Title: TRICYCLIC COMPOUNDS AS INHIBITORS OF THE HYPOXIC SIGNALING PATHWAY

Abstract: Tricyclic compounds that selectively inhibit HIF-1α activity are disclosed. Methods also are disclosed for reducing HIF-1α activity, and for inhibiting angiogenesis, tumorigenesis and/or metastasis, in a subject. In some embodiments, the tricyclic compounds surprisingly inhibit HIF-1α activity at non-cytotoxic concentrations, thereby avoiding drug side effects associated with significant cytotoxicity.
TRICYCLIC COMPOUNDS AS INHIBITORS OF THE HYPOXIC SIGNALING PATHWAY

Cross Reference to Related Application

This application claims the benefit of, and incorporates by reference, U.S. Provisional Patent Application Nos. 60/570,615, filed May 12, 2004, and 60/618,279, filed October 12, 2004.

Field

The present invention relates to tricyclic compounds that inhibit hypoxia inducible transcription factor-1 (HIF-1) activity.

Background

Solid tumor growth is dependent upon the supply of nutrients and oxygen from the blood. Typically a tumor mass will not grow beyond 2-3 cubic millimeters unless new blood vessels are formed within the tumor. Such "pre-vascular" tumors and dormant micrometastases maintain their small volume due to a balance of cell proliferation and cell death. Also, they are generally asymptomatic and hence clinically undetected. Formation of new blood vessels (vasculature) within a tumor, by a process known as angiogenesis or neovascularisation, permits further growth, and it is typically vascularised solid tumors that are detected and that require treatment. Thus, angiogenesis is an important component of tumorigenesis and the pathogenesis of cancer.

A stimulus for angiogenesis is believed to be localized tissue "hypoxia," in which tumor cells become starved of oxygen. This condition is typically observed within solid tumors, and the hypoxic environment is believed to arise largely as a result of the rapid aberrant proliferation of the cancer cell, and thus inability of the tumor to
maintain an adequate and organized vasculature to supply oxygen to cells within the
tumor. When the tumor cells become starved of oxygen, they respond by the expression
and secretion of proteins important for stimulating angiogenesis. This response, known
as hypoxia adaptation, leads to vascularization, and facilitates further tumor growth.

There are several control points influencing angiogenesis that may be considered
as targets for intervention, and one of particular interest is the transcription factor
Hypoxia-Inducible Factor 1 (HIF-1). HIF-1 has been shown to play an essential role in
cellular responses to hypoxia. Upon hypoxic stimulation, HIF-1 is known to activate
genes that contain Hypoxic Response Elements (HREs) in their promoters, and thus up-
regulate a series of gene products that promote cell survival under conditions of low
oxygen availability. The list of HIF-responsive genes is constantly expanding, but
known gene products include glycolytic enzymes such as lactate dehydrogenase, (LDH-
A), enolase-1 (ENO-1), and aldolase A; glucose transporters GLUT 1 & 3; vascular
endothelial growth factor (VEGF); inducible nitric oxide synthase (NOS-2); and
erthropoietin (EPO). The switch of the cell to anaerobic glycolysis, and the up-
regulation of angiogenesis by VEGF is geared at maximizing cell survival under
conditions of low oxygen tension by reducing the requirement for oxygen, and
increasing vasculature to maximize oxygen delivery to tissues. Induction of NOS-2,
and the subsequent increase in NO effectively promotes a state of vasodilation in the
hypoxic microenvironment, thereby maximizing blood flow and oxygen delivery to
cells. Increased EPO production by the tubular interstitial cells of the kidney is geared
at promoting erythropoiesis, and increasing red blood cell number to further facilitate
oxygen delivery to hypoxic tissues.

Recent studies on the inhibition of angiogenesis, taking several approaches, have
clearly demonstrated that efficient inhibition of this process can block tumor growth in
animal models. Using agents to inhibit angiogenesis and prevent tumor growth and
metastasis represents a mode of treatment that is distinct from using cytotoxins at
cytotoxic concentrations, and has the advantage of having a lower risk of inducing drug
resistance in the tumor cells. In contrast to drugs that interfere with the pro-angiogenic response of tumor cells to hypoxic conditions, cytotoxic agents are used to disrupt fundamental cellular processes related to cell growth and division. The capacity of such agents to interfere with normal mitosis and cell division in rapidly proliferating tissues is the basis for their therapeutic action against neoplastic cells. However, since tumor cells are not the only rapidly dividing cells in the body, this mode of treatment leads to unpleasant side effects that may include nausea, vomiting, hair loss and suppression of bone marrow function. Nonetheless, cytotoxic agents are typically administered at the maximum tolerated dose (MTD) because the deleterious side-effects are believed to be justified in an effort to stop a cancer, until the side-effects of a particular dose themselves become life-threatening.

Tricyclic compounds, especially tricyclic carboxamides, were initially promising as anti-neoplastic agents. However, their use in this mode of therapy appears to have been abandoned due to their dose-limiting toxicities. For example, Phase II clinical trials of the tricyclic carboxamide-based cytotoxic agent XR5000 (DACA), in which occurrence of severe chest pain and abdominal pain precluded the use of higher doses, revealed that the compound did not have any antitumoral activity at the MTD in systemically pretreated patients with metastatic or locally advanced non-small cell lung cancer. These results led to the conclusion that further evaluation of this compound in clinical trials was not justified (See, Dittrich et al., Eur. J. of Cancer, 39: 330-334, 2003). Similar results were obtained using cytotoxic doses of XR5000 to treat glioblastoma multiforme, colorectal cancer, and ovarian cancer (See, Twelve et al., Ann. Oncol., 13: 771-780, 2002; Caponigro et al., Eur. J. of Cancer, 38: 70-74, 2002; and Dieras et al., Proc. Am. Assoc. Cancer Res., 42: 48, abstract 253, 2001). Similarly, a study of the structure-activity relationships for the broad class of linear tricyclic carboxamides showed that unsubstituted di-benzo dioxin, phenoxazine, acridine and anthracene carboxamides were inactive against such solid tumors at all doses up to acutely toxic doses (See, Palmer et al., J. Med. Chem., 31: 707-712, 1988). Although,
in a different study, a 9-chloro derivative of dibenzo dioxin carboxamide was shown to be effective against Lewis lung carcinoma solid tumors in a mouse model, cytotoxic doses (100 mg/kg) again were employed to provide the observed therapeutic effect (See, for example, Lee et al., J. Med. Chem., 35: 258-266, 1992).

Summary

Tricyclic compounds that inhibit HIF-1α activity are disclosed. Surprisingly, in some embodiments, some of the disclosed compounds that would otherwise have cytotoxic activity can be used at reduced dosages that avoid cytotoxicity, while still providing therapeutic anti-angiogenic effects through their ability to inhibit HIF-1α activity. Accordingly, such dosages substantially avoid the adverse consequences of prior uses of such compounds, such as damage to healthy tissue, since they interfere with tumor growth by a different mechanism. In other embodiments, the disclosed compounds are administered at any dosage, including cytotoxic amounts.

In further embodiments, compounds are disclosed that have one or more, or all, of the properties of stimulating HIF-1α degradation by a novel pathway that is independent of the von Hippel-Lindau protein (VHL), increasing degradation of HIF-1α without substantially decreasing expression of HIF-2α, having substantially no effect on HIF-1α mRNA expression, inhibiting HIF-1α activity through a Topoisomerase-1-independent mechanism, decreasing VEGF mRNA expression, inhibiting HIF-1α activity through a proteosome dependent mechanism, inducing G2/M arrest, being substantially non-cytotoxic, inhibiting HIF-1α activity by a mechanism which does not involve microtubule disruption, having substantially no inhibiting effect on hsp 90, being independent of the AKT pathway, having substantially no inhibiting effect on deacetylation enzymes, being independent of the p53 gene, and increasing p21 mRNA expression.

The novel properties of the disclosed compounds make them especially attractive for combination therapies in which the compounds can be administered along
with other drugs that work by dissimilar or complementary mechanisms, thereby lowering the likelihood of the subject’s tumors developing drug resistance. For example, disclosed compounds that have no effect on HIF-1α mRNA expression can be administered in combination with topotecan, which has a direct effect on HIF-1α mRNA expression. Alternatively, disclosed compounds that inhibit HIF-1α activity through a Topoisomerase-1-independent mechanism can be administered with another agent that acts through a Topoisomerase-1-dependent mechanism. In further examples, the compound has one or more, or all, of the previous or following properties, which property is not possessed by the co-administered agent: stimulating HIF-1α degradation by a novel pathway that is independent of VHL, increasing degradation of HIF-1α without substantially decreasing expression of HIF-2α, decreasing VEGF mRNA expression, inhibiting HIF-1α activity through a proteosome dependent mechanism, inducing G2/M arrest, being substantially non-cytotoxic, inhibiting HIF-1α activity by a mechanism which does not involve microtubule disruption, having substantially no inhibiting effect on hsp 90, being independent of the AKT pathway, having substantially no inhibiting effect on deacetylation enzymes, being independent of the p53 gene, and increasing p21 mRNA expression.

In some embodiments, the tricyclic compounds have a structure defined by the following formula:

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R2
  /  \
X - Y - 1
  \  /
R3
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wherein X and Y are independently O, S, N, NR₄, CR₅ or CR₆R₇ and all valency requirements are satisfied; R₁ is one or more substituents such as alkyl, alkoxy, amino, carboxamide, carboxyl, diamine halogen (F, Cl, Br, I), hydroxyl or nitro, which in some
embodiments include at least one of a carboxamide group or a diamine group; and R₂
and R₃ are either joined to form a six-membered aromatic ring (which can be further
substituted, for example, with one or more alkyl, alkoxy, amino, carboxyl, halogen,
hydroxyl, or nitro groups) or one of R₂ and R₃ is an aryl group such as a phenyl, 3-
pyridyl or 4-pyridyl group (which can be further substituted, for example, with one or
more alkyl, alkoxy, amino, carboxyl, halogen, hydroxyl, or nitro groups) and the other
is hydrogen or a substituent such as alkyl, alkoxy, amino, carboxyl, halogen, hydroxyl,
or nitro. R₄, R₅, R₆, and R₇ are independently H or a substituent such as alkyl, alkoxy,
amino, carboxyl, halogen, hydroxyl, or nitro. In particular embodiments, substituents
are selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino,
aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl,
sulfonamido and sulfato. In more particular embodiments, substituents are selected
from the group consisting of alkoxy, alkyl, amino, aryl, aza, carboxy, carboxamide,
halogen, hydroxyl, nitro, alkylsulfonyl, alkylsulfonamido, and sulfato. In even more
particular embodiments, substituents are selected from the group consisting of alkoxy,
alkyl, -NH₂, alkylamino, aryl, aza, carboxy, alkylacylamino, alkylaminoacetyl, halogen,
hydroxyl, nitro, lower alkylsulfonyl, and lower alkylsulfonamido. In other particular
embodiments, R₄, R₅, R₆, and R₇ can be an amino-substituted alkyl group or a diamine
group. In still other particular embodiments, at least one of R₃ has the formula –
CONR'(CH₂)nNR”R‴ where n=1-5 and R’, R”, and R‴ are independently H or
alkyl. In other more particular embodiments, R₄ can be an amino-substituted alkyl
group such as an 2-(N,N'-dimethyl)amino-1-methylethyl or an 3-(N-
isopropyl)aminopropyl group and, in others, R₅ can be a diamine group. In other
particular embodiments, X and Y are as follows: X=O, Y=O; X=S, Y=O; X=O, Y=S;
X=S, Y=S; X=O, Y=NR₂; X=NR₂, Y=O; X=S, Y=NR₂; X=NR₂, Y=S; X=CR₃, Y=N;
X=N, Y=CR₃; X=N, Y=N; or X=CR₃, Y=CR₃. Examples of specific types of
compounds disclosed herein include acridine carboxamides, acridine diamines,
anthracene carboxamides, aryquinoline carboxamides, dibenzo-[1,4]dioxin
carboxamides, phenazine carboxamides, thioanthrene carboxamides, phenoxathiin carboxamides, phenoxazine carboxamides, and phenothiazine carboxamides. In yet other embodiments, the compound is not one or more of an acridine carboxamide, an acridine diamine, or a phenazine carboxamide.

Additional tricyclic compounds of the disclosure further include 2-
Arylbenezimidazoles having the structure:

\[ \text{Ar} \quad W \quad \text{Z} \quad R_8 \]

where \( R_8 \) is one or more substituents (such as alkyl, alkoxy, amino, carboxamide, carboxyl, diamine, halogen, hydroxyl, or nitro) that in some embodiments include at least one carboxamide group or diamine group; \( \text{Ar} \) is an aryl group such as phenyl, 2-furyl, 2-thienyl, 3-thienyl, or 2-pyrrolyl (any of which may be further substituted, for example, with one or more alkyl, alkoxy, amino, carboxyl, halogen, hydroxyl, or nitro groups); \( W \) and \( Z \) are independently either \( NR_9 \) or \( =N— \), but not the same; and \( R_9 \) is hydrogen or a substituent such as lower alkyl or an amino-substituted alkyl group.

Another aspect pertains to methods of inhibiting HIF-1 activity in a cell or a subject, including contacting the cell with, or administrating to the subject, a therapeutically effective concentration or amount of a disclosed compound that decreases the HIF-1 activity in the cell or subject from a first level to a second, lower level. For example, the method may include selecting a subject having a tumor that can be treated by inhibiting angiogenesis, for example inhibiting the blood supply to a tumor. In some embodiments, the therapeutically effective amount is a non-cytotoxic amount or achieves a non-cytotoxic concentration in target tissue (such as the tumor) in the subject. In other embodiments, the therapeutically effective amount may include cytotoxic amounts. The method may further include selecting the tricyclic compound
that is used in the method. The tricyclic compound can be selected to increase
degradation of HIF-1α and have substantially no effect on degradation of HIF-2α. In
other examples, the tricyclic compound is selected to increase degradation of HIF-1α in
a VHL-independent manner. In yet other embodiments, the compound is selected to
have substantially no effect on the level of HIF-1α mRNA that is expressed in the
subject or the cell. Alternatively, the compound used in the method can be selected to
inhibit HIF-1α activity through a Topoisomerase-1-independent mechanism. In further
effects, the tricyclic compound is selected because it has one or more, or all, of the
previous properties or the properties of decreasing VEGF mRNA expression, inhibiting
HIF-1α activity through a proteosome dependent mechanism, inducing G2/M arrest,
being substantially non-cytotoxic, inhibiting HIF-1α activity by a mechanism which
does not involve microtubule disruption, having substantially no inhibiting effect on hsp
90, being independent of the AKT pathway, having substantially no inhibiting effect on
deacetylation enzymes, being independent of the p53 gene, and increasing p21 mRNA
expression. Furthermore, any combination of such criteria of selection may be used to
select the compound used in the method.

Yet another aspect pertains to a pharmaceutical composition that includes a
disclosed compound and a pharmaceutically acceptable carrier for use, for example, in
treating tumors or lowering HIF-1α activity in a subject by increasing degradation of
HIF-1α, and the use of such a composition for lowering HIF-1α activity in a subject. In
certain examples, the pharmaceutical composition includes a non-cytotoxic amount of a
disclosed compound. In further examples, the pharmaceuticial composition includes
any amount of a disclosed compound, including cytotoxic amounts.

Another aspect pertains to a method of treating a proliferative condition (such as
cancer) in a subject by administering to the subject a therapeutically-effective amount of
a disclosed compound, for example a compound that increases degradation of HIF-1α in
the subject but does not increase degradation of HIF-2α in the subject, that increases
HIF-1α degradation in the subject in a VHL-independent manner, that has substantially
no effect on the level of HIF-1α mRNA expressed in the subject, and/or that inhibits HIF-1α activity in the subject through a Topoisomerase-1-independent mechanism. Further aspects pertain to such methods where the disclosed compound has one or more, or all, of these properties or the properties of decreasing VEGF mRNA expression, inhibiting HIF-1α activity through a proteosome dependent mechanism, inducing G2/M arrest, being substantially non-cytotoxic, inhibiting HIF-1α activity by a mechanism which does not involve microtubule disruption, having substantially no inhibiting effect on hsp 90, being independent of the AKT pathway, having substantially no inhibiting effect on deacetylation enzymes, being independent of the p53 gene, and increasing p21 mRNA expression. In some embodiments, the therapeutically effective amount is also a non-cytotoxic amount or achieves a non-cytotoxic concentration in the subject. In other embodiments, the therapeutically effective amount may include cytotoxic amounts.

Further aspects include the use of such a disclosed compound in a method of treatment of the human or animal body, and the use of such a disclosed compound in the manufacture of a medicament for use in the treatment of a proliferative condition such as cancer. Yet further examples include the use of a disclosed compound to treat a proliferative condition such as cancer by inhibiting angiogenesis in proliferative conditions that are responsive to anti-angiogenesis treatment.

The foregoing and other features and advantages will become more apparent from the following detailed description of several embodiments that proceeds with reference to the accompanying figures.

**Brief Description of the Drawings**

FIG. 1A is an image of an immunoblot analysis showing the time-dependent effect of NSC 644221 on hypoxia-induced HIF-1α protein levels.

FIG. 1B is an image of an immunoblot analysis showing concentration dependent effect of NSC 644221 on hypoxia-induced HIF-1α protein levels, and the lack of effect of NSC 644221 on HIF-1β protein levels.
FIG. 2 is an image of an immunoblot analysis showing the time-dependent effect of NSC 644221 on hypoxia-induced HIF-1α protein levels.

FIG. 3 is an image of an immunoblot analysis showing concentration dependent effect of NSC 644221 on hypoxia-induced HIF-1α protein levels.

FIG. 4 is a graph showing the effect of NSC 644221 on HRE-controlled luciferase activity in U251 cells (HRE/pGL3).

FIG. 5 is a set of graphs illustrating the effect of NSC 644221 on hypoxic signaling and the low cytotoxicity of NSC 644221 toward U251 cells.

FIG. 6 is a graph showing the effect of NSC 644221 on VEGF mRNA expression in U251 cells.

FIG. 7 is a composite of immunoblot images showing the effect of NSC 644221 on HIF-1α and actin levels in the presence and absence of proteosome inhibitors.

FIG. 8 is an image of an immunoblot analysis showing the effect of NSC 644221 on HIF-1α and actin levels in the presence and absence of proteosome inhibitors.

FIG. 9 is a composite of immunoblot images showing the effect of NSC 644221 on HIF-1α, HIF-2α, and actin levels in the presence and absence of VHL expression.

FIG. 10 is a composite of immunoblot images showing the effect of NSC 644221 on HIF-1α and actin levels in the presence and absence of VHL expression.

FIG. 11 is a bar graph showing the effect of NSC 644221 on VEGF mRNA expression in wild-type and mutant 145 (Topo-1) cells.

FIG. 12 is an immunoblot image showing the effects of NSC 644221, NSC 299505, and NSC 295504 on the levels of HIF-1α protein in hypoxic cells.

FIG. 13 is a bar graph comparing the effect of NSC 644221 and NSC 295504 on HRE-controlled luciferase activity in U251 cells (HRE/pGL3).

FIG. 14 is a bar graph comparing the effect of NSC 644221 and NSC 295504 on VEGF mRNA expression in U251 cells.
FIG. 15 is an image of an immunoblot analysis showing the effects of NSC 644221, NSC 24058, NSC 24568, NSC 24623, NSC 24459, NSC 24544, and NSC 24460 on hypoxia-induced HIF-1α protein levels and phosphorylated histone H2A.X levels.

FIG. 16 is a schematic representation of the chemical structures of NSC 24058, NSC 24568, NSC 24623, NSC 24459, NSC 24544, and NSC 24460.

FIG. 17 is a chart showing the effect of NSC 644221 on DNA absorbance at 260 nm.

FIG. 18 is a bar graph showing the effect of NSC 644221 on cell viability and growth of U251 and PC-3 cells.

FIG. 19 is an image of an immunoblot analysis showing the effect of NSC 644221 on hsp70 protein levels under various conditions.

FIG. 20 is an image of an immunoblot analysis showing the effect of NSC 644221 on AKT levels under various conditions.

FIG. 21 is an image of an immunoblot analysis showing the effect of NSC 644221 on phospho AKT levels under various conditions.

FIG. 22 is a photomicrograph of U251 control cells and U251 cells treated with NSC 644221, colchicine, or jasplakinolide.

FIG. 23 are flow cytometric histograms of control and NSC 644221 treated PC-3 and U251 cells.

FIG. 24 is an image of an immunoblot analysis showing the effect of NSC 644221 on acetylation of histone and tubulin.

FIG. 25 is a bar graph showing the effect of NSC 644221 on CYP1A1 luciferase expression in MCF-7 cells.

FIG. 26 is a bar graph showing the effect of NSC 644221 on p21 mRNA expression in U251 cells under various conditions.
FIG. 27 is an image of an immunoblot analysis showing the effect of NSC 644221 on p21 protein levels in U251 cells without an active p53 gene and in HCT116 cells with a wild-type p53 gene.

FIG. 28 is a table showing various cells, the nature of their p53 gene, and whether they exhibit HIF-1α inhibition in response to treatment with NSC 644221.

FIG. 29 is a bar graph showing the effect of NSC 644221 on HIF-1α mRNA expression.

**Detailed Description**

I. **Abbreviations**

**ALLNL** – N-acetyl leucyl-leucyl norlucinal  
**BSA** – bovine serum albumin  
**CHX** – cyclohexamide  
**DFO** – desferrioxamine  
**DLT** – dose limiting toxicity  
**DMEM** – Dulbecco/Vogt modified Eagle’s medium  
**ECL** – enhanced chemiluminescence  
**HIF** – hypoxia inducible transcription factor  
**HRE** – hypoxia responsive element  
**MTD** – maximum tolerated dose  
**NSC 295504** –

![Chemical Structure](image-url)
NSC 295505 –

NSC 644221 – N-[2-(Dimethylamino)ethyl]-9-nitrodibenzo[1,4]dioxin-1-carboxamide

5 PS341 – N-pyrazinecarbonyl-L-phenylalanine-L-Leucine
PVDF – polyvinylidene fluoride
RPMI – Roswell Park Memorial Institute medium
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
TDL – toxic dose low

10 VEGF – vascular endothelial growth factor
VHL – von Hippel-Lindau protein

II. Terms

In order to facilitate an understanding of the embodiments presented, the following explanations are provided.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The singular terms “a,” “an,” and “the” include plural referents unless context clearly indicates otherwise. Similarly, the word “or” is intended to include “and” unless the context clearly indicates otherwise. The term “comprising” means “including;” hence, “comprising A or B” means including A or B, or including A and B. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described herein. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.
“Acyl” refers to a group having the structure RCO-, where R may be alkyl, or substituted alkyl. “Lower acyl” groups are those that contain one to six carbon atoms.

“Acyloxy” refers to a group having the structure RCOO-, where R may be alkyl or substituted alkyl. “Lower acyloxy” groups contain one to six carbon atoms.

The term “alkoxy” refers to a group having the formula –OR, wherein R is an alkyl group. “Lower alkoxy” refers to an –OR group in which the R group has from 1 to 10 carbon atoms, such as from 1 to 5 carbon atoms. Examples of lower alkoxy groups include, but are not limited to, methoxy, ethoxy, n-propoxy, isopropoxy and butoxy groups.

“Alkenyl” refers to a cyclic, branched or straight chain hydrocarbon group including 2-25 carbon atoms and one or more double bonds that may or may not be conjugated with each other. Alkenyl groups may be unsubstituted or substituted, so the term “alkenyl” is to be understood to include both unsubstituted alkenyl groups and substituted alkenyl groups unless clearly indicated otherwise. “Lower alkenyl” groups contain one to six carbon atoms.

The term “alkyl,” by itself or as part of another substituent refers to a straight chain, branched chain, or cyclic hydrocarbon group (or some combination thereof) including 1 to about 25 carbon atoms. Alkyl groups can be fully saturated, mono-, or polyunsaturated, and can include divalent ("alkylene") and multivalent ("alkylyne") carbon-carbon bonds. Examples of saturated alkyl groups include, but are not limited to, groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, and homologs and isomers thereof. For example, n-pentyl, n-hexyl, n-heptyl, and n-octyl are homologs of smaller normal alkyl groups such as n-butyl and n-propyl, and isopentyl and neopentyl are isomers of n-pentyl. An unsaturated alkyl group is one having one or more double bonds or triple bonds. Examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butynyl, and the higher homologs and
isomers. Straight, branched, or cyclic hydrocarbon groups having from 1 to 10 carbon atoms, such as from 1 to 5 carbon atoms, are referred to herein as “lower alkyl”. Alkyl groups can be unsubstituted or substituted, so the term “alkyl” is to be understood to include both unsubstituted alkyl groups and substituted alkyl groups unless clearly indicated otherwise.

The term “alkythio” refers to a group having the structure –SR, where R is alkyl.

“Alkynyl” refers to a cyclic, branched, or straight chain hydrocarbon group including from 2 to 25 carbon atoms and one or more triple bonds. Alkynyl groups may be unsubstituted or substituted, so the term “alkynyl” is to be understood to include both unsubstituted and substituted alkynyl groups unless clearly indicated otherwise.

“Lower alkynyl” groups are those that contain one to six carbon atoms.

The term “amino” refers to an R-group having the structure –NRR’, wherein R and R’ each are independently hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, or substituted or unsubstituted alkoxy or thioalkoxy groups.

Particular examples of amino groups include “alkylamino” groups (–NHR, where R is alkyl) and “dialkylamino” groups (–NRR’, where R and R’ are both alkyl). Where used alone in reference to a specific compound (such as in the name of a compound or a particular substituent at a particular position in a structure) the term “amino” refers to the group –NH₂.

The term “amino-substituted alkyl” refers to an alkyl group substituted with at least one amino group.

The term “aryl” refers to a polyunsaturated, aromatic moiety that can be a single ring or multiple rings (for example, from 1 to 3 rings), which are fused together or linked covalently. Aryl groups can be unsubstituted or substituted. The term “aryl” also includes “heteroaryl” groups (or rings) that contain at least one heteroatom (such as from 1 to 4) selected from N, O, and S, wherein the nitrogen and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl
group can be attached to the remainder of the molecule through a heteroatom. Aryl and heteroaryl groups also can be fused to a ring of a molecule, typically at adjacent atoms in the ring of the molecule. An example of a fused aryl group is the “benz” group.

Non-limiting examples of aryl and heteroaryl groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxalinyl, 5-quinoxalinyl, 3-quinolyl, tetrazolyl, benzo[b]furanyl, benzo[b]thienyl, 2,3-dihydrobenzo[1,4]dioxin-6-yl, benzo[1,3]dioxol-5-yl, and 6-quinolyl. Aryl groups can be unsubstituted or substituted, so the term “aryl” is to be understood to include both unsubstituted aryl (and heteroaryl) groups and substituted aryl (and heteroaryl groups).

The term “carboxamide” refers to a substituent including the bivalent group –CONR– (or –NRCO–), where R is hydrogen, a substituted or unsubstituted alkyl group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted alkoxy or thiaalkoxy group). Examples of carboxamide groups include “acylamino” groups (which have the structure –NRCOR‘, where R and R’ each are independently hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, or substituted or unsubstituted alkoxy or thiaalkoxy groups) and “aminoacyl” groups (which have the structure –CONRR‘, where R and R’ each are independently hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, or substituted or unsubstituted alkoxy or thiaalkoxy groups). In particular embodiments, carboxamide groups can be “alkylacylamino” groups (which have the structure –NRCOR‘, where R and R’ each are independently hydrogen or substituted or unsubstituted alkyl) or “alkylaminoacyl” groups (which have the structure –CONRR‘, where R and R’ each are independently hydrogen or substituted or unsubstituted alkyl). Particular examples of carboxamide
groups include those having the formula \(-\text{CONR'}(\text{CH}_2)_n\text{NR''R'''},\) wherein \(n=1-5\) and \(R', R''\) and \(R'''\) each are independently hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, or substituted or unsubstituted alkoxy or thioalkoxy groups, for example, each are independently H or substituted or unsubstituted alkyl, such as each being independently H or unsubstituted or substituted lower alkyl.

The term "carboxyl" refers to a substituent having the formula -COOH or its conjugate base -COO'.

The term "diamine" refers to a substituent having the structure \(-\text{NR'}(\text{CH}_2)_n\text{NR''R'''},\) wherein \(n=1-5\) and \(R', R''\), and \(R'''\) each are independently hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, or substituted or unsubstituted alkoxy or thioalkoxy groups, for example, each are independently H or substituted or unsubstituted alkyl, such as each being independently H or unsubstituted or substituted lower alkyl. A diamine group can be further substituted, for example, with additional lower alkyl groups. Particular examples of diamine groups include:

The term "halogen" refers to fluoro, bromo, chloro, and iodo substituents.

The phrase "inhibiting HIF-1α activity" refers to a reduction in the amount or concentration of the HIF-1α protein. A reduction in the amount or concentration of the HIF-1α protein can be determined directly in the cells of a subject, for example, by a Western blot, or can be detected by a concomitant reduction in the expression of gene products under the control of a hypoxia responsive element (HRE). Alternatively, a reduction in the amount or concentration of the HIF-1α protein can be determined by a reduction in a pathological process that depends upon increased levels of this protein, for example, angiogenesis, tumorigenesis, or tumor metastasis. Suitable methods for
determining whether a compound to be administered to a subject inhibits HIF-1α activity are provided in the Examples that follow.

The term "mercapto" refers to the –SH group.

"Nitro" refers to an R-group having the structure –NO₂.

In some specific embodiments, a "non-cytotoxic dose" is a dose that is significantly less than the maximum tolerated dose (MTD) of the disclosed compounds, for example, less than 25%, less than 10%, less than 1% or less than 0.1% of the MTD. The MTD is a dose that leads to dose-limiting toxicity (DLT) in a small percentage of subjects (such as 15%, 10%, or 5% of subjects), the side effects of which cannot be managed and typically require some type of intervention (for example, a blood transfusion or surgery). DLT is the occurrence of an unacceptable toxicity, which can be hematological or non-hematological in nature, that leads to one or more Grade 3 or higher adverse events as graded according to the National Cancer Institute/Common Toxicity Criteria version 3.0 (NCI/CTC; an online version of the NCI/CTC v. 3.0 is available online at the U.S. Food and Drug Administration/Center for Drug Evaluation and Research website). Thus, in some embodiments, a non-toxic dosage is a dosage that does not lead to Grade 3 or higher hematological or non-hematological adverse events in a subject, for example, a dosage that does not result in any Grade 2 or higher hematological or non-hematological adverse events in a subject, such as a dosage that results in no hematological or non-hematological adverse events in a subject. Such non-cytotoxic doses are contrary to doses used when the compounds are used as cytotoxic anti-neoplastics, where the goal is to kill rapidly dividing cancer cells. Examples of adverse events covered by the NCI/CTC include drug induced chemical changes associated with loss of hepatic function or renal function (such as elevation of hepatic transaminases), alopecia (hair loss), neutropenia, thrombocytopenia, various hemorrhagic events (such as CNS bleeding, epistaxis, hemoptysis and hematuria), decrease of hemoglobin levels, lowering CD4 cell count, lowered bone marrow cellularity, hemolysis, lowered platelet levels, lymphopenia, cardiovascular arrhythmias,
general cardiovascular events (such as ischemia/infarction or edema), constitutional symptoms (such as fatigue, weight loss, or fever), diarrhea, dehydration, infection, pain, gastritis, vomiting, and metabolic/laboratory changes (such as acidosis, hyperglycemia, or hypoglycemia).

In other specific embodiments, a non-cytotoxic dose is less than the toxic dose low (TDL) in a large animal species (such as dog or monkey), for example 50% or less of the TDL, 25% or less of the TDL, or 10% or less of the TDL. The TDL is defined as the lowest dose that produces pathological alterations in hematological, chemical, clinical, or morphological parameters, and which when doubled, produces no lethality (See, Prieur et al., *Cancer Chemother. Rep.*, 4: 1-6, 1973). The TDL is determined on two basic schedules, single dose and daily for 5 days. Animal doses in mg/kg can be used to obtain the dose in mg/m² for human dose equivalents, by multiplying by 3.00 for monkey results and by 8.00 for dog results. The human body surface in m² can be calculated by Boyd's Formula of Body Surface Area (Boyd, E., “The growth of the surface area of the human body,” University of Minnesota Press, 1935) and used to arrive at the human dose equivalent. Additional conversion factors for other animal species can be found in *Cancer Chemother. Rep.*, 50: 219, 1966, and are available online at the U.S. Food and Drug Administration website. Additional methods for calculating body surface include the Mosteller formula (Mosteller, *N Engl J Med*, 317:1098, 1987), the DuBois and DuBois formula (DuBois D; DuBois EF, *Arch Int Med*, 17:863-71, 1916), the Haycock formula (Haycock et al., *The Journal of Pediatrics*, 93: 62-66, 1978), and the Gehan and George formula (Gehan EA, George SL, *Cancer Chemother Rep*, 54:225-35, 1970).

In further embodiments, a non-cytotoxic dose is a dose that achieves a tissue concentration at the site of action (such as at a solid tumor) that is less than a concentration that is determined to be cytotoxic in an *in vitro* cytotoxicity test. For example, a non-cytotoxic dose can be a dose that achieves a tissue concentration that is less than an IC₅₀ determined in a cytotoxicity assay, for example, less than 50% of an
IC₅₀, less than 10% of an IC₅₀, or less than 1% of an IC₅₀. Since even a single cancer cell remaining in a subject's body can lead to regrowth or spread of a tumor, using doses that achieve tissue concentrations of less than an IC₅₀ are contrary to typical anti-neoplastic dosing schedules. Examples of cytotoxicity assays include the SRB assay, the Trypan Blue Dye Exclusion Test, Differential Staining with Fluorescein Diacetate and Ethidium Bromide/Propidium Iodide, and the Neutral Red Cytotoxicity Assay, which are discussed further in Example 11. Additional cytotoxicity assays are available, for example, from Promega (Madison, WI).

In some instances, cytotoxic doses of the disclosed compounds have been determined in mouse models. Thus, in other embodiments, non-cytotoxic doses are doses that are less than such cytotoxic doses, for example, less than 25% of, less than 10% of, less than 1% of, or less than 0.1% of such determined cytotoxic doses. For example, cytotoxic doses of phenazine carboxamide compounds are provided in EP 0172744 (incorporated by reference herein), and range from 30-225 mg/kg per day. Cytotoxic doses of 2-arylbenzamidazoles are provided in Denny et al., *J. Med. Chem.*, **33**: 814-819, 1990 (incorporated by reference herein) and range from 100-150 mg/kg per day. Cytotoxic doses of acridine carboxamide compounds are provided in EP 0098098 (incorporated by reference herein), and range from 2.6-66 mg/kg per day. Cytotoxic doses of dibenzo[1,4]dioxincarboxamides are provided in Lee et al., *J. Med. Chem.*, **35**: 258-266, 1992 (incorporated by reference herein), and range from 20-225 mg/kg per day. Cytotoxic doses of a number of unsubstituted tricyclic carboxamides having various tricyclic ring systems are provided in Palmer et al., *J. Med. Chem.*, **31**: 707-712, 1988 (incorporated by reference herein) and range from 4.5-225 mg/kg per day. Cytotoxic doses of a number of 2-phenylquinoline-8-carboxamides are provided in Atwell et al., *J. Med. Chem.*, **32**: 396-401, 1989 (incorporated by reference herein) and range from 45-225 mg/kg per day. Cytotoxic doses of acridine-4-carboxamides are provided in Atwell et al., *J. Med. Chem.*, **30**: 664-669, 1987 (incorporated by reference
herein) and range from about 20-150 mg/kg per day. Such doses can be converted to human equivalents in mg/m² by multiplying by 3.

"Pharmaceutical compositions" are compositions that include an amount (for example, a unit dosage) of one or more of the disclosed compounds (or particular stereoisomers or pharmaceutically acceptable salts thereof) together with one or more non-toxic pharmaceutically acceptable excipients, including carriers, diluents, and/or adjuvants, and optionally other biologically active ingredients. Such pharmaceutical compositions can be prepared by standard pharmaceutical formulation techniques such as those disclosed in Remington's *Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA (19th Edition).

As used herein, the term "solid tumor" refers to a cancer of a body tissue other than blood, bone marrow, or the lymphatic system. Examples of solid tumors include carcinomas, sarcomas, blastomas, fibromas, astrocytomas, and melanomas, which may variously arise in breast, bladder, bone, skin, brain, neuronal, kidney, colon, esophageal, pancreatic, liver, lung, ovarian, testicular, uterine, or thyroid tissues.

The term "stereoisomer" refers to a molecule that is an enantiomer, diasteromer, or geometric isomer of a molecule. Stereoisomers, unlike structural isomers, do not differ with respect to the number and types of atoms in the molecule’s structure but with respect to the spatial arrangement of the molecule’s atoms. Examples of stereoisomers include the (+) and (-) forms of optically active molecules. Stereoisomers of all of the disclosed compounds are contemplated.

The term "subject" refers to animals, including mammals (for example, humans and veterinary animals such as dogs, cats, pigs, horses, sheep, mice, rats, and cattle).

A "substituent" or "R-group" refers to a single atom (for example, a halogen atom) or a group of two or more atoms that are covalently bonded to each other, which are in turn covalently bonded to an atom or atoms in a molecule to satisfy the valency requirements of the atom or atoms of the molecule. "Substituents" can replace one or more hydrogen atoms on the rings of the disclosed tricyclic compounds ("ring
substituents") or can replace hydrogen atoms on ring substituents (for example, a hydrogen on an alkyl, acyl, aryl, or alkoxy group that is a ring substituent).

"Substituted" or "substitution" refer to replacement of a hydrogen atom of a molecule or an R-group with one or more additional R-groups. Substituents are generally denoted as "R_2" when shown in a structural formula. Substituents include, but are not limited to: -OR, =O, =NR, =N-OR, =NR'R, =SR (thioalkoxy), -halogen (-F, -Cl, -Br, and -I), -SiR'R'R", -OC(O)R, -C(O)R, -CO_2R, -CONR'R, -OC(O)NR'R", -NR'C(O)R', -NR'-C(O)NR'R", -NR"C(O)_2R', -NR-C(NR'R'R")=NR"", -NR-C(NR'R")=NR"", -S(O)R', -S(O)_2R', -S(O)_2NR'R"", -NRSO_2R', -CN, and -NO_2 groups, which can replace any number of hydrogens in a compound from one up to the total number of hydrogen atoms that are present in the compound. R', R", R"" and R""" each independently refer to hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, and substituted or unsubstituted alkoxy or thioalkoxy groups. When a compound includes more than one R group, unless otherwise stated, each of the R groups is independently selected as are each of the R', R", R"" and R""" groups when more than one of these groups is present. When R' and R" are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. For example, -NR'R" is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholanyl. In particular embodiments, substituents can be halogen, alkyl, alkoxy, alkylthio,

 trifluoromethyl, acyl, acyloxy, hydroxyl, mercapto, carboxy, arlyoxy, aryl, arylalkyl, heteroaryl, amino, alkylamino, dialkylamino, morpholino, piperidino, pyrrolidin-1-yl, piperazin-1-yl, nitro, or sulfato, for example, halogen, alkyl (such as lower alkyl), alkoxy, acyloxy, hydroxyl, carboxy, amino, alkylamino, dialkylamino, or nitro such as alkyl, halogen, alkoxy or nitro. In other particular embodiments, "substituents" can be halogen, alkyl, alkoxy, alkylthio, trifluoromethyl, acyloxy, hydroxyl, mercapto, carboxy, arlyoxy, aryl, arylalkyl, heteroaryl, amino, alkylamino, dialkylamino, morpholino, piperidino, pyrrolidin-1-yl, piperazin-1-yl, nitro, or sulfato, for example, halogen, alkyl (such as lower alkyl), alkoxy, acyloxy, hydroxyl, carboxy, amino, alkylamino,
dialkylamino, or nitro such as alkyl, halogen, alkoxy, or nitro. In still further embodiments, "substituents" can be acyl, acyloxy, alkoxy, alkyl, alkylamino, alkylthio, amino, aryl, aza, carboxy, carboxamide, dialkylamino, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido or sulfato, for example, alkoxy, alkyl, amino, aroyl, aza, carboxy, carboxamide, halogen, hydroxyl, nitro, alkylsulfonyl, alkylsulfonamido, or sulfato, such as alkoxy, alkyl, alkylacrylamino, alkylaminoacyl, alkylamino, aryl, aza, carboxy, dialkylamino, halogen, hydroxyl, -NH₂, nitro, lower alkylsulfonyl, or lower alkylsulfonamido.

The term "sulfato" refers to the group --SO₃⁻ or its conjugate base --SO₃⁻.

The term "sulfonyl" refers to a substituent including the bivalent group --SO₂⁻, and more typically --SO₂R, where R is hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, or substituted or unsubstituted alkoxy or thioalkoxy. Sulfonyl groups include "alkylsulfonyl" groups having the structure --SO₂R, where R is unsubstituted or substituted alkyl. Similarly, "lower alkylsulfonyl" refers to --SO₂R, wherein R is unsubstituted or substituted lower alkyl.

The term "sulfonamido" refers to a substituent having the formula --NRSO₂R', where R and R' each are independently hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, or substituted or unsubstituted alkoxy or thioalkoxy groups. Sulfonamido groups include "alkylsulfonamido" groups that have the structure --NHSO₂R, where R is substituted or unsubstituted alkyl. Similarly, sulfonamido groups include "lower alkylsulfonamido" groups having the structure --NHSO₂R, where R is unsubstituted or substituted lower alkyl.

A "therapeutically effective dose, amount or concentration" of a disclosed compound is a dose, amount or concentration of the compound that is sufficient to achieve a desired therapeutic effect, such as inhibition of angiogenesis, an anti-metastatic or anti-tumorigenic effect, or inhibition of HIF-1α activity in a cell or a subject. In some examples, a therapeutically effective amount is an amount sufficient to achieve tissue concentrations at the site of action that are similar to those that are shown...
to modulate angiogenesis or HIF-1α activity in tissue culture, *in vitro*, or *in vivo*. For example, a therapeutically effective amount of a compound may be such that the subject receives a dosage of about 0.1 µg/kg body weight/day to about 1000 mg/kg body weight/day, for example, a dosage of about 1 µg/kg body weight/day to about 250 mg/kg body weight/day, a dosage of about 10 µg/kg body weight/day to about 100 mg/kg body weight/day, 10 µg/kg body weight/day to about 10 mg/kg body weight/day, or 10 µg/kg body weight/day to about 1 mg/kg body weight/day. A therapeutically effective dosage, amount, or concentration can also be a non-cytotoxic dosage, amount, or concentration.

III. Overview

One aspect of the disclosure pertains to tricyclic compounds that inhibit HIF-1α activity and/or inhibit angiogenesis. These compounds can be used to decrease angiogenesis, or to inhibit metastasis or tumorigenesis, both of which depend at least in part upon angiogenesis. Pharmaceutically acceptable salts and stereoisomers of the compounds also are contemplated.

In the structures that follow, all valency requirements are understood to be satisfied. Thus, for example, carbon atoms have four bonds to other atoms, even if all such bonds are not shown. As is understood by those of ordinary skill in the art, where all four bonds to a carbon atom are not shown, additional bonds to hydrogen atoms are implied. Further substitution of such implied hydrogen atoms is possible.

As inhibitors of the hypoxic signaling pathway, the disclosed compounds are useful in the treatment of both primary and metastatic solid tumors, including carcinomas of breast, colon, rectum, lung, oropharynx, hypopharynx, esophagus, stomach, pancreas, liver, gallbladder and bile ducts, small intestine, urinary tract (including kidney, bladder, and urothelium), female genital tract, (including cervix, uterus, and ovaries as well as choriocarcinoma and gestational trophoblastic disease), male genital tract (including prostate, seminal vesicles, testes, and germ cell tumors),
endocrine glands (including the thyroid, adrenal, and pituitary glands), and skin, as well as hemangiomas, melanomas, sarcomas (including those arising from bone and soft tissues as well as Kaposi's sarcoma), and tumors of the brain, nerves, eyes, and meninges (including astrocytomas, gliomas, glioblastomas, retinoblastomas, neuromas, neuroblastomas, Schwanomomas, and meningiomas). Such compounds may also be useful in treating solid tumors arising from hematopoietic malignancies such as leukemias (i.e. chloromas, plasmacytomas, and the plaques and tumors of mycosis fungoides and cutaneous T-cell lymphoma/leukemia) as well as in the treatment of lymphomas (both Hodgkin's and non-Hodgkin's lymphomas). In addition, these compounds may be useful in the prevention of metastases from the tumors described above, either when used alone or in combination with surgery, radiotherapy, and/or other chemotherapeutic agents.

In some embodiments, the tricyclic compound has the following structure:

![Tricyclic Compound Structure](image)

where X and Y are independently O, S, or N, R_{10} is H or one or more substituents such as acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido or sulfato, at least one of which is a carboxamide, R_{11} is H or one or more substituents such as acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido or sulfato, and all valency requirements are satisfied. In yet other embodiments, X, Y, R_{10}, and R_{11} in the structure above are as in Table 2 below, where R_{12} and R_{13} are independently H or lower alkyl. In particular embodiments, R_{12} and R_{13} are both H, or both methyl.
Table 2

<table>
<thead>
<tr>
<th>X</th>
<th>Y</th>
<th>( \mathbf{R}_{10} )</th>
<th>( \mathbf{R}_{11} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>O</td>
<td>CONH(CH₂)₂NR₁₂R₁₃</td>
<td>H, alkoxy, alkyl, halogen, NO₂ or COOH</td>
</tr>
<tr>
<td>S</td>
<td>S</td>
<td>CONH(CH₂)₂NR₁₂R₁₃</td>
<td>H, alkoxy, alkyl, halogen, NO₂ or COOH</td>
</tr>
<tr>
<td>N</td>
<td>N</td>
<td>CONH(CH₂)₂NR₁₂R₁₃</td>
<td>H, alkoxy, alkyl, halogen, NO₂ or COOH</td>
</tr>
<tr>
<td>O</td>
<td>S</td>
<td>CONH(CH₂)₂NR₁₂R₁₃</td>
<td>H, alkoxy, alkyl, halogen, NO₂ or COOH</td>
</tr>
<tr>
<td>O</td>
<td>N</td>
<td>CONH(CH₂)₂NR₁₂R₁₃</td>
<td>H, alkoxy, alkyl, halogen, NO₂ or COOH</td>
</tr>
<tr>
<td>S</td>
<td>O</td>
<td>CONH(CH₂)₂NR₁₂R₁₃</td>
<td>H, alkoxy, alkyl, halogen, NO₂ or COOH</td>
</tr>
<tr>
<td>N</td>
<td>O</td>
<td>CONH(CH₂)₂NR₁₂R₁₃</td>
<td>H, alkoxy, alkyl, halogen, NO₂ or COOH</td>
</tr>
<tr>
<td>S</td>
<td>N</td>
<td>CONH(CH₂)₂NR₁₂R₁₃</td>
<td>H, alkoxy, alkyl, halogen, NO₂ or COOH</td>
</tr>
<tr>
<td>N</td>
<td>S</td>
<td>CONH(CH₂)₂NR₁₂R₁₃</td>
<td>H, alkoxy, alkyl, halogen, NO₂ or COOH</td>
</tr>
</tbody>
</table>

In other embodiments, the tricyclic compound has a structure

![Diagram](attachment:image.png)

wherein \( T \) is CR₁₆ or NR₁₇; \( V \) is O, S, or N and all valency requirements are satisfied; \( R₁₄ \) and \( R₁₅ \) are independently H or a substituent such as acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, alyl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido, or sulfato; \( R₁₆ \) is diamine; and \( R₁₇ \) is amino-substituted alkyl. In yet other embodiments, \( T, V, R₁₄, R₁₅, R₁₆, \) and \( R₁₇ \) are as shown in Table 3 below.
Table 3

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>V</td>
<td>R\textsubscript{14}</td>
<td>R\textsubscript{15}</td>
<td>R\textsubscript{16}</td>
</tr>
<tr>
<td>C-R\textsubscript{16}</td>
<td>O</td>
<td>H, alkyl, alkoxy, halogen or nitro</td>
<td>H, alkyl, halogen or nitro</td>
<td>diamine</td>
</tr>
<tr>
<td>N-R\textsubscript{17}</td>
<td>O</td>
<td>H, alkyl, alkoxy, halogen or nitro</td>
<td>H, alkyl, halogen or nitro</td>
<td></td>
</tr>
<tr>
<td>C-R\textsubscript{16}</td>
<td>N</td>
<td>H, alkyl, alkoxy, halogen or nitro</td>
<td>H, alkyl, halogen or nitro</td>
<td>diamine</td>
</tr>
</tbody>
</table>

In more particular embodiments, the tricyclic compound is:

![Chemical Structures](image-url)
In particular embodiments, the tricyclic compounds are dibenzo-[1,4]-dioxin compounds having the formula:

where $R_{18}$ is or one or more substituents such as acyl, acyloxy, alkoxyl, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido, or sulfato, at least one of which is carboxamide, and $R_{19}$ is hydrogen or one or more substituents such as acyl, acyloxy, alkoxyl, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido, or sulfato. In particular embodiments, at least one $R_{18}$ group has the formula $-\text{CONR}'(\text{CH}_2)_n\text{NR}''\text{R}'''$ where $n=1-5$ and $R'$, $R''$, and $R'''$ are independently H or lower alkyl. Specific examples of such dibenzo-[1,4]-dioxin compounds include the compounds shown below.
Additional examples include the dibenzo-[1,4]dioxin-1-carboxamides disclosed in Lee et al. (Lee et al., *J. Med. Chem.*, **35**: 258-266, 1992, which is incorporated by reference herein). Particular examples disclosed by Lee et al. include the 3-nitro, 6-chloro, 6-methyl, 6-methoxy, 6-aza, 7-chloro, 8-chloro, 7-bromo, 7-methyl, 8-methyl, 7-nitro, 8-bromo, 8-nitro, 9-chloro, 9-bromo, 9-methyl, 9-methoxy, 9-aza, 9-nitro, 9-[CONH(CH$_2$)$_2$NMe$_2$], and 7,8-dichloro derivatives of
In general, dibenzo-[1,4]dioxin carboxamides can be synthesized using the methods outlined in Lee et al. (Lee et al., *J. Med. Chem.*, 35: 258-266, 1992). For example, 9-(and 6-) substituted dibenzo-[1,4]dioxins-1-carboxylic acids may be prepared by the synthesis of dibenzo-[1,4]dioxin-1-carboxylic acid and its regioselective metalation at the C-9 position according to the method described in Palmer et al., *J. Org. Chem.*, 55: 438-441, 1990, which is incorporated by reference herein. Alternatively, 9-(and 6-) substituted dibenzo-[1,4]dioxin-1-carboxylic acids can be prepared by cyclocondensation of the dianion of isopropyl 2,3-dihydroxybenzoate with 3-substituted 1,2-dichloro- or o-chloro-nitrobenzenes, or the dianion of methylcatechols with 1,2-nitrochloro- or 1,2-dichlorobenzoates. Similar cyclocondensation reactions may be used to generate 8-(and 7-) substituted dibenzo-[1,4]dioxin-1-carboxylic acids. Coupling of the acids with an appropriate (dialkylamino)-alkylamine (such as *N*,*N*-dimethylamino) in the presence of 1,1'-carbonyldimidazole yields the carboxamide. This general coupling reaction is shown below in Scheme 1, which shows a particular example of a synthesis of a dibenzo-[1,4]dioxin carboxamide from a carboxylic acid (see, Staab, *Angew. Chem. Int. Ed. Engl.*, 1: 351, 1962).
In other embodiments, tricyclic compounds that are sulfur analogs of the dibenzo-[1,4]dioxin compounds above are provided. Such phenoxathiin and thianthrene compounds have the following structures:

where \( R_{20} \) and \( R_{22} \) in the alternative structures each are independently one or more substituents such as acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido, or sulfato, at least one of which is carboxamide, and \( R_{21} \) and \( R_{23} \) in the alternative structures each are independently hydrogen or one or more substituents such as acyl,
acyloxy, alkoxy, alkyl, alkylthio, amino, aroyl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido, or sulfato. Particular examples of this type of compound include:

\[
\begin{align*}
\text{N} & \quad \text{O} \\
\text{S} & \quad \text{S} \\
\text{NO}_2 & \\
\text{N} & \quad \text{O} \\
\text{S} & \quad \text{S} \\
\text{NO}_2 & \\
\end{align*}
\]

Substitution of sulfur analogs of the diion of isopropyl 2,3-dihydroxybenzoate or the diion of methyl-catechols in the reactions described above for the dibenzo-[1,4]dioxin compounds above may be used to provide such sulfur analogs. Method for synthesizing such compounds also are provided in Palmer et al., *J. Med. Chem.*, 31: 707-712, 1988, which is incorporated by reference herein. In general, the parent heterocycles are available commercially, for example, from Sigma-Aldrich, St. Louis Mo., and the carboxylic acids of such heterocycles can be obtained by metatation with n-butyllithium, followed by either quenching with solid CO$_2$ or treatment with dimethylformamide, followed by oxidation, for example, with potassium permanganate.

In still other particular embodiments, the compound is a phenazine compound having the following formula:

\[
\begin{align*}
\text{N} & \quad \text{R}_{25} \\
\text{R}_{24} & \\
\end{align*}
\]

wherein $R_{24}$ is one or more substituents such as acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aroyl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido, or sulfato, at least one of which is carboxamide, and $R_{25}$ is hydrogen or one or more substituents such as acyl, acyloxy, alkoxy, alkyl, alkylthio,
amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido, or sulfato. Examples of phenazine carboxamide compounds are provided in Rewcastle et al., J. Med. Chem., 30: 843-851, 1987, which is incorporated by reference herein. Additional examples of such compounds are provided in EP 0172744, which also is incorporated by reference herein. Particular examples disclosed by Rewcastle et al. include the 2-methoxy, 2-chloro, 3-methyl, 3-methoxy, 3-chloro, 4-methyl, 4-methoxy, 6-methyl, 6-methoxy, 6-chloro, 7-methyl, 7-methoxy, 7-chloro, 8-methyl, 8-methoxy, 8-chloro, 9-methyl, 9-methoxy, and 9-chloro derivatives of:

![](image)

Phenazine carboxamide compounds may be synthesized according to the procedures described in Rewcastle et al. and in EP 0172744. Briefly, phenazine-1-carboxylic acids can be prepared by Jourdan-ULLMANN copper-catalyzed condensation of substituted anilines with 3-nitro-2-halobenzoic acids using a copper catalyst to provide N-phenylanthranilic acids, which are reductively cyclized (such as with NaBH₄ in alkali) to give the phenazine-1-carboxylic acid (see, Challand et al., J. Chem. Soc. Chem. Comm., p. 1423, 1970). 9-substituted phenylanthranilic acids also can be synthesized using symmetrical 2,6-disubstituted anilines with subsequent ring closure when the symmetrical substituents of the anilines are small and displaceable (such as F or methoxy). Reaction of fluorinated, substituted anilines with 3-nitro-2-halobenzoic acids followed by reductive ring closure can be used to provide 6- and 8-substituted
phenazine-1-carboxylic acids. Preparation of phenazine-1-carboxylic acids with at least one substituent in the 2-, 3-, or 4- utilizes highly substituted benzoic acids as starting materials. As described above, coupling of the acids with an appropriate (dialkylamino)-alkylamine (such as N,N'-dimethylethylenediamine) in the presence of 1,1'-carbonyldiimidazole yields the carboxamide compounds.

In further particular embodiments, the tricyclic compound is an arylquinoline compound having the formula:

where R_{26} and R_{29} in the alternative structures each are independently H or one or more substituents such as acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido, or sulfato, at least one of which is carboxamide, and R_{27}, R_{28}, R_{30}, and R_{31} in the alternative structures each are independently hydrogen or one or more substituents such as acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido, or sulfato. A particular example of such an arylquinoline compound is disclosed in Woynarowski et al., Anti-
cancer Drug Des., 9: 9-24, 1994, which is incorporated by reference herein. Additional aryquinoline compounds are disclosed in Atwell et al., J. Med. Chem., 32: 396-401, 1989, which is incorporated by reference herein. Particular aryquinoline compounds include the unsubstituted 2'-aza, 2'-chloro, 3'-aza, 3'-methoxy, 4'-aza, 4'-fluoro, 4'-chloro, 4'-bromo, 4'-iodo, 4'-methoxy, 4'-hydroxyl, 4'-phenyl, 4'-nitro, 4'-trifluoromethyl, 4'-SO₂CH₃, 4'-amino, 4'-NHOCH₃, 4'-NHSO₂CH₃, and 3',4'-benz derivatives of:

![Chemical结构式](image)

where n=2, 3, 4, or 5.

Aryquinoline compounds can be synthesized according to the methods disclosed in Atwell et al., J. Med. Chem., 32: 396-401, 1989, and in EP 0206802, both of which are incorporated by reference herein. 2-phenylquinoline-8-carboxylic acids can be produced by a variety of methods. For example, the unsubstituted 2-phenyl-8-carboxylic acid can be synthesized by the Doebner pyruvic acid synthesis of 8-methyl-2-phenylquinoline-4-carboxylic acid, followed by decarboxylation and oxidation with SeO₂ (see, Doebner and Giesecke, Justus Liebigs Ann. Chem., 242: 290, 1887). Substituted derivatives can be synthesized by Pfizinger reaction of substituted acetophenones with 7-methylisatin followed by decarboxylation and oxidation (see, Filippi, J. Bull. Soc. Chim. Fr., 1968, 268). 4'-nitro substituted acids can be prepared by low-temperature nitration of 2-phenylquinoline-8-carboxylic acid in fuming nitric acid to yield a mixture of mono-nitro derivatives followed by isolation of the 4'-nitro derivative by repeated recrystallization. Since oxidation is not compatible with some
substituents, an alternative route to the substituted 2-phenylquinoline-8-carboxylic acid is reaction of an ethyl benzoylacetate and a methyl anthranilate to give a Schiff base, which is then cyclized to a quinoline ester and reduced to provide the acid. The acid can then be coupled with the appropriate (dialkylamino)-alkylamine as has been described above for the previously discussed tricyclic carboxamide compounds.

In yet further particular embodiments, the tricyclic compound is an acridine compound having the structure:

where \( R_{32} \) and \( R_{33} \) are independently hydrogen or a substituent such as acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido, or sulfato; and \( R_{34} \) is one or more substituents such as acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido, or sulfato, at least one of which is carboxamide. A particular example of a compound having this type of structure is:

Acridine carboxamides can be synthesized by the methods described in Denny et al. (Denny et al., *J. Med. Chem.*, 30: 658-663, 1987), Atwell et al., (Atwell et al., *J.*

In some other particular embodiments, the tricyclic compound is a phenoxazine compound having the formula:

![Chemical Structure Image]

where $R_{35}$ is one or more substituents such as acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido, or sulfato, at least one of which is carboxamide, $R_{36}$ is hydrogen or a substituent such as lower alkyl, and $R_{37}$ is hydrogen or a substituent such as acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido, or sulfato. Synthesis of such tricyclic carboxamides may be accomplished using the methods described in WO 02/100843 (which are outlined below in Scheme 2), which is incorporated by reference herein.
Scheme 2

If a tricyclic amine is desired, \( R_{36}\)-Br in Scheme 2 can be an amino-substituted alkyl bromide, for example,
These methods also can be used to produce corresponding carboxylic acids as outlined in Scheme 3 below, which carboxylic acids can then be coupled with an appropriate (dialkylamino)-alkylamine (such as N,N'-dimethylethlenediamine) in the presence of 1,1'-carbonyldiimidazole to yield a carboxamide compound (see, Scheme 1 above for an example of the coupling reaction).

Scheme 3

Particular examples of compounds that may be made according to the reactions of Schemes 2 and 3 include
Additional methods for making various linear tricyclic compounds discussed above are found in Palmer et al., *J. Med. Chem.,* 31: 707-712, 1988, which is incorporated by reference herein.

In even further embodiments, the compound is a 2-arylbenzimidazole having the structure:

\[
\text{Ar} \quad \text{W} \quad \text{Z} \quad \text{R}_{38}
\]

where R_{38} is one or more substituents such as acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido, or sulfato, at least one of which is carboxamide; Ar is an aryl group such as phenyl, 2-furyl, 2-thienyl, 3-thienyl, or 2-pyrrolyl (which may be further substituted, for example, with one or more acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido, or sulfato groups); W and Z are independently either NR_7 or =N—, but not the same; and R_7 is hydrogen or a substituent such as alkyl. Particular examples of 2-arylbenzimidazoles include the 2'-methyl; 2'-methoxy; 2'-chloro; 2'-aza; 3'methyl; 3'-methoxy; 3'-chloro; 3'-aza; 4'-methyl; 4'-methoxy; 4'-chloro; 4'-aza; 4'phenyl; 4'-NHAc; 4'-N(CH_3)_2; 5'-methyl; 6'-methyl; 7'-methyl; 2',3'-benz; 3',4'-benz; 2',3'-(-OCH_2-); 2',3'-dimethoxy; and 2',3',4'-trimethoxy derivatives of:
which can be synthesized by the methods described in Denny et al., *J. Med. Chem.*, 33: 814-819, 1990 (incorporated by reference herein). Briefly, such compounds can be synthesized by oxidative condensation of substituted or unsubstituted 1,2-diaminobenzoic acids and substituted or unsubstituted arylaldehydes (such as benzaldehydes) with cupric ion (*see*, Weidenhagen and Weeden, *Chem. Ber.*, 69B: 2263, 1938).

Yet another aspect pertains to methods of inhibiting HIF-1 activity in a cell, comprising contacting the cell with an effective amount of a disclosed compound that decreases the HIF-1 activity in the cell from a first level to a second, lower level (for example, by reducing the amount thereof inside a cell). In one example, the disclosed compound is a dibenzo-[1,4]dioxin carboxamide other than a 6-chloro-[1,4]dioxin-1-carboxamide, such as NSC 644221, and the effective amount may be any amount, including a cytotoxic amount, but, in certain particular examples, is a non-cytotoxic amount. HIF-1 amounts can be assessed by gel electrophoresis of a cell extract followed by antibody detection as shown elsewhere in this disclosure.

Still another aspect pertains to methods of inhibiting angiogenesis, comprising contacting a cell with an effective amount of a disclosed compound to inhibit HIF-1α activity, and, for example, reduce the amount of VEGF. In one example, the disclosed compound is a dibenzo-[1,4]dioxin carboxamide other than a 6-chloro-[1,4]dioxin-1-carboxamide, such as NSC 644221, and the effective amount may be any amount, including a cytotoxic amount, but, in certain particular examples, is a non-cytotoxic
amount. Angiogenesis is the formation of new blood vessels from pre-existing vessels. Angiogenesis is prominent in solid tumor formation and metastasis, and also is part of the wound healing process. Pathological angiogenesis sometimes occurs in inappropriate anatomic locations, such as the retina or cornea, in response to disease and injury. Inhibition of angiogenesis could avoid the progression of conditions of inappropriate angiogenesis.

Tumor formation, for example, requires a network of blood vessels to sustain the nutrient and oxygen supply for continued growth. Tumors in which angiogenesis is important include most solid tumors and benign tumors, such as acoustic neuroma, neurofibroma, trachoma, and pyogenic granulomas. Inhibition of angiogenesis could halt the growth of these tumors and the resultant damage due to the presence of the tumor.

There also is a direct correlation between tumor microvessel density and the incidence of metastasis. Hypoxic tumor cells themselves can produce factors such as the HIF-1α protein that stimulate the proliferation of endothelial cells and new capillary growth. Angiogenesis is important in two stages of tumor metastasis. As discussed above, the first stage where angiogenesis stimulation is important is in the vascularization of the primary tumor, which allows tumor cells to enter the blood stream and to circulate throughout the body (metastasis). After the tumor cells have left the primary site, and have settled into the secondary, metastatic site, angiogenesis must occur before the metastasis can grow and expand (tumorigenesis). Therefore, inhibiting angiogenesis by decreasing HIF-1α activity can lead to the reduction or elimination of metastasis of tumors and possibly contain the neoplastic growth at the primary site.

Still further, a method for inhibiting angiogenesis by inhibiting HIF-1α activity in a subject is disclosed. The method includes administering to the subject a therapeutically effective amount of one or more of any of the compounds disclosed above. In one example, the disclosed compound is a dibenzo-[1,4]dioxin carboxamide other than a 6-chloro-[1,4]dioxin-1-carboxamide, such as NSC 644221, and the
effective amount may be any amount, including cytotoxic amount, but, in certain particular examples, is a non-cytotoxic amount.

In some embodiments, the therapeutically effective amount of the compound is administered to a subject with a tumor to achieve an anti-tumor effect, such as inhibition of tumor metastasis or inhibition of tumorogenesis. In other embodiments, the therapeutically effective amount of the compound is administered to a subject with a pathological angiogenesis, such as proliferative retinopathy. In still other embodiments the method further includes administering a second therapeutic agent, which can be an anti-angiogenic compound or a cytotoxic compound. In some embodiments, the therapeutically effective amount is a non-cytotoxic amount or achieves a non-cytotoxic concentration in the subject. The method may further include selecting the tricyclic compound that is used in the method. The tricyclic compound can be selected to increase degradation of HIF-1α and have substantially no effect on degradation of HIF-2α. In other embodiments, the tricyclic compound is selected to increase degradation of HIF-1α in a VHL-independent manner. In yet other embodiments, the compound is selected to have substantially no effect on the level of HIF-1α mRNA that is expressed in the subject or the cell. Alternatively, the compound used in the method can be selected to inhibit HIF-1α activity through a Topoisomerase-1-independent mechanism. In further examples, the compound is selected as having one or more, or all, of these properties or the properties of decreasing VEGF mRNA expression, inhibiting HIF-1α activity through a proteosome dependent mechanism, inducing G2/M arrest, being substantially non-cytotoxic, inhibiting HIF-1α activity by a mechanism which does not involve microtubule disruption, having substantially no inhibiting effect on hsp 90, being independent of the AKT pathway, having substantially no inhibiting effect on deacetylation enzymes, being independent of the p53 gene, and increasing p21 mRNA expression. Furthermore, any combination of such criteria of selection may be used to select the compound used in the method. Suitable methods for selecting the tricyclic compound for use in the method are described in the Examples below.
Other aspects pertain to the use of the disclosed compounds that inhibit HIF-1α activity in a method of treatment of the human or animal body, and the use of the disclosed compounds that inhibit HIF-1α activity in the manufacture of a medicament for use in the treatment of a proliferative condition such as cancer. In one example, the disclosed compound is a dibenzo-[1,4]dioxin carboxamide other than a 6-chloro-[1,4]dioxin-1-carboxamide, such as NSC 644221, and the medicament may include any amount, including cytotoxic amounts, of the disclosed compound, but, in certain particular examples, includes non-cytotoxic amounts.

The disclosed tricyclic compounds can also be used in combination therapies, for example, in conjunction with other therapies, such as in conjunction with other therapeutic agents including anti-proliferative agents or anti-neoplastic agents. In one example, the disclosed tricyclic compound is a dibenzo-[1,4]dioxin carboxamide other than a 6-chloro-[1,4]dioxin-1-carboxamide, such as NSC 644221, and may used in any amount, including cytotoxic amounts, but in certain particular examples, is used in non-cytotoxic amounts. Using the disclosed compounds in combination with other therapies can refer to using the combination of therapies in some sequence (such as alternating) or together simultaneously. For example, the disclosed compounds can be used, for example, at non-cytotoxic doses, in combination with antineoplastic agents, either simultaneously, or in a sequence such as using the antineoplastic agent followed by administration of a disclosed compound. In another example, a tumor can be treated conventionally with surgery, radiation, or chemotherapy in combination with a disclosed compound and then, optionally the compound can be further administered to the subject to extend the dormancy of micrometastases and to stabilize and inhibit the growth of any residual primary tumor. Examples of agents that can be used in combination with the disclosed compounds include alkylating agents, antimetabolites, natural products, kinase inhibitors, hormones and their antagonists, and miscellaneous other agents. Examples of alkylating agents include nitrogen mustards (such as mechlorethamine, cyclophosphamide, melphalan, uracil mustard, or chlorambucil),
alkyl sulfonates (such as busulfan), nitrosoureas (such as carmustine, lomustine, semustine, streptozocin, or dacarbazine). Examples of antimetabolites include folic acid analogs (such as methotrexate), pyrimidine analogs (such as 5-FU or cytarabine), and purine analogs, such as mercaptopurine or thioguanine. Examples of natural products include vinca alkaloids (such as vinblastine, vincristine, or vindesine), epipodophyllotoxins (such as etoposide or teniposide), antibiotics (such as dactinomycin, daunorubicin, doxorubicin, bleomycin, plicamycin, or mitomycin C), and enzymes (such as L-asparaginase). Examples of kinase inhibitors include small molecule inhibitors (such as Iressa, Tarceva, PKI-166, CI-1033, CGP-5923A, EKB-569, TAK165, GE-572016, CI-1033, SU5416, ZD4190, PTK787/ZK222584, CGP41251, CEP-5214, ZD6474, BIBF1000, VGA1102, SU6668, SU11248, CGP-57148, tricyclic quinoxalines, SU4984, SU5406, Gleevec, NSC680410, PD166326, PD1173952, CT53518, GTP14564, PKC412, PP1, PD116285, CGP77675, CGP76030, CEP-701, and CEP2583), ligand modulators (such as Bevacizumab, MV833, Soluble Fit-1 and Flk-1, VEGF Trap, GFB 116, NM3, VEGF 121-diptheria toxin conjugate, and Interferone-α), and monoclonal antibodies against receptors (such as Cetuximab, ABX-EGF, Y10, MDX-447, h-R3, EMD 72000, herceptin, MDX-H210, pertuzumab, IMC-1C11, and MF1). Examples of hormones and antagonists include adrenocorticosteroids (such as prednisone), progestins (such as hydroxyprogesterone caproate, medroxyprogesterone acetate, and maeostrol acetate), estrogens (such as diethylstilbestrol and ethinyl estradiol), antiestrogens (such as tamoxifen), and androgens (such as testosterone propionate and fluoxymesterone). Examples of miscellaneous agents include platinum coordination complexes (such as cis-diaminedichloroplatinum II, which is also known as cisplatin), substituted ureas (such as hydroxyurea), methyl hydrazine derivatives (such as procarbazine), vaccines (such as APC8024), AP22408, B43-genistein conjugate, paclitaxel, AG538, and adrenocortical suppressants (such as mitotane and aminoglutethimide). In addition, the disclosed compounds can be combined with gene therapy approaches, such as those targeting
VEGF/VEGFR (including antisense oligonucleotide therapy, Adenovirus-based Flt-1 gene therapy, Retrovirus-base Flk-1 gene therapy, Retrovirus-based VHL gene therapy, and angiozyme) and IGF-1R (including INX-4437). Examples of the most commonly used chemotherapy drugs that can be used in combination with the disclosed tricyclic compounds agent include Adriamycin, Alkeran, Ara-C, BiCNU, Busulfan, CCNU, Carboplatinum, Cisplatinum, Cytoxan, Daunorubicin, DTIC, 5-FU, Fludarabine, Hydrea, Idarubicin, Ifosfamide, Methotrexate, Mitomycin, Mitomycin, Mitoxantrone, Nitrogen Mustard, Taxol, Velban, Vincristine, VP-16, Gemcitabine (Gemzar), Herceptin, Irinotecan (Camptosar, CPT-11), Leustatin, Navelbine, Rituxan STI-571, Taxotere, Topotecan (Hycamtin), Xeloda (Capecitabine), Zevelin, and calcitriol.

The disclosed compounds also can be combined with radiotherapy employing radioisotopes (such as $^{32}$P, $^{90}$Y, $^{125}$I, $^{131}$I, and $^{177}$Lu), particle beams (such as proton, neutron and electron beams) and electromagnetic radiation (such as gamma rays, x-rays, and photodynamic therapy using photosensitizers and visible or ultraviolet rays). In one example, the disclosed compound is a dibenzo-[1,4]dioxin carboxamide other than a 6-chloro-[1,4]dioxin-1-carboxamide, such as NSC 644221, and may be used in any amount, including cytotoxic amounts, but, in certain particular examples, is used in non-cytotoxic amounts.

One of ordinary skill in the art is readily able to determine whether or not a candidate compound is active, that is, capable of inhibiting HIF-1 activity. For example, assays which may conveniently be used to readily assess the suitability of a particular compound are described in the Examples below.

Since compounds that inhibit HIF-1α activity and/or angiogenesis have antiproliferative effects, antiproliferative agents are thus provided. In particular, the disclosed compounds are applicable to proliferative conditions (e.g., cancers) which are characterized by so-called "solid" tumors, and which rely on angiogenesis, and the resulting vasculature to grow and or spread to other parts of a subject's body. In some embodiments, the antiproliferative compounds are used in non-cytotoxic concentrations,
although the antiproliferative effect can indirectly lead to a net effect of reducing the
number of cells by decreasing cell proliferation so that the rate of programmed cell
death is greater than the rate of cellular proliferation.

Use of the compounds in a method of treatment of a proliferative condition
(such as a neoplasm, for example a solid tumor) of a human or animal subject is also
disclosed. Such methods comprise administering to such a subject a therapeutically-
effective amount (such as a cytotoxic or non-cytotoxic amount) of an active compound,
for example, in the form of a pharmaceutical composition. In one example, the
disclosed compound is a dibenzo-[1,4]dioxin carboxamide other than a 6-chloro-
[1,4]dioxin-1-carboxamide, such as NSC 644221, and the therapeutically effective
amount may be any amount, including a cytotoxic amount, but, in certain particular
examples, the therapeutically effective amount is a non-cytotoxic amount. The term
"treatment," in the context of treating a condition, generally refers to treatment and
therapy, whether of a human or an animal (e.g., in veterinary applications), in which
some desired therapeutic effect is achieved, for example, the inhibition of the progress
of the condition, and includes a reduction in the rate of progress, a halt in the rate of
progress, amelioration of the condition, or cure of the condition. Treatment as a
prophylactic measure is also contemplated.

The disclosure further provides the use of a disclosed compound for the
manufacture of a medicament, for example, for the treatment of a proliferative
condition, as discussed above. In one example, the disclosed compound is a dibenzo-
[1,4]dioxin carboxamide other than a 6-chloro-[1,4]dioxin-1-carboxamide, such as NSC
644221, and may be used in any amount, including cytotoxic amounts, but in certain
particular examples, is used in non-cytotoxic amounts. Furthermore, the compounds
may also be used as part of an in vitro assay, for example, in order to determine whether
a candidate host is likely to benefit from treatment with the compound in question, and
the compounds can be used as standards, for example, in an assay, in order to identify
other active compounds, such as other antiproliferative agents.
The following examples are provided to illustrate certain particular features and/or embodiments, but these examples should not be construed to limit the invention to the particular features or embodiments described.

**Example 1 – Tricyclic carboxamide NSC 644221 modulates the hypoxic level of HIF-1α protein but not HIF-1β protein in a dose and time dependent manner**

Monolayers of U251 cells were treated under the below-specified conditions before protein extracts were obtained using RIPA lysis buffer. Cellular proteins (80μg) were then separated by SDS-PAGE. Separated proteins were transferred to a PVDF membrane before blocking (4% milk) and hybridisation (0.4% BSA) with a primary HIF-1α antibody (overnight) and then a secondary anti-mouse antibody (for 30 min). The resultant membrane was developed using an enhanced chemiluminescent (ECL) reagent. The membrane was exposed to X-ray film, which was then developed to visualize the membrane bound proteins.

The U251 cells were treated with 1% oxygen for 2, 4, 6, and 18 hours with a final concentration of NSC 644221 equivalent to 5μM, which was added 30 min prior to hypoxic (1% oxygen) exposure (FIG. 1A). Total cellular proteins were separated using SDS-PAGE and then subjected to immunoblot analysis using the HIF-1α antibody. The membrane-bound protein was then re-probed for actin with an anti-actin antibody following an antibody stripping procedure to remove the HIF-1α antibody. Detection of actin was used to detect any significant differences in loading of the gel lanes prior to the SDS-PAGE separation. The results of this analysis are shown in FIG 1A. A further study was performed, exposing the cells to up to 8 hours of hypoxia; the results are shown in FIG. 2.

Immunoblot analysis was again employed to analyze modulation of HIF-1α following the addition of 0.5, 1, 2, 4 and 8μM NSC 644221 for 30 minutes, followed by exposure to 1% oxygen for a further 6 hours. Total protein extracts were separated
using SDS-PAGE, and then subjected to immounoblot analysis using a HIF-1α antibody as described above. The membrane was then stripped and re-probed for HIF-1β using an anti-HIF-1β antibody. Protein bands were visualized as before using ECL, and the results of this analysis are shown in FIG. 1B. A similar experiment using 1, 2, 4, and 8 μM NSC 644221 for 30 minutes, followed by exposure to 1% oxygen for a further 18 hours, was performed. The results are summarized in FIG. 3.

FIG 1A and FIG. 2 demonstrate that relative to the amount of HIF-1α induced by hypoxia (1% oxygen) in a given amount of time, the amount of HIF-1α produced decreases to a greater extent with concomitant exposure to NSC 644221. In other words, a greater relative reduction in the amount of HIF-1α is seen the longer the cells are exposed to NSC 644221, an effect that can be useful for reducing the dose needed during a period of treatment.

FIG. 1B and FIG. 3 demonstrate that for a given period of exposure of the U251 cells to hypoxia, the amount of HIF-1α in the cells is reduced in proportion to the concentration of NSC 644221. In other words, as the concentration of NSC 644221 increases, the amount of hypoxia induced HIF-1α protein in the cells decreases. FIG. 1B and FIG. 3 also demonstrate that NSC 644221 does not affect the amount of HIF-1β in the cells, regardless of concentration. Thus, NSC 644221 exerts its anti-HIF activity by specifically inhibiting HIF-1α.

**Example 2 – NSC 644221 decreases hypoxic HIF-1 reporter luciferase activity with dose dependent activity**

HIF-1 inhibitory activity of NSC 644221 was also determined according to the methods described by Rapisarda et al., *Cancer Res.*, 64: 1475-1482, 2004, and Rapisarda et al., *Cancer Res.*, 62: 4316-4324, 2002, both of which are incorporated by reference herein. Briefly, genetically engineered U251 human glioma cells that stably express a recombinant vector in which the luciferase reporter gene is under control of three copies of a canonical hypoxia-responsive element (HRE) are used to determine
HIF-1 activity. These cells consistently express luciferase in a hypoxia- and HIF-dependent fashion, making it possible to detect either an increase or a decrease in HIF-1 activity by detecting the increase or decrease of luciferase activity in a sample following administration of a compound.

U251 (human glioma) cells that contained a stably transfected HRE reporter system and U251 cells containing a stably transfected pGL3 reporter system (constitutive reporter system used as a control) were grown overnight in 96-well plates in RPMI 1640 media supplemented with 5% heat inactivated fetal bovine serum (Whitakaker Bioproducts, Walkersville, MD) to which is added penicillin (50IU/mL, Invitrogen, Carlsbad, CA), streptomycin (50 μg/mL, Invitrogen, Carlsbad, CA) and glutamine (2 mM, Invitrogen, Carlsbad, CA). After the initial incubation period, a range of concentrations (0.5, 1, 2, 4, 5 and 10μM) of NSC 644221 was added to the cells 30 min prior to exposure to hypoxia or normoxia for a further 18 hours. Following treatment, the cells were lysed in situ using the Bright-Glo luciferase assay reagent (Promega, Madison, WI). Alterations in luciferase activity were measured using a Packard luminometer. Luciferase expression was then measured for normoxic and hypoxic treatments and any changes HRE luciferase activity due to NSC 64421 were normalized to total protein expression and calculated as a fold change in luciferase activity. The results are shown graphically in FIG. 4.

FIG. 4 demonstrates that significant reductions in the expression of luciferase under control of the HRE are observed at concentrations over 1.0 μM, and that the effect is observable even at concentrations as low as 0.5 μM. In other words, hypoxia-induced HIF-1α activity can be reduced at very low concentrations of NSC 644221.

FIG. 5 shows the effect of NSC 644221 on hypoxic signaling inhibition for both U251-HRE cells (upper left panel) and U251-PG3 cells (upper right panel), and the effect of NSC 644221 on cell growth in U251-HRE cells (lower left panel) and U251-PG3 (lower right panel). A comparison of the upper panels demonstrates that NSC 644221 potently inhibits hypoxic signaling (EC_{50} = 0.05 μM) in U251-HRE cells, and
has little effect on the constitutive reporter (EC$_{50}$ > 10 µM) in the U251-PG3 cells. The lower panels demonstrate that NSC 644221 has little effect on the growth of the U251 cells, both for U251-HRE cells (EC$_{50}$ > 10µM) and for U251-PG3 cells (EC$_{50}$ > 10µM). FIG. 5 further demonstrates the surprising result that NSC 644221 can significantly inhibit hypoxic signaling at non-cytotoxic concentrations. This is evidenced by the results for the pGL3 cells, which show that the inhibition of HIF-1 activity is not a generalized toxicity phenomenon, but specific to the cells containing the plasmid including the HRE. In other words, the compound is non-cytotoxic in this assay and is acting selectively on HRE.

Example 3 – NSC 644221 decreases hypoxic VEGF mRNA expression with dose dependent activity

U251 cells were grown overnight in 10cm dishes. After the initial incubation period, a range of concentrations (0.5, 1, 2, 4, 5, and 10µM) of NSC 644221 was added to the cells 30 min prior to exposure to hypoxia or normoxia for a further 18 hours. Total RNA was extracted and reverse transcribed prior to the real time amplification of the cDNA and subsequent measurement.

For real-time PCR, total RNA was extracted from the U251 cells using a Qiagen RNA extraction Kit (Qiagen, Valencia, CA). 1µg RNA was then reverse transcribed using a RT-PCR kit. The resulting cDNA was diluted 1:15 and real time PCR analysis was carried out using the human VEGF forward primer: forward 5’ TACCTCCACCAGCCACAGTG 3’, reverse 5’ ATGATTCTGCCCCTCCT CCTTC 3’ and a probe, sequence: probe 5’ FAM-TCCAGGGCTGCAACCCATGGC-TAMRA 3’ including an internal 18s rRNA control. Real time PCR analysis was measured in an ABI-Prism 7700 Sequence Detector. The results of the analysis are shown in FIG. 6, and show that as the concentration of NSC 644221 was increased from 0 µM to 10 µM, there was an approximate 2.5-fold decrease in the amount of hypoxic VEGF mRNA expression. Thus, NSC 644221 can be used to decrease VEGF mRNA expression at
non-cytotoxic concentrations (EC_{50} > 10 \mu M, see Example 3). These results demonstrate that inhibition of HIF-1 activity is followed by decreased transcriptional activity of the target sequence, in this case the sequence coding for VEGF, which is a pro-angiogenic sequence.

Example 4 – NSC 644221 increases HIF-1α degradation that is dependent upon proteosome activity.

U251 glioma cells were treated with either: A) The proteosome inhibitors ALLNL or PS341 (Sigma, St. Louis MO, and DTP-NCI, respectively) for 1 hour prior to the addition of either topotecan or NSC 644221, or B) NSC 644221 for 30 minutes and then DFO (desferroximine, used to mimic hypoxia, Sigma, St. Louis, MO) for a further 6 hours, followed by addition of cyclohexamide (CHX, a protein synthesis inhibitor, Sigma St. Louis, MO) for 30, 60 or 90 minutes, both in the presence or absence of NSC 644221. Total cellular proteins were separated using SDS-PAGE and immunoblot analysis was carried out using an HIF-1α antibody. The membrane-bound protein was reprobed for actin as a ‘loading’ control. The results of these analyses are shown in FIG. 7, in which figure the notation “MCG-1” is used for the compound NSC 644221.

As shown in panel A of FIG. 7, NSC 644221 does not inhibit HIF-1α expression if proteosome activity is blocked by either ALLNL or PS341, indicating that NSC 644221 is increasing the degradation of HIF-1α through the intact proteosome. By contrast, topotecan appears to affect HIF-1α synthesis because it is still effective for reducing HIF-1α activity in the presence of the proteosome inhibitor PS341. Thus, NSC 644221 appears to be acting via a mechanism distinct from that of topotecan.

As shown in panel B of FIG. 7, HIF-1α was stabilized using DFO, an effect that was reduced by NSC 644221. These results show that NSC 644221 also can counteract the increase in HIF-1α activity induced by iron-dependent oxygen depletion in the presence of DFO. The effect of NSC 644221 on HIF-1α accumulation also is
maintained in the presence of the protein synthesis inhibitor cyclohexamide (CHX), which stops de novo HIF-1α synthesis. This result demonstrates that NSC 644221 acts by degrading HIF-1α and is not affecting the transcription or translation (synthesis) of HIF-1α. In other words, the mode of action of NSC 644221 is dependent on the degradation but not the synthesis pathway of HIF-1α.

FIG. 8 presents another panel illustrating HIF-1α levels in the presence and absence of NSC 644221, ALLNL, and PS341. Consistent with the results summarized in FIG. 7, HIF-1α inhibition is observed when NSC 644221 is administered in the absence of the proteosome inhibitors ALLNL and PS341. When either proteosome inhibitor is co-administered, no inhibition of HIF-1α is observed.

Example 5 – NSC 644221 increases HIF-1α but not HIF-2α degradation in a VHL independent manner.

UMRC-2 cells (a renal clear cell carcinoma cell line overexpressing HIFs under normoxic conditions), both deficient and replete in VHL expression, were treated with NSC 644221 for 30 minutes, and then exposed to hypoxia for 6 hours and analyzed. Total cellular proteins were separated using SDS-PAGE and immunoblot analysis was carried out using a HIF-1α or HIF-2α antibody. The membrane-bound antibody was stripped and the protein was re-probed for actin as a ‘loading’ control. Both HIF-1α and HIF-2α are capable of dimerizing with HIF-1β to form the active transcription factor HIF-1. This example shows the differential effect of NSC-644221 on the degradation of HIF-1α and HIF-2α, and the VHL-independence of the degradation of HIF-1α induced by NSC 644221.

The results are shown in FIG. 9 (where the notation “MCG-1” is again used to denote the compound NSC 644221) and FIG. 10. As shown in panel A of FIG. 9, NSC 644221 does not affect degradation of HIF-2α in UMRC-2 cells under both normoxic and hypoxic conditions. However, NSC 644221 has been observed to slightly inhibit
HIF-2α in A549 cells. Panel B of FIG. 9 and FIG. 10 show that NSC 644221 increases degradation of HIF-1α regardless of whether or not the cells express VHL.

The VHL protein was thought to be an essential component of the cellular degradation pathway for HIF-1α and HIF-2α since VHL forms the recognition component of an E3 ligase that recognizes hydroxylated HIF-1 proteins. However, these results surprisingly demonstrate that NSC 644221 increases degradation of HIF-1α in a VHL-independent matter.

Example 6 – The inhibitory effect of NSC 644221 on VEGF mRNA expression is maintained in DU145 (Topo-1) mutant cells

DU 145 cells are prostate cancer cells that carry a mutation in the topoisomerase-1 (Top-1) binding site. The camptothecin analog topotecan (TPT), a potent HIF-1 inhibitor, exerts its action through topoisomerase-1 inhibition. In contrast, this Example demonstrates that NSC 644221 inhibits HIF-1 in a manner independent of Top-1.

DU145 cells were grown to a confluence of 80% in 10% RPMI medium as previously detailed, and the mRNA was harvested for real-time PCR analysis of VEGF mRNA, also as previously detailed. As shown in FIG. 11, there was a decrease in VEGF mRNA for both the Top-1 mutant and the wild type cells, demonstrating that the absence or presence of an intact Top-1 binding site does not affect the activity of NSC 644221. This result shows that NSC 644221 does not work through a Top-1 dependent mechanism like TPT.
Example 7 – Comparison of NSC 644221 to NSC 295504 and NSC 295505

The compounds NSC 644221, NSC 295504, and NSC 295505 were separately used to treat U251 cells and an immunoblot analysis as described in Example 1 was used to determine their relative abilities to reduce HIF-1α protein. The results are shown in FIG. 12. At the concentrations specified in FIG. 12, all of these compounds reduced the amount of HIF-1α in the cells. Again, surprisingly, these results were obtained at concentrations that were not significantly cytotoxic over the course of the experiment (6 hours).

Compounds NSC 644221 and NSC 299504 are further compared in FIGS. 13 and 14, which respectively show the results of analyzing these compounds in the assays of Examples 2 and 3. FIG. 13 shows that like NSC 644221, NSC 295504 inhibits expression of luciferase under the control of the HRE, and that it does so at non-cytotoxic concentrations. FIG. 14 shows that like NSC 644221, NSC 295504 reduces VEGF mRNA expression in U251 cells.

Example 8 – Pharmaceutical compositions

Since the disclosed tricyclic compounds can be used to treat a proliferative condition such as cancer, another aspect pertains to a pharmaceutical composition that includes a disclosed compound and a pharmaceutically acceptable excipient. The compounds can be mixed with one or more non-toxic pharmaceutically acceptable excipients, including carriers, diluents, and/or adjuvants, and optionally other biologically active ingredients. Standard pharmaceutical formulation techniques are used, such as those disclosed in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA (19th Edition), which is incorporated by reference herein.

A pharmaceutical composition according to the disclosure includes one or more of the disclosed compounds, and can also include, for example, one or more other biologically active ingredients, such as other anti-angiogenic (such as thalidomide derivatives), HIF-1 activity inhibitors (such as geldanamycin), or cytotoxic agents. The
dosage of the combined biologically active agents is sufficient to achieve tissue
concentrations at the site of action that are similar to those that are shown to inhibit
HIF-1 activity in vivo.

The compositions can be in the form of tablets, capsules, powders, granules,
lozenges, liquid, or gel preparations, such as oral, topical, or sterile parenteral solutions
or suspensions (e.g., eye or ear drops, throat or nasal sprays, etc.), ointments, creams,
transdermal patches, and other forms known in the art.

Such pharmaceutical compositions can be administered systemically or locally
in any manner appropriate to the treatment of a given condition, including orally,
parenterally, rectally, nasally, buccally, vaginally, topically, optically, by inhalation
spray, or via an implanted reservoir. The term "parenterally" as used herein includes,
but is not limited to subcutaneous, intravenous, intramuscular, intrasternal,
intrasynovial, intrathecal, intrahepatic, intralesional, and intracranial administration, for
example, by injection or infusion. For treatment of proliferative diseases in the central
nervous system, the pharmaceutical compositions preferably readily penetrate the
blood-brain barrier when peripherally administered or are administered
intraventricularly.

Pharmaceutically acceptable carriers include, but are not limited to, ion
exchangers, alumina, aluminum stearate, lecithin, serum proteins (such as human serum
albumin), buffers (such as phosphates), glycine, sorbic acid, potassium sorbate, partial
glyceride mixtures of saturated vegetable fatty acids, water, DMSO, ethanol/water
solutions, salts or electrolytes such as protamine sulfate, disodium hydrogen phosphate,
potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium
trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol,
sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-
block polymers, polyethylene glycol, and wool fat.

Tablets and capsules for oral administration can be in a form suitable for unit
dose presentation and can contain conventional pharmaceutically acceptable excipients.
Examples of these include binding agents such as syrup, acacia, gelatin, sorbitol, tragacanth, and polyvinylpyrrolidone; fillers such as lactose, sugar, corn starch, calcium phosphate, sorbitol, or glycine; tableting lubricants, such as magnesium stearate, talc, polyethylene glycol, or silica; disintegrants, such as potato starch; and dispersing or wetting agents, such as sodium lauryl sulfate. Oral liquid preparations can be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or can be presented as a dry product for reconstitution with water or other suitable vehicle before use.

The pharmaceutical compositions can also be administered parenterally in a sterile aqueous or oleaginous medium. The composition can be dissolved or suspended in a non-toxic parenterally-acceptable diluent or solvent, e.g., as a solution in 1,3-butanediol. Commonly used vehicles and solvents include water, physiological saline, Hank's solution, Ringer's solution, and sterile, fixed oils, including synthetic mono- or di-glycerides, etc.

The dosage unit involved depends, for example, on the condition treated, nature of the formulation, nature of the condition, embodiment of the claimed pharmaceutical compositions, mode of administration, and condition and weight of the patient. Dosage levels are typically sufficient to achieve a tissue concentration at the site of action that is at least the same as a concentration that has been shown to be active in vitro, in vivo, or in tissue culture. For example, a dosage of about 0.1 to about 1000 mg/kg per day of the active ingredient may be useful in the treatment of the condition.

The compounds can also be used in the form of pharmaceutically acceptable salts derived from inorganic or organic acids and bases, including, but not limited to: acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate,
nicotinate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, picate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, and undecanoate. Base salts include, but are not limited to, ammonium salts, alkali metal salts (such as sodium and potassium salts), alkaline earth metal salts (such as calcium and magnesium salts), salts with organic bases (such as dicyclohexylamine salts), N-methyl-D-glucamine, and salts with amino acids (such as arginine, lysine, etc.). Basic nitrogen-containing groups can be quaternized, for example, with such agents as C1-8 alkyl halides (such as methyl, ethyl, propyl, and butyl chlorides, bromides, and iodides), dialkyl sulfates (such as dimethyl, diethyl, dibutyl, and diamyl sulfates), long-chain halides (such as decyl, lauryl, myristyl, and stearyl chlorides, bromides, and iodides), aralkyl halides (such as benzyl and phenethyl bromides), etc. Water or oil-soluble or dispersible products are produced thereby. Furthermore, isomers, such as stereoisomers and geometric isomers of the compounds are contemplated, as are prodrugs of such compounds, which prodrugs may themselves exhibit little or no intrinsic activity.

Example 9—Inhibition of angiogenesis by tricyclic compounds

The angiogenesis modulating activity of compounds is assessed in a rat aortic ring microvessel growth assay. Briefly, twelve-well tissue culture plates are coated with 250 μl of Matrigel (Becton-Dickinson, Bedford, MA) and allowed to gel for 30 min at 37°C and 5% CO2. Thoracic aortas are excised from 8- to 10-week-old male Sprague Dawley rats. After careful removal of fibroadipose tissues, the aortas are cut into 1-mm-long cross-sections, placed on Matrigel-coated wells, and covered with an additional 250 μl of Matrigel. After the second layer of Matrigel sets, the rings are covered with EGM-II and incubated overnight at 37°C and 5% CO2. EGM-II consists of endothelial cell basal medium (EBM-II; Clonetics, San Diego, CA) plus endothelial cell growth factors provided as the EGM-II Bulletkit (Clonetics). The culture medium is subsequently changed to EBM-II supplemented with 2% fetal bovine serum, 0.25 μg/ml amphotericin B, and 10 μg/ml gentamicin. Aortic rings are then treated daily with either the vehicle (0.5%
DMSO), carboxyamidotriazole (CAI, 12 μg/ml), or a tricyclic compound (for example, at one or more concentrations between 0.1-20 μg/ml) for 4 days and photographed on the 5th day using a x2.5 objective. CAI, a known antiangiogenic agent, is used at higher than clinically achievable concentrations as a positive control. Experiments are repeated four times using aortas from four different rats. The area of angiogenic sprouting, reported in square pixels, is quantified using Adobe PhotoShop. Further details of the method are provided in Luzzio et al., J. Med. Chem.; 46:3793-9, 2003.

Example 10 – Cytotoxicity assays

Any type of assay designed to measure the relative toxicity of compounds at various concentrations may be used to determine a non-cytotoxic amount of a compound. However, specific examples of such assays include the SRB (sulforhodamine B) assay, the Trypan Blue Dye Exclusion Assay, Differential Staining with Fluorescein Diacetate and Ethidium Bromide/Propidium iodide, and the Neutral Red cytotoxicity assay. Typically, cytotoxicity is measured for the type of cells that are to be treated with a particular compound, for example, measured in cultured tumor cells of the type that are to be treated using a particular compound.

In the SRB assay, cultured cells that are fixed with trichloroacetic acid are stained for 30 min with 0.4% sulforhodamine B (SRB) dissolved in 1% acetic acid. Unbound dye is removed by four washes with 1% acetic acid, and protein-bound dye is extracted with 10 mM Tris base (pH10.5) for 5 min. The OD value is then read at 490 nm in a microplate reader. The value is a reflection of cellular protein content and provides a sensitive measure of drug-induced cytotoxicity. Further details of this method are described in Skehan et al., JNCI, 82: 1107-1112, 1990.

In the Trypan Blue Dye Exclusion Assay, which is based on the exclusion of trypan blue dye (Sigma, St. Louis, MO) from viable cells. Cells are incubated with trypan blue (final concentration, 0.2 mg/mL) for 1 minute, and then observed under a microscope. Stained (non-viable) and non-stained (viable) cells are counted separately.
and the viable cell ratio can be calculated. Dead cells take up the dye and are stained blue, whereas viable cells have yellow nuclei. This assay can be repeated at various concentrations to determine an effective concentration (EC) for a certain percentage of the cells to be rendered non-viable by the compound being tested, for example, an EC_{50} for cytotoxicity. Alternatively, a percentage viability can be calculated as (number of unstained cells multiplied by 100)/Total Number of Cells Counted. A non-cytotoxic concentration of a compound tested using the trypan blue dye exclusion test can be a concentration that results in a percentage viability of greater than 90%, for example, greater than 95%, or greater than 98%.

Differential Staining with Fluorescein Diacetate and Ethidium Bromide/Propidium Iodide measures cytotoxicity by detecting whether a particular concentration of a compound compromises cell membrane function. This assay is based on the differential staining of cells with fluorescein-diacetate (FDA), propidium-iodide (PI), and Hoechst 33342. The cytoplasm of cells with intact plasma membranes is stained green, and late stage apoptotic/ necrotic cells are stained red. The cells in early stages of apoptosis that still have an intact plasma membrane exhibit an apoptotic nuclear morphology stained blue with Hoechst dye, while the cytoplasm is stained green. This method offers a rapid and sensitive method to determine the percentage of apoptotic cells. In this method, a stock solution of 25 mL or 1 mg/mL propidium iodide solution in distilled water, 50 mL of 1.5mg/mL fluorescein diacetate in DMSO, 10 mL of 1 mg/ML Hoechst 33342 solution in distilled water, and 15 mL of phosphate buffered saline pH 8.0 is prepared. The stock solution is diluted 50 times in cell suspension (in which the cells have been exposed to a particular compound at a particular concentration) and scored under a fluorescent microscope.

The neutral red (NR) cytotoxicity assay is based on the ability of viable cells to incorporate and bind neutral red, a supravital dye. NR is a weak cationic dye that readily penetrates cell membranes and accumulates in lysosomes, where it binds with anionic sites in the lysosomal matrix. Alterations of the cell surface and/or the sensitive
lyosomal membrane brought about by the action of a cytotoxin present at a cytotoxic concentration result in a decreased uptake and binding of NR. Thus, it is possible to distinguish between viable and dead cells. Cells are maintained in culture and exposed to test compounds over a broad range of concentrations, and the cultures are visually examined after 24 hours and the highest tolerated dose (HTD) is determined as the concentration of a compound that is tolerated by the cells, at which only minimal morphological alterations are evident. An HTD value has been shown to be equivalent to an NR90 value, i.e. the concentration of toxicant that causes a 10% reduction in the uptake of NR when compared to the controls. In some embodiments, a non-cytotoxic concentration can be an HTD value or an NR90 value determined by this method. Additional details regarding the NR assay may be found in Babich and Borenfreund, “Neutral Red assay for toxicology in vitro,” in *In Vitro Methods of Toxicology*, Watson, R.R., ed, Chapter 17, pp 237-251. CRC Press, Ann Arbor, 1992.

Additional assays for cytotoxicity are available from Promega (Madison, WI).

Such cytotoxicity assays are described, for example, in U.S. Patent Nos. 5,158,450, 4,557,862, and 4,640,893, and in Promega *Cell Notes*, Issue 3, 2002, which describes the use of several assays in combination to differentiate cell death due to cytotoxicity, apoptosis, and necrosis.

It was found that some of the disclosed compounds damage DNA.

Phosphorylation of histone 2A.X is a known response to DNA damage. FIG. 15 is an image of an immunoblot analysis of cells exposed to NSC 644221, NSC 24058, NSC 24568, NSC 24623, NSC 24459, NSC 24544, or NSC 24460 (the structures of which are shown in FIG. 16). The gel was probed for histone 2A.X phosphorylation using an anti-phospho-histone-2A.X antibody (such as those available from Upstate, Charlottesville, VA) and for HIF-1α using an anti-HIF-1α antibody, as previously described.

As shown in FIG. 15, all of the tested compounds caused DNA damage, as indicated by the presence of phosphorylated histone 2A.X. However, the DNA damage
caused by a compound does not appear to be related to its HIF-1α inhibiting properties. For example, while NSC 644221 exhibits both HIF-1α inhibition and DNA damage, NSC 24058 exhibits DNA damage but no HIF-1α inhibition. While DNA damage may contribute to HIF-1α inhibition, according to the methods and results of this Example, it does not appear to be sufficient by itself to inhibit HIF-1α or indicate whether a particular compound will inhibit HIF-1α. As shown in FIG. 17, NSC 644221 binds to DNA in a dose dependent manner, as indicated by the decrease in OD$_{260}$.

Overall toxicity of NSC 644221 was assessed using a trypan blue assay, as previously described, by treating U251 and PC-3 cells with various concentrations of NSC 644221 (0 μM, 1 μM, and 5 μM) and staining the sample with trypan blue. As shown in FIG. 18 (and the additional data presented in Table 4, below), compared to controls, NSC 644221 did not generally increase the amount of dead cells observed. However, the total cell populations of samples treated with NSC 644221 were generally lower than controls. Accordingly, it appears that NSC 644221 has more of a cytostatic effect than a cytotoxic effect on U251 and PC-3 cells.

### Table 4

<table>
<thead>
<tr>
<th></th>
<th>Live cells %</th>
<th>Dead Cells %</th>
<th>Total Cells (million)</th>
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</thead>
<tbody>
<tr>
<td>U251 Control</td>
<td>93.333333</td>
<td>6.666666667</td>
<td>2.233333333</td>
</tr>
<tr>
<td>U251 + 1uM 644221</td>
<td>87.2093023</td>
<td>12.79069767</td>
<td>0.666666667</td>
</tr>
<tr>
<td>U251 + 5uM 644221</td>
<td>90</td>
<td>10</td>
<td>0.933333333</td>
</tr>
<tr>
<td>PC-3 Control</td>
<td>98.245614</td>
<td>1.785714286</td>
<td>1.7</td>
</tr>
<tr>
<td>PC-3 + 1 uM 644221</td>
<td>98.1481481</td>
<td>1.851851852</td>
<td>1.233333333</td>
</tr>
<tr>
<td>PC-3 + 5 uM 644221</td>
<td>98.1481481</td>
<td>1.851851852</td>
<td>1.233333333</td>
</tr>
</tbody>
</table>

**Example 11—Absence of hsp90 inhibition by tricyclic compounds**

In order to further elucidate the mechanism by which NSC 644221 inhibits HIF-1α, NSC 644221 was tested for inhibition of hsp90. HIF-1α is known to associate with
hsp90, and hsp90 inhibition generally results in up regulation of hsp70. Accordingly, increased hsp70 levels (and of its cognate form, hsc70), can indicate hsp90 inhibition.

In order to determine whether NSC 644221 inhibits hsp90, hsp70/hsc70 upregulation was studied in untreated control cells and cells treated with NSC 644221 (5 μM for 8 and 18 hours, and 1 μM for 24 hours) or 17-(Allylamino)-17-demethoxygeldanamycin ("17AAG", a geldamycin analog and positive control which binds to hsp90 and therefore upregulates hsp70, at 5 μM and 50 μM for 18 hours). As shown in FIG. 19, none of the NSC 64421 treated cells exhibited increased hsp70/hsc70 levels compared to the negative (untreated) or positive (17AAG treated) controls.

Accordingly, hsp90 does not appear to be involved in the inhibition of HIF-1α by NSC 644221.

AKT (also known as protein kinase B) is believed to enhance the expression of heat shock proteins, and thus stabilize HIF-1α, through a pathway involving phosphatidylinositol 3-kinase (P13K). Inhibition of the (P13K)/Akt regulated pathway is believed to inhibit HIF-1α translation. See Zhou et al., *J. Bio. Chem.*, 279(14): 13506-13513, 2004.

Total AKT was measured for untreated controls, positive controls (treated with 1 μM 17AAG), and cells treated with 1 μM NSC 644221 for U251, C2, and HCT116 cells. As shown in FIG. 20, for all cell lines, NSC 644221 treated cells showed similar AKT concentration to untreated controls. As expected, the positive controls (treated with 17AAG) exhibited lower levels of AKT, likely due to the interaction of 17AAG with hsp90 (thus decreasing AKT expression).

AKT inhibition can also be probed by measuring phosphorylation at Ser-473. FIG. 21 compares untreated controls with cells treated with 5 μM of NSC 644221 under conditions of normoxia and after exposure to 1, 2, 3, and 4 hours of hypoxia. Phosphorylation was measured using an anti-phospho-Akt1/PKBα (Ser473) antibody (such as those available from Upstate of Charlottesville, VA). Actin was used as a loading control. At all times, the phospho-AKT level of the NSC 644221 treated cells
was similar to the untreated controls. Accordingly, NSC 644221 does not appear to inhibit HIF-1α by a mechanism involving the AKT pathway.

Example 12—Absence of microtubule disruption by tricyclic compounds

Microtubules are cellular proteins that form part of the cytoskeleton and assist in transporting substances within the cell, such as vesicles, granules, organelles (e.g., mitochondria), and chromosomes. It has been observed that certain anti-tumor agents operate by disrupting the microtubules.

In order to test whether NSC 644221 inhibits HIF-1α via microtubule disruption, U251 cells were exposed to 1 μM NSC 644221 for 18 hours and compared to U251 cells exposed to 100 μM colchicine (a positive control), 100 μM jasplakinolide ("jas," a filament stabilizer, a negative control), and untreated U251 control cells. As shown in FIG. 22, the U251 cells exposed to NSC 644221 were not mitotic, and appeared very similar to the untreated U251 control cells and the jasplakinolide treated (negative control) cells. As expected, the cells treated with colchicine (positive controls) contained mitotic cells. Accordingly, it does not appear that NSC 644221 inhibits HIF-1α by microtubule disruption.

Example 13—G2 cell arrest by tricyclic compounds

The G2 checkpoint helps regulate cell growth and death, such as by determining whether a cell undergoes mitosis or apoptosis. The effect of NSC 644221 on the G2 pathway was studied in order to determine whether the method of HIF-1α inhibition by NSC 644221 occurs by an alteration to the G2 pathway.

PC-3 cells, which do not exhibit HIF-1α inhibition in response to NSC 644221, and U251 cells, which do exhibit HIF-1α inhibition when treated with NSC 644221, were treated with 1μM NSC 644221 for 24 hours and compared to untreated controls. Cell cycles were analyzed by flow cytometry measuring DNA content. As shown in FIG. 23, compared to controls, both cell lines exhibit a characteristic G2 arrest after
treatment with NSC 644221, as indicated by the increased number of cells in the G2/M phase. Accordingly, since both HIF-α active and HIF-α inactive cells experienced G2 arrest, this test does not indicate that the HIF-1α inhibiting properties of NSC 644221 occur via alteration of the G2 pathway. However, the observed G2 arrest in NSC 644221 treated cells may be related to the DNA damaging properties of NSC 644221.

Example 14–Absence of histone deacetylation inhibition by tricyclic compounds

Histone deacetylases (HDACs) are known to play a role in hypoxia-induced angiogenesis (see Kim et al., Nat. Med., 7(4): 437-444, 2001). In order to determine if the HIF-1α inhibiting properties of NSC 644221 result from HDAC inhibition, the acetylation properties of NSC 644221 were compared to those of trichostatin A (TSA, a known HDAC inhibitor). Untreated controls were compared to cells treated with either 0.5 μM TSA, 1 μM NSC 644221, or 5 μM NSC 644221 for 24 hours and then subjected to an immunoblot analysis using appropriate antibodies. As shown in FIG. 24, levels of acetylated histone and acetylated tubulin were similar between the untreated controls and the cells treated with NSC 644221. As expected, cells treated with TSA (positive controls) showed increased levels of acetylated histone and acetylated tubulin. Accordingly, it does not appear that NSC 644221 functions as an HDAC inhibitor.

Example 15–Effect of tricyclic compounds on the AhR pathway

HIF-1α dimerizes with the aryl hydrocarbon receptor ("AhR") nuclear translocator (ARNT) to initiate transcription. In order to test whether HIF-1α inhibition by NSC 644221 involves the AhR pathway, MCF-7 cells were treated with various concentrations (0 μM, 0.1 μM, 0.2 μM, 0.5 μM, and 1 μM) of NSC 644221 or 2,3,7,8-tetrachlorodibenzo-p-Dioxin (TCDD, which is known to induce binding of ARNT to the ligand bound AhR, leading to activation of the CYP1A1 gene and increased luciferase expression). As shown in FIG. 25, little increase in luciferase activity was seen in MCF-7 cells exposed to NSC 644221. As expected, increased luciferase activity was
seen as increasing amounts of the positive control TCDD were added. Accordingly, it
does not appear that NSC 644221 inhibits HIF-1\(\alpha\) by the AhR pathway in a manner
analogous to TCDD.

Example 16—Effect of tricyclic compounds on p21 mRNA expression

The p53 gene is a component of cellular response to DNA damage and helps
regulate the cell growth and death cycle. The p21 gene is regulated by p53 and
mediates G1 arrest following DNA damage. Overexpression of p53 may lead to
increased HIF-1\(\alpha\) levels.

The effect of NSC 644221 on p21 mRNA expression was studied by treating
U251 cells with various combinations of NSC 644221 and actinomycin D (“Act D,” a
RNA synthesis inhibitor) under both normoxic and hypoxic conditions. As shown in
FIG. 26, p21 mRNA expression in U251 cells is enhanced by treatment with NSC
644221 alone under both normal and hypoxic oxygen conditions. However, cells
treated with Act D, with or without co-treatment with NSC 644221, show comparable
or reduced p21 mRNA expression compared to controls. Accordingly, it appears that
NSC 644221 increases the synthesis of p21, rather than stabilizing existing p21.

The effect of NSC 644221 on de novo p21 synthesis was studied by comparing
p21 expression of U251 cells having an inactive p53 gene to HCT116 cells having a
wild type p53 gene. Actin was used as a control to detect differences in protein loading.
As shown in FIG. 27, compared to controls, NSC 644221 increased p21 production in
cells with and without a p53 gene. Accordingly, the p21 expression enhancing
properties of NSC 644221 do not appear to be related to the p53 gene.

The HIF-1\(\alpha\) inhibiting properties of NSC 644221 do not appear to correlate to
the p53 status of a particular cell line. For example, FIG. 28 presents a chart
summarizing the HIF-1\(\alpha\) activity of NSC 644221 toward a variety of cell lines having
various types of p53 genes. Examples of cells having inactive, mutant, or wild-type p53
genes can be found which exhibit HIF-1\(\alpha\) inhibition and examples can be found which
lack HIF-1α inhibition in response to NSC 644221, providing further evidence that the mechanism of HIF-1α inhibition does not involve the p53 gene.

**Example 17—Effect of Tricyclic Compounds on HIF-1α mRNA Expression**

The effect of NSC 644221 on HIF-1α mRNA expression under normoxic conditions was also studied. As shown in FIG. 29, NSC 644221, even at higher concentrations, did not decrease HIF-1α mRNA expression. Accordingly, it does not appear that NSC 644221 inhibits HIF-1α by interfering with HIF-1α mRNA expression.

It should be understood that the foregoing relates only to particular embodiments and that numerous modifications or alterations may be made without departing from the true scope and spirit of the invention as defined by the following claims.
We claim:

1. A method for inhibiting HIF-1α activity in a subject in need of such
treatment, comprising administering a non-cytotoxic, therapeutically effective amount
of a tricyclic compound having the formula:

\[
\begin{array}{c}
\text{R}_2 \\
\text{X} \\
\text{Y} \\
\text{R}_3 \\
\text{R}_1
\end{array}
\]

wherein X and Y are independently O, S, N, NR₄, CR₃, or CR₃R₂ and all valency
requirements are satisfied; R₁ is one or more substituents independently selected from
the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aroyl, aza,
carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyle,
sulfonamido and sulfato, at least one of which is carboxamide or diamine; R₂ and R₃ are
either joined to form a six-membered aromatic ring, which can optionally be further
substituted with one or more substituents independently selected from the group
consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aroyl, aza, carboxy,
carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyle, sulfonamido and
sulfato, or one of R₂ and R₃ is an aryl group, which can optionally be further substituted
with one or more substituents independently selected from the group consisting of acyl,
acyloxy, alkoxy, alkyl, alkylthio, amino, aroyl, aza, carboxy, carboxamide, diamine,
halogen, hydroxyl, mercapto, nitro, sulfonyle, sulfonamido and sulfato, and the other is
hydrogen or a substituent selected from the group consisting of acyl, acyloxy, alkoxy,
alcohol, alkylthio, amino, aroyl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl,
mercapto, nitro, sulfonyle, sulfonamido and sulfato; and R₄, R₅, R₆ and R₇ are
independently H or a substituent selected from the group consisting of acyl, acyloxy,
alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato; or having the formula:

wherein \( R_8 \) is one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxyl, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato, at least one of which is a carboxamide group or a diamine group; Ar is an aryl group, which can be further substituted with one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxyl, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato; \( W \) and \( Z \) are independently \( NR_9 \) or \( =N- \), but not the same; and \( R_9 \) is hydrogen or a substituent selected from the group consisting of acyl, acyloxy, alkoxyl, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato.

2. The method of claim 1, wherein the non-cytotoxic dose achieves a tissue concentration that is less than the IC\(_{50}\) for cytotoxicity toward cultured cells from the subject’s tumor.

3. The method of claim 1, wherein the compound increases degradation of HIF-1\( \alpha \) and has substantially no effect on degradation of HIF-2\( \alpha \).

4. The method of claim 1, wherein the compound increases degradation of HIF-1\( \alpha \) in a VHL-independent manner.
5. The method of claim 1, wherein the compound has substantially no effect on the level of HIF-1α mRNA that is expressed in the subject.

6. The method of claim 1, wherein the compound inhibits HIF-1α activity through a Topoisomerase-1-independent mechanism.

7. The method of claim 1, wherein the compound decreases VEGF mRNA expression.

8. The method of claim 1, wherein the compound inhibits HIF-1α activity through a proteosome dependent mechanism.

9. The method of claim 1, wherein the compound induces G2/M arrest.

10. The method of claim 1, wherein the compound inhibits HIF-1α activity by a mechanism which does not involve microtubule disruption.

11. The method of claim 1, wherein the compound has substantially no inhibiting effect on hsp90.

12. The method of claim 1, wherein the compound inhibits HIF-1α activity through a mechanism independent of the AKT pathway.

13. The method of claim 1, wherein the compound has substantially no inhibiting effect on deacetylation enzymes.
14. The method of claim 1, wherein the compound inhibits HIF-1α activity through a p53-independent mechanism.

15. The method of claim 1, wherein the compound increases p21 mRNA expression.

16. The method of claim 1 further comprising selecting the compound to increase degradation of HIF-1α and have substantially no effect on degradation of HIF-2α, to increase degradation of HIF-1α in a VHL-independent manner, to have substantially no effect on the level of HIF-1α mRNA that is expressed in the subject, and/or to inhibit HIF-1α activity through a Topoisomerase-1-independent mechanism.

17. The method of claim 1, wherein the least one R₁ group that is carboxamide has the formula CONR'(CH₂)ₙNR''R'''', wherein n=1-5 and R', R'', and R''' are independently H or alkyl.

18. The method of claim 1, wherein the tricyclic compound has a structure:

![Tricyclic Compound Structure]

wherein X and Y are independently O, S, or N and all valency requirements are satisfied; R₁₀ is one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alklythio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato, at least one of which is carboxamide, R₁₁ is H or one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl,
alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato.

19. The method of claim 18, wherein $R_{12}$ and $R_{13}$ are independently H or $C_1$ - $C_5$ alkyl, and $R_{12}$ and $R_{13}$ are both H.

20. The method of claim 1, wherein the compound has a structure:

wherein $T$ is CR$_{16}$ or NR$_{17}$; V is O, S, or N and all valency requirements are satisfied; $R_{14}$ and $R_{15}$ are each independently H or one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato; $R_{16}$ is diamine; and $R_{17}$ is amino-substituted alkyl.

21. The method of claim 20, wherein $T$ is NR$_{17}$, and V is O or S.

22. The method of claim 21, wherein $R_{17}$ is 2-($N,N$'-dimethyl)amino-1-methylethyl or 3-($N$-isopropyl)aminopropyl.

23. The method of claim 20, wherein $T$ is CR$_{16}$ and V is N or O.

24. The method of claim 23, wherein V is O.
25. The method of claim 23, wherein the compound is:

\[
\begin{align*}
\text{N} & \quad \text{N} \\
\text{NO}_2 & \quad \text{NO}_2 \\
\text{H} & \quad \text{H} \\
\end{align*}
\]

or

\[
\begin{align*}
\text{N} & \quad \text{N} \\
\text{NO}_2 & \quad \text{NO}_2 \\
\text{H} & \quad \text{H} \\
\end{align*}
\]

26. The method of claim 18, wherein the compound has the formula:

\[
\text{R}_{18} \quad \text{R}_{19}
\]

wherein \( R_{18} \) is one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato, at least one of which is carboxamide, and \( R_{19} \) is hydrogen or one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato.
27. The method of claim 26, wherein the at least one R₁₈ that is carboxamide has the formula \(-\text{CONR'}(\text{CH}_2)_n\text{NR''R'''}\), wherein \(n=1-5\), and R', R'', and R'''' are independently H or lower alkyl.

28. The method of claim 27, wherein the compound comprises the 3-nitro, 6-chloro, 6-methyl, 6-methoxy, 6-aza, 7-chloro, 8-chloro, 7-bromo, 7-methyl, 8-methyl, 7-nitro, 8-bromo, 8-nitro, 9-chloro, 9-bromo, 9-methyl, 9-methoxy, 9-aza, 9-nitro, 9-[\text{CONH(\text{CH}_2)_2\text{NMe}_2}], or 7,8-dichloro derivative of:
29. The method of claim 28, wherein the compound is:

![Chemical structures](image1)

30. The method of claim 18, wherein the compound has the formula:

![Chemical structures](image2)
wherein $R_{20}$ and $R_{22}$ each are independently one or more substituents that are independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato, at least one of which is carboxamide, and $R_{31}$ and $R_{23}$ each are independently hydrogen or one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato.

31. The method of claim 30, wherein the compound is:

![Chemical Structure 1](image1)

or

![Chemical Structure 2](image2)

32. The method of claim 18, wherein the compound has the formula:

![Chemical Structure 3](image3)

wherein $R_{24}$ is one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato, at least one of which is carboxamide, and $R_{25}$ is hydrogen or one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy,
alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato.

33. The method of claim 32, wherein the compound is the 2-methoxy, 2-chloro, 3-methyl, 3-methoxy, 3-chloro, 4-methyl, 4-methoxy, 6-methyl, 6-methoxy, 6-chloro, 7-methyl, 7-methoxy, 7-chloro, 8-methyl, 8-methoxy, 8-chloro, 9-methyl, 9-methoxy or 9-chloro derivative of:
The method of claim 1, wherein the compound has the formula:

![Chemical Structure](image-url)

wherein R_{26} and R_{29} each are independently one or more substituents that are independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato, at least one of which is carboxamide, and R_{27}, R_{28}, R_{30} and R_{31} each are independently hydrogen or one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato.

The method of claim 34, wherein the compound is the 2'-aza, 2'-chloro, 3'-aza, 3'-methoxy, 4'-aza, 4'-fluoro, 4'-chloro, 4'-bromo, 4'-iodo, 4'-methoxy, 4'-hydroxyl, 4'-phenyl, 4'-nitro, 4'-trifluoromethyl, 4'-SO_{2}CH_{3}, 4'-amino, 4'-NHCOC\textsubscript{H}_{3}, 4'-NHSO_{2}CH_{3} or 3',4'-benz derivative of:
wherein \( n = 2, 3, 4, \) or 5.

36. The method of claim 1, wherein the compound has the formula:

wherein \( R_{32} \) is hydrogen or a substituent selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato; \( R_{33} \) is one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato, at least one of which is carboxamide; and \( R_{34} \) is hydrogen or one or more substituents independently selected from the group consisting of halogen, alkyl, alkoxy, alkylthio, acyl, acyloxy, aroyl, hydroxyl, mercapto, carboxy, amino, alkylamino, dialkylamino, nitro and sulfato.
37. The method of claim 1, wherein the compound has the formula:

wherein $R_{35}$ is one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato, at least one of which is carboxamide, $R_{36}$ is hydrogen or lower alkyl, and $R_{37}$ is hydrogen or one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato.

38. The method of claim 1, wherein the compound has the formula:

wherein $R_{38}$ is one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato, at least one of which is carboxamide; $Ar$ is an aryl group optionally substituted with one or more substituents independently selected from the group consisting of acyl,
acyloxy, alk oxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato; W and Z are independently NR₇ or =N—, but not the same; and R₇ is hydrogen or alkyl.

39. The method of claim 38, wherein Ar comprises phenyl, 2-furyl, 2-thienyl, 3-thienyl or 2-pyrrolyl.

40. The method of claim 38, wherein the compound is the 2'-methyl; 2'-methoxy; 2'-chloro; 2'-aza; 3'-methyl; 3'-methoxy; 3'-chloro; 3'-aza; 4'-methyl; 4'-methoxy; 4'-chloro; 4'-aza; 4'-phenyl; 4'-NHAe; 4'-N(CH₃)₂; 5-methyl; 6-methyl; 7-methyl; 2',3'-benz; 3',4'-benz; 2,3',4'-trioxy; or 2',3',4'-trimethoxy derivative of:

![Chemical Structure](image)

41. The method of claim 1, wherein R₁ is one or more substituents selected from the group consisting of alkoxy, alk oxy, alkyl, amino, aryl, aza, carboxy, carboxamide, halogen, hydroxyl, nitro, alkylsulfonyl, alkylsulfonamido and sulfato, at least one of which is carboxamide; R₂ and R₃ are either joined to form a six-membered aromatic ring, which can optionally be further substituted with one or more substituents independently selected from the group consisting of alkoxy, alkyl, amino, aryl, aza, carboxy, carboxamide, halogen, hydroxyl, nitro, alkylsulfonyl, alkylsulfonamido and sulfato, or one of R₂ and R₃ is an aryl group, which can optionally be further substituted
with one or more substituents independently selected from the group consisting of alkoxy, alkyl, amino, aryl, aza, carboxy, carboxamide, halogen, hydroxyl, nitro, alkylsulfonyl, alkylsulfonamido and sulfato, and the other is hydrogen or a substituent selected from the group consisting of alkoxy, alkyl, amino, aryl, aza, carboxy, carboxamide, halogen, hydroxyl, nitro, alkylsulfonyl, alkylsulfonamido and sulfato; $R_4$, $R_5$, $R_6$ and $R_7$ are independently $H$ or a substituent selected from the group consisting of alkoxy, alkyl, amino, aryl, aza, carboxy, carboxamide, halogen, hydroxyl, nitro, alkylsulfonyl, alkylsulfonamido and sulfato; $R_8$ is one or more substituents independently selected from the group consisting of alkoxy, alkyl, amino, aryl, aza, carboxy, carboxamide, halogen, hydroxyl, nitro, alkylsulfonyl, alkylsulfonamido and sulfato, at least one of which is a carboxamide group; Ar is an aryl group, which can be further substituted with one or more substituents independently selected from the group consisting of alkoxy, alkyl, amino, aryl, aza, carboxy, carboxamide, halogen, hydroxyl, nitro, alkylsulfonyl, alkylsulfonamido and sulfato; and $R_9$ is hydrogen or a substituent selected from the group consisting of alkoxy, alkyl, amino, aryl, aza, carboxy, carboxamide, halogen, hydroxyl, nitro, alkylsulfonyl, alkylsulfonamido and sulfato.

42. The method of claim 1, wherein $R_1$ is one or more substituents selected from the group consisting of alkoxy, alkyl, $\text{-NH}_2$, alkylamino, aryl, aza, carboxy, alkylacylamino, alkylaminoacetyl, halogen, hydroxyl, nitro, lower alkylsulfonyl, and lower alkylsulfonamido, at least one of which is alkylaminoacetyl; $R_2$ and $R_3$ are either joined to form a six-membered aromatic ring, which can optionally be further substituted with one or more substituents independently selected from the group consisting of alkoxy, alkyl, $\text{-NH}_2$, alkylamino, aryl, aza, carboxy, alkylacylamino, alkylaminoacetyl halogen, hydroxyl, nitro, lower alkylsulfonyl, and lower alkylsulfonamido, or one of $R_2$ and $R_3$ is an aryl group, which can optionally be further substituted with one or more substituents independently selected from the group
consisting of alkoxy, alkyl, -NH₂, alkylamino, aryl, aza, carboxy, alkylacylamino, alkylaminoacyl halogen, hydroxyl, nitro, lower alkylsulfonyl, and lower alkylsulfonamido, and the other is hydrogen or a substituent selected from the group consisting of alkoxy, alkyl, -NH₂, alkylamino, aryl, aza, carboxy, alkylacylamino, alkylaminoacyl halogen, hydroxyl, nitro, lower alkylsulfonyl, and lower alkylsulfonamido; R₄, R₅, R₆ and R₇ are independently H or a substituent selected from the group consisting of alkoxy, alkyl, -NH₂, alkylamino, aryl, aza, carboxy, alkylacylamino, alkylaminoacyl halogen, hydroxyl, nitro, lower alkylsulfonyl, and lower alkylsulfonamido; R₈ is one or more substituents independently selected from the group consisting of alkoxy, alkyl, -NH₂, alkylamino, aryl, aza, carboxy, alkylacylamino, alkylaminoacyl halogen, hydroxyl, nitro, lower alkylsulfonyl, lower alkylsulfonamido and sulfato, at least one of which is alkylaminoacyl; Ar is an aryl group, which can be further substituted with one or more substituents independently selected from the group consisting of alkoxy, alkyl, -NH₂, alkylamino, aryl, aza, carboxy, alkylacylamino, alkylaminoacyl halogen, hydroxyl, nitro, lower alkylsulfonyl, and lower alkylsulfonamido; and R₉ is hydrogen or a substituent selected from the group consisting of alkoxy, alkyl, -NH₂, alkylamino, aryl, aza, carboxy, alkylacylamino, alkylaminoacyl, halogen, hydroxyl, nitro, lower alkylsulfonyl, and lower alkylsulfonamido.

43. The method of claim 1, further comprising administering a second therapeutic agent in a combination with the compound.

44. A method for inhibiting HIF-1α activity in a subject in need of such treatment, comprising administering a therapeutically effective amount of a compound comprising a dibenzo-[1,4]dioxin carboxamide other than a 6-chloro-[1,4]dioxin-1-carboxamide.
45. The method of claim 44, wherein the compound is:

![Chemical Structure]

wherein $R_{18}$ is one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato, at least one of which is carboxamide, and $R_{19}$ is hydrogen or one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato.

46. The method of claim 44, wherein the compound is:
47. The method of claim 44, wherein the compound is:

![ Compound Structure ]

48. The method of claim 44, wherein the method comprises treating a proliferative disorder.
49. The method of claim 44, wherein the method comprises inhibiting angiogenesis in tumors that respond to anti-angiogenesis treatment.

50. The method of claim 48, wherein the method comprises treating a tumor.

51. A pharmaceutical composition comprising a non-cytotoxic, therapeutically effective amount of a tricyclic compound having the formula:

![Chemical Structure](image)

wherein X and Y are independently O, S, N, NR₄, CR₅ or CR₆R₇ and all valency requirements are satisfied; R₁ is one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfanyl, sulfonamido and sulfato, at least one of which is carboxamide or diamine; R₂ and R₃ are either joined to form a six-membered aromatic ring, which can optionally be further substituted with one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfanyl, sulfonamido and sulfato, or one of R₂ and R₃ is an aryl group, which can optionally be further substituted with one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfanyl, sulfonamido and sulfato, and the other is hydrogen or a substituent selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl,
mercapto, nitro, sulfonyl, sulfonamido and sulfato; and R₄, R₅, R₆ and R₇ are independently H or a substituent selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato; or having the formula:

\[
\begin{align*}
\text{Ar} & \quad W \\
 & \quad Z \\
 & \quad \text{R₈}
\end{align*}
\]

wherein R₈ is one or more substituents independently selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato; W and Z are independently NR₉ or =N—, but not the same; and R₉ is hydrogen or a substituent selected from the group consisting of acyl, acyloxy, alkoxy, alkyl, alkylthio, amino, aryl, aza, carboxy, carboxamide, diamine, halogen, hydroxyl, mercapto, nitro, sulfonyl, sulfonamido and sulfato.

52. The pharmaceutical composition of claim 51, wherein the compound comprises a dibenzo-[1,4]dioxin carboxamide other than a 6-chloro-[1,4]dioxin-1-carboxamide.

53. The pharmaceutical composition of claim 51, wherein the compound is:
54. A pharmaceutical composition comprising a therapeutically effective amount of a compound comprising a dibenzo-[1,4]dioxin carboxamide other than a 6-chloro-[1,4]dioxin-1-carboxamide.

55. The pharmaceutical composition of claim 54, wherein the compound is
FIG. 1A

--- + --- +  NSC 644221 (5 microM)
--- + --- +  1% Oxygen
2 hours  4 hours  6 hours  18 hours

HIF-1 α
Actin
FIG. 1B

<table>
<thead>
<tr>
<th>Concentration of NSC 644221 (microM)</th>
</tr>
</thead>
</table>

1% O₂

0 | 0.5 | 1 | 2 | 4 | 8 |

HIF-1α

HIF-1β
FIG. 3

1% O₂

0 0 1 2 4 8

644/221 (µM)

18 hours

HHF-1α

HHF-1β
MCG-1 = NSC 644221

FIG. 7
**MCG1 = NSC 644221**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control + MCG-1 5μM</th>
<th>1% oxygen 6h</th>
<th>1% oxygen 6h + MCG-1 5μM</th>
<th>Control</th>
<th>Control + MCG-1 5μM</th>
<th>1% oxygen 18h</th>
<th>1% oxygen 18h + MCG-1 5μM</th>
</tr>
</thead>
</table>

**A**

- **HIF-2α**
- **actin**

**B**

- **HIF-1α**
- **actin**

**FIG. 9**
FIG. 10

1% Oxygen (8 hours)

UMRC2 VHL +

6444221 (5μM)

UMRC2 VHL -

HIF-1α

actin

HIF-1α

actin
The inhibitory effect of 644221 on VEGF mRNA expression is maintained in 145 (Topo-1) mutant cells.
1% oxygen

<table>
<thead>
<tr>
<th>Condition</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td></td>
</tr>
<tr>
<td>644221</td>
<td>2.5uM</td>
</tr>
<tr>
<td>644221</td>
<td>5uM</td>
</tr>
<tr>
<td>295505</td>
<td>0.5uM</td>
</tr>
<tr>
<td>295505</td>
<td>2.5uM</td>
</tr>
<tr>
<td>295504</td>
<td>1uM</td>
</tr>
<tr>
<td>295504</td>
<td>5uM</td>
</tr>
</tbody>
</table>

FIG. 12
The effect of 644221 and 295504 on HRE/pGL3 reporter activity in U251 cells (pGL3 normalised)
The effect of NSC 644221 on VEGF mRNA expression in U251 cells

FIG. 14
FIG. 15

Hypoxia + Drug 5μM

HIF-1α

H2A.X

0460
24544
24459
24623
24588
24058
64422

Control
U251 control
U251 + 644221 1μM
U251 + 17AAG 1μM
C2 control
C2 + 644221 1μM
C2 + 17AAG 1μM
HCT116 control
HCT116 + 644221 1μM
HCT116 + 17AAG 1μM

Actin  Total AKT  24 Hrs

FIG. 20
Hypoxia

644221 5µM

1h 2h 3h 4h
- - + - + - + - +

Phospho AKT (Ser 473)

Actin

FIG. 21
Cells are very similar to control cells, little damage.

U251+1µM 64221 (0% Mitotic)

U251+100µM Jas

U251 control cells (0% mitotic)

18h

U251+100µM Colchicine (46% mitotic)
PC-3 (inactive cells)  FIG. 23  U251 (active cells)

Control

644221
1μM
24h

Data acquired: 04-Nov-04
File: 11-4-04 PC-3 CONTROL,103
Source: PRBC
Case: PATIENT ID
Analysis type: Manual analysis
Prep: Fresh/Frozen

DIPLOID: 100.00 %
 Dip G2-M: 1.00 % at 54.93
 Dip S: 0.00 % at 74.26
 Dip G1/G0: 4.97
 Dip %CV: 6.48

Total S-Phase: 10.04 %
Extra Pop: %
Debris: 0.00 %
Aggregates: 15.83 %
Modal Event: 994
RCS: 4.26

Data acquired: 04-Nov-04
File: 11-4-04 US251 CONTROL,100
Source: PRBC
Case: PATIENT ID
Analysis type: Manual analysis
Prep: Fresh/Frozen

DIPLOID: 100.00 %
 Dip G2-M: 1.00 % at 54.93
 Dip S: 0.00 % at 74.26
 Dip G1/G0: 4.97
 Dip %CV: 6.48

Total S-Phase: 17.69 %
Extra Pop: %
Debris: 2.14 %
Aggregates: 18.94 %
Modal Event: 994
RCS: 3.60

Data acquired: 04-Nov-04
File: 11-4-04 PC-3 PLUS DRUG,103
Source: PRBC
Case: PATIENT ID
Analysis type: Manual analysis
Prep: Fresh/Frozen

DIPLOID: 100.00 %
 Dip G2-M: 1.00 % at 54.93
 Dip S: 0.00 % at 74.26
 Dip G1/G0: 4.97
 Dip %CV: 6.48

Total S-Phase: 10.04 %
Extra Pop: %
Debris: 0.00 %
Aggregates: 15.83 %
Modal Event: 994
RCS: 4.26

Data acquired: 04-Nov-04
File: 11-4-04 US251 PLUS DRUG,101
Source: PRBC
Case: PATIENT ID
Analysis type: Manual analysis
Prep: Fresh/Frozen

DIPLOID: 100.00 %
 Dip G2-M: 1.00 % at 54.93
 Dip S: 0.00 % at 74.26
 Dip G1/G0: 4.97
 Dip %CV: 6.48

Total S-Phase: 17.69 %
Extra Pop: %
Debris: 2.14 %
Aggregates: 18.94 %
Modal Event: 994
RCS: 3.60
FIG. 25

The effect of TCDD (dioxin) and NSC64421 on CYP1A1 luciferase expression in MCF-7 cells (48 hours)

Fold increase in basal luciferase activity

Protein normalized

Concentration (microMoles)

Control

0.1

0.2

0.5

1
FIG. 26

The induction of p21 mRNA expression by 644221 and hypoxia in the presence and absence of Actinomycin D (5 micrograms/microlitre) (U251 cells)
(P53 wt) HCT116 cells (Inactive p53) U251 cells

Control

644221 5μM

Actin p21 Actin p21

FIG. 27
FIG. 28

<table>
<thead>
<tr>
<th>644221 HIF-1α active cell lines</th>
<th>P53 status</th>
<th>644221 HIF-1α inactive cell lines</th>
<th>P53 status</th>
</tr>
</thead>
<tbody>
<tr>
<td>U251 (glioma)</td>
<td>mut/inact</td>
<td>MCF-7 (breast)</td>
<td>wt</td>
</tr>
<tr>
<td>UMRC-2 (VHL+&amp;-) (renal)</td>
<td></td>
<td>T47D (breast)</td>
<td>mut</td>
</tr>
<tr>
<td>RCC4 (VHL+&amp;-) (renal)</td>
<td>wt</td>
<td>PC-3 (prostate)</td>
<td>mut</td>
</tr>
<tr>
<td>ACHN (renal)</td>
<td>wt</td>
<td>Hela (cervical)</td>
<td>mut/inact</td>
</tr>
<tr>
<td>COLO357 pancreatic</td>
<td>wt</td>
<td>HCT116 (p21/-, wt, and p53/-)</td>
<td>+/- or wt</td>
</tr>
<tr>
<td>SN12C (renal)</td>
<td>mut</td>
<td>A549 (lung)</td>
<td>wt</td>
</tr>
<tr>
<td>CAKI (renal)</td>
<td>wt</td>
<td>TK-10 (renal)</td>
<td>mut</td>
</tr>
<tr>
<td>A498 (renal) HIF-2α (slight)</td>
<td>wt</td>
<td>SK-MEL-2 (melanoma)</td>
<td>mut</td>
</tr>
<tr>
<td>CCRF-CEM (leukemia)</td>
<td>mut</td>
<td>SK-MEL-2 (melanoma)</td>
<td>mut</td>
</tr>
<tr>
<td>UO-31 (renal)</td>
<td>wt</td>
<td>CHO (hamster ovary)</td>
<td>mut</td>
</tr>
<tr>
<td>RXF-393 (renal)</td>
<td>mut</td>
<td>SNB-75 (glioma)</td>
<td>mut</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H460 (lung)</td>
<td>wt</td>
</tr>
</tbody>
</table>