COMPOSITIONS AND METHODS FOR TREATING NEOPLASTIC DISEASES

Background of the Invention

Field of the Invention

[0001] The present invention relates to the fields of chemistry and medicine. More particularly, the present invention relates to the treatment of neoplastic diseases, such as cancer.

Description of the Related Art

[0002] Cancer is a leading cause of death in the United States. Despite significant efforts to find new approaches for treating cancer, the primary treatment options remain surgery, chemotherapy and radiation therapy, either alone or in combination. Surgery and radiation therapy, however, are generally useful only for fairly defined types of cancer, and are of limited use for treating patients with disseminated disease. Chemotherapy is the method that is generally useful in treating patients with metastatic cancer or diffuse cancers such as leukemias. Although chemotherapy can provide a therapeutic benefit, it often fails to result in cure of the disease due to the patient's cancer cells becoming resistant to the chemotherapeutic agent.

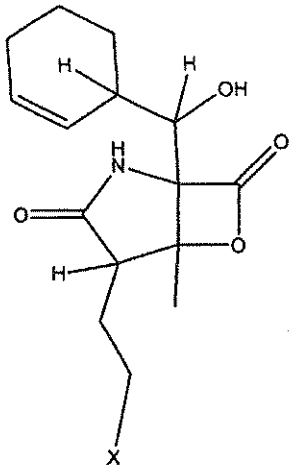
[0003] Therefore, a need exists for additional chemotherapeutics to treat cancer. A continuing effort is being made by individual investigators, academia and companies to identify new, potentially useful chemotherapeutic and anti-microbial agents.

[0004] The successful development of Bortezomib /PS-341 therapy for treatment of relapsed/refractory multiple myeloma (MM) has established proteasome inhibition as an effective therapeutic strategy. The dipeptide boronic acid analogue Bortezomib is a potent, highly selective, and reversible proteasome inhibitor which targets the 26S proteasome complex and inhibits its function. The 26S proteasome is an adenosine triphosphate (ATP)–dependent multicatalytic protease mediating intracellular protein degradation. Proteasomal degradation of misfolded or damaged proteins proceeds by recognition of polyubiquitinated proteins by the 19S regulatory subunit of the 26S protease, and subsequent hydrolysis to small polypeptides. Bortezomib primarily inhibits chymotryptic, without altering tryptic or caspase-like, proteasome activity. Besides inhibiting NF-kB, Bortezomib has pleiotropic

effects on MM biology by targeting: 1) cell-cycle regulatory proteins; 2) UPR pathway *via* modulating transcriptional activity of plasma cell differentiation factor X-box binding protein-1 (XBP-1); 3) p53-mediated apoptosis/MDM2; 4) DNA repair mechanisms; 5) classical stress-response pathways *via* both intrinsic (caspase-9 mediated) and extrinsic (caspase-8 mediated) cell death cascades. Specifically, Bortezomib activates JNK, which triggers mitochondrial apoptotic signaling: release of cytochrome-c (cyto-c) and second mitochondrial activator of caspases (Smac) from mitochondria to cytosol, followed by activation of caspase-9 and caspase-3. However, both intrinsic and acquired resistance has already been observed, and there are no therapies to overcome Bortezomib resistance at present.

Summary of the Invention

[0005] One aspect of the present invention is a method of treating a neoplastic disease, comprising administering to a patient inflicted with the neoplastic disease a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof:



(I)

[0006] wherein X is selected from the group consisting of fluorine, chlorine, bromine or iodine, and wherein the neoplastic disease is susceptible to resistance to at least one other chemotherapeutic agent.

[0007] Another aspect of the present invention is a method of treating a neoplastic disease, comprising administering to a patient inflicted with the neoplastic disease a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof wherein X

is selected from the group consisting of fluorine, chlorine, bromine or iodine, in combination with at least one additional chemotherapeutic agent.

[0008] Another aspect of the present invention is a pharmaceutical composition, comprising a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, wherein X is selected from the group consisting of fluorine, chlorine, bromine or iodine, and at least one additional chemotherapeutic agent.

[0009] Another aspect of the present invention is a method of treating a neoplastic disease, comprising administering to a patient inflicted with the neoplastic disease a synergistic combination of at least two proteosome inhibitors.

Brief Description of the Drawings

[0010] FIGURE 1 illustrates inhibition of chymotrypsin-like, caspase-like, and trypsin-like proteasome activities in human erythrocytes-derived 20S proteasome by NPI-0052.

[0011] FIGURE 2 illustrates the *in vivo* chymotrypsin-like activity of NPI-0052 in mice.

[0012] FIGURE 3 depicts the autoradiograph obtained after treating MM.1S multiple myeloma (MM) cells with NPI-0052 (7 nM) and incubating protein extracts with AdaY(^{125}I)Ahx$_3$L$_3$VS at 37°C.

[0013] FIGURE 4 depicts immunoblots obtained after treating MM.1S cells with NPI-0052 and then incubating with Dansyl-Ahx3L3VS.

[0014] FIGURE 5A illustrates cell viability of various multiple myeloma cell lines treated with indicated doses of NPI-0052 for 24h.

[0015] FIGURE 5B illustrates DNA fragmentation assays of apoptosis after treatment with NPI-0052 of MM cells obtained from patients.

[0016] FIGURE 6 illustrates DNA fragmentation assays of apoptosis after treatment with NPI-0052 of bone marrow stromal cells obtained from patients.

[0017] FIGURE 7 illustrates MTT assay of MM.1S cell viability after treatment with NPI-0052 or Dex in the presence or absence of IL-6 or IGF-I.

[0018] FIGURE 8 illustrates the effect of NPI-0052 on VEGF-induced migration of MM.1S cells.

[0019] FIGURE 9 illustrates the effect of NPI-0052 on Bcl2-overexpressing MM.1S cell viability.

[0020] FIGURES 10A and 10B depict the effect of NPI-0052 on tumor growth when administered orally to mice.

[0021] FIGURE 10C illustrates the effect of NPI-0052 on survival when administered orally to mice.

[0022] FIGURE 10D illustrates the effect of NPI-0052 on body weight when administered orally to mice.

[0023] FIGURE 10E illustrates tissue sections of inoculation sites from NPI-0052-treated and control-treated mice.

[0024] FIGURE 10F compares the effect of NPI-0052 and Bortezomib on tumor growth when administered i.v. to mice.

[0025] FIGURE 10G compares the effect of NPI-0052 and Bortezomib on survival when administered i.v. to mice.

[0026] FIGURE 11A illustrates the effect of NPI-0052 on mitochondrial membrane potential in MM.1S cells incubated with CMXRos.

[0027] FIGURE 11B illustrates the effect of NPI-0052 on superoxide generation in MM.1S cells stained with membrane permeable dye dihydroethidium (HE).

[0028] FIGURE 11C depicts immunoblots of mitochondrial and cytosolic protein fractions obtained from MM.1S cells treated with NPI-0052.

[0029] FIGURE 11D depicts immunoblots of cytosolic proteins obtained from MM.1S cells treated with NPI-0052 and analyzed with anti-caspase-9 Abs.

[0030] FIGURE 11E depicts immunoblots of cytosolic proteins obtained from MM.1S cells treated with NPI-0052 and analyzed with anti-caspase-8 Abs.

[0031] FIGURE 11F depicts immunoblots of MM.1S or MM.1R MM cells treated with NPI-0052 and assessed for apoptosis by both PARP and caspase-3 cleavage assays.

[0032] FIGURE 12A illustrates MM.1S cell viability after treatment with NPI-0052 or Bortezomib in the presence or absence of caspase-3, caspase-8, or caspase-9 inhibitor.

[0033] FIGURE 12B illustrates MM.1S cell viability for cells transfected with vector alone, DN-caspase-8, and DN-caspase-9 after treatment with NPI-0052 or Bortezomib.

[0034] FIGURE 12C depicts immunoblots of cytosolic extracts from DN-caspase-8 and DN-caspase-9 transfected MM.1S cells treated with dexamethasone or anti-Fas MoAb.

[0035] FIGURE 12D illustrates MM.1S cell viability for vector or DN-FADD transfected cells after treatment with NPI-0052 or Bortezomib.

[0036] FIGURE 12E depicts immunoblots of mitochondrial protein extracts from MM.1S MM cells treated with indicated concentration of either NPI-0052 or Bortezomib and analysed with anti-Bax or anti-Hsp60 Abs.

[0037] FIGURE 12F illustrates cell viability of mouse embryonic fibroblasts (MEFs) cells with either wild-type or deleted Bax (knock-out) treated with indicated concentrations of NPI-0052 or Bortezomib.

[0038] FIGURE 13 illustrates viability of normal lymphocytes from five healthy donors treated with indicated concentrations of NPI-0052 or Bortezomib.

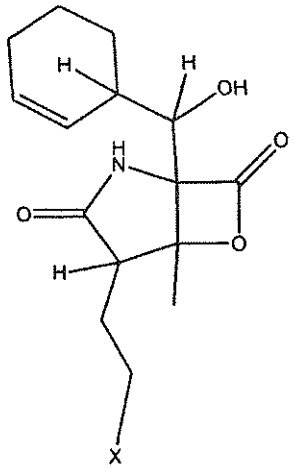
[0039] FIGURE 14A illustrates MM.1S cell viability for cells transfected with vector alone or Bcl-2 after treatment with NPI-0052 or Bortezomib.

[0040] FIGURE 14B depicts immunoblots of cytosolic extracts from vector- or Bcl-2-transfected MM.1S cells treated with NPI-0052 or Bortezomib.

[0041] FIGURE 15 illustrates cell viability of MM.1S and MM.1R MM cells treated with indicated concentration of NPI-0052, Bortezomib, or NPI-0052 + Bortezomib.

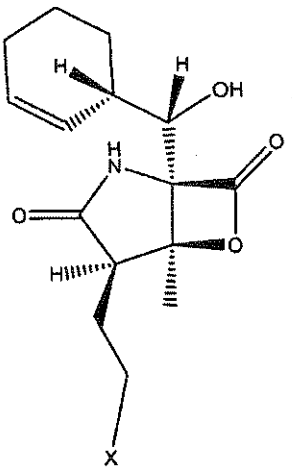
Detailed Description of the Preferred Embodiment

[0042] In one embodiment, a compound according to formula (I) is provided for use as described herein:



(I)

where X may be fluorine, chlorine, bromine or iodine. In one embodiment, X is chlorine. In one embodiment, the compound of formula (I) has stereochemistry according to formula (II):



(II)

The compound of formula (II) where X=Cl is also referred to herein as NPI-0052. Compounds according to formulae (I) or (II) may be derived from fermentation of Salinospora, a marine gram-positive actinomycete.

[0043] In some embodiments, prodrugs, metabolites, stereoisomers, and pharmaceutically acceptable salts of the compounds disclosed herein are provided for use as described herein.

[0044] A "prodrug" refers to an agent that is converted into the parent drug *in vivo*. Prodrugs are often useful because, in some situations, they may be easier to administer than the parent drug. They may, for instance, be bioavailable by oral administration whereas the parent is not. The prodrug may also have improved solubility in pharmaceutical compositions over the parent drug. An example, without limitation, of a prodrug would be a compound which is administered as an ester (the "prodrug") to facilitate transmittal across a cell membrane where water solubility is detrimental to mobility but which then is metabolically hydrolyzed to the carboxylic acid, the active entity, once inside the cell where water-solubility is beneficial. A further example of a prodrug might be a short peptide (polyaminoacid) bonded to an acid group where the peptide is metabolized to reveal the active moiety. Conventional procedures for the selection and preparation of suitable prodrug derivatives are described, for example, in *Design of Prodrugs*, (ed. H. Bundgaard, Elsevier, 1985), which is hereby incorporated herein by reference in its entirety.

[0045] The term "pro-drug ester" refers to derivatives of the compounds disclosed herein formed by the addition of any of several ester-forming groups that are hydrolyzed under physiological conditions. Examples of pro-drug ester groups include pivoyloxymethyl, acetoxymethyl, phthalidyl, indanyl and methoxymethyl, as well as other such groups known in the art, including a (5-R-2-oxo-1,3-dioxolen-4-yl)methyl group. Other examples of pro-drug ester groups can be found in, for example, T. Higuchi and V. Stella, in "Pro-drugs as Novel Delivery Systems", Vol. 14, A.C.S. Symposium Series, American Chemical Society (1975); and "Bioreversible Carriers in Drug Design: Theory and Application", edited by E. B. Roche, Pergamon Press: New York, 14-21 (1987) (providing examples of esters useful as prodrugs for compounds containing carboxyl groups). Each of the above-mentioned references is herein incorporated by reference in their entirety.

[0046] Metabolites of the compounds disclosed herein include active species that are produced upon introduction of the compounds into the biological milieu.

[0047] Where the compounds disclosed herein have at least one chiral center, they may exist as a racemate or as enantiomers. It should be noted that all such isomers and mixtures thereof are included in the scope of the present invention. Furthermore, some of the crystalline forms for the compounds of disclosed herein may exist as polymorphs. Such polymorphs are included in one embodiment of the present invention. In addition, some of the compounds of the present invention may form solvates with water (i.e., hydrates) or common organic solvents. Such solvates are included in one embodiment of the present invention.

[0048] The term "pharmaceutically acceptable salt" refers to a salt of a compound that does not cause significant irritation to an organism to which it is administered and does not abrogate the biological activity and properties of the compound. In some embodiments, the salt is an acid addition salt of the compound. Pharmaceutical salts can be obtained by reacting a compound with inorganic acids such as hydrohalic acid (e.g., hydrochloric acid or hydrobromic acid), sulfuric acid, nitric acid, phosphoric acid and the like. Pharmaceutical salts can also be obtained by reacting a compound with an organic acid such as aliphatic or aromatic carboxylic or sulfonic acids, for example acetic, succinic, lactic, malic, tartaric, citric, ascorbic, nicotinic, methanesulfonic, ethanesulfonic, p-toluensulfonic, salicylic or naphthalenesulfonic acid. Pharmaceutical salts can also be obtained by reacting a compound with a base to form a salt such as an ammonium salt, an alkali metal salt, such as a sodium or a potassium salt, an alkaline earth metal salt, such as a calcium or a magnesium salt, a salt of organic bases such as dicyclohexylamine, N-methyl-D-glucamine, tris(hydroxymethyl)methylamine, C_1-C_7 alkylamine, cyclohexylamine, triethanolamine, ethylenediamine, and salts with amino acids such as arginine, lysine, and the like.

[0049] If the manufacture of pharmaceutical formulations involves intimate mixing of the pharmaceutical excipients and the active ingredient in its salt form, then it may be desirable to use pharmaceutical excipients which are non-basic, that is, either acidic or neutral excipients.

[0050] In various embodiments, the compounds disclosed herein can be used alone, in combination with other compounds disclosed herein, or in combination with one or more other agents active in the therapeutic areas described herein.

[0051] The term "halogen atom," as used herein, means any one of the radio-stable atoms of column 7 of the Periodic Table of the Elements, *e.g.*, fluorine, chlorine, bromine, or iodine, with fluorine and chlorine being preferred.

[0052] The term "ester" refers to a chemical moiety with formula $-(R)_n-COOR'$, where R and R' are independently selected from the group consisting of alkyl, cycloalkyl, aryl, heteroaryl (bonded through a ring carbon) and heteroalicyclic (bonded through a ring carbon), and where n is 0 or 1.

[0053] An "amide" is a chemical moiety with formula $-(R)_n-C(O)NHR'$ or $-(R)_n-NHC(O)R'$, where R and R' are independently selected from the group consisting of alkyl, cycloalkyl, aryl, heteroaryl (bonded through a ring carbon) and heteroalicyclic (bonded through a ring carbon), and where n is 0 or 1. An amide may be an amino acid or a peptide molecule attached to a molecule of the present invention, thereby forming a prodrug.

[0054] Any amine, hydroxy, or carboxyl side chain on the compounds of the present invention can be esterified or amidified. The procedures and specific groups to be used to achieve this end are known to those of skill in the art and can readily be found in reference sources such as Greene and Wuts, Protective Groups in Organic Synthesis, 3rd Ed., John Wiley & Sons, New York, NY, 1999, which is incorporated herein in its entirety.

[0055] The terms "purified," "substantially purified," and "isolated" as used herein refer to compounds disclosed herein being free of other, dissimilar compounds with which the compounds of the invention are normally associated in their natural state, so that the compounds of the invention comprise at least 0.5%, 1%, 5%, 10%, or 20%, and most preferably at least 50% or 75% of the mass, by weight, of a given sample.

Methods of Use

[0056] As demonstrated by the examples presented herein, the compound of formula (I) inhibits chymotrypsin-like, trypsin-like, and caspase-like proteasome activities. In contrast, Bortezomib has been shown to inhibit only chymotrypsin-like proteasome activity. *See* Goldberg, A. L. & Rock, K. (2002) *Nat Med* **8,** 338-40 and Adams, J. (2004) *Nat Rev Cancer* **4,** 349-60; both of which are incorporated herein by reference in their entirety. It is further demonstrated that compounds of formula (I) have a different mechanism of action than bortezomib. Furthermore, the compound of formula (I) induces apoptosis in

various multiple myeloma cell lines including, but not limited to, Dexamethasone-sensitive MM.1S, Dexamethasone-resistant MM.1R, RPMI-8226, OPM2, U266, and Doxorubicin-resistant Dox-40. The compound of formula I also induced apoptosis in cell lines obtained from human multiple myloma patients that had relapsed after multiple prior therapies with Dexamethasone, Bortezomib, and thalidomide. Thus, the compound of formula (I) is effective against MM cells that are resistant to other chemotherapeutic agents, including Dexamethasone, Doxorubicin, Bortezomib/PS-341, and thalidomide.

[0057] Accordingly, in one embodiment, a method of treating a neoplastic disease that is susceptible to resistance to at least one chemotherapeutic agent is provided comprising administering to a patient, such as a human, a compound of formula (I) or a pharmaceutically acceptable salt or prodrug ester thereof. By "resistance to at least one chemotherapeutic agent," it is meant that administration of the chemotherapeutic agent to the patient does not result in significant amelioration of symptoms of the neoplastic disease. In some embodiments where the neoplastic disease is a characterized by a tumor, "resistance to at least one chemotherapeutic agent" means that administration of the chemotherapeutic agent does not result in appreciable inhibition of the growth of the tumor or reduction in the size of the tumor. "Resistance to at least one chemotherapeutic agent" can also mean that when the agent is exposed to resistant tumor cells, no appreciable apoptosis is induced. By "susceptible to" resistance to at least one chemotherapeutic agent, it is meant that the neoplastic disease currently is resistant to the at least one chemotherapeutic agent or will develop resistance upon repeated administration of the chemotherapeutic agent.

[0058] The examples herein also demonstrate that compounds of formula (I) when combined with bortezomib trigger synergistic apoptosis in MM cells. Thus, a compound of formula (I) may be administered in combination with Bortezomib/PS-341 to achieve apoptosis using lower doses of each agent than if the agents were administered separately, thus reducing the toxicity of the agents. Surprisingly, these results demonstrate that a synergistic result may be obtained by administering two different proteasome inhibitors. By "synergistic," it is meant that the combination of two or more agents yield a combination index (CI) < 1.0. It has also been demonstrated that combination of the compound of formula (I) with non-proteasome inhibitor agents provide an additive effect. By

"additive," it is meant that the combination of two or more agents yield a CI approximately equal to one. CI may be determined, for example, by the Chou-Talalay method according to the following equation: "CI = (D)1/(Dx)1 + (D)2/(Dx)2 + (D)1(D)2/(Dx)1(Dx)2", where (D)1 and (D)2 are the doses of drug 1 and drug 2 that have x effect when used in combination; and (Dx)1 and (Dx)2 are the doses of drug 1 and drug 2 that have the same x effect when used alone.

[0059] Accordingly, in one embodiment, a method is provided for treating a neoplastic disease comprising administering two or more proteasome inhibitors in synergistic combination. Non-limiting examples of classes of proteasome inhibitors that may be combined include peptide boronate proteasome inhibitors, peptide aldehyde proteasome inhibitors, and non-peptide proteasome inhibitors. A non-limiting example of a peptide boronate proteasome inhibitor is bortezomib. A non-limiting example of a peptide aldehyde proteasome inhibitor is MG-132. Non-limiting examples of non-peptide proteasome inhibitors include omuralide and the compound of formula (I). In one embodiment, at least one of the proteasome inhibitors is a compound of formula (I) or bortezomib. By administration in "combination," it is meant that the two or more agents may be found in the patient's bloodstream at the same time, regardless of when or how they are actually administered. In one embodiment, the agents are administered simultaneously. In one such embodiment, administration in combination is accomplished by combining the agents in a single dosage form. In another embodiment, the agents are administered sequentially. In one embodiment the agents are administered through the same route, such as orally. In another embodiment, the agents are administered through different routes, such as one being administered orally and another being administered i.v. In one advantageous embodiment, the pharmacokinetics of the two or more agents are substantially the same.

[0060] In one embodiment, a method is provided for treating a neoplastic disease comprising administering a compound of formula (I) in combination with another chemotherapeutic agent. In one embodiment, the other chemotherapeutic agent is dexamethasone, doxorubicin, or thalidomide. In one embodiment, the other chemotherapeutic agent is another proteasome inhibitor such as bortezomib. In one

embodiment, a pharmaceutical composition is provided that combines a compound of formula (I) with the additional chemotherapeutic agent.

[0061] In some embodiments, the neoplastic disease treated by any of the methods above may be a cancer selected from breast cancer, sarcoma, leukemia, ovarian cancer, uretal cancer, bladder cancer, prostate cancer, colon cancer, rectal cancer, stomach cancer, lung cancer, lymphoma, multiple myeloma, pancreatic cancer, liver cancer, kidney cancer, endocrine cancer, skin cancer, melanoma, angioma, and brain or central nervous system (CNS) cancer. In one embodiment, the neoplastic disease is a multiple myeloma.

Pharmaceutical Compositions

[0062] In another aspect, the present disclosure relates to a pharmaceutical composition comprising physiologically acceptable surface active agents, carriers, diluents, excipients, smoothing agents, suspension agents, film forming substances, and coating assistants, or a combination thereof; and a compound or combination disclosed herein. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Co., Easton, PA (1990), which is incorporated herein by reference in its entirety. Preservatives, stabilizers, dyes, sweeteners, fragrances, flavoring agents, and the like may be provided in the pharmaceutical composition. For example, sodium benzoate, ascorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. In addition, antioxidants and suspending agents may be used. In various embodiments, alcohols, esters, sulfated aliphatic alcohols, and the like may be used as surface active agents; sucrose, glucose, lactose, starch, crystallized cellulose, mannitol, light anhydrous silicate, magnesium aluminate, magnesium methasilicate aluminate, synthetic aluminum silicate, calcium carbonate, sodium acid carbonate, calcium hydrogen phosphate, calcium carboxymethyl cellulose, and the like may be used as excipients; magnesium stearate, talc, hardened oil and the like may be used as smoothing agents; coconut oil, olive oil, sesame oil, peanut oil, soya may be used as suspension agents or lubricants; cellulose acetate phthalate as a derivative of a carbohydrate such as cellulose or sugar, or methylacetate-methacrylate copolymer as a derivative of polyvinyl may be used as suspension agents; and plasticizers such as ester phthalates and the like may be used as suspension agents.

[0063] The term "pharmaceutical composition" refers to a mixture of a compound or combination of compounds disclosed herein with other chemical components, such as diluents or carriers. The pharmaceutical composition facilitates administration of the compound to an organism. Multiple techniques of administering a compound exist in the art including, but not limited to, oral, injection, aerosol, parenteral, and topical administration. Pharmaceutical compositions can also be obtained by reacting compounds with inorganic or organic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like.

[0064] The term "carrier" defines a chemical compound that facilitates the incorporation of a compound into cells or tissues. For example dimethyl sulfoxide (DMSO) is a commonly utilized carrier as it facilitates the uptake of many organic compounds into the cells or tissues of an organism.

[0065] The term "diluent" defines chemical compounds diluted in water that will dissolve the compound of interest as well as stabilize the biologically active form of the compound. Salts dissolved in buffered solutions are utilized as diluents in the art. One commonly used buffered solution is phosphate buffered saline because it mimics the salt conditions of human blood. Since buffer salts can control the pH of a solution at low concentrations, a buffered diluent rarely modifies the biological activity of a compound.

[0066] The term "physiologically acceptable" defines a carrier or diluent that does not abrogate the biological activity and properties of the compound.

[0067] The pharmaceutical compositions described herein can be administered to a human patient *per se*, or in pharmaceutical compositions where they are mixed with suitable carriers or excipient(s). Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, 18th edition, 1990.

[0068] Suitable routes of administration may, for example, include oral, rectal, transmucosal, topical, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intravenous, intramedullary injections, as well as intrathecal, direct intraventricular, intraperitoneal, intranasal, or intraocular injections. The compounds

can also be administered in sustained or controlled release dosage forms, including depot injections, osmotic pumps, pills, transdermal (including electrotransport) patches, and the like, for prolonged and/or timed, pulsed administration at a predetermined rate.

[0069] The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, *e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or tabletting processes.

[0070] Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen. Any of the well-known techniques, carriers, and excipients may be used as suitable and as understood in the art; *e.g.*, in Remington's Pharmaceutical Sciences, above.

[0071] Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride, and the like. In addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. Physiologically compatible buffers include, but are not limited to, Hanks's solution, Ringer's solution, or physiological saline buffer. If desired, absorption enhancing preparations (for example, liposomes), may be utilized.

[0072] For transmucosal administration, penetrants appropriate to the barrier to be permeated may be used in the formulation.

[0073] Pharmaceutical formulations for parenteral administration, *e.g.*, by bolus injection or continuous infusion, include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or other organic oils such as soybean, grapefruit or almond oils, or

synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

[0074] For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol,

and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

[0075] Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

[0076] For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

[0077] For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

[0078] Further disclosed herein are various pharmaceutical compositions well known in the pharmaceutical art for uses that include intraocular, intranasal, and intraauricular delivery. Suitable penetrants for these uses are generally known in the art. Pharmaceutical compositions for intraocular delivery include aqueous ophthalmic solutions of the active compounds in water-soluble form, such as eyedrops, or in gellan gum (Shedden et al., *Clin. Ther.*, 23(3):440-50 (2001)) or hydrogels (Mayer et al., *Ophthalmologica*, 210(2):101-3 (1996)); ophthalmic ointments; ophthalmic suspensions, such as microparticulates, drug-containing small polymeric particles that are suspended in a liquid carrier medium (Joshi, A., *J. Ocul. Pharmacol.*, 10(1):29-45 (1994)), lipid-soluble

formulations (Alm et al., *Prog. Clin. Biol. Res.*, 312:447-58 (1989)), and microspheres (Mordenti, *Toxicol. Sci.*, 52(1):101-6 (1999)); and ocular inserts. All of the above-mentioned references, are incorporated herein by reference in their entireties. Such suitable pharmaceutical formulations are most often and preferably formulated to be sterile, isotonic and buffered for stability and comfort. Pharmaceutical compositions for intranasal delviery may also include drops and sprays often prepared to simulate in many respects nasal secretions to ensure maintenance of normal ciliary action. As disclosed in Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Co., Easton, PA (1990), which is incorporated herein by reference in its entirety, and well-known to those skilled in the art, suitable formulations are most often and preferably isotonic, slightly buffered to maintain a pH of 5.5 to 6.5, and most often and preferably include antimicrobial preservatives and appropriate drug stabilizers. Pharmaceutical formulations for intraauricular delivery include suspensions and ointments for topical application in the ear. Common solvents for such aural formulations include glycerin and water.

[0079] The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

[0080] In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

[0081] For hydrophobic compounds, a suitable pharmaceutical carrier may be a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. A common cosolvent system used is the VPD co-solvent system, which is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant Polysorbate 80™, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the

co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of POLYSORBATE 80™; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, *e.g.*, polyvinyl pyrrolidone; and other sugars or polysaccharides may substitute for dextrose.

[0082] Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

[0083] Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external micro-environment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. The liposome may be coated with a tissue-specific antibody. The liposomes will be targeted to and taken up selectively by the desired organ. Alternatively, small hydrophobic organic molecules may be directly administered intracellularly.

[0084] Additional therapeutic or diagnostic agents may be incorporated into the pharmaceutical compositions. Alternatively or additionally, pharmaceutical compositions may be combined with other compositions that contain other therapeutic or diagnostic agents.

Methods of Administration

[0085] The compounds or pharmaceutical compositions may be administered to the patient by any suitable means. Non-limiting examples of methods of administration include, among others, (a) administration though oral pathways, which administration includes administration in capsule, tablet, granule, spray, syrup, or other such forms; (b) administration through non-oral pathways such as rectal, vaginal, intraurethral, intraocular, intranasal, or intraauricular, which administration includes administration as an aqueous suspension, an oily preparation or the like or as a drip, spray, suppository, salve, ointment or the like; (c) administration via injection, subcutaneously, intraperitoneally, intravenously, intramuscularly, intradermally, intraorbitally, intracapsularly, intraspinally, intrasternally, or the like, including infusion pump delivery; (d) administration locally such as by injection directly in the renal or cardiac area, e.g., by depot implantation; as well as (e) administration topically; as deemed appropriate by those of skill in the art for bringing the compound of the invention into contact with living tissue.

[0086] Pharmaceutical compositions suitable for administration include compositions where the active ingredients are contained in an amount effective to achieve its intended purpose. The therapeutically effective amount of the compounds disclosed herein required as a dose will depend on the route of administration, the type of animal, including human, being treated, and the physical characteristics of the specific animal under consideration. The dose can be tailored to achieve a desired effect, but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize. More specifically, a therapeutically effective amount means an amount of compound effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated. Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

[0087] As will be readily apparent to one skilled in the art, the useful *in vivo* dosage to be administered and the particular mode of administration will vary depending upon the age, weight and mammalian species treated, the particular compounds employed, and the specific use for which these compounds are employed. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, can

be accomplished by one skilled in the art using routine pharmacological methods. Typically, human clinical applications of products are commenced at lower dosage levels, with dosage level being increased until the desired effect is achieved. Alternatively, acceptable *in vitro* studies can be used to establish useful doses and routes of administration of the compositions identified by the present methods using established pharmacological methods.

[0088] In non-human animal studies, applications of potential products are commenced at higher dosage levels, with dosage being decreased until the desired effect is no longer achieved or adverse side effects disappear. The dosage may range broadly, depending upon the desired affects and the therapeutic indication. Typically, dosages may be between about 10 microgram/kg and 100 mg/kg body weight, preferably between about 100 microgram/kg and 10 mg/kg body weight. Alternatively dosages may be based and calculated upon the surface area of the patient, as understood by those of skill in the art.

[0089] The exact formulation, route of administration and dosage for the pharmaceutical compositions of the present invention can be chosen by the individual physician in view of the patient's condition. (See *e.g.*, Fingl *et al.* 1975, in "The Pharmacological Basis of Therapeutics", which is hereby incorporated herein by reference in its entirety, with particular reference to Ch. 1, p. 1). Typically, the dose range of the composition administered to the patient can be from about 0.5 to 1000 mg/kg of the patient's body weight. The dosage may be a single one or a series of two or more given in the course of one or more days, as is needed by the patient. In instances where human dosages for compounds have been established for at least some condition, the present invention will use those same dosages, or dosages that are between about 0.1% and 500%, more preferably between about 25% and 250% of the established human dosage. Where no human dosage is established, as will be the case for newly-discovered pharmaceutical compounds, a suitable human dosage can be inferred from ED_{50} or ID_{50} values, or other appropriate values derived from *in vitro* or *in vivo* studies, as qualified by toxicity studies and efficacy studies in animals.

[0090] It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity or organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if

the clinical response were not adequate (precluding toxicity). The magnitude of an administrated dose in the management of the disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

[0091] Although the exact dosage will be determined on a drug-by-drug basis, in most cases, some generalizations regarding the dosage can be made. The daily dosage regimen for an adult human patient may be, for example, an oral dose of between 0.1 mg and 2000 mg of each active ingredient, preferably between 1 mg and 500 mg, e.g. 5 to 200 mg. In other embodiments, an intravenous, subcutaneous, or intramuscular dose of each active ingredient of between 0.01 mg and 100 mg, preferably between 0.1 mg and 60 mg, e.g. 1 to 40 mg is used. In cases of administration of a pharmaceutically acceptable salt, dosages may be calculated as the free base. In some embodiments, the composition is administered 1 to 4 times per day. Alternatively the compositions of the invention may be administered by continuous intravenous infusion, preferably at a dose of each active ingredient up to 1000 mg per day. As will be understood by those of skill in the art, in certain situations it may be necessary to administer the compounds disclosed herein in amounts that exceed, or even far exceed, the above-stated, preferred dosage range in order to effectively and aggressively treat particularly aggressive diseases or infections. In some embodiments, the compounds will be administered for a period of continuous therapy, for example for a week or more, or for months or years.

[0092] Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the modulating effects, or minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

[0093] Dosage intervals can also be determined using MEC value. Compositions should be administered using a regimen which maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%.

[0094] In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

[0095] The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

[0096] Compounds disclosed herein can be evaluated for efficacy and toxicity using known methods. For example, the toxicology of a particular compound, or of a subset of the compounds, sharing certain chemical moieties, may be established by determining *in vitro* toxicity towards a cell line, such as a mammalian, and preferably human, cell line. The results of such studies are often predictive of toxicity in animals, such as mammals, or more specifically, humans. Alternatively, the toxicity of particular compounds in an animal model, such as mice, rats, rabbits, or monkeys, may be determined using known methods. The efficacy of a particular compound may be established using several recognized methods, such as *in vitro* methods, animal models, or human clinical trials. Recognized *in vitro* models exist for nearly every class of condition, including but not limited to cancer, cardiovascular disease, and various immune dysfunction. Similarly, acceptable animal models may be used to establish efficacy of chemicals to treat such conditions. When selecting a model to determine efficacy, the skilled artisan can be guided by the state of the art to choose an appropriate model, dose, and route of administration, and regime. Of course, human clinical trials can also be used to determine the efficacy of a compound in humans.

[0097] The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accompanied with a notice associated with the container in form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the drug

for human or veterinary administration. Such notice, for example, may be the labeling approved by the U.S. Food and Drug Administration for prescription drugs, or the approved product insert. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

EXAMPLES

Example 1 – General Procedures

[0098] Cell culture and reagents. Dex-sensitive MM.1S and Dex-resistant MM.1R human MM cell lines were obtained from Dr. Steven Rosen (Northwestern University, Chicago, IL). *See* Moalli, P. A., Pillay, S., Weiner, D., Leikin, R. & Rosen, S. T. (1992) Blood 79, 213-22 and Chauhan, D., Catley, L., Hideshima, T., Li, G., Leblanc, R., Gupta, D., Sattler, M., Richardson, P., Schlossman, R. L., Podar, K., Weller, E., Munshi, N. & Anderson, K. C. (2002) Blood 100, 2187-94; both of which are incorporated herein by reference in their entirety. RPMI-8226 and Doxorubicin (Dox)-resistant (Dox-40) cells were obtained from Dr. William Dalton (Moffit Cancer Center, Tampa, FL). U266 and OPM2 MM cell lines were obtained from the American Type Culture Collection (Rockville, MD). The human tumor cell lines DU 145, HT-29, Jurkat, LoVo, MDA-MB-231, MIA PaCa-2, NCI-H292, OVCAR-3, PANC-1, and PC-3 were purchased from ATCC (Manassas, VA). MM Cell lines were grown in RPMI-1640 media supplemented with 10% heat inactivated fetal-bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. MM cells were freshly isolated from patients relapsing after multiple prior therapies including Dexamethasone (Dex), melphalan, thalidomide or Bortezomib. MM cells were purified from patient bone marrow samples by CD138 positive selection method using CD138 (Syndecan-1) Micro Beads and the Auto MACS magnetic cell sorter (Miltenyi Biotec Inc., Auburn, CA). *See* Chauhan, D., Catley, L., Hideshima, T., Li, G., Leblanc, R., Gupta, D., Sattler, M., Richardson, P., Schlossman, R. L., Podar, K., Weller, E., Munshi, N. & Anderson, K. C. (2002) Blood 100, 2187-94; which is incorporated herein by reference in its entirety. Normal human skin fibroblasts CCD-27sk were obtained from ATCC and grown in DMEM supplemented with 10% heat inactivated FBS, 100 units/ml penicillin, 100 μg/ml

streptomycin, 4 mM L-glutamine and 1 mM sodium pyruvate. Cells were treated with various concentrations of the compound of formula II (X=Cl) (Nereus Pharmaceuticals, Inc, San Diego, CA), Bortezomib or Dex (Sigma Chemical Co, St. Louis, MO).

[0099] Cell viability and apoptosis assays. Cell viability was assessed by 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Chemicon International Inc., Temecula, CA) assay, according to manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN), and as described in Chauhan, D., Catley, L., Hideshima, T., Li, G., Leblanc, R., Gupta, D., Sattler, M., Richardson, P., Schlossman, R. L., Podar, K., Weller, E., Munshi, N. & Anderson, K. C. (2002) Blood 100, 2187-94; which is incorporated herein by reference in its entirety. Cell Death Detection ELISAplus was utilized to quantitate cell death, as per manufacturer's instructions (Roche Applied Sciences, Indianapolis, IN).

Example 2 – *In vitro* 20S proteasome activity assay

[0100] The chymotrypsin-like activity of the 20S proteasome was measured as described in Stein, R. L., Melandri, F. & Dick, L. (1996) Biochemistry 35, 3899-908 and Lightcap, E. S., McCormack, T. A., Pien, C. S., Chau, V., Adams, J. & Elliott, P. J. (2000) Clin Chem 46, 673-83; both of which are incorporated herein by reference in their entirety. Purified human erythrocyte-derived 20S proteasome were obtained from Biomol, Plymouth Meeting, PA. The chymotrypsin-like, caspase-like and trypsin-like activity activities of the 20S proteasome were determined using Suc-LLVY-AMC, Z-LLE-AMC (Boston Biochem, Cambridge, MA) and Boc-LRR-AMC (Bachem Bioscience, King of Prussia, PA) as peptide substrates, respectively. Fluorescence of the cleaved peptide substrate was measured using a Fluoroskan Ascent 96-well microplate reader (Thermo Electron, Waltham, MA). The EC_{50} values were calculated by Prism (GraphPad Software) using a sigmoidal dose-response, variable slope model. The EC_{50} values were defined as the drug concentration at which 50% of the maximal relative fluorescence is inhibited. The results, plotted in Figure 1, indicated that the compounf of formula (II) (X=Cl) inhibits all three proteasome activities, albeit at different concentrations.

Example 3 – Analysis of *ex vivo* 20S proteasome activity in whole blood cells in mice (single i.v. or oral administration)

[0101] To directly determine whether the compound of formula (II) (X=Cl) inhibits proteasome activity *in vivo*, the compound of formula (II) (X=Cl) was dissolved in 100% DMSO and serially diluted with 5% Solutol (Solutol® HS 15; polyethylene glycol 660 12- hydroxystearate, BASF, Shreveport, LA) yielding a final concentration of 2% DMSO. The vehicle control consisted of 2% DMSO and 98% (5% Solutol® HS15). Male Swiss-Webster mice (five per group, 20-25 grams in weight) were treated at Bolder BioPATH, Inc. (Boulder, CO) with various concentrations of the compound either intravenously or orally at a volume of 10 mL/kg. One group of animals was untreated to establish a baseline of proteasome activity. Ninety minutes after administration of the compound, the animals were anesthetized and blood withdrawn by cardiac puncture. Packed whole blood cells were collected by centrifugation, washed with PBS, and frozen on dry ice for determination of *ex vivo* proteasome activity. Chymotrypsin-like activity of the 20S proteasome in white blood cell (WBC) lysates was determined using the peptide substrate suc-LLVY-AMC. Relative Fluoresence Units (RFU) were normalized using the protein concentrations of the cell lysates. The 20S proteasome activity of the individual mice is shown in Figure 2 with the horizontal bar representing the average activity. Baseline represents the 20S proteasome activity observed in WBC lysates prepared from untreated mice. The results, depicted in Figure 2, indicte that the compound of formula (II) (X=Cl) inhibits chymotrypsin-like activity of 20S proteasomes in white blood cells in a dose-dependent manner. Importantly, these findings establish that the compound is orally active and inhibits proteasome activity *in vivo*.

Example 4 – Determination of triggered alterations in proteasome activity in MM cells (*in vitro*)

[0102] Determination of whether the compound of formula II (X=Cl) affects the proteasome activity in multiple myeloma cells *in vitro* was made using a competition experiment with $AdaY^{125}Iahx_3L_3VS$. In this assay, sites that are not targeted by the compound of formula II (X=Cl) are labeled by $AdaY(^{125}I)Ahx_3L_3VS$ and visualized by autoradiography, while sites that are targeted by the compound of formula II (X=Cl) can not be seen on the autoradiogram. MM.1S MM cells were incubated with the compound of formula (II) (X=Cl) (7 nM) for 30 mins, 1h, 3h, or 6h, and cell lysis was performed with glass beads. 60 μg of protein extracts was incubated for 2h with the iodinated proteasome inhibitor

AdaY^{125}Iahx$_3$L$_3$VS at 37°C. Proteins were then denatured by boiling in reducing sample buffer and separated on a 12.5% SDS-PAGE gel, followed by autoradiography. As can be seen in Figure 3, the beta-5 (β-5) subunit of the proteasome is markedly less labeled by AdaY(^{125}I)Ahx$_3$L$_3$VS in treated cells than control cells. Given that the β-5 subunit mediates the chymotrypsin-like activity, these results suggest that the compound of formula (II) (X=Cl) binds to the β-5 subunit, thereby inhibiting the chymotrypsin-like activity in MM.1S cells. Moreover, treatment of MM.1S cells with the compound (7 nM) for 6h also decreased the labeling of the β-2 subunits (tryptic-activity) and the β-1 subunits (caspase-like activity) (data not shown).

Example 5 – Determination of triggered alterations in proteasome activity in MM cells (*in vivo*)

[0103] *In vivo* determination of proteasome activity was conducted using a competition experiment with Dansyl-Ahx$_3$L$_3$VS, which covalently modifies all active proteasome subunits. This inhibitor contains a dansyl sulfonamide hexanoyl hapten that can be visualized by immunoblotting using antibodies agsint the dansyl moiety. MM.1S cells were treated with the compound of formula (II) (X=Cl) (7 nM) for 30 mins, 1h, or 3h, followed by 1h incubation with 5 μM Dansyl-Ahx$_3$L$_3$VS at 37°C. Cells were lysed by incubating them for 30 mins in NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40), followed by 5 min. centrifugation to remove membrane fractions, nuclei, and cell debris. 60 μg of protein extract was separated by 12.5% SDS-PAGE gel, followed by Immunoblot analysis using polyclonal anti-dansyl polyclonal Ab (1:7500, rabbit, Molecular Probes) and horseradish peroxidase coupled goat anti-rabbit secondary antibody (Southern Biotech). Blots were developed by enhanced chemiluminescence (Western Lightning, Perkin-Elmer). As can be seen in Figure 4, treatment of MM.1S cells with the compound of formula (II) (X=Cl) decreases the dansylAhx$_3$L$_3$VS-labeling of the β-5 subunits. Furthermore, the compound also decreased the dansylAhx$_3$L$_3$VS-labeling of the β-1 and β-2 subunits, albeit at higher concentrations: 1 nM and 20 nM, respectively. In contrast, treatment of MM.1S cells with even higher doses of Bortezomib does not inhibit the β-2 subunits (data not shown). Taken together, these findings demonstrate the ability of the compound of formula (II) (X=Cl) to inhibit all three proteasome activities in MM cells.

Example 6 – Effect on MM cell viability

[0104] Cell viability was assessed by 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Chemicon International Inc., Temecula, CA) assay, according to manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN), and as described in Chauhan, D., Catley, L., Hideshima, T., Li, G., Leblanc, R., Gupta, D., Sattler, M., Richardson, P., Schlossman, R. L., Podar, K., Weller, E., Munshi, N. & Anderson, K. C. (2002) Blood 100, 2187-94; which is incorporated herein by reference in its entirety. Cell viability after treatment of MM.1S (-■-), Dex-resistant MM.1R (-□-), RPMI-8226 (-●-), Doxorubicin-resistant Dox-40 (-◆-), OPM2 (-○-), and U266 (-◊-) cells with the compound of formula (II) (X=Cl) for 24h is illustrated in Figure 5A. Results are mean ± S.D from three independent experiments ($P < 0.005$; $n = 3$ for all cell lines). A dose-dependent significant decrease in cell viability in all cell lines was observed (IC_{50} range 7-24 nM).

[0105] Cell viability was also assessed on purified patient MM cells. Freshly isolated tumor cells from nine MM patients relapsing after multiple prior therapies including Dex, Bortezomib, and thalidomide were treated with the compound of formula (II) (X=Cl) (10 nM) for 24h and analyzed for apoptosis. As seen in Figure 5B, significant apoptosis was observed in these cells as measured by DNA fragmentation assays ($P < 0.005$; n=2). Plotted values are the mean ± SD of triplicate samples. Importantly, 4 of 9 patients examined were refractory to Bortezomib therapy, and 5 patients were resistant to Thalidomide and Dex therapies. These data suggest that 1) the compound of formula (II) (X=Cl) induces apoptosis in MM cells sensitive and resistant to conventional and Bortezomib therapies; and 2) IC_{50} of the compound for MM cells is within the nanomolar concentration.

Example 7 – Effect on Bone Marrow Stromal Cell (BMSCs) viability

[0106] MM cells predominantly localize in the bone marrow microenvironment and their interaction with BMSCs induces production of cytokines which mediate growth of MM cells, as well as protect against drug-induced apoptosis. *See* Anderson, K. C. (2003) *Cancer* **97**, 796-801; which is incorporated herein by reference in its entirety. Therefore, the effect of the compound of formula (II) (X=Cl) on five patient MM-derived BMSCs was determined. As seen in Figure 6, treatment of BMSCs (Patient#1-5) with the compound of formula (II) (X=Cl) (20 nM) for 24h does not induce apoptosis in these cells, as evidenced by

DNA fragmentation assay. Positive control shown is an internal control for the assay. Purified MM cells (CD138+) from two of the five MM patient were also examined within the same experiments. Results are mean ± SD from triplicate samples. The compound triggered a significant (10-12 fold) increase in apoptosis of purified (CD138-positive) patient MM cells. These results suggest that the compound of formula (II) (X=Cl) acts directly on MM cells, but not BMSCs.

Example 8 – Effect of recombinant human interleukin-6 (rhIL-6) and recombinant human insulin-like growth factor-I (rhIGF-I) anti-apoptotics

[0107] Adhesion of MM cells to BMSCs induces IL-6 and IGF-I secretion from BMSCs, which not only regulates the growth of MM cells, but also protects against chemotherapy. *See* Hardin, J., MacLeod, S., Grigorieva, I., Chang, R., Barlogie, B., Xiao, H. & Epstein, J. (1994) *Blood* **84,** 3063-70 and Chauhan, D., Kharbanda, S., Ogata, A., Urashima, M., Teoh, G., Robertson, M., Kufe, D. W. & Anderson, K. C. (1997) *Blood* **89,** 227-234; both of which are incorporated herein by reference in their entirety. Thus, whether rhIL-6 or rhIGF-I inhibits apoptosis in MM cells induced by the compound of Formula (II) (X=Cl) was evaluated. MM.1S cells were treated with the compound of formula (II) (X=Cl) (7 nM) or Dex (0.5 μM) for 24h, in the presence and absence of rhIL-6 (10 ng/ml) or rhIGF (50 ng/ml). At 24h cells were harvested and viability analyzed by MTT assays. As seen in Figure 7, the median cell viability was 47 ± 2.3% after treatment with the compound alone; 51.2 ± 3.2% with the compound + rhIL-6 (P = 0.26, Wilcoxon test), and 50.3% ± 2.0% with the compound + rhIGF-I (P = 0.28). Median viability was 51 ± 2.1% after treatment with Dex and 92 ± 5.5% for Dex + rhIL-6 (P = 0.05, as determined by one-sided Wilcoxon rank-sum test). Results are mean ± SD of three independent experiments. These findings suggest that neither IL-6 nor IGF-I block the anti-MM activity of the compound of formula (II) (X=Cl). In contrast and as in other studies, both IL-6 and IGF-I block Dex-induced decreased MM.1S cell viability. *See* Chauhan, D., Hideshima, T. & Anderson, K. C. (2003) *Int J Hematol* **78,** 114-20 and Mitsiades, C. S., Mitsiades, N., Poulaki, V., Schlossman, R., Akiyama, M., Chauhan, D., Hideshima, T., Treon, S. P., Munshi, N. C., Richardson, P. G. & Anderson, K. C. (2002) *Oncogene* **21,** 5673-83; both of which are incorporated herein by reference in their entirety. Thus, the data suggests that the compound of formula (II) (X=Cl)

overcomes the growth and protective effects of IL-6 and IGF-I on MM cells, and indicate distinct mechanisms of action for the compound and Dex against MM cells. Reports that high serum levels of IL-6 contribute to clinical chemoresistance and treatment failure, coupled with the ability of the compound of formula (II) (X=Cl) to induce MM cell apoptosis even in the presence of IL-6 or IGF-I, suggest that the compound may overcome drug resistance in patients with advanced MM. *See* Kyrstsonis, M. C., Dedoussis, G., Baxevanis, C., Stamatelou, M. & Maniatis, A. (1996) *Br J Haematol* **92**, 420-422; which is incorporated herein by reference in its entirety.

Example 9 – Effect on vascular endothelial growth factor (VEGF) induced migration of MM cells

[0108] VEGF is elevated in the bone marrow microenvironment and triggers migration, growth, and angiogenesis in MM cells. *See* Podar, K., Tai, Y. T., Lin, B. K., Narsimhan, R. P., Sattler, M., Kijima, T., Salgia, R., Gupta, D., Chauhan, D. & Anderson, K. C. (2002) *J Biol Chem* **277**, 7875-81; which is incorporated herein by reference in its entirety. Thus, whether the compound of formula (II) (X=Cl) alters VEGF-induced migration of MM cells was evaluated. VEGF induced migration was examined in the presence or absence of the compound (7 or 10 nM). Cell migration was assayed as described previously in Podar, K., Tai, Y. T., Davies, F. E., Lentzsch, S., Sattler, M., Hideshima, T., Lin, B. K., Gupta, D., Shima, Y., Chauhan, D., Mitsiades, C., Raje, N., Richardson, P. & Anderson, K. C. (2001) *Blood* **98**, 428-35; which is incorporated herein by reference in its entirety. As shown in Figure 8, the compound of formula (II) (X=Cl) significantly ($P < 0.05$) decreases VEGF-induced migration of MM.1S MM cells. These findings indicate that the compound may negatively regulate both homing of MM cells to the bone marrow and their egress into the peripheral blood.

Example 10 – Effect on Bcl2-mediated protective effects

[0109] Bcl2 confers resistance to conventional therapies in cancer cells, including MM. *See* Cory, S. & Adams, J. M. (2002) *Nat Rev Cancer* **2**, 647-56 and Gazitt, Y., Fey, V., Thomas, C. & Alvarez, R. (1998) *Int J Oncol* **13**, 397-405; both of which are incorporated herein by reference in their entirety. Bcl2 can modestly attenuate Bortezomib-induced apoptosis. Thus, whether ectopic expression of Bcl2 in MM.1S cells affects responsiveness

to the compound of formula (II) (X=Cl) was evaluated. MM.1S cells were stably transfected with Bcl2 construct and analyzed for alterations in cell viability using an MTT assay. As seen in Figure 9, the compound of formula (II) (X=Cl) significantly decreases cell viability of Bcl2-transfected MM.1S cells ($P < 0.005$) in a dose-dependent manner. Nonetheless, the compound induced 15 ± 1.1 % less cell death in Bcl2-transfected cells compared to empty vector-transfected MM.1S cells. Results are mean $\pm$ SD of three independent experiments. These findings suggest that the compound can overcome Bcl2-mediated protection.

Example 11 - *In vivo* evaluation in murine tumor model

[0110] Six-week-old triple immune deficient beige-nude-xid (BNX) mice were obtained from Frederick Cancer Research and Development Center (Frederick, MD). All animal studies were conducted according to protocols approved by the Animal Ethics Committee of the Dana-Farber Cancer Institute. Mice were observed daily for signs of toxicity. Terminal bleeding was done under anesthesia using isoflourane inhalation, and animals were sacrificed by CO_2 asphyxiation. To determine the *in vivo* anti-MM activity of the compound of formula (II) (X=Cl), 21 BNX mice were inoculated subcutaneously in the flank with $3x10^7$ RPMI 8226 MM cells in 100 µl of RPMI-1640 media. When tumors became measurable, mice were assigned to treatment groups receiving the compound of formula (II) (X=Cl) 0.25 mg/kg (n = 7), 0.5 mg/kg (n = 7), or to control groups (n = 7) receiving the vehicle only. Drug treatment was started after the development of measurable tumor. The drug (0.25 mg/kg or 0.5 mg/kg) was given orally twice a week. Serial caliper measurements of perpendicular diameters were done every other day to calculate tumor volume, using the following formula: 4/24 x (shortest diameter)2 x (longest diameter). Animals were sacrificed if the tumor was $\geq$ 2 cm or necrotic. For tumor growth studies, 7 mice were used in each group.

[0111] As seen in Figures 10A-C, treatment of tumor bearing mice with the compound of formula (II) (X=Cl), but not with vehicle alone, significantly inhibits MM tumor growth and prolongs survival of these mice. All mice in the control group developed progressive tumors, whereas complete regression of tumors were observed in 70% of treated mice. The mouse on the upper panel of Figure 10B received oral doses of vehicle alone, whereas the mouse on the lower panel received the compound of formula (II) (X=Cl) (0.25

mg/kg). The left panels in Figure 10B are enlargements of subcutaneous plasmacytomas growing on the right flanks of the mice. Survival was evaluated from the first day of treatment until death; mice were sacrificed when their tumor diameters reached 2 cm or they became moribund (Figure 10C). Moreover, no neurological behavioral changes were observed even after 12 weeks of treatment. The concentrations of the compound administered were well tolerated by mice, without evidence of weight loss. Mice in both untreated and treated group were weighed every week. The average changes in the mice body weight are shown in Figure 10D.

[0112] Analysis at day 300 showed no recurrence of tumor in 57% of the compound of formula (II) (X=Cl)-treated mice (Figure 10C). In addition, histologic analysis performed on the inoculation sites confirmed the disappearance of plasma cells in the compound of formula (II) (X=Cl)- versus vehicle-treated mice (Figure 10E, left and right panels, respectively). These data show that the compound is orally active; inhibits MM tumor growth *in vivo*; and prolongs survival.

Example 12 – Comparative analysis of *in vivo* antitumor activity

[0113] To compare the *in vivo* activity of the compound of formula (II) and Bortezomib, the mice models as described above were treated with the compound of formula (II) (X=Cl) (0.15 mg/kg i.v.) or Bortezomib (1.0 mg/kg i.v.) twice weekly. Both agents significantly reduced the tumor progression ($p < 0.01$) and prolonged survival ($p = 0.0137$) (Figures 10F and 10G).

Example 13 – Mechanisms mediating anti-MM activity

[0114] Mitochondria play a critical role in apoptosis induction during stress. *See* Bossy-Wetzel, E. & Green, D. R. (1999) *Mutat Res* **434**, 243-51 and Chauhan, D. & Anderson, K. C. (2003) *Apoptosis* **8**, 337-43; both of which are incorporated herein by reference in their entirety. Serum starved MM.1S cells were treated with the compound of formula (II) (X=Cl) (7 nM) for 12h and incubated with CMXRos for the last 20 min; stained with lipophilic cationic dye CMXRos (Mitotracker Red) (Molecular Probes, Eugene, OR) in phosphate-buffered saline (PBS) for 20 mins at 37°C; and analyzed by flow cytometry to assay for alterations in $\Delta\Psi m$ (mitochondrial membrane potential). Superoxide (O_2^-) production was measured by staining cells with membrane permeable dye dihydroethidium

(HE) for the last 15 min. Superoxide anions oxidize HE to fluorescent ethidium, permitting analysis by flow cytometry.

[0115] As seen in Figures 11A and 11B, the compound of formula (II) (X=Cl) decreases $\Delta\Psi m$, evidenced by an increased number of CMXRos negative cells ($P < 0.005$, $n = 2$), and triggers O_2^- production in MM.1S cells. Results are mean $\pm$ SD of two independent experiments. Alterations in $\Delta\Psi m$ are associated with release of mitochondrial proteins cyto-c and Smac to the cytosol, thereby triggering caspase 9 and caspase-3. *See* Du, C., Fang, M., Li, Y., Li, L. & Wang, X. (2000) *Cell* **102,** 33-42 and Liu, X., Naekyung Kim, C., Yang, J., Jemmerson, R. & Wang, X. (1996) *Cell* **86,** 147-157; both of which are incorporated herein by reference in their entirety.

[0116] As seen in Figure 11C, treatment of MM.1S cells with compound of formula (II) (X=Cl) triggers a decrease in mitochondrial cyto-c (upper, left panel) and smac (upper, right panel), and a concurrent increase of these proteins in the cytosolic fractions (middle, left and right panels, respectively). Reprobing the immunoblots with anti-Hsp60 (lower, left panel) and anti-tubulin (lower, right panel) Abs confirms purity of mitochondrial extracts and equal protein loading. Release of mitochondrial apoptogenic proteins cyto-c and Smac/DIABLO induce activation of caspases-9 and –3. MM.1S cells were treated with the compound of formula (II) (X=Cl) (7 nM) for 24h and harvested; mitochondrial and cytosolic protein fractions were separated by 12.5% SDS-PAGE and analyzed by immunoblotting with anti-cyto-c (upper panel) or anti- Smac (middle panel) Abs. As a control for equal loading of proteins and purity of mitochondrial fractions, filters were also reprobed with anti-tubulin (lower right panel) and anti-Hsp60 Abs (lower left panel), respectively. Blots are representative of three independent experiments.

[0117] MM.1S cells were treated with the compound of formula (II) (X=Cl) (7 nM) for 24h and harvested; cytosolic proteins were separated by 12.5% SDS-PAGE and analyzed by immunoblotting with anti-caspase-8 Abs and anti-caspase-9 Abs. As seen in Figure 11D, treatment of MM.1S cells with the compound of formula (II) (X=Cl) induces proteolytic cleavage of caspase-9. Moreover, the compound also activates caspase-8 (Figure 11E). Both caspase-9 (mitochondria-dependent) and caspase-8 (mitochondria-independent) are known to proteolytically cleave and activate a common downstream effector capsase-3,

resulting in PARP cleavage. *See* Miller, L. K. (1999) *Trends Cell Biol* **9**, 323-8; which is incorporated herein by reference in its entirety. Thus, MM.1S or MM.1R MM cells were treated with the compound of formula (II) (X=Cl) (7 nM) for 24h and assessed for apoptosis by both PARP and caspase-3 cleavage assays. Total protein lysates were subjected to SDS-PAGE analysis. Immunoblot analysis of the lysates was performed with anti-PARP (upper panel) or anti-caspase-3 (lower panel) Abs. 'FL' indicates 'full length' and 'CF' denotes cleaved fragment. This data further shows that the compound of formula (II) (X=Cl) triggers caspase-3 and PARP cleavage (Figure 11F).

[0118] Immunoblot analysis was performed using antibodies to cytochrome-c, Smac, Caspase-8, -9, or -3 (Cell Signaling, Beverly, MA), tubulin (Sigma, St. Louis, MO), PARP, Hsp60, or Bax (BD Bioscience Pharmingen, San Diego, CA). Blots were developed by enhanced chemiluminesence (ECL; Amersham, Arlington Heights, IL).

Example 14 – Mechanistic differences of MM cell apoptosis compared to bortezomib

[0119] MM.1S cells were treated with the compound of formula (II) (X=Cl) or Bortezomib in the presence or absence of caspase-9 inhibitor (LEHD-FMK), caspase-8 inhibitor (IETD-fmk) or caspase-3 inhibitor (Z-Val-Ala-Asp-fluoromethylketone, z-VAD-fmk). As seen in Figure 12A, caspase-3 inhibition markedly abrogates both the compound of formula (II) (X=Cl) and Bortezomib-induced apoptosis. Results are mean $\pm$ SD of four independent experiments ($P < 0.004$). Blockade of caspase-8 led to a significant decrease in cell death triggered by the compound of formula (II) (X=Cl) ($P < 0.005$, $n = 4$), whereas inhibition of caspase-9 only moderately blocked decreased viability in MM.1S cells triggered by the compound. In contrast, Bortezomib-induced decrease in viability of MM.1S cells is equally blocked in the presence of either caspase-8 or caspase-9 inhibitor ($P < 0.005$). Together, these data suggest that caspase-8 and caspase-9 activation equally contribute during Bortezomib-triggered cell death, whereas apoptosis trigerred by the compound of formula (II) (X=Cl) proceeds primarily *via* caspase-8 signaling pathway.

[0120] These biochemical data were confirmed by genetic studies using dominant-negative (DN) strategies. MM.1S cells were also transiently transfected using Cell line Nucleofecto kit V, according to the manufacturer's instructions (Amaxa Biosystems, Germany), with vector alone, DN-caspase-8, DN-caspase-9, or DN-FADD and cotransfected

with vector containing green fluorescence protein (GFP) alone. Following transfections, GFP-positive cells were selected by flow cytometry, treated with the compound of formula (II) (X=Cl) or Bortezomib, and analyzed for viability. Treatment of DN-caspase-8-transfected MM cells with the compound of formula (II) (X=Cl) (IC50, 7 nM) markedly increased survival of these cells, compared to the cells transfected with DN-caspase-9 (Figure 12B). In contrast, treatment of either DN-caspase-8 or DN-caspase-9-tranfected MM.1S cells with Bortezomib (IC50, 5 nM) increased the survival to a similar extent. The functional specificity of DNcaspase-8 and DN-caspase-9 was confirmed by treatment of MM.1S cells with known inducers of caspase-9 (Dex) and caspase-8 in these cells (anti-Fas MoAb) (Chauhan et al., 1997) (Figure 12C). These data suggest that (1) compound of formula (II) (X=Cl)-induced MM cell apoptosis is predominantly mediated by caspase-8; and (2) Bortezomib-induced apoptosis requires both caspase-8 and caspase-9 activation.

[0121] It was next determined whether inhibition of an upstream signaling pathway that leads to caspase-8 activation affects the response to the compound of formula (II) (X=Cl) or Bortezomib. The Fas-associated death domain (FADD) protein is an important part of the deathinducing signaling complexes (DISCs) that assemble upon engagement of TNF receptor family members, such as Fas, resulting in proteolytic processing and autoactivation of pro-caspase-8. Since both the compound of formula (II) (X=Cl) and Bortezomib trigger caspase-8 activation, the role of FADD during this event in MM cells was evaluated using DN-FADD. Blockade of FADD with DN-FADD significantly attenuated compound of formula (II) (X=Cl)-induced cytotoxicity compared to the empty vector-transfected MM.1S cells (42% ± 2.0% viable cells in vector-transfected cells versus 76% ± 5.1% viable cells in DN-FADD-transfected cells; $p < 0.05$) (Figure 12D). DN-FADD decreased compound of formula (II) (X=Cl)-induced caspase-8 activation; however, minimal caspase-8 activation was still noted (data not shown), which may be due to upstream activators of caspase-8 other than FADD. Importantly, treatment of DN-FADD-transfected MM.1S cells with Bortezomib resulted in only a 16% increase in survival compared to vector-transfected cells (39% ± 2.4% viable cells in vector-transfected cells versus 55% ± 4.1% viable cells in DN-FADD-transfected cells; $p < 0.05$) (Figure 12D). These data, coupled with caspase-8 or caspase-9 inhibition studies, suggest that the compound of formula (II)

relies more on FADD-caspase-8 signaling axis than does Bortezomib, further confirming differential mechanism of action of the compound of formula (II) versus Bortezomib in MM cells.

[0122] Previous studies have established that Bax induces mitochondrial apoptotic pathway. *See* Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B. & Korsmeyer, S. J. (2001) Science **292**, 727-30 and Lei, K., Nimnual, A., Zong, W. X., Kennedy, N. J., Flavell, R. A., Thompson, C. B., Bar-Sagi, D. & Davis, R. J. (2002) Mol Cell Biol **22**, 4929-42; both of which are incorporated herein by reference in their entirety. Thus, whether MM cell apoptosis induced by the compound of formula (II) (X=Cl) correlates with alterations in Bax was evaluated. MM.1S MM cells were treated with either the compound of formula (II) (X=Cl) or Bortezomib and mitochondrial protein extracts were subjected to immunoblot analysis with anti-Bax or anti-Hsp60 Abs. As seen in Figure 12E, the compound of formula (II) (X=Cl) induces little, if any increase in Bax levels in mitochondria. Blots are representatives of three independent experiments. Importantly, Bortezomib triggers a significant accumulation of Bax in mitochondria.

[0123] Mouse embryonic fibroblast (MEFs) carrying wild-type Bax or knock-outs were treated with the compound of formula (II) (X=Cl) or Bortezomib for 48h and analyzed for cell viability by MTT assays. As seen in Figure 12F, the compound of formula (II) (X=Cl) decreases viability in both Bax (WT) and Bax (knock-out), whereas deletion of Bax confers significant resistance to Bortezomib. Results are mean $\pm$ SD of three independent experiments ($P < 0.05$). These data show the differential requirement of Bax during apoptosis induced by the compound of formula (II) (X=Cl) and Bortezomib and suggest distinct mechanism of action of these agents.

Example 15 – Differential effects on normal lymphocytes as compared to bortezomib

[0124] Bortezomib therapy is associated with toxicity in patients. Thus, the effects of the compound of formula (II) (X=Cl) and Bortezomib on normal cells was compared. Lymphocytes from five healthy donors were treated with various concentrations (0-20 nM) of the compound of formula (II) (X=Cl) or Bortezomib (0-20 nM) for 72h and analyzed for cytotoxicity by an MTT assay. As seen in Figure 13, the compound of formula

(II) (X=Cl) does not significantly decrease the survival of normal lymphocytes (P = 0.27 from J-T trend test), even at higher doses (20 nM). Results are the mean ± SD of three independent experiments. In contrast, Bortezomib significantly decreases the viability of lymphocytes even at lower concentrations of 6-10 nM. Of note, IC_{50} of patient MM cells is reached at concentrations of the compound of formula (II) (X=Cl) that have no effect on normal lymphocytes, whereas IC_{50} of Bortezomib for MM cells triggers 50% decrease in viability of normal lymphocytes. Together, these data suggest that the compound of formula (II) (X=Cl) has selective anti-MM activity; and particularly, it is less toxic to normal cells than Bortezomib.

[0125] Whether the compound of formula (II) or Bortezomib alters proteasome activity in normal lymphocytes and skin fibroblasts was also examined. Both the compound of formula (II) (X=Cl) and Bortezomib significantly inhibited proteasome activity in these cells: 20 nM of the compound of formula (II) (X=Cl) or Bortezomib triggered 99% or 59 ± 11% inhibition of Chymotrypsin-like proteasome activity, respectively (data not shown). Thus, although 20 nM of the compound of formula (II) (X=Cl) did not trigger significant cytotoxicity in normal lymphocytes, it reduced Chymotrypsin-like proteasome activity in these cells. Similarly, treatment of normal CCD-27sk fibroblasts at the IC50 for the compound of formula (II) (X=Cl) (317 nM) or Bortezomib (15 nM) also inhibited proteasome activity (data not shown).

Example 16 – Differential effects on Bcl-2-overexpressing MM cells as compared to bortezomib

[0126] During apoptosis Bax neutralizes the antiapoptotic function of Bcl-2, thereby facilitating the cyto-c release and caspase-9 activation. Bcl-2 also confers drug resistance in cancer cells, including MM, and provides partial protection against Bortezomib-induced killing. Therefore, whether ectopic expression of Bcl-2 in MM.1S cells affects the ability of the compound of formula (II) or Bortezomib to trigger cytotoxicity and postmitochondrial apoptotic signaling in MM cells was evaluated. Overexpression of Bcl-2 promoted a modest increase in viability of cells treated with both agents: for the compound of formula (II) (X=Cl), 50% ± 2.6% viability in Bcl-2-transfected cells versus 39% ± 1.5% viability in vector-transfected cells ($p < 0.05$); and for Bortezomib, 61% ± 2.9% viability in

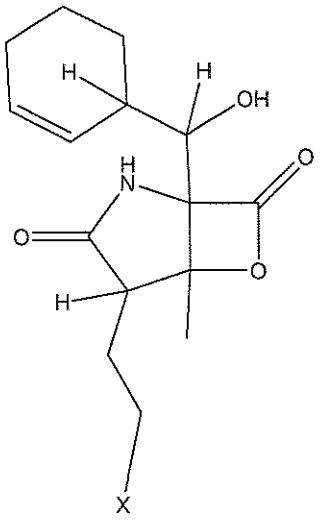
Bcl-2-transfected cells versus 40% ± 2.1% viability in vector-transfected cells ($p < 0.05$) (Figure 14A). The increased survival of Bcl-2 transfectants in response to Bortezomib was greater (21%) than that in response to the compound of formula (II) (X=Cl) (11%) ($p < 0.04$; $n = 3$) (Figure 14A). Moreover, Bortezomib triggered significant caspase-9 cleavage in control vector-transfected cells, which is markedly attenuated (3-fold decrease by densitometry) in Bcl-2-transfected cells; in contrast, compound of formula (II) (X=Cl)-induced caspase-9 cleavage is minimally affected by Bcl-2 overexpression (Figure 14B). These findings, together with the viability results, suggest that Bcl-2 provides more protection against Bortezomib than the compound of formula (II).

Example 17 – Combination treatement

[0127] As seen in Figure 15, treatment of MM.1S or MM.1R MM cells with the compound of formula (II) (X=Cl) in combination with Bortezomib for 24h induces synergistic growth inhibition. Results are mean ± SD of three independent experiments ($P < 0.005$). The interaction between anti-MM agents formula (II) (X=Cl) and Bortezomib was analyzed using isobologram analysis with "CalcuSyn" software program (Biosoft, Ferguson, MO and Cambridge, UK). Data from cell viability assay (MTT) were expressed as fraction of cells with growth affected (FA) in drug-treated *versus* untreated cells. The CalcuSyn program is based upon the Chou-Talalay method according to the following equation: "CI = (D)1/(Dx)1 + (D)2/(Dx)2 + (D)1(D)2/(Dx)1(Dx)2", where (D)1 and (D)2 are the doses of drug 1 and drug 2 that have x effect when used in combination; and (Dx)1 and (Dx)2 are the doses of drug 1 and drug 2 that have the same x effect when used alone. When CI = 1, this equation represents the conservation isobologram and indicates additive effects. CI values of < 1.0 indicate synergism. A combination index (CI) of < 1.0 was obtained for Bortezomib + NPI-0052, indicating synergism. Moreover, maximal anti-MM activity was observed when given concomitantly, rather than other treatment schedules. Low doses of combined compound of formula (II) (X=Cl) and Bortezomib does not significantly affect viability of normal PBMNCs (data not shown). Combination therapy with Bortezomib and the compound of formula (II) (X=Cl) therefore may: 1) allow use of sub-toxic concentrations of each agent; 2) delay or prevent development of drug-resistance; and 3) permit escalating synergistic doses of these agents to increase the apoptotic threshold.

WHAT IS CLAIMED IS:

1. Use of a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof:

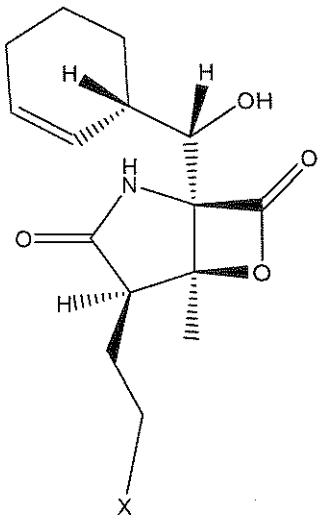


(I)

in the manufacture of a medicament for treating a neoplastic disease in a patient, wherein X is selected from the group consisting of fluorine, chlorine, bromine or iodine, and wherein the neoplastic disease is susceptible to resistance to at least one other chemotherapeutic agent.

2. The use of claim 1, wherein X is chlorine.

3. The use of claim 1, wherein the compound of formula (I) has the structure of formula (II):



(II)

4. The use of claim 1, wherein the neoplastic disease is cancer.

5. The use of claim 4, wherein the cancer is selected from the group consisting of breast cancer, sarcoma, leukemia, ovarian cancer, uretal cancer, bladder cancer, prostate cancer, colon cancer, rectal cancer, stomach cancer, lung cancer, lymphoma, multiple myeloma, pancreatic cancer, liver cancer, kidney cancer, endocrine cancer, skin cancer, melanoma, angioma, and brain or central nervous system (CNS) cancer.

6. The use of claim 5, wherein the cancer is selected from the group consisting of multiple myeloma, colorectal carcinoma, prostate carcinoma, breast adenocarcinoma, non-small cell lung carcinoma, and an ovarian carcinoma or melanoma.

7. The use of claim 6, wherein the cancer is a multiple myeloma.

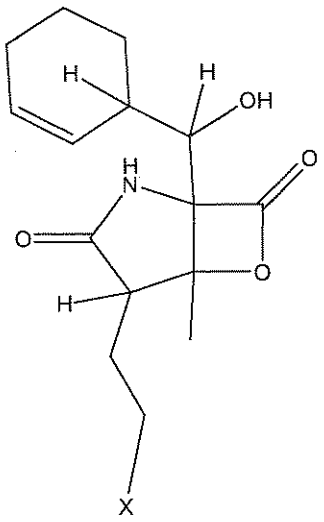
8. The use of claim 1, wherein the patient is a human.

9. The use of claim 1, wherein the other chemotherapeutic agent is selected from the group consisting of dexamethasone, doxorubicin, and thalidomide.

10. The use of claim 1, wherein the other chemotherapeutic agent is a proteosome inhibitor.

11. The use of claim 10, wherein the other chemotherapeutic agent is bortezomib.

12. Use of a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof:

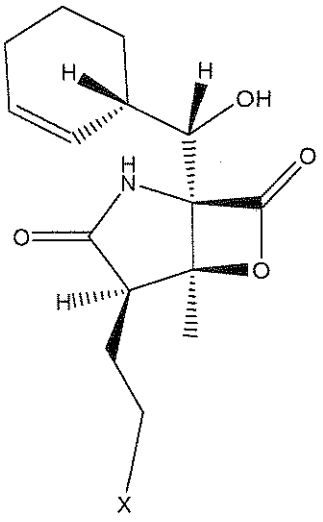


(I)

in the manufacture of a medicament for treating a neoplastic disease in a patient, wherein X is selected from the group consisting of fluorine, chlorine, bromine or iodine, in combination with at least one additional chemotherapeutic agent.

13. The use of claim 12, wherein X is chlorine.

14. The use of claim 12, wherein the compound of formula (I) has the structure of formula (II):



(II)

15. The use of claim 12, wherein the neoplastic disease is cancer.

16. The use of claim 15, wherein the cancer is selected from the group consisting of breast cancer, sarcoma, leukemia, ovarian cancer, uretal cancer, bladder cancer, prostate cancer, colon cancer, rectal cancer, stomach cancer, lung cancer, lymphoma, multiple

myeloma, pancreatic cancer, liver cancer, kidney cancer, endocrine cancer, skin cancer, melanoma, angioma, and brain or central nervous system (CNS) cancer.

17. The use of claim 16, wherein the cancer is selected from the group consisting of multiple myeloma, colorectal carcinoma, prostate carcinoma, breast adenocarcinoma, non-small cell lung carcinoma, and an ovarian carcinoma or melanoma.

18. The use of claim 17, wherein the cancer is a multiple myeloma.

19. The use of claim 12, wherein the patient is a human.

20. The use of claim 12, wherein the other chemotherapeutic agent is Selected from the group consisting of dexamethasone, doxorubicin, and thalidomide.

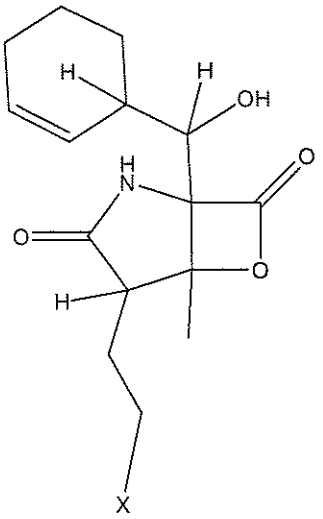
21. The use of claim 12, wherein the other chemotherapeutic agent is a proteosome inhibitor.

22. The use of claim 21, wherein the other chemotherapeutic agent is bortezomib.

23. The use of claim 12, wherein the combination is synergistic.

24. The use of claim 12, wherein the combination is additive.

25. A pharmaceutical composition, comprising:
a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof:



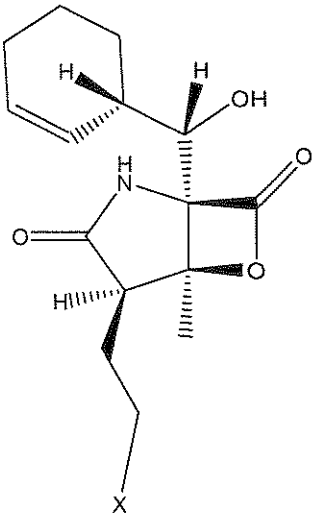
(I)

wherein X is selected from the group consisting of fluorine, chlorine, bromine or iodine; and

at least one additional chemotherapeutic agent.

26. The composition of claim 25, wherein X is chlorine.

27. The composition of claim 25, wherein the compound of formula (I) has the structure of formula (II):



(II)

28. The composition of claim 25, wherein the other chemotherapeutic agent is selected from the group consisting of dexamethasone, doxorubicin, and thalidomide.

29. The composition of claim 25, wherein the other chemotherapeutic agent is proteosome inhibitor.

30. The composition of claim 29, wherein the other chemotherapeutic agent is bortezomib.

31. Use of a synergistic combination of at least two proteosome inhibitors in the manufacture of a medicament for treating a neoplastic disease in a patient.

32. The use of claim 31, wherein the neoplastic disease is cancer.

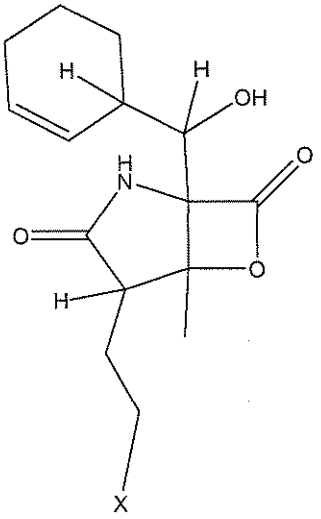
33. The use of claim 32, wherein the cancer is selected from the group consisting of breast cancer, sarcoma, leukemia, ovarian cancer, uretal cancer, bladder cancer, prostate cancer, colon cancer, rectal cancer, stomach cancer, lung cancer, lymphoma, multiple myeloma, pancreatic cancer, liver cancer, kidney cancer, endocrine cancer, skin cancer, melanoma, angioma, and brain or central nervous system (CNS) cancer.

34. The use of claim 33, wherein the cancer is selected from the group consisting of multiple myeloma, colorectal carcinoma, prostate carcinoma, breast adenocarcinoma, non-small cell lung carcinoma, and an ovarian carcinoma or melanoma.

35. The use of claim 34, wherein the cancer is a multiple myeloma.

36. The use of claim 31, wherein the patient is a human.

37. The use of claim 31, wherein at least one of the proteosome inhibitors is selected from the group consisting of bortezomib and the compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof:



(I)

wherein X is selected from the group consisting of fluorine, chlorine, bromine or iodine.