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(72) Inventors: VOGEL, Tikva; Kossyver Street 4, 76326 Rehovot (IL). PANET, Amos; HaRav Schrim Street 21/11, 96920 Jerusalem (IL).

(74) Agent: WHITE, John, P.; Cooper & Dunham LLP, 1185 Avenue of the Americas, New York, NY 10036 (US).


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(54) Title: ISOLATION OF TISSUE SPECIFIC PEPTIDE LIGANDS AND THEIR USE FOR TARGETING PHARMACEUTICALS TO ORGANS

(57) Abstract

The subject invention provides novel EGR-containing peptides and the use of these peptides in the treatment of various diseases and conditions. The novel peptides specifically bind to endothelial cells and various tumor cells and tissues.
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ISOLATION OF TISSUE SPECIFIC PEPTIDE LIGANDS
AND THEIR USE FOR TARGETING PHARMACEUTICALS TO ORGANS

This application claims priority of U.S. Serial No. 09/154,404, filed September 10, 1998 which is a continuation-in-part of PCT International Application No. PCT/US98/04188, filed March 4, 1998 which claims priority of U.S. provisional application No. 60/039,509, filed March 4, 1997, the contents of which are hereby incorporated by reference into this application.

Background

There is a need in the pharmaceutical industry for pharmaceutical agents that can be targeted to specific organs, and thus provide local drug delivery.

Advantages of local drug delivery are the lowering of the amount of drug needed to achieve therapeutic efficacy and the minimizing of undesired side effects.

Currently, the main approach to tissue specific targeting is either to infuse the drug through a catheter or a balloon (PTCA) to a site of the vasculature, or through linking of a drug to a protein ligand with affinity for a predetermined target.

For example, full-size monoclonal antibodies against predetermined cell surface antigens have been generated in order to produce cell targeting ligands. However, the complexity of isolating a specific antibody and the size of such antibody are severe limitations to their use as cell targeting ligands.

The use of a phage peptide display library facilitates an alternative means of producing unique ligands for targeting to specific, yet unrecognized cell (undetermined) surface moieties. Phage libraries have been used to select random
peptides that bind to isolated pre-determined target proteins such as antibodies, hormone receptors, and the erythropoietin receptor.

Unlike the use of a known ligand, the process of phage selection from a random peptide library does not require prior knowledge of the target cell or its receptors. This approach also has the advantage that molecular recognition and ligand selection are not dependent on the immunogenicity of the candidate target protein, as required in the monoclonal antibody approach.

Pasqualini and Ruoslahti (Nature 380: 364-366, 1996) injected phage libraries intravenously into mice and subsequently rescued the phage from individual organs. Peptides capable of mediating selective localization of phage to brain and kidney blood vessels were identified and were shown to exhibit up to 13-fold higher sensitivity for these mouse organs. One of the peptides displayed by the brain-localized phage was chemically synthesized and shown to specifically inhibit the localization of the homologous phage into the brain. When coated onto glutaraldehyde fixed red blood cells, the peptide caused selective localization of intravenously injected red blood cells into the brain of the mouse.

The subject invention discloses the use of phage display peptide (epitope) libraries to identify peptides useful as ligands for targeting drugs, cells or genes to specific human tissue and various human organs.

The novelty of the subject approach is inter alia the application of the peptide library methodology to isolated perfused human tissues.

A phage library is included in the organ perfusion fluid, and after ex vivo organ perfusion, phages are extracted from the human tissue, amplified and the displayed peptide
sequence is determined. This _ex vivo_ approach is applied to human organs such as placenta, umbilical cord artery and vein as well as blood vessels removed during surgery. This approach is further applied to diseased tissue removed during surgery and to organs such as kidney, heart and liver available following transplantation procedures.

The endothelium which lines the inner surface of blood vessels expresses multiple surface proteins and receptors for diverse types of ligands. Endothelial cells, derived from different tissues or even from veins and arteries of the same tissue, have been shown to be phenotypically and functionally distinct. The unique distinctive, characteristic surface proteins and receptors expressed by endothelial cells of the various tissues are exploited to discover novel, defined peptide ligands which are subsequently linked to drugs or radioactive isotopes for targeting to the desired tissue.

The peptide sequences of the subject invention, specific for different human organs and tissue cells are linked to various pharmaceutical agents to form drug-peptide conjugates and to radioactive isotopes for diagnostic and therapeutic purposes.

*Summary of the Invention*

The subject invention provides novel peptides comprising the sequence Glu Gly Arg and the use of these peptides in the treatment of various diseases and conditions. The novel peptides specifically bind to endothelial cells and various tumor cells and tissues.

The subject invention provides for compositions comprising novel peptides and a pharmaceutical agent linked to the peptide, wherein the pharmaceutical agent is a polypeptide and is linked to the peptide by a peptide linkage. The pharmaceutical agent may also be a toxin, an anti-cancer agent, an anti-angiogenic compound, a cardiovascular agent,
an agent used in a neurological disorder, a liver disease agent, a kidney disease agent or a radioisotope.

The subject invention provides for a method for the identification of a peptide comprising incubating a phage display peptide library with an isolated organ; washing the isolated organ to remove unbound phages; eluting bound phage from the isolated organ; amplifying the resulting bound phage; and determining the displayed peptide sequence of the bound phage so as to identify the peptide.

The organ may be an artery, a vein, placenta, tumor tissue, kidney, heart, liver, or central nervous system. The organ may also be a perfused organ.

The phage display library may be a 15-mer library or a 6-mer library.

The elution medium may be a compound selected from acid, urea, octyl, trypsin or tween.

The subject invention also provides for a method of producing the novel peptides comprising identifying the peptide as described above, and synthesizing the peptide by joining the amino acids of the peptide in the proper order.

The subject invention additionally provides an imaging agent which comprises a peptide of the subject invention with an imageable marker. Such an imaging agent may be used for diagnostic purposes.

The subject invention further provides a composition comprising an effective imaging amount of an imaging agent of the invention and a physiologically acceptable carrier.

The subject invention also encompasses a composition comprising an effective imaging amount of an imaging agent
of the invention, a pharmaceutical agent linked thereto and a physiologically acceptable carrier.

The marker may be a radioactive isotope, an element which is opaque to X-rays or a paramagnetic ion.

The radioactive isotope may be indium-111, technetium-99, iodine-123, iodine-125, iodine-131, krypton-81m, xenon-33 or gallium-67.

The subject invention also provides for a method for imaging an organ comprising contacting the organ to be imaged with an imaging agent under conditions such that the imaging agent binds to the organ, imaging bound imaging agent; and thereby imaging the organ.

The subject invention also provides for a method of treating an organ in vivo comprising contacting the organ to be treated with a composition of the invention under conditions such that the composition binds to the organ, and thereby treating the organ.

Brief Description of the Figures

Figure 1: Organ distribution of selected phages

Clone KSC#3 (KSCR3#3) is a phage clone that was enriched on Kaposi Sarcoma cells after three rounds of biopanning in culture. Clone R4B#1 (TUV-R4B*#1) is a phage clone that was enriched on umbilical vein and artery. Clone #P13 (R6P#13) is a sporadic non-enriched phage clone as a negative control. The in vivo binding of these three clones to tumor tissue and brain tissue was compared.

Figure 2: Binding of TUV-R4B-#1 (the EGR-phage) to mouse melanoma cells and to brain cells.

Figure 3: Ratio of binding of TUV-R4B-#1 to melanoma tumor cells and to brain cells.

Figure 4: Binding of TUV-R4B-#1 and of 14-4 phage to
mouse mammary tumor cells.

**Figure 5:** Binding of TUV-R4B-#1 to mouse mammary tumor cells and to brain cells.

**Figure 6:** Comparison of binding of TUV-R4B-#1 to mouse mammary tumor cells and to brain cells.

**Detailed Description of the Invention**

The subject invention provides novel peptides comprising the sequence Glu Gly Arg and the use of these peptides in the treatment of various diseases and conditions. The novel peptides specifically bind to endothelial cells and various tumor cells and tissues.

Phage display is a technique in which a peptide, antibody or protein is expressed on the surface of a bacteriophage, while the DNA encoding the displayed protein resides within the phage virion. A phage display peptide library (also termed phage peptide library or phage display library or peptide library) is constructed wherein the virions display a wide range of protein residues of specific lengths. This technology, known to one skilled in the art, is more specifically described in the following publications: Smith (1985) Science 228: 1315, Scott et al. (1990), Science 249: 386-390, Cwirla et al. (1990), P.N.A.S. 87: 6378-6382; Devlin et al. (1990), Science 249: 404-406, U.S. Patent Nos 5,427,908, 5,432,018, 5,223,409 and 5,403,484.

Biopanning is a procedure comprising many steps, one of which is selection; biopanning is carried out by incubating phages displaying protein ligand variants (a phage display library) with a target, washing away unbound phage and specifically eluting the bound phage. The eluted phage is amplified and taken through additional cycles of binding and amplification which enrich the pool of eluted specific sequences in favor of the best binding peptide bearing phages. After several rounds, individual phages are characterized, and the sequence
of the peptides displayed is determined by sequencing of the corresponding DNA of the phage virion. A peptide obtained in this manner may be called a "lead-compound".

One way of obtaining a peptide with a higher affinity relative to a lead-compound is to construct an extension phage display peptide library based on a core amino acid sequence of the lead-compound. In such an extension library, random amino acids are added to each side of the core sequence.

An additional way to obtain a peptide with a higher affinity relative to a lead-compound is the construction of a phagemid display mutagenesis library. In such a library, oligonucleotides are synthesized so that each amino acid of the core sequence is independently substituted by any other amino acid.

Cancer tissue as used herein may be obtained from any form of cancer such as carcinoma, sarcoma, leukemia, adenoma, lymphoma, myeloma, blastoma, seminoma or melanoma.

Diseased tissue as used herein may be obtained from any diseased organ such as liver, kidney, lung, heart, ovary, colon and so forth. The organ may be diseased as a result of an autoimmune disorder. The organ may be diseased as a result of any other disease, such as cardiovascular disease or cancer.

A neurologic disorder as used herein encompasses any neurologic disorder as defined and described in "The Merck Manual", sixteenth edition (1992). For example, muscular dystrophy, myasthenia gravis, multiple sclerosis, Alzheimer's disease, neuropathy, Parkinson's disease and amyotrophic lateral sclerosis (Lou Gehrig's disease) are neurologic disorders.

A vein as used herein may originate from any tissue. An
example of a vein is safenal vein or femoral vein.

An artery as used herein may originate from any tissue, e.g. radial artery, coronary artery, mammary artery and so forth.

A peptide of the subject invention may be administered to a patient, alone, radiolabeled, linked to a pharmaceutical agent (drug), or in the form of a peptidomimetic.

The mode of administration of a peptide of the subject invention is intravenous, intramuscular, subcutaneous, topical, intratracheal, intrathecal, intraperitoneal, rectal, vaginal or intrapleural.

The pharmaceutical agent may inter alia be a radioactive label (radio-isotope).

If the peptide or the peptide-drug combination is administered orally, it is administered in the form of a tablet, a pill or a capsule.

The compositions comprising the peptides produced in accordance with the invention may comprise conventional pharmaceutically acceptable diluents or carriers. Tablets, pills and capsules may include conventional excipients such as lactose, starch and magnesium stearate. Suppositories may include excipients such as waxes and glycerol. Injectable solutions comprise sterile pyrogen-free media such as saline and may include buffering agents, stabilizing agents or preservatives. Conventional enteric coatings may also be used.

Compositions for topical administration may be in the form of creams, ointments, lotions, solutions or gels.

The mode of administration of the peptide or drug-peptide linkage is a solid dosage form, a liquid dosage form, or a
-9-
sustained-release formulation.

In an additional embodiment the subject invention provides a polypeptide which comprises a peptide of the subject invention which corresponds to a peptide displayed on a phage virion and wherein both the polypeptide and the peptide have the same biological activity.

In another embodiment, a Fv fragment of a human antibody of about 100 amino acids is displayed on the N-terminus of pIII of M13 bacteriophage and contains the Glu Gly Arg sequence.

The subject invention provides peptides comprising Glu Gly Arg having the following sequences:

Gly Arg Gln His Phe Phe Leu Ala Glu Gly Arg Ser Phe Tyr Phe (Seq. ID. No. 2);
R1,-Glu Gly Arg Ser Phe-R2 (Seq. ID. No. 1)
and
R3,-Glu Gly Arg-R2

where R1 and R2 are amino acid sequences ranging from 1-50 amino acids, preferably 1-15 amino acids, most preferably 1-5 amino acids in length;

and

Gly Pos Pos Pos/Ar Ar Ar Leu Ala Glu Gly Arg Ser Ar Ar Ar wherein Pos is a positively charged amino acid and Ar is an aromatic amino acid.

In each of the sequences in this application the amino acid at the extreme left represents an amino acid on the amino terminal side of the peptide and the amino acid at the extreme right represents an amino acid on the carboxy terminal side of the peptide.

The invention further provides a composition comprising a peptide of the subject invention and a pharmaceutical agent
linked thereto. The composition optionally also comprises a 
pharmaceutically acceptable carrier.

In a preferred embodiment, the pharmaceutical agent is a 
polypeptide and is linked to the peptide by a covalent 
linkage, preferably a peptide linkage.

In another embodiment, the pharmaceutical agent is a toxin, 
an anti-cancer agent, an anti-angiogenic compound, a 
cardiovascular agent, an agent used in a neurological 
disorder, a liver disease agent or a kidney disease agent or 
a radio isotope.

In a presently preferred embodiment, the pharmaceutical agent 
is a recombinant protein.

The subject invention further provides a composition 
comprising a peptide of the subject invention and a 
pharmaceutically acceptable carrier.

The subject invention also provides a chimeric polypeptide 
comprising a first peptide and a second peptide wherein the 
first peptide is a peptide of the subject invention.

In a preferred embodiment, the second peptide is a toxin, an 
anti-cancer agent, an anti-angiogenic compound, a 
cardiovascular agent, an agent used in a neurological 
disorder, a liver disease agent or a kidney disease agent.

In another preferred embodiment, the second peptide is a 
recombinant protein.

The subject invention also encompasses a method of 
synthesizing a peptide of the subject invention which 
comprises joining the amino acids of the peptide in the proper 
order.
The subject invention further provides a method of producing a peptide which comprises identifying the peptide by the above described method and synthesizing the peptide by joining the amino acids of the peptide in the proper order.

In a preferred embodiment, the isolated organ is a perfused organ.

In another embodiment, the isolated organ is an artery, a vein, placenta, tumor tissue, kidney, heart, liver or central nervous system.

In yet another embodiment, the artery is umbilical cord artery, a radial artery, a coronary artery, a mammary artery or a damaged artery.

In an especially preferred embodiment, the damaged artery is a damaged coronary artery.

In a preferred embodiment, the vein is umbilical cord vein, safenal vein or femoral vein.

It is envisaged that the phage display peptide library is a 15-mer library, a 6-mer library or a synthetic human antibody library.

Preferred elution medium is a compound selected from acid, urea, octyl, trypsin or Tween.

The subject invention additionally provides an imaging agent which comprises a peptide of the subject invention with an imageable marker. Such an imaging agent may be used for diagnostic purposes.

The subject invention further provides a composition comprising an effective imaging amount of an imaging agent of the invention and a physiologically acceptable carrier.
The subject invention also encompasses a composition comprising an effective imaging amount of an imaging agent of the invention, a pharmaceutical agent linked thereto and a physiologically acceptable carrier.

In a preferred embodiment, the marker is a radioactive isotope, an element which is opaque to X-rays or a paramagnetic ion.

In a presently preferred embodiment, the radioactive isotope is indium-111, technetium-99, iodine-123, iodine-125, iodine-131, krypton-81m, xenon-33 or gallium-67.

The subject invention also provides a method for imaging an organ which comprises contacting the organ to be imaged with an imaging agent of the invention under conditions such that the imaging agent binds to the organ, imaging the bound imaging agent and thereby imaging the organ.

In a preferred embodiment, the organ is an artery, a vein, placenta, tumor tissue, kidney, heart or liver.

In another embodiment, the artery is umbilical cord artery, a radial artery, a coronary artery, a mammary artery or a damaged artery.

In another embodiment, the damaged artery is a damaged coronary artery.

In another embodiment, the vein is umbilical cord vein, safenal vein or femoral vein.

In another embodiment, the pharmaceutical agent is a polypeptide and is linked to the imaging agent by a peptide linkage.

In a preferred embodiment, the pharmaceutical agent is a
toxin, an anti-cancer agent, an anti-angiogenic compound, a cardiovascular agent, an agent used in a neurological disorder, a liver disease agent or a kidney disease agent.

The subject invention further provides a composition wherein the pharmaceutical agent is a recombinant protein.

The subject invention further provides a method of treating an organ *in vivo* which comprises contacting the organ to be treated with a composition of the invention under conditions such that the composition binds to the organ and thereby treating the organ.

In a preferred embodiment, the organ is an artery, a vein, placenta, tumor tissue, kidney, heart, liver, or central nervous system.

In another embodiment, the artery is umbilical cord artery, a radial artery, a coronary artery or a mammary artery or a damaged artery.

In another embodiment, the damaged artery is a damaged coronary artery.

In another embodiment, the vein is umbilical cord vein, safenal vein or femoral vein.

The novel peptides of the subject invention or their corresponding peptidomimetics are also used in the manufacture of compositions to treat various diseases and conditions.

The subject invention also provides a method for the identification of peptides or antibodies by biopanning which comprises incubating a phage display library with lymphocytes derived from blood, washing to remove unbound phages, eluting the bound phages from the lymphocytes, amplifying the resulting bound phage and determining the displayed peptide
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sequence of the bound phage so as to identify the peptide.
Examples
The Examples which follow are set forth to aid in understanding the invention but are not intended to, and should not be construed to, limit its scope in any way.

EXAMPLE 1: Isolation and Selection of Tissue-Specific Epitopes which Specifically Bind to Undetermined Targets in Umbilical Cord Vein and Artery

1. MATERIALS AND METHODS

1.1. Preparation of perfused umbilical cord vein and artery
Freshly obtained full term umbilical cord was cut into fragments of 4-5 cm in length, the blood was removed and the umbilical cord was cannulated and connected either through the vein (V) or the artery (A) to a small circulating pump for perfusion at a rate of 1ml/min (Pharmacia peristaltic pump). Perfusion temperature was either 4°C or 23°C and the perfusion buffer composition is indicated below.

1.2. Peptide display phage library source
Two phage display peptide libraries (6-mer and 15-mer) were kindly provided by G. Smith (Virology 167: 156-165, 1988). The libraries were amplified to form phage library working stock.

The libraries were originally constructed by splicing the 2.8 kilo-base pair tetracycline resistance determinant of Tn10 into the minus-strand origin of replication of the wild type fd phage. The resulting defect in minus strand synthesis reduced the intracellular replicative form (RF) copy number to 1. As a result thereof, the filamentous phage including fd-tet do not kill their host. The infected cell, which becomes resistant to tetracycline, continues to grow and secret tetracycline resistant progeny particles (about 10 tetracycline transducing units (TTU) per bacteria). The infectivity of the 6-mer and 15-mer libraries are 2.5% and 39%, respectively. The calculated theoretical number of
primary clones for the 6 and 15-mers libraries are $4 \times 10^7$ and $1 \times 10^{19}$, respectively. However, the number of primary clones obtained from each library was about $2 \times 10^9$. The phage titer is determined by infecting starved K91/kan bacterial culture and selecting tetracycline resistant clones on kan/tetracycline agar plates. The biopanning yield is calculated as the % of the output phage from the total input.

1.3. Bacterial strain: K91/Kan

The preparation of starved bacterial culture for phage infection was essentially as described by G. Smith (1993), Methods of Enzymology 217:228.

1.4. Phage selection and amplification

Phages that expressed epitopes of specific interest were selected from the libraries by biopanning in an essentially 4-step procedure:

a. Binding of phage to the perfused vessel cells
b. Removal of non-bound phage by extensive washing
c. Elution of bound phages
d. Infection and propagation of eluted phage in E. coli.

This biopanning procedure was generally repeated 4-6 times. Selected clones were individually propagated, and the single stranded DNA from the secreted phage was purified. Properties of the selected clones were examined as follows:

(i) Sequencing selected phage:
The DNA sequence of the insert was determined by the dideoxy DNA sequencing method (Sanger et al. (1977), P.N.A.S. 74: 5463-5467) using Sequenase Version 2.0 (DNA Sequencing Kit, Amersham) and a primer of 18 nucleotides (5': TGAATTTTCGATGAGG) (Seq. ID. No. 1).

(ii) Biopanning and tissue distribution in vivo (mouse or rat)
(iii) Phage binding comparison ex vivo, in culture, and in vivo

The following procedures are then carried out (Example 4):

(iv) Peptide synthesis
(v) Mutant peptide synthesis
(vi) Peptide cross linking (chemically and by genetic engineering)

(vii) Immunolocalization (using labeled antibody)
(viii) Linking of peptide to drug
(ix) Radiolabeling of peptide
(x) Radiotherapy and other therapeutic treatments.
(xi) Organic synthesis of peptidomimetic.

1.5 Basic Biopanning protocols

Several biopanning protocols were developed and used:

1.5.1. Protocol T1 (Acid/Urea/Octyl elution)
1.5.2. Protocol R1 (Trypsin-EDTA/Acid-Tween elution)
1.5.3. Protocol N1 (Acid-Tween/Tissue elution)
1.5.4. Protocol R2 (Trypsin-EDTA/Acid-Tween/Tissue elution)
1.5.5. Protocol EC-1 (Acid/Tissue/Urea elution)

1.5.1. Protocol T1 (Acid/Urea/Octyl elution)

1. Prewashing of the umbilical cord was carried out with 20 ml cold PBS\(^1\) followed by 20ml cold DMEM-5% BSA.

2. Selection was carried out at 2x10\(^{10}\) TTU\(^2\) in 3ml, DMEM\(^3\)-1% 

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\(^1\) PBS is 136 mM NaCl, 2.7 mM KCl, 100 mM Na\(_2\)HPO\(_4\), 2H\(_2\)O, 1.4 mM KH\(_2\)PO\(_4\),

\(^2\) TTU is phage infection units, conferring tetracycline resistance in the E.coli host.

\(^3\) DMEM is Dulbecco's Modified Eagle Medium with D-glucose (450 mg/l), Bet HaEmek
BSA\(^4\)-Protease inhibitors mixture (Pi\(^5\)). (50\(\mu\)l of the selection solution is used for titration of the input phage).

3. Wash 1 Wash 2 (W1,W2) was carried out in 2 x 25ml DMEM-5% BSA-Pi. When indicated, 0.2% Tween-20 was included.

4. Wash 3 (WO) was carried out in 3 ml W1+2 containing TBS\(^6\)-Octyl\(^7\) (0.05%).

Steps 2-4 were carried out at 4°C or at 23°C as indicated below.

5. Elutions: eluates were collected into 6ml TBS-1% BSA and adjusted to pH 7.4 with Trisma Base.

5a. Acid elution was carried out in 3 ml 0.2M glycine-HCl, pH 2.2.

5b. Urea elution was carried out in 3 ml 6M urea, pH 3 (in 0.2M Glycine-HCl, pH 3.0).

5c. Octyl elution was carried out in 3ml TBS containing 0.2% Octyl.

6. Concentration/dialysis of the eluates was carried out on a 30K millipore dialysis membrane.

7. Amplification: for the first biopanning round, the entire eluate was amplified. For other rounds of biopanning only 20% of the eluate was amplified. The amplification was carried

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\(^4\) BSA is bovine serum albumin fraction V (Sigma)

\(^5\) Pi is Phenyl Methyl Sulfonyl Fluoride (PMSF) 1mM, Aprotinin (20\(\mu\)g/ml), Leupeptin (1 \(\mu\)g/ml)

\(^6\) TBS is 50 mM Tris-HCl, pH 7.5 and 150 mM NaCl

\(^7\) Octyl is octyl-\(\beta\)-D-glucopyranoside (Sigma)
out in liquid medium by mixing the eluate with equal volume of $10^{10}$ starved bacteria. After 10 minutes, 100mL\(^8\) NZY solution containing 0.2 µg/ml tetracycline was added and bacterial suspension was mixed vigorously at 37°C for 30 minutes. Diluted samples were plated immediately on agar/kanamycin/tetracycline plates for titration of the output phage. To the rest of the bacterial cell suspension 100µl of 20mg/ml tetracycline were added and incubation/amplification continued over-night.

1.5.2. Protocol R1 (Trypsin-EDTA/Acid-Tween elution)

1. **Prewashing** of umbilical cord vein and artery was carried out with 30ml DMEM-heparin (5u/ml) (Laboratoire Choay)-$10^8$ M13 phage followed by 30ml, DMEM-5% human serum (Sigma).

2. **Selection** was carried out with 30ml, DMEM-5% human serum containing phage, $2\times 10^{10}$ TTU.

3. **Wash** was carried out with 40ml DMEM 5% human serum in the presence of Pi.

4. **Elutions** were carried out with:
   a. Trypsin-EDTA (2 ml, 0.25% and 0.05% respectively).
   b. Acid-Tween (5ml, 0.2M glycine-HCl (pH2.2)-0.5% Tween).

5. **Titration and amplification**:
The acidic fraction was neutralized with 2M Trisma base (about 270µl). To the combined 7ml eluate, 200µl, $10^{10}$ bacteria and 10ml NZY were added. The solution was mixed gently for 10 minutes at 37°C. 100ml prewarmed NZY and 0.2µg/ml tetracycline was added and mixed vigorously for 30 minutes. 100µl 20mg/ml tetracycline was added and steps 1-4 were continued on ice and step 5 at room temperature.

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\(^8\) NZY is a medium consisting of 10 g NZ amine A, 5 g yeast extract and 5 g NaCl.
1.5.3. Protocol N1 (Acid-Tween/Tissue elution)

1. **Prewashing** was carried out with 30ml DMEM-heparin (5u/ml) containing $10^8$ M13 phage followed by 30ml DMEM-5% human serum.

2. **Selection** was carried out with 30ml DMEM-5% human serum, containing $4 \times 10^{10}$ TTU.

3. **Wash** was carried out with 40ml DMEM-5% human serum and 2% human haemoglobin (Sigma)-Pi.

4. **Elution 1** was carried out in 3ml acid (0.2M glycine-HCl (pH2.2), 1% BSA, 0.5% Tween-20). The blood vessel was further washed with 2ml DMEM-5% human serum and combined with elution 1. 200µl $10^{10}$ bacteria and 10ml NZY were added, mixed gently and left at room temperature for 10 minutes.

**Elution 2**, tissue elution (i.e. bacteria elution), was carried out by clamping one side of the vein and adding 0.5ml NAP$^9$ buffer containing $2.5 \times 10^{10}$ starved bacteria. After clamping the other end of the vein, the blood vessel was immersed into DMEM-1% BSA solution and shaken at 37°C for 45 minutes. The bacteria were removed to a 50ml tube, the blood vessel was washed twice with 1-2ml NAP buffer, and the two washes were combined. 10ml NZY was added, mixed gently and left at room temperature for 10 minutes.

Steps 1-4 were carried out on ice and step 5 at room temperature (using a lamp from above).

5. **Titration and amplification:**

100ml prewarmed NZY containing 0.2 µg/ml tetracycline was added and mixed vigorously for 30 minutes. Samples (50 µl each) were removed for titration on plates (output), and for over-night amplification, 100µl of 20mg/ml tetracycline was

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NAP consists of 80mM NaCl and 50 mM NH$_4$H$_2$PO$_4$, pH 7.0
1.5.4. Protocol R2 (Trypsin-EDTA/Acid-Tween/Tissue elution)

Protocol R2 is essentially identical to protocol R1 apart from the addition of tissue elution with bacteria following the trypsin and acid-tween elutions.

1.5.5. Protocol EC-1

Primary human endothelial cells seeded in 35cm tissue culture bottles (3rd passage of full term umbilical cord) were used.

1. **Re-equilibration** to 37°C and CO₂ for 30 minutes.

2. **Prewashing** was carried out with 5ml serum-free medium followed by 10 ml blocking solution (DMEM-BSA(1%) - Pi) for 90 minutes at 37°C.

3. **Selection** (incubation) for 45 minutes at 37°C with 3ml DMEM-BSA(1%) - Chloroquine (100μM) mixture containing 2x10¹⁰ phages of the 15-mer initial library and a selected phage clone at a ratio of approximately 100:1, respectively.

4. **Washing** was carried out 5 times with 5ml blocking solution for 5 minutes each at room temperature.

5. **Elutions**:

   5a. Elution 1 was carried out with 2ml acid-glycine pH 2.2 containing 0.2% Tween for 10 minutes at room temperature. 170μl of 2M Trizma base was added to the bottle for neutralization, and the acid elution obtained was transferred to another tube. The bottle was washed with 2ml DMEM-BSA(1%). 0.2ml of the eluate was mixed with NAP buffer containing 1x10¹⁰ starved bacteria, and after 10 minutes absorption at room temperature, 2ml NZY-0.2% tetracycline was added and the
suspension was shaken at 37°C. Aliquots were plated on agar-
kanamycin-tetracycline plates for phage titration.

5b. Elution 2 was carried out with 2ml NaPO₄ buffer containing
5x10¹⁰ starved bacteria. Incubation was carried out at room
temperature for 45 minutes on a rocker. Cell eluate was
transferred to another tube. The bottle was washed with 2ml
medium. 0.4ml suspension was mixed with 2ml NZY 0.2%
tetracycline and incubated and titrated as described for
elution 1(6a).

5c. Elution 3 was carried out with 2ml urea (6M, pH 3). Incubation at room temperature for 10 minutes while rocking.
170µl of 2M Trizma base was added directly to the bottle and
urea eluate was transferred to another tube containing 36ml
medium. The bottle was washed with 2ml medium. 0.2ml was
mixed with 0.2ml NAP buffer containing 1x10¹⁰ starved bacteria
and then continued as described for elution 1(6a).

Testing the working stock of 15-mer library:
The working stock of the 15-mer library was the product of
several cycles of amplifications. Several clones of the
working stock (i.e. input of round 1) were isolated, amplified
and their single stranded DNA was purified and sequenced.
None of the phage clones was identical to specifically
selected clones described below.

In addition, approximately 500 colonies derived from the same
library were examined by colony hybridization to the ³²p-
labeled antisense oligonucleotide probe of clones TUV-R4B-#1
and TUV-R4B-#3 disclosed below. None of the colonies
hybridized to these probes.
2. RESULTS:

2.1. Specificity of phage library to umbilical cord

Table 1: Comparison of phage binding to umbilical cord vein and non-specific phage adsorption to peristaltic pump plastic tubes following biopanning with 15-mer library (yield after acid and urea elution, protocol T1, room temperature (23°C)).

<table>
<thead>
<tr>
<th></th>
<th>Plastic tubes¹ (TTU)</th>
<th>yield %</th>
<th>Vein² (TTU)</th>
<th>yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>input</td>
<td>2x10⁹</td>
<td></td>
<td>3x10¹⁰</td>
<td></td>
</tr>
<tr>
<td>W1 + Tween</td>
<td>1x10⁸</td>
<td>6</td>
<td>1x10⁹</td>
<td>3.8</td>
</tr>
<tr>
<td>W2 + Tween</td>
<td>2x10⁶</td>
<td>0.1</td>
<td>9x10⁶</td>
<td>0.03</td>
</tr>
<tr>
<td>WO</td>
<td>2x10⁵</td>
<td>0.001</td>
<td>1x10⁵</td>
<td>0.0004</td>
</tr>
<tr>
<td>acid elution</td>
<td>&lt;&lt;10³</td>
<td>&lt;.00001</td>
<td>7x10⁶</td>
<td>0.02</td>
</tr>
<tr>
<td>urea elution</td>
<td>&lt;&lt;10³</td>
<td>&lt;.00001</td>
<td>9x10⁶</td>
<td>0.0260</td>
</tr>
</tbody>
</table>

¹ Selection was carried out through the circulating pump without the umbilical vein.
² Selection was carried out through the umbilical vein and the circulating pump.

Thus, the results shown in Table 1 indicate that the phage yield values of the acid and the urea elutions from the umbilical vein were 2 to 3 orders of magnitude higher than the background values obtained from background binding to the plastic tubes, thus indicating a specific binding of the phage to the umbilical vein.
2.2 Effect of temperature and detergent on phage binding

Table 2: Effect of temperature and Tween-20 on the binding of the 6-mer and 15-mer phage libraries to umbilical artery and vein (acid/urea/Octyl elution, protocol T1).

<table>
<thead>
<tr>
<th></th>
<th>Artery (4°C), 6-mer-(TTU)</th>
<th>Vein 1 (4°C), 15-mer-(TTU)</th>
<th>Vein 2 (23°C), 15-mer (TTU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input</td>
<td>2.6x10^10</td>
<td>2.5x10^10</td>
<td>1.8x10^10</td>
</tr>
<tr>
<td>W1</td>
<td>1.7x10^10</td>
<td>2.5x10^10</td>
<td></td>
</tr>
<tr>
<td>W2</td>
<td>1.4x10^9</td>
<td>2.8x10^9</td>
<td></td>
</tr>
<tr>
<td>W1+Tween</td>
<td></td>
<td></td>
<td>1.3x10^10</td>
</tr>
<tr>
<td>W2+Tween</td>
<td></td>
<td></td>
<td>9x10^6</td>
</tr>
<tr>
<td>WO</td>
<td>2.8x10^8</td>
<td>6.5x10^8</td>
<td>1.3x10^4</td>
</tr>
<tr>
<td>Acid Elution</td>
<td>3.1x10^6</td>
<td>1.5x10^6</td>
<td>6.6x10^6</td>
</tr>
<tr>
<td>Urea Elution</td>
<td>3.0x10^6</td>
<td>3x10^6</td>
<td>9.0x10^6</td>
</tr>
<tr>
<td>Octyl Elution</td>
<td>3x10^8</td>
<td>2.4x10^8</td>
<td>1.1x10^6</td>
</tr>
</tbody>
</table>

The results indicate that by the addition of Tween (0.2%) in the washing solution and by increasing the temperature of the biopanning procedure to 23°C, there is a dramatic decrease in phage titers of the subsequent washing and elution steps (i.e. W2 + Tween, WO and Octyl elution) both with artery and vein. Therefore, the conditions of vein 2 (i.e. biopanning at 23°C and washing with a buffer containing 0.2% Tween followed by washing with a buffer containing 0.05% Octyl) were selected and used in the following protocol T1 based experiments.
2.3 Yield of Biopanning with Umbilical Vein and Artery

Table 3: Biopanning of 15-mer library with human umbilical vein (Vein 1 and Vein 2) and 6-mer library with human umbilical artery (Artery 1) - yield after urea elution, protocol T1 (experiment B).

<table>
<thead>
<tr>
<th>Round #</th>
<th>Artery 1 (4°C) (TTU)</th>
<th>Vein 1 (4°C) (TTU)</th>
<th>Vein 2 (23°C) (TTU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1B</td>
<td>1.7x10^{-2}</td>
<td>2x10^{-2}</td>
<td>2.3x10^{-2}</td>
</tr>
<tr>
<td>R2B</td>
<td>0.9x10^{-2}</td>
<td>0.8x10^{-2}</td>
<td>2x10^{-2}</td>
</tr>
<tr>
<td>R3B</td>
<td>1.2x10^{-1}</td>
<td>1x10^{-1}</td>
<td>1.3x10^{-1}</td>
</tr>
<tr>
<td>R4B</td>
<td>1.5x10^{-1}</td>
<td>1.3x10^{-1}</td>
<td>1.28x10^{-1}</td>
</tr>
<tr>
<td>R4B*</td>
<td>-</td>
<td>-</td>
<td>3x10^{9}</td>
</tr>
</tbody>
</table>

The protocol used was essentially as protocol T1 with indicated modifications below. Input phage in rounds R1B-R4B was 2x10^{10}-10^{11} and in R4B* was 1x10^{9}.

a. Selection was carried out for 15 minutes. Wash was carried out in buffer without Tween, followed by buffer containing Octyl.

b. Selection was carried out for 8 minutes. Vein 2 was washed with buffer containing Tween, followed by buffer containing Octyl. Artery 1 and Vein 1 were washed with buffer without Tween followed by buffer containing Octyl. Elutions were carried out for 8 minutes.

c. Selection was carried out for 3 minutes. Washing was carried out with buffer containing Tween followed by buffer containing Octyl for all blood vessels. Elution was carried out for 8 minutes.

d. and e. Selection and washing conditions were identical to
c. Elution was carried out for 3 minutes.

Twenty-eight clones were sequenced after R4B*V2, i.e. the urea eluted fraction from umbilical vein 2 after round 4:

17/28 (60%) of the clones were identical: this clone was designated TUV-R4B*-#1:

**Amino Acid Sequence displayed by clone TUV-R4B*-#1:**

<table>
<thead>
<tr>
<th>Gly</th>
<th>Arg</th>
<th>Gln</th>
<th>His</th>
<th>Phe</th>
<th>Phe</th>
<th>Leu</th>
<th>Ala</th>
<th>Glu</th>
<th>Gly</th>
<th>Arg</th>
<th>Ser</th>
<th>Phe</th>
<th>Tyr</th>
<th>Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>12</td>
<td>13</td>
<td>14</td>
<td>15</td>
</tr>
</tbody>
</table>

(Seq. ID. No. 2)

Six amino acids are aromatic (bolded) forming two hydrophobic clusters (amino acids 4,5,6,...13,14,15), accompanied by a linker peptide (underlined) region containing the charged sequence Glu Gly Arg (9,10,11) followed by Ser Phe. Peptides based on this charged core sequence, Glu Gly Arg, are discussed in Example 2. In addition, the amino end of the 15-mer peptide is positively charged having the sequence Arg, Gln, His (position 2, 3, 4, double underlining).

3/28 (11%) identical clones were designated TUV-R4B*-#3:

**Amino Acid Sequence displayed by clone TUV-R4B*-#3:**

<table>
<thead>
<tr>
<th>Ser</th>
<th>His</th>
<th>Val</th>
<th>Pro</th>
<th>Pro</th>
<th>Ile</th>
<th>Phe</th>
<th>Asn</th>
<th>Asp</th>
<th>Val</th>
<th>Tyr</th>
<th>Trp</th>
<th>Ile</th>
<th>Ala</th>
<th>Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>12</td>
<td>13</td>
<td>14</td>
<td>15</td>
</tr>
</tbody>
</table>

(Seq. ID. No. 3)

The underlined sequence also appears in clone TUV-R5D-#2 (see below).

2/28 (7%) were identical clones and were designated TUV-R4B*-#11.
Amino Acid Sequence displayed by clone TUV-R4B*-#11:
His Thr Phe Phe Leu Pro Gly Cys Ala Gly His Cys Ile Asp Ala
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
(Seq. ID. No. 4)

2/28 (7%) were identical clones and were designated TUV-R4B*-#23.

Amino Acid Sequence displayed by clone TUV-R4B*-#23:
Pro Ser Thr Thr Arg Asn Arg Thr Asp Ile Asn Lys Pro Thr Gln
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
(Seq. ID. No. 5)
4/28 were unique clones.

Furthermore, ten clones were sequenced after R4BV2, i.e. another urea eluted fraction from umbilical vein 2 after round 4:

3/10 (30%) clones were identical to clone TUV-R4B*-#1 and 7/10 were unique clones.

In addition, seven clones were sequenced after R4BA1, i.e. the urea eluted fraction from umbilical artery after round 4: all seven clones were unique clones.
Table 4: Biopanning of 15-mer library with umbilical vein - yield of acid elution fraction using Protocol T1 (Experiment B)

<table>
<thead>
<tr>
<th>Round#</th>
<th>Input (TTU)</th>
<th>Output (TTU)</th>
<th>Yield(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R3Ba¹</td>
<td>3x10^10</td>
<td>7x10^6</td>
<td>2x10^2</td>
</tr>
<tr>
<td>R4Ba</td>
<td>7x10^9</td>
<td>5x10^5</td>
<td>6x10^3</td>
</tr>
<tr>
<td>R5Ba</td>
<td>4x10^10</td>
<td>2x10^6</td>
<td>6x10^3</td>
</tr>
<tr>
<td>R6Ba</td>
<td>3x10^10</td>
<td>3x10^6</td>
<td>9x10^3</td>
</tr>
</tbody>
</table>

¹ The amplified acid elution of R2B-V2 (Table 3) was used as the input of round 3. In the subsequent rounds (R4Ba-R6Ba) the amplified acid eluted phage was used for biopanning.

Ten clones were sequenced after R6Ba, i.e. the acid eluted fraction from umbilical vein 2 after round 6:

4/10 clones were identical and displayed strong homology to clone TUV-R4B*-#3. This clone was designated TUV-R6Ba-#7.

Amino acid sequence displayed by TUV-R6Ba-#7:

Arg Asn Val Pro Pro Ile Phe Asn Asp Val Tyr Trp Ile Ala Phe
1  2  3  4  5  6  7  8  9 10 11 12 13 14 15
(Seq. ID. No. 6)

The underlined amino acid sequence appears in clone TUV-R4B*-3# as well. Moreover, this clone is identical to UV-R5D-#2 (below). Thus, TUV-R6Ba = TUV-R5D-#2.

In addition, 1/10 clone was identical to TUV-R4B*-#1.
Table 5: Biopanning of 15-mer library with umbilical vein - yield of Octyl elution fraction using Protocol T1 (Experiment B)

<table>
<thead>
<tr>
<th>Round #</th>
<th>Input (TTU)</th>
<th>Output (TTU)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R3BO²</td>
<td>$3 \times 10^{10}$</td>
<td>$1 \times 10^{6}$</td>
<td>$3 \times 10^{3}$</td>
</tr>
<tr>
<td>R4BO</td>
<td>$3 \times 10^{10}$</td>
<td>$5 \times 10^{6}$</td>
<td>$2 \times 10^{3}$</td>
</tr>
<tr>
<td>R5BO</td>
<td>$2 \times 10^{11}$</td>
<td>$7 \times 10^{5}$</td>
<td>$4 \times 10^{4}$</td>
</tr>
<tr>
<td>R6BO</td>
<td>$1 \times 10^{10}$</td>
<td>$4 \times 10^{5}$</td>
<td>$4 \times 10^{3}$</td>
</tr>
</tbody>
</table>

² The amplified Octyl eluate of R2B-V2 (Table 3) was used as the input of round 3. In the subsequent rounds (R4BO-R6BO) the amplified Octyl eluted phage was used for biopanning.

Seven clones were sequenced after R4BO, i.e. the Octyl eluted fraction from umbilical vein 2 after round 4:

6/7 (85%) clones were identical to clone TUV-R4B*-#1.
Table 6: Biopanning of 15-mer library with umbilical vein yield after acid-tween/trypsin elution, protocol R1 (experiment C):

I. Yields of Rounds 1-5 with umbilical vein

<table>
<thead>
<tr>
<th></th>
<th>Input (TTU)</th>
<th>Output (TTU)</th>
<th>Yield(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1C-Vein</td>
<td>5x10^8</td>
<td>1.5x10^6</td>
<td>0.03</td>
</tr>
<tr>
<td>R2C-Vein</td>
<td>3.3x10^10</td>
<td>2.8x10^6</td>
<td>0.0085</td>
</tr>
<tr>
<td>R3C-Vein</td>
<td>4.7x10^10</td>
<td>6.7x10^5</td>
<td>0.0014</td>
</tr>
<tr>
<td>R4C-Vein</td>
<td>2.4x10^10</td>
<td>8.4x10^5</td>
<td>0.0035</td>
</tr>
<tr>
<td>R5C-Vein</td>
<td>1.3x10^10</td>
<td>4.9x10^6</td>
<td>0.037</td>
</tr>
</tbody>
</table>

II. Yields of Round 6 with umbilical vein and artery.

<table>
<thead>
<tr>
<th></th>
<th>Input (TTU)</th>
<th>Output (TTU)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R6C-Vein</td>
<td>1.5x10^11</td>
<td>6.4x10^6</td>
<td>0.004</td>
</tr>
<tr>
<td>R6C-Artery</td>
<td>1.5x10^11</td>
<td>1.7x10^6</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Seven clones were sequenced after R4C, i.e. the Acid-Tween/Trypsin eluted fraction from umbilical vein after round 4:

6/7 (85%) clones were identical to clone TUV-R4B*-#1.

Furthermore, seven clones were sequenced after R5C, i.e. the Acid-Tween/Trypsin eluted fraction from umbilical vein after round 5:

7/7 clones were identical to clone TUV-R4B*-#1.

The binding of clone TUV-R4B*-#1 to the umbilical vein (R6C-V) and to the artery (R6C-A) was nearly identical, indicating the inability of clone TUV-R4B*-#1 to discriminate between the two types of vessels.
Table 7: Biopanning of umbilical vein with a mixture of the 6-mer and the 15-mer libraries (10^15 TTU each) - yield of tissue elution with protocol N1 (Experiment D)

<table>
<thead>
<tr>
<th>Round #</th>
<th>Input (TTU)</th>
<th>Output (TTU)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1D</td>
<td>2x10^10</td>
<td>2x10^7</td>
<td>10^4</td>
</tr>
<tr>
<td>R2D</td>
<td>5x10^10</td>
<td>4x10^7</td>
<td>8x10^2</td>
</tr>
<tr>
<td>R3D</td>
<td>3x10^11</td>
<td>2x10^6</td>
<td>10^3</td>
</tr>
<tr>
<td>R4D</td>
<td>2x10^10</td>
<td>8x10^6</td>
<td>4x10^2</td>
</tr>
<tr>
<td>R5D</td>
<td>3x10^10</td>
<td>7x10^6</td>
<td>3x10^2</td>
</tr>
</tbody>
</table>

Six clones were sequenced after R4D, i.e. the tissue eluted fraction from umbilical vein after round 4:

3/6 clones (clones #1, #2, and #4) were identical at nucleotide positions 1-27 (amino acids 1-9). Clones #1 and #2 also have an identical amino acid at position 13 (proline) and clones #1 and #4 have an identical amino acid at position 14 (leucine).

These clones were designated TUV-R4D-#1, TUV-R4D-#2, and TUV-R4D-#4.

Amino Acid Sequence displayed by clone TUV-R4D-#1
Phe Tyr Ser His Ser Ala Asp Gly Ala Arg Pro Phe Pro Leu Tyr
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
(Seq. ID. No. 7)

Amino Acid Sequence displayed by clone TUV-R4D-#2:
Phe Tyr Ser His Ser Ala Asp Gly Ala Glu Ser Ser Pro Arg Met
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
(Seq. ID. No. 8)

Amino Acid Sequence displayed by clone TUV-R4D-#4:
Phe Tyr Ser His Ser Ala Asp Gly Ala Pro Arg Arg Asp Leu Leu
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
The amino acids bolded in clones TUV-R4D-#1, TUV-R4D-#2 and TUV-R4D-#4 are the amino acids which the displayed peptide sequences have in common.

3/6 clones were unique (#3, #5, and #6) clones.

Amino Acid Sequence displayed by clone TUV-R4D-#3:
Gly Cys Gly Gly Phe His Pro Gly Ser Asn Cys Leu Val Val Ser
1  2  3  4  5  6  7  8  9 10 11 12 13 14 15
(Seq. ID. No. 10)

TUV-R4D-#3 was also identical to six of the clones selected at R5D, to two of the clones selected at R5E and to seven clones selected at R5H.

In addition, ten clones were sequenced after R5D, i.e. the tissue eluted fraction from umbilical vein after round 5:

6/10 clones were identical to clone TUV-R4D-#3.

4/10 identical clones were designated TUV-R5D-#2.

Amino Acid Sequence displayed by clone TUV-R5D-#2
Arg Asn Val Pro Pro Ile Phe Asn Asp Val Tyr Trp Ile Ala Phe
1  2  3  4  5  6  7  8  9 10 11 12 13 14 15
(Seq. ID. No. 6)

The underlined sequence appears in clone TUV-R4B*-#3 as well.
Table 8: Biopanning of umbilical artery with a mixture of the 15-mer and 6-mer libraries (4.5 x 10⁶ TTU each) - yield of tissue elution using Protocol N1 (Experiment E)

<table>
<thead>
<tr>
<th>Round #</th>
<th>Input (TTU)</th>
<th>Output (TTU)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1E</td>
<td>9x10⁹</td>
<td>3x10⁶</td>
<td>3x10⁻³</td>
</tr>
<tr>
<td>R2E</td>
<td>7x10¹⁶</td>
<td>2x10⁵</td>
<td>2x10⁻⁴</td>
</tr>
<tr>
<td>R3E</td>
<td>2x10¹⁶</td>
<td>1x10⁷</td>
<td>6x10⁻²</td>
</tr>
<tr>
<td>R4E</td>
<td>1x10¹¹</td>
<td>4x10⁶</td>
<td>4x10⁻³</td>
</tr>
<tr>
<td>R5E</td>
<td>5x10¹¹</td>
<td>3x10⁸</td>
<td>6x10⁻²</td>
</tr>
</tbody>
</table>

Ten clones were sequenced after R5E, i.e. the tissue eluted fraction from umbilical artery after round 5:

3/10 clones were identical to TUV-R4D-#3.

1/10 clone was identical to TUV-R4B*-#3.

6/10 clones were unique clones.
Table 9: Biopanning of umbilical artery with 15-mer library - yield of tissue elution using Protocol R2 (Experiment F).

<table>
<thead>
<tr>
<th>Round #</th>
<th>Input (TTU)</th>
<th>Output (TTU)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1F</td>
<td>5x10^8</td>
<td>4x10^6</td>
<td>7x10^-1</td>
</tr>
<tr>
<td>R2F</td>
<td>2x10^10</td>
<td>6x10^5</td>
<td>3x10^-3</td>
</tr>
<tr>
<td>R3F</td>
<td>2x10^10</td>
<td>2x10^5</td>
<td>1x10^-3</td>
</tr>
<tr>
<td>R4F</td>
<td>2x10^10</td>
<td>1x10^6</td>
<td>6x10^-3</td>
</tr>
<tr>
<td>R5F</td>
<td>9x10^9</td>
<td>4x10^7</td>
<td>5x10^-2</td>
</tr>
</tbody>
</table>

Ten clones were sequenced after R5F, i.e. the tissue eluted fraction from umbilical artery after round 5:

8/10 clones were identical to clone TUV-R4B*-#1.

1/10 clone was identical to clone TUV-R4B*-#3 and 1/10 clone was a unique clone.
Table 10: Biopanning of 15-mer library with umbilical artery with vein-excluded\textsuperscript{1} phage library - yield of tissue elution using protocol N1 (Experiment H).

<table>
<thead>
<tr>
<th></th>
<th>Input (TTU)</th>
<th>Output (TTU)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1H</td>
<td>1x10\textsuperscript{9}</td>
<td>2x10\textsuperscript{5}</td>
<td>2x10\textsuperscript{-2}</td>
</tr>
<tr>
<td>R2H</td>
<td>3x10\textsuperscript{9}</td>
<td>8x10\textsuperscript{6}</td>
<td>3x10\textsuperscript{4}</td>
</tr>
<tr>
<td>R3H</td>
<td>6x10\textsuperscript{9}</td>
<td>6x10\textsuperscript{7}</td>
<td>9x10\textsuperscript{4}</td>
</tr>
<tr>
<td>R4H</td>
<td>-</td>
<td>8x10\textsuperscript{6}</td>
<td>-</td>
</tr>
<tr>
<td>R5H</td>
<td>8x10\textsuperscript{10}</td>
<td>3x10\textsuperscript{7}</td>
<td>3x10\textsuperscript{-2}</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Phage library was first biopanned on the umbilical vein (8-10cm length), and only the unbound (vein-excluded) phage was then biopanned with umbilical artery (4-5 cm length). This sequence was repeated for all rounds. Tissue elution with starved bacteria was conducted according to protocol N1.

Seven clones were sequenced after R5H, i.e. the tissue eluted fraction from umbilical artery after round 5: 7/7 clones were identical to clone TUV-R4D-#3.
Table 11: Binding of clone TUV-R4B*-#1 and clone TUV-R4B*-#3 (see section 3 below) to endothelial cell culture (Protocol EC-1); results of acid, tissue and urea elutions.

<table>
<thead>
<tr>
<th></th>
<th>Total colonies</th>
<th>clone TUV-R4B*-#1 (TTU)</th>
<th>clone TUV-R4B*-#1 (%)</th>
<th>clone TUV-R4B*-#3 (TTU)</th>
<th>clone TUV-R4B*-#3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>input</td>
<td>2x10⁹</td>
<td>3.4x10⁷</td>
<td>1.7</td>
<td>1.2x10⁷</td>
<td>0.6</td>
</tr>
<tr>
<td>acid elution</td>
<td>5x10⁵</td>
<td>7x10³</td>
<td>1.4</td>
<td>5x10³</td>
<td>1.0</td>
</tr>
<tr>
<td>tissue elution</td>
<td>3x10⁶</td>
<td>5.7x10²</td>
<td>1.9</td>
<td>N.D.¹</td>
<td>N.D.</td>
</tr>
<tr>
<td>urea elution</td>
<td>5x10⁴</td>
<td>5x10⁴</td>
<td>100</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

¹ N.D. = not detectable.

The results indicate that clone TUV-R4B*-#1 binds strongly to endothelial cells or to endothelial cell extra-cellular matrix, since 100% of the phages eluted with urea were identical to clone TUV-R4B*-#1.

3. TISSUE DISTRIBUTION OF TUV-R4B*-#1

In order to analyze tissue specificity of the selected phage displayed peptides, a phage mixture (4x10¹⁰) of a selected clone, either clone TUV-R4B*-#1 or clone TUV-R4B*-#3 or both clones together, with the 15-mer library (in a ratio of 1:10-1:100) was injected through the tail vein of a rat (in 0.5ml DMEM-1% BSA). After 4 minutes, the animal was sacrificed, the chest was opened, and extensive washing of the blood vessels was carried out by flushing isotonic salt solution through the left ventricle. After approximately 10 minutes, as the lungs became discolored, various organs were dissected, weighed, and homogenized in 1ml homogenization solution (DMEM-1%BSA-Pi) using an ultra thorax grinding device. Following centrifugation (10 minutes at top speed eppendorf centrifuge), the supernatant was discarded, and the pelleted tissue was
washed three times with the same buffer. The washed tissue extract was resuspended in 0.6ml NZY and 0.4 ml starved bacteria (2x10^10) and was incubated with gentle mixing for 10 minutes at 37°C. The tissue-bacterial suspension was diluted in 10ml NZY containing 0.2% tetracycline and after 45 minutes of vigorous mixing at 37°C, aliquots were plated on agar plates containing 40μg/ml tetracycline- 20μg/ml kanamycin and incubated at 37°C for 16 hours. After monitoring tet^R colonies on each plate, the colonies were transferred to a millipore sheet for colony hybridization as described in "Molecular Cloning: A Laboratory Manual", J, Sambrook, E.S. Fritsch, and T. Maniatis, Cold Spring Harbor Laboratory Press, 2nd edition, 1989.

Synthetic DNA probes of 24 and 23 oligonucleotides derived from the antisense sequence of clones TUV-R4B*-#1 and TUV-R4B*-#3 respectively, were used.
Table 12: Tissue distribution of TUV-R4B*-#1 in a rat model (the ratio of clone TUV-R4B*-#1 to 15-mer library in the input phage was approximately 1:10).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Total No. of colonies</th>
<th>No. of TUV-R4B*-#1</th>
<th>% of TUV-R4B*-#1</th>
<th>% of 15-mer library</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input</td>
<td>167</td>
<td>15</td>
<td>8.9</td>
<td>91.1</td>
</tr>
<tr>
<td>Lung</td>
<td>145</td>
<td>144</td>
<td>99.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Vena Cava(^3)</td>
<td>70</td>
<td>47</td>
<td>67.1</td>
<td>32.9</td>
</tr>
<tr>
<td>Aorta(^4)</td>
<td>110</td>
<td>75</td>
<td>68.2</td>
<td>31.8</td>
</tr>
<tr>
<td>Liver</td>
<td>174</td>
<td>110</td>
<td>63.2</td>
<td>36.8</td>
</tr>
<tr>
<td>Spleen</td>
<td>108</td>
<td>58</td>
<td>53.7</td>
<td>46.3</td>
</tr>
<tr>
<td>Brain</td>
<td>204</td>
<td>130</td>
<td>63.7</td>
<td>36.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>198</td>
<td>135</td>
<td>68.1</td>
<td>31.9</td>
</tr>
</tbody>
</table>

1 Total bacterial colonies on agar plates.

2 Positive colonies after hybridization to the antisense radioactive probe of TUV-R4B*-#1.

3 Example of Vein.

4 Example of Artery.
Tables 13A and 13B: Tissue distribution of clone TUV-R4B*-#1 and TUV-R4B*-#3 in rat model. The ratio of TUV-R4B*-#1 and TUV-R4B*-#3 to the 15-mer library was approximately 1:1:98.

Table 13A: Tissue distribution of TUV-R4B*-#1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Total No. of colonies</th>
<th>No. of TUV-R4B*-#1</th>
<th>% of TUV-R4B*-#1</th>
<th>% of 15-mer library</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input</td>
<td>800</td>
<td>16</td>
<td>1.1</td>
<td>98.9</td>
</tr>
<tr>
<td>Lung</td>
<td>9</td>
<td>8</td>
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<td>57.3</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>Kidney</td>
<td>22</td>
<td>12</td>
<td>54.5</td>
<td>45.5</td>
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</table>

1 Total bacterial colonies on agar plates.

2 Positive colonies obtained by hybridization to the antisense radioactive probe of TUV-R4B*-#1.

The results of tables 12 and 13A demonstrate that clone TUV-R4B*-#1 is enriched in the rat lung, i.e. has highest specificity to the lung. Therefore, this peptide can be used as a specific marker for inter alia lung tissue, for drug delivery to lung tissue and to image lung tissue.
### Table 13B: tissue distribution of clone TUV-R4B*-#3

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<th>% of TUV-R4B*-#3</th>
<th>% of 15-mer library</th>
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1 Total bacterial colonies on agar plates.

2 Positive colonies by hybridization to the antisense radioactive probe of TUV-R4B*-#3.
EXAMPLE 2: Peptides comprising the Glu Gly Arg core sequence

Preferred peptides of the subject invention contain the core sequence Glu Gly Arg (EGR) which binds specifically to endothelial cells and to tumor tissue.

An example of such a peptide is a cyclic peptide Cys Glu Gly Arg Cys where the Cys residues form a -S-S- bond. Another example of such a peptide is the 15-mer peptide derived from clone TUV-R4B-#1 described in Example 1:

Gly Arg Gln His Phe Phe Leu Ala Glu Gly Arg Ser Phe Tyr Phe (Seq. ID. No. 2)

An additional example is a peptide having the sequence Glu Gly Arg Ser Phe (Seq. ID. No. 11).

Other longer peptides with higher affinity for binding to cells/tissue may be constructed based on the core sequence Glu Gly Arg. These peptides may be identified, e.g. by preparing an extension phage display library or by a phagemid display mutagenesis library. They may also be identified by varying the above 15-mer peptide by additions, deletions or mutations, while maintaining the Glu Gly Arg core sequence.

Peptides of the invention have the amino acid sequence R₁-Glu Gly Arg-R₂, where R₁ and R₂ comprise amino acid sequences from 0-50 amino acids, preferably 1-15 amino acids, most preferably 1-5 amino acids. All peptides of the invention bind to cells/tissue by means of the Glu Gly Arg core sequence.

R₁ and R₂ are amino acid sequences which do not negatively affect the specific binding of the EGR sequence to tissue/cells.
EXAMPLE 3: Preparation of a peptide

A peptide of the subject invention, which contains the Glu Gly Arg (EGR) core sequence, is prepared by the following methods:

I. Chemical (organic) synthesis

Chemical synthesis of peptides is carried out by methods well-known in the art, e.g. solid phase synthesis of Merrifield, J. Amer. Chem Soc. 85: 2149-2154 (1963); Science 150: 178-185 (1965); Ibid 232, 341-347 (1986). The solid phase synthesis provides a growing peptide chain anchored by its carboxyl terminus to a solid support, e.g. a resin such as chloromethylated polystyrene resin or p-methylbenzhydrylamin resin when synthesizing a peptide amide derivative. The use of various N-protecting groups, e.g. the carbobenzyloxy group, the t-butyloxy carbonyl group (BOC) or the N-(9-fluorenyl-methyl carbonyl) group (FMOC), various coupling reagents, e.g. dicyclohexyl carbodiimide (DCC) or carbonyldiimidazole, various cleavage reagents, e.g. trifluoracetic acid (TFA) in methylene chloride (CH$_2$Cl$_2$) and other such reagents of classical solution phase peptide synthesis are also used in conventional solid phase synthesis of peptides.

Various commercial companies prepare peptides custom-made, i.e. specifically as ordered by customers. Examples of such companies are Bio-Synthesis, Research Genetics, Inc., and AnaSpec (Science 275: 270 (1997)).

The chemically synthesized peptide is linked to a pharmaceutical agent (drug) *inter alia* by a covalent or by a non-covalent bond forming a drug-peptide conjugate. The peptide may also be incorporated into a liposome. The covalent bond may be *inter alia* a peptide, an amide, an ester, a disulfide or an anhydride covalent bond. The non covalent bond is *inter alia* an ionic bond or a hydrophobic complex. The pharmaceutical agent may be *inter alia* a radio isotope label.
The choice of the peptide linkage is determined based on the functional groups of the individual drug. The peptide may also be cyclic (see e.g. Example 2). Cyclic peptides having a disulfide bond may be prepared as described in U.S. Patent No. 4,903,773 (Partoliano and Ladner). Cyclic peptides may also be prepared as described in Schiller et al., Int. J. Peptide-Protein Res. 25:171 (1985) and in U.S. Patent No. 5,648,330 (Pierschbacher et al.).

II. Recombinant technology
Recombinant production of peptides is carried out by methods known in the art. The DNA encoding the peptide is prepared by synthetic oligonucleotides based on the amino acid sequence of the peptide and their known nucleotide codons (see e.g. U.S. Patent No. 5,221,619). The peptide is then produced by expression of the nucleotide sequence encoding the polypeptide.

The recombinantly produced peptide is linked to a pharmaceutical agent. When the pharmaceutical agent is a recombinant polypeptide, a hybrid fusion polypeptide is constructed comprising the peptide linked by a peptide bond to the drug as follows: a DNA molecule is prepared comprising DNA encoding the drug and DNA encoding the peptide. The DNA encoding the peptide is prepared by synthetic oligonucleotides based on the amino acid sequence of the peptide and their known nucleotide codons. The synthetic oligonucleotide encoding the peptide is ligated either to the 5' end of the DNA strand encoding the drug or to the 3' end. When ligated to the 5' end of the DNA encoding the drug, an ATG nucleotide sequence is added to the synthetic oligonucleotide encoding the peptide. When ligated at the 3' end of the DNA encoding the drug, a DNA termination codon (TAA or TGA) is added at the 3' end of the synthetic oligonucleotide encoding the peptide and the termination codon at the 3' end of the DNA encoding the drug is removed using conventional recombinant DNA technology methods by use of Mung bean nuclease or cleavage
with an appropriate restriction enzyme.

The hybrid polypeptide encoded by the above described recombinant DNA molecule is expressed and produced by recombinant technology by methods known in the art, e.g. in bacteria, yeast, insect, plant or mammalian cells in culture or in a genetically engineered transgenic animal or plant.

Upon injection, a recombinant protein is targeted to tissue specific endothelial cells and is converted from a soluble protein to a cell surface bound protein (ectoenzyme). In this manner, arteriosclerosis and thrombogenicity of the vessel wall in certain clinical indications is reduced. Conversion of these soluble proteins into ectoenzymes also reduces blood clearance time and hence a lower amount of recombinant protein is injected to achieve efficacy.

III. Computer-aided design technology

In recent years, intensive efforts have been made to develop peptidomimetics or peptide analogs that display more favorable pharmacological properties than their prototype native peptides. The native peptide itself, the pharmacological properties of which have been optimized, generally serves as a lead for the development of these peptidomimetics. A lead structure for development of a peptidomimetic can be optimized, for example, by molecular modeling programs.

Computer-aided design technology known in the art is used in order to produce a mimotope, i.e. a peptide mimetic of a peptide of the subject invention, the chemical structure of which is different from the peptide, but the biological activity of which remains similar to that of the peptide. U.S. Patent Nos. 4,879,313, 4,992,463 and 5,0191,396 describe examples of such peptide mimetic compounds.

To a peptide of the subject invention, produced by any one of the above three methods, amino acid residues may be added,
deleted or substituted using established well known procedures, thereby producing extended peptides.

Furthermore, the DNA encoding a peptide of the subject invention may be mutated by methods known to those skilled in the art, thereby produced mutated peptides.

As mentioned above, a peptide of the subject invention, produced by any one of the above three methods, may be administered to a patient, alone, radiolabeled, or linked to a pharmaceutical agent (drug).

The following pharmaceutical agents are examples of drugs which are linked to a peptide of the subject invention corresponding to a peptide displayed by a phage virion to form drug-peptide conjugates. Other pharmaceutical agents not mentioned below may also be used.

Peptides of the subject invention are linked to a toxin, an anti-cancer drug or an anti-angiogenic compound in order to target and destroy tumor tissue. This specific use of such drug-peptide conjugates represents an alternative approach to current attempts to apply immunotoxins against cancer.

Solid tumor growth in vivo is associated with recruitment of new blood vessels. Targeting the tumor-vasculature is an attractive possibility for anti-cancer therapy for the following reasons: First, most anti-cancer drugs are given by the systemic intravenous route and drug concentration at the tumor tissue is the crucial factor for effective therapy. Secondly, one limitation of current anti-cancer therapy is toxicity. Targeting the drug by cross-linking with a tissue specific peptide circumvents the toxicity problem. Thirdly, targeted anti-vascular or anti-angiogenesis therapy obliterates the capacity of the tumor to continue to grow and metastize as blood supply of fresh nutrient is blocked.
Examples of anti-cancer drugs that are linked to the peptides of the subject invention are adriamycin, cis-Platinum, taxol, bleomycin and so forth.

Examples of anti-angiogenic compounds that are linked to peptides of the subject invention are cortisone, heparin and so forth.

Examples of toxins that are linked to peptides of the subject invention are *Pseudomonas* exotoxin A, ricin and so forth.

Several major drugs applied in heart conditions are given by the systemic intravenous route while their target, the coronary arteries, constitute only a small portion of the vasculature. Therefore, targeting to the coronaries, or even to arteries in general concentrates the drug to the diseased vessels and reduces undesired side effects.

Examples of cardiovascular drugs that are linked to the peptides of the subject invention are thrombolytic enzymes such as tissue plasminogen activator (tPA), streptokinase (SK), and anti-thrombotic agents such as heparin, ticlopidine or antiplatelet monoclonal antibodies.

Peptides of the subject invention are further linked to recombinant proteins such as CuZnSOD, MnSOD, Factor Xa Inhibitors, erythropoietin, von Willebrand Factor or fragments thereof, ecto-enzymes such as Apyrase and so forth. Such recombinant proteins are described *inter alia* in U.S. Patent Nos. 5,126,252 and 5,360,729 (CuZnSOD), U.S. Patent Nos. 5,270,195 and 5,246,847 (MnSOD), WO 91/01416 (von Willebrand factor fragment), U.S. Patent No. 5,783,421 (Factor Xa Inhibitor), and U.S. Patent No. 4,703,008 (erythropoietin).

The most common brain disorders include tumors, stroke, head trauma, epilepsy, infectious agents, Parkinson's disease and Alzheimer's disease. Several drugs have been approved for
these indications. However, there is a need to increase the level of drug reaching the brain vasculature and developing a delivery system which transports therapeutics across the blood-brain barrier (BBB).

Examples of "CNS drugs" that are linked to the peptides of the subject invention are L-Dopa, Cortisone, tPA or phenobarbital.

Both the liver and the kidney are suitable for targeted drug delivery via the vascular system since they are well perfused tissues, involved in the metabolism of many endogenous and exogenous compounds. Targeting of liver and kidney is important particularly for cancer therapy and hepatitis B and C virus infection of the liver.

Examples of drugs for treatment of liver and kidney disease that are linked to peptides of the subject invention are anti-viral drugs such as Interferon, Iododeoxyuridine, and Adenine arabinoside. Adriamycin can be used for the treatment of renal cancer and Glucocerebrosidase 6-thioguanine for the treatment of Gaucher's disease.
EXAMPLE 4: Isolation of peptides which specifically bind to undetermined targets on the blood vessels of Kaposi sarcoma (KS) tumor-bearing nude mice in vivo.

Phage selection strategy:

For the induction of KS tumors we used a KS cell line which was isolated from a non-HIV-infected patient, and which was recently shown to carry several markers of HIV-infected KS cells (Herndier et al. (1994), Aids 8(5): 575-581). The KS cells (5×10⁶) as a mixture in Matri-gel (1:1 v/v) were injected into nude mice. After approximately 12-14 days, when well defined localized tumors were visualized (about 1.2 cm in diameter), a mixture of the 15-mer library (described in Example 1) together with M13 bacteriophage (10¹²:10¹³, respectively) was injected into the tail-vein. After 4 minutes, the animal vascular system was perfused with PBS for an additional 4 minutes and then sacrificed. Tumors were excised, weighed, and kept on ice for all subsequent manipulations. Following homogenization and extensive washing, phage elution was carried out with starved bacteria. The eluted phage was titrated, amplified, and then subjected to additional rounds of biopanning in KS tumor bearing mice.

Results of biopanning in vivo

The KS-tumor eluted phages from two independent experiments (designated MKS1 and MKS2), following three rounds of biopanning in vivo, were analyzed by DNA sequencing. The sequencing results indicated that almost 100% of the clones at round 3 in both experiments, were identical to clone TUV-R4B*#1 (Example 1), which was also selected on umbilical vein and artery, ex vivo.
Organ distribution of selected phages:

Mixtures of selected phage and M13 bacteriophage (5x10^{10} and 5x10^{11}, respectively) were injected into the tail vein of KS-tumor bearing mice, and after one round of panning, tumor and brain tissue were excised, washed, and treated for phage elution and titration as described above. The results of three parallel experiments, with various phage mixtures, are demonstrated in Figure 1.

These results demonstrate that the tumor-distribution of clone TUV-R4B*#1 is 4-5 fold higher than the brain-distribution. No such results were obtained with the two other clones, which demonstrate similar low binding to both tumor and brain tissues.

Thus clone TUV-R4B*#1, which comprises an EGR-containing peptide, binds specifically to KS-tumor.
EXAMPLE 5: Binding of EGR-containing peptides to melanoma and mammary tumor cells

Note: All results discussed below were normalized to gram tissue.

5.1 Mouse Melanoma model:

0.2 million Mouse melanoma cells, B16.F10.9 (A. Progador et al, Int. J. Cancer Suppl (1991), 6: 54-60), were injected subcutaneously into the hind leg of C57 Black female mice, 6 weeks old. Within approximately 12-15 days when tumor size was approximately 0.4-0.9g, phage mixture containing clones TUV-R4B*-#1 (the EGR-phage) and unselected control, phage 14-4, were injected at a ratio of approximately 1:20, respectively.

1:10 and 1:100 dilutions of the phage mixture were also injected into the melanoma tumor bearing mice. Phage titers in the tumor and brain eluates were determined.

The results are demonstrated in Fig. 2. The dose response curve of the binding of clone TUV-R4B-*#1 demonstrated a different binding profile for the two organs tested. At the higher doses of the injected phage (3x10¹⁰) the binding to the tumor was much higher than to the brain (Fig 2), with a tumor/brain ratio of approximately 7.5 (Fig. 3). Each point is based on an average of 3 animals.

A synthetic 15-mer-EGR-peptide having the sequence Gly Arg Gln His Phe Phe Leu Ala Glu Gly Arg Ser Phe Tyr Phe competes for the binding of the EGR-phage to the vascular tissue.
5.2 *Mouse mammary carcinoma model:*

Mouse mammary carcinoma cells, DA-3-p (Fu X.-Y. et al., Cancer Res., 1990, 50:227) were injected subcutaneously into the hind leg of Balb/C female mice, 6 weeks old, at 1 million cells/animal. After 10-15 days when tumor size was approximately 0.3-0.6g, phage mixture was injected into the tail vein in triplicates. The yield (%) of the binding [eluted phage (output) over input phage x 100] of the EGR-phage (clone TUV-R4B-#1) and of the unselected control phage, clone 14-4, are shown in Fig. 4.

The yield (%) of binding of the EGR-phage is several fold higher than that of the unselected control phage 14-4, indicating a selective enrichment of the EGR-phage on the mammary tumor vascular bed. The comparison of binding of the EGR-phage to the mammary tumor and to the brain is demonstrated in Fig. 5.

The yield (%) of the binding to the mammary tumor is many fold higher than to the brain in all three animals tested. The enrichment factor of the EGR-phage in tumor and brain (i.e., the yield (%) of the EGR-phage over the yield (%) of the 14-4 phage in the tumor and in the brain) is depicted in Fig. 6. Thus, the enrichment factor of the EGR-phage in the tumor was approximately 4 fold higher than enrichment factor in the brain, indicating a preferential homing of the EGR-phage to the mammary tumor vascular bed.

5.3 *Human mammary carcinoma model:*

They were separately injected into the foot pad of 8-10 weeks old female Balb/C nude mice at 0.1-0.5 million cells per animal. After 8-14, weeks when tumor size was approximately 0.3-0.8g, phage mixture was injected into the tail vein. Phage elution and titration was monitored as described in Example 4.

Discussion

These results and those in Example 4 show that the EGR-containing phage binds specifically to various tumor tissues. It is likely that the binding is to endothelial cells in the vascular bed. Such specific binding is a characteristic of the EGR-containing peptides of the subject invention.
What is claimed is:

1. A nonnaturally occurring pharmaceutically active peptide which comprises the tripeptide Glu-Gly-Arg.

2. A peptide according to claim 1 having the amino acid sequence:
   Gly Arg Gln His Phe Phe Leu Ala Glu Gly Arg Ser Phe Tyr Phe (Seq. ID. No. 2)

3. A peptide according to claim 1 comprising a peptide, the amino acid sequence of which is:
   Gly Pos Pos Pos/Ar Ar Ar Leu Ala Glu Gly Arg Ser Ar Ar Ar wherein Pos is a positively charged amino acid and Ar is an aromatic amino acid.

4. A peptide according to claim 1 comprising a peptide, the amino acid sequence of which is:
   $R_1$-Glu Gly Arg-$R_2$ where $R_1$ and $R_2$ comprise from 0-50 amino acids.

5. A peptide of claim 4 where $R_1$ and $R_2$ comprise 1-15 amino acids.

6. A peptide of claim 5 where $R_1$ and $R_2$ comprise 1-5 amino acids.

7. A peptide of claim 1 comprising a peptide having the amino acid sequence $R_1$-Glu Gly Arg Ser Phe-$R_2$ (Seq. ID. No. 11) where in $R_1$ and $R_2$ comprise from 0-50 amino acids.

8. A peptide of claim 7 where in $R_1$ and $R_2$ comprise 1-15 amino acids.

9. A peptide of claim 8 where in $R_1$ and $R_2$ comprise 1-5 amino acids.
10. A composition comprising a peptide according to any of claims 1 to 9 and a pharmaceutical agent linked thereto.

11. A composition according to claim 10, wherein the pharmaceutical agent is a polypeptide and is linked to the peptide by a peptide linkage.

12. A composition according to claim 10, wherein the pharmaceutical agent is a toxin, an anti-cancer agent, an anti-angiogenic compound, a cardiovascular agent, an agent used in a neurological disorder, a liver disease agent, a kidney disease agent or a radioisotope.

13. A composition according to claim 10, wherein the pharmaceutical agent is a recombinant protein.

14. A composition comprising a peptide according to any of claims 1 to 9 and a pharmaceutically acceptable carrier.

15. A composition according to claims 10-13 which additionally comprises a pharmaceutically acceptable carrier.

16. A chimeric polypeptide comprising a first peptide portion and a second peptide portion, wherein the first peptide portion is a peptide of any of claims 1 to 9.

17. A polypeptide according to claim 16, wherein the second peptide portion is a toxin, an anti-cancer agent, an anti-angiogenic compound, a cardiovascular agent, an agent used in a neurological disorder, a liver disease agent or a kidney disease agent.
18. A polypeptide according to claim 16, wherein the second peptide portion is a recombinant protein.

19. An imaging agent which comprises a peptide of any of claims 1 to 9 labeled with an imageable marker.

20. A composition comprising an effective imaging amount of the imaging agent of claim 19 and a physiologically acceptable carrier.

21. A composition comprising an effective imaging amount of the imaging agent of claim 19, a pharmaceutical agent linked thereto, and a physiologically acceptable carrier.

22. An agent according to claim 19 wherein the marker is a radioactive isotope, an element which is opaque to X-rays, or a paramagnetic ion.

23. An agent of claim 22, wherein the marker is a radioactive isotope.

24. An agent of claim 23, wherein the radioactive isotope is indium-111, technetium-99, iodine-123, iodine-125, iodine-131, krypton-81m, xenon-33 or gallium-67.

25. A method for imaging an organ which comprises:
   (i) contacting the organ to be imaged with an imaging agent according to claim 19 under conditions such that the imaging agent binds to the organ; and
   (ii) imaging bound imaging agent so as to thereby image the organ.

26. A method according to claim 25, wherein the organ is an artery, a vein, placenta, tumor tissue, kidney, heart or liver.
27. A method according to claim 26, wherein the artery is umbilical cord artery, a radial artery, a coronary artery or a mammary artery.

28. A method according to claim 26, wherein the artery is a damaged artery.

29. A method according to claim 28, wherein the damaged artery is a damaged coronary artery.

30. A method according to claim 26, wherein the vein is umbilical cord vein, safenal vein or femoral vein.

31. A composition according to claim 21, wherein the pharmaceutical agent is a polypeptide and is linked to the imaging agent by a peptide linkage.

32. A composition according to claim 21, wherein the pharmaceutical agent is a toxin, an anti-cancer agent, an anti-angiogenic compound, a cardiovascular agent, an agent used in a neurological disorder, a liver disease agent or a kidney disease agent.

33. A composition according to claim 21, wherein the pharmaceutical agent is a recombinant protein.

34. A method of treating an organ in vivo which comprises: contacting the organ to be treated with a composition according to claim 21 under conditions such that the composition binds to the organ so as to thereby treat the organ.

35. A method according to claim 34, wherein the organ is an artery, a vein, placenta, tumor tissue, kidney, heart, liver, or central nervous system.

36. A method according to claim 35, wherein the artery is
umbilical cord artery, a radial artery, a coronary artery or a mammary artery.

37. A method according to claim 35, wherein the artery is a damaged artery.

38. A method according to claim 37, wherein the damaged artery is a damaged coronary artery.

39. A method according to claim 35, wherein the vein is umbilical cord vein, safenal vein or femoral vein.

40. A method of synthesizing a peptide of any of claims 1-9 which comprises joining the amino acids of the peptide in the proper order.
FIG. 1

Peptide-phage clones injected to mice

Phage binding (TTU/gram tissue x 0.001)

- Tumor
- Brain

KSCR3#3  R4B*#1  R6P#13
FIG. 4

Mouse #

Yield (%)

0.06
0.05
0.04
0.03
0.02
0.01
0

1 2 3

□ EGR-phage
□ 14-4 phage

SUBSTITUTE SHEET (RULE 26)
Sequence Listing

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<120> ISOLATION OF TISSUE SPECIFIC PEPTIDE LIGANDS AND THEIR USE FOR TARGETING PHARMACEUTICALS TO ORGANS

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INTERNATIONAL SEARCH REPORT

INTERNATIONAL APPLICATION NO.
PCT/US99/04691

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.
US CL : 530/300, 350, 402, 333, 424/1.11;
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)
U.S. : 530/300, 350, 402, 333; 424/1.11;

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, BIOSIS, SCISEARCH, EMBASE, DERWENT, Glu-Gly-Arg

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of Box C. See patent family annex.

Date of the actual completion of the international search: 24 MAY 1999

Date of mailing of the international search report: 17 JUN 1999

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
Martha Label

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1992)*
A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):
C07H 21/04; C07K 5/08, 7/00, 14/00; A61K 43/00; C12N/15/00, 15/63