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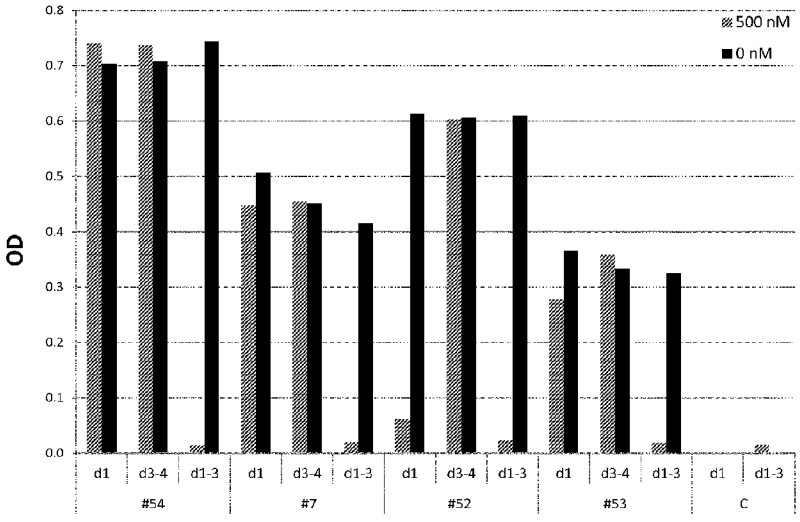
(54) Title: BINDING PROTEINS COMPRISING AT LEAST TWO REPEAT DOMAINS AGAINST HER2

Fig. 1A



(57) Abstract: The present invention relates to a recombinant binding protein comprising at least a first and a second repeat domain, wherein each of said two repeat domains binds the extracellular region of HER2 and wherein said repeat domains are covalently linked

Binding proteins comprising at least two repeat domains against HER2.

Field of the invention

The present invention relates to binding proteins comprising at least two repeat domains with binding specificity for human epidermal growth factor receptor 2 (HER2), as well as nucleic acids encoding such HER2 binding proteins, pharmaceutical compositions comprising such proteins and the use of such proteins in the treatment of diseases.

Background of the invention

Human epidermal growth factor receptor 2 (HER2; human HER2 has the UniProtKB/Swiss-Prot number P04626) also known as ErbB2 is a protein that in humans is encoded by the *ERBB2* gene. Amplification or over-expression of this gene has been shown to play an important role in the pathogenesis and progression of certain types of cancer and in recent years it has evolved to become an important biomarker and target of disease therapy. HER2 is a trans-membrane receptor tyrosine kinase (RTK) belonging to the wider family of ErbB receptors (Bublil, E.M. and Yarden, Y. Curr. Opin. Cell Biol. *19(2)*, 124-34, 2007). The ErbB receptor family is conserved across vertebrates and also includes the family founder ErbB1 (also named epidermal growth factor receptor (EGFR) or HER1; P00533 number in UniProKB/Swiss-Prot for the human protein) and the more recently identified receptors HER3 (also named ErbB3; P21860 number in UniProKB/Swiss-Prot for the human protein) and HER4 (also named ErbB4; Q15303 number in UniProKB/Swiss-Prot for the human protein). All ErbB receptors share extensive sequence and domain homologies, and form functional homodimers (e.g. ErbB1-ErbB1, HER2-HER2 and HER4-HER4) and heterodimers in all combinations. Receptor homo- and heterodimerization occurs upon ligand binding or receptor overexpression, and in turn activates intracellular receptor kinase domains by autophosphorylation. This then triggers downstream intracellular signaling and biological responses. In contrast to the other ErbB-receptors, HER2 does not have any known ligand and is able to dimerize, which is strongly pronounced after its overexpression and is thereby activated without previous ligand binding. Importantly, HER3 has no active intracellular kinase domain and is activated through heterodimerization with other ErbB receptor family members leading to very potent downstream signaling. Such heterodimerization and activation of HER3 occurs upon ligand binding to HER3 or if a partnering receptor, such as HER2, is strongly overexpressed.

HER2 as well as all the other ErbB receptor family members are composed of four extracellular domains, which are sequentially named I, II, III and IV; where domain IV is the closest to the extracellular cell membrane and domain I the most distal. In ligand-deprived conditions, domains I and III in ErbB receptors share an intramolecular interaction that occludes domain II. This prevents receptor homo-/heterodimerization and signaling, since interaction between domains II of two neighboring ErbB receptors is required for dimerization (Burguess A.W., et al., Mol. Cell *12(3)*, 541-552, 2003). Ligand binding disrupts the interaction between domains I and III, which then causes a tethered-to-extended receptor conformational change and leaves domain II exposed. This makes the receptor promiscuous to dimerize with other extended ErbB receptors and initiate signaling. Interestingly, HER2 is the only ErbB receptor family member that is constitutively found in an extended conformation; hence domain II is continuously exposed and accessible for homo- and heterodimerization.

ErbB receptor dimerization and autophosphorylation leads to the activation of a plethora of key downstream signaling molecules involved in normal physiology as well as in disease. The nature of such activated signaling molecules depends to some extend on the composition of the active ErbB receptor dimers. For instance, HER1-HER1 and HER2-HER2 homodimers preferentially activate downstream extracellular-signal-regulated kinase (ERK) signaling and proliferation, whereas HER2-HER3 heterodimers also activate the PI3K-signaling pathway (including activation of the downstream kinase AKT) and thereby cell survival. In fact, AKT activation by HER2-HER3 signaling in tumor cells promotes survival and makes tumor cells resistant to HER2 targeting drugs, such as the monoclonal antibody trastuzumab (Berns K. et al., Cancer Cell *12*, 395-402, 2007). Interestingly, inhibition of HER2-HER3 mediated PI3K-AKT signaling in these cells becomes rate-limiting and results in cell death. Apart from cell proliferation and survival, HER2 signaling has been also causally involved in other processes such as angiogenesis and migration.

HER2 is overexpressed in approximately 20% of all breast cancers. Due to its clinical relevance, HER2 became the first RTK against which a targeted biological was developed, namely trastuzumab (Herceptin®; Genentech). This antibody binds to domain IV of HER2 and inhibits HER2 signaling by several mechanisms that are not yet completely understood. These include induction of receptor internalization in tumor cells, which results in reduced HER2 expression levels and signaling and leads to an attenuated

tumorigenic phenotype. Trastuzumab has changed the life of tens of thousands of breast cancer women, expanding their lifetime and quality of life. However, trastuzumab has mainly an anti-proliferative effect and tumors may escape from such treatment in advanced disease stages. In an attempt to develop more efficacious treatments, a new antibody was generated that recognized domain II or HER2, namely pertuzumab (Omnitarg®, Perjeta®; Genentech). In contrast to trastuzumab, this antibody was not developed to reduce the membrane expression levels of HER2, but to interfere with HER2 homo- and heterodimer formation by binding to and occluding the dimerization domain II of the receptor. Pertuzumab treatment has an unexpected low therapeutic efficacy *in vitro* and *in vivo* as single agent; nevertheless, its combination with trastuzumab shows synergistic effects. Therefore, the combination of both antibodies may become a standard of care therapy for breast cancer patients (Capelan M., et al., Ann. Oncol., *24*, 273-82, 2013).

The preclinical and clinical success of the combination of trastuzumab and pertuzumab has led to the concept that dual targeting of domains II and IV in HER2 is required for superior anti-tumor efficacy. This is aligned with other molecules more recently generated to simultaneously target HER2 on domains II and IV. For instance, the Danish company Symphogen is developing antibody mixes against domains II and IV of HER2 that have shown some higher efficacy (i.e. superior to trastuzumab alone) in preclinical mouse tumor models.

Similarly, US2011/033460 describes that the combination of antibodies that bind domain I and domain IV of HER2 exhibits synergistic effects on DNA synthesis and viability of BT474 cells. Furthermore, US2011/033460 also describes bispecific antibodies that bind two different epitopes of HER2, one epitope located on domain I of HER2 and the other epitope located on domain IV of HER2.

WO 2009/068625 covers the development of biparatopic antibody constructs comprising a first antibody domain, which competes with trastuzumab for binding to HER2, and a second antibody domain, which binds to a different epitope or part of HER2. Interestingly, some constructs had an antagonistic effect of SKBR3 cell proliferation, whereas others had an agonistic effect. Especially, WO 2009/068625 covers the development of biparatopic antibody constructs comprising a first antibody domain, which competes with trastuzumab for binding to HER2 (i.e. binding domain IV of Her2) and a second antibody domain, which competes with pertuzumab for binding to HER2 (i.e. binding domain II of

HER2). Constructs where the domain IV binding antibody domain was cloned N-terminally to the domain II binding antibody domain showed blocking of map kinase activation, whereas such a blocking was not observed with the other orientation (i.e., having the domain II binding antibody domain at the N-terminus). Overall, WO 2009/068625 describes a variety of biparatopic antibody constructs targeting HER2, which have to variable extends effects on SKBR3 cell proliferation (agonistic or antagonistic) or cell signaling, but no cytotoxic nor apoptotic effects were described.

Bivalent binding proteins, such as bivalent diabody molecules or bivalent affibodies targeting HER2, are described also (Nielsen, U.B., et al., Cancer Res., *60*, 6434-6440, 2000; Steffen, A-C., Cancer Biother. Radiopharmaceut. 20, 239-248, 2005). Such molecules combine two times the same binding domain and thus are different to biparatopic molecules that comprise two binding domains each of which binds to a different epitope on the same target molecule.

As an alternative to antibody-derived therapeutics and SMIs, there are novel binding proteins or binding domains that can be used to specifically bind a target molecule (e.g. Binz, H.K., Amstutz, P. and Plückthun, A., Nat. Biotechnol. *23*, 1257-1268, 2005) and thereby act as an antagonist. One such novel class of binding proteins or binding domains not possessing an Fc are based on designed repeat proteins or designed repeat domains (WO 2002/020565; Binz, H.K., Amstutz, P., Kohl, A., Stumpp, M.T., Briand, C., Forrer, P., Grütter, M.G., and Plückthun, A., Nat. Biotechnol. *22*, 575-582, 2004; Stumpp, M.T., Binz, H.K and Amstutz, P., Drug Discov. Today *13*, 695-701, 2008).

WO 2002/020565 describes how large libraries of repeat proteins can be constructed and their general application. Such designed repeat domains harness the modular nature of repeat proteins and may possess N-terminal and C-terminal capping modules to prevent the designed repeat domains from aggregation by shielding the hydrophobic core of the domain (Forrer, P., Stumpp, M.T., Binz, H.K. and Plückthun, A., FEBS letters *539*, 2-6, 2003). This novel class of binding proteins includes designed ankyrin repeat proteins (DARPins). The generation of monospecific DARPins binding to HER2 were previously described (e.g. Steiner, D., Forrer, P. and Plückthun, A., J. Mol. Biol. *382*, 1211-1227, 2008; Zahnd, C., Pecorari, F., Straumann, N., Wyler, E. and Plückthun, A., J. Biol. Chem. 281(46), 35167-35175, 2006).

Recently, a bispecific designed ankyrin repeat protein was described, which targets HER2 (Jost, Ch., et al., Structure *21*, 1-13, 2013). The authors show that binding of two ankyrin repeat domains connected by a short linker (longer linkers do not work as well), one targeting domain I of Her2 and the other domain IV of Her2, causes stronger cytotoxic effects on BT474 cells as compared to trastuzumab alone, which targets domain IV of Her2. This biparatopic repeat protein works by intra-molecular cross-linking of two Her2 molecules; i.e., it connects two membrane-bound HER2 molecules, distorting them such that they cannot form signaling-competent dimers with any EGFR family member, preventing any kinase dimerization, and thus leading to the observed cytotoxic effects.

Even though the prior art indicates that targeting of HER2 is beneficial for the therapy of diseases, such as cancer, there is a clear need to generate binding proteins targeting HER2 with higher efficacy.

Object of the present invention

It is an object of the present invention to provide new antagonists to Her2.

It is another object of the present invention to provide a new mechanism of inhibiting HER2-related cell signaling.

It is another object of the present invention to provide a novel approach to inhibit HER2-mediated cell proliferation and/or to induce apoptosis in a cell (e.g. tumor cell), tissue, organ or patient.

It is another object of the present invention to provide a monotherapeutic approach that addresses two domains of Her2 by using biparatopic repeat proteins.

It is another object of the present invention to provide new therapeutic options for cancer.

It is another object of the present invention to provide a treatment against a neoplastic disease, which has good efficacy and/or little side effects.

It is another object of the present invention to provide an alternative treatment against neoplastic diseases which do not (or only partially) respond, or are resistant, to, therapies from the prior art.

Summary of the invention

These objects are achieved by the subject matter of the independent claims, while the dependent claims as well as the specification disclose further preferred embodiments.

While the invention has been illustrated and described in detail in the drawings and foregoing description, such illustration and description are to be considered illustrative or exemplary and not restrictive; the invention is not limited to the disclosed embodiments. Other variations to the disclosed embodiments can be understood and effected by those skilled in the art in practicing the claimed invention, from a study of the drawings, the disclosure, and the appended claims. In the claims, the word "comprising" does not exclude other elements or steps, and the indefinite article "a" or "an" does not exclude a plurality. The mere fact that certain measures are recited in mutually different dependent claims does not indicate that a combination of these measures cannot be used to advantage. Any reference signs in the claims should not be construed as limiting the scope.

Brief Description of the Figures

Figure 1. *Binding of DARPin domains to HER2*

The binding of monovalent DARPins to the HER2 extra cellular domain (domain I-IV) was tested by competition ELISA using purified HER2 domains (domain I, domain III-IV or domain I-III) as competitors, as depicted in Figure 1A and 1B. In presence of 500nM of Her2 domain I, the DARPin #51 and DARPin #52 cannot bind HER2 (domain I-IV) anymore, indicating that they bind an epitope located on domain I. DARPin #7, DARPin #53 and DARPin #54 are binding domain II as neither 500nM of Her2 domain I nor 500nM of Her2 domain III-IV can prevent their binding to the full length Her2 (domain I-IV). Figure 1.C shows that the monovalent DARPins can bind on the preformed HER2-pertuzumab complex and are thus binding a different epitope than pertuzumab on the HER2 domain II. See below for the definitions of the DARPins. OD, optical density at 450 nM minus OD at 620 nm; C, a control DARPin, which is not binding HER2; d1, domain I of HER2; d1-3; domain I-III of HER2; d3-4, domain III-IV of HER2.

Figure 2. *Inhibition of BT474 cell proliferation by monovalent and biparatopic binding proteins*

The inhibition of BT474 proliferation by monovalent DARPins (i.e. DARPin # 1and DARPin #18), a non-covalent mixture of these monovalent DARPins and biparatopic binding proteins comprising these monovalent DARPins in different orientations (DARPin # 41 and DARPin #49) was tested. Figure 2A shows the inhibition of proliferation by various concentrations of biparatopic DARPins and the corresponding fitted inhibition curves are shown for a distinct single experiment. The IC_{50} value for DARPin #41 was then calculated to be about 2 nM. The IC_{50} values for distinct DARPins are listed in Table 2. The graph in Figure 2A shows OD, optical density at 450 nm minus OD at 620 nm plotted against C, concentration of DARPins in nM. The X axis is shown in logarithmic scale. Figure 2B shows inhibition of proliferation at a concentration of 100nM for biparatopic DARPins, a mixture of both monovalent DARPins and the individual corresponding monovalent DARPins. The OD is plotted on the Y-axis. Inhibition of proliferation is reflected by a low OD. See below for the definitions of the DARPins. #41, DARPin #41; #49, DARPin #49; #18, DARPin #18; #1, DARPin #1; n.c., negative control.

Figure 3. *Inhibition of BT474 cell proliferation by various biparatopic DARPins*
Inhibition of BT474 proliferation by a subset of biparatopic DARPins (#23, #24, #33, #37, #43, #44 and #41) comprising different N-terminal and/or C-terminal ankyrin repeat domains is shown. The inhibition of proliferation by various concentrations of DARPins and the corresponding fitted inhibition curves are shown for a distinct single experiment each. The IC_{50} values for distinct DARPins are listed in Table 2. Figure 3A shows inhibition of biparatopic DARPins having DARPin #15 and Figure 3B shows inhibition of biparatopic DARPins having DARPin #18 at the C-terminus. Figure 3C and 3D show inhibition of biparatopic DARPins having DARPin #51 at the N-terminus and DARPin #18 on the C-terminus and Figure 3D shows inhibition of biparatopic DARPins having DARPin #51 at the N-terminus and DARPin #21 at the C-terminus. Graph show OD, optical density at 450nm minus OD at 620nm plotted against C, concentration of DARPins in nM. The X axis is shown in logarithmic scale. See below for the definitions of the DARPins. #23, DARPin #23; #24, DARPin #24; .#33, DARPin #33; #37, DARPin #37; #41, DARPin #41; #43, DARPin #43; #44, DARPin #44.

Figure 4. *Inhibition of cell proliferation by biparatopic DARPin #41 in different cell lines*
Inhibition of proliferation of NCI-N87 (Figure 4A) and ZR75-30 (Figure 4B) and MDA-MB175 (Figure 4C) by DARPin #41 and trastuzumab was tested. The inhibition of proliferation by various concentrations of DARPins and the corresponding fitted inhibition curves are shown for a distinct single experiment each. The IC_{50} values for distinct cell

lines are listed in Table 3. Graph shows OD, optical density at 450 nm minus OD at 620 nm plotted against C, concentration of DARPins in nM. The X axis is shown in logarithmic scale. See below for the definitions of the DARPins and reference molecules. #41, DARPin #41; T, trastuzumab.

Figure 5. *Induction of apoptosis by biparatopic DARPin #41 in different cell lines*

Induction of apoptosis in BT474 cells (Figure 5A) and NCI-N87 cells (Figure 5B) and MDA-MB175 (Figure 5C) by DARPin #41 and trastuzumab was tested. The induction of apoptosis by various concentrations of DARPins and the corresponding fitted inhibition curves are shown for a distinct single experiment each. The EC_{50} values for distinct cell lines are listed in Table 3. Graph in Figure 5A shows OD, optical density at at 450 nm minus OD at 490 nm plotted against C, concentration of DARPins of trastuzumab in nM. Graph in Figure 5B and 5C shows RLU, relative light units plotted against C, concentration of DARPins or trastuzumab in nM. The X axis is shown in logarithmic scale. See below for the definitions of DARPins. T, trastuzumab; #41, DARPin #41.

Figure 6. *Comparison of efficacy of DARPin #41 with benchmarks in inhibition of cell proliferation and induction of apoptosis.*

Inhibition of proliferation (Figure 6A) and induction of apoptosis (Figure 6B) in BT474 cells was tested for DARPin #41 and the benchmarks trastuzumab and pertuzumab and a combination of 100 nM trastuzumab and a titration of pertuzumab. Figure 6A shows inhibition of proliferation by various concentrations of DARPin, respectively benchmark concentrations and the corresponding fitted inhibition curves are shown for a distinct single experiment each. The IC_{50} values for distinct cell lines are listed in Table 3. The Graph shows OD, optical density at at 450 nm minus OD at 620 nm plotted against C, concentration of DARPin / benchmarks in nM. The X axis is shown in logarithmic scale. Figure 6B shows induction of apoptosis by various concentrations of DARPin, respectively benchmark concentrations and the corresponding fitted activation curves are shown for a distinct single experiment each. The EC_{50} values for distinct cell lines are listed in Table 3. The Graph shows relative light units (RLU) plotted against C, concentration of DARPin / benchmarks in nM. The X axis is shown in logarithmic scale. See below for the definitions of DARPins. T, trastuzumab; P, pertuzumab; #41, DARPin #41.

Figure 7. *Inhibition of BT474 cell proliferation by different formats of biparatopic binding proteins*

The inhibition of BT474 proliferation by different formats of biparatopic DARPins composed DARPin #1 at the N-terminus and DARPin #18 at the C-terminus is shown. Figure 7A shows the inhibition of proliferation by various concentrations of biparatopic DARPins, which were engineered to have a long serum half live, and the corresponding fitted inhibition curves are shown for a distinct single experiment. The biparatopic DARPin #63 is PEGylated at its C-terminal Cys residue, whereas the biparatopic DARPins #64 and #65 comprise an ankyrin repeat domain binding to serum albumin. Figure 7B shows the inhibition of proliferation by various concentrations of biparatopic DARPins comprising different linkers between the repeat domains binding HER2 and the corresponding fitted inhibition curves are shown for a distinct single experiment. The IC_{50} values for DARPins are listed in Table 2. Graph shows OD, optical density at 450 nm minus OD at 620 nm plotted against C, concentration of DARPins in nM. The X axis is shown in logarithmic scale. See below for the definitions of the DARPins. #66, DARPin #66, which comprises a short two amino acid long GS-linker between the two repeat domains; #67, DARPin #67, which comprises a five amino acid long GS-linker between the two repeat domains; #41, DARPin #41, which comprises a ten amino acid long GS-linker between the two repeat domains; #68, DARPin #68, which comprises a 24 amino acid long PT-linker between the two repeat domains.

Detailed description of the invention

According to one embodiment of the invention, a recombinant binding protein comprising at least a first and a second repeat domain, wherein each of said two repeat domains binds the extracellular region of HER2 and wherein said repeat domains are covalently linked.

It has surprisingly turned out that binding of the extracellular part of HER2 with a recombinant binding protein comprising at least two covalently linked repeat domains, each with specificity for the extracellular region of HER2, has advantageous and unexpected effects over prior art approaches as outlined above, which bind HER2 with distinct and individual binders (e.g., a combination of trastuzumab and pertuzumab; Figure 6).

Human HER2 consists of 1255 amino acids with a 21 amino acid signal sequence, a 631 amino acid extracellular region (e.g. the ectodomain comprising domains I to IV), a 23 amino acid transmembrane region, and a 580 amino acid cytoplasmic domain.

Preferably, said binding of the extracellular region of HER2 by said recombinant binding protein is a simultaneous or concurrent binding of said repeat domains to said extracellular region of HER2. Also preferably, said repeat domains bind to two different epitopes of the extracellular region of HER2. Also preferably, said repeat domains bind to two different and non-overlapping epitopes of the extracellular region of HER2.

One reason for this increased efficacy could be that a recombinant binding protein according to the invention induces a so far not described tethered conformation of the extracellular region of HER2, which seems to be the consequence of an intramolecular interaction of the biparatopic binding protein of the invention with two different epitopes on the extracellular region of HER2 (Example 8); i.e. both repeat domains of the binding protein seem to bind simultaneously to different epitopes on the same HER2 molecule and thereby forcing the extracellular region of HER2 in this new tethered conformation. Such a tethered conformation is not described by the prior art. Importantly, these two repeat domains need to be linked by being present in the same binding protein; i.e. a simple mixture of the two repeat domains does not show efficacy (Fig. 2B). Furthermore, the bivalent binding of such a binding protein to the extracellular region of HER2 could develop synergistic binding effects by exhibiting increased avidity, i.e., a combined strength of synchronous binding to different epitopes of the target. Avidity is distinct from affinity, which corresponds to the strength of a single binding interaction. Overall, this specific interaction of the binding protein with HER2 may explain the very effective inhibition of proliferation and induction of apoptosis by such molecules as shown in the examples.

According to this theory the two different repeat domains in the same protein synergistically support each other in binding their respective epitope, thus leading to an increase in overall affinity to the target.

Binding of the first repeat domain to its epitope on HER2 brings the second repeat domain into an energetically and/or sterically favorable position which facilitates it's binding to its respective epitope on HER2.

As shown in the examples the covalent linkage of the first and the second repeat domain seems to potentiate their biological activity.

In a preferred embodiment of the recombinant binding protein according to the invention a first repeat domain binds domain II of HER2 and a second repeat domain binds domain IV of HER2.

It is important to understand that the term “binds domain II” means that the respective repeat domain binds primarily domain II of HER2. This definition, however, does not exclude that the parts of said repeat domain can bind, or overlap, to other domains. The same applies for the term “binds domain IV”.

A simultaneous targeting of domains II and IV of HER2 by a biparatopic binding protein according to the present invention has particular unexpected effects over what was known from the prior art. Cell responses in terms of inhibition of proliferation and induction of cell apoptosis by such binding proteins were much more dramatic when compared to effects obtained by state of the art antibodies. For example, the extent of such responses has proved to be superior to that induced by clinical antibody benchmarks, such as the combination of trastuzumab and pertuzumab targeting domain IV and II of HER2, respectively (Fig. 4, 5 and 6). Interestingly, some biparatopic binding proteins binding to domain I and domain IV of HER2 do not show such unexpected effects (Fig. 3C and 3D).

Methods to determine the domain of the extracellular region of HER2 to which a repeat domain binds, e.g. as shown in Example 3, are well known to the person skilled in the art (e.g. Jost et al., loc. cit.).

Applicant's findings have important implications for the treatment of HER2-driven human cancers, in the sense that simultaneous targeting of domains II and IV of HER2 with a biparatopic binding protein according to the present invention could be a more efficacious alternative to current antibody targeting approaches.

The binding protein according to the present invention is thus preferably a biparatopic binding protein, i.e., it comprises two antigen repeat domains recognizing two different epitopes, or domains (e.g. domains II and IV) on the same protein target (namely HER2). However, polypeptides which are multiparatopic, i.e, containing antigen repeat domains recognizing three, four or more epitopes on the same target protein, are encompassed within the scope invention, as are polypeptides which are both bi- or multiparatopic and multivalent, i.e,, having also antigen repeat domains recognizing one or more other target proteins.

HER2, as used herein, relates to Human Epidermal Growth Factor Receptor 2, also known as Neu, ErbB-2, CD340 (cluster of differentiation 340) or p185. HER2 is a member of the epidermal growth factor receptor (EGFR/ErbB) family. HER2 is, in humans, encoded by ERBB2, a known proto-oncogene located at the long arm of human chromosome 17 (17q12). HER2 has the UniProtKB/Swiss-Prot number P04626.

According to a preferred embodiment of the invention, the first and second repeat domains are located on the same polypeptide, while the repeat domain targeting domain II of HER2 is located N-terminally to the repeat domain targeting domain IV of HER2.

These embodiments are for example shown in Fig. 2A, and the corresponding description. The inventors have, surprisingly, shown that a binding protein in which the repeat domain targeting domain II of HER2 is located C-terminally to the repeat domain targeting domain IV of HER2 is significantly less efficacious than a binding protein in which the repeat domain targeting domain II of HER2 is located N-terminally to the repeat domain targeting domain IV of HER2.

Preferably, said first repeat domain binding domain II of HER2 is not competing for binding to HER2 with pertuzumab. For example, Fig. 1C shows such repeat domains not competing for binding to HER2 with pertuzumab. Likewise preferably, said second repeat domain binding domain IV of HER2 is not competing for binding to HER2 with trastuzumab. For example, the repeat domains of DARPins #18 to 20 do not compete for binding to HER2 with trastuzumab. Methods to determine if a repeat domain does not compete for binding to HER2 with trastuzumab or pertuzumab, e.g. as shown in Example 3, are well known to the person skilled in the art.

This means that, in the first preferred embodiment, the first repeat domain binds a different epitope of domain II of HER2 than pertuzumab. Likewise, in the second preferred embodiment, the second repeat domain binds a different epitope of domain IV of HER2 than trastuzumab. Without being bound to theory, the inventors attribute at least some of the effects shown in the experimental section to these facts.

According to another preferred embodiment of the invention said first repeat domain is an ankyrin repeat domain, or a designed ankyrin repeat domain, and said second repeat domain is an ankyrin repeat domain, or a designed ankyrin repeat domain.

Preferably, said ankyrin repeat domains or designed ankyrin repeat domains comprise between 70 and 300 amino acids, in particular between 90 and 200 amino acids.

Also preferably, a repeat domain of the invention is an ankyrin repeat domain or a designed ankyrin repeat domain as described in WO 2002/020565. Examples of designed ankyrin repeat domains with biparatopic binding specificity for different domains of Her2 are shown in the Examples.

According to a preferred embodiment of the invention, the first repeat domain binds the extracellular region of HER2 in PBS with a Kd smaller than 10^{-7}M and said second repeat domain binds the extracellular region of HER2 in PBS with a Kd smaller than 10^{-7}M.

Kd is the dissociation constant and will further be defined in the text below. A Kd smaller than 10^{-7}M is required to provide sufficient affinity of the repeat domain to its target. Preferably, the repeat domains bind their target domains in PBS with a Kd smaller than 10_{-8}M, 10^{-9}M, 10^{-10}M, or, most preferably smaller than 10^{-11}M.

Recombinant binding proteins comprising proteins binding domain II and/or domain IV of Her2 with a Kd in PBS below 10^{-7}M are shown in Example 2.

According to a preferred embodiment, said binding protein inhibits stimulated proliferation of BT474 cells with an half maximal inhibitory concentration (IC50) value of smaller than 100 nM. Preferably, said binding protein inhibits stimulated proliferation of BT474 cells with an IC50 value of smaller than 90, 80, 70, 60, 50, 40, 30, 20 or 10 nM. Also preferably, said binding protein inhibits stimulated proliferation of BT474 cells by at least 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20% or 10%.

BT474 cells can be used to measure the functional capability of the binding proteins of the invention to inhibit proliferation by standard means well known to the person skilled in the art, e.g. as shown in Example 4. Preferably, BT474, SKBR-3, NCI-N87, ZR75-30, HCC1419 or MDA-MB175 cells can be used to measure the functional capability of the compounds of the invention to inhibit proliferation, e.g. as shown in Example 5.

Recombinant binding proteins which inhibit stimulated proliferation of BT474 cells with an IC50 value of smaller than 100 nM are disclosed, and discussed, in Example 4.

According to another preferred embodiment, said binding protein induces apoptosis in BT474 cells with an half maximal effective concentration (EC50) value of smaller than 100 nM. Preferably, said binding protein induces apoptosis in BT474 cells with an EC50 value of smaller than 90, 80, 70, 60, 50, 40, 30, 20 or 10 nM.

BT474 cells can be used to measure the functional capability of the binding proteins of the invention to induce apoptosis by standard means well known to the person skilled in the art, e.g. as shown in Example 5. Preferably, BT474, SKBR-3, NCI-N87, ZR75-30, HCC1419 or MDA-MB175 cells can be used to measure the functional capability of the compounds of the invention to induce apoptosis, e.g. as shown in Example 5.

Recombinant binding proteins which induce apoptosis in BT474 cells with an EC50 value of smaller than 100 nM are disclosed, and discussed, in Examples 5.

According to a preferred embodiment, said first and second repeat domains are connected by a polypeptide linker.

Such polypeptide linker may, for example, be accomplished by mere genetic fusion of the encoding cDNAs of the respective domains to be fused. Such type of embodiment qualifies as a fusion peptide protein with two different repeat domains.

The linker can for example consist of an oligopeptide comprising the amino acids G and S, or P and T, respectively, as set forth in SEQ ID Nos: 7 to 12. According to another preferred embodiment, a "multimerization moiety" as described below can be used. Alternatively, the two repeat domains can be linked to one another, e.g., by means of non-peptide based chemical linkers.

Preferably, the recombinant binding protein and/or repeat domain has a midpoint denaturation temperature (Tm) above 45°C, more preferably above 50°C, more preferably above 55°C, and most preferably above 60°C upon thermal unfolding in PBS at pH 7.4. A binding protein or a repeat domain of the invention possesses a defined secondary and tertiary structure under physiological conditions. Thermal unfolding of such a polypeptide results in a loss of its tertiary and secondary structure, which can be followed, for example, by circular dichroism (CD) measurements. The midpoint denaturation temperature of a binding protein or repeat domain upon thermal unfolding corresponds to

the temperature at the midpoint of the cooperative transition in physiological buffer upon heat denaturation of said protein or domain by slowly increasing the temperature from 10°C to about 100°C. The determination of a midpoint denaturation temperature upon thermal unfolding is well known to the person skilled in the art. This midpoint denaturation temperature of a binding protein or repeat domain upon thermal unfolding is indicative of the thermal stability of said polypeptide.

Also preferred is a recombinant binding protein and/or ankyrin repeat domain forming less than 5% (w/w) insoluble aggregates at concentrations up to 20 g/L, preferably up 40 g/L, more preferably up to 60 g/L, even more preferably up to 80 g/L, and most preferably up to 100 g/L when incubated for over 5 days, preferably over 10 days, more preferably over 20 days, more preferably over 40 days, and most preferably over 100 days at 37°C in PBS. The formation of insoluble aggregates can be detected by the appearance of visual precipitations, gel filtration or dynamic light scattering, which strongly increases upon formation of insoluble aggregates. Insoluble aggregates can be removed from a protein sample by centrifugation at 10'000 x g for 10 minutes. Preferably, a recombinant binding protein and/or ankyrin repeat domain forms less than 2%, more preferably less than 1%, 0.5%, 0.2%, 0.1%, or most preferably less than 0.05% (w/w) insoluble aggregates under the mentioned incubation conditions at 37°C in PBS. Percentages of insoluble aggregates can be determined by separation of the insoluble aggregates from soluble protein, followed by determination of the protein amounts in the soluble and insoluble fraction by standard quantification methods.

Also preferred is a recombinant binding protein and/or ankyrin repeat domain that does not lose its native three-dimensional structure upon incubation in PBS containing 100 mM dithiothreitol (DTT) for 1 or 10 hours at 37°C.

In one particular embodiment the invention relates to a recombinant binding protein comprising two ankyrin repeat domains, specifically binding to HER2 and having the indicated or preferred midpoint denaturation temperature and non-aggregating properties as defined above.

According to other preferred embodiments of the invention, it is provided that

- said first repeat domain competes for binding to HER2 with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 62 to 68, 72 and 114 to 121 and/or

- said second repeat domain competes for binding to HER2 with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 74 to 82.

The inventors have evidence that, out of these repeat domains, the first repeat domain binds domain II of HER2, whereas the second repeat domain binds domain IV of HER2

Preferably, said first repeat domain competes for binding to HER2 with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 62 to 67 and 115 to 121. More preferably, said first repeat domain competes for binding to HER2 with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 62, 115, 120, and 121, in particular SEQ ID NO: 115 and 120. Also preferably, said first repeat domain competes for binding to HER2 with a binding protein selected from the group of DARPins #1 to 6 and 54 to 60; more preferably, with a binding protein from the group of DARPins #1, 54, 59 and 60; in particular, with a binding protein from the group of DARPins #54 and 60.

Further preferred, said second repeat domain competes for binding to HER2 with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 79 to 81, in particular SEQ ID NO: 80 and 81. Also preferably, said second repeat domain competes for binding to HER2 with a binding protein selected from the group of DARPins #18 to 20; in particular, with a binding protein from the group of DARPins #19 and 20.

According to still other preferred embodiments of the invention, it is provided that

- a first repeat domain comprises an amino acid sequence that has at least 70% amino acid sequence identity with one ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 62 to 68, 72 and 114 to 121,
- a second repeat domain comprises an amino acid sequence that has at least 70% amino acid sequence identity with one ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 74 to 82,

and wherein further,

- G at position 1 and/or S at position 2 of said ankyrin repeat domain are optionally missing; and

- L at the second last position and/or N at the last position of said ankyrin repeat domain are optionally exchanged by A.

Preferably, said first repeat domain comprises an amino acid sequence that has at least 70% amino acid sequence identity with one ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 62 to 67 and 115 to 121. More preferably, said first repeat domain comprises an amino acid sequence that has at least 70% amino acid sequence identity with one ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 62, 115, 120, and 121, in particular SEQ ID NO: 115 and 120. Also preferably, said first repeat domain comprises an amino acid sequence that has at least 70% amino acid sequence identity with a binding protein selected from the group consisting of DARPins #1 to 6 and 54 to 60; more preferably, with a binding protein from the group of DARPins #1, 54, 59 and 60; in particular, with a binding protein from the group of DARPins #54 and 60.

Further preferred, said second repeat domain comprises an amino acid sequence that has at least 70% amino acid sequence identity with one ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 79 to 81, in particular SEQ ID NO: 80 and 81. Also preferably, said second repeat domain comprises an amino acid sequence that has at least 70% amino acid sequence identity with a binding protein from the group consisting of of DARPins #18 to 20; in particular, with a binding protein from the group of DARPins #19 and 20.

Preferably, the first ankyrin repeat domain comprises an amino acid sequence that has at least 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 % amino acid sequence identity with one ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 62 to 68, 72 and 114 to 121.

Preferably, the second ankyrin repeat domain comprises an amino acid sequence that has at least 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 % amino acid sequence identity with one ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 74 to 82.

Also preferably, the first ankyrin repeat domain comprises an amino acid sequence that has at least 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89,

90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 % amino acid sequence identity with one, two or three ankyrin repeat modules present between the N-terminal and C-terminal capping modules of an ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 62 to 68, 72 and 114 to 121.

Also preferably, the second ankyrin repeat domain comprises an amino acid sequence that has at least 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 % amino acid sequence identity with one, two or three ankyrin repeat modules present between the N-terminal and C-terminal capping modules of an ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 74 to 82.

According to yet other preferred embodiments of the invention, it is provided that

- said first repeat domain is selected from the group consisting of SEQ ID NOs: 62 to 68, 72 and 114 to 121,
- said second repeat domain is selected from the group consisting of SEQ ID NOs:74 to 82

and wherein further

- G at position 1 and/or S at position 2 of said ankyrin repeat domain are optionally missing; and
- L at the second last position and/or N at the last position of said ankyrin repeat domain are optionally exchanged by A.

Preferably, the first ankyrin repeat domain is selected from the group consisting of SEQ ID NOs: 62 to 67 and 115 to 121; more preferably, 115, 120, and 121; in particular, SEQ ID NO: 115 and 120.

Preferably, the second ankyrin repeat domain is selected from the group consisting of SEQ ID NOs: 79 to 81, in particular SEQ ID NO: 80 and 81.

According to yet other preferred embodiments of the invention, it is provided that

- said first repeat domain comprises an ankyrin repeat module having an amino acid sequence selected from the group consisting of SEQ ID NO: 15 to 18, 21 to 23, 37, 38, 125, 126, 129, 130, 133 and 134 and sequences, wherein up to 9 amino acid residues in SEQ ID NO: 15 to 18, 21 to 23, 37, 38, 125, 126, 129, 130, 133 and 134 are replaced by any other amino acid residues, and/or

- said second repeat domain comprises an ankyrin repeat module having an amino acid sequence selected from the group consisting of SEQ ID NO: 46, 47, 51, 52, 55 and 56, and sequences, wherein up to 9 amino acid residues in SEQ ID NO: 46, 47, 51, 52, 55 and 56 are replaced by any other amino acid residues.

Preferably, such an ankyrin repeat module of the first ankyrin repeat domain is selected from the group consisting of SEQ ID NO: 15 to 18, 125, 126, 129, 130, 133 and 134; more preferably, 15, 125, 129 and 133; and even more preferably, 125 and 133.

Preferably, such an ankyrin repeat module of the second ankyrin repeat domain is selected from the group consisting of SEQ ID NO: 46, 47, 55 and 56; more preferably, 55 and 56.

Also preferably, up to 8 amino acids in the repeat modules of SEQ ID NO: 15 to 18, 21 to 23, 37, 38, 46, 47, 51, 52, 55, 56, 125, 126, 129, 130, 133 and 134 are exchanged by another amino acid, more preferably up to 7 amino acids, more preferably up to 6 amino acids, more preferably up to 5 amino acids, even more preferably up to 4 amino acids, more preferably up to 3 amino acids, more preferably up to 2 amino acids, and most preferably 1 amino acid.

Preferably, when amino acids are exchanged in capping modules, repeat modules or repeat domains, repeat domains, or binding proteins, these amino acids are replaced by an amino acid selected from the group consisting of A, D, E, F, H, I, K, L, M, N, Q, R, S, T, V, W and Y; more preferably from the group consisting of A, D, E, H, I, K, L, Q, R, S, T, V, and Y. Also preferably, an amino acid is exchanged by a homologous amino acid; i.e. an amino acid is exchanged by an amino acid having a side chain with similar biophysical properties. For example, the negative charged amino acid D may be replaced by the negative charged amino acid E, or a hydrophobic amino acid such as L may be replaced by A, I or V. The techniques of exchanging an amino acid by another amino acid in a polypeptide are well known to the person skilled in the art.

Preferably, the repeat module according to the invention has an amino acid sequence selected from the group consisting of KDFQGITPLHIAATSGHLEIVEVLLKAGADVNA (SEQ ID NO: 16 and sequences, in which up to 9 amino acid residues in SEQ ID NO: 16 are replaced by any other amino acid residues, and wherein

- F at position 3 is optionally exchanged by A

- Q at position 4 is optionally exchanged by E;
- G at position 5 is optionally exchanged by S;
- I at position 6 is optionally exchanged by V;
- I at position 11 is optionally exchanged by L;
- T at position 14 is optionally exchanged by Q; and/or
- N at position 15 is optionally exchanged by an amino acid selected from the group consisting of S and W.

One very preferred repeat module of this group has an amino acid sequence consisting of KDFQGVTPLHIAAQSGHLEIVEVLLKAGADVNA (SEQ ID NO: 125), SEQ ID NO: 129 or SEQ ID NO: 133.

Also preferably, the ankyrin repeat module according to the invention has an amino acid sequence selected from the group consisting of KDITGETPLHHAADSGHLEIVEVLLKAGADVNA (SEQ ID NO: 18) and sequences, in which up to 9 amino acid residues in SEQ ID NO: 18 are replaced by any other amino acid residues, and wherein

- I at position 3 is optionally exchanged by V;
- E at position 6 is optionally exchanged by D;
- H at position 11 is optionally exchanged by L;
- D at position 14 is optionally exchanged by Q;
- S at position 15 is optionally exchanged by H; and/or
- E at position 19 is optionally exchanged by V.

One very preferred repeat module of this group has an amino acid sequence consisting of KDVTGDTPLHLAAQHGHLEIVEVLLKAGADVNA (SEQ ID NO: 126), SEQ ID NO: 130 or SEQ ID NO: 134.

Also preferably, the ankyrin repeat module according to the invention has an amino acid sequence selected from the group consisting of KDWEGTTPLHLAAHTGHLEIVEVLLKAGADVNA (SEQ ID NO: 21) and sequences, in which up to 9 amino acid residues in SEQ ID NO: 21 are replaced by any other amino acid residues, and wherein

- W at position 3 is optionally exchanged by F;
- W at position 4 is optionally exchanged by Q;

- T at position 6 is optionally exchanged by an amino acid selected from the group consisting of I, Y and V; preferably T;
- L at position 11 is optionally exchanged by an amino acid selected from the group consisting of I and V; preferably I and V;
- H at position 14 is optionally exchanged by an amino acid selected from the group consisting of H, Q, Y and W; preferably H; and/or
- T at position 15 is optionally deleted or exchanged by an amino acid selected from the group consisting of A and D.

Also preferably, the ankyrin repeat module according to the invention has an amino acid sequence selected from the group consisting of KDTVGTTPLHYAAEDGHLEIVEVLLKAGADVNA (SEQ ID NO: 22) and sequences, in which up to 9 amino acid residues in SEQ ID NO: 22 are replaced by any other amino acid residues, and wherein

- T at position 3 is optionally exchanged by an amino acid selected from the group consisting of S, K, E and I; equal amino acid distribution;
- V at position 4 is optionally exchanged by an amino acid selected from the group consisting of Q, I and Y; preferably Y;
- T at position 6 is optionally exchanged by an amino acid selected from the group consisting of Q, F, R and W;
- Y at position 11 is optionally exchanged by an amino acid selected from the group consisting of L, E and S; preferably S;
- E at position 14 is optionally exchanged by an amino acid selected from the group consisting of S, Q, Y and V; and/or
- D at position 15 is optionally exchanged by an amino acid selected from the group consisting of S, F and Y.
- G at position 16 is optionally exchanged by D.

Also preferably, the ankyrin repeat module according to the invention has an amino acid sequence selected from the group consisting of KDVEGWTPLHYAASSGHLEIVEVLLKAGADVNA (SEQ ID NO: 38) and sequences, in which up to 9 amino acid residues in SEQ ID NO: 38 are replaced by any other amino acid residues, and wherein

- W at position 6 is optionally exchanged by Q;
- Y at position 11 is optionally exchanged by L; and/or

- S at position 15 is optionally exchanged by Y.

Also preferably, the ankyrin repeat module according to the invention has an amino acid sequence selected from the group consisting of KDWRGFTPLHYAAYLGHLEIVEVLLKAGADVNA (SEQ ID NO: 46) and sequences, in which up to 9 amino acid residues in SEQ ID NO: 46 are replaced by any other amino acid residues, and wherein

- W at position 3 is optionally exchanged by an amino acid selected from the group consisting of W, T, V and R; preferably, T and R;
- R at position 4 is optionally exchanged by an amino acid selected from the group consisting of R, T and I; preferably, I;
- F at position 6 is optionally exchanged by F or H; preferably F;
- Y at position 11 is optionally exchanged by R;
- Y at position 14 is optionally exchanged by F;
- L at position 15 is optionally exchanged by V; and/or
- H at position 17 is optionally exchanged by Q.

Preferably, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid residues in SEQ ID NOs:16, 18, 28, 31. 21, 22, 38 and/or 46 are replaced by any other amino acid residues.

Furthermore, it is particularly preferred that said binding protein comprises a polypeptide, wherein said polypeptide comprises said first and second ankyrin repeat domains and wherein said polypeptide has at least 70% amino acid sequence identity with a polypeptide selected from the group consisting of SEQ ID NO: 83 to 98, 102, 103, 122, 123 and 136 to 141.

Preferably, said polypeptide comprises an amino acid sequence that has at least 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 % amino acid sequence identity with a polypeptide selected from the group consisting of SEQ ID NOs: 83 to 98, 102, 103, 122, 123 and 136 to 141.

Also preferably, such polypeptide is selected from the group consisting of SEQ ID NO: 84, 85, 86, 87, 90, 91, 92, 98, 102, 103, 122 and 123; more preferably, 85, 86, 87, 90, 91, 92, 102, 103, 122 and 123; even more preferably, 86, 87, 91 and 92; and most preferably, 86 and 87.

According to yet other preferred embodiment, one or more of the amino acid residues of the ankyrin repeat modules of said first and second ankyrin repeat domains are exchanged by an amino acid residue found at the corresponding position on alignment of an ankyrin repeat unit.

Another embodiment of the invention provides a nucleic acid molecule encoding at least one binding protein or a particular ankyrin repeat domain according to the above description. Further, a vector comprising said nucleic acid molecule is considered.

Not all binding compositions according to the present invention comprise polypeptides or proteins. The latter embodiment only relates to those who do. For these, applicant refrains from disclosing herein all nucleic acid molecules capable of encoding them because, due to the Degeneracy of the genetic code, many nucleic acid molecules can encode for one and the same polypeptide or protein.

However, it can unequivocally and unambiguously determined whether a given nucleic acid encodes for a given polypeptide or protein. Thus, the present embodiment is clear for the skilled person, and its scope is easily determined.

Another embodiment of the invention provides the use of a binding protein according to the above description to inhibit at least one of

- HER2-receptor dimerization,
- HER2/HER3-heterodimerization,
- HER2-receptor autophosphorylation
- HER-receptor mediated signal transduction
- HER3-receptor ligand induced phosphorylation, and/or
- HER3-receptor mediated signal transduction.

HER2-receptor dimerization (also called "homodimerization") occus in tissues overexpressing HER2 independent of a ligand. Said homodimerization leads to an intracellular autophosphrylation which can eventually lead, for example, to increased cell proliferation.

Because HER3 lacks intrinsic kinase activity, HER3 is phosphorylated in HER2-overexpressing breast cancer after formation of HER2/HER3 heterodimers, which may eventually result, for example, in apoptosis inhibition.

Said use can either take place in vitro or in vivo. As set forth above, all these processes can result in pathogenic consequences, namely by activating respective signal transduction pathways. Signal transduction pathways activated by HER2 dimerization and/or HER2/HER3-heterodimerization include mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K/Akt), phospholipase C γ, protein kinase C (PKC), Signal transducer and activator of transcription (STAT), the Ras-Map kinase pathway and the mTOR pathway.

The phosphoinositide 3-kinase (PI3K/Akt) pathway is for example considered to be one of the critical pathways that is maintaining cell survival by blocking apoptosis. Pathologic activation thereof, e.g., by HER2/HER3-heterodimerization, may thus lead to malignant proliferation (e.g. see Examples)

Pathologic activation of HER2, e.g. by HER2-homodimerization, may lead to malignant cell migration, invasion or proliferation (e.g. see Examples; Hynes NE. and Lane HA., Nat. Rev. Cancer., *5*,341-54, 2005).

Yet another embodiment of the invention provides a pharmaceutical formulation comprising a binding protein or a composition according to the above disclosure, and optionally a pharmaceutical acceptable carrier and/or diluent.

Pharmaceutical acceptable carriers and/or diluents are known to the person skilled in the art and are explained in more detail below. Even further, a diagnostic composition comprising one or more of the above mentioned recombinant binding proteins, in particular binding proteins comprising repeat domains, is considered.

A pharmaceutical formulation comprises recombinant binding proteins as described above and a pharmaceutically acceptable carrier, excipient or stabilizer, for example as described in Remington's Pharmaceutical Sciences 16^{th} edition, Osol, A. Ed. [1980]. Suitable carriers, excipients or stabilizers known to the skilled man are saline, Ringer's solution, dextrose solution, Hank's solution, fixed oils, ethyl oleate, 5% dextrose in saline, substances that enhance isotonicity and chemical stability, buffers and preservatives. Other suitable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition such as proteins,

polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids and amino acid copolymers.

The formulations to be used for *in vivo* administration must be aseptic or sterile. This is readily accomplished by filtration through sterile filtration membranes. The pharmaceutical formulation may be administered by any suitable method within the knowledge of the person skilled in the art.

Further, in another embodiment of the present invention the use of at least one binding protein, composition or pharmaceutical formulation according to the above disclosure as a medicament is provided. Likewise, a process comprising administering a binding protein, composition or pharmaceutical formulation according to the aforementioned claims to a patient is provided. In both cases, it is preferred that the disease to be treated is a neoplastic disease, preferably cancer.

In each case, an effective amount of the binding protein, composition or pharmaceutical formulation according to the aforementioned claims is preferably administered to a patient for treating the disease.

The term "neoplastic disease", as used herein, refers to an abnormal state or condition of cells or tissue characterized by rapidly proliferating cell growth or neoplasm. In a more specific meaning, the term relates to cancerous processes, e.g., tumors and/or leukemias.

The binding proteins according to the invention demonstrated apoptotic and anti-proliferative effects (see experimental section). As neoplastic diseases are often characterized by suppression of apoptosis and/or increased proliferation, it is plausible to deduce, from these experiments, that the binding proteins according to the present invention can be used in the treatment of neoplastic diseases. .

Preferably, said neoplastic disease is a disease characterized by at least one selected from the group consisting of

- Amplification of the HER2 encoding gene
- Overexpression of the HER2 encoding gene,
- Expression of a mutated form of the HER2 encoding gene, and/or
- Overexpression of the Her3 encoding gene in trastuzumab resistant tumors.

In humans, HER2 is encoded by the ERBB2 gene. The above options can be ascribed to mutations in the ERBB2 gene which can be detected by means of modern molecular diagnostics, as are currently on the market.

As used herein, the term „expression of the HER2 encoding gene“ is related to cells, tissues or organs which express the HER2 receptor protein, as for example detected by immunohistochemistry (IHC). As used herein, the term "amplification or overexpression of the HER2 encoding gene“ is related to indicate an abnormal level of expression of the HER2 receptor protein in a cell, tissue or organ, relative to the level of expression in a normal cell, tissue or organ, as for example detected by Immunohistochemistry (IHC).

Such IHC detection assays are known in the art and include the Clinical Trial Assay (CTA), the commercially available LabCorp 4D5 test, and the commercially available DAKO HercepTest® (DAKO, Carpinteria, Calif.). The latter assay uses a specific score range of 0 to 3+ cell staining (0 being normal expression, 3+ indicating the strongest positive expression) to identify cancers having overexpression of the HER2 protein. Thus, patients having a cancer characterized by overexpression of the HER2 protein in the range of 1+, 2+, or 3+, preferably 2+ or 3+, more preferably 3+ would benefit from the methods of therapy of the present invention.

Alternatively, Her2 expression and/or overexpression scores can also be detected by In Situ hybridization (ISH), RT-PCT and other methods.

According to a particularly preferred embodiment, said neoplastic disease is at least one selected from the group consisting

- breast cancers
- ovarian cancer,
- gastric cancer,
- stomach cancer, and/or
- uterine cancer.
- colorectal cancer.

Furthermore, said use is preferably complemented, in a coordinated fashion, by the administration of at least one active substance selected from the group consisting of

- an antineoplastic agent
- an endocrine drug,
- a tumor vaccine,
- immunotherapy, and/or
- cellular therapy.

The term "complemented, in a coordinated fashion", as used herein, shall refer to a co-administration, which is carried out under a given regimen. This includes synchronous administration of the different compounds as well as time-shifted administration of the different compounds (e.g., compound A is given once and compound B is given several times thereafter, or vice versa, or both compounds are given synchronously and one of the two is also given at later stages).

As used herein, the term "antineoplastic agent" relates to a drug, or a combination of drugs, which have antineoplastic or anticancer effects. This applies, above all, to chemotherapeutic agents, which work by impairing mitosis, effectively targeting fast-dividing cells, or by causing cells to undergo apoptosis. The majority of chemotherapeutic drugs can be divided into alkylating agents, antimetabolites, anthracyclines, plant alkaloids, topoisomerase inhibitors, and other antitumour agents.

Preferred antineoplastic agents are 5-fluorouracil, actinomycin, adriamycin, amsacrine, anthracyclines, azathioprine, bendamustine, bleomycin, carboplatin, chlorambucil, cisplatin, cyclophosphamide, daunorubicin, docetaxel, doxorubicin, epirubicin, etoposide, idarubicin, ifosfamide, irinotecan, mechlorethamine, mercaptopurine, methotrexate, mitomycin, oxaliplatin, paclitaxel, plicamycin, podophyllotoxin, teniposide, topotecan., valrubicin, vinblastine, vincristine, vincristine, vindesine, and/or vinorelbine.

Immunotherapy involves the isolation of proteins from cancer cells and subsequent immunization of cancer patients against those proteins, in the hope of stimulating an immune reaction that would kill the cancer cells. Another approach to therapeutic anti-cancer vaccination is to generate the immune response in situ in the patient. This enhances the anti-tumor immune response to tumor antigens released following lytic virus replication providing an in situ, patient specific anti-tumor vaccine as a result. Yet another approach is to immunize the patient with a compound that plays a physiological role in cancer genesis, so that the human body eliminates said compound.

Targeted drugs are a type of medication that blocks the growth of cancer cells by interfering with specific targeted molecules needed for carcinogenesis and tumor growth, rather than by simply interfering with rapidly dividing cells (e.g. with traditional chemotherapy). The main categories of targeted therapy are small molecules and monoclonal antibodies.

Small molecules falling under this definition encompass, but are not limited, to Lapatinib, Neratinib, Afatinib, Imatinib, Gefitinib, Erlotinib, Bortezomib, Bcl-2 inhibitors (e.g. Obatoclax, ABT-263, and Gossypol), PARP inhibitors (e.g. Iniparib, Olaparib), Janus kinase inhibitors, PI3K inhibitors, Apatinib, mTOR inhibitors (Everolimus), AN-152, AKT-inhibitors, HDAC inhibitors, proteasome inhibitors, Doxorubicin linked to [D-Lys(6)]- LHRH, Pegaptanib, Sunitinib, Sorafenib, Tivozanib and Pazopanib. Monoclonal antibodies falling under this definition encompass, but are not limited, to Rituximab, trastuzumab, trastuzumab-TDM1, pertuzumab, cetuximab and bevacizumab.

Endocrine drugs, as used herein, are drugs that are antagonistic to hormones or hormone receptors and thus interfere with cancer types that require hormones to grow. One example for such Endocrine drug is Tamoxifen, which is an antagonist of the estrogen receptor in breast tissue.

The term "cellular therapy", as used herein, shall relate to cell-based therapies such as adoptive transfer of modified, or unmodified, cytotoxic lymphocytes or dendritic cells.

The term ”tumor vaccine”, as used herein, refers to vaccines that either a) prevent infections with cancer-causing viruses (mode of action is similar to other vaccines against viral infections), b) treat existing cancer (therapeutic cancer vaccines) or c) prevent the development of cancer, or ameliorate its effects (prophylactic cancer vaccines).

In addition or alternatively thereto, said use is preferably complemented, in a coordinated fashion, by at least one other treatment selected from the group consisting of

- radiotherapy
- surgery, and/or
- laser ablation

Furthermore, a method of treatment of a human or animal subject is provided which method comprises the use according to the above disclosure. Preferably, said method of treatment relates to an indication as set forth in the above disclosure. The method comprises administering, to a human or animal in need thereof, a therapeutically effective amount of a recombinant binding protein of the invention.

The recombinant binding protein or ankyrin repeat domain according to the invention may be obtained and/or further evolved by several methods such as display on the surface of bacteriophages (WO 1990/002809, WO 2007/006665) or bacterial cells (WO 1993/010214), ribosomal display (WO 1998/048008), display on plasmids (WO 1993/008278) or by using covalent RNA-repeat protein hybrid constructs (WO 2000/032823), or intracellular expression and selection / screening such as by protein complementation assay (WO 1998/341120). Such methods are known to the person skilled in the art.

A library of ankyrin repeat proteins used for the selection/screening of a recombinant binding protein or ankyrin repeat domain according to the invention may be obtained according to protocols known to the person skilled in the art (WO 2002/020565, Binz, H.K., et al., J. Mol. Biol., *332*, 489-503, 2003, and Binz et al., 2004, loc. cit). The use of such libraries for the selection of ankyrin repeat domains with specificity for the extracellular region of HER2 is exemplified in Example 1. Furthermore, ankyrin repeat domains of the present invention may be modularly assembled from ankyrin repeat modules according to the current invention and appropriate capping modules or capping repeats (Forrer, P., et al., FEBS letters *539*, 2-6, 2003) using standard recombinant DNA technologies (e.g. WO 2002/020565, Binz et al., 2003, loc. cit. and Binz et al., 2004, loc. cit).

The invention is not restricted to the particular embodiments described in the Examples. Other sources may be used and processed following the general outline described below.

Definitions

The term "protein" refers to a polypeptide, wherein at least part of the polypeptide has, or is able to acquire a defined three-dimensional arrangement by forming secondary, tertiary, or quaternary structures within and/or between its polypeptide chain(s). If a protein comprises two or more polypeptides, the individual polypeptide chains may be linked non-covalently or covalently, e.g. by a disulfide bond between two polypeptides. A part of a

protein, which individually has, or is able to acquire, a defined three-dimensional arrangement by forming secondary or tertiary structures, is termed "protein domain". Such protein domains are well known to the practitioner skilled in the art.

The term “recombinant” as used in recombinant protein, recombinant protein domain, recombinant binding protein and the like, means that said polypeptides are produced by the use of recombinant DNA technologies well known by the practitioner skilled in the relevant art. For example, a recombinant DNA molecule (e.g. produced by gene synthesis) encoding a polypeptide can be cloned into a bacterial expression plasmid (e.g. pQE30, Qiagen), yeast expression plasmid or mammalian expression plasmid. When, for example, such a constructed recombinant bacterial expression plasmid is inserted into an appropriate bacteria (e.g. *Escherichia coli*), this bacteria can produce the polypeptide encoded by this recombinant DNA. The correspondingly produced polypeptide is called a recombinant polypeptide.

In the context of the present invention, the term "polypeptide" relates to a molecule consisting of one or more chains of multiple, i.e. two or more, amino acids linked via peptide bonds. Preferably, a polypeptide consists of more than eight amino acids linked via peptide bonds.

The term "polypeptide tag" refers to an amino acid sequence attached to a polypeptide/protein, wherein said amino acid sequence is useful for the purification, detection, or targeting of said polypeptide/protein, or wherein said amino acid sequence improves the physicochemical behavior of the polypeptide/protein, or wherein said amino acid sequence possesses an effector function. The individual polypeptide tags, moieties and/or domains of a binding protein may be connected to each other directly or via polypeptide linkers. These polypeptide tags are all well known in the art and are fully available to the person skilled in the art. Examples of polypeptide tags are small polypeptide sequences, for example, His (e.g. the His-tag of SEQ ID NO: 6), myc, FLAG, or Strep-tags or moieties such as enzymes (for example enzymes like alkaline phosphatase), which allow the detection of said polypeptide/protein, or moieties which can be used for targeting (such as immunoglobulins or fragments thereof) and/or as effector molecules.

The term "polypeptide linker" refers to an amino acid sequence, which is able to link, for example, two protein domains, a polypeptide tag and a protein domain, a protein domain

and a non-polypeptide moiety such as polyethylene glycol or two sequence tags. Such additional domains, tags, non-polypeptide moieties and linkers are known to the person skilled in the relevant art. A list of example is provided in the description of the patent application WO 2002/020565. Particular examples of such linkers are glycine-serine-linkers and proline-threonine-linkers of variable lengths; preferably, said linkers have a length between 2 and 24 amino acids; more preferably, said linkers have a length between 2 and 16 amino acids. Examples of glycine-serine-linkers are provided in SEQ ID NO: 7 to 10 and examples of a proline-threonine-linkers are provided in SEQ ID NO: 11 and 12. Preferably, the proline-threonine-linker of SEQ ID NO: 11 is preceded by GS and/or followed by GS.

The term “polymer moiety” refers to either a proteinaceous polymer moiety or a non-proteinaceous polymer moiety. A “proteinaceous polymer moiety” preferably is a polypeptide that does not form a stable tertiary structure. Examples of proteinaceous polymer moieties are XTEN® (a registered trademark of Amunix; WO 2007/103515) polypeptides, or polypeptides comprising proline, alanine and serine residues as described in WO 2008/155134. Such proteinaceous polymer moieties can be covalently attached to, for example, a repeat domain of the invention by the generation of genetic fusion polypeptides using standard DNA cloning technologies, followed by their standard expression and purification. A “non-proteinaceous polymer moiety” is a polymer moiety not built from polypeptides. Examples of non-proteinaceous polymer moieties are hydroxyethyl starch (HES), polyethylene glycol (PEG), polypropylene glycol, or polyoxyalkylene. The term “PEGylated” means that a PEG moiety is covalently attached to, for example, a polypeptide of the invention. A polymer moiety of the invention may vary widely in molecular weight. Preferably, said polymer moiety is connected by a polypeptide linker to a repeat domain.

In a specific embodiment, a PEG moiety or any other non-proteinaceous polymer can, e.g., be coupled to a cysteine thiol via a maleimide linker with the cysteine being coupled via a peptide linker to the N- or C-terminus of a repeat domain as described herein.

The term "binding protein" refers to a protein comprising one or more binding domains, one or more bioactive compounds and one or more polymer moieties as further explained below. Preferably, said binding protein comprises up to four binding domains. Furthermore, any such binding protein may comprise additional protein domains that are

not binding domains, multimerization moieties, polypeptide tags, polypeptide linkers and/or a single Cys residue.

Examples of "multimerization moieties" are immunoglobulin heavy chain constant regions which pair to provide functional immunoglobulin Fc domains, and leucine zippers or polypeptides comprising a free thiol which forms an intermolecular disulfide bond between two such polypeptides. The single Cys residue may be used for conjugating other moieties to the polypeptide, for example, by using the maleimide chemistry well known to the person skilled in the art. Preferably, said binding protein is a recombinant binding protein. Also preferably, the binding domains of binding protein possess different target specificities.

The term “compete for binding” means the inability of two different binding domains of the invention to bind simultaneously to the same target, while both are able to bind the same target individually. Thus, such two binding domains compete for binding to said target. Preferably, said two competing binding domains bind to an overlapping or the same binding epitope on said target. Methods, such as competition Enzyme-Linked Immuno Sorbent Assay (ELISA) or competition SPR measurements (e.g. by using the Proteon instrument from BioRad), to determine if two binding domains compete for binding to a target, are well known to the practitioner in the art.

The term “multiparatopic binding protein” means a binding protein directed against two or mpre different epitopes located on the same target protein. For example, a multiparatopic binding protein targeting HER2 comprises at least a first binding domain targeting a first epitope on HER2, a second binding domain targeting a different second epitope on HER2, and optionally further binding domain targeting further epitopes on HER2.

The term “biparatopic binding protein” means a binding protein directed against two different epitopes located on the same target protein. For example, a biparatopic binding protein targeting HER2 comprises at least a first binding domain targeting a first epitope on HER2 and a second binding domain targeting a different second epitope on HER2. Correspondingly, a “biparatopic DARPin” comprises a first binding domain against a first epitope and a second binding domain against a different second epitope on the same target molecule.

The term “bioactive compound” refers to a compound that is disease modifying when applied to a mammal having said disease. A bioactive compound may have antagonistic or agonistic properties and can be a proteinaceous bioactive compound or a non-proteinaceous bioactive compound. Such proteinaceous bioactive compounds can be covalently attached to, for example, a binding domain of the invention by the generation of genetic fusion polypeptides using standard DNA cloning technologies, followed by their standard expression and purification. Such non-proteinaceous bioactive compounds can be covalently attached to, for example, a binding domain of the invention by chemical means, e.g., by coupling to a cysteine thiol via a maleimide linker with a cysteine being coupled via a peptide linker to the N- or C-terminus of a binding domain as described herein. Examples of proteinaceous bioactive compounds are binding domains having a distinct target specificity (e.g. neutralizing a growth factor by binding to it), cytokines (e.g. interleukins), growth factors (e.g. human growth hormone), antibodies and fragments thereof, hormones (e.g. GLP-1) and any possible proteinaceous drug. Examples of non-proteinaceous bioactive compounds are, toxins (e.g. DM1 from ImmunoGen), small molecules targeting GPCRs, antibiotics and any possible non-proteinaceous drug.

The term “binding domain” means a protein domain exhibiting the same "fold" (three-dimensional arrangement) as a protein scaffold and having a predetermined property, as defined below. Such a binding domain may be obtained by rational, or most commonly, combinatorial protein engineering techniques, skills which are known in the art (Binz et al., 2005, loc. cit.). For example, a binding domain having a predetermined property can be obtained by a method comprising the steps of (a) providing a diverse collection of protein domains exhibiting the same fold as a protein scaffold as defined further below; and (b) screening said diverse collection and/or selecting from said diverse collection to obtain at least one protein domain having said predetermined property. The diverse collection of protein domains may be provided by several methods in accordance with the screening and/or selection system being used, and may comprise the use of methods well known to the person skilled in the art, such as phage display or ribosome display. Preferably, said binding domain is a recombinant binding domain. Also preferably, said binding domain is a repeat protein or a designed repeat protein.

Accordingly, the term "binds", as used herein, relates to a binding domain that recognizes and binds a given target, but does not substantially recognize or bind other targets. Preferably, a dissociation constant in PBS of smaller than 10^{-7}M is required for a candidate to qualify as a binding domain in the meaning of the present invention.

The term "Kd" relates to the dissociation constant, which is a specific type of equilibrium constant that measures the propensity of a larger object to separate (dissociate) reversibly into smaller components, as when a complex falls apart into its component molecules. Methods to determine dissociation constants of protein-protein interactions, such as surface plasmon resonance (SPR) based technologies (e.g. SPR equilibrium analysis) or isothermal titration calorimetry (ITC) are well known to the person skilled in the art. The measured Kd values of a particular protein-protein interaction can vary if measured under different conditions (e.g., salt concentration, pH). Thus, measurements of Kd values are preferably made with standardized solutions of protein and a standardized buffer, such as PBS.

The term “PBS” means a phosphate buffered water solution containing 137 mM NaCl, 10 mM phosphate and 2.7 mM KCl and having a pH of 7.4.

The term "protein scaffold" means a protein with exposed surface areas in which amino acid insertions; substitutions or deletions are highly tolerable. Examples of protein scaffolds that can be used to generate binding domains of the present invention are antibodies or fragments thereof such as single-chain Fv or Fab fragments, protein A from *Staphylococcus aureus*, the bilin binding protein from *Pieris brassicae* or other lipocalins, ankyrin repeat proteins or other repeat proteins, and human fibronectin. Protein scaffolds are known to the person skilled in the art (Binz et al., 2005, loc. cit.; Binz et al., 2004, loc. cit.).

The term "target" refers to an individual molecule such as a nucleic acid molecule, a polypeptide or protein, a carbohydrate, or any other naturally occurring molecule, including any part of such individual molecule, or complexes of two or more of such molecules. The target may be a whole cell or a tissue sample, or it may be any non-natural molecule or moiety. Preferably, the target is a naturally occurring or non-natural polypeptide or a polypeptide containing chemical modifications, for example modified by natural or non-natural phosphorylation, acetylation, or methylation. In the particular application of the present invention, the target is the extracellular region of HER2.

The term "predetermined property" refers to a property such as binding to a target, blocking of a target, activation of a target-mediated reaction, enzymatic activity, and related further properties. Depending on the type of desired property, one of ordinary skill

will be able to identify format and necessary steps for performing screening and/or selection of a binding domain with the desired property. Preferably, said predetermined property is binding to a target.

The definitions hereinafter for repeat proteins are based on those in patent application WO 2002/020565. Patent application WO 2002/020565 further contains a general description of repeat protein features, techniques and applications.

The term "repeat protein" refers to a protein comprising one or more repeat domains. Preferably, each of said repeat proteins comprises up to four repeat domains. More preferably, each of said repeat proteins comprises up to two repeat domains. Most preferably, each of the repeat proteins comprises only one repeat domain. Furthermore, said repeat protein may comprise additional non-repeat protein domains, polypeptide tags and/or polypeptide linkers.

The term "repeat domain" refers to a protein domain comprising two or more consecutive repeat units (modules) as structural units, wherein said structural units have the same fold, and stack tightly to create a superhelical structure having a joint hydrophobic core. Preferably, a repeat domain further comprises an N-terminal and/or a C-terminal capping unit (or module). Even more preferably, said N-terminal and/or C-terminal capping units (or modules) are capping repeats.

The term "designed repeat protein" and “designed repeat domain” refer to a repeat protein or repeat domain, respectively, obtained as the result of the inventive procedure explained in patent application WO 2002/020565. Designed repeat proteins and designed repeat domains are synthetic and not from nature. They are man-made proteins or domains, respectively, obtained by expression of correspondingly designed nucleic acids. Preferably, the expression is done in eukaryotic or prokaryotic cells, such as bacterial cells, or by using a cell-free *in vitro* expression system. Accordingly, a designed ankyrin repeat protein (i.e. a DARPin) corresponds to a recombinant binding protein of the invention comprising at least one ankyrin repeat domain.

The term "structural unit" refers to a locally ordered part of a polypeptide, formed by three-dimensional interactions between two or more segments of secondary structure that are near one another along the polypeptide chain. Such a structural unit exhibits a structural motif. The term "structural motif" refers to a three-dimensional arrangement of secondary

structure elements present in at least one structural unit. Structural motifs are well known to the person skilled in the art. Structural units alone are not able to acquire a defined three-dimensional arrangement; however, their consecutive arrangement, for example as repeat modules in a repeat domain, leads to a mutual stabilization of neighboring units resulting in a superhelical structure.

The term "repeat unit" refers to amino acid sequences comprising repeat sequence motifs of one or more naturally occurring repeat proteins, wherein said "repeat units" are found in multiple copies, and which exhibit a defined folding topology common to all said motifs determining the fold of the protein. Such repeat units correspond to the “repeating structural units (repeats)” of repeat proteins as described by Forrer et al., 2003, loc. cit. or the “consecutive homologous structural units (repeats)” of repeat proteins as described by Binz et al, 2004, loc. cit.. Such repeat units comprise framework residues and interaction residues. Examples of such repeat units are armadillo repeat units, leucine-rich repeat units, ankyrin repeat units, tetratricopeptide repeat units, HEAT repeat units, and leucine-rich variant repeat units. Naturally occurring proteins containing two or more such repeat units are referred to as "naturally occurring repeat proteins". The amino acid sequences of the individual repeat units of a repeat protein may have a significant number of mutations, substitutions, additions and/or deletions when compared to each other, while still substantially retaining the general pattern, or motif, of the repeat units.

Accordingly, the term “ankyrin repeat unit” shall mean a repeat unit, which is an ankyrin repeat as described, for example, by Forrer et al., 2003, loc. cit.. Ankyrin repeats are well known to the person skilled in the art. The term "ankyrin repeat domain" refers to a repeat domain comprising two or more consecutive ankyrin repeat units (modules) as structural units, and, preferably, an N-terminal and/or a C-terminal capping unit (or module).

The term "framework residues" relates to amino acid residues of the repeat units, or the corresponding amino acid residues of the repeat modules, which contribute to the folding topology, i.e. which contribute to the fold of said repeat unit (or module) or which contribute to the interaction with a neighboring unit (or module). Such contribution might be the interaction with other residues in the repeat unit (or module), or the influence on the polypeptide backbone conformation as found in α-helices or β-sheets, or amino acid stretches forming linear polypeptides or loops.

The term "target interaction residues" refers to amino acid residues of the repeat units, or the corresponding amino acid residues of the repeat modules, which contribute to the interaction with target substances. Such contribution might be the direct interaction with the target substances, or the influence on other directly interacting residues, e.g. by stabilizing the conformation of the polypeptide of a repeat unit (or module) to allow or enhance the interaction of directly interacting residues with said target. Such framework and target interaction residues may be identified by analysis of the structural data obtained by physicochemical methods, such as X-ray crystallography, NMR and/or CD spectroscopy, or by comparison with known and related structural information well known to practitioners in structural biology and/or bioinformatics.

Preferably, the repeat units used for the deduction of a repeat sequence motif are homologous repeat units, wherein the repeat units comprise the same structural motif and wherein more than 70% of the framework residues of said repeat units are homologous to each other. Preferably, more than 80% of the framework residues of said repeat units are homologous. Most preferably, more than 90% of the framework residues of said repeat units are homologous. Computer programs to determine the percentage of homology between polypeptides, such as Fasta, Blast or Gap, are known to the person skilled in the art. Further preferably, the repeat units used for the deduction of a repeat sequence motif are homologous repeat units obtained from repeat domains selected on a defined target.

The term "repeat sequence motif" refers to an amino acid sequence, which is deduced from one or more repeat units or repeat modules. Preferably, said repeat units or repeat modules are from repeat domains having binding specificity for the same target. Such repeat sequence motifs comprise framework residue positions and target interaction residue positions. Said framework residue positions correspond to the positions of framework residues of the repeat units (or modules). Likewise, said target interaction residue positions correspond to the positions of target interaction residues of the repeat units (or modules). Repeat sequence motifs comprise fixed positions and randomized positions. The term "fixed position" refers to an amino acid position in a repeat sequence motif, wherein said position is set to a particular amino acid. Most often, such fixed positions correspond to the positions of framework residues and/or the positions of target interaction residues that are specific for a certain target. The term "randomized position" refers to an amino acid position in a repeat sequence motif, wherein two or more amino acids are allowed at said amino acid position, for example, wherein any of the usual twenty naturally occurring amino acids are allowed, or wherein most of the twenty

naturally occurring amino acids are allowed, such as amino acids other than cysteine, or amino acids other than glycine, cysteine and proline. Most often, such randomized positions correspond to the positions of target interaction residues. However, some positions of framework residues may also be randomized.

The term "folding topology" refers to the tertiary structure of said repeat units or repeat modules. The folding topology will be determined by stretches of amino acids forming at least parts of α-helices or β-sheets, or amino acid stretches forming linear polypeptides or loops, or any combination of α-helices, β-sheets and/or linear polypeptides/loops. For example, an ankyrin repeat unit/module consists of a β-turn, followed by two antiparallel α-helices and a loop that reaches the turn of the next repeat unit/module.

The term "consecutive" refers to an arrangement, wherein the repeat units or repeat modules are arranged in tandem. In designed repeat proteins, there are at least 2, usually about 2 to 6, in particular at least about 6, frequently 20 or more repeat units (or modules). In most cases, repeat units (or modules) of a repeat domain will exhibit a high degree of sequence identity (same amino acid residues at corresponding positions) or sequence similarity (amino acid residues being different, but having similar physicochemical properties), and some of the amino acid residues might be key residues being strongly conserved. However, a high degree of sequence variability by amino acid insertions and/or deletions, and/or substitutions between the different repeat units (or modules) of a repeat domain may be possible as long as the common folding topology of the repeat units (or modules) is maintained.

Methods for directly determining the folding topology of repeat proteins by physico-chemical means such as X-ray crystallography, NMR or CD spectroscopy, are well known to the practitioner skilled in the art. Methods for identifying and determining repeat units or repeat sequence motifs or for identifying families of related proteins comprising such repeat units or motifs, such as homology searches (BLAST etc.), are well established in the field of bioinformatics, and are well known to the practitioner in the art. The step of refining an initial repeat sequence motif may comprise an iterative process.

The term "repeat modules" refers to the repeated amino acid sequences of the designed repeat domains, which are originally derived from the repeat units of naturally occurring repeat proteins. Each repeat module comprised in a repeat domain is derived from one or more repeat units of the family or subfamily of naturally occurring repeat proteins, e.g. the

family of armadillo repeat proteins or ankyrin repeat proteins. Further preferably, each repeat module comprised in a repeat domain comprises a repeat sequence motif deduced from homologous repeat units obtained from repeat domains selected on a target, for example as described in Example 1 and having the same target specificity.

Accordingly, the term “ankyrin repeat module” shall mean a repeat module, which is originally derived from the repeat units of naturally occurring ankyrin repeat proteins. Ankyrin repeat proteins are well known to the person skilled in the art.

"Repeat modules" may comprise positions with amino acid residues present in all copies of corresponding repeat modules ("fixed positions") and positions with differing or "randomized" amino acid residues ("randomized positions").

The term “capping module” refers to a polypeptide fused to the N- or C-terminal repeat module of a repeat domain, wherein said capping module forms tight tertiary interactions (i.e. tertiary structure interactions) with said repeat module thereby providing a cap that shields the hydrophobic core of said repeat module at the side not in contact with the consecutive repeat module from the solvent. Said N- and/or C-terminal capping module may be, or may be derived from, a capping unit or other structural unit found in a naturally occurring repeat protein adjacent to a repeat unit. The term “capping unit” refers to a naturally occurring folded polypeptide, wherein said polypeptide defines a particular structural unit which is N- or C-terminally fused to a repeat unit, wherein said polypeptide forms tight tertiary structure interactions with said repeat unit thereby providing a cap that shields the hydrophobic core of said repeat unit at one side from the solvent. Preferably, capping modules or capping units are capping repeats. The term “capping repeat” refers to capping module or capping unit having a similar or the same fold as said adjacent repeat unit (or module) and/or sequence similarities to said adjacent repeat unit (or module). Capping modules and capping repeats are described in WO 2002/020565 and by Interlandi et al., 2008 (loc. cit.).

Examples of N-terminal ankyrin capping modules (i.e. N-terminal capping repeats) are SEQ ID NO: 1, 2, 3, 13, 14, 20, 26, 27 36, 40, 44, 45, 50, 54, 124, 128 and 132 and examples of ankyrin C-terminal capping modules (i.e. C-terminal capping repeats) are SEQ ID NO: 4, 5, 19, 24, 25, 33, 34, 35, 39, 43, 48, 49, 53 ,57, 127, 131 and 135.

For example, the N-terminal ankyrin capping module of SEQ ID NO: 13 is encoded by the amino acids from position 1 to 32 and the C-terminal capping module of SEQ ID NO: 19 is encoded by the amino acids from position 99 to 126.

A recombinant binding protein according to the invention comprises at least one ankyrin repeat domain, wherein said ankyrin repeat domain has binding specificity for mammalian extracellular region of HER2.

The term “has binding specificity for a target”, “specifically binding to a target” or “target specificity” and the like means that a binding protein or binding domain binds in PBS to a target with a lower dissociation constant than to an unrelated protein such as the *E. coli* maltose binding protein (MBP). Preferably, the dissociation constant in PBS for the target is at least 10, more preferably at least 10^2, even more preferably at least 10^3, or most preferably at least 10^4 times lower than the corresponding dissociation constant for MBP.

The term "consensus sequence" refers to an amino acid sequence, wherein said consensus sequence is obtained by structural and/or sequence aligning of multiple repeat units. Using two or more structural and/or sequence aligned repeat units, and allowing for gaps in the alignment, it is possible to determine the most frequent amino acid residue at each position. The consensus sequence is that sequence which comprises the amino acids which are most frequently represented at each position. In the event that two or more amino acids are represented above-average at a single position, the consensus sequence may include a subset of those amino acids. Said two or more repeat units may be taken from the repeat units comprised in a single repeat protein, or from two or more different repeat proteins.

Consensus sequences and methods to determine them are well known to the person skilled in the art.

A “consensus amino acid residue” is the amino acid found at a certain position in a consensus sequence. If two or more, e.g. three, four or five, amino acid residues are found with a similar probability in said two or more repeat units, the consensus amino acid may be one of the most frequently found amino acids or a combination of said two or more amino acid residues.

Further preferred are non-naturally occurring capping modules, repeat modules, binding proteins or binding domains.

The term “non-naturally occurring” means synthetic or not from nature, more specifically, the term means made from the hand of man. The term “non-naturally occurring binding protein" or "non-naturally occurring binding domain” means that said binding protein or said binding domain is synthetic (i.e. produced by chemical synthesis from amino acids) or recombinant and not from nature. “Non-naturally occurring binding protein" or "non-naturally occurring binding domain” is a man-made protein or domain, respectively, obtained by expression of correspondingly designed nucleic acids. Preferably, the expression is done in eukaryotic or bacterial cells, or by using a cell-free *in vitro* expression system. Further, the term means that the sequence of said binding protein or said binding domain is not present as a non-artificial sequence entry in a sequence database, for example in GenBank, EMBL-Bank or Swiss-Prot. These databases and other similar sequence databases are well known to the person skilled in the art.

General modifications and derivatives of the ankyrin repeat domains according to the invention; particularly of the ankyrin repeat modules and capping modules according to the invention:

Further preferred is a N-terminal or C-terminal ankyrin capping module comprising an N-terminal or C-terminal ankyrin capping repeat, respectively, wherein one or more of the amino acids residues in said capping repeat are replaced by an amino acid residue found at the corresponding position on alignment of a corresponding ankyrin capping unit or ankyrin repeat unit.

The replacement of amino acids can be by any of the 20 most often naturally occurring amino acids, preferably by amino acids selected from the group consisting of A, D, E, F, H, I, K, L, M, N, Q, R, S, T, V, W and Y; and more preferably from the group consisting of A, D, E, H, I, K, L, Q, R, S, T, V, and Y. Also preferably, the replacement of amino acids is by a homologous amino acid; i.e. an amino acid is replaced by an amino acid having a side chain with similar biophysical properties. For example, the negative charged amino acid D may be replaced by the negative charged amino acid E, or a hydrophobic amino acid such as L may be replaced by A, I or V. The replacement of an amino acid by a homologous amino acid is well known to the person skilled in the art.

Also preferred is a C-terminal ankyrin capping module comprising the amino acid A at position 27 and 28 of any of the above C-terminal capping modules based on SEQ ID NO: 4, 5, 19, 24, 25, 33, 34, 35, 39, 43, 48, 49, 53, 57, 127, 131 or 135

Also preferred is a C-terminal capping module comprising the amino acids from position 1 to 26 or from position 1 to 27 of any of the above C-terminal capping modules based on SEQ ID NO: 4, 5, 19, 24, 25, 33, 34, 35, 39, 43, 48, 49, 53, 57, 127, 131 or 135.

Amino acids G at position 1 and/or S at position 2 of SEQ ID NO: 1, 2, 3, 13, 14, 20, 26, 27, 36, 40, 44, 45, 50, 54, 124, 128 or 132 can be removed from N-terminal ankyrin capping modules without any apparent influence on the properties. These two amino acids serve as linkers to connect the ankyrin repeat domain to further amino acids and proteins. The invention also comprises such ankyrin repeat domains comprising N-terminal ankyrin capping modules wherein G at position 1 and/or S at position 2 are removed. It is understood that the amino acid positions (e.g. “position 33") in an ankyrin repeat domain as defined herein are adapted accordingly, resulting in a number shift, e.g. “position 33” will become “position 32”, if one amino acid is missing, or “position 33” will become “position 31”, if two amino acid are missing.

An ankyrin capping module of an ankyrin repeat domain of the invention can be exchanged by an ankyrin capping module by combining techniques, such as alignment of amino acid sequences, mutagenesis and gene synthesis, known to the person skilled in the art. For example, the C-terminal capping repeat of SEQ ID NO: 79 can be replaced by the C-terminal capping repeat of SEQ ID NO: 5 by (i) determination of the C-terminal capping repeat of SEQ ID NO: 79 (i.e. sequence position 99 to 126) by sequence alignment with SEQ ID NO: 5, (ii) replacing the sequence of the determined C-terminal capping repeat of SEQ ID NO: 79 with the sequence of SEQ ID NO: 5, (iii) generation of a gene encoding the repeat domain encoding the exchanged C-terminal capping module, (iv) expressing of the modified repeat domain in the cytoplasm of *E. coli* and (v) purification of the modified repeat domain by standard means. As a further example, the N-terminal capping repeat of SEQ ID NO: 79 can be replaced by the N-terminal capping repeat of SEQ ID NO: 3 by (i) determination of the N-terminal capping repeat of SEQ ID NO: 79 (i.e. sequence position 1 to 32) by sequence alignment with SEQ ID NO: 3, (ii) replacing the sequence of the determined N-terminal capping repeat of SEQ ID NO: 79 with the sequence of SEQ ID NO: 3, (iii) generation of a gene encoding the repeat domain encoding the exchanged N-terminal capping module, (iv) expressing of the modified

repeat domain in the cytoplasm of *E. coli* and (v) purification of the modified repeat domain by standard means.

Furthermore, an ankyrin repeat domain of the invention can be constructed genetically by assembling a N-terminal ankyrin capping module (e.g. the N-terminal capping repeat of SEQ ID NO: 3) followed by one or more repeat modules (e.g. the two ankyrin repeat modules comprising the amino acid residues from position 33 to 99 of SEQ ID NO: 79) and a C-terminal capping module (e.g. the C-terminal capping repeat of SEQ ID NO: 5) by means of gene synthesis. The genetically assembled repeat domain gene can then be expressed in *E. coli* as described above.

Further preferred is a recombinant binding protein, repeat domain, repeat module, N-terminal capping module or C-terminal capping module having an amino acid sequence devoid of amino acids C, M or N.

Further preferred is a recombinant binding protein, repeat domain, repeat module, N-terminal capping module or C-terminal capping module having an amino acid sequence devoid of amino acid N followed by G.

Further preferred is a recombinant binding protein or repeat domain comprising any such N-terminal or C-terminal capping module.

In a further preferred embodiment of a recombinant binding protein comprising an ankyrin repeat domain according to the present invention, one or more of the amino acid residues of the N-terminal capping module of said repeat domain is exchanged by an amino acid residue found at the corresponding position on alignment of an N-terminal capping unit. Preferably, up to 30% of the amino acid residues are exchanged, more preferably, up to 20%, and even more preferably, up to 10% of the amino acid residues are exchanged. Most preferably, such an N-terminal capping unit is a naturally occurring N-terminal capping unit.

In a further preferred embodiment of a recombinant binding protein comprising an ankyrin repeat domain according to the present invention, one or more of the amino acid residues of the C-terminal capping module of said repeat domain is exchanged by an amino acid residue found at the corresponding position on alignment of a C-terminal capping unit. Preferably, up to 30% of the amino acid residues are exchanged, more preferably, up to

20%, and even more preferably, up to 10% of the amino acid residues are exchanged. Most preferably, such a C-terminal capping unit is a naturally occurring C-terminal capping unit.

In still another particular embodiment, up to 30% of the amino acid residues, more preferably, up to 20%, and even more preferably, up to 10% of the amino acid residues are exchanged with amino acids which are not found in the corresponding positions of repeat units, N-terminal capping units or C-terminal capping units.

In a further preferred embodiment of a recombinant binding protein comprising an ankyrin repeat domain according to the present invention, one or more of the amino acid residues of the repeat modules of said ankyrin repeat domain are exchanged by an amino acid residue found at the corresponding position on alignment of a repeat unit. Preferably, up to 30% of the amino acid residues are exchanged, more preferably, up to 20%, and even more preferably, up to 10% of the amino acid residues are exchanged. Most preferably, such a repeat unit is a naturally occurring repeat unit.

In still another particular embodiment, up to 30% of the amino acid residues, more preferably, up to 20%, and even more preferably, up to 10% of the amino acid residues are exchanged with amino acids which are not found in the corresponding positions of repeat units.

In further embodiments, any of the recombinant HER2 binding proteins or domains described herein may be covalently bound to one or more additional moieties, including, for example, a moiety that binds to a different target to create a dual-specificity binding agent, a bioactive compound, a labeling moiety (e.g. a fluorescent label such as fluorescein, or a radioactive tracer), a moiety that facilitates protein purification (e.g. a small peptide tag, such as a His- or strep-tag), a moiety that provides effector functions for improved therapeutic efficacy (e.g. the Fc part of an antibody to provide antibody-dependent cell-mediated cytotoxicity, a toxic protein moiety such as *Pseudomonas aeruginosa* exotoxin A (ETA) or a small molecular toxic agent such as maytansinoids or DNA alkylating agents) or a moiety that provides improved pharmacokinetics. Improved pharmacokinetics may be assessed according to the perceived therapeutic need. Often it is desirable to increase bioavailability and/or increase the time between doses, possibly by increasing the time that a protein remains available in the serum after dosing. In some instances, it is desirable to improve the continuity of the serum concentration of the

protein over time (e.g., decrease the difference in serum concentration of the protein between the concentration shortly after administration and the concentration shortly before the next administration). Moieties that tend to slow clearance of a protein from the blood include hydroxyethyl starch (HES), polyethylene glycol (PEG), sugars (e.g. sialic acid), well-tolerated protein moieties (e.g. Fc fragments or serum albumin), and binding domains or peptides with specificity and affinity for abundant serum proteins, such as antibody Fc fragments or serum albumin. Examples of such binding domains or repeat domains with affinity for serum albumin are provided in WO 2012/069654. The recombinant binding protein of the invention may be attached to a moiety that reduces the clearance rate of polypeptides in a mammal (e.g. in mouse, rat, or human) by greater than three-fold relative to the unmodified polypeptides.

In one particular embodiment the invention relates to a recombinant binding protein comprising the first repeat domain binding to HER2, the second repeat domain binding to HER2 and further comprising one or more ankyrin repeat domains specifically binding to human serum albumin. Examples of repeat domains with specificity for HER2 are given herein and examples of ankyrin repeat domains with specificity to human serum albumin are described in WO 2012/069654. Such domains can be linked by a polypeptide linker by genetic means by methods known to the person skilled in the art.

Another preferred embodiment is a recombinant binding protein wherein the first repeat domain and the second repeat domain are ankyrin repeat domains with binding specificity for HER2 comprising one, two, three or more internal repeat modules that will participate in binding to HER2. Preferably, such ankyrin repeat domains comprise an N-terminal capping module, one to four internal repeat modules, and a C-terminal capping module. Preferably, said capping modules are capping repeats. Also preferably, said capping modules will participate in binding to HER2.

Further, any of the above mentioned pharmaceutical composition is considered for the treatment of a disorder.

The invention further provides methods of treatment. The method comprises administering, to a patient in need thereof, a therapeutically effective amount of a recombinant binding protein of the invention.

Further, a method of treating a pathological condition in a mammal including man, comprising administering to a patient in need thereof an effective amount of the above mentioned pharmaceutical composition is considered.

Examples

All of the starting materials and reagents disclosed below are known to those skilled in the art, and are available commercially or can be prepared using well-known techniques.

Materials

Chemicals were purchased from Fluka (Switzerland). Oligonucleotides were from Microsynth (Switzerland). Unless stated otherwise, DNA polymerases, restriction enzymes and buffers were from New England Biolabs (USA) or Fermentas (Lithuania). The cloning and protein production strain was *E. coli* XL1-blue (Stratagene, USA) or BL21 (Novagen, USA). Recombinant human HER2 ectodomain (ErbB2 S22-N530-Flag and ErbB2 S22-E645-Flag produced in CHO cells by standard means) was purchased from CSIRO Enquiries (Australia). Biotinylated Her2 ectodomain was obtained chemically via coupling of the biotin moiety to primary amines of the protein using standard biotinylation reagents and methods (Pierce, USA). Cell lines were purchased from LGC/ATCC (France/USA; Cat. No: BT474 -HTB-20, SKBR-3 –HTB-30, NCI-N87 – CRL5822, ZR75-30 –CRL1504, HCC1419 -CRL2326, MDA-MB175 VII–HTB-25). Cell culture media were from Invitrogen / Lubio (Switzerland). Fetal calf serum was from PAA. Assay reagent for detection of cell proliferation, Cell Proliferation ELISA, BrdU (colorimetric) (Cat. No. 1164722900) was from Roche, Switzerland and the assay reagent for detection of apoptosis, Caspase Glo 3/7 (Cat. No. G8091) was from Promega and the Switzerland and the Cell Death Detection ELISAPLUS system (11 774 425 001) from Roche, Switzerland. Cell transfection reagent, Lipofectamin 2000 (11668027) was from Invitrogen Switzerland. FACS analyses were performed using the FACS Canto II System from Becton-Dickinson (Switzerland). The binding of DARPins to Her2 was detected using an anti-Penta-His Alexa Fluor 647 conjugate (Cat. No. A21445; Lubio Switzerland). Accutase (Cat. No: L-11-007) was from PAA. Trastuzumab was purchased from Kantonal Apotheke Zurich and pertuzumab was synthesized by Evitra (Switzerland). The expression vector for GFP-tagged Her2 (Cat. No. RG212583) was from Origene USA.

Molecular Biology

Unless stated otherwise, methods are performed according to described protocols (Sambrook J., Fritsch E.F. and Maniatis T., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory 1989, New York).

Proliferation analysis

Effects of DARPins on cell proliferation were determined by measuring DNA synthesis using BrdU-labeling (BrdU, Cell Proliferation ELISA, Roche). Briefly, 10000 BT474 cells were seeded per well in a 96 well plate in 100 ul complete medium and incubated for 24h. DARPins and benchmarks were added for an additional 72h. BrdU for cell labeling was added for the last 24h. Labeled (proliferating) cells were detected according to the manufactures protocol. The data were analyzed using the GraphPad prism software, plotting log [c] on the x-axis against OD450-602 nm on the y-axis. Data were fitted using a non-linear regression fit (log(antagonist) vs. response -- Variable slope (four parameters)).

Apoptosis analysis

Induction of apoptosis by DARPins was determined by measuring Caspase3/7 activation using the Caspase 3/7-Glo systems (Promega, Switzerland). Briefly, 10000 BT474 cells were seeded per well in a 96 well plate in100ul complete medium and incubated for 24h. DARPins and benchmarks were added for an additional 24h. Caspase Glo reagent was added according to the manufactures protocol for 1h. Caspase 3/7 activation was monitored by measuring luciferase activity.

Alternatively induction of apoptosis was determined using the Cell Death Detection ELISAPLUS system (Roche, Switzerland). The assay was performed according to the manufactures protocol. Cell number and incubations times were similar to the Caspase Glo readout.

The data were analyzed using the GraphPad prism software, plotting concentration on the x-axis against OD405/490 nm or RLU on the y-axis. Data were fitted using a non-linear regression fit (log(agonist) vs. response - Variable slope (four parameters)).

Designed ankyrin repeat protein libraries

Methods to generate designed ankyrin repeat protein libraries are described (WO 2002/020565; Binz et al. 2003, loc. cit.; Binz et al. 2004, loc. cit.). By such methods designed ankyrin repeat protein libraries having randomized ankyrin repeat modules and/or randomized capping modules can be constructed. For example, such libraries

could accordingly be assembled based on a fixed N-terminal capping module (e.g. the N-terminal capping module of SEQ ID NO: 2) or a randomized N-terminal capping module according to the sequence motif of SEQ ID NO: 60, one or more randomized repeat modules according to the sequence motif of SEQ ID NO: 58 or 59, and a fixed C-terminal capping module (e.g. the C-terminal capping module of SEQ ID NO: 5) or a randomized C-terminal capping module according to the sequence motif of SEQ ID NO: 61. Preferably, such libraries are assembled to not have the amino acids C, G, M, N (in front of a G residue) or P at randomized positions of repeat or capping modules. In addition, randomized repeat modules according to the sequence motif of SEQ ID NO: 58 or 59 could be further randomized at position 10 and/or position 17; the randomized N-terminal capping module according to the sequence motif of SEQ ID NO: 60 could be further randomized at position 7 and/or position 9; and the randomized C-terminal capping modules according to the sequence motif of SEQ ID NO: 61 could be further randomized at positions 10, 11 and/or 17.

Furthermore, such randomized modules in such libraries may comprise additional polypeptide loop insertions with randomized amino acid positions. Examples of such polypeptide loop insertions are complement determining region (CDR) loop libraries of antibodies or de novo generated peptide libraries. For example, such a loop insertion could be designed using the structure of the N-terminal ankyrin repeat domain of human ribonuclease L (Tanaka, N., Nakanishi, M, Kusakabe, Y, Goto, Y., Kitade, Y, Nakamura, K.T., EMBO J. *23(30)*, 3929-3938, 2004) as guidance. In analogy to this ankyrin repeat domain where ten amino acids are inserted in the beta-turn present close to the boarder of two ankyrin repeats, ankyrin repeat proteins libraries may contain randomized loops (with fixed and randomized positions) of variable length (e.g. 1 to 20 amino acids) inserted in one or more beta-turns of an ankyrin repeat domain.

Any such N-terminal capping module of an ankyrin repeat protein library preferably possesses the RELLKA or RILKAA motif instead of the RILLAA motif (e.g. present from position 21 to 26 in SEQ ID NO: 65) and any such C-terminal capping module of an ankyrin repeat protein library preferably possesses the KAA or KLA motif instead of the KLN motif (e.g. the last three amino acids in SEQ ID NO: 65).

The design of such an ankyrin repeat protein library may be guided by known structures of an ankyrin repeat domain interacting with a target. Examples of such structures, identified

by their Protein Data Bank (PDB) unique accession or identification codes (PDB-IDs), are 1WDY, 3V31, 3V30, 3V2X, 3V2O, 3UXG, 3TWQ-3TWX, 1N11, 1S70 and 2ZGD.

Examples of designed ankyrin repeat protein libraries, such as the N2C and N3C designed ankyrin repeat protein libraries, are described (WO 2002/020565; Binz et al. 2003, loc. cit.; Binz et al. 2004, loc. cit.). The digit in N2C and N3C describes the number of randomized repeat modules present between the N-terminal and C-terminal capping modules.

The nomenclature used to define the positions inside the repeat units and modules is based on Binz et al. 2004, loc. cit. with the modification that borders of the ankyrin repeat modules and ankyrin repeat units are shifted by one amino acid position. For example, position 1 of an ankyrin repeat module of Binz et al. 2004 (loc. cit.) corresponds to position 2 of a ankyrin repeat module of the current disclosure and consequently position 33 of a ankyrin repeat module of Binz et al. 2004, loc. cit. corresponds to position 1 of a following ankyrin repeat module of the current disclosure.

All the DNA sequences were confirmed by sequencing, and the calculated molecular weight of all described proteins was confirmed by mass spectrometry.

Example 1: *Selection of binding proteins comprising ankyrin repeat domains with binding specificity for HER2*

Using ribosome display (Hanes, J. and Plückthun, A., PNAS *94*, 4937-42, 1997) many designed ankyrin repeat proteins (DARPins) with binding specificity for the ectodomain of HER2 were selected from DARPin libraries as described by Binz et al. 2004 (loc. cit.). Their binding specificity was assessed by crude extract ELISA (see below) indicating that hundreds of HER2-specific binding proteins were selected. HER2-specififc inhibition of proliferation and induction of apoptosis of the selected clones was measured by testing biparatopic DARPins for their ability to inhibit proliferation of BT474 cells.

For example, the ankyrin repeat domains of SEQ ID NO: 62 to 82, 112 to 121 constitute amino acid sequences of selected binding proteins comprising an ankyrin repeat domain with binding specificity for HER2. Individual ankyrin repeat modules from such ankyrin repeat domains with binding specificity to HER2 are provided in SEQ ID NO: 15 to 18, 21 to 23, 28 to 32, 37, 38, 41, 42, 46, 47, 51, 52, 55, 56, 125, 126, 129, 130, 133 and 134.

Individual capping modules of such ankyrin repeat domains with binding specificity to HER2 are provided in SEQ ID NO: 13, 14, 19, 20, 24 to 27, 33 to 36, 39, 40, 43 to 45, 48 to 50, 53, 54, 57, 124, 127, 128, 131, 132 and 135.

Selection of HER2 specific ankyrin repeat proteins by ribosome display

The selection of HER2 specific ankyrin repeat proteins was performed by ribosome display (Hanes and Plückthun, loc. cit.) using human HER2 as target proteins, libraries of designed ankyrin repeat proteins as described above and established protocols (Zahnd, C., Amstutz, P. and Plückthun, A., Nat. Methods *4*, 69-79, 2007). The number of reverse transcription (RT)-PCR cycles after each selection round was constantly reduced from 45 to 30, adjusting to the yield due to enrichment of binders. The first four rounds of selection employed standard ribosome display selection, using decreasing target concentration and increasing washing stringency to increase selection pressure from round 1 to round 4 (Binz et al. 2004, loc. cit.). To enrich high affinity anti-HER2 DARPins, the output from the fourth round of standard ribosome display selection (above) was subjected to an off-rate selection round with increased selection stringency (Zahnd, 2007, loc. cit.). A final standard selection round was performed to amplify and recover the off-rate selected binding proteins.

Selected clones bind specifically to HER2 as shown by crude extract ELISA

Individual selected DARPins specifically binding the ectodomain of HER2 were identified by enzyme-linked immunosorbent assay (ELISA) using crude *Escherichia coli* extracts of DARPin expression cells using standard protocols. DARPins selected by ribosome display were cloned into the pQE30 (Qiagen) expression vector, transformed into *E. coli* XL1-Blue (Stratagene) and then grown overnight at 37°C in a 96-deep-well plate (each clone in a single well) containing 1 ml growth medium (2YT containing 1% glucose and 100 µg/ml ampicillin). 1 ml of fresh 2YT containing 50 µg/ml ampicillin was inoculated with 100 µl of the overnight culture in a fresh 96-deep-well plate. After incubation for 2 h at 37°C, expression was induced with IPTG (1 mM final concentration) and continued for 3 h. Cells were harvested, resuspended in 100 µl B-PERII (Pierce) and incubated for 15 min at room temperature with shaking. Then, 900 µl PBS-TC (PBS supplemented with 0.25% Casein hydrolysate, 0.1% Tween 20®, pH 7.4) were added and cell debris were removed by centrifugation. 100 µl of each lysed clone were applied to a well of a Neutravidin coated MaxiSorp plate containing either HER2 or the unrelated MBP immobilized via their biotin moiety and incubated for 1 h at RT. After extensive washing with PBS-T (PBS supplemented with 0.1% Tween 20®, pH 7.4) the plate was developed using standard

ELISA procedures using the monoclonal horse-radish-labeled anti-RGS(His)$_4$ antibody (34650, Qiagen) Binding was then detected by POD substrate (Roche). The color development was measured at 405 nm. Screening of several hundred clones by such a crude cell extract ELISA revealed more than hundred different DARPins with specificity for HER2. These binding proteins were chosen for further analysis. Examples of amino acid sequences of selected ankyrin repeat domains that specifically bind to the ectodomain HER2 are provided in SEQ ID NO: 62 to 82 and 112 to 121.

These ankyrin repeat domains with binding specificity for HER2 and a negative control ankyrin repeat domain with no binding specificity for HER2 (i.e. SEQ ID NO: 111) were cloned into a pQE (QIAgen, Germany) based expression vector providing an N-terminal His-tag to facilitate simple protein purification as described below. Thus, expression vectors encoding the following DARPins were constructed:

DARPin #1 (SEQ ID NO: 62 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #2 (SEQ ID NO: 63 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #3 (SEQ ID NO: 64 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #5 (SEQ ID NO: 66 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #6 (SEQ ID NO: 67 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #7 (SEQ ID NO: 68 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #8 (SEQ ID NO: 69 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #9 (SEQ ID NO: 70 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #10 (SEQ ID NO: 71 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #11 (SEQ ID NO: 72 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #12 (SEQ ID NO: 73 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #13 (SEQ ID NO: 74 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #14 (SEQ ID NO: 75 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #15 (SEQ ID NO: 76 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #16 (SEQ ID NO: 77 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #17 (SEQ ID NO: 78 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #18 (SEQ ID NO: 79 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #19 (SEQ ID NO: 80 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #20 (SEQ ID NO: 81 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #21 (SEQ ID NO: 82 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #50 (SEQ ID NO: 111 with a His-tag (SEQ ID NO: 6) fused to its N-terminus).
DARPin #51 (SEQ ID NO: 112 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);

DARPin #52 (SEQ ID NO: 113 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #53 (SEQ ID NO: 114 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #54 (SEQ ID NO: 115 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #55 (SEQ ID NO: 116 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #56 (SEQ ID NO: 117 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #57 (SEQ ID NO: 118 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #58 (SEQ ID NO: 119 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #59 (SEQ ID NO: 120 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #60 (SEQ ID NO: 121 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);

Examples of amino acid sequences of selected biparatopic ankyrin repeat proteins are provided in SEQ ID NO: 83 to 110, 122, 123, and 136 to 141. These biparatopic DARPins were cloned into a pQE (QIAgen, Germany) based expression vector providing an N-terminal His-tag to facilitate simple protein purification as described below. Thus, expression vectors encoding the following DARPins were constructed:

DARPin #22 (SEQ ID NO: 83 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #23 (SEQ ID NO: 84 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #24 (SEQ ID NO: 85 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #25 (SEQ ID NO: 86 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #26 (SEQ ID NO: 87 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #27 (SEQ ID NO: 88 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #28 (SEQ ID NO: 89 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #29 (SEQ ID NO: 90 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #30 (SEQ ID NO: 91 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #31 (SEQ ID NO: 92 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #32 (SEQ ID NO: 93 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #33 (SEQ ID NO: 94 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #34 (SEQ ID NO: 95 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #35 (SEQ ID NO: 96 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #36 (SEQ ID NO: 97 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #37 (SEQ ID NO: 98 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #38 (SEQ ID NO: 99 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #39 (SEQ ID NO: 100 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #40 (SEQ ID NO: 101 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #41 (SEQ ID NO: 102 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);

DARPin #42 (SEQ ID NO: 103 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #43 (SEQ ID NO: 104 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #44 (SEQ ID NO: 105 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #45 (SEQ ID NO: 106 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #46 (SEQ ID NO: 107 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #47 (SEQ ID NO: 108 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #48 (SEQ ID NO: 109 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #49 (SEQ ID NO: 110 with a His-tag (SEQ ID NO: 6) fused to its N-terminus)
DARPin #61 (SEQ ID NO: 122 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #62 (SEQ ID NO: 123 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #63 (SEQ ID NO: 136 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #64 (SEQ ID NO: 137 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #65 (SEQ ID NO: 138 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #66 (SEQ ID NO: 139 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #67 (SEQ ID NO: 140 with a His-tag (SEQ ID NO: 6) fused to its N-terminus);
DARPin #68 (SEQ ID NO: 141 with a His-tag (SEQ ID NO: 6) fused to its N-terminus).

High level and soluble expression of monovalent DARPins

For further analysis, DARPins #1 to 50 were expressed in *E. coli* BL21 or XL1-Blue cells and purified using their His-tag using standard protocols. 25 ml of stationary overnight cultures (LB, 1% glucose, 100 mg/l of ampicillin; 37°C) were used to inoculate 1 l cultures (same medium). At an absorbance of 0.7 at 600 nm, the cultures were induced with 0.5 mM IPTG and incubated at 37°C for 4-5 h. The cultures were centrifuged and the resulting pellets were resuspended in 40 ml of TBS500 (50 mM Tris–HCl, 500 mM NaCl, pH 8) and sonicated. The lysate was recentrifuged, and glycerol (10% (v/v) final concentration) and imidazole (20 mM final concentration) were added to the resulting supernatant. Proteins were purified over a Ni-nitrilotriacetic acid column (2.5 ml column volume) according to the manufacturer's instructions (QIAgen, Germany). Alternatively, DARPins or selected repeat domains devoid of a 6xHis-tag were purified by anion exchange chromatography followed by size exclusion chromatography according to standard resins and protocols known to the person skilled in the art. Up to 200 mg of highly soluble DARPins with binding specificity to HER2 can be purified from one liter of *E. coli* culture with a purity > 95% as estimated from SDS-15% PAGE. Such purified DARPins are used for further characterizations.

Example 2: *Characterization of the DARPins with binding for specificity for HER2 by Surface Plasmon Resonance Analysis*

Protein binding kinetics of interesting purified HER2-binding DARPins were assayed by Surface Plasmon Resonance (SPR) analysis with a ProteOn array system (BioRad) using a setup, where biotinylated human HER2 was immobilized *via* neutravidin and the interaction was measured by adding free monovalent DARPin. The determination of Kd values was performed according to standard procedures.

Biotinylated ectodomain of human HER2 molecule was immobilized in a flow cell through binding to coated Streptavidin and the interaction with various selected DARPins was analyzed.

Surface Plasmon Resonance (SPR) analysis

SPR was measured using a ProteOn instrument (BioRad) and measurement was performed according standard procedures known to the person skilled in the art. The running buffer was PBS, pH 7.4, containing 0.005% Tween 20®. Neutravidin was covalently immobilized on a GLC chip (BioRad) to a level of about 8000 resonance units (RU). Immobilization of HER2 on the neutravidin coated chip was then performed. The interaction of DARPin HER2 was then measured by injecting 100 µl running buffer (PBS containing 0.005% Tween®) containing serial dilutions of DARPins of concentration of 50, 25, 12.5, 6.25 and 3.125 nM (on-rate measurement), followed by a running buffer flow for between 10 minutes and up to 3 hours at a constant flow rate of 100 µl/min (off-rate measurement). The signals (i.e. resonance unit (RU) values) of an uncoated reference cell and a reference injection (i.e. injection of running buffer only) were subtracted from the RU traces obtained after injection of HER2 (double-referencing). From the SRP traces obtained from the on-rate and off-rate measurements the on- and off-rate of the corresponding DARPin HER2 interaction can be determined.

The results are summarized in Table 1. Dissociation constants (Kd) were calculated from the estimated on- and off-rates using standard procedures known to the person skilled in the art.

Table 1: Dissociation constants of selected DARPins for human HER2 as determined by SPR

DARPin#	Kd [M]
1	7.81E-11
2	8.75E-10
3	1.31E-11
4	1.86E-10
5	7.08E-11
6	2.92E-11
7	1.03E-09
8	4.83E-10
9	4.17E-10
10	1.03E-09
11	2.56E-10
12	1.41E-09
13	n.d.
14	1.88E-09
15	4.68E-10
16	2.67E-09
17	2.30E-09
18	3.35E-10
19	9.44E-10
20	2.58E-10
21	1.65E-09
51	1.3E-09
52	1.37E-10
53	1.46E-09
54	9.27E-12
55	8.73E-11
56	2.00E-09
57	6.04E-11
58	4.13E-11
59	3.33E-11
60	1.17E-11

n.d.: not determined.

Example 3: *Mapping repeat domain binding to specific extracellular HER2 epitopes*

The interaction of the repeat domains with the extracellular HER2 domains was analyzed by standard methods known to the person skilled in the art, such as quaternary structure analysis of the complexes by X-ray crystallography or NMR spectroscopy, or epitope mapping by using alanine mutagenesis of potential interaction residues or by using mass spectrometry and covalent tagging. Furthermore, various competition assays, such as competition enzyme-linked immunosorbent assays (ELISAs) know to the practitioner in the art were performed to identify the extracellular domains to which selected repeat protein bind or if they have overlapping epitopes on the extracellular domains of HER2 with other binding proteins, for example antibodies such as trastuzumab or pertuzumab.

The extracellular domains of HER2 were either purchased or produced as described (Jost et. al., loc. cit.)

Competition of interesting purified HER2-binding DARPins was performed by Surface Plasmon Resonance (SPR) analysis with a ProteOn array system (BioRad) using a setup, where biotinylated human ErbB2 S22-N530 and ErbB2 S22-E645 was immobilized *via* neutravidin and the competition was measured by adding the first monovalent DARPin at saturation (1 uM), followed by a 1:1 mixture of the first and the second DARPin (100 nM each). If the second DARPin bound, despite the presence of the first DARPin, the second DARPin was considered to bind a different epitope.

For example, competition ELISA (Fig 1A and 1B) data suggest that DARPin #54 binds to domain II in Her2 and DARPin #51 binds to domain I of HER2. Previously it was shown that DARPin #18 binds to domain IV of HER2 (Jost et al., loc. cit.). The DARPins (20nM) were preincubated with HER2 domain I, domain I-III or domain III-IV (in each case at a domain concentration of 500nM) in PBS for 45min at room temperature. The mixture was added to 20nM of full length Her2 coated on a F96 MaxiSorb Nunc (Cat. 442404) plate. Bound DARPins were specifically detected using a monoclonal mouse anti RGS-His antibody (Qiagen Cat.34650) as primary antibody and an anti-mouse antibody labeled with horse radish peroxidase (Pierce, Cat.31438) as secondary antibody. The primary antibody (mouse anti RGS-His antibody) was replaced by a monoclonal mouse anti-DARPin antibody for the ELISA depicted in Figure 1B.

The read out was made at 450 nm. All the incubations steps were performed in PBS at pH 7.4 containing 0.1% Tween 20® and 0.25% Casein at room temperature for 2h on a Heidolph Titramax 1000 shaker at 450 rpm except the plate coating, which was performed over-night at 4°C using PBS at pH 7.4.

These findings were confirmed by competing binding of these DARPins to Her2 overexpressing cells (BT474) with recombinant domain I, domain I-II-III and domain III-IV of Her2 by Flow Cytometry (FACS). DARPins (100 nM) were preincubated with the individual Her2 constructs (1 uM) at 25°C for 30 minutes. The mixture was applied to cells (100.000 cells in 100 ul) for 20 minutes on ice. DARPin binding to cells was monitored using an Alexa 647 labeled anti-Penta-His antibody (Qiagen Cat. No: 35370). The analyses confirmed the binding of DARPin #51 to domain I of HER2 and DARPin #1 to domain II in HER2 and DARPin#18 to domain IV of HER2.

Competition of DARPin #1 with pertuzumab and DARPin #18 with trastuzumab was also tested using Flow Cyotmetry. To this end BT474 cells were preincubated with pertuzumab, respectively trastuzumab (both 1 uM) before incubation with the respective DARPin (1 uM). Binding of DARPin to the cells was monitored using an Alexa 647 labeled anti-Penta-His antibody (Qiagen Cat. No: 35370) and binding of pertuzumab or trastuzumab was monitored using an Alexa 546 labeled anti-human-IgG antibody (Invitrogen Cat. No: A-21089). The experiment showed that none of the DARPins competes with binding of pertuzumab or trastuzumab to HER2 expressed by BT474 cells.

This finding was also observed by ELISA (Fig. 1C), where pertuzumab (coated on a F96 MaxiSorb Nunc (Cat. 442404) at 20nM) was preincubated with 20nM Her2 (domain I-IIII) before incubation with the respective DARPins (20nM). The specific binding of the DARPin on the Her2-Pertuzumab complex was detected using a monoclonal mouse anti RGS-His antibody (Qiagen, Cat.34650) and an anti-mouse antibody labeled with horse radish peroxidase (Pierce, Cat.31438) (premixed for 45min at room temperature). All the incubations steps were performed at room temperature for 2h on a Heidolph Titramax 1000 shaker at 450 rpm except the plate coating, performed over-night at 4°C. PBS, 0.1% Tween 20® pH7.4, 0.25% Casein was used a blocking agent. All the N-terminal DARPins tested in this assay (DARPin #7, DARPin #52, DARPin #53, and DARPin #54) are binding Her2 in presence of pertuzumab, showing that they all bind a different epitope than the antibody.

Overall such experiments showed that the monovalent repeat domains encoded by SEQ ID NO: 62 to 68, 72, and 114 to 121 bind to domain II of HER2, the monovalent repeat domains encoded by SEQ ID NO: 69-71, 73, 112 and 113 bind to domain I of HER2 and the monovalent repeat domains encoded by SEQ ID NO: 74 to 82 bind to domain IV of HER2. None of the monovalent repeat domains binding to domain II of HER2 (SEQ ID NO: 62 to 68, 72, and 114 to 121 compete with pertuzumab on binding to HER2. Among the monovalent repeat domain binding to domain IV of HER2, the repeat domains encoded by the SEQ ID NO: 77, 78 and 82 compete with trastuzumab for binding to HER2 whereas the repeat domains encoded by the SEC ID NO: 74 to 76 and 79 to 81 do not compete with trastuzumab.

Example 4*: Biparatopic Her2-binding DARPins block growth of Her2-overexpressing tumor cells.*

Monovalent DARPins, mixtures of DARPins and biparatopic Her2-binding DARPins were tested for inhibition of BT474 cell proliferation. Figure 2 shows that monovalent DARPins and mixtures of monovalent DARPins are not capable to block BT474 proliferation. In contrast, a subset of biparatopic DARPins induce proliferation inhibition (Figure 2, and Table 2). Interestingly, DARPins repeat domain IV of HER2 have to be located at the C-terminus of the molecule (Figure 2). Multiple combinations of monovalent DARPins in a biparatopic format resulted in proliferation inhibiting biparatopic DARPins. However, not all combinations are capable to block BT474 proliferation to 90-100% (Figure 3), which allows ranking of certain DARPin combinations. These findings indicate that targeting a distinct subset of certain epitopes in HER2 in a biparatopic format is key for achieving potency. Induction of HER2 receptor internalization and degradation as reported by trastuzumab is not sufficient to induce potent inhibition of tumour cell proliferation (Figure 3 and 5). Both DARPin #41 and DARPin #43 induce degradation of Her2 similar to trastuzumab, but only DARPins such as DARPin #41 inhibits tumour cell proliferation.

Experiments were performed as described in the Methods section. Example results are summarized in Table 2. IC_{50} values were calculated from the titration curves obtained as described above using standard procedures known to the person skilled in the art. Example titration curves are given for DARPin #41 in Figure 2 and 3.

Table 2 : *Inhibition potency by various DARPins of BTB474 cell proliferation*

DARPin # or antibody	IC50[nM]	% activity vs. DARPin # 41
32	3.29	48.0
22	4.03	60.1
27	4.57	37.8
35	4.63	63.0
38	3.30	99.3
33	4.47	65.3
23	2.99	97.3
28	5.15	82.5
36	2.56	68.8
34	3.88	95.1
24	1.97	99.9
29	1.33	95.0
37	2.19	94.8
40	2.76	91.2
42	3.77	100
45	1.55	100
46	3.34	100
41	4.01	100
47	n.i.	6.8
43	n.i.	n.i.
44	n.i.	n.i.
48	n.i.	n.i.
49	n.i	n.i
21	n.i.	n.i.
12	n.i.	n.i.
1	n.i.	n.i.
18	n.i.	n.i.
64	2.31	100
65	4.07	100
63	1.77	100
68	5.35	100
67	4.87	100
66	4.06	100
64	2.31	100
trastuzumab	3.05	52
pertuzumab	n.i	n.i

n.i.: no inhibition observed

Example 5: *Biparatopic Her2-targeting DARPins inhibit proliferation of various Her2 overexpressing cell lines and induces apoptosis*

The potency of the biparatopic DARPin #41 was tested The DARPin inhibited proliferation in cell lines overexpressing Her2 in the range from Her2 IHC 3+ to 1+ and not in cells expressing wild type HER2 levels (Figure 4; Table 3). Moreover the DARPin induces robustly apoptosis within 24h of incubation in the listed cell lines (Figure 5, Table 3).

Experiments were performed as described in the Methods section. Example results are summarized in Table 3. IC_{50} and EC_{50} values were calculated from the titration curves obtained as described above using standard procedures known to the person skilled in the art. Example titration curves are given for DARPin #41 on three different cell lines in Figure 4 and 5. The IC50 and EC50 values ranges between 0.2 – 10 nM, depending on the tested DARPin and the cell line. For example, it was shown that DARPin #41, #45 and #46 induce apoptosis in BT474, MDA-MB175 and NCI-N87 cells (Table 3). Similar results were obtained using other biparatopic binding proteins of the inventions.

Table 3: Potency of DARPin #41 on various different cell lines

Cell line	Her2 status	Inhibition of proliferation IC50[nM]	Induction of apoptosis EC50 [nM]
BT474	IHC 3+	0.98	0.69
SKBR-3	IHC3+	1.75	n.a.
NCI-N87	IHC2+	0.94	0.26
ZR75-30	IHC3+	0.60	n.a.
HCC1419	IHC 3+	3.17	n.a.
MDA-MB175	IHC 1+	3.42	5.94
MCF7	IHC 0 / wt	n.i.	n.i.

n.a.: not analyzed

n.i.: no inhibition

Example 6: *Biparatopic Her2-targeting DARPins inhibit proliferation and induces apoptosis in BT474 cells in contrast to the current standard of care therapies*

The potency of the biparatopic DARPin #41 was compared to drugs approved for the treatment of Her2 positive breast cancers, trastuzumab and pertuzumab. The DARPin efficiently inhibits proliferation and is inducing apoptosis in contrast to trastuzumab, Pertuzumab or a combination of trastuzumab and pertuzumab (Figure 6).

Experiments were performed as described in the Methods section. Example results are shown in Figure 6. IC_{50} and EC_{50} values (Table 3) were calculated from the titration curves obtained as described above using standard procedures known to the person skilled in the art. Similar results were obtained using other biparatopic binding proteins of the inventions.

Example 7: *Generation of various DARPin formats*

As an example, the potency of different formats of the biparatopic DARPin #41 were compared to DARPin #41 in inhibition of BT474 cell proliferation (Figure 7, Table 2). PEGylation or fusion to a human serum albumin binding DARPin (DARPin #41, #63, #64, #65) to the N- or C-terminus did not affect potency (Figure 7A). Moreover variation of the linkers between the DARPin moieties did not affect potency (Figure 7B). The IC50 values

range between 1.5 – 5.5 nM. Corresponding results were obtained using corresponding formats of the biparatopic DARPins #41, #66, #67, #68 was obtained. Overall, this clearly suggests that the biparatopic DARPins can be modified (by methods known to the person skilled in the art, such as PEGylation or fusion to serum albumin binding domains) to increase their *in vivo* half-life without the loss of potency. Furthermore, these experiments suggest that the linker between the two repeat domains binding to HER2 in a biparatopic construct can be varied at least from two to 24 amino acids without significantly influencing the efficacy of the biparatopic construct.

Example 8: *DARPin/Her2 interaction mapping*

The interaction of the biparatopic DARPins of the inventions with the HER2 ectodomain was further analyzed by chemical crosslinking of the complex formed by these two molecules in solution (i.e. in PBS pH 7.4), followed by a digest of the complex with a protease, and analysis of the resulting peptides by mass spectroscopy. In such an experiment regions of the DARPin can be covalently crosslinked to regions of HER2 only if they are in close proximity to the latter. The detection of peptides from the DARPin that are covalently crosslinked to a corresponding peptide of HER2 by such a mass spectroscopy analysis indicates that those peptides are in close proximity in the HER2/DRAPin complex. Such proximity analysis methods are well known to the person skilled in the art (e.g., Birch, C., et al., *Anal. Chem.*, *82,* 172–179, 2010) and are offered by various companies as a service (e.g., CovalX AG, Zürich, Switzerland).

For example, in such experiments it was found that the biparatopic DARPin #41, which binds domain II and domain IV of HER2, can form a 1 to 1 complex with HER2. Surprisingly, covalent crosslinks between the C-terminal repeat domain (binding to domain IV of HER2) and domain I of HER2 were observed, indicating close proximity of this repeat domain with domain I of HER2 in the complex, even though it binds to domain IV. Such crosslinks would not be expected to be seen if HER2 would be in a conformation as described in the prior art (e.g., Bublil and Yarden, loc. cit). Importantly, when the HER2 ectodomain was analyzed in complex with this C-terminal repeat domain binding to domain IV alone then no such crosslinks to domain I of HER2 could be observed, indicating that in the case of the complex formed by HER2 and the monomeric repeat domain binding to domain IV, no proximity of this repeat domain to domain I exists. Thus, the three dimensional domain arrangements for HER2 must be different in the complex

formed with the biparatopic binding protein of the invention compared to the complex formed with the individual repeat domain binding domain IV of HER2.

Interestingly, the known structures of the ectodomain of HER2 would not allow the simultaneous binding of both repeat domains of a biparatopic binding protein of the invention to the same HER2 molecule, when considering the short linkers in the range of 2 to 24 amino acids between two repeat domains. This indicates that HER2 may be in a yet unknown conformation allowing the simultaneous binding of both repeat domains.

Overall, such experiments indicate that the biparatopic binding proteins of the invention may be able to intramolecularly interact with the ectodomain of HER2, and that they thereby fix the HER2 ectodomain in a novel conformation not known in the prior art, namely by bringing domain I and domain IV in a steric arrangement that allows the observed crosslink between the repeat domain (binding to domain IV of HER2) and domain I to occur. Thus, this novel conformation of HER2 seems to be stabilized by a biparatopic binding protein of the invention by simultaneously binding domain II and domain IV of HER2 in an intramolecular manner.

What is claimed is:

1. A recombinant binding protein comprising at least a first and a second repeat domain, wherein each of said two repeat domains binds the extracellular region of HER2 and wherein said repeat domains are covalently linked.

2. The binding protein of claim 1, wherein said first repeat domain binds domain II of HER2 and said second repeat domain binds domain IV of HER2.

3. The binding protein according to any of claims 1 - 2, wherein the first and second repeat domains are located on the same polypeptide and wherein the repeat domain targeting domain II of HER2 is located N-terminally to the repeat domain targeting domain IV of HER2.

4. The binding protein according to any of the aforementioned claims, wherein said first repeat domain binding domain II of HER2 is not competing for binding to HER2 with pertuzumab.

5. The binding protein according to any of the aforementioned claims, wherein said second repeat domain binding domain IV of HER2 is not competing for binding to HER2 with trastuzumab.

6. The binding protein according to any of the aforementioned claims, wherein said first repeat domain is an ankyrin repeat domain and said second repeat domain is an ankyrin repeat domain.

7. The binding protein according to any of the aforementioned claims, wherein said first repeat domain binds the extracellular region HER2 in PBS with a Kd below 10^{-7}M and said second repeat domain binds the extracellular region HER2 in PBS with a Kd below 10^{-7}M.

8. The binding protein according to any of the aforementioned claims, wherein said binding protein inhibits stimulated proliferation of BT474 cells with an IC50 value of smaller than 100 nM.

9. The binding protein according to any of the aforementioned claims, wherein said binding protein induces apoptosis in BT474 cells with an EC50 value below 100 nM.

10. The binding protein according to any of the aforementioned claims, wherein said first and second repeat domains are connected by a polypeptide linker.

11. The binding protein of claim 10, wherein

- said first repeat domain competes for binding to HER2 with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 62 to 68, 72 and 114 to 121, and/or
- said second repeat domain competes for binding to HER2 with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 74 to 82.

12. The binding protein of claim 10, wherein

- said first repeat domain comprises an amino acid sequence that has at least 70% amino acid sequence identity with one ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 62 to 68, 72 and 114 to 121 and/or
- said second repeat domain comprises an amino acid sequence that has at least 70% amino acid sequence identity with one ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 74 to 82,

and wherein further,

- G at position 1 and/or S at position 2 of said ankyrin repeat domains are optionally missing; and
- L at the second last position and/or N at the last position of said ankyrin repeat domains are optionally exchanged by A.

13. The binding protein according to any of claims 10 to 12, wherein

- said first repeat domain is selected from the group of ankyrin repeat domains consisting of SEQ ID NOs: 62 to 68, 72 and 114 to 121 and/or
- said second repeat domain is selected from the group of ankyrin repeat domains consisting of SEQ ID NOs: 74 to 82

and wherein further

- G at position 1 and/or S at position 2 of said ankyrin repeat domains are optionally missing; and

- L at the second last position and/or N at the last position of said ankyrin repeat domains are optionally exchanged by A.

14. The binding protein according to any of claims 10 to 13 wherein

- said first repeat domain comprises an ankyrin repeat module having an amino acid sequence selected from the group consisting of SEQ ID NOs: 15 to 18, 21 to 23, 37, 38, 125, 126, 129, 130, 133, 134 and sequences, wherein up to 9 amino acid residues in SEQ ID NOs: 15 to 18, 21 to 23, 37, 38, 125, 126, 129, 130, 133, 134 are replaced by any other amino acid residues, and/or
- said second repeat domain comprises an ankyrin repeat module having an amino acid sequence selected from the group consisting of SEQ ID NO: 46, 47, 51, 52, 55 and 56 and sequences, wherein up to 9 amino acid residues in SEQ ID NO: 46, 47, 51, 52, 55 and 56 are replaced by any other amino acid residues.

15. The composition according to any of claims 10 to 14, wherein said ankyrin repeat module has an amino acid sequence selected from the group consisting of KDFQGITPLHIAATSGHLEIVEVLLKAGADVNA (SEQ ID NO: 16) and sequences, in which up to 9 amino acid residues in SEQ ID NO: 16 are replaced by any other amino acid residues, and wherein

- F at position 3 is optionally exchanged by A
- Q at position 4 is optionally exchanged by E;
- G at position 5 is optionally exchanged by S;
- I at position 6 is optionally exchanged by V;
- I at position 11 is optionally exchanged by L;
- T at position 14 is optionally exchanged by Q; and/or
- N at position 15 is optionally exchanged by an amino acid selected from the group consisting of S and W; most preferably S.

16. The composition according to any of claims 10 to 14, wherein said ankyrin repeat module has an amino acid sequence selected from the group consisting of KDITGETPLHHAADSGHLEIVEVLLKAGADVNA (SEQ ID NO: 18) and sequences, in which up to 9 amino acid residues in SEQ ID NO: 18 are replaced by any other amino acid residues, and wherein

- I at position 3 is optionally exchanged by V;

- E at position 6 is optionally exchanged by D;
- H at position 11 is optionally exchanged by L;
- D at position 14 is optionally exchanged by Q;
- S at position 15 is optionally exchanged by H; and/or
- E at position 19 is optionally exchanged by V.

17. The binding protein according to any of claims 10 to 15, wherein said binding protein comprises a polypeptide, wherein said polypeptide has at least 70%, preferably 90 % amino acid sequence identity with a polypeptide selected from the group consisting of SEQ ID NO: 83 to 98, 102, 103, 122, 123 and 136 to 141. .

18. A pharmaceutical formulation comprising a binding protein or a composition according to the aforementioned claims.

19. Use of at least one binding protein, composition or pharmaceutical formulation according to the aforementioned claims for the treatment of cancer

20. A process comprising administering a binding protein, composition or pharmaceutical formulation according to the aforementioned claims to a patient for treating cancer

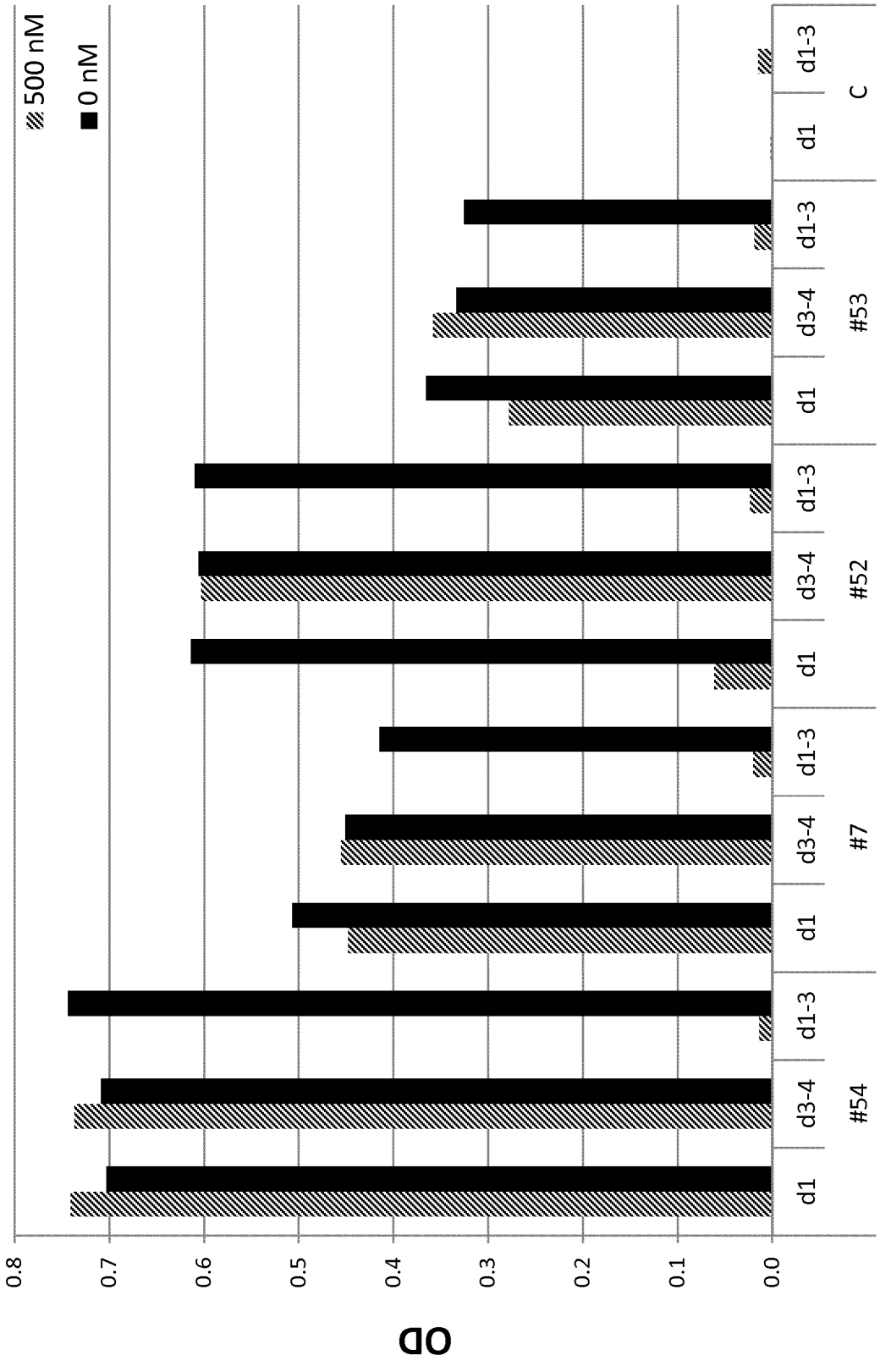
21. Use or process according to claim 19 or 20, in which use or process the disease is characterized by at least one feature selected from the group consisting of

- Amplification of the HER2 encoding gene
- Overexpression of the HER2 encoding gene,
- Expression of a mutated form of the HER2 encoding gene, and/or
- Overexpression of the Her3 encoding gene in trastuzumab resistant tumors.

22. Use or process according to any of claims 19 - 21, in which use or process the disease is at least one selected from the group consisting of

- Breast cancer
- ovarian cancer
- gastric cancer
- stomach cancer,

- uterine cancer, and/or
- colorectal cancer.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d1-3
#54
d1
d3-4
d1-3
#7
d1
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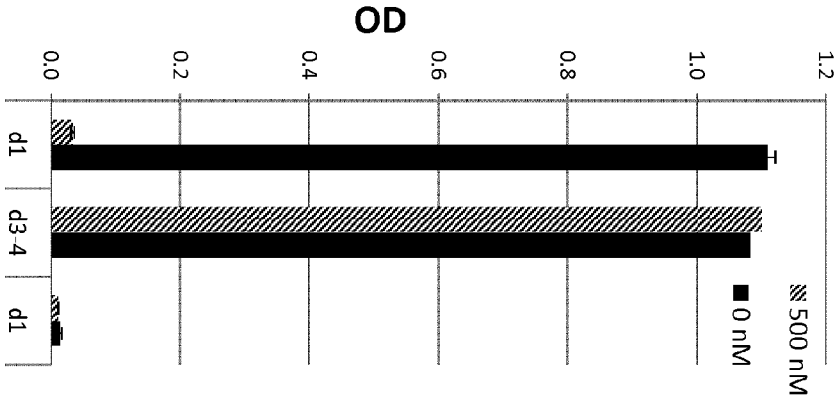
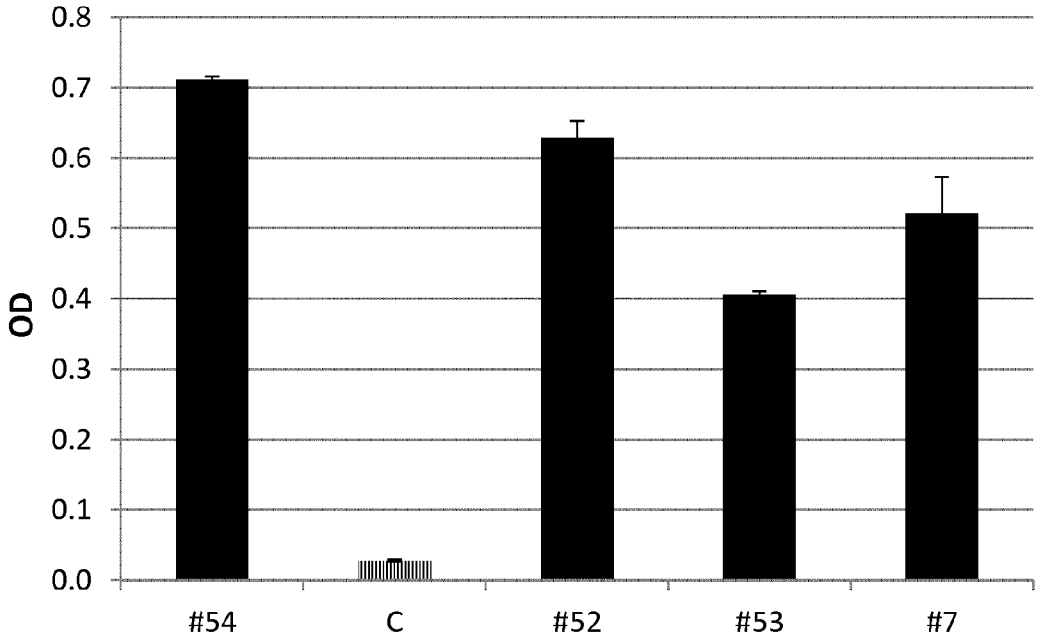
C

Fig. 1A

Fig. 1B





1C

Fig. 2A

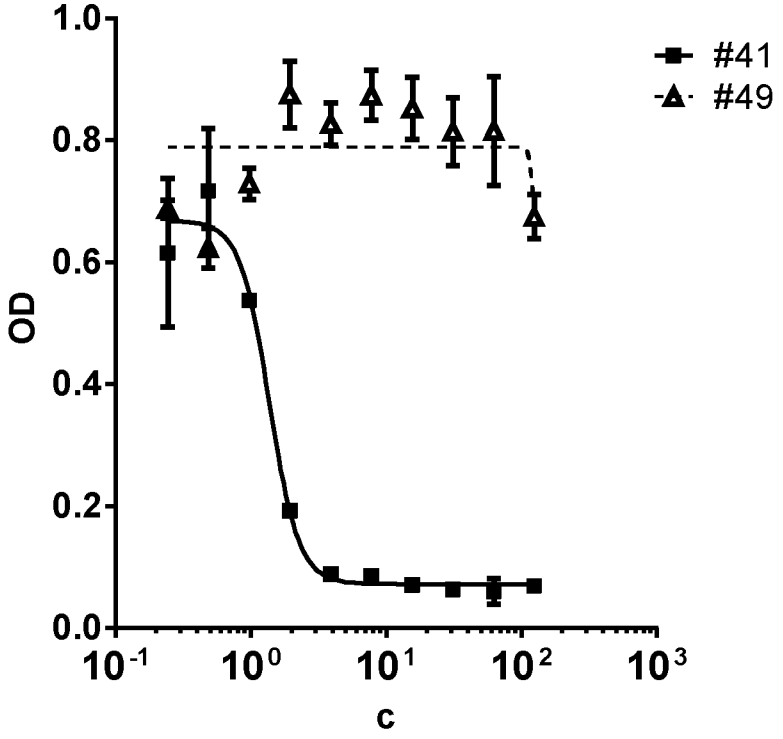

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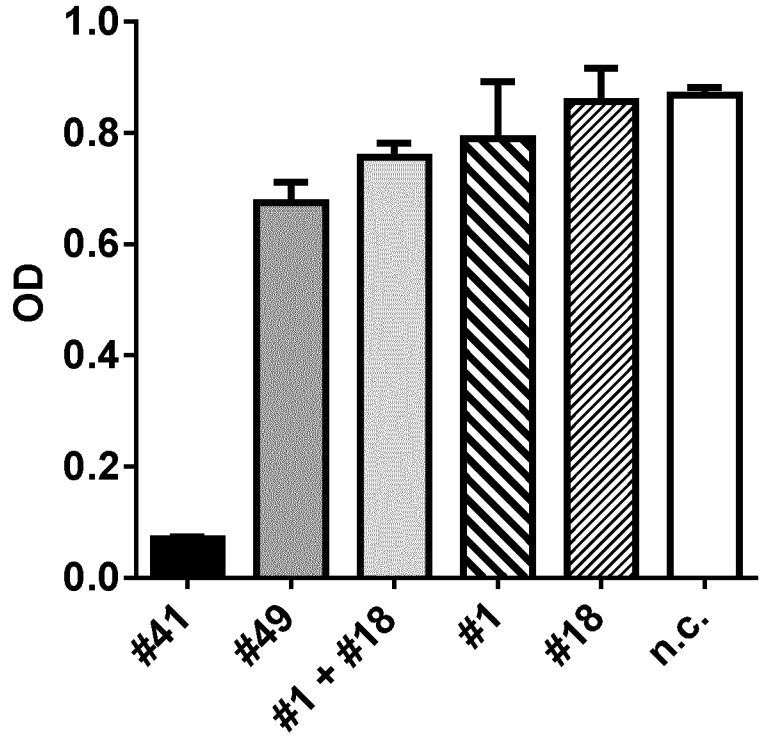
Fig. 2B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#18
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Fig. 3A

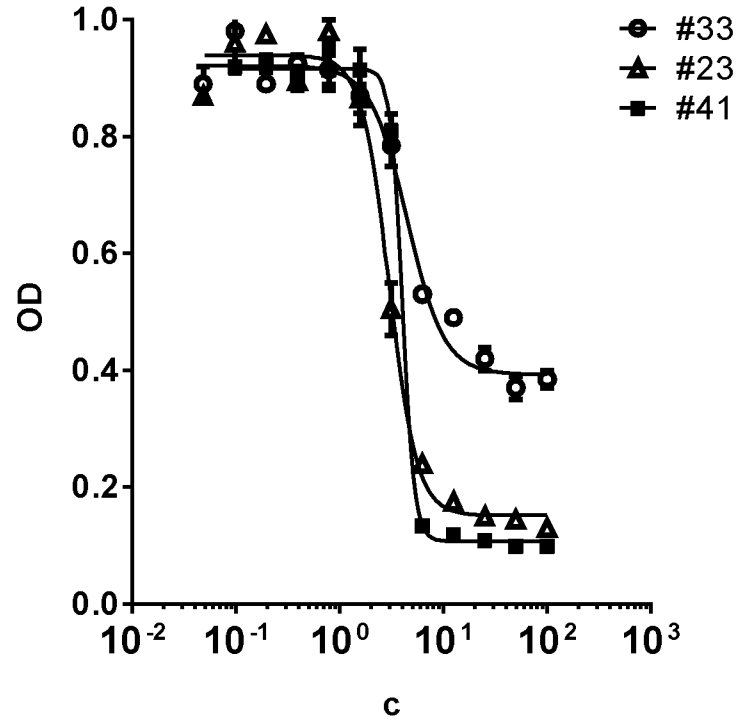
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Fig. 3B

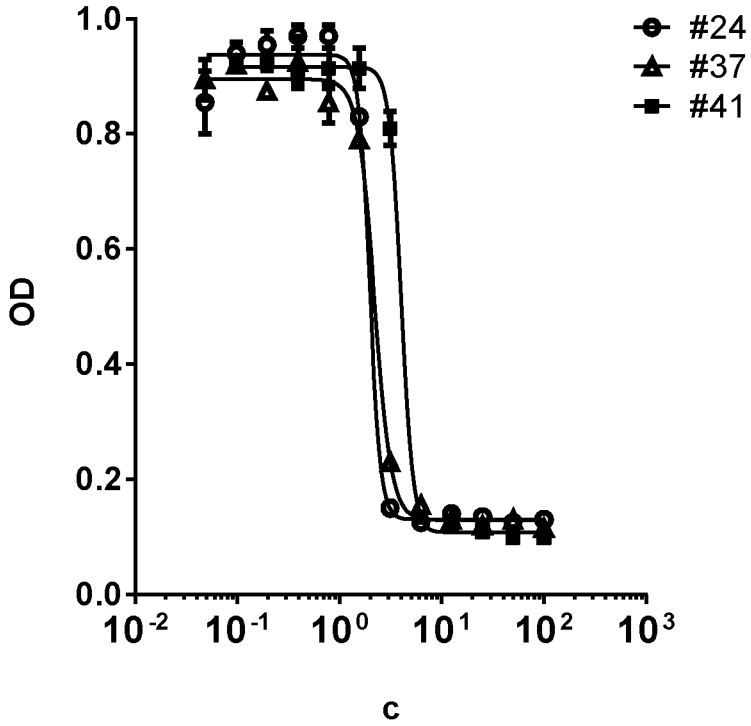
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Fig. 3C

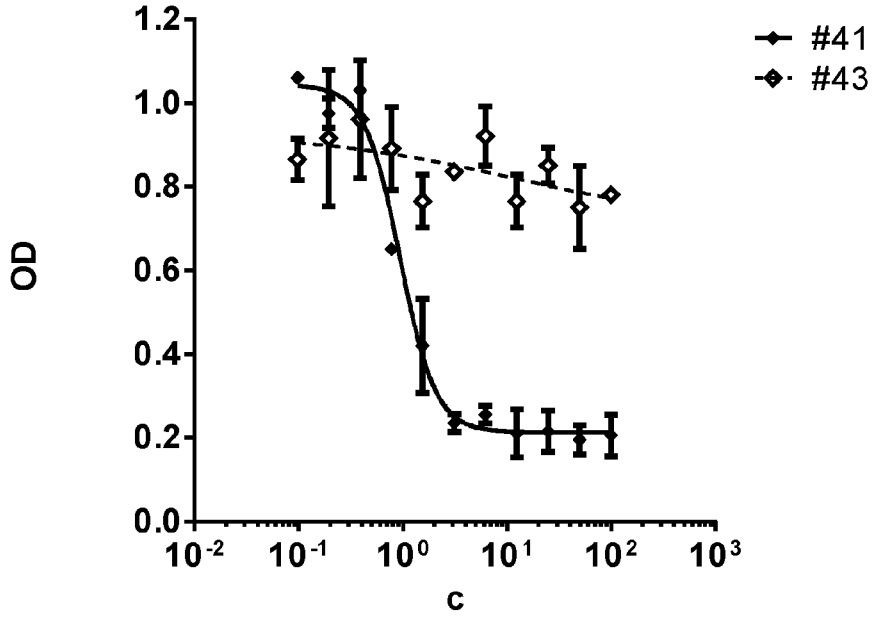
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Fig. 3D

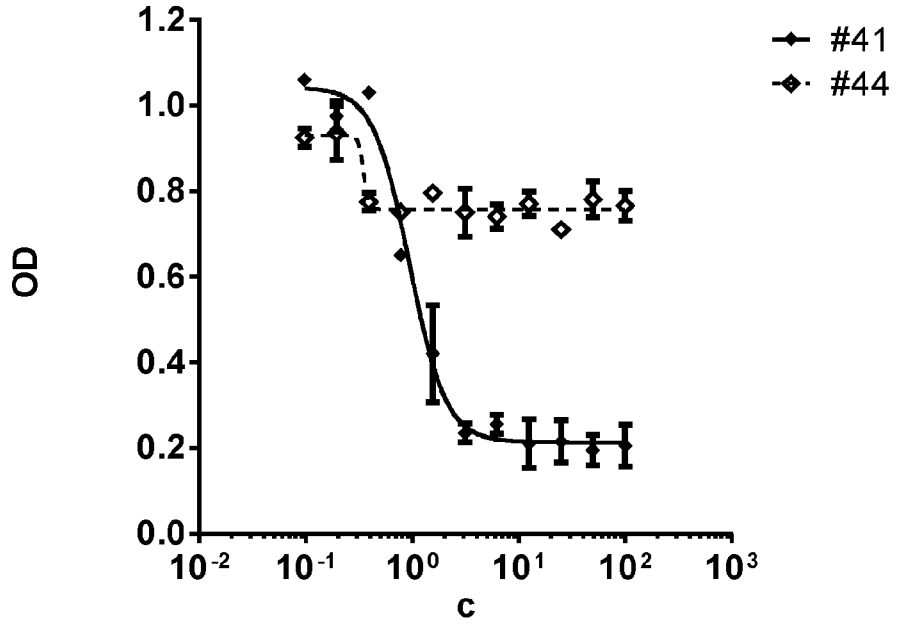
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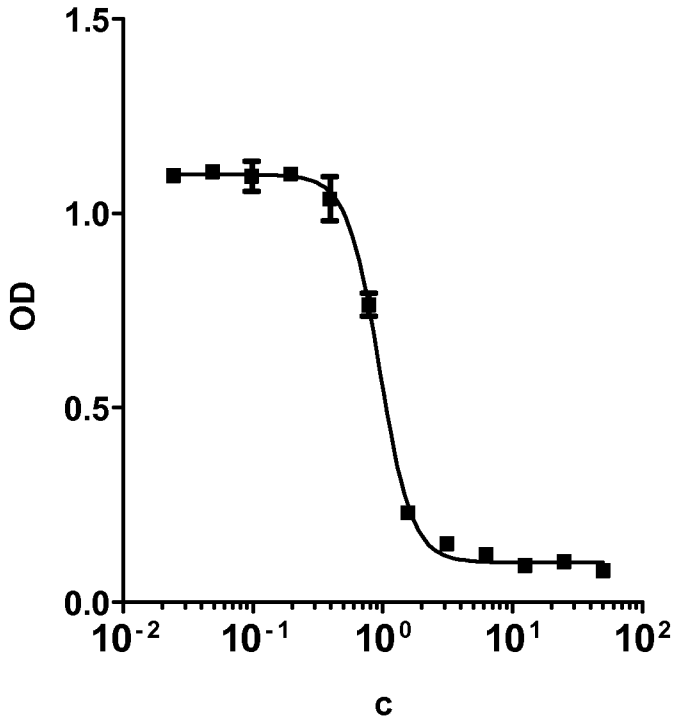
Fig. 4A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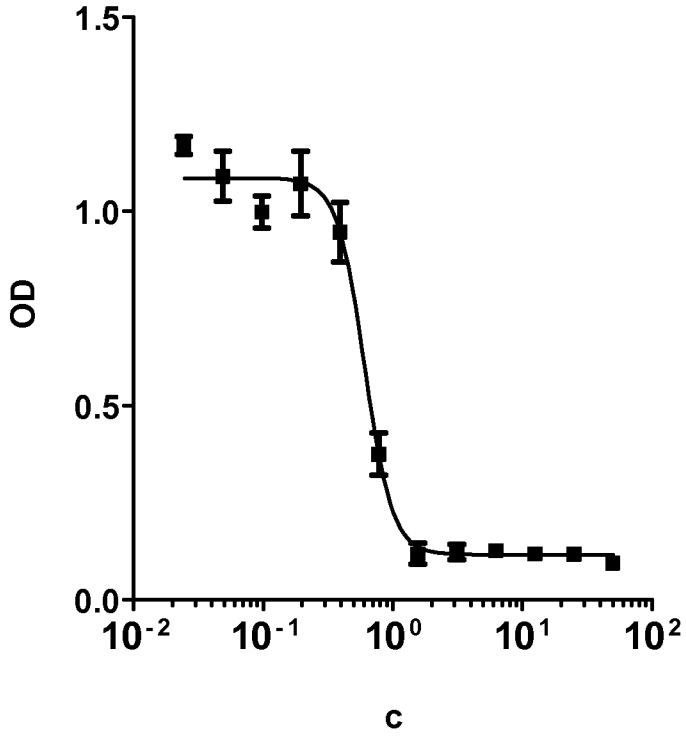
101
102
c

Fig. 4B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OD
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10-1
100
101
102
c

Fig. 4C

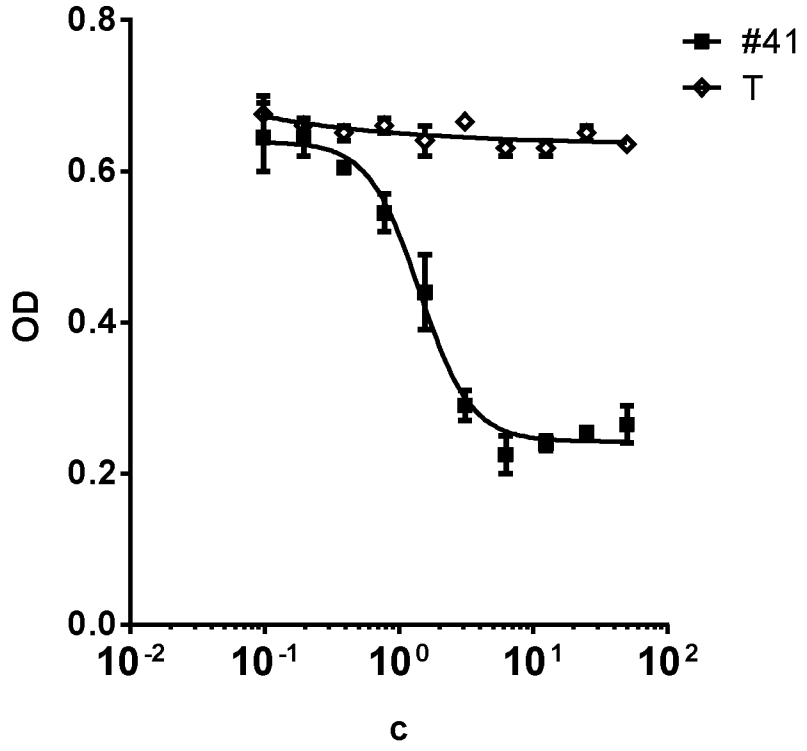
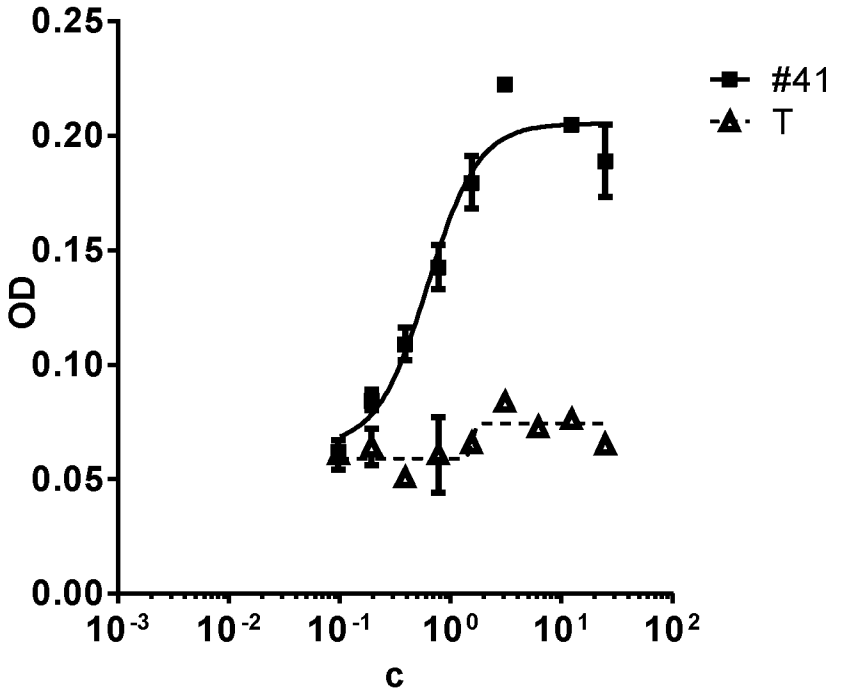


Fig. 5A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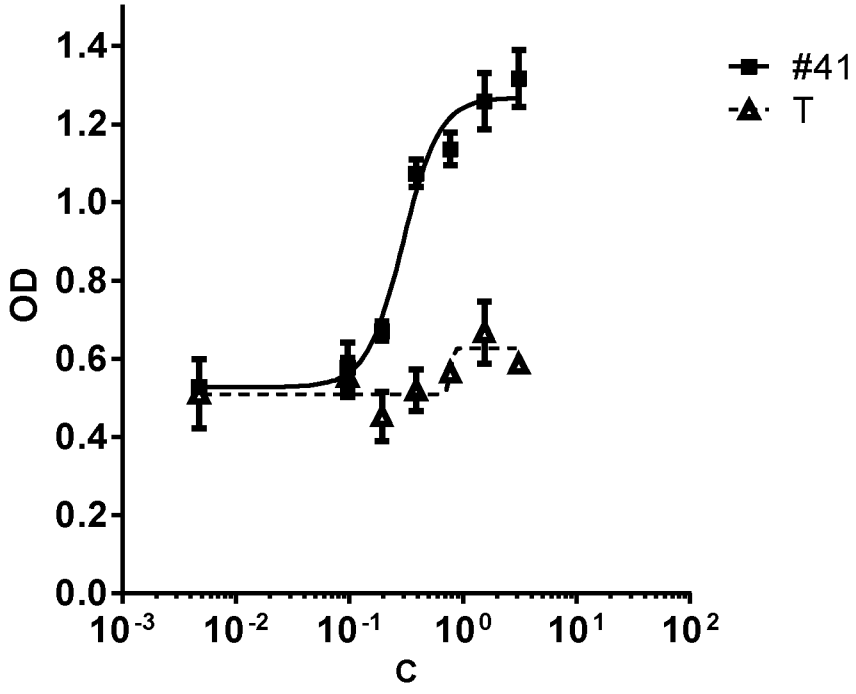
T

Fig. 5B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102
c
#41
T

Fig. 5C

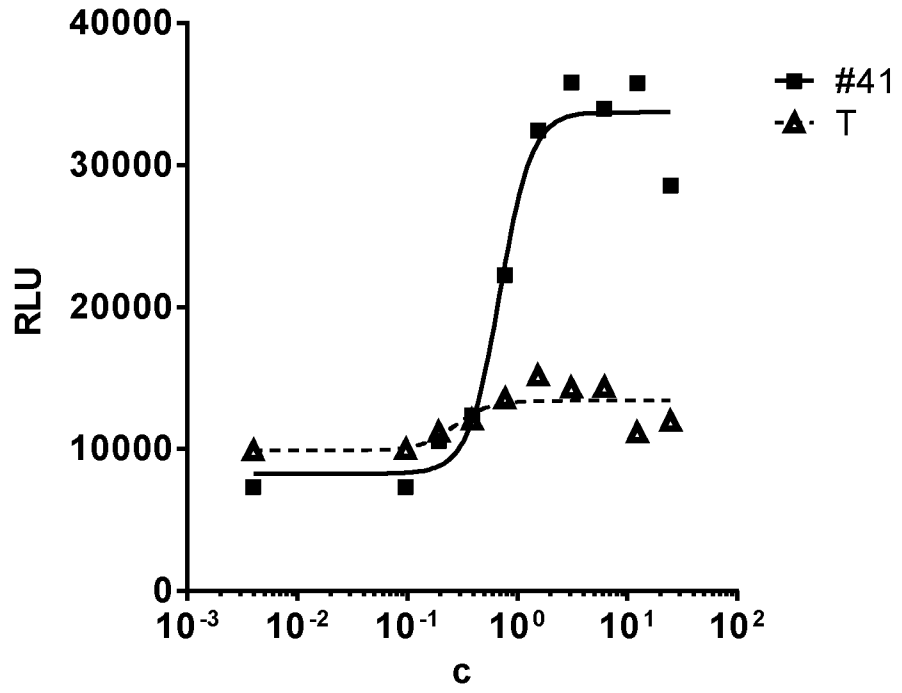
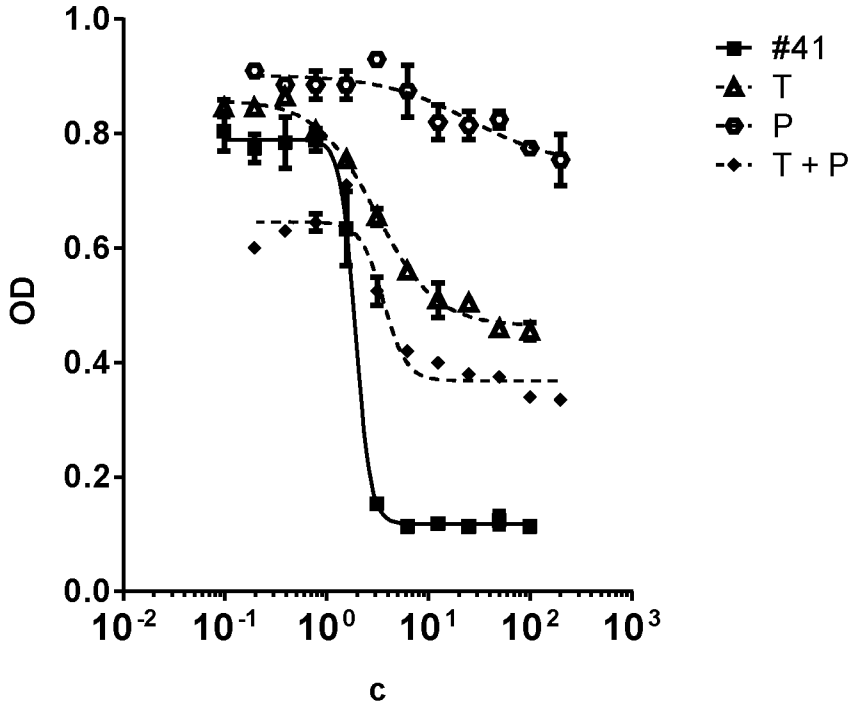


Fig. 6A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0.6
0.4
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OD
10-2
10-1
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101
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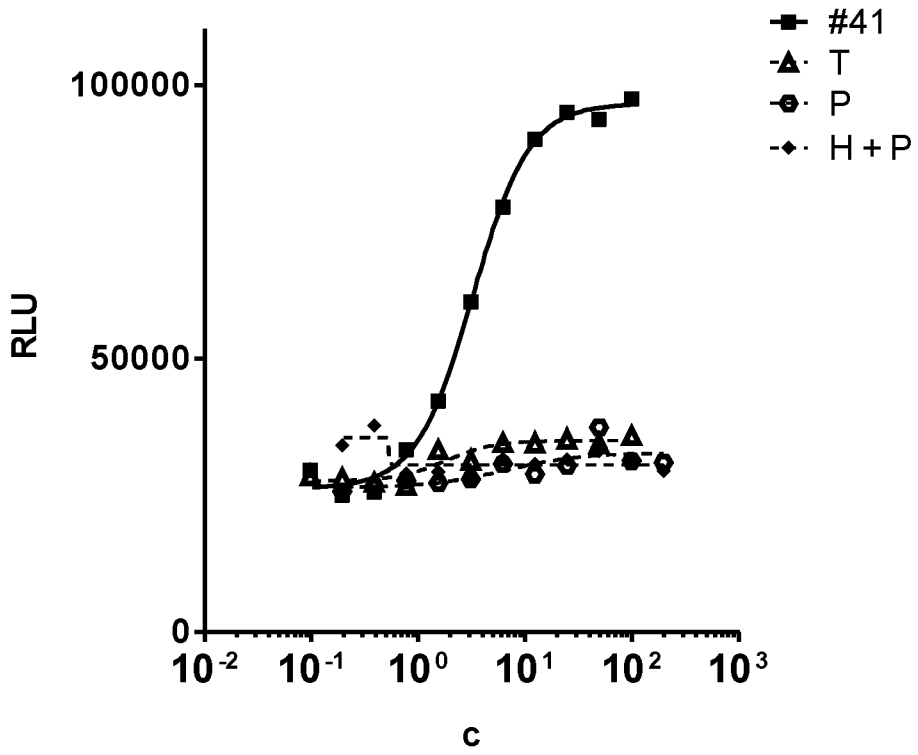
P
T + P

Fig. 6B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50000
0
RLU
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103
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P
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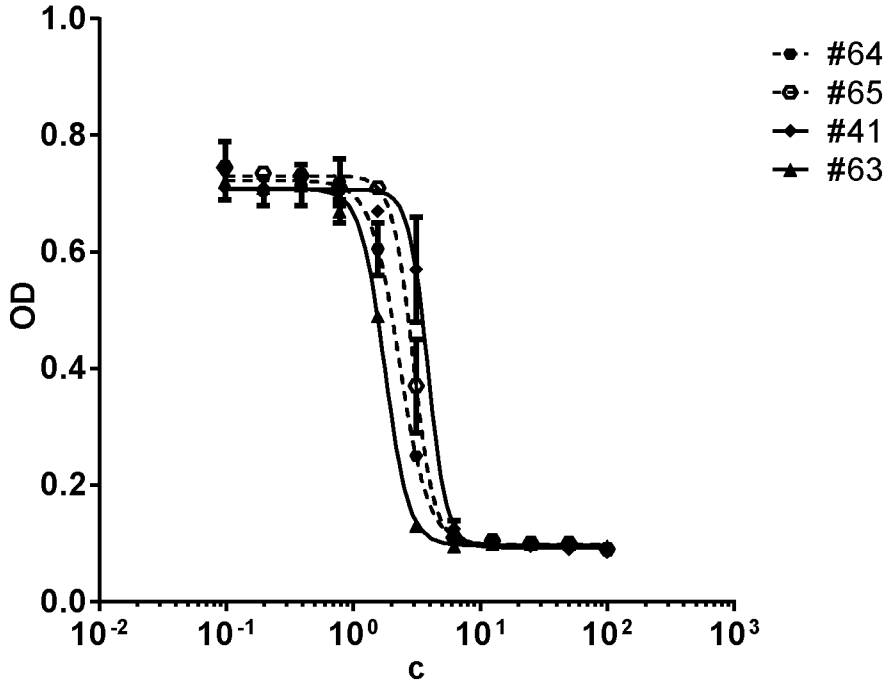
Fig. 7A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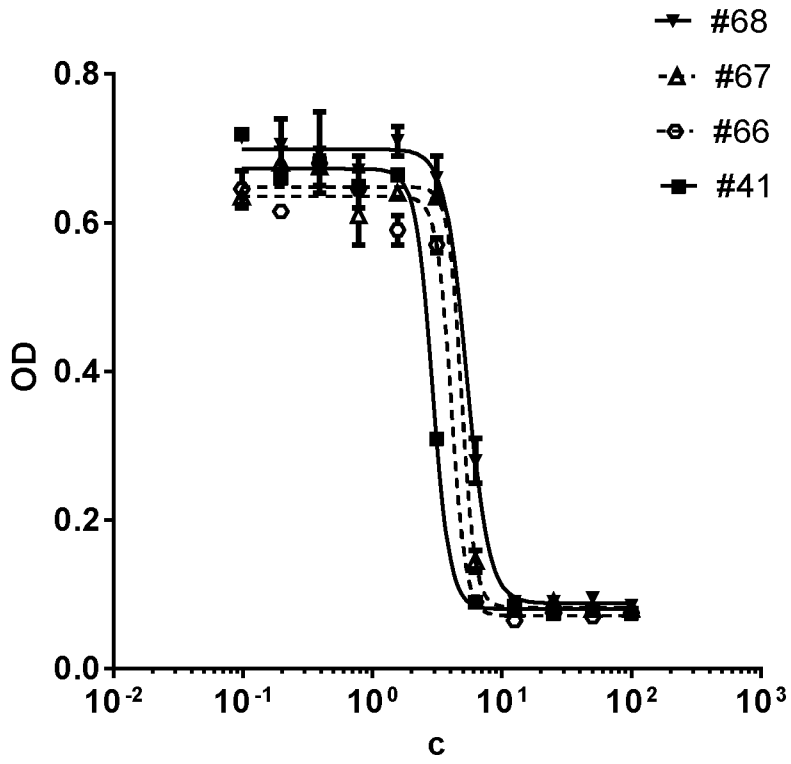
#64
#65
#41
#63

Fig. 7B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#68
#67
#66
#41

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/075290

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/32
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NIELSEN U B ET AL: "Targeting of bivalent anti-ErbB2 diabody antibody fragments to tumor cells is independent of the intrinsic antibody affinity", CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, US, vol. 60, no. 22, 15 November 2000 (2000-11-15), pages 6434-6440, XP003014072, ISSN: 0008-5472	1,7,10
Y	the whole document	2-6,8,9, 11-15,17
	----- -/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 March 2014	25/03/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Kalsner, Inge

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STEFFEN A C ET AL: "In vitro Characterization of a bivalent Anti-HER-2 Affibody with Potential for Radionuclide-Based Diagnostics", CANCER BIOTHERAPY AND RADIOPHARMACEUTICALS, LIEBERT, US, vol. 20, no. 3, 1 January 2005 (2005-01-01), pages 239-249, XP002530379, DOI: 10.1089/CBR.2005.20.239	1,10
Y	the whole document	2,3,6,8, 9,11-15, 17

Y	STUMPP MT ET AL: "DARPins: a true alternative to antibodies", CURRENT OPINION IN DRUG DISCOVERY AND DEVELOPMENT, CURRENT DRUGS, LONDON, GB, vol. 10, no. 2, 1 March 2007 (2007-03-01), pages 153-159, XP009167810, ISSN: 1367-6733 the whole document	2,3,6,8, 9,11-15, 17

Y	ZAHND ET AL: "A Designed Ankyrin Repeat Protein Evolved to Picomolar Affinity to Her2", JOURNAL OF MOLECULAR BIOLOGY, ACADEMIC PRESS, UNITED KINGDOM, vol. 369, no. 4, 17 May 2007 (2007-05-17), pages 1015-1028, XP022083577, ISSN: 0022-2836, DOI: 10.1016/J.JMB.2007.03.028 the whole document	2-6,8,9, 11-15,17

A	ZAHND CHRISTIAN ET AL: "Efficient Tumor Targeting with High-Affinity Designed Ankyrin Repeat Proteins: Effects of Affinity and Molecular Size", CANCER RESEARCH, vol. 70, 15 February 2010 (2010-02-15), page 35167, XP002693518, abstract	1-22

A	WO 2012/162418 A1 (UNIV NORTH CAROLINA [US]; LIU RIHE [US]) 29 November 2012 (2012-11-29) the whole document	1-22

A	WO 2011/054519 A1 (HOFFMANN LA ROCHE [CH]; FISCHER STEPHAN [DE]; IMHOF-JUNG SABINE [DE];) 12 May 2011 (2011-05-12) the whole document	1-22

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2013/075290

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012162418 A1	29-11-2012	NONE	
WO 2011054519 A1	12-05-2011	AR 078882 A1	07-12-2011
		AU 2010314450 A1	26-04-2012
		CA 2778203 A1	12-05-2011
		CN 102597000 A	18-07-2012
		EP 2496603 A1	12-09-2012
		JP 2013509868 A	21-03-2013
		KR 20120089487 A	10-08-2012
		RU 2012122869 A	10-12-2013
		TW 201120210 A	16-06-2011
		US 2012309940 A1	06-12-2012
		WO 2011054519 A1	12-05-2011

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
5 June 2014 (05.06.2014)

WIPO | PCT

(10) International Publication Number
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(51) **International Patent Classification**:
C07K 16/32 (2006.01)

(21) **International Application Number**:
PCT/EP2013/075290

(22) **International Filing Date**:
2 December 2013 (02.12.2013)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
12195156.0 30 November 2012 (30.11.2012) EP

(71) **Applicant**: **MOLECULAR PARTNERS AG** [CH/CH]; Wagistrasse 14, CH-8952 Zürich-Schlieren (CH).

(72) **Inventors**: **FIEDLER, Ulrike**; Leutrumweg 8, 79539 Lörrach (DE). **DOLADO, Igancio**; Binzmühlestrasse 78, CH-8050 Zürich (CH). **STROBEL, Heike**; Bachmattstrasse 4, CH-8048 Zürich (CH).

(74) **Agent**: **MICHALSKI HÜTTERMANN & PARTNER PATENTANWÄLTE MBB**; Speditionstr. 21, 40221 Düsseldorf (DE).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

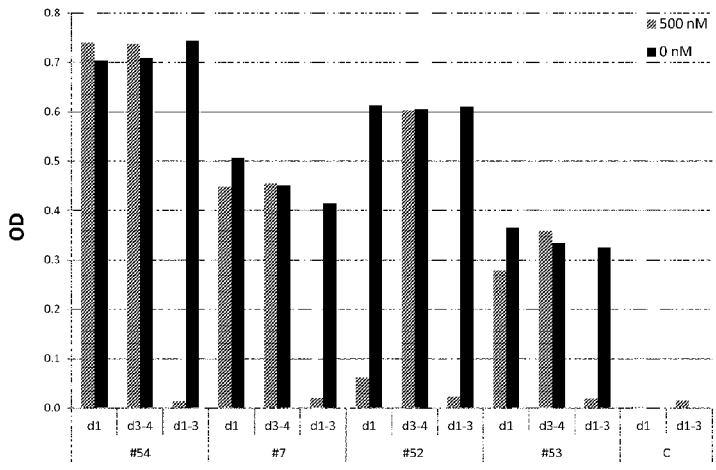
— *with sequence listing part of description (Rule 5.2(a))*

(48) **Date of publication of this corrected version**:
30 October 2014

(15) **Information about Correction**:
see Notice of 30 October 2014

(54) **Title**: BINDING PROTEINS COMPRISING AT LEAST TWO REPEAT DOMAINS AGAINST HER2

Fig. 1A



(57) **Abstract**: The present invention relates to a recombinant binding protein comprising at least a first and a second repeat domain, wherein each of said two repeat domains binds the extracellular region of HER2 and wherein said repeat domains are covalently linked

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

REVISED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
5 June 2014 (05.06.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/083208 A9

(51) International Patent Classification:
C07K 16/32 (2006.01)

(21) International Application Number:
PCT/EP2013/075290

(22) International Filing Date:
2 December 2013 (02.12.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
12195156.0 30 November 2012 (30.11.2012) EP

(71) Applicant: **MOLECULAR PARTNERS AG** [CH/CH]; Wagistrasse 14, CH-8952 Zürich-Schlieren (CH).

(72) Inventors: **FIEDLER, Ulrike**; Leutrumweg 8, 79539 Lörrach (DE). **DOLADO, Igancio**; Binzmühlestrasse 78, CH-8050 Zürich (CH). **STROBEL, Heike**; Bachmattstrasse 4, CH-8048 Zürich (CH).

(74) Agent: **MICHALSKI HÜTTERMANN & PARTNER PATENTANWÄLTE MBB**; Speditionstr. 21, 40221 Düsseldorf (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

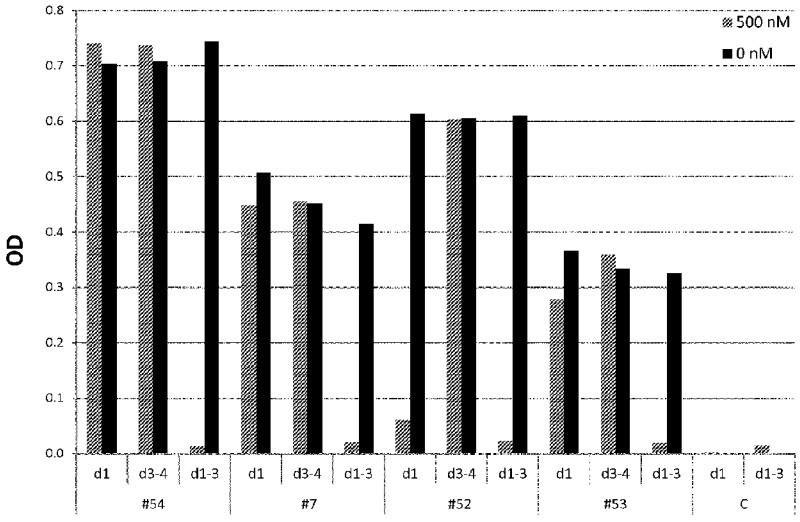
— *with sequence listing part of description (Rule 5.2(a))*

(88) Date of publication of the revised international search report:
24 December 2014

[Continued on next page]

(54) Title: BINDING PROTEINS COMPRISING AT LEAST TWO REPEAT DOMAINS AGAINST HER2

Fig. 1A



(57) Abstract: The present invention relates to a recombinant binding protein comprising at least a first and a second repeat domain, wherein each of said two repeat domains binds the extracellular region of HER2 and wherein said repeat domains are covalently linked.

WO 2014/083208 A9

(15) **Information about Corrections**:
see Notice of 24 December 2014

Previous Correction:
see Notice of 30 October 2014

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/075290

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/32
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	the whole document	2-6,8,9, 11-15,17
	----- -/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 March 2014	25/03/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Kalsner, Inge

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STEFFEN A C ET AL: "In vitro Characterization of a bivalent Anti-HER-2 Affibody with Potential for Radionuclide-Based Diagnostics", CANCER BIOTHERAPY AND RADIOPHARMACEUTICALS, LIEBERT, US, vol. 20, no. 3, 1 January 2005 (2005-01-01), pages 239-249, XP002530379, DOI: 10.1089/CBR.2005.20.239	1,10
Y	the whole document	2,3,6,8, 9,11-15, 17

Y	STUMPP MT ET AL: "DARPins: a true alternative to antibodies", CURRENT OPINION IN DRUG DISCOVERY AND DEVELOPMENT, CURRENT DRUGS, LONDON, GB, vol. 10, no. 2, 1 March 2007 (2007-03-01), pages 153-159, XP009167810, ISSN: 1367-6733 the whole document	2,3,6,8, 9,11-15, 17

Y	ZAHND ET AL: "A Designed Ankyrin Repeat Protein Evolved to Picomolar Affinity to Her2", JOURNAL OF MOLECULAR BIOLOGY, ACADEMIC PRESS, UNITED KINGDOM, vol. 369, no. 4, 17 May 2007 (2007-05-17), pages 1015-1028, XP022083577, ISSN: 0022-2836, DOI: 10.1016/J.JMB.2007.03.028 the whole document	2-6,8,9, 11-15,17

A	ZAHND CHRISTIAN ET AL: "Efficient Tumor Targeting with High-Affinity Designed Ankyrin Repeat Proteins: Effects of Affinity and Molecular Size", CANCER RESEARCH, vol. 70, 15 February 2010 (2010-02-15), page 35167, XP002693518, abstract	1-22

A	WO 2012/162418 A1 (UNIV NORTH CAROLINA [US]; LIU RIHE [US]) 29 November 2012 (2012-11-29) the whole document	1-22

A	WO 2011/054519 A1 (HOFFMANN LA ROCHE [CH]; FISCHER STEPHAN [DE]; IMHOF-JUNG SABINE [DE];) 12 May 2011 (2011-05-12) the whole document	1-22

1

CORRECTED VERSION

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2013/075290

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   - [ ] on paper
   - [X] in electronic form

   b. (time)

   - [X] in the international application as filed
   - [ ] together with the international application in electronic form
   - [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2013/075290

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012162418 A1	29-11-2012	NONE	
WO 2011054519 A1	12-05-2011	AR 078882 A1	07-12-2011
		AU 2010314450 A1	26-04-2012
		CA 2778203 A1	12-05-2011
		CN 102597000 A	18-07-2012
		EP 2496603 A1	12-09-2012
		JP 2013509868 A	21-03-2013
		KR 20120089487 A	10-08-2012
		RU 2012122869 A	10-12-2013
		TW 201120210 A	16-06-2011
		US 2012309940 A1	06-12-2012
		WO 2011054519 A1	12-05-2011

(19)中华人民共和国国家知识产权局

(12)发明专利申请

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(43)申请公布日 2015.09.16

(21)申请号 201380062520.9
(22)申请日 2013.12.02
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2015.05.29
(86)PCT国际申请的申请数据
PCT/EP2013/075290 2013.12.02
(87)PCT国际申请的公布数据
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(71)申请人 分子组合公司
地址 瑞士苏黎世-施利伦
(72)发明人 乌尔丽克·菲德勒
伊格纳西奥·多拉多
海克·施特罗贝尔
(74)专利代理机构 广州华进联合专利商标代理有限公司 44224
代理人 邓云鹏
(51)Int.Cl.
C07K 16/32(2006.01)

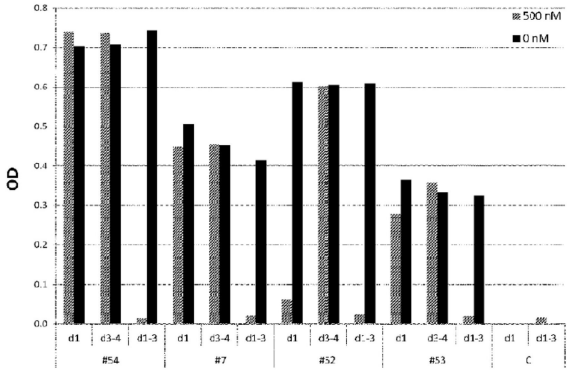
权利要求书3页 说明书38页
序列表109页 附图10页

(54)发明名称

包含至少两个针对HER2的重复序列结构域的结合蛋白

(57)摘要

本发明涉及重组结合蛋白，其至少包含第一和第二重复序列结构域，其中所述2个重复序列结构域中的每一个均结合HER2的胞外区，并且其中所述重复序列结构域通过共价键连接。



CN 104918959 A

1.一种重组结合蛋白，其包含至少第一和第二重复序列结构域，其中该第一和第二重复序列结构域中的每一个均结合至 HER2 的胞外区域，并且其中所述重复序列结构域通过共价键连接。

2.根据权利要求 1 所述的结合蛋白，其特征在于，所述第一重复序列结构域结合 HER2 的结构域 II 并且所述第二重复序列结构域结合 HER2 的结构域 IV。

3.根据权利要求 1-2 中任意一项所述的结合蛋白，其特征在于，所述第一和第二重复序列结构域位于相同的多肽上，并且其中靶向 HER2 的结构域 II 的重复序列结构域位于靶向 HER2 的结构域 IV 的重复序列结构域的 N 端。

4.根据前述任何一项权利要求所述的结合蛋白，其特征在于，结合 HER2 的结构域 II 的所述第一重复序列结构域不与帕妥珠单抗竞争结合至 HER2。

5.根据前述任何一项权利要求所述的结合蛋白，其特征在于，结合 HER2 的结构域 IV 的所述第二重复序列结构域不与曲妥珠单抗竞争结合至 HER2。

6.根据前述任何一项权利要求所述的结合蛋白，其特征在于，所述第一重复序列结构域为锚蛋白重复序列结构域，并且所述第二重复序列结构域为锚蛋白重复序列结构域。

7.根据前述任何一项权利要求所述的结合蛋白，其特征在于，所述第一重复序列结构域以低于 10^{-7}M 的 Kd 结合 HER2 的胞外区域，并且所述第二重复序列结构域以低于 10^{-7}M 的 Kd 结合 HER2 的胞外区域。

8.根据前述任何一项权利要求所述的结合蛋白，其特征在于，所述结合蛋白以小于 100nM 的 IC_{50}值抑制 BT474 的受激增殖。

9.根据前述任何一项权利要求所述的结合蛋白，其特征在于，所述结合蛋白能以低于 100nM 的 EC_{50}值诱导 BT474 细胞凋亡。

10.根据前述任何一项权利要求所述的结合蛋白，其特征在于，所述第一和第二重复序列结构域通过多肽接头连接。

11.根据前述任何一项权利要求所述的结合蛋白，其特征在于：

所述第一重复序列结构域与选自 SEQ ID NOs:62-68、72 和 114-121 的锚蛋白重复序列结构域竞争结合至 HER2；和 / 或

所述第二重复序列结构域与选自 SEQ ID NOs:74-82 的锚蛋白重复序列结构域竞争结合至 HER2。

12.根据权利要求 10 所述的结合蛋白，其特征在于：

所述第一重复序列结构域包含与选自 SEQ ID NOs:62-68、72 和 114-121 的一个锚蛋白重复序列结构域具有至少 70％氨基酸序列一致性的氨基酸序列；和 / 或

所述第二重复序列结构域包含与选自 SEQ ID NOs:74-82 的一个锚蛋白重复序列结构域具有至少 70％氨基酸序列一致性的氨基酸序列，并且其中进一步地，

在所述锚蛋白重复序列结构域的位置 1 的 G 和 / 或位置 2 的 S 可选地缺失；以及

在所述锚蛋白重复序列结构域的倒数第二位置的 L 和 / 或最后位置的 N 可选地被 A 替换。

13.根据权利要求 10 至 12 中任意一项所述的结合蛋白，其特征在于：

所述第一重复序列结构域选自 SEQ ID NOs:62-68、72 和 114-121 的锚蛋白重复序列结构域，和 / 或

所述第二重复序列结构域选自 SEQ ID NOs:74-82 的锚蛋白重复序列结构域，

并且其中进一步地，

在所述锚蛋白重复序列结构域的位置 1 的 G 和 / 或位置 2 的 S 可选地缺失 ;以及

在所述锚蛋白重复序列结构域的倒数第二位置的 L 和 / 或最后位置的 N 可选地被 A 替换。

14. 根据权利要求 10 至 13 中任意一项所述的结合蛋白，其特征在于，其中 ：

所述第一重复序列结构域包含锚蛋白重复序列模块，该锚蛋白重复序列模块具有选自 SEQ ID NO:15-18、21-23、37、38、125、126、129、130、133、134 的氨基酸序列，其中在 SEQ ID NO:15-18、21-23、37、38、125、126、129、130、133、134 中多达 9 个氨基酸被任何其它氨基酸残基取代，和 / 或

所述第二重复序列结构域包含锚蛋白重复序列模块，该锚蛋白重复序列模块具有选自 SEQ ID NO:46、47、51、52、55 和 56 的氨基酸序列，其中在 SEQ ID NO:46、47、51、52、55 和 56 中多达 9 个氨基酸被任何其它氨基酸残基取代。

15. 根据权利要求 10 至 14 中任意一项所述的结合蛋白，其特征在于，所述锚蛋白重复序列模块具有选自 KDFQGITPLHIAATSGHLEIVEVLLKAGADVNA(SEQ ID NO:16) 的氨基酸序列，其中在 SEQ ID NO:16 中多达 9 个氨基酸残基被任何其它氨基酸残基取代，并且其中

在位置 3 的 F 可选地被 A 替换 ；

在位置 4 的 Q 可选地被 E 替换 ；

在位置 5 的 G 可选地被 S 替换 ；

在位置 6 的 I 可选地被 V 替换 ；

在位置 11 的 I 可选地被 L 替换 ；

在位置 14 的 T 可选地被 Q 替换 ;和 / 或

在位置 15 的 N 可选地被选自 S 和 W 的氨基酸，最优选被 S，替换。

16. 根据权利要求 10 至 14 中任意一项所述的结合蛋白，其特征在于，所述锚蛋白重复序列模块具有选自 KDITGETPLHHAADSGHLEIVEVLLKAGADVNA(SEQ ID NO:18) 的氨基酸序列，其中在 SEQ ID NO:18 中多达 9 个氨基酸残基被任何其它氨基酸残基取代，并且其中

在位置 3 的 I 可选地被 V 替换 ；

在位置 6 的 E 可选地被 D 替换 ；

在位置 11 的 H 可选地被 L 替换 ；

在位置 14 的 D 可选地被 Q 替换 ；

在位置 15 的 S 可选地被 H 替换 ;和 / 或

在位置 19 的 E 可选地被 V 替换。

17. 根据权利要求 10 至 15 中任意一项所述的结合蛋白，其特征在于，所述结合蛋白包含多肽，所述多肽与选自 SEQ ID NO:83-98、102、103、122、123 和 136-141 的多肽具有至少 70％，优选 90％，的氨基酸序列一致性。

18. 一种药物制剂，其包含根据前述权利要求所述的结合蛋白或组合物。

19. 根据前述权利要求所述的至少一种结合蛋白、组合物或药物制剂用于治疗癌症的用途。

20. 一种方法，包括向患者进行根据前述权利要求所述的结合蛋白、组合物或药物制剂

的给药，以治疗癌症。

21. 根据权利要求 19 或 20 所述的用途或方法，其中在所述用途或方法中，疾病具有选自下组的至少一种特征 ：

HER2 编码基因的扩增，

HER2 编码基因的过度表达，

HER2 编码基因的突变形式的表达，和 / 或

在抗曲妥珠单抗的肿瘤中 HER3 编码基因的过度表达。

22. 根据权利要求 19-21 中所述的用途或方法，其中在所述用途或方法中，疾病为选自下组的至少一种疾病 ：

乳腺癌，

卵巢癌，

胃肿瘤，

胃癌，

子宫癌，和 / 或

结肠直肠癌。

包含至少两个针对 HER2 的重复序列结构域的结合蛋白

技术领域

[0001] 本发明涉及包含至少两个对人表皮生长因子受体 2(HER2) 具有结合特异性的重复序列结构域的结合蛋白质以及编码这种结合蛋白质的核酸，含有这种蛋白质的药物组合物和这种蛋白质在疾病治疗中的用途。

背景技术

[0002] 人表皮生长因子受体 2(HER2 ;人 HER2 的 UniProtKB/Swiss-Prot 编号为 P04626) 也称为 ErbB2，是由人 ERBB2 基因编码的蛋白质。该基因的扩增或过度表达表现出在某些类型的癌症的发生和发展起重要作用，并且近年来，该基因已经进展成为疾病治疗的重要生物标记和目标。HER2 是跨膜受体酪氨酸激酶 (RTK)，其属于较广泛的 ErbB 受体家族 (Bublil,E.M. 和 Yarden,Y.Curr.Opin.Cell Biol.19(2),124-34,2007)。ErbB 受体家族在脊椎动物中是保守的，并且包括家族奠基者 ErbB1(也称为表皮生长因子受体 (EGFR) 或 HER1 ;该人蛋白质在 UniProKB/Swiss-Prot 中的编号为 P00533) 以及最近识别的受体 HER3(也称为 ErbB3 ;该人蛋白质在 UniProKB/Swiss-Prot 中的编号为 P21860) 和 HER4(也称为 ErbB4 ;该人蛋白质在 UniProKB/Swiss-Prot 中的编号为 Q15303)。所有的 ErbB 受体共享广泛的序列和结构域同源性，并且形成功能性同源二聚体 (例如，ErbB1-ErbB1,HER2-HER2 和 HER4-HER4) 以及所有组合的异源二聚体。受体同源和异源二聚化在配体结合或受体过度表达时发生并且转而通过自身磷酸化激活受体激酶结构域。这接着触发下游胞内信号传导和生物反应。与其它 ErbB 受体相反，HER2 不具有任何已知的配体，并且能二聚化，在其过度表达之后，二聚化是非常明显的并且在之前没有配体结合的情况下被激活。重要的是，HER3 没有活化的胞内激酶结构域并且通过与其它 ErbB 受体家族成员的异源二聚化激活，导致非常有效的下游信号传导。HER3 的这种异源二聚化和激活在配体结合至 HER3 时或如果合作的受体，比如 HER2 极大地过度表达而发生。

[0003] HER2 以及其它 ErbB 受体家族成员由 4 个胞外结构域组成，该 4 个胞外结构域被顺序命名为 I、II、III 和 IV，其中结构域 IV 离胞外细胞膜最近并且结构域 I 离胞外细胞膜最远。在配体被剥夺条件下，ErbB 受体中的结构域 I 和 III 共享分子间的相互作用，该分子间的相互作用封闭结构域 II。这防止受体同源 / 异源二聚化和信号传导，因为二聚化需要两个邻近的 ErbB 受体的结构域 II 之间的相互作用 (Burguess A.W., 等人 ,Mol.Cell 12(3),541-552,2003)。结合配体破坏结构域 I 和 III 之间的相互作用，然后导致非激活态 - 激活态受体构象变化并且使结构域 II 暴露。这造成该受体混杂从而与其它激活态 ErbB 受体二聚化并且开始信号传导。有趣的是，HER2 是唯一的组成处于激活态构象的 ErbB 受体家族成员，因此，结构域 II 被持续暴露并可进行同源和异源二聚化。

[0004] ErbB 受体二聚化和自身磷酸化导致参与正常生理以及疾病的过多的、关键的下游信号传导分子的激活。这种被激活的信号传导分子的性质在一定程度上取决于活化的 ErbB 受体二聚体的组合物。例如，HER1-HER1 和 HER2-HER2 同源二聚体优选激活下游胞外信号调节激酶 (ERK) 信号传导和增殖，而 HER2-HER3 异源二聚体也激活 PI3K 信号传导通路 (包括

下游激酶 AKT 的激活），并由此使细胞存活。事实上，在肿瘤细胞中 AKT 被 HER2-HER3 信号传导激活促进肿瘤细胞存活并且使肿瘤细胞抵抗 HER2 靶向药物，如单克隆抗体曲妥珠单抗 (Berns K. 等人，Cancer Cell 12,395-402,2007)。有趣的是，在这些细胞中抑制 HER2-HER3 介导的 PI3K-AKT 信号传导变成速率限制，并导致细胞死亡。除了细胞增殖和存活，HER2 信号传导还有原因地参与其它过程，比如血管生成和迁移。

[0005] HER2 在约 20％的所有乳腺癌中过度表达。由于其临床相关性，HER2 成为第一 RTK，针对该第一 RTK 研发了靶向生物制剂，即曲妥珠单抗（Herceptin®; Genentech)。该抗体结合 HER2 的结构域 IV 并通过尚未完全了解的几种机制抑制 HER2 信号传导。这包括在肿瘤细胞中诱导受体内在化，导致降低 HER2 表达水平和信号传导，并导致致瘤表型减毒。曲妥珠单抗已经改变了数以万计的乳腺癌妇女的生活，扩展了她们的寿命和生活质量。然而，曲妥珠单抗主要具有抗增殖作用并且在疾病晚期阶段肿瘤可能逃避这样的治疗。在研发更有效的治疗的尝试中，产生了新抗体，其识别 HER2 的结构域 II，即帕妥珠单抗（Omnitarg®, Perjeta®; Genentech)。与曲妥珠单抗相比，该抗体不是开发成降低 HER2 的膜表达水平，而是通过结合至和阻断受体二聚化结构域 II 干扰 HER2 同源二聚体和异源二聚体的形成。帕妥珠单抗在体外和体内作为单一试剂具有意想不到的低治疗功效；然而，其与曲妥珠单抗的组合显示协同效应。因此，两种抗体的组合可以成为乳腺癌患者的标准护理治疗 (Capelan M., 等人，Ann.Oncol.,24,273-82,2013)。

[0006] 曲妥珠单抗和帕妥珠单抗的组合的临床前和临床成功造成这样的观点：良好的抗肿瘤功效需要双重靶向 HER2 结构域 II 和 IV。这与最近产生的同时靶向 HER2 的结构域 II 和 IV 的其它分子一致。例如，丹麦公司 Symphogen 正在研发的针对 HER2 的结构域 II 和 IV 的抗体混合物在临床前小鼠肿瘤模型已经显示出一些更高的功效（即优于单独的曲妥珠单抗）。

[0007] 同样地，US2011/033460 描述了结合 HER2 的结构域 I 和结构域 IV 的抗体的组合在 DNA 合成和 BT474 细胞的存活能力上显示协同效应。此外，US2011/033460 还描述了结合 HER2 的两种不同表位的双特异性抗体，其中一个表位位于 HER2 的结构域 I 并且另一表位位于 HER2 的结构域 IV。

[0008] WO 2009/068625 报道双特异性抗体 (biparatopic antibody) 构建体的研发，该抗体构建体包含第一抗体结构域和第二抗体结构域，第一抗体结构域与曲妥珠单抗竞争结合至 HER2，该第二壳体结构域结合 HER2 的不同表位或一部分。有趣的是，一些构建体具有 SKBR3 细胞增殖的拮抗作用，而另一些具有激动作用。特别是，WO 2009/068625 报道双特异性抗体构建体的研发，该抗体构建体包含第一抗体结构域和第二抗体结构域，其中该第一抗体结构域与曲妥珠单抗竞争结合 HER2（即，结合 HER2 的结构域 IV)，该第二抗体结构域与帕妥珠单抗竞争结合 HER2（即，结合 HER2 的结构域 II)。构建体，其中结合结构域 IV 的抗体结构域克隆到结合结构域 II 的抗体结构域的 N 端，显示出阻断图谱激酶激活，而在其它取向（即，结合结构域 II 的抗体结构域在 N 端）没有观察到这样阻断。总之，WO 2009/068625 描述了靶向 HER2 的多种双特异性抗体构建体，其在 SKBR3 细胞增殖或细胞信号传导上可变地扩大效果（激动或拮抗），但没有对细胞毒性和凋亡作用进行描述。

[0009] 也描述了二价结合蛋白质，如二价双抗体分子或靶向 HER2 的二价亲和体

(Nielsen,U.B.,等人,Cancer Res.,60,6434-6440,2000;Steffen,A-C.,Cancer Biother. Radiopharmaceut.20,239-248,2005)。这样的分子结合两倍的相同的结合域,因此与包含2个结合结构域(每个结合结构域结合至相同靶向分子上的不同表位)的双特异性分子不同。

[0010] 作为抗体衍生的疗法和SMI的替代,新的结合蛋白或结合结构域可用于特异性结合靶分子(例如,Binz,H.K.,Amstutz,P.和Plückthun,A.,Nat. Biotechnol.23,1257-1268,2005),并因此充当拮抗剂。一种不具有Fc的这类新型结合蛋白或结合结构域基于设计的重复序列蛋白或设计的重复序列结构域(WO 2002/020565;Binz,H.K.,Amstutz,P.,Kohl,A.,Stumpp,M.T.,Briand,C.,Forrer,P.,Grütter,M.G.,和Plückthun,A.,Nat.Biotechnol.22,575-582,2004;Stumpp,M.T.,Binz,H.K和Amstutz,P.,Drug Discov.Today 13,695-701,2008)。

[0011] WO2002/020565描述了如何构建重复序列蛋白的大文库及其一般应用。这些设计的重复序列结构域利用重复序列蛋白的模块化性质,并且可以具有N端和C端加帽模块,以防止设和Plückthun,A.,FEBS letters 539,2-6,2003)。该新型结合蛋白包括设计的锚蛋白重复序列蛋白(DARPin)。之前描述了结合至HER2的单特异性DARPin的产生(例如,Steiner,D.,Forrer,P.和Plückthun,A.,J.Mol.Biol.382,1211-1227,2008;Zahnd,C.,Pecorari,F.,Straumann,N.,Wyler,E.和Plückthun,A.,J.Biol. Chem.281(46),35167-35175,2006)。

[0012] 最近,描述了设计的双特异性锚蛋白重复序列蛋白,其靶向HER2(Jost,Ch.,等人,Structure 21,1-13,2013)。作者表示通过短接头(长接头不能达到同样好的效果)连接使两个锚蛋白重复序列结构域(一个靶向HER2的结构域I并且另一靶向HER2的结构域IV)结合相比单独的靶向HER2的结构域IV的曲妥珠单抗在BT474细胞上导致更强的细胞毒性作用。该特异性重复序列蛋白通过2个HER2分子的分子内交联而起作用,即,其连接两个膜结合型HER2分子,使其扭曲,从而使其不能与任何EGFR家族成员形成能进行信号传导的二聚体,防止任何激酶的二聚化,并因此导致所观察到的细胞毒性作用。

[0013] 即使现有技术表明HER2的靶向有利于疾病,例如癌症的治疗,还明确需要产生较高效地靶向HER2的结合蛋白。

发明内容

[0014] 本发明的目的是提供HER2的新拮抗剂。

[0015] 本发明的另一个目的是提供抑制HER2相关的细胞信号传导的新机制。

[0016] 本发明的另一目的是提供一种在细胞(例如,肿瘤细胞)、组织、器官或患者中抑制HER2介导的细胞增殖和/或诱导凋亡的新方法。

[0017] 本发明的另一目的是提供利用双特异性重复序列蛋白处理HER2的两个结构域的单一治疗方法。

[0018] 本发明的另一目的是提供癌症治疗的新选择。

[0019] 本发明的另一目的是提供针对肿瘤疾病的治疗,该治疗具有良好的功效和/或副作用小。

[0020] 本发明的另一目的是提供一种针对肿瘤疾病的可供选择的治疗,该治疗不会(或

仅部分地）对现有技术的疗法有反应，或有抗性。

[0021] 发明概要

[0022] 这些目的通过独立权利要求的主题实现，从属权利要求以及说明书进一步公开优选的实施例。

[0023] 虽然附图和前面的描述更详细地展示和描述了本发明，这些展示和描述被认为是解说或示例而非限制，本发明不限于已公开的实施例，在实践本发明时，本领域技术人员可以从附图、公布和权利要求的学习中明白并实现公布的实施例的其它变形。在权利要求中，术语“包含”不排除其它元件或步骤，并且不定冠词“一”或“一个”不排除多个。在仅凭某些措施被记载在相互不同的从属权利要求中并不表示这些措施的组合不能被有利地使用。权利要求的任何附图标记不能解释为限制本发明的范围。

附图说明

[0024] 图 1. DARPin 与 HER2 结合

[0025] 如图 1A 和 1B 所示，利用纯化的 HER2 结构域（结构域 I、结构域 III-IV 或结构域 I-III）作为竞争者通过竞争 ELISA 测试单价 DARPin 与 HER2 胞外结构域（结构域 I-IV）的结合。存在 500nM Her2 结构域 I 的情况下，DARPin#51 和 DARPin#52 不可以结合 HER2（结构域 I-IV），表明其结合在结构域 I 上的表位。DARPin#7、DARPin#53 和 DARPin#54 结合结构域 II，因为 500nM Her2 结构域 I 或 500nM Her2 结构域 III-IV 都不能防止其结合至全长 Her2（结构域 I-IV）。图 1C 展示了单价 DARPin 可以结合在预先形成的 HER2- 帕妥珠单抗复合体上并且，与帕妥珠单抗结合 HER2 结构域 II 相比，结合 HER2 上的不同的表位。参下文 DARPin 的定义。OD，在 450nM 的光密度减去 620nm 的 OD ；C 对照 DARPin，其不结合 HER2 ；d1，HER2 的结构域 I ；d1-3，HER2 的结构域 I-III ；d3-4，HER2 的结构域 III-IV。

[0026] 图 2. 单价和双特异性结合蛋白对 BT474 细胞增殖的抑制作用

[0027] 测试单价 DARPin（即，DARPin#1 和 DARPin#18）、这些单价 DARPin 的非共价混合物和包含在不同取向的这些单价 DARPin 的双特异性结合蛋白 (DARPin#41 和 DARPin#49) 对 BT474 增殖的抑制。图 2A 展示了不同浓度的双特异性 DARPin 对增殖的抑制作用并且对不同的单个实验展示了相应的抑制拟合曲线。接着计算 DARPin#41 的 IC_{50}值为约 2nM。表 2 列举了不同的 DARPin 的 IC_{50}值。图 2A 展示了 OD，在 450nM 的光密度减去 620nm 的 OD 针对 C、DARPin 浓度 (nM) 绘图。X 轴以对数标度显示。图 2B 展示了双特异性 DARPin 的浓度为 100nM 的双特异性 DARPin、单价 DARPin 二者的混合物和单独的相应的单价 DARPin 对增殖的抑制。OD 绘制在 Y 轴。低 OD 反映抑制增殖。参见下文对 DARPins. #41、DARPin#41 ；#49、DARPin#49 ；#18、DARPin#18 ；#1、DARPin#1 的定义 ；n. c. 负对照。

[0028] 图 3. 各种双特异性 DARPin 对 BT474 细胞增殖的抑制

[0029] 该图展示了包含不同 N 端和 / 或 C 端锚蛋白重复序列结构域的双特异性 DARPin（#23，#24，#33，#37，#43，#44 和 #41）分组对 BT474 增殖的抑制。对于各不同的单个试验展示了不同浓度的 DAPRin 对增殖的抑制作用以及相应的抑制拟合曲线。表 2 列举了不同 DAPRin 的 IC_{50}值。图 3A 展示了具有 DARPin#15 的双特异性 DARPin 的抑制作用并且图 3B 展示了 DARPin#18 在 C 端的双特异性 DARPin 的抑制作用。图 3C 和 3D 展示了 DARPin#51 在 N 端和 DARPin#18 在 C 端的双特异性 DARPin 的抑制作用，并且图 3D 展示了 DARPin#51

在 N 端和 DARPin#21 在 C 端的双特异性 DARPin 的抑制作用。曲线图展示了 OD，在 450nM 的光密度减去 620nm 的 OD，针对 C、DARPin 浓度 (nM) 绘图。X 轴以对数标度显示。参见下文对 DARPins.#23、DARPin#23 ;#24、DARPin#24 ;#33、DARPin#33 ;#37、DARPin#37 ;#41、DARPin#41 ;#43、DARPin#43 ;#44、DARPin#44 的定义。

[0030] 图 4. 在不同的细胞系中双特异性 DARPin#41 对细胞增殖的抑制

[0031] 测试 DARPin#41 和曲妥珠单抗对 NCI-N87(图 4A) 和 ZR75-30(图 4B) 以及 MDA-MB175(图 4C) 增殖的抑制作用。对于各不同的单个试验展示了不同浓度的 DAPRin 对增殖的抑制作用以及相应的抑制拟合曲线。表 3 列举了不同细胞系的 IC_{50}值。曲线图展示了 OD，在 450nM 的光密度减去 620nm 的 OD，针对 C、DARPin 浓度 (nM) 绘图。X 轴以对数标度显示。参见下文对 DARPin 和相关分子 #41、DARPin#41 ;T、曲妥珠单抗的定义。

[0032] 图 5. 在不同细胞系中双特异性 DARPin#41 对凋亡的诱导

[0033] 测试在 BT474 细胞 (图 5A) 和 NCI-N87 细胞 (图 5B) 以及 MDA-MB175(图 5C) 中 DARPin#41 和曲妥珠单抗对凋亡的诱导。对于各不同的单个试验展示了不同浓度的 DAPRin 对凋亡的诱导以及相应的抑制拟合曲线。表 3 列举了不同细胞系的 EC_{50}值。图 5A 的曲线图展示了 OD，在 450nM 的光密度减去 490nm 的 OD，针对 C、曲妥珠单抗或 DARPin 浓度 (nM) 绘图。图 5B 和 5C 的曲线图展示了 RLU，相对光度单位针对 C、曲妥珠单抗或 DARPin 浓度 (nM) 绘图。X 轴以对数标度显示。参见下文对 DARPin ;T、曲妥珠单抗 ;#41、DARPin#41 的定义。

[0034] 图 6. 比较 DARPin#41 和基准对细胞增殖的抑制作用和对凋亡的诱导的功效

[0035] 测试 DARPin#41 和基准曲妥珠单抗和帕妥珠单抗以及 100nM 曲妥珠单抗和滴定的帕妥珠单抗的组合对 BT474 细胞的增殖抑制作用和对凋亡的诱导。图 6A 展示了对于各不同的单个试验各种浓度的 DARPin，各基准浓度对增殖的抑制作用，以及相应的抑制拟合曲线。表 3 列举了不同细胞系的 IC_{50}值。该曲线图展示了 OD，在 450nM 的光密度减去 620nm 的 OD，针对 C、DARPin/ 基准浓度 (nM) 绘图。X 轴以对数标度显示。图 6B 展示了对于各不同的单个试验，不同浓度的 DARPin，各基准浓度对凋亡的诱导，以及相应的激活拟合曲线。表 3 列举了不同细胞系的 EC_{50}值。该曲线图展示了相对光度单位 (RLU) 针对 C、DARPin/ 基准 (nM) 浓度绘图。X 轴以对数标度显示。参见下文对 DARPin ;T、曲妥珠单抗 ;P、帕妥珠单抗 ;#41、DARPin#41 的定义。

[0036] 图 7. 不同形式的双特异性结合蛋白对 BT474 细胞增殖的抑制作用

[0037] 该图展示了由在 N 端的 DARPin#1 和在 C 端的 DARPin#18 组成的不同形式的双特异性 DARPin 对 BT474 增殖的抑制作用。图 7A 展示了对于不同的单个试验，各种浓度的双特异性 DARPin(该双特异性 DARPin 被改造成具有长血清半衰期) 对增殖的抑制以及相应的抑制拟合曲线。双特异性 DARPin#63 在其 C 端 Cys 残基被聚乙二醇化，而双特异性 DARPins#64 和 #65 包含结合至血清白蛋白的锚蛋白重复序列结构域。图 7B 展示了对于不同的单个试验，在结合 HER2 的重复序列结构域之间含有不同接头的不同浓度的双特异性 DARPin 对增殖的抑制以及相应的抑制拟合曲线。表 2 列举了 DARPin 的 IC_{50}值。该曲线图展示了 OD，在 450nM 的光密度减去 620nm 的 OD，针对 C、不同浓度的 DARPin(nM) 绘图。X 轴以对数标度显示。参见下文对 DARPin ;#66、DARPin#66(其在两个重复序列结构域之间包含 2 个氨基酸长度的短 GS- 接头) ;#67、DARPin#67(其在两个重复序列结构域之间包含 5 个氨基酸长度的 GS- 接头) ;#41、DARPin#41(其在两个重复序列结构域之间包含 10 个氨基酸长度的 GS- 接

头）;#68、DARPin#68（其在两个重复序列结构域之间包含 24 个氨基酸长度的 PT- 接头）的定义，

具体实施方式

[0038] 根据本发明的一个实施例，重组结合蛋白包含至少第一和第二重复序列结构域，其中所述两个重复序列结构域中的每个结合 HER2 的胞外区，并且其中所述重复序列结构域是通过共价键连接的。

[0039] 已经令人惊讶地证明 HER2 的胞外部分与包含至少两个共价连接的重复序列结构域（每个共价连接的重复序列结构域对 HER2 的胞外区具有特异性）的重组结合蛋白的结合比上文概括的现有技术的方法［通过不同和单独的结合物（例如，曲妥珠单体和帕妥珠单体的组合 ;图 6）结合 HER2］具有优势和意想不到的效果。

[0040] 人 HER2 由 1255 个氨基酸组成，其中 21 个氨基酸的信号序列、631 个氨基酸的胞外区（例如，包含结构域 I 至 IV 的胞外域）、23 个氨基酸的跨膜区，以及 580 个氨基酸的胞浆结构域。

[0041] 优选地，HER2 的胞外区与所述重组结合蛋白的结合和所述重复序列结构域结合至 HER2 的胞外区是同时发生的或并行发生的。同样优选地，所述重复序列结构域结合至 HER2 胞外区的 2 个不同的表位。同样优选地，所述重复序列结构域结合至 HER2 胞外区的 2 个不同的非覆盖型表位。

[0042] 功效增加的一个原因可能是根据本发明的重组结合蛋白诱导迄今未描述的 HER2 胞外区的非激活态构象，这似乎是本发明的双特异性结合蛋白与 HER2 胞外区上的 2 个不同表位的分子内相互作用的结果（实施例 8）;即，该结合蛋白的 2 个重复序列结构域似乎都同时结合至相同的 HER2 分子上的不同表位，并且从而迫使 HER2 的胞外区处于这种新的非激活态构象中。现有技术没有描述这种非激活态构象。重要的是，这 2 个重复序列结构域需要连接在相同的结合蛋白中，即，这两个重复序列结构域的简单混合不表现出疗效（图 2B）。此外，这种结合蛋白与 HER2 胞外区的二价结合可以通过显示增加的亲合力（即，同步结合至靶的不同表位的组合强度）产生协同结合效果。亲合力与亲和力不同，其对应单个的结合相互作用的强度。总之，如实施例所示，该结合蛋白与 HER2 的特异性相互作用可以解释通过这些分子对增殖非常有效的抑制作用和对凋亡的诱导。

[0043] 根据这些理论，在相同蛋白中的 2 个不同的重复序列结构域协同地支持彼此结合其各自的表位，从而导致对靶的总亲和力增加。

[0044] 第一重复序列结构域结合至 HER2 上的表位使得第二重复序列结构域进入积极和 / 或空间有利的位置，这促进该第二重复序列结构域与 HER2 上其单独的表位结合。

[0045] 如实施例所示，该第一和第二重复序列结构域的共价连接似乎增强其生物活性。

[0046] 在根据本发明的重组结合蛋白的优选实施例中，第一重复序列结构域结合 HER2 的结构域 II 并且第二重复序列结构域结合 HER2 的结构域 IV。

[0047] 重点要了解的是，术语“结合结构域 II”意味着单独的重复序列结构域主要结合 HER2 的结构域 II。但是，这个定义并不排除所述重复序列结构域的部分可以与其它结构域结合，或相交。这同样适用于术语“结合结构域 IV”。

[0048] 根据本发明的双特异性结合蛋白同时靶向 HER2 的结构域 II 和 IV 比现有技术中

的已知技术具有特别意想不到的效果。在这些结合蛋白抑制增殖和诱导细胞凋亡的情况下的细胞应答相比现有技术的抗体获得的效果要更加明显的多。例如，这些应答的程度已经证明优于临床抗体基准［比如，分别靶向 HER2 的结构域 IV 和 II 的曲妥珠单抗和帕妥珠单抗的组合（图 4、5 和 6)］诱导的程度。有趣的是，与 HER2 的结构域 I 和结构域 IV 结合的一些双特异性结合蛋白没有显示这些意想不到的效果（图 3C 和 3D)。

[0049] 本领域技术人员周知确定与重复序列结构域结合的 HER2 胞外区的结构域的方法（例如，Jost 等人，同上）。

[0050] 通过本发明的双特异性结合蛋白同时靶向 HER2 的结构域 II 和 IV 可能更有效替代当前抗体靶向方法，在此意义上，申请人的发现对 HER2 造成的人癌症有重要意义。

[0051] 因此，根据本发明的结合蛋白优选双特异性结合蛋白，即，其包括识别在相同蛋白靶（即，HER2）上的 2 个不同表位，或结构域（即，结构域 II 和 IV）的 2 个抗原重复序列结构域。但是，和那些既是双特异性或多特异性多肽、也是多价多肽（即具有能够识别一个或更多个其它靶蛋白的抗原重复序列结构域的多肽）的多肽一样，多特异性多肽，即，含有能够识别相同靶蛋白上的 3 个、4 个或更多个表位的抗原重复序列结构域的多肽，也包含在本发明的范围之内。

[0052] 在此使用的 HER2 涉及人表皮生长因子受体 2，也称为 Neu，ErbB-2，CD340（分化群 340）或 p185。HER2 为表皮生长因子受体（EGFR/ErbB）家族成员。在人中，HER2 由 ERBB2 编码，ERBB2 是位于人染色体 17 长臂（17q12）上的已知的原癌基因。HER2 的 UniProtKB/Swiss-Prot 编号为 P04626。

[0053] 根据本发明的优选实施例，该第一和第二重复序列结构域位于相同的多肽上，而靶向 HER2 的结构域 II 的重复序列结构域位于靶向 HER2 的结构域 IV 的重复序列结构域的 N 端。

[0054] 例如，图 2A 以及相应的描述展示了这些实施例。发明人已经令人惊讶地展示靶向 HER2 的结构域 II 的重复序列结构域位于靶向 HER2 的结构域 IV 的重复序列结构域 C 端的结合蛋白比靶向 HER2 的结构域 II 的重复序列结构域位于靶向 HER2 的结构域 IV 的重复序列结构域 N 端的结合蛋白的功效明显较小。

[0055] 优选地，结合 HER2 的结构域 II 的所述第一重复序列结构域不与帕妥珠单抗竞争结合至 HER2。例如，图 1C 展示了这些重复序列结构域不与帕妥珠单抗竞争结合至 HER2。同样优选地，结合 HER2 的结构域 IV 的所述第二重复序列结构域不与曲妥珠单抗竞争结合至 HER2。例如，DARPin#18 至 20 的重复序列结构域不与曲妥珠单抗竞争结合至 HER2。本领域技术人员周知确定重复序列结构域是否与曲妥珠单抗或帕妥珠单抗竞争结合至 HER2 的方法，例如，如实施例 3 所示。

[0056] 这意味着，在第一优选实施例中，与帕妥珠单抗比较，该第一重复序列结构域结合与 HER2 结构域 II 不同的表位。同样地，在第二优选实施例中，与曲妥珠单抗比较，该第二重复序列结构域结合与 HER2 结构域 IV 不同的表位。在不受限于理论的情况下，发明人将试验部分中所示的至少一些效果归因于这些事实。

[0057] 根据本发明的另一优选实施例，所述第一重复序列结构域为锚蛋白重复序列结构域，或设计的锚蛋白重复序列结构域，并且所述第二重复序列结构域为锚蛋白重复序列结构域，或设计的锚蛋白重复序列结构域。

[0058] 优选地，所述锚蛋白重复序列结构域或设计的锚蛋白重复序列结构域包括 70 ～ 300 个氨基酸，特别是 90 ～ 200 个氨基酸。

[0059] 同样优选地，本发明的重复序列结构域为锚蛋白重复序列结构域，或如在 WO 2002/020565 中所示的设计的锚蛋白重复序列结构域。实施例展示了对 HER2 的不同结构域具有双特异性结合特异性的设计的锚蛋白重复序列结构域的例子。

[0060] 根据本发明的优选实施例，该第一重复序列结构域在 PBS 中以小于 10^{-7}M 的 Kd 结合 HER2 的胞外区并且所述第二重复序列结构域在 PBS 中以小于 10^{-7}M 的 Kd 结合 HER2 的胞外区。

[0061] Kd 为解离常数，其将进一步在下文中定义。Kd 需要小于 10^{-7}M 才能使重复序列结构域对其靶具有足够的亲和力。优选的是，重复序列结构域在 PBS 中以比 10^{-8}M、10^{-9}M、10^{-10}M 小，或者，更优选比 10^{-11}M 小的 Kd 结合其靶结构域。

[0062] 实施例 2 展示了包含在 PBS 以低于 10^{-7}M 的 Kd 结合 HER2 结构域 II 和 / 或结构域 IV 的蛋白的重组结合蛋白。

[0063] 根据优选实施例，所述结合蛋白以小于 100nM 的半抑制浓度 (IC_{50}) 值抑制 BT474 细胞的受激增殖。优选地，所述结合蛋白以小于 90、80、70、60、50、40、30、20 或 10nM 的 IC_{50} 值抑制 BT474 细胞的受激增殖。同样优选地，所述结合蛋白抑制至少 100％、90％、80％、70％、60％、50％、40％、30％、20％或 10％ BT474 细胞的受激增殖。

[0064] 可以通过本领域技术人员周知的标准手段将 BT474 细胞用于测量本发明的结合蛋白抑制增殖的功能，例如，如实施例 4 所示。优选地，BT474、SKBR-3、NCI-N87、ZR75-30、HCC1419 或 MDA-MB175 细胞可以用于测量本发明的化合物抑制增殖的功能，例如，如实施例 5 所示。

[0065] 在实施例 4 中公开并论述了以小于 100nM 的 IC_{50} 值抑制 BT474 细胞的受激增殖的重组结合蛋白。

[0066] 根据本发明的另一优选实施例，所述结合蛋白以小于 100nM 的半数有效浓度 (EC_{50}) 在 BT474 细胞中诱导凋亡。优选地，所述结合蛋白以小于 90、80、70、60、50、40、30、20 或 10nM 的 EC_{50} 在 BT474 细胞中诱导凋亡。

[0067] 可以通过本领域技术人员周知的标准手段将 BT474 细胞用于测量本发明的结合蛋白诱导凋亡的功能，例如，如实施例 5 所示。优选地，BT474、SKBR-3、NCI-N87、ZR75-30、HCC1419 或 MDA-MB175 细胞可以用于测量本发明的化合物诱导凋亡的功能，例如，如实施例 5 所示。

[0068] 在实施例 5 中公开并论述了以小于 100nM 的 EC_{50} 值在 BT474 细胞中诱导凋亡的重组结合蛋白。

[0069] 根据优选实施例，所述第一和第二重复序列结构域通过多肽接头连接。

[0070] 例如，这种多肽接头可以通过仅对待融合的各结构域的编码 cDNA 进行基因融合实现。这类实施例有资格成为具有 2 个不同重复序列结构域的融合多肽蛋白。

[0071] 例如，该接头可以由分别含有氨基酸 G 和 S，或 P 和 T 的寡肽组成，如 SEQ ID Nos:7 至 12 中阐述。根据另一优选实施例，可以使用下文所述的“多聚化部分”。可替代地，该 2 个重复序列结构域可以，例如，通过基于非多肽的化学接头，互相连接。

[0072] 优选地，在 PBS、pH7.4 中该重组结合蛋白和 / 或重复序列结构域的热去折叠的中

点变性温度 (Tm) 高于 45℃,更优选高于 50℃,更优选高于 55℃,并且最优选高于 60℃时。在生理条件下,本发明的结合蛋白或重复序列结构域具有确定的二级和三级结构。这种多肽的热去折叠导致其丧失二级和三级结构,这接着,例如,可以进行圆二色性测量。该重组结合蛋白和 / 或重复序列结构域的热去折叠的中点变性温度对应在生理缓冲液中通过将温度从 10℃缓慢提高至约 100℃使所述蛋白或结构域热变性时协作转变的中点温度。本领域技术人员周知热去折叠的中点变性温度的测定。该结合蛋白或重复序列结构域在热去折叠的中点变性温度表明所述多肽的热稳定性。

[0073] 还优选的是这样的重组结合蛋白和 / 或锚蛋白重复序列结构域 :当在 37℃,PBS 中培养超过 5 天,优选超过 10 天,更优选超过 20 天,更优选超过 40 天,最优选超过 100 天,该重组结合蛋白和 / 或锚蛋白重复序列结构域在浓度高达 20g/L,优选高达 40g/L,更优选高达 60g/L,甚至更优选高达 80g/L,最优选高达 100g/L 时,形成小于 5% (w/w) 的不溶性聚集物。可通过直观沉淀的出现、凝胶过滤或动态光散射 (不溶性聚集物形成后其将极大地增加) 检测不溶性聚集物的形成。可通过在 10’000x g 离心 10 分钟从蛋白样品移除不溶性聚集物。在 PBS、37℃上述培养条件下重组结合蛋白和 / 或锚蛋白重复序列域形成少于 2%,更优选少于 1%、0.5%、0.2%、0.1%,或最优选少于 0.05% (w/w) 的不溶性聚集物。可以通过从可溶性蛋白中分离不溶性聚集物,接着用标准量化方法测定可溶性和不溶性部分中的蛋白量而确定不溶性聚集物的百分比。

[0074] 还优选的是 :在含有 100mM 二硫苏糖醇 (DTT) 的 PBS 中 37℃培养 1 或 10 小时后,不丧失其天然三维结构的重组结合蛋白和 / 或锚蛋白重复序列结构域。

[0075] 在一个特别的实施例中,本发明涉及这样的重组结合蛋白 :其包含 2 个锚蛋白重复序列结构域,特异性结合 HER2 并具有如上定义的指示的或优选的中点变性温度和不聚集性质。

[0076] 根据本发明的其它优选的实施例,提供 :

[0077] 所述第一重复序列结构域与选自 EQ ID NOs:62 至 68、72 和 114 至 121 的锚蛋白重复序列结构域竞争结合至 HER2 ;和 / 或

[0078] 所述第二重复序列结构域与选自 SEQ ID NOs:74 至 82 的锚蛋白重复序列结构域竞争结合至 HER2。

[0079] 发明人已经证实这些重复序列结构域,第一重复序列结构域结合 HER2 的结构域 II,而第二重复序列结构域结合 HER2 的结构域 IV。

[0080] 优选地,所述第一重复序列结构域与选自 SEQ ID NOs:62 至 67 和 115 至 121 的锚蛋白重复序列结构域竞争结合至 HER2。更优选地,所述第一重复序列结构域与选自 SEQ ID NOs:62、115、120 和 121,特别是 SEQ ID NO:115 和 120 的锚蛋白重复序列结构域竞争结合至 HER2。同样优选地,所述第一重复序列结构域与选自 DARPins#1 至 6 和 54 至 60 的结合蛋白,更优选地,与选自 DARPins#1、54、59 和 60 的结合蛋白,特别地,与选自 DARPins#54 和 60 的结合蛋白竞争结合至 HER2。

[0081] 另外优选地,所述第二重复序列结构域与选自 SEQ ID NOs:79 至 81,特别是 SEQ ID NO:80 和 81 的锚蛋白重复序列结构域竞争结合至 HER2。同样优选地,所述第二重复序列结构域与选自 DARPins#18 至 20 的结合蛋白,特别地,与选自 DARPins#19 和 20 的结合蛋白竞争结合至 HER2。

[0082] 根据本发明的其它优选实施例，提供：

[0083] 第一重复序列结构域包含于选自 SEQ ID NOs:62 至 68,72 和 114 至 121 的一个锚蛋白重复序列结构域具有至少 70％氨基酸序列一致性的氨基酸序列，

[0084] 第二重复序列结构域包含于选自 SEQ ID NOs:74 至 82 的一个锚蛋白重复序列结构域具有至少 70％氨基酸序列一致性的氨基酸序列，

[0085] 并且其中进一步地

[0086] 在所述锚蛋白重复序列结构域位置 1 的 G 和 / 或在位置 2 的 S 可选择地缺失；和

[0087] 在所述锚蛋白重复序列结构域的倒数第二位置的 L 和 / 或在最后位置的 N 可选择地被 A 替换。

[0088] 优选地，所述第一重复序列结构域包含与选自 SEQ ID NOs:62 至 67 和 115 至 121 的一个锚蛋白重复序列结构域具有至少 70％氨基酸序列一致性的氨基酸序列。更优选地，所述第一重复序列结构域包含与选自 SEQ ID NOs:62、115、120 和 121，特别是 SEQ ID NO:115 和 120 的一个锚蛋白重复序列结构域具有至少 70％氨基酸序列一致性的氨基酸序列。同样优选地，所述第一重复序列结构域包含与选自 DARPins#1 至 6 和 54 至 60 的结合蛋白，更优选地，与选自 DARPins#1、54、59 和 60 的结合蛋白，特别地，与选自 DARPins#54 和 60 的结合蛋白具有至少 70％氨基酸序列一致性的氨基酸序列。

[0089] 另外优选地，所述第二重复序列结构域包含与选自 SEQ ID NOs:79 至 81，特别是 SEQ ID NO:80 和 81 的一个锚蛋白重复序列结构域具有至少 70％氨基酸序列一致性的氨基酸序列。同样优选地，所述第二重复序列结构域包含与选自 DARPins#18 至 20 的结合蛋白，特别是与选自 DARPins#19 和 20 的结合蛋白具有至少 70％氨基酸序列一致性的氨基酸序列。

[0090] 优选地，所述第一锚蛋白重复序列结构域包含与选自 SEQ ID NOs:62 至 68、72 和 114 至 121 的一个锚蛋白重复序列结构域具有至少 70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、86、87、88、89、90、91、92、93、94、95、96、97、98、99，或 100％氨基酸序列一致性的氨基酸序列。

[0091] 优选地，该第二锚蛋白重复序列结构域包含与选自 SEQ ID NOs:74 至 82 的一个锚蛋白重复序列结构域具有至少 70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、86、87、88、89、90、91、92、93、94、95、96、97、98、99，或 100％氨基酸序列一致性的氨基酸序列。

[0092] 同样优选地，该第一锚蛋白重复序列结构域包含与一个、两个或三个锚蛋白重复序列模块具有至少 70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、86、87、88、89、90、91、92、93、94、95、96、97、98、99，或 100％氨基酸序列一致性的氨基酸序列，该一个、两个或三个锚蛋白重复序列模块存在于选自 SEQ ID NOs:62 至 68、72 和 114 至 121 的锚蛋白重复序列结构域的 N 端和 C 端加帽模块之间。

[0093] 同样优选地，该第二锚蛋白重复序列结构域包含与一个、两个或三个锚蛋白重复序列模块具有至少 70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、86、87、88、89、90、91、92、93、94、95、96、97、98、99，或 100％氨基酸序列一致性的氨基酸序列，该一个、两个或三个锚蛋白重复序列模块存在于选自 SEQ ID NOs:74 至 82 的锚蛋白重复序列结构域的 N 端和 C 端加帽模块之间。

[0094] 根据本发明的其它优选实施例，提供：

[0095] 所述第一重复序列结构域选自 SEQ ID NOs:62 至 68、72 和 114 至 121，

[0096] 所述第二重复序列结构域选自 SEQ ID NOs:74 至 82，

[0097] 并且其中进一步地

[0098] 在所述锚蛋白重复序列结构域位置 1 的 G 和 / 或在位置 2 的 S 可选择地缺失 ;和

[0099] 在所述锚蛋白重复序列结构域的倒数第二位置的 L 和 / 或在最后位置的 N 可选择地被 A 替换。

[0100] 优选地，所述第一锚蛋白重复序列结构域选自 SEQ ID NOs:62 至 67、72 和 115 至 121 ;更优选 SEQ ID NO:115、120，和 121，特别是，SEQ ID NO:115 和 120。

[0101] 优选地，该第二锚蛋白重复序列结构域选自 SEQ ID NOs:79 至 81，特别是 SEQ ID NO:80 和 81。

[0102] 根据本发明的其它优选实施例，提供 :

[0103] 所述第一重复序列结构域包含锚蛋白重复序列模块，该锚蛋白重复序列模块具有选自 SEQ ID NO:15 至 18、21 至 23、37、38、125、126、129、130、133 和 134 的氨基酸序列，其中在 SEQ ID NO:15 至 18、21 至 23、37、38、125、126、129、130、133 和 134 中多达 9 个氨基酸被任何其它氨基酸残基取代，和 / 或

[0104] 所述第二重复序列结构域包含锚蛋白重复序列模块，该锚蛋白重复序列模块具有选自 SEQ ID NO:46、47、51、52、55 和 56 的氨基酸序列，其中在 SEQ ID NO:46、47、51、52、55 和 56 中多达 9 个氨基酸被任何其它氨基酸残基取代。

[0105] 优选地，所述第一锚蛋白重复序列结构域的锚蛋白重复序列模块选自 SEQ ID NO:15 至 18、125、126、129、130、133 和 134，更优选 SEQ ID NO:15、125、129 和 133，并且甚至更优选 SEQ ID NO:125 和 133。

[0106] 优选地，所述第二锚蛋白重复序列结构域的锚蛋白重复序列模块选自 SEQ ID NO:46、47、55 和 56，并且更优选 SEQ ID NO:55 和 56。

[0107] 同样优选地，在 SEQ ID NO:15 至 18、21 至 23、37、38、46、47、51、52、55、56、125、126、129、130、133 和 134 的重复序列模块中多达 8 个氨基酸被另一氨基酸替换，被替换的氨基酸优选为多达 7 个氨基酸，更优选为多达 6 个氨基酸，更优选为多达 5 个氨基酸，甚至更优选为多达 4 个氨基酸，更优选为多达 3 个氨基酸，更优选为多达 2 个氨基酸，最优选为 1 个氨基酸。

[0108] 优选地，当在加帽模块、重复序列模块或重复序列结构域，重复序列结构域，或结合蛋白中替换氨基酸时，这些氨基酸被选自 A、D、E、F、H、I、K、L、M、N、Q、R、S、T、V、W 和 Y，更优选 A、D、E、H、I、K、L、Q、R、S、T、V，和 Y 的氨基酸取代。同样优选地，氨基酸被同源氨基酸替换，即，氨基酸被侧链具有相似的生物物理特性的氨基酸替换。例如，带负电荷的氨基酸 D 可以被带负电荷的氨基酸 E 取代，或疏水性氨基酸，例如 L 可被 A，I 或 V 取代。本领域技术人员周知在多肽中氨基酸被另一氨基酸替换的技术。

[0109] 优选地，根据本发明的重复序列结构域具有选自 KDFQGITPLHIAATSGHLEIVEVLLKAGADVNA(SEQ ID NO:16) 的氨基酸序列，其中在 SEQ ID NO:16 中多达 9 个氨基酸残基被任何其它氨基酸残基取代，并且其中

[0110] 在位置 3 的 F 被 A 可选择地替换 ;

[0111] 在位置 4 的 Q 被 E 可选择地替换 ;

[0112] 在位置 5 的 G 被 S 可选择地替换；

[0113] 在位置 6 的 I 被 V 可选择地替换；

[0114] 在位置 11 的 I 被 L 可选择地替换；

[0115] 在位置 14 的 T 被 Q 可选择地替换；和 / 或

[0116] 在位置 15 的 N 被选自 S 和 W 的氨基酸可选择地替换。

[0117] 该组中一个非常优选的重复序列模块具有由 KDFQGVTPLHIAAQSGHLEIVEVLLKAGADVNA(SEQ ID NO:125)、SEQ ID NO:129 或 SEQ ID NO:133 组成的氨基酸序列。

[0118] 同样优选地，根据本发明的锚蛋白重复序列模块具有选自 KDITGETPLHHAADSGHLEIVEVLLKAGADVNA(SEQ ID NO:18) 的氨基酸序列，其中在 SEQ ID NO:18 中多达 9 个氨基酸残基被任何其它氨基酸残基取代，并且其中

[0119] 在位置 3 的 I 被 V 可选择地替换；

[0120] 在位置 6 的 E 被 D 可选择地替换；

[0121] 在位置 11 的 H 被 L 可选择地替换；

[0122] 在位置 14 的 D 被 Q 可选择地替换；

[0123] 在位置 15 的 S 被 H 可选择地替换；和 / 或

[0124] 在位置 19 的 E 被 V 可选择地替换。

[0125] 该组中一个非常优选的重复序列模块具有由 KDVTGDTPLHLAAQHGHLEIVEVLLKAGADVNA(SEQ ID NO:126)、SEQ ID NO:130 或 SEQ ID NO:134 组成的氨基酸序列。

[0126] 同样优选地，根据本发明的锚蛋白重复序列结构域具有选自 KDWEGTTPLHLAAHTGHLEIVEVLLKAGADVNA(SEQ ID NO:21) 的氨基酸序列，其中在 SEQ ID NO:21 中多达 9 个氨基酸残基被任何氨基酸残基取代，并且其中

[0127] 在位置 3 的 W 被 F 可选择地替换；

[0128] 在位置 4 的 W 被 Q 可选择地替换；

[0129] 在位置 6 的 T 被选自 I、Y 和 V 的氨基酸可选择地替换，优选 T；

[0130] 在位置 11 的 L 被选自 I 和 V 的氨基酸可选择地替换，优选 I 和 V；

[0131] 在位置 14 的 H 被选自 H、Q、Y 和 W 的氨基酸可选择地替换，优选 H；和 / 或

[0132] 在位置 15 的 T 可选择地缺失或被选自 A 和 D 的氨基酸替换。

[0133] 同样优选地，根据本发明的锚蛋白重复序列结构域具有选自 KDTVGTTPLHYAAEDGHLEIVEVLLKAGADVNA(SEQ ID NO:22) 的氨基酸序列，其中在 SEQ ID NO:22 中多达 9 个氨基酸残基被任何其它氨基酸残基取代，并且其中

[0134] 在位置 3 的 T 被选自 S、K、E 和 I 的氨基酸可选择地替换；氨基酸分配相等；

[0135] 在位置 4 的 V 被选自 Q、I 和 Y 的氨基酸可选择地替换；优选 Y；

[0136] 在位置 6 的 T 被选自 Q、F、R 和 W 的氨基酸可选择地替换；

[0137] 在位置 11 的 Y 被选自 L、E 和 S 的氨基酸可选择地替换；优选 S；

[0138] 在位置 14 的 E 被选自 S、Q、Y 和 V 的氨基酸可选择地替换；和 / 或

[0139] 在位置 15 的 D 被选自 S、F 和 Y 的氨基酸可选择地替换；

[0140] 在位置 16 的 G 被 D 可选择地替换；

[0141] 同样优选地，根据本发明的锚蛋白重复序列模块选自 KDVEGWTPLHYAASSGHLEIVEVLLKAGADVNA(SEQ ID NO:38) 的氨基酸序列，其中在 SEQ ID NO:38 中多达 9 个氨基酸被任何

其它氨基酸残基取代，并且其中

[0142] 在位置 6 的 W 被 Q 可选择地替换；

[0143] 在位置 11 的 Y 被 L 可选择地替换；和 / 或

[0144] 在位置 15 的 S 被 Y 可选择地替换。

[0145] 同样优选地，根据本发明的锚蛋白重复序列模块选自 KDWRGFTPLHYAAYLGHLEIVEVL LKAGADVNA(SEQ ID NO:46) 的氨基酸序列，其中在 SEQ ID NO:46 中多达 9 个氨基酸被任何其它氨基酸残基取代，并且其中

[0146] 在位置 3 的 W 被选自 W、T、V 和 R 的氨基酸可选择地替换；优选 T 和 R；

[0147] 在位置 4 的 R 被选自 R、T 和 I 的氨基酸可选择地替换；优选 I；

[0148] 在位置 6 的 F 被选自 F 或 H 可选择地替换；优选 F；

[0149] 在位置 11 的 Y 被 R 可选择地替换；

[0150] 在位置 14 的 Y 被 F 可选择地替换；

[0151] 在位置 15 的 L 被 V 可选择地替换；和 / 或

[0152] 在位置 17 的 H 被 Q 可选择地替换。

[0153] 优选地，SEQ ID NOs:16、18、28、31、21、22、38 和 / 或 46 的 9、8、7、6、5、4、3、2，或 1 个氨基酸残基被任何其它氨基酸残基取代。

[0154] 此外，特别优选地，所述结合蛋白包含多肽，其中所述多肽包含所述第一和第二锚蛋白重复序列结构域，并且其中所述多肽与选自 SEQ ID NO:83 至 98、102、103、122、123 和 136 至 141 的多肽具有至少 70％氨基酸序列一致性。

[0155] 优选地，所述多肽包含与选自 SEQ ID NO:83 至 98、102、103、122、123 和 136 至 141 的多肽具有至少 70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、86、87、88、89、90、91、92、93、94、95、96、97、98、99，或 100％氨基酸序列一致性的氨基酸序列。

[0156] 同样优选地，所述多肽选自 SEQ ID NO:84、85、86、87、90、91、92、98、102、103、122and 123，更优选 SEQ ID NO:85、86、87、90、91、92、102、103、122 和 123，甚至更优选 SEQ ID NO:86、87、91 和 92，并且最优选 SEQ ID NO:86 和 87。

[0157] 根据其它优选实施例，所述第一和第二锚蛋白重复序列结构域的锚蛋白重复序列模块的一个或更多个氨基酸残基被在锚蛋白重复序列单元的比对上相应位置处的氨基酸残基替换。

[0158] 本发明的另一实施例提供核酸分子，该核酸分子编码上文所述的至少一个结合蛋白或特别的锚蛋白重复序列结构域。此外，考虑包含所述核酸分子的载体。

[0159] 并非所有根据本发明的结合组合物都包含多肽或蛋白。后面的实施例仅涉及那些包含多肽或蛋白的结合组合物。对于这些组合物，申请人没有在此公开能够对它们进行编码的所有核酸分子，这是因为，由于遗传密码的简并性，许多核酸分子能够对一个和相同的多肽或蛋白进行编码。

[0160] 但是，它可以明确地并且毫不含糊地确定给定的核酸是否编码给定的多肽或蛋白。因此，对技术人员来说，本实施例是清楚的，并且其范围是易于确定的。

[0161] 本发明的另一实施例是提供根据上文所述的结合蛋白抑制以下至少一种的用途：

[0162] HER2 受体二聚化，

[0163] HER2/HER3 异源二聚化，

[0164] HER2 受体自身磷酸化，

[0165] HER 受体介导的信号传导，

[0166] HER3 受体配体诱导的磷酸化，和 / 或

[0167] HER3 受体介导的信号传导。

[0168] HER2 受体二聚化（也称为“同源二聚化”）发生在独立于配体之外使 HER2 过度表达的组织中。所述同源二聚化导致胞内自身磷酸化，这可以最终导致，例如，增加细胞增殖。

[0169] 因为 HER3 缺乏内在的激酶活性，HER2/HER3 异源二聚体形成之后，在 HER2 过度表达的乳腺癌中 HER3 被磷酸化，其可以最终导致，例如，抑制细胞凋亡。

[0170] 所述用途可以发生在体外或体内。如上所述，所有这些方法可以导致致病结果，即，通过激活各自的信号转导通路。通过 HER2 二聚化和 / 或 HER2/HER3 异源二聚体激活的信号转导通路包括丝裂原活化蛋白激酶 (MAPK)，磷酸肌醇 3- 激酶 (PI3K/Akt)，磷脂酶 C γ，蛋白激酶 C(PKC)，信号转导和转录激活因子 (STAT)，Ras-Map 激酶通路和 mTOR 通路。

[0171] 例如，磷酸肌醇 3- 激酶 (PI3K/Akt) 通路被认为是通过阻断凋亡使细胞维持存活的关键通路之一。其病理激活，例如，通过 HER2/HER3 异源二聚体，可能引发恶性增殖（例如，参见实施例）。

[0172] HER2 的病理结合，例如，通过 HER2 同源二聚化，可能引发细胞恶性迁移、入侵或增殖（例如，参见实施例、Hynes NE. 和 Lane HA.，Nat.Rev.Cancer.，5，341-54，2005）。

[0173] 本发明的另一实施例，提供包含根据上述公布的结合蛋白或组合物的药物制剂，以及可选地提供可接受药物的载体和 / 或稀释剂。

[0174] 可接受药物的载体和 / 或稀释剂对本领域技术人员来说是已知的并且在下文有更详细的说明。甚至进一步地，考虑包含一种或更多种上文所述的重组结合蛋白，特别是包含重复序列结构域的结合蛋白的用于诊断的组合物。

[0175] 药物制剂包含上述的重组结合蛋白和可接收药物的载体、赋形剂或稳定剂，例如，如在 Remington's Pharmaceutical Sciences 16^{th} edition，Osol，A.Ed.[1980] 一文中所述。本领域技术人员已知的合适的载体、赋形剂或稳定剂为生理盐水、林格氏溶液、葡萄糖溶液、汉克 (Hank's) 溶液、固定油、油酸乙酯、在盐水中的 5％葡萄糖、增强等渗性和化学稳定性的物质、缓冲剂和防腐剂。其它合适的载体包括本身不诱导对接受所述组合物的个体有害的抗体的产生的任何载体，例如，蛋白质、多糖、聚乳酸、聚乙醇酸、聚合氨基酸和氨基酸共聚物。

[0176] 用于体内服用的制剂必须是消过毒的或无菌的。这经由无菌滤膜过滤轻易实现。该药物制剂可通过在本领域技术人员知识范围内的任何合适的方法来服用。

[0177] 此外，在本发明的另一实施例中提供根据上文公布的至少一种结合蛋白、组合物或药物制剂作为药剂的用途。同样地，提供包括使患者服用上述的结合蛋白、组合物或药物制剂的方法。在这两种情况下，优选，待治疗的疾病是肿瘤疾病，优选癌症。

[0178] 在每种情况下，优选使患者服用有效量的上述的结合蛋白、组合物或药物制剂以治疗疾病。

[0179] 在此使用的术语“肿瘤性疾病”是指细胞或组织的异常状态或情况，特征在于迅速增殖的细胞生长或肿瘤。在更具体的意义上，该术语涉及癌变过程，例如，瘤和 / 或白血病。

[0180] 根据本发明的结合蛋白显示出凋亡和抗增殖作用（参见实施例部分）。由于肿瘤疾病的特征通常为由凋亡受抑制和/或增殖增强，从这些实施例中合理推断出根据本发明的结合蛋白可以用于肿瘤疾病的治疗。

[0181] 优选地，所述肿瘤疾病为具有选自以下的至少一个特征的疾病：

[0182] HER2 编码基因的扩增，

[0183] HER2 编码基因的过度表达，

[0184] HER2 编码基因的突变形式的表达，和/或

[0185] 在抗曲妥珠单抗的肿瘤中 HER3 编码基因的过度表达。

[0186] 人 HER2 由 ERBB2 基因编码。上述选项可以归因于 ERBB2 基因突变，这可以通过现代分子诊断来检测，如目前市场上的现代分子诊断。

[0187] 在此使用的术语“HER2 表面具有的表达”涉及表达 HER2 受体蛋白的细胞、组织或器官，例如，通过免疫组织化学法（IHC）检测出。在此使用的术语“HER2 编码基因的扩增或过度表达”涉及与在正常细胞、组织或器官中的表达水平比较，指示在细胞、组织或器官中 HER2 受体蛋白的异常表达水平，例如，通过免疫组织化学法（IHC）检测出。

[0188] 本领域已知这样的 IHC 检测试验，包括临床试验测定（CTA），市售 LabCorp4D5 测验和市售DAKOHercepTest®（DAKO，卡平特里亚，加利福尼亚州）。后面的试验使用 0 至 3+（0 是正常表达，3+ 表示最强阳性表达）细胞染色的特定得分范围，以识别 HER2 蛋白过度表达的癌症。因此，HER2 蛋白的过度表达在 1+、2+ 或 3+，优选 2+ 或 3+，更优选 3+ 的范围特征的癌症患者将受益于本发明的治疗方法。

[0189] 可代替地，也可以通过原位杂交法（ISH）、RT-PC 和其它方法检测 HER2 表达和/或过度表达的得数。

[0190] 根据特别优选的实施例，所述肿瘤疾病为选自以下疾病中的至少一种：

[0191] • 乳腺癌，

[0192] • 卵巢癌，

[0193] • 胃肿瘤（gastric cancer），

[0194] • 胃癌（stomach cancer），和/或

[0195] • 子宫癌，

[0196] • 结肠直肠癌。

[0197] 此外，优选通过服用选自以下的至少一种活性物质以协作方式补充所述用途：

[0198] • 抗肿瘤试剂

[0199] • 内分泌药物，

[0200] • 肿瘤疫苗，

[0201] • 免疫疗法，和/或

[0202] • 细胞疗法。

[0203] 在此使用的术语“以协作方式补充”应该指的是共同服用，其在给定方案的情况下进行。这包括同步服用不同化合物，以及时移服用不同化合物（例如，化合物 A 给药一次并且在此之后化合物 B 给药几次，或反之亦然，或这两种化合物同步给药并且在稍后阶段两者之一也给药）。

[0204] 在此使用的术语“抗肿瘤试剂”涉及具有抗肿瘤或抗癌作用的药物，或药物的组

合。这首先适用于化疗剂，其通过消弱有丝分裂，有效地靶向快速分裂的细胞或者通过使细胞进行凋亡而起作用。大多数化疗药物可分为烷化剂、抗代谢物、蒽环类药物、植物生物碱、拓扑异构酶抑制剂，和其它抗肿瘤试剂。

[0205] 优选的抗肿瘤试剂是 5- 氟尿嘧啶、放线菌素、阿霉素、安吖啶、蒽环类药物、硫唑嘌呤、苯达莫司汀、博莱霉素、卡铂、苯丁酸氮芥、顺铂、环磷酰胺、柔红霉素、多西紫杉醇、阿霉素、表柔比星、依托泊苷、伊达比星、异环磷酰胺、伊立替康、氮芥、巯基嘌呤、甲氨蝶呤、丝裂霉素、奥沙利铂、紫杉醇、普卡霉素、鬼臼毒素、替尼泊苷、托泊替康、戊柔比星、长春碱、长春新碱、长春新碱、长春地辛，和 / 或长春瑞滨。

[0206] 免疫治疗涉及从癌细胞分离蛋白质并且随后使癌症患者对这些蛋白质免疫，以期刺激免疫反应，杀死癌细胞。另一种治疗性抗癌疫苗接种方法是在患者体内的原位产生免疫应答。这增强对肿瘤抗原的抗肿瘤免疫应答，随后释放裂解性病毒复制体，结果在原位产生患者特异性抗肿瘤疫苗。另一种方法是使患者对在癌症发生中起生理作用的化合物免疫，从而使人体消除所述化合物。

[0207] 靶向药物是通过干扰致癌和肿瘤生长所需的特异靶向分子，而不是简单干扰快速分裂的细胞（例如，传统的化疗），阻断癌细胞的生长的一类药剂。靶向治疗的主要种类是小分子和单克隆抗体。

[0208] 落入此定义的小分子包括，但不限于，拉帕替尼、来那替尼、阿法替尼、伊马替尼、吉非替尼、埃罗替尼、硼替佐米、Bcl-2 抑制剂（例如，奥巴克拉、ABT-263、和棉酚）、PARP 抑制剂（例如，Iniparib、奥拉帕尼）、Janus 激酶抑制剂、PI3K 抑制剂、阿帕替尼、mTOR 抑制剂（依维莫司）、AN-152、AKT 抑制剂、HDAC 抑制剂、蛋白酶体抑制剂、连接至 [D-Lys(6)]-LHRH 的多柔比星、哌加他尼、舒尼替尼、索拉非尼、Tivozanib 和帕唑帕尼。落入此定义的单克隆抗体包括，但不限于，利妥昔单抗、曲妥珠单抗、曲妥珠单抗 -TDM1、帕妥珠单抗、西妥昔单抗和贝伐珠单抗。

[0209] 在此使用的内分泌药物是对激素或激素受体有拮抗作用并因此干扰生长需要激素的癌症类型的药物。这样的内分泌药物的一个例子是它莫西芬，其为乳房组织中的雌激素受体的拮抗剂。

[0210] 在此使用的术语“细胞治疗”，应涉及基于细胞的疗法，如修饰，或未经修饰的，细胞毒性淋巴细胞或树突状细胞的过继转移。

[0211] 在此使用的术语“肿瘤疫苗”是指如下的疫苗 a) 防止感染致癌病毒（作用模式类似于其它抗病毒感染的疫苗），b) 治疗现有癌症（治疗性癌症疫苗），或 c) 防止癌症发展，或减轻其影响（预防癌症疫苗）。

[0212] 另外或可代替地，优选通过以下的至少一种其它治疗以协作方式补充所述用途：

[0213] • 放射治疗

[0214] • 手术，和 / 或

[0215] • 激光消融。

[0216] 此外，提供了治疗人或动物受试者的方法，该方法包括根据上述公开的用途。优选地，所述治疗方法涉及上文所述的指示。该方法包括使有需要的人或动物服用治疗有效量的本发明的重组结合蛋白。

[0217] 可通过比如噬菌体 (WO 1990/002809, WO 2007/006665)、或细菌细胞 (WO

1993/010214) 表面展示、核糖体展示 (WO 1998/048008)，质粒展示 (WO 1993/008278) 几种方法，或通过利用共价 RNA 重复序列蛋白杂交构建体 (WO 2000/032823)，或胞内表达和选择 / 筛选比如蛋白互补试验 (WO 1998/341120) 获得和 / 或进一步形成根据本发明的重组结合蛋白或锚蛋白重复序列结构域。本领域技术人员知晓这些方法。

[0218] 可以根据本领域技术人员已知的方案获得用于根据本发明的重组结合蛋白或锚蛋白重复序列结构域的选择 / 筛选的锚蛋白重复序列蛋白的文库 (WO 2002/020565, Binz, H.K., 等人, J.Mol.Biol., 332, 489-503, 2003 和 Binz 等人, 2004, 同上)。实施例 1 举例说明了这些文库在选择对 HER2 胞外区具有特异性的锚蛋白重复序列结构域上的用途。此外，可以利用标准的重组 DNA 技术 (例如，WO2002/020565，Binz 等人，2003，同上和 Binz 等人，2004，同上) 由根据本发明的锚蛋白重复序列模块和合适的加帽模块或加帽重复序列模块化地组装 ((Forrer, P. 等人，FEBS letters 539, 2-6, 2003) 本发明的锚蛋白重复序列结构域。

[0219] 本发明并不限于在实施例中描述的特定实施例。也可以使用其它资源，并用下面概括的方法进行处理。

[0220] 定义

[0221] 术语“蛋白”是指多肽，其中至少部分所述多肽具有或能够通过在其多肽链内和 / 或之间形成二级、三级或四级结获得确定的三维排列。如果蛋白包含两条或更多条多肽，该单独的多肽链可以非共价地或共价地连接，例如，通过两条多肽之间的二硫键连接。通过形成二级或三级结构单独具有，或能够获得确定的三维排列的蛋白部分被称为“蛋白结构域”。本领域技术从事者周知这样的蛋白质结构域。

[0222] 在重组蛋白、重组蛋白结构域、重组结合蛋白等等中使用的术语“重组”是指通过利用相关领域从事者周知的重组 DNA 技术产生所述多肽。例如，可以将编码多肽的重组 DNA 分子 (例如，通过基因合成产生) 克隆到细菌表达质粒 (例如，pQE30，Qiagen)、酵母表达质粒或哺乳动物表达质粒中。例如，当将这样构建的重组细菌表达质粒插入到合适的细菌 (例如，大肠杆菌) 时，该细菌可产生由该重组 DNA 编码的多肽。相应产生的多肽被称为重组多肽

[0223] 在本文，术语“多肽”涉及由例如，通过肽键连接的多个，即，2 个或更多个氨基酸的一条或多条链组成的分子。优选地，多肽由通过肽键连接的八个以上的氨基酸组成。

[0224] 术语“多肽标签”指的是连接至多肽 / 蛋白质的氨基酸序列，其中所述氨基酸序列用于所述多肽 / 蛋白质的纯化、检测或靶向，或其中所述氨基酸序列能够改善所述多肽 / 蛋白质的物理化学行为，或其中所述氨基酸序列具有效应子功能。单独的多肽标签，结合蛋白的部分和 / 或结构域可直接或通过多肽接头彼此连接。这些多肽标签在本领域中都是周知的，并且对本领域技术人员来说完全是可以获得的。多肽标签的例子为小多肽序列，例如，组氨酸 (His)(例如，SEQ ID NO :6 的组氨酸标签)、myc、FLAG、Strep 标签或部分，例如，酶 (例如，碱性磷酸酶)，其能对所述多肽 / 蛋白质，或可用于靶向 (如免疫球蛋白或其片段) 和 / 或作为效应分子的部分或进行检测。

[0225] 术语“多肽接头”是指氨基酸序列，其能够连接，例如两个蛋白结构域，多肽标签和蛋白结构域，蛋白结构域和非多肽部分，例如，聚乙二醇或两个序列标签。相关领域技术人员已知这些结构域、标签、非多肽部分和接头。在申请号为 WO2002/020565 的专利申请的描

述中提供了上述例子的列表。此类接头的具体例子为长度可变的甘氨酸－丝氨酸连接子及脯氨酸－苏氨酸接头，优选地，所述接头的长度在 2 和 24 个氨基酸之间；更优选地，所述接头的长度在 2 和 16 个氨基酸之间。SEQ ID NO:7 至 10 提供甘氨酸－丝氨酸接头的例子标签 SEQ ID NO:11 和 12 提供脯氨酸－苏氨酸接头的例子。优选地，SEQ ID NO:11 的脯氨酸－苏氨酸接头之前有 GS 和 / 或之后有 GS。

[0226] 术语“聚合物部分”指的是蛋白质的聚合物部分或非蛋白质的聚合物部分。“蛋白质的聚合物部分”优选为不形成稳定的三级结构的多肽。蛋白质的聚合物部分的例子是 XTEN® (Amunix 的注册商标；WO 2007/103515) 多肽，或如 WO 2008/155134 所述包含脯氨酸、丙氨酸和丝氨酸残基的多肽。通过利用标准 DNA 克隆技术产生基因融合多肽，随后通过标准的表达和纯化，蛋白质的聚合物部分可以共价连接至，例如，本发明的重复序列结构域。“非蛋白质的聚合物部分”是并非由多肽构成的聚合物部分。非蛋白质的聚合物部分的例子是羟乙基淀粉 (HES)、聚乙二醇 (PEG)、聚丙二醇，或聚氧化烯。术语“聚乙二醇化”是指 PEG 部分共价连接至，例如，本发明的多肽。本发明的聚合物部分的分子量可以广泛变化。优选地，所述聚合物部分由多肽接头连接至重复序列结构域。

[0227] 在特定的实施例中，PEG 部分或任何其它非蛋白质的聚合物可以，例如，通过马来酰亚胺接头连接到半胱氨酸巯基，并且半胱氨酸通过肽接头连接至在此所述的重复序列结构域的 N 或 C 端。

[0228] 术语“结合蛋白”是指包含一个或多个结合结构域，一种或多种生物活性化合物和如下面进一步解释的一种或多种聚合物部分的蛋白质。优选地，所述结合蛋白包含高达四个结合结构域。此外，任何此类结合蛋白可以包含并非为结合结构域的另外的蛋白结构域、多聚化部分、多肽标签、多肽接头和 / 或单个 Cys 残基。

[0229] “多聚化部分”的例子是免疫球蛋白重链恒定区，其成对提供功能性免疫球蛋白 Fc 结构域，和亮氨酸拉链或包含游离巯基（在 2 条这样的多肽之间形成分子间二硫键）的多肽。单个 Cys 残基可用于，例如，通过利用本领域技术人员周知的马来酰亚胺化学作用使其它部分结合至多肽。优选地，所述结合蛋白为重组结合蛋白。还优选地，结合蛋白的结合结构域具有不同的靶特异性。

[0230] 术语“竞争结合”表示本发明的两个不同的结合结构域都能单独地结合相同的靶，而不能同时结合至相同的靶。因此，这样的两个结合结构域竞争结合至所述靶。优选地，所述两个竞争的结合结构域结合至在所述靶上的重叠或相同的结合表位。本领域技术人员周知确定两个结合结构域是否竞争结合至靶的方法，如竞争酶联免疫吸附法 (ELISA) 或竞争 SPR 测定（例如，通过使用来自 BioRad 公司的 Proteon 仪）。

[0231] 术语“多特异性结合蛋白”是指针对位于相同靶蛋白上的两个或多个不同表位的结合蛋白。例如，靶向 HER2 的多特异性结合蛋白包含至少一个靶向 HER2 上的第一表位的第一结合结构域、靶向 HER2 上的不同的第二表位的第二结合结构域，以及任选地包含靶向 HER2 上的另外的表位的另外的结合结构域。

[0232] 术语“双特异性结合蛋白”是指针对位于相同靶蛋白上的两个不同表位的结合蛋白。例如，靶向 HER2 的双特异性结合蛋白包含至少一个靶向 HER2 第一表位的第一结合结构域和靶向 HER2 上的不同的第二表位的第二结合结构域。相应地，“双特异性结合蛋白”包含针对第一表位的第一结合结构域和针对相同靶分子上的不同的第二表位的第二结合结

构域。

[0233] 术语“生物活性化合物”指的是当适用于具有所述疾病的哺乳动物时，减轻疾病的化合物。生物活性化合物可以具有拮抗或激动性质，并且可以是蛋白质的生物活性化合物或非蛋白质的生物活性化合物。这种蛋白质的生物活性化合物通过使用标准 DNA 克隆技术产生基因融合多肽，随后通过标准的表达和纯化可以共价连接至，例如，本发明的结合结构域。这种非蛋白质的生物活性化合物通过化学方法可以共价连接至，例如，本发明的结合结构域，例如，通过由马来酰亚胺接头连接至半胱氨酸硫基并且半胱氨酸由肽接头连接至在此所述的结合结构域的 N 或 C 端。蛋白质的生物活性化合物的例子是具有不同靶特异性（例如，通过与生长因子结合使其无效）的结合结构域、细胞因子（如白介素）、生长因子（例如，人生长激素）、抗体及其片段、激素（例如，GLP-1）和任何可能的蛋白质药物。非蛋白质的生物活性化合物的例子是毒素（例如，ImmunoGen 的 DM1）、靶向 GPCR 的小分子、抗生素和任何可能的非蛋白质药物。

[0234] 术语“结合结构域”是指蛋白质支架表现出相同的“折叠”（三维排列）并且具有下文定义的预定形状的蛋白质结构域。这样的结合结构域可以通过合理的，或最常见的是，组合蛋白质工程技术获得，这是本领域已知的技术 (Binz 等人，2005，同上）。例如，可以通过包括如下步骤的方法获得具有预定性质的结合结构域 :(a) 提供蛋白质支架展示系统折叠的蛋白质结构域的各种集合 ;和 (b) 筛选所述各种集合和 / 或从所述各种集合中选择，以获得至少一种具有所述预定性质的蛋白质结构域。根据使用的筛选和 / 或选择系统，可以通过几种方法提供蛋白质结构域的各种集合，并且可以包括利用本领域技术人员已知的方法，比如，噬菌体展示或核糖体展示。优选地，所述结合域是重组结合结构域。还优选地，所述结合结构域是重复序列蛋白或设计的重复序列蛋白。

[0235] 因此，在此使用的术语“结合”涉及识别并结合给定的靶，但基本上不识别或结合其它靶的结合结构域。优选地，具有本发明意义的结合结构域资格的候选者要求解离常数在 PBS 中小于 10^{-7}M。

[0236] 术语“Kd”涉及解离常数，其为测量较大的对象可逆地分离（离解）为较小成分的性质（如当复合物分开成其组成分子时）的特定类型的平衡常数。本领域技术人员周知确定蛋白质 - 蛋白质相互作用的解离常数的方法，比如，基于表面等离子体共振 (SPR) 的技术（例如，SPR 平衡分析）或等温滴定量热法 (ITC)。如果在不同的条件（例如，盐浓度，pH 值）下测量，那么特定的蛋白质 - 蛋白质相互作用的测得的 Kd 值可以不同。因此，优选用标准化的蛋白溶液和标准化的缓冲液，例如 PBS 进行 Kd 值的测量。

[0237] 术语“PBS”意为含有 137mM NaCl、10mM 磷酸盐和 2.7mM KCl 并且 pH 为 7.4 的磷酸盐缓冲水溶液。

[0238] 术语“蛋白质支架”是指具有可以高度容忍氨基酸插入、取代或缺失的暴露表面区域的蛋白质。可以用来产生本发明的结合结构域的蛋白质支架的例子是抗体或其片段，比如单链 Fv 或 Fab 片段、来自金黄色葡萄球菌的蛋白 A、来自大菜粉蝶 (Pieris brassicae) 的胆汁三烯结合蛋白或其它脂质运载蛋白、锚蛋白重复序列蛋白或其它重复序列蛋白，和人纤连蛋白。本领域技术人员已知所述蛋白支架 (Binz 等人，2005，同上 ;Binz 等人，2004，同上）。

[0239] 术语“靶”指的是单独的分子，例如核酸分子、多肽或蛋白质、碳水化合物，或任何

其它天然存在的分子，包括这些单独分子的任何一部分，或两个或多个这些分子的复合物。靶可以是整个细胞或组织样品，或其可以为任何非天然分子或部分。优选地，所述靶是天然存在的或非天然多肽或含有化学修饰，例如，通过天然或非天然的磷酸化、乙酰化、或甲基化修饰的多肽。在本发明的具体应用中，所述靶为 HER2 的胞外区域。

[0240] 术语“预定性质”是指性质，比如，结合至靶、靶的阻断、靶介导的反应的激活、酶活性，以及相关另外的性质。取决于预期性质的类型，普通技术人员能够识别进行筛选和 / 或选择具有预期性质的结合结构域的形式和必要的步骤。优选地，所述预定性质为结合至靶。

[0241] 下文对重复序列蛋白的定义是基于申请号为 WO 2002/020565 的专利申请中的定义。申请号为 WO 2002/020565 的专利申请进一步涵盖了重复序列蛋白特征、技术和应用的概述。

[0242] 术语“重复序列蛋白”是指包含一种或多种重复序列结构域的蛋白质。优选地，每个所述重复序列蛋白包含高达四个重复序列结构域。更优选地，每个所述重复序列蛋白包含高达两个重复序列结构域。最优选地，每个重复序列蛋白包括仅一个重复序列结构域。此外，所述重复序列蛋白可以包含另外的非重复序列的蛋白结构域、多肽标签和 / 或多肽接头。

[0243] 术语“重复序列结构域”是指包含作为结构单元的两个或更多个连续的重复序列单元（模块）的蛋白结构域，其中，所述结构单元具有相同的折叠部分并紧密堆叠从而产生具有共同的疏水核心的超螺旋结构。优选地，重复序列结构域还包含 N 端和 / 或 C 端加帽单元（或模块）。甚至更优选地，所述 N 端和 / 或 C 端加帽单元（或模块）为加帽的重复序列。

[0244] 术语“设计的重复序列蛋白”和“设计的重复序列结构域”分别指的是 WO2002/020565 的专利申请中说明的发明方法的结果获得的重复序列蛋白或重复序列结构域。设计的重复序列蛋白和设计的重复序列结构域是合成的，而不是来自大天然的。其分别为人造蛋白或结构域，通过表达相应的设计的核酸而获得。优选地，该表达在真核或原核细胞中，如细菌细胞，或利用体外无细胞表达系统进行。因此，设计的锚蛋白重复序列蛋白（即，DARPin）对应包含至少一个锚蛋白重复序列结构域的本发明的重组结合蛋白。

[0245] 术语“结构单元”是指多肽的局部有序的部分，其由二级结构沿着多肽链彼此邻近的两个或更多个片段之间的三维相互作用形成。这样的结构单元呈现结构基序。术语“结构基序”指的是存在于至少一个结构单元的二级结构元件的三维排列。结构基序是本领域技术人员周知的。单独的结构单元不能获得确定的三维排列，然而，其连续结构，例如，在重复序列结构域的重复序列模块，导致相邻单元的相互稳定化，从而产生超螺旋结构。

[0246] 术语“重复序列单元”是指包含一个或多个天然存在的重复序列蛋白的重复序列基序的氨基酸序列，其中所述“重复序列单元”有多个拷贝，并且对于确定蛋白质折叠的所有所述基序，所述“重复序列单元”表现出共同的、确定的折叠拓扑结构。这样的重复序列单元对应重复序列蛋白的“重复序列结构单元（重复序列）”（如 Forrer 等人，2003，同上，一文所述）或重复序列蛋白的“连续的同源结构单元（重复序列）”（如 Binz 等人，2004，同上，一文所述）。这样的重复序列单元包含框架残基和相互作用的残基。这种重复序列单元的例子为犰狳重复序列单元、富含亮氨酸的重复序列单元，锚蛋白重复序列单位，三角形四肽 (tetratricopeptide) 重复序列单元，HEAT 重复序列单元，和富含亮氨酸的变体的重

复序列单元。含有两个或更多个这种重复序列单元的天然存在的蛋白质被称为“天然存在的重复序列蛋白”。当相互比较时，重复序列蛋白的单独的重复序列单元的氨基酸序列可具有显著数目的突变、取代、添加和 / 或缺失，同时仍基本上保持重复序列单元的一般模式，或基序。

[0247] 因此，术语“锚蛋白重复序列单位”指的是重复序列单元，其为如 Forrer 等人，2003，在上述引文中所述的锚蛋白重复序列。锚蛋白重复序列是本领域技术人员周知的。术语“锚蛋白重复序列结构域”指的是包含两个或更多个连续的作为结构单元的锚蛋白重复序列单位（模块）的重复序列结构域，并且，优选地，包含 N 端和 / 或 C 端加帽单元（或模块）的重复序列结构域。

[0248] 术语“框架残基”涉及重复序列单元的氨基酸残基，或者重复序列模块的相应的氨基酸残基，其对折叠的拓扑结构有贡献，即其对所述重复序列单元（或模块）的折叠部分有贡献或其对相邻单元（或模块）的相互作用有贡献。这样的贡献可能为与在重复序列单元（或模块）的其它残基的相互作用，或如在 α 螺旋或 β 折叠，或氨基酸段形成线性多肽或环中发现的对多肽骨架的构象的影响。

[0249] 术语“靶向相互作用残基”指的是重复序列单元的氨基酸残基或重复序列模块的相应的氨基酸残基，其对与靶物质的相互作用有贡献。这种贡献可能是与靶物质的直接的相互作用，或在其它直接相互作用的残基上的影响，例如，通过稳定重复序列单元（或模块）的多肽的构象以使直接相互作用的残基与所述靶能够或增强相互作用。这样的框架和靶相互作用残基可以通过分析用物理化学方法，比如 X 射线晶体学、NMR 和 / 或 CD 光谱获得的结构数据，或者通过与结构生物学和 / 或生物信息学的从事者周知的已知的和相关的结构信息的比较来识别。

[0250] 优选地，用于推断重复序列基序的重复序列单元是同源的重复序列单元，其中该重复序列单元包含相同的结构基序，并且其中重复序列单元 70％以上的框架残基是彼此同源的。优选地，重复序列单元 80％以上的框架残基是彼此同源的。最优选地，重复序列单元 90％以上的框架残基是彼此同源的。本领域技术人员知晓确定多肽之间的同源百分比的计算机程序，比如 FASTA、BLAST 或间 Gap。进一步优选地，用于推断重复序列基序的重复序列单元是从确定的靶上选择的重复序列结构域获得的同源重复序列单元。

[0251] 术语“重复序列基序”是指由一个或多个重复序列单元或重复序列模块推导出的氨基酸序列。优选地，所述重复序列单元或重复序列模块来自对相同靶具有结合特异性的重复序列结构域。这样的重复序列基序包括框架残基位置和靶相互作用残基位置。所述框架残基位置对应重复序列单元（或模块）的框架残基的位置。同样地，所述靶相互作用残基位置对应重复序列单元（或模块）的靶相互作用残基的位置。重复序列基序包含固定位置和随机位置。术语“固定位置”指的是氨基酸在重复序列基序的位置，其中所述位置被设定为特定的氨基酸。多数情况下，这样的固定位置与对特定靶有特异性的框架残基的位置和 / 或靶相互作用残基的位置是对应的。术语“随机位置”指的是氨基酸在重复序列基序的位置，其中，在所述氨基酸位置可以允许两个或多个氨基酸，例如，其中允许二十种天然存在的常见氨基酸中的任何一种，或者其中允许二十种天然存在的氨基酸中大多数，比如，除半胱氨酸以外的氨基酸，或除甘氨酸、半胱氨酸和脯氨酸之外的氨基酸。多数情况下，这样的随机位置对应靶相互作用残基的位置。然而，框架残基的一些位置也可以是随机化的。

[0252] 术语“折叠拓扑结构”是指所述重复序列单元或重复序列模块的三级结构。折叠拓扑结构由形成至少部分 α 螺旋或 β 折叠的一段氨基酸，或形成线性多肽或环，或 α 螺旋、β 折叠和 / 或线性多肽 / 环的任意组合的氨基酸段确定。例如，锚蛋白重复序列单元 / 模块由 β 转角，接着两个反平行的 α 螺旋和到达下一个重复序列单元 / 模块的转角的环组成。

[0253] 术语“连续的”指的是排列，其中重复序列单元或重复序列模块串联排列。在设计的重复序列蛋白中，有至少 2 个，通常约 2 至 6 个，特别是至少约 6，经常 20 个或更多个重复序列单元（或模块）。在多数情况下，重复序列结构域的重复序列单元（或模块）将呈现高度的序列一致性（在对应位置的氨基酸残基相同），或序列相似性（氨基酸残基不同，但具有相似的物理化学性质），并且一些氨基酸残基可能是高度保守的关键残基。然而，只要保持重复序列单元（或模块）的共同的折叠拓扑结构，通过在不同的重复序列单元（或模块）之间插入和 / 或缺失和 / 或取代氨基酸，高度的序列变异性也是可能的。

[0254] 本领域技术人员周知通过物理化学方法如 X 射线晶体学，核磁共振或 CD 光谱直接确定重复序列蛋白的折叠拓扑结构的方法。识别并确定重复序列单元或重复序列基序或用于识别包含这样的重复序列单元或基序的相关蛋白质的家族的方法，例如，同源性检索 (BLAST 等)，已生物信息学领域建立完善，并且被本领域技术人员周知。改进最初的重复序列基序的步骤可包括迭代过程。

[0255] 术语“重复序列模块”指的是设计的重复序列结构域的重复序列的氨基酸序列，其最初来源于天然存在的重复序列蛋白的重复序列单元。包含在重复序列结构域的每个重复序列模块来源于天然存在的重复序列蛋白的家族或亚家族（例如，犰狳重复序列蛋白或锚蛋白重复序列蛋白的家族）的一个或多个重复序列单元。进一步优选地，包含在重复序列结构域的每个重复序列模块包括重复序列基序，该重复序列基序由从在靶上选择的重复序列结构域获得的同源重复序列单元（例如，如实施例 1 所述）推导出并具有相同靶特异性的。

[0256] 因此，术语“锚蛋白重复序列模块”指的重复序列模块，其最初来源于天然存在的锚蛋白重复序列蛋白的重复序列单元。本领域技术人员周知锚蛋白重复序列蛋白。

[0257] “重复序列模块”可以包括氨基酸残基存在于相应的重复序列模块的所有拷贝中的位置（“固定位置”）和不同的或“随机”的氨基酸残基的位置（“随机位置”）。

[0258] 术语“加帽模块”指的是融合至重复序列结构域的 N 端或 C 端重复序列模块的多肽，其中所述加帽模块形成紧密的三级相互作用（即，三级结构的相互作用），并且因此所述重复序列模块在不与连续的重复序列模块接触的一侧提供使所述重复序列模块的疏水核心与溶剂隔离的帽。所述 N 端和 / 或 C 端加帽模块可以为，或可以来源于，在天然存在的重复序列蛋白中发现的邻近重复序列单元的加帽单元或其它结构单元。术语“加帽单元”指的是天然存在的折叠的多肽，其中所述多肽限定特定的结构单元，该特定的结构单元的 N 或 C 融合至重复序列单元，其中所述多肽形成紧密的三级结构相互作用，并且因此所述重复序列单元提供使在一侧的所述重复序列单元的疏水核心与溶剂隔离的帽。优选地，加帽模块或加帽单元为加帽重复序列。术语“加帽重复序列”中的是与所述相邻重复序列单元（模块）具有相似或相同的折叠和 / 或与相邻重复序列单元（或模块）具有序列相似性的重复序列单元（或模块）。WO 2002/020565 和 Interlandi 等人，2008（同上）描述了加帽

模块和加帽重复序列。

[0259] N端锚蛋白加帽模块（即,N端加帽重复序列）的例子为SEQ ID NO:1、2、3、13、14、20、26、27、36、40、44、45、50、54、124、128 和 132,并且 C 端加帽重复序列模块（即,C 端加帽重复序列）的例子为SEQ ID NO:4、5、19、24、25、33、34、35、39、43、48、49、53、57、127、131 和 135。

[0260] 例如,SEQ ID NO :13 的N 端锚蛋白加帽模块由位置 1 至 32 的氨基酸编码并且 SEQ ID NO :19 的 C 端加帽模块由位置 99 至 126 的氨基酸编码。

[0261] 根据本发明的重组结合蛋白包含至少一个锚蛋白重复序列结构域,其中所述锚蛋白重复序列结构域对哺乳动物的 HER2 胞外区具有结合特异性。

[0262] 术语“对靶具有结合特异性”、“特异性结合至靶”或“靶特异性”等等意为结合蛋白或结合结构域在 PBS 以比结合至不相关蛋白,比如大肠杆菌麦芽糖结合蛋白 (MBP) 低的解离常数结合至靶。优选地,对于靶,PBS 中的解离常数比对于 MBP 相应的解离常数低至少 10 倍,更优选至少 10^2倍,甚至更优选至少 10^3倍,或最优选至少 10^4倍。

[0263] 术语“共有序列”是指氨基酸序列,其中通过多个重复序列单元的结构和 / 或序列比对获得所述共有序列。使用两个或多个结构和 / 或序列比对的重复序列单元,并在比对中允许间隙,有可能确定各位置上最频繁的氨基酸残基。共有序列是包含最频繁表现在各位置的氨基酸的序列。两个或更多个氨基酸在单个位置以高于平均值表现的情况下,共有序列可包括的那些氨基酸的分组。所述两个或更多个重复序列单元可以取自包括在单一的重复序列蛋白的重复序列单位,或取自两个或更多个不同的重复序列蛋白。

[0264] 本领域技术人员周知共有序列和确定共有序列的方法。

[0265] “共有氨基酸残基”为在共有序列中的某位置发现的氨基酸。如果在所述两个或更多个重复序列单元中以相似概率发现 2 个或更多个,例如,3 个、4 个或 5 个氨基酸残基,那么共有氨基酸残基可以为最频繁发现的氨基酸之一,或所述 2 个或更多个氨基酸残基的结合。

[0266] 另外优选非天然存在的加帽模块、重复序列模块、结合蛋白或结合结构域。

[0267] 术语“非天然存在”是指合成的或不是来自自然界的,更具体地,该术语是指人造的。术语“非天然存在的结合蛋白”或“非天然存在的结合结构域”是指所述结合蛋白或所述结合结构域是合成的（即由氨基酸通过化学合成产生的）或重组的,而不是来自自然界。“非天然存在的结合蛋白”或“非天然存在的结合结构域”分别为人造蛋白质或结构域,通过表达相应设计的核酸而获得。优选地,该表达在真核或细菌细胞中,或者利用体外无细胞表达系统进行。此外,该术语意为所述结合蛋白或所述结合结构域的序列在序列数据库（比如,GenBank,EMBL-Bank 或 SWISS-PROT）中不是作为非人工序列而存在。这些数据库和其它类似的序列数据库是本领域技术人员周知的。

[0268] 根据本发明的锚蛋白重复序列结构域的一般修饰和衍生物,特别地根据本发明的锚蛋白重复序列模块和加帽模块 :

[0269] 进一步优选分别包含 N 端或 C 端锚蛋白加帽重复序列的 N 端或 C 端锚蛋白加帽模块,其中在所述加帽重复序列的一个或多个氨基酸残基由相应的锚蛋白加帽单元或锚蛋白重复序列单元的比对上的相应位置发现的氨基酸残基取代。

[0270] 氨基酸可以被 20 种天然存在的最常见氨基酸中的任何一种取代,优选被选自 A、

D、E、F、H、I、K、L、M、N、Q、R、S、T、V、W 和 Y，更优选被选自 A、D、E、H、I、K、L、Q、R、S、T、V，和 Y 的氨基酸取代。而且优选地，氨基酸被同源氨基酸取代，即氨基酸被侧链具有相似生物物理学性质的氨基酸取代。例如，带负电的氨基酸 D 可以被带负电的氨基酸 E 取代，或疏水氨基酸比如，L 可以被 A、I 或 V 取代。氨基酸被同源氨基酸取代是本领域技术人员周知的。

[0271] 而且优选 C 端锚蛋白加帽模块，其包含基于 SEQ ID NO:4、5、19、24、25、33、34、35、39、43、48、49、53、57、127、131 或 135 的上述 C 端加帽模块中的任何一个的位置 27 和 28 的氨基酸 A。

[0272] 而且优选 C 端锚蛋白加帽模块，其包含基于 SEQ ID NO:4、5、19、24、25、33、34、35、39、43、48、49、53、57、127、131 或 135 的上述 C 端加帽模块中的任何一个的位置 1 至 26 或位置 1 至 27 和 28 的氨基酸。

[0273] 在不对性质有明显的影响的情况下可以从 N 端锚蛋白加帽模块移除在 SEQ ID NO:1,2,3,13,14,20,26,27,36,40,44,45,50,54,124,128 或 132 的位置 1 的 G 或位置 2 的 S。这 2 个氨基酸作为接头连接锚蛋白重复序列结构域至另外的氨基酸和蛋白质。本发明也包含这样的锚蛋白重复序列结构域：其包含 N 端锚蛋白加帽模块，其中移除了在位置 1 的 G 和在位置 2 的 S。应该理解，在此定义的锚蛋白重复序列结构域中的氨基酸位置（例如，“位置 33”）被相应地调整，导致数字偏移，例如，如果缺失一个氨基酸，“位置 33”会变成“位置 32”，或者，如果缺失两个氨基酸，“位置 33”会变成“位置 31”。

[0274] 本发明的锚蛋白重复序列结构域的锚蛋白加帽模块可以通过本领域技术人员已知的组合技术，比如氨基酸序列比对、诱变和基因合成被锚蛋白加帽模块替换。例如，通过如下步骤，SEQ ID NO:79 的 C 端加帽重复序列被 SEQ ID NO:5 的 C 端加帽重复序列取代：(i) 通过与 SEQ ID NO:5 进行序列比对确定 SEQ ID NO:79 的 C 端加帽重复序列（即，序列位置 99 至 126)，(ii) 用 SEQ ID NO:5 的序列取代确定的 SEQ ID NO:79 的 C 端加帽重复序列的序列，(iii) 产生编码重复序列结构域的基因，所述重复序列结构域编码替换后的 C 端加帽模块，(iv) 在大肠杆菌的细胞质中表达修饰的重复序列结构域并且 (v) 通过标准手段纯化修饰的重复序列结构域。作为另外的实施例，SEQ ID NO:79 的 N 端加帽重复序列可以通过如下步骤被 SEQ ID NO:3 的 N 端加帽重复序列取代：通过与 SEQ ID NO:3 进行序列比对确定 SEQ ID NO:79 的 N 端加帽重复序列（即，序列位置 1 至 32)(ii) 用 SEQ ID NO:3 取代确定的 SEQ ID NO:79 的 N 加帽重复序列的序列，(iii) 产生编码重复序列结构域的基因，所述重复序列结构域编码替换后的 N 端加帽模块，(iv) 在大肠杆菌的细胞质中表达修饰的重复序列结构域并且 (v) 通过标准手段纯化修饰的重复序列结构域。

[0275] 此外，可以通过基因技术合成，组装 N 端锚蛋白加帽模块（例如，SEQ ID NO:3 的 N 端加帽重复序列)，接着一个或更多个重复序列模块（例如，2 个包含来自 SEQ ID NO:79 的位置 33 至 99 的氨基酸残基的锚蛋白重复序列模块）和 C 端加帽模块（例如，SEQ ID NO:5 的 C 端加帽重复序列)，从基因方面构建本发明的锚蛋白重复序列结构域。接着可以在大肠杆菌中表达通过基因技术组装成的重复序列结构域基因。

[0276] 此外优选氨基酸序列缺失氨基酸 C、M 或 N 的重组结合蛋白、重复序列结构域、重复序列模块、N 端加帽模块或 C 端加帽模块。

[0277] 此外优选氨基酸序列缺失氨基酸 N(紧接 G) 的重组结合蛋白、重复序列结构域、重复序列模块、N 端加帽模块或 C 端加帽模块。

[0278] 此外优选包含任何这样的 N 端或 C 端加帽模块的重组结合蛋白或重复序列结构域。

[0279] 在包含根据本发明的锚蛋白重复序列结构域的重组结合蛋白的另外的优选实施例中，所述重复序列结构域的 N 端加帽模块的一个或更多个氨基酸残基被在 N 端加帽单元的比对上的相应位置处发现的氨基酸残基替换。优选地，高达 30％的氨基酸残基被替换，更优选地，高达 20％，并且甚至更优选地，高达 10％的氨基酸残基被替换。最优选地，这样的 N 端加帽单元为天然存在的 N 端加帽单元。

[0280] 在包含根据本发明的锚蛋白重复序列结构域的重组结合蛋白的另外的优选实施例中，所述重复序列结构域的 C 端加帽模块的一个或更多个氨基酸残基被在 C 端加帽单元的比对上的相应位置处发现的氨基酸残基替换。优选地，高达 30％的氨基酸残基被替换，更优选地，高达 20％，并且甚至更优选地，高达 10％的氨基酸残基被替换。最优选地，这样的 C 端加帽单元为天然存在的 C 端加帽单元。

[0281] 在又一特定实施例中，高达 30％的氨基酸残基，更优选地，高达 20％，并且甚至更优选地，高达 10％的氨基酸残基被在重复序列单元、N 端加帽单元或 C 端加帽单元的相应位置中没有发现的氨基酸替换。

[0282] 在包含根据本发明的锚蛋白重复序列结构域的重组结合蛋白的另外的优选实施例中，所述锚蛋白重复序列结构域的重复序列模块的一个或更多个氨基酸残基被在重复序列单元的比对上的相应位置处发现的氨基酸残基替换。优选地，高达 30％的氨基酸残基被替换，更优选地，高达 20％，并且甚至更优选地，高达 10％的氨基酸残基被替换。最优选地，这样的重复序列单元为天然存在的重复序列单元。

[0283] 在又一特定实施例中，高达 30％的氨基酸残基，更优选地，高达 20％，并且甚至更优选地，高达 10％的氨基酸残基被在重复序列单元的相应位置中没有发现的氨基酸替换。

[0284] 在另外的实施例中，在此所述的任何结合 HER2 的重组蛋白或结构域可以共价绑定至一个或多个另外的部分，包括，例如，结合至不同靶以产生双特异性结合试剂的部分、生物活性化合物、标记部分（例如，荧光标记，如荧光素，或放射性示踪剂）、便于蛋白纯化的部分（例如，小肽标记，比如 His 或 Strep 标签），为提高治疗效果提供效应子功能的部分（例如，抗体的 Fc 部分提供抗体依赖性细胞介导的细胞毒作用、有毒的蛋白部分比如铜绿假单胞菌外毒素 A(ETA) 或小分子毒剂比如美登木素生物碱或 DNA 烷化剂）或提供改善的药物动力学的部分。可以根据感知的治疗需要评估改善的药物动力学。通常，理想的是（有可能通过增加服药后蛋白质保持在血清中的时间）增加生物利用度和 / 或增加剂量之间的时间。在某些情况下，理想的是改善蛋白质的血清浓度随时间的持续性（例如，降低蛋白质血清在服用后不久的浓度和在下一次服用前不久的浓度之间的浓度差异）。倾向于使从血液中清除蛋白质变慢的部分包括羟乙基淀粉 (HES)、聚乙二醇 (PEG)、糖（例如，唾液酸），耐受性良好的蛋白质部分（例如，Fc 片段或血清白蛋白），和对丰富的血清蛋白具有特异性和亲和力的结合结构域或肽，例如，抗体的 Fc 片段或血清白蛋白。WO 2012/069654 中提供了这些对血清白蛋白具有亲和力的结合结构域或重复序列结构域的例子。本发明的重组结合蛋白可以连接至使哺乳动物（例如，大鼠、小鼠，或人）中多肽的清除率相对未修饰的多肽降低大于 3 倍的部分。

[0285] 在一个特定的实施例中，本发明涉及包含结合至 HER2 的第一重复序列结构域、结

合至 HER2 的第二重复序列结构域，并且进一步包含特异性结合至人血清白蛋白的一个或更多个锚蛋白重复序列结构域的重组结合蛋白。在此给出了对 HER2 具有特异性的重复序列结构域的例子并且 WO 2012/069654 中描述了对人血清白蛋白具有特异性的锚蛋白重复序列结构域的例子。这些结构域可以通过本领域技术人员已知的方法、通过遗传手段由多肽接头连接。

[0286] 另一优选的实施例时重组结合蛋白，其中第一重复序列结构域和第二重复序列结构域为锚蛋白重复序列结构域，该锚蛋白重复序列结构域对 HER2 具有结合特异性，包含 1 个、2 个、3 个或更多个参与结合至 HER2 的内部重复序列模块。优选地，这些锚蛋白重复序列结构域包含 N 端加帽模块，一至四个内部重复序列模块，和 C 端加帽模块。优选地，所述加帽模块为加帽重复序列。而且优选地，所述加帽模块将参与结合至 HER2。

[0287] 此外，为了治疗病症考虑上述的任何药物组合物。

[0288] 本发明进一步提供治疗方法。该方法包括使有需要的患者服用治疗有效量的本发明的重组结合蛋白。

[0289] 此外，考虑治疗包括人的哺乳动物的病理状态的方法，包括使有需要的患者服用有效量的上述药物组合物。

[0290] 实施例

[0291] 下文公开的所有的起始原料和试剂是本领域技术人员已知并且是市售的或者可以利用公知的技术来制备的。

[0292] 材料

[0293] 化学制品购自 Fluka(瑞士)。寡核苷酸来自 Microsynth(瑞士)。除非另有说明，DNA 聚合酶，限制性内切酶和缓冲液来自 New England Biolabs(USA) 或 Fermentas(立陶宛)。克隆和蛋白质生产菌株是 E.coli XL1-blue(Stratagene,USA) 或 BL21(Novagen,USA)。重组人 HER2 胞外域(通过标准手段在 CHO 细胞中产生的 ErbB2S22-N530-Flag 和 ErbB2S22-E645-Flag) 购自 CSIRO Enquiries(澳大利亚)。生物素化的 HER2 胞外域使用标准生物素化试剂和方法通过将生物素部分连接至蛋白质的伯胺以化学方法获得 (Pierce,USA)。细胞系购自 LGC/ATCC(法国 /USA；商品目录号：BT474-HTB-20、SKBR-3 - HTB-30、NCI-N87 - CRL5822、ZR75-30 - CRL1504、HCC1419-CRL2326、MDA-MB175VII - HTB-25)。细胞培养基来自 Invitrogen/Lubio(瑞士)。胎牛血清来自 PAA。检测细胞增殖、细胞增殖 ELISA 的分析试剂，BrdU(色度)(商品目录号：G8091) 来自 Roche，瑞士并且检测凋亡的分析试剂，Caspase Glo 3/7(商品目录号：1164722900) 来自 Promega 和瑞士并且 Cell Death Detection ELISAPLUS 系统 (11774425001) 来自 Roche，瑞士。细胞转染试剂，Lipofectamin2000(11668027) 是来自 Invitrogen 瑞士。使用来自 Becton-Dickinson(瑞士) 的 FACS Canto II 系统进行 FACS 分析。利用 anti-Penta-His Alexa Fluor 647 轭合物(商品目录号：A21445；Lubio，瑞士) 检测 DARPins 与 Her2 的结合。Accutase(商品目录号：L-11-007) 来自 PAA。曲妥珠单抗购自 Kantonal Apotheke 苏黎世，并且帕妥珠单抗由 Evitra(瑞士) 合成。GFP- 标记的 Her2 的表达载体(商品目录号：RG212583) 来自 Origene USA。

[0294] 分子生物学

[0295] 除非另有说明，方法是按照 (Sambrook J.,Fritsch E.F. 和 Maniatis

T.,Molecular Cloning:A Laboratory Manual,Cold Spring Harbor Laboratory 1989,New York) 所述方案进行的。

[0296] Proliferation analysis

[0297] 增殖分析

[0298] 利用 BrdU 标记 (BrdU,细胞增殖 ELISA,Roche) 通过测量 DNA 合成确定 DARPin 对细胞增殖的影响。简要地说,在 96 孔板的每孔中将 10000BT474 细胞接种在 100ul 完全培养基中并培养 24 小时。加入 DARPin 和基准,再培养 72 小时。加入用于细胞标记的 BrdU,最后培养 24 小时。根据制造方案检测标记的 (增殖的) 细胞。用 GraphPad Prism 软件分析数据,以 x 轴上的 log[c] 对 y 轴上的 OD450-602nm 进行绘图。使用非线性回归拟合 (log(拮抗剂)vs. 反应—可变斜率 (4 个参数)) 对数据进行拟合。

[0299] 凋亡分析

[0300] 使用 Caspase 3/7-Glo 系统 (Promega,瑞士) 测量 Caspase3/7 的激活确定 DARPin 对凋亡的诱导。简要地说,在 96 孔板的每孔中将 10000BT474 细胞接种在 100ul 完全培养基中并培养 24 小时。加入 DARPin 和基准,保持另外的 24 小时。根据制造方案加入 Caspase Glo 试剂,保持 1h。通过测量荧光素酶活性监测 Caspase 3/7 活性。

[0301] 可代替地,使用 Cell Death Detection ELISAPLUS 系统 (Roche,瑞士) 确定对凋亡的诱导。根据制造方案进行该试验。细胞数目和培养时间与 Caspase Glo 读数相似。

[0302] 使用 GraphPad prism 软件分析数据,以 x 轴上的浓度对 y 轴上的 OD405/490nm 或 RLU 进行绘图。使用非线性回归拟合 (log(激动剂)vs. 反应—可变斜率 (4 个参数)) 对数据进行拟合。

[0303] 设计的锚蛋白重复序列蛋白文库

[0304] (WO 2002/020565 ;Binz 等人 . 2003, 同上 ;Binz 等人 . 2004, 同上) 描述了产生设计的锚蛋白重复序列蛋白文库的方法。通过这种方法,可以构建具有随机锚蛋白重复序列模块和 / 或随机加帽模块的锚蛋白重复序列蛋白文库。例如,可以基于固定的 N 端加帽模块 (例如, SEQ ID NO:2 的 N 端加帽模块) 或根据 SEQ ID NO:60 的序列基序的随机的 N 端加帽模块,根据 SEQ ID NO:58 或 59 的序列基序的一个或更多个随机的重复序列模块,和固定的 C 端加帽模块 (例如,SEQ ID NO:5 的 C 端加帽模块) 或根据 SEQ ID NO:61 的序列基序的随机的 C 端加帽模块组装这些文库。优选地,这些文库组装成在重复序列或加帽模块的随机位置不具有氨基酸 C、G、M、N(在 G 残基前面) 或 P。此外,根据 SEQ ID NO:58 或 59 的序列基序的随机重复序列模块在位置 10 和 / 或位置 17 可进一步随机化 ;根据 SEQ ID NO:60 的序列基序的随机的 N 端加帽模块在位置 7 和 / 或位置 9 可进一步随机化 ;并且根据 SEQ ID NO:61 的序列基序的随机的 C 端加帽模块在位置 10、11 和 / 或 17 可进一步随机化。

[0305] 此外,在这些文库中的这些随机模块可以包含在随机的氨基酸位置的另外的多肽环插入物。这些多肽环插入物为抗体的互补决定区 (CDR) 环文库或再次产生的肽文库。例如,可以利用人核糖核酸酶 L 的 N 端锚蛋白重复序列结构域的结构 (Tanaka,N.,Nakanishi,M,Kusakabe,Y,Goto,Y.,Kitade,Y,Nakamura,K.T.,EMBO J.23(30),3929-3938,2004) 作为引导设计这种环插入物。类似 10 个氨基酸插入到存在于 2 个锚蛋白重复序列的边界附近的 β 转角的锚蛋白重复序列结构域,锚蛋白重复序列蛋白文库可以含有插入锚蛋白重复序列

结构域的一个或更多个 β 转角的、长度可变（例如，1 至 20 个氨基酸）的随机环（具有固定的和随机的位置）。

[0306] 任何这样的锚蛋白重复序列蛋白文库的 N 端加帽模块优选具有 RELLKA 或 RILKAA 基序而非 RILLAA 基序（例如，存在于 SEQ ID NO:65 的位置 21 至 26）并且任何这样的锚蛋白重复序列蛋白文库的 C 端加帽模块优选具有 KAA 或 KLA 基序而非 KLN 基序（例如，SEQ ID NO:65 的最后 3 个氨基酸）。

[0307] 这种锚蛋白重复序列蛋白文库的设计可以通过与靶相互作用的锚蛋白重复序列结构域的已知结构来引导。这些结构（通过其 Protein Data Bank(PDB) 唯一登录号或识别代码 (PDB-IDs) 识别）的例子为 1WDY、3V31、3V30、3V2X、3V20、3UXG、3TWQ-3TWX、1N11、1S70 和 2ZGD。

[0308] (WO 2002/020565 ;Binz 等人 . 2003, 同上 ;Binz 等人 . 2004, 同上）描述了设计的锚蛋白重复序列蛋白文库的例子，比如 N2C 和 N3C 设计的锚蛋白重复序列蛋白文库。N2C 和 N3C 中的数字描述在 N 端和 C 端加帽模块之间存在的随机重复序列模块的数目。

[0309] 用于定义重复序列单元和模块内的位置的命名法基于 Binz 等人 . 2004, 同上，修饰锚蛋白重复序列模块的边界，并且锚蛋白重复序列单元通过一个氨基酸位置移位。例如，Binz 等人 2004（同上）的锚蛋白重复序列模块的位置 1 对应本发明的锚蛋白重复序列模块的位置 2，并且因此 Binz 等人 . 2004，同上，的锚蛋白重复序列模块的位置 33 对应本发明的锚蛋白重复序列模块的位置 1。

[0310] 通过测序确定所有的 DNA 序列，并且通过质谱法确定所有所述蛋白的计算的分子量

[0311] 实施例 1 :筛选包含对 HER2 具有结合特异性的锚蛋白重复序列结构域的结合蛋白

[0312] 如 Binz 等人 2004（同上）所述，利用核糖体展示技术 (Hanes, J. 和 Plückthun, A., PNAS 94, 4937-42, 1997) 从 DARPin 文库筛选许多对 HER2 胞外域具有结合特异性的设计的锚蛋白重复序列蛋白 (DARPin)。通过指示筛选成百上千的 HER2 特异性结合蛋白的粗提物 ELISA（参见下文）评价结合特异性。通过测试双特异性 DARPin 抑制 BT474 细胞增殖的能力测量选定的克隆对增殖的 HER2 特异性抑制和对凋亡的诱导。

[0313] 例如，SEQ ID NO:62 至 82、112 至 121 的锚蛋白重复序列结构域构成包含对 HER2 具有结合特异性的锚蛋白重复序列结构域的选定的结合蛋白的氨基酸序列。SEQ ID NO:15 至 18、21 至 23、28 至 32、37、38、41、42、46、47、51、52、55、56、125、126、129、130、133 和 134 提供来自这些对 HER2 具有结合特异性的锚蛋白重复序列结构域的单独的锚蛋白重复序列模块。SEQ ID NO:13、14、19、20、24 至 27、33 至 36、39、40、43 至 45、48 至 50、53、54、57、124、127、128、131、132 和 135 提供对 HER2 具有结合特异性的这些锚蛋白重复序列结构域的单独的加帽模块。

[0314] 通过核糖体展示技术筛选 HER2 特异性锚蛋白重复序列蛋白

[0315] 利用人 HER2 作为靶蛋白，上述的设计的锚蛋白重复序列蛋白文库和已定方案 (Zahnd, C., Amstutz, P. 和 Plückthun, A., Nat. Methods 4, 69-79, 2007) 通过核糖体展示技术 (Hanes, J. 和 Plückthun, 同上）进行 HER2 特异性锚蛋白重复序列蛋白的筛选。每轮筛选之后，逆转录 (RT)-PCR 循环数从 45 不断下降至 30，因结合物的富集调整产量。前四轮筛选采用标准的核糖体展示筛选，利用下降的靶浓度和增加的洗涤严格度以从第一轮至第四

轮增加筛选压力。为了富集高亲和力的抗 HER2 DARPin，将来自标准核糖体展示筛选（上文）的第四轮的产物进行解离速率筛选回合并且增加筛选严格度 (Zahnd, 2007, 同上)。进行最后的标准筛选回合以扩增并恢复解离速率筛选的结合蛋白。

[0316] 如粗提物 ELISA 所示筛选特异性结合至 HER2 的克隆

[0317] 根据标准方案利用 DARPin 表达细胞的大肠杆菌粗提物通过酶联免疫吸附测定 (ELISA) 识别特异性结合 HER2 胞外域的单独筛选出的 DARPin。将由核糖体展示技术筛选出的 DARPin 克隆到 pQE30(Qiagen) 表达载体中，转化到 E.coli XL1-Blue(Stratagene)，接着在含有 1ml 生长培养基（含有 1％葡萄糖和 100μg/ml 氨苄青霉素的 2YT) 的 96 深孔板中（每个克隆单个孔）37℃过夜生长。将 100μl 过夜培养物与含有 50μg/ml 氨苄青霉素的 1ml 新鲜 2YT 接种到新的 96 深孔板中培养。在 37℃中培养 2h 之后，用 IPTG（终浓度 1mM) 诱导表达并且持续 3h。收集细胞，重新悬浮在 100μl B-PERII(Pierce) 中并且在室温下培养并摇晃 15min。接着，加入 900μl PBS-TC（补充 0.25％酪蛋白水解物，0.1％ Tween 20®, pH 7.4 的 PBS) 并且通过离心移除细胞碎片。将 100μl 每个裂解克隆应用于含有 HER2 或通过其生物素部分固定的不相关 MBP 的 Neutravidin coated MaxiSorp 板的孔中并在 RT 下培养 1h。用 PBS-T（补充 0.1％ Tween 20®, pH 7.4 的 PBS) 充分洗涤之后，利用辣根标记的抗 RGS(His)$_4$单克隆抗体 (34650, Qiagen) 以标准 ELISA 程序展开该板，接着用 POD 底物 (Roche) 检测结合。在 405nm 测量显色。通过这种细胞粗提物 ELISA 筛选几百个克隆揭示超过百种不同的 DARPins 对 HER2 具有特异性。选择这些结合蛋白用于进一步分析。SEQ ID NO:62 至 82 和 112 至 121 提供了特异性结合至 HER2 胞外域的选定的锚蛋白重复序列结构域的氨基酸序列的例子。

[0318] 将这些对 HER2 具有结合特异性的锚蛋白重复序列结构域和对 HER2 没有结合特异性的负对照锚蛋白重复序列结构域（即，SEQ ID NO:111) 克隆到基于 pQE(QIAgen, Germany) 的表达载体（如下所述，提供 N 端 His 标签以便于简化蛋白质纯化）中。从而构建编码下面的 DARPin 的表达载体。

[0319] DARPin#1(His- 标签 (SEQ ID NO:6) 融合至其 N 端的 SEQ ID NO:62)；

[0320] DARPin#2(His- 标签 (SEQ ID NO:6) 融合至其 N 端的 SEQ ID NO:63)；

[0321] DARPin#3(His- 标签 (SEQ ID NO:6) 融合至其 N 端的 SEQ ID NO:64)；

[0322] DARPin#5(His- 标签 (SEQ ID NO:6) 融合至其 N 端的 SEQ ID NO:66)；

[0323] DARPin#6(His- 标签 (SEQ ID NO:6) 融合至其 N 端的 SEQ ID NO:67)；

[0324] DARPin#7(His- 标签 (SEQ ID NO:6) 融合至其 N 端的 SEQ ID NO:68)；

[0325] DARPin#8(His- 标签 (SEQ ID NO:6) 融合至其 N 端的 SEQ ID NO:69)；

[0326] DARPin#9(His- 标签 (SEQ ID NO:6) 融合至其 N 端的 SEQ ID NO:70)；

[0327] DARPin#10(His- 标签 (SEQ ID NO:6) 融合至其 N 端的 SEQ ID NO:71)；

[0328] DARPin#11(His- 标签 (SEQ ID NO:6) 融合至其 N 端的 SEQ ID NO:72)；

[0329] DARPin#12(His- 标签 (SEQ ID NO:6) 融合至其 N 端的 SEQ ID NO:73)；

[0330] DARPin#13(His- 标签 (SEQ ID NO:6) 融合至其 N 端的 SEQ ID NO:74)；

[0331] DARPin#14(His- 标签 (SEQ ID NO:6) 融合至其 N 端的 SEQ ID NO:75)；

[0332] DARPin#15(His- 标签 (SEQ ID NO:6) 融合至其 N 端的 SEQ ID NO:76)；

[0333] DARPin#16(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:77)；

[0334] DARPin#17(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:78)；

[0335] DARPin#18(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:79)；

[0336] DARPin#19(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:80)；

[0337] DARPin#20(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:81)；

[0338] DARPin#21(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:82)；

[0339] DARPin#50(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:111).

[0340] DARPin#51(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:112)；

[0341] DARPin#52(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:113)；

[0342] DARPin#53(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:114)；

[0343] DARPin#54(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:115)；

[0344] DARPin#55(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:116)；

[0345] DARPin#56(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:117)；

[0346] DARPin#57(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:118)；

[0347] DARPin#58(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:119)；

[0348] DARPin#59(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:120)；

[0349] DARPin#60(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:121)。

[0350] SEQ ID NO:83至110、122、123和136至141提供筛选出的双特异性锚蛋白重复序列蛋白的氨基酸序列的例子。将这些双特异性DARPin克隆到基于pQE(QIAgen,Germany)的表达载体（如下所述,提供N端His标签以便于简化蛋白质纯化）中。从而构建编码下面的DARPin的表达载体。

[0351] DARPin#22(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:83)；

[0352] DARPin#23(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:84)；

[0353] DARPin#24(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:85)；

[0354] DARPin#25(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:86)；

[0355] DARPin#26(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:87)；

[0356] DARPin#27(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:88)；

[0357] DARPin#28(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:89)；

[0358] DARPin#29(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:90)；

[0359] DARPin#30(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:91)；

[0360] DARPin#31(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:92)；

[0361] DARPin#32(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:93)；

[0362] DARPin#33(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:94)；

[0363] DARPin#34(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:95)；

[0364] DARPin#35(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:96)；

[0365] DARPin#36(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:97)；

[0366] DARPin#37(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:98)；

[0367] DARPin#38(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:99)；

[0368] DARPin#39(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:100)；

[0369] DARPin#40(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:101)；

[0370] DARPin#41(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:102)；

[0371] DARPin#42(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:103)；

[0372] DARPin#43(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:104)；

[0373] DARPin#44(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:105)；

[0374] DARPin#45(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:106)；

[0375] DARPin#46(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:107)；

[0376] DARPin#47(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:108)；

[0377] DARPin#48(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:109)；

[0378] DARPin#49(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:110)

[0379] DARPin#61(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:122)；

[0380] DARPin#62(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:123)；

[0381] DARPin#63(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:136)；

[0382] DARPin#64(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:137)；

[0383] DARPin#65(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:138)；

[0384] DARPin#66(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:139)；

[0385] DARPin#67(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:140)；

[0386] DARPin#68(His-标签(SEQ ID NO:6)融合至其N端的SEQ ID NO:141).

[0387] 单价DARPin的高水平和可溶表达

[0388] 为了进一步分析,在E.coli BL21或XL1-Blue细胞中表达DARPins#1至50并利用标准方案通过其His标签纯化。将25ml静止过夜培养物(LB,1％葡萄糖,100mg/l氨苄青霉素的;37℃)用于接种到1l培养物(相同的培养基)中。在600nm,吸光度为0.7,用0.5mM IPTG诱导培养物并在37℃培养4-5h。将培养物离心并且将得到的团块重新悬浮在40ml TBS500(50mM Tris-HCl,500mM NaCl,pH 8)中并进行超声处理。将裂解产物离心并且将甘油(终浓度10％(v/v))和咪唑(终浓度20mM)加入所得的上清液中。根据制造商的指示(QIAgen,德国)在镍-氨次氮基三乙酸柱(2.5ml柱容积)上纯化蛋白。可代替地,根据本领域技术人员已知的标准树脂和方案,通过阴离子交换色谱,接着通过体积排阻色谱纯化缺乏6xHis标签的DARPin或选定的重复序列结构域。从SDS-15％PAGE估计,1升大肠杆菌培养物可以纯化出高达200mg对HER2具有结合特异性的高度可溶的DARPin,并且纯度>95％。这样纯化的DARPin用于进一步表征。

[0389] 实施例2:通过表面等离子体共振分析表征对HER2具有结合特异性的DARPin

[0390] 通过表面等离子体共振(SPR)分析和ProteOn阵列系统(BioRad)检验纯化的、结合HER2的感兴趣的DARPin的蛋白结合动力学,其中ProteOn阵列系统这样设置:通过中和亲和素(neutravidin)固定生物素化的人HER2并且加入游离的单价DARPin测量相互作用。根据标准程序确定Kd值。

[0391] 通过结合至涂布的链霉亲和素将生物素化的人HER2分子的胞外域固定在流动池中,并且分析与各种选定的DARPin的相互作用。

[0392] 表面等离子体共振(SPR)分析

[0393] 利用ProteOn仪(BioRad)测量SPR并且根据本领域技术人员已知的标准程序进

行测量。运行缓冲液为PBS,pH 7.4,其含有0.005％Tween 20®。中和亲和素通过共价键固定在GLC芯片(BioRad)上,达到约8000个共振单位(RU)的水平。接着,将HER2固定在涂布中和亲和素的芯片上。然后,通过如下方式测量DARPin HER2的相互作用:注入100μl含连续稀释的DARPin(浓度为50、25、12.5、6.25和3.125nM)的运行缓冲液(含有0.005％Tween®的PBS)(基于速率测量),接着,使运行缓冲液以100μl/min的恒定流速流动10分钟至最多3小时(解离速率测量)。从注入HER2之后获得的RU踪迹(双参考)中去除未涂布的参考细胞和参考注入物(即,仅注入运行缓冲液)的信号(即,共振单元(RU)值)。从结合速率测量和解离速率测量获得的SRP踪迹可以确定相应的DARPin HER2相互作用的结合速率和解离速率。

[0394] 表1总结了结果。利用本领域技术人员已知的标准程序由预计的结合速率和解离速率计算解离常数(Kd)。

[0395] 表1:通过SPR确定的选定用于人HER2的DARPin的解离常数

[0396]

DARPin#	Kd [M]
1	7.81E-11
2	8.75E-10
3	1.31E-11
4	1.86E-10
5	7.08E-11
6	2.92E-11
7	1.03E-09
8	4.83E-10
9	4.17E-10
10	1.03E-09
11	2.56E-10
12	1.41E-09
13	n.d.
14	1.88E-09
15	4.68E-10
16	2.67E-09
17	2.30E-09
18	3.35E-10
19	9.44E-10
20	2.58E-10
21	1.65E-09
51	1.3E-09
52	1.37E-10
53	1.46E-09
54	9.27E-12
55	8.73E-11
56	2.00E-09
57	6.04E-11
58	4.13E-11
59	3.33E-11
60	1.17E-11

[0397] n.d.：未确定。

[0398] 实施例 3：绘制结合至特定的胞外 HER2 表位的重复序列结构域的图谱

[0399] 通过本领域技术人员已知的标准方法，分析重复序列结构域与胞外 HER2 结构域的相互作用，比如，通过 X 射线结晶技术或 NMR 光谱法分析复合物四级结构，或利用潜在的相互作用的残基的丙氨酸突变或利用质谱法和共价标记进行表位绘图。此外，进行本领域从事者已知的各种竞争试验，比如竞争酶联免疫吸附试验 (ELISA) 以识别选定的重复序列

蛋白结合的胞外结构域或者其是否具有与其它结合蛋白（例如，抗体，比如曲妥珠单抗或帕妥珠单抗）重叠的在 HER2 的胞外结构域上的表位。

[0400] HER2 的胞外结构域通过购买得到或如 (Jost 等人，同上）所述制备得到。

[0401] 通过表面等离子体共振 (SPR) 分析和 ProteOn 阵列系统 (BioRad) 进行结合 HER2 的、感兴趣的纯化的 DARPin 的竞争，ProteOn 阵列系统使用如下设置：生物素化人 ErbB2S22-N530 和 ErbB2S22-E645 通过中和亲和素固定，并且通过加入饱和状态 (1uM) 的第一单价 DARPin，接着加入第一和第二 DARPin（各 100uM) 的 1:1 混合物，测量竞争。如果尽管存在第一 DARPin，第二 DARPin 仍结合，则该第二 DARPin 被认为结合不同的表位。

[0402] 例如，竞争 ELISA（图 1A 和 1B) 数据表明 DARPin#54 结合 HER2 的结构域 II 并且 DARPin#51 结合 HER2 的结构域 I。先前已表明 DARPin#18 结合至 HER2 的结构域 IV(Jost 等人，同上）。所述 DARPins(20nM) 与 HER2 域 I，结构域 I-III 或域 III-IV（在每种情况下结构域浓度为 500nm) 在 PBS 中室温预培养 45 分钟。将混合物加入涂布在 F96MaxiSorb Nunc（商品目录号 442404) 板的 20nM 全长 HER2 中。使用小鼠抗 RGS-His 单克隆抗体 (Qiagen，商品目录号 34650) 作为第一抗体并且用辣根过氧化物酶标记的抗小鼠抗体 (Pierce，商品目录号 31438) 作为第二抗体特异性检测结合的 DARPins。在图 1B 描述的 ELISA 中，第一抗体（小鼠抗 RGS-His 单克隆抗体）被小鼠抗 DARPin 单克隆抗体代替。

[0403] 在 450nm 下进行读数。除了涂板（利用 PBS，pH 7.4 在 4℃过夜进行）之外，所有的培养步骤在含有 0.1％ Tween 20®和 0.25％酪蛋白，pH7.4 的 PBS 中、450rpm Heidolph Titramax 1000 摇床上室温下进行 2h。

[0404] 这些研究结果得到了确认：通过流式细胞术 (FACS) 确定，这些 DARPin 已竞争结合至具有 HER2 重组结构域 I、结构域 I-II-III 和结构域 III-IV 的 HER2 过表达细胞 (BT474)。DARPins(100nM) 与单独的 Her2 构建体 (1uM) 在 25℃预培养 30 分钟。将混合物施加在细胞 (100ul 中 100.000 个细胞）中，在冰上保持 20 分钟。利用 Alexa 647 标记的抗 5-His 抗体 (Qiagen 商品目录号：35370) 监测 DARPin 与细胞的结合。该试验确定 DARPin#51 结合至 HER2 的结构域 I，并且 DARPin#1 结合至 HER2 的 II，DARPin#18 结合至 HER2 的 IV。

[0405] 还利用流式细胞仪测试 DARPin#1 与帕妥珠单抗和 DARPin#18 与曲妥珠单抗的竞争。为此，在与各 DARPin(1uM) 一起培养之前，将 BT474 细胞分别与帕妥珠单抗、曲妥珠单抗（两者都为 1uM) 一起培养。利用 Alexa 647 标记的抗 5-His 抗体 (Qiagen 商品目录号：35370) 监测 DARPin 与细胞的结合，并且利用 Alexa 546 标记的抗人 -lgG 抗体 (Invitrogen 商品目录号：A-21089) 监测帕妥珠单抗或曲妥珠单抗与细胞的结合。该实验展示了 DARPin 都不与帕妥珠单抗或曲妥珠单抗竞争结合至 BT474 细胞表达的 HER2。

[0406] 也通过 ELISA（图 1C) 观察该研究结果，其中在与各 DARPin(20nM) 一起培养之前，将帕妥珠单抗（以 20nM 涂布在 F96 MaxiSorb Nunc（商品目录号 442404) 上）与 20nM Her2（结构域 I-IIII) 一起预培养。利用小鼠抗 RGS-His 单克隆抗体 (Qiagen, 商品目录号 34650) 和辣根过氧化物酶标记的抗小鼠抗体 (Pierce, 商品目录号 31438)（在室温下预混合 45min) 检测 DARPin 在 HER2- 帕妥珠单抗复合物上的特异性结合。除了涂板（在 4℃过夜进行）之外，所有的培养步骤在 450rpm Heidolph Titramax 1000 摇床上室温下进行 2h。PBS，0.1％ Tween 20® pH7.4、0.25％酪蛋白用作阻断剂。在帕妥珠单抗存在的情况下，该试验中测试的所有 N 端 DARPin(DARPin#7、DARPin#52、DARPin#53，和 DARPin#54) 结合 HER2，

表明 N 端 DARPin 都结合与该抗体不同的表位。

[0407] 总的来说，这些实验表明 SEQ ID NO:62 至 68、72，和 114 至 121 编码的单价重复序列结构域结合至 HER2 的结构域 II，SEQ ID NO:69-71、73、112 和 113 编码的单价重复序列结构域结合至 HER2 的结构域 I，并且 SEQ ID NO:74 至 82 编码的单价重复序列结构域结合至 HER2 的结构域 IV。结合至 HER2 的结构域 II 的单价重复序列结构域 (SEQ ID NO:62 至 68、72，和 114 至 121) 都不与帕妥珠单抗竞争结合至 HER2。在结合至 HER2 的结构域 IV 的单价重复序列结构域中，SEQ ID NO:77、78 和 82 编码的重复序列结构域与曲妥珠单抗竞争结合至 HER2，而 SEC ID NO:74 至 76 和 79 至 81 编码的重复序列结构域不与曲妥珠单抗竞争。

[0408] 实施例 4 :结合 HER2 的双特异性 DARPin 阻断过度表达 HER2 的肿瘤细胞的生长

[0409] 测试单价 DARPin、DARPin 的混合物和结合 HER2 的双特异性 DARPin 对 BT474 细胞增殖的抑制。图 2 展示了单价 DARPin 和单价 DARPin 的混合物不能阻断 BT474 细胞增殖。相反，双特异性 DARPin 分组诱导对增殖的抑制 (图 2，表 2)。有趣的是，DARPin HER2 的重复序列结构域 IV 必须位于分子的 C 端 (图 2)。双特异性形式的单价 DARPin 的多个组合导致对增殖有抑制作用的 DARPin。但是并非所有的组合都能将阻断 90-100％的 BT474 增殖 (图 3)，这使某些 DARPin 组合得以归类。这些研究结果表明双特异性形式是有效实现靶向不同分组的某些 HER2 表位的关键。通过曲妥珠单抗诱导 HER2 受体内在化和降解 (据报道) 不足以诱导对肿瘤细胞增殖的有效抑制 (图 3 和 5)。与曲妥珠单抗类似，DARPin#41 和 DARPin#43 都能诱导 HER2 的降解，但仅有 DARPin，比如 DARPin#41 抑制肿瘤细胞增殖。

[0410] 如方法部分所述进行实验。表 2 总结了实验结果。利用本领域技术人员已知的标准程序由如上所述获得的滴定曲线计算 IC_{50} 值。图 2 和 3 中给出了 DARPin#41 的滴定曲线例子。

[0411] 表 2: 各种 DARPin 对 BTB474 细胞增殖的抑制效力

[0412]

DARPin # 或抗体	IC50[nM]	% 活性 vs. DARPin # 41
32	3.29	48.0
22	4.03	60.1
27	4.57	37.8
35	4.63	63.0
38	3.30	99.3
33	4.47	65.3
23	2.99	97.3
28	5.15	82.5
36	2.56	68.8
34	3.88	95.1
24	1.97	99.9
29	1.33	95.0
37	2.19	94.8
40	2.76	91.2
42	3.77	100
45	1.55	100
46	3.34	100
41	4.01	100
47	n.i.	6.8
43	n.i.	n.i.
44	n.i.	n.i.
48	n.i.	n.i.
49	n.i	n.i
21	n.i.	n.i.
12	n.i.	n.i.
1	n.i.	n.i.
18	n.i.	n.i.
64	2.31	100
65	4.07	100
63	1.77	100
68	5.35	100
67	4.87	100
66	4.06	100
64	2.31	100
曲妥珠单抗	3.05	52
帕妥珠单抗	n.i	n.i

[0413] n.i.：未观察到抑制

[0414] 实施例 5：靶向 HER2 的双特异性 DARPin 抑制各种 HER2 过度表达的细胞系的增殖并诱导凋亡

[0415] 测试双特异性DARPin#41的效力。在过度表达HER2的细胞系而非表达野生型HER2水平的细胞中，该DARPin对增殖的抑制范围为Her2IHC 3+至1+(图4；表3)。此外，在所列举的细胞系中，该DARPin在24h的培养中强劲地诱导凋亡(图5；表3)。

[0416] 如方法部分所述进行实验。表3总结了实施例结果。利用本领域技术人员已知的标准程序由如上所述获得的滴定曲线计算IC_{50}和EC_{50}值。图4和5中给出了DARPin#41在三个不同细胞系上的滴定曲线例子。取决于测试的DARPin和细胞系，IC_{50}和EC_{50}值的范围在0.2-10nM之间。例如，实验表明DARPin#41、#45和#46在BT474、MDA-MB175和NCI-N87细胞中诱导凋亡(表3)。利用本发明的其它双特异性结合蛋白也获得相似的结果。

[0417] 表3::DARPin#41在各种不同的细胞系上的效力

[0418]

细胞系	Her2 状态	抑制增殖 IC_{50}[nM]	诱导凋亡 EC_{50} [nM]
BT474	IHC 3+	0.98	0.69
SKBR-3	IHC3+	1.75	n.a.
NCI-N87	IHC2+	0.94	0.26
ZR75-30	IHC3+	0.60	n.a.
HCC1419	IHC 3+	3.17	n.a.
MDA-MB175	IHC 1+	3.42	5.94
MCF7	IHC 0 / wt	n.i.	n.i.

[0419] n.a.：未分析

[0420] n.i.：无抑制

[0421] 实施例6：与当前标准护理治疗对比，靶向HER2的双特异性DARPin抑制BT474细胞的增殖并诱导凋亡

[0422] 比较双特异性DARPin#41的效力与批准用于治疗HER2阳性乳腺癌的药物、曲妥珠单抗和帕妥珠单抗的效力。与曲妥珠单抗、帕妥珠单抗或曲妥珠单抗和帕妥珠单抗的组合相比，该DARPin有效抑制增殖并且诱导凋亡。

[0423] 如方法部分所述进行实验。图6展示了实施例结果。利用本领域技术人员已知的标准程序由如上所述获得的滴定曲线计算IC_{50}和EC_{50}值(表3)。利用本发明的其它双特异性结合蛋白也获得相似的结果。

[0424] 实施例7：各种DARPin形式的产生

[0425] 作为例子，比较不同形式的双特异性DARPin#41和DARPin#41抑制BT474细胞增殖的效力(图7，表2)。N端或C端的聚乙二醇化或融合至结合人血清白蛋白的DARPin(DARPin#41、#63、#64、#65)的行为不影响效力(图7A)。此外，DARPin部分之间的

接头的变化不影响效力（图 7B）。IC_{50}值范围在 1.5 - 5.5nM 之间。利用相应形式的双特异性 DARPin#41、#66、#67、#68 获得相应的结果。总的来说，这清楚地表明可以（通过本领域技术人员已知的方法，比如聚乙二醇化或融合至结合血清白蛋白的结构域）修饰该双特异性 DARPin 以增加其体内半衰期而不损失效力。此外，这些实验表明，在双特异性构建体中结合至 HER2 的两个重复序列结构域之间的接头可以在至少 2 至 24 个氨基酸之间变化而不明显影响该双特异性构建体的功效。

[0426] 实施例 8：绘制 DARPin/Her2 相互作用图谱

[0427] 通过溶液（即，PBS，pH 7.4）中这两种分子形成的复合物的化学交联，接着用蛋白酶消化该复合物，并且通过质谱法分析得到的多肽，进一步分析本发明的双特异性 DARPin 与 HER2 胞外域的相互作用。在这样的实验中，DARPin 区域与 HER2 区域可以通过共价键交联，只要其接近后者。通过质谱法分析对来自与 HER2 的相应多肽通过共价键交联的 DARPin 的多肽的检测表明在 HER2/DRAPin 复合物中这些多肽很接近。本领域技术人员周知这些邻近分析方法（例如，Birch,C., 等人，Anal.Chem.,82,172 - 179,2010）并且是各种公司提供的服务（例如，CovalX AG,Zürich, 瑞士）。

[0428] 例如，在这些实验中发现，双特异性 DARPin#41（其结合 HER2 的结构域 II 和结构域 IV）可以与 HER2 形成 1：1 复合物。令人惊讶地，观察到 C 端重复序列结构域（结合至 HER2 的结构域 IV）和 HER2 的结构域 I 之间的共价交联，表明即使其结合至结构域 IV，但该重复序列结构域与在该复合物中的 HER2 结构域 I 接近。如果 HER2 的构象如现有技术（例如，Bublil 和 Yarden, 同上）所述，则预期不能看见这些交联。重要的是，分析 HER2 胞外域与单独结合至结构域 IV 的 C 端重复序列结构域的复合，不能观察到这种与 HER2 的结构域 I 的交联，表面在 HER2 和结合至结构域 IV 的单体重复序列结构域形成复合物的情况下，该重复序列结构域不接近结构域 I。因此，相比单独的重复序列结构域结合 HER2 的结构域 IV 形成的复合物，与本发明的双特异性结合蛋白形成的复合物中，HER2 的三维结构域排列必然不同

[0429] 有趣的是，考虑到在 2 个重复序列结构域之间的短接头的范围为 2 至 24 个氨基酸，HER2 的胞外域的已知结构使本发明的双特异性结合蛋白的两个重复序列结构域不能同时结合至相同的 HER2 分子。这表明 HER2 可能处于未知的构象中，从而能同时结合两个重复序列结构域。

[0430] 总的来说，这些实验表明本发明的双特异性结合蛋白可以与 HER2 的胞外域在分子内相互作用，并且因此本发明的双特异性结合蛋白使 HER2 胞外域处于现有技术未知的新构象，即，使结构域 I 和结构域 IV 处于这样的空间排列：能够观察到重复序列结构域（结合至 HER2 的结构域 IV）和结构域 I 之间的交联。因此，这种 HER2 新构象看起来是由本发明的双特异性结合蛋白以分子内的方式通过同时结合 HER2 的结构域 II 和结构域 IV 来稳定。

[0001]

序 列 表

<110> 分子组合公司

<120> 包含至少两个针对 HER2 的重复序列结构域的结合蛋白

<130> MD41080

<160> 141

<170> PatentIn 版本 3.5

<210> 1
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<212> PRT
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<220>
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Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Lys Asp Gly Tyr Thr Pro Leu His Leu Ala Ala Arg Glu Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Lys Asp Gly Tyr Thr Pro Leu His Leu Ala Ala Arg Glu
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile Asp
100 105 110

Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
115 120 125

[0002]

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<220>
<223> Capping module

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Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 3
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<220>
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<400> 3

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 4
<211> 28
<212> PRT
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<220>
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<400> 4

Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile Asp Asn Gly
1 5 10 15

Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
20 25

[0003]

<210> 5
<211> 28
<212> PRT
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<220>
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<400> 5

Gln Asp Lys Ser Gly Lys Thr Pro Ala Asp Leu Ala Ala Asp Ala Gly
1 5 10 15

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

<210> 6
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<212> PRT
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<220>
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<400> 6

Met Arg Gly Ser His His His His His His
1 5 10

<210> 7
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Gly Ser
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<210> 8
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<220>

[0004]

<223> GS-linker

<400> 8

Gly Gly Gly Gly Ser
1 5

<210> 9
<211> 10
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<220>
<223> GS-linker

<400> 9

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
1 5 10

<210> 10
<211> 20
<212> PRT
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<223> GS-linker

<400> 10

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
1 5 10 15

Gly Gly Gly Ser
20

<210> 11
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<400> 11

Pro Thr Pro Thr Pro
1 5

[0005]

<210> 12
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<212> PRT
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<220>
<223> PT-linker

<400> 12

Pro Thr Pro Thr Pro Thr Thr Pro Thr Pro Thr Pro Thr Thr Pro Thr
1 5 10 15

Pro Thr Pro Thr
20

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<400> 13

Gly Ser Asp Leu Gly Ile Lys Leu Leu Phe Ala Ala Ala Lys Ser Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 14
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<220>
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<400> 14

Gly Ser Asp Leu Gly Val Asn Leu Leu Trp Ala Ala Thr Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

[0006]

<210> 15
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Lys Asp Phe Gln Ser Val Thr Pro Leu His Ile Ala Ala Gln Ser Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 16
<211> 33
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<220>
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<400> 16

Lys Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Thr Ser Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 17
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[0007]

<400> 17

Lys Asp Phe Glu Gly Val Thr Pro Leu His Leu Ala Ala Gln Trp Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 18
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<220>
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<400> 18

Lys Asp Ile Thr Gly Glu Thr Pro Leu His His Ala Ala Asp Ser Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 19
<211> 28
<212> PRT
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<220>
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<400> 19

Gln Asp Lys Ala Gly Val Thr Pro Ala Asp Leu Ala Ala Ala Trp Gly
1 5 10 15

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

[0008]

<210> 20
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<400> 20

Gly Ser Asp Leu Gly Trp Lys Leu Leu Trp Ala Ala Ala His Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 21
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<400> 21

Lys Asp Trp Glu Gly Thr Thr Pro Leu His Leu Ala Ala His Thr Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 22
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<212> PRT
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<220>
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<400> 22

Lys Asp Thr Val Gly Thr Thr Pro Leu His Tyr Ala Ala Glu Asp Gly
1 5 10 15

[0009]

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 23
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<212> PRT
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<220>
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<400> 23

Lys Asp Glu Tyr Gly Phe Thr Pro Leu His Leu Ala Ala Gln Phe Asp
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 24
<211> 28
<212> PRT
<213> Artificial

<220>
<223> C-Cap module (Cr)

<400> 24

Gln Asp Trp Val Gly Gln Thr Pro Ala Asp Leu Ala Ala Ala Trp Gly
1 5 10 15

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

<210> 25
<211> 28
<212> PRT

[0010]

<213> Artificial

<220>
<223> C-Cap module (Cr)

<400> 25

Gln Asp Glu Arg Gly Trp Thr Pro Ala Asp Leu Ala Ala Asp Trp Gly
1 5 10 15

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

<210> 26
<211> 32
<212> PRT
<213> Artificial

<220>
<223> N-Cap module (Nr)

<400> 26

Gly Ser Asp Leu Gly His Lys Leu Leu Glu Ala Ala Val Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 27
<211> 32
<212> PRT
<213> Artificial

<220>
<223> N-Cap module (Nr)

<400> 27

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ser His Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 28
<211> 33

[0011]

<212> PRT
<213> Artificial

<220>
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<400> 28

Lys Asp Trp Tyr Gly Lys Thr Pro Leu His Phe Ala Ala Gly Leu Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 29
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<212> PRT
<213> Artificial

<220>
<223> AR module (M1.1b)

<400> 29

Lys Asp Phe Phe Gly Ile Thr Pro Leu His Gln Ala Ala Trp Gly His
1 5 10 15

Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 30
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<212> PRT
<213> Artificial

<220>
<223> AR module (M1.1b)

<400> 30

Lys Asp Asp Phe Gly Thr Thr Pro Leu His Ala Ala Ala Asp Tyr Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

[0012]

Ala

<210> 31
<211> 33
<212> PRT
<213> Artificial

<220>
<223> AR module (M1.1b)

<400> 31

Lys Asp Glu Asp Gly Gln Thr Pro Leu His Leu Ala Ala Ala Tyr Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 32
<211> 33
<212> PRT
<213> Artificial

<220>
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<400> 32

Lys Glu Glu Asp Gly Thr Thr Pro Leu His Leu Ala Ala Thr His Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 33
<211> 28
<212> PRT

[0013]

<213> Artificial

<220>
<223> C-Cap module (Cr)

<400> 33

Gln Asp Tyr Thr Gly His Thr Pro Ala Asp Leu Ala Ala Val Tyr Gly
1 5 10 15

His Glu Asp Ile Ala Ala Val Leu Gln Lys Leu Asn
20 25

<210> 34
<211> 28
<212> PRT
<213> Artificial

<220>
<223> C-Cap module (Cr)

<400> 34

Gln Asp Asn Asp Gly Phe Thr Pro Ala Asp Leu Ala Ala Asp Ser Gly
1 5 10 15

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

<210> 35
<211> 28
<212> PRT
<213> Artificial

<220>
<223> C-Cap module (Cr)

<400> 35

Gln Asp Trp Tyr Gly Thr Thr Pro Ala Asp Leu Ala Ala Trp Trp Gly
1 5 10 15

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

<210> 36
<211> 32

[0014]

<212> PRT
<213> Artificial

<220>
<223> N-Cap module (Nr)

<400> 36

Gly Ser Asp Leu Gly Ile Lys Leu Leu Phe Ala Ala Ser Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 37
<211> 33
<212> PRT
<213> Artificial

<220>
<223> AR module (M1.1b)

<400> 37

Lys Asp Phe Glu Gly Ile Thr Pro Leu His Ala Ala Ala Arg Ser Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 38
<211> 33
<212> PRT
<213> Artificial

<220>
<223> AR module (M1.1b)

<400> 38

Lys Asp Val Glu Gly Trp Thr Pro Leu His Tyr Ala Ala Ser Ser Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

[0015]

Ala

<210> 39
<211> 28
<212> PRT
<213> Artificial

<220>
<223> C-Cap module (Cr)

<400> 39

Gln Asp Asn His Gly Ala Thr Pro Ala Asp Leu Ala Ala Gln Trp Gly
1 5 10 15

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

<210> 40
<211> 32
<212> PRT
<213> Artificial

<220>
<223> N-Cap module (Nr)

<400> 40

Gly Ser Asp Leu Gly Asn Lys Leu Leu Ile Ala Ala Ser Val Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 41
<211> 33
<212> PRT
<213> Artificial

<220>
<223> AR module (M1.1b)

<400> 41

Lys Asp Glu Thr Gly Trp Thr Pro Leu His Leu Ala Ala Ala Trp Gly
1 5 10 15

[0016]

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 42
<211> 33
<212> PRT
<213> Artificial

<220>
<223> AR module (M1.1b)

<400> 42

Lys Asp Val Lys Gly Gln Thr Pro Leu His Leu Ala Ala Ala Tyr Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 43
<211> 28
<212> PRT
<213> Artificial

<220>
<223> C-Cap module (Cr)

<400> 43

Gln Asp Asn Asp Gly Tyr Thr Pro Ala Asp Leu Ala Ala Arg Tyr Gly
1 5 10 15

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

<210> 44
<211> 32
<212> PRT

[0017]

<213> Artificial

<220>
<223> N-Cap module (Nr)

<400> 44

Gly Ser Asp Leu Gly Lys Lys Leu Leu Asn Ala Ala Val Cys Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Val Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 45
<211> 32
<212> PRT
<213> Artificial

<220>
<223> N-Cap module (Nr)

<400> 45

Gly Ser Asp Leu Gly Thr Lys Leu Leu Asp Ala Ala Thr Tyr Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 46
<211> 33
<212> PRT
<213> Artificial

<220>
<223> AR module (M1.1b)

<400> 46

Lys Asp Trp Arg Gly Phe Thr Pro Leu His Tyr Ala Ala Tyr Leu Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

[0018]

<210> 47
<211> 33
<212> PRT
<213> Artificial

<220>
<223> AR module (M1.1b)

<400> 47

Lys Asp Thr Ile Gly His Thr Pro Leu His Arg Ala Ala Phe Val Gly
1 5 10 15

Gln Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 48
<211> 28
<212> PRT
<213> Artificial

<220>
<223> C-Cap module (Cr)

<400> 48

Gln Asp Thr Ala Gly Tyr Thr Pro Ala Asp Leu Ala Ala Trp Thr Gly
1 5 10 15

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

<210> 49
<211> 28
<212> PRT
<213> Artificial

<220>
<223> C-Cap module (Cr)

<400> 49

Gln Asp Asp Tyr Gly Trp Thr Pro Ala Asp Leu Ala Ala Asn Ser Gly
1 5 10 15

[0019]

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

<210> 50
<211> 32
<212> PRT
<213> Artificial

<220>
<223> N-Cap module (Nr)

<400> 50

Gly Ser Asp Leu Gly Ile Lys Leu Leu Gln Ala Ala Asn Leu Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Thr Gly Ala Asp Val Asn Ala
20 25 30

<210> 51
<211> 33
<212> PRT
<213> Artificial

<220>
<223> AR module (M1.1b)

<400> 51

Lys Asp Ser Ile Gly Gln Thr Pro Leu His Trp Ala Ala Arg Arg Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 52
<211> 33
<212> PRT
<213> Artificial

<220>
<223> AR module (M1.1b)

[0020]

<400> 52

Lys Asp Glu Tyr Gly Val Thr Pro Leu His Leu Ala Ala Ser Leu Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 53
<211> 28
<212> PRT
<213> Artificial

<220>
<223> C-Cap module (Cr)

<400> 53

Gln Asp Thr Ala Gly Gln Thr Pro Ala Asp Leu Ala Ala Asp Asp Gly
1 5 10 15

His Glu Asp Ile Ala Val Val Leu Gln Lys Leu Asn
20 25

<210> 54
<211> 32
<212> PRT
<213> Artificial

<220>
<223> N-Cap module (old)

<400> 54

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 55
<211> 33

[0021]

<212> PRT
<213> Artificial

<220>
<223> AR module (old)

<400> 55

Lys Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala Thr Ala His Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 56
<211> 33
<212> PRT
<213> Artificial

<220>
<223> AR module (old)

<400> 56

Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu Ala Ala Phe Ile Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 57
<211> 28
<212> PRT
<213> Artificial

<220>
<223> C-Cap module (old)

<400> 57

Gln Asp Lys Ser Gly Lys Thr Pro Ala Asp Leu Ala Ala Gly Ala Gly
1 5 10 15

[0022]

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

<210> 58
<211> 33
<212> PRT
<213> Artificial

<220>
<223> AR sequence motif

<220>
<221> misc_feature
<222> (1)..(1)
<223> Xaa can be any naturally occurring amino acid

<220>
<221> misc_feature
<222> (3)..(4)
<223> Xaa can be any naturally occurring amino acid

<220>
<221> misc_feature
<222> (6)..(6)
<223> Xaa can be any naturally occurring amino acid

<220>
<221> misc_feature
<222> (14)..(15)
<223> Xaa can be any naturally occurring amino acid

<220>
<221> misc_feature
<222> (27)..(27)
<223> Xaa can be any naturally occurring amino acid

<400> 58

Xaa Asp Xaa Xaa Gly Xaa Thr Pro Leu His Leu Ala Ala Xaa Xaa Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Xaa Gly Ala Asp Val Asn
20 25 30

Ala

[0023]

<210> 59
<211> 33
<212> PRT
<213> Artificial

<220>
<223> AR sequence motif

<220>
<221> misc_feature
<222> (3)..(4)
<223> Xaa can be any naturally occurring amino acid

<220>
<221> misc_feature
<222> (6)..(6)
<223> Xaa can be any naturally occurring amino acid

<220>
<221> misc_feature
<222> (11)..(11)
<223> Xaa can be any naturally occurring amino acid

<220>
<221> misc_feature
<222> (14)..(15)
<223> Xaa can be any naturally occurring amino acid

<400> 59

Lys Asp Xaa Xaa Gly Xaa Thr Pro Leu His Xaa Ala Ala Xaa Xaa Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 60
<211> 32
<212> PRT
<213> Artificial

<220>
<223> AR sequence motif

<220>

[0024]

<221> misc_feature
<222> (6)..(6)
<223> Xaa can be any naturally occurring amino acid

<220>
<221> misc_feature
<222> (10)..(10)
<223> Xaa can be any naturally occurring amino acid

<220>
<221> misc_feature
<222> (13)..(14)
<223> Xaa can be any naturally occurring amino acid

<400> 60

Gly Ser Asp Leu Gly Xaa Lys Leu Leu Xaa Ala Ala Xaa Xaa Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 61
<211> 28
<212> PRT
<213> Artificial

<220>
<223> AR sequence motif

<220>
<221> misc_feature
<222> (3)..(4)
<223> Xaa can be any naturally occurring amino acid

<220>
<221> misc_feature
<222> (6)..(6)
<223> Xaa can be any naturally occurring amino acid

<220>
<221> misc_feature
<222> (14)..(15)
<223> Xaa can be any naturally occurring amino acid

<400> 61

Gln Asp Xaa Xaa Gly Xaa Thr Pro Ala Asp Leu Ala Ala Xaa Xaa Gly
1 5 10 15

[0025]

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

<210> 62
<211> 126
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 62

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Gln Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His
65 70 75 80

Gly His Leu Val Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Glu Arg Gly Trp Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 63
<211> 126
<212> PRT
<213> Artificial

<220>

[0026]

<223> AR domain (one-domain)

<400> 63

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Thr Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Ile Thr Gly Glu Thr Pro Leu His His Ala Ala Asp Ser
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Lys Ala Gly Val Thr Pro Ala Asp Leu Ala Ala Ala
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 64
<211> 126
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 64

Gly Ser Asp Leu Gly Ile Lys Leu Leu Phe Ala Ala Ala Lys Ser Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

[0027]

Lys Asp Phe Gln Ser Val Thr Pro Leu His Ile Ala Ala Gln Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Glu Arg Gly Trp Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 65
<211> 126
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 65

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Gln Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His
65 70 75 80

[0028]

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Glu Arg Gly Lys Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 66
<211> 126
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 66

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Thr Asn Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Ile Thr Gly Glu Thr Pro Leu His His Ala Ala Asp Ser
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Lys Ala Gly Val Thr Pro Ala Asp Leu Ala Ala Ala
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

[0029]

<210> 67
<211> 126
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 67

Gly Ser Asp Leu Gly Val Asn Leu Leu Trp Ala Ala Thr Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Glu Gly Val Thr Pro Leu His Leu Ala Ala Gln Trp Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Glu Arg Gly Trp Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 68
<211> 126
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 68

[0030]

Gly Ser Asp Leu Gly Ile Lys Leu Leu Phe Ala Ala Ala Lys Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Glu Gly Tyr Thr Pro Leu His Val Ala Ala Tyr Asp Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Ser Gln Gly Arg Thr Pro Leu His Glu Ala Ala Tyr Ser
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Asp Ala Gly Glu Thr Pro Ala Asp Leu Ala Ala Ala
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 69
<211> 126
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 69

Gly Ser Asp Leu Gly Ile Lys Leu Leu Trp Ala Ala Ala His Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Asp Ala Asp Val Asn Ala
20 25 30

Lys Asp Trp Tyr Gly Thr Thr Pro Leu His Ile Ala Ala Val Ala Gly
35 40 45

[0031]

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Asp Phe Gly Thr Thr Pro Leu His Leu Ala Ala Tyr His
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Trp Gln Gly Gln Thr Pro Ala Asp Leu Ala Ala Gln
100 105 110

Asp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 70
<211> 126
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 70

Gly Ser Asp Leu Gly His Lys Leu Leu Glu Ala Ala Val Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Trp Tyr Gly Lys Thr Pro Leu His Phe Ala Ala Gly Leu Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Glu Asp Gly Gln Thr Pro Leu His Leu Ala Ala Ala Tyr
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

[0032]

Asn Ala Gln Asp Asn Asp Gly Phe Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Ser Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 71
<211> 125
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 71

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ser His Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Phe Gly Ile Thr Pro Leu His Gln Ala Ala Trp Gly His
35 40 45

Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
50 55 60

Lys Glu Glu Asp Gly Thr Thr Pro Leu His Leu Ala Ala Thr His Gly
65 70 75 80

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
85 90 95

Ala Gln Asp Trp Tyr Gly Thr Thr Pro Ala Asp Leu Ala Ala Trp Trp
100 105 110

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 72

[0033]

<211> 126
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 72

Gly Ser Asp Leu Gly Ile Lys Leu Leu Phe Ala Ala Ser Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Glu Gly Ile Thr Pro Leu His Ala Ala Ala Arg Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Glu Gly Trp Thr Pro Leu His Tyr Ala Ala Ser Tyr
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Asn His Gly Ala Thr Pro Ala Asp Leu Ala Ala Gln
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 73
<211> 159
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 73

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

[0034]

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Tyr Gly Ile Thr Pro Leu His Leu Ala Ala Ala Tyr Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys His Gly Ala Asp Val Asn
50 55 60

Ala His Asp Trp Asn Gly Trp Thr Pro Leu His Leu Ala Ala Lys Tyr
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys His Gly Ala Asp Val
85 90 95

Asn Ala Ile Asp Asn Ala Gly Lys Thr Pro Leu His Leu Ala Ala Ala
100 105 110

His Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Tyr Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile
130 135 140

Asp Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
145 150 155

<210> 74
<211> 93
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 74

Gly Ser Asp Leu Gly Lys Lys Leu Leu Asn Ala Ala Val Cys Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Val Ala Gly Ala Asp Val Asn Ala
20 25 30

[0035]

Lys Asp Trp Arg Gly Phe Thr Pro Leu His Tyr Ala Ala Tyr Leu Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Thr Ala Gly Tyr Thr Pro Ala Asp Leu Ala Ala Trp Thr
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 75
<211> 93
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 75

Gly Ser Asp Leu Gly Ala Lys Leu Leu Ile Ala Ala Thr Val Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Thr Ile Gly His Thr Pro Leu His Arg Ala Ala Phe Val Gly
35 40 45

Gln Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Asp Tyr Gly Trp Thr Pro Ala Asp Leu Ala Ala Asn Ser
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 76

[0036]

<211> 93
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 76

Gly Ser Asp Leu Gly Ala Lys Leu Leu Val Ala Ala Thr Ser Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Arg Ile Gly Phe Thr Pro Leu His Arg Ala Ala Phe Val Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Asp Phe Gly His Thr Pro Ala Asp Leu Ala Ala Ser Leu
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 77
<211> 126
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 77

Gly Ser Asp Leu Gly Ile Lys Leu Leu Gln Ala Ala Asn Leu Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Ser Ile Gly Gln Thr Pro Leu His Trp Ala Ala Arg Arg Gly
35 40 45

[0037]

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Glu Tyr Gly Val Thr Pro Leu His Leu Ala Ala Ser Leu
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Glu Ser Gly Glu Thr Pro Ala Asp Leu Ala Ala Leu
100 105 110

His Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 78
<211> 126
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 78

Gly Ser Asp Leu Gly Leu Lys Leu Leu Gln Ala Ala Asn Leu Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Ser Ile Gly Gln Thr Pro Leu His Trp Ala Ala Arg Arg Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Glu Tyr Gly Val Thr Pro Leu His Leu Ala Ala Ser Leu
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

[0038]

Asn Ala Gln Asp Thr Ala Gly Gln Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Asp Gly His Glu Asp Ile Ala Val Val Leu Gln Lys Leu Asn
115 120 125

<210> 79
<211> 126
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 79

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala Thr Ala His Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val Asn
50 55 60

Ala Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu Ala Ala Phe Ile
65 70 75 80

Gly His Leu Glu Ile Ala Glu Val Leu Leu Lys His Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile Gly
100 105 110

Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
115 120 125

<210> 80

[0039]

<211> 126
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 80

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala Thr Ala His Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val Asn
50 55 60

Ala Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu Ala Ala Phe Ile
65 70 75 80

Gly His Leu Glu Ile Ala Glu Val Leu Leu Lys His Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Lys Ser Gly Lys Thr Pro Ala Asp Leu Ala Ala Gly
100 105 110

Ala Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 81
<211> 126
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 81

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

[0040]

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala Thr Ala His Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu Ala Ala Phe Ile
65 70 75 80

Gly His Leu Glu Ile Ala Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Lys Phe Gly Lys Thr Pro Ala Asp Ile Ala Ala Gly
100 105 110

Ala Gly Asn Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 82
<211> 159
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain)

<400> 82

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Thr Asp Ile His Gly His Thr Pro Leu His Leu Ala Ala Ala Met Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val Asn

[0041]

50 55 60

Ala Asn Asp Trp Arg Gly Phe Thr Pro Leu His Leu Ala Ala Leu Asn
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val
85 90 95

Asn Ala Thr Asp Thr Ala Gly Asn Thr Pro Leu His Leu Ala Ala Trp
100 105 110

Phe Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile
130 135 140

Asp Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
145 150 155

<210> 83
<211> 270
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 83

Gly Ser Asp Leu Gly Asp Lys Leu Leu Gln Ser Asp Leu Gly Ile Lys
1 5 10 15

Leu Leu Phe Ala Ala Ala Lys Ser Gln Asp Asp Glu Val Arg Ile Leu
20 25 30

Leu Ala Ala Gly Ala Asp Val Asn Ala Lys Asp Phe Gln Ser Val Thr
35 40 45

Pro Leu His Ile Ala Ala Gln Ser Gly His Leu Glu Ile Val Glu Val
50 55 60

[0042]

Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Val Thr Gly Asp
65 70 75 80

Thr Pro Leu His Leu Ala Ala Gln His Gly His Leu Glu Ile Val Glu
85 90 95

Val Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Glu Arg Gly
100 105 110

Trp Thr Pro Ala Asp Leu Ala Ala Asp Trp Gly His Glu Asp Ile Ala
115 120 125

Glu Val Leu Gln Lys Leu Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
130 135 140

Arg Ser Asp Leu Gly Ile Lys Leu Leu Gln Ala Ala Asn Leu Gly Gln
145 150 155 160

Asp Asp Glu Val Arg Ile Leu Leu Ala Thr Gly Ala Asp Val Asn Ala
165 170 175

Lys Asp Ser Ile Gly Gln Thr Pro Leu His Trp Ala Ala Arg Arg Gly
180 185 190

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
195 200 205

Ala Lys Asp Glu Tyr Gly Val Thr Pro Leu His Leu Ala Ala Ser Leu
210 215 220

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
225 230 235 240

Asn Ala Gln Asp Glu Ser Gly Glu Thr Pro Ala Asp Leu Ala Ala Leu
245 250 255

His Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
260 265 270

<210> 84

[0043]

<211> 237
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 84

Gly Ser Asp Leu Gly Asp Lys Leu Leu Gln Ser Asp Leu Gly Ile Lys
1 5 10 15

Leu Leu Phe Ala Ala Ala Lys Ser Gln Asp Asp Glu Val Arg Ile Leu
20 25 30

Leu Ala Ala Gly Ala Asp Val Asn Ala Lys Asp Phe Gln Ser Val Thr
35 40 45

Pro Leu His Ile Ala Ala Gln Ser Gly His Leu Glu Ile Val Glu Val
50 55 60

Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Val Thr Gly Asp
65 70 75 80

Thr Pro Leu His Leu Ala Ala Gln His Gly His Leu Glu Ile Val Glu
85 90 95

Val Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Glu Arg Gly
100 105 110

Trp Thr Pro Ala Asp Leu Ala Ala Asp Trp Gly His Glu Asp Ile Ala
115 120 125

Glu Val Leu Gln Lys Leu Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
130 135 140

Arg Ser Asp Leu Gly Ala Lys Leu Leu Val Ala Ala Thr Ser Gly Gln
145 150 155 160

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
165 170 175

[0044]

Lys Asp Arg Ile Gly Phe Thr Pro Leu His Arg Ala Ala Phe Val Gly
180 185 190

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
195 200 205

Ala Gln Asp Asp Phe Gly His Thr Pro Ala Asp Leu Ala Ala Ser Leu
210 215 220

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
225 230 235

<210> 85
<211> 270
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 85

Gly Ser Asp Leu Gly Asp Lys Leu Leu Gln Ser Asp Leu Gly Ile Lys
1 5 10 15

Leu Leu Phe Ala Ala Ala Lys Ser Gln Asp Asp Glu Val Arg Ile Leu
20 25 30

Leu Ala Ala Gly Ala Asp Val Asn Ala Lys Asp Phe Gln Ser Val Thr
35 40 45

Pro Leu His Ile Ala Ala Gln Ser Gly His Leu Glu Ile Val Glu Val
50 55 60

Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Val Thr Gly Asp
65 70 75 80

Thr Pro Leu His Leu Ala Ala Gln His Gly His Leu Glu Ile Val Glu
85 90 95

Val Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Glu Arg Gly
100 105 110

[0045]

Trp Thr Pro Ala Asp Leu Ala Ala Asp Trp Gly His Glu Asp Ile Ala
115 120 125

Glu Val Leu Gln Lys Leu Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
130 135 140

Arg Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
145 150 155 160

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
165 170 175

Lys Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala Thr Ala His Gly
180 185 190

His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val Asn
195 200 205

Ala Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu Ala Ala Phe Ile
210 215 220

Gly His Leu Glu Ile Ala Glu Val Leu Leu Lys His Gly Ala Asp Val
225 230 235 240

Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile Gly
245 250 255

Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
260 265 270

<210> 86
<211> 272
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 86

Gly Ser Asp Leu Gly Ile Lys Leu Leu Phe Ala Ala Ala Lys Gly Gln
1 5 10 15

[0046]

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Val Thr Pro Leu His Ile Ala Ala Gln Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Glu Arg Gly Trp Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Ala Ala Gly Gly
115 120 125

Gly Gly Ser Gly Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly
130 135 140

Gly Ser Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala
145 150 155 160

Gly Gln Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val
165 170 175

Asn Ala Lys Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala Thr Ala
180 185 190

His Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp
195 200 205

Val Asn Ala Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu Ala Ala
210 215 220

[0047]

Phe Ile Gly His Leu Glu Ile Ala Glu Val Leu Leu Lys Ala Gly Ala
225 230 235 240

Asp Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Pro Ala Asp Ile Ala
245 250 255

Ala Gly Ala Gly Asn Glu Asp Ile Ala Glu Val Leu Gln Lys Ala Ala
260 265 270

<210> 87
<211> 274
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 87

Gly Ser Asp Leu Gly Ile Lys Leu Leu Phe Ala Ala Ala Lys Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Val Thr Pro Leu His Ile Ala Ala Gln Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Glu Arg Gly Trp Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Ala Ala Gly Ser
115 120 125

[0048]

Pro Thr Pro Thr Pro Thr Thr Pro Thr Pro Thr Pro Thr Thr Pro Thr
130 135 140

Pro Thr Pro Thr Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala
145 150 155 160

Arg Ala Gly Gln Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala
165 170 175

Asp Val Asn Ala Lys Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala
180 185 190

Thr Ala His Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly
195 200 205

Ala Asp Val Asn Ala Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu
210 215 220

Ala Ala Phe Ile Gly His Leu Glu Ile Ala Glu Val Leu Leu Lys Ala
225 230 235 240

Gly Ala Asp Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Pro Ala Asp
245 250 255

Ile Ala Ala Gly Ala Gly Asn Glu Asp Ile Ala Glu Val Leu Gln Lys
260 265 270

Ala Ala

<210> 88
<211> 261
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 88

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala Arg Gly Gln

[0049]

1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Thr Asn Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Ile Thr Gly Glu Thr Pro Leu His His Ala Ala Asp Ser
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Lys Ala Gly Val Thr Pro Ala Asp Leu Ala Ala Ala
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Gly Gly Gly
115 120 125

Gly Ser Gly Gly Gly Gly Ser Arg Ser Asp Leu Gly Ile Lys Leu Leu
130 135 140

Gln Ala Ala Asn Leu Gly Gln Asp Asp Glu Val Arg Ile Leu Leu Ala
145 150 155 160

Thr Gly Ala Asp Val Asn Ala Lys Asp Ser Ile Gly Gln Thr Pro Leu
165 170 175

His Trp Ala Ala Arg Arg Gly His Leu Glu Ile Val Glu Val Leu Leu
180 185 190

Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Glu Tyr Gly Val Thr Pro
195 200 205

Leu His Leu Ala Ala Ser Leu Gly His Leu Glu Ile Val Glu Val Leu
210 215 220

[0050]

Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Glu Ser Gly Glu Thr
225 230 235 240

Pro Ala Asp Leu Ala Ala Leu His Gly His Glu Asp Ile Ala Glu Val
245 250 255

Leu Gln Lys Leu Asn
260

<210> 89
<211> 228
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 89

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Thr Asn Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Ile Thr Gly Glu Thr Pro Leu His His Ala Ala Asp Ser
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Lys Ala Gly Val Thr Pro Ala Asp Leu Ala Ala Ala
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Gly Gly Gly

[0051]

115 120 125

Gly Ser Gly Gly Gly Gly Ser Arg Ser Asp Leu Gly Ala Lys Leu Leu
130 135 140

Val Ala Ala Thr Ser Gly Gln Asp Asp Glu Val Arg Ile Leu Leu Ala
145 150 155 160

Ala Gly Ala Asp Val Asn Ala Lys Asp Arg Ile Gly Phe Thr Pro Leu
165 170 175

His Arg Ala Ala Phe Val Gly His Leu Glu Ile Val Glu Val Leu Leu
180 185 190

Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Asp Phe Gly His Thr Pro
195 200 205

Ala Asp Leu Ala Ala Ser Leu Gly His Glu Asp Ile Ala Glu Val Leu
210 215 220

Gln Lys Leu Asn
225

<210> 90
<211> 261
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 90

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Thr Asn Gly
35 40 45

[0052]

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Ile Thr Gly Glu Thr Pro Leu His His Ala Ala Asp Ser
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Lys Ala Gly Val Thr Pro Ala Asp Leu Ala Ala Ala
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Gly Gly Gly
115 120 125

Gly Ser Gly Gly Gly Gly Ser Arg Ser Asp Leu Gly Lys Lys Leu Leu
130 135 140

Glu Ala Ala Arg Ala Gly Gln Asp Asp Glu Val Arg Ile Leu Met Ala
145 150 155 160

Asn Gly Ala Asp Val Asn Ala Lys Asp Glu Tyr Gly Leu Thr Pro Leu
165 170 175

Tyr Leu Ala Thr Ala His Gly His Leu Glu Ile Val Glu Val Leu Leu
180 185 190

Lys Asn Gly Ala Asp Val Asn Ala Val Asp Ala Ile Gly Phe Thr Pro
195 200 205

Leu His Leu Ala Ala Phe Ile Gly His Leu Glu Ile Ala Glu Val Leu
210 215 220

Leu Lys His Gly Ala Asp Val Asn Ala Gln Asp Lys Phe Gly Lys Thr
225 230 235 240

Ala Phe Asp Ile Ser Ile Gly Asn Gly Asn Glu Asp Leu Ala Glu Ile
245 250 255

Leu Gln Lys Leu Asn

[0053]

260

<210> 91
<211> 272
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 91

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Thr Asn Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Ile Thr Gly Glu Thr Pro Leu His His Ala Ala Asp Ser
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Lys Ala Gly Val Thr Pro Ala Asp Leu Ala Ala Ala
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Ala Ala Gly Gly
115 120 125

Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly
130 135 140

Gly Ser Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala
145 150 155 160

[0054]

Gly Gln Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val
165 170 175

Asn Ala Lys Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala Thr Ala
180 185 190

His Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp
195 200 205

Val Asn Ala Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu Ala Ala
210 215 220

Phe Ile Gly His Leu Glu Ile Ala Glu Val Leu Leu Lys Ala Gly Ala
225 230 235 240

Asp Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Pro Ala Asp Ile Ala
245 250 255

Ala Gly Ala Gly Asn Glu Asp Ile Ala Glu Val Leu Gln Lys Ala Ala
260 265 270

<210> 92
<211> 274
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 92

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Thr Asn Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

[0055]

Ala Lys Asp Ile Thr Gly Glu Thr Pro Leu His His Ala Ala Asp Ser
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Lys Ala Gly Val Thr Pro Ala Asp Leu Ala Ala Ala
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Ala Ala Gly Ser
115 120 125

Pro Thr Pro Thr Pro Thr Thr Pro Thr Pro Thr Pro Thr Thr Pro Thr
130 135 140

Pro Thr Pro Thr Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala
145 150 155 160

Arg Ala Gly Gln Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala
165 170 175

Asp Val Asn Ala Lys Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala
180 185 190

Thr Ala His Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly
195 200 205

Ala Asp Val Asn Ala Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu
210 215 220

Ala Ala Phe Ile Gly His Leu Glu Ile Ala Glu Val Leu Leu Lys Ala
225 230 235 240

Gly Ala Asp Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Pro Ala Asp
245 250 255

Ile Ala Ala Gly Ala Gly Asn Glu Asp Ile Ala Glu Val Leu Gln Lys
260 265 270

[0056]

Ala Ala

<210> 93
<211> 261
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 93

Gly Ser Asp Leu Gly Ile Lys Leu Leu Phe Ala Ala Ser Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Glu Gly Ile Thr Pro Leu His Ala Ala Ala Arg Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Glu Gly Trp Thr Pro Leu His Tyr Ala Ala Ser Tyr
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Asn His Gly Ala Thr Pro Ala Asp Leu Ala Ala Gln
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Gly Gly Gly
115 120 125

Gly Ser Gly Gly Gly Gly Ser Arg Ser Asp Leu Gly Ile Lys Leu Leu
130 135 140

Gln Ala Ala Asn Leu Gly Gln Asp Asp Glu Val Arg Ile Leu Leu Ala
145 150 155 160

[0057]

Thr Gly Ala Asp Val Asn Ala Lys Asp Ser Ile Gly Gln Thr Pro Leu
165 170 175

His Trp Ala Ala Arg Arg Gly His Leu Glu Ile Val Glu Val Leu Leu
180 185 190

Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Glu Tyr Gly Val Thr Pro
195 200 205

Leu His Leu Ala Ala Ser Leu Gly His Leu Glu Ile Val Glu Val Leu
210 215 220

Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Glu Ser Gly Glu Thr
225 230 235 240

Pro Ala Asp Leu Ala Ala Leu His Gly His Glu Asp Ile Ala Glu Val
245 250 255

Leu Gln Lys Leu Asn
260

<210> 94
<211> 228
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 94

Gly Ser Asp Leu Gly Ile Lys Leu Leu Phe Ala Ala Ser Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Glu Gly Ile Thr Pro Leu His Ala Ala Ala Arg Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

[0058]

Ala Lys Asp Val Glu Gly Trp Thr Pro Leu His Tyr Ala Ala Ser Tyr
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Asn His Gly Ala Thr Pro Ala Asp Leu Ala Ala Gln
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Gly Gly Gly
115 120 125

Gly Ser Gly Gly Gly Gly Ser Arg Ser Asp Leu Gly Ala Lys Leu Leu
130 135 140

Val Ala Ala Thr Ser Gly Gln Asp Asp Glu Val Arg Ile Leu Leu Ala
145 150 155 160

Ala Gly Ala Asp Val Asn Ala Lys Asp Arg Ile Gly Phe Thr Pro Leu
165 170 175

His Arg Ala Ala Phe Val Gly His Leu Glu Ile Val Glu Val Leu Leu
180 185 190

Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Asp Phe Gly His Thr Pro
195 200 205

Ala Asp Leu Ala Ala Ser Leu Gly His Glu Asp Ile Ala Glu Val Leu
210 215 220

Gln Lys Leu Asn
225

<210> 95
<211> 261
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

[0059]

<400> 95

Gly Ser Asp Leu Gly Ile Lys Leu Leu Phe Ala Ala Ser Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Glu Gly Ile Thr Pro Leu His Ala Ala Ala Arg Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Glu Gly Trp Thr Pro Leu His Tyr Ala Ala Ser Tyr
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Asn His Gly Ala Thr Pro Ala Asp Leu Ala Ala Gln
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Gly Gly Gly
115 120 125

Gly Ser Gly Gly Gly Gly Ser Arg Ser Asp Leu Gly Lys Lys Leu Leu
130 135 140

Glu Ala Ala Arg Ala Gly Gln Asp Asp Glu Val Arg Ile Leu Met Ala
145 150 155 160

Asn Gly Ala Asp Val Asn Ala Lys Asp Glu Tyr Gly Leu Thr Pro Leu
165 170 175

Tyr Leu Ala Thr Ala His Gly His Leu Glu Ile Val Glu Val Leu Leu
180 185 190

Lys Asn Gly Ala Asp Val Asn Ala Val Asp Ala Ile Gly Phe Thr Pro
195 200 205

[0060]

Leu His Leu Ala Ala Phe Ile Gly His Leu Glu Ile Ala Glu Val Leu
210 215 220

Leu Lys His Gly Ala Asp Val Asn Ala Gln Asp Lys Phe Gly Lys Thr
225 230 235 240

Ala Phe Asp Ile Ser Ile Gly Asn Gly Asn Glu Asp Leu Ala Glu Ile
245 250 255

Leu Gln Lys Leu Asn
260

<210> 96
<211> 261
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 96

Gly Ser Asp Leu Gly Ile Lys Leu Leu Phe Ala Ala Ala Lys Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Glu Gly Tyr Thr Pro Leu His Val Ala Ala Tyr Asp Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Ser Gln Gly Arg Thr Pro Leu His Glu Ala Ala Tyr Ser
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Asp Ala Gly Glu Thr Pro Ala Asp Leu Ala Ala Ala

[0061]

100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Gly Gly Gly
115 120 125

Gly Ser Gly Gly Gly Gly Ser Arg Ser Asp Leu Gly Ile Lys Leu Leu
130 135 140

Gln Ala Ala Asn Leu Gly Gln Asp Asp Glu Val Arg Ile Leu Leu Ala
145 150 155 160

Thr Gly Ala Asp Val Asn Ala Lys Asp Ser Ile Gly Gln Thr Pro Leu
165 170 175

His Trp Ala Ala Arg Arg Gly His Leu Glu Ile Val Glu Val Leu Leu
180 185 190

Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Glu Tyr Gly Val Thr Pro
195 200 205

Leu His Leu Ala Ala Ser Leu Gly His Leu Glu Ile Val Glu Val Leu
210 215 220

Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Glu Ser Gly Glu Thr
225 230 235 240

Pro Ala Asp Leu Ala Ala Leu His Gly His Glu Asp Ile Ala Glu Val
245 250 255

Leu Gln Lys Leu Asn
260

<210> 97
<211> 228
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 97

[0062]

Gly Ser Asp Leu Gly Ile Lys Leu Leu Phe Ala Ala Ala Lys Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Glu Gly Tyr Thr Pro Leu His Val Ala Ala Tyr Asp Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Ser Gln Gly Arg Thr Pro Leu His Glu Ala Ala Tyr Ser
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Asp Ala Gly Glu Thr Pro Ala Asp Leu Ala Ala Ala
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Gly Gly Gly
115 120 125

Gly Ser Gly Gly Gly Gly Ser Arg Ser Asp Leu Gly Ala Lys Leu Leu
130 135 140

Val Ala Ala Thr Ser Gly Gln Asp Asp Glu Val Arg Ile Leu Leu Ala
145 150 155 160

Ala Gly Ala Asp Val Asn Ala Lys Asp Arg Ile Gly Phe Thr Pro Leu
165 170 175

His Arg Ala Ala Phe Val Gly His Leu Glu Ile Val Glu Val Leu Leu
180 185 190

Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Asp Phe Gly His Thr Pro
195 200 205

Ala Asp Leu Ala Ala Ser Leu Gly His Glu Asp Ile Ala Glu Val Leu

[0063]

210 215 220

Gln Lys Leu Asn
225

<210> 98
<211> 261
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 98

Gly Ser Asp Leu Gly Ile Lys Leu Leu Phe Ala Ala Ala Lys Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Glu Gly Tyr Thr Pro Leu His Val Ala Ala Tyr Asp Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Ser Gln Gly Arg Thr Pro Leu His Glu Ala Ala Tyr Ser
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Asp Ala Gly Glu Thr Pro Ala Asp Leu Ala Ala Ala
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Gly Gly Gly
115 120 125

Gly Ser Gly Gly Gly Gly Ser Arg Ser Asp Leu Gly Lys Lys Leu Leu
130 135 140

[0064]

Glu Ala Ala Arg Ala Gly Gln Asp Asp Glu Val Arg Ile Leu Met Ala
145 150 155 160

Asn Gly Ala Asp Val Asn Ala Lys Asp Glu Tyr Gly Leu Thr Pro Leu
165 170 175

Tyr Leu Ala Thr Ala His Gly His Leu Glu Ile Val Glu Val Leu Leu
180 185 190

Lys Asn Gly Ala Asp Val Asn Ala Val Asp Ala Ile Gly Phe Thr Pro
195 200 205

Leu His Leu Ala Ala Phe Ile Gly His Leu Glu Ile Ala Glu Val Leu
210 215 220

Leu Lys His Gly Ala Asp Val Asn Ala Gln Asp Lys Phe Gly Lys Thr
225 230 235 240

Ala Phe Asp Ile Ser Ile Gly Asn Gly Asn Glu Asp Leu Ala Glu Ile
245 250 255

Leu Gln Lys Leu Asn
260

<210> 99
<211> 261
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 99

Gly Ser Asp Leu Gly His Lys Leu Leu Glu Ala Ala Val Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Trp Tyr Gly Lys Thr Pro Leu His Phe Ala Ala Gly Leu Gly
35 40 45

[0065]

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Glu Asp Gly Gln Thr Pro Leu His Leu Ala Ala Ala Tyr
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Asn Asp Gly Phe Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Ser Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Gly Gly Gly
115 120 125

Gly Ser Gly Gly Gly Gly Ser Arg Ser Asp Leu Gly Ile Lys Leu Leu
130 135 140

Gln Ala Ala Asn Leu Gly Gln Asp Asp Glu Val Arg Ile Leu Leu Ala
145 150 155 160

Thr Gly Ala Asp Val Asn Ala Lys Asp Ser Ile Gly Gln Thr Pro Leu
165 170 175

His Trp Ala Ala Arg Arg Gly His Leu Glu Ile Val Glu Val Leu Leu
180 185 190

Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Glu Tyr Gly Val Thr Pro
195 200 205

Leu His Leu Ala Ala Ser Leu Gly His Leu Glu Ile Val Glu Val Leu
210 215 220

Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Glu Ser Gly Glu Thr
225 230 235 240

Pro Ala Asp Leu Ala Ala Leu His Gly His Glu Asp Ile Ala Glu Val
245 250 255

[0066]

Leu Gln Lys Leu Asn
260

<210> 100
<211> 228
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 100

Gly Ser Asp Leu Gly His Lys Leu Leu Glu Ala Ala Val Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Trp Tyr Gly Lys Thr Pro Leu His Phe Ala Ala Gly Leu Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Glu Asp Gly Gln Thr Pro Leu His Leu Ala Ala Ala Tyr
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Asn Asp Gly Phe Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Ser Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Gly Gly Gly
115 120 125

Gly Ser Gly Gly Gly Gly Ser Arg Ser Asp Leu Gly Ala Lys Leu Leu
130 135 140

Val Ala Ala Thr Ser Gly Gln Asp Asp Glu Val Arg Ile Leu Leu Ala
145 150 155 160

[0067]

Ala Gly Ala Asp Val Asn Ala Lys Asp Arg Ile Gly Phe Thr Pro Leu
165 170 175

His Arg Ala Ala Phe Val Gly His Leu Glu Ile Val Glu Val Leu Leu
180 185 190

Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Asp Phe Gly His Thr Pro
195 200 205

Ala Asp Leu Ala Ala Ser Leu Gly His Glu Asp Ile Ala Glu Val Leu
210 215 220

Gln Lys Leu Asn
225

<210> 101
<211> 261
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 101

Gly Ser Asp Leu Gly His Lys Leu Leu Glu Ala Ala Val Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Trp Tyr Gly Lys Thr Pro Leu His Phe Ala Ala Gly Leu Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Glu Asp Gly Gln Thr Pro Leu His Leu Ala Ala Ala Tyr
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

[0068]

Asn Ala Gln Asp Asn Asp Gly Phe Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Ser Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Gly Gly Gly
115 120 125

Gly Ser Gly Gly Gly Gly Ser Arg Ser Asp Leu Gly Lys Lys Leu Leu
130 135 140

Glu Ala Ala Arg Ala Gly Gln Asp Asp Glu Val Arg Ile Leu Met Ala
145 150 155 160

Asn Gly Ala Asp Val Asn Ala Lys Asp Glu Tyr Gly Leu Thr Pro Leu
165 170 175

Tyr Leu Ala Thr Ala His Gly His Leu Glu Ile Val Glu Val Leu Leu
180 185 190

Lys Asn Gly Ala Asp Val Asn Ala Val Asp Ala Ile Gly Phe Thr Pro
195 200 205

Leu His Leu Ala Ala Phe Ile Gly His Leu Glu Ile Ala Glu Val Leu
210 215 220

Leu Lys His Gly Ala Asp Val Asn Ala Gln Asp Lys Phe Gly Lys Thr
225 230 235 240

Ala Phe Asp Ile Ser Ile Gly Asn Gly Asn Glu Asp Leu Ala Glu Ile
245 250 255

Leu Gln Lys Leu Asn
260

<210> 102
<211> 269
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

[0069]

<400> 102

Gly Ser Asp Leu Gly Ala Lys Leu Leu Ser Asp Leu Gly Val Lys Leu
1 5 10 15

Leu Trp Ala Ala Ala Arg Gly Gln Asp Asp Glu Val Arg Ile Leu Leu
20 25 30

Ala Ala Gly Ala Asp Val Asn Ala Lys Asp Phe Gln Gly Ile Thr Pro
35 40 45

Leu His Ile Ala Ala Gln Ser Gly His Leu Glu Ile Val Glu Val Leu
50 55 60

Leu Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Val Thr Gly Asp Thr
65 70 75 80

Pro Leu His Leu Ala Ala Gln His Gly His Leu Val Ile Val Glu Val
85 90 95

Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Glu Arg Gly Trp
100 105 110

Thr Pro Ala Asp Leu Ala Ala Asp Trp Gly His Glu Asp Ile Ala Glu
115 120 125

Val Leu Gln Lys Leu Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Arg
130 135 140

Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln Asp
145 150 155 160

Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala Lys
165 170 175

Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala Thr Ala His Gly His
180 185 190

Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val Asn Ala
195 200 205

[0070]

Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu Ala Ala Phe Ile Gly
210 215 220

His Leu Glu Ile Ala Glu Val Leu Leu Lys His Gly Ala Asp Val Asn
225 230 235 240

Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile Gly Asn
245 250 255

Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
260 265

<210> 103
<211> 302
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 103

Gly Ser Asp Leu Gly Ala Lys Leu Leu Ser Asp Leu Gly Val Lys Leu
1 5 10 15

Leu Trp Ala Ala Ala Arg Gly Gln Asp Asp Glu Val Arg Ile Leu Leu
20 25 30

Ala Ala Gly Ala Asp Val Asn Ala Lys Asp Phe Gln Gly Ile Thr Pro
35 40 45

Leu His Ile Ala Ala Gln Ser Gly His Leu Glu Ile Val Glu Val Leu
50 55 60

Leu Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Val Thr Gly Asp Thr
65 70 75 80

Pro Leu His Leu Ala Ala Gln His Gly His Leu Val Ile Val Glu Val
85 90 95

Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Glu Arg Gly Trp

[0071]

100 105 110

Thr Pro Ala Asp Leu Ala Ala Asp Trp Gly His Glu Asp Ile Ala Glu
115 120 125

Val Leu Gln Lys Leu Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Arg
130 135 140

Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln Asp
145 150 155 160

Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala Thr
165 170 175

Asp Ile His Gly His Thr Pro Leu His Leu Ala Ala Ala Met Gly His
180 185 190

Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val Asn Ala
195 200 205

Asn Asp Trp Arg Gly Phe Thr Pro Leu His Leu Ala Ala Leu Asn Gly
210 215 220

His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val Asn
225 230 235 240

Ala Thr Asp Thr Ala Gly Asn Thr Pro Leu His Leu Ala Ala Trp Phe
245 250 255

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val
260 265 270

Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile Asp
275 280 285

Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
290 295 300

<210> 104
<211> 270

[0072]

<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 104

Gly Ser Asp Leu Gly Asp Lys Leu Leu Gln Ser Asp Leu Gly Asn Lys
1 5 10 15

Leu Leu Ile Ala Ala Ser Val Gly Gln Asp Asp Glu Val Arg Ile Leu
20 25 30

Leu Ala Ala Gly Ala Asp Val Asn Ala Lys Asp Glu Thr Gly Trp Thr
35 40 45

Pro Leu His Leu Ala Ala Ala Trp Gly His Leu Glu Ile Val Glu Val
50 55 60

Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Val Lys Gly Gln
65 70 75 80

Thr Pro Leu His Leu Ala Ala Ala Tyr Gly His Leu Glu Ile Val Glu
85 90 95

Val Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Asn Asp Gly
100 105 110

Tyr Thr Pro Ala Asp Leu Ala Ala Arg Tyr Gly His Glu Asp Ile Ala
115 120 125

Glu Val Leu Gln Lys Leu Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
130 135 140

Arg Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
145 150 155 160

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
165 170 175

Lys Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala Thr Ala His Gly

[0073]

180 185 190

His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val Asn
195 200 205

Ala Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu Ala Ala Phe Ile
210 215 220

Gly His Leu Glu Ile Ala Glu Val Leu Leu Lys His Gly Ala Asp Val
225 230 235 240

Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile Gly
245 250 255

Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
260 265 270

<210> 105
<211> 303
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 105

Gly Ser Asp Leu Gly Asp Lys Leu Leu Gln Ser Asp Leu Gly Asn Lys
1 5 10 15

Leu Leu Ile Ala Ala Ser Val Gly Gln Asp Asp Glu Val Arg Ile Leu
20 25 30

Leu Ala Ala Gly Ala Asp Val Asn Ala Lys Asp Glu Thr Gly Trp Thr
35 40 45

Pro Leu His Leu Ala Ala Ala Trp Gly His Leu Glu Ile Val Glu Val
50 55 60

Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Val Lys Gly Gln
65 70 75 80

[0074]

Thr Pro Leu His Leu Ala Ala Ala Tyr Gly His Leu Glu Ile Val Glu
85 90 95

Val Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Asn Asp Gly
100 105 110

Tyr Thr Pro Ala Asp Leu Ala Ala Arg Tyr Gly His Glu Asp Ile Ala
115 120 125

Glu Val Leu Gln Lys Leu Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
130 135 140

Arg Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
145 150 155 160

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
165 170 175

Thr Asp Ile His Gly His Thr Pro Leu His Leu Ala Ala Ala Met Gly
180 185 190

His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val Asn
195 200 205

Ala Asn Asp Trp Arg Gly Phe Thr Pro Leu His Leu Ala Ala Leu Asn
210 215 220

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val
225 230 235 240

Asn Ala Thr Asp Thr Ala Gly Asn Thr Pro Leu His Leu Ala Ala Trp
245 250 255

Phe Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp
260 265 270

Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile
275 280 285

Asp Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn

[0075]

290 295 300

<210> 106
<211> 294
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 106

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Tyr Gly Ile Thr Pro Leu His Leu Ala Ala Ala Tyr Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys His Gly Ala Asp Val Asn
50 55 60

Ala His Asp Trp Asn Gly Trp Thr Pro Leu His Leu Ala Ala Lys Tyr
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys His Gly Ala Asp Val
85 90 95

Asn Ala Ile Asp Asn Ala Gly Lys Thr Pro Leu His Leu Ala Ala Ala
100 105 110

His Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Tyr Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile
130 135 140

Asp Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Gly Gly
145 150 155 160

[0076]

Gly Gly Ser Gly Gly Gly Gly Ser Arg Ser Asp Leu Gly Lys Lys Leu
165 170 175

Leu Glu Ala Ala Arg Ala Gly Gln Asp Asp Glu Val Arg Ile Leu Met
180 185 190

Ala Asn Gly Ala Asp Val Asn Ala Lys Asp Glu Tyr Gly Leu Thr Pro
195 200 205

Leu Tyr Leu Ala Thr Ala His Gly His Leu Glu Ile Val Glu Val Leu
210 215 220

Leu Lys Asn Gly Ala Asp Val Asn Ala Val Asp Ala Ile Gly Phe Thr
225 230 235 240

Pro Leu His Leu Ala Ala Phe Ile Gly His Leu Glu Ile Ala Glu Val
245 250 255

Leu Leu Lys His Gly Ala Asp Val Asn Ala Gln Asp Lys Phe Gly Lys
260 265 270

Thr Ala Phe Asp Ile Ser Ile Gly Asn Gly Asn Glu Asp Leu Ala Glu
275 280 285

Ile Leu Gln Lys Leu Asn
290

<210> 107
<211> 327
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 107

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

[0077]

Lys Asp Phe Tyr Gly Ile Thr Pro Leu His Leu Ala Ala Ala Tyr Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys His Gly Ala Asp Val Asn
50 55 60

Ala His Asp Trp Asn Gly Trp Thr Pro Leu His Leu Ala Ala Lys Tyr
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys His Gly Ala Asp Val
85 90 95

Asn Ala Ile Asp Asn Ala Gly Lys Thr Pro Leu His Leu Ala Ala Ala
100 105 110

His Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Tyr Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile
130 135 140

Asp Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Gly Gly
145 150 155 160

Gly Gly Ser Gly Gly Gly Gly Ser Arg Ser Asp Leu Gly Lys Lys Leu
165 170 175

Leu Glu Ala Ala Arg Ala Gly Gln Asp Asp Glu Val Arg Ile Leu Met
180 185 190

Ala Asn Gly Ala Asp Val Asn Ala Thr Asp Ile His Gly His Thr Pro
195 200 205

Leu His Leu Ala Ala Ala Met Gly His Leu Glu Ile Val Glu Val Leu
210 215 220

Leu Lys Asn Gly Ala Asp Val Asn Ala Asn Asp Trp Arg Gly Phe Thr
225 230 235 240

[0078]

Pro Leu His Leu Ala Ala Leu Asn Gly His Leu Glu Ile Val Glu Val
245 250 255

Leu Leu Lys Asn Gly Ala Asp Val Asn Ala Thr Asp Thr Ala Gly Asn
260 265 270

Thr Pro Leu His Leu Ala Ala Trp Phe Gly His Leu Glu Ile Val Glu
275 280 285

Val Leu Leu Lys Asn Gly Ala Asp Val Asn Ala Gln Asp Lys Phe Gly
290 295 300

Lys Thr Ala Phe Asp Ile Ser Ile Asp Asn Gly Asn Glu Asp Leu Ala
305 310 315 320

Glu Ile Leu Gln Lys Leu Asn
325

<210> 108
<211> 270
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 108

Gly Ser Asp Leu Gly Asp Lys Leu Leu Gln Ser Asp Leu Gly Ile Lys
1 5 10 15

Leu Leu Phe Ala Ala Ala Lys Ser Gln Asp Asp Glu Val Arg Ile Leu
20 25 30

Leu Ala Ala Gly Ala Asp Val Asn Ala Lys Asp Phe Gln Ser Val Thr
35 40 45

Pro Leu His Ile Ala Ala Gln Ser Gly His Leu Glu Ile Val Glu Val
50 55 60

Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Val Thr Gly Asp
65 70 75 80

[0079]

Thr Pro Leu His Leu Ala Ala Gln His Gly His Leu Glu Ile Val Glu
85 90 95

Val Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Glu Arg Gly
100 105 110

Trp Thr Pro Ala Asp Leu Ala Ala Asp Trp Gly His Glu Asp Ile Ala
115 120 125

Glu Val Leu Gln Lys Leu Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
130 135 140

Arg Ser Asp Leu Gly Ile Lys Leu Leu Val Ala Ala Ala Gln Gly Gln
145 150 155 160

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
165 170 175

Lys Asp Gln Gln Gly Ala Thr Pro Leu His Leu Ala Ala Trp Lys Gly
180 185 190

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
195 200 205

Ala Lys Asp Leu Ser Gly Asp Thr Pro Leu His Ile Ala Ala Trp Phe
210 215 220

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
225 230 235 240

Asn Ala Gln Asp Thr Glu Gly Tyr Thr Pro Ala Asp Leu Ala Ala Leu
245 250 255

Tyr Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
260 265 270

<210> 109
<211> 294
<212> PRT
<213> Artificial

[0080]

<220>
<223> AR domain (two-domain)

<400> 109

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala Thr Ala His Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val Asn
50 55 60

Ala Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu Ala Ala Phe Ile
65 70 75 80

Gly His Leu Glu Ile Ala Glu Val Leu Leu Lys His Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile Gly
100 105 110

Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Gly Gly Gly
115 120 125

Gly Ser Gly Gly Gly Gly Ser Arg Ser Asp Leu Gly Lys Lys Leu Leu
130 135 140

Glu Ala Ala Arg Ala Gly Gln Asp Asp Glu Val Arg Ile Leu Met Ala
145 150 155 160

Asn Gly Ala Asp Val Asn Ala Lys Asp Phe Tyr Gly Ile Thr Pro Leu
165 170 175

His Leu Ala Ala Ala Tyr Gly His Leu Glu Ile Val Glu Val Leu Leu
180 185 190

[0081]

Lys His Gly Ala Asp Val Asn Ala His Asp Trp Asn Gly Trp Thr Pro
195 200 205

Leu His Leu Ala Ala Lys Tyr Gly His Leu Glu Ile Val Glu Val Leu
210 215 220

Leu Lys His Gly Ala Asp Val Asn Ala Ile Asp Asn Ala Gly Lys Thr
225 230 235 240

Pro Leu His Leu Ala Ala Ala His Gly His Leu Glu Ile Val Glu Val
245 250 255

Leu Leu Lys Tyr Gly Ala Asp Val Asn Ala Gln Asp Lys Phe Gly Lys
260 265 270

Thr Ala Phe Asp Ile Ser Ile Asp Asn Gly Asn Glu Asp Leu Ala Glu
275 280 285

Ile Leu Gln Lys Leu Asn
290

<210> 110
<211> 269
<212> PRT
<213> Artificial

<220>
<223> AR domain (two-domain)

<400> 110

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala Thr Ala His Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val Asn
50 55 60

[0082]

Ala Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu Ala Ala Phe Ile
65 70 75 80

Gly His Leu Glu Ile Ala Glu Val Leu Leu Lys His Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile Gly
100 105 110

Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Gly Gly Gly
115 120 125

Gly Ser Gly Gly Gly Gly Ser Arg Ser Asp Leu Gly Ala Lys Leu Leu
130 135 140

Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala Arg Gly Gln Asp
145 150 155 160

Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala Lys
165 170 175

Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Gln Ser Gly His
180 185 190

Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
195 200 205

Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His Gly
210 215 220

His Leu Val Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
225 230 235 240

Ala Gln Asp Glu Arg Gly Trp Thr Pro Ala Asp Leu Ala Ala Asp Trp
245 250 255

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
260 265

[0083]

<210> 111
<211> 126
<212> PRT
<213> Artificial

<220>
<223> AR domain (one-domain) Negative Control

<400> 111

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Lys Asp Gly Tyr Thr Pro Leu His Leu Ala Ala Arg Glu Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Lys Asp Gly Tyr Thr Pro Leu His Leu Ala Ala Arg Glu
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile Asp
100 105 110

Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
115 120 125

<210> 112
<211> 126
<212> PRT
<213> Artificial Sequence

<220>
<223> AR domain (one-domain)

<400> 112

[0084]

Gly Ser Asp Leu Gly Asn Lys Leu Leu Ile Ala Ala Ser Val Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Glu Thr Gly Trp Thr Pro Leu His Leu Ala Ala Ala Trp Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Lys Gly Gln Thr Pro Leu His Leu Ala Ala Ala Tyr
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Asn Asp Gly Tyr Thr Pro Ala Asp Leu Ala Ala Arg
100 105 110

Tyr Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 113
<211> 126
<212> PRT
<213> Artificial Sequence

<220>
<223> AR domain (one-domain)

<400> 113

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala His Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Asp Ala Asp Val Asn Ala
20 25 30

Lys Asp Trp Tyr Gly Thr Thr Pro Leu His Ile Ala Ala Val Ala Gly
35 40 45

[0085]

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Asp Phe Gly Thr Thr Pro Leu His Ala Ala Ala Asp Tyr
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Tyr Thr Gly His Thr Pro Ala Asp Leu Ala Ala Val
100 105 110

Tyr Gly His Glu Asp Ile Ala Ala Val Leu Gln Lys Leu Asn
115 120 125

<210> 114
<211> 126
<212> PRT
<213> Artificial Sequence

<220>
<223> AR domain (one-domain)

<400> 114

Gly Ser Asp Leu Gly Ala Lys Leu Leu Trp Ala Ala Ala Lys Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Trp Glu Gly Val Thr Pro Leu His Ile Ala Ala His Ala Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Ile Ile Gly Trp Thr Pro Leu His Ser Ala Ala Val Tyr
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

[0086]

Asn Ala Gln Asp Trp Tyr Gly Thr Thr Pro Ala Asp Leu Ala Ala Trp
100 105 110

Trp Gly His Glu Asp Ile Ala Val Val Leu Gln Lys Leu Asn
115 120 125

<210> 115
<211> 126
<212> PRT
<213> Artificial Sequence

<220>
<223> AR domain (one-domain)

<400> 115

Gly Ser Asp Leu Gly Ile Lys Leu Leu Phe Ala Ala Ala Lys Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Val Thr Pro Leu His Ile Ala Ala Gln Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Glu Arg Gly Trp Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 116

[0087]

<211> 126
<212> PRT
<213> Artificial Sequence

<220>
<223> AR doamin (one-domain)

<400> 116

Gly Ser Asp Leu Gly Ile Lys Leu Leu Ile Ala Ala Ser His Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asn Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Val Thr Pro Leu His Ile Ala Ala Gln Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Glu Arg Gly Trp Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 117
<211> 126
<212> PRT
<213> Artificial Sequence

<220>
<223> AR domain (one-domain)

<400> 117

Gly Ser Asp Leu Gly Gln Lys Leu Leu Ile Ala Ala Ser Arg Gly Gln
1 5 10 15

[0088]

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Val Thr Pro Leu His Ile Ala Ala Gln Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Glu Arg Gly Trp Thr Pro Thr Asp Leu Ala Ala Asp
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 118
<211> 126
<212> PRT
<213> Artificial Sequence

<220>
<223> AR domain (one-domain)

<400> 118

Gly Ser Asp Leu Gly Ile Lys Leu Leu Trp Ala Ala Ala Gln Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Val Thr Pro Leu His Ile Ala Ala Gln Ser Gly
35 40 45

His Leu Glu Val Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn

[0089]

50 55 60

Ala Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Glu Arg Gly Trp Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 119
<211> 126
<212> PRT
<213> Artificial Sequence

<220>
<223> AR domain (one-domain)

<400> 119

Gly Ser Asp Leu Gly Phe Lys Leu Leu Phe Ala Ala Ala Lys Ser Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Val Thr Ser Leu His Ile Ala Ala Gln Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

[0090]

Asn Ala Gln Asp Glu Arg Gly Trp Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 120
<211> 126
<212> PRT
<213> Artificial Sequence

<220>
<223> AR domain (one-domain)

<400> 120

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Thr Asn Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Ile Thr Gly Glu Thr Pro Leu His His Ala Ala Asp Ser
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Lys Ala Gly Val Thr Pro Ala Asp Leu Ala Ala Ala
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 121
<211> 126
<212> PRT

[0091]

<213> Artificial Sequence

<220>
<223> AR domain (one-domain)

<400> 121

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Gln Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Glu Arg Gly Trp Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 122
<211> 274
<212> PRT
<213> Artificial Sequence

<220>
<223> AR domain (two-domain)

<400> 122

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala Arg Gly Gln
1 5 10 15

[0092]

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Gln Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Glu Arg Gly Trp Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Ala Ala Gly Ser
115 120 125

Pro Thr Pro Thr Pro Thr Thr Pro Thr Pro Thr Pro Thr Thr Pro Thr
130 135 140

Pro Thr Pro Thr Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala
145 150 155 160

Arg Ala Gly Gln Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala
165 170 175

Asp Val Asn Ala Lys Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala
180 185 190

Thr Ala His Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly
195 200 205

Ala Asp Val Asn Ala Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu
210 215 220

Ala Ala Phe Ile Gly His Leu Glu Ile Ala Glu Val Leu Leu Lys Ala

[0093]

225 230 235 240

Gly Ala Asp Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Pro Ala Asp
245 250 255

Ile Ala Ala Gly Ala Gly Asn Glu Asp Ile Ala Glu Val Leu Gln Lys
260 265 270

Ala Ala

<210> 123
<211> 274
<212> PRT
<213> Artificial Sequence

<220>
<223> AR domain (two-domain)

<400> 123

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Gln Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Glu Arg Gly Lys Thr Pro Ala Asp Leu Ala Ala Asp
100 105 110

[0094]

Trp Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Ala Ala Gly Ser
115 120 125

Pro Thr Pro Thr Pro Thr Thr Pro Thr Pro Thr Pro Thr Thr Pro Thr
130 135 140

Pro Thr Pro Thr Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala
145 150 155 160

Arg Ala Gly Gln Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala
165 170 175

Asp Val Asn Ala Lys Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala
180 185 190

Thr Ala His Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly
195 200 205

Ala Asp Val Asn Ala Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu
210 215 220

Ala Ala Phe Ile Gly His Leu Glu Ile Ala Glu Val Leu Leu Lys Ala
225 230 235 240

Gly Ala Asp Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Pro Ala Asp
245 250 255

Ile Ala Ala Gly Ala Gly Asn Glu Asp Ile Ala Glu Val Leu Gln Lys
260 265 270

Ala Ala

<210> 124
<211> 32
<212> PRT
<213> Artificial Sequence

<220>
<223> N-Cap module (Nr)

<400> 124

[0095]

Gly Ser Asp Leu Gly Ile Lys Leu Leu Phe Ala Ala Ala Lys Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 125
<211> 33
<212> PRT
<213> Artificial Sequence

<220>
<223> AR module (M1.1b)

<400> 125

Lys Asp Phe Gln Gly Val Thr Pro Leu His Ile Ala Ala Gln Ser Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 126
<211> 33
<212> PRT
<213> Artificial Sequence

<220>
<223> AR module (M1.1b)

<400> 126

Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

[0096]

<210> 127
<211> 28
<212> PRT
<213> Artificial Sequence

<220>
<223> C-Cap module (Cr)

<400> 127

Gln Asp Glu Arg Gly Trp Thr Pro Ala Asp Leu Ala Ala Asp Trp Gly
1 5 10 15

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

<210> 128
<211> 32
<212> PRT
<213> Artificial Sequence

<220>
<223> N-Cap module (Nr)

<400> 128

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 129
<211> 33
<212> PRT
<213> Artificial Sequence

<220>
<223> AR module (M1.1b)

<400> 129

Lys Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Thr Asn Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

[0097]

Ala

<210> 130
<211> 33
<212> PRT
<213> Artificial Sequence

<220>
<223> AR module (M1.1b)

<400> 130

Lys Asp Ile Thr Gly Glu Thr Pro Leu His His Ala Ala Asp Ser Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 131
<211> 28
<212> PRT
<213> Artificial Sequence

<220>
<223> C-Cap module (Cr)

<400> 131

Gln Asp Lys Ala Gly Val Thr Pro Ala Asp Leu Ala Ala Ala Trp Gly
1 5 10 15

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

<210> 132
<211> 32
<212> PRT
<213> Artificial Sequence

<220>
<223> N-Cap module (Nr)

[0098]

<400> 132

Gly Ser Asp Leu Gly Val Lys Leu Leu Trp Ala Ala Ala Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 133
<211> 33
<212> PRT
<213> Artificial Sequence

<220>
<223> AR module (M1.1b)

<400> 133

Lys Asp Phe Gln Gly Ile Thr Pro Leu His Ile Ala Ala Gln Ser Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 134
<211> 33
<212> PRT
<213> Artificial Sequence

<220>
<223> AR module (M1.1b)

<400> 134

Lys Asp Val Thr Gly Asp Thr Pro Leu His Leu Ala Ala Gln His Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

[0099]

<210> 135
<211> 28
<212> PRT
<213> Artificial Sequence

<220>
<223> C-Cap module (Cr)

<400> 135

Gln Asp Glu Arg Gly Trp Thr Pro Ala Asp Leu Ala Ala Asp Trp Gly
1 5 10 15

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

<210> 136
<211> 278
<212> PRT
<213> Artificial Sequence

<220>
<223> AR domain (two-domain)

<400> 136

Gly Ser Asp Leu Gly Ala Lys Leu Leu Ser Asp Leu Gly Val Lys Leu
1 5 10 15

Leu Trp Ala Ala Ala Arg Gly Gln Asp Asp Glu Val Arg Ile Leu Leu
20 25 30

Ala Ala Gly Ala Asp Val Asn Ala Lys Asp Phe Gln Gly Ile Thr Pro
35 40 45

Leu His Ile Ala Ala Gln Ser Gly His Leu Glu Ile Val Glu Val Leu
50 55 60

Leu Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Val Thr Gly Asp Thr
65 70 75 80

Pro Leu His Leu Ala Ala Gln His Gly His Leu Val Ile Val Glu Val
85 90 95

[0100]

Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Glu Arg Gly Trp
100 105 110

Thr Pro Ala Asp Leu Ala Ala Asp Trp Gly His Glu Asp Ile Ala Glu
115 120 125

Val Leu Gln Lys Leu Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Arg
130 135 140

Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln Asp
145 150 155 160

Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala Lys
165 170 175

Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala Thr Ala His Gly His
180 185 190

Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val Asn Ala
195 200 205

Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu Ala Ala Phe Ile Gly
210 215 220

His Leu Glu Ile Ala Glu Val Leu Leu Lys His Gly Ala Asp Val Asn
225 230 235 240

Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile Gly Asn
245 250 255

Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Ala Ala Gly Gly Gly
260 265 270

Ser Gly Gly Gly Ser Cys
275

<210> 137
<211> 414
<212> PRT
<213> Artificial Sequence

[0101]

<220>
<223> AR domain (three-domain)

<400> 137

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Tyr Phe Ser His Thr Pro Leu His Leu Ala Ala Arg Asn Gly
35 40 45

His Leu Lys Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Phe Ala Gly Lys Thr Pro Leu His Leu Ala Ala Asn Asp
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys His Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Ile Phe Gly Lys Thr Pro Ala Asp Ile Ala Ala Asp
100 105 110

Ala Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Gly Gly Gly
115 120 125

Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly
130 135 140

Ser Arg Ser Asp Leu Gly Ala Lys Leu Leu Ser Asp Leu Gly Val Lys
145 150 155 160

Leu Leu Trp Ala Ala Ala Arg Gly Gln Asp Asp Glu Val Arg Ile Leu
165 170 175

Leu Ala Ala Gly Ala Asp Val Asn Ala Lys Asp Phe Gln Gly Ile Thr
180 185 190

[0102]

Pro Leu His Ile Ala Ala Gln Ser Gly His Leu Glu Ile Val Glu Val
195 200 205

Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Val Thr Gly Asp
210 215 220

Thr Pro Leu His Leu Ala Ala Gln His Gly His Leu Val Ile Val Glu
225 230 235 240

Val Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Glu Arg Gly
245 250 255

Trp Thr Pro Ala Asp Leu Ala Ala Asp Trp Gly His Glu Asp Ile Ala
260 265 270

Glu Val Leu Gln Lys Leu Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
275 280 285

Arg Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
290 295 300

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
305 310 315 320

Lys Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala Thr Ala His Gly
325 330 335

His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val Asn
340 345 350

Ala Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu Ala Ala Phe Ile
355 360 365

Gly His Leu Glu Ile Ala Glu Val Leu Leu Lys His Gly Ala Asp Val
370 375 380

Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile Gly
385 390 395 400

Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn

[0103]

405 410

<210> 138
<211> 417
<212> PRT
<213> Artificial Sequence

<220>
<223> AR domain (three-domain)

<400> 138

Gly Ser Asp Leu Gly Ala Lys Leu Leu Ser Asp Leu Gly Val Lys Leu
1 5 10 15

Leu Trp Ala Ala Ala Arg Gly Gln Asp Asp Glu Val Arg Ile Leu Leu
20 25 30

Ala Ala Gly Ala Asp Val Asn Ala Lys Asp Phe Gln Gly Ile Thr Pro
35 40 45

Leu His Ile Ala Ala Gln Ser Gly His Leu Glu Ile Val Glu Val Leu
50 55 60

Leu Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Val Thr Gly Asp Thr
65 70 75 80

Pro Leu His Leu Ala Ala Gln His Gly His Leu Val Ile Val Glu Val
85 90 95

Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Glu Arg Gly Trp
100 105 110

Thr Pro Ala Asp Leu Ala Ala Asp Trp Gly His Glu Asp Ile Ala Glu
115 120 125

Val Leu Gln Lys Leu Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Arg
130 135 140

Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln Asp
145 150 155 160

[0104]

Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala Lys
165 170 175

Asp Glu Tyr Gly Leu Thr Pro Leu Tyr Leu Ala Thr Ala His Gly His
180 185 190

Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val Asn Ala
195 200 205

Val Asp Ala Ile Gly Phe Thr Pro Leu His Leu Ala Ala Phe Ile Gly
210 215 220

His Leu Glu Ile Ala Glu Val Leu Leu Lys His Gly Ala Asp Val Asn
225 230 235 240

Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile Gly Asn
245 250 255

Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn Lys Leu Gly
260 265 270

Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly
275 280 285

Gly Gly Ser Arg Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg
290 295 300

Ala Gly Gln Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp
305 310 315 320

Val Asn Ala Lys Asp Tyr Phe Ser His Thr Pro Leu His Leu Ala Ala
325 330 335

Arg Asn Gly His Leu Lys Ile Val Glu Val Leu Leu Lys Ala Gly Ala
340 345 350

Asp Val Asn Ala Lys Asp Phe Ala Gly Lys Thr Pro Leu His Leu Ala
355 360 365

Ala Asn Asp Gly His Leu Glu Ile Val Glu Val Leu Leu Lys His Gly

[0105]

370 375 380

Ala Asp Val Asn Ala Gln Asp Ile Phe Gly Lys Thr Pro Ala Asp Ile
385 390 395 400

Ala Ala Asp Ala Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu
405 410 415

Asn

<210> 139
<211> 260
<212> PRT
<213> Artificial Sequence

<220>
<223> AR domain (two-domain)

<400> 139

Gly Ser Asp Leu Gly Ala Lys Leu Leu Ser Asp Leu Gly Val Lys Leu
1 5 10 15

Leu Trp Ala Ala Ala Arg Gly Gln Asp Asp Glu Val Arg Ile Leu Leu
20 25 30

Ala Ala Gly Ala Asp Val Asn Ala Lys Asp Phe Gln Gly Ile Thr Pro
35 40 45

Leu His Ile Ala Ala Gln Ser Gly His Leu Glu Ile Val Glu Val Leu
50 55 60

Leu Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Val Thr Gly Asp Thr
65 70 75 80

Pro Leu His Leu Ala Ala Gln His Gly His Leu Val Ile Val Glu Val
85 90 95

Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Glu Arg Gly Trp
100 105 110

[0106]

Thr Pro Ala Asp Leu Ala Ala Asp Trp Gly His Glu Asp Ile Ala Glu
115 120 125

Val Leu Gln Lys Ala Ala Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu
130 135 140

Ala Ala Arg Ala Gly Gln Asp Asp Glu Val Arg Ile Leu Met Ala Asn
145 150 155 160

Gly Ala Asp Val Asn Ala Lys Asp Glu Tyr Gly Leu Thr Pro Leu Tyr
165 170 175

Leu Ala Thr Ala His Gly His Leu Glu Ile Val Glu Val Leu Leu Lys
180 185 190

Asn Gly Ala Asp Val Asn Ala Val Asp Ala Ile Gly Phe Thr Pro Leu
195 200 205

His Leu Ala Ala Phe Ile Gly His Leu Glu Ile Ala Glu Val Leu Leu
210 215 220

Lys His Gly Ala Asp Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala
225 230 235 240

Phe Asp Ile Ser Ile Gly Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu
245 250 255

Gln Lys Ala Ala
260

<210> 140
<211> 263
<212> PRT
<213> Artificial Sequence

<220>
<223> AR domain (two-domain)

<400> 140

Gly Ser Asp Leu Gly Ala Lys Leu Leu Ser Asp Leu Gly Val Lys Leu
1 5 10 15

[0107]

Leu Trp Ala Ala Ala Arg Gly Gln Asp Asp Glu Val Arg Ile Leu Leu
20 25 30

Ala Ala Gly Ala Asp Val Asn Ala Lys Asp Phe Gln Gly Ile Thr Pro
35 40 45

Leu His Ile Ala Ala Gln Ser Gly His Leu Glu Ile Val Glu Val Leu
50 55 60

Leu Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Val Thr Gly Asp Thr
65 70 75 80

Pro Leu His Leu Ala Ala Gln His Gly His Leu Val Ile Val Glu Val
85 90 95

Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Glu Arg Gly Trp
100 105 110

Thr Pro Ala Asp Leu Ala Ala Asp Trp Gly His Glu Asp Ile Ala Glu
115 120 125

Val Leu Gln Lys Ala Ala Gly Gly Gly Gly Ser Asp Leu Gly Lys Lys
130 135 140

Leu Leu Glu Ala Ala Arg Ala Gly Gln Asp Asp Glu Val Arg Ile Leu
145 150 155 160

Met Ala Asn Gly Ala Asp Val Asn Ala Lys Asp Glu Tyr Gly Leu Thr
165 170 175

Pro Leu Tyr Leu Ala Thr Ala His Gly His Leu Glu Ile Val Glu Val
180 185 190

Leu Leu Lys Asn Gly Ala Asp Val Asn Ala Val Asp Ala Ile Gly Phe
195 200 205

Thr Pro Leu His Leu Ala Ala Phe Ile Gly His Leu Glu Ile Ala Glu
210 215 220

[0108]

Val Leu Leu Lys His Gly Ala Asp Val Asn Ala Gln Asp Lys Phe Gly
225 230 235 240

Lys Thr Ala Phe Asp Ile Ser Ile Gly Asn Gly Asn Glu Asp Leu Ala
245 250 255

Glu Ile Leu Gln Lys Ala Ala
260

<210> 141
<211> 282
<212> PRT
<213> Artificial Sequence

<220>
<223> AR domain (two-domain)

<400> 141

Gly Ser Asp Leu Gly Ala Lys Leu Leu Ser Asp Leu Gly Val Lys Leu
1 5 10 15

Leu Trp Ala Ala Ala Arg Gly Gln Asp Asp Glu Val Arg Ile Leu Leu
20 25 30

Ala Ala Gly Ala Asp Val Asn Ala Lys Asp Phe Gln Gly Ile Thr Pro
35 40 45

Leu His Ile Ala Ala Gln Ser Gly His Leu Glu Ile Val Glu Val Leu
50 55 60

Leu Lys Ala Gly Ala Asp Val Asn Ala Lys Asp Val Thr Gly Asp Thr
65 70 75 80

Pro Leu His Leu Ala Ala Gln His Gly His Leu Val Ile Val Glu Val
85 90 95

Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Glu Arg Gly Trp
100 105 110

Thr Pro Ala Asp Leu Ala Ala Asp Trp Gly His Glu Asp Ile Ala Glu
115 120 125

[0109]

Val Leu Gln Lys Ala Ala Gly Ser Pro Thr Pro Thr Pro Thr Thr Pro
130 135 140

Thr Pro Thr Pro Thr Thr Pro Thr Pro Thr Pro Thr Gly Ser Asp Leu
145 150 155 160

Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln Asp Asp Glu Val
165 170 175

Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala Lys Asp Glu Tyr
180 185 190

Gly Leu Thr Pro Leu Tyr Leu Ala Thr Ala His Gly His Leu Glu Ile
195 200 205

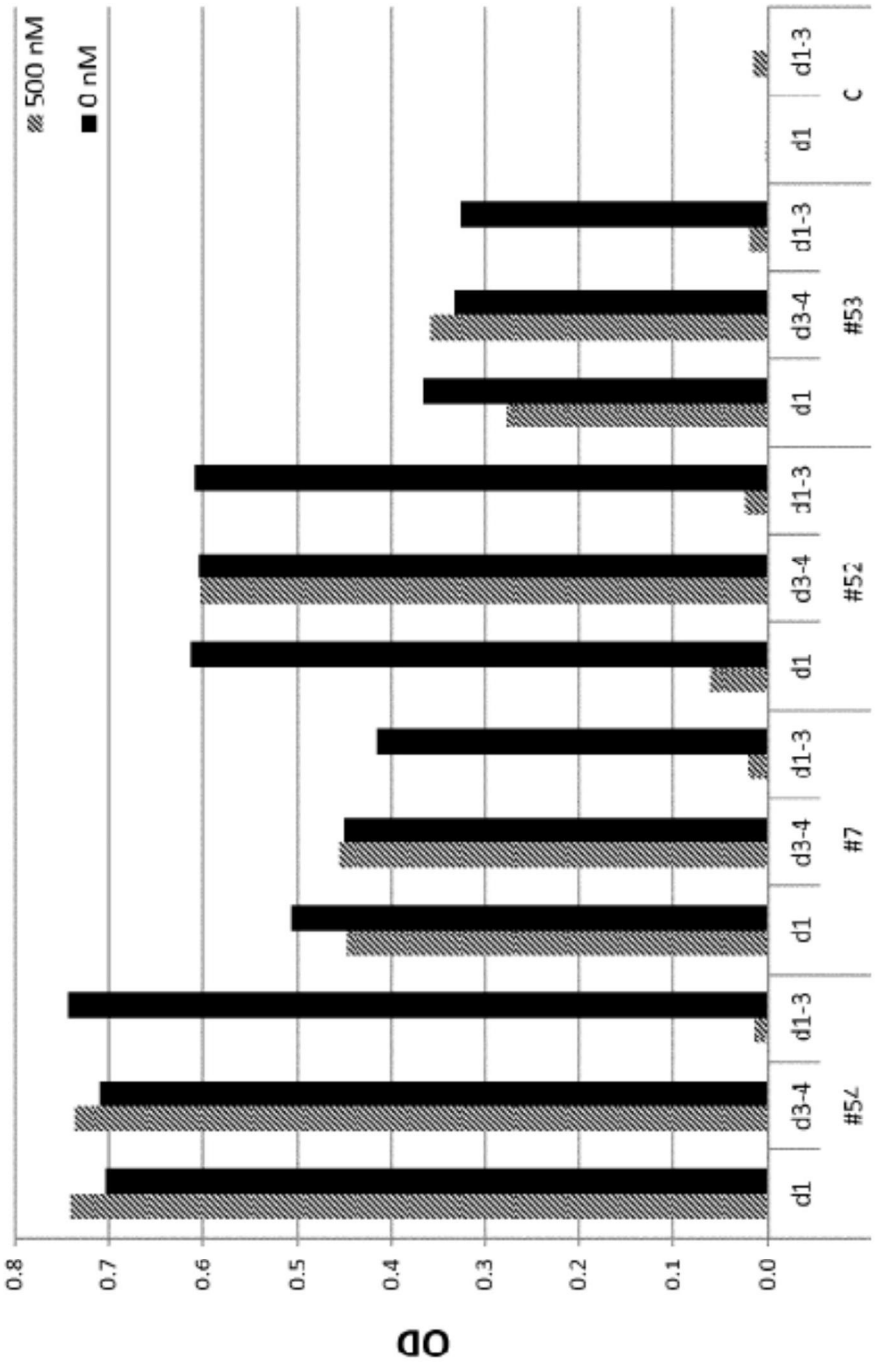
Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val Asn Ala Val Asp Ala
210 215 220

Ile Gly Phe Thr Pro Leu His Leu Ala Ala Phe Ile Gly His Leu Glu
225 230 235 240

Ile Ala Glu Val Leu Leu Lys His Gly Ala Asp Val Asn Ala Gln Asp
245 250 255

Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile Gly Asn Gly Asn Glu
260 265 270

Asp Leu Ala Glu Ile Leu Gln Lys Ala Ala
275 280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0 nM
OD
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0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
d1
d3-4
d1-3
#54
d1
d3-4
d1-3
#7
d1
d3-4
d1-3
#52
d1
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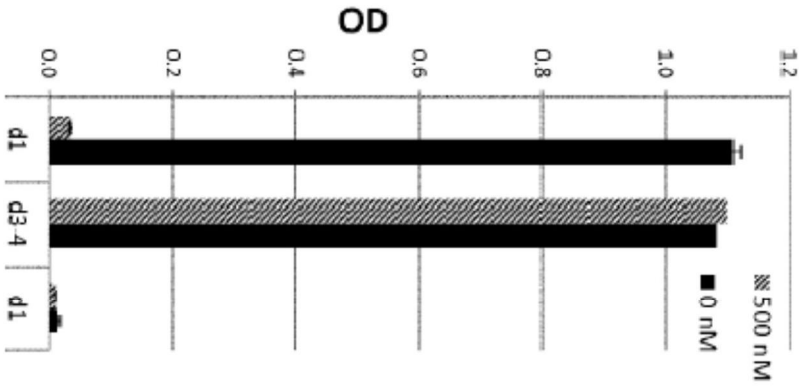
d1-3
#53
d1
d1-3
C

图 1A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0.2
0.4
0.6
0.8
1.0
1.2
d1
d3-4
d1
0 nM
500 nM

图 1B

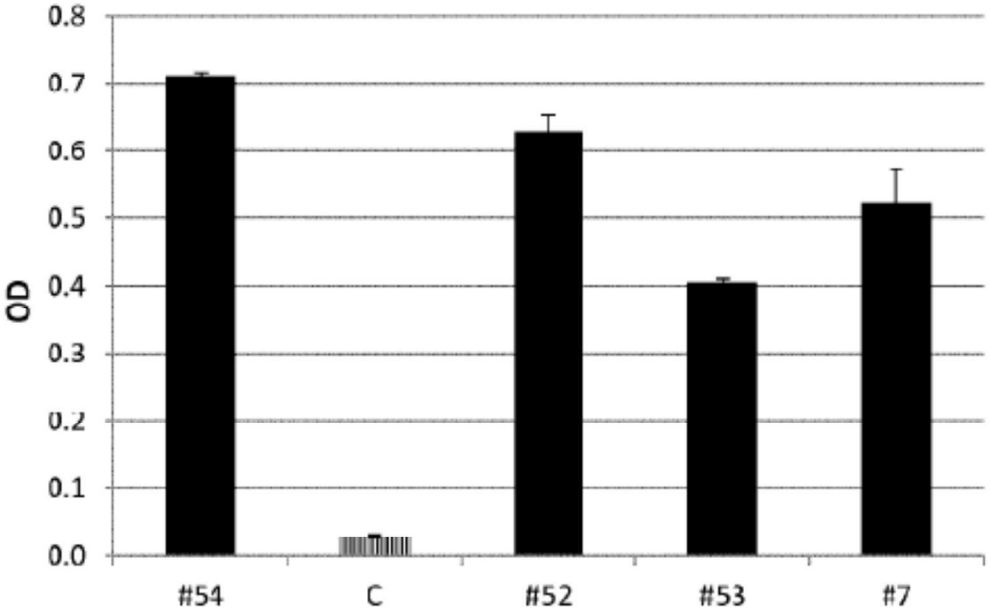
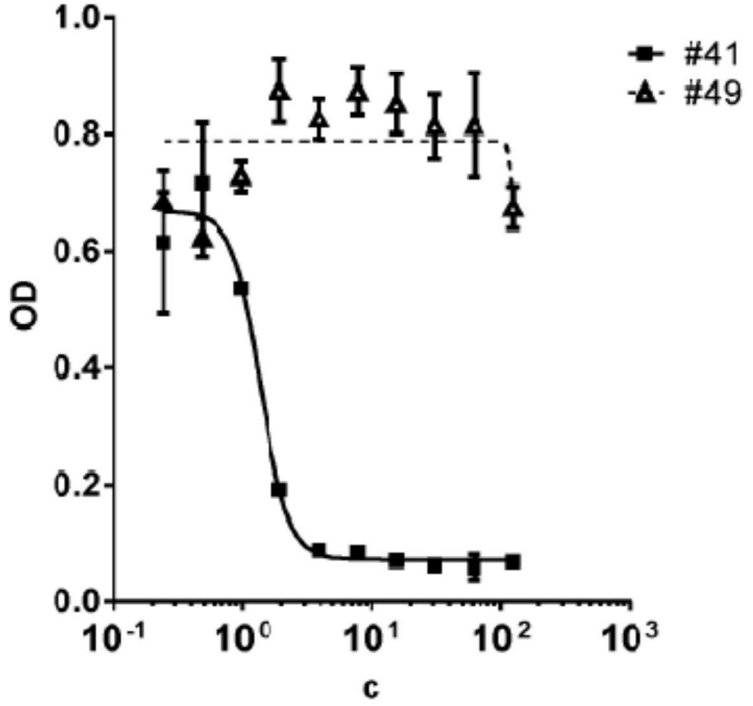

OD
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
#54
C
#52
#53
#7

图 1C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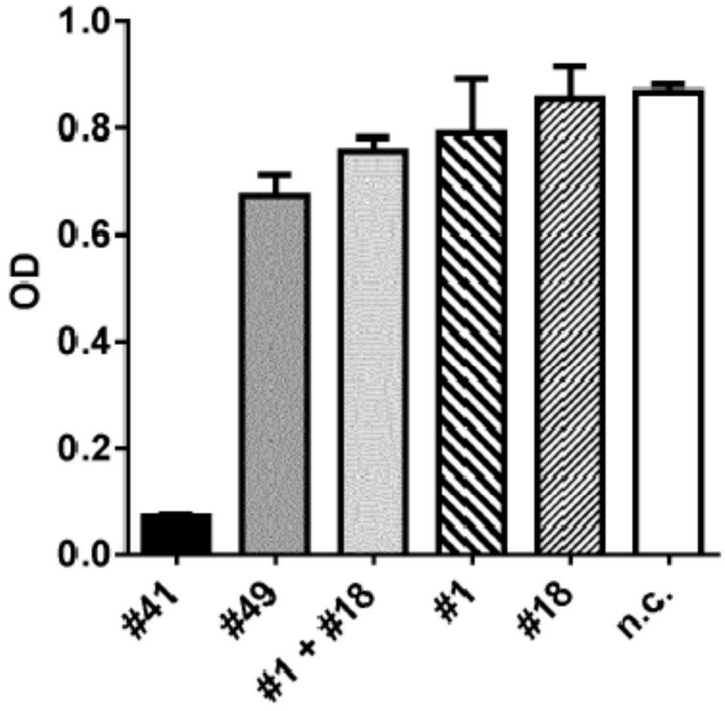
0.8
0.6
0.4
0.2
0.0
OD
#41
#49
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
c

图 2A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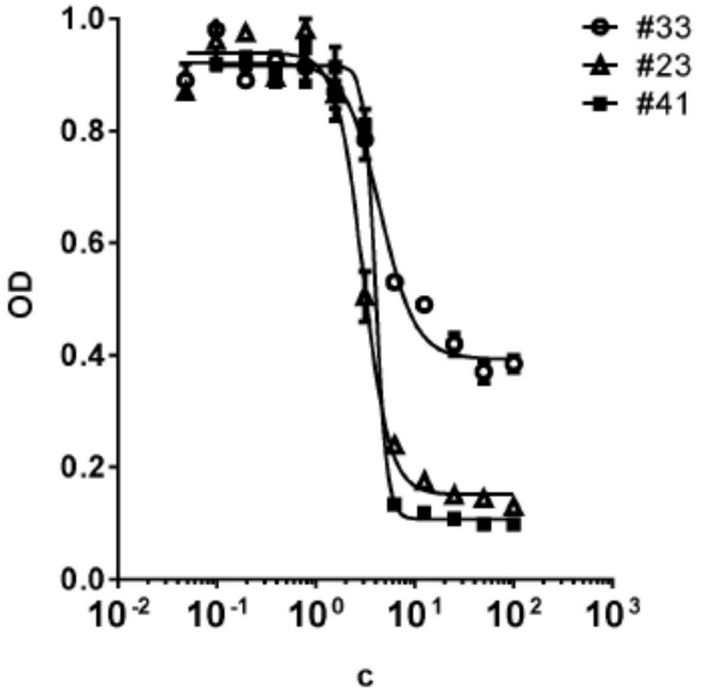
0.8
0.6
0.4
0.2
0.0
OD
#41
#49
#1 + #18
#1
#18
n.c.

图 2B


#33
#23
#41
OD
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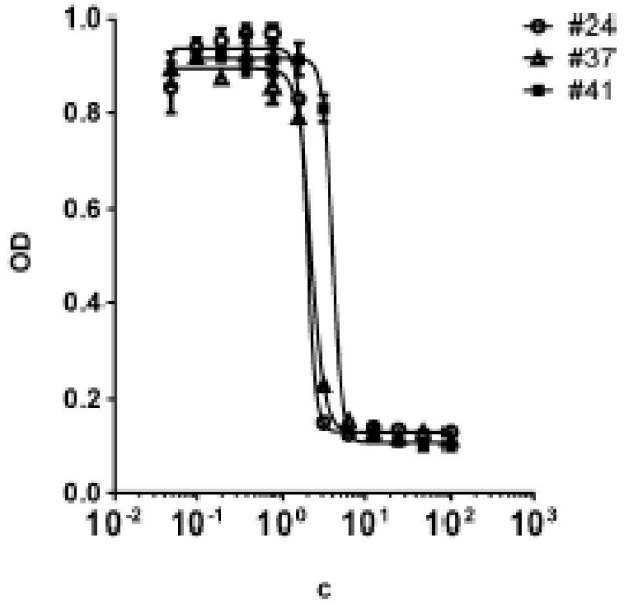
0.8
0.6
0.4
0.2
0.0
10-2
10-1
100
101
102
103
c

图 3A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#37
#41
OD
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0.8
0.6
0.4
0.2
0.0
10-2
10-1
100
101
102
103
c

图 3B

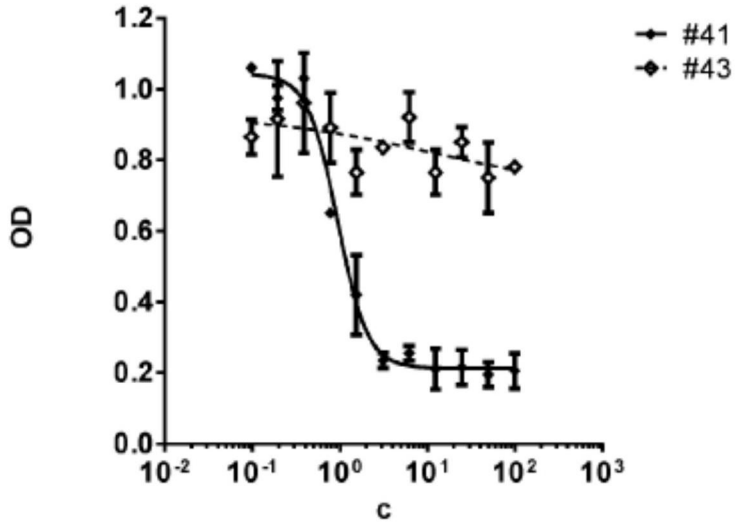
#41
#43
OD
C
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10-2
10-1
100
101
102
103

图 3C

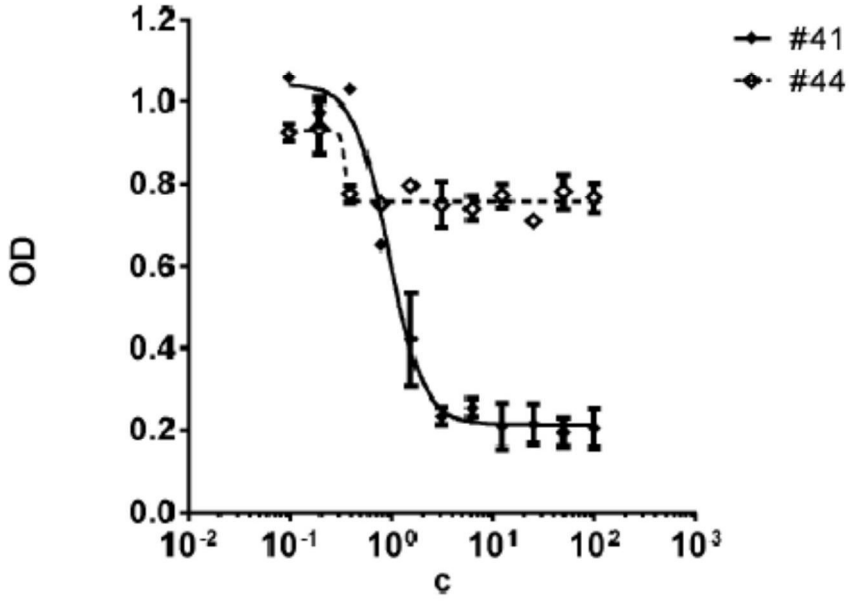
#41
#44
OD
C
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10-2
10-1
100
101
102
103

图 3D

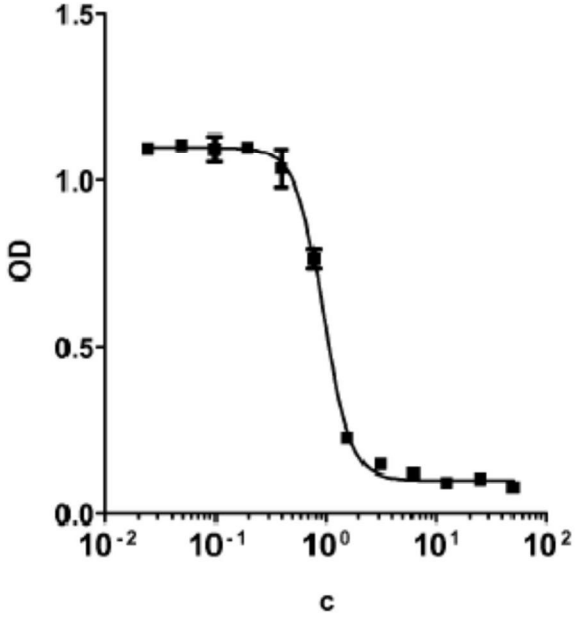
1.5
1.0
0.5
0.0
OD
10-2
10-1
100
101
102
c

图 4A

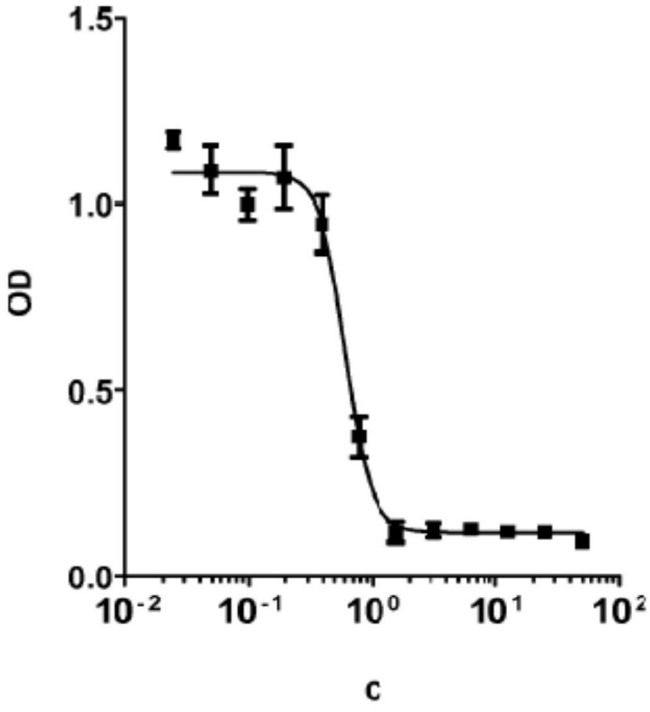
1.5
1.0
0.5
0.0
OD
10-2
10-1
100
101
102
c

图 4B

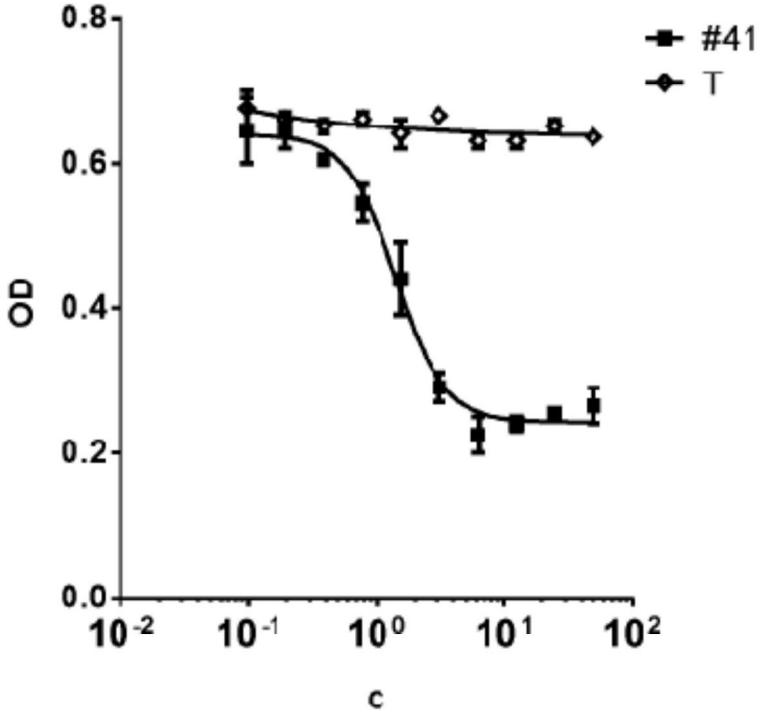
0.8
0.6
0.4
0.2
0.0
OD
#41
T
10-2
10-1
100
101
102
c

图 4C

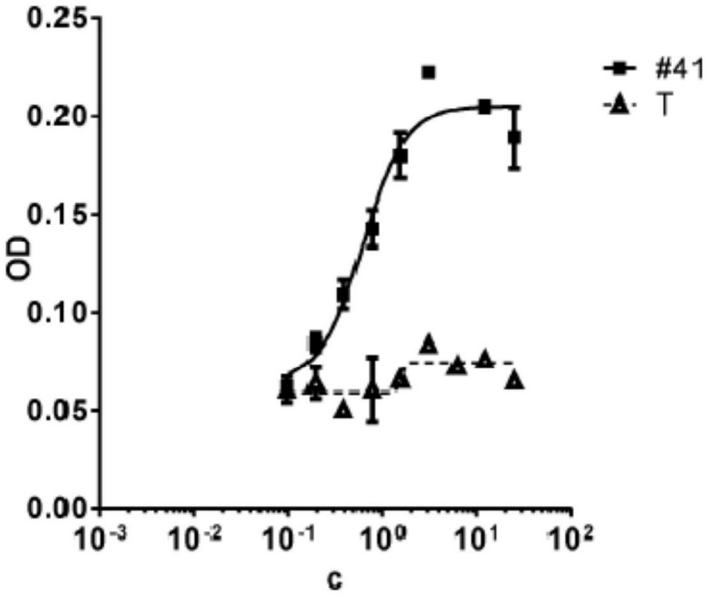
0.25
0.20
0.15
0.10
0.05
0.00
OD
#41
T
10-3
10-2
10-1
100
101
102
c

图 5A

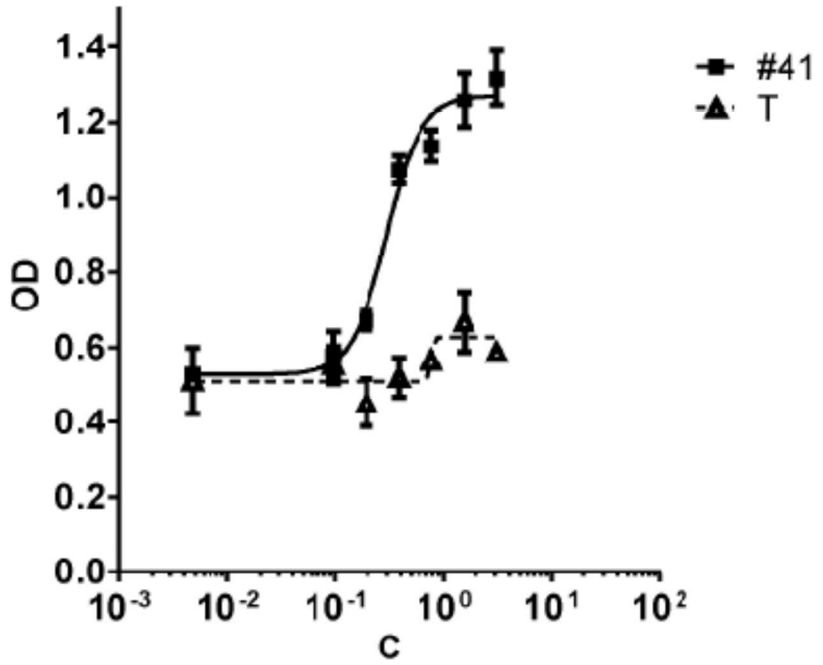
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD
10-3
10-2
10-1
100
101
102
C
#41
T

图 5B

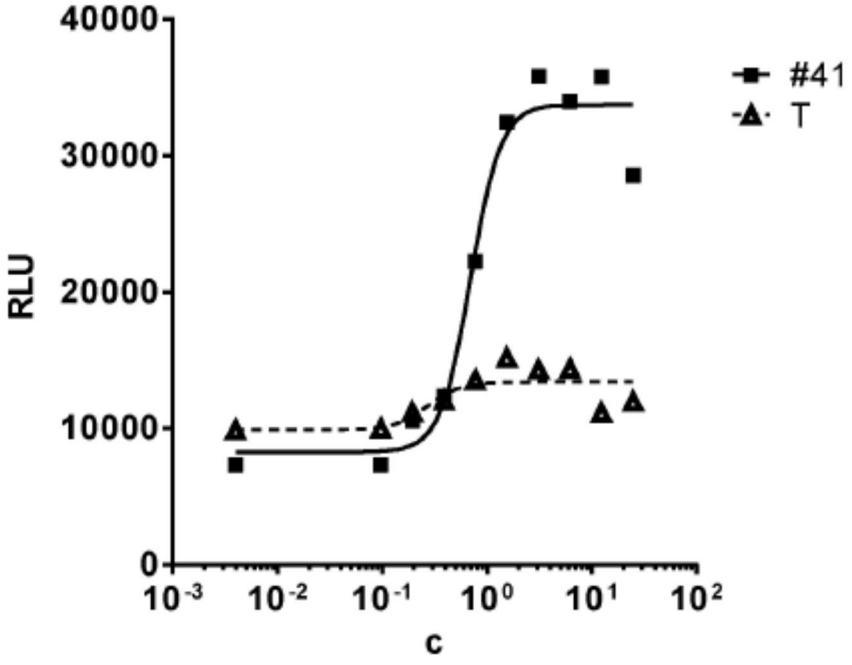
40000
30000
20000
10000
0
RLU
10-3
10-2
10-1
100
101
102
C
#41
T

图 5C

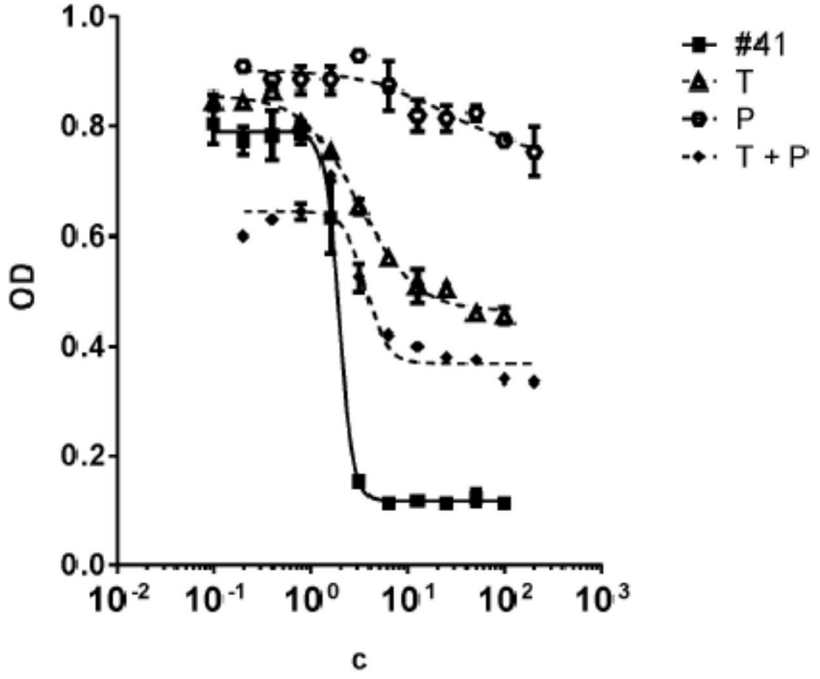
1.0
0.8
0.6
0.4
0.2
0.0
OD
10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3}
c
#41
T
P
T + P

图 6A

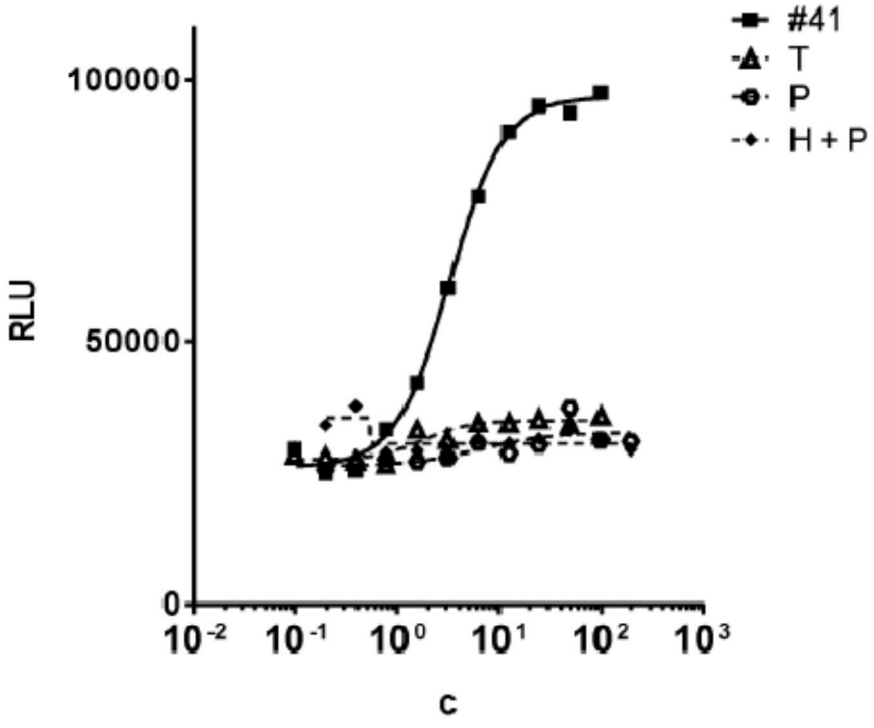
100000
50000
0
RLU
10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3}
c
#41
T
P
H + P

图 6B

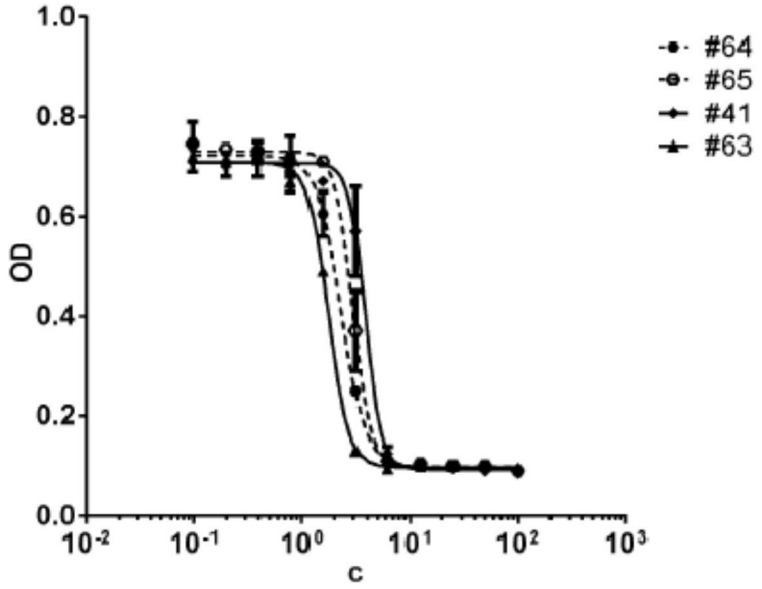
1.0
0.8
0.6
0.4
0.2
0.0
OD
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
c
#64
#65
#41
#63

图 7A

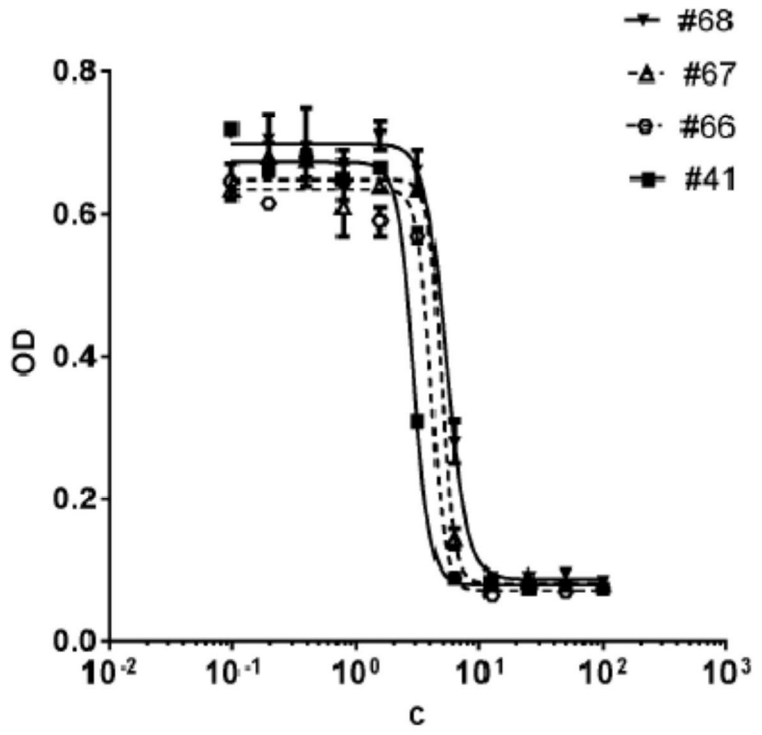
0.8
0.6
0.4
0.2
0.0
OD
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
c
#68
#67
#66
#41

图 7B

Abstract

The present invention relates to a recombinant binding protein comprising at least a first and a second repeat domain, wherein each of said two repeat domains binds the extracellular region of HER2 and wherein said repeat domains are covalently linked.