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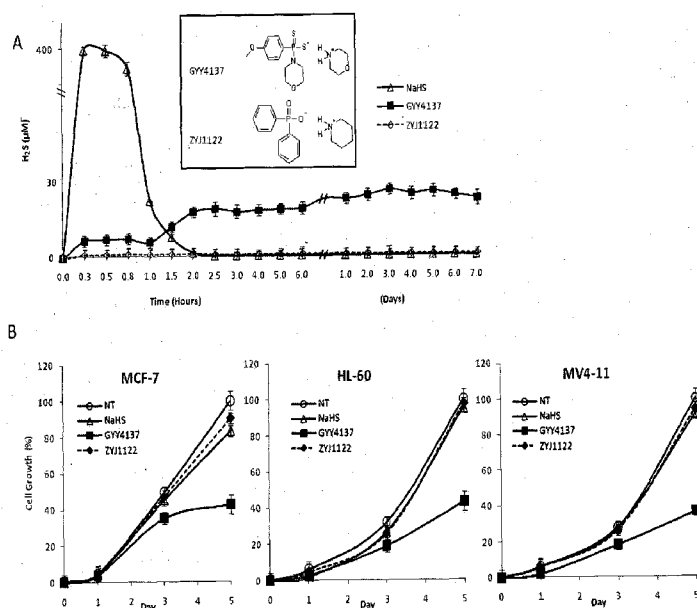
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(54) Title: A SLOW-RELEASING HYDROGEN SULPHIDE DONOR IN A METHOD FOR THE TREATMENT OF CANCER

Figures 1A-1B



(57) Abstract: The invention is directed to a method of treating cancer in an individual in need thereof comprising administering an effective amount of a slow-releasing H₂S compound. In another aspect, the invention is directed to a method (*e.g.*, *in vitro*; *in vivo*) of killing and/or inhibiting (*e.g.*, selectively killing and/or inhibiting) the growth (proliferation) of a (one or more) cancer cell(s) comprising contacting cells which comprise cancer cells (*e.g.*, a heterogeneous population of cells) with an effective amount of a slow-releasing H₂S compound. A combination is thereby produced and the combination is maintained under conditions in which H₂S is slowly released in the presence of the cells, thereby killing and/or inhibiting the growth of the cancer cells.



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Inventors: Philip Keith Moore, Choon Hong Tan, Lih Wen Deng, and Ling Li

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A slow-releasing hydrogen sulphide donor in a method for the treatment of cancer

BACKGROUND OF THE INVENTION

Hydrogen sulfide (H₂S) is synthesized naturally from cysteine by several
5 enzymes including cystathionine γ lyase (CSE), cystathionine β synthetase (CBS) and
3-mercaptosulfurtransferase (3-MST) in a wide range of mammalian and non-
mammalian cells both in vitro and in vivo. In the last decade, numerous physiological
and pathophysiological roles have been proposed for this gas along with a plethora of
cellular and molecular targets including a range of ion channels, enzymes and
10 transcription factors (Li, L. *et al. Annu Rev Pharmacol Toxicol*, 51:169-187 (2011)).

Apart from its potential roles in normal physiology there is also a
comprehensive literature describing the toxicity of H₂S and its role as an environmental
pollutant (Fiedler, N., *et al., Environ Health Perspect*, 116(1):78-85 (2008)). A number
of studies have investigated the role of H₂S in triggering cell death and evidence has
15 been presented that this gas can exert both pro- and anti-apoptotic activity in cultured
cells (HU, LF., *et al., J Neurochem*, 100(4):1121-1128 (2008); Taniguchi, S., *et al., Br
J Pharmacol*, 162(5):1171-1178 (2011)). However, the precise mechanism(s) involved
remain unclear.

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Surprisingly, there have been few studies of the effect of H₂S on cancer cells in vitro and no reports of its effect on tumor progression *in vivo*.

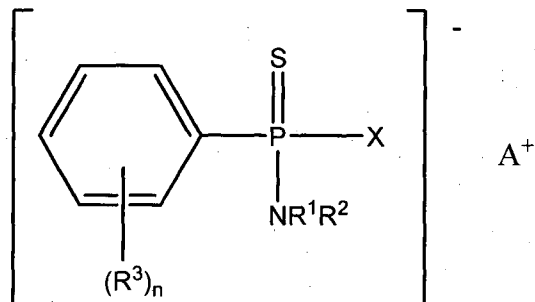
SUMMARY OF THE INVENTION

5 As shown herein, the slow-releasing hydrogen sulfide (H₂S) donor, GYY4137, caused concentration-dependent killing of seven different human cancer cell lines (HeLa, HCT-116, Hep G2, HL-60, MCF-7, MV4-11 and U2OS) but did not affect survival of normal, human lung fibroblasts (IMR90, WI-38) as determined by trypan blue exclusion. Sodium hydrosulfide (NaHS) was less potent and not active in all cell
10 lines. A structural analogue of GYY4137 (ZYY1122) lacking sulfur and thence not able to release H₂S was inactive. Similar results were obtained using a clonogenic assay. Incubation of GYY4137 (400 μM) in culture medium led to the generation of low (< 20 μM) concentrations of H₂S sustained over 7 days. In contrast, incubation of NaHS (400 μM) in the same way led to much higher (up to 400 μM) concentrations of H₂S which
15 persisted for only 1 h. Mechanistic studies revealed that GYY4137 (400 μM) incubated (5 days) with MCF-7 but not IMR90 cells caused the generation of cleaved PARP and cleaved caspase 9 indicative of a pro-apoptotic effect. GYY4137 (but not ZYY1122) also caused partial G₂/M arrest of these cells. In xenograft experiments using immunodeficient mice, GYY4137 (100-300 mg/kg/day for 14 days) significantly
20 reduced tumor growth following implantation of either HL-60 or MV4-11 cells. Thus, shown herein is that GYY4137 exhibits anti-cancer activity by releasing H₂S over a period of days, a combination of apoptosis and cell cycle arrest contributes to this effect and H₂S donors can be used as potential anti-cancer agents (see Lee, ZW., *et al.*, *PLoS One*, 6(6):e21077 (June 20, 2011) which is incorporated herein by reference).

25 Accordingly, the invention is directed to a method of treating cancer in an individual in need thereof comprising administering an effective amount of a slow-releasing H₂S compound. In one aspect, the invention is directed to a method of treating cancer in an individual in need thereof comprising administering an effective amount of

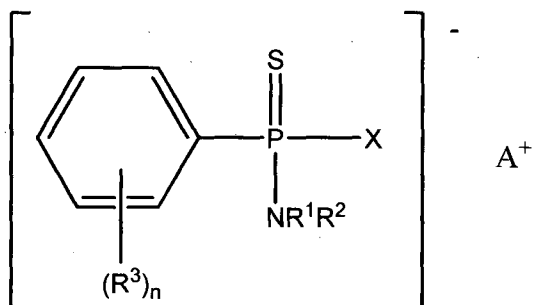
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a (one or more) slow-releasing H₂S compound wherein the compound is represented by the following structural formula:



or a pharmaceutically acceptable salt thereof.

- 5 In another aspect, the invention is directed to a method (*e.g.*, *in vitro*; *in vivo*) of killing and/or inhibiting (*e.g.*, selectively killing and/or inhibiting) the growth (proliferation) of a (one or more) cancer cell(s) comprising contacting cells which comprise cancer cells (*e.g.*, a heterogeneous population of cells) with an effective amount of a slow-releasing H₂S compound. A combination is thereby produced and the
- 10 combination is maintained under conditions in which H₂S is slowly released in the presence of the cells, thereby killing and/or inhibiting the growth of the cancer cells. In a particular aspect, the compound is represented by the following structural formula:



or a pharmaceutically acceptable salt thereof.

15

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A: H₂S-releasing profile of NaHS, GYY4137 and ZYJ1122. H₂S released from NaHS, and GYY4137 (400 μM) was determined in aliquots (100 μl) of

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medium withdrawn at timed intervals (up to 7 days) from cultured MCF-7 cells.

Concentration of H₂S amounts was assessed spectrophotometrically using N,N-dimethyl-p phenylenediamine-dihydrochloride and results show the H₂S concentration (μM). H₂S release from GYY4137 was significantly different (P<0.05) from T=0 at all time points from 0.3 h to 7 days. H₂S release from NaHS was significantly different (P<0.05) from T=0 at all time points up to 1.5 h. No detectable H₂S was released from ZYJ1122 (400 μM) under identical experimental conditions. Results show mean ± s.e. mean, n=3. The chemical structures of GYY4137 and ZYJ1122 are shown in the inset.

Figure 1B: Growth curve analyses of MCF-7, HL-60 and MV4-11 cells treated with NaHS, GYY4137 and ZYJ1122 (400 μM) over 5 days. Cell survival was determined by trypan blue staining. Results show cell growth as a percentage relative to NT cell numbers at day 5 and are mean ± s.e. mean, n=3.

Figure 2A: The effect of treatment (5 days) of a range of cancer and non-cancer cells with NaHS, GYY4137 and ZYJ1122 (400 μM or 800 μM) as determined by trypan blue staining. Results show cell number as a % of cell viability following incubation in the absence of drug treatment i.e. no treatment (NT) and are mean ± s.e. mean, n=3, (*P <0.05; *P < 0.01).

Figure 2B: Concentration-response curves showing the effect of GYY4137 treatment for 5 days on survival of MCF-7, HL-60 and MV4-11 cells. Results show mean ± s.e. mean, n=3.

Figure 2C: Representative photographs showing clonogenic survival assay of MCF-7 cells following exposure (5 days, 200-600 μM) to either NaHS (top row), GYY4137 (middle row) and ZYJ1122 (bottom row). NT= non-treatment.

Figure 3A: Cell cycle analysis of MCF-7 cells after 5 days (NT and ZYJ1122) and 5 and 8 days (GYY4137) drug treatment. Inset shows percentage distribution of cells in each cell phase. Results shown are indicative of 3 individual experiments. NT: non-treatment.

Figure 3B: Western blot analysis of apoptosis markers (α-PARP, α-cleaved-PARP, α-cleaved-caspase 9) of MCF-7 and IMR90 treated (5 days) with GYY4137 or

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ZYJ1122 (both 400 μ M). The anti-cleaved caspase-9 antibody used detects the large fragment of caspase-9 following cleavage but does not recognize uncleaved procaspase-9. α -tubulin was used as a loading control. Results shown are indicative of 3 individual experiments.

5 Figures 4A-4B: Changes in volume of established tumors in, (4A) HL-60 xenograft mice (4B) MV4-11 xenograft mice treated daily with GYY4137 (100-300 mg/kg, i.p.) or an appropriate volume of vehicle as control. Treatment with GYY4137 significantly reduced the tumor volume in both animal models, in a dose-dependent manner. Results show change in tumor volume measured and are mean $\pm$ s.e. mean,
10 n=4-6, # P<0.05; * P<0.01.

 Figures 5A-5C: Concentration-response curves showing the effect of GYY4137 treatment for 5 days on survival of (5A) epithelial ovarian cancer cell lines (OVCA433, SKOV3 and OVCAR10); (5B) gastric carcinoma cell lines (SNU1 and SNU5); (5C) Cervical carcinoma (SiHa), osteosarcoma (Saos2) and breast adenocarcinoma (MDA-
15 MB-231). Results show mean $\pm$ s.e. mean, n=3.

DETAILED DESCRIPTION OF THE INVENTION

A description of example embodiments of the invention follows.

Several years ago it was reported that H₂S protected colon cancer cells (HCT-
20 116) from apoptosis due to β -phenylethyl isothiocyanate (Rose, P., *et al.*, *World J Gastroenterol*, 11(26):3990-3997 (2005)). Others have subsequently reported that H₂S increases human colon cancer cell proliferation and reduces apoptosis in several cell lines (e.g. HCT-116) (Cai, WJ., *et al.*, *Cell Biol Int*, 34(6):565-572 (2010)) whilst decreasing survival in other human colon cell lines (e.g. WiDR) (Cao, Q., *et al.*,
25 *Antioxid Redox Signal*, 12(9):1101-1109 (2010)). These disparate observations are difficult to reconcile. However, as shown herein one explanation may lie in the choice of H₂S donor. Sulfide salts such as sodium hydrosulfide (NaHS) and sodium sulfide (Na₂S) have been widely used to study the biological effects of this gas in many cells,

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tissues and animals. On addition of water, these salts generate a large amount of H₂S over a short time period. Since cell culture takes place over a period of hours or days it is likely that little, if any, H₂S is present in medium within a short time of adding either NaHS or Na₂S. As shown herein, it is likely that the concentration of H₂S that cancer
5 (or indeed other) non-cancer cells are exposed to during culture with NaHS will be high at the start and not sustained throughout the experiment. Thus, it is difficult to draw firm conclusions about the ability of H₂S to affect cancer cell survival using sulfide salts as donor agents.

It was reported that GYY4137 releases H₂S slowly both in aqueous media and
10 when administered to intact animals over a period of hours to days. See Li, L., *et al.*, *Circulation*, 117(18):2351-2360 (2008) and Whiteman, M., *et al.*, *Antioxid Redox Signal*, 12(10):1147-1154 (2010), both of which are incorporated herein by reference. See also U.S. Application Publication No. 2010/0273743 A1 which is also incorporated herein by reference. Described herein is a comparison of the effect of GYY4137 and
15 NaHS on survival of a range of cancer and non-cancer cells in culture and a correlation of their effect with changes in concentration of H₂S in the medium. In addition, the effect of GYY4137 on tumor growth using a xenograft model in immunodeficient mice was examined. Shown herein is that GYY4137 exhibits anti-cancer activity by releasing H₂S over a period of days.

20 Accordingly, the invention is directed to a method of treating cancer in an individual in need thereof comprising administering to the individual an effective amount of a compound that generates H₂S at low concentrations over a period of one or more (*e.g.*, several) hours and/or one or more (*e.g.*, several) days, referred to herein as a slow-releasing H₂S compound or donor. In one aspect, administration of the slow-
25 releasing H₂S compound causes low H₂S concentrations that exceed basal levels of H₂S in an individual and/or cell (*e.g.*, that exceeds a physiological (normal) level) for about one or more hours, or one or more days after administration. In another aspect, administration of the slow-releasing H₂S compound results in a sustained release of a low concentration of H₂S, wherein the low concentration of H₂S is above the basal level

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of H₂S which is the level of H₂S normally present in the individual and/or cell. In one aspect, the low concentration of H₂S is less than the H₂S concentration immediately released upon administration of, for example, NaHS (*e.g.*, 400 μM). In another aspect, the low concentration of H₂S is about 20 μM. In yet another aspect, the low
5 concentration of H₂S is about 10 μM.

The slow-releasing H₂S compound can release H₂S for over one or more hours (*e.g.*, for about 70 minutes, 80 minutes, 90 minutes, 100 minutes, 110 minutes, 120 minutes, 130 minutes, 140 minutes, 150 minutes, 160 minutes, 170 minutes, 180 minutes, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12
10 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours) after administration to the individual (in contrast to “explosively” (*e.g.*, immediately or within one or several seconds or an hour or less after administration), or about one or more days (*e.g.*, for about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14
15 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, *etc.*), after administration to the individual.

In the methods described herein, treatment of cancer includes one or more of a variety of anti-cancer activities such as ameliorating one or more symptoms associated
20 with the cancer, preventing or delaying the onset or progression of the cancer, and/or lessening the severity and/or frequency of symptoms and/or side effects of the cancer.

In one aspect, the methods of treating cancer can include methods of reducing tumor cell growth in an individual. Thus, the invention is directed to a method of reducing tumor growth in an individual in need thereof comprising administering to the
25 individual an effective amount of a slow-releasing H₂S compound.

In another aspect, the invention is directed to a method (*e.g.*, *in vitro*; *in vivo*) of killing and/or inhibiting (*e.g.*, selectively killing and/or inhibiting) the growth (proliferation) of a (one or more) cancer cell(s) comprising contacting cells which comprise cancer cells with an effective amount of a slow-releasing H₂S compound. A

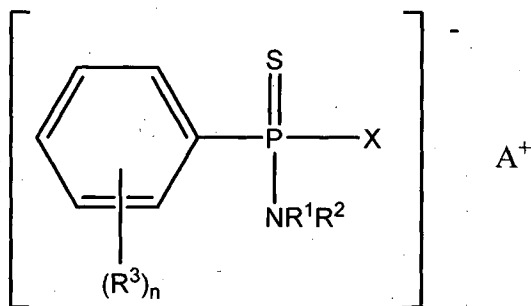
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combination is thereby produced and the combination is maintained under conditions in which H₂S is slowly released in the presence of the cells, thereby killing and/or inhibiting the growth of the cancer cells. As used herein, "selective killing and/or inhibition of growth" refers to the ability of the slow-releasing H₂S compound to kill and/or inhibit growth of cancer cells (*e.g.*, tumor cells) but not non-cancer cells (*e.g.*, normal cells). Thus, the method can be used to kill and/or inhibit the growth of cancer cells present in a homogeneous population of cells (*e.g.*, a population of cells in which all or substantially all of the cells are cancer cells) or a heterogeneous population of cells that comprise cancer and non-cancer cells (*e.g.*, a population of cells in which many or only some (*e.g.*, a few) cancer cells are present). Thus, the methods can be used to kill and/or inhibit the growth of cancer cells in a tumor which can comprise cancer and non-cancer cells or to kill and/or inhibit the growth of cancer cells that have metastasized from one or more primary cancer sites in an individual.

A variety of cancers can be treated using the methods described herein.

Examples of such cancers include cervical cancer, colorectal cancer (*e.g.*, colon cancer; rectal cancer), liver cancer, bone cancer, breast cancer (*e.g.*, breast adenocarcinoma), ovarian cancer, bone cancer (*e.g.*, osteosarcoma), gastric cancer, leukemia (*e.g.*, promyelocytic leukemia; myelomonocytic leukemia) and the like.

One or more slow-releasing H₂S compounds are used in the methods described herein. In one aspect, the compound is represented by the following structural formula:



or a pharmaceutically acceptable salt thereof.

In a particular aspect, the compound is morpholin-4-ium 4-methoxyphenyl(morpholino)phosphinodithioate (hereinafter referred to as GYY4137).

The term "alkyl", used alone or as part of a larger moiety such as "arylalkyl" or "cycloalkylalkyl" means a straight or branched hydrocarbon radical having 1-10 carbon atoms and includes, for example, methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, isobutyl, tert-butyl, n-pentyl, i-pentyl, n-hexyl, n-heptyl, n-octyl, n-nonyl, n-decyl and the like. Unless otherwise described, exemplary substituents for a substituted alkyl group include the values and specific values described for R⁴.

The term "cycloalkyl" means a monocyclic, bicyclic or tricyclic, saturated hydrocarbon ring having 3-12 carbon atoms and includes, for example, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, bicyclo[2.2.2]octyl, bicyclo[2.2.1]heptyl, spiro [4.4]nonane, adamantyl and the like. Unless otherwise described, exemplary substituents for a substituted cycloalkyl group include the values and specific values described for R⁴.

"Aryl", used alone or as part of a larger moiety as in "arylalkyl", means a 6-10 membered carbocyclic aromatic monocyclic or polycyclic ring system. Examples include phenyl and naphthyl. The term "aryl" also includes phenyl rings fused to non-aromatic carbocyclic ring or to a heterocyclyl group. The term "aryl" may be used interchangeably with the terms "aromatic group", "aryl ring", "aromatic ring", "aryl group" and "aromatic group". Unless otherwise described, exemplary substituents for a substituted aryl group include the values and specific values described for R⁴.

"Hetero" refers to the replacement of at least one carbon atom member in a ring system with at least one heteroatom selected from N, S, and O. A hetero ring may have 1, 2, 3, or 4 carbon atom members replaced by a heteroatom.

"Heterocyclyl" refers to a saturated or unsaturated, non-aromatic, monocyclic or polycyclic ring system of 3 to 20 atoms, 3 to 12 atoms, or 3 to 8 atoms, containing one to four ring heteroatoms chosen from O, N and S. Exemplary heterocyclyls include pyrrolidine, pyrrolidin-2-one, 1-methylpyrrolidin-2-one, piperidine, piperidin-2-one, 2-pyridone, 4-pyridone, piperazine, 1-(2,2,2-trifluoroethyl)piperazine, piperazin-2-one,

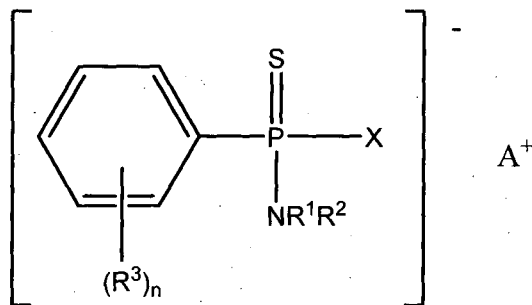
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5,6-dihydropyrimidin-4-one, pyrimidin-4-one, tetrahydrofuran, tetrahydropyran, tetrahydrothiophene, tetrahydrothiopyran, isoxazolidine, 1,3-dioxolane, 1,3-dithiolane, 1,3-dioxane, 1,4-dioxane, 1,3-dithiane, 1,4-dithiane, oxazolidin-2-one, imidazolidin-2-one, imidazolidine-2,4-dione, tetrahydropyrimidin-2(1H)-one, morpholine, N-methylmorpholine, morpholin-3-one, 1,3-oxazinan-2-one, thiomorpholine, thiomorpholine 1,1-dioxide, tetrahydro-1,2,5-thioxazole 1,1-dioxide, tetrahydro-2H-1,2-thiazine 1,1-dioxide, hexahydro-1,2,6-thiadiazine 1,1-dioxide, tetrahydro-1,2,5-thiadiazole 1,1-dioxide and isothiazolidine 1,1-dioxide. Unless otherwise described, exemplary substituents for a substituted heterocyclyl group include the values and specific values described for R⁴.

“Heterocyclyl” also includes heteroaryl groups. The term “heteroaryl” means a 5-10 membered monovalent heteroaromatic monocyclic and polycyclic ring radical containing 1 to 4 heteroatoms independently selected from N, O, and S. The term “heteroaryl” also includes monocyclic heteroaryl ring fused to non-aromatic carbocyclic ring or to a heterocyclyl group. Heteroaryl groups include furyl, thienyl, thiophenyl, pyrrolyl, oxazolyl, thiazolyl, imidazolyl, pyrazolyl, isoxazolyl, isothiazolyl, oxadiazolyl, triazolyl, thiadiazolyl, pyridinyl, pyridinyl-N-oxide, pyridazinyl, pyrimidinyl, pyrazinyl, indoliziny, indolyl, isoindolyl, benzo[b]furyl, benzo[b]thienyl, indazolyl, benzimidazolyl, benzthiazolyl, purinyl, 4H-quinoliziny, quinolinyl, isoquinolinyl, quinazolinyl, benzothienyl, benzofuranyl, 2,3-dihydrobenzofuranyl, benzodioxolyl, benzimidazolyl, indazolyl, benzisoxazolyl, benzoxazolyl, benzothiazolyl, cinnolinyl, phthalziny, quinazolinyl, quinoxaliny, 1,8-naphthyridiny, 1,2,3-triazolyl, 1,2,4-triazolyl, 1,3,4-oxadiazolyl, 1,2,5-thiadiazolyl, 1,2,5-thiadiazolyl-1-oxide, 1,2,5-thiadiazolyl-1,1-dioxide, 1,3,4-thiadiazolyl, 1,2,4-triazinyl, 1,3,5-triazinyl, tetrazolyl, and pteridinyl. The terms “heteroaryl”, “heteroaryl ring”, and “heteroaryl group” are used interchangeably herein. Unless otherwise described, exemplary substituents for a substituted heterocyclyl group include the values and specific values described for R⁴.

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One embodiment of the invention is a method of treating cancer in an individual in need thereof comprising administering an effective amount of a compound represented by Structural Formula (I):

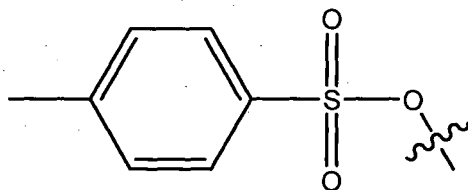


(I), or a pharmaceutically acceptable

- 5 salt thereof. Values and specific values for the variables of Structural Formula (I) are as follows:

X is O or S and A^{+} is present; or

- 10 X is halogen, OMs or OTs and A^{+} is absent. As used herein, "OMs" is a mesylate group represented by the chemical formula $-\text{OS}(\text{O})_2\text{CH}_3$. As used herein, "OTs" is tosylate group represented by the chemical formula



- 15 In one embodiment, X is O or S and A^{+} is present. In a specific embodiment, X is S and A^{+} is present. In another embodiment, X is O and A^{+} is present.

- Alternatively, X is halogen, OMs or OTs and A^{+} is absent. In a specific alternative embodiment, X is halogen and A^{+} is absent. In another specific alternative embodiment, X is Cl and A^{+} is absent. In another specific alternative embodiment, X is OMs and A^{+} is absent. In another specific alternative embodiment, X is OTs and A^{+} is
20 absent.

When present, A^+ is a monovalent cation. As used herein a "cation" is a positively charged ion. As used herein "monovalent" means that the charge of the cation is +1. Cations can be monoatomic or polyatomic.

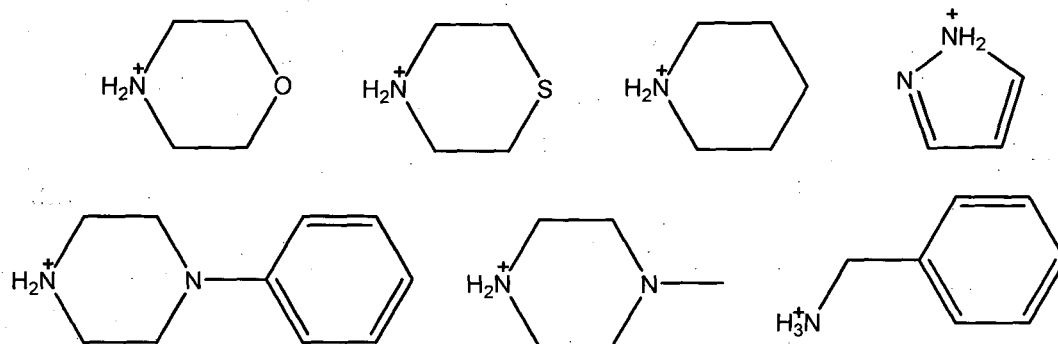
In one embodiment, the monovalent cation is a monoatomic cation. A
5 monoatomic cation contains one positively charged atom. Examples of monoatomic cations include, but are not limited to H^+ , Li^+ , Na^+ , K^+ or Cu^+ . In one embodiment, the monovalent cation is a metal cation. In a specific embodiment, A^+ is selected from lithium, sodium or potassium. In another specific embodiment, A^+ is potassium. Alternatively, A^+ is H^+ .

10 In one embodiment, the monovalent cation is a polyatomic cation. A polyatomic cation is a positively charged molecule that contains more than one atom. Unless otherwise described, exemplary substituents for a substituted a polyatomic cation include the values and specific values described for R^4 .

Examples of polyatomic cations include, but are not limited to NH_4^+ , $N(C_1-$
15 $C_4alkyl)H_3^+$ (for example, $N(methyl)H_3^+$ or $N(ethyl)H_3^+$), $N(benzyl)H_3^+$, $N(phenyl)H_3^+$, and H_3O^+ . In a specific example, A^+ is selected from NH_4^+ , $N(C_1-C_4alkyl)H_3^+$ (for example, $N(methyl)H_3^+$ or $N(ethyl)H_3^+$), $N(benzyl)H_3^+$, and $N(phenyl)H_3^+$. In another specific embodiment, A^+ is $N(benzyl)H_3^+$.

Alternatively, A^+ is a polyatomic cation that includes a heterocyclyl or a
20 heteroaryl, wherein each heterocyclyl or a heteroaryl contain a nitrogen atom that can be protonated. In one embodiment, A^+ is selected from piperidinium, morpholin-4-ium, thiomorpholin-4-ium, piperazin-1-ium, 4-methylpiperazin-1-ium, 4-phenyl-piperazin-1-ium, pyridinium, pyridazin-1-ium, pyrimidin-1-ium, pyrazin-1-ium, 1H-pyrrolium, 1H-pyrazol-1-ium, 1H-imidazol-1-ium, pyrazolidin-1-ium, pyrrolidinium, imidazolidin-1-
25 ium, each optionally substituted with one to three groups represented by R^4 . In a specific embodiment, A^+ is selected from:

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R^1 and R^2 are each independently (a) hydrogen; or (b) (C_1-C_{10}) alkyl, (C_2-C_{10}) alkenyl, (C_2-C_{10}) alkynyl, aryl(C_0-C_3)alkyl, heteroaryl(C_0-C_3)alkyl, cycloalkyl(C_0-C_3)alkyl, heterocyclyl(C_0-C_3)alkyl, heteroaryl(C_0-C_3)alkyl, each optionally substituted with one to three groups represented by R^4 ; or

R^1 and R^2 , along with the nitrogen to which they are attached, form a monocyclic heterocyclyl, wherein the heterocyclyl formed may contain 0, 1 or 2 heteroatoms in addition to the nitrogen atom, wherein the heterocyclyl is optionally substituted by one or more groups selected from halogen, hydroxy, (C_1-C_4) alkyl, (C_1-C_4) alkoxy, or phenyl, wherein the (C_1-C_4) alkyl, (C_1-C_4) alkoxy, or phenyl is optionally substituted with halogen, hydroxy, (C_1-C_4) alkyl, or (C_1-C_4) alkoxy.

In a specific embodiment, R^1 and R^2 , along with the nitrogen to which they are attached, form a heterocyclyl; and the heterocyclyl is selected from pyrrolidinyl, imidazolidinyl, pyrazolidinyl, dihydropyrrolyl, pyrrolyl, imidazolyl, pyrazolyl, triazolyl, oxazolidinyl, thiazolidinyl, oxazolyl, thiazolyl, piperidinyl, hydroypyrimidinyl, piperazinyl, pyridinyl, pyridazinyl, pyrimidinyl, triazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C_1-C_4) alkyl, (C_1-C_4) alkoxy, or phenyl. In a specific embodiment, R^1 and R^2 , along with the nitrogen to which they are attached, form a heterocyclyl, and the heterocyclyl is selected from pyrazolyl, piperidinyl, piperazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C_1-C_4) alkyl, $(C_1-$

- 14 -

C₄)alkoxy, or phenyl. Specific substituents for the heterocyclyl formed include methyl and phenyl.

In an alternate embodiment, R¹ and R² are each independently (a) hydrogen; or (b) (C₁-C₁₀)alkyl, (C₂-C₁₀)alkenyl, (C₂-C₁₀)alkynyl, aryl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, cycloalkyl(C₀-C₃)alkyl, heterocyclyl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, each optionally substituted with one to three groups represented by R⁴. In a specific embodiment, R¹ and R² are each independently (a) hydrogen; or (b) (C₁-C₄)alkyl, (C₂-C₄)alkenyl, (C₂-C₄)alkynyl, (C₃-C₈)cycloalkyl, phenyl, benzyl, each optionally substituted with one to three groups represented by R⁴. In a specific embodiment, R¹ and R² are the same. In an alternative specific embodiment, R¹ is different from R².

In a specific embodiment, R¹ is hydrogen and R² is selected from (C₁-C₁₀)alkyl, (C₂-C₁₀)alkenyl, (C₂-C₁₀)alkynyl, aryl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, cycloalkyl(C₀-C₃)alkyl, heterocyclyl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, each optionally substituted with one to three groups represented by R⁴. In another specific embodiment, R¹ is hydrogen or (C₁-C₄)alkyl; and R² is (C₁-C₄)alkyl or benzyl. In another specific embodiment, R¹ is hydrogen and R² is benzyl.

Each R³ is independently selected from halogen, hydroxy, (C₁-C₄)alkyl, halo(C₁-C₄)alkyl, hydroxy(C₁-C₄)alkyl, (C₁-C₄)alkoxy, halo(C₁-C₄)alkoxy, and hydroxy(C₁-C₄)alkoxy. In a specific embodiment, R³ is halogen, hydroxy, methyl, ethyl, propyl, isopropyl, butyl, t-butyl, hydroxy methyl, hydroxyethyl, hydroxymethoxy, hydroxyethoxy, methoxy, ethoxy, propoxy, butoxy, t-butyloxy, or -CF₃.

Each R⁴ is independently selected from halogen, hydroxy, (C₁-C₄)alkyl, halo(C₁-C₄)alkyl, hydroxy(C₁-C₄)alkyl, (C₁-C₄)alkoxy, halo(C₁-C₄)alkoxy, and hydroxy(C₁-C₄)alkoxy. In a specific embodiment, R³ is halogen, hydroxy, methyl, ethyl, propyl, isopropyl, butyl, t-butyl, hydroxy methyl, hydroxyethyl, hydroxymethoxy, hydroxyethoxy, methoxy, ethoxy, propoxy, butoxy, t-butyloxy, or -CF₃.

In another specific embodiment, n is 0. In a specific embodiment n is 0 or 1. Alternatively, n is 1, 2 or 3. In a specific embodiment, n is 1. In another specific embodiment, n is 2. In yet another specific embodiment, n is 3.

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In one embodiment of Structural Formula (I),

X is O or S and A⁺ is present; or

X is Cl, OMs or OTs and A⁺ is absent;

A⁺ is a monovalent cation;

5 R¹ and R² are each independently (a) hydrogen; or (b) (C₁-C₁₀)alkyl, (C₂-C₁₀)alkenyl, (C₂-C₁₀)alkynyl, aryl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, cycloalkyl(C₀-C₃)alkyl, heterocyclyl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, each optionally substituted with one to three groups represented by R⁴; or
10 R¹ and R², along with the nitrogen to which they are attached, form a monocyclic heterocyclyl, wherein the heterocyclyl formed may contain 0, 1 or 2 heteroatoms in addition to the nitrogen atom, wherein the heterocyclyl is optionally substituted by one or more groups selected from halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl, wherein the (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl is optionally substituted with halogen, hydroxy,
15 (C₁-C₄)alkyl, or (C₁-C₄)alkoxy;

Each R³ is independently selected from halogen, hydroxy, (C₁-C₄)alkyl, halo(C₁-C₄)alkyl, hydroxy(C₁-C₄)alkyl, (C₁-C₄)alkoxy, halo(C₁-C₄)alkoxy, and hydroxy(C₁-C₄)alkoxy;

20 Each R⁴ is independently selected from halogen, hydroxy, (C₁-C₄)alkyl, halo(C₁-C₄)alkyl, hydroxy(C₁-C₄)alkyl, (C₁-C₄)alkoxy, halo(C₁-C₄)alkoxy, and hydroxy(C₁-C₄)alkoxy; and

n is 0-3;

or a pharmaceutically acceptable salt thereof.

25 In a first specific embodiment of Structural Formula (I)

X is S;

A⁺ is selected from lithium, sodium or potassium. Specifically, A⁺ is potassium.

Alternatively, A⁺ is H⁺.

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R^1 and R^2 are each independently (a) hydrogen; or (b) (C₁-C₁₀)alkyl, (C₂-C₁₀)alkenyl, (C₂-C₁₀)alkynyl, aryl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, cycloalkyl(C₀-C₃)alkyl, heterocyclyl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, each optionally substituted with one to three groups represented by R^4 . Specifically, R^1 and R^2 are each

5 independently (a) hydrogen; or (b) (C₁-C₄)alkyl, (C₂-C₄)alkenyl, (C₂-C₄)alkynyl, (C₃-C₈)cycloalkyl, phenyl, benzyl, each optionally substituted with one to three groups represented by R^4 . More specifically, R^1 is hydrogen or (C₁-C₄)alkyl; and R^2 is (C₁-C₄)alkyl or benzyl. Even more specifically, R^1 is hydrogen and R^2 is benzyl, or a pharmaceutically acceptable salt thereof, and the values and specific values of the

10 remainder of the variables are as described for Structural Formula (I).

In a second specific embodiment of Structural Formula (I)

X is S;

A^+ is selected from NH_4^+ , $N(C_1-C_4alkyl)H_3^+$ (for example, $N(methyl)H_3^+$ or

15 $N(ethyl)H_3^+$), $N(benzyl)H_3^+$, and $N(phenyl)H_3^+$. Specifically, A^+ is $N(benzyl)H_3^+$.

R^1 and R^2 are each independently (a) hydrogen; or (b) (C₁-C₁₀)alkyl, (C₂-C₁₀)alkenyl, (C₂-C₁₀)alkynyl, aryl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, cycloalkyl(C₀-C₃)alkyl, heterocyclyl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, each optionally substituted with one to three groups represented by R^4 . Specifically, R^1 and R^2 are each

20 independently (a) hydrogen; or (b) (C₁-C₄)alkyl, (C₂-C₄)alkenyl, (C₂-C₄)alkynyl, (C₃-C₈)cycloalkyl, phenyl, benzyl, each optionally substituted with one to three groups represented by R^4 . More specifically, R^1 is hydrogen or (C₁-C₄)alkyl; and R^2 is (C₁-C₄)alkyl or benzyl. Even more specifically, R^1 is hydrogen and R^2 is benzyl, or a pharmaceutically acceptable salt thereof, and the values and specific values of the

25 remainder of the variables are as described for Structural Formula (I).

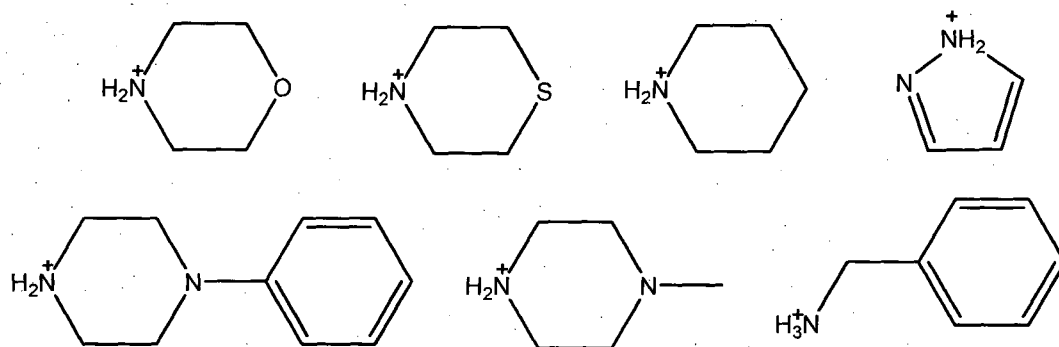
In a third specific embodiment of Structural Formula (I)

X is S;

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A^+ is selected from piperidinium, morpholin-4-ium, thiomorpholin-4-ium, piperazin-1-ium, 4-methylpiperazin-1-ium, 4-phenyl-piperazin-1-ium, pyridinium, pyridazin-1-ium, pyrimidin-1-ium, pyrazin-1-ium, 1H-pyrrolium, 1H-pyrazol-1-ium, 1H-imidazol-1-ium, pyrazolidin-1-ium, pyrrolidinium, imidazolidin-1-ium.

5 Specifically, A^+ is selected from:



R^1 and R^2 are each independently (a) hydrogen; or (b) (C_1-C_{10}) alkyl, (C_2-C_{10}) alkenyl, (C_2-C_{10}) alkynyl, aryl (C_0-C_3) alkyl, heteroaryl (C_0-C_3) alkyl, cycloalkyl (C_0-C_3) alkyl, heterocyclyl (C_0-C_3) alkyl, heteroaryl (C_0-C_3) alkyl, each optionally substituted with one to three groups represented by R^4 . Specifically, R^1 and R^2 are each independently (a) hydrogen; or (b) (C_1-C_4) alkyl, (C_2-C_4) alkenyl, (C_2-C_4) alkynyl, (C_3-C_8) cycloalkyl, phenyl, benzyl, each optionally substituted with one to three groups represented by R^4 . More specifically, R^1 is hydrogen or (C_1-C_4) alkyl; and R^2 is (C_1-C_4) alkyl or benzyl. Even more specifically, R^1 is hydrogen and R^2 is benzyl, or a pharmaceutically acceptable salt thereof, and the values and specific values of the remainder of the variables are as described for Structural Formula (I).

In a fourth specific embodiment of Structural Formula (I)

X is S;

20 A^+ is selected from lithium, sodium or potassium. Specifically, A^+ is potassium. Alternatively, A^+ is H^+ .

R^1 and R^2 , along with the nitrogen to which they are attached, form a monocyclic heterocyclyl, wherein the heterocyclyl formed may contain 0, 1 or 2

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heteroatoms in addition to the nitrogen atom, wherein the heterocyclyl is optionally substituted by one or more groups selected from halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl, wherein the (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl is optionally substituted with halogen, hydroxy, (C₁-C₄)alkyl, or (C₁-C₄)alkoxy.

- 5 Specifically, R¹ and R², along with the nitrogen to which they are attached, form a heterocyclyl; and the heterocyclyl is selected from pyrrolidinyl, imidazolidinyl, pyrazolidinyl, dihydropyrrolyl, pyrrolyl, imidazolyl, pyrazolyl, triazolyl, oxazolidinyl, thiazolidinyl, oxazolyl, thiazolyl, piperidinyl, hydropyrimidinyl, piperazinyl, pyridinyl, pyridazinyl, pyrimidinyl, triazinyl, morpholinyl, and thiomorpholinyl, each of which is
10 optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl. Even more specifically, R¹ and R², along with the nitrogen to which they are attached, form a heterocyclyl; and the heterocyclyl is selected from pyrazolyl, piperidinyl, piperazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl, or a pharmaceutically
15 acceptable salt thereof, and the values and specific values of the remainder of the variables are as described for Structural Formula (I).

In a fifth specific embodiment of Structural Formula (I)

X is S;

- 20 A⁺ is selected from NH₄⁺, N(C₁-C₄alkyl)H₃⁺ (for example, N(methyl)H₃⁺ or N(ethyl)H₃⁺), N(benzyl)H₃⁺, and N(phenyl)H₃⁺. Specifically, A⁺ is N(benzyl)H₃⁺.

- R¹ and R², along with the nitrogen to which they are attached, form a monocyclic heterocyclyl, wherein the heterocyclyl formed may contain 0, 1 or 2 heteroatoms in addition to the nitrogen atom, wherein the heterocyclyl is optionally
25 substituted by one or more groups selected from halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl, wherein the (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl is optionally substituted with halogen, hydroxy, (C₁-C₄)alkyl, or (C₁-C₄)alkoxy.

Specifically, R¹ and R², along with the nitrogen to which they are attached, form a heterocyclyl; and the heterocyclyl is selected from pyrrolidinyl, imidazolidinyl,

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pyrazolidinyl, dihydropyrrolyl, pyrrolyl, imidazolyl, pyrazolyl, triazolyl, oxazolidinyl, thiazolidinyl, oxazolyl, thiazolyl, piperidinyl, hydropyrimidinyl, piperazinyl, pyridinyl, pyridazinyl, pyrimidinyl, triazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl.

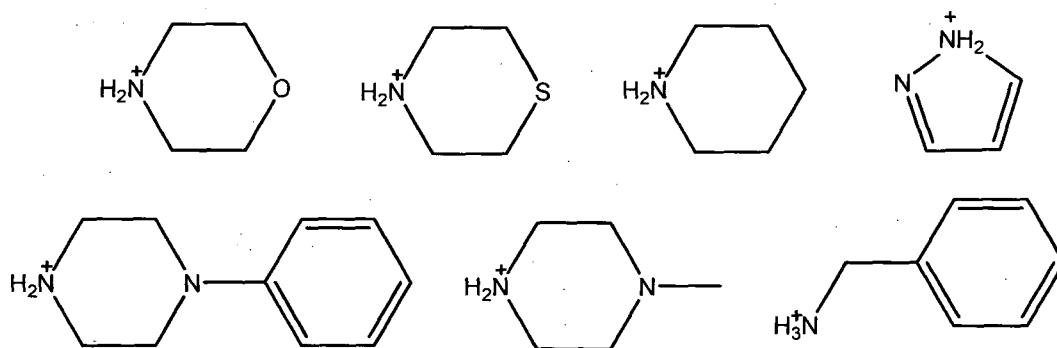
- 5 Even more specifically, R¹ and R², along with the nitrogen to which they are attached, form a heterocyclyl; and the heterocyclyl is selected from pyrazolyl, piperidinyl, piperazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl, or a pharmaceutically acceptable salt thereof, and the values and specific values of the remainder of the
- 10 variables are as described for Structural Formula (I).

In a sixth specific embodiment of Structural Formula (I)

X is S;

- A⁺ is selected from piperidinium, morpholin-4-ium, thiomorpholin-4-ium,
- 15 piperazin-1-ium, 4-methylpiperazin-1-ium, 4-phenyl-piperazin-1-ium, pyridinium, pyridazin-1-ium, pyrimidin-1-ium, pyrazin-1-ium, 1H-pyrrolium, 1H-pyrazol-1-ium, 1H-imidazol-1-ium, pyrazolidin-1-ium, pyrrolidinium, imidazolidin-1-ium.

Specifically, A⁺ is selected from:



- 20 R¹ and R², along with the nitrogen to which they are attached, form a monocyclic heterocyclyl, wherein the heterocyclyl formed may contain 0, 1 or 2 heteroatoms in addition to the nitrogen atom, wherein the heterocyclyl is optionally substituted by one or more groups selected from halogen, hydroxy, (C₁-C₄)alkyl, (C₁-

- 20 -

C₄)alkoxy, or phenyl, wherein the (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl is optionally substituted with halogen, hydroxy, (C₁-C₄)alkyl, or (C₁-C₄)alkoxy.

Specifically, R¹ and R², along with the nitrogen to which they are attached, form a heterocyclyl; and the heterocyclyl is selected from pyrrolidinyl, imidazolidinyl,

5 pyrazolidinyl, dihydropyrrolyl, pyrrolyl, imidazolyl, pyrazolyl, triazolyl, oxazolidinyl, thiazolidinyl, oxazolyl, thiazolyl, piperidinyl, hydroypyrimidinyl, piperazinyl, pyridinyl, pyridazinyl, pyrimidinyl, triazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl.

Even more specifically, R¹ and R², along with the nitrogen to which they are attached,
10 form a heterocyclyl; and the heterocyclyl is selected from pyrazolyl, piperidinyl, piperazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl, or a pharmaceutically acceptable salt thereof, and the values and specific values of the remainder of the variables are as described for Structural Formula (I).

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In a seventh specific embodiment of Structural Formula (I)

X is O;

A⁺ is selected from lithium, sodium or potassium. Specifically, A⁺ is potassium.

Alternatively, A⁺ is H⁺.

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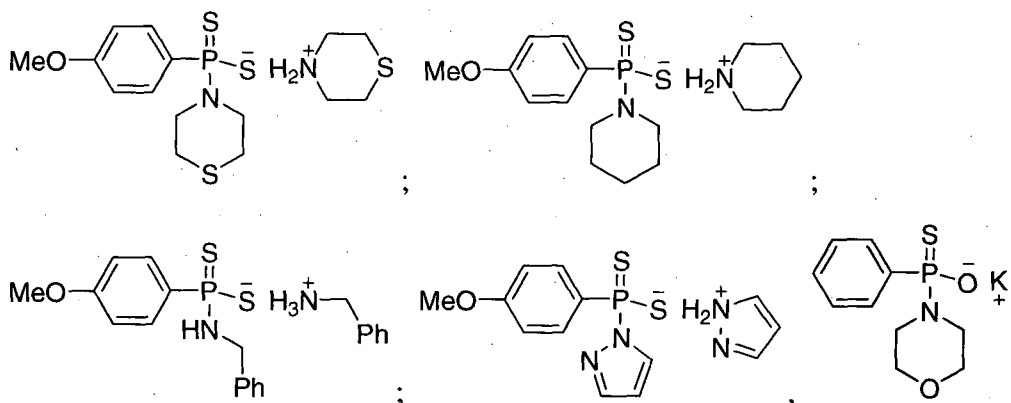
R¹ and R², along with the nitrogen to which they are attached, form a monocyclic heterocyclyl, wherein the heterocyclyl formed may contain 0, 1 or 2 heteroatoms in addition to the nitrogen atom, wherein the heterocyclyl is optionally substituted by one or more groups selected from halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl, wherein the (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl is optionally
25 substituted with halogen, hydroxy, (C₁-C₄)alkyl, or (C₁-C₄)alkoxy.

Specifically, R¹ and R², along with the nitrogen to which they are attached, form a heterocyclyl; and the heterocyclyl is selected from pyrrolidinyl, imidazolidinyl, pyrazolidinyl, dihydropyrrolyl, pyrrolyl, imidazolyl, pyrazolyl, triazolyl, oxazolidinyl, thiazolidinyl, oxazolyl, thiazolyl, piperidinyl, hydroypyrimidinyl, piperazinyl, pyridinyl,

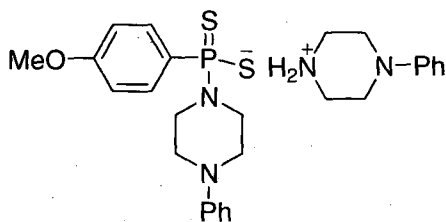
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- pyridazinyl, pyrimidinyl, triazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl. Even more specifically, R¹ and R², along with the nitrogen to which they are attached, form a heterocyclyl; and the heterocyclyl is selected from pyrazolyl, piperidinyl, piperazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl, or a pharmaceutically acceptable salt thereof, and the values and specific values of the remainder of the variables are as described for Structural Formula (I).

- Another embodiment is method of treating cancer in an individual in need thereof comprising administering an effective amount of compound represented by a one of the following structural formulas:



or



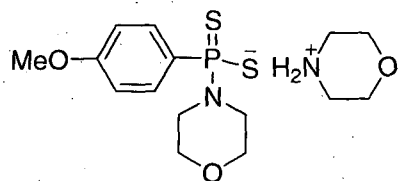
, or a pharmaceutically acceptable salt

thereof.

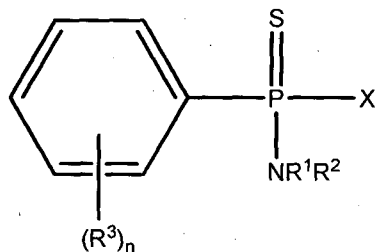
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Another embodiment is a method of treating cancer in an individual in need thereof comprising administering an effective amount of morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate (GY4137) to the individual.

Morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate, also referred to herein as GY4137, is represented by the following structural formula:



In an eighth specific embodiment of Structural Formula (I), the compound is represented by following Structural Formula (II):



10

(II), wherein

X is halogen, OMs or OTs. In a specific embodiment, X is Cl, OMs or OTs. In another specific embodiment, X is Cl. Alternatively, X is OMs. Alternatively, X is OTs.

R^1 and R^2 are each independently (a) hydrogen; or (b) (C_1-C_{10}) alkyl, (C_2-C_{10}) alkenyl, (C_2-C_{10}) alkynyl, aryl(C_0-C_3)alkyl, heteroaryl(C_0-C_3)alkyl, cycloalkyl(C_0-C_3)alkyl, heterocyclyl(C_0-C_3)alkyl, heteroaryl(C_0-C_3)alkyl, each optionally substituted with one to three groups represented by R^4 ; or

R^1 and R^2 , along with the nitrogen to which they are attached, form a monocyclic heterocyclyl, wherein the heterocyclyl formed may contain 0, 1 or 2 heteroatoms in addition to the nitrogen atom, wherein the heterocyclyl is optionally substituted by one or more groups selected from halogen, hydroxy, (C_1-C_4) alkyl, $(C_1-$

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(C₄)alkoxy, or phenyl, wherein the (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl is optionally substituted with halogen, hydroxy, (C₁-C₄)alkyl, or (C₁-C₄)alkoxy.

In a specific embodiment, R¹ and R², along with the nitrogen to which they are attached, form a heterocyclyl, wherein the heterocyclyl is selected from pyrrolidinyl, imidazolidinyl, pyrazolidinyl, dihydropyrrolyl, pyrrolyl, imidazolyl, pyrazolyl, triazolyl, oxazolidinyl, thiazolidinyl, oxazolyl, thiazolyl, piperidinyl, hydroypyrimidinyl, piperazinyl, pyridinyl, pyridazinyl, pyrimidinyl, triazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl. In a specific embodiment, R¹ and R², along with the nitrogen to which they are attached, form a heterocyclyl, wherein the heterocyclyl is selected from pyrazolyl, piperidinyl, piperazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl.

In an alternate embodiment, R¹ and R² are each independently (a) hydrogen; or (b) (C₁-C₁₀)alkyl, (C₂-C₁₀)alkenyl, (C₂-C₁₀)alkynyl, aryl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, cycloalkyl(C₀-C₃)alkyl, heterocyclyl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, each optionally substituted with one to three groups represented by R⁴. In a specific embodiment, R¹ and R² are each independently (a) hydrogen; or (b) (C₁-C₄)alkyl, (C₂-C₄)alkenyl, (C₂-C₄)alkynyl, (C₃-C₈)cycloalkyl, phenyl, benzyl, each optionally substituted with one to three groups represented by R⁴. In a specific embodiment, R¹ and R² are the same. In an alternative specific embodiment, R¹ is different from R².

In a specific embodiment, R¹ is hydrogen and R² is selected from (C₁-C₁₀)alkyl, (C₂-C₁₀)alkenyl, (C₂-C₁₀)alkynyl, aryl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, cycloalkyl(C₀-C₃)alkyl, heterocyclyl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, each optionally substituted with one to three groups represented by R⁴. In another specific embodiment, R¹ is hydrogen or (C₁-C₄)alkyl; and R² is (C₁-C₄)alkyl or benzyl. In another specific embodiment, R¹ is hydrogen and R² is benzyl.

Each R³ is independently selected from halogen, hydroxy, (C₁-C₄)alkyl, halo(C₁-C₄)alkyl, hydroxy(C₁-C₄)alkyl, (C₁-C₄)alkoxy, halo(C₁-C₄)alkoxy, and hydroxy(C₁-

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C₄)alkoxy. In a specific embodiment, R³ is halogen, hydroxy, methyl, ethyl, propyl, isopropyl, butyl, t-butyl, hydroxy methyl, hydroxyethyl, hydroxymethoxy, hydroxyethoxy, methoxy, ethoxy, propoxy, butoxy, t-butyloxy, or -CF₃.

Each R⁴ is independently selected from halogen, hydroxy, (C₁-C₄)alkyl, halo(C₁-C₄)alkyl, hydroxy(C₁-C₄)alkyl, (C₁-C₄)alkoxy, halo(C₁-C₄)alkoxy, and hydroxy(C₁-C₄)alkoxy. In a specific embodiment, R³ is halogen, hydroxy, methyl, ethyl, propyl, isopropyl, butyl, t-butyl, hydroxy methyl, hydroxyethyl, hydroxymethoxy, hydroxyethoxy, methoxy, ethoxy, propoxy, butoxy, t-butyloxy, or -CF₃.

In one embodiment, n is 0-3. In another specific embodiment, n is 0. In a specific embodiment m is 0 or 1. Alternatively, n is 1, 2 or 3. In a specific embodiment, n is 1. In another specific embodiment, n is 2. In yet another specific embodiment, n is 3.

In a first specific embodiment of Structural Formula (II)

X is Cl;

R¹ and R² are each independently (a) hydrogen; or (b) (C₁-C₁₀)alkyl, (C₂-C₁₀)alkenyl, (C₂-C₁₀)alkynyl, aryl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, cycloalkyl(C₀-C₃)alkyl, heterocyclyl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, each optionally substituted with one to three groups represented by R⁴. Specifically, R¹ and R² are each independently (a) hydrogen; or (b) (C₁-C₄)alkyl, (C₂-C₄)alkenyl, (C₂-C₄)alkynyl, (C₃-C₈)cycloalkyl, phenyl, benzyl, each optionally substituted with one to three groups represented by R⁴. More specifically, R¹ is hydrogen or (C₁-C₄)alkyl; and R² is (C₁-C₄)alkyl or benzyl. Even more specifically, R¹ is hydrogen and R² is benzyl, or a pharmaceutically acceptable salt thereof, and the values and specific values of the remainder of the variables are as described for Structural Formula (II).

In a second specific embodiment of Structural Formula (II)

X is Cl;

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R¹ and R², along with the nitrogen to which they are attached, form a monocyclic heterocyclyl, wherein the heterocyclyl formed may contain 0, 1 or 2 heteroatoms in addition to the nitrogen atom, wherein the heterocyclyl is optionally substituted by one or more groups selected from halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl, wherein the (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl is optionally substituted with halogen, hydroxy, (C₁-C₄)alkyl, or (C₁-C₄)alkoxy.

Specifically, R¹ and R², along with the nitrogen to which they are attached, form a heterocyclyl; and the heterocyclyl is selected from pyrrolidinyl, imidazolidinyl, pyrazolidinyl, dihydropyrrolyl, pyrrolyl, imidazolyl, pyrazolyl, triazolyl, oxazolidinyl, thiazolidinyl, oxazolyl, thiazolyl, piperidinyl, hydroxypyrimidinyl, piperazinyl, pyridinyl, pyridazinyl, pyrimidinyl, triazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl. Even more specifically, R¹ and R², along with the nitrogen to which they are attached, form a heterocyclyl; and the heterocyclyl is selected from pyrazolyl, piperidinyl, piperazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl, or a pharmaceutically acceptable salt thereof, and the values and specific values of the remainder of the variables are as described for Structural Formula (II).

In a third specific embodiment of Structural Formula (II)

X is OMs;

R¹ and R² are each independently (a) hydrogen; or (b) (C₁-C₁₀)alkyl, (C₂-C₁₀)alkenyl, (C₂-C₁₀)alkynyl, aryl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, cycloalkyl(C₀-C₃)alkyl, heterocyclyl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, each optionally substituted with one to three groups represented by R⁴. Specifically, R¹ and R² are each independently (a) hydrogen; or (b) (C₁-C₄)alkyl, (C₂-C₄)alkenyl, (C₂-C₄)alkynyl, (C₃-C₈)cycloalkyl, phenyl, benzyl, each optionally substituted with one to three groups represented by R⁴. More specifically, R¹ is hydrogen or (C₁-C₄)alkyl; and R² is (C₁-C₄)alkyl or benzyl. Even more specifically, R¹ is hydrogen and R² is benzyl, or a

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pharmaceutically acceptable salt thereof, and the values and specific values of the remainder of the variables are as described for Structural Formula (II).

In a fourth specific embodiment of Structural Formula (II)

5 X is OMs;

R¹ and R², along with the nitrogen to which they are attached, form a monocyclic heterocyclyl, wherein the heterocyclyl formed may contain 0, 1 or 2 heteroatoms in addition to the nitrogen atom, wherein the heterocyclyl is optionally substituted by one or more groups selected from halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl, wherein the (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl is optionally substituted with halogen, hydroxy, (C₁-C₄)alkyl, or (C₁-C₄)alkoxy.

Specifically, R¹ and R², along with the nitrogen to which they are attached, form a heterocyclyl; and the heterocyclyl is selected from pyrrolidinyl, imidazolidinyl, pyrazolidinyl, dihydropyrrolyl, pyrrolyl, imidazolyl, pyrazolyl, triazolyl, oxazolidinyl, thiazolidinyl, oxazolyl, thiazolyl, piperidinyl, hydroxypyrimidinyl, piperazinyl, pyridinyl, pyridazinyl, pyrimidinyl, triazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl. Even more specifically, R¹ and R², along with the nitrogen to which they are attached, form a heterocyclyl; and the heterocyclyl is selected from pyrazolyl, piperidinyl, piperazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl, or a pharmaceutically acceptable salt thereof, and the values and specific values of the remainder of the variables are as described for Structural Formula (II).

25 In a fifth specific embodiment of Structural Formula (II)

X is OTs;

R¹ and R² are each independently (a) hydrogen; or (b) (C₁-C₁₀)alkyl, (C₂-C₁₀)alkenyl, (C₂-C₁₀)alkynyl, aryl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, cycloalkyl(C₀-C₃)alkyl, heterocyclyl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, each optionally substituted

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with one to three groups represented by R^4 . Specifically, R^1 and R^2 are each independently (a) hydrogen; or (b) (C₁-C₄)alkyl, (C₂-C₄)alkenyl, (C₂-C₄)alkynyl, (C₃-C₈)cycloalkyl, phenyl, benzyl, each optionally substituted with one to three groups represented by R^4 . More specifically, R^1 is hydrogen or (C₁-C₄)alkyl; and R^2 is (C₁-C₄)alkyl or benzyl. Even more specifically, R^1 is hydrogen and R^2 is benzyl, or a pharmaceutically acceptable salt thereof, and the values and specific values of the remainder of the variables are as described for Structural Formula (II).

In a sixth specific embodiment of Structural Formula (II)

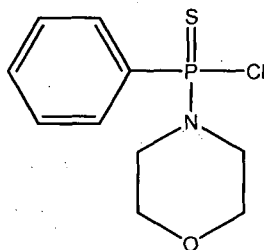
10 X is OTs;

R^1 and R^2 , along with the nitrogen to which they are attached, form a monocyclic heterocyclyl, wherein the heterocyclyl formed may contain 0, 1 or 2 heteroatoms in addition to the nitrogen atom, wherein the heterocyclyl is optionally substituted by one or more groups selected from halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl, wherein the (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl is optionally substituted with halogen, hydroxy, (C₁-C₄)alkyl, or (C₁-C₄)alkoxy.

Specifically, R^1 and R^2 , along with the nitrogen to which they are attached, form a heterocyclyl; and the heterocyclyl is selected from pyrrolidinyl, imidazolidinyl, pyrazolidinyl, dihydropyrrolyl, pyrrolyl, imidazolyl, pyrazolyl, triazolyl, oxazolidinyl, thiazolidinyl, oxazolyl, thiazolyl, piperidinyl, hydroxypyrimidinyl, piperazinyl, pyridinyl, pyridazinyl, pyrimidinyl, triazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl. Even more specifically, R^1 and R^2 , along with the nitrogen to which they are attached, form a heterocyclyl; and the heterocyclyl is selected from pyrazolyl, piperidinyl, piperazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl, or a pharmaceutically acceptable salt thereof, and the values and specific values of the remainder of the variables are as described for Structural Formula (II).

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In a specific embodiment, the Structural Formula (II) is represented by the following structure:



, or a pharmaceutically acceptable salt thereof.

- 5 Certain of the disclosed compounds may exist in various stereoisomeric forms. Stereoisomers are compounds that differ only in their spatial arrangement. Enantiomers are pairs of stereoisomers whose mirror images are not superimposable, most commonly because they contain an asymmetrically substituted carbon atom that acts as a chiral center. "Enantiomer" means one of a pair of molecules that are mirror
- 10 images of each other and are not superimposable. Diastereomers are stereoisomers that are not related as mirror images, most commonly because they contain two or more asymmetrically substituted carbon atoms. The symbol "*" in a structural formula represents the presence of a chiral carbon center. "R" and "S" represent the configuration of substituents around one or more chiral carbon atoms. Thus, "R*" and
- 15 "S*" denote the relative configurations of substituents around one or more chiral carbon atoms.

"Racemate" or "racemic mixture" means a compound of equimolar quantities of two enantiomers, wherein such mixtures exhibit no optical activity; i.e., they do not rotate the plane of polarized light.

- 20 "Geometric isomer" means isomers that differ in the orientation of substituent atoms in relationship to a carbon-carbon double bond, to a cycloalkyl ring, or to a bridged bicyclic system. Atoms (other than H) on each side of a carbon-carbon double bond may be in an E (substituents are on opposite sides of the carbon-carbon double bond) or Z (substituents are oriented on the same side) configuration.

“R,” “S,” “S*,” “R*,” “E,” “Z,” “cis,” and “trans,” indicate configurations relative to the core molecule.

The compounds of the invention may be prepared as individual isomers by either isomer-specific synthesis or resolved from an isomeric mixture. Conventional resolution techniques include forming the salt of a free base of each isomer of an isomeric pair using an optically active acid (followed by fractional crystallization and regeneration of the free base), forming the salt of the acid form of each isomer of an isomeric pair using an optically active amine (followed by fractional crystallization and regeneration of the free acid), forming an ester or amide of each of the isomers of an isomeric pair using an optically pure acid, amine or alcohol (followed by chromatographic separation and removal of the chiral auxiliary), or resolving an isomeric mixture of either a starting material or a final product using various well known chromatographic methods.

When the stereochemistry of a disclosed compound is named or depicted by structure, the named or depicted stereoisomer is at least 60%, 70%, 80%, 90%, 99% or 99.9% by weight pure relative to the other stereoisomers. When a single enantiomer is named or depicted by structure, the depicted or named enantiomer is at least 60%, 70%, 80%, 90%, 99% or 99.9% by weight optically pure. Percent optical purity by weight is the ratio of the weight of the enantiomer over the weight of the enantiomer plus the weight of its optical isomer.

When a disclosed compound is named or depicted by structure without indicating the stereochemistry, and the compound has at least one chiral center, it is to be understood that the name or structure encompasses one enantiomer of compound free from the corresponding optical isomer, a racemic mixture of the compound and mixtures enriched in one enantiomer relative to its corresponding optical isomer.

When a disclosed compound is named or depicted by structure without indicating the stereochemistry and has at least two chiral centers, it is to be understood that the name or structure encompasses a diastereomer free of other diastereomers, a pair of diastereomers free from other diastereomeric pairs, mixtures of diastereomers,

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mixtures of diastereomeric pairs, mixtures of diastereomers in which one diastereomer is enriched relative to the other diastereomer(s) and mixtures of diastereomeric pairs in which one diastereomeric pair is enriched relative to the other diastereomeric pair(s).

The compounds of the invention may be present in the form of

- 5 pharmaceutically acceptable salts. For use in medicines, the salts of the compounds of the invention refer to non-toxic "pharmaceutically acceptable salts." Pharmaceutically acceptable salt forms include pharmaceutically acceptable acidic/anionic or basic/cationic salts.

- Pharmaceutically acceptable acidic/anionic salts include, the acetate,
10 benzenesulfonate, benzoate, bicarbonate, bitartrate, bromide, calcium edetate, camsylate, carbonate, chloride, citrate, dihydrochloride, edetate, edisylate, estolate, esylate, fumarate, glyceptate, gluconate, glutamate, glycolylarsanilate, hexylresorcinate, hydrobromide, hydrochloride, hydroxynaphthoate, iodide, isethionate, lactate, lactobionate, malate, maleate, malonate, mandelate, mesylate,
15 methylsulfate, mucate, napsylate, nitrate, pamoate, pantothenate, phosphate/diphosphate, polygalacturonate, salicylate, stearate, subacetate, succinate, sulfate, hydrogensulfate, tannate, tartrate, teoclate, tosylate, and triethiodide salts.

- Pharmaceutically acceptable basic/cationic salts include, the sodium, potassium, calcium, magnesium, diethanolamine, N-methyl-D-glucamine, L-lysine, L-
20 arginine, ammonium, ethanolamine, piperazine and triethanolamine salts.

As used herein an "individual" refers to an animal, and in a particular aspect, a mammal. Examples of mammals include primates, a canine, a feline, a rodent, and the like. Specific examples include humans, dogs, cats, horses, cows, sheep, goats, rabbits, guinea pigs, rats and mice.

- 25 The term "individual in need thereof" refers to an individual who is in need of treatment or prophylaxis as determined by a researcher, veterinarian, medical doctor or other clinician. In one embodiment, an individual in need thereof is a mammal, such as a human.

The need or desire for administration according to the methods of the present invention is determined via the use of well known risk factors. The effective amount of a (one or more) particular compound is determined, in the final analysis, by the physician in charge of the case, but depends on factors such as the exact cancer to be treated, the severity of the cancer from which the patient suffers, the chosen route of administration, other drugs and treatments which the patient may concomitantly require, and other factors in the physician's judgment.

As used herein, "effective amount" or "therapeutically effective amount" means an amount of the active compound that will elicit the desired biological or medical response in a tissue, system, subject, or human, which includes alleviation of the symptoms, in whole or in part, of the cancer being treated. The novel methods of treatment of this invention are for cancer disorders known to those skilled in the art. In a particular aspect the amount (dosage) of H₂S that is administered is from about 100 mg/kg to about 300mg/kg (*e.g.*, about 100mg/kg; about 125mg/kg; about 150mg/kg; about 175mg/kg; about 200mg/kg, about 225mg/kg; about 250mg/kg; about 275mg/kg; about 300 mg/kg) . In another aspect, the dosage is from about 200μM to about 1000μM, and in other aspects, the dosage is about 200μM, 300μM, 400μM , 500μM , 600μM , 700μM, 800μM, 900μM or 1000μM.

The compound can be administered in a single dose (*e.g.*, in a day) or in multiple doses. In addition, the compound can be administered in one or more days (*e.g.* over several consecutive days or non-consecutive days). In particular aspects, the compound can be administered over 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 31 days, *etc.*).

In a particular aspect, the effective concentration of the H₂S generated in the individual and/or cell delivered by the compound is about or less than about 20μM over one or more hours or one or more days. In other aspects, the effective concentration of the H₂S generated in the individual and/or cell delivered by the compound is about or

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less than about 20 μ M, 19 μ M, 18 μ M, 17 μ M, 16 μ M, 15 μ M, 14 μ M, 13 μ M, 12 μ M, 11 μ M, 10 μ M, 9 μ M, 8 μ M, 7 μ M, 6 μ M, 5 μ M, 4 μ M, 3 μ M, 2 μ M, 1 μ M or a combination thereof over one or more hours or one or more days.

The chemical compounds used in the methods described herein can be administered to a subject as part of a pharmaceutical composition. Formulations will vary according to the route of administration selected (*e.g.*, solution, emulsion or capsule). A "pharmaceutical composition" comprises a (one or more) chemical compound described herein as the active ingredient and inert ingredient(s), such as pharmaceutically acceptable excipients, that make up the carrier. Standard pharmaceutical formulation techniques can be employed, such as those described in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA. Suitable pharmaceutical carriers for parenteral administration include, for example, sterile water, physiological saline, bacteriostatic saline (saline containing about 0.9% mg/ml benzyl alcohol), phosphate-buffered saline, Hank's solution, Ringer's lactate and the like. Formulations can also include small amounts of substances that enhance the effectiveness of the active ingredient (*e.g.*, emulsifying, solubilizing, pH buffering, wetting agents). Methods of encapsulation compositions (such as in a coating of hard gelatin or cyclodextran) are known in the art. For inhalation, the agent can be solubilized and loaded into a suitable dispenser for administration (*e.g.*, an atomizer or nebulizer or pressurized aerosol dispenser).

Any suitable route of administration can be used, for example, oral, dietary, topical, transdermal, rectal, parenteral (*e.g.*, intravenous, intraarterial, intramuscular, subcutaneous injection, intradermal injection), inhalation (*e.g.*, intrabronchial, intranasal or oral inhalation, intranasal drops), ocular, pulmonary, nasal, and the like may be employed. Administration can be local or systemic as indicated. The preferred mode of administration can vary depending on the particular agent chosen. Suitable dosage forms include tablets, troches, dispersions, suspensions, solutions, capsules, creams, ointments, aerosols, and the like.

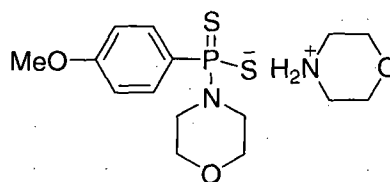
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Exemplification

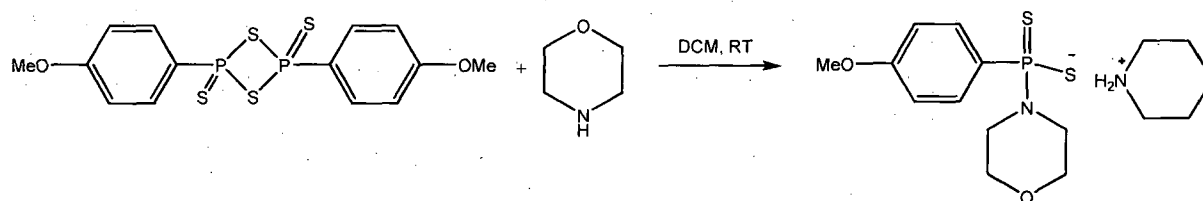
Example 1 Example Synthesis of GYY4137

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GYY4137



Chemical Synthesis of GYY4137:



10 Morpholine (20 mmol) in methyl chloride (CH_2Cl_2 , 6 mL) is added dropwise (room temperature) to a CH_2Cl_2 solution (6 mL) of 2,4-bis(4-methoxyphenyl)-2,4-dithioxo-1,3,2,4-dithiadiphosphetane (4.0 mmol). The reaction mixture is stirred at room temperature for 2 hours. The precipitate is filtered and washed several times with CH_2Cl_2 . The product is a white solid (67% yield) and pure as determined by ^1H nuclear magnetic resonance.

15

Example 2 The Slow-releasing Hydrogen Sulfide Donor, GYY4137, Exhibits Novel Anti-Cancer Effects *In Vitro* and *In Vivo*

Material and Methods

20 Chemical synthesis of GYY4137 and ZYJ1122

GYY4137 was synthesized chemically in house as described previously in Li, L., *et al.*, *Circulation*, 117(18):2351-2360 (2008) which is incorporated herein by

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reference. ZYJ1122 (morpholin-4-ium diphenylphosphinate) was synthesized as follows. To a solution of diphenylphosphinic acid (1.0 mmol, 1.0 equiv.) in dichloromethane (DCM; 2 ml) at room temperature, morpholine (2.0 mmol, 2.0 equiv.) was added drop-wise. The reaction was stirred at the same temperature for 1 h and the product subsequently collected by suction filtration. The pure product was obtained after washing with cold DCM. White solid was obtained as 56% yield. ^1H NMR (500 MHz, CDCl_3 , ppm): δ = 7.77-7.74 (m, 4H), 7.38-7.32 (m, 6H), 3.77-3.75 (m, 4H), 2.95-2.93 (m, 4H); LRMS (ESI) m/z 217.2 (M^+).

10 Measurement of H_2S

The generation of H_2S from either NaHS (Sigma), GYY4137 or ZYJ1122 (all 400 μM) was determined in aliquots (100 μl) withdrawn at timed intervals (up to 7 days) from cultured MCF-7 cells maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) as described below. The concentration of H_2S (determined as a combination of free H_2S , HS^- and S^{2-}) was measured spectrophotometrically as described previously (Huang, S., *et al.*, *J Mol Biol*, 396(3):708-718 (2010)). Briefly, medium (100 μl) was mixed with 0.85% w/v zinc acetate/3% NaOH mixture (1:1 ratio, 100 μl). Methylene blue was then formed by the addition of N,N-dimethyl-p phenylenediamine-dihydrochloride dye and FeCl_3 (final concentrations, 2.5 mM and 3.3 mM respectively) and absorbance subsequently monitored at 670 nm. The concentration of H_2S (defined as above) was determined using a standard curve of NaHS (0-160 μM ; $R^2=0.9987$).

Cell culture and cell viability

Human cervical carcinoma (HeLa), colorectal carcinoma (HCT-116), hepatocellular carcinoma (Hep G2), osteosarcoma (U2OS), breast adenocarcinoma (MCF-7) and human diploid lung fibroblasts (IMR90 and WI-38) were cultured in DMEM supplemented with 10% v/v fetal bovine serum (FBS; HyClone), penicillin/streptomycin (100 U/ml; Sigma) and L-glutamine (2 mM; Caisson) at 37°C in an atmosphere of 5% CO_2 . Human acute promyelocytic leukemia cells (HL-60) were

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cultured in DMEM containing 20% v/v FBS whilst human myelomonocytic leukemia (MV4-11) cells were cultured in RPMI with 10% v/v FBS under the same incubation conditions. Live cell populations incubated with either NaHS, GYY4137 or ZYJ1122 (400 or 800 μ M) were collected after 5 days and counted in triplicate after staining with trypan blue using a haemocytometer. In addition, concentration response for GYY4137 (100-1000 μ M) were generated in MCF-7, HL-60 and MV4-11 exposed to drugs for 5 days and the ability to reduce survival assessed as IC₅₀ values. Colony formation using MCF-7 cells was also assessed by a clonogenic survival assay as described elsewhere (Franken, NAP, *et al.*, *Nat Proc*, 1(5):2315-2319 (2006)). Briefly, MCF-7 cells (10,000) were seeded in triplicate in 6 well plates in the presence of GYY4137, NaHS or ZYJ1122 (200 to 600 μ M) for 10 days until colonies were readily visible. Colonies were then stained with crystal violet (5% w/v) and the representative pictures were captured using a ChemiGenius 2 Bio Imaging System (SynGene Ltd).

Effect of GYY4137 on tumor growth

All experimental protocols used have been described previously (Shen, J., *Exp Hematol* 35(1):75-83 (2007)). Briefly, female, severe combined immunodeficiency (SCID) mice (BALB-16, 17-20 g, 4-6 weeks old) were bred in house and maintained throughout in specific pathogen-free (SPF) isolators. Exponentially growing HL-60 and MV4-11 cells (1×10^7) cultured as described above (>95% viability) were washed twice in phosphate-buffered saline and injected subcutaneously into loose skin between the shoulder blades and left front leg of recipient mice. Animals were treated with GYY4137 (100-300 mg/kg/day, i.p.) or saline (1 ml/kg/day, i.p.) for 14 days commencing 14 days after injection of cells at which point mice displayed palpable tumors of approx. 100 mm³. All animals were closely monitored, weighed, and tumor size was measured at daily intervals. For tumor size measurement, length (L) and width (W) of the tumor were measured with a caliper, and tumor volume (TV) was calculated as $TV = (L \times W^2)/2$. All experiments were reviewed and conducted with the approval of

the Institutional Animal Care and Use Committee (IACUC) of National University of Singapore.

Cell Cycle Analysis and western blotting

5 MCF-7 cells (40,000) were incubated in 6 well plates in the presence or absence of GYY4137 (400 μ M) for either 5 or 8 days. Cells treated with ZYJ1122 were used as control. To analyze the cell cycle profile, cells were fixed with 70% v/v ethanol on ice for at least 2 h and then stained in propidium iodide solution (20 μ g/ml propidium iodide, 100 μ g/ml RNase A and 0.1% v/v Triton X-100) for 15 min at 37°C. Stained
10 cells were then subject to DNA content analysis by flow cytometry (Dako CyAn ADP) and the data obtained was processed using Summit software (Beckman Coulter). Cell lysates of MCF-7 were subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in Tris buffered saline (TBS) containing non-fat dry milk (5% w/v) and thereafter incubated with the relevant
15 primary antibody (1 μ g/ml) at 4°C overnight. Antibodies used were; α -PARP, α -cleaved-PARP, α -cleaved-caspase 9 (all Cell Signaling Ltd.) using α -tubulin (Sigma) as a loading control.

Statistical analysis

20 Cell survival, IC₅₀ and tumor volumes were expressed as mean $\pm$ standard error (SEM). For *in vitro* studies, cell survival of both non-treatment (NT) and treatment groups was analyzed using one-way ANOVA followed by a post-hoc t test. For *in vivo* studies, the comparisons between vehicle control group and different dosage treatments was analyzed using linear mixed model for longitudinal data analysis by SPSS software
25 (IBM). P < 0.05 was considered significant.

Results

Release of H₂S from NaHS and GYY4137 in culture medium

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Incubation of either NaHS or GYY4137 in culture medium resulted in the release of detectable amounts of H₂S as reflected by an increase in concentration of H₂S (μM) following removal of aliquots and assay for methylene blue formation. Release of H₂S from NaHS was rapid - peaking at or before 20 min and declining to undetectable levels by 90 min. In stark contrast, H₂S release from GYY4137 was much lower (<10% of that observed with NaHS) but was sustained, remaining higher than baseline for up to 7 days. No release of H₂S was apparent from ZYJ1122, a control for GYY4137 lacking sulfur and thus unable to form H₂S, under the same experimental conditions for up to 7 days (Figure 1A). The chemical structures of GYY4137 and ZYJ1122 are shown in the inset to Figure 1A.

Effect of NaHS and GYY4137 on cell growth and viability

The effect of NaHS, GYY4137 and ZYJ1122 on growth of three cancer cell lines i.e. MCF-7, MV4-11 and HL-60, was monitored for 5 days. At each indicated interval, the number of live cells from each treatment group was recorded in triplicates. GYY4137 (400 μM) significantly reduced cell proliferation of all three cancer lines whereas both NaHS and ZYJ1122 were inactive (Figure 1B).

To determine the effect of two different concentrations (400 μM and 800 μM) of GYY4137, NaHS and ZYJ1122 on a wider panel of human cancer cell lines, cell survival of a further four cancer cell lines of different origins i.e. cervical carcinoma (HeLa), colorectal carcinoma (HCT-116), hepatocellular carcinoma (Hep G2) and osteosarcoma (U2OS) cells was determined in comparison with two normal human diploid fibroblasts (WI-38 and IMR90) (Figure 2A). Over a 5-day culture period, NaHS (400 μM) failed to influence the survival of any of the seven cancer lines tested. In contrast, the effect of GYY4137 on cell survival was much more profound with 30-70% (P<0.01) death in all cancer cell lines at the same concentration. A higher concentration of NaHS (800 μM) resulted in a further, albeit small, reduction in HCT-116, Hep G2 and MCF-7 cell survival (approximately 15-30%) although again no significant difference in cell survival was apparent in HeLa, HL-60, U2OS and MV4-11 cells. In

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contrast, GYY4137 at the same concentration, markedly reduced survival by approximately 75-95% in all cancer cell lines. The absolute degree of cell death caused by GYY4137 varied between cancer cell lines with greatest effect in HepG2, HL-60, MV4-11, MCF-7 and U2OS cells and least effect in HCT-116 and HeLa cells. For this reason, subsequent experiments were conducted using one or more of HL-60, MCF-7 and MV4-11 cancer cells. Importantly, neither NaHS nor GYY4137 significantly changed the survival of human non-cancer WI-38 and IMR90 cells. The sulfur-lacking control compound, ZYJ1122, was without significant effect on the survival of any cell line, suggesting that the observed effects of GYY4137 on cancer cells are likely due to H₂S release. The concentration response relationship for GYY4137 (100-1000 μ M) to reduce cell survival was also examined in MCF-7, HL-60 and MV4-11 cells. The IC₅₀ values for this compound were 337.1 ± 15.4 , 389.3 ± 16.8 and 341.8 ± 21.2 μ M (all n=3) respectively (Figure 2B).

The effect of NaHS, GYY4137 and ZYJ1122 (200-600 μ M) on survival of MCF-7 cells was also assessed in vitro using a clonogenic assay. Representative photographs are shown in Figure 2C. For these experiments, MCF-7 cells were plated in the presence or absence of drugs and cultured over a 10 day period. GYY4137 caused a concentration dependent loss of cell colony formation which was close to maximal at a concentration of 600 μ M. Cell loss was not apparent in either NaHS or ZYJ1122 treated samples.

The effect of GYY4137 (400 μ M, 5 or 8 days) on MCF-7 cells was also examined using cell cycle analysis. The sub-G1 population of MCF-7 cells exposed to GYY4137 was significantly higher ($P < 0.05$) compared either to non-treated cells or cells exposed to the same concentration of ZYJ1122 on day 5 (Figure 3A). Thus, the sub-G1 population of cells treated with GYY4137 represented 7.5% of the total cell population at day 5 and 14.8% at day 8 of treatment compared with approximately 1% of cells which either did not receive treatment or were exposed to ZYJ1122 (Figure 3A). In addition, there was a significant accumulation of 4N-DNA cell population in cells treated with GYY4137 (to 18.6% and 26.6% after 5 and 8 days of incubation

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respectively) as compared to either untreated (14.8%) or ZYJ1122-treated (14%) cells (Figure 3A). In further experiments, the possibility that GYY4127 triggers cancer cell death by promoting apoptosis was also studied. A strong signal for cleaved-PARP and activated caspase 9 was detected in MCF-7 samples treated with GYY4137 (400 μ M, 5
5 days) with a greatly reduced signal in cells treated with ZYJ1122 (Figure 3B). Interestingly, no cleavage of PARP and no activation of caspase 9 were observed in IMR90 cells incubated with either GYY4137 or ZYJ1122.

Effect of GYY4137 on tumor growth *in vivo*

10 Subcutaneous transplantation of either HL-60 or MV4-11 cells resulted in time-dependent tumor growth in the SCID mouse (Figure 4A, 4B). Tumor volume at the end of the experiment was 3024 ± 220 mm and 1166 ± 199 mm ($n=4-6$) in animals receiving daily vehicle injection and administered HL-60 and MV4-11 cells respectively. Administration of GYY4137 on a daily basis resulted in a significant
15 ($P<0.05$) dose related inhibition of tumor growth in both sets of animals. GYY4137 (at the highest dose used i.e. 300 mg/kg) administered daily for 14 days reduced tumor volume by $52.5 \pm 9.2\%$ ($n=6$) and $55.3 \pm 5.7\%$ ($n=4$) in HL-60 and MV4-11 injected animals. Although not measured objectively in these experiments, GYY4137 treatment did not affect animal weight or gross behavior.

20

Discussion

Reported herein is that (i) GYY4137 (but not NaHS) causes a concentration-dependent reduction in cancer cell survival, (ii) neither GYY4137 nor NaHS, using identical concentrations and experimental conditions, affected the survival of normal
25 i.e. non-cancer cells, (iii) GYY4137 promoted cancer cell (MCF-7) but not normal cell (IMR90) apoptosis as indicated by measurement of sub-G1 population and by observation of cleaved PARP and cleaved caspase 9 and triggered cell cycle arrest of MCF-7 cells in the G₂/M phase, (iv) the H₂S concentration detected in medium containing MCF-7 cells exceeded 'basal' levels for up to 7 days after exposure to

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GY4137 but for less than 2 h after exposure to NaHS, (v) ZYJ1122, a control for GY4137 lacking sulfur and thus unable to form H₂S, was inactive in all cases and, (vi) GY4137 administered daily to immunodeficient mice for 14 days caused a dose-dependent reduction in tumor growth elicited by prior injection of one of two human leukemia cell lines.

Thus, the present data reveals, for the first time, an anti-cancer effect of GY4137. All cancer cells tested were susceptible to this compound albeit to different extents. It is now well established that NaHS releases large amounts of H₂S over a short time period. In the experiments described herein it is shown that GY4137, like NaHS, also releases H₂S following incubation in culture medium containing MCF-7 cells thereby confirming our previous observation of spontaneous H₂S generation in aqueous media (Li, LI, *et al.*, *Circulation* 117(18):2351-2360 (2008)). Since ZYJ1122 exhibited no anti-cancer activity in any of the *in vitro* models we conclude that the anti-cancer activity of both GY4137 and NaHS (at high concentration) is likely to be H₂S-dependent. Perhaps surprisingly, GY4137 exhibited greater cancer cell killing activity than did NaHS *in vitro* even though it led to markedly lower concentrations of H₂S in the cell medium. Thus, optimal killing of cancer cells by H₂S under these experimental conditions would appear to occur at low concentrations of the gas spread over a period of several days as opposed to a much higher concentration achieved over a shorter time frame following exposure of cells to NaHS. It should be noted that whilst relatively high concentrations of GY4137 (i.e. 400-800 μ M) are required for this effect the effective concentration of H₂S generated is much less i.e. <20 μ M based on measurements in culture medium. However, the possibility that GY4137 may accumulate inside cancer cells and thereby release larger amounts of H₂S intracellularly cannot be ruled out. With this in mind, the present data implies that the rate at which cells are exposed to H₂S as well as the concentration of H₂S encountered is important in determining the ability of this gas to promote cell killing.

Interestingly, neither GY4137 nor NaHS caused significant killing of normal i.e. non-cancer cells suggesting that the effect of both H₂S donors is specific for cancer

cells. The mechanism of action of the cancer cell killing effect of GYY4137 has also been investigated. Treatment of MCF-7 cells with GYY4137 resulted in cell cycle arrest in the G₂/M phase and promotion of apoptosis as evidenced by increased sub-G1 population as well as the presence of both cleaved PARP and cleaved caspase 9. No significant effect either on cell cycle or on apoptosis was apparent in ZYJ1122-treated cells again suggesting that both of these effects were secondary to the sustained exposure of cells to low levels of H₂S. The ability of H₂S to cause cancer cell killing in this way has not previously been reported. However, H₂S has previously been shown to affect both cell cycle and apoptosis. For example, H₂S promotes cell cycle entry and proliferation of intestinal IEC-18 cell *in vitro* by activating MAPK (Deplancke, B., *et al.*, *FASEB J*, 17(10):1310-1312 (2003)). H₂S can also exhibit both pro- (e.g. (Yang, G., *et al.*, *FASEB J*, 18(14):1782-1784 (2004)) and anti- (e.g. (Hu, LF, *et al.*, *Mol Pharmacol*, 75(1):27-34 (2009)) apoptotic activity depending on the cell type studied and the experimental conditions, particularly the concentration of H₂S used. A number of potential molecular targets have been implicated in the effect of H₂S on apoptosis including p38 and caspase-3 (Hu, LF, *et al.*, *Mol Pharmacol*, 75(1):27-34 (2009); Rinaldi, L., *et al.*, *Lab Invest*, 86(4):391-397 (2006)), other MAPK such as MEK and JNK (Yonezawa, D., *et al.*, *Toxicology*, 241:11-18 (2009)) as well as augmented production of heat shock protein (HSP-90) (Jha, S., *et al.*, *Am J Physiol Heart Circ Physiol*, 295(2):H801-806 (2008)).

In conclusion, GYY4137 exhibits anti-cancer cell activity both *in vitro* and *in vitro*. It is described herein that GYY4137 breaks down slowly to yield H₂S which, by a combination of cell cycle arrest and promoting apoptosis, inhibits tumor growth. No cell death was apparent in non-cancer cells. Whether such cells simply break down H₂S at a faster rate or whether cancer cells are uniquely sensitive to the killing effect of this gas requires further study. The finding that cancer cells can be killed selectively when exposed to relatively small amounts of H₂S over a relatively long time period is key. This observation needs to be borne in mind in any future work examining the part

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played by H₂S in cancer cell survival and also in the development of novel H₂S-based anti-tumor agents.

Example 3 Characteristics of GYY4137

5 1. Solubility determination

	Compound	Structure	Aqueous Solubility (mM, pH 7.4)	
1	GY Y 4137		3 h 81.59 ± 1.85	24 h 67.77 ± 1.42

Summary of data

The solubility of GYY 4137 was determined using multiscreen filter plates from Millipore.

- 10 This method gives the kinetic solubility of GYY 4137. Briefly, a saturated solution of GYY 4137 was prepared in Universal Buffer pH 7.4, agitated for a fixed period of time, filtered and the concentration of the filtrate determined to give an estimate of solubility.

GY 4137 has excellent aqueous solubility under these conditions, with solubility ranging from 68 – 82 mM, depending on agitation time.

- 15 Some remarks on the method: In view of the good solubility of GYY 4137, the solubility method was modified in the following ways : (i) Addition of DMSO was omitted from the test and calibration solutions (ii) To construct the calibration curve, it was necessary to dilute the stock solutions (80 mM – 10 mM) with acetonitrile-buffer because uv/vis absorbances of the original stock solutions were too high and did not show variation. Two dilutions (100 fold and
20 150 fold) were carried out. As expected, solubility values were not affected by the extent of dilution.

- Solubility determinations were carried out after 3 h and 24 h of gentle agitation. Solubility was time dependent and lower after 24 h than 3 h, possibly due to equilibration after the longer period of agitation. The solubility of GYY 4137 was 81.59 mM ± 1.85 at 3 hours and 67.77 ±
25 1.42 at 24 hours.

Absorbances of the test solution were read at two wavelengths (252 nm, 260 nm). There were negligible differences at these wavelengths and readings were averaged.

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Methodology employed:

Reagents

Acetonitrile (HPLC grade, Tedia, USA)

Dimethylsulfoxide (DMSO) (ACS grade, Sigma Aldrich, USA)

5 Ethanolamine (ACS grade, Sigma Aldrich, Germany)

Methanol (HPLC grade, Tedia, USA)

Milli-Q water (Millipore Corp., Direct Q-3 system, 18.2 MΩ cm resistivity)

Potassium acetate (ACS grade, Sigma Aldrich, Canada)

Potassium chloride (ACS grade, Merck, Germany)

10 Potassium dihydrogen phosphate (ACS grade, Merck, Germany)

Materials

MultiScreen_{HTS}- PCF Filter Plate MSSLBPC10 (Millipore Corp., Ireland)

96-well Corning flat bottom transparent UV plate FIS#07-200-623 (Corning, USA)

15 Nalgene Filter Unit – 500 mL with PES membrane (NALGENE, USA)

Instrumentation

MultiScreen Vacuum Manifold MAVM 0906 0R (Millipore Corp)

BOECO Microtiter Plate Shaker (BOECO)

20 Tecan Infinite M200 Microplate reader (Tecan)

Method

Preparation of universal aqueous buffer

500 mL of pH 7.4 universal aqueous buffer was prepared as follows:

25 250 mL Milli-Q water

1.36 mL (45mM) ethanolamine

3.08 g (45mM) potassium dihydrogen phosphate

2.21 g (45mM) potassium acetate

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The reagents were dissolved in water and thoroughly mixed. The pH of solution was adjusted to 7.4 ± 0.05 with 1.0 M HCl and brought to 500 mL with 0.15 M KCl. The solution was then filtered through a Filter Unit.

5 Preparation of compound standards for calibration

21.93 mg of GYY 4137 was weighed and dissolved in an 80:20 Universal Buffer: acetonitrile solution to give a 100 mM solution. Serial dilution was performed and 5 standard calibrators, 80 mM, 60 mM, 40 mM, 20 mM, 10 mM were prepared. 15 μ L of each standard calibrator was pipetted to 1485 μ L of 80:20 buffer: acetonitrile solution, i.e. a 100x dilution from the mM stock solutions which resulted in a concentration range of 800 μ M, 600 μ M, 400 μ M, 200 μ M and 100 μ M. 100 μ L of the resulting solution was pipetted into wells of a 96-well UV plate. Separately, 10 μ L of each standard calibrator was pipette to 1490 μ L of 80:20 buffer: acetonitrile solution, i.e. a 150x dilution from the mM stock solutions which resulted in a concentration range of 533 μ M, 400 μ M, 266 μ M, 133 μ M and 66 μ M. 100 μ L of the resulting solution was pipette into wells of a 96-well UV plate. The plates were covered and agitated on the plate shaker (250rpm, 30 min) at room temperature ($22.5 \pm 2.5^\circ\text{C}$). After this time, UV/Vis absorbance readings of wells were read on the Microplate Reader (wavelengths of 252 nm and 260 nm). Calibration curve was plotted for each wavelength and at each dilution.

Solubility analysis

24 hour solubility analysis

21.56 mg of GYY 4137 was weighed. 800 μ L of universal aqueous buffer was added. The mixture was sonicated and vortexed. The resulting solution was turbid. 250 μ L of the mixture was transfer to the 3 wells of MultiScreen HTS- PCF filter plate. The plate was covered and incubated with gentle shaking (250rpm, 24 h) at room temperature ($22.5 \pm 2.5^\circ\text{C}$). After the period of incubation (24 h), the filter plate was placed on a vacuum manifold and the contents filtered into a 96-well UV plate. After

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filtration, 200 μ L of filtrate was transferred from each well to another well in a 96-well UV plate. 50 μ L of acetonitrile was added to each well. This will give the 80:20 buffer: acetonitrile composition of the calibration solutions. The absorbances of the solutions were read at 252 nm/260 nm. As the absorbances were > 3.0, further dilution was made as described in Step 8. 10 μ L of the filtrate from Step 6 was added to 1490 μ L of 80:20 buffer: acetonitrile solution (150 fold dilution). Absorbance of solutions were read at two wavelengths (252 nm and 260 nm). Another dilution was attempted for confirmation. A 100 fold dilution was made by transferring 15 μ L of the filtrate (from Step 6) to 1485 μ L buffer: acetonitrile solution. Absorbance readings were taken at 252 nm and 260 nm.

3 hour solubility analysis

22.60 mg of GYY 4137 was weighed. 700 μ L of universal aqueous buffer was added. The mixture was sonicated and vortexed. 220 μ L of the mixture was transfer to the 3 wells of MultiScreen _{HTS}- PCF filter plate. Steps 4 – 9 of 2b (i) (24 hour solubility analysis) were repeated but this time after an incubation period of 3 hours.

Results:

Calibration curves

100x dilution

Concentration (μ M)	800	600	400	200	100	0
Absorbance (252nm)	2.28	1.74	1.24	0.64	0.32	0.0000
Absorbance (260nm)	1.57	1.19	0.85	0.44	0.22	0.0000

150x dilution

Concentration (μ M)	533.33	400.00	266.66	133.33	66.66	0
Absorbance (252nm)	1.58	1.18	0.86	0.43	0.21	0.0000
Absorbance (260nm)	1.08	0.809	0.59	0.29	0.15	0.0000

Solubility data

Incub	Waveleng	Dil	Vol	Absorbance	Concentration (Conc)	Back Calculate
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time (h)	th (nm)		(uL)				(uM)			Conc (mM)		
24	252	100	100	1.54	1.55	1.63	660.9	664.87	697.94	66.09	66.49	69.79
24	252	150	100	1.08	1.08	1.11	451.16	450.77	461.82	67.67	67.62	69.27
24	260	100	100	1.05	1.06	1.11	657.77	662.44	695.55	65.78	66.24	69.55
24	260	150	100	0.74	0.74	0.75	451.79	452.40	460.56	67.77	67.86	69.08
3	252	100	100	1.83	1.93	1.93	782.47	828.77	826.12	78.25	82.88	82.61
3	252	150	100	1.34	1.32	1.30	558.08	549.61	540.77	83.71	82.44	81.12
3	260	100	100	1.24	1.32	1.33	778.43	823.27	820.64	77.84	82.33	82.06
3	260	150	100	0.91	0.89	0.88	554.97	545.87	538.48	83.25	81.88	80.77

Compound	Wavelength (nm)	Aqueous Solubility (mM, pH 7.4)	
		3h	24h
GYG 4137	252	81.83 ± 1.95 ^a	67.82 ± 1.47 ^a
	260	81.36 ± 1.90 ^a	67.71 ± 1.50 ^a

5 ^a Mean and SD from readings obtained from two dilutions (150x and 100x), n = 6

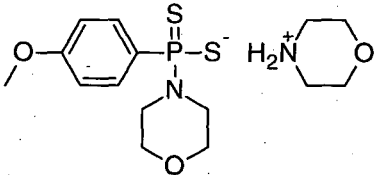
Conclusions

Compound	Aqueous Solubility (mM, pH 7.4)	
	3h	24h
GYG 4137	81.59 ± 1.85 ^a	67.77 ± 1.42 ^a

10 ^a Mean and SD from readings obtained from two dilutions (150x, 100x) and two wavelengths (252 nm, 260 nm), n= 12

2. Permeability across artificial membranes

15

Compound	Structure	P_e (pH 7.4) (10^{-6} cm/s)	
GYG 4137		Hexadecane/hexane	Lecithin/dodecane
		0.37 ± 0.02 (6 h) 0.21 ± 0.01 (15 h)	0.30 ± 0.04 (6 h) 0.12 ± 0.01 (15 h)

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Summary of data

Effective Permeability (P_e) of GYY 4137 was determined by the parallel artificial membrane permeation assay (PAMPA) which is widely employed in the pharmaceutical industry to predict oral absorption potential of early drug candidates.

- 5 The P_e of three standard compounds (quinidine, verapamil, caffeine) were determined under similar experimental conditions as GYY 4137. Permeability of these standard compounds have been reported to vary in the sequence verapamil (most permeable) > quinidine > caffeine (least permeable).

- 10 In this investigation, permeability was assessed at pH7.4 under different experimental conditions, namely (i) type of lipid employed (hexadecane/hexane or lecithin/dodecane) and (ii) time permitted for permeation (6h, 15 h at ambient temperature). Both conditions were found to influence permeation.

- 15 The P_e of GYY 4137 varied from 0.12×10^{-6} to 0.37×10^{-6} cm/s, depending on conditions (i) and (ii). Its P_e was found to be close to that of caffeine, suggesting low permeability under the present experimental conditions.

P_e determined with lecithin/dodecane as lipid barrier and after 15 hours may closely approximate physiological conditions. P_e of GYY 4137 under these conditions was 0.12 ± 0.01 ($n = 3$) as compared to 1.33 ± 0.05 ($n = 3$) for caffeine.

- 20 Methodology employed:

Compounds

GY 4137

Caffeine (Reagent, Sigma Aldrich, USA)

Quinidine (Monohydrate, Sigma Aldrich, USA)

- 25 Verapamil (Reagent, Sigma Aldrich, England)

Reagents

L- α -phosphatidylcholine (lecithin) (Lyophilized powder from egg yolk, Sigma Aldrich, USA)

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n-dodecane (ReagentPlus[®], Sigma Aldrich, USA)

n-hexane (AR, Tedia, USA)

Hexadecane (Reagent grade, Sigma Aldrich, USA)

Dimethyl sulfoxide (DMSO) (ACS grade, Sigma Aldrich, USA)

- 5 1x Phosphate buffer saline (PBS) solution (Ultra pure grade, Vivantis Inc., Malaysia)

Milli-Q water (Millipore Corp., Direct Q-3 system, 18.2 MΩ cm resistivity)

Materials

MultiScreen-IP PAMPA assay plates (MAIPNTR10) (Millipore Corp., Ireland)

- 10 MultiScreen Receiver Plate (MATRNPS50) (Millipore Corp., USA)

96-well flat bottom transparent UV plate (FIS#07-200-623) (Corning, USA)

Instrumentation

BOECO Microtiter Plate Shaker (BOECO)

- 15 Tecan Infinite M200 Microplate reader (Tecan)

Method

Calibration curve for GYY 4137

- 20 1mM stock solution of GYY 4137 was prepared in 1x PBS solution and diluted to give standard calibrator solutions of 600 μM, 500 μM, 300 μM, 200 μM, 100 μM, 50 μM and 20 μM. 300 μL of each standard calibrator solution was transferred to wells in a 96-well polypropylene, 2.4 mL deep-well plate. The plate was covered and agitated on a plate shaker (250rpm, 30 minutes) at room temperature ($22.5 \pm 2.5^{\circ}\text{C}$). UV/Vis absorbance of each well was read at λ max (269 nm) of GYY 4137 to give its
- 25 calibration curve.

Calibration curves of standards (caffeine, quinidine, verapamil)

50mM stock solution of each compound was prepared in DMSO and diluted with 1xPBS to give standard calibrator solutions of 500 μM, 400 μM, 200 μM, 100 μM,

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50 μM , 25 μM , 5 μM . The concentration of DMSO in each solution was kept at 1% (v/v). 300 μL of each standard calibrator solution was transferred to wells in a 96-well polypropylene, 2.4 mL deep-well plate. The plate was covered and agitated on a plate shaker (250rpm, 30 minutes) at room temperature ($22.5 \pm 2.5^\circ\text{C}$). UV/Vis absorbance of each well was read at λ max of the standard compound to give its calibration curve.

Permeability assay of GYY 4137 using Hexadecane in hexane as the lipid barrier

A 5% solution (w/v) of hexadecane in hexane was prepared. 15 μL of the solution was pipetted into a well on the donor plate. The hexane was removed by placing the plate in a fume hood (suction turned on and sash pulled down) for 1 hour. A uniform layer of hexadecane is formed at the base of the donor plate well. 1mM stock solution of GYY 4137 was prepared in 1x PBS. 450 μL of the 1mM stock solution was diluted with 1050 μL of 1x PBS buffer to give a 300 μM solution. 150 μL of this solution was added to the well in the donor plate (with hexadecane). 300 μL of 1x PBS buffer was added into the corresponding well in the acceptor plate. The donor plate was placed on top of the acceptor plate. The underside of the membrane in the donor plate must be in contact with the buffer in the acceptor well. The donor/acceptor plate unit was covered, placed in an air-tight container and agitated on a plate shaker (250rpm, 7 h) at room temperature ($22.5 \pm 2.5^\circ\text{C}$). After this time, 100 μL /well of the donor and 250 μL /well of the acceptor plates were transfer to separate wells in a 96-well UV plate. The absorbances of the solutions were determined at λ max of 269 nm on a microplate reader. The assay was repeated using a 2nd stock solution (1 mM) of GYY 4137

Permeability assay of GYY 4137 using lecithin in dodecane as the lipid barrier

A 1% solution (w/v) of lecithin in dodecane was prepared. 5 μL of the 1% lecithin/dodecane was pipetted into the well of the donor plate. Steps 2-7 of the preceding protocol using hexadecane as lipid barrier were repeated.

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Permeability assay of standard compounds (caffeine, quinidine and verapamil) using Hexadecane in hexane as the lipid barrier.

A 5% solution (w/v) of hexadecane in hexane was prepared. 15 μ L of the solution was pipetted into a well in the donor plate. The hexane was removed by placing the plate in a fume hood (suction turned on and sash pulled down) for 1 hour. A uniform layer of hexadecane is formed at the base of the donor plate well. 50mM stock solution of the standard compound in DMSO was prepared. 15 μ L of the 50mM stock solution was diluted with 1350 μ L of 1x PBS buffer to give a 300 μ M solution. Final concentration of DMSO in the solution is 1% (v/v). 150 μ L of this solution was added to the well in a donor plate (with hexadecane). 300 μ L of 1x PBS buffer (containing 1% v/v DMSO) was added into the corresponding well of the acceptor plate. The donor plate was placed on top of the acceptor plate. The underside of the membrane in the donor plate must be in contact with the buffer in the acceptor well. The donor/acceptor plate unit was covered, placed in an air-tight container and agitated on a plate shaker (250rpm, 7 h) at room temperature ($22.5 \pm 2.5^{\circ}\text{C}$). After this time, 100 μ L/well of the donor and 250 μ L/well of the acceptor plates were transfer to separate wells in a 96-well UV plate. The absorbance of the solutions were determined at λ max of the standard compound on a microplate reader (caffeine 247 nm, quinidine 332 nm, verapamil 278 nm). The assay was repeated using a 2nd stock solution (50 mM) of the standard compounds.

Permeability assay of standard compounds (caffeine, quinidine and verapamil) using lecithin in dodecane as the lipid barrier

A 1% solution (w/v) of lecithin in dodecane was prepared. 5 μ L of the 1% lecithin/dodecane was pipette into the well of the donor plate. Steps 2-7 of the preceding protocol using hexadecane as lipid barrier were repeated.

Results

Calibration curve results for GYY4137

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Concentration (uM)	600	500	300	200	100	50	20	0
Absorbance (260 nm)	3.07	2.67	1.65	1.10	0.55	0.28	0.11	0.00

Calibration curve results for standard compounds Caffeine, Quinidine and Verapamil

Compound	Concentration (uM)	500	400	200	100	50	25	5	0
Caffeine (274nm)	Absorbance	3.36	3.08	1.63	0.81	0.41	0.21	0.04	0.00
Quinidine (332nm)		2.14	1.78	0.91	0.46	0.22	0.11	0.02	0.00
Verapamil (278nm)		1.64	1.33	0.66	0.33	0.16	0.08	0.01	0.00

5 Calculation of Effective Permeability P_e P_e was calculated from Equation 1

Equation 1:

$$\log P_e = \log \left\{ C \times \ln \left(1 - \frac{[\text{drug}]_{\text{acceptor}}}{[\text{drug}]_{\text{equilibrium}}} \right) \right\}$$

Where :

- 10 (i) $[\text{drug}]_{\text{acceptor}}$ = concentration of test compound in acceptor well after period of shaking (6 h or 15 h).
- (ii) $[\text{drug}]_{\text{equilibrium}}$ = sum of concentrations of test compound in acceptor and donor wells after period of shaking (6 h or 15 h).
- (iii) $C = V_D \cdot V_A / [(V_D + V_A) \cdot \text{Area} \cdot \text{Time}]$
- 15 Where V_A = volume of acceptor well (150 μL), V_D = volume of donor well (300 μL),
Surface Area of lipid barrier = 0.24 cm^2 , Incubation time = 21600 s or 43200 s

Effective Permeability of test compounds with hexadecane as lipid barrier

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Compound	P_e (pH 7.4) ^a (10^{-6} cm/s)	Log P_e	P_e (pH 7.4) ^a (10^{-6} cm/s)	Log P_e
	6hr		15hr	
GY4137	0.37 ± 0.02	-6.45 ± 0.02	0.21 ± 0.01	-6.72 ± 0.02
Caffeine	0.34 ± 0.06	-6.47 ± 0.08	0.11 ± 0.02	-6.99 ± 0.07
Quinidine	0.09 ± 0.04	-7.04 ± 0.12	0.43 ± 0.22	-6.25 ± 0.10
Verapamil	5.32 ± 1.05	-5.28 ± 0.08	3.24 ± 2.01	-5.57 ± 0.30

^a Mean and SD of 3 determinations using two stock solutions of test compound.

Effective Permeability of test compounds with lecithin as lipid barrier

5

Compound	P_e (pH 7.4) ^a (10^{-6} cm/s)	Log P_e	P_e (pH 7.4) ^a (10^{-6} cm/s)	Log P_e
	6hr		15hr	
GY4137	0.30 ± 0.04	-6.52 ± 0.05	0.12 ± 0.01	-6.92 ± 0.03
Caffeine	1.46 ± 0.03	-5.83 ± 0.02	1.33 ± 0.05	-5.88 ± 0.02
Quinidine	2.69 ± 0.97	-5.59 ± 0.16	2.11 ± 0.25	-5.68 ± 0.05
Verapamil	15.23 ± 0.60	-4.82 ± 0.02	7.84 ± 0.28	-5.11 ± 0.02

^a Mean and SD of 3 determinations using two stock solutions of test compound.

10 3. Toxicity in *vitro*

Summary of data

GY4137 exhibited minimal toxicity towards TAMH or HL-1 cells with IC₅₀ values from 3 separate experiments of 6.83 mM and >10 mM respectively.

15 Methodology employed

Panels of proposed cell lines:

TAMH (Transforming growth factor- α mouse hepatocyte)

HL-1 (Mouse cardiomyocyte derived from AT-1 mouse atrial cardiomyocyte tumor lineage)

20

Reagents

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Dimethyl sulfoxide (DMSO; Solvent control)

CellTiter-Glo[®] Cell Viability Assay (Promega Corporation)

Methods

- 5 Cells were seeded at a cell density of 12,000 cells/well (60,000 cells/ml) in a 96-well plate (NUNC) a day before drug treatment. Cells were then treated across a wide concentration range as follows, starting from a concentration of 10 mM (10,000 μ M):

	Concentration (μM)	Dilution factor
1	10000	
2	1000	10
3	333.3333333	3
4	33.33333333	10
5	11.11111111	3
6	1.111111111	10
7	0.37037037	3
8	0.037037037	10
9	0.012345679	3

- Drug samples were prepared such that an equal volume of each drug concentration is added into its respective wells. In this case, 10 μ l of each drug was added to 90 μ l of cell culture medium.

- Drug-treated cells were then incubated at 37°C for 24 hours. After 24 hours, cell viability was determined with CellTiter-Glo[®] Cell Viability Assay (Promega Corporation) as per manufacturer's instructions. The cell-reagent mixture was then transferred to a solid white flat-bottom 96-well plate (Greiner) for luminescence reading. Luminescence was then recorded with an integration time of 0.25 second with a Tecan Infinite[®] M200 Microplate reader. Based on the analyses from the above mentioned concentration range, cell viability assay was repeated with a more focused drug concentration range.

20

4. Toxicity in *vivo*

Positive controls

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Carbon tetrachloride (CCl₄; 0.5 ml/kg; known hepatotoxic agent)

Gentamicin sulfate (100 mg/kg; known nephrotoxic agent)

Doxorubicin hydrochloride (25 mg/kg; known cardiotoxic agent)

5 Negative controls

Water (vehicle control for gentamicin, doxorubicin and GYY4137)

Olive oil (vehicle control for CCl₄)

Test compound

10 GYY4137 (25 mg/kg)

Serum injury markers tested

Liver

Aspartate transaminase (AST)

15 Alanine transaminase (ALT)

Kidney

Serum creatinine

Blood urea nitrogen (BUN)

20

Heart

Creatine-kinase muscle/brain (CKMB)

Cardiac troponin T (cTnT)

25 Methodology

Mice of C57BL/5 strain were used as approved according to the IACUC¹ protocol number 035/11. Animals were subjected to an acclimatization period of 2 weeks in the animal facility before testing was carried out.

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The positive and negative controls and GYY4137 were then administered intraperitoneally (i.p.) as per dose mentioned above.

Subsequently, gentamicin sulfate, GYY4137 and water were administered (i.p.) every 24 hours, for 3 days.

- 5 Blood was collected via cardiac puncture 24 hours and 72 hours (for gentamicin group) after drug administration.

Samples were centrifuged at 13,000 rpm for 5 minutes for serum collection. Serum samples were dispatched to the Veterinary Diagnostic Laboratory (MD-2) for the testing of AST, ALT, serum creatinine, BUN and CKMB.

10

Conclusion:

There were no significant toxicity effects observed in mice with the administration of GYY4137 *in vivo*. No significant effects were exerted by GYY4137 on AST and ALT in mice after 24 hours (Olive oil $n = 1$; CCl₄ (0.5 ml/kg) $n = 3$;

- 15 GYY4137 (25 mg/kg) $n = 4$. No significant effect of GYY4137 was observed on serum creatinine in mice after repeated dosing for 3 days. A significant difference was observed between BUN levels of gentamicin and GYY4137 in mice after 3 days (Water $n = 5$; Gentamicin (GEN; 100 mg/kg) $n = 5$; GYY4137 (25 mg/kg) $n = 4$).

20 5. Genotoxicity

Salmonella typhimurium strains tested:

Strain	Gene Affected	DNA-repair	Cell wall	Biotin requirement	Plasmids	Main DNA Target	Type of mutation
TA98	hisD3052	uvrB	Rfa	bio-	pKM101	GC	Frameshift
TA100	hisG46	uvrB	Rfa	bio-	pKM101	GC	Base-pair substitution

Methodology employed:

25 Materials

Reagents

Water (Solvent control)

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2-AA (Positive control)

S9 mix (MOLTOX Inc; consists of rat liver microsomes, phosphate-buffered salt solution, glucose-6-phosphate and NADP)

Histidine/biotin supplemented top agar

5 Minimal glucose agar

S. typhimurium strains (MOLTOX Inc)

Nutrient broth (Oxoid #2)

Equipment

10 Hitachi U-1800 UV Spectrophotometer

Vortex mixer

37°C shaking incubator

Growing of bacterial cultures

15 *S. typhimurium* strains were grown from bacterial discs in nutrient broth at 37°C in a shaking incubator (~150 rpm) for about 10 hours.

The cultures were then measured for absorbance with a UV spectrophotometer at 660 nm.

Cultures are ready for experiment at a density of approximately 1.0 to 1.2

20 absorbance reading

Drug treatment genotoxicity assay

The top agar was melted in a hot water bath or microwave oven and 2 ml volumes were aliquot into culture tubes. The tubes of agar were then maintained at 45°C.

25 *Controls:* 100 µl of water and 2-AA was added to separate tubes containing top agar.

GY4137: 100 µl of GYY4137 at concentrations 50 mM and 0.5 mM were added to separate tubes containing top agar

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S9 mix: To all the tubes containing either controls or drug compounds, 500 µl of S9 mix was added to the top agar.

Culture: 100 µl of *S. typhimurium* strain (either TA98 or TA100) was added to the top agar.

- 5 The top agar containing all components from Steps 2-4 were immediately mixed and decant onto Minimal Glucose Agar Plate and swirled to obtain an even distribution of plating mixture over the agar surface.

After agar was set to harden, the plates were then incubated in a 37°C incubator for 48 hours.

10

Note: Duplicates were carried out for all experimental groups.

Results

Compound	Number of His+ Revertants ⁺	
	TA98	TA100
GY4137 (50 mM)	19	91
GY4137 (0.5mM)	20	83
Water	19	67
2-aminoanthracene (Positive Control)	1632	1196

15 Conclusions

GY4137 exhibited no evidence of genotoxicity at the concentrations used.

6. *In vitro* stability

Summary of data

- 20 GY4137 was very slowly metabolized in rat liver microsomes, degrading at most 25% in 45 min. Thus, GY4137 is regarded to be very metabolic stable.

Methodology employed

Reagents

5 GYY4137 was supplied by DDU, the internal standard (IS) probenecid, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water (Millipore Corp., Milford, MA, USA) was used throughout the experiment. All other chemicals and reagents were of analytical grade and solvents were of HPLC grade.

Instrumentation

10 A LC-MS/MS system used was composed of a model 1200 HPLC instrument (Agilent Technologies, Palo Alto, CA, USA) coupled to a Q TrapTM 3200 hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada). Data processing was performed with AnalystTM 1.4.2 software package (Applied Biosystems, MA., USA).

15 Chromatographic conditions

Chromatographic separation was performed on an Eclipse Plus C18 column (4.6 X 150 mm, i.d., 3.5 μ m, Agilent Technologies, Palo Alto, CA, USA) with a Security Guard Cartridge (3.0 X 4 mm, Agilent Technologies, Palo Alto, CA, USA). The mobile phase consisted of methanol - 10mM ammonium formate and the flow rate was set at 20 0.5 mL/min. Table 1A shows the liquid chromatography gradient parameters. For sample analysis, a 10 μ L full loop sample injection was used.

MS/MS detection

25 The mass spectrometer was operated using ESI source in the negative ion detection mode. The optimized instrument parameters for monitoring GYY and IS by mass spectrometry are shown in Table 1B as follow: source temperature (TEM), 400^oC; turbo spray voltage (IS), 4500 V; curtain gas (CUR), 10; Nebulising gas (GS1), 40; turbo ion spray gas (GS3), 40; collision gas (CAD), medium; and dwell time 200 ms.

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Conditions for (A) separation and (B) detection of GYY4137 and the internal standard (IS)

(A) Gradient parameters for separation of GYY4137 and its IS (Solvent A: Methanol:water 5/95, v/v containing 10mM ammonium formate, Solvent B: Methanol:water 95/5, v/v containing 10mM ammonium formate)

Time (min)	Solvent A (%)	Solvent B (%)
0	40%	60%
0.5	0%	100%
4	0%	100%
4.2	40%	60%
7.5	40%	60%

(B) MRM transition ions and compound-dependent parameters

Analyte	MRM transition	Declustering Potential (V)	Entrance potential (V)	Collision energy (V)	Collision cell exit potential (V)
GYY4137	288.2→187.0	-50	-5	-42	-3
IS	284.3→240.1	-30	-5	-27	-3

5

Microsomal incubation procedure

Phosphate buffer (100 mM, pH 7.4) containing 1 mM EDTA was prepared from 400 mM mono- and dibasic potassium phosphate stock solution. NADPH stock solutions (10 mM) in phosphate buffer were made fresh daily.

10

Sample preparation and incubation

Liver microsomal incubations were conducted in triplicate. Incubation mixtures consisted of 7.5 μ L of 20 mg/mL FRLM and MRLM (final: 0.3 mg microsome protein/mL), 2.5 μ L of 600 μ M GYY in methanol (final: 3 μ M), 440 μ L of 0.1 M phosphate buffer (pH 7.4). The mixture was first shaken for 5 min for pre-incubation in a shaking water bath at 37°C. Reaction was initiated by adding 50 μ L of 10 mM

15

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NADPH to obtain a final concentration of 1mM NADPH in the mixture. The total volume of the reaction mixture was 500 μ L. For metabolic stability studies, aliquots of 50 μ L of the incubation sample mixture were collected at 0, 5, 15, 30, and 45 min. After collection of samples, the reaction was terminated with 100 μ L of chilled methanol containing the internal standard (4 μ g/mL probenecid). The mixture was then centrifuged at 17,000 X g to remove the protein and the supernatant was subsequently applied to LC-MS/MS analyse.

Positive control (PC) samples were prepared as described above, except the test compound was replaced with the known P450 substrate (Midazolam, 5 μ M). The samples were assayed for the degradation of midazolam to evaluate the adequacy of the experimental conditions for drug metabolism study. Negative control samples were also prepared as described above but without NADPH.

Metabolic stability calculations and statistical analysis

The corresponding MRM transition of this candidate was selected and used for peak configuration in Analyst 1.4.2 for semi-quantitation. In the determination of the *in vitro* half-life ($T_{1/2}$), the peak areas of drug were converted to parent remaining percentages, using the $t = 0$ peak area values as 100%. The remaining percentages of this candidate were plotted against the microsomal incubation time using Microsoft Excel. Data points were the average of three measurements with standard deviations as the error bars. The *in vitro* $T_{1/2}$ (in units of min) was calculated from the slope of the linear regression (k) of the natural logarithm of the parent remaining percentage versus incubation time according to the following formula.

Statistical analysis of data was performed using Microsoft Excel. All data were presented as means $\pm$ standard deviation (SD). Comparisons between GYY4137 in MRLM and FRLM were made using Student's independent-samples t-test. The statistical significance level was set at $p < 0.05$.

Results

Metabolic stability of GYY4137 in rat liver microsomes

Upon incubation at 37 °C, the mean percentages of remaining GYY4137 and PC relative to initial amount in RLM are shown in Table 2 and Fig. 1 respectively.

- 5 Percentage of remaining GYY4137 and midazolam (PC) over time in the presence of RLM

Time (min)	GYG-MRLM	SD(n=3)	GYG-FRLM	SD(n=3)	PC	SD(n=3)
0	100.00%	2.54%	100.00%	1.43%	100.00%	3.58%
5	97.68%	0.61%	100.87%	5.69%	41.06%	1.23%
15	91.87%	1.81%	99.70%	1.79%	8.57%	0.48%
30	85.51%	1.70%	97.83%	1.51%	1.55%	0.17%
45	77.95%	1.58%	97.75%	2.51%	1.14%	0.01%

- 10 The positive control (midazolam) was metabolized rapidly and the finding was consistent with the published result (1), indicating that the experimental conditions were validated to describe the metabolic stability of the test compound, GYY4137.

Significant differences were noted in K , $CL_{int, in vitro}$ and $CL_{int, app}$ describing the metabolic stability of GYY4137 between the MRLM and FRLM ($P < 0.004$), however

- 15 there was no apparent difference in $T_{1/2}$ as shown in Table 3 ($p > 0.05$). Nevertheless, based on the *in vitro* $T_{1/2}$, $CL_{int, in vitro}$ and $CL_{int, app}$ values, GYY4137 was very slowly metabolized in rat liver microsomes, degrading at most 25% in 45 min. Thus, this candidate is regarded to be very metabolic stable. The difference in metabolic stability between the males and females may not be important therapeutically.

20

k , $T_{1/2}$, $CL_{int, in vitro}$ and $CL_{int, app}$ values of 3 μ M GYY4137 upon incubation at 37 °C for 45 min

<i>in vitro</i> system	k (1/min)	$T_{1/2}$ (min)	$CL_{int, in vitro}$ (uL/min/mg)	$CL_{int, app}$ (L/h/kg)
GYG-Male				
RLM	0.0048±0.0004	209.4±18	16.0±1	1.9±0.2
GYG-Female				
RLM	0.0018±0.0007	647.7±324	5.9±2	0.7±0.3

7. Pharmacokinetic profile (i.v. and p.o.)

Summary

5 The AUCs obtained after i.v. administration of GYY4137 had a relative standard deviation (RSD) of less than 10%.

The AUCs obtained after p.o. administration had a higher variation with RSD in the region of 50%.

$(AUC)_{\text{oral}} \times \text{Dose}_{\text{IV}} / (AUC)_{\text{IV}} \times \text{Dose}_{\text{oral}}$ was quite low in the range of 8%.

10 Methodology

NB - Instrumentation, chromatographic and MS/MS analysis as described above.

Animal experimentation

15 The animals were anaesthetized before a cannula was inserted into the jugular vein two days before the pharmacokinetic study. The rats would be given sufficient time to recuperate. The animals were fasted on the day before the actual study. GYY4137 was given at 2 mg/kg and 10 mg/kg in saline for the respective intravenous and oral administration. The blood samples were then collected from the catheter at various time
20 points as indicated in Table 1. The blood samples were then processed to collect the plasma and stored at -80°C until they were analyzed for the drug levels.

Results

25 The measured plasma levels of GYY4137 after dosing at 2 mg/kg intravenously and 10 mg/kg orally are shown below.

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Plasma concentration (ng/mL) of GYY4137 in Wistar rats over time following (A) intravenous injection of 2 mg/mL GYY4137 and (B) oral administration of 10 mg/mL GYY4137

(A)

Time (h)	1#-rat	2#-rat	3#-rat
0.084	5450	5240	3490
0.25	1900	1880	1730
0.5	604	566	468
1	100	75.2	97.5
1.5	35.3	18.9	61.6
2	70.5	12.7	114
3	43.1	10.5	69.8
5	13.3	5	21.5
8	6.52	6.75	7.03
12	3.85	3.27	3.22
24	N/A	N/A	N/A

5

(B)

Time (h)	1#-rat	2#-rat	3#-rat
0.25	615	282	187
0.5	338.3	172	161
0.7	253.3	121	128
1	225.8	106	108
1.5	291.7	90.5	70.7
2	382.5	82.4	52.6
3	95.8	90.9	71.5
5	45.8	17.9	26.2
8	11.5	12	12.2
12	8.8	8.64	7.09
24	N.A.	N.A.	N.A.

The estimated pharmacokinetic parameters of GYY4137 are shown below.

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Pharmacokinetic parameters of GYY4137 in Wistar rats following (A) intravenous injection of 2 mg/mL GYY4137 and (B) oral administration of 10 mg/mL GYY4137

(A)

parameter	unit	#1-rat	#2-rat	#3-rat	Mean	SD	RSD
AUC_{0-t}	ug/L*h	1883.255	1698.839	1555.535	1712.543	164.289	9.59
$AUC_{0-\infty}$	ug/L*h	1886.721	1723.3	1557.917	1722.646	164.403	9.54
MRT	h	0.577	0.416	0.86	0.618	0.225	36.4
$t_{1/2}$	h	1.774	5.199	1.498	2.824	2.062	73.0

5 (B)

parameter	unit	#1-rat	#2-rat	#3-rat	Mean	SD	RSD
AUC_{0-t}	ug/L*h	1132.288	529.28	462.605	708.058	368.904	52.1
$AUC_{0-\infty}$	ug/L*h	1137.316	565.965	481.729	728.337	356.682	48.9
MRT	h	2.244	2.774	3.001	2.673	0.388	14.5
$t_{1/2}$	h	1.623	2.891	2.845	2.453	0.719	29.3
t_{max}	h	0.25	0.25	0.25	0.25	0	0
C_{max}	ug/L	615	282	187	361.333	224.758	62.2

Conclusions

The AUCs obtained after intravenous administration of GYY4137 were found to have a small relative standard deviation (RSD) of less than 10%. In contrast, the AUCs obtained after oral administration were shown to have a high variation with RSD in the region of 50%. This indicates that GYY4137 gave a steady plasma level once it has been introduced into the systemic circulation. However, its absorption appeared to be variable. It may take note that the time for C_{max} was always at the first time point of sample collection at 15 min after oral administration. This suggests that the drug was very rapidly absorbed. Despite that, the extent of absorption (bioavailability) which is equal to $(AUC)_{oral} \times Dose_{IV} / (AUC)_{IV} \times Dose_{oral}$ was quite low in the range of 8%. The low bioavailability is unlikely to be due to the first pass effect, as the drug has previously been found to be metabolic stable. It is more likely to be caused by its moderate permeability as a result of its hydrophilic physicochemical properties. Therefore, GYY4137 could be classified as type III under the biopharmaceutics classification with high solubility but poor permeability. This is expected as the drug is hydrophilic and may not have the degree of lipophilicity for it to pass through the lipid

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membrane. It warrants to explore if its biopharmaceutical properties could possibly be improved by either the ion pairing or formulation approach.

Summary

5 GYY 4137 has very good aqueous solubility at ambient temperature (22 deg C) in Universal Buffer pH 7.4 as determined by the filtration method using multiscreen plates. Solubility values were in the range of 68 mM – 82 mM, depending on the period of agitation (3h, 24h).

10 The PAMPA assay indicates that GYY 4137 has modest permeability across hexadecane and lecithin as lipid barriers after 6 or 15 h of incubation. Its effective permeability (Pe) approximates to that of caffeine determined under similar conditions.

GYY4137 exhibits very limited toxicity against either TAMH (transforming growth factor-alpha mouse hepatocyte) or HL-1 (mouse cardiomyocyte derived from AT-1 mouse atrial cardiomyocyte tumor lineage). IC50s are 6.83 mM and >10 mM
15 respectively.

GYY4137 exhibited negative genotoxicity (Ames test) at concentrations up to 50 mM.

GYY4137 was very slowly metabolized in rat liver microsomes, degrading at most 25% in 45 min. Thus, this candidate is regarded to be very metabolic stable.

20 In conscious rats, GYY4137 very rapidly absorbed after p.o. administration. Despite that, the extent of absorption (bioavailability) which is equal to $(AUC)_{oral} \times Dose_{iv} / (AUC)_{IV} \times Dose_{oral}$ was quite low (approx. 8%). The low bioavailability is unlikely to be due to the first pass effect, as the drug has previously been found to be metabolic stable. It is more likely to be caused by moderate permeability as a result of
25 its hydrophilic physicochemical properties. Therefore, GYY4137 is likely classified as type III under the biopharmaceutics classification with high solubility but poor permeability.

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GY4137 (50 mg/kg, i.p.) exhibited no demonstrable toxicity when administered to the mouse in vivo and monitoring plasma markers of heart, liver 24 h or kidney (72 h) injury

5 Example 4 The slow-releasing hydrogen sulfide donor, GYY4137, exhibits anti-proliferation effects *in vitro*

Material and Methods - cell culture and cell viability

Human cervical carcinoma (SiHa), gastric carcinoma cell lines (SNU1, SNU5), epithelial ovarian cancer cell lines (OVCA433, SKOV3 and OVCAR10), osteosarcoma
10 (Saos2) and breast adenocarcinoma (MDA-MB-231) were cultured in DMEM supplemented with 10% v/v fetal bovine serum (FBS; HyClone), penicillin/streptomycin (100 U/ml; Sigma) and L-glutamine (2 mM; Caisson) at 37°C in an atmosphere of 5% CO₂. Live cell populations incubated with GYY4137 (200, 400, 600, 800 or 1000µM) were collected after 5 days and counted in triplicate after staining
15 with trypan blue using a haemocytometer. The half maximal effective concentration (EC₅₀) of GYY4137 on each of the above mentioned cell lines was estimated from the respective concentration response curve.

Figure 5 Concentration-response curves showing the effect of GYY4137 treatment for 5 days on survival of (5A) epithelial ovarian cancer cell lines (OVCA433, SKOV3 and OVCAR10); (5B) gastric carcinoma (SNU1 and SNU5); (5C) cervical
20 carcinoma (SiHa), osteosarcoma (Saos2) and breast adenocarcinoma (MDA-MB-231). Results show mean ± s.e. mean, n=3. Over a 5-day culture period, GYY4137 significantly decreased epithelial ovarian cancer (OVCA433, SKOV3 and OVCAR10) survival in a concentration-dependent manner. GYY4137 (200µM) dramatically
25 decreased OVCA433 and SKOV3 cell survival to 60% and 40% respectively whilst a higher concentration (800µM) decreased survival of OVCAR10 to nearly 50% survival as compared to non-treated samples. GYY4137 similarly possessed anti-proliferative effect in two gastric carcinoma cell lines (SNU1 and SNU5). SiHa, Saos2 and MDA-

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MB-231 also showed positive anti-proliferative effect in response to GYY4137 treatment.

This work shows that GYY4137 possesses an anti-proliferative activity in a broad range of cancer cell types.

5

The teachings of all patents, published applications and references cited herein are incorporated by reference in their entirety.

While this invention has been particularly shown and described with references to example embodiments thereof, it will be understood by those skilled in the art that
10 various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

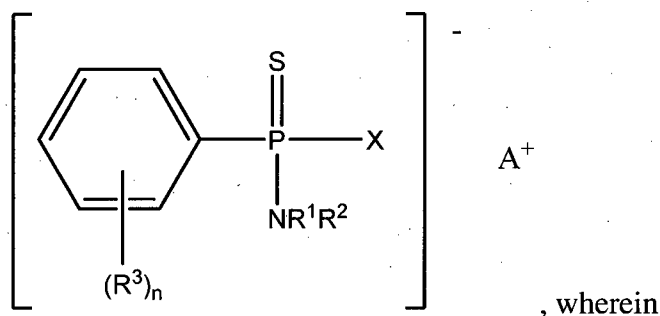
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CLAIMS

What is claimed is:

1. A method of treating cancer in an individual in need thereof comprising administering an effective amount of a slow-releasing hydrogen sulfide (H₂S) compound to the individual.

2. The method of Claim 1 wherein the compound is represented by the following structural formula:



- X is O or S;
- A⁺ is a monovalent cation;
- R¹ and R² are each independently (a) hydrogen; or (b) (C₁-C₁₀)alkyl, (C₂-C₁₀)alkenyl, (C₂-C₁₀)alkynyl, aryl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, cycloalkyl(C₀-C₃)alkyl, heterocyclyl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, each optionally substituted with one to three groups represented by R⁴; or
- R¹ and R², along with the nitrogen to which they are attached, form a monocyclic heterocyclyl, wherein the heterocyclyl formed may contain 0, 1 or 2 heteroatoms in addition to the nitrogen atom, wherein the heterocyclyl is optionally substituted by one or more groups selected from halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl, wherein the (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl is optionally substituted with halogen, hydroxy, (C₁-C₄)alkyl, or (C₁-C₄)alkoxy;

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Each R³ is independently selected from halogen, hydroxy, (C₁-C₄)alkyl, halo(C₁-C₄)alkyl, hydroxy(C₁-C₄)alkyl, (C₁-C₄)alkoxy, halo(C₁-C₄)alkoxy, and hydroxy(C₁-C₄)alkoxy;

Each R⁴ is independently selected from halogen, hydroxy, (C₁-C₄)alkyl, halo(C₁-C₄)alkyl, hydroxy(C₁-C₄)alkyl, (C₁-C₄)alkoxy, halo(C₁-C₄)alkoxy, and hydroxy(C₁-C₄)alkoxy; and

n is 0-3;

or a pharmaceutically acceptable salt thereof.

10 3. The method of Claim 2, wherein

R¹ and R², along with the nitrogen to which they are attached, form a heterocyclyl; and

the heterocyclyl is selected from pyrrolidinyl, imidazolidinyl, pyrazolidinyl, dihydropyrrolyl, pyrrolyl, imidazolyl, pyrazolyl, triazolyl, oxazolidinyl, thiazolidinyl, oxazolyl, thiazolyl, piperidinyl, hydroypyrimidinyl, piperazinyl, pyridinyl, pyridazinyl, pyrimidinyl, triazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl.

20 4. The method of Claim 3, wherein the heterocyclyl is selected from pyrazolyl, piperidinyl, piperazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl.

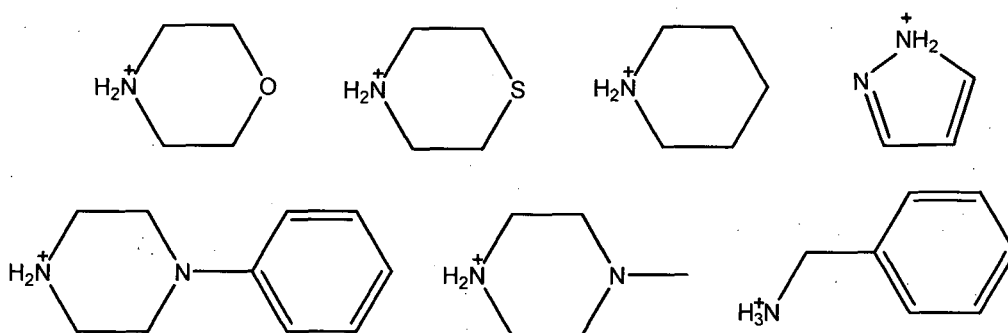
25 5. The method of Claim 2, wherein

R¹ and R² are each independently (a) hydrogen; or (b) (C₁-C₄)alkyl, (C₂-C₄)alkenyl, (C₂-C₄)alkynyl, (C₃-C₈)cycloalkyl, phenyl, benzyl, each optionally substituted with one to three groups represented by R⁴.

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6. The method of Claim 5, wherein
 R^1 is hydrogen or (C₁-C₄)alkyl; and
 R^2 is (C₁-C₄)alkyl or benzyl.
7. The method of Claim 2, wherein A⁺ is selected from lithium, sodium or potassium.
8. The method of Claim 7, wherein A⁺ is potassium.
9. The method of Claim 2, wherein A⁺ is selected from:
A⁺ is selected from piperidinium, morpholin-4-ium, thiomorpholin-4-ium, piperazin-1-ium, 4-methylpiperazin-1-ium, 4-phenyl-piperazin-1-ium, pyridinium, pyridazin-1-ium, pyrimidin-1-ium, pyrazin-1-ium, 1H-pyrrolium, 1H-pyrazol-1-ium, 1H-imidazol-1-ium, pyrazolidin-1-ium, pyrrolidinium, imidazolidin-1-ium, each optionally substituted with one to three groups represented by R⁴.

10. The method of Claim 9, wherein A⁺ is selected from:



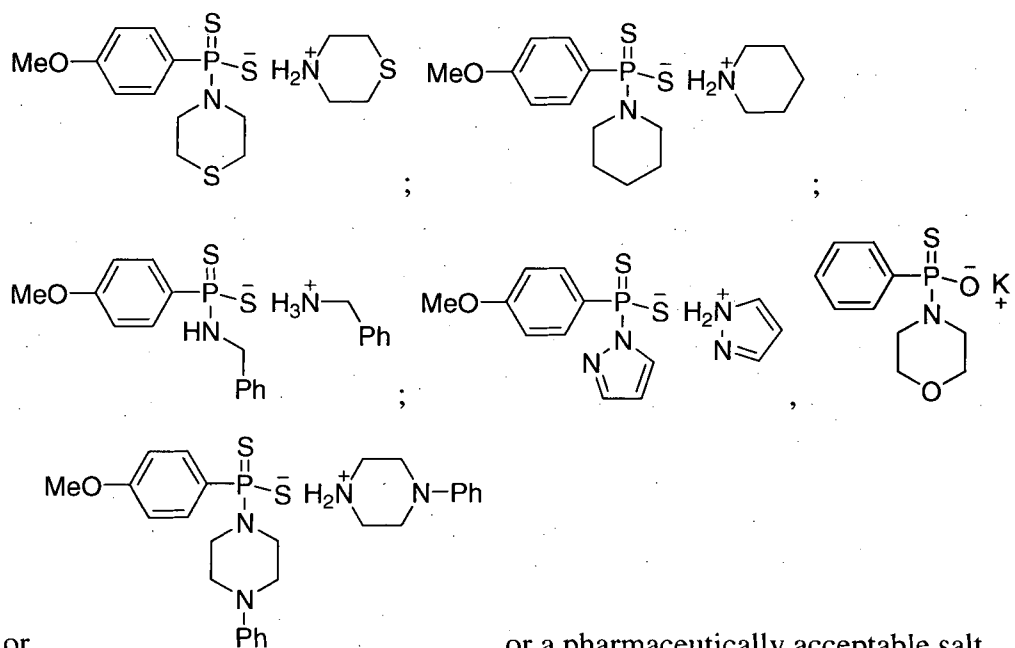
or a pharmaceutically acceptable salt thereof.

11. The method of Claim 2 wherein X is S.

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12. The method of Claim 2 wherein X is O.

13. The method of Claim 2, wherein the compound represented by a one of the following structural formula:



10 14. The method of Claim 1 wherein administration of the compound results in a low concentration of H_2S above basal level in the individual over one or more days.

15. The method of Claim 14 wherein the concentration is less than about $20\mu\text{M}$.

15 16. The method of Claim 1 wherein from about 100mg/kg to about 300mg/kg of the H_2S is administered to the individual.

17. The method of Claim 1 wherein the H_2S is administered intravenously, intraperitoneally, intraarterially, intramuscularly, intrathecally, intranasally, or subcutaneously.

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18. The method of Claim 1 wherein the individual is a mammal.

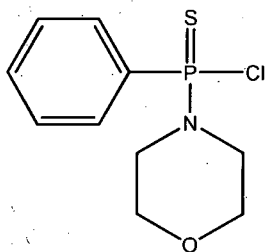
19. The method of Claim 18 wherein the mammal is a primate, a canine, a feline, or a rodent.

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20. The method of Claim 19 wherein the primate is a human.

21. A method of treating cancer in an individual in need thereof comprising administering to the individual an effective amount of compound represented by the following structure:

10



, or a pharmaceutically acceptable salt thereof.

22. A method of treating cancer in an individual in need thereof comprising administering an effective amount of morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate (GY4137) to the individual.

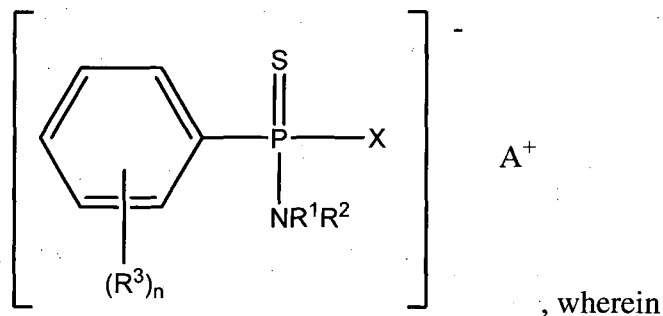
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23. A method of killing cancer cells, inhibiting the growth of cancer cells or a combination thereof, comprising contacting the cells with an effective amount of a slow-releasing hydrogen sulfide (H₂S) compound.

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24. The method of Claim 23 wherein the compound is represented by the following structural formula:

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X is O or S;

A⁺ is a monovalent cation;

R¹ and R² are each independently (a) hydrogen; or (b) (C₁-C₁₀)alkyl, (C₂-C₁₀)alkenyl, (C₂-C₁₀)alkynyl, aryl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, cycloalkyl(C₀-C₃)alkyl, heterocyclyl(C₀-C₃)alkyl, heteroaryl(C₀-C₃)alkyl, each optionally substituted with one to three groups represented by R⁴; or

R¹ and R², along with the nitrogen to which they are attached, form a monocyclic heterocyclyl, wherein the heterocyclyl formed may contain 0, 1 or 2 heteroatoms in addition to the nitrogen atom, wherein the heterocyclyl is optionally substituted by one or more groups selected from halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl, wherein the (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl is optionally substituted with halogen, hydroxy, (C₁-C₄)alkyl, or (C₁-C₄)alkoxy;

Each R³ is independently selected from halogen, hydroxy, (C₁-C₄)alkyl, halo(C₁-C₄)alkyl, hydroxy(C₁-C₄)alkyl, (C₁-C₄)alkoxy, halo(C₁-C₄)alkoxy, and hydroxy(C₁-C₄)alkoxy;

Each R⁴ is independently selected from halogen, hydroxy, (C₁-C₄)alkyl, halo(C₁-C₄)alkyl, hydroxy(C₁-C₄)alkyl, (C₁-C₄)alkoxy, halo(C₁-C₄)alkoxy, and hydroxy(C₁-C₄)alkoxy; and

n is 0-3;

or a pharmaceutically acceptable salt thereof.

25. The method of Claim 24, wherein

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R^1 and R^2 , along with the nitrogen to which they are attached, form a heterocyclyl; and

the heterocyclyl is selected from pyrrolidinyl, imidazolidinyl, pyrazolidinyl, dihydropyrrolyl, pyrrolyl, imidazolyl, pyrazolyl, triazolyl, oxazolidinyl, thiazolidinyl, oxazolyl, thiazolyl, piperidinyl, hydroypyrimidinyl, piperazinyl, pyridinyl, pyridazinyl, pyrimidinyl, triazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl.

26. The method of Claim 25, wherein the heterocyclyl is selected from pyrazolyl, piperidinyl, piperazinyl, morpholinyl, and thiomorpholinyl, each of which is optionally substituted by halogen, hydroxy, (C₁-C₄)alkyl, (C₁-C₄)alkoxy, or phenyl.

27. The method of Claim 24, wherein R^1 and R^2 are each independently (a) hydrogen; or (b) (C₁-C₄)alkyl, (C₂-C₄)alkenyl, (C₂-C₄)alkynyl, (C₃-C₈)cycloalkyl, phenyl, benzyl, each optionally substituted with one to three groups represented by R^4 .

28. The method of Claim 27, wherein R^1 is hydrogen or (C₁-C₄)alkyl; and R^2 is (C₁-C₄)alkyl or benzyl.

29. The method of Claim 24, wherein A^+ is selected from lithium, sodium or potassium.

30. The method of Claim 29, wherein A^+ is potassium.

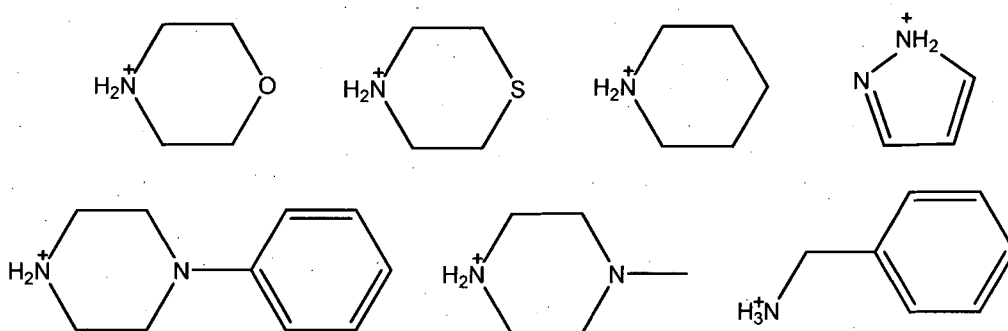
31. The method of Claim 24, wherein A^+ is selected from:

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A^+ is selected from piperidinium, morpholin-4-ium, thiomorpholin-4-ium, piperazin-1-ium, 4-methylpiperazin-1-ium, 4-phenyl-piperazin-1-ium, pyridinium, pyridazin-1-ium, pyrimidin-1-ium, pyrazin-1-ium, 1H-pyrrolium, 1H-pyrazol-1-ium, 1H-imidazol-1-ium, pyrazolidin-1-ium, pyrrolidinium, imidazolidin-1-ium, each optionally substituted with one to three groups represented by R^4 .

32. The method of Claim 31, wherein A^+ is selected from:



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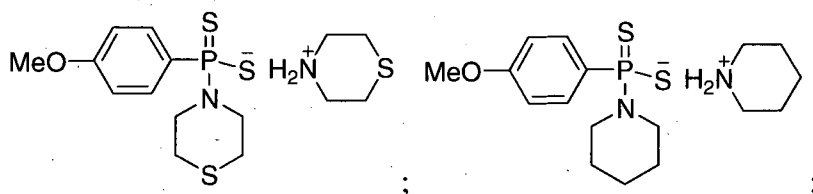
or a pharmaceutically acceptable salt thereof.

33. The method of Claim 24 wherein X is S.

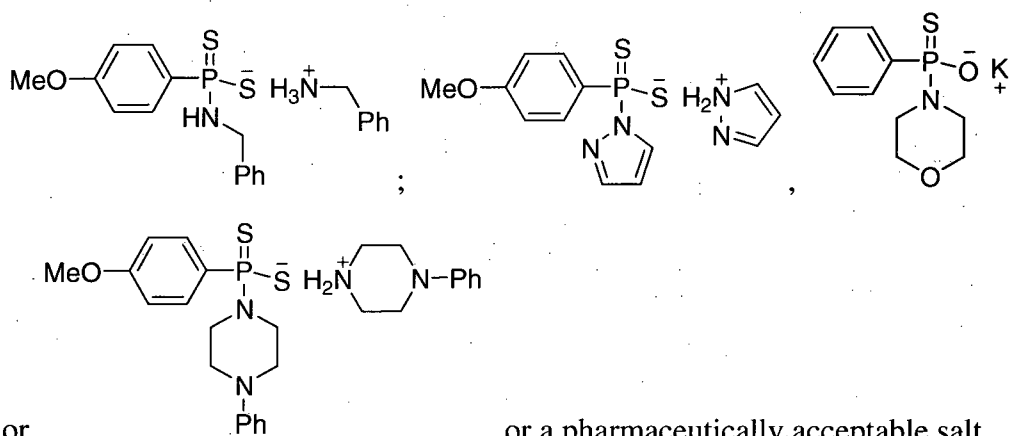
34. The method of Claim 24 wherein X is O.

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35. The method of Claim 24, wherein the compound represented by a one of the following structural formula:

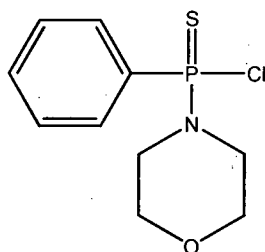


- 76 -



or
thereof. , or a pharmaceutically acceptable salt thereof.

- 5 36. The method of Claim 23 wherein contacting the cells with the compound results in a low concentration of H_2S above basal level in the cells over one or more days.
37. The method of Claim 36 wherein the concentration is less than about $20\mu M$.
- 10 38. A method of killing cancer cells, inhibiting the growth of cancer cells or a combination thereof, comprising contacting the cells with an effective amount of a compound represented by the following structure:



, or a pharmaceutically acceptable salt thereof.

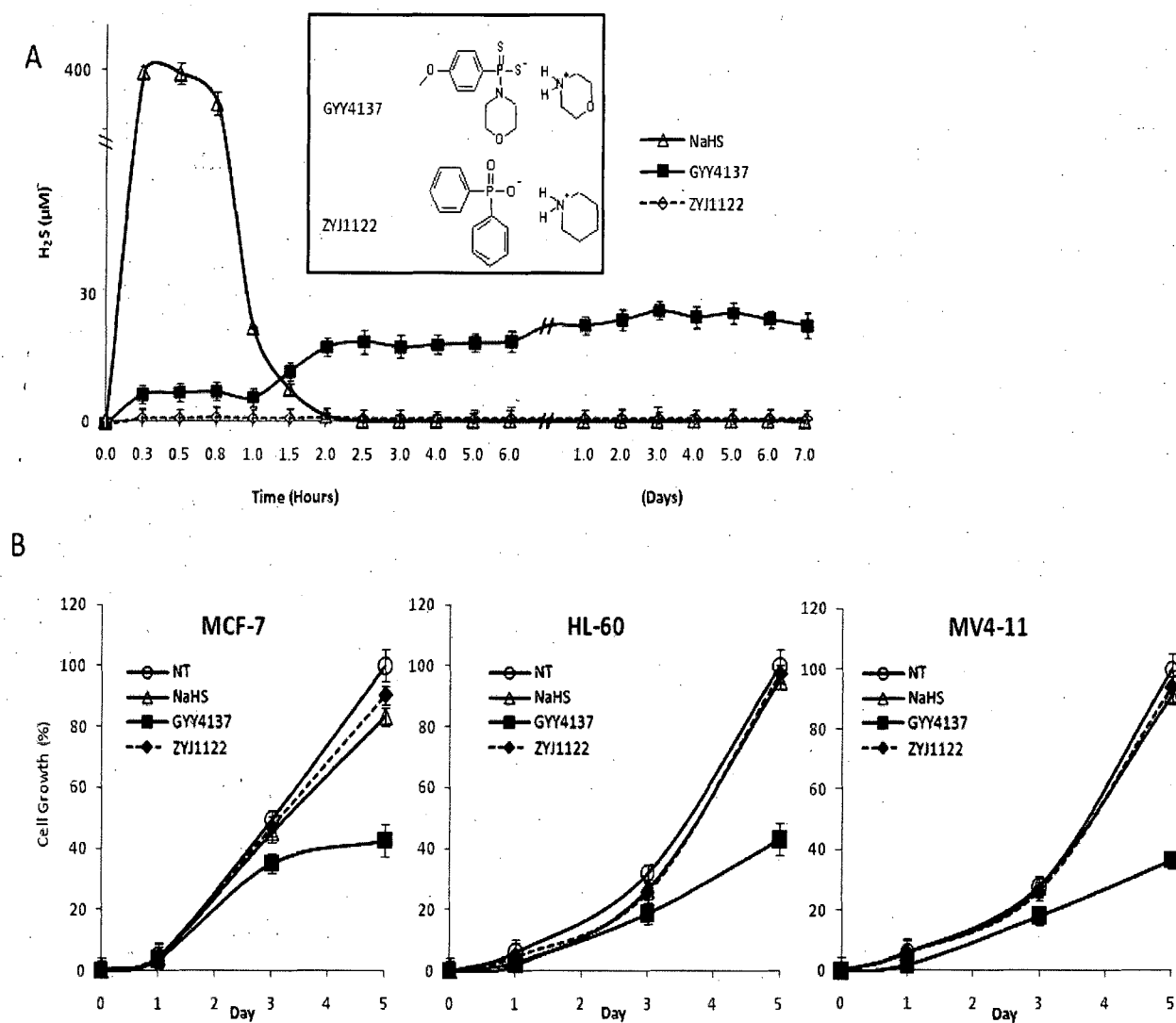
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39. A method of killing cancer cells, inhibiting the growth of cancer cells or a combination thereof, comprising contacting the cells with an effective amount of

- 77 -

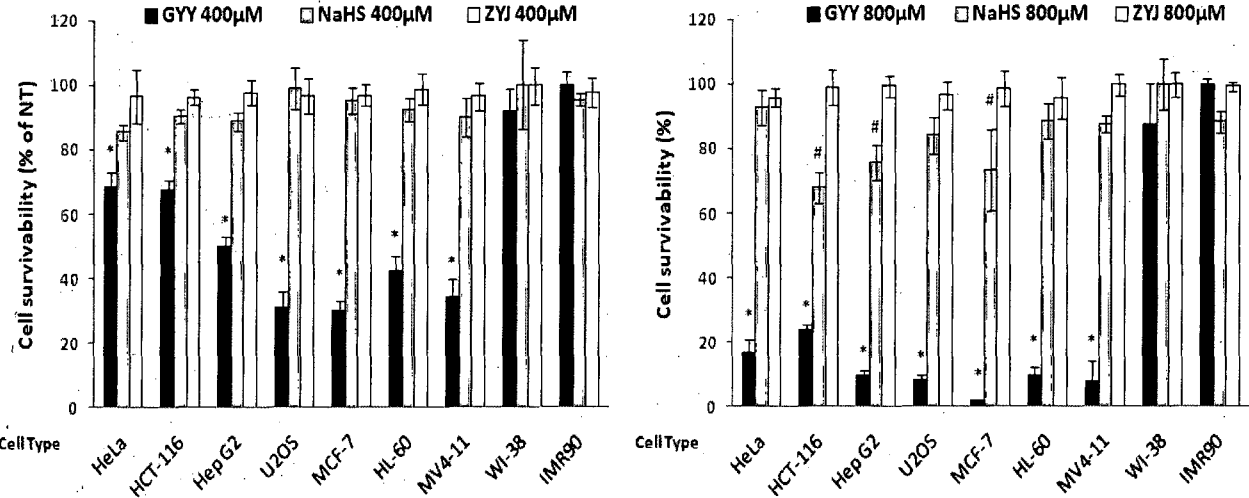
morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate
(GYY4137).

Figures 1A-1B

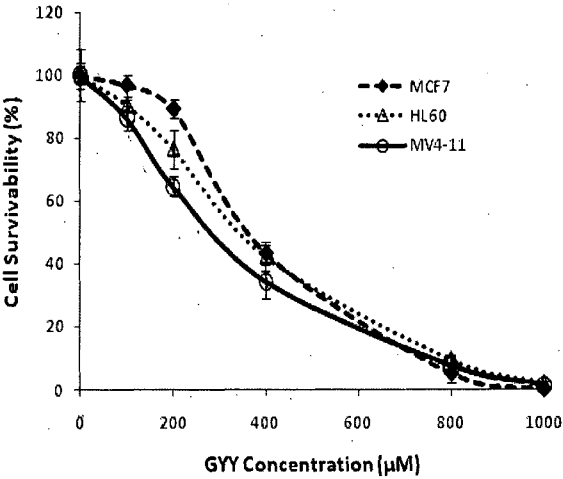


Figures 2A-2C

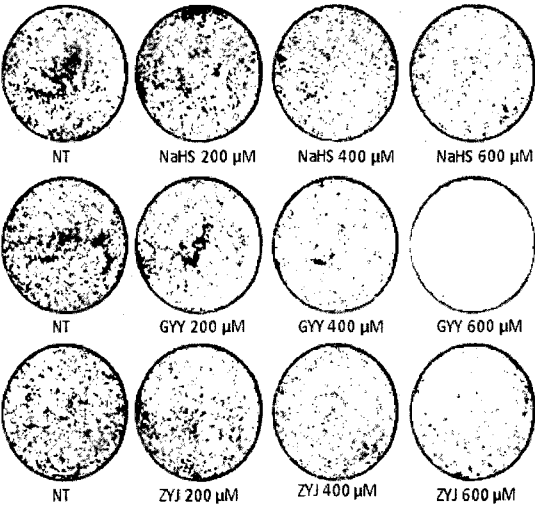
A



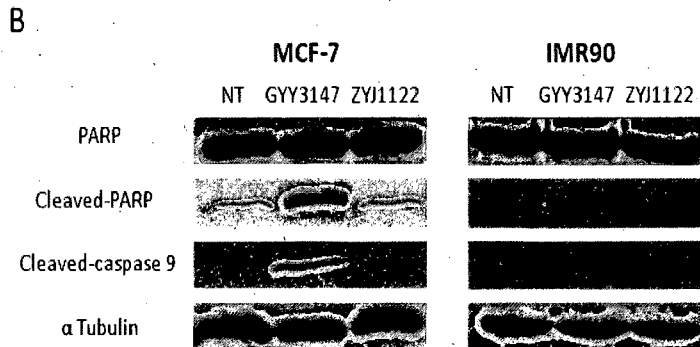
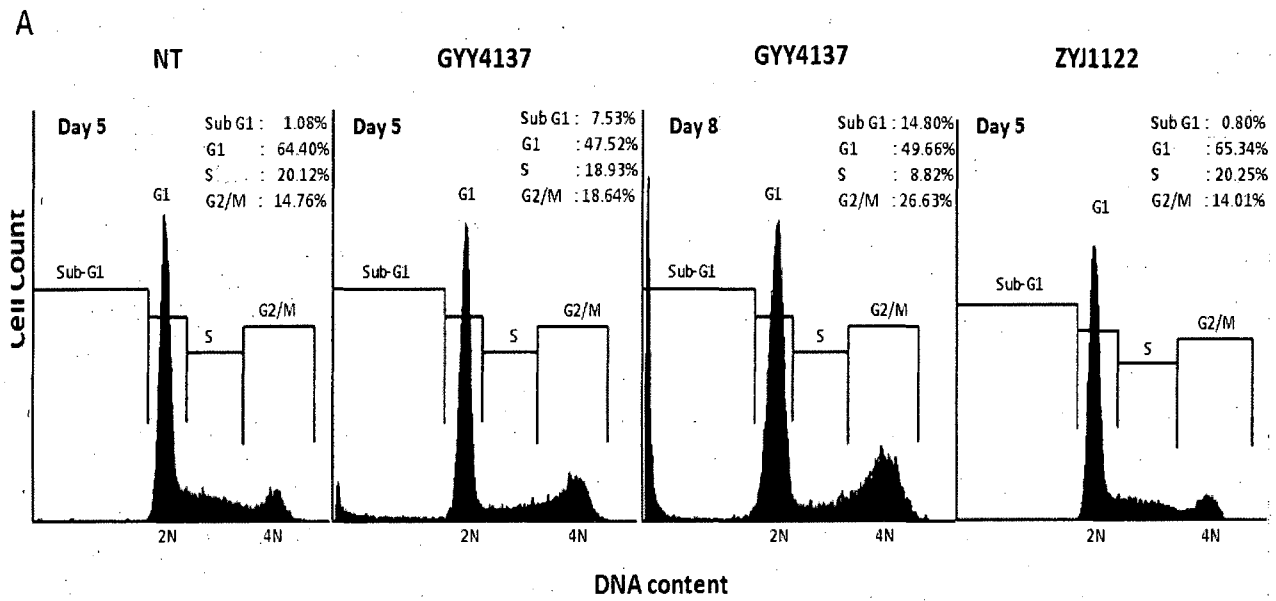
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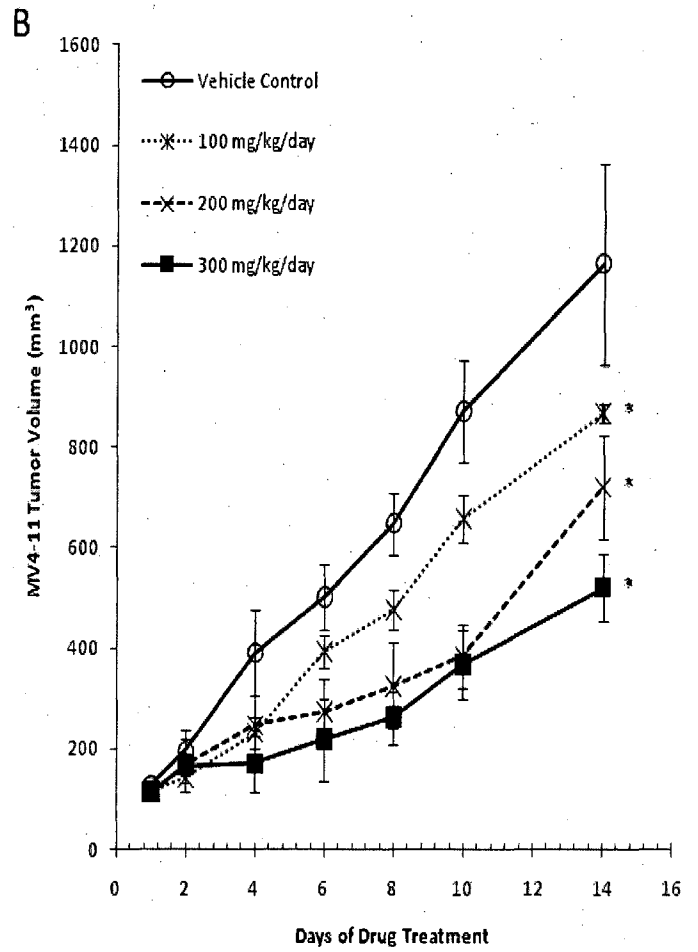
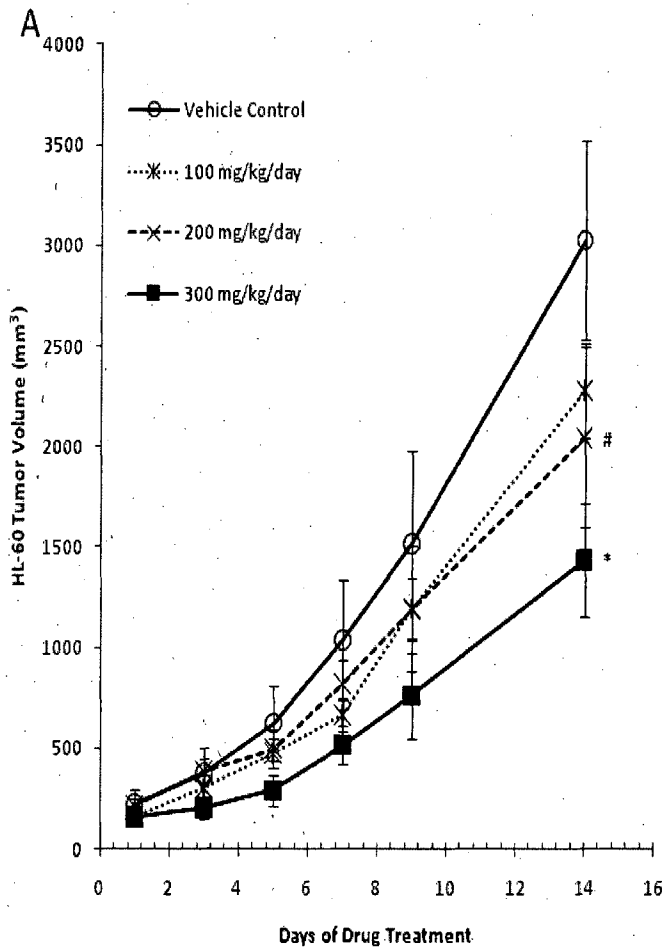
C



Figures 3A-3B

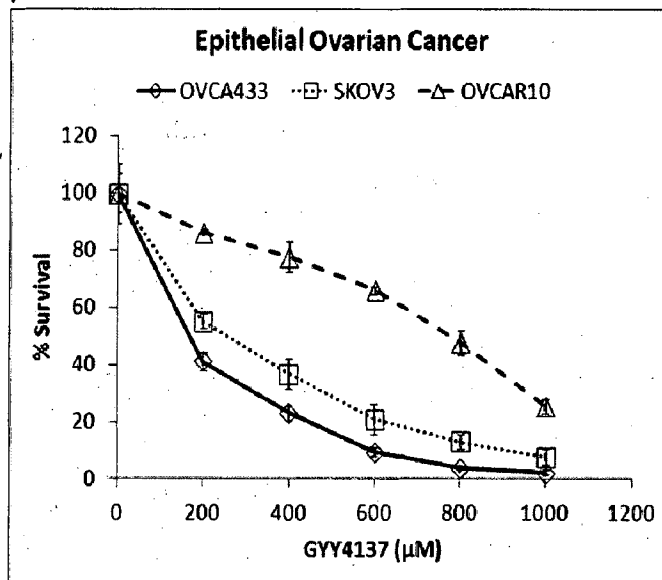


Figures 4A-4B

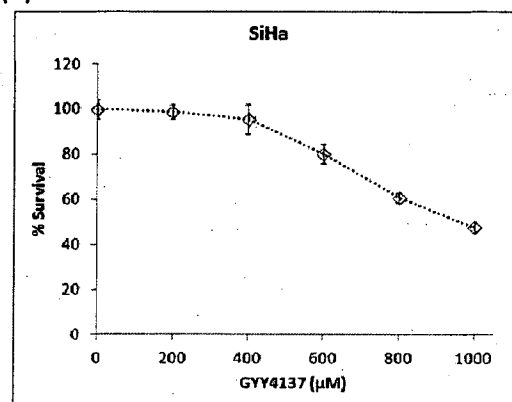


Figures 5A-5C

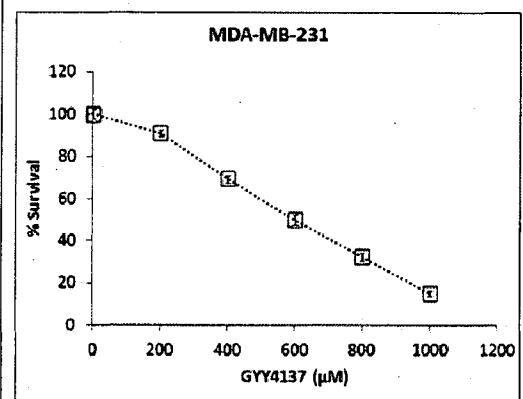
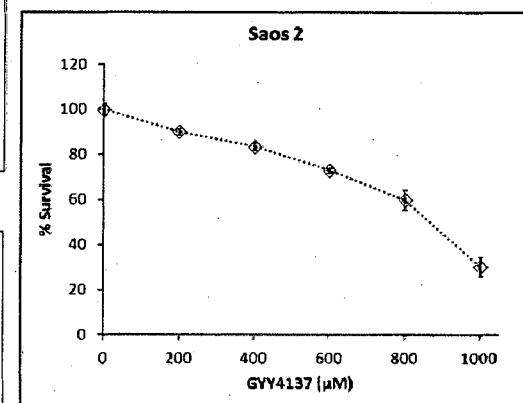
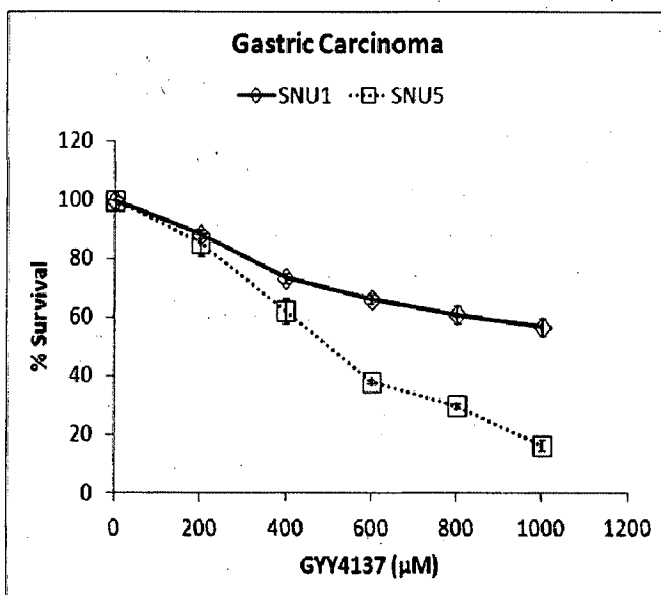
(A)



(C)



(B)



INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2012/000158

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

A61P 35/00 (2006.01)*A61K 31/66* (2006.01)*A61K 31/675* (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

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

CA (STN); WPI, EPODOC, MEDLINE, NPL (EPOQUE): [Structure Searches Cl.2 and 38], hydrogen sulfide, hydrogen sulphide, H₂S, GYY4137, cancer, metastat, leukemia, leukaemia, carcinoma, sarcoma, proliferat, neoplasm, malignan, tumor, tumour, slow

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 20060/270635 A1 (WALLACE, JOHN L. et al.) 30 November 2006 (see Claims 1, 47, 48; paragraphs [0029], [0103] to [0104], [0106])	1, 14-20, 23, 36-37
X	US 2009/0304819 A1 (GOJON-ROMANILLOS, GABRIEL) 10 December 2009 (see Claims 1, 4; paragraphs [0055] to [0056], [0058], [0068], [0077] to [0080], [0085], [0096] to [0097]; Utility Examples)	1, 14-20, 23, 36-37
X	WO 2006/024491 A1 (INTERSTITIAL THERAPEUTICS) 9 March 2006 (see Claims 1, 17; page 11 line 32 to page 12 line 6, page 26 line 13 to page 28 line 23; page 41 line 21 to page 43 line 6).	1, 14-20, 23, 36-37

☒ Further documents are listed in the continuation of Box C☒ See patent family annex

* Special categories of cited documents:	
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"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
29 May 2012Date of mailing of the international search report
31 May 2012

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2012/000158

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2009/0324682 A1 (POPOWSKI, YOURI) 31 December 2009 (see Claims 3, 17, 33; paragraphs [0097], [0241] to [0243], [0389] to [0403]).	1, 14-20, 23, 36-37

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/SG2012/000158

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
US 2006270635	US 2007197479	US 7498355	US 2009233886		
	US 7879827	US 2011130368	US 8114857		
	US 2011098257	US 8114858			
US 2009304819	US 2003235571	US 2009181081			
WO 2006024491	EP 1789029	EP 1789030	EP 1789107		
	US 2007254037	US 2007292478	US 2009324682		
	WO 2006024488	WO 2006024489	WO 2006024490		
	WO 2006024492				
US 2009324682	EP 1789029	EP 1789030	EP 1789107		
	US 2007254037	US 2007292478	WO 2006024488		
	WO 2006024489	WO 2006024490	WO 2006024491		
	WO 2006024492				
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.					
END OF ANNEX					