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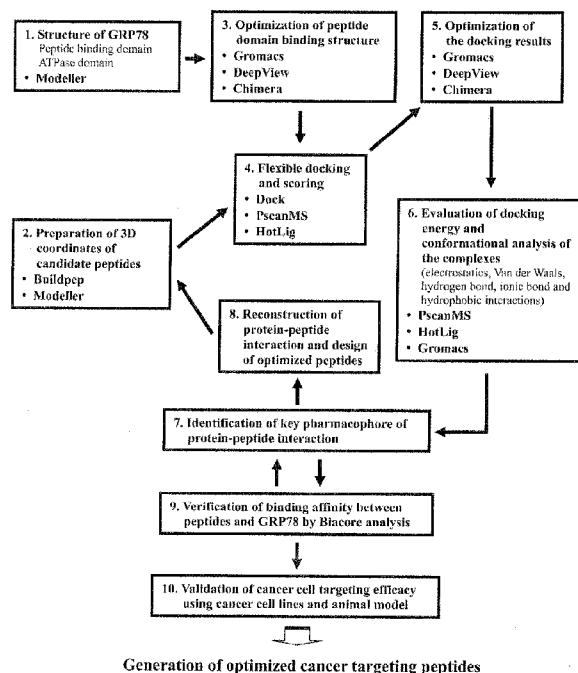
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(54) Title: CANCER-TARGETING PEPTIDES AND USES THEREOF IN CANCER TREATMENT AND DIAGNOSIS

FIGURE 1



(57) Abstract: Cancer-targeting peptides having a
PX₁LX₂ motif, in which X₁ is His or an amino acid
residue with a hydrophobic side chain and X₂ is Pro, Phe,
or Trp. Also disclosed herein are conjugates containing
the cancer-targeting peptides and uses thereof in cancer
treatment and diagnosis.



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Cancer-Targeting Peptides and Uses Thereof in Cancer Treatment and Diagnosis

RELATED APPLICATION

5 This application claims priority to U.S. Provisional Application No. 61/455,781, filed on October 25, 2010, the content of which is hereby incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

The use of peptides as targeted delivery agents is a rapidly emerging field applicable
10 to treatment of a variety of diseases, such as cancer, metabolic diseases, inflammatory autoimmune diseases, and viral infection. Wang et al. *Expert Opin. Drug Deliv.* 7:159-171 (2010); Liu, *Bioconjug. Chem.* 20: 2199-2213 (2009); Hsu et al. *BioDrugs.* 23:289-304 (2009); Bellmann-Sickert et al. *Trends Pharmacol. Sci.* 31:434-441 (2010); Zhong, *Curr. Top Med. Chem.* 10:386-396 (2010); and Briand et al. *Curr. Pharm. Des.* 16:1136-1142 (2010).
15 A number of cancer-targeting peptides have been identified, which specifically bind to various cancer markers, including integrin, vascular endothelial growth factor (VEGF), and heat-shock protein 90 (Hsp90). Wang et al., 2010; Hsu, 2009; and Horibe et al. *J. Transl. Med.* 9:8 (2011) Most of these cancer-targeting peptides have been used in the treatment of neuroendocrine tumors.

20 It is of great interest to develop new cancer-targeting peptides for use in diagnosing and treating a broad spectrum of cancers.

SUMMARY OF THE INVENTION

The present disclosure is based on the identification of a number of peptides that
25 target human glucose-regulated protein 78 (GRP78), a protein expressed on the surface of various types of cancer cells, via computational design.

Accordingly, one aspect of the present disclosure relates to an isolated peptide comprising an amino acid sequence motif PX_1LX_2 (SEQ ID NO:1) in which X_1 is H or an amino acid with a hydrophobic side chain (e.g., L, F, or W) and X_2 is P, F, or W. Preferably,
30 when X_1 is L, X_2 is not P; and when X_2 is P, X_1 is not L. In some embodiments, the isolated peptide comprises the amino acid sequence of $RLLDTNRPX_1LX_2Y$ (SEQ ID NO:2).

Examples include, but are not limited to, RLLDTNRPFLPY (P-6) (SEQ ID NO:3), RLLDTNRPHLWY (P-12) (SEQ ID NO:4), and RLLDTNRPFLFY (P-13) (SEQ ID NO:5).

In another aspect, the present disclosure provides a composition comprising (a) any of the cancer-targeting peptide disclosed herein, and (b) an anti-cancer agent (e.g., doxorubicin, vinorelbine, vincristine, paclitaxel or lurtotecan), a detectable label (e.g., a fluorescent compound such as fluorescein isothiocyanate or a luminescent compound), or both. In some 5 embodiments, the cancer-targeting peptide and the anti-cancer agent or the detectable label are conjugated (attached), either directly or via a linker (e.g., a polymer such as polyethylene glycol). The composition can further comprise a vehicle carrier such as a liposome. In some 10 embodiments, the vehicle carrier encapsulates the anti-cancer agent, the detectable label, or both. The detectable label can be an imaging agent suitable for tumor imaging (e.g., a radioactive molecule such as ^{99m}Tc or ^{188}Re . or an iron oxide nanoparticle). The cancer-targeting peptide, preferably pegylated, can be attached on the surface of the vehicle carrier.

The composition described above can be a pharmaceutical composition, which can 15 further comprise a pharmaceutically acceptable carrier. In some embodiments, the composition contains an anti-cancer agent in an amount effective in treating cancer. In other embodiments, the composition contains a detectable label such as an imaging agent in an amount effective in detecting cancerous tissues and/or cells.

In addition, the present disclosure also provides a method for delivering an anti- 20 cancer agent or a detectable label to cancer cells, e.g., breast cancer cells (such as breast cancer stem cells), hepatocellular carcinoma cells, prostate cancer cells, lung cancer cells, ovarian cancer cells, kidney cancer cells, uterine cervical cancer cells, melanoma cells, embryonal carcinoma cells, leukemia cells, or osteosarcoma cells. The method comprises contacting cancer cells or cells suspected to be cancerous with any of the compositions 25 described herein. The composition can be administered to a subject in need thereof (e.g., a human patient having or suspected of having cancer). Alternatively, it can be incubated (*in vitro*) with a sample (e.g., a tissue sample) having or suspected of having cancer cells. In some embodiments, the anti-cancer agent is delivered to a subject in an amount effective in treating cancer. In other embodiments, a detectable label, preferably an agent suitable for 30 cancer imaging, is delivered to a subject in need thereof (e.g., a human patient having or

suspected of having a solid tumor) in an amount effective in detecting cancer cells and/or cancerous tissues.

Also within the scope of this disclosure are any of the pharmaceutical compositions described herein for use in delivering one or more anti-cancer agents, one or more detectable
 5 labels, or both to cancer cells, for use in cancer treatment and/or diagnosis, as well as using these compositions in manufacturing a medicament for the above-noted purposes.

Further, the present disclosure provides a method for identification (structure-based optimization) of a cancer-targeting peptide ligand. The method comprises: (a) providing a cancer cell-surface protein (e.g., human GRP78); (b) calculating the Connolly surface of the
 10 cancer cell-surface protein by, e.g., a binding pocket analysis program such as PscanMS; (c) identifying a peptide-binding site on the protein surface; (d) estimating distance-dependent potential of paired atoms involved in polar interactions (e.g., hydrogen bonding, ionic interactions and metal-ion coordination), the estimation comprising consideration of an intermolecular surface distance; (e) estimating distance-dependent potential of paired atoms
 15 involved in non-polar interactions, the estimation comprising application of Connolly surface of protein in calculation of the intermolecular surface distance; and (f) selecting a peptide ligand optimized for binding to the binding site of the cancer cell-surface protein from a peptide database, which can be combinatorially constructed, based on the information obtained from steps (d) and/or (e). In one example, the intermolecular surface distance is
 20 determined based on the binding energy (ΔE) between protein (p) and ligand (l), which is calculated according to the following equation:

$$\begin{aligned} \Delta E_{p,l} &= \Delta E_{polar}(\delta, \theta, \phi) + \Delta E_{non-polar}(v, A), (\phi < 100^\circ) \\ &= F_{hbond} \times \sum_h (\cos(180^\circ - \theta_h) \times W_{hbond}(\delta_h)) \\ &\quad + F_{ion} \times \sum_i W_{ion}(\delta_i) \\ &\quad + F_{metal} \times \sum_m W_{metal}(\delta_m) \\ &\quad + F_{vdw} \times \sum_v (A_v \times W_{vdw}(v_v)) \end{aligned}$$

wherein, h is the pair of H-bond; i is the pair of ionic interaction; m is the pair of metal-ion coordination and v is the ligand-contacted normal vector in hydrophobic

interaction.

The distance-dependent potentials can be predicted from a statistical set comprising occurrence frequencies of paired pharmacophores in molecular interactions.

In other embodiments, the just-described method is performed *in silico* (i.e.,
5 performed on computer or via computer simulation).

The details of one or more embodiments of the invention are set forth in the description below. Other features or advantages of the present invention will be apparent from the following drawings and detailed description of several embodiments, and also from the appended claims.

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BRIEF DESCRIPTION OF THE DRAWINGS

The drawings are first described.

Figure 1 is a schematic illustration of the design of optimized peptides targeting human cancer marker proteins (using human GRP as an example) *in silico*.

15

Figure 2 is a schematic illustration of HotLig scoring function.

Figure 3 is a schematic illustration of parametric analysis of protein-ligand interactions applied in HotLig.

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Figure 4 is a diagram showing modeling of the 3D structure of human GRP78, a cancer marker protein, and optimization of peptides targeting human GRP78 using the L-peptide as a lead. **a:** modeling of human GRP78 shows that this protein is composed of a peptide-binding domain and an ATPase domain linked by a loop. **b:** the L-peptide binding site appears as a "tunnel" through which a binding peptide can pass. The peptide-binding site of the L-peptide is illustrated with identification of the A-, B-, and C-pockets, which are predicted to interact with Pro11, Leu10, and Leu9 of the L-peptide. **c:** The Connolly protein surface generated by PscanMS displays the perspective shape of the peptide-binding site and the geometric matching of the peptide molecule with the binding site. Pockets A and C are key sites for optimization of cancer-targeting peptides. **d:** Intermolecular hydrogen bonds between GRP78 and the L-peptide. Hydrogen bonds mainly occurred at the Pro8-Leu9-Leu10-Pro11 sequence of the L-peptide. **e:** The Trp and Phe amino acids were observed to be
25 promising candidates for alterations at Pro11 to fit in the A-pocket in different orientations
30

because of their rigid, planar side chains. **f**: The C-pocket for Leu9 of the L-peptide could be replaced by many other amino acids which resulted in similar orientations.

Figure 5 is a diagram showing derivation of H-bond potential from the statistic distribution of H-bond parameters. **a**: The distribution of angle donor-H-acceptor (θ) vs. atomic surface distance (δ) of H-bonding pairs shows the atomic distance is smaller than the summation of radii of H-bond donor and acceptor atoms ($\delta < 0$) when H-bond occurred. The H-bond cluster also indicated that the optimal angle of θ is 180 degree and the binding force is decreasing with the smaller angle of θ . **b**: The distribution of the H-bond cluster is not significantly correlate with the angle electron-acceptor-H ϕ . Generally, the angles ϕ were smaller than 100 degree in H-bond cluster. **c**: The distance-dependant potential for H-bond (W_{hbond}) was derived from (a) using the normalization method as Velec's approach (Velec et al., 2005) **d**: To simulate the H-bond potential from the distribution of H-bond cluster, the angle θ and ϕ were introduced in HotLig to calculate the energy score of H-bond (ΔE_{hbond}) as the following equation: $\Delta E_{hbond} = \cos(180 - \theta) \times W_{hbond}(\delta)$, $\phi < 100$

Figure 6 is a diagram showing oriented immobilization of protein GRP78 on sensor chip NTA.

Figure 7 is a diagram showing *in vitro* binding evaluation of cancer-targeting peptides. **a**: A representative Biacore sensorgram of 50 μ M peptides binding to the full-length recombinant GRP78. Peptide CdL was used as a negative control. **b**: Binding of FITC-labeled peptides to breast cancer cell line, MDA-MB-231 and NPC TW01 by flow cytometry. **c**: Binding of FITC-labeled peptides to primary human breast cancer, BC0145 and BC0244 engrafted in NOD/SCID mice. **d**: Binding of FITC-labeled peptides to clinical breast cancer specimens, BC0854 and BC0861 by flow cytometry.

Figure 8 is a diagram showing therapeutic efficacy of Lipo-Dox linked to L-peptide (n=4), P-6 (n=4), P-12 (n=7), or P-13 (n=6) in NOD/SCID mice bearing BC0244 human breast cancer xenografts. **a** and **b**: tumor size measured twice a week. **c**: body weight, measured twice a week. **d**: Kaplan Meier survival curve of mice treated with PBS, Lipo-Dox, or peptides-labeled Lipo-Dox. *, P<0.05; **, P<0.01; ***, P<0.001 of peptides-labeled Lipo-Dox compared with Lipo-Dox.

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Figure 9 is a chart showing normalized distribution of hydrophobic interactions.

Figure 10 is a diagram showing detection of binding pocket and water-contactable atoms by PscanMS for construction of a protein surface.

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DETAILED DESCRIPTION OF THE INVENTION

Disclosed herein are cancer-targeting peptides, which are capable of binding to human GRP78, compositions comprising (a) one or more of the cancer-targeting peptides, and (b) one or more anti-cancer agents, one or more detectable labels, or both. These peptides are capable of targeting GRP78, which was found to be expressed on a broad spectrum of cancer
10 cells. Accordingly, the cancer-targeting peptides described herein can be used for delivering anti-cancer agents and/or detectable labels to various cancers, particularly those that express GRP78 on cell surfaces, thereby facilitating cancer diagnosis and treatment.

(i) Cancer-targeting Peptides and Conjugates Containing Such

The isolated cancer-targeting peptides disclosed herein each comprise an amino acid
15 sequence motif Pro- X_1 -Leu- X_2 (also known as PX_1LX_2) (SEQ ID NO:1), in which X_1 is His or an amino acid residue having a hydrophobic side chain and X_2 is Pro, Phe, or Trp. X_1 can be an amino acid residue having an aliphatic hydrophobic side chain, e.g., Ala, Ile, Leu, or Val. Alternatively, X_1 can be an amino acid residue having an aromatic hydrophobic side chain, e.g., Phe, Trp, or Tyr. In addition, X_1 can also be Gly, Met, or Pro. Examples of the
20 motifs include, but are not limited to, PFLP (SEQ ID NO:6), PHLW (SEQ ID NO:7), PFLW (SEQ ID NO:8), PYLW (SEQ ID NO:9), and PFLF (SEQ ID NO:10). Preferably, when X_1 is Leu, X_2 is not Pro, and when X_2 is Pro, X_1 is not Leu. When desired, the cancer-targeting peptides can include the any of the above disclosed PX_1LX_2 (SEQ ID NO:1) motif and a R residue and a Y residue at the N-terminal and C-terminal of the motif, respectively.

25 In some embodiments, the cancer-targeting peptides described herein comprises the amino acid sequence of RLLDTNRP X_1LX_2Y (SEQ ID NO:2), in which the motif PX_1LX_2 is described above. Examples of the cancer-targeting peptides include, but are not limited to, RLLDTNRPFLPY (P-6; SEQ ID NO:3), RLLDTNRPHLWY (P-12; SEQ ID NO:4), RLLDTNRPFLFY (P-13; SEQ ID NO:5), RLLDTNRPFLWY (PB-1; SEQ ID NO:11); and
30 RLLDTNRPFLFY (PB-2; SEQ ID NO:12). In other embodiments, the cancer-targeting

peptides described herein each consist of the PX_1LX_2 motif described herein, or consists of the motif and a R residue and a Y residue at the N-terminal and C-terminal of the motif, respectively.

The term "peptide" used herein refers to a polymer composed of two or more amino acid monomers and is shorter than a protein. Preferably, each of the cancer-targeting peptides described herein includes up to 50 (e.g., up to 20 or 30) amino acids. In some examples, the cancer-targeting peptides each contain 4-20 amino acid residues (e.g., 4-10, 6-10, 6-15, or 6-20 amino acid residues). These peptides can contain naturally-occurring amino acid residues, or modified amino acids. In one example, either the N-terminus or the C-terminus of a cancer-targeting peptide is modified, e.g., containing an $-NH_2$ group at the C-terminus. An "isolated" peptide is a peptide that is substantially free from naturally associated molecules, i.e., the naturally associated molecules constituting at most 20% by dry weight of a preparation containing the polypeptide. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, and HPLC.

The cancer-targeting peptides described herein can be made by any conventional methods, e.g., recombinant technology or standard methods of solid phase peptide chemistry well known to any one of ordinary skill in the art. For example, the peptides may be synthesized by solid phase chemistry techniques following the procedures described by Stewart et al. in *Solid Phase Peptide Synthesis*, 2nd Ed., Pierce Chemical Company, Rockford, Ill., (1984) using a Rainin PTI Symphony synthesizer. For solid phase peptide synthesis, techniques may be found in Stewart et al. in "Solid Phase Peptide Synthesis", W. H. Freeman Co. (San Francisco), 1963 and Meienhofer, *Hormonal Proteins and Peptides*, 1973, 2 46. For classical solution synthesis, see for example Schroder et al. in "The Peptides", volume 1, Academic Press (New York). In general, such methods comprise the sequential addition of one or more amino acids or suitably protected amino acids to a growing peptide chain on a polymer. Normally, either the amino or carboxyl group of the first amino acid is protected by a suitable protecting group. The protected and/or derivatized amino acid is then either attached to an inert solid support or utilized in solution by adding the next amino acid in the sequence having the complimentary (amino or carboxyl) group suitably protected and under conditions suitable for forming the amide linkage. The protecting group is then

removed from this newly added amino acid residue and the next amino acid (suitably protected) is added, and so forth.

5 The cancer-targeting peptides described herein can also be prepared by conventional recombinant technology, using expression vectors comprising nucleic acids encoding the cancer-targeting peptides. Such nucleic acids and vectors (e.g., expression vectors) are also within the scope of this disclosure.

10 The cancer-targeting peptides described herein are capable of binding to human GRP78, which was reported to reside on the outer surface of cancer cells but only in the cytoplasm of normal cells (Lee et al., *Cancer Res.*, 67:3496-3499, 2007; Jakobsen et al., *Cancer Res.* 67:9507-9517, 2007; Graner et al., *Cancer Sci.* 100:1870-1879, 2009; and Ni et al., *Biochem. J.* 434:181-188, 2011). Thus, these peptides can be used to target various types of cancers for, e.g., cancer therapy or diagnosis such as imaging. Target cancers can be, but are not limited to, breast cancer, hepatocellular carcinoma, prostate cancer, lung cancer, ovarian cancer, kidney cancer, uterine cervical cancer, melanoma, embryonal carcinoma, leukemia, osteosarcoma, brain cancer, nasal cancer, pharyngeal cancer, head cancer, neck cancer, bladder cancer, pancreatic cancer, stomach cancer, colon cancer, skin cancer, colorectal, lymphoma, gastric cancer, or leukemia.

15 Any of the cancer-targeting peptides can be conjugated with (attached to) an anti-cancer agent, a detectable label, or both for cancer treatment and/or cancer diagnosis (either *in vivo* or *in vitro*). As used herein, "conjugated" or "attached" means two entities are associated, preferably with sufficient affinity that the therapeutic/diagnostic benefit of the association between the two entities is realized. The association between the two entities can be either direct or via a linker, such as a polymer linker. Conjugated or attached can include 20 covalent or noncovalent bonding as well as other forms of association, such as entrapment, e.g., of one entity on or within the other, or of either or both entities on or within a third entity, such as a micelle.

25 In one example, a cancer-targeting peptide is attached to a detectable label, which is a compound that allows recognition, either directly or indirectly, the peptide conjugated to it such that the peptide can be detected, measured, and/or qualified. Examples of such 30 "detectable labels" are intended to include, but are not limited to, fluorescent labels,

chemiluminescent labels, colorimetric labels, enzymatic markers, radioactive isotopes, and affinity tags such as biotin. Such labels can be conjugated to the peptide, directly or indirectly, by conventional methods.

In some embodiments, the detectable label is an agent suitable for cancer imaging, which can be a radioactive molecule, a radiopharmaceutical, or an iron oxide particle.

Radioactive molecules suitable for *in vivo* imaging include, but are not limited to, ^{122}I , ^{123}I , ^{124}I , ^{125}I , ^{131}I , ^{18}F , ^{75}Br , ^{76}Br , ^{76}Br , ^{77}Br , ^{211}At , ^{225}Ac , ^{177}Lu , ^{153}Sm , ^{186}Re , ^{188}Re , ^{67}Cu , ^{213}Bi , ^{212}Bi , ^{212}Pb , and ^{67}Ga . Exemplary radiopharmaceuticals suitable for *in vivo* imaging include ^{111}In Oxyquinoline, ^{131}I Sodium iodide, $^{99\text{m}}\text{Tc}$ Mebrofenin, and $^{99\text{m}}\text{Tc}$ Red Blood Cells, ^{123}I Sodium iodide, $^{99\text{m}}\text{Tc}$ Exametazime, $^{99\text{m}}\text{Tc}$ Macroaggregate Albumin, $^{99\text{m}}\text{Tc}$ Medronate, $^{99\text{m}}\text{Tc}$ Mertiatide, $^{99\text{m}}\text{Tc}$ Oxidronate, $^{99\text{m}}\text{Tc}$ Pentetate, $^{99\text{m}}\text{Tc}$ Pertechnetate, $^{99\text{m}}\text{Tc}$ Sestamibi, $^{99\text{m}}\text{Tc}$ Sulfur Colloid, $^{99\text{m}}\text{Tc}$ Tetrofosmin, Thallium-201, and Xenon-133. The reporting agent can also be a dye, e.g., a fluorophore, which is useful in detecting tumor mass in tissue samples.

In another example, one of the cancer-targeting peptides described herein is conjugated with an anti-cancer agent to form a treatment conjugate. The anti-cancer agent can be a chemotherapy agent, such as drugs that stop DNA building block synthesis (e.g., methotrexate, fluorouracil, hydroxyurea, lurtotecan, mercaptopurine, pentostatin and pirarubicin), drugs that directly damage DNA (e.g., cisplatin, daunorubicin, doxorubicin, etoposide, teniposide, camptothecin, topotecan, irinotecan, rubitecan, belotecan), drugs that affect mitotic spindle synthesis or breakdown (e.g., vinblastine, vincristine, vinorelbine, vinflunine, vindesine, docetaxel, larotaxel, ortataxel, paclitaxel, tesetaxel, ixabepilone and epithilones), or drugs that disrupt angiogenesis (e.g., anti-VEGF antibody, angiostatin, endostatin, and tumstatin). Alternatively, the anti-cancer agent can be a radiotherapy agent (e.g., ^{90}Y , ^{125}I , ^{188}Re , ^{111}In DTPA, or ^{131}I Sodium iodide).

Examples of anti-cancer drugs or antineoplastics to be attached to the cancer-targeting peptides described herein include, but are not limited to, aclarubicin, altretamine, aminopterin, amrubicin, azacitidine, azathioprine, belotecan, busulfan, camptothecin, capecitabine, carboplatin, carmofur, carmustine, chlorambucil, cisplatin, cladribine, clofarabine, cyclophosphamide, cytarabine, daunorubicin, decitabine, doxorubicin, epirubicin, etoposide, floxuridine, fludarabine, 5-fluorouracil, fluorouracil, gemcitabine,

idarubicin, ifosfamide, irinotecan, mechlorethamine, melphalan, mercaptopurine, methotrexate, mitoxantrone, nedaplatin, oxaliplatin, paclitaxel, pemetrexed, pentostatin, pirarubicin, pixantrone, procarbazine, pyrimethamine raltitrexed, rubitecan, satraplatin, streptozocin, thioguanine, triplatin tetranitrate, teniposide, topotecan, tegafur, trimethoprim, uramustine, valrubicin, vinblastine, vincristine, vindesine, vinflunine, vinorelbine, and zorubicin.

In any of the conjugates described above, the cancer-targeting peptide can be linked directly to a detectable label or an anti-cancer agent via methods known in the art.

Alternatively, the cancer-targeting peptide is linked to a vehicle carrier, which is associated with the detectable label and/or the anti-cancer agent. In one example, the vehicle carrier encapsulates the detectable label and/or the anti-cancer agent. Vehicle carriers include, but are not limited to, micelle, liposome (*e.g.*, cationic liposome), nanoparticle, microsphere, or biodegradable polymer. A cancer-targeting peptide can be tethered to a vehicle carrier by a variety of linkages (*e.g.*, a disulfide linkage, an acid labile linkage, a peptide-based linkage, an oxyamino linkage, or a hydrazine linkage). To improve the association between the peptide and the vehicle carrier, the peptide can be modified by a suitable polymer, such as PEG (peglyated). The detectable label or the anti-cancer agent can be encapsulated within the vehicle via, *e.g.*, association with lipophilic molecules, which can aid in the delivery of the detectable label or the anti-cancer agent to the interior of the vehicle.

In a preferred example, a cancer-targeting peptide described herein is linked to a liposome (as a vehicle carrier) that encapsulates one or more agents of interest (*e.g.*, a detectable label such as a cancer imaging agent or an anti-cancer agent). Liposome is a vesicle comprised of one or more concentrically ordered lipid bilayers, which encapsulate an aqueous phase. The aqueous phase typically contains an agent to be delivered to a target site such as a tumor site. Upon reaching the target site, the liposome fuses with the plasma membranes of local cells to release the agent into the cytosol. Alternatively, the liposome is endocytosed or otherwise taken in by the cells as the content of a transport vesicle (*e.g.*, an endosome or phagosome). Once in the transport vesicle, the liposome either degrades or fuses with the membrane of the vesicle and releases its contents. Liposome membranes can be constructed so that they become destabilized when the nearby environment becomes acidic (see, *e.g.*, PNAS 84:7851, 1987; Biochemistry 28:908, 1989). Thus, when liposomes enter a

target cell, they become destabilized to release their encapsulated contents. This destabilization process is termed fusogenesis. Dioleoylphosphatidylethanolamine (DOPE) is commonly used to facilitate this process.

A variety of methods are available for preparing liposomes. See, e.g., Szoka et al.,
5 Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871,
4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, 4,235,871, 4,261,975, 4,485,054,
4,501,728, 4,774,085, 4,837,028, 4,946,787, PCT Publication No. WO 91/17424, Deamer &
Bangham, Biochim. Biophys. Acta 443:629-634 (1976); Fraley, et al., PNAS 76:3348-3352
(1979); Hope et al., Biochim. Biophys. Acta 812:55-65 (1985); Mayer et al., Biochim.
10 Biophys. Acta 858:161-168 (1986); Williams et al., PNAS 85:242-246 (1988); Liposomes
(Ostro (ed.), 1983, Chapter 1); Hope et al., Chem. Phys. Lip. 40:89 (1986); Gregoriadis,
Liposome Technology (1984) and Lasic, Liposomes: from Physics to Applications (1993)).
Suitable methods include, for example, sonication, extrusion, high pressure/homogenization,
microfluidization, detergent dialysis, calcium-induced fusion of small liposome vehicles and
15 ether fusion methods, all of which are well known in the art.

*(ii) Uses of Cancer-Targeting Peptides in Delivery of Anti-Cancer Agent or
Imaging Agent to Cancer Cells*

In light of their ability of targeting cancer cells, any of the peptides described herein
can be used for target delivery of an agent of interest (e.g., an anti-cancer agent or a
20 detectable label such as an imaging agent) to cancer cells, thereby facilitating cancer
treatment and/or diagnosis.

The delivery method described herein can be performed by contacting cancer cells or
cells suspected of being cancerous a cancer-targeting peptide as described herein conjugated
with the agent of interest. Cells suspected of being cancerous are cells that display one or
25 more cancer cell characteristics, e.g., immortalization, loss of contact inhibitions, reduced
cellular adhesion, invasiveness, loss of anchorage dependence, lower serum requirements,
selective agglutination by lectins, molecular changes in cell membrane components,
disorganization of the cytoskeleton, increase in negative surface charge of cell membrane,
increased sugar transport, appearance of virus specific transplantation rejection antigens,
30 defective electrical communication, increased secretion of proteolytic enzymes, aldolases,
and increased rate of glycolysis. In some embodiments, the cancer-targeting peptide/agent

conjugate is incubated with a sample having or suspected of having cancer cells. Such a sample can be a sample containing cultured cancer cells, a tissue sample obtained from a subject who has or is suspected of having cancer, or an *in vivo* tissue sample in such a subject (e.g., a human patient).

5 Alternatively, the conjugate can be administered to a subject who has or is suspected of having cancer. A subject having cancer can be identified by routine medical procedures. A subject suspected of having cancer may show one or more symptoms associated with certain types of cancers. Cancer symptoms vary, depending upon the types of cancers. Typical cancer symptoms include, but are not limited to, cough or blood-tinged saliva (lung
10 cancer), a change in bowel habits such as continuous diarrhea or blood in stools (colon cancer), unexplained anemia (bowel cancers), breast lump or breast discharge (breast cancer), lumps in the testicles or enlarged testicles (cancer of the testicles), frequent urination or enlarged prostate gland (prostate cancer), and/or swollen lymph nodes (related to various cancers). Such subjects can be identified via routine medical procedures.

15 In some embodiments, a cancer-targeting peptide, conjugated with an anti-cancer agent or a detectable label as described herein, is mixed with a pharmaceutically acceptable carrier to form a pharmaceutical composition. The carrier in the pharmaceutical composition must be "acceptable" in the sense of being compatible with the active ingredient of the formulation (and preferably, capable of stabilizing it) and not deleterious to the subject to be
20 treated. For example, solubilizing agents such as cyclodextrins, which form more soluble complexes with the anti-viral agents described herein, or more solubilizing agents, can be utilized as pharmaceutical carriers for delivery of the anti-viral agents. Examples of other carriers include colloidal silicon dioxide, magnesium stearate, sodium lauryl sulfate, and D&C Yellow # 10. See, e.g., Remington's Pharmaceutical Sciences, Edition 16, Mack
25 Publishing Co., Easton, Pa (1980); and Goodman and Gilman's "The Pharmacological Basis of Therapeutics", Tenth Edition, Gilman, J. Hardman and L. Limbird, eds., McGraw-Hill Press, 155-173, 2001.

To deliver the anti-cancer agent or the detectable label to a target site, the composition described herein can be administered orally, parenterally, topically, rectally, nasally,
30 buccally, vaginally, via an implanted reservoir, or via inhalation spray. The term "parenteral" as used herein includes subcutaneous, intracutaneous, intravenous, intramuscular,

intraarticular, intraarterial, intrasynovial, intrasternal, intrathecal, intralesional, and intracranial injection or infusion techniques.

5 A sterile injectable composition, e.g., a sterile injectable aqueous or oleaginous suspension, can be formulated according to techniques known in the art using suitable dispersing or wetting agents (such as Tween 80) or suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are mannitol, water, Ringer's solution
10 and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium (e.g., synthetic mono- or diglycerides). Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions can also
15 contain a long-chain alcohol diluent or dispersant, or carboxymethyl cellulose or similar dispersing agents. Other commonly used surfactants such as Tweens or Spans or other similar emulsifying agents or bioavailability enhancers which are commonly used in the manufacture of pharmaceutically acceptable solid, liquid, or other dosage forms can also be used for the purposes of formulation.

20 A composition for oral administration can be any orally acceptable dosage form including, but not limited to, capsules, tablets, emulsions and aqueous suspensions, dispersions and solutions. In the case of tablets/capsules for oral use, carriers which are commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents
25 include lactose and dried corn starch. When aqueous suspensions or emulsions are administered orally, the active ingredient can be suspended or dissolved in an oily phase combined with emulsifying or suspending agents. If desired, certain sweetening, flavoring, or coloring agents can be added. A nasal aerosol or inhalation composition can be prepared according to techniques well known in the art of pharmaceutical formulation. An oxadiazole
30 compound-containing composition can also be administered in the form of suppositories for rectal administration.

A composition containing one or more of the cancer-targeting peptides described herein conjugated with an anti-cancer agent can be used in cancer treatment, particularly in treating GRP78-positive cancers. Cancer cells are cells having the capacity for autonomous growth, i.e., an abnormal state or condition characterized by rapidly proliferating cell growth.

5 It is meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. The just-noted composition, containing an effective amount of the anti-cancer agent, can be administered to such a subject as described above. Optionally, a subject who carry GRP78-positive cancer cells can be first identified via routine methods,
10 e.g., PCR or immunoassays, and then treated with the composition described herein.

Treating or treatment refers to the application or administration of a composition including one or more active agents to a subject, who has cancer, a symptom of cancer, or a predisposition toward cancer, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect cancer, the symptoms of cancer, or the predisposition toward
15 cancer.

A composition containing one or more of the cancer-targeting peptides described herein conjugated with a detectable label such as an imaging agent can be used for detecting presence of cancer cells and/or cancerous tissues (e.g., cancer diagnosis and/or cancer imaging). When such a composition is used for *in vivo* tumor imaging, a suitable
20 amount of the composition (e.g., containing about 20 μ g of a cancer-targeting peptide and about 400 MBq of a radioactive molecule) can be injected to a suspected cancer patient, e.g., a patient carrying or suspected of carrying a solid tumor. The patient is then subjected to scintigraphy at suitable periods, e.g., 2 h, 4 h, 24 h, 48 h, and/or 72 h, after injection. Radioactivities of the whole body and the regions of interest are normalized against
25 background activity and the presence/absence of tumor matter can be determined based on the results thus obtained.

The anti-cancer agent or the detectable label in the compositions described herein are administered in effective amounts. An "effective amount" is that amount of the anti-cancer agent or the detectable label that alone, or together with further doses, produces one or more
30 desired responses, e.g. inhibit cancer cell growth, induce cancer cell apoptosis, or suppress cancer cell metastasis, or signal presence of cancer cells. In the case of treating a cancer, the

desired responses include inhibiting the progression of the disease. This may involve only slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently. This can be monitored by routine methods or can be monitored according to diagnostic methods of the invention discussed
5 herein. The desired responses to treatment of the disease or condition also can be delaying the onset or even preventing the onset of the disease or condition.

Effective amounts will depend, of course, on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size, gender and weight, the duration of the treatment, the nature of concurrent
10 therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is generally preferred that a maximum dose of the individual components or combinations thereof be used, that is, the highest safe dose according to sound medical judgment. It will be
15 understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons.

(iii) Computational Methods for Identifying Cancer-Targeting Peptides

Practical software programs, including combinatorial construction of peptide-structure
20 library (Buildpep), binding pocket analyzer (PscanMS), high accuracy protein-ligand scoring program (HotLig), or combinations thereof, can be utilized in the computational methods described herein to design optimized cancer targeting peptides, preferable using a known cancer-targeting peptide as a lead. Based on these programs, a strategy of structure-based optimization of peptides targeting cancer marker proteins *in silico*, as illustrated in Figure 1
25 (using human GRP78 as an exemplary cancer marker protein), was developed to identify peptides that target GRP78. Briefly, the structures of a protein of interest (e.g., a cancer cell-surface protein) and peptide ligands are first modeled and energy-minimized. Then flexible docking is applied to produce the complexes of peptide-binding domain of the protein of interest with peptide ligands. The initial resulting complexes are then further energy-
30 optimized followed by analyzing various molecular interactions using HotLig package to

identify the key pharmacophore of protein-targeting motif. After repeated *in silico* screening from the peptide library, new peptides can be designed and examined to optimize the *in vitro* binding via Biacore analysis. The results were finally validated by *in vitro* binding with various cancer cells and *in vivo* tumor imaging and therapeutic studies in mice.

5 The computational methods described herein can include homologous modeling and molecular docking to predict structural features of a cancer-cell surface protein, particularly surface features of the protein, and binding interactions with peptides, PscanMS for detection of binding pockets and protein surface calculation, and HotLig for energy minimization and interaction scoring to identify or optimize peptides that are capable of binding to the cancer-
10 cell surface protein. Preferably, a peptide known to bind to the cancer-surface protein is used as a lead peptide for molecular docking. A peptide structural library (e.g., a database with peptide sequences and 3D-structural information) is used for identifying and optimizing peptides targeting the cancer cell-surface protein. The overall schemes of these methods are exemplified in Figures 2.

15 Structural modeling of a cancer-cell surface protein can be performed by computational tools known in the art, such as the method implemented in the PSIPRED server to predict Bryson et al., *Nucleic Acids Res.* 33:W36-38 (2005); and McGuffin et al., *Bioinformatics* 19:874-881 (2003). For example, homologs of the protein of interest can be identified from any publicly available databases, such as the protein data bank and their
20 secondary structures/sequence alignment can be predicted/determined using the method provided in the PSIPRED server. The 3D structure of the protein of interest is then predicted by a conventional computational method, such as MODELLER 9v4 using functions of the AUTOMODEL class in python scripts with multiple-template mode. Eswar et al., *Curr. Protoc. Bioinformatics*, Chapter 5: Unit 5.6 (2006). The Discrete Optimized Protein Energy (DOPE) method also described in Eswar et al. can be used to select the best model from the
25 50 initially generated models. The loop regions of the energy-optimized model can then be refined by, e.g., the functions of LOOPMODEL class. Finally, the refined model can be subjected to energy minimization further by DEEVIEW *vers.* 3.7 using the GROMOS 43B1 force field till the delta E between two steps dropped below 0.05 KJ/mol. Guex et al.,
30 Electrophoresis, 18:2714-2723 (1997).

Molecular docking of the protein of interest is then performed using, e.g. Modeller 9v4 as described above and a peptide structural library also noted above. The peptide structural library can be using Buildpep, which is a UNIX-shell script using the strategy that employs modeling and energy-minimization functions from the Modeller 9v4 package (Eswar et al.) to build a large pool of optimized 3D coordinates of peptide structures in combinatorial sequences with various lengths. The sequences of the peptides can be aligned to an alanine single amino acid template first and then energy-optimized peptides can be built by the AUTOMODEL functions of Modeller and the resulting library can be converted to a "mol2" file format by OpenBabel as described in Rajarshi et al., *J. Chem. Inf. Model.* 46:991-998 (2006).

The molecular flexible docking can then be performed by a method known in the art, such as the Dock 5.1 described in Kuntz, 1982. The Kollman partial charges (SYBYL 8.0, Tripos International, 1699 South Hanley Rd. St. Louis, Missouri 63144, USA) were applied to both protein and peptides for force field calculation. The parameters for Dock program can be set to iteratively generate 1,000 orientations and 200 conformers in binding pocket. The docked conformers can then be re-scored and ranked by HotLig to predict protein-ligand interactions, which is described below. The rendering of figures for molecular model can be performed by, e.g., Chimera (Lee et al., *Cancer Res.* 64:8002-8008; 2004).

In one example, binding pocket and water-contactable atoms can be detected by PscanMS to construct a protein surface. Figure 10. A grid box containing the region of a protein to be scanned can be set up for detection of binding cavities. Figure 10, panel a. To detect the cavity, the scanning box moves along the axes of the grid box and the probe scanned the scanning box once time every step the scanning box moved. All three dimensions can be scanned. Figure 10, panel b. Once the protein atoms are found to be involved in the scanning box, the involved atoms were selected for detection of cavity. The size of scanning box can be set to fit the diameter of the probe (r Å in radius, default is 3 Å). Figure 10, panel c. To detect the cavity inside the selected atoms involved in the scanning box, the probe moved along the scanning direction in a step length of dS Å (default is 0.5 Å). As shown in Figure 10, panel d, the positions A and B, the probe contacted at least one atom of protein, but the positions that located between A and B are not contactable to any protein atoms. The protein atoms contacted by the probe at the A and B positions are defined as the

“water-contactable atoms”. The positions A and B are also defined as the “pocket dots” which can be used to illustrate the configuration of cavity. Additionally, the distance between one “pocket dot” and one water contactable atom of protein is in the range of $(r-ds)$ to r Å. The minimum length of a space (between any two protein atoms) buried inside the protein can be detected is $2(r+ds)$ Å. Since the water contactable atoms are identified, the protein surface can thus be calculated using these surface atoms and their neighbor atoms by using their radii.

HotLig is used in computational design/optimization of cancer-targeting peptides. HotLig is a knowledge-based and empirical-based scoring program for prediction of protein-ligand interactions. Figure 2 illustrates the scheme of HotLig development. One of the innovative features in HotLig is the introduction of parameters for “intermolecular surface distance” into the distance-dependent functions for scoring various molecular interactions, such as polar interactions (hydrogen bonding, ionic interaction, metal-ion coordination) and non-polar interactions (hydrophobic effects). As illustrated in Figure 3, in order to estimate the polar interactions quantitatively, the atomic surface distances can be calculated from the distance between centers of the two interactive atoms minus the summation of van der Waals radii. Hence, the HotLig excluded the differences of various atomic van der Waals radii, and assessed exactly how close the two interactive atoms were, when hydrogen bonding, ionic interaction or metal-ion coordination was formed.

As shown in Figure 2, a statistic dataset (containing, e.g., 600 complexes) and a training set (containing, e.g., 214 complexes) from protein data bank (rcsb.org) can be used for the development of HotLig scoring functions. Examples of the statistic dataset and the training set are listed in Tables 1 and 2 below:

Table 1: Statistic set containing 600 complexes collected from PDB for the study of knowledge-based potential.

1a0j	1a42	1a46	1a4k	1a50	1a5g	1a8i	1a8t	1a94	1aaq	1abe	1abf	1acj	1acl	1acm
1aco	1adb	1add	1adf	1aec	1af2	1ah0	1ah3	1aha	1aj7	1anf	1apb	1apt	1apu	1apv
1apw	1ase	1ayx	1azm	1b05	1b0h	1b1h	1b2h	1b32	1b3f	1b3g	1b3h	1b3l	1b40	1b46
1b4h	1b4p	1b4z	1b51	1b52	1b58	1b5g	1b5h	1b5i	1b5j	1b6a	1b6h	1b7h	1b9j	1ba8
1baf	1bai	1bap	1bb0	1bbz	1bcu	1bhf	1bll	1bn1	1bn3	1bn4	1bnm	1bnn	1bnq	1bnt
1bnu	1bnv	1bnw	1bra	1bxo	1bxq	1bzm	1bzy	1c1c	1c5c	1c8k	1cbx	1cil	1cla	1cnw
1cnx	1cny	1coy	1cps	1cru	1csc	1ct8	1ctt	1cx2	1d0c	1d3d	1d3p	1d3q	1d3t	1dbb
1dbj	1dbk	1dbm	1dcy	1dhf	1did	1die	1dih	1dr1	1drf	1duv	1dwb	1dwc	1dwd	1dy3
1dyr	1e1y	1e2f	1e66	1e7v	1e8w	1e96	1eap	1ebg	1ee2	1eed	1efy	1ejb	1ela	1elc

1epb	1epo	1epp	1eqc	1eqg	1eta	1etr	1ets	1ett	1ewl	1exw	1eyq	1f2a	1f74	1f8e
1f9y	1fbc	1fbf	1fbp	1fe2	1ffq	1fjs	1fkb	1fkf	1fkg	1fki	1fl3	1fl6	1flr	1fm7
1fmo	1fq4	1fq5	1fq6	1fq8	1g1d	1g27	1g45	1g46	1g48	1g4j	1g4o	1g52	1g53	1g54
1gaf	1gg5	1ghb	1gic	1gj7	1glq	1gz8	1h1i	1h3a	1hak	1hb1	1hbv	1hdc	1hdy	1hef
1hew	1hfs	1hik	1hvp	1hri	1hsl	1ht8	1htf	1htg	1hvi	1hvj	1hvk	1hvl	1hvr	1hvs
1hw8	1hw9	1hy7	1hyt	1hyx	1hyy	1i76	1i9l	1i9m	1i9n	1i9o	1i9p	1i9q	1icn	1ida
1if8	1igj	1ij8	1ik3	1ikg	1iki	1inc	1itu	1ivd	1ive	1ix1	1iy1	1j4r	1jak	1jcx
1jet	1jeu	1jev	1jk7	1jkx	1jq3	1kel	1kgj	1ki8	1klk	1kmv	1kn2	1kn4	1kno	1kqb
1kvo	1kz8	1l82	1l83	1l86	1l87	1ld8	1ldm	1lgr	1lic	1llo	1lnm	1lpd	1lrh	1lri
1lst	1ly3	1lyb	1m17	1m2x	1m52	1m79	1m7y	1mcb	1mcf	1mch	1mcj	1mcr	1mcs	1mdq
1mdr	1me8	1mfc	1mfe	1ml4	1mnc	1moq	1mrk	1mrs	1mup	1nc1	1nhx	1nis	1njs	1nms
1nnb	1nqu	1nqx	1o9f	1odc	1ogx	1oiy	1ooq	1oq5	1ow2	1oyn	1p4f	1p6k	1p6o	1pa9
1pbd	1pgp	1pha	1phd	1phg	1phh	1pmq	1pmv	1pn9	1poc	1ppc	1pph	1ppk	1ppl	1ppm
1pso	1q6z	1q92	1qci	1qhy	1qka	1qkb	1ql7	1ql8	1qmg	1qxy	1rbp	1rds	1rgk	1rgl
1rne	1rnt	1rob	1rpa	1rus	1s2a	1s5s	1sln	1slt	1snc	1sre	1srj	1stp	1t31	1t46
1t4e	1tbb	1tdb	1tet	1tha	1the	1thz	1tka	1tlp	1tmn	1tmt	1tng	1tnh	1tni	1tnj
1tnk	1tnl	1tpp	1tt8	1ttm	1tu7	1tx2	1u0h	1u1x	1u2y	1u32	1u4g	1ulb	1uof	1us0
1uu3	1uwh	1uwz	1uzf	1v2k	1val	1vam	1vot	1w82	1w96	1w9u	1wb0	1wvm	1x7r	1x8b
1xid	1xie	1xii	1xli	1xnk	1xnz	1xo2	1xoz	1xpo	1xuo	1y57	1yda	1ydb	1ydd	1yeg
1yei	1yej	1yqy	1yuh	1yvm	1yvx	1ywn	1yyy	1zkl	1zl2	1zos	1zsb	1zz3	1zzz	2a1h
2a3i	2ab2	2ack	2ada	2ai2	2ai8	2aie	2ak3	2akw	2anj	2ao0	2b0m	2b7a	2bb7	2bik
2brc	2bua	2byi	2bz6	2c1a	2c4w	2cbu	2ccs	2cgr	2chl	2cht	2cpp	2csc	2ctc	2cvd
2dbl	2dri	2er0	2er6	2er7	2er9	2f4j	2f7d	2f8c	2f94	2fda	2fd2	2fm0	2ftc	2fp7
2fq9	2fqr	2g28	2gbp	2gfs	2gke	2gss	2h4n	2ifb	2ldb	2mcp	2msb	2olb	2pcp	2phh
2pk4	2plv	2qwb	2qwc	2qwd	2qwe	2qwf	2qwg	2r04	2r07	2rnt	2sim	2sns	2tmn	2xim
2xis	2yhx	2ypi	35c8	3cla	3cpa	3csc	3er3	3fx2	3gch	3gpb	3hvt	3mth	3pgm	3ptb
3tmn	3tpi	3ts1	43ca	4aah	4cla	4cts	4dfr	4er1	4er2	4er4	4erk	4est	4fab	4gr1
4hvp	4mdh	4pah	4phv	4sga	4tim	4tln	4tmn	4ts1	4xia	5abp	5acn	5cna	5enl	5er2
5hvp	5icd	5ldh	5p21	5p2p	5pah	5sga	5std	5tim	5tln	5tmn	5xia	6abp	6acn	6apr
6cpa	6enl	6rnt	6rsa	6tim	6tmn	7abp	7acn	7cat	7dfr	7est	7hvp	7taa	7tim	7tln
8a3h	8abp	8acn	8atc	8cpa	8gch	8hvp	8icd	8xia	9aat	9abp	9hvp	9icd	9ldt	9rub

Table 2: Training set containing 214 complexes

<i>Basic set (101 complexes)</i>														
1a50	1a8i	1ah0	1ah3	1bxo	1c1c	1c8k	1cx2	1d0c	1dyr	1e1y	1e66	1e7v	1e8w	1efy
1ewl	1eyq	1fm7	1fmo	1gg5	1gz8	1h3a	1ij8	1iki	1iy1	1j4r	1kgj	1ki8	1klk	1kmv
1kz8	1lnm	1lpd	1lri	1ly3	1m17	1m52	1m79	1me8	1moq	1nc1	1nhx	1nqu	1ogx	1oiy
1ooq	1oyn	1p4f	1pmq	1pmv	1qci	1s5s	1t46	1t4e	1tbb	1tnj	1tx2	1u0h	1u2y	1u32
1us0	1uu3	1uwh	1v2k	1vot	1w82	1w96	1w9u	1x7r	1x8b	1xnk	1xo2	1xoz	1xuo	1y57
1yvx	1ywn	1zkl	1zl2	1zos	2a1h	2a3i	2ab2	2ao0	2b7a	2bik	2brc	2byi	2bz6	2c4w
2ccs	2chl	2cvd	2f4j	2f7d	2fdd	2fm0	2gfs	4erk	5std	8a3h				
<i>Ionic set (56 complexes)</i>														
1a0j	1ayx	1b4p	1dhf	1duv	1ejb	1eqc	1eqg	1f74	1f8e	1fe2	1ffq	1fjs	1gj7	1ht8
1hw8	1hw9	1ikg	1ivd	1jak	1jcx	1jk7	1jkx	1jq3	1kqb	1m7y	1ml4	1njs	1nms	1nqx
1p6k	1pa9	1pn9	1q92	1t31	1thz	1tnl	1tt8	1tu7	1u1x	1wb0	2ai2	2akw	2anj	2b0m
2bua	2c1a	2cbu	2fda	2fp7	2fqr	2gke	2gss	2qwd	35c8	7taa				
<i>Metal set (57 complexes)</i>														
1a8t	1b6a	1bzy	1cil	1cru	1ctt	1dcy	1dy3	1e2f	1ee2	1f9y	1g27	1h1i	1hb1	1hfs
1hik	1hy7	1i76	1ik3	1itu	1ix1	1kvo	1ld8	1lri	1m2x	1mrs	1oq5	1ow2	1p6o	1q6z
1qmg	1qxy	1s2a	1sln	1snc	1ttm	1u4g	1uof	1uwz	1uzf	1xii	1xnz	1xpo	1yqy	1yvm

1zz3 2ai8 2aie 2bb7 2cpp 2f8c 2f94 2fm5 2g28 4pah 4tln 5pah

* Three subsets were classified according to the molecular interactions between protein and ligand.

The principle of derivation of distance-dependant potential from the occurrence frequencies of paired atoms is similar to Velec's approach, which had been implemented in DrugScore. In addition to analyzing molecular interactions based on "Sybyl-defined" atom types in previous studies (Velec, H.F. et al. *J. Med. Chem.* **48**(20), 6296-303. (2005); SYBYL 8.0, Tripos International, 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA), HotLig can further analyze the pharmacophore of each atom for scoring molecular interactions. Additionally, the "atomic surface distance" can be used in the parametric functions of HotLig rather than the "atomic center distance". Briefly, the predicted binding potentials can be derived from the statistic set according to the occurrence frequencies of the paired pharmacophores in molecular interactions. Then, the weight factors for each binding potential can be determined via learning from the training set.

To give a quantitative score for a protein-ligand complex, the Connolly surface of protein can be first calculated by PscanMS, which detects binding cavity and 3D patterns of structural information and also calculates solvent-accessible surface and Connolly surface of proteins. The resulting surface features can be outputted as dot surface in QCPE-compatible MS format (Connolly, 1983), which possesses a sectioned area value and a unit normal vector for each dot. The molecular interactions can then be analyzed and divided into polar- and non-polar- interactions according to their pharmacophore classifications. Polar interactions include hydrogen bonding, ionic interaction, and metal-ion coordination; whereas non-polar interactions include hydrophobic interactions (which may not distinguishable from van der Waals interactions in HotLig) that occurred on carbon-carbon contacts.

To assess the energy potentials of polar interactions, the distance-dependent scoring functions can be derived from fitting the normalized distribution of atomic surface distance (δ ; atomic surface distance between atoms N & O in Figure 3) vs. angle donor-H-acceptor (θ) and angle electron-acceptor-H (ϕ) that were measured from the paired pharmacophores (Fig. 7). The potential for non-polar interaction can be derived from fitting the distribution of normal vector length (ν) between Connolly protein surface and ligand atomic surface (Figure

3). Additionally, the integration of the contacted area (A) on Connolly surface can also be introduced in the calculation of non-polar potential.

The potential of hydrophobic interaction can be derived from the normalized distribution of the length of ligand-contacted normal vector (ν) between the protein Connolly surface and ligand atomic surface. Additionally, the sectioned area (A) associated with the normal vector on Connolly surface can be introduced into the calculation of non-polar potential. The final equation for scoring of hydrophobic interaction (ΔE_{vdw}) can be:

$$\Delta E_{vdw} = A \times W_{vdw}(\nu)$$

Overall, combining the equations of each kind of interaction, the resulting parametric equation for the binding energy (ΔE) between a protein (p) and a ligand (l) is represented as:

$$\begin{aligned} \Delta E_{p,l} &= \Delta E_{polar}(\delta, \theta, \phi) + \Delta E_{non-polar}(\nu, A), (\phi < 100^\circ) \\ &= F_{hbond} \times \sum_h (\cos(180^\circ - \theta_h) \times W_{hbond}(\delta_h)) \\ &+ F_{ion} \times \sum_i W_{ion}(\delta_i) \\ &+ F_{metal} \times \sum_m W_{metal}(\delta_m) \\ &+ F_{vdw} \times \sum_\nu (A_\nu \times W_{vdw}(\nu_\nu)) \end{aligned}$$

Here, h refers to the pair of H-bond; i refers to the pair of ionic interaction; m refers to the pair of metal-ion coordination and ν refers to the ligand-contacted normal vector in hydrophobic interaction.

To optimize the weight factor for each kind of interaction, the F_{hbond} can be set as 1 and then the other factors, F_{vdw} , F_{ion} , F_{metal} , can be determined from the training set as illustrated in Fig. 6. This training set can be classified into three subsets: a basic set, an ionic set, and a metal set. See, e.g., Figure 2. The interactions between a protein and its cognate ligands can be analyzed for the classification. In the basic set, neither charged ionic interaction nor metal coordination is contained in the interactions between the protein and ligand in the complex (Figure 2). The complexes which possess ionic interactions between a protein and its ligand without any binding through metal ion are assigned to the ionic set (Figure 2). The metal set includes complexes, in each of which the protein binds to its ligand

through metal coordination (Figure 2). The weight factor F_{vdw} can be first optimized based on the maximum success rate of prediction for the basic set while the F_{hbond} is set as 1. Once the factor F_{vdw} is determined, the optimal factor F_{ion} can be determined through the iterative analysis of the ionic set. Similarly, the factor F_{metal} can be obtained from the analysis of the metal set (Figure 2).

The peptides targeting the cancer cell-surface protein of interest identified from or optimized by the computational methods described herein can then be tested in an in vitro assay (e.g., in vitro binding assay) or in vivo assay (e.g., in vivo imaging assay) to verify their activities of targeting cancer cells expressing the surface protein of interest.

Without further elaboration, it is believed that one skilled in the art can, based on the above description, utilize the present invention to its fullest extent. The following specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. All publications cited herein are incorporated by reference for the purposes or subject matter referenced herein.

Example 1: Computational Design of Cancer-Targeting Peptides

A series of novel cancer-targeting peptides was developed based on molecular modeling of GRP78 and *in silico* molecular docking and scoring using HotLig and L-peptide (Lee et al., 2004). L-peptide has the amino acid sequence of RLLDTNRPLL PY (SEQ ID NO: 13). See US Patent No. 7,238,665.

(i) Determining Structural Features of Human GRP78

To design and optimize cancer-targeting peptides, homologous modeling and molecular docking were performed to characterize the structural features of human GRP78.

The method implemented in the PSIPRED server (Bryson et al. *Nucleic Acids Res.* 33: W36-38; 2005; and McGuffin et al. *Bioinformatics* 19: 874-881;2003) was used for predicting the secondary structures and making sequence alignments. Initially, the structural information of GRP78 homolog (Protein Data Bank code: 2QWL, 1YUW, 2V7Y, 2OP6, 1DKX and 1U00) was used as the modeling templates. The 3D structure of GRP78 was constructed by MODELLER 9v4 (Eswar et al. *Curr. Protoc. Bioinformatics*, Chapter 5: Unit 5.6; 2006)) using functions of the AUTOMODEL class in python scripts with a multiple-template mode. The Discrete Optimized Protein Energy (DOPE) method (Eswar, et al.) was

used to select the best model from the 50 initially generated models. The loop regions of the energy-optimized model were then refined by the functions of LOOPMODEL class. Finally, the refined model was subjected to energy minimization further by DEEVIEW *vers.* 3.7 (Guex et al. *Electrophoresis*, 18:2714-2723; 1997) using the GROMOS 43B1 force field till
5 the delta E between two steps dropped below 0.05 KJ/mol.

Next, the structure of human GRP78 was modeled using Modeller 9v4 as described above and the peptide structural library was constructed using Buildpep also described above. The molecular flexible docking was then performed by Dock 5.1 as described in Kuntz et al., *J. Mol. Biol.* 161:269-288 (1982). The Kollman partial charges were applied to both protein
10 and peptides for force field calculation. The parameters for Dock program were set to iteratively generate 1,000 orientations and 200 conformers in binding pocket. The docked conformers were then re-scored and ranked by HotLig to predict protein-ligand interactions. The rendering of figures for molecular model was performed by Chimera (Kuntz, 1982; and Pettersen et al. *J. Comput. Chem.* 25:1605-1612; 2004).

15 The structure of human GRP78 thus modeled is shown in Figure 4, panel a. This protein was found to contain two major structural domains, a peptide-binding domain and an ATPase domain. This structural model with respect to the ATPase domain is consistent with the an X-ray crystallography determination as described in Wisniewska et al., *PLoS One* 5:e8625 (2010) and Connolly, *Science* 221:709-713;1983. The value of the root-mean-
20 square deviation was as low as as low as 0.6 Å, when compared with the modeled ATPase domain with the crystallographic data via structural superimposition. In the model, the peptide-binding domain was found to be composed of a β -sandwich subdomain and a helix-bundle subdomain (Figure 4, panel a). The β -sandwich subdomain is made up by two layers of β -sheets stacked together, followed by several tandem helices. These helices are further
25 folded to form the helix-bundle subdomain at the C-terminus. On the other hand, the sequence from Met1 to Asp26 at the N-terminus of GRP78 was predicted to have a single helix. Since the GRP78 protein moves to the surface in cancer cells, the hydrophobicity of this N-terminal helix was also investigated according to the attributes of its amino acids. A hydrophobic region (Leu3 to Ala16) was identified within the helix (Figure 4, panel a).

30

(ii) *Novel surface-directed algorithm for scoring protein-peptide interactions*

Software HotLig (described above) was developed for structure-based optimization of peptides capable of binding to human GRP78.

The scheme for the development of HotLig algorithm was shown in Figure 2 also
5 described above. HotLig is a knowledge-based and empirical-based scoring program with
outstanding predictive power for protein-ligand interactions. The statistical distribution and
the derived function for the simulation of hydrogen-bonding pairs versus the atomic surface
distance were shown in Figure 5. For the estimation of the hydrophobic interactions, the
10 Connolly surface of protein (Connolly, Science 221:709-713; 1983), which was composed of
surface-point coordinates, areas, and normal vectors, was applied in the calculation of
intermolecular surface distance and the contact area. Figure 7. The normal vectors were the
vectors perpendicular to the protein surface, pointing toward the ligand side. Hundreds of
thousands of normal vectors on Connolly surface of protein were involved in the calculation
of molecular surface contact. The measured intermolecular surface distance and contact area
15 were then used to estimate the contribution of hydrophobic contact. Figure 9. The potential
for hydrophobic interaction was derived from fitting the normalized distribution of the length
(ν) of contacted normal vector between Connolly protein surface and ligand atomic surface.
Additionally, the integration of contacted area (A) on Connolly surface was also introduced in
calculation of non-polar potential. The final equation for scoring of hydrophobic interaction
20 (ΔE_{vdw}) is: $\Delta E_{vdw} = A \times W_{vdw}(\nu)$.

As shown in Table 3 below, the HotLig improved the docking accuracy of the
software Dock v5.1 from 44.39% to 71.96% when re-scored the docked results generated by
Dock. In addition, if the experimental coordinates of cognate ligands were also included for
scoring, the success rate of the HotLig could reach 88.32%. Table 3. Similar results were
25 also observed when validation was performed using the Gold dataset (Jones et al., J. Mol.
Biol. 267:727-748; 1997). See Table 3.

30

Table 3. Re-scoring of docked conformers by the HotLig improved the accuracy of prediction significantly ^a.

Dataset	Dock v5.1 success rate (%)	HotLig success rate (%)	
		native ligand pose excluded	native ligand pose included
HotLig training set (214 complexes)	44.39	71.96	88.32
Gold dataset (100 complexes)	35	69	87

^a The success rate were calculated at the criteria of RMSD ≤ 2.0 Å (comparing with the native pose of cognate ligand). The native pose refers to the experimental coordinates of binding conformation of the cognate ligand.

Furthermore, utilizing another Wang's dataset (Wang et al., J. Med. Chem. 46:2287-2303; 2003), the success rate of binding mode prediction of the HotLig was as high as 91%, when comparing with many other known scoring programs and HotLig was the best program in the list. See Table 4 below:

Table 4. Comparing prediction accuracy for binding mode by HotLig with 11 other scoring programs using Wang's dataset³.

scoring function ^a	Success rate (%)				
	RMSD ≤ 1.0 Å	RMSD ≤ 1.5 Å	RMSD ≤ 2.0 Å	RMSD ≤ 2.5 Å	RMSD ≤ 3.0 Å
HotLig	79	87	91	93	94
Cerius2/PLP	63	69	76	79	80
SYBYL/F-Score	56	66	74	77	77
Cerius2/LigScore	64	68	74	75	76
DrugScore	63	68	72	74	74
Cerius2/LUDI	43	55	67	67	67
X-Score	37	54	66	72	74
AutoDock	34	52	62	68	72
Cerius2/PMF	40	46	52	54	57
SYBYL/G-Score	24	32	42	49	56
SYBYL/ChemScore	12	26	35	37	40
SYBYL/D-Score	8	16	26	30	41

^a Scoring functions are ranked by their success rates at RMSD ≤ 2.0 Å (comparing with the native pose of cognate ligand).

To estimate the accuracy of HotLig in prediction of binding-affinity, the Wang's dataset was also used for evaluation. As shown in Table 5, the *R_s* value of the HotLig was

0.609, which was also better than most programs.

Table 5. Comparing prediction accuracy for binding affinity by HotLig with 11 other scoring programs using Wang's dataset

Scoring functions	Spearman correlation coefficient (R_s) based on	
	the experimentally observed conformations	the best-scored conformations
X-Score	0.660	0.698
HotLig	2. 0.609	0.606
Cerius2/PLP	0.592	0.607
DrugScore	0.587	0.601
SYBYL/G-Score	0.569	0.531
SYBYL/D-Score	0.475	0.488
SYBYL/ChemScore	0.431	0.435
Cerius2/LUDI	0.430	0.456
Cerius2/PMF	0.369	0.367
Cerius2/LigScore	0.363	0.418
SYBYL/F-Score	0.283	0.253
AutoDock	0.141	0.423

Taken together, the software HotLig provides a novel surface-directed algorithm and exhibits excellent predictive power for protein-ligand interactions.

(iii) Design of Optimized Cancer-Targeting Peptides

The L-peptide, binding to GRP78 in a dose-dependent manner with a dissociation constant (K_D) of about 1 ~ 10 μ M, was used as a lead peptide in this study.

To delineate the binding motif of the L-peptide and its detailed molecular interactions with GRP78, molecular docking, energy minimization, and interaction scoring were performed as outlined in Figure 1. It was found that the L-peptide accessed a binding site at the center of the peptide-binding domain of GRP78. See Figure 4, panel a. To depict the features of the L-peptide binding site hidden inside GRP78, a surface model of the peptide-binding domain was sliced open and shown by clipping a plane along the binding site. Figure 1b shows that the L-peptide-binding site runs through the inter-region of the β -sandwich and helix-bundle subdomains, which appears as a "tunnel" for access of the L-peptide chain.

To illustrate GRP78-L-peptide interactions, three specific binding regions, A-, B- and C-pockets were identified in GRP78, which interacted, respectively, with Pro11, Leu10, and

Leu9 of the L-peptide Figure 4, panel b. As shown in Figure 4, panel c, the Connolly surface represents the water-contactable surface of the protein (Connolly, 1983) and is shown as a dotted surface to display the perspective configuration of the peptide-binding site in GRP78. The surface structure of amino acids, Arg7-Pro8-Leu9-Leu10-Pro11-Tyr12 (RPLLPY), of the L-peptide showed a geometric matching of the peptide molecule binding site in GRP78. Figure 1c. Obviously, these pockets provided a matched configuration, which allowed the RPLLPY sequence of the L-peptide to fit inside the peptide-binding pocket in GRP78.

A schematic diagram was constructed to represent molecular interactions between the L-peptide and GRP78. Figure 4, panel d. The sequence, RPLLPY, interacted with the peptide-binding domain of GRP78 through hydrogen bonding with the L-peptide backbone, and hydrophobic contacts via side chains of the L-peptide. The hydrogen bonds mainly occurred at the PLLP peptide sequence of the L-peptide (Figure 4, panel d). Since Val429, Ser452, Ala454, Gln458, and Thr462 of the GRP78 formed intermolecular hydrogen bonding with L-peptide (Figure 1d); these amino acids play important roles in trapping peptide ligands for GRP78. Furthermore, the side chain of Leu10 of the L-peptide was found to contact with Val461, Ile426, and Phe451 of GRP78 (the radiating semicircles in Figure 1d) to form hydrophobic interactions. In contrast, except for amino acids at the 8th to 11th positions, other amino acids in the L-peptide sequence did not display significantly specific interactions with GRP78. Thus, the most significant binding sequence of the L-peptide to be docked in the peptide-binding domain of GRP78 was determined to consist of the PLLP sequence.

Practical software programs, including combinatorial construction of peptide-structure library (Buildpep), binding pocket analyzer (PscanMS) and high accuracy protein-ligand scoring program (HotLig) as described above, were utilized to design optimized cancer targeting peptides, using the L-peptide as a lead. Based on these programs, a strategy of structure-based optimization of cancer targeting peptides *in silico*, as illustrated in Figure1, was developed to identify peptides that target GRP78, using the L-peptide as a lead.

To improve binding affinity, structural modification was first focused on alterations of amino acids within the binding sequence of PLLP of the L-peptide (see discussions above). It is noted that the binding pockets A and C in GRP78 for Pro11 and Leu9, respectively, represent wide open grooves, suggesting that these two amino acids of the L-peptide are likely to be substituted during optimization. On the other hand, Leu10 of the L-peptide was

invariable because of its limited and specific binding in the B-pocket (Figure 4, panel c). Additionally, Pro8 was found to interact with GRP78 as a rigid amino acid which prevents folding to form a secondary helix structure of peptides. Therefore, Pro8 might also be conserved during optimization of the peptide sequence.

5 Thus, in order to optimize the binding sequence based on the structural features of pockets A and C in GRP78 as described above, Leu9 and Pro11 of the L-peptide were chosen for substitutions with different amino acids. As a result, a structural library of 400 peptides was built by changing Leu9 and Pro11 for *in silico* screening analysis. To prevent time-consuming docking procedures, the lengths of the peptides in the library were reduced to 6-
10 mer peptides, i.e., RPXLXY. Flexible molecular docking was performed, and the molecular interactions were then estimated and predicted by HotLig as described above.

After docking various peptides in the library, Trp and Phe (i.e. AA11 in Figure 4, panel e) with large planar side chains were observed to be the most promising candidates for substitution for Pro11 of the L-peptide, because any of these substitutions could fit into the
15 additional cavity of A-pocket, in addition to the Pro11 binding site. On the other hand, the C-pocket for Leu9 binding of the L-peptide could be replaced by many other amino acids while preserving a similar matched configuration at C-pocket (Figure 4, panel f). In addition, in consideration of the binding energy predicted by HotLig, there were 17 peptide candidates (P-1 to P-17 in Table 6 below) which exhibited low HotLig energy scores and thus
20 represented good interactions of these peptide analogs with GRP78.

To further improve the diversity of peptide-candidate selection, another 9 peptides (PA-1 to PA-6 and PB-1 to PB-3 in Table 6) below were also included for comparison. For example, PA-1 contained hydrophobic Trp9 which was comparable to P-6 and the L-peptide because of the different-sized substitutions at the same 9th position of these peptides. In
25 addition, PA-2 and PA-3 were substituted with negatively charged residues (Glu9 and Asp9, respectively), and PA-6 was altered with a positive charged His9 at the same position. Additionally, PA-4 and PA-5 had the substitution of two polar amino acids (Asn9 and Gln9), respectively, which acted as either an H-bond donor or acceptor; PB-1 and PB-2 increased the hydrophobicity by replacing the His9 of P-12. Furthermore, peptide PB-3 was modified with
30 the amidation (CONH₂) to shield the negative charge of the COOH group.

Thus the structure-activity relationships of these designed GRP78-targeting peptides were validated by an *in vitro* binding assay and *in vivo* imaging assays as described in Example 3 below.

5 Example 2: Preparation of Liposomes Conjugated with Cancer-Targeting Peptides

The cancer-targeting peptides can be conjugated with liposomes, which encapsulate one or more anti-cancer agents, one or more cancer imaging agents, or both following routine methods. See, e.g., Chen et al., *Anticancer Research* 30:65-72, 2010.

(i) *Preparation of Liposomes*

10 Liposomes were provided by Taiwan Liposome Co. Briefly, liposomes composed of distearoylphosphatidylcholine, cholesterol, and PEG-DSPE were hydrated at 55°C in ammonium sulfate solution [250 mmol/L (NH₄)₂SO₄ (pH 5.0) and 530 mOs] and extruded through polycarbonate membrane filters (Costar, Cambridge, MA) of 0.1- and 0.05- μm pore size with high-pressure extrusion equipment at 60°C. The final concentration of liposomes
15 was determined by phosphate assay. Vesicle size was measured by dynamic laser scattering with a submicron particle analyzer. After preparation, the liposomes usually had a particle size ranging from 65 to 75 nm in diameter.

(ii) *Preparation of peptide linked PEGylated liposomes*

20 The procedures for preparation of peptide linked liposome were adopted from the methods published previously. (Lee, T. Y., et al. *Cancer Res.* **64**, 8002-8008. (2004)). A peptide was coupled to NHS-PEG-DSPE [N-hydroxysuccinimido-carboxyl-PEG (MW, 3400)-derived distearoylphosphatidylethanolamine (DSPE) (NOF Corporation, Tokyo, Japan)] at a 1:1.5 molar ratio. This coupling was performed with the unique free amine group in the NH₂ terminus of the peptide to produce peptidyl-PEG-DSPE. The reaction was
25 completed and confirmed by quantitation of the remaining amino groups. The amino group was measured with trinitrobenzenesulfonate reagent. The same method was used to prepare a control peptide to replace the cancer-targeting peptides and couple to NHS-PEG-DSPE for comparison. Peptidyl-PEG-DSPE was then conjugated to pre-formed liposomes encapsulating doxorubicin after co-incubation at temperature above the transition temperature
30 of lipid bilayer. There were 300 to 500 molecules of the peptide per liposome, as determined by the method described in Kirpotin et al., *Biochemistry* 36:66-75, 1997.

(iii) *Preparation of peptide-¹⁸⁸Re- labeled pegylated liposomes*

The peptide-pegylated-liposomes (1 ml) was added to the ¹⁸⁸Re (50-250 MBq) solution and incubation at 60°C for 30 min. The peptide-¹⁸⁸Re labeled pegylated liposomes were separated from free ¹⁸⁸Re using PD-10 column (GE Healthcare) eluted with normal saline. Each 0.5 ml fraction was collected into a tube. The opacity of liposome was used to visually monitor the collection of the peptide-¹⁸⁸Re labeled pegylated liposomes. The labeling efficiency was determined by using the activity in peptide-pegylated-liposomes after separation divided by the total activity before separation. See also Chen et al., *Anticancer Research* 30:65-72 (2010).

10

Example 3: Characterization of Designed Cancer-Target Peptides

(i) *Determining Peptide Binding Activity to GRP78 in an In vitro Binding Assay*

26 candidate peptides designed by the method described in Example 1 (listed in Table 6 below) above and peptide CdL, a negative control, were synthesized by conventional chemical synthesis and subjected to the surface-plasmon-resonance based method described below to examine the binding activities of the peptides to human GRP78.

The chip NTA and HBS-P buffer were obtained from GE Healthcare. Sensor chip NTA was prepared by combining Ni-ion chelation and covalent immobilization of N-His tagged GRP78 via amine-coupling with nitrilotriacetic acid on chip NTA (Figure 6). To immobilize GRP78 protein on sensor chip for peptide-binding assay using Biacore, the methods based on Ni-ion chelation and amine coupling reaction were combined. Sensor chip NTA was prepared by chelation followed by covalent immobilization of N-His-tagged GRP78 via amine-coupling at the nitrilotriacetic acid group on chip NTA. The sensor chip NTA, HBS-P buffer and amine coupling kits were all obtained from GE Healthcare. First, the activation of chip surface using EDC and NHS reagents for amine coupling were according to the standard procedure from manufacturer's instruction. Additionally, one-minute pulse of NiCl₂ solution (500 μM) was used to attract His-tagged GRP78 to the surface of sensor chip and formed covalent bond subsequently in the condition of HBS-P buffer (pH 7.4). The resulting differences of RU were about 5,000~6,000 RU. After de-activation by ethanolamine and washed with EDTA (3 mM), the prepared sensor chip was then applied to Biacore X for binding assay. The HBS-P buffer containing 1 mM Gly was used as running

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buffer for binding assay. Solution of 20 mM sodium hydroxide dissolved in running buffer was used to regenerate the chip surface by one-minute pulse. Comparing with conventional methods using Ni-ion chelation alone, our chip surface exhibit more stable baseline and provide higher sensitivity for binding analysis of small molecules in Biacore X. This method was found to be better than that using chip CM5 because of its higher protein viability in oriented immobilization and without being exposed to low pH buffer during protein immobilization.

The sensor chip thus prepared was applied to Biacore X for determining the binding between the peptides and GRP78. The HBS-P buffer containing 1 mM Gly was used as a running buffer. A solution of 20 mM sodium hydroxide dissolved in the running buffer was used to regenerate chip surface by 1-minute pulses. The results obtained from this study were shown in Table 6 below.

Table 6. Biacore binding assay of 26 peptide analogs of L-peptide at 50 μ M against full-length recombinant GRP78

Alterations at Pro11	ID	Sequence	HotLig score (rank)	Binding index
Positive control	L-peptide	RLLDTNRPLL ¹¹ Y (SEQ ID No:13)	-27.65 (14)	1.00
Negative control	CdL	RLLDTNRPL(<i>d</i> -L)PY (SEQ ID No:13)	-	< 0.2
Hydrophobic				
Pro	P-6	RLLDTNRPF¹¹LPY (SEQ ID No:3)	-28.56 (6)	1.1-1.52
	PA-1	RLLDTNRPWLPY (SEQ ID No:14)	-	0.35
	PA-2	RLLDTNRPELPY (SEQ ID No:15)	-	< 0.2
	PA-3	RLLDTNRPDLPY (SEQ ID No:16)	-	< 0.2
	PA-4	RLLDTNRPNLPY (SEQ ID No:17)	-	0.31
	PA-5	RLLDTNRPQLPY (SEQ ID No:18)	-	0.3
	PA-6	RLLDTNRPHLPY (SEQ ID No:19)	-	0.47
	PB-3	RLLDTNRPLL¹¹Y(CONH₂) (SEQ ID No:13)	-	1.15

Trp	P-12	RLLDTNRPHLWY (SEQ ID No:4)	-27.79 (12)	1.2~3.5
	PB-1	RLLDTNRPFLWY (SEQ ID No:11)	-	1.6
	PB-2	RLLDTNRPYLWY (SEQ ID No:12)	-	3
Phe	P-4	RLLDTNRPKLFY (SEQ ID No:23)	-28.68 (4)	< 0.2
	P-7	RLLDTNRPSLFY (SEQ ID No:25)	-28.46 (7)	< 0.2
	P-13	RLLDTNRPFLFY (SEQ ID No:5)	-27.70 (13)	1.4~3.5
Leu	P-14	RLLDTNRPLLY (SEQ ID No:30)	-26.72 (29)	0.21
Val	P-2	RLLDTNRPKLVY (SEQ ID No:21)	-31.18 (2)	< 0.2
	P-3	RLLDTNRQLVY (SEQ ID No:22)	-29.66 (3)	< 0.2
Positive charged				
Lys	P-1	RLLDTNRPYLKY (SEQ ID No:20)	-33.46 (1)	< 0.2
	P-8	RLLDTNRPMLKY (SEQ ID No:26)	-28.04 (8)	< 0.2
	P-10	RLLDTNRPFLKY (SEQ ID No:28)	-27.89 (10)	< 0.2
	P-15	RLLDTNRPHLKY (SEQ ID No:31)	-27.52 (16)	< 0.2
Arg	P-16	RLLDTNRPKLRY (SEQ ID No:32)	-27.42 (20)	< 0.2
Negative charged				
Glu	P-17	RLLDTNRPRLEY (SEQ ID No:33)	-26.78 (27)	< 0.2
Other				
Met	P-5	RLLDTNRPMLMY (SEQ ID No:24)	-28.58 (5)	< 0.2
Cys	P-9	RLLDTNRPPLCY (SEQ ID No:27)	-27.99 (9)	< 0.2
Gly	P-11	RLLDTNRPLLY (SEQ ID No:29)	-27.87 (11)	0.77

A representative sensorgram of peptide binding to the full-length recombinant GRP78 is shown in Figure 4, panel a. It was shown that peptides P-12, P-6, and P-13 bound to GRP78 better than the L-peptide in the assay. The binding of various peptides to GRP78 is

also shown as a binding index in Table 6. The binding index represents the difference of resonance units (RUs) divided by the molecular weight of each peptide and was normalized by comparison to the L-peptide. Six of these peptides were found to exhibit significant binding responses in Biacore including P-6, P-12, P-13, PB-1, PB-2, and PB-3 (Table 6).

5 When compared to the L-peptide, the structure-activity relationships at the 9th amino acid of the L-peptide revealed that an increase in the hydrophobicity led to enhancement of the binding affinity (*e.g.*, L-peptide and P-6). However, a Trp-substitution at the 9th site (*e.g.*, PA-1) significantly decreased the binding, probably due to steric hindrance. For other hydrophilic peptides (*e.g.*, PA-2 to PA-6), Glu-, Asp-, Asn-, Gln-, and His-substituted
10 peptides also showed lower binding affinities than the L-peptide.

On the other hand, only Pro and aromatic residues such as Phe and Trp at the 11th amino acid were found to be able to bind to GRP78 (*e.g.*, L-peptide, P-6, P-12, P-13, PB-1, and PB-2), because any other amino acid substitution at this position led to a decrease in the binding affinity. In particular, the charged amino acids substituted at either the 9th or 11th
15 position of the L-peptide resulted in a loss of binding ability. Additionally, modification of the carboxylic acid terminus of the L-peptide, such as PB-3 in Table 6, did not significantly affect the binding affinity as compared to the L-peptide.

Three optimized peptides, P-6, P-12, and P-13, were used for further *in vitro* and *in vivo* evaluations of their cancer-targeting abilities.

20 (ii) Binding of FITC-labeled peptides to cancer cells

To evaluate the *in vitro* binding abilities of cancer-targeting peptides described herein, the FITC-labeled L-peptide, P-6, P-12, P-13, and P-16 were tested for their binding activities to cancer cells, primary breast cancer engrafted in NOD/SCID mice, and clinical breast cancer specimens by a flow cytometric analysis as described below.

25 Cells were grown to 80% confluence and harvested with 5mmol/L EDTA in PBS. Breast cancer specimens were obtained from patients who underwent initial surgery at Tri-Service General Hospital (Taipei, Taiwan). The clinical breast cancer specimens were sliced to fragments of 1 mm² in size and subjected to enzymatic digestion by collagenase (1,000 U/mL), hyaluronidase (300 U/mL), and DNase I (100 μ g/mL) at 37°C for 2 hours. Primary
30 breast tumor cells were collected after filtration through a 100 μ m cell strainer (BD

Biosciences) and re-suspended. For transplantation, tumor cells mixed with normal human breast fibroblasts and Matrigel were subcutaneously injected into mammary fat pads of female NOD/SCID mice received a sub-lethal dose of gamma irradiation in advance. After transplantation, cells of xenografted tumors were isolated and inoculated in NOD/SCID mice for serial passages. The cancer cell lines, xenograft cells, and primary breast tumor cells were re-suspended in a FACS buffer (PBS with 2% fetal bovine serum) and incubated at room temperature for 30 min with FITC-conjugated various peptides. To determine breast cancer stem cell (BCSCs) and non-breast cancer stem cell (non-BCSC) populations from xenografts, the cells were stained with a mixture of anti-H2K_d-biotin followed by streptavidin-PER-CP, anti-CD24-PE, anti-CD44-APC and FITC-conjugated various peptides. The primary breast tumor cells were stained with anti-CD45-PerCP-Cy5.5 antibodies, instead of H2K_d staining. The stained cells were then subjected to FACS analysis.

As shown in Figure 7, panel b, L-peptide, P-6, P-12, and P-13 all bound to both MDA-MB-231 cells (breast cancer cells) and TW01 cells (nasopharyngeal carcinoma).

The binding capacities of the L-peptide and the P-6, P-12, P-13, and P-16 peptides to two xenograft samples from NOD-SCID mice transplanted with human primary breast cancer cell lines BC0145 and BC0244 were evaluated with special focus on their binding to the BCSC subpopulation enriched from the breast cancer cells. Breast cancer cells harvested from engrafted tumors were stained with H2K_d to gate out mouse cells, and stained with anti-CD24 and anti-CD44 antibodies to distinguish BCSC-enriched cells (CD24⁻CD44⁺) from non-BCSC cells. These cells were co-stained with one of the above-noted peptides, which was FITC-labeled and subjected to flow cytometric analysis. As shown in Figure 7, panel c, these peptides were able to bind both the BCSC-enriched population and the non-BCSC cells isolated from the two xenografts. P-13 stained most strongly, followed by P-6 > P-12 > L > P-16, although the binding capacity of P6 was equivalent to P-12 to BCSC-enriched cells isolated from the BC0145 xenograft. The binding abilities of these peptides to clinical breast specimens were similar to those to xenograft samples. The tested peptides were found to bind to both BCSC-enriched (CD45⁻CD44⁺CD24⁻) and non-BCSC (the remaining CD45⁻ cells) cells isolated from samples BC0854 and BC0861. Figure 7, panel d. These results demonstrated that the peptides tested in this study are capable of binding to BCSCs isolated from xenografts of primary breast cancer and clinical breast cancer specimens, suggesting

that they target the BCSC-enriched subpopulation. Moreover, P-13, P-12, and P-6 displayed greater binding capacities *in vitro* than L-peptide to both BCSCs and non-BCSCs, indicating that they are more effective than the L-peptide in targeting cancer cells.

(iii) *Use of cancer-targeting peptides for tumor imaging*

5 Peptide-linked liposomes containing ^{188}Re were used to evaluate the tumor-targeting abilities of the L-peptide and the peptides identified in Example 1 above by microSPECT/CT imaging of BC0244 xenografts. Briefly, NOD-SCID female mice were subcutaneously injected with 1×10^6 BC0244 cells in the right hind flank. After one month, BC0244 xenograft-bearing mice were intravenously injected with 400 μCi of ^{188}Re -liposome-L-peptide, ^{188}Re -liposome-P-6, ^{188}Re -liposome-P-12, ^{188}Re -liposome-P-13, or ^{188}Re -liposome
10 alone, all of which were prepared following the methods described in Example 2 above. Six, twenty-four, and forty-eight hours after the intravenously injection, microSPECT images were acquired using a microSPECT/CT scanner system as described in Rajarshi Guha et al. *J. Chem. Inf. Model.* 46:991-998; 2006. The standardized uptake value (SUV) was calculated to
15 determine the uptake of radioactivity in tumors using the formula:

$$\text{SUV} = [\text{mean ROI activity } (\mu\text{Ci/g})] / [\text{injected activity } (\mu\text{Ci}) / \text{mouse body weight (g)}].$$

20 Two-way ANOVA with Bonferroni's multiple comparison test was used to analyze microSPECT/CT imaging data. Mixed model was used to analyze the differences of growth rate tendency between various groups. Kaplan-Meier method and log-rank test were used to analyze the survival data. One-way ANOVA with Bonferroni's multiple comparison test was used to analyze the body weight data. Statistical significance was taken as $p < 0.05$. All statistical analyses were done using the SPSS statistical software (SPSS Inc., Chicago, IL).

25 As shown in Table 7 below, there were significantly greater uptake of ^{188}Re -liposome-P-6 ($p < 0.05$) at 6h, ^{188}Re -liposome-L-peptide ($p < 0.01$) at 24h, as well as ^{188}Re -liposome-P-6 ($p < 0.001$), ^{188}Re -liposome-P-12 ($p < 0.01$), and ^{188}Re -liposome-P-13 ($p < 0.05$) at 48h, as compared to the ^{188}Re -liposomes in BC0244 tumors at all time points. At 48h, the uptake of P-6-linked ^{188}Re -liposomes was higher than that of the L-peptide, indicating its
30 better tumor targeting abilities ($p < 0.001$).

Table 7. Uptake of peptide-conjugated ^{188}Re -liposomes in Xenograft NOD-SCID Mice

Time points	Treatments				
	^{188}Re -liposome-L	^{188}Re -liposome-P-6	^{188}Re -liposome-P-12	^{188}Re -liposome-P-13	^{188}Re -liposome
6h	1.58±0.54	2.26±0.14 ^{a, b***}	1.59±0.37	1.79±0.21	1
24h	2.92±0.42 ^{a**}	2.09±0.10	1.71±0.15	1.79±0.45	1
48h	1.07±0.30	3.68±0.41 ^{a***}	2.21±0.39 ^{a**}	2.26±0.76 ^{a*}	1

(iv) Therapeutic efficacy of peptide-labeled liposomal doxorubicin

The cancer-targeting activities of the peptides disclosed herein in targeted cancer chemotherapy was examined as follows, using the L-peptide as a positive control. The L-peptide was found to bind to a variety of tumor cell lines and cancer tissues from cancer patients. See Tables 8-10 below:

Table 8. Binding of FITC-L-peptide to a variety of tumor cell lines

Binding ability	Cancer type	Cell line
positive	Nasopharyngeal	TW01, TW06, TW07
	Breast	MDA-MB-231, T47D, MB157, AU565
	Lung	H1299, A549
	Prostate	LNCap.FGC, PC-3
	Melanoma	M14
	Immortalized fetal kidney epithelium containing T antigen	293T
	Ovarian	2008
	Cervix epithelial	Hela
	Testicular embryonal	Ntera-2-cl.D1
	Leukemia	CEM, K562, Jurkat, HL-60
	Osteosarcoma	SaOs2
	negative	Colon
Hepatocyte		HepG2
Glioblastoma		G9T/VGH, U-373
Neuroblastoma		Be2c

Table 9. Clinical histopathological characteristics of breast cancer patients and their corresponding L-peptide binding capacities to tumors.

Coding NO.	Age	Stage	Tumor type(Histological grade)	Grade	ER*	PR*	HER2 /neu *	L peptide (Differences in MFI)*
BC0634	53	IIIA	invasive ductal carcinoma	3	—	+	+	1.39
BC0643	65	IIB	Invasive ductal carcinoma	3	—	+	—	2.12
BC0679	46	IIIB	Invasive ductal carcinoma	2	+	+	—	2.58
BC0697	50	IIA	Invasive ductal carcinoma	3	—	+	—	2.71
BC0775	68	IIA	Invasive ductal carcinoma	3	+	+	+	0
BC0854	53	IIB	Invasive ductal carcinoma	3	+	+	—	7.67
BC0859	49	IIIA	Invasive ductal carcinoma with apocrine differentiation and EIC (high grade, 30%).	3	—	—	w+	0.92
BC0861	57	IIB	Invasive ductal carcinoma	3	—	—	+	5.11
BC0866	36	IIIC	Invasive ductal carcinoma with DCIS, high grade (15%).	3	+	+	+	14.3
VBC039	71	II	Invasive ductal carcinoma	3	+	+	—	2
VBC040	68	II	Invasive ductal carcinoma	2	+	+	—	47.54

5

10

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Table 10. Biodistribution of L-peptide –linked PEG-liposome containing ¹⁸⁸Re in NOD-SCID mice

¹⁸⁸ Re-liposome-L-peptide(%ID/g)	1hr	4hr	8hr	24hr	48hr	72hr
Whole Blood	55.93±1.51	35.46±2.88	27.03±0.47	6.36±0.73	0.68±0.06	0.09±0.01
Tumor	2.12±0.42	4.64±0.67	7.28±0.01	4.01±1.11	2.31±0.27	2.11±0.05
Pituitary	2.91±0.93	5.72±1.99	4.02±0.32	1.20±0.32	1.01±0.96	0.28±0.06
L intestine	1.23±0.10	1.48±0.19	1.22±0.09	1.38±0.29	0.44±0.40	0.27±0.17
S intestine	9.63±2.76	15.59±1.53	17.43±0.06	9.88±2.20	4.97±2.34	2.85±0.87
Muscle	0.58±0.08	1.52±0.86	0.53±7.93	0.46±0.10	0.15±0.05	0.11±0.00
Bone	1.94±0.42	2.81±0.65	1.75±1.28	1.10±0.23	1.33±0.87	2.07±0.64
Pancreas	2.24±0.18	1.90±0.30	1.85±0.21	1.16±0.20	0.29±0.16	0.07±0.01
Spleen	10.76±0.12	12.05±3.31	14.62±0.08	3.08±3.10	7.77±4.01	6.80±2.06
Adrenals	5.98±0.66	8.51±1.92	4.73±0.27	1.79±0.36	3.01±1.13	3.44±2.55
Kidney	9.73±0.51	9.06±2.16	7.68±0.11	4.27±0.51	2.38±1.02	1.77±0.07
Lung	12.63±1.03	8.97±1.93	6.11±0.62	2.08±0.23	0.58±0.27	0.73±0.16
Heart	4.63±0.18	4.53±0.61	4.40±0.04	2.59±0.43	0.91±0.49	1.29±0.09
Liver	13.26±0.07	20.19±3.33	62.33±0.73	3.55±2.11	9.33±3.90	6.85±0.92
Feces	0.18±0.03	8.48±3.62	12.20±0.33	0.62±2.49	13.02±5.25	7.00±0.56
S intestine content	1.25±0.30	1.67±0.33	1.61±4.55	1.68±0.13	1.59±0.74	0.20±0.07
Brain	0.79±0.05	0.74±0.21	0.47±1.20	0.12±0.02	0.02±0.01	0.01±0.00
Bladder	1.09±0.16	1.27±0.27	1.67±3.90	0.81±0.15	1.10±0.53	0.55±0.22
Tu/Mu	3.96±0.95	14.56±1.55	14.32±6.01	6.73±2.81	34.96±20.96	24.93±12.23

5

PEGylated P-6, P-12, and P-13 were coupled with liposomal doxorubicin (P-6-Lipo-Dox, P-12-Lipo-Dox, and P-13-Lipo-Dox) following the method described in Example 2 above. NOD-SCID female mice were subcutaneously injected with 1×10^6 BC0244 xenograft cells into mammary fat pad. Mice with size-matched tumors (approximate 100 mm^3) (n=5) were randomly assigned to different treatment groups and intravenously injected with PBS, liposomal doxorubicin (Lipo-Dox), L-peptide-linked Lipo-Dox (L-peptide-Lipo-Dox), P-6-Lipo-Dox, P-12-Lipo-Dox, or P-13-Lipo-Dox. The dosage of doxorubicin was 2 mg/kg injected once every week for three weeks. Mouse body weight and tumor size were measured twice a week with calipers. The tumor volumes were calculated using the equation: length x (width)² x 0.5.

The results obtained from this study indicate that Lipo-Dox significantly suppressed tumor growth in xenografted mice as compared to PBS ($p < 0.0001$) (Figure 8, panel a) and the peptide-conjugated Lipo-Dox further suppressed tumor growth, indicating that targeted chemotherapy using any of the tested cancer-targeting peptides enhanced the efficacy of doxorubicin therapy. (Figure 8, panel b). To be more specific, the tumor growth rates in

mice treated with L-peptide-Lipo-Dox, P-6-Lipo-Dox, P-12-Lipo-Dox, and P-13-Lipo-Dox groups, and Lipo-Dox were reduced by 66.90%, 37.37%, 67.13%, and 65.00%, respectively, as compared to that in mice treated with PBS. Figure 8, panel b. Moreover, treatment with P-6-lipo-Dox suppressed the tumor growth rate to 55.86% of that of the L-peptide-Lipo-Dox group ($p=0.0012$). Figure 8, panel b. Survival rates of tumor-bearing mice after various treatments were monitored over 70 days. The results show that the survival of P-6-Lipo-Dox and P-13-Lipo-Dox groups were significantly longer than the L-peptide-Lipo-Dox group ($p=0.0388$ and $p=0.0221$, respectively). Figure 8, panel d. The body weight of tumor-bearing mice receiving PBS gradually increased due to the growing weight of the tumors; however, the body weight of the other five groups did not change significantly. Figure 8, panel c.

Taken together, the results discussed above demonstrate that the tested cancer-targeting peptides enhanced chemotherapy efficacy by targeting chemotherapy drugs to tumor sites and that the cancer-targeting activities of these peptides, such as P6 and P13, are unexpectedly higher than that of the L-peptide.

OTHER EMBODIMENTS

All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

From the above description, one skilled in the art can easily ascertain the essential characteristics of the present invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, other embodiments are also within the claims.

What is claimed is:

1. An isolated peptide comprising an amino acid sequence motif PX_1LX_2 (SEQ ID NO:1), in which X_1 is H or an amino acid with a hydrophobic side chain, X_2 is P, F, or W, wherein when X_1 is L, X_2 is not P, and when X_2 is P, X_1 is not L, and wherein the peptide binds to human glucose-regulated protein 78 (GRP-78).

2. The isolated peptide of claim 1, wherein X_1 is L, H, F, or W.

3. The isolated peptide of claim 1 or claim 2, wherein the peptide comprises an amino acid sequence of $RLLDTNRPX_1LX_2Y$ (SEQ ID NO:2).

4. The isolated peptide of claim 3, wherein the peptide comprises an amino acid sequence selected from the group consisting of $RLLDTNRPFLPY$ (P-6) (SEQ ID NO:3), $RLLDTNRPHLWY$ (P-12) (SEQ ID NO:4), and $RLLDTNRPFLFY$ (P-13) (SEQ ID NO:5).

5. The isolated peptide of claim 4, wherein the peptide is selected from the group consisting of $RLLDTNRPFLPY$ (P-6) (SEQ ID NO:3), $RLLDTNRPHLWY$ (P-12) (SEQ ID NO:4), and $RLLDTNRPFLFY$ (P-13) (SEQ ID NO:5).

6. A composition comprising (a) the peptide of any of claims 1-5, and (b) an anti-cancer agent, a detectable label, or both.

7. The composition of claim 6, wherein the composition comprises a detectable label attached to the peptide.

8. The composition of claim 7, wherein the detectable label is a fluorescent or a luminescent compound.

9. The composition of claim 8, wherein the detectable label is fluorescein isothiocyanate (FITC).

10. The composition of any of claims 6-9, wherein the composition further
5 comprises a vehicle carrier.

11. The composition of claim 10, wherein the vehicle carrier is a liposome, which encapsulates the anti-cancer agent, the detectable label, or both, and wherein the peptide is attached on the surface of the liposome.

10

12. The composition of any of claims 6-11, wherein the peptide is pegylated.

13. The composition of any of claims 6-12, wherein the composition comprises an anti-cancer agent.

15

14. The composition of claim 13, wherein the amount of the anti-cancer agent is effective in treating cancer.

15. The composition of claim 13 or claim 14, wherein the anti-cancer agent is
20 doxorubicin, vinorelbine, vincristine, paclitaxel or lurtotecan.

16. The composition of any of claims 6-12, wherein the composition comprises a detectable label, which is an imaging agent.

17. The composition of claim 16, wherein the imaging agent is a radioactive
25 molecule or an iron oxide nanoparticle.

18. The composition of claim 17, wherein the imaging agent is ^{99m}Tc or ^{188}Re .

19. The composition of any of claims 16-18, wherein the amount of the imaging agent is effective in detecting cancerous tissues or cells.

20. The composition of any of claims 6-19, wherein the composition is a pharmaceutical composition, which further comprises a pharmaceutically acceptable carrier.

21. A method for delivering an anti-cancer agent or a detectable label to cancer cells, comprising contacting cancer cells or cells that are suspected to be cancerous with the composition of any of claims 6-20.

22. The method of claim 21, wherein the contacting step is performed by administering the composition to a subject having or suspected of having cancer cells.

23. The method of claim 22, wherein the subject has or is suspected of having breast cancer cells, hepatocellular carcinoma cells, prostate cancer cells, lung cancer cells, ovarian cancer cells, kidney cancer cells, uterine cervical cancer cells, melanoma cells, embryonal carcinoma cells, leukemia cells, or osteosarcoma cells.

24. The method of claim 22, wherein the subject has or is suspected of having breast cancer stem cells.

25. The method of claim 21, wherein the composition comprises an anti-cancer agent in an amount effective in treating cancer.

26. The method of claim 21, wherein the composition comprises a detectable label, which is a cancer imaging agent, in an amount effective for detecting cancer cells.

27. The method of claim 21, wherein the contacting step is performed by incubating the composition with a sample having or suspected of having cancer cells.

28. A method for structure-based optimization of a cancer-targeting peptide ligand, the method comprising:

(a) providing a cancer cell-surface protein;

(b) calculating the Connolly surface of the cancer cell-surface protein;

5 (c) identifying a peptide-binding site on the protein surface;

(d) estimating distance-dependent potential of paired atoms involved in polar interactions comprising hydrogen bonding, ionic interactions and metal-ion coordination, the estimation comprising consideration of an intermolecular surface distance;

10 (e) estimating distance-dependent potential of paired atoms involved in non-polar interactions, the estimation comprising application of Connolly surface of protein in calculation of the intermolecular surface distance; and

(f) selecting a peptide ligand optimized for binding to the binding site of the cancer cell-surface protein from a peptide database based on the information obtained from steps (d) and/or (e).

15

29. The method of claim 28, wherein the Connolly surface of the cancer cell-surface protein is determined by the binding pocket analysis program PscanMS.

30. The method of claim 28, wherein the peptide database is combinatorially
20 constructed.

31. The method of claim 28, wherein the cancer cell-surface protein is human glucose-regulated protein 78 (GRP-78).

25

32. The method of claim 28, wherein the intermolecular surface distance is determined based on the binding energy (ΔE) between protein (p) and ligand (l), which is calculated according to the following equation:

$$\begin{aligned} \Delta E_{p,l} &= \Delta E_{polar}(\delta, \theta, \phi) + \Delta E_{non-polar}(v, A), (\phi < 100^\circ) \\ &= F_{hbond} \times \sum_h (\cos(180^\circ - \theta_h) \times W_{hbond}(\delta_h)) \\ &+ F_{ion} \times \sum_i W_{ion}(\delta_i) \\ &+ F_{metal} \times \sum_m W_{metal}(\delta_m) \\ &+ F_{vdw} \times \sum_v (A_v \times W_{vdw}(v_v)) \end{aligned}$$

wherein, h is the pair of H-bond; i is the pair of ionic interaction; m is the pair of metal-ion coordination and v is the ligand-contacted normal vector in hydrophobic interaction.

33. The method of claim 28, wherein the method is performed *in silico*.

34. The method of claim 28, wherein the distance-dependent potentials are predicted from a statistical set comprising occurrence frequencies of paired pharmacophores in molecular interactions.

35. A pharmaceutical composition for use in delivering an anti-cancer agent or a detectable label to cancer cells or cells suspected of being cancerous, the composition comprising (a) a cancer-targeting peptide of any of claims 1-5, (b) an anti-cancer agent, a detectable label, or both, and (c) a pharmaceutically acceptable carrier.

36. The pharmaceutical composition of claim 35, wherein the composition comprises a detectable label attached to the cancer-targeting peptide.

37. The pharmaceutical composition of claim 36, wherein the detectable label is a fluorescent or a luminescent compound.

38. The pharmaceutical composition of claim 37, wherein the detectable label is fluorescein isothiocyanate (FITC).

39. The pharmaceutical composition of any of claims 35-38, wherein the composition further comprises a vehicle carrier.

40. The pharmaceutical composition of claim 39, wherein the vehicle carrier is a liposome, which encapsulates the anti-cancer agent, the detectable label, or both, and wherein the peptide is attached on the surface of the liposome.

41. The pharmaceutical composition of any of claims 35-40, wherein the peptide is pegylated.

42. The pharmaceutical composition of any of claims 35-41, wherein the composition comprises an anti-cancer agent.

43. The pharmaceutical composition of claim 42, wherein the amount of the anti-cancer agent is effective in treating cancer.

44. The pharmaceutical composition of claim 42 or claim 43, wherein the anti-cancer agent is doxorubicin, vinorelbine, vincristine, paclitaxel or lurtotecan.

45. The pharmaceutical composition of any of claims 35-41, wherein the composition comprises a detectable label, which is an imaging agent.

46. The pharmaceutical composition of claim 45, wherein the imaging agent is a radioactive molecule or an iron oxide nanoparticle.

47. The pharmaceutical composition of claim 46, wherein the imaging agent is ^{99m}Tc or ^{188}Re .

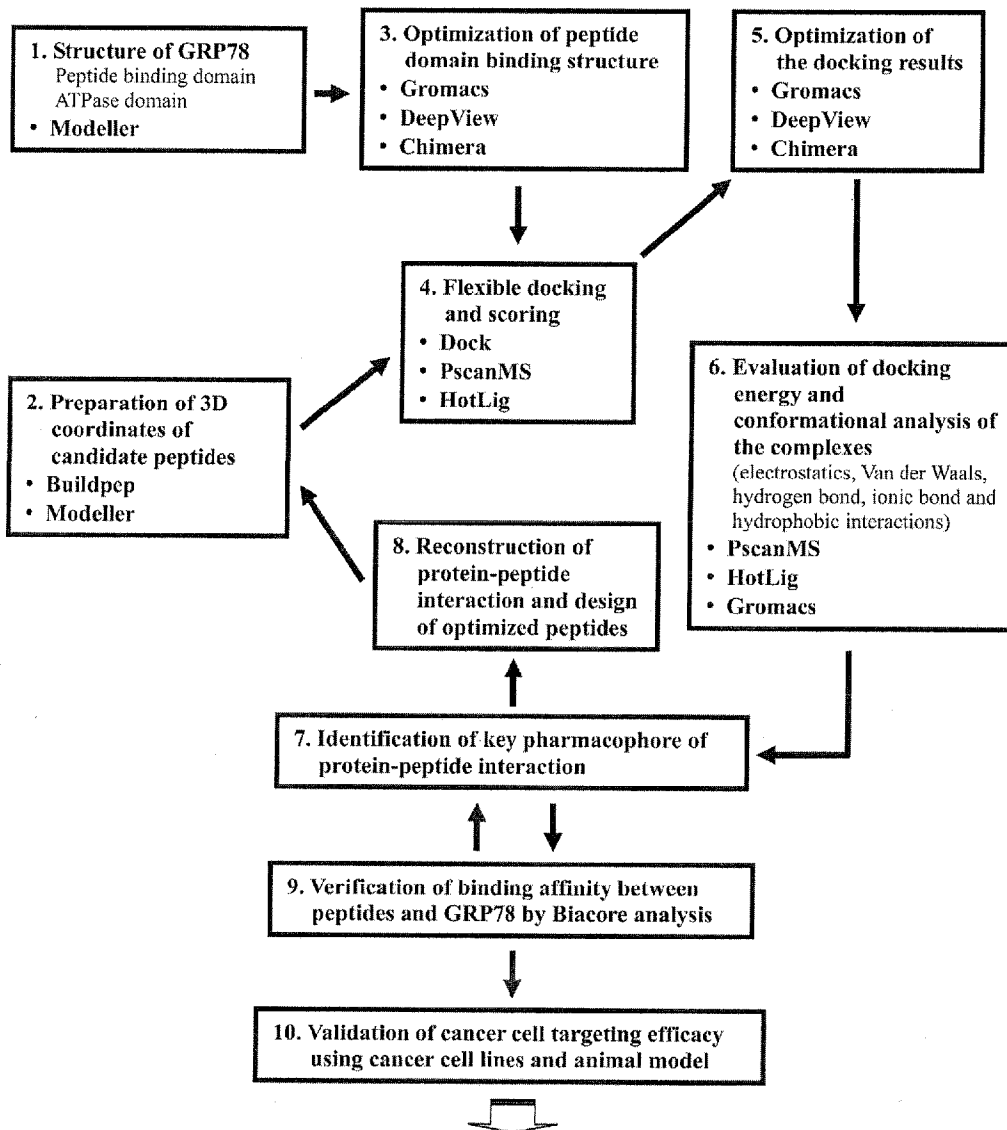
5 48. The pharmaceutical composition of any of claims 45-47, wherein the amount of the imaging agent is effective in detecting cancerous tissues or cells.

10 49. The pharmaceutical compositions of any of claims 35-48, wherein the cancer cells are breast cancer cells, breast cancer stem cells, hepatocellular carcinoma cells, prostate cancer cells, lung cancer cells, ovarian cancer cells, kidney cancer cells, uterine cervical cancer cells, melanoma cells, embryonal carcinoma cells, leukemia cells, or osteosarcoma cells.

15 50. The pharmaceutical compositions of any of claims 35-48, wherein the composition comprises an anti-cancer agent in an amount effective in treating cancer.

20 51. The pharmaceutical compositions of any of claims 35-48, wherein the composition comprises a detectable label in an amount effective in detecting the cancer cells.

FIGURE 1



Generation of optimized cancer targeting peptides

FIGURE 2

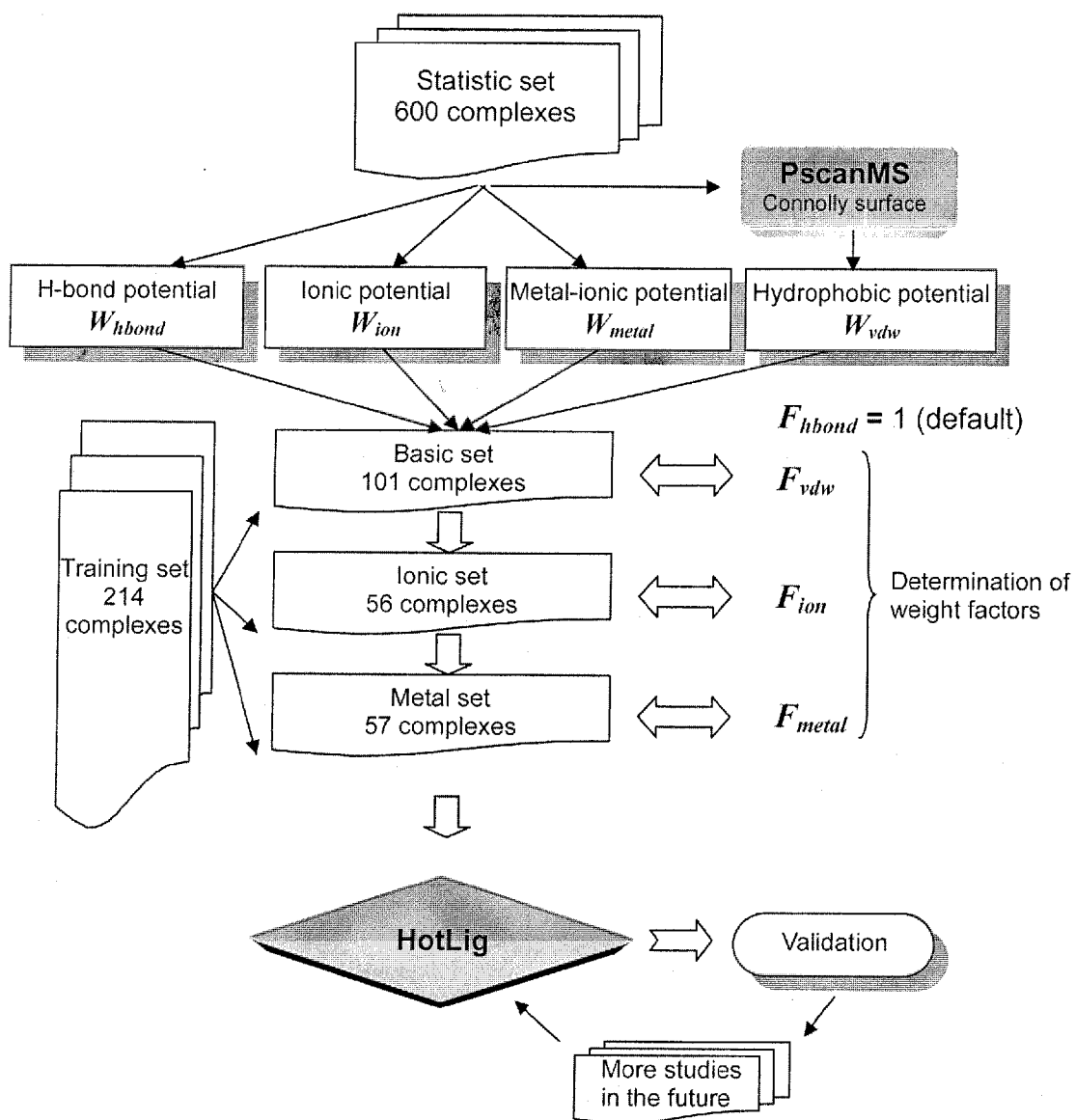


FIGURE 3

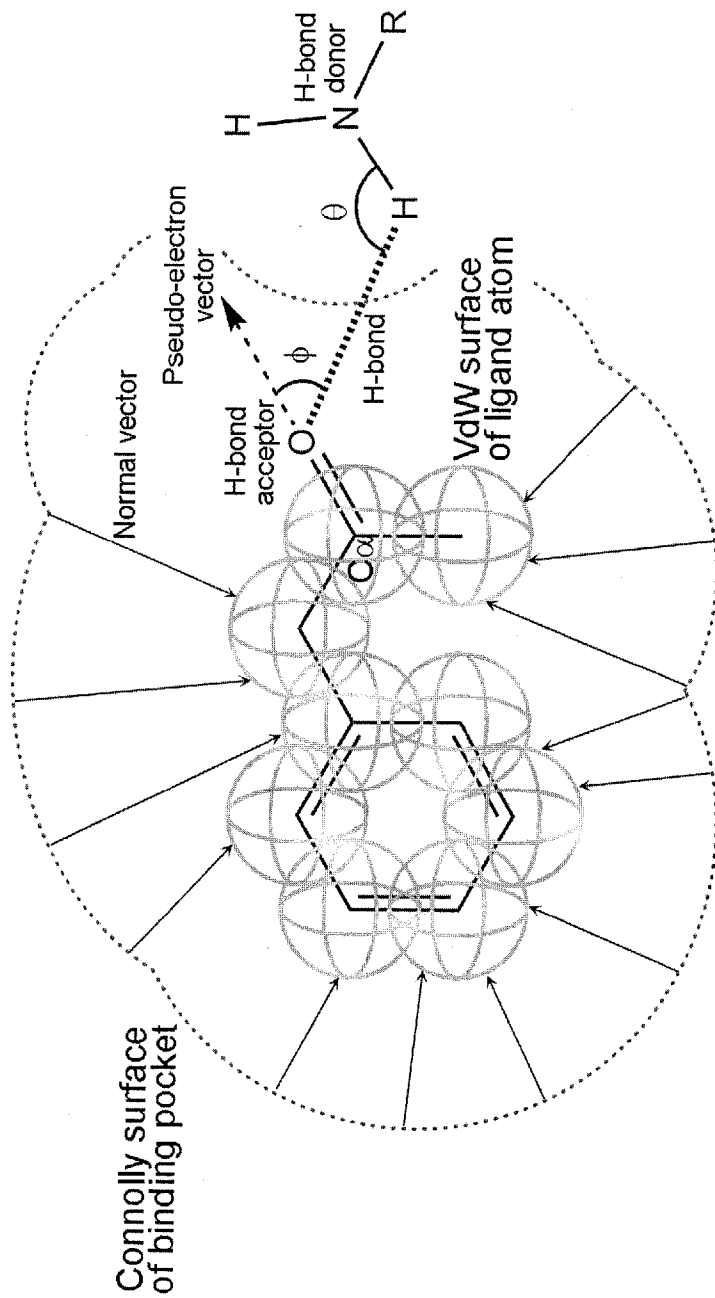


FIGURE 5

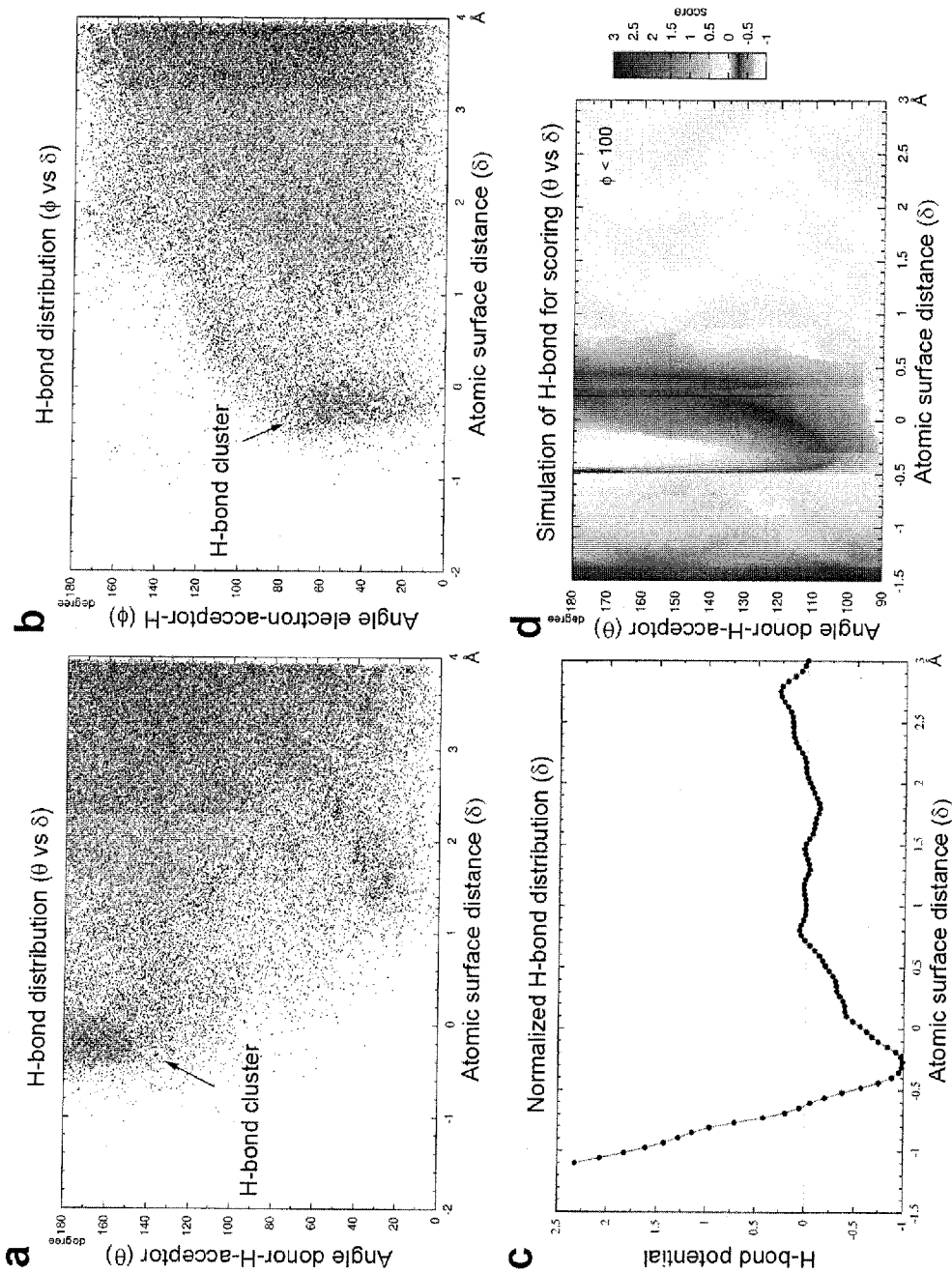
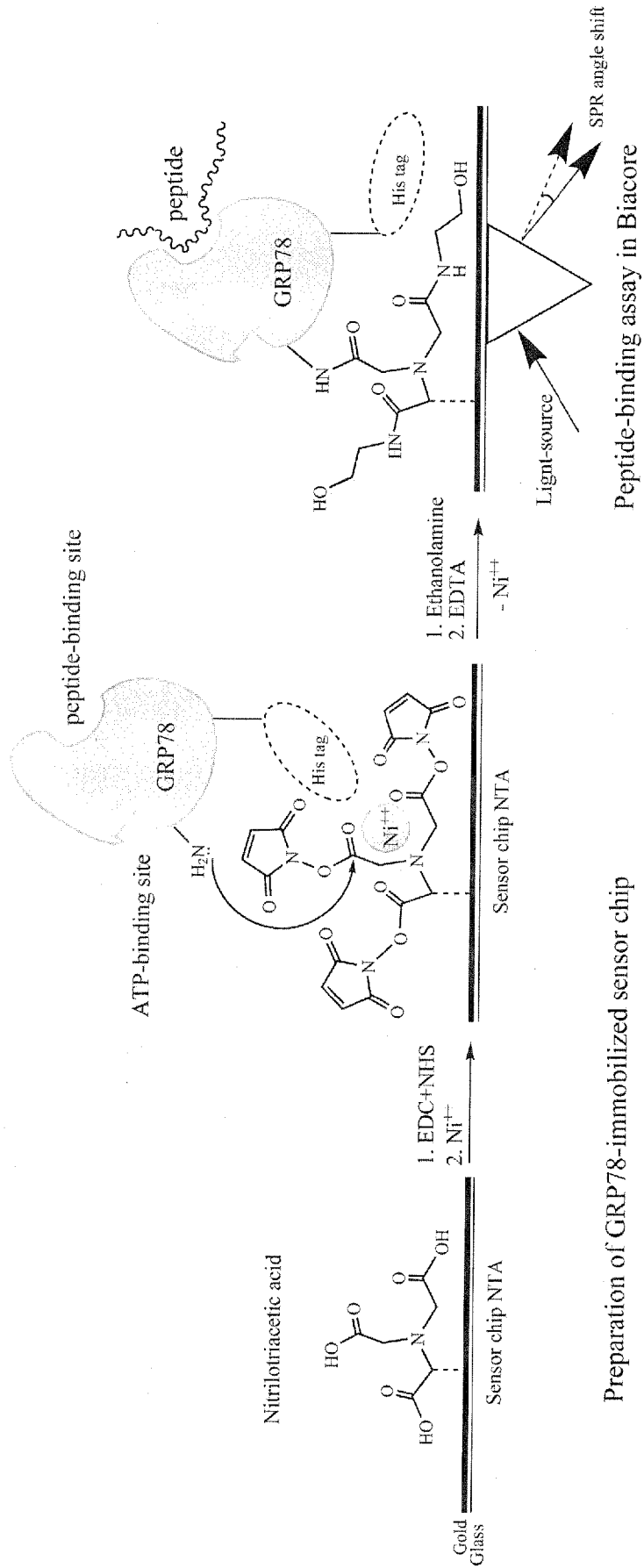
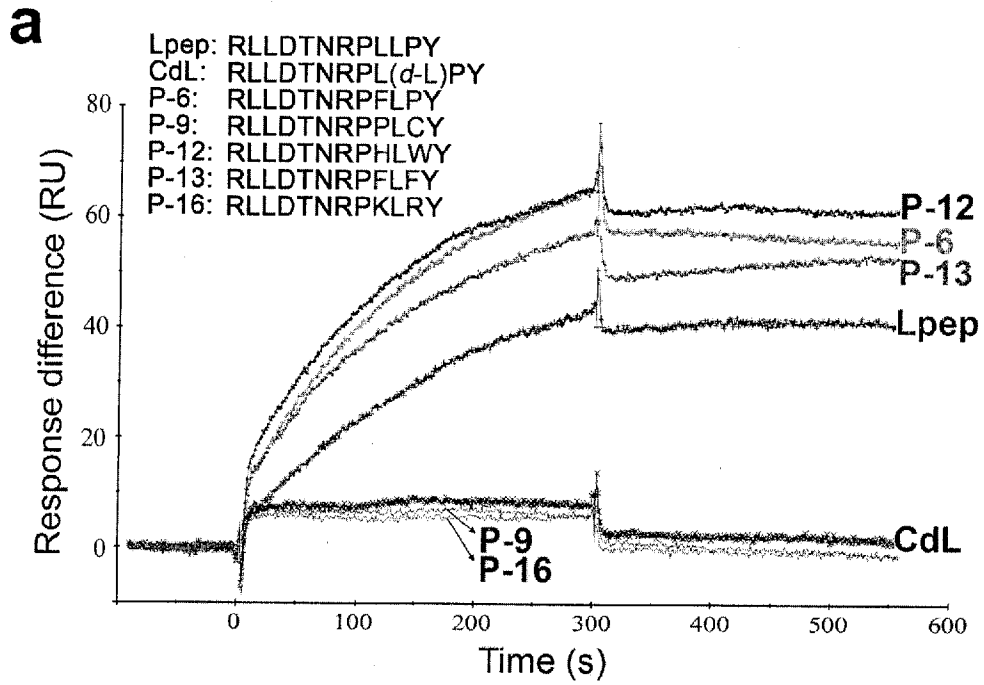


FIGURE 6



7/12

Figure 7



b

Cancer cell line

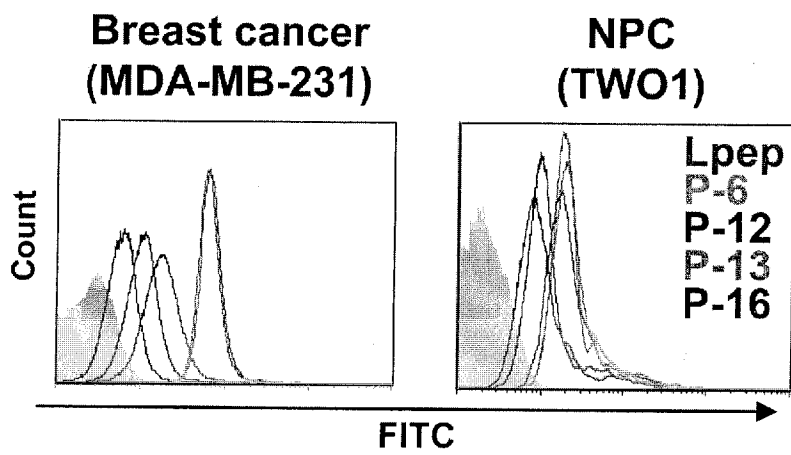


Figure 7 (Cont'd)

C

**Primary breast cancer
engrafted in NOD/SCID mice**

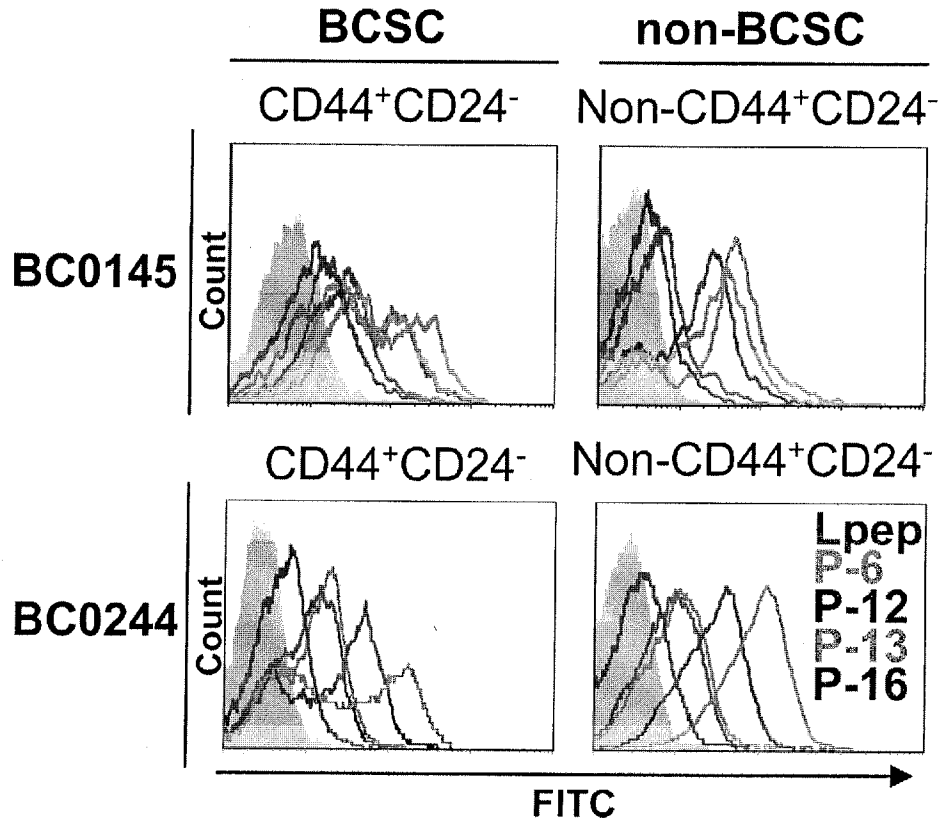


Figure 7 (Cont'd)

d

Clinical breast cancer

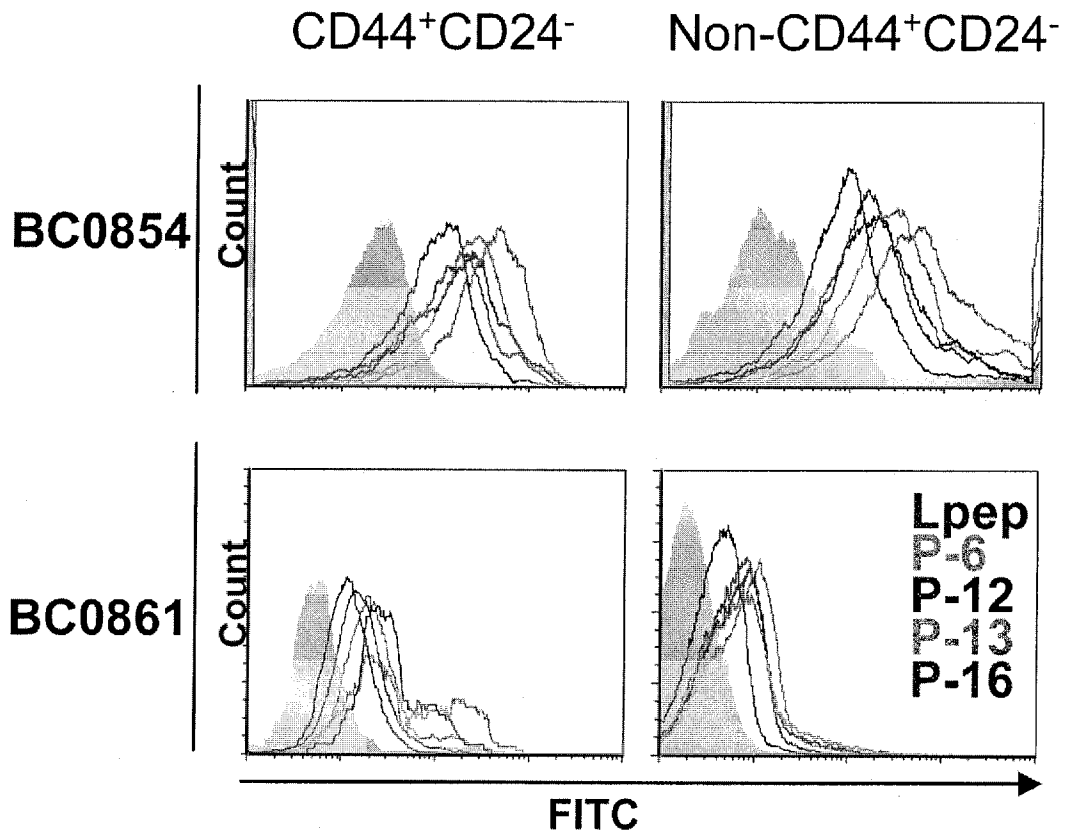


FIGURE 8

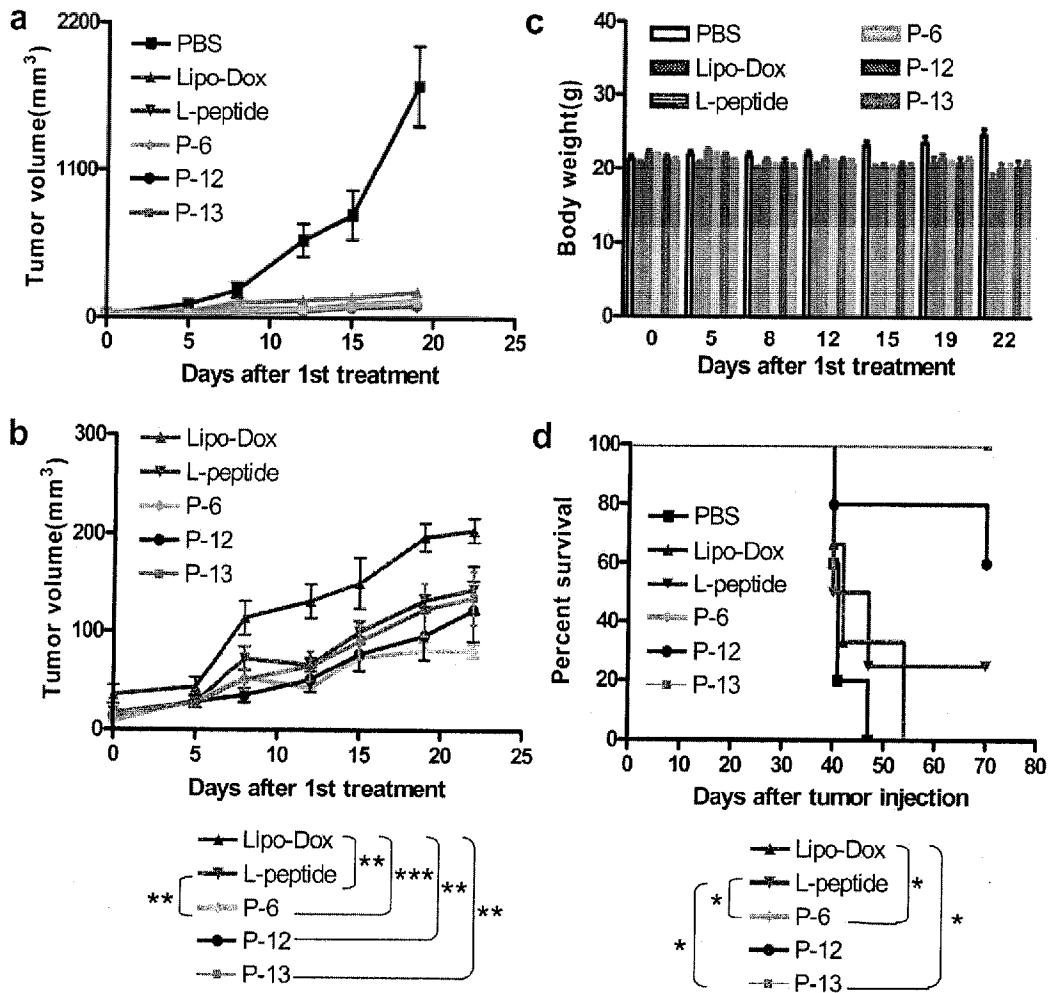


FIGURE 9

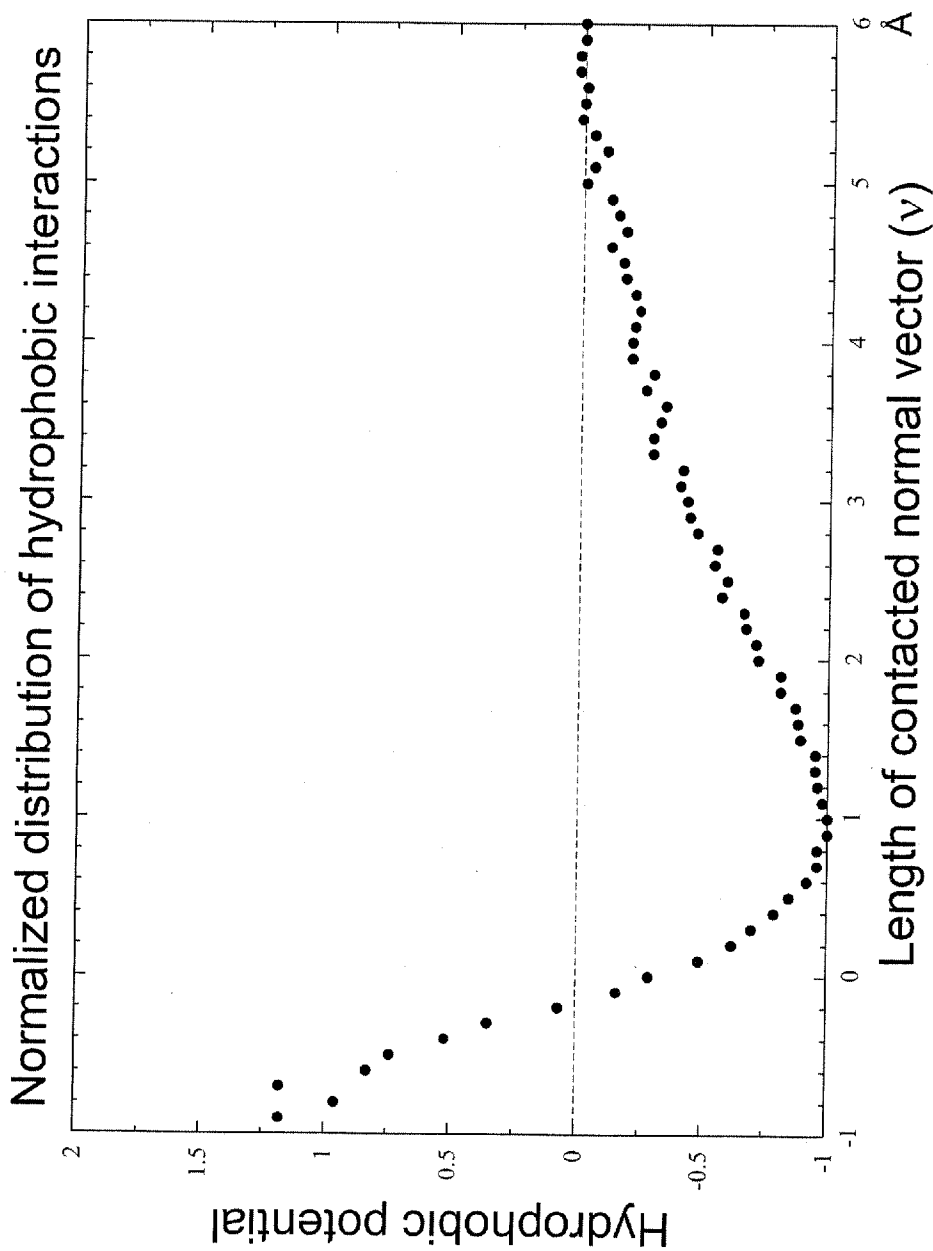


FIGURE 10

