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(54) Title: COMBINATION THERAPY FOR THE TREATMENT OF CANCER

(57) Abstract: The present invention relates to methods for treatment or prevention of neoplasia disorders using protein tyrosine kinase inhibitors in combination with cyclooxygenase inhibitors, in particular cyclooxygenase-2 selective inhibitors.

COMBINATION THERAPY FOR THE TREATMENT OF CANCER

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to methods for treatment or prevention of neoplasia disorders using protein tyrosine kinase inhibitors in combination with cyclooxygenase inhibitors, in particular cyclooxygenase-2 selective inhibitors.

State of the Art

A neoplasm, or tumor, is an abnormal, unregulated, and disorganized proliferation of cell growth. A neoplasm is malignant, or cancerous, if it has properties of destructive growth, invasiveness and metastasis. Invasiveness refers to the local spread of a neoplasm by infiltration or destruction of surrounding tissue, typically breaking through the basal laminas that define the boundaries of the tissues, thereby often entering the body's circulatory system. Metastasis typically refers to the dissemination of tumor cells by lymphotics or blood vessels. Metastasis also refers to the migration of tumor cells by direct extension through serous cavities, or subarachnoid or other spaces. Through the process of metastasis, tumor cell migration to other areas of the body establishes neoplasms in areas away from the site of initial appearance.

Cancer is now the second leading cause of death in the United States and over 8,000,000 persons in the United States have been diagnosed with cancer. In 1995, cancer accounted for 23.3% of all deaths in the United States. (See U.S. Dept. of Health and Human Services, National Center for Health Statistics, Health United States 1996-97 and Injury Chartbook 117 (1997)).

Cancer is not fully understood on the molecular level. It is known that exposure of a cell to a carcinogen such as certain viruses, certain chemicals, or radiation, leads to DNA alteration that inactivates a "suppressive" gene or activates an "oncogene". Suppressive genes are growth regulatory genes, which upon mutation, can no longer control cell growth. Oncogenes are initially normal genes (called prooncogenes) that by mutation or altered context of expression become transforming genes. The products of transforming genes cause inappropriate cell growth. More than twenty different normal cellular genes can become oncogenes by genetic alteration. Transformed cells differ from normal cells in many ways,

including cell morphology, cell-to-cell interactions, membrane content, cytoskeletal structure, protein secretion, gene expression and mortality (transformed cells can grow indefinitely).

Cancer is now primarily treated with one or a combination of three types of therapies: surgery, radiation, and chemotherapy. Surgery involves the bulk removal of diseased tissue. While surgery is sometimes effective in removing tumors located at certain sites, for example, in the breast, colon, and skin, it cannot be used in the treatment of tumors located in other areas, such as the backbone, nor in the treatment of disseminated neoplastic conditions such as leukemia.

Chemotherapy involves the disruption of cell replication or cell metabolism. It is used most often in the treatment of breast, lung, and testicular cancer.

The adverse effects of systemic chemotherapy used in the treatment of neoplastic disease is most feared by patients undergoing treatment for cancer. Of these adverse effects nausea and vomiting are the most common and severe side effects. Other adverse side effects include cytopenia, infection, cachexia, mucositis in patients receiving high doses of chemotherapy with bone marrow rescue or radiation therapy; alopecia (hair loss); cutaneous complications (see M.D. Abeloff, et al: Alopecia and Cutaneous Complications. P. 755-56. In Abeloff, M.D., Armitage, J.O., Lichter, A.S., and Niederhuber, J.E. (eds) Clinical Oncology. Churchill Livingston, New York, 1992, for cutaneous reactions to chemotherapy agents), such as pruritis, urticaria, and angioedema; neurological complications; pulmonary and cardiac complications in patients receiving radiation or chemotherapy; and reproductive and endocrine complications.

Chemotherapy-induced side effects significantly impact the quality of life of the patient and may dramatically influence patient compliance with treatment.

Additionally, adverse side effects associated with chemotherapeutic agents are generally the major dose-limiting toxicity (DLT) in the administration of these drugs. For example, mucositis, is one of the major dose limiting toxicity for several anticancer agents, including the antimetabolite cytotoxic agents 5-FU, methotrexate, and antitumor antibiotics, such as doxorubicin. Many of these chemotherapy-induced side effects if severe, may lead to hospitalization, or require treatment with analgesics for the treatment of pain.

The adverse side effects induced by chemotherapeutic agents and radiation therapy have become of major importance to the clinical management of cancer patients.

Currently, scientists are looking at treating cancers via the use of antiangiogenic agents. Angiogenesis is believed to be the mechanism via which tumors get needed nutrients to grow and metastasize to other locations in the body. Antiangiogenic agents interfere with these processes and destroy or control tumors.

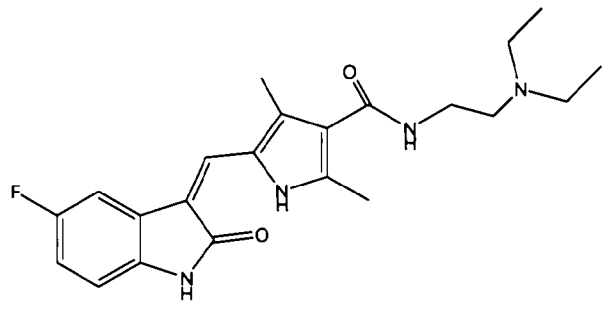
For example, U.S. Patent No. 5,854,205 describes an isolated endostatin protein that is an inhibitor of endothelial cell proliferation and angiogenesis; U.S. Patent No. 5,843,925 describes a method for inhibiting angiogenesis and endothelial cell proliferation using a 7-[substituted amino]-9-[(substituted glycyloamido]-6-demethyl-6-deoxytetracycline; U.S. Patent No. 5,861,372 describes the use of an aggregate endothelial inhibitor, angiostatin, and it use in inhibiting angiogenesis, PCT/GB97/00650 describes the use of cinnoline derivatives for use in the production of an antiangiogenic and/or vascular permeability reducing effect; Tai-Ping, D. describes potential anti-angiogenic therapies, *see* Trends Pharmacol.Sci. 16, No. 2, 57-66, 1995; Lode, H. et al. describes synergy between an antiangiogenic integrin alpha v antagonist and an antibody-cytokine fusion protein eradicates spontaneous tumor metastasis, *see* Proc. Nat. Acad. Sci. USA. , 96 (4), 1591-1596, 1999; Giannis, A. et al describes Integrin antagonists and other low molecular weight compounds as inhibitors of angiogenesis, *see* New drugs in cancer therapy. Angew. Chem. Int. Ed. Engl. 36(6), 588-590, 1997; WO 97/41,844 describes a method of using combinations of angiostatic compounds for the prevention and/or treatment of neovascularization in human patients; WO 98/22,101 describes a method of using [pyrozol-1-yl]benzenesulfonamides as anti-angiogenic agents; and US Patent 5,792,783 describes use of 3-heteroaryl-2-indolinone that are protein kinase inhibitors and antiangiogenic agents for the treatment of various cancers.

Recently, it has been reported that treatment of colorectal cancer with a cyclooxygenase-2 selective inhibitor, specifically Celecoxib®, in combination with an ART-2 inhibitor, specifically Herceptin®, is more effective than when either agent is used alone. Accordingly, there is a need to discover new combinations of chemotherapeutic agents that when used together are more efficacious than when used alone. The present invention fulfills this need.

SUMMARY OF THE INVENTION

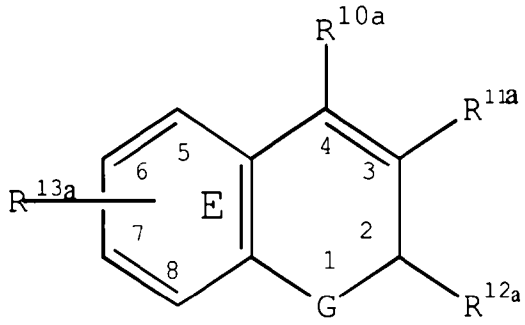
In one aspect, this invention is directed to a method for the treatment of a cancer comprising administering to a mammal in need of such treatment a therapeutically effective amount of:

a protein kinase inhibitor of the formula:



in combination with a cyclooxygenase-2 selective inhibitor or a pharmaceutically acceptable salt thereof selected from the group consisting of:

(i) a compound of Formula (II):



wherein:

G is selected from the group consisting of O, S, and -NR^a- wherein R^a is hydrogen or alkyl;

R^{10a} is selected from the group consisting of hydrogen and aryl;

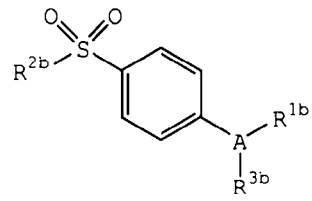
R^{11a} is selected from the group consisting of carboxyl, alkyl, aralkyl, aminocarbonyl, alkylsulfonylaminocarbonyl, and alkoxycarbonyl;

R^{12a} is selected from the group consisting of haloalkyl, alkyl, aralkyl, cycloalkyl and aryl optionally substituted with one or more radicals selected from alkylthio, nitro and alkylsulfonyl; and

R^{13a} is one or more radicals independently selected from the group consisting of hydrogen, halo, alkyl, aralkyl, alkoxy, aryloxy, heteroaryloxy, aralkyloxy, heteroaralkyloxy, haloalkyl, haloalkoxy, alkylamino, arylamino, aralkylamino, heteroarylamino, heteroarylalkylamino, nitro, amino, aminosulfonyl, alkylaminosulfonyl, arylaminosulfonyl, heteroarylaminosulfonyl, aralkylaminosulfonyl, heteroaralkylaminosulfonyl, heterocyclosulfonyl, alkylsulfonyl, hydroxyarylcarbonyl, nitroaryl, optionally substituted aryl, optionally substituted heteroaryl, aralkylcarbonyl, heteroarylcarbonyl, arylcarbonyl, aminocarbonyl, and alkylcarbonyl; or

R^{13a} together with ring E forms a naphthyl ring; or

(ii) a compound of Formula (III):



(III)

wherein:

A is selected from the group consisting of partially unsaturated or unsaturated heterocyclyl and partially unsaturated or unsaturated carbocyclic rings;

R^{1b} is selected from the group consisting of heterocyclyl, cycloalkyl, cycloalkenyl and aryl, wherein R^{1b} is optionally substituted at a substitutable position with one or more radicals independently selected from alkyl, haloalkyl, cyano, carboxyl, alkoxycarbonyl, hydroxyl, hydroxyalkyl, haloalkoxy, amino, alkylamino, arylamino, nitro, alkoxyalkyl, alkylsulfinyl, halo, alkoxy, and alkylthio;

R^{2b} is selected from the group consisting of methyl and amino; and

R^{3b} is selected from the group consisting of a radical selected from hydrogen, halo, alkyl, alkenyl, alkynyl, oxo, cyano, carboxyl, cyanoalkyl, heterocyclyloxy, alkyloxy, alkylthio, alkylcarbonyl, cycloalkyl, aryl, haloalkyl, heterocyclyl, cycloalkenyl, aralkyl, heterocyclylalkyl, acyl, alkylthioalkyl, hydroxyalkyl, alkoxycarbonyl, arylcarbonyl, aralkylcarbonyl, aralkenyl, alkoxyalkyl, arylthioalkyl, aryloxyalkyl, aralkylthioalkyl, aralkoxyalkyl, alkoxyaralkoxyalkyl, alkoxycarbonylalkyl, aminocarbonyl, aminocarbonylalkyl, alkylaminocarbonyl, N-arylaminocarbonyl, N-alkyl-N-arylaminocarbonyl, alkylaminocarbonylalkyl, carboxyalkyl, alkylamino, N-arylamino, N-aralkylamino, N-alkyl-N-aralkylamino, N-alkyl-N-arylamino, aminoalkyl, alkylaminoalkyl, N-arylaminoalkyl, N-aralkylaminoalkyl, N-alkyl-N-aralkylaminoalkyl, N-alkyl-N-arylaminoalkyl, aryloxy, aralkoxy, arylthio, aralkylthio, alkylsulfinyl, alkylsulfonyl, aminosulfonyl, alkylaminosulfonyl, N-arylaminosulfonyl, arylsulfonyl, and N-alkyl-N-arylaminosulfonyl.

Preferably, the above compounds are administered as a pharmaceutical composition comprising one or more of the above compounds and a pharmaceutically acceptable excipient. Specifically, the therapeutic agents of this invention can be formulated as separate compositions which are given at the same time or different times, or the therapeutic agents can be given as a single composition.

The methods of the present invention are useful for the treatment or prevention of neoplasia disorders including acral lentiginous melanoma, actinic keratoses, adenocarcinoma, adenoid cycstic carcinoma, adenomas, adenosarcoma, adenosquamous carcinoma, astrocytic tumors, bartholin gland carcinoma, basal cell carcinoma, bronchial gland carcinomas, capillary, carcinoids, carcinoma, carcinosarcoma, cavernous, cholangiocarcinoma, chondosarcoma, choriod plexus papilloma/carcinoma, clear cell carcinoma, cystadenoma, endodermal sinus tumor, endometrial hyperplasia, endometrial stromal sarcoma, endometrioid adenocarcinoma, ependymal, epitheloid, Ewing's sarcoma, fibrolamellar, focal nodular hyperplasia, gastrinoma, germ cell tumors, glioblastoma, glucagonoma, hemangiblastomas,

hemangioendothelioma, hemangiomas, hepatic adenoma, hepatic adenomatosis, hepatocellular carcinoma, insulinoma, intaepithelial neoplasia, interepithelial squamous cell neoplasia, invasive squamous cell carcinoma, large cell carcinoma, leiomyosarcoma, lentigo maligna melanomas, malignant melanoma, malignant mesothelial tumors, medulloblastoma, medulloepithelioma, melanoma, meningeal, mesothelial, metastatic carcinoma, mucoepidermoid carcinoma, neuroblastoma, neuroepithelial adenocarcinoma nodular melanoma, oat cell carcinoma, oligodendroglial, osteosarcoma, pancreatic polypeptide, papillary serous adenocarcinoma, pineal cell, pituitary tumors, plasmacytoma, pseudosarcoma, pulmonary blastoma, renal cell carcinoma, retinoblastoma, rhabdomyosarcoma, sarcoma, serous carcinoma, small cell carcinoma, soft tissue carcinomas, somatostatin-secreting tumor, squamous carcinoma, squamous cell carcinoma, submesothelial, superficial spreading melanoma, undifferentiatied carcinoma, uveal melanoma, verrucous carcinoma, vipoma, well differentiated carcinoma, and Wilm's tumor.

The methods of the present invention provide one or more benefits. One of the benefits is that the compositions, agents and therapies of the present invention are administered in combination at a low dose, that is, at a dose lower than has been conventionally used in clinical situations for each of the individual components administered alone.

A benefit of lowering the dose of the compounds, compositions, agents and therapies of the present invention administered to a mammal includes a decrease in the incidence of adverse effects associated with higher dosages. For example, by lowering the dosage of a chemotherapeutic agent such as methotrexate, a reduction in the frequency and the severity of nausea and vomiting will result when compared to that observed at higher dosages. Similar benefits are contemplated when a protein kinase inhibitor of the present invention is used in combination with a cyclooxygenase inhibitor, in particular a cyclooxygenase-2 selective inhibitor.

By lowering the incidence of adverse effects, an improvement in the quality of life of a patient undergoing treatment for cancer is contemplated. Further benefits of lowering the incidence of adverse effects include an improvement in patient compliance, a reduction in the number of hospitalizations needed for the treatment of adverse effects, and a reduction in the administration of analgesic agents needed to treat pain associated with the adverse effects.

Another benefit that is contemplated is that the inhibition of the tumors is greater when a protein kinase inhibitor is administered in combination with a cyclooxygenase inhibitor, preferably a cyclooxygenase-2 selective inhibitor than when either of these agents are administered alone.The present invention further includes kits comprising a COX-2 inhibitor and a protein kinase inhibitor as described above.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Unless otherwise stated the following terms used in the specification and claims have the meanings discussed below:

"Alkyl" refers to a saturated aliphatic hydrocarbon radical including straight chain and branched chain groups of 1 to 20 carbon atoms (whenever a numerical range; e.g. "1-20", is stated herein, it means that the group, in this case the alkyl group, may contain 1 carbon atom, 2 carbon atoms, 3 carbon atoms, etc. up to and including 20 carbon atoms). Alkyl groups containing from 1 to 4 carbon atoms are refered to as lower alkyl groups. When said lower alkyl groups lack substituents, they are referred to as lower alkyl groups. More preferably, an alkyl group is a medium size alkyl having 1 to 10 carbon atoms e.g., methyl, ethyl, propyl, 2-propyl, *n*-butyl, *iso*-butyl, *tert*-butyl, pentyl, and the like. Most preferably, it is a lower alkyl having 1 to 4 carbon atoms e.g., methyl, ethyl, propyl, 2-propyl, *n*-butyl, *iso*-butyl, or *tert*-butyl, and the like.

"Substituted alkyl" means the alkyl group as defined above is substituted with one or more, more preferably one to three, even more preferably one or two substituent(s) independently selected from the group consisting of halo, hydroxy, lower alkoxy, aryl optionally substituted with one or more groups, preferably one, two or three groups which are independently of each other halo, hydroxy, lower alkyl or lower alkoxy groups, aryloxy optionally substituted with one or more groups, preferably one, two or three groups which are independently of each other halo, hydroxy, lower alkyl or lower alkoxy groups, 6-member heteroaryl having from 1 to 3 nitrogen atoms in the ring, the carbons in the ring being optionally substituted with one or more groups, preferably one, two or three groups which are independently of each other halo, hydroxy, lower alkyl or lower alkoxy groups, 5-member heteroaryl having from 1 to 3 heteroatoms selected from the group consisting of nitrogen, oxygen and sulfur, the carbon and the nitrogen atoms in the group being optionally substituted with one or more groups, preferably one, two or three groups which are independently of each other halo, hydroxy, lower alkyl or lower alkoxy groups, 5- or 6-member heteroalicyclic group having from 1 to 3 heteroatoms selected from the group consisting of nitrogen, oxygen and sulfur, the carbon and nitrogen (if present) atoms in the group being optionally substituted with one or more groups, preferably one, two or three groups which are independently of each other halo , hydroxy, lower alkyl or lower alkoxy groups, mercapto, (lower alkyl)thio, arylthio optionally substituted with one or more groups, preferably one, two or three groups which are independently of each other halo, hydroxy, lower alkyl or lower alkoxy groups, cyano, acyl,

thioacyl, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, C-amido, N-amido, nitro, N-sulfonamido, S-sulfonamido, $R^{18}S(O)$-, $R^{18}S(O)_2$-, -C(O)OR^{18}, $R^{18}C(O)O$-, and -$NR^{18}R^{19}$, wherein R^{18} and R^{19} are independently selected from the group consisting of hydrogen, lower alkyl, trihalomethyl, (C_3-C_6)cycloalkyl, lower alkenyl, lower alkynyl and aryl optionally substituted with one or more, groups, preferably one, two or three groups which are independently of each other halo, hydroxy, lower alkyl or lower alkoxy groups.

Preferably, the alkyl group is substituted with one or two substituents independently selected from the group consisting of hydroxy, 5- or 6-member heteroalicyclic group having from 1 to 3 heteroatoms selected from the group consisting of nitrogen, oxygen and sulfur, the carbon and nitrogen (if present) atoms in the group being optionally substituted with one or more groups, preferably one, two or three groups which are independently of each other halo, hydroxy, lower alkyl or lower alkoxy groups, 5-member heteroaryl having from 1 to 3 heteroatoms selected from the group consisting of nitrogen, oxygen and sulfur, the carbon and the nitrogen atoms in the group being optionally substituted with one or more groups, preferably one, two or three groups which are independently of each other halo, hydroxy, lower alkyl or lower alkoxy groups, 6-member heteroaryl having from 1 to 3 nitrogen atoms in the ring, the carbons in the ring being optionally substituted with one or more groups, preferably one, two or three groups which are independently of each other halo, hydroxy, lower alkyl or lower alkoxy groups, or -$NR^{18}R^{19}$, wherein R^{18} and R^{19} are independently selected from the group consisting of hydrogen, lower alkyl. Even more preferably the alkyl group is substituted with one or two substituents which are independently of each other hydroxy, dimethylamino, ethylamino, diethylamino, dipropylamino, pyrrolidino, piperidino, morpholino, piperazino, 4-lower alkylpiperazino, phenyl, imidazolyl, pyridinyl, pyridazinyl, pyrimidinyl, oxazolyl, triazinyl, and the like.

"Cycloalkyl" refers to a 3 to 8 member all-carbon monocyclic ring, an all-carbon 5-member/6-member or 6-member/6-member fused bicyclic ring or a multicyclic fused ring (a "fused" ring system means that each ring in the system shares an adjacent pair of carbon atoms with each other ring in the system) group wherein one or more of the rings may contain one or more double bonds but none of the rings has a completely conjugated pi-electron system.

Examples, without limitation, of cycloalkyl groups are cyclopropane, cyclobutane, cyclopentane, cyclopentene, cyclohexane, cyclohexadiene, adamantane, cycloheptane, cycloheptatriene, and the like.

"Substituted cycloalkyl" means a cycloalkyl group as defined above that is substituted with one or more, more preferably one or two substituents, independently selected from the group consisting of lower alkyl, trihaloalkyl, halo, hydroxy, lower alkoxy, aryl optionally

substituted with one or more, preferably one or two groups independently of each other halo, hydroxy, lower alkyl or lower alkoxy groups, aryloxy optionally substituted with one or more, preferably one or two groups independently of each other halo, hydroxy, lower alkyl or lower alkoxy groups, 6-member heteroaryl having from 1 to 3 nitrogen atoms in the ring, the carbons in the ring being optionally substituted with one or more, preferably one or two groups independently of each other halo, hydroxy, lower alkyl or lower alkoxy groups, 5-member heteroaryl having from 1 to 3 heteroatoms selected from the group consisting of nitrogen, oxygen and sulfur, the carbon and nitrogen atoms of the group being optionally substituted with one or more, preferably one or two groups independently of each other halo, hydroxy, lower alkyl or lower alkoxy groups, 5- or 6-member heteroalicyclic group having from 1 to 3 heteroatoms selected from the group consisting of nitrogen, oxygen and sulfur, the carbon and nitogen (if present)atoms in the group being optionally substituted with one or more, preferably one or two groups independently of each other halo, hydroxy, lower alkyl or lower alkoxy groups, mercapto,(lower alkyl)thio, arylthio optionally substituted with one or more, preferably one or two groups independently of each other halo, hydroxy, lower alkyl or lower alkoxy groups, cyano, acyl, thioacyl, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, C-amido, N-amido, nitro, N-sulfonamido, S-sulfonamido, $R^{18}S(O)$-, $R^{18}S(O)_2$-, -$C(O)OR^{18}$, $R^{18}C(O)O$-, and -$NR^{18}R^{19}$ are as defined above.

"Alkenyl" refers to a lower alkyl group, as defined herein, consisting of at least two carbon atoms and at least one carbon-carbon double bond. Representative examples include, but are not limited to, ethenyl, 1-propenyl, 2-propenyl, 1-, 2-, or 3-butenyl, and the like.

"Alkynyl" refers to a lower alkyl group, as defined herein, consisting of at least two carbon atoms and at least one carbon-carbon triple bond. Representative examples include, but are not limited to, ethynyl, 1-propynyl, 2-propynyl, 1-, 2-, or 3-butynyl, and the like.

"Aryl" refers to an all-carbon monocyclic or fused-ring polycyclic (i.e., rings which share adjacent pairs of carbon atoms) groups of 1 to 12 carbon atoms having a completely conjugated pi-electron system. Examples, without limitation, of aryl groups are phenyl, naphthalenyl and anthracenyl. The aryl group may be substituted or unsubstituted. When substituted, the substituted group(s) is preferably one or more, more preferably one, two or three, even more preferably one or two, independently selected from the group consisting of lower alkyl, trihaloalkyl, halo, hydroxy, lower alkoxy, mercapto,(lower alkyl)thio, cyano, acyl, thioacyl, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, C-amido, N-amido, nitro, N-sulfonamido, S-sulfonamido, $R^{18}S(O)$-, $R^{18}S(O)_2$-, -$C(O)OR^{18}$, $R^{18}C(O)O$-, and -$NR^{18}R^{19}$, with R^{18} and R^{19} as defined above. Preferably, the aryl group is optionally substituted with one or two substituents independently selected from halo, lower alkyl, trihaloalkyl,

hydroxy, mercapto, cyano, N-amido, mono or dialkylamino, carboxy, or N-sulfonamido.

"Heteroaryl" refers to a monocyclic or fused ring (i.e., rings which share an adjacent pair of atoms) group of 5 to 12 ring atoms containing one, two, or three ring heteroatoms selected from N, O, or S, the remaining ring atoms being C, and, in addition, having a completely conjugated pi-electron system. Examples, without limitation, of unsubstituted heteroaryl groups are pyrrole, furan, thiophene, imidazole, oxazole, thiazole, pyrazole, pyridine, pyrimidine, quinoline, isoquinoline, purine and carbazole. The heteroaryl group may be substituted or unsubstituted. When substituted, the substituted group(s) is preferably one or more, more preferably one, two, or three, even more preferably one or two, independently selected from the group consisting of lower alkyl, trihaloalkyl, halo, hydroxy, lower alkoxy, mercapto, (lower alkyl)thio, cyano, acyl, thioacyl, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, C-amido, N-amido, nitro, N-sulfonamido, S-sulfonamido, $R^{18}S(O)$-, $R^{18}O)_2$-, -$C(O)OR^{18}$, $R^{18}C(O)O$-, and -$NR^{18}R^{19}$, with R^{18} and R^{19} as defined above. Preferably, the heteroaryl group is optionally substituted with one or two substituents independently selected from halo, lower alkyl, trihaloalkyl, hydroxy, mercapto, cyano, N-amido, mono or dialkylamino, carboxy, or N-sulfonamido.

"Heteroalicyclic" refers to a monocyclic or fused ring group having in the ring(s) of 5 to 9 ring atoms in which one or two ring atoms are heteroatoms selected from N, O, or $S(O)_n$ (where n is an integer from 0 to 2), the remaining ring atoms being C. The rings may also have one or more double bonds. However, the rings do not have a completely conjugated pi-electron system. Examples, without limitation, of unsubstituted heteroalicyclic groups are pyrrolidino, piperidino, piperazino, morpholino, thiomorpholino, homopiperazino, and the like. The heteroalicyclic ring may be substituted or unsubstituted. When substituted, the substituted group(s) is preferably one or more, more preferably one, two or three, even more preferably one or two, independently selected from the group consisting of lower alkyl, trihaloalkyl, halo, hydroxy, lower alkoxy, mercapto,(lower alkyl)thio, cyano, acyl, thioacyl, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, C-amido, N-amido, nitro, N-sulfonamido, S-sulfonamido, $R^{18}S(O)$-, $R^{18}S(O)_2$-, -$C(O)OR^{18}$, $R^{18}C(O)O$-, and -$NR^{18}R^{19}$, with R^{18} and R^{19} as defined above. Preferably, the heteroalicyclic group is optionally substituted with one or two substituents independently selected from halo, lower alkyl, trihaloalkyl, hydroxy, mercapto, cyano, N-amido, mono or dialkylamino, carboxy, or N-sulfonamido.

Preferably, the heteroalicyclic group is optionally substituted with one or two substituents independently selected from halo, lower alkyl, trihaloalkyl, hydroxy, mercapto, cyano, N-amido, mono or dialkylamino, carboxy, or N-sulfonamido.

"Heterocycle" means a saturated cyclic radical of 3 to 8 ring atoms in which one or two ring atoms are heteroatoms selected from N, O, or $S(O)_n$ (where n is an integer from 0 to 2), the remaining ring atoms being C, where one or two C atoms may optionally be replaced by a carbonyl group. The heterocyclyl ring may be optionally substituted independently with one, two, or three substituents selected from optionally substituted lower alkyl (substituted with 1 or 2 substituents independently selected from carboxy or ester), haloalkyl, cyanoalkyl, halo, nitro, cyano, hydroxy, alkoxy, amino, monoalkylamino, dialkylamino, aralkyl, heteroaralkyl, -COR (where R is alkyl) or –COOR where R is (hydrogen or alkyl). More specifically the term heterocyclyl includes, but is not limited to, tetrahydropyranyl, 2,2-dimethyl-1,3-dioxolane, piperidino, N-methylpiperidin-3-yl, piperazino, N-methylpyrrolidin-3-yl, 3-pyrrolidino, morpholino, thiomorpholino, thiomorpholino-1-oxide, thiomorpholino-1,1-dioxide, 4-ethyloxycarbonylpiperazino, 3-oxopiperazino, 2-imidazolidone, 2-pyrrolidinone, 2-oxohomopiperazino, tetrahydropyrimidin-2-one, and the derivatives thereof. Preferably, the heterocycle group is optionally substituted with one or two substituents independently selected from halo, lower alkyl, lower alkyl substituted with carboxy, ester hydroxy, mono or dialkylamino.

"Hydroxy" refers to an -OH group.

"Alkoxy" refers to both an -O-(alkyl) and an -O-(cycloalkyl) group. Representative examples include, but are not limited to, e.g., methoxy, ethoxy, propoxy, butoxy, cyclopropyloxy, cyclobutyloxy, cyclopentyloxy, cyclohexyloxy, and the like.

"Aryloxy" refers to both an -O-aryl and an -O-heteroaryl group, as defined herein. Representative examples include, but are not limited to, phenoxy, pyridinyloxy, furanyloxy, thienyloxy, pyrimidinyloxy, pyrazinyloxy, and the like, and derivatives thereof.

"Mercapto" refers to an -SH group.

"Alkylthio" refers to both an -S-alkyl and an -S-cycloalkyl group. Representative examples include, but are not limited to, e.g., methylthio, ethylthio, propylthio, butylthio, cyclopropylthio, cyclobutylthio, cyclopentylthio, cyclohexylthio, and the like.

"Arylthio" refers to both an -S-aryl and an -S-heteroaryl group, as defined herein. Representative examples include, but are not limited to, phenylthio, pyridinylthio, furanylthio, thientylthio, pyrimidinylthio, and the like and derivatives thereof.

"Acyl" refers to a -C(O)-R" group, where R" is selected from the group consisting of hydrogen, lower alkyl, trihalomethyl, cycloalkyl, aryl optionally substituted with one or more, preferably one, two, or three substituents selected from the group consisting of lower alkyl, trihalomethyl, lower alkoxy, halo and $-NR^{18}R^{19}$ groups, heteroaryl (bonded through a ring

carbon) optionally substituted with one or more, preferably one, two, or three substitutents selected from the group consisting of lower alkyl, trihaloalkyl, lower alkoxy, halo and -$NR^{18}R^{19}$ groups and heteroalicyclic (bonded through a ring carbon) optionally substituted with one or more, preferably one, two, or three substituents selected from the group consisting of lower alkyl, trihaloalkyl, lower alkoxy, halo and -$NR^{18}R^{19}$ groups. Representative acy groups include, but are not limited to, acetyl, trifluoroacetyl, benzoyl, and the like

"Aldehyde" refers to an acyl group in which R" is hydrogen.

"Thioacyl" refers to a -C(S)-R" group, with R" as defined herein.

"Ester" refers to a -C(O)O-R" group with R" as defined herein except that R" cannot be hydrogen.

"Acetyl" group refers to a $-C(O)CH_3$ group.

"Halo" group refers to fluorine, chlorine, bromine or iodine, preferably fluorine or chlorine.

"Trihalomethyl" group refers to a $-CX_3$ group wherein X is a halo group as defined herein.

"Trihalomethanesulfonyl" group refers to a $X_3CS(=O)_2$- groups with X as defined above.

"Cyano" refers to a -C≡N group.

"Methylenedioxy" refers to a $-OCH_2O-$ group where the two oxygen atoms are bonded to adjacent carbon atoms.

"Ethylenedioxy" group refers to a $-OCH_2CH_2O-$ where the two oxygen atoms are bonded to adjacent carbon atoms.

"S-sulfonamido" refers to a -$S(O)_2NR^{18}R^{19}$ group, with R^{18} and R^{19} as defined herein.

"N-sulfonamido" refers to a –$NR^{18}S(O)_2R^{19}$ group, with R^{18} and R^{19} as defined herein.

"O-carbamyl" group refers to a -$OC(O)NR^{18}R^{19}$ group with R^{18} and R^{19} as defined herein.

"N-carbamyl" refers to an $R^{18}OC(O)NR^{19}$- group, with R^{18} and R^{19} as defined herein.

"O-thiocarbamyl" refers to a -$OC(S)NR^{18}R^{19}$ group with R^{18} and R^{19} as defined herein.

"N-thiocarbamyl" refers to a $R^{18}OC(S)NR^{19}$- group, with R^{18} and R^{19} as defined herein.

"Amino" refers to an -$NR^{18}R^{19}$ group, wherein R^{18} and R^{19} are both hydrogen.

"C-amido" refers to a -$C(O)NR^{18}R^{19}$ group with R^{18} and R^{19} as defined herein.

"N-amido" refers to a $R^{18}C(O)NR^{19}$- group, with R^{18} and R^{19} as defined herein.

"Nitro" refers to a $-NO_2$ group.

"Haloalkyl" means an alkyl, preferably lower alkyl as defined above that is substituted with one or more same or different halo atoms, e.g., $-CH_2Cl$, $-CF_3$, $-CH_2CF_3$, $-CH_2CCl_3$, and the like.

"Aralkyl" means alkyl, preferably lower alkyl as defined above which is substituted with an aryl group as defined above, e.g., $-CH_2$phenyl, $-(CH_2)_2$phenyl, $-(CH_2)_3$phenyl, $CH_3CH(CH_3)CH_2$phenyl,and the like and derivatives thereof.

"Heteroaralkyl" group means alkyl, preferably lower alkyl as defined above which is substituted with a heteroaryl group, e.g., $-CH_2$pyridinyl, $-(CH_2)_2$pyrimidinyl, $-(CH_2)_3$imidazolyl, and the like, and derivatives thereof.

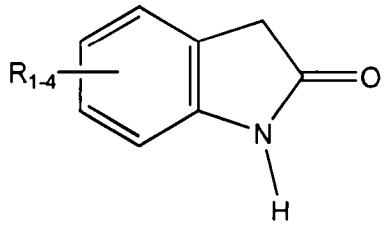
"Monoalkylamino" means a radical -NHR where R is an unsubstitued alkyl or cycloalkyl group as defined above, e.g., methylamino, (1-methylethyl)amino, cyclohexylamino, and the like.

"Dialkylamino" means a radical -NRR where each R is independently an unsubstitued alkyl or cycloalkyl group as defined above, e.g., dimethylamino, diethylamino, (1-methylethyl)-ethylamino, cyclohexylmethylamino, cyclopentylmethylamino, and the like.

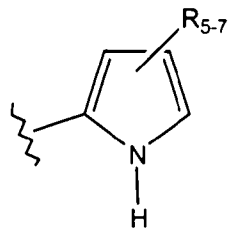
"Cyanoalkyl" means alkyl, preferably lower alkyl as defined above, which is substituted with 1 or 2 cyano groups.

"Optional" or "optionally" means that the subsequently described event or circumstance may but need not occur, and that the description includes instances where the event or circumstance occurs and instances in which it does not. For example, "heterocycle group optionally substituted with an alkyl group" means that the alkyl may but need not be present, and the description includes situations where the heterocycle group is substituted with an alkyl group and situations where the heterocyclo group is not substituted with the alkyl group.

The terms "2-indolinone","indolin-2-one" and "2-oxindole" are used interchangeably herein to refer to a molecule having the chemical structure:



The term "pyrrole" refers to a molecule having the chemical structure:



The term "pyrrole substituted 2-indolinone" and "3-pyrrolidenyl-2-indolinone" are used interchangeably herein to refer to a chemical compound having the general structure shown in Formula (I).

Compounds that have the same molecular formula but differ in the nature or sequence of bonding of their atoms or the arrangement of their atoms in space are termed "isomers". Isomers that differ in the arrangement of their atoms in space are termed "stereoisomers".

Stereoisomers that are not mirror images of one another are termed "diastereomers" and those that are non-superimposable mirror images of each other are termed "enantiomers". When a compound has an asymmetric center, for example, it is bonded to four different groups, a pair of enantiomers is possible. An enantiomer can be characterized by the absolute configuration of its asymmetric center and is described by the R- and S-sequencing rules of Cahn and Prelog, or by the manner in which the molecule rotates the plane of polarized light and designated as dextrorotatory or levorotatory (i.e., as (+) or (-)-isomers respectively). A chiral compound can exist as either individual enantiomer or as a mixture thereof. A mixture containing equal proportions of the enantiomers is called a "racemic mixture".

The compounds of this invention may possess one or more asymmetric centers; such compounds can therefore be produced as individual (R)- or (S)- stereoisomers or as mixtures thereof. For example, if the R^6 substituent in a compound of formula (I) is 2-hydroxyethyl, then the carbon to which the hydroxy group is attached is an asymmetric center and therefore the compound of Formula (I) can exist as an (R)- or (S)-stereoisomer. Unless indicated otherwise, the description or naming of a particular compound in the specification and claims is intended to include both individual enantiomers and mixtures, racemic or otherwise, thereof. The methods for the determination of stereochemistry and the separation of stereoisomers are well-known in the art (*see* discussion in Chapter 4 of "Advanced Organic Chemistry", 4th edition J. March, John Wiley and Sons, New York, 1992).

The compounds of the present invention may exhibit the phenomena of tautomerism and structural isomerism. For example, the compounds of Formula (I) described herein may

adopt an E or a Z configuration about the double bond connecting the 2-indolinone moiety to the pyrrole moiety or they may be a mixture of E and Z. This invention encompasses any tautomeric or structural isomeric form and mixtures thereof which possess the ability to modulate RTK, CTK and/or STK activity and is not limited to any one tautomeric or structural isomeric form.

A "pharmaceutical composition" refers to a mixture of one or more of the compounds described herein, or physiologically/pharmaceutically acceptable salts or prodrugs thereof, with other chemical components, such as physiologically/pharmaceutically acceptable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

The compounds of the present invention may also act as a prodrug. A "prodrug" refers to an agent which is converted into the parent drug *in vivo*. Prodrugs are often useful because, in some situations, they may be easier to administer than the parent drug. They may, for instance, be bioavailable by oral administration whereas the parent drug is not. The prodrug may also have improved solubility in pharmaceutical compositions over the parent drug. An example, without limitation, of a prodrug would be a compound of the present invention which is administered as an ester (the "prodrug") to facilitate transmittal across a cell membrane where water solubility is detrimental to mobility but then is metabolically hydrolyzed to the carboxylic acid, the active entity, once inside the cell where water solubility is beneficial.

A further example of a prodrug might be a short polypeptide, for example, without limitation, a 2 - 10 amino acid polypeptide, bonded through a terminal amino group to a carboxy group of a compound of this invention wherein the polypeptide is hydrolyzed or metabolized *in vivo* to release the active molecule. The prodrugs of the protein kinase inhibitors used with the method of the invention are within the scope of this invention.

Additionally, it is contemplated that a compound of Formula (I) would be metabolized by enzymes in the body of the organism such as human being to generate a metabolite that can modulate the activity of the protein kinases. Such metabolites are within the scope of the present invention.

As used herein, a "physiologically/pharmaceutically acceptable carrier" refers to a carrier or diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound.

An "pharmaceutically acceptable excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of a compound. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

As used herein, the term "pharmaceutically acceptable salt" refers to those salts which retain the biological effectiveness and properties of the parent compound. Such salts include:

(i) acid addition salt which is obtained by reaction of the free base of the parent compound with inorganic acids such as hydrochloric acid, hydrobromic acid, nitric acid, phosphoric acid, sulfuric acid, and perhcloric acid and the like, or with organic acids such as acetic acid, oxalic acid, (D) or (L) malic acid, maleic acid, methanesulfonic acid, ethanesulfonic acid, *p*-toluenesulfonic acid, salicylic acid, tartaric acid, citric acid, succinic acid or malonic acid and the like, preferably hydrochloric acid or (L)-malic acid such as the L-malate salt of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid(2-diethylaminoethyl)amide; or

(2) salts formed when an acidic proton present in the parent compound either is replaced by a metal ion, e.g., an alkali metal ion, an alkaline earth ion; or coordinates with an organic base. Exemplary ions include aluminum, calcium, lithium, magnesium, potassium, sodium and zinc in their usual valences. Preferred organic base include protonated tertiary amines and quaternary ammonium cations, including in part, trimethylamine, diethylamine, N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, ethylenediamine, meglumine (N-methylglucamine) and procaine.

"Method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by, practitioners of the chemical, pharmaceutical, biological, biochemical and medical arts.

"Treat", "treating" and "treatment" refers to any process, action, application, therapy, or the like, wherein a mammal, including a human being, is subject to medical aid with the object of improving the mammal's condition, directly or indirectly. With regard particularly to cancer, these terms simply mean that the life expectancy of an individual affected with a cancer will be increased or that one or more of the symptoms of the disease. This can be monitored by delayed appearance of primary or secondary tumors, slowed development of primary or secondary tumors, decreased occurrence of primary or secondary tumors, slowed or decreased severity of secondary effects of disease, arrested tumor growth and regression of tumors, among others.

The term "prevention" includes either preventing the onset of clinically evident neoplasia altogether or preventing the onset of a preclinically evident stage of neoplasia in individuals at risk. Also intended to be encompassed by this definition is the prevention of initiation for malignant cells or to arrest or reverse the progression of premalignant cells to malignant cells. This includes prophylactic treatment of those at risk of developing the

neoplasia.

The phrase "therapeutically-effective" is intended to qualify the amount of each agent that will achieve the goal of improvement in neoplastic disease severity and the frequency of neoplastic disease over treatment of each agent by itself, while avoiding adverse side effects typically associated with alternative therapies.

A "therapeutic effect" or "therapeutic effective amount" is intended to qualify the amount of an anticancer agent required to relieve to some extent one or more of the symptoms of a neoplasia disorder, including, but is not limited to: 1) reduction in the number of cancer cells; 2) reduction in tumor size; 3) inhibition (i.e., slowing to some extent, preferably stopping) of cancer cell infiltration into peripheral organs; 3) inhibition (i.e., slowing to some extent, preferably stopping) of tumor metastasis; 4) inhibition, to some extent, of tumor growth; 5) relieving or reducing to some extent one or more of the symptoms associated with the disorder; and/or 6) relieving or reducing the side effects associated with the administration of anticancer agents.

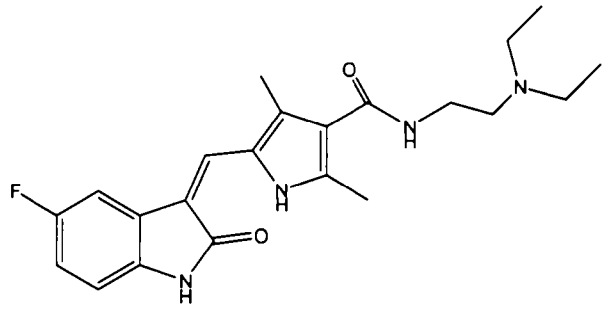
The phrase "combination therapy" (or "co-therapy") embraces the administration of a protein kinase inhibitor and a cyclooxygenase-2 inhibitor as part of a specific treatment regimen intended to provide a beneficial effect from the co-action of these therapeutic agents. The beneficial effect of the combination includes, but is not limited to, pharmacokinetic or pharmacodynamic co-action resulting from the combination of therapeutic agents. Administration of these therapeutic agents in combination typically is carried out over a defined time period (usually minutes, hours, days or weeks depending upon the combination selected). "Combination therapy" generally is not intended to encompass the administration of two or more of these therapeutic agents as part of separate monotherapy regimens that incidentally and arbitrarily result in the combinations of the present invention. "Combination therapy" is intended to embrace administration of these therapeutic agents in a sequential manner, that is, wherein each therapeutic agent is administered at a different time, as well as administration of these therapeutic agents, or at least two of the therapeutic agents, in a substantially simultaneous manner. Substantially simultaneous administration can be accomplished, for example, by administering to the subject a single capsule having a fixed ratio of each therapeutic agent or in multiple, single capsules for each of the therapeutic agents. Sequential or substantially simultaneous administration of each therapeutic agent can be effected by any appropriate route including, but not limited to, oral routes, intravenous routes, intramuscular routes, and direct absorption through mucous membrane tissues. The therapeutic agents can be administered by the same route or by different routes. For example, a first therapeutic agent of the combination selected may be administered by intravenous

injection while the other therapeutic agents of the combination may be administered orally. Alternatively, for example, both the therapeutic agents may be administered orally or both therapeutic agents may be administered by intravenous injection. The sequence in which the therapeutic agents are administered is not narrowly critical. "Combination therapy" also can embrace the administration of the therapeutic agents as described above in further combination with other biologically active ingredients (such as, but not limited to, a second and different antineoplastic agent) and non-drug therapies (such as, but not limited to, surgery or radiation treatment). Where the combination therapy further comprises radiation treatment, the radiation treatment may be conducted at any suitable time so long as a beneficial effect from the co-action of the combination of the therapeutic agents and radiation treatment is achieved. For example, in appropriate cases, the beneficial effect is still achieved when the radiation treatment is temporally removed from the administration of the therapeutic agents, perhaps by days or even weeks.

"Adjunctive therapy" encompasses treatment of a subject with agents that reduce or avoid side effects associated with the combination therapy of the present invention, including, but not limited to, those agents, for example, that reduce the toxic effect of anticancer drugs, e.g., bone resorption inhibitors, cardioprotective agents; prevent or reduce the incidence of nausea and vomiting associated with chemotherapy, radiotherapy or operation; or reduce the incidence of infection associated with the administration of myelosuppressive anticancer drugs.

PREFERRED EMBODIMENTS

The protein kinase has the formula



B. <u>Cyclooxygenase-2 selective ihbibitors- Compounds of Formulae (II) and (III):</u>

A number of the COX-2 inhibitors used in the present invention are shown in Tables II and III below. Not all COX-2 listed inhibitors in the tables are used in the present invention.

TABLE II

Compound	Trade/ Research Name	Reference	Dosage
1,5-Diphenyl-3-substituted pyrazoles		WO 97/13755	
	radicicol	WO 96/25928. Kwon et al (Cancer Res(1992) 52 6296)	
	GB-02283745		
	TP-72	Cancer Res 1998 58 4 717 -723	
1-(4-chlorobenzoyl)-3-[4-(4-fluoro-phenyl)thiazol-2-ylmethyl]-5-methoxy-2-methy lindole	A-183827.0		
	GR-253035		
4-(4-cyclohexyl-2-methyloxazol-5-yl)-2-fluorobenzenesulfonamide	JTE-522	JP 9052882	
5-chloro-3-(4-(methylsulfonyl)phenyl)-2-(methyl-5-pyridinyl)-pyridine			
2-(3,5-difluoro-phenyl)-3-4-(methylsulfonyl)-phenyl)-2-cyclopenten-1-one			
	L-768277		
	L-783003		
	MK-966; VIOXX®	US 5968974	12.5-100 mg po
Indomethacin-derived indolalkanoic acid		WO 96/374679	200 mg/kg/day
1-Methylsulfonyl-4-[1,1-dimethyl-4-(4-fluoro-phenyl)cyclopenta-2,4-dien-3-yl]benzene		WO 95/30656. WO 95/30652. WO 96/38418. WO 96/38442.	
4,4-dimethyl-2-phenyl-3-[4-(methylsulfonyl)phenyl]cyclo-			

Compound	Trade/ Research Name	Reference	Dosage
butenone			
2-(4-methoxyphenyl)-4-methyl-1-(4-sulfamoylphenyl)-pyrrole		EP 799823	
N-[5-(4-fluoro)phenoxy]thiophene-2-methanesulfon-amide	RWJ-63556		
5(E)-(3,5-di-tert-butyl-4-hydroxy)benzylidene-2-ethyl-1,2-isothiazolidine-1,1-dioxide	S-2474	EP 595546	
3-formylamino-7-methylsulfonylamino-6-phenoxy-4H-1-benzopyran-4-one	T-614	DE 38/34204	
Benzenesulfonamide, 4-(5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl)-	celecoxib	US 5466823	
CS 502	(Sankyo)		
2-[(2-chloro-6-fluorophenyl)amino]-5-methylbenzeneacetic acid	lumiracoxib (Cox-189)	WO 99/11605	
5-chloro-6'-methyl-3-[4-(methylsulfonyl)phenyl]-2,3'-bipyridine	etoricoxib (MK 663)	WO 98/03484	
	BMS 34070	US 6180651	
	meloxicam	US 4233299	15-30 mg/day
	nimesulide	US 3840597	

TABLE III

WO 99/30721	WO 99/30729	US 5760068	WO 98/15528
WO 99/25695	WO 99/24404	WO 99/23087	FR 27/71005
EP 921119	FR 27/70131	WO 99/18960	WO 99/15505
WO 99/15503	WO 99/14205	WO 99/14195	WO 99/14194
WO 99/13799	GB 23/30833	US 5859036	WO 99/12930
WO 99/11605	WO 99/10332	WO 99/10331	WO 99/09988
US 5869524	WO 99/05104	US 5859257	WO 98/47890
WO 98/47871	US 5830911	US 5824699	WO 98/45294
WO 98/43966	WO 98/41511	WO 98/41864	WO 98/41516
WO 98/37235	EP 86/3134	JP 10/175861	US 5776967
WO 98/29382	WO 98/25896	ZA 97/04806	EP 84/6,689
WO 98/21195	GB 23/19772	WO 98/11080	WO 98/06715
WO 98/06708	WO 98/07425	WO 98/04527	WO 98/03484
FR 27/51966	WO 97/38986	WO 97/46524	WO 97/44027
WO 97/34882	US 5681842	WO 97/37984	US 5686460
WO 97/36863	WO 97/40012	WO 97/36497	WO 97/29776
WO 97/29775	WO 97/29774	WO 97/28121	WO 97/28120
WO 97/27181	WO 95/11883	WO 97/14691	WO 97/13755
WO 97/13755	CA 21/80624	WO 97/11701	WO 96/41645
WO 96/41626	WO 96/41625	WO 96/38418	WO 96/37467
WO 96/37469	WO 96/36623	WO 96/36617	WO 96/31509
WO 96/25405	WO 96/24584	WO 96/23786	WO 96/19469
WO 96/16934	WO 96/13483	WO 96/03385	US 5510368
WO 96/09304	WO 96/06840	WO 96/06840	WO 96/03387
WO 95/21817	GB 22/83745	WO 94/27980	WO 94/26731
WO 94/20480	WO 94/13635	FR 27/70,131	US 5859036
WO 99/01131	WO 99/01455	WO 99/01452	WO 99/01130
WO 98/57966	WO 98/53814	WO 98/53818	WO 98/53817
WO 98/47890	US 5830911	US 5776967	WO 98/22101
DE 19/753463	WO 98/21195	WO 98/16227	US 5733909
WO 98/05639	WO 97/44028	WO 97/44027	WO 97/40012
WO 97/38986	US 5677318	WO 97/34882	WO 97/16435

WO 97/03678	WO 97/03667	WO 96/36623	WO 96/31509
WO 96/25928	WO 96/06840	WO 96/21667	WO 96/19469
US 5510368	WO 96/09304	GB 22/83745	WO 96/03392
WO 94/25431	WO 94/20480	WO 94/13635	JP 09052882
GB 22/94879	WO 95/15316	WO 95/15315	WO 96/03388
WO 96/24585	US 5344991	WO 95/00501	US 5968974
US 5945539	US 5994381	US 6180651	

Pyrazoles can be prepared by methods described in WO 95/15316. Pyrazoles can further be prepared by methods described in WO 95/15315. Pyrazoles can also be prepared by methods described in WO 96/03385. Thiophene analogs can be prepared by methods described in WO 95/00501. Preparation of thiophene analogs is also described in WO 94/15932. Oxazoles can be prepared by the methods described in WO 95/00501. Preparation of oxazoles is also described in WO 94/27980. Isoxazoles can be prepared by the methods described in WO 96/25405. Imidazoles can be prepared by the methods described in WO 96/03388. Preparation of imidazoles is also described in WO 96/03387. Cyclopentene cyclooxygenase-2 inhibitors can be prepared by the methods described in U.S. Patent No. 5,344,991. Preparation of cyclopentane Cox-2 inhibitors is also described in WO 95/00501. Terphenyl compounds can be prepared by the methods described in WO 96/16934. Thiazole compounds can be prepared by the methods described in WO 96/03,392. Pyridine compounds can be prepared by the methods described in WO 96/03392. Preparation of pyridine compounds is also described in WO 96/24585.

The celecoxib used in the therapeutic combinations of the present invention can be prepared in the manner set forth in U.S. Patent No. 5,466,823.

The valdecoxib used in the therapeutic combinations of the present invention can be prepared in the manner set forth in U.S. Patent No. 5,633,272.

The parecoxib used in the therapeutic combinations of the present invention can be prepared in the manner set forth in U.S. Patent No. 5,932,598.

The rofecoxib used in the therapeutic combinations of the present invention can be prepared in the manner set forth in U.S. Patent No. 5,968,974.

The Japan Tobacco JTE-522 used in the therapeutic combinations of the present invention can be prepared in the manner set forth in JP 90/52,882.

The lumiracoxib (Cox-189) used in the therapeutic combinations of the present invention can be prepared in the manner set forth in WO 99/11605.

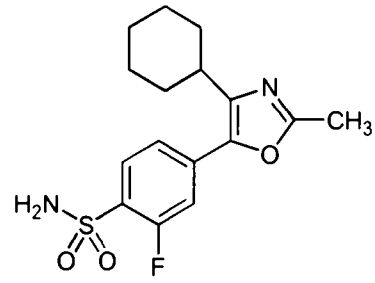
The etoricoxib (MK 663) used in the therapeutic combinations of the present invention can be prepared in the manner set forth in WO 98/03484.

Bristol Meyers Squibb's BMS 34070 used in the therapeutic combinations of the present invention can be prepared in the manner set forth in U.S. Patent No. 6,180,651.

The above references listed in Tables II and III above describe various COX-2 inhibitors suitable for use in the present invention described herein, and processes for their manufacture, are hereby individually incorporated by reference.

Preferred COX-2 inhibitors that may be used in the present invention include, but are not limited to:

C1)



JTE-522, 4-(4-cyclohexyl-2-methyloxazol-5-yl)-2-fluorobenzenesulfonamide;

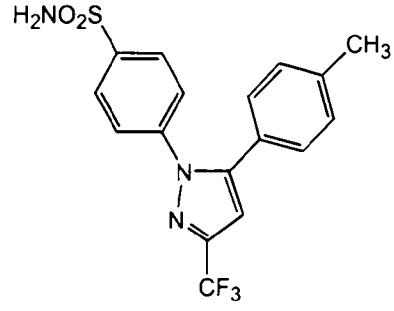
C2)

5-chloro-3-(4-(methylsulfonyl)phenyl)-2-(methyl-5-pyridinyl)pyridine;

C3)

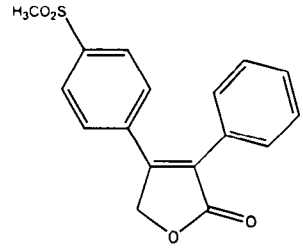
2-(3,5-difluorophenyl)-3-4-(methylsulfonyl)phenyl)-2-cyclopenten-1-one;

C4)



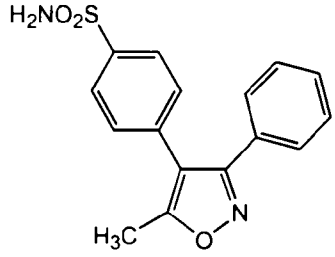
4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]-benzenesulfonamide;

C5)



rofecoxib, 4-(4-(methylsulfonyl)phenyl]-3-phenyl-2(5H)-furanone;

C6)

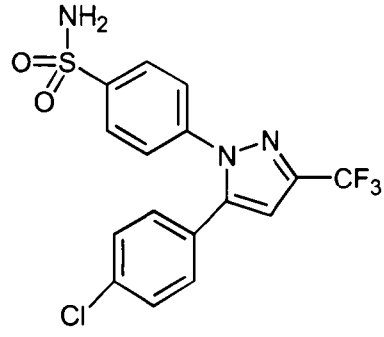


4-(5-methyl-3-phenylisoxazol-4-yl)benzenesulfonamide;

C7)

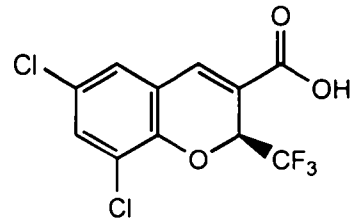
N-[[4-(5-methyl-3-phenylisoxazol-4yl]phenyl]sulfonyl]propanamide;

C8)



4-[5-(4-chorophenyl)-3-(trifluoromethyl)-1H-pyrazole-1-yl]benzenesulfonamide;

C9)



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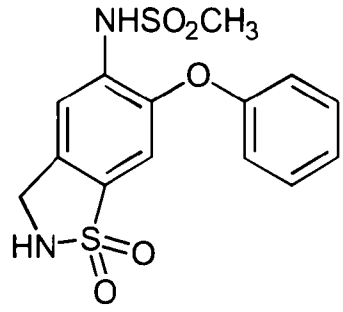
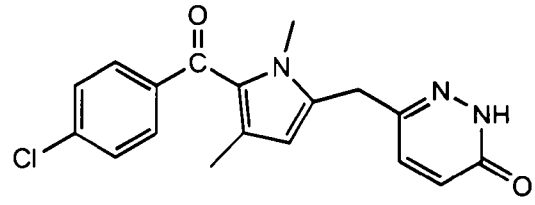
C10)



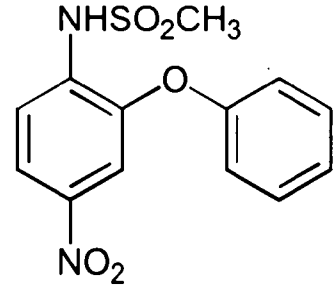
;

C11)



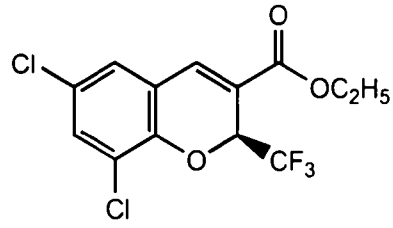
6-[[5-(4-chlorobenzoyl)-1,4—dimethyl-1H-pyrrol-2-yl]methyl]-3(2H)-pyridazinone;

C12)

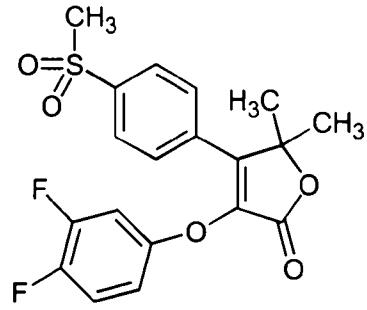


N-(4-nitro-2-phenoxyphenyl)methanesulfonamide;

C13)

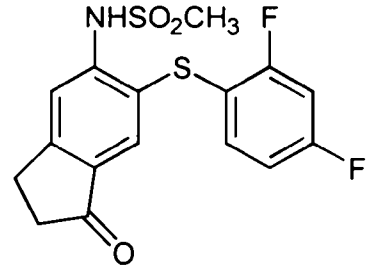


C14)



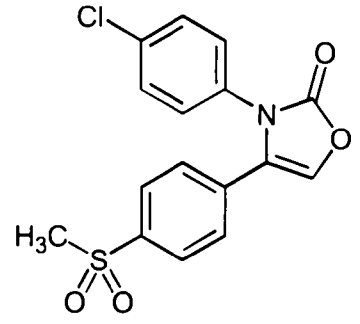
3-(3,4-difluorophenoxy)-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]-2(5H)-furanone;

C15)



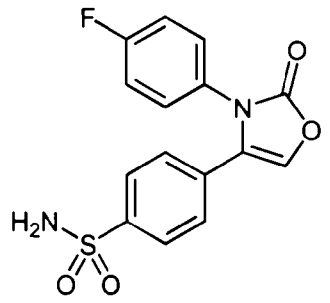
N-[6-[(2,4-difluorophenyl)thio]-2,3-dihydro-1-oxo-1H-inden-5-yl]methanesulfonamide;

C16)



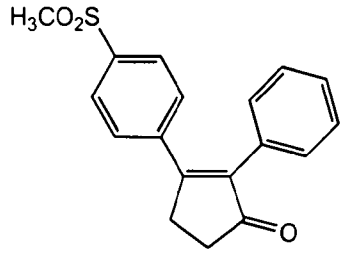
3-(4-chlorophenyl)-4-[4-(methylsulfonyl)phenyl]-2(3H)-oxazolone;

C17)



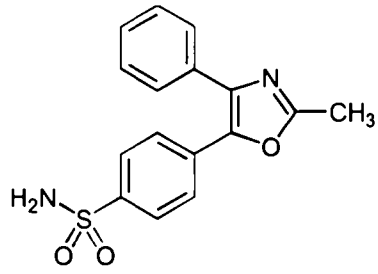
4-[3-(4-fluorophenyl)-2,3-dihydro-2-oxo-4-oxazolyl]benzenesulfonamide;

C18)



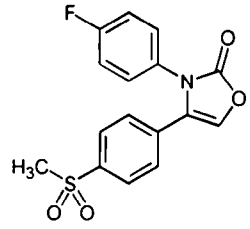
3-[4-(methylsulfonyl)phenyl]-2-phenyl-2-cyclopenten-1-one;

C19)



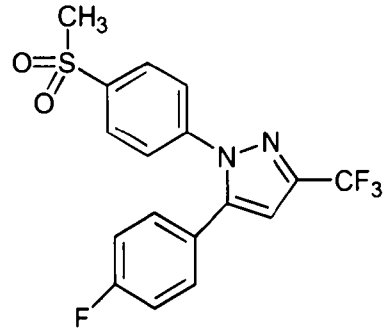
4-(2-methyl-4-phenyl-5-oxazolyl)benzenesulfonamide;

C20)



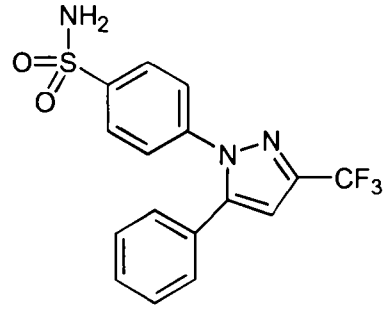
3-(4-fluorophenyl)-4-[4-(methylsulfonyl)phenyl]-2(3H)-oxazolone.

C21)



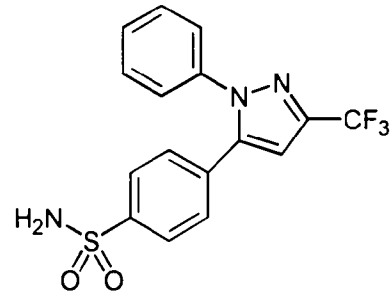
5-(4-fluorophenyl)-1-[4-(methylsulfonyl)phenyl]-3-(trifluoromethyl)-1H-pyrazole;

C22)



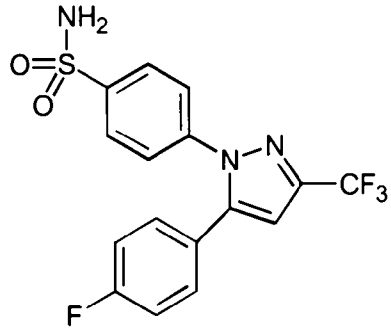
4-[5-phenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl)benzenesulfonamide;

C23)



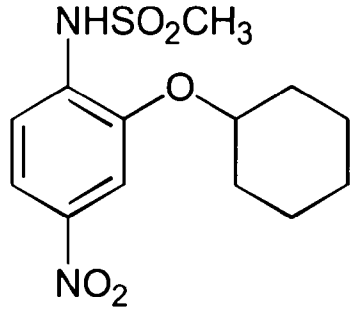
4-[1-phenyl-3-(trifluoromethyl)-1H-pyrazol-5-yl]benzenesulfonamide;

C24)



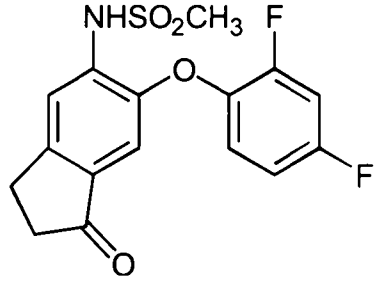
4-[5-(4-fluorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide;

C25)



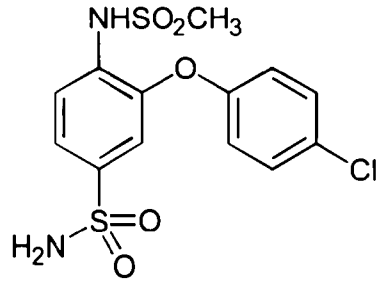
N-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide;

C26)



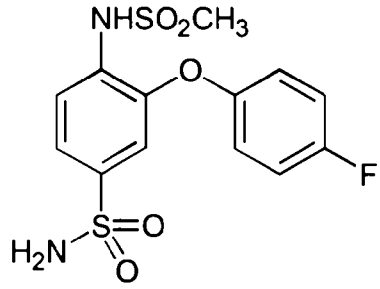
N-[6-(2,4-difluorophenoxy)-2,3-dihydro-1-oxo-1H-inden-5-yl]methanesulfonamide;

C27)



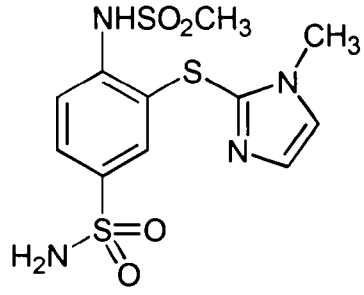
3-(4-chlorophenoxy)-4-[(methylsulfonyl)amino]benzenesulfonamide;

C28)



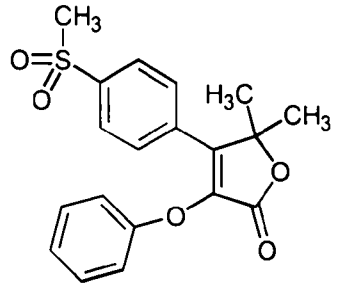
3-(4-fluorophenoxy)-4-[(methylsulfonyl)amino]benzenesulfonamide;

C29)



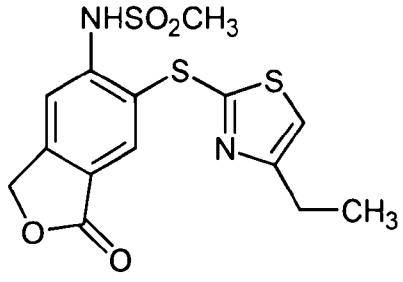
3-[(1-methyl-1H-imidazol-2-yl)thio]-4 [(methylsulfonyl) amino]benzenesulfonamide;

C30)



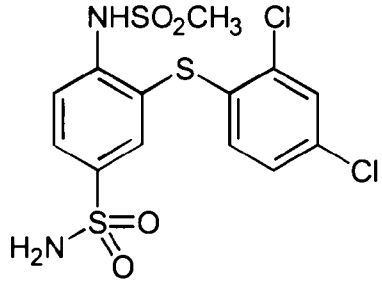
5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]-3-phenoxy-2(5H)-furanone;

C31)



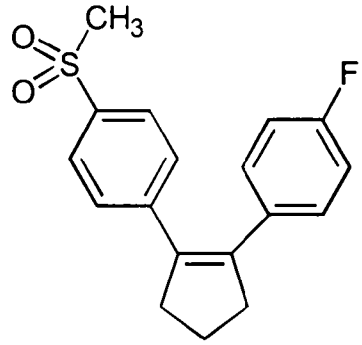
N-[6-[(4-ethyl-2-thiazolyl)thio]-1,3-dihydro-1-oxo-5-isobenzofuranyl]methanesulfonamide;

C32)



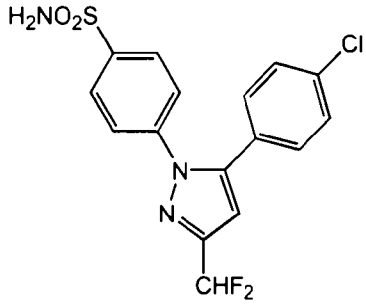
3-[(2,4-dichlorophenyl)thio]-4-[(methylsulfonyl)amino]benzenesulfonamide;

C33)



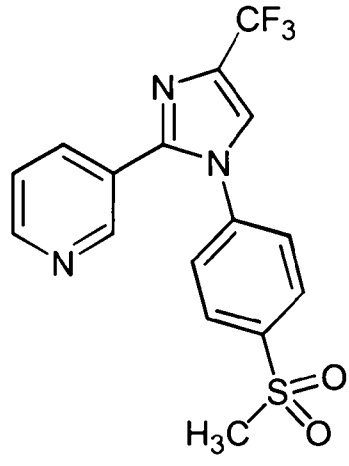
1-fluoro-4-[2-[4-(methylsulfonyl)phenyl]cyclopenten-1-yl]benzene;

C34)



4-[5-(4-chlorophenyl)-3-(difluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide;

C35)



3-[1-[4-(methylsulfonyl)phenyl]-4-(trifluoromethyl)-1H-imidazol-2-yl]pyridine;

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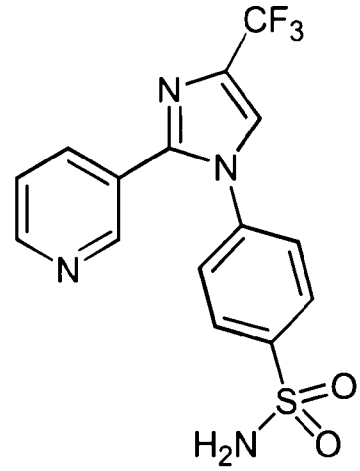
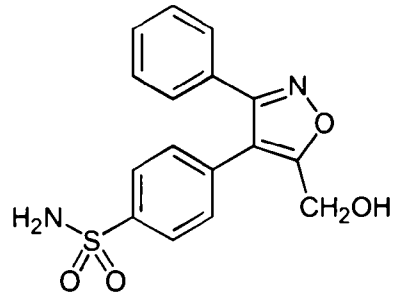
C36)



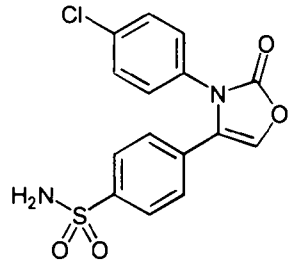
4-[2-(3-pyridinyll)-4-(trifluoromethyl)-1H-imidazol-1-yl]benzenesulfonamide;

C37)



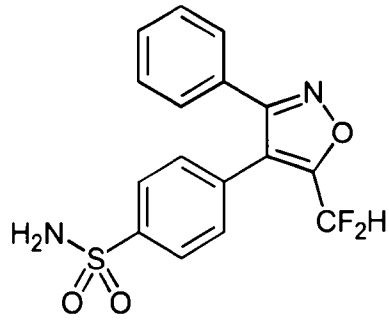
4-[5-(hydroxymethyl)-3-phenylisoxazol-4-yl]benzenesulfonamide;

C38)



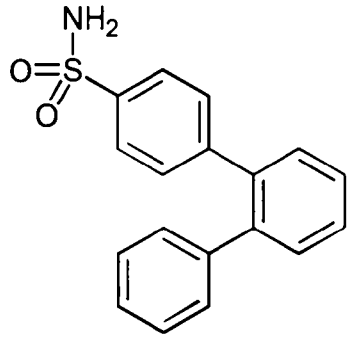
4-[3-(4-chlorophenyl)-2,3-dihydro-2-oxo-4-oxazolyl]benzenesulfonamide;

C39)



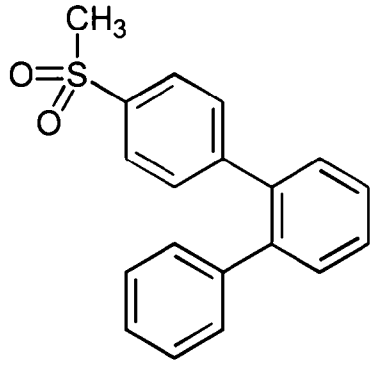
4-[5-(difluoromethyl)-3-phenylisoxazol-4-yl]benzenesulfonamide;

C40)



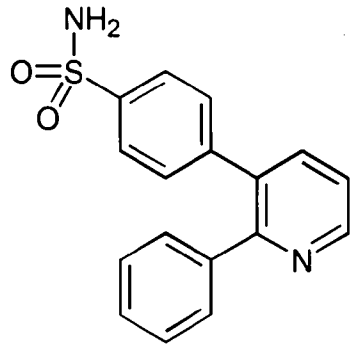
[1,1':2',1"-terphenyl]-4-sulfonamide;

C41)



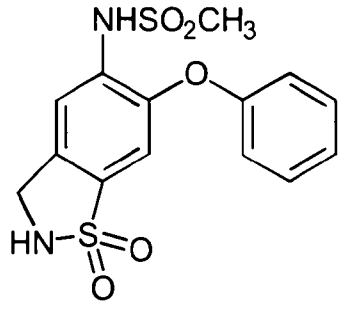
4-(methylsulfonyl)-1,1',2],1"-terphenyl;

C42)



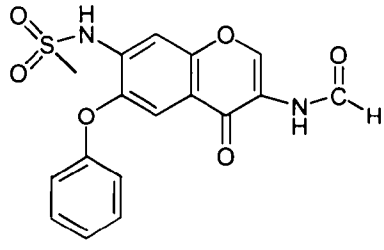
4-(2-phenyl-3-pyridinyl)benzenesulfonamide;

C43)



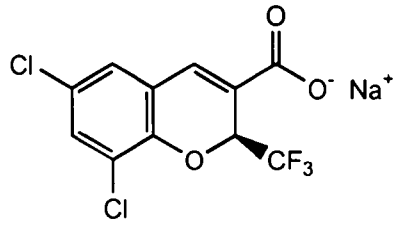
N-(2,3-dihydro-1,1-dioxido-6-phenoxy-1,2-benzisothiazol-5-yl)methanesulfonamide; and

C44)



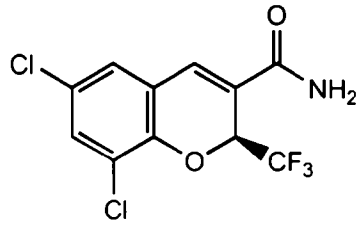
N-[3-(formylamino)-4-oxo-6-phenoxy-4H-1-benzopyran-7-yl]methanesulfonamide;

C45)



;

C46)



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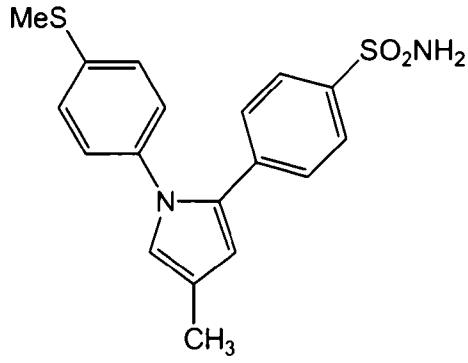
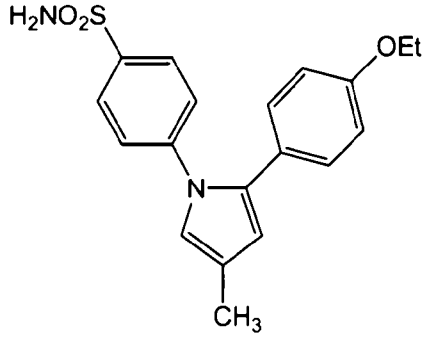
C47)



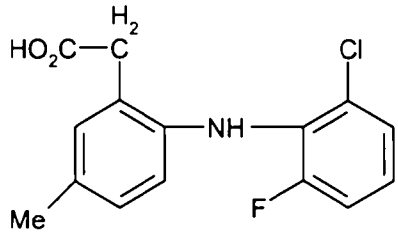
;

C48)



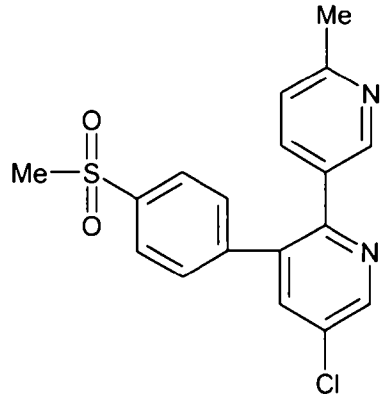
; and

C49) lumiracoxib (COX-189)



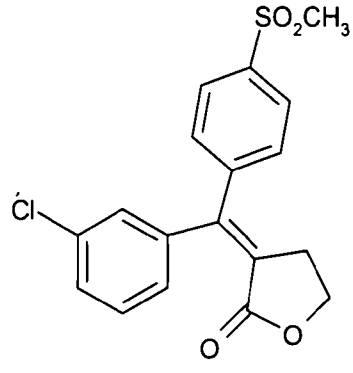
2-[(2-chloro-6-fluorophenyl)amino]-5-methylbenzeneacetic acid

C50) etoricoxib (MK 663)



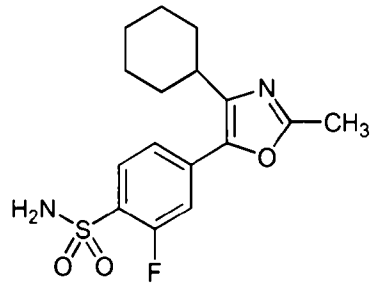
5-chloro-6'-methyl-3-[4-(methylsulfonyl)phenyl]-2,3'-bipyridine

C51) BMS 34070



More preferred COX-2 inhibitors that may be used in the present invention are selected from the group consisting of:

I)



JTE-522, 4-(4-cyclohexyl-2-methyloxazol-5-yl)-2-fluorobenzenesulfonamide;

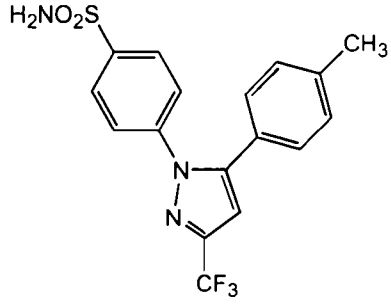
II)

5-chloro-3-(4-(methylsulfonyl)phenyl)-2-(methyl-5-pyridinyl)pyridine;

III)

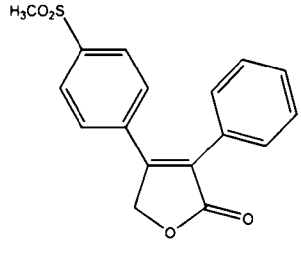
2-(3,5-difluorophenyl)-3-4-(methylsulfonyl)phenyl)-2-cyclopenten-1-one;

IV)



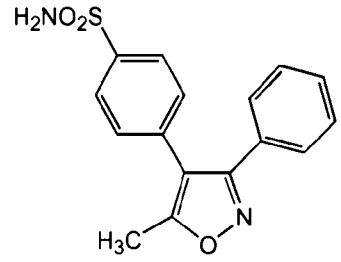
4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]-benzenesulfonamide;

V)



rofecoxib, 4-(4-(methylsulfonyl)phenyl]-3-phenyl-2(5H)-furanone;

VI)

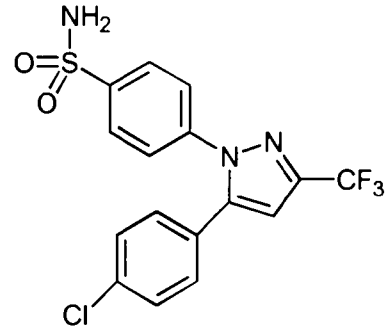


4-(5-methyl-3-phenylisoxazol-4-yl)benzenesulfonamide;

VII)

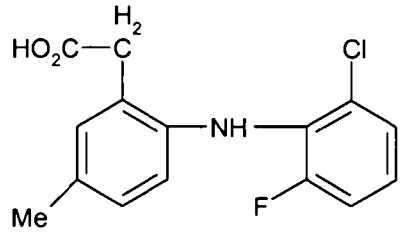
N-[[4-(5-methyl-3-phenylisoxazol-4yl]phenyl]sulfonyl]propanamide;

VIII)



4-[5-(4-chorophenyl)-3-(trifluoromethyl)-1H-pyrazole-1-yl]benzenesulfonamide; and

IX) lumiracoxib (COX-189)



2-[(2-chloro-6-fluorophenyl)amino]-5-methylbenzeneacetic acid

Still more preferably, the COX-2 inhibitors that may be used in the present invention include, but are not limited to celecoxib, valdecoxib, parecoxib, rofecoxib, lumiracoxib and Japan Tobacco JTE-522.

Also included in the combination of the invention are the isomeric forms, prodrugs and tautomers of the described compounds and the pharmaceutically-acceptable salts thereof. Illustrative pharmaceutically acceptable salts are prepared from formic, acetic, propionic, succinic, glycolic, gluconic, lactic, malic, tartaric, citric, ascorbic, glucuronic, maleic, fumaric, pyruvic, aspartic, glutamic, benzoic, anthranilic, mesylic, stearic, salicylic, p-hydroxybenzoic, phenylacetic, mandelic, embonic (pamoic), methanesulfonic, ethanesulfonic, benzenesulfonic, pantothenic, toluenesulfonic, 2-hydroxyethanesulfonic, sulfanilic, cyclohexylaminosulfonic, algenic, b-hydroxybutyric, galactaric and galacturonic acids.

Utility

The compounds of the present invention are inhibitor of protein kinases (PKs) and cyclooxygenase enzyme, in particular cyclooxygenase-2 enzyme, and are therefore useful in the treatment of cancer.

The PKs whose catalytic activity is modulated by the compounds of Formula (I) of this invention include protein tyrosine kinases such as receptor tyrosine kinases (RTKs), cellular tyrosine kinases (CTKs), and serine-threonine kinases (STKs). RTK mediated signal transduction is initiated by extracellular interaction with a specific growth factor (ligand), followed by receptor dimerization, transient stimulation of the intrinsic protein tyrosine kinase activity and phosphorylation. Binding sites are thereby created for intracellular signal transduction molecules and lead to the formation of complexes with a spectrum of cytoplasmic signaling molecules that facilitate the appropriate cellular response (e.g., cell division, metabolic effects on the extracellular microenvironment, etc.). See, Schlessinger and Ullrich, 1992, Neuron 9:303-391.

It has been shown that tyrosine phosphorylation sites on growth factor receptors function as high-affinity binding sites for SH2 (src homology) domains of signaling molecules. Fantl *et al.*, 1992, Cell 69:413-423, Songyang *et al.*, 1994, Mol. Cell. Biol. 14:2777-2785), Songyang *et al.*, 1993, Cell 72:767-778, and Koch *et al.*, 1991, Science 252:668-678. Several intracellular substrate proteins that associate with RTKs have been identified. They may be divided into two principal groups: (1) substrates that have a catalytic domain, and (2) substrates which lack such domain but which serve as adapters and associate with catalytically active molecules. Songyang *et al.*, 1993, Cell 72:767-778. The specificity of the interactions between receptors and SH2 domains of their substrates is determined by the amino acid residues immediately surrounding the phosphorylated tyrosine residue. Differences in the binding affinities between SH2 domains and the amino acid sequences surrounding the phosphotyrosine residues on particular receptors are consistent with the observed differences in

their substrate phosphorylation profiles. Songyang *et al.*, 1993, Cell 72:767-778. These observations suggest that the function of each RTK is determined not only by its pattern of expression and ligand availability but also by the array of downstream signal transduction pathways that are activated by a particular receptor. Thus, phosphorylation provides an important regulatory step which determines the selectivity of signaling pathways recruited by specific growth factor receptors, as well as differentiation factor receptors.

STKs, being primarily cytosolic, affect the internal biochemistry of the cell, often as a down-line response to a PTK event. STKs have been implicated in the signaling process which initiates DNA synthesis and subsequent mitosis leading to cell proliferation.

Thus, PK signal transduction results in, among other responses, cell proliferation, differentiation, growth and metabolism. Abnormal cell proliferation may result in a wide array of disorders and diseases, including the development of neoplasia such as carcinoma, sarcoma, glioblastoma and hemangioma, disorders such as leukemia, psoriasis, arteriosclerosis, arthritis and diabetic retinopathy and other disorders related to uncontrolled angiogenesis and/or vasculogenesis.

A precise understanding of the mechanism by which the compounds of this invention inhibit PKs is not required in order to practice the present invention. However, while not hereby being bound to any particular mechanism or theory, it is believed that the compounds of Formula (I) interact with the amino acids in the catalytic region of PKs. PKs typically possess a bi-lobate structure wherein ATP appears to bind in the cleft between the two lobes in a region where the amino acids are conserved among PKs. Inhibitors of PKs are believed to bind by non-covalent interactions such as hydrogen bonding, van der Waals forces and ionic interactions in the same general region where the aforesaid ATP binds to the PKs. More specifically, it is thought that the 2-indolinone component of the compounds of Formula (I) binds in the general space normally occupied by the adenine ring of ATP. Specificity of a particular molecule for a particular PK may then arise as the result of additional interactions between the various substituents on the 2-indolinone core and the amino acid domains specific to particular PKs. Thus, different indolinone substituents may contribute to preferential binding to particular PKs. The ability to select compounds active at different ATP (or other nucleotide) binding sites makes the compounds of Formula (I) useful for targeting any protein with such a site. The compounds of Formula (I) disclosed herein thus have utility in *in vitro* assays for such proteins as well as exhibiting *in vivo* therapeutic effects through interaction with such proteins.

Additionally, the compounds of Formula (I) provide a therapeutic approach to the treatment of many kinds of solid tumors, including but not limited to carcinomas, sarcomas

including Kaposi's sarcoma, erythroblastoma, glioblastoma, meningioma, astrocytoma, melanoma and myoblastoma. Treatment or prevention of non-solid tumor cancers such as leukemia are also contemplated by this invention. Indications may include, but are not limited to brain cancers, bladder cancers, ovarian cancers, gastric cancers, pancreas cancers, colon cancers, blood cancers, lung cancers and bone cancers.

Further examples, without limitation, of the types of disorders related to inappropriate PK activity that the compounds of present invention may be useful in preventing, treating and studying, are cell proliferative disorders, fibrotic disorders and metabolic disorders.

Cell proliferative disorders, which may be prevented, treated or further studied by the present invention include cancer, blood vessel proliferative disorders and mesangial cell proliferative disorders.

Blood vessel proliferative disorders refer to disorders related to abnormal vasculogenesis (blood vessel formation) and angiogenesis (spreading of blood vessels). While vasculogenesis and angiogenesis play important roles in a variety of normal physiological processes such as embryonic development, corpus luteum formation, wound healing and organ regeneration, they also play a pivotal role in cancer development where they result in the formation of new capillaries needed to keep a tumor alive. Other examples of blood vessel proliferation disorders include arthritis, where new capillary blood vessels invade the joint and destroy cartilage, and ocular diseases, like diabetic retinopathy, where new capillaries in the retina invade the vitreous, bleed and cause blindness.

Two structurally related RTKs have been identified to bind VEGF with high affinity: the fms-like tyrosine 1 (fit-l) receptor (Shibuya *et al.*, 1990, Oncogene,5:519-524; De Vries *et al.*, 1992, Science, 255:989-991) and the KDR/FLK-1 receptor, also known as VEGF-R2. Vascular endothelial growth factor (VEGF) has been reported to be an endothelial cell specific mitogen with *in vitro* endothelial cell growth promoting activity. Ferrara & Henzel, 1989, Biochein. Biophys. Res. Comm., 161:851-858; Vaisman *et al.*, 1990, J. Biol. Chem., 265:19461-19566. Information set forth in U.S. application Ser. Nos. 08/193,829, 08/038,596 and 07/975,750, strongly suggest that VEGF is not only responsible for endothelial cell proliferation, but also is the prime regulator of normal and pathological angiogenesis. See generally, Klagsburn & Soker, 1993, Current Biology, 3(10)699-702; Houck, *et al.*, 1992, J. Biol. Chem., 267:26031-26037.

Normal vasculogenesis and angiogenesis play important roles in a variety of physiological processes such as embryonic development, wound healing, organ regeneration and female reproductive processes such as follicle development in the corpus luteum during ovulation and placental growth after pregnancy. Folkman & Shing, 1992, J. Biological Chem.,

267(16):10931-34. Uncontrolled vasculogenesis and/or angiogenesis has been associated with diseases such as diabetes as well as with malignant solid tumors that rely on vascularization for growth. Klagsburn & Soker, 1993, Current Biology, 3(10):699-702; Folkham, 1991, J. Natl. Cancer Inst., 82:4-6; Weidner, *et al.*, 1991, New Engl. J. Med., 324:1-5.

The surmised role of VEGF in endothelial cell proliferation and migration during angiogenesis and vasculogenesis indicates an important role for the KDR/FLK-1 receptor in these processes. Diseases such as diabetes mellitus (Folkman, 198, in XIth Congress of Thrombosis and Haemostasis (Verstraeta, *et al.*, eds.), pp. 583-596, Leuven University Press, Leuven) and arthritis, as well as malignant tumor growth may result from uncontrolled angiogenesis. See e.g., Folkman, 1971, N. Engl. J. Med., 285:1182-1186. The receptors to which VEGF specifically binds are an important and powerful therapeutic target for the regulation and modulation of vasculogenesis and/or angiogenesis and a variety of severe diseases which involve abnormal cellular growth caused by such processes. Plowman, *et al.*, 1994, DN&P, 7(6):334-339. More particularly, the KDR/FLK-1 receptor's highly specific role in neovascularization make it a choice target for therapeutic approaches to the treatment of cancer and other diseases which involve the uncontrolled formation of blood vessels.

Thus, the present invention provides compounds capable of regulating and/or modulating tyrosine kinase signal transduction including KDR/FLK-1 receptor signal transduction in order to inhibit or promote angiogenesis and/or vasculogenesis, that is, compounds that inhibit, prevent, or interfere with the signal transduced by KDR/FLK-1 when activated by ligands such as VEGF. Although it is believed that the compounds of the present invention act on a receptor or other component along the tyrosine kinase signal transduction pathway, they may also act directly on the tumor cells that result from uncontrolled angiogenesis.

Although the nomenclature of the human and murine counterparts of the generic "flk-I" receptor differ, they are, in many respects, interchangeable. The murine receptor, Flk-1, and its human counterpart, KDR, share a sequence homology of 93.4% within the intracellular domain. Likewise, murine FLK-I binds human VEGF with the same affinity as mouse VEGF, and accordingly, is activated by the ligand derived from either species. Millauer *et al.*, 1993, Cell, 72:835-846; Quinn *et al.*, 1993, Proc. Natl. Acad. Sci. USA, 90:7533-7537. FLK-1 also associates with and subsequently tyrosine phosphorylates human RTK substrates (e.g., PLC-γ or p85) when co-expressed in 293 cells (human embryonal kidney fibroblasts).

Models which rely upon the FLK-1 receptor therefore are directly applicable to understanding the KDR receptor. For example, use of the murine FLK-1 receptor in methods which identify compounds that regulate the murine signal transduction pathway are directly

applicable to the identification of compounds which may be used to regulate the human signal transduction pathway, that is, which regulate activity related to the KDR receptor. Thus, chemical compounds identified as inhibitors of KDR/FLK-1 *in vitro*, can be confirmed in suitable *in vivo* models. Both *in vivo* mouse and rat animal models have been demonstrated to be of excellent value for the examination of the clinical potential of agents acting on the KDR/FLK-1 induced signal transduction pathway.

Thus, the present invention provides compounds that regulate, modulate and/or inhibit vasculogenesis and/or angiogenesis by affecting the enzymatic activity of the KDR/FLK-1 receptor and interfering with the signal transduced by KDR/FLK-1. Thus the present invention provides a therapeutic approach to the treatment of many kinds of solid tumors including, but not limited to, glioblastoma, melanoma and Kaposi's sarcoma, and ovarian, lung, mammary, prostate, pancreatic, colon and epidermoid carcinoma. In addition, data suggests the administration of compounds which inhibit the KDR/Flk-1 mediated signal transduction pathway may also be used in the treatment of hemangioma, restenois and diabetic retinopathy.

Furthermore, this invention relates to the inhibition of vasculogenesis and angiogenesis by other receptor-mediated pathways, including the pathway comprising the flt-l receptor.

Receptor tyrosine kinase mediated signal transduction is initiated by extracellular interaction with a specific growth factor (ligand), followed by receptor dimerization, transient stimulation of the intrinsic protein tyrosine kinase activity and autophosphorylation. Binding sites are thereby created for intracellular signal transduction molecules which leads to the formation of complexes with a spectrum of cytoplasmic signalling molecules that facilitate the appropriate cellular response, e.g., cell division and metabolic effects to the extracellular microenvironment. See, Schlessinger and Ullrich, 1992, Neuron, 9:1-20.

The close homology of the intracellular regions of KDR/FLK-1 with that of the PDGF-β receptor (50.3% homology) and/or the related flt-l receptor indicates the induction of overlapping signal transduction pathways. For example, for the PDGF-β receptor, members of the src family (Twamley *et al.*, 1993, Proc. Natl. Acad. Sci. USA, 90:7696-7700), phosphatidylinositol-3'-kinase (Hu *et al.*, 1992, Mol. Cell. Biol., 12:981-990), phospholipase cγ (Kashishian & Cooper, 1993, Mol. Cell. Biol., 4:49-51), ras-GTPase-activating protein, (Kashishian *et al.*, 1992, EMBO J., 11:1373-1382), PTP-ID/syp (Kazlauskas *et al.*, 1993, Proc. Natl. Acad. Sci. USA, 10 90:6939-6943), Grb2 (Arvidsson *et al.*, 1994, Mol. Cell. Biol., 14:6715-6726), and the adapter molecules Shc and Nck (Nishimura *et al.*, 1993, Mol. Cell. Biol., 13:6889-6896), have been shown to bind to regions involving different autophosphorylation sites. See generally, Claesson-Welsh, 1994, Prog. Growth Factor Res.,

5:37-54. Thus, it is likely that signal transduction pathways activated by KDR/FLK-1 include the ras pathway (Rozakis *et al.*, 1992, Nature, 360:689-692), the PI-3'-kinase, the src-mediated and the plcγ-mediated pathways. Each of these pathways may play a critical role in the angiogenic and/or vasculogenic effect of KDR/FLK-1 in endothelial cells. Consequently, a still further aspect of this invention relates to the use of the organic compounds described herein to modulate angiogenesis and vasculogenesis as such processes are controlled by these pathways.

Conversely, disorders related to the shrinkage, contraction or closing of blood vessels, such as restenosis, are also implicated and may be treated or prevented by the methods of this invention.

Fibrotic disorders refer to the abnormal formation of extracellular matrices. Examples of fibrotic disorders include hepatic cirrhosis and mesangial cell proliferative disorders. Hepatic cirrhosis is characterized by the increase in extracellular matrix constituents resulting in the formation of a hepatic scar. An increased extracellular matrix resulting in a hepatic scar can also be caused by a viral infection such as hepatitis. Lipocytes appear to play a major role in hepatic cirrhosis. Other fibrotic disorders implicated include atherosclerosis.

Mesangial cell proliferative disorders refer to disorders brought about by abnormal proliferation of mesangial cells. Mesangial proliferative disorders include various human renal diseases such as glomerulonephritis, diabetic nephropathy and malignant nephrosclerosis as well as such disorders as thrombotic microangiopathy syndromes, transplant rejection, and glomerulopathies. The RTK PDGFR has been implicated in the maintenance of mesangial cell proliferation. Floege *et al.*, 1993, Kidney International 43:47S-54S.

Many cancers are cell proliferative disorders and, as noted previously, PKs have been associated with cell proliferative disorders. Thus, it is not surprising that PKs such as, for example, members of the RTK family have been associated with the development of cancer. Some of these receptors, like EGFR (Tuzi *et al.*, 1991, Br. J. Cancer 63:227-233, Torp *et al.*, 1992, APMIS 100:713-719) HER2/neu (Slamon *et al.*, 1989, Science 244:707-712) and PDGF-R (Kumabe *et al.*, 1992, Oncogene, 7:627-633) are over-expressed in many tumors and/or persistently activated by autocrine loops. In fact, in the most common and severe cancers these receptor over-expressions (Akbasak and Suner-Akbasak *et al.*, 1992, J. Neurol. Sci., 111:119-133, Dickson *et al.*, 1992, Cancer Treatment Res. 61:249-273, Korc *et al.*, 1992, J. Clin. Invest. 90:1352-1360) and autocrine loops (Lee and Donoghue, 1992, J. Cell. Biol., 118:1057-1070, Korc *et al.*, supra, Akbasak and Suner-Akbasak *et al.*, supra) have been demonstrated. For example, EGFR has been associated with squamous cell carcinoma, astrocytoma, glioblastoma, head and neck cancer, lung cancer and bladder cancer. HER2 has been associated with breast, ovarian, gastric, lung, pancreas and bladder cancer. PDGFR has

been associated with glioblastoma and melanoma as well as lung, ovarian and prostate cancer. The RTK c-met has also been associated with malignant tumor formation. For example, c-met has been associated with, among other cancers, colorectal, thyroid, pancreatic, gastric and hepatocellular carcinomas and lymphomas. Additionally c-met has been linked to leukemia. Over-expression of the c-met gene has also been detected in patients with Hodgkins disease and Burkitts disease.

IGF-IR, in addition to being implicated in nutritional support and in type-II diabetes, has also been associated with several types of cancers. For example, IGF-I has been implicated as an autocrine growth stimulator for several tumor types, e.g. human breast cancer carcinoma cells (Arteaga *et al.*, 1989, J. Clin. Invest. 84:1418-1423) and small lung tumor cells (Macauley *et al.*, 1990, Cancer Res., 50:2511-2517). In addition, IGF-I, while integrally involved in the normal growth and differentiation of the nervous system, also appears to be an autocrine stimulator of human gliomas. Sandberg-Nordqvist *et al.*, 1993, Cancer Res. 53:2475-2478. The importance of IGF-IR and its ligands in cell proliferation is further supported by the fact that many cell types in culture (fibroblasts, epithelial cells, smooth muscle cells, T-lymphocytes, myeloid cells, chondrocytes and osteoblasts (the stem cells of the bone marrow)) are stimulated to grow by IGF-I. Goldring and Goldring, 1991, Eukaryotic Gene Expression,1:301-326. Baserga and Coppola suggest that IGF-IR plays a central role in the mechanism of transformation and, as such, could be a preferred target for therapeutic interventions for a broad spectrum of human malignancies. Baserga, 1995, Cancer Res., 55:249-252, Baserga, 1994, Cell 79:927-930, Coppola *et al.*, 1994, Mol. Cell. Biol., 14:4588-4595.

STKs have been implicated in many types of cancer including, notably, breast cancer (Cance, *et al.*, Int. J. Cancer, 54:571-77 (1993)).

The association between abnormal PK activity and disease is not restricted to cancer. For example, RTKs have been associated with diseases such as psoriasis, diabetes mellitus, endometriosis, angiogenesis, atheromatous plaque development, Alzheimer's disease, restenosis, von Hippel-Lindau disease, epidermal hyperproliferation, neurodegenerative diseases, age-related macular degeneration and hemangiomas. For example, EGFR has been indicated in corneal and dermal wound healing. Defects in Insulin-R and IGF-1R are indicated in type-II diabetes mellitus. A more complete correlation between specific RTKs and their therapeutic indications is set forth in Plowman *et al.*, 1994, DN&P 7:334-339.

As noted previously, not only RTKs but CTKs including, but not limited to, src, abl, fps, yes, fyn, lyn, lck, blk, hck, fgr and yrk (reviewed by Bolen *et al.*, 1992, FASEB J., 6:3403-3409) are involved in the proliferative and metabolic signal transduction pathway and thus could be expected, and have been shown, to be involved in many PTK-mediated disorders to which the

present invention is directed. For example, mutated src (v-src) has been shown to be an oncoprotein ($pp60^{v\text{-}src}$) in chicken. Moreover, its cellular homolog, the proto-oncogene $pp60^{c\text{-}src}$ transmits oncogenic signals of many receptors. Over-expression of EGFR or HER2/neu in tumors leads to the constitutive activation of $pp60^{c\square src}$, which is characteristic of malignant cells but absent in normal cells. On the other hand, mice deficient in the expression of c-src exhibit an osteopetrotic phenotype, indicating a key participation of c-src in osteoclast function and a possible involvement in related disorders.

Similarly, Zap70 has been implicated in T-cell signaling which may relate to autoimmune disorders.

STKs have been associated with inflamation, autoimmune disease, immunoresponses, and hyperproliferation disorders such as restenosis, fibrosis, psoriasis, osteoarthritis and rheumatoid arthritis.

PKs have also been implicated in embryo implantation. Thus, the compounds of this invention may provide an effective method of preventing such embryo implantation and thereby be useful as birth control agents. Additional disorders which may be treated or prevented using the compounds of this invention are immunological disorders such as autoimmune disease, AIDS and cardiovasular disorders such as atherosclerosis.

Finally, both RTKs and CTKs are currently suspected as being involved in hyperimmune disorders.

Examples of the effect of a number of exemplary compounds of this invention on several PTKs are shown in Table 2 below. The compounds and data presented are not to be construed as limiting the scope of this invention in any manner whatsoever.

B. Cyclooxygenase-2 inhibitor or COX-2 inhibitor or cyclooxygenase-2 inhibitor includes agents that specifically inhibit a class of enzymes, cyclooxygenase-2, with less significant inhibition of cyclooxygenase-1. Preferably, it includes compounds which have a cyclooxygenase-2 IC_{50} of less than about 0.2 μM, and also have a selectivity ratio of cyclooxygenase-2 inhibition over cyclooxygenase-1 inhibition of at least 50, and more preferably of at least 100. Even more preferably, the compounds have a cyclooxygenase-1 IC_{50} of greater than about 1 μM, and more preferably of greater than 10 μM.

Studies indicate that prostaglandins synthesized by cyclooxygenases play a critical role in the initiation and promotion of cancer. Moreover, COX-2 is overexpressed in neoplastic lesions of the colon, breast, lung, prostate, esophagus, pancreas, intestine, cervix, ovaries, urinary bladder, and head & neck. In several in vitro and animal models, COX-2 inhibitors have inhibited tumor growth and metastasis.

In addition to cancers *per se*, COX-2 is also expressed in the angiogenic vasculature within and adjacent to hyperplastic and neoplastic lesions indicating that COX-2 plays a role in angiogenesis. In both the mouse and rat, COX-2 inhibitors markedly inhibited bFGF-induced neovascularization. The utility of COX-2 inhibitors as chemopreventive, antiangiogenic and chemotherapeutic agents is described in the literature (Koki et al., Potential utility of COX-2 inhibitors in chemoprevention and chemotherapy. Exp. Opin. Invest. Drugs (1999) 8(10) pp. 1623-1638, hereby incorporated by reference). Amplification and/or overexpression of HER-2/nue (ErbB2) occurs in 20-30% of human breast and ovarian cancers as well as in 5-15% of gastric and esophageal cancers and is associated with poor prognosis. Additionally, it has been recently discovered in vitro that COX-2 expression is upregulated in cells overexpressing the HER-2/neu oncogene. (Subbaramaiah et al., Increased expression of cyclooxygenase-2 in HER-2/neu-overexpressing breast cancer. Cancer Research (submitted 1999), hereby incorporated by reference). In this study, markedly increased levels of PGE_2 production, COX-2 protein and mRNA were detected in HER-2/neu transformed mammary epithelial cells compared to a non-transformed partner cell line. Products of COX-2 activity, i.e., prostaglandins, stimulate proliferation, increase invasiveness of malignant cells, and enhance the production of vascular endothelial growth factor, which promotes angiogenesis. Further, HER-2/neu induces the production of angiogenic factors such as vascular endothelial growth factor.

Consequently, the administration of a COX-2 inhibitor in combination with an anti HER-2/neu antibodies such as trastuzumab (Herceptin®) and other therapies directed at inhibiting HER-2/neu is contemplated to treat cancers in which HER-2/neu is overexpressed.

Also, it is contemplated that COX-2 levels are elevated in tumors with amplification and/or overexpression of other oncogenes including but not limited to c-*myc*, N-*myc*, L-*myc*, K-*ras*, H-*ras*, N-*ras*. Products of COX-2 activity stimulate cell proliferation, inhibit immune surveillance, increase invasiveness of malignant cells, and promote angiogenesis. Consequently, the administration of a protein kinase inhibitor in combination with a COX-2 inhibitor of the present invention is useful to prevent or treat cancers in which oncogenes are overexpressed.

Specific COX-2 inhibitors are useful for the treatment of cancer (WO98/16227) and in several animal models reduce angiogenesis driven by various growth factors (WO98/22101). Anti-angiogenesis was achieved with a COX-2 inhibitor in rats implanted with bFGF, vascular endothelium growth factor (VEGF) or carrageenan, proteins with well-known angiogenic

properties. (Masferrer, et al., 89th Annual Meeting of the American Association for Cancer Research, March 1998.)

Pharmaceutical composition and Administration

A compound of the present invention or a pharmaceutically acceptable salt thereof, can be administered as such to a human patient or can be administered in pharmaceutical compositions in which the foregoing materials are mixed with suitable carriers or excipient(s). Techniques for formulation and administration of drugs may be found in "Remington's Pharmacological Sciences," Mack Publishing Co., Easton, PA., latest edition.

As used herein, "administer" or "administration" refers to the delivery of a compound of Formula (I) or a pharmaceutically acceptable salt thereof or of a pharmaceutical composition containing a compound of Formula (I) or a pharmaceutically acceptable salt thereof of this invention to an organism for the purpose of prevention or treatment of a PK-related disorder.

Suitable routes of administration may include, without limitation, oral, rectal, topical, transmucosal or intestinal administration or intramuscular, subcutaneous, intramedullary, intrathecal, direct intraventricular, intravenous, intravitreal, intraperitoneal, intranasal, or intraocular injections. The preferred routes of administration are oral and parenteral.

Alternatively, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into a solid tumor, often in a depot or sustained release formulation.

Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with tumor-specific antibody. The liposomes will be targeted to and taken up selectively by the tumor.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the compounds of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, lozenges, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient. Pharmaceutical preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding other suitable auxiliaries if desired, to obtain tablets or dragee cores. Useful excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol, cellulose preparations such as, for example, maize starch, wheat starch, rice starch and potato starch and other materials such as gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinyl- pyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid. A salt such as sodium alginate may also be used.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with a filler such as lactose, a binder such as starch, and/or a lubricant such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. Stabilizers may be added in these formulations, also.

The capsules may be packaged into brown glass or plastic bottles to protect the active compound from light. The containers containing the active compound capsule formulation must be stored at controlled room temperature (15-30°C).

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray using a pressurized pack or a nebulizer and a suitable propellant, e.g., without limitation, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetra- fluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be controlled by providing a valve to deliver a metered amount. Capsules and cartridges of, for example, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may also be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulating materials such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of a water soluble form, such as, without limitation, a salt, of the active compound. Additionally, suspensions of the active compounds may be prepared in a lipophilic vehicle. Suitable lipophilic vehicles include fatty oils such as sesame oil, synthetic fatty acid esters such as ethyl oleate and triglycerides, or materials such as liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers and/or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

In addition to the fomulations described previously, the compounds may also be formulated as depot preparations. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. A compound of this invention may be formulated for this route of administration with suitable polymeric or hydrophobic materials (for instance, in an emulsion with a pharamcologically acceptable oil), with ion exchange resins, or as a sparingly soluble derivative such as, without limitation, a sparingly soluble salt.

A non-limiting example of a pharmaceutical carrier for the hydrophobic compounds of the invention is a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer and an aqueous phase such as the VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant Polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:D5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system dissolves hydrophobic compounds well, and itself produces low toxicity upon systemic administration. Naturally, the proportions of such a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of Polysorbate 80, the fraction size of polyethylene glycol may be varied, other biocompatible polymers may replace polyethylene glycol, *e.g.*, polyvinyl pyrrolidone, and other sugars or polysaccharides may substitute for dextrose.

Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. In addtion, certain organic solvents such as dimethylsulfoxide also may be employed, although often at the cost of greater toxicity.

Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

The pharmaceutical compositions herein also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include, but are not limited to, calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Many of the PK modulating compounds of the invention may be provided as physiologically acceptable salts wherein the claimed compound may form the negatively or the positively charged species. Examples of salts in which the compound forms the positively charged moiety include, without limitation, quaternary ammonium (defined elsewhere herein), salts such as the hydrochloride, sulfate, carbonate, lactate, tartrate, malate, maleate, succinate wherein the nitrogen atom of the quaternary ammonium group is a nitrogen of the selected compound of this invention which has reacted with the appropriate acid. Salts in which a

compound of this invention forms the negatively charged species include, without limitation, the sodium, potassium, calcium and magnesium salts formed by the reaction of a carboxylic acid group in the compound with an appropriate base (e.g. sodium hydroxide (NaOH), potassium hydroxide (KOH), Calcium hydroxide ($Ca(OH)_2$), etc.).

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an amount sufficient to achieve the intended purpose, e.g., the modulation of PK activity or the treatment or prevention of a PK-related disorder.

More specifically, a therapeutically effective amount means an amount of compound effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any compound used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from cell culture assays. Then, the dosage can be formulated for use in animal models so as to achieve a circulating concentration range that includes the IC_{50} as determined in cell culture (i.e., the concentration of the test compound which achieves a half-maximal inhibition of the PK activity). Such information can then be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the compounds described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., by determining the IC_{50} and the LD_{50} (both of which are discussed elsewhere herein) for a subject compound. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the active species which are sufficient to maintain the kinase modulating effects. These plasma levels are referred to as minimal effective concentrations (MECs). The MEC will vary for each compound but can be estimated from *in vitro* data, e.g., the concentration necessary to achieve 50-90% inhibition of a kinase may be ascertained using the assays described herein. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals can also be determined using MEC value. Compounds should be administered using a regimen that maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%.

At present, the therapeutically effective amounts of compounds of Formula (I) may range from approximately 0.25 mg/m^2 to 1500 mg/m^2 per day; preferably about 3 mg/m^2/day. Even more preferably 50mg/qm qd till 400 mg/qd.

Therapeutically effective amounts of cyclooxygenase inhibitor is from about 0.1 mg to about 10,000 mg for the treatment of the above conditions, with preferred levels of about 1.0 mg to about 1,000 mg.

The amount of active ingredient that may be combined with other anticancer agents to produce a single dosage form will vary depending upon the host treated and the particular mode of administration.

In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration and other procedures known in the art may be employed to determine the correct dosage amount and interval.

The amount of a composition administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

The compositions may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accompanied by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or of human or veterinary administration. Such notice, for example, may be of the labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition. Suitable conditions indicated on the label may include treatment of a tumor, inhibition of angiogenesis, treatment of fibrosis, diabetes, and the like.

It is also an aspect of this invention that a compound described herein, or its salt or prodrug, might be combined with other chemotherapeutic agents for the treatment of the diseases and disorders discussed above. For instance, a compound, salt or prodrug of this

invention might be combined with alkylating agents such as fluorouracil (5-FU) alone or in further combination with leukovorin; or other alkylating agents such as, without limitation, other pyrimidine analogs such as UFT, capecitabine, gemcitabine and cytarabine, the alkyl sulfonates, e.g., busulfan (used in the treatment of chronic granulocytic leukemia), improsulfan and piposulfan; aziridines, e.g., benzodepa, carboquone, meturedepa and uredepa; ethyleneimines and methylmelamines, e.g., altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolmelamine; and the nitrogen mustards, e.g., chlorambucil (used in the treatment of chronic lymphocytic leukemia, primary macroglobulinemia and non-Hodgkin's lymphoma), cyclophosphamide (used in the treatment of Hodgkin's disease, multiple myeloma, neuroblastoma, breast cancer, ovarian cancer, lung cancer, Wilm's tumor and rhabdomyosarcoma), estramustine, ifosfamide, novembrichin, prednimustine and uracil mustard (used in the treatment of primary thrombocytosis, non-Hodgkin's lymphoma, Hodgkin's disease and ovarian cancer); and triazines, e.g., dacarbazine (used in the treatment of soft tissue sarcoma).

A compound, salt or prodrug of this invention can also be used in combination with other antimetabolite chemotherapeutic agents such as, without limitation, folic acid analogs, e.g. methotrexate (used in the treatment of acute lymphocytic leukemia, choriocarcinoma, mycosis fungiodes breast cancer, head and neck cancer and osteogenic sarcoma) and pteropterin; and the purine analogs such as mercaptopurine and thioguanine which find use in the treatment of acute granulocytic, acute lymphocytic and chronic granulocytic leukemias.

It is contemplated that a compound, salt or prodrug of this invention can also be used in combination with natural product based chemotherapeutic agents such as, without limitation, the vinca alkaloids, e.g., vinblastin (used in the treatment of breast and testicular cancer), vincristine and vindesine; the epipodophylotoxins, e.g., etoposide and teniposide, both of which are useful in the treatment of testicular cancer and Kaposi's sarcoma; the antibiotic chemotherapeutic agents, e.g., daunorubicin, doxorubicin, epirubicin, mitomycin (used to treat stomach, cervix, colon, breast, bladder and pancreatic cancer), dactinomycin, temozolomide, plicamycin, bleomycin (used in the treatment of skin, esophagus and genitourinary tract cancer); and the enzymatic chemotherapeutic agents such as L-asparaginase.

In addition to the above, a compound, salt or prodrug of this invention could also be used in combination with the platinum coordination complexes (cisplatin, etc.); substituted ureas such as hydroxyurea; methylhydrazine derivatives, e.g., procarbazine; adrenocortical suppressants, e.g., mitotane, aminoglutethimide; and hormone and hormone antagonists such as the adrenocorticosteriods (e.g., prednisone), progestins (e.g., hydroxyprogesterone

caproate); estrogens (e.g., diethylstilbesterol); antiestrogens such as tamoxifen; androgens, e.g., testosterone propionate; and aromatase inhibitors such as anastrozole.

Other antineoplastic agents known in the art can be used along with the combination therapy of the present invention. Examples of these antineoplastic agents and other are give in Applicants' United States patent application Serial No. 60/113,786, filed December 23, 1998, and PCT Application Publication No. WO 00/38716 the disclosure of which is incorporated herein by reference.

Finally, it is also contemplated that the combination of a compound of this invention will be effective in combination with mitoxantrone or paclitaxel for the treatment of solid tumor cancers or leukemias such as, without limitation, acute myelogenous (non-lymphocytic) leukemia.

EXAMPLES

Synthetic Examples

The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. They should not be considered as limiting the scope of the invention, but merely as being illustrative and representative thereof.

SYNTHESIS OF PROTEIN KINASE INHIBITOR OF THE PRESENT INVENTION-COMPOUNDS OF FORMULA (I)

General Synthetic Procedure:

The following general methodology may be employed to prepare the compounds of this invention:

The appropriately substituted 2-oxindole (1 equiv.), the appropriately substituted aldehyde (1.2 equiv.) and a base (0.1 equiv.) are mixed in a solvent (1-2 ml/mmol 2-oxindole) and the mixture is then heated for from about 2 to about 12 hours. After cooling, the precipitate that forms is filtered, washed with cold ethanol or ether and vacuum dried to give the solid product. If no precipitate forms, the reaction mixture is concentrated and the residue is triturated with dichloromethane/ether, the resulting solid is collected by filtration and then dried. The product may optionally be further purified by chromatography.

The base may be an organic or an inorganic base. If an organic base is used, preferably it is a nitrogen base. Examples of organic nitrogen bases include, but are not limited to, diisopropylamine, trimethylamine, triethylamine, aniline, pyridine, 1,8-diazabicyclo[5.4.1]undec-7-ene, pyrrolidine and piperidine.

Examples of inorganic bases are, without limitation, ammonia, alkali metal or alkaline earth hydroxides, phosphates, carbonates, bicarbonates, bisulfates and amides. The alkali metals include, lithium, sodium and potassium while the alkaline earths include calcium, magnesium and barium.

In a presently preferred embodiment of this invention, when the solvent is a protic solvent, such as water or alcohol, the base is an alkali metal or an alkaline earth inorganic base, preferably, a alkali metal or an alkaline earth hydroxide.

It will be clear to those skilled in the art, based both on known general principles of organic synthesis and on the disclosures herein which base would be most appropriate for the reaction contemplated.

The solvent in which the reaction is carried out may be a protic or an aprotic solvent, preferably it is a protic solvent. A "protic solvent" is a solvent which has hydrogen atom(s) covalently bonded to oxygen or nitrogen atoms which renders the hydrogen atoms appreciably acidic and thus capable of being "shared" with a solute through hydrogen bonding. Examples of protic solvents include, without limitation, water and alcohols.

An "aprotic solvent" may be polar or non-polar but, in either case, does not contain acidic hydrogens and therefore is not capable of hydrogen bonding with solutes. Examples, without limitation, of non-polar aprotic solvents, are pentane, hexane, benzene, toluene, methylene

chloride and carbon tetrachloride. Examples of polar aprotic solvents are chloroform, tetrahydrofuran, dimethylsulfoxide and dimethylformamide.

In a presently preferred embodiment of this invention, the solvent is a protic solvent, preferably water or an alcohol such as ethanol.

The reaction is carried out at temperatures greater than room temperature. The temperature is generally from about 30° C to about 150° C, preferably about 80°C to about 100° C, most preferable about 75° C to about 85° C, which is about the boiling point of ethanol. By "about" is meant that the temperature range is preferably within 10 degrees Celcius of the indicated temperature, more preferably within 5 degrees Celcius of the indicated temperature and, most preferably, within 2 degrees Celcius of the indicated temperature. Thus, for example, by "about 75° C" is meant 75° C ± 10° C, preferably 75° C ± 5° C and most preferably, 75° C ± 2° C.

2-Oxindoles and aldehydes, may be readily synthesized using techniques well known in the chemical arts. It will be appreciated by those skilled in the art that other synthetic pathways for forming the compounds of the invention are available and that the following is offered by way of example and not limitation.

Method A: Formylation of pyrroles

$POCl_3$ (1.1 equiv.) is added dropwise to dimethylformamide (3 equiv.)at -10° C followed by addition of the appropriate pyrrole dissolved in dimethylformamide. After stirring for two hours, the reaction mixture is diluted with H_2O and basified to pH 11 with 10 N KOH. The precipitate which forms is collected by filtration, washed with H_2O and dried in a vacuum oven to give the desired aldehyde.

Method B: Saponification of pyrrolecarboxylic acid esters

A mixture of a pyrrolecarboxylic acid ester and KOH (2 – 4 equiv.) in EtOH is refluxed until reaction completion is indicated by thin layer chromatography (TLC). The cooled reaction mixtrue is acidified to pH 3 with 1 N HCl. The precipitate which forms is collected by filtration, washed with H_2O and dried in a vacuum oven to give the desired pyrrolecarboxylic acid.

Method C: Amidation

To a stirred solution of a pyrrolecarboxylic acid dissolved in dimethylformamide(0.3M) is added 1-ethyl-3-(3-dimethylamino- propyl)carbodiimide (1.2 equiv.), 1-hydroxybenzotriazole (1.2 equiv.), and triethylamine (2 equiv.). The appropriate amine is added (1 equiv.) and the reaction stirred until completion is indicated by TLC. Ethyl acetate is then added to the reaction mixture and the solution washed with saturated $NaHCO_3$ and brine

(with extra salt), dried over anhydrous $MgSO_4$ and concentrated to afford the desired amide.

Method D: Condensation of aldehydes and oxindoles containing carboxylic acid substituents

A mixture of the oxindole (1 equivalent), 1 equivalent of the aldehyde and 1 – 3 equivalents of piperidine (or pyrrolidine) in ethanol (0.4 M) is stirred at 90-100° C until reaction completion is indicated by TLC. The mixture is then concentrated and the residue acidified with 2N HCl. The precipitate that forms is washed with H_2O and EtOH and then dried in a vacuum oven to give the product.

Method E: Condensation of aldehydes and oxindoles not containing carboxylic acid substituents

A mixture of the oxindole (1 equivalent), 1 equivalent of the aldehyde and 1 – 3 equivalents of piperidine (or pyrrolidine) in ethanol (0.4 M) is stirred at 90-100° C until reaction completion is indicated by TLC. The mixture is cooled to room temperature and the solid which forms is collected by vacuum filtration, washed with ethanol and dried to give the product. If a precipitate does not form upon cooling of the reaction mixture, the mixture is concentrated and purified by column chromatography.

C. Examples of oxindole syntheses

The following examples of the synthesis of representative oxindoles is not to be construed as limiting the scope of this invention in any manner whatsoever. Alternate routes to the oxindoles shown as well as other oxindoles to be used to make the compounds of this invention will become apparent to those skilled in the art based on the following disclosures. Such syntheses and oxindoles are within the scope and spirit of this invention.

5-Amino-2-oxindole

5-Nitro-2-oxindole (6.3 g) was hydrogenated in methanol over 10% palladium on carbon to give 3.0 g (60% yield) of the title compound as a white solid.

5-Bromo-2-oxindole

2-Oxindole (1.3 g) in 20 mL acetonitrile was cooled to –10 °C and 2.0 g N-bromosuccinimide was slowly added with stirring. The reaction was stirred for 1 hour at -10 °C and 2 hours at 0 °C. The precipitate was collected, washed with water and dried to give 1.9 g (90 % yield) of the title compound.

4-Methyl-2-oxindole

Diethyl oxalate (30 mL) in 20 mL of dry ether was added with stirring to 19 g of potassium ethoxide suspended in 50 mL of dry ether. The mixture was cooled in an ice bath and 20 mL of 3-nitro-o-xylene in 20 mL of dry ether was slowly added. The thick dark red mixture was heated to reflux for 0.5 hr, concentrated to a dark red solid, and treated with 10%

sodium hydroxide until almost all of the solid dissolved. The dark red mixture was treated with 30% hydrogen peroxide until the red color changed to yellow. The mixture was treated alternately with 10% sodium hydroxide and 30% hydrogen peroxide until the dark red color was no longer present. The solid was filtered off and the filtrate acidified with 6N hydrochloric acid. The resulting precipitate was collected by vacuum filtration, washed with water, and dried under vacuum to give 9.8 g (45% yield) of 2-methyl-6-nitrophenylacetic acid as an off-white solid. The solid was hydrogenated in methanol over 10 % palladium on carbon to give 9.04 g of the title compound as a white solid.

7-Bromo-5-chloro-2-oxindole

5-Chloro-2-oxindole (16.8 g) and 19.6 g of N-bromosuccinimide were suspended in 140 mL of acetonitrile and refluxed for 3 hours. Thin layer chromatography (silica, ethyl acetate) at 2 hours of reflux showed 5-chloro-2-oxindole or N-bromosuccinimide (Rf 0.8), product (Rf 0.85) and a second product (Rf 0.9) whose proportions did not change after another hour of reflux. The mixture was cooled to 10 °C, the precipitate was collected by vacuum filtration, washed with 25 mL of ethanol and sucked dry for 20 minutes in the funnel to give 14.1 g of wet product (56 % yield). The solid was suspended in 200 mL of denatured ethanol and slurry-washed by stirring and refluxing for 10 minutes. The mixture was cooled in an ice bath to 10 °C. The solid product was collected by vacuum filtration, washed with 25 mL of ethanol and dried under vacuum at 40 °C to give 12.7 g (51% yield) of 7-bromo-5-chloro-2-oxindole.

5-Fluoro-2-oxindole

5-Fluoroisatin (8.2 g) was dissolved in 50 mL of hydrazine hydrate and refluxed for 1.0 hr. The reaction mixtures were then poured in ice water. The precipitate was then filtered, washed with water and dried in a vacuum oven to afford the title compound.

5-Nitro-2-oxindole

2-Oxindole (6.5 g) was dissolved in 25 mL concentrated sulfuric acid and the mixture maintained at –10 to -15 °C while 2.1 mL of fuming nitric acid was added dropwise. After the addition of the nitric acid the reaction mixture was stirred at 0 °C for 0.5 hr and poured into ice-water. The precipitate was collected by filtration, washed with water and crystallized from 50% acetic acid. The crystalline product was then filtered, washed with water and dried under vacuum to give 6.3 g (70%) of 5-nitro-2-oxindole.

5-Aminosulfonyl-2-oxindole

To a 100 mL flask charged with 27 mL of chlorosulfonic acid was added slowly 13.3 g of 2-oxindole. The reaction temperature was maintained below 30 °C during the addition.

After the addition, the reaction mixture was stirred at room temperature for 1.5 hr, heated to 68 °C for 1 hr, cooled, and poured into water. The precipitate was washed with water and dried in a vacuum oven to give 11.0 g of 5-chlorosulfonyl-2-oxindole (50% yield) which was used without further purification.

5-Chlorosulfonyl-2-oxindole (2.1 g) was added to 10 mL of ammonium hydroxide in 10 mL of ethanol and stirred at room temperature overnight. The mixture was concentrated and the solid collected by vacuum filtration to give 0.4 g (20% yield) of the title compound as an off-white solid.

5-Isopropylaminosulfonyl-2-oxindole

To a 100 mL flask charged with 27 mL chlorosulfonic acid was slowly added 13.3 g 2-oxindole. The reaction temperature was maintained below 30° C during the addition. The reaction mixture was stirred at room temperature for 1.5 hour, heated to 68° C for 1 hour, cooled, and poured into water. The precipitate which formed was filtered, washed with water and dried in a vacuum oven to give 11.0 g (50%) of 5-chlorosulfonyl-2-oxindole which was used without further purification.

A suspension of 3 g 5-chlorosulfonyl-2-oxindole, 1.15 g isopropylamine and 1.2 mL of pyridine in 50 mL of dichloromethane was stirred at room temperature for 4 hours during which time a white solid formed. The solid was collected by vacuum filtration, slurry-washed with hot ethanol, cooled, collected by vacuum filtration and dried under vacuum at 40° C overnight to give 1.5 g (45%) of 5-isopropylaminosulfonyl-2-oxindole.

^{1}HNMR (360 MHz, DMSO-d6) δ 10.69 (s, br, 1H, NH), 7.63 (dd, J = 2 and 8 Hz, 1H), 7.59 (d, J = 2 Hz, 1H), 7.32 (d, J = 7 Hz, 1H, N*H*-SO_2-), 6.93 (d, J = 8 Hz, 1H), 3.57 (s, 2H), 3.14-3.23 (m, 1H, C*H*-$(CH_3)_2$), 0.94 (d, J = 7 Hz, 6H, 2xCH_3).

5-Phenylaminosulfonyl-2-oxindole

A suspension of 5-chlorosulfonyl-2-oxindole (1.62 g, 7 mmol), aniline (0.782 mL, 8.4 mmol) and pyridine (1 mL) in dichloromethane (20 ml) was stirred at room temperature for 4 hours. The reaction mixture was diluted with ethyl acetate (300 mL) and acidified with 1N hydrochloric acid (16 mL). The organic layer was washed with sodium bicarbonate and brine, dried and concentrated. The residue was washed with ethanol (3 mL) and then chromatographed on silica gel eluting with methanol/ dichloromethane 1:9 to give of 5-phenylaminosulfonyl-2-oxindole.

^{1}HNMR (360 MHz, DMSO-d6) δ 10.71 (s, br, 1H, NH), 10.10 (s, br, 1H, NH), 7.57-7.61 (m, 2H), 7.17-7.22 (m, 2H), 7.06-7.09 (m, 2H), 6.97-7.0 (m, 1H), 6.88 (d, J = 8.4 Hz, 1H), 3.52 (s, 2H).

2-Oxo-2,3-dihydro-1H-indole-5-sulfonic acid pyridin-3-ylamide

A solution of 5-chlorosufonyl-2-oxindole (3 g) and 3-aminopyridine (1.46g) in pyridine (15 mL) was stirred at room temperature overnight at which time a brown solid was present. The solid was filtered, washed with ethanol and dried under vacuum to yield 1.4 g (38%) of 2-oxo-2,3-dihydro-1H-indole-5-sulfonic acid pyridin-3-ylamide.

^{1}HNMR (360 MHz, DMSO-d6) δ 10.74 (s, 1H, NH), 10.39 (s, 1H, SO_2NH), 8.27-8.28 (d, 1H), 8.21-8.23 (m, 1H), 7.59-7.62 (m, 2H), 7.44-7.68 (m, 1H), 7.24-7.28 (m, 1H), 6.69-6.71 (d, 1H), 3.54 (s, 2H).

MS *m/z* (APCI+) 290.2.

5-Phenyloxindole

5-Bromo-2-oxindole (5 g, 23.5 mmol) was dissolved in 110 mL toluene and 110 mL ethanol with stirring and a little heat. Tetrakis(triphenylphosphine)palladium(0) (1.9 g, 1.6 mmol) was added followed by 40 mL (80 mmol) 2M aqueous sodium carbonate. To this mixture was added benzene boronic acid (3.7 g, 30.6 mmol) and the mixture was heated in a 100° C oil bath for 12 hours. The reaction was cooled, diluted with ethyl acetate (500 mL), washed with saturated sodium bicarbonate (200 mL), water (200 mL), 1N HCl (200 mL) and brine (200 mL). The organic layer was dried over magnesium sulfate and concentrated to afford a brown solid. Trituration with dichloromethane afforded 3.8 g (77%) of 5-phenyl-2-oxindole as a tan solid.

^{1}H NMR (360 MHz, DMSO-d6) δ 10.4 (br s, 1H, NH), 7.57 (dd, J = 1.8 and 7.2 Hz, 1H), 7.5 to 7.35 (m, 5H), 7.29 (m, 1H), 6.89 (d, J = 8.2 Hz, 1H), 3.51 (s, 2H, CH_2CO).

MS *m/z* 209 [M^+].

In similar fashion, the following oxindoles can be prepared:

6-(3,5-Dichlorophenyl)-1,3-dihydroindol-2-one

^{1}H NMR (360 MHz, DMSO-d6) δ 10.46 (br, 1H, NH), 7.64 (d, J = 1.8 Hz, 2H), 7.57 (m, 1H), 7.27 (m, 2H), 7.05 (d, J = 1.1 Hz, 1H), 3.5 (s, 2H).

MS-EI *m/z* 277/279 $[M]^+$.

6-(4-Butylphenyl)-1,3-dihydroindol-2-one

^{1}H NMR (360 MHz, DMSO-d6) δ 10.39 (s, 1H, NH), 7.49 (d, J = 8.0 Hz, 2H), 7.25 (d, J = 8 Hz, 3H), 7.17 (dd, J = 1.5 and 7.8 Hz, 1H), 6.99 (d, J = 1.5 Hz, 1H), 3.48 (s, 2H, CH_2CO), 2.60 (t, J = 7.5 Hz, 2Hz, CH_2CH_3), 1.57 (m, 2H, CH_2), 1.32 (m, 2H, CH_2), 0.9 (t, J = 7.5 Hz, 3H, CH_3).

6-(5-Isopropyl-2-methoxyphenyl)-1,3-dihydroindol-2-one

^{1}H NMR (360 MHz, DMSO-d6) δ 10.29 (br s, 1H, NH), 7.16-7.21 (m, 2H), 7.08 (d, *J* = 2.4 Hz, 1H), 6.97-7.01 (m, 2H), 6.89 (d, *J* = 0.8 Hz, 1H), 3.71 (s, 3H, OC*H*$_3$), 3.47 (s, 2H, CH_2CO), 2.86 (m, 1H, C*H*(CH$_3$)$_2$), 1.19 (d, *J* = 6.8 Hz, 6H, CH(C*H*$_3$)$_2$).

MS-EI *m/z* 281 $[M]^+$.

6-(4-Ethylphenyl)-1,3-dihydroindol-2-one

^{1}H NMR (360 MHz, DMSO-d6) δ 10.39 (br s, 1H, N*H*), 7.50 (d, *J* = 8.2 Hz, 2H), 7.28 (d, *J* = 8.2 Hz, 2H), 7.25 (d, *J* = 7.5 Hz, 1H), 7.17 (dd, *J* = 1.6 & 7.5 Hz, 1H), 6.99 (d, *J* = 1.6 Hz, 1H), 3.48 (s, 2H, CH_2CO), 2.63 (q, *J* = 7.6 Hz, 2H, C*H*$_2$CH$_3$), 1.20 (t, *J* = 7.6 Hz, 3H, CH_2CH_3).

MS-EI *m/z* 237 $[M]^+$.

6-(3-Isopropylphenyl)-1,3-dihydroindol-2-one

^{1}H NMR (360 MHz, DMSO-d6) δ 10.37 (br s, 1H, N*H*), 7.43 (m, 1H), 7.35-7.39 (m, 1H), 7.17-7.27 (m, 3H), 7.01 (d, *J* = 1.8 Hz, 1H), 3.49 (s, 2H, C*H*$_2$CO), 2.95 (m, 1H, C*H*(CH$_3$)$_2$), 1.24 (d, *J* = 6.8 Hz, 6H, CH(C*H*$_3$)$_2$).

MS-EI *m/z* 251 $[M]^+$.

6-(2,4-Dimethoxyphenyl)-1,3-dihydroindol-2-one

^{1}H NMR (360 MHz, DMSO-d6) δ 10.28 (br s, 1H, N*H*), 7.17 (m, 2H), 6.93 (dd, *J* = 1.6 & 7.6 Hz, 1H), 6.86 (d, *J* = 1.6 Hz, 1H), 6.63 (d, *J* = 2.4 Hz, 1H), 6.58 (dd, *J* = 2.4 & 8.5 Hz, 1H), 3.79 (s, 3H, OC*H*$_3$), 3.74 (s, 3H, OC*H*$_3$), 3.45 (s, 2H, C*H*$_2$CO).

MS-EI *m/z* 269 $[M]^+$.

6-Pyridin-3-yl-1,3-dihydroindol-2-one

^{1}H NMR (360 MHz, DMSO-d6) δ 10.51 (s, 1H, NH), 8.81 (d, *J* = 2.5 Hz, 1H), 8.55 (dd, *J* = 1.8 and 5.7 Hz, 1H), 8 (m, 1H), 7.45 (dd, *J* = 5.7 and 9.3 Hz, 1H), 7.3 (m, 2H), 7.05 (s, 1H), 3.51 (s, 2H, CH_2CO).

MS *m/z* 210 $[M]^+$.

2-Oxo-2,3-dihydro-1H-indole-4-carboxylic acid (3-chloro-4-ethoxyphenyl)-amide

To a solution of 4-carboxy-2-oxindole (200 mg, 1.13 mmol) and 3-chloro-4-methoxyphenylamine (178 mg, 1.13 mmol) in dimethylformamide (15 mL) at room temperature was added benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP reagent, 997 mg, 2.26 mmol) followed by 4-dimethylaminopyridine (206 mg, 1.69 mmol). The mixture was stirred at room temperature for 72 hours. The reaction was then diluted with ethyl acetate (300 mL), washed with saturated sodium bicarbonate (100 mL), water, 2N hydrochloric acid (100 mL), water (3x200 mL) and

brine. It was then dried over magnesium sulfate and concentrated. The residue was triturated with ethyl acetate to give 2-oxo-2,3-dihydro-1H-indole-4-carboxylic acid (3-chloro-4-methoxyphenyl)-amide as a pink solid.

^{1}HNMR (360 MHz, DMSO-d6) δ 10.50 (s, br, 1H, NH), 10.12 (s, br, 1H, NH), 7.9 (s, *J* = 2.5 Hz, 1H), 7.62 (dd, *J* = 2.5 & 9 Hz, 1H), 7.38 (d, *J* = 7.6 Hz, 1H), 7.32 (t, *J* = 7.6 Hz, 1H), 7.13 (d, *J* = 9 Hz, 1H), 6.98 (d, *J* = 7.6 Hz, 1H), 3.83 (s, 3H, OCH_3), 3.69 (s, 2H, CH_2).

MS-EI *m/z* 316 $[M]^+$.

4-Carboxy-2-oxindole

A solution of trimethylsilyldiazomethane in hexane (2 M) was added dropwise to a solution of 2.01 g 2-chloro-3-carboxy-nitrobenzene in 20 mL methanol at room temperature until no further gas evolution occurred. Acetic acid was then added to quench excess trimethylsilyldiazomethane. The reaction mixture was evaporated under vacuum and the residue was dried in an oven overnight. The 2-chloro-3-methoxycarbonylnitrobenzene obtained was pure enough for the following reaction.

Dimethyl malonate (6.0 mL) was added to an ice-cold suspension of 2.1 g sodium hydride in 15 mL DMSO. The reaction mixture was stirred at 100° C for 1 hour and then cooled to room temperature. 2-Chloro-3-methoxycarbonylnitrobenzene (2.15 g) was added in one portion and the mixture was heated to 100° C for 1.5 hours. The reaction mixture was then cooled to room temperature, poured into ice water, acidified to pH 5 and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous sodium sulfate and concentrated to give 3.0 g of the dimethyl 2-methoxycarbonyl-6-nitrophenyl- malonate.

Dimethyl 2-methoxycarbonyl-6-nitrophenylmalonate (3.0 g) was refluxed in 50 mL of 6 N hydrochloric acid overnight. The mixture was concentrated to dryness, 20 mL ethanol and 1.1 g of tin(II) chloride were added and the mixture was refluxed for 2 hours. The mixture was filtered through Celite, concentrated and chromatographed on silica gel using ethyl acetate:hexane:acetic acid as eluent to give 0.65 g (37%) of 4-carboxy-2-oxindole as a white solid.

^{1}HNMR (360 MHz, DMSO-d6) δ 12.96 (s, br, 1H, COOH), 10.74 (s, br, 1H, NH), 7.53 (d, *J* = 8Hz, 1H), 7.39 (t, *J* = 8Hz, 1H), 7.12 (d, *J* = 8Hz, 1H), 3.67 (s, 2H).

Example 1

5-(5-Fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide

5-Fluoro-1,3-dihydroindol-2-one (0.54 g, 3.8 mmol) was condensed with 5-formyl-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (2-diethylaminoethyl)amide to give 0.83 g (55%) of the title compound as a yellow green solid.

^{1}HNMR (360 MHz, DMSO-d6) δ 13.66 (s, 1H, N*H*), 10.83 (s, br, 1H, N*H*), 7.73 (dd, *J* = 2.5 & 9.4 Hz, 1H), 7.69 (s, 1H, H-vinyl), 7.37 (t, 1H, CON*H*CH_2CH_2), 6.91 (m, 1H), 6.81-6.85 (m, 1H), 3.27 (m, 2H, C*H*$_2$), 2.51 (m, 6H, 3xCH_2), 2.43 (s, 3H, C*H*$_3$), 2.41 (s, 3H, C*H*$_3$), 0.96 (t, *J* = 6.9 Hz, 6H, N(CH_2C*H*$_3$)$_2$).

MS-EI *m/z* 398 [M^+].

Example 1 (Alternative synthesis)

5-[5-Fluoro-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)-amide

Hydrazine hydrate (55 %, 3000 mL) and 5-fluoroisatin (300 g) were heated to 100 °C. An additional 5-fluoro-isatin (500 g) was added in portions (100 g) over 120 minutes with stirring. The mixture was heated to 110 °C and stirred for 4 hours. The mixture was cooled to room temperature and the solids collected by vacuum filtration to give crude (2-amino-5-fluoro-phenyl)-acetic acid hydrazide (748 g). The hydrazide was suspended in water (700 mL) and the pH of the mixture adjusted to < pH 3 with 12 N hydrochloric acid. The mixture was stirred for 12 hours at room temperature. The solids were collected by vacuum filtration and washed twice with water. The product was dried under vacuum to give 5-fluoro-1,3-dihydro-indol-2-one (600 g, 73 % yield) as as a brown powder. ^{1}H-NMR (dimethylsulfoxide-d_6) δ 3.46 (s, 2H, CH_2), 6.75, 6.95, 7.05 (3 x m, 3H, aromatic), 10.35 (s, 1H, NH). MS *m/z* 152 [M+1].

3,5-Dimethyl-1*H*-pyrrole-2,4-dicarboxylic acid 2-tert-butyl ester 4-ethyl ester (2600 g) and ethanol (7800 mL) were stirred vigorously while 10 N hydrochloric acid (3650 mL) was slowly added. The temperature increased from 25 °C to 35 °C and gas evolution began. The mixture was warmed to 54 °C and stirred with further heating for one hour at which time the temperature was 67 °C. The mixture was cooled to 5 °C and 32 L of ice and water were slowly added with stirring. The solid was collected by vacuum filtration and washed three times with water. The solid was air dried to constant weight to give of 2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid ethyl ester (1418 g, 87 % yield) as a pinkish solid. ^{1}H-NMR (dimethylsulfoxide-d_6) δ 2.10, 2.35 (2xs, 2x3H, 2xCH_3), 4.13 (q, 2H, CH_2), 6.37 (s, 1H, CH), 10.85 (s, 1H, NH). MS *m/z* 167 [M+1].

Dimethylformamide (322 g) and dichloromethane (3700 mL) were cooled in an ice bath to 4 °C and phosphorus oxychloride (684 g) was added with stirring. Solid 2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid ethyl ester (670 g) was slowly added in aliquots over 15 minutes. The maximum temperature reached was 18 °C. The mixture was heated to reflux for one hour, cooled to 10 °C in an ice bath and 1.6 L of ice water was rapidly added with vigorous stirring. The temperature increased to 15 °C. 10 N Hydrochloric acid (1.6 L) was added with vigorous stirring. The temperature increased to 22 °C. The mixture was allowed to stand for 30 minutes and the layers allowed to separate. The temperature reached a maximum of 40 °C. The aqueous layer was adjusted to pH 12-13 with 10 N potassium hydroxide (3.8 L) at a rate that allowed the temperature to reach and remain at 55 °C during the addition. After the addition was complete the mixture was cooled to 10 °C and stirred for 1 hour. The solid was collected by vacuum filtration and washed four times with water to give 5-formyl-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid ethyl ester (778 g, 100 % yield) as a yellow solid. ^{1}H-NMR (DMSO-d_6) δ 1.25 (t, 3H, CH_3), 2.44, 2.48 (2xs, 2x3H, 2xCH_3), 4.16 (q, 2H, CH_2), 9.59 (s, 1H, CHO), 12.15 (br s, 1H, NH). MS *m/z* 195 [M+1].

5-Formyl-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid ethyl ester (806 g), potassium hydroxide (548 g), water (2400 mL) and methanol (300 mL) were refluxed for two hours with stirring and then cooled to 8 °C. The mixture was extracted twice with dichloromethane. The aqueous layer was adjusted to pH 4 with 1000 mL of 10 N hydrochloric acid keeping the temperature under 15 °C. Water was added to facilitate stirring. The solid was collected by vacuum filtration, washed three times with water and dried under vacuum at 50 °C to give 5-formyl-2,4-dimethyl-1*H*-pyrrole-3-carboxylic (645 g, 93.5 % yield) acid as a yellow solid. NMR (DMSO-d_6) δ 2.40, 2.43 (2xs, 2x3H, 2xCH_3), 9.57 (s, 1H, CHO), 12.07 (br s, 2H, NH+COOH). MS *m/z* 168 [M+1].

5-Formyl-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (1204 g) and 6020 mL of dimethylformamide were stirred at room temperature while 1-(3-dimethyl-aminopropyl-3-ethylcarbodiimide hydrochloride (2071 g), hydroxybenzotriazole (1460 g), triethylamine (2016 mL) and diethylethylenediamine (1215 mL) were added. The mixture was stirred for 20 hours at room temperature. The mixture was diluted with 3000 mL of water, 2000 mL of brine and 3000 mL of saturated sodium bicarbonate solution and the pH adjusted to greater than 10 with 10 N sodium hydroxide. The mixture was extracted twice with 5000 mL each time of 10 % methanol in dichloromethane and the extracts combined, dried over anhydrous magnesium sulfate and rotary evaporated to dryness. The mixture was with diluted with 1950 mL of toluene and rotary evaporated again to dryness. The residue was triturated with 3:1 hexane:diethyl ether (4000 mL). The solids were collected by vacuum filtration, washed twice with 400 mL of ethyl acetate and dried under vacuum at 34 °C for 21 hours to give 5-formyl-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)-amide (819 g, 43 % yield) as a light brown solid. ^{1}H-NMR (dimethylsulfoxide-d_6) δ 0.96 (t, 6H, 2xCH_3), 2.31, 2.38 (2xs, 2 x CH_3), 2.51 (m, 6H 3xCH_2), 3.28 (m, 2H, CH_2), 7.34 (m, 1H, amide NH), 9.56 (s, 1H, CHO), 11.86 (s, 1H, pyrrole NH). MS *m/z* 266 [M+1].

5-Formyl-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (2-diethylaminoethyl)-amide (809 g), 5-fluoro-1,3-dihydro-indol-2-one (438 g), ethanol (8000 mL) and pyrrolidine (13 mL) were heated at 78 °C for 3 hours. The mixture was cooled to room temperature and the solids collected by vacuum filtration and washed with ethanol. The solids were stirred with ethanol (5900 mL) at 72 °C for 30 minutes. The mixture was cooled to room temperature. The solids were collected by vacuum filtration, washed with ethanol and dried under vacuum at 54 °C for 130 hours to give 5-[5-fluoro-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)-amide (1013 g, 88 % yield) as an orange solid. ^{1}H-NMR (dimethylsulfoxide-d_6) δ 0.98 (t, 6H, 2xCH_3), 2.43, 2.44 (2xs, 6H, 2xCH_3), 2.50 (m, 6H, 3xCH_2), 3.28 (q, 2H, CH_2), 6.84, 6.92, 7.42, 7.71, 7.50 (5xm, 5H, aromatic, vinyl, CONH), 10.88 (s, 1H, CONH), 13.68 (s, 1H, pyrrole NH). MS *m/z* 397 [M-1].

Biological Examples

Protein kinase inhibitors of Formula (I):

The following assays may be used to determine the level of activity and effect of the compounds of Formula (I) on one or more of the protein kinases (hereinafter PKs). Similar assays can be designed along the same lines for any PK using techniques well known in the art.

A. Assay Procedures.

Several of the assays described herein are performed in an ELISA (Enzyme-Linked Immunosorbent Sandwich Assay) format (Voller, *et al.*, 1980, "Enzyme-Linked Immunosorbent Assay," Manual of Clinical Immunology, 2d ed., Rose and Friedman, Am. Soc. Of Microbiology, Washington, D.C., pp. 359-371). The general procedure is as follows: a compound of Formula (I) is introduced to cells expressing the test kinase, either naturally or recombinantly, for a selected period of time after which, if the test kinase is a receptor, a ligand known to activate the receptor is added. The cells are lysed and the lysate is transferred to the wells of an ELISA plate previously coated with a specific antibody recognizing the substrate of the enzymatic phosphorylation reaction. Non-substrate components of the cell lysate are washed away and the amount of phosphorylation on the substrate is detected with an antibody specifically recognizing phosphotyrosine compared with control cells that were not contacted with a test compound.

The presently preferred protocols for conducting the ELISA experiments for specific PKs is provided below. However, adaptation of these protocols for determining the activity of compounds against other receptor tyrosine kinases (RTKs), as well as for cytoplasm tryrosine kinases (CTKs) and serine threonine tyrosine kinases (STKs), is well within the scope of knowledge of those skilled in the art. Other assays described herein measure the amount of DNA made in response to activation of a test kinase, which is a general measure of a proliferative response. The general procedure for this assay is as follows: a compound is introduced to cells expressing the test kinase, either naturally or recombinantly, for a selected period of time after which, if the test kinase is a receptor, a ligand known to activate the receptor is added. After incubation at least overnight, a DNA labeling reagent such as 5-bromodeoxyuridine (BrdU) or H^3-thymidine is added. The amount of labeled DNA is detected with either an anti-BrdU antibody or by measuring radioactivity and is compared to control cells not contacted with a test compound.

GST-FLK-1 BIOASSAY

This assay analyzes the tyrosine kinase activity of GST-Flk1 on poly(glu,tyr) peptides.

Materials and Reagents:

1. Corning 96-well ELISA plates (Corning Catalog No. 5805-96).
2. poly(glu,tyr) 4:1, lyophilizate (Sigma Catalog # P0275).
3. Preparation of poly(glu,tyr)(pEY) coated assay plates: Coat 2 ug/well of poly(glu,tyr)(pEY) in 100 ul PBS, hold at room temperature for 2 hours or at 4^0C overnight. Cover plates well to prevent evaporation.

4. PBS Buffer: for 1 L, mix 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 0.2 g KCl and 8 g NaCl in approx. 900ml dH_2O. When all reagents have dissolved, adjust the pH to 7.2 with HCl. Bring total volume to 1 L with dH_2O.
5. PBST Buffer: to 1 L of PBS Buffer, add 1.0 ml Tween-20.
6. TBB - Blocking Buffer: for 1 L, mix 1.21 g TRIS, 8.77 g NaCl, 1 ml TWEEN-20 in approximately 900 ml dH_2O. Adjust pH to 7.2 with HCl. Add 10 g BSA, stir to dissolve. Bring total volume to 1 L with dH_2O. Filter to remove particulate matter.
7. 1% BSA in PBS: To make a 1x working solution, add 10 g BSA to approx. 990 ml PBS buffer, stir to dissolve. Adjust total volume to 1 L with PBS buffer, filter to remove particulate matter.
8. 50 mM Hepes pH 7.5.
9. GST-Flk1cd purified from sf9 recombinant baculovirus transformation (SUGEN, Inc.).
10. 4% DMSO in dH_2O.
11. 10 mM ATP in dH_2O.
12. 40 mM $MnCl_2$
13. Kinase Dilution Buffer (KDB): mix 10 ml Hepes (pH 7.5), 1 ml 5M NaCl, 40 µL 100 mM sodium orthovanadate and 0.4 ml of 5% BSA in dH_2O with 88.56 ml dH_2O.
14. NUNC 96-well V bottom polypropylene plates, Applied Scientific Catalog # AS-72092
15. EDTA: mix 14.12 g ethylenediaminetetraacetic acid (EDTA) to approx. 70 ml dH_2O. Add 10 N NaOH until EDTA dissolves. Adjust pH to 8.0. Adjust total volume to 100 ml with dH_2O.
16. 1^0 Antibody Dilution Buffer: mix 10 ml of 5% BSA in PBS buffer with 89.5 ml TBST.
17. Anti-phosphotyrosine monoclonal antibody conjugated to horseradish peroxidase (PY99 HRP, Santa Cruz Biotech).
18. 2,2'-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS, Moss, Cat. No. ABST).
19. 10% SDS.

Procedure:

1. Coat Corning 96-well ELISA plates with 2 µg of polyEY peptide in sterile PBS as described in step 3 of Materials and Reagents.

2. Remove unbound liquid from wells by inverting plate. Wash once with TBST. Pat the plate on a paper towel to remove excess liquid.
3. Add 100 µl of 1% BSA in PBS to each well. Incubate, with shaking, for 1 hr. at room temperature.
4. Repeat step 2.
5. Soak wells with 50 mM HEPES (pH7.5) (150 µl/well).
6. Dilute test compound with dH_2O/4% DMSO to 4 times the desired final assay concentration in 96-well polypropylene plates.
7. Add 25 µl diluted test compound to ELISA plate. In control wells, place 25 µl of dH_2O/4% DMSO.
8. Add 25 µl of 40 mM $MnCl_2$ with 4x ATP (2 µM) to each well.
9. Add 25 µl 0.5M EDTA to negative control wells.
10. Dilute GST-Flk1 to 0.005 µg(5 ng)/well with KDB.
11. Add 50 µl of diluted enzyme to each well.
12. Incubate, with shaking, for 15 minutes at room temperature.
13. Stop reaction by adding 50 µl of 250 mM EDTA (pH 8.0).
14. Wash 3X with TBST and pat plate on paper towel to remove excess liquid.
15. Add 100 µl per well anti-phosphotyrosine HRP conjugate, 1:5,000 dilution in antibody dilution buffer. Incubate, with shaking, for 90 min. at room temperature.
16. Wash as in step 14.
17. Add 100 µl of room temperature ABTS solution to each well.
18. Incubate, with shaking, for 10 to 15 minutes. Remove any bubbles.
19. Stop reaction by adding 20 µl of 10% SDS to each well.
20. Read results on Dynatech MR7000 ELISA reader with test filter at 410 nM and reference filter at 630 nM.

PYK2 BIOASSAY

This assay is used to measure the *in vitro* kinase activity of HA epitope-tagged full length pyk2 (FL.pyk2-HA) in an ELISA assay.

Materials and reagents:

1. Corning 96-well Elisa plates.
2. 12CA5 monoclonal anti-HA antibody (SUGEN, Inc.)
3. PBS (Dulbecco's Phosphate-Buffered Saline (Gibco Catalog # 450-1300EB)

4. TBST Buffer: for 1 L, mix 8.766 g NaCl, 6.057 g TRIS and 1 ml of 0.1% Triton X-100 in approx. 900 ml dH_2O. Adjust pH to 7.2, bring volume to 1 L.

5. Blocking Buffer: for 1 L, mix 100 g 10% BSA, 12.1 g 100 mM TRIS, 58.44 g 1M NaCl and 10 mL of 1% TWEEN-20.

6. FL.pyk2-HA from sf9 cell lysates (SUGEN, Inc.).

7. 4% DMSO in MilliQue H_2O.

8. 10 mM ATP in dH_2O.

9. 1M $MnCl_2$.

10. 1M $MgCl_2$.

11. 1M Dithiothreitol (DTT).

12. 10X Kinase buffer phosphorylation: mix 5.0 ml 1M Hepes (pH 7.5), 0.2 ml 1M $MnCl_2$, 1.0 ml 1 M $MgCl_2$, 1.0 ml 10% Triton X-100 in 2.8 ml dH_2O. Just prior to use, add 0.1 ml 1M DTT.

13. NUNC 96-well V bottom polypropylene plates.

14. 500 mM EDTA in dH_2O.

15. Antibody dilution buffer: for 100 mL, 1 mL 5% BSA/PBS and 1 mL 10% Tween-20 in 88 mL TBS.

16. HRP-conjugated anti-Ptyr PY99), Santa Cruz Biotech Cat. No. SC-7020.

17. ABTS, Moss, Cat. No. ABST-2000.

18. 10% SDS.

Procedure:

1. Coat Corning 96 well ELISA plates with 0.5 µg per well 12CA5 anti-HA antibody in 100 µl PBS. Store overnight at 4°C.

2. Remove unbound HA antibody from wells by inverting plate. Wash plate with dH_2O. Pat the plate on a paper towel to remove excess liquid.

3. Add 150 µl Blocking Buffer to each well. Incubate, with shaking, for 30 min at room temperature.

4. Wash plate 4x with TBS-T.

5. Dilute lysate in PBS (1.5 µg lysate/100 µl PBS).

6. Add 100 µl of diluted lysate to each well. Shake at room temperature for 1 hr.

7. Wash as in step 4.

8. Add 50 µl of 2X kinase Buffer to ELISA plate containing captured pyk2-HA.

9. Add 25 μL of 400 μM test compound in 4% DMSO to each well. For control wells use 4% DMSO alone.
10. Add 25 μL of 0.5 M EDTA to negative control wells.
11. Add 25 μl of 20 μM ATP to all wells. Incubate, with shaking, for 10 minutes.
12. Stop reaction by adding 25 μl 500 mM EDTA (pH 8.0) to all wells.
13. Wash as in step 4.
14. Add 100 μL HRP conjugated anti-Ptyr diluted 1:6000 in Antibody Dilution Buffer to each well. Incubate, with shaking, for 1 hr. at room temperature.
15. Wash plate 3X with TBST and 1X with PBS.
16. Add 100 μL of ABST solution to each well.
17. If necessary, stop the development reaction by adding 20 μL 10% SDS to each well.
18. Read plate on ELISA reader with test filter at 410 nM and reference filter at 630 nM.

FGFR1 BIOASSAY

This assay is used to measure the *in vitro* kinase activity of FGF1-R in an ELISA assay.

Materials and Reagents:

1. Costar 96-well Elisa plates (Corning Catalog # 3369).
2. Poly(Glu-Tyr) (Sigma Catalog # PO275).
3. PBS (Gibco Catalog # 450-1300EB)
4. 50 mM Hepes Buffer Solution.
5. Blocking Buffer (5% BSA/PBS).
6. Purified GST-FGFR1 (SUGEN, Inc.)
7. Kinase Dilution Buffer.
   Mix 500 µl 1M Hepes (GIBCO), 20 µl 5% BSA/PBS, 10 µl 100mM sodium orthovanadate and 50 µl 5M NaCl.
8. 10mM ATP
9. ATP/$MnCl_2$ phosphorylation mix: mix 20 µL ATP, 400 µL 1M $MnCl_2$ and 9.56 ml dH_2O.
10. NUNC 96-well V bottom polypropylene plates (Applied Scientific Catalog # AS-72092).
11. 0.5M EDTA.
12. 0.05% TBST
    Add 500 µL TWEEN to 1 liter TBS.
13. Rabbit polyclonal anti-phosphotyrosine serum (SUGEN, Inc.).
14. Goat anti-rabbit IgG peroxidase conjugate (Biosource, Catalog # ALI0404).
15. ABTS Solution.
16. ABTS/H_2O_2 solution.

Procedure:

1. Coat Costar 96 well ELISA plates with 1 µg per well Poly(Glu,Tyr) in 100µl PBS. Store overnight at 4° C.
2. Wash coated plates once with PBS.
3. Add 150 µL of 5%BSA/PBS Blocking Buffer to each well. Incubate, with shaking, for 1 hr.room temperature.
4. Wash plate 2x with PBS, then once with 50mM Hepes. Pat plates on a paper towel to remove excess liquid and bubbles.
5. Add 25µL of 0.4 mM test compound in 4% DMSO or 4% DMSO alone (controls) to plate.
6. Dilute purified GST-FGFR1 in Kinase Dilution Buffer (5 ng kinase/50ul KDB/well).

7. Add 50µL of diluted kinase to each well.
8. Start kinase reaction by adding 25µl/well of freshly prepared ATP/Mn++ (0.4 ml 1M $MnCl_2$, 40 µL 10 mM ATP, 9.56 ml dH_2O), freshly prepared).
9. This is a fast kinase reaction and must be stopped with 25µL of 0.5M EDTA in a manner similar to the addition of ATP.
10. Wash plate 4x with fresh TBST.
11. Make up Antibody Dilution Buffer: Per 50 ml:
    Mix 5 ml of 5% BSA, 250 µl of 5% milk and 50 µl of 100mM sodium vanadate, bring to final volume with 0.05% TBST.
12. Add 100 µl per well of anti-phosphotyrosine (1:10000 dilution in ADB). Incubate, with shaking for 1 hr. at room temperature.
13. Wash as in step 10.
14. Add 100 µl per well of Biosource Goat anti-rabbit IgG peroxidase conjugate (1:6000 dilution in ADB). Incubate, with shaking for 1 hr. at room temperature.
15. Wash as in step 10 and then with PBS to remove bubbles and excess TWEEN.
16. Add 100 µl of ABTS/H_2O_2 solution to each well.
17. Incubate, with shaking, for 10 to 20 minutes. Remove any bubbles.
18. Read assay on Dynatech MR7000 elisa reader: test filter at 410 nM, reference filtrate 630 nM.

EGFR BIOASSAY

This assay is used to the *in vitro* kinase activity of FGF1-R in an ELISA assay.

Materials and Reagents:

1. Corning 96-well Elisa plates.
2. SUMO1 monoclonal anti-EGFR antibody (SUGEN, Inc.).
3. PBS
4. TBST Buffer
5. Blocking Buffer: for 100 ml, mix 5.0 g Carnation Instant Non-fat Milk® with 100 ml of PBS.
6. A431 cell lysate (SUGEN, Inc.).
7. TBS Buffer:
8. TBS + 10% DMSO: for 1L, mix 1.514 g TRIS, 2.192 g NaCl and 25 ml DMSO; bring to 1 liter total volume with dH_2O.
9. ATP (Adenosine-5'-triphosphate, from Equine muscle, Sigma Cat. No. A-5394), 1.0 mM solution in dH_2O. This reagent should be made up immediately prior to

use and kept on ice.

10. 1.0 mM $MnCl_2$.

11. $ATP/MnCl_2$ phosphorylation mix: to make 10 ml, mix 300 µl of 1 mM ATP, 500 µl $MnCl_2$ and 9.2 ml dH_2O. Prepare just prior to use, keep on ice.

12. NUNC 96-well V bottom polypropylene plates.

13. EDTA.

14. Rabbit polyclonal anti-phosphotyrosine serum (SUGEN, Inc.).

15. Goat anti-rabbit lgG peroxidase conjugate (Biosource Cat. No. ALI0404).

16. ABTS.

17. 30% Hydrogen peroxide.

18. $ABTS/H_2O_2$.

19. 0.2 M HCl.

Procedure:

1. Coat Corning 96 well ELISA plates with 0.5 µg SUMO1 in 100 µl PBS per well, store overnight at 4° C.

2. Remove unbound SUMO1 from wells by inverting plate to remove liquid. Wash 1x with dH_2O. Pat the plate on a paper towel to remove excess liquid.

3. Add 150 µl of Blocking Buffer to each well. Incubate, with shaking, for 30 min. at room temperature.

4. Wash plate 3x with deionized water, then once with TBST. Pat plate on a paper towel to remove excess liquid and bubbles.

5. Dilute lysate in PBS (7 µg lysate/100 µl PBS).

6. Add 100 µl of diluted lysate to each well. Shake at room temperature for 1 hr.

7. Wash plates as in 4, above.

8. Add 120 µl TBS to ELISA plate containing captured EGFR.

9. Dilute test compound 1:10 in TBS, place in well

10. Add 13.5 µl diluted test compound to ELISA plate. To control wells, add 13.5 µl TBS in 10% DMSO.

11. Incubate, with shaking, for 30 minutes at room temperature.

12. Add 15 µl phosphorylation mix to all wells except negative control well. Final well volume should be approximately 150 µl with 3 µM ATP/5 mM $MnCl_2$ final concentration in each well. Incubate with shaking for 5 minutes.

13. Stop reaction by adding 16.5 µl of EDTA solution while shaking. Shake for additional 1 min.

14. Wash 4x with deionized water, 2x with TBST.
15. Add 100 µl anti-phosphotyrosine (1:3000 dilution in TBST) per well. Incubate, with shaking, for 30-45 min. at room temperature.
16. Wash as in 4, above.
17. Add 100 µl Biosource Goat anti-rabbit IgG peroxidase conjugate (1:2000 dilution in TBST) to each well. Incubate with shaking for 30 min. at room temperature.
18. Wash as in 4, above.
19. Add 100 µl of ABTS/H_2O_2 solution to each well.
20. Incubate 5 to 10 minutes with shaking. Remove any bubbles.
21. If necessary, stop reaction by adding 100 µl 0.2 M HCl per well.
22. Read assay on Dynatech MR7000 ELISA reader: test filter at 410 nM, reference filter at 630 nM.

PDGFR BIOASSAY

This assay is used to the *in vitro* kinase activity of FGF1-R in an ELISA assay.

Materials and Reagents:

1. Corning 96-well Elisa plates
2. 28D4C10 monoclonal anti-PDGFR antibody (SUGEN, Inc.).
3. PBS.
4. TBST Buffer.
5. Blocking Buffer (same as for EGFR bioassay).
6. PDGFR-β expressing NIH 3T3 cell lysate (SUGEN, Inc.).
7. TBS Buffer.
8. TBS + 10% DMSO.
9. ATP.
10. $MnCl_2$.
11. Kinase buffer phosphorylation mix: for 10 ml, mix 250 µl 1M TRIS, 200 µl 5M NaCl, 100 µl 1M $MnCl_2$ and 50µl 100 mM Triton X-100 in enough dH_2O to make 10 ml.
12. NUNC 96-well V bottom polypropylene plates.
13. EDTA.
14. Rabbit polyclonal anti-phosphotyrosine serum (SUGEN,Inc.).
15. Goat anti-rabbit IgG peroxidase conjugate (Biosource Cat. No. ALI0404).
16. ABTS.

17. Hydrogen peroxide, 30% solution.
18. ABTS/H_2O_2.
19. 0.2 M HCl.

Procedure:

1. Coat Corning 96 well ELISA plates with 0.5 µg 28D4C10 in 100 µl PBS per well, store overnight at 4° C.
2. Remove unbound 28D4C10 from wells by inverting plate to remove liquid. Wash 1x with dH_2O. Pat the plate on a paper towel to remove excess liquid.
3. Add 150 µl of Blocking Buffer to each well. Incubate for 30 min. at room temperature with shaking.
4. Wash plate 3x with deionized water, then once with TBST. Pat plate on a paper towel to remove excess liquid and bubbles.
5. Dilute lysate in HNTG (10 µg lysate/100 µl HNTG).
6. Add 100 µl of diluted lysate to each well. Shake at room temperature for 60 min.
7. Wash plates as described in Step 4.
8. Add 80 µl working kinase buffer mix to ELISA plate containing captured PDGFR.
9. Dilute test compound 1:10 in TBS in 96-well polypropylene plates.
10. Add 10 µl diluted test compound to ELISA plate. To control wells, add 10 µl TBS + 10% DMSO. Incubate with shaking for 30 minutes at room temperature.
11. Add 10 µl ATP directly to all wells except negative control well (final well volume should be approximately 100 µl with 20 µM ATP in each well.) Incubate 30 minutes with shaking.
12. Stop reaction by adding 10 µl of EDTA solution to each well.
13. Wash 4x with deionized water, twice with TBST.
14. Add 100 µl anti-phosphotyrosine (1:3000 dilution in TBST) per well. Incubate with shaking for 30-45 min. at room temperature.
15. Wash as in Step 4.
16. Add 100 µl Biosource Goat anti-rabbit IgG peroxidase conjugate (1:2000 dilution in TBST) to each well. Incubate with shaking for 30 min. at room temperature.
17. Wash as in Step 4.
18. Add 100 µl of ABTS/H_2O_2 solution to each well.

19. Incubate 10 to 30 minutes with shaking. Remove any bubbles.
20. If necessary stop reaction with the addition of 100 µl 0.2 M HCl per well.
21. Read assay on Dynatech MR7000 ELISA reader with test filter at 410 nM and reference filter at 630 nM.

CELLULAR HER-2 KINASE ASSAY

This assay is used to measure HER-2 kinase activity in whole cells in an ELISA format.

Materials and Reagents:

1. DMEM (GIBCO Catalog #11965-092).
2. Fetal Bovine Serum (FBS, GIBCO Catalog #16000-044), heat inactivated in a water bath for 30 min. at 56° C
3. Trypsin (GIBCO Catalog #25200-056).
4. L-Glutamine (GIBCO Catalog #25030-081)
5. HEPES (GIBCO Catalog #15630-080).
6. Growth Media
   Mix 500 ml DMEM, 55 ml heat inactivated FBS, 10 ml HEPES and 5.5 ml L-Glutamine.
7. Starve Media
   Mix 500 ml DMEM, 2.5 ml heat inactivated FBS, 10 ml HEPES and 5.5 ml L-Glutamine.
8. PBS.
9. Flat Bottom 96-well Tissue Culture Micro Titer Plates (Corning Catalog # 25860).
10. 15 cm Tissue Culture Dishes (Corning Catalog #08757148).
11. Corning 96-well ELISA Plates.
12. NUNC 96-well V bottom polypropylene plates.
13. Costar Transfer Cartridges for the Transtar 96 (Costar Catalog #7610).
14. SUMO 1: monoclonal anti-EGFR antibody (SUGEN, Inc.).
15. TBST Buffer.
16. Blocking Buffer : 5% Carnation Instant Milk® in PBS.
17. EGF Ligand: EGF-201, Shinko American, Japan. Suspend powder in 100 uL of 10mM HCl. Add 100uL 10mM NaOH. Add 800 uL PBS and transfer to an Eppendorf tube, store at -20°C until ready to use.
18. HNTG Lysis Buffer

For Stock 5X HNTG, mix 23.83 g Hepes, 43.83 g NaCl, 500 ml glycerol and 100 ml Triton X-100 and enough dH_2O to make 1 L of total solution.

For 1X HNTG*, mix 2 ml HNTG, 100 µL 0.1M Na_3VO_4, 250 µL 0.2M $Na_4P_2O_7$ and 100 µL EDTA.

19. EDTA.

20. Na_3VO_4. To make stock solution, mix 1.84 g Na_3VO_4 with 90 ml dH_2O. Adjust pH to 10. Boil in microwave for one minute (solution becomes clear). Cool to room temperature. Adjust pH to 10. Repeat heating/cooling cycle until pH remains at 10.

21. 200 mM $Na_4P_2O_7$.

22. Rabbit polyclonal antiserum specific for phosphotyrosine (anti-Ptyr antibody, SUGEN, Inc.).

23. Affinity purified antiserum, goat anti-rabbit IgG antibody, peroxidase conjugate (Biosource Cat # ALI0404).

24. ABTS Solution.

25. 30 % Hydrogen peroxide solution.

26. ABTS/H_2O_2.

27. 0.2 M HCl.

Procedure:

1. Coat Corning 96 well ELISA plates with SUMO1 at 1.0 ug per well in PBS, 100 ul final volume/well. Store overnight at 4°C.

2. On day of use, remove coating buffer and wash plate 3 times with dH_2O and once with TBST buffer. All washes in this assay should be done in this manner, unless otherwise specified.

3. Add 100 ul of Blocking Buffer to each well. Incubate plate, with shaking, for 30 min. at room temperature. Just prior to use, wash plate.

4. Use EGFr/HER-2 chimera/3T3-C7 cell line for this assay.

5. Choose dishes having 80-90 % confluence. Collect cells by trypsinization and centrifuge at 1000 rpm at room temperature for 5 min.

6. Resuspend cells in starve medium and count with trypan blue. Viability above 90% is required. Seed cells in starve medium at a density of 2,500 cells per well, 90 ul per well, in a 96 well microtiter plate. Incubate seeded cells overnight at 37° under 5% CO_2.

7. Start the assay two days after seeding.

8. Test compounds are dissolved in 4% DMSO. Samples are then further diluted directly on plates with starve-DMEM. Typically, this dilution will be 1:10 or greater. All wells are then transferred to the cell plate at a further 1:10 dilution (10µl sample and media into 90 µl of starve media. The final DMSO concentration should be 1% or lower. A standard serial dilution may also be used.
9. Incubate under 5% CO_2 at 37°C for 2 hours.
10. Prepare EGF ligand by diluting stock EGF (16.5 uM) in warm DMEM to 150 nM.
11. Prepare fresh HNTG* sufficient for 100 ul per well; place on ice.
12. After 2 hour incubation with test compound, add prepared EGF ligand to cells, 50 ul per well, for a final concentration of 50 nM. Positive control wells receive the same amount of EGF. Negative controls do not receive EGF. Incubate at 37° C for 10 min.
13. Remove test compound, EGF, and DMEM. Wash cells once with PBS.
14. Transfer HNTG* to cells, 100 ul per well. Place on ice for 5 minutes. Meanwhile, remove blocking buffer from ELISA plate and wash.
15. Scrape cells from plate with a micropipettor and homogenize cell material by repeatedly aspirating and dispensing the HNTG* lysis buffer. Transfer lysate to a coated, blocked, washed ELISA plate. Or, use a Costar transfer cartridge to transfer lysate to the plate.
16. Incubate, with shaking, at room temperature for 1 hr.
17. Remove lysate, wash. Transfer freshly diluted anti-Ptyr antibody (1:3000 in TBST) to ELISA plate, 100 ul per well.
18. Incubate, with shaking, at room temperature, for 30 min.
19. Remove anti-Ptyr antibody, wash. Transfer freshly diluted BIOSOURCE antibody to ELISA plate(1:8000 in TBST, 100 ul per well).
20. Incubate, with shaking, at room temperature for 30 min.
21. Remove BIOSOURCE antibody, wash. Transfer freshly prepared ABTS/H_2O_2 solution to ELISA plate, 100 ul per well.
22. Incubate, with shaking, for 5-10 minutes. Remove any bubbles.
23. Stop reaction with the addition of 100ul of 0.2M HCl per well.
24. Read assay on Dynatech MR7000 ELISA reader with test filter set at 410 nM and reference filter at 630 nM.

CDK2/CYCLIN A ASSAY

This assay is used to measure the *in vitro* serine/threonine kinase activity of human cdk2/cyclin A in a Scintillation Proximity Assay (SPA).

Materials and Reagents.

1. Wallac 96-well polyethylene terephthalate (flexi) plates (Wallac Catalog # 1450-401).
2. Amersham Redivue [γ^{33}P] ATP (Amersham catalog #AH 9968).
3. Amersham streptavidin coated polyvinyltoluene SPA beads (Amersham catalog #RPNQ0007). The beads should be reconstituted in PBS without magnesium or calcium, at 20 mg/ml.
4. Activated cdk2/cyclin A enzyme complex purified from Sf9 cells (SUGEN, Inc.).
5. Biotinylated peptide substrate (Debtide). Peptide biotin-X-PKTPKKAKKL is dissolved in dH_2O at a concentration of 5 mg/ml.
6. Peptide/ATP Mixture: for 10 ml, mix 9.979 ml dH_2O, 0.00125 ml "cold" ATP, 0.010 ml Debtide and 0.010 ml γ^{33}P ATP. The ultimate concentration per well will be 0.5 μM "cold" ATP, 0.1 μg Debtide and 0.2 μCi γ^{33}P ATP.
7. Kinase buffer: for 10 ml, mix 8.85 ml dH_2O, 0.625 ml TRIS(pH 7.4), 0.25 ml 1M $MgCl_2$, 0.25 ml 10% NP40 and 0.025 ml 1M DTT, added fresh just prior to use.
8. 10 mM ATP in dH_2O.
9. 1M Tris, pH adjusted to 7.4 with HCl.
10. 1M $MgCl_2$.
11. 1M DTT.
12. PBS (Gibco Catalog # 14190-144).
13. 0.5M EDTA.
14. Stop solution: For 10 ml, mix 9.25 ml PBS, 0.005 ml 100 mM ATP, 0.1 ml 0.5 M EDTA, 0.1 ml 10% Triton X-100 and 1.25 ml of 20 mg/ml SPA beads.

Procedure:

1. Prepare solutions of test compounds at 5x the desired final concentration in 5% DMSO. Add 10 ul to each well. For negative controls, use 10 ul 5% DMSO alone in wells.
2. Dilute 5 μl of cdk2/cyclin A solution with 2.1 ml 2x kinase buffer.
3. Add 20 ul enzyme to each well.

4. Add 10 µL of 0.5 M EDTA to the negative control wells.
5. To start kinase reaction, add 20 µL of peptide/ATP mixture to each well. Incubate for 1 hr. without shaking.
6. Add 200 µl stop solution to each well.
7. Hold at least 10 min.
8. Spin plate at approx. 2300 rpm for 3-5 min.
9. Count plate using Trilux or similar reader.

MET TRANSPHOSPHORYLATION ASSAY

This assay is used to measure phosphotyrosine levels on a poly(glutamic acid:tyrosine (4:1)) substrate as a means for identifying agonists/antagonists of met transphosphorylation of the substrate.

Materials and Reagents:

1. Corning 96-well Elisa plates, Corning Catalog # 25805-96.
2. Poly(glu, tyr) 4:1, Sigma, Cat. No; P 0275.
3. PBS, Gibco Catalog # 450-1300EB
4. 50 mM HEPES
5. Blocking Buffer: Dissolve 25 g Bovine Serum Albumin, Sigma Cat. No A-7888, in 500 ml PBS, filter through a 4 µm filter.
6. Purified GST fusion protein containing the Met kinase domain, Sugen, Inc.
7. TBST Buffer.
8. 10% aqueous (MilliQue H_2O) DMSO.
9. 10 mM aqueous (dH_2O) Adenosine-5'-triphosphate, Sigma Cat. No. A-5394.
10. 2X Kinase Dilution Buffer: for 100 ml, mix 10 mL 1M HEPES at pH 7.5 with 0.4 mL 5% BSA/PBS, 0.2 mL 0.1 M sodium orthovanadate and 1 mL 5M sodium chloride in 88.4 mL dH_2O.
11. 4X ATP Reaction Mixture: for 10 mL, mix 0.4 mL 1 M manganese chloride and 0.02 mL 0.1 M ATP in 9.56 mL dH_2O.
12. 4X Negative Controls Mixture: for 10 mL, mix 0.4 mL 1 M manganese chloride in 9.6 mL dH_2O.
13. NUNC 96-well V bottom polypropylene plates, Applied Scientific Catalog # S-72092
14. 500 mM EDTA.

15. Antibody Dilution Buffer: for 100 mL, mix 10 mL 5% BSA/PBS, 0.5 mL 5% Carnation Instant Milk® in PBS and 0.1 mL 0.1 M sodium orthovanadate in 88.4 mL TBST.

16. Rabbit polyclonal antophosphotyrosine antibody, Sugen, Inc.

17. Goat anti-rabbit horseradish peroxidase conjugated antibody, Biosource, Inc.

18. ABTS Solution: for 1 L, mix 19.21 g citric acid, 35.49 g Na_2HPO_4 and 500 mg ABTS with sufficient dH_2O to make 1 L.

19. ABTS/H_2O_2: mix 15 mL ABST solution with 2μL H_2O_2 five minutes before use.

20. 0.2 M HCl

Procedure:

1. Coat ELISA plates with 2 μg Poly(Glu-Tyr) in 100 μL PBS, store overnight at 4 ° C.
2. Block plate with 150 μL of 5% BSA / PBS for 60 min.
3. Wash plate twice with PBS, once with 50 mM Hepes buffer pH 7.4.
4. Add 50 μl of the diluted kinase to all wells. (Purified kinase is diluted with Kinase Dilution Buffer. Final concentration should be 10 ng/well.)
5. Add 25 μL of the test compound (in 4% DMSO) or DMSO alone (4% in dH_2O) for controls to plate.
6. Incubate the kinase/compound mixture for 15 minutes.
7. Add 25 μL of 40 mM $MnCl_2$ to the negative control wells.
8. Add 25 μL ATP/ $MnCl_2$ mixture to the all other wells (except the negative controls). Incubate for 5 min.
9. Add 25 μL 500 mM EDTA to stop reaction.
10. Wash plate 3x with TBST.
11. Add 100 μL rabbit polyclonal anti-Ptyr diluted 1:10,000 in Antibody Dilution Buffer to each well. Incubate, with shaking, at room temperature for one hour.
12. Wash plate 3x with TBST.
13. Dilute Biosource HRP conjugated anti-rabbit antibody 1: 6,000 in Antibody Dilution buffer. Add 100 μL per well and incubate at room temperature, with shaking, for one hour.
14. Wash plate 1X with PBS.
15. Add 100 μl of ABTS/H_2O_2 solution to each well.

16. If necessary, stop the development reaction with the addition of 100 µl of 0.2M HCl per well.
17. Read plate on Dynatech MR7000 elisa reader with the test filter at 410 nM and the reference filter at 630 nM.

IGF-1 TRANSPHOSPHORYLATION ASSAY

This assay is used to measure the phosphotyrosine level in poly(glutamic acid:tyrosine)(4:1) for the identification of agonists/antagonists of gst-IGF-1 transphosphorylation of a substrate.

Materials and Reagents:

1. Corning 96-well Elisa plates.
2. Poly (Glu-tyr) (4:1), Sigma Cat. No. P 0275.
3. PBS, Gibco Catalog # 450-1300EB.
4. 50 mM HEPES
5. TBB Blocking Buffer: for 1 L, mix 100 g BSA, 12.1 gTRIS (pH 7.5), 58.44 g sodium chloride and 10 mL 1%TWEEN-20.
6. Purified GST fusion protein containing the IGF-1 kinase domain (Sugen, Inc.)
7. TBST Buffer: for 1 L, mix 6.057 g Tris, 8.766 g sodium chloride and 0.5 ml TWEEN-20 with enough dH_2O to make 1 liter.
8. 4% DMSO in Milli-Q H_2O.
9. 10 mM ATP in dH_2O.
10. 2X Kinase Dilution Buffer: for 100 mL, mix 10 mL 1 M HEPES (pH 7.5), 0.4 mL 5% BSA in dH_2O, 0.2 mL 0.1 M sodium orthovanadate and 1 mL 5 M sodium chloride with enough dH_2O to make 100 mL.
11. 4X ATP Reaction Mixture: for 10 mL, mix 0.4 mL 1 M $MnCl_2$ and 0.008 mL 0.01 M ATP and 9.56 mL dH_2O.

12.4X Negative Controls Mixture: mix 0.4 mL 1 M manganese chloride in 9.60 mL dH_2O.

13. NUNC 96-well V bottom polypropylene plates.
14. 500 mM EDTA in dH_2O.
15. Antibody Dilution Buffer: for 100 mL, mix 10 mL 5% BSA in PBS, 0.5 mL 5% Carnation Instant Non-fat Milk® in PBS and 0.1 mL 0.1 M sodium orthovanadate in 88.4 mL TBST.
16. Rabbit Polyclonal antiphosphotyrosine antibody, Sugen, Inc.
17. Goat anti-rabbit HRP conjugated antibody, Biosource.
18. ABTS Solution.

20. ABTS/H_2O_2: mix 15 mL ABTS with 2 µL H_2O_2 5 minutes before using.

21. 0.2 M HCl in dH_2O.

Procedure:

1. Coat ELISA plate with 2.0 µg / well Poly(Glu, Tyr) 4:1 (Sigma P0275) in 100 µl PBS. Store plate overnight at 4° C.
2. ash plate once with PBS.
3. Add 100 µl of TBB Blocking Buffer to each well. Incubate plate for 1 hour with shaking at room temperature.
4. Wash plate once with PBS, then twice with 50 mM Hepes buffer pH 7.5.
5. Add 25 µL of test compound in 4% DMSO (obtained by diluting a stock solution of 10 mM test compound in 100% DMSO with dH_2O) to plate.
6. Add 10.0 ng of gst-IGF-1 kinase in 50 µl Kinase Dilution Buffer) to all wells.
7. Start kinase reaction by adding 25µl 4X ATP Reaction Mixture to all test wells and positive control wells. Add 25µl 4X Negative Controls Mixture to all negative control wells. Incubates for 10 minutes with shaking at room temperature.
8. Add 25µl 0.5M EDTA (pH 8.0) to all wells.
9. Wash plate 4x with TBST Buffer.
10. Add rabbit polyclonal anti-phosphotyrosine antisera at a dilution of 1:10,000 in 100µl Antibody Dilution Buffer to all wells. Incubate, with shaking, at room temperature for 1 hour.
11. Wash plate as in step 9.
12. Add 100 µL Biosource anti-rabbit HRP at a dilution of 1:10,000 in Antibody dilution buffer to all wells. Incubate, with shaking, at room temperature for 1 hour.
13. Wash plate as in step 9, follow with one wash with PBS to reduce bubbles and excess Tween-20.
14. Develop by adding 100µl/well ABTS/H_2O_2 to each well
15. After about 5 minutes, read on ELISA reader with test filter at 410 nm and referenced filter at 630 nm.

BRDU INCORPORATION ASSAYS

The following assays use cells engineered to express a selected receptor and then evaluate the effect of a compound of interest on the activity of ligand-induced DNA synthesis by determining BrdU incorporation into the DNA.

The following materials, reagents and procedure are general to each of the following BrdU incorporation assays. Variances in specific assays are noted.

Materials and Reagents:

1. The appropriate ligand.
2. The appropriate engineered cells.
3. BrdU Labeling Reagent: 10 mM, in PBS (pH 7.4)(Boehringer Mannheim, Germany).
4. FixDenat: fixation solution (ready to use)(Boehringer Mannheim, Germany).
5. Anti-BrdU-POD: mouse monoclonal antibody conjugated with peroxidase (Boehringer Mannheim, Germany).
6. TMB Substrate Solution: tetramethylbenzidine (TMB, Boehringer Mannheim, Germany).
7. PBS Washing Solution : 1X PBS, pH 7.4.
8. Albumin, Bovine (BSA), fraction V powder (Sigma Chemical Co., USA).

General Procedure:

1. Cells are seeded at 8000 cells/well in 10% CS, 2 mM Gln in DMEM, in a 96 well plate. Cells are incubated overnight at 37°C in 5% CO_2.
2. After 24 hours, the cells are washed with PBS, and then are serum-starved in serum free medium (0% CS DMEM with 0.1% BSA) for 24 hours.
3. On day 3, the appropriate ligand and the test compound are added to the cells simultaneously. The negative control wells receive serum free DMEM with 0.1% BSA only; the positive control cells receive the ligand but no test compound. Test compounds are prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.
4. After 18 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration=10 μM) for 1.5 hours.
5. After incubation with labeling reagent, the medium is removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution is added (50 μl/well) and the plates are incubated at room temperature for 45 minutes on a plate shaker.
6. The FixDenat solution is thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 μl/well) as a blocking solution and the plate is incubated for 30 minutes at room temperature on a plate shaker.

7. The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:200 dilution in PBS, 1% BSA) is added (50 µl/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.
8. The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times with PBS, and the plate is dried by inverting and tapping on a paper towel.
9. TMB substrate solution is added (100 µl/well) and incubated for 20 minutes at room temperature on a plate shaker until color development is sufficient for photometric detection.
10. The absorbance of the samples are measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

EGF-Induced BrdU Incorporation Assay

Materials and Reagents:

1. Mouse EGF, 201 (Toyobo Co., Ltd., Japan).
2. 3T3/EGFRc7.

EGF-Induced Her-2-driven BrdU Incorporation Assay

Materials and Reagents:

1. Mouse EGF, 201 (Toyobo Co., Ltd., Japan).
2. 3T3/EGFr/Her2/EGFr (EGFr with a Her-2 kinase domain).

EGF-Induced Her-4-driven BrdU Incorporation Assay

Materials and Reagents:

1. Mouse EGF, 201 (Toyobo Co., Ltd., Japan).
2. 3T3/EGFr/Her4/EGFr (EGFr with a Her-4 kinase domain).

PDGF-Induced BrdU Incorporation Assay

Materials and Reagents:

1. Human PDGF B/B (Boehringer Mannheim, Germany).
2. 3T3/EGFRc7.

FGF-Induced BrdU Incorporation Assay

Materials and Reagents:

1. Human FGF2/bFGF (Gibco BRL, USA).
2. 3T3c7/EGFr

IGF1-Induced BrdU Incorporation Assay

Materials and Reagents:

1. Human, recombinant (G511, Promega Corp., USA)
2. 3T3/IGF1r.

Insulin-Induced BrdU Incorporation Assay

Materials and Reagents:

1. Insulin, crystalline, bovine, Zinc (13007, Gibco BRL, USA).
2. 3T3/H25.

HGF-Induced BrdU Incorporation Assay

Materials and Reagents:

1. Recombinant human HGF (Cat. No. 249-HG, R&D Systems, Inc. USA).
2. BxPC-3 cells (ATCC CRL-1687).

Procedure:

1. Cells are seeded at 9000 cells/well in RPMI 10% FBS in a 96 well plate. Cells are incubated overnight at 37°C in 5% CO_2.
2. After 24 hours, the cells are washed with PBS, and then are serum starved in 100 µl serum-free medium (RPMI with 0.1% BSA) for 24 hours.
3. On day 3, 25 µl containing ligand (prepared at 1 µg/ml in RPMI with 0.1% BSA; final HGF conc. is 200 ng/ml) and test compounds are added to the cells. The negative control wells receive 25 µl serum-free RPMI with 0.1% BSA only; the positive control cells receive the ligand (HGF) but no test compound. Test compounds are prepared at 5 times their final concentration in serum-free RPMI with ligand in a 96 well plate, and serially diluted to give 7 test concentrations. Typically, the highest final concentration of test compound is 100 µM, and 1:3 dilutions are used (i.e. final test compound concentration range is 0.137-100 µM).
4. After 18 hours of ligand activation, 12.5 µl of diluted BrdU labeling reagent (1:100 in RPMI, 0.1% BSA) is added to each well and the cells are incubated with BrdU (final concentration is 10 µM) for 1 hour.
5. Same as General Procedure.
6. Same as General Procedure.
7. The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) is added

(100 µl/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.

8. Same as General Procedure.

9. Same as General Procedure.

10. Same as General Procedure.

HUV-EC-C Assay

This assay is used to measure a compound's activity against PDGF-R, FGF-R, VEGF, aFGF or Flk-1/KDR, all of which are naturally expressed by HUV-EC cells.

DAY 0

1. Wash and trypsinize HUV-EC-C cells (human umbilical vein endothelial cells, (American Type Culture Collection, catalogue no. 1730 CRL). Wash with Dulbecco's phosphate-buffered saline (D-PBS, obtained from Gibco BRL, catalogue no. 14190-029) 2 times at about 1 ml/10 cm^2 of tissue culture flask. Trypsinize with 0.05% trypsin-EDTA in non-enzymatic cell dissociation solution (Sigma Chemical Company, catalogue no. C-1544). The 0.05% trypsin is made by diluting 0.25% trypsin/1 mM EDTA (Gibco, catalogue no. 25200-049) in the cell dissociation solution. Trypsinize with about 1 ml/25-30 cm^2 of tissue culture flask for about 5 minutes at 37°C. After cells have detached from the flask, add an equal volume of assay medium and transfer to a 50 ml sterile centrifuge tube (Fisher Scientific, catalogue no. 05-539-6).

2. Wash the cells with about 35 ml assay medium in the 50 ml sterile centrifuge tube by adding the assay medium, centrifuge for 10 minutes at approximately 200x g, aspirate the supernatant, and resuspend with 35 ml D-PBS. Repeat the wash two more times with D-PBS, resuspend the cells in about 1 ml assay medium/15 cm^2 of tissue culture flask. Assay medium consists of F12K medium (Gibco BRL, catalogue no. 21127-014) and 0.5% heat-inactivated fetal bovine serum. Count the cells with a Coulter Counter® (Coulter Electronics, Inc.) and add assay medium to the cells to obtain a concentration of 0.8-1.0 x 10^5 cells/ml.

3. Add cells to 96-well flat-bottom plates at 100 µl/well or 0.8-1.0 x 10^4 cells/well, incubate ~24h at 37°C, 5% CO_2.

DAY 1

1. Make up two-fold test compound titrations in separate 96-well plates, generally 50 µM on down to 0 µM. Use the same assay medium as mentioned in day 0, step 2 above. Titrations are made by adding 90 µl/well of test compound at 200 µM (4X the final well concentration) to the top well of a particular plate column. Since the stock test compound is usually 20 mM in DMSO, the 200 µM drug concentration contains 2% DMSO.

A diluent made up to 2% DMSO in assay medium (F12K + 0.5% fetal bovine serum) is used as diluent for the test compound titrations in order to dilute the test compound but keep the DMSO concentration constant. Add this diluent to the remaining wells in the column at 60 µl/well. Take 60 µl from the 120 µl of 200 µM test compound dilution in the top well of the column and mix with the 60 µl in the second well of the column. Take 60 µl from this well and mix with the 60 µl in the third well of the column, and so on until two-fold titrations are completed. When the next-to-the-last well is mixed, take 60 µl of the 120 µl in this well and discard it. Leave the last well with 60 µl of DMSO/media diluent as a non-test compound-containing control. Make 9 columns of titrated test compound, enough for triplicate wells each for: (1) VEGF (obtained from Pepro Tech Inc., catalogue no. 100-200, (2) endothelial cell growth factor (ECGF) (also known as acidic fibroblast growth factor, or aFGF) (obtained from Boehringer Mannheim Biochemica, catalogue no. 1439 600), or, (3) human PDGF B/B (1276-956, Boehringer Mannheim, Germany) and assay media control. ECGF comes as a preparation with sodium heparin.

2. Transfer 50 µl/well of the test compound dilutions to the 96-well assay plates containing the 0.8-1.0x10^4 cells/100 µl/well of the HUV-EC-C cells from day 0 and incubate ~2 h at 37° C, 5% CO_2.

3. In triplicate, add 50 µl/well of 80 µg/ml VEGF, 20 ng/ml ECGF, or media control to each test compound condition. As with the test compounds, the growth factor concentrations are 4X the desired final concentration. Use the assay media from day 0 step 2 to make the concentrations of growth factors. Incubate approximately 24 hours at 37°C, 5% CO_2. Each well will have 50 µl test compound dilution, 50 µl growth factor or media, and 100 µl cells, which calculates to 200 µl/well total. Thus the 4X concentrations of test compound and growth factors become 1X once everything has been added to the wells.

DAY 2

1. Add ^{3}H-thymidine (Amersham, catalogue no. TRK-686) at 1 µCi/well (10 µl/well of 100 µCi/ml solution made up in RPMI media + 10% heat-inactivated fetal bovine serum) and incubate ~24 h at 37°C, 5% CO_2. RPMI is obtained from Gibco BRL, catalogue no. 11875-051.

DAY 3

1. Freeze plates overnight at -20°C.

DAY 4

Thaw plates and harvest with a 96-well plate harvester (Tomtec Harvester 96®) onto filter mats (Wallac, catalogue no. 1205-401), read counts on a Wallac Betaplate™ liquid

scintillation counter.

TABLE 2 shows the results of biological testing of the protein kinase used in the present invention. The results are reported in terms of IC_{50}, the micromolar (μM) concentration of the compound being tested which causes a 50% change in the activity of the target PKT compared to the activity of the PTK in a control to which no test compound has been added. Specifically, the results shown indicate the concentration of a test compound needed to cause a 50% reduction of the activity of the target PTK. Bioassays which have been or can be used to evaluate compounds are described in detail below.

TABLE 2

Example	bio flkGST IC50 (μM)	bio FGFR1 IC50 (μM)	bio PDGF IC50 (μM)	bio EGF IC50 (μM)	cell EGF IC50 (μM)	Her2 Kinase IC50 (μM)	cdk2spa C50 (μM)	bio pyk2 IC50 (μM)
1	0.13	4.29	0.001	>100		50.19	17.19	0.28

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Example	bio flkGST IC50 (μM)	bio FGFR1 IC50 (μM)	bio PDGF IC50 (μM)	bio EGF IC50 (μM)	cell EGF IC50 (μM)	Her2 Kinase IC50 (μM)	cdk2spa C50 (μM)	bio pyk2 IC50 (μM)

Example	bio flkGST IC50 (μM)	bio FGFR1 IC50 (μM)	bio PDGF IC50 (μM)	bio EGF IC50 (μM)	cell EGF IC50 (μM)	Her2 Kinase IC50 (μM)	cdk2spa C50 (μM)	bio pyk2 IC50 (μM)

***IN VIVO* ANIMAL MODELS**

XENOGRAFT ANIMAL MODELS

The ability of human tumors to grow as xenografts in athymic mice (*e.g.*, Balb/c, nu/nu) provides a useful *in vivo* model for studying the biological response to therapies for human tumors. Since the first successful xenotransplantation of human tumors into athymic mice, (Rygaard and Povlsen, 1969, Acta Pathol. Microbial. Scand. 77:758-760), many different human tumor cell lines (*e.g.*, mammary, lung, genitourinary, gastro-intestinal, head and neck,

glioblastoma, bone, and malignant melanomas) have been transplanted and successfully grown in nude mice. The following assays may be used to determine the level of activity, specificity and effect of the different compounds of Formula (I).

Three general types of assays are useful for evaluating compounds of Formula (I): cellular/catalytic, cellular/biological and *in vivo*. The object of the cellular/catalytic assays is to determine the effect of a compound of Formula (I) on the ability of a TK to phosphorylate tyrosines on a known substrate in a cell. The object of the cellular/biological assays is to determine the effect of a compound of Formula (I) on the biological response stimulated by a TK in a cell. The object of the *in vivo* assays is to determine the effect of a compound of Formula (I) in an animal model of a particular disorder such as cancer.

Suitable cell lines for subcutaneous xenograft experiments include C6 cells (glioma, ATCC # CCL 107), A375 cells (melanoma, ATCC # CRL 1619), A431 cells (epidermoid carcinoma, ATCC # CRL 1555), Calu 6 cells (lung, ATCC # HTB 56), PC3 cells (prostate, ATCC # CRL 1435), SKOV3TP5 cells and NIH 3T3 fibroblasts genetically engineered to overexpress EGFR, PDGFR, IGF-1R or any other test kinase. The following protocol can be used to perform xenograft experiments:

Female athymic mice (BALB/c, nu/nu) are obtained from Simonsen Laboratories (Gilroy, CA). All animals are maintained under clean-room conditions in Micro-isolator cages with Alpha-dri bedding. They receive sterile rodent chow and water ad libitum.

Cell lines are grown in appropriate medium (for example, MEM, DMEM, Ham's F10, or Ham's F12 plus 5% - 10% fetal bovine serum (FBS) and 2 mM glutamine (GLN)). All cell culture media, glutamine, and fetal bovine serum are purchased from Gibco Life Technologies (Grand Island, NY) unless otherwise specified. All cells are grown in a humid atmosphere of 90-95% air and 5-10% CO_2 at 37°C. All cell lines are routinely subcultured twice a week and are negative for mycoplasma as determined by the Mycotect method (Gibco).

Cells are harvested at or near confluency with 0.05% Trypsin-EDTA and pelleted at 450 x g for 10 min. Pellets are resuspended in sterile PBS or media (without FBS) to a particular concentration and the cells are implanted into the hindflank of the mice (8 - 10 mice per group, 2 - 10 x 10^6 cells/animal). Tumor growth is measured over 3 to 6 weeks using venier calipers. Tumor volumes are calculated as a product of length x width x height unless otherwise indicated. P values are calculated using the Students t-test. Test compounds of Formula (I) in 50 - 100 µL excipient (DMSO, or VPD:D5W) can be delivered by IP injection at different concentrations generally starting at day one after implantation.

TUMOR INVASION MODEL

The following tumor invasion model has been developed and may be used for the evaluation of therapeutic value and efficacy of the compounds of Formula (I) identified to selectively inhibit KDR/FLK-1 receptor.

Procedure

Eight week old nude mice (female) (Simonsen Inc.) are used as experimental animals. Implantation of tumor cells can be performed in a laminar flow hood. For anesthesia, Xylazine/Ketamine Cocktail (100 mg/kg ketamine and 5 mg/kg Xylazine) are administered intraperitoneally. A midline incision is done to expose the abdominal cavity (approximately 1.5 cm in length) to inject 10^7 tumor cells in a volume of 100 µl medium. The cells are injected either into the duodenal lobe of the pancreas or under the serosa of the colon. The peritoneum and muscles are closed with a 6-0 silk continuous suture and the skin is closed by using wound clips. Animals are observed daily.

Analysis

After 2-6 weeks, depending on gross observations of the animals, the mice are sacrificed, and the local tumor metastases to various organs (lung, liver, brain, stomach, spleen, heart, muscle) are excised and analyzed (measurement of tumor size, grade of invasion, immunochemistry, in situ hybridization determination, etc.).

C-KIT ASSAY

This assay is used to detect the level of c-kit tyrosine phosphorylation.

MO7E (human acute myeloid leukemia) cells were serum starved overnight in 0.1% serum. Cells were pre-treated with the compound of Formula (I) (concurrent with serum starvation), prior to ligand stimulation. Cells were stimulated with 250 ng/ml rh-SCF for 15 minutes. Following stimulation, cells were lysed and immunoprecipitated with an anti-c-kit antibody. Phosphotyrosine and protein levels were determined by Western blotting.

MTT PROLIFERATION ASSAY

MO7E cells were serum starved and pre-treated with a compound of Formula (I) as described for the phosphorylation experiments. Cells were plated @ $4X10^5$ cells/well in a 96 well dish, in 100 µl RPMI + 10% serum. rh-SCF (100 ng/mL) was added and the plate was incubated for 48 hours. After 48 hours, 10 µl of 5 mg/ml MTT [3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) was added and allowed to incubate for 4 hours. Acid isopropanol (100 µl of 0.04N HCl in isopropanol) was added and the optical density was measured at a wavelength of 550 nm.

APOPTOSIS ASSAY

MO7E cells were incubated +/- SCF and +/- compound of Formula (I) in 10% FBS with rh-GM-CSF(10ng/mL) and rh-IL-3 (10ng/mL). Samples were assayed at 24 and 48 hours. To measure activated caspase-3, samples were washed with PBS and permeabilized with ice-cold 70% ethanol. The cells were then stained with PE-conjugated polyclonal rabbit anti-active caspase-3 and analyzed by FACS. To measure cleaved PARP, samples were lysed and analyzed by western blotting with an anti-PARP antibody.

Additional assays

Additional assays which may be used to evaluate the compounds of Formula (I) include, without limitation, a bio-flk-1 assay, an EGF receptor-HER2 chimeric receptor assay in whole cells, a bio-src assay, a bio-lck assay and an assay measuring the phosphorylation function of raf. The protocols for each of these assays may be found in U. S. Application Ser. No. 09/099,842, which is incorporated by reference, including any drawings, herein.

Measurement of Cell Toxicity

Therapeutic compounds should be more potent in inhibiting receptor tyrosine kinase activity than in exerting a cytotoxic effect. A measure of the effectiveness and cell toxicity of a compound can be obtained by determining the therapeutic index, i.e., IC_{50}/LD_{50}. IC_{50}, the dose required to achieve 50% inhibition, can be measured using standard techniques such as those described herein. LD_{50}, the dosage which results in 50% toxicity, can also be measured by standard techniques as well (Mossman, 1983, J. Immunol. Methods, 65:55-63), by measuring the amount of LDH released (Korzeniewski and Callewaert, 1983, J. Immunol. Methods, 64:313, Decker and Lohmann-Matthes, 1988, J. Immunol. Methods, 115:61), or by measuring the lethal dose in animal models. Compounds with a large therapeutic index are preferred. The therapeutic index should be greater than 2, preferably at least 10, more preferably at least 50.

B. Example of Cellular Assay Results Using 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1).

To confirm the potency of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) detected in biochemical assays(*vide infra*), the ability of said compound to inhibit ligand-dependent RTK phosphorylation was evaluated in cell-based assays using NIH-3T3 mouse cells engineered to overexpress Flk-1 or human PDGFRβ. 5-(5-Fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) inhibited VEGF-dependent Flk-1 tyrosine phosphorylation with an IC_{50} value of approximately

0.03 μM. This value is similar to the 0.009 μM K_i value determined for inhibition of Flk-1 by 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) determined in biochemical assays. This indicates that 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) readily penetrates into cells. Consistent with the biochemical data (*vide infra*) indicating that 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) had comparable activity against Flk-1 and PDGFRβ, it was also found that it inhibited PDGF-dependent receptor phosphorylation in cells with an IC_{50} value of approximately 0.03 μM. The ability of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) to inhibit c-kit, a closely related RTK that binds stem cell factor (SCF), was determined using MO7E cells that express this receptor. In these cells, 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) inhibited SCF-dependent c-kit phosphorylation with an IC_{50} value of 0.01–0.1 μM. This compound also inhibited SCF-stimulated c-kit phosphorylation in acute myeloid leukemia (AML) blasts isolated from the peripheral blood of patients.

In addition to testing the ability of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) to inhibit ligand-dependent receptor phosphorylation in cells, its effect on ligand-dependent proliferative response of cells was also examined *in vitro* (*see* Table 4). In these studies, cells quiesced by overnight serum starvation were induced to undergo DNA synthesis upon addition of the appropriate mitogenic ligand. As shown in Table 3, 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) inhibited the PDGF-induced proliferation of NIH-3T3 cells overexpressing PDGFRβ or PDGFRα with IC_{50} values 0.031 and 0.069 μM, respectively, and the SCF-induced proliferation of MO7E cells with an IC_{50} value of 0.007 μM.

TABLE 3

	Biochemical	Cellular IC_{50}	
Receptor	K_i[1] (μM)	Receptor Phosphorylation (μM)	Ligand-dependent Proliferation (μM)
Flk-1/KDR	0.009	0.03[2]	0.004[3]
PDGFRα	0.008	0.03[4]	0.031[4]
PDGFRβ	ND	ND	0.069[5]
FGFR	0.83	ND	0.7[3]
c-kit	ND	0.01-0.1[6]	0.007[6]

ND = Not Determined
[1] Determined using recombinant enzyme
[2] Determined using serum-starved NIH-3T3 cells expressing Flk-1
[3] Determined using serum-starved HUVECs
[4] Determined using serum-starved NIH-3T3 cells expressing PDGFR□
[5] Determined using serum-starved NIH-3T3 cells expressing PDGFR□
[6] Determined using serum-starved MO7E cells

As shown in Table 3, there is a general agreement between the biochemical and cellular activities of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) supporting the conclusion that this compound crosses cellular membranes. Further, it can be concluded that the cellular responses are a result of the activity of compound 1 against the indicated target. In contrast, when tested in the presence of complete growth medium *in vitro*, substantially higher concentrations of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) (>10 μM) were required to inhibit the growth of a variety of human tumor cells (*see* Table 4). This indicates that the compound did not directly inhibit the growth of these cells at concentrations required to inhibit ligand-dependent receptor phosphorylation and cell proliferation.

TABLE 4

Cell Line	Origin	IC_{50} (μM)	LD_{50} (μM)
HT29	Colon carcinoma	10	22
A549	Lung carcinoma	9.5	22
NCI-H460	NSC lung carcinoma	8.9	20
SF767T	Glioma	7.9	14
A431	Epidermoid carcinoma	6.0	18

Briefly, the results shown in Table 4 were obtained by incubating cells for 48 hr in complete growth medium in the presence of serial dilutions 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (2-diethylamino-

ethyl)amide. At the end of the growth period, the relative number of cells was determined. IC_{50} values were calculated as the concentration of compound that inhibited the growth of cells by 50% relative to untreated cells. LD_{50} values were calculated as the concentration of compound that caused a 50% reduction in the number of cells relative to those at the start of the experiment.

A more relevant cell-based assay in which to evaluate the anti-angiogenic potential of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) is the *in vitro* mitogenesis assay using human umbilical vein endothelial cells (HUVECs) as a model system for the endothelial cell proliferation critical to the angiogenic process. In this assay, a mitogenic response, measured as an increase in DNA synthesis, is induced in serum-starved HUVECs upon addition of VEGF or FGF. In these cells, 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) inhibited the VEGF- and FGF-induced mitogenic response in a dose-dependent manner with IC_{50} values of 0.004 μM and 0.7 μM, respectively, when compound was present throughout the 48-hr assay.

Briefly, the aforementioned results were obtained using Serum-starved HUVECs that were incubated with mitogenic concentrations of VEGF (100 ng/ml) or FGF (30 ng/ml) in the presence of serial dilutions of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) for 24 hrs. The mitogenic response during the following 24 hrs. in the presence of ligand and inhibitor was quantitated by measurement of DNA synthesis based on incorporation of bromodeoxyuridine into cellular DNA.

In separate experiments, compound 1 inhibited the VEGF-dependent phosphorylation of ERK 1/2 (p42/44MAP kinase), an early downstream target of Flk-1/KDR, in a dose-dependent manner. The inhibitory activity of compound 1 was also shown to be long-lasting in this system; inhibiting VEGF-dependent phosphorylation of ERK 1/2 for as long as 48 hours after removal of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) from the medium following a short (2 hr) exposure to micromolar concentrations of the compound.

VEGF has been recognized to be an important survival factor for endothelial cells. Since 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) inhibits the VEGF-dependent mitogenic response of HUVECs, the effect of the compound on HUVEC survival was investigated. In these experiments, cleavage of the caspase 3 substrate poly-ADP-ribosyl

polymerase (PARP) was used as a readout for apoptosis. HUVECs cultured in serum-free conditions for 24 hours exhibited substantial levels of PARP cleavage, as detected by the accumulation of the 23 kDa PARP cleavage fragment. This was largely prevented by the addition of VEGF to the cell medium, indicating that VEGF acts as a survival factor in this assay. 5-(5-Fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) has been shown to inhibit KDR signaling. Accordingly, 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) inhibited VEGF-mediated HUVEC survival in a dose-dependent manner. Thus, these data indicate that compound 1 induced apoptosis in endothelial cells in culture in the presence of VEGF.

C. *In vivo* Efficacy Studies

i. Efficacy Against Established Tumor Xenografts

The *in vivo* efficacy of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) was studied in subcutaneous (SC) xenograft models using human tumor cells implanted into the hindflank region of athymic mice. Following implantation, tumors were allowed to become established to a size of 100–550 mm^3 prior to starting oral treatment with the compound.

Daily oral administration of compound 1 caused a dose-dependent inhibition of A431 tumor growth when treatment was initiated after tumors had grown to a size of 400 mm^3. Statistically significant ($P < 0.05$) inhibition of tumor growth was seen at doses of 40 mg/kg/day (74% inhibition) and 80 mg/kg/day (84% inhibition) (*see* Table 6). In preliminary experiments, a higher (160 mg/kg/day) dose of the compound was not more efficacious against established A431 tumors than the 80 mg/kg/day dose. In addition, mice treated at the 160 mg/kg/day dose of the compound lost body weight, indicating that the higher dose was not as well tolerated. Similar results were obtained in an experiment in which A431 tumors were only allowed to reach 100 mm^3 in size (*see* Table 4). In this second experiment, complete regression of the tumors occurred in six of the eight animals treated at the 80 mg/kg/day for 21 days. In these six animals, the tumors did not regrow during a 110-day observation period following the end of treatment. In the two animals in which the tumors regrew to a large size (2000-3000 mm^3), the tumors regressed in response to a second round of treatment with compound 1. Importantly, in all efficacy experiments, 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) at 80 mg/kg/day has been well tolerated, even when dosed continuously for more than 100 days.

TABLE 5

Initial Tumor Volume (mm^3)	Compound[1] (mg/kg/day)	% Inhibition (day)	P-Value
400	80	84 (36)	0.001
	40	74 (36)	0.003
	20	51 (36)	0.130
100	80	93 (40)	0.002
	40	75 (40)	0.015
	10	61 (40)	0.059

[1] Compound 1.

Briefly, the results shown in Table 5 were obtained using A431 cells (0.5 x 106 cells/mouse) which were implanted SC into the hindflank region of athymic mice. Daily oral administration of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) in a Cremophore-based vehicle or vehicle control began when tumors reached the indicated average volume. Tumors were measured using vernier calipers and tumor volume was calculated as the product of length x width x height. P-values were calculated by comparing the size of the tumors for animals that were treated with compound 1 (n=8) to those of animals that were treated with a vehicle (n=16) on the last day of the experiment, using the two-tailed Student's t-test.

The efficacy compound 1 against established human tumors of different origins was determined using Colo205 (colon carcinoma), SF763T (glioma), and NCI-H460 (non-small cell lung carcinoma) xenografts (*see* Table 6). These experiments were conducted using 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) administered orally at 80 mg/kg/day; a dose that was effective and well tolerated.

TABLE 6

	Tumor Type	Initial Tumor Volume (mm^3)	% Inhibition (day)	P-Value
A431[1]	Epidermoid	100	93 (40)	0.002
A431[1]	Epidermoid	400	84 (36)	0.001
Colo205	Colon	370	77 (54)	0.028
NCI-H460	Lung	300	61 (54)	0.003
SF763T	Glioma	550	53 (30)	0.001

[1] Data are from experiment reported in Table 5.

In the abovementioned experiments, compound 1 was administered once daily at 80 mg/kg in a Cremophor-based vehicle once tumors reached the indicated size. Percent inhibition compared to the vehicle-treated control group was calculated at termination of the experiments. P-values were calculated by comparing tumor sizes of the animals that had been treated with the compound to tumor sizes of those animals that had been treated with the vehicle, using the two-tailed Student's t-test.

Although 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 1) inhibited the growth of all the tumor types shown in Table 7, there was a difference in the response of the different xenograft models. Specifically, the growth of NCI-H460 and SF763T tumors was arrested or greatly slowed whereas the Colo205 tumors, like A431 tumors, regressed when treated with 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide.

In order to determine the molecular basis for the difference in response between xenograft models, the SF763T tumors were studied. Therefore, SF763T tumors, which were less responsive to treatment with 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide, have been evaluated at the molecular level using immunohistological techniques to determine the effect of treatment with the compound. These studies were initially conducted in this tumor type because SF763T tumors are highly vascularized with microvessels that strongly express the endothelial cell marker CD31 and are hence well suited for studies of tumor microvessel density (MVD). Immunohistological evaluation of SF763T tumors indicated that tumors from treated animals had reduced MVD relative to vehicle-treated controls, consistent with an anti-angiogenic mechanism of action for compound 1; MVD was 24.2 ± 4.1 in animals treated with compound 1, compared to 39.3 ± 5.7 for those that were treated with just the vehicle. As anticipated from

the associated tumor growth arrest, a pronounced inhibition of tumor cell proliferation was evident in tumors that were treated with compound 1. These tumors had half the mitotic index of those in vehicle-treated tumors (data not shown). The effect compound 1 on MVD and tumor cell proliferation indicates that the compound has profound anti-angiogenic and anti-tumor effects, even under conditions in which tumors do not regress.

The ability of compound 1 to inhibit PDGFR phosphorylation and subsequent signaling *in vivo* was also evaluated in the SF763T tumors, which express high levels of PDGFRβ. Treatment of the SF763T tumors with compound 1 strongly inhibited PDGFRβ tyrosine phosphorylation in established SF763T tumors. Compound 1 also reduced the levels of phosphorylated (activated) phospholipase C gamma (PLC-γ), an immediate downstream indicator of PDGFR activation. These data demonstrate that oral administration of compound 1 causes a direct effect on target (PDGFR) activity in tumors *in vivo*.

Based on the demonstration that the ability of compound 1 to inhibit VEGF-dependent signaling in HUVECs *in vitro* was long-lasting (*vide supra*), the efficacy of the compound was evaluated when the compound was administered infrequently in the Colo205 tumor model. As shown in Table 7, 80 mg/kg (91% inhibition) and 40 mg/kg (84% inhibition) were efficacious when administered daily, but not when administered twice weekly. In contrast, a higher dose of compound 1 (160 mg/kg) did inhibit (52% inhibition) the growth of established Colo205 tumors when administered twice weekly, suggesting that this compound can demonstrate efficacy when administered infrequently at a higher dose. It should be noted that dosing regimens may be determined by those with ordinary skill in the art without undue experimentation.

TABLE 7

Dose (mg/kg)	Frequency	% Inhibition	P-Value
160	Twice weekly	52	0.085
	Once weekly	17	NS
80	Daily	91	0.039
	Twice weekly	19	NS
	Once weekly	0	NS
40	Daily	84	0.028
	Twice weekly	36	NS
NS: not significant (P >0.05)			

Briefly, the results shown in Table 7 were obtained using Colo205 cells (0.5×10^6 cells/mouse) that had been implanted SC into the hindflank region of athymic mice. Oral administration of compound 1 according to the indicated schedule began when tumors reached

400 mm^3. Tumors were measured using vernier calipers and tumor volume was calculated as the product of length x width x height. P-values were calculated by comparing the size of the tumors for animals that were treated with compound 1 to those of animals that were treated with a vehicle on the last day of the experiment, using the two-tailed Student's t-test.

ii. Efficacy of Compound 1 in a Model of Disseminated Disease

In addition to supporting the sustained growth of solid primary tumors, angiogenesis is also an essential component supporting the development of disseminated disease due to metastasis from the primary tumor. The effect of compound 1 on the development of disseminated disease was examined in the B16-F1 mouse melanoma lung colonization model. In this model, B16-F1 cells inoculated intravenously via the tail vein of athymic mice colonize the lungs and form tumors. As shown in Table 7, oral administration of compound 1 at 80 mg/kg/day effectively reduced the burden of B16-F1 cells in the lung as evaluated by measurements of total lung weight. These data suggest that compound 1 can inhibit disseminated disease *in vivo*.

TABLE 8

	Lung Weight (g)	% Inhibition	P-Value
Vehicle	0.83 ± 0.07	-	-
Compound[1]	0.41 ± 0.04	50	<0.001

[1] Compound 1

Briefly, the results shown in Table 8 were obtained using athymic mice that had been inoculated with B16-F1 tumor cells ($5x10^5$ cells/mouse) via the tail vein. Mice were treated daily with orally administered compound 1 at 80 mg/kg/day (n=10) or vehicle (n=18) for 24 days after tumor cell inoculation. At the end of the treatment period, the mice were sacrificed and their lungs removed and weighed. Percent inhibition was calculated by comparing the lung weight of those animals that had been treated with compound 1, with the lung weight of the animals that had only been treated with vehicle. P-values were determined using the two-tailed Student's t-test.

II. COX-2 Inhibitors

The ability of COX-2 inhibitors to retard the growth of tumors was tested using the Lewis Lung Model described below.

Mice were injected subcutaneously in the left paw (1 x 10^6 tumor cells suspended in 30 % Matrigel) and tumor volume was evaluated using a phlethysmometer twice a week for

30-60 days. Blood was drawn twice during the experiment in a 24 h protocol to assess plasma concentration and total exposure by AUC analysis. The data are expressed as the mean +/- SEM. Student's and Mann-Whitney tests were used to assess differences between means using the InStat software package. Celecoxib given in the diet at doses between 160-3200 ppm retarded the growth of these tumors. The inhibitory effect of celecoxib was dose-dependent and ranged from 48 % to 85 % as compared with the control tumors. Analysis of lung metastasis was done in all the animals by counting metastasis in a stereomicroscope and by histochemical analysis of consecutive lung sections. Celecoxib did not affect lung metastasis at the lower dose of 160 ppm, however surface metastasis was reduced by more than 50 % when given at doses between 480-3200 ppm. In addition, histopathological analysis revealed that celecoxib dose-dependently reduced the size of the metastasic lesions in the lung.

2. HT-29 Model:

Mice were injected subcutaneously in the left paw (1×10^6 tumor cells suspended in 30 % Matrigel) and tumor volume was evaluated using a phlethysmometer twice a week for 30-60 days. Implantation of human colon cancer cells (HT-29) into nude mice produces tumors that will reach 0.6-2 ml between 30-50 days. Blood was drawn twice during the experiment in a 24 h protocol to assess plasma concentration and total exposure by AUC analysis. The data are expressed as the mean +/- SEM. Student's and Mann-Whitney tests were used to assess differences between means using the InStat software package.

A. Mice injected with HT-29 cancer cells were treated with cytoxin i.p at doses of 50 mg/kg on days 5,7 and 9 in the presence or absence of celecoxib in the diet. The efficacy of both agents were determined by measuring tumor volume. Treatment using a celecoxib related COX-2 inhibitor (SC-58236) reduced tumor volume by 89 %. In the same assay, indomethacin given at near the maximum tolerated dose of 2 mg/kg/day in the drinking water inhibited tumor formation by 77%. Moreover, the COX-2 selective inhibitor completely inhibited the formation of lung metastasis while the non-selective NSAID indomethacin was ineffective. The results from these studies demonstrate that celecoxib administered in the diet to tumor bearing mice can delay the growth of tumors and metastasis when administered as sole therapy. Moreover, a positive benefit is observed when celecoxib is administered in combination with a cytotoxic agent such as cyclophosphamide.

B. In a second assay, mice injected with HT-29 colon cancer cells were treated with celecoxib (10, 40 or 160 ppm) in the diet beginning at day 10. An approximate dose dependent effect was observed. (Table 9).

Table 9. Celecoxib Inhibitis HT-29 Human Colon Carcinoma

Days	vehicle	10 ppm	40 ppm	160 ppm
14	0.114	0.124	0.125	0.120
22	0.25	0.25	0.19	0.14
28	0.45	0.36	0.27	0.21
35	0.79	0.57	0.4	0.3
42	1.38	0.89	0.68	0.49
50	1.9	1.49	1.04	0.8

Volume (ml)

III. *In Vivo* assays using protein kinases in combination with a Cyclooxygenase-2 selective inhibitor for treating Cancer.

The ability of a protein kinase inhibitor to retard the growth of tumors in combination with a COX-2 selective inhibitor can be tested in 1483 xenograft model described below.

1483 xenograft is an animal xenograft that models human epithelial cancers expressing cyclooxgenase-2 (COX-2) in the tumor cells and in the vasculature.

A human tumor xenograft nude mouse model of head and neck squamous cell carcinoma (1483 cell line) that expresses COX-2 in the tumor cells and in the vasculartur, similar to human epithelial cancers. We believe this model represents human epithelial cancers and would be a good model to correlate efficacy of anti-cancer drugs including COX-2 inhibitors to efficacy in humans.

Materials and Methods:

Cell Culture:

1483 human head and neck squamous cell carcinoma (HNSCC) cells are stored in frozen vials containing 3 x 10^6 cells, 90 % fetal bovine serum (FBS) and 10 % dimethyl sulfoxide (DMSO). Take a frozen vial and quickly thaw at 37°C and placed in a T-162 cm^2 (Corning) flask containing D-MEM/F12 media (GibcoBRL) with 15mM Hepes buffer, L-glutamine, pyridoxine hydrochloride and 10 % FBS. Cells are grown in an incubator with 5 % CO_2 and temperature at 37°C. Media is changed every other day and cells are passed when at 80-90 % confluence. For passing of cells, wash flask with 10 ml of phosphate buffered saline

(PBS), aspirate off and add 2 ml of trypsin/EDTA (0.25 % / 1 mM, GibcoBRL) place back in incubator, after 5 min., cells will detach. Add 8 ml of above media to flask rinse and transfer to a sterile 50 ml centrifuge tube. Add 30 ml more of media and mix and count cells using a hemacytometer, plate out cells in a T-162 cm^2 containing 3-4 x 10^6 cells.

1483 Animal Model:

Change media 24 hours before harvest of 1483 cells before injection in to nude mice. Trypsinize 1483 cells as described above in cell culture section. Count cells and determine number of cells. Centrifuge cells down at 1000 rpm for 5 minutes at room temperature. Resuspened cell pellets and pool them (if multiple 50 ml centrifuge tubes) into one 50 ml centrifuge tube with Hank's buffered saline solution (HBSS, GibcoBRL) and centrifuge as before. You will want to have about 25 % more cells then what you really need for injection to have extra cells. If you are injecting 72 mice and you have 100 x 10^6 cells, prepare all the cells for injecting into mice. Inject 1483 cells at 1 x 10^6 cells in 0.03 ml/mouse.

Inject cells with 30 % Matrigel (Collaborative Biomedical Products) and 70 % HBSS. Resuspend pooled pellet with 2.1 ml (70%) of cold HBSS then add 0.9 ml (30 %) of thawed liquefied cold Matrigel and mix well on ice. Keep this cell preparation on ice at all times prior to injecting into mice. Male nude mice age 4-6 weeks old are used in these studies (Harlen). Anesthetize the mice using CO_2/O_2 gas and then inject them in the middle of the right hind paw using a 0.5cc tuberculin syringe (Beckerson & Dickerson). Weigh the mice for body weight on day of injection (Day 0) for baseline weight for start of study. Weigh the mice on day 7 and measure the right hind paw for paw tumor volume using a plethysmometer (Stoelting Co.). The plethysmometer is a machine that measure paw volume by water displacement. Measure a few left non-injected paws and average for a background measurement to subtract from the right tumor bearing paw.

Place the animals on test compounds in chow meal when tumors are 100-200ul in size and continue on compound meal throughout study. Some mice are given only a protein kinase inhibitor or a cyclooxygenase-2 selective inhibitor. Some mice are given both a protein kinase inhibitor and a cyclooxygenase-2 selective inhibitor daily at a suitable dose determined based on the in vitro assay results.

Weigh and measure the mice throughout the study on days 7, 10, 14, 17, 21, 24 and 28. Animals can be started on compound treatment on day 0 (prophylactic) or once there is an established tumor around day 7 (therapeutic). Around day 30 vehicle (control) mice will have large tumors (~1.0 -1.5 ml) and start to lose weight, at this time terminate the vehicle animals. If tumor inhibition is seen in treated groups and they are in good health, euthanize half of the treated groups and keep half of the treated group alive to determine the delay in tumor growth.

One skilled in the art would also readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent herein. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

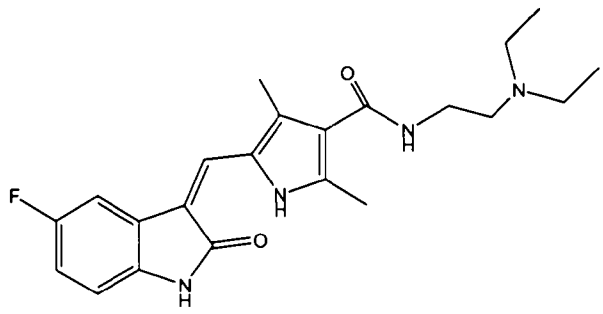
All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

This application claims priority to U.S. Serial No. 60/312,413, filed August 15, 2001, which is hereby incorporated by reference in its entirety.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

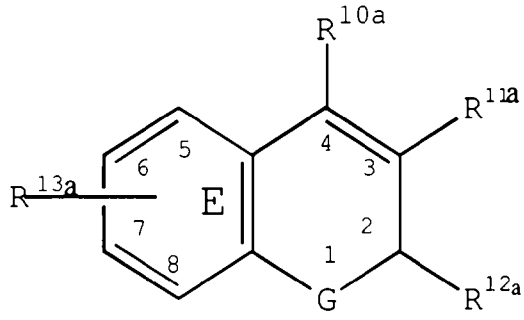
1. A method for the treatment of a cancer comprising administering to a mammal in need of such treatment a therapeutically effective amount of:

a protein kinase inhibitor of the formula:



in combination with a cyclooxygenase-2 selective inhibitor or a pharmaceutically acceptable salt thereof selected from the group consisting of:

(i) a compound of Formula (II):



wherein:

G is selected from the group consisting of O, S, and -NR^a- wherein R^a is hydrogen or alkyl;

R^{10a} is selected from the group consisting of hydrogen and aryl;

R^{11a} is selected from the group consisting of carboxyl, alkyl, aralkyl, aminocarbonyl, alkylsulfonylaminocarbonyl, and alkoxycarbonyl;

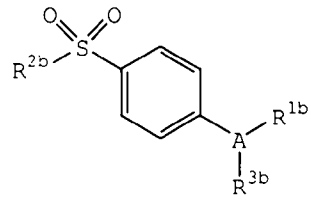
R^{12a} is selected from the group consisting of haloalkyl, alkyl, aralkyl, cycloalkyl and aryl optionally substituted with one or more radicals selected from alkylthio, nitro and alkylsulfonyl; and

R^{13a} is one or more radicals independently selected from the group consisting of hydrogen, halo, alkyl, aralkyl, alkoxy, aryloxy, heteroaryloxy, aralkyloxy, heteroaralkyloxy, haloalkyl, haloalkoxy, alkylamino, arylamino, aralkylamino, heteroarylamino, heteroarylalkylamino, nitro, amino, aminosulfonyl, alkylaminosulfonyl, arylaminosulfonyl,

heteroarylaminosulfonyl, aralkylaminosulfonyl, heteroaralkylaminosulfonyl, heterocyclosulfonyl, alkylsulfonyl, hydroxyarylcarbonyl, nitroaryl, optionally substituted aryl, optionally substituted heteroaryl, aralkylcarbonyl, heteroarylcarbonyl, arylcarbonyl, aminocarbonyl, and alkylcarbonyl; or

R^{13a} together with ring E forms a naphthyl ring; or

(ii) a compound of Formula (III):



(III)

wherein:

A is selected from the group consisting of partially unsaturated or unsaturated heterocyclyl and partially unsaturated or unsaturated carbocyclic rings;

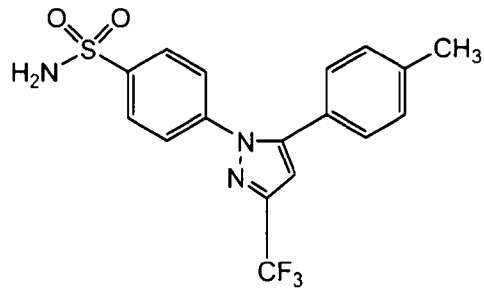
R^{1b} is selected from the group consisting of heterocyclyl, cycloalkyl, cycloalkenyl and aryl, wherein R^{1b} is optionally substituted at a substitutable position with one or more radicals independently selected from alkyl, haloalkyl, cyano, carboxyl, alkoxycarbonyl, hydroxyl, hydroxyalkyl, haloalkoxy, amino, alkylamino, arylamino, nitro, alkoxyalkyl, alkylsulfinyl, halo, alkoxy, and alkylthio;

R^{2b} is selected from the group consisting of methyl and amino; and

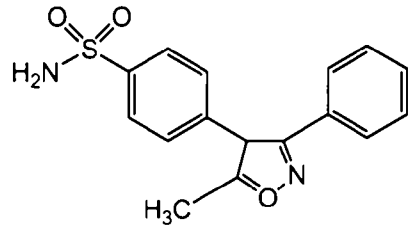
R^{3b} is selected from the group consisting of a radical selected from hydrogen, halo, alkyl, alkenyl, alkynyl, oxo, cyano, carboxyl, cyanoalkyl, heterocyclyloxy, alkyloxy, alkylthio, alkylcarbonyl, cycloalkyl, aryl, haloalkyl, heterocyclyl, cycloalkenyl, aralkyl, heterocyclylalkyl, acyl, alkylthioalkyl, hydroxyalkyl, alkoxycarbonyl, arylcarbonyl, aralkylcarbonyl, aralkenyl, alkoxyalkyl, arylthioalkyl, aryloxyalkyl, aralkylthioalkyl, aralkoxyalkyl, alkoxyaralkoxyalkyl, alkoxycarbonylalkyl, aminocarbonyl, aminocarbonylalkyl, alkylaminocarbonyl, N-arylaminocarbonyl, N-alkyl-N-arylaminocarbonyl, alkylaminocarbonylalkyl, carboxyalkyl, alkylamino, N-arylamino, N-aralkylamino, N-alkyl-N-aralkylamino, N-alkyl-N-arylamino, aminoalkyl, alkylaminoalkyl, N-arylaminoalkyl, N-aralkylaminoalkyl, N-alkyl-N-aralkylaminoalkyl, N-alkyl-N-arylaminoalkyl, aryloxy, aralkoxy, arylthio, aralkylthio, alkylsulfinyl, alkylsulfonyl, aminosulfonyl, alkylaminosulfonyl, N-arylaminosulfonyl, arylsulfonyl, and N-alkyl-N-arylaminosulfonyl.

2. The method of claim 1, wherein said cyclooxygenase-2 selective inhibitor is a compound of Formula (III) selected from the group consisting of:

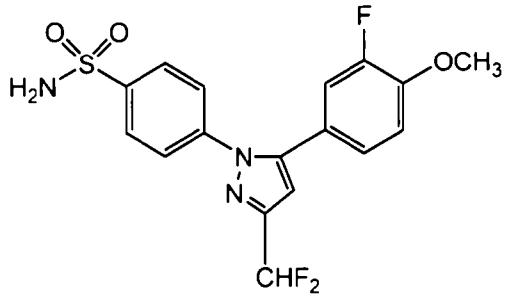
a)



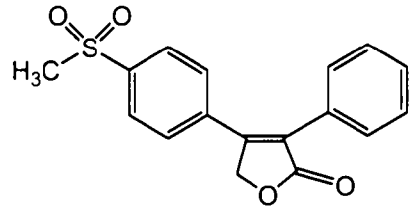
b)



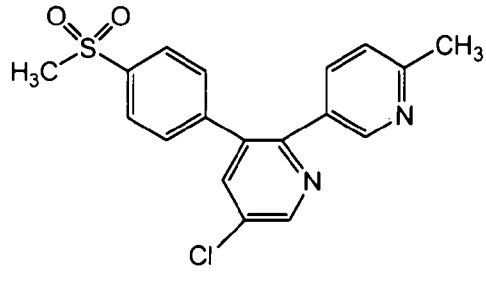
c)



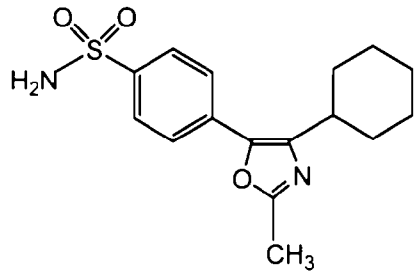
d)



e)



f)



or a pharmaceutically acceptable salt thereof; and any combination thereof.

3. The method of claim 1, wherein said cyclooxygenase-2 selective inhibitor is 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]phenylsulfonamide, or a pharmaceutically acceptable salt.