Abstract: The present invention features panoramic human epidermal receptor (HER) antagonists, pan-HER antagonists. The pan-HER antagonists of the present invention are polypeptide variant ligands of HER which may be selectively derivatized, with for example polyethylene glycol moieties, without losing antagonistic biological activity. As such, PEG-optimized pan-HER antagonists are designed to possess pan-HER antagonistic properties with improved binding and pharmacokinetic and pharmacodynamic profiles.
OPTIMIZED PAN-HER LIGANDS

GOVERNMENT SUPPORT

The invention was supported, in whole or in part, by a grant number 2R44CA095930-04 from the National Institutes of Health. The Government has certain rights in the invention.

RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 60/875,157 filed on December 15, 2006. The entire teachings of the above application are incorporated herein by reference.

ABBREVIATIONS

ACL, anti-cancer ligand; DNL, dominant negative ligand; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GPCR, G-protein coupled receptor; HER, human epidermal receptor; HER1, human epidermal receptor 1; hGH, human growth hormone; IFN, interferon; IGF, insulin-like growth factor; IR, insulin receptor; mPEG, methyl polyethylene glycol; NGF, nerve growth factor; Pan-HER antagonist, panoramic human epidermal receptor antagonist; PEG, polyethylene glycol; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

BACKGROUND OF THE INVENTION

Human Epidermal Receptors (HER), including epidermal growth factor receptors (EGFR), are well known examples of receptor tyrosine kinases. Interaction of HERs with their cognate ligands, or with structurally related ligands, leads to dimerization and activation of the kinase domain. This initiates a signaling cascade, leading to cell division. Dysregulation of HER signaling, such as the
overexpression of the genes coding for HER family members has been implicated in a number of pathologies, especially cancers of the breast, ovary, head and neck.

Epidermal growth factor (EGF), a cognate HER ligand, is a 53 amino acid cytokine which plays an important role in the growth control of mammalian cells. It is proteolytically cleaved from a large integral membrane protein precursor. The amino acid and nucleotide sequences of human EGF (EGF) are, for example, disclosed in Hollenberg, "Epidermal Growth Factor-Urogastrone, A Polypeptide Acquiring Hormonal States"; eds., Academic Press, Inc., New York (1979), pp. 69-110; or Urdea et al, Proc. Natl. Acad. Sci, USA. 80:7461 (1983). The amino acid sequence of wild-type or native EGF is also disclosed in U.S. Patent No. 5,102,789 and copending U.S. Patent Application No: 10/820,640 both of which are incorporated herein by reference in their entirety.

There is a need for novel therapies for HER-related pathologies, particularly therapeutic compounds which interfere with the entire HER receptor family in a panoramic fashion. Certain Pan-HER antagonists are disclosed in copending application Serial Number 11/172,611 filed June 30, 2005 and incorporated herein reference in its entirety. In addition, EGFR dominant negative ligands (DNLs) and anti-cancer ligands (ACLs) which may function as pan-HER antagonists, and methods of designing them, are disclosed in copending applications; Serial Numbers 60/818,735 (Attorney docket Number 3530.3004US) and 60/818,736 (Attorney Docket Number 3530.3006US), both filed July 6, 2006, each of which is incorporated herein by reference in its entirety.

Currently available methods of synthesis and expression of polypeptides provide a backdrop for the discovery, investigation and validation of new methods of designing optimized ligands or receptors having panoramic therapeutic properties. These molecules can then be exploited in the areas of drug discovery and medicine, including gene therapy.

One strategy for optimizing ligands, especially protein based ligands involves modification of the protein with bulking moieties to improve or impart certain therapeutically beneficial properties to the protein. The most common bulking moiety is the polymer or derivative of the polymer, polyethylene glycol (PEG).
Polymers, and particularly polyethylene glycol (PEG), are highly flexible and soluble and have gained widespread scientific and regulatory acceptance as a chemical modification for therapeutic proteins. Polyethylene glycol (PEG) is a hydrophilic, biocompatible and non-toxic water-soluble polymer of general formula H-(OCH₂CH₂)ₙ—OH. In typical form n ranges from about 10 to about 2000. Its molecular weight varies from 300 to 40,000 Daltons. PEG is useful in biological applications because it has properties that are highly desirable and is generally approved for biological or biotechnical applications. PEG typically is clear, colorless, odorless, soluble in water, stable to heat, inert to many chemical agents, does not hydrolyze or deteriorate, and is generally nontoxic. It is considered to be biocompatible, which is to say that PEG is capable of coexistence with living tissues or organisms without causing harm.

The attachment of polyethylene glycol (PEG) to therapeutic proteins has been a successful technique in developing drugs, improving clinical properties such as better physical and thermal stability, protection against proteolysis, increased in vivo circulation half-life, decreased clearance, reduced immunogenicity, antigenicity and toxicity, and enhanced in vivo activity. In this manner, PEGylation often improves the safety and efficacy of therapeutics. For example, PEGylation, including, but not limited to, site-specific PEGylation, can improve drug performance by optimizing pharmacokinetics, increasing bioavailability, decreasing immunogenicity and dosing frequency, and/or the like.

Several PEGylated protein therapeutics are currently on the market or in late-stage clinical trials. Schering-Plough's PEG-Intron.R™ (peginterferon alfa-2b) and Roche's PEGasys.R™ (peginterferon alfa-2a), both PEGylated variants of interferon-alpha (IFNa) used to treat hepatitis C, show significantly improved in vivo efficacy relative to the parent molecules.

One disadvantage of PEGylated protein therapeutics is that they have significantly reduced specific activity relative to the unmodified proteins. As a result, developers of PEGylated therapeutics are often faced with the difficult challenge of seeking PEG attachment sites that minimally impact the specific activity of the modified protein.
Many of the successful derivatization strategies rely on the chemically reactive epsilon amino group of lysine residues as a target for attachment. This is the most common target for both PEGylation and acylation strategies. There are at least three major issues with this target. (1) The protein must have exposed lysine; (2) The residues cannot be critical to the therapeutic function of the protein; and (3) There should not be too many lysines since this makes complete and uniform derivatization difficult, which has significant implications for producing a uniform product (This is an important issue in the production of PEGylated growth hormone, pegvisomant).

SUMMARY OF THE INVENTION

Accordingly, it is an object herein to provide novel HER ligand variants for use, among other things, as therapeutics. The compositions of the present invention function as Pan-HER antagonists and are capable of being selectively derivatized with bulking moieties, such as PEG, which impart or improve, among other things, the pharmacodynamic and pharmacokinetic properties without loss of activity. The resulting optimized pan-HER antagonists are useful therapeutics for the treatment of diseases, disorders or conditions which implicate multiple human epidermal receptors.

In one embodiment of the invention is a HER ligand variant comprising (a) one or more amino acid substitutions in the B-loop (amino acids 21-30) of the wild-type human epidermal growth factor (EGF), and (b) a polyethylene glycol (PEG) moiety attached to a lysine of the HER ligand variant of (a) wherein the lysine is either lysine 28 (K28) or lysine 48 (K48). The HER ligand variants of the present invention preferably act as Pan-HER antagonists.

In one embodiment, The HER ligand variants of the present invention comprise one or more amino acid substitutions in the B-loop of wild type EGF. In other embodiments the substitutions are in the second half of the B-loop (amino acids 26-30) and in other embodiments the substitutions are in the first half of the B-loop (amino acids 21-25). In one embodiment, a PEG moiety is attached to lysine 48 (K48) of the variants having one or more substitutions in the B-loop.

In one embodiment, substitutions are made in the B-loop and comprise replacing the amino acids of the second half of the B-loop (LDKYA) with amino
acids EPQRG or QPQRG. These variants may also be pegylated on any conjugating amino acid. These variants may be pegylated at particularly lysine 48 (K48).

In one embodiment, the HER ligand variant is SEQ ID No. 10 or SEQ ID No. 9 or SEQ ID No. 13.

5 In one embodiment, the HER ligand variants of the present invention are modified or conjugated with a PEG derivative. The PEG or PEG derivatives of the present invention may be substantially linear and have a molecular weight from about 10,000 to about 40,000 Daltons.

In one embodiment is provided a HER ligand variant of human wild-type epidermal growth factor comprising: (a) substitution of arginine (R45) with tyrosine resulting in (R45Y), (b) substitution of lysine (K48) with leucine resulting in (K48L), and (c) a polyethylene glycol (PEG) moiety attached to a wherein the lysine is either lysine 28 (K28). This variant may further comprise (a) substitution of tyrosine (Y22) with aspartic acid resulting in (Y22D), and (b) substitution of leucine (L26) with glycine resulting in (L26G).

10 In one embodiment, the HER ligand variants comprise one or more amino acid substitutions in the B-loop comprise replacing the amino acids of the first half of the B-loop (LDKYA) with amino acids EPQRG, and a PEG moiety attached to lysine 48 (K48). This variant may further comprise: (a) substitution of lysine (K28) with leucine resulting in (K28L), (b) substitution of serine (S2) with tryptophan resulting in (S2W), and (c) substitution of aspartic acid (D3) with valine resulting in (D3V).

15 In one embodiment, a HER ligand variant comprises (a) substitution of lysine (K28) and lysine (K48) of the wild-type human epidermal growth factor (EGF), with a non-reactive amino acid, and (b) substitution of one of any of the amino acids in the sequence forming the Domain I binding face, said Domain I binding face consisting of residues 1-5 (NSDSE) and residues 20-33 (MYIEALDKYACNC), with a conjugating amino acid that can be selectively conjugated with a bulking agent. In one embodiment, the non-reactive amino acid is leucine.

20 In one embodiment, the HER ligand variants described above may further comprise one or more amino acid substitutions in residues 34-48
(VGYIGERCQYRDLK) of the wild-type human epidermal growth factor (EGF), and optionally, one or more further substitutions in residues 7-19 (PLSHDGYCLHDGV).

These variants may further comprise attachment of a polyethylene glycol (PEG) moiety to the conjugating amino acid in the Domain I binding face.

In one embodiment, the HER ligand variants comprise (a) substitution of lysine (K28) and lysine (K48) of the wild-type human epidermal growth factor (EGF) with a non-reactive amino acid and (b) substitution of one of any of the amino acids in the sequence forming the Domain III binding face, said Domain III binding face consisting of residues 34-48 (VGYIGERCQYRDLK) and residues 7-19 (PLSHDGYCLHDGV) of the wild-type human epidermal growth factor (EGF) with a conjugating amino acid.

These variants may further comprise one or more amino acid substitutions in a first half of the B-loop (amino acids 21-25) of the wild-type human epidermal growth factor (EGF), and optionally, one or more further substitutions in a second half of the B-loop (amino acids 26-30).

In one embodiment, the HER ligand variants of the present invention comprise a polyethylene glycol (PEG) moiety attached or conjugated to the conjugating amino acid in the Domain III binding face.

The present invention also provides pharmaceutical compositions of HER ligand variants and a pharmaceutically acceptable carrier. Also provide are methods of treating a patient with a disease characterized by overexpression of HER comprising, administering to the patient, a therapeutically effective amount of a pharmaceutical composition of the present invention comprising a HER ligand variant of the present invention. Included in the HER ligand variants of the present invention comprising pharmaceutical compositions are any modifications or conjugations or substitutions which may have been made to the wild type EGF starting molecule.

The compounds and compositions of the present invention are useful in methods for treating diseases or disorders. In one embodiment, the disease is cancer.
In one embodiment the cancer is selected from the group consisting of gliomas, squamous cell carcinomas, breast carcinomas, melanomas, invasive bladder carcinomas, colorectal carcinomas and esophageal cancers.

In one aspect of the invention is provided methods of treating a patient with a disease characterized by overexpression of HER or a HER-mediated pathology comprising, administering to the patient, a therapeutically effective amount of a pharmaceutical composition of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

A description of embodiments of the invention follows. The present invention features novel therapeutic HER ligand variants termed Pan-HER antagonists, so named because of their capacity to antagonize signaling mediated by two or more human epidermal receptors (HERs).

As is known in the art, ligands that bind human epidermal receptors (HERs) can be grouped into three categories: 1) Those that bind to HER1 alone (EGF, TGF-a, amphiregulin), 2) those that bind to HER3 and/or HER4 (heregulins and neuregulins) and 3) those that bind to HER-I and HER-4 (betacellulin, heparin-binding EGF, NRG3, epigen, and epiregulin) (Riese and Stern 1998 Bioessays 20:41). Each human epidermal receptor exists as a monomer in the inactive state. Ligand binding promotes either homodimerization or heterodimerization between the bound receptor and other members of the HER family. The various EGF-like growth factors bind with high affinity to ErbB receptors except for HER2, which has no known ligand and has the constitutive ability to form homodimers and heterodimers. HER2 homodimers have been implicated in tumor cell growth, but also are important for cardiac muscle development and repair. (Dougall et al 1994 Oncogene 9:2109, Hynes and Stern 1994, Biochim Biophys Acta 1198:165, Tzahar and Yarden 1998 Biochim Biophys Acta 1377:M25, Negro et al. 2004, Recent Prog Horm Res. 59:1.). HER2 is the preferred heterodimeric partner of the other HER receptors (Tzahar et al. 1996, Mol Cell Biol, 16:5276, Beerli et al. 1995 Mol Cell Biol 15:6496, Karunagar et al. 1996 EMBO J 15:254, Wang et al. 1998, PNAS, 95:6809). HER3 differs from the other HER family members in that it has a deficient tyrosine kinase domain (Guy et al 1994 PNAS 91:8132) and must associate with another HER-family receptor to trigger signaling.
Examples of HER ligands include mammalian EGF (e.g. human (EGF), pig, cat, dog, mouse, horse and rat). Other examples of HER ligands include transforming growth factor-α (TGFα), betacellulin, heparin-binding EGF-like growth factor (HB-EGF), neuregulins, heregulin (HRG) including HRGα, HRGβ1, HRGβ2 and HRG-factor (NDF), amphiregulin (AR), epigen and epiрегulin.

The HER ligand variants of the present invention are ligand variants which bind HERs. Preferred HER ligand variants of the invention are based on HER ligands which are capable of selectively inhibiting HER-mediated biological activity.

According to the present invention the term "Pan-HER antagonist" encompasses any amino-acid based molecule that inhibits, suppresses or causes the cessation of at least one HER-mediated biological activity by reducing, interfering with, blocking, supplanting or otherwise preventing the interaction or binding of a native or active HER ligand to more than one human epidermal receptor (HER) thereby attenuating or inhibiting signaling via a human epidermal receptor. As used herein "amino-acid based" means that the molecule is predominantly protein in nature. It is understood that amino-acid based molecules may have non-protein moieties attached or linked to them. Specifically excluded from this definition are antibodies to a receptor and noncovalent conjugates of an antibody and an antigen for that antibody.

HERs include HER1, HER2, HER3, and/or HER4 and variant forms of these receptors. It is understood that direct interference with HER1, HER3 and HER4 can provide indirect interference with HER2, by blockading the dimerization partners implicated in much of HER2's role in cancer.

It is not necessary that the HER ligand variant of the invention target HER2, thereby suppressing HER2 biological activity that may be undesirable. It is advantageous to avoid suppression of HER2 homodimerization because such activity has been shown to cause cardiomyopathy, a life threatening side effect.

As used herein, the term "antagonist" means any molecule that blocks the ability of a given chemical to bind to its receptor, thereby preventing a biological response. The term antagonist can be used in a functional sense and is not intended to limit the invention to compounds having a particular mechanism of action. For
example, the term "antagonist" includes, but is not limited to, molecules that function as competitive antagonists. A "competitive antagonist" is one which binds the receptor but does not trigger the biological activity of the receptor.

"HER-mediated biological activity" as used herein means the intrinsic protein-tyrosine kinase activity of the HER and/or its downstream signal transduction cascade. For example, HER-mediated biological activities include reducing or inhibiting HER kinase activation, signaling, regulation, dimerization, HER-regulated cell proliferation or phosphorylation as well as any HER-mediated pathology or phenotypic manifestation evidenced as HER-mediated. The HER ligand variants of the invention are designed to act as Pan-HER antagonists. Such HER ligand variants, and nucleic acids encoding these variants, can be used therapeutically in situations in which inhibition of HER biological activity is indicated, e.g. cancer, inflammation and the like. As such the present invention encompasses therapeutic Pan-HER antagonists and variants thereof and methods for their design and use in medicine, diagnostics and drug discovery.

Others have shown that the serum half-life of EGF can be increased with PEGylation at the two lysines in the molecules (K28 and K48) (Lee, H and G. Park. 2002, Pharmaceutical Research 19(6):845-851). Unfortunately, PEGylation led to loss of activity. This is not surprising, since the two lysines are located in the two binding surfaces of the ligand to its receptor. Binding at both surfaces is required for agonist activity, but not for the antagonist activity possessed by the HER ligand variants disclosed herein. In fact, antagonists of the present invention have been created by ablating binding to one receptor binding domain (through point mutations in EGF), and enhancing binding at the other (through affinity maturation).

Therefore, by eliminating one or the other lysines in the compounds of the present invention, it is possible to interfere with binding at the desired domain of the HER receptor, and thus, PEGylation will enhance rather than diminish antagonist properties.

It is an object of the present invention to engineer variant Pan-HER Dominant Negative Ligands with only a single lysine, and then to produce, purify and PEGylate these variants Binding properties and antagonist properties (in vitro) and their serum half-lives in mice are then investigated.
The present invention is directed to at least two classes of Pan-HER DNLS: 
"EGFDI" variants that bind only to Domain I of HER receptors and "EGFDIII" 
variants that bind only to Domain III.

In the first category, EGFDI variants comprise, in addition to the other 
constructs disclosed herein, variants in which both lysines at position 28 and 48 of 
the native EGF are eliminated and substitutions of any one of the residues in the 
sequence at the Domain III binding face are made to allow modification with a 
bulking group (e.g., pegylation). For example, one of ordinary skill in the art, armed 
with the instant disclosure, will appreciate that modifications to eliminate a 
pegylation sites and add new sites need not be confined to the native lysine groups. 
Therefore, within the scope of the invention are HER ligand variants in which both 
native lysines have been substituted and in which further substitutions have been 
performed to introduce a lysine (or another residue that can be conjugated to a 
bulking agent) at any amino acid residue in the sequence on the Domain III binding 
face. This would be evident as it is shown herein that residues on the Domain III 
binding face may be changed without a loss of antagonist activity.

Likewise, it is within the scope of the invention to design the same type of 
variants (substitution of native lysines and introduction of new lysine substitutions 
on a binding face) in relation to Domain I binding. In this respect, EGFDIII variants 
are designed having both native lysines substituted and with substitutions of one of 
any of the amino acids in the sequence at the Domain I binding face of the molecule 
which will allow modification with a bulking group (e.g. pegylation).

Substitution on a binding face is preferably with a non-reactive residue. As 
used herein a "non-reactive residue or non-reactive amino acid" is any amino acid 
which is not readily derivatized with a bulking agent. As used herein a "conjugating 
residue or conjugating amino acid" is one which can be conjugated with or to a 
bulking group.

Both of these variants currently posses lysines at positions 28 (in the Domain 
I binding region) and 48 (in Domain III binding region). Both of these lysines have 
been substituted with leucines in EGF and a Pan-HER EGF variant with little or no 
loss of activity (Groenen, L et al. 1994, Growth Factors 11:253-257). This will 
allow monoPEGylation at the remaining lysine, further disrupting binding to the
desired region of the receptor. Other HER ligands (TGF-α, heregulin, etc.) can similarly be manipulated to remove and add lysines to introduce conjugation sites on their corresponding binding faces.

According to the present invention, the variants, K28L in the EGFDI class of antagonist and the K48L substitution in the EGFDIII class are studied.

In the "EGFDIII" class of Pan-HER DNL, the starting ligand variant is the R45Y mutation of panerbin disclosed by Van der Woning, S. P. et al. (2006. Negative constraints underlie the ErbB specificity of EGF-like growth factors. J. Biological Science. JBC Papers in Press: Published on line October 10, 2006 as manuscript M603 1682000).

Residues S and D at positions 2 and 3 are retained because they prevent binding of EGF to HER3 and HER4 and Y22D and L26G substitutions are made to further ablate binding to Domain I of EGFR.

To complete the protein engineering necessary for monoPEGylation the K28L mutation is created in the EGFDI variant so that PEGylation will further disrupt binding to Domain III and the K48L mutation in the EGFDIII variant so that PEGylation will further disrupt binding to Domain I.

Then the central question can be addressed, which is: can one introduce a large PEG molecule (to avoid kidney filtration) to this small protein (53 amino acids), retain binding (and clinically relevant biological activity) through the remaining binding interaction, and the Pan-HER phenotype?

To this end, the present invention contemplates the use and investigation of EGF homologs, analogs and fragments of the EGF in the creation of HER ligand variants or Pan-HER antagonists. The term "homolog" refers to the corresponding polypeptides of HER ligands from other species having substantial identity to human wild-type HER ligands. These homologs may be modified and optimized according to the present invention to produce Pan-HER antagonists. For example, homologs of EGF polypeptide sequences from various mammalian species are disclosed in Table 1.
As used herein, the term "analog" refers to compounds whose structure is related to that of another compound but whose chemical and biological properties may be quite different.

As used herein the term "ligand" is used to designate an amino acid-based molecule capable of specific binding to a receptor as herein defined. The definition includes any native (cognate) ligand for a receptor or any region or derivative thereof retaining at least a qualitative receptor binding ability. Specifically excluded from this definition are antibodies to a receptor and noncovalent conjugates of an antibody and an antigen for that antibody.

The terms "native ligand" and "wild-type ligand" are used interchangeably and refer to the amino acid sequence of a ligand occurring in nature ("native sequence ligand"), including mature, pre-pro and pro forms of such ligands, purified from natural source, chemically synthesized or recombinantly produced. Native ligands that can activate receptors are well known in the art or can be prepared by art known methods. The mature wild-type human EGF protein sequence is represented by SEQ ID. NO. 1.

In one embodiment of the invention, the HER ligand variants or Pan-HER antagonists act as dominant negative ligands (DNLs). In this regard, the term "dominant negative" is used to describe that type of ligand, when altered or

Table 1

<table>
<thead>
<tr>
<th>PROTEIN SEQUENCE</th>
<th>Species</th>
<th>SEQ ID No.</th>
</tr>
</thead>
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<td>NSDSECPLSHDGYCLHDGVMYIEALDKYACNCVYGIGERCQYRDLKW WE1R</td>
<td>Human</td>
<td>1</td>
</tr>
<tr>
<td>NSYSECFFSHDGYCLHGGMIEAVDSYACNCVFGYVGERCQHRDLKW WE1R</td>
<td>Pig</td>
<td>2</td>
</tr>
<tr>
<td>NSQECPSYDGYCLNYGCMYIEAVDREACNCVFGBGYVGERCQHRDLK - WELR</td>
<td>Cat</td>
<td>3</td>
</tr>
<tr>
<td>N QRECPSYDGYCLNGCMHIESLDSYTCNVCVIGYSQDGRCQTRDLR WELR</td>
<td>Dog</td>
<td>4</td>
</tr>
<tr>
<td>NSYPGCPSSYDGYCLNGMVHIESLDSYTCNVCVIGYSQDGRCQTRDLR WELR</td>
<td>Mouse</td>
<td>5</td>
</tr>
<tr>
<td>N QRECQSYDGYCLHGKVCYLVQVDTHACNCVVGYVGERCQHMDLRT ___</td>
<td>Horse</td>
<td>6</td>
</tr>
<tr>
<td>NSNTGCPSYDGYCLNGCMYVESVDRYVCNVCVIGYSQGRCQHRDLRW KLR</td>
<td>Rat</td>
<td>7</td>
</tr>
</tbody>
</table>

As used herein, the term "analog" refers to compounds whose structure is related to that of another compound but whose chemical and biological properties may be quite different.

As used herein the term "ligand" is used to designate an amino acid-based molecule capable of specific binding to a receptor as herein defined. The definition includes any native (cognate) ligand for a receptor or any region or derivative thereof retaining at least a qualitative receptor binding ability. Specifically excluded from this definition are antibodies to a receptor and noncovalent conjugates of an antibody and an antigen for that antibody.

The terms "native ligand" and "wild-type ligand" are used interchangeably and refer to the amino acid sequence of a ligand occurring in nature ("native sequence ligand"), including mature, pre-pro and pro forms of such ligands, purified from natural source, chemically synthesized or recombinantly produced. Native ligands that can activate receptors are well known in the art or can be prepared by art known methods. The mature wild-type human EGF protein sequence is represented by SEQ ID. NO. 1.

In one embodiment of the invention, the HER ligand variants or Pan-HER antagonists act as dominant negative ligands (DNLs). In this regard, the term "dominant negative" is used to describe that type of ligand, when altered or
modified to differ from the native or wild-type ligand in any respect, results in a ligand that retains binding affinity for a wild-type binding partner (e.g., a receptor) but inhibits the function or signaling of the wild-type binding partner. As used herein the term "dominant negative ligand activity" refers to the functions associated with dominant negative ligands (e.g., binding a receptor but inhibiting a function of the receptor).

Amino acid sequences of the HER ligand variants and Pan-HER antagonists of the invention may be obtained through various means such as chemical synthesis, phage display, cleavage of proteins or polypeptides into fragments, or by any means in which amino acid sequences of sufficient length to possess selected properties may be made or obtained.

In one embodiment, the HER ligand variants and Pan-HER antagonists of the invention are produced by expression in a suitable host of a gene coding for the relevant HER ligand variant or Pan-HER antagonist. Such a gene is most readily prepared by site-directed mutagenesis of the wild-type gene, a technique well known in the art.

As such, the present invention also provides nucleic acid molecules encoding a HER ligand variant or Pan-HER antagonist of the invention. The nucleic acid molecules of the present invention can be RNA, for example, mRNA, or DNA. DNA molecules can be double-stranded or single-stranded. The nucleic acid molecule can also be fused to a marker sequence, for example, a sequence that encodes a polypeptide to assist in isolation or purification of the polypeptide. Such sequences include, but are not limited to, those that encode a glutathione-S-transferase (GST) fusion protein, those that encode a hemagglutinin A (HA) polypeptide marker from influenza, and sequences encoding a His tag.

It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed and the level of expression of HER ligand variant or Pan-HER antagonist desired. The expression vectors of the invention can be introduced into host cells to thereby produce the modified polypeptides of the invention, including fusion polypeptides, encoded by nucleic acid molecules as described herein. Molecular biology techniques for carrying out recombinant production of the

Alternatively, the HER ligand variant or Pan-HER antagonist of the invention may be produced in whole or in part by chemical synthetic techniques such as by a Merrifield-type synthesis (*J. Am. Chem. Soc.* 85:2149 (1963), although other equivalent chemical syntheses known in the art may be used. Solid-phase synthesis is initiated from the C-terminus of the peptide by coupling a protected alpha-amino acid to a suitable resin. The amino acids are coupled the peptide chain using techniques well known in the art for the formation of peptide bonds. Chemical synthesis of all or a portion of a HER ligand variant or Pan-HER antagonist of the invention may be particularly desirable in the case of the use of a non-naturally occurring amino acid substituent in the HER ligand variant or Pan-HER antagonist.

The present invention contemplates the design of HER ligand variants, Pan-HER antagonists and dominant negative ligands, which have as their design reference point, other HER ligand variants, Pan-HER antagonists and dominant negative ligands. These further designed HER ligand variants, Pan-HER antagonists and dominant negative ligands may be the result of further optimization of properties in addition to or beyond binding and signal inhibition. For example, once optimized over a first HER ligand variant, Pan-HER antagonist or dominant negative ligand, a HER ligand variant, Pan-HER antagonist or dominant negative ligand may then be the starting point for further optimization meaning that, in the design scheme, the resultant compound would then become the starting compound. Therefore, a "HER ligand" or "Pan-HER antagonist" can, in certain contexts, be construed as a "HER ligand variant" or "Pan-HER antagonist variant", respectively, and vice versa. Furthermore, when used as a starting or reference point for design, a HER ligand variant, Pan-HER antagonist or dominant negative ligand may also be referred to or considered a druggable ligand.

Methods of designing and testing HER ligand variants are disclosed in copending applications; Serial Numbers 60/8 18,735 (Attorney docket Number 3530.3004US) and 60/818,736 (Attorney Docket Number 3530.3006US), both filed July 6, 2006, each of which is incorporated herein by reference in its entirety.
The HER ligand variants and Pan-HER antagonists of the present invention are amino acid-based molecules. These molecules may be "peptides," "polypeptides," or "proteins."

The terms "amino acid" and "amino acids" refer to all naturally occurring L-alpha-amino acids. The amino acids are identified by either the one-letter or three-letter designations as listed in Table 2.

### Table 2

<table>
<thead>
<tr>
<th>Three letter</th>
<th>One letter</th>
<th>Amino acid</th>
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<tbody>
<tr>
<td>Asp</td>
<td>D</td>
<td>aspartic acid</td>
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<tr>
<td>Ile</td>
<td>I</td>
<td>isoleucine</td>
</tr>
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<td>Thr</td>
<td>T</td>
<td>threonine</td>
</tr>
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<td>serine</td>
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<tr>
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<td>Y</td>
<td>tyrosine</td>
</tr>
<tr>
<td>Glu</td>
<td>E</td>
<td>glutamic acid</td>
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<tr>
<td>Phe</td>
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<tr>
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The amino acid sequences of the HER ligand variants and Pan-HER antagonists of the invention may comprise naturally occurring amino acids and as such may be considered to be proteins, peptides, polypeptides, or fragments thereof.

Alternatively, the HER ligand variants and Pan-HER antagonists may comprise non-naturally occurring amino acids or both naturally and non-naturally occurring amino acids.

The term "amino acid sequence variant" refers to molecules with some differences in their amino acid sequences as compared to a native sequence. The amino acid sequence variants may possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence of a native ligand.
Ordinarily, variants will possess at least about 70% homology to a native ligand, and preferably, they will be at least about 80%, more preferably at least about 90% homologous to a native ligand.

"Homology" as it applies to amino acid sequences is defined as the percentage of residues in the candidate amino acid sequence that are identical with the residues in the amino acid sequence of a native ligand after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the alignment are well known in the art. It is understood that homology depends on a calculation of percent identity but may differ in value due to gaps and penalties introduced in the calculation.

As described herein, the HER ligand variants and Pan-HER antagonists produced by the methods of the present invention, their homolog variants and analogs may have substantial sequence identity to wild-type ligands. However, it is appreciated that substantial sequence identity is not a single defining feature of the compounds of the present invention. Structural components are also factors when considering identity of a variant to the parent molecule.

As used herein, "substantial sequence identity" means at least 60% sequence identity, preferably at least 70% identity, preferably at least 80% and more preferably at least 90% sequence identity to the amino acid sequence of starting ligand (or domains thereof in the instance where the variant is a chimera produced by swapping domains), while maintaining HER-mediated biological activity. In other embodiments, the HER ligand variants and Pan-HER antagonists of the present invention have at least 91%, at least 92%, at least 93%, at least 94%, at least 95% at least 96%, at least 97%, or at least 98% sequence identity to the amino acid sequence of wild-type human ligand, while maintaining HER-mediated biological activity.

The percent identity of two amino acid sequences can be determined by aligning the sequences for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first sequence). The amino acids at corresponding positions are then compared, and the percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., \( \text{% identity} = \frac{\# \text{ of identical positions}}{\text{total } \# \text{ of positions}} \times 100 \)). The actual comparison
of the two sequences can be accomplished by well-known methods, for example, using a mathematical algorithm. A preferred, non-limiting example of such a mathematical algorithm is described in Karlin et al., Proc. Natl. Acad. Sci. USA, 90:5873-5877 (1993). Such an algorithm is incorporated into the BLASTN and BLASTX programs (version 2.2) as described in Schaffer et al., Nucleic Acids Res., 29:2994-3005 (2001).

The term "derivative" is used synonymously with the term "variant" and refers to a molecule that has been modified or changed in any way relative to a reference molecule or starting molecule. As used herein derivative and variant HER ligands or Pan-HER antagonists are amino acid-based molecules which are modified, altered, improved or optimized relative to a starting parent molecule.

The present invention contemplates several types of HER ligand and Pan-HER antagonist variants and derivatives. These modifications can be useful to alter receptor specificity (either broaden specificity such that the variant binds to additional receptors or target specificity towards a specific receptor or narrow specificity to less than all of the family of receptors but not less than two) or to alter phenotypic outcomes. Also included in the modifications of the compounds of the invention are domain swapping and/or domain modifications.

As such, included within the scope of this invention are amino acid-based molecules containing substitutions, insertions and/or additions, deletions or covalently modifications. For example, sequence tags or amino acids, such as one or more lysines, can be added to the peptide sequences of the invention (e.g., at the N-terminal or C-terminal ends). Sequence tags can be used for peptide purification or localization. Lysines can be used to increase peptide solubility or to provide sites for biotinylation or pegylation. Alternatively, amino acid residues located at the carboxy and amino terminal regions of the amino acid sequence of a peptide or protein may optionally be deleted providing for truncated sequences. Certain amino acids (e.g., C-terminal or N-terminal residues) may alternatively be deleted depending on the use of the sequence, as for example, expression of the sequence as part of a larger sequence which is soluble, or linked to a solid support.

"Substitutional variants" are those that have at least one amino acid residue in a native or starting sequence removed and a different amino acid inserted in its
place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule. Substitutions may be, therefore, one or more.

As used herein the term "conservative amino acid substitution" refers to the substitution of an amino acid that is normally present in the sequence with a different amino acid of similar size, charge, or polarity. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine and leucine for another non-polar residue. Likewise, examples of conservative substitutions include the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, and between glycine and serine. Additionally, the substitution of a basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue such as aspartic acid or glutamic acid for another acidic residue are additional examples of conservative substitutions. Examples of non-conservative substitutions include the substitution of a non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine, alanine, methionine for a polar (hydrophilic) residue such as cysteine, glutamine, glutamic acid or lysine and/or a polar residue for a non-polar residue.

In one aspect, the present invention features a HER ligand variant or Pan-HER antagonist designed from a naturally occurring HER ligand that has at least one amino acid substitution at amino acid position that corresponds to any one or more amino acids selected from the B-loop (amino acids 21-30) of wild type EGF.

In one aspect the amino acid substitutions may occur in only a portion of the B-loop such as in the first half (amino acids 21-25) or in the second half (amino acids 26-30).

In one aspect the entire B-loop, or either half may be replaced. The replacement of half of the B-loop would represent five amino acid substitutions.

As used herein, the phrase "amino acid position that corresponds to" means that when the starting HER ligand is aligned with the variant for optimal comparison, the amino acids that appear at or near the positions identified may be substituted with another amino acid. See U.S. Utility Application bearing Attorney

"Insertional variants" are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native or starting sequence. "Immediately adjacent" to an amino acid means connected to either the alpha-carboxy or alpha-amino functional group of the amino acid at the particular position.

"Deletional variants" are those with one or more amino acids in the native or starting amino acid sequence removed. Ordinarily, deletional variants will have one or more amino acids deleted in a particular region of the molecule.

"Covalent derivatives" include modifications of a native or starting ligand (which may be a molecule already validated as a HER ligand variant or Pan-HER antagonist) with an organic proteinaceous or non-proteinaceous derivatizing agent, or post-translational modifications. Covalent modifications are traditionally introduced by reacting targeted amino acid residues of the ligand with an organic derivatizing agent that is capable of reacting with selected side-chains or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells.

Covalent derivatives specifically include fusion molecules in which ligands of the invention are covalently bonded to a nonproteinaceous polymer. The nonproteinaceous polymer ordinarily is a hydrophilic synthetic polymer, i.e. a polymer not otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or in vitro methods are useful, as are polymers which are isolated from nature.

Hydrophilic polyvinyl polymers fall within the scope of this invention, e.g. polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are polyvinylalkylene ethers such a polyethylene glycol (PEG), polypropylene glycol. The ligands may be linked to various nonproteinaceous polymers, such as polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.
Alternatively, non-covalent derivatives may be attached to the HER ligand variants or Pan-HER antagonists and these can be labile (both internally labile or labily attached).

The serum half-life of the HER ligand variants and Pan-HER antagonists of the present invention is expected to be about the same as unmodified EGF (about 10 minutes). This may be acceptable or even desirable for some applications, where rapid clearing of the drug is beneficial. In order to be considered for significant oncology applications, a half-life of 5-7 days is desirable.

In other anticipated applications, longer retention time in the body is desirable. There are several very well established methods to extend the serum half life of therapeutic proteins, primarily through adding "bulking" side chains like PEG that exclude the compound from the kidney filtering system and hide it from immune surveillance (Veronese, F. and G. Pasut, 2005, Drug Discovery Today, 10(21), 1451-1458).

While polyethylene glycol is a preferred protein conjugating (bulking) reactant, a variety of additional polymer modifiers have been used to modify proteins. These include modified polyethylene glycols, branched polyethylene glycols, crosslinked polyethylene glycols, PEG derivatives, dextrans, polyvinylpyrrolidone, polyvinylalcohol, polyamino acids, albumin and gelatins.

Those skilled in the art will appreciate, once having an understanding of the present invention, that the principles and methods described herein can be applied to processes for modifying proteins with any of these additional reagents.

Addition of acyl (fatty acid) side chains or albumin binding domains can enhance the binding of the drug to other circulating proteins (primarily albumin), allowing for slow release back into the serum (Home, P. and P. Kurtzhals, 2006, Expert Opin. Pharmather., 7(3), 325-343). Examples of the bulking strategy include PEGylated TNF-alpha (Yamamoto, Y., et al, 2003, Nature Biotech. 21(May), 546-550), PEG-interferon (PEG-IntronTM, Schering) and pegvisomant (SomavertTM, Pfizer), which have been derivatized with PEG to bulk them up. Insulin detemir (LevemirTM, Novo Nordisk) is an acylated form of insulin that binds to albumin, significantly extending its serum half life (Home, P. and P. Kurtzhals, 2006, Expert Opin. Pharmather., 7(3), 325-343).
In one embodiment, it is desirable to use reversible (labile) PEG attachment to temporarily inactivate a protein while improving its half-life in vivo. This concept can be used, for example, to generate a slow-release version of a therapeutic protein or a protein that bypasses some biological systems at early time points after administration. Labile attachments will also be desirable such as where it is impossible to add a bulking agent big enough to the excluded from the kidney filtering system without also blocking important binding sites.

The linkage between the PEG moieties described herein and the therapeutic protein are preferably covalent and optionally but preferably reversible. That is, under physiological conditions, the linkage between the PEG and the protein is preferably labile. Using reversible chemistry for PEG attachment allows regeneration of active therapeutic protein over time, preferably following absorption from the site of administration. For such situations, the present invention finds use for the optimization of reversible PEG attachment sites. In some cases it is desirably that biological activity is lost when the PEG is attached; in other cases, the activity is stable or even increased.

Covalent attachment of a polyethylene glycol moiety is also referred to in the art as PEG conjugation or PEGylation. As used herein, the term "PEGylation" means and refers to modifying a protein by covalently attaching polyethylene glycol (PEG) to the protein, with "PEGylated" referring to a protein having a PEG attached.

A range of PEG, or PEG derivative sizes with optional ranges of from about 10,000 Daltons to about 40,000 Daltons may be attached to proteins using a variety of chemistries. The molecular weight of the poly(ethylene glycol) used in the present invention may be between about 10,000 and about 40,000 Dalton. The term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight and the stated molecular weight refers to the average molecular weight. It is understood that there is some degree of polydispersity associated with polymers such as poly(ethylene glycol). It is preferable to use PEGs with low polydispersity. Normally, a PEG with molecular weight of between about 10,000 and about 40,000 is used. A specific PEG molecular weight range of the present invention is from about 10,000 to about 30,000. In another specific embodiment the PEG molecular weight is greater than about 15,000
to about 40,000. In another specific embodiment the PEG molecular weight is about 20,000 to about 40,000.

Other sizes may be used, depending on the desired therapeutic profile (e.g. duration of sustained release desired, the effects, if any on biological activity, the degree or lack of antigenicity and other known effects of the polyethylene to a therapeutic protein. For example the polyethylene glycol may have an average molecular weight of about 9000, 9500, 10,000, 10,500, 11,000, 11,500, 12,000, 12,500, 13,000, 13,500, 14,000, 14,500, 15,000, 15,500, 16,000, 16,500, 17,000, 17,500, 18,000, 18,500, 19,000, 19,500, 20,000, 25,000, 30,000, 35,000, 40,000, 45,000 or 50,000 Dalton.

PEG conjugation is an established methodology for peptide and protein modification pioneered by the work of Davis and Abuchowski [Abuchowski, A. et al, J. Biol. Chem., 252, 3571 (1977) and J. Biol. Chem., 252, 3582 (1977)]. PEG conjugation to peptides or proteins generally involved the activation of PEG and coupling of the activated PEG-intermediates directly to target proteins/peptides or to a linker, which is subsequently activated and coupled to target proteins/peptides. One of the key issues with the conjugation is the chemistry used to activate PEG and PEG-linker, which in turn will determine the coupling efficiency and specificity of the activated PEG or PEG-linker to its targets.

For PEG a variety of means have been used to attach the PEG molecules to the protein. Generally, PEG molecules are connected to the protein via a reactive group found on the protein. Linkages are typically formed between PEG and primary amines (lysine side chains or the protein N-terminus), thiols (cysteine residues), or histidines. Linkages can also be formed between PEG and nonnatural amino acids, especially those involving an acetyl moiety, for instance, p-acetyl-L-phenylalanine.

In the present invention, when the number of lysines is reduced to one, PEGylation of lysine side chains produces mono-PEGylation products. Since the pKa of the N-terminus is significantly different than the pKa of a typical lysine side chain, it is possible to specifically target either for modification.

In one embodiment of the invention, lysine at position 28 (K28) is modified to any of the naturally occurring amino acids. Preferably this modification comprises
K28R or K28L. Further, variants containing a K28 modification may further be
derivatized at lysine 48 with a non-amino acid moiety, such as polyethelyene glycol
(PEG).

Similarly, as most proteins contain very few free cysteine residues, cysteines
(naturally occurring or engineered) are commonly targeted for site-specific
PEGylation.

In one embodiment, the present invention finds use for replacing specific lysine or histidine residues with alternative amino acids, such that PEGylation at
such residues is no longer possible.

In on embodiment of the invention, HER ligand variants or Pan-HER antagonists are conjugated to PEG moieties or PEG derivatives. Conjugation or
derivatization of PEG to the compositions of the present invention may be selective
as the compositions have been designed to minimize the number of PEGylation sites
while still retaining biologic activity.

PEG is commonly used as methoxy PEG-OH, or mPEG in brief, in which one terminus is the relatively inert methoxy group, while the other terminus is a
hydroxyl group that is subject to ready chemical modification.

In another embodiment the PEG moiety may be a branched PEG having
more than one PEG moiety attached thereto (see U.S. Pat. No. 5,932,462; U.S. Pat.
No. 5,342,940; U.S. Pat. No. 5,643,575; U.S. Pat. No. 5,919,455; U.S. Pat. No.
6,1 13,906; U.S. Pat. No. 5,183,660; Kodera Y., Bioconjugate Chemistry 5:283-288
(1994); and WO 02/09766. Branched polymer backbones are generally known in the
art. Typically, a branched polymer has a central branch core moiety and a plurality
of linear polymer chains linked to the central branch core. PEG is commonly used in
branched forms that can be prepared by addition of ethylene oxide to various polyols, such as glycerol, pentaerythritol and sorbitol. The central branch moiety can
also be derived from several amino acids, e.g., lysine.

Suitable branched PEGs can be prepared in accordance with International
Publication No. WO 96/21469, entitled Multi-Armed, Monofunctional, and
Hydrolytically Stable Derivatives of Poly(Ethylene Glycol) and Related Polymers
For Modification of surfaces and Molecules, which was filed Jan. 11, 1996, the
contents of which are incorporated herein in their entirety by reference (which
corresponds to U.S. Pat. No. 5,932,462, which is also incorporated by reference).

These branched PEGs can then be modified in accordance with the teachings herein.

The branched PEGs can be represented in general form as $R(\text{--PEG--OH})_n$ in which $R$ represents the central "core" molecule, such as glycerol or pentaerythritol, and $n$ represents the number of arms.

Branched PEGs can also be prepared in which two PEG "arms" are attached to a central linking moiety having a single functional group capable of joining to other molecules; e.g., Matsushima et al., (Chem. Lett., 773, 1980) have coupled two PEGs to a central cyanuric chloride moiety.

To couple PEG to a molecule such as a protein, it is often necessary to "activate" the PEG to prepare a derivative of the PEG having a functional group at the terminus. The functional group can react with certain moieties on the protein such as an amino group, thus forming a PEG-protein conjugate. Many activated derivatives of PEG have been described. An example of such an activated derivative is the succinimidyl succinate "active ester."

According to the present invention, the amino acid sequence of the active HER ligand variants can be modified without compromising antagonist properties. This is an important and novel finding that enables the development of several alternative, superior forms of HER ligand variants or Pan-HER antagonists as the active molecules may be further derivatized thereby optimizing other characteristics of the molecule, e.g., pharmacodynamic and pharmacokinetic properties.

It is an object of the present invention to produce a Pan-HER antagonist for use as an anti-cancer ligand (ACL) that can be derivatized through its lysine residues.

The starting molecule, EGF has two lysines (abbreviated in sequence nomenclature "K") at positions 28 and 48. It has been shown that PEGylation of either of these lysines significantly increases the serum half-life of the protein (Lee, H. and G. Park, 2002, *Pharmaceutical Research*, 19(6), 845-851). PEGylation of K28 greatly reduced the biological activity of the protein while PEGylation at K48 appears to totally destroy activity (Lee, H. and G. Park, 2002, *Pharmaceutical Research*, 19(6), 845-851). N-terminal PEGylation was not nearly as detrimental but

Another embodiment of the invention relates to methods for the prevention and/or treatment of a disease or disorder in which use of a HER ligand variant or pan-HER antagonist, is beneficial, comprising administering to a patient in need thereof a therapeutically effective amount of a PEG conjugated or modified pan-HER antagonist of the invention or variant thereof, alone or in combination with another therapeutic agent. The invention also relates to the use of a PEG modified pan-HER antagonist of the invention or variant thereof in the manufacture of a medicament for the prevention and/or treatment of a disease or disorder in which use of a pan-HER antagonist is beneficial.

In addition, the invention also relates to a pharmaceutical composition comprising a PEG modified pan-HER antagonist of the invention or variant thereof for the prevention and/or treatment of a disease or disorder in which use of pan-HER antagonist is beneficial.

In one embodiment of the invention are provided compounds and compositions designed by making modifications to one or more features of the HER ligand variants to alter one or more properties, said properties selected from the group consisting of optimal pH or pH-activity, digestibility, antigenicity, half-life, bioavailability, the amphipathic properties, ligand-receptor interactions, thermal or kinetic stability, solubility, folding, posttranslational modification, hydrophobicity, hydrophilicity, and combinations thereof. It will be understood by those of skill in the art that the properties listed represent considerations in developing therapeutics, diagnostics and research tools and that other properties of molecules may also need to be considered and optimized depending on the particular application.

As used herein the term "optimized or optimization" refers to the modification or alteration of a molecule such that one or more characteristics of the molecule are improved for a particular purpose as compared to a starting molecule. "Modification" is the result of modifying wherein the thing being modified is changed in form or character. The molecules of the present invention being optimized via modifications include HER ligand variants and Pan-HER antagonists.
For the purposes of the instant invention, these molecules are being optimized for
the purpose of creating therapeutic, diagnostic or research reagents.

The modifications of the present invention are herein made to one or more
features of the druggable ligands, HER ligand variants or Pan-HER antagonists.

"Features" are defined as distinct amino acid sequence-based components of a
molecule. Features of the HER ligand variants or Pan-HER antagonists of the
present invention include surface manifestations, local conformational shape, folds,
loops, half-loops, domains, half-domains, sites, termini or any combination thereof.

As used herein the term "surface manifestation" refers to an amino acid-
based component of a HER ligand variant or Pan-HER antagonist appearing on an
outermost surface.

As used herein the term "local conformational shape" means an amino acid-
based structural manifestation of a HER ligand variant or Pan-HER antagonist which
is located within a definable space of the HER ligand variant or Pan-HER
antagonist.

As used herein the term "fold" means the resultant conformation of an amino
acid sequence upon energy minimization. A fold may occur at the secondary or
tertiary level of the folding process. Examples of secondary level folds include beta
sheets and alpha helices. Examples of tertiary folds include domains and regions
formed due to aggregation or separation of energetic forces. Regions formed in this
way include hydrophobic and hydrophilic pockets, and the like.

As used herein the term "turn" as it relates to protein conformation means a
bend which alters the direction of the backbone of a peptide or polypeptide and may
involve one, two, three or more amino acid residues.

As used herein the term "loop" refers to a structural feature of a peptide or
polypeptide which reverses the direction of the backbone of a peptide or polypeptide
and comprises four or more amino acid residues. Oliva et al. have identified at least

As used herein the term "half-loop" refers to a portion of an identified loop
having at least half the number of amino acid residues as the loop from which it is
derived. It is understood that loops may not always contain an even number of
amino acid residues. Therefore, in those cases where a loop contains or is identified
to comprise an odd number of amino acids, a half-loop of the odd-numbered loop
will comprise the whole number portion or next whole number portion of the loop
(number of amino acids of the loop/2 +/- 0.5 amino acids). For example, a loop
identified as a 7 amino acid loop could produce half-loops of 3 amino acids or 4
amino acids (7/2=3.5 +/- 0.5 being 3 or 4).

As used herein the term "domain" refers to a motif of a polypeptide having
one or more identifiable structural or functional characteristics or properties (e.g.,
binding capacity, serving as a site for protein-protein interactions).

As used herein the term "half-domain" means portion of an identified
domain having at least half the number of amino acid residues as the domain from
which it is derived. It is understood that domains may not always contain an even
number of amino acid residues. Therefore, in those cases where a domain contains
or is identified to comprise an odd number of amino acids, a half-domain of the odd-
numbered domain will comprise the whole number portion or next whole number
portion of the domain (number of amino acids of the domain /2 +/- 0.5 amino acids).
For example, a domain identified as a 7 amino acid domain could produce half-
domains of 3 amino acids or 4 amino acids (7/2=3.5 +/- 0.5 being 3 or 4). It is also
understood that sub-domains may be identified within domains or half-domains,
these subdomains possessing less than all of the structural or functional properties
identified in the domains or half domains from which they were derived. It is also
understood that the amino acids that comprise any of the domain types herein need
not be contiguous along the backbone of the polypeptide (i.e., nonadjacent amino
acids may fold structurally to produce a domain, half-domain or subdomain).

As used herein the terms "site" is used synonymous with "amino acid
residue" and "amino acid side chain" and "residue". A site represents a position
within a peptide or polypeptide that may be modified, manipulated, altered,
derivatized or varied within the amino acid-based molecules of the present
invention.

As used herein the terms "termini or terminus" refers to an extremity of a
peptide or polypeptide. Such extremity is not limited only to the first or final site of
the peptide or polypeptide but may include additional amino acids in the terminal
regions. The polypeptide based molecules of the present invention may be
characterized as having both an N-terminus (terminated by an amino acid with a free amino group (NH2)) and a C-terminus (terminated by an amino acid with a free carboxyl group (COOH)). Druggable ligands are in some cases made up of multiple polypeptide chains brought together by disulfide bonds or by non-covalent forces (multimers, oligomers). These sorts of ligands will have multiple N- and C-termini. Alternatively, the termini of the polypeptides may be modified such that they begin or end, as the case may be, with a non-polypeptide based moiety such as an organic conjugate.

Once any of the features have been identified or defined as a component of a molecule of the invention, any of several manipulations and/or modifications of these features may be performed by moving, swapping, substituting, inverting, deleting, randomizing or duplicating. Furthermore, it is understood that manipulation of features may result in the same outcome as a modification to the molecules of the invention. For example, a manipulation which involved deleting a domain would result in the alteration of the length of a molecule just as modification of a nucleic acid to encode less than a full length molecule would.

Modifications and manipulations can be accomplished by methods known in the art such as site directed mutagenesis. The resulting modified molecules may then be tested for activity using in vitro or in vivo assays such as those described herein or any other suitable screening assay known in the art.

Once a HER ligand variant or Pan-HER antagonist has been identified, and optionally modified or optimized, domain binding optimization (DBO) of the druggable ligand may be performed. Domain binding optimization is described in detail in copending applications; Serial Numbers 60/818,735 (Attorney docket Number 3530.3004US) and 60/818,736 (Attorney Docket Number 3530.3006US), both filed July 6, 2006, each of which is incorporated herein by reference in its entirety.

As used herein the term "binding" includes the formation of one or more ionic, covalent, hydrophobic, electrostatic, or hydrogen bonds between a receptor binding surface of the HER ligand variants and Pan-HER antagonists of the invention and one or more amino acids of a target receptor domain of a target receptor. Binding can be considered "tight" if the HER ligand variant or Pan-HER
antagonist is not substantially displaced in an in vitro assay. The HER ligand variant or Pan-HER antagonist is not substantially displaced if at least 50%, preferably at least 70%, more preferably at least about 90%, such as 100%, of the HER ligand variant or Pan-HER antagonist remains bound to a receptor or receptor moiety when competitively challenged with a native ligand. It will be understood that the HER ligand variants of the present invention may still be therapeutically relevant even if binding is 10-100 times poorer than native ligand. Binding can also be considered tight if the HER ligand variant or Pan-HER antagonist substantially displaces the native ligand from the receptor. The HER ligand variant or Pan-HER antagonist substantially displaces the native ligand if at least 50%, preferably at least 70%, more preferably at least about 90%, such as 100%, of the native ligand is displaced from the receptor.

The binding or bioactive activity of a HER ligand variant or Pan-HER antagonist of the invention can further be assessed by any other suitable assay or other method, wherein the results or activity of such assay are compared to the binding or receptor activity from an assay which measures the binding or receptor activity of wild-type human ligands and receptors.

In one embodiment of the invention, binding studies are performed on libraries of compounds of the invention. Methods of library production can also be used to create the starting molecules of the invention.

In one embodiment of the invention, the modifications made to the HER ligand variants or Pan-HER antagonists result in or from the production of a library of modified polypeptides. The library of modified polypeptides may comprise a phage library or any other selection or grouping of polypeptide sequences independent of the manner in which they were generated.

As used herein, the term "library" means a collection of molecules. A library can contain a few or a large number of different molecules, varying from about two to about $10^{15}$ molecules or more. The chemical structure of the molecules of a library can be related to each other or be diverse. If desired, the molecules constituting the library can be linked to a common or unique tag, which can facilitate recovery and/or identification of the molecule.
The HER ligand variants and Pan-HER antagonists of the present invention can be assayed for inhibition of HER-mediated bioactivity in one or more cell lines using a number of known methods, assays, devices and kits well known in the art.

In one embodiment of the invention the one or more cell lines comprises a cancer cell line. Cancer cell lines include, but are not limited to lung, breast, liver, heart, bone, blood, colon, brain, skin, kidney, pancreatic, ovarian, uterine and prostate or any cells isolated from tissues or tumors of the cancers listed herein.

In one embodiment of the invention are methods of identifying anticancer agents or anticancer ligands (ACL) comprising assaying therapeutic Pan-HER antagonists and HER ligand variants designed by the methods described herein in a tumor xenograft system wherein a measured reduction in tumor growth rate, tumor size or tumor metastasis represents a positive hit as a candidate cancer therapeutic.

In one embodiment the disease associated with HER-mediated biological activity is a tumor. In particular the tumor is a solid tumor and/or blood or lymphatic node cancer. More specifically, tumors which can be of epithelial or mesodermal origin, can be benign or malignant types of tumors in organs such as lungs, prostate, urinary bladder, kidneys, esophagus, stomach, pancreas, brain, ovaries, skeletal system, with adenocarcinoma of breast, prostate, lungs and intestine, bone marrow cancer, melanoma, hepatoma, ear-nose-throat tumors in particular being explicitly preferred as members of so-called malignant tumors.

According to the invention, the group of blood or lymphatic node cancer types includes all forms of leukemias (e.g. in connection with B cell leukemia, mixed-cell leukemia, null cell leukemia, T cell leukemia, chronic T cell leukemia, HTLV-II-associated leukemia, acute lymphocytic leukemia, chronic lymphocytic leukemia, mast cell leukemia, and myeloid leukemia) and lymphomas.

Examples of mesenchymal malignant tumors (so-called bone and soft-tissue sarcomas) are: fibrosarcoma; malignant histiocytoma; liposarcoma; hemangiosarcoma; chondrosarcoma and osteosarcoma; Ewing sarcoma; lei- and rhabdomyosarcoma, synovialsarcoma; carcinosarcoma.

Also contemplated within the scope of the invention are neoplasms. Neoplasms include: bone neoplasms, breast neoplasms, neoplasms of the digestive system, colorectal neoplasms, liver neoplasms, pancreas neoplasms, hypophysis
neoplasms, testicle neoplasms, orbital neoplasms, neoplasms of head and throat, of
the central nervous system, neoplasms of the hearing organ, pelvis, respiratory tract
and urogenital tract.

In another embodiment the cancerous disease or tumor being treated or
prevented is selected from the group of: tumors of the ear-nose-throat region,
comprising tumors of the inner nose, nasal sinus, nasopharynx, lips, oral cavity,
 oropharynx, larynx, hypopharynx, ear, salivary glands, and paragangliomas, tumors
of the lungs, comprising non-parvicellular bronchial carcinomas, parvicellular
bronchial carcinomas, tumors of the mediastinum, tumors of the gastrointestinal
tract, comprising tumors of the esophagus, stomach, pancreas, liver, gallbladder and
biliary tract, small intestine, colon and rectal carcinomas and anal carcinomas,
urogenital tumors comprising tumors of the kidneys, ureter, bladder, prostate gland,
urethra, penis and testicles, gynecological tumors comprising tumors of the cervix,
vagina, vulva, uterine cancer, malignant trophoblast disease, ovarian carcinoma,
tumors of the uterine tube, tumors of the abdominal cavity, mammary carcinomas,
tumors of the endocrine organs, comprising tumors of the thyroid, parathyroid,
adrenal cortex, endocrine pancreas tumors, carcinoid tumors and carcinoid
syndrome, multiple endocrine neoplasias, bone and soft-tissue sarcomas,
mesotheliomas, skin tumors, melanomas comprising cutaneous and intraocular
melanomas, tumors of the central nervous system, tumors during infancy,
comprising retinoblastoma, Wilms tumor, neurofibromatosis, neuroblastoma, Ewing
sarcoma tumor family, rhabdomyosarcoma, lymphomas comprising non-Hodgkin
lymphomas, cutaneous T cell lymphomas, primary lymphomas of the central
nervous system, Hodgkin's disease, leukemias comprising acute leukemias, chronic
myeloid and lymphatic leukemias, plasma cell neoplasms, myelodysplasia
syndromes, paraneoplastic syndromes, metastases with unknown primary tumor
(CUP syndrome), peritoneal carcinomatosis, immunosuppression-related
malignancy comprising AIDS-related malignancies such as Kaposi sarcoma, AIDS-
associated lymphomas, AIDS-associated lymphomas of the central nervous system,
AIDS-associated Hodgkin disease, and AIDS-associated anogenital tumors,
transplantation-related malignancy, metastasized tumors comprising brain
metastases, lung metastases, liver metastases, bone metastases, pleural and pericardial metastases, and malignant ascites.

According to the present invention, the biological activity being assayed includes, but is not limited to; a receptor-mediated pathology such as any of the diseases or conditions noted herein, receptor-mediated cell signaling, phosphorylation, cell growth, cell proliferation and tumor growth.

As used herein the term "receptor-mediated" refers to any phenomenon or condition, the occurrence of which can be linked or traced to the function or activity of a receptor, as that term is defined herein.

In one embodiment of the invention the inhibited biological activity is a receptor-mediated pathology selected from the group consisting of cancer (including all those identified hereinabove), inflammation, cardiovascular disease, hyperlipidemia, glucose dysregulation, epilepsy, allergies, Alzheimers disease, metabolic syndrome, Cortisol resistance, Crohn's disease and Huntington disease.

In one embodiment of the invention, the inhibited biological activity is receptor-mediated cell signaling. This inhibition of receptor-mediated cell signaling may result in ablation of downstream signaling by a receptor and this effect can be determined by measuring altered phosphorylation states of one or more proteins.

According to the present invention, inhibition of receptor-mediated cell signaling can be measured using autophosphorylation assays or gene expression assays. Methods of measuring and quantifying cell signaling cascades are known in the art as are methods to measure gene expression either by measuring mRNA (e.g., RT-PCR) or measuring protein levels (e.g., Western blot analysis).

It is within the scope of the present invention to design therapeutic Pan-HER antagonists that are capable of activity which is panoramic (i.e., has an effect of the same kind on multiple receptors) over two or more receptors. Further, the level or degree panoramic inhibition of biological activity may be or is substantially the same against said two or more HERs. Identification of panoramic capacity of any Pan-HER antagonist or HER ligand variant simply involves assaying the Pan-HER antagonist or HER ligand variant for inhibition of biological activity against the two or more receptors of interest.
The Pan-HER antagonists and HER ligand variants of the invention possess a number of uses. For example, the Pan-HER antagonists of the present invention can be used to treat patients wherein dysregulation of cell signaling is implicated in the pathological process of disease (e.g., cancer, inflammation). Not only may the molecules of the present invention be administered as amino-acid based molecules, they may also be administered as nucleic acid molecules in the context of gene therapy. Furthermore, these molecules may be used in diagnostic applications as well as to further basic research.

The present invention also embraces pharmaceutical compositions comprising the therapeutic Pan-HER antagonists described herein. For instance, a Pan-HER antagonist of the invention can be formulated with a pharmaceutically acceptable carrier or excipient to prepare a pharmaceutical composition. The carrier and composition can be sterile. The formulation should suit the mode of administration. As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a human without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like.

Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions (e.g., NaCl), saline, buffered saline, alcohols, glycerol, ethanol, gum arabic, vegetable oils, benzyl alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylase or starch, dextrose, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrrolidone, etc., as well as combinations thereof.

In addition, carriers such as liposomes and microemulsions may be used. The Pan-HER antagonists of the invention may also be covalently attached to a protein carrier such as albumin, or a polymer, such as polyethylene glycol so as to minimize premature clearing of the polypeptides. The pharmaceutical preparations can, if desired, be mixed with auxiliary agents, e.g. lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or aromatic substances and the like that do not
deleteriously react with the active agent in the composition (i.e., a polypeptide and/or nucleic acid molecule of the invention).

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, polyvinyl pyrrolidone, sodium saccharine, cellulose, magnesium carbonate, etc.

Methods of introduction of these compositions include, but are not limited to, transdermal, intramuscular, intraperitoneal, intraocular, intravenous, subcutaneous, pulmonary, topical, oral and intranasal. In one embodiment, topical applications include those for treating conditions such as scarring, skin cancer and psoriasis.

Other suitable methods of introduction can also include gene therapy (as described below), rechargeable or biodegradable devices, particle acceleration devices ("gene guns") and slow release polymeric devices. The pharmaceutical compositions of this invention can also be administered as part of a combination therapy with other Pan-HER antagonists or other compounds.

The Pan-HER antagonists of the present invention can be formulated in accordance with the routine procedures as a pharmaceutical composition adapted for administration to human beings. For example, compositions for intravenous administration typically are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentration in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active compound (polypeptide and/or nucleic acid). Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water, saline or dextrose/water. Where the composition is
administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The Pan-HER antagonists described herein can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The Pan-HER antagonists of the invention are administered in a therapeutically effective amount. The amount of Pan-HER antagonist that will be therapeutically effective in the treatment of a particular disorder or conditions will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, in vitro or in vivo assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the symptoms of the disease or condition, and should be decided according to the judgment of a practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

The present invention also pertains to methods of treatment (prophylactic, diagnostic, and/or therapeutic) for conditions characterized by HER-mediated pathology, HER overexpression, or dysregulation of cell signaling. A "condition characterized by dysregulation of cell signaling" is a condition in which the presence of a Pan-HER antagonist of the invention is therapeutic. Such conditions include many types of cancer. Dysregulation of cell signaling has also been implicated in a variety of other disorders. The present invention also features a method of treating a condition characterized by HER over-expression or HER ligand-mediated pathology in a patient, comprising administering to the patient a therapeutically effective amount of a pharmaceutical composition comprising at least one Pan-HER antagonist of the invention. A single Pan-HER antagonist specific for HER1, HER3 and HER4 is very desirable as it provides a powerful therapeutic for targeting and treating diseases (such as cancer) in which undesirable HER overexpression,
overexpression of HER ligands or other HER-mediated biological activity of one or more HER family members is implicated.

The term "treatment" as used herein, refers not only to ameliorating symptoms associated with the disease or condition, but also preventing or delaying the onset of the disease, and also lessening the severity or frequency of symptoms of the disease or condition. More than one Pan-HER antagonist of the present invention can be used concurrently as a co-therapeutic treatment regimen, if desired. As used herein, a "co-therapeutic treatment regimen" means a treatment regimen wherein two drugs are administered simultaneously, in either separate or combined formulations, or sequentially at different times separated by minutes, hours or days, but in some way act together to provide the desired therapeutic response. The Pan-HER antagonists of the invention may also be used in conjunction with other drugs that inhibit various aberrant activities of HER-mediated pathologies or dysregulated cell signaling. Such additional drugs include but are not limited to receptor specific antibodies, small molecule receptor inhibitors, and traditional chemotherapeutic agents.

The therapeutic compound(s) of the present invention are administered in a therapeutically effective amount (i.e., an amount that is sufficient to treat the disease or condition, such as by ameliorating symptoms associated with the disease or condition, preventing or delaying the onset of the disease or condition, and/or also lessening the severity or frequency of symptoms of the disease or condition). The amount that will be therapeutically effective in the treatment of a particular individual's disease or condition will depend on the symptoms and severity of the disease, and can be determined by standard clinical techniques. In addition, in vitro or in vivo assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or condition, and should be decided according to the judgment of a practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

A therapeutically effective amount of a Pan-HER antagonist of this invention is typically an amount of Pan-HER antagonist such that when administered in a
physiologically tolerable composition is sufficient to achieve a plasma concentration of from about 0.1 microgram (ug) per milliliter (ml) to about 100 ug/ml, preferably from about 1 ug/ml to about 5 ug/ml, and usually about 5 ug/ml. Stated differently, the dosage can vary from about 0.1 mg/kg to about 300 mg/kg, preferably from about 0.2 mg/kg to about 200 mg/kg, most preferably from about 0.5 mg/kg to about 20 mg/kg, in one or more dose administrations daily, for one or several days. Dosages recited are on a protein basis as the bulking agent (e.g., PEG) might be much bigger than the therapeutically active agent. For example, EGF weighs approximately 5,000 Daltons, while attachment of a PEG might add 50,000 Daltons to the total weight of the composition.

Dosages may also be based on the range of serum levels of EGF (0.1-1 ng/ml) and/or relative to the affinity for the ACL. Using this starting point, compounds of the invention may be administered in doses up to ten-fold these measurements. For example, if the ACL affinity is $10^{\text{InM}}$ and the affinity of EGF is $10^{\text{M}}$, then the dosing range would be between about 10 ng/mL and about 100 ng/mL.

The therapeutic compositions containing a Pan-HER antagonist or a polypeptide of this invention may be administered via a unit dose. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

The therapeutic compounds of the present invention can be used either alone or in a pharmaceutical composition as described above. For example, the gene for a Pan-HER antagonist or HER ligand variant of the present invention, either by itself or included within a vector, can be introduced into cells (either in vitro or in vivo) such that the cells produce the desired Pan-HER antagonist polypeptide. If desired, cells that have been transfected with the nucleic acid molecule of the present invention can be introduced (or re-introduced) into an individual affected with the disease.
The therapeutic Pan-HER antagonists of the invention may also be contained within a kit. As such, the invention also relates to a kit comprising the therapeutic Pan-HER antagonist and/or the pharmaceutical composition. Furthermore, the invention also relates to an array comprising the therapeutic Pan-HER antagonist and/or the pharmaceutical composition. Kits and arrays can be used in the diagnosis and/or therapy of diseases associated with the dysregulation of cell signaling. The invention also relates to the use of said therapeutic Pan-HER antagonist, said kit, said array in the diagnosis, prophylaxis, reduction, therapy, follow-up and/or aftercare of diseases associated with an HER-mediated pathology or dysregulation of cell signaling.

EXAMPLES

EXAMPLE 1: Methods and Reagents

Cloning and gene expression.

The human epidermal growth factor gene (EGF) was synthesized chemically and ligated into the Pet-9a vector (Novagen) at the Ndel and BamHI cloning sites. The EGF gene contained the OmpA leader sequence followed by an N-terminal 6x-his tag (underlined) and a factor Xa cleavage site for future his-tag removal, (BOLDED: IEGR) if necessary, and corresponds to the following amino acid sequence:

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MKKTAIAIAVALAGFATVAQA HHHHHH IEGRNSDSECPLSHDGYCLHDGVCYIEA
LDKYACNCWGYIGERCQYRDKWWELR
```

This original clone, designated pMLPPl, was used as a basis for cloning all Pan HER ligand variants (including substitution, deletion, insertion and domain swap variants) using the QuickChange mutagenesis kit (Stratagene). For protein production the EGF plasmids were transformed into E. coli strain BL21 (DE3) pLysS (Novagen).
Production of ligand variants.

Single colonies were inoculated into shake flask cultures containing 15ml LB + Km25 + Cm30. After growth overnight, samples of culture were frozen for stocks, and for plasmid preps to confirm the identities of the EGF variant gene inserts. The remaining cultures were used to inoculate production cultures in Terrific Broth + Km25 + Cm30. Cells were induced with 0.2 mM IPTG during early log phase, and the cultures were grown overnight. Culture supernatants were collected by centrifugation and production was confirmed by dot blot using the Mouse Western Breeze Chromogenic Immunodection System (Invitrogen cat#WB7103) with primary antibody: 1:1000 mouse anti-penta his antibody (Qiagen cat#34660).

EGF protein purification.

Human EGF gene was synthesized chemically and ligated into PET9a (Novagen) at the Ndel and BamHI cloning sites. The gene contained the OmpA leader sequence followed by an N-terminal His6 tag and a factor Xa cleavage site. The plasmid, pMLPP was then mutagenized with the Quick Change mutagenesis kit (Stratagene) and selected plasmids used to transform E. coli BL21 (DE3) pLysS (Novagen). Single colonies of transformants were inoculated into shake flasks cultures containing 15mL LB plus kanamycin 25ug/mL plus chloramphenicol 30ug/mL. After 24 hours culture, they were used to inoculate IL of Terrific Broth plus kanamycin plus chloramphenicol in Fernbach flasks at 225 rpm at 37oC in a New Brunswick Shaker Incubator. Cells were induced in early log phase with 0.2 mM IPTG and grown overnight. Supernatants were collected by centrifugation, adjusted to pH 8.0, and loaded onto a 5mL nickel-NTA matrix (Qiagen #30230). After washing with PBS and PBS plus 50mM imidazole, recombinant His tagged proteins were eluted with PBS plus 250mM imidazole, dialyzed in PBS and concentrated with a 3000 MWCO centrifugation device. BioRad Coomassie Plus and 15% SDS-PAGE gel electrophoresis with Coomassie staining and anti-EGF immunoblots were used to confirm purity and identity, respectively. Recombinant proteins were secreted into the media at 25ug/mL. Competitive binding with 125I-
EGF has been accomplished in our laboratory with commercial radiolabeled EGF and A431 cells and EGFR targeted proteins (Coco et al. 2002, Nat Biotechnol. 20:1246-1250). EGF bound EGFR on A431 cells with a dissociation constant of InM and a single class of receptors.

Ligand binding assay.

Competition binding assays were performed using displacement of biotinylated panerbin binding to measure affinity. Panerbin was biotinylated using NHS-Biotin Reagent (Pierce). A431 cells and MDA-MB-453 cells were used as a source of EGFR and HER3, respectively. A431 cells were grown in DMEM with 10% FBS in an atmosphere containing 5% CO2; MDA-MB-453 cells were grown in Liebovitz's L-15 with 10% FBS in ambient CO2. Cells were trypsinized, and transferred to 96 well plates at 10^5 cells per well for A431 and 3 X 10^5 cells per well for MDA-MB-453. Each well contains a fixed amount of biotinylated panerbin and varied amounts of the competing ligand. Plates were incubated for 1 hour on ice. Unbound ligand was washed off and bound panerbin detected with horse radish peroxidase conjugated to streptavidin (Pierce), using 1-Step Ultra TMB-ELISA (Pierce).

Phosphorylation Assay.

DuoSet ELISA kits (R&D Systems) are used to measure total and phosphorylated levels of EGFR, HER3 and HER4 in T47D cell lysates. It has been observed that this cell line produces measurable amount of all four HER receptors and that these receptors can be phosphorylated in cells treated with the appropriate ligand. The cells are grown in complete medium and then serum starved overnight. They are then treated with ligand in serum-free medium containing 1 mM Na3VO4 at 37° for 15 minutes. Cells are washed and then treated with PBS containing 1 mM Na3VO4 on ice for 15 minutes. Cells are resuspended by scraping and lysed with Cellytic-M (Sigma) containing 1 mM Na3VO4 plus protease inhibitors. Lysates are stored at -80° prior to analysis.
Protein concentrate and buffer exchange.

Column eluents were dialyzed in PBS at 4 °C with one buffer exchange, and then concentrated with 3000 MWCO Macrosep centrifuge devices (ISC# OD003C41). The final product was tested for protein concentration using the BCA method and for purity by SDS-PAGE.

Phage panning.

Phage panning is performed according to the teachings of Rodi and Malowski, (Curr. Opin. Biotechnol, 10:87-93; 1999). Briefly, genes encoding HER ligand variants were cloned into the pentavalent M13 phage display system (New England Biolabs). Sequences may include those coding for pan-HER agonists (TIE, WVS, and BiR); those coding for HER ligand variants having the agonist modifications in addition to modifications that reduce binding to HER receptor domains (e.g. R41D and L47G); and those variants generated from libraries constructed using the Kpn I and Eag I restriction sites of the M13KE phage vector for expression as an N-terminus-fusion with the pill coat protein of the M13 phage.

All five copies of pill should display the cloned protein. To produce phage, the vector with insert was transformed into electrocompetent E. coli 10GF. Transformation outgrowth was used to infect E. coli and infected cells were plated on LB+tet20+xgal+IPTG. Blue plaques resulting from the infection were amplified and plasmid DNA was sequenced to verify the identity of the insert. Phage were amplified by infecting E. coli in LB culture, and cells were removed by centrifugation. Phage were harvested by PEG precipitation.

These phage are used to measure binding affinity of the HER ligand variants as well as biological activity by stimulation of HER receptor dependent cell proliferation or phosphorylation. The phage binding assay system is disclosed in co-pending applications; Serial Numbers 60/818,735 (Attorney docket Number 3530.3004US) and 60/818,736 (Attorney Docket Number 3530.3006US), both filed July 6, 2006, each of which is incorporated herein by reference in its entirety.
Phage ELISA for analysis of binding affinity

A431 cells for EGFR binding or T47D cells for HER3 binding are grown as monolayers in tissue culture flasks in media containing fetal bovine serum. Cells are trypsinized, neutralized with growth medium, washed twice with DPBS and resuspended in ice-cold PBS-Glu-T. 10^5 cells are transferred to 96 well plates and incubated on ice for 1 hour in the presence of varied concentrations of phage. Cells are centrifuged and washed 5X with PBS-T then incubated for one hour at room temperature with anti-M13 pVIII coat protein antibody conjugated with horseradish peroxidase (HRP). Cells are again centrifuged and washed 5X with PGS-T. Color is developed with TMP followed by H_2SO_4. Cells are then pelleted and supernatant transferred to optically transparent plate for measurement of absorbance at 450 nm.

Phage particles displaying ligand variants are evaluated for binding affinity to the HER1 receptor (EGFR) in T47D whole cell suspension by measuring absorbance at Abs450. Theoretical estimates may also be performed.

Additional phage particles displaying HER ligand variants may be evaluated for binding affinity to the HER3 receptor in A431 whole cell suspensions by measuring absorbance at Abs450.

Additional phage particles displaying HER ligand variants may be evaluated for inhibition of phosphorlation of HER3 receptor in A431 whole cell suspensions by measuring absorbance at Abs450.

Cell lines

**HER5 cells**

The HER5 cell line, a murine fibroblast line (derived from the NR-6 line; mouse fibroblast cells that overexpress human EGFR) that has been stably transfected to express the human EGF receptor was provided by Dr. M.C. Hung (MD Anderson Cancer Center).

Stock cultures of HER5 are propagated in D-MEM/F12 medium containing 10% fetal bovine serum, 100 units/ml of penicillin and 100 ug/ml of streptomycin in a water-jacketed incubator at 37°C in a humidified 5% CO_2 atmosphere.

For HER5 proliferation assays, the cells are changed into DMEM/F12 without serum for 24 hours. Cells are then trypsinized and suspended at 1E5
cells/ml. Serial dilutions of EGF (PeproTech, Rocky Hill, NJ), and HER ligand polypeptide variants are prepared in serum-free DMEM/F12 at 2-fold the final concentration and plated into the wells of 96-well plates. Fifty microliters of cell suspension (5000 cells) is added to appropriate wells bringing the total volume to 100 ul at the desired concentrations. Plates are incubated for a 48 hour proliferation period. Cell proliferation is determined by addition of 10 ul/well of WST-I Cell Proliferation Reagent (Roche Applied Sciences, Indianapolis, IN) for the last three hours of the proliferation period. WST-I is a tetrazolium salt that is cleaved to formazan dye by mitochondrial dehydrogenases in viable cells. The amount of formazan is measured at 450 nm using a microplate reader (Dynex Technologies) with MRX Revelation software.

**MCF-7 cells**

MCF-7 cells (human breast cancer cell lines that express HER2 and HER3) are obtained from the American Type Culture Collection (ATCC). Stock cultures of MCF-7 are maintained in Eagle's MEM supplemented with 1% ITS-X (Invitrogen) and 10% fetal bovine serum.

For proliferation assays, MCF-7 cells are transferred to serum-free medium (SFM) for 24 hours and then trypsinized and suspended at $10^5$ cells/mL in SFM.

Fifty microliters of cell suspension (5000 cells) is plated per well in 96 well microtiter plates. Serial dilutions of HER ligands or mutant proteins are prepared at twice the final concentration in SFM and 50 ul is added to wells, bringing the final volume to 100 ul at the desired final concentration. Plates are incubated for 72 hours at 37°C in a humidified 5% CO2 atmosphere. Cell proliferation is determined by addition of 10 ul/well of WST-1 Cell Proliferation Reagent (Roche Applied Sciences, Indianapolis, IN) for the last three hours of the proliferation period.

**A431 cells**

The human epidermoid carcinoma line, A431, is obtained from ATCC.

Stock cultures of A-431 are propagated in DMEM medium containing 10% fetal bovine serum. A431 cells are used to evaluate EGFR receptor binding.
**T47D cells**

Human ductal carcinoma cells are obtained from ATCC. They are maintained in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate and supplemented with 0.2 Units/ml bovine insulin, 90%; fetal bovine serum, 10%.

T47-D cells are used to evaluate HER3 receptor binding.

### EXAMPLE 2: Identification of Pan-HER antagonist

Using the phage panning assay described herein, phage libraries of the two halves of the B-loop, amino acids 21-25 and amino acids 26-30 were created. Each library, comprising over 1 billion variants, was panned against HER1 targets and HER3 targets.

For the second half of the B-loop, amino acids 26-30, the best sequences (best binders) were EPQRG and QPQRG. Sequences containing these substitutions were found to be better binders to HER1 and HER3, even though the K28 has been replaced by R. This result significantly reinforces the observation in the art that K28 can safely be substituted. The enhanced binding through the new residues makes using arginine (R) at position 28 as arginine is occasionally susceptible to derivatization with polyethylene glycol (PEG) moieties.

The sequences identified during these studies are summarized in Table 3. In the table, the B-loop motifs, EPQRG and QPQRG are **BOLDED**.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Protein Sequence</th>
<th>SEQ ID NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPQRG second half</td>
<td>NSDSECPLSHDGYCLHDGVCMYIEAE [EPQRG] CNVC VGY1GERCQYRLKWWELR</td>
<td>9</td>
</tr>
<tr>
<td>QPQRG second half</td>
<td>NSDSECPLSHDGYCLHDGVCMYIEAQ [PQRG] CNVC VGY1GERCQYRLKWWELR</td>
<td>10</td>
</tr>
</tbody>
</table>

The randomized library of the first half of the B-loop, amino acids 21-25 was then created and panned against HER1 and HER3. It has been suggested in the art that this section of the B-loop is the most important for binding to HER3. Again, the
best binding sequence was found to be EPQRG which was identified frequently in the post-panning pool.

As a result, HER ligand variant sequences including the "EPQRG motif" in the first half of the B-loop should represent a superior starting point for PEGylation, based on the expectation that it will be a better binder than the variant with the changes in residue 26-30.

In another study, variant EGFDI was designed and tested. Substitutions were made to the EPQRG-first half sequence to further improve the properties of the variant. To this end the N-terminus included substitutions of amino acids 2-3 from SD to WV as well as a substitution of lysine at position 28 (K28) to leucine (K28L). This variant was also found to possess excellent binding properties.

In another study, variant EGFDIII was designed and tested. Starting with an R45Y mutation to enhance binding to Domain III of HER3 and HER4, residues S and D at positions 2 and 3 were retained (because they prevent binding of EGF to HER3 and HER4 at Domain I) and substitutions Y22D and L26G were incorporated to further ablate binding to Domain I of EGFR. The EGFDIII variant is useful when PEGylation at the 28 position is desired. Once pegylated, this variant should not interact with Domain I.

Table 4 summarizes the B-loop (first half) HER ligand variants designed and tested. Substitutions are BOLDED and underlined.

Table 4

<table>
<thead>
<tr>
<th>Variant</th>
<th>SEQUENCE</th>
<th>SEQ ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPQRG</td>
<td>NSDSECPLSHDGYCLHDGVC {EPQRG}LDKYACNCVVGYZGER CQYRDKWWELR</td>
<td>11</td>
</tr>
<tr>
<td>EGFDI</td>
<td>NWVSECP {LDG}L {LDG}LYACNCVVGYZGER CQYRDKWWELR</td>
<td>12</td>
</tr>
<tr>
<td>EGFDIII</td>
<td>NSDSECPLSHDGYCLHDGVC {DIEAG}KYACNCVVGYZGER CQYLDLWWELR</td>
<td>13</td>
</tr>
</tbody>
</table>

The strategy employed here is particularly attractive since derivatization here will further impede ligand binding to Domain III, which is the strategy formerly used to create ACLs. It is further attractive since it minimizes (to one) the number of lysines to be derivatized, which has significant implications for the production of the final drug, its purification and standardization.
This could only be achieved if K28 could be removed or made inaccessible. Previous mutagenesis studies (Groenen, L. et al., 1994, *Growth Factors*, 11:253-257) indicated that a K28L or K28R mutation did not significantly change binding of the ligand to EGFR. However, the unchanged high affinity binding of the Domain I:C-loop interactions would have masked any real impact of this mutation. Its contribution could only be assessed in a mutant with the dominant binding interaction ablated, such as the RL mutant (native EGF wherein amino acid R at position 41 is replaced by D and amino acid L at position 47 is replaced by G). Therefore its contribution in low affinity binding at Domain I, and to the improved binding desired, could not be predicted from published results.

Although the residue at amino acid position 28 is not particularly conserved in the HER ligands, it is conserved in the EGFR ligands EGF and TGF-alpha, and the others have a positively charged residue (such as lysine) nearby.

It was therefore impossible to predict the significance of this residue for binding, especially in the context of the objective to significantly increase binding and activity through randomization and affinity maturation of the B-loop of EGF.

Reported here is the successful substitution of the K28 residue with retention of antagonist properties. These studies demonstrate that the B-loop and K28 can be significantly altered, and the K replaced, while maintaining or even enhancing antagonist activity.

This observation is in contrast to the published results in which substitutions of K28 did not significantly change binding. While not wishing to be bound by theory, it is likely that it is the unique combination of the five randomized amino acids that allows lysine to be replaced at this vital binding site and for activity to be increased.

**EXAMPLE 3: PEGylation**

In order to form PEGylated pan-HER antagonists or HER ligand variants of the present invention, polymers such as poly(alkylene oxide) are converted into activated forms, as such term is known to those of ordinary skill in the art. The reactive group, for example, is a terminal reactive group, which mediates a bond between chemical moieties on the protein and poly(ethylene glycol).
Typically, one or both of the terminal polymer hydroxyl end-groups, (i.e. the alpha and omega terminal hydroxyl groups) are converted into reactive functional groups, which allows covalent conjugation. This process is frequently referred to as "activation" and the poly(ethylene glycol) product having the reactive group is referred to as "an activated poly(ethylene glycol)."

In one embodiment, one of the terminal polymer hydroxyl end-groups is converted or capped with a non-reactive group. In one embodiment one of the terminal polymer hydroxyl end-groups is converted or capped with a methyl group. As used herein, the term "mPEG" refers to a PEG, which is capped at one end with a methyl group.

The activated polymers are thus suitable for mediating a bond between chemical moieties on the protein, such as alpha- or epsilon-amino, carboxyl or thiol groups, and poly(ethylene glycol).

Bis-activated polymers can react in this manner with two protein molecules or one protein molecule and a reactive small molecule in another embodiment to effectively form protein polymers or protein-small molecule conjugates through cross linkages.

Secondary amine or amide linkages are formed using the epsilon-amino groups of lysine of a pan-HER antagonists or HER ligand variant and the activated PEG.

A secondary amine linkage may also be formed between the lysine epsilon-amino group of a pan-HER antagonists or HER ligand variant and single or branched chain PEG aldehyde by reductive alkylation with a suitable reducing agent such as NaCNBH₃, NaBH₃, pyridine borane etc. as described in Chamow et al., Bioconjugate Chem. 5: 133-140 (1994), U.S. Pat. No. 4,002,531, WO 90/05534, and U.S. Pat. No. 5,824,784.

The chemical modification through a covalent bond may be performed under any suitable condition generally adopted in a reaction of a biologically active substance with the activated poly(ethylene glycol).

There are several suppliers of PEGylation reagents. In one embodiment, para-nitrophenyloxycarbonyl-PEG derivatives for the PEGylation studies may be used. "PNP-PEG" is a well established starting material and generates a higher
percentage of conjugated and stable product compared to other linkers (such as NHS esters). They are also easy to make (Conditions: borate-phosphate buffer (pH 8.0-8.3), room temperature with gentle stirring, overnight (Sartore, L. *et al.* 1991. *Appl. Biochem. Biotechnol.* 27(I):45-54). Dow Pharma supplies very high purity material (narrow polydispersity and very low levels of PEG diol).

Mono-PEGylated EGF can be purified by dialysis (MW cutoff = 10,000) in Tris buffer (Tris-HCl, 5mM, pH 8.0). This removes unreacted PEG, unreacted EGF and other chemical reagents (Basu, A. *et al.* 2006. *Bioconjugate Chem* 17:618-630).

The purity and physical properties of the PEGylated protein can be evaluated using standard methods. For example, a poly(ethylene glycol)-modified pan-HER antagonist or HER ligand variant may be purified from a reaction mixture by conventional methods which are used for purification of proteins, such as dialysis, salting-out, ultrafiltration, ion-exchange chromatography, hydrophobic interaction chromatography (HIC), gel chromatography and electrophoresis.

**EXAMPLE 4:** Pegylation of HER ligand variants

Sequences identified during phage panning (See Example 2) which may be pegylated include those shown in Tables 3 and 4.

First, the HER ligand variants, are produced and the proteins purified for PEGylation. Pan-HER optimized EGF variants can then be conjugated with PEG molecules big enough to provide a benefit of PEGylation (extended serum half life) while still retaining clinically relevant Pan-HER binding affinity.

Pegylation is performed using standard methods, using a relatively small PEG (5000 mol wt.) and a large PEG (40,000 mol wt). Evaluation of the 5K-PEGylated material will allow comparison to published experiments with PEGylated EGF, and the effect of a small PEG on Pan-HER antagonist activity. Testing the larger (40K-PEGylated form) will allow testing of the central hypothesis.

To confirm the functionality of the constructs, binding constants of the proteins with and without PEGylation using a standard Ligand-displacement assay can be performed. For in vivo confirmation, serum half-life in rodent models may be determined using well established methods.
EXAMPLE 5: In Vitro Activity

The EGFDI and EGFDIII with and without PEGylation can be tested for their abilities to displace panerbin in competition binding assays and to antagonize panerbin-dependent phosphorylation of EGFR, HER3 and HER4 in T47D cells using the assays described herein.

The PEG5K (HER ligand variant modified with a 5K PEG moiety) proteins should bind with an affinity similar to that reported for non-PEGylated proteins. If the binding of the PEG40K (HER ligand variant modified with a 40K PEG moiety) proteins is significantly lower than that of the 5K material, it is likely that the larger PEG is interfering with the single domain binding and further optimization may be necessary. If the binding of the PEGylated proteins is similar to that of the non-PEGylated proteins, it is expected that they will demonstrate equivalent or improved antagonism towards HER receptor phosphorylation because the PEG groups are designed to add to the interference with the conformational shift that is necessary for receptor dimerization and activation.

EXAMPLE 6: Pharmacokinetics

Following the methods of Basu et al. (2006, Bioconjugate Chem 17:618-630) the serum stability of the original and PEGylated compounds can be evaluated. Using C57BL/6 mice (7-8 week, female) supplied by Sprague Dawley Harlan (Madison, WI), mice (5/group) are injected intravenously with 100µL per mouse (0.2 mg protein/kg) of the authentic protein or the PEGylated conjugates. Following sedation with 0.09% avertin, sampling of blood will be undertaken via the retro-orbital sinus into vials containing EDTA. At 2, 15, 30, and 60 min, the mice are bled 100 µL, and at 4, 24, 48, 72, and 96 h, mice are terminally bled by cardiac puncture. The plasma is collected following centrifugation of the blood at 5000 rpm at 4 °C for 5 min and immediately frozen on dry ice. The concentrations of the compounds are analyzed by EGF- and His-tag ELISA. The data are modeled to determine pharmacokinetic parameters using a two compartment, bolus, first-order elimination model for the intravenous samples.

The objective of this exercise is to block binding to one of the HER ligand binding domains with the PEG unit, thus creating an antagonist while also benefiting
from the new desirable therapeutic properties bestowed by the PEG. Antagonist
properties of the compounds are measured using the phosphorylation assay disclosed
herein.

The patent and scientific literature referred to herein establishes the
knowledge that is available to those with skill in the art. All United States patents
and published or unpublished United States patent applications cited herein are
incorporated by reference. All published foreign patents and patent applications
cited herein are hereby incorporated by reference. All other published references,
documents, manuscripts and scientific literature cited herein are hereby incorporated
by reference.

While this invention has been particularly shown and described with
references to preferred embodiments thereof, it will be understood by those skilled
in the art that various changes in form and details may be made therein without
departing from the scope of the invention encompassed by the appended claims.
CLAIMS

What is claimed is:

1. A HER ligand variant comprising:
   (a) one or more amino acid substitutions in the B-loop (amino acids 21-30) of the wild-type human epidermal growth factor (EGF), and
   (b) a polyethylene glycol (PEG) moiety attached to a lysine of the HER ligand variant of (a) wherein the lysine is either lysine 28 (K28) or lysine 48 (K48).

2. The HER ligand variant of claim 1, which is a Pan-HER antagonist.

3. The HER ligand variant of claim 1, wherein the one or more amino acid substitutions in the B-loop are in the second half of the B-loop (amino acids 26-30) and wherein the PEG moiety is attached to lysine 48 (K48).

4. The HER ligand variant of claim 3, wherein the one or more amino acid substitutions in the B-loop comprise replacing the amino acids of the second half of the B-loop (LDKYA) with amino acids EPQRG or QPQRG, and wherein the PEG moiety is attached to lysine 48 (K48).

5. The HER ligand variant of claim 4, wherein the second half of the B-loop is QPQRG, and wherein the PEG moiety is attached to lysine 48 (K48).

6. The HER ligand variant of claim 5 having the amino acid sequence of SEQ ID No. 10.

7. The HER ligand variant of claim 1, wherein the PEG moiety is a PEG derivative.
8. The HER ligand variant of claim 5, wherein the polyethylene glycol moiety is substantially linear and has a molecular weight from about 10,000 to about 40,000 Daltons.

9. The HER ligand variant of claim 4, wherein the second half of the B-loop is EPQRG, and wherein the PEG moiety is attached to lysine 48 (K48).

10. The HER ligand variant of claim 9 having the sequence of SEQ ID NO: 9.

11. The HER ligand variant of claim 9, wherein the polyethylene glycol moiety is substantially linear and has a molecular weight from about 10,000 to about 40,000 Daltons.

12. A HER ligand variant of human wild-type epidermal growth factor comprising:
   (a) substitution of arginine (R45) with tyrosine resulting in (R45Y),
   (b) substitution of lysine (K48) with leucine resulting in (K48L), and
   (c) a polyethylene glycol (PEG) moiety attached to a wherein the lysine is either lysine 28 (K28).

13. The HER ligand variant of claim 12 further comprising:
   a) substitution of tyrosine (Y22) with aspartic acid resulting in (Y22D), and
   b) substitution of leucine (L26) with glycine resulting in (L26G).

14. The HER ligand variant of claim 13 having the amino acid sequence of SEQ ID No. 13.

15. The HER ligand variant claim 12, wherein the polyethylene glycol moiety is substantially linear and has a molecular weight from about 10,000 to about 40,000 Daltons.
16. The HER ligand variant of claim 1, wherein the one or more amino acid
substitutions in the B-loop are in the first half of the B-loop (amino acids 21-
25) and wherein the PEG moiety is attached to lysine 48 (K48).

5 17. The HER ligand variant of claim 16, wherein the one or more amino acid
substitutions in the B-loop comprise replacing the amino acids of the first
half of the B-loop (LDKYA) with amino acids EPQRG, and wherein the
PEG moiety is attached to lysine 48 (KAS).

10 18. The HER ligand variant of claim 17 further comprising:
(a) substitution of lysine (K28) with leucine resulting in (K28L),
(b) substitution of serine (S2) with tryptophan resulting in (S2W), and
(c) substitution of aspartic acid (D3) with valine resulting in (D3V).

15 19. The HER ligand variant of claim 18, wherein the polyethylene glycol moiety
is substantially linear and has a molecular weight from about 10,000 to about
40,000 Daltons.

20. A pharmaceutical composition comprising the HER ligand variant of any of
claims 1-19 and a pharmaceutically acceptable carrier.

21. A method of treating a patient with a disease characterized by overexpression
of HER comprising, administering to the patient, a therapeutically effective
amount of a pharmaceutical composition of claim 20.

22. The method of claim 21, wherein the disease is cancer.

23. The method of claim 22, wherein the cancer is selected from the group
consisting of gliomas, squamous cell carcinomas, breast carcinomas,
melanomas, invasive bladder carcinomas, colorectal carcinomas and
esophageal cancers.
24. A HER ligand variant comprising:
   (a) substitution of lysine (K28) and lysine (K48) of the wild-type human epidermal growth factor (EGF), with a non-reactive amino acid, and
   (b) substitution of one of any of the amino acids in the sequence forming the Domain I binding face, said Domain I binding face consisting of residues 1-5 (NSDSE) and residues 20-33 (MYIEALDKYACNC), with a conjugating amino acid that can be selectively conjugated with a bulking agent.

25. The HER ligand variant of claim 24, wherein the non-reactive amino acid is leucine.

26. The HER ligand variant of claim 24, wherein the conjugating amino acid is lysine.

27. The HER ligand variant of any of claims 24-26 further comprising
   (c) one or more amino acid substitutions in residues 34-48 (VVGYIGERCQYRDLK) of the wild-type human epidermal growth factor (EGF), and
   (d) optionally, one or more further substitutions in residues 7-19 (PLSHDGYCLHDGV).

28. The HER ligand variant of claim 27 further comprising attachment of a polyethylene glycol (PEG) moiety to the conjugating amino acid in the Domain I binding face.
29. The HER ligand variant of claim 28, wherein the conjugating amino acid is lysine.

30. A HER ligand variant comprising:

(a) substitution of lysine (K28) and lysine (K48) of the wild-type human epidermal growth factor (EGF) with a non-reactive amino acid and

(b) substitution of one of any of the amino acids in the sequence forming the Domain III binding face, said Domain III binding face consisting of residues 34-48 (VVGYIGERCQYRDLK) and residues 7-19 (PLSHDGYCLHDGV) of the wild-type human epidermal growth factor (EGF) with a conjugating amino acid.

31. The HER ligand variant of claim 30, wherein the conjugating amino acid is lysine.

32. The HER ligand variant of claim 30 further comprising

(c) one or more amino acid substitutions in a first half of the B-loop (amino acids 21-25) of the wild-type human epidermal growth factor (EGF), and

(d) optionally, one or more further substitutions in a second half of the B-loop (amino acids 26-30).

33. The HER ligand variant of claim 32 further comprising attachment of a polyethylene glycol (PEG) moiety to the conjugating amino acid in the Domain III binding face.

34. The HER ligand variant of claim 33, wherein the conjugating amino acid is lysine.