NOVEL COMPOUNDS FOR TREATMENT OF CANCER AND DISORDERS ASSOCIATED WITH ANGIOGENESIS FUNCTION

RELATED APPLICATIONS

The present application is a continuation-in-part of pending prior U.S. Patent Application Serial No. 11/027,465 filed on December 29, 2004. The present application also claims priority to U.S. Provisional Application Serial No. 60/624,253 filed on November 1, 2004. The contents of U.S. Patent Application Serial No. 11/027,465 and U.S. Provisional Application Serial No. 60/624,253 are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to therapeutic compounds for treatment of cancer and disorders associated with angiogenesis function. More specifically, the invention relates to novel compounds and their uses in treating cancer such as leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, breast cancer, renal cancer, and prostate cancer, as well as disorders associated with angiogenesis function such as age-related macular degeneration, macular dystrophy, and diabetes.

BACKGROUND OF THE INVENTION

Traditionally most anticancer drugs were discovered by high throughput screening with cytotoxicity as the end-point measurement (Neamati and Barchi Jr. (2002) Curr. Top. Med. Chem. 2:211-227). In general, most if not all of these drugs have multiple mechanisms of action and multiple mechanisms of resistance. With very few exceptions, their mechanisms of action were identified much later than their discovery. True mechanisms of action of certain drugs were found to be different than what they originally anticipated. Although various strategies have been used to identify drug targets, it is becoming appreciated that there

are no easy and straightforward ways to do so with current technologies. Two reasons can be presented to explain this phenomenon. The first has to do with the intrinsic natures of small molecule drugs (e.g., membrane permeability in many cell types) coupled with their lack of selectivity and specificity as compared to for example, antibody-antigen recognition. Second, there is an overwhelming redundancy built into the biological systems serving as targets, due to the abundance of sequence and structural homology. This might explain why in many cases "messy anticancer drugs" work just as well or better than targeted therapeutics. Whatever the mechanism, an initial and critical step in any drug discovery program is lead identification.

Of over 100 FDA approved anticancer drugs, fewer than 20 are widely used. By contrast, all the 19 FDA approved drugs for HIV-1 infection are used in various combinations. Although antiviral drugs are almost always administered orally, only very few anticancer drugs are orally active. Accordingly, it is desirable that most targeted therapeutics of the future are orally active.

There is a desperate need to develop highly active, well-tolerated, and easy to use (ideally orally active) drugs, which exploit our increased understanding of tumor biology. However, one major hurdle to overcome in a drug discovery program is the identification of a suitable lead compound having desired biological activity. Less than 1% of tested compounds will eventually become selected for further studies. Preclinical evaluation of pharmacokinetic and pharmacodynamic properties and a knowledge of drug metabolism are important in the drug development processes. After a drug candidate is selected for further study, detailed information from in vitro screening as well as an evaluation of in vivo efficacy and toxicity in animal models is required to predict the in vivo outcome of selected compounds in humans. Traditional pharmacokinetic studies, although essential, are cumbersome and timeconsuming and require a large number of animals. Recent technological advances in computer simulations have allowed absorption, distribution, metabolism,

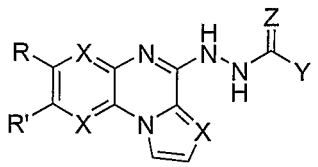
excretion, and toxicity (ADMET) prediction to become a reliable and rapid means of decreasing the time and resources needed to evaluate the therapeutic potential of a drug candidate (Neamati and Barchi Jr. (2002) Curr. Top. Med. Chem. 2:211-227).

Previously, we showed that certain of our HIV-1 integrase inhibitors exhibit significant cytotoxicity due to lack of selectivity for integrase (Hong et al. (1997) J. Med. Chem. 40:930–6, Zhao et al. (1997) J. Med. Chem. 40:937–41, Neamati et al. (1998) J. Med. Chem. 41:3202–9, and Neamati et al. (2002) J. Med. Chem. 45:5661–70). In fact, the similarities between retroviral integrases and topoisomerase prompted the first study that evaluated topoisomerase I and II poisons against integrase (Fesen et al. (1993) Proc. Natl. Acad. Sci. USA 90:2399–403). As a result, we have been routinely using topoisomerases as a counter screen for integrase inhibitors (Neamati et al. (1998) J. Med. Chem. 41:3202–9; Neamati et al. (2002) J. Med. Chem. 45:5661–70; Neamati et al. (1997) In Keystone Symposia on Molecular and Cellular Biology, Santa Fe. Keystone Symposia, p. 32; Neamati et al. (1997) Mol. Pharmacol. 52:1041–55; and Neamati et al. (1997) J. Med. Chem. 40:942–51). In a more recent study, we showed that even the most selective integrase inhibitors identified thus far also inhibit RAG1/2 enzymes that are essential for VDJ recombination (Melek et al. (2002) Proc. Natl. Acad. Sci. USA 99:134–7). All these enzymes share a similar chemistry of DNA binding, DNA cleavage, and recombination that require divalent metal (Mn^{2+} and Mg^{2+} but not Ca^{2+}; Neamati et al. (2000) Adv. Pharmacol. 49:147–65). Because integrase belongs to a large family of polynucleotidyl transferases (Rice et al. (1996) Curr. Opin. Struct. Biol. 6:76–83), it is plausible that certain of our inhibitors could target an unknown DNA-processing enzyme.

SUMMARY OF THE INVENTION

This invention is based, at least in part, on the unexpected discovery that novel compounds described below can be used for treating cancer and disorders associated with angiogenesis function.

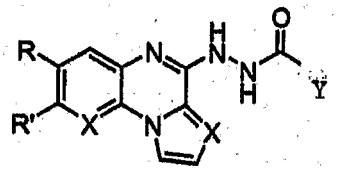
Accordingly, in one aspect, the invention features a compound of Formula I,



Formula I

wherein X = CH or N; Z = O or S; R = alkyl, halogen, acetyl, O-alkyl, or N-alkyl; R' = alkyl, halogen, acetyl, O-alkyl, or N-alkyl; and Y = alkyl, heterocyclic aromatic, aliphatic, sugar, or lipid.

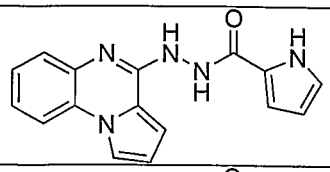
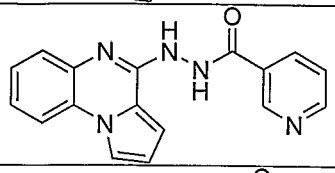
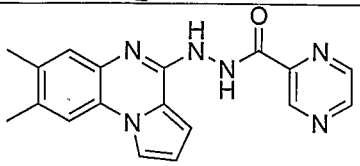
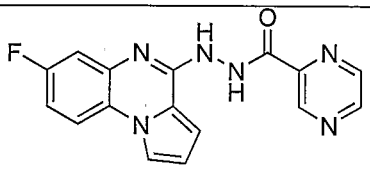
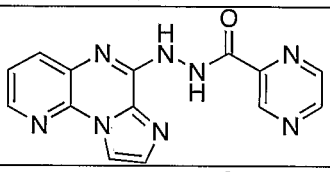
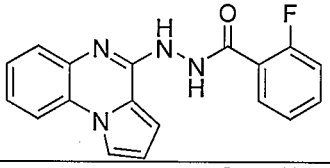
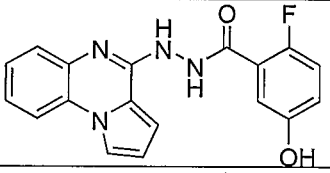
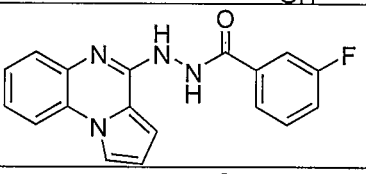
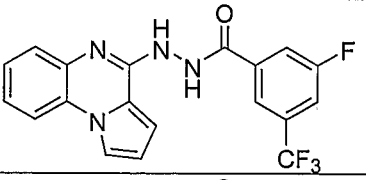
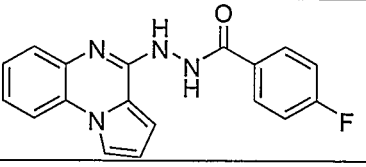
In another aspect, the invention features a compound of Formula II,

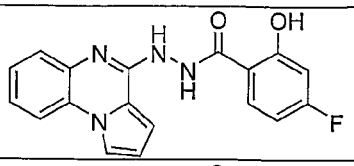
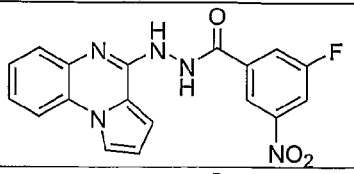
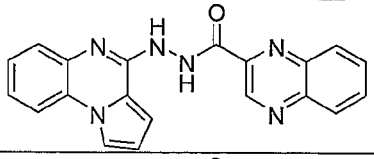
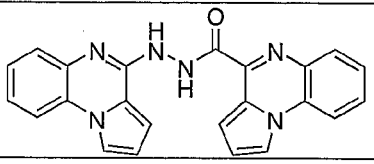


Formula II

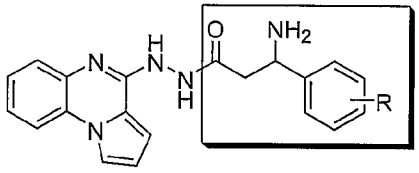
wherein R is H, alkyl, or halogen; R' is H, alkyl, or halogen; X is CH or N; and Y comprises a homocyclic or heterocyclic ring, wherein Y is 3-, 5-, or 6-pyrazinyl or 3-, 4-, 5-, or 6-pyridinyl when R is H, R' is H, X is CH, and Y is pyrazinyl or pyridinyl.

For example, the alkyl may be Me, the halogen may be F, and Y may be pyrrolyl, pyridinyl, pyrazinyl, fluorophenyl, quinoxalinyl, or pyrrolo-quinoxalinyl. More specifically, in one embodiment, R is H, R' is H, and X is CH; in another embodiment, R is Me, R' is Me, and X is CH; in still another embodiment, R is F, R' is H, and X is CH; and in yet another embodiment, R is H, R' is H, and X is N. Examples of such compounds include SC141-144, SC148, and SC166-174.

SC141		1*H*-Pyrrole-2-carboxylic acid *N*'-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide
SC142		Nicotinic acid *N*'-pyrrolo[1,2-*a*] quinoxalin-4-yl-hydrazide
SC143		Pyrazine-2-carboxylic acid *N*'-(7,8-dimethyl-pyrrolo[1,2-*a*]quinoxalin-4-yl)-hydrazide
SC144		Pyrazine-2-carboxylic acid *N*'-(7-fluoro-pyrrolo[1,2-*a*]quinoxalin-4-yl)-hydrazide
SC148		N'-Imidazo[1,2-a]pyrido[3,2-e]pyrazin-6-ylpyrazine-2-carbohydrazide
SC166		2-Fluoro-benzoic acid N'-pyrrolo[1,2-a]quinoxalin-4-yl-hydrazide
SC167		2-Fluoro-5-hydroxy-benzoic acid N'-pyrrolo[1,2-a]quinoxalin-4-yl-hydrazide
SC168		3-Fluoro-benzoic acid N'-pyrrolo[1,2-a]quinoxalin-4-yl-hydrazide
SC169		3-Fluoro-5-trifluoromethyl-benzoic acid N'-pyrrolo[1,2-a]quinoxalin-4-yl-hydrazide
SC170		4-Fluoro-benzoic acid N'-pyrrolo[1,2-a]quinoxalin-4-yl-hydrazide

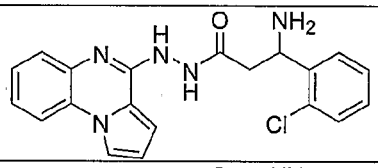
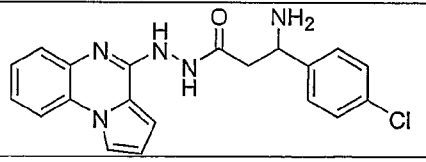
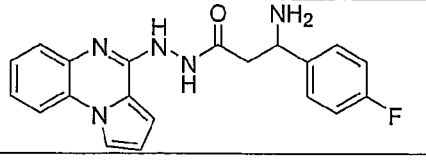
SC171		4-Fluoro-2-hydroxy-benzoic acid N'-pyrrolo[1,2-a]quinoxalin-4-yl-hydrazide
SC172		3-Fluoro-5-nitrobenzoic acid N'-pyrrolo[1,2-a]quinoxalin-4-yl-hydrazide
SC173		Quinoxaline-2-carboxylic acid N'-pyrrolo[1,2-a]quinoxalin-4-yl-hydrazide
SC174		Pyrrolo[1,2-a]quinoxaline-4-carboxylic acid N'-pyrrolo[1,2-a]quinoxalin-4-yl-hydrazide

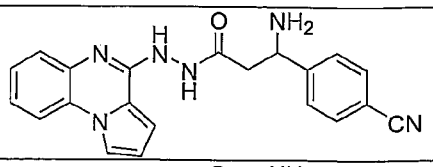
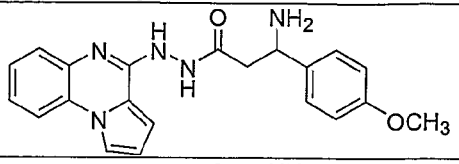
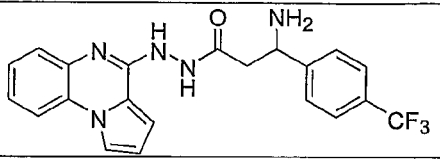
In one embodiment, the compound is of Formula III,



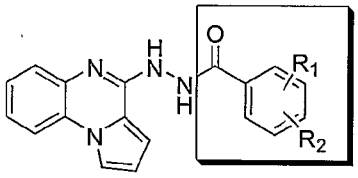
Formula III

wherein R = *o*-Cl, *p*-Cl, *p*-F, *p*-CN, *p*-OMe, or *p*-CF_3. Examples of such compounds include SC160-165.

SC160		3-Amino-3-(2-chloro-phenyl)-propionic acid *N*'-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide
SC161		3-Amino-3-(4-chloro-phenyl)-propionic acid *N*'-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide
SC162		3-Amino-3-(4-fluoro-phenyl)-propionic acid *N*'-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide

SC163		3-Amino-3-(4-cyano-phenyl)-propionic acid *N'*-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide
SC164		3-Amino-3-(4-methoxy-phenyl)-propionic acid *N'*-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide
SC165		3-Amino-3-(4-trifluoromethyl-phenyl)-propionic acid *N'*-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide

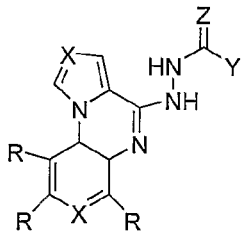
In another embodiment, the compound is of Formula IV,



Formula IV

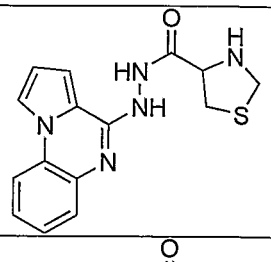
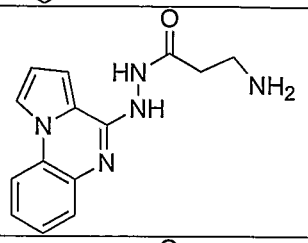
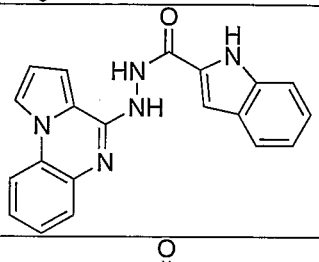
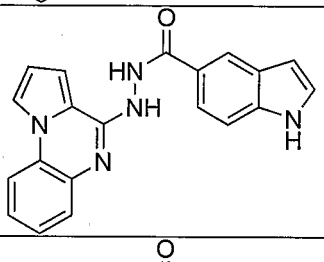
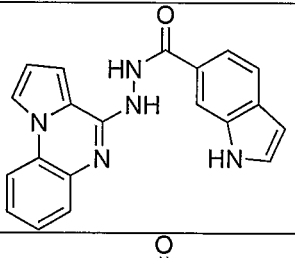
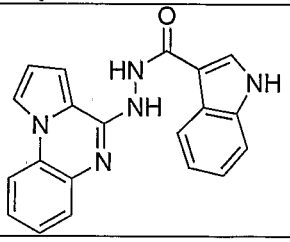
wherein R_1 = 3-NH_2, R_2 = 5-CF_3; R_1 = 5-NH_2, R_2 = 2-NO_2; R_1 = 4-NH_2, R_2 = 3-NO_2; R_1 = 2-NH_2, R_2 = 5-OH; R_1 = 4-NH_2, R_2 = H; R_1 = 3-NH_2, R_2 = H; or R_1 = 2-NH_2, R_2 = H.

The invention also features a compound of Formula V,

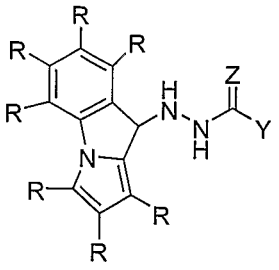


Formula V

wherein X = CH or N; Z = O or S; R = alkyl, halogen, acetyl, O-alkyl, or N-alkyl; and Y = alkyl, heterocyclic aromatic, aliphatic, sugar, or lipid. Examples of such compounds include SC153-158.

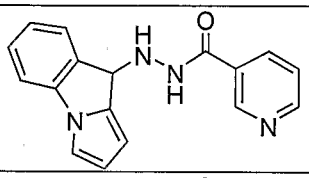
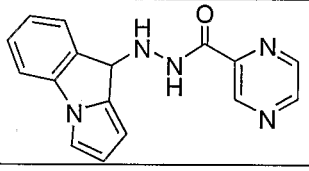
SC153		Thiazolidine-4-carboxylic acid *N'*-pyrrolo [1,2-*a*]quinoxalin-4-yl-hydrazide
SC154		3-Amino-propionic acid *N'*-pyrrolo [1,2-*a*]quinoxalin-4-yl-hydrazide
SC155		1*H*-Indole-2-carboxylic acid *N'*-pyrrolo [1,2-*a*]quinoxalin-4-yl-hydrazide
SC156		1*H*-Indole-5-carboxylic acid *N'*-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide
SC157		1*H*-Indole-6-carboxylic acid *N'*-pyrrolo [1,2-*a*]quinoxalin-4-yl-hydrazide
SC158		1*H*-Indole-3-carboxylic acid *N'*-pyrrolo [1,2-*a*]quinoxalin-4-yl-hydrazide

Another compound of the invention is of Formula VI,

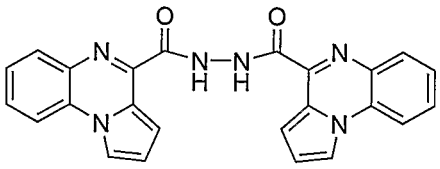


Formula VI

wherein Z = O or S; R = alkyl, halogen, acetyl, O-alkyl, or N-alkyl; and Y = alkyl, heterocyclic aromatic, aliphatic, sugar, or lipid. Examples of such compounds include SC175-176.

SC175		Nicotinic acid *N'*-9*H*-pyrrolo[1,2-a] indol-9-yl-hydrazide
SC176		Pirazine-2-carboxylic acid *N'*-9*H*-pyrrolo[1,2-a]indol-9-yl-hydrazide

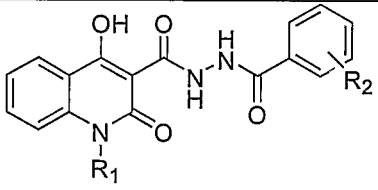
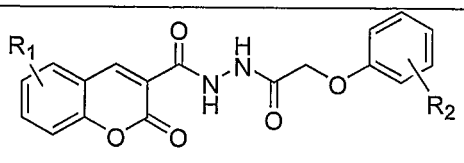
Moreover, a compound of Formula VII is also within the invention:

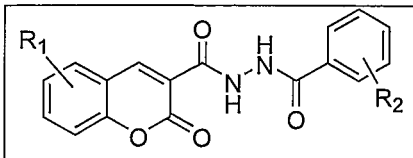


Formula VII

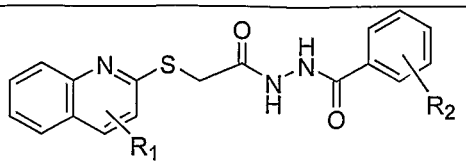
SC147

In addition, the invention features a compound of any of Formulas 1-19,

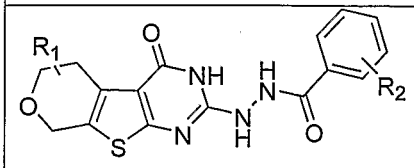
 Formula 1	 Formula 2



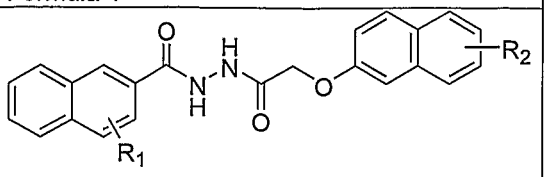
Formula 3



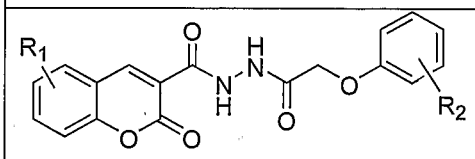
Formula 4



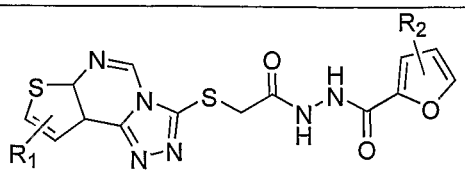
Formula 5



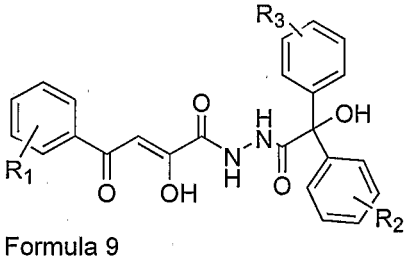
Formula 6



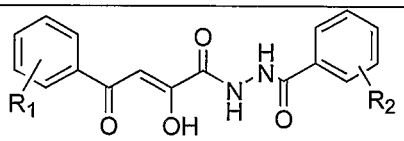
Formula 7



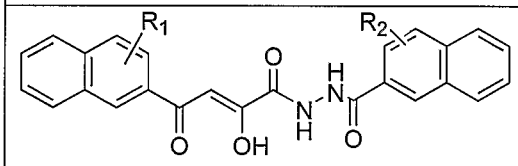
Formula 8



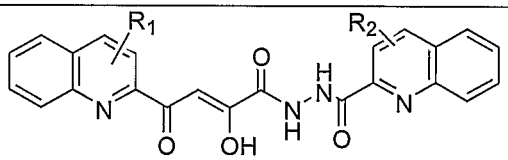
Formula 9



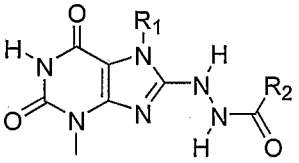
Formula 10



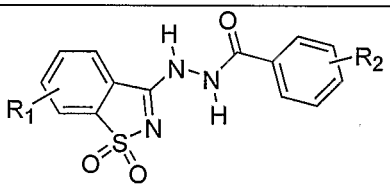
Formula 11



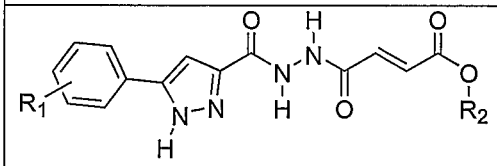
Formula 12



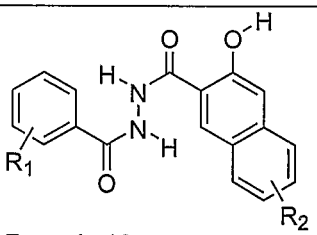
Formula 13



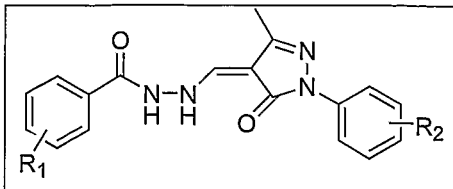
Formula 14



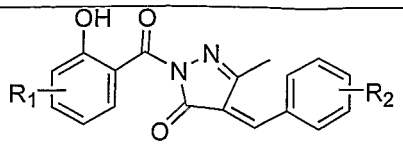
Formula 15



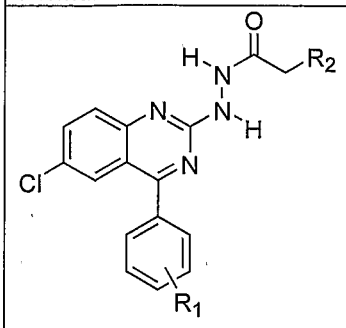
Formula 16



Formula 17



Formula 18

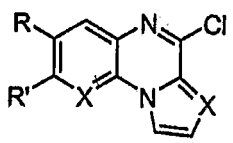


Formula 19

wherein each of R1, R2, and R3 is a hydrogen, halogen, hydroxyl, alkyl, substituted alkyl, alkenyl, substituted alkenyl, phenyl, substituted phenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, or an organic group containing 1-20 carbon atoms in a linear, branched, or cyclic structural format. The substituted alkyl, substituted alkenyl, substituted phenyl, substituted aryl, or substituted heteroaryl may contain a halo, hydroxyl, alkoxy, alkylthio, phenoxy, aroxy, cyano, isocyano, carbonyl, carboxyl, amino, amido, sulfonyl, or substituted heterocyclic, sugar, or peptide substitution. The organic group may include a heteroatom of oxygen, sulfur, or nitrogen.

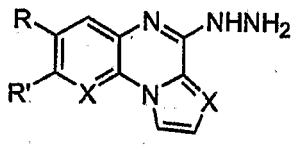
Specific examples of such compounds include SC20-37, SC201-266, SC268, and SC270-280. The structures of SC20-37, SC201-266, SC268, and SC270-280 are shown below.

The invention provides a method of preparing the compounds of the invention. For example, compounds SC141-144, SC148, SC153-158, and SC160-174 can be prepared as follows: First, contact hydrazine monohydrate with a compound (13a, 13b, 13c, or 13d) of Formula VIII,



Formula VIII

wherein R is H, R' is H, and X is CH (13a); R is Me, R' is Me, and X is CH (13b); R is F, R' is H, and X is CH (13c); or R is H, R' is H, and X is N (13d), to form a compound (14a, 14b, 14c, or 14d, respectively) of Formula IX,

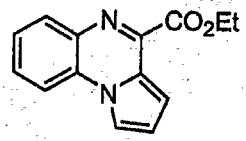


Formula IX

wherein R is H, R' is H, and X is CH (14a); R is Me, R' is Me, and X is CH (14b); R is F, R' is H, and X is CH (14c); or R is H, R' is H, and X is N (14d). SC141 can then be formed by contacting 14a with pyrrole-2-carboxylic acid chloride; SC142 by contacting 14a with nicotinoyl chloride hydrochloride; SC143, SC144, and SC148 by contacting 14b, 14c, and 14d with 2-pyrazinecarboxylic acid in the presence of 2,2'-dipyrildisulphide and triphenylphosphine, respectively; SC153 by contacting 14a with *N*-BOC-thiazolidine-4-carboxylic acid in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) / 4-(dimethylamino)pyridine (DMAP) and then trifluoroacetic acid (TFA) / anisole; SC154 by contacting 14a with *N*-BOC-β-alanine in the presence of EDC/DMAP and then TFA / anisole; SC155, SC156, SC157, and SC158 by contacting 14a with 2-indolecarboxylic acid, 5-indolecarboxylic acid, 6-indolecarboxylic acid, and 3-indolecarboxylic acid in the presence of EDC/DMAP, respectively; SC160, SC161, SC162, SC163, SC164, and SC165 by contacting 14a with Boc-3-amino-3-(2-chlorophenyl)propionic acid, Boc-3-amino-3-(4-chlorophenyl)propionic acid, Boc-3-amino-3-(4-fluorophenyl)propionic acid, Boc-3-amino-3-(4-cyanophenyl)propionic acid, Boc-3-amino-3-(4-methoxyphenyl)propionic acid, and Boc-3-amino-3-(4-trifluoromethylphenyl)propionic acid in the presence of EDC/DMAP followed by TFA and anisole, respectively; SC166, SC167, SC168, SC169, SC170, SC171, and SC172 by contacting 14a with 15a-g (15a: 2-fluorobenzoic acid, 15b: 2-fluoro-4-hydroxybenzoic acid, 15c: 3-fluorobenzoic acid, 15d: 3-fluoro-4-(trifluoromethyl)benzoic acid, 15e: 4-

fluorobenzoic acid, 15f: 4-fluoro-2-hydroxybenzoic acid, 15g: 3-fluoro-5-nitrobenzoic acid), in the presence of EDC/DMAP followed by TFA and anisole, respectively; SC173 by contacting 14a with 2-quinoxalinecarboxylic acid, dichloromethane, triphenylphosphine, and 2,2'-dipyridyl disulfide; and SC174 by contacting 14a with pyrrolo[1,2-*a*]quinoxaline-4-carboxylic acid, dichloromethane, triphenylphosphine, and 2,2'-dipyridyl disulfide.

Compound SC147 can be prepared by contacting hydrazine monohydrate with a compound of formula X.

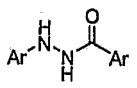


Formula X

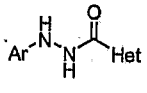
Compound SC175 can be prepared by contacting nicotinoyl chloride hydrochloride with 9-hydrazino-9*H*-pyrrolo[1,2-*a*]indole and pyridine. Compound SC176 can be prepared by contacting pyrazine-2-carbonyl chloride hydrochloride with 9-hydrazino-9*H*-pyrrolo[1,2-*a*]indole and pyrazine.

The invention further provides a pharmaceutical composition comprising an effective amount of one or more compounds of the invention and a pharmaceutically acceptable carrier. The composition may further comprise an effective amount of one or more other agents for treating cancer or a disorder associated with angiogenesis function, e.g., taxol, doxorubicin, or 5-FU.

The invention also features a packaged product comprising a container; an effective amount of a compound of formula XI or XII,



Formula XI

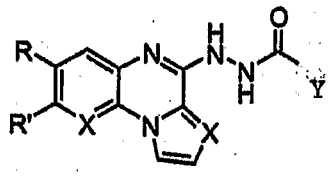


Formula XII

wherein Ar comprises an aromatic ring and Het comprises a heterocyclic ring; and an insert associated with the container, indicating administering the compound for treating non-small cell lung cancer, CNS cancer, ovarian

cancer, breast cancer, renal cancer, prostate cancer, age-related macular degeneration, macular dystrophy, or diabetes.

Furthermore, the invention provides a packaged product comprising a container; an effective amount of a compound of Formula II,



Formula II

wherein R is H, alkyl, or halogen; R' is H, alkyl, or halogen; X is CH or N; and Y comprises a homocyclic or heterocyclic ring; and an insert associated with the container, indicating administering the compound for treating cancer or a disorder associated with angiogenesis function.

Another packaged product comprises a container; an effective amount of a compound of the invention; and an insert associated with the container, indicating administering the compound for treating cancer or a disorder associated with angiogenesis function.

A product of the invention may further comprise an effective amount of one or more other agents for treating cancer or a disorder associated with angiogenesis function, e.g., taxol, doxorubicin, or 5-FU.

Examples of cancer include leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, breast cancer, renal cancer, and prostate cancer; examples of disorders associated with angiogenesis function include age-related macular degeneration, macular dystrophy, and diabetes.

Also within the scope of the invention is a method of treating a subject by administering to a subject in need thereof an effective amount of a compound described above. The subject may be identified as being suffering from or at risk for developing cancer or a disorder associated angiogenesis function. In particular, the cancer may be leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, breast cancer, renal cancer, or prostate cancer; and the disorder associated with angiogenesis function may be age-related macular

degeneration, macular dystrophy, or diabetes. The method may further comprise administering to the subject an effective amount of one or more other agents for treating cancer or a disorder associated with angiogenesis function, e.g., taxol, doxorubicin, or 5-FU. The compound and the one or more other agents may be administered simultaneously or sequentially.

In addition, the invention features a method of monitoring treatment of a subject by administering to a subject having cancer cells or cells associated with an angiogenesis function disorder a compound described above and measuring the survival of the cells, the growth of the cells, or a combination thereof using PET imaging. The subject may be suffering from leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, breast cancer, renal cancer, prostate cancer, age-related macular degeneration, macular dystrophy, or diabetes. The subject may be an animal, e.g., a mouse, and the cells may be xenografted human cells. In one embodiment, the subject is a human.

Furthermore, the invention provides a method of profiling gene expression. The method comprises contacting a test cell with a compound described above and profiling gene expression in the test cell. The test cell may be a cancer cell or a cell associated with an angiogenesis function disorder. More specifically, the test cell may be a leukemia cell, non-small cell lung cancer cell, colon cancer cell, CNS cancer cell, melanoma cell, ovarian cancer cell, breast cancer cell, renal cancer cell, prostate cancer cell; or a cell associated with age-related macular degeneration, macular dystrophy, or diabetes. The method may further comprise comparing gene expression in the test cell with that in a control cell, which may be contacted with another compound with known action or resistant to the compound used to contact the test cell.

The invention also provides a method of modulating gene expression in a cell. The method comprises contacting a cell with a compound described above, thereby modulating (increasing or decreasing) expression of one or more genes in the cell. The cell may be a cancer cell or a cell associated with an angiogenesis function disorder. Specifically,

the cell may be a leukemia cell, non-small cell lung cancer cell, colon cancer cell, CNS cancer cell, melanoma cell, ovarian cancer cell, breast cancer cell, renal cancer cell, prostate cancer cell; or a cell associated with age-related macular degeneration, macular dystrophy, or diabetes. Examples of the one or more genes include small proline-rich protein 1A; GTP binding protein overexpressed in skeletal muscle; interleukin 24; sestrin 2; hypothetical protein MGC4504; cyclin-dependent kinase inhibitor 1A (p21); early growth response 1; ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d isoform 2; AXIN1 up-regulated 1; dual specificity phosphatase 5; superoxide dismutase 2, mitochondrial; heparin-binding epidermal growth factor-like growth factor; A disintegrin and metalloproteinase domain 19 (meltrin beta); endothelial PAS domain protein 1; inositol 1,4,5-triphosphate receptor, type 1; tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor); fibrinogen, gamma polypeptide; RAB20, member RAS oncogene family; protein kinase, AMP-activated, gamma 2 non-catalytic subunit; oncostatin M receptor; cathepsin B; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; BCL2/adenovirus E1B 19kDa interacting protein 3; integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61); dual specificity phosphatase 10; cell cycle control protein SDP35; plexin C1; microphthalmia-associated transcription factor; calpain small subunit 2; hypothetical protein DKFZp434L142; MEGF 10 protein; EphA2; jagged 1 (Alagille syndrome); hemicentin; low density lipoprotein receptor (heparin-binding epidermal growth factor-like growth factor); tyrosinase-related protein 1; tyrosinase (oculocutaneous albinism IA); dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2); laminin, beta 3; MAX dimerization protein 1; CDK4-binding protein p34SEI1; Homo sapiens cDNA FLJ42435 fis, clone BLADE2006849; growth arrest and DNA-damage-inducible, beta; cycline-dependent kinase inhibitor 2B (p15, inhibits CDK4); Diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor); syntaxin binding protein 6 (amisyn); transport-secretion protein 2.2; Arg/Abl-interacting

protein ArgBP2; hypothetical protein DJ667H12.2; Homo sapiens cDNA FLJ37284 fis, clone RAMY2013590; BCL2, BCL2L1, JUN, JUNB, MAD, MAX, TNFRSF1A, TP53, NFKB1, TNFSF10, CASP1, PCNA, TNFAIP1, DAP, KDR, MAP3K14, CCNA2, CDC2, CDK7, CDK8, CDKN1A, CDKN1B, CDKN2A, CDKN2C, E2F1, E2F4, E2F5, MYC, RB1, RBL2, CCND3, CCNG1, CCNE1, CDC25C, TGFBR2, TGIF, TRAF4, CYP1A2, PTGS2, (p21,) p27, cyclin A, cdk1, p53, cyclin E, cdc25, p130, NFKB, c-MYC, COX2, Bcl-X_L, annexin V, caspase 1, TNF receptor, microtubule-associated protein 4, microtubule affinity-regulating kinase 2, microtubule affinity-regulating kinase 4, transducer of ERBB2, vascular endothelial growth factor B, vascular endothelial growth factor, ankyrin repeat and MYND domain containing 1, RAB4B, putative prostate cancer tumor suppressor, pre-B-cell leukemia transcription factor 2, T-cell leukemia translocation altered gene, leukemia inhibitory factor, interferon regulatory factor 2 binding protein, interferon stimulated gene (20kDa), interferon gamma receptor 2, 28kD interferon responsive protein, polymerase (RNA) III, peroxisomal proliferator-activated receptor A interacting complex 285, RAD50 homolog (S. cerevisiae), MAX dimerization protein 3, kruppel-like factor 16, apolipoprotein L (6), X-ray repair complementing defective repair, mitogen-activated protein kinase 3, phosphatidylinositol 4-kinase type II, mitogen-activated protein kinase 12, protein kinase (AMP-activated, alpha 2 catalytic subunit), pyruvate dehydrogenase phosphatase regulatory subunit, phospholipase D3, inositol 1,4,5-triphosphate receptor (type 3), retinoic acid receptor (alpha), tumor necrosis factor receptor superfamily, Enolase 2 (gamma, neuronal), stanniocalcin 2, apelin, plexin B2, cathepsin Z, histone 1 (H2bc), histone 1 (H3h), β-tubulin, myc promoter-binding protein (MPB-1), retinoblastoma-binding protein 7, vimentin, enolase, phosphopyruvate hydratase beta, and mitochondrial ATP synthase beta chain.

The invention further provides a method of modulating cell growth, cell cycle, or apoptosis. The method comprises contacting a cell with a compound of claim 1 or 3, thereby inhibiting cell growth, arresting cell

cycle, or inducing apoptosis. Examples of the cell include a leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, breast cancer, renal cancer, or prostate cancer cell.

The above-mentioned and other features of this invention and the manner of obtaining and using them will become more apparent, and will be best understood, by reference to the following description, taken in conjunction with the accompanying drawings. The drawings depict only typical embodiments of the invention and do not therefore limit its scope.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates flow cytometric analysis of the cell cycle profile of MDA-MB-435 cells treated with SC144. Cells were exposed for 16 h, 24 h and 48 h to SC144, stained with propidium iodide (PI) and analyzed for perturbation in the cell cycle.

Figure 2 illustrates apoptosis analysis of MDA-MB-435 cells treated with SC144 and CPT (IC_{80}). Cells were stained with annexin V/PI and analyzed by flow cytometry. Cells in the bottom left quadrant of each panel (Annexin V-negative, PI-negative) are viable, whereas cells in the bottom right quadrant (Annexin V-positive, PI-negative) are in the early stages of apoptosis, and cells in the top right quadrant (Annexin V-positive, PI-positive) are in later stages of apoptosis and necrosis.

Figure 3. (A) is a schematic outline of tumor growth and dosing in xenograft models. Athymic nude mice implanted with MDA-MB-435 cells were treated with the indicated doses of SC144 by daily i.p. administration for five-days. (B) illustrates that SC144 reduced the size of human breast cancer xenografts at doses of 0.3, 0.8 and 4 mg/kg. Tumor growth was monitored for five weeks. Values represent the median tumor weight for each group. (C) shows %T/C for each treatment group calculated on the last day of experiment (bars ± SD).

Figure 4. (A) shows representative images of SC144 treated mice. (B) shows comparison of the tumor size of SC144 treated (4 mg/kg) and control. (C) shows tumors incised from mice shown in panel B.

Figure 5 demonstrates that SC144 induce remarkable necrosis of tumor tissue. H&E staining of untreated tumor tissue (A) and SC144 treated tissue (B) were prepared at day 70. In general, greater than 80% necrosis was observed in treated tumors (left side of panel B) and the non-necrotic cells (right side of panel B) are in early stages of apoptosis.

Figure 6 demonstrates that SC144 does not exhibit organ toxicity. H&E staining of SC144 treated kidney tissue (A), liver tissue (B) and cardiac tissue (C) shows normal pattern.

Figure 7 illustrates inhibition of human CYP3A4 by ketoconazole, SC144 and its analog SC24. The metabolism of fluorescent substrates by human cDNA-expressed CYP3A4 was assessed by incubation in 96 well plate at 37°C. Metabolism of 7-benzyloxy-4-trifluoromethylcoumarin (BFC) was assayed by measuring the production of the corresponding 7-hydroxy-4-trifluoro-methylcoumarin.

Figure 8 shows PET imaging (slice thickness 0.6 mm) of a nude mouse implanted with human breast cancer (MDA-MB-435) cells. Top row, baseline scans: (A) equilibrium-phase FDG, 30 min post injection; (B) FMAU, 10 min post injection; (C) FMAU, 60 min post injection. Bottom row, follow-up scans: (D) FDG, 30 min post injection; (E) FMAU, 10 min post injection; (F) FMAU, 60 min post injection. The mouse was imaged on consecutive days with FDG and FMAU (baseline), then treated with daily i.p. injections of SC144 at 4 mg/kg. After five days of dosing, the drug treatment was discontinued and the follow-up scans were obtained on days 6 and 7.

Figure 9 illustrates comparison of gene expression profiles in two independent experiments. (A) A scatter plot of untreated control samples D565 versus D566 and (B) SC144 treated pairs D571 and D572 Chips. (C) A plot of t-statistic (x-axis), representing the significance level, versus log mean expression difference (representing fold change) in SC144 treated cells versus untreated control.

Figure 10 illustrates that SC144 shows a unique pattern of activity distinct from other classes of compounds. (A) A three-principal

components analysis of genes for all 14 observations and (B) hierarchical cluster analysis generated by Genetrix™.

Figure 11 shows bioinformatic analysis of genes by molecular function using Genetrix™ tools.

Figure 12 shows a list of genes derived from InterPro classification tools implemented in Genetrix™.

Figure 13 shows subset classification of common genes identified between SC144 and etoposide.

Figure 14 shows subset classification of genes in common among SC144, mitoxantrone, and camptothecin.

Figure 15 illustrates prediction of drug absorption. Fast polar surface area in Angstrom^2 for each compound is plotted against their corresponding calculated partition coefficient. The area encompassed by the ellipse is a prediction of good absorption with no violation of ADMET properties. On the basis of Egan et al. ((2000) J. Med. Chem. 43:3867–77) absorption model, the outer ellipse represents a 99% confidence, whereas the inner ellipse a 95% confidence.

Figure 16 shows time- (A) and concentration-dependent (B) inhibition of DU145 cells by SC21 and CPT.

Figure 17 depicts flow cytometric analysis of the cell cycle profiles of DU145, PC3, MDA-MB-435, and HEY cells treated with SC21. Cells were exposed for 24, 48, and 72 h to SC21 (IC_{50}) then harvested, stained with propidium iodide and analyzed for perturbation in the cell cycle. SC21 induced a G_0/G_1 phase arrest in DU145 and MDA-MB-435 cells and S phase arrest in PC3 and HEY cells. Control cells shown were measured at 24 h and, as expected, no significant changes were observed in the control cells at 48 and 72 h.

Figure 18 shows percentage of apoptosis calculated by measuring sub-G_0/G_1 population using flow cytometry. Apoptotic cell population increased with time in PC3 and DU145 treated with SC21 and CPT.

Figure 19 illustrates apoptosis analysis of DU145 cells treated with SC21 or CPT (IC_{80}). Cells were treated with SC21 or CPT for 24, 48,

and 72 h, harvested, stained with annexin V / propidium iodide and analyzed by flow cytometry. Untreated control cells (24 and 48 h) were also included in the analysis. Annexin VFITC signals are recorded in FL1-H or red channel and propidium iodide in FL2-H or green channel. Cells in the bottom left quadrant (annexin V-negative, propidium iodide-negative) are viable, whereas cells in the right quadrant (annexin V-positive, propidium iodide-negative) are in the early stages of apoptosis, and the cells in the top right quadrant (annexin V-positive, propidium iodide-positive) are in later stages of apoptosis and necrosis.

Figure 20. (A) is a schematic outline of tumor growth and dosing in PC3 mice xenografts. (B) shows that SC21 reduced the size of human prostate cancer xenografts. Athymic nude mice implanted with PC3 cells were treated with the indicated concentration of SC21 through daily i.p. administration for 5 d. Tumor growth was monitored for 5 wks. Values represent the tumor weight (mean $\pm$ SD) for each group. (C) depicts dose-response to SC21 in the PC3 xenograft. Values represent the %T/C from each treatment group on the last day of measurement (after 5 wks); bars, $\pm$ SD. Treatment with SC21 significantly reduced tumor growth (%T/C V50%) at both doses as compared with the control.

Figure 21 illustrates RT-PCR gene expression analysis. Total RNA form T24 cells was isolated and cDNA was synthesized with 2.5 ug of total RNA. Standardized RT-PCR was performed with GENE system I gene expression kit (Gene Express Inc.). Each kit contains a mixture of the internal competitive templates and the corresponding primers.

Figure 22 shows SC23-induced expression (number of molecules) of selected genes from Table 8 normalized against 10^6 molecule of β-actin. Total RNA form T24 cells were isolated after 3h, 6h, 12h, 24h, and 48h exposure to SC23.

Figure 23 is a schematic representation of pathways involved in cell cycle (left) and apoptosis (right).

Figure 24 is a representative example of comparison of gene expression profiles in two independent experiments. (A) A scatter plot of

untreated control samples D565 versus D566 Chips, (B) etoposide treated pairs D720 and D721 Chips, and (C) mitoxantrone treated pairs D724 and D725 Chips.

Figure 25 illustrates that SC23 shows a pattern of activity most similar to taxol. (A) A series of scatter plots comparing SC23 gene expression (18,000 genes after removing all the noise and low expressors) with 5FU, CPT, etoposide, taxol, and mitoxantrone. (B) Same as panel A but only those genes that were altered by at least five fold change are plotted.

Figure 26 is a Venn diagram showing the number of genes overlapping among three compounds. The diagram was generated from a total of 878 genes that were more than five fold altered in response to SC23, 5-FU, and taxol treatment.

Figure 27 illustrates that SC23 shows a pattern of activity most similar to taxol. (A) A three-principal components analysis of genes for all 10 observations. (B) Hierarchical cluster analysis generated by Genetrix™.

Figure 28 depicts SC23-induced alteration of protein expression. T24 cells were treated with IC_{50} (lane 2) and IC_{80} (lane 3) doses of SC23 for 72 h. Lane 1: control untreated cells.

Figure 29 shows two-dimensional gel electrophoresis of SC23 treated T24 cells. Cells were treated for 12, 24, 48 and 72 hr with SC23 (IC_{80} dose). The soluble fraction was then extracted and quantified. 50 mg of protein was loaded in the first dimension gel at 800 V for 16 h. Gels were then equilibrated, separated on a 12% SDS-PAGE gel for the second dimension, stained with CyproRuby, and imaged by Typhoon 9100.

Figure 30 illustrates a selected region of SC23 treated cells from a 2D gel (left) and quantition of spots using PDQuest.

Figure 31 shows MS / MS spectrum of β-tubulin peptide (EVDEQMLNVQNK) and myc promoter-binding protein (MPB-1) peptide (VNQIGSVTESLQACK).

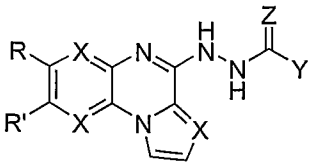
DETAILED DESCRIPTION OF THE INVENTION

A series of compounds were designed based on three-dimensional anti-tumor structural modeling (specific for inhibition of DNA processing enzymes) integrated with predictive pharmacokinetic (PK) simulations. Several of the compounds showed remarkable cytotoxicity patterns against a panel of human cancer cell lines. A series of 200 compounds were tested against several drug-resistant cancer cell lines. SC144 was selected as a lead molecule based on potency and drug like properties. The compound exhibits *in vivo* efficacy against breast cancer xenografts in nude mice with no apparent toxicity. The activity of this compound was independent of the status of the hormone receptor (HR), p53, pRb, p21 or p16. Moreover, SC144 blocked cells in S-phase and induced apoptosis in a cisplatin resistant ovarian cancer cell line (HEY) with activity comparable to that of camptothecin. Considering the cytotoxicity profile displayed by this compound in a variety of *in vitro* models, as well as its effects on cell cycle regulation and apoptosis, SC144 appears to represent a novel and promising candidate for the treatment of cancer and disorders associated with angiogenesis function.

We also evaluated the in vitro activity of SC21 and SC23 against a range of human tumor cell types and the in vivo efficacy of compound SC21 in a PC3 human prostate cancer xenograft model in mice. We determined the effects of SC21 on cell cycle regulation and apoptosis. Our in vitro results show that salicylhydrazides are highly potent compounds effective in both hormone receptor–positive and –negative cancer cells. SC21 induced apoptosis and blocked the cell cycle in G_0/G_1 or S phase, depending on the cell lines used and irrespective of p53, p21, pRb, and p16 status. SC21 effectively reduced the tumor growth in mice without apparent toxicity. Although the mechanism of action of SC21 is not completely elucidated, the effect on cell cycle, the induction of apoptosis and the activity against a panel of tumor cell lines of different origins prompted us to carry out an in-depth preclinical evaluation of SC21. These compounds are potentially useful for treating cancer.

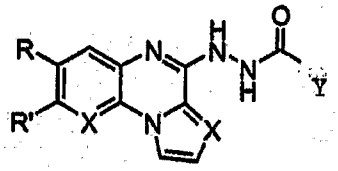
Compounds

A compound of the invention has one of the following formulas:



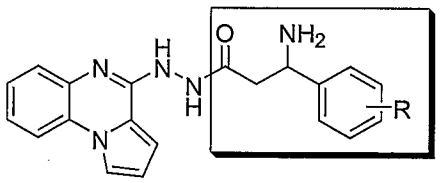
Formula I

wherein X = CH or N; Z = O or S; R = alkyl, halogen, acetyl, O-alkyl, or N-alkyl; R' = alkyl, halogen, acetyl, O-alkyl, or N-alkyl; and Y = alkyl, heterocyclic aromatic, aliphatic, sugar, or lipid;



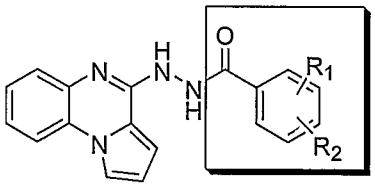
Formula II

wherein R is H, alkyl, or halogen; R' is H, alkyl, or halogen; X is CH or N; and Y comprises a homocyclic or heterocyclic ring, wherein Y is 3-, 5-, or 6-pyrazinyl or 3-, 4-, 5-, or 6-pyridinyl when R is H, R' is H, X is CH, and Y is pyrazinyl or pyridinyl;



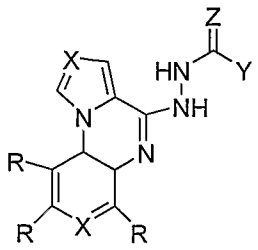
Formula III

wherein R = *o*-Cl, *p*-Cl, *p*-F, *p*-CN, *p*-OMe, or *p*-CF_3;



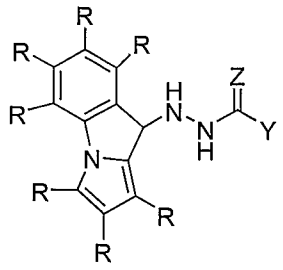
Formula IV

wherein R_1 = 3-NH_2, R_2 = 5-CF_3; R_1 = 5-NH_2, R_2 = 2-NO_2; R_1 = 4-NH_2, R_2 = 3-NO_2; R_1 = 2-NH_2, R_2 = 5-OH; R_1 = 4-NH_2, R_2 = H; R_1 = 3-NH_2, R_2 = H; or R_1 = 2-NH_2, R_2 = H;



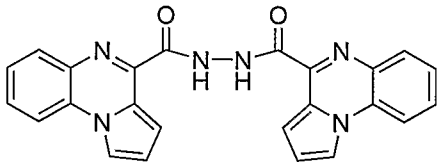
Formula V

wherein X = CH or N; Z = O or S; R = alkyl, halogen, acetyl, O-alkyl, or N-alkyl; and Y = alkyl, heterocyclic aromatic, aliphatic, sugar, or lipid;

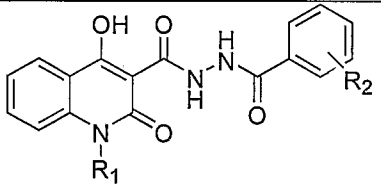


Formula VI

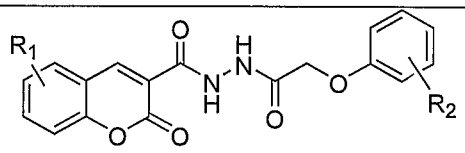
wherein Z = O or S; R = alkyl, halogen, acetyl, O-alkyl, or N-alkyl; and Y = alkyl, heterocyclic aromatic, aliphatic, sugar, or lipid; or



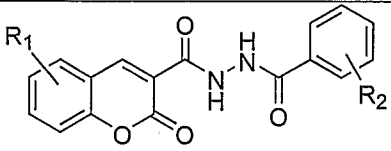
Formula VII



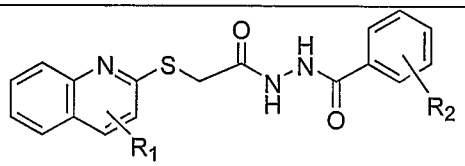
Formula 1



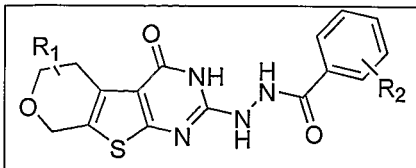
Formula 2



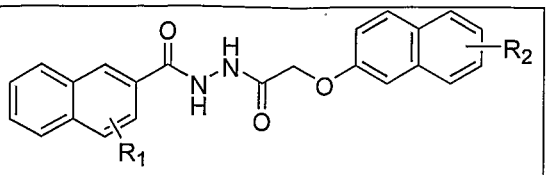
Formula 3



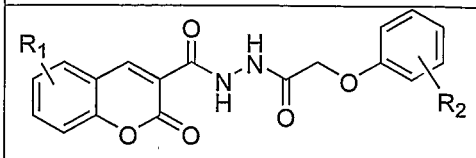
Formula 4



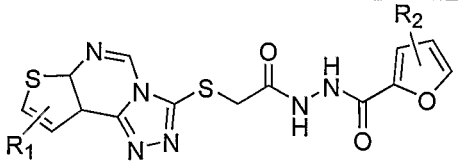
Formula 5



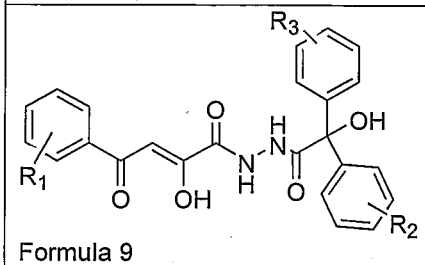
Formula 6



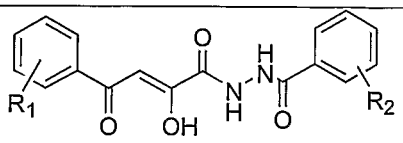
Formula 7



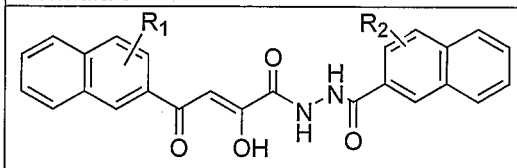
Formula 8



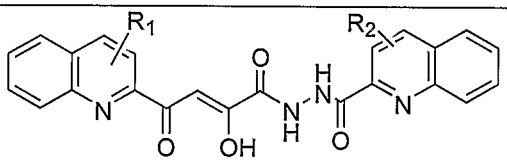
Formula 9



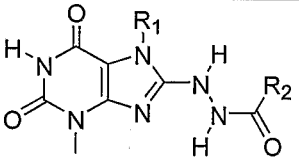
Formula 10



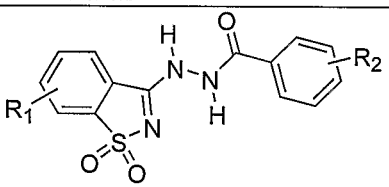
Formula 11



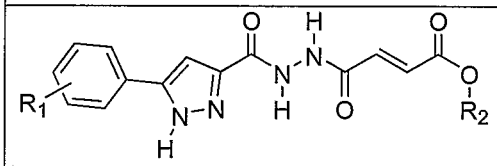
Formula 12



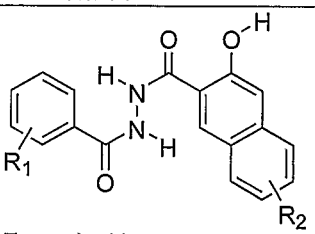
Formula 13



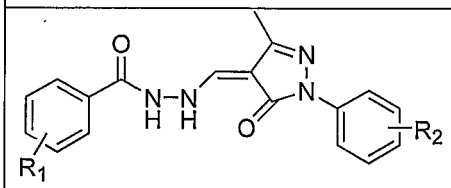
Formula 14



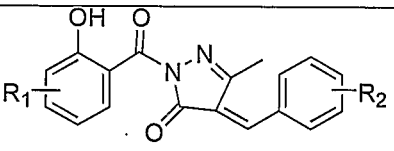
Formula 15



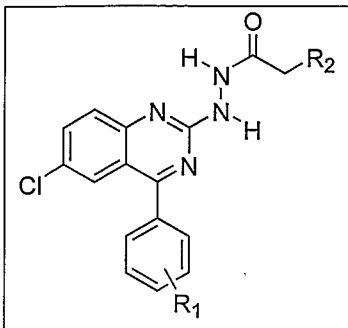
Formula 16



Formula 17



Formula 18



Formula 19

Each of R1, R2, and R3, taken independently or together, is a hydrogen atom, a halogen atom, a hydroxyl group, or any other organic group containing any number of carbon atoms, preferably 1-20 carbon atoms, and optionally including a heteroatom such as oxygen, sulfur, or nitrogen, in a linear, branched or cyclic structural format. Representative R1, R2, and R3 groups include, but are not limited to, alkyl, substituted alkyl, alkenyl, substituted alkenyl, phenyl, substituted phenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl. Representative substitutions include, but are not limited to, halo, hydroxyl, alkoxy, alkylthio, phenoxy, aroxy, cyano, isocyano, carbonyl, carboxyl, amino, amido, sulfonyl, and substituted heterocyclic, sugar, or peptide.

A "homocyclic ring" refers to a closed ring of atoms of the same kind especially carbon atoms; a "heterocyclic ring" refers to a closed ring of atoms of which at least one is not a carbon atom. An "aromatic" group contains one or more benzene rings. Sugars refer to mono, di, and tri-saccharides and lipid refers to long chain aliphatic compound with or without a hydrophilic head group.

A compound of the invention may include both substituted and unsubstituted moieties. The term "substituted" refers to moieties having one, two, three or more substituents, which may be the same or different, each replacing a hydrogen atom. Examples of substituents include, but are not limited to, alkyl, hydroxyl, protected hydroxyl, amino, protected amino, carboxy, protected carboxy, cyano, alkoxy, and nitro. The term "unsubstituted" refers to a moiety having each atom hydrogenated such

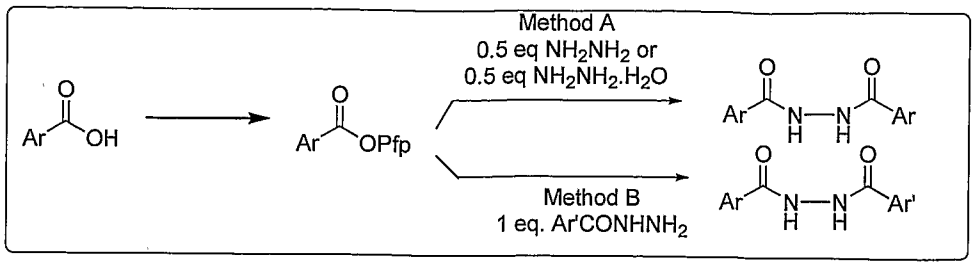
that the valency of each atom is filled. An reactive moiety is "protected" when it is temporarily and chemically transformed such that it does not react under conditions where the non-protected moiety reacts. For example, trimethylsilylation is a typical transformation used to protect reactive functional groups such as hydroxyl or amino groups from their reaction with growing anionic species in anionic polymerization.

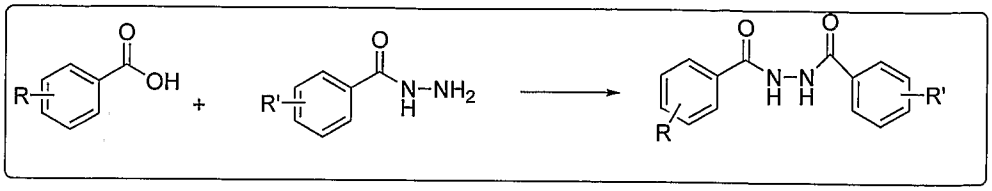
Protected forms of the compounds are included within the scope of the invention. In general, the species of protecting group is not critical, provided that it is stable to the conditions of any subsequent reactions on other positions of the compound and can be removed at the appropriate point without adversely affecting the remainder of the molecule. In addition, one protecting group may be substituted for another after substantive synthetic transformations are complete. Examples and conditions for the attachment and removal of various protecting groups are found in Greene, Protective Groups in Organic Chemistry, 1st ed., 1981, and 2nd ed., 1991. In addition, salts of the compounds are within the scope of the invention. For example, a salt can be formed between a positively charged amino substituent and a negatively charged counterion.

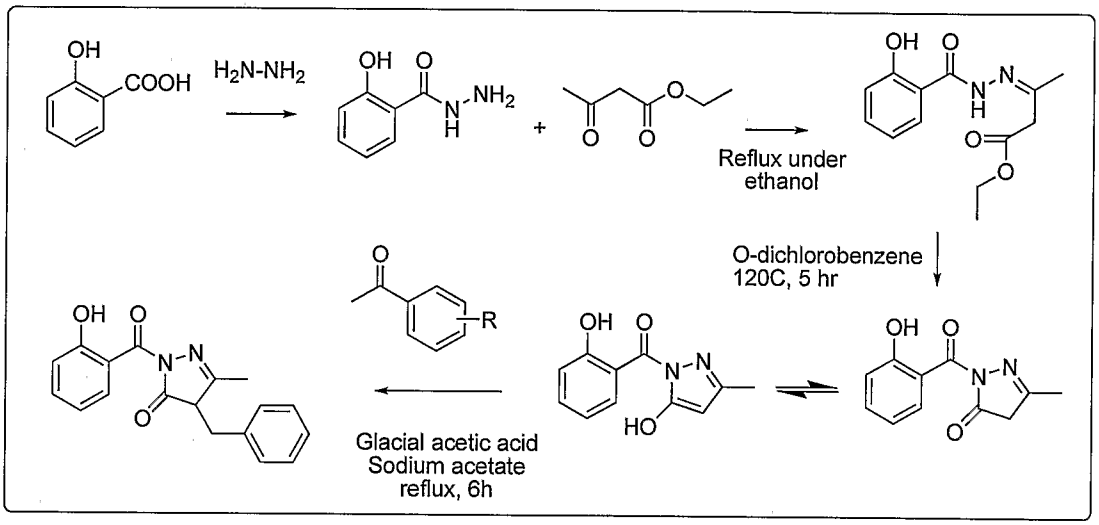
Examples of the compounds of the invention include SC141-144, SC148, SC153-158, SC160-176, SC20-37, SC201-266, SC268, and SC270-280.

Compounds of the invention may be prepared, e.g., according to the schemes described below.

Generally, salicylhydrazides (SCs) can be prepared as follows: A mixture of aromatic acid (10 mmol), pentafluorophenol (11 mmol) and dicylcohexylcarbodiimide (DCC) (10 mmol) in anhydrous dioxane (40 mL) is stirred at room temperature (overnight). Dicyclohexyl urea is removed by filtration through celite, and the filtrate taken to dryness and purified directly by crystallization or by silica gel chromatography (Zhao and Burke (1997) Tetrahedron 53:4219-30).







Pfp – pentafluorophenyl; each of R and R', taken independently or together, is a hydrogen atom, a halogen atom, a hydroxyl group, or any other organic group containing any number of carbon atoms, preferably 1-20 carbon atoms, and optionally including a heteroatom such as oxygen, sulfur, or nitrogen, in a linear, branched or cyclic structural format. Representative R and R' groups include, but are not limited to, alkyl, substituted alkyl, alkenyl, substituted alkenyl, phenyl, substituted phenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl. Representative substitutions include, but are not limited to, halo, hydroxyl, alkoxy, alkylthio, phenoxy, aroxy, cyano, isocyano, carbonyl,

carboxyl, amino, amido, sulfonyl, and substituted heterocyclic, sugar, or peptide.

To prepare salicylic acid pentafluorophenyl ester, a mixture of salicylic acid (4.14 g, 30 mmol), pentafluorophenol (5.52 g, 33 mmol) and DCC (6.3 g, 30 mmol) in dioxane (180 mL) is stirred at room temperature overnight. Dicyclohexyl urea is removed by filtration through celite, and the filtrate taken to dryness. Residue is crystallized from ether:hexane to provide salicylic acid pentafluorophenyl ester as a white solid (4.56 g, 50% yield), mp 111-111.5°C., I H NMR (CDC13) 8 9.83 (s, 1H), 8.06 (dd, J = 8.1, 1.6 Hz, 1H), 7.63-7.56 (m, 1H), 7.08-6.97 (m, 2H).

To prepare picolinic acid pentafluorophenyl ester, picolinic acid (1.23 g, 10 mmol) is reacted with pentafluorophenol (1.84 g, 10 mmol) in dioxane (30 mL) as described above. Purification by silica gel chromatography followed by crystallization provides picolinic acid pentafluorophenyl ester as a white solid (1.52 g, 53%), mp 62-64°C (ether:hexane), JH NMR (CDCI3) 8 8.87 (d, J = 4.5 Hz, IH), 8.29 (d, J = 7.9 Hz, IH), 7.99-7.92 (m, 1H), 7.64-7.56 (m, IH).

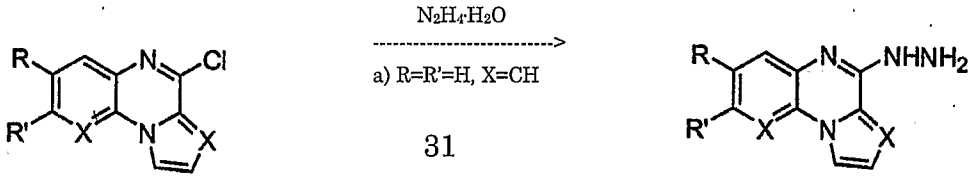
To prepare N,N'-Bis-salicyihydrazine, salicylic acid pentafluorophenyl ester (304 mg, 1.0 mmol) is reacted with anhydrous hydrazine or hydrazine monohydrate as described above. N,N'-bis-salicyihydrazine is provided as a white solid (123 mg, 90% and 130 mg, 95%, respectively), mp 315-316°C (EtOAc) (lit. 5, 302°C), IH NMR (DMSO-d 6) 5 11.78 (s, 2H), 10.89 (s, 2H), 7.92 (dd, J = 7.8, 1.3 Hz, 2H), 7.49-7.42 (m, 2H), 7.0-6.94 (m, 4H); IR (KBr) 3088, 1654, 1605, 1484, 1234, 754; FABMS m/z 273 (MH+). Analysis (CI4HIzNzO4): C, 61.76; H, 4.44; N, 10.29. Found: C, 61.66; H, 4.51; N, 10.37.

To prepare N,N'-Bis-picolinoylhydrazine, picolinic acid pentafluorophenyl ester (289 mg, 1.0 mmol) is reacted with anhydrous hydrazine or hydrazine monohydrate as described above. N,N'-bis-picolinoylhydrazine is provided as a white solid (110 mg, 91% and 96 mg, 80%, respectively), mp 224-225°C (EtOAc), I H NMR (DMSO-d 6) 8 10.63 (s, 2H), 8.70 (d, J = 4.8 Hz, 2H), 8.05-8.04 (m, 4H), 7.69-7.63 (m, 2H); IR

(KBr) 3321, 1676, 1560, 1482; FABMS m/z 243 (MH+). Analysis (CIeHIoN40:): C, 59.50; H, 4.16; N, 23.13. Found: C, 59.45; H, 4.17; N, 23.07.

The synthesis of SC141-SC144, SC148, and SC153-158 can be accomplished starting from the appropriate 4-chloropyrrolo[1,2-*a*]quinoxaline 13a-c (Nagarajan et al. (1972) Indian J. Chem. 10:344-350 and Guillon et al. (2004) J. Med. Chem. 17:1997-2009) or 6-chloroimidazo[1,2-*a*]pyrido[3,2-*e*]pyrazine 13d (Campiani et al. (1997) J. Med. Chem. 40:3670-3678) and hydrazine monohydrate to give essentially pure 4-hydrazinopyrrolo[1,2-*a*]quinoxalines 14a-c and 6-hydrazinoimidazo[1,2-*a*]pyrido[3,2-*e*]pyrazine 14d, respectively (Scheme 1). The subsequent N-acylation step can be performed in different experimental conditions: the SC141 and SC142 can be obtained by reaction of compound 14a with pyrrole-2-carboxylic acid chloride and nicotinoyl chloride hydrochloride, respectively; while SC143, SC144 and SC148 can be obtained by reaction of derivatives 14b-d with commercial 2-pyrazinecarboxylic acid by use of 2,2'-dipyrildisulphide and triphenylphosphine as condensing reagents (Di Fabio et al. (1993) Tetrahedron 43:229-2306). The condensation between hydrazine derivative 14a and an appropriate indolecarboxylic acid by a 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) / 4-(dimethylamino)pyridine (DMAP) system gives compounds SC155-158; *N*-BOC-derivatives of compounds SC153 and SC154 can be synthesized starting from compound 14a and *N*-BOC-thiazolidine-4-carboxylic acid or *N*-BOC-*β*-alanine, respectively, using again EDC / DMAP as a dehydrating system and finally deprotected by means of trifluoroacetic acid (TFA) / anisole.

Scheme 1

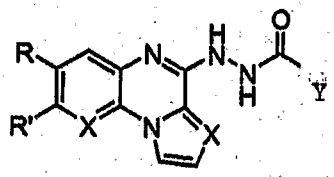


b) R=R'=Me, X=CH
c) R=F, R'=H, X=CH
d) R=R'=H, X=N

Formula VIII
13a-d

Formula IX
14a-d

1) acylation
2) deprotection (for SC153-154)
---------->

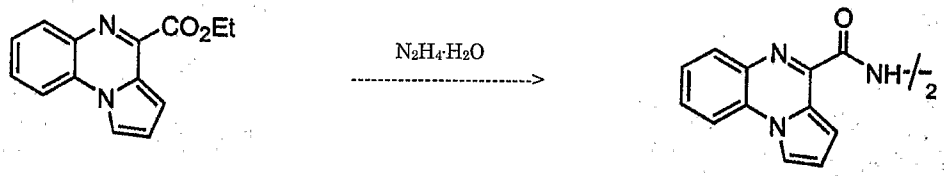


Formula II

SC141-SC144, SC148, and SC153-158

The preparation of *bis*-derivatives SC147 can be performed by direct reaction of hydrazine monohydrate with two molar equivalents of ethyl pyrrolo[1,2-*a*]quinoxaline-4-carboxylate 15, in turn obtained after the fashion of Nagarajan et al. ((1972) Indian J. Chem. 10:344-350) (Scheme 2).

Scheme 2

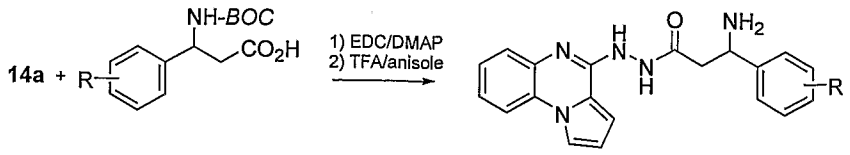


Formula X
15

SC147

SC160, SC161, SC162, SC163, SC164, and SC165 can be obtained by reaction of 14a with Boc-3-amino-3-(2-chlorophenyl)propionic acid, Boc-3-amino-3-(4-chlorophenyl)propionic acid, Boc-3-amino-3-(4-fluorophenyl)propionic acid, Boc-3-amino-3-(4-cyanophenyl)propionic acid, Boc-3-amino-3-(4-methoxyphenyl)propionic acid, and Boc-3-amino-3-(4-trifluoromethylphenyl)propionic acid in the presence of EDC/DMAP followed by TFA and anisole, respectively (Scheme 3).

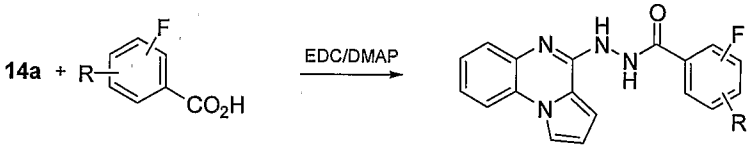
Scheme 3



Compd	R
SC160	2-Cl
SC 161	4-Cl
SC162	4-F
SC163	4-CN
SC164	4-OCH3
SC165	4-CF3

SC166, SC167, SC168, SC169, SC170, SC171, and SC172 can be obtained by reaction of 14a with corresponding acid (15a-g) shown in Scheme 4 in the presence of EDC/DMAP followed by TFA and anisole, respectively (Scheme 4).

Scheme 4

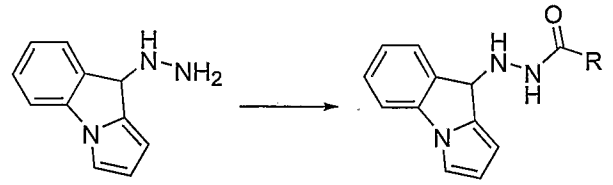


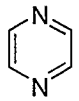
Compd	R
SC166	2-F, R= H
SC 167	2-F, R = 4-OH
SC168	3-F, R = H
SC169	3-F, R = 4-CF_3
SC170	4-F, R = H
SC171	4-F, R = 2-OH
SC 172	3-F, R = 5-NO_2

SC173 can be obtained by reaction of 14a with 2-quinoxalinecarboxylic acid, dichloromethane, triphenylphosphine, and 2,2'-dipyridyl disulfide; SC174 can be obtained by reaction of 14a with pyrrolo[1,2-*a*]quinoxaline-4-carboxylic acid, dichloromethane, triphenylphosphine, and 2,2'-dipyridyl disulfide.

SC175 can be obtained by reaction of nicotinoyl chloride hydrochloride with 9-hydrazino-9*H*-pyrrolo[1,2-*a*]indole and pyridine; SC176 can be obtained by reaction of pyrazine-2-carbonyl chloride hydrochloride or pyrazine-2-carbonyl chloride with 9-hydrazino-9*H*-pyrrolo[1,2-*a*]indole and pyrazine (Scheme 5).

Scheme 5



Compd	R
SC 175	
SC176	

Compositions

The compounds of the invention can be incorporated into pharmaceutical compositions. Such compositions typically include the compounds and pharmaceutically acceptable carriers. "Pharmaceutically acceptable carriers" include solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Other active compounds (e.g., taxol, doxorubicin, or 5-FU) can also be incorporated into the compositions.

A pharmaceutical composition is formulated to be compatible with its intended route of administration. See, e.g., U.S. Patent No. 6,756,196. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral

preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the compounds in the required amounts in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the compounds into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above.

In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. For the purpose of oral therapeutic administration, the compounds can be incorporated with excipients and used in the form of tablets, troches, or capsules, e.g., gelatin capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For

transdermal administration, the compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

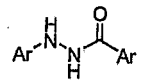
The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the compounds are prepared with carriers that will protect the compounds against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

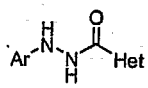
It is advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. "Dosage unit form," as used herein, refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier.

Pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration to form packaged products. For example, a packaged product may comprise a container; an effective amount of a compound of the invention; and an insert associated with the container, indicating administering the compound for treating cancer or a disorder associated with angiogenesis function.

In another example, an effective amount of a compound of formula XI or XII,



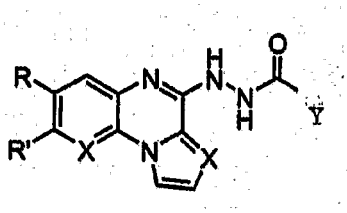
Formula XI



Formula XII

wherein Ar comprises an aromatic ring and Het comprises a heterocyclic ring, may be packaged in a container with an insert. The insert is associated with the container and contains instructions for administration of the compound for treating non-small cell lung cancer, CNS cancer, ovarian cancer, breast cancer, renal cancer, prostate cancer, age-related macular degeneration, macular dystrophy, or diabetes.

Alternatively, an effective amount of a compound of Formula II,



Formula II

wherein R is H, alkyl, or halogen; R' is H, alkyl, or halogen; X is CH or N; and Y comprises a homocyclic or heterocyclic ring, may be packaged in a container with an insert. The insert is associated with the container and contains instructions for administration of the compound for treating cancer or a disorder associated with angiogenesis function.

A packaged product may further comprise an effective amount of one or more other agents for treating cancer or a disorder associated with angiogenesis function, e.g., taxol, doxorubicin, or 5-FU.

Uses

Method of treatment

The present invention provides for both prophylactic and therapeutic methods of treating a subject in need thereof an effective amount of a compound or composition described above.

"Subject," as used herein, refers to a human or animal, including all vertebrates, e.g., mammals, such as primates (particularly higher primates), sheep, dog, rodents (e.g., mouse or rat), guinea pig, goat, pig, cat, rabbit, cow; and non-mammals, such as chicken, amphibians, reptiles, etc. In a preferred embodiment, the subject is a human. In another embodiment, the subject is an experimental animal or animal suitable as a disease model.

A subject to be treated may be identified, e.g., using diagnostic methods known in the art, as being suffering from or at risk for developing cancer or a disorder associated angiogenesis function, i.e., blood vessel formation, which usually accompanies the growth of malignant tissue. The subject may be identified in the judgment of a subject or a health care professional, and can be subjective (e.g., opinion) or objective (e.g., measurable by a test or diagnostic method). Examples of cancer include leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, breast cancer, renal cancer, or prostate cancer; examples of disorders associated with angiogenesis function include age-related macular degeneration, macular dystrophy, or diabetes.

As used herein, the term "treatment" is defined as the application or administration of a therapeutic agent to a subject, or application or administration of a therapeutic agent to an isolated tissue or cell line from a subject, who has a disease, a symptom of disease or a predisposition toward a disease, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease, the symptoms of disease or the predisposition toward disease.

An "effective amount" is an amount of the therapeutic agent that is capable of producing a medically desirable result as delineated herein in a treated subject. The medically desirable result may be objective (i.e., measurable by some test or marker) or subjective (i.e., subject gives an indication of or feels an effect).

Toxicity and therapeutic efficacy of the compounds can be determined by standard pharmaceutical procedures in cell cultures or

experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds which exhibit high therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of the compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of a compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

A therapeutically effective amount of the compounds (i.e., an effective dosage) may range from, e.g., about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram. The compounds can be administered, e.g., one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It is furthermore understood that appropriate doses of a compound depend upon the

potency of the compound. When one or more of these compounds is to be administered to a subject (e.g., an animal or a human), a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, the severity of the disease or disorder, previous treatments, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of the compounds can include a single treatment or, preferably, can include a series of treatments.

The treatment may further include administering to the subject an effective amount of one or more other agents for treating cancer or a disorder associated with angiogenesis function, e.g., taxol, doxorubicin, or 5-FU. When multiple therapeutic agents are used, the agents may be administered, simultaneously or sequentially, as mixed or individual dosages.

Method of monitoring treatment using PET technology

Miniaturized, high-resolution PET scanners employing novel detector technology have been designed specifically for small animal imaging (Holdsworth and Thornton (2002) Trends Biotechnol. 20:S34-39 and Lewis et al. (2002) Eur. J. Cancer 38:2173-2188). This approach allows the rapid testing of drug effects in human tumor xenografts implanted into mice in order to optimize drug PK and dose regimens prior to testing in humans. Such *in vivo* assessment can predict success of drug candidates, thus filtering potential clinical candidates earlier in the drug discovery pipeline. As applied to drug discovery and development, information obtainable via functional PET imaging can be divided into four categories: (1) the absorption, distribution, metabolism and elimination of the labeled drug candidate; (2) the delivery of a drug to a

specific target of interest (e.g., tumor); (3) the interaction of a drug or drug candidate with the desired molecular target (e.g., an enzyme or cell surface receptor); and (4) determination of desirable PD effects (e.g., cell killing and cell cycle arrest) or undesirable side effects. Noninvasive PET imaging techniques can enable more accurate titration of therapeutic dose and, using a labeled form of the drug, more rapid characterization of PK and PD, linking *in vivo* affinity with efficacy. This will inevitably improve data quality, reduce costs and animal numbers used and, most importantly, decrease the work-up time for new compounds.

PET imaging with the glucose analog [^{18}F]fluorodeoxyglucose ([^{18}F]FDG) has been used extensively in human patients to visualize primary cancers with a high degree of accuracy and to quantify cancer response to antineoplastic therapies; an example of this in breast cancer can be found in references (Bellon et al. (2004) Am. J. Clin. Oncol. 27:407-410 and Eubank and Mankoff (2004) Semin. Nucl. Med. 34:224-240). Early assessment of *in vivo* efficacy of new drugs in mice by PET could greatly aid selection of the right drug for future clinical studies. The generally high rate of glycolysis by tumor cells can be quantitated by PET/[^{18}F]FDG imaging. FDG is phosphorylated by hexokinase, yielding negatively charged FDG-6-phosphate, which is effectively trapped in the cell. Increased tumor uptake of FDG as measured by PET is highly correlated with viable tumor density (i.e., viable cell number per unit tissue volume). Because FDG uptake is representative of tumor cell viability (Higashi et al. (1993) J. Nucl. Med. 34:773-779) reduction in FGD uptake with effective tumor therapy reflects killing of tumor cells. Evaluation of tumor response in experimental animal models is of paramount importance in drug development, and FDG PET is an ideal tool for this purpose. In fact, a number of clinical trials have already shown that quantification of the changes in tumor [^{18}F]-FDG uptake may provide an early, sensitive, pharmacodynamic marker of the tumoricidal effect of anticancer drugs. Changes in FDG PET images during chemotherapy are predictive of response in patients with a variety of

cancers such as breast carcinoma (Avril et al. (2000) J..Clin. Oncol. 18:3495-3502), lung (Higashi et al. (2002) J. Nucl. Med. 43:39-45), head and neck carcinoma (Halfpenny et al. (2002) Br. J. Cancer 86:512-516), and lymphoma (Lowe and Wiseman (2002) J. Nucl. Med. 43:1028-1030) (for reviews, see Czernin and Phelps (2002) Annu. Rev. Med. 53:89-112, Cohade and Wahl (2002) Cancer J. 8:119-134, and Nabi and Zubeldia (2002) J. Nucl. Med. Technol. 30:3-9; quiz 10-11). These studies demonstrate that PET can identify clinical response to treatment at a much earlier stage in the therapeutic regimen than is possible using conventional procedures based on change in tumor size.

An important characteristic of highly proliferating cells is their remarkable rate of DNA synthesis. PET probes that are incorporated into the DNA synthetic pathway are ideal agents with which to measure tumor growth rate and the impact of treatment on tumor cell division. The prototype agent in this class is thymidine. Unfortunately, the utility of thymidine is limited due to its rapid catabolism *in vivo* (Conti et al. (1994) Nucl. Med. Biol. 21:1045-1051). During the past decade several radiolabeled analogs of thymidine that are resistant to enzymatic degradation and are incorporated into DNA with high specificity and affinity have been identified (see, for example, Czernin and Phelps (2002) Annu. Rev. Med. 53:89-112, Cohade and Wahl (2002) Cancer J. 8:119-134). One such radiotracer, 2'-fluoro-5-methyl-1-β-D-arabinofuranosyluracil (FMAU) labeled with C-11 (20 min half life) has shown promise for tumor imaging with PET (Conti et al. (1995) Nucl. Med. Biol. 22:783-789, Bading et al. (2000) Nucl. Med. Biol. 27:361-368, and Bading et al. (2004) Nucl. Med. Biol. 31:407-418). Following cellular uptake, FMAU is phosphorylated by thymidine kinase and incorporated into DNA.

Accordingly, the invention provides a method of monitoring treatment of a subject. The method involves administering to a subject having cancer cells or cells associated with an angiogenesis function disorder a compound described above and measuring the survival of the cells, the growth of the cells, or a combination thereof using PET imaging.

The subject may be suffering from leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, breast cancer, renal cancer, or prostate cancer. The subject may be an animal, e.g., a mouse, and the cells may be xenografted human cells. Preferably, the subject is a human.

Method of profiling gene expression

Gene expression patterns in response to drug treatment are strong indications of the mechanism of action, mechanism of resistance and cellular pathways for the drug. Profiling of gene expression, e.g., by means of DNA microarray technology, is useful for identifying and validating drug targets, and for monitoring drug treatment.

Accordingly, the invention provides a method of profiling gene expression by contacting a test cell with a compound described above and profiling gene expression in the test cell. In particular, the test cell may be a cancer cell or a cell associated with an angiogenesis function disorder, e.g., a leukemia cell, non-small cell lung cancer cell, colon cancer cell, CNS cancer cell, melanoma cell, ovarian cancer cell, breast cancer cell, renal cancer cell, prostate cancer cell, or a cell associated with age-related macular degeneration, macular dystrophy, or diabetes. Gene expression in the test cell may be compared with that in a control cell, e.g., a cell not contacted with the compound, a cell contacted with another compound with known action, or a cell resistant to the compound. Such comparison provides useful information for understanding the action of the compound.

Gene expression can be determined at mRNA and protein levels. The presence, level, or absence of a protein or nucleic acid in a biological sample can be evaluated by obtaining a biological sample from a test subject and contacting the biological sample with an agent capable of detecting the protein or nucleic acid (e.g., mRNA, genomic DNA) that encodes the protein such that the presence of the protein or nucleic acid is detected in the biological sample. The term "biological sample" includes tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. The level of expression

of a gene can be measured in a number of ways, including, but not limited to: measuring the mRNA transcribed from the gene, measuring the amount of protein encoded by the gene, or measuring the activity of the protein encoded by the gene.

The level of mRNA transcribed from the gene in a cell can be determined both by *in situ* and by *in vitro* formats. The isolated mRNA can be used in hybridization or amplification assays that include, but are not limited to, Southern or Northern analyses, polymerase chain reaction analyses and probe arrays. One preferred diagnostic method for detection of the mRNA level involves contacting the isolated mRNA with a nucleic acid molecule (probe) that can hybridize to the mRNA transcribed from the gene being detected. The probe can be disposed on an address of an array.

In one format, mRNA (or cDNA) is immobilized on a surface and contacted with the probes, for example, by running the isolated mRNA on an agarose gel and transferring the mRNA from the gel to a membrane, such as nitrocellulose. In an alternative format, the probes are immobilized on a surface and the mRNA (or cDNA) is contacted with the probes, for example, in a two-dimensional gene chip array. A skilled artisan can adapt known mRNA detection methods for use in detecting the level of mRNA transcribed from the gene.

The level of mRNA in a sample can be evaluated with nucleic acid amplification, e.g., by RT-PCR (U.S. Patent No. 4,683,202), ligase chain reaction (Barany (1991) Proc. Natl. Acad. Sci. USA 88:189-193), self sustained sequence replication (Guatelli et al. (1990) Proc. Natl. Acad. Sci. USA 87:1874-1878), transcriptional amplification system (Kwoh et al. (1989) Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi et al. (1988) Bio/Technology 6:1197), rolling circle replication (U.S. Patent No. 5,854,033) or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques known in the art. As used herein, amplification primers are defined as being a pair of nucleic acid molecules that can anneal to 5' or 3'

regions of a gene (plus and minus strands, respectively, or vice-versa) and contain a short region in between. In general, amplification primers are from about 10 to 30 nucleotides in length and flank a region from about 50 to 200 nucleotides in length. Under appropriate conditions and with appropriate reagents, such primers permit the amplification of a nucleic acid molecule comprising the nucleotide sequence flanked by the primers.

For *in situ* methods, a cell or tissue sample can be prepared/processed and immobilized on a support, typically a glass slide, and then contacted with a probe that can hybridize to mRNA transcribed from the gene being analyzed.

A variety of methods can be used to determine the level of protein encoded by the gene. In general, these methods include contacting an agent that selectively binds to the protein, such as an antibody with a sample, to evaluate the level of protein in the sample. In a preferred embodiment, the antibody bears a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (e.g., Fab or $F(ab')_2$) can be used. The term "labeled," with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (i.e., physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with a detectable substance.

The detection methods can be used to detect a protein in a biological sample *in vitro* as well as *in vivo*. *In vitro* techniques for detection of a protein include enzyme linked immunosorbent assays (ELISAs), immunoprecipitations, immunofluorescence, enzyme immunoassay (EIA), radioimmunoassay (RIA), and Western blot analysis. *In vivo* techniques for detection of a protein include introducing into a subject a labeled antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques. In another embodiment, the sample is labeled, e.g., biotinylated and then contacted to the antibody, e.g., an

antibody positioned on an antibody array. The sample can be detected, e.g., with avidin coupled to a fluorescent label.

It is now well established that DNA microarray technology allows simultaneous quantification of the expression of thousands of genes. This methodology is now robust, reproducible, and highly efficient. It can be used to evaluate cellular pathways and validate drug targets (see, for example,

Clarke et al. (2001) Biochem. Pharmacol. 62:1311-1336, Onyango (2004) Curr. Cancer Drug Targets 4:111-124, and Weinstein (2002) Curr. Opin. Pharmacol. 2:361-365).

Clustering of compounds into presumed mechanistic groupings based on the similarity in their growth inhibition profiles across the NCI 60 human cancer cell-lines was first realized by Paull et al. ((1989) J. Natl. Cancer Inst. 81:1088-1092). They developed a computer program called "COMPARE" which is based on a pattern recognition algorithm that assesses the degree of similarity of compounds based on their cytotoxicity profiles. Some of the compounds were classified according to their published and widely accepted molecular targets. Recently, Dr. John Weinstein and his colleagues at NCI have created a software package called "DISCOVERY" to compare the gene expression analysis of 60 cell lines using a cDNA chip containing 1,200 genes (Weinstein et al. (1997) Science 275:343-349). A correlation between gene expression patterns and the cytotoxic profiles against 60 cell lines in response to a particular compound could be determined (Scherf et al. (2000) Nat. Genet. 24:236-244). Using this methodology, it is possible to identify targets or pathways for these compounds. DISCOVERY then allows the identification of genes common to the pathways by correlative gene expression. This publicly available software allows comparison of compounds against a database of 5000 compounds in the NCI 60 human cancer cell-lines (see the NCI web site at discover.nci.nih.gov).

Genes identified through profiling as responsive to the treatment of a compound may be used as therapeutic markers. These markers can in

turn be used to monitor treatment of a subject with the compound. For example, genes responsive to SC144 include small proline-rich protein 1A; GTP binding protein overexpressed in skeletal muscle; interleukin 24; sestrin 2; hypothetical protein MGC4504; cyclin-dependent kinase inhibitor 1A (p21); early growth response 1; ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d isoform 2; AXIN1 up-regulated 1; dual specificity phosphatase 5; superoxide dismutase 2, mitochondrial; heparin-binding epidermal growth factor-like growth factor; A disintegrin and metalloproteinase domain 19 (meltrin beta); endothelial PAS domain protein 1; inositol 1,4,5-triphosphate receptor, type 1; tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor); fibrinogen, gamma polypeptide; RAB20, member RAS oncogene family; protein kinase, AMP-activated, gamma 2 non-catalytic subunit; oncostatin M receptor; cathepsin B; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; BCL2/adenovirus E1B 19kDa interacting protein 3; integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61); dual specificity phosphatase 10; cell cycle control protein SDP35; plexin C1; microphthalmia-associated transcription factor; calpain small subunit 2; hypothetical protein DKFZp434L142; MEGF 10 protein; EphA2; jagged 1 (Alagille syndrome); hemicentin; low density lipoprotein receptor (heparin-binding epidermal growth factor-like growth factor); tyrosinase-related protein 1; tyrosinase (oculocutaneous albinism IA); dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2); laminin, beta 3; MAX dimerization protein 1; CDK4-binding protein p34SEI1; Homo sapiens cDNA FLJ42435 fis, clone BLADE2006849; growth arrest and DNA-damage-inducible, beta; cycline-dependent kinase inhibitor 2B (p15, inhibits CDK4); Diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor); syntaxin binding protein 6 (amisyn); transport-secretion protein 2.2; Arg/Abl-interacting protein ArgBP2; hypothetical protein DJ667H12.2; and Homo sapiens cDNA FLJ37284 fis, clone RAMY2013590. One or more of these genes

may be used as markers for monitoring treatment of a subject with SC144, e.g., determining the efficacy of the compound.

Method of modulating cell growth, cell cycle, apoptosis, or gene expression

Another aspect of the invention pertains to methods of modulating cell growth, cell cycle, apoptosis, or gene expression or activity for therapeutic purposes. Accordingly, the modulatory method of the invention involves contacting a cell with a compound described above that modulates cell growth, cell cycle, apoptosis, or expression of one or more of the genes associated with the cell. Methods of measuring cell growth, cell cycle, apoptosis, or gene expression or activity are known in the art. Examples of such methods are provided in the Examples below and the description above.

Examples of the genes to be modulated include small proline-rich protein 1A; GTP binding protein overexpressed in skeletal muscle; interleukin 24; sestrin 2; hypothetical protein MGC4504; cyclin-dependent kinase inhibitor 1A (p21); early growth response 1; ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d isoform 2; AXIN1 up-regulated 1; dual specificity phosphatase 5; superoxide dismutase 2, mitochondrial; heparin-binding epidermal growth factor-like growth factor; A disintegrin and metalloproteinase domain 19 (meltrin beta); endothelial PAS domain protein 1; inositol 1,4,5-triphosphate receptor, type 1; tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor); fibrinogen, gamma polypeptide; RAB20, member RAS oncogene family; protein kinase, AMP-activated, gamma 2 non-catalytic subunit; oncostatin M receptor; cathepsin B; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; BCL2/adenovirus E1B 19kDa interacting protein 3; integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61); dual specificity phosphatase 10; cell cycle control protein SDP35; plexin C1; microphthalmia-associated transcription factor; calpain small subunit 2; hypothetical protein DKFZp434L142; MEGF 10 protein; EphA2; jagged 1 (Alagille syndrome); hemicentin; low density

lipoprotein receptor (heparin-binding epidermal growth factor-like growth factor); tyrosinase-related protein 1; tyrosinase (oculocutaneous albinism IA); dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2); laminin, beta 3; MAX dimerization protein 1; CDK4-binding protein p34SEI1; Homo sapiens cDNA FLJ42435 fis, clone BLADE2006849; growth arrest and DNA-damage-inducible, beta; cycline-dependent kinase inhibitor 2B (p15, inhibits CDK4); Diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor); syntaxin binding protein 6 (amisyn); transport-secretion protein 2.2; Arg/Abl-interacting protein ArgBP2; hypothetical protein DJ667H12.2; Homo sapiens cDNA FLJ37284 fis, clone RAMY2013590; BCL2, BCL2L1, JUN, JUNB, MAD, MAX, TNFRSF1A, TP53, NFKB1, TNFSF10, CASP1, PCNA, TNFAIP1, DAP, KDR, MAP3K14, CCNA2, CDC2, CDK7, CDK8, CDKN1A, CDKN1B, CDKN2A, CDKN2C, E2F1, E2F4, E2F5, MYC, RB1, RBL2, CCND3, CCNG1, CCNE1, CDC25C, TGFBR2, TGIF, TRAF4, CYP1A2, PTGS2, (p21,) p27, cyclin A, cdk1, p53, cyclin E, cdc25, p130, NFKB, c-MYC, COX2, Bcl-X_L, annexin V, caspase 1, TNF receptor, microtubule-associated protein 4, microtubule affinity-regulating kinase 2, microtubule affinity-regulating kinase 4, transducer of ERBB2, vascular endothelial growth factor B, vascular endothelial growth factor, ankyrin repeat and MYND domain containing 1, RAB4B, putative prostate cancer tumor suppressor, pre-B-cell leukemia transcription factor 2, T-cell leukemia translocation altered gene, leukemia inhibitory factor, interferon regulatory factor 2 binding protein, interferon stimulated gene (20kDa), interferon gamma receptor 2, 28kD interferon responsive protein, polymerase (RNA) III, peroxisomal proliferator-activated receptor A interacting complex 285, RAD50 homolog (S. cerevisiae), MAX dimerization protein 3, kruppel-like factor 16, apolipoprotein L (6), X-ray repair complementing defective repair, mitogen-activated protein kinase 3, phosphatidylinositol 4-kinase type II, mitogen-activated protein kinase 12, protein kinase (AMP-activated, alpha 2 catalytic subunit), pyruvate dehydrogenase phosphatase regulatory subunit, phospholipase D3,

inositol 1,4,5-triphosphate receptor (type 3), retinoic acid receptor (alpha), tumor necrosis factor receptor superfamily, Enolase 2 (gamma, neuronal), stanniocalcin 2, apelin, plexin B2, cathepsin Z, histone 1 (H2bc), histone 1 (H3h), β-tubulin, myc promoter-binding protein (MPB-1), retinoblastoma-binding protein 7, vimentin, enolase, phosphopyruvate hydratase beta, and mitochondrial ATP synthase beta chain.

In one embodiment, the compound stimulates expression of one or more of the genes in the cell. For example, SC144 stimulates expression of small proline-rich protein 1A; GTP binding protein overexpressed in skeletal muscle; interleukin 24; sestrin 2; hypothetical protein MGC4504; cyclin-dependent kinase inhibitor 1A (p21); early growth response 1; ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d isoform 2; AXIN1 up-regulated 1; dual specificity phosphatase 5; superoxide dismutase 2, mitochondrial; heparin-binding epidermal growth factor-like growth factor; A disintegrin and metalloproteinase domain 19 (meltrin beta); endothelial PAS domain protein 1; inositol 1,4,5-triphosphate receptor, type 1; tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor); fibrinogen, gamma polypeptide; RAB20, member RAS oncogene family; protein kinase, AMP-activated, gamma 2 non-catalytic subunit; oncostatin M receptor; cathepsin B; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; BCL2/adenovirus E1B 19kDa interacting protein 3; integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61); and dual specificity phosphatase 10. In another embodiment, the compound inhibits expression of one or more of the genes in the cell. For example, SC144 inhibits expression of cell cycle control protein SDP35, plexin C1, microphthalmia-associated transcription factor, calpain small subunit 2, hypothetical protein DKFZp434L142.

These modulatory methods can be performed *in vitro*, e.g., by culturing the cell with the compound. For example, the cell may be a cancer cell (e.g., a leukemia cell, non-small cell lung cancer cell, colon cancer cell, CNS cancer cell, melanoma cell, ovarian cancer cell, breast

cancer cell, renal cancer cell, prostate cancer cell) or a cell associated with an angiogenesis function disorder (e.g., a cell associated with age-related macular degeneration, macular dystrophy, or diabetes). Alternatively, the modulatory methods can be performed *in vivo*, e.g., by administering the compound to a subject such as a subject suffering from or at risk for developing cancer or a disorder associated with angiogenesis function. As such, the present invention provides methods of treating a subject afflicted with a disease or disorder characterized by aberrant or unwanted cell growth, cell cycle, apoptosis, or expression of one or more of the genes. Stimulation of gene expression is desirable in situations in which the gene is abnormally downregulated and/or in which increased gene expression is likely to have a beneficial effect. Likewise, inhibition of gene expression is desirable in situations in which gene expression is abnormally upregulated and/or in which decreased gene expression is likely to have a beneficial effect.

The following examples are intended to illustrate, but not to limit, the scope of the invention. While such examples are typical of those that might be used, other procedures known to those skilled in the art may alternatively be utilized. Indeed, those of ordinary skill in the art can readily envision and produce further embodiments, based on the teachings herein, without undue experimentation.

EXAMPLES

Example I

Chemistry.

All reactions were carried out under a nitrogen atmosphere. Progress of the reaction was monitored by TLC on silica gel plates (Merck 60, F_{254}, 0.2 mm). Organic solutions were dried over $MgSO_4$; evaporation refers to removal of solvent on a rotary evaporator under reduced pressure. Melting points were measured using a Gallenkamp apparatus and are uncorrected. IR spectra were recorded as thin films on Perkin-Elmer 398 and FT 1600 spectrophotometers. ^1H NMR spectra were recorded on a Brüker 300-MHz spectrometer with TMS as an internal

standard: chemical shifts are expressed in δ values (ppm) and coupling constants (*J*) in Hz. Mass spectral data were determined by direct insertion at 70 eV with a VG70 spectrometer. Merck silica gel (Kieselgel 60/230-400 mesh) was used for flash chromatography columns. Elemental analyses were performed on a Perkin-Elmer 240C elemental analyzer, and the results are within ±0.4% of the theoretical values. Yields refer to purified products and are not optimized.

General procedure for the preparation of compounds 14a-14d. The preparation of 7-fluoro-4-hydrazinopyrrolo[1,2-*a*]quinoxaline 14c is reported as a representative example.

A mixture of 7-fluoro-4-chloropyrrolo[1,2-*a*]quinoxaline 13c (100 mg, 0.45 mmol), hydrazine monohydrate (5 mL), and DMF (2 mL) was heated to 70-80°C for 1 h. Crushed ice was then added and the mixture was extracted with EtOAc. The organic layer was separated and shaken with water and brine successively. After evaporation of the volatiles, compound 14c was obtained as a solid (84 mg, 86% yield) and used in the subsequent step without further purification. An analytical sample was obtained by crystallization; mp 158°C (dec.) (dichloromethane/light petroleum); IR (KBr) 3300 cm^{-1}; ^1H NMR (DMSO-d_6) 4.56 (bs, 2 H), 6.66 (t, 1 H, J = 3.2 Hz), 7.03 (m, 2 H), 7.18 (dd, 1 H, J = 10.6, 2.7 Hz), 8.02 (dd, 1 H, J = 8.9, 5.6 Hz), 8.15 (s, 1 H), 8.87 (bs, 1 H). Anal. Calcd for $C_{11}H_9FN_4$: C, H, N.

1*H*-Pyrrole-2-carboxylic acid *N*'-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide_1 (SC141). A suspension of pyrrole-2-carboxylic acid chloride (58 mg, 0.45 mmol) and triethylamine (1 mL) in dry THF (10 mL) was added portionwise to a stirred solution of compound 14a (90 mg, 0.45 mmol) in dry THF (3 mL). The mixture was stirred overnight at room temperature. The residue obtained after evaporation of the volatiles was partitioned between ethyl acetate and water. The organic layer separated was shaken with brine and dried. Evaporation of the solvent gave compound 1 as a white solid (82 mg, 62% yield); mp 210-212°C (methanol); IR (KBr) 3255, 1675 cm^{-1}; ^1H NMR (DMSO-d_6) 6.14 (s, 1 H), 6.77 (t, 1 H, J = 3.1 Hz), 6.99 (s, 1 H), 7.13 (d, 1 H, J = 3.7 Hz), 7.25 (m, 2 H), 7.42 (m, 2

H), 8.06 (m, 1 H), 8.27 (m, 1 H), 9.32 (bs, 1 H), 10.11 (bs, 1 H) 11.58 (bs, 1 H). MS (CI) *m/z* 292 (MH^+). Anal. Calcd. for $C_{16}H_{13}N_5O$: C, H, N.

Nicotinic acid *N'*-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide 2 (SC142). Solid nicotinoyl chloride hydrochloride (155 mg, 0.90 mmol) was added portionwise to a stirred and ice-cooled solution of 4-hydrazinopyrrolo[1,2-*a*]quinoxaline 14a (200 mg, 1.01 mmol) in dry pyridine (15 mL). The mixture was stirred overnight at room temperature. After a usual work-up, compound 2 was obtained as a pale yellow solid (122 mg, 40% yield); mp 237°C (methanol/ethyl acetate); IR (KBr) 3245, 1680 cm^{-1}; ^1H NMR (DMSO-d_6) 6.70 (m, 1 H), 7.07 (m, 1 H), 7.18 (m, 2 H), 7.36 (m, 1 H), 7.48 (m, 1 H), 7.98 (m, 1 H), 8.20 (m, 2 H), 8.69 (m, 1 H), 9.05 (m, 1 H), 10.75 (bs, 1 H), 11.80 (bs, 1 H). MS (CI) *m/z* 304 (MH^+). Anal. Calcd. for $C_{17}H_{13}N_5O$: C, H, N.

Pyrazine-2-carboxylic acid *N'*-(7,8-dimethylpyrrolo[1,2-*a*]quinoxalin-4-yl)-hydrazide 3 (SC143). To a stirred suspension of 2-pyrazinecarboxylic acid (62 mg, 0.50 mmol) in dry dichloromethane (2 mL) were added, portion wise, within 1 h, triphenylphosphine (262 mg, 1.00 mmol) and 2,2'-dipyridyl disulfide (220 mg, 1.00 mmol). When the starting material disappeared (TLC) a solution of 4-hydrazino-7,8-dimethylpyrrolo[1,2-*a*]quinoxaline 14b (113 mg, 0.50 mmol) in the same solvent (6 mL) was added and the resulting mixture was stirred at room temperature overnight. The solvent was removed and the residue was partitioned between ethyl acetate and water. The organic layer was separated, shaken with brine and dried. The residue left after evaporation of the solvent was purified by flash-chromatography (chloroform:methanol:ammonium hydroxide, 89:10:1) to afford compound 3 as a pale yellow solid (63 mg, 38% yield); mp 116°C (methanol/ethyl acetate); IR (KBr) 3250, 1675 cm^{-1}; ^1H NMR (DMSO-d_6) 3.35 (s, 6 H), 6.74 (t, 1 H, J = 3.8 Hz), 7.31 (d, 1 H, J = 3.8 Hz), 7.42 (m, 1 H), 7.64 (m, 2 H), 7.87 (bs, 1 H), 8.28 (bs, 1 H), 8.71 (s, 1 H), 8.87 (m, 1 H), 9.20 (s, 1 H). MS (CI) *m/z* 333 (MH^+). Anal. Calcd. for $C_{18}H_{16}N_6O$: C, H, N.

Pyrazine-2-carboxylic acid *N'*-(7-fluoropyrrolo[1,2-*a*]quinoxalin-4-yl)-hydrazide 4 (SC144). Following a procedure identical to that described for compound 3, but using 7-fluoro-4-hydrazinopyrrolo[1,2-*a*]quinoxaline 14c (108 mg, 0.50 mmol), compound 4 was obtained as a pale yellow solid (56 mg, 35% yield); mp 196°C (methanol/ethyl acetate); IR (KBr) 3255, 1690 cm^{-1}; ^1H NMR (DMSO-d_6) 6.75 (m, 1 H), 7.15 (m, 1 H), 7.37 (bs, 1 H), 7.61 (m, 2 H), 8.15 (m, 1 H), 8.31 (m, 1 H), 8.87 (s, 1 H), 8.97 (m, 1 H), 9.26 (s, 1 H), 11.50 (bs, 1 H, exch. with D_2O). MS (CI) *m/z* 323 (MH^+). Anal. Calcd. for $C_{16}H_{11}FN_6O$: C, H, N.

N'-Imidazo[1,2-*a*]pyrido[3,2-*e*]pyrazin-6-ylpyrazine-2-carbohydrazide 5 (SC148). Following a procedure identical to that described for compound 3, but using 6-hydrazinoimidazo[1,2-*a*]pyrido[3,2-*e*]pyrazine 14d (100 mg, 0.50 mmol), compound 5 was obtained as a pale yellow solid (38 mg, 25% yield); mp 271°C (methanol); IR (KBr) 3250, 1675 cm^{-1}; ^1H NMR (DMSO-d_6) 7.52 (m, 1 H), 7.76 (s, 1 H), 8.02 (m, 1 H), 8.41 (s, 1 H), 8.57 (s, 1 H), 8.85 (s, 1 H), 8.96 (s, 1 H), 9.26 (s, 1 H), 10.76 (bs, 1 H), 13.93 (bs, 1 H). MS (CI) *m/z* 307 (MH^+). Anal. Calcd. for $C_{14}H_{10}N_8O$: C, H, N.

General procedure for the preparation of compounds 6-9 (SC 155-158). The preparation of 1*H*-indole-2-carboxylic acid *N'*-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide 6 (SC155) is reported as a representative example.

To a stirred solution of EDC (94 mg, 0.49 mmol) and DMAP (cat.) in ethyl acetate (15 mL), compound 14a (77 mg, 0.39 mmol) and 2-indolecarboxylic acid (63 mg, 0.39 mmol) were added, portion wise, within 15 minutes. The resulting mixture was stirred at room temperature for 24 h, then shaken with sodium bicarbonate saturated solution and water. Evaporation of the dried extract gave a residue which was crystallized to give compound 6 as a white solid (82 mg, 62% yield); mp 186°C (dichloromethane/light petroleum); IR (KBr) 3255, 1680 cm^{-1}; ^1H NMR (DMSO-d_6) 6.75 (s, 1 H), 7.05 (m, 1 H), 7.20 (m, 4 H), 7.40 (m, 3 H), 7.65

(m, 1 H), 8.10 (m, 1 H), 8.35 (s, 1 H), 9.55 (bs, 1 H), 10.65 (bs, 1 H),11.80 (bs, 1 H). MS (CI) *m/z* 342 (MH^+). Anal. Calcd. for $C_{20}H_{15}N_5O$: C, H, N.

1*H*-Indole-5-carboxylic acid *N'*-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide 7 (SC156). Following a procedure identical to that described for compound 6, but using 2-indolecarboxylic acid (63 mg, 0.39 mmol), compound 7 was obtained as a white solid (69 mg, 52% yield); mp 160°C (dichloromethane/light petroleum); IR (KBr) 3250, 1680 cm^{-1}; ^1H NMR (acetone-d_6) 6.60 (d, 1 H, J = 3.6 Hz), 6.75 (t, 1 H, J = 3.6 Hz), 7.23 (d, 1 H, J = 3.6 Hz), 7.29 (m, 2 H), 7.51 (m, 3 H), 7.85 (d, 1 H, J = 8.5 Hz), 8.03 (m, 1 H), 8.20 (m, 1 H), 8.39 (s, 1 H), 9.60 (bs, 1 H), 10.70 (bs, 1 H), 11.45 (bs, 1 H). MS (CI) *m/z* 342 (MH^+). Anal. Calcd. for $C_{20}H_{15}N_5O$: C, H, N.

1*H*-Indole-6-carboxylic acid *N'*-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide 8 (SC157). Following a procedure identical to that described for compound 6, but using 6-indolecarboxylic acid (63 mg, 0.39 mmol), compound 8 was obtained as a white solid (17 mg, 13% yield); mp 198.5°C (dichloromethane/light petroleum); IR (KBr) 3245, 1685 cm^{-1}; ^1H NMR (acetone-d_6) 6.55 (m, 1 H), 6.85 (m, 1 H), 7.28 (m, 1 H), 7.28 (m, 3 H), 7.45 (m, 1 H), 7.60 (d, 1 H, J = 8.1 Hz), 8.70 (m, 2 H), 8.15 (s, 1 H), 8.39 (m, 1 H), 9.44 (bs, 1 H), 10.55 (bs, 1 H), 11.51 (bs, 1 H). MS (CI) *m/z* 342 (MH^+). Anal. Calcd. for $C_{20}H_{15}N_5O$: C, H, N.

1*H*-Indole-3-carboxylic acid *N'*-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide 9 (SC158). Following a procedure identical to that described for compound 6, but using 3-indolecarboxylic acid (63 mg, 0.39 mmol), compound 9 was obtained as a white solid (42 mg, 32% yield); mp 162.5°C (dichloromethane/light petroleum); IR (KBr) 3250, 1685 cm^{-1}; ^1H NMR ($CDCl_3$) 6.80 (m, 1 H), 6.90 (t, 1 H, J = 3.3 Hz), 7.08 (d, 1 H, J = 3.2 Hz), 7.30-7.60 (m, 4 H), 7.48 (m, 1 H), 7.58 (m, 1 H), 7.90 (m, 2 H), 8.10 (m, 1 H), 8.11 (s, 1 H), 8.30 (m, 1 H), 9.20 (bs, 1 H), 10.25 (bs, 1 H), 11.60 (bs, 1 H). MS (CI) *m/z* 342 (MH^+). Anal. Calcd. for $C_{20}H_{15}N_5O$: C, H, N.

General procedure for the preparation of compounds 10 and 11 (SC153 and SC154). The preparation of compounds 10 and 11 was accomplished by a condensation step, using an EDC/DMAP procedure

identical to that described for the preceding compound but using the appropriate *N*-BOC-aminoacid, followed by deprotection.

Thiazolidine-4-carboxylic acid *N'*-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide 10 (SC153). Starting from *N*-BOC-thiazolidine-4-carboxylic acid (90 mg, 0.39 mmol), *tert*-butyl 4-[(2-pyrrolo[1,2-*a*]quinoxalin-4-ylhydrazino)carbonyl]-1,3-thiazolidine-3-carboxylate was obtained as a solid, after crystallization (hexanes), and directly used for the subsequent hydrolytic step. The solid obtained was added to a stirred mixture of TFA (2 mL) and anisole (2 mL) at 0°C. The reaction mixture was allowed to reach to room temperature and stirred for a further 50 minutes. Evaporation of the volatiles by azeotropization with toluene (3 x 3 mL) gave compound 10 as a pale yellow solid (66 mg, 55% yield based on 14a); mp 162°C (ethyl acetate/hexanes); IR (KBr) 3255, 1690 cm^{-1}; ^1H NMR (methanol-d_4) 3.15 (dd, 1 H, J = 10.9, 4.9) 3.30 (dd, 1 H, J = 10.9, 7.1 Hz), 4.11 (0.5 of ABq, 1 H, J = 9.7 Hz), 4.25 (0.5 of ABq, 1 H, J = 9.7 Hz), 4.45 (dd, 1 H, J = 7.1, 4.9 Hz), 6.92 (m, 1 H), 7.41 (m, 3 H), 7.71 (d, 1 H, J = 7.4 Hz), 8.09 (d, 1 H, J = 9.3 Hz), 8.38 (m, 1 H), 10.40 (bs, 1 H), 11.20 (bs, 1 H). MS (CI) m/z 314 (MH^+). Anal. Calcd. for $C_{15}H_{15}N_5OS$: C, H, N.

3-Amino-propionic acid *N'*-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide 11 (SC154). Following a procedure identical to that described for compound 10, but using *N*-BOC-β-alanine (74 mg, 0.39 mmol), compound 11 was obtained as a white solid (92 mg, 88% yield based on 14a); mp 164.5°C (dichloromethane/light petroleum); IR (KBr) 3255, 1680 cm^{-1}; ^1H NMR (DMSO-d_6) 2.80 (m, 2 H) 3.20 (m, 2 H), 7.05 (m, 1 H), 7.50 (m, 2 H), 7.95 (m, 2 H), 8.30 (m, 1 H), 8.60 (m, 1 H), 10.70 (bs, 1 H), 11.25 (bs, 1 H). MS (CI) m/z 270 (MH^+). Anal. Calcd. for $C_{14}H_{15}N_5O$: C, H, N.

N,N'-Bis-pyrrolo[1,2-*a*]quinoxaline-4-carbohydrazide 12 (SC147). A mixture of hydrazine monohydrate (22 uL, 0.45 mmol) and ethyl pyrrolo[1,2-*a*]quinoxaline-4-carboxylate 15 (216 mg, 0.90 mmol) in ethanol (2 mL) was heated to reflux for 3 h. The residue obtained after evaporation of the solvent was purified by chromatography (dichloromethane:ethyl acetate, 9:1) to give compound 12 as a white solid

(115 mg, 62% yield); mp 138-139°C (ethyl acetate/hexane)); IR (KBr) 1680 cm^{-1}; ^1H NMR (DMSO-d_6) 6.28 (d, 2 H, J = 1.7 Hz), 7.01 (d, 2 H, J = 1.7 Hz), 7.45 (m, 8 H), 7.95 (d, 2 H, J = 7.5 Hz), 9.95 (bs, 1 H), 10.80 (bs, 1 H). MS (CI) m/z 421 (MH^+). Anal. Calcd. for $C_{24}H_{16}N_6O_2$: C, H, N.

3-Amino-3-(2-chlorophenyl)-propionic acid *N'*-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide (SC160). To a stirred solution of EDC (94 mg, 0.49 mmol) and DMAP (cat.) in ethyl acetate (15 mL), 4-hydrazinopyrrolo[1,2-*a*]quinoxaline 14a (77 mg, 0.39 mmol) and Boc-3-amino-3-(2-chlorophenyl)propionic acid (78 mg, 0.39 mmol) were added, portion wise over 15 minutes period. The resulting mixture was stirred at room temperature for 24 h, then shaken with sodium bicarbonate saturated solution and water. Evaporation of the dried extract gave a residue which was purified by crystallization and used for the subsequent hydrolytic step without further characterization. The solid obtained was added to a stirred mixture of TFA (2 mL) and anisole (2 mL) at 0°C. The reaction mixture was allowed to reach to room temperature and stirred for an additional 50 minutes. Evaporation of the volatiles by azeotropization with toluene (3 x 3 mL) gave the title compound as a solid.

Quinoxaline-2-carboxylic acid *N'*-pyrrolo[1,2-*a*]quinoxalin-4-yl-hydrazide (SC 173). To a stirred suspension of 2-quinoxalinecarboxylic acid (87 mg, 0.50 mmol) in dry dichloromethane (2 mL) were added, portion wise, within 1 h, triphenylphosphine (262 mg, 1.00 mmol) and 2,2'-dipyridyl disulfide (220 mg, 1.00 mmol). When the starting material disappeared (TLC) a solution of 4-hydrazinopyrrolo[1,2-*a*]quinoxaline 14a (100 mg, 0.50 mmol) in the same solvent (6 mL) was added and the resulting mixture was stirred at room temperature overnight. The solvent was removed and the residue was partitioned between ethyl acetate and water. The organic layer was separated, shaken with brine and dried. The residue left after evaporation of the solvent was purified by flash-chromatography to afford the title compound as a solid.

Nicotinic acid *N'*-9*H*-pyrrolo[1,2-*a*]indol-9-yl-hydrazide (SC 175). Solid nicotinoyl chloride hydrochloride (155 mg, 0.90 mmol) was added

portion wise to a stirred and ice-cooled solution of 9-hydrazino-9*H*-pyrrolo[1,2-*a*]indole (187 mg, 1.01 mmol) in dry pyridine (15 mL). The mixture was stirred overnight at room temperature. After evaporation of the volatiles, the title compound was isolated as a solid which was purified by column chromatography or crystallization.

SC144 shows remarkable potency against a panel of hormone-dependent and -independent cell lines.

The sensitivity of a panel of seven human cancer cell lines to SC144 was assessed by MTT-assay. SC144 showed an excellent activity with CC_{50} dose range of 0.7 to 10 uM (Table 1). The sensitivity towards SC144 was time- and dose-dependent. The activity of SC144 in these cell lines appeared to be independent of HR, p53, pRb, p21 and p16 status (Table 1). SC144 showed a remarkable activity in HEY cells (CC_{50} = 1.0 ± 0.06 uM) considering that this cell line appears to be practically resistant to cisplatin, the most commonly used drug in ovarian cancer. Moreover, SC144 was ten-fold more potent in HEY cells than in the prostate cancer PC3 cell line (CC_{50} = 10.0 ± 0.2 uM). SC144 also exhibited a good activity in HR positive (MCF-7 and MDA-MB-468) and negative (MDA-MB-435) human breast cancer cells. Interestingly, the ER+ cells exhibited a 5.5-fold (MDA-MB-468, CC_{50} = 0.7 ± 0.1 uM) and 2.3-fold (MCF-7, CC_{50} = 1.7 ± 0.3 uM) more sensitivity to SC144 than the ER- cell line (MDA-MB-435, CC_{50} = 4.0 ± 1.4 uM) (Table 1).

Table 1. Sensitivity of prostate, breast and ovarian cancer cell lines to SC144

Cell line	Origin	[b]HR	p53	pRb	p16	p21	[a]CC_{50} values (mean ± SD) SC144 (μM)
PC3	Prostate	AR-	Null	WT	WT	WT	10 ± 0.2
DU145	Prostate	AR-	Mut	Null	Mut	Mut	3.0 ± 0.3
HEY	Ovarian	AR+	WT	ND	WT	ND	1.0 ± 0.1
MCF-7	Breast	ER+	WT	WT	WT	WT	2.0 ± 0.3
MCF-7/ADR	Breast	ER-	Mut	WT	ND	WT	2.5 ± 1.0
MDA-MB-435	Breast	ER-	Mut	WT	WT	WT	4.0 ± 0.1
MDA-MB-468	Breast	ER-	Mut	Null	ND	WT	0.7 ± 0.1

[a] CC_{50} is defined as drug concentration causing a 50% decrease in cell population; [b]HR: hormone receptor; AR: androgen receptor; ER: estrogen receptor; WT: wild-type; Mut: mutated; ND: not-determined. HEY cells are resistant to cisplatin and MCF/ADR cells are resistant to doxorubicin.

SC144 treatment induces S-phase arrest.

Cell cycle perturbations induced by SC144 were examined in HEY and MDA-MB-435 cells. The analysis of DNA profiles by flow cytometry indicated that SC144 induced S-phase arrest comparable to that of camptothecin (CPT). As shown in Fig. 1, 80% of the cells were retained in S-phase after 24 h of treatment with SC144 (3 uM). Similar effects were obtained on the asynchronus prostate cancer cell line DU145. The maximum arrest was observed at 24 h of SC144 exposure, which was sustained up to 48 h. This property of SC144 to induce cell cycle arrest makes it an ideal agent for combination therapy with other agents that act at different stages of cell cycle, such as taxanes.

SC144 treatment induces apoptosis.

An early event in apoptotic cell death is the translocation of the phosphatidyl-serine residues to the outer part of the cell membrane. This event precedes nuclear breakdown, DNA fragmentation, the appearance of most apoptosis-associated molecules, and is readily measured by annexin V binding assay. By this method, SC144 was compared with CPT. As shown in Fig. 2, SC144 caused a very strong apoptotic effect comparable to that induced by CPT. The percentage of early-apoptotic cells increased in treated cells reaching 37% and 34% at 48 h for SC144 and CPT, respectively. At 48 h an increase in late-apoptosis/necrosis was also observed for both compounds (16% and 39% for SC144 and CPT, respectively).

SC144 shows *in vivo* efficacy in mice xenograft models.

The *in vivo* efficacy of SC144 was evaluated in a nude mice xenograft model of human breast MDA-MB-435 cells. A schematic outline of the experimental procedure is shown in Fig. 3A. Animals were treated with daily i.p. injections of saline (controls) and SC144 at 0.3 mg/kg, 0.8 mg/kg and 4 mg/kg. After five-days of dosing, the drug treatment was discontinued and the animals were monitored bi-weekly for five weeks.

Fig. 3B shows the volume (mean ± SD) for SC144 treated MDA-MB-435 xenografts over time.

For statistical analysis, the %T/C value was calculated on the last day of dosing and is graphed for all of the treatment groups (Fig. 3C). A marginal reduction was observed at the lowest dose of SC144 in breast cancer xenografts. Significant reduction in tumor growth was observed at higher SC144 doses. SC144 reduced tumor growth by 60% at 4 mg/kg. Representative images of mice with and without SC144 treatment at the end of the study is shown in Fig. 4A. Whereas in control mice, tumor mass became bulky, spread around the chest cavity, and densely vascularized, the SC144 treated tumors were markedly decreased in size, poorly vascularized, and remained localized (Fig. 4, B and C). Treatment with SC144 was well tolerated and did not result in drug-related deaths. Furthermore, no changes in body weight compared to vehicle control were observed with SC144.

The studies were expanded to other cell lines. It was found that SC144 shows nanomolar potency in non-small cell lung cancer cells HOP-62, EKVX, and HOP-92. The CC_{50} values range from 10-20 nM, which is about 400-fold more potent than the MDA-MB-435 cell line (Table 2). Subnanomolar to low nanomolar potency was also observed in HCT-116 and HT29 colon cancer cell lines (Table 2).

Table 2. Sensitivity of various cancer cells to SC144

Cell line	Origin	CC_{50} (uM)
HOP-62	Non-small cell lung cancer	0.01
HOP-92	Non-small cell lung cancer	0.2
EKVX	Non-small cell lung cancer	0.01
HL60	Leukemia	0.27
RPMI-8226	Leukemia	0.25
SF-268	CNS cancer	0.3
SF-295	CNS	0.42
UACC-257	Melanoma	0.4
UACC-62	Melanoma	0.8
SKOV3	Ovarian cancer	0.12
UO-31	Renal cancer	0.3
HCT-116	Colon	0.017
HT29	Colon	0.078

SC144 induce a selective and remarkable tumor necrosis *in vivo*.

To evaluate the extent of tumor necrosis after drug treatment tumor samples were collected from control and treated mice on day 70. Fig. 5 shows an H&E staining of tumor samples from a representative mouse. In general, greater than 80% necrosis of tumor tissues treated with 4 mg/kg of SC144 was observed (Fig. 5B).

SC144 does not exhibit systemic toxicity.

To evaluate the possibility for systemic toxicity of the SC144, several organs were examined microscopically. Fig. 6 shows representative H&E staining of kidney, liver, and heart tissues from mice treated with 4 mg/kg injection of SC144. No necrosis of glomeruli or tubular necrosis of the kidney was observed (Fig. 6A). No significant pathology of liver tissues was observed. Fig. 6B shows cords of hepatocytes are normal. Finally, cardiac muscles were normal and no detectable damage could be observed (Fig. 6C). In summary, the H&E staining results demonstrate that there was no damage in these organs of the representative mice of each group.

SC144 does not inhibit cytochrome P450 enzymes at concentrations relevant to its antitumor activity.

The investigation of cytochrome P450 enzyme inhibition by potential drug candidates can aid in predicting drug-drug interactions and/or unfavorable PK profiles produced upon dosing. Competitive inhibition of drug metabolism mediated by important cytochrome P450 enzymes may result in undesirable elevations in plasma drug concentrations, which is of clinical importance for both therapeutic and toxicological reasons. To determine if SC144 inhibits human cytochrome P450 catalytic activity an *in vitro* assay specific for CYP3A4 comparing to ketoconazole, a well-known substrate as a control, was performed (Fig. 7). These results suggest that SC24, an analogue of SC144, does not significantly inhibit CYP3A4 activity, but SC144 had an IC_{50} value range 8-20 μM, suggesting some CYP3A4 inhibitory activity. However, this concentration is above its antitumor efficacy.

Monitoring tumor response to SC144.

[^{18}F]FDG is currently the most widely used radiotracer for imaging therapy response in oncology with PET. PET/[^{18}F]FDG measures viable cell density in tumors and also provides information on the expression of glucose transporters and hexokinase activity. FMAU labeled with C-11 (20 min half life) is also effective for imaging tumor cell division with PET (Bading et al. (2004) Nucl. Med. Biol. 31:407-418). Following cellular uptake, FMAU is phosphorylated by thymidine kinase and incorporated into DNA. Preliminary studies with this technology have indicated that it is well suited for following the effects of SC144 in a mouse human tumor xenograft model.

The baseline, equilibrium-phase FDG scan shows a viable tumor on the right shoulder of the mouse (arrow). Early on (Fig. 8B), FMAU shows a "hot" rim surrounding the tumor, suggesting a poorly perfused center. In later images at 30 and 60 min (Fig. 8C), FMAU had filled up the whole tumor, indicating the presence of dividing cells throughout.

Figs. 8D-F show a repeat study of the same mouse after 5 days of treatment. The FDG scan shows that the tumor has grown considerably (measured volume more than doubled), but now has a necrotic center, consistent with the hypoperfusion seen in the baseline FMAU study. The FMAU scan (Fig. 8E) shows a completely hypoperfused tumor at 10 min. However, the tumor pretty much fills up with FMAU by 60 min, suggesting the continued presence of dividing cells throughout the tumor. Caliper measurements of tumor size were continued for 5 weeks in this mouse and showed a marked (>50%) long-term reduction of tumor volume compared with sham-treated control mice.

The preliminary studies have demonstrated the ability to perform serial microPET studies with [^{18}F]FDG and [^{11}C]FMAU in xenografted mice treated with SC144. Interestingly, it has been observed that 5 days of SC144 appears to inhibit tumor perfusion, suggesting a possible anti-angiogenic effect.

Comparison of SC compounds with drugs with known mechanisms.

Six drugs with known mechanisms of action and mechanisms of cell cycle regulations (Table 3) were selected to compare to three SC compounds. Initially, the cytotoxic concentration 50% and 80% CC_{50} and CC_{80} values of all these drugs were determined using MTT assay under a continuous drug exposure for 48 hours (Table 3). For gene expression analysis, MDA-MB-435 cells (1 x 10^6) were treated with the CC_{80} of drugs for 24 hours. The CC_{80} at 24 h value was selected as a single concentration and a single time point because of the prior experience with gene expression analysis using Real-Time PCR studies where it was found that under this condition a significant number of genes could be consistently and reproducibly altered in response to treatment. The goal was to identify patterns of change in gene expression that are characteristic of different classes of drugs, distinct from patterns of final common pathway changes associated with apoptotic or non-apoptotic cell death.

Table 3. Activities and profile of drugs used in this study

Drug	Mechanism of action	Cell cycle profile	CC_{50} (uM)	CC_{80} (uM)
SC144	Unknown	S-phase	4 ± 1.4	10 ± 0.01
SC23	Unknown	G_0/G_1 and S-phase	0.04 ± 0.007	0.1 ± 0.01
SC24	Unknown	G_0/G_1	0.24 ± 0.03	0.97 ± 0.15
Etoposide	Topoisomerase II inhibitor	G_2/M	52.5 ± 3.5	300 ± 106
Mitoxantrone	Topoisomerase II inhibitor	G_2/M	4.5 ± 1.4	7.3 ± 0.35
Camptothecin	Topoisomerase I inhibitor	S and G_2/M	0.03 ± 0.002	0.1 ± 0.002
Cisplatin	DNA alkylating agent		39 ± 1.41	71 ± 1.4
Taxol	Microtuble stabilizer	M-phase	0.04 ± 0.003	0.07 ± 0.01
5-Fluorouracil (5FU)	Thymidylate synthase inhibitor	S-phase	29 ± 10.7	100 ± 0.01

Bioinformatic analysis.

For profiling gene expression analysis, two independent experiments were used with and without drug treatment using the 57,000 Affymetrix GeneChip (U133+2) array. Expression values were truncated below 10, and log transformed. Initial filtering removed all genes that had expression values less than 50 in more than 10% of samples: below this threshold, there is substantial "noise" in the estimates and many genes showing such low values are probably not expressed at all. By allowing

10% to be very low expressers, for a given gene, inclusion of those genes that were unexpressed in just a single group (such as the control group) was allowed. Data reproducibility was confirmed by observation of high correlations between duplicate experiments (Fig. 9, A and B). A consequence of the close correlation of duplicate experiments was that these samples tended to cluster together (see Fig. 10).

To identify genes significantly up- or down-regulated in treated samples (compared to controls) t-tests was carried out for each gene and the t-statistic against difference in mean log expression plotted (Fig. 9C). From this plot it is possible to identify genes that simultaneously are statistically significant, at a given threshold p value, and show a fold change above a defined value. Alternatively, a p-value cutoff can be selected to yield a set of genes with a predetermined false discovery rate.

Lists of genes that were substantially (10-fold) up- or down-regulated after exposure to each of the six drugs with known modes of action were obtained (see Table 4 for SC144 regulated genes). The lists were combined to create a set of 753 genes that could be expected to distinguish between the six drugs with known mechanism of action. A principal components analysis of these genes for all 14 observations (the three SC compounds, in duplicate plus six known drugs, two analyzed in duplicate) showed that the duplicates tended to cluster relatively close together, with the two topoisomerase II inhibitors forming one group, the other known drugs forming a second and the three SC compounds making up a distinct third cluster (Fig. 10A).

Table 4. A list of most significant genes, with p <0.0001 and fold change of at least 2 for SC144 versus control

Gene name	Fold change	p-value
Small proline-rich protein 1A	28.28	0.00002
GTP binding protein overexpressed in skeletal muscle	25.84	0.00003
Interleukin 24	25.83	0.00008
Sestrin 2	25.73	0.00005
Hypothetical protein MGC4504	24.88	0.00002
Cyclin-dependent kinase inhibitor 1A (p21)	19.81	0.00001
Early growth response 1	17.89	0.00006
ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d isoform 2	12.81	<0.00001
AXIN1 up-regulated 1	12.45	<0.00001

Dual specificity phosphatase 5	11.65	<0.00001
Superoxide dismutase 2, mitochondrial	11.52	<0.00001
Heparin-binding epidermal growth factor-like growth factor	9.62	0.00008
A disintegrin and metalloproteinase domain 19 (meltrin beta)	8.5	0.00003
Endothelial PAS domain protein 1	6.59	0.00005
Inositol 1,4,5-triphosphate receptor, type 1	5.96	0.00005
Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	5.4	<0.00001
Fibrinogen, gamma polypeptide	4.9	<0.00001
RAB20, member RAS oncogene family	4.87	<0.00001
Protein kinase, AMP-activated, gamma 2 non-catalytic subunit	4.78	0.00001
Oncostatin M receptor	4.36	0.00008
Cathepsin B	3.89	0.00002
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	3.78	<0.00001
BCL2/adenovirus E1B 19kDa interacting protein 3	3.63	0.00006
Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	3.35	<0.00001
Dual specificity phosphatase 10	3.3	<0.00001
Cell cycle control protein SDP35	0.19	0.00002
Plexin C1	0.19	0.00003
Microphthalmia-associated transcription factor	0.16	0.00009
Calpain small subunit 2	0.14	0.00007
Hypothetical protein DKFZp434L142	0.07	<0.00001

This pattern was supported by a hierarchical cluster analysis (distance metric: correlation; method: cluster distance computed as the average distance between points in the two clusters), based on all genes, which clustered the SC compounds separately (Fig. 10B). This provides evidence to support the hypothesis that the SC drugs have a distinct mechanism of action resulting in different downstream molecular effects on cells, and thus their gene expression profiles. There are many genes that can be identified as being distinct from patterns of final common pathway changes associated with apoptotic or non-apoptotic cell death. This further illustrates that some patterns of change in gene expression are characteristic of different classes of drugs and can be distinguished from nonspecific (e.g., stress-sensitive) genes by bioinformatic tools.

The attributes (gene ontology codes, protein classification, pathway membership) of the genes in Table 4 were compared to the attributes of the full data set to determine the features that best characterized this set of genes (Fig. 11).

From this analysis, it is possible to examine subsets of genes with particular properties of interest. One such group is the set of genes with an EGF-like domain (as an InterPro classification). Fig. 12 shows this gene list using Genetrix™.

Another category of interest is the "Subset" category, which represents user-defined gene categorizations. For this analysis, the sets of genes up- or down-regulated at least 10-fold were used for each drug to create six such categories. It can be seen from Fig. 13 that there was a significant overlap between the genes associated with SC144 treatment and the "Etoposide" subset, with 19 genes in common between the two lists (with an odds ratio of 16.1, $p<0.0001$).

A more detailed analysis that looked at all six genes (Fig. 14) showed that there was also significant overlap with mitoxantrone and CPT.

Taken together, these results indicate that, while SC144 shares some features with the topoisomerase inhibitors (specifically, an overlap in the genes with 10-fold or greater up- or down regulation), all three SC compounds cluster separately from the topoisomerase inhibitors, suggesting that these drugs have a distinct mode of action.

Example II

We built a 10,000 compound database of reported and patented integrase inhibitors, which are in some instances likely to target additional DNA processing enzymes, possibly even more potently than integrase. Using this database, we developed various pharmacophore models followed by toxicity prediction using ADMET Predictor software package (Simulations Plus, Inc., Lancaster, CA) and cluster analysis to separate a majority of antiviral compounds from cytotoxics. On the basis of these pharmacophores, we identified the salicylhydrazide class of compounds as potential leads for inclusion in our anticancer drug discovery program. Pursuing development of this class of compounds, we searched our in-house multiconformational database of ~4.5 million compounds and identified >2,200 compounds that possess common structural features and pharmacophore fragments. We then acquired 950 analogues from commercial sources and subjected them to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assays for an initial screen followed by in-depth testing of proprietary derivatives.

An additional 740 compounds that did not satisfy our ADMET calculations were not tested.

Herein, we present the activity profiles of 18 of these compounds in vitro and focus on two compounds, SC21 and SC23, for detailed analyses. Our results indicate that SC21 and SC23 show remarkable activity in a panel of tumor cell lines, including androgen receptor–positive and -negative prostate cancer cells, estrogen receptor–positive and -negative breast cancer cells and an ovarian cancer line intrinsically resistant to cisplatin. Additionally, we tested the effects of SC21 on cell cycle regulation and apoptosis and evaluated the in vivo therapeutic potential of SC21 in a human prostate cancer xenograft model.

Materials and Methods

Cell Culture

Human prostate cancer cells (PC3, p53 null, AR-; DU145, p53 mutant, AR-; and LNCaP, p53 wild-type, AR+) and breast cancer cells (MCF-7, overexpressed wild-type p53, ER+; MDA-MB-468, p53 mutant, ER+; and MDA-MB-435, p53 mutant, ER-) were obtained from American Type Cell Culture (Manassas, VA). The human ovarian carcinoma cell line (HEY) naturally resistant to cisplatin (CDDP) was kindly provided by Dr. Dubeau (University of Southern California Norris Cancer Center; Buick et al. (1985) Cancer Res. 45:3668–76 and Hamaguchi et al. (1993) Cancer Res. 53:5225–32). The results with CEM cells were previously described (Neamati et al. (1998) J. Med. Chem. 41:3202–9). Cells were maintained as monolayer cultures in RPMI 1640 supplemented with 10% fetal bovine serum (Gemini-Bioproducts, Woodland, CA) and 2 mmol/L L-glutamine at 37°C in a humidified atmosphere of 5% CO_2. To remove the adherent cells from the flask for passaging and counting, cells were washed with PBS without calcium or magnesium, incubated with a small volume of 0.25% trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO) for 5 to 10 minutes, and washed with culture medium and centrifuged. All experiments were done using cells in exponential cell growth.

Drugs

A 10 mmol/L stock solution of all compounds were prepared in DMSO and stored at 20°C. Further dilutions were freshly made in PBS.

Cytotoxicity Assay

Cytotoxicity was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as previously described (Carmichael et al. (1987) Cancer Res. 47:936–42). Briefly, cells were seeded in 96-well microtiter plates (PC3 and DU145 at 5,000 cells/well and LNCaP at 10,000 cells/well; breast and ovarian cells at 4,000 cells/well) and allowed to attach. Cells were subsequently treated with a continuous exposure to the corresponding drug for 72 hours. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (at a final concentration of 0.5 mg/mL) was added to each well and cells were incubated for 4 hours at 37°C. After removal of the medium, DMSO was added and the absorbance was read at 570 nm. All assays were done in triplicate. The IC_{50} was then determined for each drug from a plot of log (drug concentration) versus percentage of cell kill.

Cell Cycle Analysis

Cell cycle perturbations induced by SC21 and camptothecin (CPT) were analyzed by propidium iodide DNA staining. Briefly, exponentially growing PC3 and DU145 cells were treated with different doses of the drug for 24, 48, and 72 hours. At the end of each treatment time, cells were collected and washed with PBS after a gentle centrifugation at 200x g for 5 minutes. Cells were thoroughly resuspended in 0.5 mL of PBS and fixed in 70% ethanol for at least 2 hours at 4°C. Ethanol-suspended cells were then centrifuged at 200x g for 5 minutes and washed twice in PBS to remove residual ethanol. For cell cycle analysis, the pellets were suspended in 1 mL of PBS containing 0.02 mg/mL of propidiumiodide, 0.5 mg/mL of DNase-free RNase A and 0.1% of Triton X-100 and incubated at 37°C for 30 minutes. Cell cycle profiles were obtained using a FACScan flowcytometer (Becton Dickinson, San Jose, CA) and data were analyzed by ModFit LT software (Verity Software House, Inc., Topsham, ME).

Apoptosis Assay

To quantify drug-induced apoptosis, annexin V / propidium iodide staining was done followed by flow cytometry. Briefly, after drug treatments (IC_{80} for each drug for 72 hours), both floating and attached cells were combined and subjected to annexin V / propidium iodide staining using annexin V-FITC apoptosis detection kit (Oncogene Research Products, San Diego, CA) according to the protocol provided by the manufacture. Untreated control cells (24–72 hours) were maintained in parallel to the drugtreated group. In cells undergoing apoptosis, annexin V binds to phosphatidylserine, which is translocated from the inner to the outer leaflet of the cytoplasmatic membrane. Double staining is used to distinguish between viable, early apoptotic, and necrotic or late apoptotic cells (Fadok et al. (1992) J. Immunol. 148:2207–16). The resulting fluorescence (FLH-1 channel for green fluorescence and FLH-2 channel for red fluorescence) was measured by flow cytometry using a FACScan flow cytometer (Becton Dickinson). According to this method, the lower left quadrant shows the viable cells, the upper left quadrant shows cell debris, the lower right quadrant shows the early apoptotic cells and the upper right quadrant shows the late apoptotic and necrotic cells.

Animals

Fifty male athymic nude (nu/nu) mice (Charles River Laboratories, Wilmington, MA) were used for in vivo testing. The animals were fed ad libitum and kept in airconditioned rooms at 20 ± 2°C with a 12-hour light-dark period. Animal care and manipulation were in agreement with the University of Southern California Institutional Guidelines, which are in accordance with the Guidelines for the Care and Use of Laboratory Animals.

Drug Treatment of Tumor Xenografts

PC3 cells from in vitro cell culture were inoculated s.c. in both flanks of athymic nude mice (2×10^6 cells / flank) under aseptic conditions. Tumor growth was assessed by biweekly measurement of tumor diameters with a Vernier caliper (length x width). Tumor weight was calculated according to the formula: TW (mg) = tumor volume (mm^3) = $d^2 \times D/2$,

where d and D are the shortest and longest diameters, respectively. Cells were allowed to grow to an average volume of 100 mm^3. Animals were then randomly assigned for control and treatment groups, to receive control vehicle or SC21 (0.3 and 3 mg/kg, dissolved in isotonic saline solution) via i.p. injections once a day for 5 days. Treatment of each animal was based on individual body weight. After 5 days of treatment, the tumor volumes in each group were measured once a week for 4 weeks. Treated animals were checked daily for treatment toxicity / mortality. The percentage of tumor growth inhibition was calculated as %T/C = 100 x (mean TW of treated group / mean TW of control group).

Computational ADMET Analysis

Structures of all the compounds were built and minimized in the Catalyst software package (Accelrys, Inc., San Diego, CA). The possible unique conformations for each compound over a 20 kcal/mol energy range were generated using the best conformation generation method within Catconf module of Catalyst. The low-energy conformers of all the compounds were exported to Accord (Accelrys) to calculate A log P 98 and fast polar surface area. The log P values were also calculated with ADMET Predictor (Simulations Plus). The human intestinal absorption plot was constructed using the A log P 98 and the fast polar surface area values of the compounds as previously described (Egan et al. (2000) J. Med. Chem. 43:3867–77 and Egan and Lauri (2002) Adv. Drug Deliv. Rev. 54:273–89).

Statistical Analysis

Assays were set up in triplicate and the results were expressed as means $\pm$ SD. Statistical analysis and P value determination were done by two-tailed paired t test with a confidence interval of 95% for determination of the significance differences between treatment groups. $P < 0.05$ was considered to be significant. ANOVA was used to test for significance among groups. The SAS statistical software package (SAS Institute, Cary, NC) was used for statistical analysis.

Results

Selection of Compounds Based on Lipinski's Rule-of-Five

From >2,200 compounds selected using pharmacophore modeling, toxicity prediction and clustering, a selection of 950 compounds were evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay. Eighteen compounds exhibited superior activity profiles against a panel of cancer cell lines from different origins. The structures, physicochemical properties, and cytotoxicities of these compounds are presented in Table 5. All compounds satisfied Lipinski's rule-of-five. This rule was based on an analysis of 2,245 compounds from the World Drug Index database that ~90% of marketed drugs have (a) molecular weight <500, (b) C log P <5, (c) hydrogen bond donors (sum of O-H and N-H) <5, (d) hydrogen-bond acceptor (sum of N and O atoms) <10 (Lipinski et al. (1997) Adv. Drug Deliv. Rev. 23:3–25).

Table 5. Physicochemical properties and cytotoxicity of salicylhydrazides

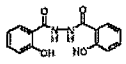
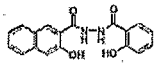
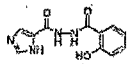
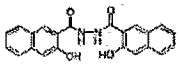
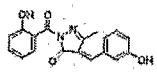
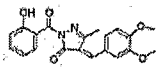
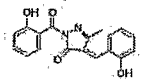
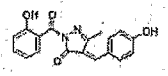
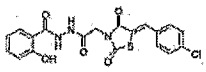
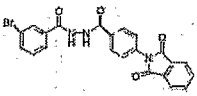
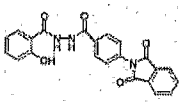
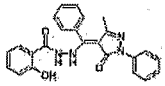
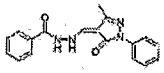
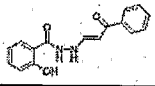
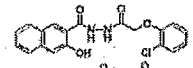
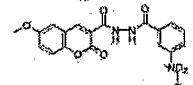
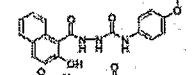
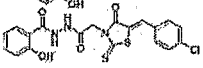
Compound	Structure	Molecular weight	HBA	HBD	Rbond	A log *P* 98	Fast polar surface area	IC_{50} (μmol/L)x
SC20		272	6	4	7	1.33 (2.02)	101.8	0.1 ± 0.01
SC21		322	6	4	7	2.24 (3.29)	101.8	0.4 ± 0.06
SC22		246	7	4	6	0.97 (0.22)	100.1	NT
SC23		372	6	4	7	3.15 (3.77)	90.9	2.3 ± 0.2
SC24		322	6	2	5	2.75 (3.56)	90.9	0.13
SC25		366	7	1	6	2.99 (3.86)	87.9	0.15
SC26		322	6	2	5	2.75 (3.47)	90.9	0.06
SC27		322	6	2	5	2.75 (3.47)	90.9	0.06
SC28		431	8	3	8	2.96 (2.66)	118.9	10 ± 2
SC29		464	7	2	6	3.84 (2.75)	98.17	7 ± 2
SC30		401	8	3	7	2.83 (2.45)	118.99	6.5 ± 1
SC31		412	7	3	7	3.02 (4.14)	95.71	10 ± 2
SC32		320	6	2	5	1.54 (3.16)	74.89	2 ± 1
SC33		282	5	3	7	1.97 (2.86)	81.03	20 ± 2

Table 5. Physicochemical properties and cytotoxicity of salicylhydrazides (Cont'd)

Compound	Structure	Molecular weight	HBA	HBD	Rbond	A log P 98	Fast polar surface area	IC_{50} (μmol/L)
SC34		370	6	3	8	3.01 (3.72)	89.9	20 ± 2
SC35		383	9	2	6	1.77 (2.30)	138.20	12 ± 2
SC36		365	7	4	9	2.75 (3.60)	102.77	20 ± 2
SC37		447	6	3	8	3.33 (2.10)	101.69	15 ± 3

All 18 compounds showed IC_{50} values ≤20 umol/L in either CEM or HEY cells. The range of activity varied >300-fold, with SC26 and SC27 being the most potent (IC_{50} = 0.06 umol/L) and SC33, SC34, and SC36 the least potent (IC_{50} = 20 umol/L).

Selection of Compounds Based on Polar Surface Area

From the original studies of Palm et al. ((1998) J. Med. Chem. 41:5382–92, (1997) Pharm. Res. 14:568–71, and (1996) J. Pharm. Sci. 85:32–9) with a small number of compounds and the more recent studies by Kelder et al. ((1999) Pharm. Res. 16:1514–9) with 1,590 orally administered drugs, it was recommended that a maximum polar surface area value of ~120 Angstrom^2 be for compounds intended to be orally absorbed by passive diffusion. Therefore, compounds with a polar surface area >140 Angstrom^2 would tend to show poor (<10%) absorption, whereas compounds with polar surface area <60 Angstrom^2 could be predicted to show complete (>90%) absorption. Several variants of polar surface area calculations such as dynamic, topological, and fast polar surface area are incorporated in various software packages (Clark and Grootenhuis (2003) Curr. Top. Med. Chem. 3:1193–203). We used fast polar surface area plots to predict absorption as described (Egan et al. (2000) J. Med. Chem. 43:3867–77 and Egan and Lauri (2002) Adv. Drug Deliv. Rev. 54:273–89) and the data are presented in Fig. 15. Compounds that fall in the area shown by the 95% confidence ellipse are expected to have favorable absorption and oral bioavailability. All compounds showed fast polar surface areas of <140 Angstrom^2 and log P value of <5. Therefore, no

obvious violations were observed using either the 99% confidence ellipse (outer ellipse) or 95% confidence ellipse (inner ellipse; Fig 1).

SC21 and SC23 Show Remarkable Potency against a Panel of Hormone-Dependent and -Independent Cell Lines

Although many of our original 950 compounds showed favorable calculated physicochemical properties, the 18 compounds presented in Table 5 were among the most potent in our initial screen. On the basis of subsequent testing against drug-resistant cell lines, we selected SC21 and SC23 for further evaluation. The sensitivity of a panel of seven human cancer cell lines to SC21 and SC23 was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-assay. Both drugs exhibit a high potency in this panel of cancer cell lines from different tumor origins (Table 6) and exhibited a time- and dose-dependent growth-inhibitory effect (Fig. 16). Thus, in vitro cell death increased with increasing concentrations and exposure time of SC21 and SC23.

Table 6. Sensitivity of breast, ovarian, and prostate cancer cell lines to SC21, SC23, and CPT

Cell line	Origin	Hormone receptor	p53	pRb	p16	p21	IC_{50} values (mean ± SD)*		
							SC21 (nmol/L)	SC23 (nmol/L)	CPT (nmol/L)
PC3	prostate	AR–	null	WT	WT	WT	3,250 ± 106	2,000 ± 500	900 ± 210
DU145	prostate	AR–	Mut	null	Mut	Mut	120 ± 50	50 ± 19	25 ± 7
LNCaP	prostate	AR+	WT	WT	WT	WT	200 ± 70	850 ± 200	25 ± 6
HEY	ovarian	AR+	WT	ND	WT	ND	400 ± 60	2,350 ± 212	35 ± 7
MCF-7	breast	ER+	WT	WT	WT	WT	40 ± 7	280 ± 35	30 ± 3
MDA-MB-435	breast	ER–	Mut	WT	WT	WT	35 ± 7	240 ± 28	27 ± 2
MDA-MB-468	breast	ER–	Mut	null	ND	WT	200 ± 2	50 ± 14	100 ± 2

Abbreviations: AR, androgen receptor; ER, estrogen receptor; WT, wild-type; Mut, mutated; ND, not determined.
*Cytotoxic concentration (IC_{50}) is defined as drug concentration causing a 50% decrease in cell population.

The activity of both agents was remarkable in prostate cancer cell lines with the exception of PC3 cells, which seemed to be the least sensitive cell line to SC21 and SC23 (IC_{50} value 3.2 ± 0.2 and 2.0 ± 0.5 umol/L, respectively). The difference in sensitivity to these agents may be independent of the status of androgen receptor (mutated in PC3 and DU145), p53 (null in PC3, mutated in DU145 and wild-type in LNCaP), p21 (mutated in DU145), or p16 (mutated in DU145; Table 6).

Interestingly, SC23 exhibits a high potency in pRb-mutated cell lines (DU145 and MDAMB468).

SC21 and SC23 also showed remarkable potency in the three breast cancer cell lines irrespective of estrogen receptor (ER+ in MCF-7 and MDA-MB-435) and p53 status (mutated in MDA-MB-435 andMDA-MB-468). The activity of SC21 in ovarian tumor–derived cell line HEY was also remarkable considering that this cell line seemed to be practically resistant to cisplatin, the most commonly used drug in ovarian cancer (Buick et al. (1985) Cancer Res. 45:3668–76 and Hamaguchi et al. (1993) Cancer Res. 53:5225–32). This cell line however seemed to be the least sensitive to SC23.

SC21 Treatment Induces a G1 and S Phase Cell Cycle Arrest

Cell cycle perturbations induced by SC21 were examined in DU145 and PC3 prostate cancer cells as well as in highly metastatic MDA-MB-435 breast cancer cells and cisplatinresistant HEY ovarian cancer cells. The analysis of DNA profiles by flow cytometry indicated that SC21 induced cell cycle arrest in G_0/G_1 phase in DU145 (Fig. 17). At 72 hours of exposure to SC21, 65% of the cells were still retained in G_0/G_1 phase compared with 46% in controls. The observed increment in G_0/G_1 was accompanied by a decrease in the number of cells in S and G2-M phases. Similar effects were obtained on asynchronous breast cancer MDA-MB-435 cells (Fig. 17).

It was noteworthy that SC21 induced S phase arrest in PC3 and HEY cell lines (Fig. 17). SC21 treatment for 72 hours resulted in 52% and 69% accumulation in S phase in PC3 and HEY cells, respectively. The effect observed on both cell lines was comparable to the arrest induced by CPT.

The maximum arrest in MDA-MB-435 and PC3 cells was observed at 48 hours of SC21 treatment, which was sustained up to 72 hours. This property of SC21 to induce cell cycle arrest makes it an ideal agent for combination with drugs acting at different stages of cell cycle, such as taxanes.

SC21Treatment Induces Apoptosis

SC21 and CPT-induced apoptosis was measured by flow cytometry (Fig. 18). SC21 at an IC_{80} dose for 72 hours induced 12% to 15% apoptosis as measured by calculating sub-G_0/G_1 population. CPT resulted in 30% apoptosis under similar conditions (Fig. 18). An early event in apoptotic cell death is the translocation of the phosphatidyl-serine residues to the outer region of the cell membrane. This event precedes nuclear breakdown, DNA fragmentation, the appearance of most apoptosis-associated molecules, and is readily measured by annexin V binding assay. By this method, we compared SC21 with CPT. As shown in Fig. 19, the percentage of early plus late apoptotic cells reached 72% and 59% after 72 hours exposure to SC21 and CPT, respectively.

SC21 Shows in vivo Efficacy in Mice Xenograft Models

The in vivo efficacy of SC21 was evaluated in nude mice inoculated with human prostate PC3 cells. A schematic outline of the experimental procedure is shown in Fig. 10A. Animals were treated with daily i.p. injections of saline (controls) and SC21 at 0.3 or 3 mg/kg. After 5 days of dosing, the drug treatment was discontinued and the animals were monitored biweekly for 5 weeks. Fig. 20B shows the volume (mean $\pm$ SD) for SC21-treated PC3 xenografts over time. SC21 significantly reduced tumor burden in prostate xenografts (Fig. 20C) without apparent toxicity. Treatment with SC21 was well-tolerated and did not result in any drug-related deaths and changes in body weight. The untreated control mice had an average weight of 33.2 $\pm$ 1.45 g before the experiments and 34.3 $\pm$ 2.79 g after the experiment. Mice treated with 0.3 mg/kg of SC21 had an average weight of 32.1 $\pm$ 1.92 g and mice treated with 3.0 mg/kg of SC21 had an average weight of 33.3 $\pm$ 1.89 g.

Discussion

Using pharmacophore models to distinguish antiviral compounds from anticancer compounds, we have successfully identified a new class of leads with remarkable activity profiles both in vitro and in vivo. Two members of this new class of compounds, SC21 and SC23, were evaluated

further against a range of human tumor-derived cancer cell lines. Both compounds inhibited cell growth in a time- and dose-dependent manner. The efficacy of SC21 and SC23 in prostate cancer cells was comparable to that of CPT and their cytotoxic effects may be independent of the androgen receptor, p53, p21, and p16 status. Interestingly, defects in pRb expression seemed to confer higher sensitivity to SC23 in DU145 and MDA-MB-468 cell lines. SC21 seemed to be 16- to 90-fold more potent in ER+ and ER- breast cancer cells as compared with PC3 prostate cancer cells, suggesting that this compound might be a potential candidate for the treatment of hormone receptor–positive and –negative breast cancers.

Consistent with the effect of SC21 on cell growth inhibition, our data also show the ability of this compound to arrest cell cycle progression. This property of SC21 opens the possibility to investigate innovative combinations with other agents acting at different stages of the cell cycle, such as taxanes. Notably, the different cell lines used in the present study displayed different cell cycle perturbations following SC21 treatment. SC21 arrested DU145 and MDA-MB-435 cells in G_0/G_1 phase, and PC3 and HEY cells in S phase. Previously, similar observations reported with different drugs were attributed to different cell cycle checkpoint status and susceptibility to apoptosis (Zuco et al. (2003) Biochem. Pharmacol. 65:1281–94, Schiff and Horwitz (1980) Proc. Natl. Acad. Sci. USA 77:1561–5, and Lanzi et al. (2001) Prostate 48:254–64.). It is well-established that p53 plays a major role on cell cycle retention in G_0/G_1 phase. We can conclude that the cell cycle arrest induced by SC21 in these cell lines may be independent of the p53 status (mutated in DU145, null in PC3). Further studies using various p53 mutant and p53 null cell lines are required to better understand the role of p53 in response to SC21 treatment.

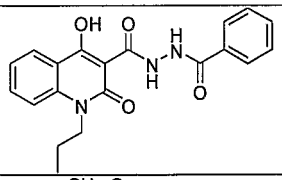
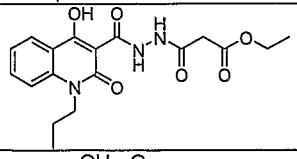
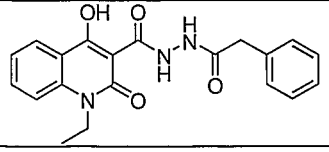
It is known that apoptosis-signaling pathways and cellular events controlling them, have a profound effect both on cancer progression and in response to chemoherapy (Sun et al. (2004) J. Natl. Cancer Inst. 96:662–72, Assuncao Guimaraes and Linden (2004) Eur. J. Biochem. 271:1638–50,

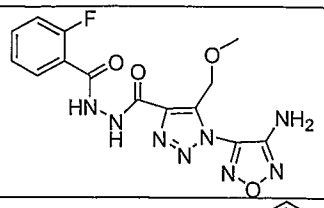
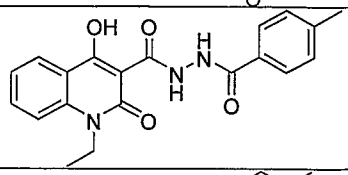
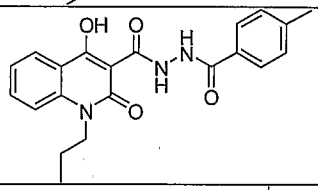
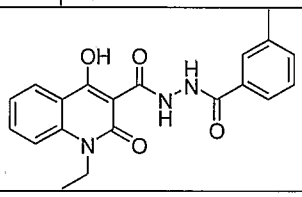
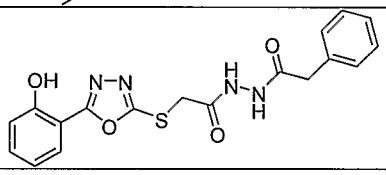
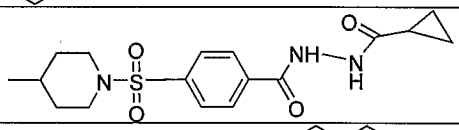
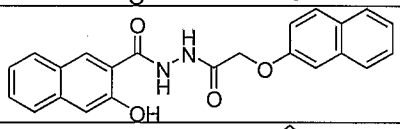
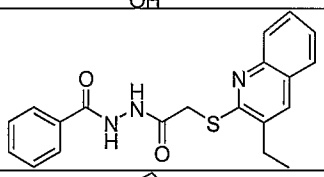
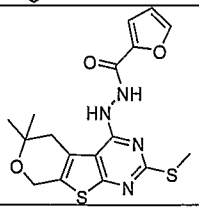
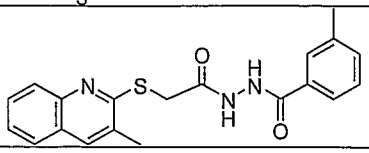
Pommier et al. (2004) Oncogene 23:2934–49, and Norbury and Zhivotovsky (2004) Oncogene 23:2797–808). Based on annexin V / propidium iodide staining and sub-G_0/G_1 fractions, it is clear that SC21 activity is mediated by apoptosis in a fashion comparable to that of CPT. SC21 also showed in vivo antitumor efficacy against PC3 tumor xenografts. Significant reduction in tumor growth was found for all doses tested. Furthermore, SC21 was well-tolerated and did not result in drug-related deaths. Finally, the fact that SC21 exhibited in vivo efficacy against the PC3 prostate cancer xenografts despite PC3 cells being the least sensitive in vitro model, clearly show its potential as a novel anticancer agent.

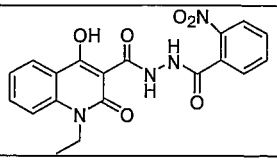
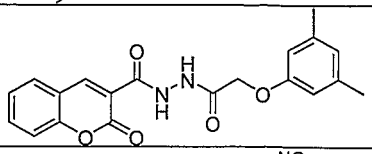
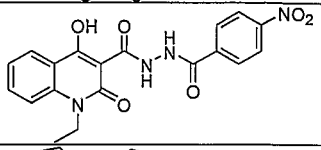
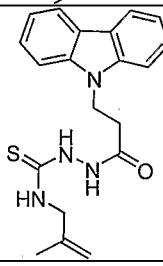
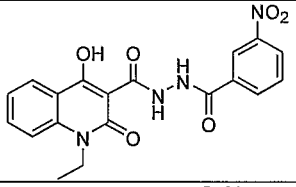
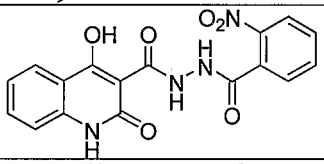
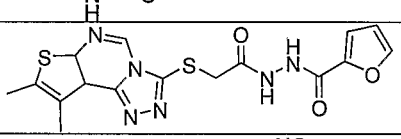
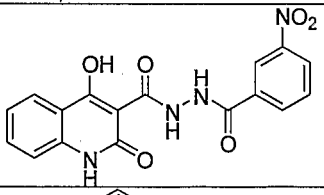
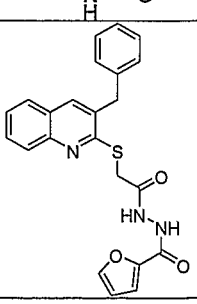
In conclusion, considering their cytotoxicity profiles in a variety of in vitro systems, including different cell lines having intrinsic or acquired resistance to known drugs, and their favorable in vivo properties, salicylhydrazides seem to represent a novel class of anticancer drugs that function by a new mechanism of action. These agents could have promising therapeutic potential.

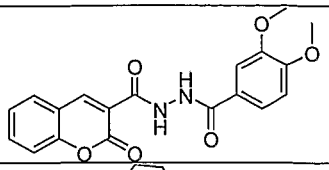
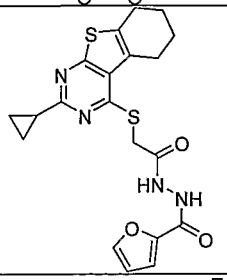
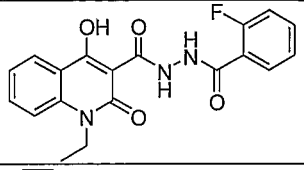
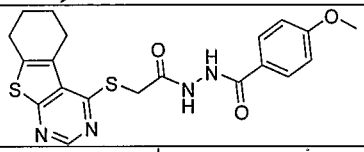
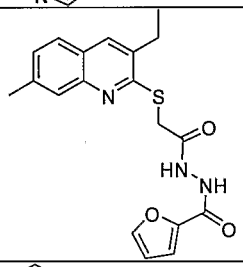
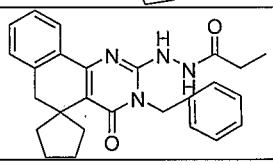
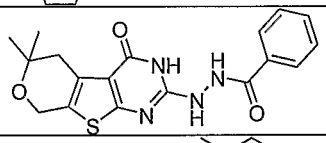
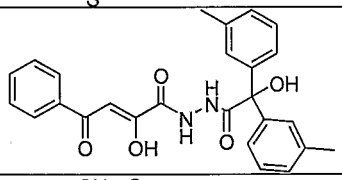
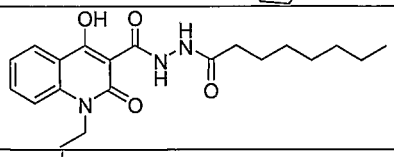
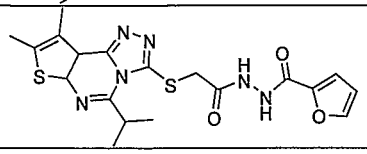
Example III

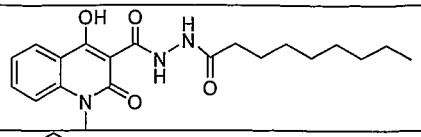
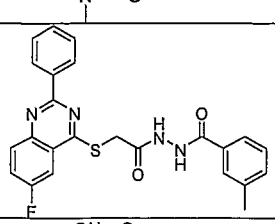
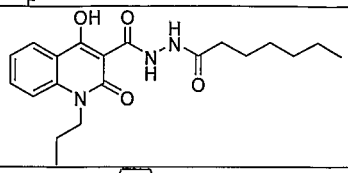
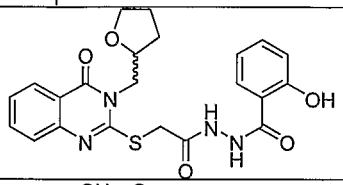
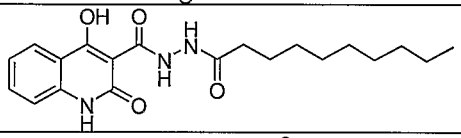
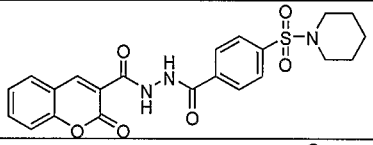
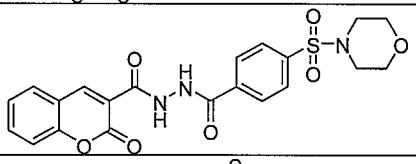
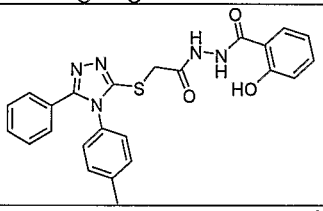
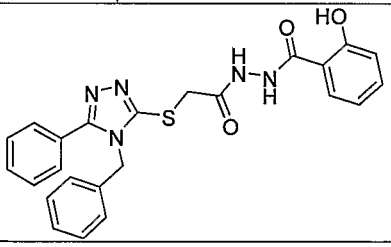
Table 7. 50% Cytotoxic concentration (IC_{50}) values of a series of SCs in HEY ovarian cancer cells

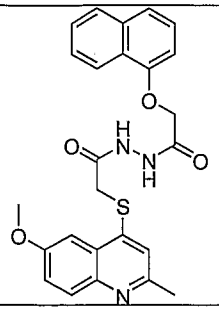
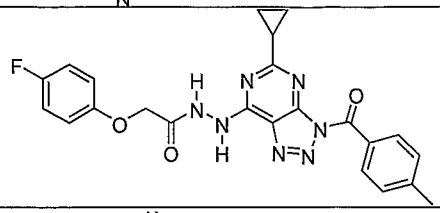
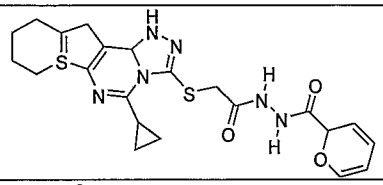
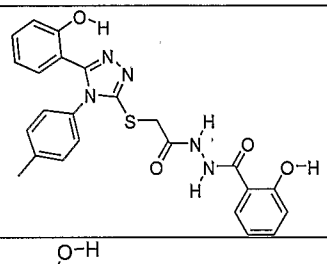
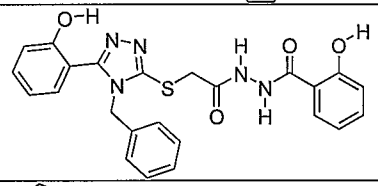
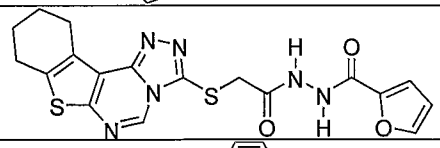
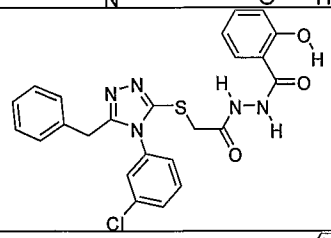
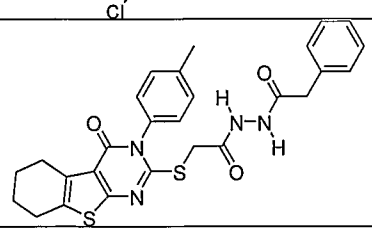
compd	Structure	IC_{50}
SC201		2
SC202		1
SC203		6

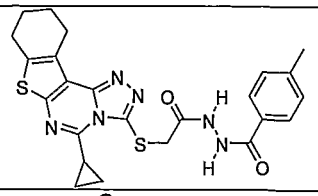
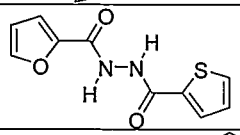
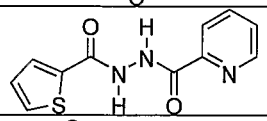
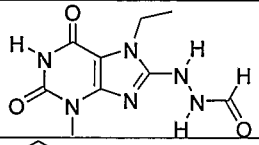
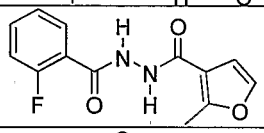
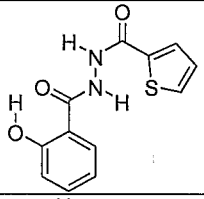
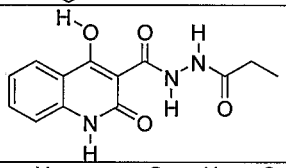
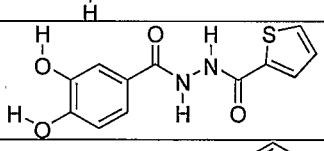
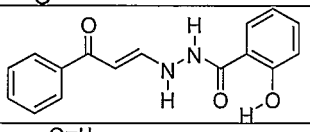
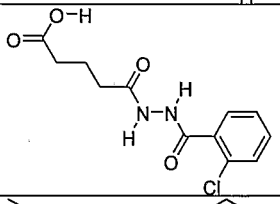
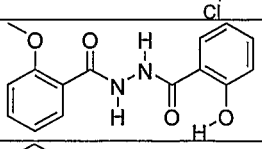
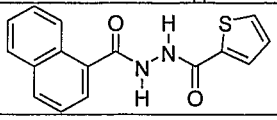
SC204		1
SC205		6
SC206		1
SC207		6
SC208		4
SC209		8
SC210		1
SC211		10
SC212		1
SC213		12

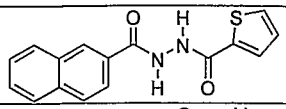
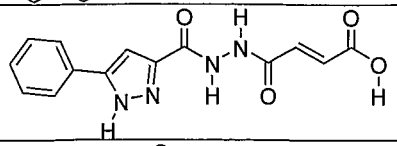
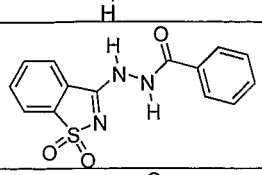
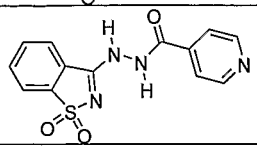
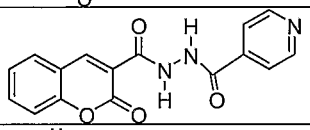
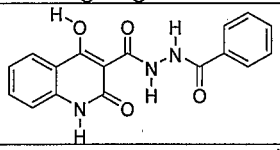
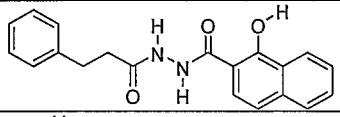
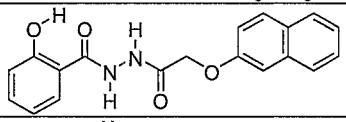
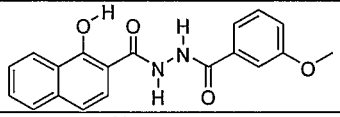
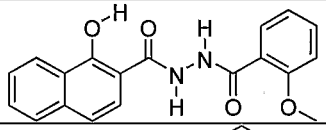
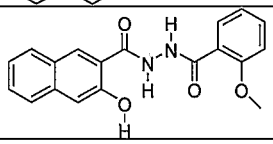
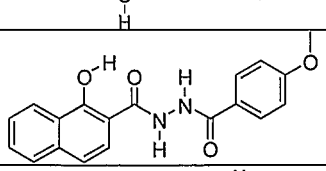
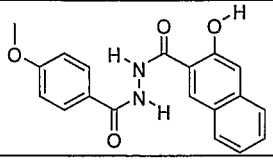
SC214		1
SC215		12
SC216		5
SC217		4
SC218		4
SC219		2
SC220		4
SC221		4
SC222		8

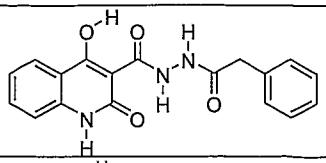
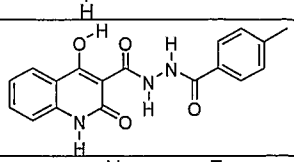
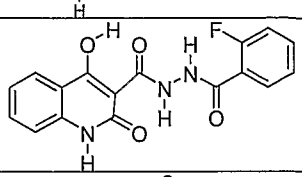
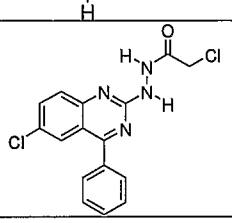
SC223		4
SC224		4
SC225		2
SC226		5
SC227		6
SC228		6
SC229		8
SC230		3
SC231		3
SC232		6

SC233		5
SC234		8
SC235		7
SC236		2
SC237		7
SC238		3
SC239		3
SC240		5
SC241		5

SC242		5
SC243		6
SC244		15
SC245		1
SC246		1
SC247		14
SC248		2
SC249		2

SC250		2
SC251		15
SC252		16
SC253		12
SC254		17
SC255		14
SC256		3
SC257		2
SC258		2
SC259		18
SC260		10
SC261		11

SC262		11
SC263		5
SC264		5
SC265		5
SC266		7
SC268		10
SC270		5
SC271		6
SC272		7
SC273		11
SC274		11
SC275		12
SC276		13

SC277		14
SC278		13
SC279		6
SC280		6

Example IV

Subsequent confirmation of the potency of SC23 against a panel of cells resistant to known drugs prompted us to investigate its mechanism of action. As a new chemical entity, SC23 is very promising for development because of its potency, selectivity, and novelty based on chemical structure and biological activities.

Mechanistic Studies. Our preliminary results show that SC23 arrests cells in G_0/G_1 and induces apoptosis. To gain further insight into the molecular mechanism(s) involved in the cytotoxicity induced by SC23, we next evaluated the expression of a panel of genes involved in cell cycle regulation, apoptosis and tumor progression (Table 8 and Fig. 22) using StaRT-PCR.

Table 8. SC23-Induced Expression of Selected Genes Important in Cell-cycle, Apoptosis, and Proliferation

Gene	Control	3 hours	6 hours	12 hours	24 hours	48 hours
BCL2	182.72	152.37	121.2	122.24	35.99	22.29
BCL2L1	20925.99	9449.42	10428.62	23160.22	30504.02	21299.99
JUN	2499.15	1846.15	4168.04	5419.7	8677.27	9510.55
JUNB	NR[a]	NR	NR	NR	NR	NR
MAD	448.91	496.75	763.05	7122.48	13204.24	12999.27
MAX	NR	NR	NR	NR	NR	NR

TNFRSF1A	26.59	15.56	20.58	107.17	26.61	21.29
TP53	1950.14	567.53	1005.63	1663.37	4238.06	2821
NFKB1	4859.08	2694.24	3274.07	6131.17	16919.99	26524.38
TNFSF10	3328.29	82.86	532.07	288.01	151.09	79.65
CASP1	496.95	68.36	80.87	113.28	142.49	184.55
PCNA	11012.48	8574.58	6433.1	5756.09	12795.59	6913.72
TNFAIP1	3865.15	4737.75	7127.73	22572.81	39858.96	49955.6
DAP	18155.74	9037.57	12572.87	38087.74	56353.65	70325.95
KDR	NR	NR	NR	NR	NR	NR
MAP3K14	25.78	55.57	68.69	158.89	143.47	720.55
CCNA2	160.4	126.84	348.88	211.27	241.1	137.61
CDC2	1863	2365.6	1650.84	1566.64	2054.95	497.44
CDK7	2690.33	1068.25	778.75	2551.62	9983.36	19212.16
CDK8	668.56	412.64	354.76	605.61	1706.26	3052.4
CDKN1A	15.07	11.42	27.06	129.69	225.4	288.56
CDKN1B	112.64	72.37	390.76	310.81	2092.37	414.6
CDKN2A	8786.98	415.63	5611.87	403.27	163.48	3382.09
CDKN2C	439	458.41	781.31	770.42	904.8	650.26
E2F1	NR	NR	NR	NR	NR	NR
E2F4	7513.16	7540.97	1285.78	1673.69	1582.79	3738.46
E2F5	618.18	268.09	243.27	486.8	1178.84	2144.23
MYC	3311.86	974.69	2650.11	5205.65	11117.04	8944.84
RB1	9989.91	7076.29	5973.3	3301.83	5935.72	6552.57
RBL2	6254.58	1287.91	2112.09	2698.44	8604.92	11571.33
CCND3	245.98	195.17	175.1	137.33	404.3	641.75
CCNG1	31936.56	2956.79	6161.69	9794.14	17499.15	25388.52
CCNE1	106.85	51.36	33.08	48.10	47.28	146.61
CDC25C	519.06	394.83	606.06	779.22	326.46	68.37
TGFBR2	3697.83	1420.02	2582.67	6799.19	15367.23	5072
TGIF	22071.87	3112.84	8002.87	13858.11	15445.36	19849.69
TRAF4	93.2	69.06	91.86	222.84	242.44	298.28
CYP1A2	17.14	59.45	10	69	36.62	37.48
PTGS2	69.22	82.69	157.43	2138.52	19413.37	26516.88

T24 bladder cancer cell lines were treated with SC23 for indicated time and samples were analyzed by StaRT-PCR. [a] NR, no results.

StaRT-PCR™ (Standardized Reverse Transcription Polymerase Chain Reaction). First described by Willey et al. ((2004) Methods Mol. Biol. 258:13-41), this technique uses standardized mixtures of competitive templates (CT) as internal standards in generating valid and reproducible numerical gene expression data for multiple genes. After the mRNA was converted to cDNA, the cDNA was mixed with a proprietary Standardized Mixture of Internal Standards™ (SMIS™, GeneExpress, Inc.). In the standard mixture, there is an internal standard CT for each gene to be measured as well as one for a reference

gene. (i.e., β-actin, GAPDH). The amplicons produced by StaRT-PCR™ was then separated on capillary electrophoresis. The amount of internal standard CT or NT amplimer was determined by measuring each peak area. All data were then reported as number of molecules of mRNA for gene of interest per 10^6 molecules of reference gene (normalizer gene). Serial dilutions of the SMIS™ allow quantitative measurements over 7 log range of gene expression observed in cells from <10 to 10^7 molecules / 10^6 molecules reference gene. Data presented in Table 8 are number of copies that have been normalized against 10^6 molecule of β-actin.

Modulation of Genes Involved in Cell Cycle Regulation and Cell Proliferation. Because SC23 induced G_0/G_1 arrest, initially we were interested in cell cycle genes. Therefore, we studied the changes in expression of key genes involved in cell-cycle regulation by SC23 using StaRT PCR. It is well established that in response to genotoxic damage, p53 is up-regulated resulting in arrest of cells in G_0/G_1, activating the repair of the DNA or driving cells to apoptosis when the injury can not be repaired. P53 arrest is mediated the activation of p21 and p27 (Fig. 21). p21 and p27 are members of the Cip1/Kip1 family of cyclin-dependent kinase inhibitors (CDKi). Together with another family of CDKi's, the INK4 (p16, p15, p18 and p19), they inhibit the activity of the cyclin / cyclin-dependent kinases (CDK) complexes. This inhibition causes the hypophosporylation of the retinoblastoma protein (Rb), preventing the release of the transcription factor E2F and inhibiting transcription of cell proliferation-associated genes.

The SC23-induced G_1 arrest correlated with the upregulation of p21 and p27. Treatment with SC23 induced a downregulation in the expression of cyclin A and cdk1 coincident with the overexpression of p53. These data correlate with the G_1 retention in SC23 treated cells. The expression of p16 was undetectable as expected because T24 cells are p16 deficient cells due to a promoter hypermethylation. Although no difference was seen in p18 expression, SC23 induced an upregulation of the expression of cdk7 and cdk8, two kinases involved in early S-phase.

The expression of cyclin E, cyclin D3 and cyclin G1 was slightly increased. The overexpression of some of these cyclins coincides with the increased expression of MYC (Fig. 22). Cdc25 was downregulated in SC23 treated cells. PCNA however remained unaltered.

Transcription factors E2F1, E2F4 and E2F5 are considered downstream mediators of $p16^{INK}$-pRB pathway. Our data revealed an upregulation of Rb-like protein 2 (also known as p130), as well as E2F5 transcription factor upon exposure of SC23 (Fig. 22). SC23 induced the expression of E2F5 but reduced E2F4 expression. These data suggest that E2F4 inhibition could be related with the profound G_1-phase arrest induced by SC23 in T24 cells. The regulation of the expression of these E2F factors reflects the importance of p107- and p103-binding receptor complexes in mediating the cell cycle arrest observed in SC23-treated T24 cells. These data also suggest that dissociation of E2F4 from pRB family proteins could play a role in the SC23-induced cytotoxicity (Fig. 23). Further studies are required to confirm this hypothesis.

The upregulation of the expression of NFKB observed, correlated with the overexpression of other genes such as proliferation genes (cyclin D3 and c-MYC), immune genes (such as COX2) or anti-apoptotic genes ($Bcl\text{-}X_L$).

Modulation of genes involved in apoptosis. In the present work, we also evaluated the expression pattern of key genes known to regulate apoptosis. SC23 induced the expression of annexin V gene, data that correlate with the flow cytometric analysis. SC23 induced the downregulation of this pro-apoptotic gene. Bcl2L1 (including $Bcl\text{-}X_L$ and $Bcl\text{-}X_S$ members) expression, however, was not substantially altered in SC23 treated cells compared to corresponding untreated control cells (Figs. 21 and 22, Table 8).

SC23 also demonstrated an effect on apoptosis pathway through the upregulation of MAD, TNF-α (TNFAIP1), JUN, MAP3K14, NFKB, annexin V, and DAP genes. SC23 also induced a significant downregulation of caspase 1 and TNF receptor, as well as the

downregulation of Bcl2 implying that apoptosis mediated by SC23 is linked to an oxidative stress where the mitochondria play a central role (Figs. 21 and 22, Table 8).

Mode of Action. To investigate the probable mode of action of SC23, we applied gene expression profiling, using the 57,000-probe set U113+2 expression array (Affymetrix) to compare expression with and without drug treatment. Expression values were truncated below 10, and log transformed. Initial filtering removed all genes that had expression values less than 50 in more than 10% of samples: below this threshold, there is substantial "noise" in the estimates and many genes showing such low values are probably not expressed at all. By allowing 10% to be very low expressers, for a given gene, we allowed inclusion of those genes that were unexpressed in just a single group (such as the control group). Data reproducibility was confirmed by observation of high correlations between duplicate experiments (Fig. 24). Five drugs of known mechanism of action were also studied, to serve as positive controls.

These data were analyzed using Genetrix software package in a number of ways as described below to provide clues as to the most probable mode of action of SC23.

Scatter Plot. At the simplest level, we examined the correlation of SC23 expression with each of the positive controls (Fig. 25), overall (Fig. 25A) and restricted to genes that were altered at least five fold following exposure to any one drug (Fig. 25B). The closest relationship was with taxol (correlation, r = 0.96), compared to mitoxantrone (r=0.84), CPT (r=0.80), etoposide (r=0.72), and 5FU (r=0.93).

Examination of Genes with Marked Changes in Expression. We next examined the genes up-regulated at least 5-fold following SC23 treatment (Table 9). Of particular interest are the first three genes, microtubule-associated protein 4, microtubule affinity-regulating kinase 2 and 4, which implies similarity in mechanism to taxol. It should be noted that taxol, although a well-known microtuble poison, has not been shown to regulate kinases 2 and 4.

Table 9. List of Selected Genes Significantly Upregulated in Response to SC23 Treatment

Code	Gene	Code	Gene
4134	Microtubule-associated protein 4	83855	Kruppel-like factor 16
2011	microtubule affinity-regulating kinase 2	80830	Apolipoprotein L, 6
57787	microtubule affinity-regulating kinase 4	7517	X-ray repair complementing defective repair
10766	Transducer of ERBB2	5595	Mitogen-activated protein kinase 3
7423	Vascular endothelial growth factor B	55361	Phosphatidylinositol 4-kinase type II
7422	Vascular endothelial growth factor	6300	Mitogen-activated protein kinase 12
51281	Ankyrin repeat and MYND domain containing 1	5563	Protein kinase, AMP-activated, alpha 2 catalytic subunit
53916	RAB4B, member RAS oncogene family	55066	Pyruvate dehydrogenase phosphatase regulatory subunit
7991	Putative prostate cancer tumor suppressor	23646	Phospholipase D3
5089	Pre-B-cell leukemia transcription factor 2	3710	Inositol 1,4,5-triphosphate receptor, type 3
6988	T-cell leukemia translocation altered gene	5914	Retinoic acid receptor, alpha
3976	Leukemia inhibitory factor	84957	Tumor necrosis factor receptor superfamily
26145	Interferon regulatory factor 2 binding protein	2026	Enolase 2, (gamma, neuronal)
3669	Interferon stimulated gene 20kDa	8614	Stanniocalcin 2
3460	Interferon gamma receptor 2	8862	Apelin
64108	28kD interferon responsive protein	23654	Plexin B2
11128	Polymerase (RNA) III	1522	Cathepsin Z
85441	Peroxisomal proliferator-activated receptor A interacting complex 285	8347	Histone 1, H2bc
10111	RAD50 homolog (S. cerevisiae)	8357	Histone 1, H3h
83463	MAX dimerization protein 3		

We also examined the overlap in genes up-regulated in response to SC23, taxol and 5FU. There were 175 genes in common among the three compounds (Fig. 26), with 29 genes common to taxol and SC23, 10 genes in common between SC23 and 5-FU, and 31 genes in common between taxol and 5-FU.

Clustering. Genes that found to be 5-fold upregulated following treatment (N=1147) with any one of the six drugs were used as the basis for a principal components and a hierarchical clustering analysis to

examine where SC23 clustered relative to the other five drugs. The principal components analysis of these genes for all the observations showed that the duplicates tended to cluster relatively close together, with the two topoisomerase II inhibitors forming one group, the other known drugs forming a second and SC23 clustered with taxol (Fig. 27). A similar pattern was apparent in the hierarchical clustering, which again identified taxol as the nearest neighbor to SC23 with respect to changes in gene expression.

In summary, our gene expression analysis suggests a mechanism for SC23 analogous to taxol, even though the two compounds are structurally distinct and arrest cells at different stages of cell cycle.

Proteomic Analysis

SC23-Treated Cells Upregulate a Variety of Proteins in the Molecular Weight Range of 8-58 kDa. Comparisons of total protein extracts of SC23 treated and untreated T24 cells on SDS-PAGE gels revealed the complexity of the protein content and a clear up-regulation of certain proteins in the molecular weight range of 8-58 KDa (Fig. 28). 2DE was then used to separate these proteins (Fig. 29). As above, treatment with SC23 led to a significant up-regulation of many proteins. Similar analysis was carried out for DU145 cells treated with SC23.

All the 2D gels were then quantified with PDQuest (BioRad) and approximately 125 spots were identified that significantly changed (> 2 fold) compared to untreated samples. A representative section of a gel is shown in Fig. 30. Proteins identified from the spots shown in Fig. 30 are β-tubulin, myc promoter-binding protein (MPB-1), retinoblastoma-binding protein 7, vimentin, enolase, phosphopyruvate hydratase beta, mitochondrial ATP synthase beta chain.

Representative tandem MS analyses of four proteins isolated from 2-D gel electrophoresis analysis of SC23 treated cells are shown in Fig. 31. Briefly, the CyproRuby stained gel spots were dissected from the gel and subjected to in-gel trypsin digestion. At the end of digestion, the peptides from the trypsin-digested gel spots were then extracted and analyzed by a

Thermofinnigan LTQ linear ion trap mass spectrometer in collaboration with Dr. Austin Yang here at the University of Southern California. Tandem MS / MS spectra were acquired with Xcalibur 1.4 software. A full MS scan was followed by three consecutive MS / MS scans of the top three ion peaks from the preceding full scan. Dynamic exclusion was enabled – after three occurrences of an ion within 1 min., the ion was placed on the exclusion list for 3 min. Other mass spectrometric data generation parameters were as follows: collision energy 35%, full scan MS mass range 400-1800 m/z, minimum MS signal $5x10^4$ counts, minimum MS / MS signal $5x10^3$ counts. Peptides were loaded onto a Michrom Bioresources peptide cap trap at 95% solvent A (2% acetonitrile, 1.0% formic acid) and 5% solvent B (95% acetonitrile, 1.0% formic acid) and then eluted with a linear gradient from 5-90% solvent B. The mass spectrometer was equipped with a nanospray ion source (Thermo Electron) using an uncoated 10 μm–ID SilicaTip™ PicoTip™ nanospray emitter (New Objective, Woburn, MA). The spray voltage of the mass spectrometer was 1.9 kV and the heated capillary temperature was 180°C.

At the end of LC / MS / MS analysis, tandem mass spectra were analyzed using Bioworks 3.1, Beta-test site version from ThermoFinnigan, utilizing the SEQUESTTM algorithm to determine cross-correlation scores between acquired spectra and an NCBI mouse protein FASTA database. The following parameters were used for the TurboSEQUEST search analyses: no enzyme will be chosen for the protease as not all proteins are digested to completion; molecular weight range: 400-4500; threshold: 1000; monoisotopic; precursor mass: 1.4; group scan: 10; minimum ion count: 20; charge state: auto; peptide: 1.5; fragment ions: 0; and differential amino acid modifications: Cys 57.0520. Results were filtered using SEQUEST cross-correlation scores greater than 1.5 for +1 ions, 2.0 for +2 ions, and 2.5 for +3 ions. Fig. 31 shows the MS / MS spectrum of β-tubulin peptide (EVDEQMLNVQNK) and myc promoter-binding protein (MPB-1) peptide (VNQIGSVTESLQACK). In general we were able to identify most of the proteins with more than 40% sequence coverage. The

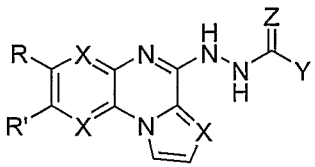
spots that did not show good peptide coverage either due to insufficient amount of sample, low protein abundance, or lack of reliable fragment were not explored further.

In summary, we were able to separate a series of proteins that were significantly changed in response to SC23 treatment. Among the several spots that were at least 4-fold overexpressed was β-tubulin, which is related to the top three genes identified from our microarray analysis as described above.

While the foregoing has been described in considerable detail and in terms of preferred embodiments, these are not to be construed as limitations on the disclosure. Modifications and changes that are within the purview of those skilled in the art are intended to fall within the scope of the invention.

WHAT IS CLAIMED IS:

1. A compound of Formula I,



Formula I

wherein

X = CH or N;

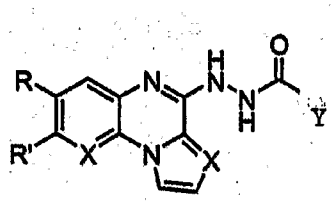
Z = O or S;

R = alkyl, halogen, acetyl, O-alkyl, or N-alkyl;

R' = alkyl, halogen, acetyl, O-alkyl, or N-alkyl; and

Y = alkyl, heterocyclic aromatic, aliphatic, sugar, or lipid.

2. A compound of Formula II,



Formula II

wherein

R is H, alkyl, or halogen;

R' is H, alkyl, or halogen;

X is CH or N; and

Y comprises a homocyclic or heterocyclic ring, wherein Y is 3-, 5-, or 6-pyrazinyl or 3-, 4-, 5-, or 6-pyridinyl when R is H, R' is H, X is CH, and Y is pyrazinyl or pyridinyl.

3. The compound of claim 2, wherein the alkyl is Me.

4. The compound of claim 2, wherein the halogen is F.

5. The compound of claim 2, wherein Y is pyrrolyl, pyridinyl, pyrazinyl, fluorophenyl, quinoxalinyl, or pyrrolo-quinoxalinyl.

6. The compound of claim 2, wherein

R is H, R' is H, and X is CH;

R is Me, R' is Me, and X is CH;

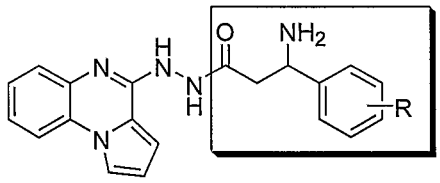
R is F, R' is H, and X is CH; or

R is H, R' is H, and X is N.

7. The compound of claim 6, wherein Y is pyrrolyl, pyridinyl, pyrazinyl, fluorophenyl, quinoxalinyl, or pyrrolo-quinoxalinyl.

8. The compound of claim 7, wherein the compound is selected from the group consisting of SC141, SC142, SC143, SC144, SC148, SC166, SC167, SC168, SC169, SC170, SC171, SC172, SC173, and SC174.

9. The compound of claim 2, wherein the compound is of Formula III,

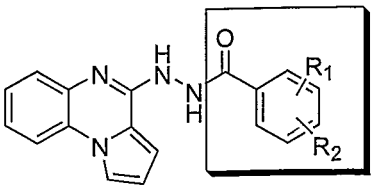


Formula III

wherein R = *o*-Cl, *p*-Cl, *p*-F, *p*-CN, *p*-OMe, or *p*-CF_3.

10. The compound of claim 9, wherein the compound is SC160, SC161, SC162, SC163, SC164, or SC165.

11. The compound of claim 2, wherein the compound is of Formula IV,



Formula IV

wherein

R_1 = 3-NH_2, R_2 = 5-CF_3;

R_1 = 5-NH_2, R_2 = 2-NO_2;

R_1 = 4-NH_2, R_2 = 3-NO_2;

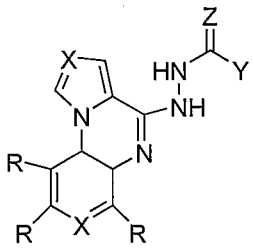
R_1 = 2-NH_2, R_2 = 5-OH;

R_1 = 4-NH_2, R_2 = H;

R_1 = 3-NH_2, R_2 = H; or

R_1 = 2-NH_2, R_2 = H.

12. A compound of Formula V,



Formula V

wherein

X = CH or N;

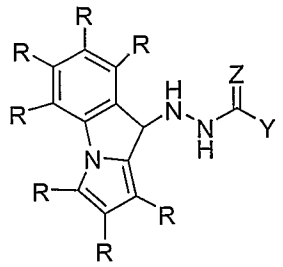
Z = O or S;

R = alkyl, halogen, acetyl, O-alkyl, or N-alkyl; and

Y = alkyl, heterocyclic aromatic, aliphatic, sugar, or lipid.

13. The compound of claim 12, wherein the compound is selected from the group consisting of SC153, SC154, SC155, SC156, SC157, and SC158.

14. A compound of Formula VI,



Formula VI

wherein

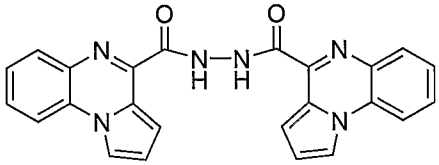
Z = O or S;

R = alkyl, halogen, acetyl, O-alkyl, or N-alkyl; and

Y = alkyl, heterocyclic aromatic, aliphatic, sugar, or lipid.

15. The compound of claim 14, wherein the compound is SC175 or SC176.

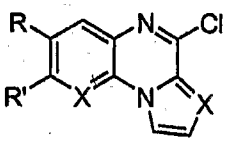
16. A compound of Formula VII,



Formula VII

17. A method of preparing a compound, comprising:

contacting hydrazine monohydrate with a compound (13a, 13b, 13c, or 13d) of Formula VIII,



Formula VIII

wherein

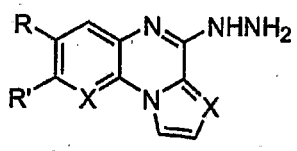
R is H, R' is H, and X is CH (13a);

R is Me, R' is Me, and X is CH (13b);

R is F, R' is H, and X is CH (13c); or

R is H, R' is H, and X is N (13d),

to form a compound (14a, 14b, 14c, or 14d, respectively) of Formula IX,



Formula IX

wherein

R is H, R' is H, and X is CH (14a);

R is Me, R' is Me, and X is CH (14b);

R is F, R' is H, and X is CH (14c); or

R is H, R' is H, and X is N (14d); and

contacting 14a with pyrrole-2-carboxylic acid chloride to form SC141,

contacting 14a with nicotinoyl chloride hydrochloride to form SC142,

contacting 14b, 14c, and 14d with 2-pyrazinecarboxylic acid in the presence of 2,2'-dipyrildisulphide and triphenylphosphine to form SC143, SC144, and SC148, respectively,

contacting 14a with *N*-BOC-thiazolidine-4-carboxylic acid in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) / 4-(dimethylamino)pyridine (DMAP) and then trifluoroacetic acid (TFA) / anisole to form SC153,

contacting 14a with *N*-BOC-β-alanine in the presence of EDC/DMAP and then TFA / anisole to form SC154,

contacting 14a with 2-indolecarboxylic acid, 5-indolecarboxylic acid, 6-indolecarboxylic acid, and 3-indolecarboxylic acid in the presence of EDC/DMAP to form SC155, SC156, SC157, and SC158, respectively,

contacting 14a with Boc-3-amino-3-(2-chlorophenyl)propionic acid, Boc-3-amino-3-(4-chlorophenyl)propionic acid, Boc-3-amino-3-(4-fluorophenyl)propionic acid, Boc-3-amino-3-(4-cyanophenyl)propionic acid,

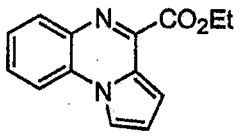
Boc-3-amino-3-(4-methoxyphenyl)propionic acid, and Boc-3-amino-3-(4-trifluoromethylphenyl)propionic acid in the presence of EDC/DMAP followed by TFA and anisole to form SC160, SC161, SC162, SC163, SC164, and SC165, respectively,

contacting 14a with 15a-g in the presence of EDC/DMAP followed by TFA and anisole to form SC166, SC167, SC168, SC169, SC170, SC171, and SC172, respectively,

contacting 14a with 2-quinoxalinecarboxylic acid, dichloromethane, triphenylphosphine, and 2,2'-dipyridyl disulfide to form SC173, and

contacting 14a with pyrrolo[1,2-*a*]quinoxaline-4-carboxylic acid, dichloromethane, triphenylphosphine, and 2,2'-dipyridyl disulfide to form SC174.

18. A method of preparing a compound, comprising contacting hydrazine monohydrate with a compound of formula X



Formula X

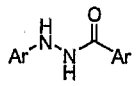
to form SC147.

19. A method of preparing a compound, comprising contacting 9-hydrazino-9*H*-pyrrolo[1,2-*a*]indole and pyridine with nicotinoyl chloride hydrochloride and pyrazine-2-carbonyl chloride, respectively, to form SC175 and SC176.

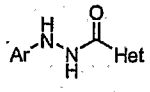
20. A method of treating a subject, comprising:

identifying a subject suffering from or at risk for developing non-small cell lung cancer, CNS cancer, ovarian cancer, breast cancer, renal cancer, prostate cancer, age-related macular degeneration, macular dystrophy, or diabetes; and

administering to the subject an effective amount of a compound of formula XI or XII,



Formula XI



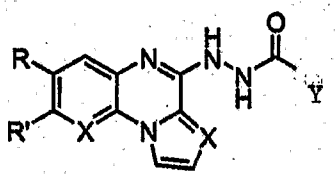
Formula XII

wherein Ar comprises an aromatic ring and Het comprises a heterocyclic ring.

21. A method of treating a subject, comprising:

identifying a subject suffering from or at risk for developing cancer or a disorder associated angiogenesis function; and

administering to the subject an effective amount of a compound of Formula II,



Formula II

wherein

R is H, alkyl, or halogen;

R' is H, alkyl, or halogen;

X is CH or N; and

Y comprises a homocyclic or heterocyclic ring.

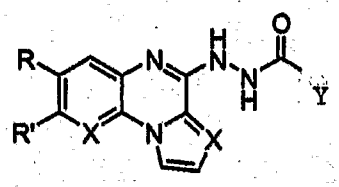
22. A method of treating a subject, comprising administering to a subject in need thereof an effective amount of the compound of claim 1, 2, 8, 9, 10, 11, 12, 13, 14, 15, or 16.

23. The method of claim 22, wherein the subject is suffering from or at risk for developing cancer or a disorder associated with angiogenesis function.

24. The method of claim 21 or 23, wherein the cancer is leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, breast cancer, renal cancer, or prostate cancer; and the disorder associated with angiogenesis function is age-related macular degeneration, macular dystrophy, or diabetes.

25. A method of monitoring treatment of a subject, comprising:

administering to a subject having cancer cells or cells associate with an angiogenesis disorder a compound of claim 1, 12, 14, or 16, or a compound of Formula II, XI or XII,



Formula II

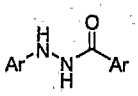
wherein

R is H, alkyl, or halogen;

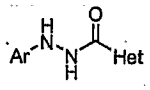
R' is H, alkyl, or halogen;

X is CH or N; and

Y comprises a homocyclic or heterocyclic ring;



Formula XI



Formula XII

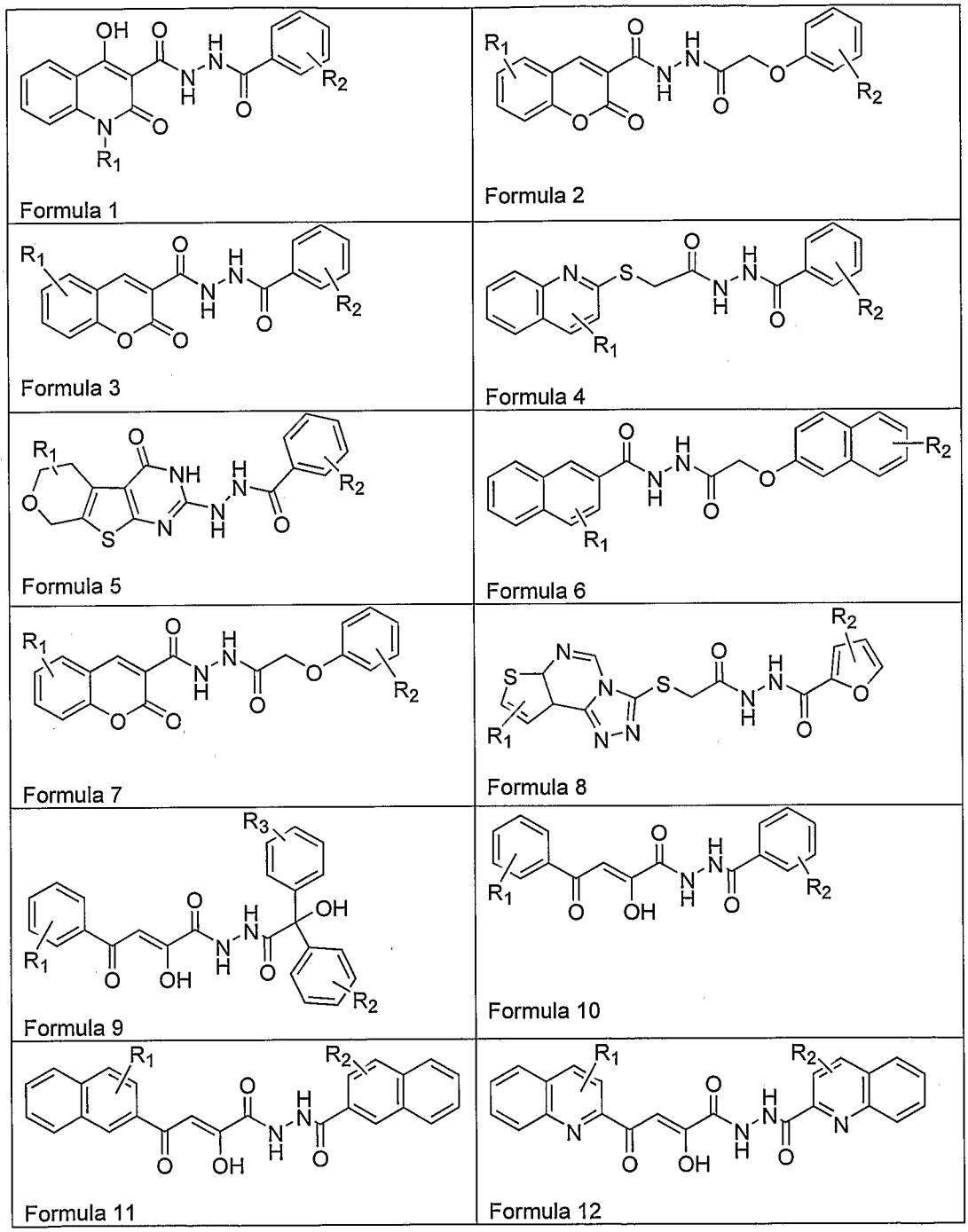
wherein Ar comprises an aromatic ring and Het comprises a heterocyclic ring; and

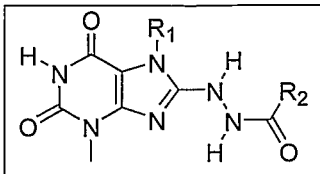
measuring the survival of the cells, the growth of the cells, or a combination thereof using PET imaging.

26. The method of claim 25, wherein the subject is suffering from leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma,

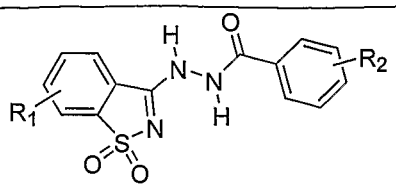
ovarian cancer, breast cancer, renal cancer, prostate cancer, age-related macular degeneration, macular dystrophy, or diabetes.

27. A compound of any of Formulas 1-19,

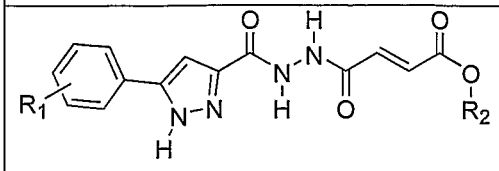




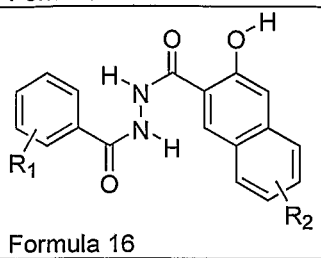
Formula 13



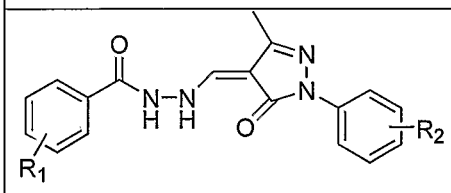
Formula 14



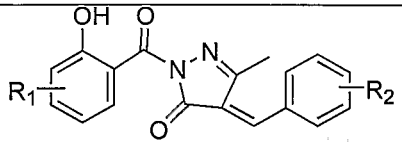
Formula 15



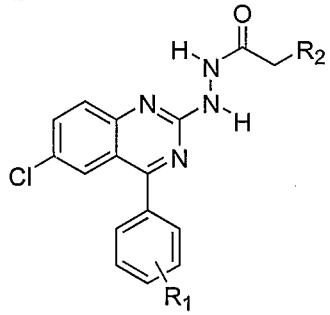
Formula 16



Formula 17



Formula 18



Formula 19

wherein each of R1, R2, and R3 is a hydrogen, halogen, hydroxyl, alkyl, substituted alkyl, alkenyl, substituted alkenyl, phenyl, substituted phenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, or an organic group containing 1-20 carbon atoms in a linear, branched, or cyclic structural format.

28. The compound of claim 27, wherein the substituted alkyl, substituted alkenyl, substituted phenyl, substituted aryl, or substituted heteroaryl contains a halo, hydroxyl, alkoxy, alkylthio, phenoxy, aroxy, cyano, isocyano, carbonyl, carboxyl, amino, amido, sulfonyl, or substituted

heterocyclic, sugar, or peptide substitution, and wherein the organic group includes a heteroatom of oxygen, sulfur, or nitrogen.

29. A compound selected from the group consisting of SC20-37, SC201-266, SC268, and SC270-280.

30. A method of modulating cell growth, cell cycle, or apoptosis, comprising contacting a cell with a compound of claim 27 or 29, thereby inhibiting cell growth, arresting cell cycle, or inducing apoptosis.

31. The method of claim 30, wherein the cell is a leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, breast cancer, renal cancer, or prostate cancer cell.

32. A method of modulating gene expressing in a cell, comprising contacting a cell with a compound of claim 27 or 29, thereby increasing or decreasing the expressiong of one or more genes in the cell.

33. The method of claim 32, wherein the one or more genes are selected from the group consisting of BCL2, BCL2L1, JUN, JUNB, MAD, MAX, TNFRSF1A, TP53, NFKB1, TNFSF10, CASP1, PCNA, TNFAIP1, DAP, KDR, MAP3K14, CCNA2, CDC2, CDK7, CDK8, CDKN1A, CDKN1B, CDKN2A, CDKN2C, E2F1, E2F4, E2F5, MYC, RB1, RBL2, CCND3, CCNG1, CCNE1, CDC25C, TGFBR2, TGIF, TRAF4, CYP1A2, PTGS2, p21, p27, cyclin A, cdk1, p53, cyclin E, cdc25, p130, NFKB, c-MYC, COX2, Bcl-X_L, annexin V, caspase 1, TNF receptor, microtubule-associated protein 4, microtubule affinity-regulating kinase 2, microtubule affinity-regulating kinase 4, transducer of ERBB2, vascular endothelial growth factor B, vascular endothelial growth factor, ankyrin repeat and MYND domain containing 1, RAB4B, putative prostate cancer tumor suppressor, pre-B-cell leukemia transcription factor 2, T-cell leukemia translocation altered gene, leukemia inhibitory factor, interferon

regulatory factor 2 binding protein, interferon stimulated gene (20kDa), interferon gamma receptor 2, 28kD interferon responsive protein, polymerase (RNA) III, peroxisomal proliferator-activated receptor A interacting complex 285, RAD50 homolog (S. cerevisiae), MAX dimerization protein 3, kruppel-like factor 16, apolipoprotein L (6), X-ray repair complementing defective repair, mitogen-activated protein kinase 3, phosphatidylinositol 4-kinase type II, mitogen-activated protein kinase 12, protein kinase (AMP-activated, alpha 2 catalytic subunit), pyruvate dehydrogenase phosphatase regulatory subunit, phospholipase D3, inositol 1,4,5-triphosphate receptor (type 3), retinoic acid receptor (alpha), tumor necrosis factor receptor superfamily, Enolase 2 (gamma, neuronal), stanniocalcin 2, apelin, plexin B2, cathepsin Z, histone 1 (H2bc), histone 1 (H3h), β-tubulin, myc promoter-binding protein (MPB-1), retinoblastoma-binding protein 7, vimentin, enolase, phosphopyruvate hydratase beta, mitochondrial ATP synthase beta chain.

34. A method of treating a subject, comprising administering to a subject in need thereof an effective amount of the compound of claim 27 or 29.

35. The method of claim 34, wherein the subject is suffering from or at risk for developing cancer.

36. The method of claim 35, wherein the cancer is leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, breast cancer, renal cancer, or prostate cancer.

37. A pharmaceutical composition comprising an effective amount of one or more compounds of claim 27 or 29 and a pharmaceutically acceptable carrier.