TREATMENT OF PROTEIN DEGRADATION DISORDERS

Related Applications

[0001] The present application claims priority under 35 U.S.C. § 119(e) to U.S. provisional patent application, USSN 60/664,470, filed March 22, 2005, which is incorporated herein by reference.

Government Support

[0002] The work described herein was supported, in part, by grants from the National Institutes of Health (CA078048, GM076262). The United States government may have certain rights in the invention.

Background of the Invention

[0003] Dynamic cellular states require a rapid, efficient mechanism of protein catabolism. Cancer cells are highly dependent on protein degradation due to continuous cell cycling, hypermutation, and chromosomal rearrangements (Adams J. The proteasome: a suitable antineoplastic target. Nat Rev Cancer. 2004;4:349-360; incorporated herein by reference). The proteasome and the aggresome are the two principal cellular structures involved in intracellular protein catabolism. The biology of the proteasome is well-characterized in normal and neoplastic cells. Proteasome complexes reside in numerous locations throughout the cell such as the endoplasmic reticulum (ER), nucleus, and cytoplasm. The primary role of the proteasome is the targeted degradation of ubiquitinated proteins. The aggresome is a juxtanuclear complex of misfolded proteins, chaperones, and proteasome components, which expands in response to proteasome inhibition or protein stress associated with certain pathologic states (Kopito RR. Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol. 2000;10:524-530; incorporated herein by reference). There are no known treatments targeting these pathologic states.

[0004] Aberrant protein catabolism is a hallmark of cancer, and is implicated in the stabilization of oncogenic proteins and the degradation of tumor suppressors (Adams J. The proteasome: a suitable antineoplastic target. Nat Rev Cancer. 2004;4:349-360; incorporated herein by reference). Thus, there is a need in the art for treatments for diseases that involve aberrant protein catabolism as well as screening methods to develop new therapeutics to treat the diseases (*e.g.*, cancer) by targeting protein degradation pathways and components.

Summary of the Invention

[0005] In one aspect, the invention provides a method of treating a subject suffering from or susceptible to a protein degradation disorder. The method includes the step of administering to a subject in need thereof a therapeutically effective amount of at least one protein degradation inhibitor. In certain embodiments, an inhibitor of the proteasome is administered in combination with an inhibitor of the aggresome. In certain embodiments, the protein degradation disorder is a cellular proliferation disorder or a protein deposition disorder. In particular, the cellular proliferation disorder is cancer. In preferred embodiments, wherein the cancer is one or more of multiple myeloma, leukemia, lymphoma, breast cancer, lung cancer, ovarian cancer, prostate cancer, and liver cancer. In other embodiments, the protein deposition disorder is Wilson's disease, spinocerebellar ataxia, prion disease, Parkinson's disease, Huntington's disease, familial amytrophic lateral sclerosis, amyloidosis, Alzheimer's disease, Alexander's disease, alcoholic liver disease, cystic fibrosis, Pick's disease, or Lewy body dementia.

[0006] In another aspect, the invention provides a method of treating a cell exhibiting symptoms of a protein degradation disorder. The method includes the step of administering a therapeutically effective amount of a protein degradation inhibitor to the cell. In certain embodiments, the cell is one or more of a cell from a subject or a cultured cell. In certain embodiments, the cell from a subject is one or more of bone marrow stromal cell (BMSC), a peripheral blood mononuclear cell (PBMC), lymphocytes, hair follicles, hematopoietic cells, blood cells, epithelial cells, bone marrow plasma cells, primary cancer cells, patient derived tumor cells, normal or cancerous hematopoietic stem cells, neural stem cells, solid tumor cells, or astrocytes. In certain embodiments, the cultured cell is one or more of MM.1S, U266, RPMI8226, DOX40, MM.1R, INA-6, LR5, primary and established cancer cell lines, primary and established normal cell lines. Inhibition of protein degradation pathways in cells under protein stress leads to cell death; therefore, treatment of diseases such as cancer in which the cancer cells are under protein stress using a protein degradation inhibitor provides a new way of killing cancer cells.

[0007] In another aspect, the invention provides a method of treating a subject suffering from or susceptible to multiple myeloma. The method includes the step of administering to a subject in need thereof a therapeutically effective amount of a protein degradation inhibitor, to thereby treat the subject suffering from or susceptible to multiple

myeloma. Due to the production of immunoglobulin, the cells are under protein stress and are susceptible to cell death upon inhibition of protein degradation.

[0008] In another aspect, the invention provides a method of treating a subject suffering from or susceptible to breast or ovarian cancer. The method includes the step of administering to a subject in need thereof a therapeutically effective amount of a protein degradation inhibitor, to thereby treat the subject suffering from or susceptible to breast or ovarian cancer.

[0009] In yet another aspect, the invention provides a method of assessing the efficacy of a protein degradation disorder treatment in a subject. The method includes the steps of determining one or more pre-treatment phenotypes; administering a therapeutically effective amount of a protein degradation inhibitor to the subject; and determining the one or more phenotypes after an initial period of treatment with the protein degradation inhibitor; wherein the modulation of the one or more phenotypes indicates efficacy of a protein degradation inhibitor treatment.

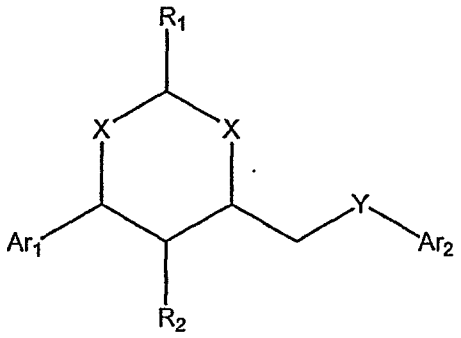
[0010] In yet another aspect, the invention provides a method of monitoring the progress of a subject being treated with an aggresome inhibitor. The method includes the steps of determining one or more pre-treatment phenotypes; administering a therapeutically effective amount of an aggresome inhibitor to the subject; and determining one or more phenotypes after an initial period of treatment with the aggresome inhibitor; wherein the modulation of one or more of the phenotypes indicates efficacy of aggresome inhibition treatment.

[0011] In a yet further aspect, the invention provides a method of selecting a subject with a protein degradation disorder for treatment with a protein degradation inhibitor. The method includes the steps of determining one or more pre-treatment phenotypes; administering a therapeutically effective amount of a protein degradation inhibitor to the subject; determining the one or more phenotypes after an initial period of treatment with the protein degradation inhibitor, wherein the modulation of the one or more phenotype is an indication that the disorder is likely to have a favorable clinical response to treatment with a protein degradation inhibitor.

[0012] In any of the above-described aspects, the protein degradation inhibitor is preferably selected from one or more of tubacin, tubacin-like compounds, tubacin derivatives, bortezomib (VELCADE®), SAHA, R115777 FTI, ^{166}Holmium-DOTMP, arsenic trioxide, 17-AAG, MG132, sapojargon, NPI-0052, or other compounds described herein. Tubacin, tubacin-like compounds, and tubacin derivatives are described in U.S. patent applications

U.S.S.N. 60/289,850, filed May 9, 2001; U.S.S.N. 10/144,316, filed May 9, 2002; and U.S.S.N. 10/621,276, filed July 17, 2003; each of which is incorporated herein by reference.

[0013] In certain preferred embodiments of the above-described aspects, the protein degradation inhibitor is a compound of formula:



wherein

each X is independently O, S, CH_2, or NR_3;

Y is O, S, CH_2, or NR_4;

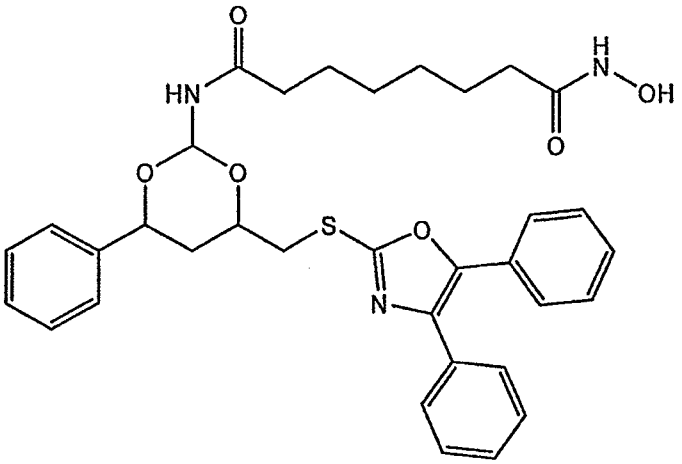
Ar_1 and Ar_2 are each independently an aryl group;

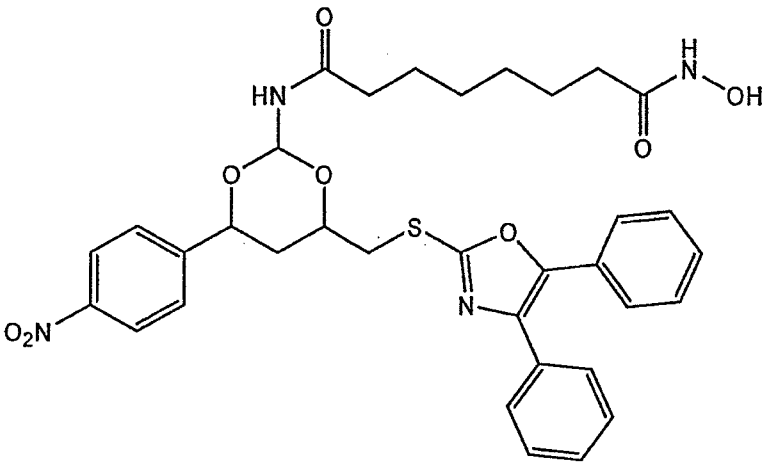
R_1 is a lower alkyl group or an aryl group;

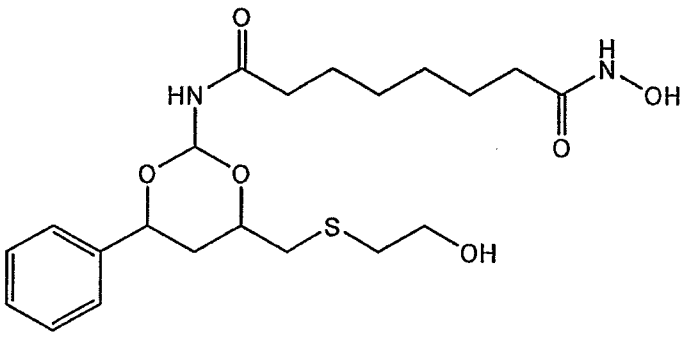
R_2 is hydrogen, a lower alkyl group or an aryl group; and

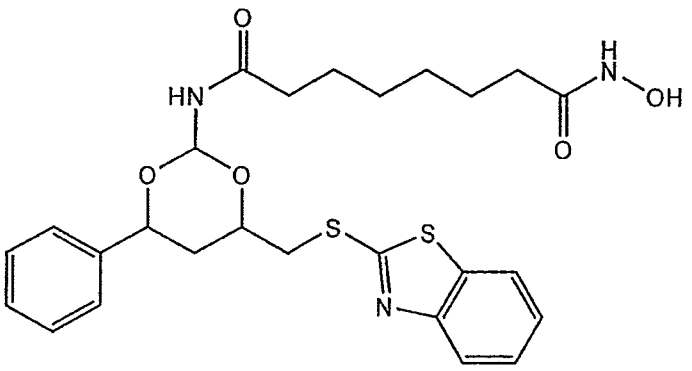
R_3 is hydrogen, a lower alkyl group, an aryl group, an alkylcarbonyl, an alkoxycarbonyl group, or an aminocarbonyl group. In certain preferred embodiments, X is for both occurrences O. In certain preferred embodiments, Y is S. In certain preferred embodiments, Ar_1 is phenyl or substituted phenyl. In certain preferred embodiments, Ar_2 is heteroaryl, more preferably optionally substituted oxazolyl. In certain preferred embodiments, R_1 is phenyl or substituted phenyl, more preferably 4-aminosubstituted phenyl. In certain preferred embodiments, R_2 is hydrogen.

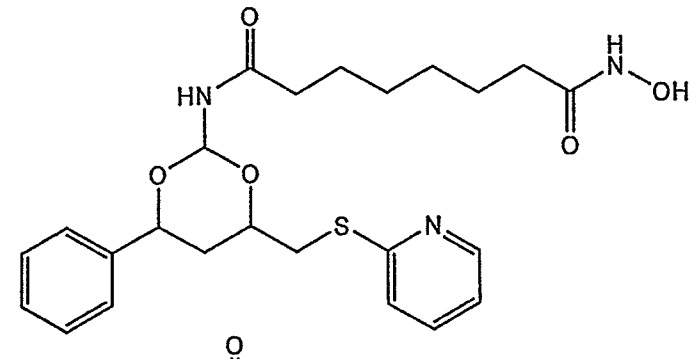
[0014] In certain embodiments, the protein degradation inhibitor is of one of the formulae:

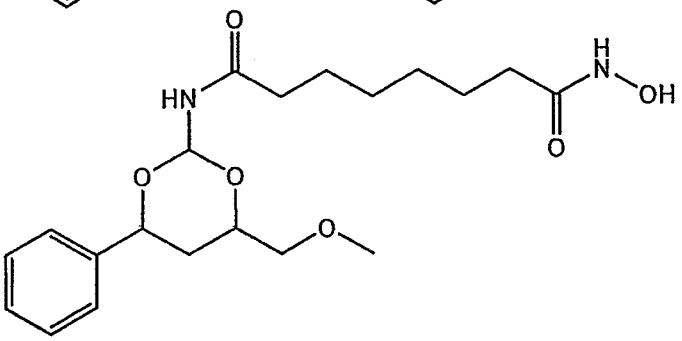




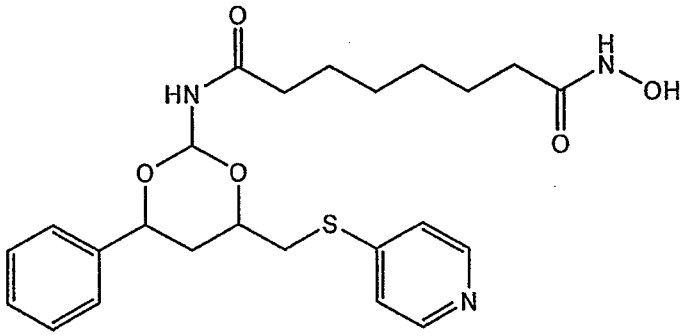




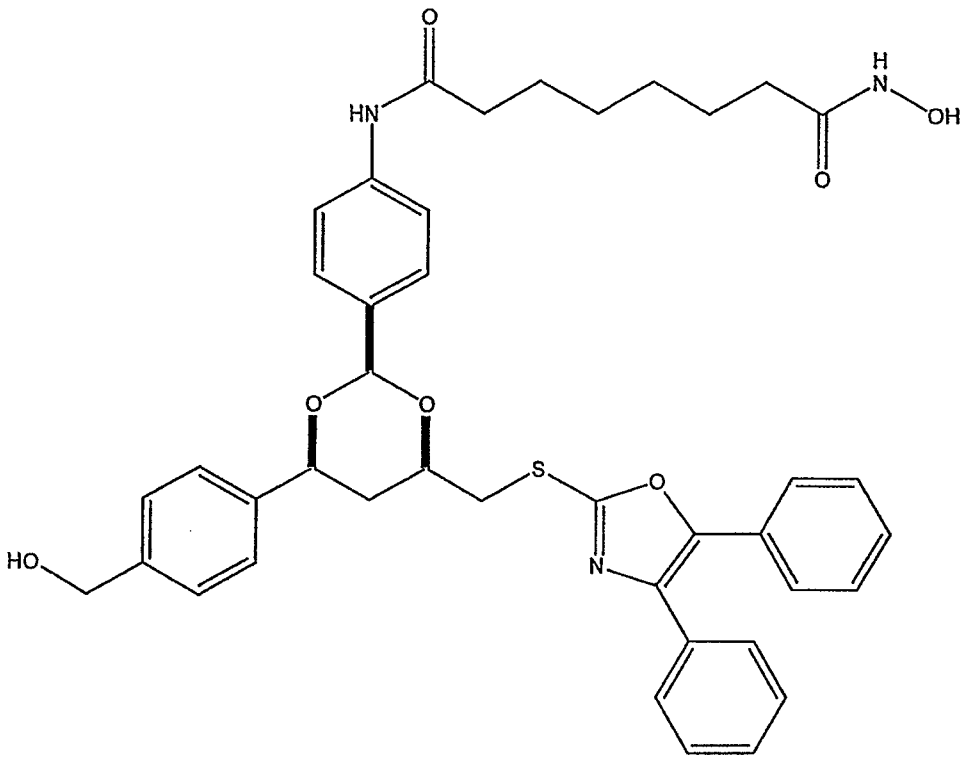




or



In certain preferred embodiments, the protein degradation inhibitor is a compound of the formula below with the stereochemistry as shown:



These compounds of the invention are particularly useful in the methods, pharmaceutical compositions, and kits of the invention.

[0015] In any of the above-described aspects, the protein degradation inhibitor is an HDAC inhibitor. Compounds known to inhibit HDACs are described in U.S. patent applications, U.S.S.N. 60/773,510, filed February 14, 2006; U.S.S.N. 60/773,172, filed February 14, 2006; U.S.S.N. 60/289,850, filed May 9, 2001; U.S.S.N. 10/144,316, filed May 9, 2002; and U.S.S.N. 10/621,276, filed July 17, 2003; each of which is incorporated herein by reference. In certain embodiments, the protein degradation inhibitor preferably inhibits HDAC6. In certain embodiments, the protein degradation inhibitor is specific for HDAC6.

[0016] In any of the above-described aspects, the protein degradation inhibitor preferably inhibits HDAC6 enzymatic activity, thereby inhibiting aggresome mediated protein degradation. In preferred embodiments, the protein degradation inhibitor inhibits the C-terminal aceylation activity of HDAC6, thereby inhibiting aggresome mediated protein degradation.

[0017] In certain embodiments, the inhibitor of HDAC6 leads to the acetylation of Hsp90. The acetylation of Hsp90 renders this protein less active towards a number of Hsp90's client proteins, thereby augmenting protein stress in the cell. In particular, for prostate and breast cancer, the inhibition of HDAC6 and the subsequent acetylation of Hsp90 leads to diminished activity of steroid-binding receptors due to the finding that glucocorticoid

receptors require Hsp90 function to engage glucorticoids. Therefore, HDAC6 inhibition leads to decreased sensitivity to estrogen in breast cancer and androgens in prostate cancer.

[0018] In any of the above-described aspects, in certain preferred embodiments, the protein degradation inhibitor is an aggresome inhibitor. In certain preferred embodiments, the aggresome inhibitor is one or more of tubacin, scriptade, or a compounds described herein. In certain other embodiments, the aggresome inhibitor is one or more of the compounds described in U.S. patent applications, U.S.S.N. 60/773,510, filed February 14, 2006; U.S.S.N. 60/773,172, filed February 14, 2006; U.S.S.N. 60/289,850, filed May 9, 2001; U.S.S.N. 10/144,316, filed May 9, 2002; and U.S.S.N. 10/621,276, filed July 17, 2003; each of which is incorporated herein by reference.

[0019] In any of the above-described aspects, in certain preferred embodiments, the protein degradation inhibitor is a proteasome inhibitor. In certain preferred embodiments, the proteasome inhibitor is one or more of bortezomib, MG132, sapojargon, and NPI-0052. In certain embodiments, the proteasome inhibitor is a compound described herein.

[0020] In any of the above-described aspects, in certain preferred embodiments, the protein degradation inhibitor is a peptide derived from HDAC6, dynein, an N-terminal peptide of HDAC6, or C-terminal peptide of HDAC6. In certain preferred embodiments, the C-terminal HDAC6 peptide is sufficient to modulate a phenotype of a cell.

[0021] In any of the above-described aspects, in certain preferred embodiments, the phenotype can be biological or clinical sequelae in response to a particular treatment or compound, anemia, thrombocytopenia, neutropenia, osteolytic lesions, bone pain, immunodeficiency, renal insufficiency, hypercalcemia, aneuploidy of mature plasma cells, percentage of malignant cells, acetylation state of tubulin, apoptosis of mature plasma cells, level of aggresomes in mature plasma cells, HDAC6 ubiquitination in mature plasma cells, HDAC6 association with dynein in mature plasma cells, cellular levels of ubiquintinated proteins in mature plasma cells, level of caspase-8 in mature plasma cells, level of PARP in mature plasma cells, thymidine uptake in mature plasma cells, dilated ER cisternae, aggregation of mature plasma cells, deposits of immunoglobulins in mature plasma cells, acetylation state of non-histone proteins, acetylation of histone proteins, global ubiquitination state of the cellular proteins, state of cell cycle regulation, necrosis, markers of apoptosis, apoptosis state, Russell body formation, cystic fibrosis transmembrane protein receptor state, and modulation of cellular protein deposits, or global acetylation state of cellular or extracellular proteins.

[0022] In preferred embodiments, a decrease in one or more of level of aggresomes, thrombocytopenia, neutropenia, osteolytic lesions, bone pain, immunodeficiency, renal insufficiency, hypercalcemia, aneuploidy of mature plasma cells, percentage of malignant cells, thymidine uptake in mature plasma cells, level of full length caspase-8 in mature plasma cells, level of full length PARP in mature plasma cells, or aggregation of mature plasma cells, indicates that the treatment is efficacious.

[0023] In preferred embodiments, an increase in acetylation state of tubulin, HDAC6 ubiquitination in mature plasma cells, level of cleaved form of caspase-8, level of cleaved form of PARP, necrosis, acetylation state of non-histone proteins, cellular ubiquitination levels, apoptosis, markers of apoptosis, cell cycle deregulation, or deposits of immunoglobulins in mature plasma cells indicates that the treatment is efficacious.

[0024] In any of the above-described aspects, in certain preferred embodiments, the method includes the further step of obtaining a biological sample from a subject, and, in certain embodiments, can include the further step of obtaining a second biological sample from the subject.

[0025] In any of the above-described aspects, in certain preferred embodiments, the method includes the further step of determining the subject's phenotype after a second period of treatment with the protein degradation inhibitor.

[0026] In any of the above-described aspects, in certain preferred embodiments, the method further includes the step of administering a therapeutically effective amount of one or more additional protein degradation inhibitors to the subject or cell. In certain preferred embodiments, at least one of the additional protein degradation inhibitors is an aggresome inhibitor. In certain preferred embodiments, at least one of the additional protein degradation inhibitors is a proteasome inhibitor. In preferred embodiments, the additional protein degradation inhibitor is one or more of bortezomib, tubacin , MG132, sapojargon, NPI-0052, or a compound described herein. In certain other embodiments, the protein degradation inhibitor is one or more of the compounds described in U.S. patent applications, U.S.S.N. 60/773,510, filed February 14, 2006; U.S.S.N. 60/773,172, filed February 14, 2006; U.S.S.N. 60/289,850, filed May 9, 2001; U.S.S.N. 10/144,316, filed May 9, 2002; and U.S.S.N. 10/621,276, filed July 17, 2003; each of which is incorporated herein by reference.

[0027] In any of the above-described aspects, in certain preferred embodiments, the method further includes monitoring the treatment or progress of the cell or subject.

[0028] In any of the above-described aspects, in certain preferred embodiments, the method further includes obtaining the protein degradation inhibitor.

[0029] In any of the above-described aspects, in certain preferred embodiments, the method further includes co-administering one or more of a chemotherapeutic agent, radiation agent, hormonal agent, biological agent, or an anti-inflammatory agent to the subject. In certain embodiments, the chemotherapeutic agent is tamoxifen, trastuzamab, raloxifene, doxorubicin, fluorouracil/5-FU, pamidronate disodium, anastrozole, exemestane, cyclophosphamide, epirubicin, letrozole, toremifene, fulvestrant, fluoxymesterone, trastuzumab, methotrexate, megastrol acetate, docetaxel, paclitaxel, testolactone, aziridine, vinblastine, capecitabine, goselerin acetate, zoledronic acid, taxol, vinblastine, or vincristine.

[0030] In any of the above-described aspects, in certain preferred embodiments, the method further includes comparing one or more of the pre-treatment or post-treatment phenotypes to a standard phenotype. In preferred embodiments, the standard phenotype is the corresponding phenotype in a reference cell or population of cells. In preferred embodiments, the reference cell is one or more of the following, cells from the subject, cultured cells, cultured cells from the subject, or cells from the subject pre-treatment. In certain embodiments, the cells from the subject are bone marrow stromal cell, (BMSC), a peripheral blood mononuclear cell (PBMC), lymphocytes, hair follicles, blood cells, other epithelial cells, bone marrow plasma cells, primary cancer cells, patient derived tumor cells, normal or cancerous hematopoietic stem cells, neural stem cells, solid tumor cells, or astrocytes.

[0031] In another aspect, the invention provides a method of inhibiting aggresome mediated protein degradation in a cell. The method includes the step of contacting the cell with an aggresome inhibitor. In preferred embodiments, the aggresome protein degradation is mediated by HDAC6. In certain preferred embodiments, the method further includes the step of inhibiting proteasome protein degradation in the cell. In certain preferred embodiments, the aggresome inhibitor is tubacin, a compound described herein, or a compound identified by a method of identifying candidate compounds as described herein below. In certain other embodiments, the aggresome inhibitor is one or more of the compounds described in U.S. patent applications, U.S.S.N. 60/773,510, filed February 14, 2006; U.S.S.N. 60/773,172, filed February 14, 2006; U.S.S.N. 60/289,850, filed May 9, 2001; U.S.S.N. 10/144,316, filed May 9, 2002; and U.S.S.N. 10/621,276, filed July 17, 2003; each of which is incorporated herein by reference.

[0032] In another aspect, the invention provides a method of identifying a candidate compound or molecule to inhibit protein degradation in a cell. The method includes the steps of contacting a cell exhibiting aggresome formation with a candidate compound, and

determining a phenotype of the cell, wherein modulation of the phenotype is indicative of the efficacy of the compound. In certain embodiments, the candidate molecule is one or more of a small molecule, a peptide (or peptidomimetic), or a nucleic acid (or a mimetic thereof (including, e.g., peptide nucleic acids (PNAs)). In certain preferred embodiments, the nucleic acid is an RNA, mRNA, RNAi, siRNA or DNA. In certain preferred embodiments, the peptide is a peptide derived from the HDAC6, the dynein-binding domain of HDAC6, the TDAC domain of HDAC6, the N-terminus of HDAC6 or the C-terminus of HDAC6. In certain preferred embodiments, the small molecule is contained in or derived from a library of compounds. In certain preferred embodiments, the step of determining the phenotype comprises using image-based multidimensional screening.

[0033] In a yet further aspect, the invention provides a method for evaluating a test compound. The method includes the steps of contacting a cell exhibiting aggresome formation with a test compound, and evaluating the cell following contact, wherein a correlation of a modulation of one or more phenotypes to a reference value is an indication that the test compound may be useful as a protein degradation disorder treatment. In certain preferred embodiments, the test compound is one or more of a small molecule, a peptide, or a nucleic acid.

[0034] In certain preferred embodiments of the above methods, the method further includes the step of determining a phenotype of the cell after an initial period of treatment with the protein degradation inhibitor. In certain preferred embodiments of the above methods, the phenotype is a biological or clinical sequelae in response to a particular treatment or compound. Phenotypes include, anemia, thrombocytopenia, neutropenia, osteolytic lesions, bone pain, immunodeficiency, renal insufficiency, hypercalcemia, aneuploidy of mature plasma cells, percentage of malignant cells, acetylation state of tubulin, apoptosis of mature plasma cells, level of aggresomes, level of aggresomes in mature plasma cells, HDAC6 ubiquitination, HDAC6 ubiquitination in mature plasma cells, HDAC6 association with dynein in mature plasma cells, cellular levels of ubiquintinated proteins in mature plasma cells, level of caspase-8 in mature plasma cells, level of PARP in mature plasma cells, thymidine uptake in mature plasma cells, dilated ER cisternae, aggregation of mature plasma cells, deposits of immunoglobulins in mature plasma cells, acetylation state of non-histone proteins, global ubiquitination state of the cellular proteins, state of cell cycle regulation, necrosis, markers of apoptosis, apoptosis state, Russell body formation, Cystic Fibrosis transmembrane protein receptor state, and modulation of cellular protein deposits, or global acetylation state of cellular and extracellular proteins. In certain preferred

embodiments, a decrease in one or more of anemia, level of aggresomes, thrombocytopenia, neutropenia, osteolytic lesions, bone pain, immunodeficiency, renal insufficiency, hypercalcemia, aneuploidy of mature plasma cells, percentage of malignant cells, thymidine uptake in mature plasma cells, level of full length caspase-8 in mature plasma cells, level of full length PARP in mature plasma cells, or aggregation of mature plasma cells, indicates that the treatment is efficacious. In certain preferred embodiments, an increase in acetylation state of tubulin, global acetylation state, acetylation state on non-histone proteins, HDAC6 ubiquitination in mature plasma cells, level of cleaved form of caspase-8, level of cleaved form of PARP, necrosis, acetylation state of non-histone proteins, cellular ubiquitination levels, apoptosis, markers of apoptosis, cell cycle deregulation, or deposits of immunoglobulins in mature plasma cells indicates that the treatment is efficacious.

[0035] In certain embodiments of any of the above-described methods, the subject or the cell is a human. In certain embodiments of any of the above-described methods, the subject or the cell is a mammalian. In certain embodiments of any of the above-described methods, the subject is or the cell is derived from a domesticated animal (*e.g.*, dog, cat, rodent, cow, pig, goat, sheep, *etc.*). In other embodiments of any of the above-described methods, the subject is or the cell is derived from an experimental animal (*e.g.*, mouse, rat, pig, dog, primate, monkey, chimpanzee, *etc.*).

[0036] In another aspect, the invention provides a kit for treating a protein degradation disorder in a subject. The kit includes a compound as described herein or pharmaceutically acceptable esters, salts, and prodrugs thereof; and instructions for use. In certain preferred embodiments, the compound is present as a pharmaceutical composition comprising a therapeutically effective amount of the compound and a pharmaceutically acceptable carrier.

[0037] In still a further aspect, the invention provides a packaged composition. The packaged compositions includes a therapeutically effective amount of an a protein degradation inhibitor and a pharmaceutically acceptable carrier or diluent, wherein the composition is formulated for treating a subject suffering from or susceptible to a protein degradation disorder, and packaged with instructions to treat a subject suffering from or susceptible to a protein degradation disorder.

[0038] In still another aspect, the invention provides an isolated nucleic acid molecule which encodes a polypeptide derived from HDAC6, wherein the polypeptide when expressed in cell exhibiting aggresomes causes an inhibition of protein degradation. In certain preferred embodiments, the nucleic acid is derived from the nucleic acid encoding the C-terminus of

HDAC6, amino acids 439-503 of HDAC6, amino acids 500-790 of HDAC 6, amino acids 781-931 of HDAC6, or the amino acids 1-460. In certain preferred embodiments, the nucleic acid is at least about 60% identical to the C-terminus of HDAC6, amino acids 439-503 of HDAC6, amino acids 500-790 of HDAC 6, amino acids 781-931 of HDAC6, or the amino acids 1-460. In certain preferred embodiments, the nucleic acid is is at least about 80% identical to the C-terminus of HDAC6, amino acids 439-503 of HDAC6, amino acids 500-790 of HDAC 6, amino acids 781-931 of HDAC6, or the amino acids 1-460. In certain preferred embodiments, the nucleic acid is is at least about 90% identical to the C-terminus of HDAC6, amino acids 439-503 of HDAC6, amino acids 500-790 of HDAC 6, amino acids 781-931 of HDAC6, or the amino acids 1-460. In certain preferred embodiments, the nucleic acid is is at least about 99.9% identical to the C-terminus of HDAC6, amino acids 439-503 of HDAC6, amino acids 500-790 of HDAC 6, amino acids 781-931 of HDAC6, or the amino acids 1-460.

[0039] In still another aspect, the invention provides an isolated polypeptide derived from HDAC6, wherein the polypeptide inhibits aggresome mediated protein degradation. In certain preferred embodiments, the isolated polypeptide comprises an amino acid sequence as identified by C-terminus of HDAC6, amino acids 439-503 of HDAC6, amino acids 500-790 of HDAC 6, amino acids 781-931 of HDAC6, or the amino acids 1-460. In certain preferred embodiments, the peptide is at least about 60% identical to any one or more of the C-terminus of HDAC6, amino acids 439-503 of HDAC6, amino acids 500-790 of HDAC 6, amino acids 781-931 of HDAC6, or the amino acids 1-460. In certain preferred embodiments, the peptide is at least about 80% identical to any one or more of the C-terminus of HDAC6, amino acids 439-503 of HDAC6, amino acids 500-790 of HDAC 6, amino acids 781-931 of HDAC6, or the amino acids 1-460. In certain preferred embodiments, the peptide is at least about 90% identical to any one or more of the C-terminus of HDAC6, amino acids 439-503 of HDAC6, amino acids 500-790 of HDAC 6, amino acids 781-931 of HDAC6, or the amino acids 1-460. In certain preferred embodiments, the peptide is at least about 99.9% identical to any one or more of the C-terminus of HDAC6, amino acids 439-503 of HDAC6, amino acids 500-790 of HDAC 6, amino acids 781-931 of HDAC6, or the amino acids 1-460.

[0040] In yet another aspect, the invention provides a vector which contains a polynucleotide capable of encoding a polypeptide having at least about 80% sequence identity to the C-terminus of HDAC6, amino acids 439-503 of HDAC6, amino acids 500-790

of HDAC 6, amino acids 781-931 of HDAC6, or the amino acids 1-460, and characterized by the ability to inhibit protein degradation.

[0041] In still another aspect, the invention provides a method of treating a protein degradation disorder or cellular proliferation disorder. The method includes the step of administering to a subject in need thereof an effective amount of an RNA to specifically bind and inactivate the HDAC6. In certain preferred embodiments, the RNA is an RNAi, siRNA, antisense RNA, or ribozyme. Examples of active siRNA reagents include, but are not limited to, double-stranded or hairpin sequences corresponding to amino acids of HDAC6 at nucleotides 211-231 or 217-237 (see, *e.g.*, Hubbert *et al.*, *Nature* (2002) 417(6887):455-8).

Brief Description of the Drawing

[0042] *Figure 1* shows the expression of HDAC6 in multiple myeloma (MM) cell lines. MM cell lines were lysed and whole cell lysates were subjected Western blotting to assess protein expression of HDAC6. *Figure 1* demonstrates MM cell lines (lane 1-7) constitutively express HDAC6 protein.

[0043] *Figure 2* shows the induction of acetylated α-tubulin by tubacin in MM cell lines. MM.1S, RPMI8226 and INA-6 cells were incubated with tubacin (0-5 μM) for 24h. Acetylation of α-tubulin was assessed by Western blotting using anti-acetylated lysine antibody. *Figure 2* demonstrates that tubacin specifically induces acetylation of α-tubulin in a dose-dependent fashion.

[0044] *Figure 3* shows the growth inhibitory effect of tubacin in MM cell lines. MM cell lines were cultured with tubacin (1.25-20 μM) for 48 h. The growth inhibitory effect of tubacin was assessed by both MTT assay (*Figure 3A*) and ^{3}H-thymidine uptake (*Figure 3B*). *Figure 3* demonstrates dose-dependent growth inhibitory effect of tubacin as single agent in MM cell lines.

[0045] *Figure 4* demonstrates that IL-6 does not overcome the effect of tubacin. MM.1S cells were cultured with tubacin (1.25-5 μM) in the presence (5-20 ng/ml) or absence of IL-6 for 48 h. DNA synthesis was measured by ^{3}H-thymidine uptake. *Figure 4* demonstrates that IL-6 which is one of the major growth factor in MM, did not overcome growth inhibitory effect of tubacin.

[0046] *Figure 5* graphically depicts protein degradation pathways and the scientific rationale for combining VELCADE with tubacin in the treatment of protein degradation disorders. There are two pathways which degrade misfolded/unfolded proteins which are

ubiquitinated. The former is the proteasome pathway, and the latter is the aggresome pathway, which requires HDAC 6 activity. Therefore inhibition of both pathways by specific inhibitors bortezomib (VELCADE®) and tubacin induced accumulation of cytotoxic misfolded/unfolded proteins.

[0047] *Figure 6* shows that tubacin significantly enhances Velcade-induced cytotoxicity in MM cell lines. MM.1S (A) and RPMI8226 (B) cells were cultured with Velcade (5 and 10 nM) in the presence (5 and 10 μM) or absence of tubacin for 24h. Cytotoxicity was assessed by MTT assay. Figure 6 demonstrates that tubacin significantly ($p < 0.01$) augmented cytotoxicity triggered by Velcade in both cell lines.

[0048] *Figure 7* shows that tubacin inhibits G2/M arrest triggered by Velcade in MM cells. MM.1S cells were incubated with Velcade (5 nM) in the presence (5 μM) or absence of tubacin for 24 h. Cell cycle was examined by flow cytometric analysis using propidium iodine staining. Tubacin significantly inhibits G2/M arrest associated down regulation of $p21^{Cip1}$, triggered by Velcade and induced sub-G0/G1 phase (apoptosis) in MM.1S cells.

[0049] *Figure 8* demonstrates that caspase and PARP cleavage is induced by the combination of Velcade with tubacin. MM.1S and RPMI8226 cells were cultured with Velcade (5 nM) in the presence (5 μM) or absence of tubacin for 24 h. Caspase-8/9/3 and PARP cleavage were assessed by Western blotting using specific antibodies. *Figure 8* demonstrates that combination of Velcade with tubacin markedly augmented (induces) caspase/PARP cleavage in both cell lines.

[0050] *Figure 9* shows that tubacin significantly enhances Velcade-induced cytotoxicity in MM patient tumor cells. Purified tumor cells from MM patients were cultured with Velcade (5 and 10 nM) in the presence (5 μM) or absence of tubacin for 24 h. Cytotoxicity was assessed by MTT assay. *Figure 9* demonstrates that tubacin significantly ($p < 0.01$) augments cytotoxicity triggered by Velcade in patient tumor cells.

[0051] *Figure 10* demonstrates that the combination treatment of Velcade with Tubacin does not trigger cytotoxicity in normal peripheral blood mononuclear cells (PBMCs). PBMCs from 3 normal volunteers were cultured with Velcade (5-20 nM) in the presence (5 μM) or absence of tubacin for 24 h. Cytotoxicity was assessed by MTT assay. *Figure 10* demonstrates that combination treatment of Velcade with tubacin did not trigger cytotoxicity in PBMCs.

[0052] *Figure 11* shows that tubacin inhibits MM.1S cell growth in the bone marrow microenvironment. MM.1S cells were cultured with tubacin (1.25-5 μM) in the presence (2.5 and 5 nM) or absence of Velcade, with or without bone marrow stromal cells (BMSCs) for 24

h. Cell growth was assessed by ^{3}H-thymidine uptake. *Figure 11* demonstrates that tubacin significantly inhibits MM.1S cell growth even in the presence of BMSCs. Moreover, tubacin further augmented cytotoxicity of Velcade.

[0053] *Figure 12* demonstrates that tubacin specifically induces acetylation of α-tubulin in MM cells. (A) Chemical structures of tubacin and an inactive analog niltubacin. (B) Western blot of baseline expression of HDAC6 in MM cell lines. (C) MM.1S and RPMI8226 cells were cultured for 24 h in the presence (2.5 and 5 μM) or absence of tubacin. (D) RPMI8226 cells were cultured for the time indicated times in the presence of tubacin (5 μM). Whole cell lysates were subjected to Western blot using anti-Ac lysine Ab. Immunoblotting with anti- α-tubulin serves confirms equal protein loading. (E) MM.1S and RPMI8226 cells were cultured for 24h in the presence (2.5 and 5 μM) or absence of SAHA. Whole cell lysates were subjected to Western blotting using anti-Ac lysine Ab. In contrast to tubacin, SAHA markedly triggers acetylation of histones H3 and H4.

[0054] *Figure 13* demonstrates that tubacin induces cytotoxicity via activation of caspases. MM.1S (•), MM.1R (•), U266 (▲), RPMI8226 (▲), RPMI-LR5 (■) and RPMI-Dox40 (■) cells were cultured in the presence of tubacin (1.25-20 μM) for 48 h (A) and 72 h (B). (C) PBMC form normal volunteers (n=3) were cultured in the presence of tubacin (2.5-20μM) for 48 h. Cell growth was assessed by MTT assay, and data represent mean (± SD) of quadruplicate cultures. (D) MM.1S and RPMI8226 cells were cultured with tubacin (10 μM) for the times indicated. Whole cell lysates were subjected to Western blotting using anti-caspase-8 and PARP Abs.

[0055] *Figure 14* demonstrates that tubacin inhibits binding of HDAC6 with dynein and when combined with bortezomib, it induces significant accumulation of polyubiquitinated proteins. (A) Hypothetical rationale whereby tubacin enhances cytotoxicity induced by bortezomib (adapted from Kawaguchi et al (17)). (B) MM.1S cells were cultured with tubacin (2.5 and 5μM) for 8h. Whole cell lysates were immunoprecipitated with anti-Ub Ab. Immununoprecipitates were subjected to Western blotting using ant-HDAC6 Ab. (C) MM.1S cells were cultured with tubacin (2.5 and 5 μM) for 8 h. Whole cell lysates were immunoprecipitated with anti-dynein Ab. Immununoprecipitates were then subjected to Western blotting using anti-HDAC6 and dynein Abs. (D) MM.1S and RPMI8226 cells were cultured with tubacin (2.5 and 5 μM) for 24h. Whole cell lysates were subjected to Western blot using anti-Ub Ab. (E) MM.1S and

RPMI8226 cells were cultured with tubacin (T: 5 μM) and/or bortezomib (B: 5 nM) for 12 h. Whole cell lysates were subjected to Western blotting using anti-Ub Ab.

[0056] *Figure 15* demonstrates that tubacin and bortezomib induce synergistic anti-tumor activity in MM cell lines. (A) MM.1S and RPMI8226 MM cells were cultured for 24 h in the presence or absence of tubacin (5 μM) in control media (□), as well as with 5 nM (■) or 10 nM (■) bortezomib; cytotoxicity was assessed by MTT assay. (B) MM.1S cells were cultured for 24 h in the presence or absence of tubacin (5 μM) and/or bortezomib (5 nM); cell cycle profile was assessed by flow cytometry using PI staining. (C) MM.1S cells were cultured for 24h in the presence or absence of tubacin (T: 5 μM) and/or bortezomib (B: 5 nM); whole cell lysates were subjected to Western blotting using anti-$p21^{Cip1}$, p-JNK (SAPK), caspase-9, caspase-8, caspase-3 and PARP Abs. MM.1S cell were transiently transfected with HDAC6 siRNA. Cells were then subjected to (D) Western blotting using anti-HDAC6 Ab or (E) MTT assay, in the presence or absence of 5 nM bortezomib (■). MM.1S cells were cultured for 24h with niltubacin (2.5 and 5 μM) or tubacin (2.5 and 5 μM). Cells were then subjected to (F) Western blotting using Ac-Lys Ab, or (G) MTT assay, in the presence or absence of 5 nM bortezomib (■). Data represent mean (± SD) of quadruplicate cultures.

[0057] *Figure 16* demonstrates that tubacin synergistically enhances bortezomib-induced cytotoxicity in patient MM cells without cytotoxicity to PBMCs. BMPCs (A, B, C) and PBMCs (D) from 3 MM patients were cultured in the presence or absence of tubacin (5 μM) in control media (□) as well as with 10 nM (■) or 20 nM (■) bortezomib for 24h; cytotoxicity was assessed by MTT assay. (E) MM patient PBMCs were cultured in the presence or absence of tubacin (5μM). Whole cell lysates were subjected to Western blotting using anti-HDAC6, Ac-Lys, or α-tubulin Abs.

[0058] *Figure 17* demonstrates that tubacin inhibits paracrine MM cell growth. MM.1S (A) and RPMI8226 (B) cells were cultured for 24 h in BMSC-coated or non-coated plates in control media (□); as well as with 1.25 μM (■), 2.5 μM (■), or 5 μM (■) tubacin, in the presence or absence of bortezomib (2.5 nM, 5 nM). DNA synthesis was assessed by [^{3}H]-thymidine uptake; data represent mean (± SD) of quadruplicate cultures.

[0059] *Figure 18* demonstrates that tubacin alone, bortezomib alone, and combinations of tubacin and bortezomib are potent inhibitors of protein catabolism in MM.1S cells and RPMI cells.

[0060] *Figure 19* shows a schematic of the high-throughput immunofluorescence quantitative assay for acetylated tubulin with resulting images.

[0061] *Figure 20* shows the toxicity and synergy of Tubacin and LBH589 with Bortezomib in MM.1S cells.

[0062] *Figure 21* shows the toxicity and synergy of Tubacin and LBH589 with Bortezomib with RPMI-8226 cells.

[0063] *Figure 22* shows the effects of LBH589 and Tubacin on acetylated tubulin versus acetylated lysine using the cytoblot assay.

[0064] *Figure 23* shows the chemical structure of Tubacin.

[0065] *Figure 24* shows the synergy between Tubacin and Velcade in myeloma cell lines (A) MM.1S, and (B) RPMI cells.

[0066] *Figure 25* demonstrates the specificity of Tubacin for tubulin acetylation versus lysine acetylation.

[0067] *Figure 26* shows the chemical structure of des(hydromethyl)-Tubacin (DHM-Tubacin). The hydroxymethyl substituent off the phenyl ring in Tubacin has been removed.

[0068] *Figure 27* shows the synergy between DHM-Tubacin and Velcade in myeloma cell lines (A) MM.1S, and (B) RPMI cells.

[0069] *Figure 28* demonstrates the specificity of DHM-Tubacin for tubulin acetylation versus lysine acetylation.

[0070] *Figure 29* shows the chemical structure of NKI-81-1.

[0071] *Figure 30* shows the synergy between NKI-81-1 and Velcade in myeloma cell lines (A) MM.1S, and (B) RPMI cells.

[0072] *Figure 31* demonstrates the specificity of NKI-81-1 for tubulin acetylation versus lysine acetylation.

[0073] *Figure 32* shows the chemical structure for NKI-94-1.

[0074] *Figure 33* shows the synergy between NKI-94-1 and Velcade in myeloma cell lines (A) MM.1S, and (B) RPMI cells.

[0075] *Figure 34* demonstrates the specificity of NKI-94-1 for tubulin acetylation versus lysine acetylation.

[0076] *Figure 35* shows the chemical structure for NKI-59-1.

[0077] *Figure 36* shows the synergy between NKI-59-1 and Velcade in myeloma cell lines (A) MM.1S, and (B) RPMI cells.

[0078] *Figure 37* demonstrates the specificity of NKI-59-1 for tubulin acetylation versus lysine acetylation.

[0079] *Figure 38* shows the chemical structure for NKI-60-1.

[0080] *Figure 39* shows the synergy between NKI-60-1 and Velcade in myeloma cell lines (A) MM.1S, and (B) RPMI cells.

[0081] *Figure 40* demonstrates the specificity of NKI-60-1 for tubulin acetylation versus lysine acetylation.

[0082] *Figure 41* shows the chemical structure for NKI-82-1.

[0083] *Figure 42* shows the synergy between NKI-82-1 and Velcade in myeloma cell lines (A) MM.1S, and (B) RPMI cells.

[0084] *Figure 43* demonstrates the specificity of NKI-82-1 for tubulin acetylation versus lysine acetylation.

[0085] *Figure 44* shows the chemical structure for NKI-84-1.

[0086] *Figure 45* shows the synergy between NKI-84-1 and Velcade in myeloma cell lines (A) MM.1S, and (B) RPMI cells.

[0087] *Figure 46* demonstrates the specificity of NKI-84-1 for tubulin acetylation versus lysine acetylation in 293T cells.

[0088] *Figure 47* shows the effect of Tubacin and NKI-84-1 on tubulin acetylation in RPMI-8226 cells.

[0089] *Figure 48* demonstrates the specificity of NKI-84-1 for tubulin acetylation versus lysine acetylation in A549 cells.

[0090] *Figure 49* demonstrates the specificity of Tubacin for tubulin acetylation versus lysine acetylation in A549 cells.

[0091] *Figure 50* shows the effect of Tubacin and NKI-84-1 on tubulin acetylation in A549 cells.

[0092] *Figure 51* shows the TDAC inhibitory activity of the compounds—tubacin, NKI-82-1, NKI-81-1, NKI-93-1, NKI-94-1, NKI-59-1, NKI-60-1, DHM-Tubacin, and MAZ-1428.

[0093] *Figure 52* is a chart showing the HDAC inhibition and TDAC inhibition of the compounds—tubacin, DHM-tubacin, NKI-59-1, NKI-60-1, NKI-82-1, NKI-84-1, NKI-94-1, and NKI-81-1.

[0094] *Figure 53A* shows the binding of various compounds to HSA. *Figure 53B* shows the structures of various compounds listed in *Figure 53A*, which were not included in previous figures.

[0095] *Figure 54* shows the solubility of tubacin in various solutions for mouse multiple myeloma model and pharmacokinetics.

[0096] *Figure 55* shows the total synthesis of tubacin.

[0097] *Figure 56* is a synthetic scheme for preparing an intermediate useful in the synthesis of des(hydroxymethyl)-tubacin. Other aldehydes may be used to begin this synthesis thereby allowing for a great deal of diversity at this site.

[0098] *Figure 57* shows another exemplary synthesis of tubacin.

[0099] *Figure 58* shows exemplary epoxide-opening reactions useful in preparing various analogs of the inventive compounds. The scheme illustrates the use of various nucleophiles to open the epoxide group to create the diol functionality later capped to create the tubacin structure.

[00100] *Figure 59* demonstrates the synergy between bortezomib (VELCADE®) and tubacin in breast cancer. The use of an HDAC6 inhibitor such as tubcin renders the breast cancer cells sensitive to proteasome inhibition (*e.g.*, bortezomib).

Detailed Description of Certain Embodiments of the Invention

[00101] The present invention provides novel HDAC and TDAC inhibitors. Certain of these compounds are inhibitors of the aggresome. The present invention also provides method of treating intracellular protein degradation disorders mediated by the aggresome and/or proteasome. The aggresome is a novel therapeutic target as are therapeutic strategies targeting both the aggresome and proteasome. The novel therapeutic strategies of the invention overcome the problems of former therapeutics, for example, drug resistance and toxicity.

[00102] The proteasome is well-characterized in cancer cell biology. The aggresome is a juxtanuclear proteolytic complex, which forms in response to proteasome inhibition or misfolded protein stress. It is directly implicated in the pathophysiology of cystic fibrosis and certain neurodegenerative diseases characterized by protein deposition. A specific role for the aggresome in cancer has not been described.

[00103] Recently, a cytoplasmic histone deacetylase protein, HDAC6, was identified as necessary for aggresome formation and for survival of cells following ubiquitinated misfolded protein stress. The aggresome is an integral component of survival in cancer cells. The mechanism of HDAC6-mediated aggresome formation is a consequence of the catalytic activity of the carboxy-terminal deacetylase domain, targeting an uncharacterized non-histone target. The present invention also provides small molecule inhibitors of HDAC6. In certain embodiments, these new compounds are potent and selective inhibitors of HDAC6.

[00104] The aggresome was first described in 1998, when it was reported that there was an appearance of microtubule-associated perinuclear inclusion bodies in cells over-expressing the pathologic ΔF508 allele of the cystic fibrosis transmembrane conductance receptor (CFTR). Subsequent reports identified a pathologic appearance of the aggresome with over-expressed presenilin-1(Johnston JA, Ward CL, Kopito RR. Aggresomes: a cellular response to misfolded proteins. J Cell Biol. 1998;143:1883-1898; incorporated herein by reference), parkin (Junn E, Lee SS, Suhr UT, Mouradian MM. Parkin accumulation in aggresomes due to proteasome impairment. J Biol Chem. 2002;277:47870-47877; incorporated herein by reference), peripheral myelin protein PMP22 (Notterpek L, Ryan MC, Tobler AR, Shooter EM. PMP22 accumulation in aggresomes: implications for CMT1A pathology. Neurobiol Dis. 1999;6:450-460; incorporated herein by reference), influenza virus nucleoprotein (Anton LC, Schubert U, Bacik I, Princiotta MF, Wearsch PA, Gibbs J, Day PM, Realini C, Rechsteiner MC, Bennink JR, Yewdell JW. Intracellular localization of proteasomal degradation of a viral antigen. J Cell Biol. 1999;146:113-124; incorporated herein by reference), a chimera of GFP and the membrane transport protein p115 (Garcia-Mata R, Bebok Z, Sorscher EJ, Sztul ES. Characterization and dynamics of aggresome formation by a cytosolic GFP-chimera. J Cell Biol. 1999;146:1239-1254; incorporated herein by reference) and notably amyloidogenic light chains (Dul JL, Davis DP, Williamson EK, Stevens FJ, Argon Y. Hsp70 and antifibrillogenic peptides promote degradation and inhibit intracellular aggregation of amyloidogenic light chains. J Cell Biol. 2001;152:705-716; incorporated herein by reference). Model systems have been established to study ubiquitinated (ΔF508 CFTR) (Johnston JA, Ward CL, Kopito RR. Aggresomes: a cellular response to misfolded proteins. J Cell Biol. 1998;143:1883-1898; incorporated herein by reference) and non-ubiquitinated (GFP-250) (Garcia-Mata R, Bebok Z, Sorscher EJ, Sztul ES. Characterization and dynamics of aggresome formation by a cytosolic GFP-chimera. J Cell Biol. 1999;146:1239-1254; incorporated herein by reference) protein aggregate transport to the aggresome. Secretory, mutated, and wild-type proteins may assume unstable kinetic intermediates resulting in stable aggregates incapable of degradation through the narrow channel of the 26S proteasome. These complexes undergo active, retrograde transport by dynein to the pericentriolar aggresome, mediated in part by a cytoplasmic histone deacetylase, HDAC6 (Kawaguchi Y, Kovacs JJ, McLaurin A, Vance JM, Ito A, Yao TP. The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. Cell. 2003;115:727-738; incorporated herein by reference).

[00105] Histone deacetylases (HDACs) are a family of at least 11 zinc-binding hydrolases, which catalyze the deacetylation of lysine residues on histone proteins. HDAC inhibition results in hyperacetylation of chromatin, alterations in transcription, growth arrest, and apoptosis in cancer cell lines. Early phase clinical trials with available nonselective HDAC inhibitors demonstrate responses in hematologic malignancies including multiple myeloma, although with significant toxicity. Of note, *in vitro* synergy of conventional chemotherapy agents (such as melphalan) with bortezomib has been reported in myeloma cell lines, though dual proteasome-aggresome inhibition was not proposed. Until recently selective HDAC inhibitors have not been realized.

[00106] HDAC6 is required for aggresome formation with ubiquitinated protein stress and is essential for cellular viability in this context. HDAC6 is believed to bind ubiquitinated proteins through a zinc finger domain and interacts with the dynein motor complex through another discrete binding motif. HDAC6 possesses two catalytic deacetylase domains. It is not presently known whether the amino-terminal histone deacetylase or the carboxy-terminal tubulin deacetylase (TDAC) domain mediates aggresome formation.

[00107] Aberrant protein catabolism is a hallmark of cancer, and is implicated in the stabilization of oncogenic proteins and the degradation of tumor suppressors (Adams J. The proteasome: a suitable antineoplastic target. Nat Rev Cancer. 2004;4:349-360; incorporated herein by reference). Tumor necrosis factor alpha induced activation of nuclear factor kappa B (NFκB) is a relevant example, mediated by NFκB inhibitor beta (IκB) proteolytic degradation in malignant plasma cells. The inhibition of IκB catabolism by proteasome inhibitors explains, in part, the apoptotic growth arrest of treated myeloma cells (Hideshima T, Richardson P, Chauhan D, Palombella VJ, Elliott PJ, Adams J, Anderson KC. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. Cancer Res. 2001;61:3071-3076; incorporated herein by reference). Multiple myeloma is an ideal system for studying the mechanisms of protein degradation in cancer. Since William Russell in 1890, cytoplasmic inclusions have been regarded as a defining histological feature of malignant plasma cells. Though the precise composition of Russell bodies is not known, they are regarded as ER-derived vesicles containing aggregates of monotypic immunoglobulins (Kopito RR, Sitia R. Aggresomes and Russell bodies. Symptoms of cellular indigestion? EMBO Rep. 2000;1:225-231; incorporated herein by reference) and stain positive for ubiquitin (Manetto V, Abdul-Karim FW, Perry G, Tabaton M, Autilio-Gambetti L, Gambetti P. Selective presence of ubiquitin in intracellular inclusions. Am J Pathol. 1989;134:505-513; incorporated herein by reference). Russell

bodies have been described with CFTR over-expression in yeast (Sullivan ML, Youker RT, Watkins SC, Brodsky JL. Localization of the BiP molecular chaperone with respect to endoplasmic reticulum foci containing the cystic fibrosis transmembrane conductance regulator in yeast. J. Histochem. Cytochem. 2003;51:545-548; incorporated herein by reference), thus raising the suspicion that these structures may be linked to overwhelmed protein catabolism, and potentially the aggresome. The role of the aggresome in cancer remains undefined.

[00108] Multiple myeloma (MM) is a plasma cell malignancy which remains incurable despite conventional treatment (Gregory, W. M., Richards, M. A. & Malpas, J. S. (1992) *J Clin Oncol* **10,** 334-342; incorporated herein by reference) as well as high dose therapy and stem cell transplantation (Attal, M., Harousseau, J. L., Facon, T., Guilhot, F., Doyen, C., Fuzibet, J. G., Monconduit, M., Hulin, C., Caillot, D., Bouabdallah, R., Voillat, L., Sotto, J. J., Grosbois, B. & Bataille, R. (2003) *N Engl J Med* **349,** 2495-2502; incorporated herein by reference). Novel agents have recently been developed which target not only MM cells, but also the bone marrow (BM) microenvironment, and can overcome conventional drug resistance (Hideshima, T. & Anderson, K. C. (2002) *Nat Rev Cancer* **2,** 927-937; incorporated herein by reference). For example, the proteasome inhibitor bortezomib (formally PS-341) induces significant anti-tumor activity in human MM cell lines and freshly isolated patient MM cells (Hideshima, T. & Anderson, K. C. (2002) *Nat Rev Cancer* **2,** 927-937; Hideshima, T., Richardson, P., Chauhan, D., Palombella, V., Elliott, P., Adams, J. & Anderson, K. C. (2001) *Cancer Res.* **61,** 3071-3076; Mitsiades, N., Mitsiades, C. S., Poulaki, V., Chauhan, D., Gu, X., Bailey, C., Joseph, M., Libermann, T. A., Treon, S. P., Munshi, N. C., Richardson, P. G., Hideshima, T. & Anderson, K. C. (2002) *Proc Natl Acad Sci U S A* **99,** 14374-14379; Hideshima, T., Chauhan, D., Richardson, P., Mitsiades, C., Mitsiades, N., Hayashi, T., Munshi, N., Dang, L., Castro, A., Palombella, V., Adams, J. & Anderson, K. C. (2002) *J Biol Chem* **277,** 16639-47; Mitsiades, N., Mitsiades, C. S., Richardson, P. G., Poulaki, V., Tai, Y. T., Chauhan, D., Fanourakis, G., Gu, X., Bailey, C., Joseph, M., Libermann, T. A., Schlossman, R., Munshi, N. C., Hideshima, T. & Anderson, K. C. (2003) *Blood* **101,** 2377-80; Chauhan, D., Li, G., Shringarpure, R., Podar, K., Ohtake, Y., Hideshima, T. & Anderson, K. C. (2003) *Cancer Res* **63,** 6174-6177; Hideshima, T., Mitsiades, C., Akiyama, M., Hayashi, T., Chauhan, D., Richardson, P., Schlossman, R., Podar, K., Munshi, N. C., Mitsiades, N. & Anderson, K. C. (2003) *Blood* **101,** 1530-1534; Hideshima, T., Chauhan, D., Hayashi, T., Akiyama, M., Mitsiades, N., Mitsiades, C., Podar, K., Munshi, N. C., Richardson, P. G. & Anderson, K. C. (2003) *Oncogene* **22,** 8386-8393;

Hideshima, T., Podar, K., Chauhan, D., Ishitsuka, K., Mitsiades, C., Tai, Y.-Z., Hamasaki, M., Raje, N., Hideshima, H., Schreiner, G., Nguyen , A. N., Navas, T., Munshi, N. C., Richardson, P. G., Higgins, L. S. & Anderson, K. C. (2004) *Oncogene* **23,** 8766-8776; each of which is incorporated herein by reference) associated with c-Jun NH_2-terminal kinase (JNK) (also known as stress-activated protein kinase) and caspase activation, followed by apoptosis (Hideshima, T., Richardson, P., Chauhan, D., Palombella, V., Elliott, P., Adams, J. & Anderson, K. C. (2001) *Cancer Res.* **61,** 3071-3076; Mitsiades, N., Mitsiades, C. S., Poulaki, V., Chauhan, D., Gu, X., Bailey, C., Joseph, M., Libermann, T. A., Treon, S. P., Munshi, N. C., Richardson, P. G., Hideshima, T. & Anderson, K. C. (2002) *Proc Natl Acad Sci USA* **99,** 14374-14379; Hideshima, T., Mitsiades, C., Akiyama, M., Hayashi, T., Chauhan, D., Richardson, P., Schlossman, R., Podar, K., Munshi, N. C., Mitsiades, N. & Anderson, K. C. (2003) *Blood* **101,** 1530-1534; each of which is incorporated herein by reference). Bortezomib also inhibits adherence of MM cells to bone marrow stromal cells (BMSCs) by downregulating adhesion molecules (ICAM-1 and VCAM-1) (Hideshima, T., Chauhan, D., Schlossman, R. L., Richardson, P. R. & Anderson, K. C. (2001) *Oncogene* **20,** 4519-4527; incorporated herein by reference); as well as induces cleavage of DNA-protein kinase catalytic subunit and ataxia telangiectasia mutated, suggesting that bortezomib also inhibits DNA repair. Neither IL-6 nor adherence of MM cells to BMSCs protects against bortezomib-induced apoptosis. Without wishing to be bound by any scientific theory, bortezomib enhances sensitivity and can overcome resistance in MM cells to conventional chemotherapeutic agents, especially to DNA damaging agents (Mitsiades, N., Mitsiades, C. S., Richardson, P. G., Poulaki, V., Tai, Y. T., Chauhan, D., Fanourakis, G., Gu, X., Bailey, C., Joseph, M., Libermann, T. A., Schlossman, R., Munshi, N. C., Hideshima, T. & Anderson, K. C. (2003) *Blood* **101,** 2377-80; incorporated herein by reference). In support of this, a phase II trial of bortezomib treatment of 202 patients with refractory relapsed MM demonstrated 35% responses, including 10% complete and near complete responses (Richardson, P. G., Barlogie, B., Berenson, J., Singhal, S., Jagannath, S., Irwin, D., Rajkumar, S. V., Srkalovic, G., Alsina, M., Alexanian, R., Siegel, D., Orlowski, R. Z., Kuter, D., Limentani, S. A., Lee, S., Hideshima, T., Esseltine, D. L., Kauffman, M., Adams, J., Schenkein, D. P. & Anderson, K. C. (2003) *N Engl J Med* **348,** 2609-2617; incorporated herein by reference); however, 65% of patients did not respond. Heat-shock protein (hsp)-27 mediates bortezomib-resistance; conversely, inhibiting hsp-27 expression using hsp-27 antisense, p38 mitogen-activated protein kinase (MAPK) siRNA, or p38 MAPK inhibitor to downregulate hsp-27 can restore MM cell susceptibility to bortezomib (Chauhan, D., Li, G.,

Shringarpure, R., Podar, K., Ohtake, Y., Hideshima, T. & Anderson, K. C. (2003) *Cancer Res* **63,** 6174-6177; Hideshima, T., Podar, K., Chauhan, D., Ishitsuka, K., Mitsiades, C., Tai, Y.-Z., Hamasaki, M., Raje, N., Hideshima, H., Schreiner, G., Nguyen , A. N., Navas, T., Munshi, N. C., Richardson, P. G., Higgins, L. S. & Anderson, K. C. (2004) *Oncogene* **23,** 8766-8776; each of which is incorporated herein by reference).

The aggresome is an alternative system to the proteasome for degradation of polyubiquitinated misfolded/unfolded proteins (Kopito, R. R. (2000) *Trends Cell Biol* **10,** 524-530; Bennett, E. J., Bence, N. F., Jayakumar, R. & Kopito, R. R. (2005) *Mol Cell* **17,** 351-365; each of which is incorporated herein by reference). Aggresome formation induces autophagic clearance, which terminates in lysosomal degradation. The aggresome pathway therefore likely provides a novel system for delivery of aggregated proteins from cytoplasm to lysosomes for degradation (Garcia-Mata, R., Gao, Y. S. & Sztul, E. (2002) *Traffic* **3,** 388-396; incorporated herein by reference). In aggresomal protein degradation, histone deacetylase6 (HDAC6) binds both polyubiquitinated proteins and dynein motors, thereby acting to recruit protein cargo to dynein motors for transport to aggresomes (Kawaguchi, Y., Kovacs, J. J., McLaurin, A., Vance, J. M., Ito, A. & Yao, T. P. (2003) *Cell* **115,** 727-738; incorporated herein by reference). In the methods of the invention include the inhibition of both proteasomal and aggresomal protein degradation systems. Without wishing to be bound by any theories, the inhibition induces accumulation of polyubiquitinated proteins and significant cell stress, followed by activation of apoptotic cascades. For example, bortezomib was utilized to inhibit the proteasome and tubacin, which specifically inhibits HDAC6 (Haggarty, S. J., Koeller, K. M., Wong, J. C., Grozinger, C. M. & Schreiber, S. L. (2003) *Proc Natl Acad Sci U S A* **100,** 4389-4394; Haggarty, S. J., Koeller, K. M., Wong, J. C., Butcher, R. A. & Schreiber, S. L. (2003) *Chem Biol* **10,** 383-396; Wong, J. C., Hong, R. & Schreiber, S. L. (2003) *J Am Chem Soc* **125,** 5586-5587; incorporated herein by reference) to block the aggresome. In the methods of the invention, tubacin or other HDAC6 inhibitors combined with bortezomib induces synergistic cytotoxicity in multiple myeloma cell lines as well as freshly isolated bone marrow plasma cells from multiple myeloma patients.

Definitions

[00109] Before further description of the present invention, and in order that the invention may be more readily understood, certain terms are first defined and collected here for convenience.

[00110] Certain compounds of the present invention, and definitions of specific functional groups are also described in more detail below. For purposes of this invention, the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, *Handbook of Chemistry and Physics*, 75th Ed., inside cover, and specific functional groups are generally defined as described therein. Additionally, general principles of organic chemistry, as well as specific functional moieties and reactivity, are described in "Organic Chemistry", Thomas Sorrell, University Science Books, Sausalito: 1999, the entire contents of which are incorporated herein by reference. Furthermore, it will be appreciated by one of ordinary skill in the art that the synthetic methods, as described herein, utilize a variety of protecting groups. By the term "protecting group", has used herein, it is meant that a particular functional moiety, *e.g.*, C, O, S, or N, is temporarily blocked so that a reaction can be carried out selectively at another reactive site in a multifunctional compound. In preferred embodiments, a protecting group reacts selectively in good yield to give a protected substrate that is stable to the projected reactions; the protecting group must be selectively removed in good yield by readily available, preferably nontoxic reagents that do not attack the other functional groups; the protecting group forms an easily separable derivative (more preferably without the generation of new stereogenic centers); and the protecting group has a minimum of additional functionality to avoid further sites of reaction. As detailed herein, oxygen, sulfur, nitrogen and carbon protecting groups may be utilized. Exemplary protecting groups are detailed herein, however, it will be appreciated that the present invention is not intended to be limited to these protecting groups; rather, a variety of additional equivalent protecting groups can be readily identified using the above criteria and utilized in the method of the present invention. Additionally, a variety of protecting groups are described in "Protective Groups in Organic Synthesis" Third Ed. Greene, T.W. and Wuts, P.G., Eds., John Wiley & Sons, New York: 1999, the entire contents of which are hereby incorporated by reference. Furthermore, a variety of carbon protecting groups are described in Myers, A.; Kung, D.W.; Zhong, B.; Movassaghi, M.; Kwon, S. *J. Am. Chem. Soc.* **1999**, *121*, 8401-8402, the entire contents of which are hereby incorporated by reference.

[00111] It will be appreciated that the compounds, as described herein, may be substituted with any number of substituents or functional moieties. In general, the term "substituted" whether preceded by the term "optionally" or not, and substituents contained in formulas of this invention, refer to the replacement of hydrogen radicals in a given structure with the radical of a specified substituent. When more than one position in any given structure may be substituted with more than one substituent selected from a specified group,

the substituent may be either the same or different at every position. As used herein, the term "substituted" is contemplated to include all permissible substituents of organic compounds. In a broad aspect, the permissible substituents include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic substituents of organic compounds. For purposes of this invention, heteroatoms such as nitrogen may have hydrogen substituents and/or any permissible substituents of organic compounds described herein which satisfy the valencies of the heteroatoms. Furthermore, this invention is not intended to be limited in any manner by the permissible substituents of organic compounds. Combinations of substituents and variables envisioned by this invention are preferably those that result in the formation of stable compounds useful in the treatment, for example of proliferative disorders, including, but not limited to cancer. The term "stable", as used herein, preferably refers to compounds which possess stability sufficient to allow manufacture and which maintain the integrity of the compound for a sufficient period of time to be detected and preferably for a sufficient period of time to be useful for the purposes detailed herein.

[00112] The term "acyl", as used herein, refers to a carbonyl-containing functionality, *e.g.*, -C(=O)R', wherein R' is an aliphatic, alycyclic, heteroaliphatic, heterocyclic, aryl, heteroaryl, (aliphatic)aryl, (heteroaliphatic)aryl, heteroaliphatic(aryl) or heteroaliphatic(heteroaryl) moiety, whereby each of the aliphatic, heteroaliphatic, aryl, or heteroaryl moieties is substituted or unsubstituted, or is a substituted (*e.g.*, hydrogen or aliphatic, heteroaliphatic, aryl, or heteroaryl moieties) oxygen or nitrogen containing functionality (*e.g.*, forming a carboxylic acid, ester, or amide functionality).

[00113] The term "aliphatic", as used herein, includes both saturated and unsaturated, straight chain (*i.e.*, unbranched) or branched aliphatic hydrocarbons, which are optionally substituted with one or more functional groups. As will be appreciated by one of ordinary skill in the art, "aliphatic" is intended herein to include, but is not limited to, alkyl, alkenyl, alkynyl moieties. Thus, as used herein, the term "alkyl" includes straight and branched alkyl groups. An analogous convention applies to other generic terms such as "alkenyl", "alkynyl" and the like. Furthermore, as used herein, the terms "alkyl", "alkenyl", "alkynyl" and the like encompass both substituted and unsubstituted groups. In certain embodiments, as used herein, "lower alkyl" is used to indicate those alkyl groups (substituted, unsubstituted, branched or unbranched) having 1-6 carbon atoms.

[00114] In certain embodiments, the alkyl, alkenyl and alkynyl groups employed in the invention contain 1-20 aliphatic carbon atoms. In certain other embodiments, the alkyl,

alkenyl, and alkynyl groups employed in the invention contain 1-10 aliphatic carbon atoms. In yet other embodiments, the alkyl, alkenyl, and alkynyl groups employed in the invention contain 1-8 aliphatic carbon atoms. In still other embodiments, the alkyl, alkenyl, and alkynyl groups employed in the invention contain 1-6 aliphatic carbon atoms. In yet other embodiments, the alkyl, alkenyl, and alkynyl groups employed in the invention contain 1-4 carbon atoms. Illustrative aliphatic groups thus include, but are not limited to, for example, methyl, ethyl, n-propyl, isopropyl, allyl, n-butyl, sec-butyl, isobutyl, tert-butyl, n-pentyl, sec-pentyl, isopentyl, tert-pentyl, n-hexyl, sec-hexyl, moieties and the like, which again, may bear one or more substituents. Alkenyl groups include, but are not limited to, for example, ethenyl, propenyl, butenyl, 1-methyl-2-buten-l-yl, and the like. Representative alkynyl groups include, but are not limited to, ethynyl, 2-propynyl (propargyl), 1-propynyl and the like.

[00115] The term "alicyclic", as used herein, refers to compounds which combine the properties of aliphatic and cyclic compounds and include but are not limited to cyclic, or polycyclic aliphatic hydrocarbons and bridged cycloalkyl compounds, which are optionally substituted with one or more functional groups. As will be appreciated by one of ordinary skill in the art, "alicyclic" is intended herein to include, but is not limited to, cycloalkyl, cycloalkenyl, and cycloalkynyl moieties, which are optionally substituted with one or more functional groups. Illustrative alicyclic groups thus include, but are not limited to, for example, cyclopropyl, -CH_2-cyclopropyl, cyclobutyl, -CH_2-cyclobutyl, cyclopentyl, -CH_2-cyclopentyl-n, cyclohexyl, -CH_2-cyclohexyl, cyclohexenylethyl, cyclohexanylethyl, norborbyl moieties and the like, which again, may bear one or more substituents.

[00116] The term "alkoxy" (or "alkyloxy"), or "thioalkyl" as used herein refers to an alkyl group, as previously defined, attached to the parent molecular moiety through an oxygen atom or through a sulfur atom. In certain embodiments, the alkyl group contains 1-20 aliphatic carbon atoms. In certain other embodiments, the alkyl group contains 1-10 aliphatic carbon atoms. In yet other embodiments, the alkyl, alkenyl, and alkynyl groups employed in the invention contain 1-8 aliphatic carbon atoms. In still other embodiments, the alkyl group contains 1-6 aliphatic carbon atoms. In yet other embodiments, the alkyl group contains 1-4 aliphatic carbon atoms. Examples of alkoxy, include but are not limited to, methoxy, ethoxy, propoxy, isopropoxy, n-butoxy, tert-butoxy, neopentoxy and n-hexoxy. Examples of thioalkyl include, but are not limited to, methylthio, ethylthio, propylthio, isopropylthio, n-butylthio, and the like.

[00117] The term "alkylamino" refers to a group having the structure -NHR'wherein R' is alkyl, as defined herein. The term "aminoalkyl" refers to a group having the structure NH_2R'-, wherein R' is alkyl, as defined herein. In certain embodiments, the alkyl group contains 1-20 aliphatic carbon atoms. In certain other embodiments, the alkyl group contains 1-10 aliphatic carbon atoms. In yet other embodiments, the alkyl, alkenyl, and alkynyl groups employed in the invention contain 1-8 aliphatic carbon atoms. In still other embodiments, the alkyl group contains 1-6 aliphatic carbon atoms. In yet other embodiments, the alkyl group contains 1-4 aliphatic carbon atoms. Examples of alkylamino include, but are not limited to, methylamino, ethylamino, iso-propylamino and the like.

[00118] Some examples of substituents of the above-described aliphatic (and other) moieties of compounds of the invention include, but are not limited to aliphatic; heteroaliphatic; aryl; heteroaryl; alkylaryl; alkylheteroaryl; alkoxy; aryloxy; heteroalkoxy; heteroaryloxy; alkylthio; arylthio; heteroalkylthio; heteroarylthio; F; C1; Br; I; -OH; $-NO_2$; -CN; $-CF_3$; $-CH_2CF_3$; $-CHC1_2$; $-CH_2OH$; $-CH_2CH_2OH$; $-CH_2NH_2$; $-CH_2SO_2CH_3$; $-C(O)R_x$; $-CO_2(R_x)$; $-CON(R_x)_2$; $-OC(O)R_x$; $-OCO_2R_x$; $-OCON(R_x)_2$; $-N(R_x)_2$; $-S(O)_2R_x$; $-NR_x(CO)R_x$ wherein each occurrence of R_x independently includes, but is not limited to, aliphatic, alycyclic, heteroaliphatic, heterocyclic, aryl, heteroaryl, alkylaryl, or alkylheteroaryl, wherein any of the aliphatic, heteroaliphatic, alkylaryl, or alkylheteroaryl substituents described above and herein may be substituted or unsubstituted, branched or unbranched, cyclic or acyclic, and wherein any of the aryl or heteroaryl substituents described above and herein may be substituted or unsubstituted. Additional examples of generally applicable substituents are illustrated by the specific embodiments shown in the Examples that are described herein.

[00119] In general, the term "aromatic moiety", as used herein, refers to a stable mono- or polycyclic, unsaturated moiety having preferably 3-14 carbon atoms, each of which may be substituted or unsubstituted. In certain embodiments, the term "aromatic moiety" refers to a planar ring having p-orbitals perpendicular to the plane of the ring at each ring atom and satisfying the Huckel rule where the number of pi electrons in the ring is (4n+2) wherein n is an integer. A mono- or polycyclic, unsaturated moiety that does not satisfy one or all of these criteria for aromaticity is defined herein as "non-aromatic", and is encompassed by the term "alicyclic".

[00120] In general, the term "heteroaromatic moiety", as used herein, refers to a stable mono- or polycyclic, unsaturated moiety having preferably 3-14 carbon atoms, each of which may be substituted or unsubstituted; and comprising at least one heteroatom selected from O, S and N within the ring (*i.e.*, in place of a ring carbon atom). In certain embodiments, the

term "heteroaromatic moiety" refers to a planar ring comprising at least on heteroatom, having p-orbitals perpendicular to the plane of the ring at each ring atom, and satisfying the Huckel rule where the number of pi electrons in the ring is (4n+2) wherein n is an integer.

[00121] It will also be appreciated that aromatic and heteroaromatic moieties, as defined herein may be attached via an alkyl or heteroalkyl moiety and thus also include –(alkyl)aromatic, -(heteroalkyl)aromatic, -(heteroalkyl)heteroaromatic, and –(heteroalkyl)heteroaromatic moieties. Thus, as used herein, the phrases "aromatic or heteroaromatic moieties" and "aromatic, heteroaromatic, –(alkyl)aromatic, -(heteroalkyl)aromatic, -(heteroalkyl)heteroaromatic, and –(heteroalkyl)heteroaromatic" are interchangeable. Substituents include, but are not limited to, any of the previously mentioned substituents, *i.e.*, the substituents recited for aliphatic moieties, or for other moieties as disclosed herein, resulting in the formation of a stable compound.

[00122] The term "aryl", as used herein, does not differ significantly from the common meaning of the term in the art, and refers to an unsaturated cyclic moiety comprising at least one aromatic ring. In certain embodiments, "aryl" refers to a mono- or bicyclic carbocyclic ring system having one or two aromatic rings including, but not limited to, phenyl, naphthyl, tetrahydronaphthyl, indanyl, indenyl and the like.

[00123] The term "heteroaryl", as used herein, does not differ significantly from the common meaning of the term in the art, and refers to a cyclic aromatic radical having from five to ten ring atoms of which one ring atom is selected from S, O and N; zero, one or two ring atoms are additional heteroatoms independently selected from S, O and N; and the remaining ring atoms are carbon, the radical being joined to the rest of the molecule via any of the ring atoms, such as, for example, pyridyl, pyrazinyl, pyrimidinyl, pyrrolyl, pyrazolyl, imidazolyl, thiazolyl, oxazolyl, isooxazolyl, thiadiazolyl, oxadiazolyl, thiophenyl, furanyl, quinolinyl, isoquinolinyl, and the like.

[00124] It will be appreciated that aryl and heteroaryl groups (including bicyclic aryl groups) can be unsubstituted or substituted, wherein substitution includes replacement of one or more of the hydrogen atoms thereon independently with any one or more of the following moieties including, but not limited to: aliphatic; alicyclic; heteroaliphatic; heterocyclic; aromatic; heteroaromatic; aryl; heteroaryl; alkylaryl; heteroalkylaryl; alkylheteroaryl; heteroalkylheteroaryl; alkoxy; aryloxy; heteroalkoxy; heteroaryloxy; alkylthio; arylthio; heteroalkylthio; heteroarylthio; F; Cl; Br; I; -OH; $-NO_2$; -CN; $-CF_3$; $-CH_2CF_3$; $-CHCl_2$; $-CH_2OH$; $-CH_2CH_2OH$; $-CH_2NH_2$; $-CH_2SO_2CH_3$; $-C(O)R_x$; $-CO_2(R_x)$; $-CON(R_x)_2$; $-OC(O)R_x$; $-OCO_2R_x$; $-OCON(R_x)_2$; $-N(R_x)_2$; $-S(O)R_x$; $-S(O)_2R_x$; $-NR_x(CO)R_x$ wherein each occurrence

of R_x independently includes, but is not limited to, aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic, heteroaromatic, aryl, heteroaryl, alkylaryl, alkylheteroaryl, heteroalkylaryl or heteroalkylheteroaryl, wherein any of the aliphatic, alicyclic, heteroaliphatic, heterocyclic, alkylaryl, or alkylheteroaryl substituents described above and herein may be substituted or unsubstituted, branched or unbranched, saturated or unsaturated, and wherein any of the aromatic, heteroaromatic, aryl, heteroaryl, -(alkyl)aryl or -(alkyl)heteroaryl substituents described above and herein may be substituted or unsubstituted. Additionally, it will be appreciated, that any two adjacent groups taken together may represent a 4, 5, 6, or 7-membered substituted or unsubstituted alicyclic or heterocyclic moiety. Additional examples of generally applicable substituents are illustrated by the specific embodiments shown in the Examples that are described herein.

[00125] The term "cycloalkyl", as used herein, refers specifically to groups having three to seven, preferably three to ten carbon atoms. Suitable cycloalkyls include, but are not limited to cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl and the like, which, as in the case of aliphatic, alicyclic, heteroaliphatic or heterocyclic moieties, may optionally be substituted with substituents including, but not limited to aliphatic; alicyclic; heteroaliphatic; heterocyclic; aromatic; heteroaromatic; aryl; heteroaryl; alkylaryl; heteroalkylaryl; alkylheteroaryl; heteroalkylheteroaryl; alkoxy; aryloxy; heteroalkoxy; heteroaryloxy; alkylthio; arylthio; heteroalkylthio; heteroarylthio; F; Cl; Br; I; -OH; $-NO_2$; -CN; $-CF_3$; $-CH_2CF_3$; $-CHCl_2$; $-CH_2OH$; $-CH_2CH_2OH$; $-CH_2NH_2$; $-CH_2SO_2CH_3$; $-C(O)R_x$; $-CO_2(R_x)$; $-CON(R_x)_2$; $-OC(O)R_x$; $-OCO_2R_x$; $-OCON(R_x)_2$; $-N(R_x)_2$; $-S(O)_2R_x$; $-NR_x(CO)R_x$ wherein each occurrence of R_x independently includes, but is not limited to, aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic, heteroaromatic, aryl, heteroaryl, alkylaryl, alkylheteroaryl, heteroalkylaryl or heteroalkylheteroaryl, wherein any of the aliphatic, alicyclic, heteroaliphatic, heterocyclic, alkylaryl, or alkylheteroaryl substituents described above and herein may be substituted or unsubstituted, branched or unbranched, saturated or usaturated, and wherein any of the aromatic, heteroaromatic, aryl or heteroaryl substituents described above and herein may be substituted or unsubstituted. Additional examples of generally applicable substituents are illustrated by the specific embodiments shown in the Examples that are described herein.

[00126] The term "heteroaliphatic", as used herein, refers to aliphatic moieties in which one or more carbon atoms in the main chain have been substituted with a heteroatom. Thus, a heteroaliphatic group refers to an aliphatic chain which contains one or more oxygen, sulfur, nitrogen, phosphorus or silicon atoms, *e.g.*, in place of carbon atoms. Heteroaliphatic

moieties may be linear or branched, and saturated o runsaturated. In certain embodiments, heteroaliphatic moieties are substituted by independent replacement of one or more of the hydrogen atoms thereon with one or more moieties including, but not limited to aliphatic; alicyclic; heteroaliphatic; heterocyclic; aromatic; heteroaromatic; aryl; heteroaryl; alkylaryl; alkylheteroaryl; alkoxy; aryloxy; heteroalkoxy; heteroaryloxy; alkylthio; arylthio; heteroalkylthio; heteroarylthio; F; C1; Br; I; -OH; $-NO_2$; -CN; $-CF_3$; $-CH_2CF_3$; $-CHC1_2$; $-CH_2OH$; $-CH_2CH_2OH$; $-CH_2NH_2$; $-CH_2SO_2CH_3$; $-C(O)R_x$; $-CO_2(R_x)$; $-CON(R_x)_2$; $-OC(O)R_x$; $-OCO_2R_x$; $-OCON(R_x)_2$; $-N(R_x)_2$; $-S(O)_2R_x$; $-NR_x(CO)R_x$ wherein each occurrence of R_x independently includes, but is not limited to, aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic, heteroaromatic, aryl, heteroaryl, alkylaryl, alkylheteroaryl, heteroalkylaryl or heteroalkylheteroaryl, wherein any of the aliphatic, alicyclic, heteroaliphatic, heterocyclic, alkylaryl, or alkylheteroaryl substituents described above and herein may be substituted or unsubstituted, branched or unbranched, saturated or unsaturated, and wherein any of the aromatic, heteroaromatic, aryl or heteroaryl substituents described above and herein may be substituted or unsubstituted. Additional examples of generally applicable substituents are illustrated by the specific embodiments shown in the Examples that are described herein.

[00127] The term "heterocycloalkyl", "heterocycle" or "heterocyclic", as used herein, refers to compounds which combine the properties of heteroaliphatic and cyclic compounds and include, but are not limited to, saturated and unsaturated mono- or polycyclic cyclic ring systems having 5-16 atoms wherein at least one ring atom is a heteroatom selected from O, S and N (wherein the nitrogen and sulfur heteroatoms may be optionally be oxidized), wherein the ring systems are optionally substituted with one or more functional groups, as defined herein. In certain embodiments, the term "heterocycloalkyl", "heterocycle" or "heterocyclic" refers to a non-aromatic 5-, 6-, or 7- membered ring or a polycyclic group wherein at least one ring atom is a heteroatom selected from O, S and N (wherein the nitrogen and sulfur heteroatoms may be optionally be oxidized), including, but not limited to, a bi- or tri-cyclic group, comprising fused six-membered rings having between one and three heteroatoms independently selected from oxygen, sulfur and nitrogen, wherein (i) each 5-membered ring has 0 to 2 double bonds, each 6-membered ring has 0 to 2 double bonds and each 7-membered ring has 0 to 3 double bonds, (ii) the nitrogen and sulfur heteroatoms may be optionally be oxidized, (iii) the nitrogen heteroatom may optionally be quaternized, and (iv) any of the above heterocyclic rings may be fused to an aryl or heteroaryl ring. Representative heterocycles include, but are not limited to, heterocycles such as furanyl, thiofuranyl, pyranyl, pyrrolyl, thienyl, pyrrolidinyl, pyrazolinyl, pyrazolidiny1, imidazolinyl,

imidazolidinyl, piperidinyl, piperazinyl, oxazolyl, oxazolidinyl, isooxazolyl, isoxazolidinyl, dioxazolyl, thiadiazolyl, oxadiazolyl, tetrazolyl, triazolyl, thiatriazolyl, oxatriazolyl, thiadiazolyl, oxadiazolyl, morpholinyl, thiazolyl, thiazolidinyl, isothiazolyl, isothiazolidinyl, dithiazolyl, dithiazolidinyl, tetrahydrofuryl, and benzofused derivatives thereof. In certain embodiments, a "substituted heterocycle, or heterocycloalkyl or heterocyclic" group is utilized and as used herein, refers to a heterocycle, or heterocycloalkyl or heterocyclic group, as defined above, substituted by the independent replacement of one, two or three of the hydrogen atoms thereon with but are not limited to aliphatic; alicyclic; heteroaliphatic; heterocyclic; aromatic; heteroaromatic; aryl; heteroaryl; alkylaryl; heteroalkylaryl; alkylheteroaryl; heteroalkylheteroaryl; alkoxy; aryloxy; heteroalkoxy; heteroaryloxy; alkylthio; arylthio; heteroalkylthio; heteroarylthio; F; Cl; Br; I; - OH; $-NO_2$; -CN; $-CF_3$; $-CH_2CF_3$; $-CHCl_2$; $-CH_2OH$; $-CH_2CH_2OH$; $-CH_2NH_2$; $-CH_2SO_2CH_3$; $-C(O)R_x$; $-CO_2(R_x)$; $-CON(R_x)_2$; $-OC(O)R_x$; $-OCO_2R_x$; $-OCON(R_x)_2$; $-N(R_x)_2$; $-S(O)_2R_x$; $-NR_x(CO)R_x$ wherein each occurrence of R_x independently includes, but is not limited to, aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic, heteroaromatic, aryl, heteroaryl, alkylaryl, alkylheteroaryl, heteroalkylaryl or heteroalkylheteroaryl, wherein any of the aliphatic, alicyclic, heteroaliphatic, heterocyclic, alkylaryl, or alkylheteroaryl substituents described above and herein may be substituted or unsubstituted, branched or unbranched, saturated or unsaturated, and wherein any of the aromatic, heteroaromatic, aryl or heteroaryl substitutents described above and herein may be substituted or unsubstituted. Additional examples or generally applicable substituents are illustrated by the specific embodiments shown in the Examples, which are described herein.

[00128] Additionally, it will be appreciated that any of the alicyclic or heterocyclic moieties described above and herein may comprise an aryl or heteroaryl moiety fused thereto. Additional examples of generally applicable substituents are illustrated by the specific embodiments shown in the Examples that are described herein.The terms "halo" and "halogen" as used herein refer to an atom selected from fluorine, chlorine, bromine and iodine.

[00129] The terms "halo" and "halogen" as used herein refer to an atom selected from fluorine, chlorine, bromine and iodine.

[00130] The term "haloalkyl" denotes an alkyl group, as defined above, having one, two, or three halogen atoms attached thereto and is exemplified by such groups as chloromethyl, bromoethyl, trifluoromethyl, and the like.

[00131] The term "amino", as used herein, refers to a primary ($-NH_2$), secondary ($-NHR_x$), tertiary ($-NR_xR_y$) or quaternary ($-N^+R_xR_yR_z$) amine, where R_x, R_y and R_z are independently an aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic or heteroaromatic moiety, as defined herein. Examples of amino groups include, but are not limited to, methylamino, dimethylamino, ethylamino, diethylamino, diethylaminocarbonyl, methylethylamino, iso-propylamino, piperidino, trimethylamino, and propylamino.

[00132] The term "alkylidene", as used herein, refers to a substituted or unsubstituted, linear or branched saturated divalent radical consisting solely of carbon and hydrogen atoms, having from one to n carbon atoms, having a free valence "-" at both ends of the radical.

[00133] The term "alkenylidene", as used herein, refers to a substituted or unsubstituted, linear or branched unsaturated divalent radical consisting solely of carbon and hydrogen atoms, having from two to n carbon atoms, having a free valence "-" at both ends of the radical, and wherein the unsaturation is present only as double bonds and wherein a double bond can exist between the first carbon of the chain and the rest of the molecule.

[00134] The term "alkynylidene", as used herein, refers to a substituted or unsubstituted, linear or branched unsaturated divalent radical consisting solely of carbon and hydrogen atoms, having from two to n carbon atoms, having a free valence "-" at both ends of the radical, and wherein the unsaturation is present only as triple bonds and wherein a triple bond can exist between the first carbon of the chain and the rest of the molecule.

[00135] Unless otherwise indicated, as used herein, the terms "alkyl", "alkenyl", "alkynyl", "heteroalkyl", "heteroalkenyl", "heteroalkynyl", "alkylidene", alkenylidene", -(alkyl)aryl, -(heteroalkyl)aryl, -(heteroalkyl)aryl, -(heteroalkyl)heteroaryl, and the like encompass substituted and unsubstituted, and linear and branched groups. Similarly, the terms "aliphatic", "heteroaliphatic", and the like encompass substituted and unsubstituted, saturated and unsaturated, and linear and branched groups. Similarly, the terms "cycloalkyl", "heterocycle", "heterocyclic", and the like encompass substituted and unsubstituted, and saturated and unsaturated groups. Additionally, the terms "cycloalkenyl", "cycloalkynyl", "heterocycloalkenyl", "heterocycloalkynyl", "aromatic", "heteroaromatic, "aryl", "heteroaryl" and the like encompass both substituted and unsubstituted groups.

[00136] The phrase, "pharmaceutically acceptable derivative", as used herein, denotes any pharmaceutically acceptable salt, ester, or salt of such ester, of such compound, or any other adduct or derivative which, upon administration to a patient, is capable of providing (directly or indirectly) a compound as otherwise described herein, or a metabolite or residue thereof. Pharmaceutically acceptable derivatives thus include among others pro-drugs. A

pro-drug is a derivative of a compound, usually with significantly reduced pharmacological activity, which contains an additional moiety, which is susceptible to removal *in vivo* yielding the parent molecule as the pharmacologically active species. An example of a pro-drug is an ester, which is cleaved *in vivo* to yield a compound of interest. Pro-drugs of a variety of compounds, and materials and methods for derivatizing the parent compounds to create the pro-drugs, are known and may be adapted to the present invention. Pharmaceutically acceptable derivatives also include "reverse pro-drugs." Reverse pro-drugs, rather than being activated, are inactivated upon absorption. For example, as discussed herein, many of the ester-containing compounds of the invention are biologically active but are inactivated upon exposure to certain physiological environments such as a blood, lymph, serum, extracellular fluid, *etc.* which contain esterase activity. The biological activity of reverse pro-drugs and pro-drugs may also be altered by appending a functionality onto the compound, which may be catalyzed by an enzyme. Also, included are oxidation and reduction reactions, including enzyme-catalyzed oxidation and reduction reactions. Certain exemplary pharmaceutical compositions and pharmaceutically acceptable derivatives will be discussed in more detail herein below.

[00137] The term "linker," as used herein, refers to a chemical moiety utilized to attach one part of a compound of interest to another part of the compound. Exemplary linkers are described herein.

[00138] Unless indicated otherwise, the terms defined below have the following meanings:

[00139] "Compound": The term "compound" or "chemical compound" as used herein can include organometallic compounds, organic compounds, metals, transitional metal complexes, and small molecules. In certain preferred embodiments, polynucleotides are excluded from the definition of compounds. In other preferred embodiments, polynucleotides and peptides are excluded from the definition of compounds. In a particularly preferred embodiment, the term compounds refers to small molecules (*e.g.*, preferably, non-peptidic and non-oligomeric) and excludes peptides, polynucleotides, transition metal complexes, metals, and organometallic compounds.

[00140] "Small Molecule": As used herein, the term "small molecule" refers to a non-peptidic, non-oligomeric organic compound either synthesized in the laboratory or found in nature. Small molecules, as used herein, can refer to compounds that are "natural product-like", however, the term "small molecule" is not limited to "natural product-like" compounds. Rather, a small molecule is typically characterized in that it contains several

carbon-carbon bonds, and has a molecular weight of less than 1500, although this characterization is not intended to be limiting for the purposes of the present invention. Examples of "small molecules" that occur in nature include, but are not limited to, taxol, dynemicin, and rapamycin. Examples of "small molecules" that are synthesized in the laboratory include, but are not limited to, compounds described in Tan *et al.*, ("Stereoselective Synthesis of over Two Million Compounds Having Structural Features Both Reminiscent of Natural Products and Compatible with Miniaturized Cell-Based Assays" *J. Am. Chem. Soc.* 120:8565, 1998; incorporated herein by reference). In certain other preferred embodiments, natural-product-like small molecules are utilized.

[00141] "Natural Product-Like Compound": As used herein, the term "natural product-like compound" refers to compounds that are similar to complex natural products which nature has selected through evolution. Typically, these compounds contain one or more stereocenters, a high density and diversity of functionality, and a diverse selection of atoms within one structure. In this context, diversity of functionality can be defined as varying the topology, charge, size, hydrophilicity, hydrophobicity, and reactivity to name a few, of the functional groups present in the compounds. The term, "high density of functionality", as used herein, can preferably be used to define any molecule that contains preferably three or more latent or active diversifiable functional moieties. These structural characteristics may additionally render the inventive compounds functionally reminiscent of complex natural products, in that they may interact specifically with a particular biological receptor, and thus may also be functionally natural product-like.

[00142] "Metal chelator": As used herein, the term "metal chelator" refers to any molecule or moiety that is is capable of forming a complex (*i.e.*, "chelates") with a metal ion. In certain exemplary embodiments, a metal chelator refers to to any molecule or moiety that "binds" to a metal ion, in solution, making it unavailable for use in chemical/enzymatic reactions. In certain embodiments, the solution comprises aqueous environments under physiological conditions. Examples of metal ions include, but are not limited to, Ca^{2+}, Fe^{3+}, Zn^{2+}, Na^{+}, *etc.* In certain embodiments, the metal chelator binds Zn^{2+}. In certain embodiments, molecules of moieties that precipitate metal ions are not considered to be metal chelators.

[00143] As used herein the term "biological sample" includes, without limitation, cell cultures or extracts thereof; biopsied material obtained from an animal (*e.g.*, mammal) or extracts thereof; and blood, saliva, urine, feces, semen, tears, or other body fluids or extracts thereof. For example, the term "biological sample" refers to any solid or fluid sample

obtained from, excreted by or secreted by any living organism, including single-celled micro-organisms (such as bacteria and yeasts) and multicellular organisms (such as plants and animals, for instance a vertebrate or a mammal, and in particular a healthy or apparently healthy human subject or a human patient affected by a condition or disease to be diagnosed or investigated). The biological sample can be in any form, including a solid material such as a tissue, cells, a cell pellet, a cell extract, cell homogenates, or cell fractions; or a biopsy, or a biological fluid. The biological fluid may be obtained from any site (*e.g.*, blood, saliva (or a mouth wash containing buccal cells), tears, plasma, serum, urine, bile, cerebrospinal fluid, amniotic fluid, peritoneal fluid, and pleural fluid, or cells therefrom, aqueous or vitreous humor, or any bodily secretion), a transudate, an exudate (*e.g.* fluid obtained from an abscess or any other site of infection or inflammation), or fluid obtained from a joint (*e.g.* a normal joint or a joint affected by disease such as rheumatoid arthritis, osteoarthritis, gout or septic arthritis). The biological sample can be obtained from any organ or tissue (including a biopsy or autopsy specimen) or may comprise cells (whether primary cells or cultured cells) or medium conditioned by any cell, tissue or organ. Biological samples may also include sections of tissues such as frozen sections taken for histological purposes. Biological samples also include mixtures of biological molecules including proteins, lipids, carbohydrates and nucleic acids generated by partial or complete fractionation of cell or tissue homogenates. Although the sample is preferably taken from a human subject, biological samples may be from any animal, plant, bacteria, virus, yeast, etc. The term *animal*, as used herein, refers to humans as well as non-human animals, at any stage of development, including, for example, mammals, birds, reptiles, amphibians, fish, worms and single cells. Cell cultures and live tissue samples are considered to be pluralities of animals. In certain exemplary embodiments, the non-human animal is a mammal (*e.g.*, a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, or a pig). An animal may be a transgenic animal or a human clone. If desired, the biological sample may be subjected to preliminary processing, including preliminary separation techniques.

[00144] The term "administration" or "administering" includes routes of introducing the compound of the invention(s) to a subject to perform their intended function. Examples of routes of administration that may be used include injection (subcutaneous, intravenous, parenterally, intraperitoneally, intrathecal), oral, inhalation, rectal and transdermal. The pharmaceutical preparations may be given by forms suitable for each administration route. For example, these preparations are administered in tablets or capsule form, by injection, inhalation, eye lotion, ointment, suppository, etc., administration by injection, infusion or

inhalation; topical by lotion or ointment; and rectal by suppositories. Oral administration is preferred. The injection can be bolus or can be continuous infusion. Depending on the route of administration, the compound of the invention can be coated with or disposed in a selected material to protect it from natural conditions which may detrimentally effect its ability to perform its intended function. The compound of the invention can be administered alone, or in conjunction with either another agent as described above or with a pharmaceutically-acceptable carrier, or both. The compounds of the invention can be administered prior to the administration of the other agent, simultaneously with the agent, or after the administration of the agent. Furthermore, the compound of the invention can also be administered in a pro-form which is converted into its active metabolite, or more active metabolite in vivo.

[00145] The language "biological activities" of a compound of the invention includes all activities elicited by compound of the inventions in a responsive cell. It includes genomic and non-genomic activities elicited by these compounds.

[00146] "Biological composition," "biological sample," or "sample" refer to a composition containing or derived from cells or biopolymers. Cell-containing compositions include, for example, mammalian blood, red cell concentrates, platelet concentrates, leukocyte concentrates, blood cell proteins, blood plasma, platelet-rich plasma, a plasma concentrate, a precipitate from any fractionation of the plasma, a supernatant from any fractionation of the plasma, blood plasma protein fractions, purified or partially purified blood proteins or other components, serum, semen, mammalian colostrum, milk, saliva, placental extracts, a cryoprecipitate, a cryosupernatant, a cell lysate, mammalian cell culture or culture medium, products of fermentation, ascites fluid, proteins induced in blood cells, and products produced in cell culture by normal or transformed cells (*e.g.*, via recombinant DNA or monoclonal antibody technology). Biological compositions can be cell-free. In a preferred embodiment, a suitable biological composition or biological sample is a red blood cell suspension. In some embodiments, the blood cell suspension includes mammalian blood cells. Preferably, the blood cells are obtained from a human, a non-human primate, a dog, a cat, a horse, a cow, a goat, a sheep or a pig. In preferred embodiments, the blood cell suspension includes red blood cells and/or platelets and/or leukocytes and/or bone marrow cells.

[00147] The term "effective amount" includes an amount effective, at dosages and for periods of time necessary, to achieve the desired result, e.g., sufficient to treat a protein degradation disorder. An effective amount of compound of the invention may vary according to factors such as the disease state, age, and weight of the subject, and the ability of the

compound of the invention to elicit a desired response in the subject. Dosage regimens may be adjusted to provide the optimum therapeutic response. An effective amount is also one in which any toxic or detrimental effects (*e.g.*, side effects) of the compound of the invention are outweighed by the therapeutically beneficial effects.

[00148] A therapeutically effective amount of compound of the invention (i.e., an effective dosage) may range from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a compound of the invention can include a single treatment or, preferably, can include a series of treatments. In one example, a subject is treated with a compound of the invention in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of a compound of the invention used for treatment may increase or decrease over the course of a particular treatment.

[00149] The term "homeostasis" is art-recognized to mean maintenance of static, or constant, conditions in an internal environment.

[00150] The language "improved biological properties" refers to any activity inherent in a compound of the invention that enhances its effectiveness in vivo. In a preferred embodiment, this term refers to any qualitative or quantitative improved therapeutic property of a compound of the invention, such as reduced toxicity, *e.g.*, reduced hypercalcemic activity.

[00151] "Inactivating," "inactivation," or "inactivate" "anti-cancer" and "treat protein degradation disorders" as used herein refers to diminishing or eliminating affected cells (e.g. per ml of a treated biological composition). In addition, these terms include reducing or abolishing the protein degradation disorder or the protein degradation disorder . The aforementioned are illustrated in detail in the examples bellow. Preferably the methods of the invention result in at least 50% of the affected cells in the treated preparation are eliminated, preferably at least 70% of the cells are eliminated, more preferably at least 80%, still more preferably at least 90%, still more preferably at least 95%. still more preferably, at least 99%,

and even still more preferably, 100% of the affected cells are eliminated. The number of affected cells in a preparation may be measured by cells counts per ml of preparation. Such a measurement may be accomplished by a variety of well known assays well known to a person of ordinary skill in the art.

[00152] An "initial period of treatment" and "period of treatment" as used herein may be the time in which it takes to establish a stable and/or therapeutically effective blood serum level of a therapeutic compound of the invention, or the time in which it takes for the subject to clear a substantial portion of the therapeutic, or any period of time selected by the subject or healthcare professional that is relevant to the treatment.

[00153] "Therapeutic," as used herein refers to a small molecule, peptide, protein, enzyme antibody, nucleic acid, etc. that is effective to treat or is suspected of being effective to treat a protein degradation disorder.

[00154] The term "modulate" refers to increases or decreases in the activity of a cell in response to exposure to a compound of the invention, e.g., the inhibition of proliferation and/or protein degradation of at least a sub-population of cells in an animal such that a desired end result is achieved, *e.g.*, a therapeutic result. In preferred embodiments, this phrase is intended to include protein degradation disorder and/or a protein degradation disorder of cells.

[00155] The term "obtaining" is intended to include purchasing, synthesizing or otherwise acquiring the compounds of the invention.

[00156] The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticulare, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

[00157] The term "prodrug" includes compounds with moieties that can be metabolized *in vivo*. Generally, the prodrugs are metabolized *in vivo* by esterases or by other mechanisms to active drugs. Prodrugs may only become active upon such reaction under biological conditions, or they may have activity in their unreacted forms. Examples of prodrugs and their uses are well known in the art (See, *e.g.*, Berge *et al.* (1977) "Pharmaceutical Salts", *J. Pharm. Sci.* 66:1-19). The prodrugs can be prepared *in situ* during the final isolation and purification of the compounds, or by separately reacting the purified compound in its free acid form or hydroxyl with a suitable esterifying agent. Hydroxyl

groups can be converted into esters *via* treatment with a carboxylic acid. Examples of prodrug moieties include substituted and unsubstituted, branched or unbranched lower alkyl ester moieties, (*e.g.*, propionoic acid esters), lower alkenyl esters, di-lower alkyl-amino lower-alkyl esters (*e.g.*, dimethylaminoethyl ester), acylamino lower alkyl esters (*e.g.*, acetyloxymethyl ester), acyloxy lower alkyl esters (*e.g.*, pivaloyloxymethyl ester), aryl esters (phenyl ester), aryl-lower alkyl esters (*e.g.*, benzyl ester), substituted (*e.g.*, with methyl, halo, or methoxy substituents) aryl and aryl-lower alkyl esters, amides, lower-alkyl amides, di-lower alkyl amides, and hydroxy amides. Other examples of prodrugs include derivatives of compounds of any of the formulae disclosed herein that comprise -NO, $-NO_2$, -ONO, or $-ONO_2$ moieties. Preferred prodrug moieties are acyl esters. Prodrugs which are converted to active forms through other mechanisms *in vivo* are also included. The compounds of the invention may be synthesized as pro-drugs that are metabolized by the subject into the compound of the invention.

[00158] The language "a prophylactically effective amount" of a compound refers to an amount of a compound of the invention of the formula (I) or otherwise described herein which is effective, upon single or multiple dose administration to the patient, in preventing or treating a protein degradation disorder.

[00159] The language "reduced toxicity" is intended to include a reduction in any undesired side effect elicited by a compound of the invention when administered *in vivo*, *e.g.*, a reduction in the hypercalcemic activity.

[00160] The term "subject" and "patient" are used interchangeably herein and include organisms which are capable of suffering from a protein degradation disorder or who could otherwise benefit from the administration of a compound of the invention, such as human and non-human animals. Preferred human animals include human patients suffering from or prone to suffering from a protein degradation disorder or associated state, as described herein. The term "non-human animals" of the invention includes all vertebrates, *e.g.*, mammals, *e.g.*, rodents, *e.g.*, mice, and non-mammals, such as non-human primates, *e.g.*, sheep, dog, cow, chickens, amphibians, reptiles, *etc.* Susceptible to a protein degradation disorder is meant to include subjects at risk of developing a protein degradation disorder, i.e., subjects suffering from myeloma, subject having a family history of a protein degradation disorder, etc.

[00161] The phrases "systemic administration," "administered systemically," "peripheral administration," and "administered peripherally" as used herein includes the administration of a compound of the invention(s), drug or other material, such that it enters

the patient's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

[00162] The language "a protein degradation disorder" is a condition or disease, which can be prevented, treated or otherwise ameliorated by administration of one or more compounds of the invention (e.g. is caused, exacerbated or characterized by the presence of aggresomes in the cells or body of a subject). Protein degradation disorders include cellular proliferation disorders and protein deposition disorders. Cellular proliferation disorders include cancer, for example, myeloma. Other cancers include cancers derived from the epithelium. Also included are solid tumors, such as breast, lung and liver. Protein deposition disorders include Wilson's disease, spinocerebellar ataxia, prion disease, Parkinson's disease, Huntington's disease, Familial amytrophic lateral sclerosis, amyloidosis, Alzheimer's disease, Alexander's disease, alcoholic liver disease, cystic fibrosis, or Lewy body dementia.

[00163] A protein degradation disorder or includes cancer. Cancers include, for example, epithelial derived cancers, and solid tumors, and cancers involving protein disregulation. Other cancers include multiple myeloma, leukemia, lymphoma, breast cancer, lung cancer and liver cancer.

[00164] A protein degradation disorder includes, for example Wilson's disease, spinocerebellar ataxia, prion disease, Parkinson's disease, Huntington's disease, Familial amytrophic lateral sclerosis, amyloidosis, Alzheimer's disease, Alexander's disease, alcoholic liver disease, cystic fibrosis, Pick's disease or Lewy body dementia.

[00165] As used herein "suffering from or susceptible to a protein degradation disorder" refers to subjects having or at risk of having such a disorder.

[00166] The compounds or other therapeutics of the invention may either directly or indirectly inhibit protein degradation. The compounds or other therapeutics of the invention may directly or indirectly inhibit aggresome formation or activity. The compounds or other therapeutics of the invention may directly or indirectly inhibit proteasome activity. Alternately, the compounds or other therapeutics of the invention may directly or indirectly inhibit aggresome and proteasome activity. The compounds or other therapeutics of the invention may directly or indirectly inhibit HDAC6 activity. Contacting cells or administering the compounds or other therapeutics of the invention to a subject is one method of treating a cell or a subject with susceptible to a protein degradation disorder or inhibiting the occurrence of a protein degradation disorder.

[00167] As used herein, "cell from a subject" include bone marrow stromal cell, (BMSC), a peripheral blood mononuclear cell (PBMC), lymphocytes, hair follicles, blood

cells, other epithelial cells, bone marrow plasma cells, primary cancer cells, patient derived tumor cells, normal or cancerous hematopoietic stem cells, neural stem cells, solid tumor cells, astrocytes, and the like. "Cultured cell" may include one or more of MM.1S, U266, RPMI8226, DOX40, MM.1R, INA-6, LR5, primary and established cancer cell lines, primary and established normal cell lines.

[00168] As used herein, "phenotype of a cell," "phenotype of the subject," or "symptoms of the subject" refer to outward, physical manifestation or characteristic of the cell or subject, e.g., an observable manifestation or observable attribute. The attribute or manifestation may be structural, biochemical, physiological and/or behavioural. The phenotype may be a biological or clinical sequelae in response to a particular treatment or compound. Phenotypes include, anemia, thrombocytopenia, neutropenia, osteolytic lesions, bone pain, immunodeficiency, renal insufficiency, hypercalcemia, aneuploidy of mature plasma cells, percentage of malignant cells, acetylation state of tubulin, apoptosis of mature plasma cells, level of aggresomes in mature plasma cells, HDAC6 ubiquitination in mature plasma cells, HDAC6 association with dynein in mature plasma cells, cellular levels of ubiquintinated proteins in mature plasma cells, level of caspase-8 in mature plasma cells, level of PARP in mature plasma cells, thymidine uptake in mature plasma cells, dilated ER cisternae, aggregation of mature plasma cells, deposits of immunoglobulins in mature plasma cells, acetylation state of non-histone proteins, global ubiquitination state of the cellular proteins, state of cell cycle regulation, necrosis, markers of apoptosis, apoptosis state, Russell body formation, Cystic Fibrosis transmembrane protein receptor state, and modulation of cellular protein deposits, or global acetylation state of cellular and extracellular proteins.

[00169] "Modulation of the phenotype," refers to a change or alteration in the observable phenotype. The modulation may be, for example, an increase or decrease in the characteristic, or the appearance or disappearance of a particular phenotype.

[00170] "Favorable clinical response," refers to any advantageous or beneficial change in the phenotype of the subject. For example, decreased symptoms or measurable indications of the disease. Favorable clinical responses may also be a total amelioration of the disease state.

[00171] "Protein degradation inhibitor" is a compound or other therapeutic that is capable of reducing protein degradation in a cell or subject. Protein degradation inhibitors may, for example inhibit HDAC6. Examples include histone acetylase inhibitors (14, 15), tubacin, bortezomib, velcade, SAHA, R115777 FTI, ^{166}Holminun-DOTMP, arsenic trioxide, 17-AAG, or compounds described herein. Protein degradation inhibitors may directly or

indirectly inhibit HDAC6 enzymatic activity, and without wishing to be bound by any particular scientific theory, to thereby inhibit aggresome mediated protein degradation. Protein degradation inhibitors may also inhibit HSP90, transcription factors, or other chaperone proteins. Protein degradation inhibitors may alternately inhibit the C-terminal acetylation activity of HDAC6, thereby inhibiting aggresome mediated protein degradation. Protein degradation inhibitors may also be aggresome inhibitors. Examples of aggresome inhibitors include tubacin, scriptade, or other compounds described herein. Protein degradation inhibitors may also inhibit, either directly or indirectly, the proteasome. Examples of proteasome inhibitors include bortezomib, MG132, sapojargon, or NPI-0052.

[00172] Additional protein degradation inhibitors include peptides derived from HDAC6 sufficient to modulate a phenotype of a cell. For example, peptides derived from the C-terminal peptide of HDAC6. Histone deacetylase inhibitors (HDI) include compounds such as hydroxamates, non-selectively targeting nearly all human HDAC enzymes (14,15).

[00173] Determination of a therapeutically effective anti-proliferative amount or a prophylactically effective anti-proliferative amount of the compound of the invention, can be readily made by the physician or veterinarian (the "attending clinician"), as one skilled in the art, by the use of known techniques and by observing results obtained under analogous circumstances. The dosages may be varied depending upon the requirements of the subject in the judgment of the attending clinician; the severity of the condition being treated and the particular compound being employed. In determining the therapeutically effective anti-proliferative amount or dose, and the prophylactically effective anti-proliferative amount or dose, a number of factors are considered by the attending clinician, including, but not limited to: the specific disease state; pharmacodynamic characteristics of the particular agent and its mode and route of administration; the desired time course of treatment; the species of mammal; its size, age, and general health; the specific disease involved; the degree of or involvement or the severity of the disease; the response of the individual patient; the particular compound administered; the mode of administration; the bioavailability characteristics of the preparation administered; the dose regimen selected; the kind of concurrent treatment (*i.e.*, the interaction of the compound of the invention with other co-administered therapeutics); and other relevant circumstances.

[00174] Treatment can be initiated with smaller dosages, which are less than the optimum dose of the compound. Thereafter, the dosage may be increased by small increments until the optimum effect under the circumstances is reached. For convenience, the total daily dosage may be divided and administered in portions during the day if desired.

A therapeutically effective amount and a prophylactically effective anti-proliferative amount of a compound of the invention of the invention is expected to vary from about 0.1 milligram per kilogram of body weight per day (mg/kg/day) to about 100 mg/kg/day.

[00175] Compounds determined to be effective for the prevention or treatment of protein degradation disorders in animals, *e.g.*, dogs, chickens, and rodents, may also be useful in treatment of the disorders in humans. Those skilled in the art of treating the disorders in humans will know, based upon the data obtained in animal studies, the dosage and route of administration of the compound to humans. In general, the dosage and route of administration in humans is expected to be similar to that in animals.

[00176] The identification of those patients who are in need of prophylactic treatment for proliferative disease states is well within the ability and knowledge of one skilled in the art. Certain of the methods for identification of patients which are at risk of developing proliferative disease states which can be treated by the subject method are appreciated in the medical arts, such as family history, the presence of risk factors associated with the development of that disease state in the subject patient. A clinician skilled in the art can readily identify such candidate patients, by the use of, for example, clinical tests, physical examination and medical/family history.

[00177] A method of assessing the efficacy of an anti-proliferative treatment in a subject includes determining a pre-treatment phenotype by methods well known in the art and then administering a therapeutically effective amount of a protein degradation inhibitor. After an appropriate period of time after the administration of the compound, e.g., 2 hours, 4 hours, 8 hours, 12 hours, or 72 hours, the phenotype is determined again. The modulation of the phenotype indicates efficacy of an treatment. The phenotype may be determined periodically throughout treatment. For example, the phenotype may be checked every few hours, days or weeks to assess the further efficacy of the treatment. Phenotypes and methods of determining phenotypes are discussed *infra*. The methods described may be used to screen or select patients that may benefit from treatment with protein degradation inhibitors.

[00178] As used herein, "obtaining a biological sample from a subject," includes obtaining a sample for use in the methods described herein. Biological samples are described herein.

[00179] In another aspect, a compound or other therapeutic of the invention is packaged in a therapeutically effective amount with a pharmaceutically acceptable carrier or diluent. The composition may be formulated for treating a subject suffering from or

susceptible to a protein degradation disorder, and packaged with instructions to treat a subject suffering from or susceptible to a protein degradation disorder.

[00180] In yet another aspect, a method of treating a subject suffering from or susceptible to a protein degradation disorder comprising administering to a subject in need thereof a therapeutically effective amount of protein degradation inhibitor, to thereby treat the subject suffering from or susceptible to a protein degradation disorder. Upon identification of a subject suffering from or susceptible to a protein degradation disorder, for example, myeloma, an protein degradation inhibitor is administered.

[00181] Methods of inhibiting protein degradation in a protein degradation disorder, comprise contacting cells or subjects with a protein degradation inhibitor. The contacting may be by addition of the inhibitor to a fluid surrounding the cells, for example, to the growth media in which the cells are living or existing. The contacting may also be by directly contacting the inhibitor to the cells. Alternately, the contacting may be by passage of the inhibitor through a subject, for example, after administration, depending on the route of administration, the inhibitor may travel through the digestive tract or the blood stream or may be applied or administered directly to cells in need of the inhibition.

[00182] In another aspect, methods of inhibiting a protein degradation disorder in a subject comprise administering an effective amount of an inhibitor to a subject. The administration may be by any route of administering known in the pharmaceutical arts. The subject may have a protein degradation disorder, may be at risk of developing a protein degradation disorder, or may need prophylactic treatment.

[00183] In one aspect, the method of treating a subject with or susceptible to a protein degradation disorder comprises contacting the cell with a protein degradation inhibitor. The contacting may be by addition of the inhibitor to a fluid surrounding the cells, for example, to the growth media in which the cells are living or existing. The contacting may also be by directly contacting the inhibitor to the cells. Alternately, the contacting may be by passage of the inhibitor through a subject, for example, after administration, depending on the route of administration, the inhibitor may travel through the digestive tract or the blood stream or may be applied or administered directly to cells in need of the treatment.

[00184] In one aspect, methods of assessing the efficacy of an anti-proliferative treatment in a subject comprise determining one or more pre-treatment phenotypes, administering a therapeutically effective amount of a protein degradation inhibitor to the subject, and determining the one or more phenotypes after an initial period of treatment with an inhibitor, wherein the modulation of the phenotype indicates efficacy of an treatment.

[00185] Efficacy of a treatment may be measured for example, as an increase, decrease, appearance or disappearance of a phenotype. Efficacy may also be measured in terms of a reduction of symptoms associated with protein degradation disorder, a stabilization of symptoms, or a cessation of symptoms associated with a protein degradation disorder.

[00186] Phenotypes include, biological or clinical sequelae in response to a particular treatment or compound. For example, anemia, thrombocytopenia, neutropenia, osteolytic lesions, bone pain, immunodeficiency, renal insufficiency, hypercalcemia, aneuploidy of mature plasma cells, percentage of malignant cells, acetylation state of tubulin, apoptosis of mature plasma cells, level of aggresomes in mature plasma cells, HDAC6 ubiquitination in mature plasma cells, HDAC6 association with dynein in mature plasma cells, cellular levels of ubiquintinated proteins in mature plasma cells, level of caspase-8 in mature plasma cells, level of PARP in mature plasma cells, thymidine uptake in mature plasma cells, dilated ER cisternae, aggregation of mature plasma cells, deposits of immunoglobulins in mature plasma cells, acetylation state of non-histone proteins, global ubiquitination state of the cellular proteins, state of cell cycle regulation, necrosis, markers of apoptosis, apoptosis state, Russell body formation, Cystic Fibrosis transmembrane protein receptor state, and modulation of cellular protein deposits, or global acetylation state of cellular and extracellular proteins. Phenotypes may be observed or measured, for example, visually, by diagnosis, or by an assay as described herein.

[00187] In certain embodiments, a reduction in certain phenotypes descried herein indicates efficacy. However, depending on the mechanism of action of the inhibitor, the phenotype may become more severe for a period of time followed by a decrease. This would also indicate efficacy of treatment.

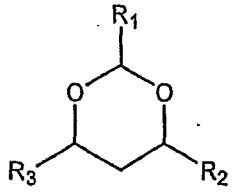
[00188] In one embodiment, the phenotype may be determined one or more times prior to treatment to establish a base-line phenotype. The phenotype may also be determined one or more times during and/or after treatment. The phenotype may alternatively be determined one or more times between treatments.

Compounds of the Invention

[00189] In another aspect, the invention provides compounds, *e.g.*, compounds useful in the methods, pharmaceutical compositions, kits, and packaged compositions of the invention. The compounds useful in the invention are inhibitors histone deacetylases and/or tubulin deacetylases. Useful compounds are described in U.S. patent applications, U.S.S.N. 60/773,510, filed February 14, 2006; U.S.S.N. 60/773,172, filed February 14, 2006; U.S.S.N.

60/289,850, filed May 9, 2001; U.S.S.N. 10/144,316, filed May 9, 2002; and U.S.S.N. 10/621,276, filed July 17, 2003; each of which is incorporated herein by reference.

[00190] Compounds useful in the present invention include compounds of the formula:



wherein

R_1 is cyclic or acyclic, substituted or unsubstituted, branched or unbranched aliphatic; cyclic or acyclic, substituted or unsubstituted, branched or unbranched heteroaliphatic; substituted or unsubstituted, branched or unbranched acyl; substituted or unsubstituted, branched or unbranched aryl; substituted or unsubstituted, branched or unbranched heteroaryl; $-OR_A$; $-C(=O)R_A$; $-CO_2R_A$; $-SR_A$; $-SOR_A$; $-SO_2R_A$; $-N(R_A)_2$; $-NHC(O)R_A$; or $-C(R_A)_3$; wherein each occurrence of R_B is independently a hydrogen, a protecting group, an aliphatic moiety, a heteroaliphatic moiety, an acyl moiety; an aryl moiety; a heteroaryl moiety; alkoxy; aryloxy; alkylthio; arylthio; amino, alkylamino, dialkylamino, heteroaryloxy; or heteroarylthio moiety;

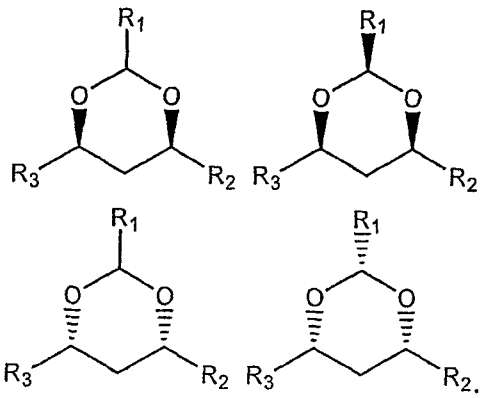
R_2 is hydrogen; halogen; cyclic or acyclic, substituted or unsubstituted, branched or unbranched aliphatic; cyclic or acyclic, substituted or unsubstituted, branched or unbranched heteroaliphatic; substituted or unsubstituted, branched or unbranched acyl; substituted or unsubstitued, branched or unbranched aryl; substituted or unsubstituted, branched or unbranched heteroaryl; $-OR_B$; $-C(=O)R_B$; $-CO_2R_B$; -CN; -SCN; $-SR_B$; $-SOR_B$; $-SO_2R_B$; $-NO_2$; $-N(R_B)_2$; $-NHC(O)R_B$; or $-C(R_B)_3$; wherein each occurrence of R_B is independently a hydrogen, a protecting group, an aliphatic moiety, a heteroaliphatic moiety, an acyl moiety; an aryl moiety; a heteroaryl moiety; alkoxy; aryloxy; alkylthio; arylthio; amino, alkylamino, dialkylamino, heteroaryloxy; or heteroarylthio moiety; and

R_3 is hydrogen; halogen; cyclic or acyclic, substituted or unsubstituted, branched or unbranched aliphatic; cyclic or acyclic, substituted or unsubstituted, branched or unbranched heteroaliphatic; substituted or unsubstituted, branched or unbranched acyl; substituted or unsubstitued, branched or unbranched aryl; substituted or unsubstituted, branched or unbranched heteroaryl; $-OR_C$; $-C(=O)R_C$; $-CO_2R_C$; -CN; -SCN; $-SR_C$; $-SOR_C$; $-SO_2R_C$; $-NO_2$; $-N(R_C)_2$; $-NHC(O)R_C$; or $-C(R_C)_3$; wherein each occurrence of R_C is independently a hydrogen, a protecting group, an aliphatic moiety, a heteroaliphatic moiety, an acyl moiety; an aryl moiety; a heteroaryl moiety; alkoxy; aryloxy; alkylthio; arylthio; amino, alkylamino,

dialkylamino, heteroaryloxy; or heteroarylthio moiety; and pharmaceutically acceptable salts and derivatives thereof. In general, R_1 comprises a metal chelating functional group (*e.g.*, hydroxyamic acids, thiols, carboxlic acids, *ortho*-aminoanilides, *etc.*). The metal chelating group is thought to bind the active site Zn^{+2} ion of deacetylase enzymes. In certain embodiments, R_2 is a substituted or unsubstituted heteroaliphatic moiety (*e.g.*, a heteroaliphatic moiety substituted with a heteroaryl ring, which may be optionally substituted). In certain embodiments, R_3 is a substituted or unsubstituted aromatic ring system (*e.g.*, a substituted or unsubstituted phenyl).

[00191] Many compounds described above have been previously disclosed in U.S. patent applications, USSN 60/289,850, filed May 9, 2001; USSN 10/144,316, filed May 9, 2002; and USSN 10/621,276, filed July 17, 2003; each of which is incorporated herein by reference. The present invention includes specific compounds and subclasses of compounds within this class. These subclasses and specific compounds within the class have been found to be particularly useful in the treatment of multiple myeloma by inhibiting HDAC6 known to play a role in the degradation of proteins by the aggresome. Methods of treating cancer (*e.g.*, multiple myeloma, breast cancer, non-Hodgkin's lymphoma, ovarian cancer, acute myelogenous leukemia), protein degradation disorders (*e.g.*, multiple myeloma, neurodegenerative disorders), protein deposition disorders (*e.g.*, neurogenerative disorders), and proliferative disorders (*e.g.*, diabetic retinopathy, inflammatory diseases, angiogenesis, infectious diseases) as well as pharmaceutical compositions and kits for treatment of these disorders using the inventive compounds are also provided. The present invention also provides new synthetic methods for preparing compounds of the invention.

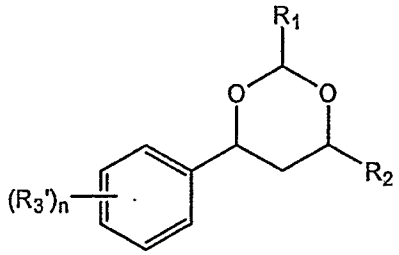
[00192] In certain embodiments, the compound is of one of the formulae below with the stereochemistry as shown:



[00193] In certain embodiments, R_3 is a substituted or unsubstituted aryl moiety. In certain embodiments, R_3 is a substituted or unsubstituted heteroaromatic moiety. In certain

embodiments, R_3 is a monocyclic moiety. In other embodiments, R_3 is a bicyclic moiety. In yet other embodiments, R_3 is a tricyclic moiety. In yet other embodiments, R_3 is a polycyclic moiety. In certain embodiments, R_3 is a substituted or unsubstituted five- or six-membered aromatic or heteroaromatic moiety. In certain embodiments, R_3 is a substituted or unsubstituted six-membered aromatic or heteroaromatic moiety. In certain embodiments, R_3 is a substituted or unsubstituted six-membered aromatic moiety. In certain embodiments, R_3 is a substituted or unsubstituted six-membered heteroaromatic moiety. In certain embodiments, R_3 is a substituted or unsubstituted non-aromatic carbocyclic or heterocyclic moiety.

[00194] In certain embodiments, the invention provides compounds of the formula:



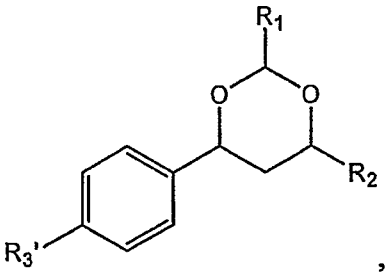
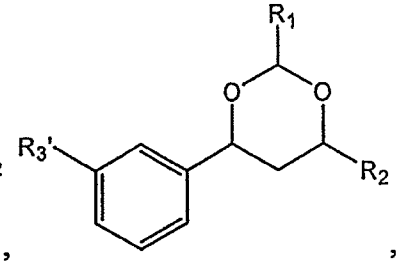
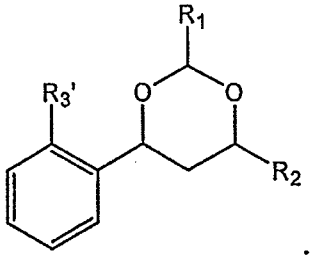
wherein R_1 and R_2 are defined as above;

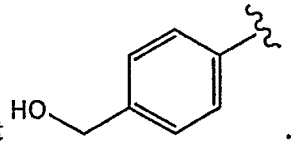
n is an integer between 1 and 5, inclusive; and

each occurrence of R_3' is independently hydrogen; halogen; cyclic or acyclic, substituted or unsubstituted, branched or unbranched aliphatic; cyclic or acyclic, substituted or unsubstituted, branched or unbranched heteroaliphatic; substituted or unsubstituted, branched or unbranched acyl; substituted or unsubstitued, branched or unbranched aryl; substituted or unsubstituted, branched or unbranched heteroaryl; $-OR_C$; $-C(=O)R_C$; $-CO_2R_C$; -CN; -SCN; $-SR_C$; $-SOR_C$; $-SO_2R_C$; $-NO_2$; $-N(R_C)_2$; $-NHC(O)R_C$; or $-C(R_C)_3$; wherein each occurrence of R_C is independently a hydrogen, a protecting group, an aliphatic moiety, a heteroaliphatic moiety, an acyl moiety; an aryl moiety; a heteroaryl moiety; alkoxy; aryloxy; alkylthio; arylthio; amino, alkylamino, dialkylamino, heteroaryloxy; or heteroarylthio moiety.

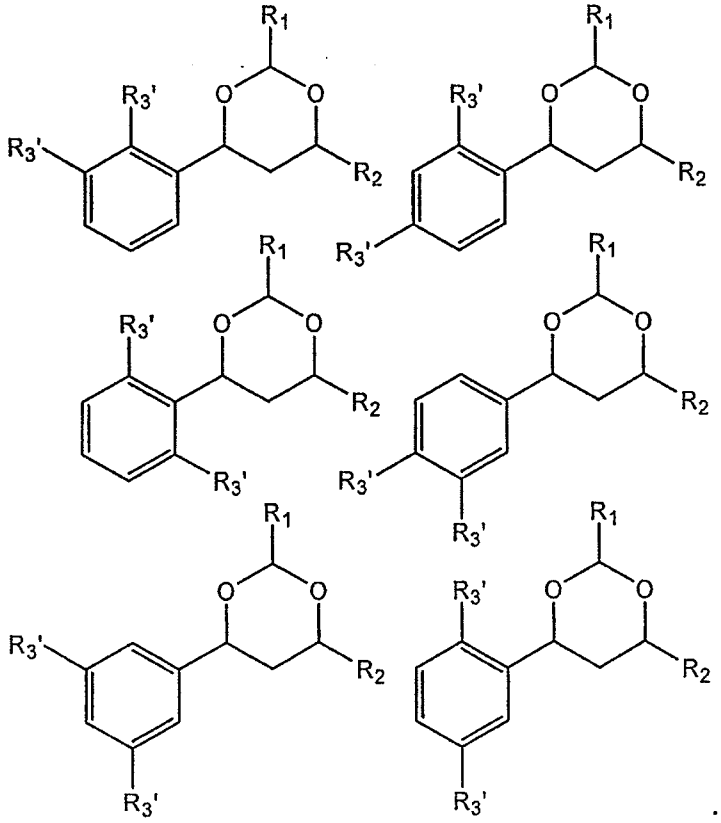
[00195] In certain embodiments, n is 0, and the phenyl ring is unsubstituted.

[00196] In other embodiments, n is 1, and the compounds are one of the formulae:

In certain embodiments, the para-substitution pattern is preferred. In other embodiments, the meta-substitution pattern is preferred. And in yet other embodiments, the ortho-substitution pattern is preferred. In certain embodiments, R_3 is not .

[00197] In other embodiments, n is 2. Compounds of the invention include compounds of one of the formulae:



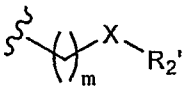
[00198] In other embodiments, n is 3. In still other embodiments, n is 4, and in other embodiments, n is 5.

[00199] In certain embodiments, R_3' is halogen, hydroxyl, protected hydroxyl, alkoxy, amino, alkylamino, dialkylamino, $-NO_2$, C_1-C_6 alkyl, C_1-C_6 alkenyl, C_1-C_6 alkynyl, or acyl. In certain embodiments, R_3' is $-NO_2$. In certain embodiments, R_3' is $-CH_2OH$. In certain

embodiments, R_3' is $-NH_2$. In certain embodiments, R_3' is –H. In other embodiments, R_3' is –OH. In other embodiments, R_3' is –CN. In yet other embodiments, R_3' is –SCN. In still other embodimetns, R_3' is acyl. In certain embodiments, R_3' is acetyl. In other embodiments, R_3' is –F. In other embodiments, R_3' is –Cl. In other embodiments, R_3' is –Br. In other embodiments, R_3' is –I. In other embodiments, R_3' is methyl, ethyl, *n*-propyl, *iso*-propyl, *n*-butyl, *tert*-butyl, or *iso*-butyl. In certain embodiments, R_3' is vinyl. In certain embodiments, R_3' is halogen-substituted alkyl (*e.g.*, trifluoromethyl). In certain embodiments, R_3' is methoxy, ethyoxy, propoxy, butoxy, or pentoxy.

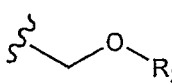
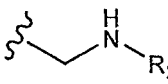
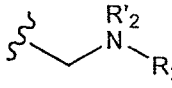
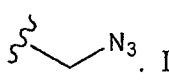
[00200] In certain compounds of this class, R_2 is a substituted or unsubstituted aliphatic group. In other embodiments, R_2 is a substituted or unsubstituted heteroaliphatic group. In certain embodiments, R_2 is a heteroaliphatic group substituted with an aryl or heteroaryl group, which may optionally be substituted. In certain embodiments, R_2 is a heteroaliphatic group substituted with a heteroaryl group, which is optionally substituted. In certain embodiments, R_2 is a heteroaliphatic group substituted with a heteroaryl group, which is substituted.

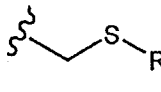
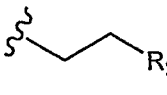
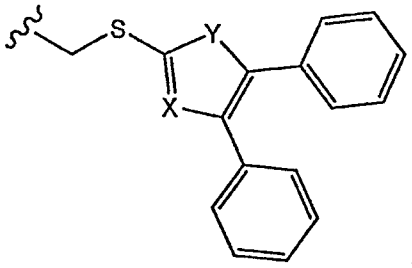
[00201] In certain embodiments, R_2 is of the formula:



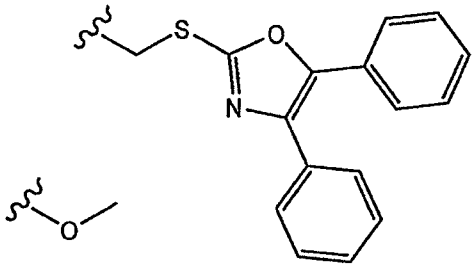
wherein m is an integer between 0 and 8, inclusive; preferably, between 1 and 6, inclusive;

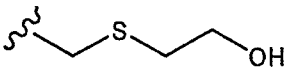
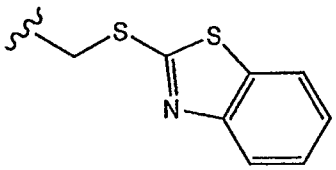
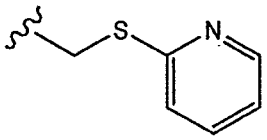
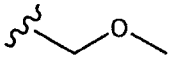
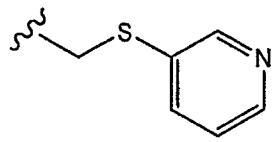
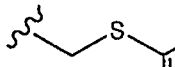
X is O, S, CH_2, NH, or NR_2'; and

R_2' is aliphatic, heteroaliphatic, acyl, substituted or unsubstituted aryl, or substituted or unsubstituted heteroaryl. In certain embodiments, m is 1, 2 or 3. In certain embodiments, m is 1. In certain embodiments, X is O. In other embodiments, X is S. In certain embodiments, X is NH. In other embodiments, X is CH_2. In certain embodiments, R_2' is C_1-C_6 alkyl. In certain embodiments, R_2' is a substituted heteroaryl moiety. In other embodiments, R_2' is an unsubstituted heteroaryl moiety. In certain particular embodiments, R_2' is a substituted oxazolyl moiety. In other embodiments, R_2' is a substituted thiazolyl moiety. In yet other embodiments, R_2' is a substituted imidazolyl moiety. In certain embodiments, R_2 is . In other embodiments, R_2 is . In yet other embodiments, R_2 is , wherein the two R_2' moieties may together form a heterocyclic group. In yet other embodiments, R_2 is . In yet other embodiments,

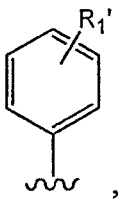
R_2 is . In yet other embodiments, R_2 is . In certain embodiments, R_2 is , wherein X is N and Y is NH, S, or O.

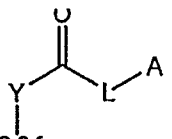
In certain embodiments, R_2 is selected from one of the following:



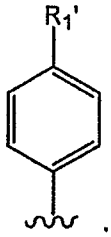







[00202] In certain embodiments, R_1 is a substituted phenyl ring. In certain particular embodiments, R_1 is of the formula:

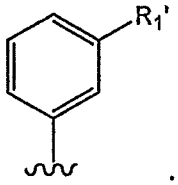
,

wherein R_1' is , wherein Y is NH or O; L is a linker moiety; and A comprises a functional group that inhibits histone or tubulin deacetylase.

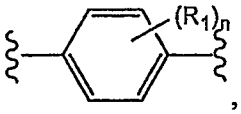
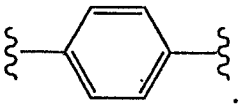
[00203] In certain embodiments, R_1 is of the formula:

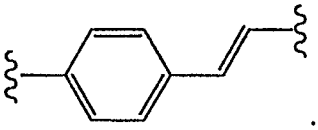


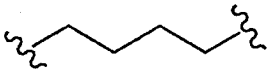
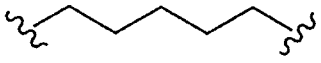
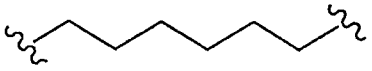
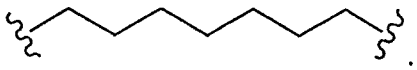
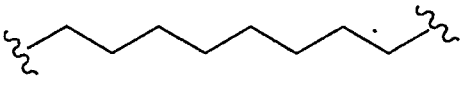
[00204] In other embodiments, R_1 is of the formula:

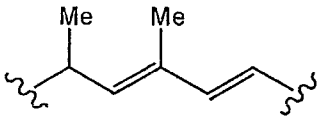


In certain embodiments, Y is NH. In other embodiments, Y is O. In certain embodiments, L is a substituted or unsubstituted, cyclic or acyclic, branched or unbranched aliphatic moiety; a substituted or unsubstituted, cyclic or acyclic, branched or unbranched heteroaliphatic moiety; a substituted or unsubstituted aryl moiety; a substituted or unsubstituted heteroaryl moiety. In certain embodiments, L is a substituted or unsubstituted, cyclic or acyclic, branched or unbranched aliphatic moiety. In certain embodiments, L is C_1-C_{20} alkylidene, preferably C_1 to C_{12} alkylidene, more preferably C_4-C_7 alkylidene. In certain embodiments, L is C_1-C_{20} alkenylidene, preferably C_1 to C_{12} alkenylidene, more preferably C_4-C_7 alkenylidene. In certain embodiments, L is C_1-C_{20} alkynylidene, preferably C_1 to C_{12} alkynylidene, more preferably C_4-C_7 alkynylidene. In certain embodiments, L is a a substituted or unsubstituted, cyclic or acyclic, branched or unbranched heteroaliphatic moiety. In certain embodiments, L comprises a cyclic ring system, wherein the rings may be aryl, heteroaryl, non-aromatic carbocyclic, or non-aromatic heterocyclic. In still other embodiments, L comprises a substituted or unsubstituted heteroaryl moiety. In certain particular embodiments, L comprises a phenyl ring. In certain embodiments, L comprises multiple phenyl rings (*e.g.*, one, two, three, or four phenyl rings).

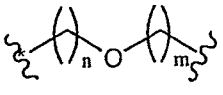
[00205] In certain embodiments, L is , wherein n is an integer between 1 and 4, inclusive; preferably, between 1 and 3, inclusive; more preferably, 1 or 2; and R_1 is is hydrogen; halogen; cyclic or acyclic, substituted or unsubstituted, branched or unbranched aliphatic; cyclic or acyclic, substituted or unsubstituted, branched or unbranched heteroaliphatic; substituted or unsubstituted, branched or unbranched acyl; substituted or unsubstitued, branched or unbranched aryl; substituted or unsubstituted, branched or unbranched heteroaryl; $-OR_A$; $-C(=O)R_A$; $-CO_2R_A$; -CN; -SCN; $-SR_A$; $-SOR_A$; $-SO_2R_A$; $-NO_2$; $-N(R_A)_2$; ; $-NHR_A$; $-NHC(O)R_A$; or $-C(R_A)_3$; wherein each occurrence of R_A is independently a hydrogen, a protecting group, an aliphatic moiety, a heteroaliphatic moiety, an acyl moiety; an aryl moiety; a heteroaryl moiety; alkoxy; aryloxy; alkylthio; arylthio; amino, alkylamino, dialkylamino, heteroaryloxy; or heteroarylthio moiety. In certain embodiments, L is .

[00206] In certain embodiments, L is .

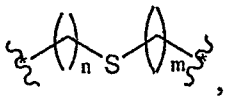
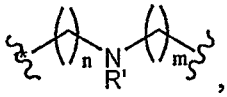
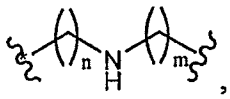
[00207] In certain embodiments, L is an unbranched, unsubstituted, acyclic alkyl chain. In certain embodiments, L is . In other embodiments, L is . In certain other embodiments, L is . In other embodiments, L is . In yet other embodiments, L is .

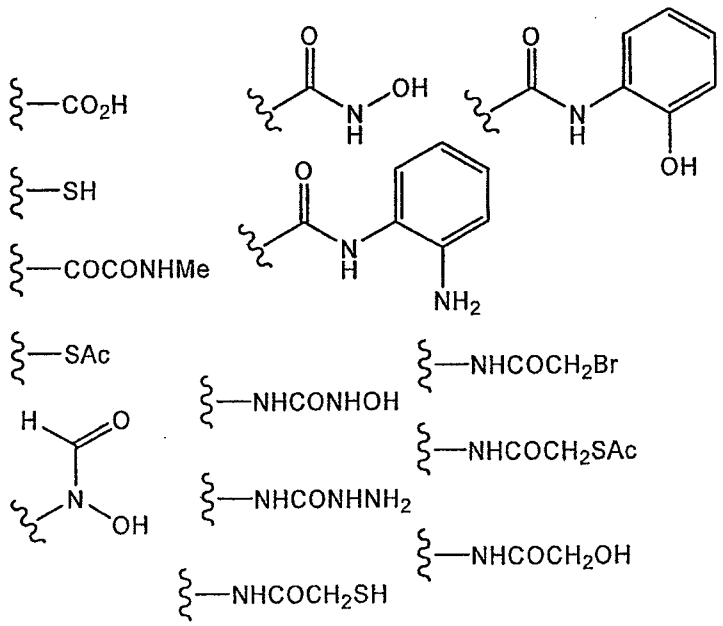
[00208] In certain embodiments, L is a substituted, acyclic aliphatic chain. In certain embodiments, L is 

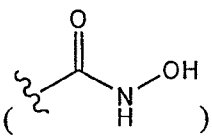
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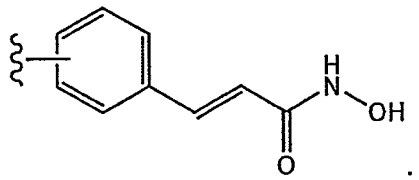
[00209] In certain embodiments, L is an unbranched, unsubstituted, acyclic heteroaliphatic chain. In certain particular embodiments, L is 

, wherein n

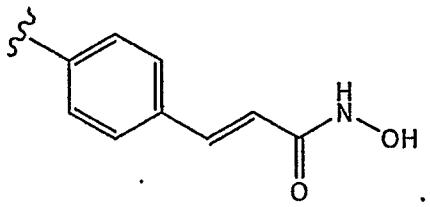
is an integer between 0 and 10, inclusive; preferably, between 0 and 5, inclusive; and m is an integer between 0 and 10, inclusive; preferably, between 0 and 5, inclusive. In certain particular embodiments, L is , wherein n is an integer between 0 and 10, inclusive; preferably, between 0 and 5, inclusive; and m is an integer between 0 and 10, inclusive; preferably, between 0 and 5, inclusive. In certain particular embodiments, L is , wherein n is an integer between 0 and 10, inclusive; preferably, between 0 and 5, inclusive; m is an integer between 0 and 10, inclusive; preferably, between 0 and 5, inclusive; and R' is hydrogen, C_1-C_6 aliphatic, heteroaliphatic, aryl, heteroaryl, or acyl. In certain particular embodiments, L is , wherein n is an integer between 0 and 10, inclusive; preferably, between 0 and 5, inclusive; and m is an integer between 0 and 10, inclusive; preferably, between 0 and 5, inclusive. In certain embodiments, A comprises a metal chelating functional group. For example, A comprises a Zn^{2+} chelating group. In certain embodiments, A comprises a functional group selected group consisting of:

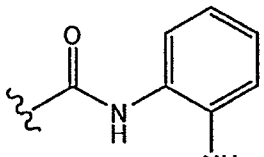
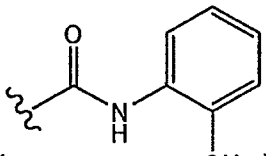
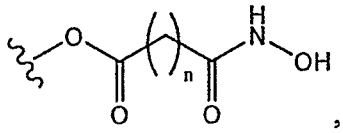
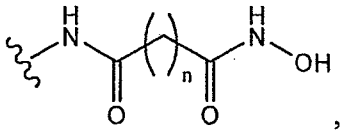


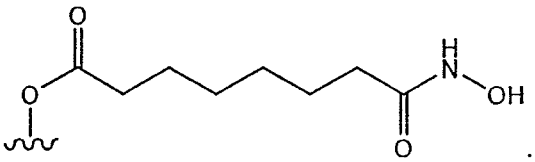
In certain embodiments, A comprises hydroxamic acid () or a salt thereof. In other embodiments, A comprises the formula:



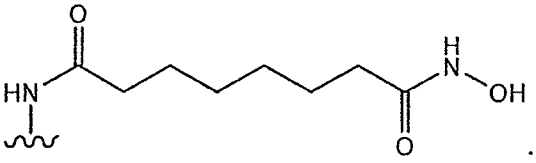
In certain particular embodiments, A comprises the formula:



In other embodiments, A comprises a carboxylic acid ($-CO_2H$). In other embodiments, A comprises an *o*-aminoanilide (). In other embodiments, A comprises an *o*-hydroxyanilide (). In yet other embodiments, A comprises a thiol (-SH). In certain embodiments, R_1' is , wherein n is an integer between 0 and 15, inclusive; preferably, between 0 and 10, inclusive; more preferably, between 1 and 8, inclusive; even more preferably, 4, 5, 6, 7, or 8. In certain embodiments, R_1' is , wherein n is an integer between 0 and 15, inclusive; preferably, between 0 and 10, inclusive; more preferably, between 1 and 8, inclusive; even more preferably, 4, 5, 6, 7, or 8. In certain embodiments, R_1' is

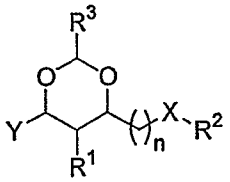


. In other particular embodiments, R_1' is



.

[00210] Certain compounds useful in the present invention include compounds of the formula:



and pharmaceutically acceptable derivatives thereof;

wherein R^1 is hydrogen, or an aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic or heteroaromatic moiety;

n is 1-5;

R^2 is hydrogen, a protecting group, or an aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic or heteroaromatic moiety;

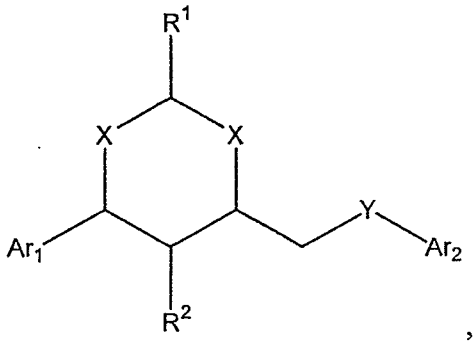
X is –O-, $-C(R^{2A})_2-$, -S-, or $-NR^{2A}-$, wherein R^{2A} is hydrogen, a protecting group, or an aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic or heteroaromatic moiety;

or wherein two or more occurrences of R^2 and R^{2A}, taken together, form an alicyclic or heterocyclic moiety, or an aryl or heteroaryl moiety;

R^3 is an aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic or heteroaromatic moiety; and

Y is hydrogen or an aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic or heteroaromatic moiety.

[00211] In certain embodiments, a compound according to the invention can be represented by formula:



wherein

each X is independently O, S, CH_2, or NR^3;

Y is O, S, CH_2, or NR^4;

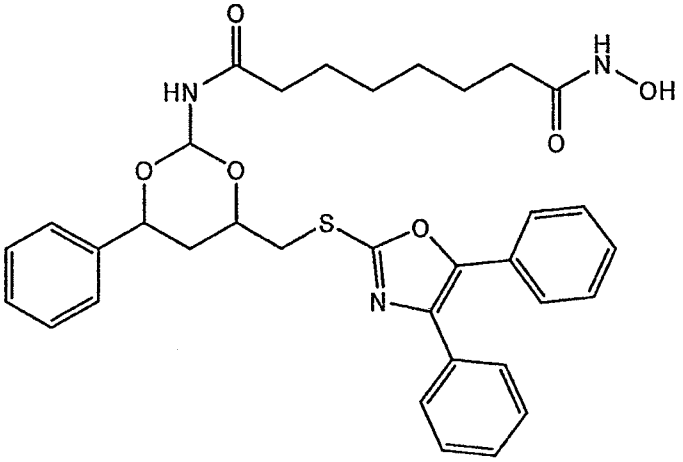
Ar_1 and Ar_2 are each independently an aryl group;

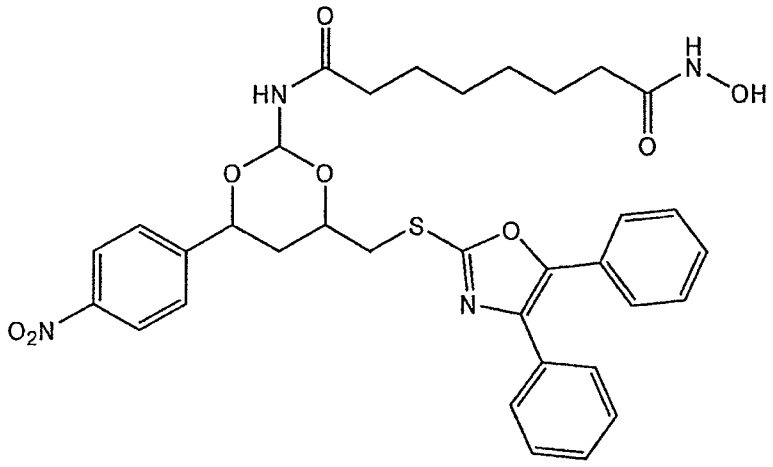
R^1 is a lower alkyl group or an aryl group;

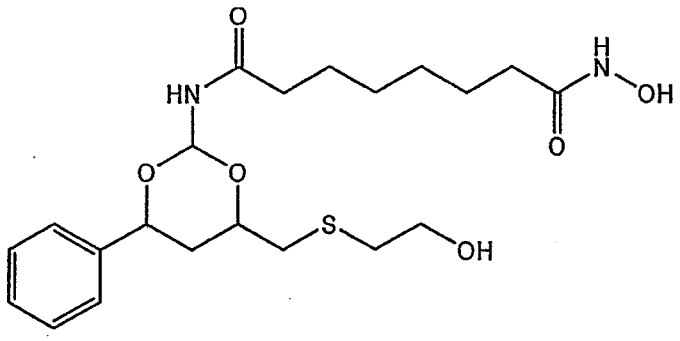
R^2 is hydrogen, a lower alkyl group or an aryl group; and

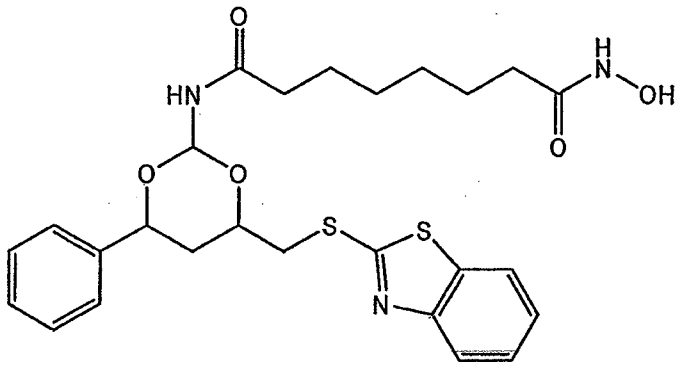
R^3 and R^4 are each independently hydrogen, a lower alkyl group, an aryl group, an alkylcarbonyl, an alkoxycarbonyl group, or an aminocarbonyl group. In certain preferred embodiments, X is for each occurrence O, S, or CH_2, more preferably O or S, and still more preferably O. In certain preferred embodiments, Y is S. In certain preferred embodiments, Ar_1 is phenyl or substituted phenyl, particularly 4-hydromethylphenyl. In certain preferred embodiments, Ar_2 is heteroaryl, more preferably optionally substituted oxazolyl, still more preferably phenyl-substituted oxazolyl, and most preferably 4,5-diphenyl-oxazol-2-yl. In certain preferred embodiments, R^1 is phenyl or substituted phenyl, more preferably 4-aminosubstituted phenyl or 4-amidosubstituted phenyl; in more preferred embodiments, R^1 is a phenyl group substituted at the 4-position with an amido group bearing an alkylene moiety in which the alkylene chain has between four and eight carbon atoms (more preferably 6 carbon atoms) in the alkylene chain, and the alkylene chain preferably bears a terminal hydroxamate group (-NHOH). In certain preferred embodiments, R_2 is hydrogen.

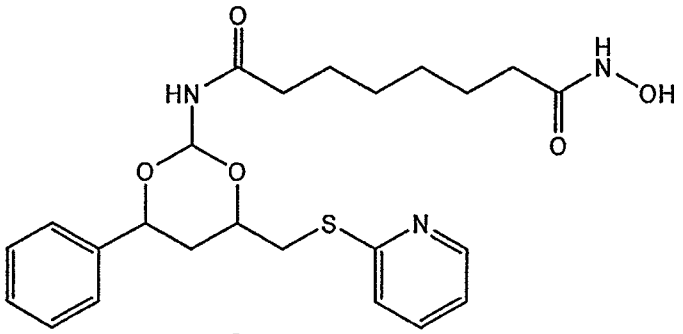
[00212] Exemplary compounds include compounds of the formulae:

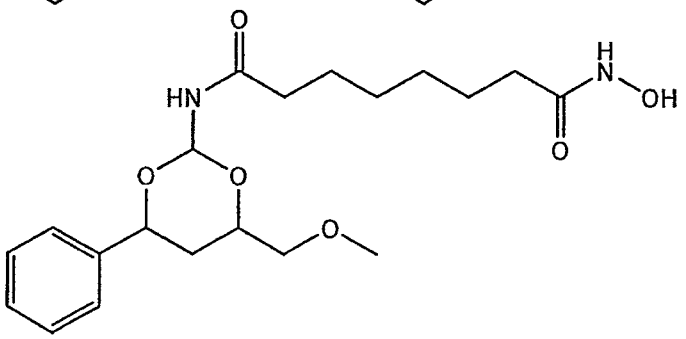


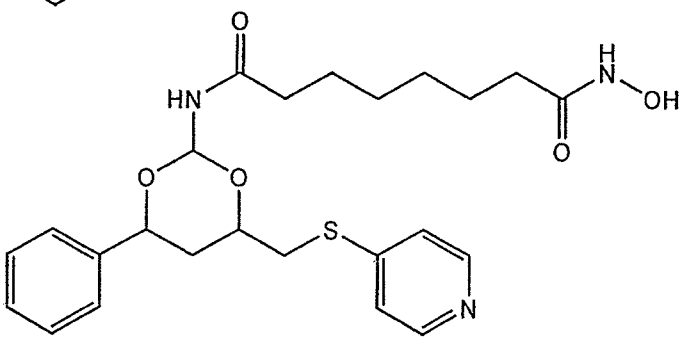


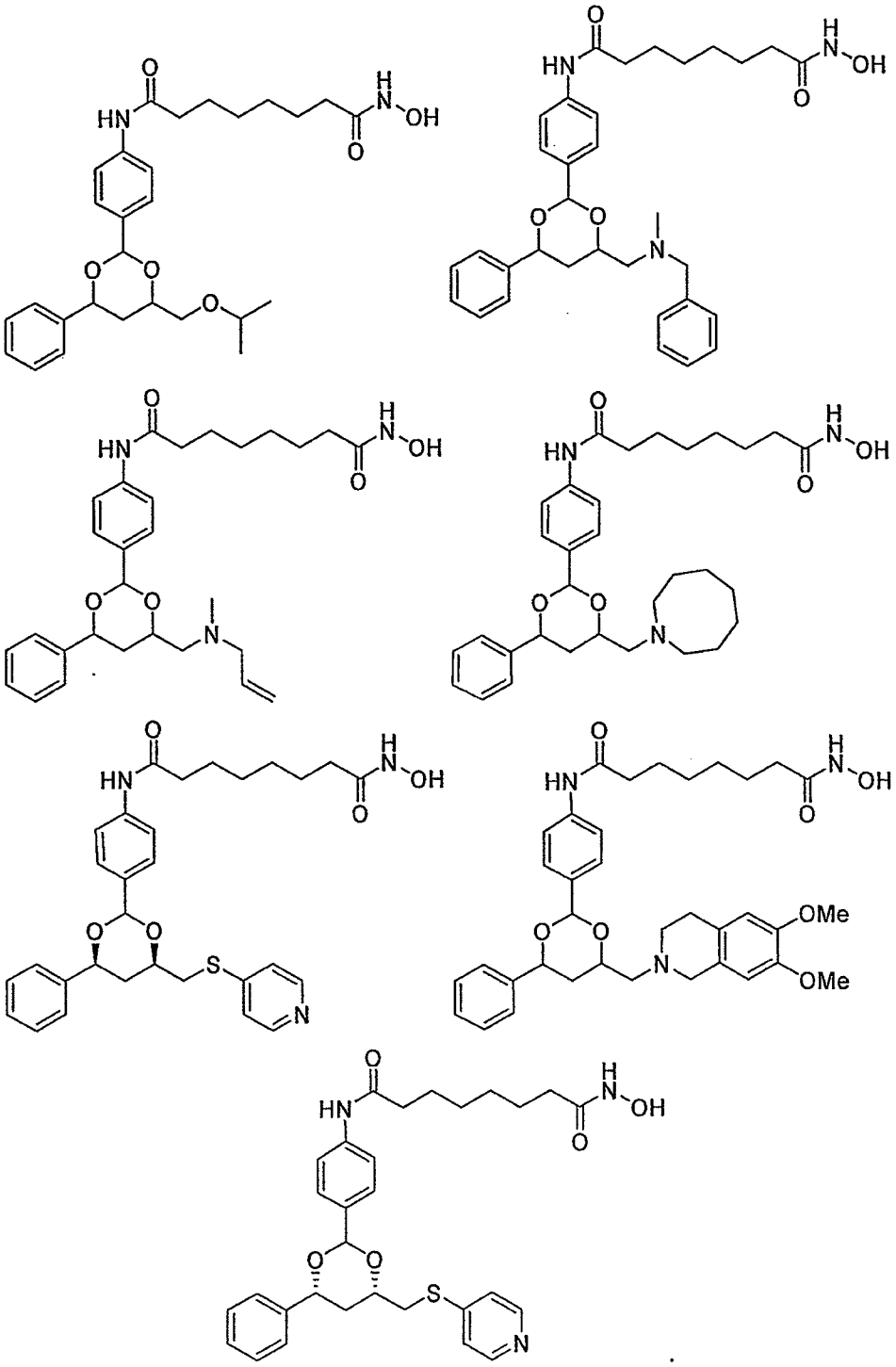




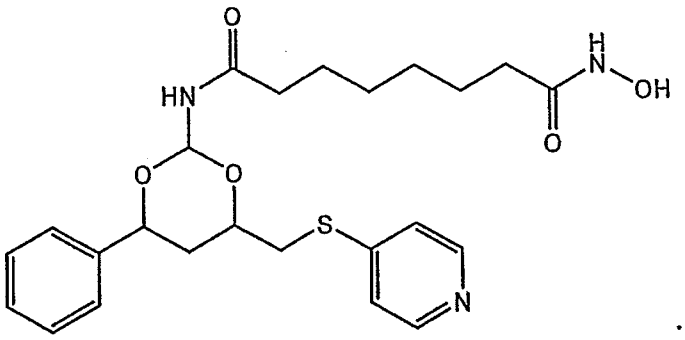




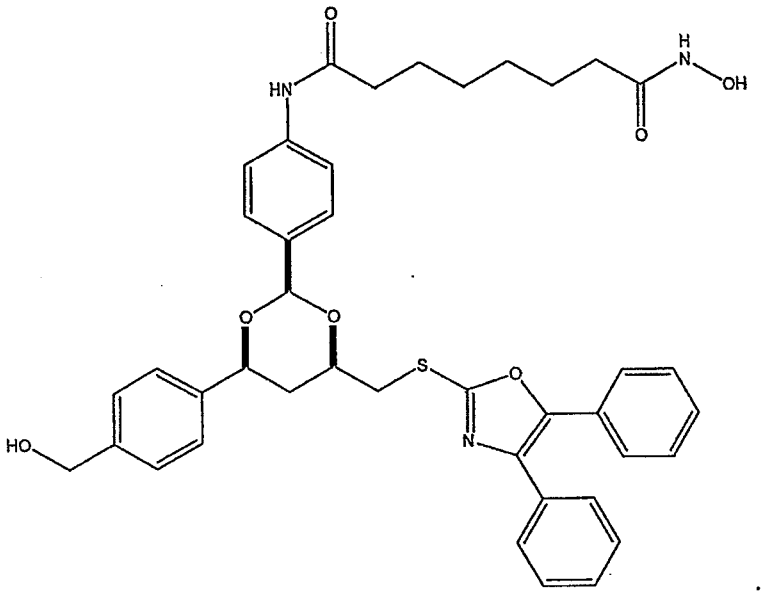




[00213] In certain embodiments, the compounds is of the formula:



[00214] In certain embodiments, the compound is tubacin:



[00215] In preferred embodiments, a compound useful in the invention has one or more of the following properties: the compound is a protein degradation inhibitor; the compound is capable of inhibiting at least one histone deacetylase; the compound is capable of inhibiting HDAC6; the compound is a selective HDAC6 inhibitor; the compound binds to the poly-ubiquitin binding domain of HDAC6; the compound is capable of inducing apoptosis in cancer cells (especially multiple myeloma cells, non-Hodgkin's lymphoma (NML) cells, breast cancer cells, acute myelogeous leukemia (AML) cells); and/or the compound is capable of inhibiting aggresome formation.

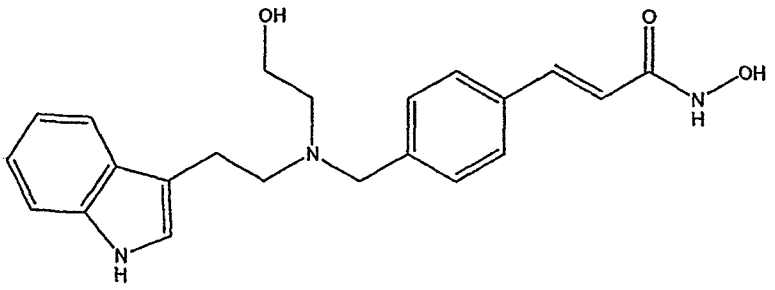
[00216] Compounds useful in at least some of the methods, pharmaceutical compositions, kits and packaged compositions of the invention can be identified, *e.g.*, according to any of the screening methods described herein.

[00217] In certain preferred embodiments, a compound of the invention comprises a metal binding moiety, preferably a zinc-binding moiety such as a hydroxamate. As noted

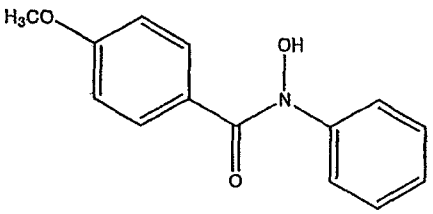
above, certain hydroxamates are potent inhibitors of HDAC6 activity; without wishing to be bound by theory, it is believed that the potency of these hydroxamates is due, at least in part, to the ability of the compounds to bind zinc. In preferred embodiments, a compound of the invention includes at least one portion or region which can confer selectivity for a biological target implicated in the aggresome pathway, e.g., a biological target having tubulin deacetylase (TDAC) or HDAC activity, e.g., HDAC6. Thus, in certain preferred embodiments, a compound includes a zinc-binding moiety spaced from other portions of the molecule which are responsible for binding to the biological target. For example, a compound of the invention may include a linker arm or other spacing moiety capable of presenting the zinc-binding moiety in a favored orientation when another portion (or portions) of the molecule is bound to a biological target such as HDAC6. Without being bound by theory, it is believed that steric 'bulk' in the capping region of a compound can contribute to TDAC specificity, although such bulk may not be required in all cases. In addition, without being bound by theory, it is believed that allosteric site inhibition contributes to TDAC activity of certain compounds of the invention.

[00218] In certain embodiments, a compound useful in at least some of the methods, pharmaceutical compositions, kits and packaged compositions of the invention is a 1,3-dioxane HDAC inhibitor compound disclosed in U.S. Patent Publication No. US2004/0072849, which is hereby incorporated by reference in its entirety. Related compounds are described in Sternson SM *et al.*, *Org Lett.* 2001 Dec 27;3(26):4239-42; Haggarty SJ *et al.*, *Chem Biol.* 2003 May;10(5):383-96; Haggarty SJ *et al.*, *Proc Natl Acad Sci U S A.* 2003 Apr 15;100(8):4389-94. Epub 2003 Apr 03; and Haggarty SJ *et al.*, *Comb Chem High Throughput Screen.* 2004 Nov;7(7):669-76; the contents of each of these references is hereby incorporated by reference in its entirety.

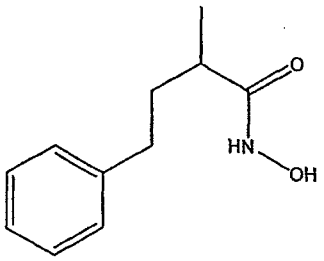
[00219] Additional compounds useful in at least some of the methods, compositions, kits, and packaged compositions of the invention include NVP-LAQ824 (a cinnamic hydroxamate having the structure shown below):



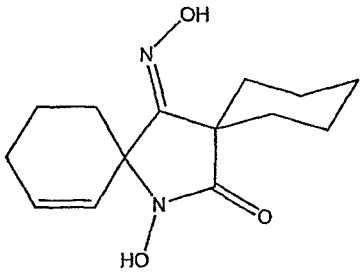
[00220] Additional compounds useful in at least some of the methods, compositions, kits, and packaged compositions of the invention include those shown below. Each of the compounds includes a hydroxamate moiety (including O-ethers of hydroxamates and cyclic hydroxamates).



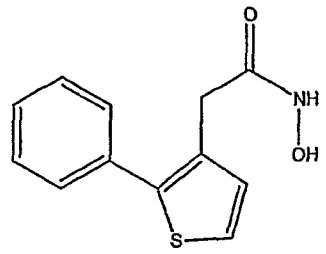
N-Hydroxy-4-methoxy-N-phenyl-benzamide



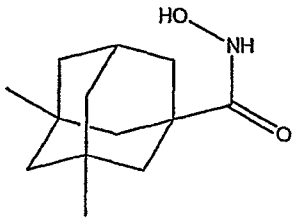
N-Hydroxy-2-methyl-4-phenyl-butyramide



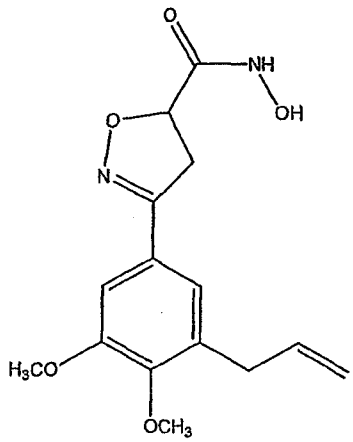
14-Hydroxy-14-aza-dispiro[5.1.5.2]-pentadec-9-ene-7,15-dione 7-oxime



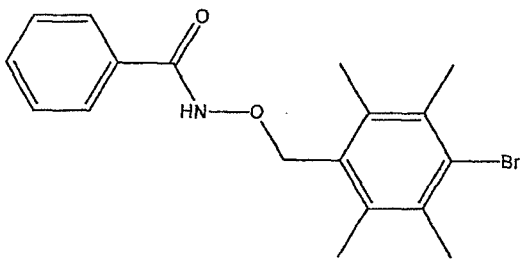
N-Hydroxy-2-(2-phenyl-thiophen-3-yl)-acetamide



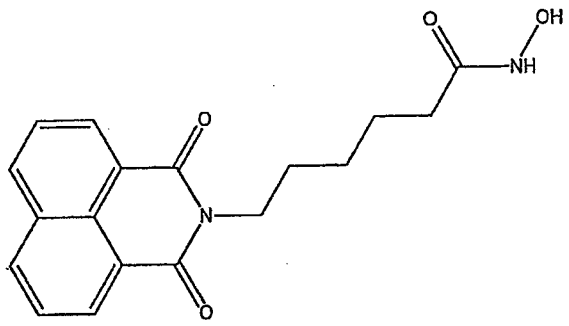
3,5-Dimethyl-adamantane-1-carboxylic acid hydroxyamide



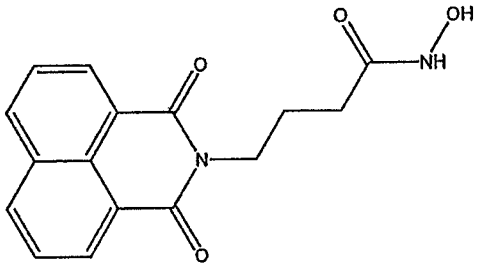
3-(3-Allyl-4,5-dimethoxy-phenyl)-4,5-dihydro-isoxazole-5-carboxylic acid hydroxyamide



N-(4-Bromo-2,3,5,6-tetramethyl-benzyloxy)-benzamide



6-(1,3-Dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid hydroxyamide



4-(1,3-Dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-N-hydroxy-butyramide

[00221] The compounds of this invention include the compounds themselves, as well as any salt (preferably pharmaceutically acceptable salt), solvate, clathrate, hydrate, polymorph, or prodrugs, if applicable. As used herein, the term "pharmaceutically acceptable salt," is a salt formed from, for example, an acid and a basic group of a compound of any one of the formulae disclosed herein. Illustrative salts include, but are not limited, to sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, isonicotinate, lactate, salicylate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, besylate, gentisinate, fumarate, gluconate, glucaronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, *p*-toluenesulfonate, and pamoate (*i.e.*, 1,1'-methylene-bis-(2-hydroxy-3-naphthoate)) salts. The term "pharmaceutically acceptable salt" also refers to a salt prepared from a compound of any one of the formulae disclosed herein having an acidic functional group, such as a carboxylic acid functional group, and a pharmaceutically acceptable inorganic or organic base. Suitable bases include, but are not limited to, hydroxides of alkali metals such as sodium, potassium, and lithium; hydroxides of alkaline earth metal such as calcium and magnesium; hydroxides of other metals, such as aluminum and zinc; ammonia,

and organic amines, such as unsubstituted or hydroxy-substituted mono-, di-, or trialkylamines; dicyclohexylamine; tributyl amine; pyridine; N-methyl,N-ethylamine; diethylamine; triethylamine; mono-, bis-, or tris-(2-hydroxy-lower alkyl amines), such as mono-, bis-, or tris-(2-hydroxyethyl)amine, 2-hydroxy-tert-butylamine, or tris-(hydroxymethyl)methylamine, N, N,-di-lower alkyl-N-(hydroxy lower alkyl)-amines, such as N,N-dimethyl-N-(2-hydroxyethyl)amine, or tri-(2-hydroxyethyl)amine; N-methyl-D-glucamine; and amino acids such as arginine, lysine, and the like. The term "pharmaceutically acceptable salt" also refers to a salt prepared from a compound of any one of the formulae disclosed herein having a basic functional group, such as an amino functional group, and a pharmaceutically acceptable inorganic or organic acid. Suitable acids include hydrogen sulfate, citric acid, acetic acid, oxalic acid, hydrochloric acid (HCl), hydrogen bromide (HBr), hydrogen iodide (HI), nitric acid, hydrogen bisulfide, phosphoric acid, lactic acid, salicylic acid, tartaric acid, bitartratic acid, ascorbic acid, succinic acid, maleic acid, besylic acid, fumaric acid, gluconic acid, glucaronic acid, formic acid, benzoic acid, glutamic acid, methanesulfonic acid, ethanesulfonic acid, benzenesulfonic acid, and *p*-toluenesulfonic acid.

[00222] As used herein, the term "polymorph" means solid crystalline forms of a compound of the present invention or complex thereof. Different polymorphs of the same compound can exhibit different physical, chemical and/or spectroscopic properties. Different physical properties include, but are not limited to stability (*e.g.*, to heat or light), compressibility and density (important in formulation and product manufacturing), and dissolution rates (which can affect bioavailability). Differences in stability can result from changes in chemical reactivity (*e.g.*, differential oxidation, such that a dosage form discolors more rapidly when comprised of one polymorph than when comprised of another polymorph) or mechanical characteristics (*e.g.*, tablets crumble on storage as a kinetically favored polymorph converts to thermodynamically more stable polymorph) or both (*e.g.*, tablets of one polymorph are more susceptible to breakdown at high humidity). Different physical properties of polymorphs can affect their processing. For example, one polymorph might be more likely to form solvates or might be more difficult to filter or wash free of impurities than another due to, for example, the shape or size distribution of particles of it.

[00223] As used herein, the term "hydrate" means a compound of the present invention or a salt thereof, which further includes a stoichiometric or non-stoichiometric amount of water bound by non-covalent intermolecular forces.

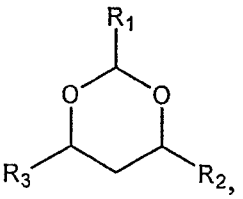
[00224] As used herein, the term "clathrate" means a compound of the present invention or a salt thereof in the form of a crystal lattice that contains spaces (*e.g.*, channels) that have a guest molecule (*e.g.*, a solvent or water) trapped within.

[00225] In addition, some of the compounds of this invention have one or more double bonds, or one or more asymmetric centers. Such compounds can occur as racemates, racemic mixtures, single enantiomers, individual diastereomers, diastereomeric mixtures, and cis- or trans- or *E*- or *Z*- double isomeric forms. All such isomeric forms of these compounds are expressly included in the present invention. The compounds of this invention may also be represented in multiple tautomeric forms, in such instances, the invention expressly includes all tautomeric forms of the compounds described herein (e.g., alkylation of a ring system may result in alkylation at multiple sites, the invention expressly includes all such reaction products). All such isomeric forms of such compounds are expressly included in the present invention. All crystal forms of the compounds described herein are expressly included in the present invention.

Synthesis of Compounds

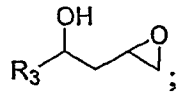
[0100] As described above, the present invention provides novel compounds. The synthesis of many of the compounds described herein has been described in previously filed U.S. patent applications, USSN 60/289,850, filed May 9, 2001; USSN 10/144,316, filed May 9, 2002; and USSN 10/621,276, filed July 17, 2003; each of which is incorporated herein by reference. As would be appreciated by one of skill in this art, the various reactions and synthetic schemes described in these patent applications may be used in preparing the inventive compounds.

[0101] A methodology for preparing the inventive compounds is shown in *Figure 55*. This synthesis provides for a greater diversity of substituents at certain positions of the 1,3-dioxane core structure. In certain embodiments, the synthesis provides for a greater variety of substituents at R_3.

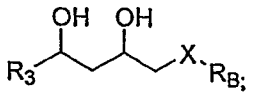
[0102] It will be appreciated that for compounds of the formula 

, a method for the synthesis of the core structure is provided, one method comprising steps of:

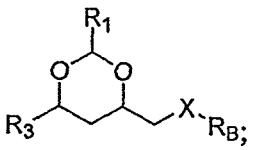
providing an epoxy alcohol having the structure:



reacting the epoxy alcohol with a reagent having the structure R_BXH under suitable conditions to generate a diol having the core structure:



reacting the diol with a reagent having the structure $R_1CH(OMe)_2$ under suitable conditions to generate a scaffold having the core structure:



wherein R_1 is a substituted or unsubstituted aromatic or heteroaromatic moiety (*e.g.*, an aryl ring substituted with a metal chelating moiety);

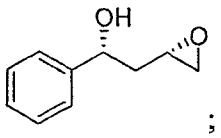
R_B is hydrogen, a protecting group, or an aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic or heteroaromatic moiety;

X is –O-, -C(R')$_2$-, -S-, or –NR'-, wherein R' is hydrogen, a protecting group, or an aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic or heteroaromatic moiety; and

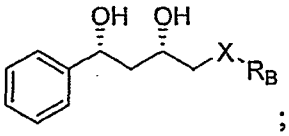
R^3 is an aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic or heteroaromatic moiety.

[0001] In certain embodiments, R_3 is aliphatic. In certain embodiments, R_3 is heteroaliphatic. In certain embodiments, R_3 is heterocyclic. In certain embodiments, R3 is carbocyclic. In certain embodiments, R_3 is aromatic. In other embodiments, R_3 is heteroaromatic. As would be appreciated by one of skill in the art, various substituents at R_3 can be introduced into the synthesis by using a different adehyde as the starting material in the synthesis. An examples of this is seen in *Figure 3* where benzaldehyde is used as the starting material. Various substituted benzaldehyde could also be used in the illustrated synthesis as well as aliphatic aldehydes, non-cyclic aldehydes, or non-aromatic aldehydes.

[0103] In certain exemplary embodiments, the epoxy alcohol has the structure:



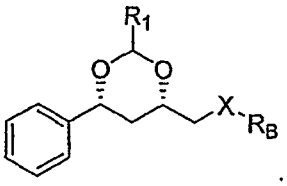
the diol has the structure:



;

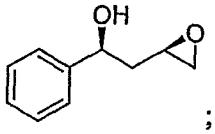
wherein X is S or O;

and the core scaffold has the structure:



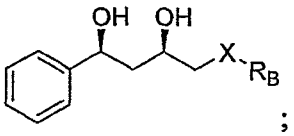
.

[0104] In certain other exemplary embodiments, the epoxy alcohol has the structure:



;

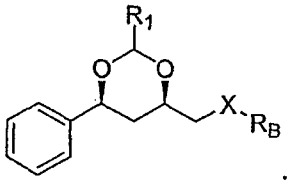
the diol has the structure:



;

wherein X is S or O;

and the core scaffold has the structure:



.

Uses of Compounds of the Invention

[00226] As described herein below, it has now surprisingly been found that the compounds of the invention and analogs can treat and prevent protein degradation disorders, especially myleoma. Thus, in one embodiment, the invention also provides methods for treating a subject for a protein degradation disorder, by administering to the subject an effective amount of a compound described herein. The compounds may inhibit protein degradation via the aggresome or the aggresome and the proteasome pathways.

[00227] Subjects may be identified as having or being susceptible to a protein degradation disorder by a health care professional or by self-identification by the subject.

[00228] The invention also provides methods of treating a cell exhibiting symptoms of a protein degradation disorder comprising administering a therapeutically effective amount of a protein degradation inhibitor to the cell. The cells include one or more of a cell from a subject or a cultured cell. The cells may be removed or isolated from a subject. Useful cells from a subject include one or more of a bone marrow stromal cell, (BMSC), a peripheral blood mononuclear cell (PBMC), lymphocytes, hair follicles, blood cells, other epithelial cells, bone marrow plasma cells, primary cancer cells, patient derived tumor cells, normal or cancerous hematopoietic stem cells, neural stem cells, solid tumor cells, astrocytes, and the like. Cultured cells include one or more of MM.1S, U266, RPMI8226, DOX40, MM.1R, INA-6, LR5, primary and established cancer cell lines, primary and established normal cell lines. The treated cells may be a pure population of cells or may be mixed with other cell types, for example other blood cells, feeder cells, or bone marrow cells. The cultured cells may be pure populations or they may be mixed with other cells. They may be mixed with other cultured cells or with cells from a subject. The cultured cells alternately be mixed with feeder cells or bone marrow cells.

[00229] The invention also provides methods for treating a subject suffering from or susceptible to multiple myeloma comprising administering to a subject in need thereof a therapeutically effective amount of a protein degradation inhibitor, to thereby treat the subject suffering from or susceptible to multiple myeloma. Multiple myeloma may be diagnosed by, for example, the detection of an M-protein in the serum or urine, the detection of more than 10% plasma cells on a bone marrow examination, the detection of lytic bone lesions or generalized osteoporosis in skeletal x-rays, and/or the presence of soft tissue plasmacytomas. In certain embodiments, an HDAC inhibitor is combined with a proteasome inhibitor in the treatment of multiple myeloma. In certain embodiments, the HDAC inhibitor is a compound of the invention. In certain embodiments, the proteasome inhibitor is bortezomib (VELCADE®).

[00230] The invention also provides methods for treating a subject suffering from or susceptible to solid tumors comprising administering to a subject in need thereof a therapeutically effective amount of a protein degradation inhibitor, to thereby treat the subject suffering from or susceptible to a solid tumor. Solid tumors that are particularly susceptible to treatment with protein degradation inhibitors include breast cancer, lung cancer, colon cancer, and prostate cancer. In certain embodiments, an HDAC inhibitor is combined with a proteasome inhibitor in the treatment of these cancers. In certain embodiments, the HDAC

inhibitor is a compound of the invention. In certain embodiments, the proteasome inhibitor is bortezomib (VELCADE®).

[00231] The invention provides methods for assessing the efficacy of a protein degradation disorder treatment in a subject, comprising determining one or more pre-treatment phenotypes, administering a therapeutically effective amount of a protein degradation inhibitor to the subject, and determining the one or more phenotypes after an initial period of treatment with the protein degradation inhibitor, wherein the modulation of the one or more phenotypes indicates efficacy of a protein degradation inhibitor treatment. The subject may be pre-diagnosed with a protein degradation disorder, or the method may further comprise diagnosing the subject with a protein degradation disorder.

[00232] "After an initial period of treatment" or after an appropriate period of time after the administration of the protein degradation inhibitor, *e.g.*, 2 hours, 4 hours, 8 hours, 12 hours, or 72 hours, weeks, or months, one or more of the proportions, levels, and/or cellular localization may be determined again. The modulation of one ore more of the phenotypes may indicate efficacy of protein degradation inhibitor. One or more of the phenotypes may be determined periodically throughout treatment. For example, one or more of the phenotypes may be checked every few hours, days or weeks to assess the further efficacy of the treatment. The method described may be used to screen or select patients that may benefit from treatment with a protein degradation inhibitor.

[00233] Provided herein are methods of monitoring the progress of a subject being treated with an aggresome inhibitor. The methods comprise determining one or more pre-treatment phenotypes; administering a therapeutically effective amount of an aggresome inhibitor to the subject; and determining one or more phenotypes after an initial period of treatment with the aggresome inhibitor, wherein the modulation of one or more of the phenotypes indicates efficacy of aggresome inhibition treatment.

[00234] Methods are also provided for selecting a subject with a protein degradation disorder for treatment with a protein degradation inhibitor. The selection methods comprise determining one or more pre-treatment phenotypes, administering a therapeutically effective amount of a protein degradation inhibitor to the subject; and determining the one or more phenotypes after an initial period of treatment with the protein degradation inhibitor wherein the modulation of the one or more phenotype is an indication that the disorder is likely to have a favorable clinical response to treatment with a protein degradation inhibitor.

[00235] Useful in the methods described herein as protein degradation inhibitors are one or more of histone acetylase inhibitors (Mitsiades *et al.*. Transcriptional signature of

histone deacetylase inhibition in multiple myeloma: biological and clinical implications. Proc Natl Acad Sci U S A. 2004;101:540-545; Rosato RR, Grant S. Histone deacetylase inhibitors in clinical development. Expert Opin Investig Drugs. 2004;13:21-38; each of which is incorporated herein by reference), tubacin, bortezomib, velcade, SAHA, R115777 FTI, ^{166}Holminun-DOTMP, arsenic trioxide, 17-AAG, MG132, sapojargon, NPI-0052, or other compounds described herein.

[00236] The protein degradation inhibitors of the invention may inhibit the activity of cellular proteins, (*e.g.*, enzymes), for example, suitable protein degradation inhibitors may inhibit the activity of HDAC6, for example, HDAC6 enzymatic activity. The inhibition of HDAC6 enzymatic activity may in turn inhibit aggresome mediated protein degradation. Suitable protein degradation inhibitors may also inhibit, for example, the C-terminal acetylation activity of HDAC6, thereby inhibiting aggresome mediated protein degradation. Other suitable protein degradation inhibitors may inhibit the activity of the aggresome.

[00237] In certain embodiments, the inhibition of HDAC6 by the inventive methods and compositions leads to the acetylation of HSP90. The acetylation of HSP90 renders this protein less active against a number of it client proteins and therefore increase protein stress in the cell. This is particularly important in cancers such as breast and prostate cancer. In these cancers, the acetylation of HSP90 lead to diminished activity of steroid-binding receptors because glucocorticoid receptors require HSP90 function to engage glucocorticoids. HSP90 inhibition has been found to diminish the glucocorticoid responsiveness of glucocorticoid receptor-containing cells. Therefore, the administration of HDAC6 inhibitors leads to the hyperacetylation of HSP90 leading to decreased sensitivity to estrogen in breast cancer cells and decreased sensitivity to androgens in prostate cancer cells.

[00238] Suitable aggresome inhibitors include tubacin, scriptade, or the compounds described herein.

[00239] Protein degradation inhibitors of the invention may also inhibit proteasome activity. Suitable proteasome inhibitors include one or more of histone acetylase inhibitors (14, 15), tubacin, bortezomib, velcade, SAHA, R115777 FTI, 166 Holminun DOTMP, arsenic trioxide, 17-AAG, MG132, sapojargon, NPI-0052, or the compound of Formula I, derivatives of the compounds of Formula I.

[00240] Additional suitable protein degradation inhibitors include peptide inhibitors, for example, a peptides derived from HDAC6, HSP 90, proteins in the aggresome pathway, both up-stream and down-stream. For example, the C-terminal portion of HDAC6, including the Buz domain.

[00241] The protein degradation inhibitors of the invention are capable of modulating one or more phenotypes of a cell. The phenotype may be a biological or clinical sequalae in response to a particular treatment or compound. Phenotypes include, anemia, thrombocytopenia, neutropenia, osteolytic lesions, bone pain, immunodeficiency, renal insufficiency, hypercalcemia, aneuploidy of mature plasma cells, percentage of malignant cells, acetylation state of tubulin, apoptosis of mature plasma cells, level of aggresomes, level of aggresomes in mature plasma cells, HDAC6 ubiquitination, HDAC6 ubiquitination in mature plasma cells, HDAC6 association with dynein in mature plasma cells, cellular levels of ubiquintinated proteins in mature plasma cells, level of caspase-8 in mature plasma cells, level of PARP in mature plasma cells, thymidine uptake in mature plasma cells, dilated ER cisternae, aggregation of mature plasma cells, deposits of immunoglobulins in mature plasma cells, acetylation state of non-histone proteins, global ubiquitination state of the cellular proteins, state of cell cycle regulation, necrosis, markers of apoptosis, apoptosis state, Russell body formation, Cystic Fibrosis transmembrane protein receptor state, and modulation of cellular protein deposits, or global acetylation state of cellular and extracellular proteins.

[00242] A decrease in one or more of anemia, level of aggresomes, thrombocytopenia, neutropenia, osteolytic lesions, bone pain, immunodeficiency, renal insufficiency, hypercalcemia, aneuploidy of mature plasma cells, percentage of malignant cells, thymidine uptake in mature plasma cells, level of full length caspase-8 in mature plasma cells, level of full length PARP in mature plasma cells, or aggregation of mature plasma cells, indicates that the treatment is efficacious.

[00243] An increase in acetylation state of tubulin, HDAC6 ubiquitination in mature plasma cells, level of cleaved form of caspase-8, level of cleaved form of PARP, necrosis, acetylation state of non-histone proteins, cellular ubiquitination levels, apoptosis, markers of apoptosis, cell cycle deregulation, or deposits of immunoglobulins in mature plasma cells indicates that the treatment is efficacious.

[00244] Phenotypes may be determined by many different methods know in the art. For example, phenotypes of a subject, e.g., anemia, thrombocytopenia, neutropenia, osteolytic lesions, bone pain, immunodeficiency, renal insufficiency, and hypercalcemia, may be determined by diagnostic methods known in the art for diagnosing these conditions. The phenotype aneuploidy of mature plasma cells may be determined by cytogenetic methods. The percentage of malignant cells may be determined, for example, by histological staining, flow cytometry, FISH, PCR, radiographic techniques, MRI, CT scan, metabolic methods, and the like. Acetylation state of tubulin, apoptosis of mature plasma cells, level of aggresomes

in mature plasma cells, HDAC6 ubiquitination in mature plasma cells, HDAC6 association with dynein in mature plasma cells, cellular levels of ubiquintinated proteins in mature plasma cells, level of caspase-8 in mature plasma cells, level of the cleaved form of caspase-8, level of PARP in mature plasma cells, level of cleaved form of PARP, and thymidine uptake in mature plasma cells may be determined by biochemical methods, for example, immunoprecipitation, Western blotting, ELISA, immunohistochemistry, mass spectrometry, or a combination of these and other methods.

[00245] According the methods of the invention, phenotypes of samples may be determined at any point before treatment, after a suspected diagnosis of a protein degradation disorder, after treatment, and during treatment. The methods may be employed one or more times on sample from a subject during any of these time periods or any other time period.

[00246] Methods of the invention may further comprise determining the subject's phenotype after a second period of treatment with the protein degradation inhibitor. The second period of treatment may of the same length as the first period or initial period of treatment of may be longer or shorter than the first or initial period of treatment. The determination as the second period of treatment may be on a second biological sample obtained from the subject.

[00247] Subjects or cells being treated with protein degradation inhibitors may be further administered a therapeutically effective amount of one or more additional protein degradation inhibitors. The additional inhibitor may be an aggresome inhibitor or a proteasome inhibitor. The additional inhibitor, may be, for example, bortezomib, tubacin , histone acetylase inhibitors, tubacin, bortezomib (VELCADE®), SAHA, R115777 FTI, ^{166}Holminun-DOTMP, arsenic trioxide, 17-AAG, or the compound of Formula I, derivatives of the compounds of Formula I.

[00248] A subject or cell may be co-administered one or more of a chemotherapeutic agent, radiation agent, hormonal agent, biological agent or an anti-inflammatory agent to the subject while being treated for a protein degradation disorder. Chemotherapeutic agents may include tamoxifen, trastuzamab, raloxifene, doxorubicin, fluorouracil/5-fu, pamidronate disodium, anastrozole, exemestane, cyclophos-phamide, epirubicin, letrozole, toremifene, fulvestrant, fluoxymester-one, trastuzumab, methotrexate, megastrol acetate, docetaxel, paclitaxel, testolactone, aziridine, vinblastine, capecitabine, goselerin acetate, zoledronic acid, taxol, vinblastine, and/or vincristine. Useful non-steroidal anti-inflammatory agents, include, but are not limited to, aspirin, ibuprofen, diclofenac, naproxen, benoxaprofen, flurbiprofen, fenoprofen, flubufen, ketoprofen, indoprofen, piroprofen, carprofen, oxaprozin,

pramoprofen, muroprofen, trioxaprofen, suprofen, aminoprofen, tiaprofenic acid, fluprofen, bucloxic acid, indomethacin, sulindac, tolmetin, zomepirac, tiopinac, zidometacin, acemetacin, fentiazac, clidanac, oxpinac, mefenamic acid, meclofenamic acid, flufenamic acid, niflumic acid, tolfenamic acid, diflurisal, flufenisal, piroxicam, sudoxicam, isoxicam; salicylic acid derivatives, including aspirin, sodium salicylate, choline magnesium trisalicylate, salsalate, diflunisal, salicylsalicylic acid, sulfasalazine, and olsalazin; para-aminophennol derivatives including acetaminophen and phenacetin; indole and indene acetic acids, including indomethacin, sulindac, and etodolac; heteroaryl acetic acids, including tolmetin, diclofenac, and ketorolac; anthranilic acids (fenamates), including mefenamic acid, and meclofenamic acid; enolic acids, including oxicams (piroxicam, tenoxicam), and pyrazolidinediones (phenylbutazone, oxyphenthartazone); and alkanones, including nabumetone and pharmaceutically acceptable salts thereof and mixtures thereof. For a more detailed description of the NSAIDs, *see Paul A. Insel, Analgesic-Antipyretic and Antiinflammatory Agents and Drugs Employed in the Treatment of Gout, in Goodman & Gilman's The Pharmacological Basis of Therapeutics* 617-57 (Perry B. Molinhoff and Raymond W. Ruddon eds., 9th ed 1996) and Glen R. Hanson, *Analgesic, Antipyretic and Anti-Inflammatory Drugs in Remington: The Science and Practice of Pharmacy Vol II* 1196-1221 (A.R. Gennaro ed. 19th ed. 1995) which are hereby incorporated by reference in their entireties.

[00249] While a subject or cell is being treated with a protein degradation inhibitor, the cell or subject may be monitored.

[00250] Methods of the invention may further comprise comparing one or more of the pre-treatment or post-treatment phenotypes to a standard phenotype. The standard phenotype is the corresponding phenotype in a reference cell or population of cells. Reference cells are one or more of the following, cells from a person or subject that is not suspected of having a protein degradation disorder, cells from the subject, cultured cells, cultured cells from the subject, or cells from the subject pre-treatment. Cells from the subject may include, for example, a bone marrow stromal cell, (BMSC), a peripheral blood mononuclear cell (PBMC), lymphocytes, hair follicles, blood cells, other epithelial cells, bone marrow plasma cells, primary cancer cells, patient derived tumor cells, normal or cancerous hematopoietic stem cells, neural stem cells, solid tumor cells, astrocytes, and the like.

[00251] Methods of the invention also include methods of inhibiting aggresome mediated protein degradation in a cell or subject comprising contacting the cell with an aggresome inhibitor. In one embodiment, the aggresome protein degradation is mediated by

HDAC6. The method may further comprise inhibiting proteasome protein degradation in the cell or subject. For example, by the administration of bortezomib and tubacin.

Pharmaceutical Compositions

[00252] The invention also provides a pharmaceutical composition, comprising an effective amount of a compound of the invention of formula I, formula II, or otherwise described herein and a pharmaceutically acceptable carrier. In a further embodiment, the effective amount is effective to treat a protein degradation disorder, as described previously.

[00253] In an embodiment, the compound of the invention is administered to the subject using a pharmaceutically-acceptable formulation, *e.g.*, a pharmaceutically-acceptable formulation that provides sustained delivery of the compound of the invention to a subject for at least 12 hours, 24 hours, 36 hours, 48 hours, one week, two weeks, three weeks, or four weeks after the pharmaceutically-acceptable formulation is administered to the subject.

[00254] In certain embodiments, these pharmaceutical compositions are suitable for topical or oral administration to a subject. In other embodiments, as described in detail below, the pharmaceutical compositions of the present invention may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin; (4) intravaginally or intrarectally, for example, as a pessary, cream or foam; or (5) aerosol, for example, as an aqueous aerosol, liposomal preparation or solid particles containing the compound.

[00255] The phrase "pharmaceutically acceptable" refers to those compound of the inventions of the present invention, compositions containing such compounds, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[00256] The phrase "pharmaceutically-acceptable carrier" includes pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject chemical from one organ, or portion of the body, to another organ, or portion of the body. Each carrier

is "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

[00257] Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

[00258] Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

[00259] Compositions containing a compound of the invention(s) include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal, aerosol and/or parenteral administration. The compositions may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will

range from about 1 per cent to about ninety-nine percent of active ingredient, preferably from about 5 per cent to about 70 per cent, more preferably from about 10 per cent to about 30 per cent.

[00260] Methods of preparing these compositions include the step of bringing into association a compound of the invention(s) with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a compound of the invention with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

[00261] Compositions of the invention suitable for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a compound of the invention(s) as an active ingredient. A compound may also be administered as a bolus, electuary or paste.

[00262] In solid dosage forms of the invention for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically-acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, acetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such a talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

[00263] A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered active ingredient moistened with an inert liquid diluent.

[00264] The tablets, and other solid dosage forms of the pharmaceutical compositions of the present invention, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

[00265] Liquid dosage forms for oral administration of the compound of the invention(s) include pharmaceutically-acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

[00266] In addition to inert diluents, the oral compositions can include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

[00267] Suspensions, in addition to the active compound of the invention(s) may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

[00268] Pharmaceutical compositions of the invention for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more compound of the invention(s) with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active agent.

[00269] Compositions of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate.

[00270] Dosage forms for the topical or transdermal administration of a compound of the invention(s) include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound of the invention(s) may be mixed under sterile conditions with a pharmaceutically-acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

[00271] The ointments, pastes, creams and gels may contain, in addition to compound of the invention(s) of the present invention, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

[00272] Powders and sprays can contain, in addition to a compound of the invention(s), excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

[00273] The compound of the invention(s) can be alternatively administered by aerosol. This is accomplished by preparing an aqueous aerosol, liposomal preparation or solid particles containing the compound. A nonaqueous (*e.g.*, fluorocarbon propellant) suspension could be used. Sonic nebulizers are preferred because they minimize exposing the agent to shear, which can result in degradation of the compound.

[00274] Ordinarily, an aqueous aerosol is made by formulating an aqueous solution or suspension of the agent together with conventional pharmaceutically-acceptable carriers and

stabilizers. The carriers and stabilizers vary with the requirements of the particular compound, but typically include nonionic surfactants (Tweens, Pluronics, or polyethylene glycol), innocuous proteins like serum albumin, sorbitan esters, oleic acid, lecithin, amino acids such as glycine, buffers, salts, sugars or sugar alcohols. Aerosols generally are prepared from isotonic solutions.

[00275] Transdermal patches have the added advantage of providing controlled delivery of a compound of the invention(s) to the body. Such dosage forms can be made by dissolving or dispersing the agent in the proper medium. Absorption enhancers can also be used to increase the flux of the active ingredient across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the active ingredient in a polymer matrix or gel.

[00276] Ophthalmic formulations, eye ointments, powders, solutions and the like, are also contemplated as being within the scope of the invention.

[00277] Pharmaceutical compositions of the invention suitable for parenteral administration comprise one or more compound of the invention(s) in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

[00278] Examples of suitable aqueous and nonaqueous carriers, which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

[00279] These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about

by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

[00280] In some cases, to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

[00281] Injectable depot forms are made by forming microencapsule matrices of compound of the invention(s) in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.

[00282] When the compound of the invention(s) are administered as pharmaceuticals, to humans and animals, they can be given per se or as a pharmaceutical composition containing, for example, 0.1 to 99.5% (more preferably, 0.5 to 90%) of active ingredient in combination with a pharmaceutically-acceptable carrier.

[00283] Regardless of the route of administration selected, the compound of the invention(s), which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically-acceptable dosage forms by conventional methods known to those of skill in the art.

[00284] Actual dosage levels and time course of administration of the active ingredients in the pharmaceutical compositions of the invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. An exemplary dose range is from 0.1 to 10 mg per day.

[00285] A preferred dose of the compound of the invention for the present invention is the maximum that a patient can tolerate and not develop serious hypercalcemia. Preferably, the compound of the invention of the present invention is administered at a concentration of about 0.001 mg to about 100 mg per kilogram of body weight, about 0.001 – about 10 mg/kg

or about 0.001 mg – about 100 mg/kg of body weight. Ranges intermediate to the above-recited values are also intended to be part of the invention.

Screening Assays

[00286] In pursuit of potency selective inhibitors (*e.g.*, effective in the nanomolar range) of protein degradation, and in particular of HDAC6, the invention provides novel screening methods. In one embodiment, the screening method is a quantitative, cellular, image-based screen of cancer cells.

[00287] Screening methods of the invention include a quantitative, high-throughput, image-based screen of cancer cells. The cells may be grown, for example in multi-well plates or chips, for example 384-well plate formats or in chips, such as the BioTrove OpenArray™ Chips (BioTrove, Woburn, MA). The cells may be grown and treated in these wells and plates. The are treated with a small molecule library, peptide library, nucleic acid library. Some of the libraries may be biased for HDAC inhibition as discussed herein. Other libraries may be biased toward inhibition of other aggresome or proteasome inhibition targets. Once treated, the cells may be monitored by phenotype as discussed herein. For example, tubulin and histone acetylation state-specific antibodies may be used and recognized by corresponding fluorescent secondary antibodies or may be directly labeled and detected if they bind. Wells or through-holes are then scored in an unbiased fashion. This may be done, for example, by an automated Axon 5000A epifluorescence microscope. Scoring takes into account, for example, the amount of fluorescence, compared with controls, the relative amount of fluorescence relative to a reference, or the amount of fluorescence. Controls may be used for reference. For example, as a control for tubacin, trichostatin, and DMSO may be used as controls. Molecules have subsequently been prioritized following assessment for direct cytotoxicity and synergy with bortezomib in the RPMI-8226 myeloma cell line.

[00288] A tubulin-selective sub-library can includes molecules derived from diversity-oriented synthetic pathways. For example, the libraries of compounds described in Sternson SM *et al.*, *Org Lett.* 2001 Dec 27;3(26):4239-42; Haggarty SJ *et al.*, *Chem Biol.* 2003 May;10(5):383-96; Haggarty SJ *et al.*, *Proc Natl Acad Sci U S A.* 2003 Apr 15;100(8):4389-94. Epub 2003 Apr 03; and Haggarty SJ *et al.*, *Comb Chem High Throughput Screen.* 2004 Nov;7(7):669-76 can be screened to identify additional active compounds.

[00289] Method for identifying a candidate compounds to inhibit protein degradation in a cell comprise contacting a cell exhibiting aggresome formation with a candidate compound, and determining a phenotype of the cell, wherein modulation of the phenotype is

indicative of the efficacy of the compound. Phenotypes of cells may be determined as described herein, for example by image-based multidimensional screening. The cell types used in the screening assays may include myeloma patient cells, myeloma cell lines, primary cell cultures of myeloma cell line. Cell cultures, primary cultures, or patient cells may be co-cultured with stromal cells or other cells. The contacting may be by adding the candidate compound to the media, directly to the cells, or as a fluid flowing over the cell, e.g., in a lateral flow or a planar flow patch clamp device. One of skill in the art would be able to identify other appropriate methods having the benefit of this disclosure.

[00290] Candidate molecules may be one or more of a small molecule, a peptide, or a nucleic acid. The nucleic acids may be, for example, an RNA or DNA molecule, e.g., mRNA, RNAi, siRNA or an oligo.

[00291] Suitable peptides may include peptides derived from HDAC6, dynenin, Ubiquitin, or chaperones. For example, peptides derived from the C-terminus of HDAC6, (amino acids 439-503), amino acids 500-790 of HDAC 6, amino acids 781-931 of HDAC6, or the amino acids 1-460. Other suitable peptides include the dynein-binding domain of HDAC6 identified by by Yao (aa 439-503); the C-terminal TDAC domain (aa 500-790), the ubiquitin-binding BUZ domain (aa 781-931); or the N-terminal 460 amino acids.

[00292] Suitable small molecules include natural and synthetic products. The molecules may be contained in a library; libraries of compounds can be obtained commercially (e.g., from ChemBridge, San Diego, CA, or may be prepared by known methods (*e.g.*, as described in herein).

[00293] Other screening methods for evaluating a test compound comprise contacting an cell exhibiting aggresome formation with a test compound, and evaluating the cell following contact, wherein a correlation of a modulation of one of more phenotypes to a reference value is an indication that the test compound may be useful as a protein degradation disorder treatment.

[00294] The method may further comprise determining a phenotype of the cell after an initial period of treatment with the protein degradation inhibitor. The initial period of treatment may be the time in which it takes to establish a stable and/or therapeutically effective blood serum level of a therapeutic compound of the invention, or the time in which it takes for the subject to clear a substantial portion of the therapeutic, or any period of time selected by the subject or healthcare professional that is relevant to the treatment.

[00295] This embodiment of the invention is well suited to screen chemical libraries for molecules which modulate, *e.g.*, inhibit protein degradation in a cell, tissue or subject, or

modulate a phenotype as described herein of a cell, tissue or subject. The chemical libraries can be peptide libraries, peptidomimetic libraries, chemically synthesized libraries, recombinant, *e.g.*, phage display libraries, and *in vitro* translation-based libraries, other non-peptide synthetic organic libraries, *etc.*

[00296] Libraries screened using the methods of the present invention can comprise a variety of types of compounds. Examples of libraries that can be screened in accordance with the methods of the invention include, but are not limited to, peptides; random biooligomers; diversomers such as hydantoins, benzodiazepines and dipeptides; vinylogous polypeptides; nonpeptidal peptidomimetics; oligocarbamates; peptidyl phosphonates; peptide nucleic acid libraries; antibody libraries; carbohydrate libraries; and small molecule libraries (preferably, small organic molecule libraries). In some embodiments, the compounds in the libraries screened are nucleic acid or peptide molecules. In a non-limiting example, peptide molecules can exist in a phage display library. In other embodiments, the types of compounds include, but are not limited to, peptide analogs including peptides comprising non-naturally occurring amino acids, *e.g.*, D-amino acids, phosphorous analogs of amino acids, such as γ-amino phosphoric acids and γ-amino phosphoric acids, or amino acids having non-peptide linkages, nucleic acid analogs such as phosphorothioates and PNAs, hormones, antigens, synthetic or naturally occurring drugs, opiates, dopamine, serotonin, catecholamines, thrombin, acetylcholine, prostaglandins, organic molecules, pheromones, adenosine, sucrose, glucose, lactose and galactose. Libraries of polypeptides or proteins can also be used in the assays of the invention.

[00297] In a preferred embodiment, the combinatorial libraries are small organic molecule libraries including, but not limited to, benzodiazepines, isoprenoids, thiazolidinones, metathiazanones, pyrrolidines, morpholino compounds, and benzodiazepines. In another embodiment, the combinatorial libraries comprise peptides; random bio-oligomers; benzodiazepines; diversomers such as hydantoins, benzodiazepines and dipeptides; vinylogous polypeptides; nonpeptidal peptidomimetics; oligocarbamates; peptidyl phosphonates; peptide nucleic acid libraries; antibody libraries; or carbohydrate libraries. Combinatorial libraries are themselves commercially available (see, *e.g.*, ComGenex, Princeton, New Jersey; Asinex, Moscow, Ru, Tripos, Inc., St. Louis, Missouri; ChemStar, Ltd, Moscow, Russia; 3D Pharmaceuticals, Exton, Pennsylvania; Martek Biosciences, Columbia, Maryland; etc.).

[00298] In a preferred embodiment, the library is preselected so that the compounds of the library are more amenable for cellular uptake. For example, compounds are selected

based on specific parameters such as, but not limited to, size, lipophilicity, hydrophilicity, and hydrogen bonding, which enhance the likelihood of compounds getting into the cells. In another embodiment, the compounds are analyzed by three-dimensional or four-dimensional computer computation programs.

[00299] The combinatorial compound library for use in accordance with the methods of the present invention may be synthesized. There is a great interest in synthetic methods directed toward the creation of large collections of small organic compounds, or libraries, which could be screened for pharmacological, biological or other activity. The synthetic methods applied to create vast combinatorial libraries are performed in solution or in the solid phase, *i.e.*, on a solid support. Solid-phase synthesis makes it easier to conduct multi-step reactions and to drive reactions to completion with high yields because excess reagents can be easily added and washed away after each reaction step. Solid-phase combinatorial synthesis also tends to improve isolation, purification and screening. However, the more traditional solution phase chemistry supports a wider variety of organic reactions than solid-phase chemistry.

[00300] Combinatorial compound libraries of the present invention may be synthesized using the apparatus described in U.S. Patent No. 6,190,619 to Kilcoin *et al.*, which is hereby incorporated by reference in its entirety. U.S. Patent No. 6,190,619 discloses a synthesis apparatus capable of holding a plurality of reaction vessels for parallel synthesis of multiple discrete compounds or for combinatorial libraries of compounds.

[00301] In one embodiment, the combinatorial compound library can be synthesized in solution. The method disclosed in U.S. Patent No. 6,194,612 to Boger *et al.*, which is hereby incorporated by reference in its entirety, features compounds useful as templates for solution phase synthesis of combinatorial libraries. The template is designed to permit reaction products to be easily purified from unreacted reactants using liquid/liquid or solid/liquid extractions. The compounds produced by combinatorial synthesis using the template will preferably be small organic molecules. Some compounds in the library may mimic the effects of non-peptides or peptides. In contrast to solid phase synthesize of combinatorial compound libraries, liquid phase synthesis does not require the use of specialized protocols for monitoring the individual steps of a multistep solid phase synthesis (Egner *et al.*, 1995, J.Org. Chem. 60:2652; Anderson *et al.*, 1995, J. Org. Chem. 60:2650; Fitch *et al.*, 1994, J. Org. Chem. 59:7955; Look *et al.*, 1994, J. Org. Chem. 49:7588; Metzger *et al.*, 1993, Angew. Chem., Int. Ed. Engl. 32:894; Youngquist *et al.*, 1994, Rapid Commun. Mass Spect.

8:77; Chu *et al.*, 1995, J. Am. Chem. Soc. 117:5419; Brummel *et al.*, 1994, Science 264:399; and Stevanovic *et al.*, 1993, Bioorg. Med. Chem. Lett. 3:431).

[00302] Combinatorial compound libraries useful for the methods of the present invention can be synthesized on solid supports. In one embodiment, a split synthesis method, a protocol of separating and mixing solid supports during the synthesis, is used to synthesize a library of compounds on solid supports (*see e.g.*, Lam *et al.*, 1997, Chem. Rev. 97:41-448; Ohlmeyer *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926 and references cited therein). Each solid support in the final library has substantially one type of compound attached to its surface. Other methods for synthesizing combinatorial libraries on solid supports, wherein one product is attached to each support, will be known to those of skill in the art (see, *e.g.*, Nefzi *et al.*, 1997, Chem. Rev. 97:449-472).

Immunofluorscence Assay for Identifying HDAC and TDAC Inhibitors

[00303] The present invention provides an immunofluorescence-based assay for identifying test agents with HDAC and/or TDAC inhibitory activity. The inventive assay is based on the use of specific antibodies for acetylated tubulin and acetylated lysine (*i.e.*, a marker for acetylated histones). The assay is particularly useful in identifying agents that specifically inhibit HDAC versus TDAC or vice versa. As described above, any type of agent, including small molecules, polymers, biomolecules, proteins, peptides, polynucleotides, *etc.*, may be screened using the inventive assay. In certain embodiments, small molecules are screened. In certain particular embodiments, the small molecules are tubacin-like or are tubacin derivatives. In certain embodiments, the small molecules are compounds of the present invention. The test agents may also be small molecules purchased, prepared through traditional synthetic techniques, prepared through combinatorial chemistry techniques, or obtained from historical chemical collections. In certain embodiments, the test agents are biomolecules. In other embodiments, the test agents are proteins or peptides. In yet other embodiments, the test agents are polynucleotides. In certain embodiments, the test agents are polymers.

[00304] The assay involves contacting cells with a specific concentration of the test agent under specific conditions. The specific conditions include type of media, concentration of agent, solvent agent is dissolved or suspended in, pH, temperature, time of incubation, *etc.* There particular parameters may be determined by the operator or scientist conducting the assay as would be appreciated by one of skill in this art. After a determined time of incubation with the test compound, the cells are treated with a first primary antibody directed

against acetylated tubulin and a second primary antibody directed against acetylated lysine. The cells are then contacted with two secondary antibodies specific for each of the primary antibodies and identifiable by a unique signal. In certain embodiments, the unique signals are unique fluorsecence signals. However, chemiluminescence, phosphorescence, colorimetric, enzymatic reaction products, or other reporters may also be used. The signal from each of the secondary antibodies is measured and optionally quantitated to determine the amount of TDAC and HDAC inhibition under the specified conditions with the test agent. Optionally, the extent of inhibition is determined relative to a control in which no test agent was added. In certain embodiments, the secondary antibody is left out and the primary antibodies are uniquely labeled for identification and optionally quantification.

[00305] Any type of cell may be used in the inventive assay. The cells may be from any species. For example, bacterial cells, yeast cells, mammalian cells, murine cells, rat cells, primate cells, or human cells may be used. In certain embodiments, the cells are human cells which may be derived from any tissue or organ system or be at any stage of development. The cells may be derived from skin, hair, nerve, muscle, bone, digestive tract, genitourinary tract, blood vessels, bone marrow, heart, lung, liver, pancreas, stomach, colon, kidneys, bladder, testes, ovaries, uterus, cervix, spleen, endocrine system, brain, spinal cord, eye, *etc.* The cells may be stem cell, embryonic stem cells, fetal cells, progenitor cells, *etc.* In certain embodiments, the cells are human cancer cells lines. Any type of cancer cell may be used. Certain exemplary cell lines include multiple myeloma, non-Hodgkin's lymphoma, acute myelogenous leukemia (AML), breast cancer, ovarian cancer, prostate cancer, lung cancer, colon cancer, leukemia, lymphoma, skin cancer, brain cancer, cervical cancer, stomach cancer, bone cancer, *etc.* Specific cell lines include MM.1S, U266, RPMI8226, DOX40, MM.1R, INA-6, LR5, *etc.*

[00306] The cells are typically plated in multi-well tissue culture plates (*e.g.* 384 well plates) and allowed to adhere to the plate before the test agent at a specified concentration is added. Multiple concentrations for each test agent may be tested to establish a dose-response curve to allow the calculation of IC_{50} values. The test agent is allowed to incubate with the cells under physioligical conditions for 1-24 hours, preferably 4-16 hours. Afterwards, the cells are fixed, blocked, and washed. The treated cells are incubated with the primary antibodies—one specific for acetylated tubulin and the other for acetylated lysine. The antibodies may be monoclonal or polyclonal. In certain embodiments, the antibodies are obtained from a commercial source. The primary antibodies are then tagged with a fluorescent secondary antibody. The plates are imaged, and the fluorescence signal from

each of the secondary antibodies is optionally quantitated. As would be appreciated by one of skill in the art, the cells may be stained for other markers, such as other proteins, nucleic acid content, DNA content, RNA content, organelles, *etc.*

[00307] The data gathered may then be used to calculate dose-response curves, to calculate IC_{50} values, to establish structure-function relationships, to calculate the ratio of HDAC to TDAC inhibition, to determine the specificity for HDAC or TDAC, *etc.*

[00308] The inventive assay is particularly amenable for use in high-throughput systems using multi-well plates, fluid handling robots, plate imagers, and computers and software developed for high-throughput screening. In certain embodiments, at least 100 test conditions (*e.g.*, test agent, concentration of test agent, type of cell, temperature, length of incubation with test agent, pH, media, *etc.*) are assayed in parallel. In other embodiments, at least 300 test conditions are assayed in parallel. In yet other embodiments, at least 500 test conditions are assayed in parallel. In still other embodiments, at least 1000 test conditions are assayed in parallel.

[00309] Compounds identified to be HDAC and/or TDAC inhbitors using the inventive assay are considered part of the invention. In certain embodiments, the assay is used to identify specific inhibitors of HDAC. In other embodiments, the assay is used to identify specific inhibitors of TDAC.

Kits

[00310] The invention provides kits for treating a protein degradation disorder in a subject. The kits may comprise one or more compound of the invention, (e.g., tubacin, a compound of Formula I) or pharmaceutically acceptable esters, salts, and prodrugs thereof, and instructions for use. The instructions for use may include dosages, administration routes, patient educational information, expiration date, storage conditions, indications, and the like.

[00311] The compounds of the present invention can be provided in therapeutically effective amounts in the kits with pharmaceutically acceptable carriers or they may be provided in bulk amounts with pharmaceutically acceptable carriers.

[00312] The invention also provides packaged composition comprising therapeutically effective amounts of an a protein degradation inhibitor and a pharmaceutically acceptable carrier or diluent. The packaged composition is formulated for treating a subject suffering from or susceptible to a protein degradation disorder, and packaged with instructions to treat a subject suffering from or susceptible to a protein degradation disorder.

[00313] The invention also provides kits for screening for protein degradation inhibitors. The kits may include control composition, for example, tubacin and niltubicin for reference. The kits may also include, reagents, for example, test compounds, buffers, media (e.g., cell growth media), cells, etc. Test compounds may include known compounds or newly discovered compounds, for example, combinatorial libraries of compounds.

[00314] Kits for assessing the efficacy of a protein degradation disorder treatment are also provided. The kits may include reagents for determining one or more of the phenotypes described herein (*e.g.*, reagents for the determining of the aceylation state of tubulin), instructions for use, and instruments for collecting subject samples.

[00315] One or more of the kit of the invention may be packaged together, for example, a kit for assessing the efficacy of a protein degradation treatment may be package with a kit for treating a protein degradation disorder.

EXAMPLES

Experimental Procedures

Bone marrow stromal cell (BMSC) cultures.

BM specimens were obtained from patients with MM. Mononuclear cells (MNCs) separated by Ficoll-Hipaque density sedimentation was used to established long-term BM cultures. When an adherent cell monolayer had developed, cells were harvested in Hank's Buffered Saline Solution containing 0.25% trypsin and 0.02% EDTA, washed, and collected by centrifugation.

Cell lines, patients BM plasma cells and BM stromal cell (SCs).

Dex-sensitive (MM.1S) and resistant (MM.1R) human MM cell lines were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). RPMI8226 and U266 human MM cell lines were obtained from American Type Culture Collection (Rockville, MD). IL-6 dependent INA-6 cell line was kindly provided by Dr M Gramatzki (Erlangen, Germany). Melphalan-resistant RPMI-LR5 and doxorubicin-resistant RPMI-Dox40 cell lines were provided by Dr William Dalton (Lee Moffitt Cancer Center, Tampa, FL). All MM cell lines were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS, Sigma Chemical Co., St. Louis, MO), 2 μM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Grand Island, NY). INA-6 cells were maintained with addition of IL-6 (1 ng/ml). MM patient plasma cells were purified from bone marrow (BM) aspirates by negative

selection using an antibody cocktail (RosetteSep Separation System, StemCell Technologies, Vancouver, Canada), as previously described (31). The purity of MM cells was >90%, as confirmed by flow cytometric analysis using anti-CD138 Ab (BD Pharmingen, San Diego, CA). Mononuclear cells (MNCs) separated by Ficoll-Hipaque density sedimentation from BM aspirates were also used to establish long-term BM stromal cells (BMSCs), as previously described (28, 43). All experiments with patient samples were performed according to the protocol approved by the Institutional Review Board.

Inhibitors.

The peptide boronate proteasome inhibitor bortezomib was provided by Millennium Pharmaceuticals (Cambridge, MA). HDAC6 specific inhibitor tubacin and its non-active derivative niltubacin are obtained from Broad Institute of Harvard University (18) and Massachusetts Institute of Technology. Both inhibitors were dissolved in DMSO and stored at –20 ^{0}C until use.

DNA synthesis.

Proliferation was measured by ^{3}H-thymidine uptake. Briefly, MM cells (3 x 10^{4}cells /well) were incubated in 96-well culture plates in the presence of media, Velcade and/or Tubacin for 48 h at 37^{o}C. DNA synthesis was measured by [^{3}H]-thymidine ([^{3}H]-TdR, Perkin Elmer, Boston, MA) uptake. Cells were pulsed with [^{3}H]TdR (0.5 μCi/well) during the last 8 h of 48 h cultures. All experiments were performed in triplicate.

Growth inhibition assay.

The inhibitory effect of bortezomib and/or tubacin on MM cell growth was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye absorbance, as described previously (33). Cells from 48 h cultures were pulsed with 10 μl of 5 mg/ml MTT to each well for the last 4 h of 48 h cultures, followed by 100 μl isopropanol containing 0.04N HCl. Absorbance was measured at 570 nm using a spectrophotometer (Molecular Devices Corp., Sunnyvale CA). All experiments were performed in quadruplicate.

Western blotting.

MM cells were cultured with Velcade and/or Tubacin; harvested; washed; and lysed using lysis buffer: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 5 mM EDTA, 5 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, 5 μg/ml leupeptine, and 5 μg/ml aprotinin. Cell lysates

were subjected to SDS-PAGE, transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA), and immunoblotted with Abs against specific proteins.

Flow cytometric analysis.

For cell cycle analysis, MM cells cultured for 24 h in Velcade (5 μM) and/or Tubacin (5 μM) were harvested, washed with phosphate-buffered saline (PBS), fixed with 70% ethanol, and treated with 10 μg/ml of RNase (Roche Diagnostics Corp., Indianapolis, IN). Cells were then stained with propidium iodine (PI, Sigma) (5 μg/ml) and cell cycle profile was determined using program M software on an Epics flow cytometer (Coulter Immunology, Hialeah, FL) (44)..

Effect of Velcade and Tubacin on paracrine MM cell growth in the BM.

To evaluate growth stimulation and signaling in MM cells adherent to BMSCs, 3 x 10^4 MM.1S cells were cultured in BMSC coated 96-well plates for 48 h, in the presence of Velcade and/or Tubacin. DNA synthesis was measured as described above.

Immunoblotting.

Cells cultured with tubacin and/or bortezomib were harvested; washed, and lysed using lysis buffer: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 5 mM EDTA, 5 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 5 μg/ml leupeptine, and 5 μg/ml aprotinin. Whole cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA), and immunoblotted with specific Abs (31). Western blotting was done using anti-HDAC6, acetylated lysine, acetylated histone H3, acetylated histone H4, ubiquitin (Ub), phospho-SAPK (JNK), caspase-8 caspase-9, caspase-3, and PARP Abs (Cell Signaling, Beverly, MA); with anti-α-tubulin Ab (Santa Cruz Biotechnology, Santa Cruz, CA); as well as with anti-dynein Ab (Sigma, Saint Louis, MO). For immunoprecipitation, whole cell lysates were incubated with anti-Ub or dynein Abs overnight at 4^0C, and then incubated with protein A/G PLUS-Agarose (Santa Cruz Biotechnology) for 2 h at 4^0C, as in our prior study (31). Immunoprecipitates were then subjected to Western blotting for detection of HDAC6 and dynein.

Transient transfection of HDAC6 siRNA.

MM.1S cells were transiently transfected with HDAC6 siRNA (Dharmacon Inc., Lafayette, CO) using "Cell Line Nucleofecto™ Kit V," according to manufacturer's (Amaxa

Biosystems, Gaithersburg, MD) instructions (33). Following transfection, MM.1S cells were subjected to Western blotting and MTT assay, in the presence or absence of bortezomib.

Growth of MM cells adherent to BMSCs.

To evaluate the effect of combined tubacin and bortezomib treatment on growth of MM cells adherent to BMSCs, MM.1S and RPMI8226 cells were cultured for 24h in BMSC coated 96-well plates, in the presence or absence of tubacin and/or bortezomib. After treatment, DNA synthesis was measured by [^{3}H]-thymidine (Perkin Elmer, Boston MA) uptake, as previously described (44). All experiments were performed in quadruplicate.

Statistical analysis.

Statistical significance of differences observed in drug-treated versus control cultures was determined using the Wilcoxon signed-ranks test. The minimal level of significance was $p < 0.05$. The interaction between tubacin and bortezomib was analyzed by isobologram analysis using the CalcuSyn software program (Biosoft, Ferguson, MO) to determine whether the combination was additive or synergistic, as previously described (45).

EXAMPLE 1

Tubacin specifically induces acetylation of α-tubulin in MM cell lines.

The baseline expression of HDAC6 was examined in several MM cell lines. Although MM.1S, U266, INA-6, RPMI8226, and RPMI-LR5 MM cell lines constitutively express HDAC6, only low levels of HDAC6 are evident in RPMI-Dox-40 cells (Fig. 1A). Since tubacin induces acetylation of α-tubulin in A549 human lung cancer cell line by specific inhibition of HDAC6 activity (40), the effect of tubacin was examined on acetylation of α-tubulin in MM.1S and RPMI8226 MM cells. As shown in Fig. 1B, tubacin significantly induces aceylation of α-tubulin in a dose-dependent fashion in both MM.1S and RPMI8226 cells, without alteration of protein expression; importantly, no other acetylated proteins were recognized by Western blotting. Similar results were observed in INA-6 and RPMI-Dox40 cells (data not shown). The dose-dependent effect of tubacin was assessed, and show that tubacin (5 μM) induces peak acetylation of α-tubulin in RPMI8226 cells at 12h (Fig. 1C). Importantly, expression of HDAC6 is not altered by tubacin treatment (data not shown).

Histone acetylation has been associated with development of malignancies (46, 47); conversely, inhibitors of histone deacetylase represent a promising new treatment strategy

(48). It has been demonstrated that both second generation of a hybrid polar compound SAHA (49) and novel hydroxamic acid derivative NVP-LAQ824 (50) mediate anti-MM activity. Since these agents non-selectively inhibit HDACs, the effect of SAHA on acetylation of lysine in MM.1S and RPMI8226 cells was also examined. In contrast to tubacin, which specifically induces acetylation of α-tubulin (Fig. 1B, 1C), SAHA triggers more potent acetylation of lysine on histone H3 and H4 than α-tubulin (Fig. 1D). These results demonstrate that HDAC6 is constitutively expressed in MM cell lines and that tubacin specifically induces acetylation of α-tubulin, confirming the specific inhibitory effect of tubacin on HDAC6 activity in MM cells.

EXAMPLE 2

Tubacin inhibits MM cell growth.

Based upon the specific inhibitory effect of tubacin on HDAC6, next examined was the cytotoxicity of tubacin against drug-sensitive (MM.1S, U266, INA-6, and RPMI8226) and -resistant (RPMI-LR5 and RPMI-Dox40) MM cell lines. These cells were treated with tubacin (1.25-20 μM) for 48h (Fig. 2A) and 72h (Fig. 2B), and cytotoxicity was assessed by MTT assay, as describe. Tubacin significantly inhibits both drug-sensitive and –resistant MM cell growth, with IC_{50} 5-20 μM at 72h. The most sensitive and resistant cell lines are RPMI8226 and MM.1R cells, respectively (Fig. 2B). Importantly, no cytotoxicity in PBMCs is induced by tubacin (Fig. 2C). These results indicate that tubacin sensitivity is independent of resistance to conventional chemotherapeutic agents (dexamethasone, melphalan and doxorubicin) and suggest a favorable therapeutic index in tumor cells versus normal cells. It was shown that HDAC inhibitors SAHA and NVP-LAQ824 trigger MM cell death via caspase-dependent apoptosis, it was studied whether tubacin-induced cytotoxicity is also mediated via apoptosis. In MM.1S and RPMI8226 cells treated with tubacin (10 μM) for 0-24h, time-dependent caspase-8/PARP cleavage is induced (Fig. 2D), confirming our MTT results. These data strongly suggest that tubacin-induced cytotoxicity in MM cells is mediated via caspase-dependent apoptosis.

EXAMPLE 3

Tubacin inhibits interaction of HDAC6 with dynein; when combined with bortezomib, it induces accumulation of ubiquitinated proteins.

To overcome bortezomib resistance in MM, novel therapeutic options are urgently needed. Based upon our preclinical studies showing that bortezomib inhibits DNA repair (29, 31), combined treatment of bortezomib has been shown to sensitize or overcome resistance to DNA damaging agents (ie, melphalan and doxorubicin) (29). It has also been shown that hsp-27 expression is associated with bortezomib resistance (30, 51); conversely, p38MAPK inhibitors can downregulate hsp-27 in bortezomib resistant MM cell lines and patient cells, and overcome bortezomib resistance. Recent studies have demonstrated that polyubiquitinated proteins are degraded via both proteasome and aggresome pathways (Fig. 3A).

HDAC6 constitutively binds both polyubiquitinated misfolded proteins and dynein, thereby recruiting misfolded protein cargo to dynein motors for transport to aggresomes along microtubules (39). It was examined whether inhibition of HDAC6 activity by tubacin alters the interaction of HDAC6 with Ub and/or dynein. HDAC6 is consistently co-immunoprecipitated with polyubiquitinated proteins in MM.1S cells (Fig. 3B) and dynein (Fig. 3C). After treatment with tubacin (2.5 μM and 5μM for 8h), co-immunoprecipitation of HDAC6 with dynein is markedly inhibited in a dose-dependent fashion, whereas co-immunoprecipitation between HDAC6 and ubiquitinated proteins is unaffected (Fig. 3B). Next examined was the impact of tubacin on polyubiquitination of proteins. As expected, polyubiquitinated proteins significantly accumulate in tubacin-treated RPMI8226 cells; however, no significant change was recognized in treated MM.1S cells (Fig. 3D), suggesting compensatory proteasomal degradation of polyubiquitinated proteins. These results also indicate that degradation of polyubiquitinqted protein in RPMI8226 cells is more dependent on aggresomes than proteasomes, consistent with MTT data demonstrating that RPMI8228 cells are more sensitive to tubacin than MM.1S cells (Fig. 2A, 2B). Importantly, combined tubacin (5 μM) and bortezomib (5 nM) dramatically augments accumulation of polyubiquitinated proteins in both MM.1S and RPMI8226 cells, compared to either agent alone (Fig. 3E). These results further indicate that degradation of polyubiquitinated occurs in both proteasomes and aggresomes; therefore, inhibiting both pathways induces significant accumulation of polyubiquitinated proteins in MM cells.

EXAMPLE 4

Synergistic anti-MM activity of tubacin with bortezomib is mediated via JNK-caspase activation.

Having shown significant accumulation of polyubiquitinated proteins after combined treatment with tubacin and bortezomib, examined was whether combination treatment could also induce significant cytotoxicity in MM cells. As expected, tubacin synergistically enhances bortezomib-induced cytotoxicity in both MM.1S and RPMI8226 cells. For example, 5 nM and 10 nM bortezomib trigger 26% and 66% RPMI8226 cell death, respectively, which is increased to 87% and 91%, respectively, when combined with 5μM tubacin (Fig. 4A). To analyze the mechanism whereby this combination treatment mediates synergistic anti-MM toxicity, next performed cell cycle profiling in MM.1S cells. Tubacin (5 μM) alone does not alter cell cycle profile, whereas bortezomib (5 nM) alone triggers increased (14.2% to 39.5%) G2M phase MM.1S cells, as in our previous studies (4); importantly, the combination of tubacin and bortezomib triggers significantly increased (5.6% to 30 4%) sub-G0/G1 phase cells, suggesting that combination treatment triggers apoptotic cell death (Fig. 4B). Further examined was expression of $p21^{Cip1}$. Consistent to cell cycle profile, tubacin does not trigger induction of $p21^{Cip1}$. Importantly, tubacin inhibits induction of $p21^{Cip1}$ induced by bortezomib (Fig. 4C).

Since accumulation of polyubiquitinated proteins induces a cell stress response, next examined whether this combination treatment of MM.1S cells triggers activation of JNK (also known as stress-activated protein kinase), a hallmark of cell stress response, followed by caspase cleavage, as described in our previous studies (9, 22). Tubacin alone does not trigger phosphorylation of JNK or caspase/PRAP cleavage, and bortezomib alone induces only modest phosphorylation of JNK as well as caspase-9, -8, -3 and PARP cleavage (Fig. 4C). Of great interest, combined tubacin and bortezomib treatment markedly augments both JNK phosphorylation and caspase/PARP cleavage in MM.1S cells (Fig. 4C), consistent with cytotoxicity assays (Fig. 4A). Other cell stress response-related proteins (ie, hsp-70 and Grp78) are also induced by this combination treatment (data not shown). These results indicate that tubacin inhibits the G2 phase arrest induced by bortezomib, thereby facilitating apoptosis mediated via stress-induced JNK activation, followed by caspase/PARP cleavage.

To identify the specific role of HDAC6 inhibition mediating synergistic MM cell cytotoxicity with bortezomib, MM.1S cells were transiently transfected with HDAC6 siRNA, as previously described. HDAC6 protein expression is significantly downregulated by transfection (Fig. 4D); importantly, bortezomib significantly increases cytotoxicity in transfectants in a dose-dependent fashion (Fig. 4E). In contrast niltubacin, an inactive carboxylic acid tubacin analog, does not affect either acetylation of α-tubulin (Fig. 4F) or

enhance cytotoxicity in MM.1S cells induced by bortezomib (Fig. 4G). Similar results were observed in RPMI8226 cells (data not shown). These results show that inhibition of HDAC6 specifically augments bortezomib-induced cytotoxicity in MM.

EXAMPLE 5

Tubacin combined with bortezomib demonstrates significant cytotoxicity in MM patient plasma cells.

Having show significant cytotoxicity of combined treatment of tubacin and bortezomib in MM cell lines, further examined was the effect of the combination in isolated CD138-positive MM patient plasma cells from BM (BMPCs). These BMPCs were cultured for 24h with or without tubacin (5 μM), in the presence or absence of bortezomib (5 nM and 10 nM). Consistent with MM cell line data, cytotoxicity in BMPCs induced by bortezomib is markedly augmented by tubacin (Fig. 5A, 5B, and 5C); importantly, no toxicity is recognized in normal PBMCs similarly treated (Fig. 5D).

We next examined the mechanism whereby combined tubacin with bortezomib specifically induces cytotoxicity in MM patient plasma cells, but not in PBMCs. Both PBMCs and BMPCs obtained from the same MM patient were treated for 12h with tubacin (5 μM). Constitutive expression of HDAC6 is relatively higher in BMPCs than PBMC; importantly, acetylation of α-tubulin is markedly enhanced by tubacin in BMPCs, but not in PBMCs (Fig. 5E). Ongoing studies are further delineating the molecular mechanisms of this observation.

EXAMPLE 6

Tubacin combined with bortezomib inhibits paracrine MM cell growth.

We have previously shown that the BM microenvironment confers cell growth and drug resistance in MM cells (3, 30, 31), and next studied was the functional sequelae of HDAC6 inhibition in the presence or absence of bortezomib, in MM cells within the BM milieu. MM.1S and RPMI8226 cells were cultured with or without BMSCs, in the presence or absence of tubacin (2.5 and 5μM) and/or bortezomib (2.5-10 nM). MM cell adherence to BMSCs triggers increased [^{3}H]-thymidine uptake of both MM.1S cells (1.75, $p < 0.01$) (Fig. 6A) and RPMI8226 cells (2.0 fold, $p < 0.01$) (Fig. 6B). Either tubacin or bortezomib alone inhibits BMSC-induced [^{3}H]-thymidine uptake in a dose-dependent fashion ($p < 0.01$).

Importantly, tubacin significantly enhances bortezomib-induced inhibition of [^{3}H]-thymidine uptake in adherent MM.1S (Fig. 6A) and RPMI8226 (Fig. 6B) cells. The viability of BMSCs, assessed by MTT assay, is not altered by combination treatment (data not shown). These data indicate that combined treatment of tubacin with bortezomib triggers synergistic selective anti-tumor activity against MM cells in the BM milieu, thereby overcoming cell adhesion mediated resistance to conventional therapies. In conclusion, these results strongly suggest that dual inhibition of the aggresome and proteasome with tubacin and bortezomib, respectively, synergistically enhances MM cytotoxicity. They provide the framework for clinical trials designed to enhance sensitivity and overcome resistance to bortezomib, thereby improving patient outcome in MM.

Tubacin selectively inhibits the carboxy-terminal domain of HDAC6, causing tubulin hyperacetylation. Notably, tubacin was previously reported to have no effect on histone acetylation, the transcriptional profile, cell cycle, or viability of A549 lung cancer cells.

Tubacin is cytotoxic to myeloma cell lines at concentrations required to exert an effect on tubulin acetylation (Figure 1a, b). Synergy is observed between tubacin and the potent proteasome inhibitor, bortezomib (Velcade), (Figure 1c). Remarkably, these molecules in combination have no effect on the viability of peripheral blood mononuclear cells (Figure 1d). These data suggest a plausible cytotoxic consequence of inhibited protein catabolism warranting further investigation. Preliminary data obtained in the RPMI-8226 cell line demonstrate an increase in cellular ubiquitinated proteins with tubacin (Figure 2a). Previous experiments with tubacin at comparable concentrations in rat astrocytes and striatal neurons have elicited features suggestive of Russell body formation (Figure 2b). These data support the hypothesis that HDAC6-mediated aggresome formation is mediated by the carboxy-terminus deacetylase domain and ascribe a mechanism for tubacin cytotoxicity.

Screening methods of the invention include a quantitative, high-throughput, image-based screen of cancer cells treated in 384-well plate format with a small molecule library biased for HDAC inhibition. Tubulin and histone acetylation state-specific antibodies were used and recognized by corresponding fluorescent secondary antibodies. Wells were scored in an unbiased fashion on an automated Axon 5000A epifluorescence microscope, for the ability of cause potent, selective tubulin acetylation. Controls of trichostatin, tubacin and DMSO were used for reference. Molecules have subsequently been prioritized following assessment for direct cytotoxicity and synergy with bortezomib in the RPMI-8226 myeloma cell line.

Tubacin is the first domain-selective HDAC inhibitor, targeting HDAC6. At low micromolar concentrations, tubacin causes tubulin hyperacetylation and marked anti-proliferative, proapoptotic effects against every myeloma cell lines. In addition, tubacin potently sensitizes myeloma cells to subtoxic concentrations of bortezomib. No adverse effect of tubacin on peripheral blood mononuclear cells was noted, with or without bortezomib. Remarkably, the robust cytotoxic effect of tubacin was maintained in all cell lines in the presence of bone marrow stroma, and interleukin-6.

EXAMPLE 7

Characterization of the role of the aggresome in the protein catabolism of malignant plasma cells.

Biochemical purification of the aggresome. The aggresome will be isolated biochemically in a panel of classical human cellular models of multiple myeloma. The presence of proteasome subunits in the pericentriolar microtubule organizing complex (MTOC) is determined as discussed below. The expression of HDAC6 in each of these cell lines has already been validated by immunoblotting. Preparation of the centrosomes will be performed as described[20]. Briefly, cells are harvested and treated with cytochalasin D and nocodazole to depolymerize the cytoskeleton and microtubules. After cell lysis and nuclear pelleting, centrosomes are purified by a sucrose gradient and assessed for 20S proteasome content. The effect of proteasome inhibition and protein folding stress on the proteosomal content of the purified aggresome will also be determined.

Fluorescence microscopic detection of the aggresome: With aggresome formation, intermediate filament reorganization occurs with the formation of a vimentin cap at the centrosome. Aggresome formation is assessed by fluorescence microscopy using antibodies against HDAC6, hsp70 and vimentin. Examination of myeloma cell lines treated with proteasome inhibitors and molecules causing misfolded protein stress will determine if aggresome formation is augmented, as reported previously.

Aggresome formation in patient-derived myeloma cells: The role of the aggresome in protein catabolism in myeloma cells is determined *in vivo*, the effects of proteasome and HDAC6 inhibition on aggresome formation is assessed in patient-derived myeloma cells. Using an immunofluorescence approach, the structure and composition of the aggresome is characterized as above. The cellular reservoirs of monotypic are examined by

immunoglobulins using fluorescence microscopy. The study of bortezomib-sensitive and resistant patient-derived cells will be examined, using peripheral blood mononuclear cell as controls. This analysis may establish the aggresome as a proteolytic hallmark of cancer cell survival, and a determinant of clinical resistance to proteasome inhibition.

EXAMPLE 8

Determine the mechanism of HDAC6-mediated aggresome formation.

Structure-function analysis of HDAC6: Characterization of the enzymatic domain relevant for aggresome formation is done by both a genetic and chemical genetic approach using the classical ubiquitinated-protein model (DF508 CFTR)[3] of aggresome formation. One method will be to knock-down wild-type HDAC6 and transfected cells with an HDAC6 protein mutated in both amino- and carboxy-terminal deacetylase domains[9]. Another approach is to knock-down HDAC6 with small, interfering RNA, preferably using separately transfected selective mutants of each enzymatic domain.

Chemical genetic analysis of HDAC6: In DF508 CFTR over-expressing cells, the effect of tubacin on ubiquitinated protein stress and aggresome formation is assessed with appropriate trichostatin and trapoxin controls. Trichostatin is a potent inhibitor of both HDAC6 domains, whereas trapoxin is a potent HDAC inhibitor, which uniquely does not inhibit HDAC6.

Proteomic analysis of protein targets of HDAC6: The non-histone targets of HDAC6 is determined. Two approaches grounded in chemical genetics and mass spectrometry are used. Adherent cells are treated with tubacin for 24 hours and lysed on culture dishes without disturbing nuclei or microtubules, enriching for cytoplasmic protein targets. An antibody-based purification detection scheme is employed using the acetylated-lysine rabbit polyclonal antibody, which is known to bind numerous cellular proteins with modified lysine residues. Selectively acetylated targets will be determined by mass spectrometry. Cytoplasmic lysates from tubacin-treated cells are prepared as above, and reacted with succinimide, covalently modifying non-acetylated lysine residues. The lysates are treated with purified HDAC6-H216A, which possesses only a functional carboxy-terminal deacetylase domain. The newly deacetylated lysines are then covalently modified with succinimidyl-biotin for streptavidin purification and mass spectrometric detection.

EXAMPLE 9

Identification of potent, selective inhibitors of HDAC6 enabling the investigation of protein catabolism and aggresome formation in mouse models of multiple myeloma.

Molecules derived from diversity-oriented synthetic pathways are used in *in silico* structure-activity relationship modeling to ascertain modular determinants of selectivity, potency and cytotoxicity.

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The contents of all references, patents, and patent applications cited herein are hereby incorporated by reference in their entirety. The contents of Appendix A (25 pages), attached hereto, are hereby incorporated by reference in their entirety.

Other embodiments are within the following claims.

Claims

What is claimed is:

1. Use of a proteasome inhibitor and an aggresome inhibitor for the manufacture of a medicament for treating a subject suffering from or susceptible to a protein degradation disorder, wherein the aggresome inhibitor selectively inhibits HDAC6.

2. The use of claim 1, wherein the protein degradation disorder is a cellular proliferation disorder or a protein deposition disorder.

3. The use of claim 2, wherein the cellular proliferation disorder is cancer.

4. The use of claim 3, wherein the cancer is one or more of multiple myeloma, leukemia, lymphoma, breast cancer, lung cancer and liver cancer.

5. The use of claim 3, wherein the cancer is multiple myeloma.

6. The use of claim 2, wherein the protein deposition disorder is Wilson's disease, spinocerebellar ataxia, prion disease, Parkinson's disease, Huntington's disease, Familial amytrophic lateral sclerosis, amyloidosis, Alzheimer's disease, Alexander's disease, alcoholic liver disease, cystic fibrosis, Pick's Disease or Lewy body dementia.

7. The use of claim 1, wherein the proteasome inhibitor is bortezomib.

8. Use of a proteasome inhibitor and an aggresome inhibitor for the manufacture of a medicament for treating a cell exhibiting symptoms of a protein degradation disorder, wherein the aggresome inhibitor selectively inhibits HDAC6.

9. The use of claim 8, wherein the cell is one or more of a cell from a subject or a cultured cell.

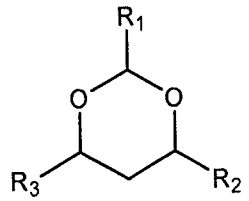
10. The use of claim 9, wherein the cell from a subject is one or more of bone marrow stromal cell, (BMSC), a peripheral blood mononuclear cell (PBMC), lymphocytes, hair follicles, blood cells, other epithelial cells, bone marrow plasma cells, primary cancer cells, patient derived tumor cells, normal or cancerous hematopoietic stem cells, neural stem cells, solid tumor cells, or astrocytes.

11. The use of claim 9, wherein the cultured cell is one or more of MM.1S, U266, RPMI8226, DOX40, MM. IR, INA-6, LR5, primary and established cancer cell lines, primary and established normal cell lines.

12. Use of a proteasome inhibitor and an aggresome inhibitor for the manufacture of a medicament for treating a subject suffering from or susceptible to multiple myeloma, wherein the aggresome inhibitor selectively inhibits HDAC6.

13. The use of any one of claims 1-12, wherein the proteasome or aggresome inhibitor is selected from the group consisting of tubacin, bortezomib, Rl 15777 FTI, ^{166}Holminun-DOTMP, arsenic trioxide, 17-AAG, MGl 32, sapojargon, and NPI-0052.

14. The use of any one of claims 1-12, wherein the aggresome inhibitor is of the formula:



wherein

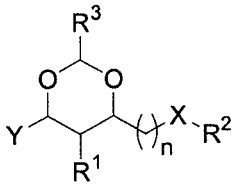
R_1 is cyclic or acyclic, substituted or unsubstituted, branched or unbranched aliphatic; cyclic or acyclic, substituted or unsubstituted, branched or unbranched heteroaliphatic; substituted or unsubstituted, branched or unbranched acyl; substituted or unsubstituted, branched or unbranched aryl; substituted or unsubstituted, branched or unbranched heteroaryl; $-OR_A$; $-C(=O)R_A$; $-CO_2R_A$; $-SR_A$; $-SOR_A$; $-SO_2R_A$; $-N(R_A)_2$;

-NHC(O)R_A; or -C(R_A)$_3$; wherein each occurrence of R_B is independently a hydrogen, a protecting group, an aliphatic moiety, a heteroaliphatic moiety, an acyl moiety; an aryl moiety; a heteroaryl moiety; alkoxy; aryloxy; alkylthio; arylthio; amino, alkylamino, dialkylamino, heteroaryloxy; or heteroarylthio moiety;

R_2 is hydrogen; halogen; cyclic or acyclic, substituted or unsubstituted, branched or unbranched aliphatic; cyclic or acyclic, substituted or unsubstituted, branched or unbranched heteroaliphatic; substituted or unsubstituted, branched or unbranched acyl; substituted or unsubstitued, branched or unbranched aryl; substituted or unsubstituted, branched or unbranched heteroaryl; -OR_B; -C(=O)R_B; -CO_2R_B; -CN; -SCN; -SR_B; -SOR_B; -SO_2R_B; -NO_2; -N(R_B)$_2$; -NHC(O)R_B; or -C(R_B)$_3$; wherein each occurrence of R_B is independently a hydrogen, a protecting group, an aliphatic moiety, a heteroaliphatic moiety, an acyl moiety; an aryl moiety; a heteroaryl moiety; alkoxy; aryloxy; alkylthio; arylthio; amino, alkylamino, dialkylamino, heteroaryloxy; or heteroarylthio moiety; and

R_3 is hydrogen; halogen; cyclic or acyclic, substituted or unsubstituted, branched or unbranched aliphatic; cyclic or acyclic, substituted or unsubstituted, branched or unbranched heteroaliphatic; substituted or unsubstituted, branched or unbranched acyl; substituted or unsubstitued, branched or unbranched aryl; substituted or unsubstituted, branched or unbranched heteroaryl; -OR_C; -C(=O)R_C; -CO_2Rc; -CN; -SCN; -SR_C; -SOR_C; -SO_2R_C; -NO_2; -N(R_C)$_2$; -NHC(O)R_C; or -C(R_C)$_3$; wherein each occurrence of R_C is independently a hydrogen, a protecting group, an aliphatic moiety, a heteroaliphatic moiety, an acyl moiety; an aryl moiety; a heteroaryl moiety; alkoxy; aryloxy; alkylthio; arylthio; amino, alkylamino, dialkylamino, heteroaryloxy; or heteroarylthio moiety; and pharmaceutically acceptable salts and derivatives thereof.

15. The use of any one of claims 1-12, wherein the aggresome inhibitor is of the formula:



and pharmaceutically acceptable derivatives thereof;

wherein R^1 is hydrogen, or an aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic or heteroaromatic moiety;

n is 1-5;

R^2 is hydrogen, a protecting group, or an aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic or heteroaromatic moiety;

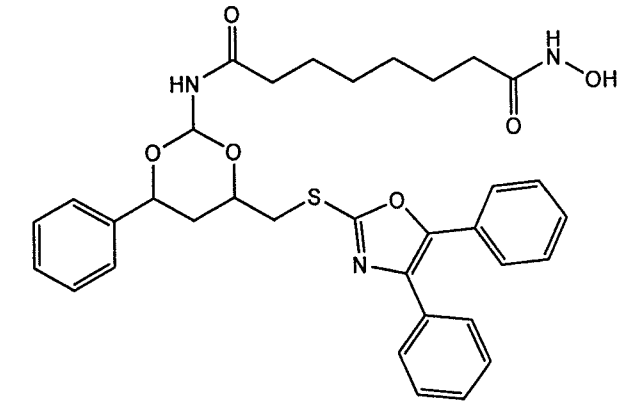
X is -O-, $-C(R^{2A})_2-$, -S-, or $-N\ R^{2A}-$, wherein R^{2A} is hydrogen, a protecting group, or an aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic or heteroaromatic moiety;

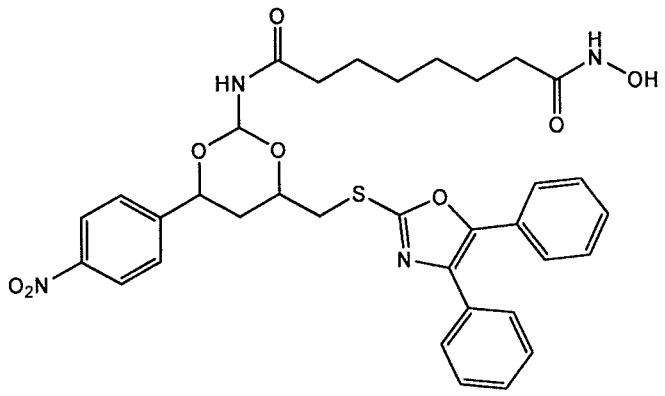
or wherein two or more occurrences of R^2 and R^{2A}, taken together, form an alicyclic or heterocyclic moiety, or an aryl or heteroaryl moiety;

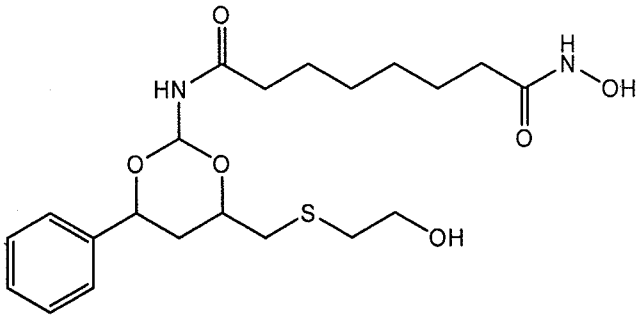
R^3 is an aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic or heteroaromatic moiety; and

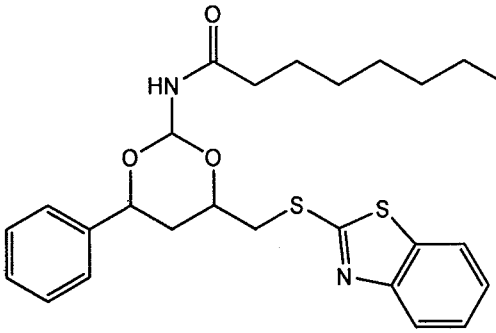
Y is hydrogen or an aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic or heteroaromatic moiety,

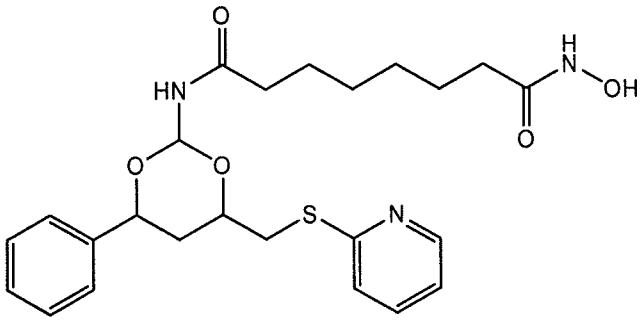
16. The use of any one of claims 1-12, wherein the aggresome inhibitor is one of the formulae:

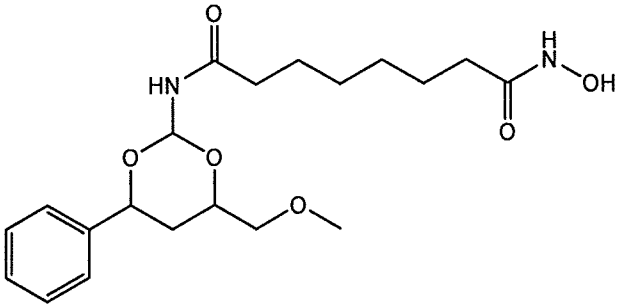


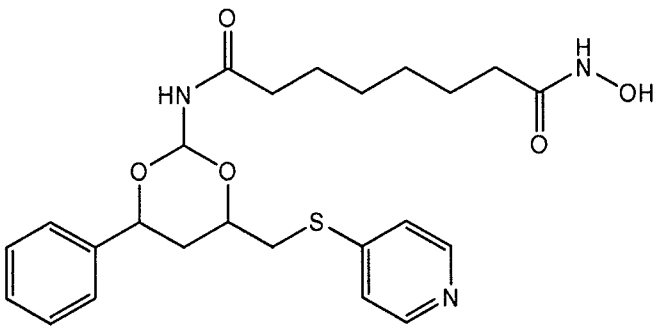




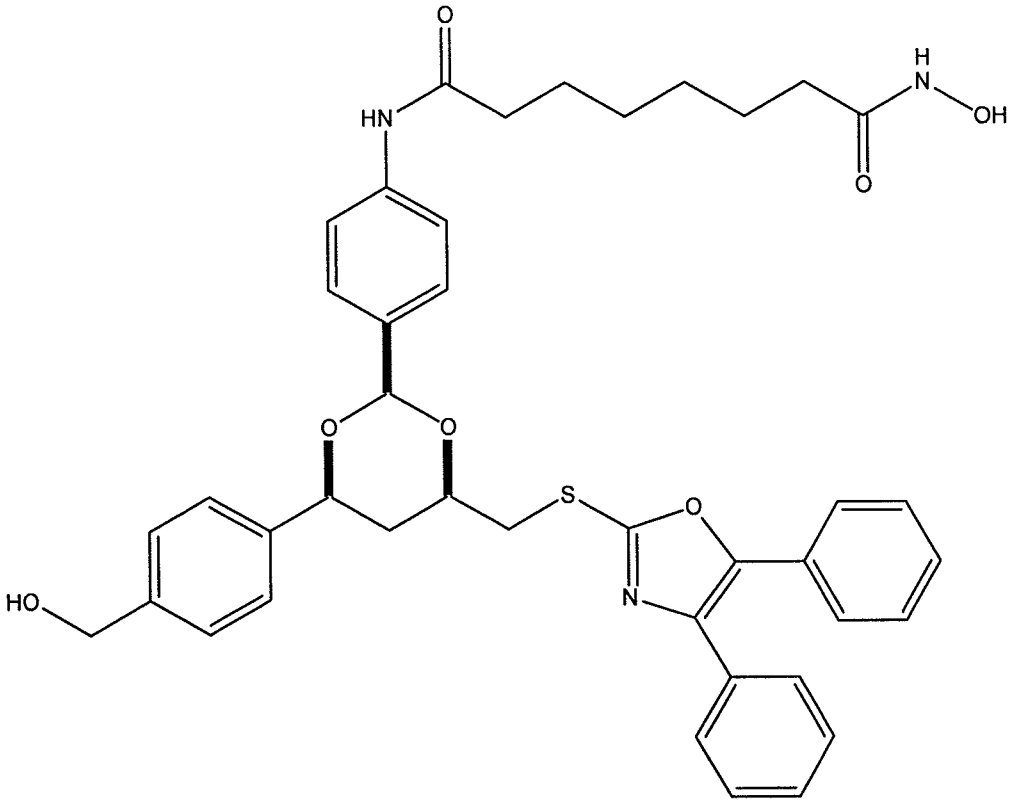




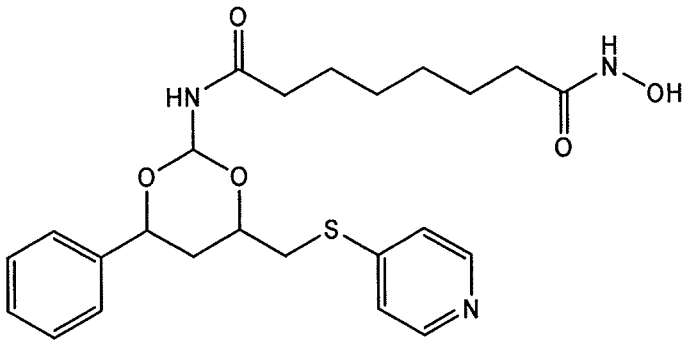




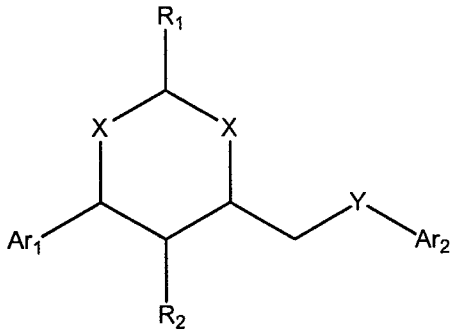
17. The use of any one of claims 1-12, wherein the aggresome inhibitor is a compound of formula:



18. The use of any one of claims 1-12, wherein the aggresome inhibitor is of the formula:



19. The use of any one of claims 1-12, wherein the aggresome inhibitor is a compound of formula:



wherein,

each X is independently O, S, CH_2, or NR_3;

Y is O, S, CH_2, or NR_4;

Ar_1 and Ar_2 are each independently an aryl group;

R_1 is a lower alkyl group or an aryl group;

R_2 is hydrogen, a lower alkyl group or an aryl group; and

R_3 is hydrogen, a lower alkyl group, an aryl group, an alkylcarbonyl, an alkoxycarbonyl group, or an aminocarbonyl group.

20. The use of any one of claims 1-12,

wherein,

X is for both occurrences O;

Y is S;

Ar_1 is phenyl or substituted phenyl;

Ar_2 is heteroaryl, more preferably optionally substituted oxazolyl;

R_1 is phenyl or substituted phenyl, more preferably 4-aminosubstituted phenyl;

R_2 is hydrogen.

21. The use of any one of claims 1-12, wherein, the aggresome inhibitor inhibits the C-terminal aceylation activity of HDAC6, thereby inhibiting aggresome mediated protein degradation.

22. The use of any one of claims 1-12, wherein the proteasome inhibitor is one or more of bortezomib, MGl32, sapojargon, or NPI-0052.

23. The use of any one of claims 1-12, wherein the aggresome inhibitor is a peptide derived from HDAC6, dyenin, an N-terminal peptide of HDAC6 or C-terminal peptide of HDAC6.

24. The use of claim 23, wherein the C-terminal HDAC6 peptide is sufficient to modulate a phenotype of a cell.

25. The use of claim 24, wherein the phenotype is biological or clinical sequelae in response to a particular treatment or compound, anemia, thrombocytopenia, neutropenia, osteolytic lesions, bone pain, immunodeficiency, renal insufficiency, hypercalcemia, aneuploidy of mature plasma cells, percentage of malignant cells, acetylation state of tubulin, apoptosis of mature plasma cells, level of aggresomes in mature plasma cells, HDAC6 ubiquitination in mature plasma cells, HDAC6 association with dynein in mature plasma cells, cellular levels of ubiquintinated proteins in mature plasma cells, level of caspase-8 in mature plasma cells, level of PARP in mature plasma cells, thymidine uptake in mature plasma cells, dilated ER cisternae, aggregation of mature plasma cells, deposits of immunoglobulins in mature plasma cells, acetylation state of non-histone proteins, global ubiquitination state of the cellular proteins, state of cell cycle regulation, necrosis, markers of apoptosis, apoptosis state, Russell body formation, Cystic Fibrosis transmembrane protein receptor state, and modulation of cellular protein deposits, or global acetylation state of cellular or extracellular proteins.

26. The use of claim 25, wherein a decrease in one or more of level of aggresomes, thrombocytopenia, neutropenia, osteolytic lesions, bone pain, immunodeficiency, renal insufficiency, hypercalcemia, aneuploidy of mature plasma cells, percentage of malignant cells, thymidine uptake in mature plasma cells, level of full length caspase-8 in mature plasma cells, level of full length PARP in mature plasma cells, or aggregation of mature plasma cells, indicates that the treatment is efficacious.

27. The use of claim 25, wherein an increase in acetylation state of tubulin, HDAC6 ubiquitination in mature plasma cells, level of cleaved form of caspase-8, level of cleaved

form of PARP, necrosis, acetylation state of non-histone proteins, cellular ubiquitination levels, apoptosis, markers of apoptosis, cell cycle deregulation, or deposits of immunoglobulins in mature plasma cells indicates that the treatment is efficacious.

28. The use of any one of claims 1-12, further comprising obtaining a biological sample from a subject.

29. The use of any one of claims 1-12,, further comprising determining the subject's phenotype after a second period of treatment with the proteasome or aggresome inhibitor.

30. The use of any one of claims 1-12, further comprising obtaining a second biological sample from the subject.

31. The use of any one of claims 1-12, further comprising administering a therapeutically effective amount of one or more additional protein degradation inhibitors to the subject or cell.

32. The use of claim 31, wherein at least one of the additional protein degradation inhibitors is an aggresome inhibitor.

33. The use of claim 31, wherein at least one of the additional protein degradation inhibitors is a proteasome inhibitor.

34. The use of claim 31, wherein the additional protein degradation inhibitor is one or more of bortezomib, tubacin, MG132, sapojargon, or NPI-0052.

35. The use of any one of claims 1-12, further comprising monitoring the treatment or progress of the cell or subject.

36. The use of any of claims 1-12, further comprising obtaining the protein degradation inhibitor.

37. The use of any one of claims 1-12, further comprising co-administering one or more of a chemotherapeutic agent, radiation agent, hormonal agent, biological agent or an anti-inflammatory agent to the subject.

38. The use of claim 37, wherein the chemotherapeutic agent is tamoxifen, trastuzamab, raloxifene, doxorubicin, fluorouracil/5-fu, pamidronate disodium, anastrozole, exemestane, cyclophosphamide, epirubicin, letrozole, toremifene, fulvestrant, fluoxymester- one, trastuzumab, methotrexate, megastrol acetate, docetaxel, paclitaxel, testolactone, aziridine, vinblastine, capecitabine, goselerin acetate, zoledronic acid, taxol, vinblastine, or vincristine.

39. The use of any one of claim 1-12, further comprising comparing one or more of the pre-treatment or post-treatment phenotypes to a standard phenotype.

40. The use of claim 39, wherein the standard phenotype is the corresponding phenotype in a reference cell or population of cells.

41. The use of claim 40, wherein the reference cell is one or more of the following, cells from the subject, cultured cells, cultured cells from the subject, or cells from the subject pre-treatment.

42. The use of claim 41, wherein the cells from the subject are bone marrow stromal cell, (BMSC), a peripheral blood mononuclear cell (PBMC), lymphocytes, hair follicles, blood cells, other epithelial cells, bone marrow plasma cells, primary cancer cells, patient derived tumor cells, normal or cancerous hematopoietic stem cells, neural stem cells, solid tumor cells, or astrocytes.

43. The use of any one of claims 1-42, wherein the subject or the cell is a human.