# INHIBITORS OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) RECEPTORS AND METHODS OF USE THEREOF

## Cross-Reference To Related Applications

This application is related and claims priority to U.S. Provisional Application Serial No. 61/290,789, filed December 29, 2009, the entire contents of which are expressly incorporated herein by this reference.

## Statement Regarding Federally Sponsored Research or Development

This invention was made with Government support under contract R01-AR 051448, R01-AR 051886, and P50 AR054086 awarded by the National Institutes of Health. The government may have certain rights in the invention.

## Background Of The Invention

Vascular endothelial growth factors (VEGF) regulate blood and lymphatic vessel development and homeostasis by binding to and activating the three members of the VEGF-receptor (VEGFR) family of receptor tyrosine kinases (RTK) (Olsson *et al., Nat. Rev. Mol. Cell. Biol.*, 7(5):359-371 (2006)). VEGFR1 (*Flt1*), VEGFR2 (KDR/*Flk1*) and VEGFR3 (Flt4) are members of type-V RTK; a family containing a large extracellular region composed of seven Ig-like domains (D1-D7), a single transmembrane (TM) helix and cytoplasmic region with a tyrosine kinase activity and additional regulatory sequences. The second and third Ig-like domains of the VEGFR ectodomain, *e.g.,* D2 and D3, function as binding sites for the five members of the VEGF family of cytokines (*i.e.* VEGF-A, B, C, D and placenta growth factor (PlGF)) (Barleon *et al., J. Biol. Chem.*, 272(16):10382-10388 (1997); and Shinkai *et al., J. Biol. Chem.*, 273(47):31283-31288 (1998)). These growth factors are covalently linked homodimers. Each protomer is composed of four stranded β-sheets arranged in an anti-parallel fashion in a structure designated cysteine-knot growth factors (Weismann *et al., Cell*, 91(5):695-704 (1997)). Other members of the cysteine-knot family of cytokines include nerve growth factor (NGF) and platelet derived growth factors (PDGF). However, the ectodomains of the PDGFR family of RTKs (type-III) are composed of five Ig-like repeats of which D1,

D2, and D3 function as the ligand binding region of PDGFR and other members of the family (*i.e.,* KIT, CSF1R, and *Flt3*). Structural and biochemical experiments have shown that SCF binding to the extracellular region induces KIT dimerization, a step followed by homotypic contacts between the two membrane proximal Ig-like domains D4 and D5 of neighboring KIT molecules (Yuzawa *et al., Cell*, 130(2):323-334(2007)). Biochemical studies of wild type and oncogenic KIT mutants have shown that the homotypic D4 and D5 contacts play a critical role in positioning the cytoplasmic regions of KIT dimers at a distance and orientation that facilitate trans-autophosphorylation, kinase activation and cell signaling. However, there is a need to better characterize the structures of VEGF receptors. Such a characterization will lead to the informed identification of regions which may be targeted with drugs, pharmaceuticals, or other biologics.

## Summary Of The Invention

The present invention provides moieties, *e.g.,* antibodies or antigen binding portions thereof, small molecules, peptidic molecules, aptamers, and adnectins, that bind to the ectodomain of vascular endothelial growth factor receptors (VEGF receptors), *e.g.,* VEGFR1 (*Flt1*), VEGFR2 (KDR/*Flk1*) and VEGFR3( Flt4). The moieties of the present invention may lock the ectodomain of the VEGF receptor in an inactive state thereby inhibiting the activity of the VEGF receptor. In one embodiment of the invention, the moiety locks the ectodomain of the VEGF receptor to a monomeric state. In another embodiment of the invention, the moiety allows the ectodomain of the VEGF receptor to dimerize but affects the positioning, orientation and/or distance between the Ig-like domains of the two monomers (*e.g.*, the D7-D7 domains of a VEGF receptor), thereby inhibiting the activity of the VEGF receptor. In other words, the moiety may allow ligand induced dimerization of the VEGF receptor ectodomains, but affect the positioning of the two ectodomains at the cell surface interface or alter or prevent conformational changes in the VEGF receptors, thereby inhibiting the activity of the VEGF receptors (*e.g.*, inhibiting receptor internalization and/or inhibiting tyrosine autophosphorylation of the receptor and/or inhibiting the ability of the receptor to activate a downstream signaling pathway). The present invention is based, at least in part, on the deciphering of the crystal structure of part of the ectodomain of the VEGF2

receptor. The deciphering of this crystal structure has allowed for the identification of epitopes, *e.g.*, conformational epitopes, which the moieties of the invention may target.

The present invention is also based, at least in part on the discovery that, rather than playing a role in receptor dimerization, the homotypic D7 interactions between neighboring receptors are required for precise positioning of the membrane proximal regions of two receptors at a distance and orientation that enable interactions between their cytoplasmic domains resulting in tyrosine kinase activation.

Accordingly, in one aspect, the present invention provides a moiety that binds to the ectodomain of a human vascular endothelial growth factor receptor (VEGF receptor), wherein the moiety locks the ectodomain of the VEGF receptor in an inactive state, thereby antagonizing the activity of the VEGF receptor. In one embodiment, the moiety binds to an Ig-like domain of a human VEGF receptor. In one embodiment, the Ig-like domain is not responsible for the binding of a ligand to the VEGF receptor. In another embodiment, the Ig-like domain is responsible for the binding of a ligand to the VEGF receptor. In one embodiment, the moiety does not block the interaction between the VEGF receptor and a ligand for the VEGF receptor. In another embodiment, the moiety blocks the interaction between the VEGF receptor and a ligand for the VEGF receptor. In one embodiment, the moiety does not prevent dimerization of the VEGF receptor. In another embodiment, the moiety prevents dimerization of the VEGF receptor.

In one embodiment, the moiety prevents the interaction between a membrane proximal region of the ectodomain from each protomer of the VEGF receptor. In another embodiment, the interaction is homotypic. In yet another embodiment, the interaction is heterotypic.

In one embodiment, the membrane proximal region of the ectodomain is the $7^{th}$ Ig-like domain (D7) of a VEGF receptor. In another embodiment, the moiety binds to the following consensus sequence for the D7 domain of a VEGF receptor: L/I $X_1$ R $\Phi$ $X_2$ $X_3$ $X_4$ D/E $X_5$ G (SEQ ID NO:158), wherein L is Leucine, I is Isoleucine, R is Arginine, $\Phi$ is a hydrophobic amino acid, D is Aspartic Acid, E is Glutamic Acid, G is Glycine, and $X_1$, $X_2$, $X_3$, $X_4$ and $X_5$ are any amino acid. In a specific embodiment, $\Phi$ is Valine; $X_1$ is selected from the group consisting of Arginine, Glutamine, Glutamic Acid and Aspartic Acid; $X_2$ is selected from the group consisting of Arginine, Lysine and Threonine; $X_3$ is selected from the group consisting of Lysine, Glutamic Acid,

Glutamine and Valine; $X_4$ is selected from the group consisting of Glutamic Acid and Valine; and $X_5$ is selected from the group consisting of Glutamic Acid, Glycine, Serine and Glutamine.

In another embodiment, the moiety causes the membrane proximal region of the ectodomain from each protomer of the VEGF receptor to be separated by a distance greater than about 16 Å, 17 Å, 18 Å, 19 Å or 20 Å. In one embodiment, the moiety locks the ectodomain of the VEGF receptor in an inactive state.

In one embodiment, the VEGF receptor is VEGFR1 (*Flt1*). In another embodiment, the VEGF receptor is VEGFR2 (KDR/*Flk1*). In another embodiment, the VEGF receptor is VEGFR3 (Flt4).

In another embodiment, the moiety binds to amino acid residue Arg726 of VEGFR2. In another embodiment, the moiety binds to amino acid residue Asp731 of VEGFR2. In another embodiment, the moiety binds to amino acid residues Arg726 and Asp731 of VEGFR2. In yet another embodiment, the moiety binds to one or more amino acid residues selected from the group consisting of amino acid residues 724, 725, 726, 727, 728, 729, 730, 731, 732 and 733 of VEGFR2. The moiety may bind within 1Å, 2Å, 3Å, 4Å or 5Å of any of the foregoing amino acid residues.

In one embodiment, the moiety binds to amino acid residue Arg720 of VEGFR1. In another embodiment, the moiety binds to amino acid residue Asp725 of VEGFR1. In another embodiment, the moiety binds to amino acid residues Arg720 and Asp725 of VEGFR1. In another embodiment, the moiety binds to one or more amino acid residues selected from the group consisting of amino acid residues 718, 719, 720, 721, 722, 723, 724, 725, 726 and 727 of VEGFR1. The moiety may bind within 1Å, 2Å, 3Å, 4Å or 5Å of any of the foregoing amino acid residues.

In one embodiment, the moiety binds to amino acid residue Arg737 of VEGFR3. In another embodiment, the moiety binds to amino acid residue Asp742 of VEGFR3. In another embodiment, the moiety binds to amino acid residues Arg737 and Asp742 of VEGFR3. In yet another embodiment, the moiety binds to one or more amino acid residues selected from the group consisting of amino acid residues 735, 736, 737, 738, 739, 740, 741, 742, 743 and 744 of VEGFR3. The moiety may bind within 1Å, 2Å, 3Å, 4Å or 5Å of any of the foregoing amino acid residues.

In one embodiment, the moiety binds to a conformational epitope on the ectodomain of the VEGF receptor. In one embodiment, the conformational epitope is

composed of two or more residues in the D7 domain of the VEGF receptor. In yet another embodiment, the conformational epitope comprises, or consists of, amino acid residues Arg726 and Asp731; Arg 720 and Asp 725; or Arg737 and Asp742. In certain embodiments, the moiety will bind within 1Å, 2Å, 3Å, 4Å or 5Å of the foregoing conformational epitopes.

In another embodiment, the moiety binds to a contiguous epitope on the VEGF receptor. In one embodiment, the contiguous epitope is composed of two or more residues in the D7 domain of the VEGF receptor. In another embodiment, the contiguous epitope is an epitope selected from the group consisting of $^{672}$VAISSS$^{677}$ of VEGFR1, $^{678}$TTLDCHA$^{684}$ of VEGFR1, $^{685}$NGVPEPQ$^{691}$ of VEGFR1, $^{700}$KIQQEPG$^{706}$ of VEGFR1, $^{707}$IILG$^{710}$ of VEGFR1, $^{711}$PGS$^{713}$ of VEGFR1, $^{714}$STLFI$^{718}$ of VEGFR1, $^{719}$ERVTEEDEGV$^{728}$ of VEGFR1, $^{689}$VNVSDS$^{694}$ of VEGFR3, $^{695}$LEMQCLV$^{701}$ of VEGFR3, $^{702}$AGAHAPS$^{708}$ of VEGFR3, $^{717}$LLEEKSG$^{723}$ of VEGFR3, $^{724}$VDLA$^{727}$ of VEGFR3, $^{728}$DSN$^{730}$ of VEGFR3, $^{731}$QKLSI$^{735}$ of VEGFR3, and $^{736}$QRVREEDAGR$^{745}$ of VEGFR3, $^{678}$TSIGES$^{683}$ of VEGFR2, $^{684}$IEVSCTA$^{690}$ of VEGFR2, $^{691}$SGNPPPQ$^{697}$ of VEGFR2, $^{706}$TLVEDSG$^{712}$ of VEGFR2, $^{713}$IVLK$^{716}$ of VEGFR2, $^{717}$DGN$^{719}$ of VEGFR2, $^{720}$RNLTI$^{724}$ of VEGFR2 and $^{725}$RRVRKEDEGL$^{734}$ of VEGFR2. In some embodiments, the moiety may bind within 1Å, 2Å, 3Å, 4Å or 5Å of any of the foregoing epitopes.

In one embodiment, the moiety blocks a ligand induced tyrosine autophosphorylation of the VEGF receptor. In another embodiment, the moiety blocks a ligand induced internalization of the VEGF receptor.

In one embodiment, the moiety which binds to the ectodomain of the VEGF receptor is an isolated antibody, or an antigen-binding portion thereof. In another embodiment, the antibody or antigen-binding portion thereof, is selected from the group consisting of a human antibody, a humanized antibody, a bispecific antibody, and a chimeric antibody. In another embodiment, the antibody, or antigen-binding portion thereof, comprises a heavy chain constant region selected from the group consisting of IgG1, IgG2, IgG3, IgG4, IgM, IgA and IgE constant regions. In one embodiment, the antibody heavy chain constant region is IgG1. In another embodiment, the antibody, or antigen-binding portion thereof, is selected from the group consisting of a Fab fragment, a F(ab')2 fragment, a single chain Fv fragment, an SMIP, an affibody, an avimer, a nanobody, and a single domain antibody. In yet another embodiment, the antibody, or

antigen-binding portion thereof, binds to an Ig-like domain of a receptor tyrosine kinase with a KD selected from the group consisting of 1 x $10^{-7}$ M or less, more preferably 5 x $10^{-8}$ M or less, more preferably 1 x $10^{-8}$ M or less, more preferably 5 x $10^{-9}$ M or less.

In one aspect, the invention provides a hybridoma which produces the antibody, or antigen binding portion thereof, which binds to the ectodomain of the VEGF receptor as described herein.

In one embodiment, the moiety which binds to the ectodomain of a VEGF receptor is a small molecule. In another embodiment, the small molecule binds to at least one of the amino acid residues Arg726 or Asp731 of VEGFR2. In another embodiment, the small molecule binds to at least one of the amino acid residues Arg720 or Asp725 of VEGFR1. In another embodiment, the small molecule binds to at least one of the amino acid residues Arg737 or Asp742 of VEGFR3.

In another embodiment, the moiety which binds to the ectodomain of the VEGF receptor is a peptidic molecule. In one embodiment, the peptidic molecule is designed based on an Ig-like domain of the VEGF receptor. In another embodiment, the peptidic molecule is designed based on the D7 domain of the human VEGF receptor. In one embodiment, the peptidic molecule comprises the structure: L/I $X_1$ R Φ $X_2$ $X_3$ $X_4$ D/E $X_5$ G (SEQ ID NO:158), wherein L is Leucine, I is Isoleucine, R is Arginine, Φ is a hydrophobic amino acid, D is Aspartic Acid, E is Glutamic Acid, G is Glycine, and $X_1$, $X_2$, $X_3$, $X_4$ and $X_5$ are any amino acid. In a specific embodiment, Φ is Valine; $X_1$ is selected from the group consisting of Arginine, Glutamine, Glutamic Acid and Aspartic Acid; $X_2$ is selected from the group consisting of Arginine, Lysine and Threonine; $X_3$ is selected from the group consisting of Lysine, Glutamic Acid, Glutamine and Valine; $X_4$ is selected from the group consisting of Glutamic Acid and Valine; and $X_5$ is selected from the group consisting of Glutamic Acid, Glycine, Serine and Glutamine.

In another embodiment, the peptidic molecule comprises a structure which is at least 80%, 85%, 90% or 95% identical to amino acid residues 724-733, 678-683, 684-690, 691-697, 706-712, 713-716, 717-719, 720-724 or 725-734 of human VEGFR2. In another embodiment, the peptidic molecule comprises a structure which is at least 80%, 85%, 90% or 95% identical to amino acid residues 718-727, 672-677, 678-684, 685-691, 700-706, 707-710, 711-713, 714-718 or 719-728 of human VEGFR1. In another embodiment, the peptidic molecule comprises a structure which is at least 80%, 85%, 90% or 95% identical to amino acid residues 735-744, 689-694, 695-701, 702-708, 717-

723, 724-727, 728-730, 731-735 or 736-745 of human VEGFR3. In another embodiment, the peptidic molecule comprises at least one D-amino acid residue.

In one embodiment, the moiety which binds to the ectodomain of the VEGF receptor is an adnectin.

In another aspect, the invention provides a moiety that binds to a conformational epitope on the D7 domain of the human VEGF receptor and antagonizes the activity of the human VEGF receptor, wherein the conformational epitope comprises residues Arg726 and Asp731 of VEGFR2; residues Arg720 and Asp725 of VEGFR1; or residues Arg737 and Asp742 of VEGFR3.

In another aspect, the invention provides a moiety that binds to amino acid residues Arg726 and Asp731 of VEGFR2; amino acid residues Arg720 and Asp725 of VEGFR1; or amino acid residues Arg737 and Asp742 of VEGFR3, thereby antagonizing the activity of a human VEGF receptor.

In another aspect, the invention provides a pharmaceutical composition comprising a moiety which binds to the ectodomain of a VEGF receptor, as described herein, and a pharmaceutically acceptable carrier.

In another aspect, the invention provides a method of treating or preventing a VEGF receptor associated disease in a subject, comprising administering to the subject an effective amount of a moiety of the invention, thereby treating or preventing the disease. In one embodiment, the VEGF receptor tyrosine kinase associated disease is selected from the group consisting of cancer, age-related macular degeneration (AMD), atherosclerosis, rheumatoid arthritis, diabetic retinopathy, a disease of the lymphatic system and pain associated diseases. In one embodiment, the cancer is selected from the group consisting of GIST, AML, SCLC, renal cancer, colon cancer, breast cancer, lymphatic cancer and other cancers whose growth is supported by stroma.

In one aspect, the invention provides a method for identifying a moiety that binds to the ectodomain, *e.g.,* an Ig-like domain, of a VEGF receptor, the method comprising: contacting a VEGF receptor with a candidate moiety; simultaneously or sequentially contacting the VEGF receptor with a ligand for the VEGF receptor; and determining whether the moiety affects the positioning, orientation and/or distance between the Ig-like domains of the ligand induced dimeric VEGF receptor, thereby identifying a moiety that binds to the ectodomain, *e.g.,* an Ig-like domain, of a VEGF receptor. In one embodiment, the moiety locks the ectodomain of the VEGF receptor in an inactive state.

In another embodiment, the moiety binds to a 7th Ig-like domain (D7) of the VEGF receptor.

In another aspect, the invention provides an isolated antibody, or an antigen-binding portion thereof, that binds to a conformational epitope on the D7 domain of a human VEGF receptor wherein the antibody, or antigen-binding portion thereof, antagonizes the activity of the human VEGF receptor, and wherein the conformational epitope comprises residues Arg726 and Asp731 of VEGFR2. In another aspect, the invention provides an isolated antibody, or an antigen-binding portion thereof, that binds to a conformational epitope on the D7 domain of a human VEGF receptor wherein the antibody, or antigen-binding portion thereof, antagonizes the activity of the human VEGF receptor, and wherein the conformational epitope comprises residues Arg720 and Asp725 of VEGFR1. In another aspect, the invention provides an isolated antibody, or an antigen-binding portion thereof, that binds to a conformational epitope on the D7 domain of a human VEGF receptor wherein the antibody, or antigen-binding portion thereof, antagonizes the activity of the human VEGF receptor, and wherein the conformational epitope comprises residues Arg737 and Asp742 of VEGFR3.

In another aspect, the invention provides an isolated antibody, or an antigen-binding portion thereof, that binds to amino acid residues 724-733 of VEGFR2, thereby antagonizing the activity of VEGFR2. In one aspect, the invention provides an isolated antibody, or an antigen-binding portion thereof, that binds to amino acid residues 718-727 of VEGFR1, thereby antagonizing the activity of VEGFR1. In another aspect, the invention provides an isolated antibody, or an antigen-binding portion thereof, that binds to amino acid residues 735-744 of VEGFR3, thereby antagonizing the activity of VEGFR3.

In one aspect, the invention provides an isolated antibody, or an antigen-binding portion thereof, that binds at least one of the amino acid residues selected from the group consisting of Arg726 and Asp731 of a human VEGFR2, thereby antagonizing the activity of the human VEGFR2. In another aspect, the invention provides an isolated antibody, or an antigen-binding portion thereof, that binds at least one of the amino acid residues selected from the group consisting of Arg720 and Asp725 of a human VEGFR1, thereby antagonizing the activity of the human VEGFR1. In another aspect, the invention provides an isolated antibody, or an antigen-binding portion thereof, that binds at least one of the amino acid residues selected from the group consisting of

Arg737 and Asp742 of a human VEGFR3, thereby antagonizing the activity of the human VEGFR3.

In another aspect, the present invention provides a moiety that binds to the ectodomain, *e.g.*, an Ig-like domain or a hinge region, of a human receptor tyrosine kinase, wherein the moiety locks the ectodomain of the receptor tyrosine kinase in an inactive state, thereby antagonizing the activity of the receptor tyrosine kinase. In one embodiment, the Ig-like domain may or may not responsible for the binding of a ligand to the receptor tyrosine kinase. In another embodiment, the moiety may or may not block the interaction between the receptor tyrosine kinase and a ligand for the receptor tyrosine kinase. In yet another embodiment, the moiety of the invention may or may not prevent dimerization of the receptor tyrosine kinase. In a further embodiment, the moiety of the invention may not prevent ligand induced receptor dimerization but will prevent the homotypic or heterotypic interactions between membrane proximal regions that are required for receptor tyrosine kinase activation.

In some embodiments, a moiety of the invention prevents a homotypic or heterotypic interaction between a membrane proximal region of the ectodomain from each protomer of the receptor tyrosine kinase. For example, a moiety of the invention may cause the termini of the ectodomain (the ends of the ectodomain closest to the plasma membrane) from each protomer of the receptor tyrosine kinase to be separated by a distance greater than about15 Å, about 20 Å, about 25 Å, about 30 Å, about 35 Å or about 40 Å.

In preferred embodiments, the receptor tyrosine kinase is a type III receptor tyrosine kinase, *e.g.*, Kit, PDGFRα, PDGFRβ, CSF1R, Fms, Flt3 or Flk2.

In other embodiments, the Ig-like domain which is bound by a moiety of the present invention is a D4 domain of a type III receptor tyrosine kinase. In one specific embodiment, the moiety binds to the following consensus sequence for the D4 interaction site: $LX_1RX_2X_3X_4X_5X_6X_7G$ wherein L is Leucine, R is Arginine, G is Glycine; , and $X_1$, $X_2$, $X_3$, $X_4$, $X_5$, $X_6$ and $X_7$ are any amino acid. In a specific embodiment, $X_1$ is selected from the group consisting of Threonine, Isoleucine, Valine, Proline, Asparagine, or Lysine; X2 is selected from the group consisting of Leucine, Valine, Alanine, and Methionine; $X_3$ is selected from the group consisting of Lysine, Histidine, Asparagine, and Arginine; $X_4$ is selected from the group consisting of Glycine, Valine, Alanine, Glutamic Acid, Proline, and Methionine; $X_5$ is selected from the group

consisting of Threonine, Serine, Glutamic Acid, Alanine, Glutamine, and Aspartic acid; $X_6$ is selected from the group consisting of Glutamic Acid, Aspartic acid, and Glutamine; and $X_7$ is selected from the group consisting of Glycine, Serine, Alanine, Lysine, Arginine, Glutamine, and Threonine.

In another embodiment, the Ig-like domain which is bound by a moiety of the present invention is a D5 domain of a type III receptor tyrosine kinase, *e.g.*, amino acid residues 309-413 or 410-519 of the human Kit. In a specific embodiment, a moiety of the present invention may bind to a consensus sequence of conserved amino acids from the D5 interaction site.

In another embodiment, the moiety of the present invention binds to mutants of the type III receptor tyrosine kinase D4 or D5 domain or to mutants of the type V receptor tyrosine kinase D7 domain. In a specific embodiment, the moiety binds a point mutation in a mutant D5 domain of human Kit, wherein the mutation is selected from the group consisting of Thr417, Tyr418, Asp419, Leu421, Arg420, Tyr503, and Ala502.

In some embodiments, the type III receptor tyrosine kinase is human Kit and the moiety of the invention binds to one or more amino acid residues, *e.g.*, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, or 18 or more amino acid residues, selected from the group consisting of those amino acid residues shown in Table 4 below. For example, moieties of the invention may bind one or more of the following residues: Y125, G126, H180, R181, K203, V204, R205, P206, P206, F208, K127, A207, V238, S239, S240, S241, H263, G265, D266, F267, N268, Y269, T295, L222, L222, L223, E306, V308, R224, V308, K310, K218, A219, S220, K218, A220, Y221, A339, D327, D398, E338, E368, E386, F312, F324, F340, F355, G311, G384, G387, G388, I371, K342, K358, L382, L379, N326, N367, N370, N410, P341, S369, T385, V325, V407, V409, Y373, Y350, Y408, T380, T390, R381, R353, T411, K412, E414, K471, F433, G470, L472, V497, F469, A431, or G432. In specific embodiments, the moiety of the invention binds at least one of the amino acid residues in the Kit receptor selected from the group consisting of K218, S220, Y221, L222, F340, P341, K342, N367, E368, S369, N370, I371, and Y373 or at least one of the amino acid residues in the Kit receptor selected from the group consisting of Y350, R353, F355, K358, L379, T380, R381, L382, E386, and T390. The moieties of the invention may bind to all of the residues forming a pocket or a cavity identified in Table 4 or they may

bind to a subset of the residues forming the pocket or the cavity. One of skill in the art will appreciate that, in some embodiments, moieties of the invention may be easily targeted to the residues corresponding to those listed above in other type III RTKs, *e.g.*, those residues that form similar pockets or cavities or those in the same position by structural alignment or sequence alignment.

In another embodiment, a moiety of the invention binds to amino acid residues $^{381}$Arg and $^{386}$Glu of human Kit. In yet another embodiment, a moiety of the invention binds to amino acid residues $^{418}$Tyr and/or $^{505}$Asn of human Kit.

In a further embodiment, the moiety of the invention binds to the PDGFRα or PDGFRβ receptor. In a similar embodiment, a moiety of the invention binds to amino acid residues $^{385}$Arg and/or $^{390}$Glu of human PDGFRβ, or the corresponding residues in PDGFRα.

In yet another embodiment, a moiety of the invention binds to a conformational epitope on a type III RTK. In specific embodiments, the conformational epitope is composed of two or more residues from the D3, D4, or D5 domain or hinge regions from a type III RTK, *e.g.*, the human Kit receptor or the PDGF receptor. In further specific embodiments, moieties of the invention may bind to conformational epitopes in the human Kit receptor composed of two or more residues, *e.g.*, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, or 18 or more amino acid residues, selected from the group consisting of those amino acid residues listed in Table 4. In a particular embodiment, a moiety of the invention binds to a conformational epitope composed of 2 or more amino acids selected from the group consisting of Y125, H180, R181, K203, V204, R205, P206. V238, S239, S240, H263, G265, D266, F267, N268, and Y269. In similar embodiments, a moiety of the invention may bind to a conformational epitope composed of 2 or more amino acids selected from one of the following groups of amino acids: P206, F208, V238, and S239; K127, A207, F208, and T295; L222, A339, F340, K342, E368, S369, N370, I371, and Y373; L222, L223, E306, V308, F312, E338, F340, and I371; R224, V308, K310, G311, F340, P341, and D398; K218, A219, S220, N367, E368, and S369; K218, A220, E368, and S369; G384, T385, T411, K412, E414, and K471; Y408, F433, G470, K471, and L472; F324, V325, N326, and N410;D327, N410, T411, K412, and V497; G384, G387, V409, and K471; L382, G387, V407, and V409; Y125, G126, H180, R181, K203, V204, R205,

P206, F208, V238, S239, S240, S241, H263, G265, D266, F267, N268, and Y269; P206, F208, V238, and S239; K218, S220, Y221, L222, F340, P341, K342, N367, E368, S369, N370, I371, and Y373; G384, G387, G388, Y408, V409, T411, F433, F469, G470, and K471; D327, T411, K412, E414, A431, G432, and K471; Y350, F355, K358, L379, T380, R381, L382, E386, and T390; Y350, R353, and F355. As indicated above, the moieties of the invention may bind to all of the amino acid residues forming a pocket or a cavity identified in Table 4 or they may bind to a subset of the residues forming the pocket or the cavity.

In a further embodiment, a moiety of the invention binds to a conformational epitope wherein the conformational epitope is composed of two or more amino acid residues selected from the peptides listed in Table 5. In a specific embodiment, the conformational epitope is composed of one or more amino acid residues selected from a first peptide and one or more amino acid residues selected from a second peptide, wherein the first and second peptides are selected from the group of peptides listed in Table 5. As such, a moiety of the invention may bind a conformational epitope wherein the first and second peptide groups are as follows: Ala219-Leu222 and Thr304-Val308; Asp309-Gly311 and Arg224-Gly226; Thr303 - Glu306 and Ala219-Leu222; Asn367–Asn370 and Ser217–Tyr221; Ala339–Pro343 and Asn396–Val399; Ala339–Pro343 and Glu368-Arg372; Lys358–Tyr362 and Val374–His378; Asp357-Glu360 and Leu377–Thr380; Met351–Glu360 and His378-Thr389; His378-Thr389 and Val323-Asp332; Val409–Ile415 and Ala493–Thr500; Val409–Ile415 and Ala431-Thr437; Val409– Ile415 and Phe469-Val473; Val409–Ile415 and Val325–Asn330; Val409–Ile415 and Arg381-Gly387; Gly466–Leu472 and Gly384–Gly388; Val325-Glu329 and Tyr494-Lys499; Thr411-leu416 and Val497-Ala502; Ile415–Leu421 and Ala502–Ala507; Ala502–Ala507 and Lys484-Thr488; and Ala502–Ala507 and Gly445–Cys450. The moieties of the invention may bind to all of the amino acid residues forming the foregoing first and second peptide groups or they may bind to a subset of the residues forming the first and second peptide groups.

In other embodiments, moieties of the present invention bind to receptor tyrosine kinases which are members of the VEGF receptor family (type V receptor tyrosine kinases), *e.g.*, VEGFR-1 (Flt1),VEGFR-2(Flk1) and VEGFR-3(Flt4). The Ig-like domain bound by moieties of the present invention may, in some embodiments, be the D7 domain of a member of the VEGF receptor family. In a specific embodiment, the

moiety binds to the following consensus sequence for the D7 domain of a member of the VEGF receptor family: $IX_1RVX_2X_3EDX_4G$ wherein I is Isoleucine, R is Arginine, E is Glutamic Acid, D is Aspartic Acid, G is Glycine; and $X_1$, $X_2$, $X_3$ and $X_4$ are any amino acid. In a specific embodiment, $X_1$ is selected from the group consisting of Glutamic Acid, Arginine, and Glutamine; X2 is selected from the group consisting of Arginine and Threonine; $X_3$ is selected from the group consisting of Glutamic Acid and Lysine; and $X_4$ is selected from the group consisting of Glutamic Acid and Alanine (SEQ ID NO: 1).

In some embodiments, the moiety of the present invention is an isolated antibody, or an antigen-binding portion thereof. The antibody or antigen-binding portion thereof, may be a human antibody, a humanized antibody, a bispecific antibody, or a chimeric antibody. In some embodiments, the antibody, or antigen-binding portion thereof, comprises a heavy chain constant region selected from the group consisting of IgG1, IgG2, IgG3, IgG4, IgM, IgA and IgE constant regions. In a preferred embodiment the antibody heavy chain constant region is IgG1. Additionally, the moiety of the present invention may be an antibody, or antigen binding portion thereof, wherein the antibody, or antigen-binding portion thereof, is selected from the group consisting of a Fab fragment, a F(ab')2 fragment, a single chain Fv fragment, an SMIP, an affibody, an avimer, a nanobody, and a single domain antibody. In particular embodiments, an antibody, or antigen-binding portion thereof, of the present invention binds to an Ig-like domain of a receptor tyrosine kinase with a $K_D$ of $1 \times 10^{-7}$ M or less, more preferably $5 \times 10^{-8}$ M or less, more preferably $1 \times 10^{-8}$ M or less, more preferably $5 \times 10^{-9}$ M or less.

In some embodiments, the isolated antibody, or an antigen-binding portion thereof, of the present invention binds to amino acid residues 309-413 and/or 410-519 of the human Kit, thereby locking the ectodomain of the human Kit in an inactive state and antagonizing the activity of human Kit.

In further embodiments, the present invention includes a hybridoma which produces the antibody, or antigen binding portion thereof, of the present invention.

In another preferred embodiment, the moiety of the present invention is a small molecule.

In some preferred embodiments, the small molecule of the invention binds to one or more amino acid residues selected from the group consisting of those amino acid residues shown in Table 4. For example, small molecules of the invention may bind one or more of the following residues: Y125, G126, H180, R181, K203, V204, R205, P206,

P206, F208, K127, A207, V238, S239, S240, S241, H263, G265, D266, F267, N268, Y269, T295, L222, L222, L223, E306, V308, R224, V308, K310, K218, A219, S220, K218, A220, Y221, A339, D327, D398, E338, E368, E386, F312, F324, F340, F355, G311, G384, G387, G388, I371, K342, K358, L382, L379, N326, N367, N370, N410, P341, S369, T385, V325, V407, V409, Y373, Y350, Y408, T380, T390, R381, R353, T411, K412, E414, K471, F433, G470, L472, V497, F469, A431, or G432. In a specific embodiment, the small molecule of the invention binds at least one of the amino acid residues in the Kit receptor selected from the group consisting of K218, S220, Y221, L222, F340, P341, K342, N367, E368, S369, N370, I371, and Y373. In a related embodiment, the small molecule of the invention binds at least one of the amino acid residues in the Kit receptor selected from the group consisting of Y350, R353, F355, K358, L379, T380, R381, L382, E386, and T390. One of skill in the art will appreciate that, in some embodiments, small molecules of the invention may be easily targeted to the residues corresponding to those listed above in other type III RTKs, *e.g.*, those residues that form similar pockets or cavities or those in the same position by structural alignment or sequence alignment.

In a further embodiment, the moiety of the present invention is a peptidic molecule. In some embodiments, the peptidic molecule is designed based on an Ig-like domain of a receptor tyrosine kinase. In a specific embodiment, the peptidic molecule of the present invention is designed based on the D4 domain of Kit. The peptidic molecule of the present invention may comprise a conserved D4 interaction site, *e.g.*, the D4 consensus sequence described above ($LX_1RX_2X_3X_4X_5X_6X_7G$), or others generated by aligning or comparing D4 domains of type III receptor tyrosine kinases. In additional embodiments, a peptidic molecule of the present invention comprises a structure which is at least 80% identical to amino acid residues 309-413 of human Kit or a structure which is at least 80% identical to amino acid residues 410-519 of human Kit. The peptidic moities may also be designed based on the D5 domain of Kit, and, in further preferred embodiments, may comprise a consensus sequence generated by aligning or comparing D5 domains of type III receptor tyrosine kinases. In alternative embodiments, the peptidic molecule may be designed based on the sequence or consensus sequence of mutant D5 domains.

The peptidic moieties of the invention may be peptides comprising or consisting of any of the amino acid sequences identified herein (*e.g.*, SEQ ID NOs: 1-89, 92, 93, and 105-157).

In some embodiments, the peptidic molecule of the present invention comprises at least one D- amino acid residue.

In another preferred embodiment, the moiety of the present invention is an adnectin.

In addition, in some embodiments the small molecules and peptidic molecules of the invention bind to conformational epitopes in the target RTKs. In other embodiments, the small molecules and peptidic molecules of the invention bind to epitopes in the target RTKs which are not conformational epitopes.

In another aspect, the present invention provides pharmaceutical compositions comprising any of the moieties of the present invention and a pharmaceutically acceptable carrier.

In additional aspects, the invention provides methods of treating or preventing a receptor tyrosine kinase associated disease in a subject. The methods include administering to the subject an effective amount of a moiety of the present invention (*e.g.*, a moiety which binds the D4 or D5 domain of a type III receptor tyrosine kinase, or a D7 domain of a type V receptor tyrosine kinase), thereby treating or preventing the disease. In preferred embodiments, the receptor tyrosine kinase associated disease is a lymphatic disease or cancer, *e.g.*, GIST, AML, SCLC, melanoma, renal cancer, colon cancer, breast cancer, lymphatic cancer and other cancers.

In another aspect, the invention provides methods of treating or preventing a receptor tyrosine kinase associated disease in a subject, by administering to the subject an effective amount of a moiety which binds the D3-D4 and/or a D4-D5 hinge region of a human type III receptor tyrosine kinase, thereby treating or preventing the disease. In specific embodiments, the receptor tyrosine kinase associated disease is cancer, *e.g.*, GIST, AML, SCLC, melanoma, renal cancer, colon cancer, breast cancer, lymphatic cancer or other cancers.

In another aspect, the invention provides methods for identifying a moiety that binds to an Ig-like domain of a receptor tyrosine kinase and locks the ectodomain of the receptor tyrosine kinase to an inactive state. The methods include contacting a receptor tyrosine kinase with a candidate moiety; simultaneously or sequentially contacting the

receptor tyrosine kinase with a ligand for the receptor tyrosine kinase; and determining whether the moiety affects the positioning, orientation and/or distance between the Ig-like domains of the ligand induced dimeric receptor tyrosine kinase, thereby identifying a moiety that binds to an Ig-like domain of a receptor tyrosine kinase and locks the ectodomain of the receptor tyrosine kinase to an inactive state.

In a further aspect, the invention provides methods for identifying a moiety that locks the ectodomain of a type III receptor tyrosine kinase to an inactive state. The methods include contacting a type III receptor tyrosine kinase with a candidate moiety; simultaneously or sequentially contacting the receptor tyrosine kinase with a ligand for the receptor tyrosine kinase; and determining whether the moiety affects the positioning, orientation and/or distance between the D4-D4 or D5-D5 domains of the ligand induced dimeric receptor tyrosine kinase, thereby identifying a moiety that locks the ectodomain of the type III receptor tyrosine kinase to an inactive state.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

## Brief Description Of The Drawings

This patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

**Figures 1A-E** depict the crystal structure of Kit ectodomain. **Figure 1A** shows a ribbon diagram (left) and surface representation (right) of Kit ectodomain monomer. Right panel shows a view following 90° rotation along the vertical axis of the view shown in the left panel. D1 is colored in blue, D2 in green, D3 in yellow, D4 in orange and D5 in pink, N and C termini are labeled. Disulfide bonds in D1 and D5 are shown in ball-and-stick rendering with sulfur atoms colored in orange. Asparagine-linked carbohydrates are shown in a stick model. **Figure 1 B-E** provides detailed views of the D1-D2 (B), D2-D3 (C), D3-D4 (D), and D4-D5 (E) interfaces. Color coding is the same as in Figure 1A. Amino acids that participate in domain-domain interactions are labeled and hydrogen bonds are drawn as dashed yellow lines. Secondary structure elements are designated according to IgSF nomenclature.

**Figures 2A-B** depict the crystal structure of the SCF-Kit ectodomain 2:2 complex. **Figure 2A** shows a ribbon diagram of SCF-Kit 2:2 complex. Color coding of D1 to D5 is the same as in Figure 1 and SCF is colored in magenta. N and C termini of Kit and SCF are labeled. Disulfide bonds in D1 and D5 are shown in ball-and-stick rendering with sulfur atoms colored in orange. Asparagine-linked carbohydrates are represented in a stick model. Arrow marks a large cavity in the SCF-Kit 2:2 complex. **Figure 2B** shows surface representations of SCF-Kit ectodomain 2:2 complex. The figure shows a top view (top), face view (center left), side view (center right) and bottom view (low). Color coding is the same as in A. The views show that a SCF dimer interacts symmetrically with D1, D2 and D3 of two corresponding Kit ectodomains. In addition, Kit ectodomains form homophylic interactions through lateral contacts between D4 (orange) and between D5 (pink) of two neighboring receptors.

**Figures 3A-E** depict SCF recognition by Kit. **Figure 3A** shows views of the SCF-Kit interface. Amino acids in the buried surfaces in SCF and Kit ectodomain are visualized by pulling apart the two molecules. The figure shows the molecular surface of Kit D1-D2-D3 (left) and SCF (right). Acidic amino acids are shown in red, basic amino acids in blue, polar amino acids in orange and hydrophobic amino acids in yellow. SCF binding site-I, site-II and site-III on Kit are circled. **Figure 3B** depicts complementarity in the electrostatic potential in the ligand-receptor interface. The right panel shows a view following a rotation of 180° along the vertical axis of the electrostatic surface presented in the left panel. Electrostatic surface potential of D1-D2-D3 superimposed on the molecular surfaces with an imprint of a cartoon diagram of bound SCF that is colored in green. Right panel depicts the electrostatic surface potential of SCF-bound Kit colored in blue (positive) and red (negative). Kit is shown in a form of ribbon diagram colored in cyan. **Figures 3C-E** show close-up views of site-I (C), site-II (D) and site-III (E) of SCF-Kit interface. SCF is colored in green and Kit in cyan. Interacting amino acids are labeled, hydrogen bonds are drawn as dashed yellow lines and secondary structure elements are marked on the ribbons and strands.

**Figures 4A-C** depict conformational changes in SCF upon binding to Kit. **Figure 4A** shows that the angle between the two SCF protomers is altered upon Kit binding. The view shows a cartoon diagram of free SCF (green) and SCF bound to Kit (magenta). Superimposition of the one SCF protomer (left) reveals an angular movement of approximately 5° of the second protomer (right), as measured for helix αC. Helices

are labeled and shown as cylinders. **Figure 4B** depicts the conformational change in the N-terminus of SCF upon Kit binding. Site-III of Kit is shown as a molecular surface (gray), the N-terminus of free SCF is shown in green and of SCF bound to Kit in magenta. Disulfide bond between Cys4' and Cys89' is shown as yellow spheres. Key amino acids are labeled and shown as a stick model. **Figure 4C** depicts the conformational change in the αC-β2 loop of SCF upon binding to site-I of Kit. Color coding is the same as in B.

**Figures 5A-B** depict the reconfiguration of Kit D4 and D5 upon SCF binding**. Figure 5A** shows the reconfiguration of D4 and D5 in the SCF-Kit complex . Superimposition of D3 from Kit monomer with D3 of Kit-bound to SCF (both colored blue) shows that D4 of the bound form (red) moves by 22° relative to the position of D4 of the free form (green). Superimposition (right panel) of D4 of the two forms (both in blue) shows that D5 of the SCF-bound form (red) moves by 27° relative to the positions of D5 of the free form (green). The two bottom panels show close views of the hinge regions of D3-D4 and D4-D5 interfaces of the monomeric (green) and homodimeric (red) forms. **Figure 5B** shows a surface representation of D4 and D5 of SCF occupied Kit (top panel), viewed in the same orientation as in Figure 2. The black outline shows the location of D4 and D5 of Kit ectodomain monomers bridged by SCF binding to the ligand binding region. Re-configuration of D4 and D5 leads to a movement of the C-termini of two neighboring ectodomains from 75 Å to 15 Å from each other. Lower panel shows a view from the cell membrane (bottom view) of SCF-Kit complex. Note a 90° rotation along the x-axis. Color coding of D1 to D5 is the same as in Figure 1.

**Figures 6A-D** depict views of the D4-D4 and the D5-D5 interfaces. **Figure 6A** (top panel) shows a 2Fo-Fc electron density map contoured at 1.1σ level showing a view of the D4-D4 interface. The backbones of Kit protomers are represented as pink and yellow tubes, respectively. A close view (bottom panel) of the D4-D4 interface of two neighboring ectodomains. Interchain hydrogren bonds formed between Arg381 and Glu386, of two adjacent D4 are colored in yellow. Key amino acids are labeled and shown as a stick model. Secondary structure elements are labeled according to the IgSF nomenclature. **Figure 6B** depicts the conservation of the D4-D4 dimerization motif across member of type-III and type-V RTK families. Residues 370-398 of human Kit (AAC50969.1) (SEQ ID NO: 94) aligned with sequences of, mouse (AAH75716.1) (SEQ ID NO: 95), chicken (NP_989692.1) (SEQ ID NO: 96), xenopus laevis

(AAH61947) (SEQ ID NO: 97), salamander (AAS91161.l) (SEQ ID NO: 98) and zebrafish (type A (SEQ ID NO: 99) and B (SEQ ID NO: 100) (NP_571128, XP_691901) homologs. Also shown amino-acid sequences of CSF1R from human (P07333) (SEQ ID NO: 101), mouse (P09581) (SEQ ID NO: 102) and torafugu type A (SEQ ID NO: 103) and B (SEQ ID NO: 104) (P79750, Q8UVR8), and sequences from PDGFRα and PDGFRβ from human (SEQ ID NOS 105 and 107, respectively) (P16234, P09619) and mouse (SEQ ID NOs:106 and 108, respectively) (NP_035188, P05622). Amino acid sequences of type-V RTKs of human VEGFR type 1-3 (SEQ ID NOs:109-111, respectively, in order of appearance) ($7^{th}$ Ig-like domain) (P17948, P35968 and P35916) are also presented. Secondary structure elements on Kit are labeled on the top of the sequence alignment. The conserved residues of Arg381 and Lys383, Leu382 and Leu379, Glu386 and Gly388 are colored in blue, yellow, red and green, respectively. **Figure 6C** depicts a ribbon diagram of a D5-D5 interface. Strands A and G of two adjacent Kit protomers participate in formation of the D5-D5 interface. The D5-D5 interface is maintained by lateral interactions between Tyr418 and Asn505 of two neighboring receptors probably through ion(s) or water molecule(s). **Figure 6D** depicts the electrostatic potential surfaces of D4 and D5 of Kit. The figures show a face view of the D4-D4 interacting surface (right) and a view following 90° rotation along the vertical axis (left). The position of acidic patch and the D4-D4 interfaces are circled and the interacting residue Arg381 and Glu386 are labeled.

**Figures 7A-C** depict Kit ectodomain mutations implicated in cancer and other diseases and mechanism of Kit and other RTK activation. **Figure 7A** depicts loss-of-function mutations responsible for the piebald trait are showin in the left panel. A ribbon diagrams of D1 (blue), D2 (green) and D3 (yellow) and surface representation of SCF (gray). Mutated amino acids are colored in red. Gain of function mutations responsible for GIST, SCLC and AML are shown in the right panel. Surface representation of D4 and D5 in the homodimeric form is colored in gray. Ala502 and Tyr503 that are duplicated in GIST are shown in blue and deletions and insertional mutations in proximity to Asp419 (AML and NCLL) are shown in green. Note that the activating Kit mutations are confined to the D5-D5 interface. **Figure 7B** shows that Kit activation is compromised by point mutants in D4-D4 interface. HEK293 cells transiently expressing wild type Kit (WT), R381A or E386A point mutations in D4 were stimulated with 10ng/ml SCF for six minutes at 37 °C as indicated (upper left panel). Lysates of

unstimulated or SCF stimulated cells were subjected to immunoprecipitation (IP) with anti-Kit antibodies followed by SDS-PAGE and immunoblotting (IB) with either anti-Kit or anti phosphotyrosine (p-Tyr) antibodies. Densitometric quantitation of tyrosine autophosphorylation of Kit from anti- p-Tyr immunoblots (upper right panel). 3T3 cells stably expressing wild type Kit (WT) or the R381A mutant were treated with different concentrations of SCF. Lysates from unstimulated or SCF stimulated cells were subjected to immunoprecipitation with anti-Kit antibodies followed by SDS-PAGE and immunoblotting with anti-Kit or anti- p-Tyr antibodies (lower left panel). Displacement assay of cell bound 125I- SCF using native SCF. 3T3 cells expressing WT (■), R381A (▼), R381A/E386A (◆), or a kinase negative Kit (▲) were treated with 125I-SCF in the presence of increasing concentrations of native SCF. The EC50 (ligand concentration that displaces 50% of $^{125}$I-SCF bound to c-Kit) of SCF towards WT Kit (1.1 nM) is comparable to the EC50 of SCF towards R381A (1.0 nM), R381A/E386A (0.8 nM) or the kinase negative Kit mutant (1.4 nM). **Figure 7C** shows models for Kit and other RTK activation driven by soluble (left panel) or membrane anchored (right panel) SCF molecules expressed on the cell surface of a neighboring cell. SCF binding to the D1-D2-D3 ligand binding module brings the C-termini of the two bound Kit ectodomain monomers within of 75Å from each other. The flexibility of the D3-D4 and D4-D5 hinges enable lateral D4-D4 and D5-D5 interactions that bring the C-termini of two neighboring ectodomains within 15 Å from each other. Consequently, the increased proximity and local concentration of Kit cytoplasmic domains leads to autophosphorylation of regulatory tyrosine residues in the kinase domain resulting in PTK activation. (Note that PTK activation is not drawn in the model.) Recruitment and activation of a complement of cell signaling molecules will proceed following phosphorylation of key tyrosines in the cytoplasmic domain. The model is based on free SCF structure, ligand-free Kit, SCF-Kit complex and Kit PTK structure (PDB entries 1QZJ, 1R01 and 1T45). Regions whose structures have not been determined were modeled using secondary structure prediction (green helices and black loops). SCF is colored in magenta, Kit ectodomain in blue and kit PTK is light blue.

**Figure 8** depicts a structure based sequence alignment of type-III RTKs, based on Kit ectodomain structure, and structure based alignment of ligands for the type-III family RTKs. Each row shows alignment of an individual Ig-like domain. Amino acid sequences were manually aligned based on the IgSF fold characteristics, as determined

by (Harpaz *et al.* (1994) J Mol Biol 238: 528-539) and within agreement with the secondary structure prediction of family members as calculated by Jpred (Cuff *et al.* (1998) Bioinformatics 14: 892-893). Amino acids marked in red represent IgSF fold determining amino acids. β strands are labeled by arrows and α-helices by springs above the sequence, along with numbering for human Kit and human SCF. Residues of the ligand binding site showing reduced solvent accessibility upon ligand binding are marked by asterisks. Site-I is colored in black, site-II in red and site-III in green. The same color code is used for labeling interacting amino acid residues in SCF. The D4 EF loop that is responsible for D4-D4 interaction is boxed in cyan. The sequences used for the alignment are: Kit human (AAC50969), Kit mouse (AAH75716), CSFR1 human (P07333), PDGFRα human (P16234), PDGFRβ human (P09619) and Flt3 human (P36888). For ligand structure alignment, the PDB entries of SCF (1EXZ), CSF (1HMC), Flt3L (1ETE) were superimposed using Lsqman (Kleywegt and Jones, 1995), while the sequence of SCF mouse (NP_038626) was aligned to the human SCF using ClustalW. Figure discloses SEQ ID NOS 112-147, respectively, in order of appearance.

**Figure 9** provides a stereo view of overall structure of the 2:2 SCF-Kit complex. Ribbon model of the 2:2 SCF-Kit complex is shown in stereo representation. The view and the color code are the same as in Figure 2A.

**Figures 10A-B** depict the amino acid conservation at the surface of SCF-Kit complex. **Figure 10A** shows the color-coded conservation pattern of the SCF-Kit crystal structure complex. Cyan through maroon are used for labeling from variable to conserved amino acids. **Figure 10B** shows a visualization of SCF and Kit by pulling away the two molecules from each other. Site I, Site II, and Site III and the D4-D4 interacting region (D4-D4 interface) are circled.

**Figures 11A-B** depict the electron densities of the SCF-Kit interface. **Figure 11A** shows a partial view of site-II of the 2:2 SCF-Kit complex with a 2Fo-Fc electron-density map drawn around Kit at 2 σ level. Kit main chain is drawn in yellow tubes except for labeled side chains. **Figure 11B** depicts the electron densities of the SCF-Kit interface, showing a partial view of free Kit with an experimental map drawn around Kit at 1.5 σ level. Orientation and color code are the same as in Figure 12A.

**Figures 12A-D** depict views of superimposed pairs of Ig-like domains from free and SCF bound Kit. Individual D1, D2, D3, and D4 from free and SCF bound Kit are superimposed. Shown are structures of pairs of Ig-like domains (A) D1 and D2, (B) D2

and D3, (C) D3 and D4 and (D) D4 and D5 in which the superimposed Ig-like domain in each pair is colored in blue and the second (not superimposed) Ig-like domain is colored in green for free ectodomain and in red for SCFbound ectodomain. These figures show that virtually no changes take place in the structures of each of the five individual Kit Ig-like domains upon SCF binding and that D1-D2-D3 function as a ligand binding unit poised towards SCF binding. By contrast, large rearrangements take place in D3-D4 and D4-D5 interfaces in SCF bound Kit.

**Figures 13A-B** depict the electrostatic surface potential of the SCF-Kit complex structure. **Figure 13A** specifically shows the SCF-Kit 2:2 complex. **Figure 13B** depicts the electrostatic surface potential of the SCF-Kit complex structure, specifically a visualization of the electrostatic surface potential of Kit after SCF was pulled away from the SCF-Kit 2:2 complex. Positively and negatively charged surfaces are colored in blue and red, respectively. The SCF binding region and the D4-D4 interface are circled.

**Figure 14** depicts the inhibition of SCF-induced Kit activation by anti Kit-D5 antibodies. 3T3 cells expressing Kit were incubated with increasing concentrations of anti-Kit D5 (directed against fifth Ig-like domain of Kit) or as controls with anti-SCF (directed against the SCF ligand), or anti-Kit ectodomain (directed against the entire Kit ectodomain).

**Figure 15** depicts the inhibition of SCF induced Kit activation using recombinant Kit D4. 3T3 cells expressing Kit were incubated with increasing concentrations of recombinant Kit-D4 for 10 minutes at room temperature followed by 10 minutes SCF stimulation.

**Figure 16A** demonstrates that PDGF-induced PDGFR activation is prevented by point mutations in D4. PDGFR-/- MEFs expressing WT PDGFR or D4 mutants (R385A and E390A) were serum starved overnight and stimulated with the indicated concentrations of PDGF BB for 5 minutes. Cell lysates were immunoprecipitated with anti-PDGFR antibodies, followed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody 4G10. Membranes were stripped off, and re-blotted with anti-flag tag antibodies to determine total PDGFR levels.

**Figure 16B** demonstrates that signaling via PDGFR is prevented by point mutations in D4. PDGFR-/- MEFs expressing WT PDGFR and D4 mutants (R385A and E390A) were serum starved overnight and stimulated with indicated concentrations of PDGF BB for 5 minutes at 23 C°. Equal amounts of total cell lysates (TCL) were

subjected to SDS-PAGE and analyzed by immunoblotting with anti-phospho-MAPK, MAPK, phospho-Akt and Akt, respectively. This experiment shows that both MAPK response and Akt activation are prevented by point mutations in D4.

**Figure 16C** demonstrates that point mutations in D4 that prevent PDGFR activation do not interfere with PDGF-induced PDGFR dimerization. PDGFR-/- MEFs expression WT or the E390A mutant were serum starved overnight, followed by incubation with the indicated amount of PDGF in DMEM/50mM Hepes buffer (pH7.4) at 4°C for 90 minutes. After removing unbound ligand, cells were incubated with 0.5mM disuccinimidyl suberate (DSS) in PBS for 30 minutes. Lysates of unstimulated or stimulated cells were subjected to immunoprecipitation with anti-PDGFR antibodies followed by SDS-PAGE analysis and by immunoblotting with anti-flag antibodies (left panel) or anti-pTyr antibodies (right panel).

**Figure 17** shows cavities in the D3-D4 hinge region. Several cavities are scattered on the D3-D4 interface in the ectodomain monomer structure. The amino acids involved in defining the cavities are summarized in Table 4 (below). Upon formation of homotypic interaction between two Kit receptors, the D3-D4 hinge region is altered resulting in formation of a shallow cavity created by the following residues: K218, S220, Y221, L222 from D3 and F340, P341, K342, N367, E368, S369, N370, I371, Y373 from D4. Figure 17 shows a ribbon diagram of the D3-D4 hinge region of unoccupied monomers (A) and SCF-bound dimers (B) and a mesh representation of the D3-D4 pocket.

**Figure 18** shows cavities in the D4-D5 hinge region. A small cavity is formed by the AB loop and the EF loop of D4, the D4-D5 connecting linker and part of DE loop and FG loop of the D5 of Kit monomer. Residues defining the cavities are summarized in Table 4 (below). The shape and size of the cavities are changed in the Kit ectodomain dimeric structure. The major cavities formed by the EF loop and strand G of D4, the D4-D5 linker and strand B and DE loop of D5 are located beneath the EF loop of D4; a region critical for formation of the D4 homotypic interface. Note that the DE loop of D5 that is located close to the cavities may have higher flexibility as revealed by the lower quality of electron densities from both unbound and occupied Kit structures. Figure 18 shows a ribbon diagram of unoccupied monomers (A) and SCF-dimers (B) and a mesh representation of a shallow cavity around the D4-D5 hinge region.

**Figure 19** shows a cavity at the region mediating D4 homotypic interactions. A concave surface formed by the CD loop and EF loop of Kit D4 is located right above the D4 homotypic interface. Residues, Y350, R353, F355, K358, L379, T380, R381, L382, E386 and T390 from D4 provide a surface area of approximately 130 $A^2$ for the concave surface in the ectodomain dimeric structure. The side chain of Glu386 that plays an important role in the D4 homotypic interface projects toward the center of the surface. A characteristic feature of the concave surface is a small hydrophobic patch surrounded by charged residues (Glu386 and Lys358). The size and accessibility of the surface is altered upon homotypic D4:D4 interactions with changes taking place in the conformation of the CD loop that becomes folded upwards to the top of the domain. Residues involved in the formation of a concave surface are summarized in Table 4 (below). Panel A in the figure below shows a ribbon diagram of the unoccupied D4 domain of Kit (gold) overlaid onto the ligand-occupied Kit D4 (not shown) with different conformations of the CD and EF loops between ligand-occupied (green) and unoccupied ectodomain structures (red). The critical residues for the D4:D4 interactions are shown in a stick model format. Panels B and C show ribbon diagrams of unoccupied Kit (Figure 19B) and SCF-occupied Kit structures (Figure 19C) and a mesh presentation of shallow cavity above D4 homotypic interface.

**Figure 20** shows a concave surface at the ligand-binding D2 and D3 regions. A shallow concave surface is located on part of the ligand-binding surface of D2 and D3. Residues involved in the small pocket are Y125, G126, H180, R181, K203, V204, R205, P206 and F208 from D2 and V238, S239, S240, S241, H263, G265, D266, F267, N268 and Y269 from D3. The pocket is created by a small hydrophobic patch surrounded by hydrophilic residues. There is no major alteration between unoccupied and SCF-occupied Kit structures with an overall buried surface area of approximately 500 $A^2$. Figures A and B show ribbon diagrams of unoccupied Kit (A) and SCF-bound Kit (B) and a mesh presentation of the D2-D3 pocket.

**Figure 21** depicts a structure-based sequence analysis and homology modeling of membrane proximal region of PDGF receptors. Figure 21A depicts an alignment of amino acid sequences (SEQ ID NOS 148-157, respectively, in order of appearance) of D4 of PDGFRα, PDGFRβ, and Kit. The amino acids of key residues of the IgSF fold and the core residues of the Ig-fold of D4 of human Kit structure are colored in red and green, correspondingly. The two key basic and acidic residues responsible for D4

homotypic interaction are boxed in blue and red, respectively. Positions corresponding to the conserved disulfide bond-forming cysteine residues on the Ig-like domain (B5 and F5) are marked by asterisks. β-strands are labeled by arrows below the Kit sequence. Secondary structure elements are marked according to the IgSF nomenclature. Figure 21B depicts a model of the membrane proximal region of extracellular domain of PDGFR. The membrane proximal region of PDGFRβ ectodomain is colored in white and shown as ribbons with a transparent molecular surface (D4 colored in orange, and D5 colored in pink; left panel). A closer view (right panel) of the D4-D4 interface of two neighboring PDGFRβ molecules demonstrates that interactions between D4 are mediated by residues Arg385 and Glu390 projected from two adjacent EF loop. Key amino acids are labeled and shown as a stick model.

**Figure 22** depicts the results of experiments demonstrating that PDGF-induced PDGFR activation is compromised by mutations in D4. Figure 22A shows the results of an experiment demonstrating that the PDGF-induced tyrosine autophosphorylation of PDGFRβ is strongly compromised in cells expressing the E390A, R385A, RE/AA, and RKE/AAA mutants of PDGFRβ. Figure 22B is a graph showing the displacement curves of wild type and mutant PDGFRβs. The IC50 values were determined by curve fitting with Prism4. Figure 22C depicts the results from an immunoblot demonstrating that the R385A, E390A or RE/AA mutations do not influence the intrinsic tyrosine kinase activity of PDGFR.

**Figure 23** depicts the results from an immunoprecipitation experiment demonstrating that PDGF-stimulated PDGFRβ mutated in the D4 domain are expressed on the cell surface in the form of inactive dimers. Cell lysates were immunoprecipitated with anti-PDGFR antibodies and immunopelletes were analyzed by SDS-PAGE and immunoblotted with anti-flag antibodies (left panel) and antiphosphotyrosine antibodies (right panel) respectively.

**Figure 24** depicts the results from an immunoprecipitation experiment demonstrating that PDGF-induced cellular responses are compromised by mutations in the PDGFRβ D4 mutant.

**Figure 25** depicts the results from an experiment demonstrating that PDGF stimulation of actin ring formation is compromised in MEFs expressing PDGFR D4 mutants. While approximately 83% of MEFs expressing WT PDGFR exhibited circular actin ring formation, only 5% of PDGFR D4 mutant cells showed similar circular actin

ring formation after 2 minutes stimulation with 50ng/ml of PDGF. Furthermore, the transient circular actin ring formation that peaks in MEFs expressing WT PDGFR after 2-5 minutes of PDGF stimulation was weakly detected in cells expressing the R385A, E390A or the RE/AA PDGFR mutants.

**Figure 26** depicts the results of experiments demonstrating that PDGFR internalization and ubiquitin-mediated PDGFR degradation are compromised by mutations in D4 of PDGFR. Figure 26A is a graph demonstrating that the kinetics of internalization of $^{125}$I labeled PDGF bound to MEFs expressing WT PDGFR is much faster than the kinetics of internalization of $^{125}$I labeled PDGF bound to cells expressing the E390A, R385A or the RE/AA PDGFR. Figure 26B shows that the kinetics of degradation of R385A, E390A or the RE/AA PDGFR mutants was strongly attenuated; and while half of WT PDGFRs were degraded within 1.5 hour of PDGF stimulation, the half-life for PDGFR D4 mutants was extended to approximately 4 to 6 hours. Figure 26C depicts an experiment showing that PDGF induced stimulation of ubiquitination of the E390A PDGFR was also strongly reduced as compared to WT PDGFR under similar conditions.

**Figure 27** depicts the results of experiments demonstrating that disruption of the D4 interface blocks oncogenic mutations in KIT. SCF stimulation of wild type KIT leads to enhancement of KIT activation revealed by enhanced tyrosine autophosphorylation of KIT. The experiment further shows that an oncogenic D5-Repeat mutant of KIT is constitutively tyrosine autophosphorylated. By contrast, the D5-Repeat/E386A mutant blocks constitutive tyrosine autophosphorylation of KIT mediated by the oncogenic D5-repeat mutation.

**Figure 28** depicts the results of an immunoblot experiment demonstrating that antibodies directed against a peptide corresponding to the homotypic interaction motif of KIT-D4, recognize the full length KIT receptor.

**Figure 29A** depicts a structure-based multiple sequence alignment of a predicted EF-loop region of D7 of VEGFR1 and VEGFR2 from different species. Key amino acids in the I-set Ig frame are highlighted in green, and the conserved Arg/Asp pair in the EF loop is highlighted in red. **Figure 29B** depicts a comparison of a predicted EF-loop region of D4 from VEGFR and D4 of KIT, CSF1R and PDGFRs (type-III RTK). Key amino acids in the I-set Ig frame are highlighted in green, and the conserved Arg/Asp or Glu pair in the EF loop is highlighted in red. Non conserved amino acids

with opposite charge in the EF-loop are highlighted in blue. The conserved Y-conner motif is marked with *.

**Figure 30** demonstrates that ligand induced activation of VEGFR2 is compromised by mutations in the EF loop region of D7 but not affected by a mutation in the EF loop region of D4. Figure 30A demonstrates that HEK293 cells transiently expressing wild-type VEGFR2, the R726A or E731A VEGFR2 mutants were stimulated with indicated amount of VEGF for 5 minutes at 37° C. Lysates from unstimulated or VEGF stimulated cells were subjected to immunoprecipitation with anti-VEGFR2 antibodies followed by immunoblotting (IB) with anti-pTyr, or with anti-VEGFR2 antibodies. Total cell lysate from the same experiment was analyzed by SDS-PAGE followed by immunoblotting with anti-phosphoMAPK (pMAPK) or anti-MAPK antibodies. Figure 30B demonstrates that serum starved 3T3 cells stably expressing WT VEGFR2-PDGFR chimeric receptor or chimeric receptors harboring mutations in D7 region (R726A, D731A or R726/D731 double mutants RD/2A) were stimulated with VEGF for 5 minutes at 37° C. Lysates from unstimulated or VEGF stimulated cells were subjected to immunoprecipitation with antibodies against the cytoplasmic region of the chimeric receptor followed by immunoblotting with either anti-pTyr or anti-tag (FLAG) antibodies, respectively. Figure 30C demonstrates that serum starved 3T3 cells stably expressing WT VEGFR1-PDGFR chimeric receptor or chimeric receptors harboring mutation in the D7 region (R721A, D725A or R721D725/2A double mutations) were stimulated with VEGF for 5 minutes at 37° C. Lysates from unstimulated or VEGF stimulated cells were subjected to immunoprecipitation with antibodies directed against the cytoplasmic region of the chimeric receptor followed by immunoblotting with either anti-pTyr or anti-tag (FLAG) antibodies, respectively. Figure 30D demonstrates that 3T3 cells expressing WT VEGFR2-PDGFR chimeric receptor or chimeric receptors harboring mutations in D4 region (D392A or D387/R391A double mutations) were analyzed as described in Figure 30A.

**Figure 31** depicts the structure of the VEGFR2 ectodomain D7 dimer. Figure 31A depicts a ribbon diagram and a transparent molecular surface of D7 homodimer structure (side view). Asp731 and Arg726 are shown as a stick model. Figure 31B depits a close view of the homotypic D7 interface of the two neighboring molecules (pink and green). Salt bridges formed by Asp731 and Arg726 are shown as dashed lines. Figure 31C depicts the charge distribution of D7 homodimer (side view) as a surface

potential model (Left panel). View of D7 surface that mediates homotypic contacts (Right panel). Figure 31D depicts a 2Fo-Fc electron density map contoured at 1.1σ level, showing a view of the D7-D7 interface. The backbones of VEGFR D7 protomers are represented as pink and yellow tubes, respectively.

**Figure 32** depicts the superposition of the structure of D7 of VEGFR2 with the structure of D4 of the dimeric KIT-SCF complex. Overlay of VEGFR D7 structure (PDB ID code: 3KVQ) and KIT dimer in complex with SCF (PDB ID code: 2E9W) (**left panel**). A closer view of superimposed D7 and D4 regions reveal high similarity in domain arrangement and homotypic contacts (**right panel**). VEGFR D7 is illustrated in green and the EF loop is in yellow. D4 of KIT is illustrated in grey and its EF loop is in orange.

**Figure 33** depicts a phylogenetic analysis of VEGFR1 and VEGFR2. Figure 33A depicts the location of the conserved EF-loop in Type-III and Type-V RTKs from various species. Ig-like domains containing a conserved EF-loop motif are marked in blue. Figure 33B depicts the color-coded conservation pattern of VEGFR2 D7 region. Amino acid sequences of human VEGFR were used as query to search non-redundant database (nr) for homologous sequences, using PSI-BLAST (Altschul *et al., J. Mol. Boiol.,* 215(3):403-410 (1990)). Sequence alignment of D7 was performed using ClustalW2 (Thompson *et al., Nucleic Acids Res.,* 22(22):4673-4680 (1994)), manually adjusted based on the IgSF fold restrains for 20 key residues. The alignment of amino acid sequences was submitted to the Consurf 3.0 server (Landau *et al., Nucleic Acids Res.,* 33 (Web Server issue):W299-302 (2005)) to generate maximum-likelihood normalized evolutionary rates for each position. Cyan through maroon is used for labeling from variable to conserved amino acids. Figure 33C depicts the phylogenetic tree of VEGFR1 and VEGFR2 are generated by the neighboring-joining method based using Clustal W2. Amino acid sequences used in the analysis include: VEGFR2_HUMAN (gi:11321597), VEGFR2_DOG (gi:114158632), VEGFR2_HORSE (gi:194209154), VEGFR2_CATTLE (gi:158508551), VEGFR2_RAT (gi:56269800), VEGFR2_MOUSE (gi:27777648), VEGFR2_CHICK (gi:52138639), VEGFR2_QUAIL (gi:1718188), VEGFR2_ZEBRARISH (gi:46401444), VEGFR1_HUMAN (gi:143811474), VEGFR1_MOUSE (gi:148673892), VEFGR1_RAT (gi:149034835), VEFGR1_HORSE (gi:149730119), VEGFR1_CHICK (gi:82105132), VEGFR1_ZEBRAFISH (gi:72535148), VEGFR_SEAURCHIN (gi:144226988),

VER1_C_ELEGANS (gi:6003694), VER3_C_ELEGANS (gi:3877967), VER4_C_ELEGANS (gi:3877968), PVR_DROSOPHILA (gi:45552252), VEGFR_SEASQUIRT (gi:198434052).

## Detailed Description Of The Invention

The present invention provides moieties, *e.g.*, antibodies or antigen binding portions thereof, small molecules, peptidic molecules, aptamers, and adnectins, that bind to the ectodomain, *e.g.*, an Ig-like domain or a hinge between Ig-like domains, of a human receptor tyrosine kinase, *e.g.,* a VEGF receptor, such as the human VEGFR1 (*Flt1*), VEGFR2 (KDR/*Flk1*) and VEGFR3 (*Flt4*). The moieties of the present invention can lock the ectodomain of the VEGF receptor in an inactive state thereby inhibiting the activity of the VEGF receptor. In one embodiment of the invention, the moiety locks the ectodomain of the VEGF receptor to a monomeric state. In another embodiment of the invention, the moiety allows the ectodomain of the VEGF receptor to dimerize but affects the positioning, orientation and/or distance between the Ig-like domains of the two monomers (*e.g.*, the D7-D7 domains of a VEGF receptor), thereby inhibiting the activity of the VEGF receptor. In other words, the moiety may allow ligand induced dimerization of the VEGF receptor ectodomains, but affect the positioning of the two ectodomains at the cell surface interface or alter or prevent conformational changes in the VEGF receptors, thereby inhibiting the activity of the VEGF receptors (*e.g.*, inhibiting receptor internalization and/or inhibiting tyrosine autophosphorylation of the receptor and/or inhibiting the ability of the receptor to activate a downstream signaling pathway). The present invention is based, at least in part, on the deciphering of the crystal structures of the entire ectodomain of the VEGF receptor VEGFR2. The deciphering of this crystal structure has allowed for the identification of epitopes, *e.g.*, conformational epitopes, which the moieties of the invention may target.

As used herein, the term "moiety" is intended to include any moiety binds to the ectodomain, *e.g.*, an Ig-like domain of a receptor tyrosine kinase, where the moiety locks the ectodomain of the receptor tyrosine kinase in an inactive state, *e.g.*, a monomeric state, thereby antagonizing the activity of the receptor tyrosine kinase. The moiety can be an isolated antibody, or antigen binding portion thereof; a small molecule; a peptidic molecule (*e.g.*, a peptidic molecule designed based on the structure of an Ig-like domain

of a receptor tyrosine kinase); an aptamer or an adnectin. In some aspects, the moiety binds to the hinge regions connecting Ig-like domains of the receptor tyrosine kinase (*e.g.*, the D3-D4 or the D4-D5 hinge regions of Type III RTKs).

In some embodiments, the moiety will bind to specific sequences of the human VEGF receptor, for example, residues 718-727 of VEGFR1, Arg720 and Asp725 of VEGFR1, residues 724-733 of VEGFR2, Arg726 and Asp731 of VEGFR2, residues 735-744 of VEGFR3, or residues Arg737 and Asp742 of VEGFR3. The moiety will alternatively bind to specific sequences of the human Kit receptor, for example, residues 309-413, residues 410-519, $^{381}$Arg and $^{386}$Glu, or $^{418}$Tyr and $^{505}$Asn of the human Kit. Residues 309-413 comprise the D4 domain and residues 410-519 comprise the D5 domain of the human Kit and are shown herein to be critical to Kit receptor dimerization. Residues $^{381}$Arg and $^{386}$Glu are residues in the D4 domain of Kit which are shown herein to be important for the non-covalent association of the D4 domain and, hence, the dimerization of the receptor. Similarily, residues $^{418}$Tyr and $^{505}$Asn are residues in the D5 domain of Kit which are shown herein to be important for dimerization of the receptor. One of skill in the art will appreciate that a moiety which specifically binds to the aforementioned residues can antagonize the activity of the receptor by, for example, preventing dimerization of the two monomeric Kit or VEGF receptor molecules.

In additional embodiments, the moiety binds to a mutated amino acid residue in the human VEGF receptor wherein the amino acid residure is at least one of Arg720 or Asp 725 of VEGFR1, Arg726 or Asp731 of VEGFR2, or Arg737 or Asp742 of VEGFR3. In additional embodiments, the moiety binds to a mutated amino acid residue in the human Kit wherein the amino acid residure is at least one of $^{417}$Thr, $^{418}$Tyr, $^{419}$Asp, $^{421}$Leu, $^{420}$Arg, $^{503}$Tyr, or $^{502}$Ala.

In a preferred embodiment, moieties of the invention bind to one or more residues in the Kit receptor which make up the small cavities or pockets described in Table 4 (below). For example, moieties of the invention may bind to one or more of the following residues in the D3-D4 hinge region of the Kit receptor: K218, S220, Y221, L222 from the D3 domain and F340, P341, K342, N367, E368, S369, N370, I371, Y373 from the D4 domain. The moieties of the invention may also bind to one or more of the following residues which make up a concave surface in the D4 domain of the Kit receptor: Y350, R353, F355, K358, L379, T380, R381, L382, E386 and T390. In

another embodiment, moieties of the invention bind to one or more of the following residues which form a pocket in the D2-D3 hinge region of the Kit receptor: Y125, G126, H180, R181, K203, V204, R205, P206 and F208 from the D2 domain and V238, S239, S240, S241, H263, G265, D266, F267, N268 and Y269 from the D3 domain.

Thus, in some embodiments, a moiety of the invention may bind to contiguous or non-contiguous amino acid residues and function as a molecular wedge that prevents the motion required for positioning of the membrane proximal region of the RTK at a distance and orientation that enables tyrosine kinase activation. The moieties of the invention may also act to prevent homotypic or heterotypic D4 or D5 receptor interactions or destabilize the ligand- receptor interaction site. In some preferred embodiments, moieties of the invention bind to one or more of the following residues on the Kit receptor: Y125, G126, H180, R181, K203, V204, R205, P206, P206, F208, K127, A207, V238, S239, S240, S241, H263, G265, D266, F267, N268, Y269, T295, L222, L222, L223, E306, V308, R224, V308, K310, K218, A219, S220, K218, A220, Y221, A339, D327, D398, E338, E368, E386, F312, F324, F340, F355, G311, G384, G387, G388, I371, K342, K358, L382, L379, N326, N367, N370, N410, P341, S369, T385, V325, V407, V409, Y373, Y350, Y408, T380, T390, R381, R353, T411, K412, E414, K471, F433, G470, L472, V497, F469, A431, or G432. One of skill in the art will appreciate that, in some embodiments, moieties of the invention may be easily targeted to the corresponding residues in other type III RTKs, *e.g.*, those residues that form similar pockets or cavities or those in the same position by structural alignment or sequence alignment.

In a specific embodiment, a moiety of the invention binds to a conformational epitope or a discontinuous epitope on a type III RTK. The conformational or discontinuous epitope may be composed of two or more residues from the D3, D4, and/or D5 domain or the D4-D5 or D3-D4 hinge regions from a type III RTK, *e.g.*, the human Kit receptor or the PDGF receptor. For example, the conformational or discontinuous epitope may be composed of two or more of the residues listed in Table 4.

In a particular embodiment, a moiety of the invention binds to a conformational epitope composed of 2 or more amino acids selected from the group consisting of Y125, H180, R181, K203, V204, R205, P206, V238, S239, S240, H263, G265, D266, F267, N268, and Y269. In similar embodiments, a moiety of the invention may bind to a conformational epitope composed of 2 or more amino acids selected from one of the

following groups of amino acids: P206, F208, V238, and S239; K127, A207, F208, and T295; L222, A339, F340, K342, E368, S369, N370, I371, and Y373; L222, L223, E306, V308, F312, E338, F340, and I371; R224, V308, K310, G311, F340, P341, and D398; K218, A219, S220, N367, E368, and S369; K218, A220, E368, and S369; G384, T385, T411, K412, E414, and K471; Y408, F433, G470, K471, and L472; F324, V325, N326, and N410;D327, N410, T411, K412, and V497; G384, G387, V409, and K471; L382, G387, V407, and V409; Y125, G126, H180, R181, K203, V204, R205, P206, F208, V238, S239, S240, S241, H263, G265, D266, F267, N268, and Y269; P206, F208, V238, and S239; K218, S220, Y221, L222, F340, P341, K342, N367, E368, S369, N370, I371, and Y373; G384, G387, G388, Y408, V409, T411, F433, F469, G470, and K471; D327, T411, K412, E414, A431, G432, and K471; Y350, F355, K358, L379, T380, R381, L382, E386, and T390; Y350, R353, and F355. As indicated above, the moieties of the invention may bind to all of the amino acid residues forming a pocket or a cavity identified in Table 4 or they may bind to a subset of the residues forming the pocket or the cavity. It is to be understood that, in certain embodiments, when reference is made to a moiety of the invention binding to an epitope, *e.g.*, a conformational epitope, the intention is for the moiety to bind only to those specific residues that make up the epitope (*e.g.*, the pocket or cavity identified in Table 4) and not other residues in the linear amino acid sequence of the receptor.

In a further embodiment, a moiety of the invention binds to a conformational epitope wherein said epitope is composed of two or more amino acid residues selected from the peptides listed in Table 5. In a specific embodiment, the conformational epitope is composed of one or more amino acid residues selected from a first peptide and one or more amino acid residues selected from a second peptide, wherein the first and second peptides are selected from the group of peptides listed in Table 5. As such, a moiety of the invention may bind a conformational epitope wherein the said first and second peptide groups from Table 5 are as follows: Ala219-Leu222 and Thr304-Val308; Asp309-Gly311 and Arg224-Gly226; Thr303 - Glu306 and Ala219-Leu222; Asn367–Asn370 and Ser217–Tyr221; Ala339–Pro343 and Asn396–Val399; Ala339–Pro343 and Glu368-Arg372; Lys358–Tyr362 and Val374–His378; Asp357-Glu360 and Leu377–Thr380; Met351–Glu360 and His378-Thr389; His378-Thr389 and Val323-Asp332; Val409–Ile415 and Ala493–Thr500; Val409–Ile415 and Ala431-Thr437; Val409– Ile415 and Phe469-Val473; Val409–Ile415 and Val325–Asn330; Val409–Ile415 and Arg381-

Gly387; Gly466–Leu472 and Gly384–Gly388; Val325-Glu329 and Tyr494-Lys499; Thr411-leu416 and Val497-Ala502; Ile415–Leu421 and Ala502–Ala507; Ala502–Ala507 and Lys484-Thr488; and Ala502–Ala507 and Gly445–Cys450. The moieties of the invention may bind to all of the amino acid residues forming the foregoing first and second peptide groups or they may bind to a subset of the residues forming the first and second peptide groups. It is to be understood that, in certain embodiments, when reference is made to a moiety of the invention binding to an epitope, *e.g.*, a conformational epitope, the intention is for the moiety to bind only to those specific residues that make up the epitope (*e.g.*, the specific peptides identified in Table 5) and not other residues in the linear amino acid sequence of the receptor.

In another embodiment, a moiety of the invention binds to a conformational or discontinuous epitope composed of 2 or more amino acids selected from the group consisting of E33, P34, D72, E76, N77, K78, Q79, K158, D159, N250, S251, Q252, T253, K254, L255, N260, W262, H264, G265, E344, N352, R353, F355, T356, D357, Y362, S365, E366, N367, N370, and G466.

In another embodiment, a moiety of the invention binds to a contiguous epitope on the VEGF receptor. In one embodiment, the contiguous epitope is composed of two or more residues in the D7 domain of the VEGF receptor. In another embodiment, the contiguous epitope is an epitope selected from the group consisting of $^{672}$VAISSS$^{677}$ of VEGFR1, $^{678}$TTLDCHA$^{684}$ of VEGFR1, $^{685}$NGVPEPQ$^{691}$ of VEGFR1, $^{700}$KIQQEPG$^{706}$ of VEGFR1, $^{707}$IILG$^{710}$ of VEGFR1, $^{711}$PGS$^{713}$ of VEGFR1, $^{714}$STLFI$^{718}$ of VEGFR1, $^{719}$ERVTEEDEGV$^{728}$ of VEGFR1, $^{689}$VNVSDS$^{694}$ of VEGFR3, $^{695}$LEMQCLV$^{701}$ of VEGFR3, $^{702}$AGAHAPS$^{708}$ of VEGFR3, $^{717}$LLEEKSG$^{723}$ of VEGFR3, $^{724}$VDLA$^{727}$ of VEGFR3, $^{728}$DSN$^{730}$ of VEGFR3, $^{731}$QKLSI$^{735}$ of VEGFR3, and $^{736}$QRVREEDAGR$^{745}$ of VEGFR3, $^{678}$TSIGES$^{683}$ of VEGFR2, $^{684}$IEVSCTA$^{690}$ of VEGFR2, $^{691}$SGNPPPQ$^{697}$ of VEGFR2, $^{706}$TLVEDSG$^{712}$ of VEGFR2, $^{713}$IVLK$^{716}$ of VEGFR2, $^{717}$DGN$^{719}$ of VEGFR2, $^{720}$RNLTI$^{724}$ of VEGFR2 and $^{725}$RRVRKEDEGL$^{734}$ of VEGFR2.

In another embodiment, a moiety of the invention binds to amino acid residues $^{385}$Arg and $^{390}$Glu of human PDGFRβ, or the corresponding residues in PDGFRα. The residues $^{385}$Arg and $^{390}$Glu of human PDGFRβ are analogous to the residues $^{381}$Arg and $^{386}$Glu of the Kit receptor and mediate homotypic D4-D4 interactions of PDGFRβ. Moieties of the invention may exert their inhibitory effect on receptor activation by preventing critical homotypic interactions (such as salt bridges formed between $^{385}$Arg

and $^{390}$Glu of human PDGFRβ) between membrane proximal regions of type-III RTKs that are essential for positioning the cytoplasmic domain at a distance and orientation essential for tyrosine kinase activation. Experiments discussed herein demonstrate that homotypic D4-D4 interactions are dispensable for PDGFRβ dimerization and that PDGFRβ dimerization is necessary but not sufficient for receptor activation. Thus, moieties of the invention may allow dimerization of PDGFRβ while preventing activation. Structure based sequence alignment has shown that the size of the EF loop, and the critical amino acids comprising the D4-D4 interface are conserved in Kit, PDGFRα, PDGFRβ, and CSF1R. Thus, in some embodiments, moieties of the invention may be targeted to the conserved regions of the D4 or D5 domains of type III RTKs. It will also be appreciated by one of skill in the art that a moiety of the invention may bind to sugar residues which may appear on a glycosylated form of an RTK. It is further possible that a moiety of the invention will bind an epitope that is composed of both amino acid residues and sugar residues.

The terms "receptor tyrosine kinase" and "RTK" are used interchangeably herein to refer to the well known family of membrane receptors that phosphorylate tyrosine residues. Many play significant roles in development or cell division. Receptor tyrosine kinases possess an extracellular ligand binding domain, a transmembrane domain and an intracellular catalytic domain. The extracellular domains bind cytokines, growth factors or other ligands and are generally comprised of one or more identifiable structural motifs, including cysteine-rich regions, fibronectin III-like domains, immunoglobulin-like domains, EGF-like domains, cadherin-like domains, kringle-like domains, Factor VIII-like domains, glycine-rich regions, leucine-rich regions, acidic regions and discoidin-like domains. Activation of the intracellular kinase domain is achieved by ligand binding to the extracellular domain, which induces dimerization of the receptors. A receptor activated in this way is able to autophosphorylate tyrosine residues outside the catalytic domain, facilitating stabilization of the active receptor conformation. The phosphorylated residues also serve as binding sites for proteins which will then transduce signals within the cell. Examples of RTKs include, but are not limited to, Kit receptor (also known as Stem Cell Factor receptor or SCF receptor), fibroblast growth factor (FGF) receptors, hepatocyte growth factor (HGF) receptors, insulin receptor, insulin-like growth factor-1 (IGF-1) receptor, nerve growth factor (NGF) receptor, vascular endothelial growth factor (VEGF) receptors, PDGF-receptor-α, PDGF-receptor-

β, CSF-1-receptor (also known as M-CSF-receptor or Fms), and the Flt3-receptor (also known as Flk2).

In a preferred embodiment of the invention, the RTK is a type III RTK. In another embodiment of the invention, the RTK is a type V RTK, *i.e.*, a member of the VEGF receptor family.

As used herein the term "type III family of receptor tyrosine kinases" or "type III RTKs" is intended to include receptor tyrosine kinases which typically contain five immunoglobulin like domains, or Ig-like domains, in their ectodomains. Examples of type III RTKs include, but are not limited to PDGF receptors, the M-CSF receptor, the FGF receptor, the Flt3-receptor (also known as Flk2) and the Kit receptor. In a preferred embodiment of the invention, the type III RTK is Kit (also known in the art as the SCF receptor). Kit, like other type III RTKs is composed of a glycosylated extracellular ligand binding domain (ectodomain) that is connected to a cytoplasmic region by means of a single transmembrane (TM) domain (reviewed in Schlessinger (2000) Cell 103: 211-225). Another hallmark of the type III RTKs, *e.g.*, Kit or PDGFR, is a cytoplasmic protein tyrosine kinase (PTK) domain with a large kinase-insert region. At least two splice isoforms of the Kit receptor are known to exist, the shorter making use of an in-frame splice site. All isoforms of Kit, and the other above described RTKs, are encompassed by the present invention.

As used herein, an "Ig-like domain" of a receptor tyrosine kinase (RTK) is intended to include the domains well known in the art to be present in the ectodomain of RTKs. In the ectodomain of the family of type III receptor tyrosine kinases (type III RTKs), *e.g.*, Kit, there are five such domains, known as D1, D2, D3, D4 and D5. The D1, D2 and D3 domains of type III RTKs are responsible for binding the ligand of the RTK (reviewed in Ullrich and Schlessinger (1990) Cell 61: 203-212). Thus, in one embodiment of the invention the term "Ig-like domain" is not intended to include a domain of a RTK which is responsible for ligand binding. In a preferred embodiment of the invention, the Ig-like domain is a D4 and/or a D5 domain of a type III RTK. In the ectodomain of the VEGF receptor family, there are seven Ig-like domains, known as D1, D2, D3, D4, D5, D6 and D7. In one preferred embodiment of the invention, the Ig-like domain is a D7 domain of the VEGF receptor family.

As used herein the term "vascular endothelial growth factor receptor", "VEGF receptor", or "VEGF receptor family", also known as type V RTKs includes RTK

receptors for the vascular endothelial growth factor. As described above, these RTKs have 7 Ig-like domains in their ectodomains. Examples of VEGF family receptors are VEGFR1 (also known as Flt-1), VEGFR2 (also known as KDR or Flk-1), and VEGFR3 (also known as Flt-4).

The term "ectodomain" of a receptor tyrosine kinase (RTK) is well known in the art and refers to the extracellular part of the RTK, *i.e.*, the part of the RTK that is outside of the plasma membrane.

The term "a membrane proximal region" of the ectodomain of a receptor tyrosine kinase refers to an extracellular part of a RTK which is in proximity to the plasma membrane and which, preferably, is not directly responsible for the binding of a ligand to the RTK. Examples of membrane proximal regions include, but are not limited to, the D4 domain of a type III receptor tyrosine kinase, the D5 domain of a type III receptor tyrosine kinase, the D3-D4 hinge region of a type III receptor tyrosine kinase, the D4-D5 hinge region of a type III receptor tyrosine kinase, and the D7 domain of a type V receptor tyrosine kinase.

The term "homotypic interaction" as used herein, refers to the interaction between two identical membrane proximal regions from two monomeric receptors.

The term "heterotypic interaction" as used herein, refers to the interaction between two different membrane proximal regions from two monomeric receptors. A heterotypic interaction may be the result of dimerization of two different types of monomeric receptors or the result of dimerization of a wild type and a mutant form of the same monomeric receptor. For example, it is well known in the art that a cancer patient may carry a wild type allele and a mutant allele for a certain receptor.

The term "monomeric state" as used herein, refers to the state of a RTK wherein the RTK molecule is composed of a single polypeptide chain which is not associated with a second RTK polypeptide of the same or different type. RTK dimerization leads to autophosphorylation and receptor activation. Thus, a RTK in a monomeric state is in an inactive state. A monomeric state is also a state wherein the D4, D5, or D7 domain of a single RTK is not associated with the D4, D5, or D7 domain, respectively, of a second, RTK.

As used herein, a "protomer" is a structural unit of an oligomeric protein, such as an RTK. A protomer is a protein subunit which may assemble in a defined stoichiometry to form an oligomer. The VEGFR family of receptor tyrosine kinases are covalently

linked homodimers, and each VEGFR protomer is composed of four stranded β-sheets arranged in an anti-parallel fashion in a structure designated "cysteine-knot growth factors".

The phrase "locks the ectodomain of the receptor tyrosine kinase in an inactive state" refers to the ability of a moiety of the invention to inhibit the activity of the receptor tyrosine kinase. In other words, this phrase includes the ability of a moiety of the invention to shift the equilibrium towards formation of an inactive or inhibited receptor configuration. For example, a moiety of the invention may inhibit the activity of a receptor tyrosine kinase by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% as compared to the activity of the receptor in the absence of the moiety.

The term "inactive state," as used herein, refers to the state of a RTK wherein the RTK molecule is unable to activate downstream signaling. An inactive state may be a state wherein the ectodomain of the receptor tyrosine kinase is allowed to dimerize but the positioning, orientation, conformation, and/or distance between the Ig-like domains of the two monomers (*e.g.*, the D4-D4 or D5-D5 domains of a type III receptor tyrosine kinase or the D7-D7 domains of a type V receptor tyrosine kinase), is altered such that the activity of the receptor tyrosine kinase is inhibited (*e.g.*, receptor internalization is inhibited and/or tyrosine autophosphorylation of the receptor is inhibited and/or the ability of the receptor to activate a downstream signaling pathway is inhibited). An inactive state also includes a monomeric state as described above. An inactive state may also be a state in which the ectodomain of the receptor tyrosine kinase is bound to a receptor ligand and is dimerized, but has not yet undergone the conformational change that allows for the activation of the receptor. Examples 22-25 further discuss experiments which show that there are specific conserved amino acid residues which are crucial for RTK activation (e.g., by mediating D4 or D5 homotypic interactions) but which are dispensable for receptor dimerization. The term "inactive state" includes a state in which a moiety of the invention may reduce or inhibit the activity of a receptor tyrosine kinase by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% as compared to the activity of the receptor in the absence of the moiety. Any of the functional assays described herein may be used to determine the ability of a moiety of the invention to inhibit the activity of a receptor tyrosine kinase. In some embodiments, a moiety of the invention may exhibit a

broad effect, *e.g.,* when most or all target RTKs are inactivated. In other embodiments, a moiety of the invention may exhibit a narrower effect, *e.g.*, when a portion of the target RTKs are inactivated. In such embodiments, at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% of the receptors are locked into an inactive state as compared to the receptors in the absence of said moiety.

As used herein, the terms "conformational epitope" or "non-linear epitope" or "discontinuous epitope" are used interchangeably to refer to an epitope which is composed of at least two amino acids which are are not consecutive amino acids in a single protein chain. For example, a conformational epitope may be comprised of two or more amino acids which are separated by a strech of intervening amino acids but which are close enough to be recognized by a moiety of the invention as a single epitope. As a further example, amino acids which are separated by intervening amino acids on a single protein chain, or amino acids which exist on separate protein chains, may be brought into proximity due to the conformational shape of a protein structure or complex to become a conformational epitope which may be bound by a moiety of the invention. Particular discontinuous and conformation epitopes are described herein (see, for example, Tables 4 and 5).

It will be appreciated by one of skill in the art that, in general, a linear epitope bound by a moiety of the invention may or may not be dependent on the secondary, tertiary, or quaternary structure of the RTK. For example, in some embodiments, a moiety of the invention may bind to a group of amino acids regardless of whether they are folded in a natural three dimensional protein structure. In other embodiments, a moiety of the invention may not recognize the individual amino acid residues making up the epitope, and may require a particular conformation (bend, twist, turn or fold) in order to recognize and bind the epitope.

As used herein, the terms "contiguous epitope" or "continuous epitope" are used interchangeably to refer to an epitope which is composed of at least two amino acids which are are consecutive amino acids in a single protein chain. Particular contiguous epitopes are described herein (see, for example, Table 8). In one embodiment, the moiety of the invention binds to a contiguous epitope on the VEGF receptor. In another embodiment, the contiguous epitope is composed of two or more residues in the D7 domain of the VEGF receptor. In another embodiment, the contiguous epitope is an

epitope selected from the group consisting of $^{672}$VAISSS$^{677}$ of VEGFR1, $^{678}$TTLDCHA$^{684}$ of VEGFR1, $^{685}$NGVPEPQ$^{691}$ of VEGFR1, $^{700}$KIQQEPG$^{706}$ of VEGFR1, $^{707}$IILG$^{710}$ of VEGFR1, $^{711}$PGS$^{713}$ of VEGFR1, $^{714}$STLFI$^{718}$ of VEGFR1, $^{719}$ERVTEEDEGV$^{728}$ of VEGFR1, $^{689}$VNVSDS$^{694}$ of VEGFR3, $^{695}$LEMQCLV$^{701}$ of VEGFR3, $^{702}$AGAHAPS$^{708}$ of VEGFR3, $^{717}$LLEEKSG$^{723}$ of VEGFR3, $^{724}$VDLA$^{727}$ of VEGFR3, $^{728}$DSN$^{730}$ of VEGFR3, $^{731}$QKLSI$^{735}$ of VEGFR3, and $^{736}$QRVREEDAGR$^{745}$ of VEGFR3, $^{678}$TSIGES$^{683}$ of VEGFR2, $^{684}$IEVSCTA$^{690}$ of VEGFR2, $^{691}$SGNPPPQ$^{697}$ of VEGFR2, $^{706}$TLVEDSG$^{712}$ of VEGFR2, $^{713}$IVLK$^{716}$ of VEGFR2, $^{717}$DGN$^{719}$ of VEGFR2, $^{720}$RNLTI$^{724}$ of VEGFR2 and $^{725}$RRVRKEDEGL$^{734}$ of VEGFR2.

As used herein, the phrase "hydrophobic amino acid" refers to an amino acid comprising hydrophobic properties *e.g.,* alanine, cysteine, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, arginine, threonine, valine, tryptophan, tyrosine, serine, proline and others listed herein.

Various aspects of the invention are described in further detail in the following subsections:

I. Antibodies Which Bind To the Ectodomain Of A Human Receptor Tyrosine Kinase

In one aspect of the invention, the moiety that binds to the ectodomain, *e.g.*, an Ig-like domain or a hinge region, of a human receptor tyrosine kinase is an antibody or an antigen binding fragment thereof.

The term “antibody” as referred to herein, includes whole antibodies and any antigen binding fragment (*i.e.*, “antigen-binding portion”) or single chains thereof. An "antibody" refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as $V_H$) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, $C_{H1}$, $C_{H2}$ and $C_{H3}$. Each light chain is comprised of a light chain variable region (abbreviated herein as $V_L$) and a light chain constant region. The light chain constant region is comprised of one domain, $C_L$. The $V_H$ and $V_L$ regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each $V_H$ and $V_L$ is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1,

FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, the D4 or D5 domains of Kit or the D7 domain of a VEGF receptor). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the $V_L$, $V_H$, $C_L$ and $C_H1$ domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fab' fragment, which is essentially an Fab with part of the hinge region (see, FUNDAMENTAL IMMUNOLOGY (Paul ed., 3rd ed. 1993); (iv) a Fd fragment consisting of the $V_H$ and $C_H1$ domains; (v) a Fv fragment consisting of the $V_L$ and $V_H$ domains of a single arm of an antibody, (vi) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a $V_H$ domain; (vii) an isolated complementarity determining region (CDR); and (viii) a nanobody, a heavy chain variable region containing a single variable domain and two constant domains. Furthermore, although the two domains of the Fv fragment, $V_L$ and $V_H$, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the $V_L$ and $V_H$ regions pair to form monovalent molecules (known as single chain Fv (scFv); *see e.g.,* Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883*).* Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

An "isolated antibody", as used herein, is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds to an Ig-like domain of an RTK is substantially free of antibodies that specifically bind antigens other than the Ig-like domain of an

RTK). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals. An "isolated antibody" may, however, include polyclonal antibodies which all bind specifically to, *e.g.*, an Ig-like domain of an RTK.

The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope.

The term "human antibody", as used herein, is intended to include antibodies having variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. Furthermore, if the antibody contains a constant region, the constant region also is derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. In one embodiment, the human monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic nonhuman animal, *e.g.*, a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

The term "recombinant human antibody", as used herein, includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (*e.g.*, a mouse) that is transgenic or transchromosomal for human immunoglobulin genes or a hybridoma prepared therefrom (described further below), (b) antibodies isolated from a host cell transformed to express the human antibody, *e.g.*, from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial human antibody library, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of human

immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable regions in which the framework and CDR regions are derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the $V_H$ and $V_L$ regions of the recombinant antibodies are sequences that, while derived from and related to human germline $V_H$ and $V_L$ sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

As used herein, "isotype" refers to the antibody class (*e.g.*, IgM or IgGl) that is encoded by the heavy chain constant region genes.

The phrases "an antibody recognizing an antigen" and "an antibody specific for an antigen" are used interchangeably herein with the term "an antibody which binds specifically to an antigen."

The term "human antibody derivatives" refers to any modified form of the human antibody, *e.g.*, a conjugate of the antibody and another agent or antibody.

The term "humanized antibody" is intended to refer to antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences. Additional framework region modifications may be made within the human framework sequences. It will be appreciated by one of skill in the art that when a sequence is "derived" from a particular species, said sequence may be a protein sequence, such as when variable region amino acids are taken from a murine antibody, or said sequence may be a DNA sequence, such as when variable region encoding nucleic acids are taken from murine DNA. A humanized antibody may also be designed based on the known sequences of human and non-human (*e.g.,* murine or rabbit) antibodies. The designed antibodies, potentially incorporating both human and non-human residues, may be chemically synthesized. The sequences may also be synthesized at the DNA level and expressed in vitro or in vivo to generate the humanized antibodies.

The term "chimeric antibody" is intended to refer to antibodies in which the variable region sequences are derived from one species and the constant region sequences are derived from another species, such as an antibody in which the variable

region sequences are derived from a mouse antibody and the constant region sequences are derived from a human antibody.

The term “antibody mimetic” or “antibody mimic” is intended to refer to molecules capable of mimicking an antibody’s ability to bind an antigen, but which are not limited to native antibody structures. Examples of such antibody mimetics include, but are not limited to, Adnectins (*i.e.*, fibronectin based binding molecules), Affibodies, DARPins, Anticalins, Avimers, and Versabodies all of which employ binding structures that, while they mimic traditional antibody binding, are generated from and function via distinct mechanisms. The embodiments of the instant invention, as they are directed to antibodies, or antigen binding portions thereof, also apply to the antibody mimetics described above.

As used herein, an antibody that “specifically binds” to an Ig-like domain of a RTK is intended to refer to an antibody that binds to an Ig-like domain of a RTK with a $K_D$ of 1 x $10^{-7}$ M or less, more preferably 5 x $10^{-8}$ M or less, more preferably 1 x $10^{-8}$ M or less, more preferably 5 x $10^{-9}$ M or less

The term “does not substantially bind” to a protein or cells, as used herein, means does not bind or does not bind with a high affinity to the protein or cells, *i.e.* binds to the protein or cells with a $K_D$ of 1 x $10^{-6}$ M or more, more preferably 1 x $10^{-5}$ M or more, more preferably 1 x $10^{-4}$ M or more, more preferably 1 x $10^{-3}$ M or more, even more preferably 1 x $10^{-2}$ M or more.

The term "$K_{assoc}$" or “$K_a$”, as used herein, is intended to refer to the association rate of a particular antibody-antigen interaction, whereas the term "$K_{dis}$" or “$K_d$,” as used herein, is intended to refer to the dissociation rate of a particular antibody-antigen interaction. The term “$K_D$”, as used herein, is intended to refer to the dissociation constant, which is obtained from the ratio of $K_d$ to $K_a$ (*i.e*,. $K_d/K_a$) and is expressed as a molar concentration (M). $K_D$ values for antibodies can be determined using methods well established in the art. A preferred method for determining the $K_D$ of an antibody is by using surface plasmon resonance, preferably using a biosensor system such as a Biacore® system.

As used herein, the term “high affinity”, when referring an IgG type antibody, refers to an antibody having a $K_D$ of $10^{-8}$ M or less, more preferably $10^{-9}$ M or less and even more preferably $10^{-10}$ M or less for an Ig-like domain of a RTK. However, “high affinity” binding can vary for other antibody isotypes. For example, “high affinity”

binding for an IgM isotype refers to an antibody having a $K_D$ of $10^{-7}$ M or less, more preferably $10^{-8}$ M or less, even more preferably $10^{-9}$ M or less.

Antibodies

The antibodies of the invention bind specifically to an Ig-like domain of a RTK, *e.g.*, member of the human type III family of receptor tyrosine kinases. In preferred embodiments, the binding of the antibodies, or antigen binding portions thereof, of the invention to an Ig-like domain of a RTK monomer locks the ectodomain in an inactive state, *e.g.*, a monomeric state, and, thus, antagonizes the ability of the RTK to dimerize and activate a downstream signaling pathway. For example, the antibody may block a ligand induced tyrosine autophosphorylation of the receptor tyrosine kinase and/or receptor internalization.

The antibodies of the invention are selected or designed to bind to specific Ig-like domains of the RTK, for example, the D4 domain or the D5 domain of the human Kit or the D7 domain of a VEGF receptor. In other embodiments the antibodies, or antigen binding portions thereof, are selected or designed to bind proteins sharing homology to a domain of the RTK, *e.g.*, the Kit receptor or the VEGF receptor. For example, an antibody may be selected or designed to bind a domain which is at least 50% identical, at least 60% identical, at least 70% identical, at least 80% identical, at least 90% identical, or at least 95%, 96%, 97%, 98% or 99% identical to a domain, *e.g.*, the D4 or D5 domain, of the Kit receptor or the D7 domain of a VEGF receptor. Such an antibody, or antigen binding portion thereof, would be able to bind protein domains, possibly in Kit, VEGF receptors, and other RTKs, which are functionally similar to the D4 or D5 domains of Kit or the D7 domains of a VEGF receptor.

The antibodies, or antigen binding portions thereof, of the present invention may also be selected or designed to bind a particular motif or consensus sequence derived from an Ig-like domain of a RTK, *e.g.*, a human type III RTK, allowing the antibodies, or antigen binding portions thereof, to specifically bind epitopes or domains which are shared among members of the human type III family of RTKs and between the type III RTKs and other RTKs, *e.g.*, type V RTKs. Such a linear consensus sequence may be found, for example, by using a sequence alignment algorithm to align domains of various RTKs, *e.g.*, domains of D4 domains across RTK types or across species (see Figure 6B). One of skill in the art may align the protein sequences of, for example, the

Kit D4 domains from various species (*e.g.*, human, mouse, rat) to determine which protein residues are conserved in at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 100% of the sequences being aligned. Such a consensus sequence may then be used to generate antibodies or other moieties which specifically bind the consensus sequence and, thus, will bind the most conserved residues of the Kit RTK. Similarily, one may also align the protein sequences of the D7 domain of type V RTKs (see Figure 6) to obtain a consensus sequence for which moieties of the present invention may be generated. One of skill in the art should appreciate that the most highly conserved residues are those which have been preserved through evolution and are most likely to be important for protein function. Alternatively, if the alignment is made across various various classes of RTKs, antibodies generated toward such consensus sequences would allow the antibodies to bind a similar Ig-like domain in multiple RTK types.

In a specific embodiment a moiety of the present invention (*e.g.*, antibodies or antigen binding portions thereof binds to the following consensus sequence for the D4 interaction site: $LX_1RX_2X_3X_4X_5X_6X_7G$ wherein L is Leucine, R is Arginine, G is Glycine; and $X_1$, $X_2$, $X_3$, $X_4$, $X_5$, $X_6$ and $X_7$ are any amino acid. In a specific embodiment, $X_1$ is selected from the group consisting of Threonine, Isoleucine, Valine, Proline, Asparagine, or Lysine; X2 is selected from the group consisting of Leucine, Valine, Alanine, and Methionine; $X_3$ is selected from the group consisting of Lysine, Histidine, Asparagine, and Arginine; $X_4$ is selected from the group consisting of Glycine, Valine, Alanine, Glutamic Acid, Proline, and Methionine; $X_5$ is selected from the group consisting of Threonine, Serine, Glutamic Acid, Alanine, Glutamine, and Aspartic acid; $X_6$ is selected from the group consisting of Glutamic Acid, Aspartic acid, and Glutamine; and $X_7$ is selected from the group consisting of Glycine, Serine, Alanine, Lysine, Arginine, Glutamine, and Threonine.

In another embodiment, a moiety of the present invention (*e.g.*, antibodies or antigen binding portions thereof) binds to the following consensus sequence for the D7 domain of a member of the VEGF receptor family: $IX_1RVX_2X_3EDX_4G$ wherein I is Isoleucine, R is Arginine, E is Glutamic Acid, D is Aspartic Acid, G is Glycine; and $X_1$, $X_2$, $X_3$ and $X_4$ are any amino acid. In a specific embodiment, $X_1$ is selected from the group consisting of Glutamic Acid, Arginine, and Glutamine; X2 is selected from the group consisting of Arginine and Threonine; $X_3$ is selected from the group consisting of

Glutamic Acid and Lysine; and $X_4$ is selected from the group consisting of Glutamic Acid and Alanine (SEQ ID NO: 1).

In another embodiment, a moiety of the present invention (e.g., antibodies or antigen binding portions thereof) binds to the following consensus sequence for the D7 domain of a VEGF receptor: L/I $X_1$ R Φ $X_2$ $X_3$ $X_4$ D/E $X_5$ G (SEQ ID NO:158), wherein L is Leucine, I is Isoleucine, R is Arginine, Φ is a hydrophobic amino acid, D is Aspartic Acid, E is Glutamic Acid, G is Glycine; and $X_1$, $X_2$, $X_3$, $X_4$, and $X_5$ are any amino acid. In a specific embodiment, Φ is Valine; $X_1$ is selected from the group consisting of Arginine, Glutamine, Glutamic Acid and Aspartic Acid; $X_2$ is selected from the group consisting of Arginine, Lysine and Threonine; $X_3$ is selected from the group consisting of Lysine, Glutamic Acid, Glutamine and Valine; $X_4$ is selected from the group consisting of Glutamic Acid and Valine; and $X_5$ is selected from the group consisting of Glutamic Acid, Glycine, Serine and Glutamine.

The antibodies of the present invention do not bind to the ligand binding site of the RTK, *e.g.*, the SCF binding site of the Kit receptor. Therefore, the antibodies described herein do not antagonize the ability of the receptor to bind its target ligand.

In some embodiments the antibody or antigen binding portion thereof binds to specific sequences of the human Kit receptor, for example, residues 309-413, residues 410-519, $^{381}$Arg and $^{386}$Glu, or $^{418}$Tyr and $^{505}$Asn of the human Kit receptor.

In other embodiments, the antibodies, or antigen binding portions thereof, bind protein motifs or consensus sequences which represent a three dimensional structure in the protein. Such motifs or consensus sequences would not represent a contiguous string of amino acids, but a non-contiguous amino acid arrangement that results from the three-dimensional folding of the RTK (*i.e.*, a "structural motif" or "non-linear epitope"). An example of such a motif would be the D4-D4 or the D5-D5 binding interface of a Kit receptor or the D7-D7 binding interface of a VEGF receptor. In one embodiment, an antibody of the present invention binds to, for example, a non-linear epitope in the D4-D4, D5-D5 or D7-D7 interface, preventing the activation of the RTK.

In a preferred embodiment, an antibody or antigen binding portion thereof of the invention may bind to one or more residues in the Kit receptor which make up the small cavities or pockets described in Table 4 (below). For example, an antibody or antigen binding portion thereof of the invention may bind to one or more of the following residues in the D3-D4 hinge region of the Kit receptor: K218, S220, Y221, L222 from

the D3 domain and F340, P341, K342, N367, E368, S369, N370, I371, Y373 from the D4 domain. An antibody or antigen binding portion thereof of the invention may also bind to one or more of the following residues which make up a concave surface in the D4 domain of the Kit receptor:Y350, R353, F355, K358, L379, T380, R381, L382, E386 and T390. In another embodiment, an antibody or antigen binding portion thereof of the invention may bind to one or more of the following residues which form a pocket in the D2-D3 hinge region of the Kit receptor:Y125, G126, H180, R181, K203, V204, R205, P206 and F208 from the D2 domain and V238, S239, S240, S241, H263, G265, D266, F267, N268 and Y269 from the D3 domain.

Thus, in some embodiments, an antibody or antigen binding portion thereof of the invention may bind to contiguous or non-contiguous amino acid residues and function as a molecular wedge that prevents the motion required for positioning of the membrane proximal region of the RTK at a distance and orientation that enables tyrosine kinase activation. An antibody or antigen binding portion thereof of the invention may also act to prevent homotypic D4 or D5 receptor interactions or destabilize the ligand-receptor interaction site. In some preferred embodiments, an antibody or antigen binding portion thereof of the invention may bind to one or more of the following residues on the Kit receptor: Y125, G126, H180, R181, K203, V204, R205, P206, P206, F208, K127, A207, V238, S239, S240, S241, H263, G265, D266, F267, N268, Y269, T295, L222, L222, L223, E306, V308, R224, V308, K310, K218, A219, S220, K218, A220, Y221, A339, D327, D398, E338, E368, E386, F312, F324, F340, F355, G311, G384, G387, G388, I371, K342, K358, L382, L379, N326, N367, N370, N410, P341, S369, T385, V325, V407, V409, Y373, Y350, Y408, T380, T390, R381, R353, T411, K412, E414, K471, F433, G470, L472, V497, F469, A431, or G432.

One of skill in the art will appreciate that, in some embodiments, an antibody or antigen binding portion thereof of the invention may be easily targeted to the corresponding residues in other type III RTKs, *e.g.*, those residues that form similar pockets or cavities or those in the same position by structural alignment or sequence alignment.

In a specific embodiment, an antibody or antigen binding portion thereof of the invention binds to a conformational epitope or a discontinuous epitope on a type III RTK. The conformational or discontinuous epitope may be composed of two or more residues from the D3, D4, or D5 domain or the D4-D5 or D3-D4 hinge regions from a

type III RTK, *e.g.*, the human Kit receptor or the PDGF receptor. For example, the conformational or discontinuous epitope may be composed of two or more of the residues listed in Table 4 below.

In a particular embodiment, an antibody or antigen binding portion thereof, of the invention binds to a conformational epitope composed of 2 or more amino acids selected from the group consisting of Y125, H180, R181, K203, V204, R205, P206. V238, S239, S240, H263, G265, D266, F267, N268, and Y269. In similar embodiments, an antibody or antigen binding portion thereof of the invention may bind to a conformational epitope composed of 2 or more amino acids selected from one of the following groups of amino acids: P206, F208, V238, and S239; K127, A207, F208, and T295; L222, A339, F340, K342, E368, S369, N370, I371, and Y373; L222, L223, E306, V308, F312, E338, F340, and I371; R224, V308, K310, G311, F340, P341, and D398; K218, A219, S220, N367, E368, and S369; K218, A220, E368, and S369; G384, T385, T411, K412, E414, and K471; Y408, F433, G470, K471, and L472; F324, V325, N326, and N410;D327, N410, T411, K412, and V497; G384, G387, V409, and K471; L382, G387, V407, and V409; Y125, G126, H180, R181, K203, V204, R205, P206, F208, V238, S239, S240, S241, H263, G265, D266, F267, N268, and Y269; P206, F208, V238, and S239; K218, S220, Y221, L222, F340, P341, K342, N367, E368, S369, N370, I371, and Y373; G384, G387, G388, Y408, V409, T411, F433, F469, G470, and K471; D327, T411, K412, E414, A431, G432, and K471; Y350, F355, K358, L379, T380, R381, L382, E386, and T390; Y350, R353, and F355. As indicated above, the antibodies of the invention may bind to all of the amino acid residues forming a pocket or a cavity identified in Table 4 or they may bind to a subset of the residues forming the pocket or the cavity. It is to be understood that, in certain embodiments, when reference is made to an antibody of the invention binding to an epitope, *e.g.*, a conformational epitope, the intention is for the antibody to bind only to those specific residues that make up the epitope (*e.g.*, the pocket or cavity identified in Table 4) and not other residues in the linear amino acid sequence of the receptor.

In a further embodiment, an antibody or antigen binding portion thereof of the invention binds to a conformational epitope wherein the conformational epitope is composed of two or more amino acid residues selected from the peptides listed in Table 5. In a specific embodiment, the conformational epitope is composed of one or more amino acid residues selected from a first peptide and one or more amino acid residues

selected from a second peptide, wherein the first and second peptides are selected from the group of peptides listed in Table 5. As such, an antibody or antigen binding portion thereof of the invention binds a conformational epitope wherein the first and second peptide groups are as follows: Ala219-Leu222 and Thr304-Val308; Asp309-Gly311 and Arg224-Gly226; Thr303 - Glu306 and Ala219-Leu222; Asn367–Asn370 and Ser217–Tyr221; Ala339–Pro343 and Asn396–Val399; Ala339–Pro343 and Glu368-Arg372; Lys358–Tyr362 and Val374–His378; Asp357-Glu360 and Leu377–Thr380; Met351–Glu360 and His378-Thr389; His378-Thr389 and Val323-Asp332; Val409–Ile415 and Ala493–Thr500; Val409–Ile415 and Ala431-Thr437; Val409– Ile415 and Phe469-Val473; Val409–Ile415 and Val325–Asn330; Val409–Ile415 and Arg381-Gly387; Gly466–Leu472 and Gly384–Gly388; Val325-Glu329 and Tyr494-Lys499; Thr411-leu416 and Val497-Ala502; Ile415–Leu421 and Ala502–Ala507; Ala502–Ala507 and Lys484-Thr488; and Ala502–Ala507 and Gly445–Cys450.

The antibodies of the invention may bind to all of the amino acid residues forming the foregoing first and second peptide groups or they may bind to a subset of the residues forming the first and second peptide groups. It is to be understood that, in certain embodiments, when reference is made to an antibody of the invention binding to an epitope, *e.g.*, a conformational epitope, the intention is for the antibody to bind only to those specific residues that make up the epitope (*e.g.*, the specific peptides identified in Table 5) and not other residues in the linear amino acid sequence of the receptor.

In another embodiment, an antibody or antigen binding portion thereof of the invention binds to a conformational or discontinuous epitope composed of 2 or more amino acids selected from the group consisting of E33, P34, D72, E76, N77, K78, Q79, K158, D159, N250, S251, Q252, T253, K254, L255, N260, W262, H264, G265, E344, N352, R353, F355, T356, D357, Y362, S365, E366, N367, N370, and G466.

In another embodiment, an antibody or antigen binding portion thereof of the invention binds to amino acid residues $^{385}$Arg and $^{390}$Glu of human PDGFRβ, or the corresponding residues in PDGFRα. The residues $^{385}$Arg and $^{390}$Glu of human PDGFRβ are analogous to the residues $^{381}$Arg and $^{386}$Glu of the Kit receptor and mediate homotypic D4-D4 interactions of PDGFRβ. Antibodies or antigen binding portions thereof of the invention may exert their inhibitory effect on receptor activation by preventing critical homotypic interactions (such as salt bridges formed between $^{385}$Arg and $^{390}$Glu of human PDGFRβ) between membrane proximal regions of type-III RTKs

that are essential for positioning the cytoplasmic domain at a distance and orientation essential for tyrosine kinase activation. Experiments discussed herein demonstrate that homotypic D4-D4 interactions are dispensable for PDGFRβ dimerization and that PDGFRβ dimerization is necessary but not sufficient for receptor activation. Thus, antibodies or antigen binding portions thereof of the invention may allow dimerization of PDGFRβ while preventing activation. Structure based sequence alignment has shown that the size of the EF loop, and the critical amino acids comprising the D4-D4 interface are conserved in Kit, PDGFRα, PDGFRβ, and CSF1R. Thus in some embodiments, antibodies or antigen binding portions thereof of the invention may be targeted to the conserved regions of the D4 or D5 domains of type III RTKs.

In some embodiments, the antibody or antigen-binding portion thereof, binds to specific sequences of a human VEGF receptor, for example, residues 718-727 of VEGFR1, Arg720 and Asp725 of VEGFR1, residues 724-733 of VEGFR2, Arg726 and Asp731 of VEGFR2, residues 735-744 of VEGFR3, or residues Arg737 and Asp742 of VEGFR3.

In another embodiment, the antibody or antigen-binding portion thereof binds to a contiguous epitope on the VEGF receptor. In one embodiment, the contiguous epitope is composed of two or more residues in the D7 domain of the VEGF receptor. In another embodiment, the contiguous epitope is an epitope selected from the group consisting of $^{672}$VAISSS$^{677}$ of VEGFR1, $^{678}$TTLDCHA$^{684}$ of VEGFR1, $^{685}$NGVPEPQ$^{691}$ of VEGFR1, $^{700}$KIQQEPG$^{706}$ of VEGFR1, $^{707}$IILG$^{710}$ of VEGFR1, $^{711}$PGS$^{713}$ of VEGFR1, $^{714}$STLFI$^{718}$ of VEGFR1, $^{719}$ERVTEEDEGV$^{728}$ of VEGFR1, $^{689}$VNVSDS$^{694}$ of VEGFR3, $^{695}$LEMQCLV$^{701}$ of VEGFR3, $^{702}$AGAHAPS$^{708}$ of VEGFR3, $^{717}$LLEEKSG$^{723}$ of VEGFR3, $^{724}$VDLA$^{727}$ of VEGFR3, $^{728}$DSN$^{730}$ of VEGFR3, $^{731}$QKLSI$^{735}$ of VEGFR3, and $^{736}$QRVREEDAGR$^{745}$ of VEGFR3, $^{678}$TSIGES$^{683}$ of VEGFR2, $^{684}$IEVSCTA$^{690}$ of VEGFR2, $^{691}$SGNPPPQ$^{697}$ of VEGFR2, $^{706}$TLVEDSG$^{712}$ of VEGFR2, $^{713}$IVLK$^{716}$ of VEGFR2, $^{717}$DGN$^{719}$ of VEGFR2, $^{720}$RNLTI$^{724}$ of VEGFR2 and $^{725}$RRVRKEDEGL$^{734}$ of VEGFR2.

In additional embodiments, the antibody, or antigen binding portion thereof, of the invention is selected or designed to bind specifically to a mutant RTK. In preferred embodiments, the mutant RTK is a tumorigenic or oncogenic mutant. In one specific embodiment, the antibody, or antigen binding portion thereof, is selected or designed to bind to an oncogenic Kit receptor mutant. Several Kit receptor mutants which may be

targeted by the antibodies of the present invention are Kit receptors with mutations in one or more of the following amino acids: Thr417, Tyr418, Asp419, Leu421, Arg420, Tyr503, or Ala502. It should be appreciated by one of skill in the art that the methods of the invention would be applicable to other mutations in Kit or to mutations in other RTKs. One advantage of targeting a mutant RTK is that a therapeutic antibody may bind to only the RTKs on cells containing the mutation, leaving healthy cells largely or entirely unaffected. Accordingly, in instances where the mutation is tumorigenic, only tumor cells would be targeted for therapy, potentially reducing side effects and dosage requirements.

Preferrably, the antibody binds to an Ig-like domain of a human RTK with a $K_D$ of 5 x $10^{-8}$ M or less, a $K_D$ of 1 x $10^{-8}$ M or less, a $K_D$ of 5 x $10^{-9}$ M or less, or a $K_D$ of between 1 x $10^{-8}$ M and 1 x $10^{-10}$ M or less. Standard assays to evaluate the binding ability of the antibodies toward an Ig-like domain of a RTK, *e.g.*, Kit or a VEGF receptor, are known in the art, including for example, ELISAs, Western blots and RIAs. The binding kinetics (*e.g.*, binding affinity) of the antibodies also can be assessed by standard assays known in the art, such as by ELISA, Scatchard and Biacore analysis.

Engineered and Modified Antibodies

The $V_H$ and/or $V_L$ sequences of an antibody prepared according the the methods of the present invention and may be used as starting material to engineer a modified antibody, which modified antibody may have altered properties from the starting antibody. An antibody can be engineered by modifying one or more residues within one or both of the original variable regions (*i.e.*, $V_H$ and/or $V_L$), for example within one or more CDR regions and/or within one or more framework regions. Additionally or alternatively, an antibody can be engineered by modifying residues within the constant region(s), for example to alter the effector function(s) of the antibody.

One type of variable region engineering that can be performed is CDR grafting. Antibodies interact with target antigens predominantly through amino acid residues that are located in the six heavy and light chain complementarity determining regions (CDRs). For this reason, the amino acid sequences within CDRs are more diverse between individual antibodies than sequences outside of CDRs. Because CDR sequences are responsible for most antibody-antigen interactions, it is possible to

express recombinant antibodies that mimic the properties of specific naturally occurring antibodies by constructing expression vectors that include CDR sequences from the specific naturally occurring antibody grafted onto framework sequences from a different antibody with different properties (see, *e.g.*, Riechmann, L. *et al.* (1998) *Nature* 332:323-327; Jones, P. *et al.* (1986) *Nature* 321:522-525; Queen, C. *et al.* (1989) *Proc. Natl. Acad. See. U.S.A.* 86:10029-10033; U.S. Patent No. 5,225,539 to Winter, and U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen *et al.*)

Framework sequences for antibodies can be obtained from public DNA databases or published references that include germline antibody gene sequences. For example, germline DNA sequences for human heavy and light chain variable region genes can be found in the "VBase" human germline sequence database (available on the Internet at mrc-cpe.cam.ac.uk/vbase), as well as in Kabat, E. A., *et al.* (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242; Tomlinson, I. M., *et al.* (1992) "The Repertoire of Human Germline $V_H$ Sequences Reveals about Fifty Groups of $V_H$ Segments with Different Hypervariable Loops" *J. Mol. Biol.* 227:776-798; and Cox, J. P. L. *et al.* (1994) "A Directory of Human Germ-line $V_H$ Segments Reveals a Strong Bias in their Usage" *Eur. J. Immunol.* 24:827-836; the contents of each of which are expressly incorporated herein by reference. As another example, the germline DNA sequences for human heavy and light chain variable region genes can be found in the Genbank database.

Antibody protein sequences are compared against a compiled protein sequence database using one of the sequence similarity searching methods called the Gapped BLAST (Altschul *et al.* (1997) Nucleic Acids *Research* 25:3389-3402), which is well known to those skilled in the art. BLAST is a heuristic algorithm in that a statistically significant alignment between the antibody sequence and the database sequence is likely to contain high-scoring segment pairs (HSP) of aligned words. Segment pairs whose scores cannot be improved by extension or trimming is called a *hit*. Briefly, the nucleotide sequences of VBASE origin (*vbase.mrc-cpe.cam.ac.uk/vbase1/list2.php*) are translated and the region between and including FR1 through FR3 framework region is retained. The database sequences have an average length of 98 residues. Duplicate sequences which are exact matches over the entire length of the protein are removed. A BLAST search for proteins using the program blastp with default, standard parameters except the low complexity filter, which is turned off, and the substitution matrix of

BLOSUM62, filters for the top 5 hits yielding sequence matches. The nucleotide sequences are translated in all six frames and the frame with no stop codons in the matching segment of the database sequence is considered the potential hit. This is in turn confirmed using the BLAST program tblastx, which translates the antibody sequence in all six frames and compares those translations to the VBASE nucleotide sequences dynamically translated in all six frames. Other human germline sequence databases, such as that available from IMGT (http://imgt.cines.fr), can be searched similarly to VBASE as described above.

The identities are exact amino acid matches between the antibody sequence and the protein database over the entire length of the sequence. The positives (identities + substitution match) are not identical but amino acid substitutions guided by the BLOSUM62 substitution matrix. If the antibody sequence matches two of the database sequences with same identity, the hit with most positives would be decided to be the matching sequence hit.

Identified $V_H$ CDR1, CDR2, and CDR3 sequences, and the $V_K$ CDR1, CDR2, and CDR3 sequences, can be grafted onto framework regions that have the identical sequence as that found in the germline immunoglobulin gene from which the framework sequence derives, or the CDR sequences can be grafted onto framework regions that contain one or more mutations as compared to the germline sequences. For example, it has been found that in certain instances it is beneficial to mutate residues within the framework regions to maintain or enhance the antigen binding ability of the antibody (see *e.g.*, U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen *et al*).

Another type of variable region modification is to mutate amino acid residues within the $V_H$ and/or $V_K$ CDR1, CDR2 and/or CDR3 regions to thereby improve one or more binding properties (*e.g.*, affinity) of the antibody of interest. Site-directed mutagenesis or PCR-mediated mutagenesis can be performed to introduce the mutation(s) and the effect on antibody binding, or other functional property of interest, can be evaluated in *in vitro* or *in vivo* assays known in the art. For example, an antibody of the present invention may be mutated to create a library, which may then be screened for binding to an Ig-like domain of an RTK, *e.g.*, a D4 or a D5 domain of the human Kit RTK or a D7 domain of a VEGF receptor. Preferably conservative modifications (as discussed above) are introduced. The mutations may be amino acid substitutions,

additions or deletions, but are preferably substitutions. Moreover, typically no more than one, two, three, four or five residues within a CDR region are altered.

Another type of framework modification involves mutating one or more residues within the framework region, or even within one or more CDR regions, to remove T cell epitopes to thereby reduce the potential immunogenicity of the antibody. This approach is also referred to as "deimmunization" and is described in futher detail in U.S. Patent Publication No. 20030153043 by Carr *et al.*

In addition or alternative to modifications made within the framework or CDR regions, antibodies of the invention may be engineered to include modifications within the Fc region, typically to alter one or more functional properties of the antibody, such as serum half-life, complement fixation, Fc receptor binding, and/or antigen-dependent cellular cytotoxicity. Furthermore, an antibody of the invention may be chemically modified (*e.g.*, one or more chemical moieties can be attached to the antibody) or be modified to alter its glycosylation, again to alter one or more functional properties of the antibody. Each of these embodiments is described in further detail below. The numbering of residues in the Fc region is that of the EU index of Kabat.

In one embodiment, the hinge region of CH1 is modified such that the number of cysteine residues in the hinge region is altered, *e.g.*, increased or decreased. This approach is described further in U.S. Patent No. 5,677,425 by Bodmer *et al.* The number of cysteine residues in the hinge region of CH1 is altered to, for example, facilitate assembly of the light and heavy chains or to increase or decrease the stability of the antibody.

In another embodiment, the Fc hinge region of an antibody is mutated to decrease the biological half life of the antibody. More specifically, one or more amino acid mutations are introduced into the CH2-CH3 domain interface region of the Fc-hinge fragment such that the antibody has impaired Staphylococcyl protein A (SpA) binding relative to native Fc-hinge domain SpA binding. This approach is described in further detail in U.S. Patent No. 6,165,745 by Ward *et al.*

In another embodiment, the antibody is modified to increase its biological half life. Various approaches are possible. For example, one or more of the following mutations can be introduced: T252L, T254S, T256F, as described in U.S. Patent No. 6,277,375 to Ward. Alternatively, to increase the biological half life, the antibody can be altered within the CH1 or CL region to contain a salvage receptor binding epitope taken

from two loops of a CH2 domain of an Fc region of an IgG, as described in U.S. Patent Nos. 5,869,046 and 6,121,022 by Presta *et al.* These strategies will be effective as long as the binding of the antibody to the Ig-like domain of the RTK is not compromised.

In yet other embodiments, the Fc region is altered by replacing at least one amino acid residue with a different amino acid residue to alter the effector function(s) of the antibody. For example, one or more amino acids selected from amino acid residues 234, 235, 236, 237, 297, 318, 320 and 322 can be replaced with a different amino acid residue such that the antibody has an altered affinity for an effector ligand but retains the antigen-binding ability of the parent antibody. The effector ligand to which affinity is altered can be, for example, an Fc receptor or the C1 component of complement. This approach is described in further detail in U.S. Patent Nos. 5,624,821 and 5,648,260, both by Winter *et al.*

In another example, one or more amino acids selected from amino acid residues 329, 331 and 322 can be replaced with a different amino acid residue such that the antibody has altered C1q binding and/or reduced or abolished complement dependent cytotoxicity (CDC). This approach is described in further detail in U.S. Patent Nos. 6,194,551 by Idusogie *et al.*

In another example, one or more amino acid residues within amino acid positions 231 and 239 are altered to thereby alter the ability of the antibody to fix complement. This approach is described further in PCT Publication WO 94/29351 by Bodmer *et al.*

In yet another example, the Fc region is modified to increase the ability of the antibody to mediate antibody dependent cellular cytotoxicity (ADCC) and/or to increase the affinity of the antibody for an Fcγ receptor by modifying one or more amino acids at the following positions: 238, 239, 248, 249, 252, 254, 255, 256, 258, 265, 267, 268, 269, 270, 272, 276, 278, 280, 283, 285, 286, 289, 290, 292, 293, 294, 295, 296, 298, 301, 303, 305, 307, 309, 312, 315, 320, 322, 324, 326, 327, 329, 330, 331, 333, 334, 335, 337, 338, 340, 360, 373, 376, 378, 382, 388, 389, 398, 414, 416, 419, 430, 434, 435, 437, 438 or 439. This approach is described further in PCT Publication WO 00/42072 by Presta. Moreover, the binding sites on human IgG1 for FcγR1, FcγRII, FcγRIII and FcRn have been mapped and variants with improved binding have been described (see Shields, R.L. *et al.* (2001) *J. Biol. Chem.* 276:6591-6604). Specific mutations at positions 256, 290, 298, 333, 334 and 339 were shown to improve binding to FcγRIII. Additionally, the following combination mutants were shown to improve

FcγRIII binding: T256A/S298A, S298A/E333A, S298A/K224A and S298A/E333A/K334A.

In still another embodiment, the C-terminal end of an antibody of the present invention is modified by the introduction of a cysteine residue as is described in U.S. Provisional Application Serial No. 60/957,271, which is hereby incorporated by reference in its entirety. Such modifications include, but are not limited to, the replacement of an existing amino acid residue at or near the C-terminus of a full-length heavy chain sequence, as well as the introduction of a cysteine-containing extension to the c-terminus of a full-length heavy chain sequence. In preferred embodiments, the cysteine-containing extension comprises the sequence alanine-alanine-cysteine (from N-terminal to C-terminal).

In preferred embodiments the presence of such C-terminal cysteine modifications provide a location for conjugation of a partner molecule, such as a therapeutic agent or a marker molecule. In particular, the presence of a reactive thiol group, due to the C-terminal cysteine modification, can be used to conjugate a partner molecule employing the disulfide linkers described in detail below. Conjugation of the antibody to a partner molecule in this manner allows for increased control over the specific site of attachment. Furthermore, by introducing the site of attachment at or near the C-terminus, conjugation can be optimized such that it reduces or eliminates interference with the antibody's functional properties, and allows for simplified analysis and quality control of conjugate preparations.

In still another embodiment, the glycosylation of an antibody is modified. For example, an aglycoslated antibody can be made (*i.e.*, the antibody lacks glycosylation). Glycosylation can be altered to, for example, increase the affinity of the antibody for antigen. Such carbohydrate modifications can be accomplished by, for example, altering one or more sites of glycosylation within the antibody sequence. For example, one or more amino acid substitutions can be made that result in elimination of one or more variable region framework glycosylation sites to thereby eliminate glycosylation at that site. Such aglycosylation may increase the affinity of the antibody for antigen. Such an approach is described in further detail in U.S. Patent Nos. 5,714,350 and 6,350,861 to Co *et al.* Additional approaches for altering glycosylation are described in further detail in U.S. Patent 7,214,775 to Hanai *et al.*, U.S. Patent No. 6,737,056 to Presta, U.S. Pub No. 20070020260 to Presta, PCT Publication No. WO/2007/084926 to Dickey *et al.*,

PCT Publication No. WO/2006/089294 to Zhu *et al.*, and PCT Publication No. WO/2007/055916 to Ravetch *et al.*, each of which is hereby incorporated by reference in its entirety.

Additionally or alternatively, an antibody can be made that has an altered type of glycosylation, such as a hypofucosylated antibody having reduced amounts of fucosyl residues or an antibody having increased bisecting GlcNac structures. Such altered glycosylation patterns have been demonstrated to increase the ADCC ability of antibodies. Such carbohydrate modifications can be accomplished by, for example, expressing the antibody in a host cell with altered glycosylation machinery. Cells with altered glycosylation machinery have been described in the art and can be used as host cells in which to express recombinant antibodies of the invention to thereby produce an antibody with altered glycosylation. For example, the cell lines Ms704, Ms705, and Ms709 lack the fucosyltransferase gene, FUT8 (alpha (1,6) fucosyltransferase), such that antibodies expressed in the Ms704, Ms705, and Ms709 cell lines lack fucose on their carbohydrates. The Ms704, Ms705, and Ms709 $FUT8^{-/-}$ cell lines were created by the targeted disruption of the FUT8 gene in CHO/DG44 cells using two replacement vectors (see U.S. Patent Publication No. 20040110704 by Yamane *et al.* and Yamane-Ohnuki *et al.* (2004) *Biotechnol Bioeng* 87:614-22). As another example, EP 1,176,195 by Hanai *et al.* describes a cell line with a functionally disrupted FUT8 gene, which encodes a fucosyl transferase, such that antibodies expressed in such a cell line exhibit hypofucosylation by reducing or eliminating the alpha 1,6 bond-related enzyme. Hanai *et al.* also describe cell lines which have a low enzyme activity for adding fucose to the N-acetylglucosamine that binds to the Fc region of the antibody or does not have the enzyme activity, for example the rat myeloma cell line YB2/0 (ATCC CRL 1662). PCT Publication WO 03/035835 by Presta describes a variant CHO cell line, Lec13 cells, with reduced ability to attach fucose to Asn(297)-linked carbohydrates, also resulting in hypofucosylation of antibodies expressed in that host cell (see also Shields, R.L. *et al.* (2002) *J. Biol. Chem.* 277:26733-26740). PCT Publication WO 99/54342 by Umana *et al.* describes cell lines engineered to express glycoprotein-modifying glycosyl transferases (*e.g.*, beta(1,4)-N-acetylglucosaminyltransferase III (GnTIII)) such that antibodies expressed in the engineered cell lines exhibit increased bisecting GlcNac structures which results in increased ADCC activity of the antibodies (see also Umana *et al.* (1999) *Nat. Biotech.* 17:176-180). Alternatively, the fucose residues of the antibody

may be cleaved off using a fucosidase enzyme. For example, the fucosidase alpha-L-fucosidase removes fucosyl residues from antibodies (Tarentino, A.L. *et al.* (1975) *Biochem.* 14:5516-23).

Additionally or alternatively, an antibody can be made that has an altered type of glycosylation, wherein that alteration relates to the level of sialyation of the antibody. Such alterations are described in PCT Publication No. WO/2007/084926 to Dickey *et al.*, and PCT Publication No. WO/2007/055916 to Ravetch *et al.*, both of which are incoporated by reference in their entirety. For example, one may employ an enzymatic reaction with sialidase, such as, for example, Arthrobacter ureafacens sialidase. The conditions of such a reaction are generally described in the U.S. Patent No. 5,831,077, which is hereby incorporated by reference in its entirety. Other non-limiting examples of suitable enzymes are neuraminidase and N-Glycosidase F, as described in Schloemer *et al.*, J. Virology, 15(4), 882-893 (1975) and in Leibiger *et al.*, Biochem J., 338, 529-538 (1999), respectively. Desialylated antibodies may be further purified by using affinity chromatography. Alternatively, one may employ methods to increase the level of sialyation, such as by employing sialytransferase enzymes. Conditions of such a reaction are generally described in Basset *et al.*, Scandinavian Journal of Immunology, 51(3), 307–311 (2000).

Another modification of the antibodies herein that is contemplated by the invention is pegylation. An antibody can be pegylated to, for example, increase the biological (*e.g.*, serum) half life of the antibody. To pegylate an antibody, the antibody, or fragment thereof, typically is reacted with polyethylene glycol (PEG), such as a reactive ester or aldehyde derivative of PEG, under conditions in which one or more PEG groups become attached to the antibody or antibody fragment. Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive PEG molecule (or an analogous reactive water-soluble polymer). As used herein, the term "polyethylene glycol" is intended to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (C1-C10) alkoxy- or aryloxy-polyethylene glycol or polyethylene glycol-maleimide. In certain embodiments, the antibody to be pegylated is an aglycosylated antibody. Methods for pegylating proteins are known in the art and can be applied to the antibodies of the invention. See for example, EP 0 154 316 by Nishimura *et al.* and EP 0 401 384 by Ishikawa *et al.* As

such, the methods of pegylation described here also apply the peptidic molecules of the invention described below.

Antibody Fragments and Antibody Mimetics

The instant invention is not limited to traditional antibodies and may be practiced through the use of antibody fragments and antibody mimetics. As detailed below, a wide variety of antibody fragment and antibody mimetic technologies have now been developed and are widely known in the art. While a number of these technologies, such as domain antibodies, Nanobodies, and UniBodies make use of fragments of, or other modifications to, traditional antibody structures, there are also alternative technologies, such as Adnectins, Affibodies, DARPins, Anticalins, Avimers, and Versabodies that employ binding structures that, while they mimic traditional antibody binding, are generated from and function via distinct mechanisms. Some of these alternative structures are reviewed in Gill and Damle (2006) 17: 653-658.

Domain Antibodies (dAbs) are the smallest functional binding units of antibodies, corresponding to the variable regions of either the heavy (VH) or light (VL) chains of human antibodies. Domain Antibodies have a molecular weight of approximately 13 kDa. Domantis has developed a series of large and highly functional libraries of fully human VH and VL dAbs (more than ten billion different sequences in each library), and uses these libraries to select dAbs that are specific to therapeutic targets. In contrast to many conventional antibodies, domain antibodies are well expressed in bacterial, yeast, and mammalian cell systems. Further details of domain antibodies and methods of production thereof may be obtained by reference to U.S. Patent 6,291,158; 6,582,915; 6,593,081; 6,172,197; 6,696,245; U.S. Serial No. 2004/0110941; European patent application No. 1433846 and European Patents 0368684 & 0616640; WO05/035572, WO04/101790, WO04/081026, WO04/058821, WO04/003019 and WO03/002609, each of which is herein incorporated by reference in its entirety.

Nanobodies are antibody-derived therapeutic proteins that contain the unique structural and functional properties of naturally-occurring heavy-chain antibodies. These heavy-chain antibodies contain a single variable domain (VHH) and two constant domains (CH2 and CH3). Importantly, the cloned and isolated VHH domain is a

perfectly stable polypeptide harbouring the full antigen-binding capacity of the original heavy-chain antibody. Nanobodies have a high homology with the VH domains of human antibodies and can be further humanized without any loss of activity. Importantly, Nanobodies have a low immunogenic potential, which has been confirmed in primate studies with Nanobody lead compounds.

Nanobodies combine the advantages of conventional antibodies with important features of small molecule drugs. Like conventional antibodies, Nanobodies show high target specificity, high affinity for their target and low inherent toxicity. However, like small molecule drugs they can inhibit enzymes and readily access receptor clefts. Furthermore, Nanobodies are extremely stable, can be administered by means other than injection (see, *e.g.*, WO 04/041867, which is herein incorporated by reference in its entirety) and are easy to manufacture. Other advantages of Nanobodies include recognizing uncommon or hidden epitopes as a result of their small size, binding into cavities or active sites of protein targets with high affinity and selectivity due to their unique 3-dimensional, drug format flexibility, tailoring of half-life and ease and speed of drug discovery.

Nanobodies are encoded by single genes and are efficiently produced in almost all prokaryotic and eukaryotic hosts, *e.g.*, *E. coli* (see, *e.g.*, U.S. 6,765,087, which is herein incorporated by reference in its entirety), molds (for example Aspergillus or Trichoderma) and yeast (for example Saccharomyces, Kluyveromyces, Hansenula or Pichia) (see, *e.g.*, U.S. 6,838,254, which is herein incorporated by reference in its entirety). The production process is scalable and multi-kilogram quantities of Nanobodies have been produced. Because Nanobodies exhibit a superior stability compared with conventional antibodies, they can be formulated as a long shelf-life, ready-to-use solution.

The Nanoclone method (see, *e.g.*, WO 06/079372, which is herein incorporated by reference in its entirety) is a proprietary method for generating Nanobodies against a desired target, based on automated high-throughout selection of B-cells and could be used in the context of the instant invention.

UniBodies are another antibody fragment technology, however this one is based upon the removal of the hinge region of IgG4 antibodies. The deletion of the hinge region results in a molecule that is essentially half the size of traditional IgG4 antibodies and has a univalent binding region rather than the bivalent binding region of IgG4

antibodies. It is also well known that IgG4 antibodies are inert and thus do not interact with the immune system, which may be advantageous for the treatment of diseases where an immune response is not desired, and this advantage is passed onto UniBodies. For example, UniBodies may function to inhibit or silence, but not kill, the cells to which they are bound. Additionally, UniBody binding to cancer cells do not stimulate them to proliferate. Furthermore, because UniBodies are about half the size of traditional IgG4 antibodies, they may show better distribution over larger solid tumors with potentially advantageous efficacy. UniBodies are cleared from the body at a similar rate to whole IgG4 antibodies and are able to bind with a similar affinity for their antigens as whole antibodies. Further details of UniBodies may be obtained by reference to patent application WO2007/059782, which is herein incorporated by reference in its entirety.

Adnectin molecules are engineered binding proteins derived from one or more domains of the fibronectin protein. Fibronectin exists naturally in the human body. It is present in the extracellular matrix as an insoluble glycoprotein dimer and also serves as a linker protein. It is also present in soluable form in blood plasma as a disulphide linked dimer. The plasma form of fibronectin is synthesized by liver cells (hepatocytes), and the ECM form is made by chondrocytes, macrophages, endothelial cells, fibroblasts, and some cells of the epithelium (see Ward M., and Marcey, D., callutheran.edu/Academic_Programs/Departments/BioDev/omm/fibro/fibro.htm). As mentioned previously, fibronectin may function naturally as a cell adhesion molecule, or it may mediate the interaction of cells by making contacts in the extracellular matrix. Typically, fibronectin is made of three different protein modules, type I, type II, and type III modules. For a review of the structure of function of the fibronectin, see Pankov and Yamada (2002) J Cell Sci.,115(Pt 20):3861-3, Hohenester and Engel (2002) 21:115-128, and Lucena *et al.* (2007) Invest Clin.48:249-262.

In a preferred embodiment, adnectin molecules are derived from the fibronectin type III domain by altering the native protein which is composed of multiple beta strands distributed between two beta sheets. Depending on the originating tissue, fibronecting may contain multiple type III domains which may be denoted, *e.g.*, $^{1}$Fn3, $^{2}$Fn3, $^{3}$Fn3, etc. The $^{10}$Fn3 domain contains an integrin binding motif and further contains three loops which connect the beta strands. These loops may be thought of as corresponding to the antigen binding loops of the IgG heavy chain, and they may be altered by methods

discussed below to specifically bind a target of interest, *e.g.*, an Ig-like domain of a RTK, such as the D4 or D5 domain of human Kit RTK or the D7 domain of a VEGF receptor. Preferably, a fibronectin type III domain useful for the purposes of this invention is a sequence which exhibits a sequence identity of at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95% to the sequence encoding the structure of the fibronectin type III molecule which can be accessed from the Protein Data Bank (PDB, rcsb.org/pdb/home/home.do) with the accession code: 1ttg. Adnectin molecules may also be derived from polymers of $^{10}$Fn3 related molecules rather than a simple monomeric $^{10}$Fn3 structure.

Although the native $^{10}$Fn3 domain typically binds to integrin, $^{10}$Fn3 proteins adapted to become adnectin molecules are altered so to bind antigens of interest, *e.g.*, an Ig-like domain of a RTK, such as the D4 or D5 domain of human Kit. In one embodiment, the alteration to the $^{10}$Fn3 molecule comprises at least one mutation to a beta strand. In a preferred embodiment, the loop regions which connect the beta strands of the $^{10}$Fn3 molecule are altered to bind to an Ig-like domain of a human receptor tyrosine kinase, *e.g.*, a VEGF receptor or a type III receptor tyrosine kinase, such as the human Kit.

The alterations in the $^{10}$Fn3 may be made by any method known in the art including, but not limited to, error prone PCR, site-directed mutagenesis, DNA shuffling, or other types of recombinational mutagenesis which have been referenced herein. In one example, variants of the DNA encoding the $^{10}$Fn3 sequence may be directly synthesized *in vitro*, and later transcribed and translated *in vitro* or *in vivo*. Alternatively, a natural $^{10}$Fn3 sequence may be isolated or cloned from the genome using standard methods (as performed, *e.g.*, in U.S. Pat. Application No. 20070082365), and then mutated using mutagenesis methods known in the art.

In one embodiment, a target protein, *e.g.*, an Ig-like domain of a RTK, such as the D4 or D5 domain of the Kit RTK or the D7 domain of a VEGF receptor, may be immobilized on a solid support, such as a column resin or a well in a microtiter plate. The target is then contacted with a library of potential binding proteins. The library may comprise $^{10}$Fn3 clones or adnectin molecules derived from the wild type $^{10}$Fn3 by mutagenesis/randomization of the $^{10}$Fn3 sequence or by mutagenesis/randomization of the $^{10}$Fn3 loop regions (not the beta strands). In a preferred embodiment the library may be an RNA-protein fusion library generated by the techniques described in Szostak *et*

*al.*, U.S. Ser. No. 09/007,005 and 09/247,190; Szostak *et al.*, WO989/31700; and Roberts & Szostak (1997) 94:12297-12302. The library may also be a DNA-protein library (*e.g.*, as described in Lohse, U.S. Ser. No. 60/110,549, U.S. Ser. No. 09/459,190, and WO 00/32823). The fusion library is then incubated with the immobilized target (*e.g.*, the D4 or D5 domain of human Kit RTK or the D7 domain of a human VEGF receptor) and the solid support is washed to remove non-specific binding moieties. Tight binders are then eluted under stringent conditions and PCR is used to amply the genetic information or to create a new library of binding molecules to repeat the process (with or without additional mutagenesis). The selection/mutagenesis process may be repeated until binders with sufficient affinity to the target are obtained. Adnectin molecules for use in the present invention may be engineered using the PROfusion™ technology employed by Adnexus, a Briston-Myers Squibb company. The PROfusion technology was created based on the techniques referenced above (*e.g.*, Roberts & Szostak (1997) 94:12297-12302). Methods of generating libraries of altered $^{10}$Fn3 domains and selecting appropriate binders which may be used with the present invention are described fully in the following U.S. Patent and Patent Application documents and are incorporated herein by reference: U.S. Pat. Nos. 7,115,396; 6,818,418; 6,537,749; 6,660,473; 7,195,880; 6,416,950; 6,214,553; 6623926; 6,312,927; 6,602,685; 6,518,018; 6,207,446; 6,258,558; 6,436,665; 6,281,344; 7,270,950; 6,951,725; 6,846,655; 7,078,197; 6,429,300; 7,125,669; 6,537,749; 6,660,473; and U.S. Pat. Application Nos. 20070082365; 20050255548; 20050038229; 20030143616; 20020182597; 20020177158; 20040086980; 20040253612; 20030022236; 20030013160; 20030027194; 20030013110; 20040259155; 20020182687; 20060270604; 20060246059; 20030100004; 20030143616; and 20020182597. The generation of diversity in fibronectin type III domains, such as $^{10}$Fn3, followed by a selection step may be accomplished using other methods known in the art such as phage display, ribosome display, or yeast surface display, *e.g.*, Lipovšek *et al.* (2007) Journal of Molecular Biology 368: 1024-1041; Sergeeva *et al.* (2006) Adv Drug Deliv Rev. 58:1622-1654; Petty *et al.* (2007) Trends Biotechnol. 25: 7-15; Rothe *et al.* (2006) Expert Opin Biol Ther. 6:177-187; and Hoogenboom (2005) Nat Biotechnol. 23:1105-1116.

It should be appreciated by one of skill in the art that the methods references cited above may be used to derive antibody mimics from proteins other than the preferred $^{10}$Fn3 domain. Additional molecules which can be used to generate antibody

mimics via the above referenced methods include, without limitation, human fibronectin modules $^{1}$Fn3-$^{9}$Fn3 and $^{11}$Fn3-$^{17}$Fn3 as well as related Fn3 modules from non-human animals and prokaryotes. In addition, Fn3 modules from other proteins with sequence homology to $^{10}$Fn3, such as tenascins and undulins, may also be used. Other exemplary proteins having immunoglobulin-like folds (but with sequences that are unrelated to the $V_H$ domain) include N-cadherin, ICAM-2, titin, GCSF receptor, cytokine receptor, glycosidase inhibitor, E-cadherin, and antibiotic chromoprotein. Further domains with related structures may be derived from myelin membrane adhesion molecule P0, CD8, CD4, CD2, class I MHC, T-cell antigen receptor, CD1, C2 and I-set domains of VCAM-1, I-set immunoglobulin fold of myosin-binding protein C, I-set immunoglobulin fold of myosin-binding protein H, I-set immunoglobulin-fold of telokin, telikin, NCAM, twitchin, neuroglian, growth hormone receptor, erythropoietin receptor, prolactin receptor, GC-SF receptor, interferon-gamma receptor, beta-galactosidase/glucuronidase, beta-glucuronidase, and transglutaminase. Alternatively, any other protein that includes one or more immunoglobulin-like folds may be utilized to create a adnecting like binding moiety. Such proteins may be identified, for example, using the program SCOP (Murzin *et al.*, J. Mol. Biol. 247:536 (1995); Lo Conte *et al.*, Nucleic Acids Res. 25:257 (2000).

An aptamer is another type of antibody-mimetic which is encompassed by the present invention. Aptamers are typically small nucleotide polymers that bind to specific molecular targets. Aptamers may be single or double stranded nucleic acid molecules (DNA or RNA), although DNA based aptamers are most commonly double stranded. There is no defined length for an aptamer nucleic acid; however, aptamer molecules are most commonly between 15 and 40 nucleotides long.

Aptamers often form complex three-dimensional structures which determine their affinity for target molecules. Aptamers can offer many advantages over simple antibodies, primarily because they can be engineered and amplified almost entirely in vitro. Furthermore, aptamers often induce little or no immune response.

Aptamers may be generated using a variety of techniques, but were originally developed using in vitro selection (Ellington and Szostak. (1990) Nature. 346(6287):818-22) and the SELEX method (systematic evolution of ligands by exponential enrichment) (Schneider et al. 1992. J Mol Biol. 228(3):862-9) the contents of which are incorporated herein by reference. Other methods to make and uses of

aptamers have been published including Klussmann. The Aptamer Handbook: Functional Oligonucleotides and Their Applications. ISBN: 978-3-527-31059-3; Ulrich et al. 2006. Comb Chem High Throughput Screen 9(8):619-32; Cerchia and de Franciscis. 2007. Methods Mol Biol. 361:187-200; Ireson and Kelland. 2006. Mol Cancer Ther. 2006 5(12):2957-62; US Pat. Nos.: 5582981; 5840867; 5756291; 6261783; 6458559; 5792613; 6111095; and US Pat. App. Nos.: 11/482,671; 11/102,428; 11/291,610; and 10/627,543 which are all incorporated herein by reference.

The SELEX method is clearly the most popular and is conducted in three fundamental steps. First, a library of candidate nucleic acid molecules is selected from for binding to specific molecular target. Second, nucleic acids with sufficient affinity for the target are separated from non-binders. Third, the bound nucleic acids are amplified, a second library is formed, and the process is repeated. At each repetition, aptamers are chosen which have higher and higher affinity for the target molecule. SELEX methods are described more fully in the following publications, which are incorporated herein by reference: Bugaut et al. 2006. 4(22):4082-8; Stoltenburg et al. 2007 Biomol Eng. 2007 24(4):381-403; and Gopinath. 2007. Anal Bioanal Chem. 2007. 387(1):171-82.

An "aptamer" of the invention also been includes aptamer molecules made from peptides instead of nucleotides. Peptide aptamers share many properties with nucleotide aptamers (*e.g.*, small size and ability to bind target molecules with high affinity) and they may be generated by selection methods that have similar principles to those used to generate nucleotide aptamers, for example Baines and Colas. 2006. Drug Discov Today. 11(7-8):334-41; and Bickle et al. 2006. Nat Protoc. 1(3):1066-91 which are incorporated herein by reference.

Affibody molecules represent a new class of affinity proteins based on a 58-amino acid residue protein domain, derived from one of the IgG-binding domains of staphylococcal protein A. This three helix bundle domain has been used as a scaffold for the construction of combinatorial phagemid libraries, from which Affibody variants that target the desired molecules can be selected using phage display technology (Nord K, Gunneriusson E, Ringdahl J, Stahl S, Uhlen M, Nygren PA, Binding proteins selected from combinatorial libraries of an α-helical bacterial receptor domain, Nat Biotechnol 1997;15:772-7. Ronmark J, Gronlund H, Uhlen M, Nygren PA, Human immunoglobulin A (IgA)-specific ligands from combinatorial engineering of protein A, Eur J Biochem

2002;269:2647-55). The simple, robust structure of Affibody molecules in combination with their low molecular weight (6 kDa), make them suitable for a wide variety of applications, for instance, as detection reagents (Ronmark J, Hansson M, Nguyen T, et al, Construction and characterization of affibody-Fc chimeras produced in Escherichia coli, J Immunol Methods 2002;261:199-211) and to inhibit receptor interactions (Sandstorm K, Xu Z, Forsberg G, Nygren PA, Inhibition of the CD28-CD80 co-stimulation signal by a CD28-binding Affibody ligand developed by combinatorial protein engineering, Protein Eng 2003;16:691-7). Further details of Affibodies and methods of production thereof may be obtained by reference to U.S. Patent No. 5,831,012 which is herein incorporated by reference in its entirety.

DARPins (Designed Ankyrin Repeat Proteins) are one example of an antibody mimetic DRP (Designed Repeat Protein) technology that has been developed to exploit the binding abilities of non-antibody polypeptides. Repeat proteins such as ankyrin or leucine-rich repeat proteins, are ubiquitous binding molecules, which occur, unlike antibodies, intra- and extracellularly. Their unique modular architecture features repeating structural units (repeats), which stack together to form elongated repeat domains displaying variable and modular target-binding surfaces. Based on this modularity, combinatorial libraries of polypeptides with highly diversified binding specificities can be generated. This strategy includes the consensus design of self-compatible repeats displaying variable surface residues and their random assembly into repeat domains.

DARPins can be produced in bacterial expression systems at very high yields and they belong to the most stable proteins known. Highly specific, high-affinity DARPins to a broad range of target proteins, including human receptors, cytokines, kinases, human proteases, viruses and membrane proteins, have been selected. DARPins having affinities in the single-digit nanomolar to picomolar range can be obtained.

DARPins have been used in a wide range of applications, including ELISA, sandwich ELISA, flow cytometric analysis (FACS), immunohistochemistry (IHC), chip applications, affinity purification or Western blotting. DARPins also proved to be highly active in the intracellular compartment for example as intracellular marker proteins fused to green fluorescent protein (GFP). DARPins were further used to inhibit viral entry with IC50 in the pM range. DARPins are not only ideal to block protein-protein

interactions, but also to inhibit enzymes. Proteases, kinases and transporters have been successfully inhibited, most often an allosteric inhibition mode. Very fast and specific enrichments on the tumor and very favorable tumor to blood ratios make DARPins well suited for *in vivo* diagnostics or therapeutic approaches.

Additional information regarding DARPins and other DRP technologies can be found in U.S. Patent Application Publication No. 2004/0132028 and International Patent Application Publication No. WO 02/20565, both of which are hereby incorporated by reference in their entirety.

Anticalins are an additional antibody mimetic technology, however in this case the binding specificity is derived from lipocalins, a family of low molecular weight proteins that are naturally and abundantly expressed in human tissues and body fluids. Lipocalins have evolved to perform a range of functions *in vivo* associated with the physiological transport and storage of chemically sensitive or insoluble compounds. Lipocalins have a robust intrinsic structure comprising a highly conserved ß-barrel which supports four loops at one terminus of the protein. These loops form the entrance to a binding pocket and conformational differences in this part of the molecule account for the variation in binding specificity between individual lipocalins.

While the overall structure of hypervariable loops supported by a conserved ß-sheet framework is reminiscent of immunoglobulins, lipocalins differ considerably from antibodies in terms of size, being composed of a single polypeptide chain of 160-180 amino acids which is marginally larger than a single immunoglobulin domain.

Lipocalins are cloned and their loops are subjected to engineering in order to create Anticalins. Libraries of structurally diverse Anticalins have been generated and Anticalin display allows the selection and screening of binding function, followed by the expression and production of soluble protein for further analysis in prokaryotic or eukaryotic systems. Studies have successfully demonstrated that Anticalins can be developed that are specific for virtually any human target protein can be isolated and binding affinities in the nanomolar or higher range can be obtained.

Anticalins can also be formatted as dual targeting proteins, so-called Duocalins. A Duocalin binds two separate therapeutic targets in one easily produced monomeric protein using standard manufacturing processes while retaining target specificity and affinity regardless of the structural orientation of its two binding domains.

Modulation of multiple targets through a single molecule is particularly advantageous in diseases known to involve more than a single causative factor. Moreover, bi- or multivalent binding formats such as Duocalins have significant potential in targeting cell surface molecules in disease, mediating agonistic effects on signal transduction pathways or inducing enhanced internalization effects via binding and clustering of cell surface receptors. Furthermore, the high intrinsic stability of Duocalins is comparable to monomeric Anticalins, offering flexible formulation and delivery potential for Duocalins.

Additional information regarding Anticalins can be found in U.S. Patent No. 7,250,297 and International Patent Application Publication No. WO 99/16873, both of which are hereby incorporated by reference in their entirety.

Another antibody mimetic technology useful in the context of the instant invention are Avimers. Avimers are evolved from a large family of human extracellular receptor domains by *in* vitro exon shuffling and phage display, generating multidomain proteins with binding and inhibitory properties. Linking multiple independent binding domains has been shown to create avidity and results in improved affinity and specificity compared with conventional single-epitope binding proteins. Other potential advantages include simple and efficient production of multitarget-specific molecules in Escherichia coli, improved thermostability and resistance to proteases. Avimers with sub-nanomolar affinities have been obtained against a variety of targets.

Additional information regarding Avimers can be found in U.S. Patent Application Publication Nos. 2006/0286603, 2006/0234299, 2006/0223114, 2006/0177831, 2006/0008844, 2005/0221384, 2005/0164301, 2005/0089932, 2005/0053973, 2005/0048512, 2004/0175756, all of which are hereby incorporated by reference in their entirety.

Versabodies are another antibody mimetic technology that could be used in the context of the instant invention. Versabodies are small proteins of 3-5 kDa with >15% cysteines, which form a high disulfide density scaffold, replacing the hydrophobic core that typical proteins have. The replacement of a large number of hydrophobic amino acids, comprising the hydrophobic core, with a small number of disulfides results in a protein that is smaller, more hydrophilic (less aggregation and non-specific binding), more resistant to proteases and heat, and has a lower density of T-cell epitopes, because the residues that contribute most to MHC presentation are hydrophobic. All four of

these properties are well-known to affect immunogenicity, and together they are expected to cause a large decrease in immunogenicity.

The inspiration for Versabodies comes from the natural injectable biopharmaceuticals produced by leeches, snakes, spiders, scorpions, snails, and anemones, which are known to exhibit unexpectedly low immunogenicity. Starting with selected natural protein families, by design and by screening the size, hydrophobicity, proteolytic antigen processing, and epitope density are minimized to levels far below the average for natural injectable proteins.

Given the structure of Versabodies, these antibody mimetics offer a versatile format that includes multi-valency, multi-specificity, a diversity of half-life mechanisms, tissue targeting modules and the absence of the antibody Fc region. Furthermore, Versabodies are manufactured in E. coli at high yields, and because of their hydrophilicity and small size, Versabodies are highly soluble and can be formulated to high concentrations. Versabodies are exceptionally heat stable (they can be boiled) and offer extended shelf-life.

Additional information regarding Versabodies can be found in U.S. Patent Application Publication No. 2007/0191272 which is hereby incorporated by reference in its entirety.

SMIPs™ (Small Modular ImmunoPharmaceuticals-Trubion Pharmaceuticals) engineered to maintain and optimize target binding, effector functions, *in vivo* half life, and expression levels. SMIPS consist of three distinct modular domains. First they contain a binding domain which may consist of any protein which confers specificity (*e.g.*, cell surface receptors, single chain antibodies, soluble proteins, etc). Secondly, they contain a hinge domain which serves as a flexible linker between the binding domain and the effector domain, and also helps control multimerization of the SMIP drug. Finally, SMIPS contain an effector domain which may be derived from a variety of molecules including Fc domains or other specially designed proteins. The modularity of the design, which allows the simple construction of SMIPs with a variety of different binding, hinge, and effector domains, provides for rapid and customizable drug design.

More information on SMIPs, including examples of how to design them, may be found in Zhao *et al.* (2007) Blood 110:2569-77 and the following U.S. Pat. App. Nos. 20050238646; 20050202534; 20050202028; 20050202023; 20050202012; 20050186216; 20050180970; and 20050175614.

The detailed description of antibody fragment and antibody mimetic technologies provided above is not intended to be a comprehensive list of all technologies that could be used in the context of the instant specification. For example, and also not by way of limitation, a variety of additional technologies including alternative polypeptide-based technologies, such as fusions of complimentary determining regions as outlined in Qui *et al.*, Nature Biotechnology, 25(8) 921-929 (2007), which is hereby incorporated by reference in its entirety, as well as nucleic acid-based technologies, such as the RNA aptamer technologies described in U.S. Patent Nos. 5,789,157, 5,864,026, 5,712,375, 5,763,566, 6,013,443, 6,376,474, 6,613,526, 6,114,120, 6,261,774, and 6,387,620, all of which are hereby incorporated by reference, could be used in the context of the instant invention.

Antibody Physical Properties

The antibodies of the present invention, which bind to an Ig-like domain of a RTK, may be further characterized by the various physical properties. Various assays may be used to detect and/or differentiate different classes of antibodies based on these physical properties.

In some embodiments, antibodies of the present invention may contain one or more glycosylation sites in either the light or heavy chain variable region. The presence of one or more glycosylation sites in the variable region may result in increased immunogenicity of the antibody or an alteration of the pK of the antibody due to altered antigen binding (Marshall *et al* (1972) *Annu Rev Biochem* 41:673-702; Gala FA and Morrison SL (2004) *J Immunol* 172:5489-94; Wallick *et al* (1988) *J Exp Med* 168:1099-109; Spiro RG (2002) *Glycobiology* 12:43R-56R; Parekh *et al* (1985) *Nature* 316:452-7; Mimura *et al.* (2000) *Mol Immunol* 37:697-706). Glycosylation has been known to occur at motifs containing an N-X-S/T sequence. Variable region glycosylation may be tested using a Glycoblot assay, which cleaves the antibody to produce a Fab, and then tests for glycosylation using an assay that measures periodate oxidation and Schiff base formation. Alternatively, variable region glycosylation may be tested using Dionex light chromatography (Dionex-LC), which cleaves saccharides from a Fab into monosaccharides and analyzes the individual saccharide content. In some instances, it may be preferred to have an antibody that does not contain variable region

glycosylation. This can be achieved either by selecting antibodies that do not contain the glycosylation motif in the variable region or by mutating residues within the glycosylation motif using standard techniques well known in the art.

Each antibody will have a unique isoelectric point (pI), but generally antibodies will fall in the pH range of between 6 and 9.5. The pI for an IgG1 antibody typically falls within the pH range of 7-9.5 and the pI for an IgG4 antibody typically falls within the pH range of 6-8. Antibodies may have a pI that is outside this range. Although the effects are generally unknown, there is speculation that antibodies with a pI outside the normal range may have some unfolding and instability under *in vivo* conditions. The isoelectric point may be tested using a capillary isoelectric focusing assay, which creates a pH gradient and may utilize laser focusing for increased accuracy (Janini *et al* (2002) *Electrophoresis* 23:1605-11; Ma *et al.* (2001) *Chromatographia* 53:S75-89; Hunt *et al* (1998) *J Chromatogr A* 800:355-67). In some instances, it is preferred to have an antibody that contains a pI value that falls in the normal range. This can be achieved either by selecting antibodies with a pI in the normal range, or by mutating charged surface residues using standard techniques well known in the art.

Each antibody will have a melting temperature that is indicative of thermal stability (Krishnamurthy R and Manning MC (2002) *Curr Pharm Biotechnol* 3:361-71). A higher thermal stability indicates greater overall antibody stability *in vivo*. The melting point of an antibody may be measure using techniques such as differential scanning calorimetry (Chen *et al* (2003) *Pharm Res* 20:1952-60; Ghirlando *et al* (1999) *Immunol Lett* 68:47-52). $T_{M1}$ indicates the temperature of the initial unfolding of the antibody. $T_{M2}$ indicates the temperature of complete unfolding of the antibody. Generally, it is preferred that the $T_{M1}$ of an antibody of the present invention is greater than 60°C, preferably greater than 65°C, even more preferably greater than 70°C. Alternatively, the thermal stability of an antibody may be measure using circular dichroism (Murray *et al.* (2002) *J. Chromatogr Sci* 40:343-9).

In a preferred embodiment, antibodies that do not rapidly degrade may be desired. Fragmentation of an antibody may be measured using capillary electrophoresis (CE) and MALDI-MS, as is well understood in the art (Alexander AJ and Hughes DE (1995) *Anal Chem* 67:3626-32).

In another preferred embodiment, antibodies are selected that have minimal aggregation effects. Aggregation may lead to triggering of an unwanted immune

response and/or altered or unfavorable pharmacokinetic properties. Generally, antibodies are acceptable with aggregation of 25% or less, preferably 20% or less, even more preferably 15% or less, even more preferably 10% or less and even more preferably 5% or less. Aggregation may be measured by several techniques well known in the art, including size-exclusion column (SEC) high performance liquid chromatography (HPLC), and light scattering to identify monomers, dimers, trimers or multimers.

Production of Polyclonal Antibodies of the Invention

Polyclonal antibodies of the present invention can be produced by a variety of techniques that are well known in the art. Polyclonal antibodies are derived from different B-cell lines and thus may recognize multiple epitopes on the same antigen. Polyclonal antibodies are typically produced by immunization of a suitable mammal with the antigen of interest, *e.g.*, an Ig-like domain of an RTK such as the D4 or D5 domain of human Kit or the D7 domain of a human VEGF. Animals often used for production of polyclonal antibodies are chickens, goats, guinea pigs, hamsters, horses, mice, rats, sheep, and, most commonly, rabbit. In Example 14 below polyclonal anti-Kit antibodies were generated by immunizing rabbits with the fourth (D4) or fifth (D5) Ig-like domain of Kit or the entire ectodomain of Kit. Standard methods to produce polyclonal antibodies are widely known in the art and can be combined with the methods of the present invention (*e.g.*, research.cm.utexas.edu/bkitto/Kittolabpage/Protocols/Immunology/PAb.html; U.S. Patent Nos. 4,719,290, 6,335,163, 5,789,208, 2,520,076, 2,543,215, and 3,597,409, the entire contents of which are incorporated herein by reference.

Production of Monoclonal Antibodies of the Invention

Monoclonal antibodies (mAbs) of the present invention can be produced by a variety of techniques, including conventional monoclonal antibody methodology *e.g.*, the standard somatic cell hybridization technique of Kohler and Milstein (1975) *Nature* 256: 495. Although somatic cell hybridization procedures are preferred, in principle, other techniques for producing monoclonal antibody can be employed *e.g.*, viral or oncogenic transformation of B lymphocytes. It should be noted that antibodies (monoclonal or polyclonal) or antigen binding portions thereof, may be raised to any epitope on an Ig-like domain of a RTK, more preferably the D4 or D5 domains of the

human Kit RTK or the D7 domain of a VEGF receptor, to the concensus sequences discussed herein, or to any conformational, discontinuous, or linear epitopes described herein.

Several methods known in the art are useful for specifically selecting an antibody or antigen binding fragment thereof that specifically binds a discontinuous epitope of interest. For example, the techniques disclosed in U.S. Publication No. 2005/0169925, the entire contents of which are incorporated herein by reference, allow for the selection of an antibody which binds to two different peptides within a protein sequence. Such methods may be used in accordance with the present invention to specifically target the conformational and discontinuous epitopes disclosed herein. If the conformational epitope is a protein secondary structure, such structures often form easily in smaller peptides (e.g., <50 amino acids). Thus, immunizing an animal with smaller peptides could capture some conformational epitopes. Alternatively, two small peptides which comprise a conformational epitope (*e.g.*, the peptides identified in Table 5) may be connected via a flexible linker (*e.g.*, polyglycol, or a stretch of polar, uncharged amino acids). The linker will allow the peptides to explore various interaction orientations. Immunizing with this construct, followed by appropriate screening could allow for identification of antibodies directed to a conformational epitope. In a preferred embodiment, peptides to specific conformational or linear epitopes may be generated by immunizing an animal with a particular domain of an RTK (*e.g.,* domain 4 or domain 5 of the Kit ectodomain or D7 of a VEGF receptor) and subsequently screening for antibodies which bind the epitope of interest. In one embodiment cryoelectron microscopy (Jiang et al. (2008) Nature 451, 1130-1134; Joachim (2006) *Oxford University Press*_ISBN:0195182189) may be used to identify the epitopes bound by an antibody or antigen binding fragment of the invention. In another embodiment, the RTK or a domain thereof may be crystallized with the bound antibody or antigen binding fragment thereof and analyzed by X-ray crystallography to determine the precise epitopes that are bound. In addition, epitopes may be mapped by replacing portions of an RTK sequence with the corresponding sequences from mouse or another species. Antibodies directed to epitopes of interest will selectively bind the human sequence regions and, thus, it is possible to sequentially map target epitopes. This technique of chimera based epitope mapping has been used successfully to identify epitopes in various settings (see Henriksson and Pettersson (1997) Journal of Autoimmunity.

10(6):559-568; Netzer et al. (1999) J Biol Chem. 1999 Apr 16;274(16):11267-74; Hsia et al. (1996) *Mol. Microbiol.* 19, 53-63, the entire contents of which are incorporated herein by reference).

It is believed that the epitopes of interest in target RTKs (*e.g.*, the Kit RTK or a VEGF receptor) are not glycosylated. However, if an RTK of interest is glycosylated, antibodies or antigen binding portions thereof (and other moieties of the invention), may be raised such that they bind to the relevant amino acid and/or sugar residues. For example, it is known in the art that the Kit protein has at least 10 sites for potential N-linked glycosylation (Morstyn, Foote, Lieschke (2004) Hematopoietic Growth Factors in Oncology: Basic Science and Clinical Therapeutics. Humana Press. ISBN:1588293025). It is further thought that Kit may exhibit O-linked glycosylation as well as attachment to sialic acid residues (Wypych J, *et al.*(1995) Blood, 85(1):66-73). Thus, it is contemplated that antibodies or antigen binding portions thereof (and other moieties of the invention), may be raised such that they also bind to sugar residues which may be attached to any epitope identified herein. For this purpose, an antigenic peptide of interest may be produced in an animal cell such that it gets properly glycosylated and the glycosylated antigenic peptide may then be used to immunize an animal. Suitable cells and techniques for producing glycosylated peptides are known in the art and described further below (see, for example, the technologies available from GlycoFi, Inc., Lebanon, NH and BioWa; Princeton, NJ). The proper glycosylation of a peptide may be tested using any standard methods such as isoelectric focusing (IEF), acid hydrolysis (to determine monosaccharide composition), chemical or enzymatic cleavage, and mass spectrometry (MS) to identify glycans. The technology offered by Procognia (procognia.com) which uses a lectin-based array to speed up glycan analysis may also be used. O-glycosylation specifically may be detected using techniques such as reductive alkaline cleavage or "beta elimination", peptide mapping, liquid chromatography, and mass spectrometry or any combination of these techniques.

The preferred animal system for preparing hybridomas is the murine system. Hybridoma production in the mouse is a very well-established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art. Fusion partners (*e.g.*, murine myeloma cells) and fusion procedures are also known.

Chimeric or humanized antibodies of the present invention can be prepared based on the sequence of a murine monoclonal antibody prepared as described above. DNA encoding the heavy and light chain immunoglobulins can be obtained from the murine hybridoma of interest and engineered to contain non-murine (*e.g.*, human) immunoglobulin sequences using standard molecular biology techniques. For example, to create a chimeric antibody, the murine variable regions can be linked to human constant regions using methods known in the art (see *e.g.*, U.S. Patent No. 4,816,567 to Cabilly *et al.*). To create a humanized antibody, the murine CDR regions can be inserted into a human framework using methods known in the art (see *e.g.*, U.S. Patent No. 5,225,539 to Winter, and U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen *et al.*). Alternatively, a humanized antibody may be designed at the DNA or protein level, given knowledge of human and non-human sequences. Such antibodies may be directly synthesized chemically, or the DNA may be synthesized and expressed in vitro or in vivo to produce a humanized antibody.

In a preferred embodiment, the antibodies of the invention are human monoclonal antibodies. Such human monoclonal antibodies directed against an Ig-like domain of an RTK, *e.g.* the D4 or D5 domain of Kit or the D7 domain of a VEGF receptor, can be generated using transgenic or transchromosomic mice carrying parts of the human immune system rather than the mouse system. These transgenic and transchromosomic mice include mice referred to herein as HuMAb mice and KM mice™, respectively, and are collectively referred to herein as "human Ig mice."

The HuMAb mouse® (Medarex, Inc.) contains human immunoglobulin gene miniloci that encode unrearranged human heavy (μ and γ) and κ light chain immunoglobulin sequences, together with targeted mutations that inactivate the endogenous μ and κ chain loci (see *e.g.*, Lonberg, *et al.* (1994) Nature 368 (6474): 856-859). Accordingly, the mice exhibit reduced expression of mouse IgM or κ, and in response to immunization, the introduced human heavy and light chain transgenes undergo class switching and somatic mutation to generate high affinity human IgGκ monoclonal (Lonberg, N. *et al.* (1994), *supra*; reviewed in Lonberg, N. (1994) *Handbook of Experimental Pharmacology* 113:49-101; Lonberg, N. and Huszar, D. (1995) *Intern. Rev. Immunol.* 13: 65-93, and Harding, F. and Lonberg, N. (1995) *Ann. N.Y. Acad. Sci.* 764:536-546). The preparation and use of HuMab mice, and the genomic modifications carried by such mice, is further described in Taylor, L. *et al.*

(1992) *Nucleic Acids Research* 20:6287-6295; Chen, J. *et al.* (1993) *International Immunology* 5: 647-656; Tuaillon *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:3720-3724; Choi *et al.* (1993) *Nature Genetics* 4:117-123; Chen, J. *et al.* (1993) *EMBO J.* 12: 821-830; Tuaillon *et al.* (1994) *J. Immunol.* 152:2912-2920; Taylor, L. *et al.* (1994) *International Immunology* 6: 579-591; and Fishwild, D. *et al.* (1996) *Nature Biotechnology* 14: 845-851, the contents of all of which are hereby specifically incorporated by reference in their entirety. See further, U.S. Patent Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877,397; 5,661,016; 5,814,318; 5,874,299; and 5,770,429; all to Lonberg and Kay; U.S. Patent No. 5,545,807 to Surani *et al.*; PCT Publication Nos. WO 92/03918, WO 93/12227, WO 94/25585, WO 97/13852, WO 98/24884 and WO 99/45962, all to Lonberg and Kay; and PCT Publication No. WO 01/14424 to Korman *et al.*

In another embodiment, human antibodies of the invention can be raised using a mouse that carries human immunoglobulin sequences on transgenes and transchomosomes, such as a mouse that carries a human heavy chain transgene and a human light chain transchromosome. Such mice, referred to herein as "KM mice™", are described in detail in PCT Publication WO 02/43478 to Ishida *et al.*

Still further, alternative transgenic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise the antibodies of the invention. For example, an alternative transgenic system referred to as the Xenomouse (Abgenix, Inc.) can be used; such mice are described in, for example, U.S. Patent Nos. 5,939,598; 6,075,181; 6,114,598; 6, 150,584 and 6,162,963 to Kucherlapati *et al.*

Moreover, alternative transchromosomic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise the antibodies of the invention. For example, mice carrying both a human heavy chain transchromosome and a human light chain tranchromosome, referred to as "TC mice" can be used; such mice are described in Tomizuka *et al.* (2000) *Proc. Natl. Acad. Sci. USA* 97:722-727. Furthermore, cows carrying human heavy and light chain transchromosomes have been described in the art (Kuroiwa *et al.* (2002) *Nature Biotechnology* 20:889-894) and can be used to raise the antibodies of the invention.

Human monoclonal antibodies of the invention can also be prepared using phage display methods for screening libraries of human immunoglobulin genes. Such phage

display methods for isolating human antibodies are established in the art. See for example: U.S. Patent Nos. 5,223,409; 5,403,484; and 5,571,698 to Ladner *et al.*; U.S. Patent Nos. 5,427,908 and 5,580,717 to Dower *et al.*; U.S. Patent Nos. 5,969,108 and 6,172,197 to McCafferty *et al.*; and U.S. Patent Nos. 5,885,793; 6,521,404; 6,544,731; 6,555,313; 6,582,915 and 6,593,081 to Griffiths *et al.*

Human monoclonal antibodies of the invention can also be prepared using SCID mice into which human immune cells have been reconstituted such that a human antibody response can be generated upon immunization. Such mice are described in, for example, U.S. Patent Nos. 5,476,996 and 5,698,767 to Wilson *et al.*

In another embodiment, antibodies of the invention may be raised using well known phage display techniques, as described in Marks, J.D., *et al.* ((1991). J. Mol. Biol. 222, 581), Nissim, A., *et al.* ((1994). EMBO J. 13, 692) and U.S. Patent Nos. 6,794,132; 6562341; 6057098; 5821047; and 6512097.

In a further embodiment, antibodies of the present invention may be found using yeast cell surface display technology as described, for example, in U.S. Patent Nos. 6,423,538; 6,300,065; 6,696,251; 6,699,658.

Generation of Hybridomas Producing Human Monoclonal Antibodies of the Invention

To generate hybridomas producing human monoclonal antibodies of the invention, splenocytes and/or lymph node cells from immunized mice can be isolated and fused to an appropriate immortalized cell line, such as a mouse myeloma cell line. The resulting hybridomas can be screened for the production of antigen-specific antibodies. For example, single cell suspensions of splenic lymphocytes from immunized mice can be fused to one-sixth the number of P3X63-Ag8. 653 nonsecreting mouse myeloma cells (ATCC, CRL 1580) with 50% PEG. Alternatively, the single cell suspension of splenic lymphocytes from immunized mice can be fused using an electric field based electrofusion method, using a CytoPulse large chamber cell fusion electroporator (CytoPulse Sciences, Inc., Glen Burnie Maryland). Cells are plated at approximately 2 x $10^5$ in flat bottom microtiter plate, followed by a two week incubation in selective medium containing 20% fetal Clone Serum, 18% "653" conditioned media, 5% origen (IGEN), 4 mM L-glutamine, 1 mM sodium pyruvate, 5mM HEPES, 0.055 mM 2-mercaptoethanol, 50 units/ml penicillin, 50 mg/ml streptomycin, 50 mg/ml gentamycin and 1X HAT (Sigma; the HAT is added 24 hours after the fusion). After

approximately two weeks, cells can be cultured in medium in which the HAT is replaced with HT. Individual wells can then be screened by ELISA for human monoclonal IgM and IgG antibodies. Once extensive hybridoma growth occurs, medium can be observed usually after 10-14 days. The antibody secreting hybridomas can be replated, screened again, and if still positive for human IgG, the monoclonal antibodies can be subcloned at least twice by limiting dilution. The stable subclones can then be cultured *in vitro* to generate small amounts of antibody in tissue culture medium for characterization.

To purify human monoclonal antibodies, selected hybridomas can be grown in two-liter spinner-flasks for monoclonal antibody purification. Supernatants can be filtered and concentrated before affinity chromatography with protein A-sepharose (Pharmacia, Piscataway, N.J.). Eluted IgG can be checked by gel electrophoresis and high performance liquid chromatography to ensure purity. The buffer solution can be exchanged into PBS, and the concentration can be determined by $OD_{280}$ using 1.43 extinction coefficient. The monoclonal antibodies can be aliquoted and stored at -80° C.

Generation of Transfectomas Producing Monoclonal Antibodies of the Invention

Antibodies of the invention also can be produced in a host cell transfectoma (a type of hybridoma) using, for example, a combination of recombinant DNA techniques and gene transfection methods as is well known in the art (*e.g.*, Morrison, S. (1985) Science 229:1202).

For example, to express the antibodies, or antibody fragments thereof, DNAs encoding partial or full-length light and heavy chains, can be obtained by standard molecular biology techniques (*e.g.*, PCR amplification or cDNA cloning using a hybridoma that expresses the antibody of interest) and the DNAs can be inserted into expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" is intended to mean that an antibody gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy chain gene can be inserted into separate vector or, more typically, both genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by

standard methods (*e.g.*, ligation of complementary restriction sites on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are present). The light and heavy chain variable regions of the described antibodies can be used to create full-length antibody genes of any antibody isotype by inserting them into expression vectors already encoding heavy chain constant and light chain constant regions of the desired isotype such that the $V_H$ segment is operatively linked to the $C_H$ segment(s) within the vector and the $V_K$ segment is operatively linked to the $C_L$ segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of the antibody chain from a host cell. The antibody chain gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the antibody chain gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (*i.e.*, a signal peptide from a non-immunoglobulin protein).

In addition to the antibody chain genes, the recombinant expression vectors of the invention carry regulatory sequences that control the expression of the antibody chain genes in a host cell. The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, for example, in Goeddel (Gene Expression Technology. Methods in Enzymology 185, Academic Press, San Diego, CA (1990)). It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences, may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, *etc.* Preferred regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV), Simian Virus 40 (SV40), adenovirus, (*e.g.*, the adenovirus major late promoter (AdMLP) and polyoma. Alternatively, nonviral regulatory sequences may be used, such as the ubiquitin promoter or β-globin promoter. Still further, regulatory elements composed of sequences from different sources, such as the SR promoter system, which contains sequences from the SV40 early promoter and the long terminal repeat of human T cell leukemia virus type 1 (Takebe, Y. *et al.* (1988) *Mol. Cell. Biol.* 8:466-472).

In addition to the antibody chain genes and regulatory sequences, the recombinant expression vectors of the invention may carry additional sequences, such as sequences that regulate replication of the vector in host cells (*e.g.*, origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (see, *e.g.*, U.S. Pat. Nos. 4,399,216, 4,634,665 and 5,179,017, all by Axel *et al.*). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in dhfr- host cells with methotrexate selection/amplification) and the neo gene (for G418 selection).

For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains is transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, *e.g.*, electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells, and most preferably mammalian host cells, is the most preferred because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody. Prokaryotic expression of antibody genes has been reported to be ineffective for production of high yields of active antibody (Boss, M. A. and Wood, C. R. (1985) *Immunology Today* 6:12-13).

Preferred mammalian host cells for expressing the recombinant antibodies of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin, (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, *e.g.*, as described in R. J. Kaufman and P. A. Sharp (1982) *Mol. Biol. 159*:601-621), NSO myeloma cells, COS cells and SP2 cells. In particular, for use with NSO myeloma cells, another preferred expression system is the GS gene expression system disclosed in WO 87/04462, WO 89/01036 and EP 338,841. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more

preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Characterization of Antibody Binding to an Ig-like Domain of a RTK

Antibodies of the invention can be tested for binding to the ectodomain, *e.g.*, an Ig-like domain of a RTK (or any chosen region such as the concensus sequences discussed herein) by, for example, standard ELISA. Briefly, microtiter plates are coated with the purified Ig-like domain (or a preferred receptor domain) at 0.25μg /ml in PBS, and then blocked with 5% bovine serum albumin in PBS. Dilutions of antibody (*e.g.*, dilutions of plasma from immunized mice, *e.g.*, mice immunized with the D4 or D5 domain of human Kit) are added to each well and incubated for 1-2 hours at 37°C. The plates are washed with PBS/Tween and then incubated with secondary reagent (*e.g.*, for human antibodies, a goat-anti-human IgG Fc-specific polyclonal reagent) conjugated to alkaline phosphatase for 1 hour at 37°C. After washing, the plates are developed with pNPP substrate (1 mg/ml), and analyzed at OD of 405-650. Preferably, mice which develop the highest titers will be used for fusions.

An ELISA assay as described above can also be used to screen for hybridomas that show positive reactivity with immunogen. Hybridomas that bind with high avidity to, *e.g.*, an Ig-like domain of an RTK, are subcloned and further characterized. One clone from each hybridoma, which retains the reactivity of the parent cells (by ELISA), can be chosen for making a 5-10 vial cell bank stored at -140 °C, and for antibody purification.

To purify anti-RTK antibodies, selected hybridomas can be grown in two-liter spinner-flasks for monoclonal antibody purification. Supernatants can be filtered and concentrated before affinity chromatography with protein A-sepharose (Pharmacia, Piscataway, NJ). Eluted IgG can be checked by gel electrophoresis and high performance liquid chromatography to ensure purity. The buffer solution can be exchanged into PBS, and the concentration can be determined by $OD_{280}$ using 1.43 extinction coefficient. The monoclonal antibodies can be aliquoted and stored at -80 °C.

To determine if the selected monoclonal antibodies bind to unique epitopes, each antibody can be biotinylated using commercially available reagents (Pierce, Rockford,

IL). Competition studies using unlabeled monoclonal antibodies and biotinylated monoclonal antibodies can be performed using RTK coated ELISA plates coated with an Ig-like domain of a RTK (*e.g.*, Kit-D4 domain, Kit-D5 domain, or a VEGF receptor D7 domain) as described above. Biotinylated mAb binding can be detected with a strep-avidin-alkaline phosphatase probe.

To determine the isotype of purified antibodies, isotype ELISAs can be performed using reagents specific for antibodies of a particular isotype. For example, to determine the isotype of a human monoclonal antibody, wells of microtiter plates can be coated with 1 μg/ml of anti-human immunoglobulin overnight at 4° C. After blocking with 1% BSA, the plates are reacted with 1 μg /ml or less of test monoclonal antibodies or purified isotype controls, at ambient temperature for one to two hours. The wells can then be reacted with either human IgG1 or human IgM-specific alkaline phosphatase-conjugated probes. Plates are developed and analyzed as described above.

Anti-RTK human IgGs can be further tested for reactivity with an Ig-like domain of a RTK or a concensus sequence presented herein by Western blotting. Briefly, an Ig-like domain of a RTK, such as the D4 or D5 domain of the Kit RTK or the D7 domain of a VEGF receptor, can be prepared and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, the separated antigens are transferred to nitrocellulose membranes, blocked with 10% fetal calf serum, and probed with the monoclonal antibodies to be tested. Human IgG binding can be detected using anti-human IgG alkaline phosphatase and developed with BCIP/NBT substrate tablets (Sigma Chem. Co., St. Louis, Mo.).

Epitope mapping may be employed to determine the binding site of an antibody or antigen binding fragment thereof of the invention. Several methods are available which further allow the mapping of conformational epitopes. For example, the methods disclosed in Timmerman *et al.* (Mol Divers. 2004;8(2):61-77) may be used. Timmerman *et al.* were able to successfully map discontinuous/conformational epitopes using two novel techniques, Domain Scan and Matrix Scan. The techniques disclosed in Ansong *et al.* (J Thromb Haemost. 2006. 4(4):842-7) may also be used. Ansong *et al.* used affinity directed mass spectrometry to map the discontinuous epitope recognized by the antibody R8B12. In addition, imaging techniques such as Protein Tomography may be used to visualize antibody or peptide binding to target RTKs. Protein Tomography has been used previously to gain insight into molecular interactions, and was used to

show that an inhibitory antibody acted by binding domain III of EGFR thereby locking EGFR into an inflexible and inactive conformation (Lammerts *et al.* Proc Natl Acad Sci U S A. 2008.105(16):6109-14). More traditional methods such as site-directed mutagenesis may also be applied to map discontinuous epitopes. Amino acid regions thought to participate in a discontinuous epitope may be selectively mutated and assayed for binding to an antibody or antigen binding fragment thereof of the invention. The inability of the antibody to bind when either region is mutated may indicate that binding is dependent upon both amino acid segments. As noted above, some linear epitopes are characterized by particular three-dimensional structures which must be present in order to bind a moiety of the invention. Such epitopes may be discovered by assaying the binding of the antibody (or another moiety) when the RTK is in its native or folded state and again when the RTK is denatured. An observation that binding occurs only in the folded state would indicate that the epitope is either a linear epitope characterized by a particular folded structure or a discontinuous epitope only present in folded protein.

In addition to the activity assays described herein, Protein Tomography may be used to determine whether an antibody or antigen binding fragment thereof of the invention is able to bind and inactivate a receptor tyrosine kinase. Visualization of the binding interaction may indicate that binding of the antibody may affect the positioning of the two ectodomains at the cell surface interface or alter or prevent conformational changes in the receptor tyrosine kinase.

II. Small Molecules Which Bind To An Ig-Like Domain or a Hinge Region Of A Human Receptor Tyrosine Kinase

In another aspect of the invention, the moiety that binds to the ectodomain, *e.g.*, an Ig-like domain or a hinge region, of a human receptor tyrosine kinase is a small molecule.

The small molecules of the instant invention are characterized by particular functional features or properties. For example, the small molecules bind to an Ig-like domain of a RTK, *e.g.*, the D4 or D5 domain of Kit RTK or the D7 domain of a VEGF receptor, or a hinge region of a RTK, *e.g.*, the D3-D4 or D4-D5 hinge regions of the Kit RTK. In preferred embodiments, the binding of small molecule inhibitors to the D3-D4 or the D4-D5 hinge regions will prevent the movement that enables the membrane proximal D4 and D5 domains to be at a distance and orientation (position) that allows

trans-autophosphorylation and activation of the tyrosine kinase domain followed by recruitment and activation of downstream signaling pathways. The small molecule binding may, in some embodiments, allow the ectodomain of the receptor tyrosine kinase to dimerize but affects the positioning, orientation and/or distance between the Ig-like domains of the two monomers (*e.g.*, the D4-D4 or D5-D5 domains of a type III receptor tyrosine kinase or the D7-D7 domains of a type V receptor tyrosine kinase), thereby inhibiting the activity of the receptor tyrosine kinase. In other words, the moiety or small molecule may allow ligand induced dimerization of the receptor tyrosine kinase ectodomains, but affect the positioning of the two ectodomains at the cell surface interface, thereby inhibiting the activity of the receptor tyrosine kinase (*e.g.*, inhibiting receptor internalization and/or inhibiting tyrosine autophosphorylation of the receptor and/or inhibiting the ability of the receptor to activate a downstream signaling pathway).

The terms "small molecule compounds", "small molecule drugs" , "small molecules", or "small molecule inhibitors" are used interchangeably herein to refer to the compounds of the present invention screened for an effect on RTKs and their ability to inhibit the dimerization or activity of the RTK, *e.g.,* the Kit RTK or a VEGF receptor. These compounds may comprise compounds in PubChem database (pubchem.ncbi.nlm.nih.gov/), the Molecular Libraries Screening Center Network (MLSCN) database, compounds in related databases, or derivatives and/or functional analogues thereof.

As used herein, "analogue" or "functional analogue" refers to a chemical compound or small molecule inhibitor that is structurally similar to a parent compound, but differs slightly in composition (*e.g.*, one or more atoms or functional groups are added, removed, or modified). The analogue may or may not have different chemical or physical properties than the original compound and may or may not have improved biological and/or chemical activity. For example, the analogue may be more hydrophobic or it may have altered activity (increased, decreased, or identical to parent compound) as compared to the parent compound. The analogue may be a naturally or non-naturally occurring (*e.g.*, recombinant) variant of the original compound. Other types of analogues include isomers (enantiomers, diasteromers, and the like) and other types of chiral variants of a compound, as well as structural isomers. The analogue may be a branched or cyclic variant of a linear compound. For example, a linear compound may have an analogue that is branched or otherwise substituted to impart certain

desirable properties (*e.g.*, improve hydrophilicity or bioavailability).

As used herein, "derivative" refers to a chemically or biologically modified version of a chemical compound or small molecule inhibitor that is structurally similar to a parent compound and (actually or theoretically) derivable from that parent compound. A "derivative" differs from an "analogue" or "functional analogue" in that a parent compound may be the starting material to generate a "derivative," whereas the parent compound may not necessarily be used as the starting material to generate an "analogue" or "functional analogue." A derivative may or may not have different chemical or physical properties of the parent compound. For example, the derivative may be more hydrophilic or it may have altered reactivity as compared to the parent compound. Derivatization (*i.e.*, modification by chemical or other means) may involve substitution of one or more moieties within the molecule (*e.g.*, a change in functional group). For example, a hydrogen may be substituted with a halogen, such as fluorine or chlorine, or a hydroxyl group (--OH) may be replaced with a carboxylic acid moiety (--COOH). The term "derivative" also includes conjugates, and prodrugs of a parent compound (*i.e.*, chemically modified derivatives which can be converted into the original compound under physiological conditions). For example, the prodrug may be an inactive form of an active agent. Under physiological conditions, the prodrug may be converted into the active form of the compound. Prodrugs may be formed, for example, by replacing one or two hydrogen atoms on nitrogen atoms by an acyl group (acyl prodrugs) or a carbamate group (carbamate prodrugs). More detailed information relating to prodrugs is found, for example, in Fleisher *et al.*, Advanced Drug Delivery Reviews 19 (1996) 115; Design of Prodrugs, H. Bundgaard (ed.), Elsevier, 1985; and H. Bundgaard, Drugs of the Future 16 (1991) 443. The term "derivative" is also used to describe all solvates, for example hydrates or adducts (*e.g.*, adducts with alcohols), active metabolites, and salts of the parent compound. The type of salt that may be prepared depends on the nature of the moieties within the compound. For example, acidic groups such as carboxylic acid groups can form alkali metal salts or alkaline earth metal salts (*e.g.*, sodium salts, potassium salts, magnesium salts, calcium salts, and salts with physiologically tolerable quaternary ammonium ions and acid addition salts with ammonia and physiologically tolerable organic amines such as triethylamine, ethanolamine, or tris-(2-hydroxyethyl)amine). Basic groups can form acid addition salts, for example with inorganic acids such as hydrochloric acid ("HCl"), sulfuric acid, or

phosphoric acid, or with organic carboxylic acids and sulfonic acids such as acetic acid, citric acid, benzoic acid, maleic acid, fumaric acid, tartaric acid, methanesulfonic acid, or p-toluenesulfonic acid. Compounds which simultaneously contain a basic group and an acidic group such as a carboxyl group in addition to basic nitrogen atoms can be present as zwitterions. Salts can be obtained by customary methods known to those skilled in the art, for example by combining a compound with an inorganic or organic acid or base in a solvent or diluent, or from other salts by cation exchange or anion exchange.

Small molecules are known to have molecular weights of 1200 or below, 1000 or below, 900 or below, 800 or below, 700 or below, 600 or below, 500 or below, 400 or below, 300 or below, 200 or below, 100 or below, 50 or below, 25 or below, or 10 or below.

The small molecule inhibitors of the present invention are selected or designed to bind to the ectodomain, *e.g.*, an Ig-like domain or a hinge region, of a RTK. In some embodiments, the small molecule inhibitors are selected or designed to bind an Ig-like domain or a hinge region of human Kit, a human VEGF receptor or PDGFR, *e.g.*, the D4 or D5 domain, or the D3-D4 and/or D4-D5 hinge regions of the human Kit receptor, thereby antagonizing the ability of the receptor to dimerize and become active, *e.g.*, autophosphorylate and activate an intracellular signal transduction pathway. In other embodiments the small molecule inhibitors are selected to bind domains sharing homology to a domain of the Kit receptor or VEGF receptor. For example, a small molecule of the present invention may be directed toward a domain which is at least 50% identical, at least 60% identical, at least 70% identical, at least 80% identical, at least 90% identical, or at least 95% or 99% identical to an Ig-like domain of a RTK, *e.g.*, the D4 or D5 domain of Kit or the D7 domain of a VEGF receptor; or a hinge region of a RTK, *e.g.*, the D3-D4 or D4-D5 hinge regions, of the Kit or PDGFR receptor. Such a small molecule would be capable of binding protein domains, possibly in Kit, VEGF receptors and other RTKs, which are functionally similar to, for example, the D4, D5 or D7 domains or the D3-D4 and/or D4-D5 hinge regions of the Kit or PDGF receptor .

The small molecule inhibitors of the present invention may also bind to a particular motif or consensus sequence derived from an Ig-like domain or a hinge region of a RTK, *e.g.*, a human VEGF receptor or a human type III RTK, allowing the small

molecule inhibitors to specifically bind domains which are shared among members of the RTK family, *e.g.*, members of the human type III family of RTKs.

In a specific embodiment, a small molecule of the present invention binds to the following consensus sequence for the D4 interaction site: $LX_1RX_2X_3X_4X_5X_6X_7G$ wherein L is Leucine, R is Arginine, G is Glycine; and $X_1$, $X_2$, $X_3$, $X_4$, $X_5$, $X_6$ and $X_7$ are any amino acid. In a specific embodiment, $X_1$ is selected from the group consisting of Threonine, Isoleucine, Valine, Proline, Asparagine, or Lysine; X2 is selected from the group consisting of Leucine, Valine, Alanine, and Methionine; $X_3$ is selected from the group consisting of Lysine, Histidine, Asparagine, and Arginine; $X_4$ is selected from the group consisting of Glycine, Valine, Alanine, Glutamic Acid, Proline, and Methionine; $X_5$ is selected from the group consisting of Threonine, Serine, Glutamic Acid, Alanine, Glutamine, and Aspartic acid; $X_6$ is selected from the group consisting of Glutamic Acid, Aspartic acid, and Glutamine; and $X_7$ is selected from the group consisting of Glycine, Serine, Alanine, Lysine, Arginine, Glutamine, and Threonine.

In another embodiment a small molecule of the present invention binds binds to the following consensus sequence for the D7 domain of a member of the VEGF receptor family: $IX_1RVX_2X_3EDX_4G$ wherein I is Isoleucine, R is Arginine, E is Glutamic Acid, D is Aspartic Acid, G is Glycine; and $X_1$, $X_2$, $X_3$ and $X_4$ are any amino acid. In a specific embodiment, $X_1$ is selected from the group consisting of Glutamic Acid, Arginine, and Glutamine; X2 is selected from the group consisting of Arginine and Threonine; $X_3$ is selected from the group consisting of Glutamic Acid and Lysine; and $X_4$ is selected from the group consisting of Glutamic Acid and Alanine (SEQ ID NO: 1).

In another embodiment, a moiety of the present invention (*e.g.,* a small molecule) binds to the following consensus sequence for the D7 domain of a VEGF receptor: L/I $X_1$ R Φ $X_2$ $X_3$ $X_4$ D/E $X_5$ G (SEQ ID NO:158), wherein L is Leucine, I is Isoleucine, R is Arginine, Φ is a hydrophobic amino acid, D is Aspartic Acid, E is Glutamic Acid, G is Glycine; and $X_1$, $X_2$, $X_3$, $X_4$ and $X_5$ are any amino acid. In a specific embodiment, Φ is Valine; $X_1$ is selected from the group consisting of Arginine, Glutamine, Glutamic Acid and Aspartic Acid; $X_2$ is selected from the group consisting of Arginine, Lysine and Threonine; $X_3$ is selected from the group consisting of Lysine, Glutamic Acid, Glutamine and Valine; $X_4$ is selected from the group consisting of Glutamic Acid and Valine; and $X_5$ is selected from the group consisting of Glutamic Acid, Glycine, Serine and Glutamine.

In other embodiments, small molecule inhibitors bind protein motifs or consensus sequences which represent the three dimensional structure of the protein. Such motifs or consensus sequences would not represent a contiguous string of amino acids, but a non-linear amino acid arrangement that results from the three-dimensional folding of the RTK (*i.e.*, a structural motif). An example of such a motif would be a motif designed based on the D3-D4 and/or D4-D5 hinge regions of the Kit receptor. Such motifs and consensus sequences may be designed according to the methods discussed in Section I regarding antibodies.

Importantly, a small molecule inhibitor of the invention does not bind to the ligand binding site of the RTK, *e.g.*, the SCF binding site of the Kit receptor. In other words, the small molecule inhibitor does not bind to the Ig-like domains of a RTK responsible for ligand binding.

In another embodiment, the small molecule inhibitor of the invention binds to a contiguous epitope on the VEGF receptor. In one embodiment, the contiguous epitope is composed of two or more residues in the D7 domain of the VEGF receptor. In another embodiment, the contiguous epitope is an epitope selected from the group consisting of $^{672}$VAISSS$^{677}$ of VEGFR1, $^{678}$TTLDCHA$^{684}$ of VEGFR1, $^{685}$NGVPEPQ$^{691}$ of VEGFR1, $^{700}$KIQQEPG$^{706}$ of VEGFR1, $^{707}$IILG$^{710}$ of VEGFR1, $^{711}$PGS$^{713}$ of VEGFR1, $^{714}$STLFI$^{718}$ of VEGFR1, $^{719}$ERVTEEDEGV$^{728}$ of VEGFR1, $^{689}$VNVSDS$^{694}$ of VEGFR3, $^{695}$LEMQCLV$^{701}$ of VEGFR3, $^{702}$AGAHAPS$^{708}$ of VEGFR3, $^{717}$LLEEKSG$^{723}$ of VEGFR3, $^{724}$VDLA$^{727}$ of VEGFR3, $^{728}$DSN$^{730}$ of VEGFR3, $^{731}$QKLSI$^{735}$ of VEGFR3, and $^{736}$QRVREEDAGR$^{745}$ of VEGFR3, $^{678}$TSIGES$^{683}$ of VEGFR2, $^{684}$IEVSCTA$^{690}$ of VEGFR2, $^{691}$SGNPPPQ$^{697}$ of VEGFR2, $^{706}$TLVEDSG$^{712}$ of VEGFR2, $^{713}$IVLK$^{716}$ of VEGFR2, $^{717}$DGN$^{719}$ of VEGFR2, $^{720}$RNLTI$^{724}$ of VEGFR2 and $^{725}$RRVRKEDEGL$^{734}$ of VEGFR2.

In additional embodiments, small molecule inhibitors of the invention are selected or designed to bind specifically to a mutant ectodomain, *e.g.*, a mutant Ig-like domain or a mutant hinge region, of a RTK. In preferred embodiments, the mutant RTK is a tumorigenic or an oncogenic mutant. In one specific embodiment, the small molecule inhibitor is selected or designed to bind to an oncogenic Kit receptor mutant. Kit receptor mutants which may be targeted by the small molecules of the instant invention are Kit receptors with mutations in one or more of the following amino acids: Thr417, Tyr418, Asp419, Leu421, Arg420, Tyr503, or Ala502. It should be appreciated

by one of skill in the art that the methods of the invention would be applicable to other mutations in Kit or to mutations in other RTKs. One advantage of targeting a mutant RTK is that a therapeutic small molecule may bind to only the RTKs on cells containing the mutation, leaving healthy cells largely or entirely unaffected. Accordingly, in instances where the mutation is tumorigenic, only tumor cells would be targeted for therapy, potentially reducing side effects and dosage requirements.

In some embodiments the small molecule binds to specific sequences of the human Kit receptor, for example, residues 309-413, residues 410-519, $^{381}$Arg and $^{386}$Glu, or $^{418}$Tyr and $^{505}$Asn of the human Kit receptor. In some embodiments, the small molecule binds to specific sequences of a human VEGF receptor, for example, residues 718-727 of VEGFR1, Arg720 and Asp725 of VEGFR1, residues 724-733 of VEGFR2, Arg726 and Asp731 of VEGFR2, residues 735-744 of VEGFR3, or residues Arg737 and Asp742 of VEGFR3.

In a preferred embodiment, a small molecule of the invention may bind to one or more residues in the Kit receptor which make up the small cavities or pockets described in Table 4 (below). For example, a small molecule of the invention may bind to one or more of the following residues in the D3-D4 hinge region of the Kit receptor: K218, S220, Y221, L222 from the D3 domain and F340, P341, K342, N367, E368, S369, N370, I371, Y373 from the D4 domain. A small molecule of the invention may also bind to one or more of the following residues which make up a concave surface in the D4 domain of the Kit receptor:Y350, R353, F355, K358, L379, T380, R381, L382, E386 and T390. In another embodiment, a small molecule of the invention may bind to one or more of the following residues which form a pocket in the D2-D3 hinge region of the Kit receptor:Y125, G126, H180, R181, K203, V204, R205, P206 and F208 from the D2 domain and V238, S239, S240, S241, H263, G265, D266, F267, N268 and Y269 from the D3 domain.

Thus, in some embodiments, a small molecule of the invention may bind to contiguous or non-contiguous amino acid residues and function as a molecular wedge that prevents the motion required for positioning of the membrane proximal region of the RTK at a distance and orientation that enables tyrosine kinase activation. A small molecule of the invention may also act to prevent homotypic D4 or D5 receptor interactions or destabilize the ligand- receptor interaction site. In some preferred embodiments, a small molecule of the invention may bind to one or more of the

following residues on the Kit receptor: Y125, G126, H180, R181, K203, V204, R205, P206, P206, F208, K127, A207, V238, S239, S240, S241, H263, G265, D266, F267, N268, Y269, T295, L222, L222, L223, E306, V308, R224, V308, K310, K218, A219, S220, K218, A220, Y221, A339, D327, D398, E338, E368, E386, F312, F324, F340, F355, G311, G384, G387, G388, I371, K342, K358, L382, L379, N326, N367, N370, N410, P341, S369, T385, V325, V407, V409, Y373, Y350, Y408, T380, T390, R381, R353, T411, K412, E414, K471, F433, G470, L472, V497, F469, A431, or G432. One of skill in the art will appreciate that, in some embodiments, a small molecule of the invention may be easily targeted to the corresponding residues in other type III RTKs, *e.g.*, those residues that form similar pockets or cavities or those in the same position by structural alignment or sequence alignment.

In a specific embodiment, a small molecule of the invention binds to a conformational epitope or a discontinuous epitope on a type III RTK or a type V RTK. The conformational or discontinuous epitope may be composed of two or more residues from the D3, D4, or D5 domain or the D4-D5 or D3-D4 hinge regions from a type III RTK, *e.g.*, the human Kit receptor or the PDGF receptor, or two or more residues from the D7 domain of a VEGF receptor. For example, the conformational or discontinuous epitope may be composed of two or more of the residues listed in Table 4 below. In a particular embodiment, a small molecule of the invention binds to a conformational epitope composed of 2 or more amino acids selected from the group consisting of Y125, H180, R181, K203, V204, R205, P206. V238, S239, S240, H263, G265, D266, F267, N268, and Y269. In similar embodiments, a small molecule of the invention may bind to a conformational epitope composed of 2 or more amino acids selected from one of the following groups of amino acids: P206, F208, V238, and S239; K127, A207, F208, and T295; L222, A339, F340, K342, E368, S369, N370, I371, and Y373; L222, L223, E306, V308, F312, E338, F340, and I371; R224, V308, K310, G311, F340, P341, and D398; K218, A219, S220, N367, E368, and S369; K218, A220, E368, and S369; G384, T385, T411, K412, E414, and K471; Y408, F433, G470, K471, and L472; F324, V325, N326, and N410;D327, N410, T411, K412, and V497; G384, G387, V409, and K471; L382, G387, V407, and V409; Y125, G126, H180, R181, K203, V204, R205, P206, F208, V238, S239, S240, S241, H263, G265, D266, F267, N268, and Y269; P206, F208, V238, and S239; K218, S220, Y221, L222, F340, P341, K342, N367, E368, S369, N370, I371, and Y373; G384, G387, G388, Y408, V409, T411, F433, F469, G470, and

K471; D327, T411, K412, E414, A431, G432, and K471; Y350, F355, K358, L379, T380, R381, L382, E386, and T390; Y350, R353, and F355. As indicated above, the small molecules of the invention may bind to all of the amino acid residues forming a pocket or a cavity identified in Table 4 or they may bind to a subset of the residues forming the pocket or the cavity. It is to be understood that, in certain embodiments, when reference is made to a small molecule of the invention binding to an epitope, *e.g.*, a conformational epitope, the intention is for the small molecule to bind only to those specific residues that make up the epitope (*e.g.*, the pocket or cavity identified in Table 4) and not other residues in the linear amino acid sequence of the receptor.

In a further embodiment, a small molecule of the invention binds to a conformational epitope wherein the conformational epitope is composed of two or more amino acid residues selected from the peptides listed in Table 5. In a specific embodiment, the conformational epitope is composed of one or more amino acid residues selected from a first peptide and one or more amino acid residues selected from a second peptide, wherein the first and second peptides are selected from the group of peptides listed in Table 5. As such, a small molecule of the invention binds a conformational epitope wherein the first and second peptide groups are as follows: Ala219-Leu222 and Thr304-Val308; Asp309-Gly311 and Arg224-Gly226; Thr303 - Glu306 and Ala219-Leu222; Asn367–Asn370 and Ser217–Tyr221; Ala339–Pro343 and Asn396–Val399; Ala339–Pro343 and Glu368-Arg372; Lys358–Tyr362 and Val374–His378; Asp357-Glu360 and Leu377–Thr380; Met351–Glu360 and His378-Thr389; His378-Thr389 and Val323-Asp332; Val409–Ile415 and Ala493–Thr500; Val409–Ile415 and Ala431-Thr437; Val409– Ile415 and Phe469-Val473; Val409–Ile415 and Val325–Asn330; Val409–Ile415 and Arg381-Gly387; Gly466–Leu472 and Gly384–Gly388; Val325-Glu329 and Tyr494-Lys499; Thr411-leu416 and Val497-Ala502; Ile415–Leu421 and Ala502–Ala507; Ala502–Ala507 and Lys484-Thr488; and Ala502–Ala507 and Gly445–Cys450. The small molecules of the invention may bind to all of the amino acid residues forming the foregoing first and second peptide groups or they may bind to a subset of the residues forming the first and second peptide groups. It is to be understood that, in certain embodiments, when reference is made to a small molecule of the invention binding to an epitope, *e.g.*, a conformational epitope, the intention is for the small molecule to bind only to those specific residues that make up the epitope (*e.g.*,

the specific peptides identified in Table 5) and not other residues in the linear amino acid sequence of the receptor.

In another embodiment, a small molecule of the invention binds to a conformational or discontinuous epitope composed of 2 or more amino acids selected from the group consisting of E33, P34, D72, E76, N77, K78, Q79, K158, D159, N250, S251, Q252, T253, K254, L255, N260, W262, H264, G265, E344, N352, R353, F355, T356, D357, Y362, S365, E366, N367, N370, and G466.

In another embodiment, a small molecule of the invention binds to amino acid residues $^{385}$Arg and $^{390}$Glu of human PDGFRβ, or the corresponding residues in PDGFRα. The residues $^{385}$Arg and $^{390}$Glu of human PDGFRβ are analogous to the residues $^{381}$Arg and $^{386}$Glu of the Kit receptor and mediate homotypic D4-D4 interactions of PDGFRβ. Small molecules of the invention may exert their inhibitory effect on receptor activation by preventing critical homotypic interactions (such as salt bridges formed between $^{385}$Arg and $^{390}$Glu of human PDGFRβ) between membrane proximal regions of type-III RTKs that are essential for positioning the cytoplasmic domain at a distance and orientation essential for tyrosine kinase activation. Experiments discussed herein demonstrate that homotypic D4-D4 interactions are dispensable for PDGFRβ dimerization and that PDGFRβ dimerization is necessary but not sufficient for receptor activation. Thus, small molecules of the invention may allow dimerization of PDGFRβ while preventing activation. Structure based sequence alignment has shown that the size of the EF loop, and the critical amino acids comprising the D4-D4 interface are conserved in Kit, PDGFRα, PDGFRβ, and CSF1R. Thus in some embodiments, small molecules of the invention may be targeted to the conserved regions of the D4 or D5 domains of type III RTKs.

In preferred embodiments, a small molecule of the invention binds to an Ig-like domain or hinge region of Kit (*e.g.*, the D3-D4 and/or D4-D5 hinge regions or the D4-D4 and/or D5-D5 interface binding site of the Kit receptor) with high affinity, for example, with an affinity of a $K_D$ of 1 x $10^{-7}$ M or less, a $K_D$ of 5 x $10^{-8}$ M or less, a $K_D$ of 1 x $10^{-8}$ M or less, a $K_D$ of 5 x $10^{-9}$ M or less, or a $K_D$ of between 1 x $10^{-8}$ M and 1 x $10^{-10}$ M or less.

Small molecule inhibitors of the invention may be made or selected by several methods known in the art. Screening procedures can be used to identify small molecules from libraries which bind desired Ig-like domains or hinge regions of a RTK, *e.g.*, the

D4 or D5 domain of human Kit RTK. One method, Chemetics® (Nuevolutions) uses DNA tags for each molecule in the library to facilitate selection. The Chemetics® system allows screening of millions of compounds for target binding. Patents related to small molecule libraries and tag based screening are U.S. Pat. Application Nos. 20070026397; 20060292603; 20060269920; 20060246450; 20060234231; 20060099592; 20040049008; 20030143561 which are incorporated herein by reference in their entirety.

Other well known methods that may be used to identify small molecules from libraries which bind desired Ig-like domains or hinge regions of a RTK, *e.g.*, the D4 or D5 domain of human Kit RTK or the D7 domain of a VEGF receptor, include methods which utilize libraries in which the library members are tagged with an identifying label, that is, each label present in the library is associated with a discreet compound structure present in the library, such that identification of the label tells the structure of the tagged molecule. One approach to tagged libraries utilizes oligonucleotide tags, as described, for example, in PCT Publication No. WO 2005/058479 A2 (the Direct Select™ technology) and in US Patent Nos. 5,573,905; 5,708,153; 5,723,598, 6,060,596 published PCT applications WO 93/06121; WO 93/20242; WO 94/13623; WO 00/23458; WO 02/074929 and WO 02/103008, and by Brenner and Lerner (*Proc. Natl. Acad. Sci. USA* 89, 5381-5383 (1992); Nielsen and Janda (*Methods: A Companion to Methods in Enzymology* 6, 361-371 (1994); and Nielsen, Brenner and Janda (*J. Am. Chem. Soc.* 115, 9812-9813 (1993)), the entire contents of each of which are incorporated herein by reference in their entirety. Such tags can be amplified, using for example, polymerase chain reaction, to produce many copies of the tag and identify the tag by sequencing. The sequence of the tag then identifies the structure of the binding molecule, which can be synthesized in pure form and tested for activity.

Preparation and screening of combinatorial chemical libraries is well known to those skilled in the art. Such combinatorial chemical libraries which may be used to identify moieties of the invention include, but are not limited to, peptide libraries (see, *e.g.*, U.S. Pat. No. 5,010,175, Furka, *Int. J. Pept. Prot. Res.* 37:487 493 (1991) and Houghton et al., *Nature* 354:84 88 (1991)). Other chemistries for generating chemical diversity libraries are well known in the art and can be used. Such chemistries include, but are not limited to: peptoids (*e.g.*, PCT Publication No. WO 91/19735), encoded peptides (*e.g.*, PCT Publication WO 93/20242), random bio-oligomers (*e.g.*, PCT

Publication No. WO 92/00091), benzodiazepines (*e.g.*, U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs et al., *Proc. Nat. Acad. Sci.* USA 90:6909 6913 (1993)), vinylogous polypeptides (Hagihara et al., *J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann et al., *J. Amer. Chem. Soc.* 114:9217 9218 (1992)), analogous organic syntheses of small compound libraries (Chen et al., *J. Amer. Chem. Soc.* 116:2661 (1994)), oligocarbamates (Cho et al., *Science* 261:1303 (1993)), and/or peptidyl phosphonates (Campbell et al., *J. Org. Chem.* 59:658 (1994)), nucleic acid libraries (see Ausubel, Berger and Russell & Sambrook, all supra), peptide nucleic acid libraries (see, *e.g.*, U.S. Pat. No. 5,539,083), carbohydrate libraries (see, *e.g.*, Liang et al., *Science*, 274:1520 1522 (1996) and U.S. Pat. No. 5,593,853), small organic molecule libraries (see, *e.g.*, benzodiazepines, Baum C&EN, Jan 18, page 33 (1993); isoprenoids, U.S. Pat. No. 5,569,588; thiazolidinones and metathiazanones, U.S. Pat. No. 5,549,974; pyrrolidines, U.S. Pat. Nos. 5,525,735 and 5,519,134; morpholino compounds, U.S. Pat. No. 5,506,337; benzodiazepines, 5,288,514, and the like). Each of the foregoing publications is incorporated herein by reference. Public databases are also available and are commonly used for small molecule screening, *e.g.*, PubChem (pubchem.ncbi.nlm.nih.gov), Zinc (Irwin and Shoichet (2005) J. Chem. Inf. Model. 45(1):177-82), and ChemBank (Seiler et al. (2008) Nucleic Acids Res. 36(Database issue): D351–D359).

Devices for the preparation of combinatorial libraries are commercially available (see, *e.g.*, 357 MPS, 390 MPS, Advanced Chem Tech, Louisville Ky., Symphony, Rainin, Woburn, Mass., 433A Applied Biosystems, Foster City, Calif., 9050 Plus, Millipore, Bedford, Mass.). In addition, numerous combinatorial libraries are themselves commercially available (see, *e.g.*, ComGenex, Princeton, N.J., Tripos, Inc., St. Louis, Mo., 3D Pharmaceuticals, Exton, Pa., Martek Biosciences, Columbia, Md., etc.). Moreover, since screening methodologies are so well defined, it is common to contract specialist firms to identifiy particular compounds for a target of interest (*e.g.*, BioFocus DPI (biofocus.com), and Quantum Lead (q-lead.com)).

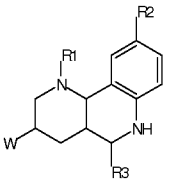
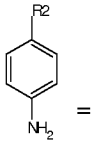
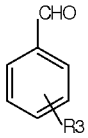
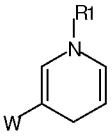
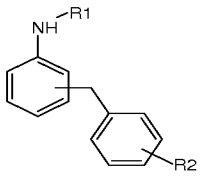
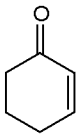
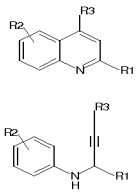
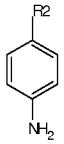
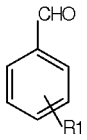
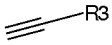
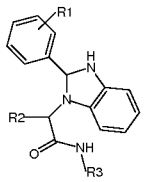
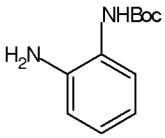
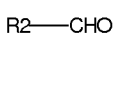
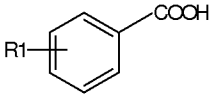
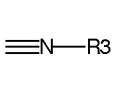
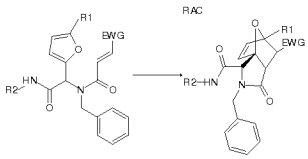
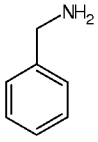
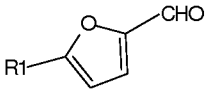
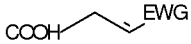
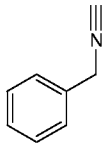
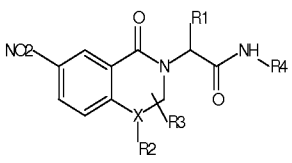
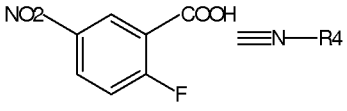
Other methods of selecting small molecules which are well known in the art, and may be applied to the methods of the present invention are Huang and Stuart L. Schreiber (1997) Proc Natl Acad Sci U S A. 94(25): 13396–13401; Hung *et al.* (2005) *Science* 310:670-674; Zhang *et al.* (2007) Proc Natl Acad Sci 104: 4606-4611; or any of

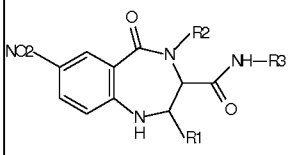
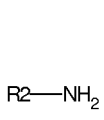
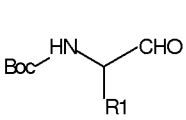
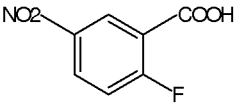
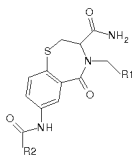
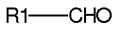
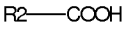
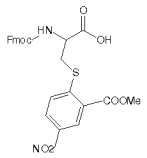
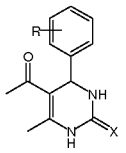
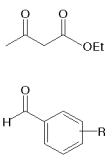
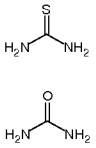
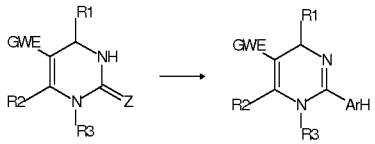
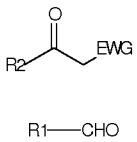
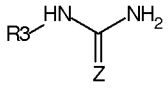
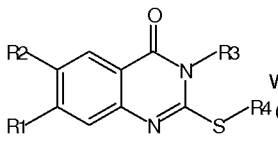
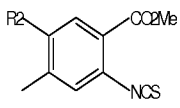
the methods reviewed in Gordon (2007) ACS Chem. Biol. 2:9–16, all of which are incorporated herein by reference in their entirety.

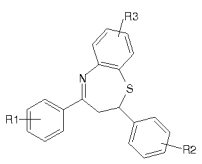
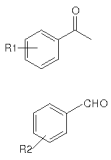
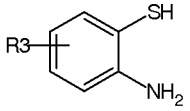
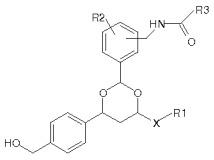
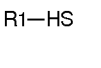
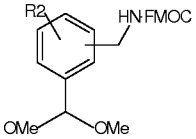
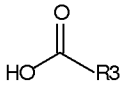
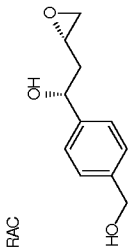
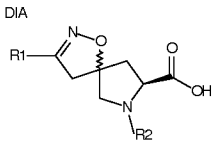
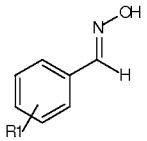
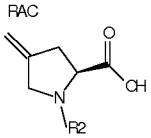
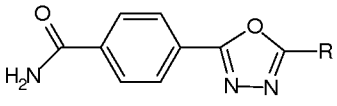
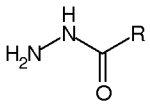
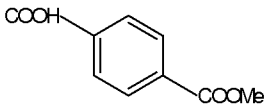
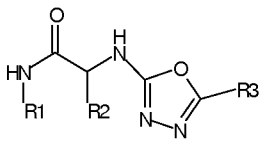
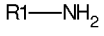
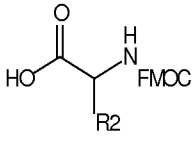
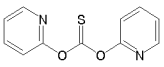
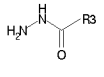
In addition to experimental screening methods, small molecules of the invention may be selected using virtual screening methods. Virtual screening technologies predict which small molecules from a library will bind to a protein, or a specific epitope therein, using statistical analysis and protein docking simulations. Most commonly, virtual screening methods compare the three-dimensional structure of a protein to those of small molecules in a library. Different strategies for modeling protein-molecule interactions are used, although it is common to employ algorithms which simulate binding energies between atoms, including hydrogen bonds, electrostatic forces, and van-der walls interactions. Typically, virtual screening methods can scan libraries of more than a million compounds and return a short list of small molecules which are likely to be strong binders. Several reviews of virtual screening methods are available, detailing the techniques which may be used to identify small molecules of the present invention (Engel et al. (2008) J. Am. Chem. Soc., 130 (15), 5115–5123;McInnes. (2007). Curr Opin Chem Biol. Oct;11(5):494-502; Reddy et al. (2007) Curr Protein Pept Sci. Aug;8(4):329-51; Muegge and Oloff. (2006) Drug Discovery Today. 3(4): 405-411; Kitchen et al. (2004) Nature Reviews Drug Discovery 3, 935-949). Further examples of small molecule screening can be found in U.S. 2005/0124678, which is incorporated herein by reference.

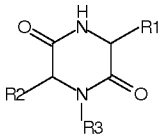
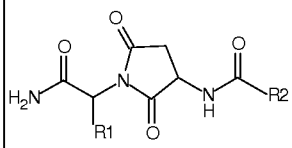
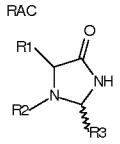
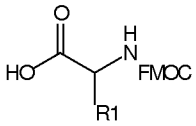
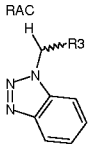
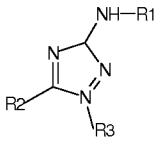
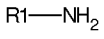
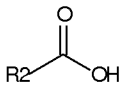
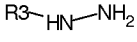
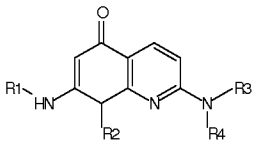
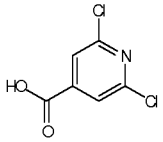
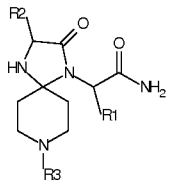
Small molecules of the invention may contain one of the scaffold structures depicted in the table below. The references cited in the table are incorporated herein by reference in their entirety. The groups $R_1$, $R_2$, $R_3$ and $R_4$ are limited only in that they should not interfere with, or significantly inhibit, the indicated reaction, and can include hydrogen, alkyl, substituted alkyl, heteroalkyl, substituted heteroalkyl, cycloalkyl, heterocycloalkyl, substituted cycloalkyl, substituted heterocycloalkyl, aryl, substituted aryl, arylalkyl, heteroarylalkyl, substituted arylalkyl, substituted heteroarylalkyl, heteroaryl, substituted heteroaryl, halogen, alkoxy, aryloxy, amino, substituted amino and others as are known in the art. Suitable substituents include, but are not limited to, alkyl, alkoxy, thioalkoxy, nitro, hydroxyl, sulfhydryl, aryloxy, aryl-S-, halogen, carboxy, amino, alkylamino, dialkylamino, arylamino, cyano, cyanate, nitrile, isocyanate, thiocyanate, carbamyl, and substituted carbamyl.

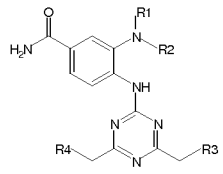
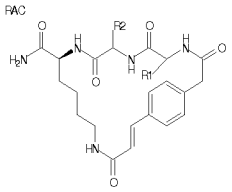
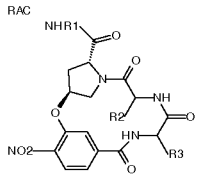
Table 6.

| Scaffolds | Amine | Aldehyde / Ketone | Carboxylic acid | Other | Reference |
|---|---|---|---|---|---|
|  |  = |  | |  | Carranco, I., et al. (2005) J. Comb. Chem. 7:33-41 |
|  | amines | benzaldehydes and furfural | |  | Rosamilia, A.E., et al. (2005) Organic Letters 7:1525-1528 |
|  |  |  | |  | Syeda Huma, H.Z., et al. (2002) Tet Lett 43:6485-6488 |
|  |  |  |  |  | Tempest, P., et al. (2001) Tet Lett 42:4959-4962 |
|  |  |  |  |  | Paulvanan, K. (1999) Tet Lett 40:1851-1854 |
|  | |  |  | | Tempest, P., et al. (2001) Tet Lett 42:4963-4968 |

| | | | | |
|---|---|---|---|---|
|  |  |  |  | Tempest, P., et al. (2003) Tet Lett 44:1947-1950 |
|  |  |  |  | Nefzi, A., et al. (1999) Tet Lett 40:4939-4942 |
|  |  |  | | Bose, A.K., et al. (2005) Tet Lett 46:1901-1903 |
|  |  |  | | Stadler, A. and Kappe, C.O. (2001) J. Comb. Chem. 3:624-630; Lengar, A. and Kappe, C.O. (2004) Organic Letters 6:771-774 |
|  wide range of primary aliphatic amines | |  |  | Ivachtchenko, A.V., et al. (2003) J. Comb. Chem. 5:775-788 |

| | | | |
|---|---|---|---|
|    | | | Micheli, F., et al. (2001) J. Comb. Chem.3 :224-228 |
|      | | | Sternson, S.M., et al. (2001) Org. Lett. 3:4239-4242 |
|    | | | Cheng, W.-C., et al. (2002) J. Org.Chem. 67:5673-5677; Park, K.-H., et al. (2001) J Comb Chem 3:171-176 |
| | | | |
|    | | | Brown, B.J., et al. (2000) Synlett 1:131-133 |
|      | | | Kilburn, J.P., et al. (2001) Tet Lett 42:2583-2586 |

| | | | | |
|---|---|---|---|---|
|  amino acid | | amino acid ester | | del Fresno, M., et al. (1998) Tet Lett 39:2639-2642 |
|  amino acid | | carboxylic acids | | Alvarez-Gutierrez, J.M., et al. (2000) Tet Lett 41:609-612 |
|  | R2—CHO |  |  | Rinnová, M., et al. (2002) J. Comb. Chem 4:209-213 |
|  |  |  |  | Makara, G.M., et al. (2002) Organic Lett 4:1751-1754 |
|  | | |  | Schell, P., et al. (2005) J. Comb. Chem 7:96-98 |
| | | | | |
|  | | amino acids | | Feliu, L., et al. (2003) J. Comb. Chem. 5:356-361 |

| | | | |
|---|---|---|---|
|  | Amines | Aldehydes | |
|  | | amino acids | Hiroshige, M., et al. (1995) J. Am. Chem. Soc. 117:11590-11591 |
|  | | amino acids | Bose, A.K., et al. (2005) Tet Lett 46:1901-1903 |

III. Peptidic Molecules Which Bind To An Ig-Like Domain Of A Human Receptor Tyrosine Kinase

In another aspect of the invention, the moiety that binds to the ectodomain, *e.g.*, an Ig-like domain or a hinge region, of a human receptor tyrosine kinase is a peptidic molecule.

The peptidic molecules may be designed based on an Ig-like domain of a RTK or a consensus sequence derived from such a domain.

In a specific embodiment the peptidic molecules bind to the following consensus sequence for the D4 interaction site: $LX_1RX_2X_3X_4X_5X_6X_7G$ wherein L is Leucine, R is Arginine, G is Glycine; and $X_1$, $X_2$, $X_3$, $X_4$, $X_5$, $X_6$ and $X_7$ are any amino acid. In a specific embodiment, $X_1$ is selected from the group consisting of Threonine, Isoleucine, Valine, Proline, Asparagine, or Lysine; X2 is selected from the group consisting of Leucine, Valine, Alanine, and Methionine; $X_3$ is selected from the group consisting of Lysine, Histidine, Asparagine, and Arginine; $X_4$ is selected from the group consisting of Glycine, Valine, Alanine, Glutamic Acid, Proline, and Methionine; $X_5$ is selected from the group consisting of Threonine, Serine, Glutamic Acid, Alanine, Glutamine, and

Aspartic acid; $X_6$ is selected from the group consisting of Glutamic Acid, Aspartic acid, and Glutamine; and $X_7$ is selected from the group consisting of Glycine, Serine, Alanine, Lysine, Arginine, Glutamine, and Threonine.

As such, in one embodiment, the peptidic molecules of the invention comprise or consist of a sequence matching the aforementioned consensus sequence ($LX_1RX_2X_3X_4X_5X_6X_7G$) wherein L is Leucine, R is Arginine, G is Glycine; and $X_1$, $X_2$, $X_3$, $X_4$, $X_5$, $X_6$ and $X_7$ are any amino acid. In a specific embodiment, $X_1$ is selected from the group consisting of Threonine, Isoleucine, Valine, Proline, Asparagine, or Lysine; X2 is selected from the group consisting of Leucine, Valine, Alanine, and Methionine; $X_3$ is selected from the group consisting of Lysine, Histidine, Asparagine, and Arginine; $X_4$ is selected from the group consisting of Glycine, Valine, Alanine, Glutamic Acid, Proline, and Methionine; $X_5$ is selected from the group consisting of Threonine, Serine, Glutamic Acid, Alanine, Glutamine, and Aspartic acid; $X_6$ is selected from the group consisting of Glutamic Acid, Aspartic acid, and Glutamine; and $X_7$ is selected from the group consisting of Glycine, Serine, Alanine, Lysine, Arginine, Glutamine, and Threonine.

In another embodiment, the peptidic molecules of the invention comprise or consist of a consensus sequence for the D7 domain of a VEGF receptor: L/I $X_1$ R Φ $X_2$ $X_3$ $X_4$ D/E $X_5$ G (SEQ ID NO:158), wherein L is Leucine, I is Isoleucine, R is Arginine, Φ is a hydrophobic amino acid, D is Aspartic Acid, E is Glutamic Acid, G is Glycine; and $X_1$, $X_2$, $X_3$, $X_4$ and $X_5$ are any amino acid. In a specific embodiment, Φ is Valine; $X_1$ is selected from the group consisting of Arginine, Glutamine, Glutamic Acid and Aspartic Acid; $X_2$ is selected from the group consisting of Arginine, Lysine and Threonine; $X_3$ is selected from the group consisting of Lysine, Glutamic Acid, Glutamine and Valine; $X_4$ is selected from the group consisting of Glutamic Acid and Valine; and $X_5$ is selected from the group consisting of Glutamic Acid, Glycine, Serine and Glutamine.

In another embodiment, the peptidic molecules bind to the following consensus sequence for the D7 domain of a member of the VEGF receptor family: $IX_1RVX_2X_3EDX_4G$ wherein I is Isoleucine, R is Arginine, E is Glutamic Acid, D is Aspartic Acid, G is Glycine; and $X_1$, $X_2$, $X_3$ and $X_4$ are any amino acid. In a specific embodiment, $X_1$ is selected from the group consisting of Glutamic Acid, Arginine, and Glutamine; X2 is selected from the group consisting of Arginine and Threonine; $X_3$ is

selected from the group consisting of Glutamic Acid and Lysine; and $X_4$ is selected from the group consisting of Glutamic Acid and Alanine (SEQ ID NO: 1).

As such, in one embodiment, the peptidic moieties of the invention comprise or consist of a sequence matching the consensus sequence $IX_1RVX_2X_3EDX_4G$ wherein I is Isoleucine, R is Arginine, E is Glutamic Acid, D is Aspartic Acid, G is Glycine; and $X_1$, $X_2$, $X_3$ and $X_4$ are any amino acid. In a specific embodiment, $X_1$ is selected from the group consisting of Glutamic Acid, Arginine, and Glutamine; X2 is selected from the group consisting of Arginine and Threonine; $X_3$ is selected from the group consisting of Glutamic Acid and Lysine; and $X_4$ is selected from the group consisting of Glutamic Acid and Alanine (SEQ ID NO: 1).

In one embodiment, the peptidic moieties of the invention may comprise an entire protein domain, for example, a D4 or a D5 domain such as the D4 domain (residues 309-413) or the D5 domain (residues 410-519) of human Kit. As a further example, the peptidic moieties of the invention may comprise a D7 domain (or fragment thereof) of a type V RTK, such as the D7 domain of a VEGFR (residues 718-727 of VEGFR1, residues 724-733 of VEGFR2 or residues 735-744 of VEGFR3). Such a peptidic molecule binds the RTK and acts as an antagonist by preventing activation of RTK (see Example 16 below). In some embodiments, the peptidic moieties of the invention may have as little as 50% identity to a domain of a RTK, such as a Type III RTK, *e.g.*, a peptidic moiety of the invention may be at least 50% identical, at least 60% identical, at least 70% identical, at least 80% identical, at least 90% identical, or at least 95%, 96%, 97%, or 98% identical to a D4, a D5 or a D7 domain of a RTK. In a specific embodiment, the peptidic moiety of the invention is at least 80% identical, at least 90% identical, or at least 95%, 96%, 97%, or 98% identical to amino acid residues 309-413 of human Kit RTK, amino acid residues 718-727 of VEGFR1, amino acid residues 724-733 of VEGFR2, or amino acid residues 735-744 of VEGFR3. In a similar embodiment, the peptidic moiety of the invention is at least 80% identical, at least 90% identical, or at least 95%, 96%, 97%, or 98% identical to amino acid residues 410-519 of human Kit RTK, amino acid residues 718-727 of VEGFR1, amino acid residues 724-733 of VEGFR2, or amino acid residues 735-744 of VEGFR3.

In some embodiments, the peptidic moiety of the invention binds to or comprises specific sequences of the human Kit receptor, for example, residues 309-413, residues 410-519, $^{381}$Arg and $^{386}$Glu, or $^{418}$Tyr and $^{505}$Asn of the human Kit receptor. In other

embodiments, the peptidic moiety of the invention binds to or comprises specific sequences of a VEGF receptor, for example, residues 718-727 of VEGFR1, Arg720 and Asp725 of VEGFR1, residues 724-733 of VEGFR2, Arg726 and Asp731 of VEGFR2, residues 735-744 of VEGFR3, or Arg737 and Asp742 of VEGFR3.

In a preferred embodiment, a peptidic moiety of the invention may bind to (or comprise or consist of) one or more residues in the Kit receptor which make up the small cavities or pockets described in Table 4 (below). For example, a peptidic molecule of the invention may bind to (or comprise or consist of) one or more of the following residues in the D3-D4 hinge region of the Kit receptor: K218, S220, Y221, L222 from the D3 domain and F340, P341, K342, N367, E368, S369, N370, I371, Y373 from the D4 domain. A peptidic molecule of the invention may also bind to (or comprise or consist of) one or more of the following residues which make up a concave surface in the D4 domain of the Kit receptor:Y350, R353, F355, K358, L379, T380, R381, L382, E386 and T390. In another embodiment, a peptidic molecule of the invention may bind to (or comprise or consist of) one or more of the following residues which form a pocket in the D2-D3 hinge region of the Kit receptor:Y125, G126, H180, R181, K203, V204, R205, P206 and F208 from the D2 domain and V238, S239, S240, S241, H263, G265, D266, F267, N268 and Y269 from the D3 domain.

A peptidic moiety of the invention may bind to contiguous or non-contiguous amino acid residues and function as a molecular wedge that prevents the motion required for positioning of the membrane proximal region of the RTK at a distance and orientation that enables tyrosine kinase activation. A peptidic molecule of the invention may also act to prevent homotypic D4, D5 or D7 receptor interactions or destabilize the ligand- receptor interaction site. In some preferred embodiments, a peptidic molecule of the invention may bind to (or comprise or consist of) one or more of the following residues on the Kit receptor: Y125, G126, H180, R181, K203, V204, R205, P206, P206, F208, K127, A207, V238, S239, S240, S241, H263, G265, D266, F267, N268, Y269, T295, L222, L222, L223, E306, V308, R224, V308, K310, K218, A219, S220, K218, A220, Y221, A339, D327, D398, E338, E368, E386, F312, F324, F340, F355, G311, G384, G387, G388, I371, K342, K358, L382, L379, N326, N367, N370, N410, P341, S369, T385, V325, V407, V409, Y373, Y350, Y408, T380, T390, R381, R353, T411, K412, E414, K471, F433, G470, L472, V497, F469, A431, or G432. The peptidic moieties of the invention may bind to (or comprise or consist of) all of the amino acid

residues forming a pocket or a cavity identified in Table 4 or they may bind to (or comprise or consist of) a subset of the residues forming the pocket or the cavity. One of skill in the art will appreciate that, in some embodiments, a peptidic molecule of the invention may be easily targeted to the corresponding residues in other type III RTKs, *e.g.*, those residues that form similar pockets or cavities or those in the same position by structural alignment or sequence alignment.

In a specific embodiment, a peptidic molecule of the invention binds to a conformational epitope or a discontinuous epitope on a type III RTK or a type V RTK. The conformational or discontinuous epitope may be composed of two or more residues from the D3, D4, D5 or D7 domain or the D4-D5 or D3-D4 hinge regions from a type III RTK, *e.g.*, the human Kit receptor or the PDGF receptor or a type V RTK, *e.g.,* a human VEGF receptor. For example, the conformational or discontinuous epitope may be composed of two or more of the residues listed in Table 4 below. In a particular embodiment, a peptidic molecule of the invention binds to a conformational epitope composed of 2 or more amino acids selected from the group consisting of Y125, H180, R181, K203, V204, R205, P206, V238, S239, S240, H263, G265, D266, F267, N268, and Y269. In similar embodiments, a peptidic molecule of the invention may bind to a conformational epitope composed of 2 or more amino acids selected from one of the following groups of amino acids: P206, F208, V238, and S239; K127, A207, F208, and T295; L222, A339, F340, K342, E368, S369, N370, I371, and Y373; L222, L223, E306, V308, F312, E338, F340, and I371; R224, V308, K310, G311, F340, P341, and D398; K218, A219, S220, N367, E368, and S369; K218, A220, E368, and S369; G384, T385, T411, K412, E414, and K471; Y408, F433, G470, K471, and L472; F324, V325, N326, and N410;D327, N410, T411, K412, and V497; G384, G387, V409, and K471; L382, G387, V407, and V409; Y125, G126, H180, R181, K203, V204, R205, P206, F208, V238, S239, S240, S241, H263, G265, D266, F267, N268, and Y269; P206, F208, V238, and S239; K218, S220, Y221, L222, F340, P341, K342, N367, E368, S369, N370, I371, and Y373; G384, G387, G388, Y408, V409, T411, F433, F469, G470, and K471; D327, T411, K412, E414, A431, G432, and K471; Y350, F355, K358, L379, T380, R381, L382, E386, and T390; Y350, R353, and F355.

In a further embodiment, a peptidic molecule of the invention binds to a conformational epitope wherein the conformational epitope is composed of two or more amino acid residues selected from the peptides listed in Table 5. In a specific

embodiment, the conformational epitope is composed of one or more amino acid residues selected from a first peptide and one or more amino acids selected from a second peptide, wherein the first and second peptides are selected from the group of peptides listed in Table 5. As such, a peptidic molecule of the invention binds a conformational epitope wherein the first and second peptide groups are as follows: Ala219-Leu222 and Thr304-Val308; Asp309-Gly311 and Arg224-Gly226; Thr303 - Glu306 and Ala219-Leu222; Asn367–Asn370 and Ser217–Tyr221; Ala339–Pro343 and Asn396–Val399; Ala339–Pro343 and Glu368-Arg372; Lys358–Tyr362 and Val374–His378; Asp357-Glu360 and Leu377–Thr380; Met351–Glu360 and His378-Thr389; His378-Thr389 and Val323-Asp332; Val409–Ile415 and Ala493–Thr500; Val409–Ile415 and Ala431-Thr437; Val409– Ile415 and Phe469-Val473; Val409–Ile415 and Val325–Asn330; Val409–Ile415 and Arg381-Gly387; Gly466–Leu472 and Gly384–Gly388; Val325-Glu329 and Tyr494-Lys499; Thr411-leu416 and Val497-Ala502; Ile415–Leu421 and Ala502–Ala507; Ala502–Ala507 and Lys484-Thr488; and Ala502–Ala507 and Gly445–Cys450. The peptidic moieties of the invention may bind to all of the amino acid residues forming the foregoing first and second peptide groups or they may bind to a subset of the residues forming the first and second peptide groups.

In another embodiment, a peptidic moiety of the invention may bind to (or comprise or consist of) 2 or more amino acids selected from the group consisting of E33, P34, D72, E76, N77, K78, Q79, K158, D159, N250, S251, Q252, T253, K254, L255, N260, W262, H264, G265, E344, N352, R353, F355, T356, D357, Y362, S365, E366, N367, N370, and G466.

In another embodiment, a peptidic moiety of the invention binds to a contiguous epitope on the VEGF receptor. In one embodiment, the contiguous epitope is composed of two or more residues in the D7 domain of the VEGF receptor. In another embodiment, the contiguous epitope is an epitope selected from the group consisting of $^{672}$VAISSS$^{677}$ of VEGFR1, $^{678}$TTLDCHA$^{684}$ of VEGFR1, $^{685}$NGVPEPQ$^{691}$ of VEGFR1, $^{700}$KIQQEPG$^{706}$ of VEGFR1, $^{707}$IILG$^{710}$ of VEGFR1, $^{711}$PGS$^{713}$ of VEGFR1, $^{714}$STLFI$^{718}$ of VEGFR1, $^{719}$ERVTEEDEGV$^{728}$ of VEGFR1, $^{689}$VNVSDS$^{694}$ of VEGFR3, $^{695}$LEMQCLV$^{701}$ of VEGFR3, $^{702}$AGAHAPS$^{708}$ of VEGFR3, $^{717}$LLEEKSG$^{723}$ of VEGFR3, $^{724}$VDLA$^{727}$ of VEGFR3, $^{728}$DSN$^{730}$ of VEGFR3, $^{731}$QKLSI$^{735}$ of VEGFR3, and $^{736}$QRVREEDAGR$^{745}$ of VEGFR3, $^{678}$TSIGES$^{683}$ of VEGFR2, $^{684}$IEVSCTA$^{690}$ of VEGFR2, $^{691}$SGNPPPQ$^{697}$ of VEGFR2, $^{706}$TLVEDSG$^{712}$ of

VEGFR2, $^{713}$IVLK$^{716}$ of VEGFR2, $^{717}$DGN$^{719}$ of VEGFR2, $^{720}$RNLTI$^{724}$ of VEGFR2 and $^{725}$RRVRKEDEGL$^{734}$ of VEGFR2.

In another embodiment, a peptidic molecule of the invention binds to, or comprises, amino acid residues $^{385}$Arg and $^{390}$Glu of human PDGFRβ, or the corresponding residues in PDGFRα. The residues $^{385}$Arg and $^{390}$Glu of human PDGFRβ are analogous to the residues $^{381}$Arg and $^{386}$Glu of the Kit receptor and mediate homotypic D4-D4 interactions of PDGFRβ. Peptidic molecules of the invention may exert their inhibitory effect on receptor activation by preventing critical homotypic interactions (such as salt bridges formed between $^{385}$Arg and $^{390}$Glu of human PDGFRβ) between membrane proximal regions of type-III RTKs that are essential for positioning the cytoplasmic domain at a distance and orientation essential for tyrosine kinase activation. Experiments discussed herein demonstrate that homotypic D4-D4 interactions are dispensable for PDGFRβ dimerization and that PDGFRβ dimerization is necessary but not sufficient for receptor activation. Thus, peptidic molecules of the invention may allow dimerization of PDGFRβ while preventing activation. Structure based sequence alignment has shown that the size of the EF loop, and the critical amino acids comprising the D4-D4 interface are conserved in Kit, PDGFRα, PDGFRβ, and CSF1R. Thus, in some embodiments, peptidic molecules of the invention may be targeted to the conserved regions of the D4 or D5 domains of type III RTKs.

The peptidic moieties of the invention may be peptides comprising or consisting of any of the amino acid sequences identified herein (*e.g.*, SEQ ID NOs: 1-89, 92, 93, and 105-157). For example, peptidic moieties of the invention may be peptides comprising or consisting of any of the following amino acid sequences: EVVDKGFIN (SEQ ID NO: 2), ASYL (SEQ ID NO: 3), TLEVV (SEQ ID NO: 4), ASYLTLEVV (SEQ ID NO: 5), DKG , REG, DKGREG (SEQ ID NO: 6), VVSVSKASYLL (SEQ ID NO: 7), VTTTLEVVD (SEQ ID NO: 8), REGEEFTVTCTI (SEQ ID NO: 9), TTLE (SEQ ID NO: 10), TTLEASYL (SEQ ID NO: 11), KSENESNIR (SEQ ID NO: 12), NESN (SEQ ID NO: 13), SKASY (SEQ ID NO: 14), NESNSKASY (SEQ ID NO: 15), AFPKP (SEQ ID NO: 16), NSDV (SEQ ID NO: 17), AFPKPNSDV (SEQ ID NO: 18), ESNIR (SEQ ID NO: 19), AFPKPESNIR (SEQ ID NO: 20), DKWEDYPKSE (SEQ ID NO: 21), IRYVSELHL (SEQ ID NO: 22), LTRLKGTEGGT (SEQ ID NO: 23), GENVDLIVEYE (SEQ ID NO: 24), MNRTFTDKWE (SEQ ID NO: 25), KWEDY (SEQ ID NO: 26), VSELH (SEQ ID NO: 27), KWEDYVSELH (SEQ ID NO: 28),

DKWE (SEQ ID NO: 29), LHLT (SEQ ID NO: 30), DKWELHLT (SEQ ID NO: 31), HLTRLKGTEGGT (SEQ ID NO: 32), MNRTFTDKWE (SEQ ID NO: 25), HLTRLKGTEGGT (SEQ ID NO: 32), MNRTFTDKWEHLTRLKGTEGGT (SEQ ID NO: 33), VFVNDGENVD (SEQ ID NO: 34), VNTKPEI (SEQ ID NO: 35), AYNDVGKT (SEQ ID NO: 36), VNTKPEIAYNDVGKT (SEQ ID NO: 37), AGFPEPT (SEQ ID NO: 38), VNTKPEIAGFPEPT (SEQ ID NO: 39), FGKLV (SEQ ID NO: 40), VNTKPEI FGKLV (SEQ ID NO: 41), VNDGEN (SEQ ID NO: 42), VNTKPEIVNDGEN (SEQ ID NO: 43), RLKGTEG (SEQ ID NO: 44), VNTKPEIRLKGTEG (SEQ ID NO: 45), GPPFGKL (SEQ ID NO: 46), GTEGG (SEQ ID NO: 47), GPPFGKLGTEGG (SEQ ID NO: 48), VNDGE (SEQ ID NO: 49), YNDVGK (SEQ ID NO: 50), VNDGEYNDVGK (SEQ ID NO: 51), TKPEILTYDRL (SEQ ID NO: 52), DRLVNGMLQC (SEQ ID NO: 53), GKTSAYFNFAFK (SEQ ID NO: 54), CPGTEQRCSAS (SEQ ID NO: 55), CSASVLPVDVQ (SEQ ID NO: 56), DSSAFKHNGT (SEQ ID NO: 57), GTVECKAYND (SEQ ID NO: 58), LNSSGPPFGKL (SEQ ID NO: 59), FAFKGNNKEQI (SEQ ID NO: 60), TKPEIL (SEQ ID NO: 61), VGKTSA (SEQ ID NO: 62), TKPEILVGKTSA (SEQ ID NO: 63), ILTYDRL (SEQ ID NO: 64), AYFNFA (SEQ ID NO: 65), ILTYDRLAYFNFA (SEQ ID NO: 66), KHNGT (SEQ ID NO: 67), AYFNFAKHNGT (SEQ ID NO: 68), GTEQRC (SEQ ID NO: 69), AYFNFAGTEQRC (SEQ ID NO: 70), YHRKVRPVSSHGDFNY (SEQ ID NO: 71), PFVS (SEQ ID NO: 72), KAFT (SEQ ID NO: 73), LAFKESNIY (SEQ ID NO: 74), LLEVFEFI (SEQ ID NO: 75), RVKGFPD (SEQ ID NO: 76), KASNES (SEQ ID NO: 77), KAES (SEQ ID NO: 78), GTTKEK (SEQ ID NO: 79), YFGKL (SEQ ID NO: 80), FVNN (SEQ ID NO: 81), DNTKV (SEQ ID NO: 82), GGVK (SEQ ID NO: 83), LGVV (SEQ ID NO: 84), YGHRKVRPFVSSSHGDFNY (SEQ ID NO: 85), PFVS (SEQ ID NO: 72), KSYLFPKNESNIY (SEQ ID NO: 86), GGGYVTFFGK (SEQ ID NO: 87), DTKEAGK (SEQ ID NO: 88), YFKLTRLET (SEQ ID NO: 89), and YRF.

A peptide molecule of the invention may be further modified to increase its stability, bioavailability or solubility. For example, one or more L-amino acid residues within the peptidic molecules may be replaced with a D-amino acid residue. The term "mimetic" as applied to the peptidic molecules of the present invention is intended to include molecules which mimic the chemical structure of a D-peptidic structure and retain the functional properties of the D-peptidic structure. The term “mimetic” is

further intended to encompass an "analogue" and/or "derivative" of a peptide as described below. Approaches to designing peptide analogs, derivatives and mimetics are known in the art. For example, see Farmer, P.S. in Drug Design (E.J. Ariens, ed.) Academic Press, New York, 1980, vol. 10, pp. 119-143; Ball. J.B. and Alewood, P.F. (1990) *J. Mol. Recognition* 3:55; Morgan, B.A. and Gainor, J.A. (1989) *Ann. Rep. Med. Chem.* 24:243; and Freidinger, R.M. (1989) *Trends Pharmacol. Sci.* 10:270. See also Sawyer, T.K. (1995) "Peptidomimetic Design and Chemical Approaches to Peptide Metabolism" in Taylor, M.D. and Amidon, G.L. (eds.) *Peptide-Based Drug Design: Controlling Transport and Metabolism*, Chapter 17; Smith, A.B. 3rd, *et al.* (1995) *J. Am. Chem. Soc.* 117:11113-11123; Smith, A.B. 3rd, *et al.* (1994) *J. Am. Chem. Soc.* 116:9947-9962; and Hirschman, R., *et al.* (1993) *J. Am. Chem. Soc.* 115:12550-12568.

As used herein, a "derivative" of a peptidic molecule of the invention refers to a form of the peptidic molecule in which one or more reaction groups on the molecule have been derivatized with a substituent group. Examples of peptide derivatives include peptides in which an amino acid side chain, the peptide backbone, or the amino- or carboxy-terminus has been derivatized (*e.g.*, peptidic compounds with methylated amide linkages). As used herein an "analogue" of a peptidic molecule of the invention to a peptidic molecule which retains chemical structures of the molecule necessary for functional activity of the molecule yet which also contains certain chemical structures which differ from the molecule. An example of an analogue of a naturally-occurring peptide is a peptide which includes one or more non-naturally-occurring amino acids. As used herein, a "mimetic" of a peptidic molecule of the invention refers to a peptidic molecule in which chemical structures of the molecule necessary for functional activity of the molecule have been replaced with other chemical structures which mimic the conformation of the molecule. Examples of peptidomimetics include peptidic compounds in which the peptide backbone is substituted with one or more benzodiazepine molecules (see *e.g.*, James, G.L. *et al.* (1993) *Science* 260:1937-1942).

Analogues of the peptidic molecules of the invention are intended to include molecules in which one or more L- or D- amino acids of the peptidic structure are substituted with a homologous amino acid such that the properties of the molecule are maintained. Preferably conservative amino acid substitutions are made at one or more amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families

of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Non-limiting examples of homologous substitutions that can be made in the structures of the peptidic molecules of the invention include substitution of D-phenylalanine with D-tyrosine, D-pyridylalanine or D-homophenylalanine, substitution of D-leucine with D-valine or other natural or non-natural amino acid having an aliphatic side chain and/or substitution of D-valine with D-leucine or other natural or non-natural amino acid having an aliphatic side chain.

The term mimetic, and in particular, peptidomimetic, is intended to include isosteres. The term "isostere" as used herein is intended to include a chemical structure that can be substituted for a second chemical structure because the steric conformation of the first structure fits a binding site specific for the second structure. The term specifically includes peptide back-bone modifications (*i.e.*, amide bond mimetics) well known to those skilled in the art. Such modifications include modifications of the amide nitrogen, the α-carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. Several peptide backbone modifications are known, including ψ[$CH_2S$], ψ[$CH_2NH$], ψ[$CSNH_2$], ψ[NHCO], ψ[$COCH_2$], and ψ [(E) or (Z) CH=CH]. In the nomenclature used above, ψ indicates the absence of an amide bond. The structure that replaces the amide group is specified within the brackets.

Other possible modifications include an N-alkyl (or aryl) substitution (ψ [CONR]), or backbone crosslinking to construct lactams and other cyclic structures. Other derivatives of the modulator compounds of the invention include C-terminal hydroxymethyl derivatives, *O*-modified derivatives (*e.g.*, C-terminal hydroxymethyl benzyl ether), N-terminally modified derivatives including substituted amides such as alkylamides and hydrazides.

Peptidic molecules of the present invention may be made by standard methods known in the art. The peptidic molecule, *e.g.*, D4 domain of the human Kit RTK or D7 domain of a human VEGF receptor, may be cloned from human cells using standard

techniques, inserted in to a recombinant vector, and expressed in an *in vitro* cell system (*e.g.*, by transfection of the vector into yeast cells). Alternatively, the peptidic molecules may be designed and synthesized *de novo* via known synthesis methods such as Atherton *et al.* (1989) Oxford, England: IRL Press. ISBN 0199630674; Stewart *et al.* (1984). 2nd edition, Rockford: Pierce Chemical Company, 91. ISBN 0935940030; Merrifield (1963) *J. Am. Chem. Soc.* 85: 2149-2154.

The peptidic molecules can then be tested for functional activity using any of the assays described herein, *e.g.*, those described in the Examples section below.

IV. Screening Assays for Identifying Moieties of the Invention

The moieties of the invention may be screened for RTK inhibitory activity using any of the assays described herein and those assays that are well known in the art. For example, assays which may determine receptor internalization, receptor autophosphorylation, and/or kinase signaling may be used to identify moieties which prevent the activation of target RTKs, *e.g.*, the Kit receptor or a human VEGF receptor. Screening for new inhibitor moieties may be accomplished by using standard methods known in the art, for example, by employing a phosphoELISA™ procedure (available at Invitrogen) to determine the phosphorylation state of the RTK or a downstream molecule. The phosphorylation state of the receptor, *e.g.*, the Kit receptor or a VEGF receptor, may be determined using commercially available kits such as, for example, C-Kit [pY823] ELISA KIT, HU (BioSource™; Catalog Number - KHO0401); c-KIT [TOTAL] ELISA KIT, HU (BioSource™; Catalog Number - KHO0391). Antibodies, small molecules, and other moieties of the invention may be screened using such kits to determine their RTK inhibitory activity. For example, after treatment with an appropriate ligand and a moiety of the invention, a phosphoELISA™ may be performed to determine the phosphorylation state and, thus, the activation state of a RTK of interest. Moieties of the invention could be identified as those which prevent RTK activation. Examples 15 and 16 below describe assays which involve the detection of RTK activation using anti-phosphotyrosine antibodies. Example 20 below describes one possible assay for detecting receptor activation using the phosphoELISA™ system. Examples 22-25 (including the methods and introduction related thereto) describe further methods used herein to determine the activation state of RTKs.

Since receptor activation may lead to endocytosis and receptor internalization, it is useful, in some embodiments, to determine the ability of moieties of the invention to inhibit target RTKs by measuring their ability to prevent receptor internalization. Example 25 below (and the methods related thereto) describes the measurement of the internalization and degradation of PDGF receptor mutants. Receptor internalization assays are well known in the art and described in, for example, Fukunaga *et al.* (2006) *Life Sciences*. 80(1). p. 17-23; Bernhagen et al. (2007) *Nature Medicine* 13, 587 – 596; natureprotocols.com/2007/04/18/receptor_internalization_assay.php), the entire contents of each of which are incorporated herein by reference. One well-known method to determine receptor internalization is to tag a ligand with a flurorecent protein, *e.g.*, Green Flurorescent Protein (GFP), or other suitable labeling agent. Upon binding of the ligand to the receptor, flurorescence microscopy may be used to visualize receptor internalization. Similarly, a moiety of the invention may be tagged with a labeling agent and flurorescence microscopy may be used to visualize receptor internalization. If the moiety is able to inhibit the activity of the receptor, lessened internalization of flurorescence in the presence of ligand as compared to appropriate controls (*e.g.*, fluorescence may be observed only at the periphery of the cell where the moity binds the receptor rather than in endosomes or vesicles).

In addition to those mentioned above, various other receptor activation assays are known in the art, any of which may be used to evaluate the function of the moieties of the invention. Further receptor activation assays which may be used in accordance with the present invention are described in U.S. Patent Nos. 6,287,784; 6,025,145; 5,599,681; 5,766,863; 5,891,650; 5,914,237; 7,056,685; and many scientific publications including, but not limited to: Amir-Zaltsman et al. (2000) *Luminescence* 15(6):377-80; Nakayama and Parandoosh (1999) *Journal of Immunological Methods*. 225(1-2), 27, 67-74; Pike et al. (1987) *Methods of Enzymology* 146: 353-362; Atienza et al. (2005) *Journal of Biomolecular Screening*. 11(6): 634-643; Hunter et al. (1982). *Journal of Biological Chemistry* 257(9): 4843-4848; White and Backer (1991) *Methods in Enzymology* 201: 65-67; Madden et al. (1991) *Anal Biochem* 199: 210-215; Cleaveland et al. (1990) Analytical Biochemistry 190: 249-253; Lazaro et al. (1991) *Analytical Biochemistry* 192: 257-261; Hunter and Cooper (1985) *Ann Rev Biochem* 54: 897-930; Ullrich and Schlessinger (1990) *Cell* 61: 203-212; Knutson and Buck (1991) *Archives of Biochemistry and Biophysics* 285(2): 197-204); King et al. (1993) *Life Sciences* 53:

1465-1472; Wang. (1985) *Molecular and Cellular Biology* 5(12): 3640-3643; Glenney et al. (1988) *Journal of Immunological Methods* 109: 277-285; Kamps (1991) *Methods in Enzymology* 201: 101-110; Kozma et al. (1991) *Methods in Enzymology* 201: 28-43; Holmes et al. (1992) *Science* 256: 1205-10; and Corfas et al. (1993) *PNAS, USA* 90: 1624-1628.

Receptor activation by ligand binding typically initiates subsequent intracellular events, *e.g.*, increases in secondary messengers such as $IP_3$ which, in turn, releases intracellular stores of calcium ions. Thus, receptor activity may be determined by measuring the quantity of secondary messengers such as $IP_3$, cyclic nucleotides, intracellular calcium, or phosphorylated signaling molecules such as STAT, PI3K,Grb2, or other possible targets known in the art. U.S. Patent No. 7,056,685 describes and references several methods which may be used in accordance with the present invention to detect receptor activity and is incorporated herein by reference.

Many of the assays described above, such as receptor internalization assays or receptor activation assays may involve the detection or quantification of a target RTK using immunological binding assays (*e.g.*, when using a radiolabeled antibody to detecting the amount of RTK on the cell surface during a receptor internalization assay). Immunological binding assays are widely described in the art (see, *e.g.*, U.S. Pat. Nos. 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, see also *Methods in Cell Biology*: *Antibodies in Cell Biology*, volume 37 (Asai, ed. 1993); *Basic and Clinical Immunology* (Stites & Terr, eds., 7th ed. 1991).

Immunoassays such as may be employed in receptor internalization studies, receptor activation studies, or receptor detection assays often use a labeling agent to specifically bind to and label the complex formed by the detecting antibody and the RTK (see U.S. Pat. No. 7,056,685 which is incorporated herein by reference). The labeling agent may itself be the antibody used to detect the receptor (the antibody here may or may not be a moiety of the invention). Alternatively, the labeling agent may be a third agent, such as a secondary or tertiary antibody (*e.g.*, and anti-mouse antibody binding to mouse monoclonal antibody specific for the target RTK). Other proteins capable of specifically binding immunoglobulin constant regions, such as protein A or protein G may also be used as the labeling agent in an immunological binding assay. These proteins exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species (see, *e.g.*, Kronval et al. (1973), *J. Immunol.* 111:1401-

1406; Akerstrom et al. (1985), *J. Immunol.* 135:2589 2542). The labeling agent can also be modified with a detectable agent, such as biotin, to which another molecule can specifically bind, such as streptavidin. A variety of detectable moieties are well known to those skilled in the art.

Commonly used assays include noncompetitive assays, *e.g.*, sandwich assays, and competitive assays. Commonly used assay formats include Western blots (immunoblots), which are used to detect and quantify the presence of protein in a sample. The particular label or detectable group used in the assay is not a critical aspect of the invention, as long as it does not significantly interfere with the specific binding of the immunoglobulin used to detect the RTK or a moiety of the invention which is designed to bind and inactivate the RTK. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of immunoassays and, in general, most any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include fluorescent dyes (*e.g.*, fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (*e.g.*, $^{3}H$, $^{125}I$, $^{35}S$, $^{14}C$, or $^{32}P$), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic beads (*e.g.*, polystyrene, polypropylene or latex).

The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. The label can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidotases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, and the like. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, *e.g.*, luminol. For a review of various labeling or signal producing systems that may be used, see U.S. Pat. No. 4,391,904.

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a

fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

In a further aspect of the invention, the moieties of the present invention may bind to epitopes on a target RTK and still allow the ectodomain of the receptor tyrosine kinase to dimerize. In this embodiment, the binding of the moiety may affect the positioning, orientation and/or distance between the Ig-like domains of the two monomers (*e.g.*, the D4-D4 or D5-D5 domains of a type III receptor tyrosine kinase or the D7-D7 domains of a type V receptor tyrosine kinase), thereby inhibiting the activity of the receptor tyrosine kinase. In other words, the moiety may allow ligand induced dimerization of the receptor tyrosine kinase ectodomains, but affect the positioning of the two ectodomains at the cell surface interface or alter or prevent conformational changes in the receptor tyrosine kinases, thereby inhibiting the activity of the receptor tyrosine kinase (*e.g.*, inhibiting receptor internalization and/or inhibiting tyrosine autophosphorylation of the receptor and/or inhibiting the ability of the receptor to activate a downstream signaling pathway).

Thus, in some embodiments, it is useful to employ assays which are able to identify moieties that allow receptor dimerization, yet render the receptor inactive. Such assays are described below. For example, Example 18 describes experiments performed with the PDGF receptor whereby receptor dimerization is detected using cross linking, and receptor activation is determined using phosphotyrosine specific antibodies. Furthermore, Example 23 shows that a mutant of PDGFR has an impairment in ligand-induced tyrosine autophosphorylation which is not caused by a deficiency in ligand-induced receptor dimerization (see also the Methods and Introducion to Examples 22-25).

The conformational state of the RTK may also be determined by Fluorescence Resonance Energy Transfer (FRET) analysis. A comprehensive review of fluorescence

methodologies for determining protein conformations and interactions can be found in Johnson (2005) Traffic. 2005 Dec;6(12):1078-92 which is incorporated herein by reference. In the FRET assay a RTK of interest is labeled with appropriate FRET fluorophores. After the RTK is labeled, cells expressing the labeled RTK are incubated with test moieties of the invention and the ligand of the RTK(*e.g.*, SCF for the Kit RTK). FRET analysis will allow the observation of conformational changes in the RTK associated with ligand binding, RTK dimerization, and/or receptor activation. By this method one of skill in the art may directly assess a protein conformational change which indicates RTK dimerization without downstream activation. There are a number of methods available to perform FRET analysis, and a large portion of the variation arises from the use of different fluorophores or different techniques to incorporate those fluorophores into proteins of interest. FRET fluorophores and analysis methods are well known in the art, and a brief review of FRET technology is available in Heyduk. (2002) Current Opinion in Biotechnology. 13(4). 292-296 and references therein. The following publications expand on the FRET method and are incorporated herein by reference: Kajihara et al. (2006*) Nat Methods*. 3(11):923-9; Biener-Ramanujan et al. (2006) Growth Horm IGF Res.16(4):247-57; Taniguchi et al. (2007*) Biochemistry*. 46(18):5349-57; U.S. Patent Nos. 6,689,574; 5,891,646; and WIPO Publication No. WO/2002/033102. FRET fluorophores may be incorporated into any domain or hinge region of a RTK to detect conformational changes (e.g., the D4 or D5 domains of a Type III RTK or the D7 domain of a Type V RTK) provided that the fluorophores do not interfere with the function of the RTK or the ability of moieties of the invention to bind the RTK.

Fluorophores useful for FRET are often the same as those useful for Bioluminescence Resonance Energy Transfer (BRET) as discussed below. The most popular FRET method is to engineer reactive cystein residues into a protein of interest. Fluorophores can then easily react with the chosen cystein residies. Often fusion proteins are constructed, whereby a protein of interest is fused to Green Fluorescent Protein (see Neininger *et al.* (2001) *EMBO Reports*. 2(8):703-708). Additional methods and useful fluorophores for FRET are described in Huebsch and Mooney (2007) *Biomaterials.* 28(15):2424-37; Schmid and Birbach (2007) *Thromb Haemost.* 97(3):378-84; Jares-Erijman AND Jovin (2006) *Curr Opin Chem Biol.* 10(5):409-16; Johansson (2006) *Methods Mol Biol.* 335:17-29; Wallrabe and Periasamy (2005) *Curr Opin*

*Biotechnol.* 16(1):19-27; and Clegg RM (1995) *Curr Opin Biotechnol.* 6(1):103-10 which are incorporated herein by reference.

In other embodiments, it may be unknown or difficult to determine (depending on the receptor) which RTK conformation is specifically indicative of dimerization without activation. In such cases, one of skill in the art may combine assays that determine receptor dimerization with those that determine receptor activation. For example, one may use traditional cross-linking studies (exemplified by Rodriguez et al. (1990) *Molecular Endocrinology*, 4(12), 1782-1790) to detect RTK dimerization in combination with any of the receptor activation assays discussed above. FRET and similar systems may also be used to directly measure receptor activation or dimerization. For example, by incorporating appropriate FRET fluorophores into the cytoplasmic domain of the RTK and into a phosphorylation target protein (i.e., a downstream signaling molecule), FRET would be capable of determining whether downstream signaling molecules were being recruited to the RTK. Therefore, in one embodiment a successful moiety of the invention is one which allows receptor dimerization, as measured by cross-linking or FRET, but which prevents receptor activation, detected as lack of fluorescence by FRET or BRET analysis or by other receptor activation assays (*e.g.*, autophosphorylation assay employing anti-phosphotyrosine antibodies and Western Blot). Thus, using the techniques described herein, one of skill in the art can easily test moieties (*e.g.*, small molecules, peptides, or antibodies.) to determine whether they inhibit RTK activity and whether they allow receptor dimerization.

In particular, Bioluminescence Resonance Energy Transfer (BRET) analysis may be used to identify moieties which inhibit the activity of RTKs. U.S. Pat. Pub. No. 20060199226, WIPO Publication No. WO/2006/094073, and Tan *et al.* (2007. Molecular Pharmacology. 72:1440-1446) specifically describe methods to identify ligands which activate RTKs and are thus incorporated herein by reference. These techniques have been employed for determining protein interactions *in vitro* and *in vivo* (Pfleger *et al.* (2006) *Nature Protocols* 1 337-345; Kroeger *et al.* (2001), *J. Biol. Chem.*, 276(16):12736-43; and Harikumar, *et al.* (2004) *Mol Pharmacol* 65:28-35; which are all incorporated herein by reference).

BRET is useful for identifying moieties of the present invention from test compounds by screening for those moieties which prevent RTK activation.

As discussed in U.S. Pat. Publication No. 2006/0199226 which is incorporated herein by reference, BRET based assays can be used to monitor the interaction of proteins having a bioluminescent donor molecule (DM) with proteins having a fluorescent acceptor moiety (AM). Briefly, cells expressing an RTK-DM fusion will convert the substrate's chemical energy into light. If there is an AM (*e.g.*, a signaling protein-AM fusion) in close proximity to the RTK-DM fusion, then the cells will emit light at a certain wavelength. For example, BRET based assays can be used to assess the interaction between a RTK-luciferase fusion and a GFP-signalling protein fusion. This differs slightly from FRET analysis, where the donor molecule may be excited by light of a specific wavelength rather than by chemical energy conversion. Examples of bioluminescent proteins with luciferase activity that may be used in a BRET analysis may be found in U.S. Pat. Nos. 5,229,285, 5,219,737, 5,843,746, 5,196,524, 5,670,356. Alternative DMs include enzymes, which can act on suitable substrates to generate a luminescent signal. Specific examples of such enzymes are beta-galactosidase, alkaline phosphatase, beta-glucuronidase and beta-glucosidase. Synthetic luminescent substrates for these enzymes are well known in the art and are commercially available from companies, such as Tropix Inc. (Bedford, Mass., USA). DMs can also be isolated or engineered from insects (U.S. Pat. No. 5,670,356).

Depending on the substrate, DMs emit light at different wavelengths. Non-limiting examples of substrates for DMs include coelenterazine, benzothiazole, luciferin, enol formate, terpene, and aldehyde, and the like. The DM moiety can be fused to either the amino terminal or carboxyl terminal portion of the RTK protein. Preferably, the positioning of the BDM domain within the RTK-DM fusion does not alter the activity of the native protein or the binding of moieties of the present invention. RTK-DM fusion proteins can be tested to ensure that it retains biochemical properties, such as ligand binding and ability to interact with downstream signaling molecules of the native protein.

AMs in BRET analysis may re-emit the transferred energy as fluorescence. Examples of AMs include Green Fluorescent Protein (GFP), or isoforms and derivatives thereof such as YFP, EGFP, EYFP and the like (R. Y. Tsien, (1998) Ann. Rev. Biochem. 63:509-544). Preferably, the positioning of the AM domain within the AM- protein fusion does not alter the activity of the native protein. AM-second protein fusion proteins can be tested to ensure that it retains biochemical properties of the cognate

native protein, such as interaction with RTKs. By way of example, an amino terminal fusion of the GFP protein to any substrate which is phosphorylated by or can bind to the target RTK can be used.

## V. Pharmaceutical Compositions Containing the Moieties of the Invention

In another aspect, the present invention provides a composition, *e.g.*, a pharmaceutical composition, containing one or a combination of the moieties of the invention (*e.g.*, monoclonal antibodies, or antigen-binding portion(s) thereof, antibody mimetics, small molecules, or peptidic molecules of the present invention), formulated together with a pharmaceutically acceptable carrier. Such compositions may include one or a combination of (*e.g.*, two or more different) antibodies, or immunoconjugates, small molecules, or peptidic molecules of the invention. For example, a pharmaceutical composition of the invention can comprise a combination of antibodies and small molecules that bind to different epitopes on the target RTK or that have complementary activities, *e.g.*, a small molecule that binds to the D3-D4 hinge region of a type III RTK together with a monoclonal antibody that binds the D4 domain of a type III RTK.

Pharmaceutical compositions of the invention also can be administered in combination therapy, *i.e.*, combined with other agents. For example, the combination therapy can include an anti-RTK antibody (or small molecule or peptidic molecule) of the present invention combined with at least one other anti-cancer agent. Examples of therapeutic agents that can be used in a combination therapy are described in greater detail below.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of administration, the active compound, *i.e.*, the moiety of the invention, may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

The pharmaceutical compounds of the invention may include one or more pharmaceutically acceptable salts. A "pharmaceutically acceptable salt" refers to a salt

that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects (see *e.g.*, Berge, S.M., *et al.* (1977) *J. Pharm. Sci.* 66:1-19). Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanoic acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

A pharmaceutical composition of the invention also may include a pharmaceutically acceptable anti-oxidant. Examples of pharmaceutically acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Examples of suitable aqueous and nonaqueous carriers that may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged

absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by sterilization microfiltration. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being treated, and

the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the composition which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 0.01 per cent to about ninety-nine percent of active ingredient, preferably from about 0.1 per cent to about 70 per cent, most preferably from about 1 per cent to about 30 per cent of active ingredient in combination with a pharmaceutically acceptable carrier.

Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

For administration of the antibody, small molecule, or peptidic molecule, the dosage ranges from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg, of the host body weight. For example dosages can be 0.3 mg/kg body weight, 1 mg/kg body weight, 3 mg/kg body weight, 5 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 mg/kg. An exemplary treatment regime entails administration once per week, once every two weeks, once every three weeks, once every four weeks, once a month, once every 3 months or once every three to 6 months. Preferred dosage regimens for a moiety of the invention include 1 mg/kg body weight or 3 mg/kg body weight via intravenous administration, with the antibody being given using one of the following dosing schedules: (i) every four weeks for six dosages, then every three months; (ii) every three weeks; (iii) 3 mg/kg body weight once followed by 1 mg/kg body weight every three weeks.

Alternatively, the antibody, small molecule, or peptidic molecule can be administered as a sustained release formulation, in which case less frequent administration is required. Dosage and frequency vary depending on the half-life of the administered substance in the patient. In general, human antibodies show the longest half life, followed by humanized antibodies, chimeric antibodies, and nonhuman antibodies. The dosage and frequency of administration can vary depending on whether the treatment is prophylactic or therapeutic. In prophylactic applications, a relatively low dosage is administered at relatively infrequent intervals over a long period of time. Some patients continue to receive treatment for the rest of their lives. In therapeutic applications, a relatively high dosage at relatively short intervals is sometimes required until progression of the disease is reduced or terminated, and preferably until the patient shows partial or complete amelioration of symptoms of disease. Thereafter, the patient can be administered a prophylactic regime.

Actual dosage levels of the active ingredients and small molecules in the pharmaceutical compositions of the present invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A "therapeutically effective dosage" of an anti-RTK moiety of the invention preferably results in a decrease in severity of disease symptoms, an increase in frequency and duration of disease symptom-free periods, or a prevention of impairment or disability due to the disease affliction. For example, for the treatment of tumors, a "therapeutically effective dosage" preferably inhibits cell growth or tumor growth by at least about 20%, more preferably by at least about 40%, even more preferably by at least about 60%, and still more preferably by at least about 80% relative to untreated subjects. The ability of a compound to inhibit tumor growth can be evaluated in an animal model

system predictive of efficacy in human tumors. Alternatively, this property of a composition can be evaluated by examining the ability of the compound to inhibit, such inhibition *in vitro* by assays known to the skilled practitioner. A therapeutically effective amount of a therapeutic compound can decrease tumor size, or otherwise ameliorate symptoms in a subject. One of ordinary skill in the art would be able to determine such amounts based on such factors as the subject's size, the severity of the subject's symptoms, and the particular composition or route of administration selected.

An anti-RTK moiety of the present invention may be tested to determine whether it is effective in antagonizing the RTK. One method of testing the anti-RTK moiety is to confirm that interaction occurs between the anti-RTK moiety and the RTK. For example, one of skill in the art may test whether an antibody, small molecule, or peptidic molecule of the invention binds to the D4 or D5 domain of human Kit RTK or D7 domain of a VEGF receptor. Such tests for binding are well known in the art and may include labeling (*e.g.,* radiolabeling) the anti-RTK moiety, incubating the anti-RTK moiety with an RTK under conditions in which binding may occur, and then isolating/visualizing the complex on a gel or phosphor screen. Similarily, the ELISA technique may be employed to determine binding.

Another method to determine whether the moiety of the invention is antagonizing a RTK is to test the phosphorylation state of the cytoplasmic domain of the RTK. In specific embodiments, effective antagonists will prevent activation and autophosphorylation of a RTK. Phosphorylation of the RTK may be tested using standard methods known in the art, for example, by using antibodies which specifically bind the phosphorylated residues of the RTK. Other methods to detect phosphorylation events include those described in U.S. Pat. Nos. 6548266; or Goshe *et al.* (2006) Brief Funct Genomic Proteomic. 4:363-76; de Graauw *et al.* (2006) Electrophoresis. 27:2676-86; Schmidt *et al.* (2007) J Chromatogr B Analyt Technol Biomed Life Sci. 849:154-62; or by the use of the FlashPlates (SMP200) protocol for the Kinase Phosphorylation Assay using [gamma-33P]ATP by PerkinElmer. One of skill in the art will appreciate that these methods, and those demonstrated in the Examples may also be used to determine the phosphorylation state of proteins which are phosphorylated by the RTK and are signal transducers within the cell. Detecing the phosphorylation state of such proteins will also indicate whether the RTK has been effectively antagonized by the moieties of the present invention.

A composition of the present invention can be administered via one or more routes of administration using one or more of a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. Preferred routes of administration for binding moieties of the invention include intravenous, intramuscular, intradermal, intraperitoneal, subcutaneous, spinal or other parenteral routes of administration, for example by injection or infusion. The phrase "parenteral administration" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

Alternatively, an anti-RTK binding moiety of the invention can be administered via a non-parenteral route, such as a topical, epidermal or mucosal route of administration, for example, intranasally, orally, vaginally, rectally, sublingually or topically.

The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. *See, e.g., Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

Therapeutic compositions can be administered with medical devices known in the art. For example, in a preferred embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in U.S. Patent Nos. 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824; or 4,596,556. Examples of well-known implants and modules useful in the present invention include: U.S. Patent No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4,486,194, which discloses a therapeutic device for administering medicants through the skin; U.S. Patent No. 4,447,233, which discloses a medication

infusion pump for delivering medication at a precise infusion rate; U.S. Patent No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Patent No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and U.S. Patent No. 4,475,196, which discloses an osmotic drug delivery system. These patents are incorporated herein by reference. Many other such implants, delivery systems, and modules are known to those skilled in the art.

V. Methods for Using the Moieties of the Invention

In another aspect, the present invention provides a method for treating a RTK associated disease in a subject, comprising administering to the subject a therapeutically effective amount of a moiety of the invention. The anti-RTK moieties, *e.g.*, antibodies, small molecules, or peptidic molecules, of the present invention have numerous *in vitro* and *in vivo* diagnostic and therapeutic utilities involving the diagnosis and treatment of a receptor tyrosine kinase associated disease. The binding moieties of the present invention can be administered to cells in culture, *in vitro* or *ex vivo*, or to human subjects, *e.g.*, *in vivo*, to treat, prevent and to diagnose a receptor tyrosine kinase associated disease.

As used herein "a receptor tyrosine kinase associated disease" is a disease or condition which is mediated by RTK activity or is associated with aberrant RTK expression or activation. Examples of receptor tyrosine kinase associated diseases include diseases or conditions that are associated with, for example, FGF receptors, HGF receptors, insulin receptors, IGF-1 receptors, NGF receptors, VEGF receptors, PDGF-receptor-α, PDGF-receptor-β, CSF-1-receptor, and Flt3-receptors, such as age-related macular degeneration (AMD), atherosclerosis, rheumatoid arthritis, diabetic retinopathy or pain associated diseases. Specific examples of receptor tyrosine kinase associated diseases include, but are not limited to, gastrointestinal stromal tumors (GIST), acute myelogenous leukemia (AML), small cell lung cancer (SCLC), breast cancer, bone metastatic breast cancer, lymphatic diseases and tenosynovial giant cell tumors. Additional examples of receptor tyrosine kinase associated diseases include colon cancer (including small intestine cancer), lung cancer, breast cancer, pancreatic cancer, melanoma (*e.g.*, metastatic malignant melanoma), acute myeloid leukemia, kidney

cancer, bladder cancer, ovarian cancer and prostate cancer. Examples of other cancers that may be treated using the methods of the invention include renal cancer (*e.g.*, renal cell carcinoma), glioblastoma, lymphatic cancer, brain tumors, chronic or acute leukemias including acute lymphocytic leukemia (ALL), adult T-cell leukemia (T-ALL), chronic myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, lymphomas (*e.g.*, Hodgkin's and non-Hodgkin's lymphoma, lymphocytic lymphoma, primary CNS lymphoma, T-cell lymphoma, Burkitt's lymphoma, anaplastic large-cell lymphomas (ALCL), cutaneous T-cell lymphomas, nodular small cleaved-cell lymphomas, peripheral T-cell lymphomas, Lennert's lymphomas, immunoblastic lymphomas, T-cell leukemia/lymphomas (ATLL), entroblastic/centrocytic (cb/cc) follicular lymphomas cancers, diffuse large cell lymphomas of B lineage, angioimmunoblastic lymphadenopathy (AILD)-like T cell lymphoma and HIV associated body cavity based lymphomas), embryonal carcinomas, undifferentiated carcinomas of the rhino-pharynx (*e.g.*, Schmincke's tumor), Castleman's disease, Kaposi's Sarcoma, multiple myeloma, Waldenstrom's macroglobulinemia and other B-cell lymphomas, nasopharangeal carcinomas, bone cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular malignant melanoma, uterine cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, solid tumors of childhood, cancer of the bladder, cancer of the kidney or ureter, carcinoma of the renal pelvis, neoplasm of the central nervous system (CNS), tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, epidermoid cancer, squamous cell cancer, environmentally induced cancers including those induced by asbestos, *e.g.,* mesothelioma and combinations of said cancers. Examples of lymphatic diseases, or "diseases of the lymphatic system", that may be treated using the methods of the invention include afibrinogenemia, anemia, aplastic anemia, hemolytic anemia, congenital nonspherocytic anemia, megaloblastic anemia, pernicious anemia, sickle cell anemia, renal anemia, angiolymphoid hyperplasia with eosinophilia, antithrombin III deficiency, Bernard-Soulier syndrome, blood coagulation disorders, blood platelet disorders, blue rubber bleb nevus syndrome,

Chediak-Higashi syndrome, cryoglobulinemia, disseminated intravascular coagulation, eosinophilia, Erdheim-Chester disease, erythroblastosis, fetal, evans syndrome, factor V deficiency, factor VII deficiency, factor X deficiency, factor XI deficiency, factor XII deficiency, fanconi anemia, giant lymph node hyperplasia, hematologic diseases, hemoglobinopathies, hemoglobinuria, paroxysmal, hemophilia a, hemophilia b, hemorrhagic disease of newborn, histiocytosis, histiocytosis, langerhans-cell, histiocytosis, non-langerhans-cell, job's syndrome, leukopenia, lymphadenitis, lymphangioleiomyomatosis, lymphedema, methemoglobinemia, myelodysplastic syndromes, myelofibrosis, myeloid metaplasia, myeloproliferative disorders, neutropenia, paraproteinemias, platelet storage pool deficiency, polycythemia vera, protein c deficiency, protein s deficiency, purpura, thrombocytopenic, purpura, thrombotic thrombocytopenic, RH-isoimmunization, sarcoidosis, sarcoidosis, spherocytosis, splenic rupture, thalassemia, thrombasthenia, thrombocytopenia, Waldenstrom macroglobulinemia, or Von Willebrand disease.

Furthermore, given the expression of type III or type V RTKs on various tumor cells, the binding moieties, compositions, and methods of the present invention can be used to treat a subject with a tumorigenic disorder, *e.g.*, a disorder characterized by the presence of tumor cells expressing Kit including, for example, gastrointestinal stromal tumors, mast cell disease, and acute myelogenous lukemia. Examples of other subjects with a tumorigenic disorder include subjects having renal cancer (*e.g.*, renal cell carcinoma), glioblastoma, brain tumors, chronic or acute leukemias including acute lymphocytic leukemia (ALL), adult T-cell leukemia (T-ALL), chronic myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, lymphomas (*e.g.*, Hodgkin's and non-Hodgkin's lymphoma, lymphocytic lymphoma, primary CNS lymphoma, T-cell lymphoma, Burkitt's lymphoma, anaplastic large-cell lymphomas (ALCL), cutaneous T-cell lymphomas, nodular small cleaved-cell lymphomas, peripheral T-cell lymphomas, Lennert's lymphomas, immunoblastic lymphomas, T-cell leukemia/lymphomas (ATLL), entroblastic/centrocytic (cb/cc) follicular lymphomas cancers, diffuse large cell lymphomas of B lineage, angioimmunoblastic lymphadenopathy (AILD)-like T cell lymphoma and HIV associated body cavity based lymphomas), embryonal carcinomas, undifferentiated carcinomas of the rhino-pharynx (*e.g.*, Schmincke's tumor), Castleman's disease, Kaposi's Sarcoma, multiple myeloma, Waldenstrom's macroglobulinemia and other B-cell lymphomas, nasopharangeal

carcinomas, bone cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular malignant melanoma, uterine cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, solid tumors of childhood, cancer of the bladder, cancer of the kidney or ureter, carcinoma of the renal pelvis, neoplasm of the central nervous system (CNS), tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, epidermoid cancer, squamous cell cancer, environmentally induced cancers including those induced by asbestos, *e.g.,* mesothelioma and combinations of said cancers.

As used herein, the term "subject" is intended to include human and non-human animals. Non-human animals includes all vertebrates, *e.g.*, mammals and non-mammals, such as non-human primates, sheep, dogs, cats, cows, horses, chickens, amphibians, and reptiles. Preferred subjects include human subjects having a receptor tyrosine kinase associated disease.

The moieties (*e.g.*, antibodies, antigen binding portions thereof, small molecules, peptidic molecules, antibody mimetics, and compositions) of the invention have additional utility in therapy and diagnosis of a RTK associated disease. For example, the human monoclonal antibodies, the multispecific or bispecific molecules, the small molecules, or the peptidic molecules can be used to elicit *in vivo* or *in vitro* one or more of the following biological activities: to inhibit the growth of and/or kill a cell expressing a RTK (*e.g.*, Kit, a VEGF receptor or PDGFR); to mediate phagocytosis or ADCC of a cell expressing a RTK (*e.g.*, Kit, a VEGF receptor or PDGFR) in the presence of human effector cells; or to lock the ectodomain of a RTK, *e.g.*, member of the type III or type V family of RTKs, to an inactive state and/or a monomeric state thereby antagonizing the activity of the receptor.

Suitable routes of administering the anti-RTK moieties of the invention *in vivo* and *in vitro* are well known in the art and can be selected by those of ordinary skill. For example, the anti-RTK moieties can be administered by injection (*e.g.*, intravenous or subcutaneous). Suitable dosages of the molecules used will depend on the age and

weight of the subject and the concentration and/or formulation of the binding moiety composition.

As previously described, the anti-RTK moieties of the invention can be co-administered with one or other more therapeutic agents, *e.g.*, a cytotoxic agent, a radiotoxic agent or an immunosuppressive agent. The moiety can be linked to the agent or can be administered separate from the agent. In the latter case (separate administration), the binding moiety can be administered before, after or concurrently with the agent or can be co-administered with other known therapies, *e.g.*, an anti-cancer therapy, *e.g.*, radiation. Such therapeutic agents include, among others, anti-neoplastic agents such as doxorubicin (adriamycin), cisplatin bleomycin sulfate, carmustine, chlorambucil and cyclophosphamide hydroxyurea which, by themselves, are only effective at levels which are toxic or subtoxic to a patient. Cisplatin is intravenously administered as a 100 mg/ dose once every four weeks and adriamycin is intravenously administered as a 60-75 mg/ml dose once every 21 days. Co-administration of the anti-RTK binding moieties, of the present invention with chemotherapeutic agents provides two anti-cancer agents which operate via different mechanisms which yield a cytotoxic effect to human tumor cells. Such co-administration can solve problems due to development of resistance to drugs or a change in the antigenicity of the tumor cells which would render them unreactive with the binding moiety.

When administering anti-RTK moiety-partner molecule conjugates of the present invention for use in the prophylaxis and/or treatment of diseases related to abnormal cellular proliferation, a circulating concentration of administered compound of about 0.001 μM to 20 μM or about 0.01 μM to 5 μM may be used.

Patient doses for oral administration of the compounds described herein, typically range from about 1 mg/day to about 10,000 mg/day, more typically from about 10 mg/day to about 1,000 mg/day, and most typically from about 50 mg/day to about 500 mg/day. Stated in terms of patient body weight, typical dosages range from about 0.01 to about 150 mg/kg/day, more typically from about 0.1 to about 15 mg/kg/day, and most typically from about 1 to about 10 mg/kg/day, for example 5 mg/kg/day or 3 mg/kg/day.

In at least some embodiments, patient doses that retard or inhibit tumor growth can be 1 μmol/kg/day or less. For example, the patient doses can be 0.9, 0.6, 0.5, 0.45, 0.3, 0.2, 0.15, or 0.1 μmol/kg/day or less (referring to moles of the drug). Preferably, the

anti-RTK moiety-drug conjugate retards growth of the tumor when administered in the daily dosage amount over a period of at least five days.

In one embodiment, conjugates of the invention can be used to target compounds (*e.g.*, therapeutic agents, labels, cytotoxins, radiotoxoins immunosuppressants, etc.) to cells which have RTK cell surface receptors by linking such compounds to the anti-RTK binding moiety. For example, an anti-RTK moiety can be conjugated to any of the toxin compounds described in US Patent Nos. 6,281,354 and 6,548,530, US patent publication Nos. 20030050331, 20030064984, 20030073852 and 20040087497 or published in WO 03/022806, which are hereby incorporated by reference in their entireties. Thus, the invention also provides methods for localizing *ex vivo* or *in vivo* cells expressing RTK (*e.g.*, with a detectable label, such as a radioisotope, a fluorescent compound, an enzyme or an enzyme co-factor).

Target-specific effector cells, *e.g.*, effector cells linked to compositions (*e.g.*, antibodies, antigen binding portions thereof, small molecules, or peptidic molecules ) of the invention can also be used as therapeutic agents. Effector cells for targeting can be human leukocytes such as macrophages, neutrophils or monocytes. Other cells include eosinophils, natural killer cells and other IgG- or IgA-receptor bearing cells. If desired, effector cells can be obtained from the subject to be treated. The target-specific effector cells can be administered as a suspension of cells in a physiologically acceptable solution. The number of cells administered can be in the order of $10^8$-$10^9$ but will vary depending on the therapeutic purpose. In general, the amount will be sufficient to obtain localization at the target cell, *e.g.*, a tumor cell expressing RTK and to effect cell killing by, *e.g.*, phagocytosis. Routes of administration can also vary.

Therapy with target-specific effector cells can be performed in conjunction with other techniques for removal of targeted cells. For example, anti-tumor therapy using the moieties of the invention and/or effector cells armed with these compositions can be used in conjunction with chemotherapy.

The invention further provides methods for detecting the presence of a human RTK antigen in a sample, or measuring the amount of human RTK antigen (*e.g.*, an Ig-like domain of human Kit RTK, human VEGF receptor or PDGFR), comprising contacting the sample, and a control sample, with and RTK binding moiety, *e.g.*, a human monoclonal antibody, or other binding moiety, which specifically binds to a human RTK, under conditions that allow for formation of a complex between the

antibody or other moiety and a human RTK such as Kit or a human VEGF receptor. The formation of a complex is then detected, wherein a difference complex formation between the sample compared to the control sample is indicative the presence of RTK, *e.g.*, human Kit RTK, a human VEGF receptor or the PDGFR RTK in the sample.

Also within the scope of the present invention are kits comprising the anti-RTK binding moieties (*e.g.*,antibodies, antigen binding portions thereof, small molecules, or peptidic molecules) and instructions for use. The kit can further contain one more additional reagents, such as an immunosuppressive reagent, a cytotoxic agent or a radiotoxic agent or one or more additional anti-RTK moieties of the invention (*e.g.*, an anti-RTK binding moiety having a complementary activity which binds to an epitope in the RTK antigen distinct from the first anti-RTK moiety). Kits typically include a label indicating the intended use of the contents of the kit. The term label includes any writing, or recorded material supplied on or with the kit, or which otherwise accompanies the kit.

The present invention is further illustrated by the following examples, which should not be construed as further limiting. The contents of all figures and all references, patents and published patent applications cited throughout this application, as well as the Figures, are expressly incorporated herein by reference in their entirety.

## EXAMPLES

### Introduction to Examples 1-19

Stem cell factor (SCF) is a cytokine that mediates its diverse cellular responses by binding to and activating the receptor tyrosine kinase Kit (also known as SCF-receptor). Kit was initially discovered as an oncogene in a feline retrovirus that captured an activated and truncated form of the surface receptor (Besmer *et al.* (1986) J Virol 60: 194-203.). SCF is encoded by the murine steel (SI) locus while Kit is encoded by the dominant white spotting (W) locus in the mouse (Copeland *et al.* (1990) Cell 63: 175-183; Huang *et al.* (1990) Cell 63: 225-233; Flanagan and Leder (1990) Cell 63: 185-194.; Tan *et al.* (1990) Science 247: 209-212; Bernstein *et al.* (1990) Ciba Found Symp 148: 158-166; discussion 166-172). SCF functions as a non-covalent homodimer and both membrane-anchored and soluble forms of SCF generated by alternative RNA

splicing and by proteolytic processing have been described (reviewed in Ashman (1999) Int J Biochem Cell Biol 31:1037-1051). Kit is a member of type-III family of receptor tyrosine kinases (RTK), which also includes PDGF-receptor-α, and β, CSF-1-receptor (also known as M-CSF-receptor or Fms), and the Flt3-receptor (also known as Flk2) (reviewed in Ullrich and Schlessinger (1990) Cell 61: 203-212; Blume-Jensen *et al.* (2001) Nature 411: 355-365). Kit is composed of a glycosylated extracellular ligand binding domain (ectodomain) that is connected to a cytoplasmic region by means of a single transmembrane (TM) domain (reviewed in Schlessinger (2000) Cell 103: 211-225). The ectodomain of Kit and other members of type-III RTKs all contain five Ig-like domains, in which the second and third membrane distal domains were shown to play a role in ligand recognition (reviewed in Ullrich and Schlessinger (1990) Cell 61: 203-212). Other RTKs whose extracellular ligand binding domains are composed exclusively of multiple Ig-like repeats include members of the VEGF-receptor family (7 Ig-like), CCK4-receptor (7 Ig-like) and FGF-receptors (3 Ig-like). The cytoplasmic region of Kit contains a protein tyrosine kinase (PTK) domain with a large kinase-insert region; another hallmark of type-III RTKs. Binding of SCF to Kit leads to receptor dimerization, intermolecular autophosphorylation and PTK activation. It was proposed that the fourth Ig-like domain of Kit is responsible for Kit dimerization in response to either monovalent or bivalent SCF binding (Lev *et al.* (1992b) J Biol Chem 267: 15970-15977; Blechman *et al.* (1995) Cell 80: 103-113). However, other studies have demonstrated that ligand induced dimerization of Kit is driven by bivalent binding of SCF (Philo *et al.* (1996) J Biol Chem 271: 6895-6902; Lemmon *et al.* (1997) J Biol Chem 272: 6311-6317).

Characterization of mice mutated at the SCF or Kit loci has shown that SCF and Kit are required for development of hematopoietic cells, melanocytes, germ cells and intestinal pacemaker cells (reviewed in Ashman (1999) Int J Biochem Cell Biol 31:1037-1051). In humans, loss of function mutations in Kit cause the piebald trait that is characterized by de-pigmentation of the ventral chest and abdomen, white fareflock of hair, deafness and constipation (Fleischman *et al.* (1991) Proc Natl Acad Sci U S A 88: 10885-10889). A variety of gain-of-function mutations in Kit were found in different types of human cancers. Activating Kit mutations were found in gastro-intestinal-stromal tumors (GIST), acute myeloid leukemia (AML) and mast cell leukemia (MCL) among

other cancers. Mutations were identified in the membrane proximal Ig-like domain (D5) (exon 8 and 9), in the juxtamembrane (JM) domain (exon 11), and in the tyrosine kinase (PTK) domain (exon 17) (see Forbes *et al.* (2006) COSMIC 2005. BR J. CANCER, 94: 318-22. Somatic mutation database: Catalogue of Somatic Mutations in Cancer http://www.sanger.ac.uk/genetics/CGP/cosmic/). While there is good evidence that the gain of function mutations in the JM and the PTK domains lead to constitutive activation of Kit, by relieving autoinhibitory constraints (Mol *et al.* (2004) J Biol Chem. 279: 31655-31663), the molecular mechanism underlying the gain of function mutations in D5 of the ectodomain is not understood. There is a need to better characterize the structures of RTKs such as Kit and PDGFR, as well as SCF, PDGFα/β, and the bound Kit/SCFcomplex. Such a characterization will lead to the informed identification of regions which may be targeted with drugs, pharmaceuticals, or other biologics.

Stem Cell Factor (SCF) initiates its multiple cellular responses by binding to the ectodomain of Kit resulting in tyrosine kinase activation. In some of the examples below the crystal structure of the entire ectodomain of Kit before and after SCF stimulation is described. The structures show that Kit dimerization is driven by SCF binding whose sole role is to bring two Kit molecules together. Receptor dimerization is followed by conformational changes that enable lateral interactions between membrane proximal Ig-like domains D4 and D5 of two Kit molecules. Experiments with cultured cells show that Kit activation is compromised by point mutations in amino acids critical for D4-D4 interaction. Moreover, a variety of oncogenic mutations are mapped to the D5-D5 interface. Since key hallmarks of Kit structures, ligand-induced receptor dimerization and the critical residues in the D4-D4 interface are conserved in other receptors, the mechanism of Kit stimulation unveiled in this report may apply for other receptor activation. This indicates that drugs or biologics targeted to these interfaces can be used as therapeutics.

The elucidation of the X-ray crystal structure of the entire ectodomain of Kit before and after SCF stimulation described herein has provided valuable insights concerning the mechanism of SCF-induced Kit dimerization and activation. The structure shows that the first three Ig-like domains of Kit designated D1, D2 and D3 are responsible for SCF binding. The main role of SCF binding is to crosslink two Kit molecules to increase the local concentration of Kit on the cell membrane. This

facilitates a large conformational change in the membrane-proximal regions of Kit resulting in homotypic interaction between D4 or D5 of neighboring Kit molecules. The lateral interactions between D4 of two neighboring Kit molecules occur via direct contacts through two pairs of salt bridges from the EF loops of each D4 protomer. The membrane proximal D5 domain provides additional indirect interactions between neighboring Kit molecules to further stabilize and position the membrane proximal part of the ectodomain at a distance and orientation that enables the activation of cytoplasmic tyrosine kinase.

In several of the examples below the crystal structures of the entire ectodomain of Kit in both monomeric and SCF-induced homodimeric (SCF-Kit 2:2 complex) forms is described. Detailed views of the unoccupied monomeric form at 3.0 Å resolution and SCF-induced homodimeric form at 3.5 Å resolution provide novel insights concerning the activation mechanism of Kit and other RTKs. It should be appreciated by one of skill in the art that the experiments described below may be performed with other RTKs. Example RTK sequences which may be used by methods of the present invention include, but are not limited to, the Genbank reference sequence for the Kit mRNA NM_000222.2 (encoding the protein NP_000213.1;

MRGARGAWDFLCVLLLLLRVQTGSSQPSVSPGEPSPPSIHPGKSDLIVRVGDEIRL
LCTDPGFVKWTFEILDETNENKQNEWITEKAEATNTGKYTCTNKHGLSNSIYVF
VRDPAKLFLVDRSLYGKEDNDTLVRCPLTDPEVTNYSLKGCQGKPLPKDLRFIPD
PKAGIMIKSVKRAYHRLCLHCSVDQEGKSVLSEKFILKVRPAFKAVPVVSVSKAS
YLLREGEEFTVTCTIKDVSSSVYSTWKRENSQTKLQEKYNSWHHGDFNYERQA
TLTISSARVNDSGVFMCYANNTFGSANVTTTLEVVDKGFINIFPMINTTVFVNDG
ENVDLIVEYEAFPKPEHQQWIYMNRTFTDKWEDYPKSENESNIRYVSELHLTRL
KGTEGGTYTFLVSNSDVNAAIAFNVYVNTKPEILTYDRLVNGMLQCVAAGFPEP
TIDWYFCPGTEQRCSASVLPVDVQTLNSSGPPFGKLVVQSSIDSSAFKHNGTVEC
KAYNDVGKTSAYFNFAFKGNNKEQIHPHTLFTPLLIGFVIVAGMMCIIVMILTYK
YLQKPMYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFGKTLGAG
AFGKVVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREALMSELKVLSYLGNHM
NIVNLLGACTIGGPTLVITEYCCYGDLLNFLRRKRDSFICSKQEDHAEAALYKNL
LHSKESSCSDSTNEYMDMKPGVSYVVPTKADKRRSVRIGSYIERDVTPAIMEDD
ELALDLEDLLSFSYQVAKGMAFLASKNCIHRDLAARNILLTHGRITKICDFGLAR
DIKNDSNYVVKGNARLPVKWMAPESIFNCVYTFESDVWSYGIFLWELFSLGSSP

YPGMPVDSKFYKMIKEGFRMLSPEHAPAEMYDIMKTCWDADPLKRPTFKQIVQ
LIEKQISESTNHIYSNLANCSPNRQKPVVDHSVRINSVGSTASSSQPLLVHDDV
(SEQ ID NO: 92)) or the Genbank reference sequence for variant 2 of the Kit mRNA
NM_001093772.1 (encoding protein NP_001087241.1;
MRGARGAWDFLCVLLLLLRVQTGSSQPSVSPGEPSPPSIHPGKSDLIVRVGDEIRL
LCTDPGFVKWTFEILDETNENKQNEWITEKAEATNTGKYTCTNKHGLSNSIYVF
VRDPAKLFLVDRSLYGKEDNDTLVRCPLTDPEVTNYSLKGCQGKPLPKDLRFIPD
PKAGIMIKSVKRAYHRLCLHCSVDQEGKSVLSEKFILKVRPAFKAVPVVSVSKAS
YLLREGEEFTVTCTIKDVSSSVYSTWKRENSQTKLQEKYNSWHHGDFNYERQA
TLTISSARVNDSGVFMCYANNTFGSANVTTTLEVVDKGFINIFPMINTTVFVNDG
ENVDLIVEYEAFPKPEHQQWIYMNRTFTDKWEDYPKSENESNIRYVSELHLTRL
KGTEGGTYTFLVSNSDVNAAIAFNVYVNTKPEILTYDRLVNGMLQCVAAGFPEP
TIDWYFCPGTEQRCSASVLPVDVQTLNSSGPPFGKLVVQSSIDSSAFKHNGTVEC
KAYNDVGKTSAYFNFAFKEQIHPHTLFTPLLIGFVIVAGMMCIIVMILTYKYLQKP
MYEVQWKVVEEINGNNYVYIDPTQLPYDHKWEFPRNRLSFGKTLGAGAFGKV
VEATAYGLIKSDAAMTVAVKMLKPSAHLTEREALMSELKVLSYLGNHMNIVNLL
GACTIGGPTLVITEYCCYGDLLNFLRRKRDSFICSKQEDHAEAALYKNLLHSKES
SCSDSTNEYMDMKPGVSYVVPTKADKRRSVRIGSYIERDVTPAIMEDDELALDL
EDLLSFSYQVAKGMAFLASKNCIHRDLAARNILLTHGRITKICDFGLARDIKNDS
NYVVKGNARLPVKWMAPESIFNCVYTFESDVWSYGIFLWELFSLGSSPYPGMP
VDSKFYKMIKEGFRMLSPEHAPAEMYDIMKTCWDADPLKRPTFKQIVQLIEKQI
SESTNHIYSNLANCSPNRQKPVVDHSVRINSVGSTASSSQPLLVHDDV (SEQ ID
NO: 93)), wherein the proteins are designated by the standard 1-letter amino acid code.

**Example 1: Expression, Purification and Crystallization of SCF and Kit**

The entire ectodomain of Kit composed of five Ig-like domains designated D1, D2, D3, D4 and D5 was expressed in insect cells using the baculovirus expression system. Purified Kit ectodomain monomers or SCF-induced Kit ectodomain homodimers (SCF-Kit 2:2 complex) were each subjected to extensive screening for crystal growth and optimization followed by determination of their crystal structures.

Protein expression and purification

A soluble Kit ectodomain (amino acids 1-519) containing a poly-histidine tag at the C-terminus was expressed in insect cells (Sf9) using the baculovirus expression system. Kit ectodomain was purified by Ni-chelate followed by size-exclusion chromatography (Superdex 200, GE Healthcare). After partial deglycosylation using endo-glycosidase F1, the ectodomain was further purified by anion exchange chromatography (MonoQ, GE Healthcare). SCF (1-141) was expressed, refolded and purified as previously described (Langley *et al.* (1994) Arch Biochem Biophys 311: 55-61; Zhang *et al.* (2000) Proc Natl Acad Sci U S A 97: 7732-7737).

Cell lines and expression vectors

HEK and NIH3T3 cells were cultured in DMEM supplemented with 10% FCS and 10% CS, respectively. Prior to SCF stimulation, cells were starved overnight in serum free medium as previously described (Kouhara et al. (1997) Cell 30: 693-702). Transfection was performed with Lipofectamin (Invitrogen) according to the manufacturer instructions. The cDNA of full length Kit was subcloned into the RK5 expression vector for transient transfection and into the pBABE/puro vector for stable expression (Kouhara et al. (1997) Cell 30: 693-702). Anti-Kit antibodies were generated by immunizing rabbits with recombinant Kit ectodomain. Monoclonal anti-Kit antibodies (Santa Cruz) were used for immunoblotting. Anti-phosphotyrosine (anti-pTyr) antibodies were purchased from Upstate Biotechnology.

Crystallization and data collection

Samples of Kit ectodomain alone or in complex with SCF were subjected to extensive screening for crystal growth and optimization. Crystals of deglycosylated ectodomain of approximate dimensions of 0.12x0.1x0.05 mm were obtained in phosphate buffer with polyethyleneglycol (PEG) as the precipitant (0.1 M Na-Pi buffer pH 6.0, 0.2 M KCl, 12% PEG 400) at 4°. All crystals were immersed in a reservoir solution supplemented with 5-18% glycerol for several seconds; flash cooled, and kept in a stream of nitrogen gas at 100° K during data collection. The crystals belonged to the rhomboidal space group $R3$ with unit cell dimensions of $a = 162.4$ Å, and $c = 67.1$ Å in hexagonal lattice setting, with one molecule per asymmetric unit. Platinum, bromine and

iodine derivatives of Kit were prepared by soaking the crystals in a reservoir solution containing heavy atom reagents in concentration ranges of 0.1 mM to 50 mM at 277 K for few seconds to 10 days.

Crystals of the SCF-Kit complex were grown with polyethyleneglycol (PEG) as the precipitant (0.2 M ammonium sulfate, 8-12% PEG 8000, 5-8% ethylene glycol at pH 7.0-8.5) at 4°C and diffraction data were collected to resolution of 3.5 Å with a ADSD quantum-210 CCD detector at the X25 beamline of NSLS, Brookhaven National Laboratory. The crystals belong to the monoclinic space group *C*2 with unit cell dimensions $a = 269.5$ Å, $b = 52.1$ Å, $c = 189.8$ Å, $\beta = 108.2°$, which is comprised of two sets of SCF and Kit molecules in the asymmetric unit. All data sets were processed and scaled using the DENZO and SCALEPACK and the HKL2000 program package (Otwinowski *et al.* (1997) Methods Enzymol. 276: 307–326). The data collection statistics are summarized in Table 1A.

**Example 2: Structure determination**

The experimental phases were calculated by using multiple isomorphous replacement with anomalous scattering (MIRAS) and by multi-wavelength anomalous diffraction (MAD) to 3.0 Å resolution (Table 1A). The resulting electron-density maps showed continuous electron density of β sandwich structures, and clear solvent-protein boundaries. The molecular model of monomeric Kit ectodomain was built manually into the experimental electron density maps. The structure was refined to a 3.0Å resolution using the native data set to a crystallographic R-factor of 25.4 % and free R-factor of 29.6% (Table 1B). The structure of SCF-Kit 2:2 complex was solved by molecular replacement using the structure of the monomeric form described in this report and the structure of SCF (Zhang *et al.* (2000) Proc Natl Acad Sci U S A 97: 7732-7737; retrievable from the Protein Data Bank with code: 1EXZ) as search models. The structure was refined to 3.5 Å resolution using the native data set to a crystallographic R-factor of 24.9% and free R-factor of 29.5 % (Tables 1A and 1B). Molecular images were produced using Pymol (pymol.sourceforge.net) and CCP4MG (Potterton *et al.* (2004) Acta Crystallogr D Biol Crystallogr 60: 2288-2294) software. The atomic coordinates and structure factors of Kit monomer and SCF-Kit complex have been

deposited in the Protein Data Bank (rcsb.org/pdb) with accession code 2EC8 and 2E9W, respectively.

Table 1A. Data collection and phasing statistics

| | Kit | | | | | | SCF-Kit |
|---|---|---|---|---|---|---|---|
| | Native | $K_2Pt(NO_2)_4$ | $NaI_2$ | $K_2Pt(NO_2)_4$ (Peak) | $K_2Pt(NO_2)_4$ (inflection) | $K_2Pt(NO_2)_4$ (remote) | Native |
| **Data collection** | | | | | | | |
| X-ray source | NSLS X26C | NSLS X8A | NSLS X8A | NSLS 8A | NSLS 8A | NSLS 8A | NSLS X25 |
| Date | 2006-Apr-3 | 2006-Jun-10 | 2006-Jun-12 | 2006-Jun-11 | 2006-Jun-11 | 2006-Jun-11 | 1999-Jun-29 |
| Wavelength (Å) | 1.1000 | 1.0716 | 1.5000 | 1.0716 | 1.0722 | 0.9600 | 1.2500 |
| Space group | *R3* | *R3* | *R3* | *R3* | *R3* | *R3* | *C2* |
| Unit Cell dimensions | | | | | | | |
| *a* (Å) | 162.25 | 162.09 | 161.61 | 162.02 | 162.44 | 162.27 | 269.48 |
| *b* (Å) | 162.25 | 162.09 | 161.61 | 162.02 | 162.44 | 162.27 | 52.07 |
| *c* (Å) | 97.59 | 96.94 | 97.62 | 99.02 | 99.13 | 99.09 | 189.79 |
| β (°) | | | | | | | 108.24 |
| Resolution (Å) | 50-3.0 (3.11-3.0) | 50-3.1 (3.2-3.1) | 50-3.0 (3.11-3.00) | 50-3.3 (3.42-3.3) | 50-3.3 (3.42-3.3) | 50-3.3 (3.42-3.3) | 50-3.5 (3.63-3.50) |
| No. of total reflections | 156126 | 68961 | 128301 | 76599 | 79043 | 79258 | 113481 |
| No. of unique reflections | 19342 | 23535 | 26283 | 19468 | 19558 | 19473 | 31962 |
| Completeness (%)[a] | 99.8 (100) | 99.2(99.6) | 99.5 (97.1) | 95.4 (97.2) | 95.4 (97.0) | 95.0 (96.4) | 96.5 (91.4) |
| I/σ(I) | 11.7 (7.5) | 11.3 (3.9) | 21.7 (9.5) | 13.0 (5.9) | 11.6 (4.8) | 11.2 (4.7) | 19.1 (6.8) |
| $R_{merge}$ (%)[b] | 7.8 (17.5) | 8.6 (32.1) | 8.5 (20.8) | 11.1 (22.8) | 10.7 (27.0) | 11.9 (27.5) | 8.1 (17.8) |
| **Phasing statistics** | | | | | | | |
| | MIRAS | | | MAD | | | |
| Resolution (Å) | 50-3.0 | 50-3.0 | 50-3.0 | 50-3.3 | 50-3.3 | 50-3.3 | |
| No. of sites | | 3 | 6 | 3 | 3 | 3 | |
| $R_{cullis}$ [c] (%) | | 22.8 | 11.6 | | | | |
| $R_{cullis}$ (iso/anom)[d] | | 0.85/0.68 | 0.80/0.61 | 0.79 | 0.74/0.81 | 0.81/0.86 | |
| Phasing power (iso/anom) [e] | | 0.86/1.48 | 1.07/0.85 | 1.10 | 1.32/1.06 | 0.97/0.16 | |
| <FOM> [f] | 0.50 | | | 0.48 | | | |

Values in parentheses indicate statistics for the highest resolution shells. (a) Completeness = (number of independent reflections) / (total theoretical reflections). (b) $R_{merge}$ = $\Sigma|I-\langle I\rangle| / \Sigma I$ , where *I* is the observed intensity and <*I*> is the averaged intensity obtained from multiple observations of symmetry related reflections. (c) $R_{iso}$ = $\Sigma||Fph| - |Fp||/\Sigma|Fp|$. (d) $R_{cullis}$ = $\Sigma|Fh - (Fph - Fp)| / \Sigma|Fph - Fp|$. (e)Phasing power = r.m.s. (|Fh|/E), where Fph and Fp are the derivative and native structure-factor amplitudes, Fh is the heavy-atom structure amplitude, respectively. E is the residual lack of closure error. (f) <FOM> is the mean figure of merit.

Table 1B. Refinement statistics

| | Kit | SCF-Kit |
|---|---|---|
| Resolution (Å) | 27.7 – 3.0 | 39.0 – 3.5 |
| No. of reflections (work/test) | 12521/650 | 30351/1594 |
| $R_{cryst}$ [a]/ $R_{free}$ [b] (%) | 25.4/29.6 | 24.9/29.5 |
| No. of atoms | | |
| Protein | 3498 | 9064 |
| Waters | 0 | 0 |
| Carbohydrate | 70 | 84 |
| Average B-factor (Å$^2$) | 31.6 | 75.2 |
| R.m.s.d. from ideal [c] | | |
| Bond lengths (Å) | 0.015 | 0.013 |
| Bond angles (°) | 1.8 | 1.9 |
| Ramachandran plot quality | | |
| Most favored (%) | 74.4 | 73.5 |
| Additionally favored (%) | 24.1 | 24.5 |
| Generously allowed (%) | 1.5 | 2.0 |
| Disallowed (%) | 0 | 0 |
| PDB code | 2EC8 | 2E9W |

(a) $R_{cryst} = \Sigma||F_{obs}|-|F_{calc}||/\Sigma|F_{obs}|$, where $F_{obs}$ and $F_{calc}$ are the observed and the calculated structure factors, respectively. (b) $R_{free}$ is calculated from 5 % of reflections removed before refinement. (c) R.m.s.d., root mean square deviation.

**Example 3: Analysis of the Structure of the Kit ectodomain**

General Analysis of Ectodomain Structure

Kit ectodomain shows an elongated serpentine shape with approximate dimensions of 170 x 60 x 50 Å (Figure 1A). The D1, D2, D3, D4 and D5 domains of Kit exhibit a typical immunoglobulin super family (IgSF) fold, composed of eight β strands, designated ABCC'DEFG, assembled into a β sandwich consisting of two anti-parallel β sheets (Figure 1A). D1, D2, D3 and D5 each contain a conserved disulfide bond connecting cysteine residues at B5 and F5 (Fifth amino acids of strand B and F, respectively); positions that bridge the two β sheets to form the center of the hydrophobic core of the Ig-like fold (Harpaz and Chothia (1994) J Mol Biol 238: 528-539). D2 and D5 contain two disulphide bonds and D4 does not contain any cysteine residue, nevertheless, the integrity of the Ig-like fold of D4 is maintained even though the conserved cysteine residues at B5 and F5 are replaced by a valine and phenylalanine residues, respectively.

The angle between D1 and D2 along the axis of the two domains is 76° (Figure 1A, B) resembling the orientation between the first and second Ig-like domains of interleukin-1β receptor (Vigers *et al.* (1997) Nature, 386: 190-194). In contrast, the angle between D2 and D3 is 150°, between D3 and D4 is 119° and between D4 and D5

is 162°. The orientations between the ABED and A'GFC β-sheets for the different Ig-like domains are ~180° for D1-D2, ~180° for D2-D3, ~90° for D3-D4, and ~180° for D4-D5 (Figure 1).

The superposition of all five Ig-like domains of the Kit ectodomain with telokin (Holden *et al.* (1992) J. Mol. Biol. 227: 840-851) used as a standard for Ig-folds reveals a root mean square (r.m.s.) deviation of 1.5-2.9 Å for equivalent Cα atoms. D2 is the most divergent among the five Kit Ig-like domains (Figure 8) as revealed by its higher r.m.s.d. values when superimposed with telokin. Based on the structural conservation of key amino acids in Ig-like domains and their secondary structural topology (Harpaz *et al.* (1994) J Mol Biol 238: 528-539; Halaby *et al.* (1999) Protein Eng 12: 563-571), D1, D2, D3 and D4 belong to the I-subset and D5 is related to the C2 and IgCAM subsets of IgSF. Furthermore, among the structurally conserved 20 finger-print residues of IgSF (Harpaz *et al.* (1994) J Mol Biol 238: 528-539), 10-14 residues are conserved in the five Ig-like domains of Kit (Table 2).

Table 2. 20 of key finger print residues of IgSF for Kit domains and Telokin (PDB code: 1TLK) as thetypical I-set IgSF(1)

| Position | D1 | D2 | D3 | D4 | D5 | Telokin | Characteristic |
|---|---|---|---|---|---|---|---|
| A'B1 | Gly51 | Asp129 | Gly226 | Gly328 | Asn423 | Gly58 | Gly |
| B1 | Ile54 | Thr132 | Phe229 | Val331 | Gly424 | Ala59 | Aliphatic |
| B3 | Leu56 | Val134 | Val231 | Leu333 | Leu426 | Phe61 | large hydrophobic |
| B5 | Cys58 | Cys136 | Cys233 | Val335 | Cys428 | Cys63 | Cys |
| B7 | Asp60 | Leu138 | Ile235 | Tyr337 | Ala430 | Val65 | Neutral or hydrophobic |
| C2 | (Phe63) | Tyr146 | Ser244 | Gln346 | Ile438 | Val73 | Hydrophobic |
| C4 | Trp66 | Leu148 | Trp246 | Trp348 | Trp440 | Trp75 | Trp |
| CD | - | (Leu156) | (Leu253) | Phe355 | - | Val82 | large hydrophobic |
| D1 | - | (Asp159) | (Gln258) | (Lys358) | - | His87 | basic and form salt bridge |
| D2 | - | (Leu160) | (Glu257) | (Trp359) | - | Phe88 | Hydrophobic |
| E4 | Trp82 | Ile170 | Leu275 | Leu377 | Ile478 | Leu100 | Hydrophobic, almost Leu |
| E6 | Thr84 | Ile172 | Ile277 | Leu379 | Ser480 | Ile102 | Hydrophobic |
| EF2 | Ala87 | Val175 | Ala280 | Leu382 | - | Val105 | Hydrophobic |
| EF6 | Asn91 | Tyr179 | Asp284 | Glu386 | - | Asp109 | Asp |
| F1 | Gly93 | Leu182 | Gly286 | Gly388 | Gly487 | Ala111 | Gly or Ala or (Asp) |
| F3 | Tyr95 | Leu184 | Phe288 | Tyr390 | Val489 | Tyr113 | Tyr |
| F5 | Cys97 | Cys186 | Cys290 | Phe392 | Cys491 | Cys115 | Cys |
| G6 | Ile107 | Phe204 | Thr303 | Phe405 | Phe504 | Ala128 | Hydrophobic |
| G8 | Val109 | Leu202 | Leu305 | Val407 | Phe506 | Leu130 | Hydrophobic |
| G10 | Val111 | Val210 | Val307 | Val409 | - | Va132 | Hydrophobic |

(1) The positions of 20 of key finger print residues and typical characteristics of each of finger print residue are shown in the first and last column, which are defined by Harpaz and Chothia, 1994.

Detailed Analysis of the Structure of Kit Ig-like domains

**Kit D1.** The D1 fold is a β sandwich composed of two β sheets. One sheet is formed by the three-strands, A, B and E and the second sheet is composed of the five-strands, A', G, F, C and C'(ABE/A'GFCC'). The first strand, interrupted by a cis-conformation at Pro41, is split into two shorter strands of A and A' which pair with strands B and G, respectively. A disulfide bond connecting Cys58 of B5 with Cys97 of F5 bridges the two β sheets. A fairly long strand C', that interacts with strand C, directs the C-terminal end of the polypeptide chain toward the upper side of D1 which is directly connected to strand E. On the basis of the Ig-like domain nomenclature, D1 belongs to the I2-subset of IgSF (Casasnovas *et al.* (1998) Proc Natl Acad Sci USA 95: 4134-4139).

**Kit D2.** D2 consists of a small β-sheet formed by strands B, E, and D and a second β-sheet composed of strands A', G, F and C (BED/A'GFC), as well as an additional helix at the crossover between strands E and F (residues 177-179). Although 11 of 20 hallmark residues of I-set of IgSF are identified on D2, this Ig-like domain differs from a standard I1-set of IgSF in a number of ways. D2 has a Leu residue at the C4 position, while other I1-set of IgSF have a conserved Trp. The pattern of hydrogen bonds in strand B is altered due to formation of two short β strands, referred as strands B and B'. The additional B' strand is aligned to strand A, forming a short β sheet with an AB' topology. The G strand is split into two short strands, G (bottom side) and G' (top side) because of an insert at amino acids 197-199, which results in formation of a β sheet with strand A'. Disruption of the hydrogen bond pattern caused by a "kink" in G strand at residues 197-199 is compensated by the hydrogen bonds between the side chains of Ser197 and the main chain amide of Cys186. Notably Ser197, is conserved as a Ser or Thr residue in Kit from different species and in other type-III RTKs. D2 contains an additional disulfide bond, between Cys151 and Cys183 bridging the CD loop with the end of the F strand to provide additional stability to strand C and the CD loop. The additional disulfide bridge may compensate for the reduced network of hydrogen bonds between strands C and F. These two Cys are highly conserved in Kit from zebrafish to humans.

**Kit D3.** D3 is composed of two sets of β sheets (ABED/A'GFC) belonging to the I1-subset of IgSF. The two β sheets are bridged by a disulfide bond between Cys233 on strand B and Cys290 on strand F. Comparison of telokin (PDB code: 1TLK) and D3

structures shows a Zscore of 10.4 and an r.m.s. deviation of 2.0 Å for the 98 aligned Cα residues of D3.

**Kit D4.** Although D4 lacks the characteristic disulfide bond between cysteines at B5 and F5, D4 maintains an IgSF topology. In addition, 13 out of 20 finger-print residues of I-set IgSF are conserved in D4. The structural integrity of D4 is preserved by interactions between buried aliphatic (Val335) and aromatic (Phe392) residues present at B5 and F5, respectively, which constitute part of the hydrophobic core of the domain. Structural comparison using DALI shows that among Kit Ig-like domains D4 is most similar to telokin (retrieve with Protein Data Bank code: 1TLK), with a Z-score of 11.9 and an r.m.s.d. of 1.5 Å for the 89 aligned Cα residues. The distance of 8.6 Å between Cα-Cα of Val335 and Phe392 is within the distance range seen between similar positions in IgSF domains lacking a disulfide bond connecting B5 and F5. For example, Titin Ig-like domain M5 (Protein Data Bank code: 1TNM); also lacking a disulfide bond, superimposes with an r.m.s.d of 2 Å with D4 and has a distance of 8.9 Å between B5 and F5 positions. D4 is composed of two β sheets each containing four strands with the arrangement ABED/A'GFC. Thr321, the first residue of the A' strand, forms Van der Waals contacts with the aromatic ring of the highly conserved Phe405. Notably, the CD loop folded upwards to the top side of the domain is stabilized by three main interactions. Side chain of Thr354 forms hydrogen bonds with side chain of Gln347 and main chain carbonyl of Trp348. The hydrophobic residues (Trp348, Tyr350, Trp359 Val377, Leu379 and Tyr390), located at the edge of the hydrophobic core provide a hydrophobic environment for Phe355. Although the CD loop does not exhibit notable sequence conservation, this loop contains eight amino acids in all type-III family RTKs.

**Kit D5.** D5 belongs to C2 and IgCAM subset of IgSF and 10 out of 20 fingerprint residues are conserved in this module. D5 exhibits a ABED/CFG topology, a disulfide bond between Cys428 of B5 and Cys491 of F5 that bridges the two β sheets and a second disulfide bond bridging the C strand and the CD loop. The two disulfide bonds are conserved in all Kit and type-III RTKs. Notably, the top half of D5 resembles the third Ig of neuronal cell adhesion molecule

Axonin-1/TAG-1 (Protein Data Bank code 1CS6). Several hallmarks can be identified, though to a lesser extent in Telokin (Protein Data Bank code 1FHG), FGFR (Protein Data Bank code 1CVS) and in the RTK Musk (Protein Data Bank code 2IEP).

These include two Ala residues (Ala430 and Ala493), in proximity to the disulfide bond connecting B5 with F5; the presence of small side chains in this region enables close packing at the top of the domain. The second hallmark is a ring arrangement of the Pro and Gly residues Pro413, Gly432, Pro436 and Gly498 in the A, B, C and G strands, respectively. The third hallmark is the presence of an Asn residue in F9 (Asn495) that forms hydrogen bonds with main chains of Val497 and Pro434 of FG and BC loop, respectively. Taken together, these three hallmarks at the top of D5 result in a tightly packed configuration similar to the configuration of of Ig-like domains of cell adhesion proteins.

**Example 4: Inter Ig-like domain interactions in Kit monomeric form**

The inter-domain interactions between the 5 Ig-like domains of Kit are responsible for maintaining the overall topology of Kit ectodomain monomers (Figure 1). The orientation of D1 relative to D2 is determined by the extensive buried surface area that is caused by the numerous interactions between the two Ig-like domains (Figure 1B). The buried surface area of 1240 $Å^2$ in the D1-D2 interface is much larger than the buried surface areas of most inter Ig-like domain interfaces of rod-like multi-domain IgSF structures (Su *et al.* (1998) Science 281: 991-995) including the three other inter Ig-like interfaces in Kit ectodomain that range between 500 and 800 $Å^2$. This interface is formed primarily by hydrophobic and electrostatic interactions between strands A' and G, loops EF and CC' of D1 with the N-terminal region of strand A, the C-terminal end of strand B, loop BC and DE of D2 (Figure 1B). Moreover, many residues in the D1-D2 interface including amino acids from strands G of D1, the linker region connecting D1 and D2 and the BC loop of D2 are conserved in Kit from different species (Figure 1B).

The buried surface area of the D2-D3 interface is approximately 780 $Å^2$. The D2-D3 interface is composed of a small hydrophobic patch surrounded by two electrostatic interactions. This interface is formed by an interaction between the EF loop of D2 and the DE loop of D3 and interactions between the D2-D3 linker region with the FG and BC loops of D3 (Figure 1C). The buried surface area of D3-D4 interface is approximately 570 $A^2$. D3 and D4 interact primarily through strands A' and G of D3 with the BC and DE loops of D4 (Figure 1D). The length of the D3-D4 interface is

approximately 20 Å due to the angular arrangement of D4 relative to D3 with an angle of 119° along the long axis of the two Ig-like domains. The D4-D5 interface forms a buried surface area of 760 $Å^2$, mainly mediated by hydrophobic interactions (Figure 1E). The interface is formed by interactions between strands A, G and F of D4, with the BC and DE loops of D5, as well as with the D4-D5 linker region (Figure 1E)

Detailed Domain-by-Domain information about Inter-Ig-like domain interactions in Kit monomers

**The D1-D2 interface.** The hydrophobic interactions between residues Ile47, Ile70, Leu71, Ala89, Tyr108 and Phe110 of D1 and Leu119, Pro137, Leu138, Pro141, Pro166 and the side chain of Lys167 of D2 stabilize the interdomain interactions (Figure 1B). There are two major electrostatic interactions in the region surrounding the hydrophobic patch including interaction between Arg112 of D1 and Asp140 of D2 and interactions between Asp72 of D1 and Arg135 of D2 (Figure 1B).

**The D2-D3 interface.** The hydrophobic patch is composed of the aliphatic part of Arg177 and side chains of Pro206, Phe208, Val238 and Phe267. The electrostatic interaction involves hydrogen bonds between side chains of Glu128 and Asp129 of D2 with Lys209 of D3 (Figure 1C). A salt bridge between the side chain of Arg177 and the side chain of Glu128 stabilize the position of the side chain of Arg177 and the side chain of Pro206 in D2 and Phe267 in D3 to create a hydrophobic environment for the aliphatic portion of the side chain of Arg177 in D2 (Figure 1C). A second electrostatic interaction is mediated by the side chains of Arg181 in D2 with the side chain of Asp266 of D3.

**The D3-D4 interface**. The hydrophobic interactions in D3-D4 interface include those between Val308 and Leu222 from D3 and Phe312, Phe340, and Ile371 from D4. The D3-D4 interface covers a smaller buried area than other inter Ig-like domain interfaces (Figure 1D).

**The D4-D5 interface**. The hydrophobic patch on the D4-D5 interface includes Phe324 and Tyr408 from the A and G strands of D4 and Phe433 from the BC loop of D5, respectively. In addition, van-der-Waals contacts contribute to the stabilization of the interface surrounding the hydrophobic patch; Phe324, Gly384, Thr389, Tyr408, Asn410,

Thr411 and Met351 of D4 interact with Val497, Phe433, Gly470, Phe649 and Lys471 of D5 (Figure 1E).

**Example 5: Analysis of the Overall structure of the Bound SCF-Kit complex**

The structure of the SCF-Kit complex shows a 2:2 stoichiometry, in which two sets of 1:1 complexes in the asymmetric unit are related by a non-crystallographic twofold symmetry (Figure 2). The observed SCF-Kit 2:2 complex in the crystal lattice is consistent with experiments demonstrating that Kit dimerization is driven by the dimeric SCF ligand (Philo *et al.* (1996) J Biol Chem 271: 6895-6902; Lemmon *et al.* (1997) J Biol Chem 272: 6311-6317). The two sets of Kit ectodomains and SCF molecules resemble an upside down "A" letter with approximate dimensions of 170 x 130 x 70 Å (Figure 2A and Figure 9).

The overall structure of SCF bound to Kit is similar to the previously described structures of free SCF (Zhang *et al.* (2000) Proc Natl Acad Sci U S A 97: 7732-7737; Jiang *et al.* (2000) Embo J 19: 3192-3203). The structure of SCF-Kit 2:2 complex shows that an individual SCF protomer binds directly to D1, D2 and D3 of an individual Kit protomer (Figure 2B). Consequently, a single receptor protomer forms a symmetric complex with a similar two-fold related surface on an SCF protomer. Dimerization of Kit is also mediated by homotypic interactions between the two membrane proximal Ig-like domains of Kit, namely, by D4-D4 and D5-D5 interactions (Figure 2B). This results in dramatically altered configurations of D4 and D5 relative to the rest of the molecule that brings the C-termini within 15 Å of each other close to the place where they connect to the transmembrane domain (Figure 2B and Figure 9). The structure is also characterized by the existence of a large cavity at the center of the complex with dimensions of ~ 50x50x15 Å (Figure 2B). The crystal structure demonstrates that each protomer of SCF binds exclusively to a single Kit molecule and that receptor dimerization is driven by SCF dimers which facilitate additional receptor-receptor interactions.

**Example 6: Analysis of the SCF binding region of Kit**

SCF is bound to a concave surface formed by D1, D2 and D3 of Kit in a configuration in which the four helix bundle of SCF is oriented perpendicularly to the long axis of D1, D2 and D3 and the C-termini of SCF and Kit are facing opposite directions (Figure 2, 3 and Figure 9). The solvent-accessible surface area buried at the interface between Kit and each of the SCF protomers is approximately 2060 $Å^2$; a buried surface area that is within the range of known ligand receptor interfaces. It is possible to divide the SCF-Kit interface into three binding sites (Figure 3A, B, Table 2, and Table 3). Site-I is located on D1, Site-II is located in D2 and in the D2-D3 linker region and Site-III is located in D3. The buried surface areas of Site I, II and III are approximately 280, 770 and 1010 $Å^2$, respectively.

Site-I

The αC- β2 loop of SCF is aligned perpendicularly to strand C' of D1, as presented in Figure 3C. Asp72, Glu73 and Thr74 of D1 and Lys99', Ser101' and Phe102' of SCF are closely located at a Cα distance of 6-8 Å, indicating that these residue could participate in the interactions between D1 and SCF. Due to poor side chain electron density of the αC- β2 loop, specific interactions could not be defined.

Site-II

SCF binding is mediated, for the most part, by complimentary electrostatic interactions of charged surfaces on Kit (Figure 3A, B, D). Salt bridges are formed between the basic amino acids Arg122, Arg181, Lys203 and Arg205 of Kit with the acidic amino acids Asp54', Asp77', Asp84' and Glu88' on SCF. The conformation of Arg122 is stabilized by a salt bridge between Glu198 of Kit and Asp54' of SCF. Figure 3D shows that three of the major interacting residues Tyr125, Arg181 and Lys203 on D2 are aligned on the same plane and form hydrogen bonds with Asp77', Asn81', Asp84', Ser53' and Thr57' of αB and αC of SCF. The van-der-Waals contacts between Ser123 and Ile201 of D2 and Val50', and Thr57' of SCF also contribute towards the formation of ligand-receptor complex. However, there are notable differences in the residues of Site-II in Kit and SCF from other species (Figure 3, Figure 8 and Figure 10). While Arg181 and Lys203 are invariant as basic amino acids in mammals, Tyr125 is substituted by a phenylalanine in the mouse and rat which most likely results in loss of a hydrogen bond. Arg205 of Kit is a highly conserved amino acid while Glu88' is

substituted by a leucine and alanine residues in the mouse and rat, respectively. Furthermore, Arg122 of Kit and Asp54' of SCF in human are substituted by a leucine or valine in the mouse and rat, respectively. These substitutions may account for the reduced affinity of rodent SCF towards human Kit (Lev *et al.* (1992b) J Biol Chem 267: 15970-15977).

Site-III

The N-terminal segment of SCF interacts with strand D of D3 (Figure 3A, E). Hydrogen bonds are formed between the side chain of Asn10' of SCF, and the main chain amide and carbonyl group of Ser261, as well as with the side chain of Asp260 and Trp262 on D3. In addition, Thr9' and Asn11'of SCF bind to the side chain and main chain amide of Ser261, and His263 of Kit, respectively. Mutational analysis of SCF has shown that substitution of Asn10' with alanine or glutamic-acid residues reduces the binding affinity of SCF towards Kit by approximately 10 fold and that Asn10' (or Asp in other species) is necessary for biological activity (Hsu *et al.* (1998) Biochemistry 37: 2251-2262). Comparison of the receptor binding interface in SCF from different species shows that Asn10' (or Asp) is a highly conserved residue (Figure 8). Additional important interactions are mediated by Asn6' and Arg7' of SCF via van-der-Waals contacts with Tyr259, Thr269, Ser240, Val242, Ser241 Ser244 on D3 of Kit.

Table 3. SCF-Kit Interactions and Homophilic Interaction between two Kit protomers

| SCF-KIT interactions 1 | | | | SCF-KIT interactions 2 | | | | KIT D4-D4 interactions | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Hydrogen bonds and salt bridges (1) | | | | Hydrogen bonds and salt bridges (1) | | | | Hydrogen bonds and salt bridges (1) | | | |
| Kit (molA) | | SCF (molC) | | Kit (molB) | | SCF (molD) | | Kit (molA) | | KIT (molB) | |
| Arg122 | Nη1 | Asp54 | Oδ1 | Arg122 | Nη1 | Asp54 | Oδ1 | Arg381 | O | Arg381 | Nη1 |
| Tyr125 | OH | Asp84 | Oδ1 | Tyr125 | OH | Asp84 | Oδ1 | Arg381 | Nη1 | Thr380 | O |
| Arg181 | Nη1 | Asp77 | Oδ2 | Arg181 | Nη1 | Asp77 | Oδ2 | Arg381 | Nη2 | Glu386 | Oε1 |
| Arg181 | Nη2 | Asn81 | Oδ1 | Arg181 | Nη2 | Asn81 | Oδ1 | Glu386 | Oε1 | Arg381 | Nη2 |
| Lys203 | Nζ | Asp84 | Oδ1 | Lys203 | Nζ | Ser53 | Oγ | | | | |
| Lys203 | Nζ | Ser53 | Oγ | Ser240 | Oγ | Asp85 | Oδ2 | | | | |
| Ser240 | Oγ | Asp85 | Oδ2 | Ser261 | O | Thr9 | Oγ1 | | | | |
| Ser261 | O | Thr9 | Oγ1 | Ser261 | O | Asn10 | Oδ1 | | | | |
| Ser261 | O | Asn10 | Oδ1 | Trp262 | Nε1 | Asn10 | Oδ1 | | | | |

| van der waals contact (2) | | van der waals contact (2) | | van der waals contact (2) | |
|---|---|---|---|---|---|
| Asp72 | Phe102 | Asp72 | Lys99 | Thr380 | Thr380, Arg381 |
| | Val87, Ser53 | Thr74 | Lys99 | Arg381 | Leu382, Lys383 |
| Tyr125 | | | | | |
| Arg181 | Leu60, Val80 | Tyr125 | Val87, Ser53 | Leu382 | Arg381 |
| Ile201 | Thr57 | Arg181 | Leu60, Val80 | Lys383 | Arg381 |
| Lys203 | Thr57 | Ile201 | Thr57 | | |
| Arg205 | Glu88 | Lys203 | Thr57, Asp84 | | |
| Ser240 | Arg7 | Arg205 | Glu88 | | |
| Trp262 | Thr9, Asn10, Asn11 | Ser240 | Arg7 | | |
| His263 | Thr9, Asn11, Lys78, Asn81, Asp85 | Trp262 | Thr9, Asn11 | | |
| Gly265 | Lys78, Asn81 | His263 | Thr9, Asn11, Lys78 | | |
| Asp266 | Asn81 | Gly265 | Lys78, Asn81 | | |
| Tyr269 | Asn6 | Tyr269 | Asn6 | | |

(1) Hydrogen bond and salt bridge and (2) van der waals contact have distance range of 2.5 – 3.5 and within 4.0 Å, respectively.

**Example 7: Analysis of the Kit/SCF Structure and the Conformational Changes Associated with Binding.**

The ligand binding domain of Kit is poised for SCF binding

Superimposition of the structures of individual D1, D2, and D3 of Kit monomeric form with corresponding structures of the SCF-induced homodimeric form reveals r.m.s.d. values of 0.5, 0.8, and 1.1 Å for 82, 92, and 100 aligned Cα residues in D1, D2, and D3, respectively. Similarly, superimposition of the structure of the entire D1-D2-D3 region of Kit monomers with the corresponding structures in the SCF-Kit 2:2 complex reveals r.m.s.d. of 1.1 Å for 274 aligned Cα residues of the D1-D2-D3 region. Remarkably, there are no significant backbone changes in the structures of the SCF binding pocket of Kit (Figure 3 and Figure 11). However, several minor structural changes were detected in the SCF binding cleft upon SCF binding. A structural change is seen in the top half of strands G, F, and C (amino acids 167-187 and 143-166) of D2 following SCF binding (Figure 1A, 2A). These strands are located at the side opposite to the SCF binding interface and are not involved in mediating any direct contacts with SCF. Overall, comparison of the structures of Kit monomers to those of SCF-occupied

Kit dimers show that the D1-D2-D3 region of Kit may be viewed as a functional unit that is poised for SCF binding followed by subsequent Kit dimerization driven by dimeric SCF molecules.

Conformational changes in SCF molecules bound to Kit

While the overall structure of SCF bound to Kit is similar to the structure of free SCF, there are notable differences in the angle between the two protomers, in the conformations of the connecting loops and in the structures of the flexible N terminus of the molecule (Figure 4). Comparison of the published structures of SCF dimers (Accession codes 1EXZ and 1SCF in the Protein Data Bank) shows that the angle between the two protomers (the angles between αC helices) of free SCF homodimers may vary by 2° to 6° in the different structures, suggesting that a certain degree of flexibility exists in the SCF dimer. The range of differences in the angles between Kit bound SCF protomers to those of free SCF was increased by 3-9°. Figure 4 shows a Kit bound SCF structure in which the angle between SCF protomers is increased by 5°.

Figure 4B shows that the N-terminus of free SCF from Cys4' to Asn11' has a random-coil configuration (Zhang *et al.* (2000) Proc Natl Acad Sci U S A 97: 7732-7737). It was also shown that deletion of the first four amino acids leads to an approximately 25% reduction in the binding affinity of SCF to Kit, suggesting that the disulfide bridge between Cys4' and Cys89' plays a role in maintaining the functional integrity of SCF (Langley *et al.* (1994) Arch Biochem Biophys 311: 55-61). Figure 4B also shows that Thr9' and Asn10' of the N-terminus region of SCF bound to Kit undergo a conformational change in which their Cα positions become displaced by 3 to 5 Å upon receptor binding (Figure 4B). The disulfide bridge between Cys4' at the N-terminus and Cys89' at the αC helix appears to play an important role in mediating the conformational change that takes place in the N-terminus of SCF. The position of Cys24' in free SCF is not altered upon receptor occupancy as revealed by root mean square deviation (r.m.s.d.) of 1.2 Å of Cα positions. Finally, the αC- β2 of free SCF is either disordered or has a different structure from the structure of the αC- β2 loop in SCF bound to Kit. Figure 4C shows that the αC-β2 loop of SCF undergoes a large conformational change upon receptor binding; a change critical for establishment of Site-I of the SCF-Kit interface.

A large rearrangement in D4 and D5 orientations in SCF bound Kit

Superimposition of the structures of individual D1, D2, D3, D4, and D5 of Kit monomeric form with corresponding individual Ig-like domains in the SCF-induced homodimeric form reveals minor changes in the structure of Kit Ig-like domains following SCF binding. By contrast, superimposition of the D3-D4-D5 region of Kit monomeric form with the corresponding region in the homodimeric form reveals a large structural change in the orientation of D4 and D5 relative to each other and relative to the ligand binding region of Kit (Figure 5A and Figure 12). Each of the individual domains D3, D4, and D5 of monomeric Kit can be superimposed with their counterparts in the SCF-occupied Kit with r.m.s.d. values of 0.9, 0.9 and 1.9 Å for 98, 101, and 85 Ca atoms of D3, D4, and D5, respectively. However, superimposition of the D3 structure of Kit monomers with the D3 structure in ligand-occupied homodimeric form reveals a dramatic movement in the orientation of D4 and D5 in the SCF bound Kit (Figure 5A). The re-orientations of D4 and D5 relative to the ligand binding region occurs by a rotation along an axis in the linker connecting D3 to D4, and a rotation along an axis in the linker connecting D4 to D5 running through the D3-D4 and D4-D5 interfaces (Figure 5A), respectively. Comparison of the free and ligand-bound Kit shows that D4 of ligand occupied Kit rotates relative to D3 by 22°, and D5 of ligand occupied Kit rotates relative to D4 by 27° (Figure 5A). The rearrangements of D4 and D5 in SCF occupied Kit result in receptor-receptor interactions that are mediated by D4-D4 and D5-D5 interactions of two neighboring Kit molecules (Figure 5B). The conformation of the DE loop of D5 is altered in the SCF occupied ectodomain. Reorientation of D4 and D5 driven by receptor dimerization imposes upon the DE loop of D5 a new configuration (Figure 5A).

D4:D4 interactions in Kit homodimers

Homotypic interactions between D4 of two neighboring Kit molecules are mediated by the D4-D4 interface in the SCF-Kit 2:2 complex. The D4-D4 interface is mediated by two β sheets formed by the ABED strands of D4 of each Kit protomer to form a nearly planar arrangement in which Arg381 of each protomer points toward each

other resulting in a buried surface area of 360A$^2$. Figure 6A shows that Arg381 and Glu386 form salt bridges and van-der-Waals contacts across the two-fold axis of the Kit dimer. In addition, the side chains of Arg381 of each protomer form hydrogen bonds with the main chain carbonyl of the corresponding residue of the neighboring Kit molecules.

Structure based sequence analysis has shown that the D4-D4 interface is conserved in most type-III RTKs including CSF1R, PDGFRα and PDGFRβ (Figure 6B and Figure 8). In PDGFRα Glu386 is replaced by an aspartic acid; a residue that could also function as a salt bridge partner. A pair of basic (Arg381) and acidic (Glu386) residues are strictly conserved in type-III RTKs of different species. The sequence motif found in the D4-D4 interface is also conserved in the membrane proximal 7$^{th}$ Ig-like domain (D7) of all members of type-V RTK (VEGFR family) including VEGFR-1 (Flt1) ,VEGFR-2(Flk1) and VEGFR-3(Flt4). In VEGFR, the basic (Arg) and acidic (Asp) residues are located in the EF loop. Although the core sequence motif that is responsible for the type-III RTK D4-D4 interface is located in a different Ig-like domain of VEGFR (*i.e.*, D7 versus D4 of type III) it is possible that receptor-receptor interactions similar to those seen in the D4-D4 interface of Kit will also take place through a similar D7-D7 interface (Figure 6A) in all members of the VEGFR family of RTKs (Ruch *et al.* (2007) Nature. Struct. Mol. Biol. 14: 249-250).

D5-D5 interactions in Kit homodimers

Figure 2B and Figure 5B, 6C show that in the SCF-Kit 2:2 complex neighboring D5 protomers are parallel and in a close proximity to each other as well as in an orientation likely to be perpendicular to the cell membrane. The β-sheet topology of D5 follows an atypical arrangement that is different from most I-set IgSF in which strand A is split into strand A and A'. Strand-A of D5 is paired with strand B resulting in the β sheet topology of ABED/CFG. Consequently, strands A and G that are located at the edge of two β sheets (ABED/CFG) are nearly parallel at a distance of 6.5-11.5Å in the Cα from each other. Moreover, strands A and G of one protomer face strands A and G of neighboring D5 in a two-fold symmetry. The side chains of Asn505 of two neighboring Kit protomers are approximately 4.2 Å from each other but water or metal

ions that may mediate indirect interactions between the two asparagines could not be detected in this area of weak electron density. Additional D5-D5 interactions are mediated by Tyr418 of two neighboring Kit molecules (Figure 6C). The interaction between hydroxyl groups of neighboring Tyr418 side chains could be mediated by water molecules. It also suggests that the relative positions of neighboring D5 domains are mediated by indirect interactions formed by Tyr418 and Asn505 of the neighboring protomers. The G-strand of D5 is connected via a short linker to the transmembrane domain of Kit.

**Example 8: Mechanism of Receptor Activation**

The structures of Kit ectodomain monomers and SCF induced dimers provide novel insights concerning the mechanism of ligand-induced activation of Kit and other RTKs containing five or seven Ig-like domains in their extracellular domains. Comparison of the structures of D1, D2 and D3 of Kit ectodomain monomers to the corresponding region in the SCF-induced ectodomain dimers shows very few structural alterations in the SCF-binding pocket and in other parts of D1, D2 and D3 following SCF binding. On the basis of their distinct biochemical functions, we have divided the ectodomain of Kit into three independent functional units. The first unit is composed of the three membrane distal Ig-like domains D1, D2, and D3. The D1-D2-D3 region acts as a separate module that functions as a specific SCF binding unit. The SCF-binding unit is connected by a flexible joint (D3-D4 interface) to D4; a second independent unit that is connected by an additional flexible joint (D4-D5 interface) to D5, defined as a third independent unit. The function of D4 and D5 is to mediate, respectively, lateral D4-D4 and D5-D5 interactions that bring together and stabilize interactions between membrane proximal region of two neighboring Kit ectodomains.

According to this view, dimerization of Kit is driven by bivalent SCF binding whose sole function is to bind SCF and to bring together two Kit molecules. SCF-induced Kit dimerization is followed by a large change in D4 and D5 orientations relative to the position of the D1-D2-D3 SCF-binding unit. The data presented herein demonstrates that the flexible joints at the D3-D4 and D4-D5 interfaces enable lateral interactions that result in a large conformational change upon receptor dimerization. Rather than inducing a conformational change in Kit, dimerization may select particular

conformations in a transition from a flexibly jointed monomer to a rigid dimer. This culminates in complex formations between two neighboring D4 and two neighboring D5 of Kit, bringing the C-termini of D5 to a point at the cell membrane in which the transmembrane domains of two neighboring Kit molecules are within 15 Å of each other. Indeed, SCF-induced tyrosine autophosphorylation of Kit (Figure 7B) and stimulation of a downstream signaling pathways are strongly compromised by a point mutation in either Arg381 or Glu386 within D4 of Kit. PDGF-receptor activation and stimulation of downstream signaling pathways are also compromised by similar point mutations in D4 of PDGFR. The data presented herein demonstrates that the homotypic interactions between membrane proximal regions of Kit are mediated primarily by the D4-D4 interface and that the D5-D5 interface plays a cooperative secondary role by facilitating exact positioning of two Kit ectodomains at the cell surface interface.

The SCF-Kit complex exhibits a strong polarization of the electrostatic field with the following characteristic: (i) an overall negatively charged surface; (ii) complementarity between SCF (negative), and the ligand binding D1-D2-D3 unit (positive); and (iii) a strongly negatively polarized surface right above and around the D4-D4 interface (Figure 6D, 3B and Figure 13). This data demonstrates that the binding of SCF to Kit occurs in at least two steps: First, the electrostatic attraction between SCF and D1-D2-D3 will align SCF along the opposing ligand binding region on Kit. The electrostatic attraction may also lead to a faster association rate of SCF due to a Steering effect (Muellera *et al.* (2002) Biochina and Biophysica Acta. 1592: 237-250). Subsequently, SCF-Kit complex formation will be stabilized by additional interactions including those mediated by a conformational change in bound SCF molecules. The strongly polarized electrostatic surface on D4 may also play a role in maintaining Kit in a monomeric inactive configuration by inducing repulsion between D4 domains of neighboring Kit receptors (Figure 6D). The binding affinities of D4 towards D4 and D5 towards D5 of neighboring receptors are probably too low to facilitate Kit ectodomain dimerization before the local receptor concentration on the cell surface is increased by SCF-driven receptor dimerization and by the effect of dimensionality. Once such a threshold of local concentration is reached, the attraction between neighboring D4 will overcome the electrostatic repulsion to the extent that two neighboring D4 units will be able to bind to each other. Interestingly, the main interactions that maintain the D4-D4

interface, *i.e.* double salt bridges between Arg381 and Glu386 in neighboring Kit molecule are also mediated by electrostatic interactions.

The ectodomains of Kit and C-cadherin (Boggon *et al.* (2002) Science 296: 1308-1313), are each composed of five tandem Ig-like domains and both exhibit a similar elongated topology; 170 Å for Kit and 185 Å for C-cadherin. Moreover, the bacterial adhesion molecule invasin exhibits a remarkably similar elongated architecture and inter-Ig-like domain topologies (Hamburger *et al.* (1999) Science 286: 291-295). Kit ectodomains may have evolved from a common ancestral gene that coded for a protein that mediates cell-cell interactions. While classical-cadherins utilize their most membrane distal Ig-like domain for homotypic binding that mediate cell-cell interactions, the ectodomain of Kit has evolved to function as a cell signaling receptor that binds membrane anchored or soluble SCF isoforms to induce receptor dimerization and activation (Figure 7C).

Since the hallmarks of Kit structure, ligand binding and receptor dimerization are conserved in other receptors, the mechanism described here for Kit activation may be a general mechanism for activation of many receptors (Figure 7C). Moreover, the structural information described here could be applied to design novel therapeutic interventions for treatment of cancers and other diseases driven by activated receptors.

**Example 9: Analysis of Kit Mutations in Human Diseases**

A variety of human diseases are caused by mutations in the Kit gene. In humans, loss of function mutations in the ectodomain of Kit cause the piebald trait (Fleischman *et al.* (1996) J Invest Dermatol 107: 703-706; Murakami *et al.* (2005) J Invest Dermatol. 124: 670-672). These exon-2 and exon-3 point mutations in the Kit locus result in Cys136 being replaced by an arginine residue and Ala178 being substituted by a threonine residue. Both mutations take place in D2, a critical component of the SCF binding site on Kit (Figure 7A). The piebald Cys136Arg mutation will cause the loss of an important disulfide bond that plays a critical role in maintaining the structural and functional integrity of D2 and hence its capacity to recognize SCF. Ala178 is located in the EF loop of D2 in close proximity to the D2-D3 interface (Figure 7A). The piebald Ala178Thr mutation may disrupt interactions that are essential for maintaining the

integrity of the D2-D3 interface and interactions that are required for D2 and/or D3 binding to SCF (Figure 7A).

A variety of gain of function mutations in the Kit locus were found in different cancers including GIST, AML and SCLC (see Forbes *et al.* (2006) COSMIC 2005. BR J. CANCER, 94: 318-22. Somatic mutation database: Catalogue of Somatic Mutations in Cancer http://www.sanger.ac.uk/genetics/CGP/cosmic/). Many oncogenic mutations were identified in the JM and in the PTK domains of Kit. A variety of oncogenic mutations were also found in Kit ectodomain (Figure 7A) including in-frame deletions, point mutations, in-frame duplications and insertions that collectively lead to formation of activated forms of Kit. In frame deletion and insertional mutations at exon-8 involving either a loss or substitution of Asp419 were described in patients with AML, while duplications of Ala502-Tyr503 and Ala502-Phe506 sequences were identified in GIST (Figure 7A). Asp419 is located in a region connecting strand A and AB loop of D5 and Ala502-Tyr503 are located on strand G of D5 of Kit. Interestingly, virtually all the activating oncogenic mutations that were found in Kit ectodomain were mapped to the D5-D5 interface (Figure 7A). The most plausible interpretation of the mode of action of the oncogenic D5 mutations is that these mutations enhance the binding affinity and homotypic interactions between neighboring D5 domains by increasing the on-rate or decreasing the off-rate or altering the rates of both processes in a fashion that facilitates enhanced D5-D5 interactions.

The analyses above demonstrate that the D4 and D5 regions are good candidates against which to target therapeutics. Drugs, pharmaceuticals, or biologics may be used to bind Kit in order to encourage Kit dimerization/activation or, more preferably, to prevent dimerization/activation.

**Example 10: Expression, purification and partial deglycosylation of Kit ectodomain**

A DNA construct coding for amino acids 1-519, of human Kit (Lemmon *et al.* (1997) J Biol Chem 272: 6311-6317) containing additional five histidine residues at the C terminus was ligated into pFastBac1 (Invitrogen, Inc.). Baculoviruses expressing the ectodomain Kit proteins were prepared according to procedures described in the Bac-to-Bac instruction manual (Invitrogen). Insect Sf9 cells were grown in 15 L culture of

Grace's insect medium supplemented with 10% heat inactivated fetal bovine serum with a Wave Bioreactor (Wave Biotech, LLC, System 20/50) to 2~3 x $10^6$ cells/ml and were then infected with recombinant baculovirus carrying the Kit ectodomain genes. Although the ectodomain Kit contained the signal sequence from human Kit, the protein was accumulated in the insect cells rather than being secreted out. After 72 hours the cells were harvested and lysed in 1.4 liter of 50 mM of potassium phosphate buffer pH 8 containing 200 mM NaC1, 10% glycerol 1% NDP-40 and 2 mM PMSF for 20 minutes on ice. After centrifugation and filtration, the ectodomain of Kit was purified using affinity chromatography with Ni-NTA beads, followed by gel filtration using Superdex 200. The purified Kit ectodomain in 25 mM Tris buffer pH8.5 containing 25 mM NaC1 and 1% glycerol was treated for 12 hours at 4°C with recombinant endoglycosylase F1 that was added to the Kit solution at a final ratio of 10:1 w/w. The endonuclease F1 treated ectodomain of Kit was then loaded onto a pre-equilibrated 16/10 Mono Q column and eluted with a shallow gradient of Tris buffer pH 8.5 containing 400 mM NaC1 and 1% glycerol. Fractions of deglycosylated Kit ectodomain were pooled and concentrated to 35mg/ml using a spin concentrator. The purified, partially deglycoslyated Kit ectodomain preparation was split into aliquots and flash-frozen in liquid N2. Using this approach, ~10 mg of partially deglycosylated Kit ectodomain was purified from 15 liters of cultured cells. SCF (1-141) was expressed, refolded and purified as previously described (Zhang *et al.* (2000) Proc Natl Acad Sci U S A 97: 7732-7737). The ectodomain of Kit (amino acids 1-514) was also expressed as a secreted form in Sf9 insect cell using the baculovirus system and purified as previously described (Lemmon *et al.* (1997) J Biol Chem 272: 6311-6317).

**Example 11: Structure determinations and refinements**

Experimental phases were determined using a combination of multi-wavelength anomalous diffraction (MAD) and multiple isomorphous replacement with anomalous scattering (MIRAS) of crystals of Kit ectodomain monomers. Heavy atom search and phasing were carried out using the CNS (Brunger *et al.* (1998) Acta Crystallogr D Biol Crystallogr 54: 905-921) and SHARP (Bricogne *et al.* (2003) Acta Crystallogr D Biol Crystallogr 59: 2023-2030) program suites. One major and two minor sites were detected for platinum derivative (K2Pt(NO2)4) and one major and five minor sites were

detected for iodine soaked crystals. MAD phases were calculated up to 3.3 Å resolution for platinum derivatives at three wavelengths using CNS. MIRAS phases were calculated up to 3.0 Å resolution for platinum and iodine derivatives using CNS and SHARP. Solvent flipping density modification resulted in electron density maps of interpretable quality with continuous electron density and very clear solvent-protein boundaries. Regions of poor electron density quality, including the top half of stands F, G and C as well as CD loop in D2 and CD loop, strand D, DE loop and EF loop and bottom half of stand F in D5, were confirmed by comparing electron density maps calculated by MIRAS and MAD phasing. The data collection and phasing statistics are summarized in Tables 1A and 1B. The molecular model of Kit was built manually into the experimental electron density maps using COOT (Emsley, and Cowtan (2004) Acta Crystallogr D Biol Crystallogr 60: 2126-2132). For the calculation of the free R-factor, 5% of the data were omitted during refinement. Refinements were carried out using CNS to 3.0Å resolution against native data. At the final stage of the refinements, translation/liberation/screw (TLS) refinements were carried out by Refmac5 (Murshudov *et al.* (1997) Acta Crystallogr D Biol Crystallogr 53: 240-255) in the CCP4 program suite with three TLS group generated using the TLSMD web server (Painter *et al.* (2006) J Appl Cryst 39: 109-111).

The structure of SCF-Kit complex was solved by molecular replacement using PHASER (McCoy *et al.* (2005) Acta Crystallogr D Biol Crystallogr 61: 458-464). A clear molecular replacement solution for D1D2D3D4 of the Kit ectodomain and SCF was found using D1D2D3 and D4 of Kit and SCF as search models against native data set, respectively, using PHASER. The Kit (D1D2D3D4)-SCF complex structures were subjected to rigid body refinement from 20 to 4Å, resulting in an Rcryst of 43.8%. Model rebuilding and refinement was performed using CNS to an Rcryst and Rfree values of 31.6 % and 34.0 %, respectively. Continuous electron density in the region of D5 was found in the 2 σ 2Fo-Fc and 3 σ Fo-Fc map. The strands for D5 were traced manually into the map using COOT, followed by application of refinements after each step. Throughout the initial refinement, non-crystallographic symmetry (NCS) constraints were imposed on the residues. Further refinements were performed to 3.5Å resolution against native X-ray diffraction data. After building almost the entire SCF-Kit complex molecule, NCS constraints were released resulting in reduced values of R and Rfree and improved electron density. At the final stage of refinements, the NCS

constraints were completely released. The stereochemistry of the models was analyzed with PROCHECK (Laskowski *et al.* (1993) J Appl Cryst 26: 283-291). A summary of the refinement statistics is shown in Table 1B.

**Example 12: Radiolabeling of SCF and ligand displacement assay**

Human SCF (10 μg) was labeled with 1mCi of $^{125}$I (PerkinElmer) using Iodo-Gen Iodination Tubes (Pierce) following the manufacturer's instructions. For the displacement binding assay, 3T3 cells expressing WT Kit or Kit mutants were grown in DMEM containing 10% FCS. Cells were washed three times with DMEM containing 10mM HEPES PH7.4 and 0.1% BSA (DMEM-BSA), and then incubated for 1 hour at room temperature with 2ng of $^{125}$I-labeled-SCF in the presence of increasing concentrations of native SCF. Cells were then washed three times with cold DMEM-BSA, lysed in 0.5 ml of 0.5M NaOH for 1 hour at room temperature, and 100 μl of the cell lysate were applied to 10ml of Opti-Fluor scintillation solution (Perkin Elmer) to measure cell associated radioactivity using a LS6500 Scintillation Counter (Beckman Coulter).

**Example 13: Conservation Analysis**

Amino acid sequences of human SCF and Kit were used as queries to search the non-redundant database (nr) for homologous sequences, using PSI-BLAST (Altschul *et al.* (1990) J Mol Biol 215: 403-410). Sequence alignment was performed using ClustalW (Higgins (1994) Methods Mol Biol 25: 307-318) on SCF sequences or Kit sequences and then, manually adjusted based on the IgSF fold restrains for 20 key residues in Kit Ig-like domains. The alignment of amino acid sequences revealed by the SCF-Kit complex crystal structure was submitted to the Consurf 3.0 server (Landau *et al.* (2005) Nucleic Acids Res 33: W299-302) to generate maximum-likelihood normalized evolutionary rates for each position of the alignment where low rates of divergence correspond to high sequence conservation. As with the Consurf output, the continuous conservation scores are partitioned into a discrete scale of 9 bins for visualization, such that bin 9 contains the most conserved (maroon) positions and bin 1 contains the most variable (cyan) positions.

**Example 14: Protein expression, purification and generation of antibodies**

DNA encoding for the fourth Ig-like domain of human Kit (residues 309-413; Kit D4) was amplified from the cDNA of full length human Kit using a PCR reaction. BL21 (DE3) *E. Coli* codon plus cells were transformed with a bacterial expression vector (pET-NusA histidine tagged) that directs the synthesis of Kit D4 followed by overnight incubation at 16 °C. The Kit D4-NusA fusion protein was purified from BL21 lysates using a metal chelating affinity column (Ni-NTA; QIAGEN) followed by further purification using anion-exchange chromatography (Source Q column; GE Healthcare). Kit D4-NusA was then incubated overnight at 4 °C with TEV protease in order to cleave NusA and the histidine tag from D4. An additional step of purification of Kit D4 was carried out using gel filtration chromatography (Superdex 200 column; GE Healthcare).

The fifth Ig-like domain of human Kit (residues 410–519; Kit D5) was expressed in the *E.coli* strain BL21 (DE3) cells and purified from bacterial inclusion bodies using a refolding step using 10 mM Tris buffer, pH 8.0 containing 6.0 M guanidine hydrochloride. Refolded Kit D5 was further purified using anion-exchange chromatography (Q sepharose column; GE Healthcare) followed by a purification using gel filtration chromatography (Superdex 200 column; GE Healthcare) and by an additional step of purification using anion-exchange chromatography (Source Q column; GE Healthcare).

Rabbit polyclonal antibodies against isolated D4, D5, or against the entire Kit ectodomain (amino acids 1-519; Kit EC) or against a GST-fusion protein containing a fragment from the C-terminal region of human Kit (residues 876-976) were generated using techniques well known in the art such as the method recited in Example 1. For example, polyclonal antibodies against the Kit ectodomain may be generated by immunizing a rabbit with a purified Kit ectodomain and collecting the produced antibodies by standard methods. The experiments in which the effect of antibodies on Kit activation were tested, such as in Example 15 and Figure 14, were performed using antibody preparations subjected to purification with protein-A affinity chromography.

**Example 15: Inhibition of SCF-induced Kit activation using antibodies against the D5 domain of Kit**

3T3 cells expressing human Kit were incubated with buffer solutions containing increasing concentrations of polyclonal rabbit antibodies generated against isolated recombinant D5 of Kit (Figure 14). As a control, the cells were treated with rabbit polyclonal antibodies against SCF or rabbit polyclonal antibodies directed against the entire Kit ectodomain that was produced in insect cells using a baculovirus expression system. Cell lysates were subjected to immunoprecipitation with anti-Kit antibodies followed by SDS-PAGE and immunoblotting with either anti-Kit or anti-pTyr antibodies (Figure 14).

This experiment shows that anti-D5 antibodies block the SCF-induced tyrosine autophosphorylation of Kit.

**Example 16: Inhibition of SCF-induced Kit activation by isolated recombinant Kit D4 domain**

3T3 cells expressing Kit were incubated for 10 minutes at 23ºC with increasing concentrations of purified recombinant D4 that was expressed in *E. Coli* followed by SCF incubation. Lysates of unstimulated or stimulated cells were subjected to immunoprecipitation with anti-Kit antibodies followed by SDS-PAGE and immunoblotting with either anti-Kit or anti-pTyr antibodies (Figure 15).

This experiment shows that the presence of isolated D4 interferes with SCF-induced tyrosine autophosphorylation of Kit.

**Example 17: SCF-induced Kit stimulation experiments**

3T3 cells expressing human Kit were grown in DMEM containing 10% Calf Serum. Prior to SCF stimulation, cells were starved overnight in serum free medium as described by Yuzawa *et al* (2007) Cell, *130*: 323. The starved cells were washed three times with cold DMEM containing 10 mM HEPES at pH 7.4 and 0.1 % BSA, followed by incubation with increasing concentration of antibodies or with Kit-D4 for 10 minutes at 23 C° as indicated in Figure 14 or Figure 15. Cells were stimulated with 100 ng/mL

SCF for 10 minutes at 23 C° and washed three times with cold PBS. Lysates of unstimulated or SCF-stimulated cells were subjected to immunoprecipitation with anti-Kit antibodies followed by SDS-PAGE and immunoblotting with anti-Kit or anti- p-Tyr antibodies.

**Example 18: PDGF-induced activation of PDGF-receptor β and signaling via PDGFRβ are prevented by point mutations in critical amino acids in D4 of PDGFRβ.**

Mouse embryonic fibroblasts (MEFs) derived from PDGFR-/- mice expressing either WT PDGFRβ or point mutants in critical amino acids in D4 (on the basis of sequence similarity with the D4-D4 interface in Kit ectodomain x-ray crystal structure) were used to demonstrate that mutations of R385 or E390 prevent PDGF-induced receptor activation (Figure 16A), or PDGF-induced MAP kinase response and Akt stimulation (Figure 16B). Moreover, using cross linking experiments with a covalent cross linking agent we demonstrate that an E390A point mutation does not interfere with PDGF-induced receptor dimerization. However, unlike the WT PDGFRβ covalently cross linked dimers that exist on the cell surface in an activated state, the covalently cross linked dimers of the E390A mutants are inactive (Figure 16C). This experiment shows that mutation of a critical E390 residue in D4 prevents D4-D4 interactions that are essential for PDGFR activation. However, PDGF-induced dimerization of PDGFR is not affected by a point mutation in D4 that prevents receptor activation indicating that D4-D4 play an important role in mediating the positioning of the membrane proximal region of the ectodomain to enable activation of the tyrosine kinase domain of PDGFR.

Thus, one embodiment of the present invention includes moieties which bind to, or target the residues R385 or E390 in PDGFR. The moieties may be employed to inactivate the receptor while preserving receptor dimerization. This example also demonstrates that information based on the crystal structure of one RTK, in this case the Kit ecodomain crystal structure, can be easily transferred to other RTKs. Here, knowledge of the Kit D4 domain was correct in identifying the amino acids which were important to activation of the PDGF receptor. A more detailed set of experiments involving PDGFR is described in Examples 22-25.

**Example 19: Molecular Surface Analysis of Kit Ectodomain**

The determination of the crystal structures of the entire ectodomain of Kit before and after SCF binding described herein has demonstrated that SCF-induced receptor dimerization is followed by homotypic lateral interactions between membrane proximal Ig-like domains D4 and D5 of two neighboring Kit molecules. The homotypic D4 and D5 interactions position the cytoplasmic tyrosine kinase domains of two neighboring receptors at a distance and orientation that enable tyrosine autophosphorylation and kinase activation. It is also demonstrated herein that mutation of a single amino acid residue critical for D4 homotypic interactions compromised SCF-induced Kit activation and PDGF-induced PDGF-receptor activation (see Examples 22-25).

The structural analyses described herein provide new insights into how to design inhibitory moieties such as monoclonal antibodies that bind to conformational or non-contiguous epitopes in shallow regions of the cavities formed by the ectodomain of RTKs (*e.g.*, the D3, D4, or D5 regions) or small molecule inhibitors that bind to the D3-D4 and D4-D5 hinge regions of the ectodomain of Kit and other type-III RTKs. Four regions in the ectodomain were initially targeted: (A) Moieties of the invention may be created that bind to the D3-D4 hinge regions and function as a molecular wedge that prevents the motion required for positioning of the membrane proximal region at a distance and orientation that enables tyrosine kinase activation (see Figure 17); (B) Moieties may be created that bind to the D4-D5 hinge regions and function as a molecular wedge that prevents the motion required for positioning of the membrane proximal region at a distance and orientation that enables tyrosine kinase activation (see Figure 18); (C) Moieties may be created that bind to the D4:D4 interface preventing homotypic D4 receptor interactions (see Figure 19), (D) Moieties may be created that bind to a concave surface at the D2-D3 hinge region resulting in destabilization of ligand-receptor interactions (see Figure 20); and (E) Moieties may be created that bind to peptide regions forming various contiguous and non-contiguous epitopes on the surface of Kit (Table 5).

The molecular surfaces of the ectodomain of Kit and SCF-Kit complex (PDB code: 2EC8 and 2E9W) were analyzed using the Computed Atlas of Surface

Topography of proteins (CASTp) server to provide information about the location, and to enable delineation and measurements, of concave surface regions on three-dimensional structures of proteins (Dundas *et al.*, (2006) Nucl Acids Res, 34: W116-W118). The identified cavities were visualized and inspected using Pymol (DeLano. (2002) DeLano Scientific, San Carlos, CA, USA).

(A) Cavities in D3-D4 hinge region (Figure 17)

Several cavities are scattered on the D3-D4 interface in the ectodomain monomer structure. The amino acids involved in defining the cavities are summarized in Table 4. Upon formation of homotypic interaction between two Kit receptors, the D3-D4 hinge region is altered resulting in the formation of a shallow cavity created by the following residues: K218, S220, Y221, L222 from D3 and F340, P341, K342, N367, E368, S369, N370, I371, Y373 from D4. Figure 17 shows a ribbon diagram of the D3-D4 hinge region of unoccupied monomers (Figure 17A) and SCF-bound dimers (Figure 17B) and a mesh representation of the D3-D4 pocket.

(B) Cavities in the D4-D5 hinge region (Figure 18)

Small cavities are formed by the AB loop and the EF loop of D4, the D4-D5 connecting linker and part of the DE loop and the FG loop of D5 in the Kit monomer. Residues defining the foregoing cavities are summarized in Table 4. The shape and size of the cavities are changed in the Kit ectodomain dimeric structure. The major cavities formed by the EF loop and strand G of D4, the D4-D5 linker and stand B and DE loop of D5 are located beneath the EF loop of D4; a region critical for formation of D4 homotypic interface. Note that the DE loop of D5 that is located close to the cavities may have higher flexibility as revealed by the lower quality of electron densities from both unbound and occupied Kit structures. Figure 18 shows a ribbon diagram of unoccupied monomers (Figure 18A) and SCF-dimers (Figure 18B) and a mesh representation of a shallow cavity around the D4-D5 hinge region.

(C) Cavity at the region mediating D4 homotypic interactions

A concave surface formed by the CD loop and the EF loop of Kit D4 is located right above the D4 homotypic interface. Residues Y350, R353, F355, K358, L379,

T380, R381, L382, E386 and T390 from D4 provide a surface area of approximately 130 $A^2$ for the concave surface in the ectodomain dimeric structure. The side chain of Glu386 plays an important role in the D4 homotypic interface projects toward the center of the surface. A characteristic feature of the concave surface is a small hydrophobic patch surrounded by charged residues (Glu386 and Lys358). The size and accessibility of the surface is altered upon homotypic D4:D4 interactions with changes taking place in the conformation of the CD loop that becomes folded upwards to the top of the domain. The residues involved in the formation of the concave surface are summarized in Table 4. Figure 19A depicts a ribbon diagram of the unoccupied D4 domain of Kit (gold) overlaid onto a ligand-occupied Kit D4 (not shown) with different conformations of the CD and EF loops between ligand-occupied (green) and unoccupied ectodomain structures (red). The critical residues for the D4:D4 interaction are shown in the stick model form. Figures 19B and 19C show a ribbon diagram of unoccupied Kit (Figure 19B) and SCF-occupied Kit structures (Figure 19C) and a mesh representation of a shallow cavity above the D4 homotypic interface.

(D) Concave surface at the ligand-binding D2 and D3 regions

A shallow concave surface is located on part of the ligand-binding surface of the D2 and the D3 domains. Residues involved in the small pocket are Y125, G126, H180, R181, K203, V204, R205, P206 and F208 from D2 and V238, S239, S240, S241, H263, G265, D266, F267, N268 and Y269 from the D3 domain of Kit. The pocket is created by a small hydrophobic patch surrounded by hydrophilic residues. There is no major alteration between unoccupied and SCF-occupied Kit structures with an overall buried surface area of approximately 500 $A^2$. Figures 20A and 20B show ribbon diagrams of unoccupied Kit (A) and SCF-bound Kit (B) and a mesh representation of the D2-D3 pocket.

Table 4

Kit free

| No | Interface | Pocket/cavity | Area ($Å^2$) | | Residues |
|---|---|---|---|---|---|
| 84 | D2-D3 | 1 | 226 | D2 | Y125, H180, R181, K203, V204, R205, P206 |
| | | | | D3 | V238, S239, S240, H263, G265, D266, F267, N268, Y269 |
| 30 | | 2 | 32 | D2 | P206, F208 |
| | | | | D3 | V238, S239 |
| 22 | | 3 | 6 | D2 | K127, A207, F208 |
| | | | | D3 | T295 |
| 81 | D3-D4 | 1 | 102 | D3 | L222, |
| | | | | D4 | A339, F340, K342, E368, S369, N370, I371, Y373 |
| 80 | | 2 | 99 | D3 | L222, L223, E306, V308 |
| | | | | D4 | F312, E338, F340, I371 |
| 74 | | 3 | 57 | D3 | R224, V308, K310 |
| | | | | D4 | G311, F340, P341, D398 |
| 63 | | 4 | 49 | D3 | K218, A219, S220 |
| | | | | D4 | N367, E368, S369 |
| 59 | | 5 | 45 | D3 | K218, A220 |
| | | | | D4 | E368, S369 |
| 65 | D4-D5 | 1 | 41 | D4 | G384, T385, |
| | | | | D5 | T411, K412, E414, K471 |
| 61 | | 2 | 20 | D4 | Y408, |
| | | | | D5 | F433, G470, K471, L472 |
| 53 | | 3 | 14 | D4 | F324, V325, N326, N410 |
| | | | | D5 | |
| 24 | | 4 | 23 | D4 | D327, N410 |
| | | | | D5 | T411, K412, V497 |
| 18 | | 5 | 17 | D4 | G384, G387, V409 |
| | | | | D5 | K471 |
| 14 | | 6 | 25 | D4 | L382, G387, V407, V409 |
| | | | | D5 | |

SCF-Kit complex molA,C (Table 4 Continued)

| No | Interface | Pocket/cavity | Area ($Å^2$) | | Residues |
|---|---|---|---|---|---|
| 60 | D2-D3 | 1 | 280 | D2 | Y125, G126, H180, R181, K203, V204, R205, P206, F208 |
| | | | | D3 | V238, S239, S240, S241, H263, G265, D266, F267, N268, Y269 |
| 47 | | 2 | 49 | D2 | P206, F208 |
| | | | | D3 | V238, S239 |
| 59 | D3-D4 | 1 | 175 | D3 | K218, S220, Y221, L222 |
| | | | | D4 | F340, P341, K342, N367, E368, S369, N370, I371, Y373 |
| 57 | D4-D5 | 1 | 126 | D4 | G384, G387, G388, Y408, V409 |
| | | | | D5 | T411, F433, F469, G470, K471 |
| 56 | | 2 | 95 | D4 | D327, |

| | | | | | |
|---|---|---|---|---|---|
| | | | | D5 | T411, K412, E414, A431, G432, K471 |
| 55 | D4-D4 | 1 | 93 | D4 | Y350, F355, K358, L379, T380, R381, L382, E386, T390 |
| 7 | | 2 | 26 | D4 | Y350, R353, F355 |

(E) Structural analysis of the KIT tyrosine kinase was conducted as described above. The analysis revealed both continuous and discontinuous epitopes which may be targets for the moieties of the invention. In Table 5, epitopes 1, 4, 5, 6, 8, 12-16, 19, 22-23, and 31-39 are continuous epitopes. These epitopes are composed of sequential amino acids in the KIT protein. Epitopes 2, 3, 7, 9-11, 17, 18, 20, 21, 24-30, and 40-43 in Table 5 are discontinuous conformational epitopes composed of at least 2 peptides of the KIT protein that are brought into proximity by the folding of the KIT protein.

**Table 5**

| # | Amino acids | sequence | Domain | Strand/ loop | Amino acids | sequence | Domain | Strand/loop |
|---|---|---|---|---|---|---|---|---|
| 1 | Glu306 - Ile313 | EVVDKGFIN (SEQ ID NO: 2) | D3-D4 linker | | | | | |
| 2 | Ala219 - Leu222 | ASYL (SEQ ID NO: 3) | D3 | A | Thr304 - Val308 | TLEVV (SEQ ID NO: 4) | D3 | G |
| 3 | Asp309 - Gly311 | DKG | D3-D4 linker | | Arg224 - Gly226 | REG | D3 | AB loop |
| 4 | Val213 - Leu222 | VVSVSKASY LL (SEQ ID NO: 7) | D3 | A | | | | |
| 5 | Val301 - Asp309 | VTTTLEVVD (SEQ ID NO: 8) | D3 | G | | | | |
| 6 | Arg224 - Ile235 | REGEEFTVT CTI (SEQ ID NO: 9) | D3 | AB loop, B | | | | |
| 7 | Thr303 - Glu306 | TTLE (SEQ ID NO: 10) | D3 | G | Ala219 - Leu222 | ASYL (SEQ ID NO: 3) | D3 | A |
| 8 | Lys364 – Arg372 | KSENESNIR (SEQ ID NO: 12) | D4 | D, DE loop | | | | |
| 9 | Asn367 – Asn370 | NESN (SEQ ID NO: 13) | D4 | DE loop | Ser217 – Tyr221 | SKASY (SEQ ID NO: 14) | D3 | A |
| 10 | Ala339 – Pro343 | AFPKP (SEQ ID NO: 16) | D4 | BC loop | Asn396 – Val399 | NSDV (SEQ ID NO: 17) | D4 | F |
| 11 | Ala339 – Pro343 | AFPKP (SEQ ID NO: 16) | D4 | BC loop | Glu368 – Arg372 | ESNIR (SEQ ID NO: 19) | D4 | DE loop |
| 12 | Asp357 – Glu366 | DKWEDYPK SE (SEQ ID NO: 21) | D4 | D | | | | |
| 13 | Ile371 – Leu379 | IRYVSELHL (SEQ ID NO: 22) | D4 | E | | | | |
| 14 | Leu379 – Thr389 | LTRLKGTEG GT (SEQ ID | D4 | EF loop | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | NO: 23) | | | | | | |
| 15 | Gly328 – Glu338 | GENVDLIVE YE (SEQ ID NO: 24) | D4 | B | | | | |
| 16 | Met351 – Glu360 | MNRTFTDK WE (SEQ ID NO: 25) | D4 | CD loop | | | | |
| 17 | Lys358 – Tyr362 | KWEDY (SEQ ID NO: 26) | D4 | D | Val374 – His378 | VSELH (SEQ ID NO: 27) | D4 | E |
| 18 | Asp357 - Glu360 | DKWE (SEQ ID NO: 29) | D4 | CD loop | Leu377 – Thr380 | LHLT (SEQ ID NO: 30) | D4 | E |
| 19 | His378 - Thr389 | HLTRLKGTE GGT (SEQ ID NO: 32) | D4 | E, EF loop | | | | |
| 20 | Met351 – Glu360 | MNRTFTDK WE (SEQ ID NO: 25) | D4 | CD loop | His378 - Thr389 | HLTRLKGTE GGT (SEQ ID NO: 32) | D4 | E, EF loop |
| 21 | His378 - Thr389 | HLTRLKGTE GGT (SEQ ID NO: 32) | D4 | E, EF loop | Val323 - Asp332 | | D4 | A, AB loop |
| 22 | Val323 - Asp332 | VFVNDGENV D (SEQ ID NO: 34) | D4 | A, AB loop | | | | |
| 23 | Val409 – Ile415 | VNTKPEI (SEQ ID NO: 35) | D4 – D5 linker | | | | | |
| 24 | Val409 – Ile415 | VNTKPEI (SEQ ID NO: 35) | D4 – D5 linker | | Ala493 – Thr500 | AYNDVGKT (SEQ ID NO: 36) | D5 | FG loop |
| 25 | Val409 – Ile415 | VNTKPEI (SEQ ID NO: 35) | D4 – D5 linker | | Ala431 - Thr437 | AGFPEPT (SEQ ID NO: 38) | D5 | B |
| 26 | Val409 – Ile415 | VNTKPEI (SEQ ID NO: 35) | D4 – D5 linker | | Phe469 - Val473 | FGKLV (SEQ ID NO: 40) | D5 | DE loop |
| 27 | Val409 – Ile415 | VNTKPEI (SEQ ID NO: 35) | D4 – D5 linker | | Val325 – Asn330 | VNDGEN (SEQ ID NO: 42) | D4 | A |
| 28 | Val409 – Ile415 | VNTKPEI (SEQ ID NO: 35) | D4 – D5 linker | | Arg381 - Gly387 | RLKGTEG (SEQ ID NO: 44) | D4 | EF loop |
| 29 | Gly466 – Leu472 | GPPFGKL (SEQ ID NO: 46) | D4 | DE loop | Gly384 – Gly388 | GTEGG (SEQ ID NO: 47) | D4 | EF loop |
| 30 | Val325 - Glu329 | VNDGE (SEQ ID NO: 49) | D4 | A | Tyr494 - Lys499 | YNDVGK (SEQ ID NO: 50) | D5 | FG loop |
| 31 | Thr411 - Leu421 | TKPEILTYDR L (SEQ ID NO: 52) | D5 | A | | | | |
| 32 | Asp419 - Cys428 | DRLVNGML QC (SEQ ID NO: 53) | D5 | AB loop | | | | |
| 33 | Gly498 - Lys509 | GKTSAYFNF AFK (SEQ ID NO: 54) | D5 | G | | | | |
| 34 | Cys443 - Ser453 | CPGTEQRC SAS (SEQ ID NO: 55) | D5 | C | | | | |
| 35 | Cys450 - Gln460 | CSASVLPVD VQ (SEQ ID NO: 56) | D5 | C | | | | |
| 36 | Asp479 - Thr488 | DSSAFKHN GT (SEQ ID NO: 57) | D5 | EF loop | | | | |
| 37 | Gly487 - Tyr496 | GTVECKAYN D (SEQ ID NO: 58) | D5 | F | | | | |
| 38 | Leu462 - Leu472 | LNSSGPPFG KL (SEQ ID | D5 | DE loop | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | NO: 59) | | | | | | |
| 39 | Phe506 – Ile515 | FAFKGNNKE QI (SEQ ID NO: 60) | D5 | C tail | | | | |
| 40 | Thr411-leu416 | TKPEIL (SEQ ID NO: 61) | D5 | A | Val497 - Ala502 | VGKTSA (SEQ ID NO: 62) | D5 | FG loop |
| 41 | Ile415 – Leu421 | ILTYDRL (SEQ ID NO: 64) | D5 | A | Ala502 – Ala507 | AYFNFA (SEQ ID NO: 65) | D5 | G |
| 42 | Ala502 – Ala507 | AYFNFA (SEQ ID NO: 65) | D5 | G | Lys484 - Thr488 | KHNGT (SEQ ID NO: 67) | D5 | EF loop |
| 43 | Ala502– Ala507 | AYFNFA (SEQ ID NO: 65) | D5 | G | Gly445 – Cys450 | GTEQRC (SEQ ID NO: 69) | D5 | C |

**Example 20: RTK Activity Assay**

Cells containing an RTK of interest are exposed to the activating ligand for the receptor and a moiety of the invention. The RTK of interest may be isolated by standard molecular biology methods (*e.g.*, antibody purification). After purification, an antibody which binds to the RTK (not a moiety of the invention but simply a structural binder, as used in purification) is pre-coated onto a 96-well mictrotiter plate. The RTK and calibrated standards are then added to separate wells wherein the RTK protein is captured. A detection antibody is added next, which may be phospho-site specific (*e.g.*, c-Kit pY823 or other residue of Kit which is phosphorylated upon activation; the phosphoELISA™ system uses rabbit antibody). The antibody-Kit complex is detected using a secondary antibody (*e.g.*, anti-rabbit Ab to detect a rabbit derived primary antibody) which is conjugated to a label or enzyme (*e.g.*, horseradish peroxidase is used in the phosphoELISA™ system) followed with a colorimetric substrate. Stop solution is then added and the plate is read (*e.g.*, using a 450nm light source and detector). Detailed protocols for the phosphoELISATM are available from Invitrogen (invitrogen.com/content.cfm?pageid=11655; invitrogen.com/downloads/F1027_BN_pELISA1006.pdf; invitrogen.com/downloads/F1028_BN_pELISA1006.pdf C-KIT [pY823] ELISA KIT, HU (BioSource™) Catalog Number - KHO0401; c-KIT [TOTAL] ELISA KIT, HU (BioSource™) Catalog Number - KHO0391).

**Example 21: Receptor Internalization Assay**

Cells expressing the RTK of interest are first incubated with an appropriate ligand (*e.g.*, Kit expressing cells are incubated with SCF), inducing receptor internalization. The process of receptor internalization is stopped by washing the cells in cold PBS. The remaining surface bound ligand is then removed by washing the cells in a solution having a salt concentration and/or pH level sufficient to dissociate the ligand. The cells are then resuspended in the appropriate buffer. The cells at this point will contain internalized receptor, and, thus, a lessened amount of receptor remaining on the cell surface.

Another set of similar experiments is run wherein the cells are exposed to an appropriate ligand and a test moiety of the invention. If the test moiety prevents the activation of the target RTK, then receptor internalization will be inhibited. When compared to the cells described in the experiment above (wherein receptor activation occurred), these cells show decreased internalization and a greater amount of receptor on the cell surface. Control groups are also set up in which cells are treated only with buffer or ethanol solution, a common vehicle for solubilization of drugs.

Determination of the amount of receptor on the cell surface in the above experiments may be accomplished by incubating the cells with mouse antibodies specific for the receptor, followed by and incubation with anti-mouse antibodies which are conjugated to a fluorophore such as Green Fluorescent Protein (GFP). Fluorescence microscopy techniques may then be used to visualize and quantitate the amount of receptor on the cell surface.

Alternative techniques for the quantitation or visualization of cell surface receptors are well known in the art and include a variety of fluorescent and radioactive techniques. For example, one method involves incubating the cells with a radiolabeled anti-receptor antibody. Alternatitively, the natural ligand of the receptor may be conjugated to a fluorescent molecule or radioactive-label and incubated with the cells. Additional receptor internalization assays and are well known in the art and described in, for example: Jiménez et al. (1999) Biochemical Pharmacology. 57(10):1125-1131; Bernhagen et al. (2007) Nature Medicine. 13(5):587-596; and Conway et al. (2001) J.

Cell Physiol. 189(3):341-55, the entire contents of each of which are incorporated herein by reference.

**Introduction to Examples 22-25**

The generally accepted mechanism of receptor tyrosine kinase (RTK) activation is that ligand-induced receptor dimerization facilitates trans-autophosphorylation of critical regulatory tyrosine residues in the activation loop of the catalytic core; a step essential for tyrosine kinase activation. This is followed by autophosphorylation of multiple tyrosine residues in the cytoplasmic domain that serve as binding sites for SH2 (Src homology 2) or PTB (phosphotyrosine binding) domains of a variety of signaling proteins, which upon recruitment and/or tyrosine phosphorylation transmit signals to variety of intracellular compartments in a regulated manner (Schlessinger, J. (2000) Cell 103, 211-225; Pawson, T. & Nash, P. (2003) Science 300, 445-452; and Hunter, T. (2000) Cell 100, 113-127).

While all RTKs are activated by dimerization, different RTK families have evolved to utilize different molecular strategies for ligand-induced receptor dimerization and activation (Burgess, A. W., et al. (2003) Mol Cell 12, 541-552; Schlessinger, J., et al. (2000) Molecular Cell 6, 743-750). All ligands of type-III RTKs including PDGFs, SCF, CSF and Flt3L are dimeric molecules capable of crosslinking their cognate receptors by bivalent binding to equivalent sites of two neighboring receptor molecules. The PDGF protomer is composed of a central four-stranded β-sheet with the characteristic cysteine-knot at one end of the molecule. Two PDGF protomers are arranged in antiparallel manner and are linked to each other by two inter-chain disulfide bridges (Oefner, C., et al. (1992) EMBO J. 11, 3921-3926.). By contrast, each SCF, CSF or Flt3L protomer is composed of short helical fold and is connected to each other by non-covalent interactions (Jiang, X., et al. (2000) Embo J 19, 3192-3203; Zhang, Z., et al. (2000) Proc Natl Acad Sci U S A 97, 7732-7737; Pandit, J., et al. (1992) Science 258, 1358-62; and Savvides, S. N., et al. (2000) Nat Struct Mol Biol 7, 486-491). Despite their diverse folds, the two growth factor subtypes bind to and activate their cognate RTKs in a virtually identical manner resulting in formation of activated ligand/RTK 2:2 complexes (Savvides, S. N., et al. (2000) Nat Struct Mol Biol 7, 486-491). All type-III RTKs are

composed of extracellular ligand binding region containing five tandem Ig-like domains followed by a single transmembrane helix and a cytoplasmic tyrosine kinase domain with a large kinase-insert region flanked by regulatory regions that are subject to autophosphorylation and to phosphorylation by heterologous protein kinases (Hubbard, S. R. (1999) Prog Biophys Mol Biol 71, 343-358).

The mechanism of PDGF-receptor β (PDGFRβ) activation was explored by analyzing the properties of mutant receptors that were designed based upon the crystal structure of the extracellular region of the related receptor tyrosine kinase Kit. Based on these experiments it was demonstrated that PDGF-induced activation of a PDGFRβ mutated in Arg385 or Glu390 in D4 (the 4th Ig-like domain of the extracellular region) was compromised resulting in impairment of a variety of PDGF-induced cellular responses. These experiments also demonstrate that homotypic D4 interactions, likely mediated by salt bridges between Arg385 and Glu390, play an important role in activation of PDGFRβ and all type-III RTKs. A chemical crosslinking agent was also used to covalently crosslink PDGF-stimulated cells to demonstrate that a Glu390Ala mutant of PDGFRβ undergoes typical PDGF-induced receptor dimerization. However, unlike WT PDGFR that is expressed on the surface of ligand-stimulated cells in an active state, PDGF-induced Glu390Ala dimers are inactive. While the conserved amino acids that are required for mediating D4 homotypic interactions are crucial for PDGFRβ activation (and similar interactions in type-III RTKs), these interactions are dispensable for PDGFRβ dimerization. Moreover, PDGFRβ dimerization is necessary but not sufficient for tyrosine kinase activation.

Similar to the D4 domain of Kit, the D4 domain of PDGFRα and PDGFRβ lack a characteristic disulfide bond that bridges cysteine residues located in B5 and F5 in Ig-like domains. The amino acid sequence alignment presented in Figure 21 shows that 13 out of 20 finger-print residues of the Iset IgSF fold are conserved in the D4 domain of PDGFRs and that the number and length of strands corresponding to the finger-print residues are highly conserved in the D4 domain of Kit, PDGFRα , PDGFRβ and CSF1R. This indicates that the inhibitors of the invention may be designed to inhibit a variety of receptor molecules including all Type III RTKs.

The D4 domain of Kit is composed of two β sheets, each containing four strands with the arrangement ABED/A'GFC and the homotypic D4 contacts are mediated by the

EF loop of D4 projecting from two neighboring Kit molecules. The Kit structure disclosed herein demonstrates that Arg381 and Glu386 in the EF loop form salt bridges and van-der-Waals contacts across a two-fold axis of the Kit dimer. In addition, the side chains of Arg381 of each protomer form hydrogen bonds with the main chain carbonyl of the corresponding residue of neighboring Kit molecules. Structure based sequence alignment has shown that the size of the EF loop, and the critical amino acids comprising the D4-D4 interface are conserved in Kit, PDGFRα, PDGFRβ, and CSF1R. In PDGFRα, Glu386 is replaced by an aspartic acid, a residue that may also function as a salt bridge partner. In addition, a pair of basic and acidic (Glu/Asp) residues is strictly conserved in PDGFRα and PDGFRβ of different species ranging from Takifugu rubripes to Homo sapien (Figure 21), providing further support for the functional importance of this region. As such, moieties of the invention targeted to RTKs, e.g. Type III RTKs, with different amino acid sequences or to variant domains of similar function to those described herein also fall within the scope of the present invention.

**Methods Related to Examples 22-25**

Sequence alignment and homology modeling

Amino acid sequence alignment was performed using the CONSEQ server (Berezin, C., et al. (2004) Bioinformatics 20, 1322-1324), as well as according to the IgSF fold characteristics (Harpaz, Y. & Chothia, C. (1994) Journal of Molecular Biology 238, 528-539) and according to the core residues of the Ig-fold of D4 of human Kit structure (Yuzawa, S., et al. (2007) Cell 130, 323-334). The accession codes of each sequence are: PDGFRα human (P16234), mouse (P26618), chicken (Q9PUF6), frog (P26619) and fugu (Q8AXC7); PDGFRβ human (P09619), dog (Q6QNF3), mouse (P05622), fugu (P79749) and Kit human (Q96RW7). A homology model of D4 of PDGFRβ was generated on the basis of D4 Kit structure (PDB code: 2E9W) using the WHAT IF server (Rodriguez, R., et al. (1998) Bioinformatics 14, 523-528). Figures were generated using PyMOL (Delano, W.L.; pymol.org).

Reagents and antibodies

L-histidinol and anti-flag antibodies were purchased from Sigma. Antibodies against MAPK, phospho-MAPK, Akt, phospho-Akt, and phospholipase Cγ were purchased from Cell Signaling Technology. Anti-phosphotyrosine (4G10) antibodies was from Upstate Technology. Antiubiquitin antibodies (P4D1) was from Santa Cruze. Antibodies against PDGFRβ were produced by immunization of rabbit with synthetic peptides from the cytoplasmic domain of PDGFRβ. PDGF BB cDNA was obtained from Stuart Aaronson. PDGF BB was purchased from Invitrogen, and produced in bacteria as previously described (Hoppe, J., et al. (1990) European Journal of Biochemistry 187, 207-214). $^{125}$I radionuclide was purchased from Perkin Elmer. Bolton-Hunter reagent and IODO-GEN pre-coated iodination tubes were from Piece. FITC-phalloidin was purchased from Invitrogen.

Cell lines and retroviral infection

Fibroblasts derived from mouse embryos deficient in both PDGFRα and PDGFRβ (PDGFRα /β) were provided by Philip Sariano and Andrius Kazlauskas. PDGFRβ cDNA was provided by Daniel DeMaio. PDGFRβ cDNA was subcloned into pLXSHD retroviral vector, and a flag-tag was added to the C terminus of the receptor. All mutants in D4 were generated by site-directed mutagenesis according to the manufacturer's instructions (Stratagen). Retrovirus encoding WT and mutant PDGFRβ were produced in 293GPG cells (Ory, D. S., et al. (1996) Proc. Nat. Acad. Sci. 93, 11400-11406). Following infection, cells were selected with L-histidinol, and pools of selected cells were used in the experiments.

Immunoprecipitation and immunoblotting

Unstimulated or PDGF-stimulated cells were lysed in a buffer solution containing 50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 25mM sodium fluoride, 1mM orthovanadate, 1mM phenyl-methylsulfonyl fluoride, 5 μg of aprotinin and leupeptin (pH 7.5). Equal amount of cell lysates were immunoprecipitated with indicated antibodies, immunopellets were resolved by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were immunoblotted with different antibodies.

Films were scanned using densitometer (Amersham) and quantitated with Imagequant software (Molecular dynamics).

*In vitro* phosphorylation assay for PDGFR

Cells were serum-starved for 16 hours and solubalized in lysis buffer containing 150 mM NaCl, 50 mM Hepes (pH 7.4), 1 mM EDTA, 25 mM NaF, 0.1 mM sodium orthovanadate, 5 μg/ml leupeptine and aprotinin, 1mM PMSF and 1% NP40. Lysates were immunoprecipitated with anti-PDGFRβ antibodies, and immunopelletes were incubated in reaction buffer containing 50mM Hepes (pH7.4), 1mM ATP and 10mM $MgCl_2$ at room temperature for 5 minutes. After incubation, pellets were analyzed by SDS-PAGE followed by immunoblotting with antiphosphotyrosine antibodies. The membrane was stripped off and re-blotted with anti-Flag tag antibodies for determination of total PDGFRβ level.

Chemical crosslinking of receptor dimers

Cells were grown in 150mm plates until an 80% confluency was reached and were serum-starved for 16 hours prior to incubation with the indicated concentration of PDGF in DMEM containing 50mM Hepes (pH 7) at 4°C. After 90 minutes, the cells were extensively washed with PBS (pH 7.4). Plates were transferred to room temperature and disuccinimidyl suberate (DSS) was added to a final concentration of 0.5mM. The crosslinking reaction was quenched after 30 minutes by incubation with 10mM Tris buffer for 15 minutes, followed by extensive wash with PBS. Cell lysates in 50mM Hepes, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 25mM sodium fluoride, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin and 5 μg/ml leupeptin (pH 7.4) were immunoprecipitated with anti-PDGFR antibodies and resolved by SDS-PAGE. Nitrocellulose membrane was immunoblotted with antibodies against flag-tag or antiphosphotyrosine (4G10) antibodies to detect the total receptor and phosphorylated receptor level respectively.

PDGF-induced actin cytoskeletal reorganization

MEFs were plated to subconfluency on glass coverslips for 24 hours, followed by overnight serum-starvation. Cells were either treated with 50 ng/ml PDGF for 2,5,10, or 30 minutes or left untreated. Cells were fixed in 4% paraformaldehyde in PBS, permeablized with 0.1% Triton in PBS and stained with FITC-phalloidin (Sigma) in PBS containing 1% BSA for 30min. Coverslip were mounted with Prolong Antifade mounting medium (Invitrogen), and images were acquired with Nikon fluorescence microscope. About 400 cells on each coverslip were analyzed, and the percentage of cells showing actin ring formation was calculated and presented linearly.

PDGF binding and internalization experiments

PDGF was labeled using Bolton-Hunter reagent (Pierce) prior to iodination using Iodo-gen Iodination tubes (Pierce) according to the manufacturer's instructions. Cells were plated on 24-well plates and allowed to grow to 80% confluency in DMEM supplemented with 10% fetal bovine serum. Cells were washed twice in cold DMEM containing 20mM Hepes (pH7.4) and 0.1% BSA. Triplicate wells were incubated with 5ng/ml of $^{125}$I-PDGF in the presence of increasing amounts of native PDGF. Binding was allowed to proceed at 25°C for 1 hour. Cells were then washed in cold PBS and solubilized in 0.5 M NaOH. The radioactive content of the samples was determined using a LS6500 scintillation counter (Beckman Coulter), and data were analyzed using PRISM software (GraphPad).

For internalization experiments, cells were seeded in 24-well plates, allowed to grow to 80% confluency and starved overnight. Cells were incubated with 5 ng/ml $^{125}$I-PDGF in DMEM/0.1% BSA/50mM Hepes, pH7.4 for 90min at 4°C. Unbound ligand was removed by washing with ice cold PBS (pH 7.4). Pre-warmed DMEM/0.1% BSA/50mM Hepes was added to the cells and incubated at 37°C for the time indicated. Cell surface-associated ligand was collected with ice-cold acidic buffer containing PBS (pH 3) and 0.1% BSA for 10 minutes. Internalized ligands were collected by solubilization with 0.5 M NaOH. The amount of degraded $^{125}$I-PDGF was determined by precipitation of the incubation medium with 10% trichloroacetic acid (TCA), and counting the supernatant for the TCA soluble fraction. Cell-surface-associated

internalized and released radioactivities were determined by liquid scintillation counter. The amounts of surface bound, intracellular and degraded PDGF were expressed as a percent of total cell associated radioactivity after a 90 minute incubation on ice (t=0 minutes). Each time point was performed in triplicate, and the results were expressed as mean ± SE.

**Example 22: PDGF-induced PDGF-receptor activation is compromised by mutations in the D4 Domain.**

The amino acid sequence alignment presented in Figure 21A demonstrates that Arg385 and Glu390 in the EF loop of PDGFR may mediate homotypic D4 interactions similar to the salt bridges formed between Arg381 and Glu386 of Kit that are responsible for mediating homotypic D4 interactions between neighboring Kit receptors. To investigate whether a similar mechanism is employed by PDGFRβ, Arg385 and Glu390, each alone (R385A, E390A) or in combination (R385E390/AA) were substituted by alanine residues. An additional conserved Lys387 residue in the loop region was also substituted by an alanine (R385K387E390/AAA) residue in order to examine its potential role in control of PDGF-induced PDGFRβ activation. Wild-type and mutant PDGFRβs were stably expressed in fibroblasts derived from mouse embryos (MEFs) deficient in both PDGFRα and PDGFRβ (Soriano, P. (1994) Genes Dev. 8, 1888-1896; Soriano, P. (1997) Development 124, 2691-2700; and Andrews, A., et al. (1999) Invest. Ophthalmol. Vis. Sci. 40, 2683-2689). MEFs expressing wild type or mutant PDGFRβs that were matched for expression level were used in the experiments described below. Cell lysates from unstimulated or PDGF-stimulated cells were subjected to immunoprecipitation with anti-PDGFR antibodies, followed by immunoblotting with anti-phosphotyrosine antibodies.

The membranes were subsequently stripped off, and re-blotted with anti-PDGFR antibodies for quantitation of PDGFR expression. The experiment presented in Figure 22A shows that PDGF-induced tyrosine autophosphorylation of PDGFRβ is strongly compromised in cells expressing the E390A, R385A, (R385E390/AA), and (R385K387E390/AAA) mutants of PDGFRβ; both the magnitude and kinetics of tyrosine autophosphorylation were reduced and attenuated, respectively. These

experiments demonstrate that Arg385 and Glu390 in the EF loop of D4 play an important role in PDGF-induced stimulation of PDGFRβ, which demonstrates that a similar pair of salt bridge to those identified in the Kit structure exists in activated PDGFRs and other Type III RTKs. Direct interaction between the D4 domain of a neighboring receptor within the ligand-receptor complex may represent a common mechanism utilized for ligand induced activation of type-III RTKs. It has consistently and reproducibly been observed that PDGF-induced receptor autophosphorylation is more strongly compromised in cells expressing the E390A in comparison to cells expressing the R385A, (R385E390/AA) or the (R385K387E390/AAA) mutants. While the precise mechanism responsible for the difference between these mutants is not clear, it is possible that the positive local surface charge at the D4 interface may cause electrostatic repulsion to maintain D4 of neighboring receptors apart prior to ligand stimulation. Whereas substitution of Arg385 by an alanine residue will prevent salt bridge formation, this change may also decrease the net positive charge in the D4-D4 interface resulting in weaker inhibition of PDGFR activation.

In order to examine the possibility of whether mutation in the D4 domain of PDGFR may have affected cell membrane expression and ligand binding affinity of mutant PDGFRβs, quantitative PDGF binding experiments to cells expressing wild type or mutant PDGFRβs were performed next. Cells expressing wild type, R385A, E390A or the (R385E390/AA) PDGFRβ mutants were incubated with a buffer solution containing $^{125}$I-PDGF for 90 minutes at 4°C in the presence of increasing concentration of native PDGF. Cell bound radioactivity was measured using a scintillation counter. The EC50 values of the displacement curves of wild type and mutant PDGFRβs were analyzed by curve fitting with Prism4 (Figure 22B). The amounts of wild type and mutant PDGFRβs that are expressed in the transfected MEFs were also compared by immunoblotting of total cell lysates with antibodies against PDGFR or anti-tag antibodies (Figures 22A and C). Taken together, these experiments demonstrate that similar amounts of wild type or mutant PDGFRβs are expressed on the cell surface of the transfected cells. Moreover, similar IC50 values (PDGF concentration that displaces 50% of $^{125}$I-PDGF binding) were obtained for cells expressing wild type (3.7nM), R385A (6.0nM), E390A (2.8nM) or the RE/AA (3.0nM) mutants. The possibility of whether the intrinsic tyrosine kinase activity of mutant PDGFRβs was adversely affected

by comparing the *in vitro* tyrosine kinase activities of wild type and mutant receptors was also examined. In this experiment, cell lysates from serum-starved cells were subjected to immunoprecipitation with anti-PDGFR antibodies, and the immobilized PDGFRs were subjected to *in vitro* kinase assays in the presence of 1mM ATP and 10mM magnesium chloride. After incubation, the samples were analyzed by immunoblotting with anti-phosphotyrosine antibodies. The experiment presented in Figure 22C demonstrates that the R385A, E390A or RE/AA mutations do not influence the intrinsic tyrosine kinase activity of PDGFR. Altogether, these experiments demonstrate that the mutations in D4 that affect PDGF-induced stimulation of PDGFRβ do not alter the expression of PDFGRβ on the cell surface, do not influence the ligand binding affinity of PDFGRβ and do not alter the intrinsic tyrosine kinase activities of mutant PDGFRβ.

**Example 23: PDGF receptor D4 point mutants are expressed on the surface of PDGF-stimulated cells in the form of inactive dimmers**

Since receptor dimerization has been established as a critical mechanism underlying receptor tyrosine kinase activation, we investigated whether reduced tyrosine autophosphorylation of mutant PDGFRβ in response to PDGF stimulation is caused by deficiency in receptor dimerization. Chemical crosslinking agents have previously been used to monitor and follow ligand-induced dimerization of several cell membrane receptors including wild type and a variety of EGF receptor mutants on the cell surface of living cells (Cochet, C., et al. (1988) J Biol Chem 263, 3290-3295). In this experiment, cells expressing wild type PDGFRβ or the E390A mutant were serum starved overnight, followed by PDGF incubation for 90 minutes at 4°C. Several washes were used to remove unbound PDGF and the cells were incubated with 0.5mM disuccinimidyl suberate (DSS) in PBS for 30 minutes at 25°C. Cell lysates from unstimulated or PDGF-stimulated cells were subjected to immunoprecipitation with anti-PDGFR antibodies followed by SDS-PAGE and immunoblotting with either anti-flag antibodies to monitor the status of PDGFR dimerization or with antiphosphotyrosine antibodies to monitor the status of PDGFR activation (Figure 23).

The experiment depicted in Figure 23 demonstrates that in lysates of unstimulated cells a band that migrates on an SDS gel with an apparent molecular weight of 180 kDa corresponding to PDGFR monomers was detected in lysates from cells expressing either wild type PDGFRβ or the E390A mutant. Upon PDGF stimulation, an additional band that migrates on an SDS gel with an apparent molecular weight of 360 kDa corresponding to PDGFR dimers was detected in cells expressing both wild type PDGFRβ and the E390A mutant. However, immunoblotting of the samples with anti-phosphotyrosine antibodies demonstrates that while the band corresponding to dimers of wild type PDGFR is strongly tyrosine phosphorylated, very weak tyrosine phosphorylation of the band corresponding to the dimers of E390A mutant is detected (Figure 23).

This experiment shows that impaired ligand-induced tyrosine autophosphorylation of the E390A mutant is not caused by a deficiency in ligand-induced receptor dimerization. This experiment also demonstrates that the covalently crosslinked wild type PDGFRβ are displayed on the cell surface of PDGF-stimulated cells in the form of active dimers while the E390A mutant is displayed on the surface of PDGF-stimulated cells in the form of inactive dimers. The foregoing data demonstrate that the D4 homotypic interactions in PDGFR are dispensable for receptor dimerization and that PDGF-induced receptor dimerization is necessary but not sufficient for tyrosine kinase activation.

**Example 24: Impaired stimulation of cells signaling in cells expressing D4 PDGF-receptor mutants**

The impact of PDGFR D4 mutations on cell signaling in response to PDGF stimulation was examined. Lysates from unstimulated or PDGF-stimulated cells expressing either WT or PDGFR D4 mutants were subjected to immunoprecipitation with anti-phospholipase Cγ (anti- PLCγ) antibodies followed by SDS-PAGE and immunoblotting with either anti- PLCγ or antipTyr antibodies. The experiment presented in Figure 24A shows that tyrosine phosphorylation of PLCγ is severely compromised in cells expressing the R385A, E390A, RE/AA or the RKE/AAA PDGFR mutants. Impaired stimulation of additional PDGF induced cellular responses are observed in

cells expressing PDGFR D4 mutants. The experiment presented in Figure 24B shows that MAP-kinase response and Akt stimulation were strongly compromised in cells expressing the R385A, E390A, R385E390/AA or R385K387E390/AAA PDGFR mutants, as compared to similar responses induced by PDGF in MEFs expressing WT PDGFRs. Overall, approximately 10-fold higher concentrations of PDGF were required for a similar level of MAP kinase response and Akt stimulation in cells expressing the E390A, R385E390/AA (*i.e.*, RE/AA) or R385K387E390/AAA (*i.e*, RKE/AAA) PDGFR mutants.

One of the hallmarks of PDGF stimulation of cultured fibroblasts is a typical formation of membrane ruffles and circular actin ring structures on the dorsal surface of PDGF-stimulated cells. The experiment presented in Figure 25 shows that PDGF stimulation of actin ring formation is compromised in MEFs expressing PDGFR D4 mutants. While approximately 83% of MEFs expressing WT PDGFR exhibited circular actin ring formation, only 5% of PDGFR D4 mutant cells showed similar circular actin ring formation after a 2 minute stimulation with 50ng/ml of PDGF. Furthermore, the transient circular actin ring formation that peaks in MEFs expressing WT PDGFR after 2-5 minutes of PDGF stimulation, was weakly detected in cells expressing the R385A, E390A or the RE/AA PDGFR mutants.

**Example 25: Reduced internalization and degradation of D4 PDGF receptor mutants**

The effect of PDGFR D4 mutations on PDGF internalization, PDGFR degradation and PDGFR ubiquitination was also examined. MEFs expressing WT PDGFR or the PDGFR D4 mutants were treated with 5ng/ml of $^{125}$I labeled PDGF for 90 minutes at 4° C followed by brief washes with PBS (pH7.4) to remove the excess ligand in the medium. Pre-labeled cells were warmed to 37°C to initiate the endocytosis of ligand-receptor complex for various time intervals up to 4 hours. Cell surface-bound, intracellular and degraded $^{125}$I-PDGF in medium were collected, quantitated using a scintillation counter, and presented as percent of total cell-associated $^{125}$IPDGF radioactivity after a 90 minute incubation (t=0) at 4°C (mean ± SD). The experiment presented in Figure 26A shows that the kinetics of internalization of $^{125}$I labeled PDGF

bound to MEFs expressing WT PDGFR is much faster than the kinetics of internalization of $^{125}$I labeled PDGF bound to cells expressing the E390A, R385A or the R385E390/AA PDGFR mutants. After 30 minutes, ~75-80% of $^{125}$I-PDGF was removed from cell surface and accumulated inside the cells expressing WT receptors compared to less than 50% in cells expressing mutant receptors.

The low molecular weight degradation product of $^{125}$I-PDGF became detectable after 30 minutes. The release of degraded $^{125}$I-PDGF was much slower in E390A mutant cells than in WT cells (Figure 26A). Reduced PDGF internalization and degradation were reflected in reduced degradation of PDGFR D4 mutants. Cells expressing WT or the R385A, E390A or R385E390/AA PDGFR mutants were first incubated for 30 minutes with cycloheximide, in order to prevent the biosynthesis of new PDGFR molecules during the degradation experiment. Lysates of unstimulated or PDGF stimulated cells were subjected to immunoprecipitation with anti-PDGFR antibodies followed by SDS-PAGE and immunoblotting with antibodies directed against a tag attached to the C-termini of WT or PDGFR D4 mutants. The experiment presented in Figure 26B shows that the kinetics of degradation of R385A, E390A or the R385E390/AA PDGFR mutants was strongly attenuated; while half of WT PDGFRs were degraded within 1.5 hour of PDGF stimulation, the half-life for PDGFR D4 mutants was extended to approximately 4 to 6 hours. The experiment presented in Figure 26C shows that PDGF induced stimulation of ubiquitination of the E390A PDGFR was also strongly reduced as compared to WT PDGFR under similar conditions. Taken together these experiments demonstrate that PDGFR internalization and ubiquitin-mediated PDGFR degradation are compromised by mutations in D4 of PDGFR.

**Discussion of Examples 22-25**

The extracellular domains of all members of type-III RTKs, including PDGFRα, PDGFRβ, CSF1R, Flt3 and Kit are composed of five Ig-like domains of which the first three function as binding site for dimeric ligand molecule which, upon binding, stimulates receptor dimerization and activation. As the molecular architecture, ligand binding characteristics and mechanism of receptor dimerization of type-III RTKs are highly conserved, the mechanism of SCF induced Kit activation revealed by the crystal

structures of the complete extracellular domain of Kit before and after SCF stimulation represents a general mechanism of activation of all type-III RTKs. Moreover, phylogenic analysis of RTKs containing Ig-like domains in their extracellular domains indicates a common evolutionary origin for type-III and type-IV RTK; a family including VEGFR1 (Flt1), VEGFR2 (KDR) and VEGFR3 (Flt4). Moreover, both VEGF and PDGF belong to the same cystein-knot family; homodimeric growth factors, sharing similar topology, size and receptor binding strategy. The salient features of Kit activation revealed by the x-ray structural analysis of its extracellular domain (disclosed for the first time herein) may, therefore, also apply for ligand-induced activation of type-V RTKs.

The structural analysis of Kit has shown that a pair of salt bridges formed between Glu386 and Arg381 of two neighboring D4 domains, are responsible for mediating homotypic D4 interactions that are essential for SCF-induced Kit activation. Comparison of the amino acid sequences of type-III RTKs demonstrates that an identical sequence motif exists in the EF loop region of D4 of PDGFRα, PDGFRβ and CSF1R (Figure 21), providing evidence that a similar salt bridge is also formed between D4 of type-III RTKs. Indeed, substitution of Arg385 or Asp390 in the D4 domain of PDGFRβ by alanines has compromised PDGF stimulation of PDGFRβ activation resulting in impairment of a variety of cellular responses that are stimulated by PDGF in cells expressing WT PDGFRβ. The mechanism of ligand induced Kit activation revealed by analysis of Kit structure applies for the activation of all type-III RTKs. A sequence motif identical to the sequence motif responsible for D4 homotypic interactions was also identified in the EF loop of the membrane proximal $7^{th}$ Ig-like domain (D7) of all three members of VEGFR family (type-IV) of RTK. Although the conserved sequence motif that is responsible for mediating homotypic D4 interactions in Kit and other type-III RTK is located in the D7 domain of type-IV RTKs, D7 of VEGFRs likely plays a role similar to D4 in mediating homotypic interactions between membrane proximal regions of type-IV RTKs. Indeed, an electron microscopic analysis of the structure of the extracellular domain of VEGFR2 has revealed a direct contact between D7 in VEGF-bound VEGFR2 dimers (Ruch, C., et al. (2007) Nat Struct Mol Biol 14, 249-250). Direct contacts between membrane proximal Ig-like domains represents a general mechanism employed by both type-III and type-IV RTKs.

Studies exploring a variety of receptor mutants or employing monoclonal antibodies that bind specifically to individual Ig-like domains of Kit (Blechman, et al. (1995) Cell 80, 103-113), PDGF-receptors (Miyazawa, K., et al. (1998) J. Biol. Chem. 273, 25495-25502) and other type-III RTKs have proposed that D4 plays a role in mediating receptor dimerization even when Kit is stimulated by monovalent SCF ligands (Lev, S., et al. (1992) J Biol Chem 267, 15970-15977). However, quantitative analyses employing microcalorimetry of SCF binding and SCF stoichiometry towards the purified extracellular domain of Kit composed of either the first three Ig-like domains (D1-D3) or all five Ig-like domains (D1-D5) have shown that D4 and D5 are dispensable for SCF stimulation of Kit dimerization. In other words these reports have shown that Kit dimerization is primarily driven by the dimeric nature of SCF binding to Kit (Lemmon, M. A., et al. (1997) J. Biol. Chem. 272, 6311-6317.).

However, the work presented herein demonstrates that, rather than playing a role in receptor dimerization, the homotypic D4 (and also homotypic D5) interactions between neighboring receptors are required for precise positioning of the membrane proximal regions of two receptors at a distance and orientation that enable interactions between their cytoplasmic domains resulting in tyrosine kinase activation. Therefore, rather than interfering with receptor dimerization, the moieties, *e.g.*, monoclonal antibodies, of the invention exert their inhibitory effect on receptor activation by preventing critical homotypic interactions between membrane proximal regions of type-III RTK that are essential for positioning the cytoplasmic domain at a distance and orientation essential for tyrosine kinase activation.

The experiments presented herein demonstrate that dimerization of PDGFRβ, Kit and other type-III RTKs is entirely driven by ligand binding and that the sole role of ligand binding is to crosslink two receptor molecules in order to increase their local concentration in the cell membrane. The two salt bridges (with interface of a buried surface area of 360Å$^2$) responsible for mediating homotypic D4 interactions are too weak to support receptor interactions without the support of ligand mediated receptor dimerization which in the case of Kit is mediated by a variety of strong interactions with a total buried surface area of 2060 Å$^2$ for each SCF protomer. The apparent concentration of a receptor in the cell membrane of an unstimulated cell expressing 20,000 receptors per cell has been estimated to be approximately 1-3 μM (Klein, P., et al.

(2004) Proc Natl Acad Sci U S A 101, 929-934; Chandrasekhar, S. (1943) Reviews of Modern Physics 15, 1). Upon binding a dimeric ligand such as SCF, two occupied receptors are held together at a distance of 75Å. Under these conditions, the apparent receptor concentration in the cell membrane calculated using the average distance to nearest neighbor approach is increased by more than two orders of magnitude to 4 – 6 x $10^{-4}$M. This calculation shows that even weak interactions with a dissociation constant in the range of $10^{-4}$ – $10^{-5}$M, such as those mediated by the two salt bridges, could mediate association and direct contacts between membrane proximal regions of two neighboring receptors. The high local concentration in the cell membrane together with the flexibility of the joints connecting D4 and D5 to the rest of the receptor molecule enable movement and formation of homotypic D4 as well as homotypic D5 contacts that position the membrane proximal region of the receptor at a precise orientation and distance (15Å in the case of Kit) that enable interactions between neighboring cytoplasmic domains, tyrosine autophosphorylation, and stimulation of tyrosine kinase activity.

Finally, applying a chemical crosslinking agent to covalently crosslink WT or mutant receptors on unstimulated or PDGF-stimulated cells it has been demonstrated herein that an E390A PDGFRβ mutant undergoes PDGF-induced dimerization similar to PDGF-induced dimerization of WT receptors. However, by contrast to WT PDGFRβ that is expressed on the cell surface of PDGF-stimulated cells in the form of activated dimers, the E390A mutant is expressed on the surface of PDGF-stimulated cells in the form of inactive dimers. This experiment demonstrates that homotypic D4-D4 interactions are dispensable for PDGFRβ dimerization and that PDGFRβ dimerization is necessary but not sufficient for receptor activation.

**Example 26: Disruption of the D4-D4 interface overcomes oncogenic KIT activation**

Murine 3T3 cells stably expressing wild type (WT) KIT, an oncogenic KIT mutant in which Ala502 and Tyr503 of D5 were duplicated (D5-Repeat mutant), or a KIT mutant in which Ala502 and Tyr503 of D5 (D5-Repeat) were duplicated together with an additional point mutation in which Glu386 of D4 was substituted by an Ala residue (D5-Repeat/E386A mutant) were stimulated with 1, 5 or 10 ng/ml of SCF for 5 minutes at 37° C.

Lysates of unstimulated or SCF stimulated cells were subjected to immunoprecipitation with anti-KIT antibodies followed by SDS-PAGE and immunobloting with either anti-KIT or anti-phosphotyrosine (anti-pY) antibodies.

The experiment presented in Figure 27 demonstrates that SCF stimulation of wild type KIT leads to enhancement of KIT activation revealed by enhanced tyrosine autophosphorylation of KIT in response to SCF stimulation. The experiment also shows that an oncogenic D5-Repeat mutant of KIT is constitutively tyrosine autophosphorylated (*i.e.*, it is activated in the absence of SCF stimulation). By contrast, the D5-Repeat/E386A mutant which carries an additional point mutation in D4 (which was shown to impair SCF activation of KIT in a background of normal receptor protein) blocks constitutive tyrosine autophosphorylation of KIT mediated by the oncogenic D5-repeat mutation.

This experiment provides a genetic validation for the importance of D4-D4 homotypic interactions in mediating KIT activation by an oncogenic mutation in D5 and presumably by other oncogenic mutations in different parts of theKIT molecule. Furthermore, this experiment provides further validation to the notion that disruption of the D4-D4 interface by pharmacological intervention by a moiety of the invention, e.g., an antibody, or antigen binding portion thereof, a small molecule or a peptidic molecule, will block the activity of oncogenic mutations in D5, oncogenic mutations in other parts of KIT molecule and in oncogenic type-III and type-IV RTKs.

**Example 27: Antibodies directed against a synthetic peptide corresponding to the signature motif of KIT, involved in mediating D4 homotypic interactions, recognize intact KIT protein**

In this example, rabbit polyclonal antibodies were raised against three different KIT antigens:

1. The full-length extracellular domain of human KIT (amino acids 1-510).
2. KIT Ig-like domain 4 (D4) composed of amino acids 308- 411 (KIT-D4)
3. A 17-mer peptide corresponding to amino acids 375-391 including the signature motif of KIT D4 (SELHLTRLKGTEGGTYT) conjugated to KLH.

Rabbits were immunized in two week intervals with each of the three antigens, and test bleeds were analyzed. The results presented are from a serum sample that was collected after the third immunization.

Lysates of 3T3 cells expressing wild type human KIT were incubated with 30µls of serum containing one of the following antibodies: 1. Anti-KIT, directed against the full-length KIT extracellular domain. 2.Anti-D4, directed against KIT-D4 and 3.Anti-peptide, directed against a peptide corresponding to amino acids 375-391 of KIT D4. Lysates of 3T3 cells expressing wild type KIT, with each of the antibodies, were incubated together with protein A Sepharose for 2 hours at 4°C and then washed three times with washing buffer containing 20mM Hepes, 150mM NaCl, 0.1% TritonX-100 and 5% glycerol. Immunoprecipitates were separated on SDS-PAGE, transferred to nitrocellulose and immunobloted with each of the antibodies as described in Figure 28. The data presented in Figure 28 show that each of the antibodies, including the anti-peptide antibodies directed against the homotypic interaction region of D4 that is essential for positioning KIT dimers in its activated configuration, recognize intact native KIT in the immunoprecipitation and the immunobloting steps of the experiment.

Remarkably, this experiment shows that the anti-peptide antibodies recognize wild type KIT as efficiently as antibodies directed against the intact extracellular or the D4 regions of KIT.

**Example 28: Direct contacts between extracellular membrane proximal domains are required for VEGF-receptor activation and cell signaling**

Structural analyses of the extracellular region of KIT in complex with SCF revealed a sequence motif in the EF-loop of the 4$^{th}$ Ig-like domain (D4) that is responsible for forming homotypic receptor contacts and for ligand induced KIT activation and cell signaling. An identical motif was identified in the most membrane proximal 7$^{th}$ Ig-like domain (D7) of VEGFR1, 2 and 3. This example demonstrates that ligand induced tyrosine autophosphorylation and cell signaling via VEGFR1 or VEGFR2 harboring mutations in critical residues (Arg726 or Asp731) in D7 are strongly impaired. The crystal structure of D7 of VEGFR2 is also described to a resolution of 2.7Å. The structure shows that homotypic D7 contacts are mediated by salt bridges and

van der-Waals contacts formed between Arg726 of one protomer and Asp731 of the other protomer. The structure of D7 dimer is very similar to the structure of D4 dimers seen in the crystal structure of KIT extracellular region in complex with SCF. The positions of the EF loop and the salt bridges in the two structures are nearly identical and the distance between their C-termini is approximately 15 Å in both structures. The high similarity between VEGFR D7 and KIT D4 in both structure and function provides further evidence for common ancestral origins of type III and type V RTKs. It also reveals a conserved mechanism for RTK activation and a novel target for pharmacological intervention of pathologically activated RTKs.

Vascular endothelial growth factors (VEGF) regulate blood and lymphatic vessel development and homeostasis by binding to and activating the three members of the VEGF-receptor (VEGFR) family of receptor tyrosine kinases (RTK) (Olsson *et al., Nat. Rev. Mol. Cell. Biol.*, 7(5):359-371 (2006)). VEGFR1 (*Flt1*), VEGFR2 (KDR/*Flk1*) and VEGFR3 (Flt4) are members of type-V RTK; a family containing a large extracellular region composed of seven Ig-like domains (D1-D7), a single transmembrane (TM) helix and cytoplasmic region with a tyrosine kinase activity and additional regulatory sequences. The second and third Ig-like domains, D2 and D3 of VEGFR ectodomains function as binding sites for the five members of the VEGF family of cytokines (*i.e.* VEGF-A, B, C, D and placenta growth factor (PlGF)) (Barleon *et al., J. Biol. Chem.*, 272(16):10382-10388 (1997); and Shinkai *et al., J. Biol. Chem.*, 273(47):31283-31288 (1998)). These growth factors are covalently linked homodimers. Each protomer is composed of four stranded β-sheets arranged in an anti-parallel fashion in a structure designated cysteine-knot growth factors (Weismann *et al., Cell*, 91(5):695-704 (1997)). Other members of the cysteine-knot family of cytokines include nerve growth factor (NGF) and platelet derived growth factors (PDGF). However, the ectodomains of the PDGFR family of RTKs (type-III) are composed of five Ig-like repeats of which D1, D2, and D3 function as ligand binding region of PDGFR and other members of the family (*i.e.,* KIT, CSF1R, and *Flt3*). Structural and biochemical experiments have shown that SCF binding to the extracellular region induces KIT dimerization, a step followed by homotypic contacts between the two membrane proximal Ig-like domains D4 and D5 of neighboring KIT molecules (Yuzawa *et al., Cell*, 130(2):323-334(2007)). Biochemical studies of wild type and oncogenic KIT mutants have shown that the homotypic D4 and D5 contacts play a critical role in positioning the cytoplasmic regions

of KIT dimers at a distance and orientation that facilitate trans-autophosphorylation, kinase activation and cell signaling.

In this example, structural and biochemical evidence demonstrates that homotypic contacts between the most membrane-proximal Ig-like domain of the ectodomain (D7) of VEGF-receptors plays a critical role in VEGF induced activation and cell signaling via VEGF-receptors.

***Sequence analysis of VEGFR2 D4 and D7***

An evolutionarily conserved sequence motif (**L/I**x**RΦ**xxx**D/E**x**G**) responsible for mediating homotypic contacts between Ig-like domains was identified by structure based sequence alignment of D4 of KIT (Yuzawa *et al., Cell*, 130(2):323-334(2007)). A similar motif was found in D4 of PDGFRα, PDGFRβ, and CSF1R as well as in the most membrane proximal Ig-like domain (D7) of VEGFR1, VEGFR2 and VEGFR3 (Figure 29). The **L/I**x**RΦ**xxx**D/E**x**G** motif is located at the loop region linking βE and βF strands of D7; a region shown to be responsible for mediating salt bridges required for homotypic D4 KIT contacts. The Arg and Asp are evolutionarily conserved from sea urchin to human in both VEGFR1 and VEGFR2 (Figure 29), indicating functional importance of these residues in VEGFR activity. However, in contrast to D4 of KIT and PDGFR, D7 of VEGFR1 and VEGFR2 contain two conserved cysteine residues at positions B5 and F5 that form a disulfide bond between the β strands, an interaction contributing to the hydrophobic coreof I-set Ig-like domains (Harpaz and Chothia, *J. Mol. Biol.*, 238(4):528-539(1994)).

Similar to D4 of PDGFR and KIT, D4 of VEGFR2 lacks the conserved cysteines responsible for disulfide bond formation between β strands at position B5 and F5. In D4 of VEGFR2, the region connecting βC with βE is shorter compared to other typical I-set Ig domains, possibly because this region lacks one of the β–strands. Amino acid sequence analysis showed that VEGFR2 D4 is homologous to myomesin domain D13 (2R15) and telokin (1TLK) with sequence identity of 30% and 33%, respectively. Manual sequence alignment revealed 20% sequence identity between D4 of VEGFR2 and D4 of PDGFRα. Both D4 of KIT and D4 of PDGFR contain a conserved "D/E-x-G" amino acids around the "Y-corner motif" in βF consisting **D/E**-x-**G/A/D**-x-**Y**-x-**C** motif (Hemmingsen *et al., Protein Sci.*, 3(11):1927-1937 (1994)) (in D4 the Cys residue is replaced by hydrophobic amino acids). In D4 a salt bridge is formed between a Glu

residue on one molecule with an Arg residue at the -5 position of a second KIT molecule (Figure 29B). An Asp residue is found in D4 of VEGFR2, but instead of an Arg at the -5 position this Ig-like domain contains a pair of amino acids with opposite charges at the -2 and -6 positions relative to the Asp residue (Figure 29B). While direct contacts between D4 have been observed in electron microscopy (EM) images of VEGF-A induced dimers of the ectodomain of VEGFR2 (Ruch *et al., Nat. Struct. Mol. Biol.,* 14(3):249-250 (2007)), the function of VEGFR2 D4 remains unclear. D7 is thus more similar than D4 of VEGFR2 in the EF loop region to corresponding sequences in D4 of KIT and PDGFR.

***Homotypic D7 contacts are essential for ligand induced VEGFR2 activation***

This sequence analysis and comparison with KIT suggests that residues R726 and D731 of VEGFR2 can mediate inter-receptor salt bridge formation and may alter response to ligand. To investigate the role of the conserved residues in D7 region in ligand induced VEGFR2 activation and signal transduction, VEGFR2 mutants were generated in which Arg726, Asp731 or both amino acids were replaced by Ala residues (R726A, D731A and RD2A). HEK293 cells were transiently transfected with pCDNA3 expression vectors that direct the expression of WT VEGFR2 or VEGFR2 harboring D7 mutations. After incubation for 24 hours the transfected cells were starved overnight prior to VEGF-A stimulation. Tyrosine autophosphorylation of VEGFR2 and MAPK response of unstimulated or VEGF-A stimulated or unstimulated cells were analyzed using anti-phosphotyrosine antibodies (anti-pTyr) or anti-phosho-MAPK antibodies, respectively. Figure 30A shows that mutations of the Arg or Glu residues predicted to be involved in mediating inter-receptor salt bridge formation markedly reduced VEGF-A induced VEGFR2 autophosphorylation and MAPK stimulation.

To overcome the relatively weak kinase activity of VEGFR2, a chimeric receptor approach was employed (Fambrough *et al., Cell,* 97(6):727-741 (1999) and Meyer *et al., J. Biol. Chem.,* 281(2):867-875 (2006)). A chimeric receptor composed of the ectodomain of VEGFR2 (amino acid 1-761) connected to the transmembrane and the cytoplasmic region of the PDGFR (amino acid 528-1106) was prepared and used to further explore the role played by D7 in VEGF-A induced VEGFR2 activation. Lysates from VEGF-A stimulated or unstimulated NIH-3T3 cells stably expressing a chimeric VEGFR2/PDGFR or chimeric VEGFR2/PDGFR harboring D7 mutations were subjected to immunoprecipitation with anti-PDGFR antibodies followed by

immunoblotting with anti-pTyr antibodies. Figure 30B demonstrates robust tyrosine autophosphorylation of the chimeric VEGFR2/PDGFR in response to VEGF-A stimulation (Figure 30B, WT). In contrast, VEGF-A induced tyrosine autophosphorylation of chimeric receptor harboring the D7 mutations (R726A, D731A, RD2A) was strongly compromised (Figure 30B). A chimeric receptor composed of the extracellular region of VEGFR1 fused to the TM and intracellular region of PDGFR was also generated. 3T3 cells stably expressing wild type chimeric receptors showed autophosphorylation in response to ligand stimulation (Figure 30C, WT). By contrast, ligand induced stimulation of kinase activity was strongly compromised in 3T3 cells expressing chimeric receptor, harboring mutations in Arg720, Asp725 or in both amino acids, R720A, D725A and RD2A, respectively (Figure 30C). The foregoing data demonstrate that homotypic contacts between the membrane proximal Ig-like domains of type-III and type-V RTKs are essential for ligand induced receptor activation and cell signaling.

A covalent cross linking agent was utilized to explore the effect of D7 mutations on ligand induced receptor dimerization by cross linking ligand stimulated cells followed by SDS-PAGE analysis of lysates from ligand stimulated or unstimulated cells (see, *e.g.,* Cochet *et al., J. Biol. Chem.,* 263(7):3290-3295 (1988)). This experiment demonstrated that VEGF-A induced dimerization of the chimeric receptors was not affected by the D7 mutations (data not shown). Similar to previously reports for PDGFR and KIT (Yuzawa *et al., Cell,* 130(2):323-334 (2007) and Yang *et al., Proc. Nat'l. Acad. Sci. U.S.A.,* 105(220:7681-7686 (2008)), D7 mediated homotypic contacts are necessary for receptor activation but dispensable for receptor dimerization. Moreover, ligand induced receptor dimerization is necessary but not sufficient for tyrosine autophosphorylation and receptor activation. By contrast, VEGF-A induced tyrosine autophosphorylation of chimeric receptor harboring mutations in D4 of VEGFR2 including a D392A mutation or mutations in which both Asp387 and Arg391 were substituted by Ala residues (DR2A) remained unchanged (Figure 30D) demonstrating that a different interface might be involved in mediating D4 interactions seen in EM images of VEGF-A induced VEGFR2 ectodomain dimmers (Ruch *et al., Nat. Struct. Mol. Biol.,* 14(3):249-250 (2007)).

Analytical centrifugation was utilized to determine the dissociation constant for dimerization of isolated D7 region. Analytical centrifugation experiments performed

using $4x10^{-5}$, $8x10^{-5}$ and $1.6x10^{-4}$ M protein concentrations showed that isolated D7 remained monomeric in solution at a concentration as high as $10^{-4}$ M indicating that the dissociation constant of D7 dimerization exceeds $10^{-4}$ M. A similar high dissociation constant was found for dimerization of isolated D4 or D5 of KIT or PDGFR. It has previously been shown that, following SCF or PDGF induced dimerization, the local concentration of two neighboring KIT or PDGFR protomers in the cell membrane is in the range of 4 -6 $x10^{-4}$ M. This together with the reduced dimensionality enables efficient lateral interactions and formation of stable homotypic contacts between pairs of Ig-like domains which bind to each other with low affinity in the cell membrane. Moreover, the homotypic contacts between membrane proximal Ig-like domain in type-III and type-V RTKs are supported by additional lateral interactions that take place between the TM and cytoplasmic regions of neighboring receptors in a cooperative manner.

***Structure of VEGFR extracellular domain D7***

In order to determine the molecular basis underlying the role played by D7 in ligand induced VEGFR2 activations the crystal structure of this Ig-like domain was determined. Crystals were obtained in space group $I2_12_12_1$, with a single D7 molecule per asymmetric unit together with 28 water molecules. D7 structure consists of amino acids 667 to 756 of VEGFR2 and diffracts X-rays to 2.7 Å resolution. The structure was determined by molecular replacement with model based on the structure of telokin (PDB code: 1TLK) (Holden *et al., J. Mol. Biol.,* 227(3):840-851 (1992)). The two copies of D7 in the complex are very similar to each other with r.m.s. deviation of 0.1 Å. D7 assumes a typical IgSF fold that consists of a β-sandwich formed by two four-stranded sheets, one comprising of strands A, B, D and E, and the second comprising of strands A', G, F and C. The first half of the A strand forms a hydrogen bond with the B strand and the A' strand forms hydrogen bonds with G strand, similar to the structure of Ig-like domain Ig1 and Ig2 from the extracellular region at receptor tyrosine kinase MuSK (Stiegler *et al., J. Mol. Biol.,* 364(3):424-433 (2006)). The crossover connection between strand βE and βF includes a single helical turn at residues 729-731. D7 of VEGFR2 displays several characteristics of the IgSF fold including a conserved disulfide bond between Cys688 of βB and Cys737 of βF, and a signature tryptophan residue that packs against disulfide bond to form the hydrophobic core. Structural comparison using DALI (Holm *et al., Curr. Protoc. Bioinformatics,* Chapter 5, Unit 5 5

(2006)) shows that among the Ig-like domains of VEGFR2, D7 is most similar to telokin (PDB code: 1TLK) (Holden *et al., J. Mol. Biol.,* 227(3):840-851 (1992)), with a Z-score of 13.4 and an r.m.s.d. of 1.5 Å for the 89 aligned Cβ residues. D7 contains 16 of the 20 key positions in the V-frame profile that defines the I-set (Harpaz and Chothia, *J. Mol. Biol.*, 238(4):528-539(1994)). An additional exposed cross-strand disulfide bond is formed by a pair of Cys located in the βF (Cys740) and βG (Cys745). This feature is highly conserved in VEGFR2 and VEGFR3, but not in VEGFR1.

The crystal structure demonstrates that homotypic D7 contacts are mediated by two β sheets formed by the ABED strands of D7 of each protomer in which Arg726 of one protomer points toward Asp731 of the other resulting in a buried surface area of approximately 360 $A^2$. Figure 31B shows that Arg726 and Asp731 form salt bridges and van der Waals contacts. The structure of D7 dimer is very similar to the homotypic D4 contacts seen in KIT extracellular dimer structure (PDB code: 2E9W) (Yuzawa *et al., Cell,* 130(2):323-334 (2007)). In addition D7 of VEGFR2 exhibits strong polarization of electrostatic field with an overall negatively charged surface with the exception of a positively charged center strip right along the D7-D7 interface (Figure 31C). The strongly charged interface may prevent aberrant association of monomeric receptor molecules prior to ligand stimulation.

Comparison of the structure of D4 of KIT to the structure of D7 of VEGFR2 using DALI (Holm *et al., Curr. Protoc. Bioinformatics,* Chapter 5, Unit 5 5 (2006)) showed a remarkable similarity with a Z-score of 10.4 and an r.m.s.d. of 1.8 Å for the 83 aligned Cα residues. The position of the EF loop in the two structures is nearly identical and the distance between the C-termini is approximately 15 Å for both D4 and D7 dimeric structures (Figure 33). The high similarity between VEGFR D7 and KIT D4 in both structure and function suggests a well conserved mechanism for RTK activation, and provides further evidence for common ancestral origins of type III and type V RTKs. Interestingly the Drosophila (Cho *et al., Cell,* 108(6):865-876 (2002)), C. elegans (Plowman *et al., Proc. Nat'l. Acad. Sci. U.S.A.,* 96(24):13603-13610 (1999)), sea squirt (Satou *et al., Dev. Genes Evol.,* 213(5-6):254-263 (2003)) and sea urchin (Duloquin *et al., Development,* 134(12):2293-2302 (2007)) genomes contain a single family of VEGFR/PDGFR like RTK which contains seven Ig-like domains in its extracellular region. Type-III and type-V RTK genes were functionally segregated in vertebrates but are located adjacent to each other on the chromosomes (Shibuya, *Biol.*

*Chem.*, 383(10):1573-1579 (2002) and Grassot *et al., Mol. Biol. Evol.*, 23(6):1232-1241 (2006)). In human, the genes for class III and class V RTK are found in three clusters on chromosomes 4q12 (KIT, PDGFRα and VEGFR2), 5q33 (FMS, PDGFRβ and VEGFR3) and 13q12 (FLT3 and VEGFR1). Phylogeny of class III and class V RTKs suggests that these 8 RTKs were generated by 2 rounds of cis duplication and 2 rounds of trans duplication (Grassot *et al., Mol. Biol. Evol.*, 23(6):1232-1241 (2006)). The highly conserved motif in EF-loop region is also identified in D7 of VER3 and VER4; two VEGFR/PDGFR like receptor genes of *C. elegans*. A similar motif was found in D7 of VEGFR/PDGFR like receptor of sea urchin, but not in a VEGFR/PDGFR like receptor of *Drosophila*. Interestingly, three of the ten Ig-like domains of the VEGFR/PDGFR like receptor of sea squirt contain typical EF-loop motifs. Homotypic contacts between D3, D6 and D9 of the VEGFR/PDGFR like sea squirt receptor may be required for ligand induced activation of an RTK containing 10 Ig-like domains in its extracellular region.

The experiments presented in this example demonstrate that type-III and type-V RTK are activated by a common mechanism in which homotypic contacts mediated by membrane proximal Ig-like domains ensure that the TM and cytoplasmic regions of two receptor monomers are brought to a close proximity and correct orientation to enable efficient trans-autophosphorylation, kinase activation and cell signaling. The combination of ligand induced receptor dimerization together with multiple low affinity homotypic associations between membrane proximal Ig-like domains provide a simple but efficient mechanism for ligand induced transmembrane signaling. Moreover, the low binding affinity of individual Ig-like domains towards each other prevents accidental receptor activation of receptor monomers prior to ligand engagement. The homotypic contact regions provide ideal targets for pharmacological intervention of pathological RTK activation and cell signaling.

***Contiguous Epitopes of the D7 Regions of VEGFR1, VEGFR2 and VEGFR3***

A structure-based sequence alignment was performed. This alignment revealed potential contiguous epitopes on VEGFR1, VEGFR2 and VEGFR3 D7 regions which may be recognized by moieties of the invention (Table 8). The epitopes are located in strand B, D, E, the A'B loop, the CD loop, the DE loop and the EF loop. These epitopes are located in the interface mediating homotypic D7 contacts.

**Table 8**: Contiguous Epitopes on VEGFR1, VEGFR2 and VEGFR3 D7 Regions

| | VEGFR1 amino acid sequence | VEGFR2 amino acid sequence | VEGFR3 amino acid sequence |
|---|---|---|---|
| **A'B loop** | $^{672}$VAISSS$^{677}$ | $^{678}$TSIGES$^{683}$ | $^{689}$VNVSDS$^{694}$ |
| **B strand** | $^{678}$TTLDCHA$^{684}$ | $^{684}$IEVSCTA$^{690}$ | $^{695}$LEMQCLV$^{701}$ |
| **BC loop** | $^{685}$NGVPEPQ$^{691}$ | $^{691}$SGNPPPQ$^{697}$ | $^{702}$AGAHAPS$^{708}$ |
| **CD loop** | $^{700}$KIQQEPG$^{706}$ | $^{706}$TLVEDSG$^{712}$ | $^{717}$LLEEKSG$^{723}$ |
| **D strand** | $^{707}$IILG$^{710}$ | $^{713}$IVLK$^{716}$ | $^{724}$VDLA$^{727}$ |
| **DE loop** | $^{711}$PGS$^{713}$ | $^{717}$DGN$^{719}$ | $^{728}$DSN$^{730}$ |
| **E strand** | $^{714}$STLFI$^{718}$ | $^{720}$RNLTI$^{724}$ | $^{731}$QKLSI$^{735}$ |
| **EF loop** | $^{719}$ERVTEEDEGV$^{728}$ | $^{725}$RRVRKEDEGL$^{734}$ | $^{736}$QRVREEDAGR$^{745}$ |

***Materials and Methods for Example 28***

**1. Protein expression, purification and crystallization**

D7 of VEGFR2 (amino acid 657-765) containing an N-terminal 6xHis-tag was expressed in *E. Coli* using PET28a vector. Inclusion bodies were collected and solubilized in 6M guanidine hydrochloride (pH8.0). D7 was refolded by drop-wise dilution of the protein into refolding buffer containing 10mM Tris (pH8.0), 2mM reduced glutathione and 0.2mM oxidized glutathione with final protein concentration at 80-100µg/ml. The refolding was carried out at 4°C for 48hrs with stirring. The refolding solution was cleared by filtration using 0.45µm filter unit and purified by FastQ sepharose column followed by size exclusion (S200, GE Healthcare) and anion exchange chromatography (Mono Q, GE Healthcare). N-terminal 6xHis tag was removed by thrombin digestion. D7 protein was concentrated to 15mg/ml in buffer containing 25mM Tris (pH 8.0) and 200mM NaCl; and was subjected to extensive screening for crystallization and optimization (Hampton research, crystal screening). Crystals of ectodomain D7 of approximate dimensions of 300 x 75 x 20 µM were grown in 0.2M succinic acid and 16% PEG3350 at 4°C. All crystals were immersed in a reservoir solution supplemented with 5-18% glycerol for several seconds, flash cooled and kept in a stream of nitrogen gas at 100K during data collection. The crystals belonged to the $I2_12_12_1$space group with unit cell dimersions of $a$=39.476Å, $b$=76.991Å, and $c$=102.034 Å with one molecule per asymmetric unit. Diffraction data was collected to a resolution of 2.7Å with an ADSD quantum-210 CCD detector at the X29A beamline of NSLS, Brookhaven National Laboratory. All data sets were processed and scaled

using the HKL2000 program package (Otwinowski and Minor, *Methods in Enzymology,* 276(part A):307-326 (1997)). The data collection statistics are summarized in Table 7. The structure was solved by molecular replacement with Phaser.using models based on the structures of Ig domains from MUSK (2IEP) (Stiegler *et al., J. Mol. Biol.,* 364(3):424-433 (2006)), telokin (1TLK) (Holden *et al., J. Mol. Biol.,* 227(3):840-851 (1992)) and D4 of KIT (2E9W) (Yuzawa *et al., Cell,* 130(2):323-344 (2007)) as search models. The structure was refined to 2.7Å resolution with a crystallographic R-factor of 22.7% and free R-factor of 27.7% (Table 7). The atomic coordinates of VEGFR2 D7 were deposited in Protein Data Bank with accession code XXX. Molecular images were produced using Pymol and CCP4MG software (Potterton *et al., Acta Crystallogr. D. Biol. Crystallogr.,* 60 (Pt. 12 Pt. 1):2288-2294 (2004)).

**Table 7: Data Collection and Refinement Statistics for VEGFR-D7**

| | |
|---|---|
| Space group | $I2_12_12_1$ |
| Unit cell dimensions | |
| a (Å) | 39.476 |
| b (Å) | 76.991 |
| c (Å) | 102.034 |
| Resolution (Å) | 50-2.7 |
| Unique reflections | 4561 |
| Completeness (%)[a] | 99.8 (97.8) |
| $R_{sym}$ (%)[ab] | 4.3 (7.5) |
| Redundancy | 13.4 (12.9) |
| | |
| Refinement | |
| $R_{work}$ (%)[c] | 22.7 |
| $R_{free}$ (%)[d] | 27.7 |
| Protein residues | 89 |
| Water molecules | 27 |
| Average B factors (Å$^2$) | 37.39 |
| RMS deviations | |
| Bond lengths (Å) | 0.007 |
| Bond angles (degree) | 1.2 |
| Ramachandran plot statistics | |
| Core (%) | 91.2 |
| Allowed (%) | 8.8 |
| Generous (%) | 0 |

[a] Values in parentheses are statistics of the highest resolution shell for SEB (2.8-2.7Å).

[b] $R_{merge} = \Sigma\, Ij - \langle I \rangle / \Sigma Ij$, where $Ij$ is the intensity of an individual reflection and is the average intensity of the reflection.

[c] $R_{work} = \Sigma\, ||F_{o}| - \langle |F_c| \rangle / \Sigma\, |F_o|$, where $F_c$ is the calculated structure factor.

[d] $R_{free}$ is as $R_{work}$ but calculated for a randomly selected 10% of the reflection not included in the refinement.

**2. Amino acid sequence alignment**

Amino acid sequence alignment of D7 was performed using ClustalW (Thompson *et al., Nucleic Acids Res.,* 22(220:4673-4680 (1994)) and then manually adjusted based on the I-set IgSF fold restrains for 20 key residues. Amino acid sequences of human VEGFRs were used as query to search the non-redundant database (nr) for homologous sequences, using PSI-BLAST (Altschul *et al., J. Mol. Biol.,* 215(3):403-410 (1990)). The alignment of amino acid sequences as well as D7 PDB file were submitted to the Consurf 3.0 server (Landau *et al., Nucleic Acids Res.,* 33 (Web Server Issue), W299-302 (2005)) to generate maximum-likelihood normalized evolutionary rates for each position of the alignment where low rates of divergence correspond to high sequence conservation. As with the Consurf output, the continuous 9 conservation scores are partitioned into a discrete scale of 9 bins for visualization, such that bin 9 contains the most conserved (maroon) positions and bin 1 contains the most variable (cyan) positions.

**3. VEGFR expression vectors and generation of chimeric receptors**

cDNA of human VEGFR1 and VEGFR2 were kindly provided by Dr. Masabumi Shibuya (Sawano *et al., Blood,* 97(3):785-791 (2001)). VEGFR2 cDNA was subcloned into pcDAN3 expression vector by PCR and inserted into XhoI/XbaI sites. Chimeric receptors composed of the extracellular regions of either VEGFR1 or VEGFR2 were fused to the transmembrane and cytoplasmic region of PDGFR-β. A flag-tag was added to the C-terminus and the chimeric receptor was cloned into EcoRI/ XhoI sites of pLXSHD retroviral expression vector.

**4. Cell lines and expression vectors**

3T3 cell lines stably expressing the VEGFR1/2-PDGFR chimeric receptor were generated by retroviral infection as previously described (Yuzawa *et al.,* 2007 and Cochet *et al.,* 1988). Cells were selected with L-histidinol and pools matched for similar expression level were used in the experiments.

HEK293 cells were transiently transfected with 1 μg of DNA and serum starved overnight prior to VEGF stimulation. Cells were treated with 200ng/ml VEGF and cell lysates were immuoprecipitated with antibodies against VEGFR1 or VEGFR2 followed by immunobloting with anti-pTyr antibodies (PY20, Santa Cruz). Total cell lysates were analysed by SDS-PAGE and subjected to immunobloting with anti- phosphoMAPK, and anti- MAPK antibodies (Cell Signaling) respectively.

VEGF was produced in sf9 cells using baculovirus expression vector pFastBac1 as previously described (Cohen *et al., Growth Factors,* 7(2):131-138 (1992)). VEGF was purified using heparin sepharose beads to >80% purity by Commassie blue stained SDS PAGE experiments.

**5. Analytical ultracentrifugation**

Sedimentation velocity experiments were performed with a Beckman Optima XL-I at the Center for Analytical Ultracentrifugation of Macromolecular Assemblies (Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX). D7 protein at concentration of $4x10^{-5}$ M, $8x10^{-5}$ M, and $1.6x10^{-4}$ M in buffer containing 25 mM Tris, pH 8 and 100 mM NaCl were subjected to centrifugation at 50,000 rpm at 20 °C. Velocity data were analyzed with 2-dimesional spectrum analysis combine with Monte Carlo analysis.

**Equivalents**

Those skilled in the art will recognize, or be able to ascertain using no more that routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

# CLAIMS

We Claim:

1. A moiety that binds to the ectodomain of a human vascular endothelial growth factor receptor (VEGF receptor), wherein said moiety antagonizes the activity of the VEGF receptor.

2. The moiety of claim 1, wherein said moiety binds to an Ig-like domain of a human VEGF receptor.

3. The moiety of claim 2, wherein said Ig-like domain is not responsible for the binding of a ligand to the VEGF receptor.

4. The moiety of claim 2, wherein said Ig-like domain is responsible for the binding of a ligand to the VEGF receptor.

5. The moiety of claim 1, wherein said moiety does not block the interaction between the VEGF receptor and a ligand for the VEGF receptor.

6. The moiety of claim 1, wherein said moiety blocks the interaction between the VEGF receptor and a ligand for the VEGF receptor.

7. The moiety of claim 1, wherein said moiety does not prevent dimerization of the VEGF receptor.

8. The moiety of claim 1, wherein said moiety prevents dimerization of the VEGF receptor.

9. The moiety of claim 1, wherein said moiety prevents the interaction between a membrane proximal region of the ectodomain from each protomer of the VEGF receptor.

10. The moiety of claim 9, wherein said interaction is homotypic.

11. The moiety of claim 9, wherein said interaction is heterotypic.

12. The moiety of claim 9, wherein said membrane proximal region of the ectodomain is the 7th Ig-like domain (D7) of a VEGF receptor.

13. The moiety of claim 12, wherein said moiety binds to the following consensus sequence for the D7 domain of a VEGF receptor:
L/I $X_1$ R Φ $X_2$ $X_3$ $X_4$ D/E $X_5$ G (SEQ ID NO:158), wherein L is Leucine, I is Isoleucine, R is Arginine, Φ is a hydrophobic amino acid, D is Aspartic Acid, E is Glutamic Acid, G is Glycine; and $X_1$, $X_2$, $X_3$, $X_4$ and $X_5$ are any amino acid.

14. The moiety of claim 13, wherein Φ is Valine; $X_1$ is selected from the group consisting of Arginine, Glutamine, Glutamic Acid and Aspartic Acid; $X_2$ is selected from the group consisting of Arginine, Lysine and Threonine; $X_3$ is selected from the group consisting of Lysine, Glutamic Acid, Glutamine and Valine; $X_4$ is selected from the group consisting of Glutamic Acid and Valine; and $X_5$ is selected from the group consisting of Glutamic Acid, Glycine, Serine and Glutamine (SEQ ID NO:159).

15. The moiety of claim 9, wherein the moiety causes the membrane proximal region of the ectodomain from each protomer of the VEGF receptor to be separated by a distance greater than 16 Å.

16. The moiety of claim 1, wherein said VEGF receptor is VEGFR1.

17. The moiety of claim 1, wherein said VEGF receptor is VEGFR2.

18. The moiety of claim 1, wherein said VEGF receptor is VEGFR3.

19. The moiety of claim 1, wherein the moiety locks the ectodomain of the VEGF receptor in an inactive state.

20. The moiety of claim 1, wherein said moiety binds to amino acid residue Arg726 of VEGFR2.

21. The moiety of claim 1, wherein said moiety binds to amino acid residue Asp731 of VEGFR2.

22. The moiety of claim 1, wherein said moiety binds to amino acid residues Arg726 and Asp731 of VEGFR2.

23. The moiety of claim 1, wherein said moiety binds to one or more amino acid residues selected from the group consisting of amino acid residues 724, 725, 726, 727, 728, 729, 730, 731, 732 and 733 of VEGFR2.

24. The moiety of claim 1, wherein said moiety binds to amino acid residue Arg720 of VEGFR1.

25. The moiety of claim 1, wherein said moiety binds to amino acid residue Asp725 of VEGFR1.

26. The moiety of claim 1, wherein said moiety binds to amino acid residues Arg720 and Asp725 of VEGFR1.

27. The moiety of claim 1, wherein said moiety binds to one or more amino acid residues selected from the group consisting of amino acid residues 718, 719, 720, 721, 722, 723, 724, 725, 726 and 727 of VEGFR1.

28. The moiety of claim 1, wherein said moiety binds to amino acid residue Arg737 of VEGFR3.

29. The moiety of claim 1, wherein said moiety binds to amino acid residue Asp742 of VEGFR3.

30. The moiety of claim 1, wherein said moiety binds to amino acid residues Arg737 and Asp742 of VEGFR3.

31. The moiety of claim 1, wherein said moiety binds to one or more amino acid residues selected from the group consisting of amino acid residues 735, 736, 737, 738, 739, 740, 741, 742, 743 and 744 of VEGFR3.

32. The moiety of claim 1, wherein the moiety binds to a conformational epitope on the VEGF receptor.

33. The moiety of claim 32, wherein said conformational epitope is composed of two or more residues in the D7 domain of the VEGF receptor.

34. The moiety of claim 32, wherein said conformational epitope comprises amino acid residues Arg726 and Asp731; Arg 720 and Asp 725; or Arg737 and Asp742.

35. The moiety of claim 1, wherein said moiety blocks a ligand induced tyrosine autophosphorylation of the VEGF receptor.

36. The moiety of claim 1, wherein said moiety blocks a ligand induced internalization of the VEGF receptor.

37. The moiety of claim 1, wherein the moiety binds to a contiguous epitope on the VEGF receptor.

38. The moiety of claim 37, wherein said contiguous epitope is composed of two or more residues in the D7 domain of the VEGF receptor.

39. The moiety of claim 38, wherein said contiguous epitope is an epitope selected from the group consisting of $^{672}$VAISSS$^{677}$ of VEGFR1, $^{678}$TTLDCHA$^{684}$ of VEGFR1, $^{685}$NGVPEPQ$^{691}$ of VEGFR1, $^{700}$KIQQEPG$^{706}$ of VEGFR1, $^{707}$IILG$^{710}$ of VEGFR1, $^{711}$PGS$^{713}$ of VEGFR1, $^{714}$STLFI$^{718}$ of VEGFR1, $^{719}$ERVTEEDEGV$^{728}$ of VEGFR1, $^{689}$VNVSDS$^{694}$ of VEGFR3, $^{695}$LEMQCLV$^{701}$ of VEGFR3, $^{702}$AGAHAPS$^{708}$ of

VEGFR3, $^{717}$LLEEKSG$^{723}$ of VEGFR3, $^{724}$VDLA$^{727}$ of VEGFR3, $^{728}$DSN$^{730}$ of VEGFR3, $^{731}$QKLSI$^{735}$ of VEGFR3, and $^{736}$QRVREEDAGR$^{745}$ of VEGFR3, $^{678}$TSIGES$^{683}$ of VEGFR2, $^{684}$IEVSCTA$^{690}$ of VEGFR2, $^{691}$SGNPPPQ$^{697}$ of VEGFR2, $^{706}$TLVEDSG$^{712}$ of VEGFR2, $^{713}$IVLK$^{716}$ of VEGFR2, $^{717}$DGN$^{719}$ of VEGFR2, $^{720}$RNLTI$^{724}$ of VEGFR2 and $^{725}$RRVRKEDEGL$^{734}$ of VEGFR2.

40. The moiety of claim 1, wherein said moiety is an isolated antibody, or an antigen-binding portion thereof.

41. The moiety of claim 40, wherein said antibody or antigen-binding portion thereof, is selected from the group consisting of a human antibody, a humanized antibody, a bispecific antibody, and a chimeric antibody.

42. The moiety of claim 41, wherein said antibody, or antigen-binding portion thereof, comprises a heavy chain constant region selected from the group consisting of IgG1, IgG2, IgG3, IgG4, IgM, IgA and IgE constant regions.

43. The moiety of claim 42, wherein the antibody heavy chain constant region is IgG1.

44. The moiety of claim 40, wherein said antibody, or antigen-binding portion thereof, is selected from the group consisting of a Fab fragment, a F(ab')2 fragment, a single chain Fv fragment, an SMIP, an affibody, an avimer, a nanobody, and a single domain antibody.

45. The moiety of claim 40, wherein said antibody, or antigen-binding portion thereof, binds to an Ig-like domain of a receptor tyrosine kinase with a KD selected from the group consisting of 1 x $10^{-7}$ M or less, more preferably 5 x $10^{-8}$ M or less, more preferably 1 x $10^{-8}$ M or less, more preferably 5 x $10^{-9}$ M or less.

46. A hybridoma which produces the antibody, or antigen binding portion thereof, of any one of claim 40 to claim 45.

47. The moiety of claim 1, wherein said moiety is a small molecule.

48. The moiety of claim 47, wherein said moiety binds to at least one of the amino acid residues selected from the group consisting of amino acid residue Arg 726 of VEGFR2, Asp731 of VEGFR2, Arg720 of VEGFR1, Asp725 of VEGFR1, Arg737 of VEGFR3, and Asp742 of VEGFR3.

49. The moiety of claim 1, wherein said moiety is a peptidic molecule.

50. The moiety of claim 49, wherein said peptidic molecule is designed based on an Ig-like domain of the VEGF receptor.

51. The moiety of claim 50, wherein said peptidic molecule is designed based on the D7 domain of the human VEGF receptor.

52. The moiety of claim 51, wherein said peptidic molecule comprises the structure: L/I $X_1$ R Φ $X_2$ $X_3$ $X_4$ D/E $X_5$ G (SEQ ID NO:158), wherein L is Leucine, I is Isoleucine, R is Arginine, Φ is a hydrophobic amino acid, D is Aspartic Acid, E is Glutamic Acid, G is Glycine; and $X_1$, $X_2$, $X_3$, $X_4$ and $X_5$ are any amino acid.

53. The moiety of claim 52, wherein Φ is Valine; $X_1$ is selected from the group consisting of Arginine, Glutamine, Glutamic Acid and Aspartic Acid; $X_2$ is selected from the group consisting of Arginine, Lysine and Threonine; $X_3$ is selected from the group consisting of Lysine, Glutamic Acid, Glutamine and Valine; $X_4$ is selected from the group consisting of Glutamic Acid and Valine; and $X_5$ is selected from the group consisting of Glutamic Acid, Glycine, Serine and Glutamine (SEQ ID NO:159).

54. The moiety of claim 50, wherein said peptidic molecule comprises a structure which is at least 80% identical to amino acid residues 724-733 of human VEGFR2.

55. The moiety of claim 50, wherein said peptidic molecule comprises a structure which is at least 80% identical to amino acid residues 718-727 of human VEGFR1.

56. The moiety of claim 50, wherein said peptidic molecule comprises a structure which is at least 80% identical to amino acid residues 735-744 of human VEGFR3.

57. The moiety of claim 50, wherein said peptidic molecule comprises at least one D-amino acid residue.

58. The moiety of claim 1, wherein said moiety is an adnectin.

59. A moiety that binds to a conformational epitope on a 7$^{th}$ Ig-like domain of the human VEGF receptor antagonizes the activity of the human VEGF receptor, and wherein said conformational epitope comprises residues Arg726 and Asp731 of VEGFR2; residues Arg720 and Asp725 of VEGFR1; or residues Arg737 and Asp742 of VEGFR3.

60. A moiety that binds to amino acid residues Arg726 and Asp731 of VEGFR2; amino acid residues Arg720 and Asp725 of VEGFR1; or amino acid residues Arg737 and Asp742 of VEGFR3, thereby antagonizing the activity of human VEGF receptor.

61. A pharmaceutical composition comprising the moiety of any one of claims 1 to claim 60 and a pharmaceutically acceptable carrier.

62. Use of an effective amount of the moiety of any one of claims 1 to 60 in the preparation of a medicament for the treatment or prevention of a VEGF receptor tyrosine kinase associated disease in a subject.

63. The use of claim 62, wherein said VEGF receptor tyrosine kinase associated disease is selected from the group consisting of cancer, age-related macular degeneration (AMD), atherosclerosis, rheumatoid arthritis, diabetic retinopathy, a lymphatic disease and pain associated diseases.

64. The use of claim 63, wherein the cancer is selected from the group consisting of GIST, AML, SCLC, renal cancer, colon cancer, lymphatic cancer and breast cancer.

65. A method for identifying a moiety that binds to an Ig-like domain of a VEGF receptor, the method comprising:

contacting a VEGF receptor with a candidate moiety;

simultaneously or sequentially contacting said VEGF receptor with a ligand for the VEGF receptor; and

determining whether said moiety affects the positioning, orientation and/or distance between the Ig-like domains of the ligand induced dimeric VEGF receptor, thereby identifying a moiety that binds to an Ig-like domain of a VEGF receptor.

66. The method of claim 65, wherein the moiety locks the ectodomain of the VEGF receptor in an inactive state.

67. The method of claim 65, wherein the moiety binds to a 7$^{th}$ Ig-like domain (D7) of the VEGF receptor.

68. An isolated antibody, or an antigen-binding portion thereof, that binds to a conformational epitope on a 7$^{th}$ Ig-like domain of a human VEGF receptor wherein said antibody, or antigen-binding portion thereof, antagonizes the activity of the human VEGF receptor, and wherein said conformational epitope comprises residues Arg726 and Asp731 of VEGFR2; residues Arg720 and Asp725 of VEGFR1; or residues Arg737 and Asp742 of VEGFR3.

69. An isolated antibody, or an antigen-binding portion thereof, that binds to amino acid residues 724-733 of VEGFR2, thereby antagonizing the activity of VEGFR2.

70. An isolated antibody, or an antigen-binding portion thereof, that binds to amino acid residues Arg720 and Asp725 of VEGFR1, thereby antagonizing the activity of VEGFR1.

71. An isolated antibody, or an antigen-binding portion thereof, that binds to amino acid residues Arg737 and Asp742 of VEGFR3, thereby antagonizing the activity of VEGFR3.

72. An isolated antibody, or an antigen-binding portion thereof, that binds at least one of the amino acid residues selected from the group consisting of Arg726 and Asp731 of a human VEGF receptor, thereby antagonizing the activity of the human VEGF receptor.

73. An isolated antibody, or an antigen-binding portion thereof, that binds at least one of the amino acid residues selected from the group consisting of Arg720 and Asp725 of a human VEGF receptor, thereby antagonizing the activity of the human VEGF receptor.

74. An isolated antibody, or an antigen-binding portion thereof, that binds at least one of the amino acid residues selected from the group consisting of Arg737 and Asp742 of a human VEGF receptor, thereby antagonizing the activity of the human VEGF receptor.