Abstract:

Title: COMBINATION TREATMENT OF CANCER WITH CETUXIMAB AND TETRAC

Results

Total cell count (median)

Combinations of both drugs show a large anti-proliferative effect than each drug on its own

FIG. 11

(57) Abstract: Provided herein are compositions and methods for treating cancer by increasing the inhibitory effect of cetuximab on HIF-1α expression by administering cetuximab in combination with anti-angiogenic thyroid hormone analogs such as tetrac or triac.
COMBINATION TREATMENT OF CANCER WITH CETUXIMAB AND TETRAC

RELATED APPLICATIONS

This application claims priority to USSN 61/165,119, filed March 31, 2009, which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

The invention described herein pertains to tetraiodothyroacetic acid-like compounds and other compounds that inhibit the expression of hypoxia-inducible factor 1-α (HIF1α) gene as well as various formulations thereof and methods for the prevention or treatment of cancer.

BACKGROUND

Thyroid hormones, such as L-thyroxine (T4) and 3,5,3’-triiodo-L-thyronine (T3), and their analogs such as GC-I, DITPA, Tetrac and Triac, regulate many different physiological processes in different tissues in vertebrates. It was previously known that many of the actions of thyroid hormones are mediated by the thyroid hormone receptor ("TR"). However, a novel cell surface receptor for thyroid hormone (L-thyroxine, T4; T3) has been described on integrin αvβ3. This receptor is at or near the Arg-Gly-Asp (RGD) recognition site on the integrin. The αvβ3 receptor is not a homologue of the nuclear thyroid hormone receptor (TR), but activation of this cell surface receptor results in a number of nucleus-mediated events, including the recently-reported pro-angiogenic action of the hormone and fibroblast migration in vitro in the human dermal fibroblast monolayer model of wound-healing.

Evidence that thyroid hormone can act primarily outside the cell nucleus has come from studies of mitochondrial responses to T3 and diiodothyronine (T2), from rapid onset effects of the hormone at the cell membrane, and from actions on cytoplasmic proteins. The recent description of a plasma membrane receptor for thyroid hormone on integrin αvβ3 has provided some insight into effects of the hormone on membrane ion pumps, such as the Na+/H+ antiporter, and has led to the description of interfaces between actions initiated at the membrane thyroid hormone receptor and nuclear events that underlie important cellular or tissue processes, such as, for example, angiogenesis and proliferation of certain tumor cells.
Integrin αvβ3 binds thyroid hormone near the Arg-Gly-Asp (RGD) recognition site of the integrin protein. The RGD site is involved in the protein-protein interactions linking the integrin to extracellular matrix (ECM) proteins such as vitronectin, fibronectin and laminin. (See Plow et al, 2000. J. Biol. Chem. 275:21785-88). Also initiated at the cell surface integrin receptor is the complex process of angiogenesis, which can be monitored in either a standard chick blood vessel assay or with human endothelial cells in a sprouting assay. This hormone-dependent process requires mitogen-activated protein kinase (MAPK; extracellular regulated kinase [ERK] 1/2) activation and the elaboration of vascular growth factors, including, but not limited to basic fibroblast growth factor (bFGF; FGF2), which is the downstream mediator of thyroid hormone's effect on angiogenesis. Tetrac blocks this action of T4 and T3, as does RGD peptide and other small molecules (such as XT-199) that mimic RGD peptide(s). Thus, it is possible that desirable neovascularization can be promoted with local application of thyroid hormone analogs, for example, in wound-healing, or that undesirable angiogenesis, such as that which supports tumor growth, can be antagonized with tetrac or triac.

SUMMARY OF THE INVENTION

The invention provided pharmaceutical compositions for treating cancer involving a combination of cetuximab and tetrac, where the combination induces apoptosis in cancer cells. In some embodiments, the tetrac can be used in a nanoparticulate form. When in nanoparticulate form, the nanoparticulates may also target additional chemotherapeutic agents to the cancer cells. Additionally, in various embodiments, the composition also contains an anti-estrogen compound. Moreover, the combination of cetuximab and tetrac can be used to inhibit phosphatidylinositol 3-kinase (PI3K)-dependent hypoxia-inducible factor 1-α (HIF1α) gene expression.

More specifically, provided herein are pharmaceutical compositions for treating cancer that contain a combination of cetuximab and an anti-angiogenic thyroid hormone analog, wherein the combination inhibits expression of HIF1α gene in cancer cells. Those skilled in the art will recognize that the HIF1α gene product is a survival factor in cancer cells. For example, the cancer cells may be selected from breast cancer, lung cancer, kidney cancer, thyroid cancer, brain cancer (glioma), ovarian cancer, pancreatic cancer, prostate cancer, plasma cell cancer (myeloma), squamous cell head-and-neck cancer, liver cancer, muscle cancer (sarcoma), colon cancer, neuroblastoma, lymphoma, stomach cancer, adenoid
cystic carcinoma, and skin cancer including melanoma, basal cell carcinoma, and squamous cell carcinoma.

In various embodiments described herein, thyroid hormone analog is tetrac or triac (triiodothyroacetic acid). By way of non-limiting example, one preferred thyroid hormone analog that is used in the compositions of the invention is tetrac.

In any of the pharmaceutical compositions described herein, the thyroid hormone analog (such as tetrac) can be conjugated via a covalent bond to a polymer selected from polyvinyl alcohol, acrylic acid ethylene co-polymer, methoxypolyethylene, polyethyleneglycol (PEG), polyacrylic acid, polylactic acid, agarose, polyglycolide, polylactide, PEO, m-PEG, PVA, PLLA, PGA, poly-L-lysine, Human Serum Albumin, cellulose derivatives, carbomethoxy/ethyl/hydroxypropyl, hyaluronic acid, folate linked cyclodextrin/dextran, sarcosine/aminoc acid spaced polymer, alginate, carrageenan, pectin/chitosan, chitosan, dextran, collagen, polyamine, poly aniline, poly alanine, polytrytophan, poly tyrosine, polylactide-co-glycolide (PLG), poly(lactic-co-glycolic acid) (PLGA), polylysyl glycolide, polyglycolide, polylactic acid, or co-polymers thereof, wherein the polymer is formulated into a nanoparticle, wherein the nanoparticle is between 150 and 250 nanometers in size, and wherein the tetrac binds to the cell surface receptor for thyroid hormone on integrin αvβ3.

In some embodiments, a linker between about 4 and 15 (i.e., 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15) atoms long is used between the polymer (or the synthesized nanoparticle) and the thyroid hormone or thyroid hormone analog. For example, the linker may be attached to the thyroid hormone or thyroid hormone analog via a covalent or non-covalent bond. Moreover, the point of attachment for the linker may be the outer ring hydroxyl of the thyroid hormone or thyroid hormone analog. Those skilled in the art will recognize that, because tetrac and triac do not have an amino group on their outer rings (in contrast to T4 and T3), the use of a linker is necessary in order to insure that conjugation to the nanoparticle occurs via the outer ring of the thyroid hormone analog. When a linker is used, the amide bond for the linker must be imbedded in the nanoparticle to prevent lysis of that bond by circulating peptidases.

The pharmaceutical compositions may also contain one or more anti-estrogen compounds (e.g., tamoxifen and/or aromatase inhibitors).

Alternatively (or additionally), the nanoparticles may also contain one or more additional chemotherapeutic agents. In such embodiments, the one or more additional chemotherapeutic agents are targeted to the cancer cells.
Those skilled in the art will recognize that the combination of cetuximab and an anti-angiogenic thyroid hormone analog inhibits PI3K-dependent HIF1α gene expression in cancer cells.

Also provided herein are methods of treating cancer comprising administering a therapeutically effective amount of a combination of cetuximab and an anti-angiogenic thyroid hormone analog to a patient suffering therefrom. In various embodiments, the thyroid hormone analog is tetrac or triac. In one preferred embodiment, the thyroid hormone analog is tetrac.

In any of the methods disclosed herein, tetrac can be conjugated via a covalent bond to a polymer selected from polyvinyl alcohol, acrylic acid ethylene co-polymer, methoxypolyethylene, polyethylene glycol (PEG), polyacrylic acid, polylactic acid, agarose, polyglycolide, polylactide, PEO, m-PEG, PVA, PLLA, PGA, poly-L-lysine, Human Serum Albumin, cellulose derivatives, carbomethoxy/ethyl/hydroxypropyl, hyaluronic acid, folate linked cyclodextrin/dextran, sarcosine/amino acid spaced polymer, alginate, carrageenan, pectin/chitosan, chitosan, dextran, collagen, polyamine, poly aniline, poly alanine, polytrytophan, poly tyrosine, polylactide-co-glycolide (PLG), poly(lactic-co-glycolic acid) (PLGA), polylysyl glycolide, polyglycolide, polylactic acid, or co-polymers thereof, wherein said polymer is formulated into a nanoparticle, wherein said nanoparticle is between 150 and 250 nanometers in size, and wherein said tetrac binds to the cell surface receptor for thyroid hormone on integrin αvβ3.

Additionally, the methods of the invention may also involve administering one or more anti-estrogen compounds to the subject. By way of non-limiting example, the anti-estrogen compounds are tamoxifen and/or aromatase inhibitors.

Moreover, the nanoparticles used in the methods of the invention may also contain one or more additional chemotherapeutic agents.

Those skilled in the art will recognize that in some embodiments, any of the pharmaceutical compositions and/or methods described herein, cetuximab can be encapsulated within nanoparticle that is linked to the thyroid hormone analog.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram of the perfusion bellows pharmacodynamic culture system. Cells of cancer lines of interest are grown on plastic flakes suspended in a flow-through, bellows-agitated system that allows for homogeneous exposure of cells to drug/drug
metabolite solutions and frequent sampling of cells for viability. Direction of arrow indicates
the flow of influx/efflux medium.

Figure 2A is a diagram of a mathematical model that assumes two populations of cells
in different states of the cell cycle: cells that are preparing for replication (state 1) and cells
that are immediately "pre-replication" (state 2). Cells transition from state 1 to state 2 by a
first-order growth rate constant, while replication from state 2 to state 1 is assumed to be fast.
Figure 2B is a diagram of a pharmacodynamic model. This model assumes that both drugs
act on independent pathways and includes a small antagonistic term. The observed effect of
the combination is larger than a model which assumes both drugs acting on the same pathway
would predict.

Figure 3 is a series of graphs showing that tetrac induces anti-proliferation in cancer
cells. Figure 3A shows U87MG cells and Figure 3B shows MDA-MB cells treated daily with
tetrac (10^{-9} to 10^{-3} M). In Figure 3C, MDA-MB cells were treated daily with different
concentrations of tetrac (10^{-9} to 10^{-3} M). Cells were harvested at the time points indicated.
The total cell numbers taken after each treatment were used as indicators of tetrac-induced
anti-proliferation.

Figure 4 is a series of graphs showing the time course distribution of [^{125}\text{I}]-tetrac in
human glioblastoma U87MG cells. In Figure 4A, cells were seeded in 100 mm Petri dishes
and refed MEM containing 0.25% stripped serum for 2 days. [^{125}\text{I}]-tetrac (0.227 x10^{-6} M)
was added to Petri dishes at time points indicated. The final concentration of [^{125}\text{I}]-tetrac was
2.27x10^{-6} M. Cells were harvested immediately after final tetrac adding. Cytosolic and
nuclear proteins were separated as described previously. 10 µl of media, cytosolic and
nuclear proteins were prepared for scintillation counting. The results are the average of four
experiments. In Figure 4B, the distribution of [^{125}\text{I}]-tetrac in cytosolic and nuclear fractions
is shown.

Figure 5 is a series of graphs showing that tetrac and nano-tetrac induce
antiproliferation. Figure 5A shows MDA-MB cells that were treated daily with different
concentrations of tetrac and nano-tetrac (2.5x10^{-6} and 10^{-6} M). Figure 5B shows MDA-MB
cells that were treated daily with different concentrations of tetrac nano (10^{-9} to 10^{-6} M).
Cells were harvested at the time points indicated. Total cell numbers taken after each
treatment were used as indicators of tetrac and nano-tetrac-induced anti-proliferation. In
Figure 5C, U87MG cells were treated daily with different concentrations of tetrac or nano-
tetrac (10^{-9} to 10^{-6} M). Cells were harvested at the time points indicated. Total cell numbers
taken after each treatment were used as indicators of tetrac or nano-tetrac-induced anti-proliferation.

Figure 6 is a series of graphs showing the effects of tetrac and nano-tetrac in human adenoid cystic carcinoma (TGSI 12T). In Figure 6A, TGSI 12T cancer cells were treated with $10^{-7}$ M $T_4$ in the presence or absence of $10^{-7}$ M tetrac for 24 h. $T_4$-induced PCNA expression was inhibited by tetrac. In Figure 6B, TGSI 12T cancer cells were treated with $10^{-7}$ M $T_4$ in the presence or absence of $10^{-7}$ M tetrac or nano-tetrac, daily and 1 µCi $[^3H]$-thymidine (final concentration, 13 nM) was added for 24 h. $T_4$ increased thymidine incorporation which was inhibited by tetrac and nano-tetrac. Tetrac itself increased thymidine incorporation slightly. In Figure 6C, TGSI 12T cells were treated daily with $10^{-6}$ M tetrac or nano-tetrac. Cells were harvested at the time points as indicated. Total cell numbers taken after each treatment were used as indicator for tetrac- or nano-tetrac-induced anti-proliferation.

Figure 7 is a series of graphs showing that tetrac induces apoptosis in MDA-MB cells. MDA-MB cells grown in perfusion bellows cell culture were treated with different concentrations of tetrac ($10^{-7}$ M to $10^{-5}$ M) daily. Cells were harvested on the day indicated. Two million cells of each sample were prepared for flow cytometry. Flow cytometry was conducted as described in Example 1, infra.

Figure 8 shows the expression of pro-apoptotic genes by tetrac and nano-tetrac. MDA-MB cells were treated daily with $10^{-6}$ M of tetrac or nano-tetrac in bellows perfusion culture system. Cells were harvested after 3 days of treatment and total RNA was extracted. RT-PCR was conducted as described in Example 1, infra.

Figure 9 is a series of graphs showing the comparison of nano-tetrac and tetrac on cell proliferation in non-malignant cells. CV-I cells (Figure 9A) and 293 T cells (Figure 9B) were treated daily with $10^{-6}$ M tetrac or nano-tetrac. Cells were harvested at the time points indicated. Total cell numbers taken after each treatment were used as an indicator for tetrac- or nano-tetrac-induced anti-proliferation.

Figure 10 is a graph showing the effect of tetrac and cetuximab-induced anti-proliferation. Human breast cancer MDA-MB cells were treated daily with 0.1 µg/ml of cetuximab, tetrac ($10^{-7}$ M), or the combination in the bellows perfusion culture system. Cells were harvested at the time points indicated. Cetuximab, tetrac, or combination-induced anti-proliferation was determined by cell number count.

Figure 11 is a graph showing that the combination of cetuximab and nano-tetrac shows a larger anti-proliferative effect than either drug on its own in colon carcinoma cells.
Figure 12A is a graph showing the total cell counts different concentrations of cetuximab, nano-tetrac, and the combination of cetuximab and nano-tetrac on K-ras mutant colon carcinoma cells. Figure 12B is a graph showing the total cell counts for 0.2 µg/mL cetuximab plus nano-tetrac in varying concentrations. Figure 12C is a graph showing the total cell counts for 1.5 µg/mL cetuximab plus 6nM nano-tetrac. Figure 12D is a graph showing the total cell counts for 10 µg/mL cetuximab plus varying concentrations of nano-tetrac.

Figure 13 is a graph showing the drug effect on growth rate constant from pharmacodynamic modeling for all treatments.

Figures 14A and 14B are graphs showing the effects of cetuximab or nano-tetrac individually, or in combination at varying concentrations on total cell counts for HCT16 cells.

Figures 15A-C are graphs showing the results of flow cytometry experiments for apoptosis.

Figures 16A and 16B are a series of graphs showing the results of flow cytometry experiments for non-apoptotic cells.

DETAILED DESCRIPTION

The details of one or more embodiments of the invention have been set forth in the accompanying description below. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. Other features, objects, and advantages of the invention will be apparent from the description and from the claims. In the specification and the appended claims, the singular forms include plural references unless the context clearly dictates otherwise. All patents and publications cited in this specification are incorporated by reference in their entirety.

For convenience, certain terms used in the specification, examples and claims are collected here. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains.

The term "agent" is used herein to denote a chemical compound, a mixture of chemical compounds, a biological macromolecule (such as a nucleic acid, an antibody, a protein or portion thereof, e.g., a peptide), or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. The activity of
such agents may render it suitable as a "therapeutic agent" which is a biologically, physiologically, or pharmacologically active substance (or substances) that acts locally or systemically in a subject.

As used herein, the terms "angiogenesis agent" or "angiogenic agent" include any compound or substance that promotes or encourages angiogenesis, whether alone or in combination with another substance. Examples include, but are not limited to, T3, T4, T3 or T4-agarose, polymeric analogs of T3, T4, 3,5-dimethyl-4-(4'-hydroxy-3'-isopropylbenzyl)-phenoxy acetic acid (GC-I), or DITPA. In contrast, the terms "anti-angiogenesis agent" or "anti-angio gene agent", as used herein, refer to any compound or substance that inhibits or discourages angiogenesis, whether alone or in combination with another substance. Examples include, but are not limited to, tetrac, triac, XT 199, and mAb LM609. The structures of representative angiogenic and anti-angiogenic agents are provided herein:

![Chemical structures](image)

DITPA

GC-I
The term "RGD", as used herein, refers to the single letter amino acid code and references the tripeptide amino acid sequence arginine-glycine-aspartic acid (Arg-Gly-Asp).

A "small molecule" or "small molecule chemical compound" as used herein, is meant to refer to a composition that has a molecular weight of less than 2000 Daltons, preferably less than 1000 Daltons, more preferably less than 750 Daltons, most preferably less than 500 Daltons. Small molecules are organic or inorganic and are distinguished from polynucleotides, polypeptides, carbohydrates and lipids.

The terms "peptide mimetic", "mimetic", or "peptidomimetic" as used herein refer to an agent or a compound that mimics at least one activity of a peptide or compound or a peptide analog in which one or more peptide bonds have been replaced with an alternative type of covalent bond that is not susceptible to cleavage by peptidases.

When referring to a compound, a "form that is naturally occurring" means a compound that is in a form, e.g., a composition, in which it can be found naturally. A compound is "not in a form that is naturally occurring" if, for example, the compound has been purified and separated from at least some of the other molecules that are typically found with the compound in nature. Thus, a "naturally occurring compound" refers to a compound that can be found in nature, i.e., a compound that has not been designed by man. A naturally occurring compound may be harvested from nature or refined from a complex mixture of naturally occurring products or it may be reproduced synthetically.

A "patient," "individual," "subject" or "host" refers to either a human or a non-human animal.

The term "modulation" is art-recognized and refers to up regulation (i.e., activation or stimulation), down regulation (i.e., inhibition or suppression) of a response, or any combination thereof.

The terms "prophylactic" or "therapeutic" treatment are art-recognized and refer to the administration of one or more drugs or compounds to a host. If administration occurs prior to clinical manifestation of the unwanted condition (e.g., disease or other unwanted state of the host animal), the treatment is prophylactic, i.e., it protects the host against developing the unwanted condition. If administration occurs after the manifestation of the unwanted
condition, the treatment is therapeutic, \textit{i.e.}, it is intended to diminish, ameliorate or maintain the existing unwanted condition or other side effects.

The term "mammal" is known in the art and includes humans, primates, bovines, porcines, canines, felines, and rodents (\textit{e.g.}, mice and/or rats).

As used herein, the term "pharmaceutically-acceptable salt" is art-recognized and refers to the relatively non-toxic, inorganic and organic acid addition salts of compounds, including, for example, those contained in the compositions described herein.

The term "pharmaceutically acceptable carrier" is art-recognized and refers to a pharmaceutically-acceptable material, composition or vehicle, such as, for example, a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting any subject composition or component thereof from one organ or portion of the body, to another organ or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the subject composition and its components and not injurious to the patient. Some non-limiting examples of materials which may serve as pharmaceutically acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

The terms "systemic administration," "administered systemically," "peripheral administration" and/or "administered peripherally", as used herein, are all art-recognized and refer to the administration of a subject composition, therapeutic or other material other than directly into the central nervous system, such that it enters the patient's system and, thus, is subject to metabolism and other like processes.

Likewise, the terms "parenteral administration" and "administered parenterally" are also art-recognized and refer to modes of administration other than enteral and topical administration, usually by injection, and include, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal,
intraperitoneal, transtracheal, subcutaneous, subcuticular, intra-articulare, subcapsular, subarachnoid, intraspinal, and/or intrasternal injection and infusion.

As used herein, "treating" a condition or disease refers to curing as well as ameliorating at least one symptom of the condition or disease.

The term "therapeutic agent" is art-recognized and refers to any chemical moiety that is a biologically, physiologically, or pharmacologically active substance that acts locally or systemically in a subject. This term also refers to any substance intended for use in the diagnosis, cure, mitigation, treatment or prevention of disease or in the enhancement of desirable physical or mental development and/or conditions in an animal or human.

Moreover, the term "therapeutic effect" is art-recognized and refers to a local or systemic effect in animals, particularly mammals, and more particularly humans, caused by a pharmacologically active substance. The phrase "therapeutically-effective amount" means that amount of such a substance that produces some desired local or systemic effect at a reasonable benefit/risk ratio and is applicable to any treatment. The therapeutically effective amount of such substance will vary depending upon the subject and disease or condition being treated, the weight and age of the subject, the severity of the disease or condition, the manner of administration and the like, which can readily be determined by one of ordinary skill in the art. For example, certain compositions described herein may be administered in a sufficient amount to produce a desired effect at a reasonable benefit/risk ratio applicable to such treatment.

The term "synthetic" is art-recognized and refers to production by in vitro chemical or enzymatic synthesis.

Throughout this application, the terms "nanoparticle", "nanoparticulate" and "nanoparticulate form" are used interchangeably to refer to a modification of any of the active compound(s) of the invention (i.e., cetuximab and/or the anti-angiogenic thyroid hormone analogs (i.e., tetrac and/or triac)), where the active compound(s) are covalently bound (e.g., by an ester, ether, or sulfur linkage) to a polymer wherein the polymer is formulated into a nanoparticle, wherein the active compound is located on the surface of the nanoparticle and wherein the nanoparticle is between 150 and 250 nm in size. Conjugation of the anti-angiogenic thyroid hormone analogs via covalent bond to a polymer increases the half life of the compound and/or insures that the compound does not gain access to the interior of the cells (thus, limiting their action to the integrin binding site). The preparation and use of nanoparticulate forms of the anti-angiogenic thyroid hormone analogs are described in the art (see, e.g., WO2008/140507, which is herein incorporated by reference in
its entirety). As used herein, the terms "nano-tetrac", "nano tetrac", "nanoparticulate tetrac", "NP-tetrac", and "NP-T" are used interchangeably to refer to a nanoparticulate form of the thyroid hormone analog tetrac.

Those skilled in the art will recognize that, in some embodiments, cetuximab can be encapsulated within the nanoparticulate form of tetrac.

Cetuximab (marketed in North America by ImClone and Bristol-Myers Squibb and in the rest of the world by Merck KGaA) under the name Erbitux), is a chimeric (mouse/human) monoclonal antibody that blocks activation of the epidermal growth factor (EGF) receptor (EGFR). Currently, cetuximab is given by intravenous infusion for treatment of metastatic colorectal cancer and head and neck cancers. Specifically, cetuximab is indicated for the treatment of patients with epidermal growth factor receptor (EGFR)-expressing, KRAS wild-type metastatic colorectal cancer (mCRC), in combination with chemotherapy, and as a single agent in patients who have failed oxaliplatin- and irinotecan-based therapy and who are intolerant to irinotecan. In addition, cetuximab (Erbitux) is also indicated for the treatment of patients with squamous cell cancer of the head and neck in combination with platinum-based chemotherapy for the first line treatment of recurrent and/or metastatic disease and in combination with radiation therapy for locally advanced disease. Cetuximab and other EGFR inhibitors only work on tumors that are not mutated.

A diagnostic immunohistochemistry assay (EGFR pharmDx) can be used to detect EGFR expression in the tumor material. Approximately 75% of patients with metastatic colorectal cancer have an EGFR-expressing tumor and are therefore considered eligible for treatment with cetuximab. In mCRC, biomarkers, including KRAS (a small G protein on the EGFR pathway), are indicative of a patient's response to cetuximab (Erbitux). Data have shown that sixty percent of patients who express the KRAS wild-type tumor are significantly more likely to benefit from treatment with cetuximab or a combination of cetuximab plus chemotherapy. Assessment for EGFR expression is required for the use of cetuximab (Erbitux) in Colorectal Cancer, but not in Head & Neck Cancer. Cetuximab and other EGFR inhibitors only work on tumors that are not mutated.

Two recent studies demonstrated that patients with KRAS wild-type tumors demonstrated significantly increased response rates and disease free survival when treated with cetuximab and standard chemotherapy, as compared to patients receiving chemotherapy alone. (See Bokemeyer et al, 2009. J. Clin. Oncol. 27(5):663-71; Van Cutsem et al, 2009 N. Engl. J. Med. 360(14): 1408-17).
Cetuximab was approved by the FDA in March 2006 for use in combination with radiation therapy for treating squamous cell carcinoma of the head and neck (SCCHN) or as a single agent in patients who have had prior platinum-based therapy. Two landmark studies have evaluated the benefits of cetuximab (Erbitux) in patients with SCCHN in both the locally advanced (Bonner trial) and the recurrent and/or metastatic (EXTREME trial) settings. The EXTREME trial marks the first time in 30 years that a Phase III trial has demonstrated a survival benefit in 1st-line recurrent and/or metastatic disease.

Associated with, or independently of, its action on EGFR, cetuximab is known to inhibit the expression of the HIF1α gene. The gene product of the HIF1α gene is a survival factor in cancer cells. This gene product stimulates tumor cell proliferation, is pro-angiogenic, and supports the process of metastasis. Moreover, it has been shown that cetuximab decreases HIF1α gene expression by a phosphatidinositol 3-kinase (PBK)-requiring process. (See Li et al., 2008. Mol. Cancer Ther 7:1207-17 (incorporated by reference in its entirety).


Integrin αvβ3 has been shown to be expressed on tumor cells, on endothelial and vascular smooth muscle cells, on osteoclasts, and on angiogenically active blood vessel cells. (See Davis et al., 2008. Front Neuroendocrinol 29:21 1-18; Davis et al., 2009. Am J Physiol Endocrinol Metab 297:E1238-E1246). This limited expression makes this integrin an attractive target for the development of cancer treatment strategies because nano-tetrac has little effect on proliferation of non-malignant cells. The observed additive effects of
combinations of nano-tetrac and other chemotherapeutic agents observed here suggests that lower dosages of agents are possible with conjoint therapy.

Combination of modified or unmodified cetuximab and modified or unmodified tetrac (i.e., tetrac or nano-tetrac) in a vehicle, for example, a single nanoparticle, can also be used together with one or more additional conventional cancer chemotherapeutic agents as a delivery system to target cancer cells for the additional chemotherapeutic agents. That is, tetrac is recognized and liganded by integrin αvβ3-bearing tumor cells, and this fact can be used to bring the tumor cells into contact with the additional chemotherapeutic agents. Thus, in this model, three (or more) anti-cancer agents are transported directly to the cancer cell.

Accordingly, those skilled in the art will recognize that cetuximab, whether unmodified or as nanoparticulates, in conjunction with tetrac or triac, whether unmodified or as nanoparticulates, in combination with one or more conventional chemotherapeutic agents can be fabricated and used.

Tetraiodothyroacetic acid (tetrac) is a deaminated thyroid hormone analogue that binds to the integrin αvβ3 receptor for the hormone. (See Bergh et al., 2005. Endocrinology 146:2864-71; Davis et al., 2006. Cancer Res. 66:7270-75). Tetraiodothyroacetic acid (tetrac) is a thyroid hormone derivative with anti-proliferative activity in cancer cells that are initiated at a cell surface receptor for thyroid hormone on integrin αvβ3. (See Davis et al., 2009. Am J Physiol 297:E1238-E1246; Lin et al., 2009. Am J Physiol 296:C980-C991; Yalcin et al., 2009. Anticancer Res 10:3825-3831; and Glinskii et al., 2009. Cell Cycle 8:3554-3562). Tetrac is pro-apoptotic (see AB Glinskii et al., ibid.). Tetrac is also a polyfunctional anti-angiogenic agent. Tetrac is an antagonist at the receptor, inhibiting binding of agonist L-thyroxine (T₄), and 3,5,3'-triiodo-L-thyronine (T₃) to the integrin of cultured cells (see Berg et al. 2005. 146:2864-71) and blocking nongenomically initiated effects of T₄ and T₃ on signal transduction pathways (see Davis et al., 2006. Cancer Res. 7270-75; Lin et al., 2007. Steroids 72:180-87; and Lin et al., 2008. Carcinogenesis 29:62-69).

Inhibition of the angiogenic action of thyroid hormone by tetrac has been shown in the chick choioallantoic membrane (CAM) model and in the vessel sprouting model involving human dermal microvascular endothelial cells (HDMEC). Tetrac is effective in the CAM and HDMEC models. This inhibitory action of tetrac is thought to reflect its influence at the RGD recognition site on the integrin, which is relevant to cell surface pro-angiogenic growth factor receptors with which the integrin engages in cross talk and whose activities may be modulated by the integrin.
Tetrac also decreases basic fibroblast growth factor (bFGF) and vascular growth factor (VEGF)-induced angiogenesis in the absence of agonist thyroid hormone analogues (see Davis et al., 2004. Circ. Res. 94:1500-1506; and Mousa et al., 2008. Angiogenesis 11:183-90), thereby supporting cross talk between the integrin and the clustered receptors for VEGF, bFGF, and other pro-angiogenic peptides. Tetrac inhibits thyroid hormone-induced activation of mitogen-activated protein kinase (MAPK, ERK1/2) and cell proliferation in a variety of cancer cell lines (see Davis et al., 2006. Cancer Res. 7270-75; Lin et al., 2007. Steroids 72:180-87; and Lin et al., 2008. Carcinogenesis 29:62-69) and also induces the production of a pro-apoptotic protein, BcL-x short form, in human bronchial adenocarcinoma cells (see Tzirogiannis et al., 2007. Abstract # P1-602 in 89th Annual Meeting, The Endocrine Society), rodent glioma C6 cells (see Lin et al., 2008. Carcinogenesis 29:62-69) and human follicular thyroid cancer cells (see Yalcin et al., 2010. Thyroid 20:281-86). These latter studies suggest that tetrac treatment supports apoptosis in cancer cells, as do RNA microarray observations. (See Glinskii et al., 2009. Cell Cycle 8(21):3544-62; 2009; J Clin Endocrinol Metab, Feb. 2010, Epub only).

In addition to the ability of tetrac to block angiogenesis induced by VEGF and bFGF, tetrac has also been shown to enhance the response in vitro to either doxorubicin, etoposide, cisplatin, or trichostatin A of chemotherapy-resistant human tumor cell lines derived from neuroblastoma (SKN-SH/R), osteosarcoma (SaOS2/R), and breast carcinoma (MCF-7/R) cells. (See Rebbaa et al., 2008. Angiogenesis 11:269-76).

Because tetrac is known to have thyromimetic activity within cells (see Lameloise et al., 2001. Eur. J. Endocrinology 144:145-54; Moreno et al., 2008. Thyroid 18:239-253), a plasma membrane-impermeable form of tetrac (i.e., nano-tetrac) has been formulated in which nonreactive nanoparticles are covalently bound to the outer ring hydroxyl group of tetrac (either directly or via a linker). The presence of the nanoparticle does not inhibit the binding of the exposed ligand (tetrac) to the plasma membrane integrin αvβ3 (Cell Cycle, 2009). Cetuximab did not inhibit proliferation of ER-negative breast cancer MDA-MB cells significantly, however, simultaneous treatment with tetrac and cetuximab increased inhibition of cell proliferation. (See Figure 10).

Any of the thyroid hormone analogs used herein can be reformulated into a nanoparticle (e.g., by conjugation to a polymer). Conjugation of any of the compounds described herein can be accomplished via a covalent bond, e.g., an anhydride bond, an ester bond, an ether bond, or a sulfur linkage or any other construct that limits action of the thyroid hormone analog to the cell surface receptor. Those skilled in the art will recognize that such
reformulation prevents transport of the agent into the cell. The use of thyroid hormone analog nanoparticles is contemplated in order to limit their action to the integrin receptor and to increase the half life of the thyroid hormone analog. Thus, these thyroid hormone analog nanoparticles represent novel structures of choice for induction of apoptosis in cancer cells.

Exemplary polymers that the agents such as the thyroid hormone analogs can be conjugated to include, but are not limited to polyvinyl alcohol, acrylic acid ethylene co-polymer, methoxypolyethylene, polyethyleneglycol (PEG), polyacrylic acid, polylactic acid, agarose, polyglycolide, polylactide, PEO, m-PEG, PVA, PLLA, PGA, poly-L-lysine, Human Serum Albumin, cellulose derivatives, carbomethoxy/ethyl/hydroxypropyl, hyaluronic acid, folate linked cyclodextrin/dextran, sarcosine/amino acid spaced polymer, alginate, carrageenan, pectin/chitosan, chitosan, dextran, collagen, polyamine, poly aniline, poly alanine, polytrytophan, poly tyrosine, polylactide-co-glycolide (PLG), poly(lactic-co-glycolic acid) (PLGA), or polysyl glycolide having different molecular weights ranging from 2,000 - 20,000 Dalton. Other suitable polymers include, by way of non-limiting example, polylactiglode, polylactide, or copolymers thereof.

Thyroid hormone has been shown to stimulate HIF1α gene expression by a PB K3-dependent process. (See, Lin et al, 2009. Am J. Physiol Cell Physiol 296:C980-C991 (incorporated herein by reference)). Thus, thyroid hormone, at least in the form of the 3, 5, 3'-triiodo-L-thyronine (T3) analogue (but not necessarily limited to this form) is a natural and endogenous inhibitor of the crucial action of cetuximab on HIF1α. Thyroid hormone is anti-apoptotic via its action on integrin αvβ3. Moreover, tetrac blocks the anti-apoptotic effect of endogenous thyroid hormone. Therefore, combination treatment with cetuximab and tetrac or nano-tetrac in order to decrease HIF1α gene expression by a PI3K-requiring process, is proposed. Such a combination has an additional advantage of summated anti-angiogenic actions.

This action of thyroid hormone on HIF1α can be blocked by tetraiodothyroacetic acid (tetrac) and nanoparticulate tetrac, which have been shown to inhibit the actions of thyroid hormone analogs at the cell surface receptor for the hormone that have been described on the integrin αvβ3. (See Bergh et al., 2005. Endocrinology 146:2864-71 (incorporated by reference)). Thus, the combination of cetuximab and unmodified or nanoparticulate tetrac (i.e., nano-tetrac), when administered concurrently (i.e., in combination), can be used for the treatment of cancer and is intended specifically and selectively to protect the clinically desirable inhibitory activity of cetuximab on PI3K-depedent expression of the HIFIα gene.
Additionally, it will be readily apparent to those skilled in the art that cetuximab, whether unmodified or in nanoparticle form, can be used in combination with other anti-angiogenic thyroid hormone analogs, such as, for example, triiodothyroacetic acid (triac), the deaminated analog of 3, 5, 3’-triiodothyronine (T3). Again, triac can be used either unmodified or as a nanoparticulate.

As shown in Figure 1, a bellows perfusion cell culture system has been developed in which to define *in vitro* the anti-proliferative pharmacodynamics (PD) of tetrac in cancer cells. The system is based on a perfusion ("hollow fiber") model used to estimate pharmacokinetics (PK) and PD of antimicrobial agents (e.g., antibiotics) against epidemiologically important infectious pathogens. (See Bilello et al., 1994. Antimicrob Agents Chemother 38:1386-1391; Drusano et al. 2002. Antimicrob Agents Chemother 46:464-70; Louie et al., 2009. Antimicrob Agents Chemother 53:3325-3330). The standard hollow fiber (dialysis) system has been modified by replacing the hollow fiber ad chamber paradigm with a flow-through Bellco perfusion bottle pumping station system (Bellco Biotechnology, Vineland, NJ). In this system, cells of cancer lines of interest are grown on plastic flakes suspended in a flow-through, bellows-agitated system that allows for homogeneous exposure of cells to drug/drug metabolite solutions and frequent sampling of cells for viability. Harvesting often flakes provides sufficient numbers of cells for analytic purposes, including flow cytometric studies. Cellular outcomes that are measurable include cell cycle arrest, stages of apoptosis, and total cell numbers. The system can also be used to estimate PK and PD of single new biopharmaceutical cancer chemotherapeutic agents.

Using this system, nano-tetrac was found to inhibit cell proliferation more effectively than tetrac does. Specifically, unmodified tetrac inhibits the proliferation of cancer cells and does so with differing IC50's in different cell lines. When covalently linked to poly (lactic-co-glycolic) acid nanoparticles (PLGA), tetrac does not enter the cell, acts exclusively at the cell surface integrin receptor, and suppresses cancer cell proliferation to a greater degree than unmodified tetrac. Moreover, tetrac and nano-tetrac induced apoptosis by suppressing the proliferative activity of thyroid hormone and by differentially affecting expression of anti-apoptotic and pro-apoptotic cells to reduce cancer cell survival.

This perfusion bellows system also permitted analysis of treatment of cancer cells with tetrac or nano-tetrac in combination with other chemotherapeutic agents. For example, in the present studies, both unmodified and nano-tetrac were tested for anti-proliferative efficacy in combination with other anticancer agents such as the commercially-available monoclonal anti-epidermal growth factor receptor (EGFR) antibody Erbitux® (cetuximab).
Additive pharmacodynamic results using tetrac in combination with Erbitux® (cetuximab) were observed. Moreover, when tested in an in vitro model, tetrac and nano-tetrac were shown to have favorable pharmacodynamics as anti-cancer agents, whether acting alone or in conjunction with other agents such as cetuximab.

Tetrac has been shown to block thyroid hormone-induced biological activities such as activation of MAPK and PI-3K signal transduction pathways [see Bergh et al., 2005. Endocrinology 146:2864-71; Davis et al., 2006. Cancer Res. 66:7270-75; and Lin et al., 2009. Am. J. Physiol. Cell Physiol. 296:C980-91], cell proliferation [see Davis et al., 2006. Cancer Res. 66:7270-752], thyroid hormone inhibition of resveratrol-induced apoptosis [see Lin et al., 2008. Carcinogenesis 29:62-69], and sensory neuron sodium current [see Yonkers et al., 2008. J. Neurophysiol. 100:2719-25] through interference with the binding of thyroid hormone to integrin αvβ3. [See Bergh et al., 2005. Endocrinology 146:2864-71; Davis et al., 2006. Cancer Res. 66:7270-75; Lin et al., 2009. Am. J. Physiol. Cell Physiol. 296:C980-91; and Rebbaa et al., 2008. Angiogenesis 11:269-76]. However, tetrac alone can actually stimulate cell proliferation when it gains access to the cell interior. [See Figures 3 and 6].

However, the use of a nanoparticulate formulation of tetrac that effectively prevents access of tetrac to the interior of cells. Thus, the anti-proliferative effects induced by these tetrac equivalents can be compared. Nanoparticulate tetrac is two times more effective than unmodified tetrac as an anti-proliferative agent [see Figure 5A] and is five times more effective than tetrac in opposing T4-induced [3H]-thymidine incorporation (DNA replication) [see Figure 6B]. Morphological studies also indicate that nano-tetrac causes a change in cell shape after 5 days of incubation. Neither nano-tetrac nor tetrac affect normal, non-cancer cell proliferation even when a high concentration (10^-6 M) is used. [See Figure 9].

Radiolabeled doxorubicin uptake in cells is enhanced by tetrac, suggesting that one or more export mechanisms for chemotherapeutic agents are inhibited. [See Rebbaa et al., 2008. Angiogenesis 11:269-76]. Tetrac is anti-proliferative in several cancer cell lines and has been shown to enhance the cellular response in vitro to doxorubicin, etoposide, cisplatin, and trichostatin A in resistant tumor cell lines derived from neuroblastoma, osteosarcoma, and breast cancer. [See Rebbaa et al., 2008. Angiogenesis 11:269-76]. Tetrac also enhances cancer cell susceptibility to apoptosis, suggesting that the agent may target multiple drug resistance mechanisms.

The anti-proliferative effect of tetrac and/or nano-tetrac on cancer cells in the perfusion bellows cell system shown in Figure 1 was seen in as early as 3 days after tetrac or nano-tetrac were added to the system [See Figure 3]. The anti-cancer effects of tetrac and
nano-tetrac in xenografts has been established in 3 days after the onset of drug administration. (See D'Arezzo et al, 2004. Endocrinology 145:5694-5703). While the effects of tetrac in the xenograft model have been shown to involve both primary effects on tumor cell proliferation as well as an anti-angiogenesis effect, the effect of tetrac and nano-tetrac in the perfusion bellows cell system is the suppression of cell proliferation.

Moreover, in the bellows perfusion cell culture system, the cells are alternately exposed to fresh medium and air. (See Figure 1). This provides optimized growth conditions for cancer cells by maximizing nutrient uptake and oxygen transfer. Cells can therefore be studied over longer time periods, e.g., up to three weeks as shown in the experiment with tetrac and MDA-MB cells in Figure 3C and the phase where cell counts have reached a plateau can be observed. By obtaining information about both the slope and the plateau of the cell count with respect to time and by utilizing mathematical modeling (described in Example 1, infra), two different effects of tetrac on cancer cells can be distinguished. (See Figure 2).

In addition to allowing for the treatment of the cells with constant drug concentrations (reflecting in vivo continuous infusion treatment), other dosing regimens such as multiple short-term or intermittent infusions or brief injections can also be studied in the perfusion bellows cell culture system by adjusting the flow rate of the medium and the dosing schedule. In this way, drug concentration/time profiles such as those expected in human or animal studies can be simulated and the effects on cancer cells of changing drug concentrations as anticipated in vivo may be observed in the in vitro bellows system. Taken together with mathematical modeling, these in vitro systems can support optimal design of animal and human studies thereby saving time and costs in drug development. Likewise, because a wider range of drug concentrations can be studied in vitro than in animal models, selection of appropriate concentrations or in vivo studies may become more efficient.

As described in detail in Example 1, infra, mathematical modeling was utilized to increase the amount of information gained from the reported experiments. By considering the whole time course of cell counts in response to multiple concentrations of tetrac and control treatment simultaneously, more insight can be gained into the dose-response relationship and the mechanism of action of a drug. Also mechanism-based models are more useful in making predictions (e.g., for other dosage regimens) than empirical growth models.

For human glioblastoma (U87MG) cells, mathematical modeling suggested a higher maximum effect but lower sensitivity of the effect on probability of successful replication compared to the effect on rate of growth for both unmodified and nano-tetrac. For both
effects, this sensitivity was higher towards nano-tetrac than unmodified tetrac. This
difference may be explained by the ability of unmodified tetrac to penetrate into cells and
thereby exert proliferative effects in addition to the anti-proliferative effects initiated at the
cell surface receptor. Therefore, the net anti-proliferative effect of unmodified tetrac is
decreased, whereas because nano-tetrac does not gain access to the cell interior, the overall
anti-proliferative effect is unchanged.

In human breast cancer (MDA-MB) cells two different modeling results are available. For the first model sparse data from two different experiments were pooled (see Table 1, study \( \text{infra} \), whereas the second set of parameter estimates is based on a single large experiment with rich sampling (see Table 1, study 2, \( \text{infra} \)). Therefore the results from study 2 appear more reliable. MDA-MB cells appeared to have similar (or lower) sensitivity to nano-tetrac compared to unmodified tetrac for the effect on growth rate and a higher sensitivity for the effect on success of replication. These mechanism-based models adequately described the cell counts over time and can be used to support the design future experiments with tetrac and nano-tetrac.

Inhibition by nano-tetrac of thyroxine-induced MAPK activation and PCNA expression in cultured cells correlates well with the anti-proliferative effects induced by tetrac and nano-tetrac in the perfusion bellows cell culture system. Results of anti-proliferation studies with nano-tetrac analogues in the bellows system are likely to be more predictive of \textit{in vivo} effects than studies performed in Petri dish cell cultures. The continuous cell perfusion studies provide useful pharmacodynamic information for the application of new drugs to the treatment of a variety of neoplastic diseases.

This, in combination with pharmacodynamic modeling and by including information about the expected pharmacokinetics of a drug, allows the perfusion bellows cell culture system to be used to study the dose-response relationships of anti-neoplastic agents over a very wide concentration range \textit{in vitro}, and can support translation from \textit{in vitro} models to animal models and human clinical trials.

Thus, combination of tetrac and cetuximab, whether unmodified or modified as a nanoparticulate, represents a novel treatment for the inhibition of PI3K-dependent HIF1\( \alpha \) gene expression in cancer cells. Moreover, the combination excludes the antagonistic action of host endogenous thyroid hormone on the HIF1\( \alpha \) gene expression. (See, Lin et al., 2009. Am J. Physiol Cell Physiol 296:C980-C991). Likewise, an anti-estrogen compound can be used in conjunction with the combination of tetrac and cetuximab, \textit{e.g.}, in estrogen-bearing breast cancers or lung cancers that are ER-positive. (See Koutras et al. \textit{Mol Cancer} 8(1): 109,
2009). That is, the combination of agents is directed at multiple sites of vulnerability in such cancer cells.

Additionally, the combination of cetuximab and tetrac in a vehicle, such as a nanoparticle, in further combination with one or more conventional cancer chemotherapeutic agents, permits delivery of such chemotherapeutic agents directly to integrin αvβ3-bearing tumor cells. Suitable chemotherapeutic agents include, but are not limited to, doxorubicin, etoposide, cyclophophamide, 5-fluoracil, cisplatin, trichostatin A, paclitaxel, gemcitabine, taxotere, cisplatinum, carboplatinum, irinotecan, topotecan, adrimycin, bortezomib, and atoposide or any combinations or derivatives thereof.

The agents described herein (e.g., cetuximab and the anti-angiogenic thyroid hormone analog) are preferably administered in a formulation (including the analogs, polymeric forms, and/or any derivatives thereof) together with a pharmaceutically acceptable carrier. Any formulation or drug delivery system containing the active ingredients, which is suitable for the intended use that are generally known to those of skill in the art, can be used. Suitable pharmaceutically acceptable carriers for oral, rectal, topical, or parenteral (including subcutaneous, intraperitoneal, intramuscular and intravenous) administration are known to those of skill in the art. Those skilled in the art will recognize that the carrier must be pharmaceutically acceptable in the sense of being compatible with the other ingredients of the formulation and not being deleterious to the recipient thereof.

Formulations suitable for parenteral administration may include sterile aqueous preparations of the active compound, which are preferably isotonic with the blood of the recipient. Thus, such formulations may contain distilled water, 5% dextrose in distilled water or saline. Useful formulations may also include concentrated solutions or solids containing any of the compositions or compounds described herein, which upon dilution with an appropriate solvent, give a solution suitable for parental administration.

For enteral administration, a compound can be incorporated into an inert carrier in discrete units such as capsules, cachets, tablets, or lozenges, each containing a predetermined amount of the active compound(s); as a powder or granules; or a suspension or solution in an aqueous liquid or non-aqueous liquid, e.g., a syrup, an elixir, an emulsion or a draught. Suitable carriers may be starches or sugars and may include lubricants, flavorings, binders, and other materials of the same nature.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active compound in a free-flowing form, e.g., a powder or granules, optionally
mixed with accessory ingredients, e.g., binders, lubricants, inert diluents, surface active or dispersing agents. Molded tablets may be made by molding in a suitable machine, a mixture of the powdered active compound with any suitable carrier.

A syrup or suspension may be made by adding the active compound to a concentrated, aqueous solution of a sugar, e.g., sucrose, to which may also be added any accessory ingredients. Such accessory ingredients may include flavoring, an agent to retard crystallization of the sugar or an agent to increase the solubility of any other ingredient, e.g., as a polyhydric alcohol, for example, glycerol or sorbitol.

Formulations for rectal administration may be presented as a suppository with a conventional carrier, e.g., cocoa butter or Witepsol S55 (trademark of Dynamite Nobel Chemical, Germany), for a suppository base.

Alternatively, the compounds may be administered in liposomes, microspheres (or microparticles), or attached to nanoparticles. Methods for preparing liposomes and microspheres for administration to a patient are well known to those of skill in the art. For example, U.S. Pat. No. 4,789,734, the contents of which are hereby incorporated by reference, describes methods for encapsulating biological materials in liposomes. Essentially, the material is dissolved in an aqueous solution, the appropriate phospholipids and lipids added, along with surfactants if required, and the material dialyzed or sonicated, as necessary. A review of known methods is provided by G. Gregoriadis, Chapter 14, "Liposomes," Drug Carriers in Biology and Medicine, pp. 287-341 (Academic Press, 1979).

Microspheres formed of polymers or proteins are well known to those skilled in the art, and can be tailored for passage through the gastrointestinal tract directly into the bloodstream. Alternatively, the compound can be incorporated and the microspheres, or composite of microspheres, implanted for slow release over a period of time ranging from days to months. (See, for example, U.S. Pat. Nos. 4,906,474; 4,925,673; and 3,625,214, and Jein, 1998. TIPS 19:155-157), the contents of which are hereby incorporated by reference.

Any of the compounds described herein, (e.g., cetuximab, tetrac or triac, and/or the polymeric forms thereof) can be formulated into nanoparticles. Preferred nanoparticles are those prepared from biodegradable polymers, such as, for example, polyethylene glycols, polyglycolide, polylactide and copolymers thereof. Those of skill in the art can readily determine an appropriate carrier system (i.e., the polymer) used for preparation of nanoparticulate forms of cetuximab and/or tetrac (or triac) depending on various factors, including, for example, the desired rate of drug release and the desired dosage.
In some embodiments, the formulations are administered via catheter directly to the inside of blood vessels. The administration can occur, for example, through holes in the catheter. In those embodiments wherein the active compounds have a relatively long half life (on the order of 1 day to a week or more), the formulations can be included in biodegradable polymeric hydrogels, such as those disclosed in U.S. Pat. No. 5,410,016 to Hubbell et al. These polymeric hydrogels can be delivered to the inside of a tissue lumen and the active compounds released over time as the polymer degrades. If desirable, the polymeric hydrogels can have microparticles or liposomes which include the active compound dispersed therein, providing another mechanism for the controlled release of the active compounds.

The formulations may also be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. For example, such methods include the step of bringing the active compound(s) into association with a carrier, which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing the active compound(s) into association with a liquid carrier or a finely divided solid carrier and then, if necessary, shaping the product into desired unit dosage form.

The formulations can optionally include one or more additional components, such as various biologically active substances including antivirals, antibacterials, antiinflammatories, immuno-suppressants, analgesics, vascularizing agents, and/or cell adhesion molecules.

In addition, any of the formulations of the invention may further include one or more optional accessory ingredient(s) routinely utilized in the art of pharmaceutical formulations, e.g., diluents, buffers, flavoring agents, binders, surface active agents, thickeners, lubricants, suspending agents, and/or preservatives (including antioxidants) and the like.

The invention will be further illustrated in the following non-limiting examples.

EXAMPLES

Example 1: Pharmacodynamic Modeling of Tetraiodothyroacetic Acid-induced Anti-Proliferation in Cancer Cells using a Perfusion Bellows Cell Culture System

Materials and Methods

Cell lines. Human glioblastoma cells (U87MG), human breast cancer MDA-MB-231 cells (MDA-MB), African green monkey kidney epithelial CV-I cells and human embryonic kidney 293T cells were purchased from ATCC. Human follicular thyroid cancer FTC236
cells were generously provided by Dr. Orlo Clark (University of California at San Francisco-Mt. Zion Medical Center, San Francisco, CA). Human adenoid cystic carcinoma cells (TGS1 12T) were made available from the laboratory of Dr. L. Queimado at the University of Oklahoma. U87MG cells were maintained for study in MEM supplemented with 10% fetal bovine serum (FBS), and MDA-MB, CV-I and 293T cells were maintained in DMEM that was supplemented with 10% FBS. Follicular thyroid cancer cells were maintained in 50% DMEM/50% Ham's F-12 plus 10 mU/ml of TSH (Sigma). TGS1 12T cells were maintained in RPMI medium that contained 20 ng/mL epidermal growth factor (EGF) (200 µL of stock at 10 µg/mL), 400 ng/mL hydrocortisone (800 µL of stock at 50 µg/mL) and 5000 ng/mL insulin (50 µL of stock at 10 mg/mL). Cultured cells were maintained in a 5% CO<sub>2</sub>/95% air incubator at 37°C.

Pharmacodynamics (PD) of tetrac. Figure 1 shows a newly developed bellows bottle cell culture system that is a disposable bioreactor capable of high density cell culture for studies of anti-cancer drugs. Each cell culture system is a compressible (bellows) 500 mL bottle which contains cell culture medium and specially-treated polymer flakes to which cells spontaneously attach and grow. Through moving bellows and porous membranes, the level of the medium in the bottle changes periodically. Consequently, the cells are alternately submerged in the culture medium, then exposed to 5% CO<sub>2</sub>/95% air which creates a dynamic interface between air and medium on the cell surface that maximizes nutrient uptake and oxygen transfer. The system provides a low shear, high aeration and foam-free culture environment. Proprietary treatment of the surfaces of the flakes enables seating and harvesting of cells and secreted proteins are readily isolated from the perfusate.

In a non-perfusion bellows cell culture system that was also used, the medium in each bottle was replaced by fresh medium every 24 h. In the perfusion bellows cell culture system, medium was progressively refreshed over 24 h, i.e., one complete change of medium occurred over 24 h.

In establishing the system, 5 x 10<sup>7</sup> cells were seeded in perfusion and non-perfusion bellows bottles and incubated overnight at 37°C. After that, flakes were harvested, trypsinized and cells were collected. Cell numbers were counted. The numbers of cells that attached to were 10-15 x10<sup>6</sup> per bottle. For experiments, the perfusion bellows cell culture system was run for 2 d prior to starting experiments. The cell numbers at this point were about 30-50x 10<sup>6</sup> cells/ bottle. Cell cultures were then exposed to 1% FBS-containing medium. Tetrac or nano-tetrac was added to the medium in the reservoir bottle to achieve the final concentrations reported for each experiment.
Liquid chromatography-tandem mass spectrometry (LC/MS/MS) In LC/MS/MS experiments, medium samples (20 µL) were injected onto an HP 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a narrow-bore column Zorbax Eclipse XDB-C18 (5 µm, 150 x 2.1 mm; Agilent). Separation was performed using a mobile phase of 0.1% (v/v) acetic acid (A) and 100% acetonitrile (B), with a linear gradient of 20-60% B over 25 min. Flow rate was maintained at 0.2 mL min⁻¹ and elution was monitored by a diode array detector (200-600 nm). The LC effluent was then introduced into a turbo ion-spray source on a Q/STAR-XL quadruple/time-of-flight (TOF) hybrid mass spectrometer (Applied Biosystems, Foster City, CA, USA). Negative ESI mass spectra were acquired over the range from m/z 100 to 400. The electrospray voltage was set at -4.5 kV and the source temperature was maintained at 475°C. CID spectra were acquired using nitrogen as the collision gas under collision energies of 25-55 V. High purity nitrogen gas (99.995%) was used as the nebulizer, curtain, heater and collision gas source.

Thymidine incorporation. TGS1 12T cells were seeded in 24-well trays and exposed to 10% hormone-depleted FBS-supplemented medium for 2 d, then treated with 0.25% hormone-depleted FBS-supplemented medium prior to starting the experiments. Aliquots of cells were treated with T₄, tetrac or nano-tetrac as indicated, as well as 1 µCi [³H]-thymidine (final concentration, 13 nM) for 24 h. Cells were then washed twice with cold PBS. TCA (5%, 1 mL) was added and the plate was held at 4°C for 30 min. The precipitate was washed twice with cold ethanol; 2% SDS (1 mL) was added to each well and the TCA-precipitable radioactivity was quantitated in a liquid scintillation counter.

Immunoblotting. The techniques have been described in a number of publications. (See Davis et al, 2006. Cancer Res. 66:7270-7275; Lin et al, 2007. Steroids. 72:180-187; Lin et al, 2008. Carcinogenesis. 29:62-69; and Davis et al., 2004. Circ Res. 94:1500-1506, each of which is herein incorporated by reference in its entirety). Nucleoproteins were separated on discontinuous SDS-PAGE (9% gels) and the proteins transferred by electroblotting to nitrocellulose membranes (Millipore, Bedford, MA). After blocking with 5% milk in Tris-buffered saline containing 0.1% Tween, the membranes were incubated with selected primary antibodies overnight. The secondary antibodies were either goat anti-rabbit IgG (1:1000, Dako, Carpenteria, CA) or rabbit anti-mouse IgG (1:1000, Dako), depending upon the origin of the primary antibody. Immunoreactive proteins were then detected by chemiluminescence.

RT-PCR. Total RNA was isolated as described previously. (See Lin et al., 2008. Carcinogenesis. 29:62-69). First strand complementary DNAs were synthesized from 1 µg
of total RNA using oligo dT and AMV Reverse Transcriptase (Promega, Madison, WI). First-strand cDNA templates were amplified for GAPDH, c-fos, PIG3, c-Jun, and BAD mRNAs by polymerase chain reaction (PCR), using a hot start (Ampliwax, Perkin Elmer, Foster City, CA). Primer sequences were GAPDH [5'-AAGAAGATGCGGCTGACTGCGAGCCACA-3’ (forward) (SEQ ID NO: 1) and 5'-TCTCATGTTCACACCCATGAGCAACATG-3’ (reverse) (SEQ ID NO:2)], c-fos [5'-GAATAAGATGGCTGCAGCCAATGCAGCGAA-3’ (forward) (SEQ ID NO:3) and 5'-CAGTCA-GATCAAGGAAGCAGACACATCT-3’ (reverse) (SEQ ID NO:4)], PIG3 [5'-TGGTCACAG-CTGGCTCCCAGAA-3’ (forward) (SEQ ID NO:5) and 5'-CCGTGGAGAAGTGAGGCAATTT-3’ (reverse) (SEQ ID NO:6)], c-jun [5'-GGAACGACCTTCTATGAGCAGCCCTCAA-3’ (forward) (SEQ ID NO:7) and 5'-GAACCCCTCTGCTCATGTCACGTTCTT-3’ (reverse) (SEQ ID NO:8)] and BAD [5'-GTT-TGAGCCGAGTGAGC AGG-3’ (forward) (SEQ ID NO:9) and 5’-ATAGCGCTGTGCTGCCAGA-3’ (reverse) (SEQ ID NO:10)]. The PCR cycle was an initial step of 95°C for 3 min, followed by 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, then 25 cycles and a final cycle of 72°C for 8 min. PCR products were separated by electrophoresis through 2% agarose gels containing 0.2 µg of ethidium bromide/ml. Gels were visualized under UV light and photographed with Polaroid film (Polaroid Co., Cambridge, MA). Photographs were scanned under direct light for quantitation and illustration. Results from PCR products were normalized to the GAPDH signal.

Flow cytometry analysis. Cells were harvested from flakes by trypsinization, washed with PBS and were resuspended in 200 µL PBS (1 x 10^5 - 1 x 10^6 cells). To quantify cellular DNA content, cells were permeabilized by fixation with 70% ethanol for 30 min at 4°C. Samples can be stored in 70 % ethanol at -20°C for several weeks prior to propidium iodide (PI) staining and flow cytometric analysis. If cellular DNA quantification was performed on the same day of cell harvest, the cells were washed after permeabilization in PBS and resuspended in 500 µL PBS. Then 2.5 µL RNase (DNase-free) was added to the cell suspension and incubation was carried out at 37°C for 30 min. The cell suspension was chilled on ice to 4°C and 50 µL propidium iodide (PI) was added to the cell suspension. Samples were then kept in the dark at room temperature for 30 min, after which they were subjected to flow cytometry. Samples were analyzed on FACSCalibur™ (Becton Dickinson), using CellQuest software to determine DNA content. Fluorescence-activated cell sorting (FACS) analysis was performed using Annexin V-FITC and PI. The relative
percentages of cells in G₁, S, or G/M phase were calculated from FL-2 histograms using ModFit LT software.

**Statistical methods and calculations.** Immunoblot and nucleotide densities were measured with a Storm 860 phosphorimager, followed by analysis with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Student's t test, with P <0.05 as the threshold for significance, was used to evaluate the significance of the hormone and inhibitor effects.

**Mathematical modeling.** The time course of cell counts of the various cancer lines treated with different concentrations of tetrac or nano-tetrac was modeled by utilizing the pooled approach in NONMEM VI (version 6.2). All time points and treatment arms within each experiment were modeled simultaneously. A mechanism-based model (see Bulitta et al., 2009. *Antimicrob. Agents Chemother.* 53:46-56) was adapted to describe the proliferation of cancer cells and the inhibition of proliferation by tetrac. This model assumes two populations of cells in different phases of the cell cycle: cells that are preparing for replication (phase 1) and cells that are immediately "pre-replication" (phase 2). Cells transition from phase 1 to phase 2 by a first-order growth rate constant, while replication from phase 2 to phase 1 is assumed to be fast (see Figure 2).

The number of cells in phase 1 and 2 are described by:

\[
\frac{dC_1}{dt} = Rep \cdot \text{InhR} \cdot k_{21} \cdot C_Z - k_{12} \cdot \text{Inhk} \cdot C_1
\]

\[
\frac{dC_2}{dt} = - k_{21} \cdot C_2 - k_{12} \cdot \text{Inhk} \cdot C_1
\]

\[
C_t = C_1 + C_2
\]

where C₁ is the number of cells in phase 1, C₂ the number of cells in phase 2, k₂₁ the first order rate constant for replication (transition from phase 2 to phase 1), and k₁₂ the first-order growth rate constant for transition from phase 1 to phase 2. The total number of cells Cₜ is the sum of C₁ and C₂. Rep is the replication efficiency factor which is described by:

\[
Re-p = 2 \cdot \left(1 - \frac{C_t}{C_{max} + C_t}\right)
\]
where Cmax is the maximum number of cells. Without tetrac, the replication efficiency factor approaches 2, which reflects a 100% probability of successful replication. InhR describes the inhibitory effect of tetrac on the probability of successful replication:

\[ I_{\text{InhR}} = \left\{ 1 - \frac{I_{\text{maxR}} \cdot \text{Tetrac}}{\text{iCSQR} - \text{Tetrac}} \right\} \]

Where ImaxR is the maximum effect of tetrac on probability of successful replication and IC50R is the tetrac concentration needed to achieve a half-maximal effect.

Inhk describes the inhibitory effect of tetrac on the rate of growth:

\[ I_{\text{InhR}} = \left\{ 1 - \frac{I_{\text{maxk}} \cdot \text{Tetrac}}{\text{iCSQk} - \text{Tetrac}} \right\} \]

Where Imaxk is the maximum effect of tetrac on rate of growth and IC50k is the tetrac concentration needed to achieve a half-maximal effect.

**Results**

Tetraiodothyroacetic acid inhibits cancer cell proliferation. The pharmacodynamics of tetrac as an anti-proliferative agent versus different cancer cells has been studied in the bellows cell culture system shown in Figure 1.

Human glioblastoma U87MG cells were treated with different concentrations of tetrac (10^{-9} - 10^{-3} M) for 7 days and tetrac was replenished daily. The turnover rate of tetrac in the culture system was measured. Tetrac detected was 75% of the original concentration after 24 h incubation in medium with 10% FBS in the absence of cells at both room temperature and 37°C. There was 12% tetrac decay when tetrac was incubated with cell cultures at 37°C. These results indicate that tetrac is stable in the perfusion bellows cell system.

A model including effects of tetrac on both rate of growth and probability of successful replication (see Figure 2) and lag-time for growth during the first 2 days adequately described the time course of the cell counts. As shown in Figure 3A, tetrac caused a concentration-dependent reduction in U87MG cell proliferation. While 10^{-9} M tetrac was the least effective, 10^{-8} and 10^{-7} M tetrac caused more than 15% and 28% decreases in cell counts when compared with the untreated control cells after 7-day treatment. (See Figure 3A). Higher concentrations of the agent were proportionately more effective. The parameter estimates for IC50k and IC50R (shown in Table 1) suggested that the U87MG cells were more sensitive to
the effect on rate of growth than to the effect on success of replication. However the capacity (Imax) was higher for the effect on success of replication (ImaxR > Imax).

Table 1. Parameter estimates for effects of tetrac on proliferation of cancer cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Formulation</th>
<th>Effect on rate of growth</th>
<th>Effect on success of replication</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Imax</td>
<td>IC50k (µM)</td>
</tr>
<tr>
<td>U87MG</td>
<td>Tetrac</td>
<td>0.57</td>
<td>0.047</td>
</tr>
<tr>
<td>MDA-MB 231</td>
<td>Tetrac (study 1)</td>
<td>0.19</td>
<td>0.0076</td>
</tr>
<tr>
<td>MDA-MB 231</td>
<td>Tetrac (study 2)</td>
<td>0.85</td>
<td>5.1</td>
</tr>
<tr>
<td>U87MG</td>
<td>nano-Tetrac</td>
<td>0.34</td>
<td>0.0001b</td>
</tr>
<tr>
<td>MDA-MB 231</td>
<td>nano-Tetrac</td>
<td>1</td>
<td>6.3</td>
</tr>
</tbody>
</table>

a Imax at time = 0, Imax decreases with time (potentially due to functional adaption of the cells or the presence of two or more subpopulations with different sensitivities towards tetrac
b fixed as the lowest concentration studied was 0.001 µM
c sparse sampling, data pooled from two different studies
d rich data from one single study over 19 days

The IC50 estimates for tetrac nanoparticles (nano-tetrac) are hypothetical concentrations assuming all of the tetrac bound on the nanoparticle is available for binding to the integrin receptor.

The anti-proliferative effect of tetrac in human estrogen receptor (ER)-negative breast cancer MDA-MB cells was also studied. Aliquots of cells were treated with several concentrations of tetrac (10⁻⁹ - 10⁻³ M) for 9 d and tetrac was replenished daily. Cells were then harvested and counted. The results presented in Figure 3B reveal a concentration-dependent effect of tetrac on MDA-MB cell proliferation. The highest tetrac concentration (10⁻³ M) induced a large anti-proliferative effect whereas at lower concentrations tetrac did not show anti-proliferative effects during the earlier days of treatment. (See Figure 3B). The results shown in Fig. 3B are pooled data from two experiments (one with sampling on days 1 and 2, and one with sampling on days 3, 5, 7, and 9) in MDA-MB cells with the same tetrac concentrations investigated. Modeling suggested a higher sensitivity (lower IC50) for the effect on growth rate and a larger capacity (larger Imax) of the effect on replication. The model assumed a decrease in ImaxR over time which could be due to functional adaptation of the cells or the presence of two or more subpopulations with different sensitivities towards tetrac.

In another study, MDA-MB cells were treated with 7 different concentrations of tetrac ranging from 10⁻⁹ to 10⁻³ M or with control medium for 19 d and total cell counts were
determined every one or two days. (See Figure 3C). A model including effects on both rate of growth and success of replication (see Figure 2) and a lag-time of growth adequately described the data. The parameter estimates suggest a higher sensitivity (lower IC50) for the effect on probability of successful replication and a larger capacity (larger Imax) of the effect on rate of growth.

Although tetrac does have a growth-suppressive effect late in the treatment period, it may also have a proliferative effect on cancer cells. This presumably reflects access of the agent to the cell interior where it is a thyroid hormone agonist (thyromimetic), rather than an inhibitor (see Lameloise et al., 2001. Eur J Endocrinology 144:145-154), as it is exclusively at the cell surface receptor. In order to examine whether tetrac enters cells, human glioblastoma U87MG cells were incubated over different time periods with [125I]-labeled tetrac. Cells were harvested and medium, plasma membranes, cytosols and nuclear extracts were prepared for scintillation counting. [125I]-Labeled tetrac reached the maximal concentration in the cytosolic fraction after 4 h incubation and increased in nuclear fractions after 36 h incubation. (See Figure 4). Thus, tetrac enters relatively quickly into cytosol and more slowly into nuclei.

**Nano-tetrac shows a consistent anti-proliferative effect in cancer cells.** In order to prevent uptake of tetrac by cancer cells, the hormone analogue was reformulated as a nanoparticle. The anti-proliferative effect of the resulting tetrac nanoparticles was then studied in MDA-MB cells. Cells were treated with constant concentrations of 10^{-6} and 2.5x10^{-6} M tetrac or nano-tetrac for 9 d. Results indicate that the anti-proliferative effect of nano-tetrac in MDA-MB cells is more prominent than that of unmodified tetrac. (See Figure 5A). There was a 100-fold concentration difference in anti-proliferation efficacy between tetrac and nano-tetrac. In another study MDA-MB cells were treated with 4 different concentrations of nano-tetrac (10^{-9} to 10^{-5} M) for 9 d. (See Figure 5B). Based on mathematical modeling, the sensitivity of the MDA-MB cells for the nano-tetrac effect on probability of successful replication was considerably higher than that for the effect on rate of growth, while the capacity was similar for both effects (see Table 1, supra).

Studies of nano-tetrac-induced antiproliferation were also conducted in U87MG cells and results indicate that the anti-proliferative effect of nano-tetrac was concentration-dependent. Concentrations of the nanoparticulate as low as 10^{-9}M reduced cell number by 36% (control vs. 10^{-9} M nano-tetrac = 1.641x10^{8} vs. 2.264x10^{8}) after 7 d of treatment. (See Figure 5C). Modeling suggested a higher sensitivity (lower IC50) for the effect on replication and a higher capacity for the effect on rate of growth (see, Table 1, supra). Both
IC50k and IC50R were lower for nano-tetrac than for unmodified tetrac in U87MG cells indicating a higher sensitivity to nano-tetrac (see, Table 1, supra). For both MDA-MB cells and U87MG cells, the model assumes a decrease in ImaxR over time and a lag-time of growth during the first two days.

**Tetrac and nano-tetrac inhibit thyroid hormone-induced proliferation of human adenoid cystic carcinoma cells.** Human adenoid cystic carcinoma TGS1 12T cells were cultured in medium that contained 0.25% hormone-stripped serum for 2 d and then treated with $10^{-7}$ M tetrac or nano-tetrac for 30 min prior institution of $10^{-7}$ M T$_4$ treatment for 24 h. Nuclear proteins were separated by SDS-PAGE followed by western blot analysis with proliferating-cell nuclear antigen (PCNA) antibody. Thyroid hormone-induced PCNA accumulation was inhibited by tetrac. (*See Figure 6A*). Proliferation of TGS1 12T cells was stimulated by $10^{-7}$ M T$_4$, while tetrac at the same concentration had little effect alone, but blocked the T$_4$ effect. (*See Figure 6A*). Inhibition of thyroid hormone-induced proliferation was confirmed by inhibition by tetrac and nano-tetrac of thyroid hormone-induced accumulation and [$^{3}$H]-thymidine incorporation. (*See Figure 6B*). In studies of thymidine incorporation in the same cell line, nano-tetrac alone reduced baseline thymidine incorporation, and reduced T$_4$-induced thymidine incorporation by more than 14-fold that of tetrac (70-fold reduction vs. 5-fold reduction). (*See Figure 6B*). On the other hand, although tetrac inhibited thyroxine-induced thymidine incorporation, unmodified tetrac when added *alone* stimulated thymidine incorporation (*see Figure 6B*) to a limited degree in this relatively brief study. The effect of nano-tetrac and tetrac on cell proliferation was examined by counting TGS1 12T cells which were treated daily with $10^{-6}$ M tetrac or $10^{-6}$ M nano-tetrac. The decrease in cell count in the nano-tetrac-treated cells ($1.69 \times 10^8$ cells) after 7 d was 2-fold more than that of the tetrac-treated culture ($3.65 \times 10^8$ cells), compared to the untreated control cultures. (*See Figure 6C*).

**Role of apoptosis in the tetrac effect on cancer cells.** Cells were harvested for FACS analysis 1-5 d after treatment with $10^{-7}$ to $10^{-5}$ M tetrac. There was a 10-fold increase of apoptotic cells with $10^{-5}$ M tetrac treatment as compared to untreated control cells at 1 d and $10^{-7}$ M and $10^{-6}$ M tetrac also induced apoptosis. (*See Figure 7*). By day 2, tetrac, $10^{-7}$ M and $10^{-6}$ M, induced apoptosis by 2.5-fold as compared to untreated control. Similar results were obtained after 4 d of treatment of tetrac. That is, cells treated with $10^{-7}$ M tetrac showed highest proportion of apoptotic cells. (*See Figure 7*). These results raise the possibility that there are two dose-dependent types of tetrac-induced apoptosis: one is induced transiently by $10^{-5}$ M tetrac and the other is induced by $10^{-6}$ and $10^{-7}$ M tetrac.
The pro-apoptotic gene expression in tetrac- and nano-tetrac-treated MDA-MB cells and U87MG cells was also examined. RNA was extracted from cells harvested from the perfusion bellows cell culture system at the end of treatment. Primers for PIG3, c-jun and c-fos were constructed for RT-PCR studies. Treatment of cells for 1 day with tetrac (10^-6 M) increased expression of c-fos and c-jun. (See Figure 8). Nano-tetrac increased expression of PIG3, c-fos and c-jun. (See Figure 8).

**Effects of tetrac and nano-tetrac on non-malignant cells.** In order to confirm that tetrac and nano-tetrac only suppress cell proliferation in cancer cells, tetrac and nano-tetrac were also examined for potential anti-proliferative effects on immortalized non-malignant cells, using monkey kidney epithelial CV-I cells and human embryonic kidney 293T cells. The cells were treated daily with either 10^-6 M tetrac or 10^-5 M nano-tetrac for 7 days, then were harvested, counted and examined microscopically. There was no significant change in either cell numbers (see Figure 9) or in morphology comparing the untreated control cells and those treated with either tetrac or nano-tetrac. These results suggest that nano-tetrac and tetrac only affect malignant cell proliferation and not that of non-neoplastic cells.

**Tetrac potentiates cetuximab-induced apoptosis in human breast cancer cells.** The EGFR antibody, cetuximab, has been used in clinical trials either alone or combination with other anti-cancer drug. Cetuximab inhibits binding of the endogenous ligand for the receptor (EGF) and decreases cell motility, invasiveness and metastasis and also promotes apoptosis. (See Kalofonos et al., 2006. Curr. Top. Med. Chem. 6:1687-1705 (incorporated herein by reference)). Combining cetuximab with various chemotherapeutic agents has revealed additive or potentiated growth inhibition in various cancer cell lines, such as those from colon, head and neck, breast, kidney and bladder. (See Balin-Gauthier et al., 2006. Cancer Chemother. Pharmacol. 57:709-718; Martens et al., 2008. Clin. Cancer Res. 14:5447-5458). Approved for clinical use against head-and-neck and colorectal cancers, cetuximab has recently been shown to be effective against invasive glioblastoma xenografts in the mouse. (See Prichard et al., 2007. Laryngoscope. 117:674-679).

In order to examine whether tetrac or nano-tetrac potentiate cetuximab-induced anti-proliferation, MDA-MB cells were treated with cetuximab (0.1 µg/ml) in the presence or absence of 10^-6 M tetrac. Both agents suppressed cell proliferation in MDA-MB cells. (See Figure 10). The inhibitory effects on cell growth of cetuximab and tetrac after 8 d treatment were 34% and 38%, respectively. The combination of tetrac and cetuximab reduced total cell number by up to 63%. These results suggest that the combination of tetrac and cetuximab may increase efficacy of cancer chemotherapy when compared to effects of either drug alone.
Example 2: Anti-Proliferative Effects of Erbitux® (Cetuximab) and Tetrac Nanoparticles on Colon Cancer Cells

Using the bellows perfusion cell culture system shown in Figure 1, the effects of tetrac nanoparticles (NP-Tetrac) plus cetuximab on proliferation of colon cancer cells in the flasks were examined.

**Effects of NP-Tetrac Plus Cetuximab on Proliferation of Colon Cancer Cells.** Cells were grown on specially treated flakes in cell culture flasks. The cell culture medium contained 10% fetal bovine serum and various concentrations of cetuximab and NP-Tetrac. The medium was refreshed every 24 hours. The results of these studies are shown in Figure 11, which demonstrates that the combinations of both drugs showed a larger anti-proliferative effect than each drug on its own.

**Effects of NP-Tetrac Plus Cetuximab on Proliferation of Colon Cancer Cells in the Bellows Perfusion System.** Cells were grown on specially treated flakes in the bellows perfusion cell culture system. The cell culture medium contained 10% fetal bovine serum and various constant concentrations of cetuximab and NP-Tetrac. The system was constantly perfused by fresh medium. The results are shown in Figures 12-13.

**Results**

Mathematical modeling (see Figure 2B) suggests that the growth rate constant is decreased in a concentration-dependent manner during the first five days of treatment. Additional effects might be present but likely were not large enough to be identified in this model. The model shown in Figure 2B, which assumes independent pathways of action for the two drugs (NP-Tetrac and cetuximab) provided adequate fits to the data.

The parameter estimates used herein are as follows:

\[
\text{Cetuximab: } I_{\text{max}} = 0.86, \text{ IC}_{50} = 0.01 \mu g/mL \\
\text{NP-Tetrac: } I_{\text{max}} = 0.87, \text{ IC}_{50} = 0.08 \text{ nM}
\]

cells, a fast growing a malignant cancer cell, have a mutation in the \textit{K-ras} protooncogene and are resistant to cetuximab.

In the experiments described herein, cells were treated with constant concentration of drugs. Cetuximab alone did not show a large effect. In one experiment, between days 12 and 18, the $10^{-6}$ M NP-Tetrac + 0.1 $\mu$g/mL cetuximab appeared to have a larger effect on cell counts than $10^{-6}$ M NP-Tetrac + 1.0 $\mu$g/mL cetuximab. In another experiment, between days 10 and 20, $10^{-6}$ M NP-Tetrac + 0.1 $\mu$g/mL cetuximab appeared to have a similar effect on cell counts as $10^{-6}$ M NP-Tetrac + 1.0 $\mu$g/mL cetuximab. (See Figures 14A and 14B).

Figures 15A-C show the results of flow cytometry experiments on apoptosis following treatment with cetuximab, NP-Tetrac, or a combination. Cells in S phase were most sensitive to apoptosis. Figures 16A-B shows the results of flow cytometry experiments on non-apoptotic cells following treatment with cetuximab, NP-Tetrac, or a combination thereof.

\textit{Results}

These results show that the combined treatment with NP-Tetrac and cetuximab had a larger anti-proliferative effect on \textit{K-ras} mutant cancer cells than cetuximab alone. A model assuming that both drugs act by separate pathways on success of replication adequately described the total cell counts from all treatments simultaneously.

The parameter estimates used herein are as follows:

\begin{align*}
\text{Cetuximab: } & I_{\text{max}} = 0.01, \text{ IC}_{50} = 1.72 \text{ }\mu\text{g/mL} \\
\text{NP-Tetrac: } & I_{\text{max}} = 0.053, \text{ IC}_{50} = 0.104\text{x}10^{-6} \text{ M}
\end{align*}

The fraction of apoptotic cells was increased by up to a factor of 5 with the combination treatment versus treatment with cetuximab alone.

\textit{Conclusion}

Accordingly, based on the results presented herein, combined treatment with NP-Tetrac and cetuximab has a larger anti-proliferative effect on cancer cells than treatment with cetuximab alone. Moreover, NP-Tetrac induces apoptosis in cetuximab-resistant \textit{K-ras} mutant colon cancer cells.
In combination with PD modeling, the use of the perfusion bellows cell culture system allows one to study the dose-response relationship of anti-neoplastic agents over a wide concentration range in vitro, and can support translation from in vitro to animal models and human clinical trials.

The addition of a cytotoxic drug after pretreatment with NP-Tetrac and cetuximab may also be promising in the treatment of cancer.

OTHER EMBODIMENTS

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.
What is claimed is:

1. A pharmaceutical composition for treating cancer comprising a combination of cetuximab and an anti-angiogenic thyroid hormone analog, wherein the combination inhibits expression of hypoxia-inducible factor 1-α (HIF1 α) in cancer cells.

2. The pharmaceutical composition of claim 1, wherein the cancer cells are selected from the group consisting of: breast cancer, lung cancer, kidney cancer, thyroid cancer, brain cancer (glioma), ovarian cancer, pancreatic cancer, prostate cancer, plasma cell cancer (myeloma), squamous cell head-and-neck cancer, liver cancer, muscle cancer (sarcoma), colon cancer, neuroblastoma, lymphoma, adenoid carcinoma, stomach cancer, skin cancer, melanoma, basal cell carcinoma, and squamous cell carcinoma.

3. The pharmaceutical composition of claim 1, wherein the thyroid hormone analog is tetrac or triac (triiodothyroacetic acid).

4. The pharmaceutical composition of claim 3, wherein the thyroid hormone analog is tetrac.

5. The pharmaceutical composition of claim 3, wherein the thyroid hormone analog is triac.

6. The pharmaceutical composition of claim 4, wherein the tetrac is conjugated via a covalent bond to a polymer selected from polyvinyl alcohol, acrylic acid ethylene co-polymer, methoxypolyethylene, polyethylene glycol (PEG), polyacrylic acid, polylactic acid, agarose, polyglycolide, polylactide, PEO, m-PEG, PVA, PLLA, PGA, poly-L-lysine, Human Serum Albumin, cellulose derivatives, carbomethoxy/ethyl/hydroxypropyl, hyaluronic acid, folate linked cyclodextrin/dextran, sarcosine/amino acid spaced polymer, alginate, carrageenan, pectin/chitosan, chitosan, dextran, collagen, polyamine, poly aniline, poly alanine, polytrytophan, poly tyrosine, polylactide-co-glycolide (PLG), poly(lactic-co-glycolic) acid (PLGA), polyglycolide, polyllysyl glycolide, polylactic acid, or co-polymers thereof, wherein said polymer is formulated into a nanoparticle, wherein said nanoparticle is
between 150 and 250 nanometers in size, and wherein said tetrac binds to the cell surface receptor for thyroid hormone on integrin $\alpha\beta_3$.

7. The pharmaceutical composition of claim 6, wherein the tetrac is attached to the nanoparticle via a linker.

8. The pharmaceutical composition of claim 7, wherein the linker is between 4 and 15 atoms long.

9. The pharmaceutical composition of claim 1, further comprising an anti-estrogen compound.

10. The pharmaceutical composition of claim 9, wherein the anti-estrogen compound is selected from the group consisting of tamoxifen and aromatase inhibitors.

11. The pharmaceutical composition of claim 6, wherein the nanoparticles further comprise one or more additional chemotherapeutic agents.

12. The pharmaceutical composition of claim 11, wherein the one or more additional chemotherapeutic agents are targeted to the cancer cells.

13. The pharmaceutical composition of claim 1, wherein the combination inhibits PI3K-dependent HIF1$\alpha$ gene expression.

14. The pharmaceutical composition of claim 13, wherein the combination blocks the inhibitory action of endogenous thyroid hormone on the actions of cetuximab on HIF1$\alpha$.

15. A method of treating cancer comprising administering a therapeutically effective amount of a combination of cetuximab and an anti-angiogenic thyroid hormone analog to a patient suffering therefrom.

16. The method of claim 15, wherein the thyroid hormone analog is tetrac or triac.

17. The method of claim 16, wherein the thyroid hormone analog is tetrac.
18. The method of claim 17, wherein tetrac is conjugated via a covalent bond to a polymer selected from polyvinyl alcohol, acrylic acid ethylene co-polymer, methoxypolyethylene, polyethyleneglycol (PEG), polyacrylic acid, polylactic acid, agarose, polyglycolide, polylactide, PEO, m-PEG, PVA, PLLA, PGA, poly-L-lysine, Human Serum Albumin, cellulose derivatives, carbomethoxy/ethyl/hydroxypropyl, hyaluronic acid, folate linked cyclodextrin/dextran, sarcosine/amino acid spaced polymer, alginate, carrageenan, pectin/chitosan, chitosan, dextran, collagen, polyamine, poly aniline, poly alanine, polytrytophan, poly tyrosine, polylactide-co-glycolide (PLG), poly(lactic-co-glycolic) acid (PLGA), polylysyl glycolide, polylactic acid, polyglycolide, or co-polymers thereof, wherein said polymer is formulated into a nanoparticle, wherein said nanoparticle is between 150 and 250 nanometers in size, and wherein said tetrac binds to the cell surface receptor for thyroid hormone on integrin αvβ3.

19. The method of claim 15, further comprising administering an anti-estrogen compound to the subject.

20. The method of claim 19, wherein the anti-estrogen compound is selected from the group consisting of tamoxifen and aromatase inhibitors.

21. The method of claim 18, wherein the nanoparticles further comprise one or more additional chemotherapeutic agents.

22. The method of claim 18, wherein cetuximab is encapsulated within the nanoparticle.

23. The pharmaceutical composition of claim 6, wherein cetuximab is encapsulated within the nanoparticle.
* Syringe A: Injecting Anti-cancer Drugs
* Syringe B: Harvesting media for LC-MS-MS Examination

FIG. 1
Tetrac inhibits success rate of replication

Replication: $k_{21}$, fast

Cells in state 2

Growth rate constant: $k_{12}$

Cells in state 1

Tetrac inhibits rate of growth

1st order transfer

Inhibition

FIG. 2A
The model assumes that both drugs act on independent pathways and includes a small antagonistic term. The observed effect of the combination is larger than a model which assumes both drugs acting on the same pathway would predict.
FIG. 3C
Uptake of tetrac into cells

FIG. 4A

Uptake of tetrac into cells

FIG. 4B
U87MG cells

- Control
- Nano Tetrac (10^{-9} M)
- Nano Tetrac (10^{-8} M)
- Nano Tetrac (10^{-7} M)
- Nano Tetrac (10^{-6} M)

Total cell count (log_{10})

Treatment Days

FIG. 5C
TGS 112T cells

**FIG. 6C**

- **Control**
- **Tetrac (10^{-6} M)**
- **Nano Tetrac (10^{-6} M)**

**Total Cell Counts (x10^6)**

**Treatment Days**

0 1 2 3 4 5 6 7
FIG. 7
SUBSTITUTE SHEET (RULE 26)
FIG. 8

MDA-MB cells

- PIG3

- c-jun

- c-fos

Control
Tetrac (10^{-6} M)
nano-Tetrac (10^{-6} M)
Effect of Tetrac and Nano Tetrac on Cell Proliferation in CV-1 cells

![Bar Chart for CV-1 cells]

FIG. 9A

Effect of Tetrac and Nano Tetrac on Cell Proliferation in 293T cells

![Bar Chart for 293T cells]

FIG. 9B
Effect of Cetuximab and Tetrac on Cell Proliferation in MDA-MB cells

- Control
- Cetuximab (0.1 μg/mL)
- Tetrac (10⁻⁷ M)
- Tetrac + Cetuximab

Total Cell Counts (x10⁶)

Treatment Days

FIG. 10
Combinations of both drugs show a larger anti-proliferative effect than each drug on its own.

FIG. 11
Cetuximab 0.2 μg/mL + NP-Tetrac

Median total cell count vs Time (days)

- Control
- Cetuximab 0.2 μg/mL
- NP-Tetrac 2 nM
- NP-Tetrac 100 nM
- NP-T 2 nM + C 0.2 μg/mL
- NP-T 100 nM + C 0.2 μg/mL

FIG. 12B
Cells were treated with constant concentrations of drugs. Cetuximab alone did not show a large effect. Between days 12 and 18 the $10^{-6}$ M NP-Tetrac + 0.1 μg/mL cetuximab appeared to have a larger effect on cell counts than $10^{-6}$ M NP-Tetrac + 1.0 μg/mL cetuximab.
Cells were treated with constant concentrations of drugs. Cetuximab alone did not show a large effect. Between days 10 and 20 the $10^{-6}$ M NP-Tetrac + 0.1 μg/mL cetuximab appeared to have a similar effect on cell counts as $10^{-6}$ M NP-Tetrac + 1.0 μg/mL cetuximab.
Flow cytometry - apoptosis
Repeat study

% Apoptotic cells

Treatment days

Cetuximab 1.0 µg/mL + NP-Tetrac 10-6M
Cetuximab 0.1 µg/mL + NP-Tetrac 10-7M
NP-Tetrac 10-7 M
Cetuximab 1 µg/mL + NP-Tetrac 10-7M
NP-Tetrac 10-6 M
Cetuximab 0.1 µg/mL
Cetuximab 1 µg/mL
Control
Flow cytometry - non-apoptotic cells

Cells in G2/M phase (% of viable cells)

Treatment days

Cells in S phase (% of viable cells)

Treatment days

Cells in G1 phase (% of viable cells)

Treatment days

- Cetuximab 0.1 µg/mL + NP-Tetrac 10.7 M
- Cetuximab 0.1 µg/mL + NP-Tetrac 10.6 M
- Cetuximab 1 µg/mL + NP-Tetrac 10.7 M
- Cetuximab 1 µg/mL + NP-Tetrac 10.6 M
- Control
- NP-Tetrac 10.6 M
- NP-Tetrac 10.6 M
Flow cytometry - non-apoptotic cells
Repeat study

Cells in G1 phase (% of viable cells)

Cells in S phase (% of viable cells)

Cells in G2/M phase (% of viable cells)

% Viable cells in G1

% Viable cells in S

% Viable cells in G2/M

Treatment days

Treatment days

Treatment days

Control

Cetuximab 0.1 μg/mL

Cetuximab 1 μg/mL

NP-Tetrac 10-7 M

NP-Tetrac 10-6 M

Cetuximab 0.1 μg/mL + NP-Tetrac 10-7 M

Cetuximab 0.1 μg/mL + NP-Tetrac 10-6 M

Cetuximab 1 μg/mL + NP-Tetrac 10-7 M

Cetuximab 1.0 μg/mL + NP-Tetrac 10-6 M

FIG. 16B
### A. CLASSIFICATION OF SUBJECT MATTER

**INV.** A61K39/395

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**

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

Electronic database consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
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<tbody>
<tr>
<td>X</td>
<td>PAUL J DAVIS, FAITH B DAVIS, HUNG-YUN LIN, JOEL J BERGH, ET AL: &quot;Cell-surface receptor for thyroid hormone and tumor cell proliferation&quot; EXPERT REVIEWS IN ENDOCRINOLOGY AND METABOLISM, vol. 1, no. 6, 1 January 2006 (2006-01-01) - 31 December 2006 (2006-12-31) pages 753-761, XP001525260 Future Drugs ISSN: 1744-6651 DOI: 10.1586/17446651.1.6.753</td>
<td>1,3-23</td>
</tr>
<tr>
<td>Y</td>
<td>The whole document, in particular Fig.1, Table 1, p.756, col.1 para.3, and the section entitled 'Five year review' on p.758</td>
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**X** Further documents are listed in the continuation of Box C

**D** See patent family annex

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Date of the actual completion of the international search: 10 August 2010

Date of mailing of the international search report: 24/08/2010

Name and mailing address of the ISA:

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<tr>
<td>A</td>
<td>MOELLER LARS C ET AL: &quot;Thyroid hormone mediated changes in gene expression can be initiated by cytosolic action of the thyroid hormone receptor beta through the phosphatidyl inositol 3-kinase pathway.&quot; NUCLEAR RECEPTOR SIGNALING 2006 LNKD-PUBMED: 16862226, vol. 4, 2006, page E020, XP009137447 ISSN: 1550-7629 the whole document</td>
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<td>Y</td>
<td>MARTENS TOBIAS ET AL: &quot;Inhibition of glioblastoma growth in a highly invasive nude mouse model can be achieved by targeting epidermal growth factor receptor but not vascular endothelial growth factor receptor-2&quot; CLINICAL CANCER RESEARCH, THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, US, vol. 14, no. 17, 1 September 2008 (2008-09-01), pages 5447-5458, XP009137368 ISSN: 1078-0432 the whole document</td>
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<td>Category</td>
<td>Citation of document, with indication, where appropriate, of the relevant passages</td>
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<td>Y</td>
<td>ABDELHADI REBBAA ET AL: &quot;Novel function of the thyroid hormone analog tetralodothyroacetic acid: a cancer chemosensitizing and anti-cancer agent&quot; ANGIogenesis, KLUWER ACADEMIC PUBLISHERS, DO, vol. 11, no. 3, 4 April 2008 (2008-04-04), pages 269-276, XP019598622 ISSN: 1573-7209 the whole document</td>
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