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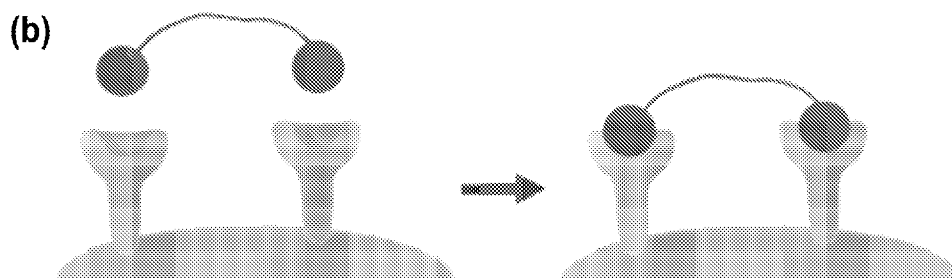


FIG . 1B

(57) Abstract: Methods for probing the distribution and distance of the cell surface receptors and using the same to make multivalent ligands targeting said receptors are disclosed. Methods for making the multivalent ligands and detecting or imaging cells expressing receptors using the compounds are also provided.

MULTIVALENT LIGANDS TARGETING CELL SURFACE RECEPTORS AND FORCE MEASUREMENT PLATFORM FOR MAKING THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims priority to U.S. Provisional Patent Application No. 62/967,329, filed January 29, 2020, which is incorporated herein by reference in its entirety.

FIELD

The disclosed subject matter relates generally to force measurement platform to probe the 10 distribution and distance of the cell surface receptors and using the same to make multivalent ligands targeting said receptors.

BACKGROUND

Multivalency governs many biological interactions and has been widely applied to synthetic compounds in support of targeted molecular imaging and drug therapy. A number of 15 multivalent binding mechanisms could be used to acquire high binding affinity, including chelate effect, statistical effect, steric stabilization, subsite binding, and receptor clustering. The type of multivalent binding is largely determined by the selection of linkers tethering active entities. Although proper architectural design to support multiple binding plays a key role for the affinity, the length of the linker largely determines the statistical or the chelate effect in the interactions 20 (Figure 1). In terms of multivalent ligands targeting cell-surface receptors, however, lack of knowledge on receptor distribution and distance in live cells often impedes the rational selection of linkers and scaffold architectures to fit into the relevant binding mechanisms for construction of multivalent ligands.

What are needed are measurement platforms to determine spatial information of cell-25 surface receptors. For example, spatial information on the interaction of drug compounds with vascular endothelial growth factor receptors (VEGFR) in cells are needed. Further, compounds that bind selectively to VEGFR expressing cells are also needed. The compositions and methods disclosed herein address these and other needs.

SUMMARY

30 Interaction of multiple moieties and receptors, or multivalency is widely applied to achieve high affinity ligands for diagnostic and therapeutic purposes. However, lack of knowledge on receptor distribution in living subjects remains a challenge for rational structure design. Disclosed herein is a force measurement platform to probe the distribution and distance

of cell surface receptors in live cells. For example, the spatial distribution and distance of vascular endothelial growth factor receptors (VEGFR) were used to assess the geometry of appropriate linkers for distinct multivalent binding modes. The tetravalent compound, ZD-4, yielded about 2000-fold improvement in the binding affinity to VEGFR with IC_{50} value of 25 pM. The improved affinity was confirmed by the associated increase of tumor uptake in the VEGFR-targeting positron emission tomography (PET) imaging using U87 tumor xenograft mouse model. In particular, radiolabeled ZD-4 resulted in 12 times increase in the tumor uptake than radiolabeled antitumor drug ZD6474 (vandetanib). Overall, this disclosure combines statistical and chelate effect determined by receptor-receptor distance on live cells for the magnification of multivalent binding to develop effective compounds that bind selectively to VEGFR expressing cells. The force measurement platform is also amenable to other cell-surface multivalent ligand design. The methods can also be used to determine the spatial information of other cell-surface receptors such as PD-L1, EphB4 in cancer cells, PD1 CTLA4 in T cells, integrin, angiotensin-converting enzyme (ACE) (such as in SARS-Cov-2 infection), estrogen receptor (ER), or a combination thereof.

In some aspects of the disclosure, the method for determining the spatial distribution of receptors on a cell comprises functionalizing an AFM tip with one or more receptor binding moieties to form a functionalized AFM tip; contacting the functionalized AFM tip with the cell to facilitate binding of the one or more receptor binding moieties with the receptors and form a binder-receptor complex; determining the number of each receptor distributed in an area of the AFM tip, and deriving a maximum distance between two neighboring receptors. In specific aspects, the method includes conjugating a receptor binding moiety to a functional group to form a functional-binder conjugate; functionalizing an AFM tip with the functional-binder conjugate or a diluted solution of the functional-binder conjugate to form a functionalized AFM tip; contacting the functionalized AFM tip with the cell to facilitate binding of the functional-binder conjugate with the receptors and form a binder-receptor complex; using adhesive force measurements to determine a dissociative force of an ensemble of the binder-receptor complex and of a single binder-receptor complex, determining the number of each receptor distributed in an area of the AFM tip, and deriving a maximum distance between two neighboring receptors using maximizing minimum algorithm.

The multivalent ligands can be such that each ligand target the same or a different receptor. Therefore, the receptors used in probing the spatial distribution can be the same or different. For example, the receptors can be selected from VEGFR, PD-L1, EphB4 (such as in cancer cells), EphA4, PD1 CTLA4 (such as in T cells), integrin, angiotensin-converting enzyme

(ACE) (such as in SARS-Cov-2 infection), estrogen receptor (ER), or a combination thereof. The receptor binding moiety can be selected from a small molecule therapeutic agent, a peptide, an antibody, an antibody fragment, a carbohydrate, an siRNA, a protein, a nucleic acid, an aptamer, a nanoparticle, a cytokine, a chemokine, a lymphokine, a lipid, a lectin, or a combination thereof.

In certain aspects of the disclosure, the method for determining the spatial distribution of VEGFR in a cell comprises conjugating a VEGFR binding moiety to a functional group such as a thiol group to form a functional-binder conjugate; functionalizing an AFM tip with the functional-binder conjugate or a diluted solution of the functional-binder conjugate to form a functionalized AFM tip; contacting the functionalized AFM tip with the cell to facilitate binding of the functional-binder conjugate with VEGFR and form a binder-VEGFR complex; using adhesive force measurements to determine a dissociative force of an ensemble of the binder-VEGFR complex and of a single binder-VEGFR complex; determining the number of VEGFR distributed in an area of the AFM tip; and deriving a maximum distance between two neighboring VEGFR using maximizing minimum algorithm.

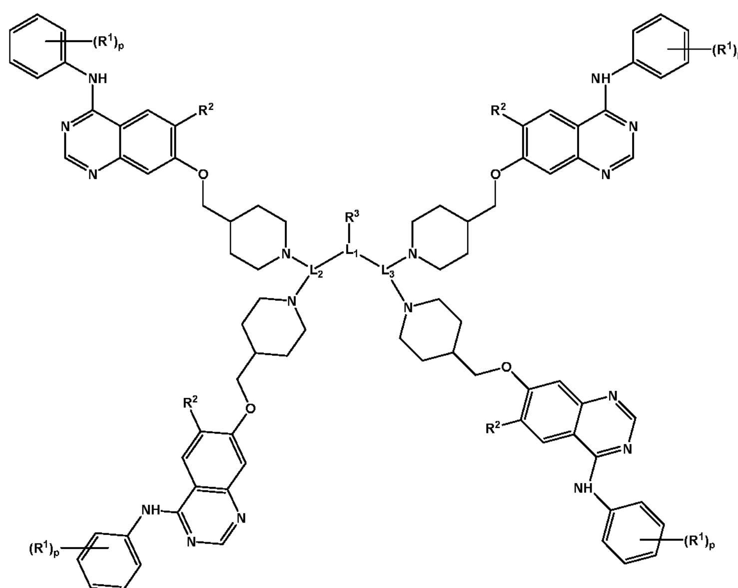
Methods for making multivalent compounds that target receptors on a cell surface are also disclosed herein. The methods for making the multivalent compounds can comprise determining the proximal receptor distance between two or more receptors on the cell surface using AFM; synthesizing a multivalent compound comprising a first moiety and a second moiety covalently linked by a first linker, L_1, wherein the first moiety comprises one or more receptor binding moieties, and the second moiety comprises an additional one or more receptor binding moieties, and wherein the first linker has a length not substantially less than, equal to, or greater than the proximal receptor distance. As described herein, the two or more receptors can be the same or different, based on the receptors being targeted. Thus, the first moiety and the second moiety can be the same or different. The first linker can have a length within 20%, preferably within 15%, more preferably within 10%, of the proximal receptor distance. Preferably, the first linker has a length within 5% of the proximal receptor distance. The multivalent compounds can have a binding affinity to the receptor that is greater than 10 times, greater than 100 times, greater than 1,000 times, or greater than 2,000 times the binding affinity of an equivalent single-valent compound to the receptor.

Also disclosed herein are multivalent compounds that target receptors for therapy and imaging of receptor expression. In some aspect, the compound is produced by a spatial distribution method as described herein and comprises at least a first moiety and a second moiety covalently linked by a first linker, L_1. Each of the first moiety and the second moiety can

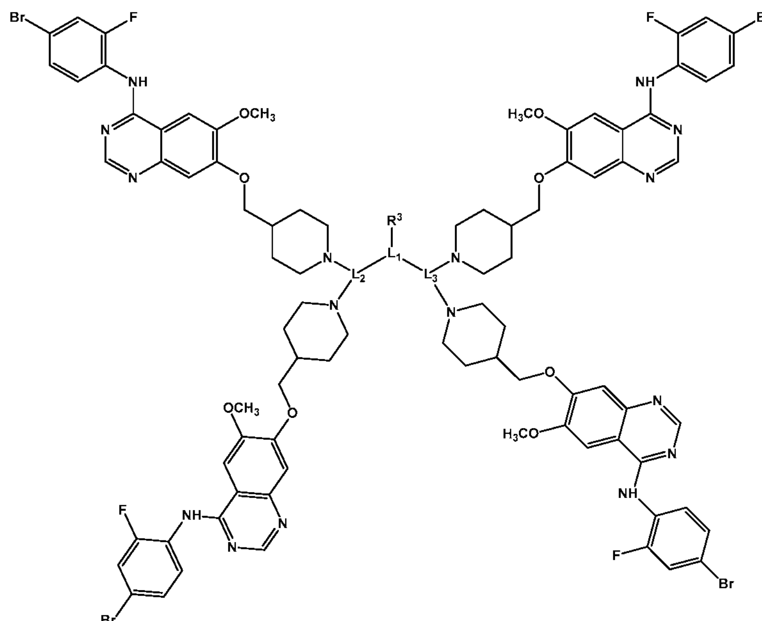
comprise a receptor binding moiety for a receptor selected from VEGFR, PD-L1, EphB4 (such as in cancer cells), PD1 CTLA4 (such as in T cells), integrin, angiotensin-converting enzyme (ACE) (such as in SARS-Cov-2 infection), estrogen receptor (ER), or a combination thereof.

In certain aspects, multivalent compounds that target vascular endothelial growth factor receptors (VEGFR) for therapy and imaging of VEGFR expression. For example, the multivalent compounds can be tetravalent and comprises a first moiety and a second moiety covalently linked by a first linker, L_1, wherein the first moiety comprises two or more VEGFR binding moieties linked by a second linker, L_2, and the second moiety comprises an additional two or more VEGFR binding moieties linked by a third linker, L_3. The geometry of the linkers, L_1, L_2, or L_3, are such that they promote multi-binding of the VEGFR binding moieties to multiple receptors. In some embodiments, each of L_1, L_2, or L_3 can independently have a length from 7 to 60 Å, such as from 40 to 60 Å, or from 7 to 25 Å. The VEGFR binding moieties can be selected from the group consisting of bevacizumab; sunitinib; aflibercept; pazopanib; axitinib; sorafenib; vandetanib; regorafenib; ramucirumab, and combination thereof.

In a specific aspect, compounds of Formula I and Formula II are disclosed:



Formula I



Formula II

wherein,

R^1, independently for each occurrence, is selected from hydroxyl, halogen, C_{1-3} alkyl, C_{1-3} alkoxyl, C_{1-3} alkanoyloxy, trifluoromethyl, cyano, amino, or nitro;

R^2, independently for each occurrence, is selected from hydrogen, hydroxyl, halogen, nitro, trifluoromethyl, cyano, C_{1-3} alkyl, C_{1-3} alkoxyl, C_{1-3} alkylthio, or $-NR^7R^8$, wherein R^7 and R^8, which can be the same or different, each represents hydrogen or C_{1-3} alkyl;

R^3 is absent or comprises a detectable moiety or therapeutic moiety;

p is an integer from 1 to 4; and

L_1, L_2, and L_3 are linkers, independently selected from $N(R^{14})_3$, $CH(R^{14})_3$, $Ar(R^{14})_3$, wherein Ar is aryl, and wherein R^{14} for each occurrence, is independently selected from a bond, hydrogen, amido, C_1-C_{20} alkyl; C_1-C_{20} heteroalkyl, C_1-C_{20} alkylamine, C_1-C_{20} alkoxy, polyalkyleneoxy, C_1-C_{20} alkanoyloxy, C_1-C_{20} alkylamido, aryl, or heteroaryl; wherein each R^{14} independent of the other, is optionally substituted with one or more substituents selected from the group consisting of halogen; hydroxyl; cyano; carbonyl; nitro; amino; amido; alkylamino; dialkylamino; alkylamido; =O; $-S(O)_2$; -SO-; -S-; $-S(O)_2N-$; haloalkyl; hydroxyalkyl; carboxy; alkyl, alkoxyl; aryloxyl; alkoxycarbonyl; aminocarbonyl; alkylaminocarbonyl; dialkylaminocarbonyl; aryl; heteroaryl; and combinations thereof;

wherein R^3 when present is bonded to at least one R^{14}.

The detectable moiety can be a near-infrared label, a fluorescent label, a radiolabel, a magnetic spin resonance label, a chromophore, a VEGFR ligand, or any combination thereof.

The therapeutic moiety can be radioisotopes for radiation therapy such as Y-90 or Lu-177 etc. or a chemotherapy drug.

Methods for making the disclosed compounds and detecting or imaging cells expressing VEGFR using these compounds are also disclosed. The cells expressing VEGFR can be cancer cells, hyperproliferative cells, or any combination thereof. In other embodiments, the cells expressing VEGFR can be present in an animal diagnosed with pulmonary hypertension or who had a transplantation procedure.

Additional advantages will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the subject matter disclosed herein. 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.

BRIEF DESCRIPTION OF THE FIGURES

The accompanying figures, which are incorporated in and constitute a part of this specification, illustrate several embodiments of the invention and together with the description, serve to explain the principles of the disclosed subject matter.

Figures 1A-1B show two effects that govern bivalent binding to cell-surface receptors: (Figure 1A) statistical effect, arisen from increased local concentration of active entities tethered by short linker in bivalent ligands; (Figure 1B) chelate effect, arisen from simultaneously binding multiple receptors by active entities tethered by long linker in bivalent.

Figures 2A-2B show structures of the ZD6474 probe and thiol PEG that were used in the functionalization of ATM tips (Figure 2A), and a schematic drawing for AFM force measurement of ZD6474 binding to VEGFR (Figure 2B). Tips functionalized by various concentrations of ZD6474 probe diluted with thiol PEG, such as the 100%-tip and 5%-tip depicted in Figure 2B were prepared.

Figures 3A-3F show the force measurement of ZD6474 to VEGFR in live HUVECs. Measurements that used AFM tips functionalized with 100% (Figures 3A, 3C, and 3E) and 5% (Figures 3B, 3D, and 3F) concentration of ZD6474 probe are shown and compared. Figures 3A and 3B show 3D height images of the cells obtained during the AFM force measurement; Figures 3C and 3D show adhesive force mapping presented as 3D images corresponding to the height images in each group; Figures 3E and 3F show histograms of ZD6474/VEGFR specific dissociative forces obtained from statistics of the area presented as the specific binding. 1024 histogram bins were used.

Figure 4 is a scheme showing chemical structures of compounds ZD-2, ZD-3, ZD-4, and ZD-5.

Figure 5 shows competition binding curves of compounds ZD-1 through -5 to HUVECs. In the binding assay, ^{64}Cu-ZD6474 was used as binding probe and the ZD compound from the design was individually supplemented as competition agent. The IC_{50} was calculated from the competitive binding curve. Data shown are represented as mean ± SD (n = 6 per group).

Figures 6A-6C show whole-body microPET/CT image of U87 glioblastoma-bearing mice. Figure 6A shows representative PET/CT images of the tumor-bearing mice were displayed 24 h p.i. of ^{64}Cu-ZD6474 and ^{64}Cu-ZD4 radiotracers. Tumor is indicated by a white arrowhead. Figure 6B shows comparison of decay-corrected ROI analysis of ^{64}Cu-ZD6474 and ^{64}Cu-ZD4 in tumor. Data shown are represented as mean ± SD (n = 6 per group, $P < 0.0001$). Figure 6C shows the imaged U87 glioblastoma xenograft was histologically studied using hematoxylin and eosin (H&E) and VEGFR2-antibody immunohistochemistry (VEGFR2 IHC) staining.

Figure 7 shows therapy data obtained from a triple native breast cancer model after administering ^{177}Lu-DiZD and anti-PD1.

Figure 8 is a flow diagram showing method of using AFM to design conforming compounds.

Figure 9 shows AFM height and force mapping of HUVEC cells using 100% IA monomer functionalized AFM tip.

Figure 10 shows AFM height and force mapping of MDA-MB-468 cells using 100% ephb4 monomer functionalized AFM tip.

DETAILED DESCRIPTION

The disclosed subject matter can be understood more readily by reference to the following detailed description and the Examples included herein and to the Figures and their previous and following description.

Definitions

Before the present compounds, compositions, and methods are disclosed and described, it is to be understood that this invention is not limited to specific synthetic methods, or specific route of administration, 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.

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 invention pertains.

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 compound” includes mixtures of such compounds and the like.

Ranges may be expressed herein as from “about” one particular value, and/or to “about” another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent “about,” it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. Unless stated to the contrary, the term “about” means within 5%, *e.g.*, within 1, 2, 3, or 4 % of the stated value, or less.

In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined to have the following meanings:

“Optional” or “optionally” means that the subsequently described event or circumstance does or does not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not. For example, the phrase “optionally substituted alkyl” means that the alkyl group is or is not substituted and that the description includes both unsubstituted lower alkyl and lower alkyl where there is substitution.

An “effective amount”, *e.g.*, of the compounds or compositions described herein, refers to an amount of the compound in a composition or formulation which, when administered as part of a desired dosage regimen, brings about a change, *e.g.*, in the rate of cell proliferation and/or the state of differentiation of a cell and/or rate of survival of a cell according to clinically acceptable standards for the disorder to be treated or, *e.g.*, is taken up in a sufficient amount by VEGFR expressing cells such that the cells can be imaged by confocal microscopic imaging, CT imaging, PET imaging, MRI, or any combination thereof according to clinically acceptable standards for imaging cells.

“Pharmaceutically acceptable” refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues, organs, and/or bodily fluids of human beings and animals without excessive toxicity, irritation, allergic response, or other problems or complications

commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable materials are known to those of ordinary skill in the art.

"Half maximal inhibitory concentration" or "IC_{50}", as used herein, refers to a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, *i.e.* an enzyme, cell, cell receptor or microorganism) by half. According to the FDA, IC_{50} represents the concentration of a drug that is required for 50% inhibition *in vitro*. The IC_{50} can be determined using a variety of assays known in the art.

"Analog" and "derivative" are used herein interchangeably and refer to a compound that possesses the same core as the parent compound, but differs from the parent compound in bond order, the absence or presence of one or more atoms and/or groups of atoms, and combinations thereof. The derivative can differ from the parent compound, for example, in one or more substituents present on the core, which can include one or more atoms, functional groups, or substructures. In general, a derivative can be imagined to be formed, at least theoretically, from the parent compound via chemical and/or physical processes.

"Aliphatic", as used herein, refers to saturated or unsaturated groups containing carbon and hydrogen, including straight-chain alkyl, alkenyl, or alkynyl groups, branched-chain alkyl, alkenyl, or alkynyl groups, cycloalkyl, cycloalkenyl, or cycloalkynyl (alicyclic) groups, alkyl substituted cycloalkyl, cycloalkenyl, or cycloalkynyl groups, and cycloalkyl substituted alkyl, alkenyl, or alkynyl groups. Unless otherwise indicated, a straight chain or branched chain aliphatic group has 30 or fewer carbon atoms in its backbone (*e.g.*, C_1-C_{30} for straight chain, C_3-C_{30} for branched chain), more preferably 20 or fewer carbon atoms, more preferably 12 or fewer carbon atoms, and most preferably 8 or fewer carbon atoms. In some embodiments, the chain has 1-6 carbons. Likewise, preferred cycloalkyls have from 3-10 carbon atoms in their ring structure, and more preferably have 5, 6, or 7 carbons in the ring structure. The ranges provided above are inclusive of all values between the minimum value and the maximum value.

The term "alkyl" includes both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having one or more substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents include, but are not limited to, halogen, hydroxyl, carbonyl (such as a carboxyl, alkoxycarbonyl, formyl, or an acyl), thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), alkoxyl, phosphoryl, phosphate, phosphonate, a phosphinate, amino, amido, amidine, imine, cyano, nitro, azido,

sulfhydryl, alkylthio, sulfate, sulfonate, sulfamoyl, sulfonamido, sulfonyl, heterocyclyl, aralkyl, or an aromatic or heteroaromatic moiety.

Unless the number of carbons is otherwise specified, “lower alkyl” as used herein means an alkyl group, as defined above, but having from one to ten carbons, more preferably from one to six carbon atoms in its backbone structure. Likewise, “lower alkenyl” and “lower alkynyl” have similar chain lengths. Preferred alkyl groups are lower alkyls.

The alkyl groups can also contain one or more heteroatoms within the carbon backbone. Examples include oxygen, nitrogen, sulfur, and combinations thereof. In certain embodiments, the alkyl group contains from one to four heteroatoms.

The term “heteroalkyl”, as used herein, refers to straight or branched chain, or cyclic carbon-containing radicals, or combinations thereof, containing at least one heteroatom. Suitable heteroatoms include, but are not limited to, O, N, Si, P, Se, B, and S, wherein the phosphorous and sulfur atoms are optionally oxidized, and the nitrogen heteroatom is optionally quaternized. Heteroalkyls can be substituted as defined above for alkyl groups.

The term “alkylthio” refers to an alkyl group, as defined above, having a sulfur radical attached thereto. In preferred embodiments, the “alkylthio” moiety is represented by one of -S-alkyl, -S-alkenyl, and -S-alkynyl. Representative alkylthio groups include methylthio, ethylthio, and the like. The term “alkylthio” also encompasses cycloalkyl groups, alkene and cycloalkene groups, and alkyne groups. “Arylthio” refers to aryl or heteroaryl groups. Alkylthio groups can be substituted as defined above for alkyl groups.

“Alkenyl” and “Alkynyl”, as used herein, refer to unsaturated aliphatic groups containing one or more double or triple bonds analogous in length (*e.g.*, C_2-C_{30}) and possible substitution to the alkyl groups described above.

“Aryl”, as used herein, refers to 5-, 6- and 7-membered aromatic rings. The ring can be a carbocyclic, heterocyclic, fused carbocyclic, fused heterocyclic, bicarbocyclic, or biheterocyclic ring system, optionally substituted as described above for alkyl. Broadly defined, “Ar”, as used herein, includes 5-, 6- and 7-membered single-ring aromatic groups that can include from zero to four heteroatoms. Examples include, but are not limited to, benzene, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, triazole, pyrazole, pyridine, pyrazine, pyridazine and pyrimidine. Those aryl groups having heteroatoms in the ring structure can also be referred to as “heteroaryl”, “aryl heterocycles”, or “heteroaromatics”. The aromatic ring can be substituted at one or more ring positions with such substituents as described above, for example, halogen, azide, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, alkoxyl, amino, nitro, sulfhydryl, imino, amido, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl,

sulfonamido, ketone, aldehyde, ester, heterocyclyl, aromatic or heteroaromatic moieties, --CF_3, and --CN. The term “Ar” also includes polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings (the rings are “fused rings”) wherein at least one of the rings is aromatic, *e.g.*, the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls and/or heterocycles, or both rings are aromatic.

“Alkylaryl”, as used herein, refers to an alkyl group substituted with an aryl group (*e.g.*, an aromatic or hetero aromatic group).

“Heterocycle” or “heterocyclic”, as used herein, refers to a cyclic radical attached via a ring carbon or nitrogen of a monocyclic or bicyclic ring containing 3-10 ring atoms, and preferably from 5-6 ring atoms, containing carbon and one to four heteroatoms each selected from non-peroxide oxygen, sulfur, and N(Y) wherein Y is absent or is H, O, (C_{1-4}) alkyl, phenyl or benzyl, and optionally containing one or more double or triple bonds, and optionally substituted with one or more substituents. The term “heterocycle” also encompasses substituted and unsubstituted heteroaryl rings. Examples of heterocyclic ring include, but are not limited to, benzimidazolyl, benzofuranyl, benzothiofuranyl, benzothiophenyl, benzoxazolyl, benzoxazolinyl, benzthiazolyl, benztriazolyl, benztetrazolyl, benzisoxazolyl, benzisothiazolyl, benzimidazolinyl, carbazolyl, 4a*H*-carbazolyl, carbolinyl, chromanyl, chromenyl, cinnolinyl, decahydroquinolinyl, 2*H*,6*H*-1,5,2-dithiazinyl, dihydrofuro[2,3-*b*]tetrahydrofuran, furanyl, furazanyl, imidazolidinyl, imidazolinyl, imidazolyl, 1*H*-indazolyl, indolenyl, indolinyl, indolizinyl, indolyl, 3H-indolyl, isatinoyl, isobenzofuranyl, isochromanyl, isoindazolyl, isoindolinyl, isoindolyl, isoquinolinyl, isothiazolyl, isoxazolyl, methylenedioxyphenyl, morpholinyl, naphthyridinyl, octahydroisoquinolinyl, oxadiazolyl, 1,2,3-oxadiazolyl, 1,2,4-oxadiazolyl, 1,2,5-oxadiazolyl, 1,3,4-oxadiazolyl, oxazolidinyl, oxazolyl, oxindolyl, pyrimidinyl, phenanthridinyl, phenanthrolinyl, phenazinyl, phenothiazinyl, phenoxathinyl, phenoxazinyl, phthalazinyl, piperazinyl, piperidinyl, piperidonyl, 4-piperidonyl, piperonyl, pteridinyl, purinyl, pyranyl, pyrazinyl, pyrazolidinyl, pyrazolinyl, pyrazolyl, pyridazinyl, pyridooxazole, pyridoimidazole, pyridothiazole, pyridinyl, pyridyl, pyrimidinyl, pyrrolidinyl, pyrrolinyl, 2H-pyrrolyl, pyrrolyl, quinazolinyl, quinolinyl, 4*H*-quinolizinyl, quinoxalinyl, quinuclidinyl, tetrahydrofuranyl, tetrahydroisoquinolinyl, tetrahydroquinolinyl, tetrazolyl, 6*H*-1,2,5-thiadiazinyl, 1,2,3-thiadiazolyl, 1,2,4-thiadiazolyl, 1,2,5-thiadiazolyl, 1,3,4-thiadiazolyl, thianthrenyl, thiazolyl, thienyl, thienothiazolyl, thienooxazolyl, thienoimidazolyl, thiophenyl and xanthenyl.

"Heteroaryl", as used herein, refers to a monocyclic aromatic ring containing five or six ring atoms containing carbon and 1, 2, 3, or 4 heteroatoms each selected from non-peroxide oxygen, sulfur, and N(Y) where Y is absent or is H, O, (C_1-C_8) alkyl, phenyl or benzyl. Non-limiting examples of heteroaryl groups include furyl, imidazolyl, triazolyl, triazinyl, oxazoyl, isoxazoyl, thiazolyl, isothiazoyl, pyrazolyl, pyrrolyl, pyrazinyl, tetrazolyl, pyridyl, (or its N-oxide), thienyl, pyrimidinyl (or its N-oxide), indolyl, isoquinolyl (or its N-oxide), quinolyl (or its N-oxide) and the like. The term "heteroaryl" can include radicals of an ortho-fused bicyclic heterocycle of about eight to ten ring atoms derived therefrom, particularly a benz-derivative or one derived by fusing a propylene, trimethylene, or tetramethylene diradical thereto. Examples of heteroaryl include, but are not limited to, furyl, imidazolyl, triazolyl, triazinyl, oxazoyl, isoxazoyl, thiazolyl, isothiazoyl, pyraxolyl, pyrrolyl, pyrazinyl, tetrazolyl, pyridyl (or its N-oxide), thientyl, pyrimidinyl (or its N-oxide), indolyl, isoquinolyl (or its N-oxide), quinolyl (or its N-oxide), and the like.

The terms "alkoxyl" or "alkoxy" as used herein refers to an alkyl group, as defined above, having an oxygen radical attached thereto. Representative alkoxyl groups include methoxy, ethoxy, propyloxy, tert-butoxy and the like. An "ether" is two hydrocarbons covalently linked by an oxygen. Accordingly, the substituent of an alkyl that renders that alkyl an ether is or resembles an alkoxyl, such as can be represented by one of -O-alkyl, -O-alkenyl, and -O-alkynyl. Aroxy can be represented by –O-aryl or O-heteroaryl, wherein aryl and heteroaryl are as defined below. The alkoxy and aroxy groups can be substituted as described above for alkyl.

The terms "amine" and "amino" are art-recognized and refer to both unsubstituted and substituted amines, *e.g.*, a moiety that can be represented by the general formula: $-NR_9R_{10}$ or $NR_9R_{10}R'_{10}$, wherein R_9, R_{10}, and R'_{10} each independently represent a hydrogen, an alkyl, an alkenyl, $-(CH_2)_m-R'_8$ or R_9 and R_{10} taken together with the N atom to which they are attached complete a heterocycle having from 4 to 8 atoms in the ring structure; R'_8 represents an aryl, a cycloalkyl, a cycloalkenyl, a heterocycle or a polycycle; and m is zero or an integer in the range of 1 to 8. In some embodiments, only one of R_9 or R_{10} can be a carbonyl, *e.g.*, R_9, R_{10} and the nitrogen together do not form an imide. In some embodiments, the term "amine" does not encompass amides, *e.g.*, wherein one of R_9 and R_{10} represents a carbonyl. In some embodiments, R_9 and R_{10} (and optionally R'_{10}) each independently represent a hydrogen, an alkyl or cycloalkyl, an alkenyl or cycloalkenyl, or alkynyl. Thus, the term "alkylamine" as used herein means an amine group, as defined above, having a substituted (as described above for alkyl) or unsubstituted alkyl attached thereto, *i.e.*, at least one of R_9 and R_{10} is an alkyl group.

The term “amido” is art-recognized as an amino-substituted carbonyl and includes a moiety that can be represented by the general formula $-CONR_9R_{10}$ wherein R_9 and R_{10} are as defined above.

“Halogen”, as used herein, refers to fluorine, chlorine, bromine, or iodine.

5 “Nitro”, as used herein, refers to $-NO_2$.

“Sulfhydryl”, as used herein, refers to –SH.

“Hydroxyl”, as used herein, refers to –OH.

“Sulfonyl” as used herein, refers to $-SO_2-$.

The term “carbonyl” is art-recognized and includes such moieties as can be represented

10 by the general formula $-CO-XR_{11}$, or $–X-CO- R'_{11}$, wherein X is a bond or represents an oxygen or a sulfur, and R_{11} represents a hydrogen, an alkyl, a cycloalkyl, an alkenyl, an cycloalkenyl, or an alkynyl, R'_{11} represents a hydrogen, an alkyl, a cycloalkyl, an alkenyl, an cycloalkenyl, or an alkynyl. Where X is an oxygen and R_{11} or R'_{11} is not hydrogen, the formula represents an “ester”. Where X is an oxygen and R_{11} is as defined above, the moiety is referred to herein as a

15 carboxyl group, and particularly when R_{11} is a hydrogen, the formula represents a “carboxylic acid”. Where X is an oxygen and R'_{11} is hydrogen, the formula represents a “formate”. In general, where the oxygen atom of the above formula is replaced by sulfur, the formula represents a “thiocarbonyl” group. Where X is a sulfur and R_{11} or R'_{11} is not hydrogen, the formula represents a “thioester.” Where X is a sulfur and R_{11} is hydrogen, the formula represents

20 a “thiocarboxylic acid.” Where X is a sulfur and R'_{11} is hydrogen, the formula represents a “thioformate.” On the other hand, where X is a bond, and R_{11} is not hydrogen, the above formula represents a “ketone” group. Where X is a bond, and R_{11} is hydrogen, the above formula represents an “aldehyde” group.

The term “substituted” as used herein, refers to all permissible substituents of the

25 compounds described herein. In the broadest sense, the permissible substituents include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic substituents of organic compounds. Illustrative substituents include, but are not limited to, halogens, hydroxyl groups, or any other organic groupings containing any number of carbon atoms, preferably 1-14 carbon atoms, and optionally include one or more heteroatoms such as

30 oxygen, sulfur, or nitrogen grouping in linear, branched, or cyclic structural formats. Representative substituents include alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, phenyl, substituted phenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, halo, hydroxyl, alkoxy, substituted alkoxy, phenoxy, substituted phenoxy, aryloxy, substituted aryloxy, alkylthio, substituted alkylthio, phenylthiol, substituted phenylthio, arylthio,

substituted arylthio, cyano, isocyano, substituted isocyano, carbonyl, substituted carbonyl, carboxyl, substituted carboxyl, amino, substituted amino, amido, substituted amido, sulfonyl, substituted sulfonyl, sulfonic acid, phosphoryl, substituted phosphoryl, phosphonyl, substituted phosphonyl, polyaryl, substituted polyaryl, C_3-C_{20} cyclic, substituted C_3-C_{20} cyclic, heterocyclic, 5 substituted heterocyclic, amino acid, peptide, and polypeptide groups.

It is understood that “substitution” or “substituted” includes the implicit proviso that such substitution is in accordance with permitted valence of the substituted atom and the substituent, and that the substitution results in a stable compound, *i.e.* a compound that does not spontaneously undergo transformation such as by rearrangement, cyclization, elimination, etc.

10 ***Compounds***

Compounds that bind to cell surface receptors (including transmembrane receptors) are disclosed herein. Such receptors can include vascular endothelial growth factor receptor (VEGFR), PD-L1, Ephrin type-B receptor 4 (EphB4), PD1 CTLA4 in T cells, integrin, angiotensin-converting enzyme (ACE) (such as in SARS-Cov-2 infection), estrogen receptor 15 (ER), or a combination thereof. The compounds can contain two or more (such as 2, 3, or 4) receptor binding moieties (such as two or more VEGFR binding moieties) and at least one linker. In some instances, the compounds can contain two or more receptor binding moieties (such as two or more VEGFR binding moieties), at least one linker, and one or more detectable moieties. In further instances, the compounds can contain two or more receptor binding moieties (such as 20 two or more VEGFR binding moieties), at least one linker, and one or more therapeutic moieties. In other instances, the compounds can contain two or more receptor binding moieties (such as two or more VEGFR binding moieties), at least one linker, one or more detectable moieties, and one or more therapeutic moieties. The detectable moiety can be the therapeutic moiety. The at least one linker covalently couples the moieties in the compounds.

25 The general structure of the compounds disclosed herein can be represented as $(RBM)_x$-L_n or $(RBM)_x$-L_n-$(DTM)_y$, where RBM is a receptor binding moiety, L is a linker, DTM is a detectable moiety and/or a therapeutic moiety, x is an integer from 2 to 5, n is an integer from 1 to 5, and y is an integer from 1 to 5. For example, the compounds can be represented as $(VBM)_x$-L_n or $(VBM)_x$-L_n-$(DTM)_y$, where VBM is a VEGFR binding moiety, L is a linker, 30 DTM is a detectable moiety and/or a therapeutic moiety, x is an integer from 2 to 5, n is an integer from 1 to 5, and y is an integer from 1 to 5. In preferred examples, the compounds disclosed herein can be represented as $(RBM)_2$-L, $(RBM)_3$-L_2, $(RBM)_4$-L_3, $(RBM)_2$-L-DTM, $(RBM)_3$-L_2-DTM, $(RBM)_4$-L_3-DTM, $(RBM)_2$-L-$(DTM)_2$, $(RBM)_2$-L-$(DTM)_3$, RBM-L-$(DTM)_3$,

$(VBM)_2$-L, $(VBM)_3$-L_2, $(VBM)_4$-L_3, $(VBM)_2$-L-DTM, $(VBM)_3$-L_2-DTM, $(VBM)_4$-L_3-DTM, $(VBM)_2$-L-$(DTM)_2$, $(VBM)_2$-L-$(DTM)_3$, and VBM-L-$(DTM)_3$.

In some aspects, the compounds can contain two or more receptor binding moieties and at least one linker. The receptor binding moieties can be selected from a small molecule therapeutic agent, a peptide, an antibody, an antibody fragment, a carbohydrate, an siRNA, a protein, a nucleic acid, an aptamer, a nanoparticle, a cytokine, a chemokine, a lymphokine, a lipid, a lectin, or a combination thereof. The compounds can also contain two or more receptor binding moieties, at least one linker, and one or more detectable moieties. The compounds can also contain two or more receptor binding moieties, at least one linker, and one or more therapeutic moieties. The compounds can also contain two or more receptor binding moieties, at least one linker, one or more detectable moieties, and one or more therapeutic moieties.

In some aspects, the compounds can contain two or more EphB4 binding moieties and at least one linker. The compounds can also contain two or more EphB4binding moieties, at least one linker, and one or more detectable moieties. The compounds can also contain two or more EphB4binding moieties, at least one linker, and one or more therapeutic moieties. The compounds can also contain two or more EphB4binding moieties, at least one linker, one or more detectable moieties, and one or more therapeutic moieties.

In some aspects, the compounds can contain four or more VEGFR binding moieties and at least one linker. The compounds can also contain four or more VEGFR binding moieties, at least one linker, and one or more detectable moieties. The compounds can also contain four or more VEGFR binding moieties, at least one linker, and one or more therapeutic moieties. The compounds can also contain four or more VEGFR binding moieties, at least one linker, one or more detectable moieties, and one or more therapeutic moieties. In some embodiments, the compounds include at least two linkers or at least three linkers. In some embodiments, the compounds contain at least four VEGFR binding moieties and at least three linkers.

In some examples, the compounds disclosed herein are tetravalent compounds comprising a first moiety and a second moiety covalently linked by a first linker, L_1. The first moiety in the compound can comprise two or more VEGFR binding moieties, wherein the two or more VEGFR binding moieties are linked by a second linker, L_2. The second moiety in the compound can comprise an additional two or more VEGFR binding moieties, wherein the additional two or more VEGFR binding moieties are linked by a third linker, L_3.

EphB4 binding moiety

Ephrin type-B receptor 4 (EphB4) makes up the largest subgroup of the receptor tyrosine kinase family and emerges as critical regulators postnatal angiogenic remodeling and tumor

neovascularization. The EphB4 binding moieties is preferably any compound that is a potent antagonist of EphB4, with inhibits the binding of ephrin-B2 to murine EphB4 receptors. For example, the EphB4 binding moiety can include a peptide such as Asn-Tyr-Leu-Phe-Ser-Pro-Asn-Gly-Pro-Ile-Ala-Arg-Ala-Trp or Tyr-Asn-Tyr-Leu-Phe-Ser-Pro-Asn-Gly-Pro-Ile-Ala-Arg-
5 Ala-Trp (TNYLFSPNGPIARAW, designated as TNYL-RAW).

As disclosed herein, two EphB4 binding moieties can be coupled to one or more linkers, optionally comprising a detectable moiety or therapeutic moiety. In some instances, one or more EphB4 binding moieties can be coupled to one or more ephrin type-A receptor 4 (EphA4) binding moieties via a linker. The EphA4 binding moiety can be a peptide. In some
10 embodiments, the EphB4 binding moiety can include a peptide such as

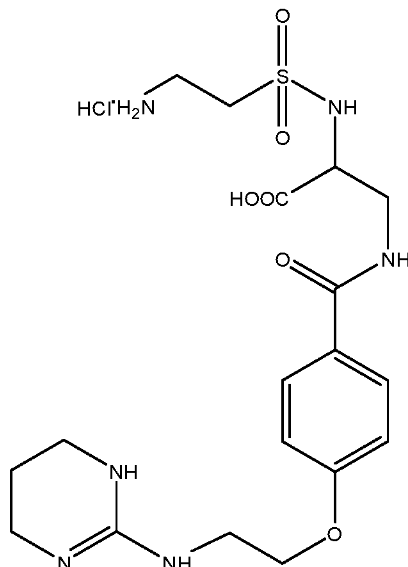
EphA2 EphB4

H-Gly-Tyr-Ser-Ala-Tyr-Pro-Asp-Ser-Val-Pro-Met-Met-Ser-Thr-Asn-Tyr-Leu-Phe-Ser-Pro-Asn-Gly-Pro-Ile-Ala-Arg-Ala-Trp-NH_2

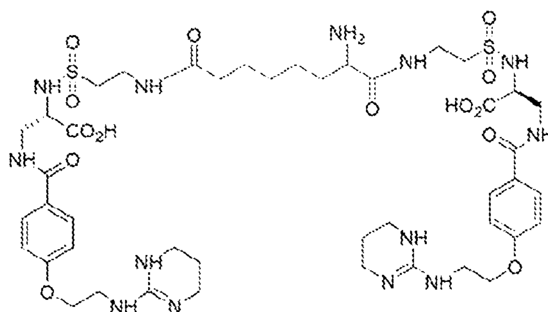
Other Ephrin type-B receptor 4 (EphB4) binding moieties (EphB4 inhibitors) include kinase inhibitors (such as anilinopyrimidine derivatives, benzenesulfonamide derivatives, XL647 also known as EXEL-7647, bis-anilino-pyrimidine derivatives, and xanthine derivatives);
15 inhibitors of Eph expression (such as oligonucleotides); inhibitors of Eph–ephrin interactions (such as TNYL-RAW peptide, APY-d2-4, and small linear peptides (MW 600–700 Da), and monoclonal antibodies (such as mAb 131, mAb 147).

Integrin binding moiety

Integrin is a transmembrane receptor and activate signal transduction pathways that
20 mediate cellular signals such as regulation of the cell cycle, organization of the intracellular cytoskeleton, and movement of new receptors to the cell membrane. The integrin binding moieties is preferably any compound that is a potent antagonist of integrin. For example, the integrin binding moiety can include a small molecule having a structure as shown below:



As disclosed herein, two integrin binding moieties can be coupled to one or more linkers, optionally comprising a detectable moiety or therapeutic moiety. In some embodiments, the integrin binding moiety can include a small molecule dimer such as



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Other integrin binding moieties can include the anti-αVβ3 antibody etaracizumab (MEDI-522); anti-αV antibodies (such as intetumumab (CNTO95) or abituzumab (EMD 525797/DI17E6)); anti-α5β1 integrin antibody M200/volociximab; endogenous antagonists such as the peptides endostatin, tumstatin, or angiostatin; Arg–Gly–Asp-based cyclic peptide cilengitide (EMD121974) targeting αVβ3/αVβ5; α5β1-blocking non Arg–Gly–Asp-based peptide ATN-161; peptidomimetics targeting αVβ3, αVβ5, and α5β1 (such as SCH221153, BCH-15046, SJ749, and JSM6427).

Angiotensin-Converting Enzyme (ACE)

Angiotensin-Converting Enzyme (ACE) acts as a host receptor for example in SARS-Cov-2 infection, which occurs by using the viral S (spike) protein receptor-binding domain binding to ACE2 to enter the host cell. Some examples of ACE inhibitors include benazepril

(Lotensin), captopril, enalapril (Vasotec), fosinopril, lisinopril (Prinivil, Zestril), moexipril, perindopril, quinapril (Accupril), Ramipril (Altace), and trandolapril.

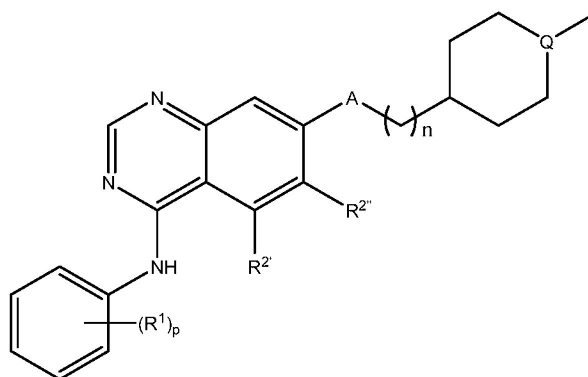
Estrogen Receptor (ER)

Estrogens are a class of steroid hormones that regulate the growth, development, and physiology of the human reproductive system. Estrogens are also involve in the neuroendocrine, skeletal, adipogenesis, and cardiovascular systems. Estrogen signaling pathways are selectively stimulated or inhibited depending on a balance between the activities of estrogen receptor (ER) α or ERβ in target organs. Research has identified membrane ER signaling mediating many physiological and pathological processes and functions in organs, such as fertility, reproductive organs, mammary gland, male reproduction, bone development and maintenance, cardiovascular tissues and metabolism, brain, and behavior.

Some examples of estrogen receptor binding moieties (ER inhibitors) can be found in Sharma D. et al, Chem Cent J. 2018; 12: 107 which is hereby incorporated herein by reference. Specific examples include heterocyclic analogues (such as dibenzo[b, f]thiepines analogues, diphenylmethane skeleton, conjugated heterocyclic scaffolds, aromatase inhibitors/selective estrogen receptor modulator, norendoxifen, furan derivatives, fulvestrant, diphenylmethane, diphenylmethyelene, diphenylheptane, diphenyl amine analogs and triarylethylene analogs, coumarin conjugates, coumarin-chalcone hybrids, inverse agonist, steroidal analogs, reseveratrol (phytoestrogen) analogs, triarylethylene analogs, isoflavone analogs, indole derivatives, pyrazole derivatives, hydrazones, isoquinoline derivatives, anilinonicotinyl linked pyrazolo[1,5-a]pyrimidine conjugate, bis(hydroxyphenyl)azoles, quinoline analogues, isoflavone derivatives as aromatase inhibitor, amino acid residues, diphenylheptane skeleton, 3, 2′-dihydroxy-19-norpregna-1, 3, 5(10)-trienes analogs, and metal based analogs.

VEGFR binding moiety

The VEGFR binding moieties is preferably any compound that inhibits VEGFR. For examples, the VEGFR binding moieties can be independently selected from the group consisting of bevacizumab; sunitinib; aflibercept; pazopanib; axitinib; sorafenib; vandetanib; regorafenib; ramucirumab, and combination thereof. In some examples, the VEGFR binding moieties has a structure as shown in Formula 1,



Formula 1

where

R^1 can be hydroxyl, halogen, $C_{1\text{-}3}$ alkyl, $C_{1\text{-}3}$ alkoxyl, $C_{1\text{-}3}$ alkanoyloxyl, trifluoromethyl,
5 cyano, amino, or nitro;

$R^{2'}$ and $R^{2''}$ can be, independent of one another, hydrogen, hydroxyl, halogen, nitro, trifluoromethyl, cyano, $C_{1\text{-}3}$ alkyl, $C_{1\text{-}3}$ alkoxyl, $C_{1\text{-}3}$ alkylthio, or $-NR^7R^8$, wherein R^7 and R^8, which can be the same or different, each represents hydrogen or $C_{1\text{-}3}$ alkyl;

A can be oxygen, $-CH_2-$, -S-, -SO-, $-SO_2-$, $-NR^7CO-$, $-CONR^7-$, $-SO_2NR^7-$, $-NR^7SO_2-$ or
10 $-NR^7-$;

Q can be nitrogen or -CH-;

n is an integer from 1 to 5, for example, n can be 1, 2, 3, 4, or 5; and

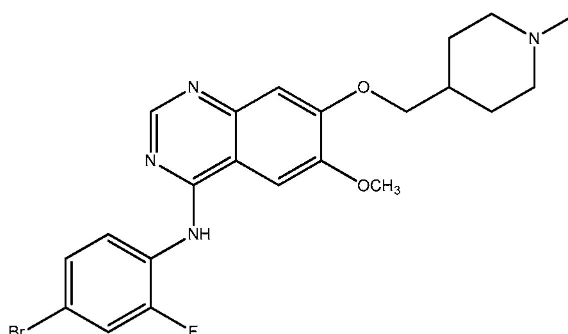
p is an integer from 1 to 4, for example, p can be 1, 2, 3, or 4.

In a preferred embodiment, of the VEGFR binding moiety, R^1 can be a halogen, for
15 example, Br or F. In other examples, R^1 can be hydroxyl, $C_{1\text{-}3}$ alkyl, or $C_{1\text{-}3}$ alkyl.

In other examples $R^{2'}$ is preferably hydrogen. In still other examples, $R^{2''}$ is preferably $C_{1\text{-}3}$ alkyl, or $C_{1\text{-}3}$ alkoxyl. In yet further examples, A is preferably –O- and Q is preferably N.

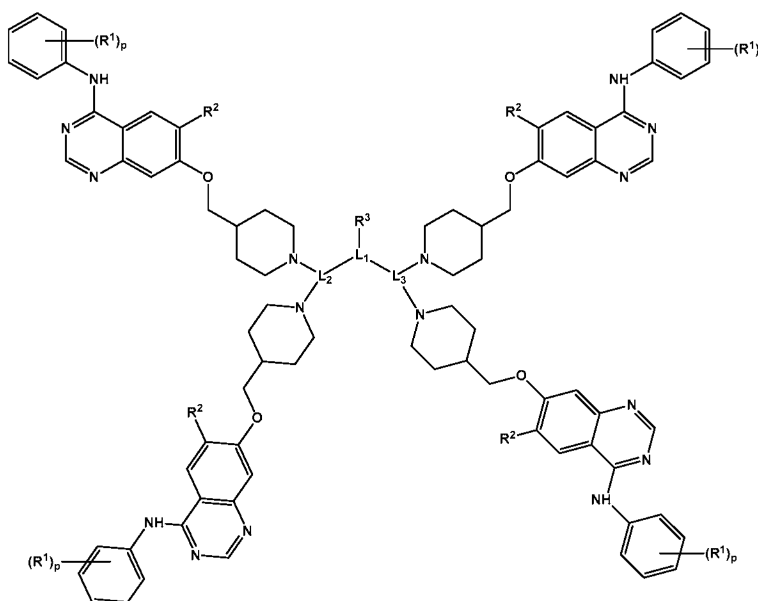
Also, in the disclosed compounds n is preferably 1 or 2 and p is preferably 1 or 2.

In one example, the VEGFR binding moiety has Formula 1-A, which corresponds to
20 vandetanib (ZD-1).



Formula 1-A.

As disclosed herein, four VEGFR binding moieties can be coupled to one or more linkers, optionally comprising a detectable moiety or therapeutic moiety (R^3). Compounds with such a configuration are shown in Formula I:



5

Formula I

wherein,

R^1, independently for each occurrence, is selected from hydroxyl, halogen, C_{1-3} alkyl, C_{1-}
10 $_3$ alkoxyl, C_{1-3} alkanoyloxy, trifluoromethyl, cyano, amino, or nitro;

R^2, independently for each occurrence, is selected from hydrogen, hydroxyl, halogen, nitro, trifluoromethyl, cyano, C_{1-3} alkyl, C_{1-3} alkoxyl, C_{1-3} alkylthio, or $-NR^7R^8$, wherein R^7 and R^8, which can be the same or different, each represents hydrogen or C_{1-3} alkyl;

R^3 is absent or comprises a detectable moiety or therapeutic moiety;

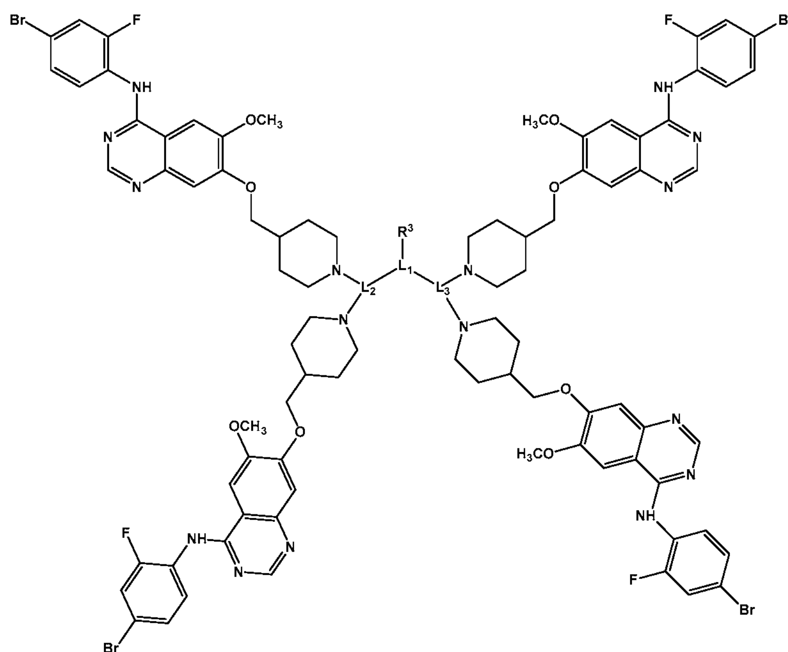
15 p is an integer from 1 to 4; and

L_1, L_2, and L_3 are linkers, independently selected from $N(R^{14})_3$, $CH(R^{14})_3$, $Ar(R^{14})_3$, wherein Ar is aryl, and wherein R^{14} for each occurrence, is independently selected from a bond, hydrogen, amido, C_1-C_{20} alkyl; C_1-C_{20} heteroalkyl, C_1-C_{20} alkylamine, C_1-C_{20} alkoxy, polyalkyleneoxy, C_1-C_{20} alkanoyloxy, C_1-C_{20} alkylamido, aryl, or heteroaryl; wherein each R^{14}
20 independent of the other, is optionally substituted with one or more substituents selected from the group consisting of halogen; hydroxyl; cyano; carbonyl; nitro; amino; amido; alkylamino; dialkylamino; alkylamido; =O; $-S(O)_2$; -SO-; -S-; $-S(O)_2N-$; haloalkyl; hydroxyalkyl; carboxy;

alkyl, alkoxyl; aryloxyl; alkoxycarbonyl; aminocarbonyl; alkylaminocarbonyl; dialkylaminocarbonyl; aryl; heteroaryl; and combinations thereof;

wherein R^3 when present is bonded to at least one R^{14}.

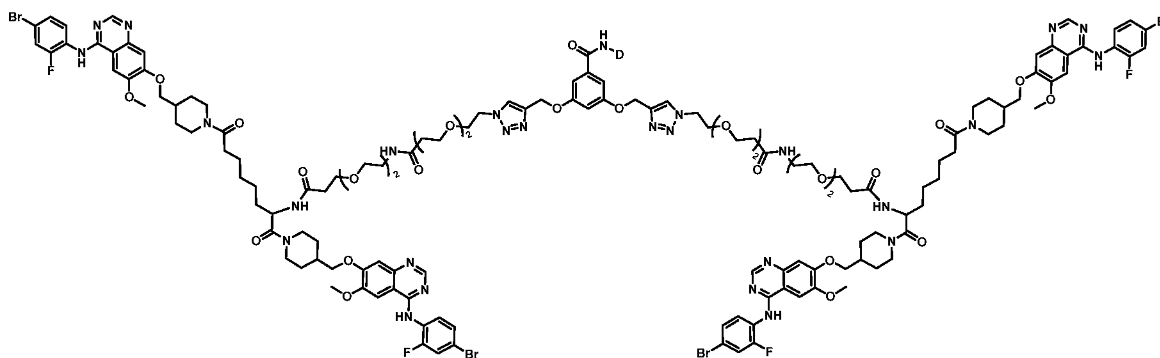
The compound can have the Formula II, which corresponds to Formula I, where p is 2, R^1 is halogen (fluro and bromo), and R^2 is methoxy:



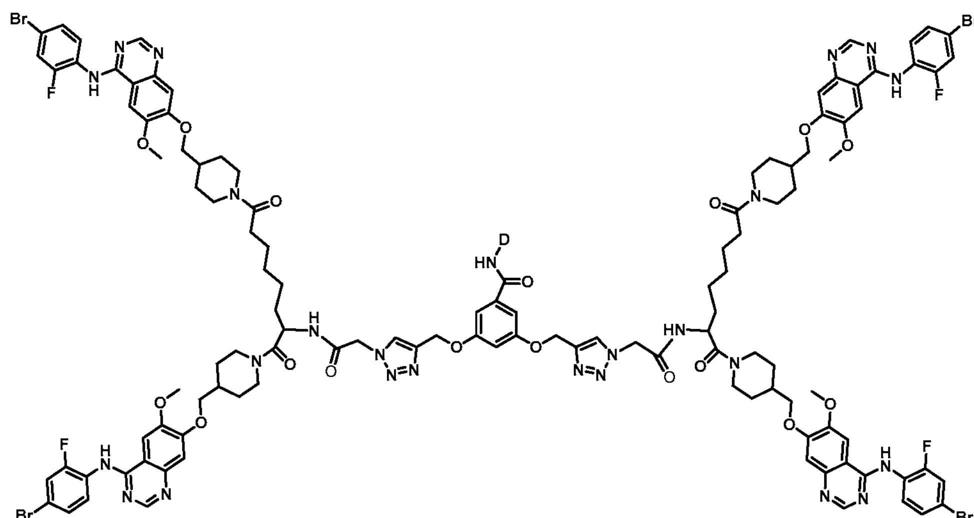
Formula II.

R^3, L_1, L_2, and L_3 in Formula II can be as described herein.

In some embodiments, the compounds have Formula IIIA or IIIB:



Formula IIIA, or

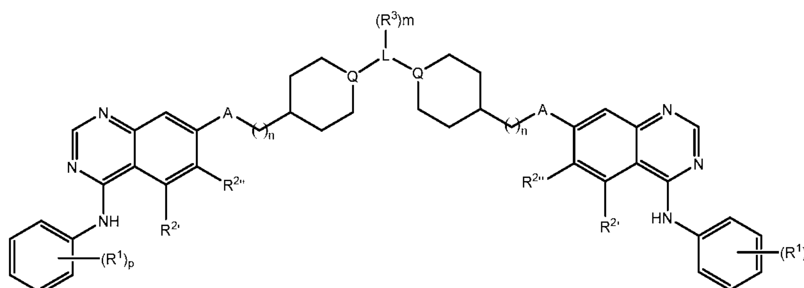


Formula IIIB,

wherein,

D comprises the detectable moiety.

Compounds of Formula IV are also described herein. Bivalent compounds that bind to VEGFR have been disclosed in U.S. Patent No. 10,011,587, the disclosure of which is hereby incorporated by reference in its entirety. As further described herein, the bivalent compounds include a linker, the geometry of which is such that it promotes multi-binding of the VEGFR binding moieties to multiple receptors.



Formula IV

wherein,

R^1 can be hydroxyl, halogen, C_{1-3} alkyl, C_{1-3} alkoxyl, C_{1-3} alkanoyloxyl, trifluoromethyl, cyano, amino, or nitro;

$R^{2'}$ and $R^{2''}$ can be, independent of one another, hydrogen, hydroxyl, halogen, nitro, trifluoromethyl, cyano, C_{1-3} alkyl, C_{1-3} alkoxy, C_{1-3} alkylthio, or $-NR^7R^8$, wherein R^7 and R^8, which can be the same or different, each represents hydrogen or C_{1-3} alkyl;

A can be oxygen, $-CH_2-$, -S-, -SO-, $-SO_2-$, $-NR^7CO-$, $-CONR^7-$, $-SO_2NR^7-$, $-NR^7SO_2-$ or $-NR^7-$;

Q can be nitrogen, or -CH-;

L is a linker;

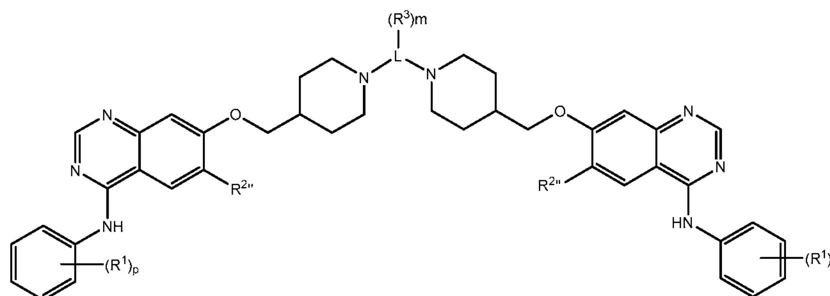
R^3 contains a detectable moiety, a therapeutic moiety, or both;

n is an integer from 1 to 5, for example, n can be 1, 2, 3, 4, or 5;

5 m is an integer from 1 to 5, for example, m can be 1, 2, 3, 4, or 5; and

p is an integer from 1 to 4, for example, p can be 1, 2, 3, or 4.

The compound can have the Formula V, which correspond to Formula IV, where A is oxygen, Q is nitrogen, n is 1, and R^2 and R^5 are both hydrogen:



10 Formula V

wherein,

R^1 can be hydroxyl, halogen, C_{1-3} alkyl, C_{1-3} alkoxyl, C_{1-3} alkanoyloxyl, trifluoromethyl, cyano, amino or nitro;

$R^{2''}$ can be, independent of one another, hydrogen, hydroxyl, halogen, nitro,

15 trifluoromethyl, cyano, C_{1-3} alkyl, C_{1-3} alkoxyl, C_{1-3} alkylthio, or $-NR^7R^8$, wherein R^7 and R^8, which can be the same or different, each represents hydrogen or C_{1-3} alkyl;

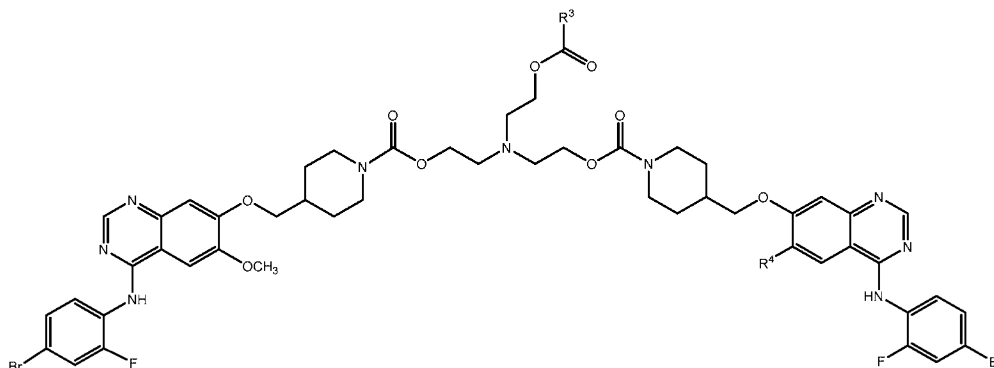
L is a linker;

R^3 contains a detectable moiety, a therapeutic moiety, or both;

m is an integer from 1 to 5, for example, m can be 1, 2, 3, 4, or 5; and

20 p is an integer from 1 to 4, for example 1, 2, 3, or 4.

In some embodiments, the compounds have Formula VI:

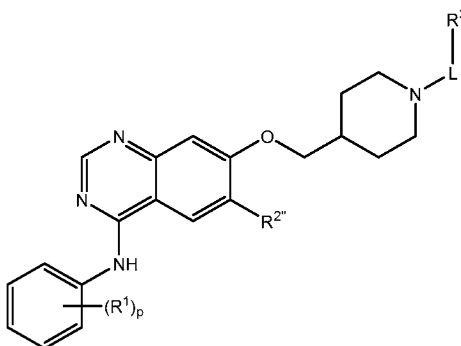


Formula VI

wherein,

R^6 is a detectable moiety, a therapeutic moiety, or both.

Compounds of Formula VII are also described herein:



5

Formula VII

wherein,

R^1 can be hydroxyl, halogen, C_{1-3} alkyl, C_{1-3} alkoxyl, C_{1-3} alkanoyloxyl, trifluoromethyl, cyano, amino, or nitro;

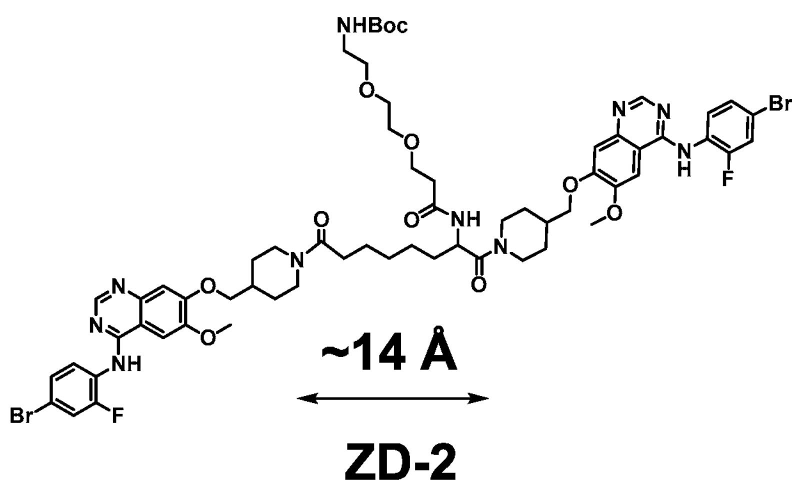
10 $R^{2''}$ can be hydrogen, hydroxy, halogen, nitro, trifluoromethyl, cyano, C_{1-3} alkyl, C_{1-3} alkoxy, C_{1-3} alkylthio, or $-NR^7R^8$, wherein R^7 and R^8, which can be the same or different, each represents hydrogen or C_{1-3} alkyl;

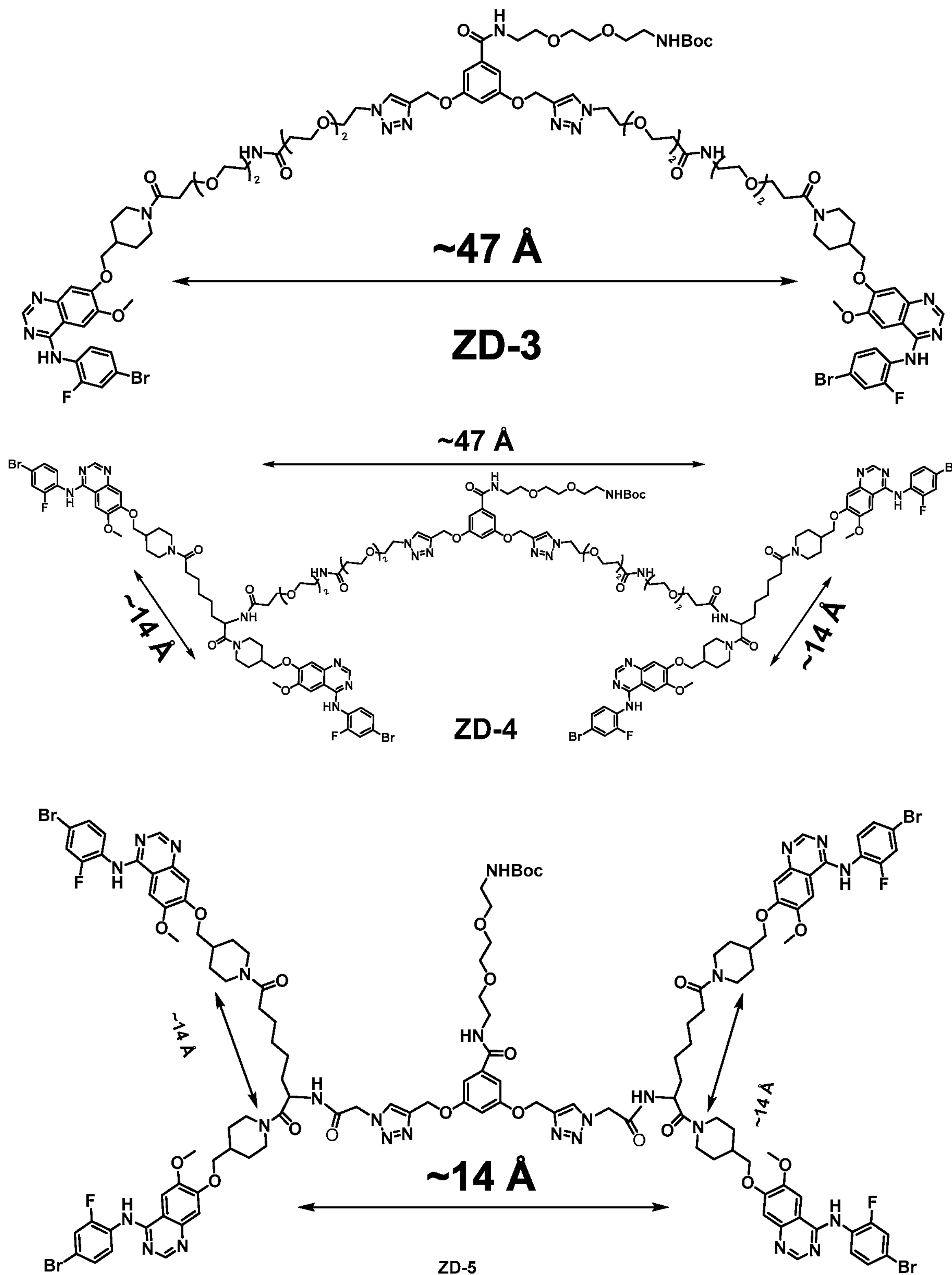
L is a linker;

R^3 contains a detectable moiety, a therapeutic moiety, or both; and

15 p is an integer from 1 to 4, for example, p can be 1, 2, 3, or 4.

In some embodiments, the compounds have the following structures:





Linker (L)

The compounds described herein contain a linker (L). As described herein, a force measurement platform to probe the distribution and distance of the cell surface receptors (such as

vascular endothelial growth factor receptors (VEGFR)) in live cells has been disclosed, which has been used to determine the geometry of appropriate linkers for distinct multivalent binding modes. In general, the linker can be of any nature, but provides a particular geometry for the multivalent binding moieties. In some aspects, the length of the linker can be important in determining the statistical or the chelate effect for the interaction of the binding moieties to the receptors (such as VEGFR).

The multivalent binding compounds can include one or more linkers. In some embodiments, the compounds include a first linker, L_1, a second linker, L_2, and a third linker, L_3. The linkers can each independently have a length from 40 to 60 Å, which defines the distance between two adjacent receptors (such as VEGFR) on the cell's periphery. For example, each linker can have a length from 40 to 58 Å, from 40 to 56 Å, from 40 to 55 Å, from 40 to 54 Å, from 40 to 52 Å, from 40 to 50 Å, from 40 to 49 Å, from 40 to 48 Å, from 40 to 47 Å, from 42 to 58 Å, from 42 to 55 Å, from 42 to 54 Å, from 42 to 52 Å, from 42 to 50 Å, from 42 to 48 Å, from 45 to 60 Å, from 45 to 56 Å, from 45 to 55 Å, from 45 to 54 Å, from 45 to 52 Å, from 45 to 50 Å, from 45 to 49 Å, from 45 to 48 Å, or from 45 to 47 Å. The binding of multivalent compounds comprising linkers with length similar or greater than the proximal receptor (such as VEGFR) distance in cells is believed to be dominated in binding by the chelating effect.

In other embodiments, each linker can independently have a length from 7 to 25 Å, which defines a distance shorter that the proximal receptor (such as VEGFR) distance on the cell's periphery. For example, each linker can independently have a length from 7 to 22 Å, from 7 to 20 Å, from 7 to 18 Å, from 7 to 16 Å, from 7 to 14 Å, from 8 to 25 Å, from 8 to 24 Å, from 8 to 22 Å, from 8 to 20 Å, from 8 to 18 Å, from 8 to 16 Å, from 8 to 14 Å, from 10 to 25 Å, from 10 to 24 Å, from 10 to 22 Å, from 10 to 20 Å, from 10 to 18 Å, from 10 to 16 Å, from 10 to 14 Å, from 12 to 25 Å, from 12 to 24 Å, from 12 to 22 Å, from 12 to 20 Å, from 12 to 18 Å, from 12 to 16 Å, or from 12 to 14 Å. The binding of multivalent compounds comprising linkers with length that was greatly shorter than the proximal receptor (such as VEGFR) distance in cells is believed to be dominated in binding by the statistical effect.

In some examples, L_1 can have a length from 40 to 60 Å (for e.g., from 40 to 58 Å, from 40 to 56 Å, from 40 to 55 Å, from 40 to 54 Å, from 40 to 52 Å, from 40 to 50 Å, from 40 to 49 Å, from 40 to 48 Å, from 40 to 47 Å, from 42 to 58 Å, from 42 to 55 Å, from 42 to 54 Å, from 42 to 52 Å, from 42 to 50 Å, from 42 to 48 Å, from 45 to 60 Å, from 45 to 56 Å, from 45 to 55 Å, from 45 to 54 Å, from 45 to 52 Å, from 45 to 50 Å, from 45 to 49 Å, from 45 to 48 Å, or from 45 to 47 Å).

In some examples, each of L_2 and L_3 can independently have a length of from 7 to 60 Å. In some instances, L_2 and L_3 can independently have similar lengths compared to the first linker (for e.g., from 40 to 60 Å, from 40 to 58 Å, from 40 to 56 Å, from 40 to 55 Å, from 40 to 54 Å, from 40 to 52 Å, from 40 to 50 Å, from 40 to 49 Å, from 40 to 48 Å, from 40 to 47 Å, from 42 to 58 Å, from 42 to 55 Å, from 42 to 54 Å, from 42 to 52 Å, from 42 to 50 Å, from 42 to 48 Å, from 45 to 60 Å, from 45 to 56 Å, from 45 to 55 Å, from 45 to 54 Å, from 45 to 52 Å, from 45 to 50 Å, from 45 to 49 Å, from 45 to 48 Å, or from 45 to 47 Å). In other instances, L_2 and L_3 can independently have shorter lengths compared to the first linker (for e.g., from 7 to 25 Å, from 7 to 22 Å, from 7 to 20 Å, from 7 to 18 Å, from 7 to 16 Å, from 7 to 14 Å, from 8 to 25 Å, from 8 to 24 Å, from 8 to 22 Å, from 8 to 20 Å, from 8 to 18 Å, from 8 to 16 Å, from 8 to 14 Å, from 10 to 25 Å, from 10 to 24 Å, from 10 to 22 Å, from 10 to 20 Å, from 10 to 18 Å, from 10 to 16 Å, from 10 to 14 Å, from 12 to 25 Å, from 12 to 24 Å, from 12 to 22 Å, from 12 to 20 Å, from 12 to 18 Å, from 12 to 16 Å, or from 12 to 14 Å).

The linker is polyfunctional, such as bi-functional, tri-functional, or tetra-functional molecules, and can be used to covalently couple the two or more receptor (such as VEGFR) binding moieties, the one or more detectable moieties, and one or more therapeutic moieties of the disclosed compounds. The linker can be attached to any part of the receptor binding moiety so long as the point of attachment does not interfere with the biological activity, for example, the anti-tumor and/or anti-inflammatory activity of the compounds described herein. In some instances, L_1 covalently links the first moiety to the second moiety in the compounds disclosed; L_2 links the two or more receptor binding moieties in the first moiety; and L_3 links the two or more additional receptor binding moieties in the second moiety. In some cases, L_1, L_2, and L_3 can be structurally similar, having similar chemical identity. In some cases, L_1, L_2, and L_3 can be segments (components) of the same (a single) molecule, but are so described to differentiate connections. For example, the compound can include a single linker that is a branched compound (such as a branched alkyl or branched amide), wherein each branch represents a linker that connects the receptor (such as VEGFR) moiety to the main chain of the linker. Thus, such a linker comprising L_1, L_2, and L_3 can be the same molecule with different segments.

In some embodiments, the linker is flexible. In some embodiment, the linker is stable and biocompatible. In some embodiments, the covalent bond formed between the linker and the receptor binding moiety and/or the detectable moiety is stable. Stable, as used herein refers to a covalent bond that remains at least 70%, preferably at least 80%, more preferably at least 90% intact in aqueous solution at temperatures ranging from about 0 °C to about 100 °C, at a pH ranging from about 2 to about 12, for at least 1 hour. The covalent bond formed between the

linker and the receptor binding moiety and/or the detectable moiety is hydrolytically and reductively stable.

The linker can be a single atom, such as a heteroatom (*e.g.*, O, N, or S), a group of atoms, such as a functional group (*e.g.*, amine, -C(=O)-, $-CH_2-$), or multiple groups of atoms, such as an alkylene chain. Suitable linkers include but are not limited to oxygen, sulfur, carbon, nitrogen, substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted cycloalkenyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted alkoxyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted ether, substituted or unsubstituted amine, substituted or unsubstituted diamine, substituted or unsubstituted amide, substituted or unsubstituted alkylamine, substituted or unsubstituted thioether, substituted or unsubstituted carboxylates, substituted or unsubstituted polymer, derivatives or combinations thereof.

The linkers, L_1, L_2, and L_3, can be selected from R^{14}, $C(O)R^{14}C(O)$, $C(O)OR^{14}OC(O)$, $C(O)R^{14}N$, $C(O)OR^{14}NH$, $NHR^{14}NH$, $C(O)NHR^{14}NHC(O)$, $HNC(O)R^{14}C(O)NH$, $C(O)CHNHR^{14}NHCHC(O)$, $C(S)OR^{14}OC(S)$; wherein R^{14} is O, S, C_1-C_{20} alkyl; C_1-C_{20} heteroalkyl; C_1-C_{20} alkylamine; C_1-C_{20} alkoxyl; C_1-C_{20} alkanoyloxyl; polyalkyleneoxy; or C_1-C_{20} alkylamido, any of which can be optionally substituted with one or more substituents including halogen, alkoxyl, alkyl, alkenyl, cycloalkyl, cycloalkenyl, heterocycloalkyl, aryl, heteroaryl, amine, alkylamine, dialkylamine, cyano, nitro, hydroxyl, carbonyl, acyl, carboxylic acid (-COOH), $-C(O)R^{12}$, $-C(O)OR^{12}$, carboxylate ($-COO^-$), primary amide (*e.g.*, $-CONH_2$), secondary amide (*e.g.*, $-CONHR^{12}$), $-C(O)NR^{12}R^{13}$, $-NR^{12}R^{13}$, $-NR^{12}S(O)_2R^{13}$, $-NR^{12}C(O)R^{13}$, $-S(O)_2R^{12}$, $-SR^{12}$, and $-S(O)_2NR^{12}R^{13}$, sulfinyl group (*e.g.*, $-SOR^{12}$), and sulfonyl group (*e.g.*, $-SOOR^{12}$);

wherein R^{12} and R^{13} can each independently be hydrogen, halogen, hydroxyl, alkyl, haloalkyl, alkenyl, alkynyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl, carbonyl, cyano, amino, alkylamino, dialkylamino, alkoxyl, aryloxyl, cycloalkyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, or dialkylaminocarbonyl.

In some embodiments, the linkers, L_1, L_2, and L_3, can be selected from $N(R^{14})_3$, $CH(R^{14})_3$, or $Ar(R^{14})_3$; wherein the receptor binding moiety or detectable moiety are bonded to at least one R^{14}, and wherein each R^{14} can be the same or different and for each occurrence, independently selected from a bond, hydrogen, amido, C_1-C_{20} alkyl; C_1-C_{20} heteroalkyl; C_1-C_{20} alkylamine; C_1-C_{20} alkoxy; polyalkyleneoxy; C_1-C_{20} alkanoyloxy; or C_1-C_{20} alkylamido; any of which can be optionally substituted with one or more substituents independently selected from

the group consisting of halogen; hydroxyl; cyano; nitro; amino; alkylamino; dialkylamino; amido; alkylamido; =O; $-S(O)_2$; -SO-; -S-; $-S(O)_2N-$; haloalkyl; hydroxyalkyl; carboxy; alkoxy; aryloxy; alkoxycarbonyl; aminocarbonyl; alkylaminocarbonyl; dialkylaminocarbonyl, aryl, heteroaryl, and combinations thereof. In some embodiment, C_{1-20} refers to alkyl groups
5 containing 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 carbons.

In some embodiments, the linkers, L_1, L_2, and L_3, can be selected from $-(CO-R^{14})_2N(R^{14})$, $-(R^{14})_3N$, $-(SO_2R^{14})_2NR^{14}$, $-(SOR^{14})_2NR^{14}$, $-(OR^{14})_2NR^{14}$, $-(O-CO-R^{14})_2NR^{14}$, $-(CO-O-R^{14})_2NR^{14}$, $-(CO-R^{14})_2CH(R^{14})$, $-(R^{14})_3CH$, $-(SO_2R^{14})_2CH(R^{14})$, $-(SOR^{14})_2CH(R^{14})$, $-(O-CO-R^{14})_2CH(R^{14})$, or $-(OR^{14})_2CH(R^{14})$, wherein R^{14} is independently selected from, for each
10 occurrence, a bond, C_1-C_{20} alkyl; C_1-C_{20} heteroalkyl, C_1-C_{20} alkylamine, C_1-C_{20} alkoxy, polyalkyleneoxy, C_1-C_{20} alkanoyloxy, or C_1-C_{20} alkylamido; wherein R^{14} is optionally substituted with one or more substituents independently selected from the group consisting of amino; alkylamino; dialkylamino; amido; alkylamido; alkoxyamido; polyalyleneoxyamido; aryl; heteroaryl; or combinations thereof.

15 In some embodiments, the linkers L_1, L_2, and L_3 are selected from is $-(NHCO-R^{14})_3Ar$, $-(CONH-R^{14})_3Ar$, $-(CO-R^{14})_3Ar$, wherein R^{14} is independently selected from, for each occurrence, a bond, C_1-C_{20} alkyl; C_1-C_{20} heteroalkyl, C_1-C_{20} alkylamine, C_1-C_{20} alkoxy, polyalkyleneoxy, C_1-C_{20} alkanoyloxy, or C_1-C_{20} alkylamido; wherein R^{14} is optionally substituted with one or more substituents independently selected from the group consisting of amino;
20 alkylamino; dialkylamino; amido; alkylamido; alkoxyamido; polyalyleneoxyamido; aryl; heteroaryl; or combinations thereof.

In further embodiments, the linkers L_1, L_2, and L_3 are selected from $-(R^{14})_3CH$, wherein each of R^{14} is independently selected from a bond, carboxyl, C_{2-10} alkylcarboxyl.

Amino acids

25 In some embodiments, the linker can be an amino acid. The amino acid can be a natural or non-natural amino acid. The term “non-natural amino acid” refers to an organic compound that is a congener of a natural amino acid in that it has a structure similar to a natural amino acid so that it mimics the structure and reactivity of a natural amino acid. The non-natural amino acid can be a modified amino acid, and/or amino acid analog, that is not one of the 20 common
30 naturally occurring amino acids or the rare natural amino acids selenocysteine or pyrrolysine. Examples of suitable amino acids include, but are not limited to, alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, a derivative, or combinations thereof.

Aminodicarboxylic acids

In some embodiments, the linker is an amino dicarboxylic acid. In some embodiments, the amino dicarboxylic acid can have from 2 to 30 carbon atoms. Examples of suitable amino dicarboxylic acids include, but are not limited to, 1,6-dicarboxylic-2-amino hexanoic acid, 1,7-dicarboxylic-2-amino heptanoic acid, 1,8-dicarboxylic-2-amino octanoic acid, α-aminosuccinic acid, β-aminoglutaric acid, β-aminosebacic acid, 2,6-piperidine dicarboxylic acid, 2,5-pyrrole dicarboxylic acid, 2-carboxypyrrole-5-acetic acid, 2-carboxypiperidine-6-propionic acid, 2-aminoadipic acid, 3-aminoadipic acid, α-aminoazelaic acid, and 4-aminobenzene-1,3-dicarboxylic acid.

Dicarboxylic acids and derivatives

In some embodiments, the linker can be a dicarboxylic acid. In some embodiments, the dicarboxylic acid can have from 2 to 20 carbon atoms. Examples of dicarboxylic acid include, but are not limited to, butanedioic acid, pentanedioic acid, hexanedioic acid, heptanedioic acid, octanedioic acid, nonanedioic acid, decanedioic acid, undecanedioic acid, dodecanedioic acid, tridecanedioic acid, 1,12-dodecanedicarboxylic acid, 1,15-pentadecanedicarboxylic acid, hexadecanedioic acid, and 1,15-pentadecanedicarboxylic acid. In some embodiments, the dicarboxylic acid is an halogenated dicarboxylic acid, hydroxy dicarboxylic acid, or ether dicarboxylic acid.

Tricarboxylic acids and derivatives

In some embodiments, the linker can be a tricarboxylic acid or a derivative thereof. In some embodiments, the tricarboxylic acid can have from 2 to 30 carbon atoms. The tricarboxylic acid can be aliphatic or cyclic. Examples of tricarboxylic acid include, but are not limited to, 2-phosphonobutane-1,2,4-tricarboxylic acid and 1,2,3-propane tricarboxylic acid.

Alcohols

In some embodiments, the linker can be an alcohol or a derivative thereof. The alcohol can be a diol, triol, amino alcohol, amino dialcohol, amino trialcohol, ethylene glycol, propylene glycol, or a derivative. In some embodiments, the alcohol can have from 2 to 30 carbon atoms. Examples of suitable alcohols include, but are not limited to, triethanolamine, 2-aminoethanol, diisopropanolamine, triisopropanolamine, amino hexanol, 2-[(2-methoxyethyl)methylamino]-ethanol, propanolamine, *N*-methylethanolamine, diethanolamine, butanol amine, isobutanolamine, pentanol amine, 1-amino-3-(2-methoxyethoxy)- 2-propanol, 2-methyl-4-(methylamino)- 2-butanol, 6-amino-1-hexanol, heptaminol, isoetarine, norepinephrine, sphingosine, phenylpropanolamine, derivatives, and combinations thereof.

Polymers

In other embodiments, the linker can be a polymer. A wide variety of polymers and methods for forming the polymers are known in the art of polymer science. Polymers can be degradable or non-degradable polymers. Polymers can be natural or unnatural (synthetic) polymers. Polymers can be homopolymers or copolymers comprising two or more monomers. In terms of sequence, copolymers can be random, block, or comprise a combination of random and block sequences. The polymers can in some embodiments be linear polymers, branched polymers, or hyperbranched/dendritic polymers. The polymers can also be present as a crosslinked particle or surface functionalized inorganic particle. Suitable polymers include, but are not limited to poly(vinyl acetate), copolymers of styrene and alkyl acrylates, and copolymers of vinyl acetate and acrylic acid, polyvinylpyrrolidone, dextran, carboxymethylcellulose, polyethylene glycol, polyalkylene, polyanhydrides, poly(ester anhydrides), polyhydroxy acids, such as polylactide (PLA), polyglycolide (PGA), poly(lactide-co-glycolide) (PLGA), poly-3-hydroxybutyrate (PHB), poly-4-hydroxybutyrate (P4HB), polycaprolactone, polyacrylates and polymethacrylates; polyanhydrides; polyorthoesters; polysytyrene (PS), poly(ethylene-co-maleic anhydride), poly(ethylene maleic anhydride-co-L-dopamine), poly(ethylene maleic anhydride-co-phenylalanine), poly(ethylene maleic anhydride-co-tyrosine), poly(butadiene-co-maleic anhydride), poly(butadiene maleic anhydride-co-L-dopamine) (pBMAD), poly(butadiene maleic anhydride-co-phenylalanine), poly(butadiene maleic anhydride-co-tyrosine), poly(bis carboxy phenoxy propane-co-sebacic anhydride) (poly (CCP:SA)), alginate; and poly(fumaric anhydride-co-sebacic anhydride (p[FA:SA]), copolymers of p[FA:SA], polyacrylates, and polyacrylamides, and copolymers thereof, and combinations thereof.

Other suitable linkers include, but are not limited to, diamino compounds such as ethylenediamine, 1,2-propylenediamine, 1,5-pentanediamine, 1,6-hexanediamine, and the like.

Detectable Moiety and/or Therapeutic Moiety (R^3)

The disclosed compounds can also contain one or more detectable moieties and/or one or more therapeutic moieties, R^3. In some embodiments, the detectable moiety can be the therapeutic moiety. The detectable moiety can contain any detectable label. Examples of suitable detectable labels include, but are not limited to, a UV-Vis label, a near-infrared label, a luminescent group, a phosphorescent group, a magnetic spin resonance label, a photosensitizer, a photocleavable moiety, a chelating center, a heavy atom, a radioactive isotope, a isotope detectable spin resonance label, a paramagnetic moiety, a chromophore, or any combination thereof. In some embodiment, the label is detectable without the addition of further reagents.

In some embodiments, the detectable moiety is a biocompatible detectable moiety, such that the compounds can be suitable for use in a variety of biological applications. "Biocompatible" and "biologically compatible", as used herein, generally refer to compounds that are, along with any metabolites or degradation products thereof, generally non-toxic to cells and tissues, and which do not cause any significant adverse effects to cells and tissues when cells and tissues are incubated (*e.g.*, cultured) in their presence.

The detectable moiety can contain a luminophore such as a fluorescent label or near-infrared label. Examples of suitable luminophores include, but are not limited to, metal porphyrins; benzoporphyrins; azabenzoporphyrine; napthoporphyrin; phthalocyanine; polycyclic aromatic hydrocarbons such as perylene, perylene diimine, pyrenes; azo dyes; xanthene dyes; boron dipyoromethene, aza-boron dipyoromethene, cyanine dyes, metal-ligand complex such as bipyridine, bipyridyls, phenanthroline, coumarin, and acetylacetonates of ruthenium and iridium; acridine, oxazine derivatives such as benzophenoxazine; aza-annulene, squaraine; 8-hydroxyquinoline, polymethines, luminescent producing nanoparticle, such as quantum dots, nanocrystals; carbostyril; terbium complex; inorganic phosphor; ionophore such as crown ethers affiliated or derivatized dyes; or combinations thereof. Specific examples of suitable luminophores include, but are not limited to, Pd (II) octaethylporphyrin; Pt (II)-octaethylporphyrin; Pd (II) tetraphenylporphyrin; Pt (II) tetraphenylporphyrin; Pd (II) meso-tetraphenylporphyrin tetrabenzoporphine; Pt (II) meso-tetrapheny metrylbenzoporphyrin; Pd (II) octaethylporphyrin ketone; Pt (II) octaethylporphyrin ketone; Pd (II) meso-tetra(pentafluorophenyl)porphyrin; Pt (II) meso-tetra (pentafluorophenyl) porphyrin; Ru (II) tris(4,7-diphenyl-1,10-phenanthroline) (Ru $(dpp)_3$); Ru (II) tris(1,10-phenanthroline) $(Ru(phen)_3)$, tris(2,2'-bipyridine)ruthenium (II) chloride hexahydrate $(Ru(bpy)_3)$; erythrosine B; fluorescein; eosin; iridium (III) ((N-methyl-benzimidazol-2-yl)-7-(diethylamino)-coumarin)); indium (III) ((benzothiazol-2-yl)-7- (diethylamino)-coumarin))-2-(acetylacetonate); Lumogen dyes; Macroflex fluorescent red; Macrolex fluorescent yellow; Texas Red; rhodamine B; rhodamine 6G; sulfur rhodamine; m-cresol; thymol blue; xylenol blue; cresol red; chlorophenol blue; bromocresol green; bromcresol red; bromothymol blue; Cy2; a Cy3; a Cy5; a Cy5.5; Cy7; 4-nitrophenol; alizarin; phenolphthalein; *o*-cresolphthalein; chlorophenol red; calmagite; bromo-xylenol; phenol red; neutral red; nitrazine; 3,4,5,6-tetrabromphenolphtalein; congo red; fluorescein; eosin; 2',7'-dichlorofluorescein; 5(6)-carboxy-fluorescein; carboxynaphtofluorescein; 8-hydroxypyrene-1,3,6-trisulfonic acid; semi-naphthorhodafluor; semi-naphthofluorescein; tris (4,7-diphenyl-1,10-phenanthroline) ruthenium (II) dichloride; (4,7-

diphenyl-1,10-phenanthroline) ruthenium (II) tetraphenylboron; platinum (II) octaethylporphyin; dialkylcarbocyanine; and dioctadecylcycloxacarbocyanine; derivatives or combinations thereof.

The detectable moiety can contain a radiolabel, also referred to herein as radioisotope. The radiolabel can also be a therapeutic moiety, i.e., a radiolabel comprising a therapeutic
5 radionuclide such as, ^{90}Y or ^{177}Lu. Other examples of suitable radiolabels include, but are not limited to, isotopes such as ^{18}F, ^{68}Ga, ^{64}Cu, ^{67}Cu, ^{89}Zr, ^{111}In, ^{124}I, ^{123}I, and ^{99m}Tc. In some embodiments, the radiolabel can be chelated by a macrocyclic molecule. Examples of such macrocyclic molecules include, but are not limited to, 2,2',2''-(10-(2-((2,5-dioxopyrrolidin-1-yl)oxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (DOTA) -based
10 chelators, diethylene triamine pentaacetic acid (DTPA)-based chelators, and a derivative or a combination thereof.

The detectable moiety can contain a magnetic spin resonance label. Examples of suitable spin resonance label include free radicals such as nitroxide-stable free radicals. Stable free radicals of nitroxides are known in the art, see for example Keana, "Newer Aspects of Synthesis
15 and Chemistry of Nitroxide Spin Labels", *Chemical Reviews,* 1978, Vol. 78 No. 1, pp. 37-64, which disclosure is incorporated herein by reference. Suitable nitroxides include, but are not limited to, those derived from 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO), 2,2,5,5-tetramethylpyrroline-N-oxyl, and 4,4-dimethyloxazolidine-N-oxyl which is a doxyl nitroxide. All of these compounds are paramagnetic and hence capable of excitation or changes in
20 magnetic resonance energy levels and therefore provide imaging. Other nitroxides include, but are not limited to, doxyl nitroxides, proxyl nitroxides, azethoxyl nitroxides, imidazoline derived nitroxides, tetrahydrooxazine derived nitroxides, and the recently synthesized steroid nitroxides, and the like.

Spin labeling, as used herein, is understood to mean "spin label" as that is defined in the
25 Keana article, namely when a nitroxide bearing molecule that is covalently attached to another molecule of interest, the nitroxide grouping does not significantly disturb the behavior of the system under study. Thus, the nitroxide molecule being paramagnetic, simply enhances the energy or excitation level subjected to the magnetic field during the magnetic resonance.

Therapeutic Moiety

30 The disclosed compounds can also contain a therapeutic moiety. The detectable moiety can be linked to a therapeutic moiety. Therapeutic moiety refers to a group that when administered to a subject, will cure, or at least relieve to some extent, one or more symptoms of, a disease or disorder. Therapeutic moieties include a wide variety of drugs, including antagonists, for example enzyme inhibitors, and agonists, for example a transcription factor

which results in an increase in the expression of a desirable gene product (although as will be appreciated by those in the art, antagonistic transcription factors can also be used), are all included. In addition, therapeutic moiety includes those agents capable of direct toxicity and/or capable of inducing toxicity towards healthy and/or unhealthy cells in the body. Also, the therapeutic moiety can be capable of inducing and/or priming the immune system against potential pathogens. A number of mechanisms are possible including without limitation, (i) a radioisotope linked to a protein as is the case with a radiolabeled protein, (ii) an antibody linked to an enzyme that metabolizes a substance, such as a prodrug, thus rendering it active in vivo, (iii) an antibody linked to a small molecule therapeutic agent, (iv) a radioisotope, (v) a carbohydrate, (vi) a lipid, (vii) a thermal ablation agent, (viii) a photosensitizing agent, and (ix) a vaccine agent.

The therapeutic compound or moiety can be one that kills or inhibits cancer cells directly (e.g., cisplatin) or it can be one that can kill or inhibit a cancer cell indirectly (e.g., gold nanoparticles that kill or destroy cancer cells when heated using a light source). In one aspect, the compounds can include therapeutic moieties including without limitation small molecules or drugs. In some embodiments, the drug is doxorubicin. In some embodiments, doxorubicin can be substituted with a doxorubicin analog such as fluorescein. The spectroscopic signature of fluorescein (UV absorbance (229 nm), visible absorbance (495 nm) and strong fluorescence (520 nm)) makes it an inexpensive and easy molecule to monitor. In some embodiments, the therapeutic moiety can comprise a targeting moiety, such as a peptide. In a specific example, the therapeutic moiety is a VEGFR ligand, such as vandetanib.

Pharmaceutical Compositions

The compounds described herein can be formulated for enteral, parenteral, topical, or pulmonary administration. The compounds can be combined with one or more pharmaceutically acceptable carriers and/or excipients that are considered safe and effective and can be administered to an individual without causing undesirable biological side effects or unwanted interactions. The carrier is all components present in the pharmaceutical formulation other than the active ingredient or ingredients.

The compounds described herein can be formulated for parenteral administration. "Parenteral administration", as used herein, means administration by any method other than through the digestive tract or non-invasive topical or regional routes. For example, parenteral administration can include administration to a patient intravenously, intradermally, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostatically, intrapleurally, intratracheally, intravitreally, intratumorally, intramuscularly, subcutaneously,

subconjunctivally, intravesicularly, intrapericardially, intraumbilically, by injection, and by infusion.

Parenteral formulations can be prepared as aqueous compositions using techniques known in the art. Typically, such compositions can be prepared as injectable formulations, for example, solutions or suspensions; emulsions, such as water-in-oil (w/o) emulsions, oil-in-water (o/w) emulsions, and microemulsions thereof, liposomes, or emulsomes.

The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, one or more polyols (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol), oils, such as vegetable oils (*e.g.*, peanut oil, corn oil, sesame oil, etc.), and combinations thereof.

Solutions and dispersions of the active compounds can be prepared in water or another solvent or dispersing medium suitably mixed with one or more pharmaceutically acceptable excipients including, but not limited to, surfactants, dispersants, emulsifiers, pH modifying agents, viscosity modifying agents, and combination thereof.

Suitable surfactants can be anionic, cationic, amphoteric or nonionic surface active agents. Suitable anionic surfactants include, but are not limited to, those containing carboxylate, sulfonate and sulfate ions. Examples of anionic surfactants include sodium, potassium, ammonium of long chain alkyl sulfonates and alkyl aryl sulfonates such as sodium dodecylbenzene sulfonate; dialkyl sodium sulfosuccinates, such as sodium dodecylbenzene sulfonate; dialkyl sodium sulfosuccinates, such as sodium bis-(2-ethylthioxyl)-sulfosuccinate; and alkyl sulfates such as sodium lauryl sulfate. Cationic surfactants include, but are not limited to, quaternary ammonium compounds such as benzalkonium chloride, benzethonium chloride, cetrimonium bromide, stearyl dimethylbenzyl ammonium chloride, polyoxyethylene and coconut amine. Examples of nonionic surfactants include ethylene glycol monostearate, propylene glycol myristate, glyceryl monostearate, glyceryl stearate, polyglyceryl-4-oleate, sorbitan acylate, sucrose acylate, PEG-150 laurate, PEG-400 monolaurate, polyoxyethylene monolaurate, polysorbates, polyoxyethylene octylphenylether, PEG-1000 cetyl ether, polyoxyethylene tridecyl ether, polypropylene glycol butyl ether, Poloxamer™ 401, stearoyl monoisopropanolamide, and polyoxyethylene hydrogenated tallow amide. Examples of amphoteric surfactants include sodium N-dodecyl-.beta.-alanine, sodium N-lauryl-.beta.-iminodipropionate, myristoamphoacetate, lauryl betaine and lauryl sulfobetaine.

The formulation can contain a preservative to prevent the growth of microorganisms. Suitable preservatives include, but are not limited to, parabens, chlorobutanol, phenol, sorbic acid, and thimerosal. The formulation can also contain an antioxidant to prevent degradation of the active agent(s).

The formulation is typically buffered to a pH of 3-8 for parenteral administration. Suitable buffers include, but are not limited to, phosphate buffers, acetate buffers, and citrate buffers.

Water soluble polymers are often used in formulations for parenteral administration. Suitable water-soluble polymers include, but are not limited to, polyvinylpyrrolidone, dextran, carboxymethylcellulose, and polyethylene glycol.

Sterile injectable solutions can be prepared by incorporating the active compounds in the required amount in the appropriate solvent or dispersion medium with one or more of the excipients listed above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those listed above.

For parenteral administration, the compound can be incorporated into microparticles, nanoparticles, or combinations thereof. For example, the compound can be incorporated into polymeric microparticles.

Suitable oral dosage forms include tablets, capsules, solutions, suspensions, syrups, and lozenges. Tablets can be made using compression or molding techniques well known in the art. Gelatin or non-gelatin capsules can be prepared as hard or soft capsule shells, which can encapsulate liquid, solid, and semi-solid fill materials, using techniques well known in the art. Formulations can be prepared using a pharmaceutically acceptable carrier. As generally used herein "carrier" includes, but is not limited to, diluents, preservatives, binders, lubricants, disintegrators, swelling agents, fillers, stabilizers, and combinations thereof.

Suitable dosage forms for topical administration include creams, ointments, salves, sprays, gels, lotions, emulsions, and transdermal patches. The formulation can be formulated for transmucosal, transepithelial, transendothelial, or transdermal administration. The compounds can also be formulated for intranasal delivery, pulmonary delivery, or inhalation. The compositions can further contain one or more chemical penetration enhancers, membrane permeability agents, membrane transport agents, emollients, surfactants, stabilizers, and combination thereof.

Suitable classes of penetration enhancers are known in the art and include, but are not limited to, fatty alcohols, fatty acid esters, fatty acids, fatty alcohol ethers, amino acids, phospholipids, lecithins, cholate salts, enzymes, amines and amides, complexing agents (liposomes, cyclodextrins, modified celluloses, and diimides), macrocyclics, such as macrocylic lactones, ketones, and anhydrides and cyclic ureas, surfactants, N-methyl pyrrolidones and derivatives thereof, DMSO and related compounds, ionic compounds, azone and related

compounds, and solvents, such as alcohols, ketones, amides, polyols (*e.g.*, glycols). Examples of these classes are known in the art.

In one embodiment, the compounds are formulated for pulmonary delivery, such as intranasal administration or oral inhalation. The respiratory tract is the structure involved in the exchange of gases between the atmosphere and the blood stream. The lungs are branching structures ultimately ending with the alveoli where the exchange of gases occurs. The alveolar surface area is the largest in the respiratory system and is where drug absorption occurs. The alveoli are covered by a thin epithelium without cilia or a mucus blanket and secrete surfactant phospholipids. Carriers for pulmonary formulations can be divided into those for dry powder formulations and for administration as solutions. Aerosols for the delivery of therapeutic agents to the respiratory tract are known in the art. For administration via the upper respiratory tract, the formulation can be formulated into a solution, *e.g.*, water or isotonic saline, buffered or unbuffered, or as a suspension, for intranasal administration as drops or as a spray. Preferably, such solutions or suspensions are isotonic relative to nasal secretions and of about the same pH, ranging *e.g.*, from about pH 4.0 to about pH 7.4 or, from pH 6.0 to pH 7.0. Buffers should be physiologically compatible and include, simply by way of example, phosphate buffers. For example, a representative nasal decongestant is described as being buffered to a pH of about 6.2. One skilled in the art can readily determine a suitable saline content and pH for an innocuous aqueous solution for nasal and/or upper respiratory administration.

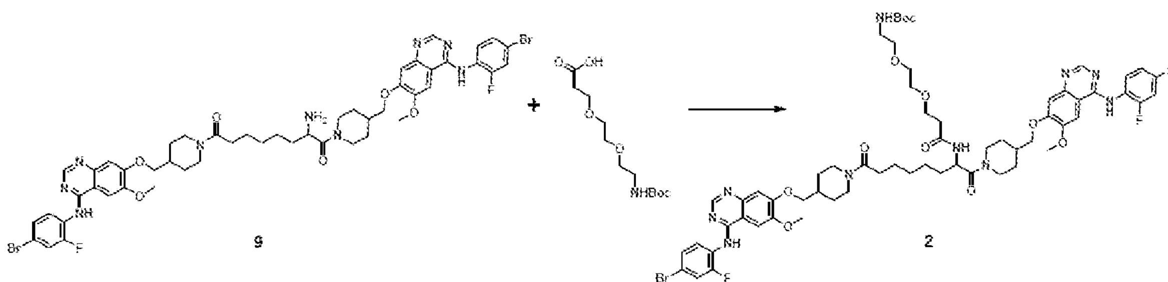
The compounds described herein can be co-administered with one or more additional active agents, such as diagnostic agents, therapeutic agents, and/or prophylactic agents.

Methods of Making

The synthetic route for preparing compounds as disclosed herein is disclosed in Yang et al., Single-Molecule Force Measurement Guides the Design of Multivalent Ligands with Picomolar Affinity, *Angew.Chem. Int. Ed.* **2019**, 58: 5272-5276. Briefly, the linker can be prepared initially followed by covalently binding the VEGFR binding moiety (*e.g.* **ZD-1**) to the linker. The detection moiety can then be linked to the linker. Alternatively, additional VEGFR binding moieties can be added to produce the trimer (ZD-3) or tetramer (ZD-4 or ZD-5). Briefly, the functional-binder (such as a thiol-binder) probe used in the methods disclosed herein can be synthesized by mixing a solution of compound ZD6474 and thioctic acid in a solvent followed by cooling to about 0°C. A solution of EDC can be added followed by stirring at room temperature until all the reactants have been reacted. A basic solution such as aqueous NH_4Cl can be was added to the mixture, and the aqueous solvent removed followed by addition of an organic solvent. The solution can be washed and then dried to yield the crude product, which can

be purified by silica gel column chromatography (5% v/v methanol/CH_2Cl_2) to get the pure product of ZD6474 probe as a yellow solid.

The divalent compound, ZD-2 can be prepared by adding EDC-HCl to a solution of the compound 9 below and tBoc-N-amido-PEG2-acid in DMF cooled to 0°C. The mixture can be stirred at room temperature for 10 h. The aqueous solvent can be removed followed by addition of an organic solvent. The solution can be washed and then dried to yield the crude product, which can be purified by silica gel column chromatography to get the pure product of ZD-2 probe as a yellow solid.



The tetravalent compound, ZD-4 can be prepared from ZD-2 by reacting with an additional linker moiety to join two molecules of ZD-2.

Other methods for coupling the VEGFR binding moiety and detectible moiety to a linker are disclosed herein and/or can be ascertained by the skilled artisan without undue experimentation. The particular method will depend on the specific detectible moiety, VEGFR binding moiety, and linker. Generally, the VEGFR binding moiety can be treated with a linker that can form a bond with the VEGFR binding moiety. That product can then be coupled with the detectable moiety. Alternatively, the linker and the detectible moiety can be coupled beforehand and then coupled with the VEGFR binding moiety. Alternatively, the linker, VEGFR binding moiety, and the detectible moiety can be coupled simultaneously.

The synthetic routes for preparing integrin antagonist (compound IA) and integrin antagonist dimer (compound IA dimer) as disclosed herein are disclosed in Schemes 1 and 3. The integrin antagonist or integrin antagonist dimer can be coupled to the thiol-PEG spacer as described in Schemes 6 or 7. The EphB4 antagonist or EphB4 antagonist dimer can be coupled to the thiol-PEG spacer as described in Schemes 8 or 9.

Methods of Use

VEGFR ligands stimulate cellular responses by binding to tyrosine kinase receptors on the cell surface, known as VEGFR1 (Flt-1), VEGFR2 (Flk-1, KDR), and VEGFR3 (Flt-4). The VEGFR-1 and VEGFR-2 receptors are over expressed in a variety of tumors and are associated

with advanced tumor growth and induction of tumor angiogenesis. For example, when VEGFR ligands bind to VEGFR-1 and VEGFR-2, a tyrosine kinase signaling cascade begins in endothelial cells that stimulate the production of factors that stimulate vessel permeability, proliferation/survival, migration, and finally differentiation into mature blood vessels. This is a fundamental step in the transition of tumors from a benign state to a malignant one.

Erythropoietin-producing hepatocellular (Eph) Type-B receptor 4 (EphB4) is part of the largest family of membrane-bound receptor tyrosine kinases (RTK) which consists of 14 different receptors which are classed as EphA or EphB. Their ligands, the ephrins, are also cell membrane-bound, either via glycosylphosphatidylinositol (GPI)-linkage (ephrin-A ligands) or transmembrane-embedded (ephrin-B ligands). Interaction between Eph receptors and their ligands normally takes place in trans through the binding of 2 ligands on one cell to 2 receptors on an adjacent cell forming a heterotetramer that is the basic complex required for signaling. Depending on the cell-environment conditions, EphB4 demonstrates the ability to be both a tumor promoter, when over-expressed and in the absence of stimulation by its sole cognate ligand, ephrin-B2, as well as a tumor suppressor stimulated by ephrin-B2. EphB4 is overexpressed in 66% of prostate cancer clinical samples and has been implicated in prostate cancer development and progression.

Integrins are obligate heterodimeric cell surface receptors, which are present in all nucleated cells of the human body. Each integrin consists of one of 18 α- and one of eight β-subunits, giving rise to a repertoire of 24 different integrins in mammals. Integrins are involved in key developmental processes such as cell differentiation, cell adhesion, cell migration, cell proliferation and cell survival and are expressed in all metazoans. Each cell type exhibit a specific range of integrins and this repertoire changes according to the cellular or environmental input. In cancer, malignant cells change this repertoire in response to changes in the components or stiffness of the extracellular matrix, in response to growth factors or due to intracellular alterations such as activation of oncogenes.

In some embodiments, the compounds disclosed herein bind to receptors in their target receptor expressing cells. In some embodiments, the compounds bind to receptor- (such as VEGFR2 in VEGFR2) expressing cells. The compounds can bind to receptors with a mean equilibrium dissociation constant (K_d) value from about 100 nM to about 0.01 nM, for *e.g.* about 95 nM, about 90 nM, about 85 nM, about 80 nM, about 75 nM, about 70 nM, about 65 nM, about 60 nM, about 55 nM, about 50 nM, about 45 nM, about 40 nM, about 35 nM, about 30 nM, about 25 nM, about 20 nM, about 15 nM, about 10 nM, about 9 nM, about 8 nM, about 8 nM, about 7 nM, about 6 nM, about 5 nM, about 4 nM, about 3 nM, about 2 nM, about 1 nM,

about 0.9 nM, about 0.8 nM, about 0.7 nM, about 0.6 nM, about 0.5 nM, about 0.4 nM, about 0.3 nM, about 0.2 nM, about 0.1 nM, about 0.05 nM, about 0.01 nM, or about 44.7 nM or about 0.45 nM.

In some embodiments, the compounds disclosed can selectively inhibit the activity of receptors (such as tyrosine kinase activity of VEGFR or EphB4). In some embodiments, the compounds disclosed can selectively inhibit tyrosine kinase activity of VEGFR2. In some embodiments, the compounds disclosed can inhibit activity of receptors (such as tyrosine kinase activity of VEGFR2 or EphB4) with 50% inhibitory concentration (IC_{50}) values of less than about 40 nM, less than about 35 nM, less than about 30 nM, less than about 25 nM, less than about 20 nM, less than about 19 nM, less than about 18 nM, less than about 17 nM, less than about 16 nM, less than about 15 nM, less than about 14 nM, less than about 13 nM, less than about 10 nM, less than about 9 nM, less than about 8 nM, less than about 7 nM, less than about 6 nM, less than about 5 nM, less than about 4 nM, less than about 3 nM, less than about 2 nM, less than about 1 nM, less than about 0.9 nM, less than about 0.8 nM, less than about 0.7 nM, less than about 0.6 nM, less than about 0.5 nM, less than about 0.4 nM, less than about 0.3 nM, less than about 0.2 nM, less than about 0.1 nM, less than about 0.09 nM, less than about 0.08 nM, less than about 0.07 nM, less than about 0.06 nM, less than about 0.05 nM, less than about 0.04 nM, less than about 0.03 nM, less than about 0.02 nM, less than about 0.01 nM, less than about 0.001 nM, or less than about 0.0001 nM. In some embodiments, the disclosed compounds can block VEGF-stimulated endothelial cell proliferation and migration. In some embodiments, the disclosed compounds can reduce tumor vessel permeability. In some embodiments, the disclosed compounds can be used to treat late-stage (metastatic) medullary thyroid cancer, prostate cancer, or triple negative breast cancer. In some embodiments, the disclosed compounds inhibit growth of experimental lung metastasis.

In some embodiments, the compounds disclosed can accumulate in selected receptor (such as VEGFR) expressing cells post administration. The compounds can be administered via systemic administration, such as intravenous administration or subcutaneous administration, oral administration or by intratumoral injection. In some embodiments, the compounds disclosed can accumulate in selected receptor (such as VEGFR) expressing cells, for example, tumor cells, within about 36 hours, about 48 hours, about 24 hours, about 23 hours, about 22 hours, about 20 hours, about 19 hours, about 18 hours, about 17 hours, about 16 hours, about 15 hours, about 14 hours, about 13 hours, about 12 hours, about 11 hours, about 10 hours, about 9 hours, about 8 hours, about 7 hours, about 6 hours, about 5 hours, about 4 hours, about 3 hours, about 2 hours, about 1.5 hours, about 1 hours, about 50 minutes, about 45 minutes, about 40 minutes, about 35

minutes, about 30 minutes, about 25 minutes, about 20 minutes, about 15 minutes, about 10 minutes, about 5 minutes post administration. In some embodiments, the selected receptor expressing cells, e.g., tumor cells exhibit sufficient uptake of the compounds disclosed, post administration. Sufficient, as used herein refers to uptake of the disclosed compounds into selected receptor expressing cells such that optical imaging of the selected receptor expressing cells exhibit low background noise. In some embodiments, the selected receptor expressing cells exhibit uptake of greater than about 5%ID/g, greater than about 4.7%ID/g, greater than about 4.5%ID/g, greater than about 4.3%ID/g, greater than about 4%ID/g, greater than about 3.7%ID/g, greater than about 3.5%ID/g, greater than about 3.3%ID/g, greater than about 3%ID/g, greater than about 2.7%ID/g, greater than about 2.5%ID/g, greater than about 2.3%ID/g, greater than about 2%ID/g, greater than about 1.8%ID/g, greater than about 1.5%ID/g, greater than about 1.3%ID/g, greater than about 1%ID/g, greater than about 0.9%ID/g, greater than about 0.8%ID/g, greater than about 0.6%ID/g, greater than about 0.5%ID/g, greater than about 0.4%ID/g, greater than about 0.3%ID/g, greater than about 0.2%ID/g, greater than about 0.1%ID/g, for example about 2. 7%ID/g, about 3.70%ID/g, about 3.8%ID/g, about 0.3%ID/g, about 0.7%ID/g, or about 0.5%ID/g of the disclosed compounds post administration.

Because of the high uptake of the compounds in selected receptor (such as VEGFR) expressing cells, the compounds disclosed herein can be used to image receptor expression, for example, by confocal microscopic imaging, CT imaging, PET imaging, MRI, or any combination thereof. In some embodiments, the compounds disclosed can be used to image selected receptor (such as VEGFR) expression in a disease. In some embodiments, the disclosed compounds can be used to image selected receptor (such as VEGF) stimulated endothelial cell proliferation and migration. In some embodiments, the disclosed compounds can be used to image tumor vessel permeability. In some embodiments, the disclosed compounds can be used to image late-stage (metastatic) medullary thyroid cancer. In some embodiments, the disclosed compounds can be used to image growth of experimental lung metastasis. In some embodiments, the disclosed compounds can be used to image pulmonary hypertension.

In some embodiments, non-targeted cells and/or tissues such as the kidney, muscles, heart, blood, lung, gastrointestinal tract, and/or spleen exhibit low uptake of the disclosed compounds. In some embodiments, uptake of the disclosed compounds in the non-targeted tissues such as the kidney is less than about 2.5%ID/g, less than about 2.3%ID/g, less than about 2%ID/g, less than about 1.8%ID/g, less than about 1.5%ID/g, less than about 1.3%ID/g, less than about 1%ID/g, less than about 0.9%ID/g, less than about 0.8%ID/g, less than about

0.6%ID/g, less than about 0.5%ID/g, less than about 0.4%ID/g, less than about 0.3%ID/g, less than about 0.2%ID/g, or less than about 0.1%ID/g at about 24 hours, about 23 hours, about 22 hours, about 20 hours, about 19 hours, about 18 hours, about 17 hours, about 16 hours, about 15 hours, about 14 hours, about 13 hours, about 12 hours, about 11 hours, about 10, about 9 hours, about 8 hours, about 7 hours, about 6 hours, about 5 hours, about 4 hours, about 3 hours, about 2 hours, about 1.5 hour, about 1 hour, about 50 minutes, about 45 minutes, about 40 minutes, about 35 minutes, about 30 minutes, about 25 minutes, about 20 minutes, about 15 minutes, about 10 minutes, about 5 minutes post administration.

The ratio of compound uptake in the targeted cells to non-targeted cells, for example, tumor cells to muscle cells can be high. In some embodiments, the ratio of compound uptake in targeted cells to non-targeted cells, for example, tumor cells to muscle cells can be greater than 5, greater than 6, greater than 7, greater than 8, greater than 9, greater than 10, greater than 11, greater than 12, greater than 13, greater than 14, greater than 15, greater than 16, greater than 17, greater than 18, greater than 19, greater than 20, greater than 25, greater than 30, greater than 35, greater than 40, greater than 45, or greater than 50. The ratio of compound uptake in targeted cells to non-targeted cells can remain high for as long as about 2 hours, about 3 hours, about 4 hours, about 6 hours, about 10 hours, about 15 hours, about 18 hours, about 20 hours, about 24 hours, about 36 hours, about 46 hours, or as long as the compound is in a subject, post administration.

In one embodiment, the compounds disclosed herein exhibit rapid clearance from the blood. For example, in some embodiments, the liver exhibit high compound uptake at early time points of about 10 hours, about 9 hours, about 8 hours, about 7 hours, about 6 hours, about 5 hours, about 4 hours, about 3 hours, about 2 hours, about 1.5 hour, about 1 hour, about 50 minutes, about 45 minutes, about 40 minutes, about 35 minutes, about 30 minutes post administration. In these embodiments, the liver uptake can be about 20%ID/g, about 18%ID/g, about 15%ID/g, about 12%ID/g, about 10%ID/g, about 8%ID/g, or about 5%ID/g. As time progresses, the amount of compound in the liver is decreased to less than about 5%ID/g, less than about 4.7%ID/g, less than about 4.5%ID/g, less than about 4.3%ID/g, less than about 4%ID/g, less than about 3.7%ID/g, less than about 3.5%ID/g, less than about 3.3%ID/g, less than about 3%ID/g, less than about 2.7%ID/g, less than about 2.5%ID/g, less than about 2.3%ID/g, less than about 2%ID/g, less than about 1.8%ID/g, less than about 1.5%ID/g, less than about 1.3%ID/g, less than about 1%ID/g, less than about 0.9%ID/g, less than about 0.8%ID/g, less than about 0.6%ID/g, less than about 0.5%ID/g, less than about 0.4%ID/g, less than about 0.3%ID/g, less than about 0.2%ID/g, or less than about 0.1%ID/g at about 24 hours,

about 23 hours, about 22 hours, about 20 hours, about 19 hours, about 18 hours, about 17 hours, about 16 hours, about 15 hours, about 14 hours, about 13 hours, about 12 hours, about 11 hours, about 10 hours post administration.

In some embodiments, the disclosed compounds exhibit good extravasations and diffusion into the extracellular space. In some embodiments, the disclosed compounds can selectively bind to tumor cells, can exhibit a high diffusion rate in fluids, can exhibit fast clearance from blood, and/or exhibit good metabolic stability compared to labeled-VEGFR antibodies.

Methods for detecting or imaging cells expressing vascular endothelial growth factor receptor (VEGFR) in a mammal are disclosed. In some embodiments, the method can noninvasively determine VEGFR expression levels, by optical imaging, in VEGFR expressing cells. In some embodiments, the method can be used to identify patients that respond to anti-angiogenic drug. In some embodiments, the method can be used to diagnose and monitor the proliferation and development of angiogenic tumors. The method comprise administering to the mammal one or more of the disclosed compound, in an amount and for a time sufficient to detect or image at least a population of the cells expressing VEGFR in the mammal to which the detectable moiety is bound. The detectable moiety can be identified by confocal microscopic imaging, CT imaging, PET imaging, MRI, or any combination thereof.

Methods for imaging a population of cells expressing VEGFR within or about the body of an animal are also disclosed. These methods comprise administering to the animal an amount of one or more of the disclosed compound for a time effective to image a population of cells expressing VEGFR within or about the body of the animal. In some embodiments, the population of cells expressing VEGFR includes cancer cells, tumor cells, hyperproliferative cells, or any combination thereof. In some embodiments, the animal is a human diagnosed with cancer.

Methods of treating or ameliorating a symptom of a disease, dysfunction, or abnormal condition in a mammal are disclosed. These methods comprise administering to the mammal one or more of the compounds disclosed herein in an amount, and for a time sufficient to treat or ameliorate the symptom of the disease, dysfunction, or abnormal condition in the mammal. The compounds can contain a detectable moiety and/or therapeutic moiety that kills or inhibits an infected, dysfunctional, or abnormal cell and/or tissue directly (e.g., cisplatin) or indirectly (e.g., radioisotope or gold nanoparticle that kill or destroy cells when irradiated with a light source). If the detectable and/or therapeutic moiety is one that kills or inhibits a cell or tissue indirectly, then the method further comprises a step of taking appropriate action to “activate” or otherwise

implement the activity of the moiety. For example, the detectable/therapeutic moiety attached to the disclosed compounds can be a gold nanoparticle and following administration to the patient and binding of the compound to cancer cells, the nanoparticles are irradiated, e.g., using a laser light, to kill or destroy the nearby cancer cells. In some embodiments, the method involves image guided surgery using a compound comprising a detectable moiety to detect and resect cancer from a subject followed by the use of the same or a different compound to kill the remaining cancer cells.

The compounds disclosed herein contain an effective amount of the one or more of the compounds disclosed. The amount to be administered can be readily determined by the attending physician based on a variety of factors including, but not limited to, age of the patient, weight of the patient, disease or disorder to be imaged or treated, and presence of a pre-existing condition, and dosage form to be administered (*e.g.*, immediate release versus modified release dosage form). Typically, the effective amount is from about 0.1 mBq/kg to about 200 mBq/kg (e.g., less than about 5 mBq/kg, less than about 10 mBq/kg, less than about 15 mBq/kg, less than about 20 mBq/kg, less than about 25 mBq/kg, less than about 30 mBq/kg, less than about 40 mBq/kg, less than about 50 mBq/kg, less than about 75 mBq/kg, less than about 100 mBq/kg, less than about 125 mBq/kg, less than about 150 mBq/kg, less than about 175 mBq/kg, less than about 200 mBq/kg. Dosages greater or less than this can be administered depending on the diseases or disorder to be treated or imaged.

The compounds disclosed herein can be administered in an effective amount to image or treat a variety of diseases and disorders including but not limited to, proliferative disorders (*e.g.*, cancers), diabetes, psoriasis, rheumatoid arthritis, pulmonary hypertension, Kaposi's sarcoma, hemangioma, acute and chronic nephropathies, atheroma, arterial restenosis, autoimmune diseases, acute inflammation, ocular diseases with retinal vessel proliferation, diabetic retinopathy, macular degeneration, and angiosarcoma.

The disclosed compounds can be used for PET imaging of tumor. For example, VEGF pathway plays a significant role in formation of new vessels which lead to increasing tumor size and metastatic spread of cancer. VEGF inhibitors target this important pathway in cancer progression. Further, immunotherapy has emerged one the most effective treatment changing paradigm in cancer. VEGF receptor PET imaging has a role in determining how immunotherapy is clinically used. Studies have shown that the efficacy of immunotherapy in the treatment of lung cancer is dependent on tumor vascularity which in turn can be measured by VEGF receptor expression. Measuring the uptake of VEGF using the compounds disclosed herein (such as 18F-ZD-2) could be used to screen patients with lung cancer to determine which ones would have a

greater chance of responding to immunotherapy beyond traditional genetic markers, such a PD-L1. Figure 7 shows therapy data generated for ^{177}Lu-DiZD (divalent compound) compared with anti-PD1 on triple native breast cancer model.

Compounds with two or more receptor (such as VEGFR) binding moieties have significantly increased receptor (such as VEGFR2) binding affinity compared with those compounds having only one receptor binding moiety. In the noninvasive small-animal PET imaging studies, ^{64}Cu-DOTA-ZD-2 showed rapid and good tumor uptake, low organs accumulations and quick liver washout, and good tumor-to-background contrast in U87MG xenograft. Overall, bivalency strategy has an efficient effect on the receptor-binding interaction and in vivo kinetics of ^{64}Cu-DOTA-ZD-2. The results indicate that the ^{64}Cu-DOTA-ZD-2 radio conjugate can be used to image tumor angiogenesis, select patient for antiangiogenic treatment, and monitor VEGFR2-targeted cancer therapy. Thus, disclosed herein are methods whereby the disclosed compounds, having a radiolabeled detectable moiety, are administered to an individual and then the individual is scanned to detect the presence, location, and/or concentration of the compound.

The disclosed compounds are particularly advantageous in treating and/or imaging the growth of primary and recurrent solid tumors. Exemplary cancers which can be treated and/or imaged include, but are not limited to, cancer of the skin, colon, uterine, ovarian, pancreatic, lung, bladder, breast, renal system, and prostate. Other cancers include, but are not limited to, cancers of the brain, liver, stomach, esophagus, head and neck, testicles, cervix, lymphatic system, larynx, esophagus, parotid, biliary tract, rectum, endometrium, kidney, and thyroid; including squamous cell carcinomas, adenocarcinomas, small cell carcinomas, gliomas, neuroblastomas, and the like. More particularly the compounds are expected to inhibit the growth of those primary and recurrent solid tumors which are associated with different receptors such as VEGF, EphB4, or integrin, especially those tumors which are significantly dependent on receptors for their growth and spread, including for example, certain tumors of the colon, breast, prostate, lung, vulva and skin.

The compounds described herein can also be used to treat or image metastatic cancer. The compounds can be used in patients who have received prior chemo, radio, or biological therapy or in previously untreated patients. In one embodiment, the patient has received previous chemotherapy. The compounds can be administered using a variety of routes including systemic administration, such as intravenous administration or subcutaneous administration, oral administration or by intratumoral injection. The compounds disclosed can also be used for

imaging in patients who have been rendered free of clinical disease by surgery, chemotherapy, and/or radiotherapy.

The disclosed compounds are also particularly advantageous in treating and/or imaging pulmonary hypertension such as Chronic Thromboembolic Pulmonary Hypertension (CTEPH) and PH associated with interstitial lung disease (WHO group III). The standard test used to diagnose CTEPH is a pulmonary angiogram, usually performed at the time of right heart catheterization. A ventilation-perfusion (V/Q) lung scanning is generally used in distinguishing chronic thromboembolic pulmonary hypertension from other non-embolic causes of PHT PET imaging can replace the V/Q scan.

The disclosed compounds are also particularly advantageous in transplantation procedures. Although transplantation science continues to improve short term outcomes and survival of transplant patients, long term outcomes are plagued by different forms of chronic rejection in all organ transplantation including heart, lung and kidney. A sign of chronic rejection is neovascularization, which is mediated by VEGF pathway. Currently, invasive testing from heart catheterization to invasive biopsies are needed to confirm these diagnoses depending on the organ involved. VEGFR imaging can lead to earlier detection of chronic rejection via PET imaging with the compounds disclosed herein, such as 18F-ZD-2.

The disclosed compounds are also particularly advantageous in vascular applications. Additional uses in cardiovascular patient care include vascular imaging of aortic aneurysms and vascular access graft imaging to assess for disease progression.

Methods for determining the spatial distribution of a receptor in a cell.

As described herein, atomic force microscopy (AFM), by the virtue of its spatial resolution and its ability to function in aqueous systems, is a powerful tool to probe biological systems in their native state at a nanoscale resolution. Disclosed herein are methods based on AFM force measurements to determine the spatial information of cell-surface receptors. Figure 8 is a flow diagram showing method of using AFM to design conforming compounds based on spatial information of cell-surface receptors. In the methods disclosed, the interaction of drug compounds, such as ZD6474 (vandetanib or ZD-1), with its targeting to vascular endothelial growth factor receptors (VEGFR) in live human umbilical vein endothelial cells (HUVECs) was used in determining the spatial information of VEGFR. Other exemplary receptors in which the spatial information can be determined include PD-L1, EphB4 in cancer cells, PD1 CTLA4 in T cells, integrin, angiotensin-converting enzyme (ACE) (such as in SARS-Cov-2 infection), estrogen receptor (ER), or a combination thereof. For example, the interaction of the integrin antagonists (compounds IA and IA dimer in Schemes 2 and 4), with its targeting to integrin

receptors in live human umbilical vein endothelial cells (HUVECs) were used in determining the spatial information of integrin. The interaction of the Ephb4 antagonists (compounds EphB4-007 monomer and EphB4 dimer in Schemes 8 and 9), with its targeting to EphB4 receptors in MDA-MB-468 human triple negative breast cancer cells were used in determining the spatial information of EphB4.

The method for determining the spatial distribution of receptors on a cell can include functionalizing an AFM tip with one or more receptor binding moieties to form a functionalized AFM tip; contacting the functionalized AFM tip with the cell to facilitate binding of the one or more receptor binding moieties with the receptors and form a binder-receptor complex; determining the number of each receptor distributed in an area of the AFM tip, and deriving a maximum distance between two neighboring receptors. As described herein, the receptors on the cell surface can be the same or different. In particular, the methods can be used to determine the spatial distribution of different receptors on a cell for the design of bifunctional multivalent ligands that target different receptors at one time. For example, the methods can be used to determine the spatial distribution of the same or different receptors selected from VEGFR, PD-L1, EphB4 (such as in cancer cells), PD1 CTLA4 (such as in T cells), integrin, angiotensin-converting enzyme (ACE) (such as in SARS-Cov-2 infection), estrogen receptor (ER), or a combination thereof. In some examples, the methods can be used to determine the spatial distribution of EphB4 and EphA4. The receptor binding moiety can be a monomer or a dimer and may be selected from a small molecule therapeutic agent, a peptide, an antibody, an antibody fragment, a carbohydrate, an siRNA, a protein, a nucleic acid, an aptamer, a nanoparticle, a cytokine, a chemokine, a lymphokine, a lipid, a lectin, or a combination thereof.

In some aspects, the method for determining the spatial distribution of receptors on a cell can include conjugating a receptor binding moiety to a functional moiety to form a functional-binder conjugate. For example, the method for determining the spatial distribution of VEGFR, integrin, or EphB4 in a cell can include conjugating a VEGFR binding moiety, an integrin binding moiety, or an EphB4 binding moiety, respectively, to a functional moiety to form a functional-binder conjugate. The functional moiety can be chosen based on the nature of the AFM tips. For example, when the tip is silicon, the functional moiety can include a thiol group. However, the AFM silicon tip can be coated with various metals, such as Au, Pt, Cr, and Ni to impart electrical conductivity or even magnetic conductivity. For metal coated AFM tips, a metal-affinity ligand can be conjugated to the binder and subsequently used to functionalize the AFM tip. Methods of conjugating the binding moiety to a thiol moiety to form the thiol-binder conjugate are described herein.

The method for determining the spatial distribution of receptors on a cell can further comprise functionalizing the AFM tip with the functional-binder conjugate (100% solution) or a diluted solution of the functional-binder conjugate to form a functionalized AFM tip. For example, the method for determining the spatial distribution of VEGFR in a cell can further include functionalizing an AFM tip with the functional-binder (e.g., thiol-binder) conjugate or a diluted solution of the functional-binder conjugate to form a functionalized AFM tip. The diluted solution of the functional-binder conjugate can comprise up to 95% by weight polyethylene glycol functionalized with a functional group (e.g., thiol group). In some examples, the diluted solution of the functional-binder conjugate can comprise 3% or greater, 5% or greater, 10% or greater, 15% or greater, or 25% or greater by weight functional-binder conjugate and 97% or less, 95% or less, 90% or less, 85% or less, or 75% or less by weight polyethylene glycol functionalized with a functional group.

AFM tips can be functionalized with the thiol-binder conjugate by first rinsing and then drying AFM tips prior to use. The tips can then be immersed overnight in an aqueous solution containing the thiol-binder conjugate diluted with various ratios of thiol PEG functionalized with thiol. The functionalized AFM tips can be rinsed with water and then used immediately in the following steps.

In the methods, the functionalized AFM tip can then be contacted with the cell to bind the functional-binder conjugate to the receptor (such as VEGFR, integrin, EphB4, or EphA4) present on the cell and form a binder-receptor complex. In some cases, the cell can be cultured with the functional-binder conjugate or the diluted solution of functional-binder conjugate until the cell reaches 50% or greater confluence.

Adhesive force measurements can be used to determine a dissociative force (force required to dissociate) of an ensemble of the binder-receptor (such as binder-VEGFR) complex and of a single binder-receptor complex. AFM adhesive force measurements can be conducted at room temperature and in the native environment of live cells (such as live HUVECs or MDA-MB-468). To determine the dissociative force, the method can include scanning a surface of the cell using the adhesive force measurement mode, mapping the adhesive force of the binder-receptor complex, selecting a region of the cell with the highest adhesive force signal, deriving a histogram of the dissociative forces obtained from statistics of the adhesive force over the region, and analyzing the histograms and differentiate individual force peaks to determine the dissociative force of the ensemble of the binder-receptor complex and a single binder-receptor complex. The spring constant, kc, (which is proportional to the tip-sample interaction force) of each individual compound can be calibrated in solution using the software, the thermal

fluctuation method. For ZD6474/VEGFR specific unbinding force measurements on live HUVEC, modified tips prepared as described can be used to probe the ZD6474/VEGFR specific binding events at the retraction velocity of 1200 nm/s. For peptide/EphB4 specific unbinding force measurements on live HUVEC modified tips prepared as described can be used to probe the peptide/EphB4 specific binding events. The loading rates were then calculated by multiplying the retraction velocity (nm/s) by the effective spring constant of the cantilever (nN/nm), which in the example below, resulted in a value of loading rate ranging from 60-66 nN/s.

The method can further include determining the number of receptors (such as VEGFR) distributed in an area of the AFM tip. The number of receptors (such as VEGFR) can be determined by dividing the dissociative force of the ensemble of the binder-receptor complex by the dissociative force of the single binder-receptor complex. In the example below, the number of VEGFR within the detection area was estimated around 16 and the detection area of the AFM tip was estimated to be about 250 nm^2 .

A maximum distance between two neighboring VEGFR can be derived using a maximizing minimum algorithm. In particular, the maximal distance of proximal VEGFR can be transformed into a pure mathematical problem that optimizes the random location of the given N points by maximizing their inter-point distances. Specifically, by maximizing the total distance the function:

$$D = \frac{1}{2}\sum_{i=1}^{N}\sum_{j=1, j\neq i}^{N}\left\|\mathbf{x}_i - \mathbf{x}_j\right\|^2, \text{ subject to } \|\mathbf{x}_i\| < r,$$

with random point initialization, the point locations converge to a configuration with maximal distances. Then, the average minimal distances among the points are computed. This experiment can be repeated to get the mean and SD values of point distances for given N and r. Similar calculations can be made for determining the maximum distance between two similar or different neighboring receptors by using a maximizing minimum algorithm.

Methods for making multivalent compounds that target receptors on a cell surface are also described. As described herein, the receptors can be the same or different. The methods can utilize information obtained from the AFM methods that provide the proximal receptor distance between two or more receptors on the cell surface. Once the spatial distribution of the receptors are obtained, multivalent compounds can be synthesized having two or more binding moieties. In some embodiments, the multivalent compounds can comprise a first moiety and a second moiety covalently linked by a first linker, L_1, wherein the first moiety comprises one or more receptor binding moieties, and the second moiety comprises an additional one or more receptor

binding moieties. The first linker can have a length not substantially less than, equal to, or greater than the proximal receptor distance. As used herein, the term "substantially" refers to the complete or nearly complete extent or degree of an action, characteristic, property, state, structure, item, or result. For example, where the first linker can have a length not substantially less than the proximal receptor distance would mean that the first linker has a length within 10%, preferably within 8%, more preferably within 5% of the proximal receptor distance. In some examples, the first linker can have a length within 20%, preferably within 15%, more preferably within 10%, of the proximal receptor distance. Preferably, the first linker has a length within 5% of the proximal receptor distance. The multivalent compounds can have a binding affinity to the receptor that is greater than 10 times, greater than 100 times, greater than 1,000 times, or greater than 2,000 times the binding affinity of a single-valent compound alone. Depending on the nature of the receptors, the first moiety and the second moiety can be the same or different.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (*e.g.*, amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

Example 1: Single-Molecule Force Measurement Guides the Design of Multivalent Ligand with Picomolar Affinity

Interaction of multiple entities and receptors, or multivalency is widely applied to achieve high affinity ligands for diagnostic and therapeutic purpose. However, lack of knowledge on receptor distribution in living subjects remains a challenge for rational structure design. In this example, a force measurement platform to probe the distribution and distance of the cell surface vascular endothelial growth factor receptors (VEGFR) in live cells, and its use to assess the geometry of appropriate linkers for distinct multivalent binding modes are described. A tetravalent lead ZD-4 with combined hybrid binding effects, yielded about 2000-fold improvement in the binding affinity to VEGFR with IC_{50} value of 25 pM. The improved affinity was confirmed by the associated increase of tumor uptake in the VEGFR-targeting positron

emission tomography (PET) imaging using U87 tumor xenograft mouse model. It showed that radiolabeled ZD-4 resulted in 12 times increase in the tumor uptake than radiolabeled parent drug ZD6474. This example demonstrates that combining statistical and chelate effect determined by receptor-receptor distance on live cells for the magnification of multivalent binding is effective. The force measurement platform described would be also amenable to other cell-surface multivalent ligand design.

Introduction: Atomic force microscopy (AFM), by the virtue of its spatial resolution and its ability to function in aqueous systems, has emerged as a powerful tool to probe biological systems in their native state at a nanoscale resolution. In this example, a force measurement platform is described based on AFM to tackle the challenge in spatial information of cell-surface receptors. Specifically, the interaction of drug compound, ZD6474 (vandetanib), with its targeting to vascular endothelial growth factor receptors (VEGFR) in live human umbilical vein endothelial cells (HUVECs) was studied. For the force measurement, the molecular probe ZD6474 was first conjugated with thioctic acid (Figure 2A) and then attached to a gold-coated AFM tip by means of a strong Au-S bond (Figure 2B). The ligand-functionalized tip was brought into contact with the surface of the HUVECs where the binding of ZD6474/VEGFR took place. The tip was then withdrawn from the surface, pulling ligand out of the binding pocket. The force required to separate the ligand from its specific binding site, the "ligand dissociation force" was determined. As shown in Figure 2, thiol modified polyethylene glycol (Thiol PEG) was used as a competing molecule to reduce the number of ZD6474 probe attached to the AFM tip, which could reduce the number of binding events even at a molecular level during each contact by changing the ratio between the ZD6474 probe and the thiol PEG.

For comparison, AFM tips functionalized with 100% ZD6474 probe and 5% ZD6474 probe diluted with thiol PEG were used for the force measurement. As shown in Figures 3A-3F, both AFM height images and adhesion force mapping images were recorded. Interestingly, the protruding area in the force mapping, which represented the specific binding, seemed to be non-uniformly distributed across the native cell surface when comparing the height and force mapping images (Figures 3A-3B, 3C-3D). The specific dissociation events were more concentrated along the cellular periphery (figure not shown). It indicated more concentrated VEGFR distribution at the cell periphery, which is in agreement with independent immunolabeling observations. In view of this heterogeneous distribution of VEGFR, the AFM force measurement was mostly focused on the cell periphery where there appeared to be abundant expression of the receptors.

To decipher the dissociating force of ZD6474/VEGFR, histograms were analyzed from statistics of the dissociation force measured in Figure 3C and 3D. For the group associated with the 100%-tip, the histogram revealed a unimodal distribution of the dissociating forces with a maxima at 728 ± 16 pN (Figure 3E). This showed an ensemble result of multiple binding events between ZD6474 and VEGFR that occurred in the contact experiments. Since the AFM tip was saturated with the probe molecules, this force value was solely determined by the contact area of the AFM tip, which in turn confined the accessible number of receptors to the given contact area. When the probe coverage on the AFM tip was diluted, the accessible number of ligand molecules in the given contact area was dramatically reduced while the number of receptors remained unchanged. This would prominently decrease the number of binding events involved during each contact measurement. With the dilution to 5% of ZD6474, the histogram revealed a quartet distribution of the dissociation force with peak values at 66 ± 28 pN, 110 ± 12 pN, 156 ± 24 pN and 201 ± 17 pN (Figure 3F). As expected, the maximum dissociation force was reduced to 201 ± 17 pN compared to 728 ± 16 pN in the 100% tip. More importantly, each peak in the quartet distribution represented a different number of binding events occurring during the contact measurements, and the difference between each pair of adjacent peaks was 45 ± 1 pN, which was in the range of the smallest peak value of 66 ± 28 pN. These results provided strong evidence that a single binding of ZD6474 to VEGFR was associated with a force of 45 ± 1 pN.

As mentioned above, the contact area of the AFM tip restricted the number of receptors accessed in the force measurements, and the number of receptors within the detecting area (N_{VEGFR}) could be readily calculated from the AFM force measurement as

$$N_{VEGFR}=F_{ensemble}/F_{single},$$

where $F_{ensemble}$ was the ensemble of multiple dissociation forces obtained by the 100%-tip, and F_{single} was the dissociation force of ZD6474/VEGFR at a single-molecule level that was obtained by the 5%-tip. As determined from the data in Figure 3, the value of $F_{ensemble}$ was 728 ± 16 pN and F_{single} was 45 ± 1 pN, and consequently N_{VEGFR} was calculated to be 16 within the effective contact area of the AFM tip measuring about 250 nm^2. Using a maximizing minimum algorithm, the maximal distance between the two adjacent VEGFR on cell periphery in live HUVECs was determined as about 48 Å. This is believed to be the first report of receptor distance in live cells measured by AFM.

With the actual distribution information of the receptors in hand, the lengths of the linkers can be rationally selected to design statistical and chelate bivalent binding. Figure 4 presented a series of multivalent ligands that were used in this example of hybrid multivalent binding. All these compounds were prepared and characterized accordingly (see Yang et al.,

Single-Molecule Force Measurement Guides the Design of Multivalent Ligands with Picomolar Affinity, *Angew.Chem. Int. Ed.* **2019**, 58: 5272-5276 for details), and their binding affinity to VEGFR was measured by the IC_{50} values required to reduce the binding of the parent entity, ZD6474 (ZD-1), in the binding assay of ligand to cell surface receptors as previously described
5 in Kim, et al., *Bioconjugate Chem.* **2013**, 24, 1937-1944 and Li, et al., *J. Nucl. Med.* **2014**, 55, 1525-1531. The inhibitory binding curve is presented in Figure 5, and the measured IC_{50} values are summarized in Table 1. As designed, a linker with length of ~14 Å that was greatly shorter than the proximal VEGFR distance in HUVECs, was used to tether ZD6474 entities and construct a bivalent ZD-2, in which the statistical effect was believed to be dominated in the
10 binding. A second bivalent ZD-3 with linker of ~47 Å that corresponded to the estimated distance of neighboring VEGFR was also constructed to assess the chelate effect. The IC_{50} of the monovalent ZD-1 was determined to be 47 ± 3.0 nM, whereas IC_{50} of the bivalent designs ZD-2 and ZD-3 were found to be 1.8 ± 0.2 nM and 2.2 ± 0.2 nM, respectively. The decreased IC_{50} of ZD-2 and ZD-3 thus represented an approximate 20-fold improvement in the affinity compared
15 to that of ZD-1. It suggests the distinction of statistical and chelate effects in the bivalent binding by using the right lengths of linkers, and both statistical and chelate effects lead to increased receptor binding.

Table 1. Linker length and IC_{50} of compounds ZD1-5.

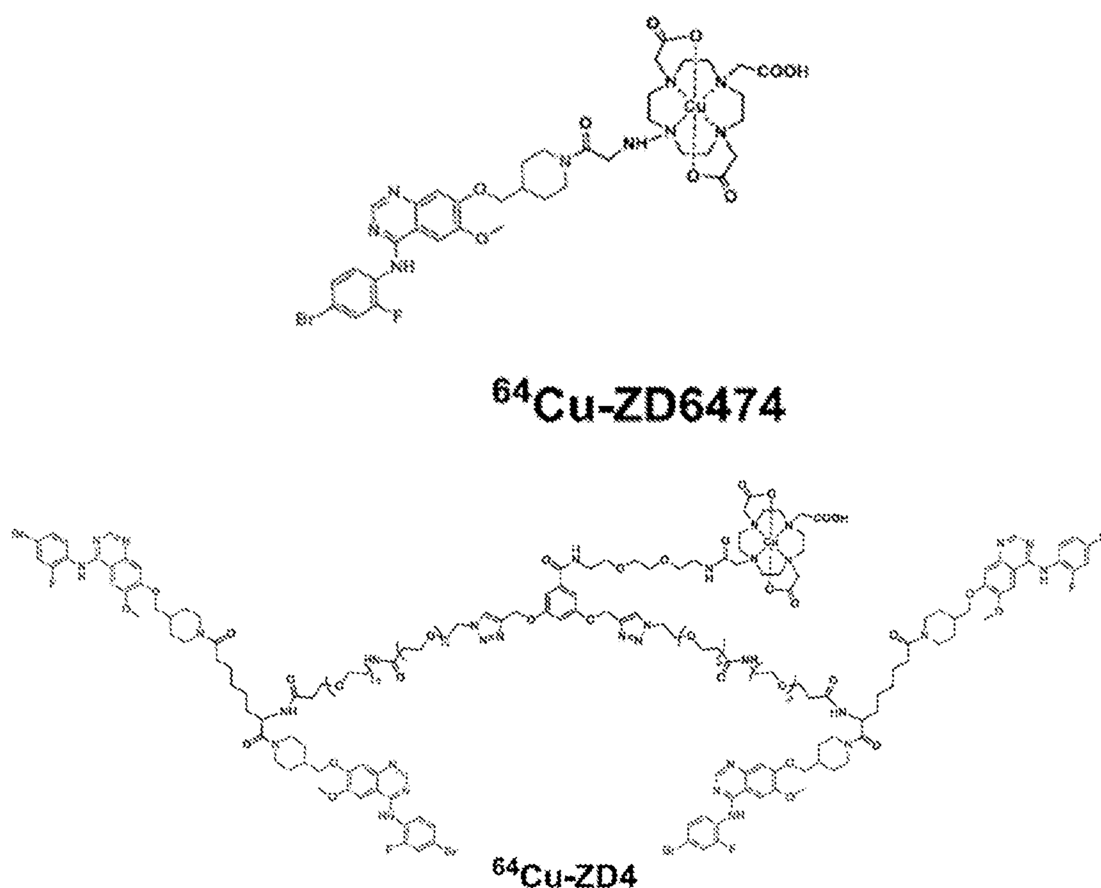
Compounds	Valency	Linker Length[a] [Å]	IC_{50} [b] [nM]
ZD-1	1	n/a	47 ± 3.0
ZD-2	2	~14	1.8 ± 0.2
ZD-3	2	~47	2.2 ± 0.2
ZD-4	4	~47 and ~14	0.025 ± 0.002
ZD-5	4	~14	0.89 ± 0.08

[a] Linker length is calculated by distance measurement using Chem3D Ultra 8.0. [b] IC_{50} is the concentration of an inhibitor required to reduce the specific binding by 50%.

With availability of distinguished linkers to arise different multivalent bindings, the
20 hybrid tetravalent ZD-4 was constructed to effectively combine the statistical and chelate effect in one structure for further optimization of binding affinity. As evaluated by the displacement/competition assay, ZD-4 showed unprecedentedly increased binding affinity with measured IC_{50} as low as 0.025 ± 0.002 nM, which presents almost 2000-fold improvement over ZD-1, and nearly 100-fold increase compared to bivalent ZD-2 and ZD-3. As a comparison,
25 another tetravalent ZD-5 that only used short linkers of ~14 Å tethering the active entities, in which only a statistical effect would be expected in the multivalent binding, was also studied. Its IC_{50} was revealed 0.89 ± 0.08 nM, which showed a 2-fold increase compared to the bivalent

ligands and 35-fold less potent than the hybrid ZD-4. The comparison of ZD-4 with ZD-5 supports the position that optimized multivalent binding can be achieved by hybrid multivalent design.

Furthermore, the improved binding affinity of ZD-4 to VEGFR was investigated using *in* 5 *vivo* VEGFR-targeting positron emission tomography (PET) imaging of U87 glioblastoma-bearing mice. Increased tumor uptake of the imaging tracer will be associated with improved VEGFR targeting (Folkman, J., *New England J. Med.* **1971**, 285, 1182-1186; and Ferrara, N. et al., *Nat. Med.* **2003**, 9, 669-676). Upon radiolabeling with ^{64}Cu, the PET radiotracer ^{64}Cu-ZD4 (shown below) with specific activity of 0.3-0.5 MBq/nmol was compared with its parent drug 10 ZD6474 derived radiotracer ^{64}Cu-ZD6474 for their tumor uptake.



Specifically, microPET/CT scans were performed on a U87 xenograft mouse model at 2 h, 6 h and 24 h after ~3.7 MBq (100uCi) injection of ^{64}Cu-ZD4 or ^{64}Cu-ZD6474 (n=6). Representative 15 whole-body 3D images at 24 h post injection (p.i.) are shown in Figure 6A. High tumor uptake of ^{64}Cu-ZD4 was observed as indicated by the arrow, and revealed ^{64}Cu-ZD4 to be a suitable PET probe for *in vivo* tumor VEGFR imaging. In comparison, the tumor uptake of ^{64}Cu-ZD6474 was revealed by the PET imaging to be much less. For each PET scan, regions of interest (ROIs)

quantification analysis of tumor uptake was performed to obtain an imaging ROI–derived percentage of the injected radioactive dose per gram of tissue (%ID/g). From ROI analysis of decay-corrected PET images, the tumor uptake of ^{64}Cu-ZD4 probe was 6.16 ± 0.30 %ID/g as early as 2 h p.i., and increased to 7.24 ± 0.23 %ID/g and 7.90 ± 0.39 %ID/g at 6 h and 24 h p.i., respectively. For the parent monomer radiotracer, the tumor uptake of ^{64}Cu-ZD6474 was only 0.30 ± 0.11 %ID/g at 2 h, 0.66 ± 0.04 %ID/g at 6 h, and 0.46 ± 0.14 %ID/g at 24 h p.i.. The following histological study confirmed the abundant expression of VEGFR in the U87 tumor, which accounted for the high uptake of VEGFR-specific radiotracers. The *in vivo* PET imaging clearly showed that the improved binding of hybrid ZD-4 led to a significantly increased tumor uptake of ^{64}Cu-ZD4, which was 12-times higher than that of radiolabeled parent monomer ^{64}Cu-ZD6474 ($P < 0.0001$). The high tumor uptake and favorable pharmacological properties suggest ^{64}Cu-ZD4 a suitable PET radiopharmaceutical. Promising radionuclide-based therapeutic products could be produced by incorporation into developed agents with appropriate radionuclides.

Other uses for ZD-G2VEGFR2 imaging: VEGF targeted imaging can be useful in other groups of pulmonary hypertension such as Chronic Thromboembolic Pulmonary Hypertension (CTEPH) and PH associated with interstitial lung disease (WHO group III). The standard test used to diagnose CTEPH is a pulmonary angiogram, usually performed at the time of right heart catheterization. V/Q scan is used to screen patients. PET imaging can replace V/Q scan.

Cancer: VEGF pathway plays a significant role in formation of new vessels which lead to increasing tumor size and metastatic spread of cancer. VEGF inhibitors (8 FDA approved drugs) target this important pathway in cancer progression. Immunotherapy has emerged one the most effective treatment changing paradigm in cancer. VEGF receptor PET imaging has a role in determining how immunotherapy is clinically used. Studies have shown that the efficacy of immunotherapy in the treatment of lung cancer is dependent on tumor vascularity which in turn can be measured by VEGF receptor expression. Measuring the uptake of VEGF using 18F-ZD-G2 could be used to screen patients with lung cancer to determine which ones would have a greater chance of responding to immunotherapy beyond traditional genetic markers, such a PD-L1. Figure 7 shows therapy data generated of ^{177}Lu-DiZD compared with anti-PD1 on triple native breast cancer model.

Transplantation: Although transplantation science continues to improve short term outcomes and survival of transplant patients, long term outcomes are plagued by different forms of chronic rejection in all organ transplantation including heart, lung and kidney. Hallmark of chronic rejection is neovascularization, which is mediated by VEGF pathway. Currently,

invasive testing from heart catheterization to invasive biopsies are needed to confirm these diagnoses depending on the organ involved. We believe VEGFr2 imaging can lead earlier detection of chronic rejection via PET imaging with 18F-ZD-G2, like PAH.

Vascular Applications: Additional uses in cardiovascular patient care include vascular imaging of aortic aneurysms and vascular access graft imaging to assess for disease progression.

Figure 8 is a flow diagram showing method of using AFM to design conforming compounds.

Summary: In summary, an AFM force measurement platform that was applied to guide the efficient design of VEGFR-targeting hybrid ZD-4 from drug ZD6474 was successfully demonstrated. Compound ZD-4 exhibited picomolar receptor affinity, increased tumor uptake and favorable pharmacological properties, which showed high potential in diagnostic imaging and therapeutic treatment of VEGFR-related diseases. This example showed a useful strategy to create ligands with dramatic increased binding affinity including from known drugs by effectively combining various multivalent binding mechanisms. And the force measurement platform that was capable of probing the actual distribution of cell-surface receptors could greatly facilitate the rational design of multivalent drugs.

Example 2: Single-Molecule Force Measurement Platform Guides the Design of High Affinity Multivalent Ligand

Objective: Generalize application of atomic force microscope based single-molecule measurement in multivalent ligand design of ligands to other cell-surface receptors.

Drug Candidates:

a) Peptide targeting ephrin type-B receptor 4, which makes up the largest subgroup of the receptor tyrosine kinase family and emerges as critical regulators postnatal angiogenic remodeling and tumor neovascularization.

b) Synthetic antagonist for integrin, which is another transmembrane receptor and activate signal transduction pathways that mediate cellular signals such as regulation of the cell cycle, organization of the intracellular cytoskeleton, and movement of new receptors to the cell membrane.

Experimental:

Chemical synthesis:

1) Thiol (SH)-tagged PEG spacer, which will attach the functional ligand to AFM tip were synthesized. Instead of the 2 PEG linker units described in the example above, an eleven (11) PEG linker unit was used as spacer to improve the flexibility of the ligands attached

to the AFM tip, which could facilitate the ligands-to-receptor binding event in the force measurement.

2) The IA monomer (see Schemes 1 and 2) was synthesized followed by tagging with SH-PEG spacer (see Schemes 5 and 6) for AFM tip functionalization.
3) The IA dimer (see Schemes 3 and 4) was synthesized followed by tagging with SH-PEG spacer (see Scheme 7) for AFM tip functionalization.
4) The ephb4 peptide (see Scheme 8) was synthesized followed by tagging with SH-PEG spacer for AFM tip functionalization.
5) The ephb4 peptide dimer (see Scheme 9) was synthesized followed by tagging with SH-PEG spacer for AFM tip functionalization.

AFM tip functionalization:

1) The four compounds (including IA monomer, IA dimer, ephb4 monomer and ephb4 dimer) synthesized above were probed for the force measurement.
2) Two types of tips were prepared for each compound, including 100% functionalized tip and 5% functionalized tip.

Cell Preparation

1) HUVEC cells that express abundant integrin receptors for integrin-towards ligand-to-receptor force mapping were cultured.
2) Similarly, MDA-MB-468 human triple negative breast cancer cells that express abundant ephb4 receptor for ephb4-towards ligand-to-receptor force mapping were cultured.

AFM force measurement

1) The ligand functionalized AFM tip was installed and the AFM instrument tuned to obtain the force mapping of the various ligands functionalized AFM tip along the targeted cell surface.

Schemes 1 and 2 show synthetic procedure of compound IA and its structure, respectively.

Scheme 1. Synthesis of IA monomer

IA.HCl

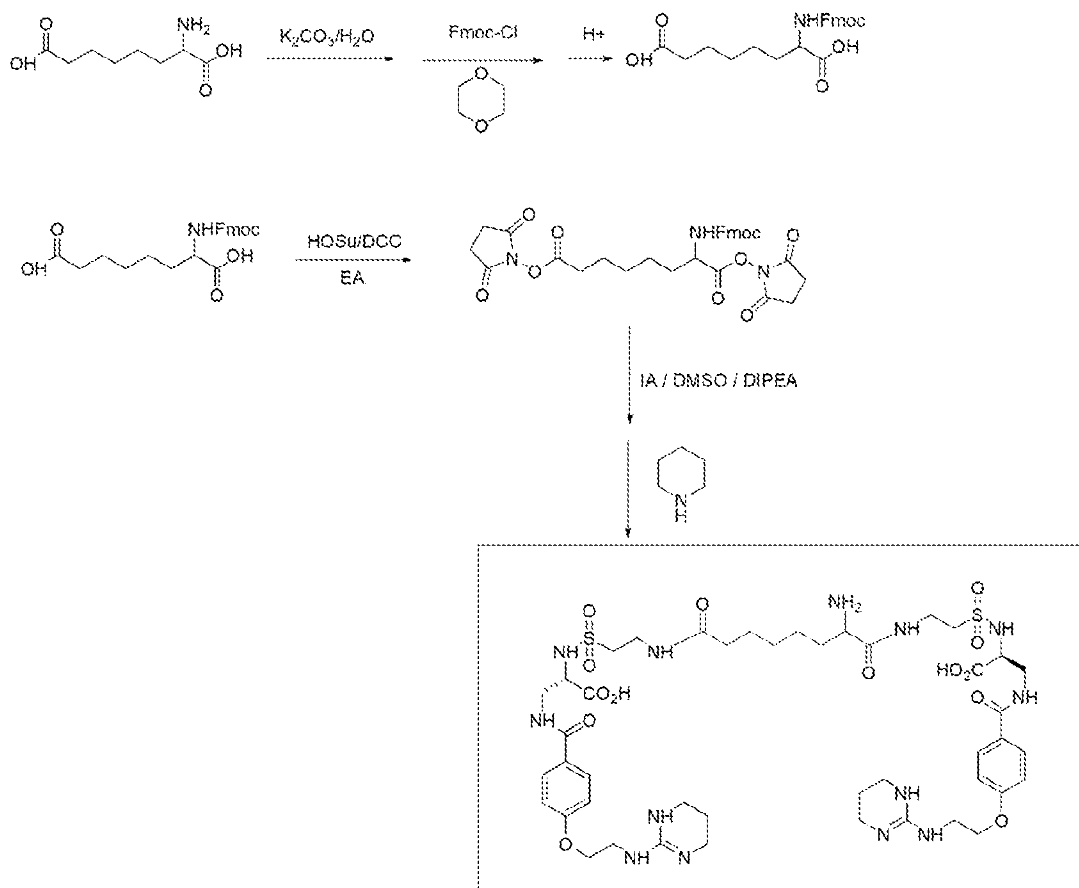
$C_{18}H_{29}ClN_6O_6S$

Exact Mass: 492.16

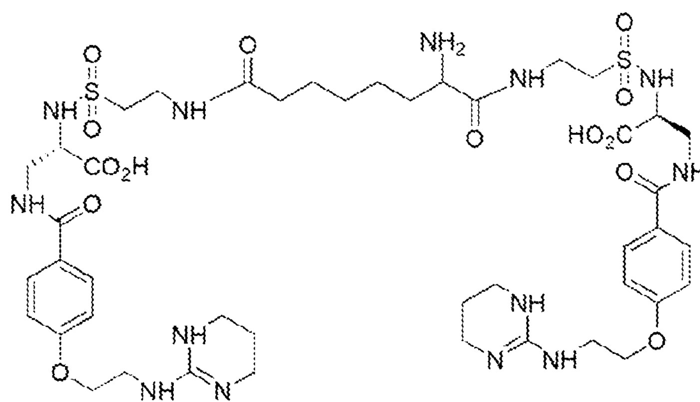
Mol. Wt.: 492.98

Scheme 2. IA monomer structure

Schemes 3 and 4 show synthetic procedure of compound IA dimer and its structure, respectively.



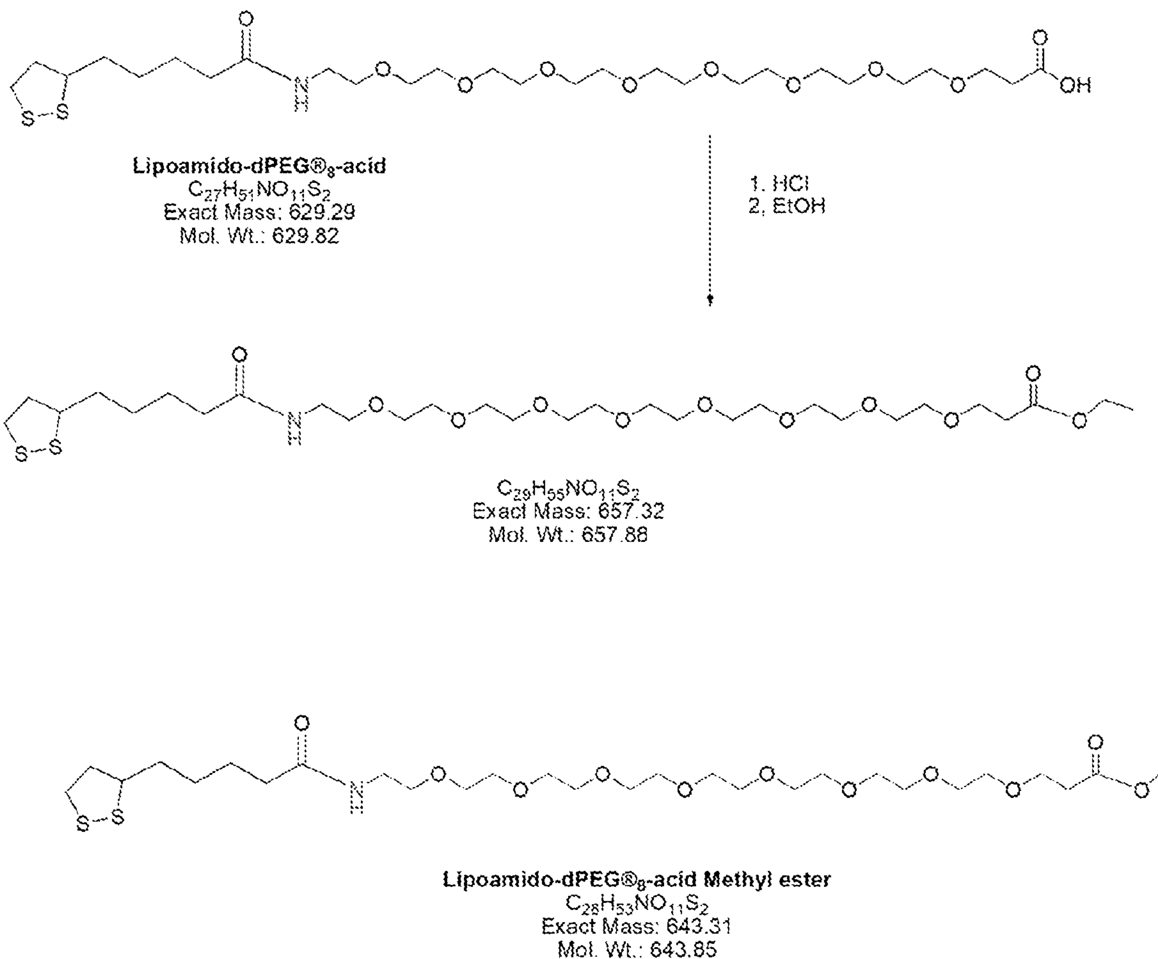
Scheme 3. Synthesis of IA dimer.



IA dimer

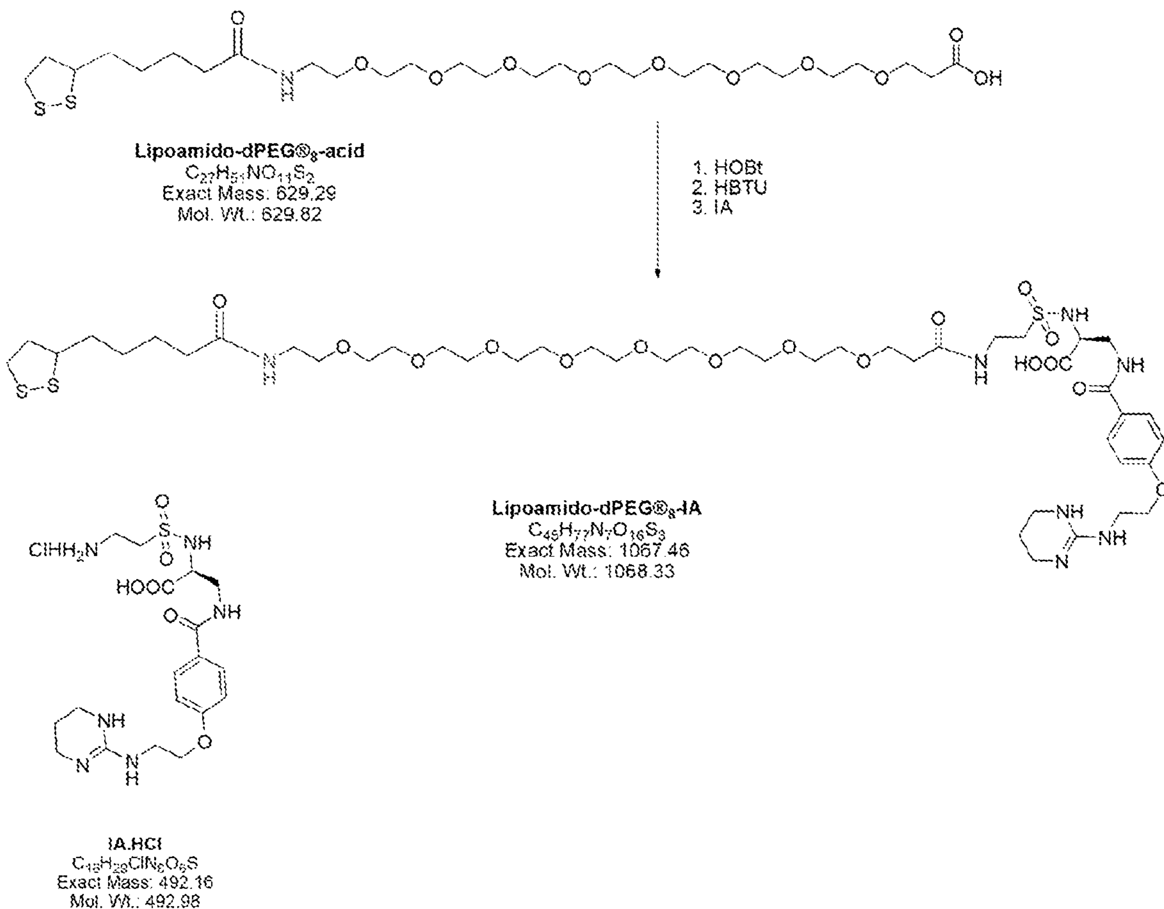
Scheme 4. IA chemical structure

5 Scheme 5 shows PEG linker being covalently conjugated with thiol (SH) functional group, for attachment of the functional ligands to the gold-made AFM tips.

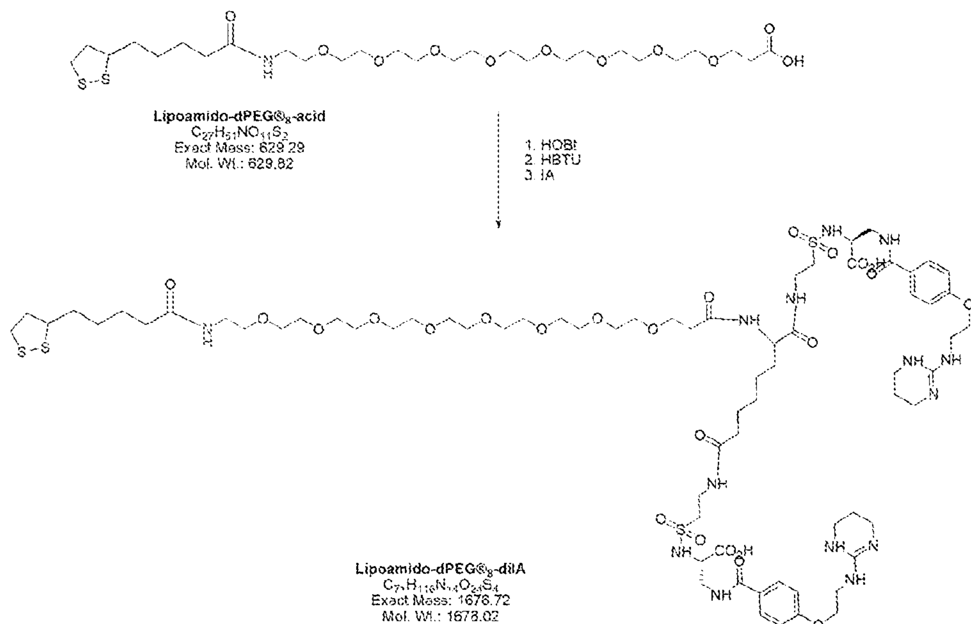


Scheme 5. Synthesis of SH-PEG spacer for AFM functionalization.

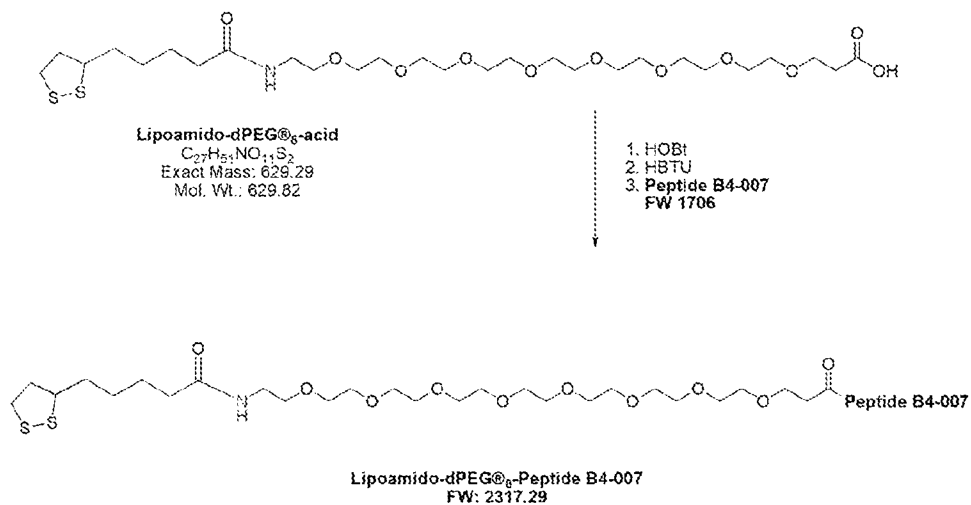
Scheme 6 details synthesis of SH-PEG-IA monomer which 5% and 100% functionalized AFM tips were prepared, and the cells were cultured. Scheme 7 details synthesis of SH-PEG tagged IA dimer. Scheme 8 details synthesis of SH-PEG tagged ephb4 monomer. Scheme 9 details synthesis of SH-PEG tagged ephb4 dimer.



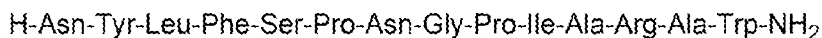
Scheme 6. Preparation of SH-PEG tagged IA monomer for AFM tip functionalization.



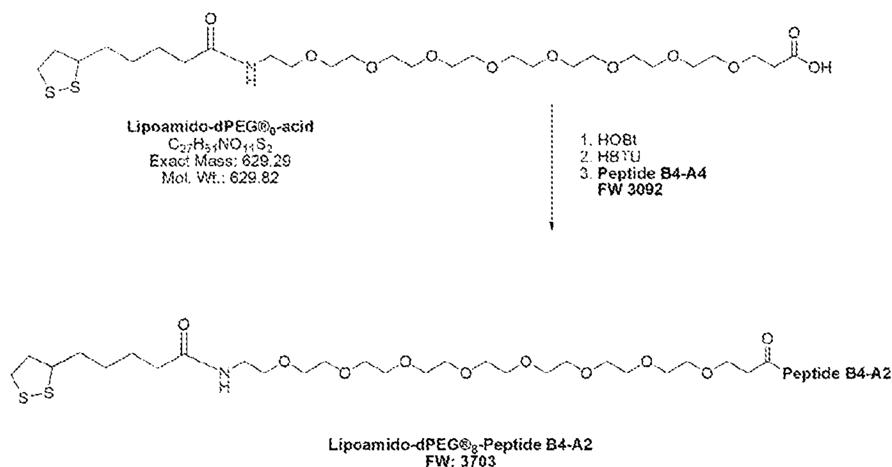
5 Scheme 7. Prepare SH-PEG tagged IA dimer for AFM tip functionalization.



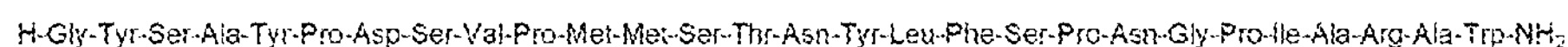
EphB4-007



Scheme 8. Prepare SH-PEG tagged ephb4 monomer for AFM tip functionalization.



EphA2 EphB4



Chemical Formula: $C_{141}H_{203}N_{35}O_{40}S_2$
Exact Mass: 3090.4368
Molecular Weight: 3092.5000
Elemental Analysis: C, 54.76; H, 6.62; N, 15.85; O, 20.69; S, 2.07

5

Scheme 9. Prepare SH-PEG tagged ephb4 dimer for AFM tip functionalization.

Figures 9 and 10 show AFM height and force mapping of HUVEC cells using 100% IA monomer functionalized AFM tip (Figure 9) and of MDA-MB-468 cells using 100% ephb4 monomer functionalized AFM tip (Figure 10).

CLAIMS

What is claimed is:

1. A tetravalent compound comprising a first moiety and a second moiety covalently linked by a first linker, L_1,

   wherein the first moiety comprises two or more vascular endothelial growth factor receptor (VEGFR) binding moieties, wherein the two or more VEGFR binding moieties are linked by a second linker, L_2, and

   wherein the second moiety comprises an additional two or more VEGFR binding moieties linked by a third linker, L_3.

2. The compound of claim 1, wherein L_1 has a length from 40 to 60 Å, from 40 to 55 Å, or from 40 to 50 Å.

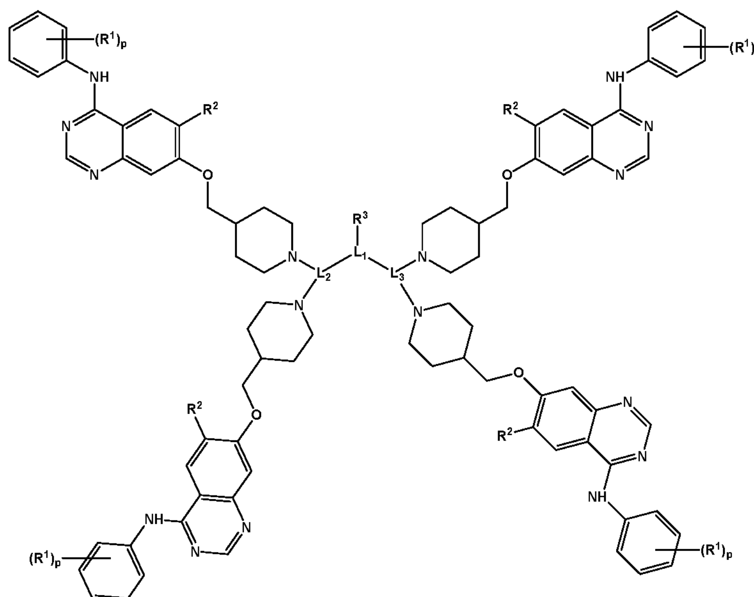
3. The compound of claim 1, wherein each of L_2 and L_3 independently has a length from 40 to 60 Å or from 40 to 55 Å.

4. The compound of claim 1, wherein each of L_2 and L_3 independently has a length from 7 to 25 Å or from 10 to 20 Å.

5. The compound of claim 1, wherein the VEGFR binding moieties are independently selected from the group consisting of bevacizumab; sunitinib; aflibercept; pazopanib; axitinib; sorafenib; vandetanib; regorafenib; ramucirumab, and combination thereof.

6. The compound of claim 5, wherein the VEGFR binding moieties comprise vandetanib.

7. The compound of claim 1, having Formula I,



Formula I

wherein,

R^1, independently for each occurrence, is selected from hydroxyl, halogen, C_{1-3} alkyl, C_{1-3} alkoxyl, C_{1-3} alkanoyloxy, trifluoromethyl, cyano, amino, or nitro;

R^2, independently for each occurrence, is selected from hydrogen, hydroxyl, halogen, nitro, trifluoromethyl, cyano, C_{1-3} alkyl, C_{1-3} alkoxyl, C_{1-3} alkylthio, or $-NR^7R^8$, wherein R^7 and R^8, which can be the same or different, each represents hydrogen or C_{1-3} alkyl;

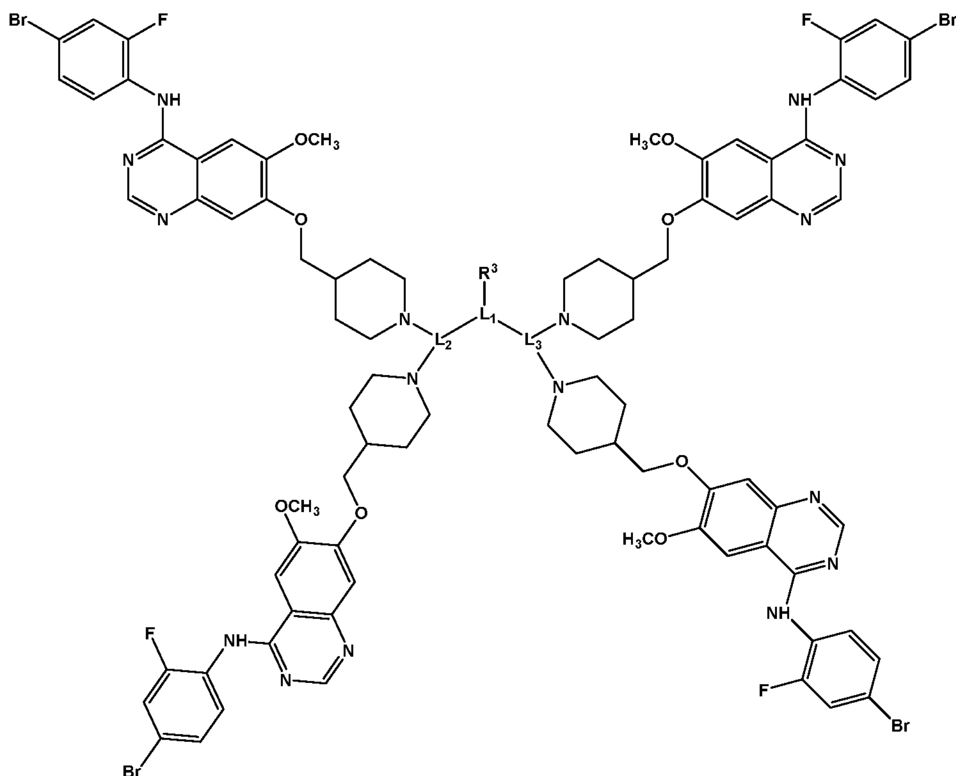
R^3 is absent or comprises a detectable moiety or therapeutic moiety;

p is an integer from 1 to 4; and

L_1, L_2, and L_3 are linkers, independently selected from $N(R^{14})_3$, $CH(R^{14})_3$, $Ar(R^{14})_3$, wherein Ar is aryl, and wherein R^{14} for each occurrence, is independently selected from a bond, hydrogen, amido, C_1-C_{20} alkyl; C_1-C_{20} heteroalkyl, C_1-C_{20} alkylamine, C_1-C_{20} alkoxy, polyalkyleneoxy, C_1-C_{20} alkanoyloxy, C_1-C_{20} alkylamido, aryl, or heteroaryl; wherein each R^{14} independent of the other is optionally substituted with one or more substituents independently selected from the group consisting of halogen; hydroxyl; cyano; carbonyl; nitro; amino; amido; alkylamino; dialkylamino; alkylamido; =O; $-S(O)_2$-; -SO-; -S-; $-S(O)_2N$-; haloalkyl; hydroxyalkyl; carboxy; alkyl, alkoxyl; aryloxyl; alkoxycarbonyl; aminocarbonyl; alkylaminocarbonyl; dialkylaminocarbonyl; aryl; heteroaryl; and combinations thereof;

wherein R^3 when present is bonded to at least one of R^{14}.

8. The compound of claim 7, having Formula II,



Formula II.

9. The compound of claim 7, wherein L_1, is $-(NHCO-R^{14})_3Ar$, $-(CONH-R^{14})_3Ar$, $-(CO-R^{14})_3Ar$,

wherein R^{14} for each occurrence, is independently selected from a bond, C_1-C_{20} alkyl; C_1-C_{20} heteroalkyl, C_1-C_{20} alkylamine, C_1-C_{20} alkoxy, polyalkyleneoxy, C_1-C_{20} alkanoyloxy, or C_1-C_{20} alkylamido; wherein R^{14} is optionally substituted with one or more substituents independently selected from the group consisting of amino; alkylamino; dialkylamino; amido; alkylamido; alkoxyamido; polyalyleneoxyamido; aryl; heteroaryl; or combinations thereof.

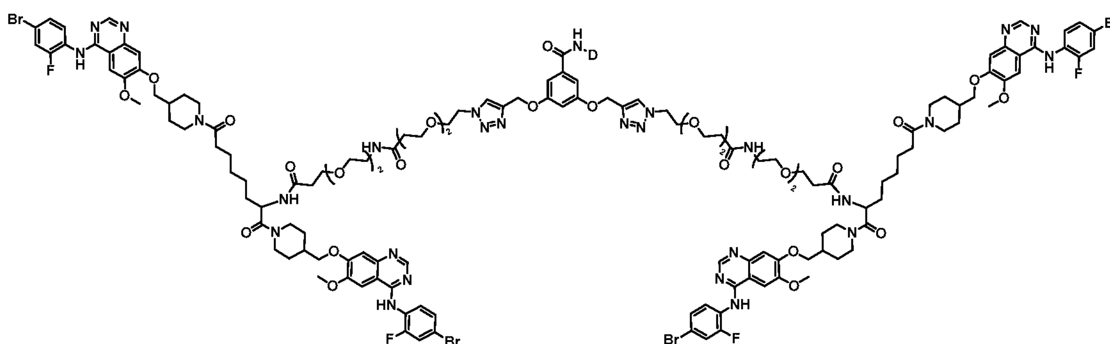
10. The compound of claim 7, wherein L_2, and L_3 are each independently selected from $-(CO-R^{14})_2N(R^{14})$, $-(R^{14})_3N$, $-(SO_2R^{14})_2NR^{14}$, $-(SOR^{14})_2NR^{14}$, $-(OR^{14})_2NR^{14}$, $-(O-CO-R^{14})_2NR^{14}$, $-(CO-O-R^{14})_2NR^{14}$, $-(CO-R^{14})_2CH(R^{14})$, -

$(R^{14})_3CH$, $-(SO_2R^{14})_2CH(R^{14})$, $-(SOR^{14})_2CH(R^{14})$, $-(O\text{-}CO\text{-}R^{14})_2CH(R^{14})$, or $-(OR^{14})_2CH(R^{14})$,

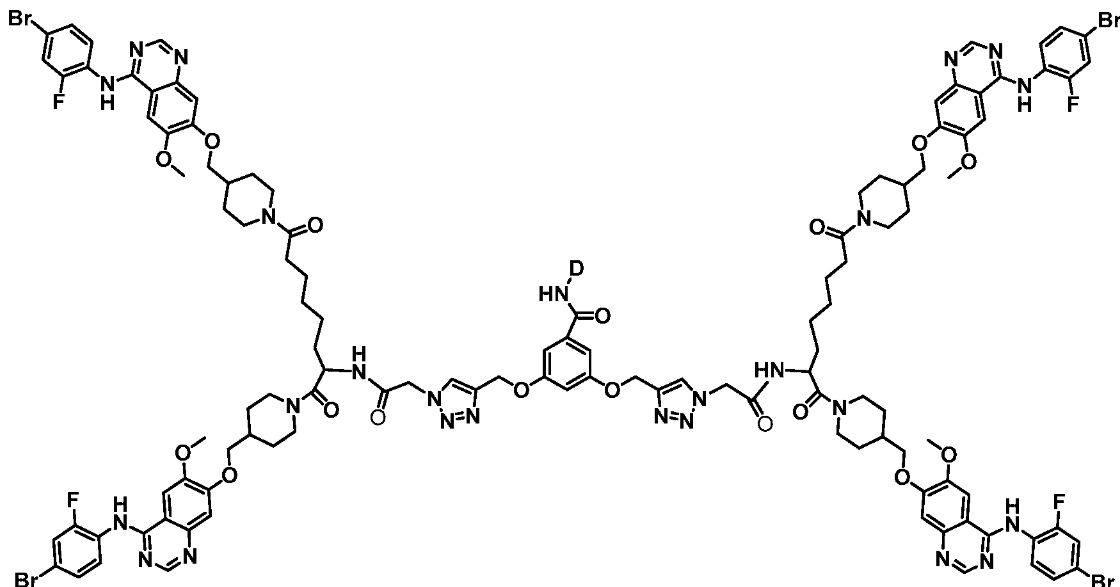
wherein R^{14} for each occurrence, is independently selected from a bond, C_1-C_{20} alkyl; C_1-C_{20} heteroalkyl, C_1-C_{20} alkylamine, C_1-C_{20} alkoxy, polyalkyleneoxy, C_1-C_{20} alkanoyloxy, or C_1-C_{20} alkylamido; and wherein R^{14} is optionally substituted with one or more substituents independently selected from the group consisting of amino; alkylamino; dialkylamino; amido; alkylamido; alkoxyamido; polyalyleneoxyamido; aryl; heteroaryl; or combinations thereof.

11. The compound of claim 10, wherein L_2, and L_3 are each independently selected from $-(R^{14})_3CH$, wherein each of R^{14} is independently selected from a bond, carboxyl, $C_{4\text{-}6}$ alkylcarboxyl.

12. The compound of claim 1, having Formula IIIA or IIIB,



Formula IIIA, or



Formula IIIB,

wherein,

D comprises a detectable moiety or a therapeutic moiety.

13. The compound of claim 7, wherein the detectable moiety comprises a near-infrared label, a fluorescent label, a radiolabel, a magnetic spin resonance label, a chromophore, or any combination thereof.

14. The compound of claim 7, wherein the detectable moiety is fluorescein, rhodamine, Texas Red, a Cy2 moiety, a Cy3 moiety, a Cy5 moiety, a Cy5.5 moiety, a Cy7 moiety, a cyanine dye, DOTA-based chelators, DTPA-based chelators, or a derivative or a combination thereof.

15. The compound of claim 7, wherein the detectable moiety comprises a radiolabel selected from the group consisting of ^{18}F, ^{64}Cu, ^{68}Ga, ^{67}Cu, ^{89}Zr, ^{111}In, ^{124}I, ^{123}I, and ^{99m}Tc.

16. A method for imaging a population of cells expressing vascular endothelial growth factor receptor within or about the body of an animal, the method comprising administering to the animal an amount of a compound of claim 1

for a time effective to image a population of cells expressing vascular endothelial growth factor receptor within or about the body of the animal.

17. The method of claim 16, wherein the population of cells expressing vascular endothelial growth factor receptor comprises cancer cells, tumor cells, hyperproliferative cells, or any combination thereof.

18. The method of claim 16, wherein the animal is a human diagnosed with pulmonary hypertension.

19. The method of claim 16, wherein the animal is a transplant patient.

20. A method of treating or ameliorating at least one symptom of a disease, dysfunction, or abnormal condition in a mammal, comprising administering to the mammal a compound claim 1, in an amount and for a time sufficient to treat or ameliorate the at least one symptom of the disease, dysfunction, or abnormal condition in the mammal.

21. A method for determining the spatial distribution of VEGFR in a cell comprising:

conjugating a VEGFR binding moiety to a functional group to form a functional-binder conjugate;

functionalizing an AFM tip with the functional-binder conjugate or a diluted solution of the functional-binder conjugate to form a functionalized AFM tip;

contacting the functionalized AFM tip with the cell to facilitate binding of the functional-binder conjugate with VEGFR and form a binder-VEGFR complex;

using adhesive force measurements to determine a dissociative force of an ensemble of the binder-VEGFR complex and of a single binder-VEGFR complex,

determining the number of VEGFR distributed in an area of the AFM tip, and

deriving a maximum distance between two neighboring VEGFR using maximizing minimum algorithm.

22. The method of claim 21, wherein the diluted solution of the functional-binder conjugate comprises up to 95% by weight polyethylene glycol functionalized with a functional group.

23. The method of claim 21 or 22, wherein the functional group is a thiol group.

24. The method of any one of claims 21-23, wherein the dissociative force is determined by,

scanning a surface of the cell using adhesive force measurement mode;

mapping the adhesive force of the binder-VEGFR complex;

selecting a region of the cell with highest adhesive force signal, and deriving a histogram of the dissociative forces obtained from statistics of the adhesive force over the region, and

analyzing the histograms and differentiating individual force peaks to determine the dissociative force of the ensemble of the binder-VEGFR complex and a single binder-VEGFR complex.

25. The method of any one of claims 21-24, wherein the number of VEGFR is determined by dividing the dissociative force of the ensemble of the binder-VEGFR complex by the dissociative force of the single binder-VEGFR complex.

26. A method for determining the spatial distribution of receptors on a cell comprising,

functionalizing an AFM tip with one or more receptor binding moieties to form a functionalized AFM tip;

contacting the functionalized AFM tip with the cell to facilitate binding of the one or more receptor binding moieties with the receptors and form a binder-receptor complex;

determining the number of each receptor distributed in an area of the AFM tip, and

deriving a maximum distance between two neighboring receptors.

27. The method of claim 26, comprising:

conjugating a receptor binding moiety to a functional group to form a functional-binder conjugate;

functionalizing an AFM tip with the functional-binder conjugate or a diluted solution of the functional-binder conjugate to form a functionalized AFM tip;

contacting the functionalized AFM tip with the cell to facilitate binding of the functional-binder conjugate with the receptors and form a binder-receptor complex;

using adhesive force measurements to determine a dissociative force of an ensemble of the binder-receptor complex and of a single binder-receptor complex,

determining the number of each receptor distributed in an area of the AFM tip, and

deriving a maximum distance between two neighboring receptors using maximizing minimum algorithm.

28. The method of claim 26 or 27, wherein the receptors are the same.

29. The method of claim 26 or 27, wherein the receptors are different.

30. The method of any one of claims 26-29, wherein the receptors are selected from VEGFR, PD-L1, EphB4 (such as in cancer cells), PD1 CTLA4 (such as in T cells), angiotensin-converting enzyme (ACE) (such as in SARS-Cov-2 infection), estrogen receptor (ER), integrin, or a combination thereof.

31. The method of any one of claims 26-29, wherein the receptor binding moiety is a monomer.

32. The method of any one of claims 26-29, wherein the receptor binding moiety is a dimer.

33. The method of any one of claims 26-29, wherein the receptor binding moiety is selected from a small molecule therapeutic agent, a peptide, an antibody, an antibody fragment, a carbohydrate, an siRNA, a protein, a nucleic acid, an aptamer, a nanoparticle, a cytokine, a chemokine, a lymphokine, a lipid, a lectin, or a combination thereof.

34. A method for making multivalent compounds that target receptors on a cell surface, the method comprising:

determining the proximal receptor distance between two or more receptors on the cell surface using AFM;

synthesizing a multivalent compound comprising a first moiety and a second moiety covalently linked by a first linker, L_1,

wherein the first moiety comprises one or more receptor binding moieties, and the second moiety comprises an additional one or more receptor binding moieties, and

wherein the first linker has a length not substantially less than, equal to, or greater than the proximal receptor distance.

35. The method of claim 34, wherein the two or more receptors are the same.

36. The method of claim 34, wherein the two or more receptors are different.

37. The method of any one of claims 34-36, wherein the first moiety and the second moiety are the same.

38. The method of any one of claims 34-37, wherein the first moiety and the second moiety are different.

39. The method of any one of claims 34-38, wherein the first moiety and the second moiety are selected from a small molecule therapeutic agent, a

peptide, an antibody, an antibody fragment, a carbohydrate, an siRNA, a protein, a nucleic acid, an aptamer, a nanoparticle, a cytokine, a chemokine, a lymphokine, a lipid, a lectin, or a combination thereof.

40. The method of any one of claims 34-39, wherein the first moiety and the second moiety are selected from a small molecule therapeutic agent, a peptide, an antibody, or a combination thereof.

41. The method of any one of claims 34-40, wherein the first linker has a length within 20%, preferably within 15%, more preferably within 10%, of the proximal receptor distance.

42. The method of any one of claims 34-41, wherein the first linker has a length within 5% of the proximal receptor distance.

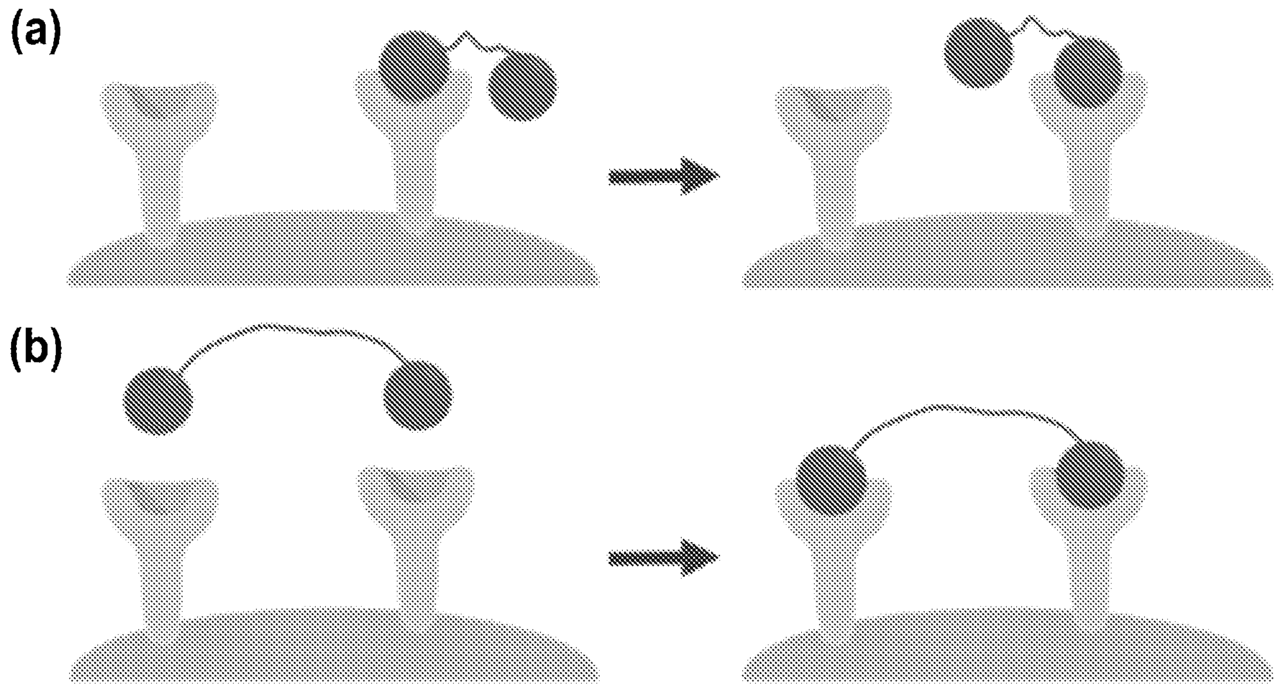
43. The method of any one of claims 34-42, wherein determining the proximal receptor distance between the two or more receptors is conducted using a method of any one of claims 21-33.

44. The method of any one of claims 35-43, wherein the receptors are selected from VEGFR, PD-L1, EphB4 (such as in cancer cells), PD1 CTLA4 (such as in T cells), integrin, angiotensin-converting enzyme (ACE) (such as in SARS-Cov-2 infection), estrogen receptor (ER), or a combination thereof.

45. The method of any one of claims 35-44, wherein the multivalent compounds have a binding affinity to the receptor that is greater than 10 times, greater than 100 times, greater than 1,000 times, or greater than 2,000 times the binding affinity of an equivalent single-valent compound to the receptor.

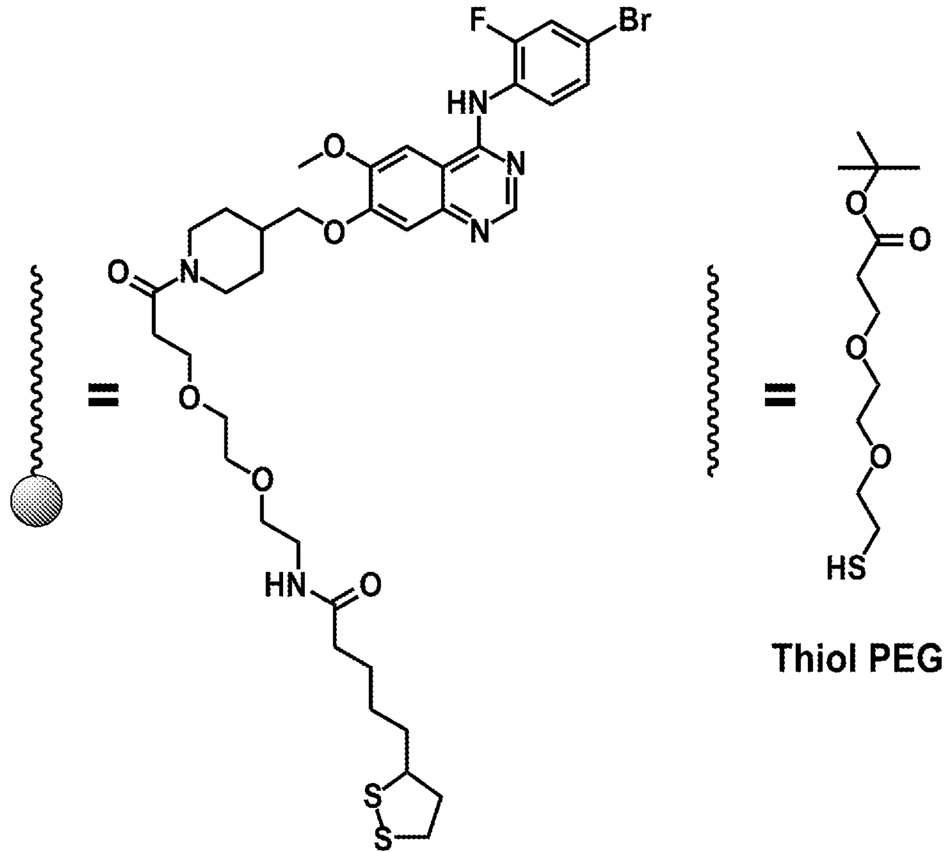
46. A compound, produced by a process according to any one of claims 21-33, wherein the compound is a multivalent compound comprising at least a first moiety and a second moiety covalently linked by a first linker, L_1,

wherein each of the first moiety and the second moiety comprises a receptor binding moiety for a receptor selected from VEGFR, PD-L1, EphB4 (such as in cancer cells), PD1 CTLA4 (such as in T cells), integrin, angiotensin-converting enzyme (ACE) (such as in SARS-Cov-2 infection), estrogen receptor (ER), or a combination thereof.

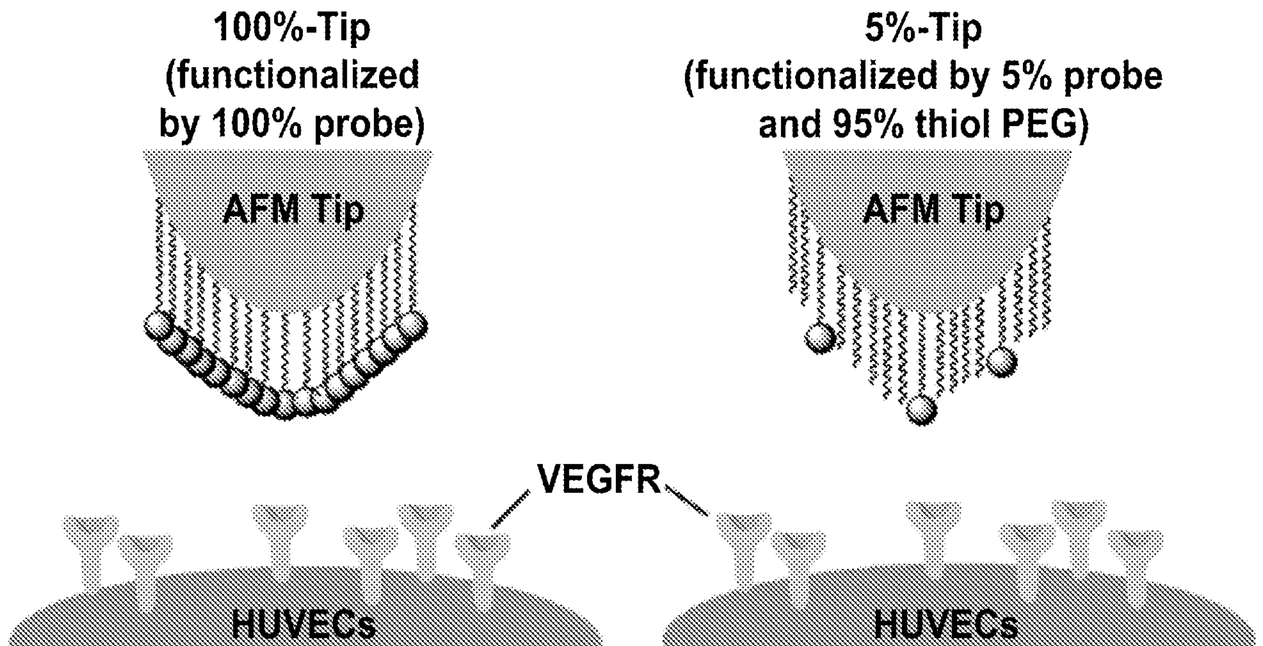


FIGS. 1A-1B

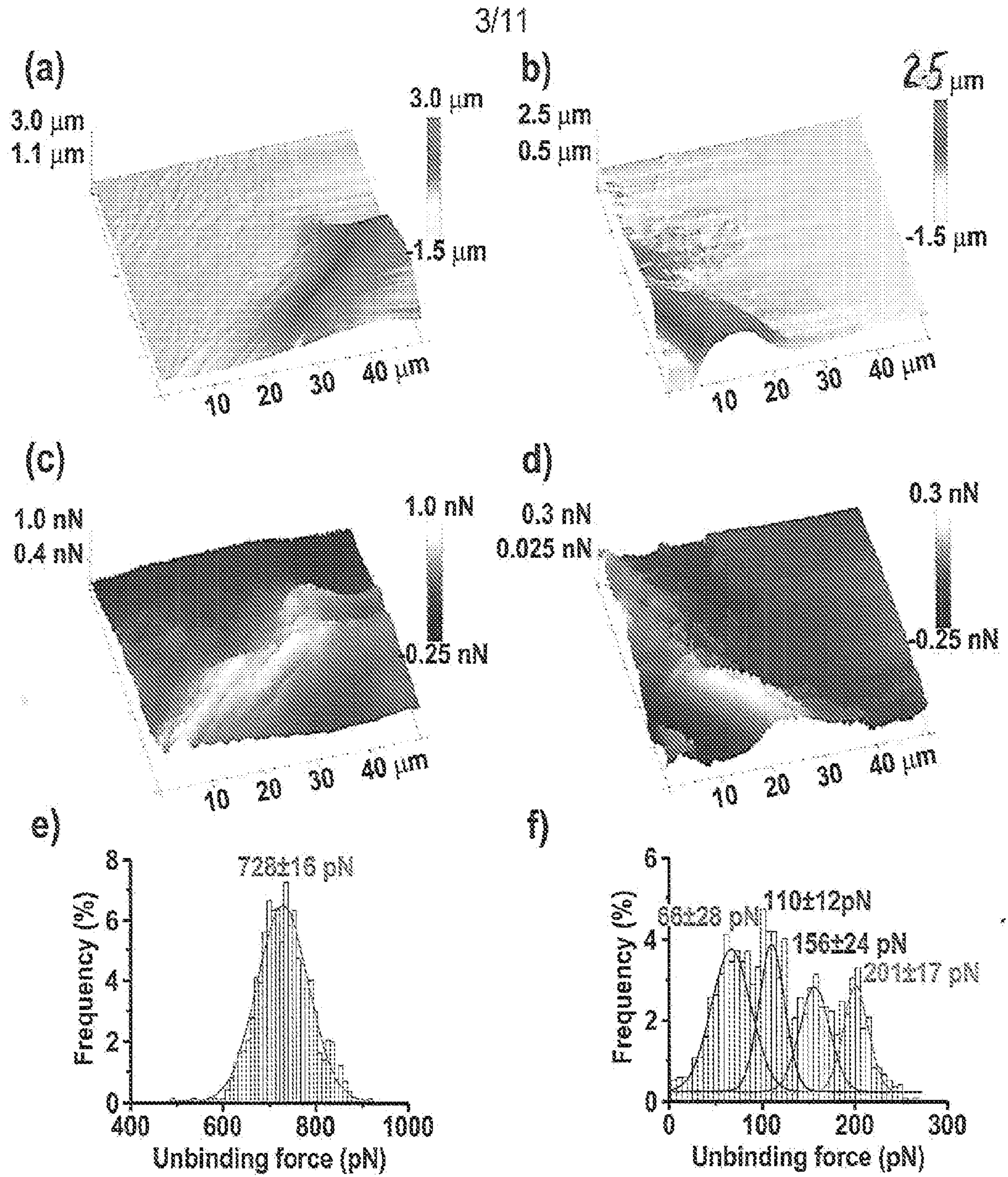
(a)



(b)



FIGS. 2A-2B



FIGS. 3A-3F

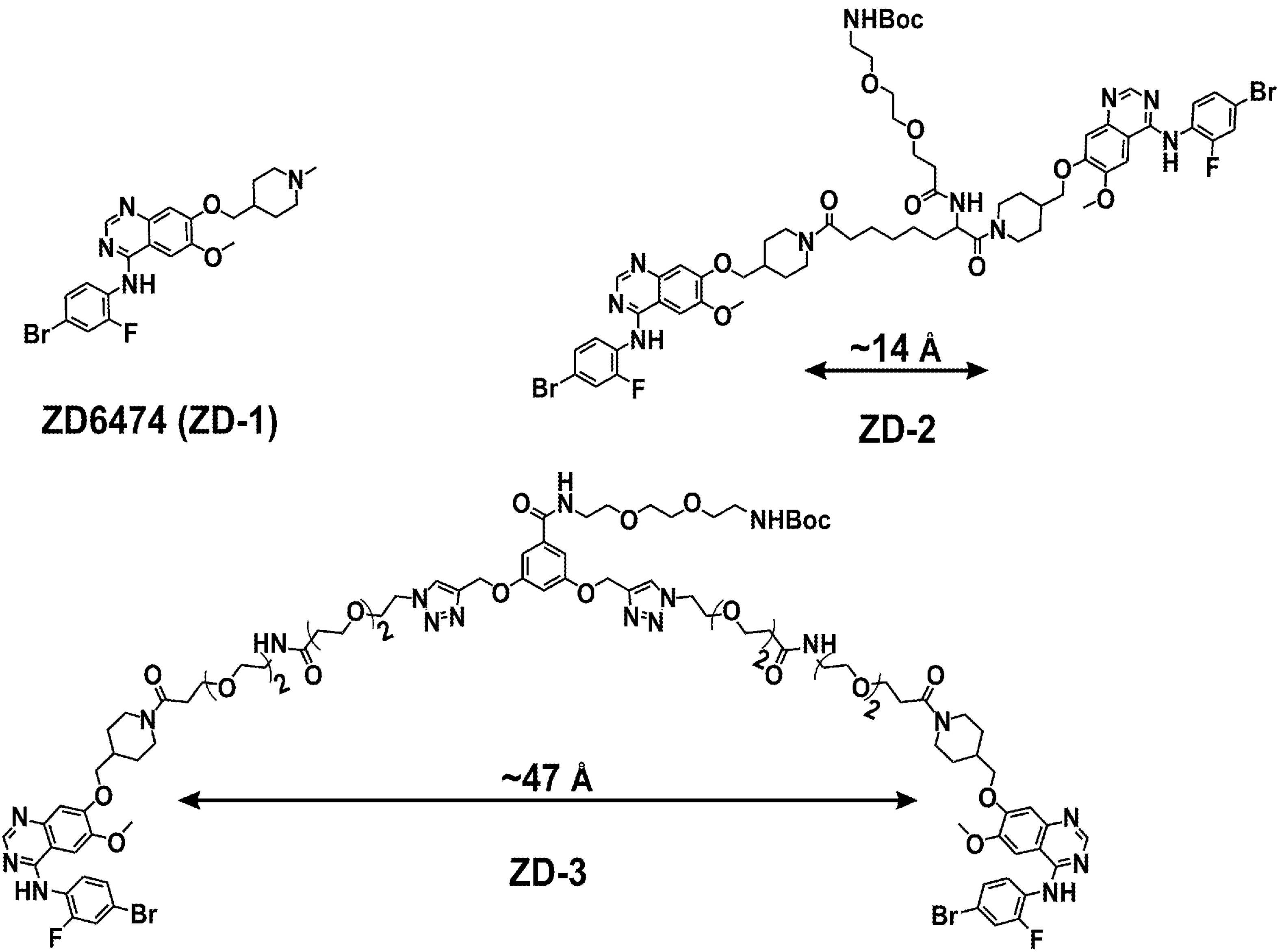


FIG. 4

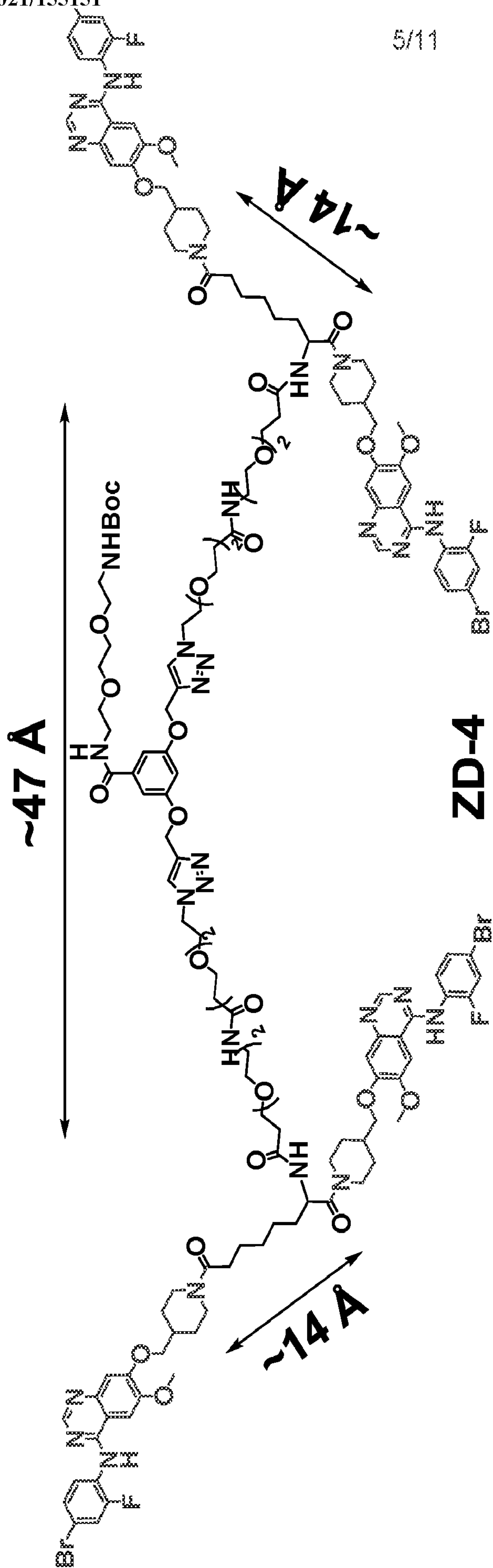


FIG. 4 CONT.

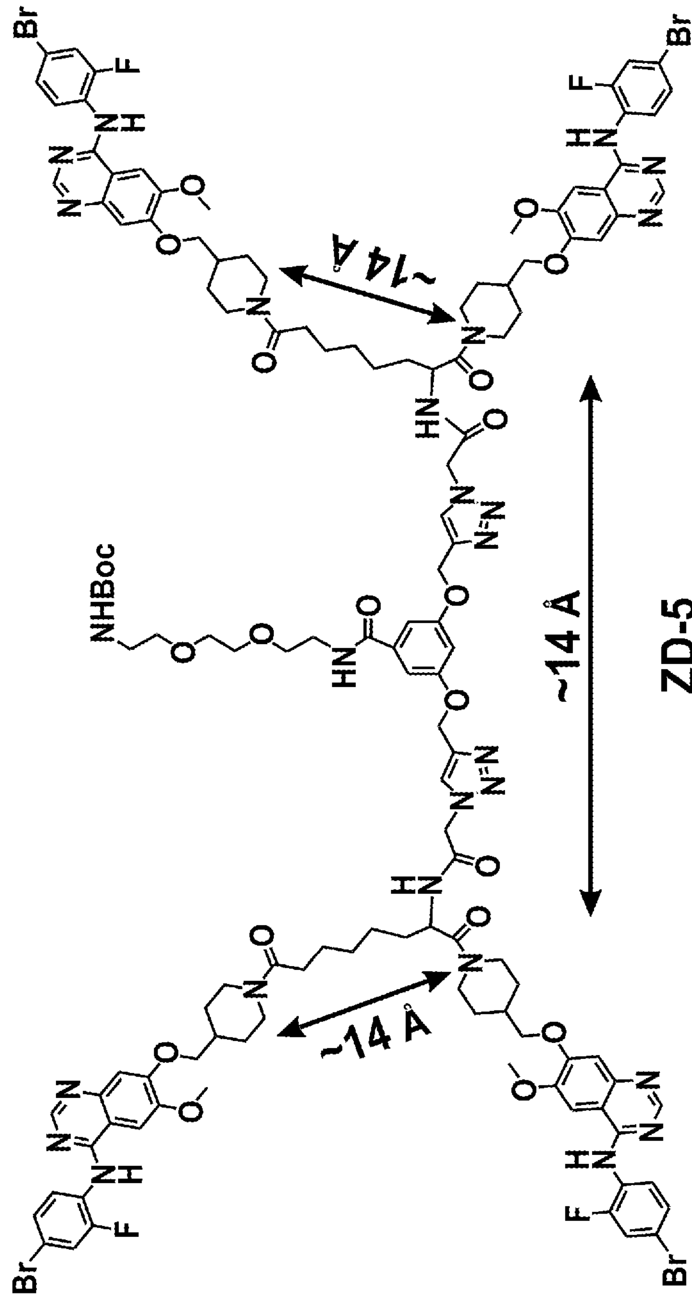


FIG. 4 CONT.

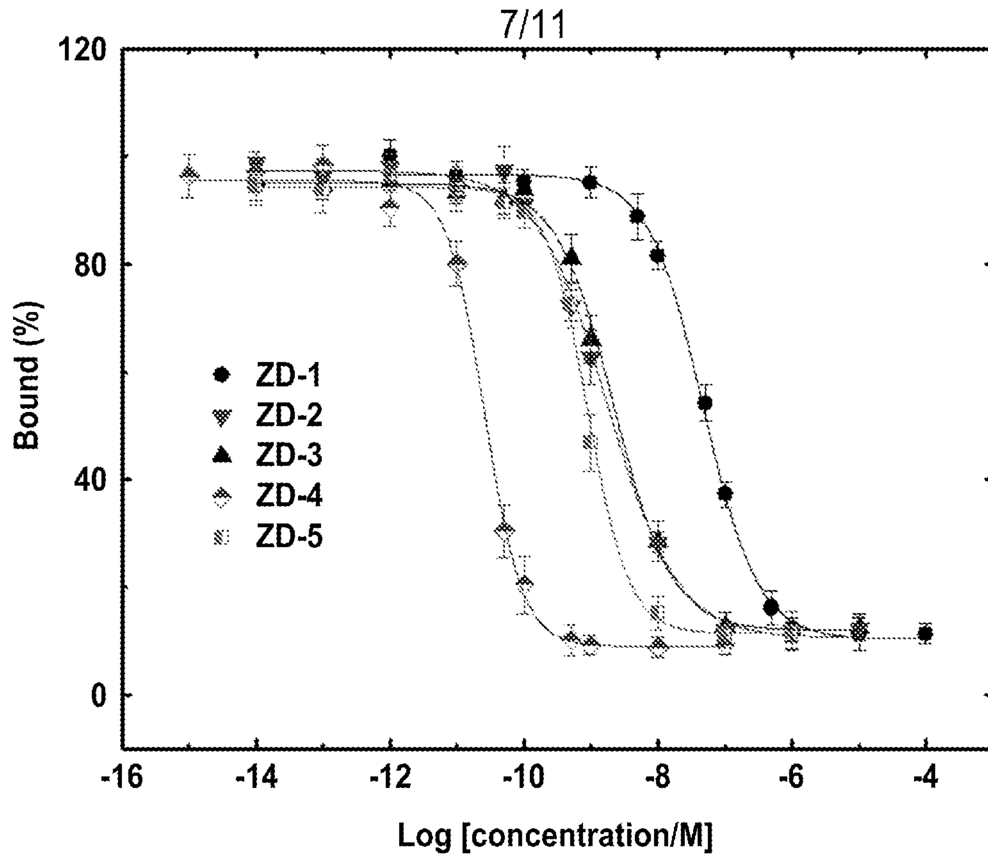
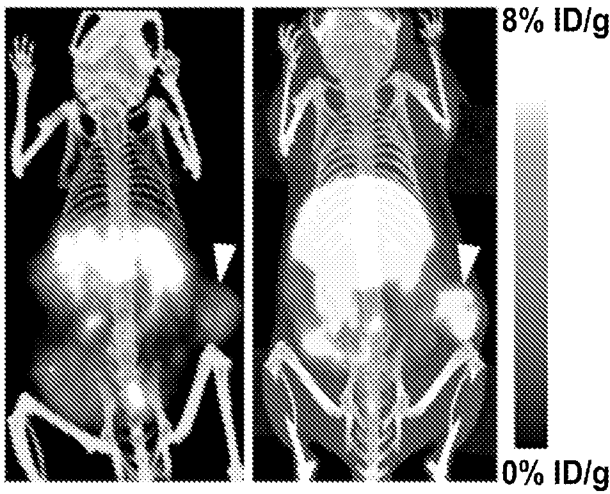
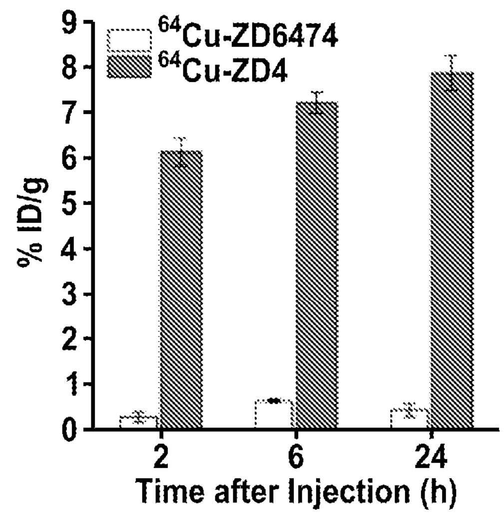


FIG. 5

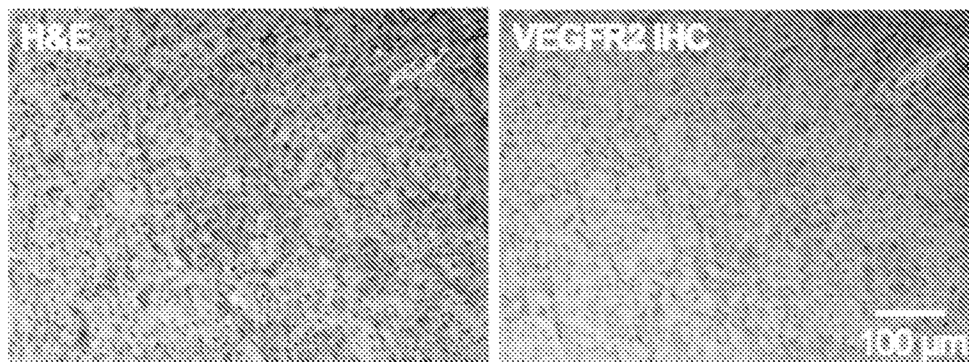
(a)



(b)



(c)



FIGS. 6A-6C

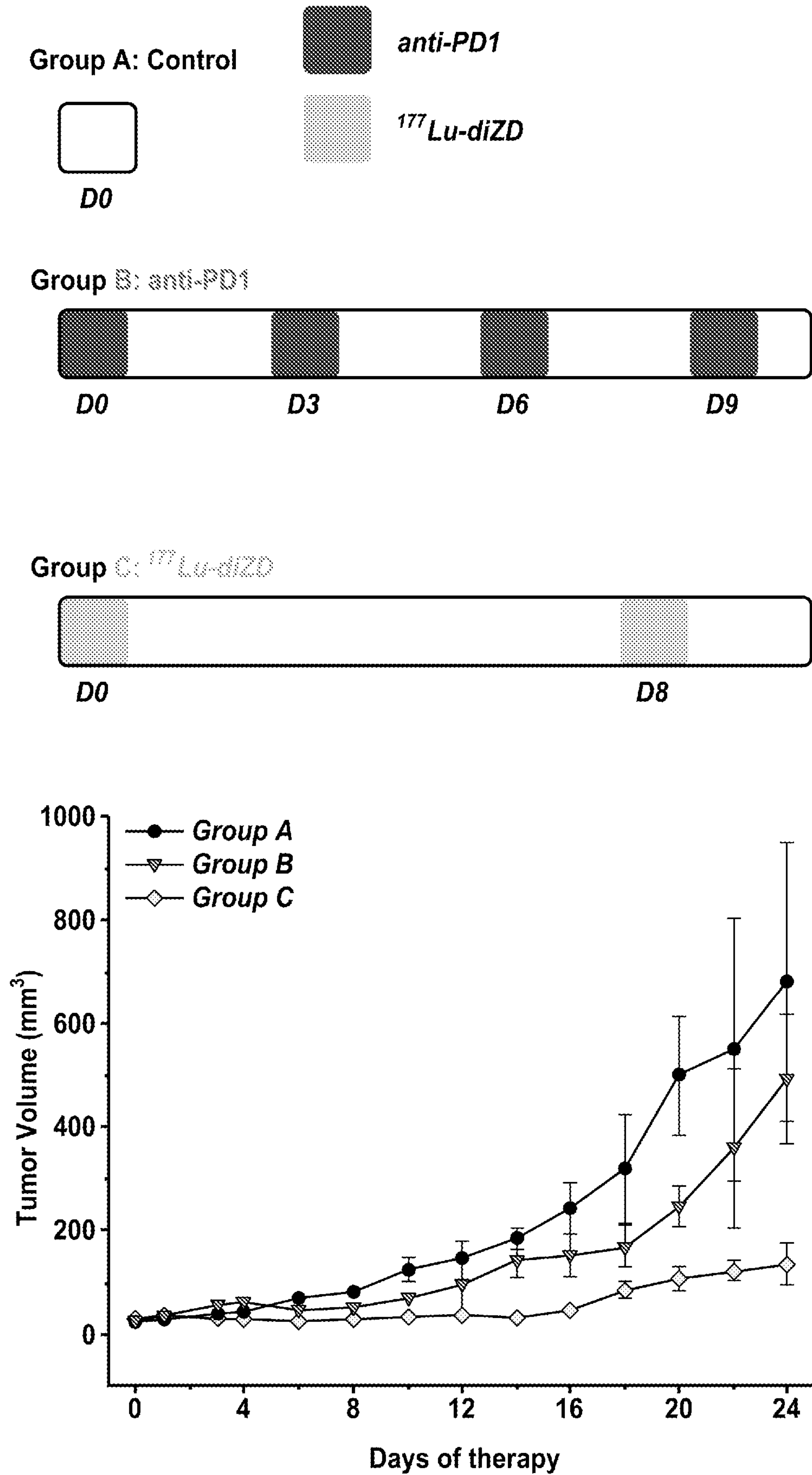


FIG. 7

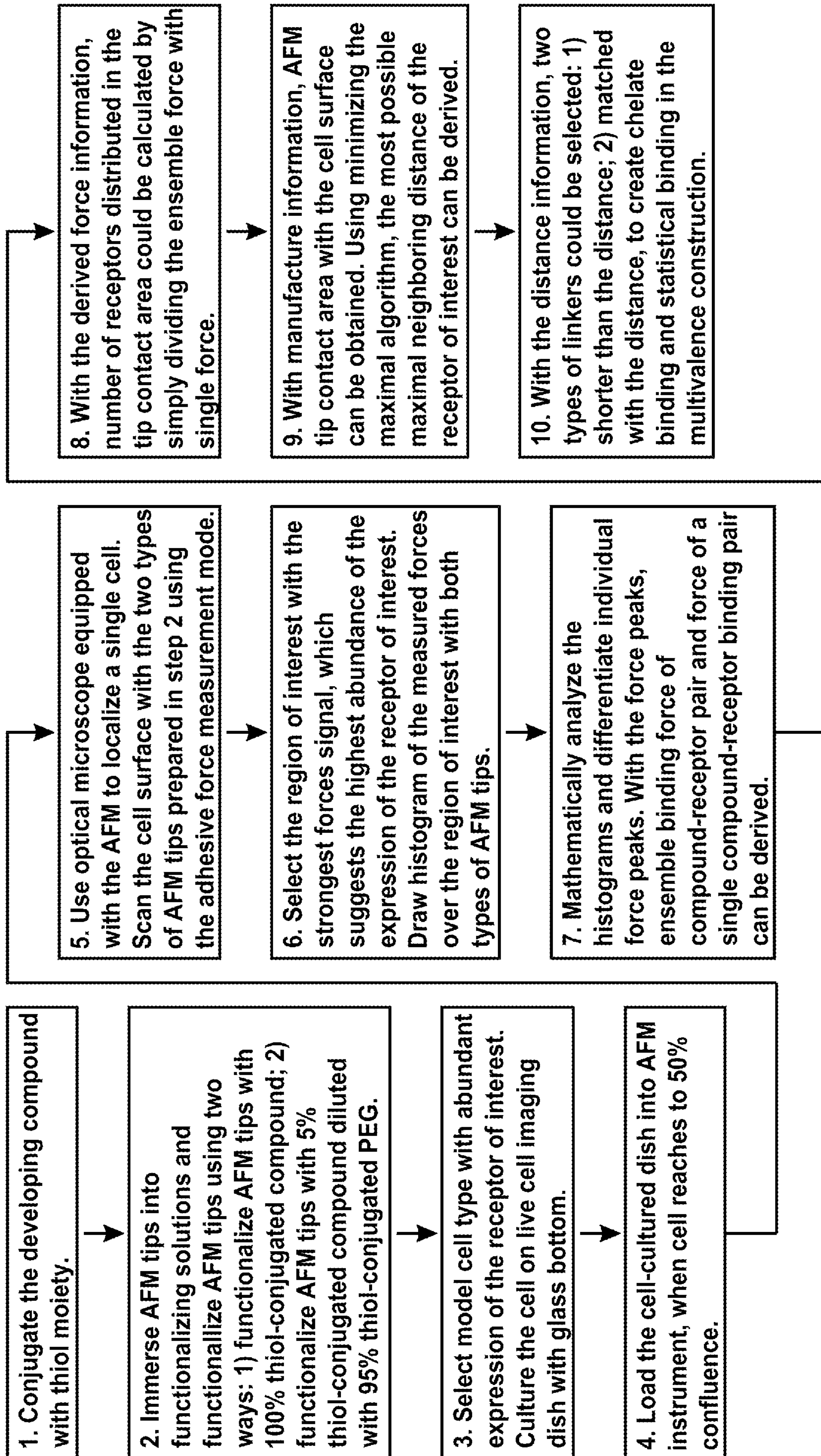
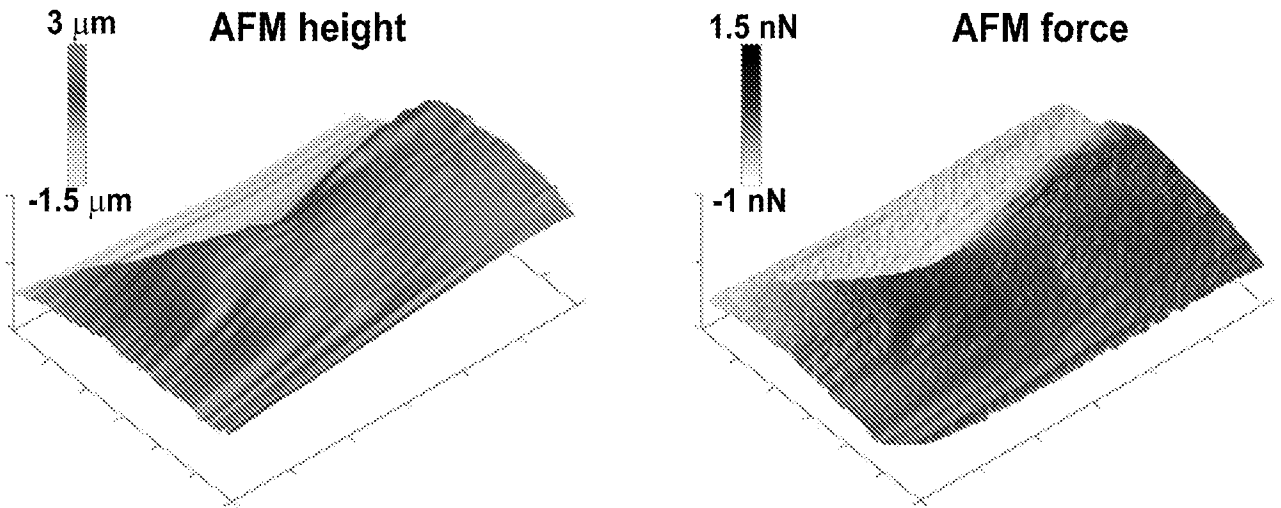
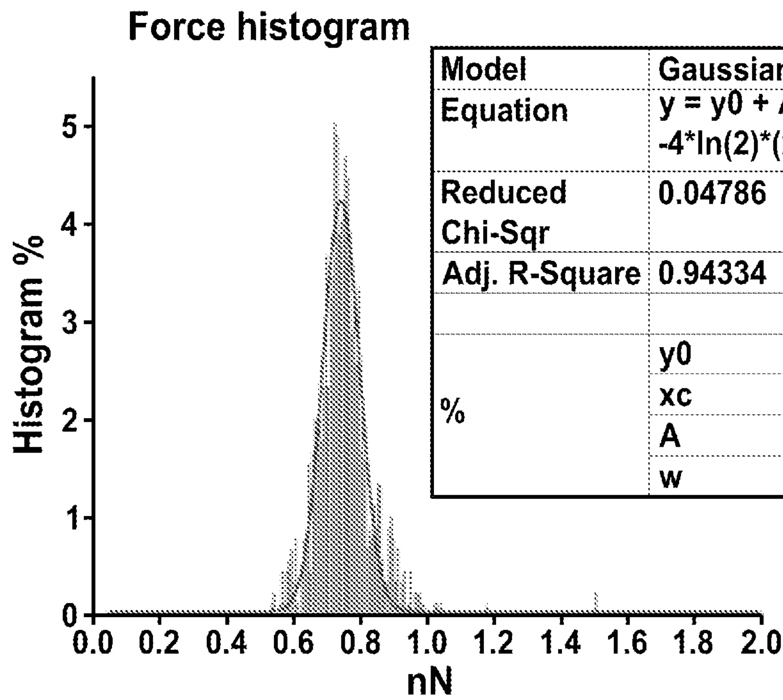


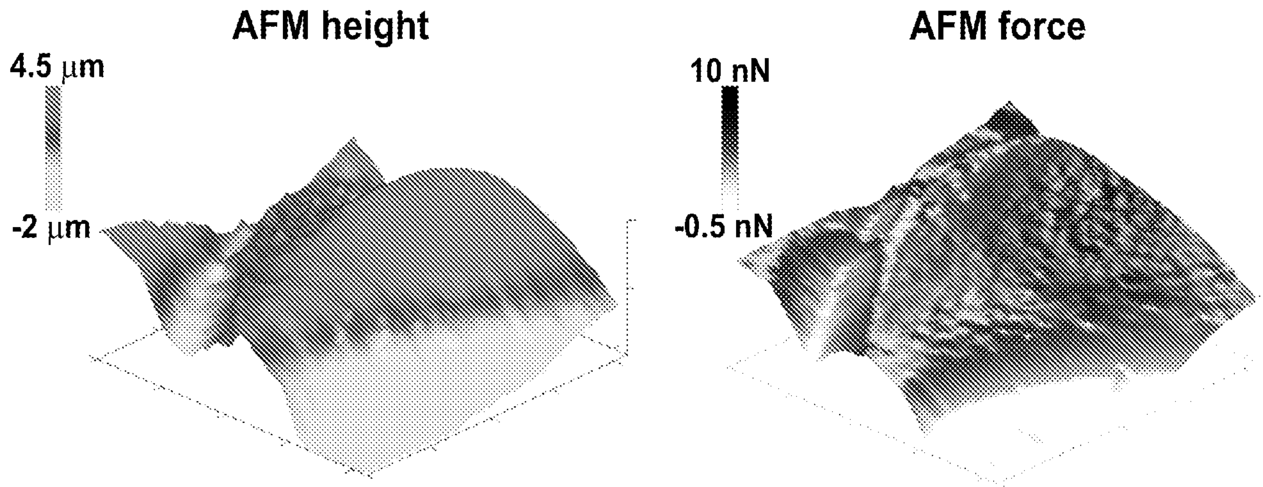
FIG. 8





Model	Gaussian		
Equation	y = y0 + A/(w*sqrt(PI/(4*ln(2))))*exp(-4*ln(2)*(x-xc)^2/w^2)		
Reduced Chi-Sqr	0.04786		
Adj. R-Square	0.94334		
		Value	Standard Error
%	y0	0.03632	0.0137
	xc	0.74175	0.00105
	A	0.5792	0.01034
	w	0.12944	0.00253

FIG. 9



Force histogram

Model	Gaussian		
Equation	y = y0 + A/(w*sqrt(PI/(4*ln(2))))*exp(-4*ln(2)*(x-xc)^2/w^2)		
Reduced Chi-Sqr	0.07501		
Adj. R-Square	0.74257		
		Value	Standard Error
B	y0	0.01835	0.02175
	xc	2.78677	0.03544
	A	3.16218	0.14994
	w	2.09634	0.09505

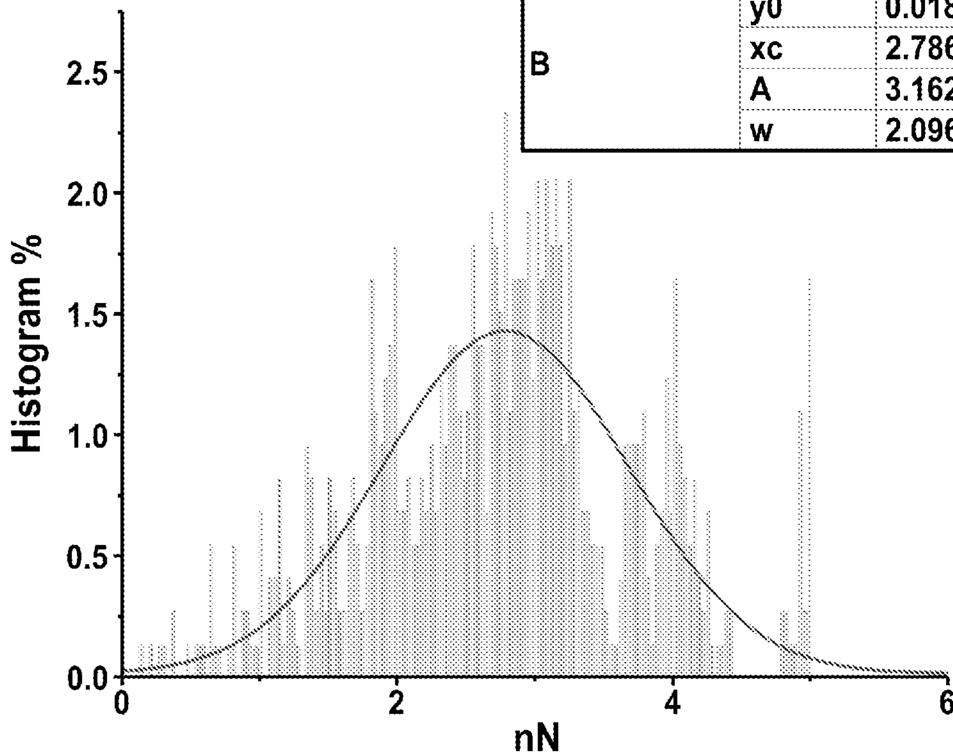


FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 21/15713

A. CLASSIFICATION OF SUBJECT MATTER
IPC - C07D 401/14; C07D 405/14; A61K 49/00 (2021.01)

CPC - C07D 401/14; C07D 403/14; C07D 239/94

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2017/0073328 A1 (THE METHODIST HOSPITAL SYSTEM) 16 March 2017 (16.03.2017); abstract, Fig. 1	1-20
A	US 2017/0247475 A1 (Boehringer Ingelheim International GmbH) 31 August 2017 (31.08.2017); abstract; para [0172]	1-20
A	US 2015/0284416 A1 (Zhao) 08 October 2015 (08.10.2015); abstract, para [0165]	1-20
A	WO 2014/071074 A2 (ABBVIE INC) 08 May 2014 (08.05.2014); entire document	1-20
A	WO 2017/165464 A1 (ELSTAR THERAPEUTICS, INC) 28 September 2017 (28.09.2017); entire document	1-20

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 May 2021	JUN 03 2021

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee Young Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/15713

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 24-25, 30-33, 38-46
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(see supplemental page)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-20

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

--continued from Box No. III--

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I: Claims 1-20, directed to a tetravalent compound comprising a first moiety and a second moiety covalently linked by a first linker, L1, wherein the first moiety comprises two or more vascular endothelial growth factor receptor (VEGFR) binding moieties, wherein the two or more VEGFR binding moieties are linked by a second linker, L2, and wherein the second moiety comprises an additional two or more VEGFR binding moieties linked by a third linker, L3.

Group II: Claims 21-23, 26-29 and 34-37, directed to a method for determining the spatial distribution of VEGFR in a cell / method for determining the spatial distribution of receptors on a cell / functionalizing an AFM tip.

The group of inventions listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I includes the technical feature of a tetravalent compound comprises four vascular endothelial growth factor receptor (VEGFR) binding moieties, which is not required by any other invention of Group II.

Group II includes the technical feature of a method comprising determining the spatial distribution of receptors on a cell / functionalizing an AFM tip, which is not required by any other invention of Group I.

Common technical features:

The inventions of Groups I and II share the technical feature of a receptor binding moiety.

These shared technical features, however, do not provide a contribution over the prior art, as being anticipated by US 2017/0073328 A1 to THE METHODIST HOSPITAL SYSTEM (hereinafter Methodist). Methodist discloses a compound comprising a receptor binding moiety (para [0007], [0070])

As said compound was known in the art at the time of the invention, these cannot be considered special technical features that would otherwise unify the inventions of Groups I or II. The inventions of Group I and II thus lack unity under PCT Rule 13.

Note: Claims 24-25, 30-33, 38-46 have been found to be unsearchable because they are not drafted in accordance with the second and third sentences of Rule 6.4(a).