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(72) Inventors; and

(74) Agent: GUTERMAN, Sonia, K.; Lawson & Weitzen, LLP, 88 Black Falcon Avenue, Suite 345, Boston, MA 02210-2414 (US).


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(54) Title: POLYMALIC ACID-BASED NANOBIOPOLYMER COMPOSITIONS AND METHODS FOR TREATING CANCER

Figure 7

(57) Abstract: Nanobiopolymeric conjugates based on biodegradable, non-toxic and non-immunogenic poly (β-L-malic acid) PMLA covalently linked to molecular modules that include morpholino oligonucleotides (AONa), an siRNA or an antibody specific for an oncogenic protein in a cancer cell, and an antibody specific for a transferrin receptor protein, are efficient for treatment a cancer in a subject.
Polymalic Acid-Based Nanobiopolymer Compositions and Methods for Treating Cancer

Technical field
The present invention generally relates to compositions and methods for treating patients having cell proliferative disorders with polymalic acid-based nanobiopolymeric compositions that inhibit synthesis and activity of an oncogenic protein.

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Background
Breast cancer is a disease affecting a significant population of women around the world. About 1 in 8 women in the United States (between 12 and 13%) will develop invasive breast cancer over the course of her lifetime. Prognosis and survival rate varies greatly depending on cancer type and staging. Breast cancers expressing genetic characteristics such as human epidermal growth factor receptor-2 (HER2) are associated with a poor prognosis.

Research has focused on the use of recombinant humanized monoclonal antibodies for the treatment of cancers with cells that overexpress protein pl85HER2. This 185-kDa growth factor receptor is encoded by the her-2 proto-oncogene, also referred to as neu and c-erbB-2 (Slamon et al. 1987 Science 235: 177). The her-2 gene is closely related to the gene encoding epidermal growth factor receptor (EGFR). Amplification of the her-2 gene has been linked to neoplastic transformation in human breast cancer cells (Slamon et al. 1987 Science 235:177). Overexpression of the HER2 protein has been identified in 20-30% of breast cancer patients, and has been correlated with regionally advanced disease, increased probability of tumor recurrence, and reduced patient survival. As many as 30-40% of patients having gastric, endometrial, salivary gland, non-small cell lung, pancreatic, ovarian, peritoneal, prostate, or colorectal cancers may also exhibit overexpression of this protein.

A more difficult-to-treat form of HER2-negative breast cancer known as "triple-
negative," affects some patients. This form tests negative for three primary receptors: HER2, estrogen receptor and progesterone receptor.

Humanized mti-HER2/neu monoclonal antibody trastuzumab (Herceptin®, Genentech Inc., San Francisco, CA) is used alone or combined with chemotherapy for treatment of patients with advanced breast cancer overexpressing HER2/neu (Baselga J. 2006 Science 312:1175; Baselga J et al. 1999 Semin Oncol 26:78; Slamon DJ et al. 2009 J Natl Cancer Inst 101:615), with significant anti-tumor effect. However, serious adverse effects on normal organs have been reported (Keef DL. 2002 Cancer 95:1592; Vahid B et al. 2008 Chest 133:528). Moreover, many patients develop resistance to Herceptin® within one year of treatment, which renders this treatment ineffective (Tseng PH et al. 2006 Mol Pharmacol 70:1534). Therefore, new drugs with minimal side effects for non-tumor tissues are urgently needed to improve HER2/«ew-positive tumor therapy.

Summary of embodiments

An embodiment of the invention provided herein is a drug delivery composition for treating a cancer in a subject, the drug including a plurality of biologically active molecular modules having at least one module that targets a tumorigenic cell or a cancer cell, at least one module that inhibits synthesis or activity of a human epidermal growth factor receptor (HER) protein in the cell, and at least one module for cytoplasmic delivery, and a polymalic acid-based molecular scaffold, such that the molecular modules are covalently linked to the scaffold. For example, the HER protein is at least one protein selected from the group of: HER1, HER2, HER3 and HER4. For example, the at least one module that inhibits synthesis or activity of the protein is selected from the group of: an antisense oligonucleotide (AON), an siRNA oligonucleotide, an antibody, a polypeptide, an oligopeptide and a low molecular weight drug. The scaffold in a related embodiment includes a poly-P-L-malic acid (PMLA) also denoted poly(-P-L-malic acid).

The AON in related embodiments is a Morpholino AON having a sequence complementary to a sequence contained in an mRNA transcript of HER2/neu protein. For example, the AON is selected from: 5'-AGGGAGCCGACTTCATGTCTG-3' (SEQ ID NO: 1), and 5'-CATGGTGCTCAGCGCTCCG-3' (SEQ ID NO:2).

In a related embodiment the at least one module that targets the cell includes an antibody that binds specifically to a vasculature protein in the cell. For example, the vasculature protein includes a transferrin receptor protein. In general, the antibody is
selected from at least one of: anti-human, rat anti-mouse, rat anti-human, rabbit anti-human and goat anti-human.

In an alternative embodiment the at least one module that inhibits activity of the protein includes an antibody binding specifically to a HER2/neu protein. For example, the antibody is Herceptin®.

The composition in alternative embodiments includes a Morpholino AON having a sequence complementary to a sequence contained in an mRNA transcript of an epidermal growth factor receptor (EGFR) protein. For example, the sequence includes 5’-TCGCTCCGCTCTCCCCGATCAATAC-3’ (SEQ ID NO:3).

The composition in related embodiments includes a Morpholino AON having a sequence complementary to a sequence contained in an mRNA transcript of at least one subunit of laminin-41. For example, the subunit is selected from an α4 subunit and a β1 subunit. The sequence complimentary to the transcript of the α4 subunit includes 5’-AGCTCAAGCCATTTCTCCGCTGAC-3’ (SEQ ID NO:4). The sequence complimentary to the transcript of the β1 subunit includes 5’-CTAGCAACTGGAGAAGCCCATGCCC-3’ (SEQ ID NO:5).

In an alternative embodiment the composition includes the siRNA oligonucleotide having a sequence complementary to a gene encoding an EGFR protein. For example, the sequence includes a sense sequence 5’-CCUAUAAUUGCUACGAAAUUt-3’ (SEQ ID NO:6) and an antisense sequence 5’-AUUUUCGUAGCAUUAUGGag-3’ (SEQ ID NO:7).

The siRNA oligonucleotide in a related embodiment includes a sequence complementary to a gene encoding a HER2 protein. For example, the sequence includes a sense sequence 5’-GUUGGAUGAUUGACUCUGAAtt-3’ (SEQ ID NO:8) and an antisense sequence 5’-UCAGAGUCAAUCAUCCCCAcat-3’ (SEQ ID NO:9).

In a related embodiment the at least one module for cytoplasmic delivery includes an endosome escape unit. In general, the endosome escape unit is selected from a group of: leucine residues, valine residues and a leucine ethylester. i.e., a plurality of leucine or valine residues, or a single or a plurality of leucine residues, or mixture of any of these. For example, a concentration of the leucine ethylester in a related embodiment is about 40% of the drug delivery composition.

The plurality of modules in related embodiments further includes a polyethylene glycol (PEG). For example, the PEG has a molecular weight of about 1,000 Da, about 5,000
Da, about 10,000 Da, about 15,000 Da, about 20,000 Da, about 25,000 Da and about 30,000 Da.

An embodiment provides the composition in a unit dose effective for treatment of the cancer in the patient. For example, the unit dose is at least one selected from: 1 μg/kg, 50 μg/kg, 100 μg/kg, 500 μg/kg, 1 mg/kg, 5 mg/kg, 10 mg/kg, 50 mg/kg, and 100 mg/kg. For example, the unit dose is at least 1 mg/kg. For example, the unit dose is less than about 10 mg/kg.

In related embodiments, the cancer is at least one selected from the list of: gastric, endometrial, salivary gland, lung, non-small cell lung, pancreatic, ovarian, peritoneal, prostate, colorectal, breast, cervical, uterine, ovarian, brain, head and neck, testicular and teratoma cancers. For example, the breast cancer is a triple-negative breast cancer. For example, the cancer includes either a primary cancer or a metastatic cancer, or both. The cancer in another embodiment includes cells overexpressing a HER2/neu receptor protein.

An embodiment of the invention herein is a drug delivery composition for treating a cancer in a subject including: a polymerized carboxylic acid molecular scaffold such as a poly-p-L-malic acid (PMLA), and a plurality of biologically active molecular modules including an antisense molecule that substantially inhibits synthesis of a HER2/neu receptor protein, a molecular module to facilitate delivery of the antisense molecule to cytoplasm, at least one antibody specific for the receptor protein that inhibits activity of the receptor protein, at least one antibody targeting a tumor vasculature protein, and a molecular module that prolongs circulation of the composition, such that the molecular modules are covalently linked to the scaffold.

The invention in an embodiment provides a drug delivery composition for treating a cancer in a subject including: a polymerized carboxylic acid molecular scaffold such as a poly-p-L-malic acid (PMLA), and a plurality of biologically active molecular modules including an antisense molecule that substantially inhibits synthesis of an epidermal growth factor receptor (EGFR) protein, an antisense molecule that substantially inhibits at least one subunit of laminin-41, a molecular module to facilitate delivery of the antisense molecule to cytoplasm, at least one antibody targeting a tumor vasculature protein, and a molecular module that prolongs circulation of the composition, such that the molecular modules are covalently linked to the scaffold.

The invention in another embodiment provides a kit for treating a patient having a cancer that includes a drug delivery composition including a nanobiopolymeric conjugate of
a scaffold such as a poly-\textit{P-L-malic} acid (PMLA) and molecular modules including an antisense molecule that substantially inhibits synthesis or activity of a human epidermal growth factor receptor (HER) protein, a molecular module to facilitate delivery of the antisense molecule to cytoplasm, at least one targeting antibody specific for the HER protein, at least one antibody specific for a tumor vasculature protein, and a molecular module that prolongs circulation of the composition, such that the PMLA is covalently linked to the molecular modules, in a container. The kit in related embodiments further includes a pharmaceutically acceptable buffer and instructions for use.

An embodiment of the invention herein is a method for treating a cancer in a subject, the method including: contacting the subject with a drug delivery composition that includes a poly-\textit{P-L-malic} acid (PMLA) covalently linked to a plurality of molecular modules including at least one module that targets a tumorigenic cell or a cancer cell, at least one module that inhibits synthesis or activity of a human epidermal growth factor receptor (HER) protein in the cell, and at least one module for cytoplasmic delivery, and analyzing at least one of: inhibition of tumor growth, tumor regression and elimination of cancer in the subject, thereby treating the cancer in the subject. For example, the HER protein is selected from a group comprising: HER1, HER2, HER3, and HER4.

In a related embodiment, the module that inhibits synthesis or activity of the HER protein is at least one selected from the group of: an antisense oligonucleotide (AON), an siRNA oligonucleotide, an antibody, a polypeptide, an oligopeptide and a low molecular weight drug. In general, the AON has a sequence complementary to an mRNA transcript of at least one protein selected from the group of: HER2, an epidermal growth factor receptor (EGFR) protein, and a subunit of laminin-41. For example, the sequence complementary to the HER2 transcript includes 5'-AGGGAGCCGCAGCTTCATGTCTGTG-3' (SEQ ID NO: 1), and 5'-CATGGTGCTCACTGCGGCTCCGGC-3' (SEQ ID NO:2). For example, the sequence complementary to the EGFR transcript includes 5'-TCGCTCCGGCTTCCCGATCAATAC-3' (SEQ ID NO:3). The subunit of laminin-41 in related embodiments is selected from a4 and $\beta^1$ subunits. For example, the a4 transcript sequence includes 5'-AGCTAAAGCCATTCTCCGCTGAC-3' (SEQ ID NO:4). For example, the $\beta^1$ transcript sequence includes 5'-CTAGCAACTGGAGAAGCCCC ATGCC-3' (SEQ ID NO:5).
In related embodiments of the method the siRNA oligonucleotide includes a sequence complementary to a gene encoding at least one of an epidermal growth factor receptor (EGFR) protein and HER2. For example, the EGFR sequence is selected from: 5’-CCUAUAAUGCUACGAAUAUtt-3’ (SEQ ID NO:6), and 5’-AUAUUCGUAGCAUUAUGGag-3’ (SEQ ID NO:7). For example, the HER2 sequence is selected from: 5’-GUUGGAUGAUUGACUCUGAtt-3’ (SEQ ID NO:8), and 5’-UCAGAGUCAAUCAUCCAACat-3’ (SEQ ID NO:9).

In an alternative embodiment of the method the antibody binds specifically to HER2/neu protein. For example, the antibody is Trastuzumab Herceptin®. In a related embodiment of the method the at least one module that targets the cell includes an antibody that binds specifically to a transferrin receptor protein. In general, the antibody is selected from at least one of: anti-human, rat anti-mouse, rat anti-human, rabbit anti-human and goat anti-human. In a related embodiment of the method the at least one module for cytoplasmic delivery includes an endosome escape unit. For example, the endosome escape unit is a leucine ethylester.

In related embodiments of the method the plurality of modules further includes a polyethylene glycol (PEG). For example, the PEG has a molecular weight of about 1,000 Da. For example, the PEG has a molecular weight of about 5,000 Da.

An embodiment of the method involves analyzing inhibition of tumor growth by observing more than about 60%, 70%, 80% or about 90% inhibition of tumor growth in the subject. The method in a related embodiment further involves observing with inhibition of tumor growth the inhibition of HER2/neu receptor signaling with suppression of Akt phosphorylation.

In general, the subject is a mammal such as a human, a simian, an equine, a bovine, or a high value agricultural or zoo animal For example, the mammal is a rodent. For example, the rodent is an experimental human-breast tumor-bearing nude mouse. The mammal in an alternative embodiment is a human.

The method in a related embodiment further includes administering an additional therapeutic agent. For example, the additional therapeutic agent is selected from the group consisting of: an antibody, an enzyme inhibitor, an antibacterial agent, an antiviral agent, a steroid, a non-steroid-inflammatory agent, an antimitabolite, a cytokine, a cytokine blocking agent, an adhesion molecule blocking agent, and a soluble cytokine receptor.
In an embodiment of the method contacting the subject with the composition further includes providing the composition in a unit dose effective for treatment the cancer in the subject. For example, the effective dose is at least one selected from: 1 µg/kg, 50 µg/kg, 100 µg/kg, 200 µg/kg, 300 µg/kg, 400 µg/kg, 500 µg/kg, 600 µg/kg, 700 µg/kg, 800 µg/kg, 900 µg/kg, 1 mg/kg, 50 mg/kg, 100 mg/kg, 200 mg/kg, 300 mg/kg, 400 mg/kg, 500 mg/kg, 600 mg/kg, 700 mg/kg, 800 mg/kg, 900 mg/kg, and 1 g/kg.

In a related embodiment of the method the cancer is selected from the list of: gastric, endometrial, salivary gland, lung, non-small cell lung, pancreatic, ovarian, peritoneal, prostate, colorectal, breast, cervical, uterine, ovarian, brain, head and neck, testicular and teratoma cancers. For example, the cancer includes either a primary cancer, a metastatic cancer, or both.

**Brief description of the drawings**

Figure 1 is a chemical structure and schematic drawings showing a nanobiopolymeric conjugate designed to inhibit HER2/neu expression by antisense oligonucleotides (AON) and to attenuate HER2/new-mediated cell signaling by Herceptin®. The modules are HER1/neu morpholino AON (indicated 1 in Figure) conjugated to the PMLA scaffold by disulfide bonds (S-S) that are cleaved by cytoplasmic glutathione to release the free drugs; targeting and/or effector antibodies that include antibody specific to a transferrin receptor protein (TfR) either alone or in combination with monoclonal antibodies (mAbs) to mouse TfR (indicated 2a in Figure), human TfR (indicated 2b) and Herceptin® (indicated 2c) for tumor endothelial and cancer cell targeting, receptor-mediated endocytosis, and anti-tumor effect, polyethylene glycol (PEG) for drug protection (indicated 3), stretches of conjugated L-leucine ethyl ester (LOEt) for endosomal escape of the drug (indicated 4), and optional fluorescent reporter dye (Alexa Fluor 680) for imaging (indicated 5). The nanopolymer also contained free unsubstituted pendant carboxyl groups for enhancing solubility and nonfunctional disulfides originating from chemical masking of excess sulphydryls with 3-(2-pyridyldithio)-propionates.

Figure 2 is a set of bar graphs showing data obtained from an *in vitro* cell viability assay. HER2/neu overexpressing breast cancer cells (BT-474 and SKBR-3; also shown in Figure 3 panel A) were treated with various drugs as indicated (top row). After 72 hours, cell viability was determined using a Trypan Blue exclusion assay. Percentage of cell
growth was calculated as average cell counts for each group and expressed relative to
parallel samples treated with PBS (control) set to 100%. Growth of tumor cells treated with
lead compound P/mPEG/LOEt/AON/Herceptin ®/TfR(M) was observed to be significantly
inhibited compared with other treatments in both cell lines. In cell lines expressing low
amounts of HER2/neu (Figure 3 panel A), the data show that the lead compound had
greatest ability to inhibit cell growth (bottom row). One asterisk indicates that P<0.05; two
asterisks indicate that P<0.01; three asterisks indicate that P<0.003 compared to PBS control
treatment. The lead compound also showed significant differences at P<0.005 when
compared to all treatment groups (top row), and at P<0.02 when compared to Herceptin®
(bottom row).

Figure 3 is a set of photographs of immunoblots showing changes observed in
HER2/neu expression, Akt phosphorylation, and apoptosis resulting from various treatments
of breast cancer cells in vitro.

Figure 3 panel A shows a comparison of HER2/neu and TfR expression in various
cell lines. Breast cancer cell lines used in Examples herein were observed to express high
levels of TfR.

Figure 3 panel B shows expression analysis of various markers in cell line SKBR-3.

Figure 3 panel C shows expression of the markers in cell line BT-474. HER2/neu
overexpressing breast cancer cells shown in panel A were treated with various compounds.

Western blot analyses showed decreased BER2/neu and phosphorylated Akt after
treatment with each of Herceptin®, P/mPEG/LOEt/Herceptin®, AON or
P/mPEG/LOEt/AON/TfR(H/M)-treated tumor cells, and not with control treatment PBS or
Endoporter in both cell lines. Treatment with lead compound
P/mPEG/LOEt/AON/Herceptin ®/TfR(M) further reduced both HER2/neu and p-Akt. Assay
of generation of cleaved poly(ADP-ribose) polymerase (PARP) as a measure of apoptosis
was observed at highest levels in P/mPEG/LOEt/AON/Herceptin ®/TfR(M)-treated cells.
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal loading
control.

Figure 4 is set of photographs showing distribution of various compounds herein
labeled with Alexa Fluor 680 in live mice with BT-474 breast tumors and in tumors in
isolated organs. Major organ analysis compared breast tumors and organs before injection
(left panel) with those twenty-four hours after intravenous injection (right panels). Live mice herein were injected with each of the lead drug P/mPEG/LOEt/AON/Herceptin®/TfR(M) (bottom row), positive control P/mPEG/LOEt with Herceptin® (middle row) and control conjugate P/mPEG/LOEt/IgG (top row). Control mice (top row) had little BT-474 tumor accumulation, and most of the control polymer accumulated in drug clearing organs, liver and kidneys. Polymer P/mPEG/LOEt with Herceptin® alone had a moderate tumor accumulation (middle row). The highest accumulation in breast tumor cells was observed in mice treated with the lead compound P/mPEG/LOEt/AON/Herceptin®/TfR(M). Arrows mark tumor implantation site.

Figure 5 is a set of photographs showing distribution of various compounds in BT-474 breast tumor cells. Animals were administered compounds intravenously as shown in Figure 4, were sacrificed 24 hours after drug injection, tumors were excised, and sections were analyzed by confocal microscopy. Nuclei were counterstained with DAPI (grey area). Animals injected with control conjugate P/mPEG/LOEt/IgG with attached Alexa Fluor 680 tracking dye (grey) showed little if any tumor cell accumulation (top row). Animals injected with P/mPEG/LOEt/Herceptin® displayed considerable accumulation in tumor cells, and the highest accumulation was observed in animals injected with the lead drug P/mPEG/LOEt/AON/Herceptin®/TfR(M), consistent with live animal imaging data shown in Figure 4. Scale bar = 50 μm.

Figure 6 is a set of photographs and a line graph showing mouse tumor inhibition, pathology, signaling and apoptosis marker expression.

Figure 6 panel A is a set of photographs showing data obtained and histopathological analysis of respective tumors from two representative animals for each group administered with different drugs. Variable amounts of dead tissue were observed to be present in all treated groups. Tumor size reduction data and pronounced disappearance of tumor cells were observed following treatment with the lead drug P/mPEG/LOEt/AON/Herceptin®/TfR(M), and mostly necrotic areas were observed to be present.

Figure 6 panel B is a line graph showing extent of tumor growth inhibition in mice. Animals treated with each of unconjugated Herceptin® (squares) and with positive control P/mPEG/LOEt/Herceptin® (triangles), or with P/mPEG/LOEt/AON/TfR(H/M) (circles)
showed significant inhibition compared with PBS control (diamonds) (P<0.03). P/mPEG/LOEt/AON/Herceptin ®/TfR(M) treatment (large squares) was observed to produce the greatest inhibition of tumor growth compared to other treatment groups, resulting in 80 to 95% tumor regression observed during the follow-up period (P<0.02 vs. Herceptin® and other drugs; P<0.001 vs. PBS). Error bars denote standard error of the mean (SEM).

Figure 6 panel C is a photograph of an immunoblot showing expression of select markers after treatment of HER2/neu positive tumors in vivo. Western blot analysis data showed a decrease in HER2/neu and p-Akt (but not total Akt) expression in each of Herceptin®, P/mPEG/LOEt/Herceptin®, or P/mPEG/LOEt/AON/TfR(H/M)-treated mice and not in control PBS-treated ones. P/mPEG/LOEt/AON/Herceptin®/TfR(M) further inhibited HER2/neu expression, with near disappearance of a p-Akt band. PARP cleavage as a measure of apoptosis was observed also to be substantially greater in P/mPEG/LOEt/AON/Herceptin®/TfR(M)-treated mice than that in other groups. GAPDH was an internal control to normalize gel loading.

Figure 7 is a line graph showing extent of tumor growth inhibition by compositions herein in subjects bearing triple-negative breast tumors. Animals treated with each of P/mPEG/LOEt/AON-EGFR/ TIR(H/M; squares), or with P/mPEG/LOEt/AON-EGFR/a4pl/TIR(H/M; triangles) showed significant inhibition compared with PBS negative control (diamonds). P=0.002 vs. α4β1; P=0.0001 vs. PBS. P/mPEG/LOEt/ AON-α4pi/TIR(H/M) treatment inhibited tumor growth compared to control PBS treatment, and was observed to be less effective compared to data obtained with other nanobiopolymers, shown in the figure. (P=0.01 vs. PBS). Error bars denote SEM.

Detailed description

Compositions and methods of the present invention provide a nanobiopolymeric drugs based on poly-β-L-malic acid (PMLA) platform specifically designed for delivery into HER2/neu-positive tumors. Targeted nanobiopolymeric conjugates based on poly-p-L-malic acid (PMLA) are biodegradable, non-toxic, and non-immunogenic. The PMLA nanoplatform was synthesized for repetitive systemic treatments of HER2/neu-positive human breast tumors in a xenogeneic mouse model. Various moieties were covalently attached to PMLA, including a combination of morpholino antisense oligonucleotides
(AON) directed against HER2/neu mRNA, to block HER2/neu synthesis; anti-HER2/neu antibody trastuzumab (Herceptin®), to target breast cancer cells and inhibit receptor activity simultaneously; and transferrin receptor antibody, to target the tumor vasculature and mediate delivery of the nanobiopolymer through the host endothelial system.

The Examples herein include tests of the lead compound, and data show that this compound significantly inhibited growth of HER2/new-positive breast cancer cells in vitro and in vivo, and enhanced apoptosis and inhibition of HER2/neu receptor signaling with suppression of Akt phosphorylation was observed in treated cells and animals. In vivo imaging analysis and confocal microscopy demonstrated selective accumulation of the nanodrug in tumor cells as a result of an active delivery mechanism resulting from design of the lead compound. Systemic treatment of human breast tumor-bearing nude mice resulted in more than 90% inhibition of tumor growth and tumor regression, compared to partial (50%) tumor growth inhibition in mice treated with control trastuzumab alone or control AON alone, either free or attached to PMLA. Data from Examples herein offer a preclinical demonstration of use of the PMLA nanoplatform for combination cancer therapy.

The epidermal growth factor receptor or ErbB family of receptor tyrosine kinases is exemplified by an epidermal growth factor receptor (also called HER1 or ErbB1), HER2 (ErbB2 or neu), HER3 (ErbB3), and HER4 (ErbB4). Upon ligand binding, ErbB family members form homodimers and heterodimers followed by the phosphorylation within intracellular kinase domains (Yarden et al. 2001 Nat Rev Mol Cell Biol 2:127). Upon ErbB1 and ErbB2 activation, phosphotyrosylated sites in Src-homology 2 (SH2) domains in these proteins serve as docking sites for adaptor proteins such as She, Grb2, and Sos resulting in the activation of the of Ras/Raf/mitogen-activated protein kinase (MAPK) kinase (MEK)/MAPK and PKB/protein kinase B (PKB) pathways and promotion of proliferation and mitogenesis (Yarden et al. 2001 Nat Rev Mol Cell Biol 2:127).

The HER2/neu proto-oncogene, also known as erbB-2, encodes a 185-kDa type I transmembrane receptor tyrosine kinase that is member of the epidermal growth factor receptor family (Hynes NE et al., 2005 Nat Rev Cancer 5:341; Bargmann CI et al. 1986. Nature 319:226; Coussens L et al. 1985 Science 230:1 132). Early studies have identified HER2/neu protein overexpression in several human carcinomas, including subsets of ovarian and breast cancers (Hynes NE et al. 1994. Biochim Biophys Acta 1198: 165; D’Emilia J et al. 1989 Oncogene 4:1233; Slamon DJ et al. 1989 Science 244:707). HER2/neu overexpression has been linked to a short relapse time and poor survival of breast
cancer patients (Slamon DJ et al. 1987 Science 235:177), as this protein plays a role in the molecular mechanisms of human cancers.

The ErbB2 gene is amplified and overexpressed in up to 30% of primary breast cancers and this is associated with poor patient prognosis (Slamon DJ et al., 1989 Science 244:707). ErbB1 is also overexpressed in up to 30% of primary invasive breast cancers and this is coTelated with reduced overall survival, proliferation, and higher metastatic potential (Tsutsui S et al. 2002 Breast Cancer Res Treat 71:67). Inhibition of ErbB1 signaling reduces both ErbB1 and ErbB2 activity and delays tumorigenesis in MMTV/Neu mice (Lenferink AEG et al. 2000 Proc Natl Acad Sci 97: 9609). The cooperative activation of proliferative pathways by these two receptors has stimulated the development of a number of small molecule inhibitors of members of the ErbB family for use as anticancer agents.

Newly diagnosed estrogen positive breast cancers are commonly treated with the antiestrogen agent tamoxifen. In estrogen-positive breast cancers, overexpression of both ErbB1 and ErbB2 is associated with resistance to tamoxifen therapy. It was shown that administration of such anticancer agents as lapatinib (GW572016) and tamoxifen together was advantageous and restored tamoxifen-mediated cell cycle arrest and inhibited tamoxifen-resistant breast tumor growth (Chu I et al. 2005 Cancer Res 65:18).

Characteristics such as extracellular accessibility, high expression, and association with poor prognosis make HER2/neu an attractive candidate for antibody therapy.

Metastatic breast cancer patients are currently being treated with Trastuzumab (also known as Herceptin; Genentech, Inc., San Francisco, CA), a Food and Drug Administration-approved humanized monoclonal anti-HER2/neu (Kaptain S et al. 2001 Diagn Mol Pathol 10: 139). Breast cancer clinical trials for patients with advanced disease expressing high levels of HER2/neu showed that use of Trastuzumab as a single immunotherapeutic agent resulted in an objective response rate of 12% to 26% (Cobleigh MA et al. 1999 J Clin Oncol 17:2639; Baselga J et al. 1996 J Clin Oncol 14:737; Vogel CL et al. 2002 J Clin Oncol 20:719). Subsequent clinical trials in patients with advanced disease have also shown that targeting metastatic breast cancer with Trastuzumab in combination with chemotherapy resulted in a 30% objective response, but disease relapse still affected most cases (Slamon DJ et al. 2001 N Engl J Med 344:783). In addition, Trastuzumab lacks considerable activity against tumors expressing HER2/neu that are not of breast origin (Burstein HJ 2005 N Engl J Med 353: 1652). Furthermore, resistance to Trastuzumab is a growing problem in patients
with breast tumors. Novel treatments for patients with HER2/neu-expressing tumors are still needed.

In 66% to 88% of cases, HER2/neu-overexpressing tumors demonstrate primary resistance to Herceptin® (Baselga J et al. 1999 Semin Oncol 26:78; Nahta R et al. 2004 Cancer Res. 64:398). This resistance may be due to epitope masking by overexpressed mucins, loss of receptor ability to influence pro-survival signaling through PI3K-Akt axis, or loss of protein phosphatase PTEN leading to the activation of PI3K-Akt signaling (Tseng PH et al. 2006 Mol Pharmacol.70:1534-41; Nagy P et al 1998 Cytometry32:120; Tanner M et al. 2004 Cancer Ther. 3:1585-92).

Antineoplastic agents for overcoming trastuzumab resistance

A variety of agents including monoclonal antibodies, recombinant proteins, and drugs, are known to have activity in treating breast cancer, and are here contemplated to be useful in combination with compositions described herein.

Combination of Herceptin® with other agents, such as paclitaxel (taxol, Bristol-Myers Squibb) and docetaxel (taxotere, Sanofi-Aventis), yielded increases in response rates, time to disease recurrence, and overall survival (Esteva FJ et al. 2002 J Clin Oncol. 20:1800; Slamon DJ et al. 2001 N Engl J Med.344:783; Wardley AM et al. 2009. J Clin Oncol 49:976).

Combining targeting of HER2 and other tyrosine kinases presents an alternative strategy. Tyrosine kinases are associated with breast cancer tumorigenesis and are of substantial interest as potential drag targets (Ocana A et al. 2008 Clin Cancer Res 14:961). The insulinlike growth factor 1 receptor (IGF-1R), a receptor tyrosine kinase (RTK), has been shown to increase the growth of breast cancer cells and is also implicated in developing resistance to trastuzumab (Nahta R et al. 2006 Nat Clin Pract Oncol 3:269).

Cotargeting or simultaneous targeting of IGF-1R and HER2 offers an advantage compared to targeting of the individual RTKs in breast cancer cells (Esparis-Ogando A et al. 2008 Ann Oncol 19:1860). The v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (c-KIT) RTK is overexpressed in triple-negative breast cancers (those that do not express estrogen receptor, progesterone receptor, and HER2) (Nielsen TO et al. 2004 Clin Res 10:5367). The activation of two nonreceptor cytosolic tyrosine kinases, c-abl oncogene 1 (ABL1) and c-SRC tyrosine kinase (CSK), is associated with the aggressiveness of breast cancer (Finn RS. 2008 Ann Oncol 19:1379) and proliferation of triple-negative breast

Dasatinib, (Sprycel®, Bristol-Myers Squibb) a small-molecule tyrosine kinase inhibitor, targets the cytosolic c-SRC and ABL1 kinases, and RTKs c-KIT and platelet-derived growth factor receptors alpha and beta (Finn RS et al. 2007 Breast Cancer Res Treat 105:319; Rix U et al. 2007 Blood 110: 4055; Huang F. et al. 2007 Cancer Res 67: 2226; Huang F. et al. 2007 Cancer Res 67: 2226). The activity of dasatinib for treatment of triple-negative breast cancer not expressing estrogen receptor, progesterone receptor, or HER2/neu (Finn RS et al. 2007 Breast Cancer Res Treat 105:319; Huang F. et al. 2007 Cancer Res 67: 2226), and favorable antitumoral activity in head and neck cancer in combination with gefitinib (Koppikar P et al. 2008 Clin Cancer Res 14:4284), led to combining trastuzumab and dasatinib for treatment of HER2-positive breast cancers. This combination was found to be highly effective against breast cancer cells overexpressing HER2 receptors. Both drugs individually inhibited cell proliferation in vitro and exhibited antitumoral action, and the combination resulted in a more potent effect on HER2-overexpressing cells.

The drug combination decreased levels of phosphorylated HER2 and phosphorylated HER3, and a decrease was observed in the total amount of these receptors. The combined treatment affected downstream signaling routes, such as the ERK1 or AKT pathways that regulate cell proliferation and survival (Garcia-Echeverria C et al. 2008 Oncogene 27:5511; Roberts PJ et al. 2007 Oncogene 26:3291). Dasatinib alone was as inhibitory for phosphorylated levels of ERK1 as the combined drug treatment. Treatment with dasatinib also inhibited SRC or FAK phosphorylation to the same degree as the combined drug treatment. These two kinases are known targets of dasatinib (Huang F. et al. 2007 Cancer Res 67: 2226) and participate in several oncogenic processes (Kim LC et al. 2009 Nat Rev Clin Oncol 6:587). Combined treatment and not the individual drugs was observed to decrease the level of phosphorylated AKT. Downstream targets of AKT such as p70S6K and BAD were also affected by the combined drug treatment, and not by the individual drugs, as the resting phosphorylated levels of these proteins were reduced by treatment with trastuzumab and dasatinib.

The drug combination also induced caspase-independent apoptosis as determined by the lack of an effect of caspase inhibitors on apoptosis induced by the drug combination.
One of the possible mediators in caspase-independent apoptosis is NAIF1, a protein that is released from the mitochondrial intermembrane space by certain apoptotic stimuli. The release of NAIF1 from mitochondria to the cytosol, by treatment with the drug combination, indicated that this mechanism could be responsible for caspase-independent apoptosis.

The drug combination also affected DNA repair machinery and led to accumulation of double-stranded breaks (DSBs) which indicate control of DNA repair machinery by tyrosine kinases and potential clinical implications.

Erlotinib (Tarceva, Roche), an inhibitor of EGFR, blocked homologous recombination repair of the DSBs in breast cancer cells through reduction of RAD51 foci formation (Li L et al 2008 Cancer Res 68:9141). Previous studies have also indicated that RTKs may regulate DNA repair (Tanaka T et al, 2008 Clin Cancer Res 14:1266; Ganapathipillai SS et al. 2008 Cancer Res 68:5769).

Gefitinib (Iressa, Astra Zeneca and Teva) is an EGFR inhibitor that impedes DNA repair in response to ionizing radiations in macrocytic lung cancer cells (Tanaka T et al, 2008 Clin Cancer Res 14:1266). Mutated forms of MET protein, an RTK implicated in several oncogenic processes such as invasion and metastasis (Benvenuti S et al. 2007 J Cell Physiol 213:316) or drug resistance (Engelman JA et al. 2007 Science 316:1039), have been reported to bind to and phosphorylate RAD51, facilitating DNA repair in tumor cells (Ganapathipillai SS et al. 2008 Cancer Res 68:5769).

A transcription factor associated with Williams-Beuren syndrome (WSTF; also known as BAZ1B), a tyrosine kinase component of the WICH complex (WSTF-ISWI ATP-dependent chromatin-remodeling complex), regulates the DNA damage response through phosphorylation of Tyr142 of H2AX (Xiao A et al. 2009 Nature 457:57).

It is here envisioned that drugs such as dasatinib in combination with other antineoplastic agents such as gefitinib and erlotinib (Koppikar P et al. 2008 Clin Cancer Res 14:4284), are further combined with compositions described herein.

Lapatinib (Tyverb®, GSK) is a dual EGFR/HER2 tyrosine kinase inhibitor (Rusnak DW et al. 2001 Mol Cancer Ther 1:85) which is highly selective to EGFR and HER2 (Karaman MW et al. 2008 Nat Biotechnol 26:127). In preclinical models of trastuzumab resistance, lapatinib inhibited phosphorylation of HER2 and overall growth in HER2 overexpressing breast cancer cell lines specifically chosen for extent of in vitro resistance to trastuzumab (Konechny GE et al. 2006 Cancer Res 66:1630). Further, treatment with lapatinib combined with trastuzumab resulted in a greater degree of survival and greater
apoptosis induction than either agent alone (Xia et al. 2005 Oncogene 24: 6213).

A substantial number of HER2-positive metastatic breast cancer patients treated with trastuzumab experience symptomatic central nervous system (CNS) metastasis, which unlike visceral diseases, are not well controlled by trastuzumab. Lapatinib and not trastuzumab has been shown to cross the blood-brain barrier, providing rationale for testing lapatinib in patients with CNS metastases (Nielsen DL et al. 2009 Cancer Treat Rev 35:121).

Trastuzumab in combination with lapatinib was found to be superior to lapatinib alone in HER2-positive metastatic breast cancer patients (Blackwell KL et al. 2010 J Clin Oncol 28:1124).

Pertuzumab (2c4, omnitarg, Genentech), another monoclonal antibody specific for the extracellular domain of HER2 protein, attaches to a different epitope of HER2 compared to trastuzumab. Pertuzumab was observed to inhibit heterodimer formation between HER2 and EGFR or HER3 (Agus DB et al. 2002 Cancer Cell 2:127). Although the HER2/HER3 heterodimer is important in HER2-driven cell signaling, the heregulin-dependent HER2/HER3 heterodimer is disrupted by pertuzumab and is not disrupted by trastuzumab (Jitunttila et al. 2009 Cancer Cell 15:429). In a phase II clinical trial involving combination treatment with pertuzumab and trastuzumab in HER2-positive breast cancer patients, treatment produced a response rate of 24.2%, and disease control rate of 50% (Baselga J et al. 2010 J Clin Oncol 28: 1138).

Trastuzumab-DM1 is comprised of trastuzumab and DM1, an agent that is an inhibitor of tubulin polymerization derived from maytansine. A stable MCC linker conjugates the DM1 to the trastuzumab. The compound is designed to deliver DM1 to HER2-overexpressing cancer cells. Preclinical studies have indicated the growth-inhibitory effect of trastuzumab-DM1 in HER2-overexpressing and trastuzumab-resistant cells (Lewis Phillips GD et al. 2008 Cancer Res 68:9280). In a phase II clinical trial involving HER2-positive metastatic breast cancer patients with disease progression despite trastuzumab-based therapy, trastuzumab-DM1 yielded an independently reviewed response rate and progression-free survival of 26.9% and 4.6 months, respectively (Vogel CL et al. 2009 J Clin Oncol 27: 15s (suppl; abstr 1017). Importantly, trastuzumab-DM1 had similar antitumor activity and an independently reviewed response rate of 24.2% even in patients previously treated with lapatinib and trastuzumab (n = 66).

PI3K pathway inhibitors are also used for treating HER2 expressing tumors. HER2-
overexpressing breast cancer cells are believed to be dependent on the PI3K signaling pathway, and a number of genetic or epigenetic alterations in PI3K signaling molecules have been shown to cause resistance to trastuzumab or small-molecule HER2 kinase inhibitors. HER2-overexpression and PIK3CA mutations frequently occur simultaneously in breast cancer cells (Oda K et al. 2008 Cancer Res 68:8127), and cell lines with either HER2 amplification or PIK3CA mutation are equally Akt-dependent (She QB et al. 2008 PLoS ONE 3:e3065). PI3K pathway inhibitors may therefore be useful in overcoming resistance to anti-HER2 agents. Indeed, PI3K/mTOR dual inhibitor and Akt inhibitor were shown to effectively inhibit cellular growth in trastuzumab-and lapatinib resistant cells. At present, many classes of PI3K pathway inhibitors are in clinical development, and their roles in overcoming trastuzumab resistance will be tested in the future.

Inhibitors of alternative signaling molecules are also used to treat trastuzumab resistant cancer cells. Alternative signaling from IGF-1R or MET may be associated with trastuzumab resistance. Small-molecular weight inhibitors of IGF-1R or MET receptor tyrosine kinase, and anti-IGF-1 antibody and anti-HGF antibody are in clinical development at present. Monotherapy or combination therapy with these agents and trastuzumab is therefore an attractive therapeutic strategy.

HER2 vaccines and adoptive immunotherapy targeting the HER2 extracellular domain have been tested in clinical trials, with results showing that significant levels of durable T-cell HER2 immunity can be generated with active immunization without significant consequences with regard to autoimmunity against normal tissues (Bernhard H et al 2002 Endoctr Relat Cancer 9:33). Early data from clinical trials testing the potential use of HER2-specific vaccines in adjuvant therapy for high-risk breast cancer patients show promising results (Peoples GE et al. 2008 Clin Cancer Res 14:797).

Ertumaxomab (Rexomum, Fresenius Biotech GmbH, phase II study) is an intact bispecific antibody targeting HER2 and CD3 on T cells with preferential binding to activating Fee type I/III receptors and redirecting T cells, macrophages, dendritic cells, and natural killer cells to HER2expressing tumor sites (Kiewe P et al. 2008 Expert Opin Investig Drugs 17: 1553). In a phase I trial, ertumaxomab treatment was associated with one complete response, two partial responses, and two stable diseases in patients with metastatic breast cancer who had received extensive prior treatment (Kiewe P et al. 2006 Clin Cancer Res 12:3085). The effects of ertumaxomab are being evaluated in phase II studies.
Defucosylated trastuzumab is also used to treat trastuzumab resistant cancer cells. Removal of fucose from antibody oligosaccharides attached to the heavy chain of Asn^{297} (defucosylation) has been shown to significantly enhance antibody-dependent cellular cytotoxicity (ADCC) compared to the activity of regular antibodies. In addition, defucosylation of trastuzumab was also found to enhance ADCC in an in vitro assay as compared to regular trastuzumab (Suzuki E et al. 2007 Clin Cancer Res 13:1875).

Defucosylated trastuzumab more than doubled the median progression-free survival compared with conventional trastuzumab in preclinical models of HER2-amplified breast cancer (Juntilla et al. 2010 Cancer Res 70: 4481).

Any of the above agents including paclitaxel, docetaxel, dasatinib, erlotinib, gefitinib, lapatinib, pertuzumab, trastuzumab, erumaxomab, trazasumab-DM1, defucosylated trastuzumab, PI3K pathway inhibitors and HER2 vaccines are here envisioned to be useful in combination with nanobiopolymer conjugate compositions herein to treat breast cancers by methods described herein.

Nanobiopolymers as a platform for carrying multiple drugs for treatment of HER2/neu cancers

Advantages of drug combinations can be offered in a single molecular entity such as a nanobiopolymeric conjugate. These compounds offer enhanced cancer cell specificity because of the presence of tumor targeting antibodies, bypass drug resistance by delivering polymer-bound drugs into cancer cell cytoplasm, and can carry multiple drugs on a single platform (Wu K et al. 2010 Angew Chem Int Ed Engl. 9:1451). Efficient delivery of nanobiopolymer-attached drugs to tumors is increased by passive targeting through enhanced permeability and retention (EPR) effect typical for tumors (Maeda H et al. 2009 Eur J Pharm Biopharm 71:409), and additionally, by active targeting using antibodies, such as anti-TfR (Maeda H et al. 2009 Eur J Pharm Biopharm 71:409; Liu X, et al. 2008 Cancer Gene Ther. 15:126; Peterson CM et al. 2003 Adv Exp Med Biol. 519:101). Table 1 shows the size (smaller than 30 nm) of conjugates used in Examples herein.

The slightly negative ζ potentials promote interaction of the conjugate with the cell membrane and enhance intracellular internalization (Wilhelm C et al. 2003 Biomaterials 24:1001-11).

A general problem with anti-cancer drugs is lack of specific tumor targeting, resulting in an extent of random tissue accumulation and significant side effects for normal
tissues (Shukla R et al. 2008 Nanotech 19:1; Shukla R et al. 2006 Bioconjug Chem 17:1 109). To circumvent this drawback, tumor-targeting antibodies have been used as drug carriers or directly as therapeutics (e.g., Herceptin®). Dendrimer nanoconjugates with attached Herceptin® displayed enhanced accumulation in breast cancer cells in animal models (Shukla R et al. 2006 Bioconjug Chem 17:1109). Methotrexate-loaded dendrimers produced a cytotoxic effect in tumor cells in vitro resulting from Herceptin®-mediated complex internalization (Shukla R et al. 2008 Nanotech 19:1). However, the efficacy of these nanodrugs was limited because of lack of efficient endosome release unit (Shukla R et al. 2008 Nanotech 19:1). Drugs were specifically delivered to cancer cells and tumor growth was inhibited as was angiogenesis in brain glioma-bearing animals (Fujita M et al. 2006 Angiogenesis 9:183; Ljubimova JY et al. 2008 Chem Biol Interact. 171:195). The efficiency of the polymers was associated with properties of tumor targeting, use of AON drugs to more than one tumor marker at the same time, and the presence of endosome disruption moiety ensuring drug release inside the target cell (Gasslmaier B et al. 2000 Eur J Biochem 267:5101).

Table 1. Nanobiopolymer drugs and controls for treatment of cancers overexpressing HER2/neu, molecular sizes, and ζ potentials

<table>
<thead>
<tr>
<th>Nanobiopolymer variant</th>
<th>Version</th>
<th>Size (nm)</th>
<th>ζ potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P/mPEG/LOEt/AON/Herceptin®/TfR(M)</td>
<td>Lead drug with AON, Herceptin® and TfR(M)</td>
<td>22.1 (±2.3)</td>
<td>-5.2 ± (0.4)</td>
</tr>
<tr>
<td>P/mPEG/LOEt/AON/TfR(H/M)</td>
<td>with AON and TfR (Human/Mouse)</td>
<td>20.1 (±2.4)</td>
<td>-5.7 (±0.6)</td>
</tr>
<tr>
<td>P/mPEG/LOEt/Herceptin®</td>
<td>with Herceptin® alone</td>
<td>15.1 (±1.2)</td>
<td>-4.1 (±0.4)</td>
</tr>
<tr>
<td>P/mPEG/LOEt/IgG</td>
<td>Control version for imaging study with IgG</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

PMLA is a natural polymer obtained from the slime mold Physarum polycephalum (Lee BS et al. 2006 Bioconjug Chem 17:317; Lee BS et al. 2002 Water-soluble aliphatic polyesters :poly(malic acid)s, in: Doi YSA, eds, Biopolymers, Weinheim: Wiley-VCH, 2002 pp.75-103). PMLA is non-toxic, non-immunogenic, and biodegradable in vitro and in vivo, stable in the bloodstream, and highly water-soluble (Gasslmaier B et al. 1997 Eur J Biochem...
250:308; Gsslmeier B et al. 2000 Eur J Biochem 267:5101). Systemic delivery of morpholino AONs having nucleotide sequences specific to e4t and βi chains of a tumor vasculature-specific protein, laminin-411 (formerly, laminin-8), to intracranial glioblastoma was shown to result in marked inhibition of tumor angiogenesis and growth (Ljubimova JY et al. 2008 Nanomedicine 3:247; Ding H et al. 2010 Proc Natl Acad Sci online publication). Further, to target tumor vasculature, a mAb to transferrin receptor (TfR) was attached to the same nanoparticle. The nanobiopolymer composition carrying each of anti-HER2/neu antibody (Herceptin®), anti-TfR antibody, and AON to WSR2/neu is shown herein to enhance the specificity and anti-tumor effect towards HER2/neu positive breast cancer.

Without being limited by any specific theory or molecular mechanism, the lead compound tested herein is a nanoparticle designed to work on several molecular levels, to inhibit the synthesis of new BER2/neu receptors with AON, and to block the activity of existing HER2/neu on the tumor cell membrane with Herceptin®.


Nanoparticles are used in drug delivery as carriers for small and large molecules. Nanoparticles are defined as particulate dispersions or solid particles with a size in the range of 10-1000 nm. The drug is dissolved, entrapped, encapsulated or attached to a nanoparticle matrix (Langer R. 2000 Acc Chem Res 33:94). Nanobiopolymers of the present invention
differ from nanoparticles in that nanoparticles have no covalent bonds between the particle and drug cargo, generally merely leak the drug, and accordingly cannot directly transport cargo to and release the cargo inside tumor cells.

Contrary to nanoparticles, nanobiopolymer compositions provided herein comprise a single unitary molecular entity having functional modules including a plurality of the following: tumor cell-targeting antibodies, two or more anti-tumor drugs, an endosomal disruption moiety, and a glutathione-cleavable bond to release the drug inside tumor cell cytoplasm, covalently attached. Such a construct functions to eliminate leakiness, suppresses non-tumor accumulation thereby minimizing side effects, and increase drug half-life dwell time of the composition in plasma. As a result, tumor uptake and drug specificity were observed in examples herein to be enhanced, leading to a significant reduction of tumor growth and volume. Moreover, the combined drug action through inhibiting Akt activation and increase of tumor cell apoptosis was also observed in examples herein.

Nanobiopolymers of the present invention offer a great potential in cancer therapy. Table 1. summarizes nanobiopolymer drugs synthesized for use in Examples herein.

Definitions

As used herein the term "molecular scaffold" refers to a molecule having at least two or more modules that transport a covalently conjugated drug to a targeted tissue; bind to cell surface receptors of the tissue; internalize into endosomes; escape the endosomes into the cytoplasm; and release reactive free drug in the cytoplasm by chemical reaction with glutathione and other sulphydryl groups of the cytoplasmic content. The specificity of high molecular mass drug vehicles and particles rests primarily on the tumor tissue targeting by tumor-specific conjugated targeting molecules and their enhanced permeability and retention in tumors that originates from high molecular mass such as greater than 20000 (Duncan R. 1999 Research Focus 2:441; Seymour LW et al., 1995 Eur J Cancer Res 31A:766).

The term "polymalic acid" or PMLA as used herein refers to a polymer, e.g., a homopolymer that contains a main chain ester linkage, is biodegradable and of a high molecular flexibility, soluble in water (when ionized) and organic solvents (in its acid form), non-toxic, and non-immunogenic (Lee Bs et al., Water-soluable aliphatic polyesters: poly(malic acid)s, in: Biopolymers, vol.3a (Doi Y, Steinbuchel A eds., pp 75-103, Wiley-VCH, New York 2002). Drug carrying PMLA is synthesized by ring-opening
polymerization of derivatized malic acid lactones. Doxorubicin-poly-malic acid has been synthesized from synthetic poly-P-D, L-malic acid (Abdellaoui K et al., 1998 Eur J Pharmaceutical Sciences 6:61). The carrier consists of poly(β ,L-malic acid), herein referred to as poly-β -L-malic acid or PMLA, representing the molecular backbone or scaffold that is chemically conjugated at its carboxylic groups at defined ratios with a variety of modules each of which performs at least one of the following functions: delivery of a pro-drug via a releasable functional module that becomes effective in the cytoplasm; directing the carrier towards a specific tissue by binding to the surfaces of cells, e.g., a monoclonal antibody (mAB); internalization into the targeted cell through endosomes (usually via internalization of a targeted surface receptor); promoting escape from endosomes into the cytoplasm by virtue of hydrophobic functional units that integrate into and finally disrupt endosomal membranes; increasing effectiveness during acidification of endosomes en route to lysosomes; and protection by polyethylene glycol (PEG) against degradative enzyme activities, e.g., peptidases, proteases, etc.

The term "module" as used herein refers to a biologically active molecular structure that forms a part of a composition herein, for example, a small drug molecule or a chromophore molecule; a protein molecule such as an antibody or lectin; or a portion thereof that are covalently joined to PMLA in constructing the composition. In the examples herein a biologically active module is exemplified by morpholino antisense oligonucleotides (AON) that are specific to HER2/neu receptor protein. Tissue targeting is exemplified by use of a monoclonal antibody (mAB) module that specifically recognizes and binds a transferrin receptor protein.

The term "transferrin receptor protein" as used herein refers to the receptor expressed on endothelium cell surfaces, and at elevated levels on certain tumors (Lee JH et al. 2001 Eur J Biochem 268:2004; Kovar MK et al, 2003 J Drug Targeting 10:23). Transferrin receptors are used as a target for a drug delivery system in compositions herein, to chemically bind to transferring, for example using a monoclonal antibody that binds the transferrin receptor and thereby achieves transcytosis through endothelium associated with blood brain barrier. Antibody binding to transferrin receptor and internalization into endosomes has been demonstrated (Broadwell RD et al, 1996 Exp Neurol 142:47). It will be appreciated that in the case of the transferrin receptor any appropriate antibody monoclonal antibody, for example, a humanized or chimeric antibody, or a lectin or another ligand specific to the transferrin receptor can be used. Other appropriate ligands to any
number of cell surface receptors or antigens can be used as targets in the compositions herein and transferrin receptor is merely examplary.

The phrase "endosomal escape unit" as used herein refers to a carrier module attached to the PMLA scaffold that becomes active by acidification during maturation of the endosomal vesicles towards lysosomes (Bulmus V et al, 2001 Cancer Research 61:5601; Lackey CA et al, 2002 Bioconjugate Chem 13:996). The carrier module includes a plurality of leucine or valine residues, or a leucine ethylester linked to the PMLA scaffold by amide bonds. During acidification of the endosomes en route to lysosomes, these stretches of the carrier molecule become charge-neutralized and hydrophobic, and capable of disrupting membranes. Other molecules that become charge neutralized at lysosomal pH's may be used in place of leucine or valine residues, or a leucine ethylester in construction of the compositions containing PMLA and an endosomal escape unit module.

PEGylation is generally used in drug design to increase the in vivo half-life of conjugated proteins, to prolong the circulation time, and enhance extravasation into targeted solid tumors (Arpicco S et al. 2002 Bioconjugate Chem 13:757; Maruyama K et al., 1997 FEBS Letters 413:1771). Other molecules known to increase half-life may be used in design of compositions herein.

As used herein, the terms "cancer" and "cancerous" refer to the physiological condition in mammals in which a population of cells are characterized by unregulated cell growth. Examples of cancers include, without limitation, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancers.

The terms "proliferative disorder" and "proliferative disease" refer to disorders associated with abnormal cell proliferation such as cancer.

The terms "tumor" and "neoplasm" as used herein refer to any mass of tissue that result from excessive cell growth or proliferation, either benign (noncancerous) or malignant (cancerous) including pre-cancerous lesions.
The term "primary cancer" refers to the original site at which a cancer originates. For example, a cancer originating in the breast is called a primary breast cancer. If it metastasizes, i.e., spreads to the brain, the cancer is referred to as a primary breast cancer metastatic to the brain.

The term "metastasis" as used herein refers to the process by which a cancer spreads or transfers from the site of origin to other regions of the body with the development of a similar cancerous lesion, i.e., having the same or substantially the same biochemical markers at the new location. A "metastatic" or "metastasizing" cell is one that has a reduced activity for adhesive contacts with neighboring cells and migrates by the bloodstream or within lymph from the primary site of disease to additional distal sites, for example, to invade neighboring body structures or distal structures.

The terms "cancer cell", "tumor cell" and grammatical equivalents refer to a cell derived from a tumor or a pre-cancerous lesion including both a non-tumorigenic cell and a tumorigenic cell, i.e., cancer stem cell.

As used herein "tumorigenic" refers to the functional features of a solid tumor stem cell including the properties of self-renewal i.e., giving rise to additional tumorigenic cancer cells, and proliferation to generate other tumor cells i.e., giving rise to differentiated and thus non-tumorigenic tumor cells, such that cancer cells form a tumor.

The phrase "target a tumorigenic cell or a cancer cell" as used herein refers to delivery of a composition to a population of tumor-forming cells within tumors, i.e., tumorigenic cells. The preferential delivery of the composition to the tumorigenic population of cancer cells in comparison to other populations of cells within tumors is referred herein as targeting to eliminate cancer cells, a property that improves specificity and efficacy of the composition.

The term "antibody" is used herein to mean an immunoglobulin molecule that is a functional module included in compositions herein for ability to recognize and specifically bind to a target, such as a protein, polypeptide, peptide, carbohydrate, polynucleotide, lipid, or combinations of the foregoing through at least one antigen recognition site within the variable region of the immunoglobulin molecule. In certain embodiments, antibodies included as functional modules of compositions herein include a class described as antagonist antibodies, which specifically bind to a cancer stem cell marker protein and interfere with, for example, ligand binding, receptor dimerization, expression of a cancer stem cell marker protein, and/or downstream signaling of a cancer stem cell marker protein.
In alternative embodiments, antibodies as functional modules in compositions herein include agonist antibodies that specifically bind to a cancer stem cell marker protein and promote, for example, ligand binding, receptor dimerization, and/or signaling by a cancer stem cell marker protein. In alternative embodiments, antibodies that do not interfere with or promote the biological activity of a cancer stem cell marker protein instead function to inhibit tumor growth by, for example, antibody internalization and/or recognition by the immune system.

As used herein, the term "antibody" encompasses intact polyclonal antibodies, intact monoclonal antibodies, antibody fragments (such as Fab, Fab', F(ab')2, and Fv fragments), single chain Fv (scFv) mutants, multispecific antibodies such as bispecific antibodies generated from at least two intact antibodies, chimeric antibodies, humanized antibodies, human antibodies, fusion proteins comprising an antigen determination portion of an antibody, and any other modified immunoglobulin molecule comprising an antigen recognition site so long as the antibodies exhibit the desired biological activity. An antibody includes any of the five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, or subclasses (isotypes) thereof (e.g. IgGl, IgG2, IgG3, IgG4, IgA1 and IgA2), based on the identity of their heavy-chain constant domains referred to as alpha, delta, epsilon, gamma, and mu, respectively. Antibodies can be naked or conjugated to other molecules such as toxins, radioisotopes, etc. In other embodiments an antibody is a fusion antibody.

As used herein, the term "antibody fragment" refers to a portion of an intact antibody and refers to the antigenic determining variable regions of an intact antibody. Examples of antibody fragments include, but are not limited to Fab, Fab', F(ab')2, and Fv fragments, linear antibodies, single chain antibodies, and multispecific antibodies formed from antibody fragments.

An "Fv antibody" refers to the minimal antibody fragment that contains a complete antigen-recognition and -binding site either as two-chains, in which one heavy and one light chain variable domain form a non-covalent dimer, or as a single-chain (scFv), in which one heavy and one light chain variable domain are covalently linked by a flexible peptide linker so that the two chains associate in a similar dimeric structure. In this configuration the complementarity determining regions (CDRs) of each variable domain interact to define the antigen-binding specificity of the Fv dimer. Alternatively a single variable domain (or half of an Fv) can be used to recognize and bind antigen, although generally with lower affinity.

A "monoclonal antibody" as used herein refers to homogenous antibody population involved in specific recognition and binding of a single antigenic determinant, or epitope.
Polyclonal antibodies include a population of antibody species each directed to a different antigenic determinant. The term "monoclonal antibody" encompasses both and full-length monoclonal antibodies and antibody fragments (such as Fab, Fab', F(ab')2, Fv), single chain (scFv) mutants, fusion proteins comprising an antibody portion, and any other modified immunoglobulin molecule comprising an antigen recognition site. Furthermore, "monoclonal antibody" refers to those obtained without limitation by methods including and not limited to hybridoma expression, phage selection, recombinant expression, and by transgenic animals.

Pharmaceutical Compositions

In one aspect of the present invention, a pharmaceutical composition is provided that includes a nanobiopolymeric conjugate of poly (P-L-malic acid) referred to as poly-p-L-malic acid or PMLA herein, covalently linked to an antisense molecule that is a functional module that inhibits expression of an oncogenic protein, and at least one module that is an antibody specific for the protein, and optionally further comprises a module that is an antibody specific for an oncogenic vascular protein, and a pharmaceutically acceptable carrier. In certain embodiments, the composition optionally further comprises one or more additional modules that are additional therapeutic agents. In certain embodiments, the additional therapeutic agent or agents is selected from the group consisting of growth factors, anti-inflammatory agents, vasopressor agents, collagenase inhibitors, topical steroids, matrix metalloproteinase inhibitors, ascorbates, angiotensin II, angiotensin III, calreticulin, tetracyclines, fibronectin, collagen, thrombospondin, transforming growth factors (TGF), keratinocyte growth factor (KGF), fibroblast growth factor (FGF), insulin-like growth factors (IGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), neu differentiation factor (NDF), hepatocyte growth factor (HGF), and hyaluronic acid.

As used herein, the term "pharmaceutically acceptable carrier" includes any and all solvents, diluents, or other liquid vehicle, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired. Remington's Pharmaceutical Sciences Ed. by Gennaro, Mack Publishing. Easton, PA, 1995 discloses various carriers used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Some examples of materials which can serve as pharmaceutically
acceptable carriers include, but are not limited to, sugars such as lactose, glucose, and
sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as
sodium carboxymethyl cellulose, ethyl cellulose, and cellulose acetate; powdered
traganth; malt; gelatin; tål; excipients such as cocoa butter and suppository waxes; oils
such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil, and soybean
oil; glycols; such a propylene glycol; esters such as ethyl oleate and ethyl laurate; agar;
buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid;
pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol, and phosphate buffer
solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and
magnesium stearate, as well as coloring agents, releasing agents, coating agents, sweetening,
flavoring and perfuming agents, preservatives and antioxidants can also be present in the
composition, according to the judgment of the formulator.

**Therapeutically Effective Dose**

In yet another aspect, according to the methods of treatment of the present invention,
methods for treatment of a specific type of cancer, are described herein. Thus, the invention
provides methods for the treatment of a cancer associated with a particular receptor
comprising administering a therapeutically effective amount of a pharmaceutical
composition comprising active agents that inhibits expression of at least one ligand of the
receptor to a subject in need thereof, in such amounts and for such time as is necessary to
achieve the desired result. It will be appreciated that this encompasses administering an
inventive pharmaceutical as a therapeutic measure to promote regression of a cancer or
prevent further development or metastasis, or as a prophylactic measure to minimize
complications associated with development of a tumor or cancer. In certain embodiments of
the present invention a "therapeutically effective amount" of the pharmaceutical
composition is that amount effective for preventing further development of a cancer or
transformed growth, and even to effect regression of the cancer. The compositions,
according to the method of the present invention, may be administered using any amount
and any route of administration effective for prevention of development of a cancer. Thus,
the expression "amount effective for inhibiting expression or activity of the oncogenic
protein", as used herein, refers to a sufficient amount of composition to prevent or retard
development of a cancer, and even cause regression of a cancer or solid tumor. The cancer
need not be limited to a solid tumor, and includes various types of lymphomas and
leukemias.

The exact dosage is chosen by the individual physician with regard to the need of the patient to be treated. Dosage and administration are adjusted to provide sufficient levels of the active agent(s) or to maintain the desired effect. Additional factors which may be taken into account include the severity of the disease state, e.g., cancer size and location; age, weight and gender of the patient; diet, time and frequency of administration; drug combinations; reaction sensitivities; and tolerance/response to therapy. Long acting pharmaceutical compositions might be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular composition.

The active agents of the invention are preferably formulated in dosage unit form for ease of administration and uniformity of dosage. The expression "dosage unit form" as used herein refers to a physically discrete unit of active agent appropriate for the patient to be treated. It will be understood, however, that the total daily usage of the compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. For any active agent, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs as shown in Examples herein. The animal model is also used to achieve a desirable concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans. A therapeutically effective dose refers to that amount of active agent which ameliorates the symptoms or condition.

Therapeutic efficacy and toxicity of active agents can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose is therapeutically effective in 50% of the population) and LD50 (the dose is lethal to 50% of the population). The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions herein exhibit large therapeutic indices. The data obtained from the animal studies herein is used in formulating a range of dosage for human use.

An initial dose of Herceptin® for human treatment accepted by the FDA is 4 mg/kg followed by 2 mg/kg weekly for a total of 52 doses. An efficient dose of the composition herein for treatment of a mouse was 100 µl of observed 40 µg/ml, which is equivalent to about 3.2 mg/kg for human use.

Administration of Pharmaceutical Compositions
After formulation with an appropriate pharmaceutically acceptable carrier in a desired dosage, the pharmaceutical compositions of this invention can be administered to humans and other mammals topically (as by powders, ointments, or drops), orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, or intravenously, depending on the severity and location of the cancer or other condition being treated. Intravenous administration includes injection as a bolus, or as a drip.

Dosage forms for topical or transdermal administration of an inventive pharmaceutical composition include ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants, or patches. The active agent is admixed under sterile conditions with a pharmaceutically acceptable carrier and any needed preservatives or buffers as may be required. For example, ocular or cutaneous infections may be treated with aqueous drops, a mist, an emulsion, or a cream. Administration may be therapeutic or it may be prophylactic. Prophylactic formulations may be present or applied to the site of potential tumors, or to sources of tumors. The invention includes devices, surgical devices, audiological devices or products which contain disclosed compositions (e.g., gauze bandages or strips), and methods of making or using such devices or products. These devices may be coated with, impregnated with, bonded to or otherwise treated with a disclosed composition.

The ointments, pastes, creams, and gels may contain, in addition to an active agent of this invention, excipients such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc, zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to the agents of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates, polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants such as chlorofluorohydrocarbons.

Transdermal patches have the added advantage of providing controlled delivery of the active ingredients to the body. Such dosage forms can be made by dissolving or dispensing the compound in the proper medium. Absorption enhancers can also be used to increase the flux of the compound across the skin. The rate can be controlled by either providing a rate controlling membrane or by dispersing the compound in a polymer matrix or gel.

Injectable preparations, for example, sterile injectable aqueous solutions or
oleaginous suspensions may be formulated according to the known art using suitable
dispersing or wetting agents and suspending agents. The sterile injectable preparation may
be formulated a sterile injectable solution, suspension or emulsion in a nontoxic parenterally
acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the
acceptable vehicles and solvents that may be employed are water, Ringer’s solution, U.S.P.
and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally
employed as a solvent or suspending medium. For this purpose any bland fixed oil can be
employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic
acid are used in the preparation of injectables. The injectable formulations can be sterilized,
for example, by filtration through a bacterial-retaining filter, or by incorporating sterilizing
agents in the form of sterile solid compositions which can be dissolved or dispersed in
sterile water or other sterile injectable medium prior to use. To prolong the effect of an
active agent, it is often desirable to slow the absorption of the agent from subcutaneous or
intramuscular injection. Delayed absorption of a parenterally administered active agent may
be accomplished by dissolving or suspending the agent in an oil vehicle. Injectable depot
forms are made by forming microencapsule matrices of the agent in biodegradable polymers
such as polylactide-polyglycolide as described herein, and in Ljubimova et al., US patent
number 7,547,511 issued June 16, 2009, Ljubimova et al., US patent application number
10/580,999 published November 8, 2007, and Ding et al, International patent application
PCT/US2009/40252 filed April 10, 2009. The rate of active agent release is controlled by
the ratio of active agent to polymer and the nature of the particular polymer employed.
Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides).
Depot injectable formulations are also prepared by entrapping the agent in liposomes or
microemulsions which are compatible with body tissues.

Compositions for rectal or vaginal administration are preferably suppositories which
can be prepared by mixing the active agent(s) of this invention with suitable non-irritating
excipients or carriers such as cocoa butter, polyethylene glycol or a suppository wax which
are solid at ambient temperature but liquid at body temperature and therefore melt in the
rectum or vaginal cavity and release the active agent(s).

Uses of Pharmaceutical Compositions

As discussed above and described in greater detail in the Examples in a manuscript
attached in an appendix hereto, accepted for publication in Cancer Research, at least one of
Inhibition of expression or activity of an oncogenic protein is useful to prevent development or metastasis of a cancer condition. In general, it is believed that these inhibitors are clinically useful in preventing further growth of a particular cancer type, including but not limited to the breast cancer; skin cancer; ovarian cancer; cervical cancer; the retinoblastoma; colon cancer and other such conditions such as those arising from the lining of the gastrointestinal tract; lung cancer and cancers of the respiratory tract; renal carcinoma and other tumors arising from the inner surface of kidney tubules; leukemias and lymphomas and such disorder of blood; and other types of genital cancer including those associated with various strains of papilloma virus; brain tumors; and cancers of the uterus, of the vagina, of the urethra.

It will be appreciated that the diagnostic, prognostic and therapeutic methods encompassed by the present invention are not limited to treating conditions in humans, but may be used to treat similar conditions in any mammal including but not limited to bovine, canine, feline, caprine, ovine, porcine, murine, and equine species. When treating tumors in a given species, it is preferred, but not required, that the antisense oligonucleotides have a nucleotide sequence that is substantially identical in base sequence to that as it occurs naturally in the species.

The invention having been fully described is it further exemplified in a research paper by Satoshi Inoue et al. entitled "Polymalic acid-based nanobiopolymer provides efficient systemic breast cancer treatment by inhibiting both HER2/neu receptor synthesis and activity", which is accepted for publication in Cancer Research and is incorporated herein by reference hereby in its entirety.

The invention now having been fully described it is exemplified in Examples below and in the claims, which are not to be construed as further limiting. References cited herein are hereby incorporated by reference in their entireties.

Examples

Example 1. Reagents

\( \text{\textit{Moffato}} \text{\textsuperscript{TM}} -\text{NH}_2 \) antisense oligonucleotides (AONs) used in Examples herein were custom made by Gene Tools (Philomath, OR).

AONs specific for \( \text{HER2/neu} \) included two sequences:

version 1: 5'-'AGGGAGCCGCAGCTTCATGTCTGTG-3' (SEQ ID NO:1), and

version 2: 5'-CATGGTGCTC ACTGCGGCTCCGGC-3' (SEQ ID NO:2).
AONs specific for an epidermal growth factor receptor (EGFR) included:
5'-TCGCTCCGGCTCTCCCGATCAATAC-3' (SEQ ID NO:3).
AONs specific for α4 and β1 subunits of laminin-411 included:
a4 subunit: 5'-AGCTCAAAGGCCATTTTCTCCGCTGAC-3' (SEQ ID NO:4), and
β1 subunit: 5'-CTAGCAACTGGAGAAGCCCCATGCC-3' (SEQ ID NO:5).

siRNA specific for EGFR included sequences as follows:
sense: 5'-CCUAUAAUGCUACGAAUAUt-3' (SEQ ID NO:6), and
antisense: 5'-AUAUUCGUAGCAUUUAUGGag-3'(SEQ ID NO:7).

siRNA specific for HER2 receptor protein included:
sense: 5'-GUUGGAUGAUUGACUCUGAtt-3' (SEQ ID NO:8), and
antisense: 5'-UCAGAGUCAAUCAUCCAACat-3' (SEQ ID NO:9).

Small letters "tt", "ag" and "at" at the 3'-terminus of the siRNA sequence denote DNA oligonucleotides that are synthesized to anneal siRNA to a DNA molecule.

Highly purified, endotoxin-free poly-β-L-malic acid, Mw (weight-averaged) = 100 kDa, polydispersity = 1.1, was obtained from the culture broth of *Physarum polycephalum*. Rat anti-mouse TfR mAb R17217 (mTfR) was purchased from Southern Biotech (Birmingham, AL). Cysteamine (2-mercaptoethyl-l-amine hydrochloride), N-hydroxysuccinimide, other reagents and solvents were of highest available purity and purchased from Sigma-Aldrich (St. Louis, MO).

Example 2. Synthesis of polyvalent acid nanobiopolymers

The nanobiopolymers contain five to six components (Figure 1): PMLA as the backbone; functional modules include: morpholino AON to inhibit HER2/neu protein synthesis; targeting anti-TfR mAb; anti-tumor Herceptin®, 40% leucine ethyl ester (LOEt) as endosome escape unit to achieve cytoplasmic AON delivery, and 5% PEG5000 to increase stability in the bloodstream. Anti-mouse TfR mAb on Herceptin®-containing conjugate was used to target tumor vasculature. The conjugate with AON without Herceptin® included an anti-human TfR mAb attached to it to promote drug binding to human tumor cells and its internalization. The preconjugate containing 40% LOEt, 5% PEG5000 and 10% of cysteamine (%) referring to the total amount of pendant carboxyl groups in polyvalent acid) was synthesized by the methods described previously (Lee BS et al. 2006 Bioconjug Chem 17:317). The antibodies conjugated with the preconjugate were qualitatively and quantitatively assayed by size exclusion HPLC. ELISA with purified TfR and HER2/neu
was used to assess functional reactivity of attached antibodies as described (Fujita M et al. 2007 J Control Release. 122:356).

Conjugates for imaging were fluorescently labeled with Alexa Fluor® 680 C2-maleimide (Invitrogen, Carlsbad, CA) by forming thioether with sulphydryl groups. Antibody conjugates were then reacted with HER2/neu AON (Figure 1). A control conjugate contained Herceptin® (Figure 1) and not Her2/neu-specific AON.

Example 3. The nanobiopolymer characterization

Chemical and physical characterization of polymeric nanobioconjugate was performed by various methods including L-malate dehydrogenase assay after nanobiopolymer hydrolysis at 100°C in the presence of 6 M HCl, PEG colorimetric determination and protein quantification, size and ζ potential, HPLC, and ELISA. HPLC was performed on a Hitachi analytical Elite LaChrom HPLC-UV system (Hitachi, Pleasanton, CA) and size exclusion, on a BioSep-SEC-S 3000 column (Phenomenex, Torrance, CA). The nanobiopolymer variants were characterized by their size (hydrodynamic diameter) on the basis of noninvasive back-scattering (NIBS), and ζ potential from electrophoretic mobility based on the Helmholtz-Smoluchowski formula, using electrophoresis M3-PALS (Gasslaier B et al. 1997 Eur J Biochem 250:308). Both measurements were performed in a Zetasizer Nano System ZS90 (Malvern Instruments, Malvern, UK). Data on molecular size and ζ potential represent mean ± standard deviation obtained from three independent measurements.

Example 4. Cell lines and culture conditions

Human breast cancer cell lines BT-474, SKBR-3, MDA-MB-231, MDA-MB-435, MDA-MB-468, and MCF-7 were obtained from American Type Culture Collection (Manassas, VA). BT-474, MDA-MB-231, MDA-MB-435, MDA-MB-468, and MCF-7 were cultured in DMEM with 10% fetal bovine serum and antibiotics. SKBR-3 was cultured in McCoy's 5A medium with 10% fetal bovine serum and antibiotics.

Example 5. Nomenclature

The term "nanobiopolymer" denotes a drug delivery system with PMLA as a nanoplatform and functional module groups covalently attached to the PMLA, including an AON, a rat anti-mouse or a mouse anti-human targeting TfR mAbs (M and H, respectively),
and LOEt as the endosomal escape unit module. The nanobiopolymer drags (Figure 1 and Table 1) described herein to treat HER2/neu-positive breast cancer contained either a drug HER2/neu AON or drug Herceptin® or both HER2/neu AON + Herceptin®.

5 Example 6. Cell proliferation assay

HER2/new-overexpressing breast cancer cells each of BT-474 or SKBR-3 were seeded into six-well plates at 3 x 10^5 cells/well. The next day, cells were treated with one of Endoporter (4 µM; control); Herceptin (40 µg/ml); P/mPEG/LOEt/Herceptin® (40 µg/ml); Endoporter (4 µM) and AON (4 µM); P/mPEG/LOEt/AON/Herceptin®/TfR(H/M); P/mPEG/LOEt/AON/Herceptin®/TfR(M); and PBS control. Seventy-two hours after treatment, the cells were stained with Trypan Blue. Cell viability was determined by calculating the mean of cell counts for each treatment group (in triplicate) and was expressed as a percentage of the total number of cells treated normalized to the number of cells treated with PBS.

15 Example 7. Western blotting

BT-474 and SKBR-3 breast cancer cells were treated with Herceptin® (40 µg/ml); P/mPEG/LOEt/Herceptin® (40 µg/ml equivalent to Herceptin®); Endoporter (4 µM) and AON (4 µM); P/mPEG/LOEt/AON/TfR(H/M); P/mPEG/LOEt/AON/Herceptin®/TfR(M); PBS control, or 4 µM Endoporter. Cell lysates were collected after 72 hours and were analyzed by western blotting as described previously (Inoue S et al. 2005 Mol Ther. 12:707-15). Lysates of excised breast tumors after various treatments were analyzed by these methods. The following anti-human primary antibodies were used: HER1/neu, Akt, phosphorylated Akt (p-Akt), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, to normalize gel load) (all from Cell Signaling Technology, Beverly, MA), and poly(ADP ribose) polymerase (PARP; BD Biosciences, San Jose, CA).

Example 8. Tumor xenografts in nude mice

Animal experiments were performed in accordance with the protocols approved by the Cedars-Sinai Medical Center Institutional Animal Care and Use Committee. Athymic mice (Crtac: Ncr-Foxn1nu Homozygous; Taconic, Hudson, NY) were used. A 0.72-mg, 90-day release, 17β-estradiol pellet (Innovative Research of America, Sarasota, FL) was inserted subcutaneously into the back of each mouse seven days prior to injection. An
amount of $1 \times 10^7$ BT-474 cells suspended in 150 µl of Matrigel (BD Biosciences, Bedford, MA) were injected into the right flank of each of 35 mice (5 mice per group), and treatment was initiated when tumors achieved an average size of $>120$ mm$^3$ (21 days after injection). Mice were divided into five treatment groups and each group was administered either of: sterile PBS (control); Herceptin® ($40 \mu$g/ml equivalent to Herceptin®); P/mPEG/LOEt/AON/TfR(H/M); or P/mPEG/LOEt/AON/Herceptin®/TfR(M); into the tail vein twice a week. Treatments were performed six times during a period of three weeks.

Tumor xenografts were measured with calipers twice a week, and tumor volumes were determined using the formula: (length x width$^2$) x ($\pi$/6).

Eighteen days after the last treatment, the animals were anesthetized with 3% isoflurane-air mixture and were euthanized. Tumor samples were stained with hematoxylin and eosin for morphological observation. The data are the average of two independent examples.

Example 9. Confocal microscopy

Alexa Fluor 680-labeled nanobiopolymers (P/mPEG/LOEt/IgG, control); P/mPEG/LOEt/Herceptin®, $40 \mu$g/ml; or P/mPEG/LOEt/AON/Herceptin®/TfR(M)) was each injected into the tail vein of mice. Twenty-four hours after drug administration, mice were euthanized; the tumors were harvested to detect the fluorescent signal, snap-frozen in liquid nitrogen and embedded in OCT compound for confocal microscopy (TCS SP5 X microscope; Leica Microsystems, Mannheim, Germany).

Example 10. In vivo imaging

BT-474 human breast cancer cells were implanted into the right thigh of mice as described. When tumor size attained 120 mm$^3$, 160 µl of Alexa Fluor 680 labeled nanobiopolymers was injected intravenously (4 µM). P/mPEG/LOEt/IgG was used as a negative control. Drug distribution and localization was assessed in tumor-bearing mice using Xenogen IVIS 200 imager (Caliper Life Sciences, Hopkinton, MA), at different time points before drug administration, 1 h, 3 h, 6 h, and 24 h after the drug injection. Twenty-four hours after drug administration, mice were euthanized and the circulating drugs eliminated by intraarterial PBS perfusion. The tumor and major organs were harvested to detect the fluorescent signal.
Example 11. Statistical analysis

Student's t-test (for two groups) and analysis of variance (ANOVA, for three and more groups) were used to calculate significance of the experimental results. GraphPad Prism4 program (GraphPad Software, statistical San Diego, CA) was utilized for all calculations. Data are presented as mean ± standard error of mean (SEM). The significance level was set at PO.05.

Example 12. Synthesis of polymer conjugates

Of the HER2/neu-specific AON sequences, a version that did not inhibit HER2/neu expression well in comparison with another version was observed; therefore, only the effective version was conjugated to the polymer platform. The absolute molecular weight of the lead version of nanobiopolymer (Figure 1) was 1,300 kDa by light scattering and close to the calculated value based on design. Hydrodynamic diameters (nano sizes) and ζ potentials of the nanobiopolymers in Figure 1 are summarized in Table 1. Parameters for ζ potentials in the range of -4.1 to - 5.7 mV have been reported for other nanoparticles as compatible with cell membrane attachment and nanoparticle internalization (Lorenz MR et al. 2006. Biomaterials 27:2820; Wilhelm C et al. 2003 Biomaterials. 24:1001).

Example 13. The lead nanobiopolymer carrying both Herceptin® and HER2/neu AON (P/mPEG/LOEi/AON/Herceptin®/TfR(M)) inhibited growth of breast cancer cells in vitro

Breast cancer cell growth inhibition following administration of anti-HER2/neu AON and Herceptin® was first examined. Based on optimization experiments, each of AON at 4 µM with 4 µM Endoporter (in vitro AON delivery agent, GeneTools), and Herceptin®, at 40 µg/ml was analyzed. Results in Figure 2 are shown for HER2/neu high-expressing cells BT474 and SKBR-3, as well as for low-expressing cells, MDA-MB-231 and MDA-MB-435. At the concentrations used, it was observed that each of free AON and Herceptin® resulted in some growth inhibition in HER2/neu high-expressing cells. Low-expressing cell lines were observed to be significantly less responsive to these treatments.

These nanobiopolymeric conjugates (a two-drug compound and single-drug compounds shown in Figure 1) were then tested for tumor cell growth inhibitory effect. The nanobiopolymers, Herceptin®, and free AON caused significant growth inhibition compared to PBS control in HER2/neu high-expressing cells (Figure 2 top, P<0.01). The lead two-
drug compound produced the strongest inhibitory effect that was significantly higher than that of the other nanobiopolymers tested and higher than Herceptin® (P<0.005 compared to all groups). In HERI/neu low-expressing cells, only the lead compound with AON, Herceptin® and TfR(M) was able to induce statistically significant inhibition of tumor growth compared to PBS (Figure 2 bottom, PO.02).


A phosphatidylinositol-3 kinase (PI3K) and its downstream target, the serine/threonine kinase Akt, play an important role in HERI/neu positive breast cancer cell growth and proliferation, as well as in anti-tumor effect of Herceptin® (Tseng PH et al, 2006 Mol Pharmacol. 70:1534; Yakes FM et al. 2002 Cancer Res. 62:4132). HERI/neu signaling can activate the PI3K/Akt/mTOR cascade, and activated Akt stimulates increases in cell size, metabolism and survival (Plas DR et al. 2005 Oncogene 24:7435).

Therefore, to examine the mechanism responsible for the enhanced growth inhibitory effect of the lead nanobiopolymer, drug effects on the expression and phosphorylation of pertinent signaling markers HERI/neu, Akt, and p-Akt were assessed. HERI/neu high-expressing cell lines BT-474 and SKBR-3 were used (Figure 3 panel A). To determine whether the nanobiopolymer carrying both HERI/neu AON and Herceptin® induces apoptosis, PARP cleavage was examined by western blot analysis.

In HERI/neu high-expressing cell lines, HERI/neu expression was inhibited to different extents by each of Herceptin®, AON, and the single-drug versions of the nanobiopolymer [P/mPEG/LOEt/Herceptin and P/mPEG/LOEt/AON/TfR(H/M)] in comparison with controls. The strongest inhibition of HERI/neu expression was observed upon treatment with the lead nanobiopolymer having AON and Herceptin® attached to the PMLA carrier molecule.

Expression of p-Akt, a key downstream mediator of HERI/neu signaling (Tseng PH et al. 2006 Mol Pharmacol. 70:1534), was inhibited to different extents in tumor cells treated with Herceptin®, AON, or single-drug versions of nanobiopolymer compared to control cells treated with PBS or AON transduction reagent Endoporter. The p-Akt signal upon treatment of both breast cancer cell lines with the lead drug carrying both Herceptin® and HER2/neu AON was observed to be markedly lower in comparison to treatment with any other agent.
(Figure 3 panel B). The amount of total Akt on western blots remained unchanged by each of the treatment.

Apoptosis assessed by PARP cleavage was induced to some extent by each of Herceptin®, AON, and single-drug nanobiopolymers in HER2/neu high-expressing cells, for example in BT-474 cell line. Significantly, the lead compound, P/mPEG/LOEt/AON/Herceptin®/TfR(M), triggered apoptosis to a greater extent than the other agents in both cell lines, as shown by increased PARP cleavage compared to the other agents (Figure 3 panel B).

Example 15. The lead compound P/mPEG/LOEt/AON/Herceptin®/TfR(M) specifically accumulates in HER2/neu-overexpressing breast tumors in vivo

Imaging studies in vivo showed that the lead compound P/mPEG/LOEt/AON/Herceptin®/TfR(M) having anti-mouse TfR and anti-human HER2/neu combined on the same PMLA molecule provided tumor-specific drug delivery through host endothelial system into subcutaneous human breast tumors. Twenty-four hours after injection of drugs, the compounds were observed to accumulate mostly in the tumor and draining organs, kidney and liver (Figure 4). The nanobiopolymer with only Herceptin® accumulated to a lesser extent in tumors than the version with Herceptin®, AON and anti-TfR mAb (the lead drug). These data show the enhanced targeting of tumor vasculature with anti-TfR mAb compared to Herceptin®. Control nanobiopolymer with IgG showed only a small amount of tumor accumulation (Figure 4).

Confocal microscopy was performed on sections of brain tumors removed 24 hours after intravenous injection of Alexa Fluor 680-labeled drugs. A significantly stronger signal in tumor cells for P/mPEG/LOEt/Herceptin® was observed than for the control conjugate P/mPEG/LOEt/IgG, and the highest tumor accumulation was observed with the lead compound compared to other nanobiopolymers (Figure 5).

Example 16. The lead compound P/mPEG/LOEt/AON/Herceptin®/TfR(M) significantly inhibits HER2/neu positive breast tumor growth in vivo

The therapeutic effect of compositions herein following intravenous administration in subcutaneous mouse models of human breast tumor xenografts was investigated. Cell line BT-474 was selected for in vivo analysis because of its high HER2/neu expression and
tumorigenicity. Treatment of BT-474 tumor-bearing mice with Herceptin®, single-drug nanobiopolymers and the lead compound P/mPEG/LOEt/AON/Herceptin®/TfR(M) was performed and compared to negative control PBS. No decreases in body weight or morbidity, or death was observed, indicating that each treatment was well tolerated.

Each the compounds inhibited tumor growth after six treatments (from days 21-38 post tumor implantation) and during follow-up to 56 days (Figure 6 panel B). Control unconjugated Herceptin® showed a similar tumor growth inhibition as a function of time as PMLA-bound Herceptin®. Both these drugs produced a somewhat stronger effect than HER2/neu AON bound to PMLA (Figure 6 panel B). This effect was significant for all three of these single drug compounds (P<0.03 vs. PBS). The compound having both Herceptin® and HER2/neu AON combined on one nanobiopolymer showed the highest degree of inhibition of tumor growth, with a clear synergistic effect compared to single-drug treatments (Figure 6 panel ; P<0.001 vs. PBS; P<0.03 vs. other treatment groups). The observed tumor regression following treatment with

P/mPEG/LOEt/AON/Herceptin®/TfR(M) was 80% at the start of follow-up to 95% at the end of this period (day 56; Figure 6 panel B). Moreover, tumors in the group treated with this lead compound started to regress within the two weeks after the initial treatment, and tumors in this group remained suppressed for an additional 20 days, at which time the treatment was terminated.

Hematoxylin and eosin staining revealed that the tumors treated with each of Herceptin®, P/mPEG/LOEt/Herceptin®, or P/mPEG/LOEt/AON/TfR(H/M) showed some areas of cell death compared with PBS (control) treated tumor. Significantly, treatment with the lead compound led to the appearance of massive morphologically necrotic areas with little unaffected tumor tissue remaining (Figure 6 panel A).

The mechanism of this antitumor effect was further investigated by western blot analysis using lysates of subcutaneous BT-474 breast tumors after different treatments. Tumor HER2/neu expression was partially inhibited by each of Herceptin®, AON, and single-drug versions of the PMLA nanobiopolymer [P/mPEG/LOEt/Herceptin® and P/mPEG/LOEt/AON/TfR(H/M)] in comparison with PBS controls (Figure 6 panel C). The lead compound P/mPEG/LOEt/AON/Herceptin®/TfR(M) produced the highest inhibition of HER2/neu tumor expression, consistent with the in vitro western blot analysis. Phosphorylated Akt was also reduced after drug treatments. Again, lead drug P/mPEG/LOEt/AON/Herceptin®/TfR(M) resulted in the most pronounced decrease, with
little p-Akt signal observed remaining (Figure 6 panel C). Total Akt remained unchanged upon treatments, as in the in vitro experiments.

Apoptosis assessed by PARP cleavage was induced to some extent by each of the compounds in HER2/neu high-expressing tumors compared to PBS treatment. Significantly, lead P/mPEG/LOEt/AON/Herceptin®/TfR(M) markedly increased PARP cleavage compared to the other treatments indicating that this nanobiopolymer induced apoptosis to a greater extent than the other used drugs (Figure 6 panel C).

Example 17. Nanobiopolymer conjugates significantly inhibited triple negative breast cancer growth in vivo

Potential therapeutic effects of each of the compounds in Table 2 following intravenous administration using subcutaneous mouse models of human triple-negative breast cancer (TNBC) xenografts were investigated. Cell line MDA-MB-468 was selected for in vivo analysis because it lacked expression of estrogen and progesterone, and the HER2 protein in these cells is expressed normally. Treatment of TNBC tumor-bearing mice was performed with a single-drug nanobiopolymer containing AONs specific for a4 and b1 subunits of laminin-411; or with a single-drug nanobiopolymer containing AONs specific for an epidermal growth factor receptor (EGFR) protein; or with a two-drug nanobiopolymer conjugate combing AONs specific for EGFR protein with AONs specific for a3 and b1 subunits of laminin-411, in comparison with negative control PBS using the treatment protocol schedule shown in Table 2.

Table 2. Nanobiopolymer drugs and controls for treatment of triple-negative breast cancers.

<table>
<thead>
<tr>
<th>Group 1 (n = 6)</th>
<th>Group 2 (n = 6)</th>
<th>Group 3 (n = 6)</th>
<th>Group 4 (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS P/PEG(5%)/LOEt(40%)/EGFR(2.1%)/HuTfR(0.12%)/MsTfR(0.12)</td>
<td>P/PEG(5%)/LOEt(40%)/α4β1(2.0%)/HuTfR(0.12%)/MsTfR(0.12)</td>
<td>P/PEG(5%)/LOEt(40%)/EGFR,α4β1(2.0%)/HuTfR(0.12%)/MsTfR(0.12)</td>
<td></td>
</tr>
<tr>
<td>IV twice a week</td>
<td>IV twice a week</td>
<td>IV twice a week</td>
<td></td>
</tr>
<tr>
<td>Amount of drug</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5 mg/kg (drug)</td>
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<tr>
<td>2.5 mg/kg of each AON</td>
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It was observed that single-drug compound carrying AONs specific for EGFR and the two-drug compound carrying both AONs specific for EGFR and AONs specific α4β1-subunits of laminin-411 inhibited tumor growth after six treatments that were administered during days 19-52 after implantation of tumor cells (Figure 7). The two-drug compound was observed to have produced a stronger therapeutic effect than the single-drug compound carrying AONs specific for EGFR alone, and the data were statistically significant for each single drag compound and the two-drug compound (P<0.1 vs. PBS). Further, administration on a schedule of the eight treatments was observed to be more effective than six treatments for greater regression of tumors.

Example 18. Advantages of nanobiopolymers

A set of nanobiopolymeric conjugates specifically tailored for HER2/neu expressing breast cancer treatment was designed and tested in vitro and in vivo. The drug was based on UER2/neu inhibition by simultaneously blocking the synthesis of HER2/neu with specific AON and internalizing the receptor by binding to Herceptin®. The lead drug P/mPEG/LOEt/AON/Herceptin®/TfR(M) was thus designed to more efficiently inhibit HER2/neu expression and function. In vitro data showed that indeed, the lead drug, P/mPEG/LOEt/AON/Herceptin®/TfR(M), suppressed proliferation of HER2/neu-positive breast cancer cell lines significantly more than Herceptin®, P/mPEG/LOEt/Herceptin® or P/mPEG/LOEt/AON/TfR(H/M) (Figure 2). Surprisingly, the lead nanobiopolymer was effective for both HER2/neu high- and low-expressing HER2/neu breast cancer cell lines. With regard to HER2/neu low-expressing cells, the lead drug was also superior to previously used HER2/neu AON, which did not inhibit their growth in vitro (Roh H et al. 2000 Cancer Res. 60:560). Moreover, the lead drug carrying both Herceptin® and HER2/neu AON produced the highest inhibition of both RER2/neu expression and Akt phosphorylation, as well as enhanced tumor cell apoptosis, compared to other treatments. Herceptin® mediates anti-proliferative effects in HER2/neu-positive cells by facilitating either HER2/neu degradation or endocytic destruction of the HER2/neu receptor or downregulation of PDK-Akt signaling (Clark AS et al. 2002. Mol Cancer Ther 1:707-17) by inhibiting HER2/neu receptor dimerization, and also by inducing immune activation (Hudis CA 2007 N Engl J Med 357:39).

Examples herein show that the in vitro growth-inhibiting effect of the lead drug carrying both Herceptin® and HER2/neu AON on tumor cells was enhanced by
simultaneous AON-mediated inhibition of HER2/neu synthesis and by downregulation of surface HER2/neu through its binding to Herceptin®. The stronger inhibition of Akt phosphorylation in this case could result from a significant attenuation of HER2/neu signaling.

The lead drug P/mPEG/LOEt/AON/Herceptin ®/TfR(M) was observed in examples herein to readily accumulate in breast tumors and dramatically inhibit human breast cancer growth in nude mice (Figure 6). Importantly, the magnitude of anti-tumor effect of this lead drug indicates synergy between HER2/neu AON and Herceptin® in vivo (Figure 6). In comparison, the in vitro effect showed about 50% growth inhibition in high HER/ /neu-expressing cells, in contrast to nearly complete in vivo inhibition. Without being limited by any particular theory or mechanism of action, the synergistic anti-tumor action in vivo could result from a combination of several effects: enhanced reduction in HER2/?/ew-mediated tumor growth by AON together with Herceptin®, preferential tumor accumulation mediated by combined EPR effect (Maeda H et al. 2009 Eur J Pharm Biopharm 71:409) and active targeting with antibodies (Ljubimova JY et al. 2008 Chem Biol Interact 171:195), and maintenance of effective drug concentration due to multiple treatments. Compared to the previously used combination of HER2/ren AON with doxorubicin that was similarly effective against xenogeneic BT-474 tumors (Roh H et al. 2000 Cancer Res. 60:560), the nanobiopolymeric conjugate herein is envisioned as free of side effects because of absence of toxic doxorubicin and of its efficient tumor targeting via Herceptin® and anti-TfR.

Examples herein confirmed that the lead nanobiopolymer P/mPEG/LOEt/AON/Herceptin ®/TfR(M) efficiently blocked HER/ /neu positive breast tumor growth through dual inhibition of HER2/neu and Akt phosphorylation, and as a result promoted enhanced tumor cell apoptosis. The combination of features of the nanobiopolymer resulted in highly specific drug accumulation in the tumor tissue and inside tumor cells.

Example 19. Nanobiopolymer platforms for combinatios of drugs to treat breast cancers

Nanobiopolymer compositions herein can be engineered to include any of at least one of functional modules: an antibody, drug, or AON, alone or in combination. By this characteristic, the nanobiopolymer conjugates herein are nanodmgs that are tailored to target simultaneously different molecular tumor markers typical of particular tumor cells and therefore are highly efficient against various tumors.
For more efficient cancer treatment, nanobiopolymer conjugates of the present invention are covalently linked to one or more antineoplastic agents selected from the following group: a tyrosine kinase inhibitor lapatinib targeting EGFR and HER2 receptor proteins; pertuzumab, a monoclonal antibody (mAb) specific for HER2 receptor; ertumaxomab, a bispecific antibody specific for HER2 and FcRI/III; trastuzumab-DM1, mAb-toxin specific for HER2; CP-751,871, mAb specific for IFG-1R; foretinib (GSK1 36089), a tyrosine kinase inhibitor targeting MET and VEGFRs; BEZ235 targeting proteins of mTOR/PI3K pathway; perifistone targeting Akt pathway; temsirolimus targeting mTOR; everolimus targeting mTOR; HER2 peptide-based vaccines; defucosylated trastuzumab, mAb specific for HER2; dasatinib, a small-molecule tyrosine kinase inhibitor targeting the cytosolic c-SRC and ABL1 kinases, as well as the RTKs c-KIT and platelet-derived growth factor receptors, alpha and beta; and gefitinib and erlotinib, inhibitors of EGFR. It is envisioned that these nanodrags and nanodrug combinations are effective for treatment of cancer cells in vivo in subjects including human patients.

The nanobiopolymer-based therapy used for treatment of BER2/neu expressing cancer cells and/or triple-negative cancer cells should make a significant clinical impact. The following claims are exemplary only and are not to be construed as further limiting. One of ordinary skill in the art would readily determine from the examples and claims numerous equivalents that are within the scope of the invention herein.
What is claimed is:

1. A drug delivery composition for treating a cancer in a subject comprising:
   a plurality of biologically active molecular modules comprising at least one module
   that targets a tumorigenic cell or a cancer cell, at least one module that inhibits synthesis or
   activity of a human epidermal growth factor receptor (HER) protein in the cell, and at least
   one module for cytoplasmic delivery; and
   a polymeric acid-based molecular scaffold, wherein the molecular modules are
   covalently linked to the scaffold.

2. The composition according to claim 1 wherein the HER protein is at least one
   protein selected from the group comprising: HER1, HER2, HER3 and HER4.

3. The composition according to claim 1 wherein the at least one module that inhibits
   synthesis or activity of the protein is selected from the group comprising: an antisense
   oligonucleotide (AON), an siRNA oligonucleotide, an antibody, a polypeptide, an
   oligopeptide and a low molecular weight drug.

4. The composition according to claim 3 wherein the AON is a Morpholino AON
   having a sequence complementary to a sequence contained in an mRNA transcript of
   HER2/neu protein.

5. The composition according to claim 4 wherein the AON is selected from:
   5'-AGGGAGCCGCAGCTTCATGTCTGTG-3' (SEQ ID NO: 1), and
   5'-CATGGTGCTCACTGCGGCTCCGGC-3' (SEQ ID NO:2).

6. The composition according to claim 1 wherein the at least one module that targets
   the cell comprises an antibody that binds specifically to a vasculature protein in the cell.

7. The composition according to claim 6 wherein the vasculature protein comprises a
   transferrin receptor protein.

8. The composition according to claim 6 wherein the antibody is anti-human.
9. The composition according to claim 6 wherein the antibody is selected from at least one of: rat anti-mouse, rat anti-human, rabbit anti-human and goat anti-human.

10. The composition according to claim 1 wherein the at least one module that inhibits activity of the protein comprises an antibody that binds specifically to a HER2/neu protein.

11. The composition according to claim 10 wherein the antibody comprises Herceptin®.

12. The composition according to claim 3 wherein the AON is a Morpholino AON having a sequence complementary to a sequence contained in an mRNA transcript of an epidermal growth factor receptor (EGFR) protein.

13. The composition according to claim 12 wherein the sequence comprises 5'-TCGCTCCGGCTCTCCCGATCAATAC-3' (SEQ ID NO:3).

14. The composition according to claim 3 wherein the AON is a Morpholino AON having a sequence complementary to a sequence contained in an mRNA transcript of at least one subunit of laminin-41.

15. The composition according to claim 14 wherein the at least one subunit is selected from a 4 subunit and β1 subunit.

16. The composition according to claim 15 wherein the sequence complimentary to the transcript of a 4 subunit comprises 5'-AGCTAAAGCCATTCTCCGCTGAC-3' (SEQ ID NO:4).

17. The composition according to claim 15 wherein the sequence complimentary to the transcript of the β1 subunit comprises 5'-CTAGCAACTGGAGAAGCCCCATGCC-3' (SEQ ID NO:5).

18. The composition according to claim 3 wherein the siRNA oligonucleotide comprises a sequence complementary to a gene encoding an EGFR protein.
19. The composition according to claim 18 wherein the sequence is selected from a sense sequence and an antisense sequence.

20. The composition according to claim 19 wherein the sense sequence comprises

5'-CCUAUAUGCUACGAAUAtt-3' (SEQ ID NO:6).

21. The composition according to claim 19 wherein the antisense sequence comprises

5'-AUAUUCGUAGCAUUUAUGGag-3' (SEQ ID NO:7).

22. The composition according to claim 3 wherein the siRNA oligonucleotide comprises a sequence complementary to a gene encoding a HER2 protein.

23. The composition according to claim 22 wherein the sequence is selected from a sense sequence and an antisense sequence.

24. The composition according to claim 23 wherein the sense sequence comprises

5'-GUUGGAUGAUUGACUCUGAtt-3' (SEQ ID NO:8).

25. The composition according to claim 23 wherein the antisense sequence comprises

5'-UCAGAGUCAAUCAUCCAACat-3' (SEQ ID NO:9).

26. The composition according to claim 1 wherein the at least one module for cytoplasmic delivery comprises an endosome escape unit.

27. The composition according to claim 26 wherein the endosome escape unit comprises a leucine ethylester.

28. The composition according to claim 27 wherein a concentration of the leucine ethylester comprises about 40% of the drug delivery composition.

29. The composition according to claim 1 wherein the plurality of modules further comprises a polyethylene glycol (PEG).
30. The composition according to claim 29 wherein the PEG has a molecular weight of about 1,000 Da, about 5,000 Da, about 10,000 Da, about 15,000 Da, about 20,000 Da, about 25,000 Da and about 30,000 Da.

31. The composition according to claim 1 wherein the scaffold comprises a poly-p-L-malic acid (PMLA).

32. The composition according to claim 1 present in a unit dose effective for treatment of the cancer in the subject.

33. The composition according to claim 32 wherein the unit dose is at least one selected from: 1 µg/kg, 50 µg/kg, 100 Hg/kg, 500 µg/kg, 1 mg/kg, 5 mg/kg, 10 mg/kg, 50 mg/kg, and 100 mg/kg.

34. The composition according to claim 32 wherein the unit dose is at least 1 mg/kg.

35. The composition according to claim 32 wherein the unit dose is less than about 10 mg/kg.

36. The composition according to claim 1 wherein the cancer is at least one selected from the list of: gastric, endometrial, salivary gland, lung, non-small cell lung, pancreatic, ovarian, peritoneal, prostate, colorectal, breast, cervical, uterine, ovarian, brain, head and neck, testicular and teratoma cancers.

37. The composition according to claim 36 wherein the cancer comprises a primary cancer.

38. The composition according to claim 36 wherein the cancer comprises a metastatic cancer.

39. The composition according to claim 36 wherein the cancer comprises cells overexpressing a HER2/neu receptor protein.
40. The composition according to claim 36 wherein the breast cancer is a triple-negative breast cancer.

41. A drug delivery composition for treating a cancer in a subject comprising:

a polymerized carboxylic acid molecular scaffold wherein the scaffold comprises a poly-P-L-malic acid (PMLA); and

a plurality of biologically active molecular modules comprising:

an antisense molecule that substantially inhibits synthesis of a HER2/neu receptor protein,

a molecular module to facilitate delivery of the antisense molecule to cytoplasm,

at least one antibody specific for the receptor protein that inhibits activity of the receptor protein,

at least one antibody targeting a tumor vasculature protein, and

a molecular module that prolongs circulation of the composition,

wherein the molecular modules are covalently linked to the scaffold.

42. A drug delivery composition for treating a cancer in a subject comprising:

a polymerized carboxylic acid molecular scaffold wherein the scaffold comprises a poly-P-L-malic acid (PMLA); and

a plurality of biologically active molecular modules comprising:

an antisense molecule that substantially inhibits synthesis of an epidermal growth factor receptor (EGFR) protein,

an antisense molecule that substantially inhibits at least one subunit of laminin-411,

a molecular module to facilitate delivery of the antisense molecule to cytoplasm,

at least one antibody targeting a tumor vasculature protein, and

a molecular module that prolongs circulation of the composition,

wherein the molecular modules are covalently linked to the scaffold.

43. A kit for treating a patient having a cancer comprising a drug delivery composition comprising a nanobiopolymeric conjugate of a scaffold comprising a poly-P-L-malic acid
(PMLA) and molecular modules comprising an antisense molecule that substantially inhibits synthesis or activity of a human epidermal growth factor receptor (HER) protein, a molecular module to facilitate delivery of the antisense molecule to cytoplasm, at least one targeting antibody specific for the HER protein, at least one antibody specific for a tumor vasculature protein, and a molecular module that prolongs circulation of the composition, wherein the PMLA is covalently linked to the molecular modules, in a container.

44. The kit according to claim 43 further comprising a pharmaceutically acceptable buffer.

45. The kit according to claim 43 further comprising instructions for use.

46. A method for treating a cancer in a subject, comprising:

- contacting the subject with a drug delivery composition comprising a poly-p-L-malic acid (PMLA) covalently linked to a plurality of molecular modules comprising at least one module that targets a tumorigenic cell or a cancer cell, at least one module that inhibits synthesis or activity of a human epidermal growth factor receptor (HER) protein in the cell, and at least one module for cytoplasmic delivery; and

- analyzing at least one of: inhibition of tumor growth, tumor regression and elimination of cancer in the subject, thereby treating the cancer in the subject.

47. The method according to claim 46 wherein the HER protein is selected from a group comprising: HER1, HER2, HER3, and HER4.

48. The method according to claim 46 wherein the module that inhibits synthesis or activity of the HER protein is at least one selected from the group of: an antisense oligonucleotide (AON), an siRNA oligonucleotide, an antibody, a polypeptide, an oligopeptide and a low molecular weight drug.

49. The method according to claim 46 wherein the AON has a sequence complementary to an mRNA transcript of at least one protein selected from the group of: HER2, an epidermal growth factor receptor (EGFR) protein, and a subunit of laminin-411.
50. The method according to claim 49 wherein the sequence complementary to the HER2 transcript comprises: 5'-AGGGAGCCGC AGCTTCATGTCTGTG-3' (SEQ ID NO: 1), and 5'-CATGGTGCTCACTGCGGCTCCG-3' (SEQ ID NO:2).

51. The method according to claim 49 wherein the sequence complementary to the EGFR transcript comprises 5'-TCGCTCCGGCTCTCCGATCAATAC-3' (SEQ ID NO:3).

52. The method according to claim 49 wherein the subunit of laminin-411 comprises an (x4 subunit.

53. The method according to claim 52 wherein the sequence comprises 5'-AGCTCAAAGCCATTTCTCCGCTGAC-3' (SEQ ID NO:4).

54. The method according to claim 49 wherein the subunit of laminin-411 comprises a β1 subunit.

55. The composition according to claim 54 wherein the sequence comprises 5'-CTAGCAACTGGAGAAGCCCC ATGCC-3' (SEQ ID NO:5).

56. The method according to claim 46 wherein the siRNA oligonucleotide comprises a sequence complementary to a gene encoding at least one of an epidermal growth factor receptor (EGFR) protein and HER2.

57. The method according to the claim 56 wherein the siRNA sequence complementary to the EGFR gene is selected from: 5'-CCUAUAAUGCUACGAAUAUtt-3' (SEQ ID NO:6), and 5'-AUAAUCGUAGCAUUUAUGGag-3' (SEQ ID NO:7).

58. The method according to claim 56 wherein the siRNA sequence complementary to the HER2 gene is selected from: 5'-GUUGGAUGAUUGACUCUAAtt-3' (SEQ ID NO:8), and 5'-UCAGAGUCAAUCAUCCAACat-3' (SEQ ID NO:9).

59. The method according to claim 48 wherein the antibody binds specifically to HER2/neu protein.
60. The method according to claim 59 wherein the antibody comprises Trastuzumab Herceptin®.

61. The method according to claim 46 wherein the at least one module that targets the cell comprises an antibody that binds specifically to a transferrin receptor protein.

62. The method according to claim 61 wherein the antibody is anti-human.

63. The method according to claim 61 wherein the antibody is selected from at least one of: rat anti-mouse, rat anti-human, rabbit anti-human and goat anti-human.

64. The method according to claim 46 wherein the at least one module for cytoplasmic delivery comprises an endosome escape unit.

65. The method according to claim 64 wherein the endosome escape unit comprises a leucine ethylester.

66. The method according to claim 46 wherein the plurality of modules further comprises a polyethylene glycol (PEG).

67. The method according to claim 66 wherein the PEG has a molecular weight of about 1,000 Da.

68. The method according to claim 66 wherein the PEG has a molecular weight of about 5,000 Da.

69. The method according to claim 46 wherein analyzing inhibition of tumor growth comprises observing more than about 60%, 70%, 80% or about 90% inhibition of tumor growth in the subject.

70. The method according to claim 46 wherein the subject is a mammal.
71. The method according to claim 70 wherein the mammal is a rodent.

72. The method according to claim 71 wherein the rodent is an experimental human-breast tumor-bearing nude mouse.

73. The method according to claim 70 wherein the subject is a human.

74. The method according to claim 46 further comprising observing with inhibition of tumor growth the inhibition of HER2/neu receptor signaling with suppression of Akt phosphorylation.

75. The method according to claim 46 further comprising administering an additional therapeutic agent.

76. The method according to claim 75 wherein the additional therapeutic agent is selected from the group consisting of: an antibody, an enzyme inhibitor, an antibacterial agent, an antiviral agent, a steroid, a non-steroid-inflammatory agent, an antimetabolite, a cytokine, a cytokine blocking agent, an adhesion molecule blocking agent, and a soluble cytokine receptor.

77. The method according to claim 46 wherein contacting the subject with the composition further includes providing the composition in a unit dose effective for treatment the cancer in the subject.

78. The method according to claim 77 wherein the unit dose is at least one selected from:

1 µg/kg, 50 µg/kg, 100 µg/kg, 500 µg/kg, 1 mg/kg, 5 mg/kg, 10 mg/kg, 50 mg/kg, and 100 mg/kg.

79. The method according to claim 77 wherein the unit dose is at least one 1 mg/kg.

80. The method according to claim 77 wherein the unit dose is less than about 10 mg/kg.
81. The method according to claim 46 wherein the cancer is selected from the list of: gastric, endometrial, salivary gland, lung, non-small cell lung, pancreatic, ovarian, peritoneal, prostate, colorectal, breast, cervical, uterine, ovarian, brain, head and neck, testicular and teratoma cancers.

82. The method according to claim 81 wherein the cancer comprises a primary cancer.

83. The method according to claim 81 wherein the cancer comprises a metastatic cancer.
Figure 4

Before injection
24 hours after

PhmPEGLO(EO)nIgG
PhmPEGLO(EO)nHepcpl
PhmPEGLO(EO)nAM\(\text{Hepcpl}\)
A. CLASSIFICATION OF SUBJECT MATTER

A61K 39/395(2006.01)i, A61K 38/16(2006.01)i, A61K 47/30(2006.01)i, A61P 35/00(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K 39/395; A61K 31/337; A61K 9/00; C07K 16/00; A61K 47/48

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: drug delivery, cancer, EGFR, HER, HER2, transferrin receptor, Herceptin, laminin-41, endosome escape unit, leucine ethylester, PEG, PMLA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>WO 2009/126913 A1 (CEDARS-SINAI MEDICAL CENTER) 15 October 2009 &lt;br&gt;see abstract; claims 1, 3, 5-8, 16, and 15-17; p. 5, line 31 - p. 6, line 8; p. 9, line 27 - p. 10, line 9; example 14.</td>
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Further documents are listed in the continuation of Box C.

See patent family annex.

Date of the actual completion of the international search 25 OCTOBER 2011 (25.10.2011)

Date of mailing of the international search report 25 OCTOBER 2011 (25.10.2011)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
Government Complex-Daejeon, 189 Cheongsaro-ro, Seo-gu, Daejeon 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer

Choi Sung Hee
Telephone No. 82-42-481-8740

Form PCT/ISA/210 (second sheet) (July 2009)
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**INTERNATIONAL SEARCH REPORT**

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

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

1. ❌ Claims Nos.: 46-83
   because they relate to subject matter not required to be searched by this Authority, namely:
   Claims 46-83 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

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

3. □ Claims Nos.:  
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:

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

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

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

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

**Remark on Protest**

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

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

☒ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)
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