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(54) Title: ALLOSTERIC REVERSIBLE PAN-CASPASE INHIBITORS

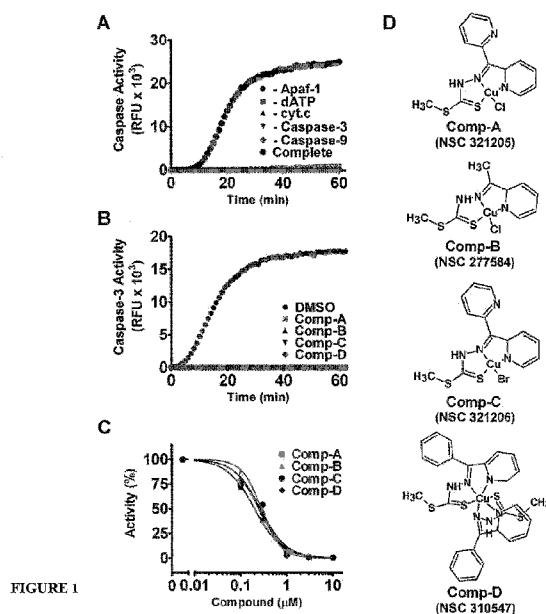


FIGURE 1

(57) Abstract: The invention provides a method for the use of a group of compounds as allosteric reversible caspase inhibitors. This invention is in the field of medicinal chemistry and relates to novel compounds, and pharmaceutical compositions and methods of use thereof that inhibit caspases and/or tumor necrosis factor alpha (TNFα) that mediate cell apoptosis and inflammation and inhibit pathophysiologic effects of excessive amounts of TNFα. The invention also relates to methods of using the compounds and pharmaceutical compositions of this invention to treat diseases where caspase and/or TNFα activity is implicated. The present invention also relates to methods for the use of allosteric, reversible pan-caspase inhibitors with pyridinyl, copper-containing molecules with a multi-ring structure.

ALLOSTERIC REVERSIBLE PAN-CASPASE INHIBITORS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of U.S. Provisional Patent Application No.
5 61/468,324, filed on. March 28, 2011, which is incorporated herein by reference [1].

STATEMENT OF GOVERNMENTAL SUPPORT

This invention was made with government support awarded by the National Institutes of Health (NIH Grant Numbers R01CA136513, U54CA137788/U54CA132378, and R01CA113890,
10 and an NIH/NCI Cancer Center Support Grant 5P30CA008748-44). The government has certain rights in the invention.

FIELD OF THE INVENTION

This invention is in the field of medicinal chemistry and relates to novel compounds, and
15 pharmaceutical compositions and methods of use thereof that inhibit caspases and/or tumor necrosis factor alpha (TNF α) that mediate cell apoptosis and inflammation and inhibit pathophysiologic effects of excessive amounts of TNF α . The invention also relates to methods of using the compounds and pharmaceutical compositions of this invention to treat diseases where caspase and/or TNF α activity is implicated. The present invention also relates to methods for the
20 use of allosteric, reversible pan-caspase inhibitors with pyridinyl, copper-containing molecules with a multi-ring structure.

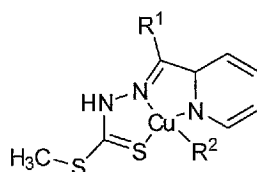
BACKGROUND OF THE INVENTION

While a number of caspase and TNF α inhibitors have been reported, it is not clear
25 whether they possess the appropriate pharmacological properties to be therapeutically useful. Therefore, there is a continued need for small molecule caspase and TNF α inhibitors to provide effective inhibition of apoptosis. Such compounds would be extremely useful in treating the disease states where caspase enzymes and/or TNF α cytokines play a role.

SUMMARY OF THE INVENTION

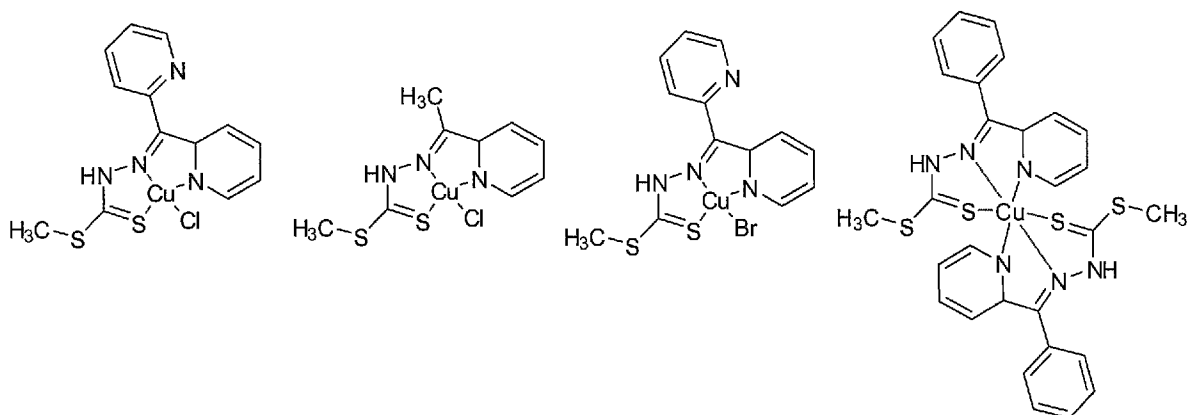
This invention is in the field of medicinal chemistry and relates to novel compounds, and pharmaceutical compositions and methods of use thereof that inhibit caspases and/or tumor necrosis factor alpha (TNF α) that mediate cell apoptosis and inflammation and inhibit pathophysiologic effects of excessive amounts of TNF α . The invention also relates to methods of using the compounds and pharmaceutical compositions of this invention to treat diseases where caspase and/or TNF α activity is implicated. The present invention also relates to methods for the use of allosteric, reversible pan-caspase inhibitors with pyridinyl, copper-containing molecules with a multi-ring structure.

In one embodiment, the invention contemplates a method comprising; a) providing; i) an allosteric pan-caspase inhibitor of Formula I; ii) a subject suffering from a disease state, wherein said disease has at least one symptom, wherein said subject comprises a caspase; and b) treating said subject with said allosteric pan-caspase inhibitor of Formula I so as to reduce at least one symptom of said disease state. In one embodiment, said allosteric pan-caspase inhibitor of Formula I comprises:



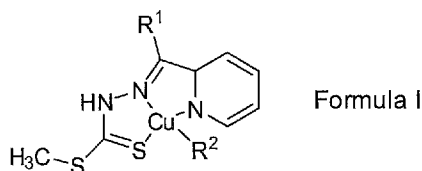
wherein R₁ is CH₃ (Me), -CH₂CH₃ (Et), -CH₂CH₂CH₃ (*n*-Pr), -CH(CH₃)₂ (*iso*-Pr), -CH(CH₂)₂ (cyclopropyl), -CH₂CH₂CH₂CH₃ (*n*-Bu), -CH(CH₃)CH₂CH₃ (*sec*-butyl), -CH₂CH(CH₃)₂ (*iso*-butyl), -C(CH₃)₃ (*tert*-butyl), -CH₂C(CH₃)₃ (*neo*-pentyl), cyclobutyl, cyclopentyl, cyclohexyl, cyclohexylmethyl, adamant-1-yl, adamant-2-yl, phenyl, and substituted phenyl; R₂ is at least one of the following groups comprising: Cl, Br, F, I, and (E)-methyl 2-((1,2-dihydropyridin-2-yl)(phenyl)methylene)hydrazinecarbodithioate. In one embodiment, said subject has a symptom of cell death. In one embodiment, symptom of cell death is mediated by an intrinsic pathway of apoptosis. In one embodiment, said allosteric pan-caspase inhibitor of Formula I provides protection from cell death. In one embodiment, said subject further comprises a cell containing a caspase, and a step of isolating said cell from said subject that is capable of undergoing apoptosis induced by ultra violet light. In one embodiment, said disease state results from exposure to at least one of the group comprising: tumor necrosis factor- α .

(TNF α) and cycloheximide. In one embodiment, said allosteric pan-caspase inhibitor of Formula I is selected from the group consisting of:



In one embodiment, said disease state is selected from the group consisting of neurodegeneration, radiation syndrome, ultra violet light exposure, osteoarthritis, pancreatitis, rheumatoid arthritis, chronic active hepatitis, inflammatory bowel disease, Crohn's disease, psoriasis, organ transplant rejection, sepsis, septic shock, cerebral ischemia, myocardial ischemia, myocardial infarction, amyotrophic lateral sclerosis, multiple sclerosis, neurological damage due to stroke, hepatitis-B, hepatitis-C, hepatitis-G, and liver disease.

In one embodiment, the invention contemplates a pharmaceutical composition of Formula I:

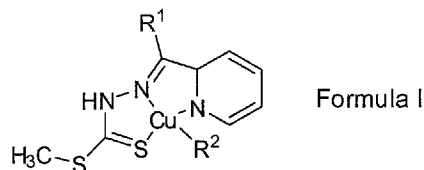


wherein R_1 is CH_3 (Me), $-CH_2CH_3$ (Et), $-CH_2CH_2CH_3$ (*n*-Pr), $-CH(CH_3)_2$ (*iso*-Pr), $-CH(CH_2)_2$ (cyclopropyl), $-CH_2CH_2CH_2CH_3$ (*n*-Bu), $-CH(CH_3)CH_2CH_3$ (*sec*-butyl), $-CH_2CH(CH_3)_2$ (*iso*-butyl), $-C(CH_3)_3$ (*tert*-butyl), $-CH_2C(CH_3)_3$ (*neo*-pentyl), cyclobutyl, cyclopentyl, cyclohexyl, cyclohexylmethyl, adamant-1-yl, adamant-2-yl, phenyl, and substituted phenyl; R_2 is at least one of the following groups comprising: Cl, Br, F, I, and (E)-methyl 2-((1,2-dihydropyridin-2-yl)(phenyl)methylene)hydrazinecarbodithioate.

This invention further provides pharmaceutical compositions comprising caspase inhibitor compound according to Formula I. This invention also relates to methods of using said pharmaceutical compositions for treatment of caspase-mediated diseases including inflammatory

and degenerative diseases.

In one embodiment, the invention contemplates an allosteric pan-caspase inhibitor of Formula I:



- 5 wherein R₁ is CH₃ (Me), -CH₂CH₃ (Et), -CH₂CH₂CH₃ (*n*-Pr), -CH(CH₃)₂ (*iso*-Pr), -CH(CH₂)₂ (cyclopropyl), -CH₂CH₂CH₂CH₃ (*n*-Bu), -CH(CH₃)CH₂CH₃ (*sec*-butyl), -CH₂CH(CH₃)₂ (*iso*-butyl), -C(CH₃)₃ (*tert*-butyl), -CH₂C(CH₃)₃ (*neo*-pentyl), cyclobutyl, cyclopentyl, cyclohexyl, cyclohexylmethyl, adamant-1-yl, adamant-2-yl, phenyl, and substituted phenyl; R₂ is at least one of the following groups comprising: Cl, Br, F, I, and (E)-methyl
- 10 2-((1,2-dihydropyridin-2-yl)(phenyl)methylene)hydrazinecarbodithioate.

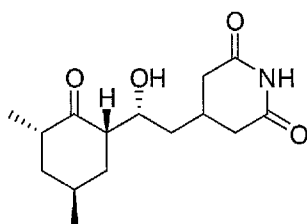
DEFINITIONS

To facilitate the understanding of this invention, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as “a”, “an” and “the” are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not delimit the invention, except as outlined in the claims.

The term “tumor necrosis factor” (TNF, cachexin or cachectin and known as tumor necrosis factor-alpha or TNFα) as used herein refers to a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. The primary role of TNF is in the regulation of immune cells. TNF is able to induce apoptotic cell death, to induce inflammation, and to inhibit tumorigenesis and viral replication. Dysregulation of TNF production has been implicated in a variety of human diseases, including major depression [2] Alzheimer's disease [3] and cancer [4] Recombinant TNF is used as an immunostimulant under the INN tasonermin. Tumor necrosis factor-α can be produced ectopically in the setting of malignancy and parallels parathyroid hormone both in causing secondary hypercalcemia and in the cancers with which excessive production is associated.

The term “cycloheximide” as used herein refers to an inhibitor of protein biosynthesis in eukaryotic organisms, produced by the bacterium *Streptomyces griseus*. Cycloheximide exerts its effect by interfering with the translocation step in protein synthesis (movement of two tRNA molecules and mRNA in relation to the ribosome) thus blocking translational elongation.

5 Cycloheximide is widely used in biomedical research to inhibit protein synthesis in eukaryotic cells studied *in vitro* (i.e. outside of organisms). It is inexpensive and works rapidly. Its effects are rapidly reversed by simply removing it from the culture medium. Cycloheximide has the structure:



10 The term “ultra violet light” (UV) as used herein refers to electromagnetic radiation with a wavelength shorter than that of visible light, but longer than X-rays, in the range 10 nm to 400 nm, and energies from 3eV to 124 eV. It is so named because the spectrum consists of electromagnetic waves with frequencies higher than those that humans identify as the color violet. Although ultraviolet is invisible to the human eye, most people are aware of the effects

15 of UV through the painful condition of sunburn, but the UV spectrum has many other effects, both beneficial and damaging, to human health.

The term “neurodegeneration” as used herein refers to any progressive loss of structure or function of neurons, including death of neurons. Many neurodegenerative diseases including Parkinson’s, Alzheimer’s, and Huntington’s occur as a result of neurodegenerative processes.

20 The terms “radiation syndrome” or “acute radiation syndrome” (ARS) also known as radiation poisoning, radiation sickness or radiation toxicity, as used herein refer to a constellation of health effects which occur within several months of exposure to high amounts of ionizing radiation [5, 6]. The term generally refers to acute medical problems rather than ones that develop after a prolonged period. The onset and type of symptoms depends on the radiation

25 exposure. Relatively smaller doses result in gastrointestinal effects such as nausea and vomiting and symptoms related to falling blood counts such as infection and bleeding. Relatively larger doses can result in neurological effects and rapid death. Treatment of acute radiation syndrome is

generally supportive with blood transfusions and antibiotics [5].

Classically acute radiation syndrome is divided into three main presentations: hematopoietic, gastrointestinal and neurological/vascular. These symptoms may or may not be preceded by a prodrome [5]. The speed of onset of symptoms is related to radiation exposure, with greater doses resulting in a shorter delay in symptom onset [5].

1. Hematopoietic: This syndrome is marked by a drop in the number of blood cells. This may result in infections due to low white blood cells, bleeding due to low platelets, and anemia due to low red blood cells [5].
2. Gastrointestinal: This syndrome typically occurs at exposure doses of 600–1000 rad (6–10 Gy) [5]. Nausea, vomiting, loss of appetite, and abdominal pain are usually seen within one to two hours [5].
3. Neurovascular: This syndrome typically occurs at exposure doses greater than 1000 rad (10 Gy) [5]. It presents with neurological symptoms such as dizziness, headache, or decreased level of consciousness with an absence of vomiting [5].

The prodrome, an early symptom (or set of symptoms) that might indicate the start of a disease before specific symptoms occur, associated with ARS typically includes nausea and vomiting, headaches, fatigue, fever and short period of skin reddening [5]. These symptoms may occur at radiation doses as low as 35 rad (0.35 Gy). Thus, they may not be followed by acute radiation sickness [5].

The term “osteoarthritis” (OA), also known as degenerative arthritis or degenerative joint disease, as used herein refers to a group of mechanical abnormalities involving degradation of joints, including articular cartilage and subchondral bone.

The term “pancreatitis” as used herein refers to inflammation of the pancreas that can occur in two very different forms. Acute pancreatitis is sudden while chronic pancreatitis "is characterized by recurring or persistent abdominal pain with or without steatorrhea or diabetes mellitus."

The term “rheumatoid arthritis” (RA) as used herein refers to a chronic, systemic inflammatory disorder that may affect many tissues and organs, but principally attacks synovial joints. The process produces an inflammatory response of the synovium (synovitis) secondary to hyperplasia of synovial cells, excess synovial fluid, and the development of pannus in the synovium.

The term “chronic active hepatitis” as used herein refers to a recurrent inflammation of the liver characterized by the presence of inflammatory cells in the tissue of the organ.

The term “inflammatory bowel disease” (IBD) as used herein refers to a group of inflammatory conditions of the colon and small intestine. The major types of IBD are Crohn's disease and ulcerative colitis.

The term “crohn's disease,” also known as regional enteritis, as used herein refers to an inflammatory disease of the intestines that may affect any part of the gastrointestinal tract from mouth to anus, causing a wide variety of symptoms. It primarily causes abdominal pain, diarrhea (which may be bloody if inflammation is at its worst), vomiting, or weight loss, but may also cause complications outside the gastrointestinal tract such as skin rashes, arthritis, inflammation of the eye, tiredness, and lack of concentration.

The term “psoriasis” as used herein refers to a chronic autoimmune disease that appears on the skin. It occurs when the immune system sends out faulty signals that speed up the growth cycle of skin cells. Psoriasis is not contagious.

The term “organ transplant rejection” as used herein refers to when a transplanted organ or tissue is not accepted by the body of the transplant recipient. This is explained by the concept that the immune system of the recipient attacks the transplanted organ or tissue.

The term “sepsis” as used herein refers to a potentially serious medical condition that is characterized by a whole-body inflammatory state (called a systemic inflammatory response syndrome or SIRS) and the presence of a known or suspected infection. The body may develop this inflammatory response by the immune system to microbes in the blood, urine, lungs, skin, or other tissues. A lay term for sepsis is blood poisoning, more aptly applied to septicemia. Severe sepsis is the systemic inflammatory response, plus infection, plus the presence of organ dysfunction.

The term “septic shock” as used herein refers to a medical emergency caused by decreased tissue perfusion and oxygen delivery as a result of severe infection and sepsis, though the microbe may be systemic or localized to a particular site. It can cause multiple organ dysfunction syndrome (formerly known as multiple organ failure) and death.

The term “cerebral ischemia” as used herein refers to a condition in which there is insufficient blood flow to the brain to meet metabolic demand.

The term “myocardial ischemia” as used herein refers to an imbalance between

myocardial oxygen supply and demand resulting in angina pectoris, myocardial stunning, myocardial hibernation, ischemic preconditioning, postconditioning, or under the most severe instances, acute coronary syndrome and myocardial infarction.

5 The term “myocardial infarction” (MI) or “acute myocardial infarction” (AMI), commonly known as a heart attack, as used herein refers to the interruption of blood supply to a part of the heart, causing heart cells to die.

10 The term “amyotrophic lateral sclerosis” (abbreviated ALS, also referred to as Lou Gehrig's disease) as used herein refers to a form of motor neuron disease. ALS is a progressive, fatal, neurodegenerative disease caused by the degeneration of motor neurons, the nerve cells in the central nervous system that control voluntary muscle movement.

The term “multiple sclerosis” (abbreviated MS, also known as disseminated sclerosis or encephalomyelitis disseminata) as used herein refers to an inflammatory disease in which the fatty myelin sheaths around the axons of the brain and spinal cord are damaged, leading to demyelination and scarring as well as a broad spectrum of signs and symptoms.

15 The term “hepatitis B” as used herein refers to an infectious illness caused by hepatitis B virus (HBV) which infects the liver of Hominoidea, including humans, and causes an inflammation called hepatitis.

The term “hepatitis C” as used herein refers to an infectious disease affecting the liver, caused by the hepatitis C virus (HCV).

20 The term “hepatitis G” as used herein refers to a form of liver inflammation caused by hepatitis G virus (HGV), a distant relative of the hepatitis C virus.

The term “liver disease” Liver disease (also called hepatic disease) as used herein refers to a broad term describing any single number of diseases affecting the liver. Many are accompanied by jaundice caused by increased levels of bilirubin in the system.

25 The terms “reduce,” “inhibit,” “diminish,” “suppress,” “decrease,” “prevent” and grammatical equivalents (including “lower,” “smaller,” etc.) when in reference to the expression of any symptom in an untreated subject relative to a treated subject, mean that the quantity and/or magnitude of the symptoms in the treated subject is lower than in the untreated subject by any amount that is recognized as clinically relevant by any medically trained personnel. In one embodiment, the quantity and/or magnitude of the symptoms in the treated subject is at least 10%
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lower than, at least 25% lower than, at least 50% lower than, at least 75% lower than, and/or at least 90% lower than the quantity and/or magnitude of the symptoms in the untreated subject.

The term "Epimers" as used herein refers to diastereomers that differ in configuration of only one stereogenic center. Diastereomers are a class of stereoisomers that are
5 non-superposable, non-mirror images of one another, unlike enantiomers that are non-superposable mirror images of one another.

The term "Sugar" as used herein refers to a monosaccharide, disaccharide, trisaccharides, or polysaccharides. Monosaccharides have the general formula $(CH_2O)_n$, in which n is an integer larger than 2. Disaccharides have the general formula $C_n(H_2O)_{n-1}$, with n larger than 5.
10 Polysaccharides include such substances as cellulose, dextrin, glycogen, and starch.

The term "pharmaceutically acceptable monosaccharide" as used herein refers to a pharmaceutically acceptable aldose sugar, a pharmaceutically acceptable ketose sugar, or other specified sugar. Among the pharmaceutically acceptable aldose sugars within the contemplation of the present invention are erythrose, threose, ribose, arabinose, xylose, lyxose,
15 allose, altrose, glucose, mannose, gulose, idose, galactose and talose. Among the pharmaceutically acceptable ketose sugars preferred for use in the composition of the present invention are erythrulose, ribulose, xylulose, psicose, fructose, sorbose, tagatose, and sedoheptulose. Among the other specified sugars preferred for use in the composition of the present invention are fucose, fuculose, rhamnose, or any other deoxy sugar. Although either (D)
20 or (L) isomers may be employed, the (D) form is generally preferable.

The pharmaceutical compositions of the present invention may be prepared by formulating them in dosage forms, which are suitable for peroral, rectal or nonparenteral administration, the last-mentioned including intravenous injection and administration into the cerebrospinal fluid. For this purpose, common carriers and routine formulation techniques may
25 be employed.

The term "API" or "active pharmaceutical ingredient" as used herein refers to the substance in a pharmaceutical drug that is biologically active.

The term "Common carriers" as used herein refers to those which are employed in standard pharmaceutical preparations and includes excipients, binders and disintegrators the
30 choice of which depends on the specific dosage form used. Typical examples of the excipient are starch, lactose, sucrose, glucose, mannitol and cellulose; illustrative binders are

polyvinylpyrrolidone, starch, sucrose, hydroxypropyl cellulose and gum arabic; illustrative disintegrators include starch, agar, gelatin powder, cellulose, and CMC. Any other common excipients, binders and disintegrators may also be employed.

In addition to the carriers described above, the pharmaceutical composition of the present invention preferably contains antioxidants for the purpose of stabilizing the effective ingredient. Appropriate antioxidants may be selected from among those which are commonly incorporated in pharmaceuticals and include ascorbic acid, N-acetylcysteine, acetylcysteine, L-cystein, D, L- α -tocopherol, and natural tocopherol.

Formulations of the pharmaceutical composition of the present invention which are suitable for peroral administration may be provided in the form of tablets, capsules, powders, granules, or suspensions in non-aqueous solutions such as syrups, emulsions or drafts, each containing one or more of the active compounds in predetermined amounts.

The granule may be provided by first preparing an intimate mixture of one or more of the active ingredients with one or more of the auxiliary components shown above, then granulating the mixture, and classifying the granules by screening through a sieve.

The tablet may be prepared by compressing or otherwise forming one or more of the active ingredients, optionally with one or more auxiliary components.

The capsule may be prepared by first making a powder or granules as an intimate mixture of one or more of the active ingredients with one or more auxiliary components, then charging the mixture into an appropriate capsule on a packing machine, etc.

The pharmaceutical composition of the present invention may be formulated as a suppository (for rectal administration) with the aid of a common carrier such a cocoa butter. The pharmaceutical composition of the present invention may also be formulated in a dosage form suitable for non-parenteral administration by packaging one or more active ingredients as dry solids in a sterile nitrogen-purged container. The resulting dry formulation may be administered to patients non-parenterally after being dispersed or dissolved in a given amount of aseptic water.

The dosage forms are preferably prepared from a mixture of the active ingredients, routine auxiliary components and one or more of the antioxidants listed above. If desired, the formulations may further contain one or more auxiliary components selected from among excipients, buffers, flavoring agents, binders, surfactants, thickening agents, and lubricants.

The dose of the various pro-drugs will of course vary with the route of administration, the severity of the disease to be treated, and the patient to be treated, but the exact dose ultimately chosen should be left to the good discretion of the doctor responsible for the treatment. If a desired dose is determined, the active ingredient may be administered once a day or, alternatively, it may be administered in up to as many portions as deemed appropriate at suitable intervals. The active ingredient may be straightforwardly administered without being mixed with any other components. However, for several reasons, typically for the purpose of providing ease in controlling the dose level, the active compound is preferably administered in a pharmaceutical dosage form.

The term "salts", as used herein, refers to any salt that complexes with identified compounds contained herein while retaining a desired function, e.g., biological activity. Examples of such salts include, but are not limited to, acid addition salts formed with inorganic acids (e.g. hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid, and the like), and salts formed with organic acids such as, but not limited to, acetic acid, oxalic acid, tartaric acid, succinic acid, malic acid, fumaric acid, maleic acid, ascorbic acid, benzoic acid, tannic acid, pamoic acid, alginic acid, polyglutamic acid, naphthalene sulfonic acid, naphthalene disulfonic acid, and polygalacturonic acid. Pharmaceutically acceptable salts also include base addition salts which may be formed when acidic protons present are capable of reacting with inorganic or organic bases. Suitable pharmaceutically-acceptable base addition salts include metallic salts, such as salts made from aluminum, calcium, lithium, magnesium, potassium, sodium and zinc, or salts made from organic bases including primary, secondary and tertiary amines, substituted amines including cyclic amines, such as caffeine, arginine, diethylamine, N-ethyl piperidine, histidine, glucamine, isopropylamine, lysine, morpholine, N-ethyl morpholine, piperazine, piperidine, triethylamine, trimethylamine. All of these salts may be prepared by conventional means from the corresponding compound of the invention by reacting, for example, the appropriate acid or base with the compound of the invention. Unless otherwise specifically stated, the present invention contemplates pharmaceutically acceptable salts of the considered pro-drugs.

As used herein, "hydrogen" means $-H$; "hydroxy" means $-OH$; "oxo" means $=O$; "halo" means independently $-F$, $-Cl$, $-Br$ or $-I$; "amino" means $-NH_2$ (see below for definitions of groups containing the term amino, e.g., alkylamino); "hydroxyamino" means $-NHOH$; "nitro"

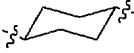
means $-\text{NO}_2$; “imino” means $=\text{NH}$ (see below for definitions of groups containing the term imino, *e.g.*, alkylamino); “cyano” means $-\text{CN}$; “azido” means $-\text{N}_3$; “mercapto” means $-\text{SH}$; “thio” means $=\text{S}$; “sulfonamido” means $-\text{NHS}(\text{O})_2-$ (see below for definitions of groups containing the term sulfonamido, *e.g.*, alkylsulfonamido); “sulfonyl” means $-\text{S}(\text{O})_2-$ (see below for definitions of groups containing the term sulfonyl, *e.g.*, alkylsulfonyl); and “silyl” means $-\text{SiH}_3$ (see below for definitions of group(s) containing the term silyl, *e.g.*, alkylsilyl).

As used herein, “methylene” means a chemical species in which a carbon atom is bonded to two hydrogen atoms. The $-\text{CH}_2-$ group is considered to be the standard methylene group. Methylene groups in a chain or ring contribute to its size and lipophilicity. In this context dideoxy also refers the methylene groups. In particular a 2,3-dideoxy compound is the same as 2,3-methylene (2,3-methylene- glycoside =2,3-dideoxy- glycoside).

For the groups below, the following parenthetical subscripts further define the groups as follows: “(C_n)” defines the exact number (n) of carbon atoms in the group; “(C_{≤n})” defines the maximum number (n) of carbon atoms that can be in the group; (C_{n-n'}) defines both the minimum (n) and maximum number (n') of carbon atoms in the group. For example, “alkoxy_(C_{≤10})” designates those alkoxy groups having from 1 to 10 carbon atoms (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, or any range derivable therein (*e.g.*, 3-10 carbon atoms)). Similarly, “alkyl_(C₂₋₁₀)” designates those alkyl groups having from 2 to 10 carbon atoms (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, or 10, or any range derivable therein (*e.g.*, 3-10 carbon atoms)).

The term “alkyl” when used without the “substituted” modifier refers to a non-aromatic monovalent group with a saturated carbon atom as the point of attachment, a linear or branched, cyclo, cyclic or acyclic structure, no carbon-carbon double or triple bonds, and no atoms other than carbon and hydrogen. The groups, $-\text{CH}_3$ (Me), $-\text{CH}_2\text{CH}_3$ (Et), $-\text{CH}_2\text{CH}_2\text{CH}_3$ (*n*-Pr), $-\text{CH}(\text{CH}_3)_2$ (*iso*-Pr or *i*-Pr), $-\text{CH}(\text{CH}_2)_2$ (cyclopropyl), $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ (*n*-Bu), $-\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$ (*sec*-butyl or *sec*-Bu), $-\text{CH}_2\text{CH}(\text{CH}_3)_2$ (*iso*-butyl or *i*-Bu), $-\text{C}(\text{CH}_3)_3$ (*tert*-butyl or *t*-Bu), $-\text{CH}_2\text{C}(\text{CH}_3)_3$ (*neo*-pentyl), cyclobutyl, cyclopentyl, cyclohexyl, cyclohexylmethyl are non-limiting examples of alkyl groups. The term “substituted alkyl” refers to a non-aromatic monovalent group with a saturated carbon atom as the point of attachment, a linear or branched, cyclo, cyclic or acyclic structure, no carbon-carbon double or triple bonds, and at least one atom independently selected from the group consisting of N, O, F, Cl, Br, I, Si, P, and S. The following groups are non-limiting examples of substituted alkyl groups: $-\text{CH}_2\text{OH}$, $-\text{CH}_2\text{Cl}$, $-\text{CH}_2\text{Br}$,

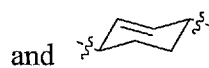
$-\text{CH}_2\text{SH}$, $-\text{CF}_3$, $-\text{CH}_2\text{CN}$, $-\text{CH}_2\text{C}(\text{O})\text{H}$, $-\text{CH}_2\text{C}(\text{O})\text{OH}$, $-\text{CH}_2\text{C}(\text{O})\text{OCH}_3$, $-\text{CH}_2\text{C}(\text{O})\text{NH}_2$,
 $-\text{CH}_2\text{C}(\text{O})\text{NHCH}_3$, $-\text{CH}_2\text{C}(\text{O})\text{CH}_3$, $-\text{CH}_2\text{OCH}_3$, $-\text{CH}_2\text{OCH}_2\text{CF}_3$, $-\text{CH}_2\text{OC}(\text{O})\text{CH}_3$, $-\text{CH}_2\text{NH}_2$,
 $-\text{CH}_2\text{NHCH}_3$, $-\text{CH}_2\text{N}(\text{CH}_3)_2$, $-\text{CH}_2\text{CH}_2\text{Cl}$, $-\text{CH}_2\text{CH}_2\text{OH}$, $-\text{CH}_2\text{CF}_3$, $-\text{CH}_2\text{CH}_2\text{OC}(\text{O})\text{CH}_3$,
 $-\text{CH}_2\text{CH}_2\text{NHCO}_2\text{C}(\text{CH}_3)_3$, and $-\text{CH}_2\text{Si}(\text{CH}_3)_3$.

- 5 The term “alkanediyl” when used without the “substituted” modifier refers to a non-aromatic divalent group, wherein the alkanediyl group is attached with two σ -bonds, with one or two saturated carbon atom(s) as the point(s) of attachment, a linear or branched, cyclo, cyclic or acyclic structure, no carbon-carbon double or triple bonds, and no atoms other than carbon and hydrogen. The groups, $-\text{CH}_2-$ (methylene), $-\text{CH}_2\text{CH}_2-$, $-\text{CH}_2\text{C}(\text{CH}_3)_2\text{CH}_2-$, $-\text{CH}_2\text{CH}_2\text{CH}_2-$,
 10 and , are non-limiting examples of alkanediyl groups. The term “substituted alkanediyl” refers to a non-aromatic monovalent group, wherein the alkanediyl group is attached with two σ -bonds, with one or two saturated carbon atom(s) as the point(s) of attachment, a linear or branched, cyclo, cyclic or acyclic structure, no carbon-carbon double or triple bonds, and at least one atom independently selected from the group consisting of N, O, F, Cl, Br, I, Si, P, and S.
 15 The following groups are non-limiting examples of substituted alkanediyl groups: $-\text{CH}(\text{F})-$, $-\text{CF}_2-$, $-\text{CH}(\text{Cl})-$, $-\text{CH}(\text{OH})-$, $-\text{CH}(\text{OCH}_3)-$, and $-\text{CH}_2\text{CH}(\text{Cl})-$.

- The term “alkenyl” when used without the “substituted” modifier refers to a monovalent group with a nonaromatic carbon atom as the point of attachment, a linear or branched, cyclo, cyclic or acyclic structure, at least one nonaromatic carbon-carbon double bond, no carbon-carbon triple bonds, and no atoms other than carbon and hydrogen. Non-limiting examples of alkenyl groups include: $-\text{CH}=\text{CH}_2$ (vinyl), $-\text{CH}=\text{CHCH}_3$, $-\text{CH}=\text{CHCH}_2\text{CH}_3$, $-\text{CH}_2\text{CH}=\text{CH}_2$ (allyl), $-\text{CH}_2\text{CH}=\text{CHCH}_3$, and $-\text{CH}=\text{CH}-\text{C}_6\text{H}_5$. The term “substituted alkenyl” refers to a monovalent group with a nonaromatic carbon atom as the point of attachment, at least one nonaromatic carbon-carbon double bond, no carbon-carbon triple bonds, a linear or branched, cyclo, cyclic or
 20 acyclic structure, and at least one atom independently selected from the group consisting of N, O, F, Cl, Br, I, Si, P, and S. The groups, $-\text{CH}=\text{CHF}$, $-\text{CH}=\text{CHCl}$ and $-\text{CH}=\text{CHBr}$, are non-limiting examples of substituted alkenyl groups.
 25

- The term “alkenediyl” when used without the “substituted” modifier refers to a non-aromatic divalent group, wherein the alkenediyl group is attached with two σ -bonds, with two
 30 carbon atoms as points of attachment, a linear or branched, cyclo, cyclic or acyclic structure, at

least one nonaromatic carbon-carbon double bond, no carbon-carbon triple bonds, and no atoms other than carbon and hydrogen. The groups, $-\text{CH}=\text{CH}-$, $-\text{CH}=\text{C}(\text{CH}_3)\text{CH}_2-$, $-\text{CH}=\text{CHCH}_2-$,



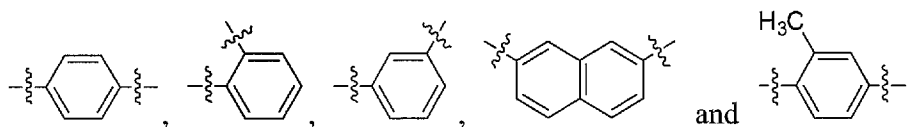
The term “substituted alkenediyl” refers to a non-aromatic divalent group, wherein the alkenediyl group is attached with two σ -bonds, with two carbon atoms as points of attachment, a linear or branched, cyclo, cyclic or acyclic structure, at least one nonaromatic carbon-carbon double bond, no carbon-carbon triple bonds, and at least one atom independently selected from the group consisting of N, O, F, Cl, Br, I, Si, P, and S. The following groups are non-limiting examples of substituted alkenediyl groups: $-\text{CF}=\text{CH}-$, $-\text{C}(\text{OH})=\text{CH}-$, and $-\text{CH}_2\text{CH}=\text{C}(\text{Cl})-$.

The term “alkynyl” when used without the “substituted” modifier refers to a monovalent group with a nonaromatic carbon atom as the point of attachment, a linear or branched, cyclo, cyclic or acyclic structure, at least one carbon-carbon triple bond, and no atoms other than carbon and hydrogen. The groups, $-\text{C}\equiv\text{CH}$, $-\text{C}\equiv\text{CCH}_3$, $-\text{C}\equiv\text{CC}_6\text{H}_5$ and $-\text{CH}_2\text{C}\equiv\text{CCH}_3$, are non-limiting examples of alkynyl groups. The term “substituted alkynyl” refers to a monovalent group with a nonaromatic carbon atom as the point of attachment and at least one carbon-carbon triple bond, a linear or branched, cyclo, cyclic or acyclic structure, and at least one atom independently selected from the group consisting of N, O, F, Cl, Br, I, Si, P, and S. The group, $-\text{C}\equiv\text{CSi}(\text{CH}_3)_3$, is a non-limiting example of a substituted alkynyl group.

The term “alkynediyl” when used without the “substituted” modifier refers to a non-aromatic divalent group, wherein the alkynediyl group is attached with two σ -bonds, with two carbon atoms as points of attachment, a linear or branched, cyclo, cyclic or acyclic structure, at least one carbon-carbon triple bond, and no atoms other than carbon and hydrogen. The groups, $-\text{C}\equiv\text{C}-$, $-\text{C}\equiv\text{CCH}_2-$, and $-\text{C}\equiv\text{CCH}(\text{CH}_3)-$ are non-limiting examples of alkynediyl groups. The term “substituted alkynediyl” refers to a non-aromatic divalent group, wherein the alkynediyl group is attached with two σ -bonds, with two carbon atoms as points of attachment, a linear or branched, cyclo, cyclic or acyclic structure, at least one carbon-carbon triple bond, and at least one atom independently selected from the group consisting of N, O, F, Cl, Br, I, Si, P, and S. The groups $-\text{C}\equiv\text{CCFH}-$ and $-\text{C}\equiv\text{CCH}(\text{Cl})-$ are non-limiting examples of substituted alkynediyl groups.

The term “aryl” when used without the “substituted” modifier refers to a monovalent group with an aromatic carbon atom as the point of attachment, said carbon atom forming part of a six-membered aromatic ring structure wherein the ring atoms are all carbon, and wherein the monovalent group consists of no atoms other than carbon and hydrogen. Non-limiting examples of aryl groups include phenyl (Ph), methylphenyl, $\text{C}_6\text{H}_3(\text{CH}_3)_2$ (dimethylphenyl), $-\text{C}_6\text{H}_4\text{CH}_2\text{CH}_3$ (ethylphenyl), $-\text{C}_6\text{H}_4\text{CH}_2\text{CH}_2\text{CH}_3$ (propylphenyl), $-\text{C}_6\text{H}_4\text{CH}(\text{CH}_3)_2$, $-\text{C}_6\text{H}_4\text{CH}(\text{CH}_2)_2$, $-\text{C}_6\text{H}_3(\text{CH}_3)\text{CH}_2\text{CH}_3$ (methylethylphenyl), $-\text{C}_6\text{H}_4\text{CH}=\text{CH}_2$ (vinylphenyl), $-\text{C}_6\text{H}_4\text{CH}=\text{CHCH}_3$, $-\text{C}_6\text{H}_4\text{C}\equiv\text{CH}$, $-\text{C}_6\text{H}_4\text{C}\equiv\text{CCH}_3$, naphthyl, and the monovalent group derived from biphenyl. The term “substituted aryl” refers to a monovalent group with an aromatic carbon atom as the point of attachment, said carbon atom forming part of a six-membered aromatic ring structure wherein the ring atoms are all carbon, and wherein the monovalent group further has at least one atom independently selected from the group consisting of N, O, F, Cl, Br, I, Si, P, and S. Non-limiting examples of substituted aryl groups include the groups: $-\text{C}_6\text{H}_4\text{F}$, $-\text{C}_6\text{H}_4\text{Cl}$, $-\text{C}_6\text{H}_4\text{Br}$, $-\text{C}_6\text{H}_4\text{I}$, $-\text{C}_6\text{H}_4\text{OH}$, $-\text{C}_6\text{H}_4\text{OCH}_3$, $-\text{C}_6\text{H}_4\text{OCH}_2\text{CH}_3$, $-\text{C}_6\text{H}_4\text{OC}(\text{O})\text{CH}_3$, $-\text{C}_6\text{H}_4\text{NH}_2$, $-\text{C}_6\text{H}_4\text{NHCH}_3$, $-\text{C}_6\text{H}_4\text{N}(\text{CH}_3)_2$, $-\text{C}_6\text{H}_4\text{CH}_2\text{OH}$, $-\text{C}_6\text{H}_4\text{CH}_2\text{OC}(\text{O})\text{CH}_3$, $-\text{C}_6\text{H}_4\text{CH}_2\text{NH}_2$, $-\text{C}_6\text{H}_4\text{CF}_3$, $-\text{C}_6\text{H}_4\text{CN}$, $-\text{C}_6\text{H}_4\text{CHO}$, $-\text{C}_6\text{H}_4\text{C}(\text{O})\text{CH}_3$, $-\text{C}_6\text{H}_4\text{C}(\text{O})\text{C}_6\text{H}_5$, $-\text{C}_6\text{H}_4\text{CO}_2\text{H}$, $-\text{C}_6\text{H}_4\text{CO}_2\text{CH}_3$, $-\text{C}_6\text{H}_4\text{CONH}_2$, $-\text{C}_6\text{H}_4\text{CONHCH}_3$, and $-\text{C}_6\text{H}_4\text{CON}(\text{CH}_3)_2$.

The term “arenediyl” when used without the “substituted” modifier refers to a divalent group, wherein the arenediyl group is attached with two σ -bonds, with two aromatic carbon atoms as points of attachment, said carbon atoms forming part of one or more six-membered aromatic ring structure(s) wherein the ring atoms are all carbon, and wherein the monovalent group consists of no atoms other than carbon and hydrogen. Non-limiting examples of arenediyl groups include:



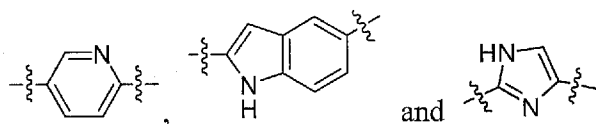
The term “substituted arenediyl” refers to a divalent group, wherein the arenediyl group is attached with two σ -bonds, with two aromatic carbon atoms as points of attachment, said carbon atoms forming part of one or more six-membered aromatic rings structure(s), wherein the ring atoms are all carbon, and wherein the divalent group further has at least one atom independently selected from the group consisting of N, O, F, Cl, Br, I, Si, P, and S.

The term "aralkyl" when used without the "substituted" modifier refers to the monovalent group -alkanediyl-aryl, in which the terms alkanediyl and aryl are each used in a manner consistent with the definitions provided above. Non-limiting examples of aralkyls are: phenylmethyl (benzyl, Bn), 1-phenyl-ethyl, 2-phenyl-ethyl, indenyl and 2,3-dihydro-indenyl, provided that indenyl and 2,3-dihydro-indenyl are only examples of aralkyl in so far as the point of attachment in each case is one of the saturated carbon atoms. When the term "aralkyl" is used with the "substituted" modifier, either one or both the alkanediyl and the aryl is substituted. Non-limiting examples of substituted aralkyls are: (3-chlorophenyl)-methyl, 2-oxo-2-phenyl-ethyl (phenylcarbonylmethyl), 2-chloro-2-phenyl-ethyl, chromanyl where the point of attachment is one of the saturated carbon atoms, and tetrahydroquinolinyl where the point of attachment is one of the saturated atoms.

The term "heteroaryl" when used without the "substituted" modifier refers to a monovalent group with an aromatic carbon atom or nitrogen atom as the point of attachment, said carbon atom or nitrogen atom forming part of an aromatic ring structure wherein at least one of the ring atoms is nitrogen, oxygen or sulfur, and wherein the monovalent group consists of no atoms other than carbon, hydrogen, aromatic nitrogen, aromatic oxygen and aromatic sulfur. Non-limiting examples of aryl groups include acridinyl, furanyl, imidazoimidazolyl, imidazopyrazolyl, imidazopyridinyl, imidazopyrimidinyl, indolyl, indazolyl, methylpyridyl, oxazolyl, phenylimidazolyl, pyridyl, pyrrolyl, pyrimidyl, pyrazinyl, quinolyl, quinazolyl, quinoxalinyl, tetrahydroquinolinyl, thienyl, triazinyl, pyrrolopyridinyl, pyrrolopyrimidinyl, pyrrolopyrazinyl, pyrrolotriazinyl, pyrroloimidazolyl, chromenyl (where the point of attachment is one of the aromatic atoms), and chromanyl (where the point of attachment is one of the aromatic atoms). The term "substituted heteroaryl" refers to a monovalent group with an aromatic carbon atom or nitrogen atom as the point of attachment, said carbon atom or nitrogen atom forming part of an aromatic ring structure wherein at least one of the ring atoms is nitrogen, oxygen or sulfur, and wherein the monovalent group further has at least one atom independently selected from the group consisting of non-aromatic nitrogen, non-aromatic oxygen, non aromatic sulfur F, Cl, Br, I, Si, and P.

The term "heteroarenediyl" when used without the "substituted" modifier refers to a divalent group, wherein the heteroarenediyl group is attached with two σ -bonds, with an aromatic carbon atom or nitrogen atom as the point of attachment, said carbon atom or nitrogen atom two

aromatic atoms as points of attachment, said carbon atoms forming part of one or more six-membered aromatic ring structure(s) wherein the ring atoms are all carbon, and wherein the monovalent group consists of no atoms other than carbon and hydrogen. Non-limiting examples of heteroarenediyl groups include:



The term “substituted heteroarenediyl” refers to a divalent group, wherein the heteroarenediyl group is attached with two σ -bonds, with two aromatic carbon atoms as points of attachment, said carbon atoms forming part of one or more six-membered aromatic rings structure(s), wherein the ring atoms are all carbon, and wherein the divalent group further has at least one atom independently selected from the group consisting of N, O, F, Cl, Br, I, Si, P, and S.

The term “heteroaralkyl” when used without the “substituted” modifier refers to the monovalent group –alkanediyl–heteroaryl, in which the terms alkanediyl and heteroaryl are each used in a manner consistent with the definitions provided above. Non-limiting examples of aralkyls are: pyridylmethyl, and thienylmethyl. When the term “heteroaralkyl” is used with the “substituted” modifier, either one or both the alkanediyl and the heteroaryl is substituted.

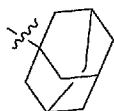
The term “acyl” when used without the “substituted” modifier refers to a monovalent group with a carbon atom of a carbonyl group as the point of attachment, further having a linear or branched, cyclo, cyclic or acyclic structure, further having no additional atoms that are not carbon or hydrogen, beyond the oxygen atom of the carbonyl group. The groups, –CHO, –C(O)CH₃, –C(O)CH₂CH₃, –C(O)CH₂CH₂CH₃, –C(O)CH(CH₃)₂, –C(O)CH(CH₂)₂, –C(O)C₆H₅, –C(O)C₆H₄CH₃, –C(O)C₆H₄CH₂CH₃, –COC₆H₃(CH₃)₂, and –C(O)CH₂C₆H₅, are non-limiting examples of acyl groups. The term “acyl” therefore encompasses, but is not limited to groups sometimes referred to as “alkyl carbonyl” and “aryl carbonyl” groups. The term “substituted acyl” refers to a monovalent group with a carbon atom of a carbonyl group as the point of attachment, further having a linear or branched, cyclo, cyclic or acyclic structure, further having at least one atom, in addition to the oxygen of the carbonyl group, independently selected from the group consisting of N, O, F, Cl, Br, I, Si, P, and S. The groups, –C(O)CH₂CF₃, –C(O)CH₂Cl, –CO₂H (carboxyl), –CO₂CH₃ (methylcarboxyl), –CO₂CH₂CH₃, –CO₂CH₂CH₂CH₃, –CO₂C₆H₅, –CO₂CH(CH₃)₂, –CO₂CH(CH₂)₂, –C(O)NH₂ (carbamoyl), –C(O)NHCH₃, –C(O)NHCH₂CH₃, –CONHCH(CH₃)₂, –CONHCH(CH₂)₂, –CON(CH₃)₂, –CONHCH₂CF₃, –CO–pyridyl,

–CO–imidazolyl, and –C(O)N₃, are non-limiting examples of substituted acyl groups. The term “substituted acyl” encompasses, but is not limited to, “heteroaryl carbonyl” groups.

The term “alkylidene” when used without the “substituted” modifier refers to the divalent group =CRR', wherein the alkylidene group is attached with one σ-bond and one π-bond, in which R and R' are independently hydrogen, alkyl, or R and R' are taken together to represent alkanediyl. Non-limiting examples of alkylidene groups include: =CH₂, =CH(CH₂CH₃), and =C(CH₃)₂. The term “substituted alkylidene” refers to the group =CRR', wherein the alkylidene group is attached with one σ-bond and one π-bond, in which R and R' are independently hydrogen, alkyl, substituted alkyl, or R and R' are taken together to represent a substituted alkanediyl, provided that either one of R and R' is a substituted alkyl or R and R' are taken together to represent a substituted alkanediyl.

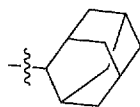
The term “alkoxy” when used without the “substituted” modifier refers to the group –OR, in which R is an alkyl, as that term is defined above. Non-limiting examples of alkoxy groups include: –OCH₃, –OCH₂CH₃, –OCH₂CH₂CH₃, –OCH(CH₃)₂, –OCH(CH₂)₂, –O–cyclopentyl, and –O–cyclohexyl. The term “substituted alkoxy” refers to the group –OR, in which R is a substituted alkyl, as that term is defined above. For example, –OCH₂CF₃ is a substituted alkoxy group.

The term “adamant-1-yl” when used without the “substituted” modifier refers to the group,



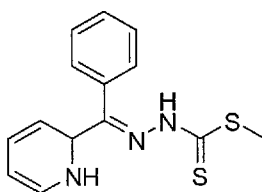
in which R is:

The term “adamant-2-yl” when used without the “substituted” modifier refers to the group,



in which R is:

The term “(E)-methyl 2-((1,2-dihydropyridin-2-yl)(phenyl)methylene)hydrazinecarbodithioate” refers to a compound with the following structure:



In addition, atoms making up the compounds of the present invention are intended to include all isotopic forms of such atoms. Isotopes, as used herein, include those atoms having the same atomic number but different mass numbers. By way of general example and without limitation, isotopes of hydrogen include tritium and deuterium, and isotopes of carbon include ^{13}C and ^{14}C . Similarly, it is contemplated that one or more carbon atom(s) of a compound of the present invention may be replaced by a silicon atom(s). Furthermore, it is contemplated that one or more oxygen atom(s) of a compound of the present invention may be replaced by a sulfur or selenium atom(s).

In structures wherein stereochemistry is not explicitly indicated, it is assumed that either stereochemistry is considered and both isomers claimed.

Any undefined valency on an atom of a structure shown in this application implicitly represents a hydrogen atom bonded to the atom. Bonds to copper (Cu) metal may be coordinate bonds and are not necessarily considered covalent.

The term “effective,” as that term is used in the specification and/or claims, means adequate to accomplish a desired, or hoped for result.

The term “hydrate” when used as a modifier to a compound means that the compound has less than one (*e.g.*, hemihydrate), one (*e.g.*, monohydrate), or more than one (*e.g.*, dihydrate) water molecules associated with each compound molecule, such as in solid forms of the compound.

An “isomer” of a first compound is a separate compound in which each molecule contains the same constituent atoms as the first compound, but where the configuration of those atoms in three dimensions differs.

As used herein, the term “patient” or “subject” refers to a living mammalian organism, such as a human, monkey, cow, sheep, goat, dog, cat, mouse, rat, guinea pig, or transgenic species thereof. In certain embodiments, the patient or subject is a primate. Non-limiting examples of human subjects are adults, juveniles, infants and fetuses.

In biochemistry, Michaelis-Menten kinetics is a model of enzyme kinetics. In particular, the Michaelis-Menten equation describes the rates of irreversible enzymatic reactions by relating reaction rate to the concentration of the substrate.

Michaelis Menten Equation:

$$v_0 = \frac{v_{\max}[S]}{K_M + [S]}$$

- 5 Wherein V_0 = current reaction rate = $k_2[ES]$ = the rate of bound substrate conversion to product (k_2) * the concentration of enzyme currently binding substrate ($[ES]$). $V_{\max}$ = maximum reaction rate = $k_2[E_0]$ = the rate of bound substrate conversion to product * the concentration of enzyme binding substrate when all of the enzyme is binding substrate. K_m = inverse of enzyme affinity = $[k_1 + k_2]/k_1$ = the rate at which bound enzyme is unbound by substrate (either by becoming product (k_2) or just unbinding (k_1)) / the rate at which enzyme binds substrate. $[S]$ = substrate concentration

15 The term "Pharmaceutically acceptable" means that which is useful in preparing a pharmaceutical composition that is generally safe, non-toxic and neither biologically nor otherwise undesirable and includes that which is acceptable for veterinary use as well as human pharmaceutical use.

20 "Pharmaceutically acceptable salts" means salts of compounds of the present invention which are pharmaceutically acceptable, as defined above, and which possess the desired pharmacological activity. Such salts include acid addition salts formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like; or with organic acids such as 1,2-ethanedisulfonic acid, 2-hydroxyethanesulfonic acid, 2-naphthalenesulfonic acid, 3-phenylpropionic acid, 4,4'-methylenebis(3-hydroxy-2-ene-1-carboxylic acid), 4-methylbicyclo[2.2.2]oct-2-ene-1-carboxylic acid, acetic acid, aliphatic mono- and dicarboxylic acids, aliphatic sulfuric acids, aromatic sulfuric acids, benzenesulfonic acid, benzoic acid, camphorsulfonic acid, carbonic acid, cinnamic acid, citric acid, cyclopentanepropionic acid, ethanesulfonic acid, fumaric acid, glucoheptonic acid, gluconic acid, glutamic acid, glycolic acid, heptanoic acid, hexanoic acid, hydroxynaphthoic acid, lactic acid, laurylsulfuric acid, maleic acid, malic acid, malonic acid, mandelic acid, methanesulfonic acid, muconic acid, *o*-(4-hydroxybenzoyl)benzoic acid, oxalic acid, *p*-chlorobenzenesulfonic acid, phenyl-substituted alkanolic acids, propionic acid, *p*-toluenesulfonic acid, pyruvic acid, salicylic

acid, stearic acid, succinic acid, tartaric acid, tertiarybutylacetic acid, trimethylacetic acid, and the like. Pharmaceutically acceptable salts also include base addition salts which may be formed when acidic protons present are capable of reacting with inorganic or organic bases. Acceptable inorganic bases include sodium hydroxide, sodium carbonate, potassium hydroxide, aluminum
5 hydroxide and calcium hydroxide. Acceptable organic bases include ethanolamine, diethanolamine, triethanolamine, tromethamine, *N*-methylglucamine and the like. It should be recognized that the particular anion or cation forming a part of any salt of this invention is not critical, so long as the salt, as a whole, is pharmacologically acceptable. Additional examples of pharmaceutically acceptable salts and their methods of preparation and use are presented in
10 *Handbook of Pharmaceutical Salts: Properties, and Use* (P. H. Stahl & C. G. Wermuth eds., Verlag Helvetica Chimica Acta, 2002) [7]. Unless otherwise specifically stated, the present invention contemplates pharmaceutically acceptable salts of the considered pro-drugs.

As used herein, “predominantly one enantiomer” means that a compound contains at least about 85% of one enantiomer, or more preferably at least about 90% of one enantiomer, or even
15 more preferably at least about 95% of one enantiomer, or most preferably at least about 99% of one enantiomer. Similarly, the phrase “substantially free from other optical isomers” means that the composition contains at most about 15% of another enantiomer or diastereomer, more preferably at most about 10% of another enantiomer or diastereomer, even more preferably at most about 5% of another enantiomer or diastereomer, and most preferably at most about 1% of another
20 enantiomer or diastereomer.

“Prevention” or “preventing” includes: (1) inhibiting the onset of a disease in a subject or patient which may be at risk and/or predisposed to the disease but does not yet experience or display any or all of the pathology or symptomatology of the disease, and/or (2) slowing the onset of the pathology or symptomatology of a disease in a subject or patient which may be at risk and/or
25 predisposed to the disease but does not yet experience or display any or all of the pathology or symptomatology of the disease.

The term “saturated” when referring to an atom means that the atom is connected to other atoms only by means of single bonds.

A “stereoisomer” or “optical isomer” is an isomer of a given compound in which the same
30 atoms are bonded to the same other atoms, but where the configuration of those atoms in three dimensions differs. “Enantiomers” are stereoisomers of a given compound that are mirror images

of each other, like left and right hands. "Diastereomers" are stereoisomers of a given compound that are not enantiomers.

Enantiomers are compounds that individually have properties said to have "optical activity" and consist of molecules with at least one chiral center, almost always a carbon atom. If
5 a particular compound is dextrorotary, its enantiomer will be levorotary, and vice-versa. In fact, the enantiomers will rotate polarized light the same number of degrees, but in opposite directions. "Dextrorotation" and "levorotation" (also spelled laevorotation) refer, respectively, to the properties of rotating plane polarized light clockwise (for dextrorotation) or counterclockwise (for levorotation). A compound with dextrorotation is called "dextrorotary," while a compound with
10 levorotation is called "levorotary".

A standard measure of the degree to which a compound is dextrorotary or levorotary is the quantity called the "specific rotation" "[α]". Dextrorotary compounds have a positive specific rotation, while levorotary compounds have negative. Two enantiomers have equal and opposite specific rotations. A dextrorotary compound is prefixed "(+)-" or "d-". Likewise, a
15 levorotary compound is often prefixed "(-)-" or "l-". These "d-" and "l-" prefixes should not be confused with the "D-" and "L-" prefixes based on the actual configuration of each enantiomer, with the version synthesized from naturally occurring (+)-compound being considered the D-form. A mixture of enantiomers of the compounds is prefixed "(±)-". An equal mixture of enantiomers of the compounds is considered "optically inactive".

20 The invention contemplates that for any stereocenter or axis of chirality for which stereochemistry has not been defined, that stereocenter or axis of chirality can be present in its *R* form, *S* form, or as a mixture of the *R* and *S* forms, including racemic and non-racemic mixtures.

The present invention contemplates the above-described compositions in "therapeutically effective amounts" or "pharmaceutically effective amounts", which means that amount which,
25 when administered to a subject or patient for treating a disease, is sufficient to effect such treatment for the disease or to ameliorate one or more symptoms of a disease or condition (e.g. ameliorate pain).

As used herein, the terms "treat" and "treating" are not limited to the case where the subject (e.g. patient) is cured and the disease is eradicated. Rather, the present invention also
30 contemplates treatment that merely reduces symptoms, improves (to some degree) and/or delays

disease progression. It is not intended that the present invention be limited to instances wherein a disease or affliction is cured. It is sufficient that symptoms are reduced.

“Subject” refers to any mammal, preferably a human patient, livestock, or domestic pet.

In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient or vehicle with which the active compound is administered. Such pharmaceutical vehicles can be liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The pharmaceutical vehicles can be saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica, urea, and the like. In addition, auxiliary, stabilizing, thickening, lubricating and coloring agents can be used. When administered to a subject, the pharmaceutically acceptable vehicles are preferably sterile. Water can be the vehicle when the active compound is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid vehicles, particularly for injectable solutions. Suitable pharmaceutical vehicles also include excipients such as starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol and the like. The present compositions, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

Pharmaceutically acceptable sugars include but are not limited to sucrose, dextrose, maltose, galactose, rhamnose, and lactose. Pharmaceutically acceptable sugar alcohols include but are not limited to mannitol, xylitol, and sorbitol.

DESCRIPTION OF THE FIGURES

Figure 1 presents exemplary data that shows the identification of compounds that inhibit cytochrome c-mediated caspase activation in vitro: (A) Time-course of the in vitro reconstituted cytochrome c-mediated caspase activity assay. For the reaction labeled “Complete,” Apaf-1, cytochrome c, dATP, procaspase-3, caspase-9, and a fluorogenic caspase-3 DEVD substrate were added in buffer ASC. For other reactions, individual component was omitted as indicated. (B)

Inhibition of caspase activation by 10 μ M of each compound. The reaction was performed as in (A), in the presence of compound or DMSO. (C) Dose response of compound inhibition of caspase activation. Compounds were added to the in vitro reconstituted assay at the concentrations indicated. Activity is shown relative to DMSO control at the 20 minute time-point.

5 (D) Structure of Compounds A, B, C, and D. NSC numbers are included for reference.

Figure 2 presents exemplary data that shows initial characterization of compounds. (A) Compounds A, B, C, D do not have quenching effect on rhodamine fluorescence. Free Rhodamine110 was incubated with DMSO or compound as indicated. Fluorescence was measured at 30°C by SpectraFluor Plus Spectrometry Reader (Tecan) with an excitation
10 wavelength of 485 nm and an emission wavelength of 535 nm. (B) Mass confirmation of Compounds A, B, C, and D. Electrospray ionization mass spectrometry was used to determine the masses of compounds (negative ion spectra are shown for Comp-A and Comp-C, positive ion spectra are shown for Comp-B and Comp-D). Numbers in parentheses indicate calculated masses of the compounds. Prominent peaks are labeled. Mass spectrometric data were recorded using
15 Electrospray API-100 Quadrupole mass spectrometer.

Figure 3 presents exemplary data that shows the identified compounds are pan-caspase inhibitors. (A) Inhibition of caspase-3 activity by compounds. 2 nM of active recombinant caspase-3 were incubated with either 10 μ M compound or DMSO in the presence of a fluorogenic DEVD substrate. (B) Inhibition of caspase-9 activity by compounds. Left panel: 20
20 nM of recombinant caspase-9 were incubated with Apaf-1, cytochrome c, dATP, and either 10 μ M compound or DMSO in the presence of a fluorogenic LEHD substrate. Right panel: 200 nM of caspase-9 Leucine-Zipper (LZ) recombinant protein were incubated with either 10 μ M compound or DMSO in presence of a fluorogenic LEHD substrate. (C) Normalized compound dose-response curves for caspases-3, -7, and -9-LZ. Recombinant caspase proteins (15 nM
25 caspase-3, 20 nM caspase-7, 100 nM caspase-9-LZ) were incubated with compounds at the indicated concentrations in the presence of their corresponding fluorogenic substrates. Reaction rates are expressed relative to the DMSO control. (D) Protease specificity of compound inhibition. Recombinant caspase-7 (20 nM), cathepsin C (20 nM), papain (20 nM), and trypsin (20 nM) were incubated with compounds at the indicated concentrations in the presence of their
30 corresponding fluorogenic substrates. Reaction rates are expressed relative to the DMSO control.

Figure 4 presents exemplary data that shows compounds inhibit multiple members of the

caspase family. Normalized compound dose-response curves for caspases-1, -2, and -8. Recombinant caspase proteins (100 nM caspase-1, 200 nM caspase-2, 200 nM caspase-8) were incubated with compounds at the indicated concentrations in the presence of their corresponding fluorogenic substrates. Reaction rates are expressed relative to the DMSO control.

Figure 5 presents exemplary data that shows the Identified Compounds Inhibit Cellular Caspase Activation. (A) Compounds diminish UV-induced apoptotic morphology in HeLa cells. Cells were irradiated with UV and either DMSO or 100 nM of each compound was added to the culture medium. Cells were imaged by light microscopy 6 hours following irradiation. (B) Inhibition of caspase activation in HeLa cells. Cells were irradiated with UV and either DMSO or compounds were added to the culture medium following irradiation. Cells were collected after 6 hours and caspase activity in cell extracts was measured. Compound concentrations were as follows: 0.5 μ M, 1 μ M, 3 μ M, as indicated from left to right. Error bars represent the SEM from experiments performed in triplicate. (C) Annexin V/Propidium Iodide double-staining of UV-treated HeLa cells. Cells were irradiated with UV in the presence or absence of Compound-A (100 nM). Cells were collected 6 hours following irradiation, stained with Annexin V-FITC and PI, and subjected to FACS analysis. Bars represent the mean of five experiments; error bars represent the SEM. (D) MCF10A cell growth following TNF- α + cycloheximide (Chx) induction. Cells were treated with TNF- α and cycloheximide in the presence of either DMSO or Comp-A at the indicated concentrations. After 3 hours of induction (denoted by gray bar), TNF- α , cycloheximide, DMSO and Comp-A were removed and growth medium was replaced, and the number of viable cells was determined at the indicated time points using Resazurin dye as described under Experimental Procedures. Error bars represent the SEM from experiments performed in triplicate. (E) Long-term survival of MCF10A cells following TNF- α + cycloheximide induction in the presence of Compound-A. Cells were treated with TNF- α and cycloheximide in the presence of either DMSO or Comp-A (100 nM) as in (D). Cells were imaged by light microscopy before induction, 3 hours after induction, and 8 days after induction. (F) Inhibition of Interleukin-1 β secretion from J774 cells following LPS stimulation. J774 cells were stimulated with 1 μ g/ml LPS in the presence of DMSO or compounds. Cell supernatants were collected after 24 hours and analyzed by ELISA for IL-1 β . Compound concentrations were as follows: 2 μ M, 1 μ M, 0.5 μ M, as indicated from left to right. Error bars represent the SEM from experiments performed in triplicate.

Figure 6 presents exemplary data that shows effect of compounds on cellular apoptosis and cell proliferation. (A) Compound-A diminishes apoptosis of TNF α -treated U937 cells, as measured by Annexin V and Propidium Iodide staining. Cells were treated with DMSO or with TNF α + cycloheximide in the presence or absence of 150 nM Comp-A. Cells were collected 1 hr following induction, stained with Annexin V-FITC and PI, and analyzed by FACS. Error bars represent the SEM of three experiments. (B) Inhibition of caspase activation in U937 cells treated with TNF α and cycloheximide. U937 cells were treated with TNF α + cycloheximide in the presence of either DMSO or Comp-A at the indicated concentrations. Cells were harvested 1 hr following treatment and caspase activity was measured in cell extracts as described in the Experimental Procedures. (C) Inhibition of caspase activation in mouse embryonic fibroblast (MEF) cells. Cells were irradiated with UV (at 2000 J/m²) and either DMSO or Comp-A was added to the growth medium following irradiation. Cells were collected 4 hours following irradiation and caspase activity was measured in cell extracts as described in the Experimental Procedures. (D) Cell viability following treatment with compounds. HeLa, U937, MEF and MCF10A cells were treated with DMSO or Comp-A at the indicated concentrations for 12 hours. Following treatment, culture medium was replaced and cell growth was measured by counting viable cells at 24 hour intervals using Resazurin dye, as described under Experimental Procedures. Error bars represent SEM from experiments performed in triplicate.

Figure 7 presents exemplary data that shows kinetic analysis of inhibition of caspase-7 and caspase-9 activity by Comp-A. (A) Recombinant caspase-7 (20 nM) and (B) recombinant caspase-9 (20 nM, with Apaf-1, cytochrome c, and dATP as in Figure 1) activity was determined using the DEVD and the LEHD substrates, respectively, in the presence of Compound-A at the indicated concentrations. (i) Substrate concentration-response curves of caspase activity in the presence of the indicated amount of Comp-A. The curves and numerical values of V_{max} and K_m represent nonlinear fitting of the data to the Michaelis-Menten equation using Prism software. (ii) Lineweaver-Burk double reciprocal transformation of the concentration-response curves in (i). Lines represent a linear least-squares fitting of the data using Prism software. (iii) Specific velocity plot for the inhibition of caspases by Comp-A. The ratio of caspase activation rate in the absence of compound (V₀) to the caspase activation rate in the presence of varying concentrations of Comp-A (V_i) was plotted as a function of the specific velocity $\sigma/(1 + \sigma)$, where $\sigma = [S]/K_m$. (iv) Replot of the specific velocity plot. Two sets of intercepts on the ordinate axes

of (iii) at abscissa value = 0 (defined as a) and 1 (defined as b) results in the resolution of kinetic parameters α , β , and K_i , shown in Table 2. Lines represent a linear least-squares fitting of the data using Prism software.

Figure 8 presents exemplary data that shows kinetic analysis of Inhibition of Caspase-3 and Caspase-9 (LZ) Activity by Comp-A. (A) Recombinant caspase-3 (5 nM) and (B) recombinant caspase-9-LZ (200 nM) activity was determined using the DEVD and the LEHD substrates, respectively, in the presence of Compound-A at the indicated concentrations. (i) – (iv) Determination of kinetic mechanism and inhibition parameters were performed as described in Figure 7.

Figure 9 presents exemplary data that shows caspase-7 activity is inhibited by Comp-A in the presence and absence of substrate. Caspase-7 (10 nM) was pre-incubated with Comp-A (2 μ M) in the presence or absence of substrate peptide (DEVDGA, 2 μ M) as indicated. Excess compound and substrate were removed through dialysis against buffer A for 2 hrs and caspase activity was measured using the fluorogenic DEVD substrate. Error bars represent SEM from experiments performed in triplicate.

Figure 10 presents exemplary data that shows crystal structure of caspase-7 in complex with Comp-A. (A) Ball and stick model of the crystal structure of Comp-C. Carbon: yellow; nitrogen: blue; sulfur: gold; copper: orange; bromine: dark red; hydrogen: pink. Atom names are labeled. (B) Ribbon representation of the structure of caspase-7 in complex with Comp-A. The two p20 and p10 subunits are shown in different shades of green. Comp-A is shown in stick models with chloride atoms in light blue. (C) Comp-A superimposed with the Fo-Fc difference Fourier density contoured at 3.0 σ . (D) Surface diagram of caspase-7 (shown with carbon atoms in gray, oxygen atoms in red, nitrogen atoms in blue and sulfur atoms in gold) in complex with stick models of Comp-A bound at the dimerization interface. (E) Detailed interaction between caspase-7 and Comp-A. The different caspase-7 subunits are shown in shades of green. Carbon atoms are shown in the same shades of green as the subunits.

Figure 11 presents exemplary data that shows a comparison of unliganded caspase-7 structure with DEVD-CHO-bound caspase-7 structure. Superposition of the unliganded caspase-7 structure (in this study, cyan and 1IBF, light blue) with the DEVD-CHO-bound active caspase-7 structure (1F1J, magenta). DEVD is shown in dark blue.

Figure 12 presents exemplary data that shows conformational changes and disordering in

the caspase-7 and structure in complex with Comp-A. (A) Superposition of the structure in complex with Comp-A (green) with that in complex with DEVD-CHO in the active conformation (magenta, accession code 1F1J). The L1, L3 and L4 loops and the L2 and L2' regions are labeled in magenta for 1F1J. For caspase-7 in complex with Comp-A, end residues in these loops are labeled in black and with arrows to indicate the breaking points or last residues in them. Relevant secondary structures are also labeled. (B) Superposition of the structure in complex with Comp-A (green) with a procaspase-7 structure (yellow, accession code 1K88). (C) Comp-A would have been in clash with active conformation of caspase-7. Active site Cys186 and caspase-7 residues in direct clash with Comp-A are shown and labeled. (D) Atoms in Comp-A that would have been in clash with caspase-7 are shown within the oval. (E) Previously reported allosteric inhibitor DICA (blue, accession code 1SHJ) would have caused similar clash with the active conformation of caspase-7. (F) Superposition of the structure in complex with Comp-A (green) with DICA-bound caspase-7 (blue, accession code 1SHJ).

Figure 13 shows sequence alignment of the residues at the dimer interfaces of vertebrate caspase-3 and caspase-7. Cys290 is outlined with a box, other residues explored by mutagenesis are indicated with *.

Figure 14 presents exemplary data that shows Comp-A inhibits effective dimerization of caspases in a noncovalent, reversible manner. (A) Mutation of C290, F221, and V292 affect caspase-7 inhibition by Comp-A. Initial reaction rates of caspase-7 wild-type (10 nM) and mutants (100 nM of each) were measured in the presence of Comp-A at the indicated concentrations. Reaction rates are expressed relative to the DMSO control. Error bars represent mean $\pm$ SEM from experiments performed in triplicate. (B) Inhibition of caspase-7 activity by Comp-A is reversible. 200 nM of caspase-7 were incubated with either DMSO (labeled "DMSO") or 10 μ M Comp-A (labeled "Comp-A") in a final volume of 300 μ l in buffer ASC. The samples were dialyzed against 4 liters of buffer ASC. Aliquots were removed at the indicated time-points and caspase-7 activity was measured by adding the fluorogenic DEVD substrate. Caspase-7 protein that was incubated with Comp-A and dialyzed ("Comp-A") recovered activity during the course of dialysis and could subsequently be inhibited by the addition of 10 μ M of exogenous Comp-A (labeled "Comp-A Add-back"). Activity is expressed relative to un-dialyzed Caspase-7. Error bars represent mean $\pm$ SEM from experiments performed in triplicate. (C) Compound-A disrupts dimerization of caspase-8. 100 nM of

recombinant caspase-8 (DED domain-deleted) were incubated with DMSO, 10 μ M Comp-A, or 10 μ M Ac-VAD-CHO for 15 minutes at 30°C and then subjected to gel filtration on a 25-ml Superdex 200 column (Amersham) to resolve the dimeric and the monomeric form of the enzyme. Collected fractions were subjected to SDS-PAGE analysis and stained with Coomassie Brilliant Blue (middle). Activity in each fraction was measured using the DEVD substrate (bottom plot). The volume (mL) labels on the top and bottom plots indicate the elution volume of the chromatographic run (starting from sample injection). The elution positions of protein mass standards are labeled inside of the top Abs₂₈₀ plot.

Table 1 presents exemplary data that shows the kinetic parameters of caspase activity in the presence of compounds.

Table 2 presents exemplary data that shows kinetic parameters of caspase activity in the presence of compounds from replots of intercepts of the specific velocity plot.

Table 3 presents exemplary data that shows the crystallographic statistics for Comp-C

Table 4 presents exemplary data that shows the crystallographic statistics of unliganded Caspase-7 and its complex with Comp-A.

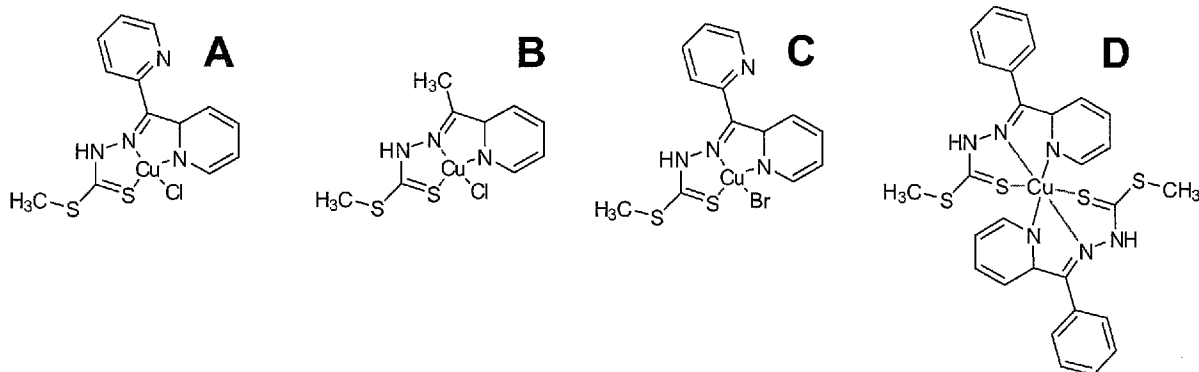
Table 5 presents exemplary data that shows Caspase-7 mutagenesis.

DESCRIPTION OF THE INVENTION

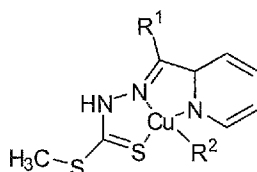
This invention is in the field of medicinal chemistry and relates to novel compounds, and pharmaceutical compositions and methods of use thereof that inhibit caspases and/or tumor necrosis factor alpha (TNF α) that mediate cell apoptosis and inflammation and inhibit pathophysiologic effects of excessive amounts of TNF α . The invention also relates to methods of using the compounds and pharmaceutical compositions of this invention to treat diseases where caspase and/or TNF α activity is implicated. The present invention also relates to methods for the use of allosteric, reversible pan-caspase inhibitors with pyridinyl, copper-containing molecules with a multi-ring structure.

The invention describes the identification and characterization of a proposed class of small molecule inhibitors for caspases. The new inhibitors have unusual characteristics in that (1) they are not peptide-based, (2) they are cell-permeable and reversible inhibitors, and (3) they function in an allosteric manner by binding to the dimerization interface and thus may interfere

with the conformation of catalytic site of caspases. The original identification of these compounds (A-D shown below) was by high-throughput screening, and their subsequent kinetic and cellular characterization.



These identified compounds are potentially new tools for basic apoptosis studies and as a base for potential therapeutic compounds. The similarity of the central scaffold structure of these compounds has led to a minimal basic structure for an allosteric reversible caspase inhibitor:



1. APOPTOSIS

Apoptosis, or programmed cell death, is a principal mechanism by which organisms maintain tissue homeostasis and to eliminate unwanted or damaged cells. It plays a critical role in development, immune responses and many other physiological processes (Ellis *et al.*, 1991; Green, 2000; Vaux and Korsmeyer, 1999) [8-10]. Molecularly, apoptosis is executed by a subfamily of cysteine proteases known as caspases (Degterev *et al.*, 2003; Pop and Salvesen, 2009; Riedl and Shi, 2004; Thornberry and Lazebnik, 1998) [11-14]. The deregulation of apoptosis, either excessive apoptosis or the failure to undergo it, has been implicated in a number of diseases such as cancer, acute inflammatory and autoimmune disorders, ischemic diseases and certain neurodegenerative disorders, see generally Golstein 1998 [15]; and Ellis *et al.* 1991 [8].

Inhibition of apoptosis is a promising therapeutic approach for treating diseases including neurodegeneration, stroke, and radiation syndrome. Using a *de novo* reconstituted assay for

cytochrome c-mediated caspase activation and high-throughput screening, a group of non-peptide inhibitors for caspases were identified from a chemical library containing over 300,000 compounds. These caspase inhibitors share common chemical scaffolds, suggesting same mechanism of action. Further, they are able to inhibit apoptosis in various cell types induced by multiple stimuli. While these compounds inhibit all the tested caspases *in vitro*, kinetic analysis indicates that they do not function by competing with caspase substrates for the catalytic sites of the enzymes. The co-crystal structure of one of these compounds with caspase-7 reveals that these compounds bind to the dimerization interface of active caspases, another common structural element shared by all active caspases. By doing so, compound considered in the present invention may alter the conformation of the catalytic loop of caspases and thus may inhibit caspase activity via an allosteric mechanism.

2. CASPASES

Caspases are a family of cysteine protease enzymes that are key mediators in the signaling pathways for apoptosis and cell disassembly [14]. These signaling pathways vary depending on cell type and stimulus, but all apoptosis pathways appear to converge at a common effector pathway leading to proteolysis of key proteins. Caspases are involved in both the effector phase of the signaling pathway and further upstream at its initiation. The upstream caspases involved in initiation events become activated and in turn activate other caspases that are involved in the later phases of apoptosis.

The utility of caspase inhibitors to treat a variety of mammalian disease states associated with an increase in cellular apoptosis has been demonstrated using peptidic caspase inhibitors. For example, in rodent models, caspase inhibitors have been shown to reduce infarct size and inhibit cardiomyocyte apoptosis after myocardial infarction, to reduce lesion volume and neurological deficit resulting from stroke, to reduce post-traumatic apoptosis and neurological deficit in traumatic brain injury, to be effective in treating fulminant liver destruction, and to improve survival after endotoxic shock (Yaoita *et al.* 1998; Endres *et al.*, 1998; Cheng *et al.*, 1998; Yakovlev *et al.*, 1997; Rodriguez *et al.*, 1996; and Grobmyer *et al.* 1999) [16-21]. The invention provides a method for the use of a group of compounds as allosteric reversible caspase inhibitors. As apoptosis is a major form of programmed cell death utilized by multicellular organisms to maintain tissue homeostasis and to eliminate unwanted or damaged cells, it plays a

role in development, immune responses and many other physiological processes. Molecularly, apoptosis is mediated by a subfamily of cysteine proteases known as caspases. Caspases usually exist in their inactive, pro-zymogen forms in living cells; however, upon apoptotic stimulation, caspases are cleaved into their active, mature forms and may implement the apoptotic process. In mammals, there are two well-characterized caspase activation pathways: the intrinsic mitochondria-mediated pathway and the extrinsic death receptor-mediated pathway (Creagh *et al.*, 2003; Jiang and Wang, 2004; Peter and Krammer, 2003) [22-24].

In the mitochondria-mediated caspase activation pathway, caspase activation is initiated by cytochrome c release from mitochondria, a process closely regulated by the Bcl-2 family of proteins (Garrido *et al.*, 2006; Green and Reed, 1998; Jiang and Wang, 2004; Youle and Strasser, 2008) [23, 25-27]. Released cytochrome c binds to the essential mediator Apaf-1 (Zou *et al.*, 1997) [28], activates the nucleotide exchanging activity of Apaf-1 (Bao *et al.*, 2007; Jiang and Wang, 2000; Kim *et al.*, 2005) [23, 29, 30], and consequently triggers the assembly of a multimeric protein complex, the apoptosome, which is the central caspase activation machinery (Srinivasula *et al.*, 1998; Zou *et al.*, 1999) [31, 32]. The apoptosome recruits and activates the initiator caspase, caspase-9. Caspase-9 needs to associate with the apoptosome to be active (Jiang and Wang, 2000; Rodriguez and Lazebnik, 1999) [33, 34] and it subsequently activates downstream executioner caspases, such as caspase-3 and caspase-7, which mediate apoptotic cell death by targeting and cleaving a variety of cellular substrates. In cells, there is a family of endogenous proteins named Inhibitors of Apoptosis Proteins (IAP) that functions to inhibit caspase activity; whereas Smac/Diablo and Omi/HtrA2 are mitochondrial proteins that are released from mitochondria during apoptosis to antagonize the inhibitory activity of IAP (Du *et al.*, 2000; Suzuki *et al.*, 2001; Verhagen *et al.*, 2000) [35-37].

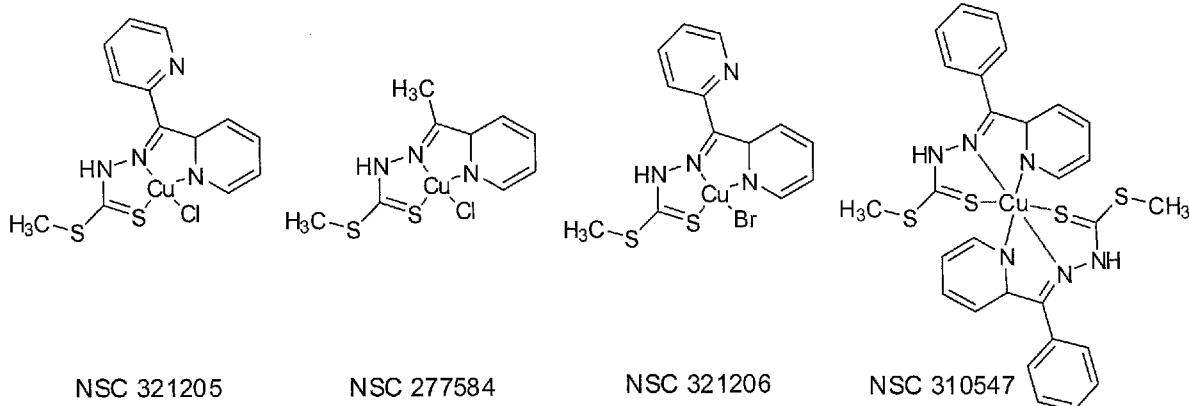
Deregulation of the intrinsic apoptotic pathway is involved in various human diseases, such as cancer and autoimmune disorders (when apoptosis is defective), and neurodegenerative diseases and strokes (when apoptosis is improperly activated) (Hotchkiss and Nicholson, 2006; Reed, 2003; Yuan and Yankner, 2000) [38, 39]. Conversely, targeting apoptotic components by both enhancing and attenuating apoptosis represents important therapeutic approaches to treat various human diseases. For example, many conventional cancer therapies kill cancer cells via activating the cytochrome c apoptotic pathway, and several promising targeted compounds, including small molecule inhibitors of Bcl-2 (Oltersdorf *et al.*, 2005) [40] and Smac mimetics (Li

et al., 2004) [41], are designed to specifically activate or potentiate this pathway. On the other hand, inhibition of this pathway should also be effective in treating symptoms with pathologically enhanced apoptosis such as neurodegeneration, stroke, and ischemic diseases. Previous efforts in developing potential anti-apoptotic agents focused on caspase inhibition. Although caspase inhibitors have been developed with some degree of specificity and are used for basic research, because of their chemical nature as peptides, they possess poor potency and are rapidly degraded *in vivo*. Another undesirable feature of most of these peptide-based inhibitors is that they are irreversible inhibitors. These weaknesses preclude their realistic clinical usage.

3. IDENTIFICATION OF CHEMICAL INHIBITORS

Cytochrome c-mediated caspase activation *in vitro* by using purified protein components, nucleotide dATP, and a fluorogenic substrate of caspase-3 was reconstituted. A high-throughput screening approach to identify small molecule inhibitors of this pathway was employed. Four structurally similar compounds were identified as caspase inhibitors. The compounds were able to inhibit cellular apoptosis. Further kinetic and co-crystallization studies revealed that the compounds inhibit caspases via an allosteric mechanism, by binding to the caspase dimerization interface and subsequently altering the conformation of the catalytic site of the enzymes.

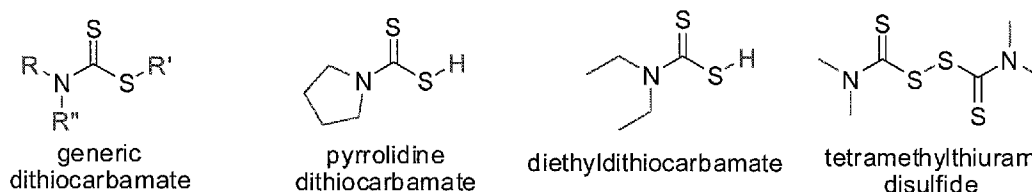
Developmental Therapeutics Program NCI/NIH (dtp.nci.nih.gov) is an online research database which discloses the existence of the four compounds shown below. These compounds are a subset of available compounds for activity screening. The database does not provide anything except the larger activity screens of which the compounds participated and data deposited. Most of the screens involved tumor cell lines.



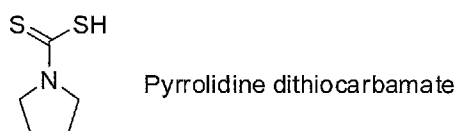
This database does not disclose the minimal basic structure, per se, but does reveal the example

compounds from which the minimal basic structure was derived. Further, the database does not directly suggest that these compounds have any caspase inhibitory activity.

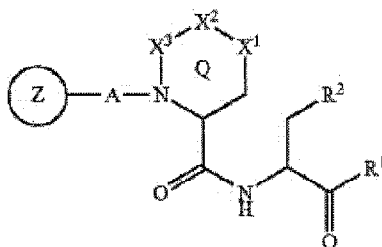
Nobel *et al.* 1997 discloses that dithiocarbamates (DCs) (shown below generically and with specific examples, central structure darkened) have been reported to be potent inhibitors of apoptosis in several different model systems suggesting a target common to the apoptotic machinery [42]. The reference investigates antiapoptotic actions of DCs and found that inclusion of a membrane-impermeable copper chelator severely compromised the inhibitory activity of reduced DCs. Since copper can promote DC oxidation to the respective DC disulfides, the inhibitory effect on lymphocyte apoptosis might be mediated by the DC disulfides. The reference concludes that (1) DC disulfides are the active agents behind DC inhibition of apoptosis and (2) their site of action is the proteolytic activation of caspase-3 proenzyme.



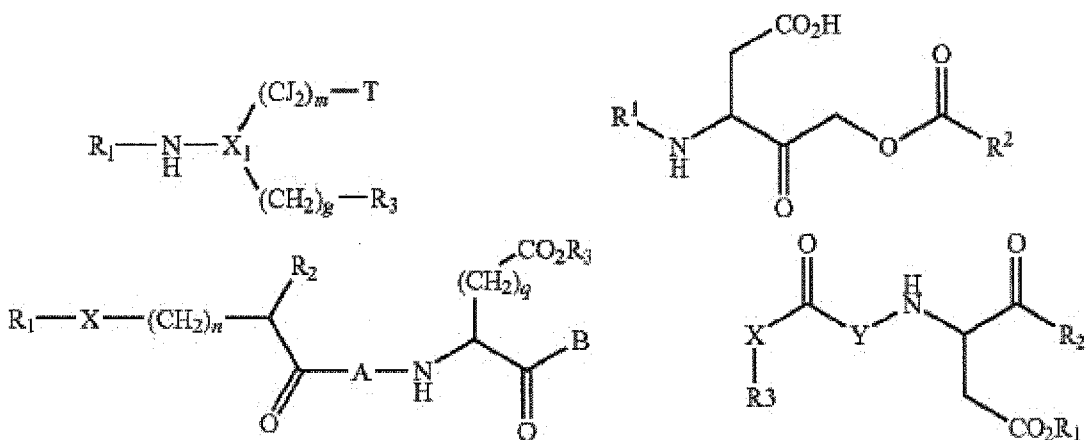
Thompson *et al.* 2010 also discusses the apoptosis blocking effect of pyrrolidine dithiocarbamate in exposure to ionizing radiation [43]. Rather than providing an antioxidant prophylactic effect, the result of pyrrolidine dithiocarbamate in exposure to ionizing radiation leads to an increase in radical species and results in greater cell necrosis compared to apoptosis.



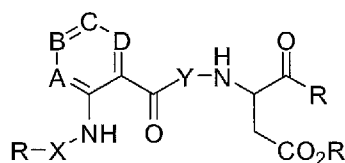
Some patents have discussed caspase inhibitors including United States Patent 7,807,659 [44]. This patent discloses a formula for a caspase inhibitor:



Additionally, the 7,807,659 patent also refers to previously disclosed formulas for caspase inhibitors:



United States Patent 7,410,956 describes compounds of a formula (shown below) which are prodrugs of caspase inhibitors and pharmaceutically acceptable salts thereof [45]. Y is the caspase inhibitor. This invention further relates to pharmaceutical compositions comprising these compounds, which are particularly well-suited for treatment of caspase-mediated diseases, including inflammatory and degenerative diseases.



Prodrug formula where
Y = caspase inhibitor

Ekert *et al.* 1999 discloses various known caspase inhibitors, including synthetic caspase inhibitors [46]. A number of specific caspase inhibitors have been developed based upon the substrate cleavage sites of the caspases. These peptides act as pseudosubstrates for active caspases and are therefore competitive inhibitors. They range from those containing a single aspartate residue (e.g. Boc-aspartyl(OMe)-fluoromethylketone: Boc-Asp-FMK) to trimers (e.g. Benzyloxycarbonyl-val-alasp (OMe) fluoromethylketone: z-VAD-FMK) to tetramers (e.g. YVAD-FMK). The inhibitors described in this reference are peptide derivatives and are described as competitive inhibitors, not as allosteric inhibitors to caspases. Linton, S. D. 2005 also describes the various caspase inhibitors found and used within the pharmaceutical industry [47]. Most of the compounds disclosed are peptide derivatives and pro-drug peptide derivatives.

Inhibition of caspase activation is a promising therapeutic approach for treating diseases including neurodegeneration, stroke, radiation syndrome, and immune disorders. Using a *de novo* reconstituted assay for cytochrome c-mediated caspase activation and high-throughput

screening, a group of non-peptide inhibitors for caspases from a chemical library containing over 300,000 compounds were identified. These caspase inhibitors share common chemical scaffolds, suggesting same mechanism of action. Further, they are able to inhibit apoptosis in various cell types induced by multiple stimuli; they can also inhibit caspase-1-mediated interleukin-1 β generation in macrophages, suggesting the potential application of these compounds in antiinflammation.

While these compounds inhibit all the tested caspases, kinetic analysis indicates that they do not compete with caspase substrates for the catalytic sites of the enzymes. The co-crystal structure of one of these compounds with caspase-7 reveals that these compounds bind to the dimerization interface of active caspases, another common structural element shared by all active caspases. Consistently, biochemical analysis demonstrates that the compounds abate dimerization of caspase-8. Therefore, via an allosteric mechanism of binding to the dimerization site, these compounds alter the conformation of the catalytic loop of caspases and thus inhibit caspase activity.

In the mitochondria-mediated caspase activation pathway, caspase activation is initiated by cytochrome c release from mitochondria, a process closely regulated by the Bcl-2 family of proteins (Garrido et al., 2006 [25]; Green and Reed, 1998 [26]; Jiang and Wang, 2004 [23]; Youle and Strasser, 2008 [27]). Although it is not necessary to understand the mechanism of an invention, it is believed that, released cytochrome c binds to the essential mediator Apaf-1 (Zou et al., 1997 [28]), activates the nucleotide binding/exchanging activity of Apaf-1 (Bao et al., 2007 [29]; Jiang and Wang, 2000 [33]; Kim et al., 2005 [30]), and consequently triggers the assembly of a multimeric protein complex, the apoptosome, which is the central caspase activation machinery (Srinivasula et al., 1998 [31]; Zou et al., 1999 [32]). Although it is not necessary to understand the mechanism of an invention, it is believed that, the apoptosome recruits and activates the initiator caspase, caspase-9. Caspase-9 needs to associate with the apoptosome to be active (Jiang and Wang, 2000 [33]; Rodriguez and Lazebnik, 1999 [34]) and it subsequently activates downstream executioner caspases, such as caspase-3 and caspase-7, which mediate apoptotic cell death by targeting and cleaving a variety of cellular substrates. Although it is not necessary to understand the mechanism of an invention, it is believed that, in cells, there is a family of endogenous proteins named Inhibitors of Apoptosis Proteins (IAP) that functions to inhibit caspase activity; whereas Smac/Diablo and Omi/HtrA2 are mitochondrial

proteins that are released from mitochondria during apoptosis to antagonize the inhibitory activity of IAP (Du et al., 2000 [35]; Suzuki et al., 2001 [36]; Verhagen et al., 2000 [37]).

Although it is not necessary to understand the mechanism of an invention, it is believed that, Deregulation of the intrinsic apoptotic pathway is involved in various human diseases, such as cancer and autoimmune disorders (when apoptosis is defective), and neurodegenerative diseases and strokes (when apoptosis is improperly activated) (Hotchkiss and Nicholson, 2006 [38]; Reed, 2003 [48]; Yuan and Yankner, 2000 [39]). Conversely, targeting apoptotic components by both enhancing and attenuating apoptosis represents important therapeutic approaches to treat various human diseases. For example, many conventional cancer therapies kill cancer cells via activating the cytochrome c apoptotic pathway, and several promising targeted compounds, including small molecule inhibitors of Bcl-2 (Oltersdorf et al., 2005 [40]) and Smac mimetics (Li et al., 2004 [41]), are designed to specifically activate or potentiate this pathway. On the other hand, inhibition of this pathway should also be effective in treating symptoms with pathologically enhanced apoptosis such as neurodegeneration, stroke, and ischemic diseases. Previous efforts in developing potential anti-apoptotic agents focused on caspase inhibition. Notably, caspase inhibition can also be used for treating inflammation, which requires caspase-1-mediated interleukin-1 β maturation (Martinon and Tschopp, 2006 [49]; Talanian et al., 2000 [50]). To date, although caspase inhibitors have been developed with some degree of specificity and are used for basic research, because of their chemical nature as peptides, they possess poor potency and are rapidly degraded in vivo. Another undesirable feature of most of these peptide-based inhibitors is that they are covalent, irreversible inhibitors. These weaknesses preclude their realistic clinical usage. Cytochrome c-mediated caspase activation in vitro by using purified protein components (Apaf-1, cytochrome c, caspase-9, and procaspase-3), nucleotide dATP, and a fluorogenic substrate of caspase-3 were reconstituted. A high-throughput screening approach to identify small molecule inhibitors of this pathway was employed. Four structurally similar compounds were identified as caspase inhibitors. The compounds were able to inhibit apoptosis and caspase-1-mediated interleukin-1 β generation. Further kinetic and structural studies revealed that the compounds inhibit caspases via an allosteric mechanism, by binding to the caspase dimerization interface and subsequently altering the conformation of the catalytic site of the enzyme.

4. Identification of Chemical Inhibitors of Cytochrome c-mediated Caspase Activation

The cytochrome c-mediated caspase activation pathway was reconstituted *in vitro* using purified recombinant proteins at their near-physiological concentrations (Jiang and Wang, 2000; Kim *et al.*, 2005; Zou *et al.*, 1999) [30, 32, 33]. In the presence of Apaf-1, cytochrome c, caspase-9, procaspase-3 and dATP, robust caspase-3 activation was achieved, monitored using a fluorogenic peptide substrate of caspase-3 (Figure 1A). As expected, omission of any single component in the reaction completely abated caspase-3 activation (Figure 1A). After adapting this highly sensitive assay system to a fully automated high-throughput screening (HTS) format, a collection of 317,856 chemical compounds was screened at a single compound concentration of 10 μ M in 1% DMSO (v/v). The optimized assay exhibited a high signal-to-noise ratio of 19:1, as well as good reproducibility with Z'-value ranging from 0.59 to 0.75. Out of this large compound collection, 34 molecules were identified as initial positive hits with a threshold of at least 30% inhibition.

Four of the 34 compounds were selected after further analysis. These compounds, named Comp-A, B, C, D thereafter for simplicity, are also included in the chemical repository of the Developmental Therapeutics Program (DTP) of National Cancer Institute (NCI), with the codes NSC321205, NSC277584, NSC321206, and NSC310547, respectively. At 10 μ M concentrations, these compounds all completely inhibited cytochrome c-mediated caspase activation (Figure 1B). Further titration of the compounds in this assay indicates their potent inhibition activity with submicromolar IC₅₀ values (Figure 1D). A striking feature of these compounds is that they share a very similar molecular scaffold, suggesting a common structure-function relationship. They are pyridinyl, copper-containing molecules with a multi-ring structure (Figure 1C). Although Comp-D is more complex structurally, it appears to be a covalent dimer of the other complexes. Mass spectrometry analysis yielded a molecular mass of each compound consistent with its molecular formula (Figure 2).

5. The Newly Identified Inhibitors Target Caspases

Because a multi-component biochemical assay was used for HTS, a deconvolution analysis was performed to identify the direct target of the inhibitors. Although it is not necessary to understand the mechanism of an invention, it is believed that the results demonstrate that these compounds may directly inhibit both caspase-9 and caspase-3. As shown in Figure 3, the activity

of recombinant caspase-3 (fully activated when expressed and purified from bacteria) was completely inhibited by 10 μ M of each compound (Figure 3A), and consistent with the result obtained from the cytochrome c-mediated caspase activation assay, these compounds inhibit caspase-3 activity with submicromolar IC₅₀ values (Figure 3C). Although it is not necessary to understand the mechanism of an invention, it is believed that the direct effect of these compounds on caspase-9 activity was measured using an engineered caspase-9 dimer, containing a leucine-zipper dimerization domain (Caspase-9/LZ) (Yin et al., 2006) [51]. Unlike native caspase-9 whose activity requires an active apoptosome complex even after complete proteolytic processing (Jiang and Wang, 2000; Rodriguez and Lazebnik, 1999) [33, 34], recombinant caspase-9/LZ is constitutively active independent of the apoptosome complex (Yin et al., 2006) [51]. Similar to caspase-3 activity, caspase-9/LZ activity was completely inhibited by 10 μ M of each compound (Figure 3C). To validate that inhibition of the enzyme by the compounds is not an artificial result caused by the engineered leucine-zipper, an apoptosome complex was used to activate unmodified caspase-9 and subsequently assessed the effect of the compounds on caspase-9 activity using a fluorogenic peptide substrate of caspase-9. In such an assay, the activity of caspase-9 was also inhibited by all four compounds (Figure 3B).

Although it is not necessary to understand the mechanism of an invention, it is believed that since the compounds were able to inhibit both caspase-9 and caspase-3, it is possible that this inhibitory effect might extend to other members of the caspase family as well. The potential inhibitory effect of the four compounds on the activity of recombinant caspase-8 and caspase-2 was tested; both caspase-8 and caspase-2 are initiator caspases that share only limited similarity with caspase-9 or caspase-3. Caspase-7, an effector caspase that is highly similar to caspase-3, was also inhibited by each of the four compounds with submicromolar IC₅₀ values (Figure 3C). Recombinant caspase-8 and caspase-2, both initiator caspases that share only limited similarity with caspase-9 or caspase-3, and caspase-1, which is involved in the inflammatory response, were inhibited by the compounds with submicromolar IC₅₀ values as well (Figure 4). Therefore, these four compounds are pan-caspase inhibitors whose chemical nature is different from the previously developed peptide-based caspase inhibitors.

To determine the specificity of these compounds, their inhibitory activity on other proteases, including cathepsin C, papain, and trypsin. Cathepsin C and papain are cysteine proteases was tested. Although they were inhibited by the compounds in a dose-dependent

manner, the IC₅₀ values were 5-10 μ M, which is about 10-fold higher than for caspases (Figure 3D). Trypsin, a serine protease, was not significantly inhibited by the compounds (Figure 3D). One exception is Comp-D, which inhibits papain and caspase-7 with similar potency. Although it is not necessary to understand the mechanism of an invention, it is believed that that this “offtarget” effect of Comp-D might be due to its more bulky and complex structure compared to the other three compounds.

6. Inhibition of Cellular Apoptosis by the Compounds

Although it is not necessary to understand the mechanism of an invention, to determine whether these compounds were able to block cellular apoptosis, several different mammalian cell lines were used and tested the effect of the compounds on both intrinsic apoptosis and extrinsic apoptosis. Because these compounds are pan-caspase inhibitors, if they are cell-permeable, they should be able to inhibit both pathways.

The intrinsic apoptotic pathway was triggered by UV radiation in HeLa cells. Following UV irradiation, cells that had not been treated with compound displayed typical apoptotic morphology, while cells treated with each of the four compounds showed either diminished apoptotic morphology or resembled non-irradiated cells (Figure 5A). Compound concentrations from 1 μ M to as low as 100 nM were tested, and it was found that even at 100 nM, the compounds still possess apparent activity in preventing UV-induced apoptosis (Figure 5A). Although it is not necessary to understand the mechanism of an invention, it is believed that because in the in vitro assays 100 nM of compounds only yielded very mild inhibition of caspases, it is possible that the cells might actively take in the compounds from culture medium, resulting in a higher cellular concentration of the compounds. Subsequent measurement of caspase-3 activity in cytosolic extracts of these cells confirmed that caspase activity was inhibited by the compounds in a dose-dependent manner (Figure 5B). Annexin V and propidium iodide (PI) staining confirmed the inhibition of UV-induced apoptosis in HeLa cells in the presence of compound (Figure 5C). Similar to HeLa cells, UV-induced caspase activation in mouse embryonic fibroblast (MEF) cells can also be inhibited by these compounds in a dose-dependent manner (Figure 6).

The effect of the compounds on the extrinsic apoptotic pathway was tested in U937 cells, a human leukemic monocyte lymphoma cell line. The extrinsic apoptotic pathway was induced

by tumor necrosis factor- α (TNF- α) plus cellular protein synthesis inhibitor cycloheximide. The compounds were able to inhibit the extrinsic apoptotic pathway in U937 cells in a dosedependent manner, as measured by both Annexin V/PI staining and cellular caspase-3 activity assay (Figure 6). Therefore, the newly identified pan-caspase inhibitory compounds can inhibit both intrinsic and extrinsic apoptotic pathways.

The ability of these compounds to enhance long-term survival of cells that have been transiently treated with an apoptotic trigger was next tested. Although inhibition of caspase activation often fails to prevent eventual cell death due to loss of mitochondrial integrity (for intrinsic apoptosis) or switching to programmed necrosis (for extrinsic apoptosis), under certain conditions, caspase inhibition can restore cell viability permanently. This could be particularly relevant in vivo where apoptotic stimulation is often transient and milder than the conditions used in experimental settings. Indeed, it has been firmly established recently that intrinsic apoptosis-associated mitochondrial outer membrane permeability (MOMP) in a cell can be partial or incomplete (iMOMP); and under such conditions, caspase inhibition can completely restore cell viability (Tait et al., 2010 [52]). Further, in cells with specific genetic backgrounds, such as lack of RIP3 expression, TNF- α -induced programmed necrosis can be defective (He et al., 2009 [53]; Oberst et al., 2011 [54]; Vandenabeele et al., 2010 [55]). It was determined that human mammary epithelial MCF10A cells appear to be defective in TNF- α -induced necrosis because inhibition of caspase activation completely restored long-term viability in these cells. As shown in Figure 5, apoptosis was induced in MCF10A cells by the addition of TNF- α and cycloheximide for 3 hours in the presence or absence of Comp-A. Growth medium was subsequently replaced, and cell numbers were monitored daily for a period of eight days. While TNF- α -treated cells in the absence of Comp-A all died, growth of MCF10A cells following TNF- α induction in the presence of Comp-A was recovered to the level of untreated cells (Figure 5D and Figure 5E). This result demonstrates that under certain specific contexts, inhibition of apoptosis by these compounds can significantly restore long-term cell viability.

To investigate whether the compounds would be effective in inhibiting cellular caspase activation outside of the context of apoptosis, J774 cells, a murine macrophage cell line, were treated with lipopolysaccharide (LPS). LPS treatment can activate inflammatory caspase-1, which results in the cleavage and subsequent secretion of the proinflammatory cytokine Interleukin (IL)-1 β (Martinon et al., 2002) [56]. Indeed, when added to the culture medium

during LPS stimulation, the compounds were able to decrease the secretion of IL-1 β in a dose-dependent manner (Figure 5F).

It should be noted that when higher concentrations of these four compounds were used, noticeable cell toxicity was observed. In culture dishes, concentrations up to 5 μ M of these compounds caused attached cells (such as HeLa cells) to detach in a short period of time. If the compounds were removed rapidly, cells were still able to re-attach and grow. However, longer treatment with high doses of the compounds caused the cells to lose viability. Although it is not necessary to understand the mechanism of an invention, it is believed that it is possible that such toxicity is due to their chemical nature as transition metal complexes. Although it is not necessary to understand the mechanism of an invention, it is believed that it is also possible that the contaminating free copper ions in the solution of the compounds caused the toxicity. Although it is not necessary to understand the mechanism of an invention, it is believed that alternatively, the compounds might exert the toxicity by targeting other biological molecules. For example, it has been reported that Comp-C can target Cdc25B phosphatase (Vogt et al., 2003)[57] and X-IAP (Glover et al., 2003) [58], although both with a lower potency (IC₅₀ over 10 μ M) than what was observed for caspase inhibition. It should also be noted that at the low concentrations sufficient for apoptosis inhibition in HeLa cells, the compounds do not affect HeLa cell viability (Figure 6).

7. Non-competitive Inhibition of Caspase-7 by Comp-A

Although it is not necessary to understand the mechanism of the invention, a kinetic analysis was performed in order to understand the mechanism of action of these compounds in inhibiting caspase activity, and to subsequently conduct medicinal chemical modification. Although it is not necessary to understand the mechanism of an invention, it is believed that because these four compounds share a similar structure, it is most likely that they inhibit caspases via the same mechanism. For this reason, there was a focus on Comp-A (which showed the strongest activity in inhibiting cellular apoptosis), and the kinetics of its inhibition of caspase-7 activity were examined. The kinetic analysis of all four compounds and multiple caspases (Figure 7 and Figure 8; Table 1 and Table 2) was later expanded.

TABLE 1

Kinetic Parameters of Caspase Activity in the Presence of Compounds

Comp-A		0 nM	50 nM	100 nM	250 nM	400 nM
Caspase-3	V_{max}	152.1 ± 0.5	136.6 ± 0.6	124.1 ± 0.4	99.9 ± 0.3	83.7 ± 0.3
	K_m	6.3 ± 0.1	6.4 ± 0.1	6.3 ± 0.1	6.5 ± 0.9	6.4 ± 0.1
Caspase-7	V_{max}	71.4 ± 0.4	64.7 ± 0.4	57.6 ± 0.3	46.1 ± 0.3	38.2 ± 0.2
	K_m	30.8 ± 0.4	31.7 ± 0.4	31.0 ± 0.4	31.9 ± 0.4	31.9 ± 0.3
Caspase-9-LZ	V_{max}	98.6 ± 0.9	68.6 ± 0.5	51.3 ± 0.3	33.1 ± 0.2	25.9 ± 0.2
	K_m	54.3 ± 0.9	58.2 ± 0.7	56.0 ± 0.6	57.4 ± 0.5	57.8 ± 0.9
Caspase-9-apoptosome	V_{max}	110.8 ± 0.9	81.3 ± 0.5	66.1 ± 0.6	43.9 ± 0.4	34.8 ± 0.2
	K_m	53.2 ± 0.8	53.2 ± 0.6	54.3 ± 0.8	55.1 ± 0.9	56.5 ± 0.6
Caspase-1	V_{max}	120.0 ± 0.4	114.2 ± 0.4	109.4 ± 0.5	97.0 ± 0.4	87.7 ± 0.2
	K_m	4.9 ± 0.1	4.9 ± 0.1	4.9 ± 0.1	5.0 ± 0.1	5.2 ± 0.1
Caspase-2	V_{max}	187.1 ± 0.7	165.1 ± 0.8	150.1 ± 0.8	118.4 ± 0.8	97.6 ± 0.4
	K_m	17.1 ± 0.2	17.0 ± 0.2	17.6 ± 0.2	18.4 ± 0.3	18.3 ± 0.2
Caspase-8	V_{max}	85.0 ± 0.3	80.2 ± 0.2	76.2 ± 0.2	66.5 ± 0.2	59.0 ± 0.2
	K_m	3.3 ± 0.1	3.3 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1
Comp-B		0 nM	50 nM	100 nM	250 nM	400 nM
Caspase-3	V_{max}	152.7 ± 0.5	136.9 ± 0.5	125.6 ± 0.5	100.6 ± 0.3	83.7 ± 0.2
	K_m	6.5 ± 0.1	6.2 ± 0.1	6.2 ± 0.1	6.3 ± 0.1	6.1 ± 0.1
Caspase-7	V_{max}	71.2 ± 0.4	63.3 ± 0.3	57.8 ± 0.3	45.9 ± 0.3	36.7 ± 0.3
	K_m	30.5 ± 0.4	30.2 ± 0.4	30.5 ± 0.4	31.5 ± 0.4	30.0 ± 0.4
Caspase-9-LZ	V_{max}	98.1 ± 0.8	76.5 ± 0.5	63.5 ± 0.4	41.2 ± 0.3	31.8 ± 0.3
	K_m	54.1 ± 0.7	53.3 ± 0.6	53.7 ± 0.6	52.1 ± 0.6	54.1 ± 0.9
Caspase-1	V_{max}	120.7 ± 0.4	114.1 ± 0.5	107.0 ± 0.4	92.8 ± 0.3	82.0 ± 0.3
	K_m	5.0 ± 0.1	5.0 ± 0.1	4.8 ± 0.1	4.9 ± 0.1	5.0 ± 0.1
Caspase-2	V_{max}	185.4 ± 0.8	164.3 ± 0.6	147.9 ± 0.7	110.7 ± 0.5	90.0 ± 0.5
	K_m	16.8 ± 0.2	17.0 ± 0.2	17.2 ± 0.2	17.0 ± 0.2	17.1 ± 0.3
Caspase-8	V_{max}	84.7 ± 0.3	81.0 ± 0.2	77.7 ± 0.2	68.4 ± 0.2	61.2 ± 0.2
	K_m	3.2 ± 0.1	3.3 ± 0.1	3.4 ± 0.1	3.3 ± 0.1	3.2 ± 0.1
Comp-C		0 nM	50 nM	100 nM	250 nM	400 nM
Caspase-3	V_{max}	151.6 ± 0.5	135.1 ± 0.4	123.3 ± 0.4	98.3 ± 0.4	82.7 ± 0.2
	K_m	6.4 ± 0.1	6.2 ± 0.1	6.2 ± 0.1	6.4 ± 0.1	6.4 ± 0.1
Caspase-7	V_{max}	72.0 ± 0.3	61.7 ± 0.3	55.8 ± 0.3	42.4 ± 0.3	34.2 ± 0.2
	K_m	31.2 ± 0.3	30.5 ± 0.3	31.8 ± 0.3	31.9 ± 0.5	31.1 ± 0.4
Caspase-9-LZ	V_{max}	97.4 ± 0.5	67.3 ± 0.4	52.0 ± 0.4	33.4 ± 0.2	26.7 ± 0.2
	K_m	53.5 ± 0.5	55.5 ± 0.6	55.1 ± 0.7	54.6 ± 0.5	55.0 ± 0.6
Caspase-1	V_{max}	121.6 ± 0.5	113.9 ± 0.4	108.1 ± 0.3	93.8 ± 0.3	82.9 ± 0.3
	K_m	5.2 ± 0.1	5.0 ± 0.1	5.1 ± 0.1	5.0 ± 0.1	5.0 ± 0.1
Caspase-2	V_{max}	187.8 ± 0.7	161.8 ± 0.6	141.2 ± 0.4	106.0 ± 0.4	86.8 ± 0.4
	K_m	17.2 ± 0.2	17.5 ± 0.2	17.3 ± 0.1	17.5 ± 0.2	17.5 ± 0.2
Caspase-8	V_{max}	84.5 ± 0.3	79.4 ± 0.3	74.2 ± 0.2	63.2 ± 0.2	55.6 ± 0.2
	K_m	3.2 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	3.4 ± 0.1
Comp-D		0 nM	50 nM	100 nM	250 nM	400 nM
Caspase-3	V_{max}	152.1 ± 0.6	143.4 ± 0.5	135.8 ± 0.4	119.6 ± 0.5	106.0 ± 0.3
	K_m	6.4 ± 0.1	6.3 ± 0.1	6.3 ± 0.1	6.7 ± 0.1	6.8 ± 0.1
Caspase-7	V_{max}	72.2 ± 0.5	66.4 ± 0.3	59.8 ± 0.3	49.9 ± 0.3	42.8 ± 0.2
	K_m	31.3 ± 0.4	31.7 ± 0.3	30.5 ± 0.3	32.1 ± 0.4	33.3 ± 0.3
Caspase-9-LZ	V_{max}	97.9 ± 0.6	82.2 ± 0.8	68.9 ± 0.5	49.3 ± 0.4	38.7 ± 0.3
	K_m	53.9 ± 0.6	57.8 ± 0.9	57.5 ± 0.8	60.8 ± 0.9	62.2 ± 0.7
Caspase-1	V_{max}	120.9 ± 0.4	116.2 ± 0.4	112.4 ± 0.3	101.6 ± 0.5	92.2 ± 0.3
	K_m	5.0 ± 0.1	5.1 ± 0.1	5.2 ± 0.1	5.2 ± 0.1	5.2 ± 0.1
Caspase-2	V_{max}	189.1 ± 0.7	173.5 ± 0.7	165.6 ± 1.0	139.9 ± 0.6	120.8 ± 0.5
	K_m	17.4 ± 0.2	17.2 ± 0.2	18.0 ± 0.3	18.2 ± 0.2	18.6 ± 0.2
Caspase-8	V_{max}	84.6 ± 0.2	80.9 ± 0.3	76.8 ± 0.2	67.3 ± 0.2	60.3 ± 0.2
	K_m	3.2 ± 0.1	3.3 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.5 ± 0.1

 V_{max} is expressed in RFU/min; K_m is expressed in μ M.Values are expressed as mean $\pm$ SEM from experiments performed in triplicate as described in the Experimental Procedures.

TABLE 2
Kinetic Parameters of Caspase Activity in the Presence of Compounds
from Replots of Intercepts of the Specific Velocity Plot

		α	β	K_i (μM)
Caspase-3	Comp-A	1.06	0.14	0.35
	Comp-B	0.94	0.14	0.71
	Comp-C	0.99	0.19	0.67
	Comp-D	1.06	0.28	0.95
Caspase-7	Comp-A	1.01	0.06	0.34
	Comp-B	0.94	0.09	0.41
	Comp-C	0.84	0.15	0.31
	Comp-D	1.04	0.08	0.34
Caspase-9-LZ	Comp-A	1.06	0.08	0.08
	Comp-B	0.92	0.04	0.18
	Comp-C	1.04	0.10	0.08
	Comp-D	1.08	0.04	0.17
Caspase-9-apoptosome	Comp-A	1.04	0.04	0.12
Caspase-1	Comp-A	0.90	0.25	1.02
	Comp-B	1.02	0.60	0.44
	Comp-C	0.99	0.34	0.80
	Comp-D	1.01	0.46	0.56
Caspase-2	Comp-A	0.96	0.16	0.37
	Comp-B	0.98	0.02	0.38
	Comp-C	1.04	0.05	0.22
	Comp-D	0.97	0.06	0.35
Caspase-8	Comp-A	0.98	0.23	0.69
	Comp-B	1.04	0.11	0.28
	Comp-C	1.03	0.19	0.40
	Comp-D	1.07	0.32	0.67

Because these novel inhibitors are pan-caspase inhibitors, it was predicted that they may act upon a common functional moiety shared by all caspases. The catalytic center of all known caspases shares a similar conformation and is a common site of inhibition for most known pharmacological caspase inhibitors (Ivachtchenko et al., 2009) [59]. The catalytic site contains a substrate binding groove that is shaped by four peptide loops (L1, L2, L3, L4) that harbor the catalytic cysteine and determine substrate specificity (Shi, 2002) [60]. Although it is not necessary to understand the mechanism of an invention, it is believed that although individual caspases show different protein substrate preferences, aspartate at the P1 position is universally

required for all caspases (Pop and Salvesen, 2009) [12]. Because of this universal specificity for aspartate residues, most pan-caspase inhibitors utilize amino-acid sequences containing aspartate and compete with substrate binding at the active site. If indeed Comp-A binds to the catalytic site of caspase-7, it should then inhibit the enzyme by competitively inhibiting substrate binding to the enzyme, thus Michaelis-Menten analysis would yield a constant V_{max} but increased K_m values when the inhibitor is present in the reaction. Although it is not necessary to understand the mechanism of an invention, kinetic analysis surprisingly showed that the addition of Comp-A caused a drastic decrease of V_{max} values in a Comp-A concentration-dependent manner, whereas the change of K_m values was modest (Figure 7A panel i and Table 1). Such concentration-dependent decrease of V_{max} and the relative constant K_m values were also confirmed by the double reciprocal analysis (Figure 7A, panel ii). Therefore, Comp-A is not a competitive inhibitor of caspase-7; but rather, Comp-A can decrease the V_{max} of caspase-7, and has minor effect on binding of caspase-7 with its substrates; thus Comp-A is a non-competitive inhibitor.

The mechanism of inhibition type was investigated in greater detail using the specific velocity plot (Baici, 1981 [61]). This method offers many advantages for the analysis of non-tightbinding, reversible inhibitors, and has been previously used to elucidate the mechanism of a caspase-2 inhibitor (Schweizer et al., 2007 [62]). The specific velocity (defined under Experimental Procedures) was plotted versus the ratio V_0/V_i at different concentrations of Comp-A to obtain a series of linear curves parallel to the abscissa (Figure 7A, panel iii), indicating no effect of the compound on substrate binding. Using the replots of the specific velocity plots (Figure 7A, panel iv), the values of α and β and an approximate value of K_i were calculated graphically (Table 2). For caspase-7 and Comp-A, $\alpha = 1.01$ and $\beta = 0.06$, and $K_i = 0.34 \mu\text{M}$. These values suggest pure non-competitive inhibition (for which $\alpha = 1$ and $\beta = 0$, theoretically).

Kinetic parameters for other caspases in combination with all four compounds (Figure 8; Table 1, and Table 2) were measured. Consistent with the observation for caspase-7 and Comp-A, caspase-3, caspase-9-LZ, caspase-1, caspase-2, and caspase-8 all showed a decrease in V_{max} and a constant K_m upon the addition of increasing concentrations of each of the four compounds. Specific velocity replots yielded α values close to 1 and β values between 0 and 1. The deviation of β from a value of 0 (indicative of pure non-competitive inhibition) to a value between 0 and 1

is suggestive of partial non-competitive inhibition: a hyperbolic system in which the inhibitor will convert the enzyme into a modified enzyme-substrate-inhibitor complex with a decreased rate of product formation (Baici, 1981 [61]). This partial non-competitive inhibition is especially evident in the β values for caspase-1.

5 In agreement with a non-competitive inhibition mechanism, the compound appears to bind to caspase-7 with similar affinity regardless of the presence of substrate, thus generating the same degree of inhibition (Figure 9).

Kinetic parameters for caspase-9 that is associated with and activated by the apoptosome, and compared it with Caspase-9-LZ, whose activity requires artificial dimerization driven by
10 leucine-zipper were also measured. It is under debate whether the apoptosome activates caspase-9 by triggering its dimerization or by a dimerization-independent, novel conformational change (Jiang and Wang, 2004 [23]; Renatus et al., 2001 [63]). It was reason that a comparison between caspase-9-LZ and apoptosome-caspase-9 could provide insight into the functional conformation of apoptosome-activated caspase-9. The kinetic profiles of Comp-A inhibition of
15 caspase-9-LZ and apoptosome-activated caspase-9 are almost identical (Figure 7B and Figure 8B, Table 1 and Table 2), indicating the compound inhibits the two forms of caspase-9 enzyme with same mechanism. A further analysis (described later) shows that the compounds inhibit caspase activity by interfering with caspase dimerization, this kinetic analysis lends support that caspase-9 dimerization is required for the activity of the apoptosome-caspase-9 holoenzyme, in
20 agreement with a model proposed previously (Renatus et al., 2001 [63]).

8. Crystal Structure of Caspase-7 in Complex with Comp-A

How can these compounds be pan-caspase inhibitors but not function by binding to the conserved active site of caspases? To identify the exact nature of the caspase-compound
25 interaction, crystal structures of Comp-C at 0.81 Å resolution, unliganded caspase-7 at 2.8 Å resolution, and caspase-7 in complex with Comp-A at 3.8 Å resolution (Table 3 and Table 4, Figure 10A and Figure 10B) were determined. Because Comp-A is identical to Comp-C except for the replacement of Br with Cl, a Cl atom was substituted in the crystal structure of Comp-C to generate the structure of Comp-A. The complex crystals contain a caspase-7 dimer per
30 crystallographic asymmetric unit with each monomer consisting of a p20 (large) and a p10 (small) subunit. Because all compounds contain a Cu atom, Cu anomalous difference Fourier to

locate Comp-A molecules in the soaked caspase-7 crystals was used. This yielded two high peaks of 11.5 σ and 7.4 σ , respectively, symmetrical with respect to the two chains of the caspase-7 dimer. These difference Fourier peaks were used as Cu positions to model the Comp-A structure rigidly into the Fo-Fc difference electron density map followed by refinement (Figure 10C).

5 Comp-A is bound to the solvent-exposed dimer interface in an edge-to-edge fashion (Figure 10B, Figure 10D, Figure 10E), and is away from the catalytic active site Cys186. It lays essentially flat on β 5 and β 6 of the central β -sheet at the dimerization interface (Figure 10B, Figure 10E). Comp-A contacts only the p10 subunits, including residues Tyr223 and Cys290 from one subunit and Glu216, Phe221, Tyr223, Val292 and Met294 from the neighboring
10 subunits (Figure 10E). Previously reported allosteric caspase-7 inhibitors DICA and FICA interact with the caspase dimerization interface and form covalent bonds with the thiol of Cys290 (Hardy et al., 2004) [64]. In the crystal structure of caspase-7 in complex with comp-A, the thiols of Cys290 in both monomers of the caspase-7 dimer are too far away from any atom in Comp-A to allow covalent bonding (Figure 10E).

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TABLE 3

Crystallographic Statistics for Comp-C

Empirical formula	C ₁₃ H ₁₂ Br Cu N ₄ S ₂
Formula weight	431.8 Dalton
Temperature	149 K
Wavelength	0.7107 Å
Space group	P-1
Unit cell dimensions	a = 8.6 Å, b = 9.8 Å, c = 9.9 Å $\alpha = 69.2^\circ$, $\beta = 71.5^\circ$, $\gamma = 81.9^\circ$
Z	2
Number of reflections collected	11,391
Independent reflections	2,898
Resolution Range	∞ - 0.81 Å
Completeness to $\theta = 26.00^\circ$	99.2 %
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	2,898 / 0 / 190
Goodness-of-fit on F ²	1.064
Final R, (I > 2 σ (I))	0.1134
Final R, (all data)	0.1456

TABLE 4

Crystallographic Statistics of Unliganded Caspase-7 and its Complex with Comp-A

	Caspase7	Caspase7 with Comp-A
Data Collection		
Space group	P3 ₂ 21	P3 ₂ 21
Cell dimensions (Å)	88.4, 88.4, 185.5	88.7, 88.7, 185.7
Wavelength (Å)	1.000	1.378
Resolution (Å)	37.0 - 2.8	45.0 - 3.8
Rsym (%)	8.3 (46.2)	10.8 (70.0)
<i>I</i> / σ <i>I</i>	30.5 (2.0)	11.9 (2.8)
Completeness (%)	88.7 (46.0)	94.1 (96.0)
Redundancy	3.6 (2.5)	5.1 (5.1)
Refinement		
Resolution (Å)	20.0 - 2.8	20 - 3.8
No. reflections	17,179	8,046
<i>R</i> _{work} / <i>R</i> _{free} (%)	18.7 / 23.1	20.6 / 26.8
No. atoms		
Protein	3,750	2,927
Cu	0	2
R.m.s. Deviations		
Bond lengths (Å)	0.006	0.008
Bond angles (°)	1.323	1.317
Ramachandran plot		
Most favored (%)	95.2	85.3
Allowed (%)	4.8	12.4

* Values in parenthesis are for highest-resolution shell. One crystal was used for each data set.

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9. Comp-A Binding Induces Conformational Changes and Disordering of Active Site Loops

The substrate binding groove of caspase-7 is composed of flexible surface loops that include L1 (residues 75-89, between β 1 and α 1), L2 (residues 185-196, after β 4), L3 (residues 224-232, between β 5 and α 4), and L4 (272-289) from one chain (A), and L2' (residues 212-218) from the other chain (B), among which L2 harbors the catalytic Cys186 in caspase-7 (Chai et al., 2001; Riedl et al., 2001) [65, 66]. While L3 and L4 form the base and one side of the catalytic groove, respectively, the interaction between L4, L2 and L2' is important for maintaining a stable active conformation for substrate binding and catalysis. There are two unliganded caspase-7 structures in the Protein Data Bank (PDB) (www.pdb.org) (accession codes 1IBF and 1K86) (Agniswamy et al., 2009; Chai et al., 2001) [65, 67]. The unliganded structure of the present

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invention is highly similar to the 1IBF structure; both structures are essentially in a catalytically productive conformation shown by the resemblance to the DEVD-CHO-bound conformation (accession code 1F1J) (Figure 11), but is different from the 1K86 structure, which has a reversed conformation for the L2' loop and an unproductive conformation for the L2 loop. It is worth noting that crystallization condition of the present invention example is also highly similar to that used for the 1IBF structure.

Given that the present invention's unliganded structure essentially assumes an active conformation, it was striking to see that extensive conformational changes and disorder were induced in all loops upon Comp-A binding (Figure 12A). L2 has only two residues with the catalytic Cys186 as the last ordered residue; L3 has a few residues at the beginning and the disorder continues to residue 236; L4 has a few residues each at the beginning and the end of the loop; and L2' is two residues shorter. In one of the monomers of the caspase-7 dimer, even L1, which is usually conserved in conformation in different forms of caspase-7 structures, has only two ordered residues at the beginning of the loop, and a neighboring region from residues 144-155 is also invisible. In the other monomer of the caspase-7 dimer, L1 and its neighboring region are involved in crystal packing and are ordered in the crystal data set. However, the tendency of the region to become disordered upon Comp-A soaking explains deterioration of diffraction quality and the need to compromise soaking conditions to both preserve some diffraction and to allow reasonable occupancy of the compound.

Structural comparison showed that the ordered parts of L2, L3 and L4 of the Comp-A bound caspase-7 are more similar to procaspase-7 structures (accession code 1K88 and 1GQF) (Chai et al., 2001; Riedl et al., 2001) (Figure 12B), with the exception that L2 of Comp-A bound caspase-7 is in an open conformation instead of an inverted conformation as in procaspase-7 structures. This change of conformation upon Comp-A binding is induced by steric clash. In particular, Comp-A is in direct clash with Arg187 of L2 and Thr225, Val226 and Pro227 of L3 in the active conformation (Figure 12C), pushing these residues away to assume a conformation more similar to procaspase-7. L2 and the end of L3 coming from the β 5 strand are both almost 90° away from the conformation in active caspases (Figure 12A). Although the key inducing event in procaspase-7, which is the linkage between L2 and L2', is different from Comp-A binding, the consequence is similar as they both disrupt the highly interdependent conformations of the active site loops. Strikingly, Comp-A atoms that are in clash with active caspase-7

conformation are all from the common core of the compound scaffold (Figure 12D), which in turn supports that all compounds would interact with caspase-7 in a manner similar to the interaction between caspase-7 and Comp-A observed here.

Analysis of the previously reported caspase-7 structures inhibited by DICA and FICA (1SHJ and 1SHL) (Hardy et al., 2004) [64] revealed a highly similar pattern of clashes induced by inhibitor binding. DICA clashes with Arg187 at the L2 loop, and Tyr223 and Pro227 of the L3 loop (Figure 12E). FICA clashes with exactly the same residues, except that one FICA molecule clashes with Pro227 of the monomer it is bound to, and with Arg187 and Tyr223 of the neighboring monomer. Similar to Comp-A inhibited structure, distortion of the L2 and L3 loops by inhibitor binding leads to massive conformational changes and disordering of the active site loops (Figure 12F). In DICA and FICA-inhibited caspase-7 structures, L2' is inverted towards the dimerization interface and interacts with both DICA and FICA directly. In any case, while DICA and FICA are covalent inhibitors that target Cys290, and Comp-A noncovalently interacts with the region of Cys290 at the dimerization interface, they all cause clashing with an active caspase conformation and therefore distortion of the active site.

10. Inhibition of Caspase-7 by Comp-A is Reversible

Although it is not necessary to understand the mechanism of an invention, it is believed that because the dimerization interface is a common structural feature of all caspases, inhibitors binding to this site, such as those identified in this study, can act as pan-caspase inhibitors. On the other hand, alignment of the primary amino acid sequences that construct the dimerization interface of individual caspases does not yield obvious conserved residues across the board, although this region is highly conserved for vertebrate caspase-3 and caspase-7 (Figure 13). Consistently, mutagenesis of residues at the caspase-7 dimerization interface that are proximal to Comp-A (Figure 10E and Table 5) indicates that only some of them are involved in inhibition by Comp-A. Notably, some of the mutations yield inactive caspase, indicating that proper dimerization is essential for caspase activity (Table 5). Of those mutants that yielded functional recombinant caspase-7, F221W, C290T/R, and V292Q/E/R showed a significant lower sensitivity to Comp-A inhibition when compared to wild-type caspase-7 (Figure 14A and Table 5), indicating that the interactions of these residues with Comp-A contribute to the inhibitory function of Comp-A.

TABLE 5

Caspase-7 Mutagenesis

Caspase-7 Mutant	Caspase Activity ^a	Inhibition by Comp-A ^b	Caspase-7 Mutant	Caspase Activity ^a	Inhibition by Comp-A ^b
WT	+	1.0	C290A	-	N/A
E216A	*	N/A	C290M	*	N/A
E216L	+	1.1	C290F	-	N/A
F221A	+	1.0	C290N	*	N/A
F221L	+	1.0	C290R	+	0.1
F221Q	-	N/A	C290T	+	0.2
F221R	*	N/A	V292I	+	1.1
F221W	+	0.3	V292E	+	0.3
Y223A	-	N/A	V292L	+	1.0
Y223D	*	N/A	V292Q	+	0.3
Y223E	-	N/A	V292R	+	0.3
Y223R	-	N/A	V292W	-	N/A
Y223T	-	N/A	M294A	+	1.0
Y223W	+	0.9	M294F	-	N/A
P227A	+	1.0	M294R	-	N/A
P227R	+	0.9	M294W	-	N/A

^a Caspase activity was measured using 100 nM caspase-7 in the presence of the fluorogenic DEVD substrate, as described under Experimental Procedures

^b Inhibition by Comp-A of mutant caspase-7 is expressed relative to that of wild-type caspase-7 in the presence of 5 μ M Comp-A. The mean of three experiments is shown. The mutations that significantly diminished the inhibitory effect of Comp-A are in bold

*Caspase-7 recombinant protein was not in the soluble fraction during purification
N/A, not applicable

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The Cys290 residue of caspase-7 is of particular interest. It is conserved in mammalian caspase-7 and caspase-3 (Figure 13) and is in close contact with Comp-A in the co-crystal structure (Figure 10E). Caspase-1 also contains a cysteine at the dimer interface, Cys331, which is structurally positioned on a different β -strand but is nonetheless located in a position close to Cys290 of caspase-7 (Scheer et al., 2006) [68]. Likewise, caspase-2 contains a cysteine at the dimer interface, Cys390, which coordinates the interaction between the two dimers (Schweizer et al., 2003) [69]. Although caspase-8 and caspase-9 do not appear to have a cysteine residue at the dimer interface, dimerization is nevertheless essential for the activation of these initiator caspases which exist primarily as monomers in solution (Acehan et al., 2002; Boatright et al., 2003) [70, 71].

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Although it is not necessary to understand the mechanism of an invention, it is believed

that the presence of a cysteine residue in the dimerization interface of multiple caspases suggests that covalent modification of this residue might be inhibitory. Indeed, previously reported caspase-7 inhibitors DICA and FICA interact with caspase-7 at the dimerization interface, and their disulfide bonding with the Cys290 residue is required for their inhibitory activity (Hardy et al., 2004; Hardy and Wells, 2009) [64, 72]. On the other hand, because Cys290 is not conserved among all caspases, DICA and FICA can only inhibit caspase-7 and caspase-3. Unlike DICA and FICA, Comp-A contacts Cys290 in a noncovalent manner, thus mutating the Cys290 residue did not fully ablate the ability of Comp-A to inhibit caspase activity (Figure 14A). However, both C290T and C290R mutants show significantly decreased inhibition by Comp-A compared to WT caspase-7, indicating that Cys290, while not functioning to covalently interact with Comp-A, is able to confer a certain degree of selectivity for inhibitor binding to caspases. Further, inhibition by Comp-A can be reversed upon dialysis (Figure 14B), demonstrating a noncovalent, reversible inhibitory mechanism.

11. Inhibition of Caspase-8 Dimerization by Comp-A

Because the dimerization interfaces of caspases are not generally similar to each other at the level of primary amino acid sequence, is the interference of proper caspase dimerization a common mechanism for these compounds to inhibit individual caspases? To test this possibility, recombinant caspase-8 was subjected to gel filtration analysis. Active recombinant caspase-8 is a dimer, which is in equilibrium with inactive monomer composed of only one copy of large and small subunits. The caspase-8 dimer and monomer can be resolved as two distinct peaks by gel filtration, with enzymatic activity present only in the earlier, dimeric peak (Figure 14C). Using this method, it was found that Comp-A caused diminishment of dimerized caspase-8, yielding only the smaller, monomeric species, with enzymatic activity completely abrogated. In contrast, incubation of caspase-8 with the substrate-mimicking inhibitor Ac-VAD-CHO resulted in a shift to the dimeric species, as the enzyme is “locked” in the active conformation upon inhibitor binding (Figure 14C). The disappearance of the dimer peak in the presence of Comp-A is indicative of the compound not only preventing de novo caspase dimer formation, but also dismantling caspase dimers that are already present.

While Comp-A can disrupt the functional dimerization conformation of both caspase-7 and caspase-8, Comp-A-associated caspase-7 can still maintain dimerization, albeit in a distorted,

non-productive conformation. However, Comp-A binding effectively disrupted the physical dimerization of caspase-8. This difference might reflect the dissimilarity of the dimerization interfaces of the two caspases at the level of primary sequence.

5 12. Discussion

Targeting the intrinsic apoptotic pathway presents an attractive approach for attenuating several pathological conditions in which apoptosis is upregulated, such as neurodegeneration, stroke, and radiation syndrome. Using an *in vitro* reconstituted caspase activation assay and a high-throughput screening approach, four pan-caspase inhibitors that share a similar chemical scaffold were identified and are distinct from the commonly used peptide inhibitors. A crystal structure of caspase-7 in complex with one of the inhibitors, Comp-A, revealed that the interaction between caspase and the inhibitor occurs at an allosteric site, the dimerization interface of the active caspase. Active caspase-7 consists of two molecules of cleaved caspase, and the dimerization interface resembles a deep cavity between the two dimerizing caspases; importantly, similar cavity exists in all caspases and is required for caspase activity (Hardy et al., 2004 [64]). Residues present within the cavity mediate the dimerization interaction and direct the orientation of the L2 loop which is critical for substrate binding. Therefore, inhibitors binding within the conserved cavity can abolish the ability of these residues to support active site catalysis. To further support this mechanism, a biochemical analysis demonstrates that Comp-A can disrupt caspase-8 dimerization.

Although it is not necessary to understand the mechanism of an invention, it is believed that mechanistically, binding of Comp-A to the dimerization interface of caspase-7 is somewhat reminiscent of the interaction of DICA and FICA with caspase-7 (Hardy *et al.*, 2004; Hardy and Wells, 2009) [64, 72]. However, unlike Comp-A that is a reversible inhibitor, DICA and FICA are irreversible inhibitors that covalently modify a cysteine residue located within the cavity of caspase-7 and caspase-3 (Hardy *et al.*, 2004; Scheer *et al.*, 2006) [64, 68]. Although it is not necessary to understand the mechanism of an invention, it is believed that because this cysteine residue is not conserved in other caspases, DICA and FICA can only inhibit caspase-3 and caspase-7, while the four compounds identified in this study are pan-caspase inhibitors. Further, the irreversible nature of DICA and FICA and lack of evidence that they can function in cells limited their potential as pharmacological tools or therapeutic leads. In contrast, the inhibitors

identified in this study are reversible caspase inhibitors and can also inhibit cellular apoptosis at submicromolar levels. Further, the irreversible nature of DICA and FICA and lack of evidence that they can function in cells limited their potential as pharmacological tools or therapeutic leads. In contrast, the inhibitors identified herein are reversible caspase inhibitors and can also inhibit cellular caspase activation at sub-micromolar levels, thus warranting further exploration.

Importantly, the compounds identified studied herein are also able to inhibit the cellular activation of caspase-1. Caspase-1 is a prominent member of the pro-inflammatory class of caspases, and is responsible for the proteolytic activation of IL-1 β and IL-18. IL-1 β and IL-18 are cytokines that play a major role in the immune response and within numerous autoimmune and inflammatory diseases (Braddock et al., 2004 [73]). Therefore, inhibitors of caspase-1, such as these newly-identified compounds, could potentially be used as intervention strategies for inflammation disorders such as rheumatoid arthritis and inflammatory bowel disease.

Strikingly, all four compounds identified here contain a transition metal atom, copper, in their molecular structures. Although excess copper is known to be toxic in mammalian tissues, the general consensus is that free copper ions, rather than complexed copper, is the major contributor to this cytotoxicity (Nor, 1987) [74]. In fact, complexed copper is present in a variety of native biological enzymes, including cytochrome c oxidase and copper nitrite reductase (MacPherson and Murphy, 2007) [75]. Therefore, it is likely that contaminating free copper ion in these complexes is what causes cell toxicity. Further, although the presence of a transition metal is a concern for the potential therapeutic application of the future derivatives of this group of compounds, there are precedents of transition metal compounds being used therapeutically. For example, cisplatin, a widely-used anticancer agent, contains at its core a transition platinum atom. Cisplatin and its derivatives function to bind to and crosslink DNA, ultimately triggering apoptosis in cancer cells. Other metal complexes, including ruthenium, titanium and gallium, have also been explored for their therapeutic properties, and are currently under clinical trials (Hannon, 2007) [76].

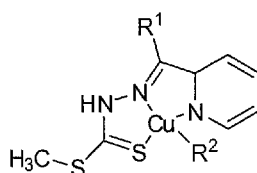
It should be noted that these compounds, when used at higher concentrations, possess cellular toxicity and can cause detachment of cultured adherent cells. Whether such toxicity is caused by the presence of copper is not clear. Further medicinal chemistry is needed to reduce the toxicity.

In order to develop derivative compounds that possess higher efficacy and lower toxicity,

the co-crystal structure of caspase-7 in complex with Comp-A may be used as guidance for chemical modification of these compounds. Although the dimerization interface is a common and essential structural moiety shared by all caspases, primary sequence homology among individual caspases at this region is limited. This property presents a unique opportunity: it is possible to develop derivative inhibitors with a certain degree of specificity toward each caspase by conducting chemical modification based on high resolution structures of the dimer interface of each caspase.

In one embodiment, the small molecule inhibitors of apoptosis, such as Compounds A-D and those of Formula I, might be developed into therapeutic agents for treating relevant human diseases. In one embodiment, the common structural features shared by these four compounds and the crystal structure of Comp-A in complex with caspase-7 provide insights and guidance for their future medicinal chemistry, which hopefully will lead to development of a more potent and less toxic inhibitor that can be used therapeutically.

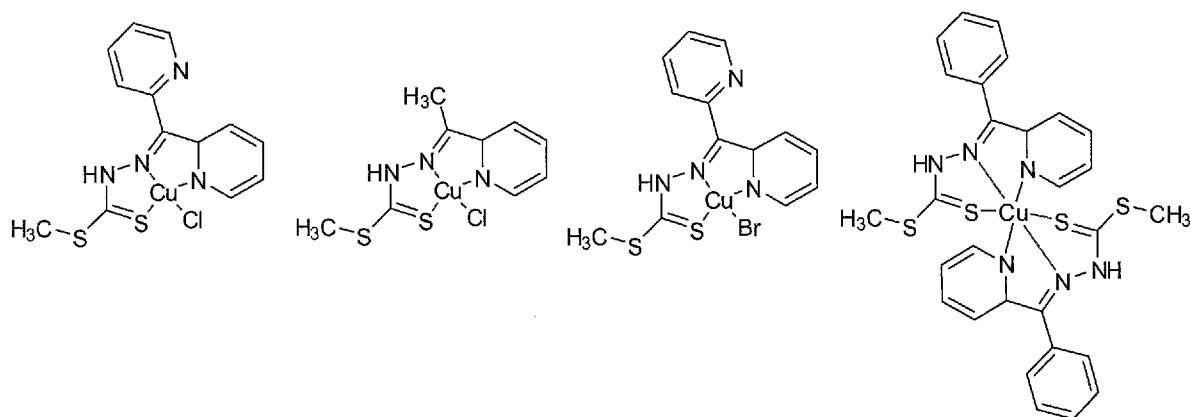
In one embodiment, the invention contemplates a method comprising; a) providing; i) an allosteric pan-caspase inhibitor of Formula I; ii) a subject suffering from a disease state, wherein said disease has at least one symptom, wherein said subject comprises a caspase; and b) treating said subject with said allosteric pan-caspase inhibitor of Formula I so as to reduce at least one symptom of said disease state. In one embodiment, said allosteric pan-caspase inhibitor of Formula I comprises:



wherein R_1 is CH_3 (Me), $-\text{CH}_2\text{CH}_3$ (Et), $-\text{CH}_2\text{CH}_2\text{CH}_3$ (*n*-Pr), $-\text{CH}(\text{CH}_3)_2$ (*iso*-Pr), $-\text{CH}(\text{CH}_2)_2$ (cyclopropyl), $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ (*n*-Bu), $-\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$ (*sec*-butyl), $-\text{CH}_2\text{CH}(\text{CH}_3)_2$ (*iso*-butyl), $-\text{C}(\text{CH}_3)_3$ (*tert*-butyl), $-\text{CH}_2\text{C}(\text{CH}_3)_3$ (*neo*-pentyl), cyclobutyl, cyclopentyl, cyclohexyl, cyclohexylmethyl, adamant-1-yl, adamant-2-yl, phenyl, and substituted phenyl; R_2 is at least one of the following groups comprising: Cl, Br, F, I, and (E)-methyl 2-((1,2-dihydropyridin-2-yl)(phenyl)methylene)hydrazinecarbodithioate. In one embodiment, said subject has a symptom of cell death. In one embodiment, symptom of cell death is mediated by an intrinsic pathway of apoptosis. In one embodiment, said allosteric pan-caspase inhibitor of Formula I provides protection from cell death. In one embodiment, said subject further

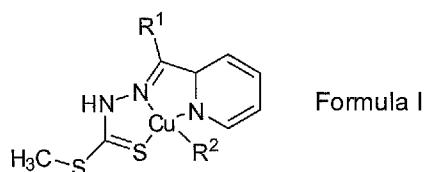
comprises a cell containing a caspase, and a step of isolating said cell from said subject that is capable of undergoing apoptosis induced by ultra violet light. In one embodiment, said disease state results from exposure to at least one of the group comprising: tumor necrosis factor- α (TNF α) and cycloheximide. In one embodiment, said allosteric pan-caspase inhibitor of Formula

5 I is selected from the group consisting of:



In one embodiment, said disease state is selected from the group consisting of neurodegeneration, radiation syndrome, ultra violet light exposure, osteoarthritis, pancreatitis, rheumatoid arthritis, chronic active hepatitis, inflammatory bowel disease, Crohn's disease, psoriasis, organ transplant rejection, sepsis, septic shock, cerebral ischemia, myocardial ischemia, myocardial infarction, amyotrophic lateral sclerosis, multiple sclerosis, neurological damage due to stroke, hepatitis-B, hepatitis-C, hepatitis-G, and liver disease.

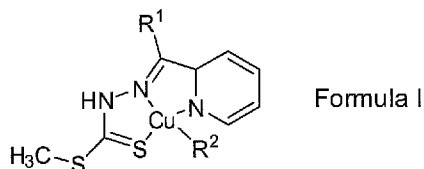
In one embodiment, the invention contemplates a pharmaceutical composition of Formula I:



wherein R₁ is CH₃ (Me), -CH₂CH₃ (Et), -CH₂CH₂CH₃ (*n*-Pr), -CH(CH₃)₂ (*iso*-Pr), -CH(CH₂)₂ (cyclopropyl), -CH₂CH₂CH₂CH₃ (*n*-Bu), -CH(CH₃)CH₂CH₃ (*sec*-butyl), -CH₂CH(CH₃)₂ (*iso*-butyl), -C(CH₃)₃ (*tert*-butyl), -CH₂C(CH₃)₃ (*neo*-pentyl), cyclobutyl, cyclopentyl, cyclohexyl, cyclohexylmethyl, adamant-1-yl, adamant-2-yl, phenyl, and substituted phenyl; R₂ is at least one of the following groups comprising: Cl, Br, F, I, and (E)-methyl 2-((1,2-dihydropyridin-2-yl)(phenyl)methylene)hydrazinecarbodithioate.

This invention further provides pharmaceutical compositions comprising caspase inhibitor compound according to Formula I. This invention also relates to methods of using said pharmaceutical compositions for treatment of caspase-mediated diseases including inflammatory and degenerative diseases.

In one embodiment, the invention contemplates an allosteric pan-caspase inhibitor of Formula I:



wherein R_1 is CH_3 (Me), $-\text{CH}_2\text{CH}_3$ (Et), $-\text{CH}_2\text{CH}_2\text{CH}_3$ (*n*-Pr), $-\text{CH}(\text{CH}_3)_2$ (*iso*-Pr), $-\text{CH}(\text{CH}_2)_2$ (cyclopropyl), $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ (*n*-Bu), $-\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$ (*sec*-butyl), $-\text{CH}_2\text{CH}(\text{CH}_3)_2$ (*iso*-butyl), $-\text{C}(\text{CH}_3)_3$ (*tert*-butyl), $-\text{CH}_2\text{C}(\text{CH}_3)_3$ (*neo*-pentyl), cyclobutyl, cyclopentyl, cyclohexyl, cyclohexylmethyl, adamant-1-yl, adamant-2-yl, phenyl, and substituted phenyl; R_2 is at least one of the following groups comprising: Cl, Br, F, I, and (E)-methyl 2-((1,2-dihydropyridin-2-yl)(phenyl)methylene)hydrazinecarbodithioate.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

Experimental Procedures

Reagents

dATP, TNF- α , and cycloheximide were purchased from Sigma. Caspase substrates DEVD-Rhodamine110, DEVD-AFC, LEHD-AFC and YVAD-AFC were purchased from Anaspec. Trypsin and papain were purchased from Sigma; Cathepsin C was purchased from R&D. Trypsin/papain substrate BA-AMC was purchased from Sigma. Cathepsin C substrate GR-AMC was purchased from MP Biomedicals. Caspase inhibitors DEVD-CHO and Ac-VADCHO were purchased from Calbiochem. Rhodamine110 standard was purchased from

Marker Gene.

EXAMPLE 1

RECOMBINANT PROTEINS

For recombinant full-length Apaf-1 purification, Hi-Five cells (Invitrogen) were infected with baculovirus expressing Apaf-1 (Jiang and Wang, 2000; Zou *et al.*, 1999)[32, 33]. Cells were collected 48 hours after infection and resuspended in buffer T (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 2 mM β -mercaptoethanol) supplemented with protease inhibitors. The cell suspension was homogenized in a B-type douncer with 25 strokes twice and centrifuged at 20000 g for 20 minutes. The supernatant was loaded to a Nickel-NTA (nitriloacetate) resin (Novagen) equilibrated with buffer T. The resin was washed with buffer T, followed by buffer T supplemented with 1 M NaCl, and then followed by buffer T. Recombinant protein was eluted using buffer T containing 250 mM imidazole, then diluted with buffer A (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) and further purified using FPLC (fast protein liquid chromatography) on HiTrap Q column (GE Healthcare) equilibrated with buffer A. The column was eluted with a 15-mL linear gradient from 150 mM to 450 mM KCl in buffer A. Aliquots were flash-frozen and stored at -80°C.

For procaspase-3 purification, Hi-Five cells were infected with baculovirus expressing procaspase-3 (C-terminal 6xHis tag). Cells were collected 24 hours after infection and purified using Nickel-NTA resin as described above for Apaf-1. The eluted protein was diluted with buffer A and loaded on a HiTrap Q column equilibrated with buffer A. The column was eluted with a 15-mL linear gradient from 50 mM to 350 mM KCl in buffer A. Aliquots were flash frozen and stored at -80°C.

Caspase-9 was expressed with pET28-Caspase-9 (N-terminal 6xHis tag) in *Escherichia coli* BL21(DE3) (Novagen) induced with 0.3 mM IPTG for 4 hours at 30°C after the culture reached OD₆₀₀ of 0.6–0.8. The cells were collected, resuspended in buffer T supplemented with protease inhibitors, lysed by sonication, and centrifuged at 20000 g for 20 minutes. The supernatant was loaded to Nickel-NTA resin, washed and eluted as described above for full length Apaf-1. The eluted protein was dialyzed in buffer A, and aliquots were flash-frozen and stored at -80°C. Caspase-1, caspase-2, caspase-3, and caspase-8 were expressed with pET28 with an N-terminal 6xHis tag under the same conditions as caspase-9. Purified horse cytochrome c was prepared as described previously (Liu *et al.*, 1996) [77].

EXAMPLE 2

Cytochrome c–Mediated Caspase Activation Assay

A fluorogenic assay was used to measure cytochrome c–mediated caspase-3 activation *in vitro*. Recombinant procaspase-3 (50 nM) and caspase-9 (20 nM) were mixed with Apaf-1 (5 nM), cytochrome c (0.5 μ M) and the nucleotide dATP (10 μ M) in buffer ASC (buffer A supplemented with 20 mM β -mercaptoethanol, 5% w/v sucrose, 0.1% w/v CHAPS, and 1 mg/mL BSA) in a final volume of 20 μ L. The conversion of a fluorogenic caspase-3 Rhodamine-DEVD substrate (15 μ M) was measured at 30°C by SpectraFluor Plus Spectrometry Reader (Tecan) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

EXAMPLE 3

Primary Screening and Compound Identification

Compounds or high/low controls were pre-plated in 1536-well microtiter plates (#3724, Corning) in a volume of 1 μ L using the automated pipetting system TPS-384 (Total Pipetting Solution; Apricot Designs). Compounds were tested at a final concentration of 10 μ M in 1% DMSO (v/v). Control wells were present on each plate to assess the statistical performance of the assay, as well as to calculate the percentage inhibition induced by each compound; low controls consisted of 2 μ M DEVD-CHO in 1% DMSO (v/v) (final concentration) and high controls consisted of 1% DMSO (v/v) (final concentration). The automated sequential reagent addition on the robotic platform was coordinated by the Polara software (Thermo CRS) to ensure consistent incubation times across the 238 assay plates. Assay plates were stored at room temperature in the Ambi automated incubators (Thermo Scientific) between reagent additions. Four microliters of Mix A, containing Apaf-1, dATP, and procaspase-3 in buffer ASC were added to each well using the Flexdrop precision reagent dispensers (Perkin Elmer). After a 15-minute pre-incubation period at room temperature, five microliters of Mix B, containing caspase-9, cytochrome c and the Rhodamine-DEVD substrate in buffer ASC were added to each well using Flexdrop. The fluorescence signal of the converted Rhodamine-DEVD substrate was read using the CCD-based LEADseeker Multimodality Imaging System (GE Healthcare) equipped with fluorescein excitation/emission filters and FLINT epi-mirror. Screening data files were loaded onto the ORIS HTS Core Screening Data Management System, a custom built suite of modules for

compound registration, plating, and data management powered by ChemAxon Cheminformatic tools (ChemAxon). ORIS handled data processing, allowing automated and unbiased control analysis and identification of initial positive hits. The statistical performance of the assay during screening was assessed by calculating the Z' factor based on the data for control wells present on each plate, as previously described (Antczak et al., 2007 [78]; Seideman et al., 2010 [79]; Shelton et al., 2009 [80]; Shum et al., 2008 [81]).

Chemical libraries – The Sloan Kettering Institute (SKI) corporate compound library collection was comprised of 317,856 natural and synthetic compounds from various sources including Analyticon Discovery (Potsdam, Germany), BioFocus (Saffron Walden, UK), ChemBridge (San Francisco, CA), and SPECS (Delft, The Netherlands) (Somwar *et al.*, 2009) [82].

Criteria for identification of initial positives – The percentage inhibition (% I) for each tested compound was calculated based on the average (AVG) of the high and low controls present on each plate, according to: $\% I = (\text{AVG high controls} - X) / (\text{AVG high controls} - \text{AVG low controls}) \times 100$. Compounds inducing greater than 30% inhibition in the assay were selected as initial positives for further studies.

Quench test to rule out fluorescence interference – To confirm that the decrease in fluorescence intensity induced by positive compounds was not due to direct quenching of the fluorescent substrate, free Rhodamine110 (5 μM in 1% DMSO (v/v)) was incubated with either DMSO (1% v/v) or compound (10 μM in 1% DMSO (v/v)) in buffer ASC in a final volume of 20 μL . Fluorescence was measured at 30°C with an excitation wavelength of 485 nm and an emission wavelength of 535 nm at an interval of 5 minutes over the course of three hours.

Immunoblotting to validate primary screen results – To confirm that the compounds that diminished fluorescence could alter caspase activity, caspase activation was assayed by the cleavage of Bid in an *in vitro* caspase reconstitution assay. 200 ng of recombinant Bid were combined with recombinant procaspase-3 (50 nM), caspase-9 (20 nM), Apaf-1 (5 nM), cytochrome c (0.5 μM) and the nucleotide dATP (10 μM) in buffer ASC in the presence or absence of compound (10 μM in 1% v/v DMSO) in a final volume of 20 μL . The reaction mix was incubated at 30°C for 1 hr and subjected to SDS-PAGE and Western blotting analysis using an anti-Bid antibody.

EXAMPLE 4

Caspase Activity Measurements

Caspase activity of recombinant caspases was measured by turnover of a fluorogenic tetrapeptide substrate. Indicated amounts of recombinant caspase were combined with the appropriate fluorogenic substrate (Rhodamine-DEVD for caspase-3, -7, -2, and -8; AFC-LEHD for caspase-9; AFC-YVAD for caspase-1) in the presence of DMSO or compound in a volume of 20 μ l in buffer ASC. Fluorescence was measured at 30°C by SpectraFluor Plus Spectrometry Reader with excitation/emission wavelengths of 485/535 nm for Rhodamine-DEVD and 400/505 nm for AFC-LEHD and AFC-YVAD. Reactions were monitored at an interval of 30 s. Reaction rates were calculated as ratios of relative fluorescence (RFU) over time during the initial 15 minutes of the reaction.

EXAMPLE 5

Apoptosis in Mammalian Cells

The inhibitory effect of compounds in cell-based models of apoptosis was evaluated in HeLa cells, U937 cells, and in mouse embryonic fibroblasts (MEFs). For HeLa and MEF cells, apoptosis was induced by irradiation with UV light (2000 J/m²; Stratagene UV Stratalinker 1800) in the presence or absence of compounds. Floating and attached cells were collected 6 hrs after irradiation and washed with PBS. To quantify caspase activity in cell lysates, thirty micrograms of total protein were incubated with 15 μ M fluorogenic caspase-3 Rhodamine-DEVD substrate in a final volume of 50 μ L, and fluorescence measurements were taken at an interval of 2 min for 3 hours using SpectraFluor Plus Spectrometry Reader (Tecan) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. For U937 cells, apoptosis was induced by the addition of TNF (10 ng/mL) and cycloheximide (10 μ g/mL) in the presence or absence of compounds, as indicated. Cells were collected after 1.5 hrs and washed with PBS. Apoptosis was measured by Annexin V and Propidium Iodide staining and cellular caspase activity assay.

EXAMPLE 6

ELISA for Interleukin-1 β

The levels of IL-1 β of culture supernates of J774 cells were determined by ELISA using a commercially available DuoSet ELISA Development kit (R&D). The assay was performed according to the instruction manual provided by the manufacturer. All samples and standards were measured in triplicate.

EXAMPLE 7

Evaluation of Cell Viability

Cell viability was measured using Resazurin (R&D), a redox-sensitive dye. Resazurin changes from a blue, non-fluorescent state to a pink, highly-fluorescent state upon its reduction to resorufin. Resazurin is converted to its reduced state by viable cells by an unknown mechanism. Cell viability and number are proportional to the value of fluorescence, measured with an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Cells were plated in triplicate in 96-well plates (Becton Dickinson) at the same cell density in a volume of 100 μ l. Viable cells were detected by adding 15 μ l of Resazurin dye, and measuring fluorescence using SpectraFluor Plus Spectrometry Reader (Tecan) after 3 hours.

EXAMPLE 8

Caspase-7 Expression and Purification

Caspase-7 was expressed with pET28-Caspase-7 (N-terminal 6xHis tag) in *Escherichia coli* BL21(DE3) induced with 0.3 mM IPTG for 4 hours at 30°C after the culture reached OD600 of 0.6. The cells were collected, resuspended in buffer T supplemented with protease inhibitors, lysed by sonication, and centrifuged at 20000 g for 20 minutes. The supernatant was loaded to a Nickel-NTA resin equilibrated with buffer T. The resin was washed with buffer T, followed by buffer T supplemented with 1 M NaCl, and then followed by buffer T. Recombinant protein was eluted using buffer T containing 250 mM imidazole, then diluted with buffer A and further purified using FPLC on HiTrap Q column (GE Healthcare) equilibrated with buffer A. The column was eluted with a 15-mL linear gradient from 50 mM to 350 mM KCl in buffer A. Fractions containing caspase-7 were pooled and further purified using Superdex200 gel filtration column equilibrated with buffer G (20 mM Tris, pH 7.5; 150 mM KCl; 10 mM DTT).

EXAMPLE 9

Caspase-7 Mutagenesis

Caspase-7 mutants were generated by site-directed mutagenesis of the wild-type construct pET28-Caspase-7. All mutations were confirmed by sequencing. Mutant caspase-7 was expressed and purified as wild-type caspase-7.

EXAMPLE 10

Enzyme Kinetic Assays

Initial reaction rates of substrate conversion by caspase-7 and caspase-3 were measured using a fluorogenic AFC-DEVD substrate. The concentration of recombinant active caspase-7 and caspase-3 was kept constant at 20 nM and 5 nM, respectively. Recombinant caspase was incubated in buffer ASC in the presence of DMSO or Comp-A for 15 minutes on ice before the addition of substrate at various concentrations. Substrate conversion was measured at 30°C by SpectraFluor Plus Spectrometry Reader with excitation/emission wavelengths of 400/505 nm. Reactions were monitored at an interval of 30 s. Reaction rates were calculated as ratios of relative fluorescence (RFU) over time during the initial 15 minutes of the reaction. Curves and kinetic values representing nonlinear fitting of the data to the Michaelis-Menten equation were generated using Prism software (GraphPad).

EXAMPLE 11

Crystallization and Structure Determination of Comp-C

Crystals of Comp-C were obtained using the hanging-drop vapor diffusion method by mixing and equilibrating 1 μ l of 10 mM Comp-C solution and a mother-liquor solution (2.5 M NaCl and 100 mM imidazole at pH 8.0). Under this condition, crystals appeared after 2 days and grew to maximum sizes within 7 days. A crystal suitable for X-ray diffraction was mounted on a Bruker SMART diffractometer equipped with a graphite monochromated Mo K α ($\lambda = 0.71073$ Å) radiation source and a CCD detector. Two-dimensional diffraction images were collected and processed to give structure factors by the program SAINT (Sheldrick, 2006) [83]. The structure was solved by a combination of direct method and difference Fourier method provided by the

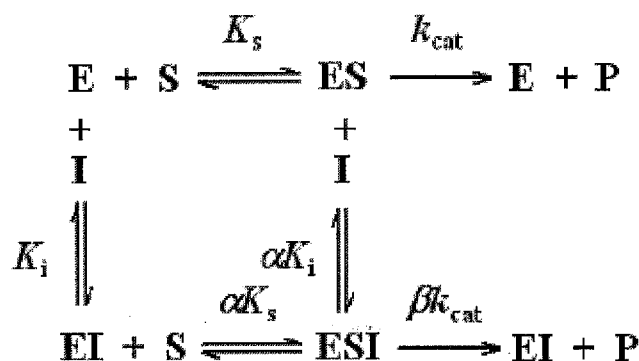
program package SHELXTL (Sheldrick, 1998) [84] and refined using full matrix least square against F^2 for all data. All the non-H atoms were refined anisotropically. All hydrogen atoms were included in calculated positions with isotropic thermal parameters 1.2 times those of attached atoms. Crystallographic statistics are summarized in Table 3.

5 Initial reaction rates of substrate conversion by caspases were measured using their respective fluorogenic substrates. The concentration of recombinant active were kept constant as follows: caspase-7, 20 nM; caspase-3, 5 nM; caspase-9 (LZ), 200 nM; caspase-1, 100 nM; caspase-2, 200 nM; caspase-8, 200 nM. For caspase-9 kinetic measurements in the presence of the components of the apoptosome, 20 nM of recombinant caspase-9 were used, in combination
10 with 5 nM Apaf-1, 0.5 μ M cytochrome c, and 10 μ M dATP. . Recombinant caspase was incubated in buffer ASC in the presence of DMSO or Comp-A for 15 minutes on ice before the addition of substrate at various concentrations. Substrate conversion was measured at 30°C by SpectraFluor Plus Spectrometry Reader with excitation/emission wavelengths of 400/505 nm. Reactions were monitored at an interval of 30 s. Reaction rates were calculated as ratios of
15 relative fluorescence (RFU) over time during the initial 15 minutes of the reaction.

Curves representing nonlinear fitting of the data to the Michaelis-Menten equation $V = V_{\max}K_m/(K_m + [S])$ were constructed. Kinetic values for $V_{\max}$ and K_m were generated using Prism software (GraphPad).

Double-reciprocal plots (Lineweaver-Burk) were constructed to provide an initial
20 analysis of the mechanism of inhibition. The reciprocal of the initial reaction rate ($1/V_0$) was plotted as a function of the reciprocal of substrate concentration ($1/[S]$). Lines representing a linear least-squares fitting of the data were generated using Prism software (GraphPad). Kinetic parameters were estimated by observing the intercepts on the ordinate axis ($1/V_{\max}$) and on the abscissa ($-1/K_m$).

25 Further kinetic analysis was performed using the specific velocity plot, a graphical method suitable for estimating linear and hyperbolic inhibition mechanisms. (Baici, 1981 [61]). Data obtained from inhibition studies of Comp-A, B, C, and D on caspase activity were fit to the following general scheme for enzyme-catalyzed reactions.



E = enzyme; S = substrate; I = inhibitor; P = product; $K_s = [\text{E}][\text{S}]/[\text{ES}]$, the substrate dissociation constant; $K_i = [\text{E}][\text{I}]/[\text{EI}]$, the competitive inhibitor constant; $\alpha K_i = [\text{ES}][\text{I}]/[\text{ESI}]$, the uncompetitive inhibitor constant; k_{cat} = catalytic constant. The coefficients α and β are dimensionless and characterize the proportion of competitive and uncompetitive character in the inhibition mechanism. The specific velocity equation is as follows:

$$\frac{V_0}{V_i} = \frac{[I] \left(\frac{1}{\alpha K_i} - \frac{1}{K_i} \right)}{1 + \beta \frac{[I]}{\alpha K_i}} \frac{\sigma}{(1 + \sigma)} + \frac{1 + \frac{[I]}{K_i}}{1 + \beta \frac{[I]}{\alpha K_i}} \quad (1)$$

V_0 and V_i are initial velocities in the absence and presence of compounds, respectively. The ratio $\sigma/(1+\sigma)$ is the specific velocity, where $\sigma = [\text{S}]/K_m$. Data collected at different concentrations of compounds and substrate were plotted as V_0/V_i versus $\sigma/(1 + \sigma)$, giving a family of straight lines. The intercepts of the graphically extrapolated lines at abscissa = 0 (intercept a) and abscissa = 1 (intercept b) were replotted as $a/(a - 1)$ and $b/(b - 1)$, respectively, versus $1/[I]$. From these replots, the α and β coefficients and an estimate of K_i were determined. Briefly, Eqn can be rearranged into a reciprocal form, yielding:

$$\frac{a}{a - 1} = \frac{\alpha K_i}{\alpha - \beta} \frac{1}{[I]} + \frac{\alpha}{\alpha - \beta} \quad (2)$$

and

$$\frac{b}{b - 1} = \frac{\alpha K_i}{1 - \beta} \frac{1}{[I]} + \frac{1}{1 - \beta} \quad (3)$$

Plots of Eqn (2) and Eqn (3) are straight lines with an intercept on the ordinate axis corresponding to $\alpha/(\alpha-\beta)$ for Eqn (2) and $1/(1-\beta)$ for Eqn (3), and an intercept on the abscissa

corresponding to $-1/K_i$ for Eqn (2) and $1/\alpha K_i$ for Eqn (3).

EXAMPLE 12

Gel Filtration

5 Gel filtration of caspase-8 was performed on an Amersham Pharmacia Superdex 200 column (25-ml column volume) in 20 mM Tris, 100 mM NaCl (pH 8) buffer with a flow rate of 0.4 ml/min; 0.25 ml fractions were collected for analysis.

EXAMPLE 13

10 Crystallization and Structure Determination of Caspase-7 in Complex with Comp-A

 Crystals of active, unliganded caspase-7 were grown at room temperature using the hanging drop vapor diffusion method from 6-9 mg/mL of caspase-7 with a well solution of 0.1 M sodium citrate buffer at pH 5.0 to 5.7 and 1.9 M sodium formate. Because co-crystallization of caspase-7 and Comp-A was not successful, Comp-A was soaked into unliganded caspase-7
15 crystals. Extensive soaking trials were performed with different concentrations of Comp-A from 0.1 to 1.0 mM at variable time periods of 5, 10, 15, 30, 35, 45, and 60 minutes. All crystals were quickly cryo-protected in 25% glycerol with the mother liquor and flash frozen in liquid nitrogen. Data for the unliganded caspase-7 and the caspase-7 in complex with Comp-A were collected at the Cu edge wavelength at the X4A beam line of BNL and GMCA beam line of
20 APS and processed and scaled using XDS (Kabsch, 1993) [85] and HKL2000 (Otwinowski and Minor, 1997) [86], respectively. The structures were solved using the program PHASER (McCoy *et al.*, 2007) [87] using a native unliganded caspase-7 structure (3IBF) as the search model without the residues 186-196, 212-217 to minimize any bias.

 Because Comp-A contains a Cu atom, presence of Comp-A in the crystals was evaluated
25 using Cu anomalous difference Fourier. Unliganded crystals diffracted to 2.8 Å resolution. However, soaking of Comp-A led to progressive worsening of the diffraction, yielding either low resolution data with significant Cu anomalous difference peaks or better resolution data with insignificant Cu anomalous difference peaks. A final Comp-A concentration of 0.4 mM at 30 minutes yielded a reasonable data set at 3.8 Å resolution. Because Comp-A is identical to
30 Comp-C except for the replacement of Br with Cl, a Cl atom was substituted for the Br atom in the crystal structure of Comp-C to generate the structure of Comp-A. Comp-A was fitted to the

Fo-Fc difference density by matching the Cu atom in the structure with the Cu anomalous difference peak.

Residues omitted in the search model were rebuilt if there were densities for them and the models were subjected to several rounds of model building and refinement using COOT (Emsley and Cowtan, 2004) [88] and CNS (Brunger *et al.*, 1998) [89]. At the final round of refinement
5 water molecules were added to the unliganded caspase-7 and no water was added to the caspase-7- compound-A complex. Crystallographic statistics are shown in Table 3 and Table 4.

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CLAIMS:

We claim:

1. A method comprising:

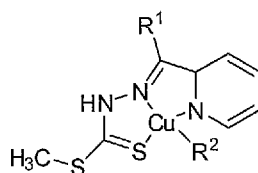
a) providing;

i) an allosteric pan-caspase inhibitor of Formula I;

ii) a subject suffering from a disease state, wherein said disease has at least one symptom, wherein said subject comprises a caspase; and

b) treating said subject with said allosteric pan-caspase inhibitor of Formula I so as to reduce at least one symptom of said disease state.

2. The method of Claim 1, wherein said allosteric pan-caspase inhibitor of Formula I comprises:



wherein R_1 is CH_3 (Me), $-\text{CH}_2\text{CH}_3$ (Et), $-\text{CH}_2\text{CH}_2\text{CH}_3$ (*n*-Pr), $-\text{CH}(\text{CH}_3)_2$ (*iso*-Pr), $-\text{CH}(\text{CH}_2)_2$ (cyclopropyl), $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ (*n*-Bu), $-\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$ (*sec*-butyl), $-\text{CH}_2\text{CH}(\text{CH}_3)_2$ (*iso*-butyl), $-\text{C}(\text{CH}_3)_3$ (*tert*-butyl), $-\text{CH}_2\text{C}(\text{CH}_3)_3$ (*neo*-pentyl), cyclobutyl, cyclopentyl, cyclohexyl, cyclohexylmethyl, adamant-1-yl, adamant-2-yl, phenyl, and substituted phenyl; R_2 is at least one of the following groups comprising: Cl, Br, F, I, and (E)-methyl 2-((1,2-dihydropyridin-2-yl)(phenyl)methylene)hydrazinecarbodithioate.

3. The method of Claim 1, wherein said subject has a symptom of cell death.

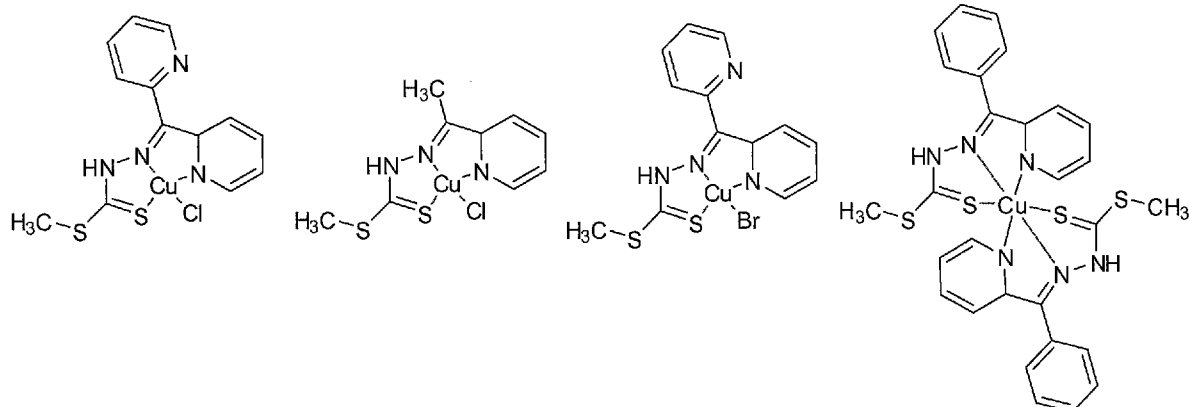
4. The method of Claim 3, wherein said symptom of cell death is mediated by an intrinsic pathway of apoptosis.

5. The method of Claim 1, wherein said allosteric pan-caspase inhibitor of Formula I provides protection from cell death.

6. The method of Claim 3, wherein said subject further comprises a cell containing a caspase, and a step of isolating said cell from said subject that is capable of undergoing apoptosis induced by ultra violet light.

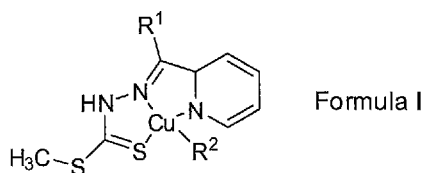
7. The method of Claim 1, wherein said disease state results from exposure to at least one of the group comprising: tumor necrosis factor- α (TNF α) and cycloheximide.

8. The method of Claim 1, wherein said allosteric pan-caspase inhibitor of Formula I is selected from the group consisting of:



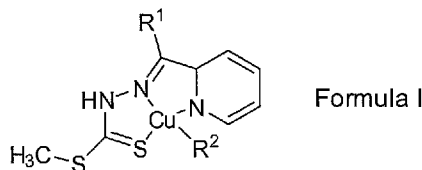
9. The method of Claim 1, wherein said disease state is selected from the group consisting of neurodegeneration, radiation syndrome, ultra violet light exposure, osteoarthritis, pancreatitis, rheumatoid arthritis, chronic active hepatitis, inflammatory bowel disease, Crohn's disease, psoriasis, organ transplant rejection, sepsis, septic shock, cerebral ischemia, myocardial ischemia, myocardial infarction, amyotrophic lateral sclerosis, multiple sclerosis, neurological damage due to stroke, hepatitis-B, hepatitis-C, hepatitis-G, and liver disease.

10. The pharmaceutical composition of Formula I:



wherein R_1 is CH_3 (Me), $-\text{CH}_2\text{CH}_3$ (Et), $-\text{CH}_2\text{CH}_2\text{CH}_3$ (*n*-Pr), $-\text{CH}(\text{CH}_3)_2$ (*iso*-Pr), $-\text{CH}(\text{CH}_2)_2$ (cyclopropyl), $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ (*n*-Bu), $-\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$ (*sec*-butyl), $-\text{CH}_2\text{CH}(\text{CH}_3)_2$ (*iso*-butyl), $-\text{C}(\text{CH}_3)_3$ (*tert*-butyl), $-\text{CH}_2\text{C}(\text{CH}_3)_3$ (*neo*-pentyl), cyclobutyl, cyclopentyl, cyclohexyl, cyclohexylmethyl, adamant-1-yl, adamant-2-yl, phenyl, and substituted phenyl; R_2 is at least one of the following groups comprising: Cl, Br, F, I, and (E)-methyl 2-((1,2-dihydropyridin-2-yl)(phenyl)methylene)hydrazinecarbodithioate.

11. An allosteric pan-caspase inhibitor of Formula I:



wherein R_1 is CH_3 (Me), $-\text{CH}_2\text{CH}_3$ (Et), $-\text{CH}_2\text{CH}_2\text{CH}_3$ (*n*-Pr), $-\text{CH}(\text{CH}_3)_2$ (*iso*-Pr), $-\text{CH}(\text{CH}_2)_2$ (cyclopropyl), $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ (*n*-Bu), $-\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$ (*sec*-butyl), $-\text{CH}_2\text{CH}(\text{CH}_3)_2$ (*iso*-butyl), $-\text{C}(\text{CH}_3)_3$ (*tert*-butyl), $-\text{CH}_2\text{C}(\text{CH}_3)_3$ (*neo*-pentyl), cyclobutyl, cyclopentyl, cyclohexyl, cyclohexylmethyl, adamant-1-yl, adamant-2-yl, phenyl, and substituted phenyl; R_2 is at least one of the following groups comprising: Cl, Br, F, I, and (E)-methyl 2-((1,2-dihydropyridin-2-yl)(phenyl)methylene)hydrazinecarbodithioate.

FIGURE 1

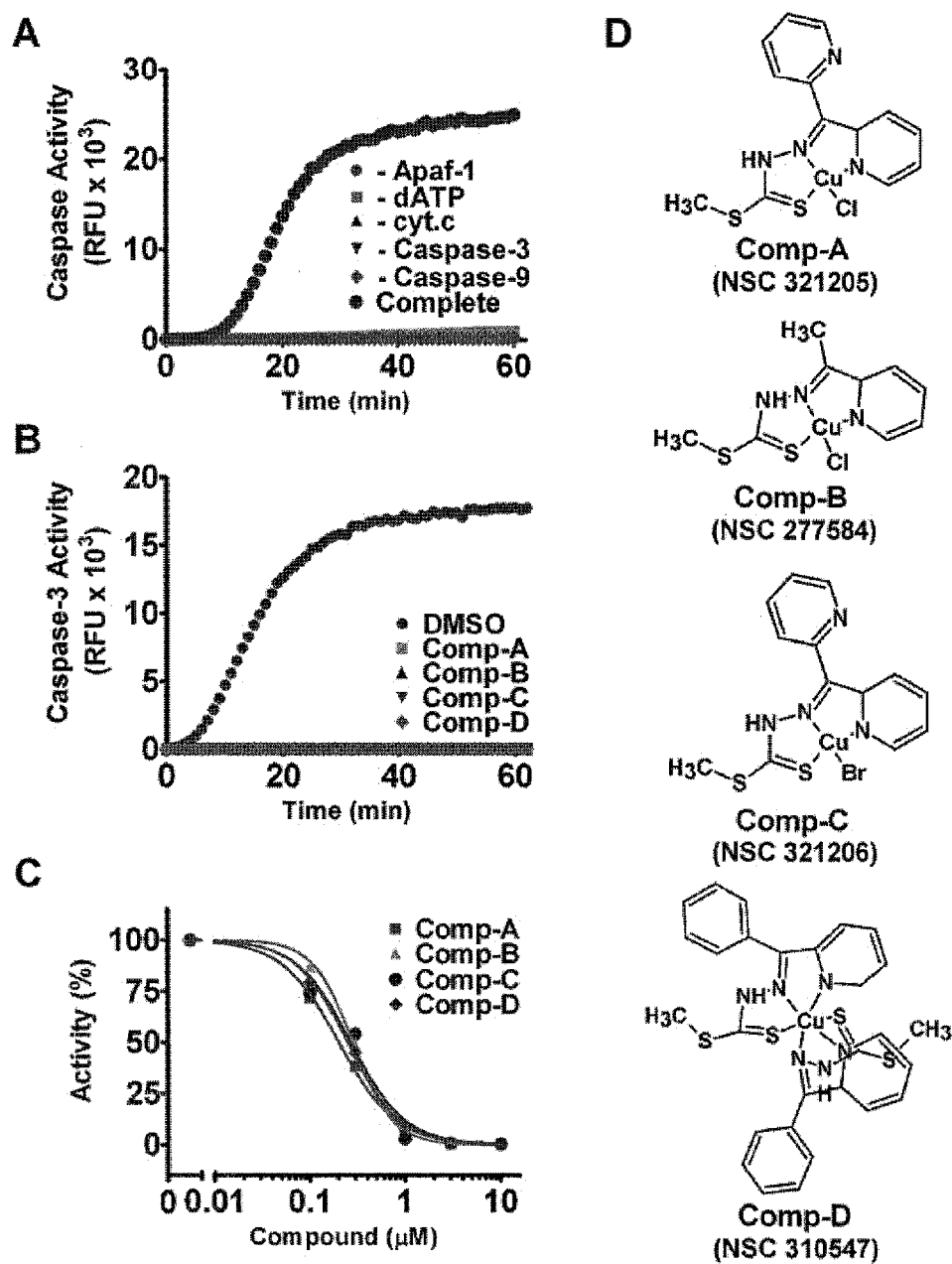


FIGURE 2

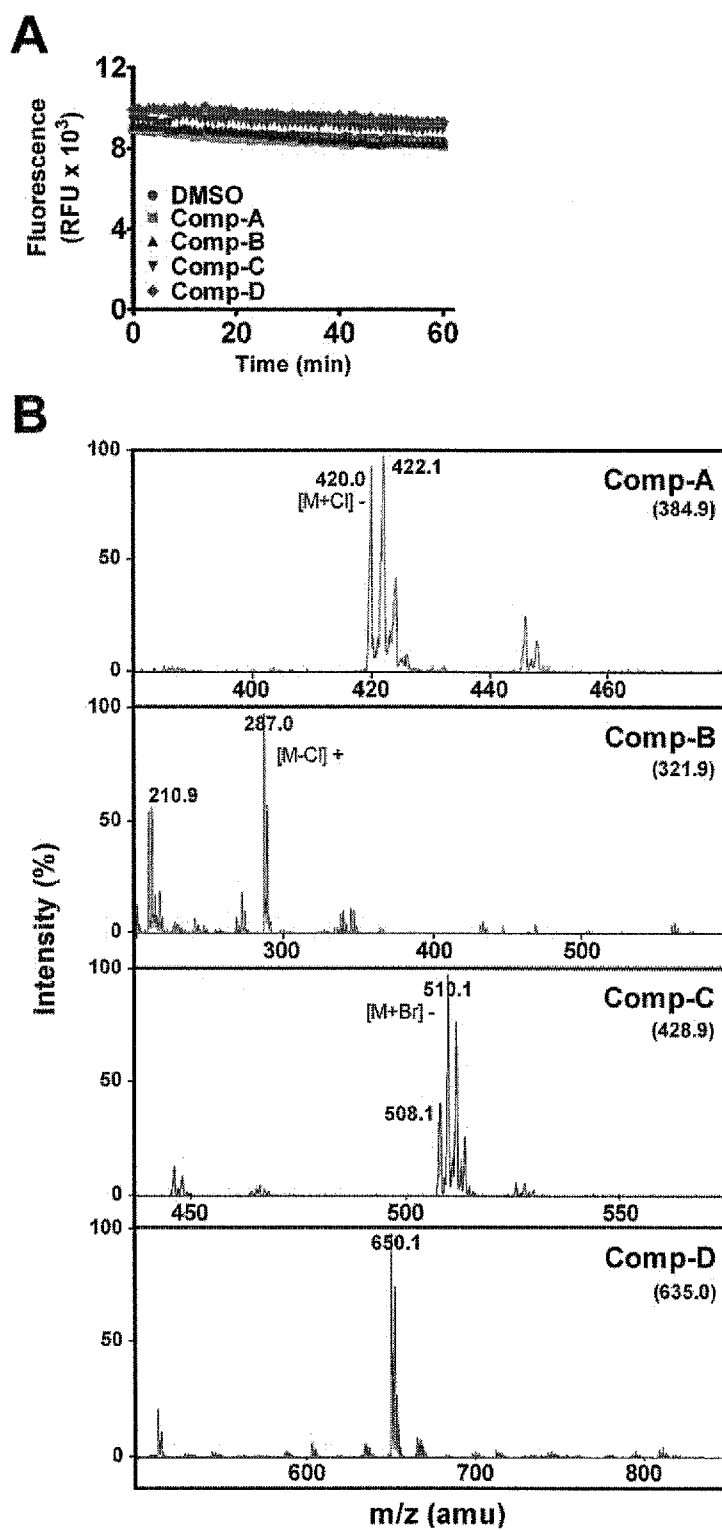


FIGURE 3

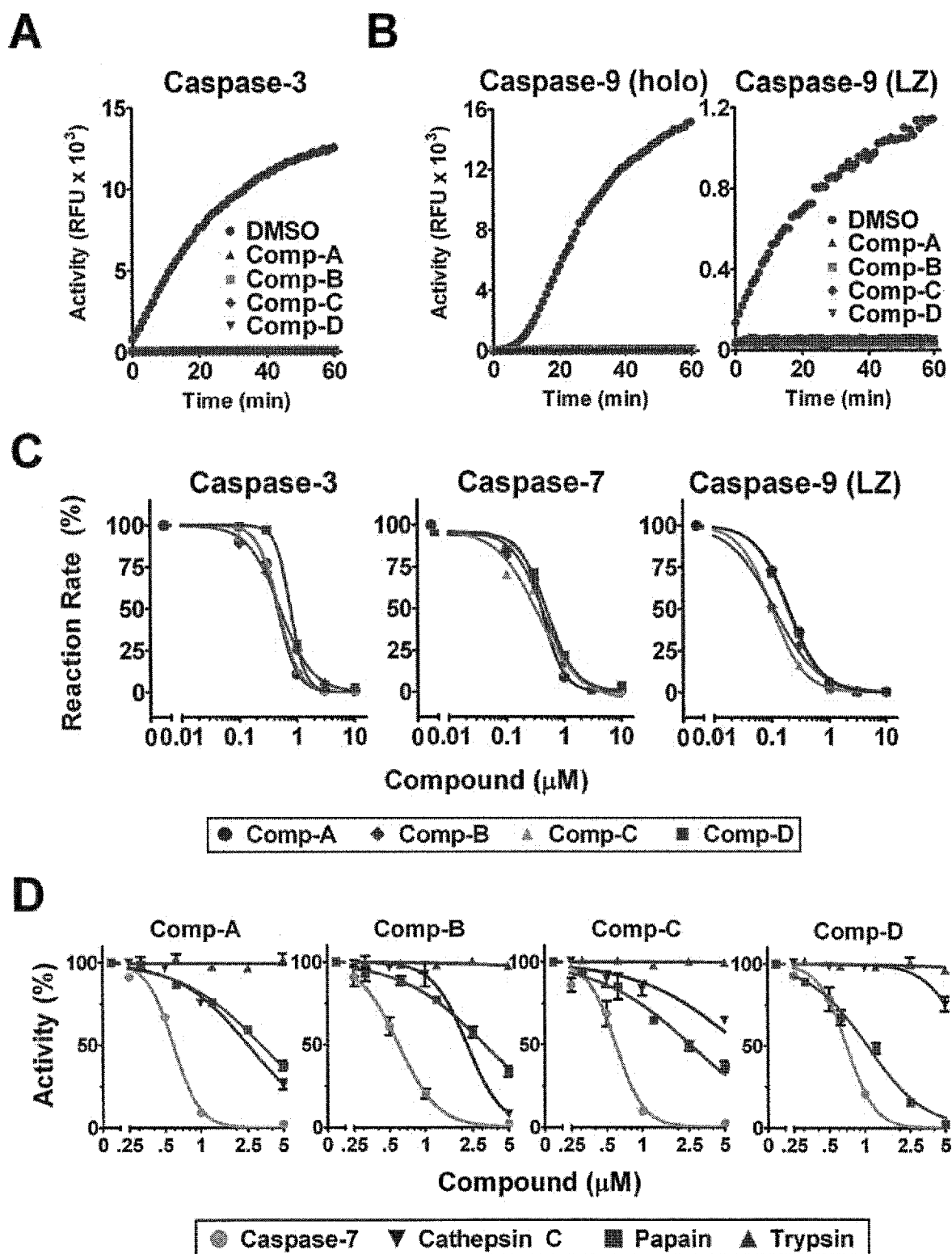


FIGURE 4

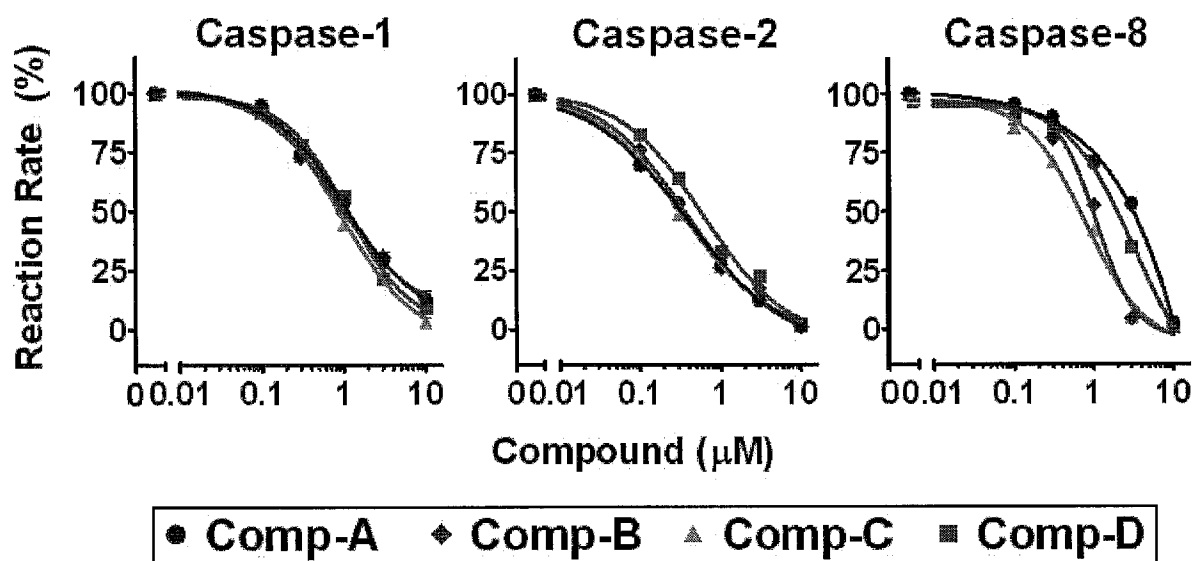


FIGURE 5

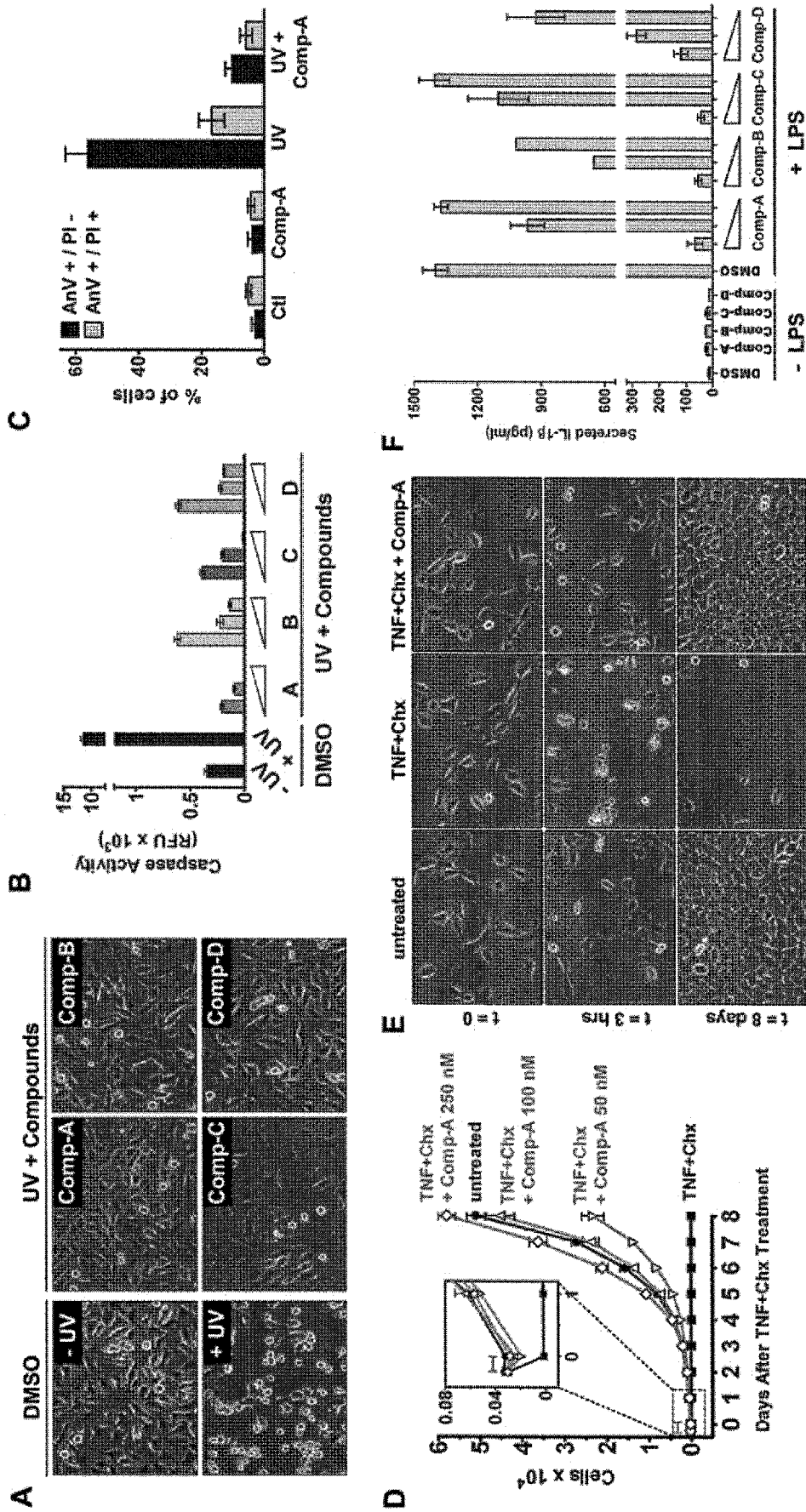


FIGURE 6

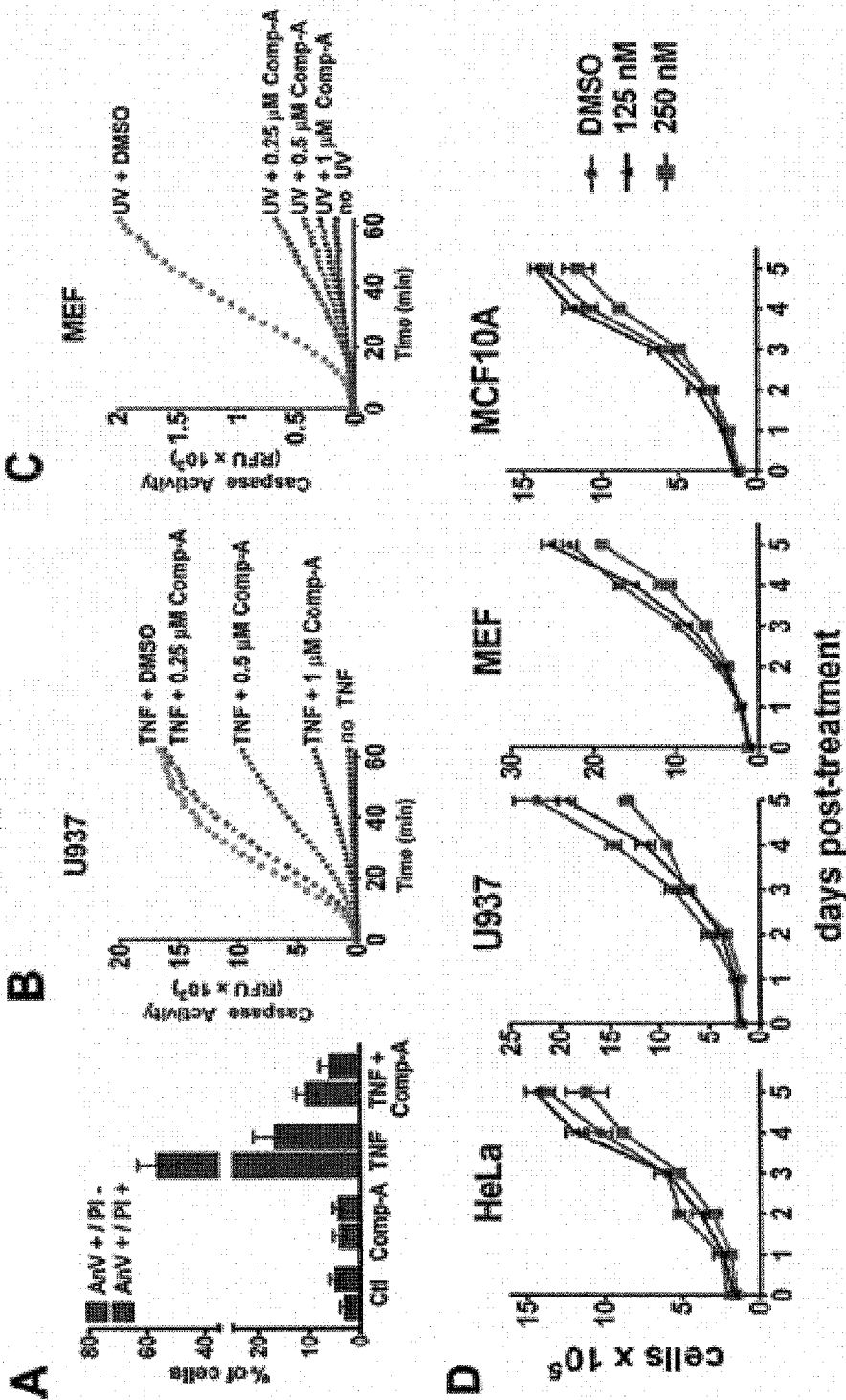


FIGURE 7

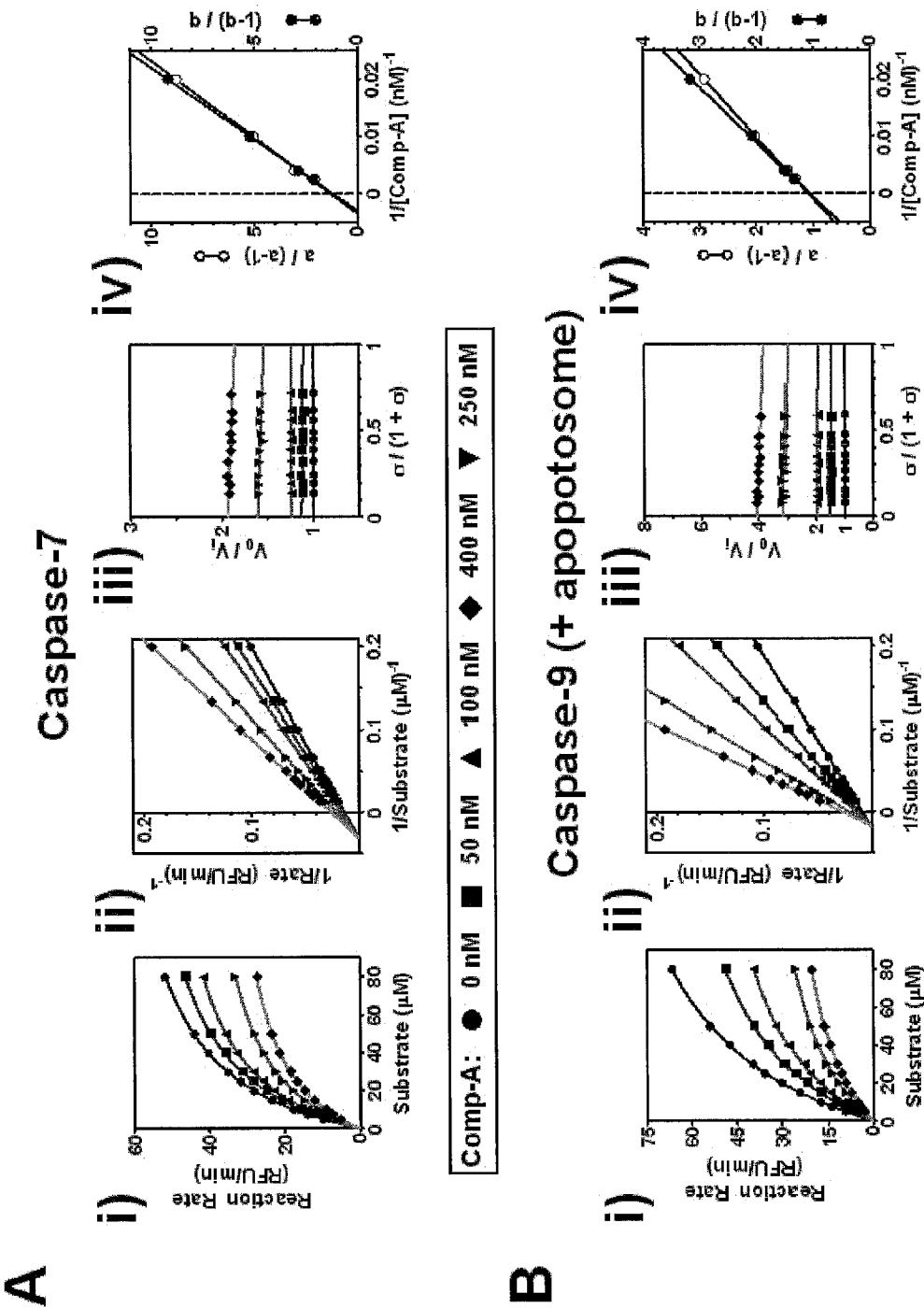


FIGURE 8

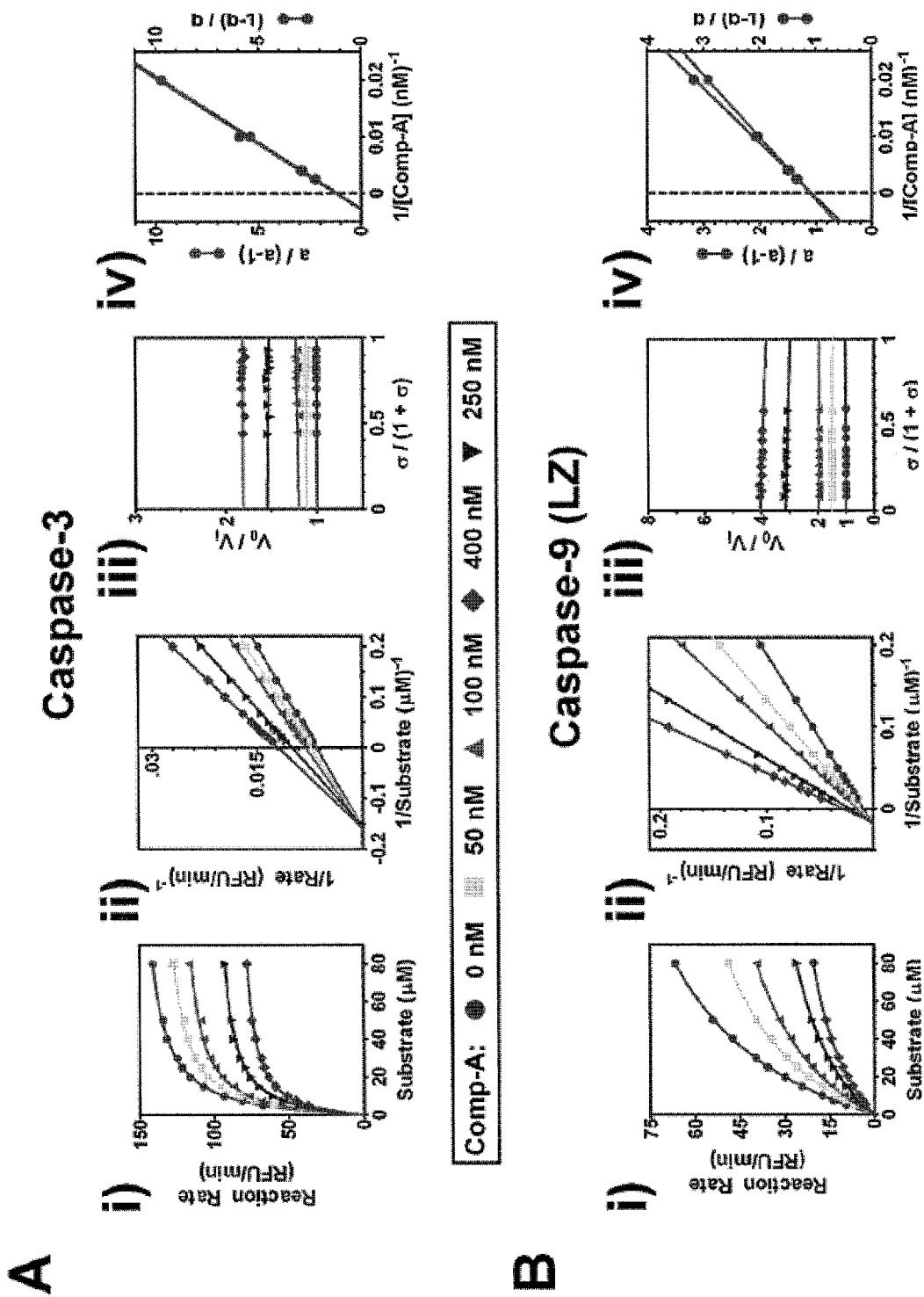


FIGURE 9

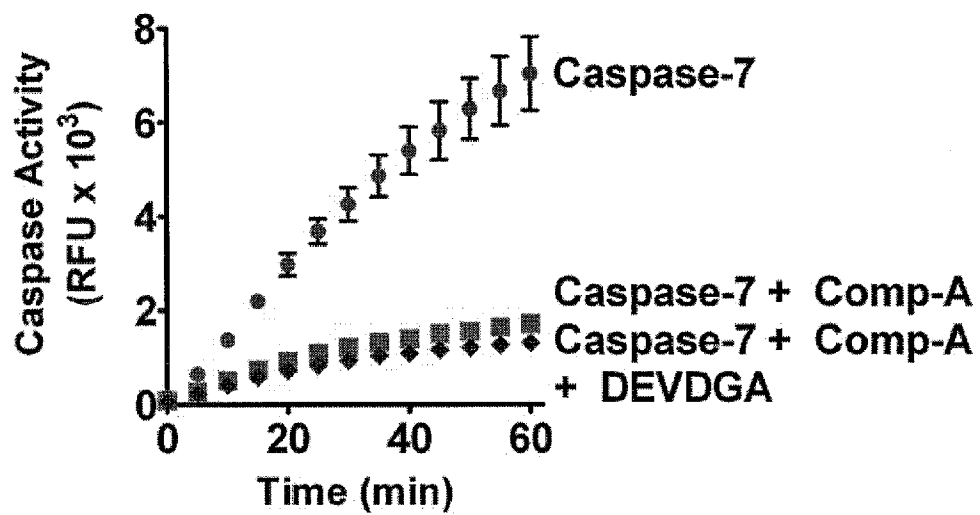


FIGURE 10

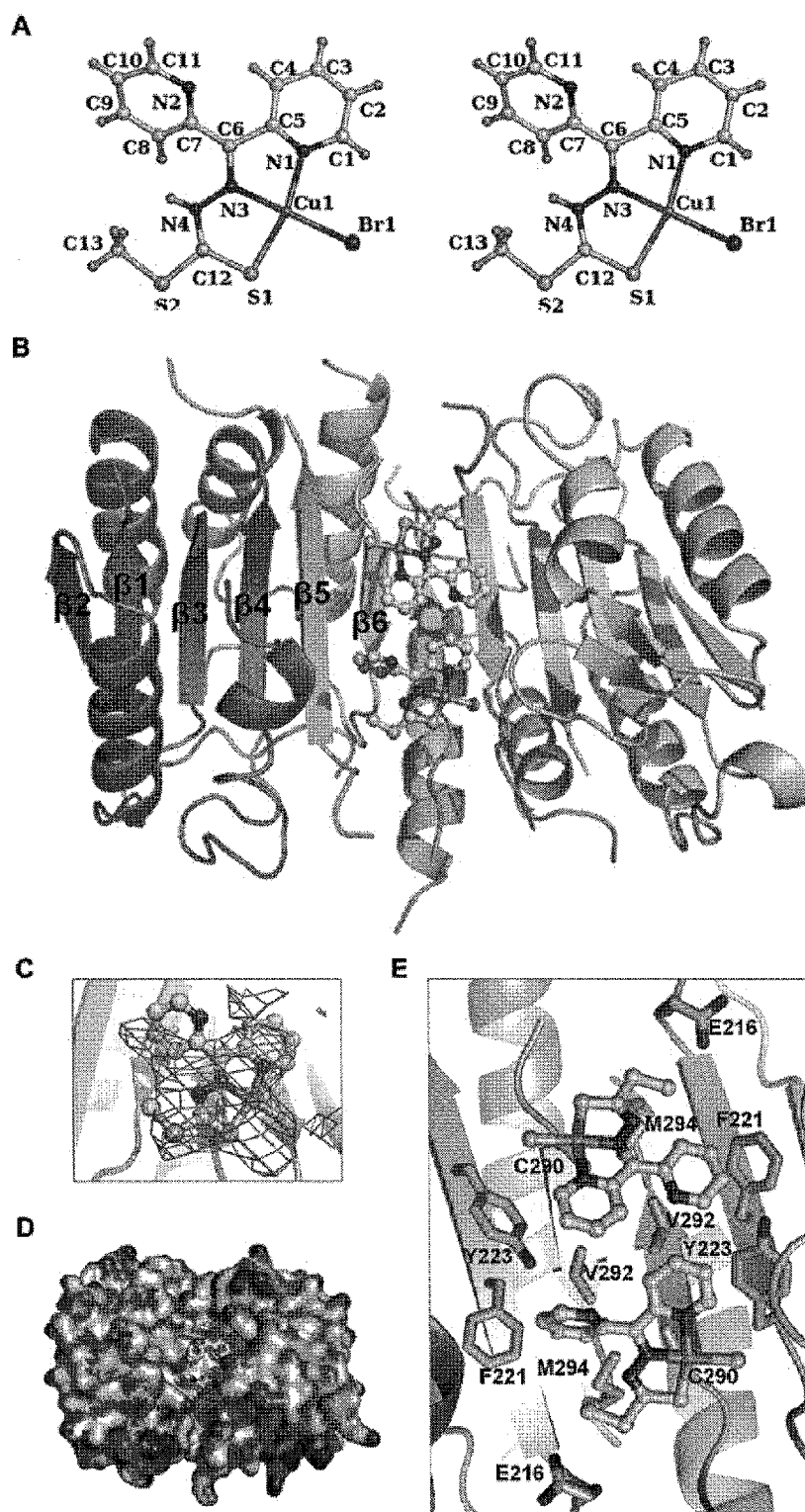


FIGURE 11

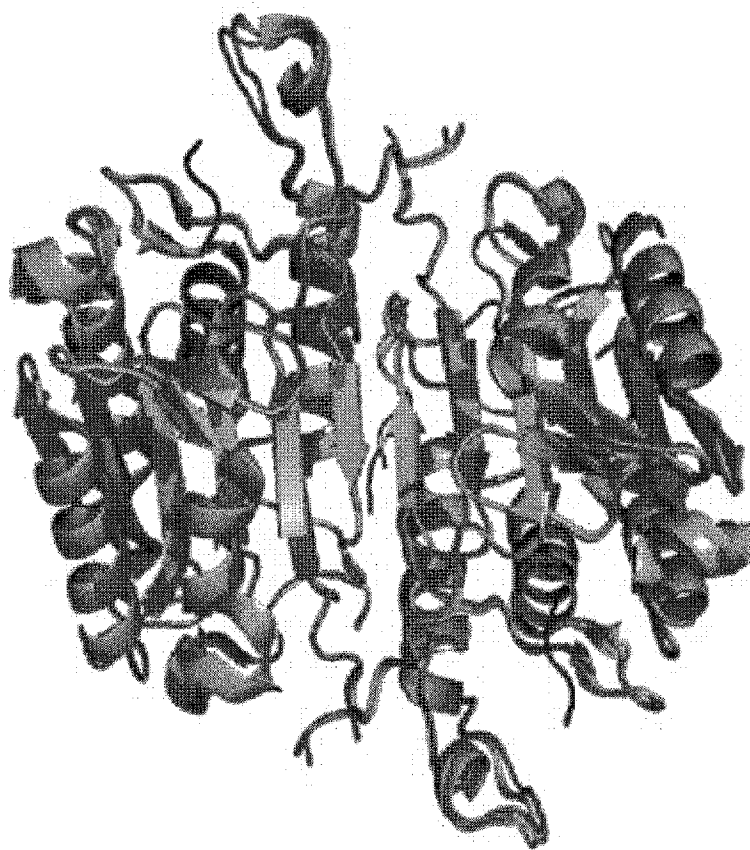


FIGURE 12

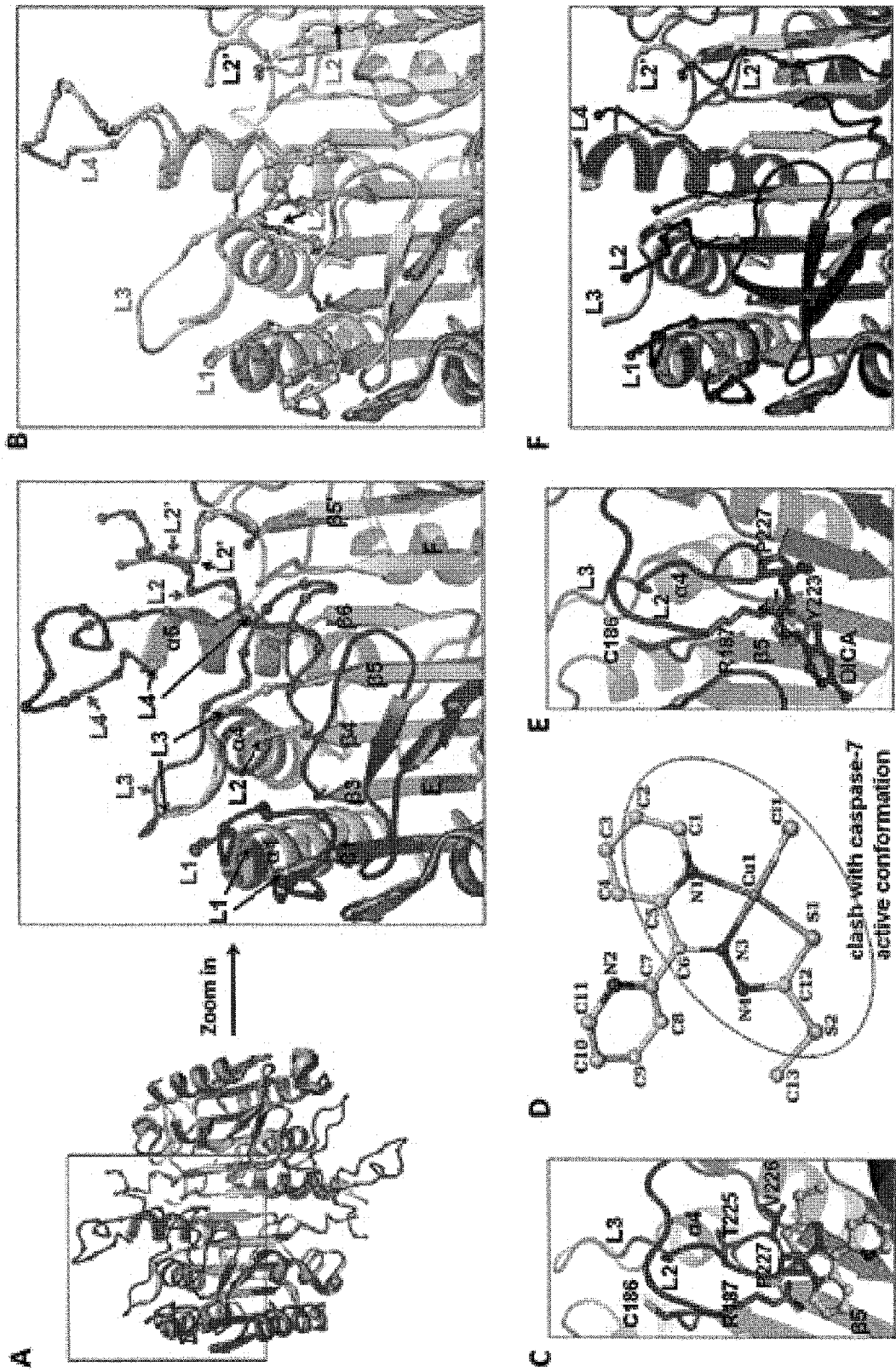
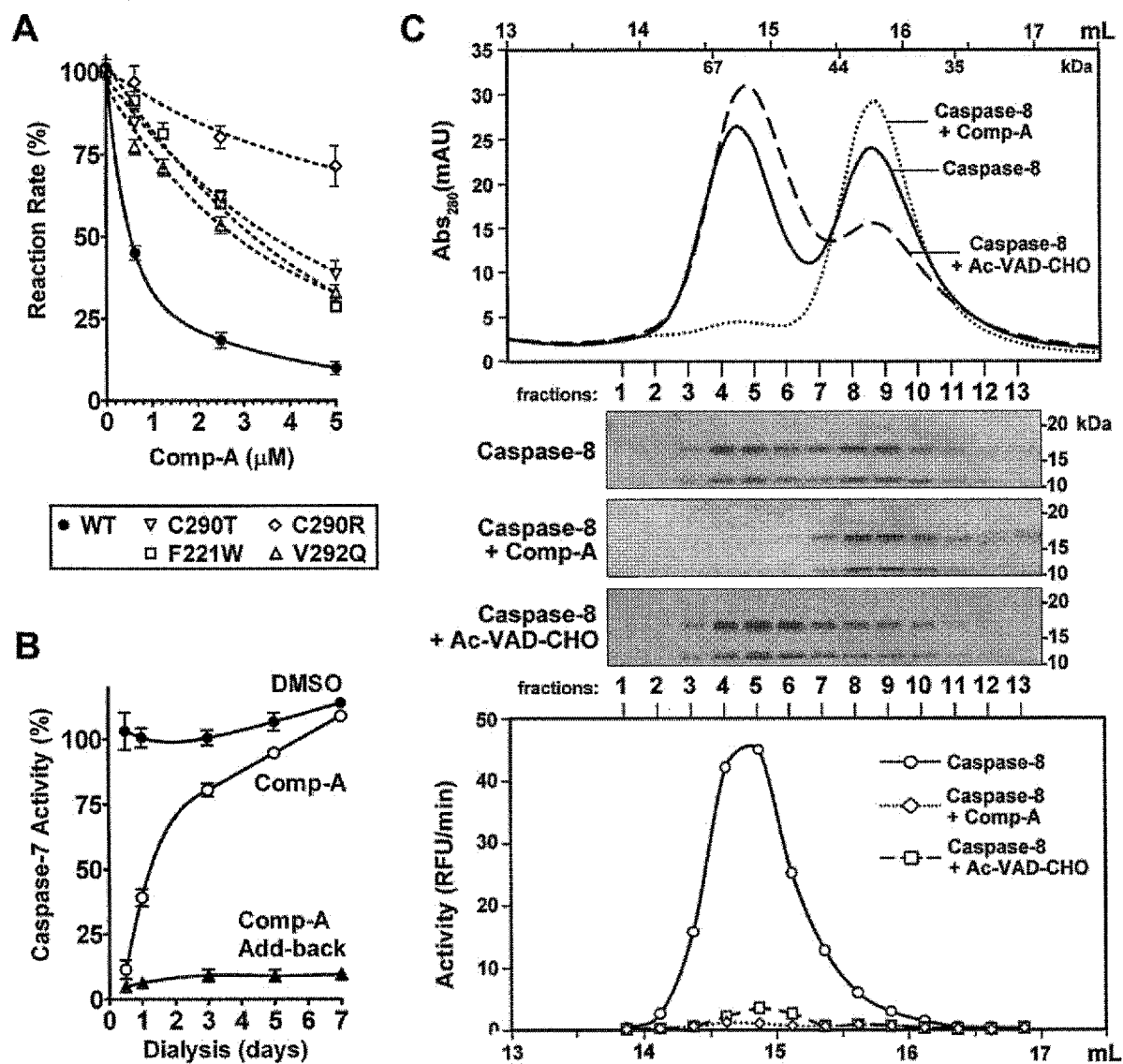


FIGURE 13

<i>H. sapiens</i>	219	ELFAYSTVPGY	229...	287	QIP	C	VVSMLTK	297	Caspase-7
<i>R. norvegicus</i>	219	ELFAYSTVPGY	229...	287	QIP	C	MVSMLTK	297	
<i>M. musculus</i>	219	ELFAYSTVPGY	229...	287	QIP	C	MVSMLTK	297	
<i>X. laevis</i>	233	FLYAYSTVPGY	243...	301	QIP	C	VVSMLTK	311	
<i>D. rerio</i>	233	FLFAYSTVPGY	243...	301	QIP	C	VVSMLTK	311	
<i>H. sapiens</i>	193	FLYAYSTAPGY	203...	261	QIP	C	IVSMLTK	271	Caspase-3
<i>R. norvegicus</i>	193	FLYAYSTAPGY	203...	261	QIP	C	IVSMLTK	271	
<i>M. musculus</i>	193	FLYAYSTAPGY	203...	261	QIP	C	IVSMLTK	271	
<i>X. laevis</i>	204	FLYAYSTVPGY	214...	266	QIP	C	VVSMLTK	276	
<i>D. rerio</i>	198	FLYAYSTVPGY	208...	266	QIP	C	IVSMLTK	276	
								*	
								*	
								*	

FIGURE 14



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/29206

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A01N 55/02 (2012.01)

USPC - 514/499

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)

USPC: 514/499

IPC(8):A01N 55/02 (2012.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 514/492; 536/22.1; 536/17.1 (see search words below)

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

WEST: PGPB, USPT, USOC, EPAB, JPAB

Google Scholar/Patents: Allosteric reversible pan-caspase inhibitor copper pyridinyl thiol chelator chelates carbodithioate pyridin% tn\$
inhibitor ultraviolet light apoptosis cell

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2003/0176496 A1 (MEDFORD et al.) 18 September 2003 (18.09.2003) para[0046]-[0051];[0319];[0320]; [0334]-[0335]; [0337], Table 1; [0425];[0428];[0438];[0454];[0472];[0473]	1-11
Y	ISKANDER et al. in Coordination Compounds of Hydrazine Derivatives with Transition Metals. Part 24. Coordination Chemistry of Hydrazine-S-Methyl Carbodithioate Schiff Bases Derived from [3-Dicarbonyl Compounds, Transition Metal Chemistry, 1982, Vol 7, 135-140. pg 136, Col 1, para 2, compound 5a and 5b ;pg 137, Col 2, para 3-4;pg 138, Col 1, para 2	1-11
Y	LUENG-TOUNG et al. in Thiol Proteases: Inhibitors and Potential Therapeutic Targets, Current Medicinal Chemistry, 2006, Vol 13, 547-581 . pg 564, Col 2, para 2-3;pg 565, Col 1, para 1)	1-11
Y	HANCOCK et al. A Copper Chelate of Thiosemicarbazone NSC 689534 induces Oxidative/ER Stress and Inhibits Tumor Growth In Vitro and In Vivo, Free Radic Biol Med. 2011 January 1; 50(1): 110-121. pg 7, para 4; pg 12, para 2; pg 15, reference 50; pg 17, Figure 1	1-11

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

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"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

09 June 2012 (09.06.2012)

Date of mailing of the international search report

02 JUL 2012

Name and mailing address of the ISA/US

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