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(57) Abstract: Disclosed herein are compositions and methods relating to detecting breast cancer. Further provided are methods and compositions for treating breast cancer.



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COMPOSITIONS AND METHODS FOR DETECTION AND TREATMENT OF BREAST CANCER

I. CROSS-REFERENCE TO RELATED APPLICATIONS

1. This application claims benefit of U.S. Provisional Application No. 61/234,163, filed August 14, 2009, which is hereby incorporated herein by reference in its entirety.

II. STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

2. This invention was made with government support under federal grant BC076376 awarded by the Department of Defense-Breast Cancer Research Program. The Government has certain rights to this invention.

III. REFERENCE TO SEQUENCE LISTING

3. The Sequence Listing submitted August 16, 2010 as a text file named "24716_12_9001_2010_08_16_MIC_AFD_Sequence_listing_text_file.txt," created on August 13, 2010, and having a size of 7,691 bytes is hereby incorporated by reference pursuant to 37 C.F.R. § 1.52(e)(5).

IV. Background

4. Breast cancer is the second leading cancer killer of women resulting in 40,000 deaths each year. There are approximately 180,000 new cases of breast cancer diagnosed each year. However, follow-up studies on breast cancer patients show a clear correlation between early detection and patient survival. It is estimated that 1 in 8 women have a lifetime risk of developing breast cancer. The age-specific probability of developing breast cancer increases considerably after age 40 (Jemal et al. CA Cancer J Clin 2008, 58:71096). For younger women, the disease can be especially devastating, affecting not only women, but also their husbands and their children.

5. The data strongly suggest that at its inception breast cancer is a progressive disease, rather than a systemic disease, and early diagnosis and treatment can have the most significant outcome on reducing patient mortality. Currently, high-quality mammography is the most effective method presently available for breast cancer screening. However, the caveats in mammography include the following: false-positive detection results in up to 85% of non-malignant lesions requiring invasive biopsies, and false-negative detection results in up to 20% of cancers being missed, particularly in younger women who have denser breast tissue that obstruct detection of the cancer. Furthermore, the technique of mammography involves taking an

X-ray of each breast. The radiation from X-ray imaging is considered harmful with a greater risk in younger women, and therefore mammography is usually only recommended for women over 40 years of age unless the younger patient has a significant risk for the cancer (Tabar et al. *Int J Gynaecol Obstet* 2003).

6. Magnetic resonance imaging (MRI) provides a solution to the limitations of mammography. MRI is virtually uninfluenced by breast density, a problem met in mammography of younger women. In comparing clinical breast examination, mammography, and MRI for detecting invasive breast cancer, the sensitivity was 18%, 33%, and 80%, respectively (Keana et al. *Magn Reson Med* 2005). However, MRI is not without its own limitations. The same study showed that specificity with MRI is lower than with mammography, meaning that the ability to distinguish between benign and cancerous images was lower for MRI. Lower specificity results in more findings judged as uncertain and requiring a follow-up exam.

7. Presented herein is a new detection strategy with the option of simultaneous drug delivery. The advantages of this system are as follows: 1. Direct imaging of intracellular chemical events can signify the presence of cancer to address the problems with false-positive and false-negative detection in mammography and MRI. 2. Fluorescence imaging can be employed, which uses harmless non-ionizing radiation as opposed to the X-rays used in mammography. 3. The imaging agents can be designed to work in tandem response to chemical environments in cancer cells, giving double confirmation of the presence of cancer. 4. An organic spin-label can be used as the T1-contrast enhancement agent in place of toxic transition metals normally found in MRI contrast agents. 5. The system can be tissue-specific allowing for directed distribution of the agent specifically to the breast. 6. A modular synthetic assembly of the system can allow for the option of attaching a therapeutic moiety to the detection system. 7. And, by design the entire system can be peptide-based so that the system is readily degraded in cancer cells, where peptidase activity is the highest, resulting in specific release of the therapeutic agent at the target.

8. A breast homing peptide that incorporates a fluorescent chromophore, a non-metallic MRI contrast agent, and a therapeutic component can act as a tissue-specific theranostic that can detect cancer by dual modality MRI fluorescence imaging, and treat the cancer by specific release of a therapeutic agent in the cancer cell.

V. Summary

9. In accordance with the purposes of this invention, as embodied and broadly described herein, this invention, in one aspect, relates to compositions and methods related to detecting and treating cancer, particularly breast cancer.

10. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

VI. Brief Description of the Figures

11. Figure 1 shows two routes to the fluorophore containing amino acids. Methods of attaching the fluorophore to an amino acid residue that can be employed in SPPS are shown. Method 1 is accomplished by treating the O-succinimide ester of the carboxyl containing fluorophore with Fmoc-Lys-OH. This gives selective functionalization on the side-chain amine group. Fmoc-protected amino acid (2a) has been synthesized by this method in an overall 51% yield. The same method is used to make Fmoc-protected amino acid (2b), which has an overlapping emission bandwidth with (2a) but has a much higher quantum yield.

12. A near IR (NIR) dye encapsulated in an α -cyclodextrin molecule. The synthesis of the encapsulated NIR dye is shown in equation 2 of Figure 1 following the protocol of Anderson and co-workers (Simon et al. Chem Commun 2008, 2897-2899).

13. Figure 2 outlines how commercially available nitroxide (7) is condensed with N-hydroxysuccinimide under DCC coupling conditions. Treatment with Fmoc-Lys-OH in the presence of Hünig's base gives the Fmoc-protected amino acid building block in 97% yield.

14. Figure 3 shows the attachment of the fluorophore and the spin label.

15. Figure 4 outlines how commercially available maytansinol (10) is conjugated to azido functionalized N-methylalanine (11) by DCC coupling to give compound (12). Carboxylic acid (11) is synthesized in one step from N-methylalanine and the hydroxysuccinimide ester of 2-azidoacetic acid.

16. Figure 5 shows the copper chelate of bleomycin A5, marketed as Bleocin, attachment to the delivery system. In the case of bleomycin A5-copper chelate, the primary amine is exposed and easily functionalized. Following the method of Xu and co-workers, bleomycin A5-copper chelate is treated with 2-azidoacetic acid, DCC, and HOBt to give the azido-conjugated compound. Treatment with 15% aqueous EDTA liberates the bleomycin conjugate from the copper (Kriege et al. NEJM 2004, 351, 427-437)

17. Figure 6 demonstrates a simple one-step reaction by click chemistry for addition of a theranostic agent to the fully functional diagnostic agent.

18. Figure 7 outlines the Solid Phase Peptide Synthesis (SPPS) of the complete diagnostic system.

19. Figure 8 outlines the incorporation of the drug molecule. The diagnostic system is treated with the azido-functionalized maytansinol in the presence of a copper sulfate and sodium ascorbate in a DMSO-water solvent system to give the completed theranostic agent. Synthesis of the complete theranostic agent.

20. Figure 9 identifies the structure of disclosed drug entities that can be attached to the diagnostic system as theranostic agents.

21. Figure 10 shows the azido functionalized dicarboxylic acid (13) condensed with the boronic acid under Dean-Stark conditions to give the boronic esters of YK-3-250 and YK-3-237 following the method of Burke and co-workers. These azido-functionalized drug agents are attached to the theranostic system via click chemistry. Boronic ester functionalization for click chemistry attachment.

22. Figure 11 shows the functionalization of the dansyl moiety of YK-4-272 and SCG-3-285 with mono-demethylation of the dimethylamine group followed by conjugation with 2-azidoacetic acid. Mono-demethylation of the tertiary amine can be accomplished using ACE-CI (Olofson et al. J Org Chem 1984, 49:2081-2082). Conjugation with O-succinimide ester of 2-azido-acetic acid gives the requisite azide for attachment to the theranostic system via click chemistry.

23. Figure 12 shows Compound MP-201 which is a lead structure optimized to give nitro-containing quinazolinone. Dihydroquinazolinone tubulin inhibitors.

24. Figure 13 shows the expression of ApaseP in cancer cells by immunohistostaining using an antibody against ApaseP. ApaseP is richly expressed on MCF-7 and MDA-MB-231 breast cancer cells. However, ApaseP expression was not detected on PC-3 prostate and A-549 lung cancer cells. A) Fluorescence ApaseP antibody shown as light gray areas. B) DIC. C) DAPI staining of the nuclei shown as light gray areas.

25. Figure 14 shows the delivery efficiency of the delivery system and the importance of the Tat sequence. The distribution of the homing peptide with or without the Tat cell penetrating sequence was observed in MCF7 breast cancer cells. The dansylated breast homing peptide that

incorporates the Tat cell-penetrating sequence efficiently penetrates MCF-7 breast cancer cells. However, the dansylated breast homing peptide without a Tat sequence shows poor penetration of MCF-7 breast cancer cells. Panel A shows the fluorescence of the peptide (light gray staining). Panel B shows the DIC image of the cells. Panel C shows a merge of A and B.

26. Figure 15 shows the homing peptide with and without Tat on another metastatic breast cancer cell line, MDA-MB-231. The dansylated PEGA breast homing peptide conjugated to the Tat sequence shows penetration into MDA-MB-231 cells. However, the dansylated PEGA breast homing peptide that does not incorporate the Tat sequence gave a considerably weaker signal under the same treatment and imaging conditions. Panel A shows the fluorescence of the peptide (bright color staining). Panel B shows the DIC image of the cells. Panel C shows a merge of A and B.

27. Figure 16 shows the homing peptide with and without Tat on the prostate cancer cell line, PC-3. Regardless of the presence or absence of Tat on the dansylated homing peptide, no cell penetration was exhibited. This shows the cell-type specificity of the homing peptide.

28. Figure 17 shows the synthesis of the homing peptide component.

29. Figure 18 shows the conjugation of the homing peptide and STAT3-Hel2A-2.

30. Figure 19 shows the immunohistostaining for ApaseP expression in cancer cells. A) fluorescence ApaseP antibody shown by bright staining. B) DIC. C) DAPI staining of the nuclei in blue.

31. Figure 20 shows images from confocal microscopy cell penetration of MCF7 and MDA-MB-231 cells. A) fluorescence of peptide shown by light gray staining. B) DIC. C) merge.

32. Figure 21 shows images from confocal microscopy cell penetration of A-549 cells. A) fluorescence of peptide shown by bright staining. B) DIC. C) merge.

33. Figure 22 shows live-imaging in MCF-7 xenograft model.

34. Figure 23 shows STAT3-Hel2A-2 peptide growth inhibition assay in breast cancer and non-cancerous breast cells.

35. Figure 24 shows FRET selectivity assay.

36. Figure 25 shows the inhibition of STAT3 transcriptional activity.

VII. Detailed Description

37. The present disclosed embodiment may be understood more readily by reference to the following detailed description of preferred embodiments of the disclosed methods and compositions and the Examples included therein and to the Figures and their previous and following description.

38. Before the present compounds, compositions, articles, devices, and/or methods are disclosed and described, it is to be understood that the disclosed methods and compositions are not limited to specific synthetic methods, specific recombinant biotechnology methods unless otherwise specified, or to particular reagents unless otherwise specified, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

A. Compositions and methods

1. Theranostics

39. The disclosed compositions and methods allow for the diagnosis and treatment of cancer (breast, lung, liver, prostate or intestinal) such that a theranostic approach can be taken. Thus, in addition to diagnosing or confirming the presence of cancer, the methods and compositions disclosed herein also provide a means of treating the a subject. The disclosed methods and compositions provide a theranostic approach to treating a disorder, such as cancer, by integrating diagnostics and therapeutics to improve the real-time treatment of a subject having, for example, breast cancer.

40. Theranostics are useful in clinical diagnosis and management of a variety of diseases and disorders, which include, but are not limited to, e.g., cardiovascular disease, cancer, infectious diseases, Alzheimer's Disease and the prediction of drug toxicity or drug resistance.

41. Disclosed herein are compositions valuable for the diagnosis (or confirmation) and treatment of a disease or disorder, such as breast cancer. The compositions comprise all or some of the following: a detection moiety, a contrasting agent, a homing moiety, a cell-penetrating moiety and a therapeutic agent.

42. In many instances, the components of theranostics can be considered toxic. In one embodiment of the disclosed composition, the composition is non-toxic.

a) 5 parts

(1) detection moiety

43. The disclosed compositions are comprised of a detection moiety and a contrasting agent. The detection moiety can be used for identification purposes. Disclosed herein, the detection moiety can be used to identify cancer present in breast tissue. The detection moiety, for example a fluorescent label, can specifically define a particular location of interest (i.e. breast cancer cells).

44. Any detection moiety known in the art or otherwise contemplated by a person having ordinary skill in the art for use with the presently disclosed and claimed invention is encompassed by the scope of the presently disclosed and claimed invention. Particular non-limiting examples of detection moieties that may be utilized in accordance with the presently disclosed and claimed invention have been described in detail herein above.

45. In one embodiment, the disclosed composition comprises a detection moiety wherein the detection moiety can be a fluorophore.

46. In one embodiment, the disclosed composition comprises a fluorophore wherein the fluorophore can be Dansyl, TAMRA, a cyanine dye, or a cyanine dye encapsulated in a cyclodextrin.

(2) contrasting agent

47. In order to achieve effective contrast between magnetic resonance (MR) images of different tissue types, it has long been known to administer to the subject contrast agents which affect relaxation times in the zones in which they are administered or at which they congregate. By shortening the relaxation times of the imaging nuclei (the nuclei whose MR signal is used to generate the image) the strength of the MR signal is changed and image contrast is enhanced.

48. The above disclosed composition comprises a contrasting agent wherein the contrasting agent can be an MRI contrasting agent.

49. In one embodiment the composition comprises a MRI contrast agent wherein the contrasting agent can be non-metallic.

50. In one embodiment the disclosed composition comprises a non-metallic contrast agent wherein the contrasting agent can be nitroxide radical or derivative thereof.

(3) homing moiety

51. The disclosed compositions can be used for targeting or homing to specific tissues or cell types based on the presence of a homing moiety. The homing moiety targets a specific ligand or environment and therefore can be used to transport different molecules or compositions

to that location. For example, a homing moiety, specific to breast tissue, can be linked to a contrasting agent and thus transport the contrasting agent to the targeted breast tissue.

52. The disclosed composition comprises a detection moiety and a contrasting agent and can further comprise a homing peptide.

53. In one embodiment, the above disclosed composition comprises a breast tissue-specific homing peptide.

54. In one embodiment, the disclosed breast tissue-specific homing peptide can be CPGPEGAGC.

(4) cell-penetrating molecule and cell penetrating moiety

55. A cell-penetrating molecule is a molecule that can comprise a cell-penetrating moiety. A moiety refers to a part of a molecule. Examples of cell-penetrating molecules are Tat, penetratin, pVEC, transportan etc. A cell penetrating moiety is a part of a molecule or functional group that is responsible for allowing the molecule containing moiety to penetrate the cell. A cell-penetrating molecule is capable of penetrating the cell unlike most homing moieties that target a cell-surface molecule but can not enter the cell. The presence of a cell-penetrating molecule allows access in to the cell. Thus, linking a composition to a cell-penetrating molecule can ensure intracellular delivery of the composition.

56. The disclosed composition can comprise a cell-penetrating molecule.

57. In one embodiment, the cell-penetrating molecule is Tat.

58. In one embodiment, the disclosed composition comprises a homing peptide that can localize the composition to the target tissue, such as breast tissue, wherein the cell-penetrating molecule transports the composition into the cells of the tissue.

(5) therapeutic agent.

59. The therapeutic agent of the present compositions comprises one or more therapeutic agents, such as chemical compounds, macromolecules, proteins, and the like, which are effective in treating diseases and disorders, such as a cancer.

60. The therapeutic agent is present in an amount effective in providing a desired therapeutic effect to an individual, such as a human or animal patient, when the composition is administered to the individual.

61. Therapeutic agents provided in the therapeutic component of the present compositions can be obtained from public sources or can be synthesized using routine chemical

procedures known to persons of ordinary skill in the art. Thus, the therapeutic agent of the present compositions can comprise a variety of therapeutic agents, including chemotherapeutic agents, anti-inflammatory agents, anti-proliferative agents, and the like.

62. The disclosed composition comprises a therapeutic agent.

63. In one embodiment, the therapeutic agent can be an anti-cancer agent.

64. In one embodiment, the anti-cancer agent can be bleomycin.

65. In one embodiment, the anti-cancer agent can be a STAT3 inhibitor. The STAT3 inhibitor can be STAT3-Hel2A-2.

2. Homing Peptides

66. Disclosed herein are peptides that target, bind to and/or home to normal tissue, such as normal breast tissue. For example, the cyclic nonapeptide CPGPEGAGC (SEQ ID NO:1) specifically homes to breast tissue and the peptide as well as peptide conjugates are known to target aminopeptidase P.

67. Disclosed herein are peptides that target, bind to and/or home to breast tissue, normal and cancerous.

68. Disclosed herein are peptides that target lung (CGFECVRQCPCRC; SEQ ID NO:3), breast (CDCRGDCFC; SEQ ID NO:4), prostate (CGRRAGGSC; SEQ ID NO:5), pancreas (SWCEPGWCR; SEQ ID NO:6 or CRVASVLPC; SEQ ID NO:7) or intestine (YSGKKGW; SEQ ID NO:8).

69. Also disclosed herein are homing peptides that each have specific homing properties. Homing peptides are disclosed in US patent applications 11/670318, 11/967509, 11/951819, 10/158566, 09/910582, 12/322371, 09/765086, 11/979624 and 11/777382 and are specifically incorporated herein by reference at least for homing peptide sequences and structures.

70. The disclosed peptide can have any suitable length. For example, the peptide can have a length of up to 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acids. The disclosed polypeptides can be, for example, 4 to about 50 amino acids in length. The disclosed polypeptides can be, for example, less than about 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, or 4 amino acids in length.

71. For example, the disclosed peptide can have a length of from 4 to about 10 amino acids, from 4 to about 15 amino acids, from 4 to about 20 amino acids, from 4 to about 25 amino

acids, from 4 to about 30 amino acids, from 4 to about 35 amino acids, from 4 to about 40 amino acids, from 4 to about 45 amino acids, from 4 to about 50 amino acids. For example, the disclosed peptide can have a length of from 5 to about 10 amino acids, from 5 to about 15 amino acids, from 5 to about 20 amino acids, from 5 to about 25 amino acids, from 5 to about 30 amino acids, from 5 to about 35 amino acids, from 5 to about 40 amino acids, from 5 to about 45 amino acids, from 5 to about 50 amino acids. For example, the disclosed peptide can have a length of from 6 to about 10 amino acids, from 6 to about 15 amino acids, from 6 to about 20 amino acids, from 6 to about 25 amino acids, from 6 to about 30 amino acids, from 6 to about 35 amino acids, from 6 to about 40 amino acids, from 6 to about 45 amino acids, from 6 to about 50 amino acids. For example, the disclosed peptide can have a length of from 7 to about 10 amino acids, from 7 to about 15 amino acids, from 7 to about 20 amino acids, from 7 to about 25 amino acids, from 7 to about 30 amino acids, from 7 to about 35 amino acids, from 7 to about 40 amino acids, from 7 to about 45 amino acids, from 7 to about 50 amino acids. For example, the disclosed peptide can have a length of from 8 to about 10 amino acids, from 8 to about 15 amino acids, from 8 to about 20 amino acids, from 8 to about 25 amino acids, from 8 to about 30 amino acids, from 8 to about 35 amino acids, from 8 to about 40 amino acids, from 8 to about 45 amino acids, from 8 to about 50 amino acids. For example, the disclosed peptide can have a length of from 9 to about 10 amino acids, from 9 to about 15 amino acids, from 9 to about 20 amino acids, from 9 to about 25 amino acids, from 9 to about 30 amino acids, from 9 to about 35 amino acids, from 9 to about 40 amino acids, from 9 to about 45 amino acids, from 9 to about 50 amino acids.

72. The disclosed peptides can be artificial sequences and can be synthesized in vitro and/or recombinantly. The disclosed polypeptides can be peptides that are not naturally occurring proteins and can be peptides that have at least two contiguous sequences that are not contiguous in a naturally occurring protein.

73. The disclosed peptides and compositions also can comprise any combination of two, three, or more of the disclosed peptides or amino acid sequences. Thus, disclosed are peptides comprising any one, two, three, or more of the herein disclosed peptides or amino acid sequences. The peptides can be combined in any suitable manner, including, for example, as a single amino acid chain (that is a fusion of the peptides), via linkers, via branched linkers, and attached individually or together to a structure. Also disclosed are bifunctional peptides, which contain one or more of the disclosed peptides fused to one or more second peptides having one

or more separate functions. Such bifunctional peptides can have at least two functions conferred by different portions of the full-length molecule and can, for example, display pro-apoptotic activity in addition to the ability to target the tumor lymphatic.

74. Also disclosed are multivalent peptides that can include at least two of the disclosed peptides each independently containing one or more of the disclosed amino acid sequences. The multivalent peptide can have, for example, at least three, at least five or at least ten of such peptides each independently containing a disclosed amino acid sequence. In some aspects, the multivalent peptide can have two, three, four, five, six, seven, eight, nine, ten, fifteen or twenty identical or non-identical peptides and/or amino acid sequences. In some aspects, the multivalent peptide can contain identical peptides and/or amino acid sequences. In some aspects, the multivalent peptide can contain contiguous identical or non-identical peptides and/or amino acid sequences, which are or are not separated by any intervening amino acids.

3. Antibodies

75. The term “antibodies” is used herein in a broad sense and includes both polyclonal and monoclonal antibodies. In addition to intact immunoglobulin molecules, also included in the term “antibodies” are fragments or polymers of those immunoglobulin molecules, and human or humanized versions of immunoglobulin molecules or fragments thereof, as described herein. The antibodies are tested for their desired activity using the *in vitro* assays described herein, or by analogous methods, after which their *in vivo* therapeutic and/or prophylactic activities are tested according to known clinical testing methods.

76. As used herein, the term “antibody” encompasses, but is not limited to, whole immunoglobulin (i.e., an intact antibody) of any class. Native antibodies are usually heterotetrameric glycoproteins, composed of two identical light (L) chains and two identical heavy (H) chains. Typically, each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V (H)) followed by a number of constant domains. Each light chain has a variable domain at one end (V (L)) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an

interface between the light and heavy chain variable domains. The light chains of antibodies from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains. Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of human immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2. One skilled in the art would recognize the comparable classes for mouse. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively.

77. The term "variable" is used herein to describe certain portions of the variable domains that differ in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not usually evenly distributed through the variable domains of antibodies. It is typically concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of the variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β -sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of antibodies (see Kabat E. A. et al., "Sequences of Proteins of Immunological Interest," National Institutes of Health, Bethesda, Md. (1987)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

78. As used herein, the term "antibody or fragments thereof" encompasses chimeric antibodies and hybrid antibodies, with dual or multiple antigen or epitope specificities, and fragments, such as scFv, sFv, F (ab')₂, Fab', Fab and the like, including hybrid fragments. Thus, fragments of the antibodies that retain the ability to bind their specific antigens are provided. Such antibodies and fragments can be made by techniques known in the art and can be screened for specificity and activity according to the methods set forth in the Examples and in general methods for producing antibodies and screening antibodies for specificity and activity (See

Harlow and Lane. *Antibodies, A Laboratory Manual*. Cold Spring Harbor Publications, New York, (1988)).

79. Also included within the meaning of "antibody or fragments thereof" are conjugates of antibody fragments and antigen binding proteins (single chain antibodies) as described, for example, in U.S. Pat. No. 4,704,692, the contents of which are hereby incorporated by reference.

80. The fragments, whether attached to other sequences or not, can also include insertions, deletions, substitutions, or other selected modifications of particular regions or specific amino acids residues, provided the activity of the antibody or antibody fragment is not significantly altered or impaired compared to the non-modified antibody or antibody fragment. These modifications can provide for some additional property, such as to remove/add amino acids capable of disulfide bonding, to increase its bio-longevity, to alter its secretory characteristics, etc. In any case, the antibody or antibody fragment must possess a bioactive property, such as specific binding to its cognate antigen. Functional or active regions of the antibody or antibody fragment may be identified by mutagenesis of a specific region of the protein, followed by expression and testing of the expressed polypeptide. Such methods are readily apparent to a skilled practitioner in the art and can include site-specific mutagenesis of the nucleic acid encoding the antibody or antibody fragment. (Zoller, M.J. *Curr. Opin. Biotechnol.* 3:348-354, 1992).

81. As used herein, the term "antibody" or "antibodies" can also refer to a human antibody and/or a humanized antibody. Many non-human antibodies (e.g., those derived from mice, rats, or rabbits) are naturally antigenic in humans, and thus can give rise to undesirable immune responses when administered to humans. Therefore, the use of human or humanized antibodies in the disclosed methods serves to lessen the chance that an antibody administered to a human can evoke an undesirable immune response.

(a) Human antibodies

82. The human antibodies of the disclosed methods and compositions can be prepared using any technique. Examples of techniques for human monoclonal antibody production include those described by Cole et al. (*Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77, 1985) and by Boerner et al. (*J. Immunol.*, 147 (1):86-95, 1991). Human antibodies of the disclosed methods and compositions (and fragments thereof) can also be produced using

phage display libraries (Hoogenboom et al., *J. Mol. Biol.*, 227:381, 1991; Marks et al., *J. Mol. Biol.*, 222:581, 1991).

83. The human antibodies of the disclosed methods and compositions can also be obtained from transgenic animals. For example, transgenic, mutant mice that are capable of producing a full repertoire of human antibodies, in response to immunization, have been described (see, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551-255 (1993); Jakobovits et al., *Nature*, 362:255-258 (1993); Bruggermann et al., *Year in Immunol.*, 7:33 (1993)). Specifically, the homozygous deletion of the antibody heavy chain joining region (*J(H)*) gene in these chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production, and the successful transfer of the human germ-line antibody gene array into such germ-line mutant mice results in the production of human antibodies upon antigen challenge. Antibodies having the desired activity are selected using Env-CD4-co-receptor complexes as described herein.

(b) Humanized antibodies

84. Optionally, the antibodies are generated in other species and "humanized" for administration in humans. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as scFv, sFv, Fv, Fab, Fab', F(ab')₂, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody can comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also can comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human

immunoglobulin (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992)).

85. Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source that is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeven et al., *Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

86. The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993) and Chothia et al., *J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immunol.*, 151:2623 (1993)).

87. It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate

immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen (s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding (see, WO 94/04679, published 3 March 1994). Also, disclosed are chimeric antibodies as well as fully human antibodies, such as monoclonal antibodies.

(c) Monoclonal Antibodies

88. The term monoclonal antibody as used herein refers to an antibody obtained from a substantially homogeneous population of antibodies, i.e., the individual antibodies within the population are identical except for possible naturally occurring mutations that may be present in a small subset of the antibody molecules. The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain (s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, as long as they exhibit the desired antagonistic activity (See, U.S. Pat. No. 4,816,567 and Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)).

89. Monoclonal antibodies of the disclosed methods and compositions can be prepared using hybridoma methods, such as those described by Kohler and Milstein, *Nature*, 256:495 (1975). In a hybridoma method, a mouse or other appropriate host animal is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that can specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*, e.g., using the complexes described herein.

90. Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (J (H)) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line

immunoglobulin gene array in such germ-line mutant mice can result in the production of human antibodies upon antigen challenge (see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggemann et al., Year in Immuno., 7:33 (1993)). Human antibodies can also be produced in phage display libraries (Hoogenboom et al., J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)). The techniques of Cote et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); Boerner et al., J. Immunol., 147 (1):86-95 (1991)).

91. Generally, either peripheral blood lymphocytes ("PBLs") are used in methods of producing monoclonal antibodies if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, "Monoclonal Antibodies: Principles and Practice" Academic Press, (1986) pp. 59-103). Immortalized cell lines are usually transformed mammalian cells, including myeloma cells of rodent, bovine, equine, and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically can include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells. Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, Calif. and the American Type Culture Collection, Rockville, Md. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., "Monoclonal Antibody Production Techniques and Applications" Marcel Dekker, Inc., New York, (1987) pp. 51-63). The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against tissue-specific antigens, for example. Preferably, the binding specificity of

monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art, and are described further in the Examples below or in Harlow and Lane "Antibodies, A Laboratory Manual" Cold Spring Harbor Publications, New York, (1988).

92. After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution or FACS sorting procedures and grown by standard methods. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

93. The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, protein G, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

94. The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Pat. No. 4,816,567 (Cabilly et al.). DNA encoding the monoclonal antibodies of the disclosed methods and compositions can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). Libraries of antibodies or active antibody fragments can also be generated and screened using phage display techniques, e.g., as described in U.S. Patent No. 5,804,440 to Burton et al. and U.S. Patent No. 6,096,441 to Barbas et al.

95. *In vitro* methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348 published Dec. 22, 1994 and U.S. Pat. No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields a fragment that has two antigen combining sites and is still capable of cross-linking antigen.

(d) Antibody fragments

96. Also disclosed are fragments of antibodies which have bioactivity. The polypeptide fragments of the disclosed composition can be recombinant proteins obtained by cloning nucleic acids encoding the polypeptide in an expression system capable of producing the polypeptide fragments thereof, such as an adenovirus or baculovirus expression system. For example, one can determine the active domain of an antibody from a specific hybridoma that can cause a biological effect associated with the interaction of the antibody with the antigen. For example, amino acids found to not contribute to either the activity or the binding specificity or affinity of the antibody can be deleted without a loss in the respective activity. For example, in various embodiments, amino or carboxy-terminal amino acids are sequentially removed from either the native or the modified non-immunoglobulin molecule or the immunoglobulin molecule and the respective activity assayed in one of many available assays. In another example, a fragment of an antibody comprises a modified antibody wherein at least one amino acid has been substituted for the naturally occurring amino acid at a specific position, and a portion of either amino terminal or carboxy terminal amino acids, or even an internal region of the antibody, has been replaced with a polypeptide fragment or other moiety, such as biotin, which can facilitate in the purification of the modified antibody. For example, a modified antibody can be fused to a maltose binding protein, through either peptide chemistry or cloning the respective nucleic acids encoding the two polypeptide fragments into an expression vector such that the expression of the coding region results in a hybrid polypeptide. The hybrid polypeptide can be affinity purified by passing it over an amylose affinity column, and the modified antibody receptor can then be separated from the maltose binding region by cleaving the hybrid polypeptide with the specific protease factor Xa. (See, for example, New England Biolabs Product Catalog, 1996, pg. 164.). Similar purification procedures are available for isolating hybrid proteins from eukaryotic cells as well.

97. The fragments, whether attached to other sequences or not, include insertions, deletions, substitutions, or other selected modifications of particular regions or specific amino acids residues, provided the activity of the fragment is not significantly altered or impaired compared to the nonmodified antibody or antibody fragment. These modifications can provide for some additional property, such as to remove or add amino acids capable of disulfide bonding, to increase its bio-longevity, to alter its secretory characteristics, etc. In any case, the fragment

must possess a bioactive property, such as binding activity, regulation of binding at the binding domain, etc. Functional or active regions of the antibody may be identified by mutagenesis of a specific region of the protein, followed by expression and testing of the expressed polypeptide. Such methods are readily apparent to a skilled practitioner in the art and can include site-specific mutagenesis of the nucleic acid encoding the antigen. (Zoller MJ et al. Nucl. Acids Res. 10:6487-500 (1982).

98. A variety of immunoassay formats may be used to select antibodies that selectively bind with a particular protein, variant, or fragment. For example, solid-phase ELISA immunoassays are routinely used to select antibodies selectively immunoreactive with a protein, protein variant, or fragment thereof. See Harlow and Lane. *Antibodies, A Laboratory Manual*. Cold Spring Harbor Publications, New York, (1988), for a description of immunoassay formats and conditions that could be used to determine selective binding. The binding affinity of a monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.*, 107:220 (1980).

(e) Administration of antibodies

99. Antibodies of the disclosed compositions are preferably administered to a subject in a pharmaceutically acceptable carrier. Suitable carriers and their formulations are described in *Remington: The Science and Practice of Pharmacy* (19th ed.) ed. A.R. Gennaro, Mack Publishing Company, Easton, PA 1995. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the pharmaceutically-acceptable carrier include, but are not limited to, saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of antibody being administered.

100. The antibodies can be administered to the subject, patient, or cell by injection (e.g., intravenous, intraperitoneal, subcutaneous, intramuscular), or by other methods such as

infusion that ensure its delivery to the bloodstream in an effective form. Local or intravenous injection is preferred.

101. Effective dosages and schedules for administering the antibodies may be determined empirically, and making such determinations is within the skill in the art. Those skilled in the art will understand that the dosage of antibodies that must be administered will vary depending on, for example, the subject that will receive the antibody, the route of administration, the particular type of antibody used and other drugs being administered. Guidance in selecting appropriate doses for antibodies is found in the literature on therapeutic uses of antibodies, e.g., Handbook of Monoclonal Antibodies, Ferrone et al., eds., Noyes Publications, Park Ridge, N.J., (1985) ch. 22 and pp. 303-357; Smith et al., Antibodies in Human Diagnosis and Therapy, Haber et al., eds., Raven Press, New York (1977) pp. 365-389. A typical daily dosage of the antibody used alone might range from about 1 $\mu\text{g/kg}$ to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above.

4. Compositions identified by screening with disclosed compositions / combinatorial chemistry

(1) Combinatorial chemistry

102. The disclosed compositions can be used as targets for any combinatorial technique to identify molecules or macromolecular molecules that interact with the disclosed compositions in a desired way. The nucleic acids, peptides, and related molecules disclosed herein can be used as targets for the combinatorial approaches. Also disclosed are the compositions that are identified through combinatorial techniques or screening techniques in which the compositions have the sequences disclosed herein, or portions thereof, are used as the target in a combinatorial or screening protocol.

103. It is understood that when using the disclosed compositions in combinatorial techniques or screening methods, molecules, such as macromolecular molecules, can be identified that have particular desired properties such as inhibition or stimulation or the target molecule's function. The molecules identified and isolated when using the disclosed compositions are also disclosed. Thus, the products produced using the combinatorial or screening approaches that involve the disclosed compositions are also considered herein disclosed.

5. Conjugate

104. Also provided herein is a conjugate comprising any one or more of the herein disclosed peptides and one or more moieties. In general, the moiety can be a substance that acts upon the target cell(s) or tissue to bring about a desired effect. In some aspects, the disclosed conjugate can target, bind to and/or home to breast, lung, liver, pancreas or intestine.

105. Tumor lymphatics, such as lymphatic vessels in and around tumors, and/or lymphangiogenic vessels. The disclosed peptides preferably selectively bind to tumor lymphatics. Thus, the effect can, for example, be the labeling, activating, repressing, or killing of the target cell(s) or tissue.

106. The moiety can be, for example, a therapeutic moiety or a detectable moiety, a cytotoxic agent, an anti-lymphangiogenic agent, a cancer chemotherapeutic agent, a pro-apoptotic polypeptide, a grafted polypeptide, a virus, a cell, or a liposome. Thus, the moiety can be a small molecule, pharmaceutical drug, toxin, fatty acid, detectable marker, conjugating tag, nanoparticle, or enzyme. For example, the moiety of the disclosed conjugate can be an anti-cancer agent, such as bleomycin, or pro-apoptotic peptide. Examples of pro-apoptotic peptides are tumor necrosis factor (Curnis et al., Cancer Res. 64, 565-71, 2004) and tachyplesin (Chen et al., Cancer res. 61, 2434-8, 2001). Many other anti-cancer agents and pro-apoptotic peptides and compounds are known and can be used with and in the disclosed compositions, conjugates and methods.

107. Examples of small molecules and pharmaceutical drugs that can be conjugated to a peptide are known in the art. The moiety can be a cytotoxic small molecule or drug that kills the target cell. The small molecule or drug can be designed to act on any critical cellular function or pathway. For example, the small molecule or drug can inhibit the cell cycle, activate protein degradation, induce apoptosis, modulate kinase activity, or modify cytoskeletal proteins. Any known or newly discovered cytotoxic small molecule or drugs is contemplated for use with the peptides.

108. The moiety can be a toxin that kills the targeted cell. Non-limiting examples of toxins include abrin, modeccin, ricin and diphtheria toxin. Other known or newly discovered toxins are contemplated for use with the provided conjugates.

109. Fatty acids (i.e., lipids) that can be conjugated to the provided conjugates include those that allow the efficient incorporation of the peptide into liposomes. Generally, the fatty acid is a polar lipid. Thus, the fatty acid can be a phospholipid. The provided conjugates can

comprise either natural or synthetic phospholipid. The phospholipids can be selected from phospholipids containing saturated or unsaturated mono or disubstituted fatty acids and combinations thereof. These phospholipids can be dioleoylphosphatidylcholine, dioleoylphosphatidylserine, dioleoylphosphatidylethanolamine, dioleoylphosphatidylglycerol, dioleoylphosphatidic acid, palmitoyloleoylphosphatidylcholine, palmitoyloleoylphosphatidylserine, palmitoyloleoylphosphatidylethanolamine, palmitoyloleoylphosphatidylglycerol, palmitoyloleoylphosphatidic acid, palmitelaidoyloleoylphosphatidylcholine, palmitelaidoyloleoylphosphatidylserine, palmitelaidoyloleoylphosphatidylethanolamine, palmitelaidoyloleoylphosphatidylglycerol, palmitelaidoyloleoylphosphatidic acid, myristoleoyloleoylphosphatidylcholine, myristoleoyloleoylphosphatidylserine, myristoleoyloleoylphosphatidylethanolamine, myristoleoyloleoylphosphatidylglycerol, myristoleoyloleoylphosphatidic acid, dilinoleoylphosphatidylcholine, dilinoleoylphosphatidylserine, dilinoleoylphosphatidylethanolamine, dilinoleoylphosphatidylglycerol, dilinoleoylphosphatidic acid, palmiticlinoleoylphosphatidylcholine, palmiticlinoleoylphosphatidylserine, palmiticlinoleoylphosphatidylethanolamine, palmiticlinoleoylphosphatidylglycerol, palmiticlinoleoylphosphatidic acid. These phospholipids may also be the monoacylated derivatives of phosphatidylcholine (lysophosphatidylcholine), phosphatidylserine (lysophosphatidylserine), phosphatidylethanolamine (lysophosphatidylethanolamine), phosphatidylglycerol (lysophosphatidylglycerol) and phosphatidic acid (lysophosphatidic acid). The monoacyl chain in these lysophosphatidyl derivatives may be palmitoyl, oleoyl, palmitoleoyl, linoleoyl, myristoyl or myristoleoyl. The phospholipids can also be synthetic. Synthetic phospholipids are readily available commercially from various sources, such as AVANTI Polar Lipids (Albaster, Ala.); Sigma Chemical Company (St. Louis, Mo.). These synthetic compounds may be varied and may have variations in their fatty acid side chains not found in naturally occurring phospholipids. The fatty acid can have unsaturated fatty acid side chains with C14, C16, C18 or C20 chains length in either or both the PS or PC. Synthetic phospholipids can have dioleoyl (18:1)-PS; palmitoyl (16:0)-oleoyl (18:1)-PS, dimyristoyl (14:0)-PS; dipalmitoleoyl (16:1)-PC, dipalmitoyl (16:0)-PC, dioleoyl (18:1)-PC, palmitoyl (16:0)-oleoyl (18:1)-PC, and myristoyl (14:0)-oleoyl (18:1)-PC as constituents. Thus, as an example, the provided conjugates can comprise palmitoyl 16:0.

110. The moiety of the disclosed conjugate can be a detection moiety. Detectable moieties/ markers include any substance that can be used to label or stain a target tissue or cell(s). Non-limiting examples of detectable markers include radioactive isotopes, enzymes, fluorophores, and quantum dots (Qdot®). For example, the detection moiety can be an enzyme, biotin, metal, or epitope tag. Other known or newly discovered detectable markers are contemplated for use with the provided conjugates.

111. Fluorophores are compounds or molecules that luminesce. Typically fluorophores absorb electromagnetic energy at one wavelength and emit electromagnetic energy at a second wavelength. Representative fluorophores include, but are not limited to, 1,5 IAEDANS; 1,8-ANS; 4- Methylumbelliferone; 5-carboxy-2,7-dichlorofluorescein; 5-Carboxyfluorescein (5-FAM); 5-Carboxynaphthofluorescein; 5-Carboxytetramethylrhodamine (5-TAMRA); 5-Hydroxy Tryptamine (5-HAT); 5-ROX (carboxy-X-rhodamine); 6-Carboxyrhodamine 6G; 6-CR 6G; 6-JOE; 7-Amino-4-methylcoumarin; 7-Aminoactinomycin D (7-AAD); 7-Hydroxy-4- I methylcoumarin; 9-Amino-6-chloro-2-methoxyacridine (ACMA); ABQ; Acid Fuchsin; Acridine Orange; Acridine Red; Acridine Yellow; Acriflavin; Acriflavin Fcugen SITSA; Aequorin (Photoprotein); AFPs - AutoFluorescent Protein - (Quantum Biotechnologies) see sgGFP, sgBFP; Alexa Fluor 350™; Alexa Fluor 430™; Alexa Fluor 488™; Alexa Fluor 532™; Alexa Fluor 546™; Alexa Fluor 568™; Alexa Fluor 594™; Alexa Fluor 633™; Alexa Fluor 647™; Alexa Fluor 660™; Alexa Fluor 680™; Alizarin Complexon; Alizarin Red; Allophycocyanin (APC); AMC, AMCA-S; Aminomethylcoumarin (AMCA); AMCA-X; Aminoactinomycin D; Aminocoumarin; Anilin Blue; Anthrocyll stearate; APC-Cy7; APTRA-BTC; APTS; Astrazon Brilliant Red 4G; Astrazon Orange R; Astrazon Red 6B; Astrazon Yellow 7 GLL; Atabrine; ATTO- TAG™ CBQCA; ATTO-TAG™ FQ; Auramine; Aurophosphine G; Aurophosphine; BAO 9 (Bisaminophenyloxadiazole); BCECF (high pH); BCECF (low pH); Berberine Sulphate; Beta Lactamase; BFP blue shifted GFP (Y66H); Blue Fluorescent Protein; BFP/GFP FRET; Bimane; Bisbenzamide; Bisbenzimidazole (Hoechst); bis-BTC; Blancophor FFG; Blancophor SV; BOBO™ -1; BOBO™-3; Bodipy492/515; Bodipy493/503; Bodipy500/510; Bodipy; 505/515; Bodipy 530/550; Bodipy 542/563; Bodipy 558/568; Bodipy 564/570; Bodipy 576/589; Bodipy 581/591; Bodipy 630/650-X; Bodipy 650/665-X; Bodipy 665/676; Bodipy FI; Bodipy FL ATP; Bodipy FI-Ceramide; Bodipy R6G SE; Bodipy TMR; Bodipy TMR-X conjugate; Bodipy TMR-X, SE; Bodipy TR; Bodipy TR ATP;

Bodipy TR-X SE; BO-PRO™ -1; BO-PRO™ -3; Brilliant Sulphoflavin FF; BTC; BTC-5N;
 Calcein; Calcein Blue; Calcium Crimson - ; Calcium Green; Calcium Green-1 Ca²⁺ Dye;
 Calcium Green-2 Ca²⁺; Calcium Green-5N Ca²⁺; Calcium Green-C18 Ca²⁺; Calcium Orange;
 Calcofluor White; Carboxy-X-rhodamine (5-ROX); Cascade Blue™; Cascade Yellow;
 Catecholamine; CCF2 (GeneBlazer); CFDA; CFP (Cyan Fluorescent Protein); CFP/YFP FRET;
 Chlorophyll; Chromomycin A; Chromomycin A; CL-NERF; CMFDA; Coelenterazine;
 Coelenterazine cp; Coelenterazine f; Coelenterazine fcp; Coelenterazine h; Coelenterazine hcp;
 Coelenterazine ip; Coelenterazine n; Coelenterazine O; Coumarin Phalloidin; C-phycoerythrin;
 CPM I Methylcoumarin; CTC; CTC Formazan; Cy2™; Cy3.1 8; Cy3.5™; Cy3™; Cy5.1 8;
 Cy5.5™; Cy5™; Cy7™; Cyan GFP; cyclic AMP Fluorosensor (FicRhR); Dabcyl; Dansyl;
 Dansyl Amine; Dansyl Cadaverine; Dansyl Chloride; Dansyl DHPE; Dansyl fluoride; DAPI;
 Dapoxyl; Dapoxyl 2; Dapoxyl 3'DCFDA; DCFH (Dichlorodihydrofluorescein Diacetate);
 DDAO; DHR (Dihydrohodamine 123); Di-4-ANEPPS; Di-8-ANEPPS (non-ratio); DiA (4-Di
 16-ASP); Dichlorodihydrofluorescein Diacetate (DCFH); DiD- Lipophilic Tracer; DiD
 (DiIc18(5)); DIDS; Dihydrohodamine 123 (DHR); Dil (DiIc18(3)); I Dinitrophenol; DiO
 (DiOC18(3)); DiR; DiR (DiIc18(7)); DM-NERF (high pH); DNP; Dopamine; DsRed; DTAF;
 DY-630-NHS; DY-635-NHS; EBFP; ECFP; EGFP; ELF 97; Eosin; Erythrosin; Erythrosin ITC;
 Ethidium Bromide; Ethidium homodimer-1 (EthD-1); Euchrysin; EukoLight; Europium (111)
 chloride; EYFP; Fast Blue; FDA; Feulgen (Pararosaniline); FIF (Formaldehyd Induced
 Fluorescence); FITC; Flazo Orange; Fluo-3; Fluo-4; Fluorescein (FITC); Fluorescein Diacetate;
 Fluoro-Emerald; Fluoro-Gold (Hydroxystilbamidine); Fluor-Ruby; FluorX; FM 1-43™; FM 4-
 46; Fura Red™ (high pH); Fura Red™/Fluo-3; Fura-2; Fura-2/BCECF; Genacryl Brilliant Red
 B; Genacryl Brilliant Yellow 10GF; Genacryl Pink 3G; Genacryl Yellow 5GF; GeneBlazer;
 (CCF2); GFP (S65T); GFP red shifted (rsGFP); GFP wild type' non-UV excitation (wtGFP);
 GFP wild type, UV excitation (wtGFP); GFPuv; Gloxalic Acid; Granular blue;
 Haematoporphyrin; Hoechst 33258; Hoechst 33342; Hoechst 34580; HPTS; Hydroxycoumarin;
 Hydroxystilbamidine (FluoroGold); Hydroxytryptamine; Indo-1, high calcium; Indo-1 low
 calcium; Indodicarbocyanine (DiD); Indotricarbocyanine (DiR); Intrawhite Cf; JC-1; JO JO-1;
 JO-PRO-1; LaserPro; Laurodan; LDS 751 (DNA); LDS 751 (RNA); Leucophor PAF; Leucophor
 SF; Leucophor WS; Lissamine Rhodamine; Lissamine Rhodamine B; Calcein/Ethidium
 homodimer; LOLO-1; LO-PRO-1; ; Lucifer Yellow; Lyso Tracker Blue; Lyso Tracker Blue-

White; Lyso Tracker Green; Lyso Tracker Red; Lyso Tracker Yellow; LysoSensor Blue; LysoSensor Green; LysoSensor Yellow/Blue; Mag Green; Magdala Red (Phloxin B); Mag-Fura Red; Mag-Fura-2; Mag-Fura-5; Mag-Indo-1; Magnesium Green; Magnesium Orange; Malachite Green; Marina Blue; I Maxilon Brilliant Flavin 10 GFF; Maxilon Brilliant Flavin 8 GFF; Merocyanin; Methoxycoumarin; Mitotracker Green FM; Mitotracker Orange; Mitotracker Red; Mitramycin; Monobromobimane; Monobromobimane (mBBBr-GSH); Monochlorobimane; MPS (Methyl Green Pyronine Stilbene); NBD; NBD Amine; Nile Red; Nitrobenzoxedidole; Noradrenaline; Nuclear Fast Red; i Nuclear Yellow; Nylosan Brilliant lavin E8G; Oregon Green™; Oregon Green™ 488; Oregon Green™ 500; Oregon Green™ 514; Pacific Blue; Pararosaniline (Feulgen); PBFI; PE-Cy5; PE-Cy7; PerCP; PerCP-Cy5.5; PE-TexasRed (Red 613); Phloxin B (Magdala Red); Phorwite AR; Phorwite BKL; Phorwite Rev; Phorwite RPA; Phosphine 3R; PhotoResist; Phycoerythrin B [PE]; Phycoerythrin R [PE]; PKH26 (Sigma); PKH67; PMIA; Pontochrome Blue Black; POPO-1; POPO-3; PO-PRO-1; PO- I PRO-3; Primuline; Procion Yellow; Propidium Iodid (PI); PyMPO; Pyrene; Pyronine; Pyronine B; Pyrozal Brilliant Flavin 7GF; QSY 7; Quinacrine Mustard; Resorufin; RH 414; Rhod-2; Rhodamine; Rhodamine 110; Rhodamine 123; Rhodamine 5 GLD; Rhodamine 6G; Rhodamine B; Rhodamine B 200; Rhodamine B extra; Rhodamine BB; Rhodamine BG; Rhodamine Green; Rhodamine Phallicidine; Rhodamine: Phalloidine; Rhodamine Red; Rhodamine WT; Rose Bengal; R-phycoyanine; R-phycoerythrin (PE); rsGFP; S65A; S65C; S65L; S65T; Sapphire GFP; SBF1; Serotonin; Sevron Brilliant Red 2B; Sevron Brilliant Red 4G; Sevron I Brilliant Red B; Sevron Orange; Sevron Yellow L; sgBFP™ (super glow BFP); sgGFP™ (super glow GFP); SITS (Primuline; Stilbene Isothiosulphonic Acid); SNAFL calcein; SNAFL-1; SNAFL-2; SNARF calcein; SNARF1; Sodium Green; SpectrumAqua; SpectrumGreen; SpectrumOrange; Spectrum Red; SPQ (6-methoxy- N-(3 sulfopropyl) quinolinium); Stilbene; Sulphorhodamine B and C; Sulphorhodamine Extra; SYTO 11; SYTO 12; SYTO 13; SYTO 14; SYTO 15; SYTO 16; SYTO 17; SYTO 18; SYTO 20; SYTO 21; SYTO 22; SYTO 23; SYTO 24; SYTO 25; SYTO 40; SYTO 41; SYTO 42; SYTO 43; SYTO 44; SYTO 45; SYTO 59; SYTO 60; SYTO 61; SYTO 62; SYTO 63; SYTO 64; SYTO 80; SYTO 81; SYTO 82; SYTO 83; SYTO 84; SYTO 85; SYTOX Blue; SYTOX Green; SYTOX Orange; Tetracycline; Tetramethylrhodamine (TRITC); Texas Red™; Texas Red-X™ conjugate; Thiadicarbocyanine (DiSC3); Thiazine Red R; Thiazole Orange; Thioflavin 5; Thioflavin S; Thioflavin TON; Thiolyte; Thiozole Orange;

Tinopol CBS (Calcofluor White); TIER; TO-PRO-1; TO-PRO-3; TO-PRO-5; TOTO-1; TOTO-3; TriColor (PE-Cy5); TRITC TetramethylRhodamine IsoThioCyanate; True Blue; Tru Red; Ultralite; Uranine B; Uvitex SFC; wt GFP; WW 781; X-Rhodamine; XRITC; Xylene Orange; Y66F; Y66H; Y66W; Yellow GFP; YFP; YO-PRO-1; YO-PRO 3; YOYO-1; YOYO-3; Sybr Green; Thiazole orange (interchelating dyes); semiconductor nanoparticles such as quantum dots; or caged fluorophore (which can be activated with light or other electromagnetic energy source), or a combination thereof.

112. The moiety can be a nanoparticle, such as a heat generating nanoshell. As used herein, "nanoshell" is a nanoparticle having a discrete dielectric or semi-conducting core section surrounded by one or more conducting shell layers. U.S. Patent No. 6,530,944 is hereby incorporated by reference herein in its entirety for its teaching of the methods of making and using metal nanoshells. Nanoshells can be formed with a core of a dielectric or inert material such as silicon, coated with a material such as a highly conductive metal which can be excited using radiation such as near infrared light (approximately 800 to 1300 nm). Upon excitation, the nanoshells emit heat. The resulting hyperthermia can kill the surrounding cell(s) or tissue. The combined diameter of the shell and core of the nanoshells ranges from the tens to the hundreds of nanometers. Near infrared light is advantageous for its ability to penetrate tissue. Other types of radiation can also be used, depending on the selection of the nanoparticle coating and targeted cells. Examples include x-rays, magnetic fields, electric fields, and ultrasound. The particles can also be used to enhance imaging, especially using infrared diffuse photon imaging methods. Targeting molecules can be antibodies or fragments thereof, ligands for specific receptors, or other proteins specifically binding to the surface of the cells to be targeted.

113. The moiety can be covalently linked to the disclosed peptide. The moiety can be linked to the amino terminal end of the disclosed peptide. The moiety can be linked to the carboxy terminal end of the disclosed peptide. The moiety can be linked to an amino acid within the disclosed peptide. The herein provided conjugates can further comprise a linker connecting the moiety and disclosed peptide. The disclosed peptide can also be conjugated to a coating molecule such as bovine serum albumin (BSA) (see Tkachenko et al., (2003) J Am Chem Soc, 125, 4700-4701) that can be used to coat the Nanoshells with the peptide.

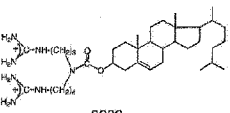
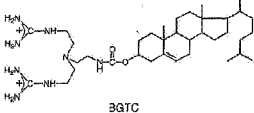
114. Protein crosslinkers that can be used to crosslink the moiety to the disclosed peptide are known in the art and are defined based on utility and structure and include DSS

(Disuccinimidylsuberate), DSP (Dithiobis(succinimidylpropionate)), DTSSP (3,3'-Dithiobis(sulfosuccinimidylpropionate)), SULFO BSOCEES (Bis[2-(sulfosuccinimidooxycarbonyloxy)ethyl]sulfone), BSOCEES (Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone), SULFO DST (Disulfosuccinimidyltartrate), DST (Disuccinimidyltartrate), SULFO EGS (Ethylene glycolbis(succinimidylsuccinate)), EGS (Ethylene glycolbis(sulfosuccinimidylsuccinate)), DPDPB (1,2-Di[3'-(2'-pyridyldithio) propionamido]butane), BSSS (Bis(sulfosuccinimidyl) suberate), SMPB (Succinimidyl-4-(p-maleimidophenyl) butyrate), SULFO SMPB (Sulfosuccinimidyl-4-(p-maleimidophenyl) butyrate), MBS (3-Maleimidobenzoyl-N-hydroxysuccinimide ester), SULFO MBS (3-Maleimidobenzoyl-N-hydroxysulfosuccinimide ester), SIAB (N-Succinimidyl(4-iodoacetyl) aminobenzoate), SULFO SIAB (N-Sulfosuccinimidyl(4-iodoacetyl)aminobenzoate), SMCC (Succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate), SULFO SMCC (Sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate), NHS LC SPDP (Succinimidyl-6-[3-(2-pyridyldithio) propionamido] hexanoate), SULFO NHS LC SPDP (Sulfosuccinimidyl-6-[3-(2-pyridyldithio) propionamido] hexanoate), SPDP (N-Succinimidyl-3-(2-pyridyldithio) propionate), NHS BROMOACETATE (N-Hydroxysuccinimidylbromoacetate), NHS IODOACETATE (N-Hydroxysuccinimidyliodoacetate), MPBH (4-(N-Maleimidophenyl) butyric acid hydrazide hydrochloride), MCCH (4-(N-Maleimidomethyl) cyclohexane-1-carboxylic acid hydrazide hydrochloride), MBH (m-Maleimidobenzoic acid hydrazidehydrochloride), SULFO EMCS (N-(epsilon-Maleimidocaproyloxy) sulfosuccinimide), EMCS (N-(epsilon-Maleimidocaproyloxy) succinimide), PMPI (N-(p-Maleimidophenyl) isocyanate), KMHU (N-(kappa-Maleimidoundecanoic acid) hydrazide), LC SMCC (Succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxy(6-amidocaproate)), SULFO GMBS (N-(gamma-Maleimidobutyroxy) sulfosuccinimide ester), SMPH (Succinimidyl-6-(beta-maleimidopropionamido)hexanoate)), SULFO KMUS (N-(kappa-Maleimidoundecanoyloxy)sulfosuccinimide ester), GMBS (N-(gamma-Maleimidobutyroxy) succinimide), DMP (Dimethylpimelimidate hydrochloride), DMS (Dimethylsuberimidate hydrochloride), MHBH (Wood's Reagent) (Methyl-p-hydroxybenzimidate hydrochloride, 98%), DMA (Dimethyladipimidate hydrochloride).

115. The moiety of the disclosed conjugate can be a cellular internalization transporter or sequence. The cellular internalization sequence can be any internalization sequence known or newly discovered in the art, or conservative variants thereof. Non-limiting examples of cellular

internalization transporters and sequences include Antennapedia sequences, TAT, HIV-Tat, Penetratin, Antp-3A (Antp mutant), Buforin II, Transportan, MAP (model amphipathic peptide), K-FGF, Ku70, Prion, pVEC, Pep-1, SynB1, Pep-7, HN-1, BGSC (Bis-Guanidinium-Spermidine-Cholesterol), and BGTC (Bis-Guanidinium-Tren-Cholesterol) (see Table 1).

Table 1: Cell Internalization Transporters

Name	Sequence	SEQ ID NO
Antp	RQPKIWFPNRRKPWKK	(SEQ ID NO:10)
HIV-Tat	GRKKRRQRPPQ	(SEQ ID NO:11)
Penetratin	RQKIWFQNRRMKWKK	(SEQ ID NO:12)
Antp-3A	RQIAIWFQNRRMKWAA	(SEQ ID NO:13)
Tat	RKKRRQRRR	(SEQ ID NO:14)
Buforin II	TRSSRAGLQFPVGRVHRLLRK	(SEQ ID NO:15)
Transportan	GWTLNSAGYLLGKINKALAALAKKIL	(SEQ ID NO:16)
model amphipathic peptide (MAP)	KLALKLALKALKAAALKLA	(SEQ ID NO:17)
K-FGF	AAVALLPAVLLALLAP	(SEQ ID NO:18)
Ku70	VPMLK-PMLKE	(SEQ ID NO:19)
Prion	MANLGYWLLALFVIMWTDVGLCKKR PKP	(SEQ ID NO:20)
pVEC	LLILRRRIRKQAHASK	(SEQ ID NO:21)
Pep-1	KETWWETWWTEWSQPKKKRKV	(SEQ ID NO:22)
SynB1	RGGRLSYSRRRFSTSTGR	(SEQ ID NO:23)
Pep-7	SDLWEMMMVSLACQY	(SEQ ID NO:24)
HN-1	TSPLNIHNGQKL	(SEQ ID NO:25)
BGSC (Bis-Guanidinium-Spermidine-Cholesterol)		
BGTC (Bis-Guanidinium-Tren-Cholesterol)		

116. Thus, the provided polypeptide can further comprise the amino acid sequence SEQ ID NO:10, SEQ ID NO:11 (Bucci, M. et al. 2000. Nat. Med. 6, 1362-1367), SEQ ID NO:12 (Derossi, D., et al. 1994. Biol.Chem. 269, 10444-10450), SEQ ID NO:13 (Fischer, P.M. et al.

2000. *J. Pept. Res.* 55, 163-172), SEQ ID NO:14 (Frankel, A. D. & Pabo, C. O. 1988. *Cell* 55,1189-1193; Green, M. & Loewenstein, P. M. 1988. *Cell* 55, 1179-1188), SEQ ID NO:15 (Park, C. B., et al. 2000. *Proc. Natl Acad. Sci. USA* 97, 8245-8250), SEQ ID NO:16 (Pooga, M., et al. 1998. *FASEB J.* 12, 67-77), SEQ ID NO:17 (Oehlke, J. et al. 1998. *Biochim. Biophys. Acta.* 1414, 127-139), SEQ ID NO:18 (Lin, Y. Z., et al. 1995. *J. Biol. Chem.* 270, 14255-14258), SEQ ID NO:19 (Sawada, M., et al. 2003. *Nature Cell Biol.* 5, 352-357), SEQ ID NO:20 (Lundberg, P. et al. 2002. *Biochem. Biophys. Res. Commun.* 299, 85-90), SEQ ID NO:21 (Elmqvist, A., et al. 2001. *Exp. Cell Res.* 269, 237-244), SEQ ID NO:22 (Morris, M. C., et al. 2001. *Nature Biotechnol.* 19, 1173-1176), SEQ ID NO:23 (Rousselle, C. et al. 2000. *Mol. Pharmacol.* 57,679-686), SEQ ID NO:24 (Gao, C. et al. 2002. *Bioorg. Med. Chem.* 10, 4057-4065), or SEQ ID NO:25 (Hong, F. D. & Clayman, G. L. 2000. *Cancer Res.* 60, 6551-6556). The provided polypeptide can further comprise BGSC (Bis-Guanidinium-Spermidine-Cholesterol) or BGTC (Bis-Guanidinium-Tren-Cholesterol) (Vigneron, J.P. et al. 1998. *Proc. Natl. Acad. Sci. USA.* 93, 9682-9686). The preceding references are hereby incorporated herein by reference in their entirety for the teachings of cellular internalization vectors and sequences. Any other internalization sequences now known or later identified can be combined with a polypeptide disclosed herein.

6. Polypeptides and Peptides

a) Protein variants

117. Protein variants and derivatives are well understood by those of skill in the art and in can involve amino acid sequence modifications. For example, amino acid sequence modifications typically fall into one or more of three classes: substitutional, insertional or deletional variants. Insertions include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple amino acid residues. Insertions ordinarily can be smaller insertions than those of amino or carboxyl terminal fusions, for example, on the order of one to four residues. Immunogenic fusion protein derivatives, such as those described in the examples, are made by fusing a polypeptide sufficiently large to confer immunogenicity to the target sequence by cross-linking in vitro or by recombinant cell culture transformed with DNA encoding the fusion. Deletions are characterized by the removal of one or more amino acid residues from the protein sequence. Typically, no more than about from 2 to 6 residues are deleted at any one site within the protein molecule. These variants ordinarily are prepared by site

specific mutagenesis of nucleotides in the DNA encoding the protein, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis and PCR mutagenesis. Amino acid substitutions are typically of single residues, but can occur at a number of different locations at once; insertions usually can be on the order of about from 1 to 10 amino acid residues; and deletions can range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e. a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof can be combined to arrive at a final construct. The mutations must not place the sequence out of reading frame and preferably can not create complementary regions that could produce secondary mRNA structure. Substitutional variants are those in which at least one residue has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Table 2 and are referred to as conservative substitutions.

TABLE 2: Amino Acid Substitutions
Original Residue Exemplary Conservative Substitutions,
others are known in the art.

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

118. Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table 2, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or

hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the protein properties can be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine, in this case, (e) by increasing the number of sites for sulfation and/or glycosylation.

119. For example, the replacement of one amino acid residue with another that is biologically and/or chemically similar is known to those skilled in the art as a conservative substitution. For example, a conservative substitution would be replacing one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as, for example, Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Such conservatively substituted variations of each explicitly disclosed sequence are included within the mosaic polypeptides provided herein.

120. Substitutional or deletional mutagenesis can be employed to insert sites for N-glycosylation (Asn-X-Thr/Ser) or O-glycosylation (Ser or Thr). Deletions of cysteine or other labile residues also may be desirable. Deletions or substitutions of potential proteolysis sites, e.g. Arg, is accomplished for example by deleting one of the basic residues or substituting one by glutaminyl or histidyl residues.

121. Certain post-translational derivatizations are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, *Proteins: Structure and Molecular Properties*, W. H. Freeman & Co., San Francisco pp 79-86 [1983]), acetylation of the N-terminal amine and, in some instances, amidation of the C-terminal carboxyl.

122. Specifically disclosed are variants of these and other polypeptides herein disclosed which have at least, 65%, 70% or 75% or 80% or 85% or 90% or 95% homology to the stated sequence. Those of skill in the art readily understand how to determine the homology of two proteins. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

123. Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison can be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

124. The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, *M. Science* 244:48-52, 1989, Jaeger et al. *Proc. Natl. Acad. Sci. USA* 86:7706-7710, 1989, Jaeger et al. *Methods Enzymol.* 183:281-306, 1989 which are herein incorporated by reference for at least material related to nucleic acid alignment.

125. It is understood that the description of conservative mutations and homology can be combined together in any combination, such as embodiments that have at least 70% homology to a particular sequence wherein the variants are conservative mutations.

126. As this specification discusses various proteins and protein sequences it is understood that the nucleic acids that can encode those protein sequences are also disclosed. This would include all degenerate sequences related to a specific protein sequence, i.e. all nucleic acids having a sequence that encodes one particular protein sequence as well as all nucleic acids, including degenerate nucleic acids, encoding the disclosed variants and derivatives of the protein sequences. Thus, while each particular nucleic acid sequence may not be written out herein, it is understood that each and every sequence is in fact disclosed and described herein through the disclosed protein sequence.

127. It is understood that there are numerous amino acid and peptide analogs which can be incorporated into the disclosed peptides. For example, there are numerous D amino acids or amino acids which have a different functional substituent than the amino acids shown in Table

2. The opposite stereo isomers of naturally occurring peptides are disclosed, as well as the stereo isomers of peptide analogs. These amino acids can readily be incorporated into polypeptide chains by charging tRNA molecules with the amino acid of choice and engineering genetic constructs that utilize, for example, amber codons, to insert the analog amino acid into a peptide chain in a site specific way (Thorson et al., *Methods in Molec. Biol.* 77:43-73 (1991), Zoller, *Current Opinion in Biotechnology*, 3:348-354 (1992); Ibba, *Biotechnology & Genetic Engineering Reviews* 13:197-216 (1995), Cahill et al., *TIBS*, 14(10):400-403 (1989); Benner, *TIB Tech*, 12:158-163 (1994); Ibba and Hennecke, *Bio/technology*, 12:678-682 (1994) all of which are herein incorporated by reference at least for material related to amino acid analogs).

128. Molecules can be produced that resemble peptides, but which are not connected via a natural peptide linkage. For example, linkages for amino acids or amino acid analogs can include $\text{CH}_2\text{NH--}$, $\text{--CH}_2\text{S--}$, $\text{--CH}_2\text{--CH}_2\text{--}$, --CH=CH-- (cis and trans), $\text{--COCH}_2\text{--}$, $\text{--CH(OH)CH}_2\text{--}$, and $\text{--CHH}_2\text{SO--}$ (These and others can be found in Spatola, A. F. in *Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins*, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A. F., *Vega Data* (March 1983), Vol. 1, Issue 3, *Peptide Backbone Modifications* (general review); Morley, *Trends Pharm Sci* (1980) pp. 463-468; Hudson, D. et al., *Int J Pept Prot Res* 14:177-185 (1979) ($\text{--CH}_2\text{NH--}$, $\text{CH}_2\text{CH}_2\text{--}$); Spatola et al. *Life Sci* 38:1243-1249 (1986) ($\text{--CH H}_2\text{--S}$); Hann J. *Chem. Soc Perkin Trans. I* 307-314 (1982) (--CH--CH-- , cis and trans); Almquist et al. *J. Med. Chem.* 23:1392-1398 (1980) ($\text{--COCH}_2\text{--}$); Jennings-White et al. *Tetrahedron Lett* 23:2533 (1982) ($\text{--COCH}_2\text{--}$); Szelke et al. *European Appln*, EP 45665 CA (1982): 97:39405 (1982) ($\text{--CH(OH)CH}_2\text{--}$); Holladay et al. *Tetrahedron. Lett* 24:4401-4404 (1983) ($\text{--C(OH)CH}_2\text{--}$); and Hruby *Life Sci* 31:189-199 (1982) ($\text{--CH}_2\text{--S--}$); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is $\text{--CH}_2\text{NH--}$. It is understood that peptide analogs can have more than one atom between the bond atoms, such as β -alanine, γ -aminobutyric acid, and the like.

129. Amino acid analogs and analogs and peptide analogs often have enhanced or desirable properties, such as, more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others.

130. D-amino acids can be used to generate more stable peptides, because D amino acids are not recognized by peptidases and such. Systematic substitution of one or more amino

acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) can be used to generate more stable peptides. Cysteine residues can be used to cyclize or attach two or more peptides together. This can be beneficial to constrain peptides into particular conformations. (Rizo and Gierasch *Ann. Rev. Biochem.* 61:387 (1992), incorporated herein by reference).

131. There are a variety of actions, such as determining homology/identity of nucleic acids or proteins, hybridization, expression, delivery, and pharmaceutical formulations which are applicable for the general and specific compositions disclosed.

7. Homology/identity

132. It is understood that one way to define any known variants and derivatives or those that might arise, of the disclosed genes and proteins herein is through defining the variants and derivatives in terms of homology to specific known sequences. Specifically disclosed are variants of these and other genes and proteins herein disclosed which have at least, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 percent homology to the stated sequence. Those of skill in the art readily understand how to determine the homology of two proteins or nucleic acids, such as genes. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

133. In general, it is understood that one way to define any known variants and derivatives or those that might arise, of the disclosed genes and proteins herein, is through defining the variants and derivatives in terms of homology to specific known sequences. This identity of particular sequences disclosed herein is also discussed elsewhere herein. In general, variants of genes and proteins herein disclosed typically have at least, about 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent homology to the stated sequence or the native sequence. Those of skill in the art readily understand how to determine the homology of two proteins or nucleic acids, such as genes. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

134. Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment

algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

135. The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, *M. Science* 244:48-52, 1989, Jaeger et al. *Proc. Natl. Acad. Sci. USA* 86:7706-7710, 1989, Jaeger et al. *Methods Enzymol.* 183:281-306, 1989 which are herein incorporated by reference for at least material related to nucleic acid alignment. It is understood that any of the methods typically can be used and that in certain instances the results of these various methods may differ, but the skilled artisan understands if identity is found with at least one of these methods, the sequences would be said to have the stated identity, and be disclosed herein.

136. For example, as used herein, a sequence recited as having a particular percent homology to another sequence refers to sequences that have the recited homology as calculated by any one or more of the calculation methods described above. For example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using the Zuker calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by any of the other calculation methods. As another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using both the Zuker calculation method and the Pearson and Lipman calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by the Smith and Waterman calculation method, the Needleman and Wunsch calculation method, the Jaeger calculation methods, or any of the other calculation methods. As yet another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using each of calculation methods (although, in practice, the different calculation methods will often result in different calculated homology percentages).

8. Hybridization/selective hybridization

137. The term hybridization typically means a sequence driven interaction between at least two nucleic acid molecules, such as a primer or a probe and a gene. Sequence driven interaction means an interaction that occurs between two nucleotides or nucleotide analogs or nucleotide derivatives in a nucleotide specific manner. For example, G interacting with C or A interacting with T are sequence driven interactions. Typically sequence driven interactions occur on the Watson-Crick face or Hoogsteen face of the nucleotide. The hybridization of two nucleic acids is affected by a number of conditions and parameters known to those of skill in the art. For example, the salt concentrations, pH, and temperature of the reaction all affect whether two nucleic acid molecules can hybridize.

138. Parameters for selective hybridization between two nucleic acid molecules are well known to those of skill in the art. For example, in some embodiments selective hybridization conditions can be defined as stringent hybridization conditions. For example, stringency of hybridization is controlled by both temperature and salt concentration of either or both of the hybridization and washing steps. For example, the conditions of hybridization to achieve selective hybridization may involve hybridization in high ionic strength solution (6X SSC or 6X SSPE) at a temperature that is about 12-25°C below the T_m (the melting temperature at which half of the molecules dissociate from their hybridization partners) followed by washing at a combination of temperature and salt concentration chosen so that the washing temperature is about 5°C to 20°C below the T_m . The temperature and salt conditions are readily determined empirically in preliminary experiments in which samples of reference DNA immobilized on filters are hybridized to a labeled nucleic acid of interest and then washed under conditions of different stringencies. Hybridization temperatures are typically higher for DNA-RNA and RNA-RNA hybridizations. The conditions can be used as described above to achieve stringency, or as is known in the art. (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989; Kunkel et al. *Methods Enzymol.* 1987:154:367, 1987 which is herein incorporated by reference for material at least related to hybridization of nucleic acids). A preferable stringent hybridization condition for a DNA:DNA hybridization can be at about 68°C (in aqueous solution) in 6X SSC or 6X SSPE followed by washing at 68°C. Stringency of hybridization and washing, if desired, can be reduced accordingly as the degree of complementarity desired is decreased, and further,

depending upon the G-C or A-T richness of any area wherein variability is searched for. Likewise, stringency of hybridization and washing, if desired, can be increased accordingly as homology desired is increased, and further, depending upon the G-C or A-T richness of any area wherein high homology is desired, all as known in the art.

139. Another way to define selective hybridization is by looking at the amount (percentage) of one of the nucleic acids bound to the other nucleic acid. For example, in some embodiments selective hybridization conditions would be when at least about, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the limiting nucleic acid is bound to the non-limiting nucleic acid. Typically, the non-limiting primer is in for example, 10 or 100 or 1000 fold excess. This type of assay can be performed at under conditions where both the limiting and non-limiting primer are for example, 10 fold or 100 fold or 1000 fold below their k_d , or where only one of the nucleic acid molecules is 10 fold or 100 fold or 1000 fold or where one or both nucleic acid molecules are above their k_d .

140. Another way to define selective hybridization is by looking at the percentage of primer that gets enzymatically manipulated under conditions where hybridization is required to promote the desired enzymatic manipulation. For example, in some embodiments selective hybridization conditions would be when at least about, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the primer is enzymatically manipulated under conditions which promote the enzymatic manipulation, for example if the enzymatic manipulation is DNA extension, then selective hybridization conditions would be when at least about 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the primer molecules are extended. Preferred conditions also include those suggested by the manufacturer or indicated in the art as being appropriate for the enzyme performing the manipulation.

141. Just as with homology, it is understood that there are a variety of methods herein disclosed for determining the level of hybridization between two nucleic acid molecules. It is understood that these methods and conditions may provide different percentages of hybridization between two nucleic acid molecules, but unless otherwise indicated meeting the parameters of any of the methods would be sufficient. For example if 80% hybridization was required and as

long as hybridization occurs within the required parameters in any one of these methods it is considered disclosed herein.

142. It is understood that those of skill in the art understand that if a composition or method meets any one of these criteria for determining hybridization either collectively or singly it is a composition or method that is disclosed herein.

a) Sequences

143. There are a variety of sequences related to the homing peptide and cell-penetrating sequence, for example, and other disclosed genes, these sequences and others are herein incorporated by reference in their entireties as well as for individual subsequences contained therein.

144. It is understood that the description related to this sequence is applicable to any sequence disclosed herein unless specifically indicated otherwise. Those of skill in the art understand how to resolve sequence discrepancies and differences and to adjust the compositions and methods relating to a particular sequence to other related sequences.

9. Delivery of the compositions to cells

145. There are a number of compositions and methods which can be used to deliver nucleic acids to cells, either in vitro or in vivo. These methods and compositions can largely be broken down into two classes: viral based delivery systems and non-viral based delivery systems. For example, the nucleic acids can be delivered through a number of direct delivery systems such as, electroporation, lipofection, calcium phosphate precipitation, plasmids, viral vectors, viral nucleic acids, phage nucleic acids, phages, cosmids, or via transfer of genetic material in cells or carriers such as cationic liposomes. Appropriate means for transfection, including viral vectors, chemical transfectants, or physico-mechanical methods such as electroporation and direct diffusion of DNA, are described by, for example, Wolff, J. A., et al., Science, 247, 1465-1468, (1990); and Wolff, J. A. Nature, 352, 815-818, (1991) Such methods are well known in the art and readily adaptable for use with the compositions and methods described herein. In certain cases, the methods can be modified to specifically function with large DNA molecules. Further, these methods can be used to target certain diseases and cell populations by using the targeting characteristics of the carrier.

10. Pharmaceutical carriers/Delivery of pharmaceutical products

146. As described above, the compositions can also be administered *in vivo* in a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject, along with the nucleic acid or vector, without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art.

147. The compositions may be administered orally, parenterally (e.g., intravenously), by intramuscular injection, by intraperitoneal injection, transdermally, extracorporeally, topically or the like, although topical intranasal administration or administration by inhalant is typically preferred. As used herein, "topical intranasal administration" means delivery of the compositions into the nose and nasal passages through one or both of the nares and can comprise delivery by a spraying mechanism or droplet mechanism, or through aerosolization of the nucleic acid or vector. The latter may be effective when a large number of animals is to be treated simultaneously. Administration of the compositions by inhalant can be through the nose or mouth via delivery by a spraying or droplet mechanism. Delivery can also be directly to any area of the respiratory system (e.g., lungs) via intubation. The exact amount of the compositions required can vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the allergic disorder being treated, the particular nucleic acid or vector used, its mode of administration and the like. Thus, it is not possible to specify an exact amount for every composition. However, an appropriate amount can be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein.

148. Parenteral administration of the composition, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves use of a slow release or sustained release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which is incorporated by reference herein.

149. The materials may be in solution or suspension (for example, incorporated into microparticles, liposomes, or cells). These may be targeted to a particular cell type via antibodies, receptors, or receptor ligands. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Senter, et al., Bioconjugate Chem., 2:447-451, (1991); Bagshawe, K.D., Br. J. Cancer, 60:275-281, (1989); Bagshawe, et al., Br. J. Cancer, 58:700-703, (1988); Senter, et al., Bioconjugate Chem., 4:3-9, (1993); Battelli, et al., Cancer Immunol. Immunother., 35:421-425, (1992); Pietersz and McKenzie, Immunolog. Reviews, 129:57-80, (1992); and Roffler, et al., Biochem. Pharmacol., 42:2062-2065, (1991)). Vehicles such as "stealth" and other antibody conjugated liposomes (including lipid mediated drug targeting to colonic carcinoma), receptor mediated targeting of DNA through cell specific ligands, lymphocyte directed tumor targeting, and highly specific therapeutic retroviral targeting of murine glioma cells *in vivo*. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Hughes et al., Cancer Research, 49:6214-6220, (1989); and Litzinger and Huang, Biochimica et Biophysica Acta, 1104:179-187, (1992)). In general, receptors are involved in pathways of endocytosis, either constitutive or ligand induced. These receptors cluster in clathrin-coated pits, enter the cell via clathrin-coated vesicles, pass through an acidified endosome in which the receptors are sorted, and then either recycle to the cell surface, become stored intracellularly, or are degraded in lysosomes. The internalization pathways serve a variety of functions, such as nutrient uptake, removal of activated proteins, clearance of macromolecules, opportunistic entry of viruses and toxins, dissociation and degradation of ligand, and receptor-level regulation. Many receptors follow more than one intracellular pathway, depending on the cell type, receptor concentration, type of ligand, ligand valency, and ligand concentration. Molecular and cellular mechanisms of receptor-mediated endocytosis has been reviewed (Brown and Greene, DNA and Cell Biology 10:6, 399-409 (1991)).

a) Pharmaceutically Acceptable Carriers

150. The compositions, including antibodies, can be used therapeutically in combination with a pharmaceutically acceptable carrier.

151. Pharmaceutical carriers are known to those skilled in the art. These most typically would be standard carriers for administration of drugs to humans, including solutions such as sterile water, saline, and buffered solutions at physiological pH. The compositions can

be administered intramuscularly or subcutaneously. Other compounds can be administered according to standard procedures used by those skilled in the art.

152. Pharmaceutical compositions may include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like in addition to the molecule of choice. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, anti-inflammatory agents, anesthetics, and the like.

153. The pharmaceutical composition may be administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. Administration may be topically (including ophthalmically, vaginally, rectally, intranasally), orally, by inhalation, or parenterally, for example by intravenous drip, subcutaneous, intraperitoneal or intramuscular injection. The disclosed antibodies can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally.

154. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

155. Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

156. Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be desirable.

157. Some of the compositions may potentially be administered as a pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic

acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

b) Therapeutic Uses

158. The dosage ranges for the administration of the compositions are those large enough to produce the desired effect in which the symptoms disorder are effected. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage can vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days.

11. Sequence Similarities

159. It is understood that as discussed herein the use of the terms homology and identity mean the same thing as similarity. Thus, for example, if the use of the word homology is used between two non-natural sequences it is understood that this is not necessarily indicating an evolutionary relationship between these two sequences, but rather is looking at the similarity or relatedness between their nucleic acid sequences. Many of the methods for determining homology between two evolutionarily related molecules are routinely applied to any two or more nucleic acids or proteins for the purpose of measuring sequence similarity regardless of whether they are evolutionarily related or not.

160. In general, it is understood that one way to define any known variants and derivatives or those that might arise, of the disclosed genes and proteins herein, is through defining the variants and derivatives in terms of homology to specific known sequences. This identity of particular sequences disclosed herein is also discussed elsewhere herein. In general, variants of genes and proteins herein disclosed typically have at least about 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent homology to the stated sequence or the native sequence. Those of skill in the art readily understand how to determine the homology of two proteins or nucleic acids, such as genes. For

example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

161. Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison can be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

162. The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, M. *Science* 244:48-52, 1989, Jaeger et al. *Proc. Natl. Acad. Sci. USA* 86:7706-7710, 1989, Jaeger et al. *Methods Enzymol.* 183:281-306, 1989 which are herein incorporated by reference for at least material related to nucleic acid alignment. It is understood that any of the methods typically can be used and that in certain instances the results of these various methods may differ, but the skilled artisan understands if identity is found with at least one of these methods, the sequences would be said to have the stated identity, and be disclosed herein.

163. For example, as used herein, a sequence recited as having a particular percent homology to another sequence refers to sequences that have the recited homology as calculated by any one or more of the calculation methods described above. For example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using the Zuker calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by any of the other calculation methods. As another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using both the Zuker calculation method and the Pearson and Lipman calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by the Smith and Waterman calculation method, the Needleman and Wunsch calculation method, the Jaeger calculation methods, or any of the other calculation methods. As yet another example, a first sequence has 80 percent homology, as

defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using each of calculation methods (although, in practice, the different calculation methods can often result in different calculated homology percentages).

B. Methods

1. Targeting

164. Provided herein is a method of targeting one or more moieties to different regions, breast tissue for example, in a subject. The method can involve administering to the subject a conjugate comprising any one or more of the herein disclosed peptides and the one or more moieties. The one or more moieties can be detection moieties, such as those disclosed herein. Thus, the method can further comprise detecting cancer in the subject by detecting the presence of the conjugate in the subject's cells.

165. The detection moiety can be a fluorophore, such as TAMRA, DANSYL, a cyanine dye, or a cyanine dye encapsulated in a cyclodextrin.

166. The one or more moieties can be homing peptides, such as those disclosed herein. The homing peptide can be breast tissue-specific, such as CPGPEGAGC.

167. The one or more moieties can be cell-penetrating peptides, such as those disclosed herein. The cell-penetrating peptide allows the composition to enter the cell, such as Tat.

168. The one or more moieties can be a contrasting agent, such as those disclosed herein. The contrasting agent can be an MRI contrasting agent. The MRI contrast agent can be non-metallic, such as a nitroxide radical or derivative thereof.

169. The one or more moieties can be therapeutic moieties, such as those disclosed herein. Thus, wherein the subject has cancer, targeting of the moiety to the cancer cells of the subject can provide a therapeutic effect in the subject. The therapeutic moiety can be an anti-cancer therapeutic such as the chemotherapeutic agent, bleomycin.

170. The composition disclosed herein can be non-toxic.

2. Detecting

171. Also provided is a method of detecting cancer, such as a dual modality detection method. The method can involve administering a composition comprising a detectable moiety and a contrasting agent to the subject, performing MRI on the subject, recording the output from

the MRI and the fluorescence imaging, and comparing the output of the MRI and the output of the fluorescence imaging to a control.

172. In one embodiment, the presence of cancer can be detected by the presence of a kinetic decay in the contrasting agent followed by the emergence of a fluorescent signal. For cancer cells the MRI contrast can kinetically decay on the order of a 1 to 8 minute time interval followed by concomitant emergence of a fluorescent signal. However, for normal cells, the MRI contrast can continue and the delayed fluorescence response can be realized. Because these agents are not specific to cancer, but specific to breast tissue, administration of the agent to a patient can result in enhanced MRI signal from the entire breast. However, regions that are cancerous can have reduced MRI signal compared to the other regions of the breast. These regions can also result in a fluorescent signal, whereas non-cancerous regions of the breast can have minimal to no fluorescent signal.

173. In one embodiment, the disclosed method can detect breast cancer.

174. In one embodiment, the disclosed method comprises a contrast agent linked to a homing peptide. In one embodiment of the disclosed method, the homing peptide can be breast tissue-specific. In one embodiment, the disclosed method comprises the breast tissue-specific homing peptide CPGPEGAGC.

175. In one embodiment, the disclosed method comprises a non-metallic contrast agent. In one embodiment, the non-metallic contrast agent can be a nitroxide radical or derivative thereof.

176. In one embodiment, the method comprises a detection moiety. In one embodiment, the detection moiety can be a fluorophore. In one embodiment, the fluorophore can be DANSYL, TAMRA, a cyanine dye, or a cyanine dye encapsulated in a cyclodextrin.

177. In one embodiment, the disclosed method comprises a peptide-based dual modality detection system.

3. Treating

178. Also provided is a method of treating cancer in a subject comprising administering to the subject a composition comprising a detection moiety, a contrasting agent, a homing peptide, a cell-penetrating molecule and a therapeutic agent.

179. In one embodiment, the method of treating cancer in a subject further comprises detecting cancer in a subject.

180. In one embodiment, the method of treating cancer in a subject further comprising detecting cancer in a subject comprises a dual modality detection method of detecting cancer in a subject comprising administering a composition to the subject, performing Magnetic Resonance Imaging (MRI) on the subject; performing fluorescence imaging on the subject, recording the output from the MRI and the fluorescence imaging, and comparing the output of the MRI and the output of the fluorescence imaging to a control.

181. In one embodiment, the method of treating cancer in a subject further comprising detecting cancer in a subject comprises a composition comprising a breast tissue specific homing peptide wherein the homing peptide can be CPGPEGAGC.

182. In one embodiment, the method of treating cancer in a subject further comprising detecting cancer in a subject comprises a composition comprising a fluorophore as the detection moiety. In one embodiment of the disclosed method, the fluorophore can be Dansyl, TAMRA, a cyanine dye, or a cyanine dye encapsulated in a cyclodextrin.

183. In one embodiment, the method of treating cancer in a subject further comprising detecting cancer in a subject comprises a composition comprising a non-metallic MRI contrasting agent. In one embodiment, the non-metallic contrasting agent is a nitroxide radical or derivative thereof.

184. In one embodiment, the method of treating cancer in a subject further comprising detecting cancer in a subject comprises a composition comprising an anti-cancer therapeutic agent. In one embodiment, the anti-cancer therapeutic agent can be bleomycin.

185. In one embodiment, the method of treating cancer in a subject further comprising detecting cancer in a subject comprises a composition comprising Tat as the cell-penetrating molecule.

186. In one embodiment, the disclosed method of treating cancer in a subject uses a non-toxic composition.

187. The disclosed compositions can be used to treat any disease where uncontrolled cellular proliferation occurs such as cancers. A non-limiting list of different types of cancers can be as follows: lymphomas (Hodgkins and non-Hodgkins), leukemias, carcinomas, carcinomas of solid tissues, squamous cell carcinomas, adenocarcinomas, sarcomas, gliomas, high grade gliomas, blastomas, neuroblastomas, plasmacytomas, histiocytomas, melanomas, adenomas,

hypoxic tumors, myelomas, AIDS-related lymphomas or sarcomas, metastatic cancers, or cancers in general.

188. A representative but non-limiting list of cancers that the disclosed compositions can be used to treat is the following: lymphoma, B cell lymphoma, T cell lymphoma, mycosis fungoides, Hodgkin's Disease, myeloid leukemia, bladder cancer, brain cancer, nervous system cancer, head and neck cancer, squamous cell carcinoma of head and neck, kidney cancer, lung cancers such as small cell lung cancer and non-small cell lung cancer, neuroblastoma/glioblastoma, ovarian cancer, pancreatic cancer, prostate cancer, skin cancer, liver cancer, melanoma, squamous cell carcinomas of the mouth, throat, larynx, and lung, colon cancer, cervical cancer, cervical carcinoma, breast cancer, and epithelial cancer, renal cancer, genitourinary cancer, pulmonary cancer, esophageal carcinoma, head and neck carcinoma, large bowel cancer, hematopoietic cancers; testicular cancer; colon and rectal cancers, prostatic cancer, or pancreatic cancer.

4. Administration

189. A composition disclosed herein, such as the disclosed peptides and conjugates, may be administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. For example, the compositions may be administered orally, parenterally (e.g., intravenous, subcutaneous, intraperitoneal, or intramuscular injection), by inhalation, extracorporeally, topically (including transdermally, ophthalmically, vaginally, rectally, intranasally) or the like.

190. As used herein, "topical intranasal administration" means delivery of the compositions into the nose and nasal passages through one or both of the nares and can comprise delivery by a spraying mechanism or droplet mechanism, or through aerosolization of the nucleic acid or vector. Administration of the compositions by inhalant can be through the nose or mouth via delivery by a spraying or droplet mechanism. Delivery can also be directly to any area of the respiratory system (e.g., lungs) via intubation.

191. Parenteral administration of the composition, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves use of a

slow release or sustained release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which is incorporated by reference herein.

192. The exact amount of the compositions required can vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the allergic disorder being treated, the particular nucleic acid or vector used, its mode of administration and the like. Thus, it is not possible to specify an exact amount for every composition. However, an appropriate amount can be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein. Thus, effective dosages and schedules for administering the compositions may be determined empirically, and making such determinations is within the skill in the art. Useful dosage ranges for the administration of the compositions are those large enough to produce the desired effect. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage can vary with the age, condition, sex and extent of the disease in the patient, route of administration, or whether other drugs are included in the regimen, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counter indications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature for appropriate dosages for given classes of pharmaceutical products.

193. For example, a typical daily dosage of the disclosed peptides used alone might range from about 1 $\mu\text{g/kg}$ to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above.

194. Following administration of a disclosed composition, the efficacy of a therapeutic moiety can be assessed in various ways well known to the skilled practitioner. For instance, one of ordinary skill in the art will understand that a composition disclosed herein is efficacious in treating or inhibiting cancer in a subject by observing that the composition reduces tumor growth or prevents a further increase in lymphangiogenesis.

C. Kits

195. The materials described above as well as other materials can be packaged together in any suitable combination as a kit useful for performing, or aiding in the performance of, the disclosed method. It is useful if the kit components in a given kit are designed and adapted for use together in the disclosed method. For example disclosed are kits for administering

compositions, such as those disclosed herein, the kit comprising a composition and a means for administering the composition to a subject. The kits also can contain protocols for administering the compositions..

196. Disclosed is a kit comprising a composition, comprising a detection moiety and contrasting agent, and articles for delivery to a subject.

D. Uses

197. The disclosed compositions can be used in a variety of ways as research tools. Other uses are disclosed, apparent from the disclosure, and/or will be understood by those in the art.

E. Methods of making the compositions

198. The compositions disclosed herein and the compositions necessary to perform the disclosed methods can be made using any method known to those of skill in the art for that particular reagent or compound unless otherwise specifically noted.

1. Nucleic acid synthesis

199. For example, the nucleic acids, such as, the oligonucleotides to be used as primers can be made using standard chemical synthesis methods or can be produced using enzymatic methods or any other known method. Such methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Edition (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) Chapters 5, 6) to purely synthetic methods, for example, by the cyanoethyl phosphoramidite method using a Milligen or Beckman System 1Plus DNA synthesizer (for example, Model 8700 automated synthesizer of Milligen-Bioscience, Burlington, MA or ABI Model 380B). Synthetic methods useful for making oligonucleotides are also described by Ikuta et al., *Ann. Rev. Biochem.* 53:323-356 (1984), (phosphotriester and phosphite-triester methods), and Narang et al., *Methods Enzymol.*, 65:610-620 (1980), (phosphotriester method). Protein nucleic acid molecules can be made using known methods such as those described by Nielsen et al., *Bioconjug. Chem.* 5:3-7 (1994).

2. Peptide synthesis

200. One method of producing the disclosed proteins is to link two or more peptides or polypeptides together by protein chemistry techniques. For example, peptides or polypeptides can be chemically synthesized using currently available laboratory equipment using either Fmoc

(9-fluorenylmethyloxycarbonyl) or Boc (*tert*-butyloxycarbonyl) chemistry. (Applied Biosystems, Inc., Foster City, CA). One skilled in the art can readily appreciate that a peptide or polypeptide corresponding to the disclosed proteins, for example, can be synthesized by standard chemical reactions. For example, a peptide or polypeptide can be synthesized and not cleaved from its synthesis resin whereas the other fragment of a peptide or protein can be synthesized and subsequently cleaved from the resin, thereby exposing a terminal group which is functionally blocked on the other fragment. By peptide condensation reactions, these two fragments can be covalently joined via a peptide bond at their carboxyl and amino termini, respectively, to form an antibody, or fragment thereof. (Grant GA (1992) *Synthetic Peptides: A User Guide*. W.H. Freeman and Co., N.Y. (1992); Bodansky M and Trost B., Ed. (1993) *Principles of Peptide Synthesis*. Springer-Verlag Inc., NY (which is herein incorporated by reference at least for material related to peptide synthesis). Alternatively, the peptide or polypeptide is independently synthesized *in vivo* as described herein. Once isolated, these independent peptides or polypeptides may be linked to form a peptide or fragment thereof via similar peptide condensation reactions.

201. For example, enzymatic ligation of cloned or synthetic peptide segments allow relatively short peptide fragments to be joined to produce larger peptide fragments, polypeptides or whole protein domains (Abrahmsen L et al., *Biochemistry*, 30:4151 (1991)). Alternatively, native chemical ligation of synthetic peptides can be utilized to synthetically construct large peptides or polypeptides from shorter peptide fragments. This method consists of a two step chemical reaction (Dawson et al. *Synthesis of Proteins by Native Chemical Ligation*. *Science*, 266:776-779 (1994)). The first step is the chemoselective reaction of an unprotected synthetic peptide--thioester with another unprotected peptide segment containing an amino-terminal Cys residue to give a thioester-linked intermediate as the initial covalent product. Without a change in the reaction conditions, this intermediate undergoes spontaneous, rapid intramolecular reaction to form a native peptide bond at the ligation site (Baggiolini M et al. (1992) *FEBS Lett.* 307:97-101; Clark-Lewis I et al., *J.Biol.Chem.*, 269:16075 (1994); Clark-Lewis I et al., *Biochemistry*, 30:3128 (1991); Rajarathnam K et al., *Biochemistry* 33:6623-30 (1994)).

202. Alternatively, unprotected peptide segments are chemically linked where the bond formed between the peptide segments as a result of the chemical ligation is an unnatural (non-peptide) bond (Schnolzer, M et al. *Science*, 256:221 (1992)). This technique has been used

to synthesize analogs of protein domains as well as large amounts of relatively pure proteins with full biological activity (deLisle Milton RC et al., Techniques in Protein Chemistry IV. Academic Press, New York, pp. 257-267 (1992)).

F. Machines, Apparati, and Systems

203. Disclosed herein are machines, apparati, and systems, which are designed to perform the various methods disclosed herein. It is understood that these can be multipurpose machines having modules and/or components dedicated to the performance of the disclosed methods. For example, a whole body imaging system, such as a MRI, can be modified as described herein so that it contains a module and/or component which for example, a) produces a tissue-specific record, which identifies the decay of contrasting agents, identifies one or more detection moieties, creates a set of data, and/or performs a dual modality analysis, such as a dual modality analysis alone or in any combination. In particular, the modules and components within the imaging system responsible for determining the presence of cancer, can be linked to the modules and/or components responsible for identifying and/or manipulating tissue-specific data sets. In certain embodiments the presence of cancer can be determined by the moment the decay of the contrasting agent starts and quenching of the fluorophore stops.

204. Thus, the methods and systems herein can have the data, in any form uploaded by a person operating a device capable of performing the methods disclosed herein. The methods can also be associated with the whole body imaging system as described herein, either incorporated into these systems or being on device which is connected to them.

205. Disclosed herein is a method of detecting cancer in a subject wherein the method is a computer implemented method.

206. In one embodiment, the method further comprises the step of outputting results from the dual modality detection.

207. Disclosed herein is a method of analyzing a subject comprising; receiving a tissue-specific record of the subject, wherein the record contains the kinetic decay of the contrasting agent; measuring the amount of decay and the amount of fluorescence, and outputting results from the dual modality detection.

208. In one embodiment the method of analyzing a subject comprises a computer implemented method.

209. In one embodiment, the method of analyzing a subject comprises receiving the tissue-specific record wherein the tissue-specific record can be from a storage medium.

210. In one embodiment, the method of analyzing a subject comprises receiving the tissue-specific record wherein the tissue-specific record can be from a computer system.

211. In one embodiment, the method of analyzing a subject comprises receiving the tissue-specific record wherein the tissue-specific record can be from a whole body imaging system.

212. In one embodiment, the method of analyzing a subject comprises receiving the tissue-specific record wherein the tissue-specific record can be via a computer network.

213. Disclosed herein is a method of analyzing the presence of cancer in a subject comprising, recommending the performance of receiving a tissue-specific record of the subject, wherein the record contains the kinetic decay of the contrasting agent; measuring the amount of decay and the amount of fluorescence, and outputting results from the dual modality detection.

214. In one embodiment, the disclosed method comprises the steps of receiving an output from any of the disclosed methods of analyzing and recommending treatment by administering a composition comprising a detection moiety, a contrasting agent, a homing peptide, a cell-penetrating peptide and a therapeutic agent.

215. Disclosed herein is one or more computer readable media storing program codes that, upon execution by one or more computer systems, causes the computer systems to perform any of the disclosed methods.

216. Disclosed herein is a computer program product comprising a computer usable memory adapted to be executed to implement any of the disclosed methods.

217. In one embodiment, the computer program disclosed above, comprises a logic processing module, a configuration file processing module, a data organization module, and data display organization module, that are embodied upon a computer readable medium.

218. Disclosed herein is a computer program product, comprising a computer usable medium having a computer readable program code embodied therein, said computer readable program code adapted to be executed to implement a method for generating the dual modality detection of any of the previously disclosed methods, said method further comprising: providing a system, wherein the system comprises distinct software modules, and wherein the distinct

software modules comprise a logic processing module, a configuration file processing module, a data organization module, and a data display organization module.

219. In one embodiment, the computer program product further comprises a computerized system configured for performing the method.

220. In one embodiment, the computer program product further comprises the outputting of the results from the dual modality detection.

221. Disclosed herein is a computer-readable medium having stored thereon instructions that, when executed on a programmed processor perform any of the disclosed methods.

222. Disclosed herein is a dual modality detection system, the system comprising: a data store capable of storing tissue-specific data; a system processor comprising one or more processing elements, the one or more processing elements programmed or adapted to: receive tissue-specific data comprising the kinetic decay of the contrasting agent and the presence of fluorescence; store the tissue-specific data in the data store; compare the reduction in the contrast agent to the increase in fluorescence; and output a treatment recommendation based upon the comparison of the decay in contrasting enhancement with the increased fluorescence.

223. In one embodiment, the dual modality detection system receives the tissue-specific data from a computer system.

224. In one embodiment, the dual modality detection system receives the tissue-specific data via a computer network.

225. In one embodiment, the dual modality detection system further comprises a whole body imaging system.

1. Systems, machines, and computer readable medium

226. In addition, or instead, the functionality and approaches discussed above, or portions thereof, can be embodied in instructions executable by a computer, where such instructions are stored in and/or on one or more computer readable storage media. Such media can include primary storage and/or secondary storage integrated with and/or within the computer such as RAM and/or a magnetic disk, and/or separable from the computer such as on a solid state device or removable magnetic or optical disk. The media can use any technology as would be known to those skilled in the art, including, without limitation, ROM, RAM, magnetic, optical, paper, and/or solid state media technology.

227.

G. Definitions

1. “a”, “an”, and “the”

228. As used in the specification and the appended claims, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a pharmaceutical carrier” includes mixtures of two or more such carriers, and the like.

2. Optional or optionally

229. The subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

3. control or control levels or control cells

230. The standard by which a change is measured, for example, the controls are not subjected to the experiment, but are instead subjected to a defined set of parameters, or the controls are based on pre- or post-treatment levels. They can either be run in parallel with or before or after a test run, or they can be a pre-determined standard. For example, a control can refer to the results from an experiment in which the subjects or objects or reagents etc are treated as in a parallel experiment except for omission of the procedure or agent or variable etc under test and which is used as a standard of comparison in judging experimental effects. Thus, the control can be used to determine the effects related to the procedure or agent or variable etc. For example, if the effect of a test compound on a cell was in question, one could a) simply record the characteristics of the cell in the presence of the compound, b) perform a and then also record the effects of adding a control compound with a known activity or lack of activity and then compare effects of the test compound to the control compound. In certain circumstances once a control is performed the control can be used as a standard, in which the control experiment does not have to be performed again and in other circumstances the control experiment should be run in parallel each time a comparison can be made.

4. targeting or homing

231. The preferential movement, binding and/or accumulation of a targeted compound or composition, such as the disclosed compositions, at a site or a location as compared to a non-targeted compound or composition. For example, in the context of *in vivo* administration to a subject, “targeting” or “homing” can refer to the preferential movement, binding, and/or

accumulation of a compound or composition, such as the disclosed compositions, in or at, for example, target tissue, target cells, and/or target structures as compared to non-target tissue, cells and/or structures.

5. Molecule

232. As used herein, the terms "molecule" or like terms refers to a biological or chemical entity that exists in the form of a chemical molecule or molecules. Many molecules are of the type referred to as organic molecules (compounds containing carbon atoms, among others, connected by covalent bonds), although some molecules do not contain carbon (including simple molecular gases such as molecular oxygen and more complex molecules such as some sulfur-based polymers). The general term "molecule" includes numerous descriptive classes or groups of molecules, such as proteins, nucleic acids, carbohydrates, steroids, organic pharmaceuticals, receptors, antibodies, and lipids. When appropriate, one or more of these more descriptive terms (many of which, such as "protein," themselves describe overlapping groups of compounds) can be used herein because of application of the method to a subgroup of molecules, without detracting from the intent to have such compounds be representative of both the general class "molecules" and the named subclass, such as proteins. Unless specifically indicated, the word molecule would include the specific compound and salts thereof, such as pharmaceutically acceptable salts.

6. subject

233. As used throughout, by a "subject" is meant an individual. Thus, the "subject" can include, for example, domesticated animals, such as cats, dogs, etc., livestock (e.g., cattle, horses, pigs, sheep, goats, etc.), laboratory animals (e.g., mouse, rabbit, rat, guinea pig, etc.) mammals, non-human mammals, primates, non-human primates, rodents, birds, reptiles, amphibians, fish, and any other animal. The subject can be a mammal such as a primate or a human. The subject can also be a non-human.

7. treating, treat or treatment

234. The medical management of a patient with the intent to cure, ameliorate, stabilize, or prevent a disease, pathological condition, or disorder. This term includes active treatment, that is, treatment directed specifically toward the improvement of a disease, pathological condition, or disorder, and also includes causal treatment, that is, treatment directed toward removal of the cause of the associated disease, pathological condition, or disorder. In addition, this term

includes palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the disease, pathological condition, or disorder; preventative treatment, that is, treatment directed to minimizing or partially or completely inhibiting the development of the associated disease, pathological condition, or disorder; and supportive treatment, that is, treatment employed to supplement another specific therapy directed toward the improvement of the associated disease, pathological condition, or disorder.

8. computer readable media, computer program product, processors.

Computer usable memory, computer systems

235. In some embodiments, instructions stored on one or more computer readable media that, when executed by a system processor, cause the system processor to perform the methods described above, and in greater detail below. Further, some embodiments may include systems implementing such methods in hardware and/or software. A typical system may include a system processor comprising one or more processing elements in communication with a system data store (SDS) comprising one or more storage elements. The system processor may be programmed and/or adapted to perform the functionality described herein. The system may include one or more input devices for receiving input from users and/or software applications. The system may include one or more output devices for presenting output to users and/or software applications. In some embodiments, the output devices may include a monitor capable of displaying to a user graphical representation of the described analytic functionality.

236. The described functionality may be supported using a computer including a suitable system processor including one or more processing elements such as a CELERON, PENTIUM, XEON, CORE 2 DUO or CORE 2 QUAD class microprocessor (Intel Corp., Santa Clara, CA) or SEMPRON, PHENOM, OPTERON, ATHLON X2 or ATHLON 64 X2 (AMD Corp., Sunnyvale, CA), although other general purpose processors could be used. In some embodiments, the functionality, as further described below, may be distributed across multiple processing elements. The term processing element may refer to (1) a process running on a particular piece, or across particular pieces, of hardware, (2) a particular piece of hardware, or either (1) or (2) as the context allows. Some implementations can include one or more limited special purpose processors such as a digital signal processor (DSP), application specific integrated circuits (ASIC) or a field programmable gate arrays (FPGA). Further, some implementations can use combinations of general purpose and special purpose processors.

237. The environment further includes a system data store (SDS) that could include a variety of primary and secondary storage elements. In one preferred implementation, the SDS would include registers and RAM as part of the primary storage. The primary storage may in some implementations include other forms of memory such as cache memory, non-volatile memory (e.g., FLASH, ROM, EPROM, etc.), etc. The SDS may also include secondary storage including single, multiple and/or varied servers and storage elements. For example, the SDS may use internal storage devices connected to the system processor. In implementations where a single processing element supports all of the functionality a local hard disk drive may serve as the secondary storage of the SDS, and a disk operating system executing on such a single processing element may act as a data server receiving and servicing data requests.

238. It will be understood by those skilled in the art that the different information used in the systems and methods for respiratory analysis as disclosed herein may be logically or physically segregated within a single device serving as secondary storage for the SDS; multiple related data stores accessible through a unified management system, which together serve as the SDS; or multiple independent data stores individually accessible through disparate management systems, which may in some implementations be collectively viewed as the SDS. The various storage elements that comprise the physical architecture of the SDS may be centrally located or distributed across a variety of diverse locations.

9. Computer network

239. A computer network or like terms are one or more computers in operable communication with each other.

10. Computer implemented

240. Computer implemented or like terms refers to one or more steps being actions being performed by a computer, computer system, or computer network.

11. Computer program product

241. A computer program product or like terms refers to product which can be implemented and used on a computer, such as software.

12. Dual modality analysis

242. A dual modality analysis or like terms is the analysis of two components in a system.

13. Obtaining

243. Obtaining as used in the context of data or values, such as tissue-specific data or values refers to acquiring this data or values. It can be acquired by, for example, collection, such as through a machine, such as an MRI machine and system. It can also be acquired by downloading or getting data that has already been collected, and for example, stored in a way in which it can be retrieved at a later time.

14. Outputting results

244. 37. Outputting or like terms means an analytical result after processing data by an algorithm.

15. Tissue-specific record

245. A tissue-specific record or like terms is any collection of tissue-specific data.

16. Tissue-specific data

246. A tissue-specific data series or like terms refers to any collection of tissue-specific data.

17. therapeutically effective

247. The amount of the composition used is of sufficient quantity to ameliorate one or more causes or symptoms of a disease or disorder. Such amelioration only requires a reduction or alteration, not necessarily elimination. The term "carrier" means a compound, composition, substance, or structure that, when in combination with a compound or composition, aids or facilitates preparation, storage, administration, delivery, effectiveness, selectivity, or any other feature of the compound or composition for its intended use or purpose. For example, a carrier can be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject.

18. comprise, comprising, comprises

248. Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises," means "including but not limited to," and is not intended to exclude, for example, other additives, components, integers or steps.

19. Cell

249. The term "cell" as used herein also refers to individual cells, cell lines, or cultures derived from such cells. A "culture" refers to a composition comprising isolated cells of the same

or a different type. The term co-culture is used to designate when more than one type of cell are cultured together in the same dish with either full or partial contact with each other.

20. Stable

250. When used with respect to pharmaceutical compositions, the term "stable" is generally understood in the art as meaning less than a certain amount, usually 10%, loss of the active ingredient under specified storage conditions for a stated period of time. The time required for a composition to be considered stable is relative to the use of each product and is dictated by the commercial practicalities of producing the product, holding it for quality control and inspection, shipping it to a wholesaler or direct to a customer where it is held again in storage before its eventual use. Including a safety factor of a few months time, the minimum product life for pharmaceuticals is usually one year, and preferably more than 18 months. As used herein, the term "stable" references these market realities and the ability to store and transport the product at readily attainable environmental conditions such as refrigerated conditions, 2°C to 8°C.

21. Components

251. It is understood that wherever the word cancer appears without breast as a modifier, it is understood that breast cancer is also disclosed.

252. Disclosed are the components to be used to prepare the disclosed compositions as well as the compositions themselves to be used within the methods disclosed herein. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. Thus, if a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if each is not individually recited each is individually and collectively contemplated meaning combinations, A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are considered disclosed. Likewise, any subset or combination of these is also disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E would be considered disclosed. This concept applies to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be

performed with any specific embodiment or combination of embodiments of the disclosed methods.

22. References

253. Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this pertains. The references disclosed are also individually and specifically incorporated by reference herein for the material contained in them that is discussed in the sentence in which the reference is relied upon.

H. Examples

1. Example 1 Breast tissue-specific cancer theranostics: Therapeutic dual-modality MRI/NIR imaging agents

254. A new smart agent as a theranostic for the diagnosis and treatment of breast cancer is described. The agent incorporates a dual modality magnetic resonance imaging/near infrared imaging component and a releasable therapeutic component. This agent can enable research opportunities in tissue selectivity by interactions with cell-surface proteins, imaging by MRI and NIR of dynamic chemical events in cells, and methods to deliver cancer drugs directly to the site of interest. Because of the dual modality imaging component and tissue specificity, this system can facilitate in vivo mechanistic studies of these drugs in breast cancer modeling.

255. The detection system is a key feature of the proposed theranostic, which is designed to dynamically respond to the intracellular chemical environment of cancer cells. The detection system is based on three physical properties of paramagnetic nitroxide radicals: nitroxide radicals can serve as T1 contrast enhancement agents for MRI (Keana et al. *Magn Reson Med* 2005, 5:525-536), nitroxide radicals quench fluorescence (Blough et al. *J Am Chem Soc* 1988, 110:1915-1917), and nitroxide radicals are reduced to the diamagnetic hydroxylamine in cancer cells (Hyodo et al. *Cancer Res* 2006, 66:9921-9928). The reduction of nitroxides to the hydroxylamine constitutes the dynamic chemical event which can be used for detection, because the resulting hydroxylamine is neither MRI active nor able to quench fluorescence. Therefore, the detection system reports the presence of cancer in a patient by displaying a kinetic decay in MRI contrast enhancement followed by the emergence of a fluorescent signal.

256. Nitroxide paramagnetic spin labels are derived from secondary amines and exist as stable radicals. The T1 relaxivities of nitroxides result in MRI contrast enhancement. Murali

and co-workers showed a preferential reduction of nitroxides in tumors compared with normal tissues by MRI (Hyodo et al. *Cancer Res* 2006, 66:9921-9928). This property of nitroxides is key in the disclosed detection system.

257. The detection system and theranostic agent can be evaluated in animal models to verify the ability of the conjugated homing peptide to distribute in the breast tissue. Mice containing breast cancer xenografts in the mammary glands can then be treated with this agent to verify dual modality detection of the tumor. Imaging can be accomplished using a Bruker 7 Tesla MRI instrument and a CRi Maestro In-Vivo Fluorescence Imaging System. These instruments allow for noninvasive whole animal imaging of live mice; desflurane is used for anesthesia during imaging of the mice.

258. Fluorescence imaging is a simple and cost effective method to determine the parameters outlined above. However, these agents are designed for dual modality imaging and the strategy relies on double confirmation of cancer. Therefore, once the half-life of the agent is determined by fluorescence imaging, MRI imaging is employed. Contrast enhancement is quantified and the kinetics for loss of T1-weighted contrast enhancement are measured, which is due to reduction of the spin label. The loss of MRI contrast correlates with the kinetics for the emerging fluorescence signal.

259. MRI inherently gives high resolution at the expense of sensitivity. Although MRI allows accurate monitoring of tumor growth independent to the level of contrast enhancement, dual confirmation of the presence of a cancerous chemical environment is best. Gadolinium, a very common transition metal used for MRI contrast enhancement, has 7 unpaired electrons in its 4f orbitals giving it a very large magnetic moment. The effect of this element on spin-lattice (T_1) relaxation rates in tissues results in enhanced T_1 weighted images (Weinmann et al. *Am J Roentgenol* 1984, 142:619-624). However, the disclosed agent contains an organic nitroxide radical that contains a single unpaired electron.

260. Disclosed herein is a single-agent theranostic that addresses detection, diagnosis, and therapy of breast cancer. Also disclosed is the use of fluorescence imaging as a new modality for detecting breast cancer that can be coupled with MRI for dual confirmation of the presence of breast cancer. A treatment option is also disclosed that utilizes the same detection system for detecting the cancer, which allows monitoring the distribution and efficacy of the therapy. And, this system does not employ ionizing radiation or toxic metals, which are current problems faced

with mammography and MRI imaging agents, respectively. Also disclosed is a simple peptide platform that can be quickly adapted for the implementation of new and emerging technologies.

261. The disclosed methods and compositions involve the design of 'intelligent' agents that can discriminate between tissue types and are activated by the chemical environment that is exclusive to cancer. These agents are also valuable tools for biologists and enable them to visualize biochemical events by MRI and fluorescence imaging. In particular, the dynamics of cell penetration can be directly observed with these agents. A spin-radical is incorporated in the agent, which has the dual role of quenching fluorescence and enhancing T1-weighted contrast by MRI. This agent enables physicists to examine energy transfer within the cell that results in reduction of the radical to the anion. For example, the energy required by cancer cells to proliferate is derived from an elaborate system of redox reactions that involves formation of NADH. NADH participates in the electron transport chain, which eventually results in the production of ATP, the main energy carrier of the cell. The agent can be used to monitor redox chemistry in living systems by MRI imaging of the spin-label T1 contrast agent and concomitantly by fluorescent imaging.

262. The disclosed methods and compositions have broad impact on health. Currently, strategies to increase success in diagnosis and treatment in the clinic are receiving considerable attention. This agent can aid in diagnosing a malignant lesion and concurrently report the effectiveness of the drug during treatment of the lesion. The use of the agent can result in fewer invasive surgeries for benign lesions due to false-positive detection by mammography, because the agent contains a dual reporting component that can assess the chemical environment that is particular to cancer. A reduced toxicity in treating the patient is expected, because the agent is specific to breast tissue, resulting in a higher effective dose at the breast at an overall reduced systemic dosage. And, the designed agent allows for accurate detection of early breast tumors in younger at-risk patients, who would normally be poor candidates for mammography, because of the increased density of breast tissue and the hazards associated with X-ray radiation. Overall, the versatility of the disclosed methods and compositions is expected to stimulate further research in adapting our technology to other cancers as well as other diseases that have peculiar chemical characteristics.

263. The designed agent is used for breast cancer screening. Although screening by MRI is currently not economically feasible, fluorescence-imaging equipment is low cost and can

easily become standard. And, fluorescence imaging does not involve radiation as does mammography, the current standard for breast cancer screening. For this system, a positive detection by fluorescence imaging would require secondary confirmation by MRI using the same agent. In addition, treatment of breast cancer patients using this technology can allow immediate assessment of drug delivery to the disease site. And, the agent readily allows for imaging of the tumor to evaluate the effectiveness of treatment over time.

264. The disclosed methods and compositions are the development of a new smart biomaterial, and more specifically a combined therapeutic-diagnostic agent coined as a theranostic. The advantages of this type of system include the ability to monitor drug distribution to the disease site and rapidly assess the efficacy of drug treatment in individual patients. The agent allows for the optional attachment of a variety of drug entities. These advantages constitute a personalized medicine approach for treating disease.

265. The disclosed methods and compositions specifically address breast cancer, a disease that can have a very promising prognosis if detected early. However, remaining problems in breast cancer include: false-positive detection by mammography and MRI resulting in unnecessary invasive surgery, false-negative detection especially in younger women with denser breast tissue, systemic toxicity of first line therapies for advanced breast cancer, and early assessment of the effectiveness of a breast cancer treatment.

266. The disclosed methods and compositions have a dual modality MRI/NIR imaging agent that incorporates a therapeutic component. It is designed to give smart agents that can doubly confirm the presence of breast cancer by dual modality magnetic resonance imaging/near infrared imaging (MRI/NIR) and deliver a therapeutic agent specifically to the cancer. Currently, a radiologist can examine X-ray and/or MRI images and assess the presence of cancerous lesions. The detection of dynamic chemical processes to confirm the presence of a cancerous chemical environment are also disclosed herein.

267. The disclosed agent contains 5 distinct components as follows: **1.** A spin-radical T1 contrast component gives MRI image enhancement. **2.** A near infrared (NIR) chromophore can allow for fluorescence imaging. **3.** A breast homing peptide sequence results in specific distribution of the agent to breast tissue. **4.** A Tat sequence allows for cell penetration. And, in combination with the breast homing sequence, Tat confers tissue/cell-type specificity (see preliminary results). **5.** A therapeutic agent is covalently attached to the system. The peptide-

based feature of the proposed agent may impart additional specificity for cancer cells. Proteases are more active in cancer cells than normal cells, which are expected to confer specificity for release of the therapeutic agent to cancer cells (Koblinski et al. Clin Chim Acta 2000, 291:113-135). Additionally, the only by-products of our agent other than the therapeutic moiety are simple amino acids.

268. The key to the dynamic detection component of this system is the nature of the covalent grouping of the MRI and NIR contrast agents. Briefly, the spin-label of the MRI contrast component quenches the fluorescent signal from the chromophore (Green et al. J Am Chem Soc 1990, 112:7337-7346). However, the stability of the spin-label is sensitive to the redox environment of the cancer cell and undergoes chemical reduction, which can be measured by MRI (Hyodo et al. Cancer Res 2006, 66:9921-9928). Therefore, as the MRI contrast is lost by chemical reduction of the spin-label, a concomitant fluorescent signal is formed. The redox chemistry of cancer cells is more reducing than in normal cells. Therefore, a kinetic loss of contrast in the MRI with concomitant contrast enhancement in fluorescence in cells is exhibit a cancerous chemical environment.

269. The disclosed methods and compositions are a multi-faceted approach to breast cancer detection and treatment using a new smart material that combines diagnosis and therapy in a single agent. Within the theranostics paradigm, the disclosed methods and compositions include the optional attachment of a variety of drug entities, which ultimately leads toward personalized medicine.

270. Currently, existing challenges and barriers to the field of theranostics include the design and syntheses of multi-faceted agents. The disclosed peptide-based template can be used to incorporate various imaging agents and therapeutics. In addition, this system is amenable to solid-phase peptide synthesis, which can easily be automated. Because of the simplicity of this system, a number of new theranostics are possible. Our initial entry can consist of a redox-sensitive MRI/NIR reporter group and a synthetic handle for general attachment of various therapeutic agents.

271. Breast cancer detection has advanced considerably. And, follow-up studies on breast cancer patients show a clear correlation between early detection and patient survival. The data strongly suggest that at its inception breast cancer is a progressive disease, rather than a systemic disease, and early diagnosis and treatment can have the most significant outcome on

reducing patient mortality. Currently, high-quality mammography is the most effective method presently available for breast cancer screening. However, the caveats in mammography include the following: false-positive detection results in up to 85% of non-malignant lesions requiring invasive biopsies, and false-negative detection results in up to 20% of cancers being missed, particularly in younger women who have denser breast tissue that obstruct detection of the cancer. Furthermore, the technique of mammography involves taking an X-ray of each breast. The radiation from X-ray imaging is considered harmful with a greater risk in younger women, and therefore mammography is usually only recommended for women over 40 years of age unless the younger patient has a significant risk for the cancer (Tabar et al. *Int J Gynaecol Obstet* 2003, 82:319-326). The disclosed methods and compositions do not employ ionizing radiation such as X-ray, and the problems with false-positive and false-negative detection by monitoring the chemical environment of the cell to determine if a lesion is cancerous is alleviated with our dual modality system..

272. The disclosed agent is sensitive to the chemical environment of cancer cells. In conjunction with the fluorescence imaging, a dual-confirmation strategy to increase the specificity of detection is used. Additionally, the nature of the disclosed imaging agents can also avoid two other properties associated with MRI imaging agents, namely the toxicity of transition metals normally used in MRI imaging and distribution of the agent to the region of interest.

273. Tissue-specificity is addressed with the homing peptide backbone that makes up the bulk the proposed agent. Homing peptides are short sequences derived from phage display libraries that have the unique property of homing to specific organs. The discovery of homing peptide technology by Ruoslahti and Pasquali revealed that different organs have distinct zip codes within the endothelium vascular, and appropriately programmed peptide sequences can be used to home to these zip codes (Pasqualini et al. *Nature* 1996, 380:Z364-366). However, these zip codes refer to cell-surface interactions and do not reflect the intracellular delivery of these peptides.

274. A breast homing peptide with a Tat sequence, derived from HIV that allows for cell penetration, can be used to deliver the disclosed agents into cells (Deshayes et al. *Cell Mol Life Sci* 2005, 62:1839-1849). Myrberg and co-workers published a related study using a different cell-penetrating peptide, *pVEC*, for intracellular delivery of breast homing peptides (Myrberg et al. *Bioconjugate Chem* 2008, 19:70-75). The breast homing peptide is a cyclic

nonapeptide with the sequence cCPGPEGAGC. The peptide and even peptide conjugates of the homing sequence have been shown to distribute to breast tissue possibly via interaction with a membrane-bound proline-specific aminopeptidase P (APaseP) (Essler et al. PNAS 2002, 99:2252-2257). In agreement with Myrberg's work, the homing peptide sequence alone does not penetrate the cell surface of breast cancer cells.

275. The disclosed delivery system is based on a 9-amino acid peptide that was discovered by phage display libraries. The unique property of this peptide is its ability to 'home' to breast tissue, presumably by interacting with aminopeptidase P (APaseP) protein expressed in breast tissue (Essler et al. PNAS 2002, 99:2252-2257). Trastuzumab (Herceptin) is a monoclonal antibody that targets the HER2 receptor tyrosine kinase and was approved by the FDA in 1998 for the treatment of breast cancer. Analogous to the system herein, monoclonal antibodies are designed to recognize an extracellular protein motif and effectively home to its target. Recently, trastuzumab covalently attached to the cytotoxic tubulin inhibitor maytansinoid (DM1) entered phase II clinical trials and shows considerable potential as a new therapy for metastatic breast cancer (Vukelja et al. Cancer Res 2009, 69(2 Suppl), Abstract nr33). However, despite the incredible success of trastuzumab and the potential of trastuzumab-DM1, there are several drawbacks to using monoclonal antibodies for therapy such as stability, manufacturability, and cost. Disclosed is a 21-amino acid peptide-based agent.

276. Monoclonal antibodies are an incredible strategy for cancer treatment. However, antibodies must be refrigerated, have a short shelf life, and require fermentation technology for manufacture. These special requirements all contribute to a major barrier to this strategy, high cost. A Canadian study showed that in 2005, trastuzumab treatment cost was between \$28,350 and \$49,915 per patient, adding a significant cost burden to their health care system (Druker et al. Current Oncology 2008, 15:136-142). In the US, trastuzumab costs \$2928.89 for 440 mg, which translates to a yearly regiment costing approximately \$50,000 for a 70-Kg woman (Fleming T, ed. Redbook. 2005 Ed. Montvale, NJ: Thomson PDR; 2005). One of the major challenges associated with monoclonal antibody therapy is manufacture and storage of the agent. Classical chemical synthesis of drugs average less than \$5 per gram. However, antibody production incurs costs between \$100 and \$1000 per gram (Molowa et al. 2001). Peptides can be manufactured as low as \$1 per gram per amino acid residue, which calculates to approximately \$21 per gram for our proposed agent.

277. The manufacture of antibodies involves a fermentation process using live cells, which require cell culture medium and medium supplements. The complexity of the fermentation process introduces several routes for contamination. Modifications to the antibody cannot be achieved readily. However, unexpected post-transcriptional modifications including various glycosylation events can occur due to slight changes to the fermentation environment. The one advantage of antibodies is the reduced toxicity associated with the agent. The disclosed compositions, methods, and systems takes advantage of using a peptide-based material, which has low toxicity. Unlike antibodies, the relatively small size of the agent is amenable to chemical synthesis, and can be reproducibly manufactured. Chemical synthesis also allows for controlled modifications and easy adjustments to the structure. And, unlike with antibodies, incorporation of various imaging and therapeutic moieties to this system can readily be achieved by standard solid-phase peptide synthesis (SPPS) protocols.

278. Tat is a transcription-activating factor derived from HIV-1, and is essential for viral gene expression. The 86 amino acid sequence contains a basic region (amino acids 49 - 58) that is responsible for cell penetration (Deshayes et al. Cell Mol Life Sci 2005, 62:1839-1849). This cell-penetrating Tat sequence (amino acids 49 -57) was incorporated into the homing peptide and induced cell penetration of the peptides. Further, the combination of the Tat sequence with the breast homing peptide conferred tissue specificity for breast-derived cells. In addition, this specificity has been correlated with the expression of ApaseP, the putative recognition protein of the breast homing peptide. And, examination of human tissue arrays revealed that ApaseP expression is especially high in human breast tissue indicating that mouse-derived breast homing sequence can have utility in humans.

279. Disclosed herein, a theranostic agent is defined as a single agent that can be used to diagnose and simultaneously treat a disease. However, the question arises whether it is reasonable to include a therapeutic component to a diagnostic agent, and in effect unnecessarily medicate patients that are diagnosed as cancer free. The single-agent theranostic has clinical utility and is designed as a fully functional diagnostic system with the option of attaching a drug molecule. Once the disease is diagnosed, the therapeutic agent is attached to the same system used for the diagnosis. To attach the drug, a one-step [3+2] Huisgen cycloaddition is used. This methodology falls under the paradigm of 'click chemistry,' which is a synthetic strategy that can accommodate the attachment of a variety of different drugs (Kolb et al. Angew Chem Int Ed

2001, 40:2004-2021). The advantage of this system is that the physician can monitor the effectiveness of the treatment with the same detection system that was used to diagnose the disease. And, the physician can have a number of treatment options tailored to the patient, because different drugs can be attached to the system using click chemistry.

280. There is a need of a personalized medicine approach to patient care by creating a theranostic agent that can simultaneously detect cancer, doubly confirm the presence of a cancerous chemical environment, and treat the tumor with an interchangeable therapeutic component. This approach is exceptionally innovative, because the latest techniques and strategies for both cancer detection and treatment have been combined into a simple single agent that is amenable to process-scale manufacture. This strategy is unconventional, because the ability to monitor and manipulate intricate processes of biology, chemistry, and physics were incorporating while maintaining a practicality that can allow significant progress within the allotted two-year time frame. This strategy is very logical and codifies the strategies that are essential to the theranostics concept.

281. The disclosed agents require three amino acids as follows: the fluorophore, the spin-label, and the therapeutic agent. Each route is very general, and can be adapted to the synthesis of the other disclosed amino acids.

282. Modern solid-phase peptide synthesis (SPPS) is a convenient method to assemble peptides of increasing lengths. The best methods available utilize Fmoc-protected amino acids that are attached to a solid support in a step-wise fashion. Peptides containing 20 or more amino acids are routinely synthesized by this technique. The disclosed peptide agent is a 21-mer made up of 8 commercially available amino acids and the 3 disclosed amino acids.

283. Figure 1 provides an example of how the fluorophore containing amino acids are made. Method 1 is accomplished by treating the O-succinimide ester of the carboxyl containing fluorophore with Fmoc-Lys-OH. This gives selective functionalization on the side-chain amine group. The synthesis of Fmocprotected amino acid (2a) was accomplished by this method in an overall 51% yield. The same method can be used to make Fmoc-protected amino acid (2b), which has an overlapping emission bandwidth with (2a) but has a much higher quantum yield.

284. Three problems that arise with fluorescence imaging are depth of light penetration, stability of available fluorophores under physiological conditions, and the solubility of fluorophores that have the appropriate photophysical properties (Frangioni et al. Curr Opin

Chem Biol 2003, 7:626-634). For depth of light penetration, the two fluorophores above are suitable for mouse modeling. However, for increased tissue penetration, a near IR (NIR) fluorophore such as a cyanine dye with an emission wavelength above 700 nm is required (Jaffer et al. J Am Med Assoc 2005, 293:855-862). Unfortunately, cyanine dyes tend to be unstable in vivo, not soluble, and considerably toxic. The increased toxicity with NIR dyes is partly attributed to their lipophilicity. Indocyanine green is an FDA-approved NIR dye that addresses this toxicity by attaching several hydrophilic sulfate groups to the dye. However, indocyanine green is still susceptible to photobleaching and radical formation by redox mechanisms. To address this problem, the disclosed agent uses an NIR dye encapsulated in an α -cyclodextrin molecule. The synthesis of the encapsulated NIR dye is shown in equation 2 of Figure 1 following the protocol of Anderson and co-workers (Simon et al. Chem Commun 2008, 2897-2899). This encapsulation procedure gives rotaxane (5a) a 34% yield. In order to attach the dye to the agent, monofunctionalization of the dye is required. Mono-azido α -cyclodextrin is commercially available and is expected to give the monofunctionalized rotaxane (5b). The click chemistry protocol for [3+2] cycloaddition with Fmoc-protected propargylglycine gives the requisite amino acid for SPPS attachment to the proposed peptide.

285. The spin-labeled amino acid is the next building block needed for the synthesis of the disclosed detection system. This synthetic reaction is outlined in Figure 2.

286. Attachment of a fluorophore, specifically nitrobenzoxadiazole, to the spin label is shown in Figure 3. Condensation of 4-chloro-7-nitrobenzofur-azane with 6-aminocaproic acid (35% yield) is followed by activation of the carboxylic acid by formation of the N-hydroxysuccinimide ester (62% yield). Coupling with the amine side chain of Fmoc-Lys-OH gives the appropriately functionalized building block for incorporation in the proposed agent by SPPS. In an analogous fashion, the spin-labeled amino acid building block is assembled by initial activation of commercially-available 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid by formation of the N-hydroxysuccinimide ester (61 %) followed by coupling to the amine side chain of Fmoc-Lys-OH

287. The final building block required is the therapeutic moiety. This can be attached by click chemistry under Huisgen [3+2] cycloaddition conditions and allows the drug to be attached to the completed peptide. The strategy for this approach is to give a fully functional diagnostic system with optional attachment of the therapeutic agent. In order to accomplish this,

the synthesis of the diagnostic system on solid support includes a propargylglycine residue. Unlike in the case for the NIR fluorophore, which is attached to the amino acid by click chemistry before SPPS, the therapeutic moiety is attached to the completed reagent after synthesis, cleavage from the solid support, and purification. This gives the option of attaching a variety of therapeutic agents in a simple one-step procedure. The only prerequisite for attachment is that the drug agent must be functionalized with an azide group.

288. Maytansine is a natural product originally isolated from the bark of the African shrub *Maytenus ovatus* by Kupchan and co-workers (Kupchan et al. J Am Chem Soc 1972, 94:1354-1356.) Maytansine has several advantages: 1. The maytansine analog DM1 attached to the antibody trastuzumab is currently in phase II clinical trials. 2. Maytansine is reported to be a tubulin inhibitor with the same mechanism of action as the clinically used agents vincristine and vinblastine. 3. Maytansine is commercially available and can be selectively functionalized at the secondary alcohol. And, 4. Maytansine activity requires an N-methylalanine residue that can readily be functionalized with an azide.

289. As shown in Figure 4, conjugation to the diagnostic system involves one step. Briefly, the azido-containing drug agent (12) in the presence of the peptide system is treated with a copper (I) source in a DMSO-H₂O solvent system. As mentioned above, this strategy gives a general one-step attachment of a variety of drug entities.

290. Also disclosed herein is the use of bleomycin as the therapeutic component of the proposed theranostic agent. Bleomycin was chosen for several reasons to include the following: bleomycin is an established and well-characterized chemotherapeutic drug in the clinic; bleomycin is itself a pentapeptide; a targeted delivery system to the breast can immediately give a new indication for this drug; selective modification to give azido-bleomycin can be achieved in a simple one-step reaction; and its molecular mechanism of action suggests that chemical modification can not alter the effectiveness of this drug.

291. Bleomycin is administered as a mixture of structurally related compounds marketed under the name Blenoxane (Hecht, In Cancer Chemotherapeutic Agents 1995). Blenoxane is administered intravenously and is used to treat lymphomas, squamous cell carcinomas, testicular carcinomas, and malignant pleural effusions (http://www.fda.gov/CDER/foi/label/2003/50443slr035_blenoxane_lbl.pdf). The most severe side effect of bleomycin treatment is late-onset pulmonary fibrosis, which usually presents

several months after therapy has been completed (Abid et al. *Curr Opin Oncol* 2001, 13:242-248). Conjugation of bleomycin to the disclosed system can result in lower pulmonary toxicity, because the agent distributes specifically to the breast and does not accumulate in the lung. In addition, overall systemic toxicity from bleomycin treatment is predicted, because of an increased effective concentration of the drug at reduced dosages.

292. The copper chelate of bleomycin A5, marketed as Bleocin, can be used for attachment to the disclosed delivery system. In general, the bleomycins are complex glycopeptides and chemical modification can be challenging. However, bleomycins strongly chelate transition metals at their N-terminus domain. In the case of bleomycin A5-copper chelate, the primary amine is exposed and easily functionalized (Figure 5). Following the method of Xu and co-workers, bleomycin A5-copper chelate is treated with 2-azidoacetic acid, DCC, and HOBt to give the azido-conjugated compound. Treatment with 15% aqueous EDTA liberates the bleomycin conjugate from the copper (Xu et al. *Bioorg Med Chem Lett* 2005, 15:3996-3999).

293. The functionalized bleomycin is conjugated to the proposed delivery system in one step under click chemistry conditions for the Huisgen [3+2] cycloaddition. The cycloaddition requires Cu (I) for the cycloaddition. In the event the cycloaddition reaction is not facile due to chelation of the Cu (I) cation with bleomycin (Oppenheimer et al. *J Biol Chem* 1981, 256:1514-1517), the copper saturated adduct can be investigated for conjugation to the proposed delivery system.

294. The antitumor activity of bleomycin is attributed to its ability to induce double-strand DNA damage. The region of bleomycin that was modified is at the bis-thiazole tether extending from the C-terminus of bleomycin, which has been shown to thread between double-stranded DNA before inducing cleavage. Analogs of bleomycin attached to a solid support at the C-terminus showed reactivity identical to that of free bleomycin for sequence-selective cleavage of duplex DNA, suggesting that the threading mechanism is not important for the activity of bleomycin (Abraham et al. *J Amer Chem Soc* 2001, 123:5167-5175). Therefore, the modified bleomycin retains antitumor activity even when attached to the disclosed system. Because bleomycin is required to enter the cell membrane and then the nuclear membrane in order to cleave DNA, this system can efficiently deliver the bleomycin into the cell and proved a more efficacious effect on cancer cells.

295. A further component of the disclosed theranostic agent is the attachment of the drug molecule. This comprises a one-step reaction to connect the drug molecule to the detection system. Click chemistry via a Huisgen 1,3-dipolar cycloaddition is a convenient method to connect two highly functionalized groups in a single step with high yield and purity. This design strategizes the synthesis of a fully functional dual modality detection system that can be elaborated with a number of therapeutic components by covalently attaching new drug entities using a single-step click reaction. The complete detection system is assembled by SPPS using commercially available Fmoc-propargylglycine as the initial residue. After cleavage from the bead and HPLC purification, the agent is fully functional as the proposed detection system. Modular addition of the therapeutic agent involves a simple one-step reaction by click chemistry as shown in Figure 6. The only requirement is that the drug is labeled with an azide functionality. The fully functional detection system can be used for initial patient diagnosis, and then an appropriate therapeutic agent is attached to the same detection system for treatment of the patient.

296. Figure 7 outlines the SPPS of the complete diagnostic system. the agent is based on a peptide sequence that incorporates the following: a cell-penetrating Tat sequence, tissue-specific PEGA breast homing motif, a fluorescent chromophore, a chemosensitive MRI contrast/fluorescence quencher moiety, and a therapeutic agent. As shown, the peptide is a 21-mer, which can easily be accommodated on solid phase. Eight of the 11 required amino acids are commercially available. One disulfide bridge must be installed, which can be accomplished by treatment of the unprotected cysteine side chains with iodine in the presence of air. Because our agent only contains two cysteine residues, selectivity is not an issue. Cleavage from the solid support and global deprotection is effected by treatment with TFA. Note the alkyne of the propargylglycine residue allows for mild and selective attachment of our azido-functionalized drug molecule. However, even without the attachment of the drug molecule, all elements of the diagnostic system is in place and is expected to be fully functional for the detection of cancer lesions.

297. By convention, peptide sequences are written from N to C termini. However, as shown in Figure 7, current SPPS methods require synthesis of the peptides from C to N. Synthesis of the proposed agents can commence as follows. A rink resin is used to give the non-charged terminal amide at the C-terminus. The cyclic PEGA homing motif is formed by

oxidative disulfide bond formation of the unprotected cysteine side chain residues with iodine. And finally, the completed compound is cleaved from the resin with TFA, which results in global deprotection of the amino acid side-chain protecting groups. The PEGA homing moiety with a C-terminal dansyl fluorescent tag, the PEGA homing moiety with a C-terminal TAMRA fluorescent tag, and the PEGA homing moiety with both a C-terminal dansyl fluorescent tag and an N-terminal Tat sequence have been made.

298. Incorporation of the drug molecule is accomplished as shown in Figure 8. The diagnostic system is treated with the azido-functionalized maytansinol in the presence of a copper sulfate and sodium ascorbate in a DMSO-water solvent system to give the completed theranostic agent.

299. The theranostic agents have a pharmacological effect. The homing peptide, the homing peptide-Tat conjugate, the homing peptide-Tat-maytansinoid conjugate, and the homing peptide-Tat-maytansinoid-NIR fluorophore-MRI nitroxide conjugate, as well as the diagnostic agent comprising other therapeutics (i.e. bleomycin), can be evaluated on breast cancer cells (MCF-7 and MDA-MB-231) in vitro by MTT assay to obtain the GI_{50} -values. The results provide an in vitro toxicity profile for the different segments of the theranostic agent as well as a reference for in vivo studies. Normal endothelial cell lines, HUVEC and HMVEC, can be used as controls to assess potential systemic toxicity. Comparing the GI_{50} values for HUVEC or HMVEC cells with breast cancer cells provides a therapeutic index. The therapeutic index is defined as the toxic dose divided by the effective dose for 50% of the population (TD_{50}/ED_{50}). Cancer drugs tend to have a relatively low therapeutic index. The maytansinoid family of compounds is 100 to 1000-fold more cytotoxic than vincristine and vinblastine toward cancer cell lines in vitro (Widdiso et al. J Med Chem 2006, 49:4392-4408), and has a 10% lethal dose of 0.40 mg/kg in mice (Issell et al. Cancer Treat Rev 1978, 5:199-207). The therapeutic index for maytansine is not reported. However, several phase I and II clinical trials failed to demonstrate therapeutic benefits at tolerable doses (Widdiso et al. J Med Chem 2006, 49:4392-4408). Bleomycin is reported to have an in vitro therapeutic index of 8.6 when comparing the IC_{90} value for the non-leukemic permanent murine hematopoietic progenitor (PMHP) cells as the 'normal cells' and the IC_{90} value for breast tumor cells (Tueni et al. Cancer Res 1989, 49:1099-1102). In comparison, gleevec, a clinically used targeted cancer therapeutic, is very well tolerated in patients. Gleevec's therapeutic index ranged from 57 to 243, as defined by the ratio

of ED₅₀ in BCR-ABL-negative cells (dose-limiting side effect) to ED₅₀ in BCR-ABL-positive cells (therapeutic effect) (Topaly et al. Br J Cancer 2002, 86:1487-1493). Cytotoxic drugs in general tend to have a very narrow therapeutic index. For example, the chemotherapeutic agent 5-FU has an in vitro therapeutic index of only 2.3 when administered by IP injection on tumor bearing mice (Iigo et al. Biochem Pharmacol 1988, 37:1609-1613). Ideally, an in vitro therapeutic index in the range of that shown for gleevec, greater than 50, and an in vivo therapeutic index of greater than 20, a 10-fold improvement over currently used cytotoxic agents, is achieved.

300. The toxicity of breast homing peptides has briefly been addressed. Myrberg and co-workers concluded that the toxicity of a breast homing peptide-pVec-chlorambucil conjugate was due predominately to the cytotoxic chlorambucil moiety. The experiment also verified the homing properties of this system in vivo (Myrberg et al. Bioconjugate Chem 2008, 19:70-75). However, the disclosed molecule comprises a different cell-penetrating peptide sequence, Tat in place of pVec, and a different cytotoxic moiety..

301. According to Chari and co-workers, the cytotoxicity of the trastuzumab-DM1 conjugate requires cleavage of the maytansinoid group. Two possible scenarios for a narrow therapeutic index for disclosed agent are as follows: 1. high toxicity is met at low concentrations due to rapid cleavage from the peptide, or 2. high concentrations of the agent is required to have an effect, because of slow cleavage. In both cases, the therapeutic index is narrowed. However, both scenarios can be addressed by attenuating the linker region. To strengthen the conjugation, non-peptidic linkers can be explored as is used for the trastuzumab-DM1 conjugate. For more labile connections, peptidic or disulfide bond linkages can be explored. Disulfide linkages are attractive, because they can be further fine-tuned by increasing steric bulk adjacent to the disulfide linkage. And, disulfide bond cleavage is redox sensitive, which follows the mechanism for reducing the spin-label MRI contrast moiety of the disclosed system.

302. The synthesized agents can be subjected to wild-type mice to determine acute toxicity. The method can be performed following the Acute Oral Toxicity- Up and Down Procedure published by the EPA (OECD Guides for testing of chemicals, <http://www.epa.gov/oppead1/harmonization/>). An estimated LD50 can be calculated from this procedure, which allows for determination of an approximate dosage to give the animals. An

LD50 is calculated for both the diagnostic system and the theranostic agent that incorporates a drug moiety.

303. Animal studies can be performed to study human breast tumor models in mice. The breast tumors can be placed in the mammary fat pad of the mice. Female nude athymic mice can be used. Because of reduced estrogen levels in these mice, estrogen supplementation can be used for MCF-7 breast cancer xenograft models.

304. Based on the estimated LD50 value from the Acute Oral Toxicity- Up and Down Procedure, the dosage for treating the animals can be determined. In order to achieve maximal therapeutic effect and minimal toxicity, the animals at can be dosed at 30% the LD50 value which is assumed to be the maximally tolerated dose. This rough calculation is based on a 6-animal per experiment study assuming a linear relationship between dosage and lethality. It is expected that 30% of the dosage that kills 50% of the mice is approximately the highest dosage that will not kill any of the mice in a study of 6 animals per experiment.

305. Normal wild-type mice can be treated with the detection system that does not incorporate the therapeutic component. The detection system can be treated with sodium borohydride prior to administration to the animal, to reduce the nitroxide radical and remove its ability to reduce fluorescence. This agent can then be used to verify localization of the homing peptide to the breast tissue by fluorescence imaging. The limit of detection for the agent can be defined as the concentration (mg of agent / kg animal weight) that results in a signal-to-noise ratio of 3:1. The quantity of agent delivered can be estimated by fluorescence imaging using the Lambert-Beer law. The practical limits of quantification can be determined as the lowest concentration to give a relative standard deviation of < 10%.

306. Secondly, the mice can be treated with the fully functional detection system with the nitroxide radical intact. The half-life for the free radical by non-specific degradation in a normal mouse can be determined by plotting the relative intensity of the fluorescent signal over a 96-h time period.

307. Finally, the plasma levels of the agent over a 96-h time period can be determined by HPLC. Degradation and/or metabolism of the agent in the plasma can be determined by HPLC.

308. The minimal amount of agent necessary for detection can be determined. In combination with the calculated LD50 values, the concentration range that can be used for

treating the animals for optimal results is determined. A time-course experiment determines the half-life of the free radical and the peptide system in normal mice.

309. Imaging of nitroxide radicals by MRI and characterizing redox activity by measuring the intensity of T1-weighted images as a function of time in mouse xenograft models is reported (Matsumoto et al. Clin Cancer Res 2006, 12:2455-2462). The SPPS methodology can be used to modify the disclosed agents in order to improve their half-life, if necessary. SPPS allows rapid synthesis of the disclosed agents, and therefore modifications to the system can be made fairly quickly. To increase the signal, multiple residues of the nitroxide-containing amino acid can be conjugated to the peptide to attenuate the signal. To increase the half-life, spacing the nitroxide-containing residues with pegylated lysines can protect the radical. In a related imaging strategy that utilizes protease activation of fluorescent probes, Weissleder and co-workers report selective protease-mediated degradation of pegylated lysine-containing peptides at tumor sites in mouse models (Weissleder et al. Nat Biotechnol 1999, 17:375-378). Disclosed herein, unveiling the nitroxide in tumors allows for chemical reduction of the nitroxide in a second step by the redox environment of the cancer.

310. Disclosed are cancer therapeutics that can be used with the disclosed theranostic system. There are a number of potential cancer drugs with various mechanisms of action. These drugs can be used in place of maytansinoid and bleomycin as the therapeutic component of the theranostic agent.

311. Optimization of the detection system and theranostic agent can be performed in xenograft models utilizing the conditions for imaging determined with the wild-type mice. Mice at various stages of tumor growth can be subjected to tail-vein injection of the diagnostic agent that does not incorporate the therapeutic moiety. MRI and fluorescence imaging can be used to ascertain the minimal size tumor that can be detected.

312. Mice bearing breast tumor xenografts (tumor volume=100 mm³) can be subjected to tail-vein injection of the theranostic agent at 30% the LD50. The time course for dosing can be determined based on the half-life of the agent in the circulatory system as determined in the wild-type mice. MRI and fluorescence imaging can be used to monitor tumor growth.

313. The disclosed agent can be used for diagnosing and treating breast cancer by homing to the breast tissue, identifying the presence of cancer, and impeding tumor growth.

314. Disclosed are cancer therapeutics that can be incorporated to the theranostic agent in place of the maytansinoid moiety. Some compounds that can be used are tubulin inhibitors (YK-3-250 and MP-201), a SIRT1 activator (YK-3-237), an HDAC inhibitor (YK-4-272), and an MLCP inhibitor (SCG-3-285) (Figure 9).

315. YK-3-250 is a boronic acid bioisostere of combretastatin A-4 developed by Dr. Yali Kong (Kong et al. Chem Biol 2005, 12:1007-1014). This compound has a GI50 value of less than 10 nM across a panel of breast cancer cell lines, and is a very potent inhibitor of tubulin polymerization with an $IC_{50}=1.5 \pm 0.2 \mu M$. In comparison, combretastatin A-4 has an IC_{50} value of $2.0 \pm 0.2 \mu M$. Phase II clinical trials on a related phosphate analog of combretastatin A-4, fosbretabulin, suggest that fosbretabulin is safe and can be advanced to phase III clinical trials for anaplastic thyroid cancer (Mooney et al. Thyroid 2009, 19:233-240).

316. Dr. Kong developed a related boronic acid chalcone analog, YK-3-237, that surprisingly showed little inhibition of tubulin polymerization ($IC_{50}=31.0 \pm 3.4 \mu M$). However, YK-3-237 was considerably potent against a number of cancer cell lines with GI50 values in the sub nanomolar range for several breast cancer cell lines. This compound can act as a SIRT1 activator. In comparison with resveratrol, a well-known SIRT1 activator, YK-3-237 is 10 times more potent in activating SIRT1 deacetylase activity.

317. In order to attach YK-3-250 or YK-3-237 to the theranostic system, the boronic acid group is functionalized. The free boronic acid is necessary for activity, and therefore requires a handle for conjugation to the peptide. Burke and co-workers recently reported a sp^3 -hybridized boronate ester that is cleaved under mild aqueous conditions (Burke et al. J Am Chem Soc 2007, 129:6716-6717). The strategy involves an internal coordination between a nitrogen atom and the boron atom. Following the method of Burke and co-workers, the azido-functionalized dicarboxylic acid (13) is condensed with the boronic acid under Dean-Stark conditions to give the boronic esters of YK-3-250 and YK-3-237 as shown in Figure 10. These azido-functionalized drug agents can be attached to the theranostic system via click chemistry. The release of the drug can occur by kinetic hydrolysis of the agent in the cell to give the free boronic acid.

318. YK-4-272 is a fluorescent HDAC inhibitor developed by Dr. Kong. This drug inhibits pan-HDAC activity with an IC_{50} value of 125 nM. More interestingly, this agent shows some selectivity toward class II isoforms of HDAC, especially HDAC 6. In addition, a crystal

structure of the HDAC inhibitor SAHA bound to HDAC 8 reveals that the aliphatic chain reaches into a cavity of the protein and the hydroxamic acid moiety binds to a zinc atom (Vannini et al. PNAS 2004,101:15064-15069). Importantly, the opposite end of the molecule, corresponding to the dansyl group in YK-4-272, is in the solvent exposed region of the protein. Therefore the drug can be attached to the theranostic system via the dimethylamine on the dansyl group. Functionalization of the dansyl group is described below.

319. A myosin light chain phosphatase (MLCP) inhibitor, SCG-3-285, that also incorporates a dansyl moiety has been developed.. 1 μ M of SCG-3-285 causes considerable increase in phosphorylation of myosin light chain (MLC) in PC-3 cells. Cell cycle analysis shows that treatment with 1 μ M of SCG-3-285 causes G2/M arrest in PC-3 cells. In addition, the NCI 60 cell line screen revealed that SCG-3-285 is a potent inhibitor of cell proliferation for several breast cancer cell lines with GI50 values around 500 nM.

320. Functionalization of the dansyl moiety of YK-4-272 and SCG-3-285 can commence with mono-demethylation of the dimethylamine group followed by conjugation with 2-azidoacetic acid. Mono-demethylation of the tertiary amine can be accomplished using ACE-Cl as reported (Figure 11) (Olofson et al. J Org Chem 1984, 49:2081-2082). Conjugation with O-succinimide ester of 2-azido-acetic acid gives the requisite azide for attachment to the theranostic system via click chemistry.

321. Biaryl-substituted dihydroquinazolinones are a class of potent tubulin inhibitors. Compound MP-201 is a lead structure optimized to give the nitro-containing quinazolinone shown in Figure 9. MP-201 induces 100% depolymerization of tubulin at 15 μ M, inhibits tubulin polymerization with an IC50 value of 0.76 ± 0.01 μ M, and displaces [3 H]colchicine $33 \pm 3.3\%$ at 5 μ M and $60 \pm 2.8\%$ at 50 μ M. An asymmetric synthesis to the optimized compound has recently been reported (Chinigo et al. J Med Chem 2008, 51:4620-4631). The *S* enantiomer of the optimized nitro-containing analog inhibits tubulin assembly with an IC50 of 1.1 ± 0.2 μ M, displaces [3 H]colchicine $28 \pm 1\%$ at 5 μ M and $66 \pm 1\%$ at 50 μ M, and inhibits proliferation of MDA-MB-435 cancer cells with a GI50 of 0.10 nM. Two regions have been identified that are amenable to functionalization on the 6-position of the dihydroquinazolinone ring and on the ortho-position of the terminal phenyl ring. Modifications at these positions are straightforward. MP-201 has been modified by replacing the methyl group on the terminal phenyl group with - [18 F]CH₂ group for evaluation as a PET agent. There are at least two possible methods of

connecting this potential drug to the disclosed theranostic system. First, synthesis of the agent with an -OH substituent on the terminal phenyl group can allow for condensation with 2-azidoacetic acid to install the requisite azido group for attachment to the theranostic system via click chemistry. Secondly, considering the success with boronic acid bioisosteres, a boronic acid moiety can be incorporated in place of the nitro group. This agent can be evaluated for its ability to inhibit tubulin assembly, displace [^3H]colchicine, and inhibit breast cancer cell proliferation. The same strategy shown above for attachment of the drug to the theranostic system can be used for the boronic acid (Figure 12).

322. The incorporation of different cancer therapeutics to the disclosed theranostic system can enable further development of these agents. Successful implementation of these agents can lead to further funding and eventual drug development.

a) Results

323. The agents disclosed above can be evaluated for two aspects, tissue specificity and pharmacological effectiveness.

324. In order to demonstrate tissue specificity, ApaseP was determined to be differentially expressed in human tissues. A tissue microarray analysis showed that ApaseP was indeed highly expressed in human breast tissue (both normal and cancerous), but not in the lung, esophagus, nor the stomach. APaseP is the putative recognition protein for the breast homing peptide. These results indicate that the breast homing peptide can also distribute to the breast in humans. Most notably, the low expression levels of ApaseP in the lung suggest that the lungs may be completely bypassed as an accumulation site, and therefore reduce the pulmonary toxicity of bleomycin.

325. The breast homing peptide can also be a substrate for APaseP, because it contains potential recognition motifs for cleavage by APaseP. APaseP cleaves at N-terminal X-P-Z sequences, and the breast homing peptide contains two X-P-Z sequences, albeit not at the N-terminal. The location of these sequences and the cyclic nature of the breast homing peptide can increase the half-life of this peptide (Essler et al. PNAS 2002, 99:2252-2257). The X-P-Z sequence can be placed at different locations in the cyclic system to maximize stability but maintain recognition.

326. Expression of ApaseP in cancer cells was determined by immunohistostaining using an antibody against ApaseP. The results show clearly that ApaseP is richly expressed on

MCF-7 and MDA-MB-231 breast cancer cells (Figure 13). However, ApaseP expression was not detected on PC-3 prostate and A-549 lung cancer cells. Binding to ApaseP can be a prerequisite for cell penetration.

327. The delivery efficiency of the proposed delivery system and the importance of the Tat sequence were assessed. The distribution of the homing peptide with or without the Tat cell-penetrating sequence was observed in MCF7 breast cancer cells. As shown in Figure 14, the dansylated breast homing peptide that incorporates the Tat cell-penetrating sequence efficiently penetrates MCF-7 breast cancer cells. However, the dansylated breast homing peptide without a Tat sequence shows poor penetration of MCF-7 breast cancer cells.

328. Another breast cancer cell line, MDA-MB-231 was also examined. As shown in Figure 15, the dansylated PEGA breast homing peptide conjugated to the Tat sequence shows penetration into MDA-MB-231 metastatic breast cancer cells. However, the dansylated PEGA breast homing peptide that does not incorporate the Tat sequence gave a considerably weaker signal under the same treatment and imaging conditions. The homogeneity of the signal that is observed can be due to non-specific binding and no penetration into the cell. The breast homing peptide did not penetrate ApaseP negative PC-3 prostate cancer cells (Figure 16) or A-549 lung cancer cells, regardless of the presence of Tat. The experiments were repeated with the homing peptide containing a TAMRA fluorophore but no Tat sequence. This agent allowed evaluation at a different excitement and emission channel. The data indicate that incorporation of Tat is required for penetration of the disclosed agent into breast cancer cells. An apparent correlation with ApaseP expression and cell penetration is noted. These results are further confirmed with a breast homing peptide that contains a TAMRA fluorophore and Tat.

b) Conclusion

329. The synthesis and evaluation of a new theranostic agent is disclosed. The disclosed methods and compositions are designed to detect cancer and give double confirmation by dual modality MRI/fluorescence imaging of a dynamic chemical event that occurs in cancer cells. Secondly, an optional therapeutic agent can be covalently attached to the agent by a simple one-step click chemistry reaction. Finally, the platform in which this agent is synthesized is an 18-amino acid peptide that homes to breast tissue and can penetrate ApaseP positive breast cancer cells.

330. This agent has clinical utility for the detection of breast cancer. In the event breast cancer is detected, the disclosed agent can then be used for the simultaneous treatment and monitoring of the cancer. The proposed theranostic agent is designed to accommodate a variety of drug entities by a simple chemical attachment, which allows for a personal medicine approach to patient treatment. And, finally a versatile platform is introduced that can be adapted for new and emerging technologies as they are developed.

2. Example 2 :Tissue-specific STAT3 Inhibition for Breast Cancer

Therapy

a) Background

331. Early detection and treatment has resulted in a dramatic reduction in breast cancer mortality. Despite this success, breast cancer continues to be the second leading cancer killer of women resulting in 40,000 deaths each year. The oncogenic transcription factor Signal Transducer and Activator of Transcription 3 (STAT3) is activated in more than 60% of malignant breast tumors. (Garcia et al., Cell Growth Differ. 1997; Bowman et al., Oncogene 2000; Dechow et al. PNAS 2004) Activation of STAT3 correlates with a poorer prognosis, presumably due to concomitant elevation of activated Src and Survivin expression (Diaz et al. Clin Cancer Res 2006).

332. Constitutive STAT3 activity promotes breast cancer aggressiveness through suppression of pro-apoptotic genes (Battle et al. Curr Mol Med 2002; Niu et al. Cancer Res 2001; Zhang et al. PNAS 2005) and activation of genes that promote proliferation, survival, invasion, and angiogenesis (Wei et al. Oncogene 2003; Ling et al. Cancer Res 2005; Song et al. PNAS 2005). STAT3 is especially critical for breast cancer stem-like cell survival and proliferation (Zhoe et al. PNAS 2007). In addition, STAT3 regulates cell motility and invasiveness through non-transcriptional mechanisms (Gao et al. Sci STKE 2006). Inhibition of STAT3 signaling in breast cancer can have enhanced anti-tumor effects, mediated by cell-cycle arrest and induction of stem-like cancer cell death, disruption of angiogenesis, and interference with tumor-cell migration and invasion (Turkson Expert Opin Ther Targets 2004). Therefore, STAT3 is a potential drug target for breast cancer.

333. Current strategies in targeting STAT3 include inhibiting tyrosine phosphorylation of STAT3, SH2-domain dependent dimerization of STAT3, translocation of STAT3 to the nucleus, or binding of STAT3 to DNA (Desrivieres et al. J Mammary Gland Biol Neoplasia

2006). However, STAT3 inhibition in general has resulted in only mildly potent agents that induce 50% growth inhibition of breast cancer cells in the range of 13.4 to 100 μ M (Song et al. PNAS 2005; Coleman et al. J Med Chem 2005; Schust et al. Anal Biochem 2004; Schust et al. Chem Biol 2006; Siddiquee et al. PNAS 2007; Bhasin et al. Bioorg Med Chem Lett 2008; Jing et al. Cancer Res 2004; Xu et al. PLoS ONE 2009). A novel strategy targeting STAT3 N-domain protein-protein interactions has resulted in a more potent STAT3 inhibitor named STAT3-Hel2A-2. STAT3-Hel2A-2 is a retro-inverso peptide with GI₅₀ values in the low micromolar range (<10 μ M) against breast cancer cells (Timofeeva et al. ACS Chem Biol 2007). Despite the increased potency of STAT3-Hel2A-2, Yue and Turkson have indicated that a major impediment to clinical development for STAT3 inhibitors is that the modest potency that can be achieved with drugs that disrupt protein-protein interactions does not provide a sufficiently wide therapeutic index to achieve clinical efficacy (Yue et al. Expert Opin Investig Drugs 2009). Herein, a new strategy is presented to increase the therapeutic index of STAT3-Hel2A-2 retro-inverso peptide by specifically increasing its concentration at breast tissues with an overall reduced systemic concentration using a breast tissue-specific molecular homing device.

334. Homing peptide technology refers to short peptide sequences derived from phage display libraries that have the unique property of homing to specific organs. Ruoslahti and Pasqualini revealed that different organs have distinct zip codes within the endothelium vascular, and appropriately programmed peptide sequences can be used to home to these zip codes (Pasqualini et al. Nature 1996). The peptide and even peptide conjugates of the homing sequence have been shown to distribute to breast tissue presumably via interaction with membrane-bound proline-specific aminopeptidase P (APaseP) (Essler et al. PNAS 2002). The STAT3-Hel2A-2 retro-inverso peptide can be conjugated to a breast homing peptide for development as a potential breast cancer therapeutic.

335. A breast homing peptide conjugated to the STAT3-Hel2A-2 retro-inverso peptide can distribute specifically to the breast for increased effective drug concentration at the breast with an overall reduced systemic concentration.

b) Materials and Methods

336. Described herein is a novel delivery system for a STAT3 inhibitor that targets the N-terminal domain of STAT3. A modular synthesis that allows for simple attachment of the delivery component to the therapeutic component in a single step can be performed.

Incorporating a fluorescent label on the homing peptide sequence will allow for following the distribution of the agent by live imaging in vivo.

337. The disclosed methods can be divided into two parts as follows: 1. Chemistry (linker optimization): the design, chemical synthesis, and characterization of the homing properties of the proposed agents; 2. Biology (in vitro and in vivo): a. in vitro characterization of the agents against STAT3 and b. in vivo evaluation in tumor models.

(1) Chemistry

338. Disclosed herein is the design and synthesis of a series of breast homing peptide – STAT3-Hel2A-2 – fluorescent label conjugates for biological evaluation. A series of linking groups can be used to synthesize conjugates of the breast homing peptide and the STAT3-Hel2A-2 retro-inverso peptide. The disclosed agents can be evaluated for cell penetration of MCF-7 and MDA-MB-231 cancer cells, specificity for breast-derived cells, and homing potential.

(a) Design and chemical synthesis of the agent

339. A schematic for synthesis of the disclosed compounds is outlined in Figure 17. As shown, the agent is based on a peptide sequence that incorporates the following: a cell-penetrating Tat sequence, the tissue-specific PEGA breast homing motif, a fluorescent chromophore, and a synthetic handle for attachment of the STAT3-Hel2A-2 peptide.

340. The cell-penetrating Tat component is necessary for internalization of an agent into the cytoplasm of the cell. As detailed below, preliminary results indicate that the promiscuity of the Tat sequence is not conferred to the homing peptide when the two units are conjugated together.

341. In order to attach the STAT3-Hel2A-2 retro-inverso peptide to the breast homing peptide, a 'click' chemistry approach can be used. Click chemistry is a new paradigm in organic synthesis that allows for efficient coupling of complex entities in high yield. The Huisgen [3+2] cycloaddition is the most common application of the click chemistry concept and can be utilized for the disclosed compounds. The linker region can be optimized by synthesizing a series of connecting groups and evaluating each compound for its ability to selectively penetrate the cell membrane of breast cancer cells and home to the breast in mouse models.

342. Figure 18 outlines a series of linkers that are proposed for synthesis and biological evaluation. An aliphatic tether and polyethylene glycol tether can determine the distance

requirement between the delivery peptide system and the STAT3-Hel2A-2 peptide that can be necessary to retain the homing properties and STAT3 inhibition activity of each entity. A poly-lysine tether can be a potential proteolytic site for release of the STAT3-Hel2A-2 peptide in vivo. Poly-lysine can be targeted for proteolytic cleavage at tumor sites (Weissleder et al. Nature 1999), and this can provide an increased selectivity in treating the cancer. A squarane tether can be a rigid system that can be an uncleavable linker with reduced rotational degrees of freedom in comparison to the aliphatic or PEG tethers. These methods can help prevent premature proteolytic cleavage of the STAT3 inhibitor from the homing peptide before distributing to the breast and help retain the function of each group by sufficiently separating the flexible aliphatic (or PEG) tether and the homing and STAT3 inhibitory moieties.

343. In one embodiment, the drug can be cleaved from the homing peptide once in the cancerous tissue. A selective release strategy can be implemented. To that end, a disulfide linkage can be used to connect the two units. The disulfide bond can serve as a selective releasing moiety by taking advantage of the hypoxic intracellular redox chemistry that is particular to cancer cells, which would chemically reduce the S-S bond into two fragments (Hyodo et al. Cancer Res 2006).

(b) Biological evaluation (homing properties)

344. The disclosed compounds can be biologically evaluated. The disclosed compounds can be evaluated for their ability to induce apoptosis, to specifically associate with STAT3 over STAT1, and to decrease STAT3-driven transcription. In vivo modeling can be used to determine biodistribution, toxicity, and efficacy in reducing tumor volume

345. The homing property of the PEGA sequence for breast tissue is presumed to be due to affinity for aminopeptidase P (ApaseP). MCF-7 and MDA-MB-231 human breast cancer cells indeed show considerable ApaseP expression as shown by immunohistochemistry, whereas PC-3 human prostate cancer cells and A-549 human lung cancer cells show minimal expression of ApaseP (Figure 19). Therefore, the disclosed analogs, which contain the same amino acid sequence for association with ApaseP can also have affinity for breast-derived cells.

346. The ability of the disclosed agent to cross the cell membrane and its specificity for breast-derived cells can be assessed as follows. The peptide can be conjugated to a fluorescent reporter group and then exposed to MCF-7 or MDA-MB-231 breast cancer cells. The

distribution of the peptide can be observed by multi-photon confocal microscopy. Preliminary data is outlined below. The disclosed compounds can be evaluated using the disclosed methods.

347. Designing compounds that can penetrate the cell membrane is a great concern when designing peptide-based drugs. The 9-amino acid Tat cell-penetrating sequence can be used to address the concern of penetrating the cell membrane. As shown in Figure 20, the Tat sequence is sufficient to allow specific penetration of the homing peptide into the cell. Using a dansylated substrate for visualization, the peptide containing the Tat sequence distributes to the cytoplasm of MCF-7 cells. However, the peptide that does not contain the Tat sequence shows very poor penetration into the cytoplasm of MCF-7 cells. A similar phenomenon is observed for MDA-MB-231 cells, where treatment with the homing peptide conjugated to the Tat sequence shows clear penetration into the cell. For the cells treated with the homing peptide not conjugated to the Tat sequence a fluorescent signal is observed, but the homogeneity of the fluorescence signifies non-specific binding and not penetration into the cell. These results indicate that the Tat sequence is sufficient to provide penetration across the cell membrane of breast cancer cells. The disclosed compounds can be subjected to this screen to ensure that the disclosed agents are able to cross the cell membrane for drug delivery.

348. A concern with using Tat for cell penetration is potential non-selective penetration, thereby overcoming the homing properties of the peptide. It was determined that the Tat-sequence did not confer non-specific penetration into cells. As shown in Figure 21, A-549 human lung cancer cells were treated with the dansylated peptide containing the Tat sequence or the dansylated peptide without the Tat sequence. In both cases, the peptide treatment showed no difference from control, indicating that the Tat sequence does not impart non-selective penetration of the peptide into any type of cell. This is in agreement with immunohistostaining experiments that showed low expression of ApaseP in A-549 cells. New compounds can be subjected to this screen to ensure cell-type specificity.

349. Specific distribution to the breast tissue can be evaluated in vivo. The fluorescent properties of the dansyl reporter group are not sufficient for live imaging in mouse models. Therefore, a TAMRA reporter group was used, which has an absorption λ_{max} at 544 nm and an emission λ_{max} at 572 nm. the breast homing peptide containing the Tat sequence and a TAMRA fluorophore was found to be distributed to the breast of nude mice bearing MCF-7 breast tumors surgically implanted in the breast tissue (Figure 22). This model requires the implantation of an

estrogen-releasing pellet, because of the low estrogen levels in female athymic nude mice. The disclosed compounds can be evaluated in this model to determine the homing potential of these compounds.

350. For in vivo evaluation of the homing properties of the drug, the limit of detection for this agent will be defined as the concentration (mg of agent / kg animal weight) that results in a signal-to-noise ratio of 3:1. The quantity of agent delivered can be estimated by fluorescence imaging based on the intensity of the fluorescent signal. The practical limits of quantification will be determined as the lowest concentration to give a relative standard deviation of < 10%.

(2) Biology

(a) Biological evaluation (STAT3 inhibition)

351. STAT3-Hel2A-2 is an optimized peptide with impressive specificity for breast cancer cells over normal cells and modest potency with GI₅₀-values in the low micromolar range. As shown in Figure 23, the cancerous cell lines are more sensitive to STAT3-Hel2A-2 treatment than the non-cancerous breast cell line MCF-10A. This screen can be used for the disclosed analogs to determine an in vitro therapeutic index. Loss of cell viability due to caspase-dependent apoptosis can also be determined by analysis of PARP cleavage. MCF-7 cells are caspase-3 deficient and therefore apoptosis can be determined by annexin V binding.

352. The disclosed analogs can then be evaluated for interaction with STAT3 in living cells by fluorescence resonance energy transfer (FRET) microscopy. The TAMRA fluorophore can act as an acceptor to the donor eGFP tag, which results in FRET (a change in fluorescence wavelength) when the two components are in close proximity. Indeed this was observed when HEK293 cells stably transfected with eGFP on the N-terminus of STAT3 were treated with TAMRA-labeled STAT3-Hel2A-2. The disclosed analogs can contain a TAMRA fluorophore, and this assay can be used to determine if the analogs interact with STAT3.

353. In order to determine specificity for STAT3 over STAT1, FRET efficiency can be compared in HEK293 cells expressing eGFP-STAT1, eGFP-STAT3, or eGFP. As shown in Figure 24, the TAMRA-labeled STAT3-Hel2A-2 peptide showed highest FRET efficiency in cells expressing eGFP-STAT3, and reduced FRET efficiency in cells that express eGFP-STAT1 or just eGFP. This assay indicates that the STAT3-Hel2A-2 retro-inverso peptide is specific for STAT3. This assay can be used to confirm whether this specificity is conferred to the disclosed analogs.

354. To evaluate the effect of the disclosed agents on STAT3-driven transcription, the luciferase reporter vector can be employed for the acute phase response element (APRE) containing STAT3 DNA-binding elements and a renilla luciferase reporter vector. The APRE reporter group has been demonstrated to be activated in MCF-7 cells by leukemia inhibitory factor (LIF). As shown in Figure 25, pretreatment with STAT3-Hel2A-2 for 1 hour inhibited basal and LIF-stimulated expression when compared to control (treatment with DMSO). The luciferase activity was measured and normalized against renilla luciferase activity. This system can be used to evaluate the ability of the disclosed agents to decrease STAT3-driven transcription.

(b) Biological evaluation (xenograft modeling)

355. Athymic female Balb/c nude mice can be purchased from the National Cancer Institute (NCI). The MCF-7 and MDA-MB-231 tumor xenografts, presenting different levels of STAT3 activation, are the models of choice, as indicated by favorable cellular data. In one embodiment, surgical implantation of the breast cancer cells into the breast tissue of the mice can be required. MCF-7 cells are estrogen-dependent, and athymic nude mice are estrogen deficient. Therefore, this model can require the implantation of an estrogen-releasing pellet. As shown in Figure 22, these models have been generated previously. Tumors can be grown to 72-94 mm³ before the treatment begins.

(i) Biodistribution

356. Biodistribution experiments can be performed to determine how selectively the drugs are delivered into tumor cells versus normal cells and organs. Balb/c nude mice bearing MCF-7 breast tumors can be administered intravenously (IV) by tail-vein injection. Animals can then be evaluated by fluorescence imaging.

(ii) Toxicity screen

357. The disclosed analogs can be subjected to acute toxicity studies in wild-type mice using the Acute Oral Toxicity- Up and Down Procedure (OECD 2001). This method allows for the determination of estimated LD₅₀ values for the compounds with minimal amounts of material. The LD₅₀ can be used to determine the dosage for treating the animals that will give us maximal therapeutic effect and minimal toxicity. The animals can be dosed at 30% the LD₅₀ value. This rough calculation is based on a 6-animal experiment, where 30% of the LD₅₀ is approximately the highest dosage that will not kill any of the mice in the study of 6 animals per

experiment. This estimate can be adjusted downward if it appears that the animals are experiencing toxicity, or the dosage can be adjusted upwards if the animals tolerate this dosage well but the fluorescent signal in the imager is insufficient. Previous experiences have shown that these mice models can tolerate 30 mg/kg dosage of the fluorescently-tagged homing peptide and give a sufficient fluorescence signal when imaged. The Acute Oral Toxicity- Up and Down Procedure can allow an estimate of the best dosage for the homing peptide conjugated to the STAT3-Hel2A-2 retro-inverso peptide.

(iii) Efficacy screen

358. Tumors can be measured with an external caliper to a significance of 0.1 mm, and volumes can be calculated ($V=H \times L \times W$). When palpable tumors grow ($72-94 \text{ mm}^3$), the mice can be divided into treatment groups. The test concentrations of the disclosed agents can be obtained by diluting with PBS. Tumor-bearing mice can be injected with either the disclosed agents or vehicle control, once every other day for 4 weeks by tail-vein injection. Tumor volumes and distribution of the agent by fluorescence imaging can be monitored at least twice weekly. Tumor and normal tissues can be obtained from treated and untreated animals, fixed in 10% buffered formalin, blocked in paraffin, sectioned and stained with hematoxylin and eosin for histopathological examination.

(iv) Statistical Analysis

359. Tumor volumes measured at different time points for each mouse can be correlated. For the tumor volumes measured at each time point, percentages with respect to the initial tumor volume (day 0, the first day of dosing) can be calculated and used as the outcome for further analysis. Changes in tumor volumes can be analyzed using linear mixed- effect models to compare differences in these changes among groups. A pair-wise comparison of interest can be made among these subgroups. Each comparison can be performed with a two-group univariate repeated measures ANOVA with Greenhouse-Geisser correction. Six tumor volume measurements from the same mouse can be correlated.

(v) Power Calculation

360. The baseline tumor volume is estimated to be 83 mm^3 ($72-94 \text{ mm}^3$). It is assumed that the decreases in tumor volume at the end of the study can be approximately 50-100% for each drug ($0-42 \text{ mm}^3$). In the power calculation, tumor growth (percentage) between the subsets can be compared. The different dose levels for each treatment can be combined. The

data from subsets can be analyzed using a univariate analysis of variance with the Greenhouse-Geisser correction. The power analyses can be performed using the nQuery software.

c) Summary

361. Disclosed herein is a new breast cancer therapeutic, wherein a breast homing peptide is conjugated to a novel STAT3 inhibitor. STAT3 inhibitors have shown promise as potential therapeutic agents for breast cancer. The STAT3-Hel2A-2 retro-inverso peptide targets the N-domain protein-protein interaction of STAT3. However, the modest potency of STAT3 inhibitors in general has limited their translation to the clinic. To address this limitation, the disclosed compounds and methods provide a new strategy to increase the effective concentration of the therapeutic agent at an overall decreased systemic concentration.

362. ApaseP expression, the putative receptor protein for the breast homing peptide, is elevated in breast cancer cells in comparison to lung or prostate cancer cells. It has also been shown that the Tat cell-penetrating peptide sequence was necessary for cell penetration of the breast homing peptide. And, then it was shown that the breast homing peptide conferred cell-type specificity to the Tat sequence, such that the breast homing peptide conjugated to the Tat sequence did not penetrate A-549 lung cancer cells. And, finally, live imaging of mice bearing MCF-7 breast cancer xenografts implanted in the breast tissue of the mouse with an estrogen-releasing pellet showed that the homing peptide effectively homed to the breast tissue.

363. The STAT3-Hel2A-2 retro-inverso peptide has been evaluated for growth inhibition of breast cancer cells, selectivity for STAT3 over STAT1 by FRET analysis, and inhibition of the transcriptional activity of STAT3. Disclosed herein is the combination of a novel STAT3 inhibition strategy to a homing peptide for development as a new breast cancer therapeutic.

I. SEQUENCES

SEQ ID NO:1 cCPGPEGAGC (breast tissue-specific homing peptide)

SEQ ID NO:2 RKKRRQRRR (Tat)

SEQ ID NO:3 CGFECVRQCPERC (lung homing peptide)

SEQ ID NO:4 CDCRGDCFC (breast homing peptide)

SEQ ID NO:5 CGRRAGGSC (prostate homing peptide)

SEQ ID NO:6 SWCEPGWCR (pancreas homing peptide)

SEQ ID NO:7 CRVASVLPC (pancreas homing peptide)

SEQ ID NO:8 YSGKWW (intestine homing peptide)

SEQ ID NO:9 LDTRYLEQLHKLY (STAT3-Hel2A-2 peptide)

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VIII. Claims

We claim:

1. A composition comprising a detection moiety and a contrasting agent.
2. The composition of claim 1, further comprising a homing peptide.
3. The composition of claim 2, further comprising a cell-penetrating molecule.
4. The composition of claim 3, further comprising a therapeutic agent.
5. The composition of claim 1, wherein the contrasting agent is an MRI contrasting agent.
6. The composition of claim 1, wherein the MRI contrast agent is non-metallic.
7. The composition of claim 6, wherein the non-metallic contrast agent is a nitroxide radical or derivative thereof.
8. The composition of claim 2, wherein the homing peptide is breast tissue specific.
9. The composition of claim 8, wherein the breast tissue specific homing peptide is CPGPEGAGC.
10. The composition of claim 1, wherein the detection moiety is a fluorophore.
11. The composition of claim 10, wherein the fluorophore is Dansyl, TAMRA, a cyanine dye, or a cyanine dye encapsulated in a cyclodextrin.
12. The composition of claim 4, wherein the therapeutic agent is an anti-cancer agent.
13. The composition of claim 12, wherein the anti-cancer agent is bleomycin.
14. The composition of claim 12, wherein the anti-cancer agent is a STAT3 inhibitor.
15. The composition of claim 14, wherein the STAT3 inhibitor is STAT3-Hcl2A-2.
16. The composition of claim 3, wherein the cell-penetrating molecule is Tat.
17. The composition of claim 1, wherein the composition is non-toxic.
18. A dual modality detection method of detecting cancer in a subject comprising:
a) administering a composition of claim 1 to the subject, b) performing Magnetic Resonance Imaging (MRI) on the subject; c) performing fluorescence imaging on the subject, c) recording

the output from the MRI and the fluorescence imaging, comparing the output of the MRI and the output of the fluorescence imaging to a control.

19. The method of claim 18, further comprising determining the presence of cancer by the presence of a kinetic decay in MRI contrast enhancement followed by the emergence of a fluorescent signal.

20. The method of claim 19, wherein the kinetic decay in MRI contrast occurs on the order of a 1 to 8 minute time interval and is followed by concomitant emergence of a fluorescent signal in cancer cells.

21. The method of claim 18, wherein the cancer is breast cancer.

22. The method of claim 18, wherein the contrast agent is linked to a homing peptide

23. The method of claim 22, wherein the homing peptide is specific to breast tissue, lung tissue, prostate tissue, pancreatic tissue or intestinal tissue

24. The method of claim 23, wherein the breast tissue specific homing peptide is CPGPEGAGC.

25. The method of claim 22, wherein the contrast agent is non-metallic.

26. The method of claim 25, wherein the non-metallic contrast agent is a nitroxide radical or derivative thereof.

27. The method of claim 18, wherein the dual modality detection system is peptide based.

28. The method of claim 18, wherein the fluorophore is Dansyl, TAMRA, a cyanine dye, or a cyanine dye encapsulated in a cyclodextrin.

29. A method of treating cancer in a subject comprising administering the composition of claim 4 to the subject.

30. The method of claim 29, further comprising detecting cancer in the subject.

31. The method of claim 30, wherein detecting cancer comprises the method of claim 18.

32. The method of claim 29, wherein the homing peptide is specific to breast tissue,

lung tissue, prostate tissue, pancreatic tissue or intestinal tissue.

33. The method of claim 32, wherein the breast tissue specific homing peptide is CPGPEGAGC.

34. The method of claim 29, wherein the composition comprises a fluorophore as the detection moiety.

35. The method of claim 33, wherein the fluorophore is Dansyl, TAMRA, a cyanine dye, or a cyanine dye encapsulated in a cyclodextrin.

36. The method of claim 29, wherein the composition comprises a non-metallic MRI contrasting agent.

37. The method of claim 36, wherein the non-metallic contrasting agent is a nitroxide radical or derivative thereof.

38. The method of claim 29, wherein the composition comprises an anti-cancer therapeutic agent.

39. The method of claim 38, wherein the anti-cancer agent is bleomycin.

40. The method of claim 38, wherein the anti-cancer agent is a STAT3 inhibitor.

41. The method of claim 40, wherein the STAT3 inhibitor is STAT3-Hel2A-2.

42. The method of claim 29, wherein the composition comprises Tat as the cell-penetrating molecule.

43. The method of claim 29, wherein the composition is non-toxic.

44. A kit comprising the composition of claim 1 and articles for delivery to a subject.

45. The method of claim 18, wherein the method is a computer implemented method.

46. The method of claim 45, further comprising the step of outputting results from the dual modality detection.

47. A method of analyzing a subject comprising; receiving a tissue-specific record of the subject, wherein the record contains the kinetic decay of the contrasting agent; measuring the amount of decay and the amount of fluorescence, and outputting results from the dual modality

detection.

48. The method of claim 47, wherein the method is a computer implemented method.

49. The method of claim 47, wherein receiving the tissue-specific record comprises receiving the tissue-specific record from a storage medium.

50. The method of claim 47, wherein receiving the tissue-specific record comprises receiving the record from a computer system.

51. The method of claim 47, wherein receiving the tissue-specific record comprises receiving the record from a whole body imaging system.

52. The method of claim 47, wherein receiving the tissue-specific record comprises receiving the tissue-specific record via a computer network.

53. A method of analyzing the presence of cancer in a subject comprising, recommending the performance of methods in claim 47 to be performed.

54. A method comprising the steps of receiving an output from any of claims 41 and recommending treatment with the method of claim 29.

55. One or more computer readable media storing program code that, upon execution by one or more computer systems, causes the computer systems to perform the method of claims 18, 19, or 47.

56. A computer program product comprising a computer usable memory adapted to be executed to implement the method of claims 18, 19, or 47.

57. The computer program of claim 56, comprising a logic processing module, a configuration file processing module, a data organization module, and data display organization module, that are embodied upon a computer readable medium.

58. A computer program product, comprising a computer usable medium having a computer readable program code embodied therein, said computer readable program code adapted to be executed to implement a method for generating the dual modality detection of claims 18, 30, and 47, said method further comprising: providing a system, wherein the system comprises distinct software modules, and wherein the distinct software modules comprise a logic processing module, a configuration file processing module, a data organization module, and a

data display organization module.

59. The method of claim 58, further comprising a computerized system configured for performing the method.

60. The method of claim 58, further comprising the outputting of the results from the dual modality detection.

61. A computer-readable medium having stored thereon instructions that, when executed on a programmed processor perform the methods of claim 18, 19, or 47.

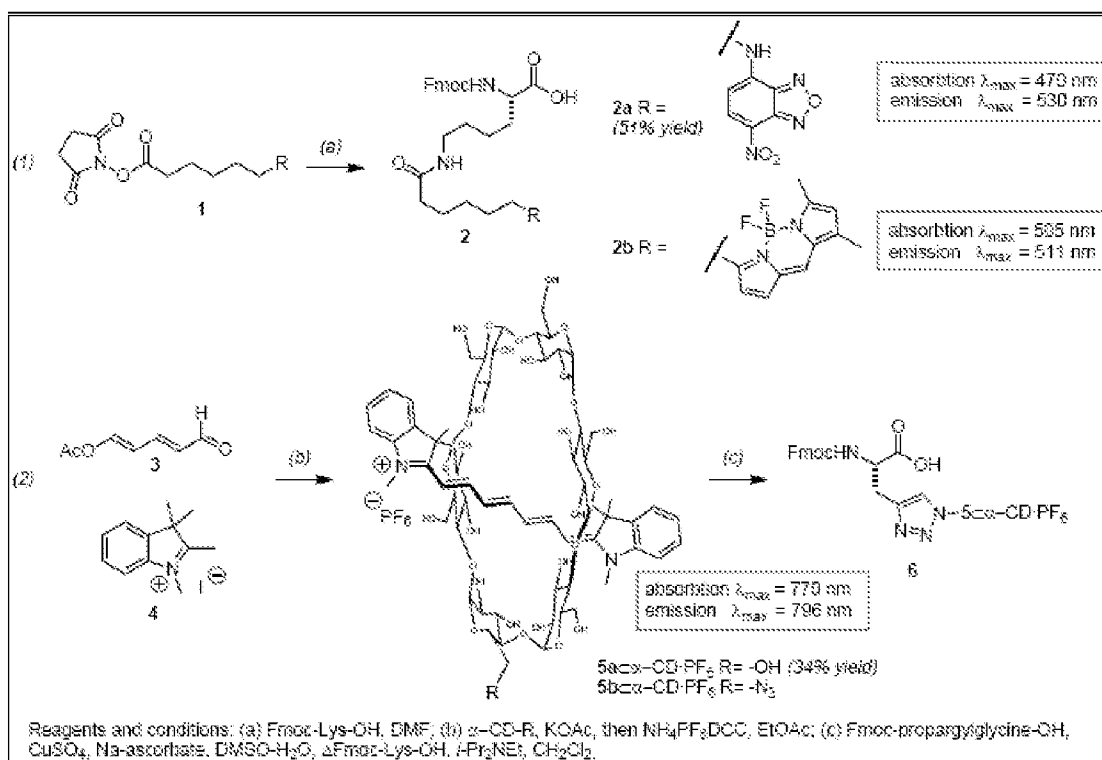
62. A dual modality detection system, the system comprising: a data store capable of storing tissue-specific data; a system processor comprising one or more processing elements, the one or more processing elements programmed or adapted to: receive tissue-specific data comprising the kinetic decay of the contrasting agent and the presence of fluorescence; store the tissue-specific data in the data store; compare the reduction in the contrast agent to the increase in fluorescence; and output a treatment recommendation based upon the comparison of the decay in contrasting enhancement with the increased fluorescence.

63. The system of claim 62, wherein the system receives the tissue-specific data from a computer system.

64. The system of claim 62, wherein the system receives the tissue-specific data via a computer network.

65. The system of claim 62, further comprising a whole body imaging system.

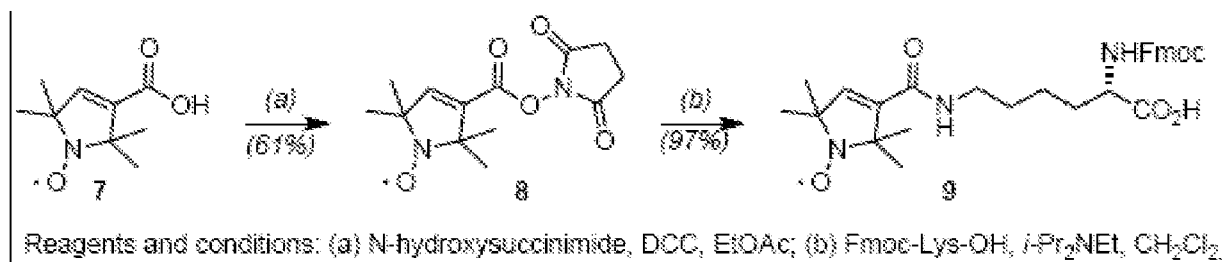
1/26



Synthesis of fluorophore-containing amino acids

FIG. 1

2/26



Synthesis of the spin-labeled amino acid

FIG. 2

3/26

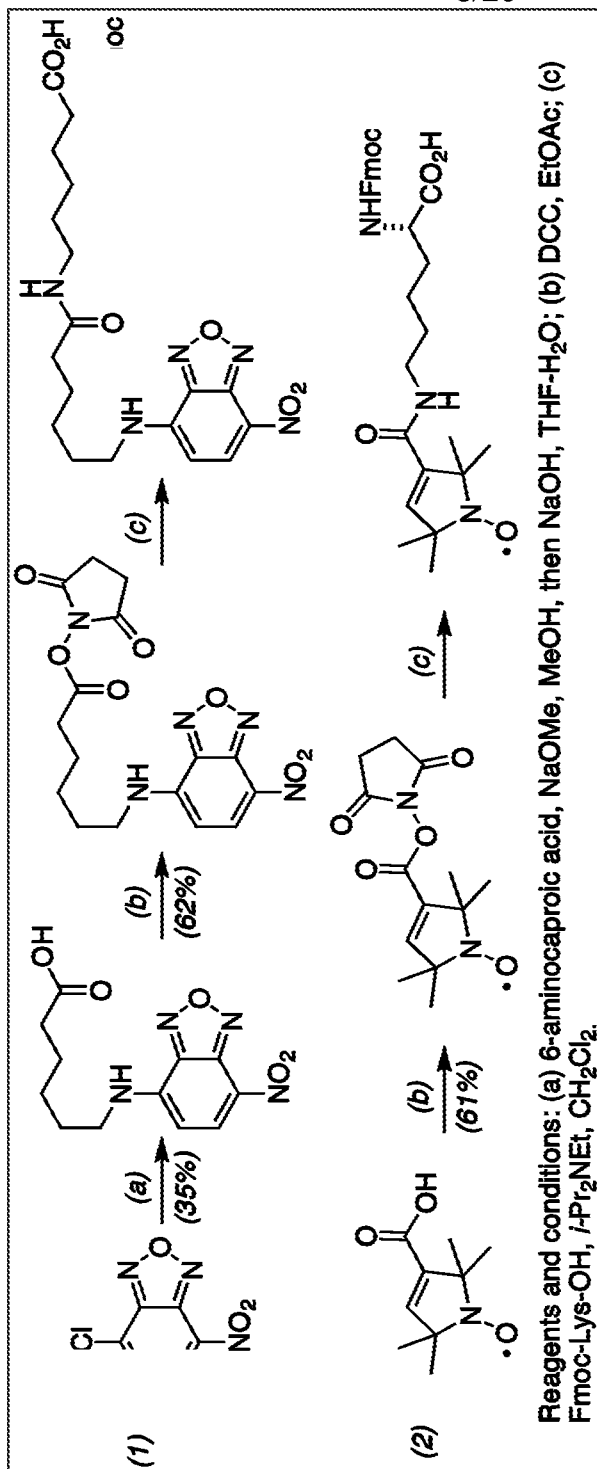
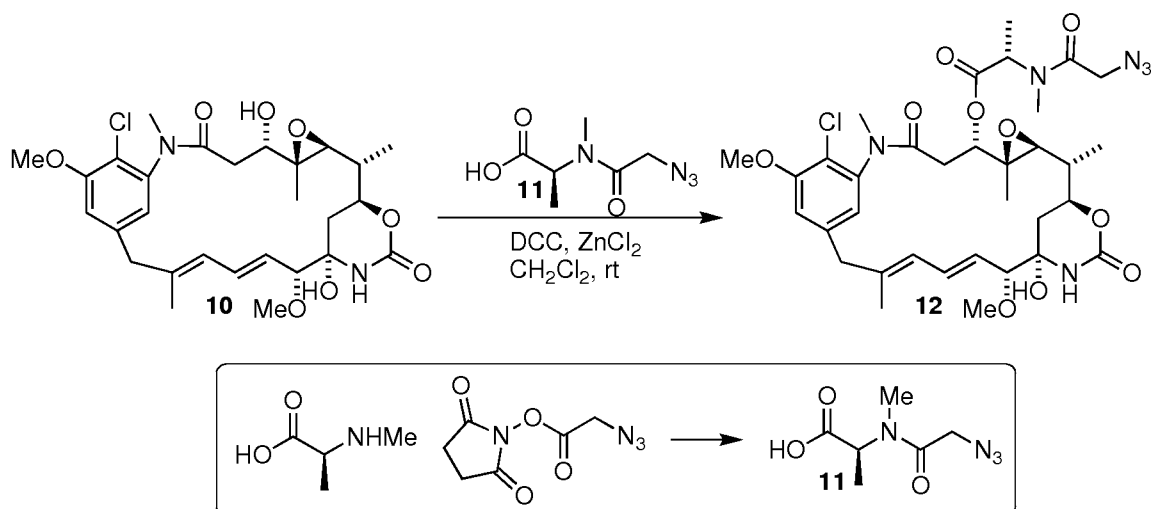


FIG. 3

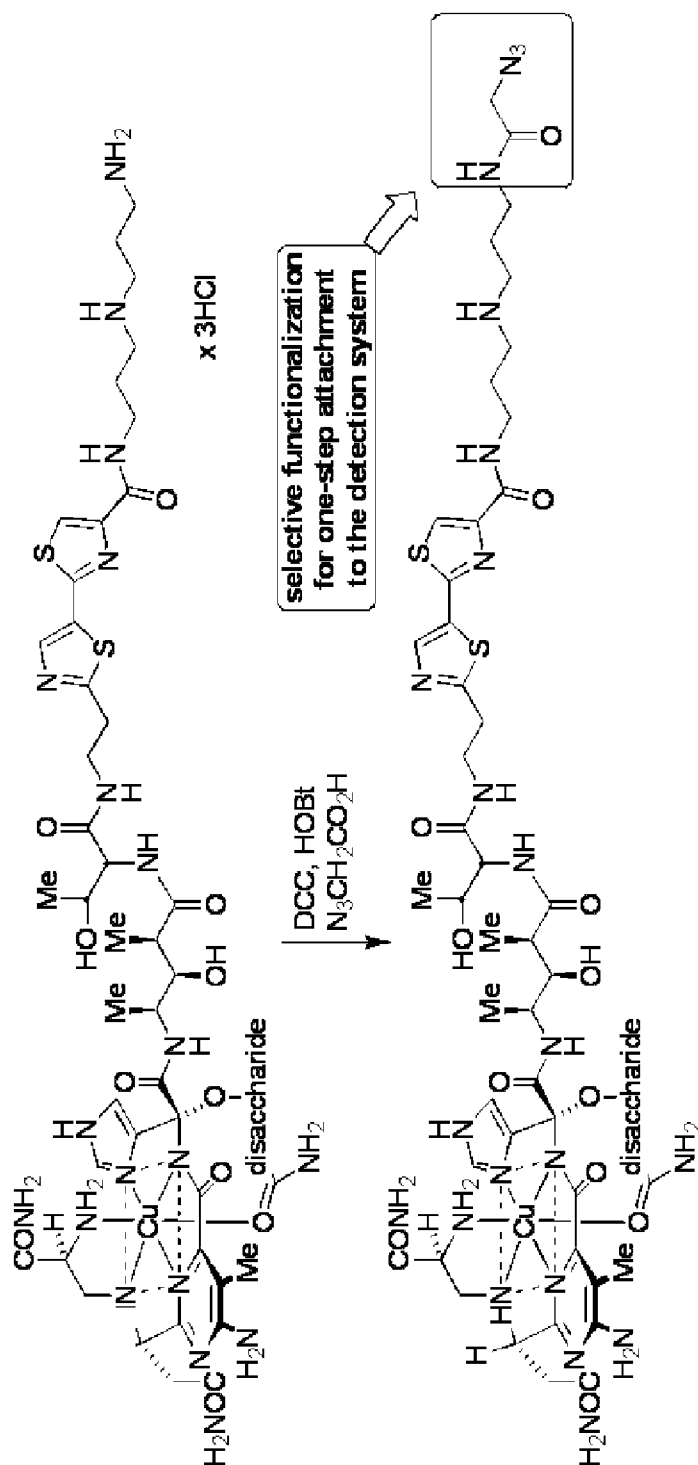
4/26



Azido-functionalized maytansine

FIG. 4

5/26



One-step functionalization of bleomycin

FIG. 5

6/26

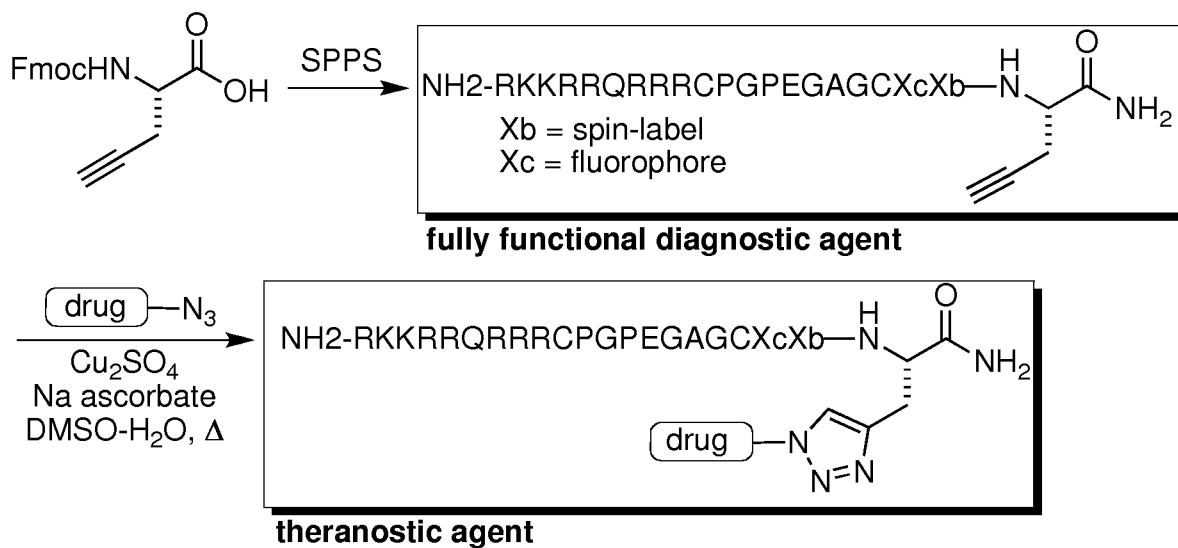
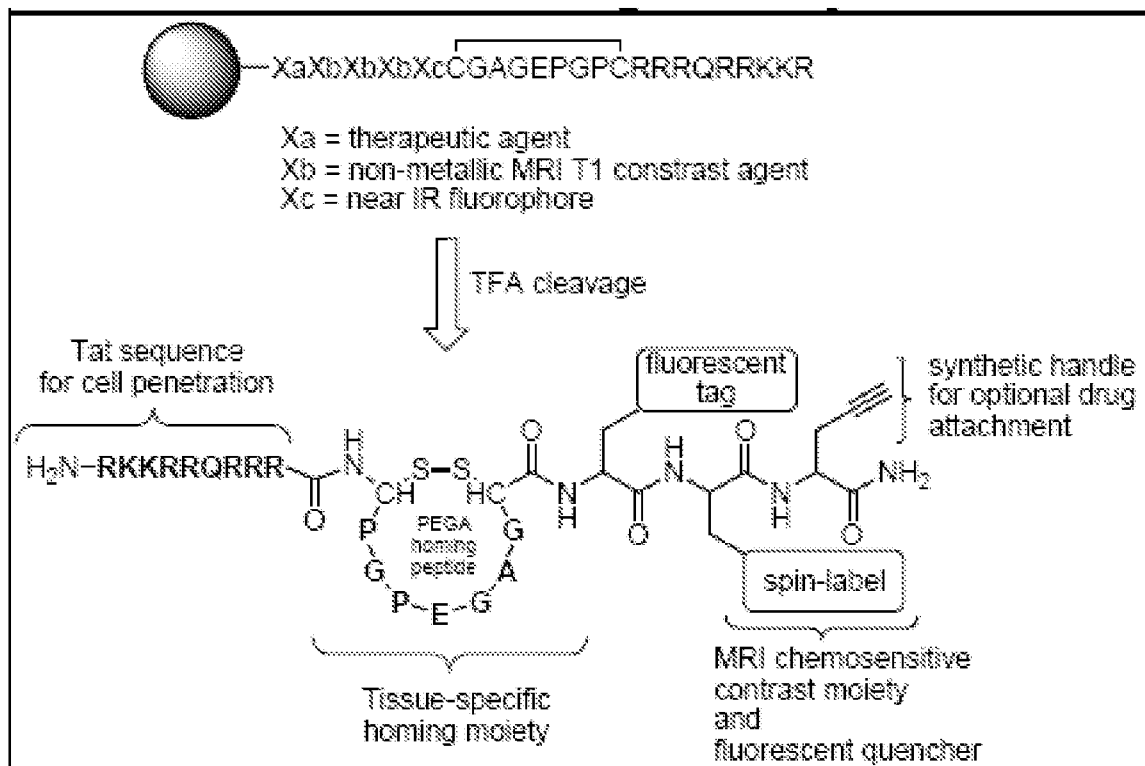


FIG. 6

7/26



Solid phase peptide synthesis of the diagnostic system

FIG. 7

8/26

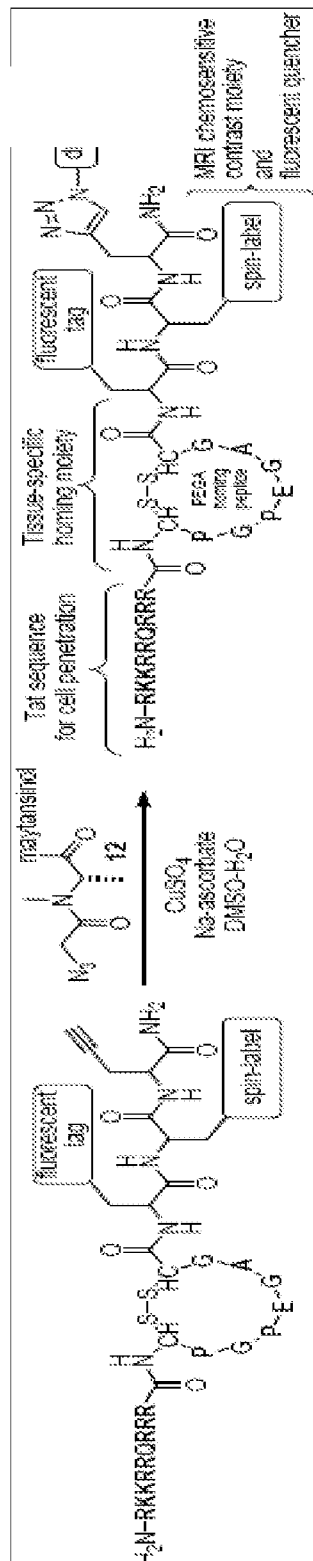


FIG. 8

9/26

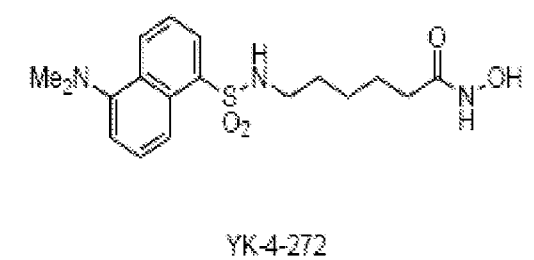
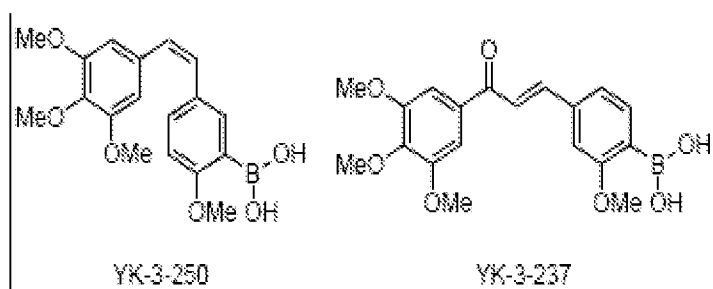
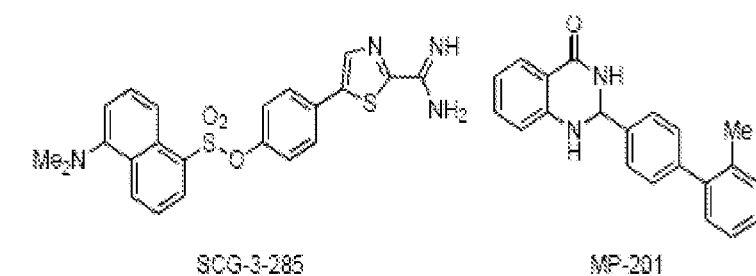


FIG. 9

10/26

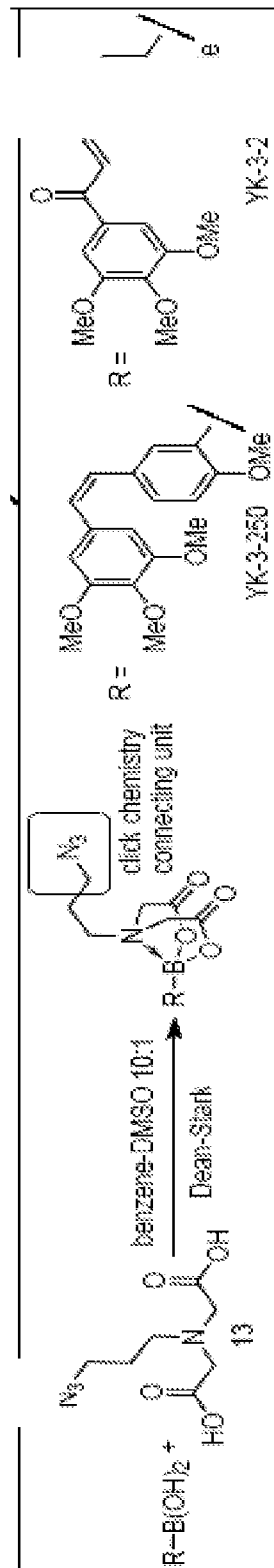


FIG. 10

11/26

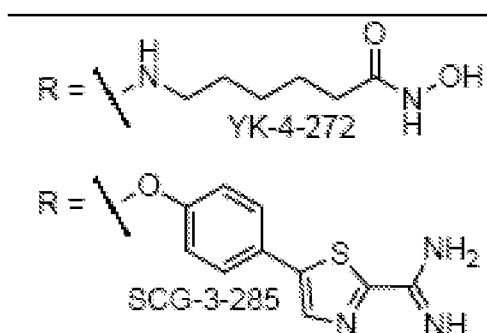
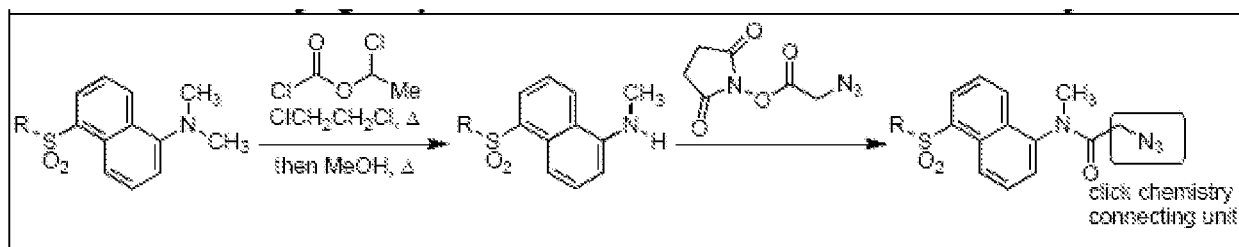


FIG. 11

12/26

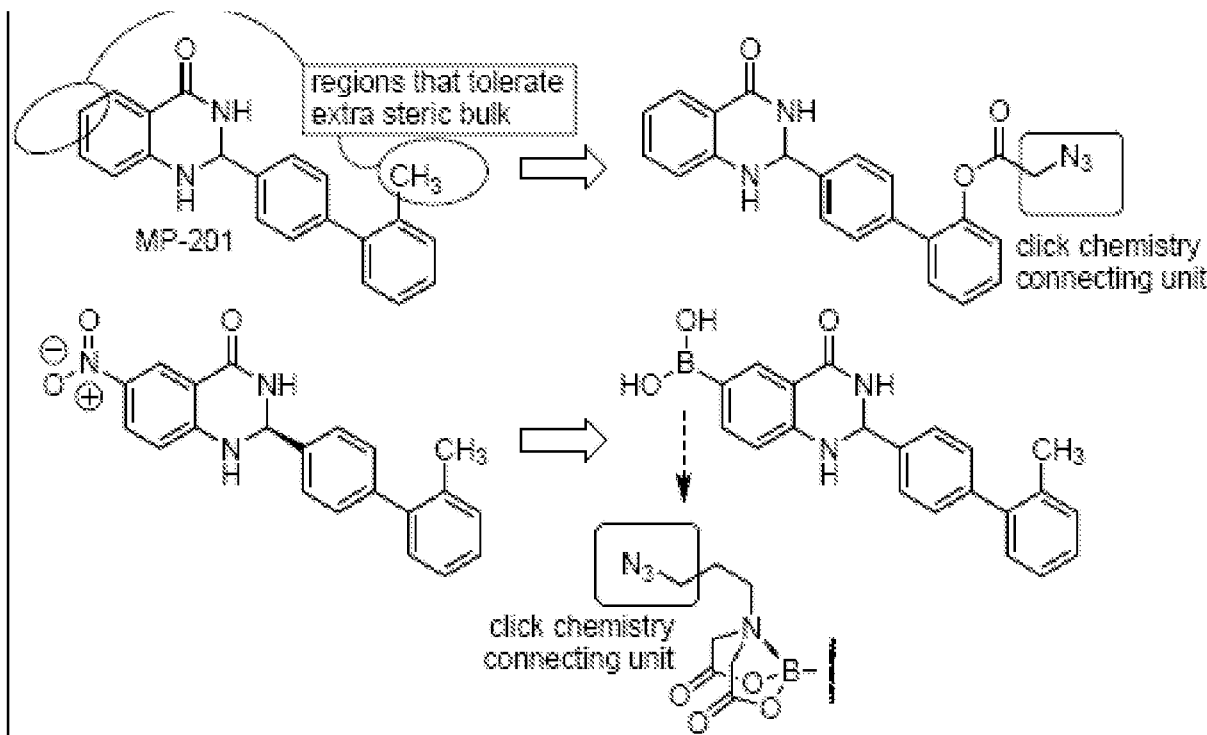
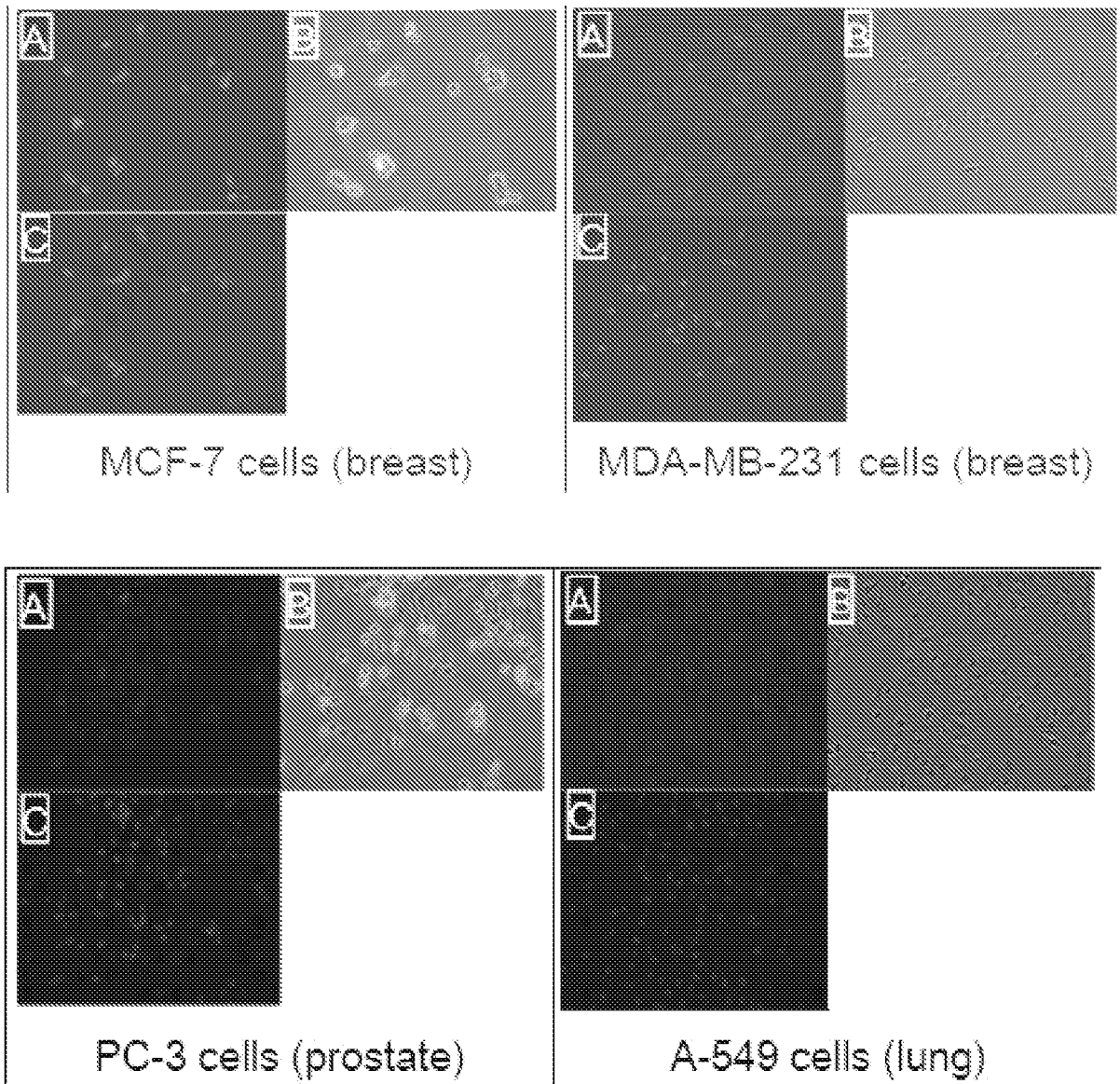


FIG. 12

13/26



14/26

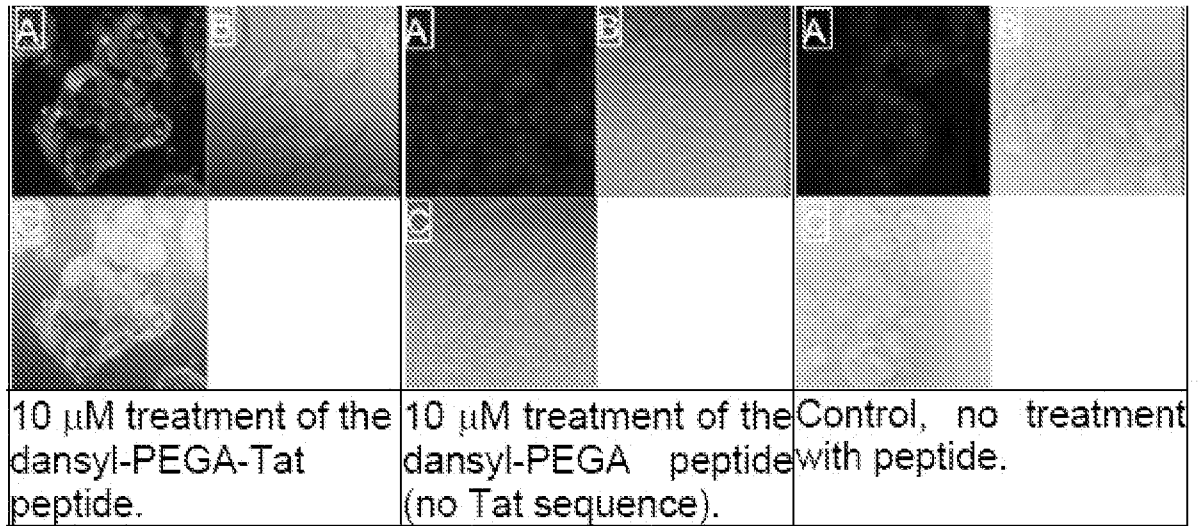


FIG. 14A, FIG. 14B and FIG. 14C

15/26

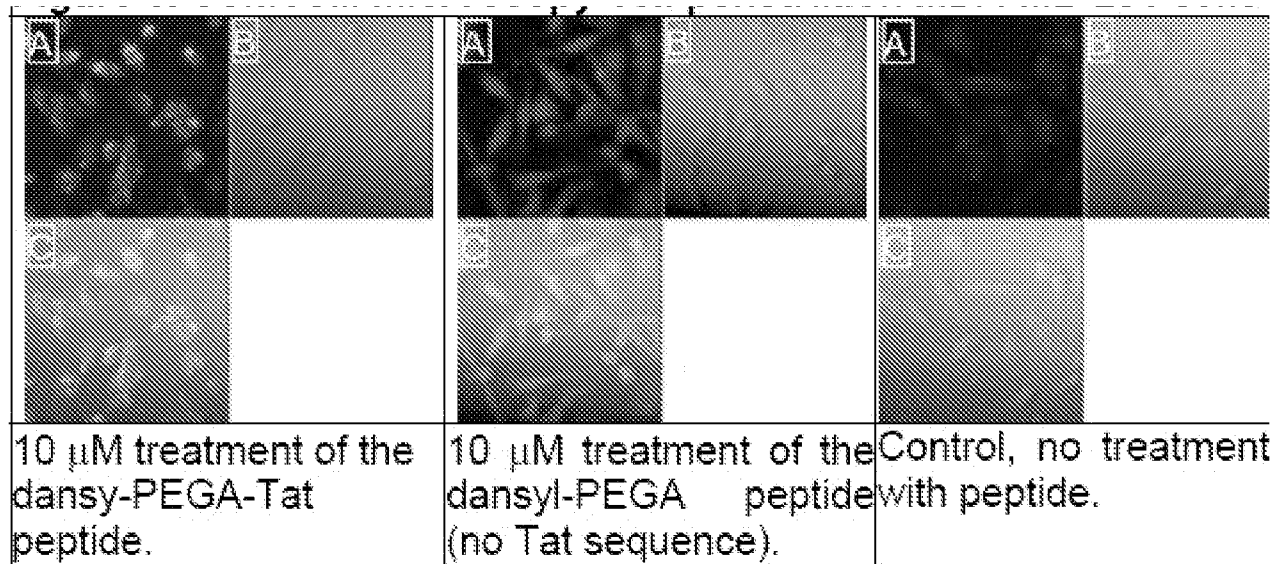


FIG. 15A, FIG. 15B and FIG. 15C

16/26

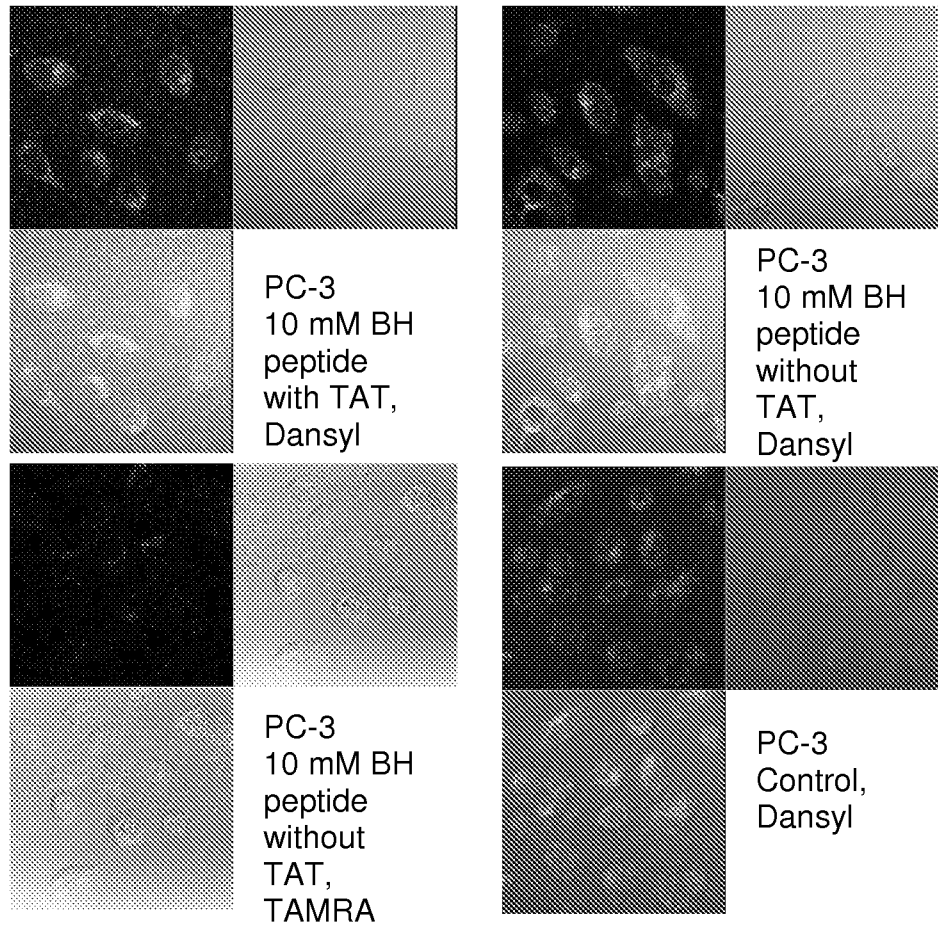


FIG. 16

17/26

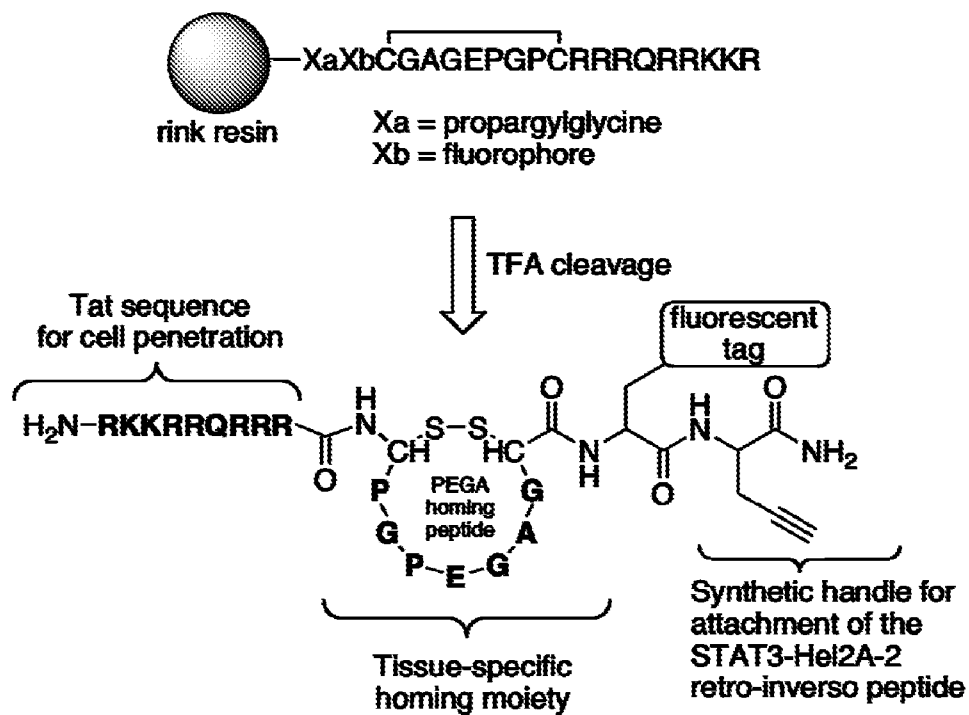


FIG. 17

18/26

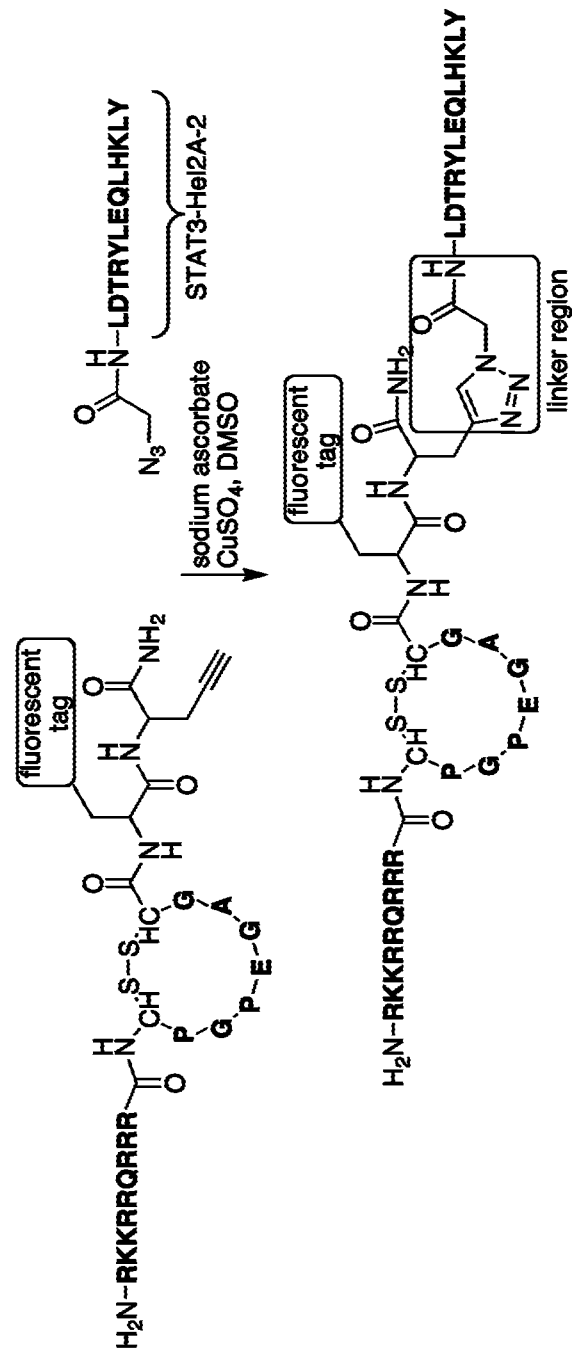


FIG. 18

19/26

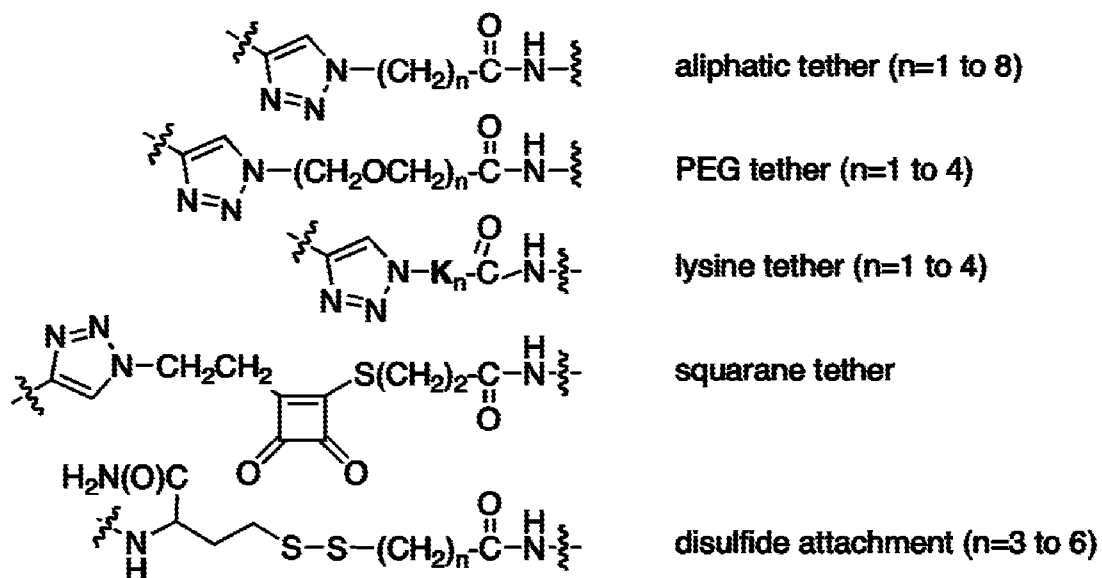


FIG. 18 continued

20/26

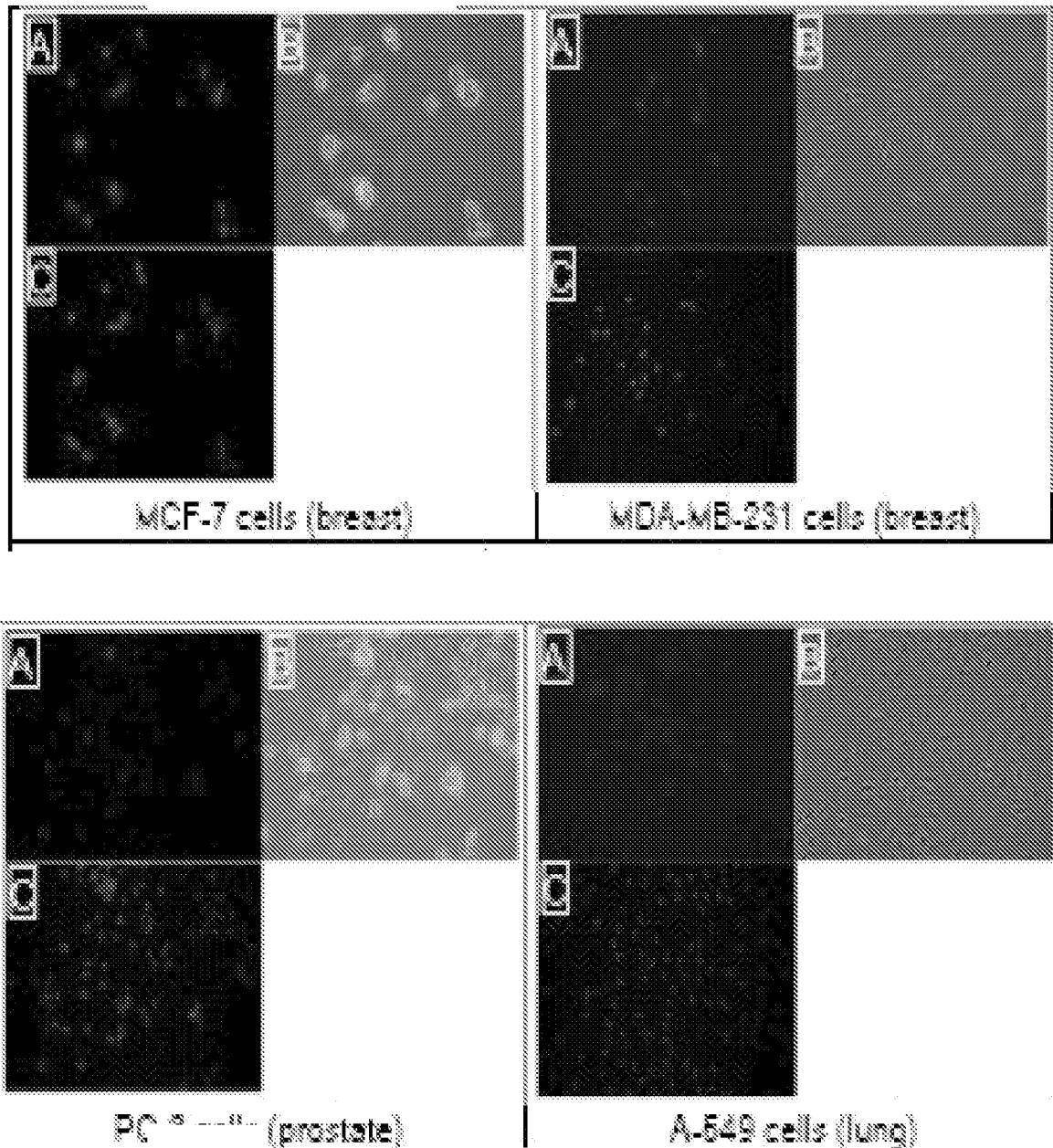


FIG. 19A, FIG. 19B, FIG. 19C

21/26

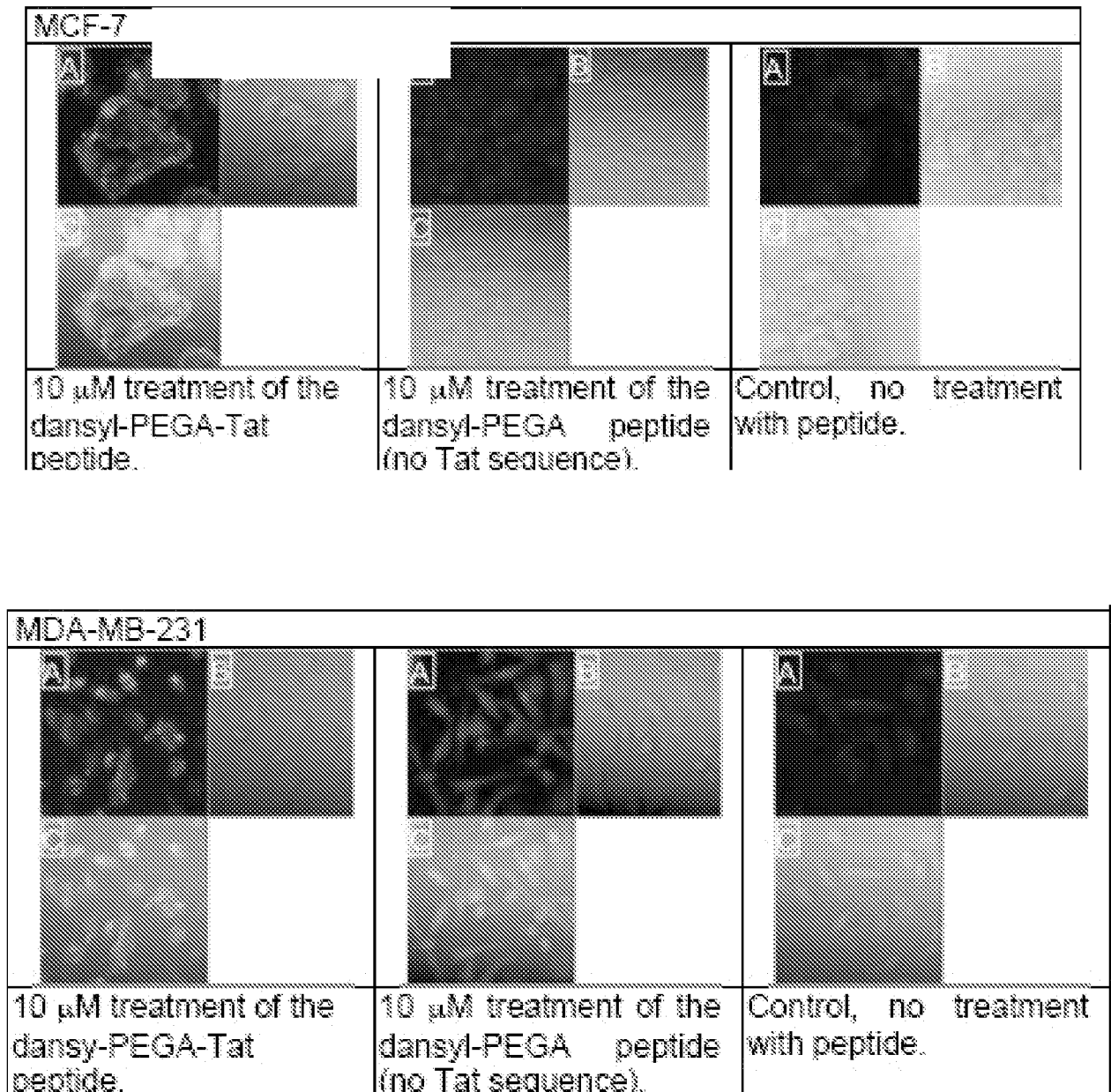


FIG. 20A, FIG. 20B and FIG. 20C

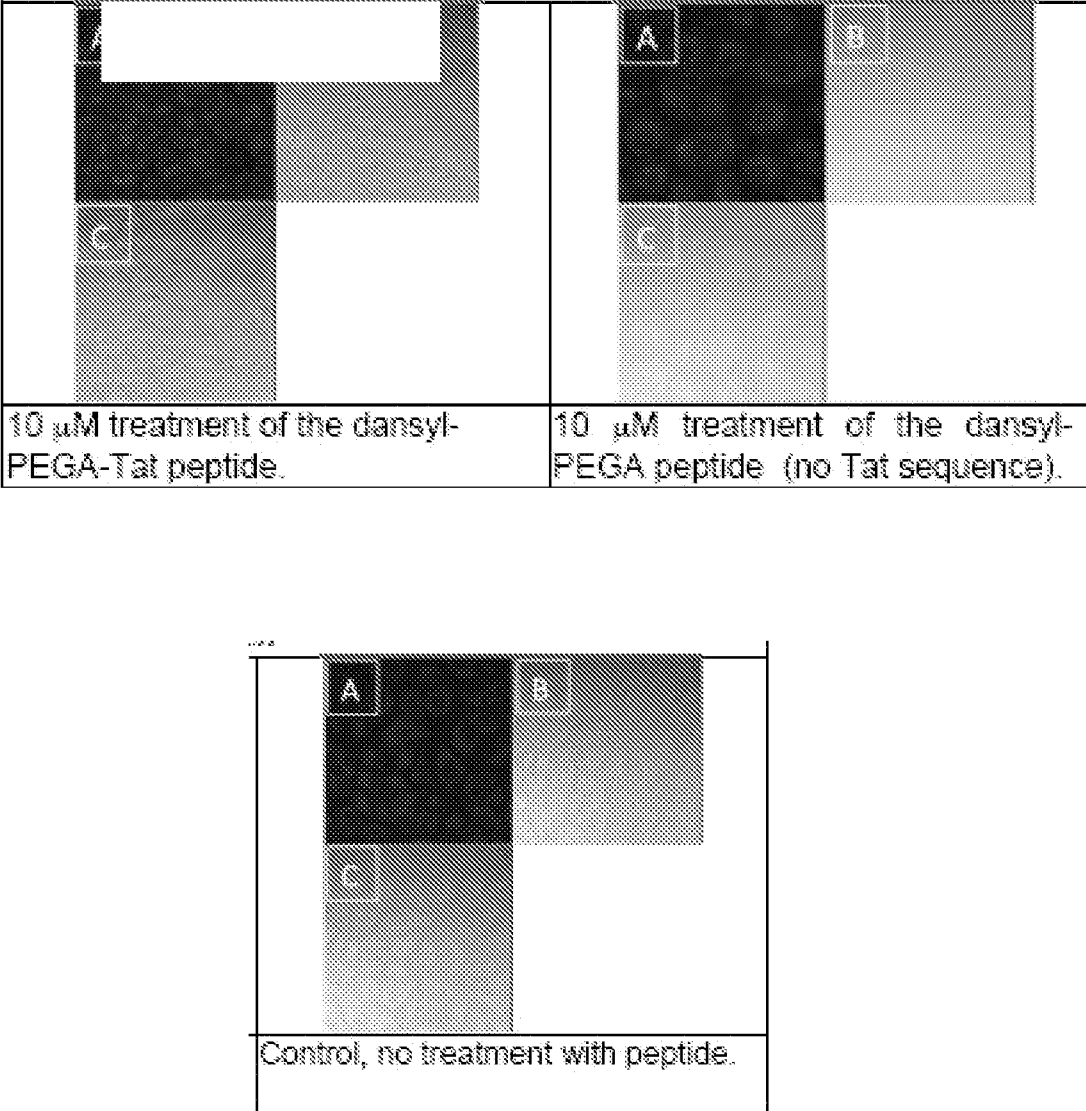


FIG. 21A, FIG. 21B, FIG. 21C

23/26

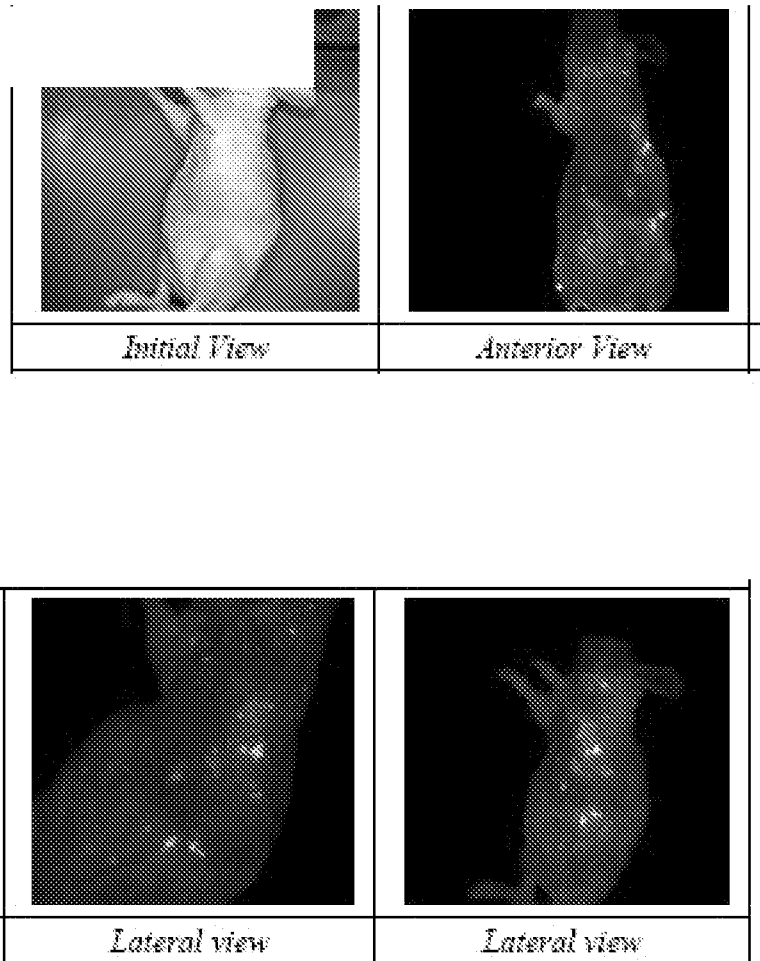


FIG. 22

24/26

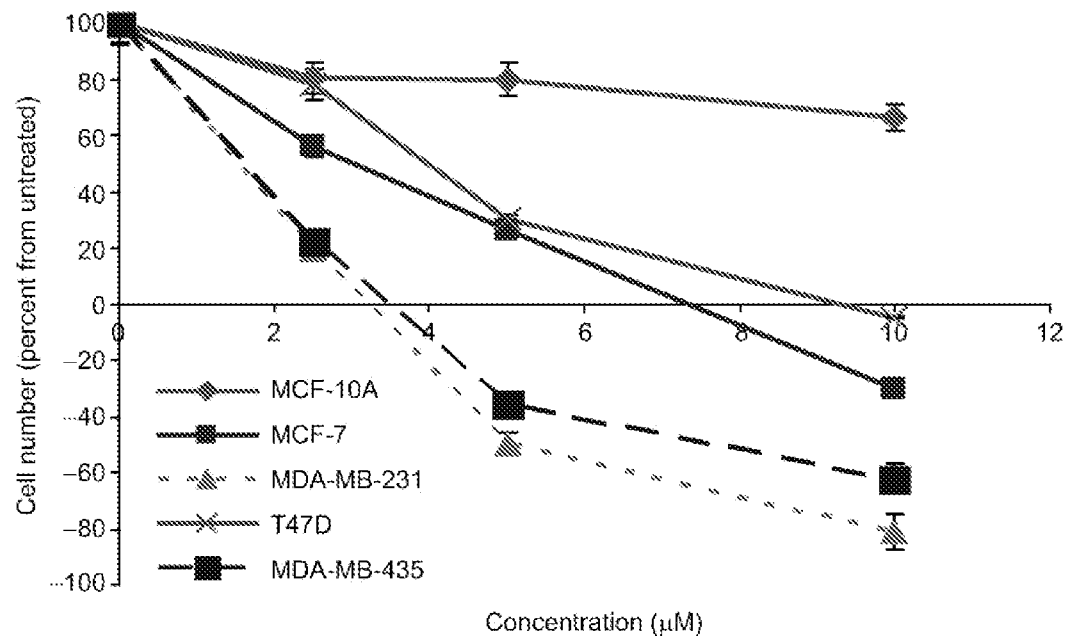


FIG. 23

25/26

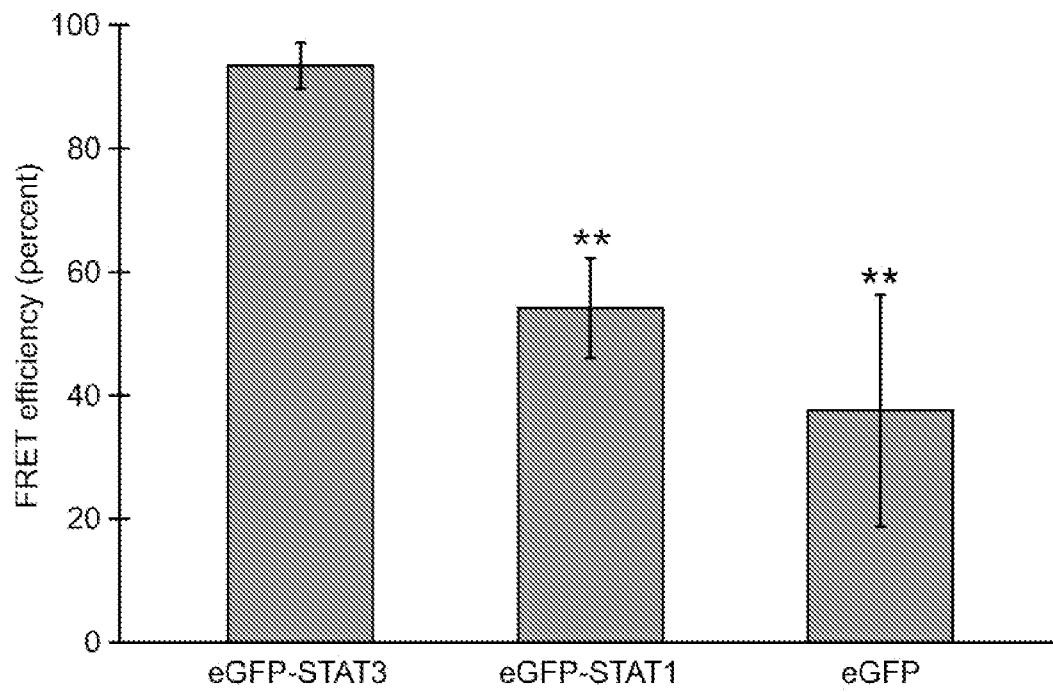


FIG. 24

26/26

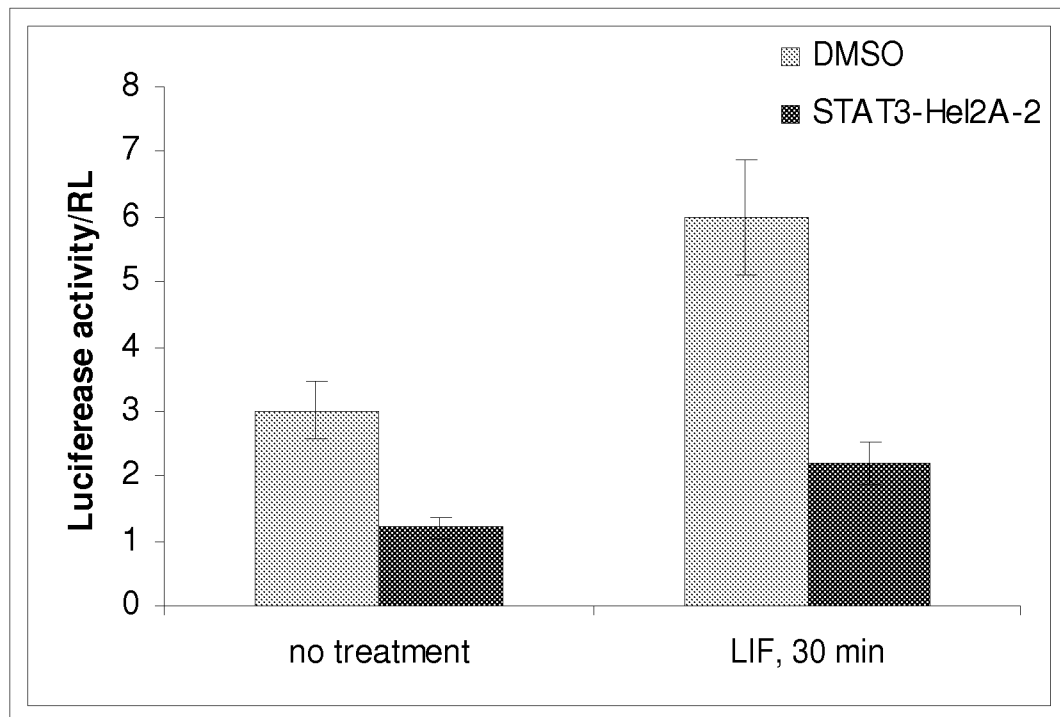


FIG. 25