDR-BP#1 is SEQ ID NO:294 and KDR-BP#2 is SEQ ID NO:286 and link1 is between 10 and 20 amino acids and link2 is also between ten and twenty amino acids. A suitable sequence for link1 is GGSGGEGRPGEGGSG (SEQ ID NO:491) and a suitable sequence for link2 is GSESGGRPEGGSGEGG (SEQ ID NO:492). Other sequences rich in Gly, Ser, Glu, Asp, Thr, Gln, Arg, and Lys are suitable. To reduce the risk of proteolysis, it is preferred to follow Arg or Lys with Pro. To avoid difficulties in production and poor solubility, it is preferred to avoid long stretches (more than twelve) of uncharged residues. Since the peptides are displayed at the amino termini of LC and HC, the combined linker length will allow them to bind to KDR simultaneously.

Additionally, in case (15)(KDR-BP#1::link1::LC::link2::KDR-BP#2 / KDR-BP#3::link3::HC::link4::KDR-BP#4), KDR-BP#1 and KDR-BP#2 are SEQ ID NO:294 and KDR-BP#3 and KDR-BP#4 are DX-912. Link1 and link3 are 10 to 20 amino acids and link2 and link4 are each 15 to 30 amino acids. Link2 and link4 are longer because they need to allow a peptide on the carboxy terminus of LC to reach a peptide on the carboxy terminus of HC.

The fusion protein is produced in eukaryotic cells so that the constant parts of the HC will be glycosylated. Preferably, the cells are mammalian cells, such as CHO cells.

The fusion proteins are injected into a patient and time is allowed for the fusion protein to accumulate at the tumor. A clearing agent is injected so that fusion protein that has not become immobilized at the tumor will be cleared. In previous pretargeting methods, the antibody combining site has been used to target to the tumor and biotin/avidin or biotin/streptavidin has been used to attach the radioactive or toxic agent to the immobilized antibody. The biotin/avidin or streptavidin binding is essentially irreversible. Here we fuse a target-binding peptide to the antibody which is picked to bind a radioactive or toxic agent. Because the fusion protein contains 2, 4, 6, or 8 KDR-BPs, binding of the fusion protein to the tumor is very avid. A clearing agent that will cause fusion protein not immobilized at the tumor to clear can be administered between 2 and 48 hours of the injection of the fusion protein. Because the clearance agent is monomeric in the moiety that binds the antibody, complexes of clearance agent and immobilized fusion protein will not have very long life times. Within 4 to 48 hours of injecting clearance agent, the immobilized antibody will have lost any clearance agent that binds there. The active agent is, preferably, dimeric in the moiety that binds the fusion protein. The active agent is injected between 2 and ~ 48 hours of injection of clearance agent.

Table 22: Amino-acid sequence of Mature HSA from GenBank entry AAN17825

DAHKSEVAHR FKDLGEENFK ALVLIAFAQY LQQCPFEDHV KLVNEVTEFA
KTCVADESAE NCDKSLHTLF GDKLCTVATL RETYGEMADC CAKQEPERNE
CFLQHKDDNP NLPRLVRPEV DVMCTAFHDN EETFLKKYLY EIARRHPYFY
APELLFFAKR YKAAFTECCQ AADKAACLLP KLDELRDEGK ASSAKQRLKC
ASLQKFGERA FKAWAVARLS QRFPKAEFAE VSKLVTDLTK VHTECCHGDL
LECADDRADL AKYICENQDS ISSKLKECCE KPLLEKSHCI AEVENDEMPA
DLPSLAADFV ESKDVCKNYA EAKDVFLGMF LYEYARRHPD YSVVLLLRLA
KTYKTTLEKC CAAADPHECY AKVFDEFKPL VEEPQNLIKQ NCELFEQLGE

YKFQNALLVR YTKKVPQVST PTLVEVSRNL GKVGSKCCKH PEAKRMPCAE
DYLSVVLNQL CVLHEKTPVS DRVTKCCTES LVNRRPCFSA LEVDETYVPK
EFNAETFTFH ADICTLSEKE RQIKKQTALV ELVKHKPKAT KEQLKAVMDD
FAAFVEKCCK ADDKETCFAE EGKKLVAASR AALGL (SEQ ID NO:500)

Table 23: SEQ ID NO:286::linker1::HSA::linker2::SEQ ID NO:294

AGDWWVECRVGTGLCYRYDTGTGGGK
PGGSGGEGGSGGEGGRPGGSEGGTGG
DAHKSEVAHR FKDLGEENFK ALVLIAFAQY LQQCPFEDHV KLVNEVTEFA
KTCVADESAE NCDKSLHTLF GDKLCTVATL RETYGEMADC CAKQEPERNE
CFLQHKDDNP NLPRLVRPEV DVMCTAFHDN EETFLKKYLY EIARRHPYFY
APELLFFAKR YKAAFTECCQ AADKAACLLP KLDELRDEGK ASSAKQRLKC
ASLQKFGERA FKAWAVARLS QRFPKAEFAE VSKLVTDLTK VHTECCHGDL
LECADDRADL AKYICENQDS ISSKLKECCE KPLLEKSHCI AEVENDEMPA
DLPSLAADFV ESKDVCKNYA EAKDVFLGMF LYEYARRHPD YSVVLLLRLA
KTYKTTLEKC CAAADPHECY AKVFDEFKPL VEEPQNLIKQ NCELFEQLGE
YKFQNALLVR YTKKVPQVST PTLVEVSRNL GKVGSKCCKH PEAKRMPCAE
DYLSVVLNQL CVLHEKTPVS DRVTKCCTES LVNRRPCFSA LEVDETYVPK
EFNAETFTFH ADICTLSEKE RQIKKQTALV ELVKHKPKAT KEQLKAVMDD
FAAFVEKCCK ADDKETCFAE EGKKLVAASR AALGL
GGSGGEGGSGGEGGSGPGEGGEGSGGRP
GDSRVCWEDSWGGEVCFRYDPGGGK (SEQ ID NO:501)

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims. The publications, patents and other references cited herein are incorporated by reference herein in their entirety.

CLAIMS

What is claimed is:

1. An isolated polypeptide having the ability to bind to KDR or VEGF/KDR complex comprising an amino acid sequence of one of the following:

Consensus Sequence I: Cys-X_2-X_3-X_4-X_5-X_6-X_7-Cys (TN8), wherein

X_2 is Ala, Arg, Asn, Asp, Gln, Glu, His, Ile, Lys, Phe, Pro, Ser, Trp, or Tyr (preferably Asp, Glu, or Tyr);

X_3 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Glu, Met, or Tyr);

X_4 is Ala, Asn, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Asp);

X_5 is Ala, Asp, Glu, Gly, Leu, Phe, Pro, Ser, Thr, Trp, or Tyr (preferably Trp or Thr);

X_6 is Arg, Gln, Glu, Gly, Ile, Leu, Met, Pro, Thr, Trp, Tyr, or Val (preferably Gly or Tyr); and

X_7 is Ala, Arg, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Trp, or Tyr (preferably Lys or Tyr); or

Consensus Sequence II:Cys-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-Cys (TN12), wherein

X_2 is Arg, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Thr, Trp, Tyr, or Val (preferably Glu, Ile, or Tyr);

X_3 is Ala, Arg, Asn, Cys, Glu, Ile, Leu, Met, Phe, Ser, Trp, or Tyr (preferably Glu, Phe, or Tyr);

X_4 is Arg, Asn, Asp, Gln, Glu, His, Ile, Leu, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Glu);

X_5 is Ala, Asn, Asp, Gln, Glu, Gly, His, Met, Phe, Pro, Ser, Trp, Tyr, or Val (preferably Gln or Ser);

X_6 is Asp, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, or Tyr (preferably Asp);

X_7 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Lys or Ser);

X_8 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Lys, Trp, Tyr, or Val (preferably Gly or Tyr);

X_9 is Ala, Arg, Gln, Gly, His, Ile, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Trp or Thr);

X_{10} is Arg, Gln, Glu, His, Leu, Lys, Met, Phe, Pro, Thr, Trp, or Val (preferably Glu or Trp); and

X_{11} is Arg, Asn, Asp, Glu, His, Ile, Leu, Met, Phe, Pro, Thr, Trp, Tyr, or Val (preferably Phe); or

Consensus Sequence III: Cys-X_2-X_3-X_4-Gly-X_6-Cys (TN7), wherein

X_2 is Asn, Asp, or Glu;

X_3 is Glu, His, Lys, or Phe;

X_4 is Asp, Gln, Leu, Lys, Met, or Tyr; and

X_6 is Arg, Gln, Leu, Lys, or Val; or

Consensus Sequence IV: Cys-X_2-X_3-X_4-X_5-X_6-X_7-X_8-Cys (TN9), wherein

X_2 is Ala, Asp, Lys, Ser, Trp, or Val (preferably Lys);

X_3 is Asn, Glu, Gly, His, or Leu;

X_4 is Gln, Glu, Gly, Met, Lys, Phe, Tyr, or Val (preferably Met);

X_5 is Ala, Asn, Asp, Gly, Leu, Met, Pro, Ser, or Thr;

X_6 is His, Pro, or Trp (preferably Pro or Trp);

X_7 is Ala, Gly, His, Leu, Trp, or Tyr (preferably Trp); and

X_8 is Ala, Asp, Gln, Leu, Met, Thr, or Trp; or

Concensus Sequence V: Cys-X_2-X_3-X_4-X_5-Ser-Gly-Pro-X_9-X_{10}-X_{11}-X_{12}-Cys (MTN13; SEQ ID NO:1), wherein

X_2 is Asp, Glu, His, or Thr;

X_3 is Arg, His, Lys, or Phe;

X_4 is Gln, Ile, Lys, Tyr, or Val;

X_5 is Gln, Ile, Leu, Met, or Phe;

X_9 is Asn, Asp, Gly, His, or Tyr;

X_{10} is Gln, Gly, Ser, or Thr;

X_{11} is Glu, Lys, Phe, or Ser; and

X_{12} is Glu, Ile, Ser, or Val.

2. The polypeptide according to Claim 1, wherein the polypeptide comprises an amino acid sequence of one of the following:

Consensus Sequence VI: Cys-X_2-X_3-X_4-X_5-X_6-Tyr-Cys (TN8), wherein

X_2 is Ala, Arg, Glu, Lys, or Ser (preferably Glu);

X_3 is Ala, Asp, Gln, Glu, Thr, or Val (preferably Asp or Glu);

X_4 is Asp or Glu;

X_5 is Trp or Tyr; and

X_6 is Thr or Tyr (preferably Tyr); or

Consensus Sequence VII: Cys-X_2-X_3-X_4-Gly-X_6-X_7-Cys (TN8), wherein

X_2 is Asp, Gln, or His;

X_3 is His or Tyr (preferably Tyr);

X_4 is His, Ile, or Tyr;

X_6 is Ile, Met, or Val; and

X_7 is Gly or Tyr; or

Consensus Sequence VIII: Cys-X_2-X_3-X_4-X_5-Gly-X_7-Cys (TN8), wherein

X_2 is Ala, Arg, Asn, Asp, His, Phe, Trp, or Tyr (preferably Tyr, Trp, or Phe);

X_3 is Ala, Asp, Gln, His, Lys, Met, Ser, Thr, Trp, Tyr, or Val;

X_4 is Ala, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Pro, Ser, Thr, or Val;

X_5 is Asp, Phe, Ser, Thr, Trp, or Tyr (preferably Thr, Ser, or Asp); and

X_7 is Ala, Arg, Gln, His, Ile, Leu, Lys, Met, Phe, Trp, or Tyr (preferably Arg, or Lys).

3. The polypeptide according to Claim 1, wherein the polypeptide comprises an amino acid sequence of one of the following:

Consensus Sequence IX: Cys-X_2-X_3-X_4-X_5-Trp-Gly-Gly-X_9-X_{10}-Cys (SEQ ID NO:3; TN11, *i.e.*, 11-mers based on isolates of the TN12 library), wherein

X_2 is Ala, Phe, or Trp (preferably Trp or Phe);

X_3 is Glu or Lys (preferably Glu);

X_4 is Asp, Ser, Trp, or Tyr (preferably Asp, Trp, or Tyr);

X_5 is Phe, Pro, or Ser (preferably Ser);

X_9 is Gln or Glu (preferably Glu); and

X_{10} is Ile, Phe, or Val; or

Consensus Sequence X: Cys-X_2-Glu-X_4-Ser-X_6-Ser-X_8-X_9-X_{10}-Phe-Cys (SEQ ID NO:4; TN12), wherein

X_2 is His or Tyr;

X_4 is Leu, His, or Thr;

X_6 is Asp or Leu (preferably Asp);

X_8 is Gly or Val (preferably Val);

X_9 is Thr or Val (preferably Thr); and

X_{10} is Arg or Trp (preferably Arg); or

Consensus Sequence XI: Cys-X_2-X_3-X_4-X_5-X_6-X_7-Gly-X_9-Trp-X_{11}-Cys (TN12; SEQ ID NO:5), wherein

X_2 is Glu, Met, or Thr (preferably Glu);

X_3 is Ile, Leu, Met, or Phe (preferably Met, Leu, Phe);

X_4 is Arg, Asp, Glu, Met, Trp, or Val;

X_5 is Asn, Gln, Gly, Ser, or Val;

X_6 is Glu or Asp;

X_7 is Lys, Ser, Thr, or Val (preferably Lys);

X_9 is Arg, Gln, Lys, or Trp (preferably Trp, Arg, or Lys); and

X_{11} is Asn, Leu, Phe, or Tyr (preferably Tyr, Phe, or Asn); or

Consensus Sequence XII: Cys-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-Cys (TN12), wherein

X_2 is Glu or Gly;

X_3 is Trp or Tyr;

X_4 is Ser or Thr;

X_5 is Asn or Gln;

X_6 is Gly or Met;

X_7 is Phe or Tyr;

X_8 is Asp or Gln;

X_9 is Lys or Tyr;

X_{10} is Glu or Thr; and

X_{11} is Glu or Phe.

4. The polypeptide according to Claim 1, wherein the polypeptide comprises an amino acid sequence of the following:

Cys-X_2-X_3-X_4-Gly-X_6-Cys (TN7), wherein

X_2 is Asn, Asp, or Glu;

X_3 is Glu, His, Lys, or Phe;

X_4 is Asp, Gln, Leu, Lys, Met, or Tyr; and

X_6 is Arg, Gln, Leu, Lys, or Val.

5. The polypeptide according to Claim 1, wherein the polypeptide comprises an amino acid sequence of the following:

Cys-X_2-X_3-X_4-X_5-X_6-X_7-X_8-Cys (TN9), wherein

X_2 is Ala, Lys, Ser, Trp, or Val (preferably Lys);

X_3 is Asn, Glu, Gly, His, or Leu;

X_4 is Glu, Gly, Lys, Met, or Tyr (preferably Met);

X_5 is Ala, Asn, Asp, Leu, Met, Pro, or Ser;

X_6 is His, Pro, or Trp (preferably Pro);

X_7 is His, Leu, Trp or Tyr (preferably Trp or His); and

X_8 is Ala, Asp, Gln, Leu, Met, Thr, or Trp.

6. The polypeptide according to Claim 1, wherein the polypeptide comprises an amino acid sequence of the following:

Cys-X_2-X_3-X_4-X_5-Ser-Gly-Pro-X_9-X_{10}-X_{11}-X_{12}-Cys (SEQ ID NO:1; MTN13), wherein

X_2 is Asp, Glu, His, or Thr;

X_3 is Arg, His, Lys, or Phe;

X_4 is Gln, Ile, Lys, Tyr, or Val;

X_5 is Gln, Ile, Leu, Met, or Phe;

X_9 is Asn, Asp, Gly, His, or Tyr;

X_{10} is Gln, Gly, Ser, or Thr;

X_{11} is Glu, Lys, Phe, or Ser; and

X_{12} is Glu, Ile, Ser, or Val.

7. An isolated polypeptide having the ability to bind to KDR or VEGF/KDR complex comprising an amino acid sequence of one of the following:

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-Cys-X_{12}-X_{13}-X_{14} (TN8), wherein

X_1 is Ala, Arg, Asp, Gly, His, Leu, Lys, Pro, Ser, Thr, Trp, Tyr, or Val;

X_2 is Asn, Asp, Glu, Gly, Ile, Leu, Lys, Phe, Ser, Thr, Trp, Tyr, or Val;

X_3 is Asn, Asp, Gln, Glu, Ile, Leu, Met, Thr, Trp, or Val;

X_5 is Ala, Arg, Asn, Asp, Gln, Glu, His, Ile, Lys, Phe, Pro, Ser, Trp, or Tyr;

X_6 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X_7 is Ala, Asn, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X_8 is Ala, Asp, Glu, Gly, Leu, Phe, Pro, Ser, Thr, Trp, or Tyr;

X_9 is Arg, Gln, Glu, Gly, Ile, Leu, Met, Pro, Thr, Trp, Tyr, or Val;

X_{10} is Ala, Arg, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Trp, or Tyr;

X_{12} is Arg, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X_{13} is Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, or Tyr; and

X_{14} is Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, or Tyr; or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}-X_{14}-Cys-X_16-X_{17}-X_{18} (TN12), wherein

X_1 is Ala, Asn, Asp, Gly, Leu, Pro, Ser, Trp, or Tyr (preferably Asn, Asp, Pro, or Tyr);

X_2 is Ala, Arg, Asn, Asp, Gly, His, Phe, Pro, Ser, Trp, or Tyr (preferably Asp, Gly, Pro, Ser, or Trp);

X_3 is Ala, Asn, Asp, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Trp);

X_5 is Arg, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Thr, Trp, Tyr, or Val (preferably Glu, Ile, or Tyr);

X_6 is Ala, Arg, Asn, Cys, Glu, Ile, Leu, Met, Phe, Ser, Trp, or Tyr (preferably Glu, Phe, or Tyr);

X_7 is Arg, Asn, Asp, Gln, Glu, His, Ile, Leu, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Glu);

X_8 is Ala, Asn, Asp, Gln, Glu, Gly, His, Met, Phe, Pro, Ser, Trp, Tyr, or Val (preferably Gln or Ser);

X_9 is Asp, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, or Tyr (preferably Asp);

X_{10} is Ala, Arg, Asn, Asp, Gln, Glu, Gly, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Lys or Ser);

X_{11} is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Lys, Trp, Tyr, or Val (preferably Gly or Tyr);

X_{12} is Ala, Arg, Gln, Gly, His, Ile, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Trp or Thr);

X_{13} is Arg, Gln, Glu, His, Leu, Lys, Met, Phe, Pro, Thr, Trp, or Val (preferably Glu or Trp);

X_{14} is Arg, Asn, Asp, Glu, His, Ile, Leu, Met, Phe, Pro, Thr, Trp, Tyr, or Val (preferably Phe);

X_{16} is Ala, Asn, Asp, Gln, Glu, Gly, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Asp);

X_{17} is Arg, Asn, Asp, Cys, Gly, His, Phe, Pro, Ser, Trp, or Tyr (preferably Pro or Tyr); and

X_{18} is Ala, Asn, Asp, Gly, His, Leu, Phe, Pro, Ser, Trp, or Tyr (preferably Asn, Pro, or Trp); or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-Gly-X_9-Cys-X_{11}-X_{12}-X_{13} (TN7), wherein

X_1 is Gly or Trp;

X_2 is Ile, Tyr, or Val;

X_3 is Gln, Glu, Thr, or Trp;

X_5 is Asn, Asp, or Glu;

X_6 is Glu, His, Lys, or Phe;

X_7 is Asp, Gln, Leu, Lys, Met, or Tyr;

X_9 is Arg, Gln, Leu, Lys, or Val;

X_{11} is Arg, Phe, Ser, Trp, or Val;

X_{12} is Glu, His, or Ser; and

X_{13} is Glu, Gly, Trp, or Tyr; or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-Cys-X_{13}-X_{14}-X_{15} (TN9), wherein

X_1 is Arg, Asp, Gly, Ile, Met, Pro, or Tyr (preferably Tyr);

X_2 is Asp, Gly, His, Pro, or Trp (preferably Gly or Trp);

X_3 is Gly, Pro, Phe, Thr, or Trp (preferably Pro);

X_5 is Ala, Asp, Lys, Ser, Trp, or Val (preferably Lys);

X_6 is Asn, Glu, Gly, His, or Leu;

X_7 is Gln, Glu, Gly, Met, Lys, Phe, Tyr, or Val (preferably Met);

X_8 is Ala, Asn, Asp, Gly, Leu, Met, Pro, Ser, or Thr;

X_9 is His, Pro, or Trp (preferably Pro);

X_{10} is Ala, Gly, His, Leu, Trp, or Tyr (preferably His or Trp);

X_{11} is Ala, Asp, Gln, Leu, Met, Thr, or Trp;

X_{13} is Ala, Lys, Ser, Trp, or Tyr (preferably Trp);

X_{14} is Asp, Gly, Leu, His, Met, Thr, Trp or Tyr (preferably His, Trp, or Tyr); and

X_{15} is Asn, Gln, Glu, Leu, Met, Pro, or Trp (preferably Glu, Met or Trp); or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-Ser-Gly-Pro-X_{12}-X_{13}-X_{14}-X_{15}-Cys-X_{17}-X_{18}-X_{19} (SEQ ID NO:1; MTN13), wherein

X_1 is Arg, Glu, His, Ser, or Trp;

X_2 is Asn, Asp, Leu, Phe, Thr, or Val;

X_3 is Arg, Asp, Glu, His, Lys, or Thr;

X_5 is Asp, Glu, His, or Thr;

X_6 is Arg, His, Lys, or Phe;

X_7 is Gln, Ile, Lys, Tyr, or Val;

X_8 is Gln, Ile, Leu, Met, or Phe;

X_{12} is Asn, Asp, Gly, His, or Tyr;

X_{13} is Gln, Gly, Ser, or Thr;

X_{14} is Glu, Lys, Phe, or Ser;

X_{15} is Glu, Ile, Ser, or Val;

X_{17} is Glu, Gly, Lys, Phe, Ser, or Val;

X_{18} is Arg, Asn, Ser, or Tyr; and

X_{19} is Asp, Gln, Glu, Gly, Met, or Tyr.

8. The polypeptide according to Claim 7, wherein the polypeptide comprises an amino acid sequence of on of the following:

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-Tyr-Cys-X_{12}-X_{13}-X_{14}, wherein

X_1 is Ala, Arg, Asp, Leu, Lys, Pro, Ser, or Val;

X_2 is Asn, Asp, Glu, Lys, Thr, or Ser (preferably Asn, Asp, Glu, or Lys);

X_3 is Ile, Leu, Trp;

X_5 is Ala, Arg, Glu, Lys, or Ser (preferably Glu);

X_6 is Ala, Asp, Gln, Glu, Thr, or Val (preferably Asp or Glu);

X_7 is Asp or Glu;

X_8 is Trp or Tyr;

X_9 is Thr or Tyr (preferably Tyr);

X_{12} is Glu, Met, Phe, Trp, or Tyr (preferably Trp, Phe, Met, or Tyr);

X_{13} is Ile, Leu, or Met; and

X_{14} is Ile, Leu, Met, Phe or Thr (preferably Thr or Leu); or

Trp-Tyr-Trp-Cys-X_5-X_6-X_7-Gly-X_9-X_{10}-Cys-X_{12}-X_{13}-X_{14}, wherein

X_5 is Asp, Gln, or His;

X_6 is His or Tyr (preferably Tyr);

X_7 is Ile, His, or Tyr;

X_9 is Ile, Met, or Val;

X_{10} is Gly or Tyr;

X_{12} is Asp, Lys, or Pro;

X_{13} is Gln, Gly, or Trp; and

X_{14} is Phe, Ser, or Thr; or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-Gly-X_{10}-Cys-X_{12}-X_{13}-X_{14}, wherein

X_1 is Gly, Leu, His, Thr, Trp, Tyr, (preferably Trp, Tyr, Leu, His);

X_2 is Ile, Leu, Thr, Trp, or Val (preferably Val, Ile, or Leu);

X_3 is Asp, Glu, Gln, Trp, or Thr, (preferably Glu, Asp, or Gln);

X_5 is Ala, Arg, Asn, Asp, His, Phe, Trp, or Tyr, (preferably Tyr, Trp, or Phe);

X_6 is Ala, Asp, Gln, His, Lys, Met, Ser, Thr, Trp, Tyr, or Val;

X_7 is Ala, Asn, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, or Val;

X_8 is Asp, Phe, Ser, Thr, Trp, or Tyr (preferably Thr, Ser, or Asp);

X_{10} is Ala, Arg, Gln, His, Ile, Leu, Lys, Met, Phe, Trp, or Tyr (preferably Arg or Lys);

X_{12} is Arg, Gln, His, Ile, Lys, Met, Phe, Thr, Trp, Tyr, or Val (preferably Tyr, Trp, Phe, Ile, or Val);

X_{13} is Arg, Asn, Asp, Glu, His, Met, Pro, Ser, or Thr; and

X_{14} is Arg, Gln, Glu, Gly, Phe, Ser, Trp, or Tyr.

9. The polypeptide according to Claim 7, wherein the polypeptide comprises an

amino acid sequence of one of the following:

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-Trp-Gly-Gly-X_{12}-X_{13}-Cys-X_{15}-X_{16}-X_{17} (SEQ ID NO:3), wherein

X_1 is Ser, Phe, Trp, Tyr, or Gly (preferably Ser);

X_2 is Arg, Gly, Ser, or Trp (preferably Arg);

X_3 is Ala, Glu, Ile, or Val (preferably Val or Ile);

X_5 is Ala, Phe, or Trp (preferably Trp or Phe);

X_6 is Glu or Lys (preferably Glu);

X_7 is Asp, Ser, Trp, or (preferably Asp, Trp, or Tyr);

X_8 is Phe, Pro, or Ser (preferably Ser);

X_{12} is Gln or Glu (preferably Glu);

X_{13} is Ile, Phe, or Val;

X_{15} is Gln, Ile, Leu, Phe, or (preferably Phe, Tyr, or Leu);

X_{16} is Arg, Gly, or Pro (preferably Arg); and

X_{17} is Gln, His, Phe, Ser, Tyr, or Val (preferably Tyr, Phe, His, or Val); or

Tyr-Pro-X_3-Cys-X_5-Glu-X_7-Ser-X_9-Ser-X_{11}-X_{12}-X_{13}-Phe-Cys-X_{16}-X_{17}-X_{18} (SEQ ID NO:4; TN12), wherein

X_3 is Gly or Trp (preferably Trp);

X_5 is His or Tyr (preferably His, or Tyr);

X_7 is His, Leu, or Thr;

X_9 is Asp or Leu (preferably Asp);

X_{11} is Gly or Val (preferably Val);

X_{12} is Thr or Val (preferably Thr);

X_{13} is Arg or Trp (preferably Arg);

X_{16} is Ala or Val (preferably Val);

X_{17} is Asp or Pro (preferably Pro); and

X_{18} is Gly or Trp (preferably Trp),

wherein the polypeptide binds KDR or a VEGF/KDR complex; or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-Gly-X_{12}-Trp-X_{14}-Cys-X_{16}-X_{17}-X_{18} (SEQ ID NO:5; TN12), wherein

X_1 is Asp, Gly, Pro, or Ser (preferably Asp);

X_2 is Arg, Asn, Asp, Gly, or Ser (preferably Asp, Asn, or Ser);

X_3 is Gly, Thr, Trp, or Tyr (preferably Trp or Tyr);

X_5 is Glu, Met, or Thr (preferably Glu);

X_6 is Ile, Leu, Met, or Phe (preferably Met, Leu, or Phe);

X_7 is Arg, Asp, Glu, Met, Trp, or Val;

X_8 is Asn, Gln, Gly, Ser, or Val;

X_9 is Asp or Glu;

X_{10} is Lys, Ser, Thr, or Val (preferably Lys);

X_{12} is Arg, Gln, Lys, or Trp (preferably Trp, Arg, or Lys);

X_{14} is Asn, Leu, Phe, or Tyr (preferably Tyr, Phe, or Asn);

X_{16} is Gly, Phe, Ser, or Tyr (preferably Tyr or Phe);

X_{17} is Gly, Leu, Pro, or Ser (preferably Pro or Ser); and

X_{18} is Ala, Asp, Pro, Ser, Trp, or Tyr; or

$Asn\text{-}Trp\text{-}X_3\text{-}Cys\text{-}X_5\text{-}X_6\text{-}X_7\text{-}X_8\text{-}X_9\text{-}X_{10}\text{-}X_{11}\text{-}X_{12}\text{-}X_{13}\text{-}X_{14}\text{-}Cys\text{-}X_{16}\text{-}X_{17}\text{-}X_{18}$

(SEQ ID NO:6; TN12), wherein

X_3 is Glu or Lys;

X_5 is Glu or Gly;

X_6 is Trp or Tyr;

X_7 is Ser or Thr;

X_8 is Asn or Gln;

X_9 is Gly or Met;

X_{10} is Phe or Tyr;

X_{11} is Asp or Gln;

X_{12} is Lys or Tyr;

X_{13} is Glu or Thr;

X_{14} is Glu or Phe;

X_{16} is Ala or Val;

X_{17} is Arg or Tyr; and

X_{18} is Leu or Pro.

10. An isolated polypeptide having the ability to bind to KDR or VEGF/KDR complex comprising an amino acid sequence of one of the following:

$Z_1\text{-}X_1\text{-}X_2\text{-}X_3\text{-}X_4\text{-}X_5\text{-}Z_2$ (Lin20); wherein,

Z_1 is a polypeptide of at least one amino acid or is absent;

X_1 is Ala, Asp, Gln, or Glu (preferably Gln or Glu);

X_2 is Ala, Asp, Gln, Glu, Pro (preferably Asp, Glu, or Gln);

X_3 is Ala, Leu, Lys, Phe, Pro, Trp, or Tyr (preferably Trp, Tyr, Phe, or Leu);

X_4 is Asp, Leu, Ser, Trp, Tyr, or Val (preferably Tyr, Trp, Leu, or Val);

X_5 is Ala, Arg, Asp, Glu, Gly, Leu, Trp, or Tyr (preferably Trp, Tyr, or Leu); and

Z_2 is a polypeptide of at least one amino acid or is absent; or

X_1–X_2–X_3–Tyr–Trp–Glu–X_7–X_8–X_9–Leu (Lin20; SEQ ID NO:7), wherein, the sequence can optionally have a N-terminal polypeptide, C-terminal polypeptide, or a polypeptide at both termini of at least one amino acid (SEQ ID NO:7; Lin20); wherein,

X_1 is Asp, Gly, or Ser (preferably Gly);

X_2 is Ile, Phe, or Tyr;

X_3 is Ala, Ser, or Val;

X_7 is Gln, Glu, Ile, or Val;

X_8 is Ala, Ile, or Val (preferably Ile or Val);

X_9 is Ala, Glu, Val, or Thr; and

11. The polypeptide according to Claim 7, wherein the polypeptide comprises an amino acid sequence selected from the group consisting of: SEQ ID NOS: 20-86, 87-136, 187-192, 193-203, and 207-259.

12 The polypeptide according to Claim 10, wherein the polypeptide comprises an amino acid sequence selected from the group consisting of: SEQ ID NOS: 137-186.

13. The polypeptide according to any of Claim 1, wherein the polypeptide further comprises N-terminal and/or C-terminal flanking peptides of one or more amino acids.

14. The polypeptide according to any of Claim 1, wherein the polypeptide comprises a modification selected from the group consisting of: an amino acid substitution, and amide bond substitution, a D-amino acid substitution, a glycosylated amino acid, a disulfide mimetic substitution, an amino acid

translocation, a retroinverso peptide, a peptoid, a retro-inverso peptoid, and a synthetic peptide.

15. The polypeptide according to any one of Claim 1, conjugated to a detectable label or a therapeutic agent, optionally further comprising a linker or spacer between the polypeptide and the detectable label or the therapeutic agent.

16. The polypeptide of Claim 15, wherein the detectable label or the therapeutic agent is selected from the group consisting of: an enzyme, a fluorescent compound, a liposome, an optical dye, a paramagnetic metal ion, an ultrasound contrast agent and a radionuclide.

17. The polypeptide of Claim 16, wherein the therapeutic agent or detectable label comprises a radionuclide.

18. The polypeptide of Claim 17, wherein the radionuclide is selected from the group consisting of: ^{18}F, ^{124}I, ^{125}I, ^{131}I, ^{123}I, ^{77}Br, ^{76}Br, ^{99m}Tc, ^{51}Cr, ^{67}Ga, ^{68}Ga, ^{47}Sc, ^{51}Cr, ^{167}Tm, ^{141}Ce, ^{111}In, ^{168}Yb, ^{175}Yb, ^{140}La, ^{90}Y, ^{88}Y, ^{153}Sm, ^{166}Ho, ^{165}Dy, ^{166}Dy, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{97}Ru, ^{103}Ru, ^{186}Re, ^{188}Re, ^{203}Pb, ^{211}Bi, ^{212}Bi, ^{213}Bi, ^{214}Bi, ^{105}Rh, ^{109}Pd, ^{117m}Sn, ^{149}Pm, ^{161}Tb, ^{177}Lu, ^{198}Au and ^{199}Au.

19. The polypeptide of Claim 18, wherein the therapeutic agent or detectable label further comprises a chelator.

20. The polypeptide of Claim 19, wherein the chelator comprises a compound selected from the group consisting of: formula 20, 21, 22, 23a, 23b, 24a, 24b, and 25.

21. The polypeptide of Claim 19, wherein the radionuclide is ^{99m}Tc or ^{111}In.

22. The polypeptide of Claim 19, wherein the radionuclide is selected from the group consisting of: ^{177}Lu, ^{90}Y, ^{153}Sm and ^{166}Ho.

23. The polypeptide of Claim 16, wherein the detectable label comprises an

ultrasound contrast agent.

24. The polypeptide of Claim 23, wherein the ultrasound contrast agent comprises a phospholipid stabilized microbubble or an ultrasound contrast agent comprising a gas.

25. The polypeptide of Claim 23 or 24, wherein the ultrasound contrast agent comprises a fluorinated gas.

26. The polypeptide of Claim 16, wherein the detectable label comprises a paramagnetic metal ion and a chelator.

27. The polypeptide of Claim 15, wherein the therapeutic agent is selected from the group consisting of: a bioactive agent, a cytotoxic agent, a drug, a chemotherapeutic agent or a radiotherapeutic agent.

28. The polypeptide according to Claim 1 or 7, wherein the polypeptide has an apparent K_D for KDR or VEGF/KDR complex of less than 10 μM.

29. The polypeptide according to Claim 1 or 7, wherein the polypeptide has an apparent K_D for KDR or VEGF/KDR complex of less than 1.0 μM.

30. The polypeptide according to Claim 1 or 7, wherein the polypeptide has an apparent K_D for KDR or VEGF/KDR complex of less than 0.1 μM.

31. The polypeptide according to Claim 1 or 7, wherein the polypeptide has an apparent K_D for KDR or VEGF/KDR complex of less than 0.05 μM.

32. A method for isolating phage that bind KDR or a VEGF/KDR complex, comprising the steps of:

(a) immobilizing a KDR or VEGF/KDR complex target on a solid support;

(b) contacting a library of potential KDR or VEGF/KDR complex binding phage with the solid support to bind KDR or a VEGF/KDR complex binding phage in the library; and

(c) removing the unbound portion of the phage library from the solid support,

thereby isolating phage that bind KDR or a VEGF/KDR complex.

33. A method of detecting KDR or VEGF/KDR complex in an animal or human subject and optionally imaging at least a portion of the animal or human subject comprising the steps of:

(a) detectably labeling a polypeptide according to any one of Claim 1;

(b) administering to the subject the labeled polypeptide; and,

(c) detecting the labeled polypeptide in the subject, and, optionally,

constructing an image.

34. The method according to Claim 33, wherein the label is selected from the group consisting of: an enzyme, a fluorescent compound, an ultrasound contrast agent, a liposome and an optical dye, wherein the label optionally further comprises a linker a spacer.

35. The method according to Claim 34, wherein the ultrasound contrast agent is a phospholipid stabilized microbubble or an ultrasound contrast agent comprising a gas.

36. The method of Claim 35, wherein the ultrasound contrast agent comprises a fluorinated gas.

37. The method according to Claim 33, wherein the label is a radioactive label or a paramagnetic metal atom, and optionally further comprises a linker or a spacer.

38. The method of Claim 37, wherein the radioactive label comprises a radionuclide selected from the group consisting of: ^{18}F, ^{124}I, ^{125}I, ^{131}I, ^{123}I, ^{77}Br, ^{76}Br, ^{99m}Tc, ^{51}Cr, ^{67}Ga, ^{68}Ga, ^{47}Sc, ^{51}Cr, ^{167}Tm, ^{141}Ce, ^{111}In, ^{168}Yb, ^{175}Yb, ^{140}La, ^{90}Y, ^{88}Y, ^{153}Sm, ^{166}Ho, ^{165}Dy, ^{166}Dy, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{97}Ru,

^{103}Ru, ^{186}Re, ^{188}Re, ^{203}Pb, ^{211}Bi, ^{212}Bi, ^{213}Bi, ^{214}Bi, ^{105}Rh, ^{109}Pd, ^{117m}Sn, ^{149}Pm, ^{161}Tb, ^{177}Lu, ^{198}Au and ^{199}Au.

39. The method of Claim 38, wherein the radioactive label further comprises a chelator.

40. The method of Claim 39, wherein the chelator is selected from the group consisting of: formula 20, 21, 22, 23a, 23b, 24a, 24b, and 25.

41. The method of Claim 39, wherein the radionuclide is ^{99m}Tc or ^{111}In.

42. The method of Claim 37, wherein the paramagnetic label comprises a paramagnetic metal atom selected from the group consisting of: Mn^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Gd^{3+}, Eu^{3+}, Dy^{3+}, Pr^{3+}, Cr^{3+}, Co^{3+}, Fe^{3+}, Ti^{3+}, Tb^{3+}, Nd^{3+}, Sm^{3+}, Ho^{3+}, Er^{3+}, Pa^{4+} and Eu^{2+}.

43 The method of Claim 42, wherein the paramagnetic label further comprises a chelator.

44. The method of Claim 43, wherein the chelator is selected from the group consisting of: DTPA, DO3A, DOTA, EDTA, TETA, EHPG, HBED, NOTA, DOTMA, TETMA, PDTA, TTHA, LICAM, and MECAM.

45. The method according to Claim 35, wherein detection of the labeled polypeptide is indicative of the presence of a pathogen selected from the group consisting of: malaria strains, HIV, SIV, simian hemorrhagic fever virus and enterohemorrhagic *E. coli* strains.

46. The method of according to Claim 33, wherein detection of the labeled polypeptide is indicative of angiogenesis or neovascularization.

47. The method according to Claim 35, wherein the ultrasound contrast agent comprises a fluorinated gas selected from the group of: SF_6 freons, CF_4, C_2F_6, C_3F_8, C_4F_{10}, $CBrF_3$, CCl_2F_2, C_2ClF_5, $CBrClF_2$ and perfluorocarbons.

48. The method according to Claim 47, wherein the ultrasound contrast agent comprises a perfluorocarbon gas having the formula C_nF_{n+2} wherein n is from 1 to 12.

49. A method of treating a condition involving activation of KDR, comprising administering to an animal or human subject in need of treatment for such a condition a composition comprising at least one polypeptide according to Claim 1.

50. A method of treating malaria, HIV infection, SIV infection, simian hemorrhagic fever virus infection, and enterohemorrhagic *E. coli* infection comprising administering to an animal or human subject in need of treatment for such condition a composition comprising a polypeptide of Claim 1.

51. The method according to Claim 49, wherein the condition is solid tumor growth.

52. The method according to Claim 49, wherein the polypeptide is conjugated with a tumorcidal agent.

53. A recombinant bacteriophage displaying a KDR binding or VEGF/KDR complex binding polypeptide, which polypeptide comprises an amino acid sequence of one of the following:

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-Cys-X_{12}-X_{13}-X_{14} (TN8), wherein

X_1 is Ala, Arg, Asp, Gly, His, Leu, Lys, Pro, Ser, Thr, Trp, Tyr, or Val;

X_2 is Asn, Asp, Glu, Gly, Ile, Leu, Lys, Phe, Ser, Thr, Trp, Tyr, or Val;

X_3 is Asn, Asp, Gln, Glu, Ile, Leu, Met, Thr, Trp, or Val;

X_5 is Ala, Arg, Asn, Asp, Gln, Glu, His, Ile, Lys, Phe, Pro, Ser, Trp, or Tyr;

X_6 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X_7 is Ala, Asn, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr,

Trp, Tyr, or Val;

X_8 is Ala, Asp, Glu, Gly, Leu, Phe, Pro, Ser, Thr, Trp, or Tyr;

X_9 is Arg, Gln, Glu, Gly, Ile, Leu, Met, Pro, Thr, Trp, Tyr, or Val;

X_{10} is Ala, Arg, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Trp, or Tyr;

X_{12} is Arg, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X_{13} is Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, or Tyr; and

X_{14} is Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, or Tyr; or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}-X_{14}-Cys-X_{16}-X_{17}-X_{18} (TN12), wherein

X_1 is Ala, Asn, Asp, Gly, Leu, Pro, Ser, Trp, or Tyr (preferably Asn, Asp, Pro, or Tyr);

X_2 is Ala, Arg, Asn, Asp, Gly, His, Phe, Pro, Ser, Trp, or Tyr (preferably Asp, Gly, Pro, Ser, or Trp);

X_3 is Ala, Asn, Asp, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Trp);

X_5 is Arg, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Thr, Trp, Tyr, or Val (preferably Glu, Ile, or Tyr);

X_6 is Ala, Arg, Asn, Cys, Glu, Ile, Leu, Met, Phe, Ser, Trp, or Tyr (preferably Glu, Phe, or Tyr);

X_7 is Arg, Asn, Asp, Gln, Glu, His, Ile, Leu, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Glu);

X_8 is Ala, Asn, Asp, Gln, Glu, Gly, His, Met, Phe, Pro, Ser, Trp, Tyr, or Val (preferably Gln or Ser);

X_9 is Asp, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, or Tyr (preferably Asp);

X_{10} is Ala, Arg, Asn, Asp, Gln, Glu, Gly, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Lys or Ser);

X_{11} is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Lys, Trp, Tyr, or Val (preferably Gly or Tyr);

X_{12} is Ala, Arg, Gln, Gly, His, Ile, Lys, Met, Phe, Ser, Thr, Trp, Tyr,

or Val (preferably Trp or Thr);

X_{13} is Arg, Gln, Glu, His, Leu, Lys, Met, Phe, Pro, Thr, Trp, or Val (preferably Glu or Trp);

X_{14} is Arg, Asn, Asp, Glu, His, Ile, Leu, Met, Phe, Pro, Thr, Trp, Tyr, or Val (preferably Phe);

X_{16} is Ala, Asn, Asp, Gln, Glu, Gly, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Asp);

X_{17} is Arg, Asn, Asp, Cys, Gly, His, Phe, Pro, Ser, Trp, or Tyr (preferably Pro or Tyr); and

X_{18} is Ala, Asn, Asp, Gly, His, Leu, Phe, Pro, Ser, Trp, or Tyr (preferably Asn, Pro, or Trp); or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-Gly-X_9-Cys-X_{11}-X_{12}-X_{13} (TN7), wherein

X_1 is Gly or Trp;

X_2 is Ile, Tyr, or Val;

X_3 is Gln, Glu, Thr, or Trp;

X_5 is Asn, Asp, or Glu;

X_6 is Glu, His, Lys, or Phe;

X_7 is Asp, Gln, Leu, Lys, Met, or Tyr;

X_9 is Arg, Gln, Leu, Lys, or Val;

X_{11} is Arg, Phe, Ser, Trp, or Val;

X_{12} is Glu, His, or Ser; and

X_{13} is Glu, Gly, Trp, or Tyr; or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-Cys-X_{13}-X_{14}-X_{15} (TN9), wherein

X_1 is Arg, Asp, Gly, Ile, Met, Pro, or Tyr (preferably Tyr);

X_2 is Asp, Gly, His, Pro, or Trp (preferably Gly or Trp);

X_3 is Gly, Pro, Phe, Thr, or Trp (preferably Pro);

X_5 is Ala, Asp, Lys, Ser, Trp, or Val (preferably Lys);

X_6 is Asn, Glu, Gly, His, or Leu;

X_7 is Gln, Glu, Gly, Met, Lys, Phe, Tyr, or Val (preferably Met);

X_8 is Ala, Asn, Asp, Gly, Leu, Met, Pro, Ser, or Thr;

X_9 is His, Pro, or Trp (preferably Pro);

X_{10} is Ala, Gly, His, Leu, Trp, or Tyr (preferably His or Trp);

X_{11} is Ala, Asp, Gln, Leu, Met, Thr, or Trp;

X_{13} is Ala, Lys, Ser, Trp, or Tyr (preferably Trp);

X_{14} is Asp, Gly, Leu, His, Met, Thr, Trp or Tyr (preferably His, Trp, or Tyr); and

X_{15} is Asn, Gln, Glu, Leu, Met, Pro, or Trp (preferably Glu, Met or Trp); or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-Ser-Gly-Pro-X_{12}-X_{13}-X_{14}-X_{15}-Cys-X_{17}-X_{18}-X_{19} (SEQ ID NO:1; MTN13), wherein

X_1 is Arg, Glu, His, Ser, or Trp;

X_2 is Asn, Asp, Leu, Phe, Thr, or Val;

X_3 is Arg, Asp, Glu, His, Lys, or Thr;

X_5 is Asp, Glu, His, or Thr;

X_6 is Arg, His, Lys, or Phe;

X_7 is Gln, Ile, Lys, Tyr, or Val;

X_8 is Gln, Ile, Leu, Met, or Phe;

X_{12} is Asn, Asp, Gly, His, or Tyr;

X_{13} is Gln, Gly, Ser, or Thr;

X_{14} is Glu, Lys, Phe, or Ser;

X_{15} is Glu, Ile, Ser, or Val;

X_{17} is Glu, Gly, Lys, Phe, Ser, or Val;

X_{18} is Arg, Asn, Ser, or Tyr; and

X_{19} is Asp, Gln, Glu, Gly, Met, or Tyr,

and wherein the polypeptide is displayed on the surface of the recombinant bacteriophage.

54. A magnetic resonance imaging contrast agent comprising a KDR or VEGF/KDR complex binding polypeptide comprising an amino acid sequence of one of the following and optionally further comprising N-terminal and/or C-terminal flanking peptides of one or more amino acids:

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-Cys-X_{12}-X_{13}-X_{14} (TN8), wherein

X_1 is Ala, Arg, Asp, Gly, His, Leu, Lys, Pro, Ser, Thr, Trp, Tyr, or Val;

X_2 is Asn, Asp, Glu, Gly, Ile, Leu, Lys, Phe, Ser, Thr, Trp, Tyr, or Val;

X_3 is Asn, Asp, Gln, Glu, Ile, Leu, Met, Thr, Trp, or Val;

X_5 is Ala, Arg, Asn, Asp, Gln, Glu, His, Ile, Lys, Phe, Pro, Ser, Trp,

or Tyr;

X_6 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X_7 is Ala, Asn, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X_8 is Ala, Asp, Glu, Gly, Leu, Phe, Pro, Ser, Thr, Trp, or Tyr;

X_9 is Arg, Gln, Glu, Gly, Ile, Leu, Met, Pro, Thr, Trp, Tyr, or Val;

X_{10} is Ala, Arg, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Trp, or Tyr;

X_{12} is Arg, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X_{13} is Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, or Tyr; and

X_{14} is Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, or Tyr; or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}-X_{14}-Cys-X_{16}-X_{17}-X_{18} (TN12), wherein

X_1 is Ala, Asn, Asp, Gly, Leu, Pro, Ser, Trp, or Tyr (preferably Asn, Asp, Pro, or Tyr);

X_2 is Ala, Arg, Asn, Asp, Gly, His, Phe, Pro, Ser, Trp, or Tyr (preferably Asp, Gly, Pro, Ser, or Trp);

X_3 is Ala, Asn, Asp, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Trp);

X_5 is Arg, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Thr, Trp, Tyr, or Val (preferably Glu, Ile, or Tyr);

X_6 is Ala, Arg, Asn, Cys, Glu, Ile, Leu, Met, Phe, Ser, Trp, or Tyr (preferably Glu, Phe, or Tyr);

X_7 is Arg, Asn, Asp, Gln, Glu, His, Ile, Leu, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Glu);

X_8 is Ala, Asn, Asp, Gln, Glu, Gly, His, Met, Phe, Pro, Ser, Trp, Tyr, or Val (preferably Gln or Ser);

X_9 is Asp, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, or Tyr (preferably Asp);

X_{10} is Ala, Arg, Asn, Asp, Gln, Glu, Gly, Leu, Lys, Met, Phe, Pro,

Ser, Thr, Trp, Tyr, or Val (preferably Lys or Ser);

X_{11} is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Lys, Trp, Tyr, or Val (preferably Gly or Tyr);

X_{12} is Ala, Arg, Gln, Gly, His, Ile, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Trp or Thr);

X_{13} is Arg, Gln, Glu, His, Leu, Lys, Met, Phe, Pro, Thr, Trp, or Val (preferably Glu or Trp);

X_{14} is Arg, Asn, Asp, Glu, His, Ile, Leu, Met, Phe, Pro, Thr, Trp, Tyr, or Val (preferably Phe);

X_{16} is Ala, Asn, Asp, Gln, Glu, Gly, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Asp);

X_{17} is Arg, Asn, Asp, Cys, Gly, His, Phe, Pro, Ser, Trp, or Tyr (preferably Pro or Tyr); and

X_{18} is Ala, Asn, Asp, Gly, His, Leu, Phe, Pro, Ser, Trp, or Tyr (preferably Asn, Pro, or Trp); or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-Gly-X_9-Cys-X_{11}-X_{12}-X_{13} (TN7), wherein

X_1 is Gly or Trp;

X_2 is Ile, Tyr, or Val;

X_3 is Gln, Glu, Thr, or Trp;

X_5 is Asn, Asp, or Glu;

X_6 is Glu, His, Lys, or Phe;

X_7 is Asp, Gln, Leu, Lys, Met, or Tyr;

X_9 is Arg, Gln, Leu, Lys, or Val;

X_{11} is Arg, Phe, Ser, Trp, or Val;

X_{12} is Glu, His, or Ser; and

X_{13} is Glu, Gly, Trp, or Tyr; or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-Cys-X_{13}-X_{14}-X_{15} (TN9), wherein

X_1 is Arg, Asp, Gly, Ile, Met, Pro, or Tyr (preferably Tyr);

X_2 is Asp, Gly, His, Pro, or Trp (preferably Gly or Trp);

X_3 is Gly, Pro, Phe, Thr, or Trp (preferably Pro);

X_5 is Ala, Asp, Lys, Ser, Trp, or Val (preferably Lys);

X_6 is Asn, Glu, Gly, His, or Leu;

X_7 is Gln, Glu, Gly, Met, Lys, Phe, Tyr, or Val (preferably Met);

X_8 is Ala, Asn, Asp, Gly, Leu, Met, Pro, Ser, or Thr;

X_9 is His, Pro, or Trp (preferably Pro);

X_{10} is Ala, Gly, His, Leu, Trp, or Tyr (preferably His or Trp);

X_{11} is Ala, Asp, Gln, Leu, Met, Thr, or Trp;

X_{13} is Ala, Lys, Ser, Trp, or Tyr (preferably Trp);

X_{14} is Asp, Gly, Leu, His, Met, Thr, Trp or Tyr (preferably His, Trp, or Tyr); and

X_{15} is Asn, Gln, Glu, Leu, Met, Pro, or Trp (preferably Glu, Met or Trp); or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-Ser-Gly-Pro-X_{12}-X_{13}-X_{14}-X_{15}-Cys-X_{17}-X_{18}-X_{19}

(SEQ ID NO:1; MTN13), wherein

X_1 is Arg, Glu, His, Ser, or Trp;

X_2 is Asn, Asp, Leu, Phe, Thr, or Val;

$X_3$is Arg, Asp, Glu, His, Lys, or Thr;

X_5 is Asp, Glu, His, or Thr;

X_6 is Arg, His, Lys, or Phe;

X_7 is Gln, Ile, Lys, Tyr, or Val;

X_8 is Gln, Ile, Leu, Met, or Phe;

X_{12} is Asn, Asp, Gly, His, or Tyr;

X_{13} is Gln, Gly, Ser, or Thr;

X_{14} is Glu, Lys, Phe, or Ser;

X_{15} is Glu, Ile, Ser, or Val;

X_{17} is Glu, Gly, Lys, Phe, Ser, or Val;

X_{18} is Arg, Asn, Ser, or Tyr; and

X_{19} is Asp, Gln, Glu, Gly, Met, or Tyr,

wherein the polypeptide is coupled to at least one chelator capable of complexing a paramagnetic metal.

55. A magnetic resonance imaging contrast agent comprising at least one paramagnetic metal atom and at least one KDR or VEGF/KDR complex binding moiety comprising a polypeptide according to Claim 1.

56. The magnetic resonance imaging contrast agent according to Claim 55, wherein the magnetic resonance imaging contrast agent further comprises at least one chelator selected from the group consisting of: DTPA, DOTA,

EDTA, TETA, EHPG, HBED, NOTA, DOTMA, TETMA, PDTA, TTHA, LICAM, and MECAM.

57. The magnetic resonance imaging contrast agent according to Claim 56, wherein the chelator is selected from the group consisting of: diethylenetriamine, tetraazacyclododecane and a carboxymethyl-substituted derivative thereof.

58. A magnetic resonance imaging contrast agent according to Claim 55, wherein the paramagnetic metal atom is selected from the group consisting of: Mn^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Gd^{3+}, Eu^{3+}, Dy^{3+}, Pr^{3+}, Cr^{3+}, Co^{3+}, Fe^{3+}, Ti^{3+}, Tb^{3+}, Nd^{3+}, Sm^{3+}, Ho^{3+}, Er^{3+}, Pa^{4+} and Eu^{2+}.

59. A magnetic resonance imaging contrast agent according to Claim 55, wherein the multivalent cation is Gd^{3+}.

60. A method for identifying KDR or VEGF/KDR complex binding compounds comprising the steps of:

(a) utilizing a KDR or VEGF/KDR complex binding polypeptide according to any one of Claim 1 to form a complex with a KDR or VEGF/KDR complex target;

(b) contacting the complex with one or more potential KDR or VEGF/KDR complex binding compounds; and

(c) determining whether the potential KDR or VEGF/KDR complex binding compound competes with the KDR or VEGF/KDR complex binding polypeptide to form a complex with the KDR or VEGF/KDR complex target.

61. A diagnostic imaging contrast agent comprising a polypeptide according to Claim 1.

62. A method of medical imaging comprising the steps of administering to an animal or human subject a pharmaceutical preparation of a contrast agent comprising at least one polypeptide according to Claim 1 and imaging the

contrast agent by a method selected from the group consisting of: magnetic resonance imaging, ultrasound imaging, optical imaging, sonoluminescence imaging, photoacoustic imaging, and nuclear imaging.

63. A method of radiotherapy comprising administering to an animal or human subject in need of such therapy a compound comprising at least one polypeptide according to Claim 1 conjugated to a radionuclide useful for radiotherapy.

64. The method of Claim 63, wherein the compound further comprises a chelator.

65. The method of Claim 64, wherein the compound further comprises a spacer or linker.

66. The method of Claims 64, wherein the chelator is a compound selected from the group consisting of: formula 20, 21, 22, 23, 24 and 25.

67. The method of Claim 66, wherein the radionuclide is ^{186}Re, ^{188}Re, ^{177}Lu, ^{90}Y, ^{153}Sm or ^{166}Ho.

68. A kit for preparation of a radiopharmaceutical comprising a polypeptide according to any one of Claim 1, a chelator for a radionuclide, and a reducing agent.

69. A method of targeting genetic material to KDR-expressing cells comprising administering to an animal or a human in need of such genetic material a polypeptide according to any one of Claim 1 conjugated to or associated with the genetic material or a delivery vehicle containing such genetic material.

70. A method of screening binding polypeptides identified by phage display for their ability to bind to cells expressing the target comprising the steps of preparing multimeric constructs including one or more binding polypeptides; contacting the multimeric constructs with cells expressing the target and

assessing the ability of the multimeric constructs to bind to the target.

71. The method of claim 70, wherein the cells have been engineered by recombinant DNA technology to express the target.

72. The method of Claim 70, wherein the multimeric constructs are detectably labeled.

73. The method of Claim 70 wherein the ability of the multimeric constructs to bind to the target is assessed in the presence of serum.

74. The method of Claim 70 wherein the multimeric construct comprises biotinylated binding polypeptides complexed with avidin, streptavidin or neutravidin.

75. The method of Claim 70 wherein the target is KDR or the KDR/VEGF complex.

76. A multimeric polypeptide construct having the ability to bind to KDR or VEGF/KDR complex comprising at least one amino acid selected from the group consisting of:

Consensus Sequence I: Cys-X2-X3-X4-X5-X6-X7-Cys (TN8), wherein

X2 is Ala, Arg, Asn, Asp, Gln, Glu, His, Ile, Lys, Phe, Pro, Ser, Trp, or Tyr (preferably Asp, Glu, or Tyr);

X3 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Glu, Met, or Tyr);

X4 is Ala, Asn, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Asp);

X5 is Ala, Asp, Glu, Gly, Leu, Phe, Pro, Ser, Thr, Trp, or Tyr (preferably Trp or Thr);

X6 is Arg, Gln, Glu, Gly, Ile, Leu, Met, Pro, Thr, Trp, Tyr, or Val (preferably Gly or Tyr); and

X7 is Ala, Arg, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Trp, or Tyr (preferably Lys or Tyr); or

Consensus Sequence II:Cys-X2-X3-X4-X5-X6-X7-X8-X9-X10-X11-Cys (TN12), wherein

X2 is Arg, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Thr, Trp, Tyr, or Val (preferably Glu, Ile, or Tyr);

X3 is Ala, Arg, Asn, Cys, Glu, Ile, Leu, Met, Phe, Ser, Trp, or Tyr (preferably Glu, Phe, or Tyr);

X4 is Arg, Asn, Asp, Gln, Glu, His, Ile, Leu, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Glu);

X5 is Ala, Asn, Asp, Gln, Glu, Gly, His, Met, Phe, Pro, Ser, Trp, Tyr, or Val (preferably Gln or Ser);

X6 is Asp, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, or Tyr (preferably Asp);

X7 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Lys or Ser);

X8 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Lys, Trp, Tyr, or Val (preferably Gly or Tyr);

X9 is Ala, Arg, Gln, Gly, His, Ile, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Trp or Thr);

X10 is Arg, Gln, Glu, His, Leu, Lys, Met, Phe, Pro, Thr, Trp, or Val (preferably Glu or Trp); and

X11 is Arg, Asn, Asp, Glu, His, Ile, Leu, Met, Phe, Pro, Thr, Trp, Tyr, or Val (preferably Phe); or

Consensus Sequence III: Cys-X2-X3-X4-Gly-X6-Cys (TN7), wherein

X2 is Asn, Asp, or Glu;

X3 is Glu, His, Lys, or Phe;

X4 is Asp, Gln, Leu, Lys, Met, or Tyr; and

X6 is Arg, Gln, Leu, Lys, or Val; or

Consensus Sequence IV: Cys-X2-X3-X4-X5-X6-X7-X8-Cys (TN9), wherein

X2 is Ala, Asp, Lys, Ser, Trp, or Val (preferably Lys);

X3 is Asn, Glu, Gly, His, or Leu;

X4 is Gln, Glu, Gly, Met, Lys, Phe, Tyr, or Val (preferably Met);

X5 is Ala, Asn, Asp, Gly, Leu, Met, Pro, Ser, or Thr;

X6 is His, Pro, or Trp (preferably Pro or Trp);

X7 is Ala, Gly, His, Leu, Trp, or Tyr (preferably Trp); and

X8 is Ala, Asp, Gln, Leu, Met, Thr, or Trp; and

Consensus Sequence V: Cys-X2-X3-X4-X5-Ser-Gly-Pro-X9-X10-X11-X12-Cys (MTN13; SEQ ID NO:1), wherein

X2 is Asp, Glu, His, or Thr;

X3 is Arg, His, Lys, or Phe;

X4 is Gln, Ile, Lys, Tyr, or Val;

X5 is Gln, Ile, Leu, Met, or Phe;

X9 is Asn, Asp, Gly, His, or Tyr;

X10 is Gln, Gly, Ser, or Thr;

X11 is Glu, Lys, Phe, or Ser; and

X12 is Glu, Ile, Ser, or Val.

77. A multimeric polypeptide construct having the ability to bind to KDR or VEGF/KDR complex comprising at least one amino acid sequence selected from:

X1-X2-X3-Cys-X5-X6-X7-X8-X9-X10-Cys-X12-X13-X14 (TN8), wherein

X1 is Ala, Arg, Asp, Gly, His, Leu, Lys, Pro, Ser, Thr, Trp, Tyr, or Val;

X2 is Asn, Asp, Glu, Gly, Ile, Leu, Lys, Phe, Ser, Thr, Trp, Tyr, or Val;

X3 is Asn, Asp, Gln, Glu, Ile, Leu, Met, Thr, Trp, or Val;

X5 is Ala, Arg, Asn, Asp, Gln, Glu, His, Ile, Lys, Phe, Pro, Ser, Trp, or Tyr;

X6 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X7 is Ala, Asn, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X8 is Ala, Asp, Glu, Gly, Leu, Phe, Pro, Ser, Thr, Trp, or Tyr;

X9 is Arg, Gln, Glu, Gly, Ile, Leu, Met, Pro, Thr, Trp, Tyr, or Val;

X10 is Ala, Arg, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Trp, or Tyr;

X12 is Arg, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X13 is Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, or Tyr; and

X14 is Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, or Tyr;

X1-X2-X3-Cys-X5-X6-X7-X8-X9-X10-X11-X12-X13-X14-Cys-X16-X17-X18 (TN12), wherein

X1 is Ala, Asn, Asp, Gly, Leu, Pro, Ser, Trp, or Tyr (preferably Asn, Asp, Pro, or Tyr);

X2 is Ala, Arg, Asn, Asp, Gly, His, Phe, Pro, Ser, Trp, or Tyr (preferably Asp, Gly, Pro, Ser, or Trp);

X3 is Ala, Asn, Asp, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Trp);

X5 is Arg, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Thr, Trp, Tyr, or Val (preferably Glu, Ile, or Tyr);

X6 is Ala, Arg, Asn, Cys, Glu, Ile, Leu, Met, Phe, Ser, Trp, or Tyr (preferably Glu, Phe, or Tyr);

X7 is Arg, Asn, Asp, Gln, Glu, His, Ile, Leu, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Glu);

X8 is Ala, Asn, Asp, Gln, Glu, Gly, His, Met, Phe, Pro, Ser, Trp, Tyr, or Val (preferably Gln or Ser);

X9 is Asp, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, or Tyr (preferably Asp);

X10 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Lys or Ser);

X11 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Lys, Trp, Tyr, or Val (preferably Gly or Tyr);

X12 is Ala, Arg, Gln, Gly, His, Ile, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Trp or Thr);

X13 is Arg, Gln, Glu, His, Leu, Lys, Met, Phe, Pro, Thr, Trp, or Val (preferably Glu or Trp);

X14 is Arg, Asn, Asp, Glu, His, Ile, Leu, Met, Phe, Pro, Thr, Trp, Tyr, or Val (preferably Phe);

X16 is Ala, Asn, Asp, Gln, Glu, Gly, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Asp);

X17 is Arg, Asn, Asp, Cys, Gly, His, Phe, Pro, Ser, Trp, or Tyr (preferably Pro or Tyr); and

X18 is Ala, Asn, Asp, Gly, His, Leu, Phe, Pro, Ser, Trp, or Tyr (preferably Asn, Pro, or Trp);

X1-X2-X3-Cys-X5-X6-X7-Gly-X9-Cys-X11-X12-X13 (TN7), wherein

X1 is Gly or Trp;

X2 is Ile, Tyr, or Val;

X3 is Gln, Glu, Thr, or Trp;

X5 is Asn, Asp, or Glu;

X6 is Glu, His, Lys, or Phe;

X7 is Asp, Gln, Leu, Lys, Met, or Tyr;

X9 is Arg, Gln, Leu, Lys, or Val;

X11 is Arg, Phe, Ser, Trp, or Val;

X12 is Glu, His, or Ser; and

X13 is Glu, Gly, Trp, or Tyr;

X1-X2-X3-Cys-X5-X6-X7-X8-X9-X10-X11-Cys-X13-X14-X15 (TN9), wherein

X1 is Arg, Asp, Gly, Ile, Met, Pro, or Tyr (preferably Tyr);

X2 is Asp, Gly, His, Pro, or Trp (preferably Gly or Trp);

X3 is Gly, Pro, Phe, Thr, or Trp (preferably Pro);

X5 is Ala, Asp, Lys, Ser, Trp, or Val (preferably Lys);

X6 is Asn, Glu, Gly, His, or Leu;

X7 is Gln, Glu, Gly, Met, Lys, Phe, Tyr, or Val (preferably Met);

X8 is Ala, Asn, Asp, Gly, Leu, Met, Pro, Ser, or Thr;

X9 is His, Pro, or Trp (preferably Pro);

X10 is Ala, Gly, His, Leu, Trp, or Tyr (preferably His or Trp);

X11 is Ala, Asp, Gln, Leu, Met, Thr, or Trp;

X13 is Ala, Lys, Ser, Trp, or Tyr (preferably Trp);

X14 is Asp, Gly, Leu, His, Met, Thr, Trp or Tyr (preferably His, Trp, or Tyr); and

X15 is Asn, Gln, Glu, Leu, Met, Pro, or Trp (preferably Glu, Met or Trp); and

X1-X2-X3-Cys-X5-X6-X7-X8-Ser-Gly-Pro-X12-X13-X14-X15-Cys-X17-X18-X19 (SEQ ID NO:1; MTN13), wherein

X1 is Arg, Glu, His, Ser, or Trp;

X2 is Asn, Asp, Leu, Phe, Thr, or Val;

X3 is Arg, Asp, Glu, His, Lys, or Thr;

X5 is Asp, Glu, His, or Thr;

X6 is Arg, His, Lys, or Phe;

X7 is Gln, Ile, Lys, Tyr, or Val;

X8 is Gln, Ile, Leu, Met, or Phe;

X12 is Asn, Asp, Gly, His, or Tyr;

X13 is Gln, Gly, Ser, or Thr;

X14 is Glu, Lys, Phe, or Ser;

X15 is Glu, Ile, Ser, or Val;

X17 is Glu, Gly, Lys, Phe, Ser, or Val;

X18 is Arg, Asn, Ser, or Tyr; and

X19 is Asp, Gln, Glu, Gly, Met, or Tyr.

78. A multimeric polypeptide construct having the ability to bind to KDR or VEGF/KDR complex comprising at least one amino acid sequence of one of the following:

Z1-X1-X2-X3-X4-X5-Z2 (Lin20); wherein,

Z1 is a polypeptide of at least one amino acid or is absent;

X1 is Ala, Asp, Gln, or Glu (preferably Gln or Glu);

X2 is Ala, Asp, Gln, Glu, Pro (preferably Asp, Glu, or Gln);

X3 is Ala, Leu, Lys, Phe, Pro, Trp, or Tyr (preferably Trp, Tyr, Phe, or Leu);

X4 is Asp, Leu, Ser, Trp, Tyr, or Val (preferably Tyr, Trp, Leu, or Val);

X5 is Ala, Arg, Asp, Glu, Gly, Leu, Trp, or Tyr (preferably Trp, Tyr, or Leu); and

Z2 is a polypeptide of at least one amino acid or is absent; or

X1–X2–X3–Tyr–Trp–Glu–X7–X8–X9–Leu (Lin20; SEQ ID NO:7), wherein, the sequence can optionally have a N-terminal polypeptide, C-terminal polypeptide, or a polypeptide at both termini of at least one amino acid (SEQ ID NO:7; Lin20); wherein,

X1 is Asp, Gly, or Ser (preferably Gly);

X2 is Ile, Phe, or Tyr;

X3 is Ala, Ser, or Val;

X7 is Gln, Glu, Ile, or Val;

X8 is Ala, Ile, or Val (preferably Ile or Val);

X9 is Ala, Glu, Val, or Thr.

79. The multimeric polypeptide construct according to Claim 77, comprising at least one amino acid sequence selected from the group consisting of: SEQ ID NOS: 20-86, 87-136, 187-192, 193-203, and 207-259.

80. The multimeric polypeptide construct according to Claim 78, comprising at least one amino acid sequence selected from the group consisting of: SEQ ID NOS: 137-186.

81. The multimeric polypeptide construct according to any one of Claims 76, 77 or 78, wherein at least one amino acid sequence further comprises N-terminal and/or C-terminal flanking peptides of one or more amino acids.

82. The multimeric polypeptide construct according to any of Claims 76, 77 or 78, wherein at least one amino acid sequence comprises a modification selected from the group consisting of: an amino acid substitution, and amide bond substitution, a D-amino acid substitution, a glycosylated amino acid, a disulfide mimetic substitution, an amino acid translocation, a retroinverso peptide, a peptoid, a retro-inverso peptoid, and a synthetic peptide.

83. The multimeric polypeptide construct according to any one of Claims 76, 77 or 78, conjugated to a detectable label or a therapeutic agent, optionally further comprising a linker or spacer between the polypeptide and the detectable label or the therapeutic agent.

84. The multimeric polypeptide construct of Claim 83, wherein the detectable label or the therapeutic agent is selected from the group consisting of: an enzyme, a fluorescent compound, a liposome, an optical dye, a paramagnetic metal ion, an ultrasound contrast agent and a radionuclide.

85. The multimeric polypeptide construct of Claim 84, wherein the therapeutic agent or detectable label comprises a radionuclide.

86. The multimeric polypeptide construct of Claim 85, wherein the radionuclide is selected from the group consisting of: ^{18}F, ^{124}I, ^{125}I, ^{131}I, ^{123}I, ^{77}Br, ^{76}Br, ^{99m}Tc, ^{51}Cr, ^{67}Ga, ^{68}Ga, ^{47}Sc, ^{51}Cr, ^{167}Tm, ^{141}Ce, ^{111}In, ^{168}Yb, ^{175}Yb, ^{140}La, ^{90}Y, ^{88}Y, ^{153}Sm, ^{166}Ho, ^{165}Dy, ^{166}Dy, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{97}Ru, ^{103}Ru, ^{186}Re, ^{188}Re, ^{203}Pb, ^{211}Bi, ^{212}Bi, ^{213}Bi, ^{214}Bi, ^{105}Rh, ^{109}Pd, ^{117}mSn, ^{149}Pm, ^{161}Tb, ^{177}Lu, ^{198}Au and ^{199}Au.

87. The multimeric polypeptide construct of Claim 86, wherein the therapeutic agent or detectable label further comprises a chelator.

88. The multimeric polypeptide construct of Claim 87, wherein the chelator comprises a compound selected from the group consisting of: formula 20, 21, 22, 23a, 23b, 24a, 24b, and 25.

89. The multimeric polypeptide construct of Claim 87, wherein the radionuclide is ^{99m}Tc or ^{111}In.

90. The multimeric polypeptide construct of Claim 87, wherein the radionuclide is selected from the group consisting of: ^{177}Lu, ^{90}Y, ^{153}Sm and ^{166}Ho.

91. The multimeric polypeptide construct of Claim 84, wherein the detectable label comprises an ultrasound contrast agent.

92. The multimeric polypeptide construct of Claim 91, wherein the ultrasound contrast agent comprises a phospholipid stabilized microbubble or a microballoon comprising a gas.

93. The multimeric polypeptide construct of Claim 91, wherein the ultrasound contrast agent comprises a fluorinated gas.

94. The multimeric polypeptide construct of Claim 84, wherein the detectable

label comprises a paramagnetic metal ion and a chelator.

95. The multimeric polypeptide construct of Claim 84, wherein the therapeutic agent is selected from the group consisting of: a bioactive agent, a cytotoxic agent, a drug, a chemotherapeutic agent or a radiotherapeutic agent.

96. An ultrasound contrast agent comprising at least one KDR or VEGF/KDR complex binding polypeptide comprising an amino acid sequence of one of the following and optionally further comprising N-terminal and/or C-terminal flanking peptides of one or more amino acids:

X1-X2-X3-Cys-X5-X6-X7-X8-X9-X10-Cys-X12-X13-X14 (TN8), wherein

X1 is Ala, Arg, Asp, Gly, His, Leu, Lys, Pro, Ser, Thr, Trp, Tyr, or Val;

X2 is Asn, Asp, Glu, Gly, Ile, Leu, Lys, Phe, Ser, Thr, Trp, Tyr, or Val;

X3 is Asn, Asp, Gln, Glu, Ile, Leu, Met, Thr, Trp, or Val;

X5 is Ala, Arg, Asn, Asp, Gln, Glu, His, Ile, Lys, Phe, Pro, Ser, Trp, or Tyr;

X6 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X7 is Ala, Asn, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X8 is Ala, Asp, Glu, Gly, Leu, Phe, Pro, Ser, Thr, Trp, or Tyr;

X9 is Arg, Gln, Glu, Gly, Ile, Leu, Met, Pro, Thr, Trp, Tyr, or Val;

X10 is Ala, Arg, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Trp, or Tyr;

X12 is Arg, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X13 is Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, or Tyr; and

X14 is Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, or Tyr; or

X1-X2-X3-Cys-X5-X6-X7-X8-X9-X10-X11-X12-X13-X14-Cys-X16-X17-X18 (TN12), wherein

X1 is Ala, Asn, Asp, Gly, Leu, Pro, Ser, Trp, or Tyr (preferably Asn, Asp, Pro, or Tyr);

X2 is Ala, Arg, Asn, Asp, Gly, His, Phe, Pro, Ser, Trp, or Tyr (preferably Asp, Gly, Pro, Ser, or Trp);

X3 is Ala, Asn, Asp, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Trp);

X5 is Arg, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Thr, Trp, Tyr, or Val (preferably Glu, Ile, or Tyr);

X6 is Ala, Arg, Asn, Cys, Glu, Ile, Leu, Met, Phe, Ser, Trp, or Tyr (preferably Glu, Phe, or Tyr);

X7 is Arg, Asn, Asp, Gln, Glu, His, Ile, Leu, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Glu);

X8 is Ala, Asn, Asp, Gln, Glu, Gly, His, Met, Phe, Pro, Ser, Trp, Tyr, or Val (preferably Gln or Ser);

X9 is Asp, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, or Tyr (preferably Asp);

X10 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Lys or Ser);

X11 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Lys, Trp, Tyr, or Val (preferably Gly or Tyr);

X12 is Ala, Arg, Gln, Gly, His, Ile, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Trp or Thr);

X13 is Arg, Gln, Glu, His, Leu, Lys, Met, Phe, Pro, Thr, Trp, or Val (preferably Glu or Trp);

X14 is Arg, Asn, Asp, Glu, His, Ile, Leu, Met, Phe, Pro, Thr, Trp, Tyr, or Val (preferably Phe);

X16 is Ala, Asn, Asp, Gln, Glu, Gly, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Asp);

X17 is Arg, Asn, Asp, Cys, Gly, His, Phe, Pro, Ser, Trp, or Tyr (preferably Pro or Tyr); and

X18 is Ala, Asn, Asp, Gly, His, Leu, Phe, Pro, Ser, Trp, or Tyr (preferably Asn, Pro, or Trp); or

X1-X2-X3-Cys-X5-X6-X7-Gly-X9-Cys-X11-X12-X13 (TN7), wherein

X1 is Gly or Trp;

X2 is Ile, Tyr, or Val;

X3 is Gln, Glu, Thr, or Trp;

X5 is Asn, Asp, or Glu;

X6 is Glu, His, Lys, or Phe;

X7 is Asp, Gln, Leu, Lys, Met, or Tyr;

X9 is Arg, Gln, Leu, Lys, or Val;

X11 is Arg, Phe, Ser, Trp, or Val;

X12 is Glu, His, or Ser; and

X13 is Glu, Gly, Trp, or Tyr; or

X1-X2-X3-Cys-X5-X6-X7-X8-X9-X10-X11-Cys-X13-X14-X15 (TN9), wherein

X1 is Arg, Asp, Gly, Ile, Met, Pro, or Tyr (preferably Tyr);

X2 is Asp, Gly, His, Pro, or Trp (preferably Gly or Trp);

X3 is Gly, Pro, Phe, Thr, or Trp (preferably Pro);

X5 is Ala, Asp, Lys, Ser, Trp, or Val (preferably Lys);

X6 is Asn, Glu, Gly, His, or Leu;

X7 is Gln, Glu, Gly, Met, Lys, Phe, Tyr, or Val (preferably Met);

X8 is Ala, Asn, Asp, Gly, Leu, Met, Pro, Ser, or Thr;

X9 is His, Pro, or Trp (preferably Pro);

X10 is Ala, Gly, His, Leu, Trp, or Tyr (preferably His or Trp);

X11 is Ala, Asp, Gln, Leu, Met, Thr, or Trp;

X13 is Ala, Lys, Ser, Trp, or Tyr (preferably Trp);

X14 is Asp, Gly, Leu, His, Met, Thr, Trp or Tyr (preferably His, Trp, or Tyr); and

X15 is Asn, Gln, Glu, Leu, Met, Pro, or Trp (preferably Glu, Met or Trp); or

X1-X2-X3-Cys-X5-X6-X7-X8-Ser-Gly-Pro-X12-X13-X14-X15-Cys-X17-X18-X19 (SEQ ID NO:1; MTN13), wherein

X1 is Arg, Glu, His, Ser, or Trp;

X2 is Asn, Asp, Leu, Phe, Thr, or Val;

X3is Arg, Asp, Glu, His, Lys, or Thr;

X5 is Asp, Glu, His, or Thr;

X6 is Arg, His, Lys, or Phe;

X7 is Gln, Ile, Lys, Tyr, or Val;

X8 is Gln, Ile, Leu, Met, or Phe;

X12 is Asn, Asp, Gly, His, or Tyr;

X13 is Gln, Gly, Ser, or Thr;

X14 is Glu, Lys, Phe, or Ser;

X15 is Glu, Ile, Ser, or Val;

X17 is Glu, Gly, Lys, Phe, Ser, or Val;

X18 is Arg, Asn, Ser, or Tyr; and

X19 is Asp, Gln, Glu, Gly, Met, or Tyr,

wherein at least one polypeptide is conjugated to microvesicles filled with gas or material useful for preparing microvesicles filled with gas.

97. The ultrasound contrast agent according to Claim 96, wherein the gas filled microvesicles comprise phospholipid stabilized microbubbles or microballoons.

98. The ultrasound contrast agent according to Claim 97, wherein the phospholipid stabilized microbubbles or microballoons further comprise a fluorinated gas.

99. A scintigraphic imaging agent comprising at least one KDR or VEGF/KDR complex binding polypeptide comprising an amino acid sequence of one of the following and optionally further comprising N-terminal and/or C-terminal flanking peptides of one or more amino acids:

X1-X2-X3-Cys-X5-X6-X7-X8-X9-X10-Cys-X12-X13-X14 (TN8), wherein

X1 is Ala, Arg, Asp, Gly, His, Leu, Lys, Pro, Ser, Thr, Trp, Tyr, or Val;

X2 is Asn, Asp, Glu, Gly, Ile, Leu, Lys, Phe, Ser, Thr, Trp, Tyr, or Val;

X3 is Asn, Asp, Gln, Glu, Ile, Leu, Met, Thr, Trp, or Val;

X5 is Ala, Arg, Asn, Asp, Gln, Glu, His, Ile, Lys, Phe, Pro, Ser, Trp, or Tyr;

X6 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X7 is Ala, Asn, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr,

Trp, Tyr, or Val;

X8 is Ala, Asp, Glu, Gly, Leu, Phe, Pro, Ser, Thr, Trp, or Tyr;

X9 is Arg, Gln, Glu, Gly, Ile, Leu, Met, Pro, Thr, Trp, Tyr, or Val;

X10 is Ala, Arg, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Trp, or Tyr;

X12 is Arg, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X13 is Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, or Tyr; and

X14 is Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, or Tyr; or

X1-X2-X3-Cys-X5-X6-X7-X8-X9-X10-X11-X12-X13-X14-Cys-X16-X17-X18 (TN12), wherein

X1 is Ala, Asn, Asp, Gly, Leu, Pro, Ser, Trp, or Tyr (preferably Asn, Asp, Pro, or Tyr);

X2 is Ala, Arg, Asn, Asp, Gly, His, Phe, Pro, Ser, Trp, or Tyr (preferably Asp, Gly, Pro, Ser, or Trp);

X3 is Ala, Asn, Asp, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Trp);

X5 is Arg, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Thr, Trp, Tyr, or Val (preferably Glu, Ile, or Tyr);

X6 is Ala, Arg, Asn, Cys, Glu, Ile, Leu, Met, Phe, Ser, Trp, or Tyr (preferably Glu, Phe, or Tyr);

X7 is Arg, Asn, Asp, Gln, Glu, His, Ile, Leu, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Glu);

X8 is Ala, Asn, Asp, Gln, Glu, Gly, His, Met, Phe, Pro, Ser, Trp, Tyr, or Val (preferably Gln or Ser);

X9 is Asp, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, or Tyr (preferably Asp);

X10 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Lys or Ser);

X11 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Lys, Trp, Tyr, or Val (preferably Gly or Tyr);

X12 is Ala, Arg, Gln, Gly, His, Ile, Lys, Met, Phe, Ser, Thr, Trp, Tyr,

or Val (preferably Trp or Thr);

X13 is Arg, Gln, Glu, His, Leu, Lys, Met, Phe, Pro, Thr, Trp, or Val (preferably Glu or Trp);

X14 is Arg, Asn, Asp, Glu, His, Ile, Leu, Met, Phe, Pro, Thr, Trp, Tyr, or Val (preferably Phe);

X16 is Ala, Asn, Asp, Gln, Glu, Gly, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Asp);

X17 is Arg, Asn, Asp, Cys, Gly, His, Phe, Pro, Ser, Trp, or Tyr (preferably Pro or Tyr); and

X18 is Ala, Asn, Asp, Gly, His, Leu, Phe, Pro, Ser, Trp, or Tyr (preferably Asn, Pro, or Trp); or

X1-X2-X3-Cys-X5-X6-X7-Gly-X9-Cys-X11-X12-X13 (TN7), wherein

X1 is Gly or Trp;

X2 is Ile, Tyr, or Val;

X3 is Gln, Glu, Thr, or Trp;

X5 is Asn, Asp, or Glu;

X6 is Glu, His, Lys, or Phe;

X7 is Asp, Gln, Leu, Lys, Met, or Tyr;

X9 is Arg, Gln, Leu, Lys, or Val;

X11 is Arg, Phe, Ser, Trp, or Val;

X12 is Glu, His, or Ser; and

X13 is Glu, Gly, Trp, or Tyr; or

X1-X2-X3-Cys-X5-X6-X7-X8-X9-X10-X11-Cys-X13-X14-X15 (TN9), wherein

X1 is Arg, Asp, Gly, Ile, Met, Pro, or Tyr (preferably Tyr);

X2 is Asp, Gly, His, Pro, or Trp (preferably Gly or Trp);

X3 is Gly, Pro, Phe, Thr, or Trp (preferably Pro);

X5 is Ala, Asp, Lys, Ser, Trp, or Val (preferably Lys);

X6 is Asn, Glu, Gly, His, or Leu;

X7 is Gln, Glu, Gly, Met, Lys, Phe, Tyr, or Val (preferably Met);

X8 is Ala, Asn, Asp, Gly, Leu, Met, Pro, Ser, or Thr;

X9 is His, Pro, or Trp (preferably Pro);

X10 is Ala, Gly, His, Leu, Trp, or Tyr (preferably His or Trp);

X11 is Ala, Asp, Gln, Leu, Met, Thr, or Trp;

X13 is Ala, Lys, Ser, Trp, or Tyr (preferably Trp);

X14 is Asp, Gly, Leu, His, Met, Thr, Trp or Tyr (preferably His, Trp, or Tyr); and

X15 is Asn, Gln, Glu, Leu, Met, Pro, or Trp (preferably Glu, Met or Trp); or

X1-X2-X3-Cys-X5-X6-X7-X8-Ser-Gly-Pro-X12-X13-X14-X15-Cys-X17-X18-X19 (SEQ ID NO:1; MTN13), wherein

X1 is Arg, Glu, His, Ser, or Trp;

X2 is Asn, Asp, Leu, Phe, Thr, or Val;

X3is Arg, Asp, Glu, His, Lys, or Thr;

X5 is Asp, Glu, His, or Thr;

X6 is Arg, His, Lys, or Phe;

X7 is Gln, Ile, Lys, Tyr, or Val;

X8 is Gln, Ile, Leu, Met, or Phe;

X12 is Asn, Asp, Gly, His, or Tyr;

X13 is Gln, Gly, Ser, or Thr;

X14 is Glu, Lys, Phe, or Ser;

X15 is Glu, Ile, Ser, or Val;

X17 is Glu, Gly, Lys, Phe, Ser, or Val;

X18 is Arg, Asn, Ser, or Tyr; and

X19 is Asp, Gln, Glu, Gly, Met, or Tyr,

wherein at least one polypeptide is coupled to at least one chelator capable of complexing a radionuclide useful for scintigraphic imaging.

100. A scintigraphic imaging agent comprising at least one radionuclide useful in scintigraphic imaging and at least one KDR or VEGF/KDR complex binding moiety comprising a polypeptide according to Claim 99.

101. The scintigraphic imaging agent according to Claim 100, further comprising at least one chelator selected from the group consisting of: formula 20, 21, 22, 23a, 23b, 24a, 24b and 25.

102. The scintigraphic imaging agent according to Claim 101, wherein the radionuclide is selected from the group consisting of ^{99m}Tc and ^{111}In.

103. An agent useful in radiotherapy comprising at least one KDR or VEGF/KDR complex binding polypeptide comprising an amino acid sequence of one of the following and optionally further comprising N-terminal and/or C-terminal flanking peptides of one or more amino acids:

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-Cys-X_{12}-X_{13}-X_{14} (TN8), wherein

X_1 is Ala, Arg, Asp, Gly, His, Leu, Lys, Pro, Ser, Thr, Trp, Tyr, or Val;

X_2 is Asn, Asp, Glu, Gly, Ile, Leu, Lys, Phe, Ser, Thr, Trp, Tyr, or Val;

X_3 is Asn, Asp, Gln, Glu, Ile, Leu, Met, Thr, Trp, or Val;

X_5 is Ala, Arg, Asn, Asp, Gln, Glu, His, Ile, Lys, Phe, Pro, Ser, Trp, or Tyr;

X_6 is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X_7 is Ala, Asn, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X_8 is Ala, Asp, Glu, Gly, Leu, Phe, Pro, Ser, Thr, Trp, or Tyr;

X_9 is Arg, Gln, Glu, Gly, Ile, Leu, Met, Pro, Thr, Trp, Tyr, or Val;

X_{10} is Ala, Arg, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Trp, or Tyr;

X_{12} is Arg, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val;

X_{13} is Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, or Tyr; and

X_{14} is Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, or Tyr; or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}-X_{14}-Cys-X_{16}-X_{17}-X_{18} (TN12), wherein

X_1 is Ala, Asn, Asp, Gly, Leu, Pro, Ser, Trp, or Tyr (preferably Asn, Asp, Pro, or Tyr);

X_2 is Ala, Arg, Asn, Asp, Gly, His, Phe, Pro, Ser, Trp, or Tyr (preferably Asp, Gly, Pro, Ser, or Trp);

X_3 is Ala, Asn, Asp, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Trp);

X_5 is Arg, Asp, Gln, Glu, Gly, His, Ile, Lys, Met, Thr, Trp, Tyr, or Val (preferably Glu, Ile, or Tyr);

X_6 is Ala, Arg, Asn, Cys, Glu, Ile, Leu, Met, Phe, Ser, Trp, or Tyr (preferably Glu, Phe, or Tyr);

X_7 is Arg, Asn, Asp, Gln, Glu, His, Ile, Leu, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Glu);

X_8 is Ala, Asn, Asp, Gln, Glu, Gly, His, Met, Phe, Pro, Ser, Trp, Tyr, or Val (preferably Gln or Ser);

X_9 is Asp, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, or Tyr (preferably Asp);

X_{10} is Ala, Arg, Asn, Asp, Gln, Glu, Gly, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val (preferably Lys or Ser);

X_{11} is Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Lys, Trp, Tyr, or Val (preferably Gly or Tyr);

X_{12} is Ala, Arg, Gln, Gly, His, Ile, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Trp or Thr);

X_{13} is Arg, Gln, Glu, His, Leu, Lys, Met, Phe, Pro, Thr, Trp, or Val (preferably Glu or Trp);

X_{14} is Arg, Asn, Asp, Glu, His, Ile, Leu, Met, Phe, Pro, Thr, Trp, Tyr, or Val (preferably Phe);

X_{16} is Ala, Asn, Asp, Gln, Glu, Gly, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val (preferably Asp);

X_{17} is Arg, Asn, Asp, Cys, Gly, His, Phe, Pro, Ser, Trp, or Tyr (preferably Pro or Tyr); and

X_{18} is Ala, Asn, Asp, Gly, His, Leu, Phe, Pro, Ser, Trp, or Tyr (preferably Asn, Pro, or Trp); or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-Gly-X_9-Cys-X_{11}-X_{12}-X_{13} (TN7), wherein

X_1 is Gly or Trp;

X_2 is Ile, Tyr, or Val;

X_3 is Gln, Glu, Thr, or Trp;

X_5 is Asn, Asp, or Glu;

X_6 is Glu, His, Lys, or Phe;

X_7 is Asp, Gln, Leu, Lys, Met, or Tyr;

X_9 is Arg, Gln, Leu, Lys, or Val;

X_{11} is Arg, Phe, Ser, Trp, or Val;

X_{12} is Glu, His, or Ser; and

X_{13} is Glu, Gly, Trp, or Tyr; or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-Cys-X_{13}-X_{14}-X_{15} (TN9), wherein

X_1 is Arg, Asp, Gly, Ile, Met, Pro, or Tyr (preferably Tyr);

X_2 is Asp, Gly, His, Pro, or Trp (preferably Gly or Trp);

X_3 is Gly, Pro, Phe, Thr, or Trp (preferably Pro);

X_5 is Ala, Asp, Lys, Ser, Trp, or Val (preferably Lys);

X_6 is Asn, Glu, Gly, His, or Leu;

X_7 is Gln, Glu, Gly, Met, Lys, Phe, Tyr, or Val (preferably Met);

X_8 is Ala, Asn, Asp, Gly, Leu, Met, Pro, Ser, or Thr;

X_9 is His, Pro, or Trp (preferably Pro);

X_{10} is Ala, Gly, His, Leu, Trp, or Tyr (preferably His or Trp);

X_{11} is Ala, Asp, Gln, Leu, Met, Thr, or Trp;

X_{13} is Ala, Lys, Ser, Trp, or Tyr (preferably Trp);

X_{14} is Asp, Gly, Leu, His, Met, Thr, Trp or Tyr (preferably His, Trp, or Tyr); and

X_{15} is Asn, Gln, Glu, Leu, Met, Pro, or Trp (preferably Glu, Met or Trp); or

X_1-X_2-X_3-Cys-X_5-X_6-X_7-X_8-Ser-Gly-Pro-X_{12}-X_{13}-X_{14}-X_{15}-Cys-X_{17}-X_{18}-X_{19} (SEQ ID NO:1; MTN13), wherein

X_1 is Arg, Glu, His, Ser, or Trp;

X_2 is Asn, Asp, Leu, Phe, Thr, or Val;

$X_3$is Arg, Asp, Glu, His, Lys, or Thr;

X_5 is Asp, Glu, His, or Thr;

X_6 is Arg, His, Lys, or Phe;

X_7 is Gln, Ile, Lys, Tyr, or Val;

X_8 is Gln, Ile, Leu, Met, or Phe;

X_{12} is Asn, Asp, Gly, His, or Tyr;

X_{13} is Gln, Gly, Ser, or Thr;

X_{14} is Glu, Lys, Phe, or Ser;

X_{15} is Glu, Ile, Ser, or Val;

X_{17} is Glu, Gly, Lys, Phe, Ser, or Val;

X_{18} is Arg, Asn, Ser, or Tyr; and

X_{19} is Asp, Gln, Glu, Gly, Met, or Tyr,

wherein at least one polypeptide is coupled to at least one chelator capable of complexing a radionuclide useful in radiotherapy.

104. An agent useful in radiotherapy comprising at least one radionuclide useful in radiotherapy and at least one KDR or VEGF/KDR complex binding moiety comprising a polypeptide according to Claim 1.

105. The agent useful in radiotherapy according to Claim 103, further comprising at least one chelator selected from the group consisting of: formula 20, 21, 22, 23a, 23b, 24a, 24b and 25.

106. The agent useful in radiotherapy according to Claim 103, wherein the radionuclide is selected from the group consisting of: ^{177}Lu, ^{90}Y, ^{153}Sm and ^{166}Ho.

107. The method of claim 49, wherein the composition further comprises a therapeutic agent.

108. A method of synthesizing a polypeptide or a multimeric polypeptide construct having the ability to bind KDR or VEGF/KDR complex comprising a cyclic polypeptide formed by introducing an amide bond between two side chains.

109. A method of synthesizing a polypeptide or a multimeric polypeptide construct having the ability to bind KDR or VEGF/KDR complex comprising a polypeptide and a linker comprising at least one glycosylated amino acid selected from the group consisting or serine, threonine and homoserine.

110. A method of synthesizing a multimeric polypeptide construct having the ability to bind KDR or VEGF/KDR complex selected from the group consisting of D1, 2, D3, D4, D5, D8, D9, D10, D11, D12, D13, D14, D15, D16, D17, D18, D19, D20, D21, D22, D23, D24, D25, D26 and D27, wherein the method comprises the steps set forth in Example 12.

FIG. 1A

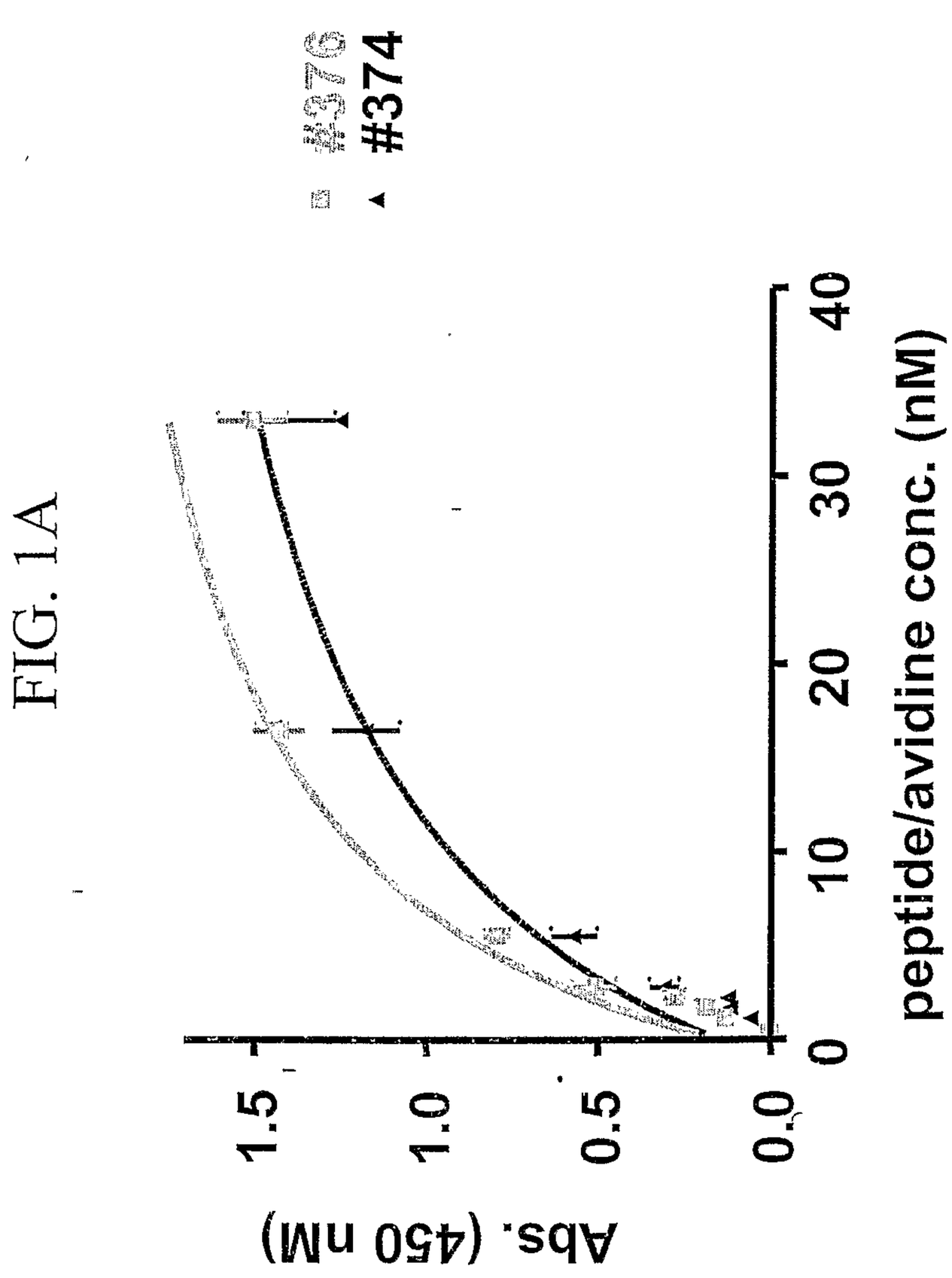

#376
#374
Abs. (450 nM)
1.5
1.0
0.5
0.0
0
10
20
30
40
peptide/avidine conc. (nM)

FIG. 1B

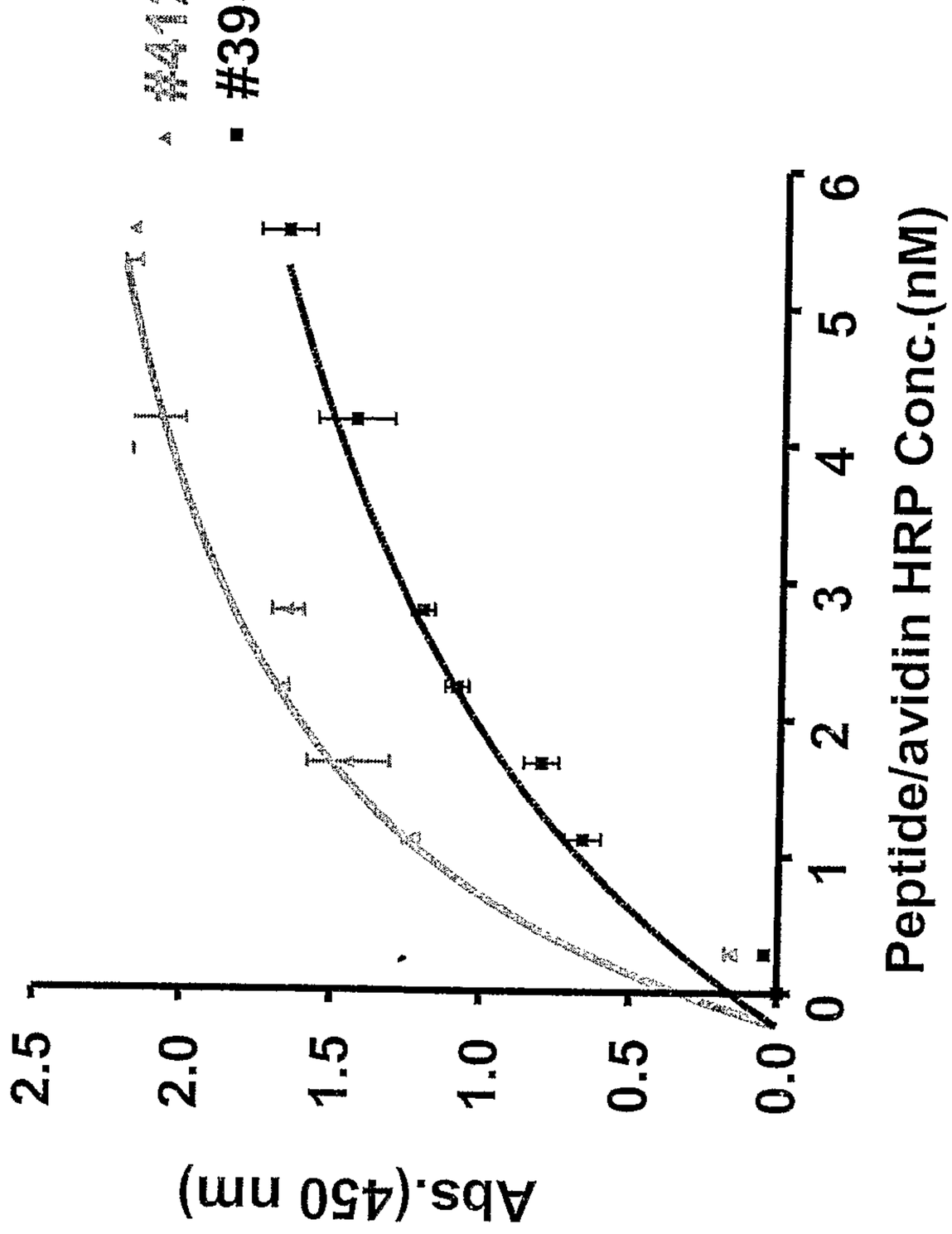

#39
Abs.(450 nm)
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
6
Peptide/avidin HRP Conc.(nM)

FIG. 2

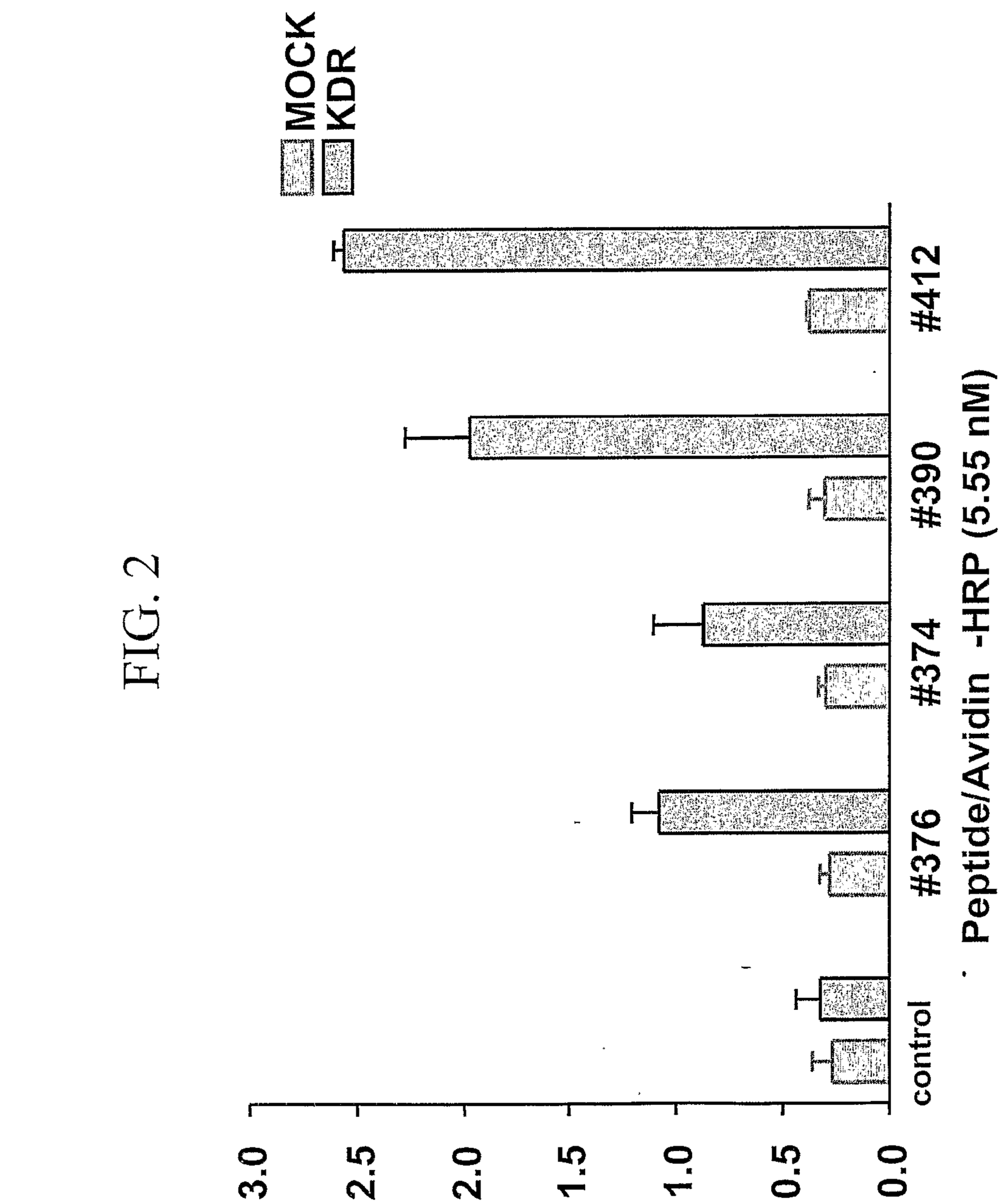

Abs. (450 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
control
#376
#374
#390
#412
Peptide/Avidin -HRP (5.55 nM)
M
K

FIG. 3

(a) Ac-AGPKWCEEDWYYCMITGT-GGGK(Biotin-di(aminodioxaocta)-)-NH_2 (SEQ ID NO:374)

(b) Ac-AGPKWCEEDWYYCMITGT-GGGK(Biotin-)-NH_2 (SEQ ID NO:373)

(c) Ac-GDSRVCWEDSWGGEVCFRYDP-GGGK(Biotin-di(aminodioxaocta)-)-NH_2 (SEQ ID NO:376)

(d) Ac-GDSRVCWEDSWGGEVCFRYDP-GGGK-(Biotin-)-NH_2 (SEQ ID NO:363)

FIG. 4

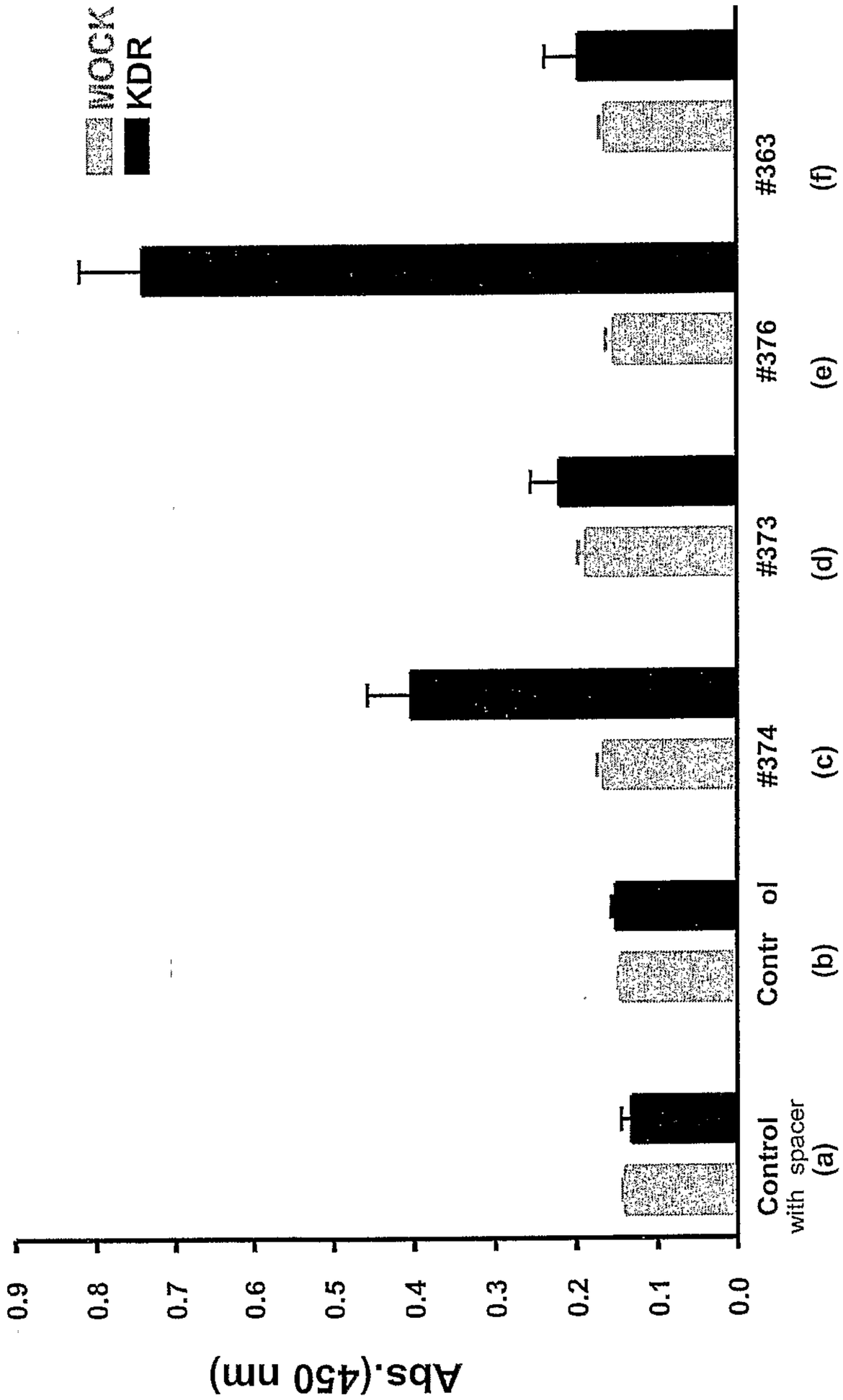

MOCK
KDR
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Abs.(450 nm)
Control with spacer (a)
Control (b)
#374 (c)
#373 (d)
#376 (e)
#363 (f)

FIG. 5

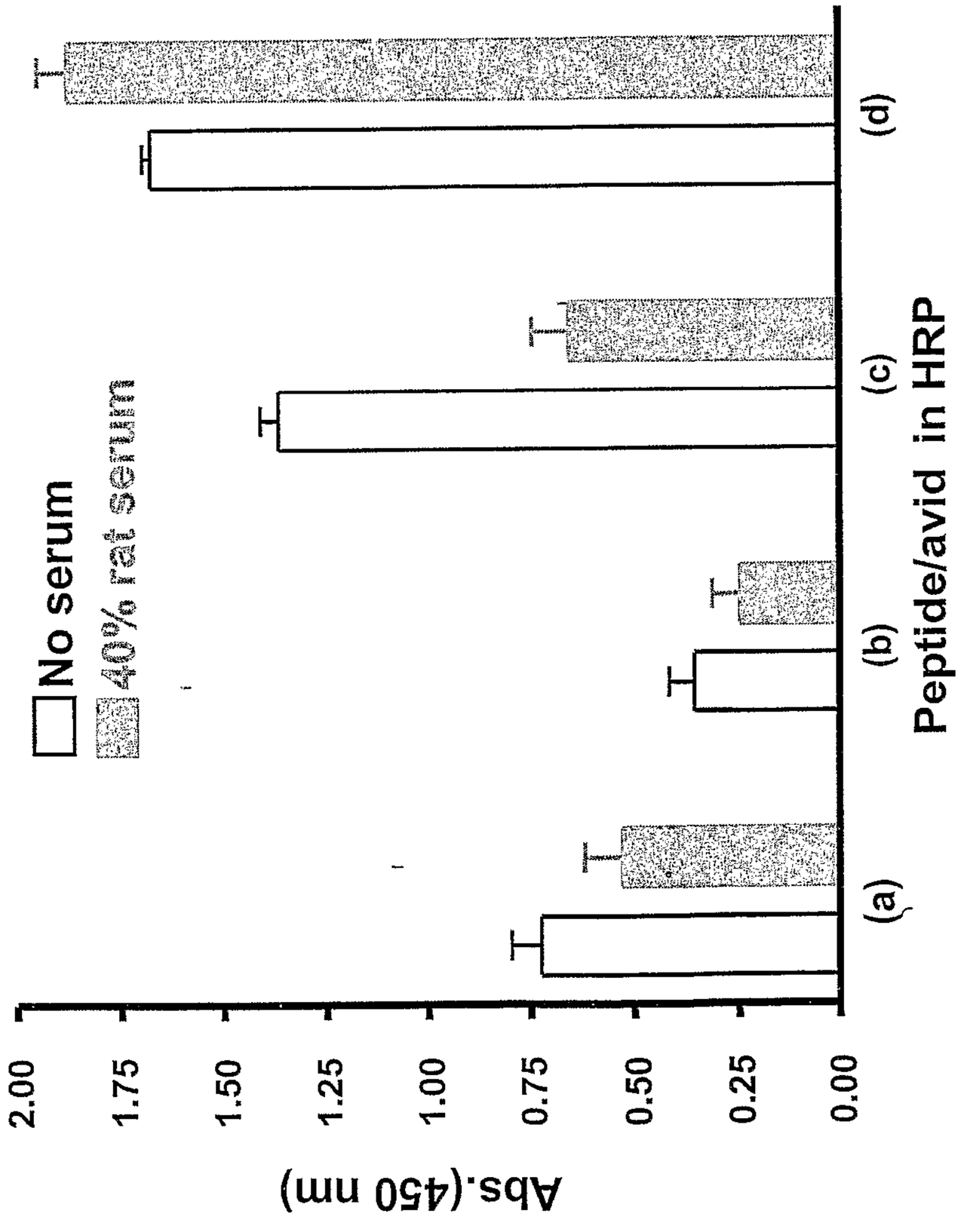

No serum
40% rat serum
Peptide/avid in HRP
Abs.(450 nm)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
(a)
(b)
(c)
(d)

FIG. 6

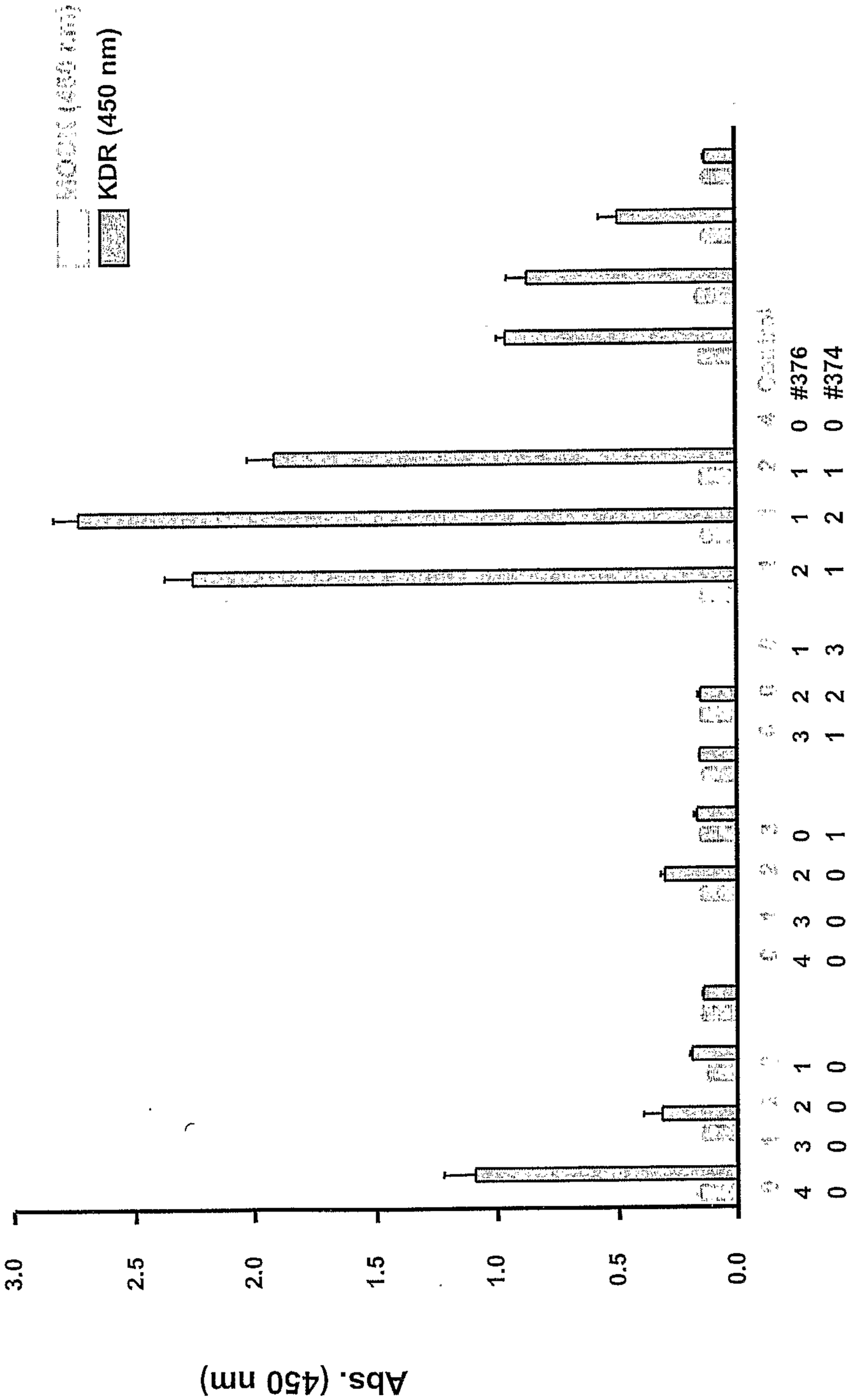


FIG. 7

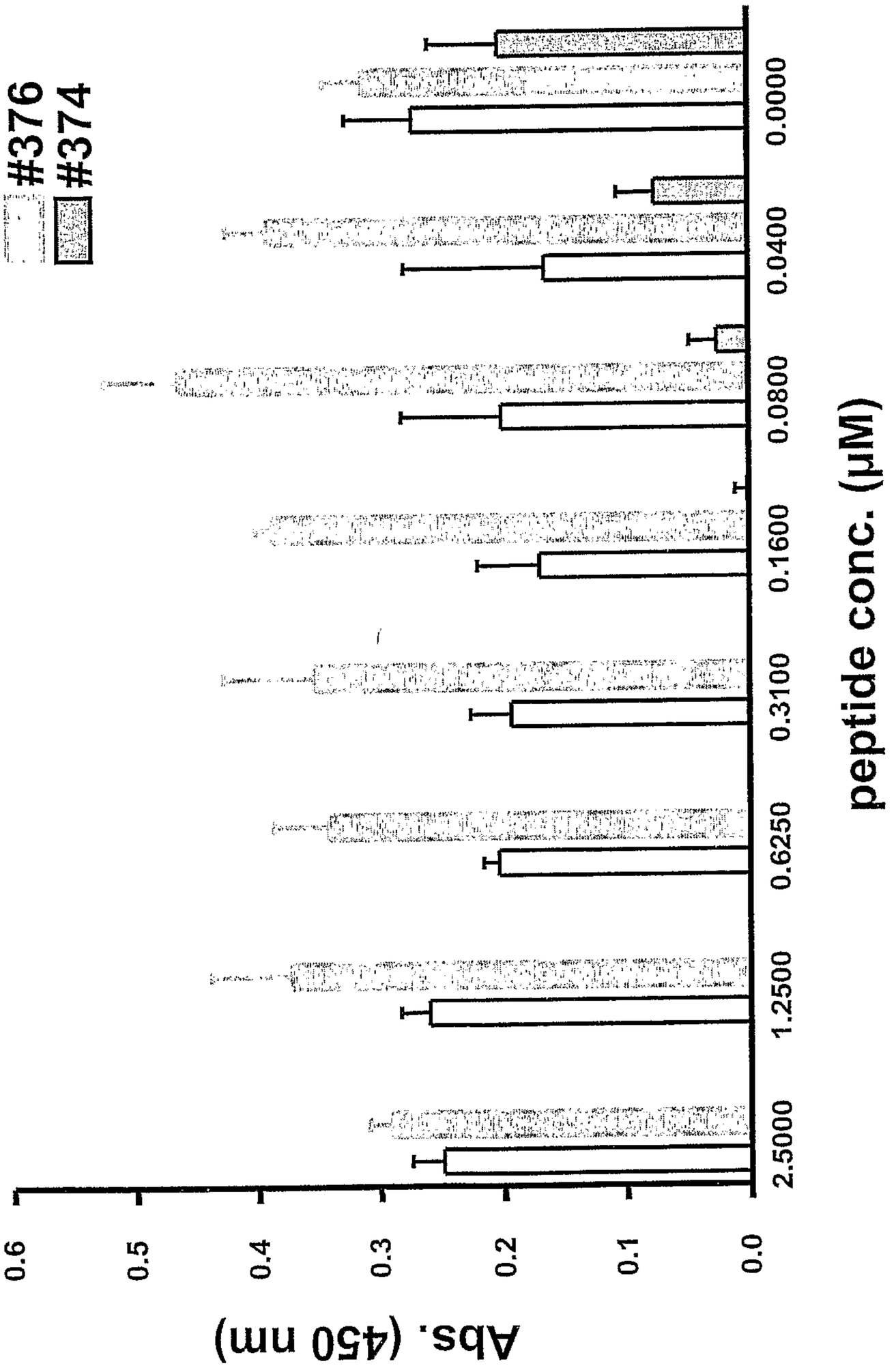

control
#376
#374
0.0000
0.0400
0.0800
0.1600
0.3100
0.6250
1.2500
2.5000
peptide conc. (μM)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Abs. (450 nm)

FIG. 8

Sequence	SEQ ID NO:	K_D, B (μM)
GDSRVCWEDSWGGEVCFRYDPGGGK	428	0.069
VCWEDSWGGEVCFGGGK	446	0.91
GDSRVCWEDSWGGEVCFGGGK	447	1.30
VCWEDSWGGEVCFRYDPGGGK	448	0.040
SRVCWEDSWGGEVCFRYGGGGK	449	0.035
GDSRVCWEDSWGGEVCFRYGGGK	450	0.060

CA 02477836 2004-08-31

FIG. 9

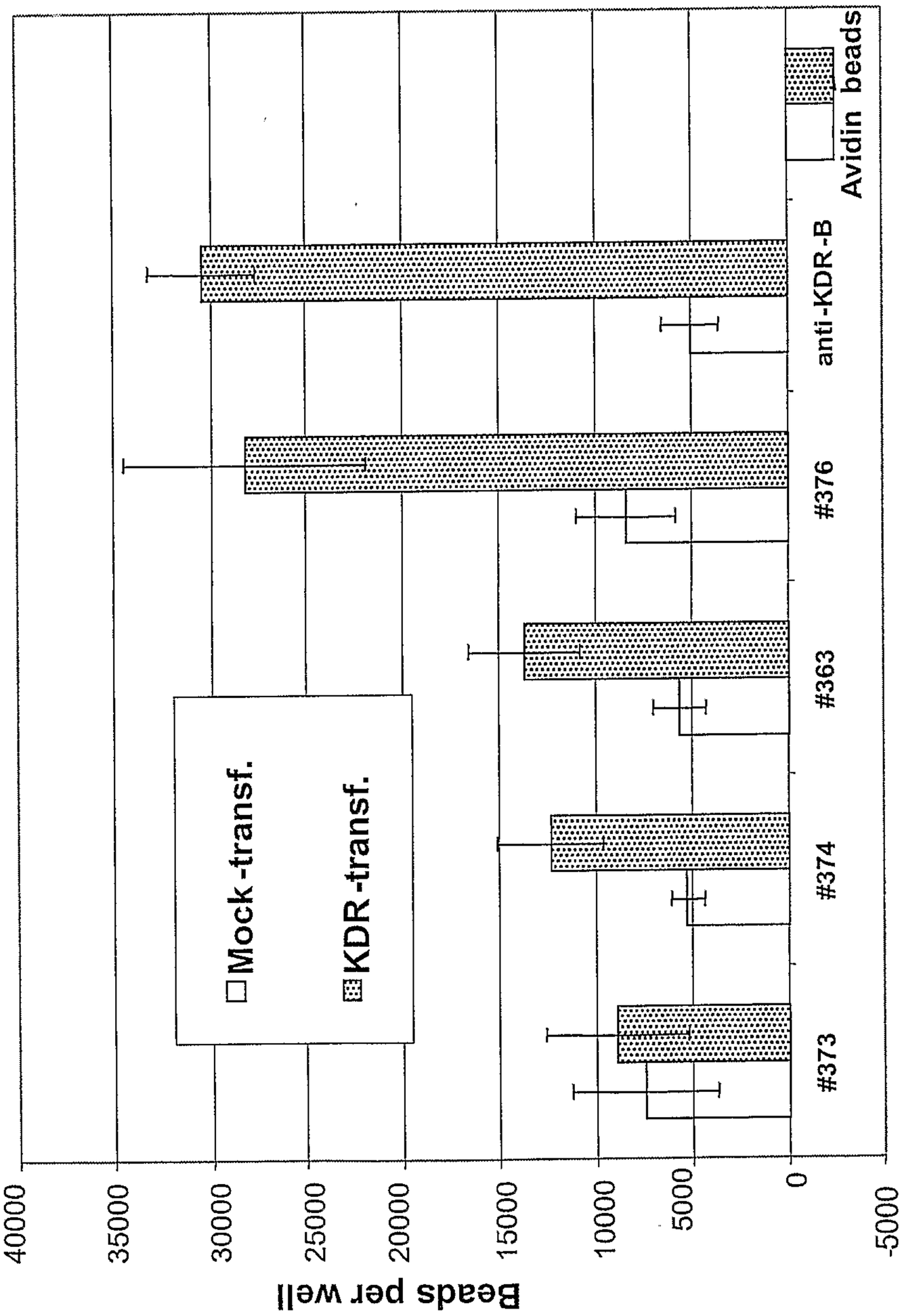

Beads per well
40000
35000
30000
25000
20000
15000
10000
5000
0
-5000
Mock -transf.
KDR -transf.
#373
#374
#363
#376
anti-KDR-B
Avidin beads

FIG. 10

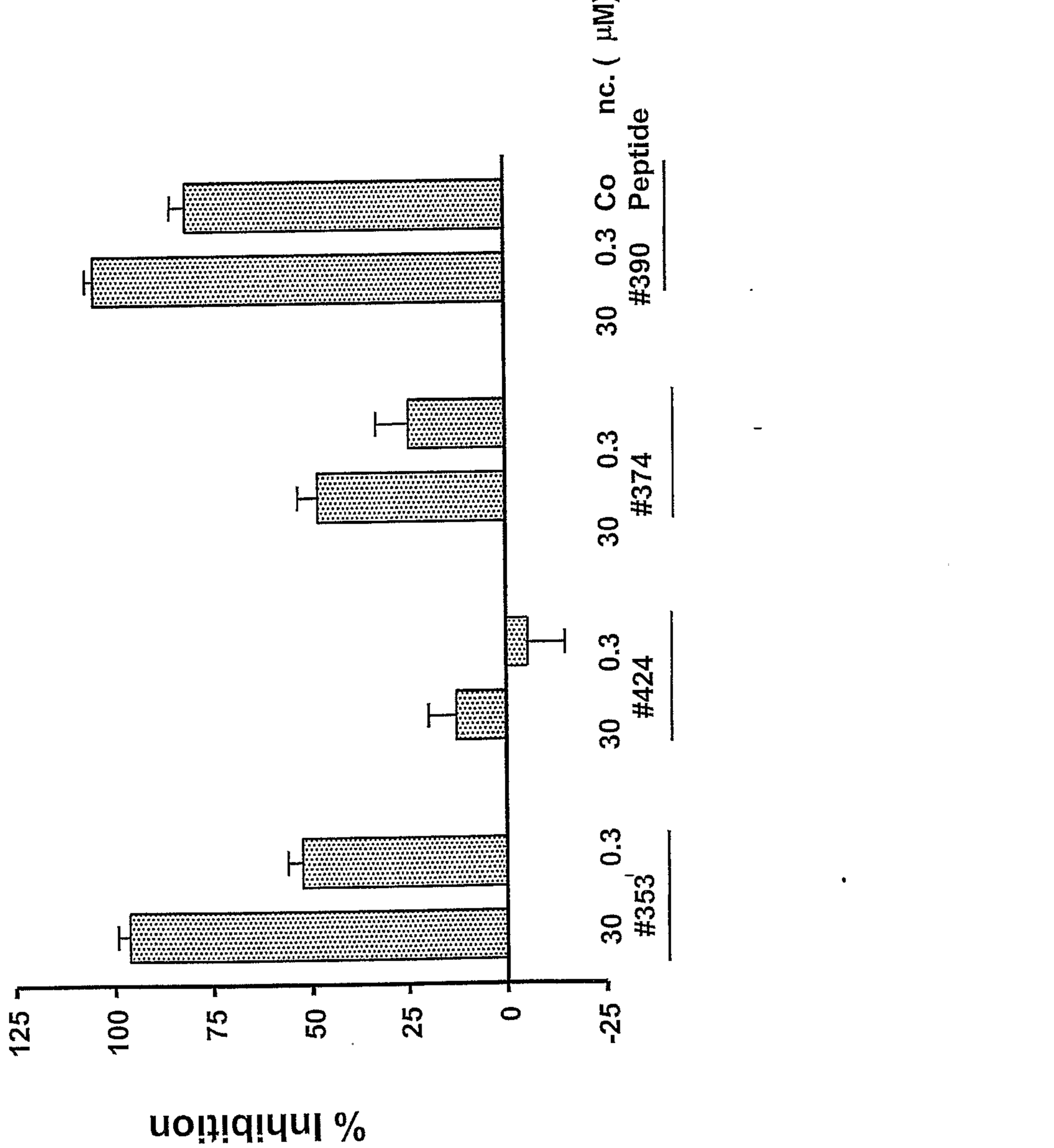

% Inhibition
125
100
75
50
25
0
-25
30 0.3
#353
30 0.3
#424
30 0.3 Co nc. (µM
#390 Peptide
30 0.3
#374

FIG. 11

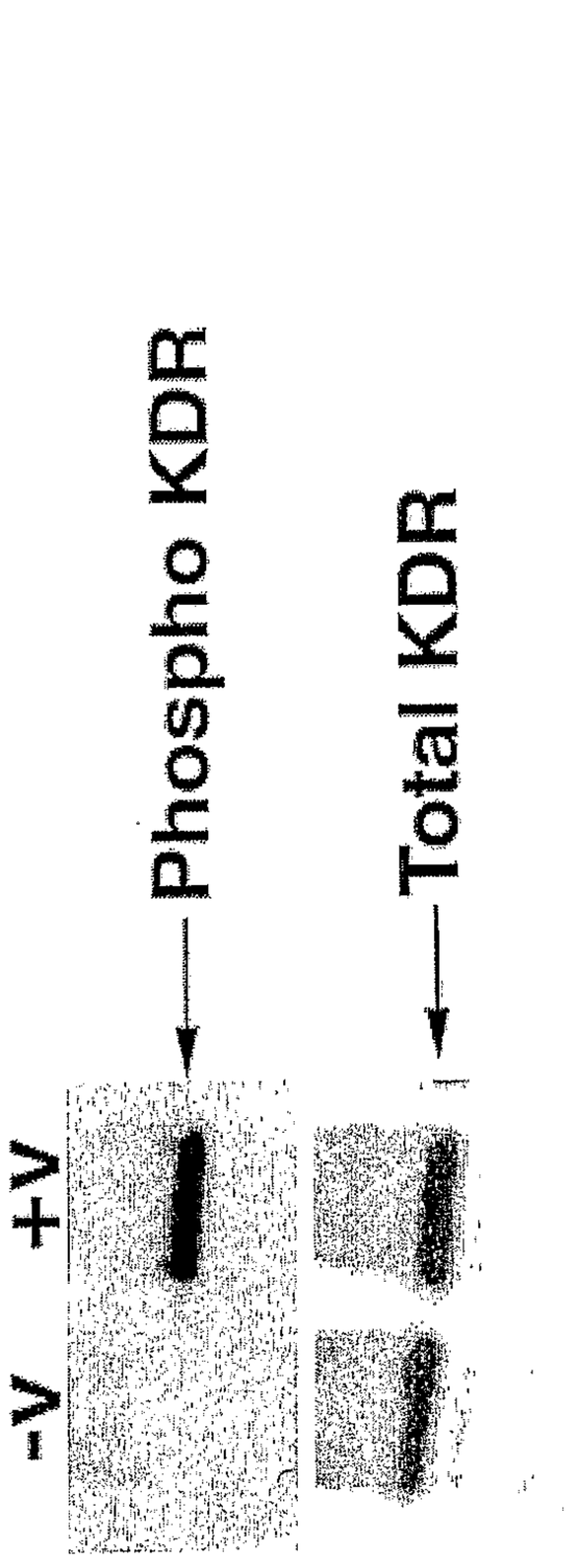
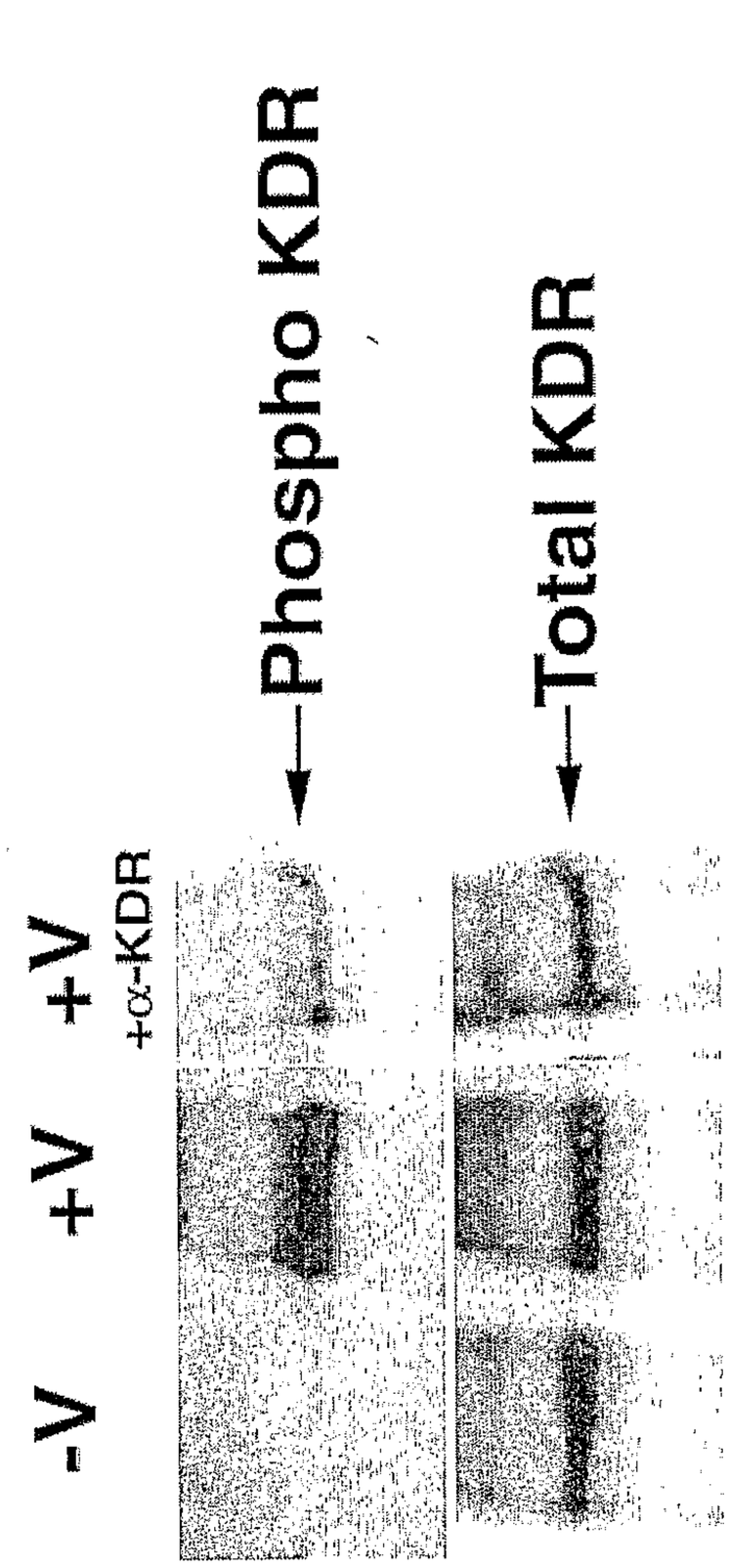


FIG. 12



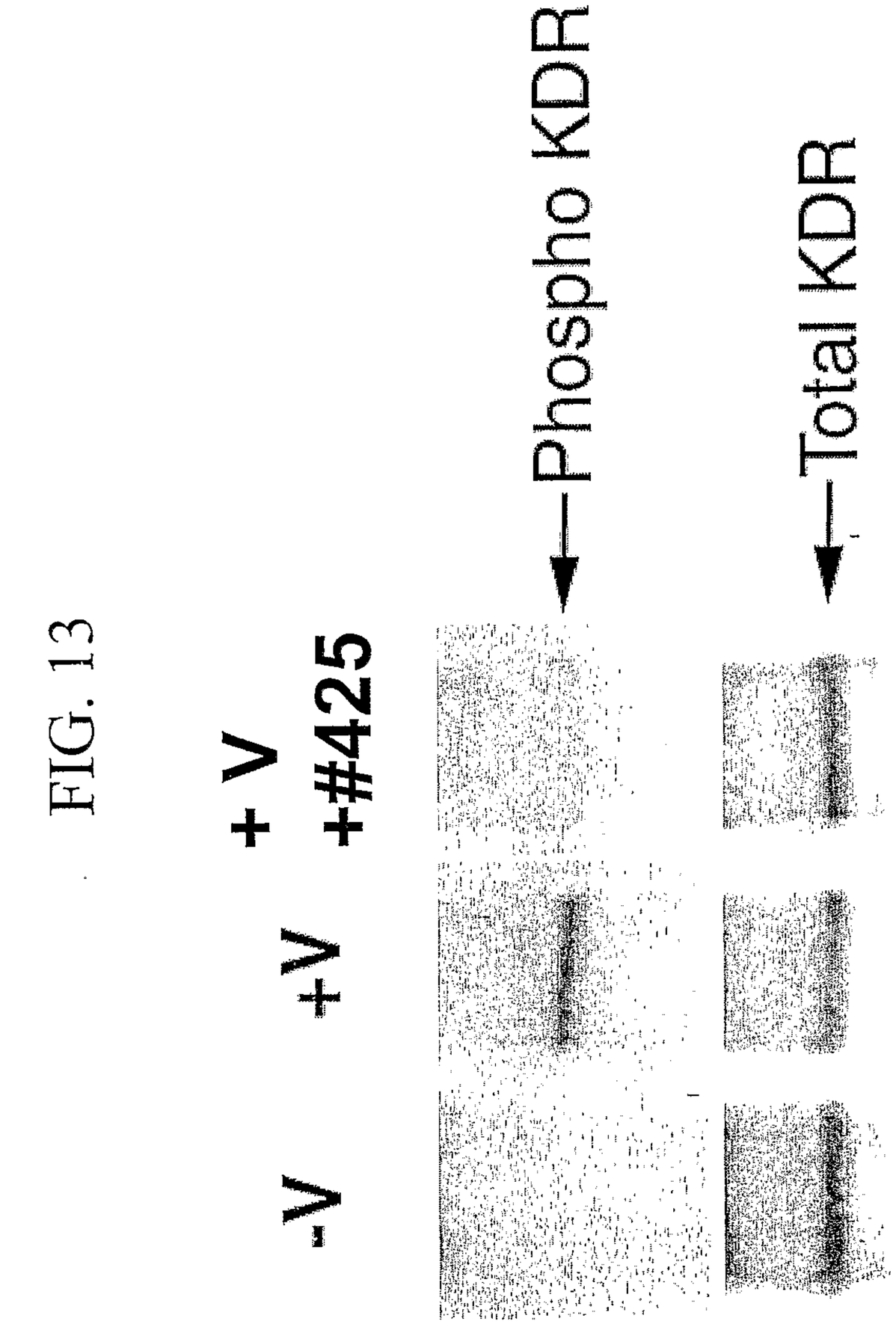


FIG. 13

FIG. 14

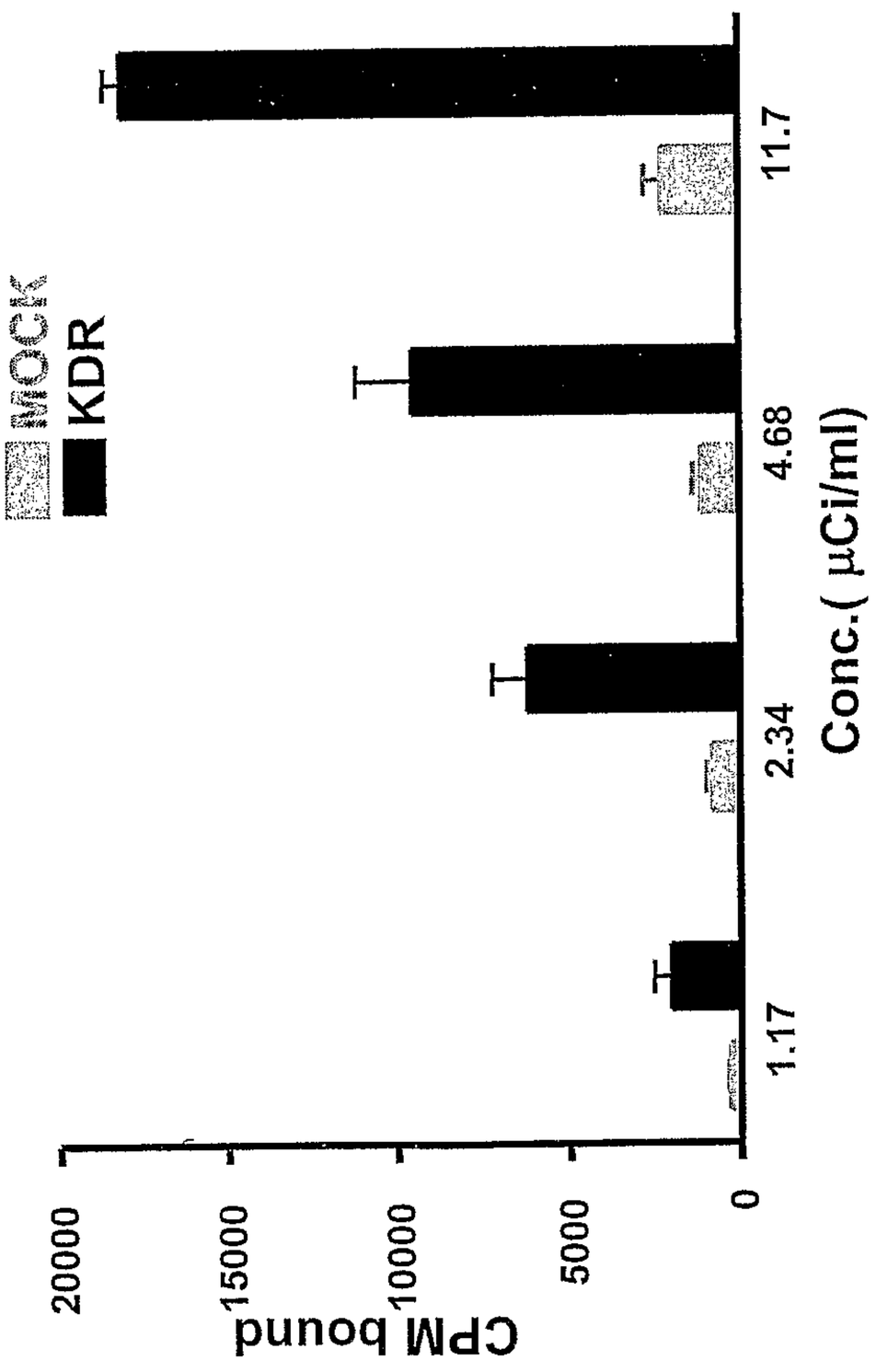

MOCK
KDR
CPM bound
20000
15000
10000
5000
0
1.17
2.34
4.68
11.7
Conc.(µCi/ml)

FIG. 15

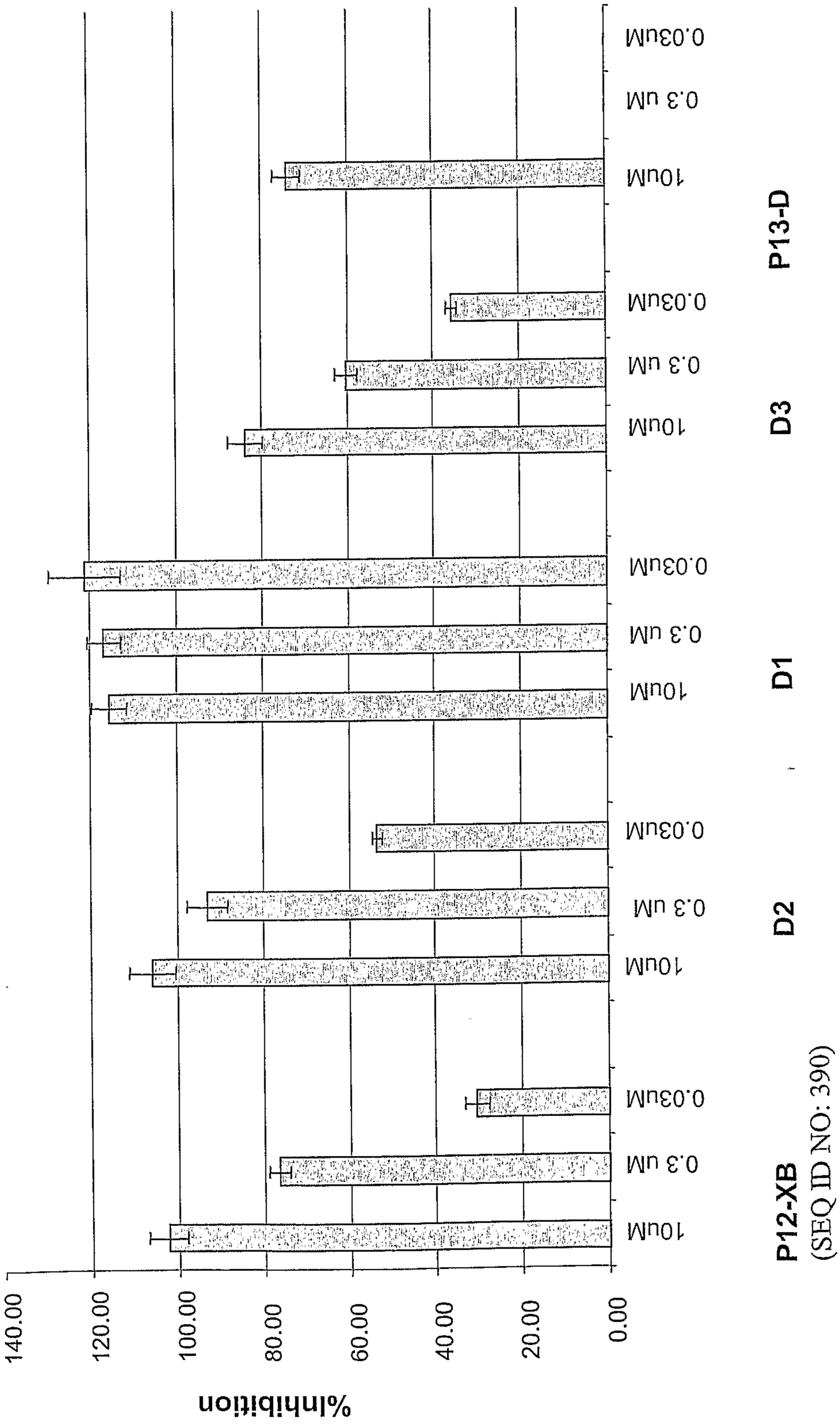

%Inhibition
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
P12-XB
(SEQ ID NO: 390)
10uM
0.3 uM
0.03uM
D2
10uM
0.3 uM
0.03uM
D1
10uM
0.3 uM
0.03uM
D3
10uM
0.3 uM
0.03uM
P13-D
10uM
0.3 uM
0.03uM

FIG. 16


BRU-888 BRU-889 BRU-892
1 10 10 100 100
- + + + + +
<-- Phospho KDR
<-- Total KDR
Conc (nM)
VEGF

FIG. 17

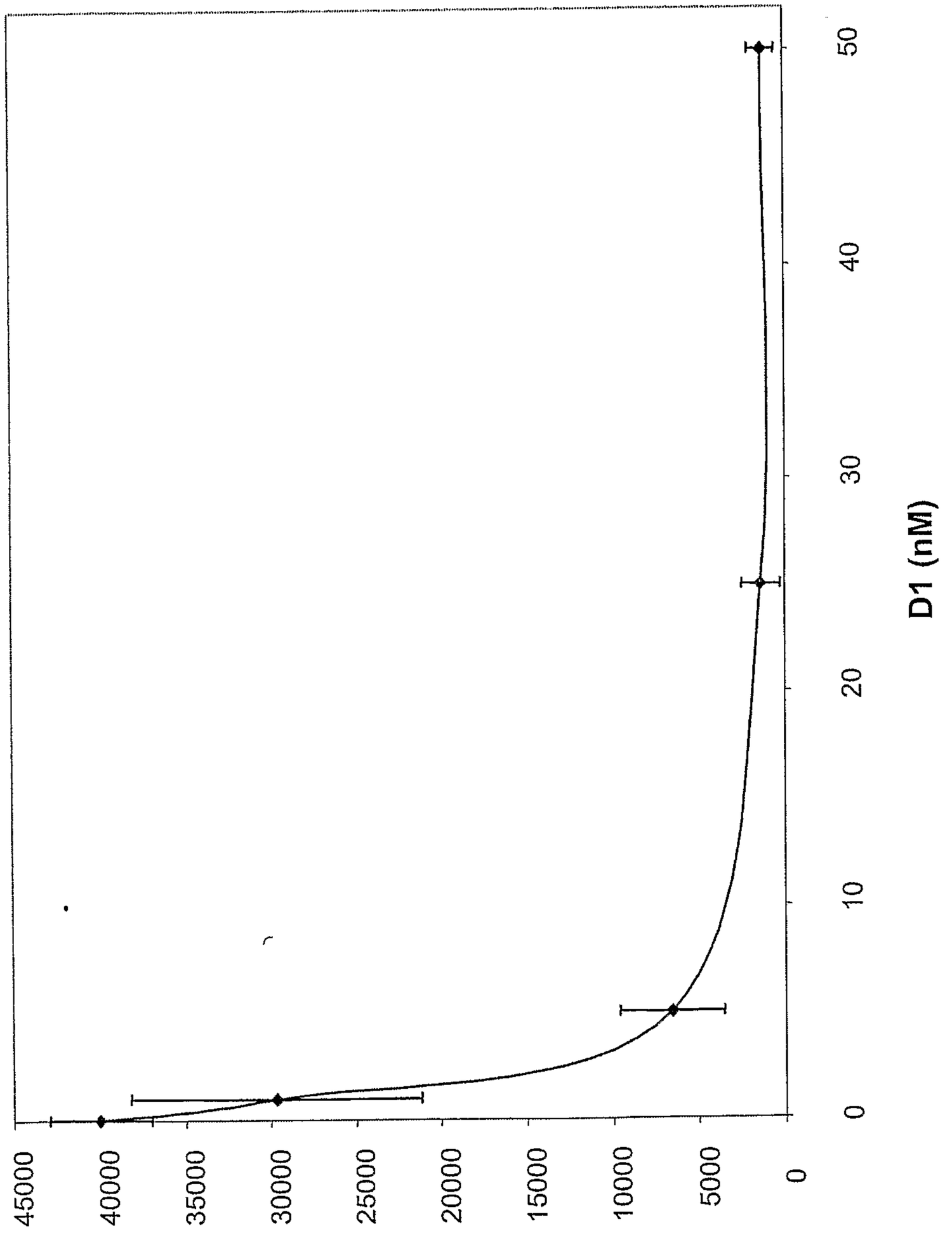

Migration (fluor)
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
D1 (nM)
0
10
20
30
40
50

FIG. 18

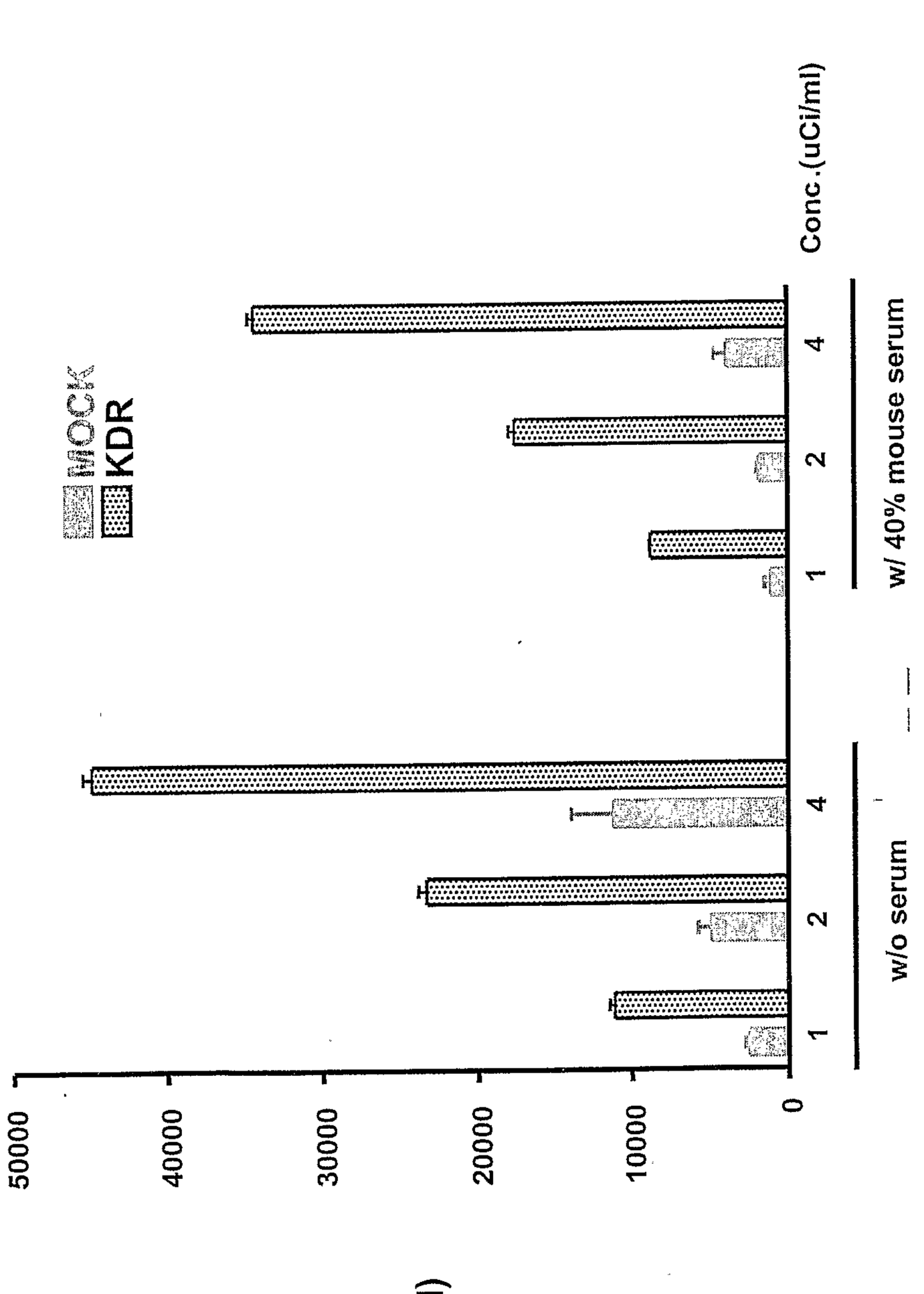
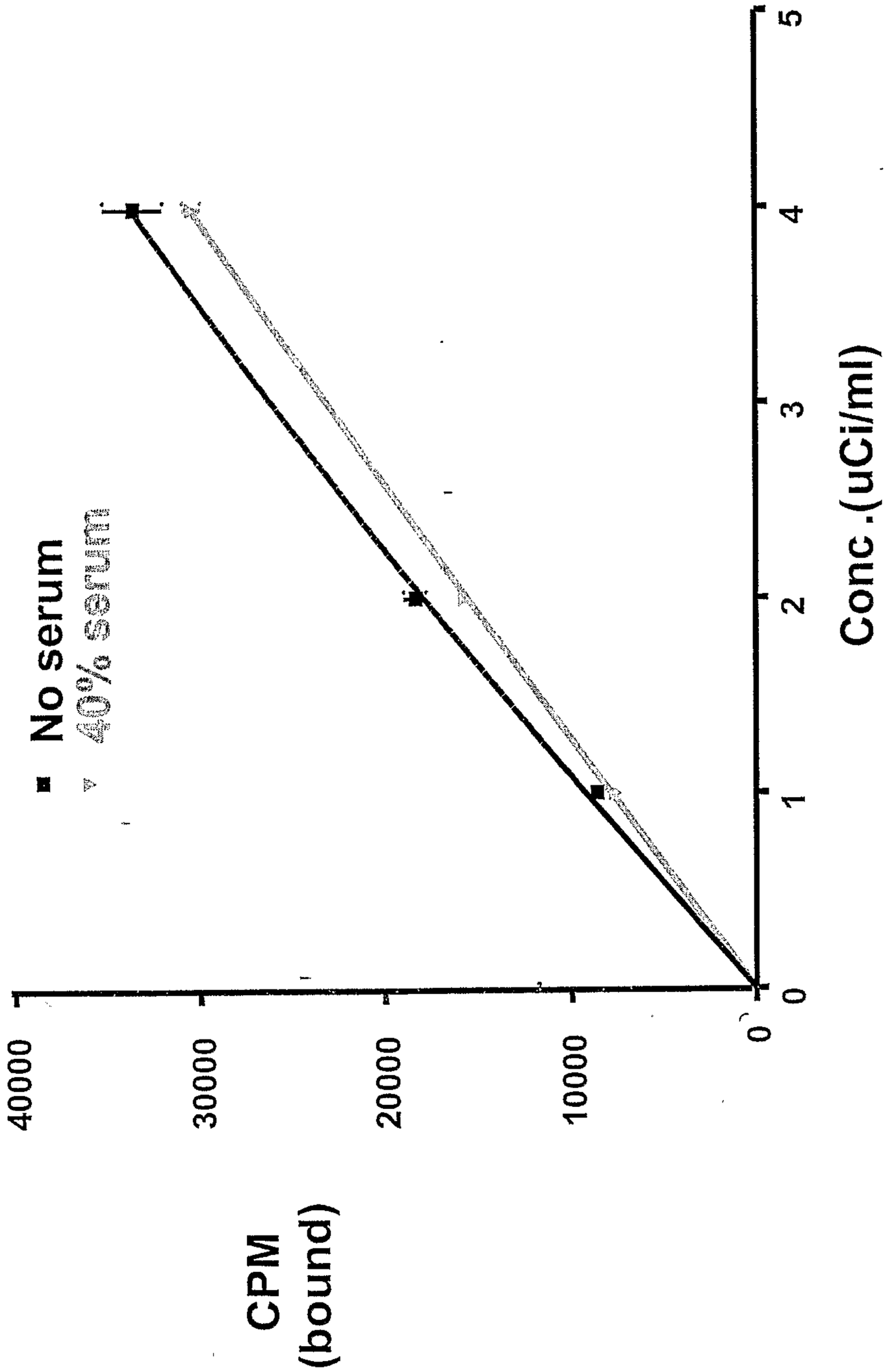

mock
KDR
CPM (bound)
50000
40000
30000
20000
10000
0
1
2
4
w/o serum
1
2
4
w/ 40% mouse serum
Conc.(uCi/ml)

FIG. 19


CPM (bound)
40000
30000
20000
10000
0
0
1
2
3
4
5
Conc.(uCi/ml)
No serum
40% serum

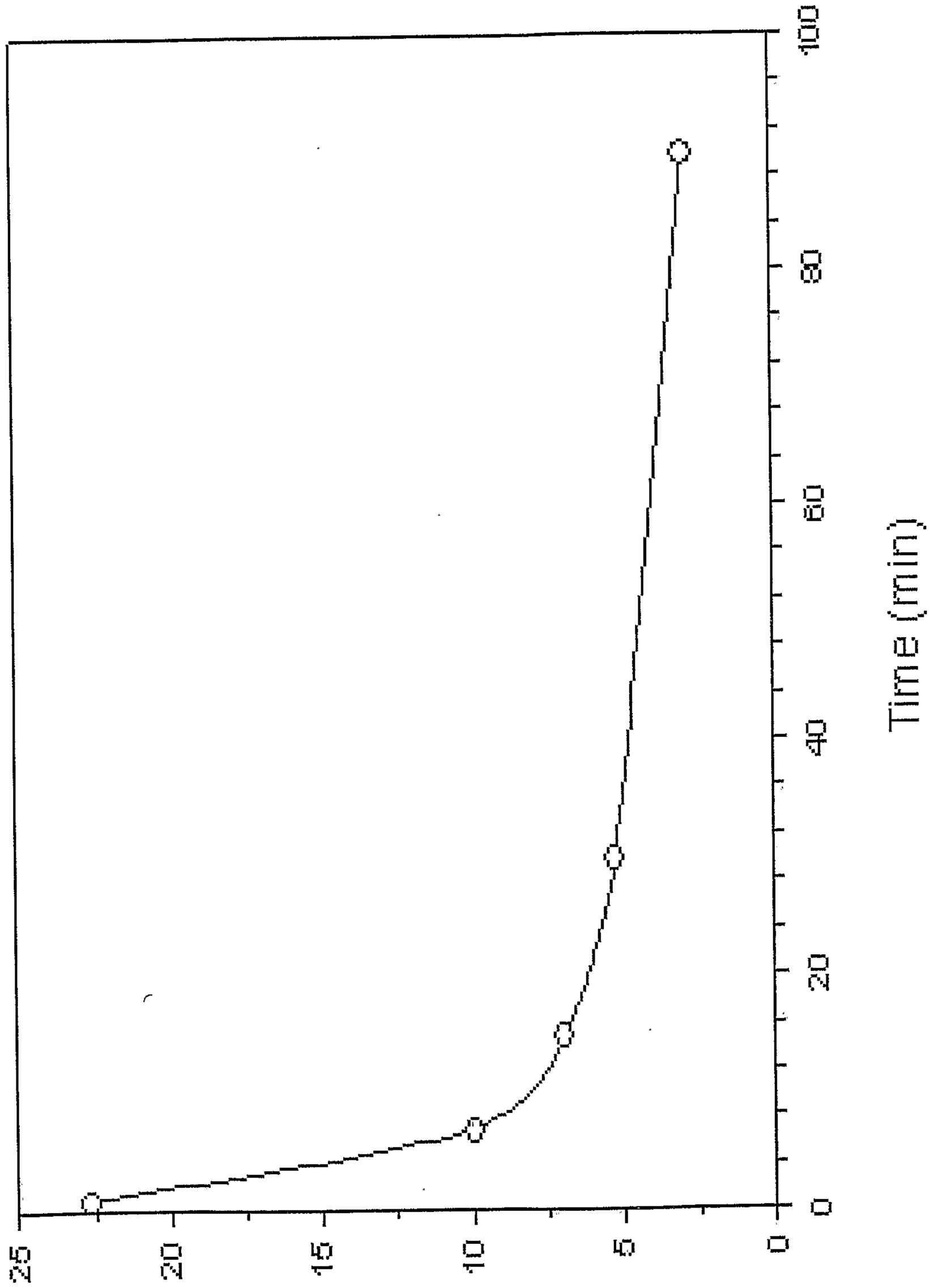


FIG. 20

FIG. 21

SEC Chromatograms of ^{125}I-DX-1235 in Mouse Plasma

FIG. 22

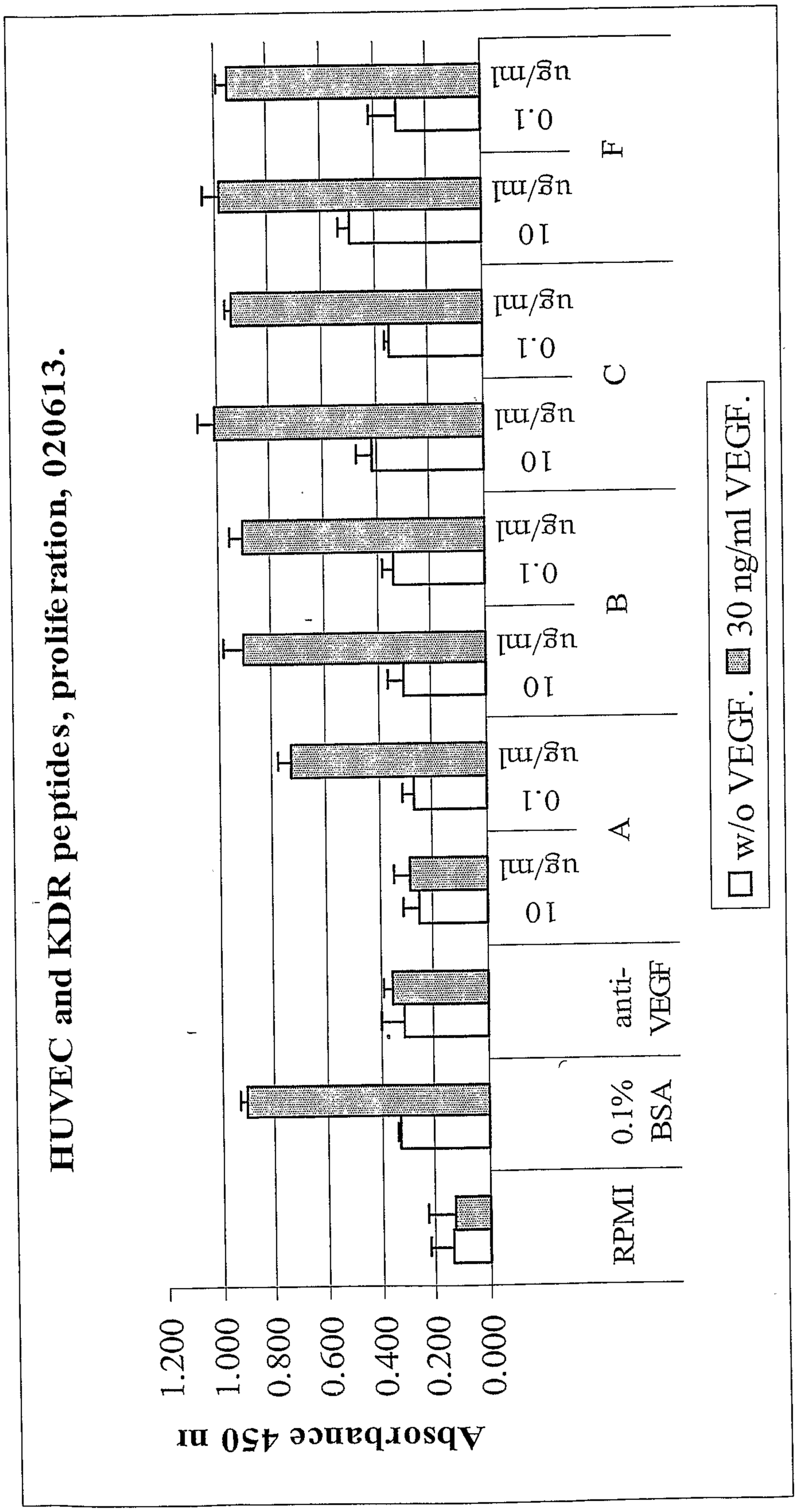

HUVEC and KDR peptides, proliferation, 020613.
Absorbance 450 nm
1.200
1.000
0.800
0.600
0.400
0.200
0.000
RPMI
0.1% BSA
anti-VEGF
A
B
C
D
0.1 ug/ml
10 ug/ml
□ w/o VEGF. ■ 30 ng/ml VEGF.

FIG. 23A

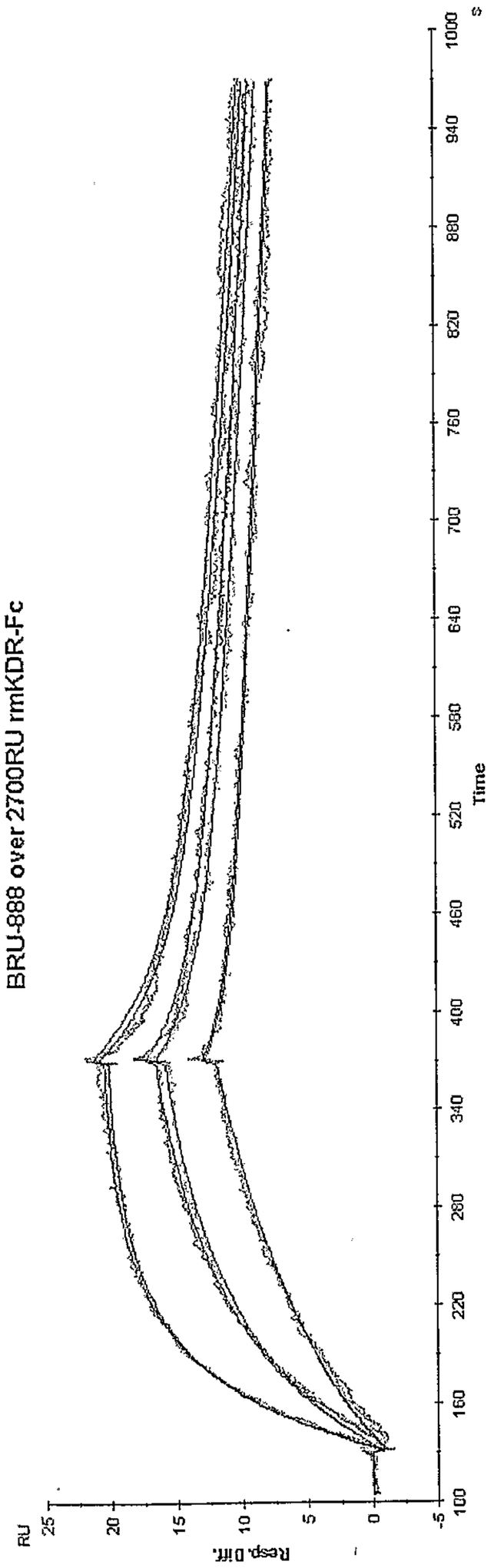

BRU-888 over 2700RU rmKDR-Fc
Resp Diff.
RU
Time
25
20
15
10
5
0
-5
100
160
220
280
340
400
460
520
580
640
700
760
820
880
940
1000
s

FIG. 23B

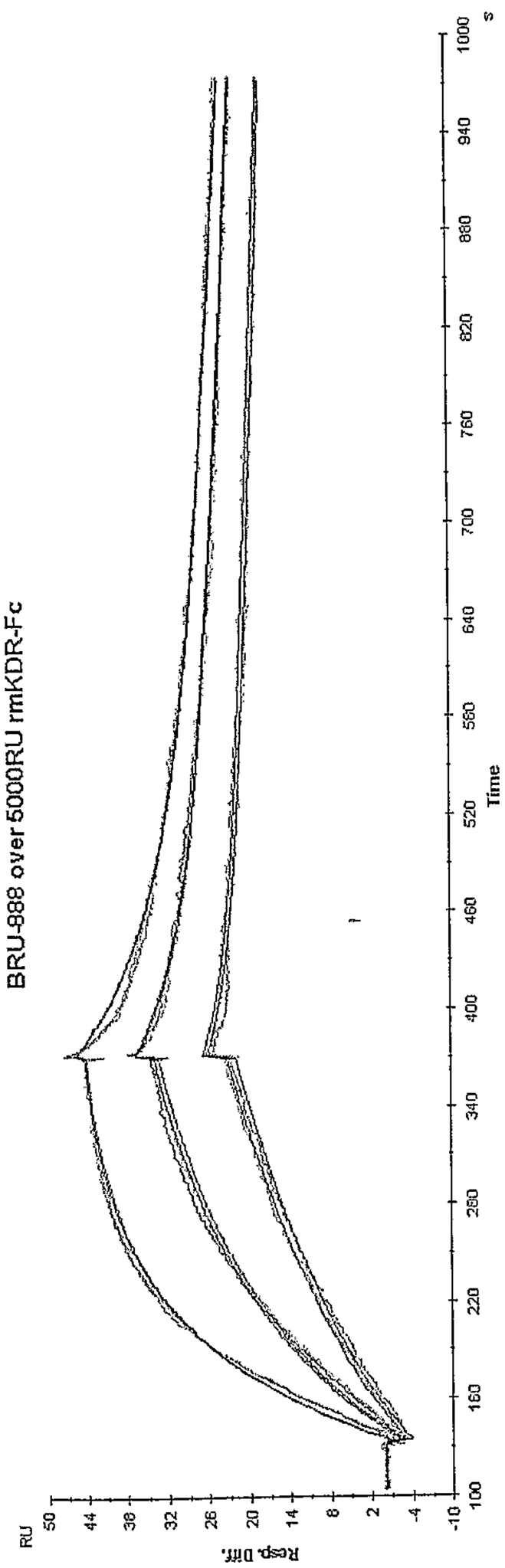

BRU-888 over 5000RU rmKDR-Fc
Resp Diff.
RU
Time
s
50
44
38
32
26
20
14
8
2
-4
-10
100
160
220
280
340
400
460
520
560
640
700
760
820
880
940
1000

FIG. 24A

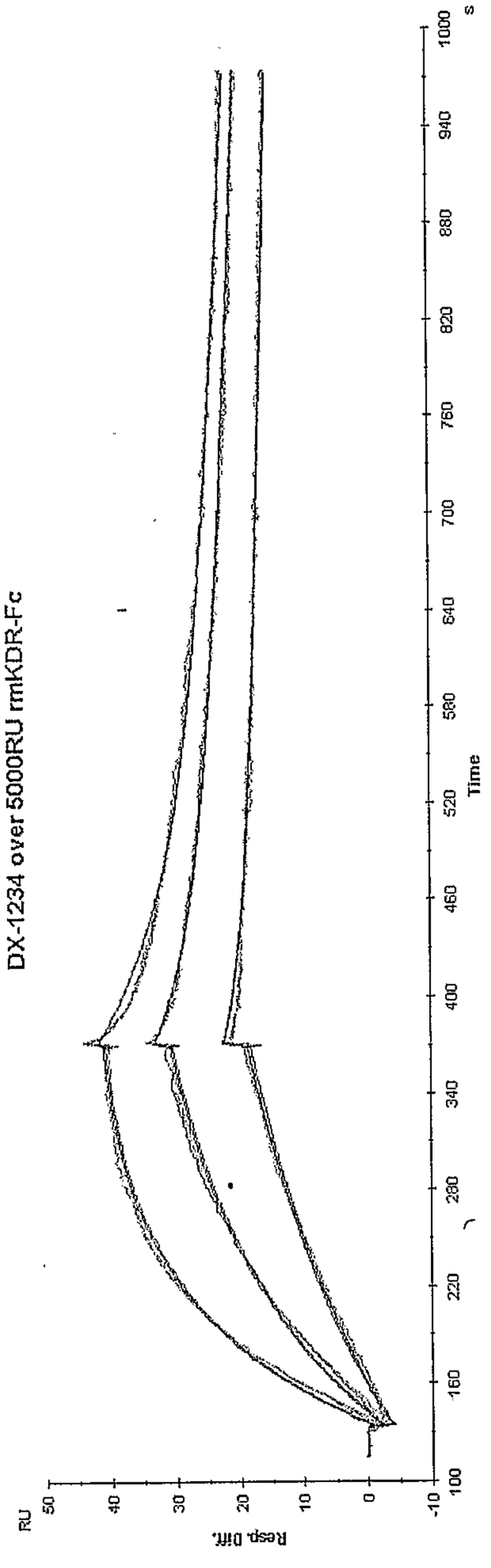


FIG. 24B

DX-1234 over 2700RU rmKDR-Fc
RU
Resp. Diff.
Time
25
20
15
10
5
0
-5
100
160
220
280
340
400
460
520
580
640
700
760
820
880
940
1000

FIG. 25A

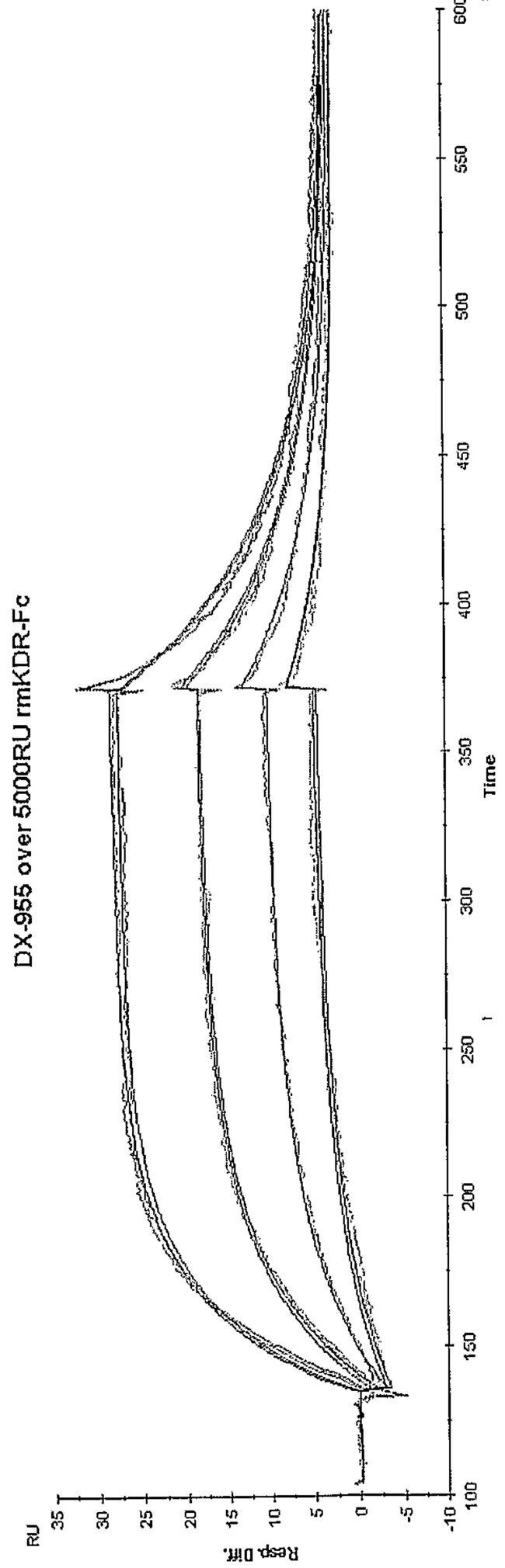

DX-955 over 5000RU rmKDR-Fc
RU
Resp Diff.
35
30
25
20
15
10
5
0
-5
-10
100
150
200
250
300
350
400
450
500
550
600
Time

FIG. 25B

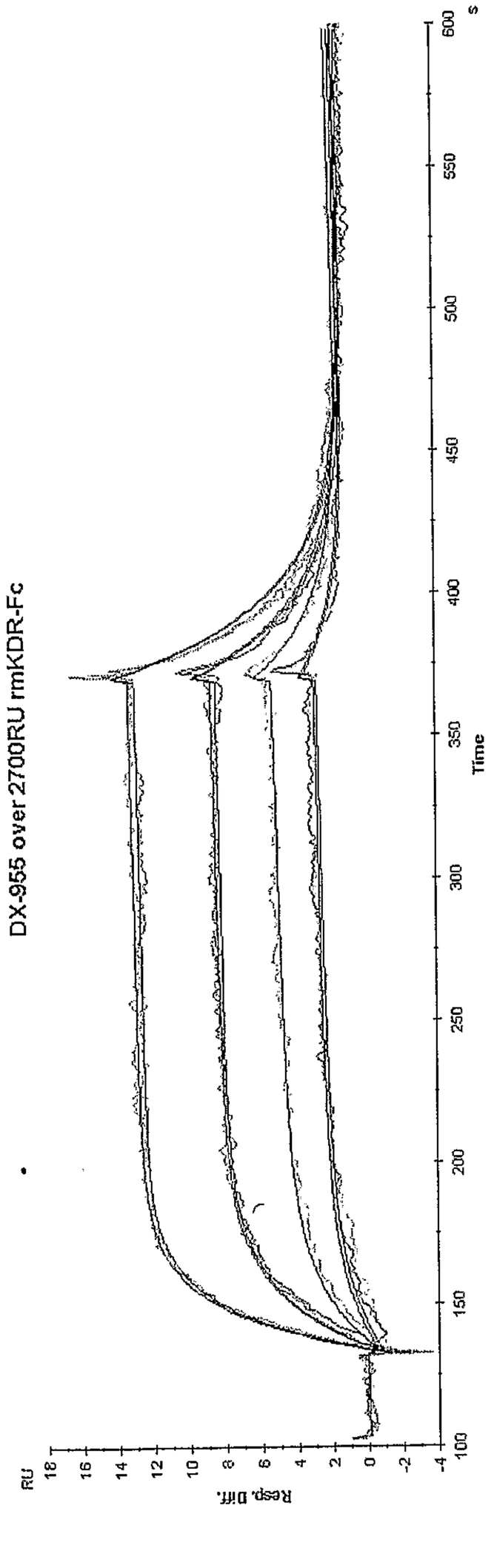

DX-955 over 2700RU rmKDR-Fc
Resp. Diff.
RU
Time
s
18
16
14
12
10
8
6
4
2
0
-2
-4
100
150
200
250
300
350
400
450
500
550
600

FIG. 26

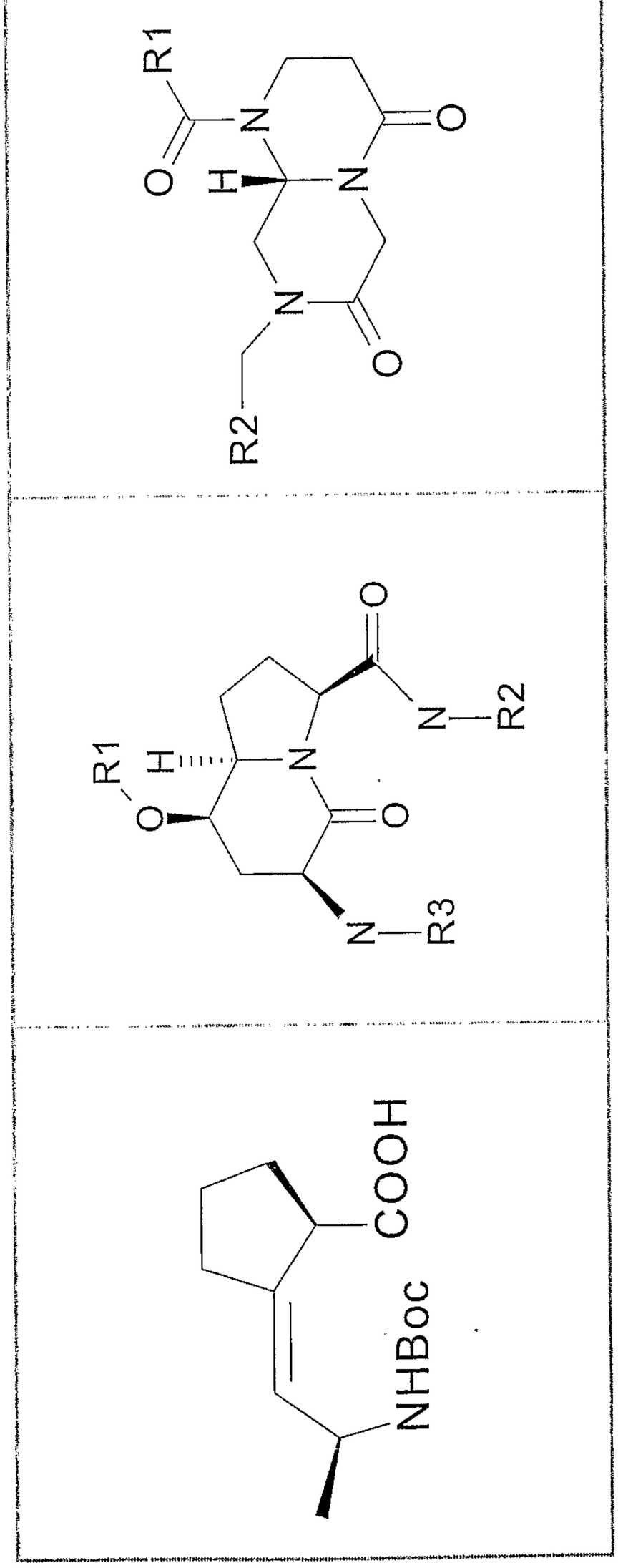
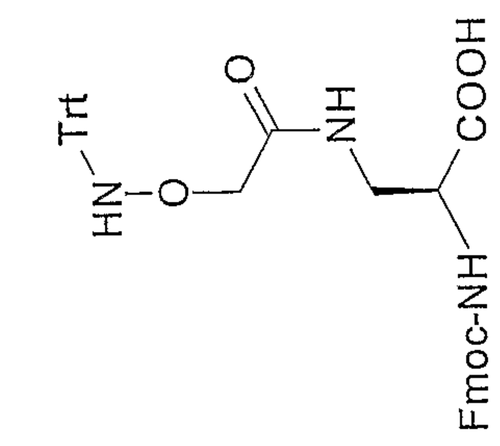
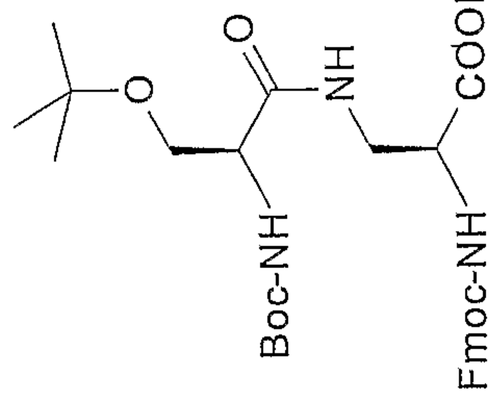


FIG. 27



Fmoc-Dap(Trt-Aoa)-OH

5



Fmoc-Dap(Boc-Ser(t-Bu))-OH

4

FIG. 28

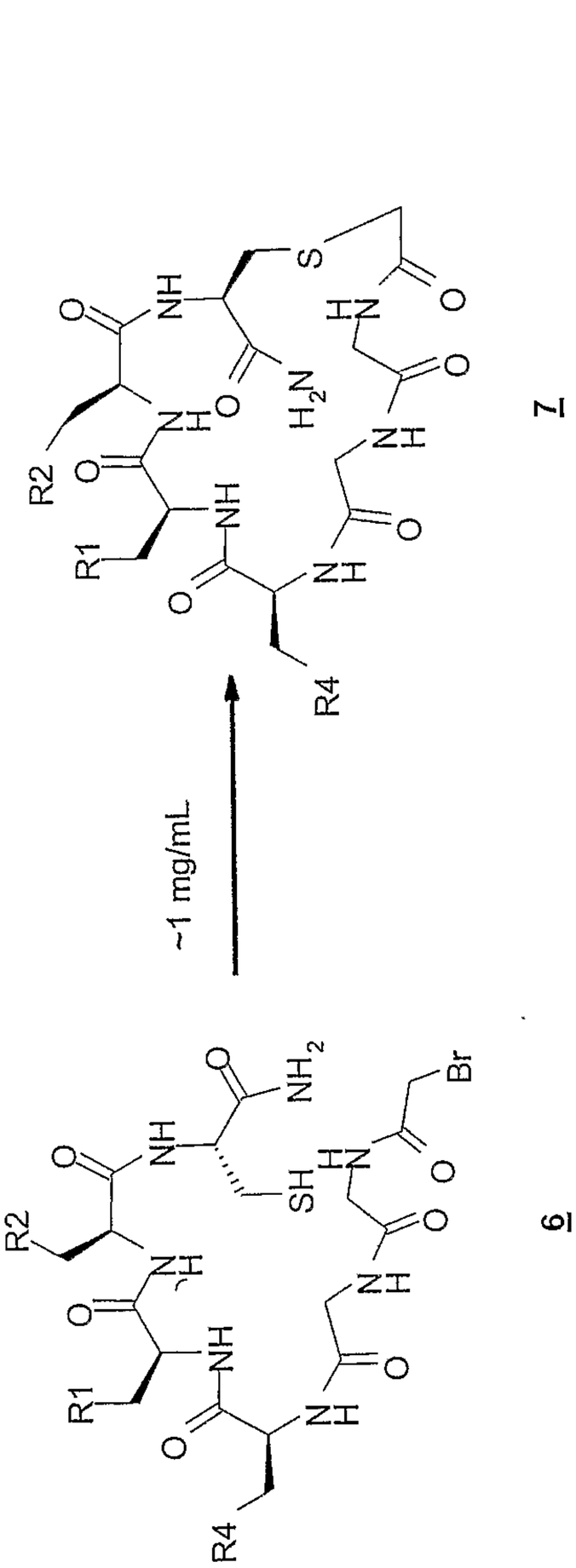


FIG. 29

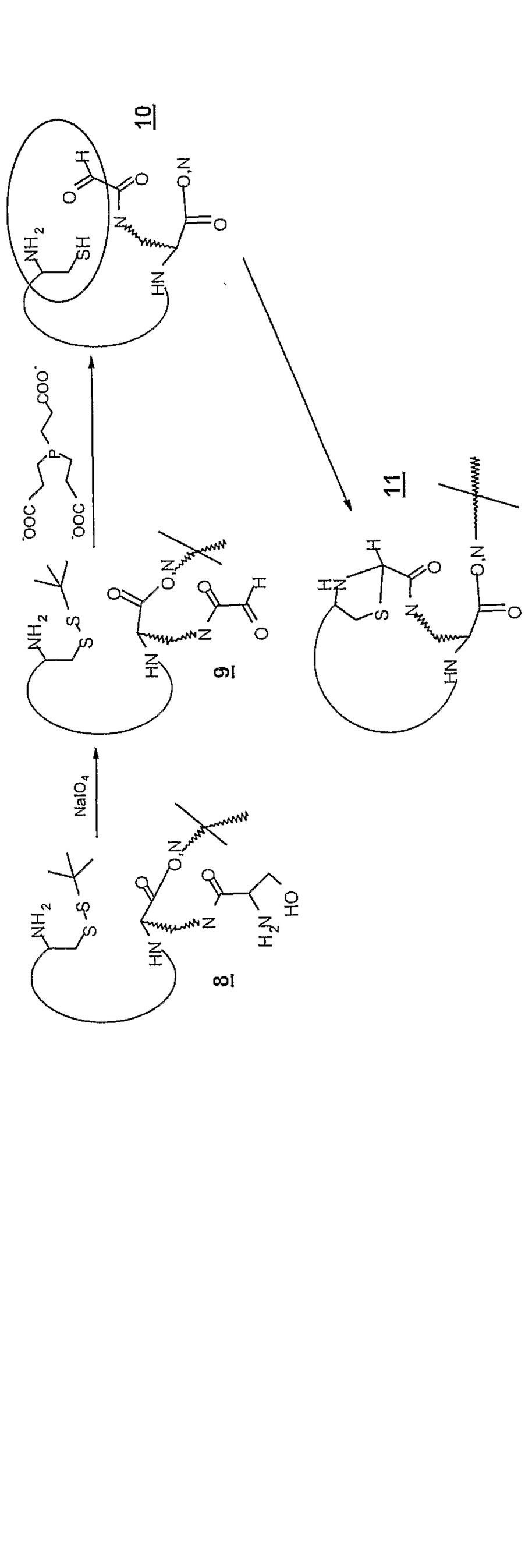


FIG. 30

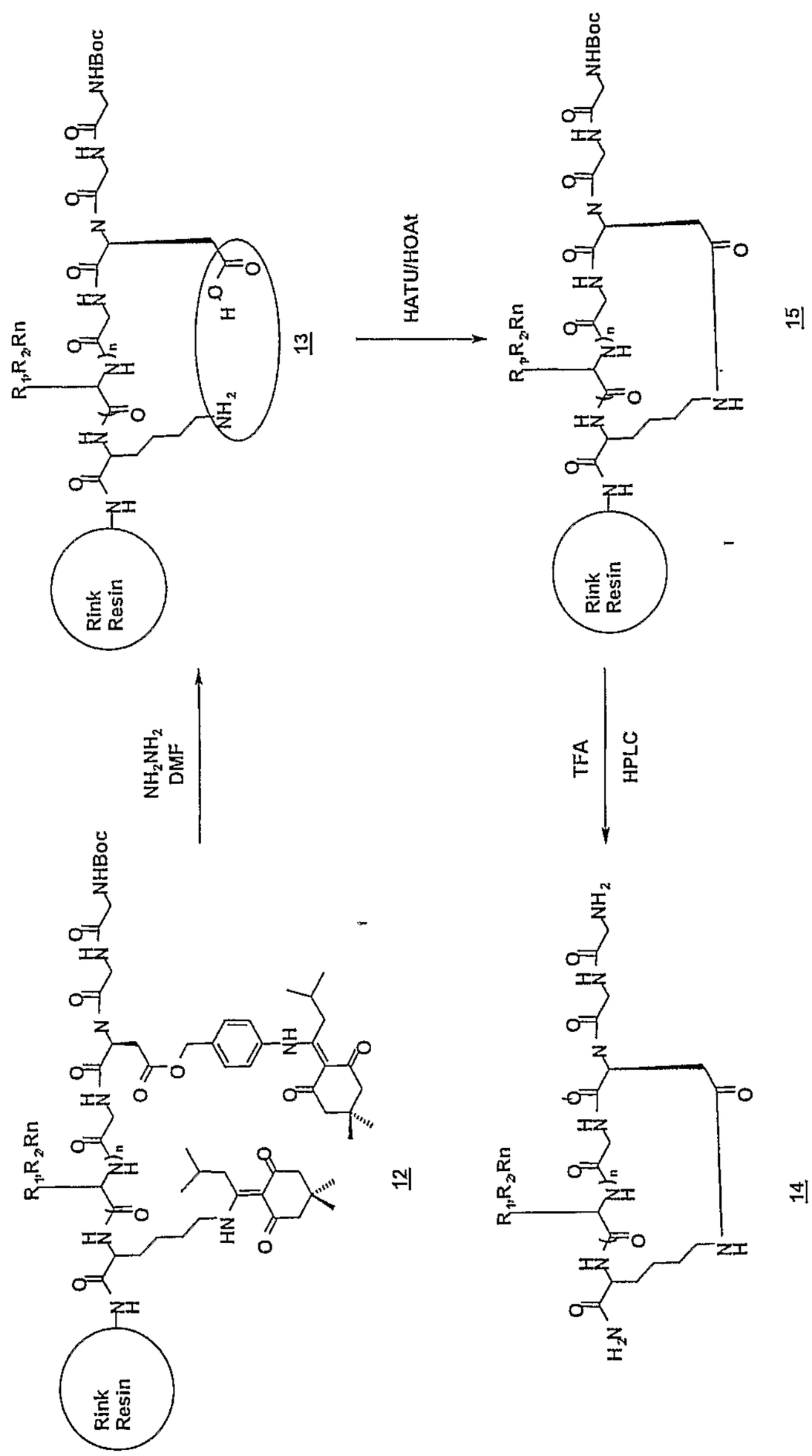

Rink Resin
R_1R_2Rn
NHBoc
NH_2
13
HATU/HOAt
15
NH_2NH_2
DMF
12
TFA
HPLC
14

FIG. 31

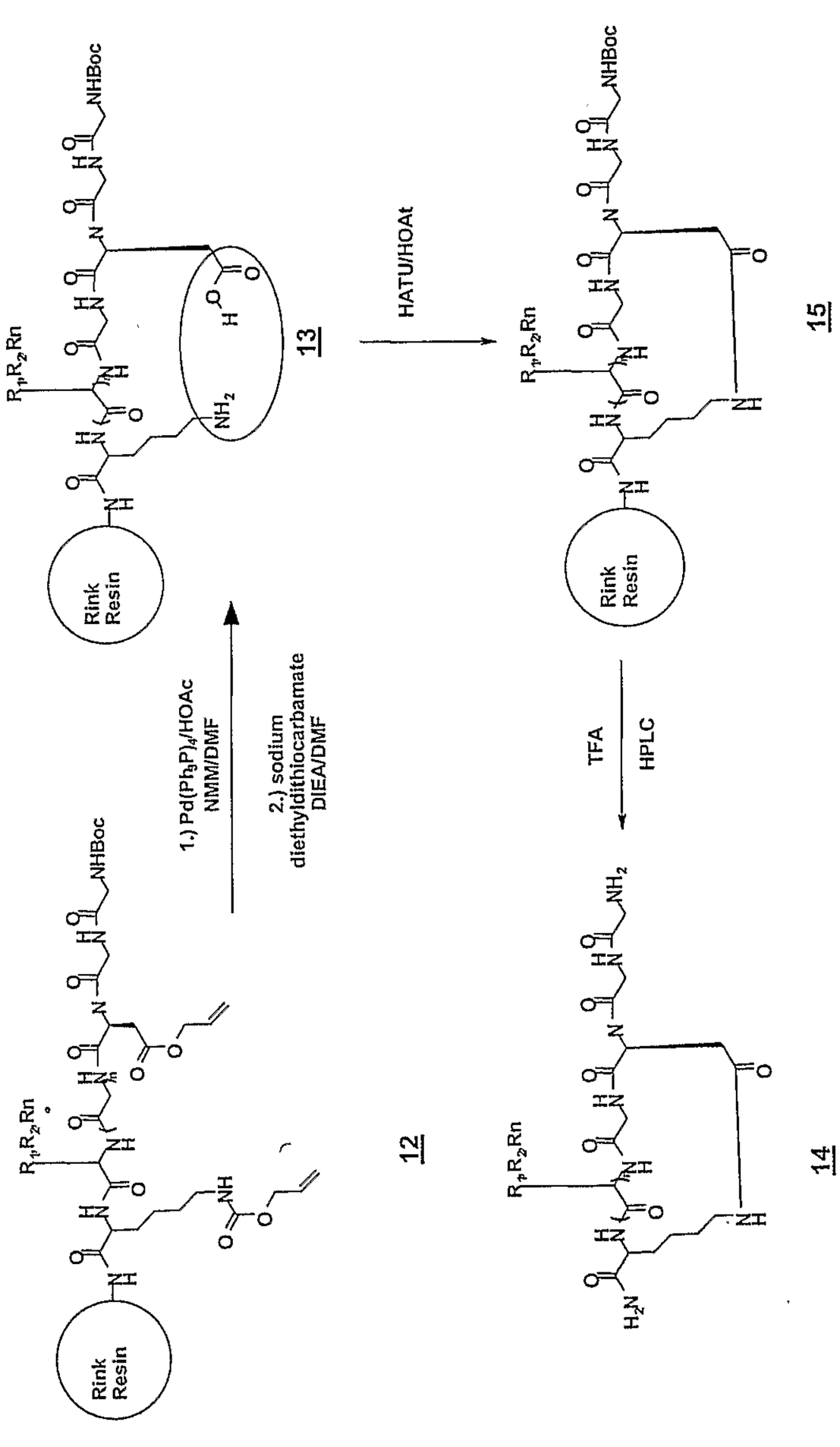

Rink Resin
R_1R_2Rn
NHBoc
NH_2
13
HATU/HOAt
15
1.) $Pd(Ph_3P)_4$/HOAc
NMM/DMF
2.) sodium diethyldithiocarbamate
DIEA/DMF
12
TFA
HPLC
14

FIG. 3

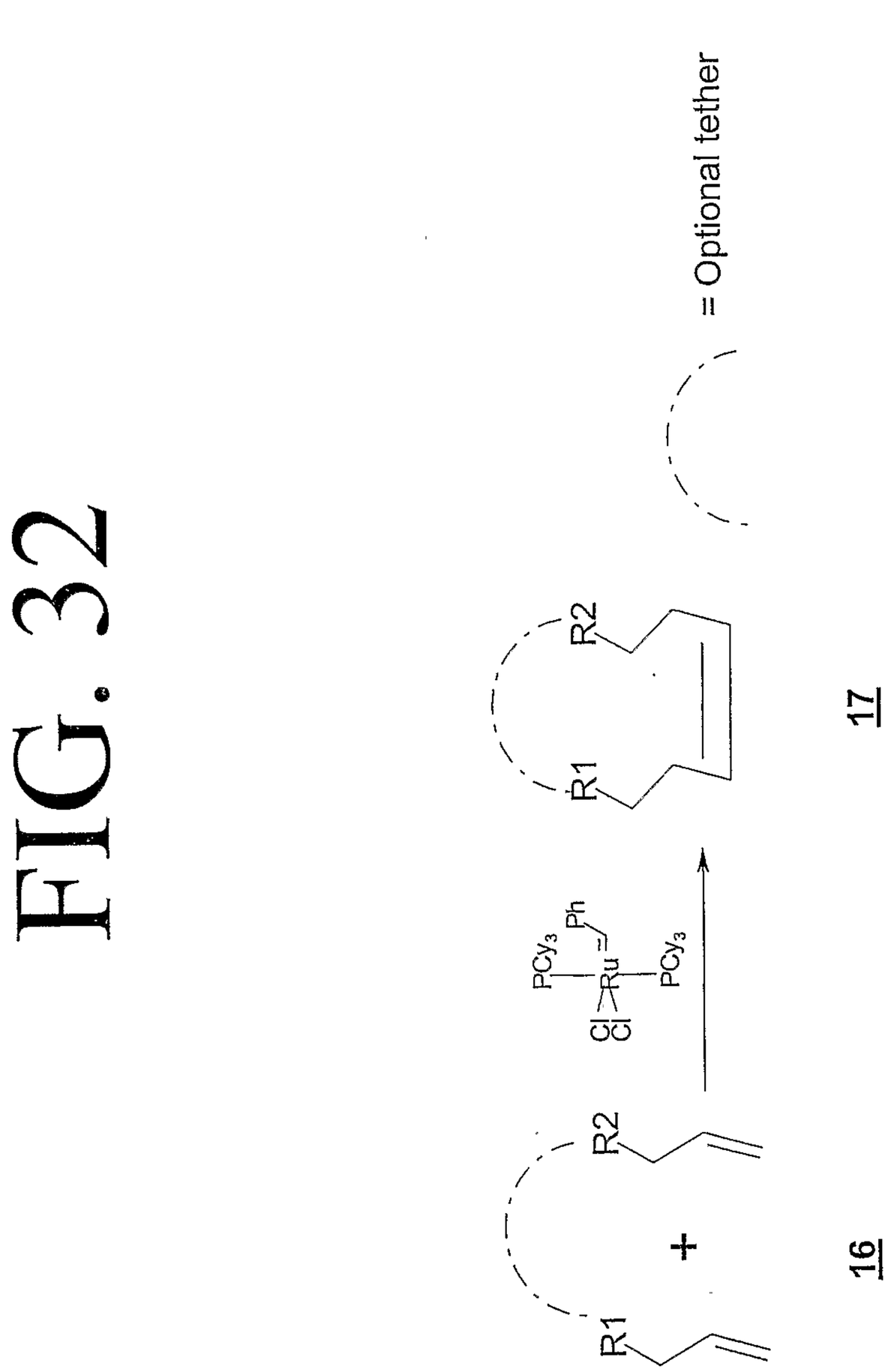


FIG. 33

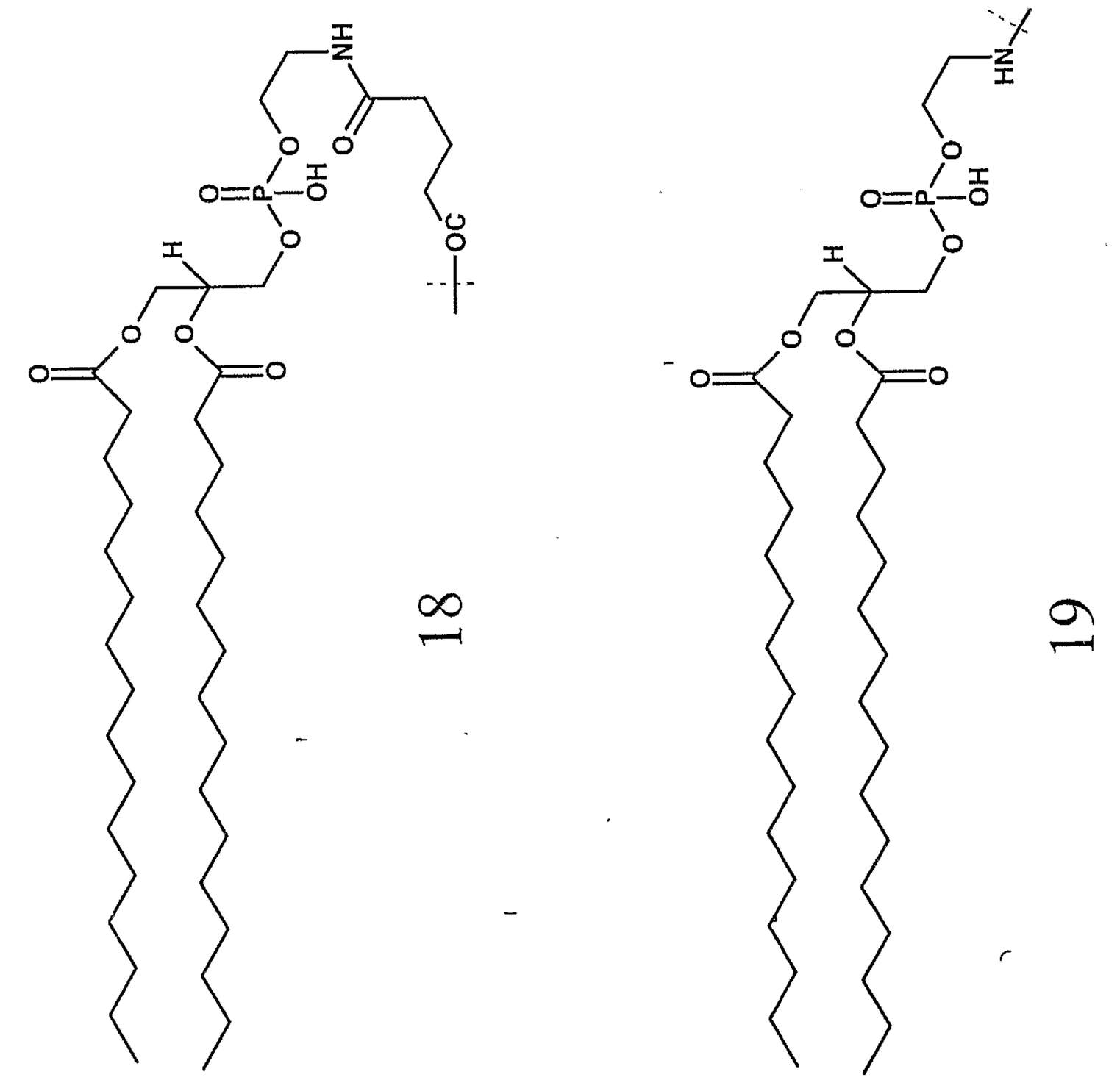
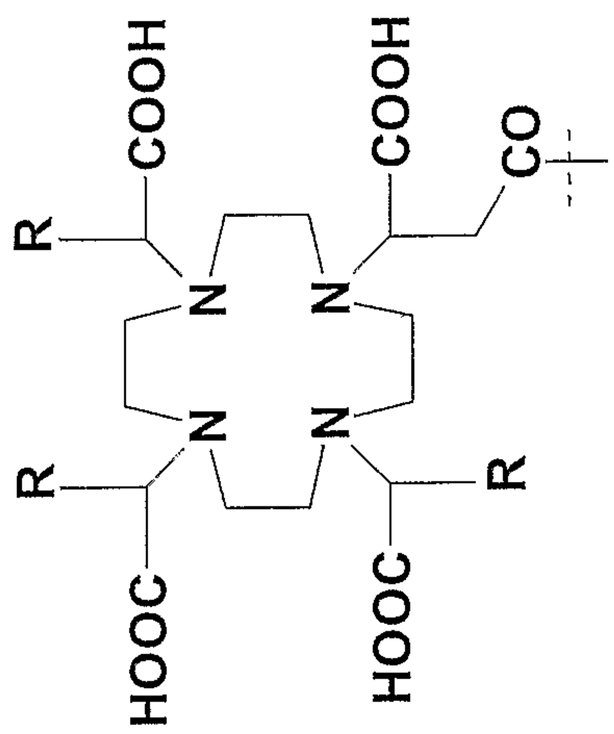
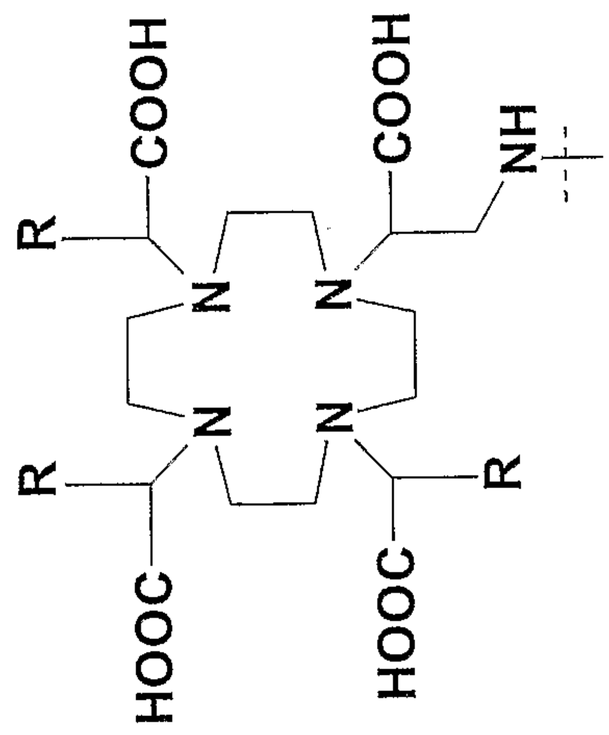


FIG. 34A



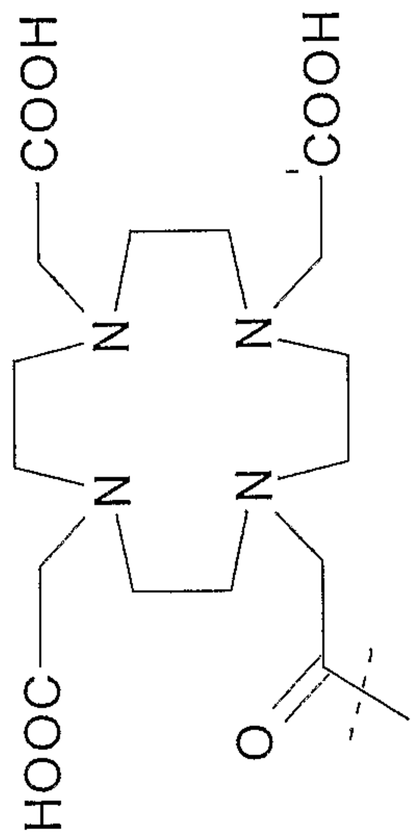
(20)

FIG. 34B



(21)

FIG. 34C



(22)

FIG. 34D

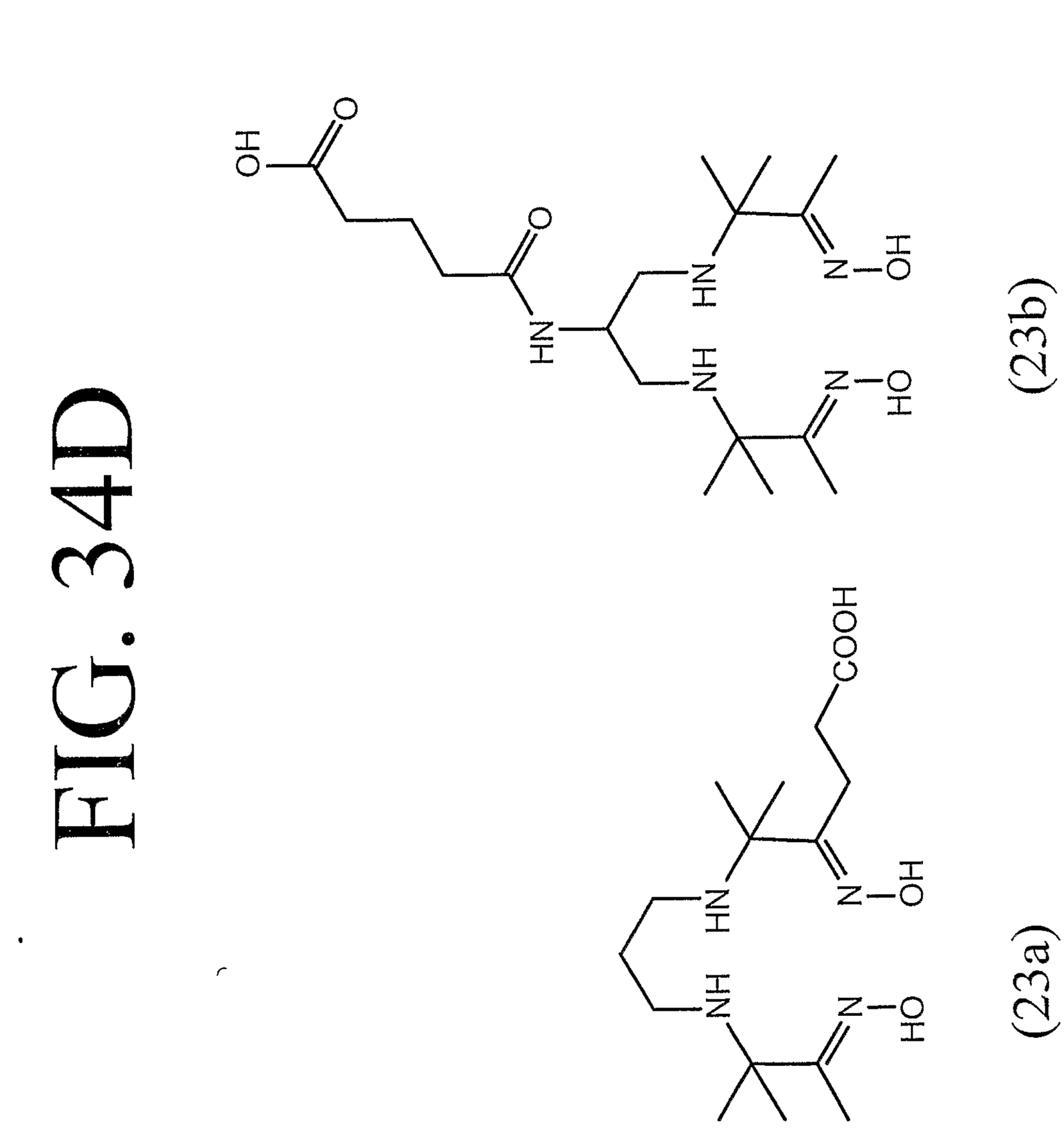
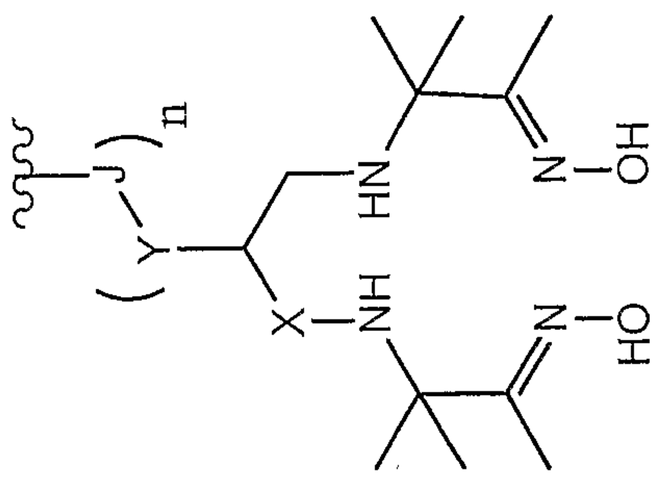
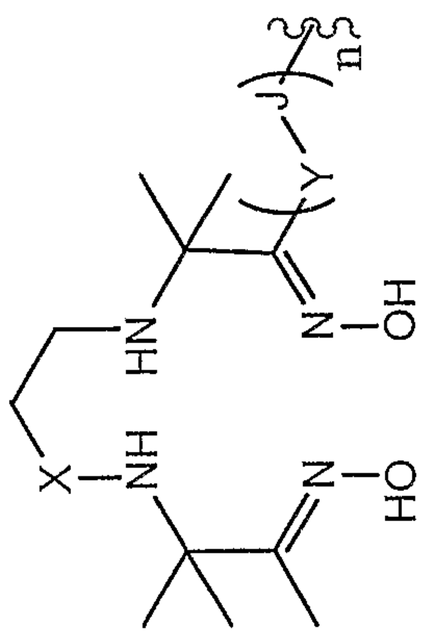


FIG. 34E

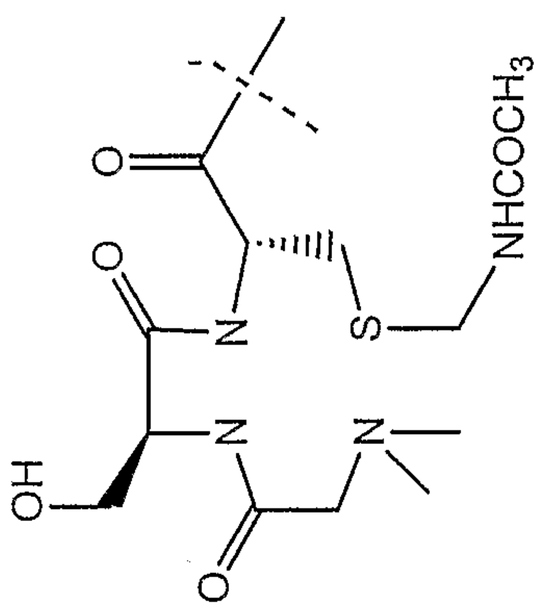


(24b)



(24a)

FIG. 34F



(25)

FIG. 35

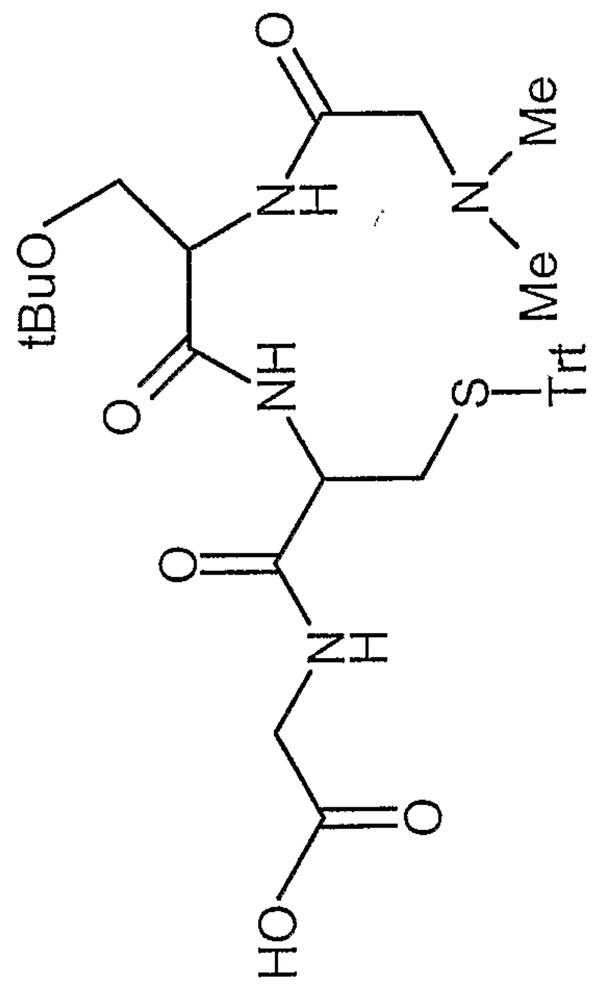


FIG. 36

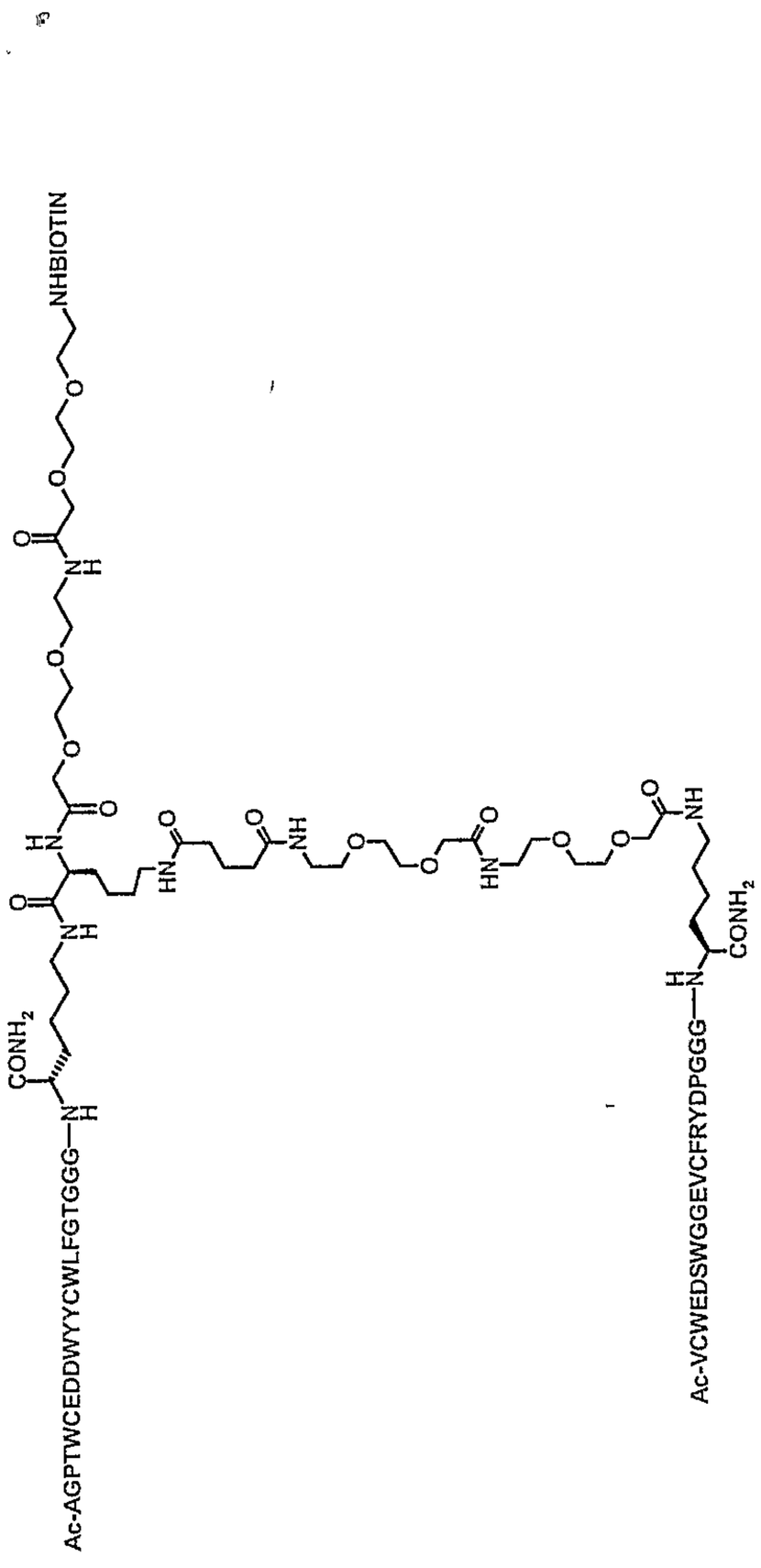

NHBIOTIN
Ac-AGPTWCEDDWYYCWLFGTGGG—
CONH2
Ac-VCWEDSWGGEVCFRYDPGGG—
CONH2

FIG. 37

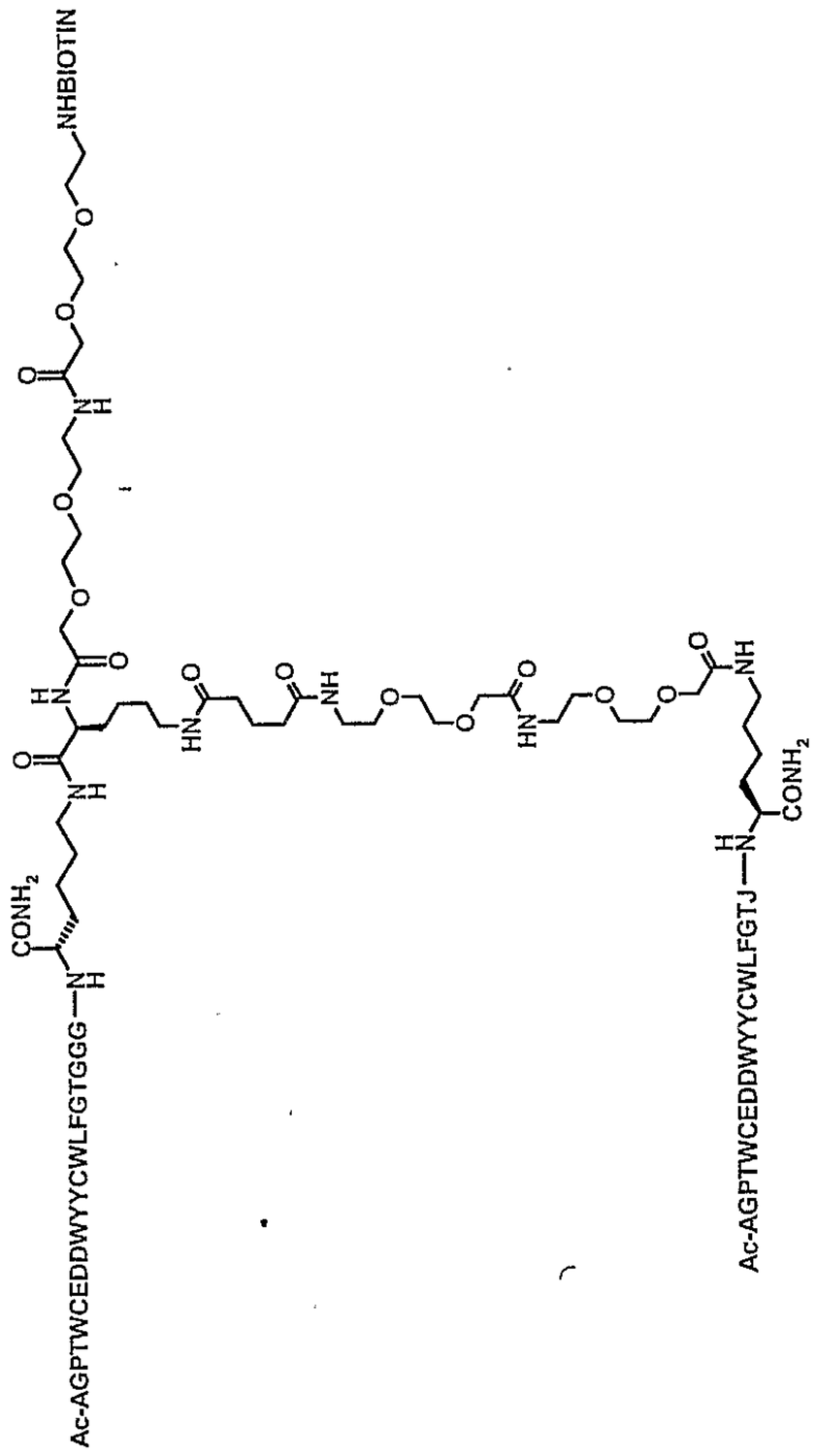

NHBIOTIN
Ac-AGPTWCEDDWYYCWLFGTGGG
Ac-AGPTWCEDDWYYCWLFGTJ
CONH2
CONH2

FIG. 38

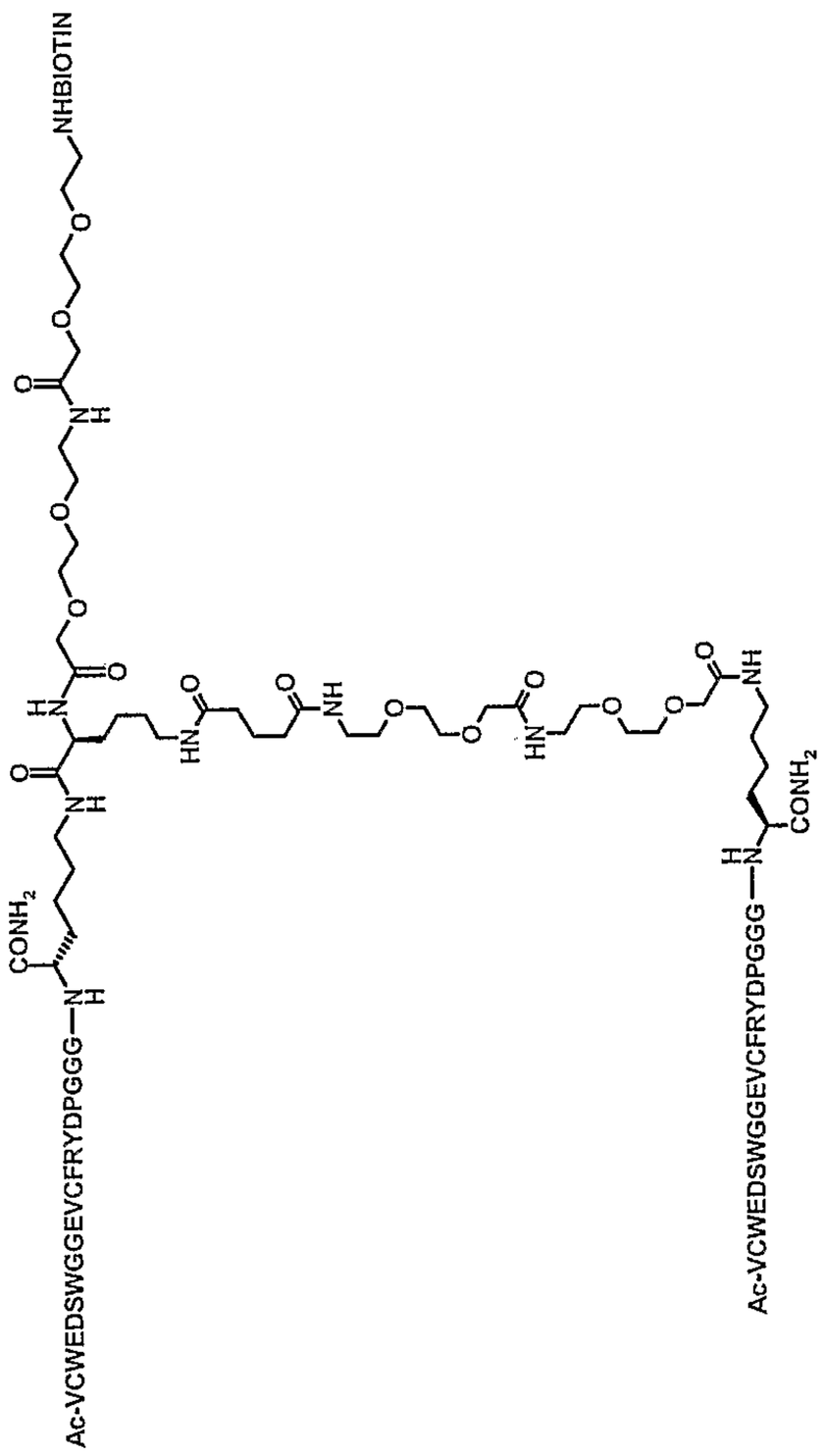

NHBIOTIN
Ac-VCWEDSWGGEVCFRYDPGGG
Ac-VCWEDSWGGEVCFRYDPGGG
CONH2
CONH2

FIG. 39

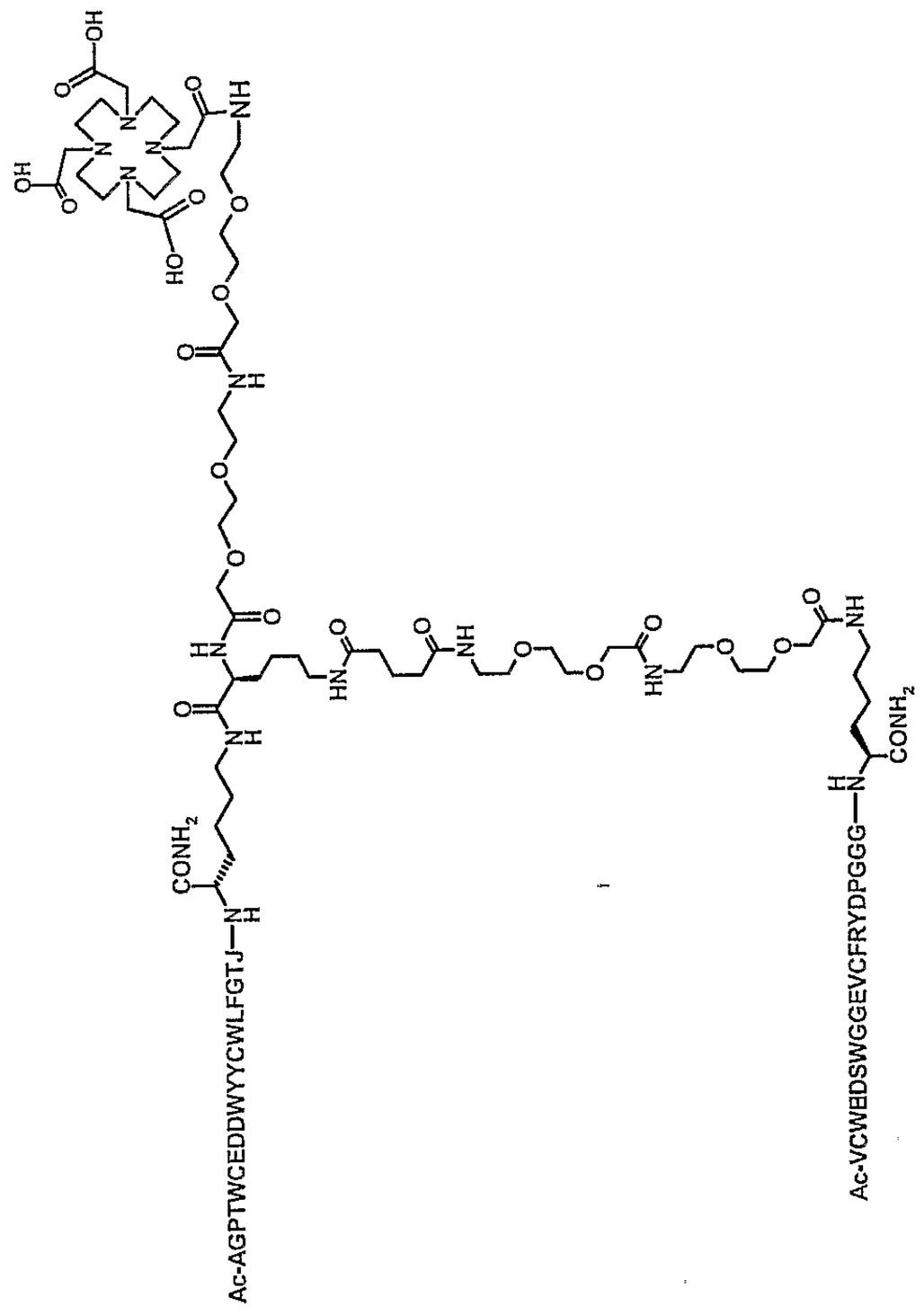

Ac-AGPTWCEDDWYYCWLFGTJ
Ac-VCWEDSWGGEVCFRYDPGGG
CONH2
CONH2

FIG. 40

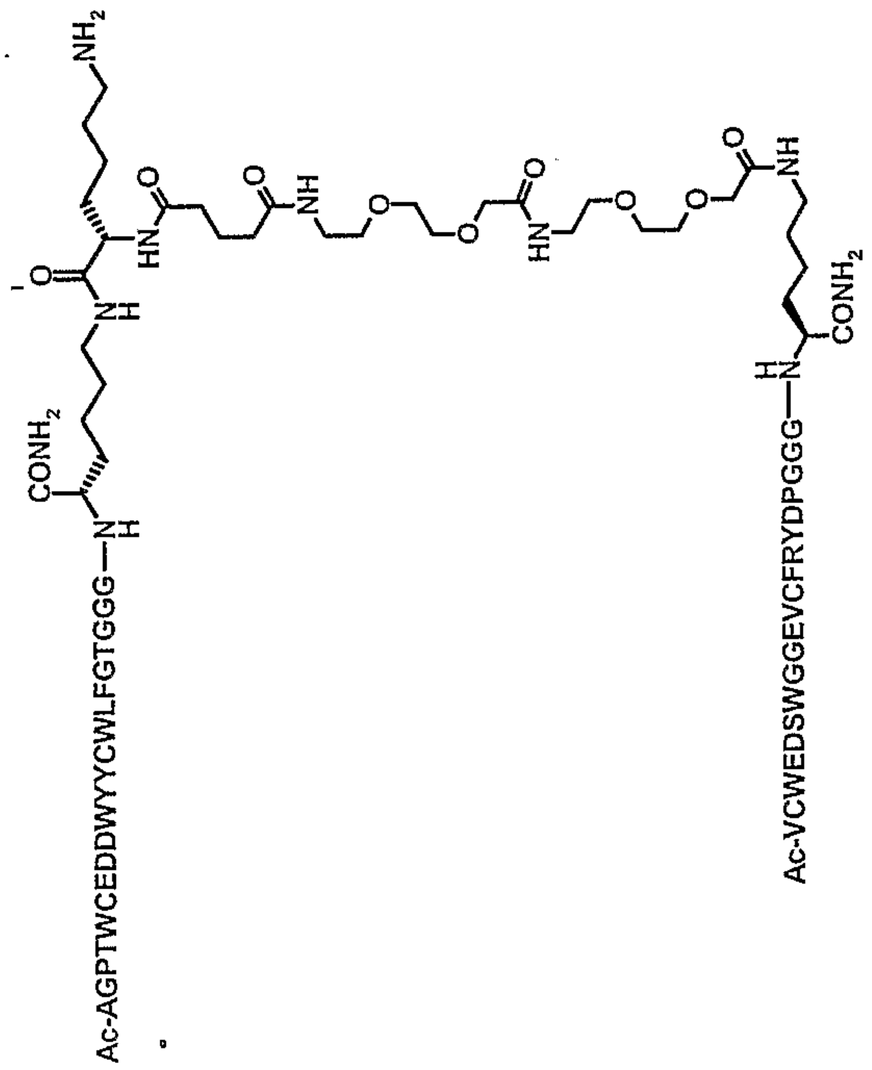
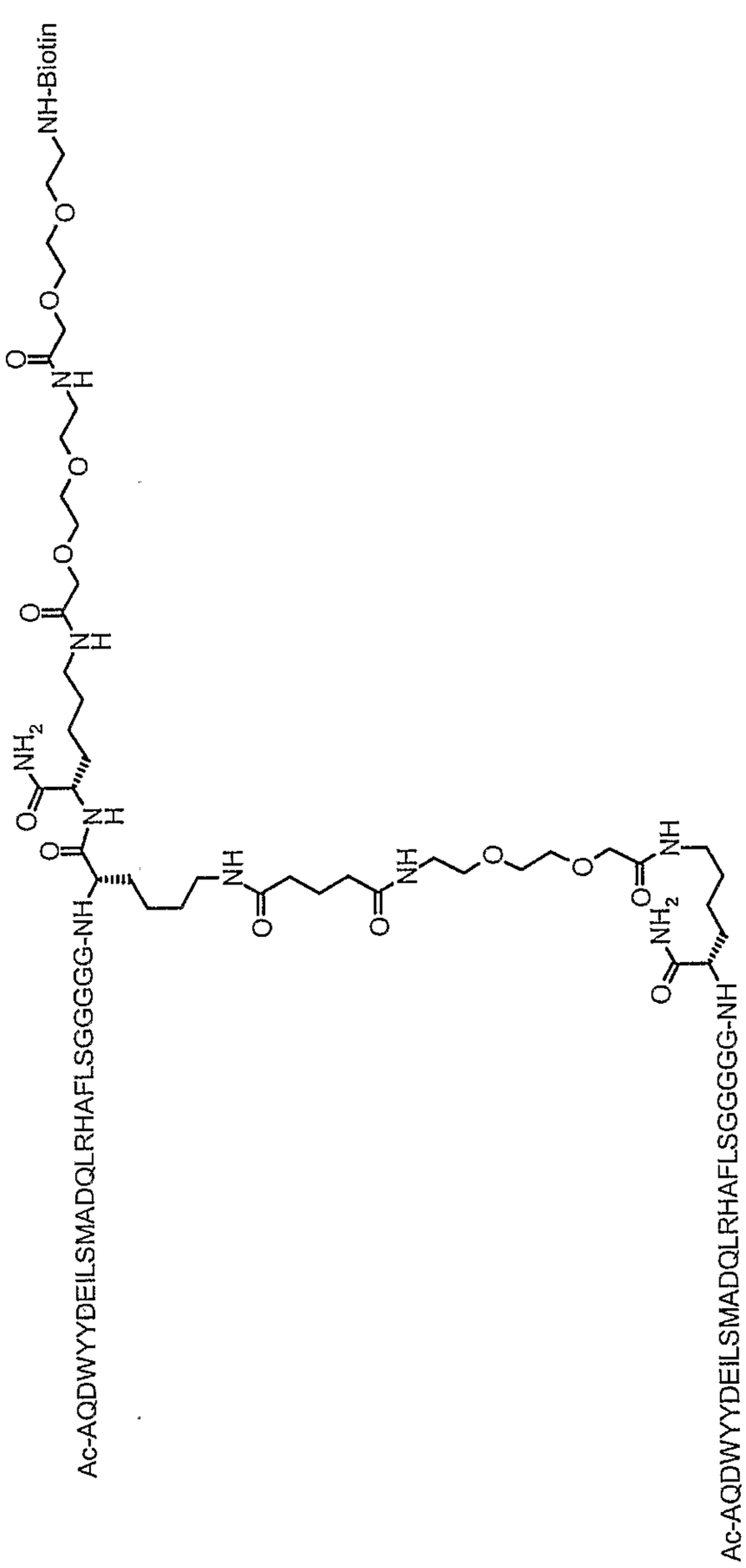


FIG. 41


Ac-AQDWYYDEILSMADQLRHAFLSGGGGG-NH
NH-Biotin
Ac-AQDWYYDEILSMADQLRHAFLSGGGGG-NH

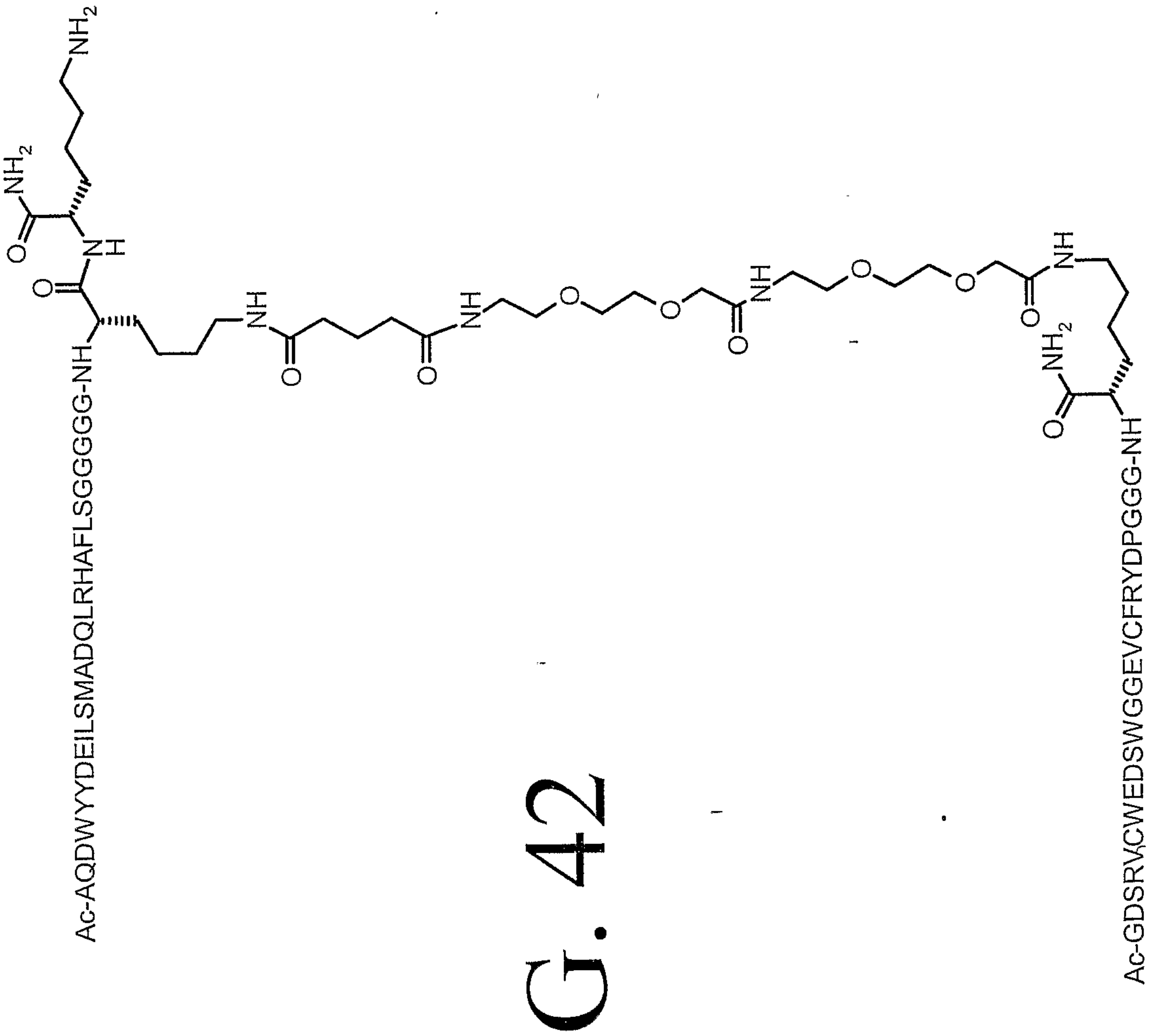

Ac-AQDWYYDEILSMADQLRHAFLSGGGG-NH
NH2
O
NH
O
NH2
NH
O
O
NH
O
O
NH
O
O
NH
O
O
NH
O
O
NH2
Ac-GDSRVCWEDSWGGEVCFRYDPGGG-NH

FIG. 42

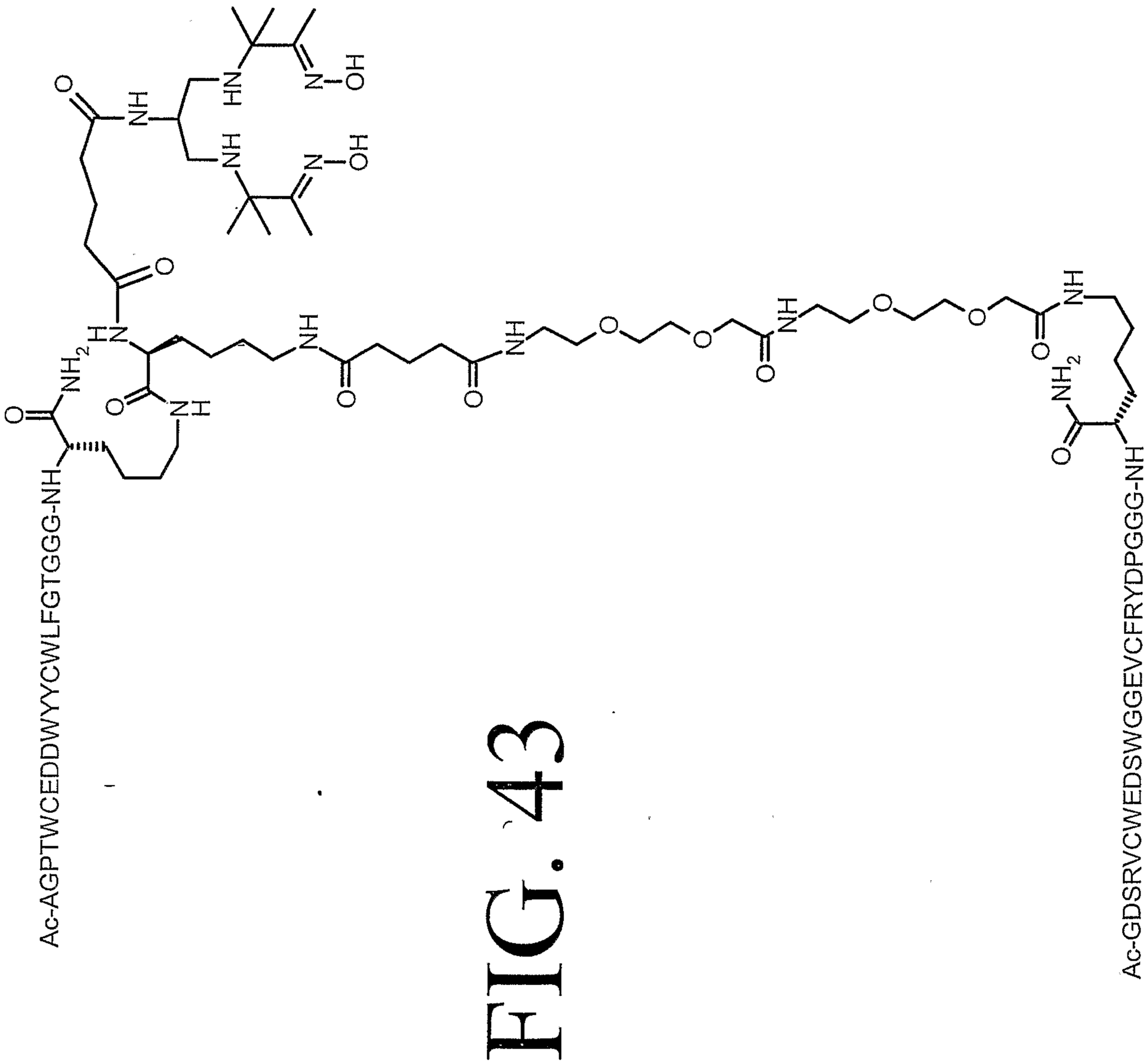

Ac-AGPTWCEDDWYYCWLFGTGGG-NH

FIG. 43

Ac-GDSRVCWEDSWGGEVCFRYDPGGG-N

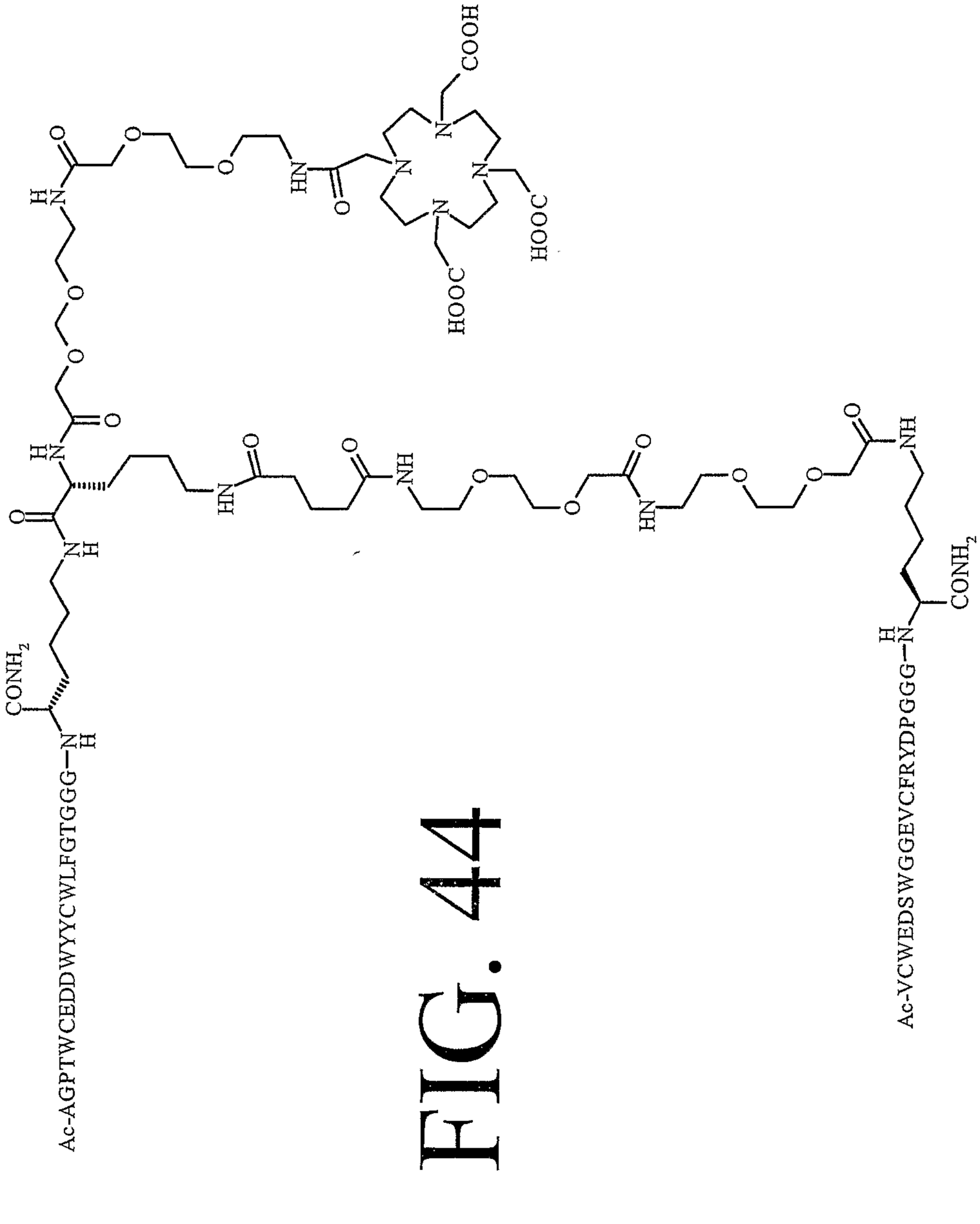

Ac-AGPTWCEDDWYYCWLFGTGGG—
CONH2
HOOC
COOH
Ac-VCWEDSWGGEVCFRYDPGGG—
CONH2

FIG. 44

FIG. 45

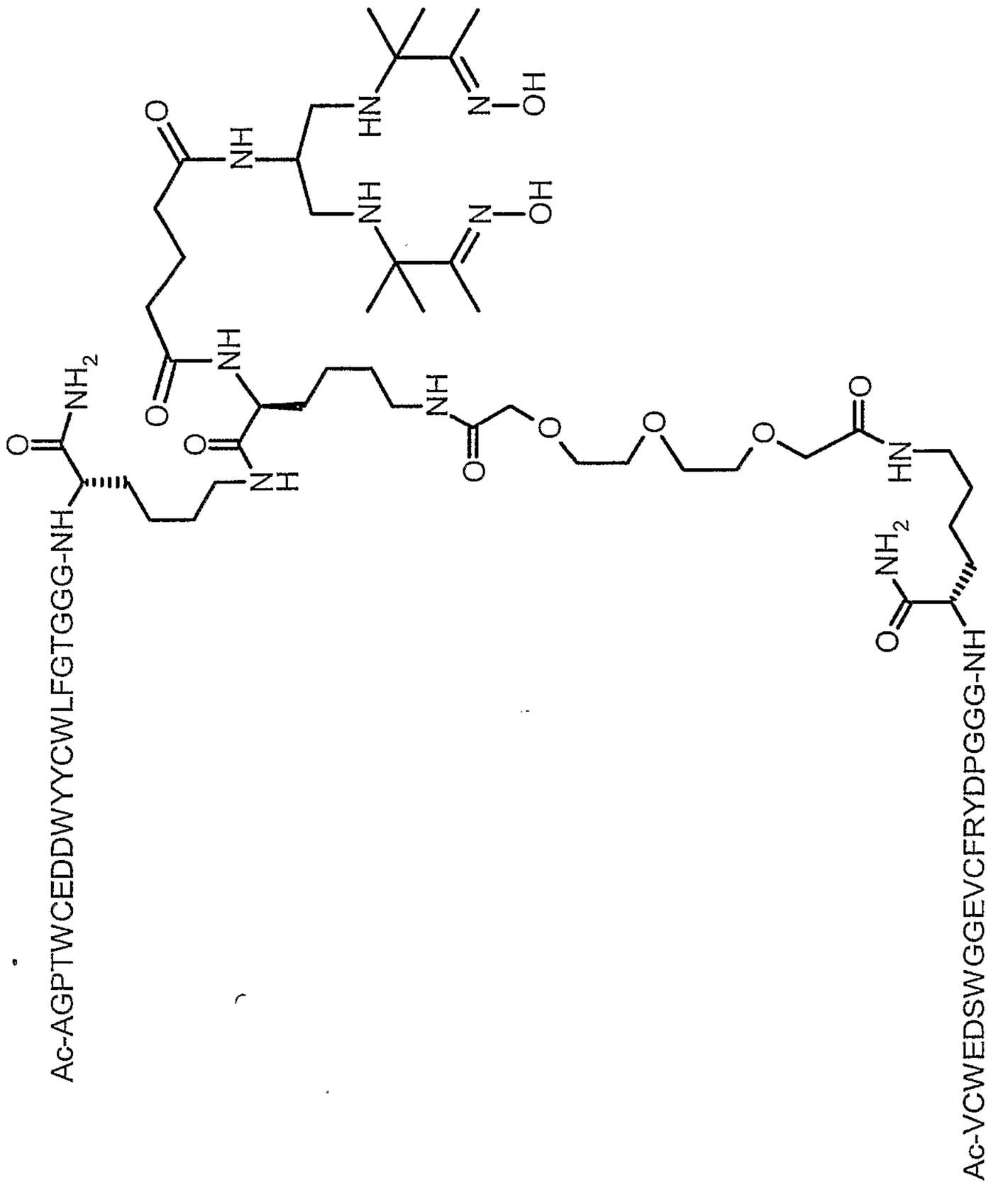
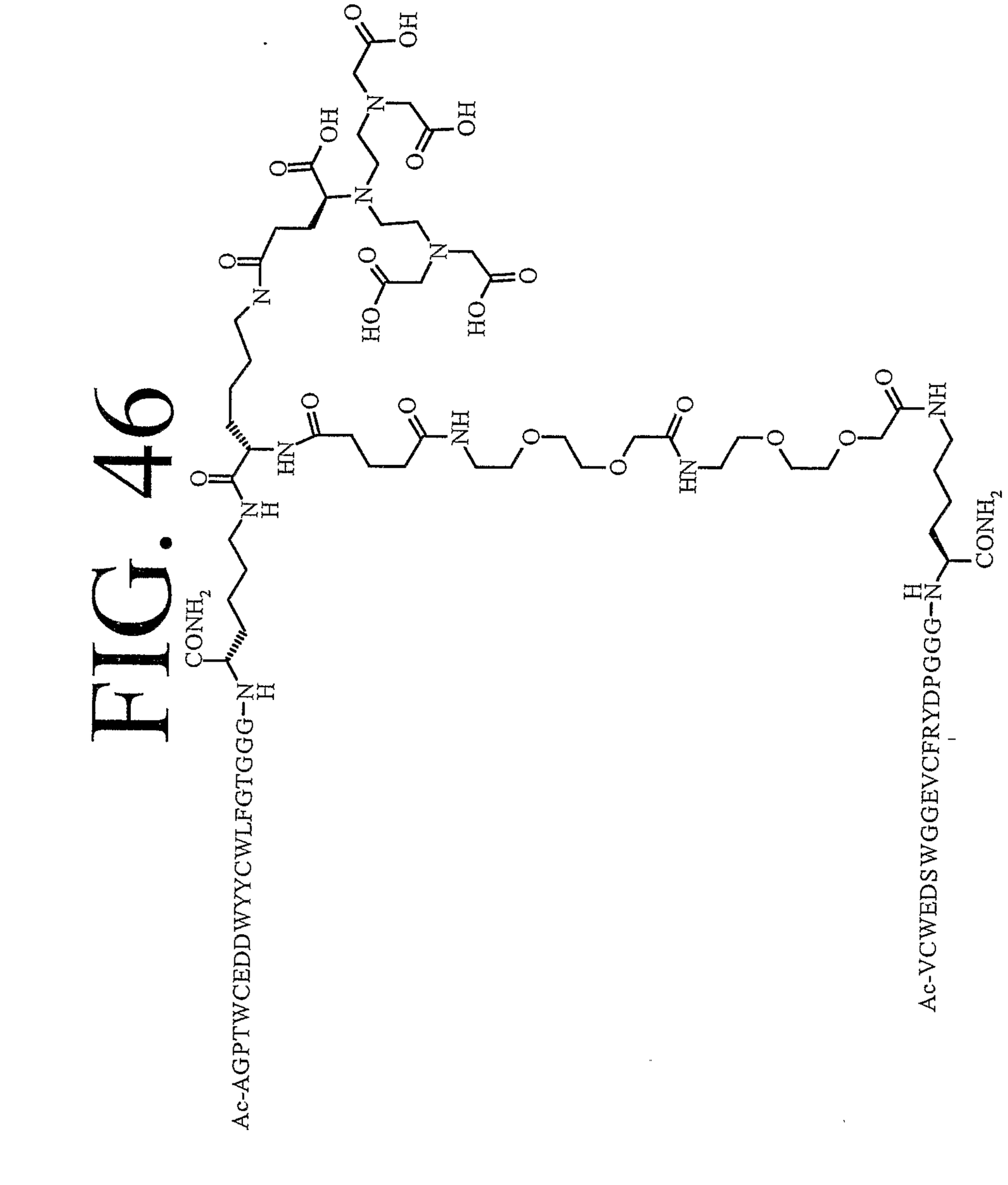
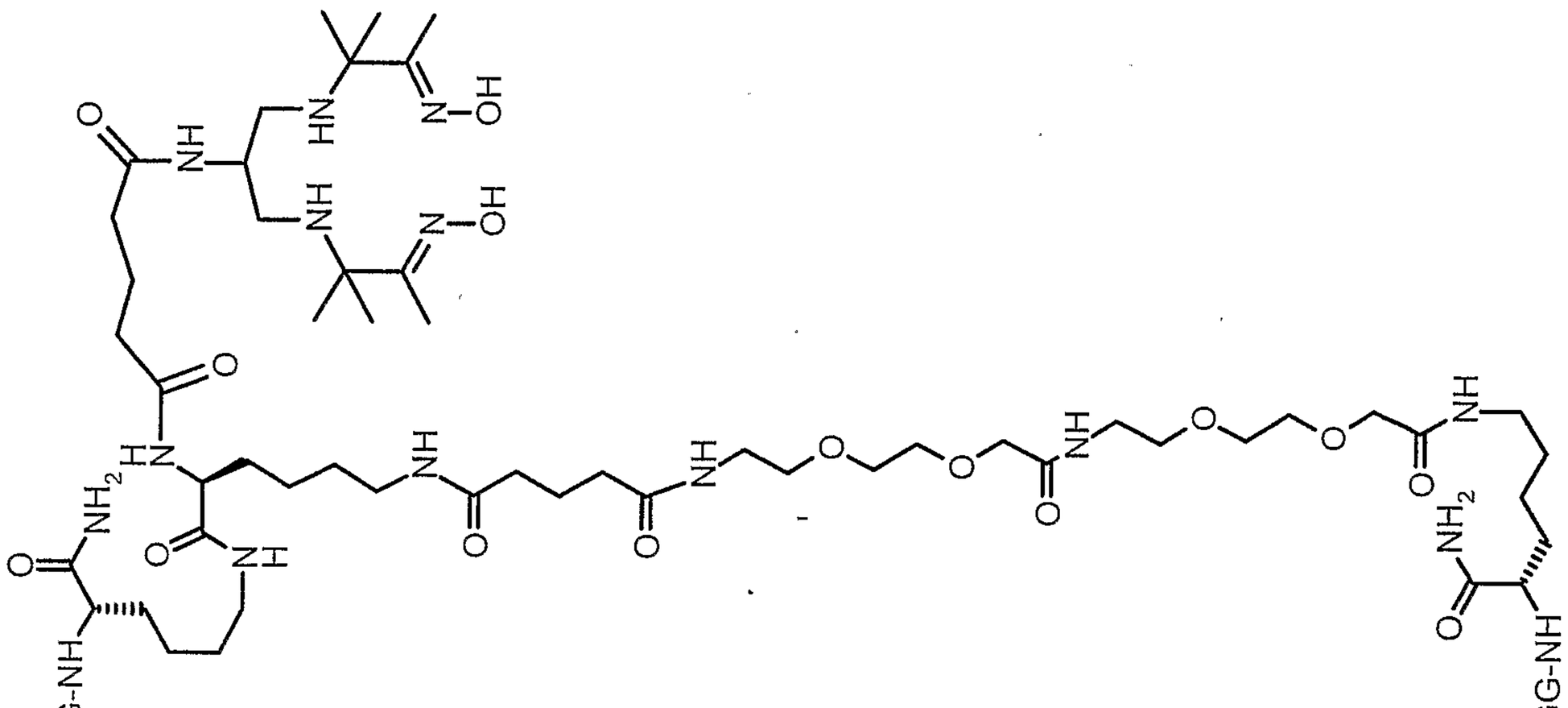

Ac-AGPTWCEDDWYYCWLFGTGGG-NH2
Ac-VCWEDSWGGEVCFRYDPGGG-NH2

FIG. 46


Ac-AGPTWCEDDWYYCWLFGTGGG-N
H
CONH2
HN
O
NH
HO
OH
N
Ac-VCWEDSWGGEVCFRYDPGGG-N
H
CONH2
NH2



Ac-AQDWYYDEILSMADQLRHAFLSGGGG-NH-CH(CH2CH2CH2CH2-NH-C(=O)CH2CH2C(=O)-NH-CH2CH2-O-CH2CH2-O-CH2-C(=O)-NH-CH2CH2-O-CH2CH2-O-CH2-C(=O)-NH-CH(CH2CH2CH2CH2-NH-C(=O)CH2CH2CH2C(=O)-NH-CH(C(=O)-NH-CH(CH2CH2CH2-NH-C(=NH)-NH2)-C(=O)-NH-CH(CH2CH2CH2CH2-NH-C(=O)CH2CH2CH2-N(CH2-CH(NH-C(CH3)2-C(=NOH)-CH3)-NH-C(CH3)2-C(=NOH)-CH3)-C(=O)CH2CH2CH2C(=O))-NH2)-C(=O)-NH-CH(CH2CH2CH2-NH-C(=NH)-NH2)-C(=O)-NH-CH(CH2CH2CH2CH2-NH-C(=O)CH2CH2CH2C(=O))-NH2)-C(=O)-GDSRVCWEDSWGGEVCFRYDPGG-NH2

FIG. 47

Ac-AGPTWCEDDWYYCWLFGTGGG-NH

FIG. 48

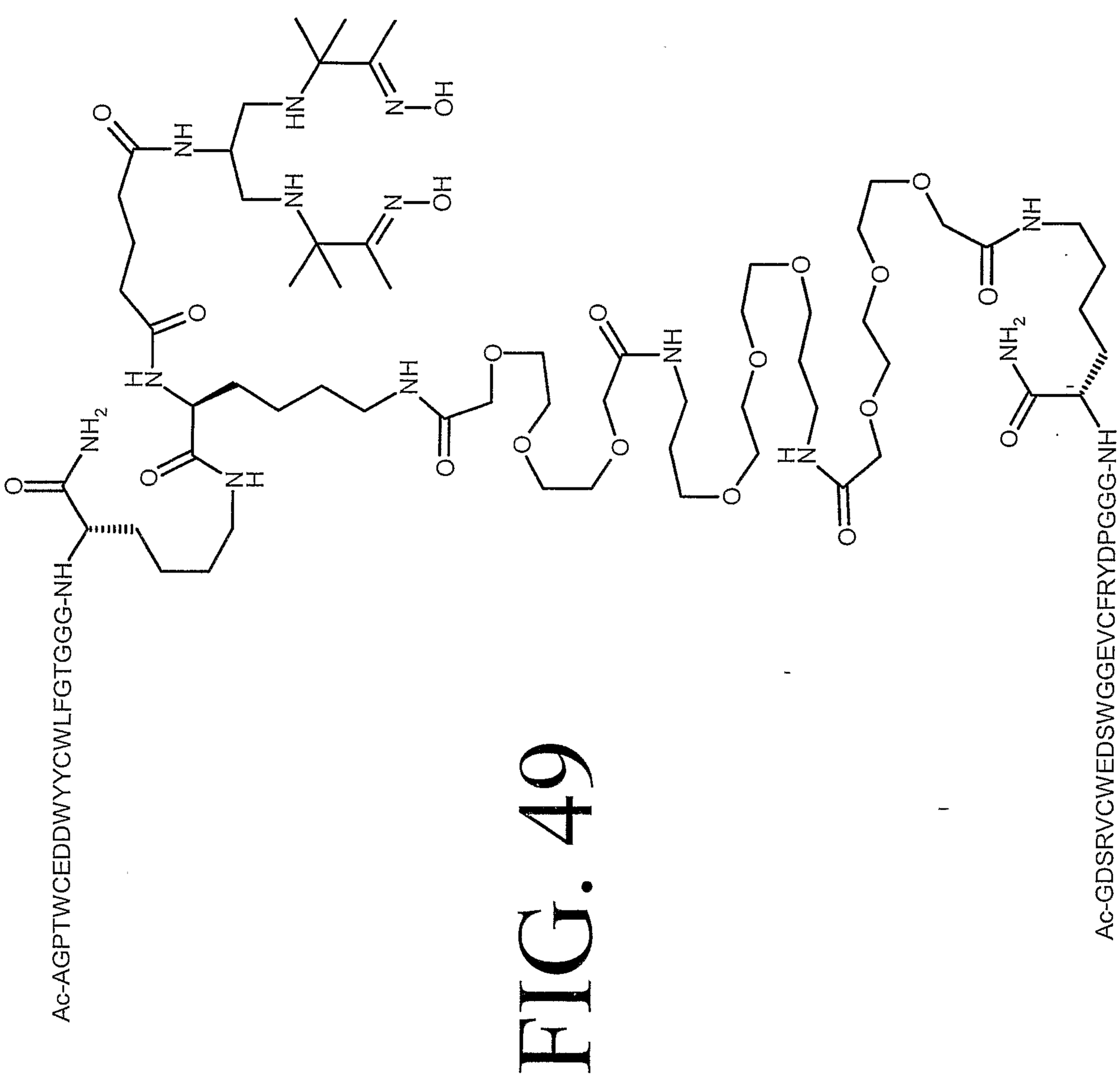

Ac-AGPTWCEDDWYYCWLFGTGGG-NH
Ac-GDSRVCWEDSWGGEVCF
RYDPGGG-NH

FIG. 49

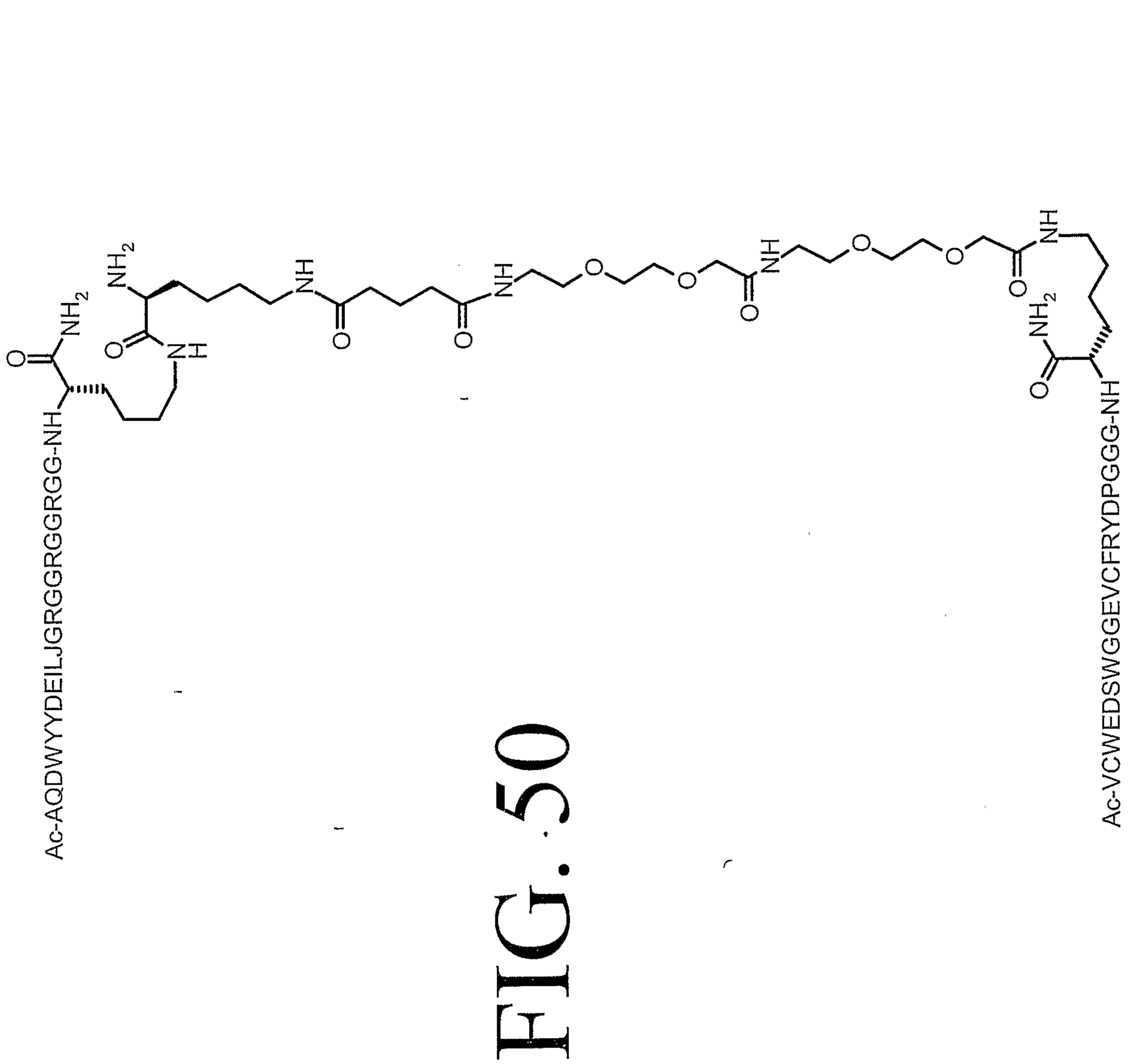
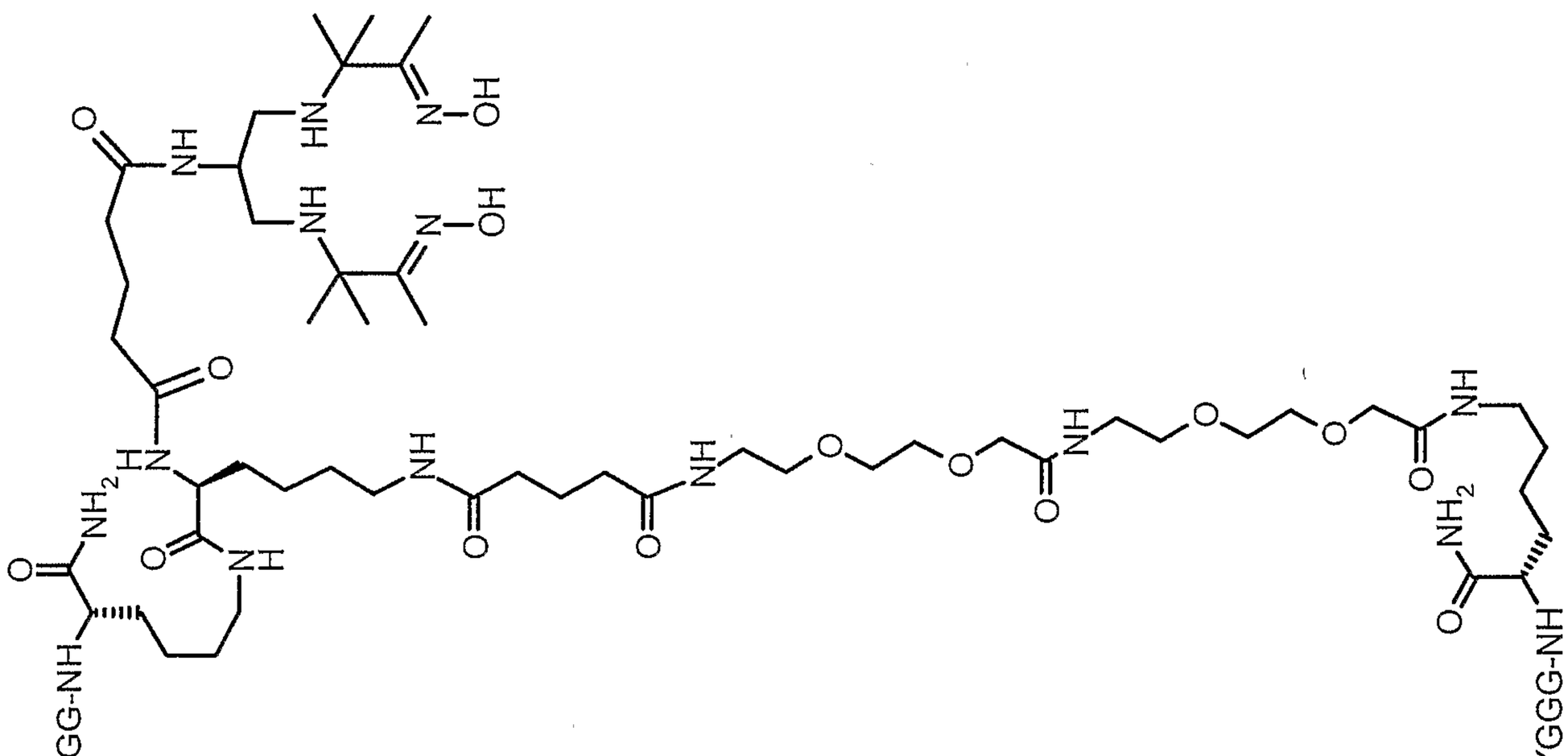

Ac-AQDWYYDEILJGRGGRGG-NH
NH2
O
NH
NH2
O
NH
O
NH
O
NH
O
O
O
NH
O
O
O
NH
O
NH2
O
NH
RYDPGGG-NH
Ac-VCWEDSWGGEVC

FIG. 50



Ac-APGTWCDYDWEYCWLGTFGGG-NH-GVDFRCEWSDWGEVGCRSPDYGGG-NH

FIG. 51

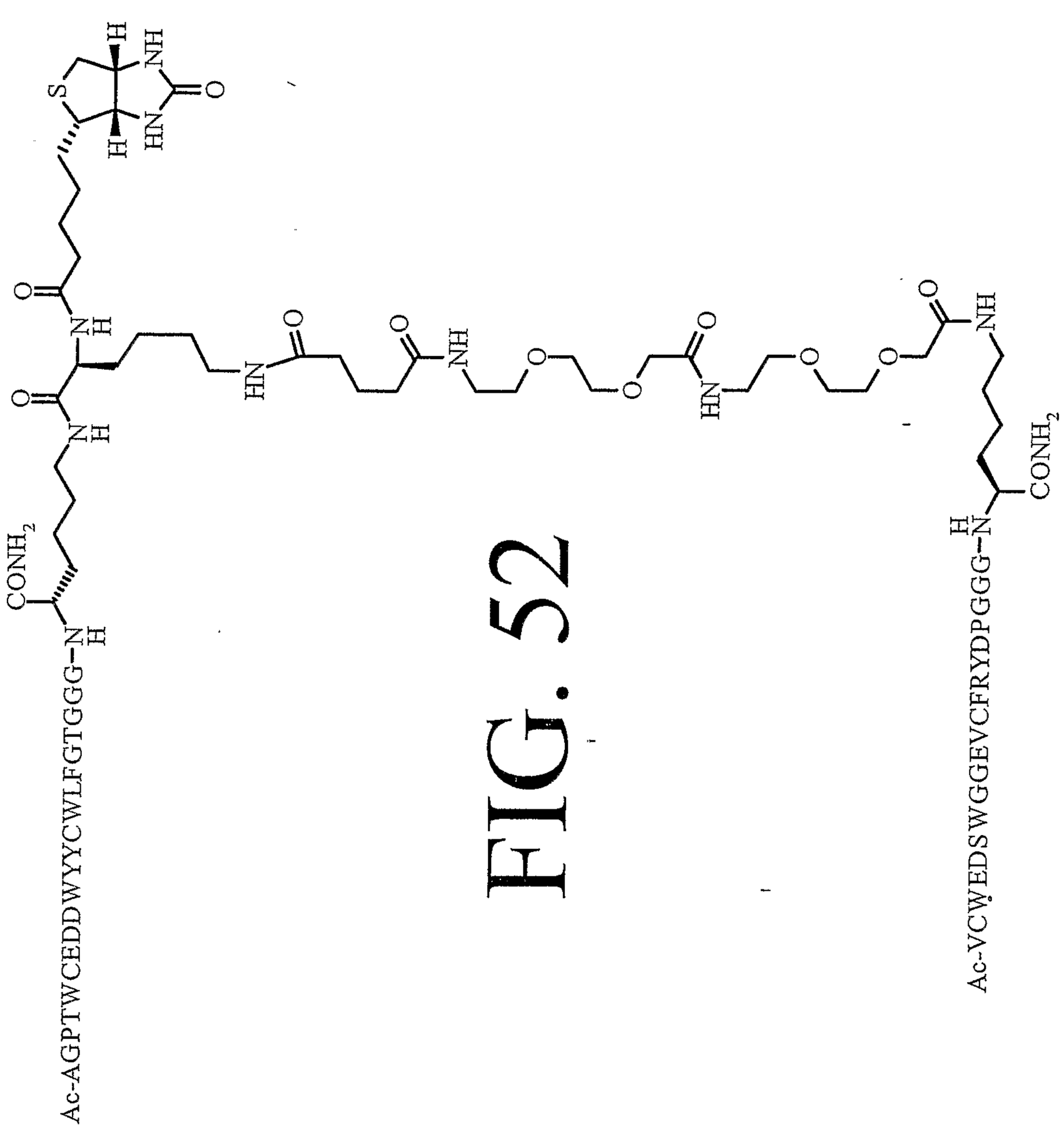

Ac-AGPTWCEDDWYYCWLFGTGGG-N
H
CONH2
O
N
H
O
N
H
HN
O
O
NH
O
O
O
HN
O
O
O
O
NH
H
N
CONH2
Ac-VCWEDSWGGEVCFRYDPGGG-N
H
H
S
NH
O
HN
H
H

FIG. 52

FIG. 53

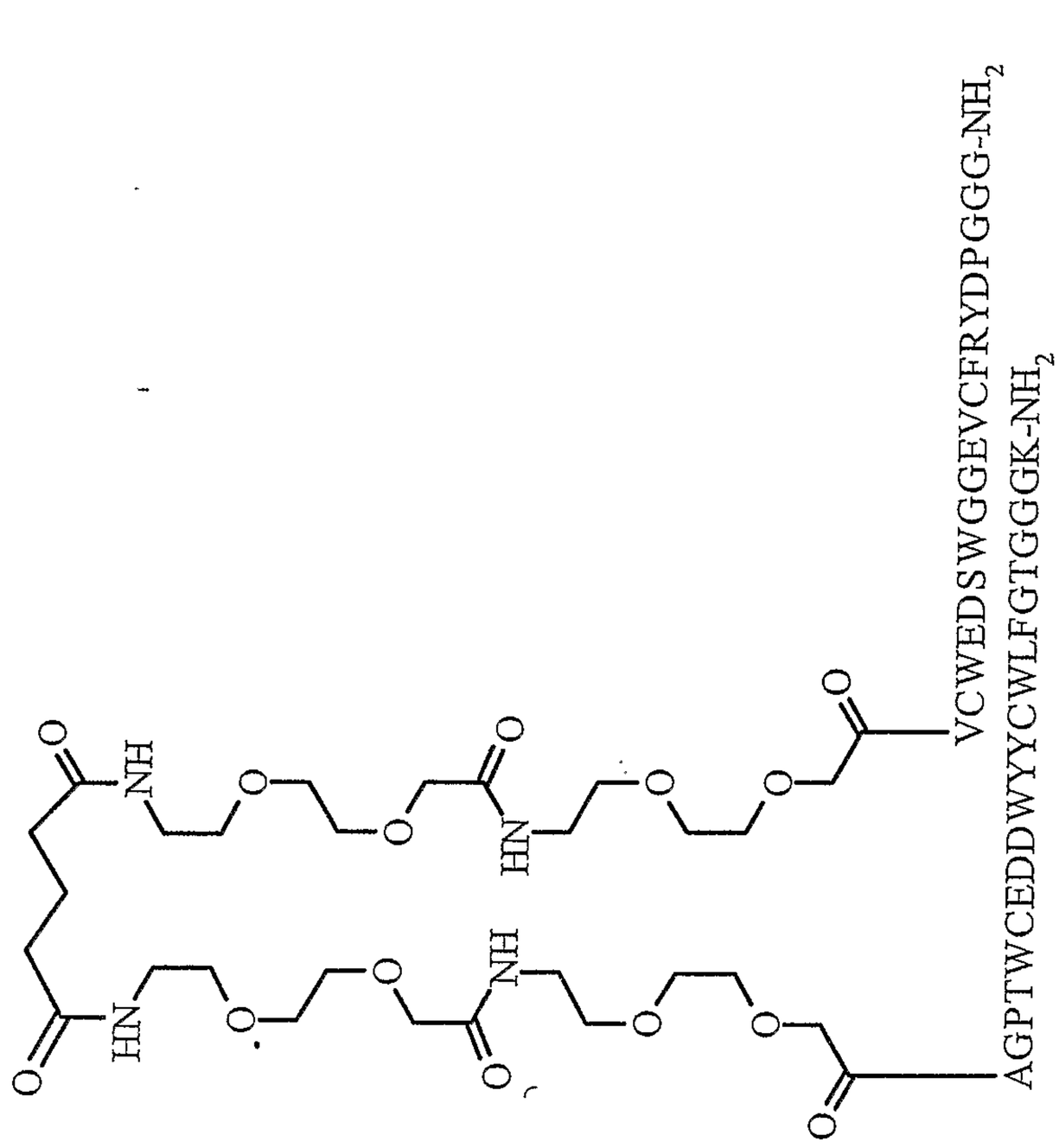


FIG. 54

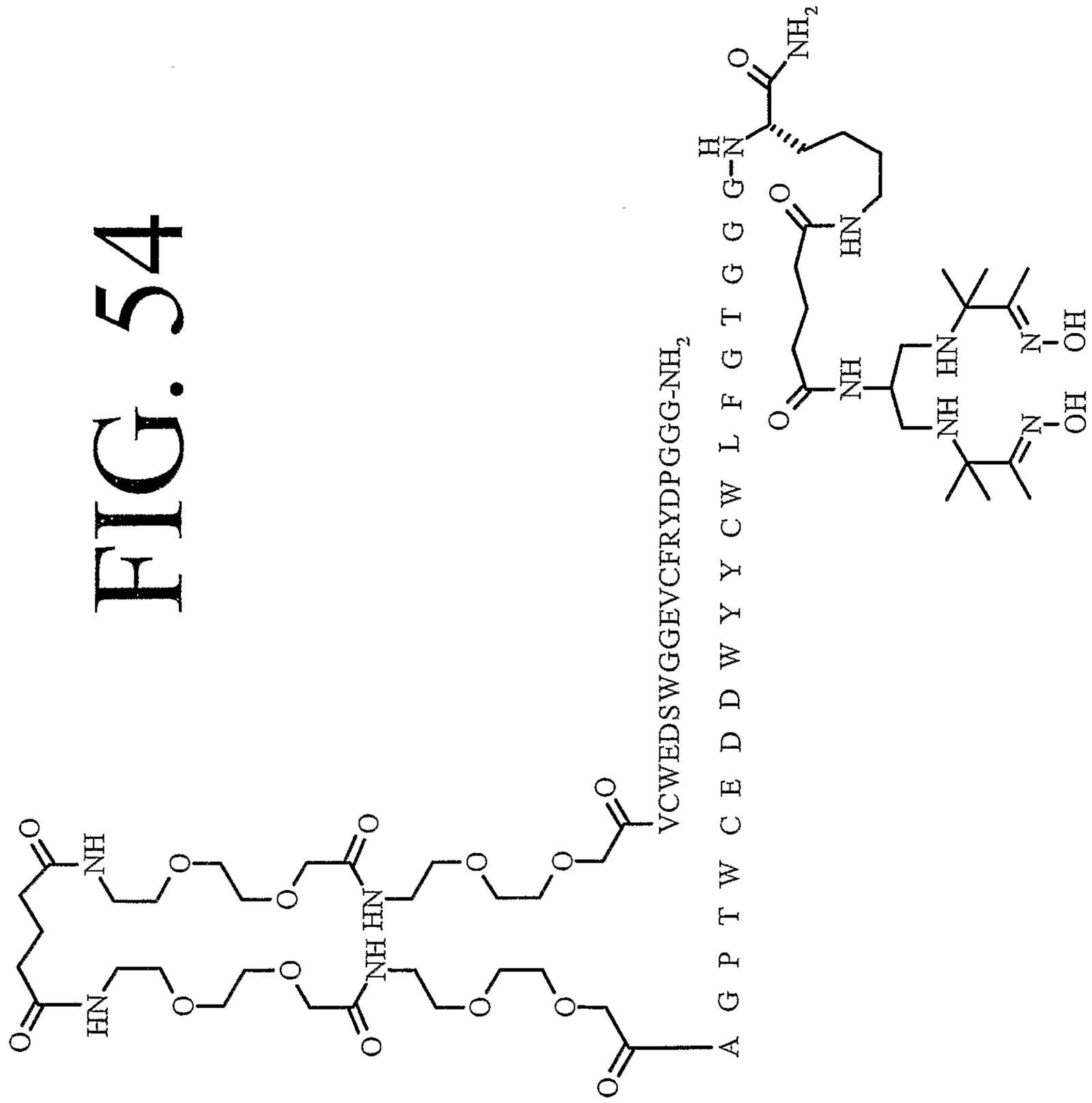
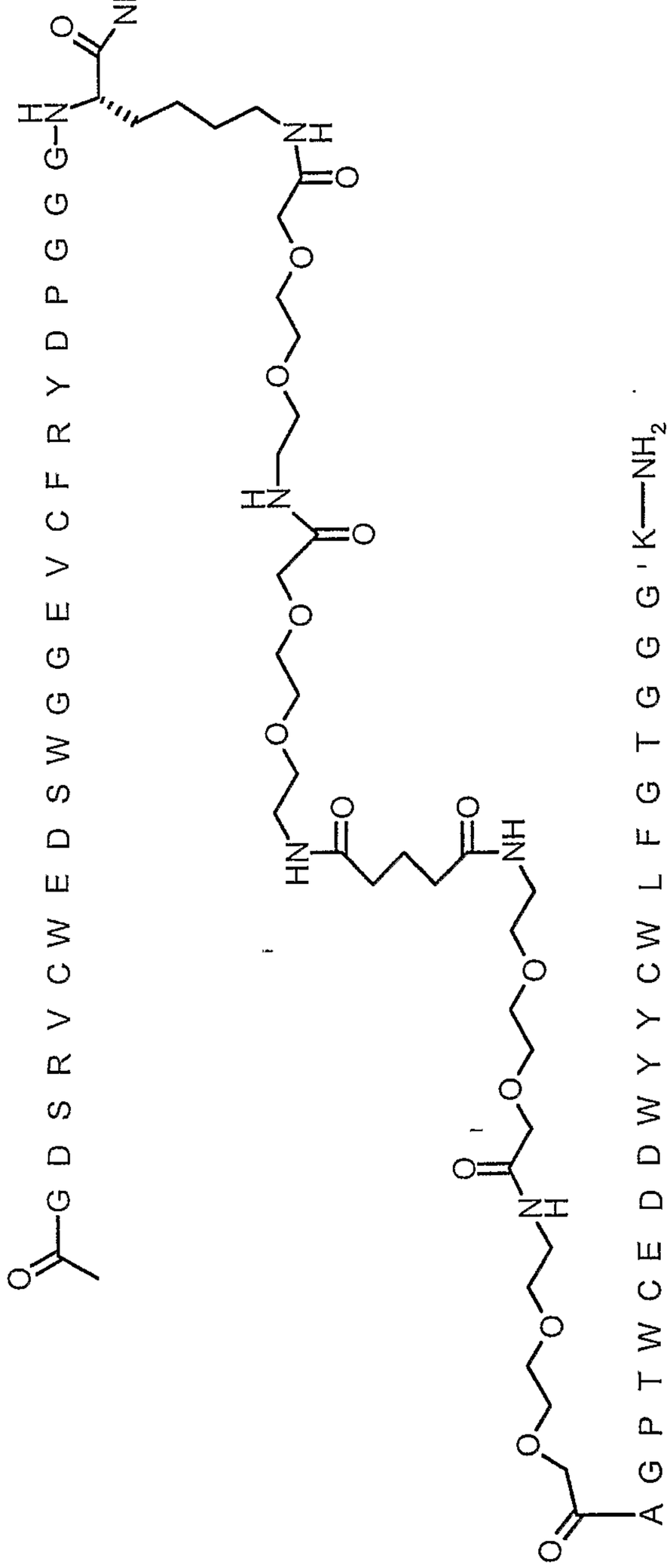

A G P T W C E D D W V C W E D S W G G E V C F R Y D P G G G-NH2
Y Y C W L F G T G G G G
O
NH2
H
N
O
HN
O
NH
O
HN
NH HN
N
OH
N
OH
O
NH
O
O
O
HN
NH HN
O
O
O
O
O
O
O
O
O

FIG. 55


G G E V C F R Y D P G G G G—N
H
O
N
H
O
O
O
O
N
H
O
O
G G G ' K—NH2
G D S R V C W E D S W
O
G A G P T W C E D D W Y Y C W L F G T

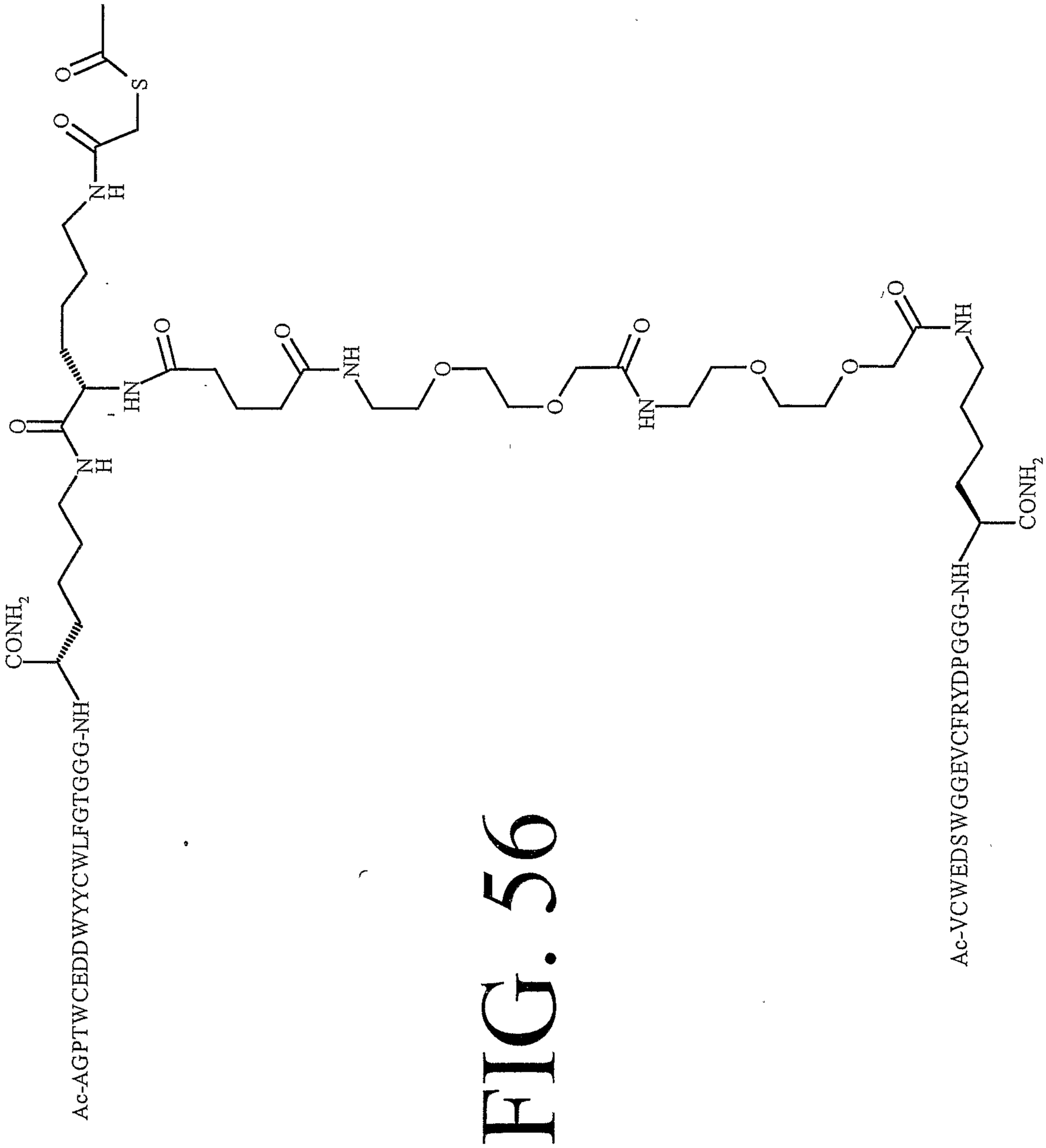

Ac-AGPTWCEDDWYYCWLFGTGGG-NH
CONH2
Ac-VCWEDSWGGEVCFRYDPGGG-NH
CONH2

FIG. 56

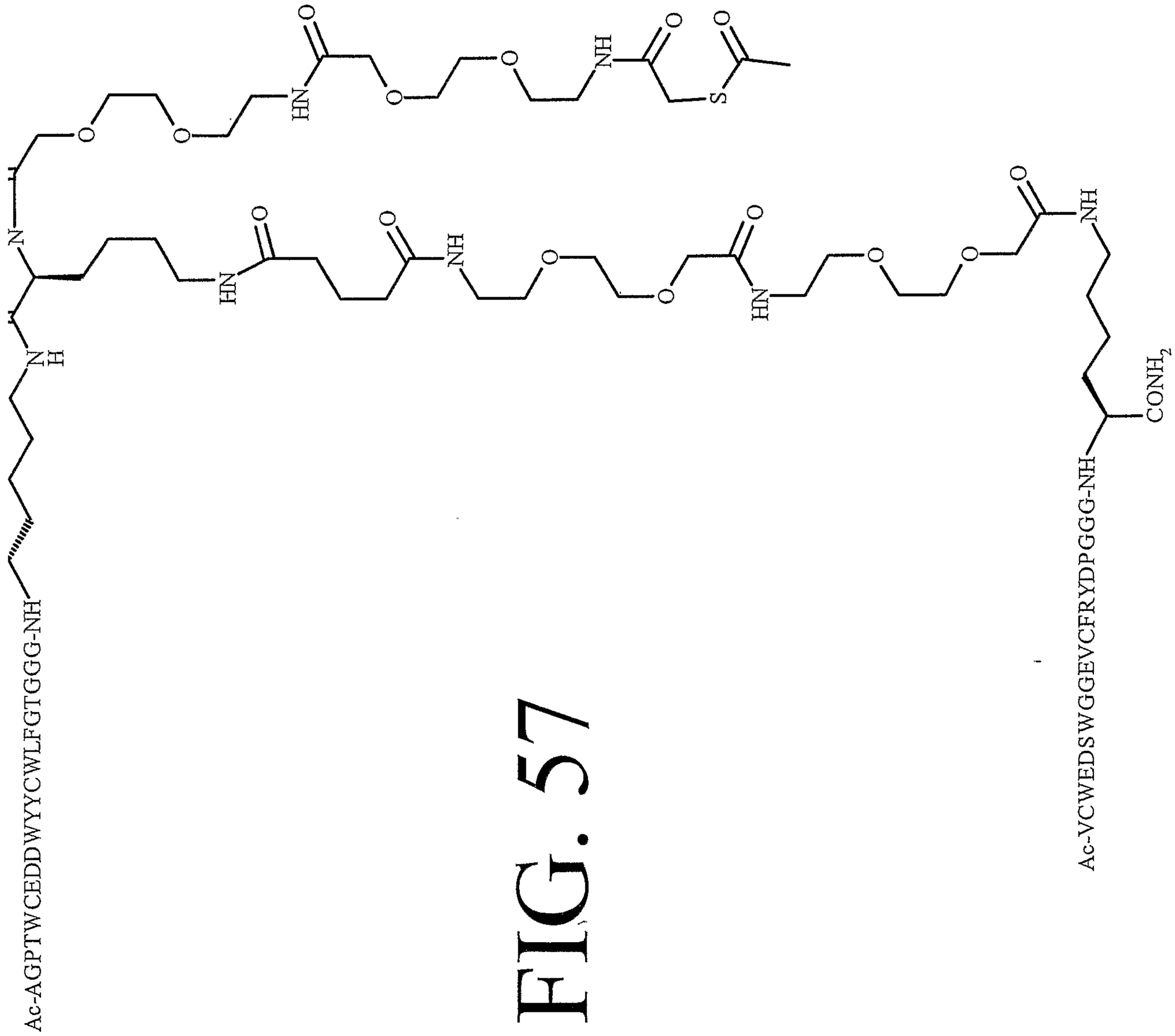

Ac-AGPTWCEDDWYYCWLFGTGGG-NH
Ac-VCWEDSWGGEVCFRYDPGGG-NH
CONH2

FIG. 57

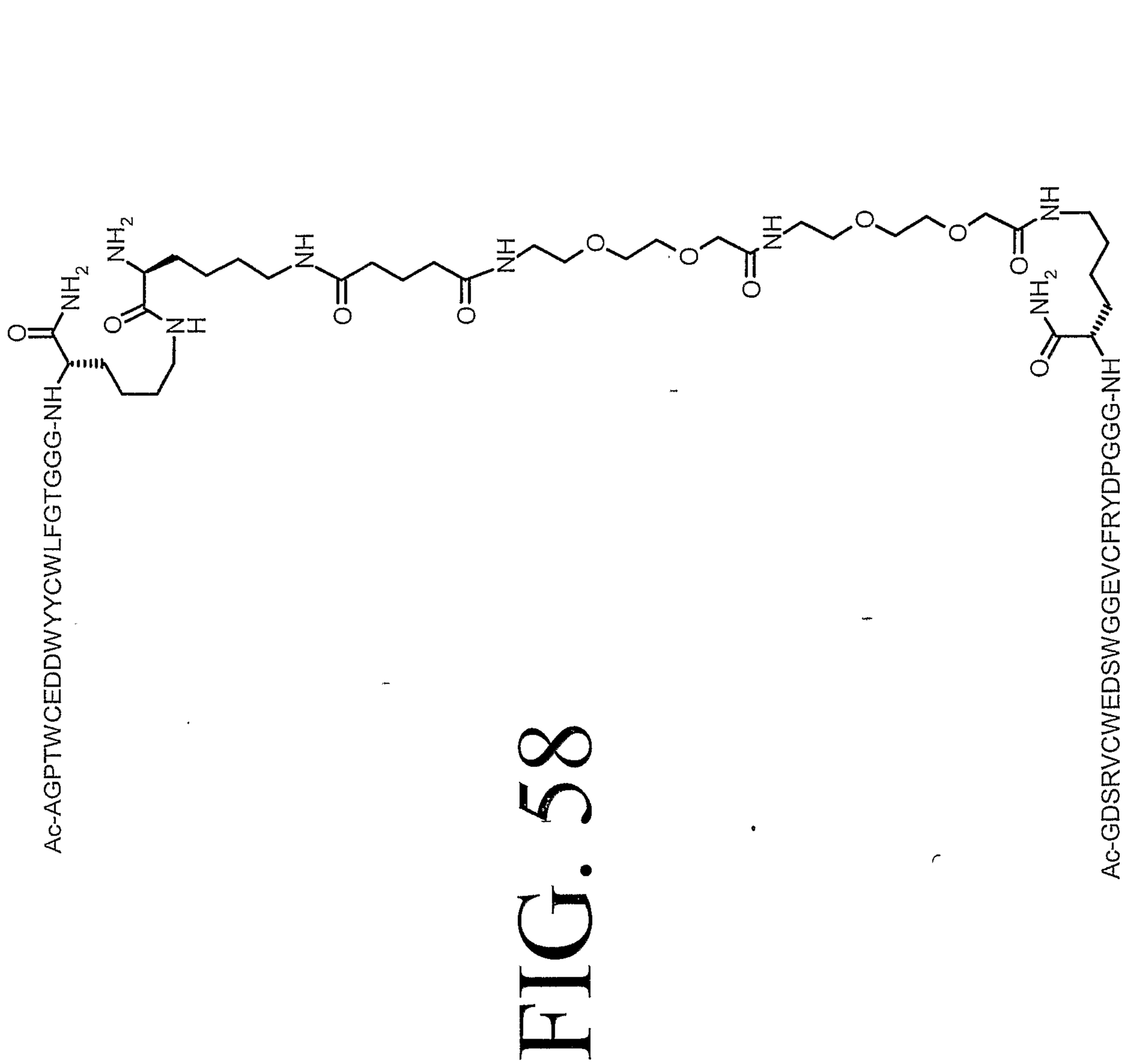

Ac-AGPTWCEDDWYYCWLFGTGGG-NH
NH2
O
NH
NH2
O
NH
O
NH
O
NH
O
O
O
NH
O
O
O
NH
O
NH2
O
NH
GEVCFRYDPGGG-NH
Ac-GDSRVCWEDSWGGE

FIG. 58

FIG. 59

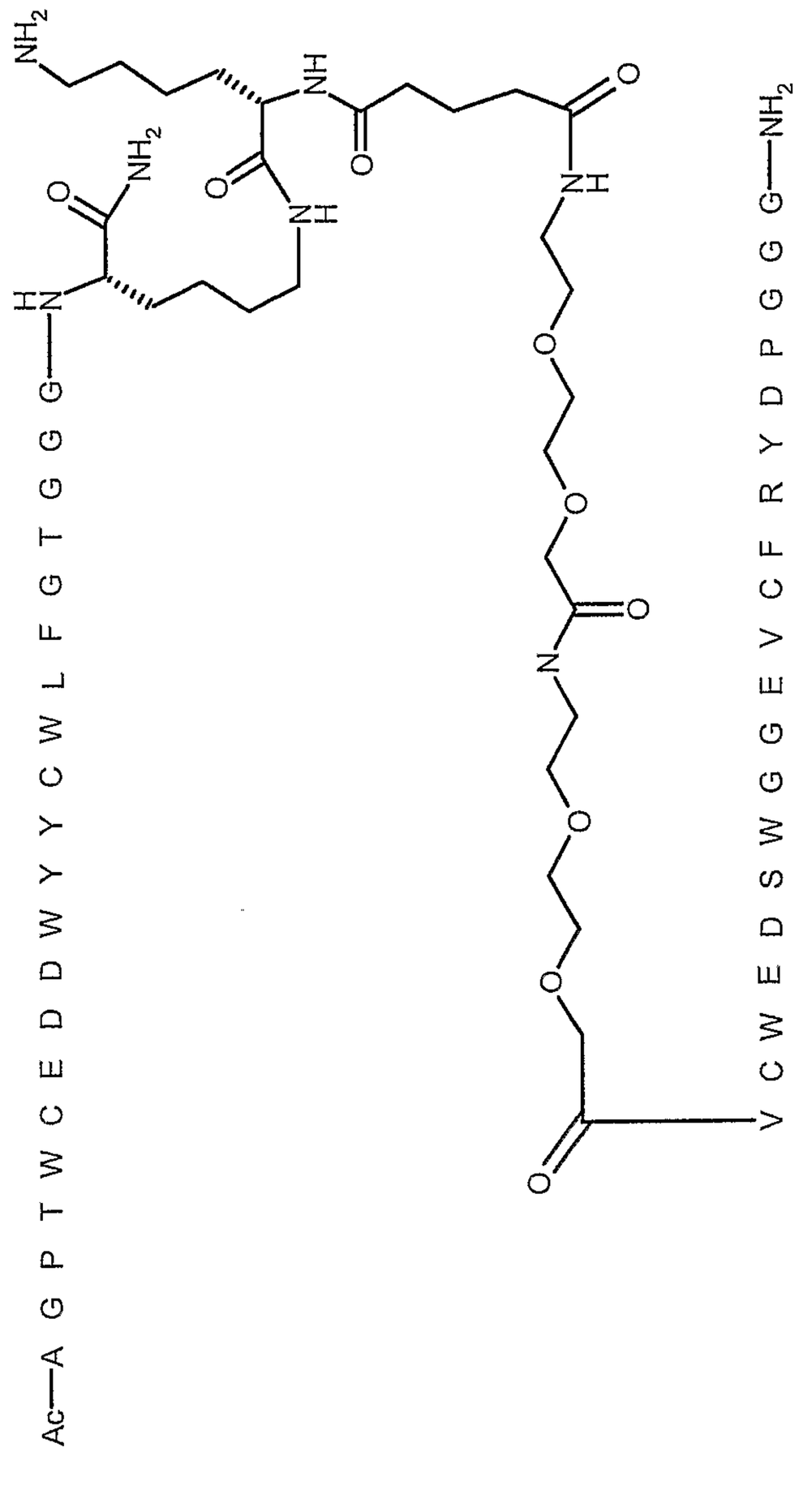

Ac—A G P T W C E D D W Y Y C W L F G T G G G G—NH2
V C F R Y D P G G G G—NH2
V C W E D S W G G E

FIG. 60

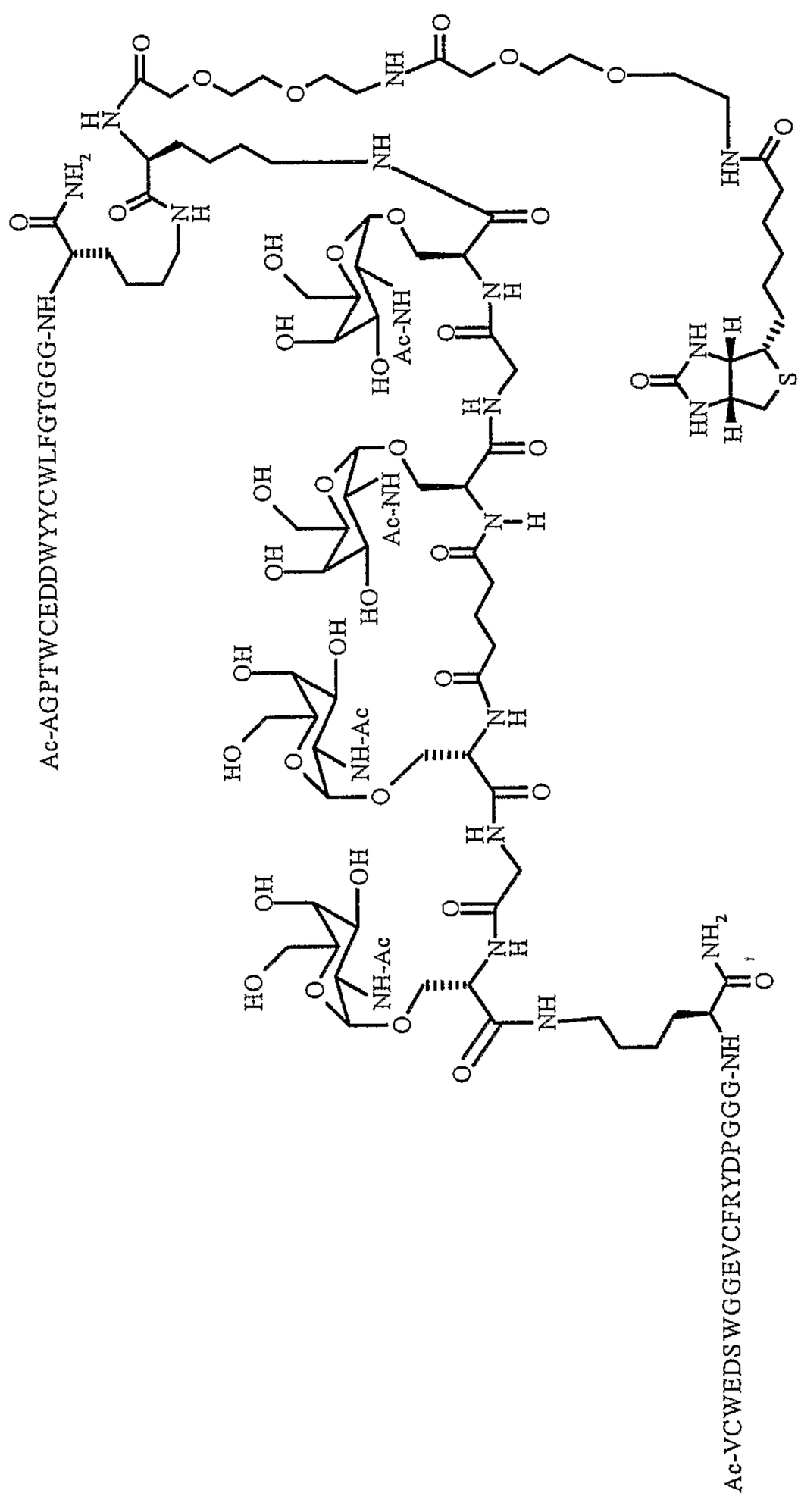

Ac-AGPTWCEDDWYYCWLFGTGGG-NH
Ac-NH
NH-Ac
Ac-CWEDSWGGEVCFRYDPGGG-NH

FIG. 61

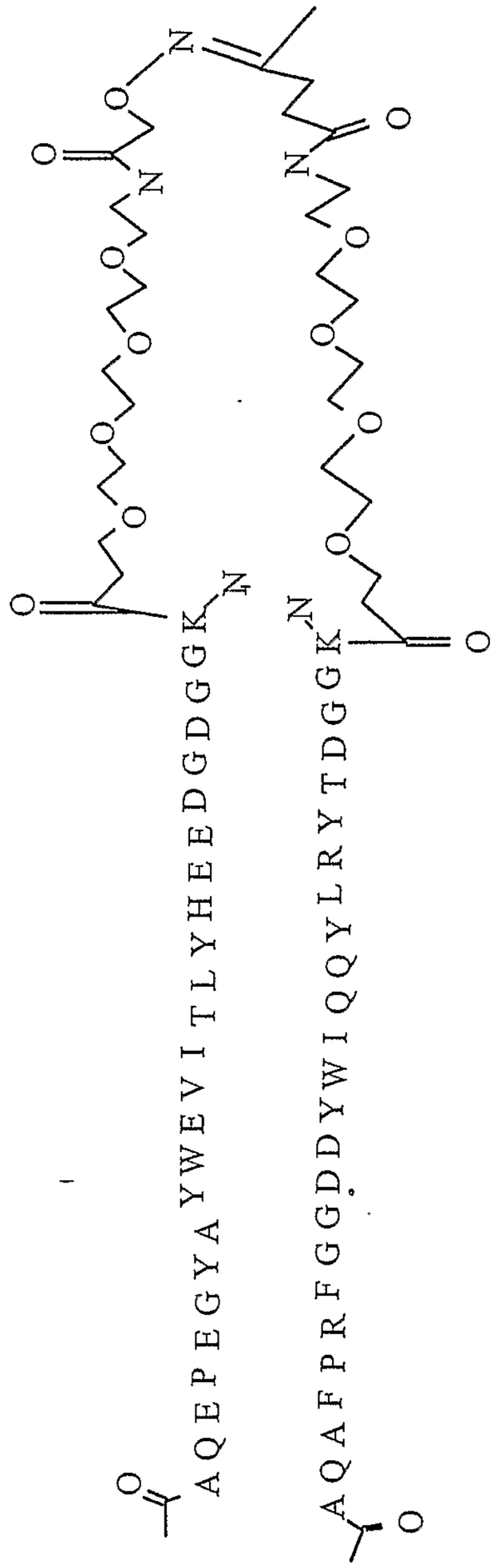
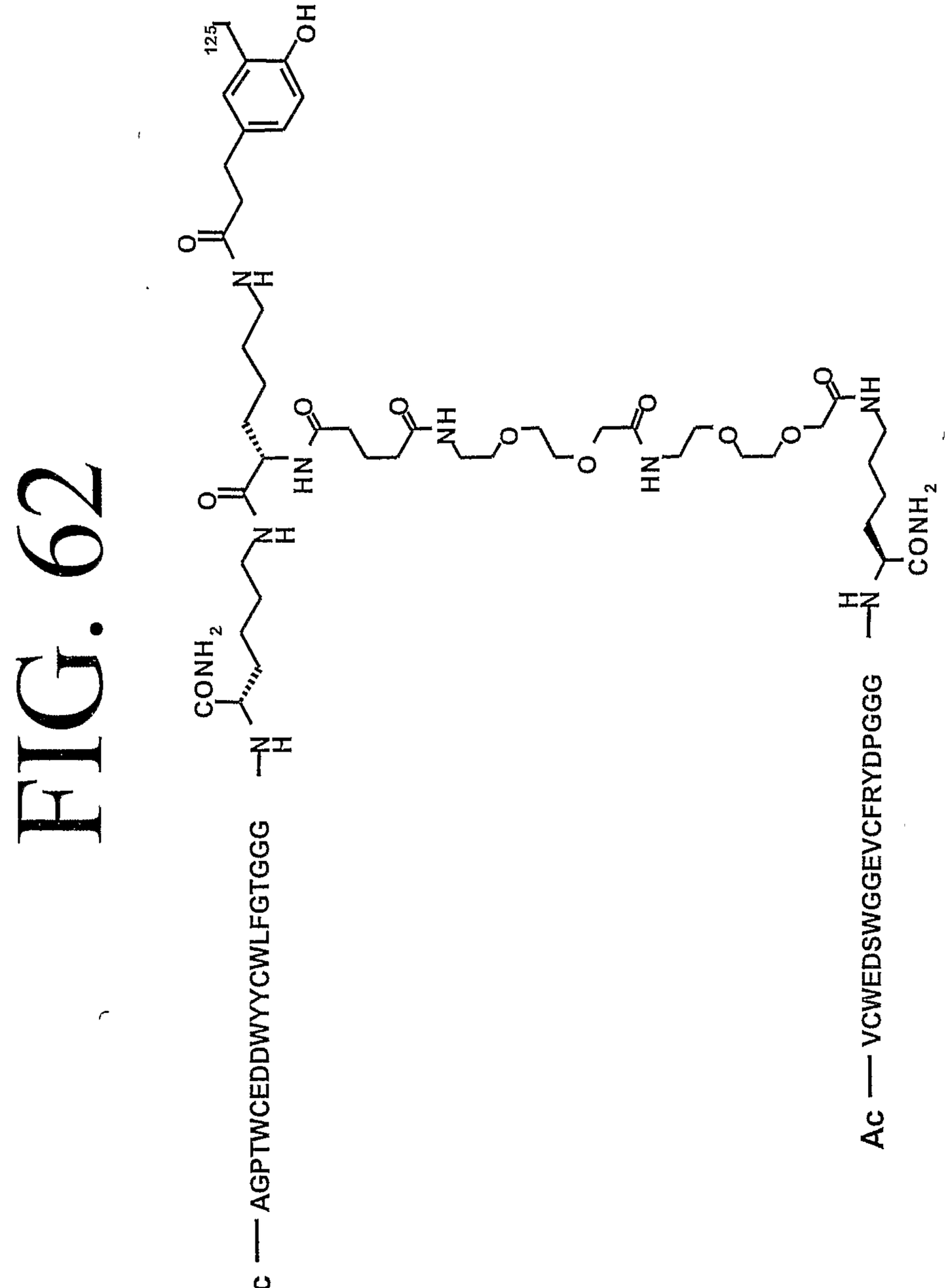
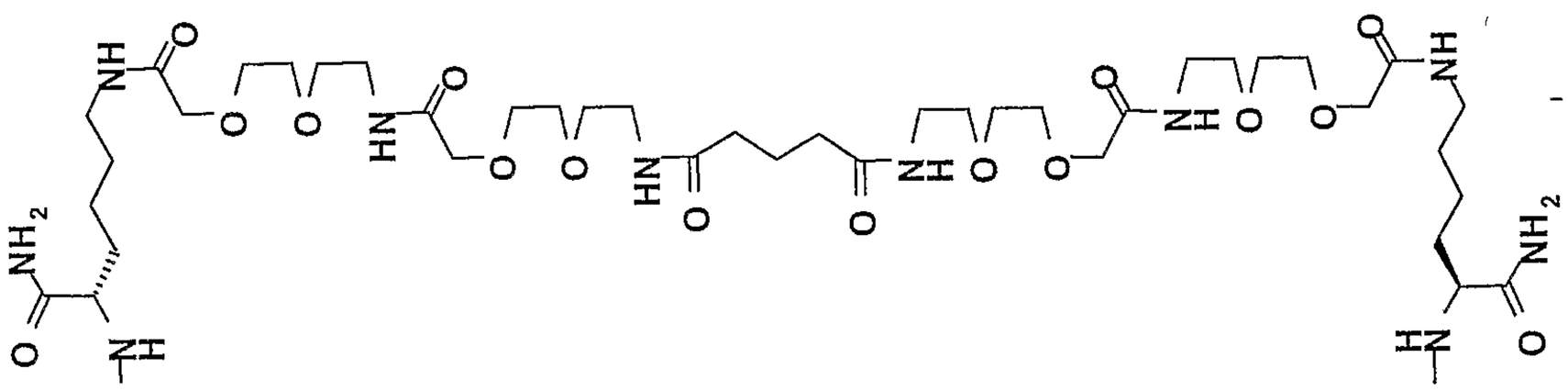

AQEPEGYAYWEVITLYHENRYTDGGKN
AQAFPRFGGDDYWIQQYLNEEDGDGGKN

FIG. 62


Ac — VCWEDSWGGEVCFRYDPAGPTWCEDDWYYCWLFGTGGG —
CONH2
OH
125
O
NH
HN
O
NH
O
NH
O
HN
O
NH
O
NH
CONH2
GGG



Ac-GDSRVCWEDSWGGEVCFRYDPGGG

Ac-AGPTWCEDDWYYCWLFGTGGG

FIG. 63

FIG. 64

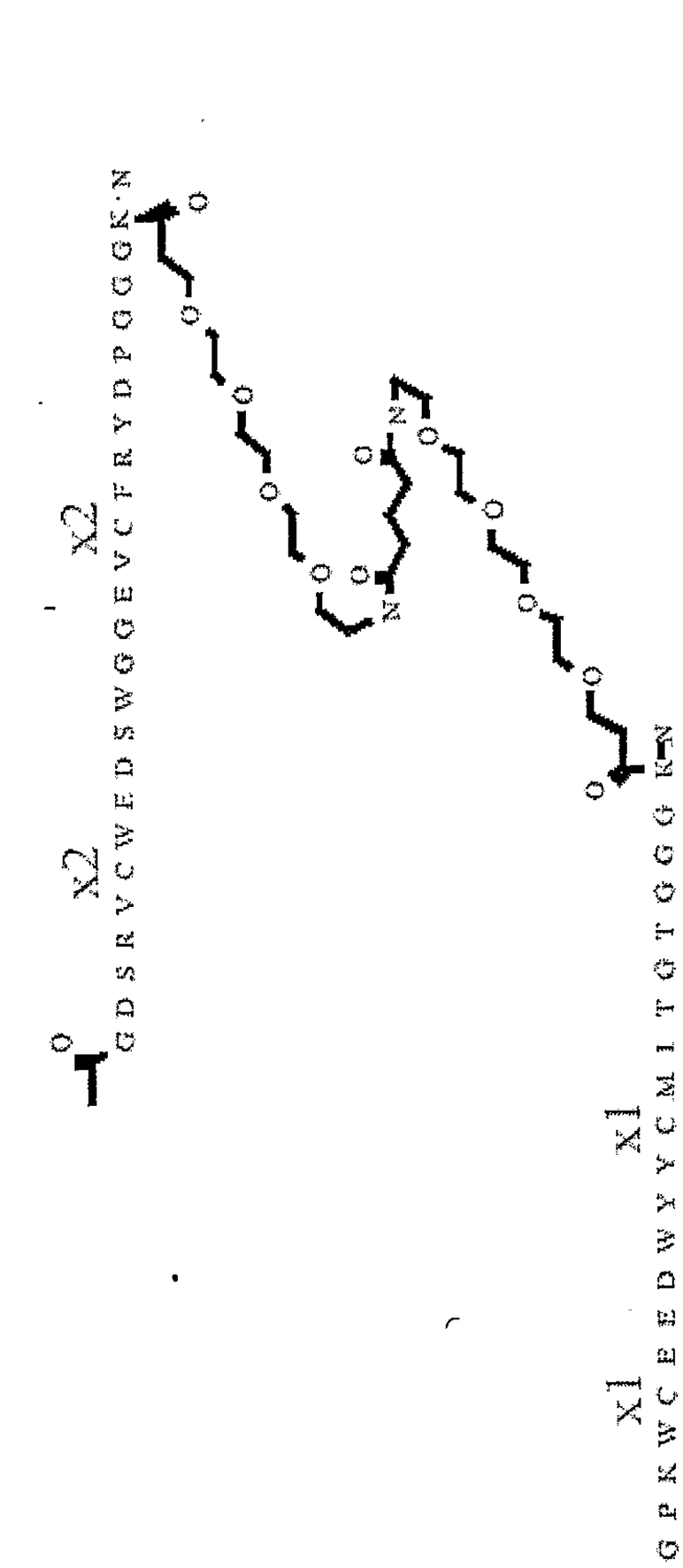

X2
G D S R V C W E D S W G G E V C F R Y D P G G G K N
X2
X1
G P K W C E E D W Y Y C M I T G T G G G K N
X1

FIG. 65

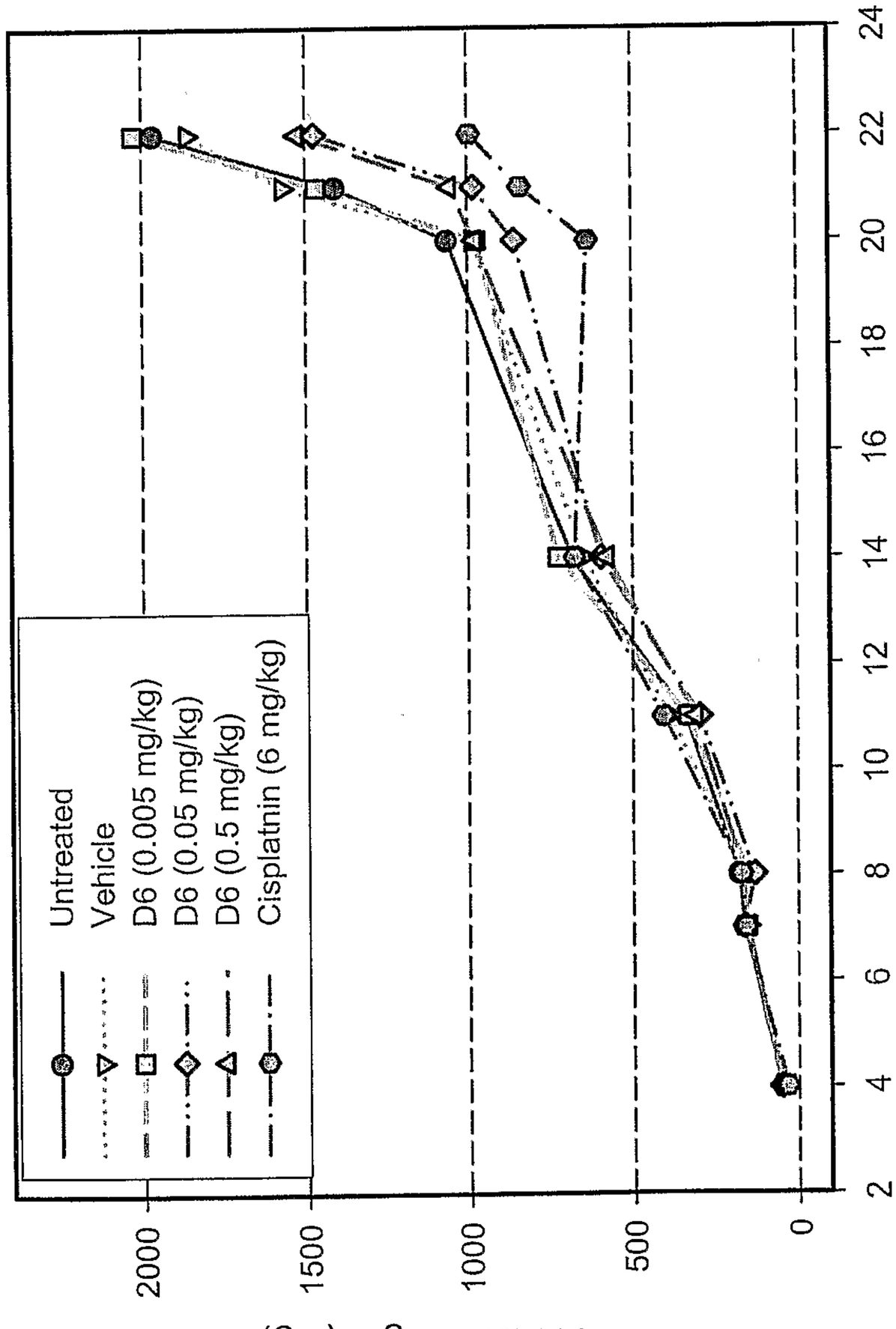

Tumor Weight (mg)
2000
1500
1000
500
0
2
4
6
8
10
12
14
16
18
20
22
24
Days (post-injection)
Untreated
Vehicle
D6 (0.005 mg/kg)
D6 (0.05 mg/kg)
D6 (0.5 mg/kg)
Cisplatnin (6 mg/kg)

FIG. 66

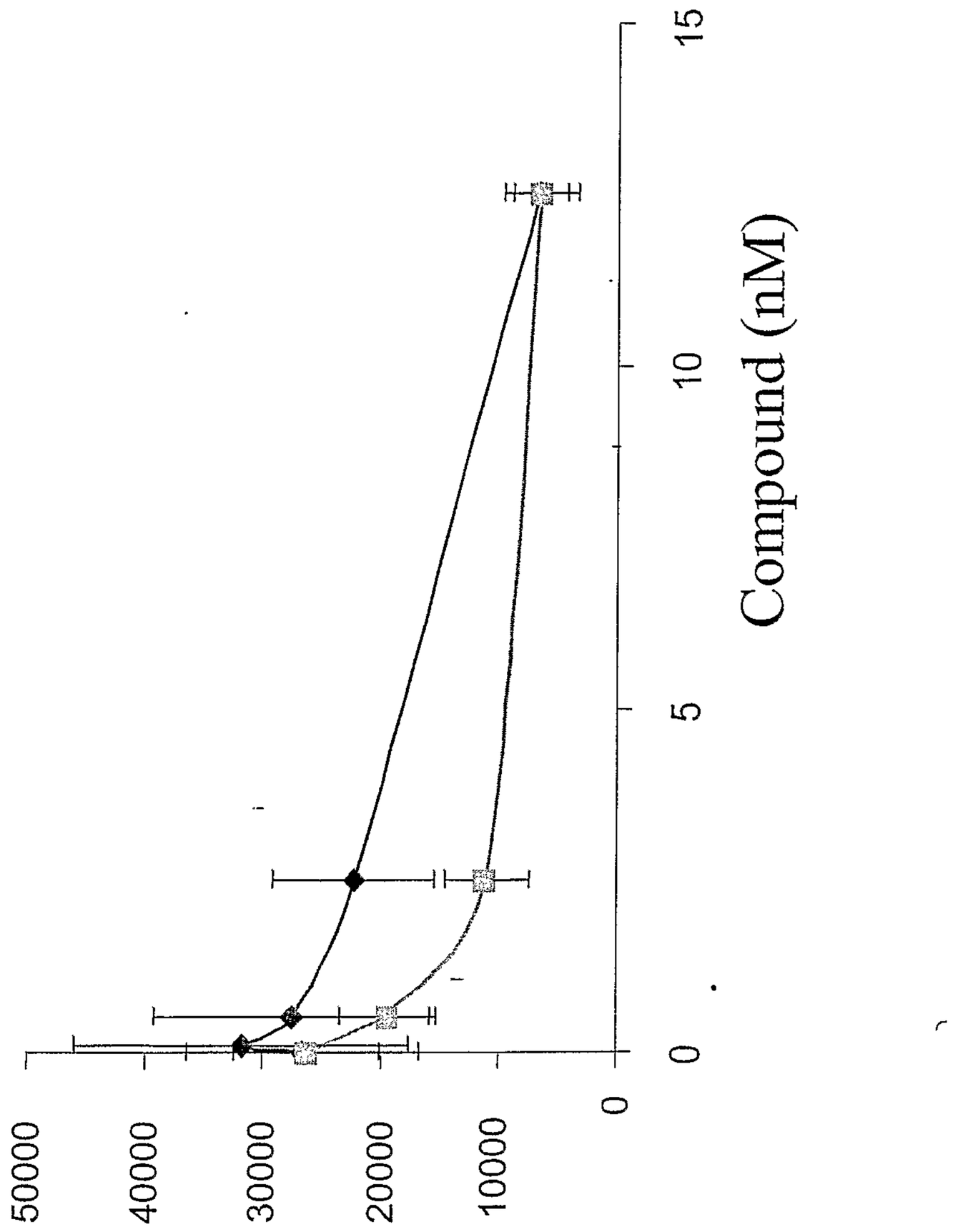

Fluor Units
50000
40000
30000
20000
10000
0
0
5
10
15
Compound (nM)

FIG. 67

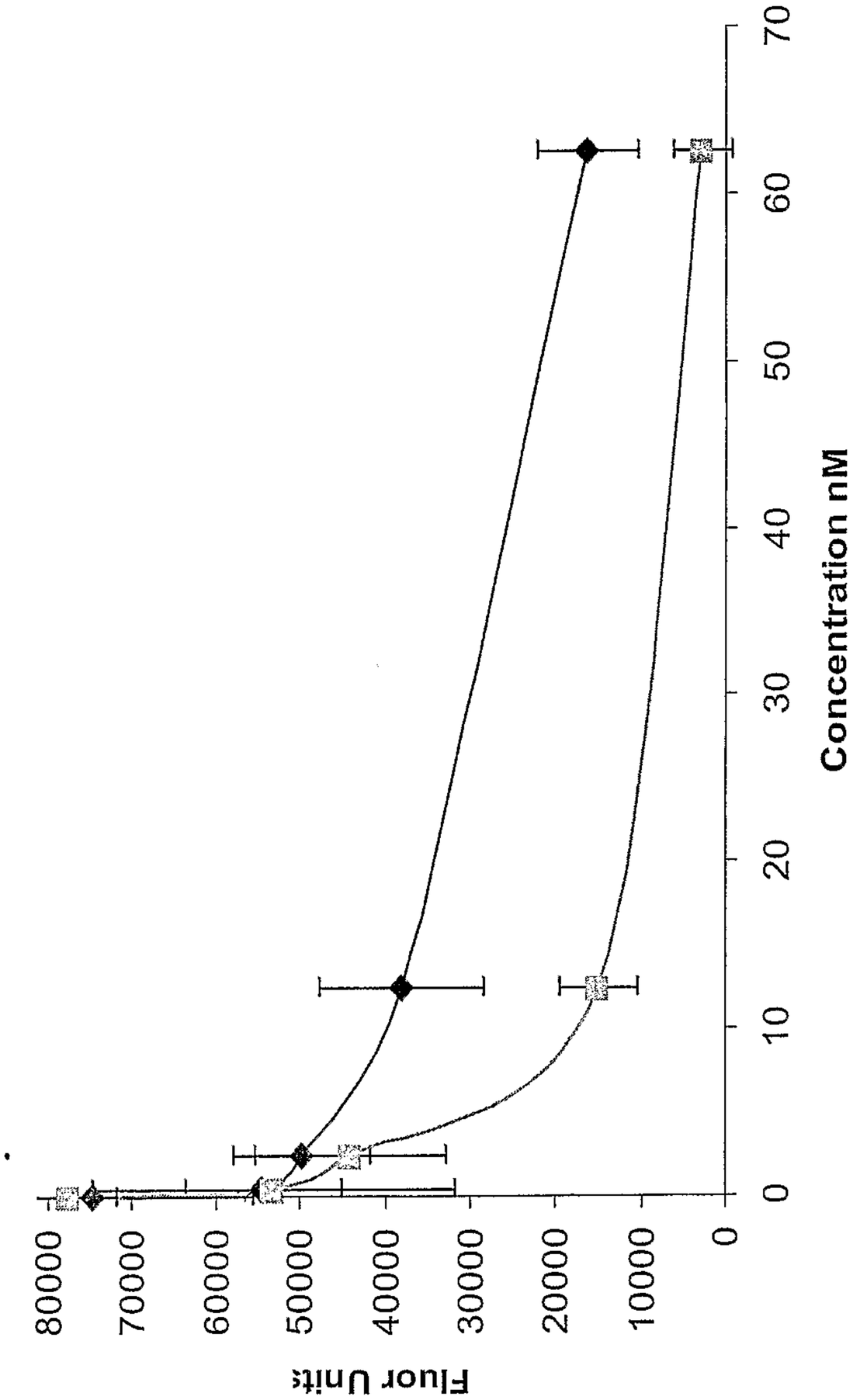
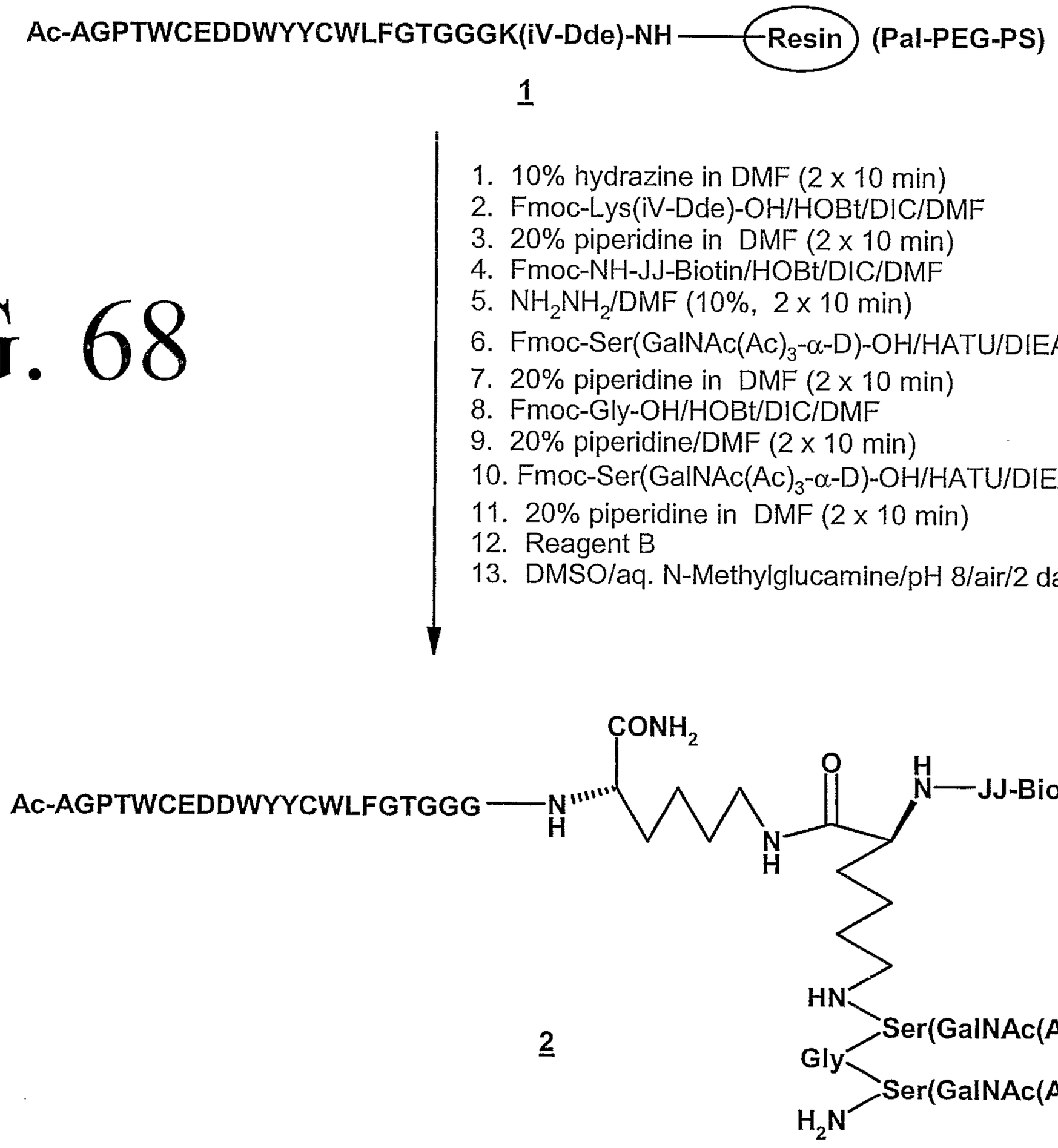

Fluor Units
80000
70000
60000
50000
40000
30000
20000
10000
0
0
10
20
30
40
50
60
70
Concentration nM

FIG. 68


Ac-AGPTWCEDDWYYCWLFGTGGGK(iV-Dde)-NH
Resin
(Pal-PEG-PS)
1
1. 10% hydrazine in DMF (2 x 10 min)
2. Fmoc-Lys(iV-Dde)-OH/HOBt/DIC/DMF
3. 20% piperidine in DMF (2 x 10 min)
4. Fmoc-NH-JJ-Biotin/HOBt/DIC/DMF
5. NH_2NH_2/DMF (10%, 2 x 10 min)
6. Fmoc-Ser(GalNAc(Ac)$_3$-α-D)-OH/HATU/DIEA/DMF
7. 20% piperidine in DMF (2 x 10 min)
8. Fmoc-Gly-OH/HOBt/DIC/DMF
9. 20% piperidine/DMF (2 x 10 min)
10. Fmoc-Ser(GalNAc(Ac)$_3$-α-D)-OH/HATU/DIEA/DMF
11. 20% piperidine in DMF (2 x 10 min)
12. Reagent B
13. DMSO/aq. N-Methylglucamine/pH 8/air/2 days
CONH$_2$
Ac-AGPTWCEDDWYYCWLFGTGGG
JJ-Biotin
HN
Ser(GalNAc(Ac)$_3$-alpha-D)
Gly
Ser(GalNAc(Ac)$_3$-alpha-D)
H$_2$N
2

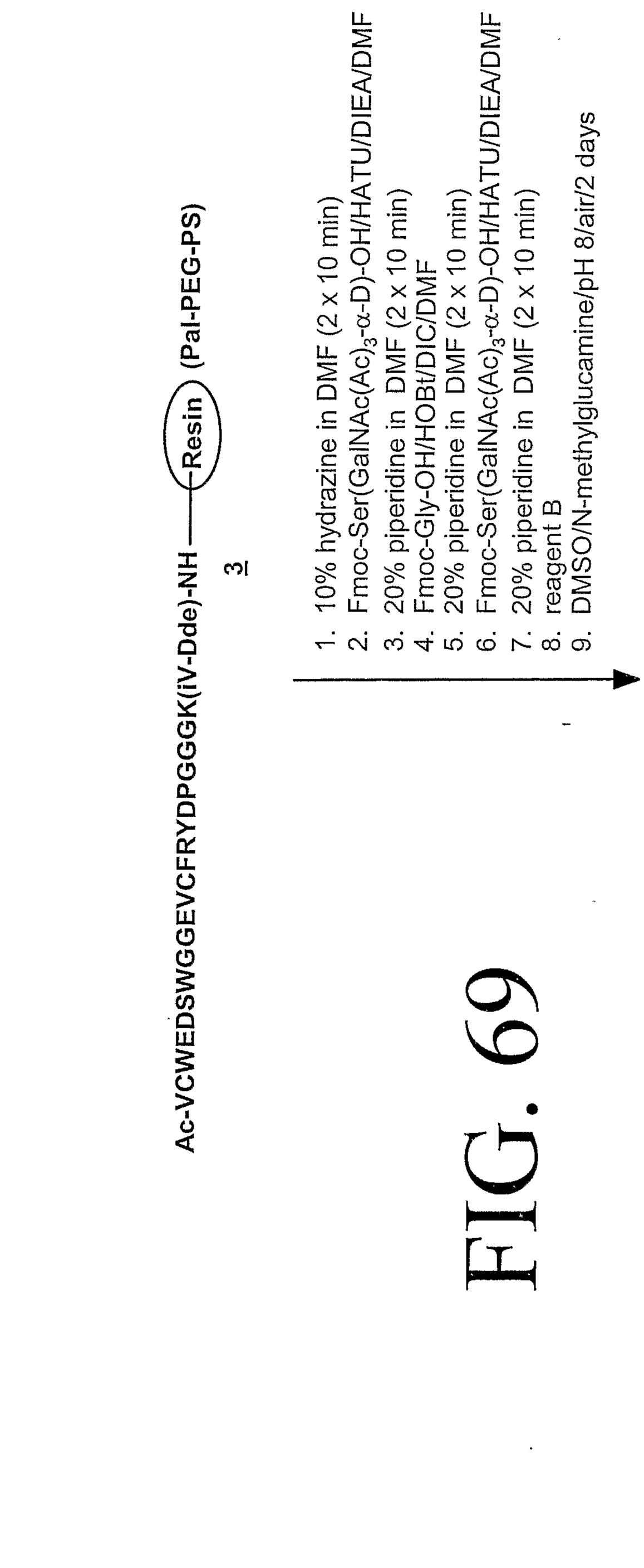

Ac-VCWEDSWGGEVCFRYDPGGGK(iV-Dde)-NH—Resin (Pal-PEG-PS)
3
1. 10% hydrazine in DMF (2 x 10 min)
2. Fmoc-Ser(GalNAc(Ac)3-α-D)-OH/HATU/DIEA/DMF
3. 20% piperidine in DMF (2 x 10 min)
4. Fmoc-Gly-OH/HOBt/DIC/DMF
5. 20% piperidine in DMF (2 x 10 min)
6. Fmoc-Ser(GalNAc(Ac)3-α-D)-OH/HATU/DIEA/DMF
7. 20% piperidine in DMF (2 x 10 min)
8. reagent B
9. DMSO/N-methylglucamine/pH 8/air/2 days
69
Ac-VCWEDSWGGEVCFRYDPGGG—N(H)—CONH2
HN—Ser(GalNAc(Ac)3-alpha-D)
Gly—Ser(GalNAc(Ac)3-alpha-D)
H2N
4

FIG.

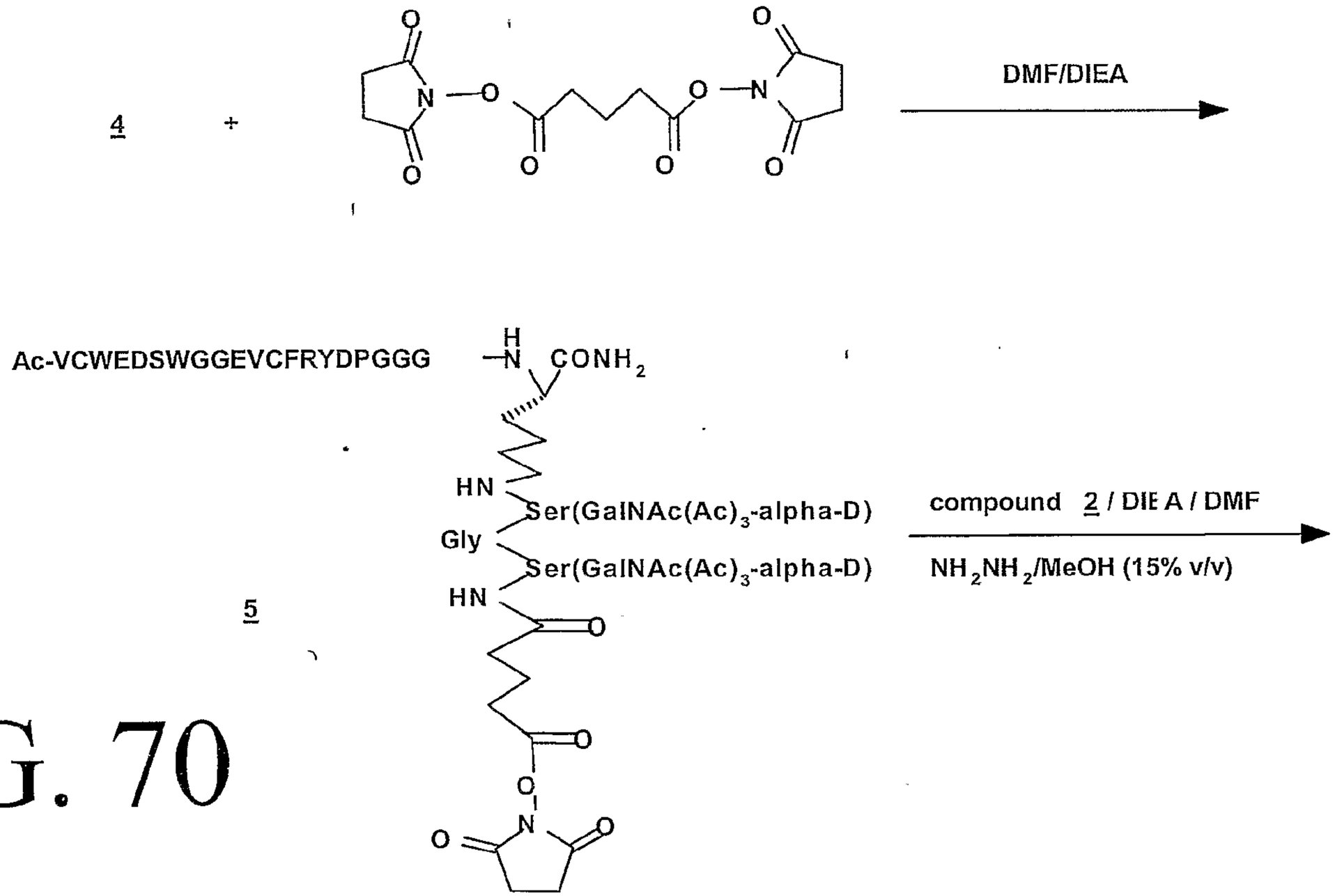

4
DMF/DIEA
Ac-VCWEDSWGGEVCFRYDPGGG
HN
Ser(GalNAc(Ac)3-alpha-D)
Gly
Ser(GalNAc(Ac)3-alpha-D)
HN
5
compound 2 / DIEA / DMF
NH2NH2/MeOH (15% v/v)

FIG. 70

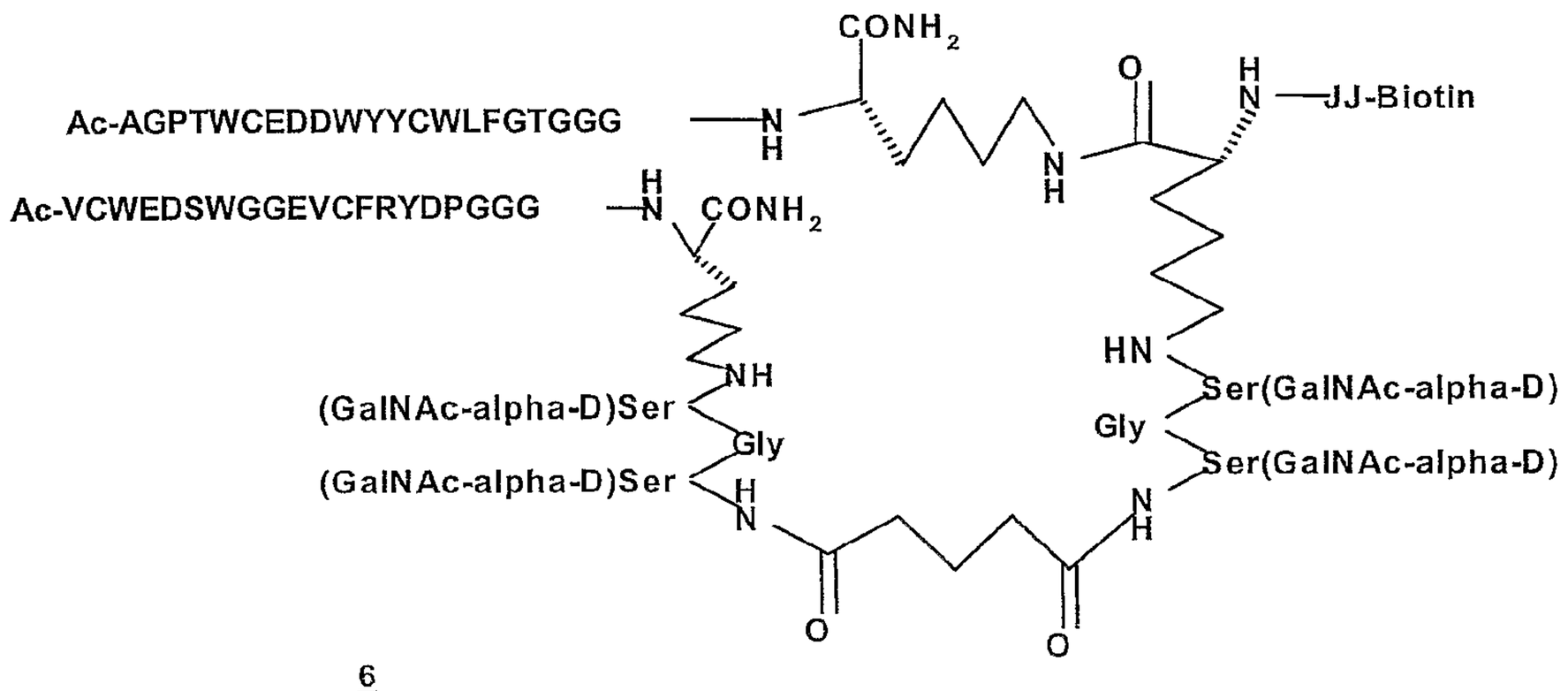

Ac-AGPTWCEDDWYYCWLFGTGGG
Ac-VCWEDSWGGEVCFRYDPGGG
JJ-Biotin
(GalNAc-alpha-D)Ser
(GalNAc-alpha-D)Ser
Gly
Ser(GalNAc-alpha-D)
Gly
Ser(GalNAc-alpha-D)
6

FIG. 71

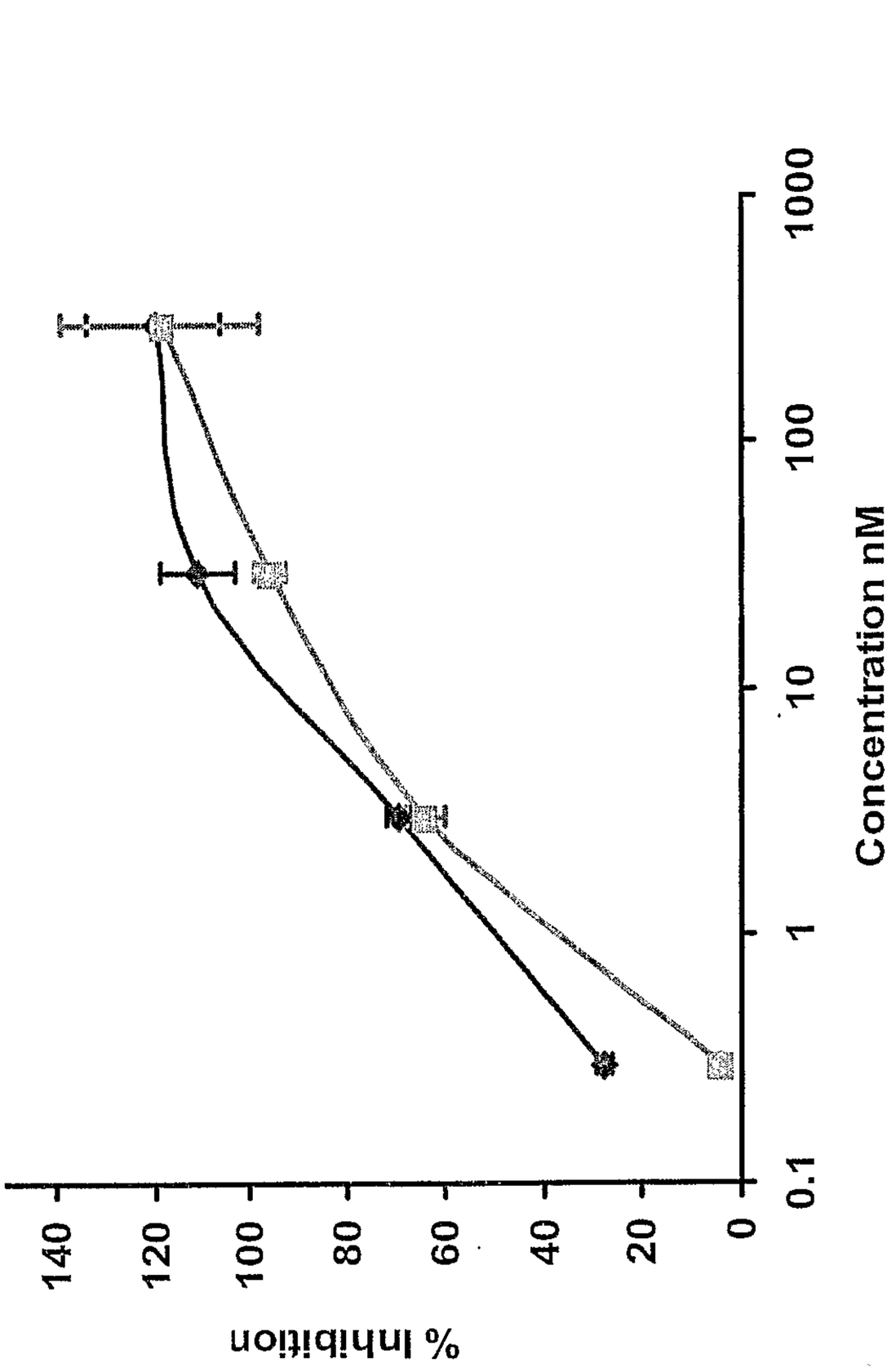

% Inhibition
140
120
100
80
60
40
20
0
0.1
1
10
100
1000
Concentration nM

FIG. 72

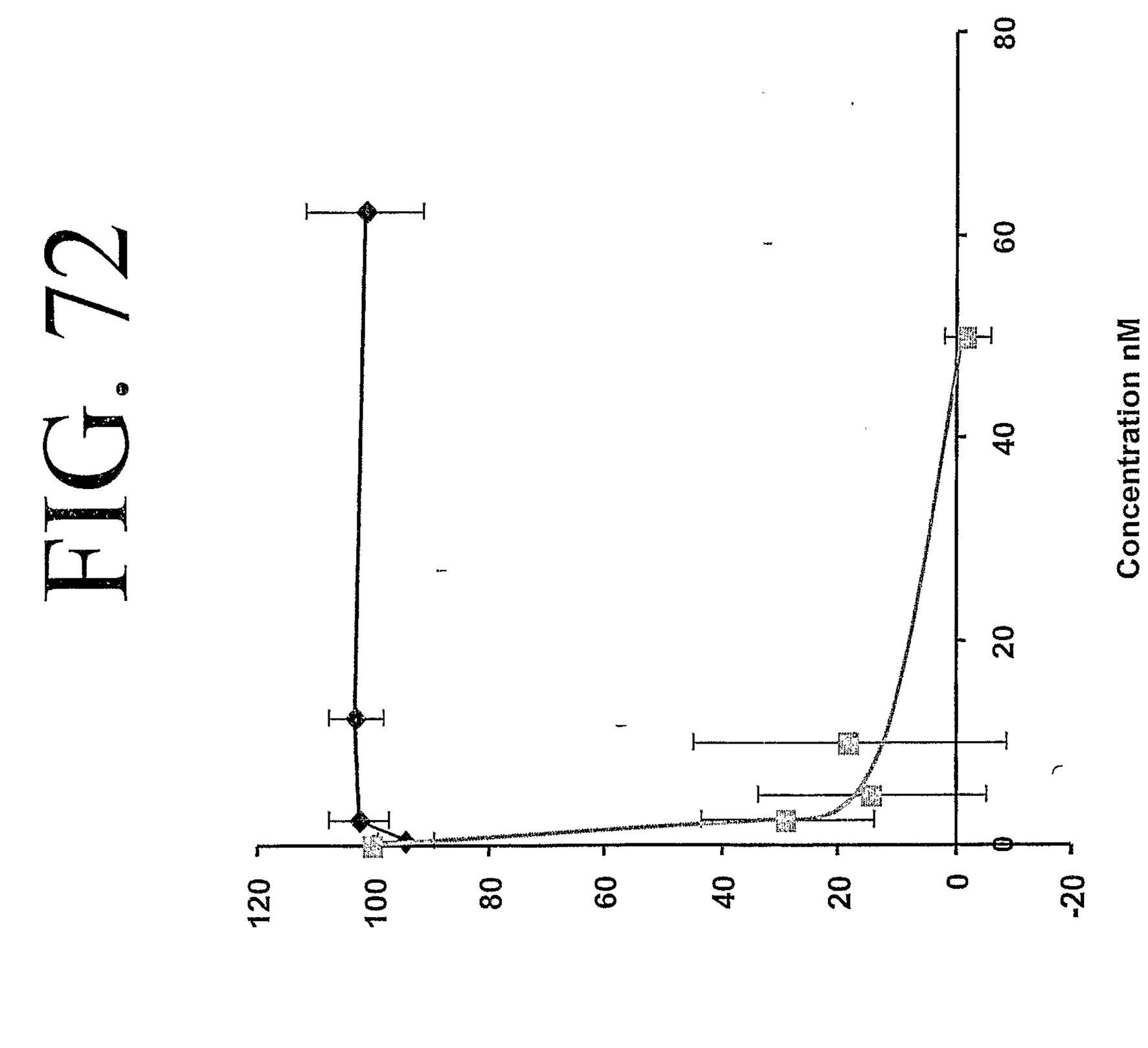


FIG. 73

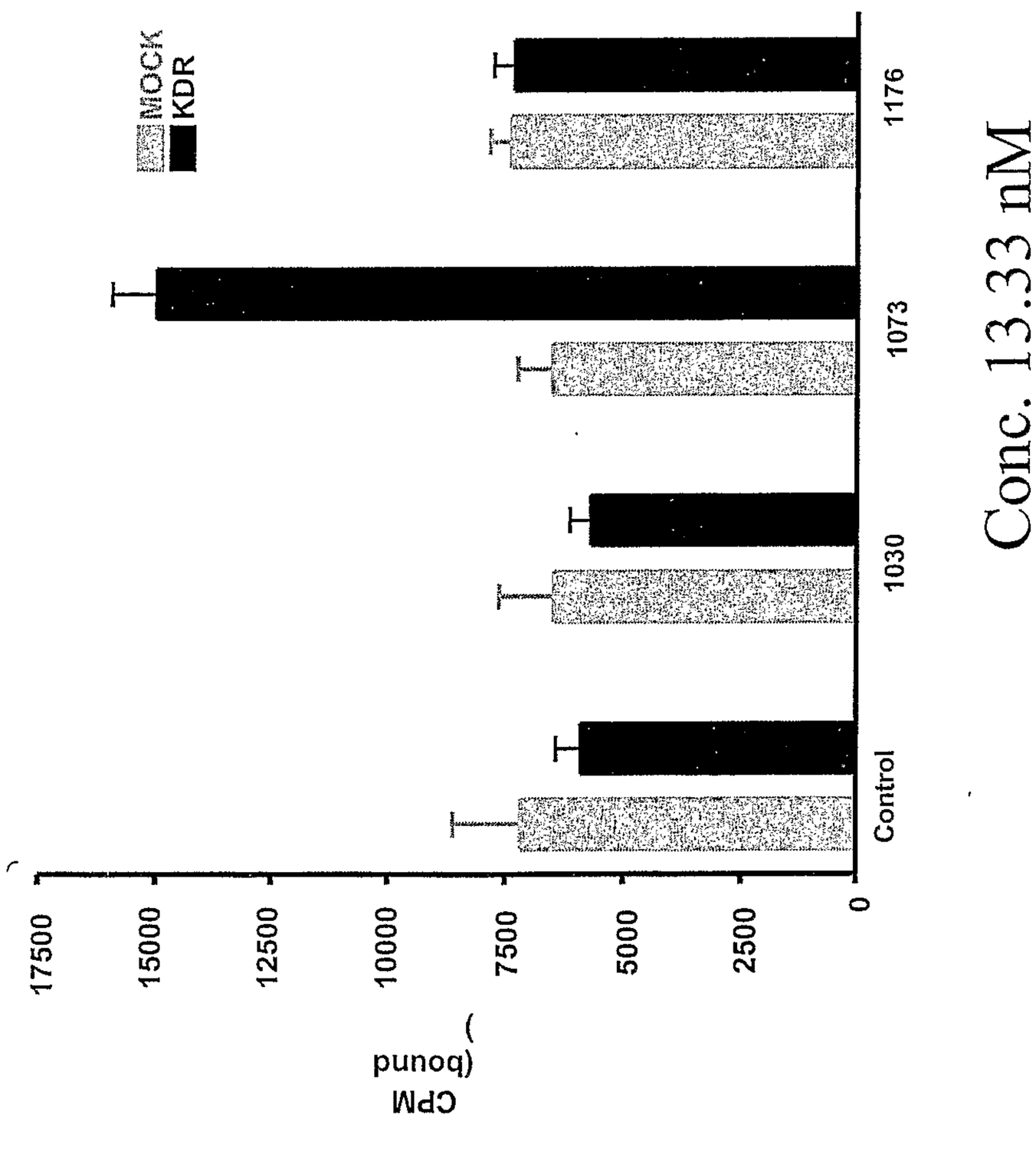

MOCK
KDR
17500
15000
12500
10000
7500
5000
2500
0
CPM (bound)
Control
1030
1073
1176
Conc. 13.33 nM

FIG. 74

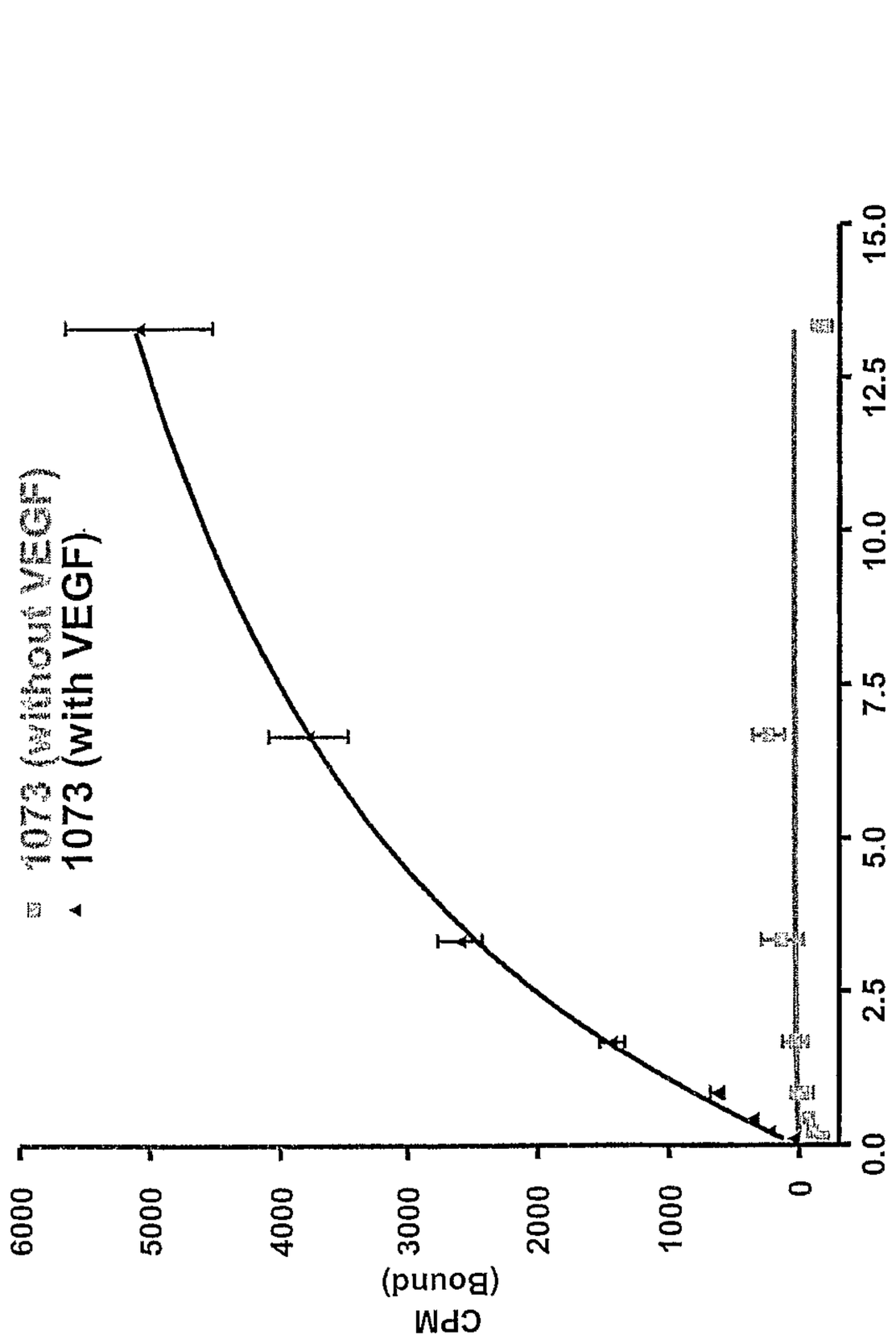

10 nM 1073 (without VEGF)
10 nM 1073 (with VEGF)
CPM (Bound)
6000
5000
4000
3000
2000
1000
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
Conc. (nM)

FIG. 75

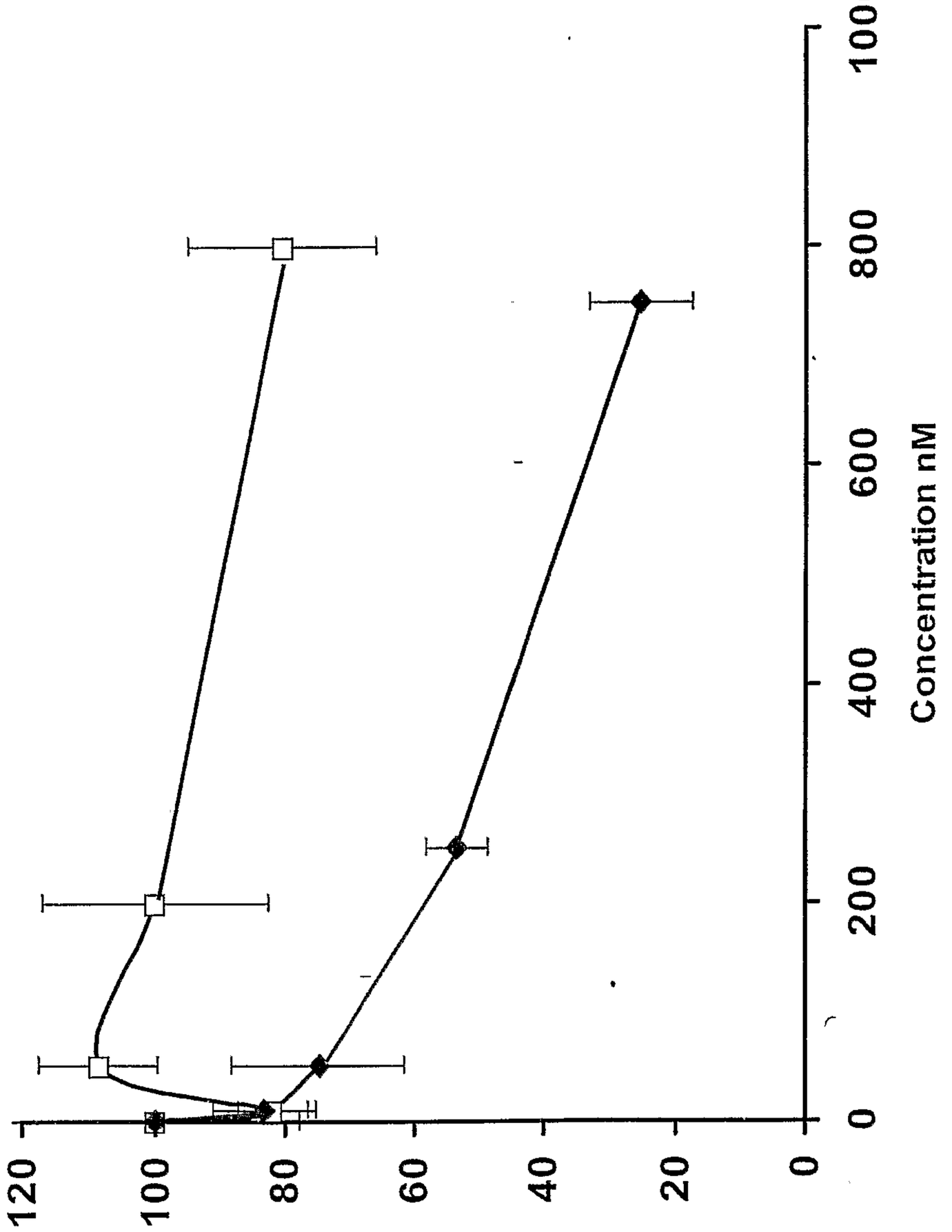

% Maximum
120
100
80
60
40
20
0
0
200
400
600
800
1000
Concentration nM

FIG. 76

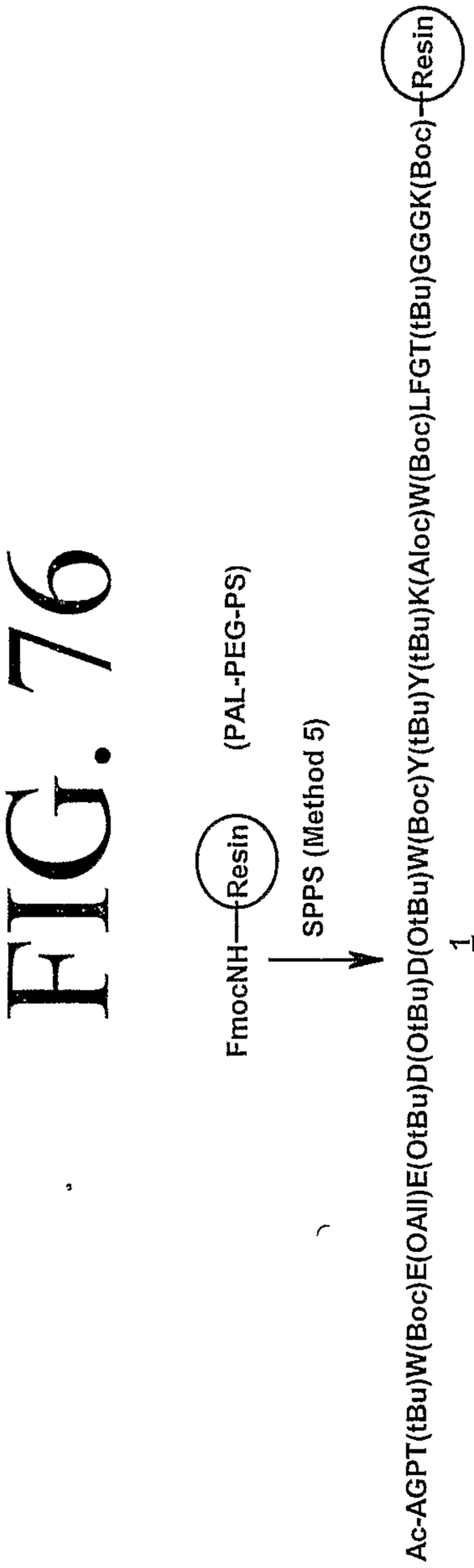

FmocNH—(Resin)
SPPS (Method 5)
(PAL-PEG-PS)
1
Ac-AGPT(tBu)W(Boc)E(OAll)E(OtBu)D(OtBu)D(OtBu)Y(tBu)K(Aloc)W(Boc)LFGT(tBu)GGGK(Boc)—(Resin)
Pd(PPh3)4
NMM/HOAc/DMF/Argon
2
Ac-AGPT(tBu)W(Boc)EE(OtBu)D(OtBu)D(OtBu)Y(tBu)KW(Boc)LFGT(tBu)GGGK(Boc)—(Resin)
HATU/NMM/DMF
3
Ac-AGPT(tBu)W(Boc)EE(OtBu)D(OtBu)D(OtBu)Y(tBu)KW(Boc)LFGT(tBu)GGGK(Boc)—(Resin)
reagent B
4
Ac-AGPTWEEDDWYYKWLFGTGGGK-NH2

FIG. 77

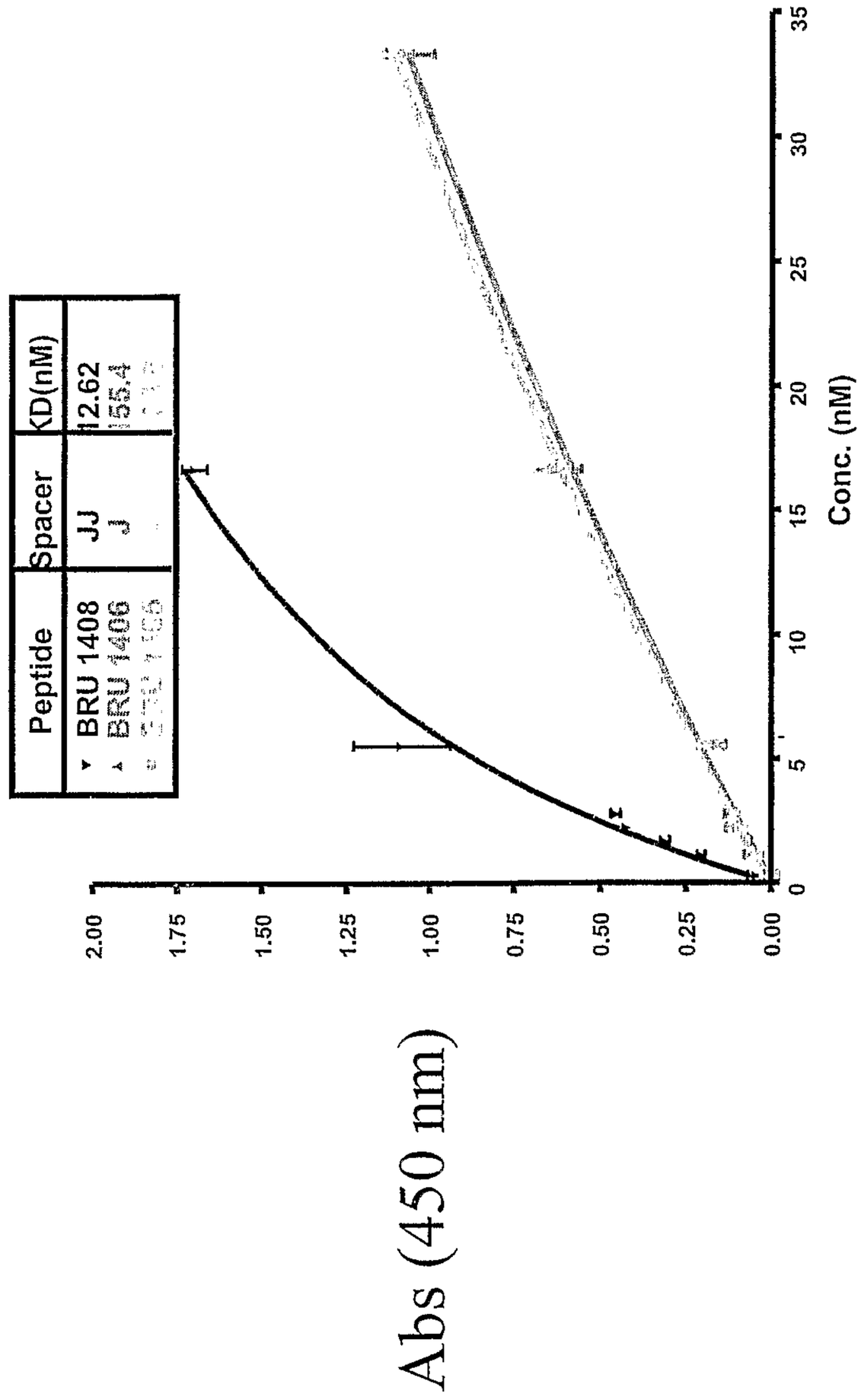
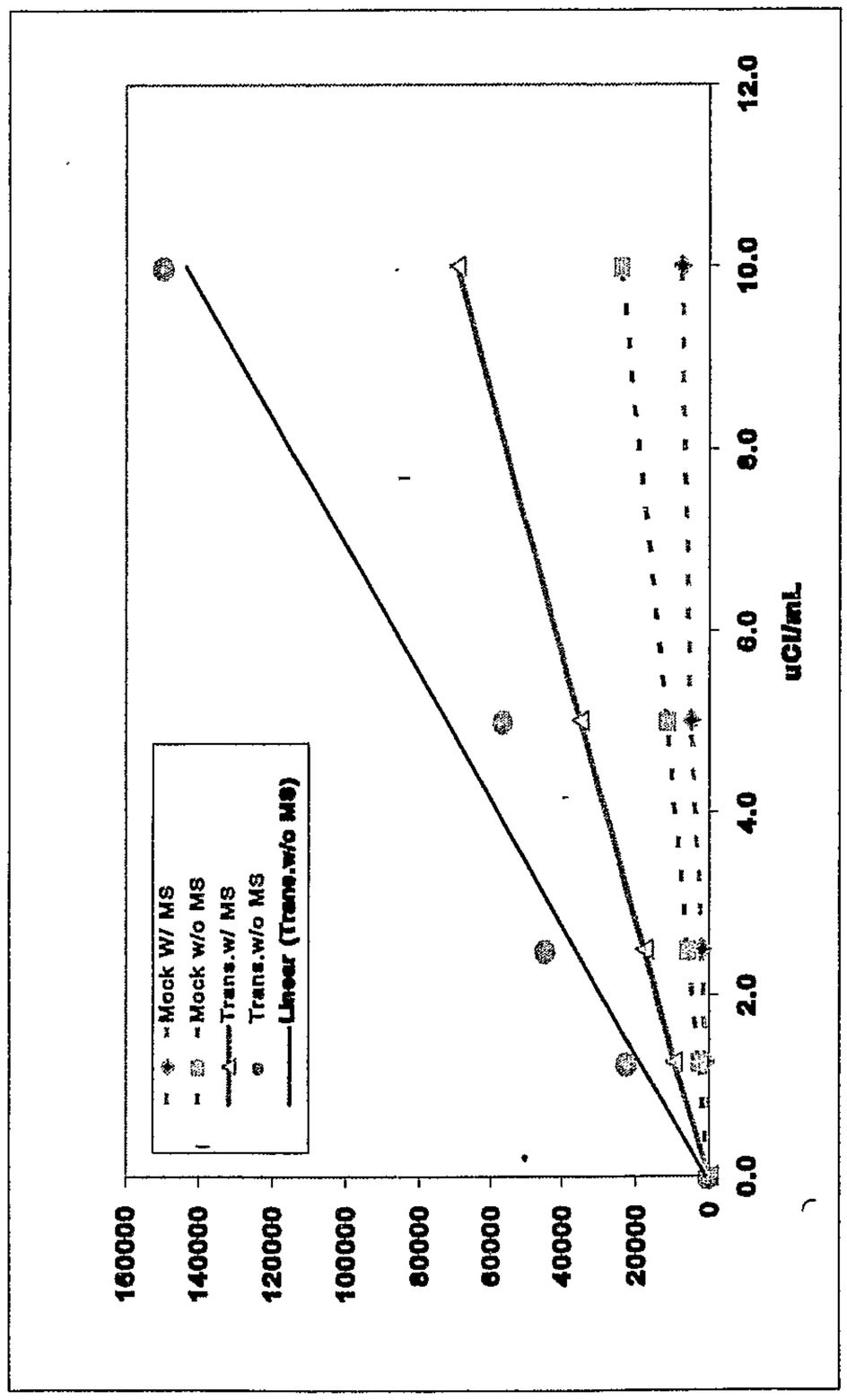

Peptide
Spacer
KD(nM)
BRU 1408
JJ
12.62
BRU 1406
J
155.4
Abs (450 nm)
Conc. (nM)
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
0
5
10
15
20
25
30
35

FIG. 78


160000
140000
120000
100000
80000
60000
40000
20000
0
0.0
2.0
4.0
6.0
8.0
10.0
12.0
uCi/mL
Bound (CPM)
Mock W/ MS
Mock w/o MS
Trans.w/ MS
Trans.w/o MS
Linear (Trans.w/o MS)

Explanation of Abbreviations: Other abbreviations: BOA = (S)-2-{Bis-[2-(bis-carboxymethyl-amino)-ethyl]-amino}-pentanedioic-4-oyl; Glut- = glutaryl or 1,5-pentanedi-oyl , SATA = S-acetyl-alpha-thioacetyl, GalNAc(Ac)3-alpha-D = O-Beta[2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-alpha-D-galactopyranosyl)-L-serinyl, iV-Dde = 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl, 5CF = 5-carboxyfluoresceinyl; J = 3,6-dioxa-8-aminooctanoyl, Dpr = 2,3-diaminopropanoyl, Adca3 = (3β,5α,7α,12α)-3-amino-7,12-dihydroxycholan-24-oyl, PnAO6 = 4-{2-(2-Hydroxyimino-1,1-dimethyl-ethylamino)-1-[(2-hydroxyimino-1,1-dimethyl-ethylamino)-methyl]-ethylcarbamoyl}-butanoyl.

Compound Sequence/Structure (Parent Sequence in red text)	Obtained ? Y/N	SEQ ID NO	MS Data†
Ac-AGPTWCEDDWYYCWLFGTGGGK(Tc-Chelator)-NH_2		277	
Ac-AGPTWCEDDWYYCWLFGTGGGK(PnAO6-NH-(O=)C(CH_2)$_3$C(=O)-JJ)-NH_2	Y	277	1611.7 [M-2H]/2,1074.4 [M-3H]/3[a]
Ac-AGPTWCEDDWYYCWLFGTGGGK-NH_2		277	
H_2N-JJK(ivDde)-AGPTWCEDDWYYCWLFGTGGG-NH_2	Y	277	1501.5 [M-2H]/2[a]
Ac-AGPTWCEDDWYYCWLFGTGGGK(BOA-K)-NH_2	Y	277	1561.9 [M-3H]/3[a]
NH_2-JJVCWEDSWGGEVCFRYDPGGG-NH_2	Y	999 (337 - C term K)	2505.4 [M-H], 1251.9 [M-2H]/2[a]
H_2N-JJAGPTWCEDDWYYCWLFGTGGGK(iV-Dde)-NH_2	Y	277	1501.5 [M-2H]/2, 1000.8 [M-3H]/3[a]
Ac-AGPTWCEDDWYYCWLFGTGGGK-NH_2	Y	277	1274.4 [M-2H]/2[a]
Ac-AGPTWCEDDWYYCWLFGTGGGK-NH_2	Y	277	1274.4 [M-2H]/2[a]
Ac-AGPTWCEDDWYYCWLFGTGGGK(5CF)-NH_2	Y	277	1453.5 [M-2H]/2, 968.7 [M-3H]/3[a]
Ac-AGPTWCEDDWYYCWLFATGGGK(Biotin-JJ)-NH_2;	Y	379	1539.8 [M-2H]/2[a]
Ac-AQXXXXXXXXXXXXXXXXXXGGGGGK(Biotin-JJ)-NH_2		380	

FIG. 79A

Compound Sequence/Structure (Parent Sequence in red text)	Obtained ? Y/N	SEQ ID NO	MS Data†
Ac-AQPDNWKEFYESGWKYPSLYKPLGGGGGK(Biotin-JJ)-NH_2	Y	381	1878.9 [M+2H]/2[b]
Ac-AQQIEYVNDKWYWTGGYWNVPFGGGGGK(Biotin-JJ)-NH_2	Y	382	1866.6 [M-2H]/2[a]
Ac-AQDALEAPKRDWYYDWFLNHSPGGGGGK(Biotin-JJ)-NH_2	Y	383	1845.5 [M-2H]/2[a]
Ac-AQWYHDGLHNERKPPSHWIDNVGGGGGK(Biotin-JJ)-NH_2	Y	384	1833.7 [M-2H]/2[a]
Ac-AQDWYWQRERDKLREHYDDAFWGGGGGK(Biotin-JJ)-NH_2	Y	385	1990.8 [M-2H]/2[a]
Ac-AGPTWCEDDWYYCWLFGTGGGK-NH_2		277	
Ac-AAPTWCEDDWYYCWLFGTGGGK-NH_2	Y	386	2563.5 [M-H], 1281.8 [M-2H]/2[a]
Ac-AGATWCEDDWYYCWLFGTGGGK-NH_2	Y	387	2523.6 [M-H], 1261.5 [M-2H]/2[a]
Ac-AGPAWCEDDWYYCWLFGTGGGK-NH_2	Y	388	2519.8 [M-H], 1259.5 [M-2H]/2[a]
Ac-AGPTACEDDWYYCWLFGTGGGK-NH_2	Y	389	2434.6 [M-H], 1216.8 [M-2H]/2[a]
Ac-AGPTWCADDWYYCWLFGTGGGK-NH_2	Y	390	1244.9 [M-2H]/2a
Ac-AGPTWCEADWYYCWLFGTGGGK-NH_2	Y	391	2434.6 [M-H], 1216.6 [M-2H]/2[a]
Ac-AGPTWCEDAWYYCWLFGTGGGK-NH_2	Y	392	1252.7 [M-2H]/2[a]
Ac-AGPTWCEDDAYYCWLFGTGGGK-NH_2	Y	393	2434.3 [M-H], 1216.8 [M-2H]/2[a]
Ac-AGPTWCEDDWAYCWLFGTGGGK-NH_2	Y	394	2457.5 [M-H], 1239.0 [M-3H+Na], 1228.3 [M-2H]/2[a]
Ac-AGPTWCEDDWYACWLFGTGGGK-NH_2	Y	395	2456.8 [M-H], 1228.2 [M-2H]/2[a]
Ac-AGPTWCEDDWYYCALFGTGGGK-NH_2	Y	396	2505.4 [M-H], 1252.5 [M-2H]/2[a]
Ac-AGPTWCEDDWYYCWAFGTGGGK-NH_2	Y	397	1253.3 [M-2H]/2[a]
Ac-AGPTWCEDDWYYCWLAGTGGGK-NH_2	Y	398	1236.4 [M-2H]/2[a]
Ac-AGPTWCEDDWYYCWLFATGGGK-NH_2	Y	399	2564.6 [M-H],1281.6 [M-2H]/2[a]
Ac-AGPTWCEDDWYYCWLFGAGGGK-NH_2	Y	400	2519.7 [M-H], 1259.6 [M-2H]/2[a]
Ac-AGPTWCEDDWYYCWLFGTGGGK-NH_2		277	
Ac-aGPTWCEDDWYYCWLFGTGGGK-NH_2	Y	277	2549.7 [M-H], 1274.7 [M-2]/2[a]
Ac-AaPTWCEDDWYYCWLFGTGGGK-NH_2	Y	401	2564.7 [M-H], 1292.7 [M-3H+Na]/2, 1281.2 [M 2H]/2[a]
Ac-AGaTWCEDDWYYCWLFGTGGGK-NH_2	Y	402	1261.4 [M-2H]/2[a]
Ac-AGPaWCEDDWYYCWLFGTGGGK-NH_2	Y	403	2519.2 [M-H], 1259.1 [M-2H]/2[a]
Ac-AGPTaCEDDWYYCWLFGTGGGK-NH_2	Y	404	2434.6 [M-H], 1217.1 [M-2H]/2[a]
Ac-AGPTWCaDDWYYCWLFGTGGGK-NH_2	Y	405	2490.8 [M-H], 1245.6 [M-2H]/2[a]

FIG. 79B

Compound Sequence/Structure (Parent Sequence in red text)	Obtained ? Y/N	SEQ ID NO	MS Data†
Ac-AGPTWCEaDWYYCWLFGTGGGK-NH_2	Y	406	2505.8 [M-H], 1252.1 [M-2H]/2[a]
Ac-AGPTWCEDaWYYCWLFGTGGGK-NH_2	Y	407	2506.0 [M-H], 1252.0 [M-2H]/2[a]
Ac-AGPTWCEDDaYYCWLFGTGGGK-NH_2	Y	408	2434.4 [M-H], 1217.1 [M-2H/2][a]
Ac-AGPTWCEDDWaYCWLFGTGGGK-NH_2	Y	409	2458 [M-H], 1228.6 [M-2H]/2[a]
Ac-AGPTWCEDDWYaCWLFGTGGGK-NH_2	Y	410	2457.6 [M-H], 1228.5 [M-2H]/2[a]
Ac-AGPTWCEDDWYYCaLFGTGGGK-NH_2	Y	411	2434.8 [M-H], 1228.1 [M-3H+Na]/2, 1217.0 [M 2H]/2[a]
Ac-AGPTWCEDDWYYCWaFGTGGGK-NH_2	Y	412	2507.7 [M-H], 1264.1 [M-3H+Na]/2, 1253.6 [M 2H]/2[a]
Ac-AGPTWCEDDWYYCWLaGTGGGK-NH_2	Y	413	2473.6 [M-H], 1247.6 [M-3H+Na]/2, 1236.2 [M 2H]/2[a]
Ac-AGPTWCEDDWYYCWLFaTGGGK-NH_2	Y	414	2563.7 [M-H], 1709.3 [unassigned], 1292.7 [M-3H+Na]/2, 1281.9 [M 2H]/2[a]
Ac-AGPTWCEDDWYYCWLFGaGGGK-NH_2	Y	415	2519.0 [M-H], 1259.6 [M-2H]/2[a]
Ac-GDSRVCWEDSWGGEVCFRYDPGGGK-NH_2		294	
Ac-GDSRVCWEDaWGGEVCFRYDPGGGK-NH_2	Y	416	1401.9 [M-3H+Na], 1391.7 [M-2H]/2[a]
Ac-GDSRVCWEDSWaGEVCFRYDPGGGK-NH_2	Y	417	1664.4 [M-2H]/2[a]
Ac-GDSRVCWEDSWGaEVCFRYDPGGGK-NH_2	Y	418	1664.7 [M-2H]/2[a]
Ac-AGPTWCEDDWYYCWLFGTGGGK-NH_2		277	
Ac-AGjTWCEDDWYYCLFTGTGGGK-NH_2	Y	419	1267.9 [M-2H]/2[a]
Minimum Number of AA for DWYY Motif		420	
Ac-GDWYYGGGK-NH_2	Y	421	1041.2 [M-H][a]
Ac-EDDWYYGGGK-NH_2	Y	422	1228.3 [M-H], 612.8 [M-2H/2][a]
Ac-AQDWYYAWLFTGGGGK-NH_2	Y	423	1859.7 [M-H], 986.4 [M-2H]/2[a]
Ac-AQDWYYAWL-NH_2	Y	424	1254.4 [M-H][a]
Ac-AGPTWCEDDWYYCWLFGTGGGK-NH_2		277	
Ac-AGPTWCEDEWYYCWLFGTGGGK-NH_2	Y	425	1281.5 [M-2H/2], 853.6 [M-3H/3][a]
Ac-AGPTWCEDDWWYCWLFGTGGGK-NH_2	Y	426	1285.4 [M-2H/2], 856.8 [M-3H/3][a]

FIG. 79C

Compound Sequence/Structure (Parent Sequence in red text)	Obtained ? Y/N	SEQ ID NO	MS Data†
Ac-AGPTWCEDDWFYCWLFGTGGGK-NH_2	Y	427	1265.8 [M-2H/2][a]
Ac-AGPTWCEDDWYYCWLFGTGGGK-NH_2		277	
Ac-AGPTWAEDDWYYAWLFGTGGGK-NH_2	Y	428	2486.9 [M-H], 1243.6 [M-2H]/2[a]
Ac-AAPAWCAADWYYCWLFGTGGGK-NH_2	Y	429	2432.7 [M-H], 1272.5 [M+TFA-2H]/2[a]
Ac-AGPTWCaDDWYYCWLFGTGGGK-NH_2	Y	430	2192.6 [M-H], 1096.0 [M-2H/2][a]
Ac-AGPTWCEDDWYYCWLFGTGGGK-NH_2		277	
Ac-CEDDWYYCWLFGTGGGK-NH_2	Y	431	2037.6 [M-H], 1018.4 [M-2H]/2, 520.8 [M-6H+2Na]/4[a]
Ac-WCEDDWYYCWLFGTGGGK-NH_2	Y	432	2221.8 [M-H], 1111.6 [M-2H]/2, 740.7 [M-3H]/3[a]
Ac-WCAADWYYCWLF-NH_2	Y	433	1663.5 [M-H][a]
Ac-WCEDDWYYCWLF-NH_2	Y	434	1766.5 [M-H], 882.1 [M-2H]/2[a]
Ac-AGPTWCEDDWYYCWLFGTGGGK(Biotin-JJ)-NH_2		277	
Ac-AGPTWCEDDWYYCWLFGTGGGKK(iV-Dde)-Adca3-NH_2	Y	373	1665.2 [M-2H]/2[a]
Ac-GDSRVCWEDSWGGEVCFRYDPGGGK(Biotin-JJ)-NH_2		294	
Ac-VCWEDSWGGEVCFRYDPGGGK(Biotin-JJ)-NH_2	Y	337	1449.3 [M-2H]/2, 965.8 [M-3H]/3[a]
Ac-VCWEDSWGGEVCFRYGGGK(Biotin-JJ)-NH_2	Y	435	2689.4 [M-H], 1344.0 [M-2H]/2[a]
Ac-GDSRVCWEDSWGGEVCFRYDPGGGK-NH_2		294	
Ac-GDSRVAWEDSWGGEVAFRYDPGGGK-NH_2	Y	436	1368.4 [M-2H]/2[a]
Ac-VCWEDSWGGEVCFRYGGGK-NH_2	Y	437	1085.8 [M-2H]/2[a]
Ac-VCWEDSWGGEVCFRYGGGK-NH_2	Y	437	2172.0 [M-H], 1086.0 [M-2H]/2[a]
Ac-GDSRVCWEDaWGGEVCFRYDPGGGK-NH_2	Y	438	1401.9 [M-3H+Na]/2, 1391.7 [M-2H]/2[a]
Ac-GDSRVCWEDfWGGEVCFRYDPGGGK-NH_2	Y	439	1429.7 [M-2H]/2[a]
Ac-GDSRVCWEDkWGGEVCFRYDPGGGK-NH_2	Y	440	1430.7 [M-3H+Na]/2, 1420.1 [M-2H]/2[a]
Ac-GDSRVCWEDSWGfEVCFRYDPGGGK-NH_2	Y	441	1444.5 [M-2H]/2[a]
Ac-GDSRVCWEDSWGkEVCFRYDPGGGK-NH_2	Y	442	1435.1 [M-2H]/2[a]
Ac-GDSRVCWEDSWGeEVCFRYDPGGGK-NH_2	Y	443	1435.5 [M-2H]/2[a]
Sequences Binding to KDR-VEGF Complex			
Ac-AGPGPCKGYMPHQCWYMGTGGGK(5CF)-NH_2	Y	321	1543.7 [M-2H]/2, 1028.8 [M-3H]/3, 771.3 [M-4H]/4, 617.0 [M-5H]/5[a]

FIG. 79D

Compound Sequence/Structure (Parent Sequence in red text)	Obtained ? Y/N	SEQ ID NO	MS Data†
Ac-AGPGPCKGYMPHQCWYMGTGGGK(Biotin-JJ)-NH_2	Y	321	2937.4 [M-H] , 1468.2 [M-2]/2[a]
Ac-AGMPWCVEKDHWDCWWWGTGGGK(Biotin-JJ)-NH_2	Y	444	1622.5 [M-2H]/2[a]
Ac-AGYGPCKNMPPWMCWHEGTGGGK(5CF)-NH_2	Y	323	2860.1 [M-H], 1429.8 [M-2H]/2[a]
Ac-AGYGPCKNMPPWMCWHEGTGGGK(Biotin-JJ)-NH_2	Y	323	1058.6 [M-2H]/2[a]
Pathogenic Sequences			
Ac-GDGSWCEMRQDVGKWNCFSDDPGGGK(Biotin-JJ)-NH_2	Y	445	1537.5 [M-2H]/2[a]
Ac-GCKTKISKVKKKWNCYSNNKVTGGGK(Biotin-JJ)-NH_2	Y	446	1706.8 [M+2H]/2, 1138.6 [M+3H]/3, 854:0 [M+4H]/4, 683.7 [M+5H]/5, 569.8 [M+6H]/6, 488.5 [M+7H]/7[b]
Ac-KQFCEENWERGRNHYYCLTTLSGGGK(Biotin-JJ)-NH_2	Y	447	1817.5 [M+2H]/2, 1211.8 [M+3H]/3, 909.1[M+4H]/4, 727.5 [M+5H]/5[b]
Ac-GDSRVCWEDWGGVVCRYRYDAGGGK(Biotin-JJ)-NH_2	Y	448	1675.2 [M+2H]/2, 1116.9 [M+3H]/3, 838.2 [M+4H]/4[b]
AGPTWCEDDWYYCWLFGTGGGK(Biotin-JJ)-NH_2		277	
Ac-AGPTWCEDDWYYCWLFGTGGGk(nSbGJJ)-NH_2	Y	277-nSbGJJ	1621.5 [M-2H]/2[a]
AGPTWCEDDWYYCWLFGTGGGK-NH_2		277	
Dansyl-NH -AGPTWCEDDWYYCWLFGTGGGK(5CF)-NH_2	Y	277-5CF	1549.1 [M-2H]/2[a]
Other KDR Compounds - Hangovers from Year 2001 such as DX-684 truncations etc.			
Ac-CEEDWYYCMITGTGGGK(Biotin-JJ)-NH_2	Y	449	1232.5 [M-2H]/2[a]
Ac-AGPKWCEEDWYYCMITaT-NH_2	Y	450	1509.6 [M-2H]/2[a]
Ac-AaPKWCEEDYYCMITGTGGGK-NH_2	Y	451	2504.2 [M-H], 1251.6 [M-2H]/2[a]
Ac-AaPKWCEEDYYCMITGTGGGK(Biotin-JJ)-NH_2	Y	451	1509.6 [M-2H]/2[a]
Ac-AGPDWCAADWYYCYITG-NH_2	Y	452	1992.5 [M-H] , 995.8 [M-2H]/2[a]
Ac-AGPTWCEDDWYYCWLFGTGGGK-NH_2		277	
Ac-AGPTWEEDDWYYKWLFGTGGGK-NH_2 (6-13 lactam)	Y	453	1291.9 [M-2H]/2[a]
Ac-AGPTWKEDDWYYEWLFGTGGGK-NH_2 (6-13 lactam)	Y	454	1291.9 [M-2H]/2[a]
Ac-AGPTW-Dpr-EDDWYYDWLFGTGGGK-NH_2 (6-13 lactam)	Y	455	1263.9 [M-2H]/2[a]
Ac-AGPTWDEDDWYY-Dpr-WLFGTGGGK-NH_2 (6-13 lactam)	Y	456	1263.9 [M-2H]/2[a]
Ac-AGPTWDEDDWYYKWLFGTGGGK-NH_2 (6-13 lactam)	Y	457	1285.1 [M-2H]/2[a]
Ac-AGPTWDEDDWYYKWLFGTGGGK-NH_2	Y	457	1294.1 [M-2H]/2[a]
Ac-AQDWYYDEILSMADQLRHAFLSGGGGGK(Biotin-JJ)-NH_2	Y/N	356	

FIG. 79E

Compound Sequence/Structure (Parent Sequence in red text)	Obtained ? Y/N	SEQ ID NO	MS Data†
Ac-AQDWYYDEILSMADQLR-NH_2	Y	458	2156.9 [M-H], 1077.9 [M-2H]/2[a]
Ac-DWYYDEILSMADQL-NH_2	Y	459	1800.5 [M-H], 900.2 [M-2H]/2[a]
Ac-AQDWYYDEILSMADQLRHAFLS-NH_2	Y	460	1355.2 [M-2H]/2[a]
Ac-AQDWYYGGGK-NH_2	Y	461	1183.3 [M-H][a]
Ac-DWYYGGGK-NH_2	Y	462	984.2 [M-H][a]
Ac-AQDWYYDEIL-NH_2	Y	463	1354.5 [M-H][a]
Ac-AEWSYQDMIRLDYADLQLSHFAGGGGGK(Biotin-JJ)-NH_2;	Y	464	1820.1 [M+2H]/2[b]
Ac-AQDWYYDEILSMADQLRHAFLSGGGGGK-NH_2	Y	356	1562.1 [M-2H]/2[a]
Ac-AQDWYYDEILSMADQLRHAFLSGGGGGK-NH_2	Y	356	1562.3 (M-2H]/2[a]
Ac-AQDWYYDEILSMADQLRHAFLSGGGGGK(iV-Dde)-NH_2	Y	356	1729.7 [M-2H]/2, 1152.5 [M-3H]/3[a]
Ac-AQDWY-YDEILSMADQLRHAFLSGGGGGK(SATA)-NH_2	Y	356-SATA	1620.2 [M-2H]/2[a]
Ac-AEWSYQDMIRLDYADLQLSHFAGGGGGK(SATA)-NH_2	Y	464	1620.4 [M+2H]/2[b]
Various Parent Sequences			
Ac-AQDWYYDEILJGRGRGGRGG-NH_2	Y	465	1185.0 [M+2H]/2, 790.8 [M+3H]/3[b]
Ac-EDDWYYJGRGGRGGRGG-NH_2	Y	466	972.3 [M+2H]/2, 648.0 [M+3H]/3[b]
Ac-GDWYYJGRGGRGGRGG-NH_2	Y	467	879.3 [M+2H]/2, 586.6 [M+3H]/3[b]
Ac-AQDWYYAWLFTJGRGGRGGRGG-NH_2	Y	468	1259.7 [M+2H]/2, 840.1 [M+3H]/3[b]
Ac-AQDWYYAWLJGRGGRGGRGG-NH_2	Y	469	1135.5 [M+2H]/2, 757.5 [M+3H]/3[b]

FIG. 79F

Compound Sequence/Structure (Parent Sequence in red text)	Obtained ? Y/N	SEQ ID NO	MS Data†
Ac-AQDWYYDEILJGRGGRGGRGGKK(iV-Dde)-NH_2	Y	470	1416.8 [M+2H]/2, 944.8 [M+3H]/3, 708.9 [M+4H]/4, 571.5 [M+4H+Na]/5[b]
Ac-GDSRVCWPDSWGGEVCFRYDP-NH_2	Y	471	1234.1 [M-2H]/2[a]
Ac-GDSRVCWEDSWGGVECFRYDP-NH_2	Y	472	1250.0 [M-2H]/2[a]
Ac-AQDWYYDEILJGRGGRGGRGGK(JJ)-NH_2	Y	473	930.23 [M+3H]/3, 697.9 [M+4H]/4, 558.5 [M+5H]/5[b]
Ac-AQDWYYDEILSMADQLRHAFLSGGGGGK(Biotin-JJ)-NH_2		356	
Ac-WYLDRQADFMYSAQAEDSLILHGGGGGK(Biotin-JJ)-NH_2	Y	474	1820.5 [M-2H]/2, 1213.4 [M-3H]/3[a]

FIG. 79G

FIG. 80

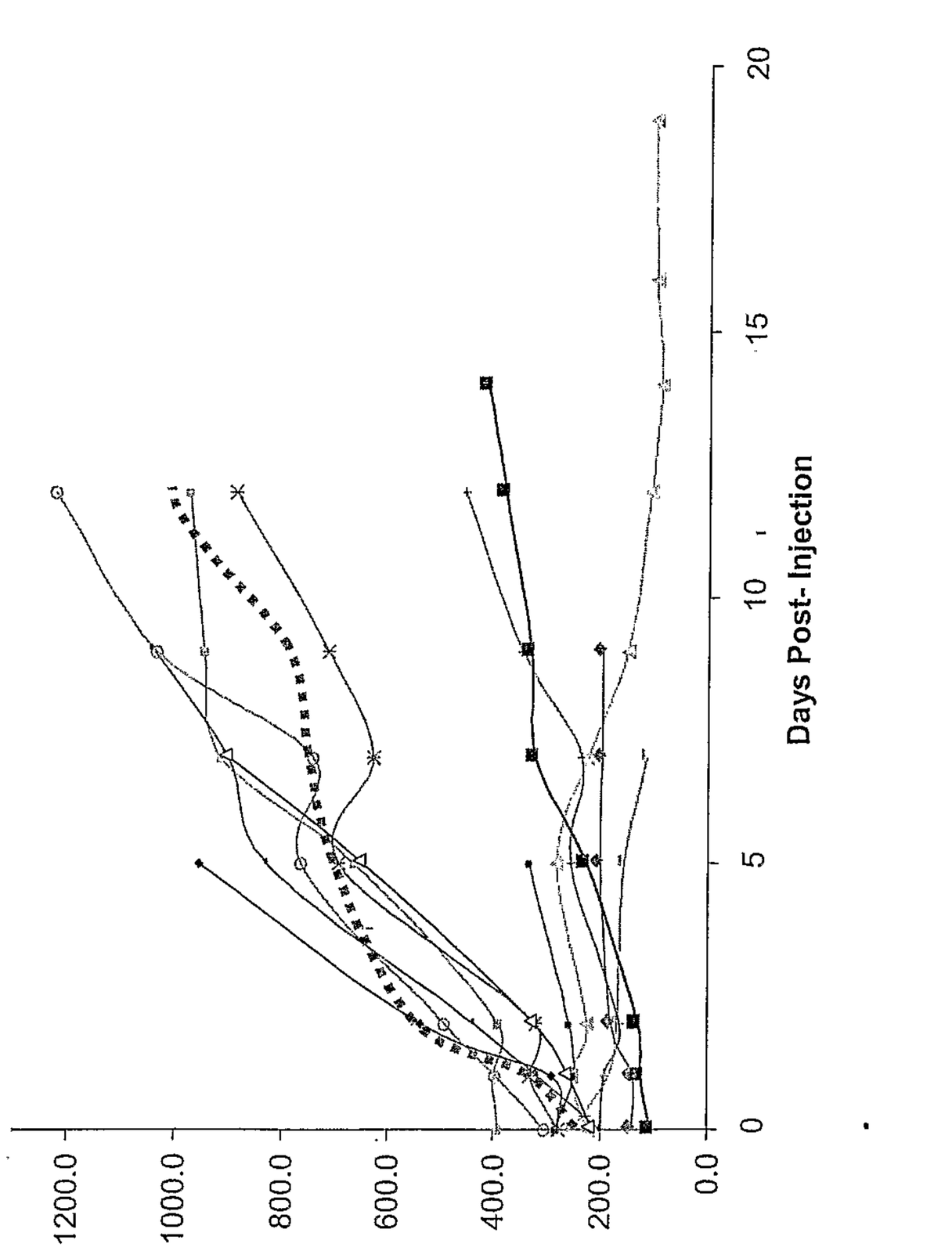

Tumor Size
mm3
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
0
5
10
15
20
Days Post- Injection

FIG. 81

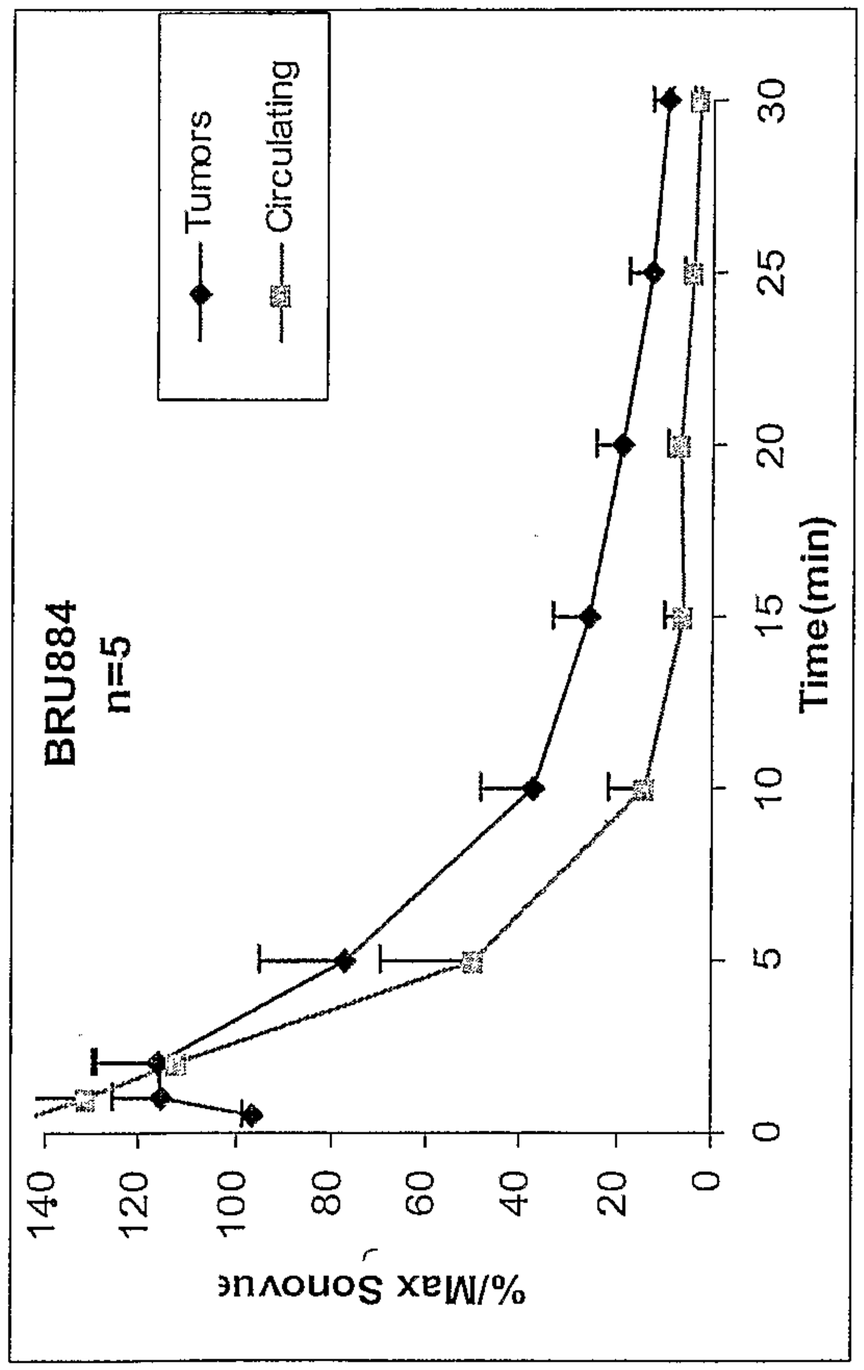

Tumors
Circulating
BRU884
n=5
%/Max Sonovue
Time(min)
0
20
40
60
80
100
120
140
0
5
10
15
20
25
30

FIG. 82

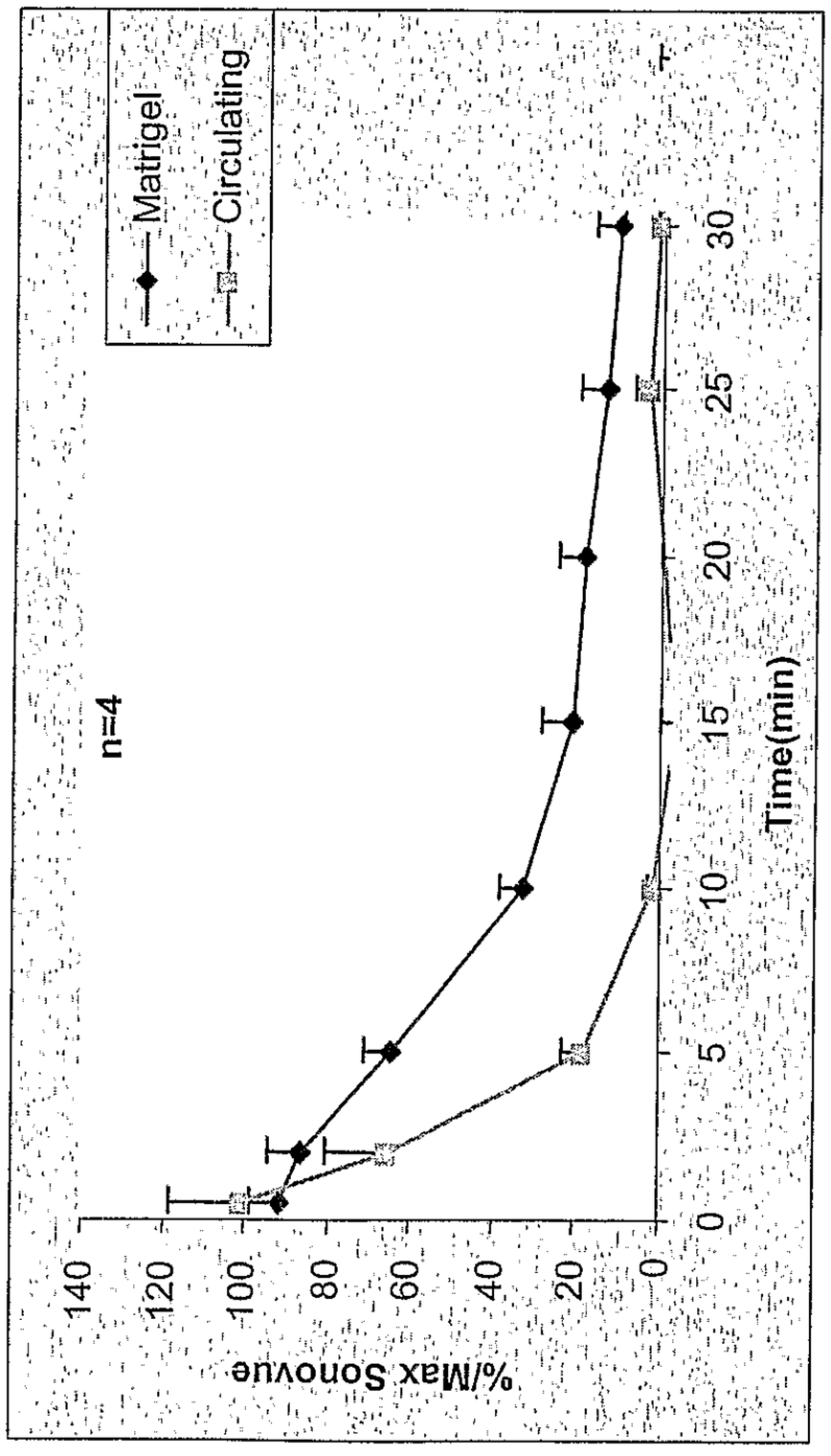

Matrigel
Circulating
n=4
%/Max Sonovue
Time(min)
0
20
40
60
80
100
120
140
0
5
10
15
20
25
30

FIG. 83

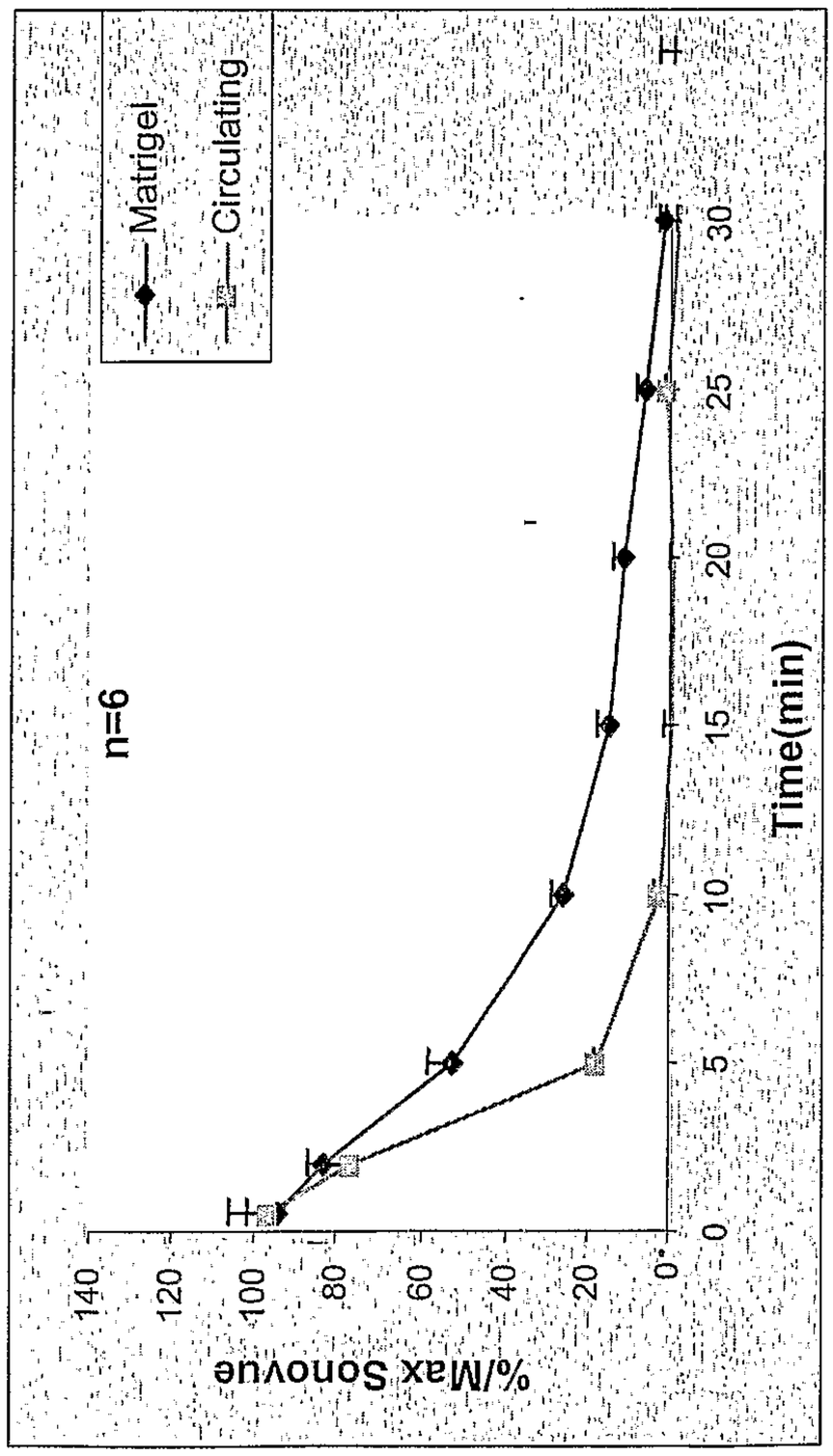

Matrigel
Circulating
n=6
%/Max Sonovue
Time(min)
0
20
40
60
80
100
120
140
0
5
10
15
20
25
30

FIG. 84

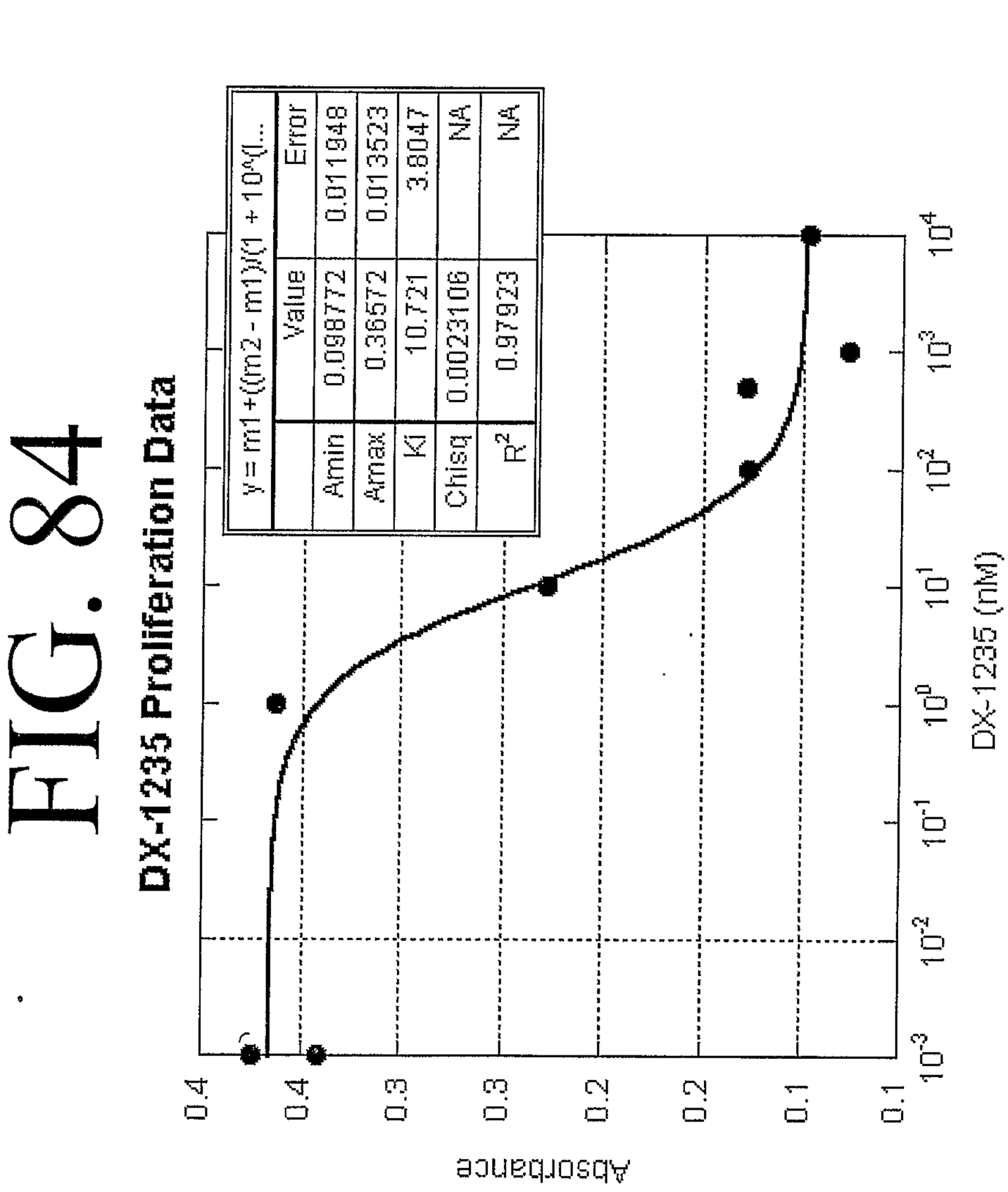
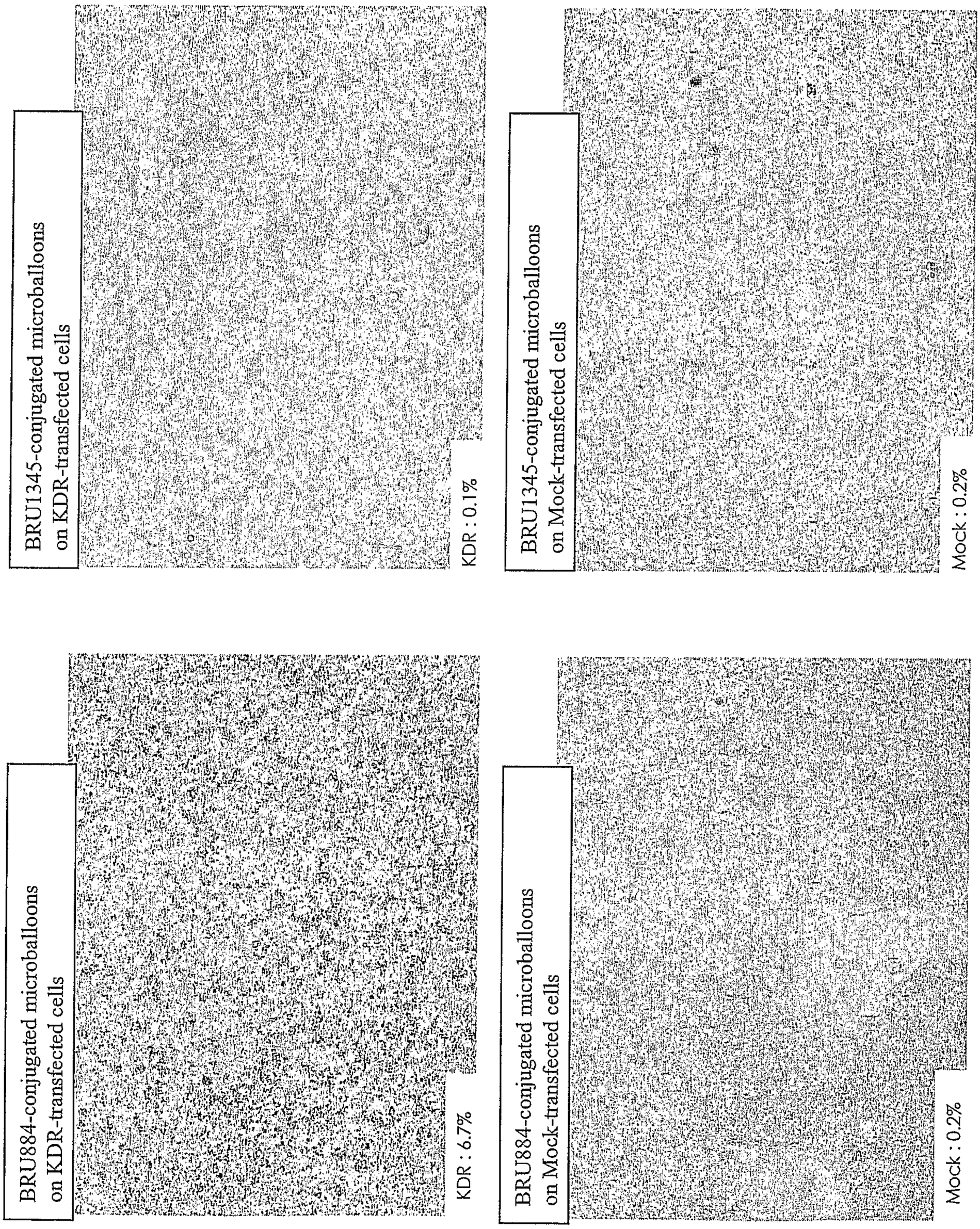

DX-1235 Proliferation Data
y = m1+((m2 - m1)/(1 + 10^(l...
Value
Error
Amin
0.098772
0.011948
Amax
0.36572
0.013523
KI
10.721
3.8047
Chisq
0.00231106
NA
R2
0.97923
NA
Absorbance
0.4
0.4
0.3
0.3
0.2
0.2
0.1
0.1
10-3
10-2
10-1
100
101
102
103
104
DX-1235 (nM)

FIG. 85


BRU1345-conjugated microballoons on KDR-transfected cells
KDR : 0.1%
BRU1345-conjugated microballoons on Mock-transfected cells
Mock : 0.2%
BRU884-conjugated microballoons on KDR-transfected cells
KDR : 6.7%
BRU884-conjugated microballoons on Mock-transfected cells
Mock : 0.2%