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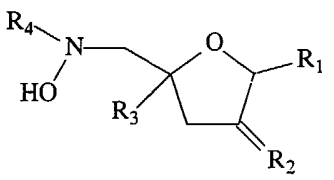
(54) Title: DIACYGLYCEROL MIMETICS AND METHODS OF USING THE SAME AS PROTEIN KINASE C ALPHA ACTIVATORS AND APOPTOSIS INDUCERS



(I)

(57) Abstract: Disclosed herein are methods for inducing apoptosis by exposing cells to an apoptic inducing amount of one or more of newly created DAG-lactone mimetics. In one embodiment of the new class of DAG compounds the new DAG-mimetic compound satisfies general formula (I) as follows:, wherein R_1, R_2, R_3, and R_4 are independently selected from the group consisting essentially of hydrogen, oxygen, nitrogen, hydroxyls, halogens, branched or unbranched, substituted or unsubstituted, aliphatic groups, heteroatom (e.g., O, S, N, hydroxyls, halogens) substituted aliphatic groups, aromatic groups, and all major functional groups. Preferably, R_1 is O and R_3 is a hydroxyalkyl such as CH_2OH. Also preferably, R_2 is an alkylidene group derived from an aldehyde and R_4 is an alkyl group derived from an acid chloride. The size of the lactone ring may be varied by either increasing or decreasing the ring size. Preferably, R_1, R_2, R_3, and R_4 when comprising carbon chains or rings, comprise groups of carbon chains or rings of 12 carbon atoms or less.

DIACYLGLYCEROL MIMETICS AND METHODS OF USING THE SAME AS PROTEIN KINASE C ALPHA ACTIVATORS AND APOPTOSIS INDUCERS

Field of the Invention

The present invention in general relates to diacylglycerol analogs containing an N-OH group, pharmaceutical compositions comprising such analogs, and methods of using the same.

Background of the Invention

Protein kinase C (PKC) is a family of phospholipid-dependent serine/theronine-specific kinases that play an important role in the regulation of cellular growth and differentiation. Phorbol esters are natural compounds which have been used as pharmacological tools for studying protein kinase C (PKC). These compounds mimic the action of a lipid second messenger diacylglycerol (DAG), a relatively simple and highly flexible molecule generated by cellular phospholipases. Activation of PKC by diacylglycerols has been shown to be an important physiological event that mediates the actions of a wide variety of hormones, neurotransmitters, and other biological control factors. In particular circumstances, PKC, when activated by some DAG or DAG mimetics, has been known to initiate apoptosis, or cell death. Some have shown hope that particular DAG compounds that activate PKC may be used as chemotherapeutic agents.

The PKC family comprises at least 10 related kinases with differential expression, subcellular distribution, and biochemical regulation. PKC isozymes have been classified into 3 subclasses according to their structure and regulation: "classical" or calcium-dependent ("cPKCs" α, β, and γ), "novel" or calcium-independent ("nPKCs" δ, ε, η, and θ) and "atypical" PKCs. While aPKCs are insensitive to DAG and the phorbol esters, cPKCs and nPKCs bind phorbol esters with high affinity in the presence of phospholipids as cofactors. PKC isozymes are subject to exquisite regulatory mechanisms and can have

either overlapping or opposite biological functions. Jaken, S., *Current Opinion in Cell Biology,* 1996, 8, 168-173.

It has been established that activation of PKC by phorbol esters triggers an apoptotic response in androgen-dependent prostate cancer cells, such as LNCaP cells. Day, M.L. et al., *Cell. Growth. Diff.*, 1994, 5, 735-741. The subcellular redistribution or translocation of PKCα and PKCδ from cytosol to plasma membrane is a hallmark of their activation. There is a need for compounds that can differentially activate particular PKC isozymes.

Synthesis of DAG compounds is generally easier than synthesis of phorbol esters due, at least in part, to the presence of multiple chiral centers in the phorbol esters. However, previously known DAG analogs do not show sufficient pharmaceutical potency as compared to phorbol esters to be useful as PKC activators and/or apoptosis inducers. Introduction of certain structural changes to particular DAG compounds has been shown to increase binding affinities (K_i) of DAG-lactones to PKC in *in vitro* competition binding assays. Marquez, et al., *Pharmacol. Ther.*, 1999, 82(2-3) 251-61. The first structural change involved a cyclization of a DAG compound to form a DAG-lactone, which led to an order of magnitude decrease in K_i (10-fold increase potency). Marquez, et al., *Pharmacol. Ther.*, 1999, 82(2-3) 251-61. Tethering a hydrophobic alkyl chain as an α-alkylidene substituent to the lactone further provided an additional order of magnitude decrease in K_i (10-fold increase potency). *Id.* While these structural changes have created somewhat effective *in vitro* PKC activators, there is a need for the synthesis of DAG-analogs having superior *in vivo* efficacy and superior apoptopic inducing abilities.

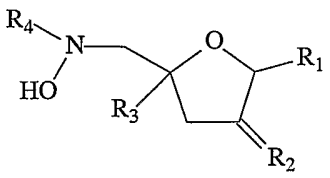
Summary of the Invention

Disclosed herein are compounds having superior *in vivo* apoptotic inducing abilities. More specifically, disclosed is a new class of DAG-lactone compounds created by "replacing" an ester moiety with a nitrogen/hydroxyl group ("N-hydroxyl" or "N-OH" group). Not only do the new DAG compounds possess superior binding

affinity, the new compounds also possess a superior degree of lipophilicity ("log P"). A superior degree of lipophilicity will allow the N-hydroxyl DAG mimetics to effectively traverse the cell membrane and, thus, interact with PKC. The synthesis and study of these new DAG compounds will show that certain members of the new class of DAG compounds disclosed herein are superior inducers of apoptosis in cancer cells, such as androgen-sensitive LNCaP prostate cancer cells.

Without being tied to any particular theory, it is suggested that this induction of apoptosis will occur through activating specific PKC isozymes via translocation of the isozyme from the cytosol to the cellular or nuclear membrane. Remarkably, particular of the new DAG compounds selectively activate one PKC isozyme over another. When treated with one of the new DAG-lactone compounds disclosed herein, N-hydroxy-N-({2-(hydroxymethyl)-4-[5-methyl-3-(2-methylpropyl)hexylidene]-5-oxo(2-2,3-dihydrofuryl)}methyl-2,2-dimethylpropanamide ("compound **6a**") PKC-alpha translocates to the plasma membrane in androgen-sensitive LNCaP prostate cancer cells. Also present in the cell, PKC-delta only translocates to the nuclear membrane by particular of the new DAG-lactone compounds.

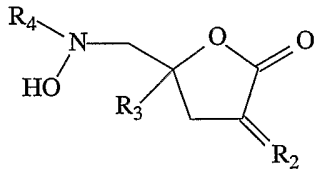
The present methods may be utilized to activate an apoptopic response in a human or animal by administration of a composition comprising an effective amount of an active DAG analog of the new class of DAG-lactone compounds. Specifically, disclosed herein are methods for inducing apoptosis by exposing cells to an apoptotic inducing amount of one or more of the newly created DAG-lactone mimetics or pharmaceutically acceptable salts of such compounds. In one embodiment of the new class of DAG compounds the new DAG-mimetic compound satisfies general formula (1) as follows:



(1)

wherein R_1, R_2, R_3, and R_4 are independently selected from the group consisting essentially of hydrogen, oxygen, nitrogen, hydroxyls, halogens, branched or unbranched, substituted or unsubstituted, aliphatic groups, heteroatom (e.g., O, S, N, hydroxyls, halogens) substituted aliphatic groups, aromatic groups, and all major functional groups. Preferably, R_1 is O and R_3 is a hydroxyalkyl such as CH_2OH. Also preferably, R_2 is an alkylidene group derived from an aldehyde and R_4 is an alkyl group derived from an acid chloride. The size of the lactone ring may be varied by either increasing or decreasing the ring size. Preferably, R_1, R_2, R_3, and R_4, when comprising carbon chains or rings, comprise groups of carbon chains or rings of 12 carbon atoms or less.

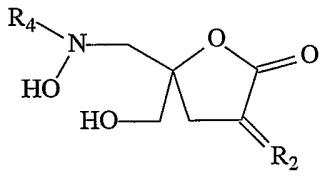
Another embodiment comprises DAG-lactone mimetics that satisfy general formula (2) as follows:



(2)

wherein R_2, R_3, and R_4 are as set forth above in relation to formula (1).

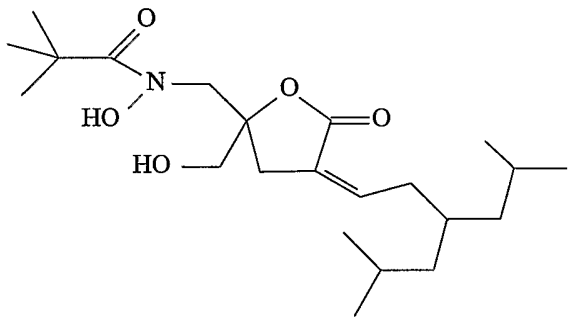
Another embodiment comprises DAG-lactone mimetics that satisfy general formula (3) as follows:



(3)

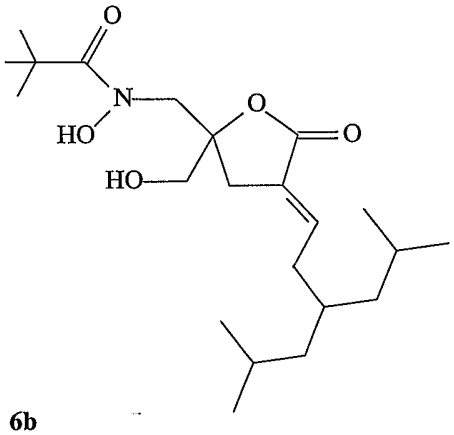
wherein R_2 and R_4 as set forth above in relation to formula (1).

An apoptotically active DAG-lactone analog (referred to in Scheme 1 below as "**6a**" (with inactive isomer "**6b**")) satisfies formula (4) as follows:



(4)

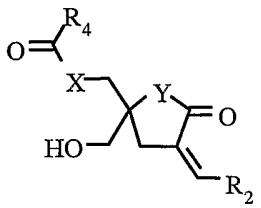
6a



6b

The lactone rings of all embodiments may be varied in size.

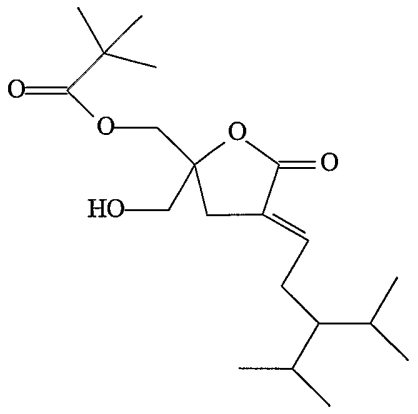
In another embodiment, the DAG-analogs (and variations of the same) satisfy general formula (5) as follows:



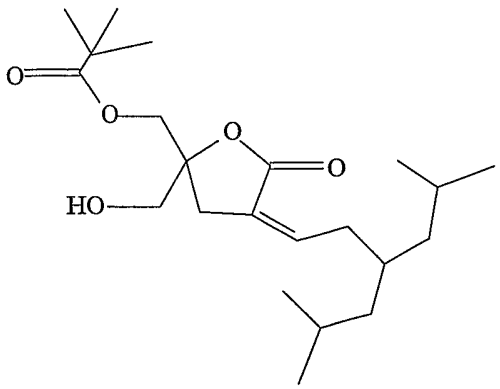
(5)

wherein X = O, Y= O, NH or NOH, R_1 and R_2 are independently selected from the group consisting essentially of branched or unbranched, substituted or unsubstituted, aliphatic groups, substituted aliphatic groups, and aromatic groups, more particularly, hydrogen, hydroxyls, halogens, branched or unbranched, substituted or unsubstituted alkyls, alkenyls, alkynyls, alkoxies, amino groups, or aryl groups that may also, for example, optionally be substituted with one or more alkyl, alkenyl, alkynyl, amino groups, hydroxyl groups, or alkoxy groups or wherein, R_4 is either $(CH_3)_3C$ or $(i\text{-}Pr)_2CHCH_2$ or wherein R_2 is either $CH_2CH(i\text{-}Pr)_2$ or $CH_2CH[CH_2(i\text{-}Pr)]_2$.

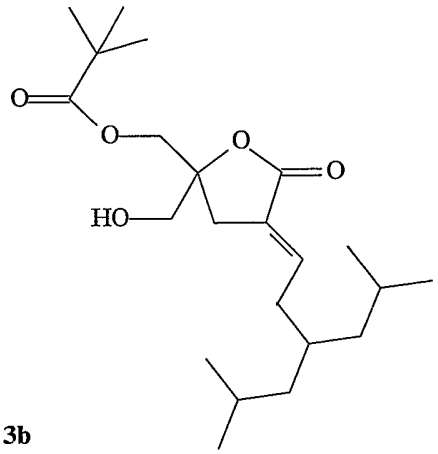
For example, the DAG mimetic compounds disclosed herein comprise compounds **2a, 2b, 3a, 3b, 4a, 4b, 7a, 7b, 8a, 8b** (as in the Schemes shown below) that satisfy formulas **2a, 3a, 3b, 4a, 4b, 7a, 7b, 8a, 8b** as follows:



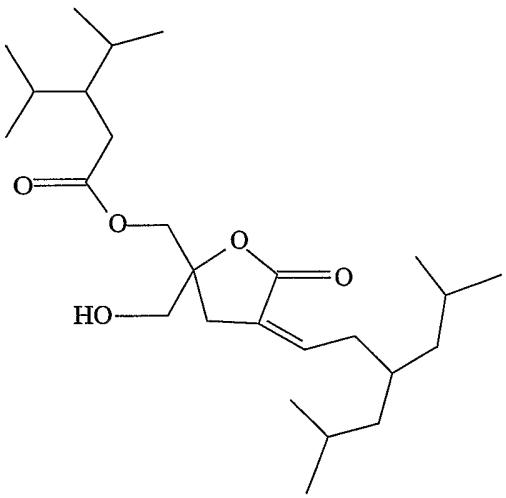
2a (and isomer 2b)



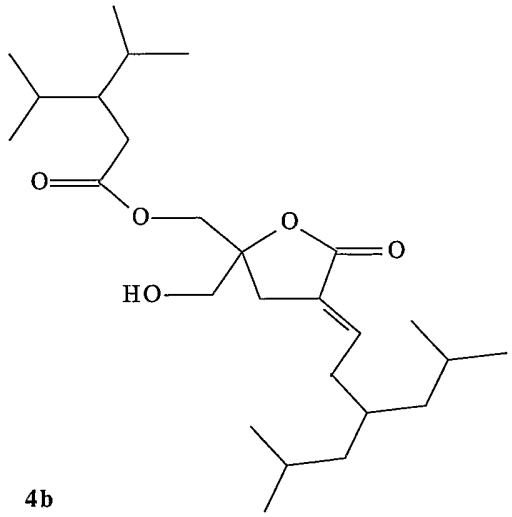
3a



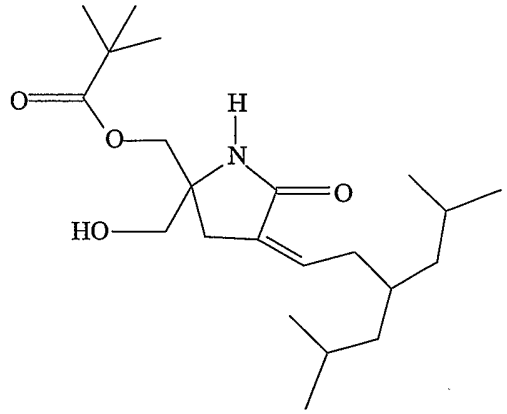
3b



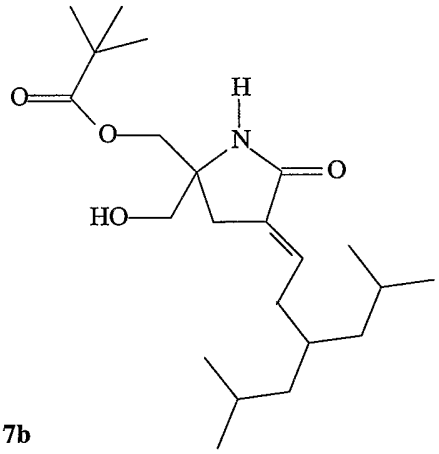
4a



4b

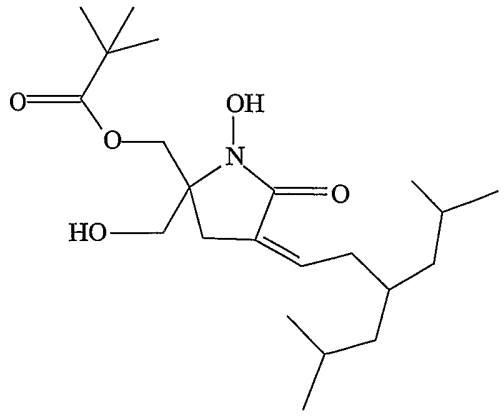


7a

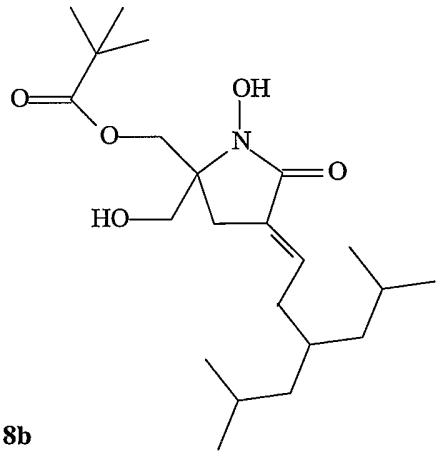


7b

5



8a



8b

In another embodiment the new DAG-lactone analog compounds will effectively bind to PKC and induce apoptosis by possessing binding affinities for PKC ("K_i") under about 12 nM, low enough lipophilicity values ("Log P") ca. 3.6 that are still compatible with high binding affinities and sufficient apoptotic-inducing activity in the low micromolar range as would be measured in an *in vitro* model system (see FIGS. 2A and 2B).

Also disclosed herein are pharmaceutical compositions that include one or more of the new compounds described above, or pharmaceutically acceptable salts thereof, and pharmaceutically acceptable carriers. Further, it is to be understood that the compounds disclosed herein are shown generally above, but include all other compounds that are members of the genus described by such general formulas.

The foregoing and other objects, features and advantages of the invention will become more apparent from the following detailed description of particular examples that proceed with reference to the accompanying figures.

Brief Description of the Figures

FIG. 1 is a table summarizing actual or expected log P and K_i values for particular embodiments of the new DAG-lactone compounds disclosed herein.

FIGS. 2A and 2B illustrate particular DAG-lactone compound embodiments' actual or expected efficacy at inducing apoptosis in LNCaP cells. LNCaP cells are

treated with different compounds for 1 hour. Apoptosis is determined 24 hours later. Concentration-dependence analysis is of actual or expected apoptosis induced by phorbol esters (PMA 4α-PMA, and PDBu), OAG, and DAG-lactones. The incidence of apoptosis in each preparation is analyzed by counting 500 cells and determining the percentage of apoptotic cells. Results are the actual or expected mean value results ± S.E. of 3 independent experiments.

FIGS. 3A and 3B illustrate the expected inhibition of DAG-lactones-induced apoptosis by a caspase inhibitor and Bcl-2 over expression using an embodiment of the new class of DAG-lactone compounds. LNCaP cells are treated with 100 nM PMA (*open bars*), 10 μM compound **6a** (*solid bars*) or vehicle (*hatched bars*) in the absence or presence of a pan-caspase inhibitor z-VAD (50 μM), to be added 1 hour before and during treatment. LNCaP cells over expressing Bcl-2 (LNCaP-Neo/Bcl-2) or mock-transfected cells (LNCaP-Neo) are treated with 100 nM PMA (*open bars*), 10 μM compound **6a** (*solid bars)* or vehicle (*hatched bars*). Cells are to be collected 24 hours later and stained with DAPI. The incidence of apoptosis expected in each preparation is to be analyzed by counting 500 cells and determining the percentage of apoptotic cells. The expected results are reported as the expected mean ± S.E. of 3 independent experiments.

FIGS. 4A and 4B illustrate the expected effect of a PKC inhibitor (GF 109203X) using embodiments of the new class of DAG-lactone compounds. LNCaP cells are to be treated with vehicle, 100 nM PMA, 10 μM compound **6a** and were treated with vehicle, 100 nM PMA 10 μM compound **3a** either in the absence or presence of 5 μM GF 109203X, added 1 hour before and during the treatment with DAG-lactones or PMA. Cells were (or "are" in the case of compound **6a**) collected 24 hours after treatment and stained with DAPI. The incidence of apoptosis in each compound **3a** preparation was analyzed by counting 500 cells and determining the percentage of apoptotic cells. Results are the mean ± S.E. The expected incidence of apoptosis in each compound **6a** preparation will be analyzed by counting 500 cells and determining the percentage of apoptotic cells. Expected results are the expected mean ± S.E. of 3

independent experiments. After staining with propidium iodine, DNA content was (or "are" in the case of compound **6a**) analyzed by flow cytometry.

FIGS. 5A and 5B illustrate the expected effect of Gö6976 and rottlerin. LNCaP is treated with increasing concentrations of either Gö6976 or rotterlin, 1 hour before and during PMA or compound **6a** treatment. Twenty-four hours later cells treated with Gö6976 and rottlerin are stained with DAPI. The incidence of apoptosis in each Gö6976 and rottlerin preparation is then assessed by county 500 cells and determining the percentage of apoptotic cells. The expected results for compound **6a** treatment are also listed. Gö6976 or rotterlin results are mean ± S.E. of 3 independent experiments. *Open bars*, 100 nM PMA; *solid bars*, and the expected results for 10 μM compound **6a**; *hatched bars*, vehicle.

FIGS. 6A and 6B illustrate the expected effects of over expression of PKCα or PKCδ on compound **6a** and PMA-induced apoptosis. LNCaP cells are infected with PKCα AdV (*open bars*), PKCδ AdV (*solid bars*) or LacZAdV (*hatched bars*) at different MOIs (1-30 pfu/cell) for 14 hours. Twenty-four hours later cells were treated with either 3nM PMA or are treated with 1 μM compound **6a** for 1 hour. Cells are collected 24 hours later and stained with DAPI. The incidence of actual (or expected in the case of compound **6a**) apoptosis in each preparation was (or is) analyzed by counting 500 cells and determining the percentage of apoptotic cells. Results and expected results are presented as the mean ± S.E. of 3 independent experiments. Representative Western blots for the expression of PKCα or PKCδ after infection with PKCαAdV or PKCδAdV, respectively using the MOIs is indicated in the figure. Expression of the corresponding PKC isozyme in cell extracts is evaluated using specific PKCα and PKCδ antibodies, as described under "Experimental Procedures."

FIG. 7 illustrates the expected results of competition of [^{3}H]PDBu binding to PKCδ by compound **6a**. Binding to recombinant PKCδ is to be performed using a fixed concentration of [^{3}H]PDBu ([^{3}H] phorbol 12, 13-dibutyrate) (5nM) in the presence of 100 μg/ml phosphatidylserine, and six increasing concentrations (in triplicate) of non-radioactive compound **6a**. Each point represents the expected mean value result ± S.E.

FIG. 8 illustrates expected activation of PKCα and PKCδ by compound **6a**. PKCα and PKCδ activities are to be assayed in the presence of 100 μg/ml phospholipid vesicles (20% phosphatidylserine/80% phosphatidylcholine) and increasing concentrations of compound **6a**. Expected results are expressed as percentages of maximum activation to be observed with 1 μM PMA (dotted line), and represent the expected mean value result ± S.E.

FIGS. 9A-9D illustrate the expected translocation of PKC isozymes by DAG-lactones. Actual quantitative changes in the fluorescent distribution of GFP-PKCα and GFP-PKCδ at the plasma membrane (*open symbols*) and nuclear membrane (*closed symbols*) in response to different doses of PMA and expected quantitative changes in response to different doses of compound **6a** are shown. Results and expected results (for compound **6a**) are expressed as changes in the ratios of plasma membrane and nuclear membrane translocation as a function of time.

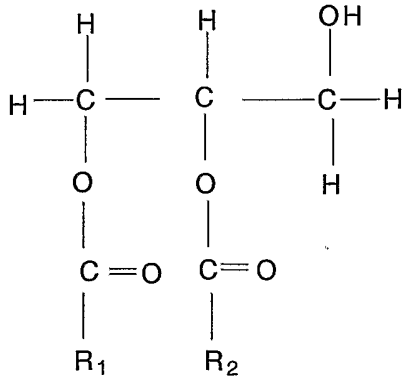
Detailed Description of Several Embodiments

Definitions

A "pharmaceutical agent" or "drug" refers to a chemical compound or composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject.

All chemical compounds include both the (+) and (-) stereoisomers, as well as either the (+) or (-) stereoisomer unless otherwise indicated.

"Diacylglycerol" typically refers to compounds satisfying the following generic chemical structure:



where R_1 and R_2 are usually, but not necessarily, aliphatic, alicyclic, or aromatic groups.

A diacylglycerol (DAG) "analog" refers to a synthetic chemical compound having a diacylglycerol backbone (i.e., side groups have been added or such groups have been deleted from the parent diacylglycerol structure). The DAG analogs differ in structure from natural diacylglycerol such as by a difference in the length of an alkyl chain, a molecular fragment, by one or more functional groups, or a change in ionization. Diacylglycerol analogs generally are not naturally occurring compounds. That is, diacylglycerol analogs generally cannot be enzymatically or nonenzymatically formed in the body.

The term "apoptosis" refers to programmed cell death as signaled by the nuclei in normally functioning human and animal cells when age or state of cell health and condition dictates.

The term "aliphatic" refers to any organic compound of hydrogen and carbon characterized by a straight chain of the carbon atoms, including alkanes, alkenes, alkynes, alcohols, dienes, aliphatic cyclic hydrocarbons, and all major functional groups, such as alkyl halides, ethers, aldehydes, ketones, acids, and esters.

The term "alcohol" refers to any member of a class of organic compounds in which a hydroxy (-OH) group has replaced a hydrogen atom of a hydrocarbon.

The term "acid" refers to a compound capable of transferring a hydrogen atom in solution.

The term "acyl" refers to the general formula RCO, where R may be an aliphatic, alicyclic, or aromatic group.

The term "alkyl" refers to a cyclic, branched, or straight chain alkyl groups containing only carbon and hydrogen, and unless otherwise mentioned contains one to twelve carbon atoms. Groups such as methyl, ethyl, isopropyl (i-Pr) and pivaloyl further exemplify this term. Alkyl groups can either be unsubstituted or substituted with one or more substituents.

The term "alkyl residue" refers to a branched or straight chain alkyl group containing only carbon and hydrogen, and unless otherwise mentioned contains one to

twelve carbon atoms. Groups such as methyl, ethyl, n-propyl, isobutyl, pentyl and pivaloyl further exemplify the term. Alkyl groups can either be substituted or unsubstituted.

The term "hydroxyl" is a chemical prefix indicating the OH group. "Hydroxy" is an alternate spelling.

The term "lactam" refers to an internal cyclic amide.

The term "lactone" refers to an internal cyclic ester.

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

A "pharmaceutical agent" or "drug" refers to a chemical compound or composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject.

The pharmaceutically acceptable salts or carriers of the compounds of this invention include those formed from cations such as sodium, potassium, aluminum, calcium, lithium, magnesium, zinc, and from bases such as ammonia, ethylenediamine, N-methyl-glutamine, lysine, arginine, ornithine, choline, N,N'-dibenzylethylenediamine, chloroprocaine, diethanolamine, procaine, N-benzylphenethylamine, diethylamine, piperazine, tris(hydroxymethyl)aminomethane, and tetramethylammonium hydroxide. These salts may be prepared by standard procedures, for example by reacting the free acid with a suitable organic or inorganic base. Any chemical compound recited in this specification may alternatively be administered as a pharmaceutically acceptable salt thereof.

A "heteroatom" refers to an organic compound including any atom other than carbon or hydrogen.

A "major functional group" is an atom or group of atoms acting as a unit that has replaced a hydrogen atom in a hydrocarbon molecule and whose presence imparts characteristic properties to the molecule.

An "alkoxy" is an alkyl radical attached to a molecule by oxygen.

An "acid chloride" is a compound containing the radical -COCl, such as benzoyl chloride.

An "aldehyde" is a class of organic compounds containing the radical CHO.

Other chemistry terms herein are used according to conventional usage in the art, as exemplified by The McGraw-Hill Dictionary of Chemical Terms (1994), The Condensed Chemical Dictionary (1981), Dorland's Illustrated Medical Dictionary (1974) and CancerWEB's On-Line Medical Dictionary (2001).

A "mammal" includes both human and non-human mammals. Similarly, the term "subject" includes both human and veterinary subjects.

An "animal" is a living multicellular vertebrate organism, a category that includes, for example, mammals and birds.

Materials and Methods

All chemical reagents were and are commercially available. Melting points were or are determined on a MelTemp II apparatus, Laboratory Devices, USA and are uncorrected. Silica gel chromatography was or is performed on silica gel 60, 230-400 mesh (E. Merck). The ^{1}H and ^{13}C NMR data were or are recorded on a Bruker AC-250 NMR instrument at 250 and 62.9 Mhz, respectively. Spectra are referenced to the solvent in which they were or are to be run (7.24 ppm for $CDCl_3$). Infrared spectra were or are recorded on a Perkin-Elmer 1600 series FT-IR. Optical rotations were or are recorded on a Perkin-Elmer model 241 polarimeter at room temperature with a path length of 1 dm. Positive ion fast-atom bombardment mass spectra (FAB-MS) were or are to be obtained on a VG 7070E mass spectrometer at an accelerating voltage of 6kV and a resolution of 2000. Glycerol was or is used as the sample matrix and ionization was or is effected by a beam of xenon atoms. Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, GA.

Syntheses

Each of the following syntheses begin with 1-(benzyloxy)-3-(4-methoxyphenoxy)acetone, to be synthesized from glycidyl 4-methoxyphenyl ether, as shown in Lee, Jeewoo et al., *J. Med. Chem.*, 1999, 42:4129, which is incorporated

herein by reference. To form 1-(benzyloxy)-3-(4-methoxyphenoxy)acetone, a solution of benzyl alcohol (8.6 mL, 83.2 mmol) in DMF (40 mL) is treated portion wise with NaOH (60%, 3.33g, 83.2 mmol) maintained at about 0 ° C. After 30 minutes of stirring at room temperature, the mixture is treated with glycidyl 4-methoxyphenyl ether (10g, 55.5 mmol) portion wise. The reaction mixture is heated at 80°C for 4 hours and cooled to room temperature. The mixture is diluted with H_2O and extracted with EtOAc several times. The combined organic layer is concentrated in vacuo. The residue is purified by flash column chromatography on silica gel with EtOAc/hexane (1:2) as eluant to give the corresponding alcohol as an oil (14.46g, 90%). ^{1}H NMR ($CDC1_3$) δ 7.27-7.37 (m, 5H, phenyl), 6.80-6.86 (m, 4H, 4-meth oxyphenyl), 4.58 (s, 2H, ph CH_2O), 4.17 (m, 1H, CHOH), 4.00 (dd of AB, 1H, CH_2OAr, J= 4.8 and $10H_z$), 3.96 (dd of AB, 1H, CH_2OAr, J = 6.0 and 10 H_z), 3.76 (s, 3H, OCH_3), 3.67 (DD of AB, 1H, $BnOCH_2$, J = 4.4 and $10H_z$) 3.61 (dd of AB, 1H, $BnOCH_2$, J = 6.0 and $10H_z$), 2.60 (bs, 1H, OH).

A suspension of 4-A molecular sieves (32.3 g) and pyridinium chloroformate 32.3 g, 150 mmol) in CH_2 CL_2 (150 mL) is slowly treated with alcohol 14.46 g, 50 mmol) in CH_2CL_2 (50 mL) via syringe. After 24 hours of stirring at room temperature, the reaction mixture is quenched with ether and Celite and stirred for 30 min. The mixture is filtered through a short pad of silica gel, and the filtrate is concentrated by conventional means. The residue is purified by flash column chromatography on silica gel with EtOAc/hexane (1:2) as eluant to give compound 10 as a white solid (13.26 g, 92%): mp 58.8 °C; IR (neat) 1753 (C-O) cm^{-1}, ^{1}H NMR ($CDC1_3$) δ 7.30-7.40 (m, 5H, phenyl), 6.83 (bs, 4H, 4-methoxyphenyl), 4.71 (s, 2 H, CH_2OAr) , 4.62 (s, 2 H, $PhCH_2O$), 4.36 (s, 2 H, CH_2OBn), 3.77 (s, 3 H, OCH_3).

An example for this type of conversion is set forth in Sharma, Rajiv et al., Conformationally Constrained Analogues of Diacylglycerol Ultra-potent PKC Ligands Based on a Racemic 5-Disubstituted Tetrahydro-2-furanone Template, *J. Med,. Chem.*, 1996, 19-28, which is incorporated herein by reference.

EXAMPLE 1

Synthesis of Compounds 6a and 6b

A stirred solution of lactone compound **10** (shown in Scheme 3) in THF (5mL/mmol) was cooled to -78 ° C in an argon atmosphere and treated drop wise with lithium diisopropylamide (1.4 equiv., 2M solution in a mixture of heptane/THF/ethylbenzene). The mixture was stirred at -78 ° C for 1.5-2.0 hours. A solution of the aldehyde (R_2CHO, 1.4 to 4 equiv.) in THF (1mL/mmol) was added drop wise to the lithium enolate at the same temperature. Stirring at -78 ° C continued for 2 hours. The reaction was quenched by slow addition of a saturated aqueous solution of ammonium chloride (1.25 mL/mmol) and then warmed to room temperature.

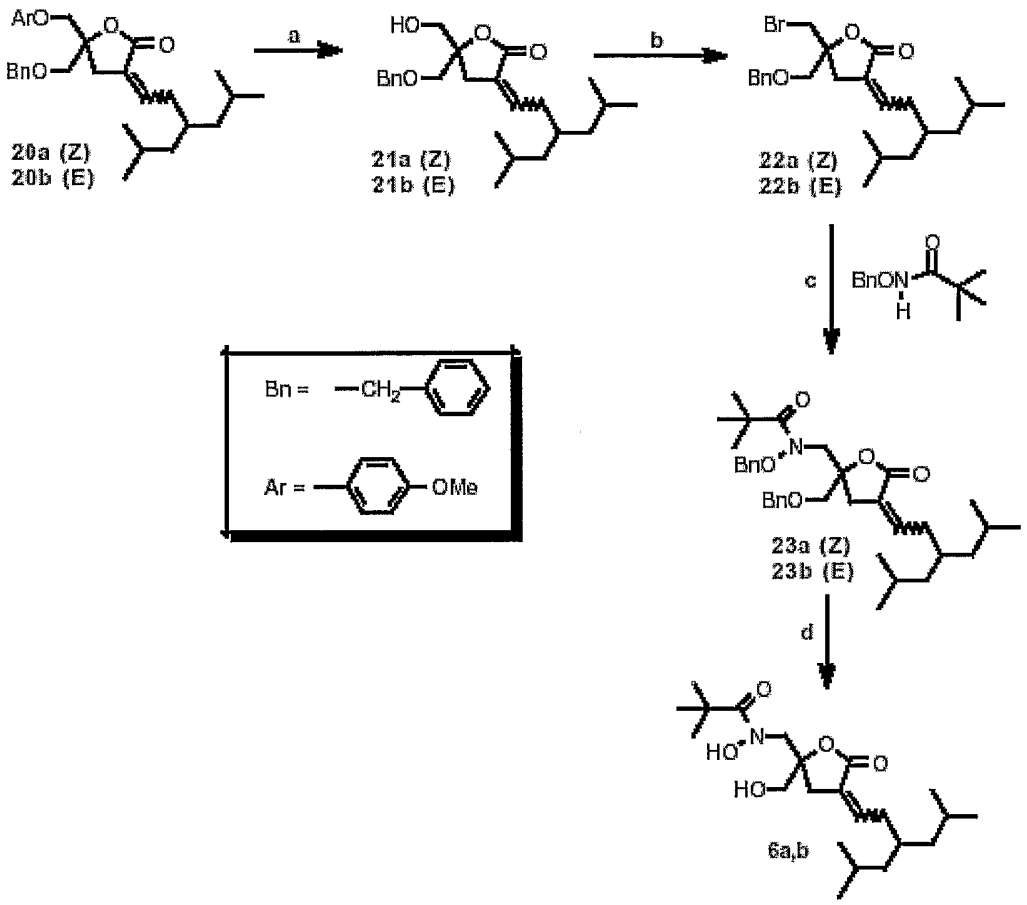
The aqueous layer was extracted three times with Et_2O (4.15mL/mmol) and the combined organic extracts were washed three times with water (2.50 mL/mmol), twice with brine (2.50 mL/mmol), dried using $MgSO_4$, and filtered by conventional means. The filtrate was concentrated under vacuum to afford the crude alkylation reaction product, which was purified by flash column chromatography after eluting with the appropriate solvent as known to those persons skilled in the art. The obtained mixture of diastereomers was typically used directly in the next step without further purification. This mixture was placed in dichloromethane (10 mL/mmol) at 0 ° C and was treated with methanesulfonyl chloride (2 equiv.) and triethylamine (4 equiv.). This mixture was stirred at 0 ° C for 30 min and then for 2 hours at about room temperature. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU, 5 eq) was added at 0 ° C, and the resulting solution was stirred overnight at ambient temperature. The reaction mixture was concentrated to a brown syrup and filtered through a pad of silica gel. The filtrate was concentrated by conventional means, and the residue was purified by flash column chromatography after eluding with the appropriate solvent providing resulting in "compound **14**."

For the N-hydroxyl amides, the geometric isomers of compound **14** are separated (to form "compounds **20a** and **20b**" shown in Scheme 1 below) and reacted individually. Selective oxidation of one of the branches gives compounds **21a** and **21b**,

which are reacted separately with Ph_3P, CBr_4, DMF at room temperature to give the corresponding compounds **22a** and **22b**. (Steps a and b, as shown in Scheme 1 below, are described further in the *Encyclopedia of Reagents for Organic Synthesis*, 8, 1995, 5364, Leo Paquette, John Wiley & Sons, incorporated herein by reference.)

Compounds **22a** and **22b** are treated with O-benzyl-N-pivaloyl-hydroxylamine in the presence of K_2CO_3 and acetone at room temperature to form compounds **23a** and **23b** to have benzyl protecting groups such that the N-alkylation compounds are formed rather than O-alkylation compounds (step c in Scheme 1). Step c in Scheme 1 is also described, e.g., in Maurer et al., *J. Am. Chem. Soc.*, 104, 1982, 3096-3101, which article is incorporated herein by reference. The benzyl protective group is removed (step d in Scheme 1) using $Pd(OH)_2$ to produce the corresponding N-hydroxyl amide compounds **6a** and its E-isomer **6b**. Other synthesis methodologies, such as use of BCl_3 may be used to synthesize compounds **6a** and **6b,** as known to those persons skilled in the art.

SCHEME 1



The reagents employed in Scheme 1 above are as follows: (a) ceric ammounium nitrate (CAN), CH_3CN/H_2O, 4/1, at 0 °C; (b) Ph_3P, CBr_4, DMF, at room temp.; (c) K_2CO_3, acetone, at room temp.; (d) [H2], $Pd(OH)_2$, cyclohexane.

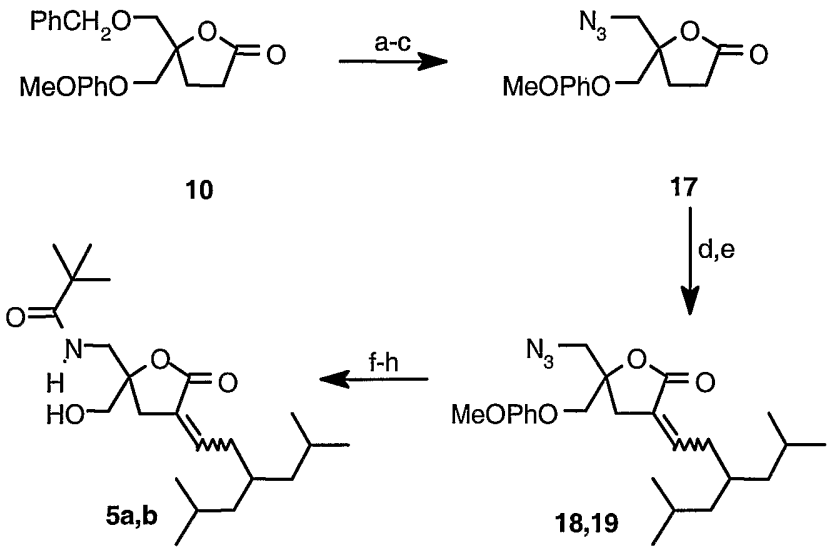
EXAMPLE 2

Synthesis of Compounds 5a and 5b

Compounds **5a** and **5b** are shown in the table of FIG. 1 as well as in Scheme 2 below. Synthesis of the side-chain amides **5a,b** required introduction of an azido group in one of the branches of compound **15** (shown in Scheme 3) to give compound **17** (shown in Scheme 2). The procedure to introduce the alkylidene chain on compound **17**

was essentially the same as that shown in Scheme 3 in Example 3. Following reduction of the azide with H_2 and Lindlar's catalyst (step f in Scheme 2 below), the mixture of amines was acylated with pivaloyl chloride, at which stage the geometric isomers 5a and 5b were separated by column chromatography (Scheme 2).

SCHEME 2



The reagents utilized in Scheme 2 were as follows: (a) BCl_3, CH_2Cl_2, at about -78 °C; (b) MsCl, Et_3N, CH_2Cl_2; (c) NaN_3, DMF, at 100 °C; (d) LiHDMS, THF, at about -78 °C, $[(i\text{-Pr})CH_2]_2CHCH_2CHO$; (e) MsCl, Et_3N, CH_2Cl_2, DBU; (f) H_2, Lindlar catalyst, EtOH; (g) $(CH_3)_3COCl$, Et_3N, CH_2Cl_2; and (h) CAN, CH_3CN-H_2O (4:1), at 0 °C.

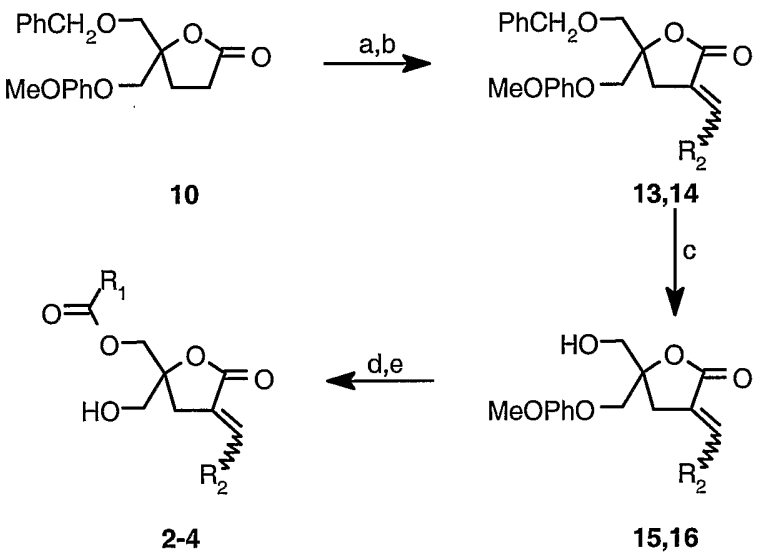
EXAMPLE 3

Synthesis of Compounds 2a, 2b, 3a, 3b, 4a, 4b, 7a, 7b, 8a, and 8b

Synthesis of compound **14** from compound **10** is shown in Scheme 3 below.

DAG-lactones 2a and b through 4a and b were synthesized essentially by the methodology shown in Scheme 3 and essentially as is shown in, *supra*, *J. Med. Chem.*, 1996, 39: 19-28, as would be known to those persons skilled in the art.

SCHEME 3



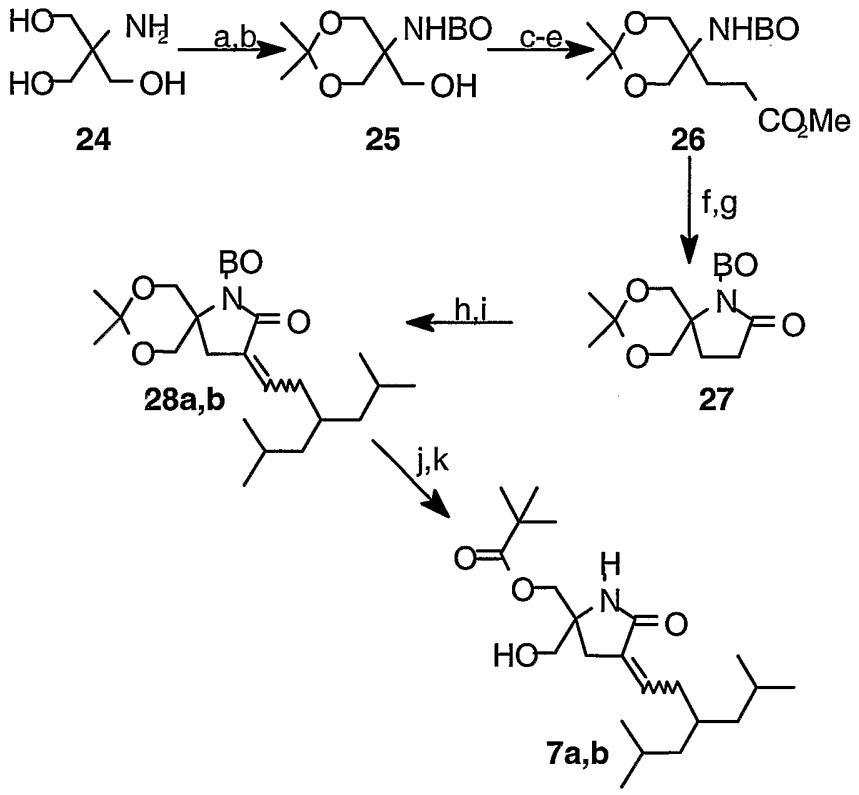
Reagents: (a) LiHDMS, THF, -78 °C, R_2CHO; (b) MsCl, Et_3N, CH_2Cl_2, DBU; (c) BCl_3, CH_2Cl_2, -78 °C; (d) R_1CO_2Cl, Et_3N, DMAP, CH_2Cl_2; (e) CAN, CH_3CN-H_2O (4:1), 0 °C.

Synthesis of Compounds 7a and 7b

The oxygen was replaced with nitrogen in the lactone ring (as shown in Scheme 4 below). The *t*-BOC protected amine of trishydroxymethylaminomethane compound **24** was reacted in acetone with catalytic amounts of *p*-toluenesulfonic acid to give compound **25**. Oxidation of the primary alcohol followed by Wittig olefination with methyl (triphenylphosphoranylidene)acetate gave compound **26** after catalytic hydrogenation of the double bond. Formation of the lactam ensued after treatment with sodium methoxide and the resulting amide was protected with the *t* BOC group to give compound **27**. This protection was necessary to implement the incorporation of the

alkylidene chain, which proceeded as planned to give compound **28** as a *Z/E*-mixture of isomers. Compounds **28a** and **28b** isomers were separated by column chromatography using methods known to those skilled in the art and reacted individually to give compounds **7a** and **7b**.

SCHEME 4

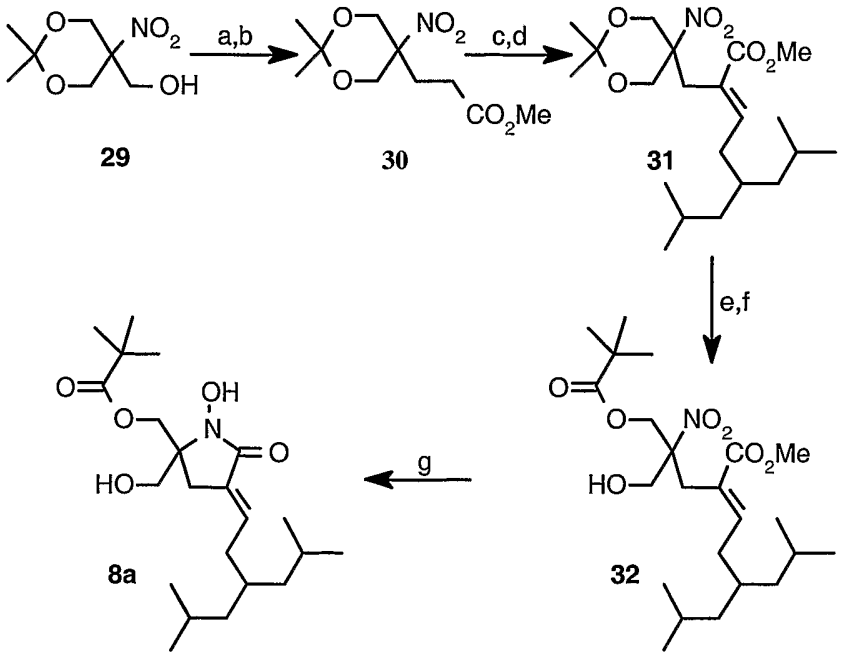


In Scheme 4, the following reagents were utilized: (a) $(t\text{-BuOCO})_2O$, Et_3N, MeOH; (b) *p*-TsOH, acetone; (c) PCC, CH_2Cl_2; (d) $Ph_3P{=}CHCO_2Me$, CH_2Cl_2; (e) H_2, Pd/C, EtOAc; (f) NaOMe, MeOH; (g) $(t\text{-BuOCO})_2O$, DMAP, Et_3N, THF; (h) LiHDMS, THF, -78 °C, $[(i\text{-Pr})CH_2]_2CHCH_2CHO$; (i) MsCl, Et_3N, CH_2Cl_2, DBU; (j) CF_3CO_2H, THF-H_2O (4:1); (k) $(CH_3)_3COCl$, Et_3N, CH_2Cl_2.

Synthesis of Compound 8a

The synthesis of the N-hydroxyl lactams required a different approach starting from tris(hydroxymethyl)nitromethane, which was converted to 2,2-dimethyl-5-nitro-1,3-(dioxan-5 yl)methanol, compound **29,** as set forth in Scheme 5 (see also, Linden G. B., et al., *J. Med. Chem.*, 1996, 39, 19-28). Similar Wittig olefination and reduction steps as to the ones used in Scheme 4, provided compound **30**. The conditions for these reactions are essentially identical; see under reagents, both part (c) and (d) in Scheme 5 which are the same as part (a) and (b) in Scheme 3. The only difference is the substrate that is used for these reactions. In the case of Scheme 5 these reactions were performed on an open substrate (compound **31**) instead of a cyclic lactone. Introduction of the alkylidene chain was performed at this stage, prior to cyclization, which produced essentially one isomer compound **31**. This compound was to become the *E*-isomer of target compound **8a**. Removal of the acetonide group from compound **31** and selective monoacylation with pivaloyl chloride gave the penultimate intermediate compound **32.** Compound **32** underwent reductive cyclization in the presence of Zn to give compound **8a**.

SCHEME 5



The reagents utilized in Scheme 5 were as follows: (a) i. $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, at about -78 ° C; ii. $Ph_3P{=}CHCO_2Me$, CH_2Cl_2, (b) H_2, Pd/C, EtOH, (c) LiHDMS, THF, at about -78 ° C, $[(i\text{-Pr})CH_2]_2CHCH_2CHO$, (d) MsCl, Et_3N, CH_2Cl_2, DBU, (e) *p*-TsOH, MeOH, (f) $(CH_3)_3COCl$, pyridine, CH_2Cl_2, and (g) Zn, NH_4Cl, EtOH.

EXAMPLE 4

Determination of DAG Compounds PKC Binding Affinity (K_i)

The binding affinity, K_i, for each compound is measured in the following manner: [^{3}H]PDBu binding to PKC isozymes is measured using polyethylene glycol precipitation assay procedures known to those persons skilled in the art. A detailed description of the methodology is presented in Kazanietz, et al, *Mol. Pharmacol.*, 1993, 44, 298-307, which is incorporated herein by reference. To measure competition of [^{3}H]PDBu binding by different analogs, a fixed concentration of [^{3}H]PDBu (3 nM) and increasing concentrations (in triplicate) of the competing non-radioactive ligand may be used. ID_{50} values are determined from the competition curve (a curve identical to that shown in FIG. 7) and the K_i for the competing ligand is calculated from the ID_{50} by using the relationship

$$K_i = ID_{50} / (1 + L/K_d),$$

wherein, K_d is the dissociation constant for [^{3}H]PDBu and L is the concentration of free [^{3}H]PDBu at the ID_{50}. PKCs used in these assays are generated by baculovirus infection of Sf9 insect cells and subsequent purification, as described in Kazanietz, et al., *Mol. Pharmacol.*,1993, 44, 298-307, which is incorporated herein by reference.

As can be seen in FIG. 1, expected Ki measurements of certain embodiments of the new DAG mimetics reveal that insertion of an O or NOH group at the X position of the generalized structure shown in Formula 5 above (with Y=O) provides tight binding with PKC in vitro; these compounds have or are expected to have Ki values below

12 nM. The compounds that did not possess these constraints, will see a drop off in binding affinity of between two and four orders of magnitude. See FIG. 8.

EXAMPLE 5

Calculation of DAG Compounds' Lipophilicity Values (Log P)

The octanol/water partition coefficient (log P) is calculated according to the fragment-based program described in KOWWIN 1.63. Meylan, et al., *J. Pharm. Sci.*, 1995, 20:84, which is incorporated herein by reference. The lipophilicity value is correlated with the corresponding PKC binding affinity by plotting $\log(1/K_i)$ versus log P.

Control of lipophilicity is important for several reasons. A compound with too high of an affinity for lipids will likely become trapped in the cellular membrane and be unable to interact effectively with PKC. A compound with too low of an affinity for lipids will likely not even enter the cell. Compounds with a high log P are very oily, are, thus, not water-soluble and therefore make poor pharmaceutical formulations.

Substitution of an NOH group for the O at the X position of the structure shown in Formula 5 above is expected to lead to a drop in the log P values by over two full units, from about 5.89 to 3.58. The log P values for these compounds are expected to range from approximately 7 to 3.5. The lower the log P values the better as long as the compound maintains good binding affinity. Superior results are expected to be achieved at a log P value of about 3.5 and no lower than about 3. For compound **6a**, a log P value of 3.6 is optimal but other log P values are acceptable.

EXAMPLE 6

Apoptosis Assays

To assess morphological changes in chromatin structure, cells are stained with DAPI (4', 6-diamidino-2-phenylindole) available from Sigma of St. Louis, Missouri. Cell culture reagents and media may be purchased from Life Technologies, Inc. of Gaithersburg, Maryland. Cells are trypsinized, mounted on glass slides, fixed in 70%

ethanol, and stained for 20 min with 1 mg/ml DAPI. Apoptosis is characterized by chromatin condensation and fragmentation when examined by fluorescence microscopy. The incidence of apoptosis in each preparation is analyzed by counting 500 cells and determining the percentage of apoptotic cells as described in Fujii, et al., *J. Biol. Chem.*, 2000, 275, 7574-7582, which reference is incorporated herein by reference.

DNA laddering is measured using the Apoptotic DNA-Ladder kit available from Boehringer Mannheim Corp (Indianapolis, IN). For flow cytometry analysis, cells are fixed in 70% ethanol and re-suspended in PBS containing propidium iodide (1 mg/ml) and RNase (40 μg/ml). Cell cycle progression and apoptosis are analyzed in an EPICS XL flow cytometer (Coulter Corp, Hialeah, FL). For each treatment 7,500 events were or are recorded.

Human prostate cancer cell line LNCaP is purchased from the American Type Culture Collection of Rockville, Maryland. The LNCaP cells are cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere. The LNCaP cells are treated with different synthetic DAG-mimetics at a single concentration (10 μM) for 1 hour, and apoptosis is assessed 24 hours later by counting the number of apoptotic cells after DAPI staining. After such time, a maximum apoptotic response may be observed following PKC activation.

Replication-deficient adenoviruses (AdV) are used for individual PKC isozymes. Generation of the PKCαAdV and PKCδAdV are produced as described in the Fuji article referenced above. Kinase inactive PKCs are generated to an Arg to Lys substitution at the ATP-binding site of the catalytic domain, and the corresponding AdVs are then generated as described in Onba et al, Mol. Cell Biol., 18, 1998, 5199-5207, which is incorporated herein by reference. AdVs are amplified in HEK 293 cells using standard techniques. Titers of viral stocks are normally higher than 1×10^9 pfu/cell. The absence of wild type AdV is confirmed by PCR using primers for the El region. An AdV for the LacZ gene (LacZAdV) is used as a control.

Subconfluent LNCaP cells in 6- or 12-well plates are infected with AdVs for 14 hours at multiplicities of infection (MOIs) ranging from 1 to 30 pfu/cell (in RPMI 1640 medium supplemented with 2% FBS). Following infection, the media is replaced with fresh RPMI 1640 medium supplemented with 10% FBS and the cells are grown for an additional 24 hours. Maximum expression of PKC isozymes after AdV infection is to be achieved with this protocol.

Synthetic DAG-lactones, PMA or vehicle (ethanol), are added for 1 hour at different concentrations to either non-infected cells or to cells infected with different AdVs. After treatment, cells are grown in RPMI 1640 supplemented with 10% FBS for 24 hours. PKC inhibitors (GF109203X, Gö6976 or rotterlin) or the pan-caspase inhibitor z-VAD are added 1 hour before and during DAG-lactones or PMA treatment.

Cells are harvested and lysed in a buffer containing 50mM Tris-HCI, pH 6.8, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol. Equal amounts of protein (10 μg) are subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes are blocked with 5% non-fat milk and 0.1% Tween 20 in phosphate-buffered saline (PBS), and then incubated with a monoclonal anti-PKCα antibody (1:3000, UBI, Lake Placid, NY) or a polyclonal anti-PKCδ antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). Membranes are washed three times with 0.2% Tween 20/PBS and incubated with anti-mouse or anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:10,000, Bio-Rad Laboratories, Hercules, CA). Bands are visualized by the enhanced chemiluminescense (ECL) Western blotting detection system (Amersham Pharmacia Biotech, Arlington Heighs, IL).

LNCaP cells are transfected with vectors encoding for GFP fusion proteins for PKCα ahd PKCδ using Lipofect AMINE PLUS (Life Technologies, Inc.) according to the manufacturer's instructions. The fluorescence becomes detectable 24 hours after transfection, and all experiments are performed 3 days after transfection. Prior to observation, transiently tranfected LNCaP cells are washed twice with standard medium (Dulbecco's modified Eagle's medium without phenol red supplemented with 1% FBS) pre-warmed to 37°C. All PKC activators are diluted to specified concentrations in the

same medium, and the final concentration of solvent (ethanol) is preferably less than 0.01%.

For live cell imaging, the Bioptechs Focht Chamber System (FCS2) is inverted and attached to the microscope stage with a custom stage adapter. The cells cultured on a 40-mm round coverslip are introduced into a chamber system that is connected to a temperature controller set at 37°C, and a medium is perfused through the chamber with a model P720 microperfusion pump (Instech, Plymouth Meeting, PA). As indicated, the perfustate to the chamber is changed to that containing the specified ligand for PKC and sequential images of the same cell are then collected at 1 minute intervals using LaserSharp software through a BioRad MRC 1024 confocal scan head mounted on a Nikon Optiphot microscope with a planapochromat lens. Excitation at 488 nm is provided by a krypton-argon gas laser with a 522/32 emission filter for green fluorescence.

From the particular DAG analogs, the following four were or are tested for apoptotic activity: compounds **3a**, **3b**, **6a**, **and 6b.** Compound **3a** induced approximately 30% of apoptosis and **6a** is expected to induce approximately 30% of apoptosis, which equals the maximum response observed with some phorbol esters. On the other hand, DAG-lactone **3b** (E isomer of **3a**) showed only a modest response, although both E and Z isomers had essentially identical K_i and log P values. The same performance is expected of compound **6b** (E isomer of **6a**). Therefore, there is apparently no direct correlation between the K_i value (PKC binding) and apoptosis-inducing activity, although the most potent compounds are good PKC ligands. Actual compound **3a** results and expected results of the **6a** and **6b** compounds of the apoptosis efficacy tests are shown in FIGS. 2-9.

EXAMPLE 7

Methods of Treatment

The present invention includes a treatment for cancer in a subject such as an animal, for example a rat or human, by inducement of apoptosis. The method includes administering one or more of the compounds of the present invention, or a combination of one or more of the compounds and one or more other pharmaceutical agents, to the subject in a pharmaceutically compatible carrier. The administration is made in an amount effective to inhibit the development or progression of cancer, specifically prostate cancer without excluding and other types of malignancies. Although the treatment can be used prophylactically in any patient in a demographic group at significant risk for such diseases, subjects can also be selected using more specific criteria, such as a definitive diagnosis of the condition.

The vehicle in which the drug is delivered can include pharmaceutically acceptable compositions of the drugs, using methods well known to those with skill in the art. Any of the common carriers, such as sterile saline or glucose solution, can be utilized with the drugs provided by the invention. Routes of administration include but are not limited to oral and parenteral routes, such as intravenous (iv), intraperitoneal (ip), rectal, topical, ophthalmic, nasal, and transdermal.

The drugs may be administered in a suitable manner now known or later developed, e.g., orally or intravenously, in any conventional medium. For example, intravenous injection may be by an aqueous saline medium. The medium may also contain conventional pharmaceutical adjunct materials such as, for example, pharmaceutically acceptable salts to adjust the osmotic pressure, lipid carriers such as cyclodextrins, proteins such as serum albumin, hydrophilic agents such as methyl cellulose, detergents, buffers, preservatives and the like. A more complete explanation of parenteral pharmaceutical carriers can be found in *Remington: The Science and Practice of Pharmacy* (19th Edition, 1995) in chapter 95.

Embodiments of other pharmaceutical compositions can be prepared with conventional pharmaceutically acceptable carriers, adjuvants and counterions as would

be known to those of skill in the art. The compositions are preferably in the form of a unit dose in solid, semi-solid and liquid dosage forms such as tablets, pills, powders, liquid solutions or suspensions.

The compounds of the present invention are ideally administered as soon as possible after the diagnosis of cancer. For example, once unwanted angiogenesis has been confirmed or the presence of a tumor has been identified, a therapeutically effective amount of the drug is administered to induce apoptosis. The dose can be given orally or by frequent bolus administration. The subject may then be monitored for signs of induced apoptosis as, for example, shown by a decrease in tumor size

Therapeutically effective doses of the compounds of the present invention can be determined by one of skill in the art, with a goal of achieving a desired level of apoptosis as illustrated in the foregoing examples and figures. The relative toxicities of the compounds make it possible to administer in various dosage ranges. An example of such a dosage range is from about 0.5 to about 50 mg/kg body weight orally in single or divided doses. Another example of a dosage range is from about 0.5 to about 50 mg/kg body weight orally in single or divided doses. For oral administration, the compositions are, for example, provided in the form of a tablet containing from about 25 to about 500 mg of the active ingredient, particularly 100 mg of the active ingredient for the symptomatic adjustment of the dosage to the subject being treated.

The specific dose level and frequency of dosage for any particular subject may be varied and will depend upon a variety of factors, including the activity of the specific compound, the staging of the existing cancer, the age, body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, and severity of the condition of the host undergoing therapy.

The pharmaceutical compositions can be used in the treatment of a variety of malignancies. Examples of such diseases include all types of cancer, ocular neovascular disease, solid tumor formation and metastasis in solid tumors.

EXAMPLE 8

Combination Therapy

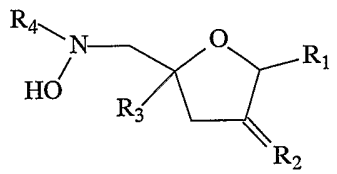
The present invention also includes combinations of the DAG analogs of the present invention and/or combinations of the same with various other anticancer agents and/or apoptotic inducing compounds. The term "administration" refers to both concurrent and sequential administration of the active agents. For example, the DAG analogs may be combined with phorbol esters for the treatment of cancer or like diseases. In addition, the DAG analogs of this invention may be used in combination with other forms of cancer therapy, e.g., chemotherapy, radiation therapy, hormonal therapy).

In view of the many possible embodiments to which the principles of our invention may be applied, it should be recognized that the illustrated embodiment is only a preferred example of the invention and should not be taken as a limitation on the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

We claim:

1. A method of inducing apoptosis comprising:

administering to a subject an effective amount of one or more compounds satisfying the formula:



(1)

wherein R_1, R_2, R_3, and R_4 are independently selected from the group consisting essentially of oxygen, nitrogen, hydrogen, hydroxyls, halogens, branched or unbranched, substituted or unsubstituted, aliphatic groups, heteroatom substituted aliphatic groups, aromatic groups, major functional groups or one or more pharmaceutically acceptable salts pharmaceutically acceptable or pharmaceutically acceptable carriers thereof.

2. The method of claim 1 wherein, R_1 is a carbonyl.

3. The method of claim 1 wherein, R_3 is CH_2OH.

4. The method of claim 1 wherein, R_1 is oxygen and hydrogen.

5. The method of claim 1 wherein, R_3 includes an alkylhydroxyl group and R_1 is a lactone carbonyl.

6. The method of claim 1 wherein, R_3 is CH_2OH and R_1 is oxygen.

7. The method of claim 1 wherein, R_4 is an alkyl group derived from an acid chloride.

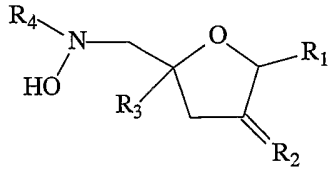
8. The method of claim 1 wherein, R_2 is an alkylidene group derived from an aldehyde.

9. The method of claim 1, wherein the compound administered is directly responsible for inducing apoptosis in the subject.

10. The method of claim 1, wherein the size of the lactone ring is increased or decreased.

11. A method of inducing apoptosis comprising:

administering to a subject one or more compounds satisfying the formula:



(1)

wherein R_1, R_2, R_3, and R_4 are independently selected from the group consisting essentially of oxygen, nitrogen, hydrogen, hydroxyls, halogens, branched or unbranched, substituted or unsubstituted, aliphatic groups, heteroatom substituted aliphatic groups, aromatic groups, and major functional groups or one or more pharmaceutically acceptable salts or pharmaceutically acceptable carriers thereof; and

monitoring the subject for indications of apoptosis.

12. The method of claim 11 wherein, R_1 is a carbonyl.

13. The method of claim 11 wherein, R_3 is CH_2OH.

14. The method of claim 11 wherein, R_1 is oxygen.

15. The method of claim 11 wherein, R_3 includes an alkylhydroxyl group and R_1 is a lactone carbonyl.

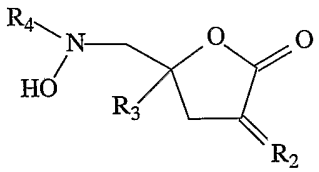
16. The method of claim 11 wherein, R_4 is an alkyl group derived from an acid chloride.

17. The method of claim 11 wherein, R_2 is an alkylidene group derived from an aldehyde.

18. The method of claim 11 wherein the size of the lactone ring is increased or decreased.

19. A method of inducing apoptosis comprising:

administering to a subject an effective amount of one or more compounds satisfying the formula:



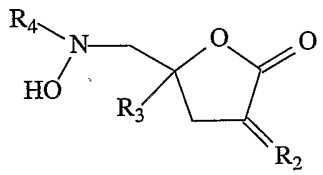
(2)

wherein R_2, R_3, and R_4 are independently selected from the group consisting essentially of oxygen, nitrogen, hydrogen, hydroxyls, halogens, branched or unbranched, substituted or unsubstituted, aliphatic groups, heteroatom substituted aliphatic groups, aromatic groups, or one or more pharmaceutically acceptable salts or pharmaceutically acceptable carriers thereof.

20. The method of claim 19 wherein, R_3 is CH_2OH or OH.

21. A method of inducing apoptosis comprising:

administering to a subject one or more compounds satisfying the formula:



(2)

wherein R_2, R_3, and R_4 are independently selected from the group consisting essentially of oxygen, nitrogen, hydrogen, hydroxyls, halogens, branched or unbranched, substituted or unsubstituted, aliphatic groups, heteroatom substituted aliphatic groups, and aromatic groups, or one or more pharmaceutically acceptable salts or pharmaceutically acceptable carriers thereof; and

monitoring the subject for indications of apoptosis.

22. The method of claim 21 wherein, R_3 is CH_2OH.

23. The method of claim 21 wherein, R_4 is an alkyl group derived from an acid chloride.

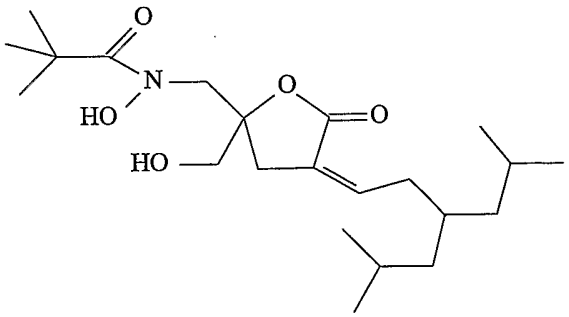
24. The method of claim 21 wherein, R_2 is an alkylidene group derived from an aldehyde.

25. The method of claim 21 wherein, R_2 is an aldehyde and R_3 is an alkylhydroxyl.

26. The method of claim 21, wherein the compound administered is directly responsible for inducing apoptosis in the subject.

27. A method of inducing apoptosis comprising:

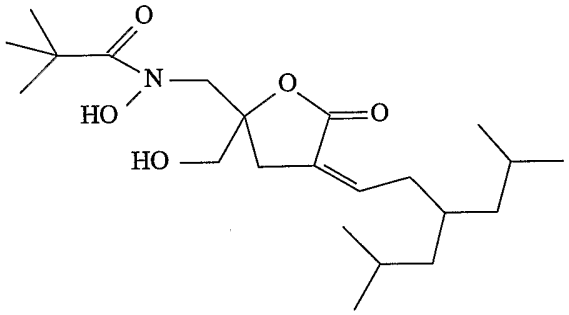
administering to a subject an effective amount of a compound satisfying the formula:



or one or more pharmaceutically acceptable salts or pharmaceutically acceptable carriers thereof.

28. A method of inducing apoptosis comprising:

administering to a subject one or more compounds satisfying the formula:

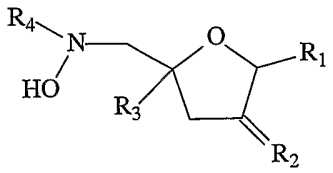


and

monitoring the subject for indications of apoptosis.

29. The method of claim 28, wherein the compound is administered is directly responsible for inducing apoptosis in the subject.

30. A composition that induces apoptosis in a subject when administered in an effective amount, the composition comprising one or more compounds satisfying the formula:



(1)

wherein R_1, R_2, R_3, and R_4 are independently selected from the group consisting essentially of oxygen, nitrogen, hydrogen, hydroxyls, halogens, branched or unbranched, substituted or unsubstituted, aliphatic groups, heteroatom substituted aliphatic groups, aromatic groups, and functional groups or one or more pharmaceutically acceptable salts or pharmaceutically acceptable carriers thereof.

31. The composition of claim 30 wherein, R_1 is a carbonyl.

32. The composition of claim 30 wherein, R_3 is CH_2OH.

33. The composition of claim 30 wherein, R_1 is oxygen.

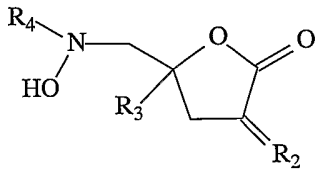
34. The composition of claim 30 wherein, R_3 includes an alkylhydroxyl group and R_1 is a lactone carbonyl.

35. The composition of claim 30 wherein, R_3 is CH_2OH and R_1 is oxygen.

36. The composition of claim 30 wherein, R_4 is an alkyl group derived from an acid chloride.

37. The composition of claim 30 wherein, R_2 is an alkylidene group derived from aldehyde.

38. A composition for inducing apoptosis comprising one or more compounds satisfying the formula:



(2)

wherein R_2, R_3, and R_4 are independently selected from the group consisting essentially of oxygen, nitrogen, hydrogen, hydroxyls, halogens, branched or unbranched, substituted or unsubstituted, aliphatic groups, heteroatom substituted aliphatic groups, aromatic groups, and functional groups or one or more pharmaceutically acceptable salts or pharmaceutically acceptable carriers thereof.

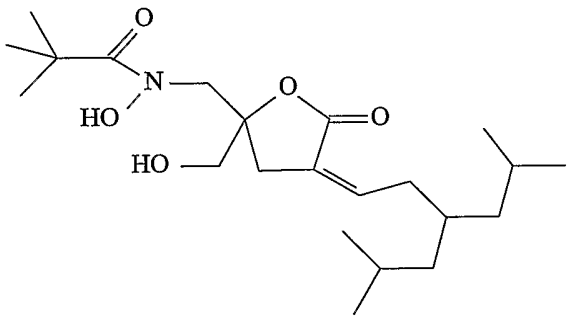
39. The composition of claim 38 wherein, R_3 is CH_2OH .

40. The composition of claim 38 wherein, R_4 is an alkyl group derived from acid chloride.

41. The composition of claim 38 wherein, R_2 is an alkylidene group derived from aldehyde.

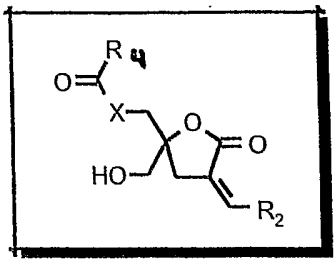
42. The composition of claim 38 wherein, R_2 is an aldehyde and R_3 is an alkylhydroxyl.

43. A composition for inducing apoptosis in a subject when an effective amount is administered thereto, the composition comprising one or more compounds satisfying the formula:



or one or more pharmaceutically acceptable salts or carriers thereof.

Table 1. Structure Activity Analysis of log P and Ki as a function of size of the acyl and α alkylidene chains (log P was or will be calculated according to the fragment-based program KOWWIN 1.63).



#	R_4	R_2	X	*E/Z*	Log P	K_i (nM)
1a	$CH_2CH(i\text{-}Pr)_2$	$CH_2CH(i\text{-}Pr)_2$	O	*Z*	5.89	2.89±0.2
1b	$CH_2CH(i\text{-}Pr)_2$	$CH_2CH(i\text{-}Pr)_2$	O	*E*	5.89	2.70±0.4
2a	$(CH_3)_3C$	$CH_2CH(i\text{-}Pr)_2$	O	*Z*	4.04	8.32±0.7
2b	$(CH_3)_3C$	$CH_2CH(i\text{-}Pr)_2$	O	*E*	4.04	11.25±0.7
3a	$(CH_3)_3C$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	O	*Z*	5.03	2.90±0.4
3b	$(CH_3)_3C$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	O	*E*	5.03	4.51±0.5
4a	$(i\text{-}Pr)_2CHCH_2$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	O	*Z*	6.88	6.87±0.6
4b	$(i\text{-}Pr)_2CHCH_2$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	O	*E*	6.88	4.46±0.4
5a	$(CH_3)_3C$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	NH	*Z*	3.95	429.7±5.5
5b	$(CH_3)_3C$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	NH	*E*	3.95	1263±90
6a	$(CH_3)_3C$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	NOH	*Z*	3.58	5.42±0.3
6b	$(CH_3)_3C$	$CH_2CH[CH_2(i\text{-}Pr)]_2$	NOH	*E*	3.58	4.81±0.4

Structures 9-12

FIG. 1

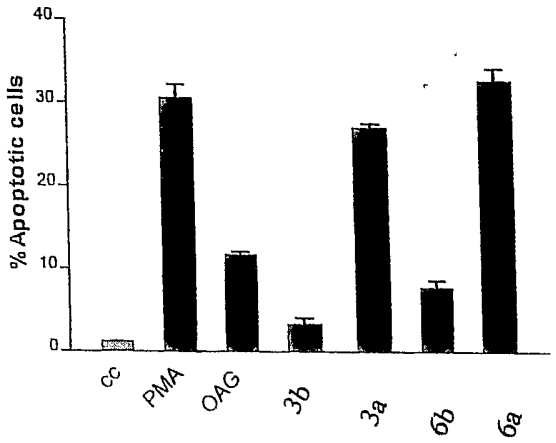

% Apoptotic cells
40
30
20
10
0
cc
PMA
OAG
3b
3a
6b
6a

FIG. 2A

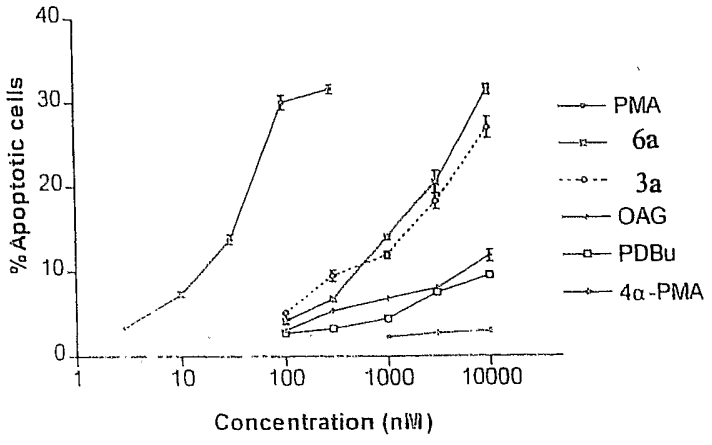

% Apoptotic cells
40
30
20
10
0
1
10
100
1000
10000
Concentration (nM)
PMA
6a
3a
OAG
PDBu
4α-PMA

FIG. 2B

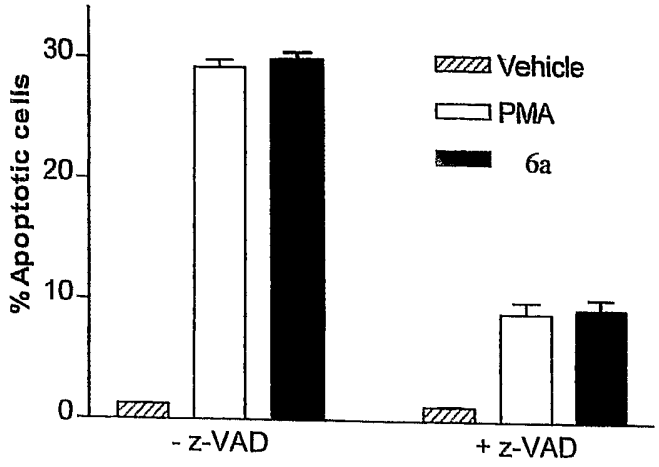

% Apoptotic cells
30
20
10
0
Vehicle
PMA
6a
- z-VAD
+ z-VAD

FIG. 3A

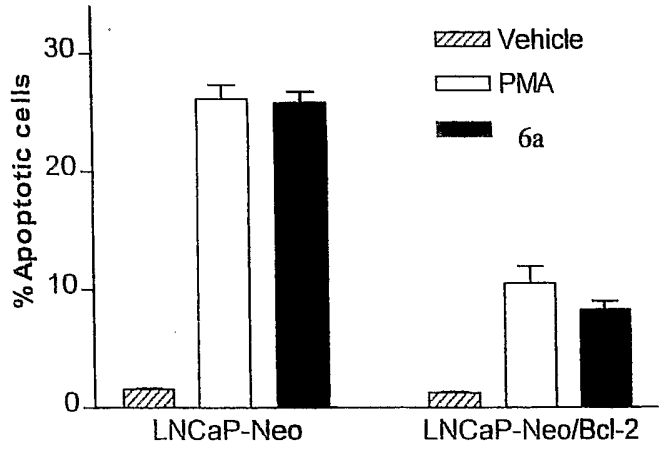

% Apoptotic cells
30
20
10
0
Vehicle
PMA
6a
LNCaP-Neo
LNCaP-Neo/Bcl-2

FIG. 3B

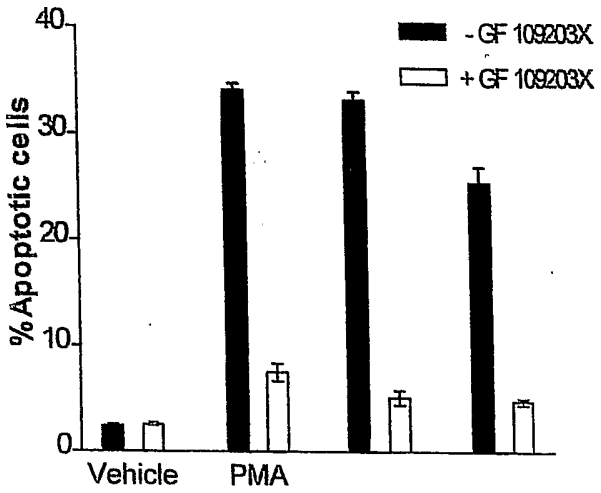

40
30
20
10
0
% Apoptotic cells
- GF 109203X
+ GF 109203X
Vehicle
PMA

FIG. 4A

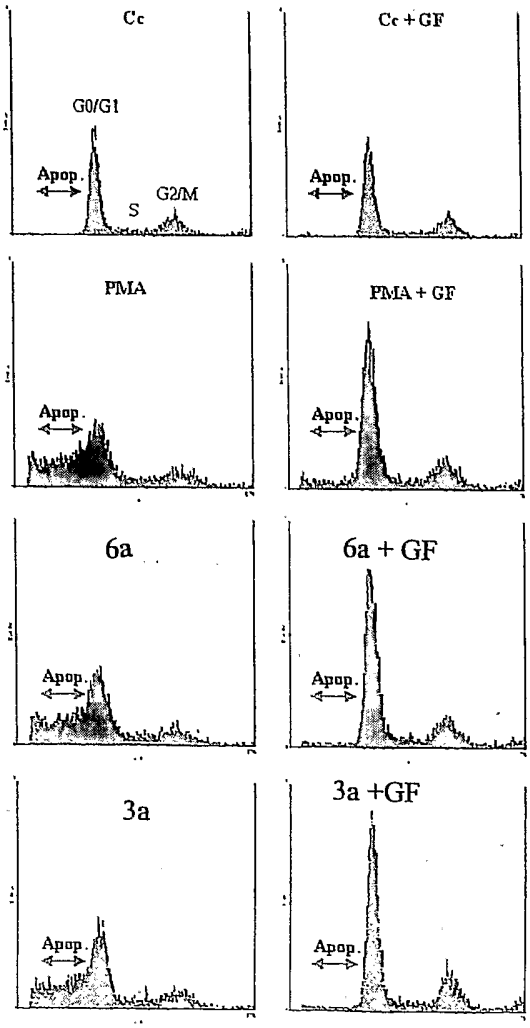

Cc
Cc + GF
G0/G1
Apop.
S
G2/M
PMA
PMA + GF
6a
6a + GF
3a
3a +GF

FIG. 4B

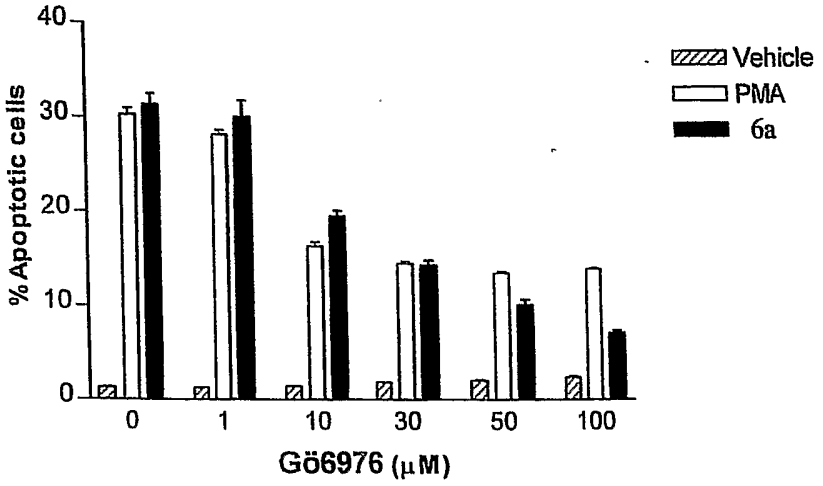

40
30
20
10
0
% Apoptotic cells
0
1
10
30
50
100
Gö6976 (μM)
Vehicle
PMA
6a

FIG. 5A

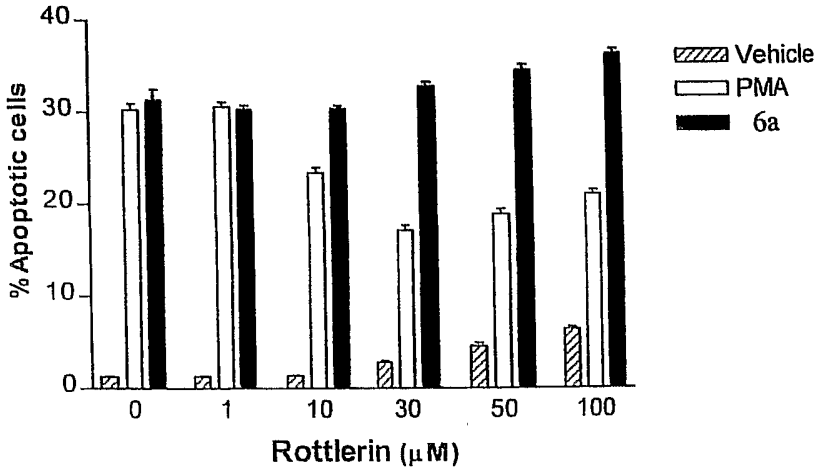

40
30
20
10
0
% Apoptotic cells
0
1
10
30
50
100
Rottlerin (μM)
Vehicle
PMA
6a

FIG. 5B

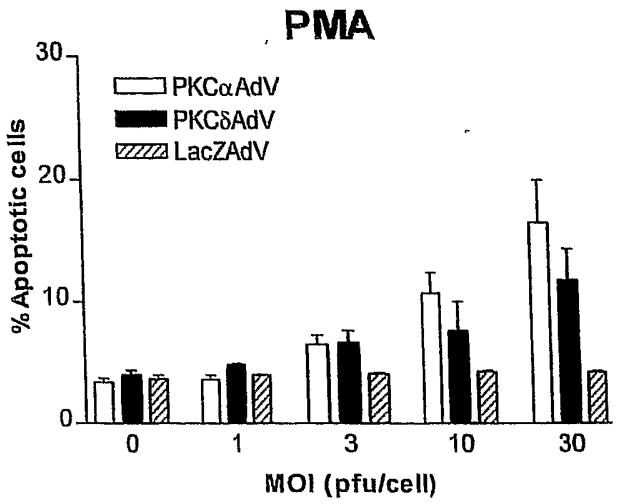

PMA
30
20
10
0
% Apoptotic cells
PKCαAdV
PKCδAdV
LacZAdV
0
1
3
10
30
MOI (pfu/cell)

FIG. 6A

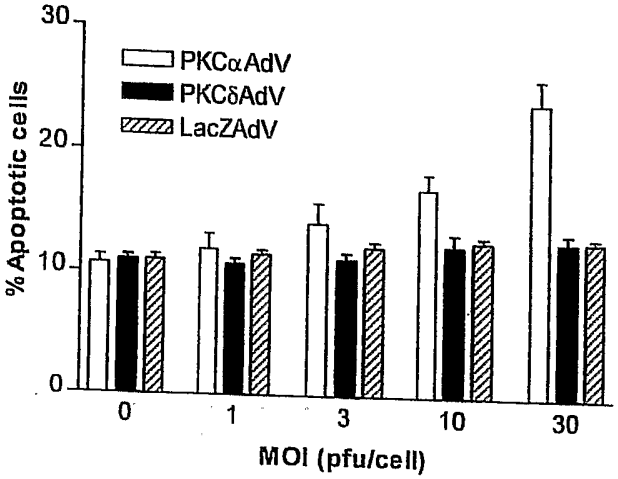

30
20
10
0
% Apoptotic cells
PKCαAdV
PKCδAdV
LacZAdV
0
1
3
10
30
MOI (pfu/cell)

FIG. 6B

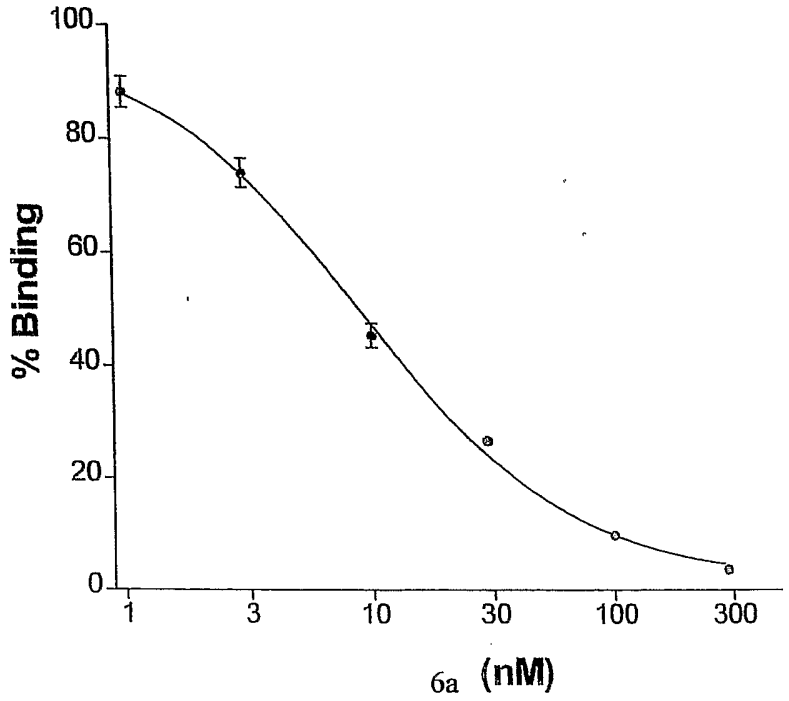

100
80
60
40
20
0
% Binding
1
3
10
30
100
300
6a (nM)

FIG. 7

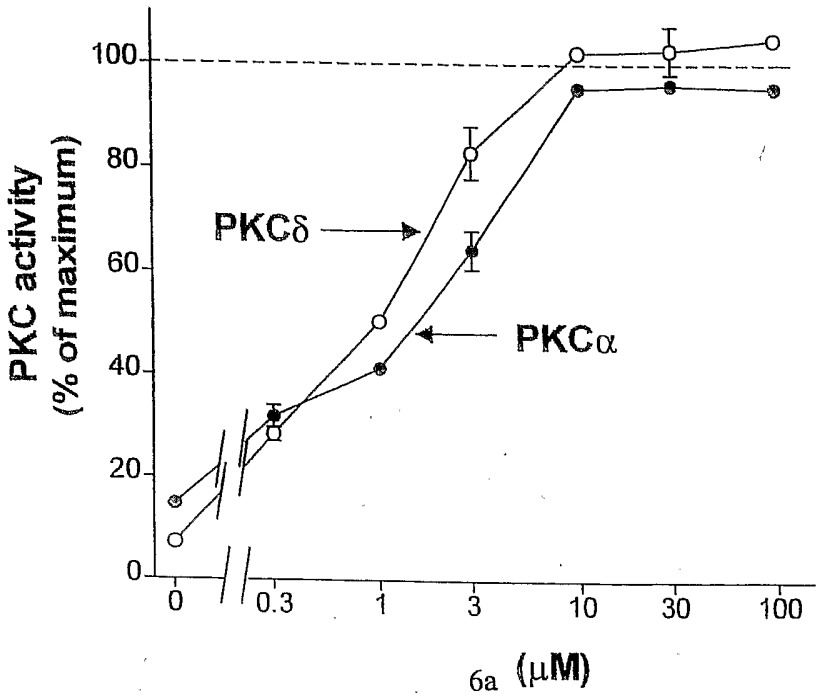

100
80
60
40
20
0
PKC activity
(% of maximum)
PKCδ
PKCα
0
0.3
1
3
10
30
100
6a (μM)

FIG. 8

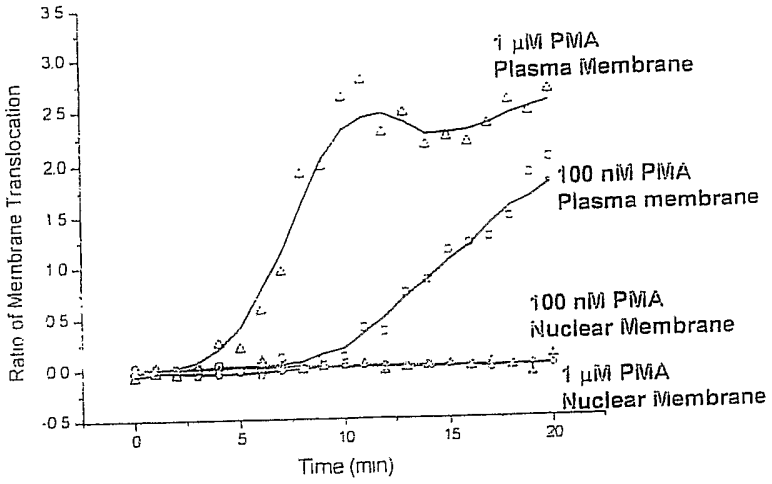

Ratio of Membrane Translocation
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0
5
10
15
20
Time (min)
1 μM PMA
Plasma Membrane
100 nM PMA
Plasma membrane
100 nM PMA
Nuclear Membrane
1 μM PMA
Nuclear Membrane

FIG. 9A

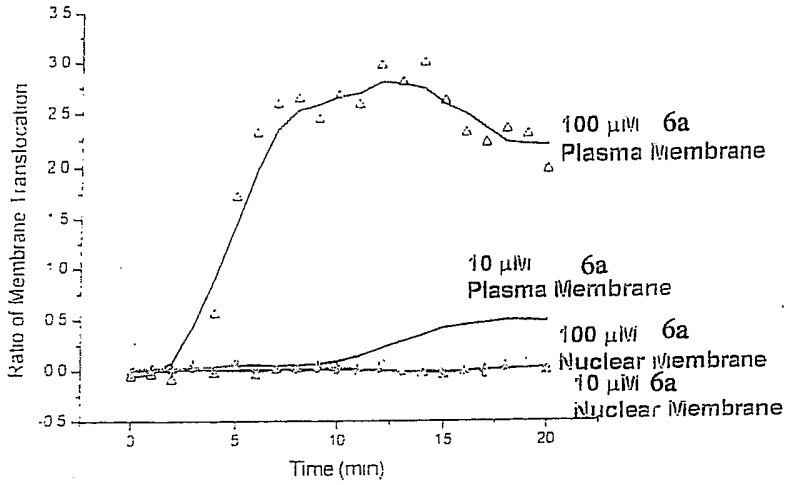

Ratio of Membrane Translocation
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0
5
10
15
20
Time (min)
100 μM 6a
Plasma Membrane
10 μM 6a
Plasma Membrane
100 μM 6a
Nuclear Membrane
10 μM 6a
Nuclear Membrane

FIG. 9B

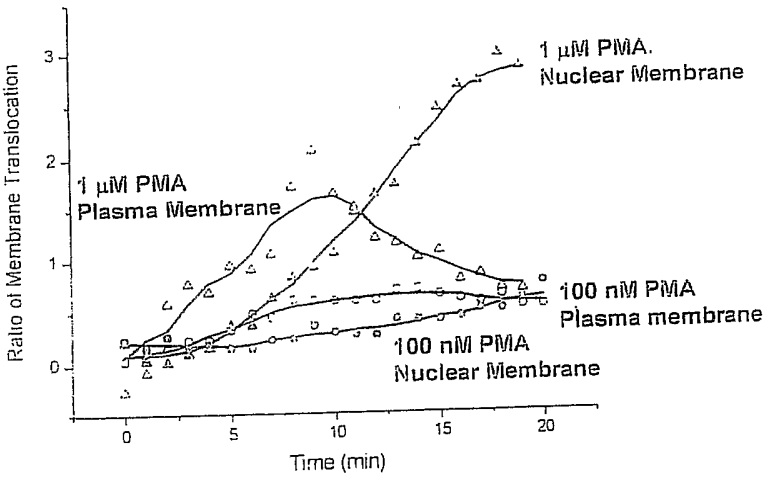

Ratio of Membrane Translocation
Time (min)
1 µM PMA.
Nuclear Membrane
1 µM PMA
Plasma Membrane
100 nM PMA
Plasma membrane
100 nM PMA
Nuclear Membrane

FIG. 9C

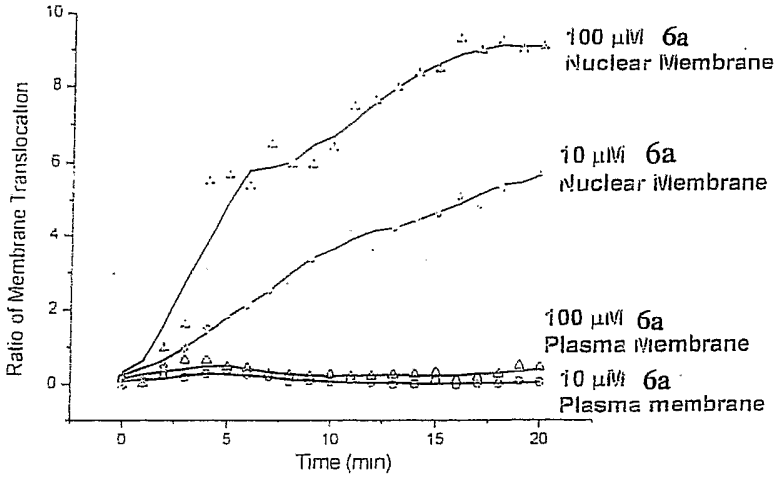

Ratio of Membrane Translocation
Time (min)
100 µM 6a
Nuclear Membrane
10 µM 6a
Nuclear Membrane
100 µM 6a
Plasma Membrane
10 µM 6a
Plasma membrane

FIG. 9D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/25088

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 31/34
US CL : 514/461, 473

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)
U.S. : 514/461, 473

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)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 6,294,573 B1 (CURTIN et al.) 25 September 2001 (25.09.01), see entire document.	1-43

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"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)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"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

"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

"&" document member of the same patent family

Date of the actual completion of the international search
04 December 2002 (04.12.2002)

Date of mailing of the international search report
12 DEC 2002

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Authorized officer
Cybille Delacroix-Muirheid
Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

Continuation of B. FIELDS SEARCHED Item 3:
STN; Reg; supatfull
structure search, apoptosis