

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
23 February 2006 (23.02.2006)

PCT

(10) International Publication Number
WO 2006/020681 A2

(51) International Patent Classification:

A61K 31/5383 (2006.01) A61K 31/506 (2006.01)
A61K 31/53 (2006.01)

(21) International Application Number:

PCT/US2005/028358

(22) International Filing Date: 10 August 2005 (10.08.2005)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

10/914,848 10 August 2004 (10.08.2004) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: COMPOUNDS AND METHODS FOR THE TREATMENT OF UBIQUITIN CONJUGATING DISORDERS



(57) Abstract: The present invention provides methods for identifying compounds that selectively bind one or more active sites within an ubiquitin conjugating enzyme. The compounds identified by the methods are useful in the treatment of disorders attributed to dysregulated ubiquitin conjugating enzyme function, specifically in hyperproliferative disorders.



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COMPOUNDS AND METHODS FOR THE
TREATMENT OF UBIQUITIN CONJUGATING DISORDERS

RELATED APPLICATION

This application claims the benefit of U.S. Application No. 10/914,848, filed on
5 August 10, 2004. The entire teachings of the above application is incorporated herein by
reference.

GOVERNMENT SUPPORT

The invention was supported, in whole or in part by a grant NIH R01-GM59467 from
the National Institutes of Health. The Government has certain rights in the invention.

10 BACKGROUND

The ubiquitin system is the major pathway for the controlled degradation of
intracellular proteins in eukaryotic cells. The ubiquitin system regulates the half-life of
cellular proteins including, cyclins, cyclin dependent kinase inhibitors, histones, oncoproteins
and tumor suppressors and is an important regulatory component of cell cycle progression,
15 endocytosis, receptor regulation, transcription, signal transduction, oncogenesis, apoptosis
and antigen presentation (Hershko et al., *J Biol. Chem.*, 269:4940-46). Abnormalities in the
ubiquitin pathway are implicated in many pathological conditions including cancer (Johnston,
1999).

In the ubiquitination pathway targeted proteins are marked with a chain of four or more
20 ubiquitins that are covalently attached through the formation of an isopeptide bond between
the C-terminal glycyl residue of ubiquitin and a specific lysl residue in the substrate protein.
The chain of ubiquitins mark the protein for degradation by a multi-subunit ATP-dependent
protease known as the 26S proteasome enzyme complex. The degradation process is
catalyzed in a sequential reaction involving three enzymes: E1 a ubiquitin activating enzyme,
25 E2 an ubiquitin-conjugating enzyme, and E3 an ubiquitin-protein ligase enzyme. E1, in an
ATP dependent process, forms a thiol ester bond with the C-terminus of ubiquitin. The
activated ubiquitin is then transferred to a catalytic cysteine on one of several E2 enzymes.
The E2 then donates the ubiquitin directly to the protein substrate or by association with an
E3 protein (ubiquitin ligase; in most cases a polyprotein complex). After ligation of the first
30 ubiquitin to the substrate protein, further ubiquitins are usually targeted to the first ubiquitin

to form multi-ubiquitin chains. The 26S proteasome then catalyzes the degradation of the ubiquitin tagged proteins. Disruptions in the regulation of protein degradation by the ubiquitin pathway can significantly impact pathways in which correct protein concentration and half-life are essential, e.g., cell cycle.

5 Thus, defects in the ubiquitin pathway are likely to cause excessive proliferation and transformation in cells and thus inhibitors of this pathway are needed. However, because the ubiquitin pathway plays an essential role in many biological pathways, non-selective compounds are likely to be toxic. Thus, targeting specific ubiquitin enzymes may overcome the obstacle of toxicity. An attractive target in this pathway are the E2, ubiquitin conjugating
10 enzymes. However, there are currently no compounds that selectively target E2. Thus there is a need in the art to develop safe and effective compounds that selectively inhibit ubiquitin conjugating enzymes or E2s.

SUMMARY OF THE INVENTION

 The present invention to provide methods for identifying compounds that selectively
15 bind one or more domains of the active sites within an ubiquitin conjugating enzyme and inhibit its activity. These compounds of the invention are useful in the treatment of ubiquitin conjugation associated disorders, particularly hyperproliferative disorders.

 In one aspect, the present invention provides a method for inhibiting an ubiquitin conjugating enzyme with an organic compound with a molecular weight of less than about
20 2000 daltons, the method comprising selectively targeting to one or more active sites of the ubiquitin conjugating enzyme with the compounds. Preferably, the molecular weights of the organic compounds of the invention are less than about 2000 daltons, and more preferably, less than about 500 daltons. In one embodiment of the invention, the selectively targeted active site is within the catalytic site.

25 In another embodiment, the organic compound binds to a binding domain in an E1 binding site on the ubiquitin conjugating enzyme. In another embodiment, the ubiquitin conjugating enzyme is Ubc1, Ubc2, Ubc3, Ubc4, Ubc5, Ubc6, Ubc7, Ubc8, Ubc10, Ubc13. In a further embodiment, the ubiquitin conjugating enzyme is a yeast ubiquitin conjugating enzyme or a mouse, rat or human homolog.

In different embodiments of the invention, the compounds of the invention selectively target one or more active sites of the ubiquitin conjugating enzyme, including the following: amino acid residues corresponding to Lys64, Pro66, Lys67, Ile68, Asn84, Ile85, Leu90, Lys91, Lys91 and Leu120 of SEQ ID NO:1; amino acid residues corresponding to Pro65, Pro66, Lys67, Ile68, Asn84, Leu90, Lys91 and Leu120 of SEQ ID NO:1; amino acid residues corresponding to Lys66, Ile67, Ala68, Ser83, Cys85, Leu86, Leu89 and Arg90 of SEQ ID NO:2; amino acid residues corresponding to Pro64, Pro65, Lys66, Ile67, Ser83, Ile84, Cys85, Leu86, Leu89, Arg90 and Leu119 of SEQ ID NO:2; amino acid residues corresponding to Pro68, Lys70, Tyr83, Glu87, Val88, Cys89, Leu93 and His94 of SEQ ID NO:3; and amino acid residues corresponding to Pro68, Pro69, Lys70, Leu71, Tyr83, Glu87, Val88, Cys89, Leu93 and His94 of SEQ ID NO:3.

In further embodiments of the invention, the compounds of the invention selectively targets one or more binding domains within the active sites, including the following: amino acid residues corresponding to Pro66, Ile85 and Leu90 of SEQ ID NO:1; amino acid residues corresponding to Pro66, Ile68, Leu90 and Lys91 of SEQ ID NO:1; amino acid residues corresponding to Ile67 and Leu89 of SEQ ID NO:2; amino acids corresponding to Pro65, Ile67, and Ser 83 of SEQ ID NO:2; amino acids corresponding to Glu87, Val88, Leu93 and His94 of SEQ ID NO:3; and amino acids corresponding to Pro69, Leu71 and Val88 of SEQ ID NO:3.

In one embodiment, the present invention provides a selective ubiquitin conjugating enzyme inhibitors of formula (I):



wherein:

Ar is a five or six membered unsubstituted or substituted aromatic ring that is optionally fused to an aromatic or heteroaromatic ring;

B is a bond, CO, SO₂ or (CH₂)_n wherein n = 1 – 5; and

R₁ and R₂ are each independently H, alkyl or aryl groups that are optionally substituted;

wherein the Formula I compound selectively binds to one or more catalytic domains in the ubiquitin conjugating enzyme. Preferably, Ar is phenyl, pyridyl, naphthyl, triazine, triazole, quinoxaline, dibenzofuran, benzimidazole, indene, indeno oxadiazine, indazole or an indole ring; B is a bond, CO or (CH₂)_n wherein n = 1; and
 5 R₁ and R₂ are both H.

In a preferred embodiment, the formula I compound is 3-amino-1,2,4-Triazine, 3-amino-1,2,4-triazole, 2-methyl-4-nitroaniline, 2-iodo-4-nitroaniline, 4-amino-3-chloro-5-methylbenzoic acid, 1-(4'Aminophenyl)-1,2,4-triazole, 2-acetamidophenol, 5-chloro-2,3-dihydroxypyridine, 2-methyl-3-(1H-pyrazol-5-yl)imidazo(1,2-a)pyridine, 5-nitro-2,3-
 10 dihydro-1H-benzo(d)imidazol-2-one, 4-(methylamino)pyridine, 2-Chloro-4-nitrobenzamide, 2-ethylformanilide, 6-aminoindazole, 2,3-diaminobenzoic acid, 1-(5-chloro-2-methylphenyl)-2-thiourea, 4,5-diiodo-1H-imidazole, 1H-indene-1,3(2H)-dione 1-methylhydrazone, 3-hydroxyindole, 3,4-dihydro-1H-quinoxalin-2-one, 1S,6S,7R,8R,8aR)-1,6,7,8-Tetrahydroxyoctahydroindolizidine, 2-naphthalen-1-yl-2,3-dihydro-1H-pyrimidine,
 15 benzo(b)thiophen-3-ylmethylamine, 1-allyl-2,4-dioxo-1,2,3,4-tetrahydro-5-pyrimidinecarbonitrile, Methyl (S)-N-(7-chloro-2,3,4a,5-tetrahydro-4a-(methoxycarbonyl)indeno(1,2-e)(1,3,4)oxadiazin-2-ylcarbonyl)-4'-(trifluoromethoxy)carbanilate (Indoxacarb-MP), and 4-acetylpyridine and thioisonicotinamide.

20 In a currently preferred embodiment, the formula I compound of the invention is 4-(Aminomethyl)piperidine. Typically, the formula I compounds of the invention have a molecular weight of less than about 2000 daltons, and preferably, a molecular weight of less than about 500 daltons.

In another embodiment, the present invention provides a selective ubiquitin
 25 conjugating enzyme inhibitor of formula (II):



wherein:

A is a 3-6 membered substituted or unsubstituted cycloaliphatic or a heterocycloaliphatic ring, each of which is optionally fused to an aromatic ring;

B is a bond, CO, SO₂ or (CH₂)_n wherein n = 1-3; and

R₁ and R₂ are each independently H, alkyl or aryl groups that are optionally substituted, and the compound selectively binds to one or more catalytic domains in the ubiquitin conjugating enzyme.

- 5 Preferably, the A of the compound of formula II is a heterocycloaliphatic ring comprising, at least one nitrogen atom and optionally, one or more additional heteroatoms selected from the group consisting of: nitrogen (N), oxygen (O) and sulfur (S); B is a bond, CO or (CH₂)_n wherein n = 1; and R₁ and R₂ are both H. In another embodiment of the invention, the cycloaliphatic or heterocycloaliphatic ring of formula II comprise one or more
10 substituents selected from the group consisting of hydroxyl, halogen, CO and alkyl.

In a preferred embodiment, the formula II compound of the invention is 1-phenyl-4-methyl-3-pyrazolidone, 4-(aminomethyl)piperidine, N-Phenyl-p-phenylenediamine, 5-(aminomethyl)-3-(2H)-isoxazolone (Muscimol), (R)-2-aminomethylpyrrolidine, 2-pyrrolidinone oxime and 1-cyclopropylethylamine.

- 15 The formula II compounds of the invention typically have a molecular weight of less than about 2000 daltons, and preferably, a molecular weight of less than about 500 daltons.

- In another embodiment of the invention, the organic compound is an aromatic or heteroaromatic compound comprising a piperidinyl, phenyl, quinolinyl and isoquinolinyl ring and having one or more nitrogen containing substituents selected from the group consisting
20 of NR₃R₄, (CH₂)_nN R₃R₄, CONH₂, NH-NH-R₅ and C(S)-NH-R₆, wherein R₃, R₄, R₅ and R₆ are independently H, alkyl, cycloalkyl and aryl, and n is 1-5, and the compound selectively interacts with the E1 binding site of the ubiquitin conjugating enzyme. In further embodiment of the invention, the aromatic or heteroaromatic compound is 4-methylcyclohexylamine, 3-(dimethylamino)-1-(5-fluoro-2-hydroxyphenyl)prop-2-en-1-one,
25 3-ethoxyphenethylamine, 4-fluorobenzenesulfonamide, 2-((4-fluorophenyl)-hydrazono)malononitrile, 3-fluoro-4-hydroxybenzaldehyde or 1-aminoisoquinoline. In still another embodiment of the invention, the aromatic or heteroaromatic compound is less than about 2000 daltons. In another embodiment, the compound the aromatic or heteroaromatic compound is less than about 500 daltons.

In still another embodiment of the invention, the organic compound is a 7 or 8 membered monocyclic or bridged bicyclic compound with a cycloheptyl, cyclooctyl, and bicyclo(2,2,1) heptenyl having one or more substituents selected from the group consisting of NR_3R_4 , $(\text{CH}_2)_n\text{NR}_3\text{R}_4$, CONH_2 , NH-NH- R_5 and C(S)-NH-R_6 , wherein R_3 , R_4 , R_5 and R_6 are independently H, alkyl, cycloalkyl and aryl, and n is 1-5, and the compound selectively interacts with the E1 binding site of the enzyme. In a further embodiment of the invention, the 7 or 8-membered monocyclic or bridged bicyclic compound is 6,7-dihydro-5H-dibenzo(a,c)cycloheptene-6-carboxylic acid, N-bicyclo(2.2.1)hept-5-en-2-ylthiourea, or N1-cyclooctyl-4-hydroxy-1-piperidinecarbothioamide. Typically, the 7 or 8-membered monocyclic or bridged bicyclic compound of the invention has a molecular weight of less than about 2000 daltons, and preferably, less than about 500 daltons.

In another aspect of the invention, a method is disclosed for determining a selective interaction between a ubiquitin conjugating enzyme and an organic compound. The method includes the steps of inputting 3-D co-ordinates of at least a fragment of the ubiquitin conjugating enzyme into an electronic storage medium, determining one or more active sites in the fragment, modifying a library of organic compounds to have hydrogens creating a modified library of organic compounds, simulating a binding interaction between the active sites in the fragments and the modified library of organic compounds, and identifying compounds in the modified library of organic compounds that interact with the active sites in the fragments.

In yet another aspect of the invention, a method is disclosed for determining a selective interaction between a ubiquitin conjugating enzyme and an organic compound. The method includes the steps of inputting 3-D co-ordinates of at least a fragment of the ubiquitin conjugating enzyme into an electronic storage medium, determining one or more active sites in the fragment, simulating a binding interaction between the active sites in the fragments and a library of organic compounds, and identifying compounds in the library of organic compounds that interact with the active sites in the fragments. In another embodiment of the invention, the ubiquitin conjugating enzyme is selected from the group consisting of: Ubc1, Ubc2, Ubc3, Ubc4, Ubc5, Ubc6, Ubc7, Ubc8, Ubc10, Ubc11 and Ubc13. In a further embodiment, the ubiquitin conjugating enzyme is a yeast ubiquitin conjugating enzyme or a mouse, rat or human homolog. In another embodiment, the 3-D coordinates are 1AYZ, 1A3S, 1QCQ, 2UCZ, 1I7K 1J7D, 1JAT, 1JBB and variants thereof. In another embodiment,

a molecular topology and charge visualization program performs the step of determining one or more active sites. In a further embodiment, the molecular topology and charge visualization program is the InsightII or Weblab Viewer programs. In another embodiment, the step of simulating a binding interaction is performed with the LUDI program. In a further
 5 embodiment, the library of organic compounds is the Available Chemicals Directory.

In yet another embodiment, the present invention provides compounds and methods for selectively targeting the active site in the ubiquitin conjugating enzyme comprising amino acid residues corresponding to Lys64, Pro66, Lys67, Ile68, Asn84, Ile85, Leu90, Lys91 and Leu120 of SEQ ID NO:1. In another embodiment, the active selectively targeted site
 10 comprise amino acid residues corresponding to Pro65, Pro66, Lys67, Ile68, Asn84, Leu90, Lys91 and Leu120 of SEQ ID NO:1. In another embodiment of the invention, the selectively targeted active site comprises the amino acid residues corresponding to Lys66, Ile67, Ala68, Ser83, Cys85, Leu86, Leu89 and Arg90 of SEQ ID NO:2. In another embodiment of the invention, the selectively targeted active site comprises the amino acid residues
 15 corresponding to Pro64, Pro65, Lys66, Ile67, Ser83, Ile84, Cys85, Leu86, Leu89, Arg90 and Leu119 of SEQ ID NO:2. In another embodiment of the invention, the selectively targeted active site comprises the amino acid residues corresponding to Pro68, Lys70, Tyr83, Glu87, Val88, Cys89, Leu93 and His94 of SEQ ID NO:3. In another embodiment of the invention, the selectively targeted active site comprises the amino acid residues corresponding to Pro68,
 20 Pro69, Lys70, Leu71, Tyr83, Glu87, Val88, Cys89, Leu93 and His94 of SEQ ID NO:3.

In a further aspect, the present invention discloses a method for treating or preventing a hyperproliferative disorder by administering an effective amount of a compound of formula (I):



25 wherein:

Ar is a five or six membered unsubstituted or substituted aromatic ring that is optionally fused to an aromatic or heteroaromatic ring;

B is a bond, CO, SO₂ or (CH₂)_n wherein n = 1 – 5; and

R₁ and R₂ are each independently H, alkyl or aryl groups that are optionally substituted. In another embodiment of the invention, Ar of formula I is phenyl, pyridyl, naphthyl, triazine, triazole, quinoxaline, dibenzofuran, benzimidazole, indene, indeno oxadiazine, indazole or an indole ring; B is a bond, CO or (CH₂)_n wherein n = 1; and R₁ and R₂ are both H.

In another embodiment of the invention, formula I is 3-amino-1,2,4-Triazine, 3-amino-1,2,4-triazole, 2-methyl-4-nitroaniline, 2-iodo-4-nitroaniline, 4-amino-3-chloro-5-methylbenzoic acid, 1-(4' Aminophenyl)-1,2,4-triazole, 2-acetamidophenol, 5-chloro-2,3-dihydropyridine, 2-methyl-3-(1H-pyrazol-5-yl)imidazo(1,2-a)pyridine, 5-nitro-2,3-dihydro-1H-benzo(d)imidazol-2-one, 4-(methylamino)pyridine, 2-Chloro-4-nitrobenzamide, 2-ethylformanilide, 6-aminoindazole, 2,3-diaminobenzoic acid, 1-(5-chloro-2-methylphenyl)-2-thiourea, 4,5-diiodo-1H-imidazole, 1H-indene-1,3(2H)-dione 1-methylhydrazone, 3-hydroxyindole, 3,4-dihydro-1H-quinoxalin-2-one, 1S,6S,7R,8R,8aR)-1,6,7,8-Tetrahydrooctahydroindolizidine, 2-naphthalen-1-yl-2,3-dihydro-1H-pyrimidine, benzo(b)thiophen-3-ylmethylamine, 1-allyl-2,4-dioxo-1,2,3,4-tetrahydro-5-pyrimidinecarbonitrile, Methyl (*S*)-*N*-(7-chloro-2,3,4a,5-tetrahydro-4a-(methoxycarbonyl)indeno(1,2-*e*)(1,3,4)oxadiazin-2-ylcarbonyl)-4'-(trifluoromethoxy)carbanilate (Indoxacarb-MP), or 4-acetylpyridine and thioisonicotinamide.

In another embodiment, formula I is 4-(aminomethyl)piperidine. Typically, the formula 1 compound has a molecular weight of less than about 2000 daltons; in another embodiment of the invention, and preferably, less than about 500 daltons.

In another aspect of the invention, a method is disclosed the method for treating or preventing a hyperproliferative disorder with a compound of formula (II):



wherein:

A is a 3-6 membered substituted or unsubstituted cycloaliphatic or a heterocycloaliphatic ring, each of which is optionally fused to an aromatic ring;

B is a bond, CO, SO₂ or (CH₂)_n wherein n = 1-3; and

R₁ and R₂ are each independently H, alkyl or aryl groups that are optionally substituted.

Preferably, A is a heterocycloaliphatic ring comprising, at least one nitrogen atom and optionally, one or more additional heteroatoms selected from the group consisting of:
5 nitrogen (N), oxygen (O) and sulfur (S); B is a bond, CO or (CH₂)_n wherein n = 1; and R₁ and R₂ are both H. In another embodiment of the invention, the cycloaliphatic or heterocycloaliphatic ring of formula II comprise one or more substituents selected from the group consisting of hydroxyl, halogen, CO and alkyl.

In a preferred embodiment, the formula II compound is 1-phenyl-4-methyl-3-
10 pyrazolidone, 4-(aminomethyl)piperidine, N-Phenyl-p-phenylenediamine, 5-(aminomethyl)-3-(2H)-isoxazolone (Muscimol), (R)-2-aminomethylpyrrolidine, 2-pyrrolidinone oxime and 1-cyclopropylethylamine.

Typically, the formula II compounds have a molecular weight of less than about 2000 daltons, and preferably, less than about 500 daltons.

15 In another aspect of the invention, a method is disclosed for treating or preventing a hyperproliferative disorder with an effective amount of the compound which is an aromatic or heteroaromatic compound comprising a piperidinyl, phenyl, quinolinyl and isoquinolinyl ring and having one or more nitrogen containing substituents selected from the group consisting of NR₃R₄, (CH₂)_nN R₃R₄, CONH₂, NH-NH-R₅ and C(S)-NH-R₆, wherein R₃, R₄, R₅ and R₆
20 are independently H, alkyl, cycloalkyl and aryl, and n is 1-5. In further embodiment of the invention, the aromatic or heteroaromatic compound is 4-methylcyclohexylamine, 3-(dimethylamino)-1-(5-fluoro-2-hydroxyphenyl)prop-2-en-1-one, 3-ethoxyphenethylamine, 4-fluorobenzenesulfonamide, 2-((4-fluorophenyl)-hydrazono)malononitrile, 3-fluoro-4-hydroxybenzaldehyde or 1-aminoisoquinoline. Typically, the aromatic or heteroaromatic
25 compound of the invention is less than about 2000 daltons, and preferably, less than about 500 daltons.

In still another aspect of the invention, a method is disclosed the method for treating or preventing a hyperproliferative disorder with an effective amount of a compound that is a
7 or 8 membered monocyclic or bridged bicyclic compound with a cycloheptyl, cyclooctyl,
30 and bicyclo(2,2,1) heptenyl having one or more substituents selected from the group consisting of NR₃R₄, (CH₂)_nNR₃R₄, CONH₂, NH-NH- R₅ and C(S)-NH-R₆, wherein R₃, R₄,

R₅ and R₆ are independently H, alkyl, cycloalkyl and aryl, and n is 1-5. In a further embodiment of the invention, the 7 or 8-membered monocyclic or bridged bicyclic compound is 6,7-dihydro-5H-dibenzo(a,c)cycloheptene-6-carboxylic acid, N-bicyclo(2.2.1)hept-5-en-2-ylthiourea, or N1-cyclooctyl-4-hydroxy-1-

- 5 piperidinecarbothioamide. Typically, the 7 or 8-membered monocyclic or bridged bicyclic compound has a molecular weight of less than about 2000 daltons, and preferably, less than about 500 daltons.

In a final aspect of the invention, a method is disclosed to treat or prevent a ubiquitin conjugating enzyme disorder by administering an effective amount of a composition of the invention. In one embodiment, the ubiquitin conjugating related disorder is a
10 hyperproliferative disorders, Cystic Fibrosis, Huntington's, Alzheimer's, or Parkinson's disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a 3-D computer representation of Ubc4 binding HBS01.

- 15 Figure 2 shows the results from Example 2 measuring IC₅₀ values. The bar graph indicates the IC₅₀ values as determined by an MTS assay for HBS01, MTX, DOX and 5FU in MCF-7 and MCF-7-Adr. IC₅₀ concentrations are indicated on the y-axis in nM concentrations.

- Figure 3 shows the results from Example 3 measuring tumor volume. The bar graph
20 indicates the tumor volume on day 10, 12, 15, 17 and 19 in untreated, pretreated (HBS01) and treated (HBS01) mice injected with C85 colorectal adenocarcinoma tumor cells. Tumor volume is indicated on the y-axis in cm³.

- Figure 4 shows the results from Example 4 measuring tumor volume. The bar graph indicates the tumor volume on day 10, 12, 15, 24, 26 and 31 in untreated, pretreated (HBS01)
25 and treated (HBS01) mice injected MCF-7 breast cancer tumors. Tumor volume is indicated on the y-axis in cm³.

Figure 5 shows the results from Example 7 measuring protein ubiquitination in control and HBS01 treated cells. The gels were 12% Laemmli gels. The gel on the left panel was stained with Commassie Blue R-250 and the gel on the right panel is a Western Blot utilizing

the 12CA5 monoclonal antibody specific for the HA-tagged ubiquitin proteins. Lane 1 depicts cell lysates from cells treated with HBS01 for 24 hrs; Lane 2 depicts cell lysates from cells untreated for 24 hrs; Lane 3 depicts cell lysates from cells treated with HBS01 for 48 hrs; and Lane 4 depicts cell lysates from cells untreated for 48 hrs.

5 Figure 6 shows the results from Example 8 measuring protein ubiquitination in control and HBS01 treated cells. The results were analyzed on a 12% Laemmli gel Western blotted with anti-cyclin-b. Lane 1 depicts cell lysates from cells treated with HBS01 for 24 hrs while Lane 2 depicts cell lysates from cells untreated for 24 hrs. The arrow indicates non-ubiquitinated cyclin-B.

10 DEFINITIONS

As used herein an "ubiquitin conjugating enzyme," "Ubc" or "E2" is a family of proteins which are capable of forming a thiol ester adduct with the C- terminal carboxyl group of ubiquitin and transferring the ubiquitin to an amino group in an acceptor protein by formation of an isopeptide bond. The biological activity of "ubiquitin conjugating enzymes" can be characterized by an ability to mediate ubiquitination of cellular and/or viral proteins, e.g. cell-cycle regulatory proteins such as p53, myc, fos, and cyclins. Such a feature will typically be marked by an ability of "Ubc" to mediate ubiquitin-dependent degradation or inactivation of such regulatory proteins, in normal proliferating cells, in virally-infected cells, e.g. by papillomavirus or adenovirus, or in transformed cells, e. g. in cancerous cells.

20 The "Ubcs" include those present in yeast and mammalian organisms such as humans, rat and mice. "Ubcs" may be selected from those available in the art by one of skill in the art. Non-limiting examples include yeast Ubc1, Ubc2, Ubc3, Ubc4, Ubc5, Ubc6, Ubc7, Ubc8, Ubc10, Ubc13, and their human, mouse and rat homologs.

25 A "variant" of a "Ubc" refers to a "Ubc" substantially similar to either the entire protein, or a fragment thereof. A "fragment" of a "Ubc", such as any of the "Ubc" discussed herein, is meant to refer to any "Ubc" protein or the corresponding 3-D co-ordinates of a subset of an individual "Ubc" that contains at least one "active site."

30 As used herein an "active site" is a region on a "Ubc" within 20 Å radius, as determined by 3-D computer modeling, of the "catalytic cysteine" or the "E1 binding consensus," that is physically and energetically favorable for binding an organic compound.

The "catalytic cysteine" is located at the amino acid position corresponding to Cys86 of SEQ ID NO:1. The "E1 binding consensus" is located at the amino acid positions corresponding to Lys5, Arg6, Lys9 and Glu10 of SEQ ID NO:1. An "active site" is identified by the methods disclosed herein, preferably by a molecular topology and charge visualization technique. The "catalytic site" is a region within a 20 Å radius of the "catalytic cysteine." As used herein, the "E1 binding site" is a region within a 20 Å radius of the "E1 binding consensus."

As used herein a "binding domain" is a region within the "active site" containing one or more amino acids which bind an organic compound. Typically, the "binding domain" allows for one or more hydrogen binding interactions with the organic compound such that an energetically favorable binding event occurs. The "binding domain" may also include hydrophobic interactions. An example of a "binding domain" is the amino acid residues corresponding to Pro66, Ile85 and Leu90 of SEQ ID NO:1.

As used herein a "library of organic compounds" is a collection of data which represents the 3-D coordinates of multiple chemical organic compounds. Examples of a "library of organic compounds" include the Available Chemical Directory (MDL Inc., San Leandro, CA), the Derwent World Drug Index (WDI), BioByteMasterFile, and the National Cancer Institute database (NCI). A "modified library of organic compounds" is a "library of organic compounds" which have been altered to include hydrogens.

As used herein, "pharmacologically effective amount," "therapeutically effective amount" or simply "effective amount" refers to that amount of an agent effective to produce the intended pharmacological, therapeutic or preventive result. For example, if a given clinical treatment is considered effective when there is at least a 25% reduction in a measurable parameter associated with a disease or disorder, a therapeutically effective amount of a drug for the treatment of that disease or disorder is the amount necessary to effect that at least 25% reduction.

As used herein, a "hyperproliferative disorder" means cancer, neoplastic growth, hyperplastic or proliferative growth or a pathological state of abnormal cellular development and includes solid tumors, non-solid tumors, and any abnormal cellular proliferation, such as that seen in leukemia. As used herein, "hyperproliferative disorder" also means angiogenesis-dependent cancers and tumors, *i.e.*, tumors that require for their growth

(expansion in volume and/or mass) an increase in the number and density of the blood vessels supplying them with blood.

As used herein, the term "homology" refers to the optimal alignment of sequences (either nucleotides or amino acids), which may be conducted by computerized implementations of algorithms. "Homology" with regard to polynucleotides, for example, may be determined by analysis with BLASTN version 2.0 using the default parameters. "Homology" with respect to polypeptides (*i.e.*, amino acids), may be determined using a program, such as BLASTP version 2.2.2 with the default parameters, which aligns the polypeptides or fragments being compared and determines the extent of amino acid identity or similarity between them. It will be appreciated that amino acid "homology" includes conservative substitutions, *i.e.* those that substitute a given amino acid in a polypeptide by another amino acid of similar characteristics. Typically seen as conservative substitutions are the following replacements: replacements of an aliphatic amino acid such as Ala, Val, Leu and Ile with another aliphatic amino acid; replacement of a Ser with a Thr or vice versa; replacement of an acidic residue such as Asp or Glu with another acidic residue; replacement of a residue bearing an amide group, such as Asn or Gln, with another residue bearing an amide group; exchange of a basic residue such as Lys or Arg with another basic residue; and replacement of an aromatic residue such as Phe or Tyr with another aromatic residue.

As used herein in relation to the position of an amino acid, e.g., Cys86 of SEQ ID NO:1, the term "corresponding to" refers to an amino acid in a first polypeptide sequence that aligns with a given amino acid in a reference polypeptide sequence when the first polypeptide and reference polypeptide sequences are aligned. Alignment is performed by one of skill in the art using software designed for this purpose, for example, BLASTP version 2.2.2 with the default parameters for that version. As an example of amino acids that "correspond," Cys86 of Ubc4 of SEQ ID NO: 1 "corresponds to" Cys89 of Ubc7 of SEQ ID NO:3, and *vice versa*.

As herein defined, the term "selectively binds" means that the interaction between the organic compound and the Ubc are specific, that is, in the event that a number of molecules are presented to the organic compound, the latter will only bind to one or a few of those molecules presented. Preferably, the compound will only target a single Ubc enzyme. Advantageously, the compound-Ubc interaction will be of high affinity. Non-covalent interactions such as hydrogen bonding and Van der Waals interactions will mediate the discriminate interaction between the compound and a specific Ubc only.

DETAILED DESCRIPTION

As described herein the present invention provides methods and compositions for selectively targeting one or more active sites of a Ubc. The invention also relates to structure based screening methods to identify organic compounds that selectively bind one or more of the Ubc's active sites with in silico techniques, thus providing an inexpensive and quick method for the identification of compounds that selectively modulate Ubcs. It is expected that modulating protein degradation via the targeting of specific Ubc active sites will treat a variety of disorders in which Ubcs have been implicated, particularly hyperproliferative disorders.

The present invention provides methods for specifically targeting Ubcs. The invention utilizes the structure based design programs (InsightII, Accelrys, Inc., San Diego, CA) and LUDI (Boehm, *J. Comp. Aid. Mol. Des.* 6:61-78; Accelrys, Inc., San Diego, CA) to identify domains within active sites that selectively bind chemical compounds and interfere with Ubc function. InsightII is used to recognize molecular topology and electrostatic charges in specific active sites in the Ubcs that are physically and energetically favorable to bind a compound in an organic compound library, while LUDI (Accelrys, Inc., San Diego, CA) is used to analyze and score binding between a compound in an organic compound library and the active site identified by InsightII. The structure based design methods of the present invention have identified efficacious compounds, e.g., HBS01, for the treatment of Ubc disorders, e.g., cancer.

These compounds of the invention specifically target the Ubc catalytic site and E1 binding sites. Many of these compounds have been tested *in vivo* and *in vitro* and have proved to selectively target Ubcs and inhibit cellular proliferation in cancer models. A compound designated HBS01, which is 4-(aminomethyl)piperidine, was identified by the in silico screening techniques described herein. *In vitro* testing revealed HBS01 was a potent cytotoxic agent with an IC50 of 30 nm against the human colon cancer cell line C85 (Longo et al. *Oncol. Res.*, 12(8):309-14), and the breast cancer cell lines MCF-7 and MCF-7Adr. HBS01 inhibited the growth of established tumors in nude mice and delayed tumor growth in mice pretreated with HBS01. These studies also revealed that the HBS01 agent is well tolerated with a maximum tolerated dose in mice of 1.4g/kg, whereas the therapeutic dose being only 17 mg/kg.

Ubcs of the invention

There are 42 human Ubcs and 13 yeast Ubcs that have been identified to date. The Ubc protein family is structurally well characterized. The E2 core domain consists of: four standard α helices, a 3_{10} helix and four to seven stranded antiparallel β sheets. The catalytic cysteine lies in a long loop that connects the β sheet and $\alpha 2$ helix. This catalytic cysteine is located at the amino acid position corresponding to Cys86 of SEQ ID NO:1. SEQ ID NO:1 shows the amino acid sequence of *S. cerevisiae* Ubc4 (Accession No. P15731), SEQ ID NO:2 shows the amino acid sequence of human Ubc5a (Accession No. P51668) and SEQ ID NO:3 shows the amino acid sequence of *S. cerevisiae* Ubc7 (Accession No. S28951). In some Ubcs like Ubc 7 and CDC34 (Ubc3), a long and flexible loop is present near the catalytic site. Some E2 enzymes have C and N terminal extensions and which have been shown to be catalytically important. The presence or absence of these extensions can be used to classify the E2s into 4 classes: Class I Ubcs consist of a highly conserved core catalytic domain and share 25% identity between members; class II Ubc enzymes have an additional C terminal extension; class III Ubcs have an additional N terminal extension; and class IV Ubcs have both C and N terminal extensions.

Any known or unknown Ubc can be used in the methods described herein by one of ordinary skill in the art. Additionally, Ubcs can be identified based on sequence alignments of sequences described herein. For example, Ubcs can be identified based on sequence alignments of sequences which include amino acid residues corresponding to Gly49, Tyr61, Pro62, Pro66, His76 to Asn78, Gly83, Cys86, Leu87, Trp94 and Pro96 of SEQ ID NO:1. Non-limiting examples of Ubcs include yeast Ubc1, Ubc2, Ubc3, Ubc4, Ubc5, Ubc6, Ubc7, Ubc8, Ubc10, Ubc13, and their human, mouse and rat homologs. In some embodiments, the Ubcs are mammalian human Ubcs. In other embodiments the Ubcs are yeast Ubcs. In a preferred embodiment the Ubc is yeast Ubc4 of SEQ ID NO:1. Additional, Ubcs that may be utilized in the present invention include: Ubiquitin-conjugating enzyme E2 4 (*S. cerevisiae*) (Accession No. P15731), Ubiquitin-conjugating enzyme E2-17 kDa 2 (*Homo sapiens*) (Accession No. P51669), ubiquitin-conjugating enzyme E2D 3 (*Homo sapiens*) (Accession No. Q9Y2X8), Ubiquitin-conjugating enzyme E2-18 kDa UbcH7 (*Homo sapiens*) (Accession No. P51966), Ubiquitin-conjugating enzyme E2-18 kDa (*S. cerevisiae*) (Accession No. P23567), Ubiquitin-conjugating enzyme E2-20 kDa (*S. cerevisiae*) (Accession No. P06104), Ubiquitin-conjugating enzyme E2-21.2 kDa (*S. cerevisiae*; Accession No. P52491),

Ubiquitin-conjugating enzyme E2-18 kDa (*S. cerevisiae*, Accession No. P50623), Ubiquitin-conjugating enzyme E2-24 kDa (*S. cerevisiae*, Accession No. P21734), Ubiquitin-conjugating enzyme E2 13 (*S. cerevisiae*, Accession No. P52490), Ubc5a (*H. Sapiens*) (Accession No. P51668) and variants thereof. The amino acid sequences can be accessed at the NCBI's protein data base (<http://www.ncbi.nlm.nih.gov/entrez>). Additional, Ubc Accession Nos. can be accessed at <http://www.ubiquitin-resource.org> (login: ubq2e1, password: sentrinE2).

Ubc 3-D Co-ordinates

The invention involves the inputting of 3-D co-ordinates of Ubcs into an electronic storage medium. In one embodiment, the complete 3-D co-ordinates of the Ubc are input. In an alternative embodiment, a fragment, or less than the complete 3-D co-ordinates are inputted. Preferably the 3-D co-ordinates at least those of the catalytic site and/or the E1 binding site. The 3-D co-ordinates may be known in the art or based on homology modeling. Known Ubc 3-D co-ordinates include Ubc2 (PDB ID No. 1AYZ), Ubc9 (PDB ID No. 1A3S), Ubc4 (PDB ID No. 1QCQ), Ubc7 (PDB ID No. 2UCZ), UbcH10 (PDB ID No. 1I7K) and Ubc13 (PDB ID No. 1JBB, 1J7D, 1JAT) as well as others known in the art. 3-D co-ordinates for many known Ubc can be obtained from the Protein Data Bank ("PDB") (Research Collaboratory for Structural Bioinformatics; <http://www.rcsb.org> or at: <http://www.ubiquitin-resource.org> (login: ubq2e1, password: sentrinE2). In one preferred embodiment, the 3-D co-ordinates are PDB ID No. 1QCQ. Variants of the 3-D-coordinates can also be used in the invention, such as variants in which the r.m.s. deviation of the x, y and z co-ordinates for all heavy (*i.e.*, not hydrogen) atoms are less than 2.5 Å. In some preferred embodiments the 3-D co-ordinates are based on homology modeling with known ubiquitin 3-D co-ordinates as described herein.

The 3-D coordinates are used to generate a 3-D structural model (or a representation thereof) for performing the method of the invention. Preferably, the 3-D coordinates may be used to generate a computer model for the structure. The 3-D coordinates comprising the Ubcs is built from all or a fraction of the 3-D coordinates of the Ubcs. Thus, for example, the 3-D co-ordinates provided in the 3-D structure and/or model structure may comprise the amino acid residues of the Ubcs, or a portion of the Ubcs or a homologue thereof useful in the modeling and design of test compounds capable of binding to domains within active sites.

Homology modeling (also known as comparative modeling or knowledge-based modeling) methods develop a three dimensional model from a polypeptide sequence based on the structures of known proteins (e.g., native or mutated Ubcs). In the present invention the method utilizes a computer representation of a Ubc structure or a complex of same, a
5 computer representation of the amino acid sequence of a polypeptide with an unknown structure (additional native or mutated Ubc), and standard computer representations of the structures of amino acids. In particular, the method comprises the steps of: (a) identifying structurally conserved and variable regions in the known structure; (b) aligning the amino acid sequences of the known structure and unknown structure (c) generating coordinates of
10 main chain atoms and side chain atoms in structurally conserved and variable regions of the unknown structure based on the coordinates of the known structure thereby obtaining a homology model and (d) refining the homology model to obtain a three dimensional structure for the unknown structure. Similar methods are known to those skilled in the art (Greer, 1985, Science 228, 1055; Bundell et al 1988, Eur. J. Biochem. 172, 513; Knighton et al., 1992, Science 258:130-135, <http://biochem.vt.edu/courses/modeling/homology.htm>). Computer programs that can be used in homology modeling include Quanta and the homology module in the Insight II modeling package (Accelrys, Inc., San Diego, CA) or MODELLER (Rockefeller University, www.iucr.ac.uk/sinris-top/logical/prg-modeller.html, Sali's Modeller also from Accelrys, Inc., San Diego, CA).

20 In step (a) of the homology modeling method, the known Ubc structure is examined to identify the structurally conserved regions (SCRs) from which an average structure, or framework, can be constructed for these regions of the protein variable regions (VRs), in which known structures may differ in conformation, also must be identified. SCRs generally correspond to the elements of secondary structure, such as alpha-helices and beta-sheets, and
25 to ligand and substrate-binding sites (e.g., acceptor and donor binding sites). The VRs usually lie on the surface of the proteins and form the loops where the main chain turns. Many methods are available for sequence alignment of known structures and unknown structures. Sequence alignments generally are based on the dynamic programming algorithm of Needleman and Wunsch (J. Mol. Biol. 48: 442-453, 1970). Current methods include
30 FASTA, Smith-Waterman, and BLASTP, with the BLASTP method differing from the other two in not allowing gaps. Scoring of alignments typically involves construction of a 20×20 matrix in which identical amino acids and those of similar character (*i.e.*, conservative substitutions) may be scored higher than those of different character. Substitution schemes

which may be used to score alignments include the scoring matrices PAM (Dayhoff et al., Meth Enzymol. 91: 524- 545, 1983), and BLOSUM (Henikoff and Henikoff, Proc. Nat. Acad. Sci. USA 89: 10915-1'0919, 1992), and the matrices based on alignments derived from three-dimensional structures including that of Johnson and Overington (JO matrices) (J. Mol. Biol. 233: 716-738, 1993). Other methods of determining sequence alignment are described herein.

Alignment based solely on sequence may be used; however, other structural features also may be taken into account. In Quanta, multiple sequence alignment algorithms are available that may be used when aligning a sequence of the unknown with the known structures. Four scoring systems (*i.e.*, sequence homology, secondary structure homology, residue accessibility homology; CA-CA distance homology) are available, each of which may be evaluated during an alignment so that relative statistical weights may be assigned.

When generating coordinates for the unknown structure, main chain atoms and side chain atoms, both in SCRs and VRs need to be modeled. A variety of approaches known to those skilled in the art may be used to assign coordinates to the unknown. In particular, the coordinates of the main chain atoms of SCRs will be transferred to the unknown structure. VRs correspond most often to the loops on the surface of the polypeptide and if a loop in the known structure is a good model for the unknown, then the main chain coordinates of the known structure may be copied. Side chain coordinates of SCRs and VRs are copied if the residue type in the unknown is identical to or very similar to that in the known structure. For other side chain coordinates, a side chain rotamer library may be used to define the side chain coordinates. When a good model for a loop cannot be found fragment databases may be searched for loops in other proteins that may provide a suitable model for the unknown. If desired, the loop may then be subjected to conformational searching to identify low energy conformers.

Once a homology model has been generated it is analyzed to determine its correctness. A computer program available to assist in this analysis is the Protein Health module in Quanta which provides a variety of tests. Other programs that provide structure analysis along with output include PROCHECK and 3-D-Profiler (Luthy R. et al, Nature 356: 83-85, 1992; and Bowie, J. U. et al, Science 253: 164-170, 1991). Once any irregularities have been resolved, the entire structure may be further refined. Refinement may consist of energy minimization with restraints, especially for the SCRS. Restraints may be gradually

removed for subsequent minimizations. Molecular dynamics may also be applied in conjunction with energy minimization.

Storage Medium

The storage medium in which the 3-D co-ordinates are provided is preferably random-
5 access memory (RAM), but may also be read only memory (ROM e.g. CDROM), or a diskette. The storage medium may be local to the computer, or may be remote (e.g., a networked storage medium, including the internet). Any suitable computer can be used in the present invention.

Active Site Determination

10 Active sites can be determined from the 3-D coordinates of the Ubcs utilizing the present invention. Molecular modeling techniques can be applied to the 3-D coordinates of at least a fragment of an Ubc to derive 3-D models and identify active sites within the Ubc catalytic and E1 binding sites. In a preferred embodiment, the active sites are within a 20 Å radius of the amino acid corresponding to Cys86 of SEQ ID NO:1 as determined in a 3-D model. In
15 another preferred embodiment, the active sites are within a 20 Å radius of the putative E1 binding site which includes the amino acids corresponding to Lys5, Arg6, Lys9 and Glu10 of SEQ ID NO:1.

The molecular modeling techniques identify Van der Waals contacts, electrostatic interactions, and/or hydrogen bonding opportunities. InsightII (Accelrys, Inc., San Diego,
20 CA) is used to recognize molecular topology and electrostatic charges in specific active sites in the Ubcs and/or multiple copy simultaneous search (MCSS) techniques which map favorable interaction positions for functional groups. The InsightII program identifies contours for the various functional probes which identify the shape of the pocket and energetically favorable active sites. This preferably reveals positions in the Ubcs for
25 interactions such as, but not limited to, those with protons, hydroxyl groups, amine groups, hydrophobic groups (e.g., methyl, ethyl, benzyl) and/or divalent cations. In a preferred embodiment, the active sites are determined using the InsightII (Accelrys, Inc., San Diego, CA) program.

Identification of Compounds that Bind to Ubc Active Sites

Once the specific active sites have been identified an in silico binding interaction is performed between the active sites and a library of organic compounds. In a preferred embodiment, the library of organic compounds is a digital library. The binding interaction is performed with a database searching program which is capable of scanning a database of

5 small molecules of known three-dimensional structure for candidates which fit into the active site. Suitable software programs include CATALYST (Molecular Simulations Inc., San Diego, CA), UNITY (Tripos Inc., St Louis, MO), FLEXX (Rarey et al., *J. Mol. Biol.* 261: 470- 489 (1996)), CHEM-3-DBS (Oxford Molecular Group, Oxford, UK), DOCK (Kuntz et al., *J. Mol. Biol* 161: 269-288 (1982)), and MACCS-3-D (MDL Information Systems Inc.,

10 San Leandro, CA) and LUDI (Boehm, *J. Comp. Aid. Mol. Des.* 6:61-78 (1992)), CAVEAT (Bartlett et al. in "Molecular Recognition in Chemical and Biological Problems", special publication of *The Royal Chem. Soc.*, 78:182-196 (1989)) and MCSS (Miranker et al. *Proteins* 11: 29-34 (1991)). Prior to performing the binding interaction it is preferable to add hydrogens to the library of organic compounds. Those of ordinary skill in the art will

15 understand that hydrogens can be added to structures by most molecular graphics software, including Insight II (InsightII, Accelrys, Inc., San Diego, CA). Relevant binding domains are identified using the software by: (1) identifying chemical groups on amino acids within the active site that allow for binding interactions, thus identifying possible interaction sites; (2) screening a library of organic compounds that interact with one or more interaction sites in

20 the active sites, thus identifying a binding domain; and (3) scoring the Ubc-organic compound complex. In one preferred embodiment, the library of organic compounds has been modified to contain hydrogen atoms. In another preferred embodiment, the binding interaction is performed with the LUDI program (Boehm, 1992).

A set of interaction sites are generated for each chemical group or functional groups

25 of a Ubc. This set of interaction sites encompasses the range of suitable positions for a ligand atom or functional group involved in the putative interaction. There are four classes of interaction sites which include: H bond-donor; H bond-acceptor; Lipophilic-aliphatic; and Lipophilic-aromatic. The H bond-donor and H bond-acceptor interaction sites are suitable sites for hydrogen bond formation. The aliphatic and aromatic interaction sites are suitable

30 sites for hydrophobic interactions. The interaction sites are then analyzed for van der Waals interactions with the Ubc. The organic compounds are then fitted into the interaction sites by RMS superposition of the atoms by the software program, thus identifying binding sites with the organic compound. A method of using LUDI to search for chemicals which bind 3-D

protein structures is taught in Boehm. *Journal of Computer-Aided Molecular Design*, 8:623-632 (1994), which is herein incorporated by reference.

Suitable libraries of organic compounds for use in the invention include the Available Chemicals Directory (MDL Inc), the Derwent World Drug Index (WDI), BioByteMasterFile, the National Cancer Institute database (NCI), and the Maybridge catalog, Aldrige/Sigma Rare Chemicals library as well as others known in the art. In a preferred embodiment, the library of organic compounds is the Available Chemical Directory. After binding events occur the results are scored based on a scoring function which indicates binding affinity and formation of hydrogen bonds and ionic interactions. In one embodiment the scoring function is the Boehm scoring function as described in Boehm. *Journal of Computer-Aided Molecular Design*, 12:309-323 (1998), which is herein incorporated by reference. The highest scored compounds are then manually selected to eliminate compounds which excessively protrude out of the binding site, as visualized by the computer 3-D structure of the Ubc and organic compound. The compounds remaining can then be tested *in vitro* and *in vivo* as described herein.

Compounds Identified by the Method of the Invention

The selective ubiquitin conjugating enzyme inhibitor compounds of the invention function by binding selectively to the catalytic domain or the E1 binding site present in the Ubc. Without wishing to be bound by theory, such selective binding is accomplished by phobic-phobic interactions between the hydrogen atoms present in the inhibitor compounds and hydrophobic domains on the enzyme, which is facilitated by the optimal size and conformation of the inhibitor compounds of the invention with respect to enzyme hydrophobic domains. Compounds of the invention are known by those of ordinary skill in the art. The compounds of the invention can be obtained from commercial suppliers including: Sigma-Aldrich (St. Louis, MO), Maybridge (Cornwall, England), Acros Organics (Morris Plains, NJ), Oackwood Products, Inc. (West Columbia, SC), Specs (Wakefield RI), Combi-Blocks, Inc (San Diego, CA), and CombiChem.net (Sudbury, UK).

In one embodiment of the present invention, the ubiquitin conjugating enzyme inhibitor compounds that selectively bind to the enzyme catalytic site are described by formula (I):



wherein:

Ar is a five or six membered unsubstituted or substituted aromatic ring that is optionally fused to an aromatic or heteroaromatic ring;

B is a bond, CO, SO₂ or (CH₂)_n wherein n = 1 – 5; and

5 R₁ and R₂ are each independently H, alkyl or aryl groups that are optionally substituted.

Preferred compounds of formula I include, but are not limited to compounds in which Ar comprises a phenyl, pyridyl, naphthyl, triazine, triazole, quinoxaline, dibenzofuran, benzimidazole, indene, indeno oxadiazine, indazole or an indole ring; B is a bond, CO or
 10 (CH₂)_n wherein n = 1; and R₁ and R₂ are both H.

Currently preferred compounds of formula (I) include:

3-amino-1,2,4-Triazine;
 3-amino-1,2,4-Triazole;
 2-methyl-4-nitroaniline;
 15 2-iodo-4-nitroaniline;
 4-amino-3-chloro-5-methylbenzoic acid;
 1-(4'Aminophenyl)-1,2,4-triazole;
 2-acetamidophenol;
 5-chloro-2,3-dihydroxypyridine;
 20 2-methyl-3-(1H-pyrazol-5-yl)imidazo(1,2-a)pyridine;
 5-nitro-2,3-dihydro-1H-benzo(d)imidazol-2-one;
 4-(methylamino)pyridine;
 2-chloro-4-nitrobenzamide;
 2-ethylformanilide;
 25 6-aminoindazole;
 2,3-diaminobenzoic acid;
 1-(5-chloro-2-methylphenyl)-2-thiourea;
 4,5-diiodo-1H-imidazole;
 1H-indene-1,3(2H)-dione 1-methylhydrazone;
 30 3-hydroxyindole;

- 3,4-dihydro-1H-quinoxalin-2-one;
 1S,6S,7R,8R,8aR)-1,6,7,8-Tetrahydroxyoctahydroindolizidine;
 2-naphthalen-1-yl-2,3-dihydro-1H-pyrimidine;
 benzo(b)thiophen-3-ylmethylaniline;
 5 1-allyl-2,4-dioxo-1,2,3,4-tetrahydro-5-pyrimidinecarbonitrile; Methyl (*S*)-*N*-(7-chloro-2,3,4a,5-tetrahydro-4a-(methoxycarbonyl)indeno(1,2-*e*)(1,3,4)oxadiazin-2-ylcarbonyl)-4'-(trifluoromethoxy)carbanilate (Indoxacarb-MP);
 4-acetylpyridine; and
 thioisonicotinamide.

- 10 In another embodiment of the present invention, the ubiquitin conjugating enzyme inhibitor compounds that selectively bind to the enzyme catalytic site are described by formula (II):



wherein:

- 15 A is a 3-6 membered substituted or unsubstituted cycloaliphatic or heterocycloaliphatic ring that is optionally fused to an aromatic ring;
 B is a bond, CO, SO₂ or (CH₂)_n wherein n = 1-3; and
 R₁ and R₂ are each independently H, alkyl or aryl groups that are optionally substituted.

- 20 Preferred compounds of formula II include, but are not limited to compounds in which the heterocycloaliphatic ring comprises at least one nitrogen atom and optionally, additional heteroatoms including nitrogen (N), oxygen (O) and sulfur (S); B is a bond, CO or (CH₂)_n wherein n = 1; and R₁ and R₂ are both H. Preferred ring substituents in the cycloaliphatic or heterocycloaliphatic ring in compounds of formula II include one or more
 25 atoms or groups chosen from hydroxyl, halogen, CO and alkyl.

Currently preferred compounds of formula (II) include:

- 1-Phenyl-4-methyl-3-pyrazolidone;
 4-(Aminomethyl)piperidine;

- N-Phenyl-p-phenylenediamine;
 5-(aminomethyl)-3-(2H)-isoxazolone (Muscimol);
 (R)-2-aminomethylpyrrolidine;
 2-pyrrolidinone oxime; and
 1-cyclopropylethylamine.

In another embodiment of the present invention, the ubiquitin conjugating enzyme inhibitor compounds that selectively bind to the E1 binding site in the enzyme are substituted or unsubstituted cycloaliphatic or aromatic ring compounds preferably containing one or more nitrogen atoms in the ring, in one or more of the substituents groups, or both.

- Preferred enzyme E1 binding site compounds of the invention comprise monocyclic and bridged 7 or 8-membered carbocyclic rings, including cycloheptyl, cyclooctyl and bicyclo(2,2,1) heptenyl rings; piperidinyl; phenyl; quinolinyl and isoquinolinyl rings. Preferred nitrogen containing substituents included NR_3R_4 , $(\text{CH}_2)_n\text{N R}_3\text{R}_4$, CONH_2 , NH-NH-R_5 and C(S)-NH-R_6 , wherein R_3 , R_4 , R_5 and R_6 are independently H, alkyl, cycloalkyl and aryl, and n is 1-5.

Currently preferred ubiquitin conjugating enzyme E1 binding site compounds of the invention include:

- N-1-cyclooctyl-4-hydroxy-1-piperidinecarbothioamide;
 6,7-dihydro-5H-dibenzo(a,c)cycloheptene-6-carboxylic acid;
 4-Methylcyclohexylamine;
 3-(dimethylamino)-1-(5-fluoro-2-hydroxyphenyl)prop-2-en-1-one;
 3-Ethoxyphenethylamine;
 N-bicyclo(2.2.1)hept-5-en-2-ylthiourea;
 4-Fluorobenzenesulfonamide;
 2-((4-fluorophenyl)hydrazono)malononitrile;
 3-Fluoro-4-hydroxybenzaldehyde; and
 1-Aminoisoquinoline.

In Vitro and In Vivo testing of the Identified Compounds

The compounds of the invention may be demonstrated to inhibit tumor cell proliferation, cell transformation and tumorigenesis *in vitro* or *in vivo* using a variety of assays known in the art, or described herein. Such assays can use cells of a cancer cell line or cells from a patient. Many assays well-known in the art can be used to assess such survival and/or growth, for example, cell proliferation can be assayed by measuring (3H) thymidine incorporation, by direct cell count, by detecting changes in transcription, translation or activity of known genes such as proto-oncogenes (e.g., fos, Myc) or cell cycle markers (Rb, cdc2, cyclin A, cyclin B, D1, D2, or E). The levels of such protein and mRNA and activity can be determined by any method well known in the art. For example, protein can be quantitated by known immunodiagnostic methods such as Western blotting or immunoprecipitation using commercially available antibodies (for example, many cell cycle marker antibodies can be obtained from Santa Cruz, Inc.). mRNA can be quantitated by methods that are well known and routine in the art, for example by Northern analysis, RNase protection, the polymerase chain reaction in conjunction with reverse transcription, etc. Cell viability can be assessed by using trypan-blue staining or other cell death or viability markers known in the art. Differentiation can be assessed visually based on changes in morphology, etc. The present invention provides for cell cycle and cell proliferation analysis by a variety of techniques known in the art, including but not limited to the following those disclosed herein. As one example, bromodeoxyuridine ("BRDU") incorporation may be used as an assay to identify proliferating cells. The BRDU assay identifies a cell population undergoing DNA synthesis by incorporation of BRDU into newly synthesized DNA. Newly synthesized DNA may then be detected using an anti-BRDU antibody (see Hoshino et al., 1986, Int. J. Cancer 38, 369; Campana et al., 1988, J. Immunol. Meth. 107, 79).

Cell proliferation may also be examined using (3H)-thymidine incorporation (see, e.g., Chen, J., 1996, Oncogene 13:1395-403; Jeoung, J., 1995, J. Biol. Chem. 270:18367-73). This assay allows for quantitative characterization of S-phase DNA synthesis. In this assay, cells synthesizing DNA will incorporate (3H)-thymidine into newly synthesized DNA. Incorporation may then be measured by standard techniques in the art such as by counting of radioisotope in a Scintillation counter (e.g., Beckman LS 3800 Liquid Scintillation Counter).

Detection of proliferating cell nuclear antigen (PCNA) may also be used to measure cell proliferation. PCNA is a 36 kilodalton protein whose expression is elevated in

proliferating cells, particularly in early G1 and S phases of the cell cycle and therefore may serve as a marker for proliferating cells. Positive cells are identified by immunostaining using an anti-PCNA antibody (see Li et al., 1996, *Curr. Biol.* 6:189-199; Vassilev et al., 1995, *J. Cell Sci.* 108:1205-15).

5 Cell proliferation may be measured by counting samples of a cell population over time (e.g., daily cell counts). Cells may be counted using a hemacytometer and light microscopy (e.g., HyLite hemacytometer, Hausser Scientific). Cell number may be plotted against time in order to obtain a growth curve for the population of interest. In a preferred embodiment, cells counted by this method are first mixed with the dye Trypan-blue (Sigma),
10 such that living cells exclude the dye, and are counted as viable members of the population. DNA content and/or mitotic index of the cells may be measured, for example, based on the DNA ploidy value of the cell. For example, cells in the G1 phase of the cell cycle generally contain a 2N DNA ploidy value. Cells in which DNA has been replicated but have not progressed through mitosis (e.g., cells in S-phase) will exhibit a ploidy value higher than 2N
15 and up to 4N DNA content. Ploidy value and cell-cycle kinetics may be further measured using propidium iodide assay (see, e.g., Turner, T., et al., 1998, *Prostate* 34:175-81). Alternatively, the DNA ploidy may be determined by quantitation of DNA Feulgen staining (which binds to DNA in a stoichiometric manner) on a computerized microdensitometry staining system (see, e.g., Bacus, S., 1989, *Am. J. Pathol.* 135:783-92).

20 In another embodiment, DNA content may be analyzed by preparation of a chromosomal spread (Zabalou, S., 1994, *Hereditas* 120:127-40; Pardue, 1994, *Meth. Cell Biol.* 44:333-351). The expression of cell-cycle proteins (e.g., CycA, CycB, CycE, CycD, cdc2, Cdk4/6, Rb, p21 or p27) provide crucial information relating to the proliferative state of a cell or population of cells. For example, identification in an anti-proliferation signaling
25 pathway may be indicated by the induction of p21^{cip1}. Increased levels of p21 expression in cells results in delayed entry into G1 of the cell cycle (Harper et al., 1993, *Cell* 75:805 to 816; Li et al., 1996, *Curr. Biol.* 6:189-199). p21 induction may be identified by immunostaining using a specific anti-p21 antibody available commercially (e.g. from Santa Cruz, Inc.). Similarly, cell-cycle proteins may be examined by Western blot analysis using
30 commercially available antibodies. In another embodiment, cell populations are synchronized prior to detection of a cell cycle protein. Cell-cycle proteins may also be

detected by FACS (fluorescence-activated cell sorter) analysis using antibodies against the protein of interest.

Detection of changes in length of the cell cycle or speed of cell cycle may also be used to measure inhibition of cell proliferation by a Organic compound. In one embodiment the length of the cell cycle is determined by the doubling time of a population of cells (e.g., using cells contacted or not contacted with one or more compounds of the invention). In another embodiment, FACS analysis is used to analyze the phase of cell cycle progression, or purify G1, S. and G2/M fractions (see, e.g., Delia, D. et al., 1997, Oncogene 14:2137-47).

Lapse of cell cycle checkpoint(s), and/or induction of cell cycle checkpoint(s), may be examined by the methods described herein, or by any method known in the art. Without limitation, a cell cycle checkpoint is a mechanism which ensures that a certain cellular events occur in a particular order. Checkpoint genes are defined by mutations that allow late events to occur without prior completion of an early event (Weiner, T., and Hartwell, L., 1993, Genetics, 134:63-80). Induction or inhibition of cell cycle checkpoint genes may be assayed, for example, by Western blot analysis, or by immunostaining, etc. Lapse of cell cycle checkpoints may be further assessed by the progression of a cell through the checkpoint without prior occurrence of specific events (e.g. progression into mitosis without complete replication of the genomic DNA).

Activity of signaling and cell cycle proteins and/or protein complexes is often mediated by a kinase activity. The present invention provides for analysis of kinase activity by assays such as the histone H1 assay (see, e.g., Delia, D. et al., 1997, Oncogene 14:2137-47). The compounds of the invention can also be demonstrated to alter cell proliferation in cultured cells *in vitro* using methods which are well known in the art. Specific examples of cell culture models include, but are not limited to, for lung cancer, primary rat lung tumor cells (Swafford et al., 1997, Mol. Cell. Biol., 17:1366-1374) and large-cell undifferentiated cancer cell lines (Mabry et al., 1991, Cancer Cells, 3:53-58); colorectal cell lines for colon cancer (Park and Gazdar, 1996, J. Cell Biochem. Suppl. 24:131-141); multiple established cell lines for breast cancer (Hambly et al., 1997, Breast Cancer Res. Treat. 43:247-258; Gierthy et al., 1997, Chemosphere 34:1495-1505; Prasad and Church, 1997, Biochem. Biophys. Res. Commun. 232:14-19); a number of well-characterized cell models for prostate cancer (Webber et al., 1996, Prostate, Part 1, 29:386-394; Part 2, 30:58-64; and Part 3, 30:136-142; Boulikas, 1997, Anticancer Res. 17:1471-1505); for genitourinary cancers,

continuous human bladder cancer cell lines (Ribeiro et al., 1997, Int. J. Radial. Biol. 72:11-20); organ cultures of transitional cell carcinomas (Booth et al., 1997, Lab Invest. 76:843-857) and rat progression models (Vet et al., 1997, Biochim. Biophys Acta 1360:39-44); and established cell lines for leukemias and lymphomas (Drexler, 1994, Leuk. Res. 18:919-927, 5 Tohyama, 1997, Int. J. Hematol. 65:309-317).

The compounds of the invention can also be demonstrated to inhibit cell transformation (or progression to malignant phenotype) *in vitro*. In this embodiment, cells with a transformed cell phenotype are contacted with one or more compounds of the invention, and examined for change in characteristics associated with a transformed 10 phenotype (a set of *in vitro* characteristics associated with a tumorigenic ability *in vivo*), for example, but not limited to, colony formation in soft agar, a more rounded cell morphology, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, release of proteases such as plasminogen activator, increased sugar transport, decreased serum requirement, or expression of fetal antigens, etc. (see Luria et al., 1978, General 30 Virology, 3-D Ed., John Wiley & Sons, New York, pp. 436-446). Loss of invasiveness or decreased adhesion may also be used to demonstrate the anti-cancer effects of the compounds of the invention. For example, a critical aspect of the formation of a metastatic cancer is the ability of a precancerous or cancerous cell to detach from primary site of disease and establish a novel colony of growth at a secondary site. The ability of a cell to invade 20 peripheral sites is reflective of a potential for a cancerous state.

Loss of invasiveness may be measured by a variety of techniques known in the art including, for example, induction of E-cadherin-mediated cell-cell adhesion. Such E-cadherin-mediated adhesion can result in phenotypic reversion and loss of invasiveness (Hordijk et al., 1997, Science 278:1464-66). Loss of invasiveness may further be examined 25 by inhibition of cell migration. A variety of 2-dimensional and 3-dimensional cellular matrices are commercially available (Calbiochem-Novabiochem Corp. San Diego, CA). Cell migration across or into a matrix may be examined by microscopy, time-lapsed photography or videography, or by any method in the art allowing measurement of cellular migration. In a related embodiment, loss of invasiveness is examined by response to hepatocyte growth 30 factor (HGF). HGF induced cell scattering is correlated with invasiveness of cells such as Madin-Darby canine kidney (MDCK) cells. This assay identifies a cell population that has lost cell scattering activity in response to HGF (Hordijk et al., 1997, Science 278:1464-66).

Alternatively, loss of invasiveness may be measured by cell migration through a chemotaxis chamber (Neuroprobe/ Precision Biochemicals Inc. Vancouver, BC). In such assay, a chemo-attractant agent is incubated on one side of the chamber (e.g., the bottom chamber) and cells are plated on a filter separating the opposite side (e.g., the top chamber).

- 5 In order for cells to pass from the top chamber to the bottom chamber, the cells must actively migrate through small pores in the filter. Checkerboard analysis of the number of cells that have migrated may then be correlated with invasiveness (see, e.g., Ohnishi, T., 1993, Biochem. Biophys. Res. Commun. 193:518-25).

- The compounds of the invention can also be demonstrated to inhibit tumor formation
- 10 *in vivo*. A vast number of animal models of hyperproliferative disorders, including tumorigenesis and metastatic spread, are known in the art and are disclosed herein (see Chapter 317, "Principals of Neoplasia," in Harrison's: Principals of Internal Medicine, 13th Edition, Isselbacher et al., eds., McGraw-Hill, New York, p. 1814, and Lovejoy et al., 1997, J. Pathol. 181:130-135). Specific examples include for lung cancer, transplantation of tumor
- 15 nodules into rats (Wang et al., 1997, Ann. Thorac. Surg. 64:216-219) or establishment of lung cancer metastases in SCID mice depleted of NK cells (Yono and Sone, 1997, Gan To Kagaku Ryoho 24:489-494); for colon cancer, colon cancer transplantation of human colon cancer cells into nude mice (Gutman and Fidler, 1995, World J. Surg. 19:226-234), the cotton top tamarin model of human ulcerative colitis (Warren, 1996, Aliment. Pharmacol. Ther. Supp
- 20 12:45-47) and mouse models with mutations of the adenomatous polyposis tumor suppressor (Polakis, 1997, Biochim. Biophys. Acta 1332:F127-F147); for breast cancer, transgenic models of breast cancer (Dankort and Muller, 1996, Cancer Treat. Res. 83:71-88; Amundadittir et al., 1996, Breast Cancer Res. Treat. 39:119-135) and chemical induction of tumors in rats (Russo and Russo, 5 1996, Breast Cancer Res. Treat. 39:7-20); for prostate
- 25 cancer, chemically-induced and transgenic rodent models, and human xenograft models (Royal et al., 1996, Semin. Oncol. 23:35-40), for genitourinary cancers, induced bladder neoplasm in rats and mice (Oyasu, 1995, Food Chem. Toxicol 33:747-755) and xenografts of human transitional cell carcinomas into nude rats (Jarrett et al., 1995, J. Endourol. 9:1 -7); and for hematopoietic cancers, transplanted allogeneic marrow in animals (Appelbaum, 1997,
- 30 Leukemia 11 (Suppl. 4):S15- S17). Further, general animal models applicable to many types of cancer have been described, including, but not restricted to, the p53-deficient mouse model (Donehower, 1996, Semin. Cancer Biol. 7:269-278), the Min mouse (Shoemaker et al., 1997,

Biochem. Biophys. Acta, 1332:F25-F48), and immune responses to tumors in rat 15 (Frey, 1997, Methods, 12:173-188).

For example, a organic compound can be administered to a test animal, in one embodiment a test animal predisposed to develop a type of tumor, and the test animal
5 subsequently examined for an decreased incidence of tumor formation in comparison with an animal not administered the Organic compound. Alternatively, a Organic compound can be administered to test animals having tumors (e.g., animals in which tumors have been induced by introduction of malignant, neoplastic, or transformed cells, or by administration of a carcinogen) and subsequently examining the tumors in the test animals for tumor regression
10 in comparison to animals not administered the Organic compound.

Modes of Administration

The organic compounds are advantageously useful in veterinary and human medicine. For example, the compounds of the invention are useful for the treatment or prevention of hyperproliferative disorders such as cancer and neoplastic disorders. The compounds are also
15 useful for the treatment or prevention of cystic fibrosis, neurodegenerative disorders and viral disorders.

The present pharmaceutical compositions, which comprise an effective amount of an organic compound, can be administered by any convenient route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa,
20 rectal and intestinal mucosa, etc.) and can be administered together with another therapeutic agent. Administration can be systemic or local. Various delivery systems are known, e.g., encapsulation in liposomes, microparticles, microcapsules or capsules, and can be used to administer an Organic compound. In certain embodiments, more than one Organic compound is administered to a patient.

25 Methods of administration include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, oral, sublingual, intranasal, intracerebral, intravaginal, transdermal, rectal, by inhalation, or topically to the ears, nose, eyes, or skin. The preferred mode of administration is left to the discretion of the practitioner, and will depend in-part upon the site of the medical condition (such as the site of
30 cancer).

The method of administering the ubiquitin conjugating enzyme inhibiting compounds of the invention may be suitably chosen depending on the nature and/or the site of the hyperproliferative disorder to be treated. For example, it may be desirable to administer one or more compounds of the invention locally to the area in need of treatment. This can be
5 achieved, for example, and not by way of limitation, by local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as silastic membranes, or fibers. In one embodiment, administration can be by direct injection at the
10 site (or former site) of a cancer, tumor or neoplastic or pre-neoplastic tissue.

In certain other situations, it may be desirable to administer one or more organic compounds using any suitable route, including intraventricular and intrathecal injection. Intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

15 Pulmonary administration can also be employed, e.g. by use of an inhaler or nebulizer, and formulation with an aerosolizing agent, or via perfusion in a fluorocarbon or synthetic pulmonary surfactant. In certain embodiments, the organic compound can be formulated as a suppository, with traditional binders and carriers such as triglycerides. In another embodiment, the organic compound can be delivered in a vesicle, in particular a
20 liposome (see Langer, *Science* 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp.353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*)

In yet another example, the compound of the invention can be delivered in a controlled-release system. In one embodiment, a pump may be used (see Langer, *supra*;
25 Sefton, *CRC Crit. Ref. Biomed. Eng.* 14:201 (1987); Buchwald et al., *Surgery* 88:507 (1980); Saudek et al., *N. Engl. J. Med.* 321:574 (1989)). In another embodiment, polymeric materials can be used (see *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J.
30 *Macromol. Sci. Rev. Macromol. Chem.* 23:61 (1983); see also Levy et al., *Science* 228:190 (1985); During et al., *Ann. Neurol.* 25:351 (1989); Howard et al., *J. Neurosurg.* 71:105 (1989)). In yet another embodiment, a controlled- release system can be placed in proximity

of the target of the organic compound, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in *Medical Applications of Controlled Release*, Sahara, vol. 2, pp. 115-138 (1984)). Other controlled-release systems discussed in the review by Langer (*Science* 249: 1527-1533 (1990)) may be used.

- 5 The present pharmaceutical compositions contain an effective amount of an organic compound, preferably in purified form, together with a suitable amount of a pharmaceutically acceptable carrier so as to provide the form for proper administration to the patient.

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

- 25 The present pharmaceutical compositions can be in the form of solutions, suspensions, emulsion, tablets, pills, pellets, capsules, capsules containing liquids, powders, sustained-release formulations, suppositories, emulsions, aerosols, sprays, suspensions, or any other form suitable for use. In one embodiment, the pharmaceutically acceptable composition is a capsule comprising the compounds of the invention. Other examples of
30 suitable pharmaceutical carriers include auxiliary agents, and exigents such as, for example, those described in "Remington's Pharmaceutical Sciences" 20th Ed. (2002).

In one embodiment, the compounds of the invention are formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compounds of the invention for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the compositions may also include a solubilizing agent. Compositions for intravenous administration may optionally include a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the organic compound is to be administered by infusion, it can be dispensed, for example, with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the organic compound is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration. Compositions for oral delivery may be in the form of tablets, lozenges, aqueous or oily suspensions, granules, powders, emulsions, capsules, syrups, or elixirs, for example, orally administered compositions may contain one or more optional agents, for example, sweetening agents such as fructose, aspartame or saccharin; flavoring agents such as peppermint, oil of wintergreen, or cherry; coloring agents; and preserving agents, to provide a pharmaceutically palatable preparation. Moreover, where in tablet or pill form, the compositions may be coated to delay disintegration and absorption in the gastrointestinal tract thereby providing a sustained action over an extended period of time. Selectively permeable membranes surrounding an osmotically active driving compound are also suitable for orally administered compounds of the invention. In these later platforms, fluid from the environment surrounding the capsule is imbibed by the driving compound, which swells to displace the agent or agent composition through an aperture. These delivery platforms can provide an essentially zero order delivery profile as opposed to the spiked profiles of immediate release formulations. A time delay material such as glycerol monostearate or glycerol stearate may also be used. Oral compositions can include standard carriers such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Such carriers are preferably of pharmaceutical grade.

Dosage of the Organic Compound

The amount of the organic compound that will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges. The precise dose will also
5 depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges, particularly for intravenous administration, are generally about 20-500 micrograms of an organic compound per kilogram body weight. In specific preferred embodiments of the invention, the i.v. dose is about 10-40, 30-60, 60-
10 100, or 100-200 micrograms per kilogram body weight. In other embodiments, the i.v. dose is about 75-150, 150-250, 250-375 or 375-500 micrograms per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 mg/kg body weight to 1 mg/kg body weight. Suppositories generally contain an organic compound in the range of about 0.5 % to 10% by weight. Oral compositions preferably contain an organic
15 compound about 10% to 95% by weight of an organic compound. In specific preferred embodiments of the invention, suitable dose ranges for oral administration are generally about 1-500 micrograms of an Organic compound per kilogram body weight. In specific preferred embodiments, the oral dose is about 1-10, 10-30, 30-90, or 90-150 micrograms per kilogram body weight.

20 In other embodiments, the oral dose is about 150-250, 250-325, 325-450 or 450-1000 micrograms per kilogram body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems. Such animal models and systems are well known in the art and are disclosed herein.

The invention also provides pharmaceutical kits comprising a container containing a
25 organic compound. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration; or instructions for use. The kit can also comprise a container containing a chemotherapeutic agent useful for treating cancer or a
30 neoplastic disease.

The compounds of the invention are preferably assayed *in vitro*, and then *in vivo* for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *in vitro*

assays can be used to determine whether administration of one or more compounds of the invention is preferred.

In one embodiment, a patient tissue sample is grown in culture, and contacted or otherwise administered with an organic compound, and the effect of such organic compound upon the tissue sample is observed and compared to a non-contacted tissue. In other 5 embodiments, a cell-culture model is used in which the cells of the cell culture are contacted or otherwise administered with a organic compound, and the effect of such organic compound upon the cell-culture is observed and compared to a non-contacted cell culture. Generally, a lower level of proliferation or survival of the contacted cells compared to the 10 non-contacted cells indicates that the organic compound is effective to treat the patient. Such compounds of the invention may also be demonstrated effective and safe using animal model systems.

Assays for measuring the *in vitro* and *in vivo* activity are described in Examples 2-4. Other methods will be known to the skilled artisan and are within the scope of the invention.

15 Combination Therapy

A hyperproliferative disorder, including but not limited to cancer or a neoplastic disease, a neoplasm, a tumor, a metastasis, or any disease or disorder characterized by uncontrolled cell growth, can be treated or prevented by administration of an effective amount of an organic compound. In one embodiment, a composition comprising an effective 20 amount of one or more organic compounds or a pharmaceutically acceptable salt thereof, is administered. In certain embodiments, the invention encompasses methods for treating or preventing cancer or a neoplastic disease comprising administering to a patient in need thereof an effective amount of a organic compound and another therapeutic agent. In one embodiment, the therapeutic agent is a chemotherapeutic agent including, but not limited to, 25 methotrexate, taxol, mercaptopurine, thioguanine, hydroxyurea, cytarabine, cyclophosphamide, ifosfamide, nitrosoureas, cisplatin, carboplatin, mitomycin, dacarbazine, procarbazine, etoposides, camptothecins, bleomycin, doxorubicin, idarubicin, daunorubicin, dactinomycin, plicamycin, mitoxantrone, asparaginase, vinblastine, vincristine, vinorelbine, paclitaxel, and docetaxel. In one embodiment, the organic compound exerts its activity at the 30 same time the other therapeutic agent exerts its activity.

Treatable and Preventable Hyperproliferative Disorders

Hyperproliferative disorders include cancerous disease states. Cancerous disease states may be categorized as pathologic, *i.e.*, characterizing or constituting a disease state, *e.g.*, malignant tumor growth, or may be categorized as non-pathologic, *i.e.*, a deviation from normal but not associated with a disease state, *e.g.*, cell proliferation associated with wound repair. The term is meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. The term "cancer" includes malignancies of the various organ systems, such as those affecting lung, breast, thyroid, lymphoid, gastrointestinal, and genito-urinary tract, as well as adenocarcinomas which include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer and/or testicular tumors, non-small cell carcinoma of the lung, cancer of the small intestine and cancer of the esophagus. The term "carcinoma" is art recognized and refers to malignancies of epithelial or endocrine tissues including respiratory system carcinomas, gastrointestinal system carcinomas, genitourinary system carcinomas, testicular carcinomas, breast carcinomas, prostatic carcinomas, endocrine system carcinomas, and melanomas. Exemplary carcinomas include those forming from tissue of the cervix, lung, prostate, breast, head and neck, colon and ovary. The term "carcinoma" also includes carcinosarcomas, *e.g.*, which include malignant tumors composed of carcinomatous and sarcomatous tissues. An "adenocarcinoma" refers to a carcinoma derived from glandular tissue or in which the tumor cells form recognizable glandular structures. The term "sarcoma" is art recognized and refers to malignant tumors of mesenchymal derivation.

In one embodiment of the invention the compounds of the invention can be used to treat or prevent a variety of hyperproliferative disorders. Preferentially, the compounds of the invention can be used to treat breast cancer, colon cancer and prostate cancer. Additional hyperproliferative disorders include but are not limited to, cancer of the head, neck, eye, skin, mouth, throat, esophagus, chest, bone, lung, colon, sigmoid, rectum, stomach, prostate, ovary, testicle, kidney, liver, pancreas, brain, intestine, heart or adrenals (for a review of such disorders, 10 see Fishman et al., 1985, *Medicine*, 2d Ed., J.B. Lippincott Co., Philadelphia).

Treatable and Preventable Ub conjugating Disorders

In addition to hyperproliferative disorders, dysfunction of the ubiquitin system has also been implicated in Huntington's, Parkinson's and Alzheimer's disease (Reboud-Ravaux, M. *Protein Degradation in Health and Disease*. Volume 29 (Heidelberg: Springer Verlag,

2002). In one embodiment, compounds of the invention can be used to treat a Ub conjugating disorder, e.g., cancer, Huntington's, Parkinson's and Alzheimer's disease.

Parkinson's disease is a slowly progressing, degenerative nervous system disorder characterized by tremors when at rest, sluggish initiation of movements and muscle rigidity.

- 5 In Parkinson's disease, nerve cells in the basal ganglia degenerate, resulting in lower production of dopamine and fewer connections with other nerve cells and muscles.

- Generally, Parkinson's disease begins with a tremor in the resting hand that decreases when the hand moves purposefully and disappears during sleep. Eventually, the tremor may progress to the other hand, the arms, the legs, and effect the jaw, tongue, forehead and
10 eyelids. Individuals with Parkinson's disease often have difficulty initiating a movement, and experience muscle stiffness. As a result of these symptoms, any type of minor physical activity becomes extremely difficult.

- A variety of drugs may be used to treat Parkinson's disease. These drugs include levodopa, bromocriptine, pergolide, selegiline, anticholinergics, antihistamines,
15 antidepressants, propranolol and amantadine. Although these drugs do not stop the progression of, or cure Parkinson' disease, they may make physical activity easier.

The mobility of a Parkinson's patient can be maintained by continuing to perform daily activities, exercising, physical therapy and the use of mechanical aids such as wheeled walkers.

- 20 Parkinson's disease is characterized by the idiopathic and progressive loss of mesencephalic dopaminergic neurons of the substantia nigra leading to a loss of dopamine in the striatura, the main projection field of these neurons. This neuronal degeneration results in the major symptoms of Parkinson's disease, *i.e.*, tremor, muscular rigidity, difficulty in movement initiation, and loss of postural reflex (Pratt and McPherson, 1997, Cytokine and
25 Growth Factor Reviews, 8:267-292).

- Alzheimer's disease is the most common form of dementia. Although the exact cause of Alzheimer's disease is not known, genetic factors play a role in the occurrence and development of this disease. Furthermore, the ubiquitin system has been implicated in the development of Alzheimer's disease. Patient's with Alzheimer's disease suffer from
30 degeneration of parts of the brain, resulting in the destruction of cells and the reduction of the

responsiveness of the remaining cells to the chemicals that transmit signals in the brain. Abnormal tissues, termed senile plaques, neurofibrillary tangles, and abnormal proteins appear in the brain. During an autopsy, Alzheimer's disease can be diagnosed by the presence of abnormal brain tissue, characterized by a loss of nerve cells, the presence of
5 tangles within the remaining nerve cells, and plaques made of amyloid scattered throughout the brain tissue.

The progression of Alzheimer's disease can be slowed but not stopped with a number of drugs including tacrine, and donepezil.

Dementia resulting from Alzheimer's disease normal begins subtly and results in a
10 gradual deterioration of memory. The first sign of dementia is usually forgetfulness (Berkow et al., *supra*).

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by neuronal loss primarily in the temporal lobes and neocortex. This neuronal loss causes progressive loss of cognitive function and ultimately leads to severe dementia. Histological
15 analysis of brains from AD patients has revealed that they contain numerous neuritic plaques consisting substantially of aggregates of β amyloid peptide ($A\beta$), a 39-43 amino acid peptide derived from a larger transmembrane protein referred to as the amyloid precursor protein (APP). Numerous investigators have shown that $A\beta$ is neurotoxic *in vitro* and *in vivo*, which has led to the conclusion that $A\beta$ accumulation is a key factor in the development of AD.
20 The cause of increased accumulation of $A\beta$ in the brains of AD patients is not known but is believed to be a consequence of increased production of APP and/or abnormal processing of the protein ultimately leading to elevated levels of $A\beta$.

The suitability of the compounds of the invention for treatment of Huntington's, Parkinson's and Alzheimer's disease can be assessed in any of a number of animal models.
25 For example, animal models for Huntington's disease (see, e.g., Mangiarini et al., 1996, Cell 87: 493-506, Lin et al., 2001, Hum. Mol. Genet. 10: 137-144), Alzheimer's disease (Hsiao, 1998, Exp. Gerontol. 33: 883-889; Hsiao et al., 1996, Science 274: 99-102), Parkinson's disease (Kim et al., 2002, Nature 418: 50-56) are known and can also be used to evaluate the efficacy of compounds of the invention.

30 Animals administered the compounds are evaluated for symptoms relative to animals not administered the compounds. A change in the severity of symptoms (e.g., a 10% or

greater improvement in one or more symptoms), or a delay in the onset of symptoms, in treated versus untreated animals is indicative of therapeutic efficacy.

EXAMPLES

Example 1: Identification of Ubc inhibitors by structure based screening.

5 A structure based screening assay was performed on yeast Ubc4 to identify compounds that bind to the active sites of these molecules. 3-D coordinates for yeast Ubc4 (PDB ID No. 1QCQ) were entered into the InsightII program (InsightII, Accelrys, Inc., San Diego, CA). Calculations were performed on the molecule to identify the active sites. Yeast Ubc4 active sites identified with the InsightII program included amino acids Lys64, Pro66,
10 Lys67, Ile68, Asn84, Ile85, Leu90, Lys91 and Leu120. Similarly, another identified active site included amino acids Pro65, Pro66, Lys67, Ile68, Asn84, Leu90, Lys91 and Leu120 of *S. cerevisiae* Ubc4. Active sites in human Ubc5a included Lys66, Ile67, Ala68, Ser83, Cys85, Leu86, Leu89 and Arg90 of SEQ ID NO:2 and Pro64, Pro65, Lys66, Ile67, Ser83, Ile84, Cys85, Leu86, Leu89, Arg90 and Leu119 of SEQ ID NO:2. Active sites in *S. cerevisiae* Ubc7
15 included Pro68, Lys70, Tyr83, Glu87, Val88, Cys89, Leu93 and His94 of SEQ ID NO:3; and Pro68, Pro69, Lys70, Leu71, Tyr83, Glu87, Val88, Cys89, Leu93 and His94 of SEQ ID NO:3.

 After identifying the active sites a screen for identifying binding compounds was performed with the LUDI program using compounds contained in the Available Chemical
20 Directory (ACD). The ACD was converted to a LUDI compatible library using the Genfra program and hydrogens were added to all compounds of the library. The center of search for the LUDI run was defined based upon active sites. A search radius of 9-12 Å was chosen for the different E2s. In LUDI, a rigid body protein-ligand docking method was used to reduce time, and a standard protonation states at pH 7.0 was used for all Ubcs/E2s and ligands.

25 LUDI identified numerous binding compounds which were scored by the Boehm scoring function (Boehm, 1998). The highest scoring compounds were manually selected to remove those that protruded from the binding site, displaying hydrophobic moieties pointing into solution. Additionally only those that formed at least two hydrogen bonds with a good contact score were selected. FIG. 1 shows the binding interaction between yeast Ubc4 and
30 one of the compounds identified by the screen (HBS01). HBS01 was found to interact with Pro66, Ile85 and Leu90 of SEQ ID NO:1. Compound OBWP was found to interact with

Pro66, Ile68, Leu90 and Lys91 of SEQ ID NO:1. Compound 05RB was found to interact with Ile67 and Leu89 of SEQ ID NO:2. Compound 0464 was found to interact with Pro65, Ile67, and Ser 83 of SEQ ID NO:2. Compound OC7K was found to interact with Glu87, Val88, Leu93 and His94 of SEQ ID NO:3. Compound 8EAG was found to interact with
5 Pro69, Leu71 and Val88 of SEQ ID NO:3. HBS01 was further evaluated by *in vivo* and *in vitro* assays as described herein.

Example 2: Determination of IC50 values for HBS01 in the breast cell lines MCF-7 and MCF-7-Adr.

In order to determine the IC50 values of HBS01 an MTS assay was performed on
10 MCF-7 and MCF-7/ADR (adramycin resistance) cell lines. Cells were plated in a 96 well plate at 5,000 cells/well. After plating, cells were treated for 96 hours with either HBS01, methotrexate (MTX), doxorubicin (DOX), or 5-Fluorouracil (5FU) and an MTS assay was performed according to manufacturer's protocol with the CELL TITER 96 AQ non-radioactive cell proliferation assay kit (Promega, Cat. No. G5421). Viable cells were
15 quantitated based on absorbance at 490 nm. IC50 values for HBS01, MTX, DOX and 5FU are shown in FIG. 2. The results indicated that HBS01 had an IC50 value of 30 nM in MCF-7 and MCF-7Adr. Thus, HBS01 was an extremely potent inhibitor of MCF-7 and MCF-7Adr proliferation.

Example 3: Effect of HBS01 in C85 colorectal tumors in mice.

C85 human colorectal cancer cell growth was assessed in nude mice (NIH, Cr:(MCR)-Fox1nu(nu/nu homologous), Taconic Inc., Germantown, NY) treated or pretreated with HBS01 over a 19 day period. Briefly, pretreated animals were administered three daily doses of HBS01 at 17mg/Kg given on day -3, -2, and -1 prior to tumor cell injection on day 0. No further treatment was given to the pretreated animals. Nude mice were injected in the flanks
25 with 1 million C85 human colorectal cancer cells. Treated animals were treated with daily injections of HBS01 at 17 mg/kg per at days 10, 11, 12, 15, 16 and 17 following appearance of palpable tumors on day 10.

Tumor diameters were measured with calipers every alternate day and tumor volumes were calculated by $1/2ab^2$ where a is the larger diameter (Friedman, HS., et al. Cancer Res.
30 46,2827-2833). The results are shown in FIG. 3 as tumor volume in cm^3 . The results indicated that treatment with HBS01 significantly inhibited the growth of established tumors

in animals treated with HBS01. Additionally, pretreating mice with HBS01 delayed tumor growth.

Example 4: Effect of HBS01 in MCF-7 breast cancer tumors in mice.

MCF-7 breast cancer cell growth was assessed in nude mice (NIH, Cr:(MCR)-
5 Fox1nu(nu/nu homogous), Taconic Inc., Germantown, NY) treated or pretreated with
HBS01 over 31 days. Briefly, pretreated animals were administered three daily doses of
HBS01 at 17mg/Kg given on day -3, -2, and -1 prior to tumor cell injection on day 0. No
further treatment was given to the pretreated animals. Nude mice were injected in the flanks
with 200,000 MCF-7 breast cancer cells. Treated animals were treated with daily injections
10 of HBS01 at 17 mg/kg per at days 10, 11, 12, 15, 16 and 17 following appearance of palpable
tumors on day 10. Tumor diameters were measured with calipers every alternate day and
tumor volumes were calculated by $1/2ab^2$ where a is the larger diameter (Friedman,HS., et al.
Cancer Res. 46,2827-2833). The results are shown in FIG 4 as tumor volume in cm^3 . The
results showed that HBS01 significantly inhibited tumor growth in animals treated with
15 HBS01 following MCF-7 palpable tumors. Additionally, pretreating mice with HBS01
delayed the onset of MCF-7 tumors.

Example 5: Clonogenic assays.

Compounds identified by structure based screening were assayed in a clonogenic
assay with C85 colorectal adenocarcinoma cells. Briefly, C85 cells were plated at 200 cells
20 per well in six well plates. Seventy-two hours after plating, compounds 05RB, 0BWP,
0FZK, 0RZL, HBS01, 01LX, 0464, 1BJO AND 27NT were added at various concentrations
(See Table 1). Colonies were counted 96 hours after treatment and IC50 concentrations
determined (Table 1). Results indicate that many of the compounds identified by structure
based screening were effective at inhibiting C85 tumor cell growth.

TABLE 1

CLONOGENIC ASSAY OF COLON C85s WITH Ubc/E2 INHIBITORS

Compound	ID No.	CONCENTRATIONS (μ M)								Target	IC-50
		0	625	312	156	78	39	19.5	9.7		
N-Phenyl-4-phenylenediamine	05RB	0	625	312	156	78	39	19.5	9.7	Ubc5a	37.5
2-Chloro-4-nitrobenzamide	0BWP	0	2000	1500	1000	750	500			Ubc4	600
5-chloro-2,3-dihydroxypyridine	0FZK	0	400	250	150	75	15			Ubc3	175
1-Phenyl-4-methyl-3-pyrazolidone	0RZL	0	70	35	15	7.5	3.75			Ubc3	50
4-(Aminomethyl)piperidine	HBS01*	0	100	50	25	12.5	6.25			Ubc4/ Ubc10	30nM*
2-Acetamidophenol	01LX	0	500	400	300	150	50			Ubc3	375
6-AMINOINDAZOLE	0464	0	1300	650	300	150	75			Ubc5a	70

Compound	ID No.	CONCENTRATIONS (μ M)								Target	IC-50
		0	200	150	100	50	25				
2-ethylformanilide	1BJO	0	200	150	100	50	25			Ubc5a	Non toxic
4-(Methylamino)pyridine	27NT	0	700	350	175	75	15			Ubc4	Non toxic

* denotes nM

Example 6: Effect of HBS01 on metastatic cancer growth.

The effect of HBS01 on the metastatic growth of enhanced green fluorescent protein (EGFP) marked C85 cells was assayed in nude mice (NIH, Cr:(MCr)-Fox1nu(nu/nu

5 homogygous), Taconic Inc., Germantown, NY). Briefly, nude mice were pre-treated with 14.5mg/kg of HBS01 every 24 hrs for five days. At the completion of pre-treatment the pre-treated or control non-treated mice were injected in the tail vein with 1×10^6 EGFP marked C85 cells. Ten days post-C85 injection, the mice were dissected and tumor size assessed based on intravital fluorescence imaging and liver biopsies. Results indicated that metastatic

10 tumors were not detectable in pre-treated mice compared with controls.

Example 7: Selective targeting by HBS01.

The ability of HBS01 to specifically inhibit selected protein ubiquitination pathways was assayed in C85 cell lines. Briefly, C85 cells were plated at 200 cells per well in six well plates.

15 Cells were either untreated (controls) or treated for 24 or 48 hrs with HBS01. After the treatment period, cells were harvested and extracts were prepared from the cells by freeze fracturing in liquid nitrogen in breakage buffer (50 mM Tris pH 7.5, 1 mM DTT, "Complete" protease inhibitor cocktail (Roche, Cat.# 1697498), 10 μ M MG132). Roughly 300 mg of total protein extracts in 300 μ l of breakage buffer was supplemented with 20 μ g of PHH-Ub

20 (HA and His6 tagged Ubiquitin) and incubated at 30°C for 60 min. 3X SDS-PAGE sample buffer was added to 40 μ l of this reaction and boiled for 3 min. The extracts were run on

12% Laemmli gels that were either stained with Coomassie Blue R-250 or blotted with HRP conjugated 12CA5 monoclonal antibody (Roche, Inc., Indianapolis, IN) and visualized by ECL, with the ECL plus western blot detection kit (Amersham Biosciences, Piscataway, NJ) according to manufacturers instructions.

- 5 As seen in FIG. 5, results indicated that control and treated cell lysates had similar ubiquitin intensities and banding patterns. This demonstrated that general inactivation of the ubiquitin conjugation pathways did not occur with HBS01 treatment.

Example 8: Blockage of ubiquination and accumulation of cyclin-B by HBS01.

- The ability of HBS01 to inhibit protein ubiquination of cyclin-B was assayed in C85
10 cell lines. Briefly, C85 cells were plated at 200 cells per well in six well plates. Cells were either untreated (controls) or treated for 24hrs with HBS01. After the treatment period, cells were harvested and extracts were prepared from the cells by freeze fracturing in liquid nitrogen in breakage buffer (50 mM Tris pH 7.5, 1 mM DTT, "Complete" protease inhibitor cocktail (Roche, Cat.# 1697498), 10 μ M MG132). Roughly 300 mg of total protein extracts
15 in 300 μ l of breakage buffer was supplemented with 20 μ g of PHH-Ub (HA and His6 tagged Ubiquitin) and incubated at 30°C for 60 min. 3X SDS-PAGE sample buffer was added to 40 μ l of this reaction and boiled for 3 min. The extracts were run on 12% Laemmli gels and Western Blots were performed with anti-cyclin-B antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Results were visualized by ECL, with the ECL plus western blot detection
20 kit (Amersham Biosciences, Piscataway, NJ) according to manufacturers instructions.

The results are shown in FIG. 6. The data indicated that a smear of ubiquinated cyclin-B is absent in control cell lysates but present in the lysates of cells treated with HBS01. Thus, cyclin-B accumulates in cells treated with HBS01. These results indicate that HBS01 selectively inhibited the ubiquination of cyclin-B.

- 25 All patents, patent applications, and published references cited herein are hereby incorporated by reference in their entirety. While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

We claim:

1. A method of inhibiting an ubiquitin conjugating enzyme with an organic compound comprising, selectively targeting said organic compound to one or more active sites of said
5 ubiquitin conjugating enzyme.
2. The method of claim 1, wherein said active site is within a catalytic site.
3. The method of claim 2, wherein said active site comprises the amino acid residues corresponding to Lys64, Pro66, Lys67, Ile68, Asn84, Ile85, Leu90, Lys91 and Leu120 of SEQ ID NO:1.
- 10 4. The method of claim 1, wherein said organic compound interacts with a binding domain comprising the amino acids corresponding to Pro66, Ile85 and Leu90 of SEQ ID NO:1.
5. The method of claim 2, wherein said active site comprises the amino acid residues corresponding to Pro65, Pro66, Lys67, Ile68, Asn84, Leu90, Lys91 and Leu120 of SEQ ID NO:1.
- 15 6. The method of claim 1, wherein said organic compound interacts with a binding domain comprising the amino acids corresponding to Pro66, Ile68, Leu90 and Lys91 of SEQ ID NO:1.
7. The method of claim 2, wherein said active site comprises the amino acid residues corresponding to Lys66, Ile67, Ala68, Ser83, Cys85, Leu86, Leu89 and Arg90 of SEQ ID
20 NO:2.
8. The method of claim 1, wherein said organic compound interacts with a binding domain comprising the amino acids corresponding to Ile67 and Leu89 of SEQ ID NO:2.
9. The method of claim 2, wherein said active site comprises the amino acid residues corresponding to Pro64, Pro65, Lys66, Ile67, Ser83, Ile84, Cys85, Leu86, Leu89, Arg90 and
25 Leu119 of SEQ ID NO:2.
10. The method of claim 1, wherein said organic compound interacts with a binding domain comprising the amino acids corresponding to Pro65, Ile67, and Ser 83 of SEQ ID NO:2.

11. The method of claim 1, wherein said organic compound binds to a binding domain in a E1 binding site.
12. The method of claim 1, wherein the ubiquitin conjugating enzyme is selected from the group consisting of: Ubc1, Ubc2, Ubc3, Ubc4, Ubc5, Ubc6, Ubc7, Ubc8, Ubc10, Ubc13.
- 5 13. The method of claim 12, wherein said ubiquitin conjugating enzyme is a yeast ubiquitin conjugating enzyme or a mouse, rat, or human homolog.
14. The method of claim 2, wherein said organic compound is a compound of formula (I):



wherein:

- 10 Ar is a five or six membered unsubstituted or substituted aromatic ring that is optionally fused to an aromatic or heteroaromatic ring;
- B is a bond, CO, SO₂ or (CH₂)_n wherein n = 1 – 5; and
- R₁ and R₂ are each independently H, alkyl or aryl groups that are optionally substituted,
- 15 wherein said compound selectively binds to one or more catalytic domains in the ubiquitin conjugating enzyme.
15. The formula I compound of claim 14, wherein Ar is phenyl, pyridyl, naphthyl, triazine, triazole, quinoxaline, dibenzofuran, benzimidazole, indene, indeno oxadiazine, indazole or an indole ring; B is a bond, CO or (CH₂)_n wherein n = 1; and R₁ and R₂ are both H.
- 20 16. The formula I compound of claim 14, which is 3-amino-1,2,4-Triazine, 3-amino-1,2,4-triazole, 2-methyl-4-nitroaniline, 2-iodo-4-nitroaniline, 4-amino-3-chloro-5-methylbenzoic acid, 1-(4' Aminophenyl)-1,2,4-triazole, 2-acetamidophenol, 5-chloro-2,3-dihydroxypyridine, 2-methyl-3-(1H-pyrazol-5-yl)imidazo(1,2-a)pyridine, 5-nitro-2,3-dihydro-1H-benzo(d)imidazol-2-one, 4-(methylamino)pyridine, 2-Chloro-4-nitrobenzamide, 2-
- 25 ethylformanilide, 6-aminoindazole, 2,3-diaminobenzoic acid, 1-(5-chloro-2-methylphenyl)-2-thiourea, 4,5-diiodo-1H-imidazole, 1H-indene-1,3(2H)-dione 1-methylhydrazone, 3-hydroxyindole, 3,4-dihydro-1H-quinoxalin-2-one, 1S,6S,7R,8R,8aR)-1,6,7,8-

- Tetrahydroxyoctahydroindolizidine, 2-naphthalen-1-yl-2,3-dihydro-1H-pyrimidine, benzo(b)thiophen-3-ylmethylamine, 1-allyl-2,4-dioxo-1,2,3,4-tetrahydro-5-pyrimidinecarbonitrile, Methyl (*S*)-*N*-(7-chloro-2,3,4a,5-tetrahydro-4a-(methoxycarbonyl)indeno(1,2-*e*)(1,3,4)oxadiazin-2-ylcarbonyl)-4'-(trifluoromethoxy)carbanilate (Indoxacarb-MP), 4-acetylpyridine and thioisonicotinamide.
17. The formula I compound of claim 14, which is 4-(Aminomethyl)piperidine.
18. The formula I compound of claim 14, whose molecular weight is less than about 2000 daltons.
19. The formula I compound of claim 14, whose molecular weight is less than about 500 daltons.
20. The method of claim 2, wherein said organic compound is a compound of formula (II):



wherein:

- A is a 3-6 membered substituted or unsubstituted cycloaliphatic or a heterocycloaliphatic ring, each of which is optionally fused to an aromatic ring;

B is a bond, CO, SO₂ or (CH₂)_n wherein n = 1-3; and

R₁ and R₂ are each independently H, alkyl or aryl groups that are optionally substituted,

- wherein said compound selectively binds to one or more catalytic domains in the ubiquitin conjugating enzyme.

21. The formula II compound of claim 20, wherein A is a heterocycloaliphatic ring comprising, at least one nitrogen atom and optionally, one or more additional heteroatoms selected from the group consisting of: nitrogen (N), oxygen (O) and sulfur (S); B is a bond, CO or (CH₂)_n wherein n = 1; and R₁ and R₂ are both H.
22. The formula II compound of claim 20, wherein the cycloaliphatic or heterocycloaliphatic ring comprise one or more substituents selected from the group consisting of hydroxyl, halogen, CO and alkyl.

23. The formula II compound of claim 20, which is 1-phenyl-4-methyl-3-pyrazolidone, 4-(aminomethyl)piperidine, N-Phenyl-p-phenylenediamine, 5-(aminomethyl)-3-(2H)-isoxazolone (Muscimol), (R)-2-aminomethylpyrrolidine, 2-pyrrolidinone oxime and 1-cyclopropylethylamine.
- 5 24. The formula II compound of claim 20, whose molecular weight is less than about 2000 daltons.
25. The formula II compound of claim 20, whose molecular weight is less than about 500 daltons.
26. The method of claim 11, wherein said organic compound is an aromatic or
10 heteroaromatic compound comprising a piperidinyl, phenyl, quinolinyl and isoquinolinyl ring and having one or more nitrogen containing substituents selected from the group consisting of NR_3R_4 , $(\text{CH}_2)_n\text{NR}_3\text{R}_4$, CONH_2 , NH-NH-R_5 and C(S)-NH-R_6 , wherein R_3 , R_4 , R_5 and R_6 are independently H, alkyl, cycloalkyl and aryl, and n is 1-5, and wherein said compound selectively interacts with the E1 binding site of the enzyme.
- 15 27. The method of claim 26, wherein the aromatic or heteroaromatic compound is 4-methylcyclohexylamine, 3-(dimethylamino)-1-(5-fluoro-2-hydroxyphenyl)prop-2-en-1-one, 3-ethoxyphenethylamine, 4-fluorobenzenesulfonamide, 2-((4-fluorophenyl)-hydrazono)malononitrile, 3-fluoro-4-hydroxybenzaldehyde and 1-aminoisoquinoline.
28. The method of claim 26, wherein the molecular weight of the aromatic or heteroaromatic
20 compound is less than about 2000 daltons.
29. The method of claim 26, wherein the molecular weight of the aromatic or heteroaromatic compound is less than about 500 daltons.
30. The method of claim 11, wherein said organic compound is a 7 or 8 membered monocyclic or bridged bicyclic compound comprising a cycloheptyl, cyclooctyl, and
25 bicyclo(2,2,1) heptenyl having one or more substituents selected from the group consisting of NR_3R_4 , $(\text{CH}_2)_n\text{NR}_3\text{R}_4$, CONH_2 , NH-NH-R_5 and C(S)-NH-R_6 , wherein R_3 , R_4 , R_5 and R_6 are independently H, alkyl, cycloalkyl and aryl, and n is 1-5, and wherein said compound selectively interacts with the E1 binding site of the enzyme.

31. The method of claim 30, wherein the 7 or 8-membered monocyclic or bridged bicyclic compound is 6,7-dihydro-5H-dibenzo(a,c)cycloheptene-6-carboxylic acid, N-bicyclo(2.2.1)hept-5-en-2-ylthiourea, N1-cyclooctyl-4-hydroxy-1-piperidinecarbothioamide.
32. The method of claim 31, wherein the molecular weight of the 7 or 8 membered
5 monocyclic or bridged bicyclic compound is less than about 2000 daltons.
33. The method of claim 31, wherein the molecular weight of the 7 or 8 membered monocyclic or bridged bicyclic compound is less than about 500 daltons.
34. The method of determining a selective interaction between an ubiquitin conjugating enzyme and an organic compound comprising the steps of:
- 10 a) inputting 3-D co-ordinates of at least a fragment of the ubiquitin conjugating enzyme into an electronic storage medium;
- b) determining one or more active sites in said fragment of the ubiquitin conjugating enzyme;
- c) simulating a binding interaction between said active sites in said fragments of the
15 ubiquitin conjugating enzyme and a library of organic compounds;
- d) identifying compounds in said library of organic compounds that interact with said active sites in said fragments of the ubiquitin conjugating enzyme.
35. The method of claim 34, wherein said ubiquitin conjugating enzyme is selected from the group consisting of: Ubc1, Ubc2, Ubc3, Ubc4, Ubc5, Ubc6, Ubc7, Ubc8, Ubc10, Ubc11,
20 Ubc13.
36. The method of claim 35, wherein said ubiquitin conjugating enzyme is a yeast ubiquitin conjugating enzyme or a mouse, rat, or human homolog.
37. The method of claim 34, wherein said atomic 3-D coordinates are selected from the group consisting of: 1AYZ, 1A3S, 1QCQ, 2UCZ, 1I7K 1J7D, 1JAT, 1JBB and variants
25 thereof.
38. The method of claim 34, wherein step (b) is performed by a molecular topology and charge visualization program.

39. The method of claim 34, wherein said step (c) is performed with LUDI.
40. The method of claim 34, wherein said library of organic compounds is the Available Chemical Directory.
41. The method of claim 34, wherein said active site comprises the amino acid residues corresponding to Lys64, Pro66, Lys67, Ile68, Asn84, Ile85, Leu90, Lys91 and Leu120 of SEQ ID NO:1.
42. The method of claim 34, wherein said active site comprises the amino acid residues corresponding to Pro65, Pro66, Lys67, Ile68, Asn84, Leu90, Lys91 and Leu120 of SEQ ID NO:1.
43. The method of claim 34, wherein said active site comprises the amino acid residues corresponding to Lys66, Ile67, Ala68, Ser83, Cys85, Leu86, Leu89 and Arg90 of SEQ ID NO:2.
44. The method of claim 34, wherein said active site comprises the amino acid residues corresponding to Pro64, Pro65, Lys66, Ile67, Ser83, Ile84, Cys85, Leu86, Leu89, Arg90 and Leu119 of SEQ ID NO:2.
45. A method of treating or preventing a hyperproliferative disorder by administering an effective amount of a compound of formula (I):



wherein:

- Ar is a five or six membered unsubstituted or substituted aromatic ring that is optionally fused to an aromatic or heteroaromatic ring;
- B is a bond, CO, SO₂ or (CH₂)_n wherein n = 1 – 5; and
- R₁ and R₂ are each independently H, alkyl or aryl groups that are optionally substituted.
- wherein said compound treats or prevents a hyperproliferative disorder.

46. The formula I compound of claim 45, wherein Ar is phenyl, pyridyl, naphthyl, triazine, triazole, quinoxaline, dibenzofuran, benzimidazole, indene, indeno oxadiazine, indazole or an indole ring; B is a bond, CO or (CH₂)_n wherein n = 1; and R₁ and R₂ are both H.
47. The formula I compound of claim 45, which is 3-amino-1,2,4-Triazine, 3-amino-1,2,4-triazole, 2-methyl-4-nitroaniline, 2-iodo-4-nitroaniline, 4-amino-3-chloro-5-methylbenzoic acid, 1-(4' Aminophenyl)-1,2,4-triazole, 2-acetamidophenol, 5-chloro-2,3-dihydroxypyridine, 2-methyl-3-(1H-pyrazol-5-yl)imidazo(1,2-a)pyridine, 5-nitro-2,3-dihydro-1H-benzo(d)imidazol-2-one, 4-(methylamino)pyridine, 2-Chloro-4-nitrobenzamide, 2-ethylformanilide, 6-aminoindazole, 2,3-diaminobenzoic acid, 1-(5-chloro-2-methylphenyl)-2-thiourea, 4,5-diiodo-1H-imidazole, 1H-indene-1,3(2H)-dione 1-methylhydrazone, 3-hydroxyindole, 3,4-dihydro-1H-quinoxalin-2-one, 1S,6S,7R,8R,8aR)-1,6,7,8-Tetrahydroxyoctahydroindolizidine, 2-naphthalen-1-yl-2,3-dihydro-1H-pyrimidine, benzo(b)thiophen-3-ylmethylamine, 1-allyl-2-4-dioxo-1-2-3-4-tetrahydro-5-pyrimidinecarbonitrile, Methyl (*S*)-*N*-(7-chloro-2,3,4a,5-tetrahydro-4a-(methoxycarbonyl)indeno(1,2-*e*)(1,3,4)oxadiazin-2-ylcarbonyl)-4'-(trifluoromethoxy)carbanilate (Indoxacarb-MP), 4-acetylpyridine and thioisonicotinamide.
48. The formula I compound of claim 45, which is 4-(Aminomethyl)piperidine.
49. The formula I compound of claim 45, whose molecular weight is less than about 2000 daltons.
50. The formula I compound of claim 45, whose molecular weight is less than about 500 daltons.
51. A method of treating or preventing a hyperproliferative disorder by administering an effective amount of a compound of formula (II):



wherein:

A is a 3-6 membered substituted or unsubstituted cycloaliphatic or a heterocycloaliphatic ring, each of which is optionally fused to an aromatic ring;

B is a bond, CO, SO₂ or (CH₂)_n wherein n = 1-3; and

R₁ and R₂ are each independently H, alkyl or aryl groups that are optionally substituted.

52. The formula II compound of claim 51, wherein A is a heterocycloaliphatic ring comprising, at least one nitrogen atom and optionally, one or more additional heteroatoms selected from the group consisting of: nitrogen (N), oxygen (O) and sulfur (S); B is a bond, CO or (CH₂)_n wherein n = 1; and R₁ and R₂ are both H.

53. The formula II compound of claim 51, wherein the cycloaliphatic or heterocycloaliphatic ring comprise one or more substituents selected from the group consisting of hydroxyl, halogen, CO and alkyl.

54. The formula II compound of claim 51, which is 1-phenyl-4-methyl-3-pyrazolidone, 4-(aminomethyl)piperidine, N-Phenyl-p-phenylenediamine, 5-(aminomethyl)-3-(2H)-isoxazolone (Muscimol), (R)-2-aminomethylpyrrolidine, 2-pyrrolidinone oxime and 1-cyclopropylethylamine.

55. The formula II compound of claim 51, whose molecular weight is less than about 2000 daltons.

56. The formula II compound of claim 51, whose molecular weight is less than about 500 daltons.

57. A method of treating or preventing a hyperproliferative disorder by administering an effective amount of an aromatic or heteroaromatic compound comprising a piperidinyl, phenyl, quinolinyl and isoquinolinyl ring and having one or more nitrogen containing substitutents selected from the group consisting of NR₃R₄, (CH₂)_nN R₃R₄, CONH₂, NH-NH-R₅ and C(S)-NH-R₆, wherein R₃, R₄, R₅ and R₆ are independently H, alkyl, cycloalkyl and aryl, and n is 1-5.

58. The method of claim 57, wherein the aromatic or heteroaromatic compound is 4-methylcyclohexylamine, 3-(dimethylamino)-1-(5-fluoro-2-hydroxyphenyl)prop-2-en-1-one, 3-ethoxyphenethylamine, 4-fluorobenzenesulfonamide, 2-((4-fluorophenyl)-hydrazono)malononitrile, 3-fluoro-4-hydroxybenzaldehyde and 1-aminoisoquinoline.

59. The method of claim 57, wherein the molecular weight of the 7 or 8 membered monocyclic or bridged bicyclic compound is less than about 2000 daltons.

60. The method of claim 57, wherein the molecular weight of the 7 or 8 membered monocyclic or bridged bicyclic compound is less than about 500 daltons.
61. A method of treating or preventing a hyperproliferative disorder by administering an effective amount of an 7 or 8 membered monocyclic or bridged bicyclic compound
- 5 comprising a cycloheptyl, cyclooctyl, and bicyclo(2,2,1) heptenyl having one or more substituents selected from the group consisting of NR_3R_4 , $(\text{CH}_2)_n\text{NR}_3\text{R}_4$, CONH_2 , NH-NH-R_5 and C(S)-NH-R_6 , wherein R_3 , R_4 , R_5 and R_6 are independently H, alkyl, cycloalkyl and aryl, and n is 1-5, and wherein said compound selectively interacts with the E1 binding site of the enzyme.
- 10 62. The method of claim 61, wherein the 7 or 8-membered monocyclic or bridged bicyclic compound is 6,7-dihydro-5H-dibenzo(a,c)cycloheptene-6-carboxylic acid, N-bicyclo(2.2.1)hept-5-en-2-ylthiourea, N1-cyclooctyl-4-hydroxy-1-piperidinecarbothioamide.
63. The method of claim 61, wherein the molecular weight of the 7 or 8 membered monocyclic or bridged bicyclic compound is less than about 2000 daltons.
- 15 64. The method of claim 61, wherein the molecular weight of the 7 or 8 membered monocyclic or bridged bicyclic compound is less than about 500 daltons.
65. The method of claim 45, 51, 57 or 61, wherein the hyperproliferative disorder is cancer.
66. The method of claim 65, wherein the cancer is colon cancer.
67. The method of claim 65, wherein the cancer is breast cancer.
- 20 68. A method of treating or preventing a Ubc related disorder by administering an effective amount of a compound of the invention.
69. The method of claim 59, wherein said Ubc related disorder is selected from the group consisting of: hyperproliferative disorders, cystic fibrosis, neurodegenerative disorders and viral disorders.

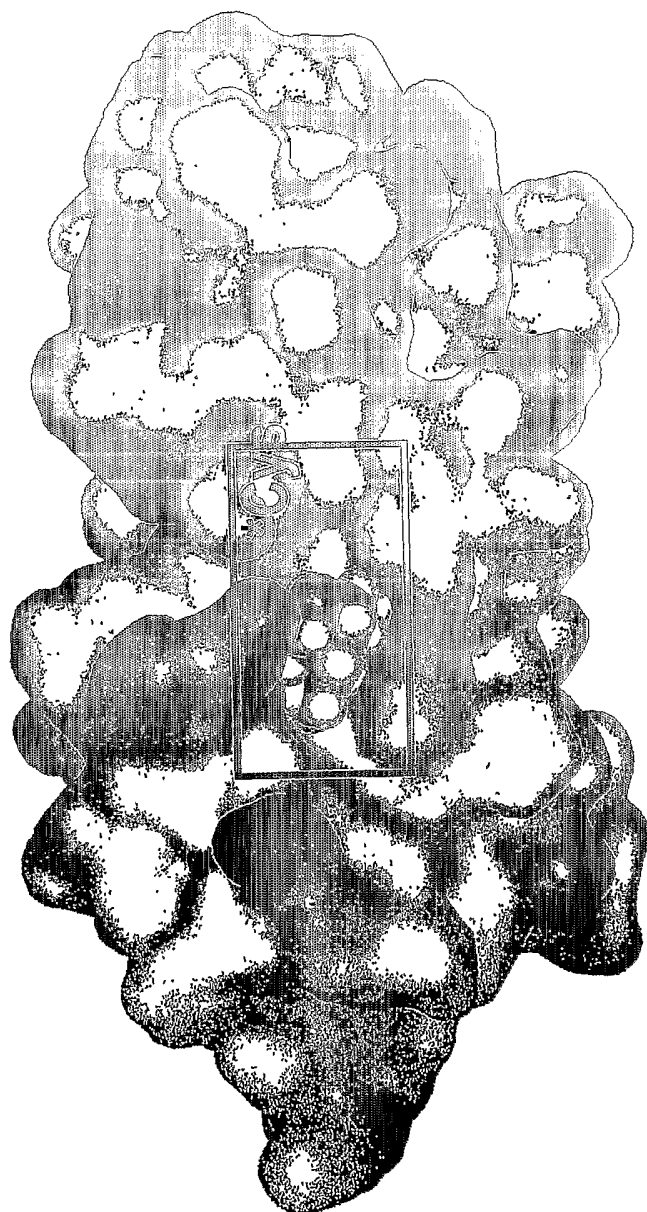


FIGURE 1

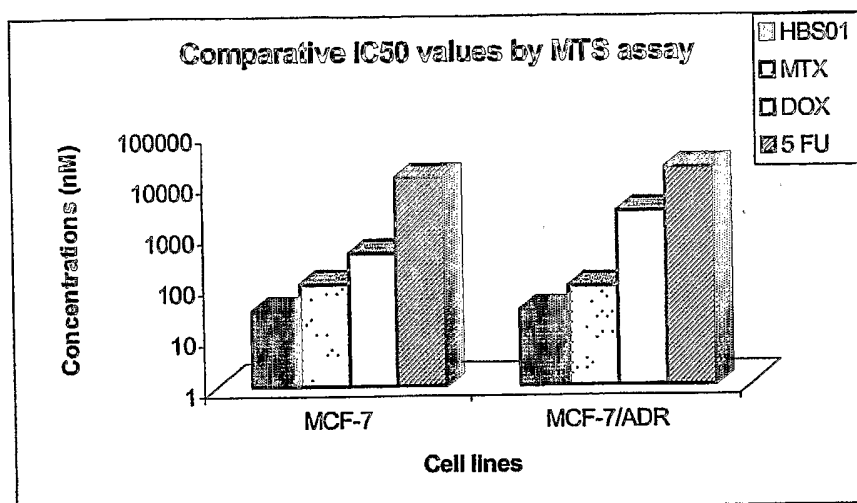
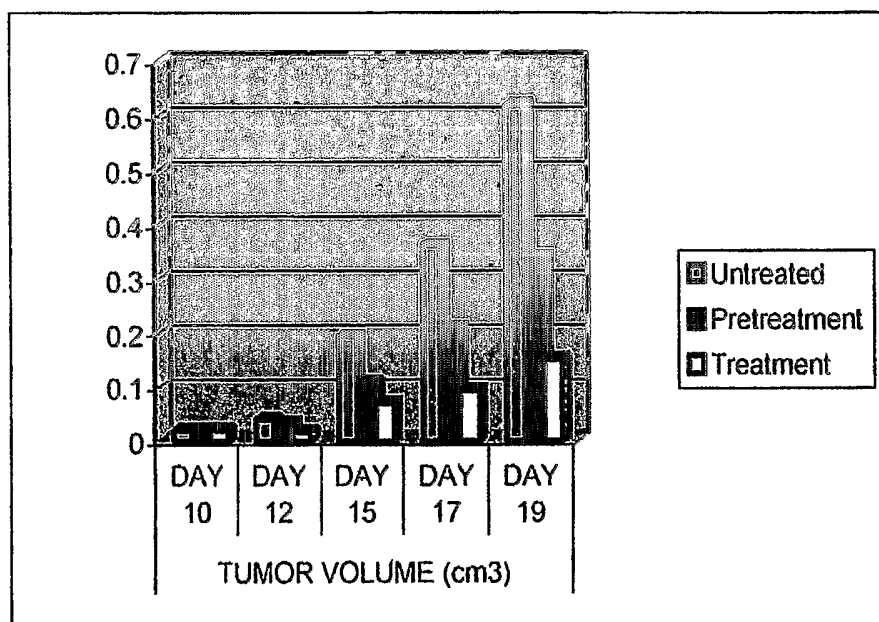
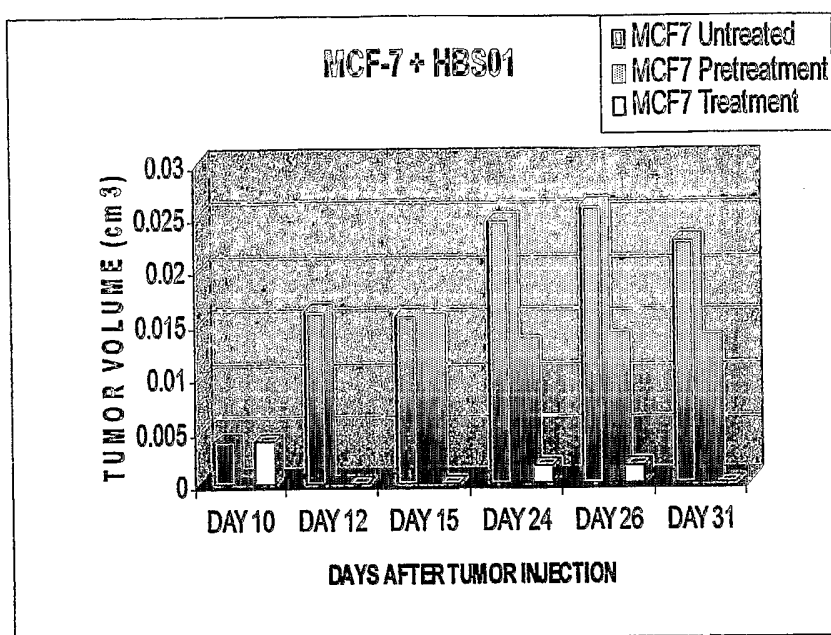
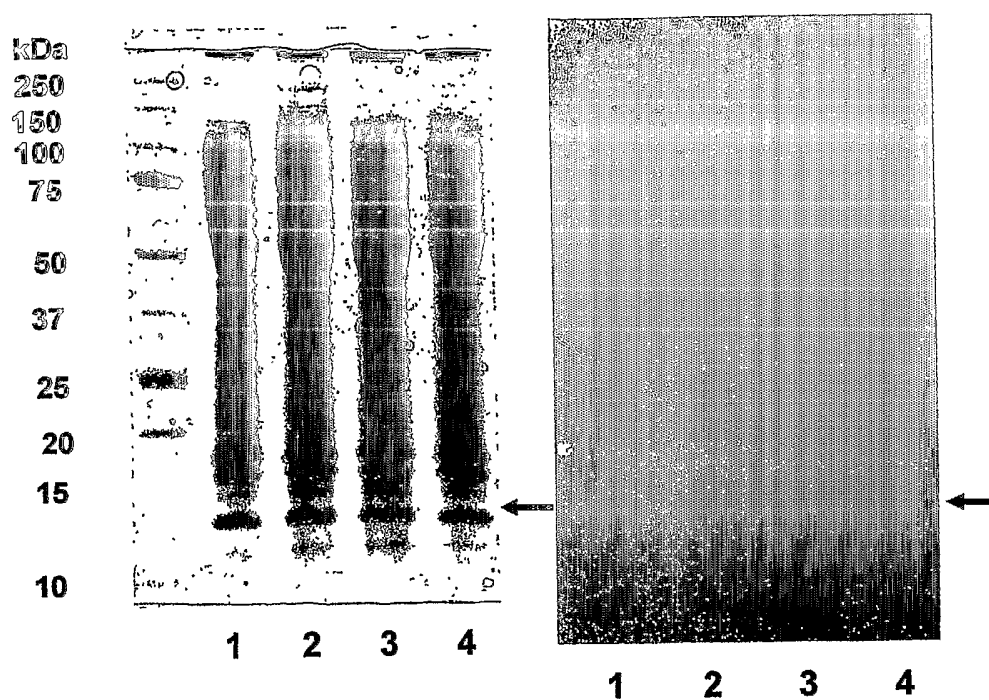


FIGURE 2

**FIGURE 3**

**FIGURE 4**

**FIGURE 5**

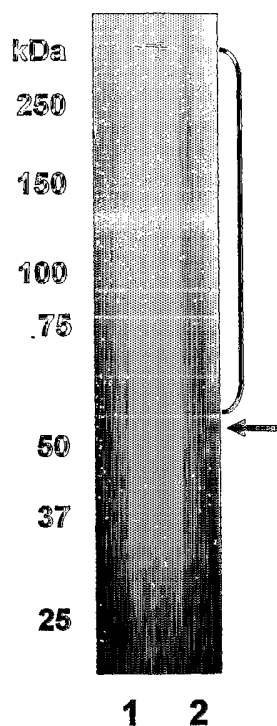


FIGURE 6

<110> Banerjee, Amit

<120> COMPOUNDS AND METHODS FOR THE TREATMENT OF UBIQUITIN CONJUGATING
DISORDERS

<130> 12667/2008

<150> US 10/914,848

<151> 2004-08-10

<160> 3

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20 25 30

His Trp Gln Ala Ser Ile Met Gly Pro Ala Asp Ser Pro Tyr Ala Gly

35 40 45

50 55 60

Pro Pro Lys Ile Ser Phe Thr Thr Lys Ile Tyr His Pro Asn Ile Asn
65 70 75 80

Ala Asn Gly Asn Ile Cys Leu Asp Ile Leu Lys Asp Gln Trp Ser Pro
85 90 95

Ala Leu Thr Leu Ser Lys Val Leu Leu Ser Ile Cys Ser Leu Leu Thr
100 105 110

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Lys Tyr Ala Val
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<213> Homo sapiens

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20

25

30

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35

40

45

Val Phe Phe Leu Thr Val His Phe Pro Thr Asp Tyr Pro Phe Lys Pro

50

55

60

Pro Lys Ile Ala Phe Thr Thr Lys Ile Tyr His Pro Asn Ile Asn Ser

65

70

75

80

Asn Gly Ser Ile Cys Leu Asp Ile Leu Arg Ser Gln Trp Ser Pro Ala

85

90

95

Leu Thr Val Ser Lys Val Leu Leu Ser Ile Cys Ser Leu Leu Cys Asp

100

105

110

Pro Asn Pro Asp Asp Pro Leu Val Pro Asp Ile Ala Gln Ile Tyr Lys

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120

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Ser Asp Lys Glu Lys Tyr Asn Arg His Ala Arg Glu Trp Thr Gln Lys

130

135

140

Tyr Ala Met

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<213> *Saccharomyces cerevisiae*

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20 25 30

Asn Ile Phe Ile Trp Asp Cys Leu Ile Gln Gly Pro Pro Asp Thr Pro

35 40 45

Tyr Ala Asp Gly Val Phe Asn Ala Lys Leu Glu Phe Pro Lys Asp Tyr

50 55 60

Pro Leu Ser Pro Pro Lys Leu Thr Phe Thr Pro Ser Ile Leu His Pro

65 70 75 80

Asn Ile Tyr Pro Asn Gly Glu Val Cys Ile Ser Ile Leu His Ser Pro

85 90 95

Gly Asp Asp Pro Asn Met Tyr Glu Leu Ala Glu Glu Arg Trp Ser Pro

100 105 110

Glu Pro Asn Ile Glu Ser Gly Ala Asn Ile Asp Ala Cys Ile Leu Trp

130

135

140

Arg Asp Asn Arg Pro Glu Phe Glu Arg Gln Val Lys Leu Ser Ile Leu

145

150

155

160

Lys Ser Leu Gly Phe

165