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(54) Title: COMPOSITIONS AND METHODS FOR INHIBITING EZH2

(57) Abstract: The present invention relates to therapeutic targets for cancer. In particular, the present invention relates to small molecules and nucleic acids that target EZH2 expression in cancer (e.g., prostate cancer, breast cancer, other solid tumors, multiple myeloma).



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COMPOSITIONS AND METHODS FOR INHIBITING EZH2

This application claims priority to application serial number 61/306,255, filed February 19, 2010, which is herein incorporated by reference in its entirety.

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STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under CA69568 awarded by the National Institutes of Health. The government has certain rights in the invention.

10

FIELD OF THE INVENTION

The present invention relates to therapeutic targets for cancer. In particular, the present invention relates to small molecules and nucleic acids that target EZH2 expression in cancer (e.g., prostate cancer, breast cancer, other solid tumors, multiple myeloma).

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BACKGROUND OF THE INVENTION

Afflicting one out of nine men over age 65, prostate cancer (PCA) is a leading cause of male cancer-related death, second only to lung cancer (Abate-Shen and Shen, Genes Dev 14:2410 [2000]; Ruijter *et al.*, Endocr Rev, 20:22 [1999]). The American Cancer Society estimates that about 184,500 American men will be diagnosed with prostate cancer and 39,200 will die in 2001.

20

Prostate cancer is typically diagnosed with a digital rectal exam and/or prostate specific antigen (PSA) screening. An elevated serum PSA level can indicate the presence of PCA. PSA is used as a marker for prostate cancer because it is secreted only by prostate cells. A healthy prostate will produce a stable amount -- typically below 4 nanograms per milliliter, or a PSA reading of "4" or less -- whereas cancer cells produce escalating amounts that correspond with the severity of the cancer. A level between 4 and 10 may raise a doctor's suspicion that a patient has prostate cancer, while amounts above 50 may show that the tumor has spread elsewhere in the body.

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When PSA or digital tests indicate a strong likelihood that cancer is present, a transrectal ultrasound (TRUS) is used to map the prostate and show any suspicious areas. Biopsies of various sectors of the prostate are used to determine if prostate cancer is present. Treatment options depend on the stage of the cancer. Men with a 10-year life
5 expectancy or less who have a low Gleason number and whose tumor has not spread beyond the prostate are often treated with watchful waiting (no treatment). Treatment options for more aggressive cancers include surgical treatments such as radical prostatectomy (RP), in which the prostate is completely removed (with or without nerve sparing techniques) and radiation, applied through an external beam that directs the dose
10 to the prostate from outside the body or via low-dose radioactive seeds that are implanted within the prostate to kill cancer cells locally. Anti-androgen hormone therapy is also used, alone or in conjunction with surgery or radiation. Hormone therapy uses luteinizing hormone-releasing hormones (LH-RH) analogs, which block the pituitary from producing hormones that stimulate testosterone production. Patients must have
15 injections of LH-RH analogs for the rest of their lives.

While surgical and hormonal treatments are often effective for localized PCA, advanced disease remains essentially incurable. Androgen ablation is the most common therapy for advanced PCA, leading to massive apoptosis of androgen-dependent malignant cells and temporary tumor regression. In most cases, however, the tumor
20 reemerges with a vengeance and can proliferate independent of androgen signals.

The advent of prostate specific antigen (PSA) screening has led to earlier detection of PCA and significantly reduced PCA-associated fatalities. However, the impact of PSA screening on cancer-specific mortality is still unknown pending the results of prospective randomized screening studies (Etzioni *et al.*, J. Natl. Cancer Inst., 91:1033
25 [1999]; Maattanen *et al.*, Br. J. Cancer 79:1210 [1999]; Schroder *et al.*, J. Natl. Cancer Inst., 90:1817 [1998]). A major limitation of the serum PSA test is a lack of prostate cancer sensitivity and specificity especially in the intermediate range of PSA detection (4-10 ng/ml). Elevated serum PSA levels are often detected in patients with non-malignant conditions such as benign prostatic hyperplasia (BPH) and prostatitis, and
30 provide little information about the aggressiveness of the cancer detected. Coincident with increased serum PSA testing, there has been a dramatic increase in the number of

prostate needle biopsies performed (Jacobsen *et al.*, JAMA 274:1445 [1995]). This has resulted in a surge of equivocal prostate needle biopsies (Epstein and Potter J. Urol., 166:402 [2001]). Thus, development of new therapeutic targets and agents is needed.

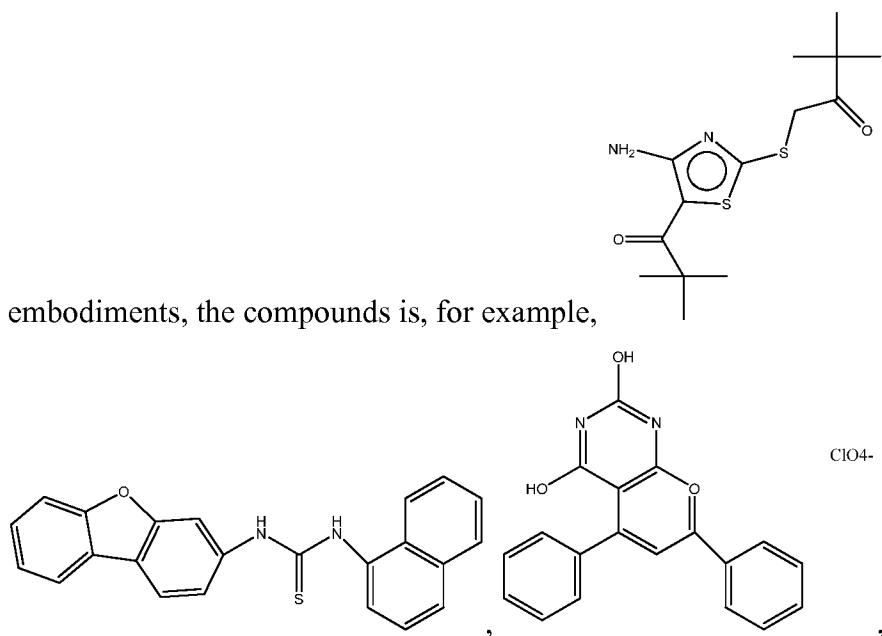
5 SUMMARY OF THE INVENTION

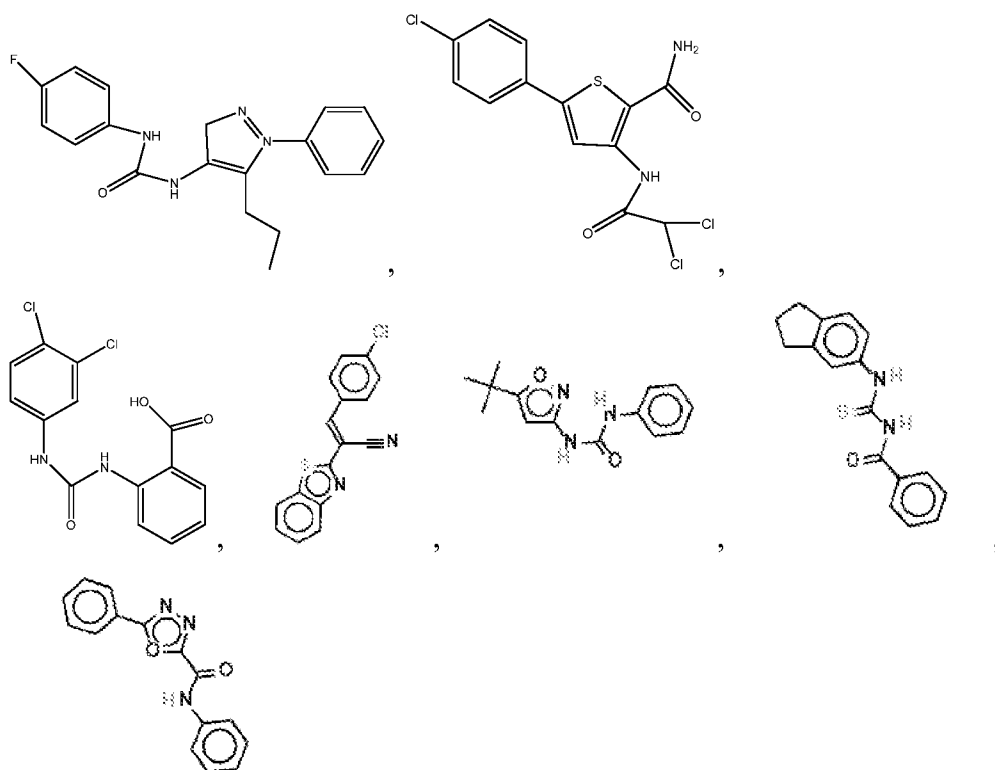
The present invention relates to therapeutic targets for cancer. In particular, the present invention relates to small molecules and nucleic acids that target EZH2 expression in cancer (e.g., prostate cancer, breast cancer, other solid tumors, multiple myeloma).

10 For example, in some embodiments, the present invention provides a method of inhibiting the growth of cells, comprising contacting a cell expressing EZH2 with a miRNA under conditions such that the expression of EZH2 in the cell is inhibited. In some embodiments, the miRNA is miR-101. In some embodiments, the cell is a cancer cell. In some embodiments, the cell is in an organism (e.g., an animal or an animal
15 diagnosed with cancer (e.g., prostate, breast, or bladder cancer)).

In some embodiments, the present invention provides compounds that inhibit the growth of cells (e.g., by inhibiting one or more activities of EZH2). In some

embodiments, the compounds is, for example,





or or a derivative, mimetic, variant, etc. thereof.

In other embodiments, the present invention provides a method of inhibiting the growth of cells (e.g., by inhibiting one or more activities of EZH2), comprising contacting a cell expressing EZH2 with a small molecule compound under conditions such that the expression of EZH2 in the cell is inhibited. In some embodiments, the small molecule is isoliquiritigenin or related compounds or the compounds described in Tables 1-3 or mimetics, variants, derivatives, etc. thereof. In some embodiments, the cell is a cancer cell. In some embodiments, the cell is in an organism (e.g., an animal or an animal diagnosed with cancer (e.g., prostate, breast, or bladder cancer)).

DESCRIPTION OF THE FIGURES

Figure 1 shows the sequence database entry for mir-101 from Sanger's Registry. The cartoon depicts the predicted stem-loop hairpin. miR-101 is predicted to target the 3' UTR of EZH2 at 2 independent sites and both predictions are the top ranked hits from the Sanger Registry.

Figure 2 shows that miR-101 down regulates EZH2. Immunoblot analysis of the breast cancer cell line SKBr3 transfected with precursor miR-101 or controls and non-

EZH2 targeting precursor miR's as well as other EZH2 targeting predicted miR's with low scores.

Figure 3 shows that miR-101 inhibits invasion. SKBr3 cells were transfected with either control miR, miR-101 which targets EZH2, siRNA duplex against
5 EZH2 or luciferase duplex. A reconstituted basement membrane invasion chamber assay (Chemicon) was used to assess invasion.

Figure 4 shows that EZH2 mediates down regulation of E-cadherin (CDH1). **A**, Histogram of E-cadherin expression from expression profiling experiment using RNA from EZH2 overexpressing breast cells and vector control. **B**, Immunoblot analysis of
10 EZH2 and E-cadherin using the lysates from the breast cell line H16N2, MCF10A, HME and primary prostate cell PrEC infected with adenovirus encoding EZH2, EZH2 SET mutant, control virus infected cells using EZH2 and E-cadherin antibody. β -Tubulin was included as a loading control. **C**, Co-immunostaining and confocal imaging of breast cell line H16N2 infected with EZH2. Panel on the right is uninfected cells and panel on
15 the right EZH2 overexpressing cells. **D**, Adaptation of E-cadherin promoter-luciferase reporter assay for high throughput screening assay (96 well format). Asterisk (*) highlights the significant down regulation of luciferase activity in EZH2 overexpressing cells.

Figure 5 shows that isoliquiritigenin inhibits EZH2 mediated gene repression. **A**,
20 Chemical structure of the flavonoid isoliquiritigenin. **B**, Quantitative SYBR green RT-PCR of EZH2 and E cadherin transcripts in cell lines over expressing EZH2 and control adenoviruses. RT-PCR on each sample was performed in duplicate, and a ratio was calculated relative to the housekeeping genes GAPDH. Transcripts were also measured in cells that were treated with isoliquiritigenin or other small molecules.

Figure 6 shows that isoliquiritigenin inhibits EZH2 activity. **A**, Dose response of
25 isoliquiritigenin on inhibiting EZH2 mediated repression. **B**, A reconstituted basement membrane invasion chamber assay was used to assess the invasion of breast epithelial cell line infected with EZH2 and control adenoviruses as well as SET domain mutant EZH2 adenovirus. EZH2 treated cells were also treated with SAHA, the HDAC inhibitor
30 and isoliquiritigenin, the small molecule inhibitor of EZH2 and the control small molecule phloretin.

Figure 7 shows that tumor development is repressed by EZH2 shRNA knockdown.

Figure 8 shows Focal genomic loss of miR-101-1 locus in gastric and breast cancers.

5 Figure 9 shows that Genomic aberration in cancer leads to the down regulation of miR-101.

Figure 10 shows the development of a primary assay for drug screening.

Figure 11 shows the development of a primary assay for drug screening.

Figure 12 shows results of a small molecule screen for inhibitors of EZH2.

10 Figure 13 shows selection of a subset of inhibitors by secondary screening.

Figure 14 shows a small molecule inhibitor dose response curve.

Figure 15 shows a small molecule inhibitor dose response curve.

Figure 16 shows small molecule inhibitors with IC₅₀ <50uM by DU145 cell Proliferation Assay.

15 Figure 17 shows small molecule inhibitors with no affect on DU145 cell proliferation.

Figure 18 shows compounds effective in inhibiting cancer cell proliferation.

Figure 19 shows a tertiary screen to test small molecules affecting H3 methylation.

20 Figure 20 shows small molecule inhibitors of EZH2 mediated invasion.

Figure 21 shows small molecule inhibitors of invasion of an aggressive prostate cell line.

Figure 22 shows that MCTP65 inhibits EZH2 mediated repression.

Figure 23 shows that MCTP65 restores E-cadherin expression.

25 Figure 24 shows that MCTP65 inhibits invasion mediated by EZH2.

Figure 25 shows that MCTP65 inhibits invasion of aggressive cell lines.

Figure 26 shows that MCTP65 inhibits invasion of aggressive cell lines.

Figure 27 shows that MCTP-65 decreases trimethylation of H3K27.

30 Figure 28 shows the effect of MCTP65 on Du145-Luc xenografts bearing Balb/C nu/nu mice.

Figure 29 shows the effect of MCTP65 on Du145-Luc xenografts bearing Balb/C nu/nu mice.

Figure 30 shows multiple secondary assays for EZH2 inhibitor MCTP1. DU145 cells were treated with multiple doses of MCTP1 and cell viability was monitored after 4 days (A). Cell invasion was monitored in EZH2 overexpressing HME cells after the addition of MCTP1 (B). Histone H3- K27 trimethylation was monitored in DU145 cells treated with MCTP1 (C). A table representing the characteristics of MCTP1 in secondary assays is shown in D. Chemical structure and name of MCTP1 (E).

Figure 31 shows multiple secondary assays for EZH2 inhibitor MCTP2. DU145 cells were treated with multiple doses of MCTP2 and cell viability was monitored after 4 days (A). Cell invasion was monitored in EZH2 overexpressing HME cells after the addition of MCTP2 (B). Histone H3- K27 trimethylation was monitored in DU145 cells treated with MCTP2 (C). A table representing the characteristics of MCTP2 in secondary assays is shown in D. Chemical structure and name of MCTP2 (E).

Figure 32 shows multiple secondary assays for EZH2 inhibitor MCTP3. DU145 cells were treated with multiple doses of MCTP3 and cell viability was monitored after 4 days (A). Cell invasion was monitored in EZH2 overexpressing HME cells after the addition of MCTP3 (B). Histone H3- K27 trimethylation was monitored in DU145 cells treated with MCTP3 (C). A table representing the characteristics of MCTP3 in secondary assays is shown in D. Chemical structure and name of MCTP3 (E).

Figure 33 shows multiple secondary assays for EZH2 inhibitor MCTP12. DU145 cells were treated with multiple doses of MCTP12 and cell viability was monitored after 4 days (A). Cell invasion was monitored in EZH2 overexpressing HME cells after the addition of MCTP12 (B). Histone H3- K27 trimethylation was monitored in DU145 cells treated with MCTP12 (C). A table representing the characteristics of MCTP12 in secondary assays is shown in D. Chemical structure and name of MCTP12 (E).

Figure 34 shows multiple secondary assays for EZH2 inhibitor MCTP15. DU145 cells were treated with multiple doses of MCTP15 and cell viability was monitored after 4 days (A). Cell invasion was monitored in EZH2 overexpressing HME cells after the addition of MCTP15 (B). Histone H3- K27 trimethylation was monitored in DU145 cells

treated with MCTP15 (C). A table representing the characteristics of MCTP15 in secondary assays is shown in D. Chemical structure and name of MCTP15 (E)

Figure 35 shows multiple secondary assays for EZH2 inhibitor MCTP28. DU145 cells were treated with multiple doses of MCTP28 and cell viability was monitored after 4 days (A). Histone H3- K27 trimethylation was monitored in DU145 cells treated with MCTP28 (B). A table representing the characteristics of MCTP28 in secondary assays is shown in C. Chemical structure and name of MCTP28 (D).

Figure 36 shows the chemical structure and name of MCTP11, MCTP18, MCTP19 and MCTP20.

DEFINITIONS

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

As used herein, the term “inhibits at least one biological activity of EZH2” refers to any agent that decreases any activity of EZH2 (*e.g.*, including, but not limited to, the activities described herein), via directly contacting EZH2 protein, contacting EZH2 mRNA or genomic DNA, causing conformational changes of EZH2 polypeptides, decreasing EZH2 protein levels, or interfering with EZH2 interactions with signaling partners, and affecting the expression of EZH2 target genes. Inhibitors also include molecules that indirectly regulate EZH2 biological activity by intercepting upstream signaling molecules.

As used herein, the term "subject" refers to any animal (*e.g.*, a mammal), including, but not limited to, humans, non-human primates, rodents, and the like, which is to be the recipient of a particular treatment. Typically, the terms "subject" and "patient" are used interchangeably herein in reference to a human subject.

As used herein, the term "cancer marker genes" refers to a gene whose expression level, alone or in combination with other genes, is correlated with cancer or prognosis of cancer. The correlation may relate to either an increased or decreased expression of the gene. For example, the expression of the gene may be indicative of cancer, or lack of expression of the gene may be correlated with poor prognosis in a cancer patient. In some embodiments, cancer marker genes serve as targets for anticancer therapeutics.

As used herein, the term "subject diagnosed with a cancer" refers to a subject who has been tested and found to have cancerous cells. The cancer may be diagnosed using any suitable method, including but not limited to, biopsy, x-ray, blood test, and the diagnostic methods of the present invention.

5 As used herein, the term "non-human animals" refers to all non-human animals including, but are not limited to, vertebrates such as rodents, non-human primates, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, aves, etc.

10 As used herein, the term "gene transfer system" refers to any means of delivering a composition comprising a nucleic acid sequence to a cell or tissue. For example, gene transfer systems include, but are not limited to, vectors (*e.g.*, retroviral, adenoviral, adeno-associated viral, and other nucleic acid-based delivery systems), microinjection of naked nucleic acid, polymer-based delivery systems (*e.g.*, liposome-based and metallic particle-based systems), biolistic injection, and the like. As used herein, the term "viral
15 gene transfer system" refers to gene transfer systems comprising viral elements (*e.g.*, intact viruses, modified viruses and viral components such as nucleic acids or proteins) to facilitate delivery of the sample to a desired cell or tissue. As used herein, the term "adenovirus gene transfer system" refers to gene transfer systems comprising intact or altered viruses belonging to the family Adenoviridae.

20 As used herein, the term "nucleic acid molecule" refers to any nucleic acid containing molecule, including but not limited to, DNA or RNA. The term encompasses sequences that include any of the known base analogs of DNA and RNA including, but not limited to, 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylicytosine, pseudoisocytosine, 5-(carboxyhydroxymethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-
25 carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil,
30 beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester,

uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine.

5 The term "gene" refers to a nucleic acid (*e.g.*, DNA) sequence that comprises coding sequences necessary for the production of a polypeptide, precursor, or RNA (*e.g.*, rRNA, tRNA). The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired activity or functional properties (*e.g.*, enzymatic activity, ligand binding, signal transduction, immunogenicity, etc.) of the full-length or fragment is retained. The term also encompasses the coding region of a structural gene and the sequences located adjacent to the coding region on both the 5' and 3' ends for a distance of about 1 kb or more on either end such that the gene corresponds to the length of the full-length mRNA. Sequences located 5' of the coding region and present on the mRNA are referred to as 5' non-translated sequences. Sequences located 3' or downstream of the coding region and present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene that are transcribed into nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

As used herein, the term "gene expression" refers to the process of converting genetic information encoded in a gene into RNA (*e.g.*, mRNA, rRNA, tRNA, or snRNA) through "transcription" of the gene (*i.e.*, via the enzymatic action of an RNA polymerase), and for protein encoding genes, into protein through "translation" of mRNA. Gene expression can be regulated at many stages in the process. "Up-regulation" or "activation" refers to regulation that increases the production of gene expression products (*i.e.*, RNA or protein), while "down-regulation" or "repression" refers to regulation that decrease production. Molecules (*e.g.*, transcription factors) that

are involved in up-regulation or down-regulation are often called "activators" and "repressors," respectively.

In addition to containing introns, genomic forms of a gene may also include sequences located on both the 5' and 3' end of the sequences that are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5' flanking region may contain regulatory sequences such as promoters and enhancers that control or influence the transcription of the gene. The 3' flanking region may contain sequences that direct the termination of transcription, post-transcriptional cleavage and polyadenylation.

The term "wild-type" refers to a gene or gene product isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene or gene product that displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally occurring mutants can be isolated; these are identified by the fact that they have altered characteristics (including altered nucleic acid sequences) when compared to the wild-type gene or gene product.

As used herein, the terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

As used herein, the term "oligonucleotide," refers to a short length of single-stranded polynucleotide chain. Oligonucleotides are typically less than 200 residues long (*e.g.*, between 15 and 100), however, as used herein, the term is also intended to encompass longer polynucleotide chains. Oligonucleotides are often referred to by their length. For example a 24 residue oligonucleotide is referred to as a "24-mer". Oligonucleotides can form secondary and tertiary structures by self-hybridizing or by

hybridizing to other polynucleotides. Such structures can include, but are not limited to, duplexes, hairpins, cruciforms, bends, and triplexes.

As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (*i.e.*, a sequence of nucleotides) related by the base-pairing rules. For example, for the sequence "A-G-T," is complementary to the sequence "T-C-A." Complementarity may be "partial," in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods that depend upon binding between nucleic acids.

The term "homology" refers to a degree of complementarity. There may be partial homology or complete homology (*i.e.*, identity). A partially complementary sequence is a nucleic acid molecule that at least partially inhibits a completely complementary nucleic acid molecule from hybridizing to a target nucleic acid is "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (*i.e.*, the hybridization) of a completely homologous nucleic acid molecule to a target under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (*i.e.*, selective) interaction. The absence of non-specific binding may be tested by the use of a second target that is substantially non-complementary (*e.g.*, less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

When used in reference to a double-stranded nucleic acid sequence such as a cDNA or genomic clone, the term "substantially homologous" refers to any probe that can hybridize to either or both strands of the double-stranded nucleic acid sequence under conditions of low stringency as described above.

A gene may produce multiple RNA species that are generated by differential splicing of the primary RNA transcript. cDNAs that are splice variants of the same gene will contain regions of sequence identity or complete homology (representing the presence of the same exon or portion of the same exon on both cDNAs) and regions of complete non-identity (for example, representing the presence of exon "A" on cDNA 1 wherein cDNA 2 contains exon "B" instead). Because the two cDNAs contain regions of sequence identity they will both hybridize to a probe derived from the entire gene or portions of the gene containing sequences found on both cDNAs; the two splice variants are therefore substantially homologous to such a probe and to each other.

When used in reference to a single-stranded nucleic acid sequence, the term "substantially homologous" refers to any probe that can hybridize (*i.e.*, it is the complement of) the single-stranded nucleic acid sequence under conditions of low stringency as described above.

As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (*i.e.*, the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementarity between the nucleic acids, stringency of the conditions involved, the T_m of the formed hybrid, and the G:C ratio within the nucleic acids. A single molecule that contains pairing of complementary nucleic acids within its structure is said to be "self-hybridized."

As used herein, the term " T_m " is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the T_m of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\% G + C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (See *e.g.*, Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization [1985]). Other references include more sophisticated computations that take structural as well as sequence characteristics into account for the calculation of T_m .

As used herein the term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds such as organic solvents, under which nucleic acid hybridizations are conducted. Under "low stringency conditions" a nucleic acid sequence of interest will hybridize to its exact complement, sequences with single base mismatches, closely related sequences (*e.g.*, sequences with 90% or greater homology), and sequences having only partial homology (*e.g.*, sequences with 50-90% homology). Under "medium stringency conditions," a nucleic acid sequence of interest will hybridize only to its exact complement, sequences with single base mismatches, and closely related sequences (*e.g.*, 90% or greater homology). Under "high stringency conditions," a nucleic acid sequence of interest will hybridize only to its exact complement, and (depending on conditions such as temperature) sequences with single base mismatches. In other words, under conditions of high stringency the temperature can be raised so as to exclude hybridization to sequences with single base mismatches.

As used herein the term "portion" when in reference to a nucleotide sequence (as in "a portion of a given nucleotide sequence") refers to fragments of that sequence. The fragments may range in size from four nucleotides to the entire nucleotide sequence minus one nucleotide (10 nucleotides, 20, 30, 40, 50, 100, 200, etc.).

As used herein, the term "vector" is used in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another. The term "vehicle" is sometimes used interchangeably with "vector." Vectors are often derived from plasmids, bacteriophages, or plant or animal viruses.

The term "expression vector" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

The terms "overexpression" and "overexpressing" and grammatical equivalents, are used in reference to levels of mRNA to indicate a level of expression approximately

3-fold higher (or greater) than that observed in a given tissue in a control or non-transgenic animal. Levels of mRNA are measured using any of a number of techniques known to those skilled in the art including, but not limited to Northern blot analysis. Appropriate controls are included on the Northern blot to control for differences in the amount of RNA loaded from each tissue analyzed (*e.g.*, the amount of 28S rRNA, an abundant RNA transcript present at essentially the same amount in all tissues, present in each sample can be used as a means of normalizing or standardizing the mRNA-specific signal observed on Northern blots). The amount of mRNA present in the band corresponding in size to the correctly spliced transgene RNA is quantified; other minor species of RNA which hybridize to the transgene probe are not considered in the quantification of the expression of the transgenic mRNA.

The term "transfection" as used herein refers to the introduction of foreign DNA into eukaryotic cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics.

The term "stable transfection" or "stably transfected" refers to the introduction and integration of foreign DNA into the genome of the transfected cell. The term "stable transfectant" refers to a cell that has stably integrated foreign DNA into the genomic DNA.

The term "transient transfection" or "transiently transfected" refers to the introduction of foreign DNA into a cell where the foreign DNA fails to integrate into the genome of the transfected cell. The foreign DNA persists in the nucleus of the transfected cell for several days. During this time the foreign DNA is subject to the regulatory controls that govern the expression of endogenous genes in the chromosomes. The term "transient transfectant" refers to cells that have taken up foreign DNA but have failed to integrate this DNA.

As used herein, the term "cell culture" refers to any in vitro culture of cells. Included within this term are continuous cell lines (*e.g.*, with an immortal phenotype), primary cell cultures, transformed cell lines, finite cell lines (*e.g.*, non-transformed cells), and any other cell population maintained in vitro.

As used, the term "eukaryote" refers to organisms distinguishable from "prokaryotes." It is intended that the term encompass all organisms with cells that exhibit the usual characteristics of eukaryotes, such as the presence of a true nucleus bounded by a nuclear membrane, within which lie the chromosomes, the presence of membrane-bound organelles, and other characteristics commonly observed in eukaryotic organisms. Thus, the term includes, but is not limited to such organisms as fungi, protozoa, and animals (*e.g.*, humans).

As used herein, the term "*in vitro*" refers to an artificial environment and to processes or reactions that occur within an artificial environment. *In vitro* environments can consist of, but are not limited to, test tubes and cell culture. The term "*in vivo*" refers to the natural environment (*e.g.*, an animal or a cell) and to processes or reaction that occur within a natural environment.

The terms "test compound" and "candidate compound" refer to any chemical entity, pharmaceutical, drug, and the like that is a candidate for use to treat or prevent a disease, illness, sickness, or disorder of bodily function (*e.g.*, cancer). Test compounds comprise both known and potential therapeutic compounds. A test compound can be determined to be therapeutic by screening using the screening methods of the present invention. In some embodiments of the present invention, test compounds include antisense compounds.

As used herein, the term "sample" is used in its broadest sense. In one sense, it is meant to include a specimen or culture obtained from any source, as well as biological and environmental samples. Biological samples may be obtained from animals (including humans) and encompass fluids, solids, tissues, and gases. Biological samples include blood products, such as plasma, serum and the like. Environmental samples include environmental material such as surface matter, soil, water, crystals and industrial samples. Such examples are not however to be construed as limiting the sample types applicable to the present invention.

The term "chemical moiety" refers to any chemical compound containing at least one carbon atom. Examples of chemical moieties include, but are not limited to, aromatic chemical moieties, chemical moieties comprising Sulfur, chemical moieties comprising Nitrogen, hydrophilic chemical moieties, and hydrophobic chemical moieties.

As used herein, the term "aliphatic" represents the groups including, but not limited to, alkyl, alkenyl, alkynyl, alicyclic.

As used herein, the term "aryl" represents a single aromatic ring such as a phenyl ring, or two or more aromatic rings (*e.g.*, bisphenyl, naphthalene, anthracene), or an aromatic ring and one or more non-aromatic rings. The aryl group can be optionally substituted with a lower aliphatic group (*e.g.*, alkyl, alkenyl, alkynyl, or alicyclic). Additionally, the aliphatic and aryl groups can be further substituted by one or more functional groups including, but not limited to, -NH₂, -NHCOCH₃, -OH, lower alkoxy (C₁-C₄), halo (-F, -Cl, -Br, or -I).

As used herein, the term "substituted aliphatic," refers to an alkane, alkene, alkyne, or alicyclic moiety where at least one of the aliphatic hydrogen atoms has been replaced by, for example, a halogen, an amino, a hydroxy, a nitro, a thio, a ketone, an aldehyde, an ester, an amide, a lower aliphatic, a substituted lower aliphatic, or a ring (aryl, substituted aryl, cycloaliphatic, or substituted cycloaliphatic, *etc.*). Examples of such include, but are not limited to, 1-chloroethyl and the like.

As used herein, the term "substituted aryl" refers to an aromatic ring or fused aromatic ring system consisting of at least one aromatic ring, and where at least one of the hydrogen atoms on a ring carbon has been replaced by, for example, a halogen, an amino, a hydroxy, a nitro, a thio, a ketone, an aldehyde, an ester, an amide, a lower aliphatic, a substituted lower aliphatic, or a ring (aryl, substituted aryl, cycloaliphatic, or substituted cycloaliphatic). Examples of such include, but are not limited to, hydroxyphenyl and the like.

As used herein, the term "cycloaliphatic" refers to an aliphatic structure containing a fused ring system. Examples of such include, but are not limited to, decalin and the like.

As used herein, the term "substituted cycloaliphatic" refers to a cycloaliphatic structure where at least one of the aliphatic hydrogen atoms has been replaced by a halogen, a nitro, a thio, an amino, a hydroxy, a ketone, an aldehyde, an ester, an amide, a lower aliphatic, a substituted lower aliphatic, or a ring (aryl, substituted aryl, cycloaliphatic, or substituted cycloaliphatic). Examples of such include, but are not

limited to, 1-chlorodecalyl, bicyclo-heptanes, octanes, and nonanes (*e.g.*, nonbornyl) and the like.

As used herein, the term "heterocyclic" represents, for example, an aromatic or nonaromatic ring containing one or more heteroatoms. The heteroatoms can be the same or different from each other. Examples of heteroatoms include, but are not limited to nitrogen, oxygen and sulfur. Aromatic and nonaromatic heterocyclic rings are well-known in the art. Some nonlimiting examples of aromatic heterocyclic rings include pyridine, pyrimidine, indole, purine, quinoline and isoquinoline. Nonlimiting examples of nonaromatic heterocyclic compounds include piperidine, piperazine, morpholine, pyrrolidine and pyrazolidine. Examples of oxygen containing heterocyclic rings include, but not limited to furan, oxirane, 2H-pyran, 4H-pyran, 2H-chromene, and benzofuran. Examples of sulfur-containing heterocyclic rings include, but are not limited to, thiophene, benzothiophene, and parathiazine. Examples of nitrogen containing rings include, but not limited to, pyrrole, pyrrolidine, pyrazole, pyrazolidine, imidazole, imidazoline, imidazolidine, pyridine, piperidine, pyrazine, piperazine, pyrimidine, indole, purine, benzimidazole, quinoline, isoquinoline, triazole, and triazine. Examples of heterocyclic rings containing two different heteroatoms include, but are not limited to, phenothiazine, morpholine, parathiazine, oxazine, oxazole, thiazine, and thiazole. The heterocyclic ring is optionally further substituted with one or more groups selected from aliphatic, nitro, acetyl (*i.e.*, -C(=O)-CH₃), or aryl groups.

As used herein, the term "substituted heterocyclic" refers to a heterocyclic structure where at least one of the ring carbon atoms is replaced by oxygen, nitrogen or sulfur, and where at least one of the aliphatic hydrogen atoms has been replaced by a halogen, hydroxy, a thio, nitro, an amino, a ketone, an aldehyde, an ester, an amide, a lower aliphatic, a substituted lower aliphatic, or a ring (aryl, substituted aryl, cycloaliphatic, or substituted cycloaliphatic). Examples of such include, but are not limited to 2-chloropyranyl.

As used herein, the term "linker" refers to a chain containing up to and including eight contiguous atoms connecting two different structural moieties where such atoms are, for example, carbon, nitrogen, oxygen, or sulfur. Ethylene glycol is one non-limiting example.

As used herein, the term "lower-alkyl-substituted-amino" refers to any alkyl unit containing up to and including eight carbon atoms where one of the aliphatic hydrogen atoms is replaced by an amino group. Examples of such include, but are not limited to, ethylamino and the like.

5 As used herein, the term "lower-alkyl-substituted-halogen" refers to any alkyl chain containing up to and including eight carbon atoms where one of the aliphatic hydrogen atoms is replaced by a halogen. Examples of such include, but are not limited to, chlorethyl and the like.

10 As used herein, the term "acetylamino" shall mean any primary or secondary amino that is acetylated. Examples of such include, but are not limited to, acetamide and the like.

15 As used herein, the term "a moiety that participates in hydrogen bonding" or "a chemical moiety that participates in hydrogen bonding" as used herein represents a group that can accept or donate a proton to form a hydrogen bond thereby. Some specific non-limiting examples of moieties that participate in hydrogen bonding include a fluoro, oxygen-containing and nitrogen-containing groups that are well-known in the art. Some examples of oxygen-containing groups that participate in hydrogen bonding include: hydroxy, lower alkoxy, lower carbonyl, lower carboxyl, lower ethers and phenolic groups. The qualifier "lower" as used herein refers to lower aliphatic groups (C₁-C₄) to which the respective oxygen-containing functional group is attached. Thus, for example, 20 the term "lower carbonyl" refers to *inter alia*, formaldehyde, acetaldehyde. Some nonlimiting examples of nitrogen-containing groups that participate in hydrogen bond formation include amino and amido groups. Additionally, groups containing both an oxygen and a nitrogen atom can also participate in hydrogen bond formation. Examples 25 of such groups include nitro, N-hydroxy and nitrous groups. It is also possible that the hydrogen-bond acceptor in the present invention can be the π electrons of an aromatic ring.

30 The term "derivative" of a compound, as used herein, refers to a chemically modified compound wherein the chemical modification takes place either at a functional group of the compound or backbone. Such derivatives include, but are not limited to, esters of alcohol-containing compounds, esters of carboxy-containing compounds,

amides of amine-containing compounds, amides of carboxy-containing compounds, imines of amino-containing compounds, acetals of aldehyde-containing compounds, ketals of carbonyl-containing compounds, and the like.

As used herein, the term “pharmaceutically acceptable salt” refers to any
5 pharmaceutically acceptable salt (*e.g.*, acid or base) of a compound of the present invention which, upon administration to a subject, is capable of providing a compound of this invention or an active metabolite or residue thereof. As is known to those of skill in the art, “salts” of the compounds of the present invention may be derived from inorganic or organic acids and bases. Examples of acids include, but are not limited to,
10 hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycolic, lactic, salicylic, succinic, toluene-p-sulfonic, tartaric, acetic, citric, methanesulfonic, ethanesulfonic, formic, benzoic, malonic, naphthalene-2-sulfonic, benzenesulfonic acid, and the like. Other acids, such as oxalic, while not in themselves pharmaceutically acceptable, may be employed in the preparation of salts useful as
15 intermediates in obtaining the compounds of the invention and their pharmaceutically acceptable acid addition salts.

Examples of bases include, but are not limited to, alkali metals (*e.g.*, sodium) hydroxides, alkaline earth metals (*e.g.*, magnesium), hydroxides, ammonia, and compounds of formula NW_4^+ , wherein W is C_{1-4} alkyl, and the like.

20 Examples of salts include, but are not limited to: acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, flucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate,
25 methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, palmoate, pectinate, persulfate, phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, undecanoate, and the like. Other examples of salts include anions of the compounds of the present invention compounded with a suitable cation such as Na^+ , NH_4^+ , and NW_4^+ (wherein W is a C_{1-4} alkyl group), and the like.

30 For therapeutic use, salts of the compounds of the present invention are contemplated as being pharmaceutically acceptable. However, salts of acids and bases

that are non-pharmaceutically acceptable may also find use, for example, in the preparation or purification of a pharmaceutically acceptable compound.

As used herein, the term "siRNAs" refers to small interfering RNAs. In some embodiments, siRNAs comprise a duplex, or double-stranded region, of about 18-25
5 nucleotides long; often siRNAs contain from about two to four unpaired nucleotides at the 3' end of each strand. At least one strand of the duplex or double-stranded region of a siRNA is substantially homologous to, or substantially complementary to, a target RNA molecule. The strand complementary to a target RNA molecule is the "antisense strand;" the strand homologous to the target RNA molecule is the "sense strand," and is also
10 complementary to the siRNA antisense strand. siRNAs may also contain additional sequences; non-limiting examples of such sequences include linking sequences, or loops, as well as stem and other folded structures. siRNAs appear to function as key intermediaries in triggering RNA interference in invertebrates and in vertebrates, and in triggering sequence-specific RNA degradation during posttranscriptional gene silencing
15 in plants.

The term "RNA interference" or "RNAi" refers to the silencing or decreasing of gene expression by siRNAs. It is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by siRNA that is homologous in its duplex region to the sequence of the silenced gene. The gene may be endogenous or exogenous
20 to the organism, present integrated into a chromosome or present in a transfection vector that is not integrated into the genome. The expression of the gene is either completely or partially inhibited. RNAi may also be considered to inhibit the function of a target RNA; the function of the target RNA may be complete or partial.

25 DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to therapeutic targets for cancer. In particular, the present invention relates to small molecules and nucleic acids that target EZH2 expression in cancer (e.g., prostate cancer, breast cancer, other solid tumors, multiple myeloma).

30

I. EZH2 Targeted Cancer Therapies

In some embodiments, the present invention provides therapies for cancer (*e.g.*, prostate cancer and other solid tumors). In some embodiments, therapies target EZH2.

The Enhancer of Zeste Homolog 2 (EZH2) was identified as a cancer marker with altered expression in cancer (*e.g.* prostate cancer) in previous studies (*e.g.*, U.S. Patent application 2003-0175736 A1; herein incorporated by reference in its entirety). EZH2 belongs to the Polycomb group protein family (PcG). The polycomb group proteins help in maintaining cellular identity by transcriptional repression of target genes (Jacobs et al., *Semin Cell Dev Biol* 1999;10(2):227-35; Jacobs et al., *Biochim Biophys Acta* 2002;1602(2):151-61.). DNA microarrays identified EZH2 as being up-regulated in hormone-refractory metastatic prostate cancer (Dhanasekaran et al., *Nature* 2001;412(6849):822-6; Varambally et al., *Nature* 2002;419(6907):624-9). EZH2 is upregulated in aggressive breast tumors and is a mediator of a pro-invasive phenotype (Kleer et al., *Proc Natl Acad Sci U S A* 2003;100(20):11606-11). Overexpression of EZH2 in immortalized human mammary epithelial cell lines promotes anchorage-independent growth and cell invasion (Kleer et al., *supra*). EZH2-mediated cell invasion required an intact SET domain and histone deacetylase activity. Previous studies provided evidence for a functional link between dysregulated EZH2 expression, transcriptional repression, and neoplastic transformation (Varambally et al., *supra*; Kleer et al., *supra*).

The present invention is not limited to a particular mechanism. Indeed, an understanding of the mechanism is not necessary to understand the present invention. Nonetheless, based upon previous studies on Polycomb group proteins, several models have been hypothesized to explain how PcG proteins exert their function. They are: 1) inhibition of the transcriptional machinery and alteration of the transcriptional state of cells; 2) forming a complex to prevent chromatin from binding to other proteins; and 3) recruiting target genes to repressive nuclear structures (Satijn et al., *Biochim Biophys Acta* 1999;1447(1):1-16). Previous studies indicated EZH2 upregulation in breast cancer and that EZH2 mediates invasion (Kleer et al., *supra*).

Metastatic prostate disease almost universally overexpresses EZH2. Furthermore, aggressive localized tumors of the prostate, bladder, and breast and other solid tumors also express high levels of EZH2. Accordingly, it is contemplated that anti EZH2

therapies find use in the treatment of cancer, in particular metastatic cancers such as prostate, breast and bladder cancer.

A. miRNA Therapies

5 In some embodiments, the present invention provides MicroRNAs that inhibit the expression of EZH2. MicroRNAs are regulatory, non-protein-coding, endogenous RNAs that have recently gained considerable attention in the scientific community. They are 18-24 nucleotides in length and are thought to regulate gene expression through translational repression by binding to a target mRNA (Lim et al., Science
10 2003;299(5612):1540; Chen et al., Semin Immunol 2005;17(2):155-65; Sevignani et al., Mamm Genome 2006;17(3):189-202). They are also proposed to regulate gene expression by mRNA cleavage, and mRNA decay initiated by miRNA-guided rapid deadenylation (Wu et al., Proc Natl Acad Sci U S A 2006;103(11):4034-9). miRNAs are abundant, highly conserved molecules and predicted to regulate a large number of
15 transcripts. To date the international miRNA Registry database has more than 600 human identified microRNAs (Griffiths-Jones et al., Nucleic Acids Res 2006;34 (Database issue):D140-4) and their total number in humans has been predicted to be as high as 1,000 (Berezikov et al., Cell 2005;120(1):21-4). Many of these microRNAs exhibit tissue-specific expression (Sood et al., Proc Natl Acad Sci U S A
20 2006;103(8):2746-51) and many are defined to be either tumor suppressors or oncogenes (Lee et al., Curr Opin Investig Drugs 2006;7(6):560-4; Zhang et al., Dev Biol 2006; Calin et al., Nat Rev Cancer 2006;6(11):857-66) and play a crucial role in variety of cellular processes such as cell cycle control, apoptosis, and haematopoiesis. Dysregulation of several miRNAs are thought to play a significant role in human disease
25 processes including tumorigenesis (Hwang et al., Br J Cancer 2006;94(6):776-80; Thomson et al., Genes Dev 2006;20(16):2202-7).

Several microRNAs are located in the region of hot spots for chromosomal abnormalities (Calin et al., Oncogene 2006;25(46):6202-10; Calin et al., Proc Natl Acad Sci U S A 2004;101(9):2999-3004). This results in abnormal expression of miRNAs
30 which affect cellular functions. Recent studies indicate that selected miRNAs may play a role in human cancer pathogenesis. For example, deletions or mutations in genes that

code for miRNA tumor suppressors lead to loss of a miRNA or miRNA cluster, and thereby contribute to oncogene deregulation (Zhang et al., supra; Calin et al., supra). The results of large-scale miRNA profiling studies using normal and cancer tissues show that a number of microRNAs are either overexpressed or downregulated in tumors (Alvarez-Garcia et al., Development 2005;132(21):4653-62; Volinia et al., Proc Natl Acad Sci U S A 2006;103(7):2257-61; Cummins et al., Proc Natl Acad Sci U S A 2006;103(10):3687-92; Yanaihara et al., Cancer Cell 2006;9(3):189-98; Iorio et al., Cancer Res 2005;65(16):7065-70; Calin et al., Proc Natl Acad Sci U S A 2004;101(32):11755-60; Calin et al., N Engl J Med 2005;353(17):1793-801; Pallante et al., Endocr Relat Cancer 2006;13(2):497-508). It has been shown that miRNA genes are frequently located in cancer-associated genomic regions or fragile sites (Calin et al., Proc Natl Acad Sci U S A 2004;101(9):2999-3004). The genes encoding mir-15 and mir-16 are located at chromosome 13q14, a region that is deleted in the majority of B-cell chronic lymphocytic leukemias (B-CLL) indicating that mir-15 and mir-16 may function as tumor suppressors. let-7 miRNA family members are known to down regulate the oncogene RAS (Johnson et al., Cell 2005;120(5):635-47). Its expression is reduced in tumors which in turn contributes to the elevated activity of the RAS pathway (Yanaihara et al., Cancer Cell 2006;9(3):189-98). Expression levels of miR-143 and miR-145 were decreased in colon cancer tissues as well as in cancer cell lines (Michael et al., Mol Cancer Res 2003;1(12):882-91). In contrast, several microRNAs are upregulated in cancer. Members of the miR-17 cluster provide an oncogenic function via their upregulated expression by c-Myc leading to effects on downstream genes which are mediators of cell cycle and apoptosis events (O'Donnell et al., Nature 2005;435(7043):839-43).

Many microRNAs play a role during development and tissue differentiation (Pasquinelli et al., Curr Opin Genet Dev 2005;15(2):200-5). miR-181, a microRNA that is strongly upregulated during differentiation, participates in establishing the muscle phenotype. Recent studies suggest that miR-181 down regulates the homeobox protein Hox-A11 (Naguibneva et al., Nat Cell Biol 2006;8(3):278-84). Similarly miR-196 is involved in regulating HOXB8 confirming the significant roles played by microRNA during developmental processes. A recent study from Lim et al., (Yekta et al., Science

2004;304(5670):594-6) showed that a few microRNAs can regulate large numbers of target mRNA and their studies also indicated that the miRNA can downregulated not only the proteins, but the transcript level of the target mRNA. Specific expression of microRNA are of prognostic significance, indicating that miRNAs are determinants of clinical aggressiveness (Volinia et al., supra, Iorio et al. Cancer Res 2005;65(16):7065-70; Lu et al., Nature 2005;435(7043):834-8). Thus, microRNA expression profiles can serve as a new class of cancer biomarkers. Breast cancer microRNA profiling studies by Iorio et al., (supra) indicated the expression patterns of several microRNAs were significantly different between normal and neoplastic tissues. This profiling study indicated miR-21 and miR-155 to be consistently up regulated and miR-10b, miR-125b and miR-145 to be down regulated. Further, breast tumor microRNA profiling distinguished normal from malignant breast tissue and correlated with breast cancer histopathologic features such as tumor size, nodal involvement, proliferative capacity and vascular invasiveness.

During experiments conducted during the course of development of embodiments of the present invention a search of the miRNA Registry database for microRNA that would target EZH2 indicated has-miR-101. Further experiments conducted during the course of development of embodiments of the present invention demonstrated that EZH2 expression is inhibited by miR-101.

Accordingly, in some embodiments, the present invention provides methods of inhibiting EZH2 expression and/or activity using microRNAs (e.g., miR-101). In some embodiments, miRNAs inhibit the expression of EZH2 protein. In other embodiments, miRNAs inhibit EZH2 activity (e.g., cell invasion activity).

The present invention is not limited to miR-101. Additional miRNAs can be screened for their activity against EZH2 using any suitable method, including, but not limited to, those disclosed in Example 1 below.

Suitable nucleic acids for use in the methods described herein include, but are not limited to, pri-miRNA, pre-miRNA, mature miRNA or fragments of variants thereof that retain the biological activity of the miRNA and DNA encoding a pri-miRNA, pre-miRNA, mature miRNA, fragments or variants thereof, or DNA encoding regulatory elements of the miRNA.

In some embodiments the nucleic acid encoding the disclosed inhibitory nucleic acids, for example an miRNA molecule, is on a vector. These vectors include a sequence encoding a mature microRNA and in vivo expression elements. In a preferred embodiment, these vectors include a sequence encoding a pre-miRNA and in vivo expression elements such that the pre-miRNA is expressed and processed in vivo into a mature miRNA. In other embodiments, these vectors include a sequence encoding the pri- miRNA gene and in vivo expression elements. In this embodiment, the primary transcript is first processed to produce the stem-loop precursor miRNA molecule. The stem-loop precursor is then processed to produce the mature microRNA. Vectors include, but are not limited to, plasmids, cosmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the nucleic acid sequences for producing the microRNA, and free nucleic acid fragments which can be attached to these nucleic acid sequences. Viral and retroviral vectors are a preferred type of vector and include, but are not limited to, nucleic acid sequences from the following viruses: retroviruses, such as: Moloney murine leukemia virus; Murine stem cell virus, Harvey murine sarcoma virus; murine mammary tumor virus; Rous sarcoma virus; adenovirus; adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes viruses; vaccinia viruses; polio viruses; and RNA viruses such as any retrovirus. One of skill in the art can readily employ other vectors known in the art.

Viral vectors are generally based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the nucleic acid sequence of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA.

Retroviruses have been approved for human gene therapy trials. Genetically altered retroviral expression vectors have general utility for the high- efficiency transduction of nucleic acids in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from

tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, M., "Gene Transfer and Expression, A Laboratory Manual," W.H. Freeman Co., New York (1990) and Murray, E. J. Ed. "Methods in Molecular Biology," vol. 7, Humana Press, Inc., Clifton, N.J. (1991).

5 In some embodiments, vectors comprise in vivo expression elements, which are any regulatory nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient expression of the nucleic acid to produce the microRNA. The in vivo expression element may, for example, be a mammalian or viral promoter, such as a constitutive or inducible promoter or a tissue specific promoter, examples of which are well known to one of ordinary skill in the art. Constitutive
10 mammalian promoters include polymerase promoters as well as the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPTR), adenosine deaminase, pyruvate kinase, and beta.-actin. Exemplary viral promoters which function constitutively in eukaryotic cells include promoters from the simian virus, papilloma
15 virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus, the long terminal repeats (LTR) of moloney leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other constitutive promoters are known to those of ordinary skill in the art. Inducible promoters are expressed in the presence of an inducing agent and include metal-
20 inducible promoters and steroid- regulated promoters. For example, the metallothionein promoter is induced to promote transcription in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

 Examples of tissue-specific promoters include the promoter for creatine kinase, which has been used to direct expression in muscle and cardiac tissue and
25 immunoglobulin heavy or light chain promoters for expression in B cells. Other tissue specific promoters include the human smooth muscle alpha-actin promoter. Exemplary tissue-specific expression elements for the liver include, but are not limited to, HMG-COA reductase promoter, sterol regulatory element 1, phosphoenol pyruvate carboxy kinase (PEPCK) promoter, human C-reactive protein (CRP) promoter, human
30 glucokinase promoter, cholesterol 7-alpha hydroxylase (CYP-7) promoter, beta-galactosidase alpha-2,6 sialyltransferase promoter, insulin-like growth factor binding

protein (IGFBP-1) promoter, aldolase B promoter, human transferrin promoter, and collagen type I promoter. Exemplary tissue- specific expression elements for the prostate include but are not limited to the prostatic acid phosphatase (PAP) promoter, prostatic secretory protein of 94 (PSP 94) promoter, prostate specific antigen complex promoter, and human glandular kallikrein gene promoter (hgt-1). Exemplary tissue-specific expression elements for gastric tissue include but are not limited to the human H⁺/K⁺-ATPase alpha subunit promoter. Exemplary tissue-specific expression elements for the pancreas include but are not limited to pancreatitis associated protein promoter (PAP), elastase 1 transcriptional enhancer, pancreas specific amylase and elastase enhancer promoter, and pancreatic cholesterol esterase gene promoter. Exemplary tissue-specific expression elements for the endometrium include the uteroglobin promoter. Exemplary tissue-specific expression elements for adrenal cells include cholesterol side-chain cleavage (SCC) promoter. Exemplary tissue- specific expression elements for the general nervous system include gamma- gamma enolase (neuron-specific enolase, NSE) promoter. Exemplary tissue- specific expression elements for the brain include the neurofilament heavy chain (NF-H) promoter. Exemplary tissue-specific expression elements for lymphocytes include the human CGL-1/granzyme B promoter, the terminal deoxy transferase (TdT), lambda 5, VpreB, and ick (lymphocyte specific tyrosine protein kinase p56lck) promoter, the human CD2 promoter and its 3' transcriptional enhancer, and the human NK and T cell specific activation (NKG5) promoter. Exemplary tissue-specific expression elements for the colon include pp60c-src tyrosine kinase promoter, organ- specific neoantigens (OSNs) promoter, and colon specific antigen-P promoter. Exemplary tissue-specific expression elements for breast cells include the human alpha-lactalbumin promoter. Exemplary tissue-specific expression elements for the lung include the cystic fibrosis transmembrane conductance regulator (CFTR) gene promoter. Other elements aiding specificity of expression in a tissue of interest can include secretion leader sequences, enhancers, nuclear localization signals, endosmolytic peptides, etc. Preferably, these elements are derived from the tissue of interest to aid specificity.

In general, the in vivo expression element includes, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription. They optionally include enhancer sequences or upstream activator sequences.

The miRNA can be isolated from cells or tissues, recombinantly produced, or
5 synthesized in vitro by a variety of techniques well known to one of ordinary skill in the art. In one embodiment, miRNA is isolated from cells or tissues. Techniques for isolating miRNA from cells or tissues are well known to one of ordinary skill in the art. For example, miRNA can be isolated from total RNA using the mirVana miRNA isolation kit from Ambion, Inc. Another technique utilizes the flashPAGE™ Fractionator
10 System (Ambion, Inc.) for PAGE purification of small nucleic acids.

The miRNA can be obtained by preparing a recombinant version thereof (e.g., by using the techniques of genetic engineering to produce a recombinant nucleic acid which can then be isolated or purified by techniques well known to one of ordinary skill in the art). This embodiment involves growing a culture of host cells in a suitable culture
15 medium, and purifying the miRNA from the cells or the culture in which the cells are grown. For example, the methods include a process for producing a miRNA in which a host cell containing a suitable expression vector that includes a nucleic acid encoding an miRNA is cultured under conditions that allow expression of the encoded miRNA. The miRNA can be recovered from the culture, from the culture medium or from a lysate
20 prepared from the host cells, and further purified. The host cell can be a higher eukaryotic host cell such as a mammalian cell, a lower eukaryotic host cell such as a yeast cell, or the host cell can be a prokaryotic cell such as a bacterial cell. Introduction of a vector containing the nucleic acid encoding the miRNA into the host cell can be effected by calcium phosphate transfection, DEAE, dextran mediated transfection, or
25 electroporation (Davis, L. et al., Basic Methods in Molecular Biology (1986)).

Any host/vector system can be used to express one or more of the miRNAs. These include eukaryotic hosts such as HeLa cells and yeast, as well as prokaryotic hosts such as *E. coli* and *B. subtilis*. miRNA can be expressed in mammalian cells, yeast, bacteria, or other cells where the miRNA gene is under the control of an appropriate promoter.
30 Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., in Molecular Cloning: A Laboratory Manual, Second

Edition, Cold Spring Harbor, N.Y. (1989). In some embodiments, the miRNA is expressed in mammalian cells. Examples of mammalian expression systems include C 127, monkey COS cells, Chinese Hamster Ovary (CHO) cells, human kidney 293 cells, human epidermal A43 1 cells, human Colo205 cells, 3T3 cells, CV-1 cells, other
5 transformed primate cell lines, normal diploid cells, cell strains derived from in vitro culture of primary tissue, primary explants, HeLa cells, mouse L cells, BHK, HL-60, U937, HaK or Jurkat cells. In some embodiments, mammalian expression vectors will comprise an origin of replication, a suitable promoter, polyadenylation site, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA
10 sequences derived from the SV40 viral genome, for example, SV40 origin, early promoter, enhancer, splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

Suitable yeast strains include, but are not limited to, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces* spp. strains, *Candida* spp., or any yeast
15 strain capable of expressing miRNA. Suitable bacterial strains include, but are not limited to, *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, or any bacterial strain capable of expressing miRNA.

In a preferred embodiment, genomic DNA encoding a miRNA is isolated, the genomic DNA is expressed in a mammalian expression system, and RNA is purified and
20 modified as necessary for administration to an individual. In some embodiments the miRNA is in the form of a pre-miRNA, which can be modified as desired (i.e. for increased stability or cellular uptake).

Knowledge of DNA sequences of miRNA allows for modification of cells to permit or increase expression of an endogenous miRNA. Cells can be modified (e.g., by
25 homologous recombination) to provide increased miRNA expression by replacing, in whole or in part, the naturally occurring promoter with all or part of a heterologous promoter so that the cells express the miRNA at higher levels. The heterologous promoter is inserted in such a manner that it is operatively linked to the desired miRNA encoding sequences. See, for example, PCT International Publication No. WO 94/12650
30 by Transkaryotic Therapies, Inc., PCT International Publication No. WO 92/20808 by Cell Genesys, Inc., and PCT International Publication No. WO 9 1/09955 by Applied

Research Systems, each of which is herein incorporated by reference. Cells also may be engineered to express an endogenous gene comprising the miRNA under the control of inducible regulatory elements, in which case the regulatory sequences of the endogenous gene may be replaced by homologous recombination. Gene activation techniques are
5 described in U.S. Patent No. 5,272,071 to Chappel; U.S. Patent No. 5,578,461 to Sherwin et al.; PCT/US92/09627 (W093/09222) by Selden et al.; and PCT/US90/06436 (W091/06667) by Skoultchi et al., each of which is herein incorporated by reference.

The miRNA may be prepared by culturing transformed host cells under culture conditions suitable to express the miRNA. The resulting expressed miRNA may then be
10 purified from such culture (i.e., from culture medium or cell extracts) using known purification processes, such as gel filtration and ion exchange chromatography. The purification of the miRNA may also include an affinity column containing agents which will bind to the protein; one or more column steps over such affinity resins as concanavalin A-agarose, HEPARINTOYOPEARL or Cibacrom blue 3GA
15 SEPHAROSE; one or more steps involving hydrophobic interaction chromatography using such resins as phenyl ether, butyl ether, or propyl ether; immunoaffinity chromatography, or complementary eDNA affinity chromatography.

The miRNA may also be expressed as a product of transgenic animals, which are characterized by somatic or germ cells containing a nucleotide sequence encoding the
20 miRNA. A vector containing DNA encoding miRNA and appropriate regulatory elements can be inserted in the germ line of animals using homologous recombination (Capecchi, Science 244:1288-1292 (1989)), such that the animals express the miRNA. Transgenic animals, preferably non-human mammals, are produced using methods as described in U.S. Patent No 5,489,743 to Robinson, et al., and PCT Publication No. WO
25 94/28 122 by Ontario Cancer Institute, each of which is herein incorporated by reference. miRNA can be isolated from cells or tissue isolated from transgenic animals as discussed above.

In some embodiments, the miRNA can be obtained synthetically, for example, by chemically synthesizing a nucleic acid by any method of synthesis known to the skilled
30 artisan. The synthesized miRNA can then be purified by any method known in the art. Methods for chemical synthesis of nucleic acids include in vitro chemical synthesis using

phosphotriester, phosphate or phosphoramidite chemistry and solid phase techniques, or via deoxynucleoside H-phosphonate intermediates (see U.S. Patent No. 5,705,629 to Bhongle, herein incorporated by reference in its entirety).

In some circumstances, for example, where increased nuclease stability is desired, nucleic acids having nucleic acid analogs and/or modified internucleoside linkages are utilized. Nucleic acids containing modified internucleoside linkages may also be synthesized using reagents and methods that are well known in the art. For example, methods of synthesizing nucleic acids containing phosphonate phosphorothioate, phosphorodithioate, phosphoramidate methoxyethyl phosphoramidate, formacetal, thioformacetal, diisopropylsilyl, acetamidate, carbamate, dimethylene-sulfide (-CH₂-S-CH₂), diethylene- sulfoxide (-CH₂-SO-CH₂), dimethylene-sulfone (-CH₂-SO₂-CH₂), 2'-O-alkyl, and 2'-deoxy-2'-fluoro phosphorothioate internucleoside linkages are well known in the art (see Uhlman et al., 1990, Chem. Rev. 90:543-584; Schneider et al., 1990, Tetrahedron Lett. 31:335 and references cited therein). U.S. Patent Nos. 5,614,617 and 5,223,618 to Cook, et al., 5,714,606 to Acevedo, et al., 5,378,825 to Cook, et al., 5,672,697 and 5,466,786 to Buhr, et al., 5,777,092 to Cook, et al., 5,602,240 to De Mesmaeker, et al., 5,610,289 to Cook, et al. and 5,858,988 to Wang, each of which is herein incorporated by reference in its entirety, also describe nucleic acid analogs for enhanced nuclease stability and cellular uptake. Additional methods for the administrations of miRNAs can be found, for example, in US20050261218A1, US20060105360A1, WO06119365A2, and WO05078096A2, each of which is herein incorporated by reference in its entirety.

The compounds can be administered to a host in an amount effective to treat or inhibit cancer or tumor growth (e.g., prostate cancer). The compositions are administered to an individual in need of treatment or prophylaxis of at least one symptom or manifestation (since disease can occur/progress in the absence of symptoms) of cancer. In some embodiments, the compositions are administered in an effective amount to inhibit gene expression of EZH2.

The present invention also includes pharmaceutical compositions and formulations that include the miRNA compounds of the present invention as described below.

B. Small Molecule Therapies

In other embodiments, the present invention provides small molecule inhibitors of EZH2 expression or activity. Experiments conducted during the course of development of embodiments of the present invention utilized cDNA expression microarray analysis using the RNA isolated from EZH2 overexpressing cells along with control RNA. The expression microarray of the present invention is also suitable for use in high-throughput experiments.

It was observed that the tumor suppressor protein E-cadherin was specifically downregulated in EZH2 overexpressing cells. These observations were further confirmed by immunoblot assays as well as co-immunostainings. Furthermore, the inverse correlation between increased EZH2 expression and E-cadherin down regulation was observed in aggressive breast tumors as well.

Further experiments conducted during the course of development of embodiments of the present invention identified isoliquiritigenin as an inhibitor of EZH2 expression. Accordingly, in some embodiments, the present invention provides methods of treating cancer (e.g., metastatic cancer) using isoliquiritigenin or related compounds.

Isoliquiritigenin, one of the components in the root of *Glycyrrhiza glabra* L., is a member of the flavonoids, which are known to have an anti-tumor activity in vitro and in vivo. (Kanazawa et al., Eur Urol. 2003 May;43(5):580-6.). Isoliquiritigenin has also been shown to be a soluble guanylate cyclase activator (Yu et al., Brit. J. Pharmacol. 114 (1995), 1587) and to possess estrogen-like activity (see, for example, S. Tamir, J. Steroid Biochem. Mol. Biol. (2001), 78(3): 291-8). Isoliquiritigenin has been shown to activate estrogen receptor-alpha and -beta and trigger biochemical reactions in cancer cells. The COX-2 inhibitory activity of isoliquiritigenin has also been demonstrated. (See e.g., WO 03/075943; US 6,696,407; and 4,952,564, each of which is herein incorporated by reference).

As used herein, isoliquiritigenin refers to CAS Reg. No. 961-29-5; also known as 2',J,d'- trihydroxychalcone, a pharmaceutically acceptable salt or ester of isoliquiritigenin, a selectively substituted analog of isoliquiritigenin, an extract of *Glycyrrhiza uralensis*

or Glycyrrhiza glabra, or a combination comprising one or more of the foregoing compounds. An ester of isoliquiritigenin is preferably a glycoside of isoliquiritigenin.

There is no particular limit on the monosacharide or polysaccharide used to form the glycoside of isoliquiritigenin. Suitable monosaccharides sugars include, for example, glucose, glucuronic acid, mannose, fructose, galactose, xylose, rutinose, rhamnose, and the like, and combinations comprising one or more of the foregoing monosaccharides. Suitable polysaccharides include, for example, dimers, trimers, oligomers, and polymers formed from one or more of the above monosaccharides.

An isoliquiritigenin analog includes, for example, phloretin, 2', 4,4' trihydroxychalcone, or the like, or a combination comprising one or more of the foregoing isoliquiritigenin analogs.

Methods for synthesizing or isolating isoliquiritigenin, its pharmaceutically acceptable salts or esters, its selectively substituted analogs, are known in the art. See, for example, S. K. Srivastava et al., Indian J. Chem., Sect. B (1981), 20B(4): 347-8; Macias et al., Phytochemistry (1998), 50(1): 35-46, each of which is herein incorporated by reference.

In some embodiments, when isoliquiritigenin is present, the isoliquiritigenin comprises greater than or equal to 0.5 weight percent, more preferably greater than or equal to about 1 weight percent, still more preferably greater than or equal to about 2 weight percent, even more preferably greater than or equal to about 5 weight percent, even more preferably greater than or equal to about 10 weight percent, still more preferably greater than or equal to about 20 weight percent of the total weight of the composition.

In some embodiments, the cancer is prostate. In other embodiments, the cancer is bladder, breast, or other solid tumors. Additional small molecule EZH2 inhibitors are identified, for example, using the compositions and methods of the present invention. The present invention additionally contemplates mimetics, analogs and modified forms of isoliquiritigenin.

The present invention is not limited to a particular mechanism. Indeed, an understanding of the mechanism is not necessary to practice the present invention. Nonetheless, it is contemplated that a small molecule inhibitor against EZH2 finds use in

the treatment of metastatic disease, which almost universally overexpresses EZH2. Furthermore, aggressive localized tumors of the prostate, bladder, and breast and other solid tumors expressing high levels of EZH2 are also therapeutically targeted by EZH2 inhibitors (e.g., isoliquiritigenin).

5 Additional small molecule inhibitors were identified using a screening assaying. Exemplary compounds are shown in Tables 1-3. In some embodiments, these compounds find use in the inhibition of EZH2 (e.g., as cancer therapeutics), alone or in combination with additional therapeutic agents described herein.

The present invention also includes pharmaceutical compositions and
10 formulations that include the small molecule compounds of the present invention as described below.

C. RNA Interference and Antisense Therapies

In some embodiments, the present invention targets the expression of EZH2. For
15 example, in some embodiments, the present invention employs compositions comprising oligomeric antisense or RNAi compounds, particularly oligonucleotides (e.g., those described herein), for use in modulating the function of nucleic acid molecules encoding EZH2, ultimately modulating the amount of EZH2 expressed.

20 1. RNA Interference (RNAi)

In some embodiments, RNAi is utilized to inhibit EZH2 protein function. RNAi represents an evolutionary conserved cellular defense for controlling the expression of foreign genes in most eukaryotes, including humans. RNAi is typically triggered by double-stranded RNA (dsRNA) and causes sequence-specific mRNA degradation of
25 single-stranded target RNAs homologous in response to dsRNA. The mediators of mRNA degradation are small interfering RNA duplexes (siRNAs), which are normally produced from long dsRNA by enzymatic cleavage in the cell. siRNAs are generally approximately twenty-one nucleotides in length (e.g. 21-23 nucleotides in length), and have a base-paired structure characterized by two nucleotide 3'-overhangs. Following the
30 introduction of a small RNA, or RNAi, into the cell, it is believed the sequence is delivered to an enzyme complex called RISC (RNA-induced silencing complex). RISC

recognizes the target and cleaves it with an endonuclease. It is noted that if larger RNA sequences are delivered to a cell, RNase III enzyme (Dicer) converts longer dsRNA into 21-23 nt ds siRNA fragments.

Chemically synthesized siRNAs have become powerful reagents for genome-wide
5 analysis of mammalian gene function in cultured somatic cells. Beyond their value for validation of gene function, siRNAs also hold great potential as gene-specific therapeutic agents (Tuschl and Borkhardt, *Molecular Intervent.* 2002; 2(3):158-67, herein incorporated by reference).

The transfection of siRNAs into animal cells results in the potent, long-lasting
10 post-transcriptional silencing of specific genes (Caplen et al, *Proc Natl Acad Sci U.S.A.* 2001; 98: 9742-7; Elbashir et al., *Nature.* 2001; 411:494-8; Elbashir et al., *Genes Dev.* 2001;15: 188-200; and Elbashir et al., *EMBO J.* 2001; 20: 6877-88, all of which are herein incorporated by reference). Methods and compositions for performing RNAi with siRNAs are described, for example, in U.S. Pat. 6,506,559, herein incorporated by
15 reference.

siRNAs are extraordinarily effective at lowering the amounts of targeted RNA, and by extension proteins, frequently to undetectable levels. The silencing effect can last several months, and is extraordinarily specific, because one nucleotide mismatch between the target RNA and the central region of the siRNA is frequently sufficient to prevent
20 silencing (Brummelkamp et al, *Science* 2002; 296:550-3; and Holen et al, *Nucleic Acids Res.* 2002; 30:1757-66, both of which are herein incorporated by reference).

An important factor in the design of siRNAs is the presence of accessible sites for siRNA binding. Bahoia et al., (*J. Biol. Chem.*, 2003; 278: 15991-15997; herein incorporated by reference) describe the use of a type of DNA array called a scanning
25 array to find accessible sites in mRNAs for designing effective siRNAs. These arrays comprise oligonucleotides ranging in size from monomers to a certain maximum, usually Comers, synthesized using a physical barrier (mask) by stepwise addition of each base in the sequence. Thus the arrays represent a full oligonucleotide complement of a region of the target gene. Hybridization of the target mRNA to these arrays provides an exhaustive
30 accessibility profile of this region of the target mRNA. Such data are useful in the design of antisense oligonucleotides (ranging from 7mers to 25mers), where it is important to

achieve a compromise between oligonucleotide length and binding affinity, to retain efficacy and target specificity (Sohail et al, Nucleic Acids Res., 2001; 29(10): 2041-2045). Additional methods and concerns for selecting siRNAs are described for example, in WO 05054270, WO05038054A1, WO03070966A2, J Mol Biol. 2005 May 13;348(4):883-93, J Mol Biol. 2005 May 13;348(4):871-81, and Nucleic Acids Res. 2003 Aug 1;31(15):4417-24, each of which is herein incorporated by reference in its entirety. In addition, software (*e.g.*, the MWG online siMAX siRNA design tool) is commercially or publicly available for use in the selection of siRNAs.

In some embodiments, the present invention utilizes siRNA including blunt ends (See *e.g.*, US20080200420, herein incorporated by reference in its entirety), overhangs (See *e.g.*, US20080269147A1, herein incorporated by reference in its entirety), locked nucleic acids (See *e.g.*, WO2008/006369, WO2008/043753, and WO2008/051306, each of which is herein incorporated by reference in its entirety). In some embodiments, siRNAs are delivered via gene expression or using bacteria (See *e.g.*, Xiang et al., Nature 24: 6 (2006) and WO06066048, each of which is herein incorporated by reference in its entirety).

In other embodiments, shRNA techniques (See *e.g.*, 20080025958, herein incorporated by reference in its entirety) are utilized. A small hairpin RNA or short hairpin RNA (shRNA) is a sequence of RNA that makes a tight hairpin turn that can be used to silence gene expression via RNA interference. shRNA uses a vector introduced into cells and utilizes the U6 promoter to ensure that the shRNA is always expressed. This vector is usually passed on to daughter cells, allowing the gene silencing to be inherited. The shRNA hairpin structure is cleaved by the cellular machinery into siRNA, which is then bound to the RNA-induced silencing complex (RISC). This complex binds to and cleaves mRNAs which match the siRNA that is bound to it. shRNA is transcribed by RNA polymerase III.

The present invention also includes pharmaceutical compositions and formulations that include the RNAi compounds of the present invention as described below.

2. Antisense

In other embodiments, EZH2 protein expression is modulated using antisense compounds that specifically hybridize with one or more nucleic acids encoding EZH2. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds that specifically hybridize to it is generally referred to as "antisense." The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity that may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of cancer markers of the present invention. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. For example, expression may be inhibited to prevent tumor proliferation.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of the present invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding EZH2. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, *e.g.*, detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and

5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). Eukaryotic and prokaryotic genes
5 may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the present invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding a tumor antigen of the present
10 invention, regardless of the sequence(s) of such codons.

Translation termination codon (or "stop codon") of a gene may have one of three sequences (*i.e.*, 5'-UAA, 5'-UAG and 5'-UGA; the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that
15 encompasses from about 25 to about 50 contiguous nucleotides in either direction (*i.e.*, 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (*i.e.*, 5' or 3') from a translation termination codon.

20 The open reading frame (ORF) or "coding region," which refers to the region between the translation initiation codon and the translation termination codon, is also a region that may be targeted effectively. Other target regions include the 5' untranslated region (5' UTR), referring to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the
25 translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3' UTR), referring to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue
30 joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap

region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," that are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites (*i.e.*, intron-exon junctions) may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

In some embodiments, target sites for antisense inhibition are identified using commercially available software programs (*e.g.*, Biognostik, Gottingen, Germany; SysArris Software, Bangalore, India; Antisense Research Group, University of Liverpool, Liverpool, England; GeneTrove, Carlsbad, CA). In other embodiments, target sites for antisense inhibition are identified using the accessible site method described in PCT Publ. No. WO0198537A2, herein incorporated by reference.

Once one or more target sites have been identified, oligonucleotides are chosen that are sufficiently complementary to the target (*i.e.*, hybridize sufficiently well and with sufficient specificity) to give the desired effect. For example, in preferred embodiments of the present invention, antisense oligonucleotides are targeted to or near the start codon.

In the context of this invention, "hybridization," with respect to antisense compositions and methods, means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases that pair through the formation of hydrogen bonds. It is understood that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient

degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired (*i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed).

5 Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with specificity, can be used to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway.

10 The specificity and sensitivity of antisense is also applied for therapeutic uses. For example, antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotides have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides are useful therapeutic modalities
15 that can be configured to be useful in treatment regimes for treatment of cells, tissues, and animals, especially humans.

 While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense
20 compounds in accordance with this invention preferably comprise from about 8 to about 30 nucleobases (*i.e.*, from about 8 to about 30 linked bases), although both longer and shorter sequences may find use with the present invention. Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 12 to about 25 nucleobases.

25 Specific examples of preferred antisense compounds useful with the present invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this
30 specification, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including
5 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

10 Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar
15 portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

20 In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage (*i.e.*, the backbone) of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a
25 peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not
30 limited to, U.S. Pat. Nos.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein

incorporated by reference. Further teaching of PNA compounds can be found in Nielsen *et al.*, Science 254:1497 (1991).

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular --CH₂, --NH--O--CH₂--, --CH₂--N(CH₃)--O--CH₂-- [known as a methylene (methylinino) or MMI backbone], --CH₂--O--N(CH₃)--CH₂--, --CH₂--N(CH₃)--N(CH₃)--CH₂--, and --O--N(CH₃)--CH₂--CH₂-- [wherein the native phosphodiester backbone is represented as --O--P--O--CH₂--] of the above referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above referenced U.S. Pat. No. 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C1 to C10 alkyl or C2 to C10 alkenyl and alkynyl. Particularly preferred are O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C1 to C10 lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O--CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, Helv. Chim. Acta 78:486 [1995]) *i.e.*, an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoethoxyethoxy (*i.e.*, a O(CH₂)₂ON(CH₃)₂ group), also known as 2'-DMAOE, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), *i.e.*, 2'-O--CH₂--O--CH₂--N(CH₂)₂.

Other preferred modifications include 2'-methoxy (2'-O--CH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2. °C and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Another modification of the oligonucleotides of the present invention involves chemically linking to the oligonucleotide one or more moieties or conjugates that enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety, cholic

acid, a thioether, (*e.g.*, hexyl-S-tritylthiol), a thiocholesterol, an aliphatic chain, (*e.g.*, dodecandiol or undecyl residues), a phospholipid, (*e.g.*, di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate), a polyamine or a polyethylene glycol chain or adamantane acetic acid, a palmityl moiety, or an
5 octadecylamine or hexylamino-carbonyl-oxcholesterol moiety.

One skilled in the relevant art knows well how to generate oligonucleotides containing the above-described modifications. The present invention is not limited to the antisense oligonucleotides described above. Any suitable modification or substitution may be utilized.

10 It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds that are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of the present invention, are antisense
15 compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, *i.e.*, a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased
20 binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNaseH is a cellular endonuclease that cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of
25 gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the present invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above.

The present invention also includes pharmaceutical compositions and
5 formulations that include the antisense compounds of the present invention as described below.

D. Genetic Therapy

The present invention contemplates the use of any genetic manipulation for use in
10 modulating the expression of EZH2. Examples of genetic manipulation include, but are not limited to, gene knockout (*e.g.*, removing the EZH2 gene from the chromosome using, for example, recombination), expression of antisense constructs with or without inducible promoters, and the like. Delivery of nucleic acid construct to cells *in vitro* or *in vivo* may be conducted using any suitable method. A suitable method is one that
15 introduces the nucleic acid construct into the cell such that the desired event occurs (*e.g.*, expression of an antisense construct). Genetic therapy may also be used to deliver siRNA or other interfering molecules that are expressed *in vivo* (*e.g.*, upon stimulation by an inducible promoter (*e.g.*, an androgen-responsive promoter)).

Introduction of molecules carrying genetic information into cells is achieved by
20 any of various methods including, but not limited to, directed injection of naked DNA constructs, bombardment with gold particles loaded with said constructs, and macromolecule mediated gene transfer using, for example, liposomes, biopolymers, and the like. Preferred methods use gene delivery vehicles derived from viruses, including, but not limited to, adenoviruses, retroviruses, vaccinia viruses, and adeno-associated
25 viruses. Because of the higher efficiency as compared to retroviruses, vectors derived from adenoviruses are the preferred gene delivery vehicles for transferring nucleic acid molecules into host cells *in vivo*. Adenoviral vectors have been shown to provide very efficient *in vivo* gene transfer into a variety of solid tumors in animal models and into human solid tumor xenografts in immune-deficient mice. Examples of adenoviral vectors
30 and methods for gene transfer are described in PCT publications WO 00/12738 and WO 00/09675 and U.S. Pat. Appl. Nos. 6,033,908, 6,019,978, 6,001,557, 5,994,132,

5,994,128, 5,994,106, 5,981,225, 5,885,808, 5,872,154, 5,830,730, and 5,824,544, each of which is herein incorporated by reference in its entirety.

Vectors may be administered to subjects in a variety of ways. For example, in some embodiments of the present invention, vectors are administered into tumors or tissue associated with tumors using direct injection. In other embodiments, administration is via the blood or lymphatic circulation (*See e.g.*, PCT publication 99/02685 herein incorporated by reference in its entirety). Exemplary dose levels of adenoviral vector are preferably 10^8 to 10^{11} vector particles added to the perfusate.

10 E. Antibody Therapy

In some embodiments, the present invention provides antibodies that target prostate tumors that express EZH2. Any suitable antibody (*e.g.*, monoclonal, polyclonal, or synthetic) may be utilized in the therapeutic methods disclosed herein. In preferred embodiments, the antibodies used for cancer therapy are humanized antibodies. Methods for humanizing antibodies are well known in the art (*See e.g.*, U.S. Pat. Nos. 6,180,370, 5,585,089, 6,054,297, and 5,565,332; each of which is herein incorporated by reference).

In some embodiments, the therapeutic antibodies comprise an antibody generated against EZH2, wherein the antibody is conjugated to a cytotoxic agent. In such embodiments, a tumor specific therapeutic agent is generated that does not target normal cells, thus reducing many of the detrimental side effects of traditional chemotherapy. For certain applications, it is envisioned that the therapeutic agents will be pharmacologic agents that will serve as useful agents for attachment to antibodies, particularly cytotoxic or otherwise anticellular agents having the ability to kill or suppress the growth or cell division of endothelial cells. The present invention contemplates the use of any pharmacologic agent that can be conjugated to an antibody, and delivered in active form. Exemplary anticellular agents include chemotherapeutic agents, radioisotopes, and cytotoxins. The therapeutic antibodies of the present invention may include a variety of cytotoxic moieties, including but not limited to, radioactive isotopes (*e.g.*, iodine-131, iodine-123, technetium-99m, indium-111, rhenium-188, rhenium-186, gallium-67, copper-67, yttrium-90, iodine-125 or astatine-211), hormones such as a steroid, antimetabolites such as cytosines (*e.g.*, arabinoside, fluorouracil, methotrexate or

aminopterin; an anthracycline; mitomycin C), vinca alkaloids (*e.g.*, demecolcine; etoposide; mithramycin), and antitumor alkylating agent such as chlorambucil or melphalan. Other embodiments include agents such as a coagulant, a cytokine, growth factor, bacterial endotoxin or the lipid A moiety of bacterial endotoxin. For example, in
5 some embodiments, therapeutic agents include plant-, fungus- or bacteria-derived toxin, such as an A chain toxins, a ribosome inactivating protein, α -sarcin, aspergillin, restrictocin, a ribonuclease, diphtheria toxin or pseudomonas exotoxin, to mention just a few examples. In some preferred embodiments, deglycosylated ricin A chain is utilized.

In any event, it is proposed that agents such as these may, if desired, be
10 successfully conjugated to an antibody, in a manner that will allow their targeting, internalization, release or presentation to blood components at the site of the targeted tumor cells as required using known conjugation technology (*See, e.g.*, Ghose *et al.*, Methods Enzymol., 93:280 [1983]).

For example, in some embodiments the present invention provides immunotoxins
15 targeting EZH2. Immunotoxins are conjugates of a specific targeting agent typically a tumor-directed antibody or fragment, with a cytotoxic agent, such as a toxin moiety. The targeting agent directs the toxin to, and thereby selectively kills, cells carrying the targeted antigen. In some embodiments, therapeutic antibodies employ crosslinkers that provide high *in vivo* stability (Thorpe *et al.*, Cancer Res., 48:6396 [1988]).

20 In other embodiments, particularly those involving treatment of solid tumors, antibodies are designed to have a cytotoxic or otherwise anticellular effect against the tumor vasculature, by suppressing the growth or cell division of the vascular endothelial cells. This attack is intended to lead to a tumor-localized vascular collapse, depriving the tumor cells, particularly those tumor cells distal of the vasculature, of oxygen and
25 nutrients, ultimately leading to cell death and tumor necrosis.

In preferred embodiments, antibody based therapeutics are formulated as pharmaceutical compositions as described below. In preferred embodiments, administration of an antibody composition of the present invention results in a measurable decrease in cancer (*e.g.*, decrease or elimination of tumor).

The present invention also includes pharmaceutical compositions and formulations that include the antibody compounds of the present invention as described below.

5 **F. Pharmaceutical Compositions**

The compounds are preferably employed for therapeutic uses in combination with a suitable pharmaceutical carrier. Such compositions comprise an effective amount of the compound, and a pharmaceutically acceptable carrier or excipient. The formulation is made to suit the mode of administration. Pharmaceutically acceptable carriers are
10 determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions containing the nucleic acids some of which are described herein.

For the use of miRNA therapeutics, it is understood by one of ordinary skill in the
15 art that nucleic acids administered in vivo are taken up and distributed to cells and tissues (Huang, et al., FEBSLett. 558(1-3):69-73 (2004)). For example, Nyce et al. have shown that antisense oligodeoxynucleotides (ODNs) when inhaled bind to endogenous surfactant (a lipid produced by lung cells) and are taken up by lung cells without a need for additional carrier lipids (Nyce and Metzger, Nature, 385:721-725 (1997). Small
20 nucleic acids are readily taken up into T24 bladder carcinoma tissue culture cells (Ma, et al., Antisense Nucleic Acid Drug Dev. 8:415-426 (1998). siRNAs have been used for therapeutic silencing of an endogenous genes by systemic administration (Soutschek, et al., Nature 432, 173-178 (2004)).

The compounds may be in a formulation for administration topically, locally or
25 systemically in a suitable pharmaceutical carrier. Remington's Pharmaceutical Sciences, 15th Edition by E. W. Martin (Mark Publishing Company, 1975), discloses typical carriers and methods of preparation. The compound may also be encapsulated in suitable biocompatible microcapsules, microparticles or micro spheres formed of biodegradable or non-biodegradable polymers or proteins or liposomes for targeting to cells. Such
30 systems are well known to those skilled in the art and may be optimized for use with the appropriate nucleic acid.

Various methods for nucleic acid delivery are described, for example in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York; and Ausubel et al., 1994, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York. Such nucleic acid delivery systems comprise the desired nucleic acid, by way of example and not by limitation, in either "naked" form as a "naked" nucleic acid, or formulated in a vehicle suitable for delivery, such as in a complex with a cationic molecule or a liposome forming lipid, or as a component of a vector, or a component of a pharmaceutical composition. The nucleic acid delivery system can be provided to the cell either directly, such as by contacting it with the cell, or indirectly, such as through the action of any biological process. By way of example, and not by limitation, the nucleic acid delivery system can be provided to the cell by endocytosis, receptor targeting, coupling with native or synthetic cell membrane fragments, physical means such as electroporation, combining the nucleic acid delivery system with a polymeric carrier such as a controlled release film or nanoparticle or microparticle, using a vector, injecting the nucleic acid delivery system into a tissue or fluid surrounding the cell, simple diffusion of the nucleic acid delivery system across the cell membrane, or by any active or passive transport mechanism across the cell membrane. Additionally, the nucleic acid delivery system can be provided to the cell using techniques such as antibody-related targeting and antibody-mediated immobilization of a viral vector.

Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, or thickeners can be used as desired.

Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions, solutions or emulsions that can include suspending agents, solubilizers, thickening agents, dispersing agents, stabilizers, and preservatives. Formulations for injection may be presented in unit dosage form, e.g., in ampules or in

multi-dose containers, with an added preservative. The compositions may take such forms as.

Preparations include sterile aqueous or nonaqueous solutions, suspensions and emulsions, which can be isotonic with the blood of the subject in certain embodiments.

5 Examples of nonaqueous solvents are polypropylene glycol, polyethylene glycol, vegetable oil such as olive oil, sesame oil, coconut oil, arachis oil, peanut oil, mineral oil, injectable organic esters such as ethyl oleate, or fixed oils including synthetic mono or diglycerides. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium
10 chloride solution, 1,3-butandiol, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antirmicrobials, antioxidants, chelating agents and inert gases and the like. In addition,
15 sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa. Those of skill in the art can readily determine the
20 various parameters for preparing and formulating the compositions without resort to undue experimentation.

The compound alone or in combination with other suitable components, can also be made into aerosol formulations (i.e., they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants,
25 such as dichlorodifluoromethane, propane, nitrogen, and the like. For administration by inhalation, the compounds are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant.

In some embodiments, the compound described above may include pharmaceutically acceptable carriers with formulation ingredients such as salts, carriers,
30 buffering agents, emulsifiers, diluents, excipients, chelating agents, fillers, drying agents, antioxidants, antimicrobials, preservatives, binding agents, bulking agents, silicas,

solubilizers, or stabilizers. In one embodiment, the compounds are conjugated to lipophilic groups like cholesterol and laurie and lithocholic acid derivatives with C32 functionality to improve cellular uptake. For example, cholesterol has been demonstrated to enhance uptake and serum stability of siRNA in vitro Lorenz, et al., Bioorg. Med. 5 Cheni. Lett. 14(19):4975-4977 (2004)) and in vivo (Soutschek, et al., Nature 432(7014):173-178 (2004)). In addition, it has been shown that binding of steroid conjugated oligonucleotides to different lipoproteins in the bloodstream, such as LDL, protect integrity and facilitate biodistribution (Rump, et al., Biochem. Pharmacol. 59 (11):1407-1416 (2000)). Other groups that can be attached or conjugated to the 10 compound described above to increase cellular uptake, include acridine derivatives; cross-linkers such as psoralen derivatives, azidophenacyl, proflavin, and azidoproflavin; artificial endonucleases; metal complexes such as EDTA-Fe(II) and porphyrin-Fe(II); alkylating moieties,; nucleases such as alkaline phosphatase; terminal transferases; abzymes; cholesteryl moieties; lipophilic carriers; peptide conjugates; long chain 15 alcohols; phosphate esters; radioactive markers; non- radioactive markers; carbohydrates; and polylysine or other polyamines.

U.S. Patent No. 6,919,208 to Levy, et al., herein incorporated by reference, also described methods for enhanced delivery. These pharmaceutical formulations may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, 20 dissolving, granulating, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

The formulations described herein of the nucleic acids embrace fusions of the nucleic acids or modifications of the nucleic acids, wherein the nucleic acid is fused to another moiety or moieties, e.g., targeting moiety or another therapeutic agent. Such 25 analogs may exhibit improved properties such as activity and/or stability. Examples of moieties which may be linked or unlinked to the nucleic acid include, for example, targeting moieties which provide for the delivery of nucleic acid to specific cells, e.g., antibodies to pancreatic cells, immune cells, lung cells or any other preferred cell type, as well as receptor and ligands expressed on the preferred cell type. Preferably, the moieties 30 target cancer or tumor cells. For example, since cancer cells have increased consumption of glucose, the nucleic acids can be linked to glucose molecules. Monoclonal humanized

antibodies that target cancer or tumor cells are preferred moieties and can be linked or unlinked to the nucleic acids. In the case of cancer therapeutics, the target antigen is typically a protein that is unique and/or essential to the tumor cells.

5 In general, methods of administering compounds, including nucleic acids, are well known in the art. In particular, the routes of administration already in use for nucleic acid therapeutics, along with formulations in current use, provide preferred routes of administration and formulation for the nucleic acids described above.

10 Compositions can be administered by a number of routes including, but not limited to: oral, intravenous, intraperitoneal, intramuscular, transdermal, subcutaneous, topical, sublingual, or rectal means. Compounds can also be administered via liposomes. Such administration routes and appropriate formulations are generally known to those of skill in the art.

Administration of the formulations described herein may be accomplished by any acceptable method which allows the compounds, for example miRNA or nucleic acid
15 encoding the miRNA, to reach its target.

The particular mode selected will depend of course, upon factors such as the particular formulation, the severity of the state of the subject being treated, and the dosage required for therapeutic efficacy. As generally used herein, an "effective amount" is that amount which is able to treat one or more symptoms of EZH2-regulated disorder,
20 reverse the progression of one or more symptoms of EZH2-regulated disorder, halt the progression of one or more symptoms of EZH2-regulated disorder, or prevent the occurrence of one or more symptoms of EZH2-regulated disorder in a subject to whom the formulation is administered, as compared to a matched subject not receiving the compound.

25 The actual effective amounts of compound can vary according to the specific compound or combination thereof being utilized, the particular composition formulated, the mode of administration, and the age, weight, condition of the individual, and severity of the symptoms or condition being treated.

Any acceptable method known to one of ordinary skill in the art may be used to
30 administer a formulation to the subject. The administration may be localized (i.e., to a

particular region, physiological system, tissue, organ, or cell type) or systemic, depending on the condition being treated.

5 Injections can be e.g., intravenous, intradermal, subcutaneous, intramuscular, or intraperitoneal. The composition can be injected intraderminally for treatment or prevention of EZH2-regulated disorder, for example. In some embodiments, the injections can be given at multiple locations. Implantation includes inserting implantable drug delivery systems, e.g., microspheres, hydrogels, polymeric reservoirs, cholesterol matrixes, polymeric systems, e.g., matrix erosion and/or diffusion systems and non-polymeric systems, e.g., compressed, fused, or partially- fused pellets. Inhalation
10 includes administering the composition with an aerosol in an inhaler, either alone or attached to a carrier that can be absorbed. For systemic administration, it may be preferred that the composition is encapsulated in liposomes.

15 The nucleic acid may be delivered in a manner which enables tissue- specific uptake of the agent and/or nucleic acid delivery system. Techniques include using tissue or organ localizing devices, such as wound dressings or transdermal delivery systems, using invasive devices such as vascular or urinary catheters, and using interventional devices such as stents having drug delivery capability and configured as expansive devices or stent grafts.

20 The formulations may be delivered using a bioerodible implant by way of diffusion or by degradation of the polymeric matrix. In certain embodiments, the administration of the formulation may be designed so as to result in sequential exposures to the miRNA over a certain time period, for example, hours, days, weeks, months or years. This may be accomplished, for example, by repeated administrations of a formulation or by a sustained or controlled release delivery system in which the miRNA
25 is delivered over a prolonged period without repeated administrations. Administration of the formulations using such a delivery system may be, for example, by oral dosage forms, bolus injections, transdermal patches or subcutaneous implants. Maintaining a substantially constant concentration of the composition may be preferred in some cases.

30 Other delivery systems suitable include time-release, delayed release, sustained release, or controlled release delivery systems. Such systems may avoid repeated administrations in many cases, increasing convenience to the subject and the physician.

Many types of release delivery systems are available and known to those of ordinary skill in the art. They include, for example, polymer-based systems such as polylactic and/or polyglycolic acids, polyanhydrides, polycaprolactones, copolyoxalates, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and/or combinations of these.

5 Microcapsules of the foregoing polymers containing nucleic acids are described in, for example, U.S. Patent No. 5,075,109, herein incorporated by reference. Other examples include nonpolymer systems that are lipid-based including sterols such as cholesterol, cholesterol esters, and fatty acids or neutral fats such as mono-, di- and triglycerides; hydrogel release systems; liposome-based systems; phospholipid based-
10 systems; silastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; or partially fused implants. Specific examples include erosional systems in which the miRNA is contained in a formulation within a matrix (for example, as described in U.S. Patent Nos. 4,452,775, 4,675, 189, 5,736,152, 4,667,013, 4,748,034 and 5,239,660, herein incorporated by reference), or diffusional
15 systems in which an active component controls the release rate (for example, as described in U.S. Patent Nos. 3,832,253, 3,854,480, 5,133,974 and 5,407,686). The formulation may be as, for example, microspheres, hydrogels, polymeric reservoirs, cholesterol matrices, or polymeric systems. In some embodiments, the system may allow sustained or controlled release of the composition to occur, for example, through control of the
20 diffusion or erosion/degradation rate of the formulation containing the miRNA. In addition, a pump-based hardware delivery system may be used to deliver one or more embodiments.

 Examples of systems in which release occurs in bursts includes, e. g., systems in which the composition is entrapped in liposomes which are encapsulated in a polymer
25 matrix, the liposomes being sensitive to specific stimuli, e.g., temperature, pH, light or a degrading enzyme and systems in which the composition is encapsulated by an ionically-coated microcapsule with a microcapsule core degrading enzyme. Examples of systems in which release of the inhibitor is gradual and continuous include, e.g., erosional systems in which the composition is contained in a form within a matrix and effusional systems in
30 which the composition permeates at a controlled rate, e.g., through a polymer. Such sustained release systems can be e.g., in the form of pellets, or capsules.

Use of a long-term release implant may be particularly suitable in some embodiments. "Long-term release," as used herein, means that the implant containing the composition is constructed and arranged to deliver therapeutically effective levels of the composition for at least 30 or 45 days, and preferably at least 60 or 90 days, or even
5 longer in some cases. Long-term release implants are well known to those of ordinary skill in the art, and include some of the release systems described above.

Dosages for a particular individual can be determined by one of ordinary skill in the art using conventional considerations, (e.g. by means of an appropriate, conventional pharmacological protocol). A physician may, for example, prescribe a relatively low dose
10 at first, subsequently increasing the dose until an appropriate response is obtained. The dose administered to a individual is sufficient to effect a beneficial therapeutic response in the individual over time, or, e.g., to reduce symptoms, or other appropriate activity, depending on the application. The dose is determined by the efficacy of the particular formulation, and the activity, stability or serum half-life of the miRNA employed and the
15 condition of the individual, as well as the body weight or surface area of the individual to be treated. The size of the dose is also determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular vector, formulation, or the like in a particular individual.

Therapeutic compositions comprising one or more nucleic acids are optionally
20 tested in one or more appropriate in vitro and/or in vivo animal models of disease, to confirm efficacy, tissue metabolism, and to estimate dosages, according to methods well known in the art. In particular, dosages can be initially determined by activity, stability or other suitable measures of treatment vs. non-treatment (e.g., comparison of treated vs. untreated cells or animal models), in a relevant assay. Formulations are administered at a
25 rate determined by the LD50 of the relevant formulation, and/or observation of any side-effects of the nucleic acids at various concentrations, e.g., as applied to the mass and overall health of the individual. Administration can be accomplished via single or divided doses.

In vitro models can be used to determine the effective doses of the nucleic acids
30 as a potential EZH2-regulated disorder treatment, as described in the examples. In determining the effective amount of the compound to be administered in the treatment or

prophylaxis of disease the physician evaluates circulating plasma levels, formulation toxicities, and progression of the disease. For nucleic acids, the dose administered to a 70 kilogram individual is typically in the range equivalent to dosages of currently-used therapeutic antisense oligonucleotides such as Vitravene® (fomivirsen sodium injection) which is approved by the FDA for treatment of cytomegaloviral RNA, adjusted for the altered activity or serum half- life of the relevant composition.

The formulations described herein can supplement treatment conditions by any known conventional therapy, including, but not limited to, antibody administration, vaccine administration, administration of cytotoxic agents, natural amino acid polypeptides, nucleic acids, nucleotide analogues, and biologic response modifiers. Two or more combined compounds may be used together or sequentially. For example, the nucleic acids can also be administered in therapeutically effective amounts as a portion of an anti-age- related disorder cocktail.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more nucleic acid or small molecule compounds and (b) one or more other chemotherapeutic agents. Examples of such chemotherapeutic agents include, but are not limited to, anticancer drugs such as daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil (5-FU), floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin and diethylstilbestrol (DES). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

F. Combination therapy

In some embodiments, the present invention provides therapeutic methods comprising one or more compositions described herein in combination with an additional

agent (e.g., a chemotherapeutic agent). The present invention is not limited to a particular chemotherapy agent.

Various classes of antineoplastic (*e.g.*, anticancer) agents are contemplated for use in certain embodiments of the present invention. Anticancer agents suitable for use with
5 embodiments of the present invention include, but are not limited to, agents that induce apoptosis, agents that inhibit adenosine deaminase function, inhibit pyrimidine biosynthesis, inhibit purine ring biosynthesis, inhibit nucleotide interconversions, inhibit ribonucleotide reductase, inhibit thymidine monophosphate (TMP) synthesis, inhibit dihydrofolate reduction, inhibit DNA synthesis, form adducts with DNA, damage DNA,
10 inhibit DNA repair, intercalate with DNA, deaminate asparagines, inhibit RNA synthesis, inhibit protein synthesis or stability, inhibit microtubule synthesis or function, and the like.

In some embodiments, exemplary anticancer agents suitable for use in compositions and methods of embodiments of the present invention include, but are not
15 limited to: 1) alkaloids, including microtubule inhibitors (*e.g.*, vincristine, vinblastine, and vindesine, *etc.*), microtubule stabilizers (*e.g.*, paclitaxel (TAXOL), and docetaxel, *etc.*), and chromatin function inhibitors, including topoisomerase inhibitors, such as epipodophyllotoxins (*e.g.*, etoposide (VP-16), and teniposide (VM-26), *etc.*), and agents that target topoisomerase I (*e.g.*, camptothecin and irinotecan (CPT-11), *etc.*); 2)
20 covalent DNA-binding agents (alkylating agents), including nitrogen mustards (*e.g.*, mechlorethamine, chlorambucil, cyclophosphamide, ifosfamide, and busulfan (MYLERAN), *etc.*), nitrosoureas (*e.g.*, carmustine, lomustine, and semustine, *etc.*), and other alkylating agents (*e.g.*, dacarbazine, hydroxymethylmelamine, thiotepa, and mitomycin, *etc.*); 3) noncovalent DNA-binding agents (antitumor antibiotics), including
25 nucleic acid inhibitors (*e.g.*, dactinomycin (actinomycin D), *etc.*), anthracyclines (*e.g.*, daunorubicin (daunomycin, and cerubidine), doxorubicin (adriamycin), and idarubicin (idarubicin), *etc.*), anthracenediones (*e.g.*, anthracycline analogues, such as mitoxantrone, *etc.*), bleomycins (BLENOXANE), *etc.*, and plicamycin (mithramycin), *etc.*; 4)
antimetabolites, including antifolates (*e.g.*, methotrexate, FOLEX, and MEXATE, *etc.*),
30 purine antimetabolites (*e.g.*, 6-mercaptopurine (6-MP, PURINETHOL), 6-thioguanine (6-TG), azathioprine, acyclovir, ganciclovir, chlorodeoxyadenosine, 2-

chlorodeoxyadenosine (CdA), and 2'-deoxycoformycin (pentostatin), *etc.*), pyrimidine antagonists (*e.g.*, fluoropyrimidines (*e.g.*, 5-fluorouracil (ADRUCIL), 5-fluorodeoxyuridine (FdUrd) (floxuridine)) *etc.*), and cytosine arabinosides (*e.g.*, CYTOSAR (ara-C) and fludarabine, *etc.*); 5) enzymes, including L-asparaginase, and
5 hydroxyurea, *etc.*; 6) hormones, including glucocorticoids, antiestrogens (*e.g.*, tamoxifen, *etc.*), nonsteroidal antiandrogens (*e.g.*, flutamide, *etc.*), and aromatase inhibitors (*e.g.*, anastrozole (ARIMIDEX), *etc.*); 7) platinum compounds (*e.g.*, cisplatin and carboplatin, *etc.*); 8) monoclonal antibodies conjugated with anticancer drugs, toxins, and/or radionuclides, *etc.*; 9) biological response modifiers (*e.g.*, interferons (*e.g.*, IFN- α ,
10 *etc.*) and interleukins (*e.g.*, IL-2, *etc.*), *etc.*); 10) adoptive immunotherapy; 11) hematopoietic growth factors; 12) agents that induce tumor cell differentiation (*e.g.*, all-trans-retinoic acid, *etc.*); 13) gene therapy techniques; 14) antisense therapy techniques; 15) tumor vaccines; 16) therapies directed against tumor metastases (*e.g.*, batimastat, *etc.*); 17) angiogenesis inhibitors; 18) proteasome inhibitors (*e.g.*, VELCADE); 19)
15 inhibitors of acetylation and/or methylation (*e.g.*, HDAC inhibitors); 20) modulators of NF kappa B; 21) inhibitors of cell cycle regulation (*e.g.*, CDK inhibitors); 22) modulators of p53 protein function; and 23) radiation.

Any oncolytic agent that is routinely used in a cancer therapy context finds use in the compositions and methods of embodiments of the present invention. For example,
20 the U.S. Food and Drug Administration maintains a formulary of oncolytic agents approved for use in the United States. International counterpart agencies to the U.S.F.D.A. maintain similar formularies. The below Table provides a list of exemplary antineoplastic agents approved for use in the U.S. Those skilled in the art will appreciate that the “product labels” required on all U.S. approved chemotherapeutics describe
25 approved indications, dosing information, toxicity data, and the like, for the exemplary agents.

Aldesleukin (des-alanyl-1, serine-125 human interleukin-2)	Proleukin	Chiron Corp., Emeryville, CA
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Alemtuzumab (IgG1κ anti CD52 antibody)	Campath	Millennium and ILEX Partners, LP, Cambridge, MA
Alitretinoin (9-cis-retinoic acid)	Panretin	Ligand Pharmaceuticals, Inc., San Diego CA
Allopurinol (1,5-dihydro-4 H -pyrazolo[3,4- d]pyrimidin-4-one monosodium salt)	Zyloprim	GlaxoSmithKline, Research Triangle Park, NC
Altretamine (N,N,N',N',N'',N'',- hexamethyl-1,3,5- triazine-2, 4, 6-triamine)	Hexalen	US Bioscience, West Conshohocken, PA
Amifostine (ethanethiol, 2-[(3-aminopropyl)amino]-, dihydrogen phosphate (ester))	Ethyol	US Bioscience
Anastrozole (1,3-Benzenediacetonitrile, a, a, a', a'- tetramethyl-5-(1H-1,2,4-triazol-1- ylmethyl))	Arimidex	AstraZeneca Pharmaceuticals, LP, Wilmington, DE
Arsenic trioxide	Trisenox	Cell Therapeutic, Inc., Seattle, WA
Asparaginase (L-asparagine amidohydrolase, type EC- 2)	Elspar	Merck & Co., Inc., Whitehouse Station, NJ
BCG Live (lyophilized preparation of an attenuated	TICE BCG	Organon Teknika, Corp., Durham,

strain of <i>Mycobacterium bovis</i> (<i>Bacillus Calmette-Guérin</i> [BCG], substrain Montreal)		NC
bexarotene capsules (4-[1-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl) ethenyl] benzoic acid)	Targretin	Ligand Pharmaceuticals
bexarotene gel	Targretin	Ligand Pharmaceuticals
Bleomycin (cytotoxic glycopeptide antibiotics produced by <i>Streptomyces verticillus</i> ; bleomycin A ₂ and bleomycin B ₂)	Blenoxane	Bristol-Myers Squibb Co., NY, NY
Capecitabine (5'-deoxy-5-fluoro-N-[(pentyloxy)carbonyl]-cytidine)	Xeloda	Roche
Carboplatin (platinum, diammine [1,1-cyclobutanedicarboxylato(2-)-0, 0']-, (SP-4-2))	Paraplatin	Bristol-Myers Squibb
Carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea)	BCNU, BiCNU	Bristol-Myers Squibb
Carmustine with Polifeprosan 20 Implant	Gliadel Wafer	Guilford Pharmaceuticals, Inc., Baltimore, MD
Celecoxib (as 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]	Celebrex	Searle Pharmaceuticals, England

benzenesulfonamide)		
Chlorambucil (4-[bis(2chloroethyl)amino]benzenebutanoic acid)	Leukeran	GlaxoSmithKline
Cisplatin (PtCl ₂ H ₆ N ₂)	Platinol	Bristol-Myers Squibb
Cladribine (2-chloro-2'-deoxy-b-D-adenosine)	Leustatin, 2-CdA	R.W. Johnson Pharmaceutical Research Institute, Raritan, NJ
Cyclophosphamide (2-[bis(2-chloroethyl)amino] tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide monohydrate)	Cytosan, Neosar	Bristol-Myers Squibb
Cytarabine (1-b-D-Arabino-furanosylcytosine, C ₉ H ₁₃ N ₃ O ₅)	Cytosar-U	Pharmacia & Upjohn Company
cytarabine liposomal	DepoCyt	Skye Pharmaceuticals, Inc., San Diego, CA
Dacarbazine (5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide (DTIC))	DTIC-Dome	Bayer AG, Leverkusen, Germany
Dactinomycin, actinomycin D (actinomycin produced by <i>Streptomyces parvullus</i> , C ₆₂ H ₈₆ N ₁₂ O ₁₆)	Cosmegen	Merck

Darbepoetin alfa (recombinant peptide)	Aranesp	Amgen, Inc., Thousand Oaks, CA
daunorubicin liposomal ((8S-cis)-8-acetyl-10-[(3-amino-2,3,6-trideoxy- α -L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedione hydrochloride)	DanuoXome	Nexstar Pharmaceuticals, Inc., Boulder, CO
Daunorubicin HCl, daunomycin ((1 S ,3 S)-3-Acetyl-1,2,3,4,6,11-hexahydro-3,5,12-trihydroxy-10-methoxy-6,11-dioxo-1-naphthacenyl 3-amino-2,3,6-trideoxy-(α)-L- <i>lyxo</i> -hexopyranoside hydrochloride)	Cerubidine	Wyeth Ayerst, Madison, NJ
Denileukin diftitox (recombinant peptide)	Ontak	Seragen, Inc., Hopkinton, MA
Dexrazoxane ((S)-4,4'-(1-methyl-1,2-ethanediyl)bis-2,6-piperazinedione)	Zinecard	Pharmacia & Upjohn Company
Docetaxel ((2R,3S)-N-carboxy-3-phenylisoserine, N-tert-butyl ester, 13-ester with 5b-20-epoxy-12a,4,7b,10b,13a-hexahydroxytax-11-en-9-one 4-acetate 2-benzoate, trihydrate)	Taxotere	Aventis Pharmaceuticals, Inc., Bridgewater, NJ
Doxorubicin HCl (8S,10S)-10-[(3-amino-2,3,6-trideoxy- α -L-lyxo-hexopyranosyl)oxy] -8-glycolyl-	Adriamycin, Rubex	Pharmacia & Upjohn Company

7,8,9,10-tetrahydro-6,8,11- trihydroxy-1-methoxy-5,12-naphthacenedione hydrochloride)		
doxorubicin	Adriamycin PFS Intravenous injection	Pharmacia & Upjohn Company
doxorubicin liposomal	Doxil	Sequus Pharmaceuticals, Inc., Menlo park, CA
dromostanolone propionate (17b-Hydroxy-2a-methyl-5a-androstan-3-one propionate)	Dromostanolone	Eli Lilly & Company, Indianapolis, IN
dromostanolone propionate	Masterone injection	Syntex, Corp., Palo Alto, CA
Elliott's B Solution	Elliott's B Solution	Orphan Medical, Inc
Epirubicin ((8S-cis)-10-[(3-amino-2,3,6-trideoxy-a-L-arabino- hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione hydrochloride)	Ellence	Pharmacia & Upjohn Company
Epoetin alfa (recombinant peptide)	Epogen	Amgen, Inc
Estramustine (estra-1,3,5(10)-triene-3,17-diol(17(beta))-, 3-[bis(2-chloroethyl)carbamate] 17-(dihydrogen	Emcyt	Pharmacia & Upjohn Company

phosphate), disodium salt, monohydrate, or estradiol 3-[bis(2-chloroethyl)carbamate] 17-(dihydrogen phosphate), disodium salt, monohydrate)		
Etoposide phosphate (4'-Demethylepipodophyllotoxin 9-[4,6-O-(R)-ethylidene-(beta)-D-glucopyranoside], 4'-(dihydrogen phosphate))	Etopophos	Bristol-Myers Squibb
etoposide, VP-16 (4'-demethylepipodophyllotoxin 9-[4,6-O-(R)-ethylidene-(beta)-D-glucopyranoside])	Vepesid	Bristol-Myers Squibb
Exemestane (6-methylenandrosta-1,4-diene-3, 17-dione)	Aromasin	Pharmacia & Upjohn Company
Filgrastim (r-metHuG-CSF)	Neupogen	Amgen, Inc
floxuridine (intraarterial) (2'-deoxy-5-fluorouridine)	FUDR	Roche
Fludarabine (fluorinated nucleotide analog of the antiviral agent vidarabine, 9-b -D-arabinofuranosyladenine (ara-A))	Fludara	Berlex Laboratories, Inc., Cedar Knolls, NJ
Fluorouracil, 5-FU (5-fluoro-2,4(1H,3H)-pyrimidinedione)	Adrucil	ICN Pharmaceuticals, Inc., Humacao, Puerto Rico
Fulvestrant	Faslodex	IPR

(7- α -[9-(4,4,5,5,5-pentafluoropentylsulphonyl) nonyl]estra-1,3,5-(10)-triene-3,17- β -diol)		Pharmaceuticals, Guayama, Puerto Rico
Gemcitabine (2'-deoxy-2', 2'-difluorocytidine monohydrochloride (b-isomer))	Gemzar	Eli Lilly
Gemtuzumab Ozogamicin (anti-CD33 hP67.6)	Mylotarg	Wyeth Ayerst
Goserelin acetate (acetate salt of [D-Ser(But) ⁶ , Azgly ¹⁰]LHRH; pyro-Glu-His-Trp-Ser-Tyr-D-Ser(But)-Leu-Arg-Pro-Azgly-NH ₂ acetate [C ₅₉ H ₈₄ N ₁₈ O ₁₄ •(C ₂ H ₄ O ₂) _x])	Zoladex Implant	AstraZeneca Pharmaceuticals
Hydroxyurea	Hydrea	Bristol-Myers Squibb
Ibritumomab Tiuxetan (immunoconjugate resulting from a thiourea covalent bond between the monoclonal antibody Ibritumomab and the linker-chelator tiuxetan [N-[2-bis(carboxymethyl)amino]-3-(p-isothiocyanatophenyl)-propyl]-[N-[2-bis(carboxymethyl)amino]-2-(methyl)-ethyl]glycine)	Zevalin	Biogen IDEC, Inc., Cambridge MA
Idarubicin (5, 12-Naphthacenedione, 9-acetyl-7-[(3-amino-2,3,6-trideoxy-(α)-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,9,11-trihydroxyhydrochloride, (7S-cis	Idamycin	Pharmacia & Upjohn Company

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Ifosfamide (3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide)	IFEX	Bristol-Myers Squibb
Imatinib Mesilate (4-[(4-Methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl]benzamide methanesulfonate)	Gleevec	Novartis AG, Basel, Switzerland
Interferon alfa-2a (recombinant peptide)	Roferon-A	Hoffmann-La Roche, Inc., Nutley, NJ
Interferon alfa-2b (recombinant peptide)	Intron A (Lyophilized Betaseron)	Schering AG, Berlin, Germany
Irinotecan HCl ((4S)-4,11-diethyl-4-hydroxy-9-[(4-piperidinopiperidino)carbonyloxy]-1H-pyrano[3', 4': 6,7] indolizino[1,2-b]quinoline-3,14(4H, 12H) dione hydrochloride trihydrate)	Camptosar	Pharmacia & Upjohn Company
Letrozole (4,4'-(1H-1,2,4-Triazol-1-ylmethylene) dibenzonitrile)	Femara	Novartis
Leucovorin (L-Glutamic acid, N[4[[[(2-amino-5-formyl)-1,4,5,6,7,8 hexahydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl], calcium salt (1:1))	Wellcovorin, Leucovorin	Immunex, Corp., Seattle, WA

Levamisole HCl ((-)-(S)-2,3,5, 6-tetrahydro-6-phenylimidazo [2,1-b] thiazole monohydrochloride C ₁₁ H ₁₂ N ₂ S·HCl)	Ergamisol	Janssen Research Foundation, Titusville, NJ
Lomustine (1-(2-chloro-ethyl)-3-cyclohexyl-1-nitrosourea)	CeeNU	Bristol-Myers Squibb
Mecllorethamine, nitrogen mustard (2-chloro-N-(2-chloroethyl)-N-methylethanamine hydrochloride)	Mustargen	Merck
Megestrol acetate 17 α (acetyloxy)- 6- methylpregna- 4,6-diene- 3,20- dione	Megace	Bristol-Myers Squibb
Melphalan, L-PAM (4-[bis(2-chloroethyl) amino]-L-phenylalanine)	Alkeran	GlaxoSmithKline
Mercaptopurine, 6-MP (1,7-dihydro-6 H -purine-6-thione monohydrate)	Purinethol	GlaxoSmithKline
Mesna (sodium 2-mercaptoethane sulfonate)	Mesnex	Asta Medica
Methotrexate (N-[4-[(2,4-diamino-6-pteridiny)methyl]methylamino]benzoyl]-L-glutamic acid)	Methotrexate	Lederle Laboratories
Methoxsalen (9-methoxy-7H-furo[3,2-g][1]-benzopyran-7-one)	Uvadex	Therakos, Inc., Way Exton, Pa

Mitomycin C	Mutamycin	Bristol-Myers Squibb
mitomycin C	Mitozytrex	SuperGen, Inc., Dublin, CA
Mitotane (1,1-dichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl) ethane)	Lysodren	Bristol-Myers Squibb
Mitoxantrone (1,4-dihydroxy-5,8-bis[[2- [(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione dihydrochloride)	Novantrone	Immunex Corporation
Nandrolone phenpropionate	Durabolin-50	Organon, Inc., West Orange, NJ
Nofetumomab	Verluma	Boehringer Ingelheim Pharma KG, Germany
Oprelvekin (IL-11)	Neumega	Genetics Institute, Inc., Alexandria, VA
Oxaliplatin (cis-[(1R,2R)-1,2-cyclohexanediamine-N,N'] [oxalato(2-)-O,O'] platinum)	Eloxatin	Sanofi Synthelabo, Inc., NY, NY
Paclitaxel (5 β , 20-Epoxy-1,2a, 4,7 β , 10 β , 13a-hexahydroxytax-11-en-9-one 4,10-diacetate 2- benzoate 13-ester with (2R, 3 S)- N-benzoyl-3-phenylisoserine)	TAXOL	Bristol-Myers Squibb
Pamidronate	Aredia	Novartis

(phosphonic acid (3-amino-1-hydroxypropylidene) bis-, disodium salt, pentahydrate, (APD))		
Pegademase ((monomethoxypolyethylene glycol succinimidyl) 11 - 17 -adenosine deaminase)	Adagen (Pegademase Bovine)	Enzon Pharmaceuticals, Inc., Bridgewater, NJ
Pegaspargase (monomethoxypolyethylene glycol succinimidyl L-asparaginase)	Oncaspar	Enzon
Pegfilgrastim (covalent conjugate of recombinant methionyl human G-CSF (Filgrastim) and monomethoxypolyethylene glycol)	Neulasta	Amgen, Inc
Pentostatin	Nipent	Parke-Davis Pharmaceutical Co., Rockville, MD
Pipobroman	Vercyte	Abbott Laboratories, Abbott Park, IL
Plicamycin, Mithramycin (antibiotic produced by <i>Streptomyces plicatus</i>)	Mithracin	Pfizer, Inc., NY, NY
Porfimer sodium	Photofrin	QLT Phototherapeutics, Inc., Vancouver, Canada
Procarbazine	Matulane	Sigma Tau

(N-isopropyl-μ-(2-methylhydrazino)-p-toluamide monohydrochloride)		Pharmaceuticals, Inc., Gaithersburg, MD
Quinacrine (6-chloro-9-(1-methyl-4-diethyl-amine)butylamino-2-methoxyacridine)	Atabrine	Abbott Labs
Rasburicase (recombinant peptide)	Elitek	Sanofi-Synthelabo, Inc.,
Rituximab (recombinant anti-CD20 antibody)	Rituxan	Genentech, Inc., South San Francisco, CA
Sargramostim (recombinant peptide)	Prokine	Immunex Corp
Streptozocin (streptozocin 2-deoxy-2-[[[(methylnitrosoamino)carbonyl]amino]-a(and b)-D-glucopyranose and 220 mg citric acid anhydrous)	Zanosar	Pharmacia & Upjohn Company
Talc (Mg ₃ Si ₄ O ₁₀ (OH) ₂)	Sclerosol	Bryan, Corp., Woburn, MA
Tamoxifen ((Z)-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-N,N-dimethylethanamine 2-hydroxy-1,2,3-propanetricarboxylate (1:1))	Nolvadex	AstraZeneca Pharmaceuticals
Temozolomide (3,4-dihydro-3-methyl-4-oxoimidazo[5,1-d]-as-tetrazine-8-carboxamide)	Temodar	Schering
Teniposide, VM-26	Vumon	Bristol-Myers

(4'-demethylepipodophyllotoxin 9-[4,6-O-(R)-2-thenylidene-(beta)-D-glucopyranoside])		Squibb
Testolactone (13-hydroxy-3-oxo-13,17-secoandrosta-1,4-dien-17-oic acid [dgr]-lactone)	Teslac	Bristol-Myers Squibb
Thioguanine, 6-TG (2-amino-1,7-dihydro-6 H - purine-6-thione)	Thioguanine	GlaxoSmithKline
Thiotepa (Aziridine, 1,1',1''-phosphinothioylidynetris-, or Tris (1-aziridiny) phosphine sulfide)	Thioplex	Immunex Corporation
Topotecan HCl ((S)-10-[(dimethylamino) methyl]-4-ethyl-4,9-dihydroxy-1H-pyrano[3', 4': 6,7] indolizino [1,2-b] quinoline-3,14-(4H,12H)-dione monohydrochloride)	Hycamtin	GlaxoSmithKline
Toremifene (2-(p-[(Z)-4-chloro-1,2-diphenyl-1-butenyl]-phenoxy)-N,N-dimethylethylamine citrate (1:1))	Fareston	Roberts Pharmaceutical Corp., Eatontown, NJ
Tositumomab, I 131 Tositumomab (recombinant murine immunotherapeutic monoclonal IgG _{2a} lambda anti-CD20 antibody (I 131 is a radioimmunotherapeutic antibody))	Bexxar	Corixa Corp., Seattle, WA
Trastuzumab (recombinant monoclonal IgG ₁ kappa	Herceptin	Genentech, Inc

anti-HER2 antibody)		
Tretinoin, ATRA (all-trans retinoic acid)	Vesanoid	Roche
Uracil Mustard	Uracil Mustard Capsules	Roberts Labs
Valrubicin, N-trifluoroacetyl Adriamycin- 14-valerate ((2S-cis)-2- [1,2,3,4,6,11-hexahydro- 2,5,12-trihydroxy-7 methoxy-6,11-dioxo- [[4 2,3,6-trideoxy-3- [(trifluoroacetyl)- amino- α -L-lyxo-hexopyranosyl]oxyl]-2- naphthacenyl]-2-oxoethyl pentanoate)	Valstar	Anthra --> Medeva
Vinblastine, Leurocristine (C ₄₆ H ₅₆ N ₄ O ₁₀ •H ₂ SO ₄)	Velban	Eli Lilly
Vincristine (C ₄₆ H ₅₆ N ₄ O ₁₀ •H ₂ SO ₄)	Oncovin	Eli Lilly
Vinorelbine (3',4'-didehydro-4'-deoxy-C'- norvincal leukoblastine [R-(R*,R*)-2,3- dihydroxybutanedioate (1:2)(salt)])	Navelbine	GlaxoSmithKline
Zoledronate, Zoledronic acid ((1-Hydroxy-2-imidazol-1-yl- phosphonoethyl) phosphonic acid monohydrate)	Zometa	Novartis

II. Antibodies

The present invention provides isolated antibodies. In some embodiments, the
5 present invention provides monoclonal antibodies that specifically bind to an isolated

polypeptide comprised of at least five amino acid residues of EZH2. These antibodies find use in the therapeutic and drug screening methods described herein.

An antibody against a protein of the present invention may be any monoclonal or polyclonal antibody, as long as it can recognize the protein. Antibodies can be produced
5 by using a protein of the present invention as the antigen according to a conventional antibody or antiserum preparation process.

The present invention contemplates the use of both monoclonal and polyclonal antibodies. Any suitable method may be used to generate the antibodies used in the methods and compositions of the present invention, including but not limited to, those
10 disclosed herein. For example, for preparation of a monoclonal antibody, protein, as such, or together with a suitable carrier or diluent is administered to an animal (*e.g.*, a mammal) under conditions that permit the production of antibodies. For enhancing the antibody production capability, complete or incomplete Freund's adjuvant may be administered. Normally, the protein is administered once every 2 weeks to 6 weeks, in
15 total, about 2 times to about 10 times. Animals suitable for use in such methods include, but are not limited to, primates, rabbits, dogs, guinea pigs, mice, rats, sheep, goats, etc.

For preparing monoclonal antibody-producing cells, an individual animal whose antibody titer has been confirmed (*e.g.*, a mouse) is selected, and 2 days to 5 days after the final immunization, its spleen or lymph node is harvested and antibody-producing
20 cells contained therein are fused with myeloma cells to prepare the desired monoclonal antibody producer hybridoma. Measurement of the antibody titer in antiserum can be carried out, for example, by reacting the labeled protein, as described hereinafter and antiserum and then measuring the activity of the labeling agent bound to the antibody. The cell fusion can be carried out according to known methods, for example, the method
25 described by Koehler and Milstein (*Nature* 256:495 [1975]). As a fusion promoter, for example, polyethylene glycol (PEG) or Sendai virus (HVJ), preferably PEG is used.

Examples of myeloma cells include NS-1, P3U1, SP2/0, AP-1 and the like. The proportion of the number of antibody producer cells (spleen cells) and the number of myeloma cells to be used is preferably about 1:1 to about 20:1. PEG (preferably PEG
30 1000-PEG 6000) is preferably added in concentration of about 10% to about 80%. Cell

fusion can be carried out efficiently by incubating a mixture of both cells at about 20°C to about 40°C, preferably about 30°C to about 37°C for about 1 minute to 10 minutes.

Various methods may be used for screening for a hybridoma producing the antibody (*e.g.*, against a tumor antigen or autoantibody of the present invention). For example, where a supernatant of the hybridoma is added to a solid phase (*e.g.*, microplate) to which antibody is adsorbed directly or together with a carrier and then an anti-immunoglobulin antibody (if mouse cells are used in cell fusion, anti-mouse immunoglobulin antibody is used) or Protein A labeled with a radioactive substance or an enzyme is added to detect the monoclonal antibody against the protein bound to the solid phase. Alternately, a supernatant of the hybridoma is added to a solid phase to which an anti-immunoglobulin antibody or Protein A is adsorbed and then the protein labeled with a radioactive substance or an enzyme is added to detect the monoclonal antibody against the protein bound to the solid phase.

Selection of the monoclonal antibody can be carried out according to any known method or its modification. Normally, a medium for animal cells to which HAT (hypoxanthine, aminopterin, thymidine) are added is employed. Any selection and growth medium can be employed as long as the hybridoma can grow. For example, RPMI 1640 medium containing 1% to 20%, preferably 10% to 20% fetal bovine serum, GIT medium containing 1% to 10% fetal bovine serum, a serum free medium for cultivation of a hybridoma (SFM-101, Nissui Seiyaku) and the like can be used. Normally, the cultivation is carried out at 20°C to 40°C, preferably 37°C for about 5 days to 3 weeks, preferably 1 week to 2 weeks under about 5% CO₂ gas. The antibody titer of the supernatant of a hybridoma culture can be measured according to the same manner as described above with respect to the antibody titer of the anti-protein in the antiserum.

Separation and purification of a monoclonal antibody (*e.g.*, against a cancer marker of the present invention) can be carried out according to the same manner as those of conventional polyclonal antibodies such as separation and purification of immunoglobulins, for example, salting-out, alcoholic precipitation, isoelectric point precipitation, electrophoresis, adsorption and desorption with ion exchangers (*e.g.*, DEAE), ultracentrifugation, gel filtration, or a specific purification method wherein only

an antibody is collected with an active adsorbent such as an antigen-binding solid phase, Protein A or Protein G and dissociating the binding to obtain the antibody.

Polyclonal antibodies may be prepared by any known method or modifications of these methods including obtaining antibodies from patients. For example, a complex of
5 an immunogen (an antigen against the protein) and a carrier protein is prepared and an animal is immunized by the complex according to the same manner as that described with respect to the above monoclonal antibody preparation. A material containing the antibody against is recovered from the immunized animal and the antibody is separated and purified.

10 As to the complex of the immunogen and the carrier protein to be used for immunization of an animal, any carrier protein and any mixing proportion of the carrier and a hapten can be employed as long as an antibody against the hapten, which is crosslinked on the carrier and used for immunization, is produced efficiently. For example, bovine serum albumin, bovine cycloglobulin, keyhole limpet hemocyanin, etc.
15 may be coupled to an hapten in a weight ratio of about 0.1 part to about 20 parts, preferably, about 1 part to about 5 parts per 1 part of the hapten.

In addition, various condensing agents can be used for coupling of a hapten and a carrier. For example, glutaraldehyde, carbodiimide, maleimide activated ester, activated ester reagents containing thiol group or dithiopyridyl group, and the like find use with the
20 present invention. The condensation product as such or together with a suitable carrier or diluent is administered to a site of an animal that permits the antibody production. For enhancing the antibody production capability, complete or incomplete Freund's adjuvant may be administered. Normally, the protein is administered once every 2 weeks to 6 weeks, in total, about 3 times to about 10 times.

25 The polyclonal antibody is recovered from blood, ascites and the like, of an animal immunized by the above method. The antibody titer in the antiserum can be measured according to the same manner as that described above with respect to the supernatant of the hybridoma culture. Separation and purification of the antibody can be carried out according to the same separation and purification method of immunoglobulin
30 as that described with respect to the above monoclonal antibody.

The protein used herein as the immunogen is not limited to any particular type of immunogen. For example, a cancer marker of the present invention (e.g., EZH2) (further including a product of a gene having a nucleotide sequence partly altered) can be used as the immunogen. Further, fragments of the protein may be used. Fragments may be
5 obtained by any methods including, but not limited to expressing a fragment of the gene, enzymatic processing of the protein, chemical synthesis, and the like.

III. Drug Screening Applications

In some embodiments, the present invention provides drug screening assays (e.g.,
10 to screen for anticancer drugs). The screening methods of the present invention utilize EZH2. For example, in some embodiments, the present invention provides methods of screening for compounds that alter (e.g., decrease) the expression of EZH2. The compounds or agents may interfere with transcription, by interacting, for example, with the promoter region. The compounds or agents may interfere with mRNA produced from
15 EZH2 (e.g., by RNA interference, antisense technologies, etc.). The compounds or agents may interfere with pathways that are upstream or downstream of the biological activity of EZH2. In some embodiments, candidate compounds are antisense or interfering RNA agents (e.g., oligonucleotides) directed against EZH2. In other embodiments, candidate compounds are antibodies or small molecules that specifically
20 bind to an EZH2 regulator or expression products of the present invention and inhibit its biological function.

In one screening method, candidate compounds are evaluated for their ability to alter EZH2 expression by contacting a compound with a cell expressing EZH2 and then assaying for the effect of the candidate compounds on expression. In some embodiments,
25 the effect of candidate compounds on expression of an EZH2 gene is assayed for by detecting the level of EZH2 mRNA expressed by the cell. mRNA expression can be detected by any suitable method.

In other embodiments, the effect of candidate compounds on expression of EZH2 genes is assayed by measuring the level of polypeptide encoded by the cancer markers.
30 The level of polypeptide expressed can be measured using any suitable method, including but not limited to, those disclosed herein.

Specifically, the present invention provides screening methods for identifying modulators, *i.e.*, candidate or test compounds or agents (*e.g.*, proteins, peptides, peptidomimetics, peptoids, small molecules or other drugs) which bind to EZH2, have an inhibitory (or stimulatory) effect on, for example, EZH2 expression or EZH2 activity, or
5 have a stimulatory or inhibitory effect on, for example, the expression or activity of a EZH2 substrate. Compounds thus identified can be used to modulate the activity of target gene products (*e.g.*, EZH2) either directly or indirectly in a therapeutic protocol, to elaborate the biological function of the target gene product, or to identify compounds that disrupt normal target gene interactions. Compounds that inhibit the activity or expression
10 of EZH2 are useful in the treatment of proliferative disorders, *e.g.*, cancer, particularly prostate cancer.

In one embodiment, the invention provides assays for screening candidate or test compounds that are substrates of an EZH2 protein or polypeptide or a biologically active portion thereof. In another embodiment, the invention provides assays for screening
15 candidate or test compounds that bind to or modulate the activity of an EZH2 protein or polypeptide or a biologically active portion thereof.

The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including biological libraries; peptoid libraries (libraries of molecules having the functionalities of
20 peptides, but with a novel, non-peptide backbone, which are resistant to enzymatic degradation but which nevertheless remain bioactive; see, *e.g.*, Zuckermann *et al.*, J. Med. Chem. 37: 2678-85 [1994]); spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography
25 selection. The biological library and peptoid library approaches are preferred for use with peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam (1997) Anticancer Drug Des. 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the
30 art, for example in: DeWitt *et al.*, Proc. Natl. Acad. Sci. U.S.A. 90:6909 [1993]; Erb *et al.*, Proc. Natl. Acad. Sci. USA 91:11422 [1994]; Zuckermann *et al.*, J. Med. Chem.

37:2678 [1994]; Cho *et al.*, Science 261:1303 [1993]; Carrell *et al.*, Angew. Chem. Int. Ed. Engl. 33:2059 [1994]; Carell *et al.*, Angew. Chem. Int. Ed. Engl. 33:2061 [1994]; and Gallop *et al.*, J. Med. Chem. 37:1233 [1994].

Libraries of compounds may be presented in solution (*e.g.*, Houghten, Biotechniques 13:412-421 [1992]), or on beads (Lam, Nature 354:82-84 [1991]), chips (Fodor, Nature 364:555-556 [1993]), bacteria or spores (U.S. Pat. No. 5,223,409; herein incorporated by reference), plasmids (Cull *et al.*, Proc. Natl. Acad. Sci. USA 89:1865-1869 [1992]) or on phage (Scott and Smith, Science 249:386-390 [1990]; Devlin Science 249:404-406 [1990]; Cwirla *et al.*, Proc. Natl. Acad. Sci. 87:6378-6382 [1990]; Felici, J. Mol. Biol. 222:301 [1991]).

EXPERIMENTAL

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

Example 1

miRNA Inhibition of EZH2 Expression

A. Experimental Approach

The primary structure of precursor miR-101 is shown in Fig. 1. Fig. 1 shows the sequence database entry for mir-101 from Sanger's Registry. The cartoon depicts the predicted stem-loop hairpin. miR-101 is predicted to target the 3' UTR of EZH2 at 2 independent sites and both predictions are the top ranked hits from the Sanger Registry.

The functional consequences of perturbing miR-101 levels in cells was evaluated. Expression of EZH2 protein was measured by immunoblot analysis. Invasion assays were carried out as previously described (Kleer *et al.*, *supra*) and pre-miR-101 was transfected along with siRNA against EZH2 (as a positive control) and luciferase siRNA (as a negative control) as well as several unrelated miRs.

B. Results

It was assessed whether miR-101 regulates EZH2 expression in cell lines. Upon transfection of the precursor miR-101 in SKBr3 breast cancer cells a marked decrease in EZH2 protein expression was observed (Fig. 2). Control miRs and other miRs predicted to regulate EZH2 with high scores (as per the Sanger Registry) did not decrease EZH2 protein levels.

To assess the functional relevance of miR-101 relative to EZH2 function a cell invasion assay was utilized. Previous studies have shown that knock-down of EZH2 in cancer cell lines expressing high levels of EZH2 abrogates cell invasion (Fig 3). Over-expression of miR-101 in SKBr3 cells induced marked reduction of cell invasion by knocking down EZH2 protein levels. Thus microRNA 101 serves as a therapeutic for knocking down EZH2 in aggressive tumors which overexpress EZH2.

Example 2

Small Molecule Inhibition of EZH2

In order to understand the mechanism of EZH2 mediated invasion, cDNA expression microarray analysis was performed using the RNA isolated from EZH2 overexpressing cells along with control RNA (Fig. 4A). It was observed that the tumor suppressor protein E-cadherin was specifically downregulated. These observations were further confirmed by immunoblot assays as well as coimmunostainings (Fig. 4 B, C). Furthermore, the inverse correlation between increased EZH2 expression and E-cadherin down regulation was observed in aggressive breast tumors as well. The studies showed that the oncogenic function of EZH2 works by activating a pro-invasion program through transcriptional repression of E-cadherin among other factors.

A. Experimental Approach

A high throughput screening protocol was used to identify small molecule inhibitors of EZH2. Primary breast cancer cells were transfected with the E-cadherin promoter luciferase reporter gene and infected with the EZH2 adenovirus to suppress luciferase expression 48 hours prior to compound addition. Eighteen hours prior to compound addition, cells were trypsinized and distributed into 384-well plates in

60 µl of medium using the Multidrop equipment. At time zero, compounds were transferred from 1.5 mM DMSO stocks to the cell plates in a final compound concentration of about 5 µM. This concentration was chosen based on other cell-based assays in which higher concentrations caused substantial cell toxicity and did not yield significantly more “hits”. After 24 hours, the expressed luciferase activity was measured by adding 50 µl of the medium and 10 µl of Steady-Glo luciferase reagent (Promega). Sample plates were read in the Pherastar plate reader (BMG Labtech). Each plate in the screen contains 320 compounds to be tested plus 64 control wells placed in the outer two columns on each side of the plate (>50,000 compounds are screened). The “Positive” control was EZH2 adenovirus infected cells followed by treatment with the HDAC inhibitor SAHA (500 nM) which shows the activity expected in the presence of an inhibitor. More than 4000 small molecules were screened, which included synthetic chemicals as well as natural products that are available in the Center for Chemical Genomics (CCG) library at the University of Michigan.

15

B. Results

The E-cadherin promoter–luciferase reporter construct was utilized in a high throughput screening assay using a chemical library. Initial screens indicated the utility of the gain of function assay with a good Z’-score. Isoliquiritigenin (Fig. 5A) was identified as a potent small molecule inhibitor of EZH2 activity.

20

The effect of this small molecule inhibitor on inhibiting the gene repression mediated by EZH2 was further analyzed. As shown in Fig. 5B, isoliquiritigenin was able to significantly inhibit the EZH2 mediated E cadherin repression. Evaluation of the dose response indicated that 16 µM isoliquiritigenin could optimally inhibit the EZH2 mediated E cadherin repression (Fig. 6A).

25

Further studies confirmed the utility of this plant flavonoid in inhibiting the EZH2 activity in cancer cell invasion. While the breast cell line HME acquires invasive potential upon EZH2 overexpression, addition of isoliquiritigenin inhibited this invasion. A control small molecule with similar structure did not inhibit the invasion mediated by EZH2 overexpression demonstrating the specificity of isoliquiritigenin in inhibiting the EZH2 activity (Fig. 6B).

30

The preliminary screen was extended to 70,000 compounds. Table 1 below shows a list of compounds identified as having EZH2 inhibitory activity. Table 2 shows 33 compounds selected as candidates for dose response screens as well as secondary screens such as invasion, apoptosis, and xenograft models.

5

Table 1**EZH2 Inhibitors****EZH2 Inhibitor- IUPAC name**

1-[[4-amino-5-(2,2-dimethylpropanoyl)-1,3-thiazol-2-yl]sulfanyl]-3,3-dimethylbutan-2-one
 4-[4-(4-methyl-1,3-thiazol-2-yl)phenyl]-1,2,3-thiadiazole
 2-[[3-(3,4-dichlorophenyl)carbamoyl]amino]benzoic acid
 N-(2-methylquinolin-6-yl)quinoxaline-2-carboxamide
 2-[(4-tert-butylphenyl)carbonyl]-1H-imidazole
 1-(2-hydroxyphenyl)-3-[4-(methoxymethoxy)phenyl]propane-1,3-dione
 N-(3-acetylphenyl)-8-methoxy-2-oxo-2H-chromene-3-carboxamide
 1-{3-[4-(2-phenylethynyl)phenyl]-1H-pyrazol-1-yl}ethan-1-one
 3-(thiophen-2-yl)benzoic acid
 5-(6-methoxynaphthalen-2-yl)-1H-pyrazole
 4-methyl-5-[3-(methylsulfanyl)-1H-pyrazol-5-yl]-2-(thiophen-2-yl)-1,3-thiazole
 2-[[2-(2-chloro-6-fluorophenyl)methyl]sulfanyl]-1-(2,3-dihydro-1,4-benzodioxin-6-yl)ethan-1-one
 3-(3-chlorophenyl)-5-(thiophen-3-yl)-1,2,4-oxadiazole
 2,3-dihydro-1-benzofuran-5-ylmethanimidamido thiophene-2-carboxylate
 N-(2,3-dihydro-1,4-benzodioxin-6-yl)[(furan-2-ylmethyl)carbamothioyl]formamide
 N-[4-(diethylamino)phenyl]-3-methylbenzamide
 3-[5-(1,2-oxazol-3-yl)thiophen-2-yl]-5-phenyl-1,2,4-oxadiazole
 ethyl (2E)-2-cyano-3-[[4-(dimethylamino)phenyl]methylidene]amino(methane)sulfinimidamido}prop-2-enoate
 (2Z)-2-(4-ethylphenyl)-3-(4-methoxyphenyl)prop-2-enenitrile
 5-tert-butyl-3-methyl-N-phenylthieno[3,2-b]thiophene-2-carboxamide
 5-(1-butyl-2-oxo-2,3-dihydro-1H-indol-3-ylidene)-2-(piperidin-1-yl)-4,5-dihydro-1,3-thiazol-4-one
 (2E,6E)-2,6-bis(thiophen-2-ylmethylidene)cyclohexan-1-one
 2-[(E)-2-(3,4-dimethoxyphenyl)ethenyl]-1,3-benzothiazole
 2-chloro-N-[3-hydroxy-4-(5-methyl-1,3-benzoxazol-2-yl)phenyl]-5-nitrobenzamide
 6-chloro-2-phenyl-4H-thiochromen-4-one
 methyl 2-(3,4-dihydro-2H-1,5-benzodioxepine-7-amido)benzoate
 3-chloro-N,N-dimethyl-4-[(1E)-[2-(quinoxalin-2-yl)hydrazin-1-ylidene]methyl]aniline
 (2E)-1-(2-methyl-1H-indol-3-yl)-3-(thiophen-2-yl)prop-2-en-1-one
 N-(2,3-dihydro-1,4-benzodioxin-6-yl)-2-(thiophen-2-yl)-1,3-thiazole-4-carboxamide
 4-[(E)-2-(1-methyl-1H-1,3-benzodiazol-2-yl)ethenyl]-1,3-thiazole
 3-(4-bromophenyl)-3,4-dihydro-1,2,3-benzotriazin-4-one
 N-(2,4-dichlorophenyl)-3,4-dihydro-2H-1-benzopyran-2-carboxamide
 N,N-dimethyl-4-[(E)-2-phenylethenyl]aniline
 2-(3,4-dichlorophenyl)quinoxaline
 N-(3-tert-butyl-1H-pyrazol-5-yl)-2,3-dihydro-1,4-benzodioxine-2-carboxamide
 (2E)-2-(1,3-benzothiazol-2-yl)-3-(4-chlorophenyl)prop-2-enenitrile
 (4-tert-butylphenyl)methanimidamido 2-(thiophen-2-yl)acetate

5-[4-(3-methyl-1-benzothiophen-2-yl)-1,3-thiazol-2-yl]-1,2-oxazole
1-(4-fluorophenyl)-3-(1-phenyl-5-propyl-1H-pyrazol-4-yl)urea
2-[(2Z)-2-phenyl-2-[(2E)-2-(thiophen-2-ylmethylidene)hydrazin-1-ylidene]ethyl]-1H-1,3-benzodiazole
N-{7-oxo-8-oxa-4-thiatricyclo[7.4.0.0^{2,6}]}trideca-1(9),2,5,10,12-pentaen-5-yl}thiophene-2-carboxamide
2-(2-chlorophenyl)-1-[4-(dimethylamino)phenyl]ethan-1-one
ethyl 4-cyano-1-(4-methylphenyl)-1H-pyrazole-3-carboxylate
3-hydrazinylquinoxaline-2-thiol
1-[(5-tert-butylthiophen-2-yl)carbonyl]piperidine
3-[5-(2-phenylethynyl)thiophen-2-yl]-1-(thiophen-2-ylcarbonyl)-1H-pyrazole
2,5-dichloro-N-(2,3-dihydro-1,4-benzodioxin-2-ylmethyl)thiophene-3-carboxamide
1-tert-butyl-N-(2,3-dihydro-1,4-benzodioxin-6-yl)-5-methyl-1H-pyrazole-3-carboxamide
4-(5-propylpyridin-2-yl)benzonitrile
5-(4-chlorophenyl)-3-(2,2-dichloroacetamido)thiophene-2-carboxamide
(4-methanesulfonamidophenyl)methanimidamido thiophene-2-carboxylate
ethyl 7-methyl-2-phenylpyrazolo[1,5-a]pyrimidine-6-carboxylate
6-(4-chlorophenyl)-3-phenylthieno[2,3-e][1,2,4]triazine
1-{1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl}-2,3-dihydro-1H-indole
5-(4-chlorophenyl)-2-(4-methylphenyl)-2H-1,2,3,4-tetrazole
4-[(1E)-[2-(3,5-dichloropyridin-4-yl)hydrazin-1-ylidene]methyl]-N,N-dimethylaniline
3-(5-tert-butyl-1,2-oxazol-3-yl)-1-phenylurea
(4-chlorophenyl)methanimidamido 3-chlorothiophene-2-carboxylate
N-{4-[(E)-2-phenyldiazen-1-yl]phenyl}acetamide
methyl 4-[(pyrimidin-2-ylsulfanyl)methyl]benzoate
2-phenylimidazo[1,2-a]pyridine
6-chloro-2-phenyl-4H-thiochromen-4-one
2-[[4-(4-methylphenyl)methyl]sulfanyl]-5-(pyrazin-2-yl)-1,3,4-thiadiazole
5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one
(E)-[1-(1H-pyrrol-2-yl)ethylidene]amino N-(4-chlorophenyl)carbamate
1-benzoyl-3,2,3-dihydro-1H-inden-5-ylthiourea
1-{1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl}-2,3-dihydro-1H-indole
N,5-diphenyl-1,3,4-oxadiazole-2-carboxamide
(3Z)-3-(2,3-dihydro-1-benzofuran-5-ylmethylidene)-2,3-dihydro-1H-indol-2-one
[(3-methylbutyl)sulfanyl]-N-phenylformamide
2,4-dihydroxy-5,7-diphenylpyrano[2,3-d]pyrimidin-8-ium perchlorate
ethyl 7-hydroxy-9-oxo-9H-xanthene-2-carboxylate
(E)-[(1-tert-butyl-3,5-dimethyl-1H-pyrazol-4-yl)methylidene]amino N-[3-(trifluoromethyl)phenyl]carbamate
5-[(4-iodophenyl)amino]-3-phenyl-1,3-thiazolidine-2,4-dione
N-(furan-2-ylmethyl)-2-[methane(4-phenoxyphenyl)sulfonamido]acetamide
N-(3-methoxyphenyl)-6-phenylpyridazin-3-amine
ethyl (2E)-3-[(2-chlorophenyl)amino]-2-cyanoprop-2-enoate
1-[(3-chloro-1-benzothiophen-2-yl)carbonyl]-1H,2H,3H,4H,6H,10bH-pyrimido[2,1-a]isoindol-6-one
2-(4-chlorophenyl)-5-[(cyclopropylmethyl)sulfanyl]-1,3,4-oxadiazole
1-[6-(benzyloxy)-3-tert-butyl-2-hydroxyphenyl]ethan-1-one
3-[(1E)-1-[(2,2-dichloroethenyl)imino]-2,2-dimethylpropyl]-1-(4-methylphenyl)thiourea
6,7-dimethyl-2-phenylquinoxaline
5-(2,3-dihydro-1-benzofuran-5-yl)-3-(4-fluorophenyl)-1,2,4-oxadiazole
2-{4-[(4-methylphenyl)methoxy]phenyl}acetonitrile
1-cyclohexyl-3-8-oxatricyclo[7.4.0.0^{2,7}]}trideca-1(9),2(7),3,5,10,12-hexaen-5-ylurea
5-(1,2,3-thiadiazol-4-yl)-3-[4-(trifluoromethyl)phenyl]-1,2,4-oxadiazole

N-(2-methylquinolin-6-yl)-2-phenylacetamide
3-(piperidin-1-ylcarbonyl)-5-(thiophen-2-yl)-1,2-oxazole
N-(3,4-dimethylphenyl)[(E)-N'-(thiophen-2-ylmethylidene)hydrazinecarbonyl]formamide
2-(2,3-dimethoxyphenyl)-2,3-dihydro-1,3-benzothiazole
2-methyl-5-(naphthalen-2-yl)-1,3-thiazole hydrobromide
(cyclohexylcarbamothioyl)-N-(4-fluorophenyl)formamide
4-(1,3-benzothiazol-2-yl)-1-methyl-1H-pyrazol-3-amine
(4-tert-butylphenyl)methanimidamido 5-methyl-1,2-oxazole-3-carboxylate
N-[2-(methylsulfanyl)-1,3-benzothiazol-6-yl]thiophene-2-carboxamide
N-(5-cyclopropyl-1,3,4-thiadiazol-2-yl)-2H-1,3-benzodioxole-5-carboxamide
(2E)-3-(2-chlorophenyl)-N-(2-methylbut-3-yn-2-yl)prop-2-enamide
1-naphthalen-1-yl-3-8-oxatricyclo[7.4.0.0^{2,7}]trideca-1(13),2,4,6,9,11-hexaen-5-ylthiourea
3-methyl-N-phenyl-1-benzothiophene-2-carbothioamide
5-(2,5-dichlorophenyl)-N-[2-(trifluoromethyl)phenyl]furan-2-carboxamide
3-(5-methyl-1,2-oxazol-3-yl)-5-(thiophen-2-yl)-1,2,4-oxadiazole
N-(1H-indazol-3-yl)-3-methoxybenzamide
2-(4-tert-butylphenyl)-5-[(propane-1-sulfonyl)methyl]-1,3,4-oxadiazole
1-[2-(4-chlorophenoxymethyl)-4-methyl-1,3-thiazol-5-yl]ethan-1-one
(4-methanesulfonamidophenyl)methanimidamido N-(4-methylphenyl)carbamate
N-phenyl-4,5,6,7-tetrahydro-1,3-benzothiazol-2-amine hydrochloride
7-nitro-N-(2-phenylethyl)-1H-indole-2-carboxamide
2-[[2E)-4-(pyridin-2-ylsulfanyl)but-2-en-1-yl]sulfanyl]pyridine
4-[(E)-2-(3-methylthiophen-2-yl)ethenyl]-2-[(3-nitropyridin-2-yl)sulfanyl]pyrimidine
4-(4-chlorophenyl)-2-[(4-methoxyphenyl)methyl]-1,3-thiazole
(3Z)-3-[[5-(thiophen-2-yl)thiophen-2-yl]methylidene]-2,3-dihydro-1H-indol-2-one
N-(4-bromo-2,5-difluorophenyl)-2,3-dimethylbenzamide
sodium N-phenyl(phenylamino)carboximidate
2-(benzylsulfanyl)-N-(2,3-dihydro-1H-inden-2-yl)acetamide
(5Z)-5-[(5-methylfuran-2-yl)methylidene]-3-phenyl-1,3-thiazolidine-2,4-dione
N-{4-[(3-chlorophenyl)carbamoyl]phenyl}thiophene-2-carboxamide
N-[(3-chlorophenyl)methyl]-5-(methylsulfanyl)-1,3,4-thiadiazol-2-amine
(E)-2-(phenylamino)-3-(phenylimino)guanidine
(2Z)-3-methyl-2-[2-(3-methyl-2,3-dihydro-1,3-benzoxazol-2-ylidene)hydrazin-1-ylidene]-2,3-dihydro-1,3-benzoxazole
3-[2-(2H-1,4-benzothiazin-3-yl)hydrazin-1-yl]-2H-1,4-benzothiazine
3-(3,4-dimethyl-1,2-oxazol-5-yl)-1-[4-(dimethylamino)-3,5-difluorophenyl]carbonylurea
(3Z)-3-[2-(2,5-difluorophenyl)hydrazin-1-ylidene]piperidin-2-one
N'-[(E)-[1-(1-benzofuran-2-yl)ethylidene]amino](methylsulfanyl)methanimidamide
(2Z)-3-(9H-fluoren-2-ylcarbamoyl)prop-2-enoic acid
4-[2-(2,3-dihydro-1,4-benzodioxin-6-yl)diazene-1-yl]-N,N-diethylaniline
4,5-dichloro-N-(3-chloro-4-fluorophenyl)-1,2-thiazole-3-carboxamide
5-[4-(4-methoxyphenoxy)phenyl]-1H-pyrazole
1-cyclohexyl-3-[(Z)-(1H-pyrazol-3-ylmethylidene)amino]thiourea
[5-(4-chlorophenyl)-3-methyl-2-sulfanylidene-1,3,4-thiadiazinan-6-ylidene]amino 5-tert-butylthiophene-2-carboxylate
N-(2-phenylethyl)benzenecarbothioamide
5-amino-3-methyl-2-N-phenylthiophene-2,4-dicarboxamide
3-amino-5-(thiophen-3-yl)thiophene-2-carboxamide
(2E)-2-[[4-(trifluoromethoxy)phenyl]imino]-3,4-dihydro-2H-1,3-benzoxazin-4-one
3-hydroxy-9H-xanthen-9-one
4-[(E)-2-(3,5-dihydroxyphenyl)ethenyl]benzene-1,2-diol

(3-chlorophenyl)methanimidamido 6-(2,2,2-trifluoroethoxy)pyridine-3-carboxylate
 5-phenyl-3-(pyrrolidin-1-yl)-1,2-thiazole-4-carbonitrile
 7-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one
 2-(4-fluorophenyl)-2H,3H,5H,6H,7H,8H-[1,2,4]triazolo[4,3-a]pyrimidin-3-one
 (4-chlorophenyl)methanimidamido 2,6-difluorobenzoate
 2-(2-amino-3-methoxyphenyl)-4H-chromen-4-one
 6,7-dimethoxy-2-phenylquinoxaline
 6-methoxy-3-phenyl-[1,2,4]triazolo[4,3-a]pyridazine
 5-[4-(furan-2-ylcarbonyl)piperazin-1-yl]-3-(thiophen-2-yl)-1,2,4-thiadiazole
 (E)-{1-[2-(4-chlorophenoxymethyl)-1,3-thiazol-4-yl]ethylidene}amino benzoate
 N-[(2-chloro-6-fluorophenyl)carbonyl]-N'-(4-methylpyridin-2-yl)ethanediamide
 (E)-hydroxy[1-(2-phenyl-1,3-thiazol-4-yl)ethylidene]amine
 ethyl 1-[[4-(trifluoromethoxy)phenyl]carbonyl]piperidine-4-carboxylate
 3-(3-methyl-1H-indol-1-yl)-N-[4-(morpholin-4-yl)phenyl]propanamide
 6,8-dimethyl-1-methylidene-2-(4-methylphenyl)-1,4-dihydronaphthalene
 N'-[(2-methyl-1,3-thiazol-4-yl)methoxy]-4-(trifluoromethyl)benzene-1-carboximidamide
 1-[4-(benzyloxy)phenyl]-3-[(3-cyanopyridin-2-yl)amino]urea
 2-phenylimidazo[1,2-a]pyridine
 3-(morpholin-4-yl)-5-[4-(trifluoromethyl)phenyl]-1,2-thiazole-4-carbonitrile
 N-(2-chlorophenyl)-2-[(3-cyano-6-acetylpyridin-2-yl)sulfanyl]acetamide
 3-[4-(4-methoxyphenyl)-1,3-thiazol-2-yl]-5-methyl-1,2-oxazole
 N-(3-bromo-5-methylpyridin-2-yl)-4-ethylbenzamide
 2-(5-methyl-1,2-oxazol-3-yl)-5-[3-(trifluoromethyl)phenyl]-1,3,4-oxadiazole
 (E)-[1-(3-methyl-1-benzothiophen-2-yl)ethylidene]amino N-phenylcarbamate
 N-(2,3-dihydro-1H-inden-2-yl)-3-(3-methyl-1H-indol-1-yl)propanamide
 1,3-dimethanesulfonyl-2,3-dihydro-1H-1,3-benzodiazole
 methyl 2-[5-methyl-2-(thiophene-2-amido)-1,3-thiazol-4-yl]acetate
 4-[(5-[(4-chlorophenyl)sulfanyl]methyl)furan-2-yl)carbonyl]morpholine
 2-oxo-2-phenylethyl 2,3-dimethoxybenzoate
 N-(4-chlorophenyl)-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carboxamide
 (2,4-dichlorophenyl)methyl N-[(2-fluorophenyl)carbonyl]carbamate
 2-[(4-chlorophenyl)carbonyl]-1-benzofuran
 4-chlorophenyl 2,3-dihydro-1-benzofuran-5-carboxylate
 2-[4-(dimethylamino)phenyl]-1,2,3,4-tetrahydroquinolin-4-one
 [6-(ethylsulfanyl)pyridin-3-yl]methanimidamido thiophene-2-carboxylate
 Natural extract 1
 Natural extract 2

Table 2

- 1) N-(3-acetylphenyl)-8-methoxy-2-oxo-2H-chromene-3-carboxamide
- 2) 2-chloro-N-[3-hydroxy-4-(5-methyl-1,3-benzoxazol-2-yl)phenyl]-5-nitrobenzamide
- 3) 5-(1-butyl-2-oxo-2,3-dihydro-1H-indol-3-ylidene)-2-(piperidin-1-yl)-4,5-dihydro-1,3-thiazol-4-one
- 4) N-(4-fluorophenyl)-N'-(1-phenyl-5-propyl-1H-pyrazol-4-yl)urea

- 5) 6-(4-chlorophenyl)-3-phenylthieno[2,3-e][1,2,4]triazine
- 6) N,5-diphenyl-1,3,4-oxadiazole-2-carboxamide
- 7) ethyl 7-hydroxy-9-oxo-9H-xanthene-2-carboxylate
- 8) 1-(tert-butyl)-3,5-dimethyl-4-{{[3-
- 5 (trifluoromethyl)anilino]carbonyl}oxyimino]methyl}-1H-pyrazole
- 9) 2,4-dihydroxy-5,7-diphenylpyrano[2,3-d]pyrimidin-8-iumperchlorate
- 10) 1-[6-(benzyloxy)-3-(tert-butyl)-2-hydroxyphenyl]ethan-1-one
- 11) N-(3,4-dimethylphenyl)[(E)-N'-(thiophen-2-ylmethylidene)hydrazinecarbonyl]formamide
- 10 12) (2E)-3-(2-chlorophenyl)-N-(2-methylbut-3-yn-2-yl)prop-2-enamide
- 13) 1-naphthalen-1-yl-3,8-oxatricyclo[7.4.0.0^{2,7}]trideca-1(13),2,4,6,9,11-hexaen-5-ylthiourea
- 14) 5-(2,5-dichlorophenyl)-N-[2-(trifluoromethyl)phenyl]furan-2-carboxamide
- 15) 7-nitro-N-phenethyl-1H-indole-2-carboxamide
- 15 16) 1,2-di(3-methyl-2,3-dihydro-1,3-benzoxazol-2-yliden)hydrazine
- 17) N-[4-(dimethylamino)-3,5-difluorobenzoyl]-N'-(3,4-dimethyl-5-isoxazolyl)urea
- 18) (2Z)-3-(9H-fluoren-2-ylcarbamoyle)prop-2-enoic acid
- 19) O1-{[6-(2,2,2-trifluoroethoxy)-3-pyridyl]carbonyl}-3-chlorobenzene-1-carbohydroximamide
- 20 20) 3-hydroxy-9H-9-xanthenone
- 21) 6-methoxy-3-phenyl-[1,2,4]triazolo[4,3-a]pyridazine
- 22) ethyl 1-{[4-(trifluoromethoxy)anilino]carbonyl}-4-piperidinecarboxylate
- 23) 2-furyl{4-[3-(2-thienyl)-1,2,4-thiadiazol-5-yl]piperazino}methanone
- 24) 4-[(benzoyloxy)ethanimidoyl]-2-[(4-chlorophenoxy)methyl]-1,3-thiazole
- 25 25) 1-[4-(benzyloxy)phenyl]-3-[(3-cyanopyridin-2-yl)amino]urea
- 26) 3-(morpholin-4-yl)-5-[4-(trifluoromethyl)phenyl]-1,2-thiazole-4-carbonitrile
- 27) (E)-[1-(3-methyl-1-benzothiophen-2-yl)ethylidene]aminoN-phenylcarbamate
- 28) 1,3-dimethanesulfonyl-2,3-dihydro-1H-1,3-benzodiazole
- 29) 2-oxo-2-phenylethyl 2,3-dimethoxybenzoate
- 30 30) 4-[(5-{[(4-chlorophenyl)sulfonyl]methyl}furan-2-yl)carbonyl]morpholine
- 31) (2,4-dichlorophenyl)methylN-[(2-fluorophenyl)carbonyl]carbamate

32) 4-chlorophenyl-2,3-dihydro-1-benzofuran-5-carboxylate

33) N-(4-chlorophenyl)-4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carboxamide

Example 3

- 5 This Example describes a high throughput screen for molecules that inhibit the activity of EZH2.

Experimental Approach

- 10 A high throughput screening protocol was used to identify small molecule inhibitors of EZH2. Primary breast cancer cells were transfected with the E-cadherin promoter luciferase reporter gene and infected with the EZH2 adenovirus to suppress luciferase expression 48 hours prior to compound addition. Eighteen hours prior to compound addition, cells were trypsinized and distributed into 384-well plates in 60ul of medium using the Multidrop equipment. At time zero, compounds were transferred from
- 15 1.5 mM DMSO stocks to the cell plates in a final compound concentration of about 5uM. This concentration was chosen based upon other cell-based assays in which higher concentrations caused substantial cell toxicity and did not yield significantly more candidate compounds. After 24hours, the expressed luciferase activity was measured by adding 50 µl of the medium and 10ul of Steady-Glo luciferase reagent (Promega).
- 20 Sample plates were read in the Pherastar plate reader (BMG Labtech). Each plate in the screen contains 320 compounds to be tested plus 64 control wells placed in the outer two columns on each side of the plate. "Positive" control was EZH2 adenovirus infected cells followed by treatment with the HDAC inhibitor SAHA (50 0nM) which shows the activity expected in the presence of an inhibitor. More than 4000 small molecules, which
- 25 included synthetic chemicals as well as natural products that are available in the Center for Chemical Genomics (CCG) library at the University of Michigan.

Luciferase Assay.

- 30 In order to test the activity of EZH2 inhibitor individually, a promoter –luciferase assay was used. EZH2 overexpression will inhibit E cadherin promoter- luciferase reporter activity. EZH2 inhibitor inhibits this repression and reactivates the promoter and

hence increases the luciferase activity. The breast cell lines H16N2 was transfected with wild-type or E-box mutant E cadherin luciferase construct as well as pRL-TK vector as internal control for luciferase activity, then subsequently infected with either EZH2 or control viruses. Following two days of incubation, the cells were lysed and luciferase assays conducted using the dual luciferase assay system (Promega, Madison, WI). For testing the inhibitory effect of small molecules, the cells were treated with different doses of small molecules dissolved in DMSO for 24 hours before performing the luciferase activity. Each experiment was performed in triplicate and luciferase activity was measured after two days as previously described.

Cell Viability assay.

In order to test the activity of EZH2 inhibitor on cell viability, a WST-1 cell viability assay (Roche) was used. The aggressive prostate cell line DU145 was treated with different doses of small molecules dissolved in DMSO for 48 hours before performing the WST-1 assay according to manufacturer's protocol. Each experiment was performed in triplicate and absorbance was measure at 450 nm.

Basement Membrane Matrix Invasion Assay.

For invasion assays, the breast cell lines HME was infected with EZH2 adenovirus. Forty-eight hours post-infection, cells were seeded onto the basement membrane matrix (EC matrix, Chemicon, Temecula, CA) present in the insert of a 24-well culture plate. Fetal bovine serum was added to the lower chamber as a chemoattractant with or without 2 doses of small molecule inhibitors. After 48 hours, the non-invading cells and EC matrix were gently removed with a cotton swab. Invasive cells located on the lower side of the chamber were measured colorimetrically by treating with 150 μ l of 10% acetic acid and the absorbance measured at 560nm using a spectrophotometer.

Immunoblot Analyses

DU145 cells were incubated with 2 doses of EZH2 small molecule inhibitors for 96 hours and homogenized in NP40 lysis buffer (50 mM Tris-HCl, 1% NP40, pH 7.4,

Sigma, St. Louis, MO), and complete proteinase inhibitor mixture (Roche, Indianapolis, IN). Ten micrograms of each protein extract were boiled in sample buffer, separated by SDS-PAGE, and transferred onto Polyvinylidene Difluoride membrane (GE Healthcare). The membrane was incubated for one hour in blocking buffer and incubated overnight at 4°C with the following: anti-EZH2 mouse monoclonal (1:1000, BD Biosciences, San Jose, CA, #612666), anti-trimethyl histone H3 lysine 27 mouse monoclonal antibody (Abcam, ab6002). Following a wash with TBS-T, the blot was incubated with horseradish peroxidase-conjugated secondary antibody and the signals visualized by enhanced chemiluminescence system as described by the manufacturer (GE Healthcare).

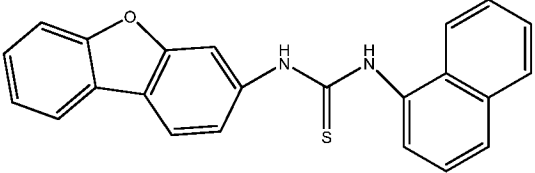
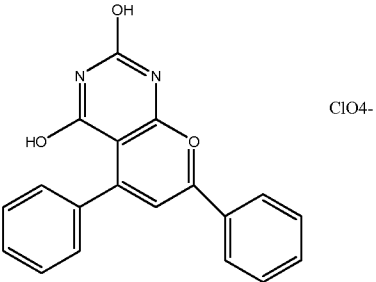
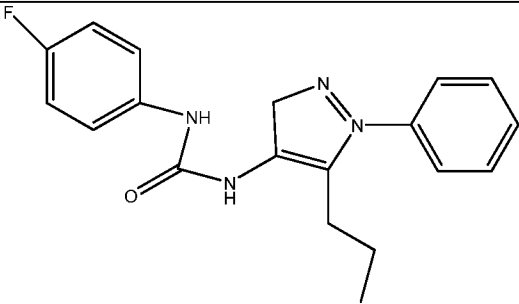
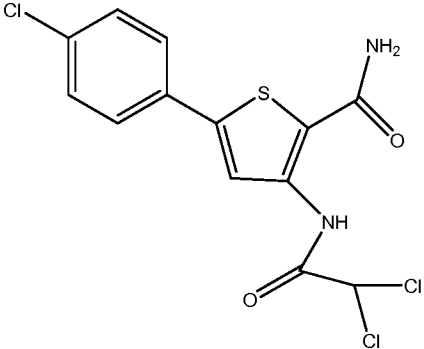
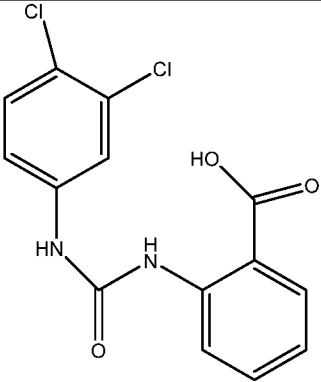
The blots were re-probed with β -actin for confirmation of equal loading.

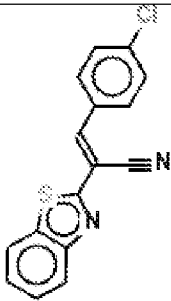
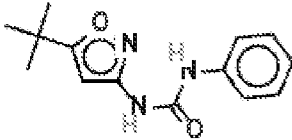
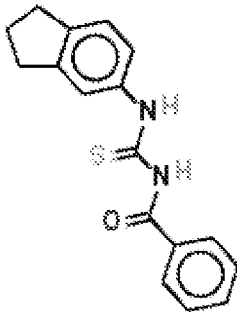
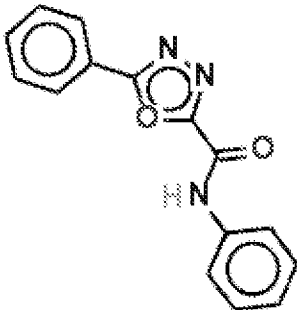
Results

By performing secondary screenings for small molecule inhibitor against EZH2, 10 compounds that showed significant inhibition of EZH2 activity by one or multiple *in vitro* assays like proliferation and invasion assay as well as trimethyl histone H3- K27 mark were identified. The data from *in vitro* assays, structure of the small molecule inhibitors and their chemical names are shown in Figures 30 to 36 and Table 3.

Table 3

Compound	Chemical Name	Structure
MCTP1	1-{[4-amino-5-(2,2-dimethylpropanoyl)-1,3-thiazol-2-yl]thio}-3,3-dimethylbutan-2-one	

MCTP2	N-dibenzo[b,d]furan-s-yl-N'-1-naphthylthiourea	
MCTP3	2,4-dihydroxy-5,7-diphenylpyrano[2,3-d]pyrimidin-8-ium perchlorate	
MCTP12	N-(4-fluorophenyl)-N'-(1-phenyl-5-propyl-1H-pyrazol-4-yl) urea	
MCTP15	5-(4-chlorophenyl)-3-[(2,2-dichloroacetyl)amino]thiophene-carboxamide	
MCTP28	2-([(3,4-dichlorophenyl)amino]carbonyl}amino)benzoic acid	

MCTP11	2-(1,3-benzothiazol-2-yl)-3-(4-chlorophenyl)acrylonitrile	
MCTP18	N-[5-(tert-butyl)-3-isoxazolyl]-N'-phenylurea	
MCTP19	N-benzoyl-N'-(2,3-dihydro-1H-inden-5-yl)thiourea	
MCTP20	N,5-diphenyl-1,3,4-oxadiazole-2-carboxamide	

All publications and patents mentioned in the above specification are herein
 5 incorporated by reference. Various modifications and variations of the described method

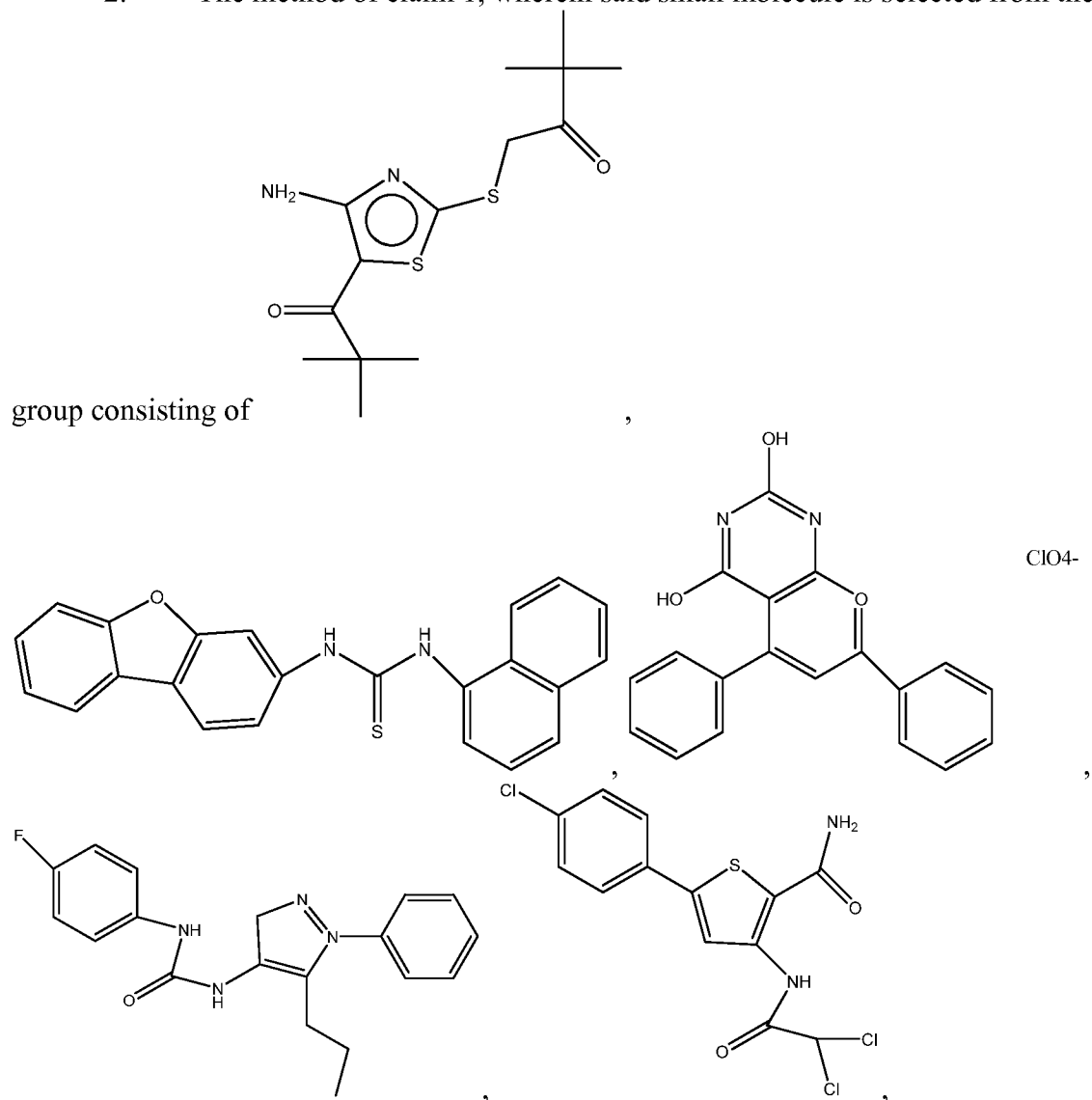
and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, 5 various modifications of the described modes for carrying out the invention which are obvious to those skilled in the relevant fields are intended to be within the scope of the following claims.

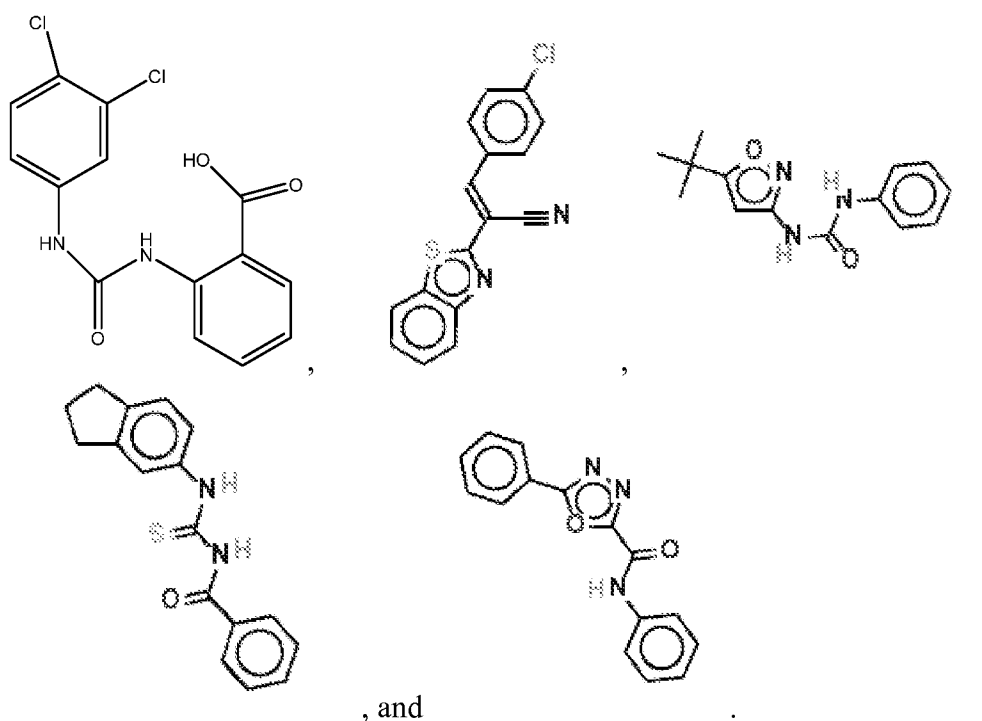
CLAIMS

We claim:

1. A method of inhibiting the growth of cells, comprising contacting a cell
 5 expressing EZH2 with a small molecule compound under conditions such that at least one activity of EZH2 in said cell is inhibited.

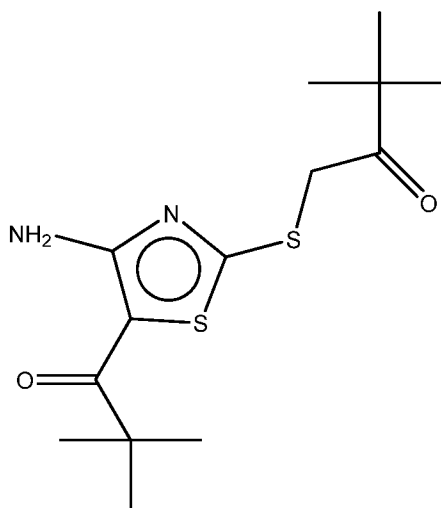
2. The method of claim 1, wherein said small molecule is selected from the



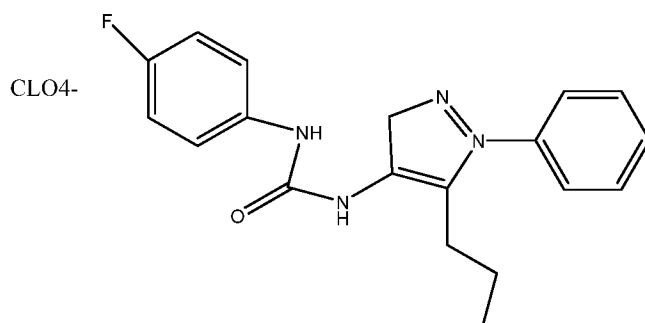
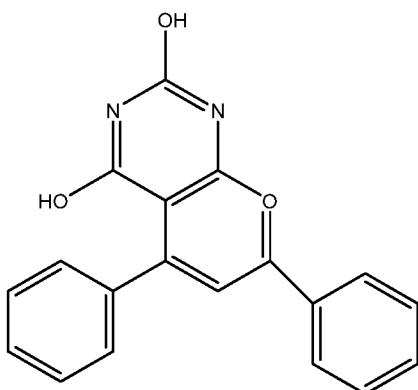
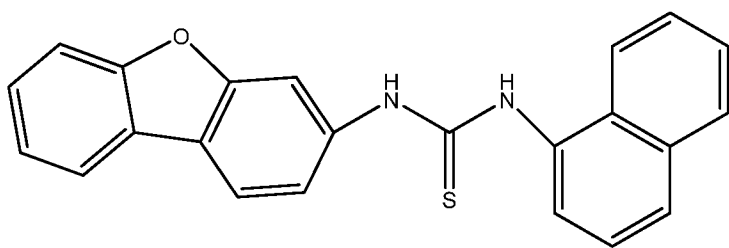


- 5 3. The method of claim 1, wherein said cell is a cancer cell.
4. The method of claim 1, wherein said cell is in an organism.
5. The method of claim 4, wherein said organism is an animal.
- 10 6. The method of claim 5, wherein said animal has been diagnosed with cancer.
7. The method of claim 6, wherein said cancer is prostate cancer.
- 15 8. The method of claim 6, wherein said cancer is selected from the group consisting of breast cancer and bladder cancer.
9. A pharmaceutical composition comprising a small molecule compound
- 20 that inhibits at least one activity of EZH2 in a cell.

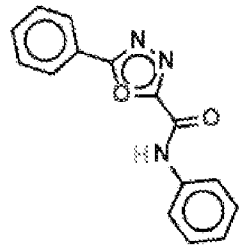
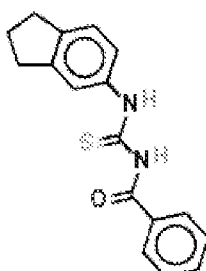
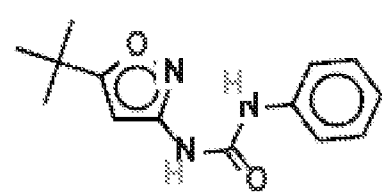
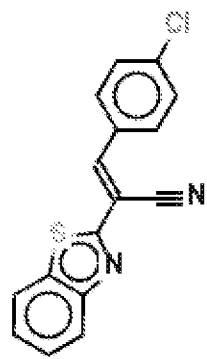
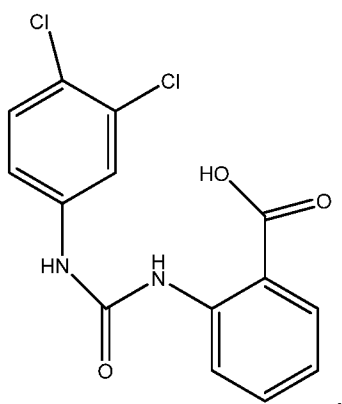
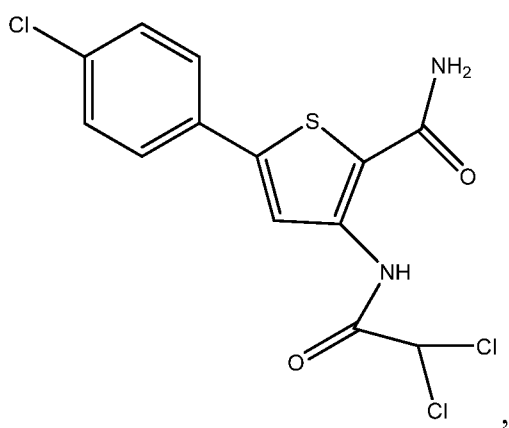
10. The pharmaceutical composition of claim 9, wherein said compound is



selected from the group consisting of



5



, and

Figure 1

Position 40-66 of EZH2 3' UTR	5'	...AGCUUCAGGAACCUCG-AGUACUGUG...	
		:	
hsa-miR-101	3'	GAAGUC--AAUAGUGUCAUGACAU	7mer-m8
Position 87-121 of EZH2 3' UTR	5'	...GCAGUUUGAAAUUCUGAAUUUGCAAAGUACUGUA...	
hsa-miR-101	3'	GAAGUCAA-----UAGUGUCAUGACAU	8mer

Figure 2

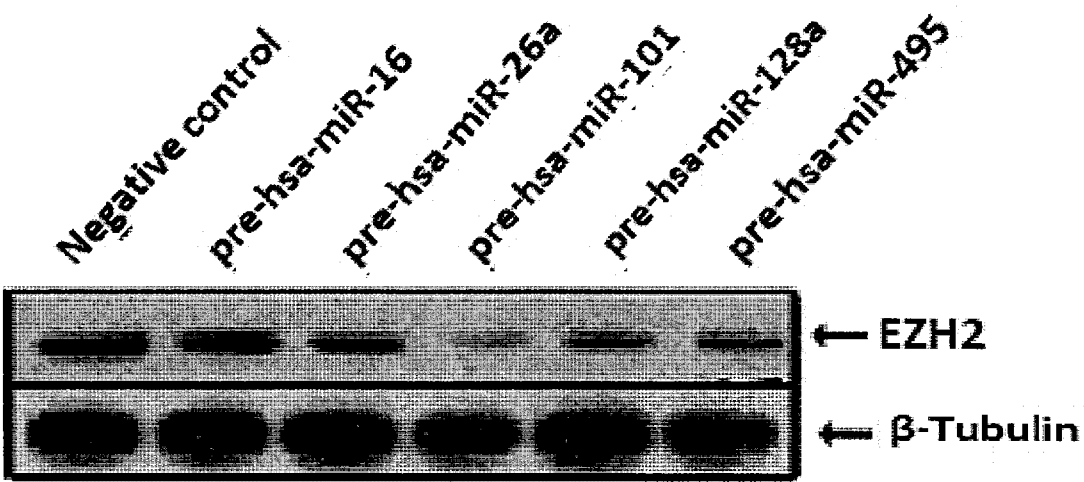


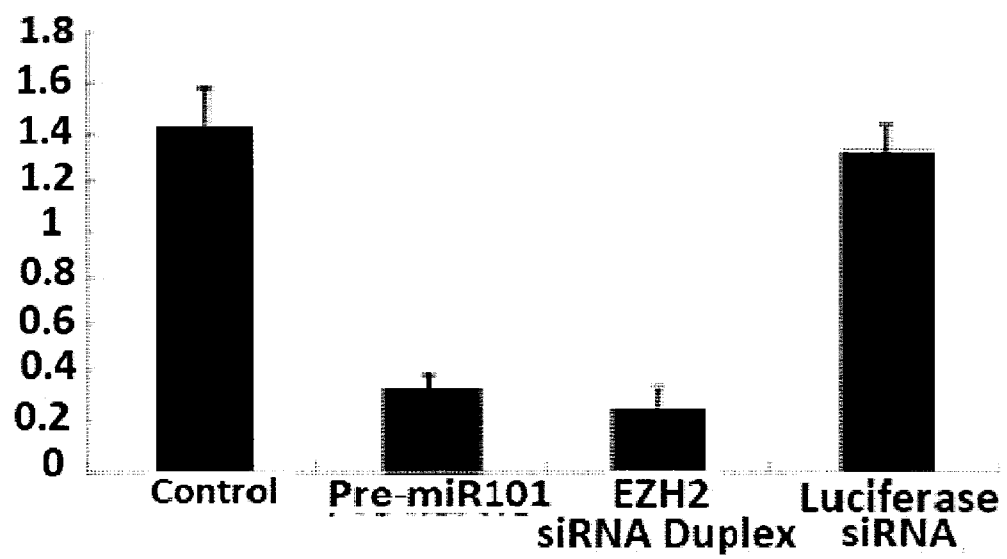
Figure 3

Figure 4

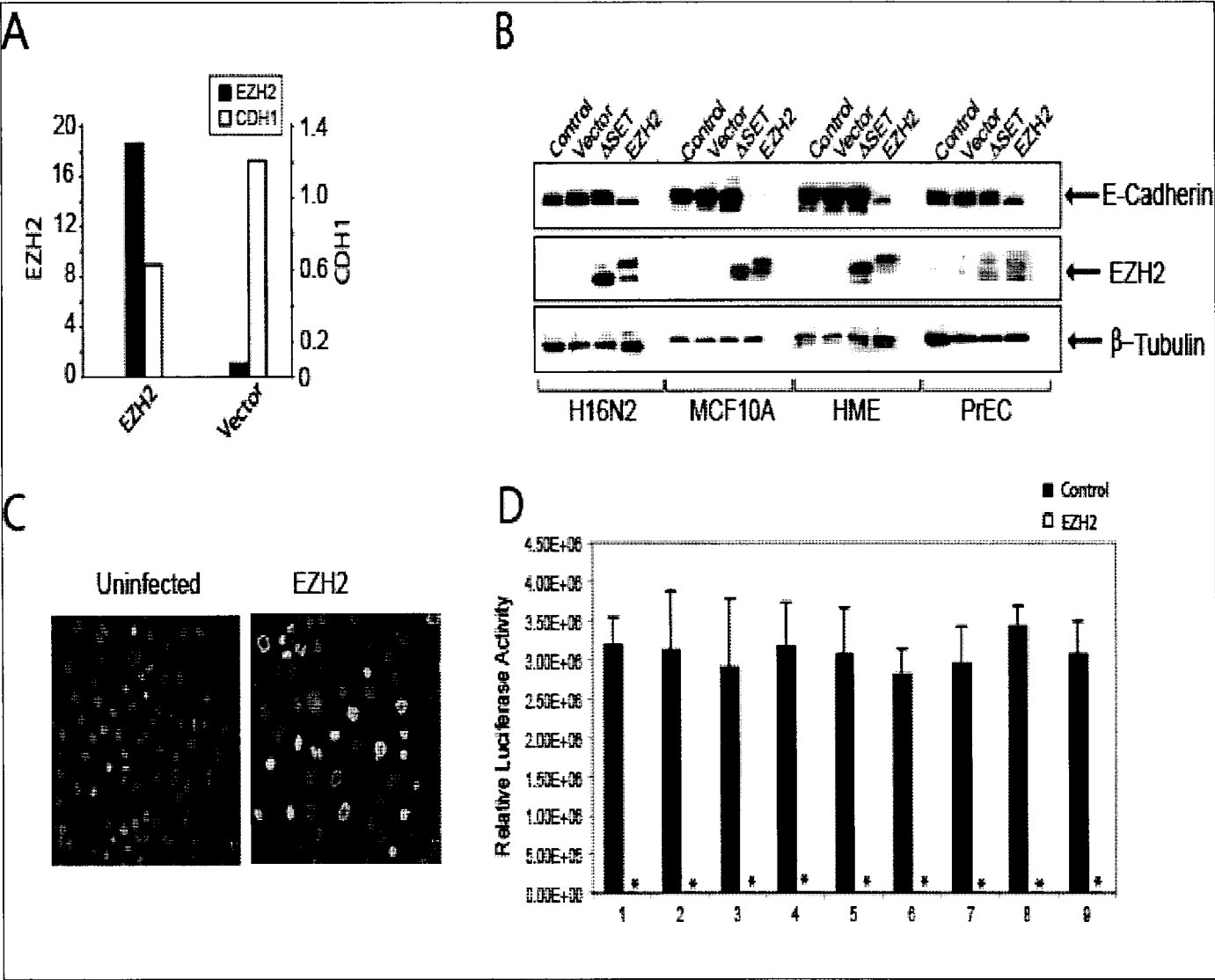


Figure 5

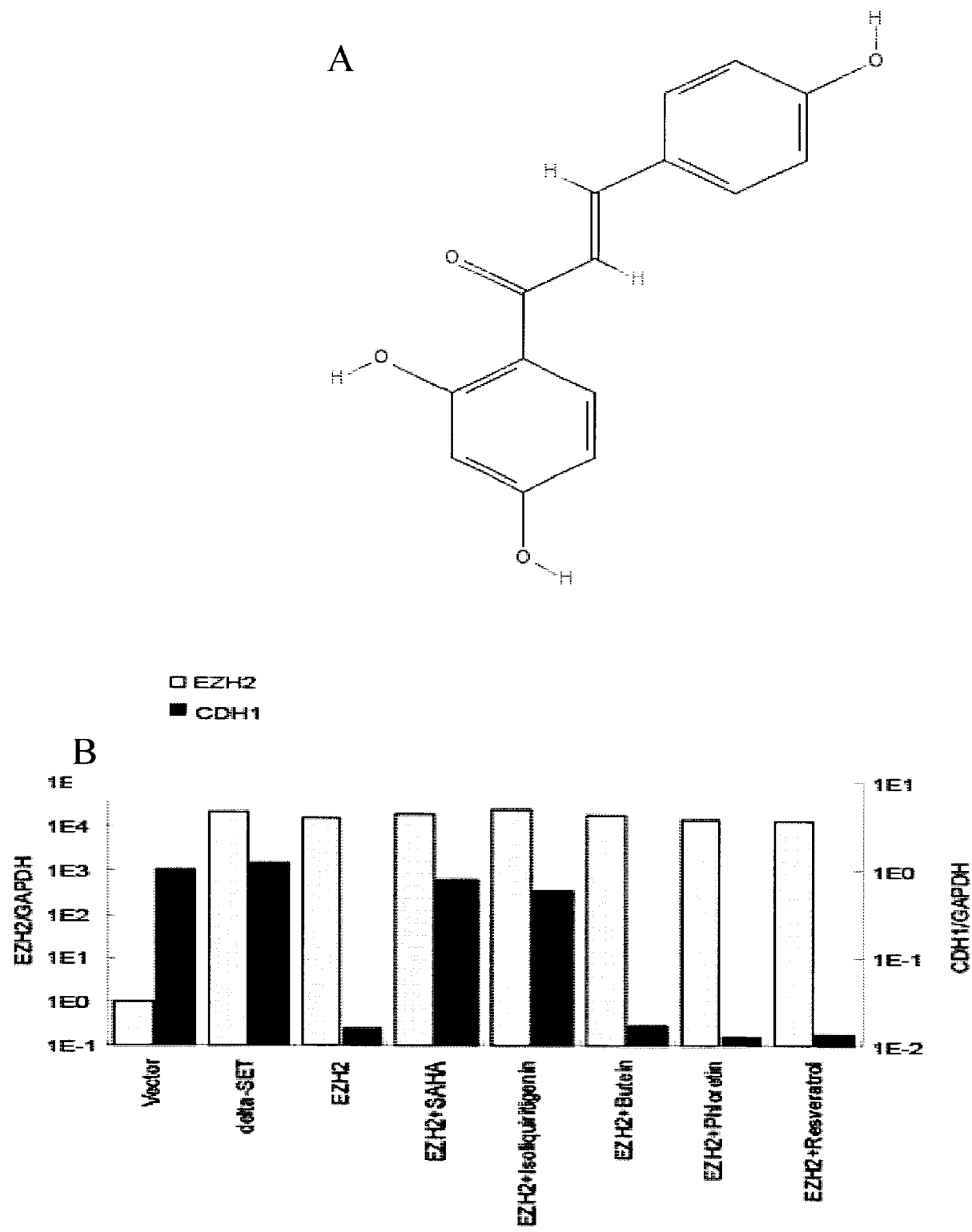
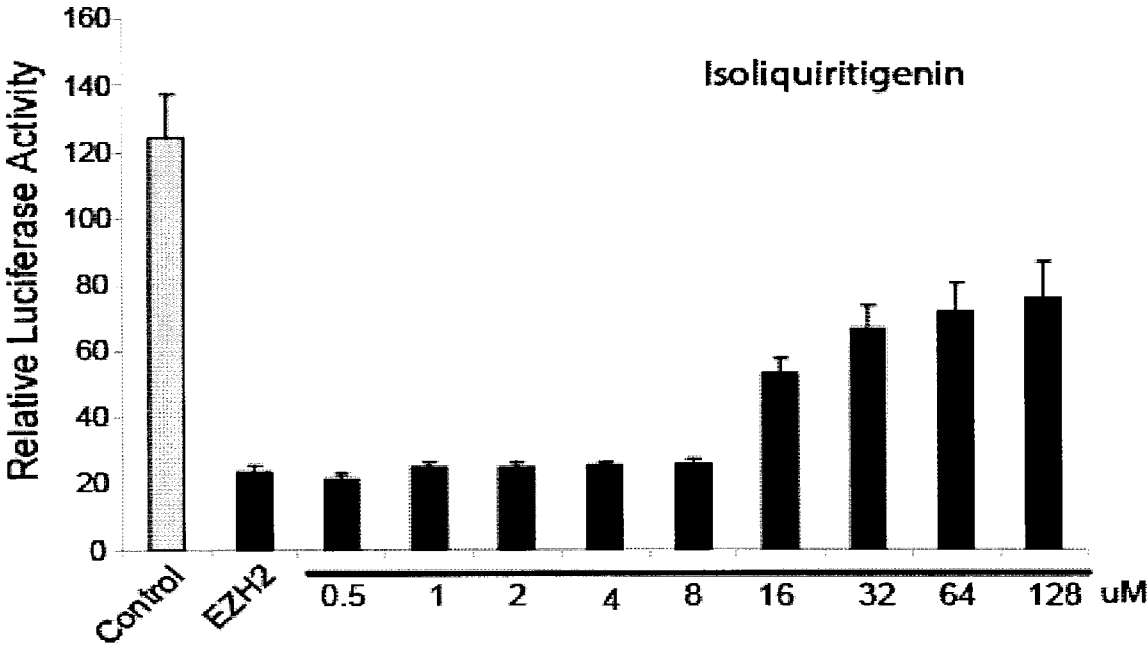


Figure 6

A



B

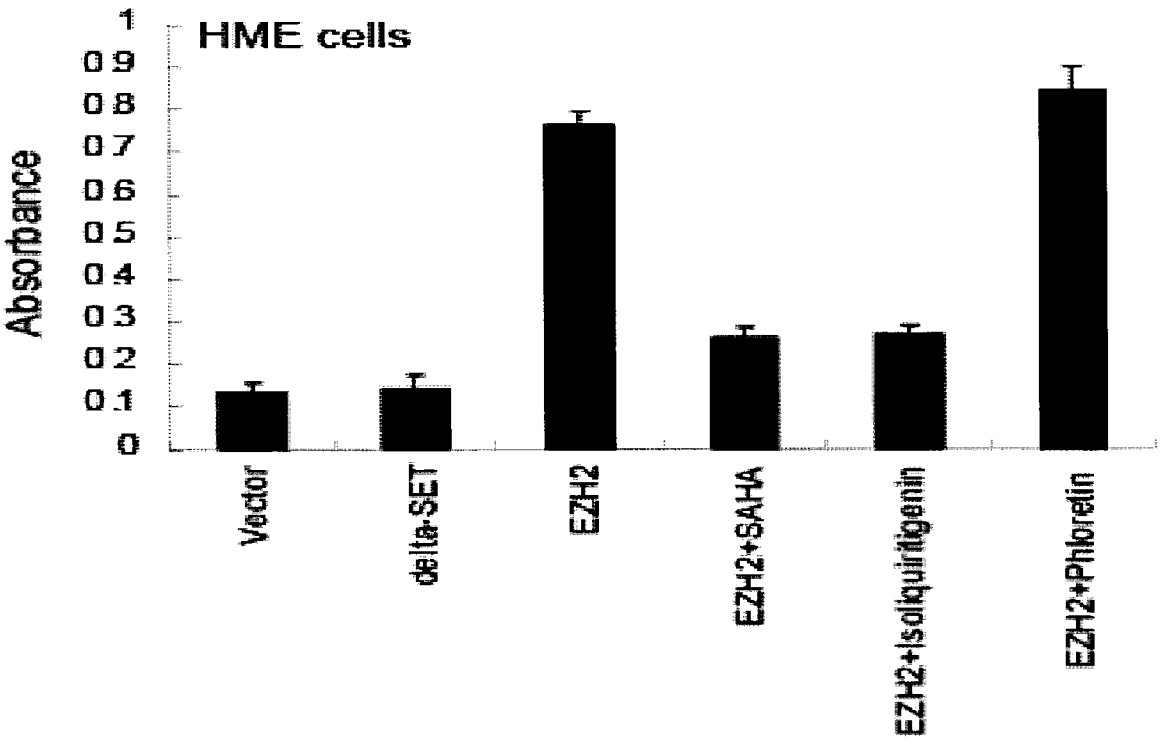


Figure 7

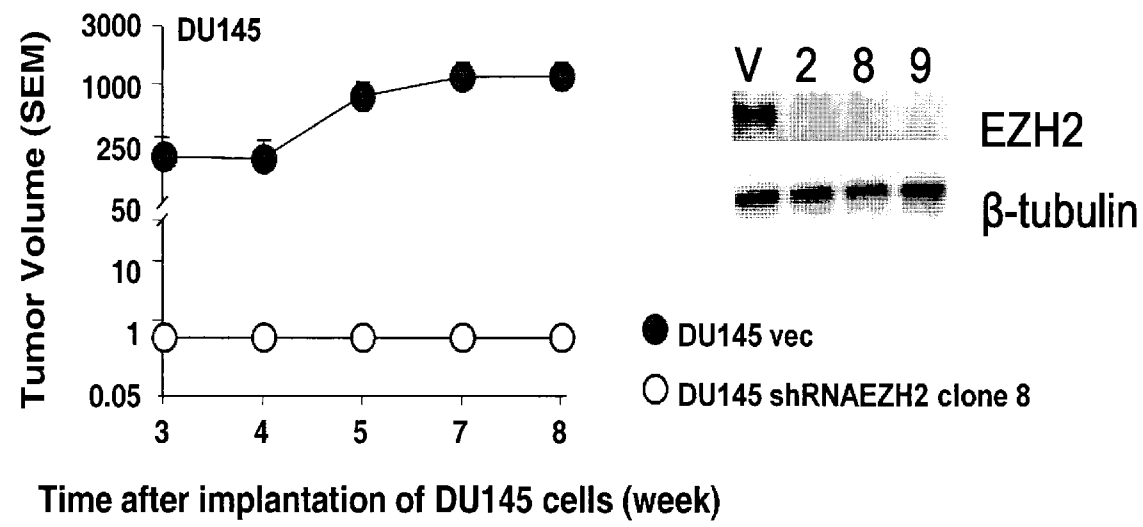


Figure 8

gastric

breast

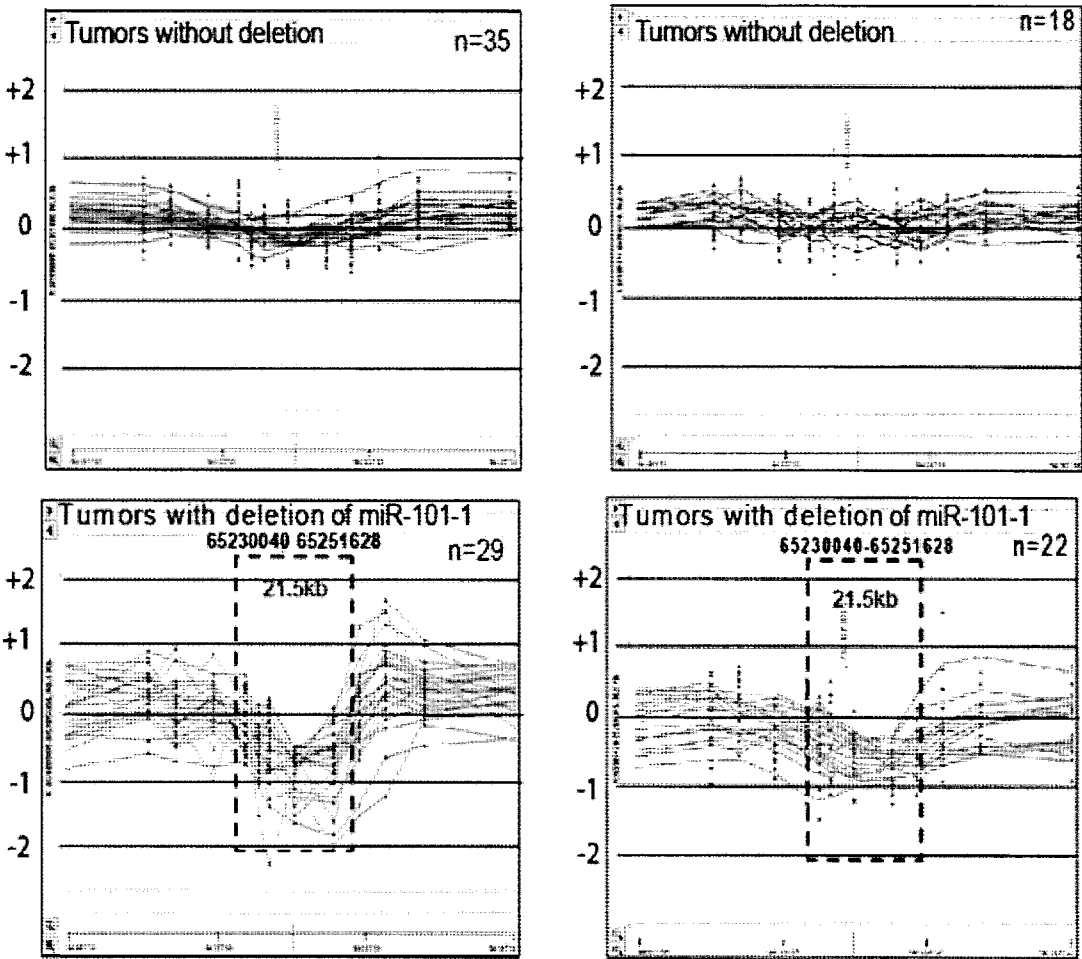


Figure 9

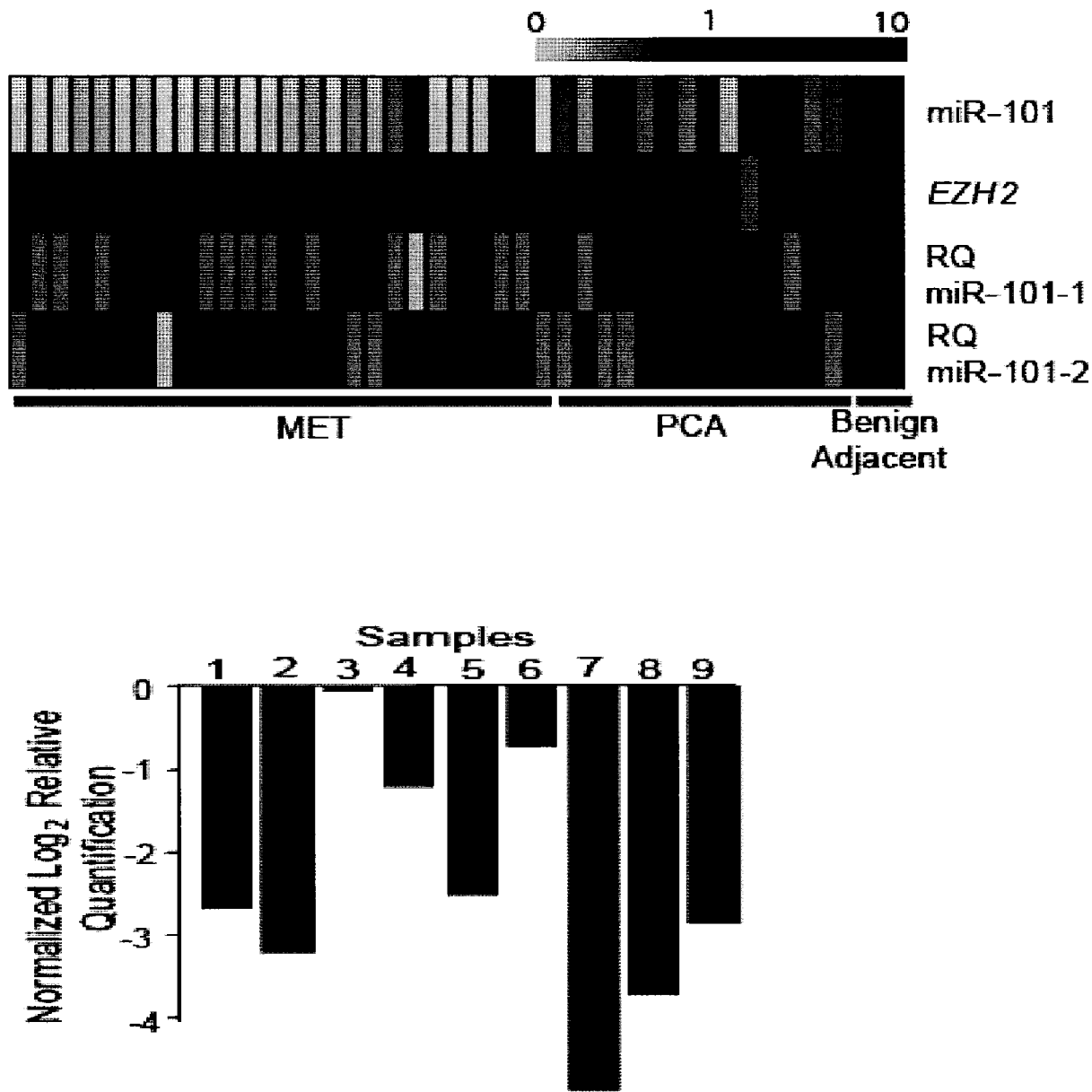


Figure 10

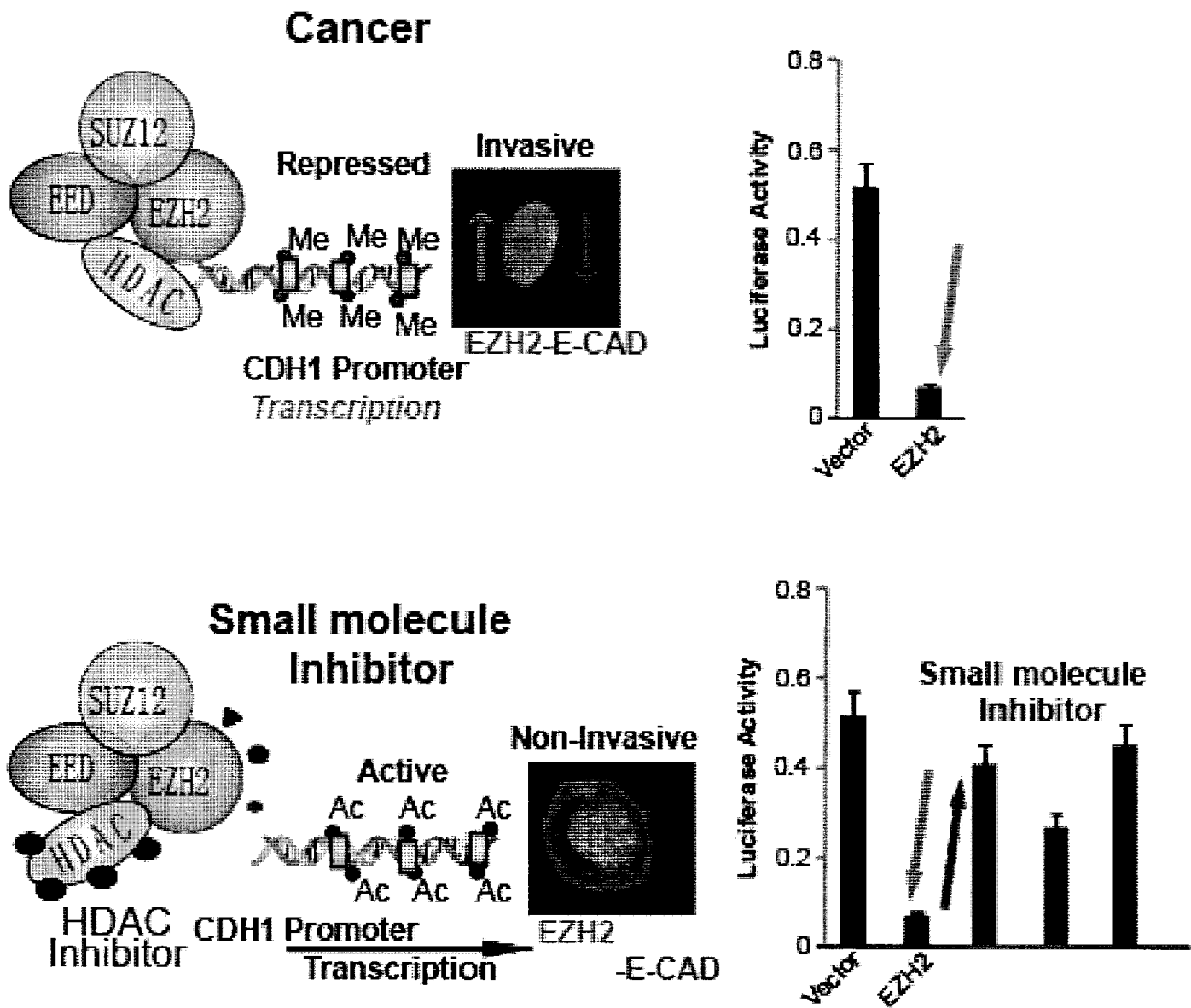
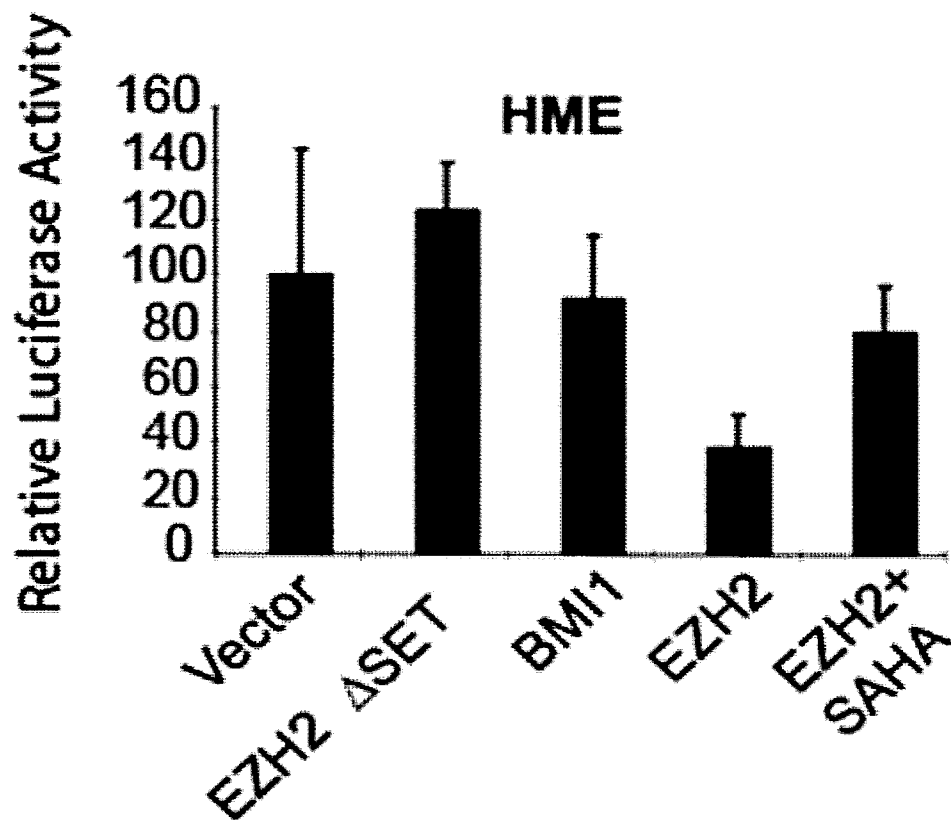


Figure 11



Gain of function assay

Figure 12

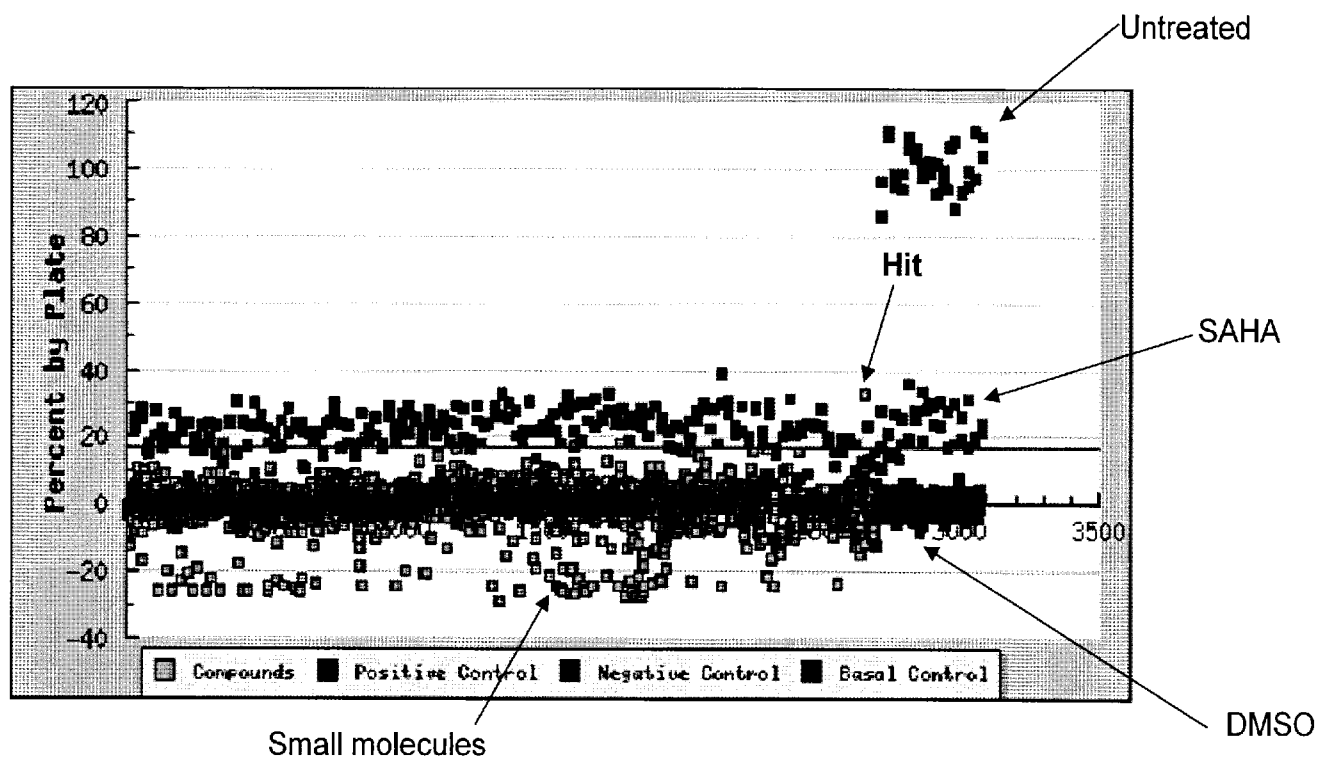


Figure 13

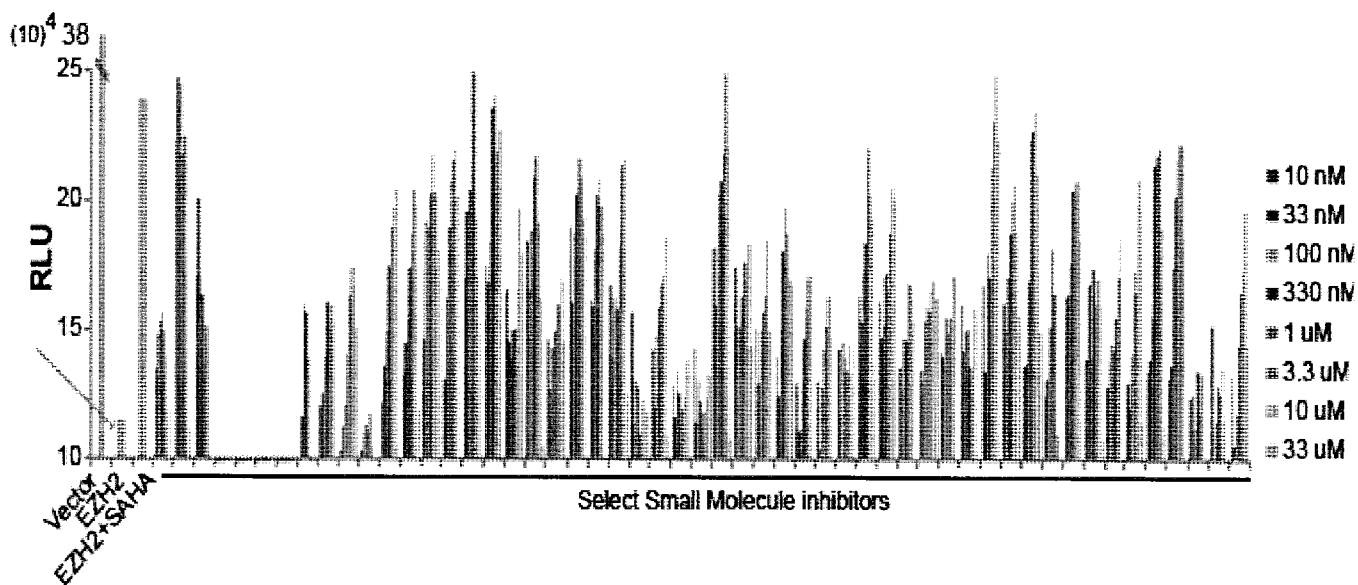


Figure 14

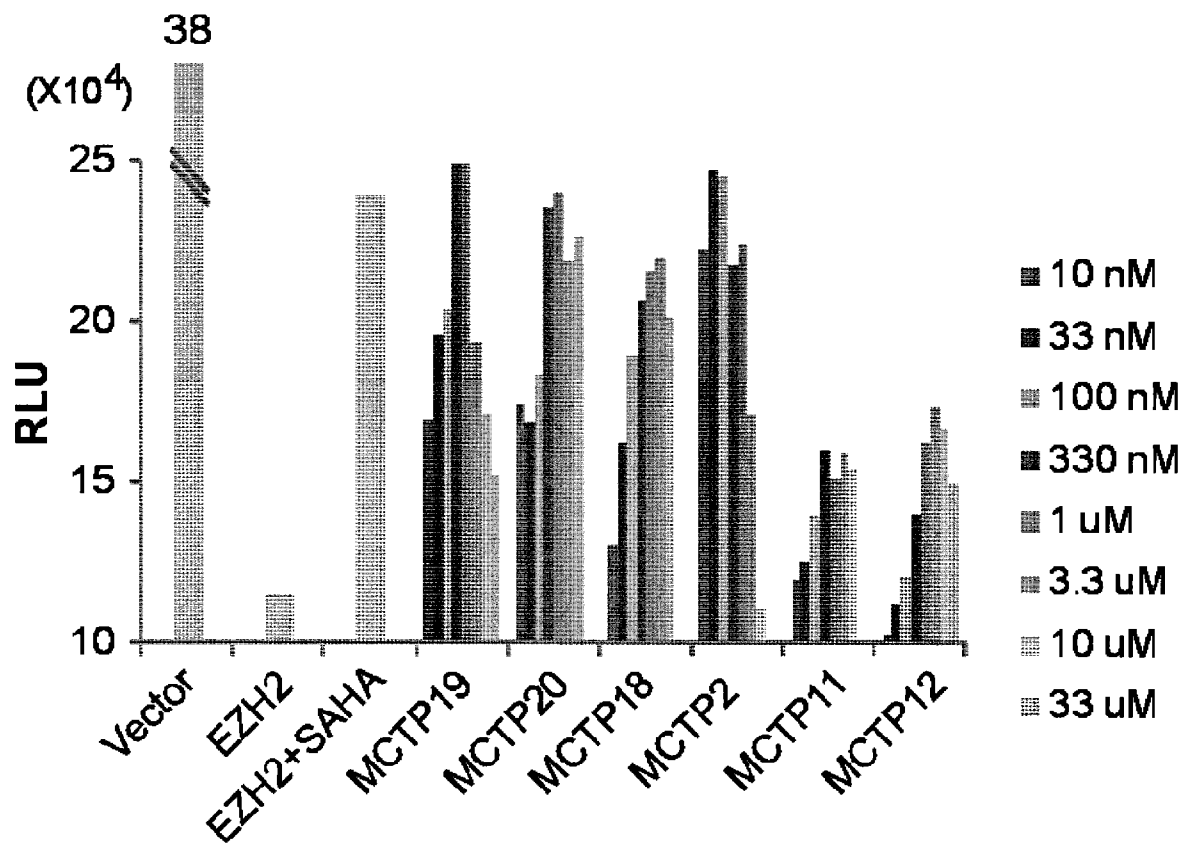


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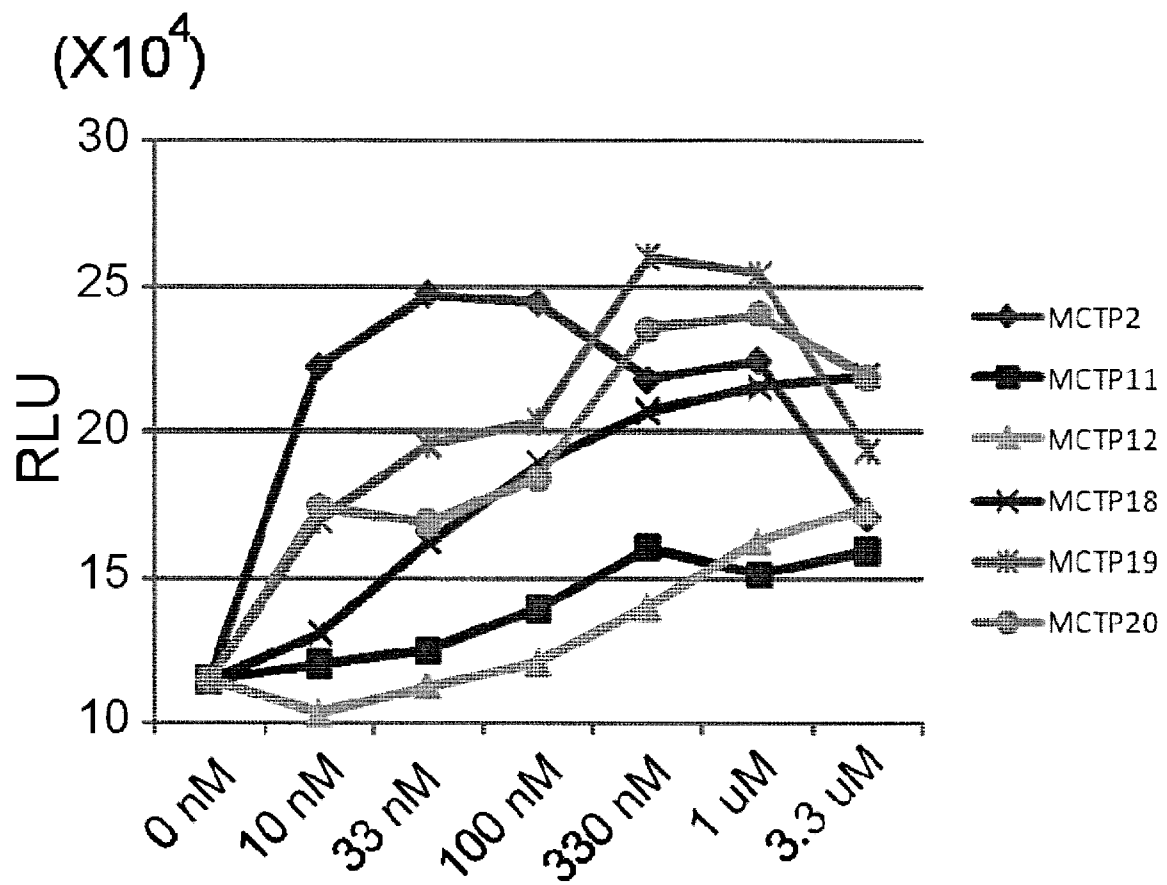


Figure 16

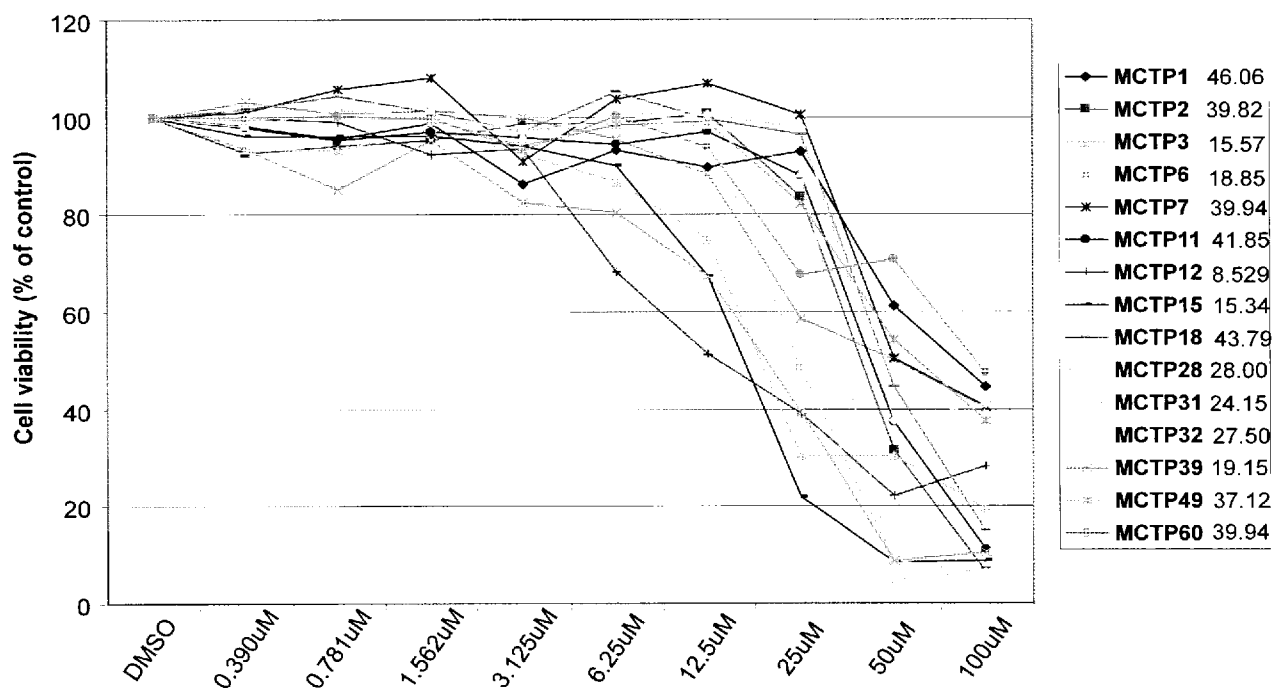


Figure 17

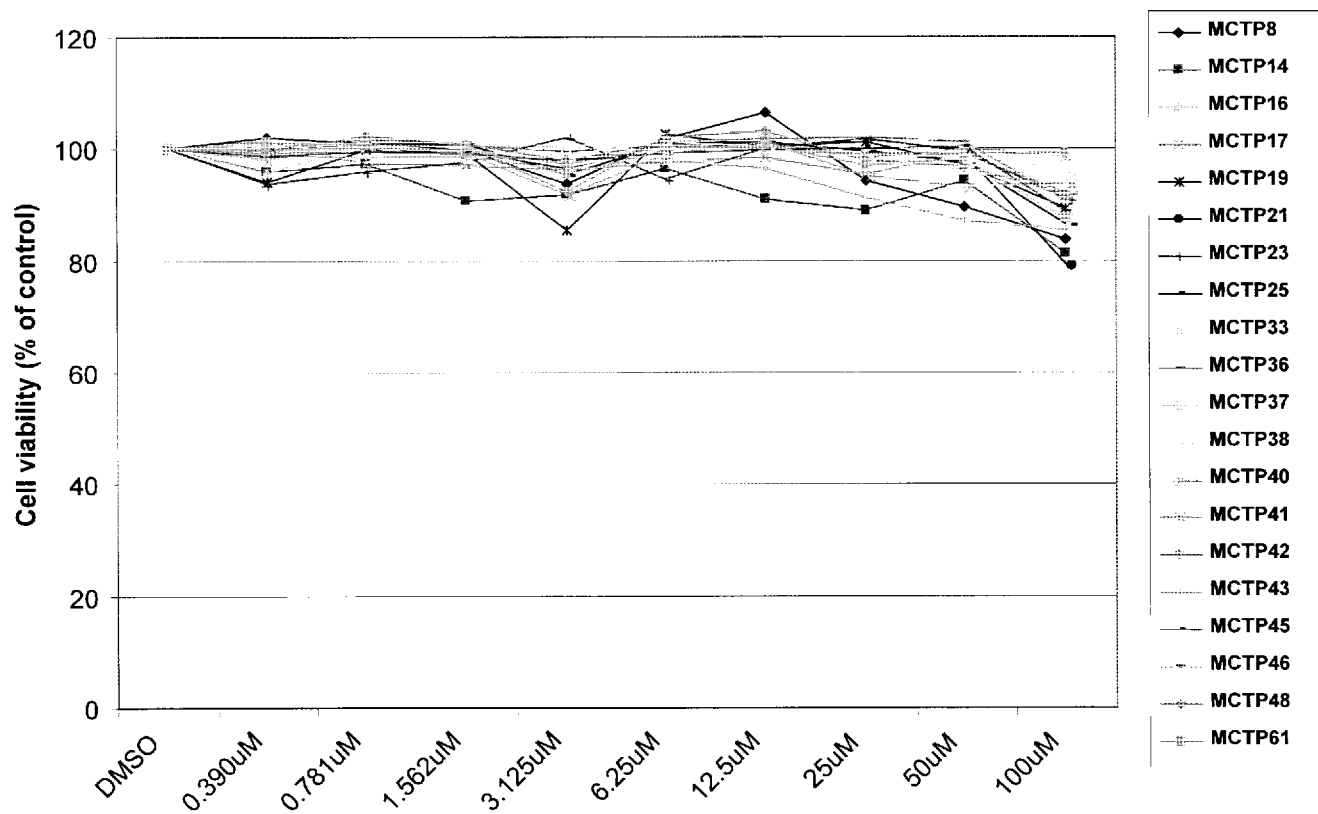
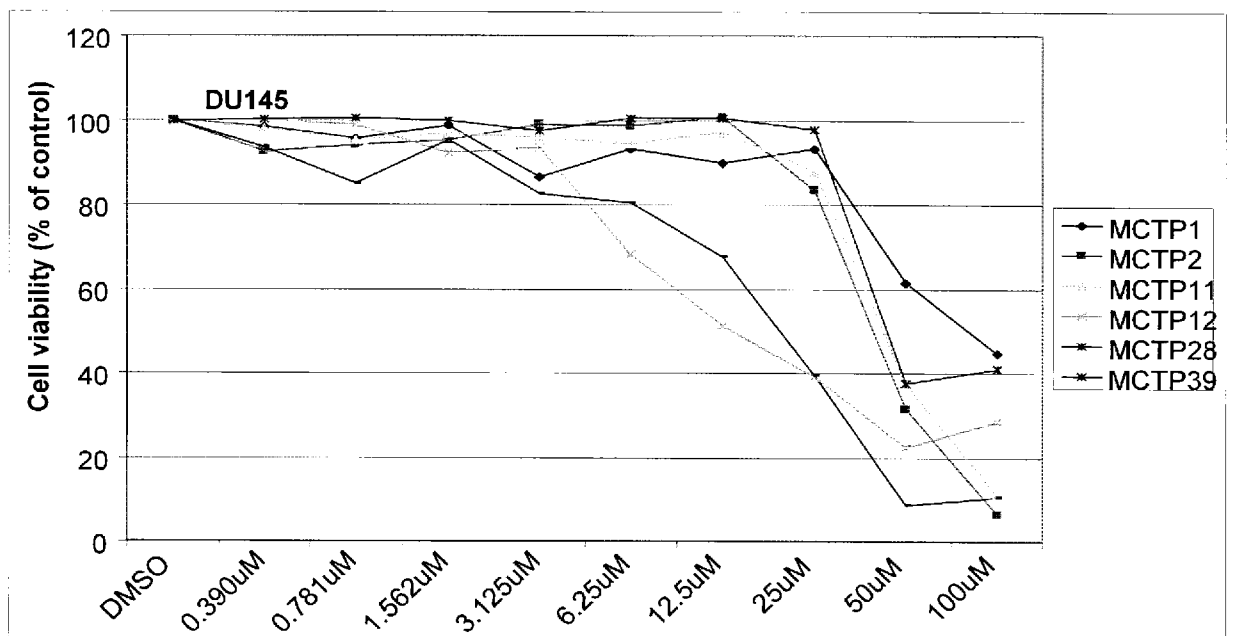
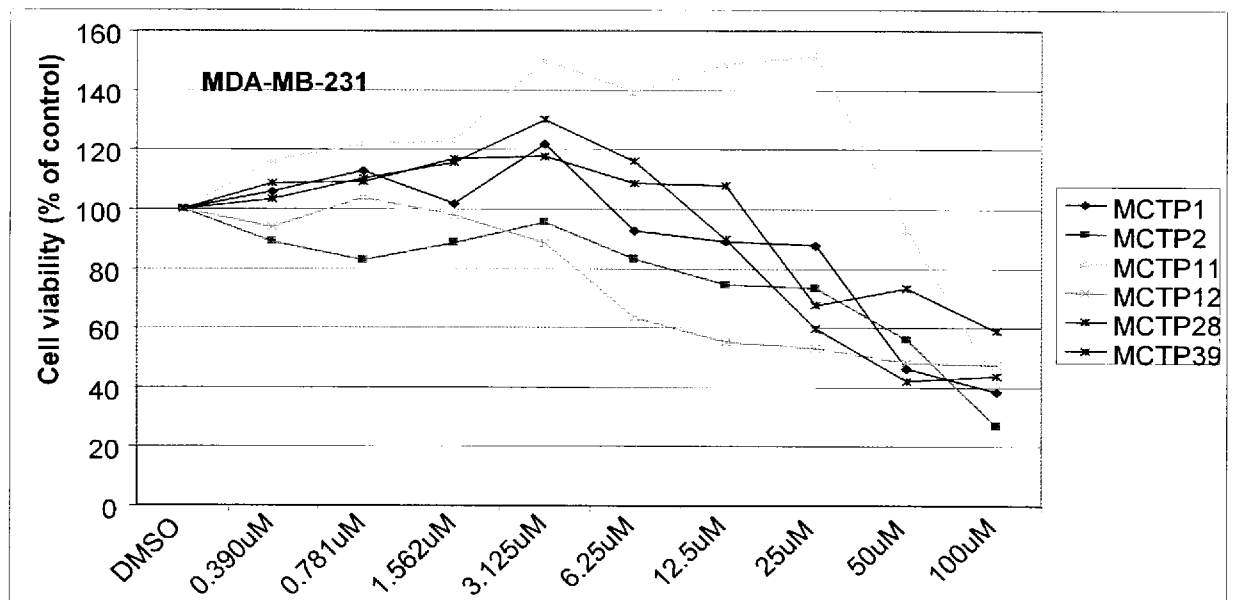


Figure 18



		N1	N2	N11	N12	N28	N39
MDA-MB-231	IC50	35.48	48.13	~ 50.56	4.679	16.57	17.13
DU145	IC50	46.06	39.82	41.85	8.529	28.00	19.15

Figure 19

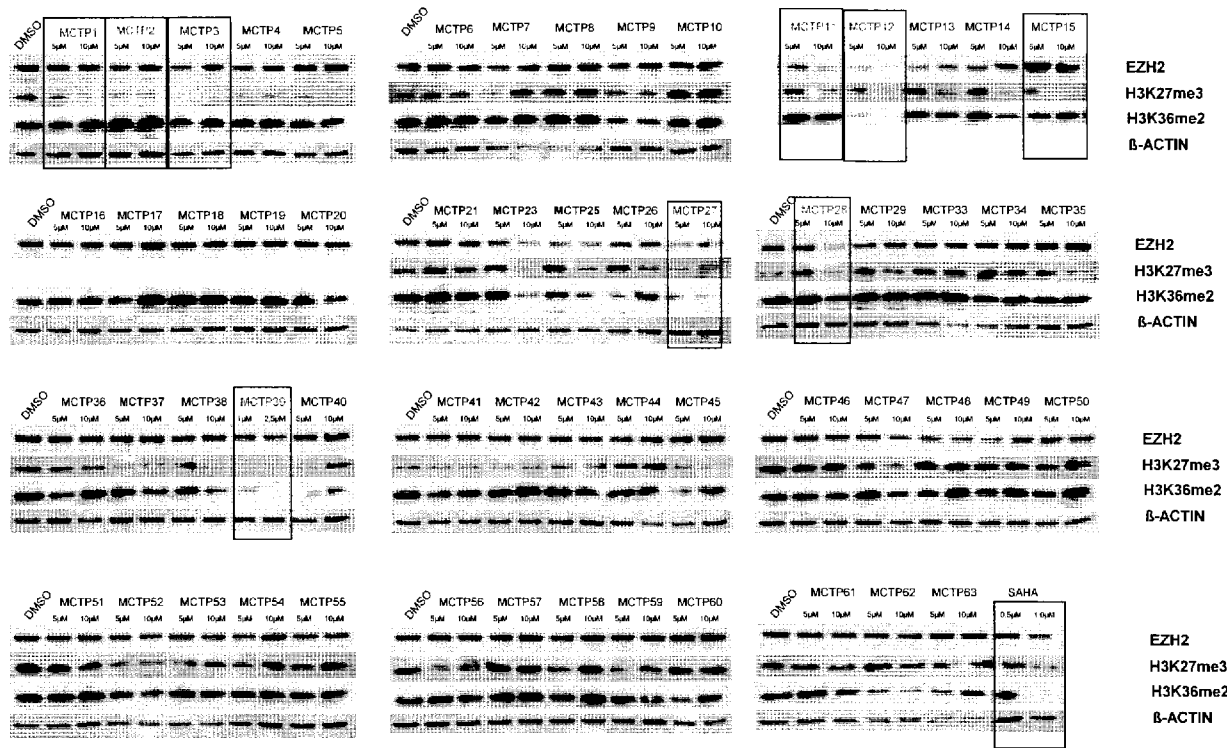


Figure 20

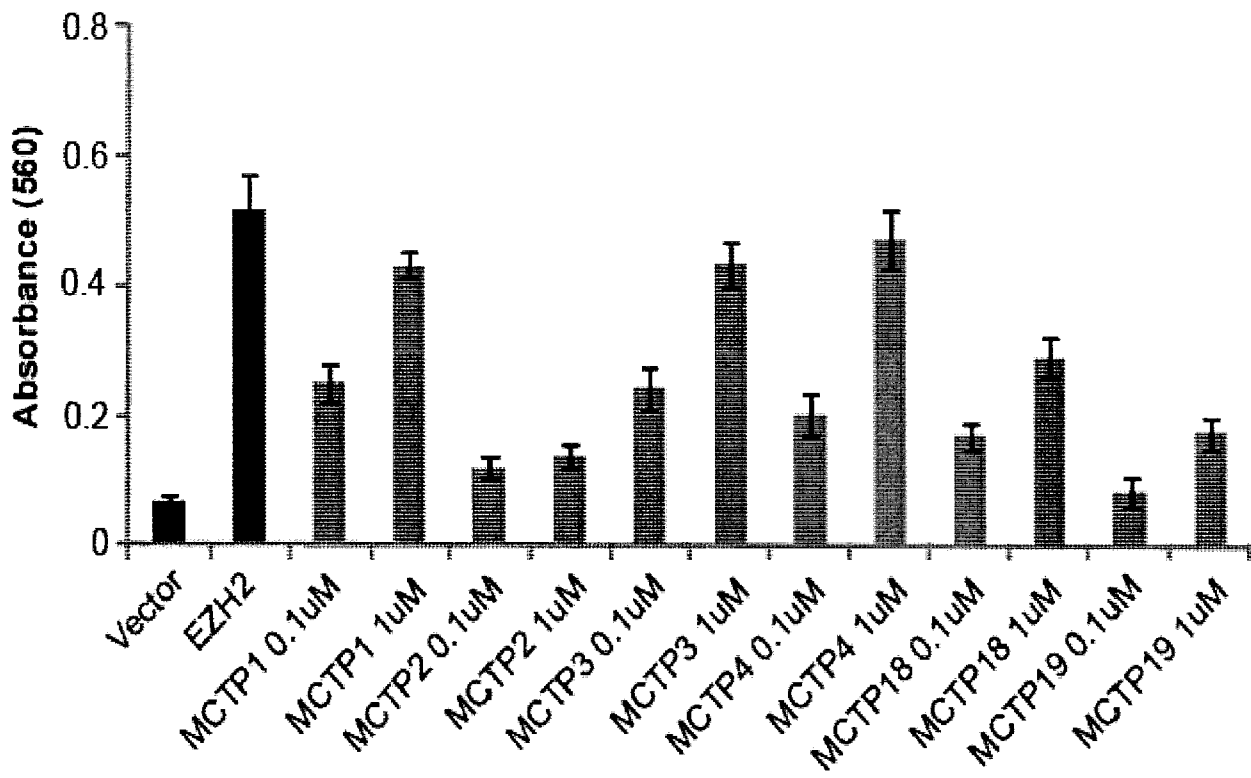


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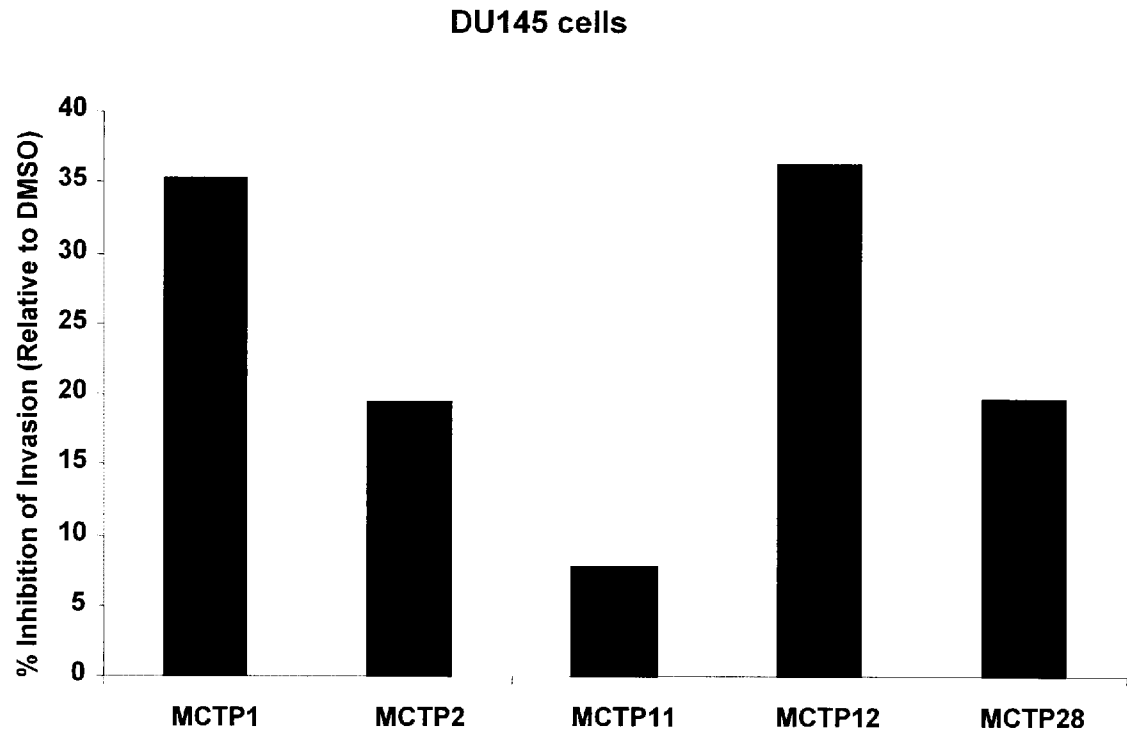


Figure 22

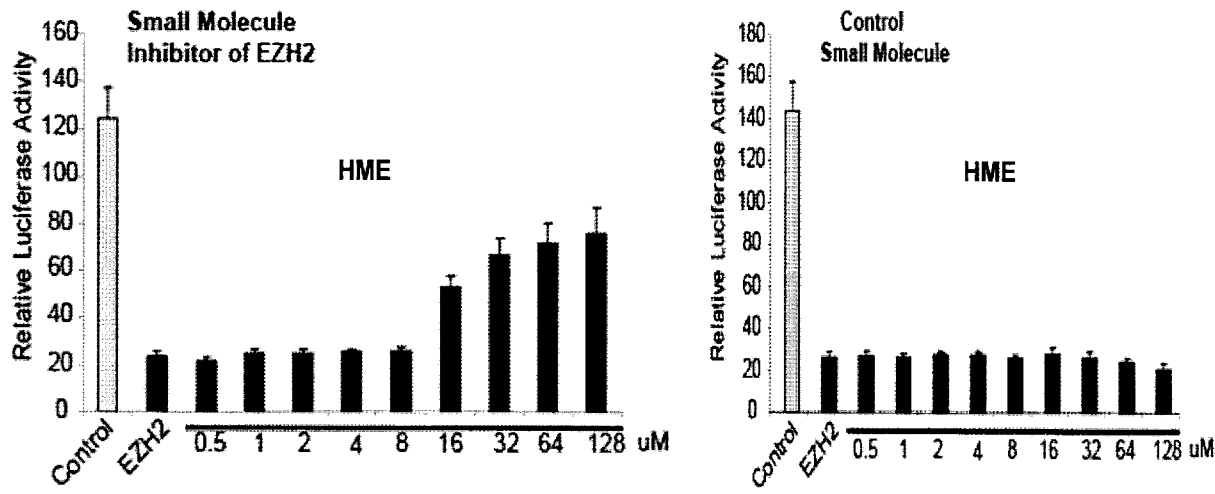


Figure 23

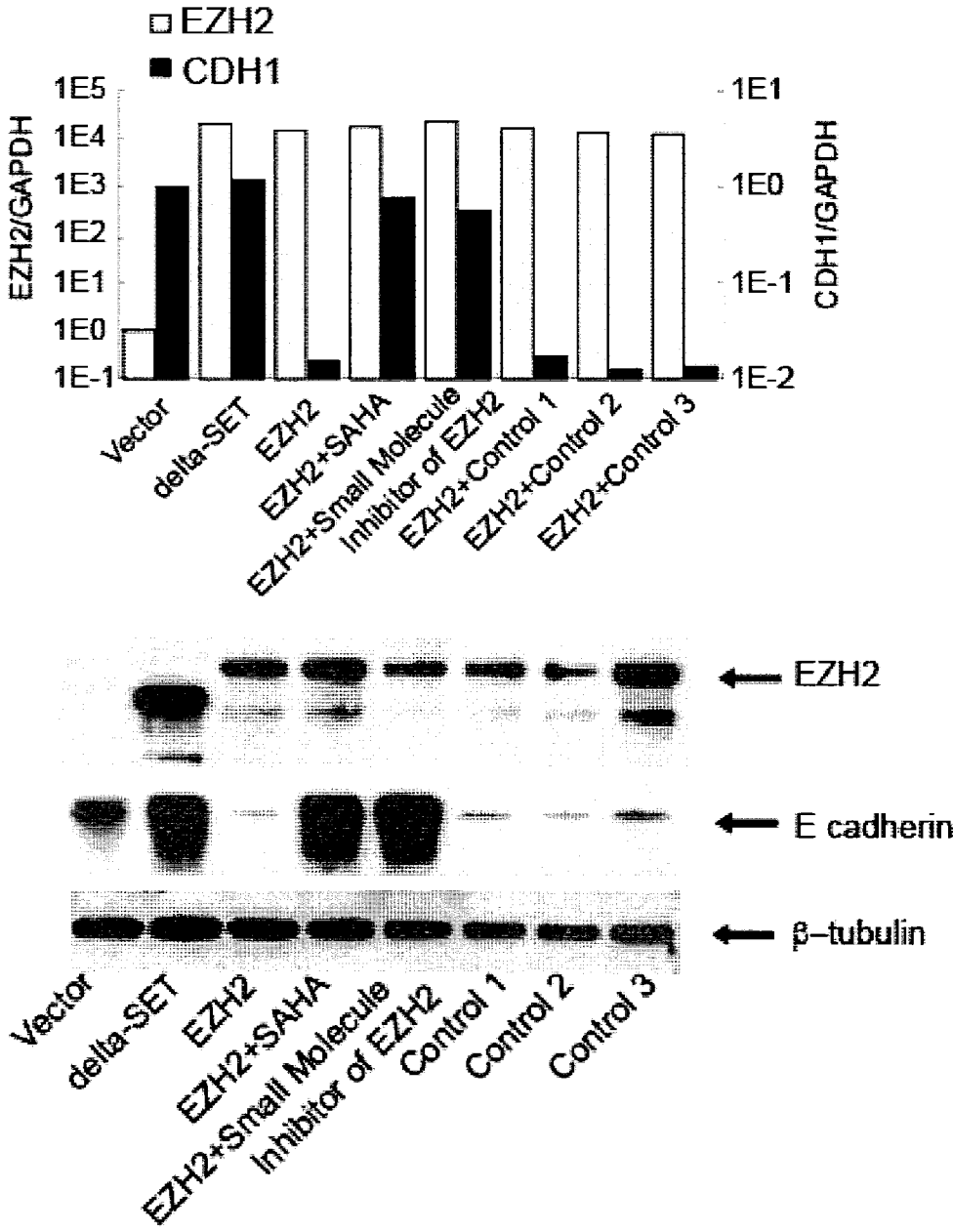


Figure 24

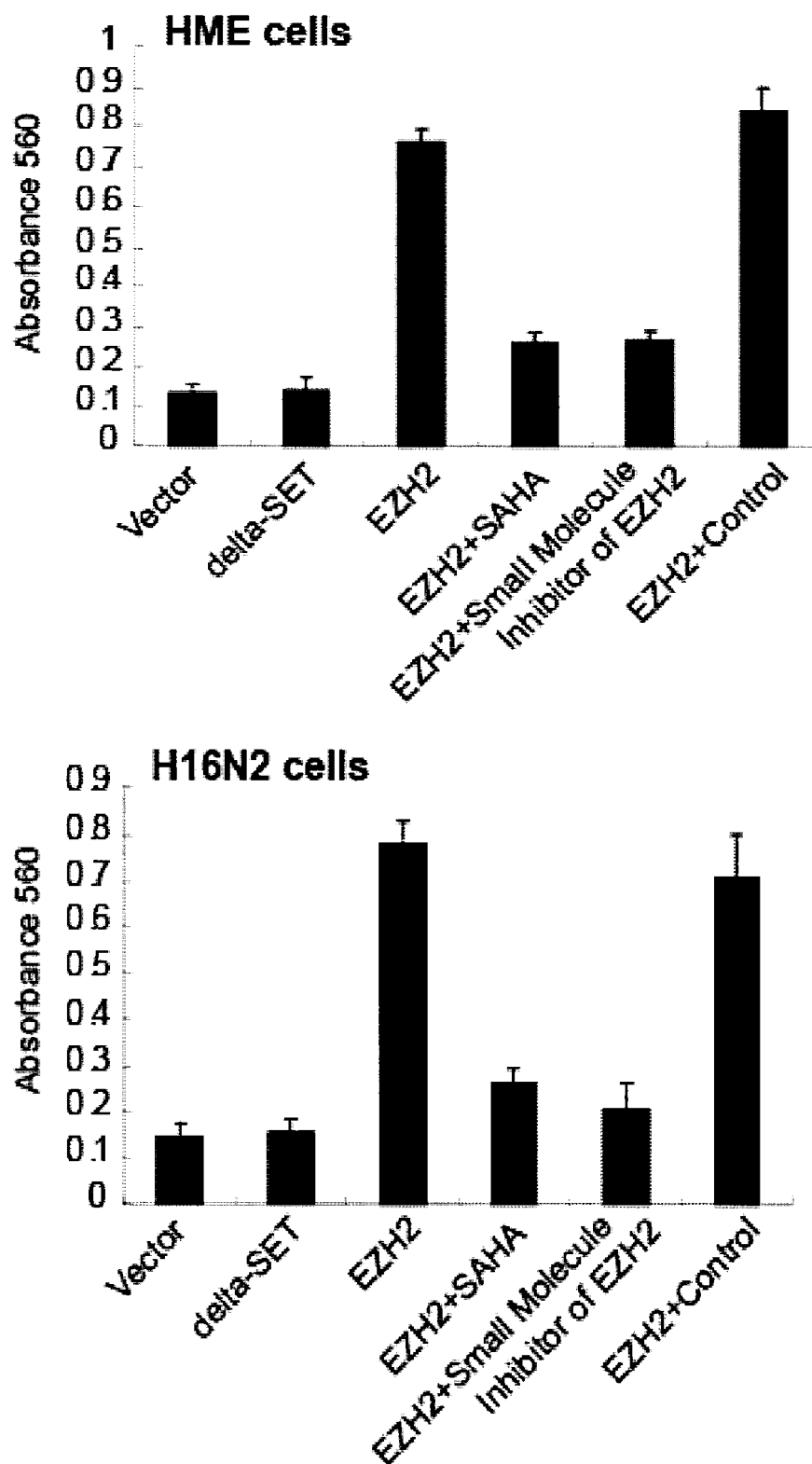


Figure 25

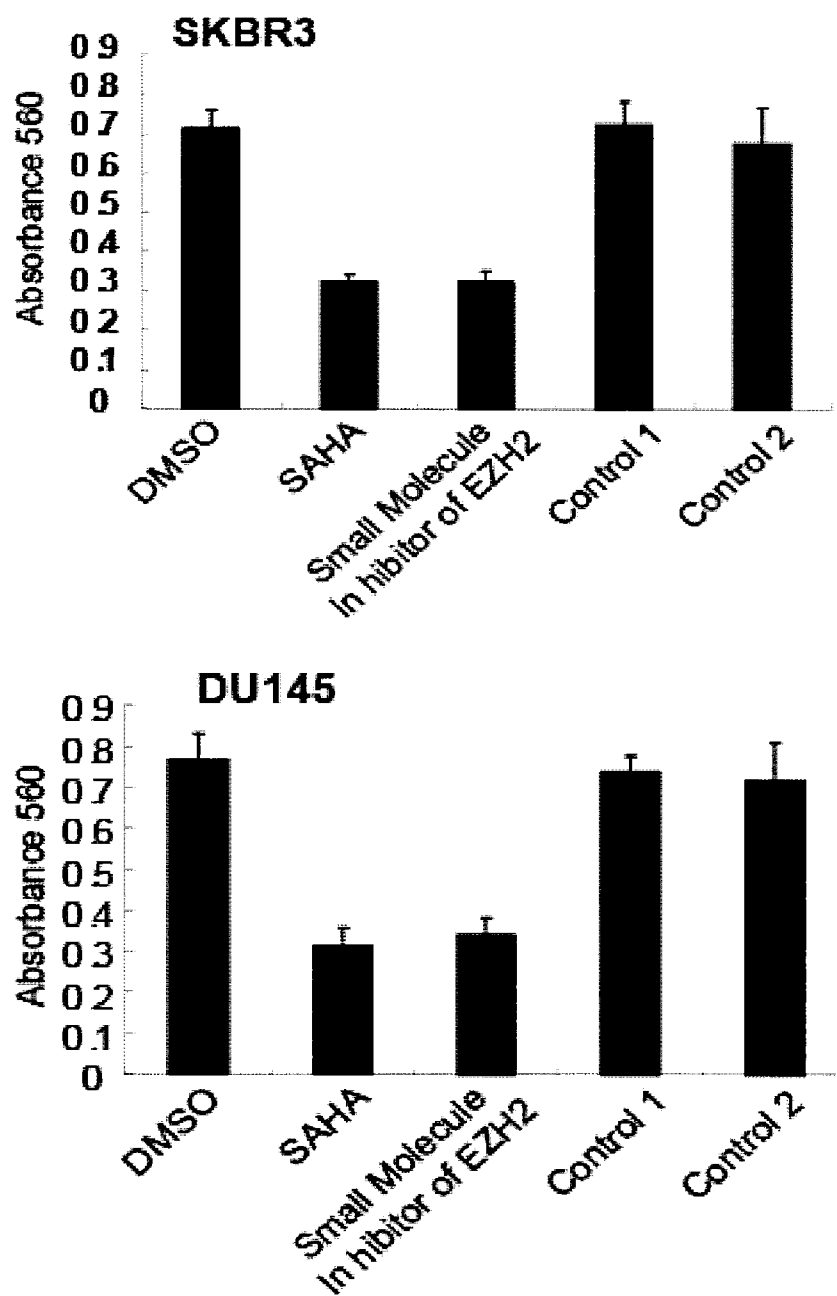
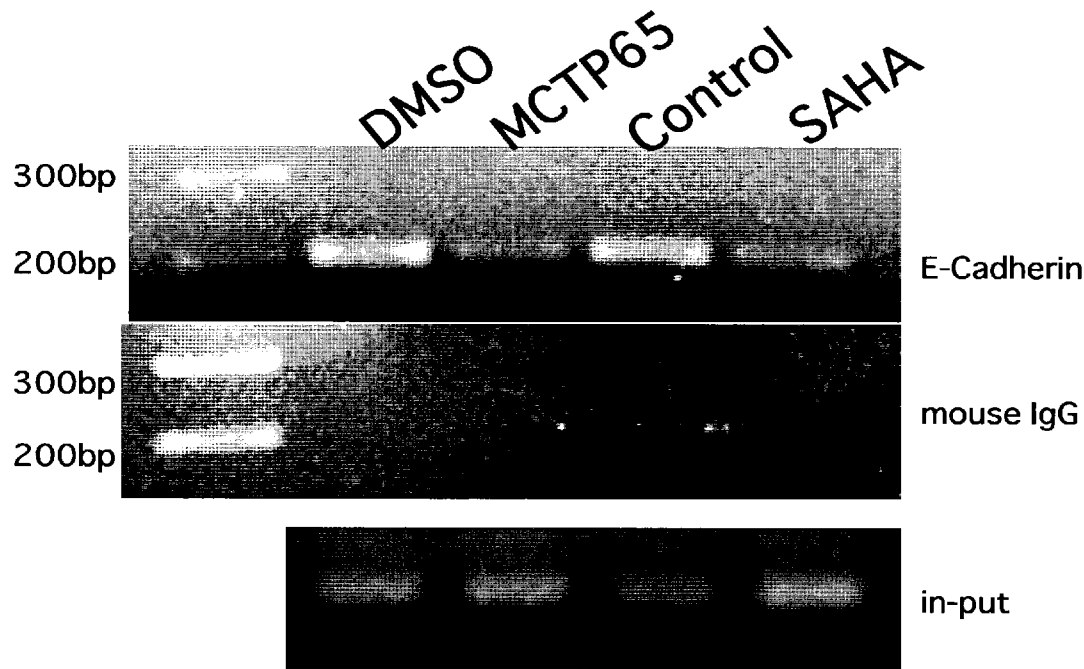


Figure 26



Chromatin IP with 3mH3K27

Figure 27

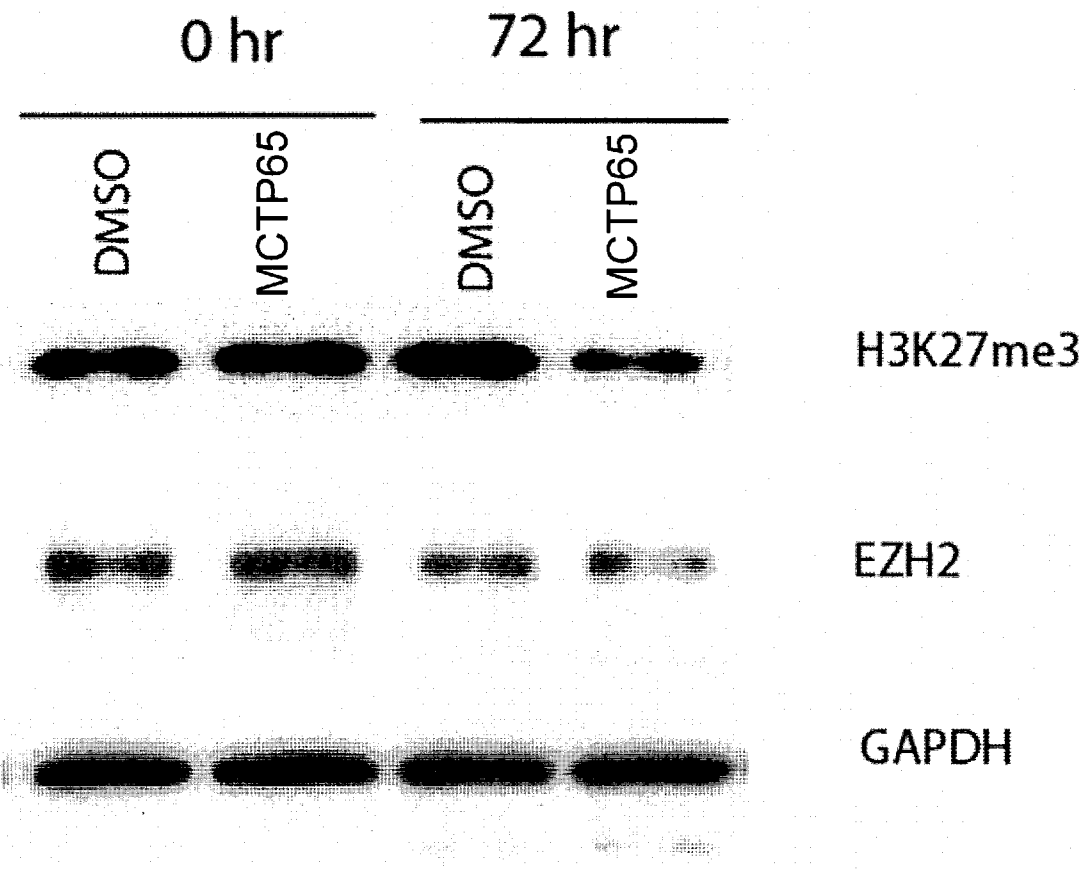


Figure 28

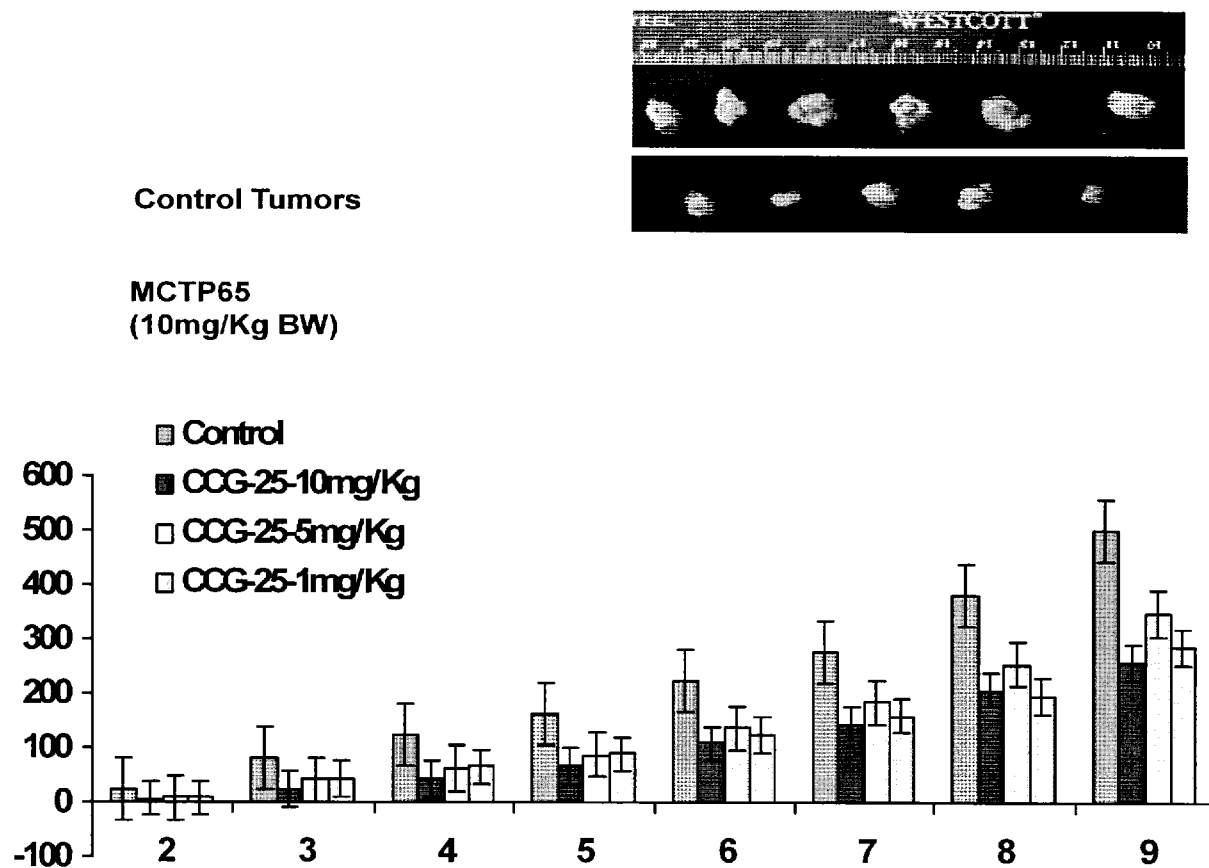


Figure 29

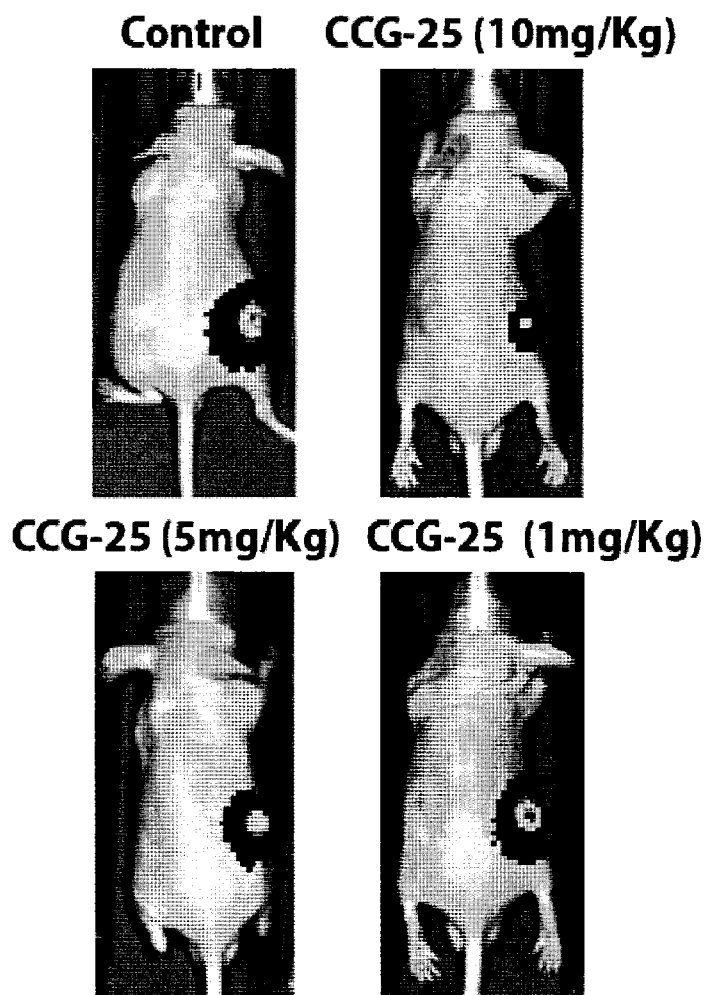
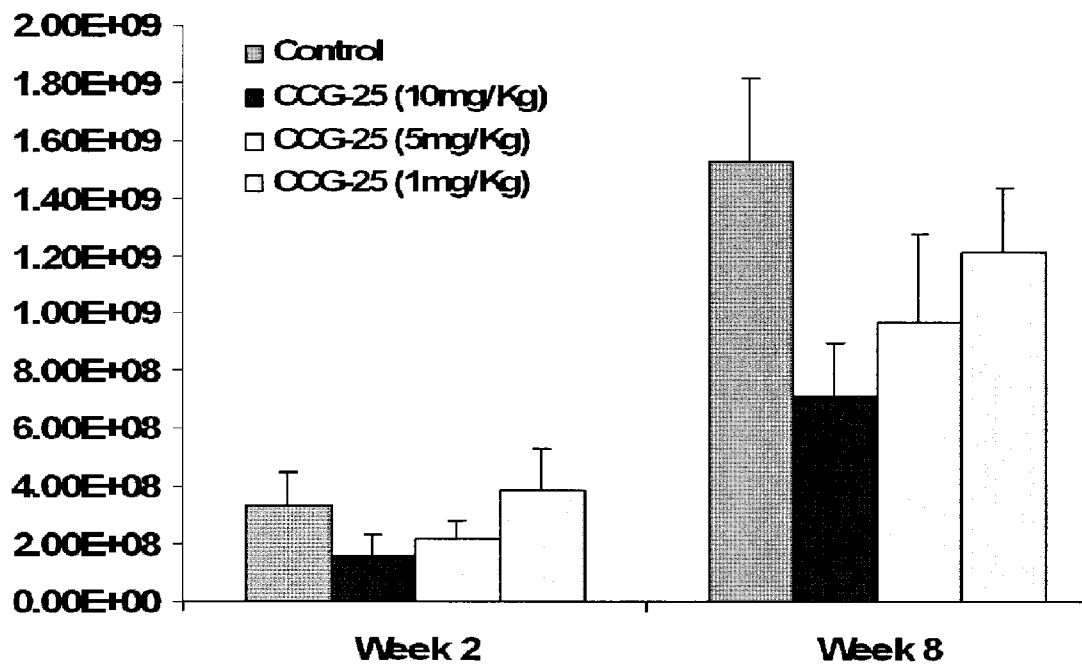
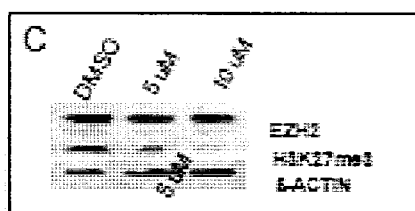
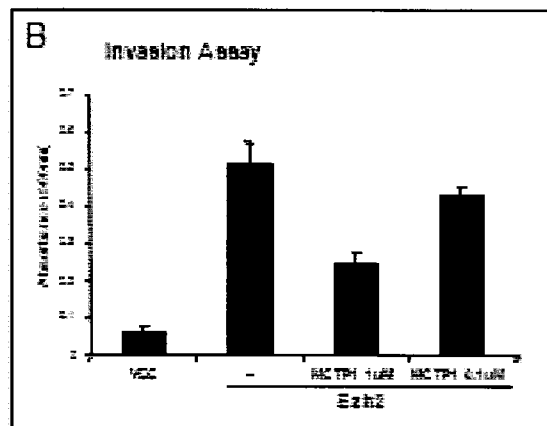
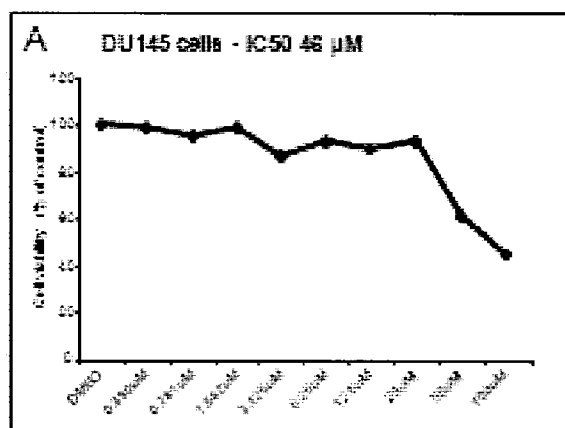
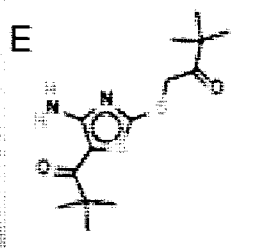


Figure 30



D

MCTP1	
Secondary Screen	+
Blocks Ezh2 mediated invasion	+
Effect on DU145 proliferation	+
Effect on Ezh2 protein level	-
Effect on H3K27me3	+



1-[[4-amino-5-(2,2-dimethylpropanoyl)-1,3-thiazol-2-yl]thio]-3,3-dimethylbutan-2-one

Figure 31

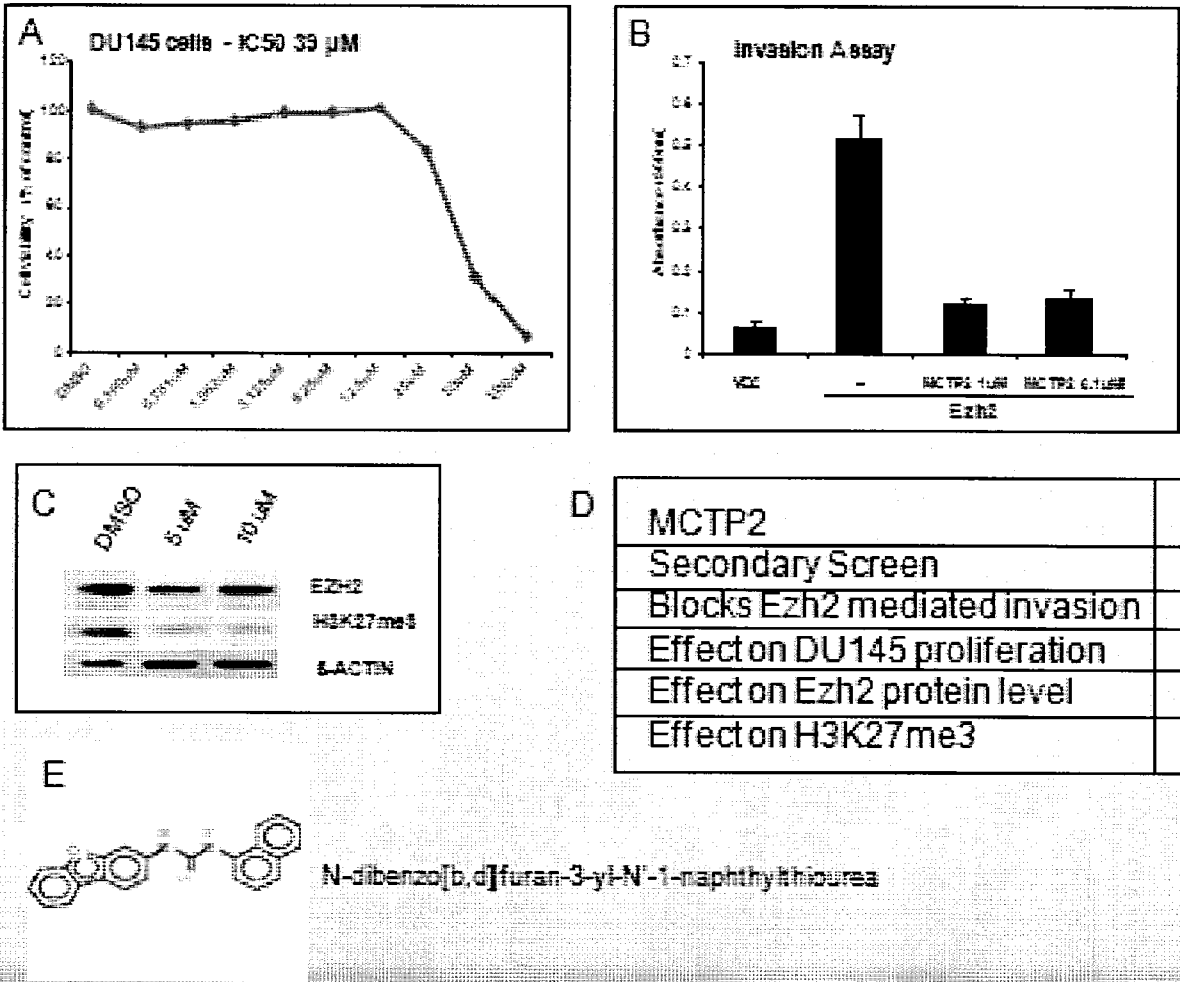


Figure 32

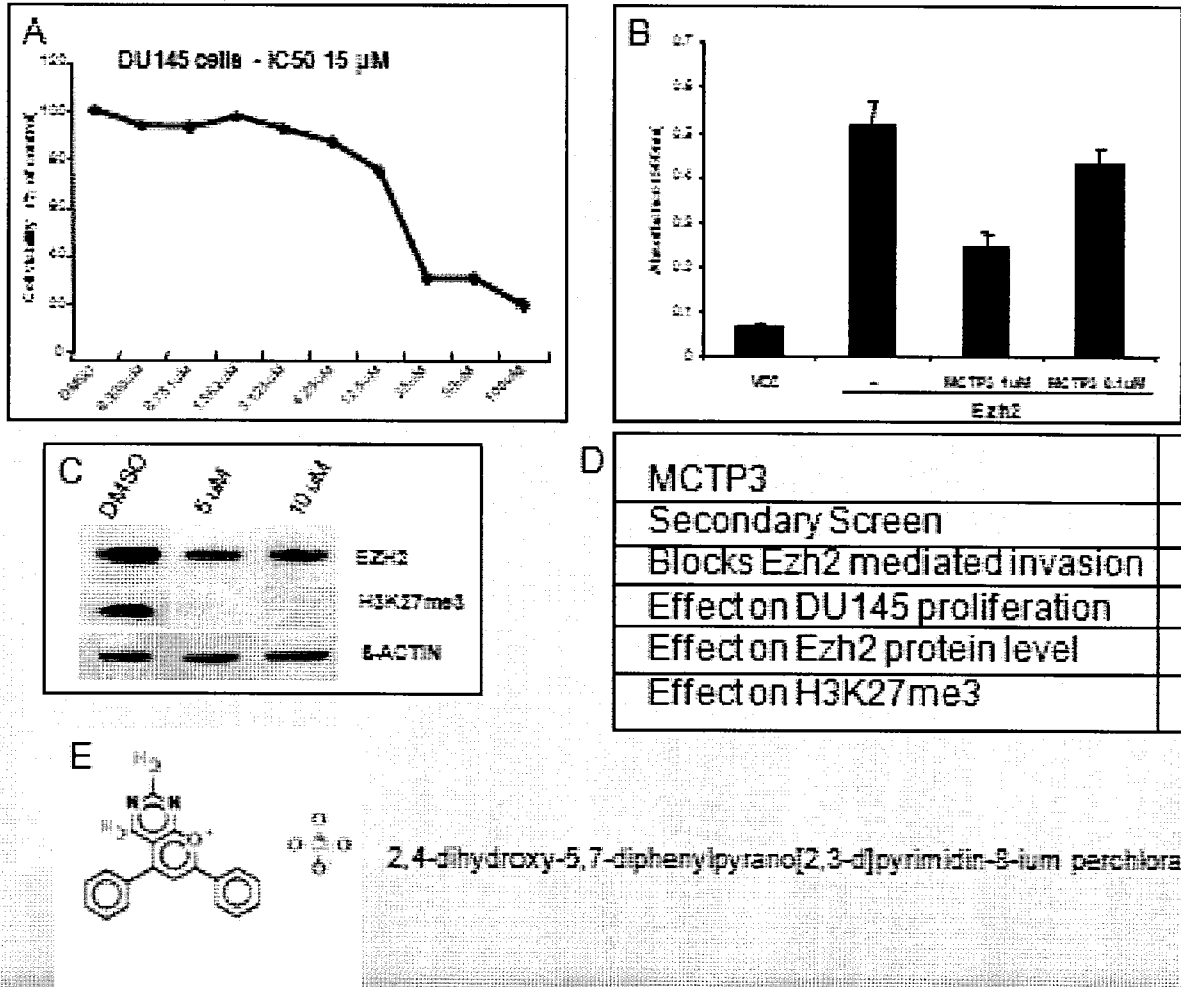


Figure 33

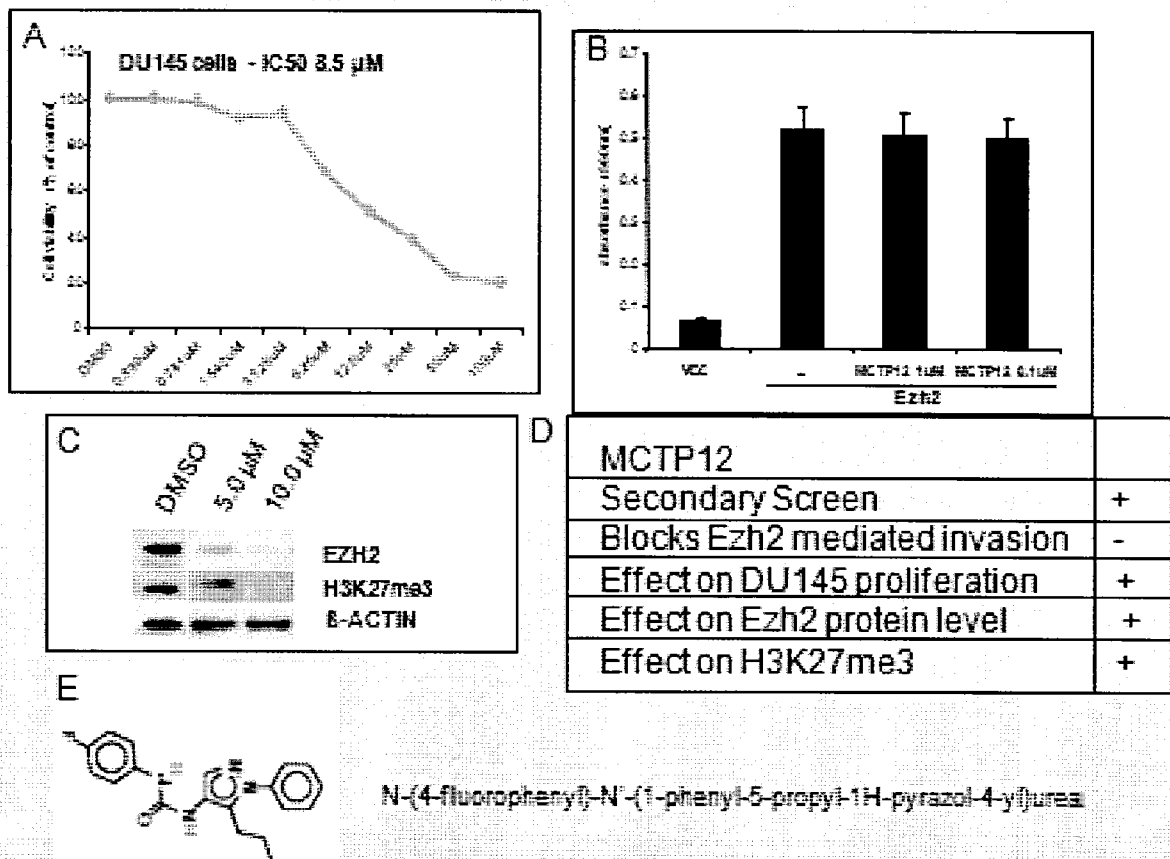


Figure 34

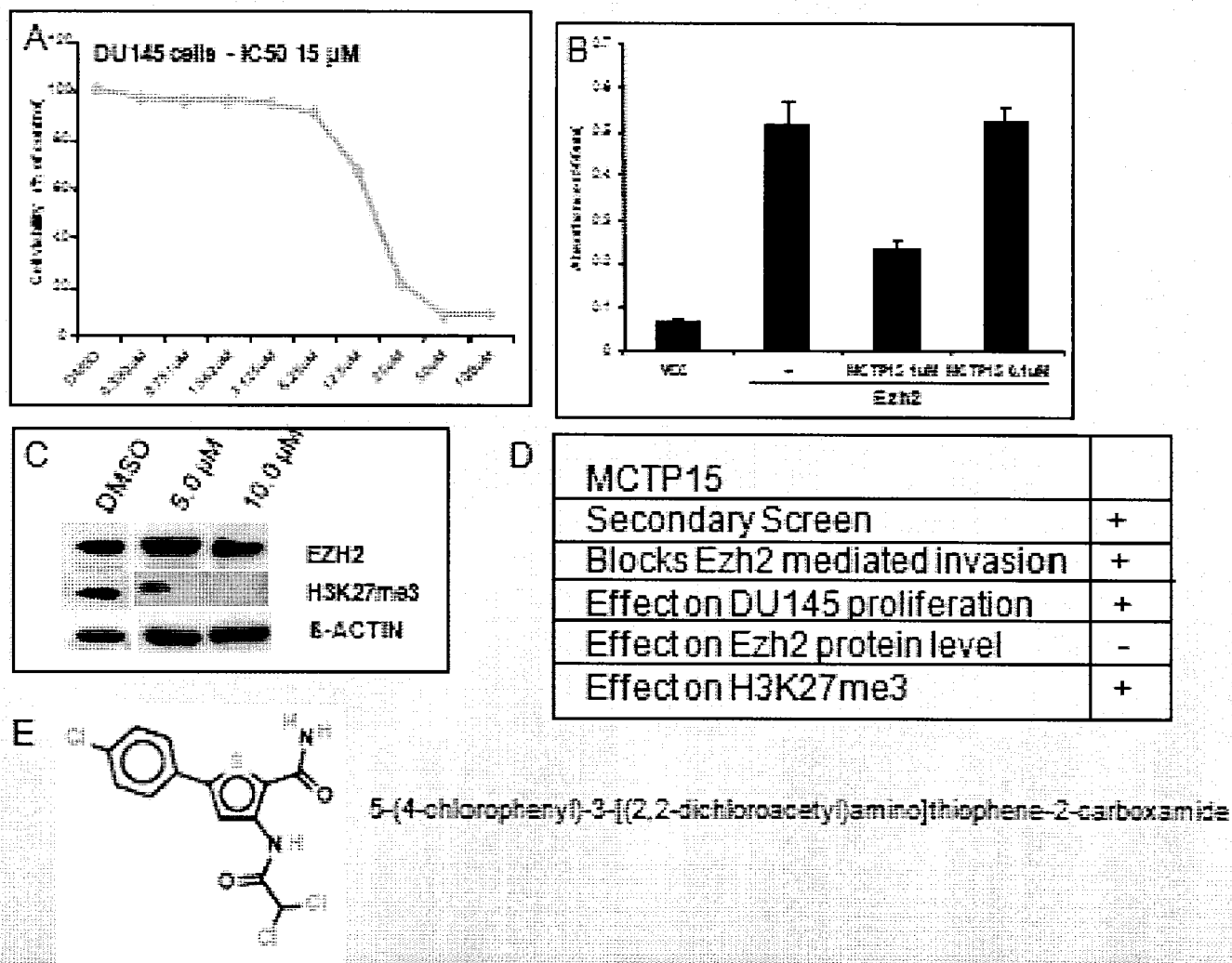


Figure 35

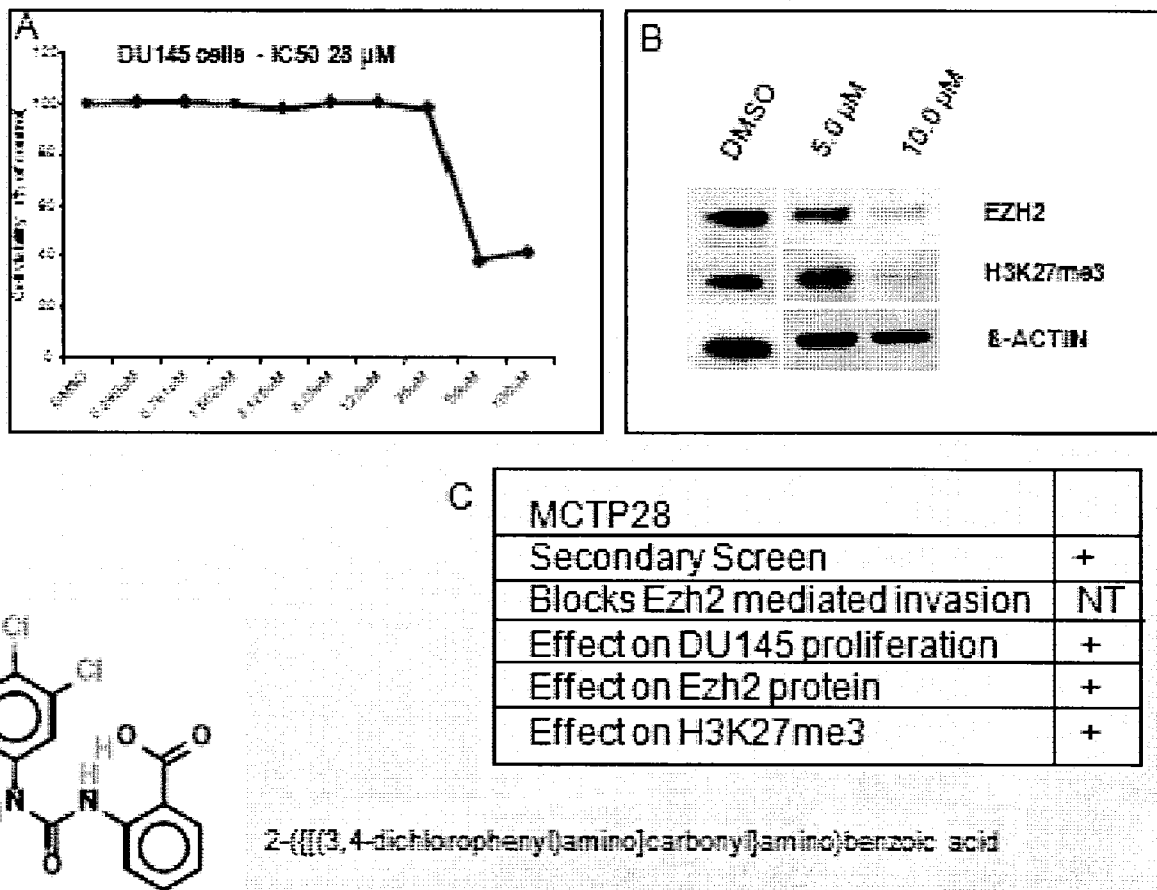
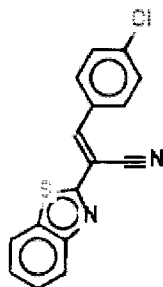


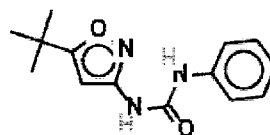
Figure 36

MCTP 11

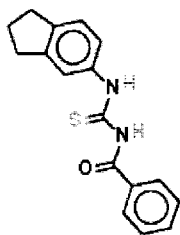
2-(1,3-benzothiazol-2-yl)-3-(4-chlorophenyl)acrylonitrile

**MCTP18**

N-[5-(tert-butyl)-3-isoxazolyl]-N'-phenylurea

**MCTP 19**

N-benzoyl-N'-(2,3-dihydro-1H-inden-5-yl)thiourea

**MCTP 20**

N,5-diphenyl-1,3,4-oxadiazole-2-carboxamide

