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(54) Title: COMPOSITIONS AND METHODS FOR REGULATING LET-7 MICRO RNA TARGETS

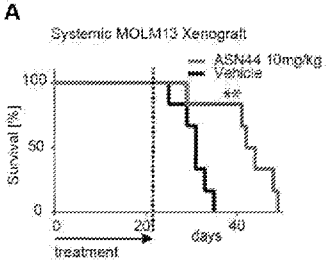


FIG. 8A

(57) Abstract: The present disclosure relates to compounds that are capable of suppressing let-7 targets, inhibiting phosphodiesterase, stimulating cAMP, and inhibiting growth activity of cancer cells. The disclosure further relates to methods of treating cancers.

COMPOSITIONS AND METHODS FOR REGULATING LET-7 MICRO RNA TARGETS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 62/566,054, filed September 29, 2017, the contents of which are hereby incorporated by reference in their entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under Grant Number GM099134, awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

There is a need for targeted therapies in the current AML treatment landscape. Current treatment strategies are dominated by generic chemotherapeutic agents, predominantly cytarabine and daunorubicin, which have been the treatment of choice for the past four decades. For patients whose leukemia cells have an FLT3 gene mutation, the drug midostaurin might be given along with chemotherapy. However, these current strategies are at best too ill-equipped to target LSCs. While chronic myeloid leukemia and even some *de novo* AML may retain a relatively clear developmental hierarchy, upon AML disease progression and/ or relapse following therapy there can be a dramatic loss of developmental structure and specific targets leading to outgrowth of LSCs. Traditional but also new therapeutic approaches for AML mainly focus on single targets which are thought to be uniquely expressed in LSCs. However, given the LSCs heterogeneity and clonal evolution a single targeted therapy might not be successful.

SUMMARY OF THE INVENTION

In certain aspects, the present disclosure provides compounds that offer a novel targeted therapeutic approach for AML. In other aspects, the present disclosure provides methods of treating disease associated with cancer and cell-cycle regulation. Compounds **44**, **61**, and **62** disclosed herein show robust upregulation of let-7 micro RNAs (miRNA) levels in various, cytogenetically different AML cell lines. The small non-coding let-7 micro RNA family is suggested to play a key role as tumor suppressor by targeting a wide variety of oncogenes such as HMGA2, cMYC and KRAS. Compound **44** offers high clinical and therapeutic significance

by restoring normal levels of the endogenous tumor suppressor miRNA let-7 ultimately leading differentiation of leukemic blast cells, inhibition of LSC growth and disease progression.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGs 1A-1E show the design of screen to identify regulators of let-7 activity. **FIG. 1A**: Schematic of the let-7 Luciferase Screen. Human liver cancer cell line (HUH) is transfected each with the let-7-Luciferase and PsiCheck2-control reporter plasmids. Each transfected line is plated on its own 384 plate and both pinned with its respective compound set. The treated cells are incubated at 37C for 48 hours. ViviRen reads out the Renilla luciferase and Cell Titer Glo is a readout of cell health. **FIG. 1B**: Schematic of the *let-7* Luciferase Assay. Psicheck2 plasmid was manipulated to contain the *let-7* seed sequence 8 times in tandem and linked to the renilla sequence. Therefore, when let-7 activity is increased, the renilla luminescence will be decreased. **FIG. 1C**: Transfection of let-7 mimics silences a variety of let-7 target genes as measured by RT-PCR. **FIG. 1D**: Example of the fidelity of the let-7 reporter construct. **FIG. 1E**: Cells transfected with the let-7 mimic showed a reduction in the readings of renilla, but the constitutive firefly luciferase was stable. **FIG. 1F**: RT-PCR for mature let-7s in response to let-7 transfection demonstrated the efficacy of the induction of let-7 levels. All RT-qPCR experiments are graphed as mean +/- s.e.m. (n=3), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FIGs. 2A-2E show a secondary screen to identify compounds that suppress *let-7* targets. **FIG. 2A**: a secondary screen using both constitutive and *let-7* sensitive reporter to identify high confidence hits. **FIG. 2B**: tertiary screening of compounds for effect on expression of HMGA2, an established *let-7* target gene by RT-PCR. **FIG. 2C**: RT-PCR for additional *let-7* target genes after treatment with compounds that came out of tertiary screen. **FIG. 2D**: RT-PCR for small RNAs to determine whether any compounds regulate *let-7* levels. **FIG. 2E**: RT-PCR for detectable mature let-7 family members in AML cell lines in response to treatment with two doses of **44**. All RT-qPCR experiments are graphed as mean +/- s.e.m. (n=3), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FIGs. 3A-3D show the identification of a compound that reliably suppresses expression of HMGA2. **FIG. 3A**: A dose curve of treatment with **44**, as measured by the expression level of HMGA2-long. **FIG. 3B**: Treating Huh7 cells with **44** at 1uM for various times showed specific activity at just 8 hours. **FIG. 3C**: Treatment with **44** for two days showed specific

suppression of HMGA2-long (PULSE), whereas two days after removal of these compounds, levels of HMGA2 returned to baseline (CHASE), indicating the effect of the compound is reversible. **FIG. 3D**: RT-PCR for let-7 target genes in response to **44**. All RT-qPCR experiments are graphed as mean +/- s.e.m. (n=3), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FIG. 4: **61** and **62** show similar effect on expression of let-7 targets such as HMGA2, NMYC and LIN28B.

FIGs. 5A-5D show that **44**, **61** and **62** are PDE inhibitors that activate CREB. **FIG. 5A**: Huh cells were treated with **44** or **61** for 48 hours and then immunostained with an antibody that recognizes the phosphorylated version of CREB, which is typically translocated to the nucleus when active. Quantification shown on the right. **FIG. 5B**: RNA-sequencing of Huh cells treated with **44** in triplicate showed strong induction of CREB target genes (FOSB, FOS, CREB5, and ATF3). **FIG. 5C**: Dose response of **44**, **61**, and **62** for an effect on *let-7* target gene, HMGA2. **FIG. 5D**: RT-PCR for direct CREB targets *FOSB* and *CFOS* shows that **44**, **61**, and **62** all stimulate CREB target gene expression. **Fig. 5E**: RT-PCR for CREB target genes in AML cell lines in response to treatment with **44**. All RT-qPCR experiments are graphed as mean +/- s.e.m. (n=3), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FIGs. 6A-6C show that extended treatment of cancer cells with let-7 inducing compounds blocks their growth. **FIG. 6A**: Equal numbers of Huh were plated across replicate wells and treated with the indicated compounds. Each day, several wells of each treatment condition were counted. Low dose of **44** slowed the growth of this cancer cell line. **FIG. 6B**: Treatment of Huh and three human squamous cell carcinoma cell lines (TSU, 22B, and 686) also showed a strong effect of **44** on cell growth at 5 days of treatment. **FIG. 6C**: Various lung, liver, and AML cancer cell lines were plated with escalating doses of **44**, and cell viability was assayed by ATP-luciferase at 48 hours . All growth proliferation assays are graphed as mean +/- SD (n=3), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FIGs. 7A-7E show that **44** induces apoptosis and AML cell differentiation via inhibition of PI3K signaling and unfolded protein response pathway. **FIG. 7A** shows fold changes of direct let-7 target genes (*HMGA1, IL6, MYC*) of genes involved in the PI3K and unfolded protein response pathway in Kasumi-1 AML cells treated with 5 μM **44** and compared to control. **FIG. 7B** shows the fold change of mRNA expression levels of direct let-7 target genes (*MYC, KRAS, HMGA1*) as well as genes involved in the PI3K and oxidative stress response pathway in primary

AML patient samples after treatment with 10 μM **44**, normalized to control, n = 3, statistics: two-tailed Student's *t*-test. **FIG. 7C** shows fold changes of let-7a-1, -b and f-1 of Kasumi-1 cells transduced with sh*C/EBPα*, sh*C/EBPε* or sh*CHOP*, normalized to sh*Control* and selected with puromycin for 3 days. Statistics: two-way Anova. **FIG. 7D** shows flow Cytometry showing % of $CD34^+$ $CD38^-$ cells (left) and mean percentage numbers of $CD34^+CD38^-$ Kasumi-1 cells 16h post treatment with 5 μM **44** or control. **FIG. 7E** shows cytotoxicity assessment via relative ATP production 12h post treatment with controls, clinically approved demethylating agent 5-Azacitidine (5-Aza, 5 μM) or **44** (5 – 1 μM). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

FIGs. 8A-8C show that **44** prolongs survival in AML Xenograft models and decreases tumor burden. **FIG. 8A** shows survival of systemic MOLM13 AML Xenograft daily injected intraperitoneally (IP) with 10 mg/kg **44** or vehicle, n = 7, statistics: log-rank test. **FIG. 8B** shows bioluminescence imaging (BLI) of NSG mice subcutaneously transplanted with Kasumi-1-Luciferase expressing AML tumors after two single intravenous (IV) injections of **44** at 10 mg/kg, n = 5, statistics: two-tailed Student's *t*-test. **FIG. 8C** shows survival of systemic MV4-11 AML Xenograft after three single IV injection of 10 mg/kg CasMa-nanoparticle formulated **44** (n = 7), CasMA-vehicle (n = 7) or vehicle (n =5). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

FIGs. 9A-9C show that **44** inhibits leukemic stem cell proliferation in vivo. **FIG. 9A** Schematic representation of NSG mice transplanted with AML patient cells treated with 10 μM **44** or control for 16 h. **FIG. 9B** Flow cytometry of hCD45+ cells (human AML) and hCD33+CD34+CD38-CD45RA+ leukemic stem cells (LSCs) in NSGs 12 weeks post transplantation of primary AML cells treated with **44** or control. **FIG. 9C** % human AML engraftment (top, overall engraftment) and % LSCs of overall engraftment (bottom, LSC engraftment) in NSG bone marrow 12 weeks post transplantation of primary AML patient cells treated with 10 μM **44** or control. Statistics: two-tailed Student's *t*-test. *** $P < 0.001$

DETAILED DESCRIPTION OF THE INVENTION

The Let-7 micro RNA family exerts its tumor suppressor and antiproliferative activities by repressing several oncogenes and by regulating key regulators of the cell cycle, cell differentiation, and apoptotic pathways (Bussing I, et al. Trends Mol Med. 2008;14(9):400-9;

Johnson SM, et al. Cell. 2005;120(5):635-47). Downregulation of Let-7 is a common phenomenon in several cancers including acute myeloid leukemia (AML), and restoration of normal Let-7 expression has been found to prevent cancer growth (Viswanathan SR, et al. Nat Genet. 2009;41(7):843-8). Cancer cells have been shown to exhibit reduced malignancy and motility when LIN28 is suppressed and *let-7* activity is elevated (Viswanathan SR, et al. Nat Genet. 2009;41(7):843-8).

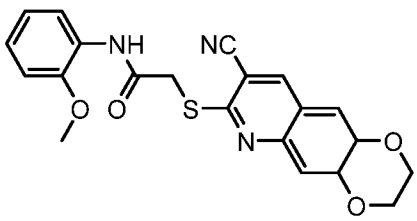
As further background, the CCAAT/enhancer binding proteins (C/EBPs) belong to a family of basic region leucine zipper (bYP) transcription factors which are involved in tissue differentiation, metabolism and immune response and play important roles in growth regulation of tissues[1,2]. In the hematopoietic system, all members of the C/EBP family (α,β,γ,δ,ε,ζ) are expressed during myeloid development[3,4]. C/EBPα, C/EBPβ and C/EBPγ are predominantly expressed in granulocytes, monocytes and eosinophils while C/EBPε is found at later stages of differentiation of granulocytes and T cells[5-7]. Thus, C/EBPs are key mediators of normal myeloid differentiation as they proper control of cell cycle progression, metabolism and differentiation. In leukemia, AML included, C/EBP expression is suppressed as a result of common leukemia-associated genetic and epigenetic alterations, such as oncogenic fusion proteins RUNX1-ETO[8], FLT3-ITD[9] or *CEBPA* promoter methylation[10]. Alterations in the CEBPα, -ε, -δ gene or in pathways that down-modulate C/EBP expression at the transcriptional, translational, or post-translational levels likely contribute to myeloid transformation by inhibiting myeloid differentiation while favoring myeloid progenitor cell cycle progression.

MicroRNAs (miRNAs) are 19-22 nucleotide (nt) short non-coding RNAs that hybridize to complementary mRNA targets and either lead to their decay, cleavage or transcriptional inhibition[11-13]. Aberrant miRNA expression has been shown to play an active role in malignant transformation including leukemia [14-16]. Systematic evaluation of the prognostic value of miRNA expression in human cancers, including several AML subtypes, found that decreased expression of let-7 miRNAs is most frequently associated with poor prognosis [16-18]. The tumor suppressor miRNA let-7 family, comprising 12 members expressed from 9 different loci (on chromosomes 3, 9, 12, 19, 21, 22 and X), represses several oncogenes including RAS, MYC, LIN28B and IL6 [19] as well as cell cycle regulators such as CyclinD and E2F [20]. At the transcriptional level, it has been reported that let-7 family members can be inhibited by oncogenic transcription factor MYC in lymphoma and hepatocellular carcinoma[21,22] and jumonji AT-rich interactive domain 1B

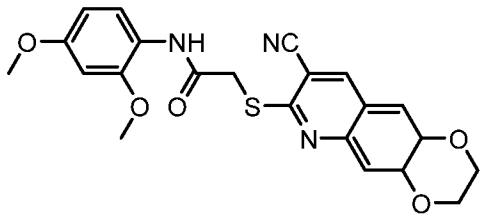
(JARID1B) proteins in breast tumor tissue[23]. More recently, two publications provided evidence that C/EBPs control expression of tumor suppressor microRNA let-7 by binding to their promoters. In lung cancer cells, it was found that C/EBPα binds to the promoter of the let-7a1, -f1 and –d cluster located on chromosome 9, and consequently promotes let-7 miRNA expression[24]. In human endocervical cells, chromatin immunoprecipitation (CHIP) showed let-7f promoter as a direct target of C/EBPβ[25]. Moreover, at the posttranscriptional level, a rush of papers described LIN28A and its homologue LIN28B as key regulators of let-7 biogenesis [26-30] through direct binding to either pre-let-7 and/or pri-let-7, thereby impairing their processing into mature, functional miRNAs. LIN28A/B is upregulated in over 15% of human cancers [31] and cancer stem cells [32-36] and is associated with poor clinical outcome [34,37-40], AML included [41]. Moreover, LIN28 [42-44] overexpression and let-7 [45-47] loss have been associated with resistance of cancer stem cells to radiation treatment and chemotherapy.

Given that C/EBPs are frequently inhibited and correlate with low let-7 microRNA transcription, it is conceivable that correction of aberrant C/EBP expression may override the activities of oncogenic fusion proteins, and promote let-7 microRNA expression at the transcriptional level which – in turn – target and downregulate a panoply of oncogenic driver genes ultimately leading to AML death.

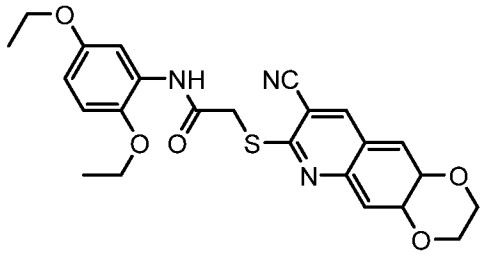
Therefore, Let-7 is a molecular marker and a potential therapeutic in cancer therapy such as AML. The present disclosure identifies three compounds that upregulate the endogenous expression of tumor suppressor micro RNAs Let-7, efficiently suppress Let-7 target genes involved in cancer pathogenesis such as HMGA2, cMYC and Lin28b (Figures 3B-3E, 4C), show strong growth inhibitory effects (Figures 6A-6C), and abolish colony forming capacity in multiple cancer cell types including Leukemic stem cells in a variety of cytogenetically different AML cell Lines.



44



61

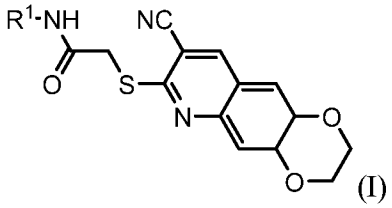


62

These compounds induce apoptosis through inhibition of phosphodiesterases (PDE) (Figure 4D) and the multi-client chaperone heat shock protein 90 (HSP90) by regulating cAMP signaling. These compounds lead to the loss of pro-survival signaling through degradation of HSP90 client proteins and inhibition of pro-survival signaling downstream of phosphodiesterase 10A (PDE10a) and CREB. These compounds affected genes related to CREB signaling (Figure 5A), particularly induction of ATF3, C-FOS, and FOSB (Figures 5B, 5D-5E).

Furthermore, these compounds upregulate let-7 microRNA levels and induce apoptosis of AML cells through inhibition of UPR and PI3K pathway. Moreover, **44** inhibits AML tumor growth in AML Xenografts, significantly prolongs animals' survival and inhibits human leukemic stem cell proliferation in vivo.

In some aspects, the present invention relates to a pharmaceutical composition comprising a compound of formula I:



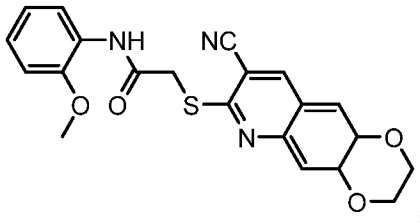
(I)

wherein R^1 is substituted or unsubstituted phenyl;

or a pharmaceutically acceptable salt thereof;

and wherein the pharmaceutical composition further comprises a pharmaceutically acceptable excipient.

In certain embodiments, R^1 is phenyl substituted with 1, 2, 3, or 4 lower alkoxy groups, such as methoxy or ethoxy. In certain embodiments, R^1 is phenyl substituted at the 2- and 4- positions with lower alkoxy groups, such as methoxy or ethoxy. In certain embodiments, R^1 is phenyl substituted at the 2-position with a lower alkoxy group, such as methoxy or ethoxy. In certain embodiments, the compound is selected from the following compounds: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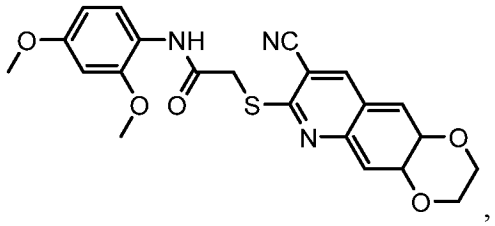
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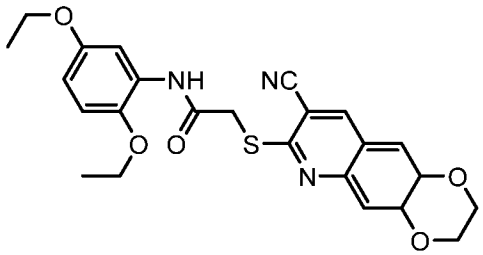
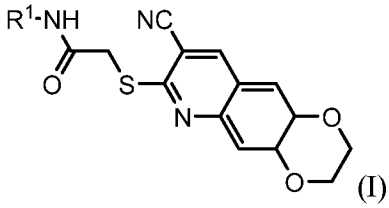


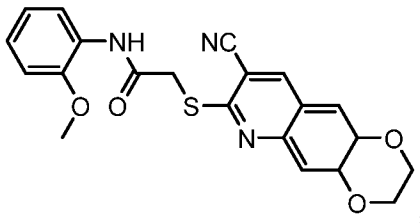
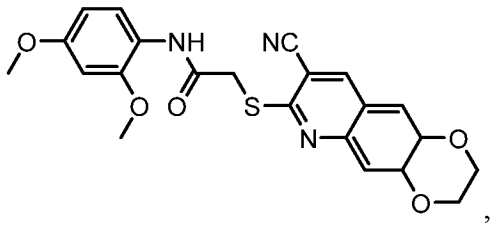
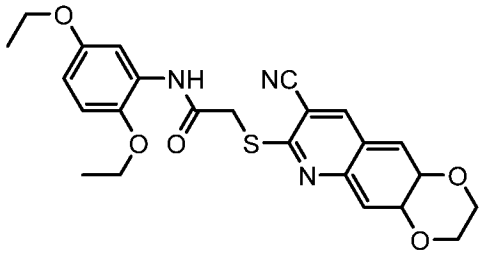
or a pharmaceutically acceptable salt thereof.

In other aspects, the present invention relates to a method of increasing let-7 micro RNA levels in a patient, comprising administering an effective amount a compound of formula I:


(I)

wherein R^1 is substituted or unsubstituted phenyl;
or a pharmaceutically acceptable salt thereof.

In certain embodiments, R^1 is phenyl substituted with 1, 2, 3, or 4 lower alkoxy groups, such as methoxy or ethoxy. In certain embodiments, R1 is phenyl substituted at the 2- and 4- positions with lower alkoxy groups, such as methoxy or ethoxy. In certain embodiments, R1 is phenyl substituted at the 2-position with a lower alkoxy group, such as methoxy or ethoxy. In certain embodiments, the compound is selected from the following compounds:

or a pharmaceutically acceptable salt thereof.

In certain embodiments, the patient has a cancer. In some embodiments, the cancer is acute myeloid leukemia, colon cancer, breast cancer, prostate cancer, lung cancer, skin cancer, liver cancer, pancreatic cancer, ovarian cancer, bladder cancer, kidney cancer, esophageal cancer, cervical cancer, endometrial cancer, melanoma, brain cancer, glioma, neuroblastoma, osteosarcoma, chondrosarcoma, gastric carcinoma, glioma, mesothelioma, Kaposi sarcoma, liposarcoma, synovial sarcoma, or Wilm's tumor. In certain embodiments, the cancer is liver cancer, skin cancer, such as squamous cell carcinoma, lung cancer, or acute myeloid leukemia.

Pharmaceutical Compositions

The compositions and methods of the present invention may be utilized to treat an individual in need thereof. In certain embodiments, the individual is a mammal such as a human, or a non-human mammal. When administered to an animal, such as a human, the composition or the compound is preferably administered as a pharmaceutical composition comprising, for example, a compound of the invention and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known in the art and include, for example, aqueous solutions such as water or physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, oils such as olive oil, or injectable organic esters. In preferred embodiments, when such pharmaceutical compositions are for human administration, particularly for invasive routes of administration (i.e., routes, such as injection or implantation, that circumvent transport or diffusion through an epithelial barrier), the aqueous solution is pyrogen-free, or substantially pyrogen-free. The excipients can be chosen, for example, to effect delayed release of an agent or

to selectively target one or more cells, tissues or organs. The pharmaceutical composition can be in dosage unit form such as tablet, capsule (including sprinkle capsule and gelatin capsule), granule, lyophile for reconstitution, powder, solution, syrup, suppository, injection or the like. The composition can also be present in a transdermal delivery system, e.g., a skin patch. The composition can also be present in a solution suitable for topical administration, such as a lotion, cream, or ointment.

A pharmaceutically acceptable carrier can contain physiologically acceptable agents that act, for example, to stabilize, increase solubility or to increase the absorption of a compound such as a compound of the invention. Such physiologically acceptable agents include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. The choice of a pharmaceutically acceptable carrier, including a physiologically acceptable agent, depends, for example, on the route of administration of the composition. The preparation or pharmaceutical composition can be a selfemulsifying drug delivery system or a selfmicroemulsifying drug delivery system. The pharmaceutical composition (preparation) also can be a liposome or other polymer matrix, which can have incorporated therein, for example, a compound of the invention. Liposomes, for example, which comprise phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl

cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

A pharmaceutical composition (preparation) can be administered to a subject by any of a number of routes of administration including, for example, orally (for example, drenches as in aqueous or non-aqueous solutions or suspensions, tablets, capsules (including sprinkle capsules and gelatin capsules), boluses, powders, granules, pastes for application to the tongue); absorption through the oral mucosa (e.g., sublingually); subcutaneously; transdermally (for example as a patch applied to the skin); and topically (for example, as a cream, ointment or spray applied to the skin). The compound may also be formulated for inhalation. In certain embodiments, a compound may be simply dissolved or suspended in sterile water. Details of appropriate routes of administration and compositions suitable for same can be found in, for example, U.S. Pat. Nos. 6,110,973, 5,763,493, 5,731,000, 5,541,231, 5,427,798, 5,358,970 and 4,172,896, as well as in patents cited therein.

The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient that can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred percent, this amount will range from about 1 percent to about ninety-nine percent of active ingredient, preferably from about 5 percent to about 70 percent, most preferably from about 10 percent to about 30 percent.

Methods of preparing these formulations or compositions include the step of bringing into association an active compound, such as a compound of the invention, with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by

uniformly and intimately bringing into association a compound of the present invention with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

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

To prepare solid dosage forms for oral administration (capsules (including sprinkle capsules and gelatin capsules), tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such a talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; (10) complexing agents, such as, modified and unmodified cyclodextrins; and (11) coloring agents. In the case of capsules (including sprinkle capsules and gelatin capsules), tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or

hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

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

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

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

Dosage forms for the topical or transdermal administration include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants that may be required.

The ointments, pastes, creams and gels may contain, in addition to an active compound, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to an active compound, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

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

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intraocular (such as intravitreal), intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion. Pharmaceutical compositions suitable for parenteral administration comprise one or more active compounds in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile

injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

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

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

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

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

For use in the methods of this invention, active compounds can be given per se or as a pharmaceutical composition containing, for example, 0.1 to 99.5% (more preferably, 0.5 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

Methods of introduction may also be provided by rechargeable or biodegradable devices. Various slow release polymeric devices have been developed and tested *in vivo* in recent years for the controlled delivery of drugs, including proteinaceous biopharmaceuticals. A variety of biocompatible polymers (including hydrogels), including both biodegradable and non-degradable polymers, can be used to form an implant for the sustained release of a compound at a particular target site.

Actual dosage levels of the active ingredients in the pharmaceutical compositions may be varied so as to obtain an amount of the active ingredient that is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

The selected dosage level will depend upon a variety of factors including the activity of the particular compound or combination of compounds employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound(s) being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compound(s) employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the therapeutically effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the pharmaceutical composition or compound at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. By "therapeutically effective amount" is meant the concentration of a compound that is sufficient to elicit the desired therapeutic effect. It is generally understood that the effective amount of the compound will vary according to the weight, sex, age, and medical history of the subject. Other factors which influence the effective amount may include, but are not limited to, the severity of the patient's condition, the disorder being treated, the stability of the compound, and, if desired, another type of therapeutic agent being administered with the compound of the invention. A larger total dose

can be delivered by multiple administrations of the agent. Methods to determine efficacy and dosage are known to those skilled in the art (Isselbacher et al. (1996) Harrison's Principles of Internal Medicine 13 ed., 1814-1882, herein incorporated by reference).

In general, a suitable daily dose of an active compound used in the compositions and methods of the invention will be that amount of the compound that is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above.

If desired, the effective daily dose of the active compound may be administered as one, two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms. In certain embodiments of the present invention, the active compound may be administered two or three times daily. In preferred embodiments, the active compound will be administered once daily.

The patient receiving this treatment is any animal in need, including primates, in particular humans; and other mammals such as equines, cattle, swine, sheep, cats, and dogs; poultry; and pets in general.

In certain embodiments, compounds of the invention may be used alone or conjointly administered with another type of therapeutic agent.

The present disclosure includes the use of pharmaceutically acceptable salts of compounds of the invention in the compositions and methods of the present invention. In certain embodiments, contemplated salts of the invention include, but are not limited to, alkyl, dialkyl, trialkyl or tetra-alkyl ammonium salts. In certain embodiments, contemplated salts of the invention include, but are not limited to, L-arginine, benenthamine, benzathine, betaine, calcium hydroxide, choline, deanol, diethanolamine, diethylamine, 2-(diethylamino)ethanol, ethanolamine, ethylenediamine, N-methylglucamine, hydrabamine, 1H-imidazole, lithium, L-lysine, magnesium, 4-(2-hydroxyethyl)morpholine, piperazine, potassium, 1-(2-hydroxyethyl)pyrrolidine, sodium, triethanolamine, tromethamine, and zinc salts. In certain embodiments, contemplated salts of the invention include, but are not limited to, Na, Ca, K, Mg, Zn or other metal salts. In certain embodiments, contemplated salts of the invention include, but are not limited to, 1-hydroxy-2-naphthoic acid, 2,2-dichloroacetic acid, 2-hydroxyethanesulfonic acid, 2-oxoglutaric acid, 4-acetamidobenzoic acid, 4-aminosalicylic acid, acetic acid, adipic acid, l-ascorbic acid, l-aspartic acid, benzenesulfonic acid, benzoic acid, (+)-camphoric acid, (+)-

camphor-10-sulfonic acid, capric acid (decanoic acid), caproic acid (hexanoic acid), caprylic acid (octanoic acid), carbonic acid, cinnamic acid, citric acid, cyclamic acid, dodecylsulfuric acid, ethane-1,2-disulfonic acid, ethanesulfonic acid, formic acid, fumaric acid, galactaric acid, gentisic acid, d-glucoheptonic acid, d-gluconic acid, d-glucuronic acid, glutamic acid, glutaric acid, glycerophosphoric acid, glycolic acid, hippuric acid, hydrobromic acid, hydrochloric acid, isobutyric acid, lactic acid, lactobionic acid, lauric acid, maleic acid, l-malic acid, malonic acid, mandelic acid, methanesulfonic acid , naphthalene-1,5-disulfonic acid, naphthalene-2-sulfonic acid, nicotinic acid, nitric acid, oleic acid, oxalic acid, palmitic acid, pamoic acid, phosphoric acid, proprionic acid, l-pyroglutamic acid, salicylic acid, sebacic acid, stearic acid, succinic acid, sulfuric acid, l-tartaric acid, thiocyanic acid, p-toluenesulfonic acid, trifluoroacetic acid, and undecylenic acid acid salts.

The pharmaceutically acceptable acid addition salts can also exist as various solvates, such as with water, methanol, ethanol, dimethylformamide, and the like. Mixtures of such solvates can also be prepared. The source of such solvate can be from the solvent of crystallization, inherent in the solvent of preparation or crystallization, or adventitious to such solvent.

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

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

Definitions

Unless otherwise defined herein, scientific and technical terms used in this application shall have the meanings that are commonly understood by those of ordinary skill in the art. Generally, nomenclature used in connection with, and techniques of, chemistry, cell and tissue culture, molecular biology, cell and cancer biology, neurobiology, neurochemistry, virology,

immunology, microbiology, pharmacology, genetics and protein and nucleic acid chemistry, described herein, are those well known and commonly used in the art.

The methods and techniques of the present disclosure are generally performed, unless otherwise indicated, according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout this specification. See, e.g. "Principles of Neural Science", McGraw-Hill Medical, New York, N.Y. (2000); Motulsky, "Intuitive Biostatistics", Oxford University Press, Inc. (1995); Lodish et al., "Molecular Cell Biology, 4th ed.", W. H. Freeman & Co., New York (2000); Griffiths et al., "Introduction to Genetic Analysis, 7th ed.", W. H. Freeman & Co., N.Y. (1999); and Gilbert et al., "Developmental Biology, 6th ed.", Sinauer Associates, Inc., Sunderland, MA (2000).

Chemistry terms used herein, unless otherwise defined herein, are used according to conventional usage in the art, as exemplified by "The McGraw-Hill Dictionary of Chemical Terms", Parker S., Ed., McGraw-Hill, San Francisco, C.A. (1985).

All of the above, and any other publications, patents and published patent applications referred to in this application are specifically incorporated by reference herein. In case of conflict, the present specification, including its specific definitions, will control.

The term "agent" is used herein to denote a chemical compound (such as an organic or inorganic compound, a mixture of chemical compounds), a biological macromolecule (such as a nucleic acid, an antibody, including parts thereof as well as humanized, chimeric and human antibodies and monoclonal antibodies, a protein or portion thereof, e.g., a peptide, a lipid, a carbohydrate), or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. Agents include, for example, agents whose structure is known, and those whose structure is not known.

A "patient," "subject," or "individual" are used interchangeably and refer to either a human or a non-human animal. These terms include mammals, such as humans, primates, livestock animals (including bovines, porcines, etc.), companion animals (e.g., canines, felines, etc.) and rodents (e.g., mice and rats).

"Treating" a condition or patient refers to taking steps to obtain beneficial or desired results, including clinical results. As used herein, and as well understood in the art, "treatment" is an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or

more symptoms or conditions, diminishment of extent of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

The term "preventing" is art-recognized, and when used in relation to a condition, such as a local recurrence (e.g., pain), a disease such as cancer, a syndrome complex such as heart failure or any other medical condition, is well understood in the art, and includes administration of a composition which reduces the frequency of, or delays the onset of, symptoms of a medical condition in a subject relative to a subject which does not receive the composition. Thus, prevention of cancer includes, for example, reducing the number of detectable cancerous growths in a population of patients receiving a prophylactic treatment relative to an untreated control population, and/or delaying the appearance of detectable cancerous growths in a treated population versus an untreated control population, e.g., by a statistically and/or clinically significant amount.

"Administering" or "administration of" a substance, a compound or an agent to a subject can be carried out using one of a variety of methods known to those skilled in the art. For example, a compound or an agent can be administered, intravenously, arterially, intradermally, intramuscularly, intraperitoneally, subcutaneously, ocularly, sublingually, orally (by ingestion), intranasally (by inhalation), intraspinally, intracerebrally, and transdermally (by absorption, e.g., through a skin duct). A compound or agent can also appropriately be introduced by rechargeable or biodegradable polymeric devices or other devices, e.g., patches and pumps, or formulations, which provide for the extended, slow or controlled release of the compound or agent. Administering can also be performed, for example, once, a plurality of times, and/or over one or more extended periods.

Appropriate methods of administering a substance, a compound or an agent to a subject will also depend, for example, on the age and/or the physical condition of the subject and the chemical and biological properties of the compound or agent (e.g., solubility, digestibility, bioavailability, stability and toxicity). In some embodiments, a compound or an agent is administered orally, e.g., to a subject by ingestion. In some embodiments, the orally

administered compound or agent is in an extended release or slow release formulation, or administered using a device for such slow or extended release.

As used herein, the phrase "conjoint administration" refers to any form of administration of two or more different therapeutic agents such that the second agent is administered while the previously administered therapeutic agent is still effective in the body (e.g., the two agents are simultaneously effective in the patient, which may include synergistic effects of the two agents). For example, the different therapeutic compounds can be administered either in the same formulation or in separate formulations, either concomitantly or sequentially. Thus, an individual who receives such treatment can benefit from a combined effect of different therapeutic agents.

A "therapeutically effective amount" or a "therapeutically effective dose" of a drug or agent is an amount of a drug or an agent that, when administered to a subject will have the intended therapeutic effect. The full therapeutic effect does not necessarily occur by administration of one dose, and may occur only after administration of a series of doses. Thus, a therapeutically effective amount may be administered in one or more administrations. The precise effective amount needed for a subject will depend upon, for example, the subject's size, health and age, and the nature and extent of the condition being treated, such as cancer or MDS. The skilled worker can readily determine the effective amount for a given situation by routine experimentation.

The term "acyl" is art-recognized and refers to a group represented by the general formula hydrocarbylC(O)-, preferably alkylC(O)-.

The term "acylamino" is art-recognized and refers to an amino group substituted with an acyl group and may be represented, for example, by the formula hydrocarbylC(O)NH-.

The term "acyloxy" is art-recognized and refers to a group represented by the general formula hydrocarbylC(O)O-, preferably alkylC(O)O-.

The term "alkoxy" refers to an alkyl group having an oxygen attached thereto. Representative alkoxy groups include methoxy, ethoxy, propoxy, tert-butoxy and the like.

The term "alkoxyalkyl" refers to an alkyl group substituted with an alkoxy group and may be represented by the general formula alkyl-O-alkyl.

The term "alkyl" refers to saturated aliphatic groups, including straight-chain alkyl groups, branched-chain alkyl groups, cycloalkyl (alicyclic) groups, alkyl-substituted cycloalkyl

groups, and cycloalkyl-substituted alkyl groups. In preferred embodiments, a straight chain or branched chain alkyl has 30 or fewer carbon atoms in its backbone (e.g., C_{1-30} for straight chains, C_{3-30} for branched chains), and more preferably 20 or fewer.

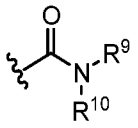
Moreover, the term "alkyl" as used throughout the specification, examples, and claims is intended to include both unsubstituted and substituted alkyl groups, the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone, including haloalkyl groups such as trifluoromethyl and 2,2,2-trifluoroethyl, etc.

The term "C_{x-y}" or "C_x-C_y", when used in conjunction with a chemical moiety, such as, acyl, acyloxy, alkyl, alkenyl, alkynyl, or alkoxy is meant to include groups that contain from x to y carbons in the chain. C_0alkyl indicates a hydrogen where the group is in a terminal position, a bond if internal. A C_{1-6}alkyl group, for example, contains from one to six carbon atoms in the chain.

The term "alkylamino", as used herein, refers to an amino group substituted with at least one alkyl group.

The term "alkylthio", as used herein, refers to a thiol group substituted with an alkyl group and may be represented by the general formula alkylS-.

The term "amide", as used herein, refers to a group



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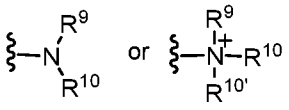
wherein R^9 and R^{10} each independently represent a hydrogen or hydrocarbyl group, or R^9 and R^{10} taken together with the N atom to which they are attached complete a heterocycle having from 4 to 8 atoms in the ring structure.

The terms "amine" and "amino" are art-recognized and refer to both unsubstituted and substituted amines and salts thereof, e.g., a moiety that can be represented by



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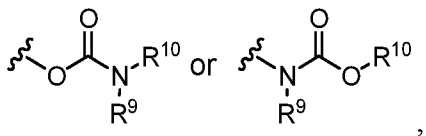
wherein R^9, R^{10}, and $R^{10'}$ each independently represent a hydrogen or a hydrocarbyl group, or R^9 and R^{10} taken together with the N atom to which they are attached complete a heterocycle having from 4 to 8 atoms in the ring structure.

The term "aminoalkyl", as used herein, refers to an alkyl group substituted with an amino group.

The term "aralkyl", as used herein, refers to an alkyl group substituted with an aryl group.

The term "aryl" as used herein include substituted or unsubstituted single-ring aromatic groups in which each atom of the ring is carbon. Preferably the ring is a 5- to 7-membered ring, more preferably a 6-membered ring. The term "aryl" also includes polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings wherein at least one of the rings is aromatic, e.g., the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryls, and/or heterocyclyls. Aryl groups include benzene, naphthalene, phenanthrene, phenol, aniline, and the like.

The term "carbamate" is art-recognized and refers to a group



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wherein R^9 and R^{10} independently represent hydrogen or a hydrocarbyl group.

The term "carbocyclylalkyl", as used herein, refers to an alkyl group substituted with a carbocycle group.

The terms "carbocycle", "carbocyclyl", and "carbocyclic", as used herein, refers to a non-aromatic saturated or unsaturated ring in which each atom of the ring is carbon. Preferably a carbocycle ring contains from 3 to 10 atoms, more preferably from 5 to 7 atoms.

The term "carbocyclylalkyl", as used herein, refers to an alkyl group substituted with a carbocycle group.

The term "carbonate" is art-recognized and refers to a group $-OCO_2-$.

The term "carboxy", as used herein, refers to a group represented by the formula $-CO_2H$.

The term "ester", as used herein, refers to a group $-C(O)OR^9$ wherein R^9 represents a hydrocarbyl group.

The term "ether", as used herein, refers to a hydrocarbyl group linked through an oxygen to another hydrocarbyl group. Accordingly, an ether substituent of a hydrocarbyl group may be hydrocarbyl-O-. Ethers may be either symmetrical or unsymmetrical. Examples of ethers include, but are not limited to, heterocycle-O-heterocycle and aryl-O-heterocycle. Ethers include "alkoxyalkyl" groups, which may be represented by the general formula alkyl-O-alkyl.

The terms "halo" and "halogen" as used herein means halogen and includes chloro, fluoro, bromo, and iodo.

The terms "hetaralkyl" and "heteroaralkyl", as used herein, refers to an alkyl group substituted with a hetaryl group.

The terms "heteroaryl" and "hetaryl" include substituted or unsubstituted aromatic single ring structures, preferably 5- to 7-membered rings, more preferably 5- to 6-membered rings, whose ring structures include at least one heteroatom, preferably one to four heteroatoms, more preferably one or two heteroatoms. The terms "heteroaryl" and "hetaryl" also include polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings wherein at least one of the rings is heteroaromatic, e.g., the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryls, and/or heterocyclyls. Heteroaryl groups include, for example, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, pyrazole, pyridine, pyrazine, pyridazine, and pyrimidine, and the like.

The term "heteroatom" as used herein means an atom of any element other than carbon or hydrogen. Preferred heteroatoms are nitrogen, oxygen, and sulfur.

The term "heterocyclylalkyl", as used herein, refers to an alkyl group substituted with a heterocycle group.

The terms "heterocyclyl", "heterocycle", and "heterocyclic" refer to substituted or unsubstituted non-aromatic ring structures, preferably 3- to 10-membered rings, more preferably 3- to 7-membered rings, whose ring structures include at least one heteroatom, preferably one to four heteroatoms, more preferably one or two heteroatoms. The terms "heterocyclyl" and "heterocyclic" also include polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings wherein at least one of the rings is heterocyclic, e.g., the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryls, and/or heterocyclyls. Heterocyclyl groups include, for example, piperidine, piperazine, pyrrolidine, morpholine, lactones, lactams, and the like.

The term "hydrocarbyl", as used herein, refers to a group that is bonded through a carbon atom that does not have a =O or =S substituent, and typically has at least one carbon-hydrogen bond and a primarily carbon backbone, but may optionally include heteroatoms. Thus, groups like methyl, ethoxyethyl, 2-pyridyl, and even trifluoromethyl are considered to be hydrocarbyl for the purposes of this application, but substituents such as acetyl (which has a =O substituent

on the linking carbon) and ethoxy (which is linked through oxygen, not carbon) are not. Hydrocarbyl groups include, but are not limited to aryl, heteroaryl, carbocycle, heterocycle, alkyl, alkenyl, alkynyl, and combinations thereof.

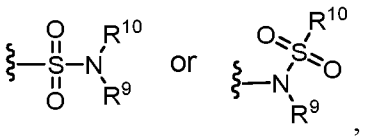
The term "hydroxyalkyl", as used herein, refers to an alkyl group substituted with a hydroxy group.

The term "lower" when used in conjunction with a chemical moiety, such as, acyl, acyloxy, alkyl, alkenyl, alkynyl, or alkoxy is meant to include groups where there are ten or fewer atoms in the substituent, preferably six or fewer. A "lower alkyl", for example, refers to an alkyl group that contains ten or fewer carbon atoms, preferably six or fewer. In certain embodiments, acyl, acyloxy, alkyl, alkenyl, alkynyl, or alkoxy substituents defined herein are respectively lower acyl, lower acyloxy, lower alkyl, lower alkenyl, lower alkynyl, or lower alkoxy, whether they appear alone or in combination with other substituents, such as in the recitations hydroxyalkyl and aralkyl (in which case, for example, the atoms within the aryl group are not counted when counting the carbon atoms in the alkyl substituent).

The terms "polycyclyl", "polycycle", and "polycyclic" refer to two or more rings (e.g., cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryls, and/or heterocyclyls) in which two or more atoms are common to two adjoining rings, e.g., the rings are "fused rings". Each of the rings of the polycycle can be substituted or unsubstituted. In certain embodiments, each ring of the polycycle contains from 3 to 10 atoms in the ring, preferably from 5 to 7.

The term "sulfate" is art-recognized and refers to the group $-OSO_3H$, or a pharmaceutically acceptable salt thereof.

The term "sulfonamide" is art-recognized and refers to the group represented by the general formulae



wherein R^9 and R^{10} independently represents hydrogen or hydrocarbyl.

The term "sulfoxide" is art-recognized and refers to the group–S(O)-.

The term "sulfonate" is art-recognized and refers to the group SO_3H, or a pharmaceutically acceptable salt thereof.

The term "sulfone" is art-recognized and refers to the group $-S(O)_2-$.

The term "substituted" refers to moieties having substituents replacing a hydrogen on one or more carbons of the backbone. It will be understood that "substitution" or "substituted with" includes the implicit proviso that such substitution is in accordance with permitted valence of the substituted atom and the substituent, and that the substitution results in a stable compound, e.g., which does not spontaneously undergo transformation such as by rearrangement, cyclization, elimination, etc. As used herein, the term "substituted" is contemplated to include all permissible substituents of organic compounds. In a broad aspect, the permissible substituents include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and non-aromatic substituents of organic compounds. The permissible substituents can be one or more and the same or different for appropriate organic compounds. For purposes of this invention, the heteroatoms such as nitrogen may have hydrogen substituents and/or any permissible substituents of organic compounds described herein which satisfy the valences of the heteroatoms. Substituents can include any substituents described herein, for example, a halogen, a hydroxyl, a carbonyl (such as a carboxyl, an alkoxycarbonyl, a formyl, or an acyl), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an alkoxyl, a phosphoryl, a phosphate, a phosphonate, a phosphinate, an amino, an amido, an amidine, an imine, a cyano, a nitro, an azido, a sulfhydryl, an alkylthio, a sulfate, a sulfonate, a sulfamoyl, a sulfonamido, a sulfonyl, a heterocyclyl, an aralkyl, or an aromatic or heteroaromatic moiety. It will be understood by those skilled in the art that the moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate.

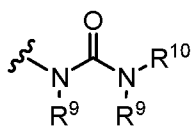
The term "thioalkyl", as used herein, refers to an alkyl group substituted with a thiol group.

The term "thioester", as used herein, refers to a group $-C(O)SR^9$ or $-SC(O)R^9$

wherein R^9 represents a hydrocarbyl.

The term "thioether", as used herein, is equivalent to an ether, wherein the oxygen is replaced with a sulfur.

The term "urea" is art-recognized and may be represented by the general formula



,

wherein R^9 and R^{10} independently represent hydrogen or a hydrocarbyl.

The term "modulate" as used herein includes the inhibition or suppression of a function or activity (such as cell proliferation) as well as the enhancement of a function or activity.

The phrase "pharmaceutically acceptable" is art-recognized. In certain embodiments, the term includes compositions, excipients, adjuvants, polymers and other materials and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

"Pharmaceutically acceptable salt" or "salt" is used herein to refer to an acid addition salt or a basic addition salt which is suitable for or compatible with the treatment of patients.

The term "pharmaceutically acceptable acid addition salt" as used herein means any non-toxic organic or inorganic salt of any base compounds represented by Formula I. Illustrative inorganic acids which form suitable salts include hydrochloric, hydrobromic, sulfuric and phosphoric acids, as well as metal salts such as sodium monohydrogen orthophosphate and potassium hydrogen sulfate. Illustrative organic acids that form suitable salts include mono-, di-, and tricarboxylic acids such as glycolic, lactic, pyruvic, malonic, succinic, glutaric, fumaric, malic, tartaric, citric, ascorbic, maleic, benzoic, phenylacetic, cinnamic and salicylic acids, as well as sulfonic acids such as p-toluene sulfonic and methanesulfonic acids. Either the mono or di-acid salts can be formed, and such salts may exist in either a hydrated, solvated or substantially anhydrous form. In general, the acid addition salts of compounds of Formula I are more soluble in water and various hydrophilic organic solvents, and generally demonstrate higher melting points in comparison to their free base forms. The selection of the appropriate salt will be known to one skilled in the art. Other non-pharmaceutically acceptable salts, e.g., oxalates, may be used, for example, in the isolation of compounds of Formula I for laboratory use, or for subsequent conversion to a pharmaceutically acceptable acid addition salt.

The term "pharmaceutically acceptable basic addition salt" as used herein means any non-toxic organic or inorganic base addition salt of any acid compounds represented by Formula I or any of their intermediates. Illustrative inorganic bases which form suitable salts include lithium, sodium, potassium, calcium, magnesium, or barium hydroxide. Illustrative organic bases which form suitable salts include aliphatic, alicyclic, or aromatic organic amines such as methylamine, trimethylamine and picoline or ammonia. The selection of the appropriate salt will be known to a person skilled in the art.

Many of the compounds useful in the methods and compositions of this disclosure have at least one stereogenic center in their structure. This stereogenic center may be present in a R or a S configuration, said R and S notation is used in correspondence with the rules described in Pure Appl. Chem. (1976), 45, 11-30. The disclosure contemplates all stereoisomeric forms such as enantiomeric and diastereoisomeric forms of the compounds, salts, prodrugs or mixtures thereof (including all possible mixtures of stereoisomers). See, e.g., WO 01/062726.

Furthermore, certain compounds which contain alkenyl groups may exist as Z (zusammen) or E (entgegen) isomers. In each instance, the disclosure includes both mixture and separate individual isomers.

Some of the compounds may also exist in tautomeric forms. Such forms, although not explicitly indicated in the formulae described herein, are intended to be included within the scope of the present disclosure.

"Prodrug" or "pharmaceutically acceptable prodrug" refers to a compound that is metabolized, for example hydrolyzed or oxidized, in the host after administration to form the compound of the present disclosure (e.g., compounds of formula I). Typical examples of prodrugs include compounds that have biologically labile or cleavable (protecting) groups on a functional moiety of the active compound. Prodrugs include compounds that can be oxidized, reduced, aminated, deaminated, hydroxylated, dehydroxylated, hydrolyzed, dehydrolyzed, alkylated, dealkylated, acylated, deacylated, phosphorylated, or dephosphorylated to produce the active compound. Examples of prodrugs using ester or phosphoramidate as biologically labile or cleavable (protecting) groups are disclosed in U.S. Patents 6,875,751, 7,585,851, and 7,964,580, the disclosures of which are incorporated herein by reference. The prodrugs of this disclosure are metabolized to produce a compound of Formula I. The present disclosure includes within its scope, prodrugs of the compounds described herein. Conventional procedures for the selection and preparation of suitable prodrugs are described, for example, in "Design of Prodrugs" Ed. H. Bundgaard, Elsevier, 1985.

The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filter, diluent, excipient, solvent or encapsulating material useful for formulating a drug for medicinal or therapeutic use.

The term "Log of solubility", "LogS" or "logS" as used herein is used in the art to quantify the aqueous solubility of a compound. The aqueous solubility of a compound significantly affects its absorption and distribution characteristics. A low solubility often goes along with a poor absorption. LogS value is a unit stripped logarithm (base 10) of the solubility measured in mol/liter.

EXAMPLES

The invention now being generally described, it will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example 1: Identification of Let-7 modulators

A luciferase assay was conducted on screening compounds to determine their effectiveness in activating Let-7 miRNA against Psi-let-7-transfected Huh7 cells, and Psi-check2-transfected cells (control) (figures 1A-1B, 1D-1E, 2A). Let-7 activity was also confirmed via measuring a variety of Let-7 target genes by RT-PCR (Figures 1C, 1F, 2B-2E). Change in Let-7 activity by disclosed compounds were confirmed in the RT-PCR (Figures 3B-3E, 4C) with a dose response (Figure 5C)

Generation of a Huh7 cell line stably expressing the let-7 activity reporter

let-7 activity can be precisely assayed using a luciferase-based method (PSI-Check2 *let-7* 8X, Fig 1A). In short, the Renilla luciferase is flanked by 8 repeats of *let-7* target sequence and its mRNA will be subject to a higher rate of degradation in the presence of a higher *let-7* activity. The control Firefly luciferase was driven by a constitutive promoter (Fig 1A). A handful of breast cancer and hepatocarcinoma cell lines (MCF7, MCF15, Huh7 and Huh7.5.1) were anylyzed to assay the detectable *let-7* activity. In Human Hepatocarcinoma (Huh), a high level of LIN28B expression was observed at both the RNA and protein level; and as a result, a low level of *let-7* activity, as shown by *let-7*-luc luciferase assay. In addition, it was found that the Huh cell line expressed a number of *let-7* targets that could be tightly regulated by changes in *let-7* levels (Fig 1C).

To facilitate reproducible results in both screening and validation assays, a cell line with stable integration of the *let-7* reporter construct was created. A Neomycin resistance cassette

was cloned into the PSI-Check2 *let-7*-luciferase, and then the reporter plasmid was stably introduced into the Huh7.5.1 cell line and selected with G418 for 3 weeks (Fig 1B). The stable cell line was subjected to dual-glo luciferase assay, where it displayed a stable luciferase unit per cell in both Firefly and Renilla (Fig 1D). To demonstrate the dynamic range of detection in *let-7* activity, this Huh7.5.1 *let-7* luciferase reporter line (Huh7.5.1 L7L) was transfected with siRNA against LIN28B, as well as *let-7* mimics (Fig 1D). siRNA effectively reduced LIN28B expression by at least 90%. In response to the downregulation of LIN28B, mature microRNA levels rose about 2 to 3 fold for all *let-7* family members. As a result, the *let-7* activity was reduced by 25-50%, as assayed by dual-glo luciferase. In addition, transfection of mimics of let-7s was used to determine how sensitive the reporter was to changes in let-7 levels (Fig 1E and F). This demonstrated that strong induction of let-7 levels by direct transfection was able to effectively silence the reporter (Fig 1E).

High Throughput Screening of Small Molecules

The initial screens with the *let-7* reporter stably introduced into Huh cells generated significant numbers of false positives in both directions. As expected, many of the false positive appeared to target luciferase enzymes, and not *let-7* activity. As an alternative method designed to minimize the identification of molecules that target luciferase, replicate wells were transiently transfected with a PSI-Check2 plasmid that either contained the *let-7* seed sequence or a clean version that should not be regulated by *let-7*. The signal change in the screen was then quantified as a function of the effect on the luciferase without *let-7* sites, and as a function of internal controls on each reporter consisting of alternate luciferase gene (firefly) driven by a constitutive promoter. As a result, it was possible to screen for molecules that affected *let-7* activity directly, after controlling for both luciferase and transfection efficiency (Fig 1A). The assay protocol was also validated based on its performance for high throughput screening (HTS) suitability. For the optimized HTS reporter screen a Z'-factor of 0.65 was derived which is indicative of a reliable assay activity.

Screening using expression of let-7 target genes

To identify candidate regulators of *let-7* activity from the screen more directly, a tertiary screen was performed that measured levels of the *let-7* target HMGA2. We chose this gene because it is expressed in several different isoforms, only one of which has more than one *let-7* target site in its 3' UTR. By quantifying the relative expression of the HMGA2 isoform with

many *let-7* sites versus all HMGA2 isoforms, specific activation of *let-7* activity could be identified without the use of an exogenous reporter. 60 candidates from the original screen were assayed in this way (Fig 2). With this screening approach, we identified compounds able to directly affect expression levels of the long form of HMGA2 (Fig 2B). This led to the identification of a compound we labeled **44**. RT-PCR was used to test whether IMP3 (IGF2BP3), PLAG2, LIN28B or MYC were affected by treatment of HUH7 cells with **44** (Fig 2C). In Huh7 cells, **44** appeared to suppress the expression of HMGA2, N-MYC, and IMP2, while LIN28B and PLAG2 did not seem to change significantly (Fig 2C). The fact that 3 out of 5 let-7 targets were suppressed by **44** could suggest that *let-7* activity is induced in these cells, and let-7 levels are altered depending on their endogenous expression levels. As let-7 miRNAs are highly expressed in Huh7 cells, endogenous changes of mature let-7miRNA levels are difficult to detect. Perhaps consistent with this notion, treatment of cells with **44** did not have a strong impact on the level of mature *let-7*s (Fig 2D).

To determine the general applicability of **44** to influence let-7 target expression, the effect of this compound on various Acute Myeloid Leukemia (AML) cell lines was measured, each with well-characterized expression levels of *let-7s* and *LIN28*. Most AML cell lines do not express high levels of let-7 miRNA levels. Perhaps as a consequence, treatment of AML cells significantly upregulated mature let-7 levels in MOLM-13, THP-1 and HL60 cell lines.

Focusing on **44**, dose curve, time course, and pulse-chase experiments were performed (Fig 3A-D). Varying concentrations of these compounds were applied to Huh7 cells and assayed by RT-PCR for the *let-7* sensitive version of HMGA2. These dose curve experiments showed that **44** was effective at 1uM, and maximally effective at 5uM (Fig 3A). To determine the time course for activity of **44**, cells were treated for various times. RT-PCR for HMGA2 showed that **44** could suppress expression of this *let-7* target gene in as few as 8 hours (Fig 3B). Finally, a pulse-chase of treatment was performed with **44** to determine if the effect on *let-7* targets was permanent or instigated a feed forward program of suppression of *let-7* targets. In fact, treating with **44** for 2 days followed by treatment withdrawal for 2 days completely reversed the effect of this compound on various *let-7* target genes (Fig 3C), suggesting that this compound transiently regulated expression of *let-7* targets. Treating a AML cell line with **44** also showed a dose-responsive effect on those let-7 targets that are expressed (Fig 3D).

Compounds 61 and 62 were also identified as regulators of *let-7* targets. Treatment of Huh cells with these compounds also suppressed levels of HMGA2 and NMYC without affecting LIN28 (Fig 4). SEA analysis predicts the top targets of compounds 44, 61, and 62 as PDE inhibitors:

Compound #44 Similarity ensemble approach (http://sea.bkslab.org/) -cross target similarity map identifier			
	Code	Reference Name	E-value
1	PDE10 MOUSE 10000.0	cAMP and cAMP-inhibited cGMP 3',5'-cyclic phosphodiesterase 10A	3.39E-54
2	PDE10 MOUSE 1000.0	cAMP and cAMP-inhibited cGMP 3',5'-cyclic phosphodiesterase 10A	1.77E-46
3	HS90B HUMAN 1.0	Heat shock protein HSP 90-beta	1.81E-44
4	HS90B HUMAN 10.0	Heat shock protein HSP 90-beta	3.97-E32
5	PDE10 MOUSE 100.0	cAMP and cAMP-inhibited cGMP 3',5'-cyclic phosphodiesterase 10A	4.11E-29
6	HS90B HUMAN 100.0	Heat shock protein HSP 90-beta	3.94E-17
7	HS90B HUMAN 1000.0	Heat shock protein HSP 90-beta	9.98E-11
8	HS90B HUMAN 10000.0	Heat shock protein HSP 90-beta	8.53E-09
9	PTN7 HUMAN 10000.0	Protein-tyrosine phosphatase LC-PTP	8.01E-08
10	DEF PSEAE 100.0	Peptide deformylase	1.06E-07
Compound #61 Similarity ensemble approach (http://sea.bkslab.org/) -cross target similarity map identifier			
	Code	Reference Name	E-value
1	HS90B HUMAN 1.0	Heat shock protein HSP 90-beta	3.34E-43
2	HS90B HUMAN 10.0	Heat shock protein HSP 90-beta	4.11E-31
3	HS90B HUMAN 100.0	Heat shock protein HSP 90-beta	1.52E-16
4	PDE10 MOUSE 10000.0	cAMP and cAMP-inhibited cGMP 3',5'-cyclic phosphodiesterase 10A	1.08E-13
5	HS90B HUMAN 1000.0	Heat shock protein HSP 90-beta	2.54E-10
6	PDE10 MOUSE 1000.0	cAMP and cAMP-inhibited cGMP 3',5'-cyclic phosphodiesterase 10A	9.78E-10
7	PDE10 MOUSE 100.0	cAMP and cAMP-inhibited cGMP 3',5'-cyclic phosphodiesterase 10A	3.83E-09
8	HS90B HUMAN 10000.0	Heat shock protein HSP 90-beta	1.91E-08
9	PTN7 HUMAN 10000.0	Protein-tyrosine phosphatase LC-PTP	4.69E-07
10	MDHM PIG 1000.0	Malate dehydrogenase mitochondrial	5.64E-06
Compound #62 Similarity ensemble approach (http://sea.bkslab.org/) -cross target similarity map identifier			
	Code	Reference Name	E-value
1	HS90B HUMAN 1.0	Heat shock protein HSP 90-beta	1.03E-11
2	PTN7 HUMAN 10000.0	Protein-tyrosine phosphatase LC-PTP	1.19E-11
3	HS90B HUMAN 10.0	Heat shock protein HSP 90-beta	1.36E-04
4	PDE10 MOUSE 1000.0	cAMP and cAMP-inhibited cGMP 3',5'-cyclic phosphodiesterase 10A	1.24E-03
5	PDE10 MOUSE 10000.0	cAMP and cAMP-inhibited cGMP 3',5'-cyclic phosphodiesterase 10A	4.27E-03
6	EBNA1 EBVB9 10000.0	Epstein-Barr nuclear antigen 1	1.39E-02
7	EDNRA RAT 100.0	Endothelin receptor ET-A	1.53E-01
8	PDE10 MOUSE 100.0	cAMP and cAMP-inhibited cGMP 3',5'-cyclic phosphodiesterase 10A	2.17E-01
9	HS90B HUMAN 100.0	Heat shock protein HSP 90-beta	3.10E-01
10	MK09 HUMAN 10.0	c-Jun N-terminal kinase 2	3.37E-01

Example 2: Measurement of CREB activation

Phosphodiesterase 10A was identified as a potential target of **44**, **61** and **62**. The role of phosphodiesterase is to regulate levels of cyclic-AMP (cAMP)(Fig 4). Therefore, if **44** inhibits PDE10, one would expect an increase in cAMP levels leading to CREB activation. Huh cells were treated with **44** and **61** and then stained with an antibody that recognizes phosphorylated CREB, consistent with activation of cAMP signaling. Both **44** and **61** strongly induced levels of nuclear phosopho-CREB (Fig 5A). To measure the degree to which **44** could regulate gene expression in Huh cells, RNA-seq was carried out to identify which genes are changed in response to treatment with these compounds and whether *let-7* targets are enriched amongst these gene expression changes (Fig 5B). A wide variety of genes appeared to be both induced and suppressed. The table below shows a list of genes up- and down regulated by at least 2 fold in response to treatment with **44**:

Compound #44 RNA-Seq Results in Huh7 cells							
Genes Up-Regulated >2.0 Fold				Genes Down Regulated <0.5 fold			
ABLIM3	DUSP5	MAP2	**SAMD4A**	**ACY3**	**D2HGDH**	**IRAK1BP1**	**NOTO**
ACRC	EGR1	**MR198**	SELPLG	**AGFG2**	**DGCR6**	**KANK4**	**NTSM**
ADAM19	FHL2	**MUC12**	SERPINB8	**AMN**	**DKK1**	**KAZALD1**	**NLUT6**
ANKRD1	FOS	**MYOF**	SERPINE1	**ATP6VOE2**	**ENHO**	**KIF12**	**PPP1R1A**
ANXA3	FOSB	NEDD9	STX3	**BRP44**	**FBX04**	**LCN12**	**PRR1SL**
ARHGDIB	FST1L1	**NLRC3**	TAB2	**C14orf142**	**FTCD**	**LINC00087**	**SARDH**
ATF3	GAS7	NNMT	**TBX15**	**C1orf186**	**GABRB1**	**LOC100128191**	**SHO**
ATP2A1	GJA1	NPPB	THBS1	**C2orf82**	**GALK1**	**LOC388588**	**SLC22A7**
C1QTNF2	HDAC9	**NSRP1**	THSD1	**C8ORFK29**	**GLYCTK**	**MIR25**	**SLC27A2**
C7orfS3	ICAM1	OOZ1	TMC7	**CCDC154**	**GNG10**	**MIRLET7BHG**	**SLC30A10**
CIDEC	IL18	PITPNA	**UBASH38**	**CEBPA**	**HIST1H3I**	**MLIP**	**SLC38A3**
CREB5	IL8	PLA2G4C	**UCA1**	**CUC3**	**HIST2H3D**	**MST1P9**	**SNORD27**
CXCR6	**KLF10**	PMFBP1	**WDR66**	**CXorf22**	**HIST3H2A**	**MYCN**	**SPATA25**

CYR61	LSAMP	PRSS23	ZNF165	**CYP4F24P**	**HOGA1**	**NGEF**	**TLE6**
DUSP10	MAK	S100A2		**CYP4F3**	**INPPSF**	**NIPSNAP38**	**TMEM191A**

At the top of the list were genes related to CREB signaling, particularly induction of ATF3, C-FOS and FOSB, suggesting that **44** activates CREB (Fig 5). We also performed dose-response assays on cells treated with **44**, **61** and **62**. These compounds again appeared to silence HMGA2 in a dose dependent manner (Fig 5C), but also these compounds induced typical CREB target genes such as FOSB and CFOS in Huh7 cells and ATF3, FOSB and CFOS in HL60, MOLM13 and THP-1 cells (AML lines)(Fig 5D-E).

Example 3: Growth inhibition of cancer cells

The growth inhibitory effect of **44** on cancer cells was confirmed by counting the number of cells treated with disclosed compounds against DMSO (control) over a period of time (Figures 6A-6B). An ATP-luciferase cell survival assay was conducted on disclosed compounds to determine their dose-response properties in different cancer cells (Figure 6C). **44** appeared to dramatically slow the growth of Huh cells (Fig. 6A) and squamous cell carcinoma lines (Fig. 6B) at 1uM, the same dose used to effectively suppress *let-7* targets. With a distinct cell growth assay, compound **44** showed significant toxicity towards several lung, liver and AML cancer cells lines with an IC50 of 0.1μM (Fig 6C). These data are consistent with previous studies showing that PDE inhibition can be an effective mediator of growth rate in cancer cell lines.

Example 4: **44** inhibits UPR and PI3K signaling pathways

A small molecule, **44**, has been identified that significantly upregulates let-7 microRNA levels in AML cell lines[48]. To measure the complete degree to which **44** could regulate gene expression in AML cells, RNAseq was carried out to identify which genes are changed in response to treatment with these compounds and whether let-7 targets are enriched amongst these genes. These studies found a panel of direct let-7 target genes more than 1.5-fold downregulated (*HMGA1, IL6* and *MYC*) and confirmed suppression of these genes in 3 primary AML patient samples (FIG. 7B).

Moreover, the table below shows a list of direct let-7 genes down-regulated in AML Kasumi-1 cells by at least 1 fold:

Compound # 44 RNA-Seq Results in AML Kasumi-1 cells	
direct let-7 genes down-regulated > 1.0 fold, p < 0.001	
Gene	fold change
LRRC37A	-311.4562374
PRR18	-107.7359195
PALM2-AKAP2	-53.09922758
GOLGA4	-13.7327063
MAP4K3	-11.82477497
GPR132	-6.606995509
ARG2	-5.923322157
RNF7	-4.935784139
EIF2S2	-4.744874359
ELF4	-4.663968742
BCL7	-4.126229886
NFKBIL1	-2.694811168
EIF1	-2.624691757
IL6	-2.498694865
EIF2S2	-2.171158298
ALDH6A1	-2.106214754
IRS2	-1.89230091
BCL2L1	-1.87879562
NFKBIA	-1.784940565
H19	-1.775752605
KLF8	-1.765526435
KLF9	-1.765526435
TRIM67	-1.729766906
SMAD2	-1.726013593
DHX57	-1.669160709
CDKN1A	-1.574128723
CDK6	-1.557702387
ACAT2	-1.529487538
INSR	-1.518495041
TRIM41	-1.477586607
POU2F	-1.47385161
TRIM71	-1.407707101
CCND2	-1.396442009
HK1	-1.392784899
E2F1	-1.338514006
E2F5	-1.287685397
IGF2BP2	-1.271594875
ATPAF1	-1.267607062
KRAS	-1.260717599
ELF4	-1.25045109

NFKB1	-1.232245039
NRAS	-1.206793668
HOXA9	-1.205671617
CTPS2	-1.186950114
CRY2	-1.162883998
ABCA5	-1.151604937
MYC	-1.129405664
E2F3	-1.084335093
CCND3	-1.079712163

Moreover, pathway analysis of Kasumi-1 cells treated with **44** predicted induced C/EBPε signaling and significant inhibition of the unfolded protein response (UPR) and PI3K pathway (FIG. 7A). In primary AML cell samples, experiments confirmed that compound **44** induced apoptosis through upregulation of the pro-apoptotic regulators CCAAT/enhancer binding protein homologue CHOP, BCL-2, BAX and BIM as well as inhibition of the heat shock proteins HSP40 and HSP90, all of which are major regulators of UPR (FIG. 7B). Overexpression of C/EBPs correlate with suppression of a wide variety of microRNAs, let-7 miRNAs included [16]. Two C/EBP family members, CHOP and C/EBPε, were found to be significantly upregulated after AML cell treatment with **44**. Subsequent experiments therefore tested if inhibition of C/EBPε and CHOP can upregulate let-7 microRNAs. Indeed, shRNA mediated silencing of CHOP and C/EBPε upregulated let-7 microRNAs in Kasumi-1 cells (FIG. 7C). Taken together, **44** inhibits UPR and PI3K signaling pathways leading to upregulation of let-7 microRNA levels, possibly through increased transcriptional activity of several C/EBP family members.

Phenotypically, treatment with **44** at 10 μM for 16h induces differentiation in Kasumi-1 cells leading to a significant decrease of CD34+CD38- expressing cells (FIG. 7D). To confirm that induction of differentiation and apoptosis is not mediated through cytotoxicity, cellular ATP levels were measured via GloTiter assay in HepG2 cells 12h after treatment with various doses of **44** and results were compared to the clinically approved demethylating agent 5-Azacitidine (5-Aza, 5 μM). Compound **44** was not toxic at dosing regimens of 5 μM and lower (FIG. 7E) and exhibited similar or better tolerability than 5-Aza.

Example 5: Effect of **44** in vivo in AML Xenograft models

Next, the effect of **44** in vivo in AML Xenograft models was determined. In a systemic MOLM-13 AML Xenograft model, daily intraperitoneal (IP) injections of **44** at 10mg/kg

significantly prolonged survival of mice when compared to vehicle injected Xenografts (Fig. 8A). In a subcuataneous AML model of Kasumi-1 cells (core binding factor (CBF) leukemia-, CD34+CD38- enriched cell line with t(8;21)-Kit(q22;q22), stably overexpressing a Luciferase plasmid (Kasumi-1^{Luc}), two single intravenous injections of **44** decreased tumor proliferation as quantified by bioluminescence imaging and intensity dynamic analysis (BLI, Figure 8B, left) at the study end point 24 days post cell transplantation. Finally, **44** was prepared as a nanoparticle suspension with Casein to optimize solubility and delivery of **44**. A single injection of **44** formulated with Casein nanoparticles (CasMA) significantly prolonged survival of mice compared to control/PBS injected mice (Fig. 8c). These results suggest that **44** inhibit AML proliferation in vivo and optimized solubility and delivery of **44** will enhance its anti-tumor activity.

Example 6: Inhibition of leukemic stem cell proliferation in vivo

In order to determine if **44** can inhibit leukemic stem cell proliferation in vivo, primary AML cells from three different donors were treated with 10 μM **44** for 16h and the progeny were transplanted into NSG mice to assess leukemic stem cell engraftment over time FIG. 9A). Mice transplanted with **44** treated primary AML cells showed 20 fold less engraftment of human CD45+ cells and human CD45+CD33+CD34+CD38-CD45RA+ leukemic stem cells compared to mice transplanted with control treated AML cells (FIGs. 9B-9C). These results suggest that **44** inhibits proliferation of LSCs and abrogate human AML tumor burden in vivo.

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INCORPORATION BY REFERENCE

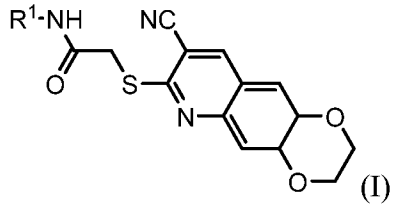
All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

EQUIVALENTS

While specific embodiments of the subject invention have been discussed, the above specification is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

We claim:

1. A pharmaceutical composition comprising a compound of formula I:



(I)

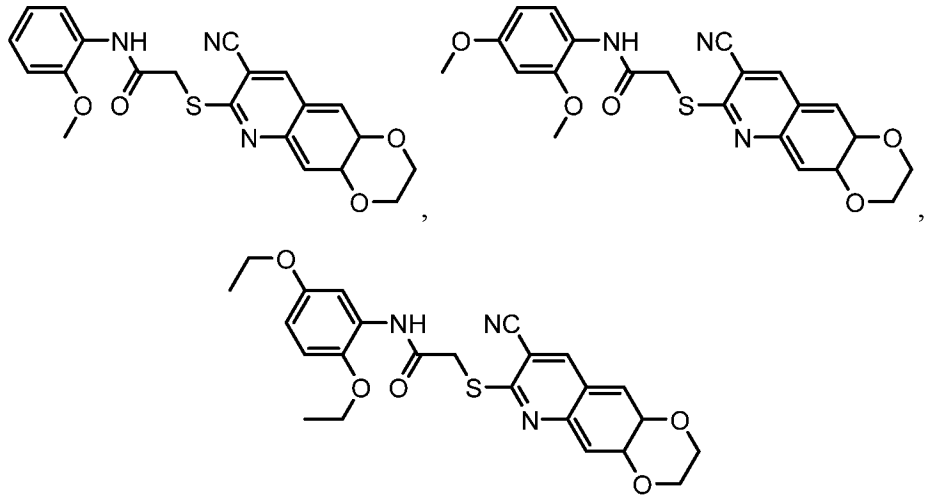
wherein R^1 is substituted or unsubstituted phenyl;

or a pharmaceutically acceptable salt thereof;

and wherein the pharmaceutical composition further comprises a pharmaceutically acceptable excipient.

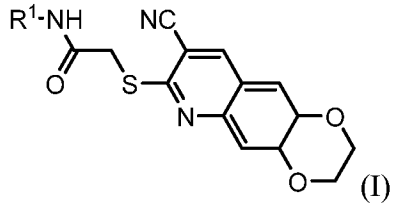
2. The pharmaceutical composition of claim 1, wherein R^1 is phenyl substituted with 1, 2, 3, or 4 lower alkoxy groups, such as methoxy or ethoxy.

3. The pharmaceutical composition of claim 1, wherein the compound is selected from the following compounds:



or a pharmaceutically acceptable salt thereof.

4. A method of increasing let-7 micro RNA levels in a patient, comprising administering an effective amount a compound of formula I:

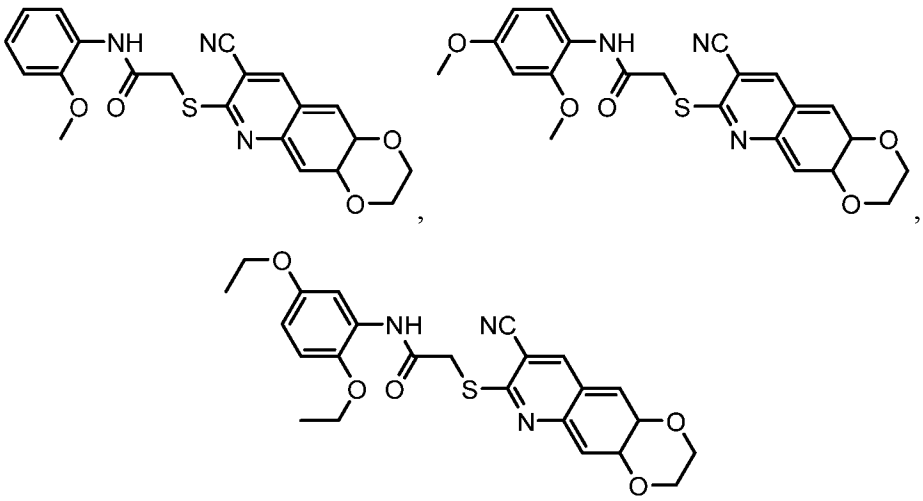


(I)

wherein R^1 is substituted or unsubstituted phenyl;
or a pharmaceutically acceptable salt thereof.

5. The method of claim 4, wherein R^1 is phenyl substituted with 1, 2, 3, or 4 lower alkoxy groups, such as methoxy or ethoxy.

6. The method of claim 5, wherein the compound is selected from the following compounds:



or a pharmaceutically acceptable salt thereof.

7. The method of any one of claims 4-6, wherein the patient has a cancer.

8. The method of claim 7, wherein the cancer is acute myeloid leukemia, colon cancer, breast cancer, prostate cancer, lung cancer, skin cancer, liver cancer, pancreatic cancer, ovarian cancer, bladder cancer, kidney cancer, esophageal cancer, cervical cancer, endometrial cancer, melanoma, brain cancer, glioma, neuroblastoma, osteosarcoma, chondrosarcoma, gastric

carcinoma, glioma, mesothelioma, Kaposi sarcoma, liposarcoma, synovial sarcoma, or Wilm's tumor.

9. The method of claim 8, wherein the cancer is liver cancer, skin cancer, such as squamous cell carcinoma, lung cancer, or acute myeloid leukemia.

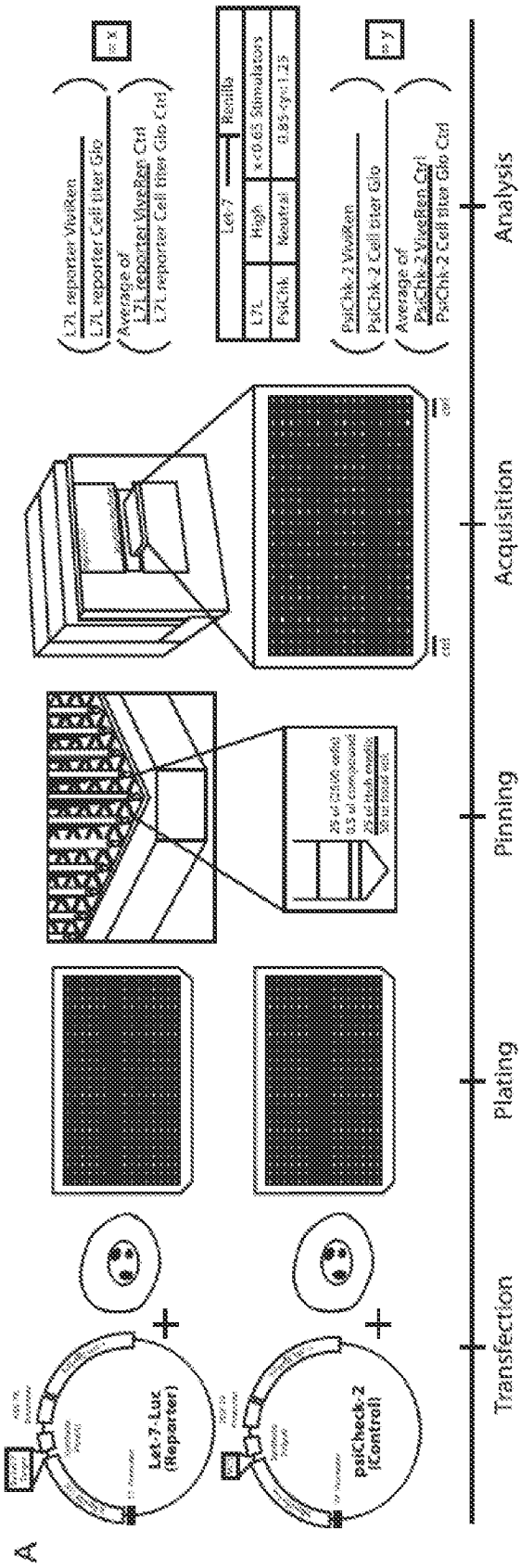

A
Let-7-Lux (Reporter)
psiCheck-2 (Control)
Transfection
Plating
Pinning
Acquisition
Analysis
Let-7
Results
Let-7 High Neutral
PsiChk
PsiChk-2 ViviRen
PsiChk-2 Cell titer Glo
Average of PsiChk-2 ViviRen Ctrl PsiChk-2 Cell titer Glo Ctrl
n = 7
L7L reporter ViviRen
L7L reporter Cell titer Glo
Average of L7L reporter ViviRen Ctrl L7L reporter Cell titer Glo Ctrl
n = 7

FIG. 1A

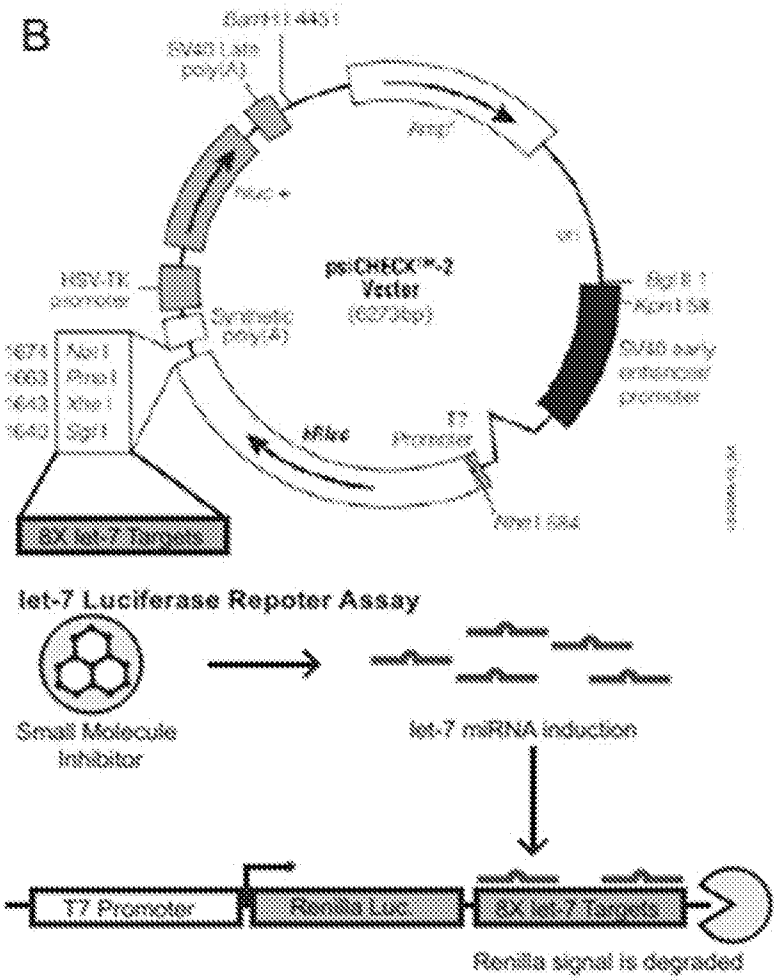

B
let-7 Luciferase Repoter Assay
Small Molecule Inhibitor
let-7 miRNA induction
T7 Promoter
Renilla signal is degraded

FIG. 1B

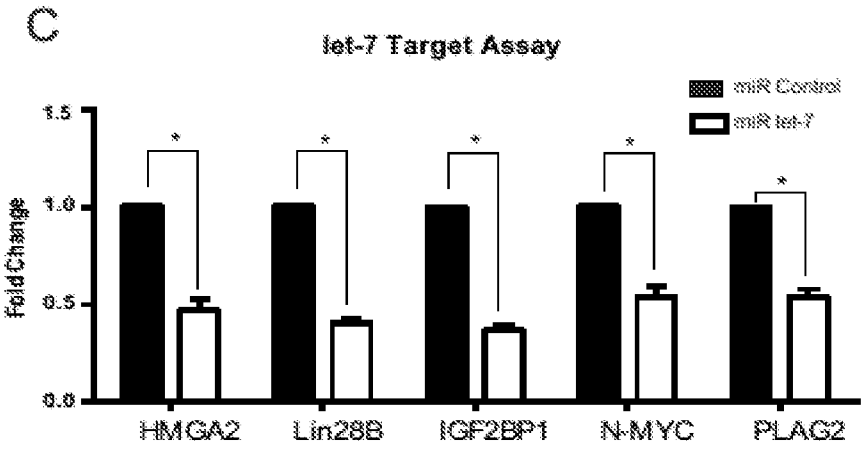

C
let-7 Target Assay
miR Control
miR let-7
Fold Change
1.5
1.0
0.5
0.0
*
HMGA2
Lin28B
IGF2BP1
N-MYC
PLAG2

FIG. 1C

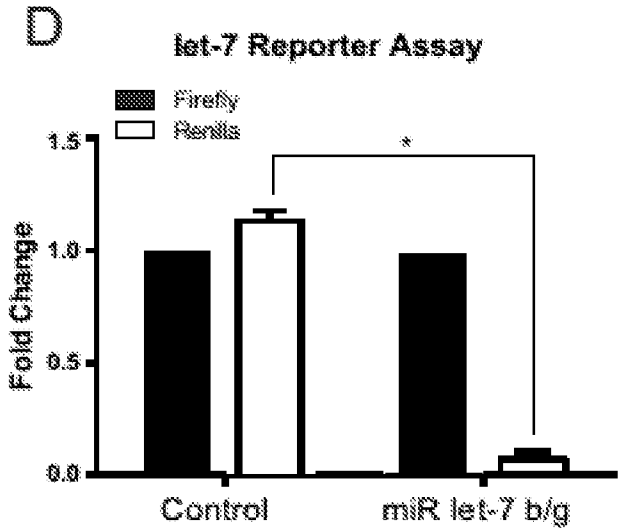

D
let-7 Reporter Assay
Firefly
Renilla
*
1.5
1.0
0.5
0.0
Fold Change
Control
miR let-7 b/g

FIG. 1D

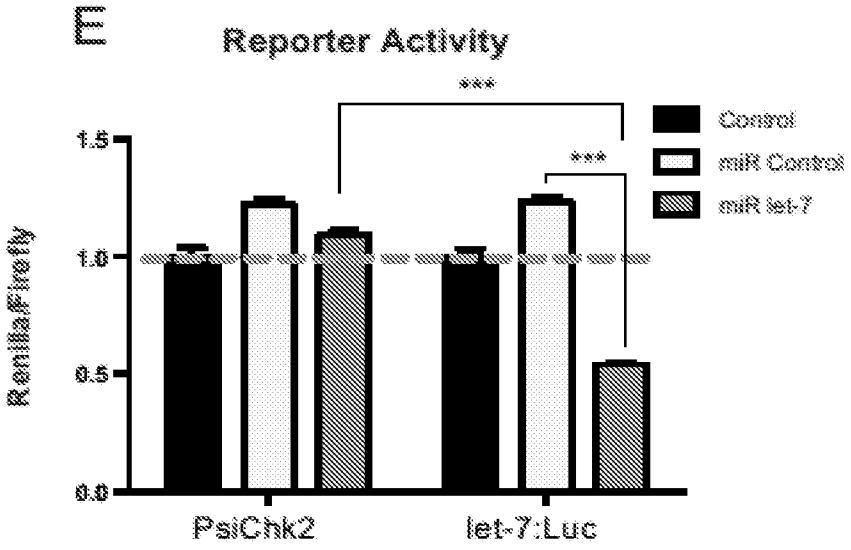

E
Reporter Activity
***
***
Control
miR Control
miR let-7
1.5
1.0
0.5
0.0
Renilla/Firefly
PsiChk2
let-7:Luc

FIG. 1E

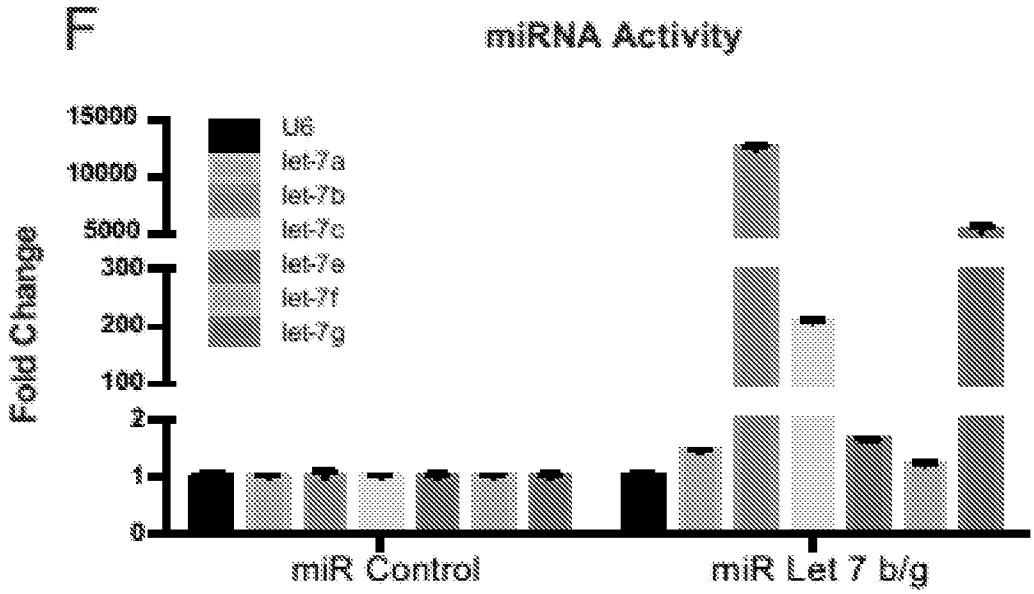

F
miRNA Activity
Fold Change
15000
10000
5000
300
200
100
2
1
0
U6
let-7a
let-7b
let-7c
let-7e
let-7f
let-7g
miR Control
miR Let 7 b/g

FIG. 1F

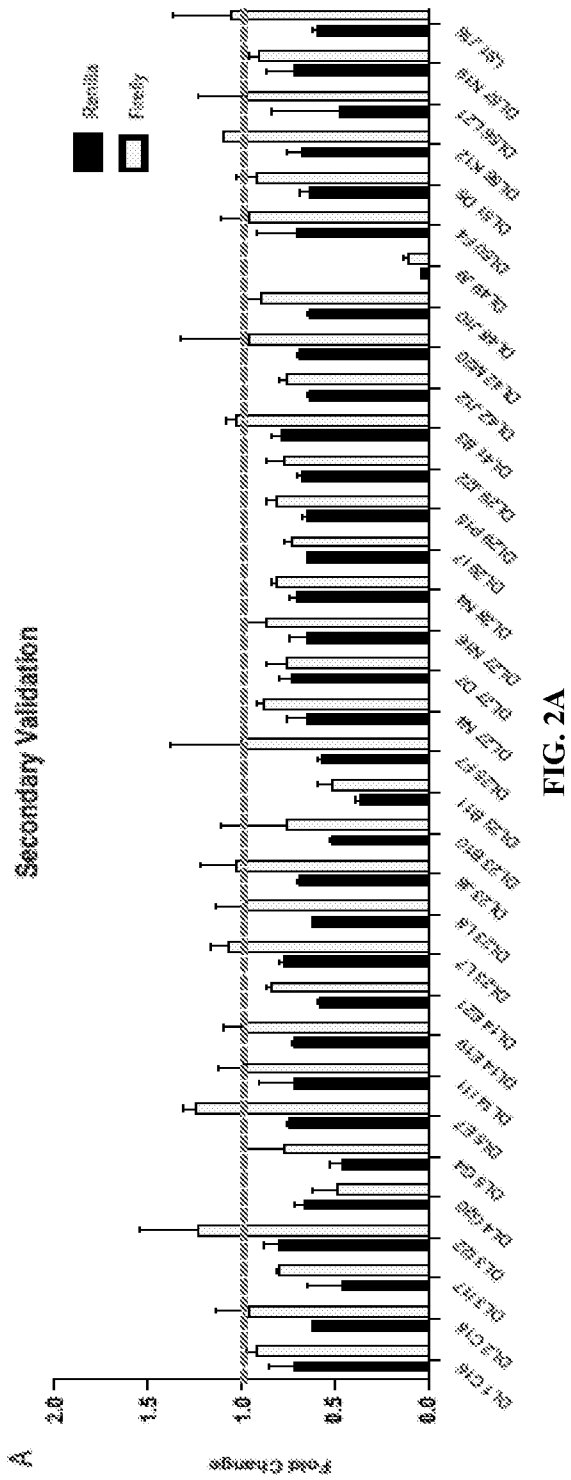

A
Secondary Validation
Renilla
Firefly
Fold Change
2.0
1.5
1.0
0.5
0.0

FIG. 2A

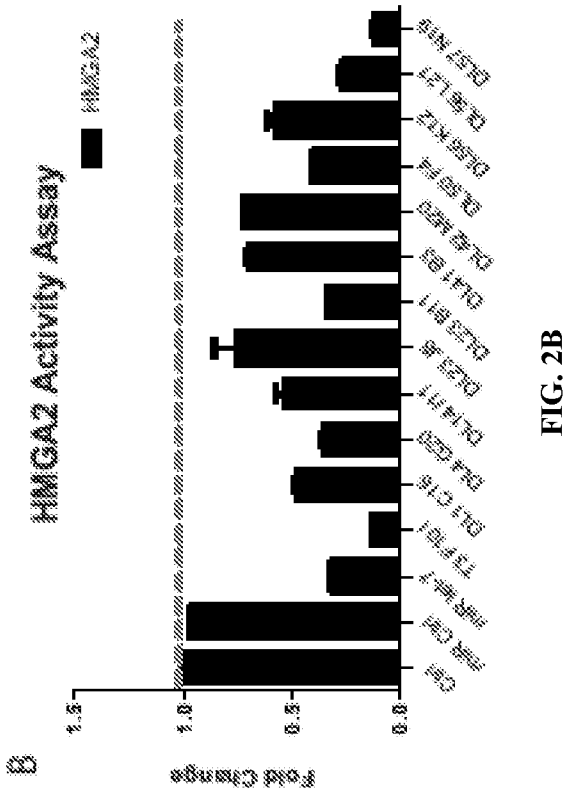

B
HMGA2 Activity Assay
HMGA2
Fold Change
1.5
1.0
0.5
0.0

FIG. 2B

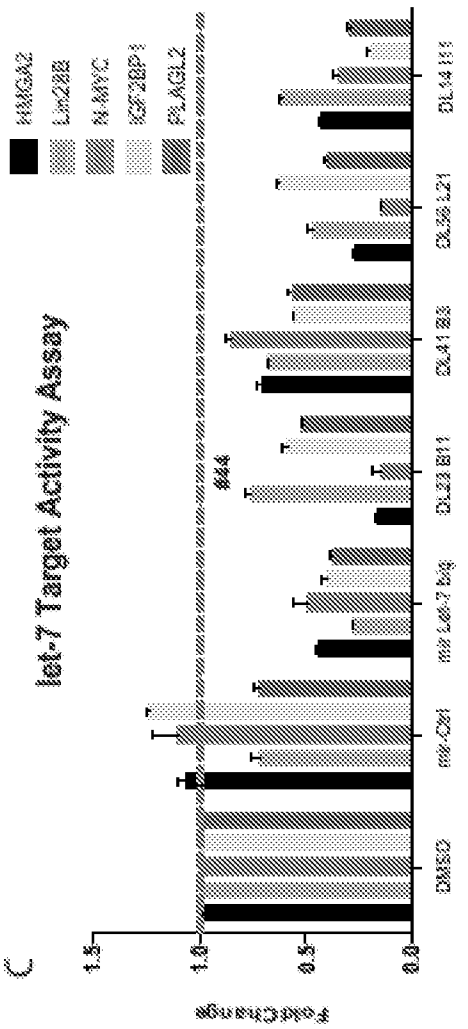

C
let-7 Target Activity Assay
HMGA2
Lin28B
N-MYC
IGF2BP1
PLAGL2
Fold change
1.5
1.0
0.5
0.0
DMSO
miR-Ctrl
mir Let-7 b/g
DL23 B11
DL41 B3
DL56 L21
DL14 I11

FIG. 2C

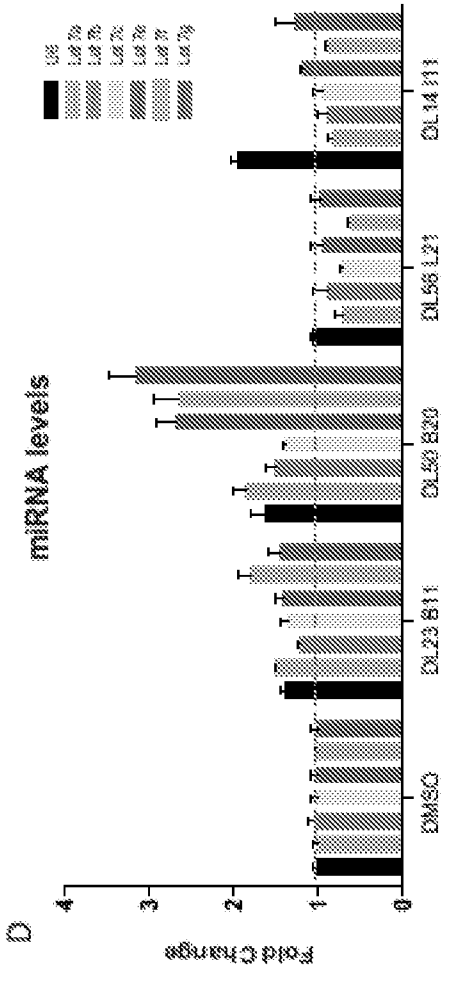

D
miRNA levels
Fold change
4
3
2
1
0
DMSO
DL23 B11
DL50 B20
DL56 L21
DL14 I11

FIG. 2D

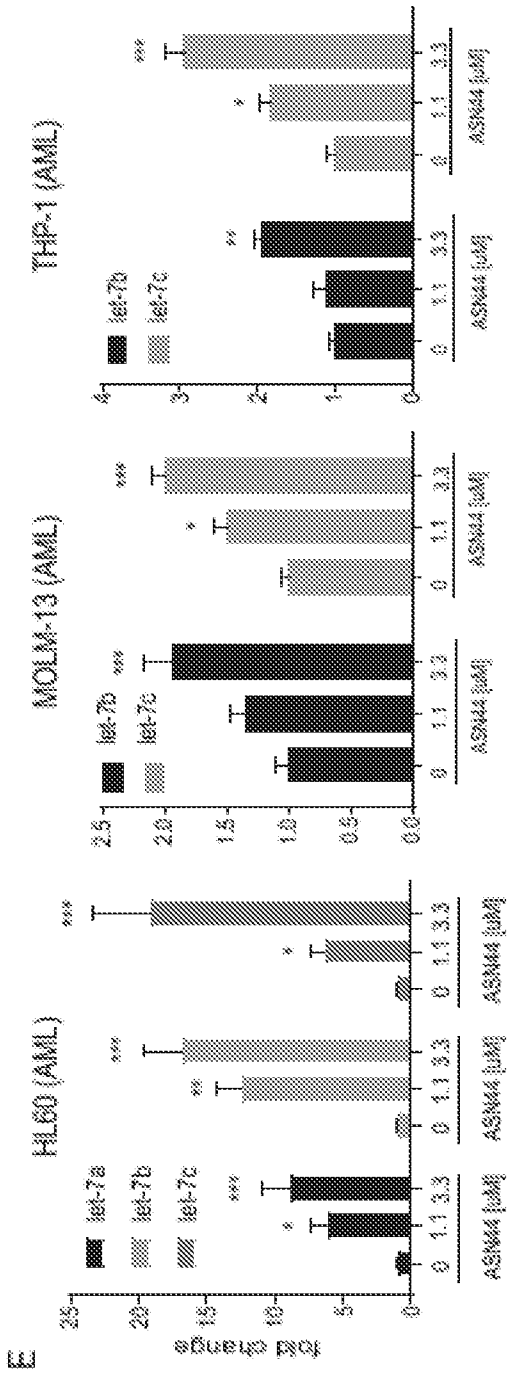

E
HL60 (AML)
MOLM-13 (AML)
THP-1 (AML)
let-7a
let-7b
let-7c
fold change
ASN44 [μM]
0 1.1 3.3

FIG. 2E

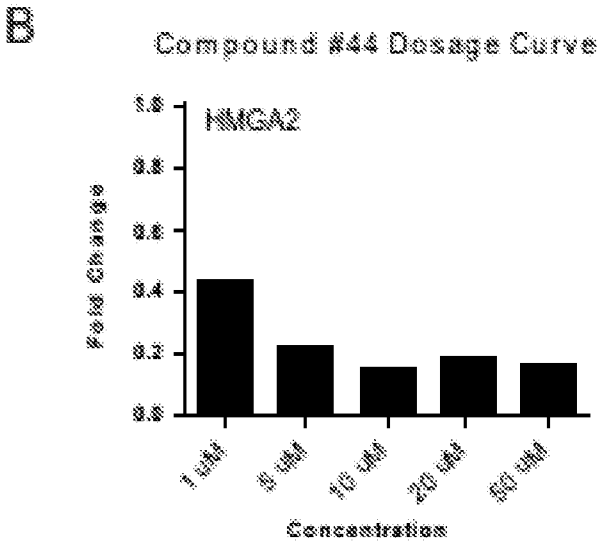

B
Compound #44 Dosage Curve
HMGA2
Fold Change
1 uM
5 uM
10 uM
20 uM
50 uM
Concentration

FIG. 3A

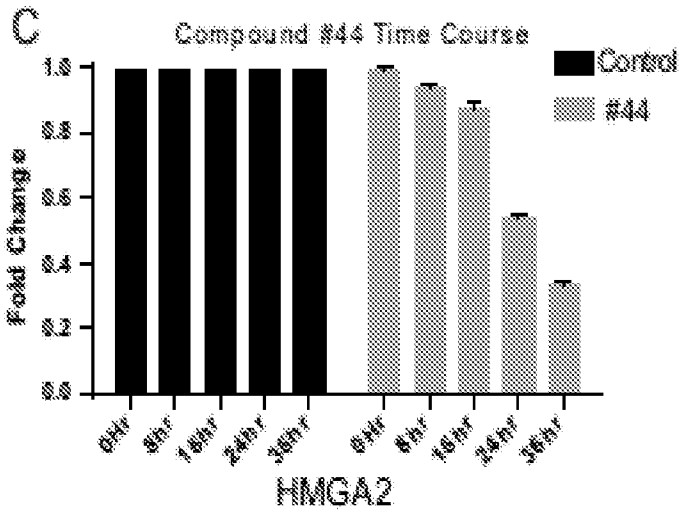

C
Compound #44 Time Course
Control
#44
Fold Change
0Hr
8hr
16hr
24hr
36hr
HMGA2

FIG. 3B

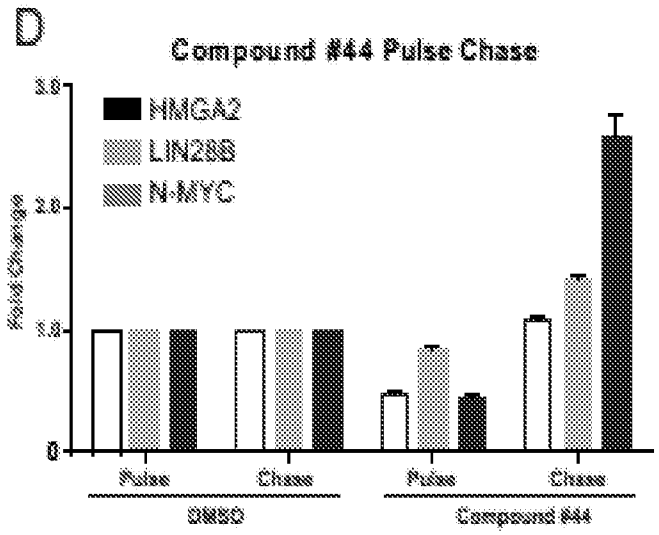

D
Compound #44 Pulse Chase
HMGA2
LIN28B
N-MYC
Fold Change
3.5
2.8
1.8
0
Pulse
Chase
Pulse
Chase
DMSO
Compound #44

FIG. 3C

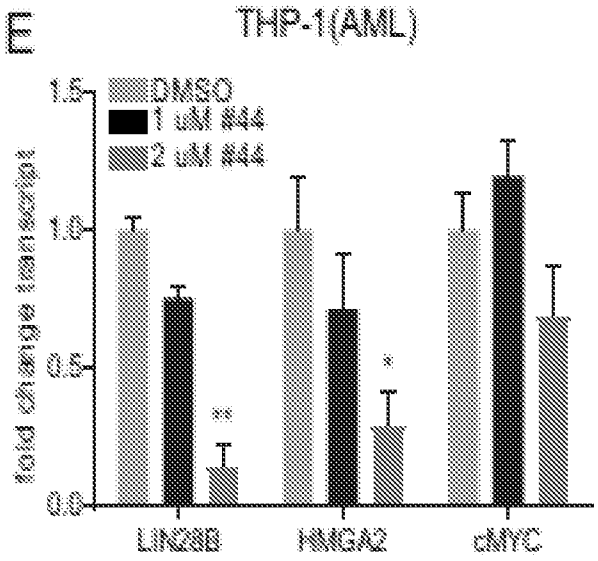

E
THP-1(AML)
DMSO
1 uM #44
2 uM #44
fold change transcript
1.5
1.0
0.5
0.0
**
*
LIN28B
HMGA2
cMYC

FIG. 3D

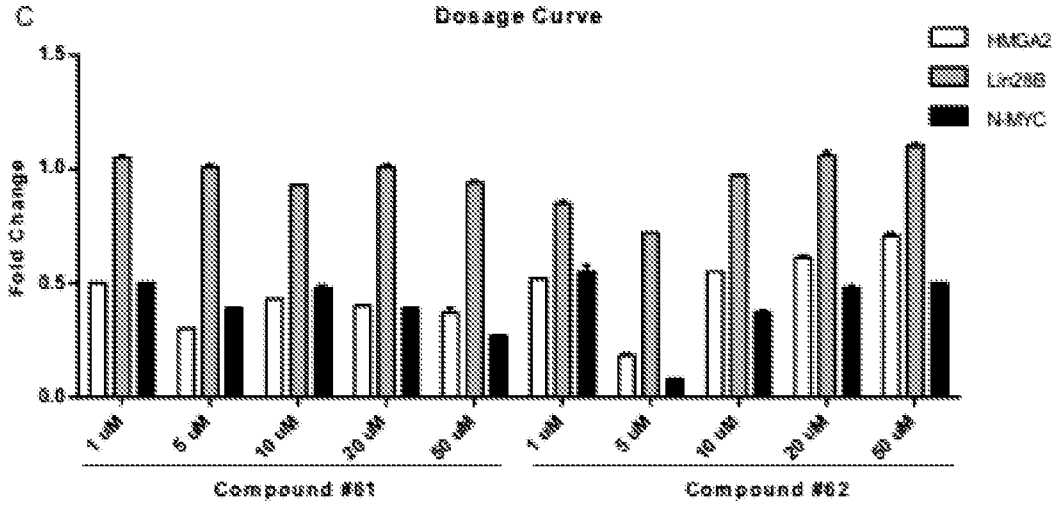

C
Dosage Curve
MKN42
Lin28B
NMYC
Fold Change
1.5
1.0
0.5
0.0
1 uM
5 uM
10 uM
20 uM
50 uM
1 uM
5 uM
10 uM
20 uM
50 uM
Compound #61
Compound #62

FIG. 4

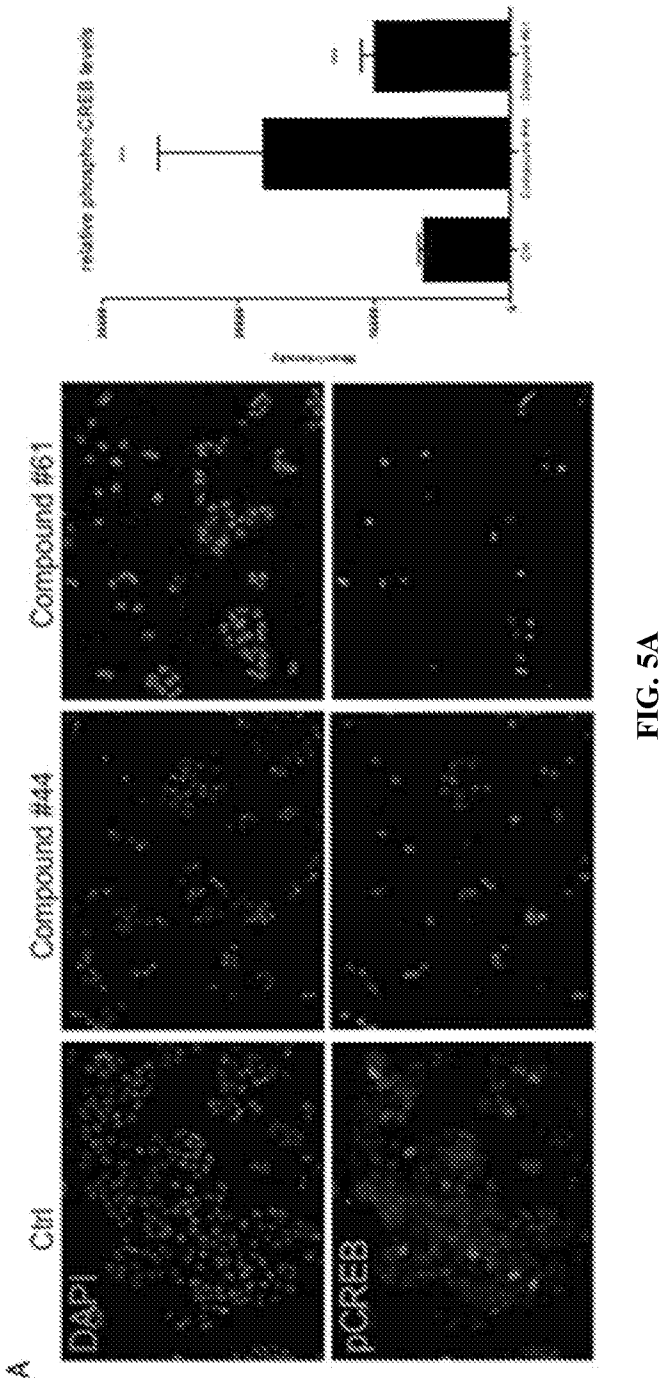


FIG. 5A

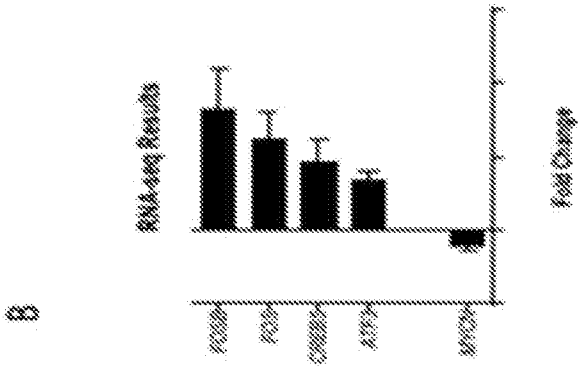


FIG. 5B

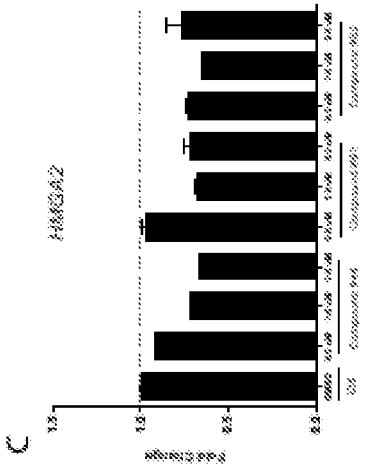


FIG. 5C

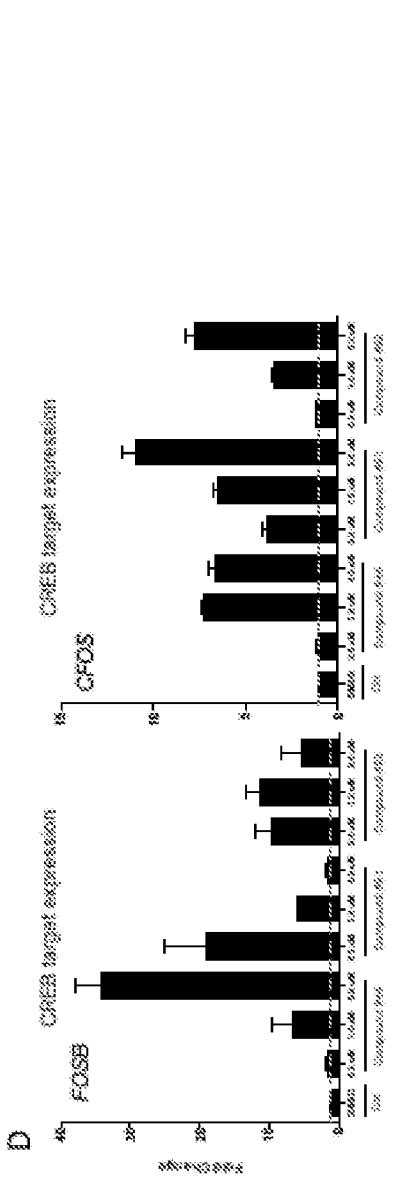

D
FOSB
CREB target expression
CFOS
CREB target expression

FIG. 5D

E
MOLM-13 (AML)
DMSO
1 uM ASN44
2 uM ASN44
ATF3
CFOS
THP-1 (AML)
FOSB
CFOS
ATF3
HL60 (AML)
ATF3
FOSB
CFOS

FIG. 5E

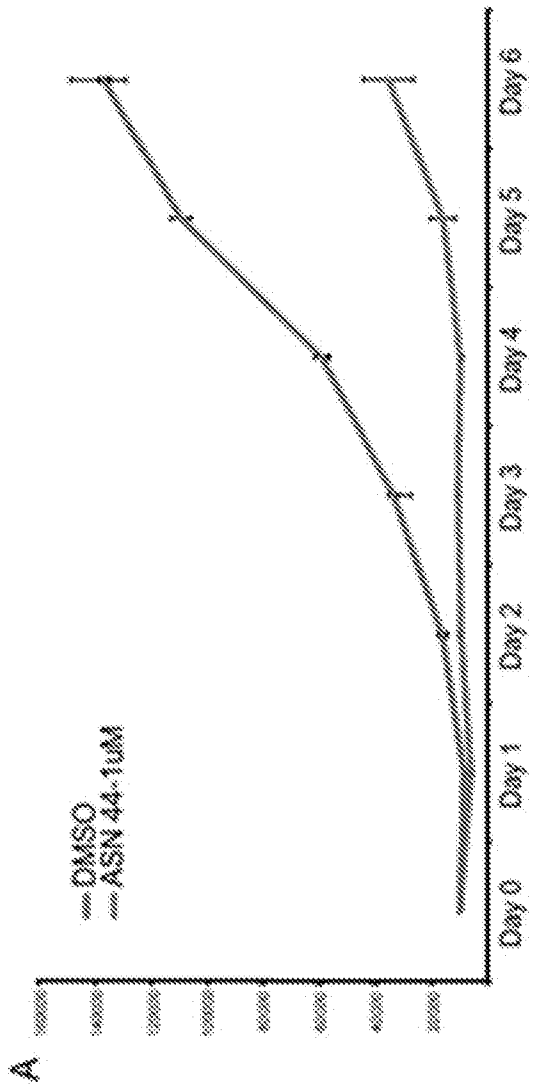

A
DMSO
ASN 44-1uM
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6

FIG. 6A

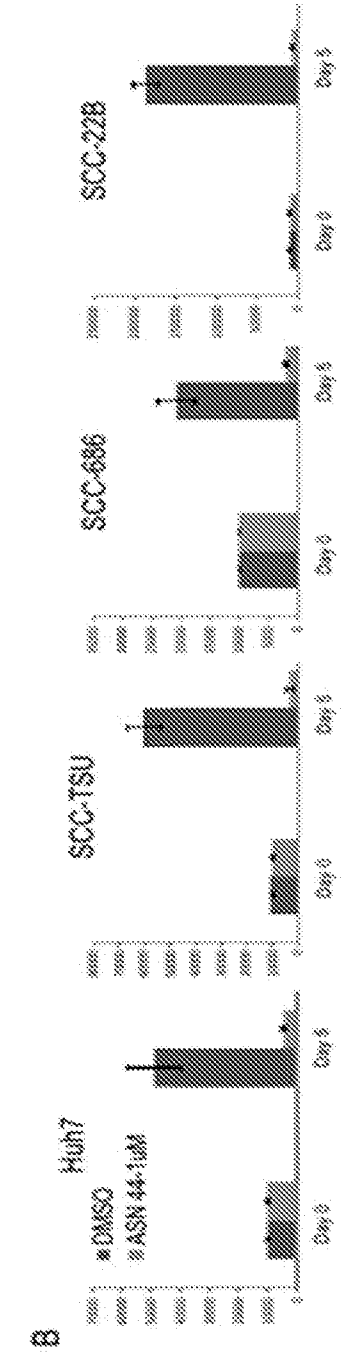

B
Huh7
SCC-TSU
SCC-686
SCC-22B
DMSO
ASN 44-1uM

FIG. 6B

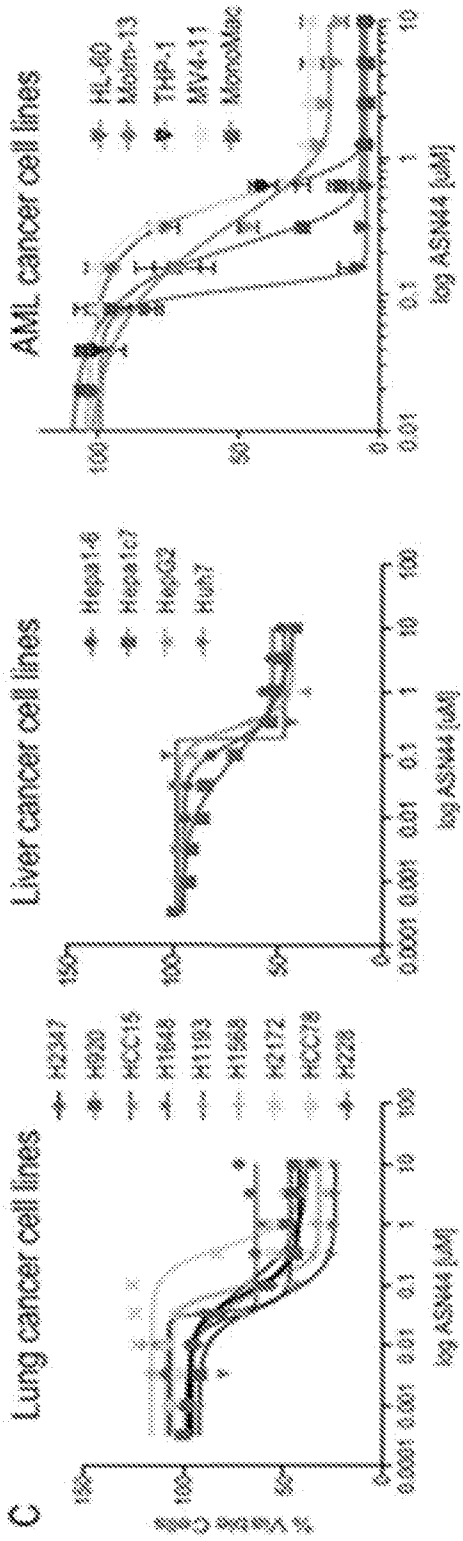

C
Lung cancer cell lines
Liver cancer cell lines
AML cancer cell lines
log ASW44 [μM]
HL-60
Mono-13
THP-1
MV4-11
Monolike

FIG. 6C

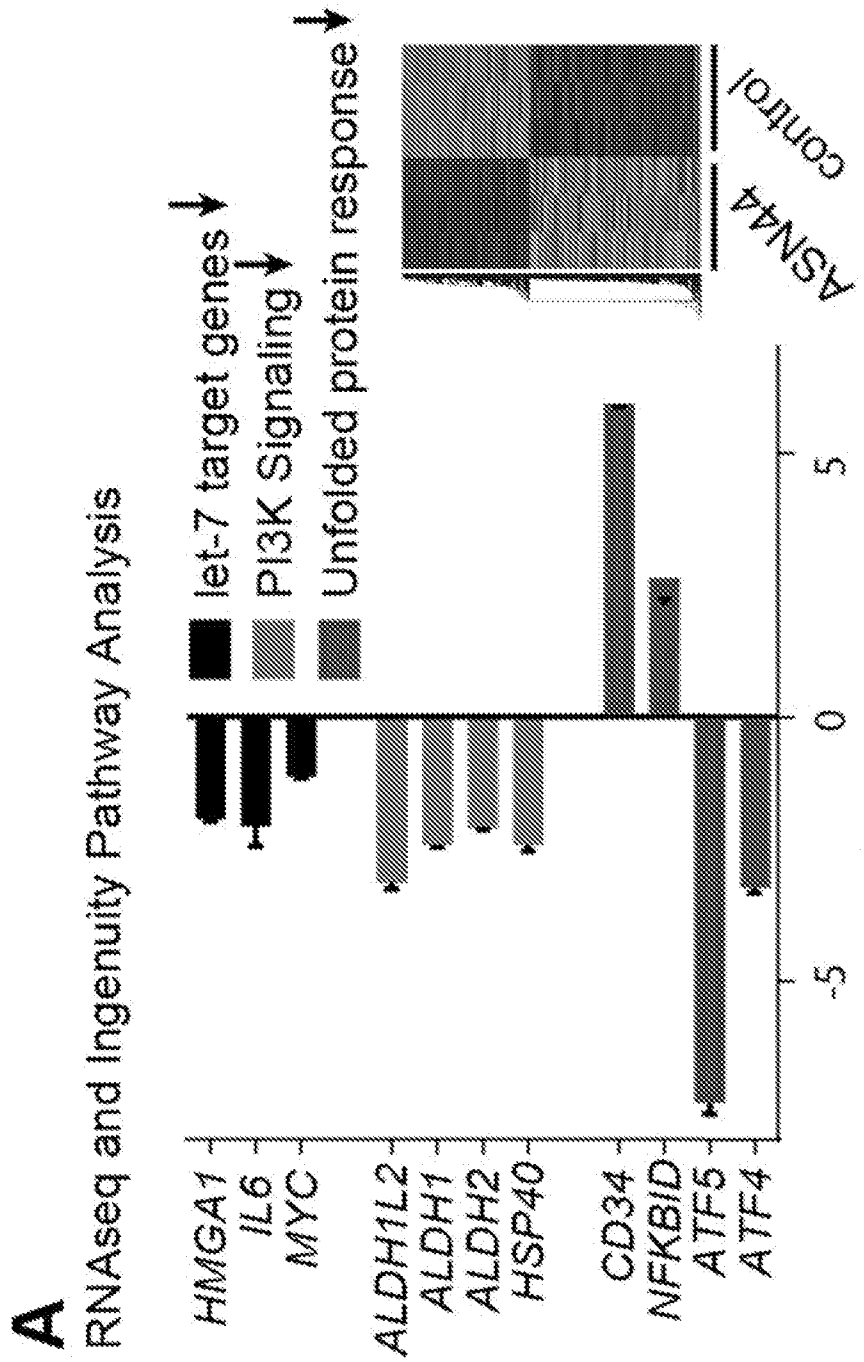

A
RNAseq and Ingenuity Pathway Analysis
let-7 target genes
PI3K Signaling
Unfolded protein response
HMGA1
IL6
MYC
ALDH1L2
ALDH1
ALDH2
HSP40
CD34
NFKBID
ATF5
ATF4
-5
0
5
fold change
ASNA4
Control

FIG. 7A

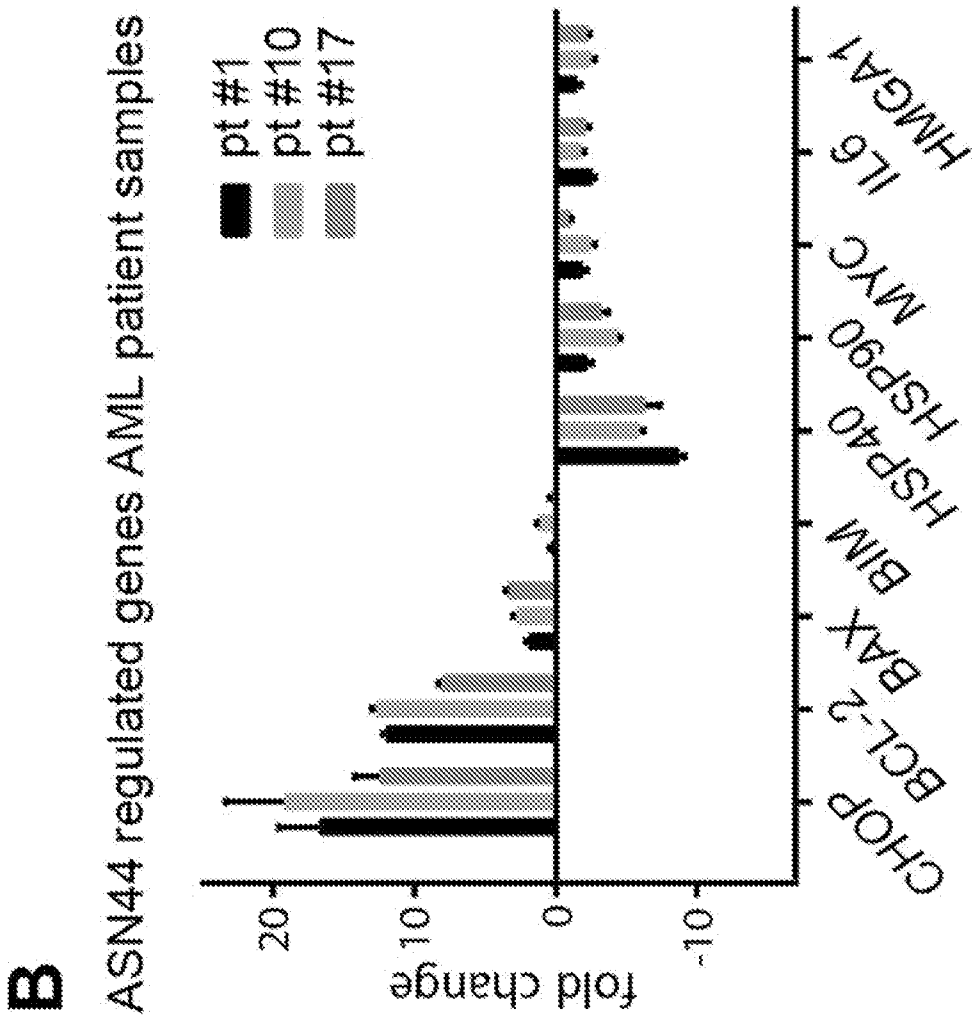

B
ASN44 regulated genes AML patient samples
pt #1
pt #10
pt #17
fold change
20
10
0
-10
CHOP
BCL-2
BAX
BIM
HSPA0
HSP90
MYC
IL6
HMGA1

FIG. 7B

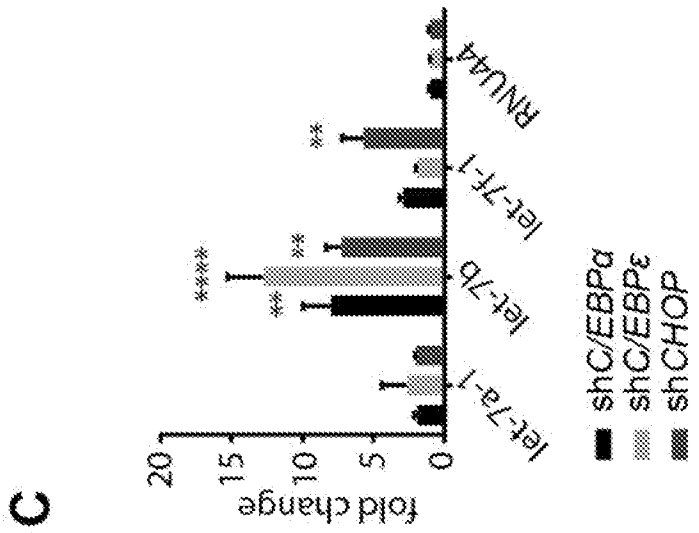

C
fold change
20
15
10
5
0
****
**
**
**
let-7a-1
let-7b
let-7f-1
RNU44
shC/EBPα
shC/EBPε
shCHOP

FIG. 7C

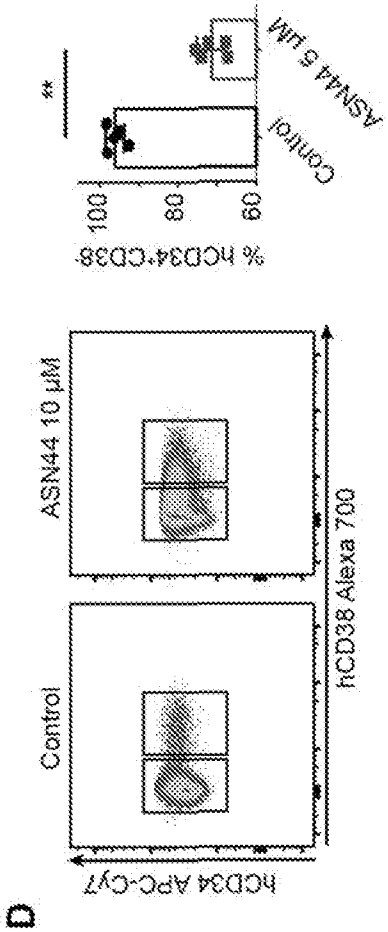

D
hCD34 APC-Cy7
hCD38 Alexa 700
Control
ASN44 10 µM
% hCD34+CD38-
100
80
60
Control
ASN44 5 µM
**

FIG. 7D

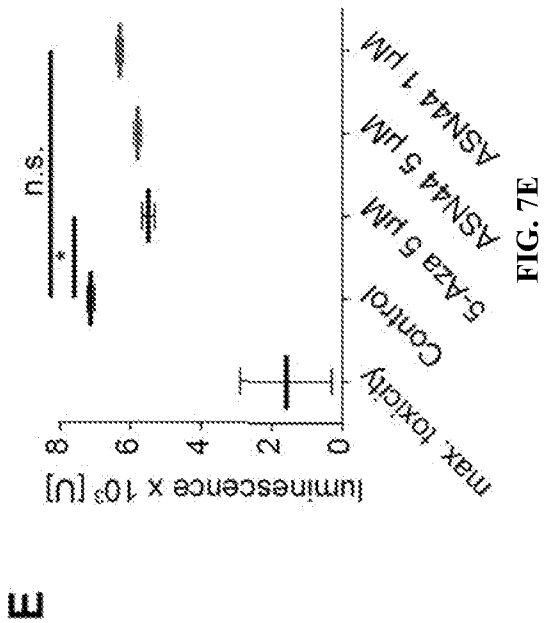

E
luminescence x 10³ [U]
0
2
4
6
8
n.s.
*
max. toxicity
Control
5-AZa 5 µM
ASNá4 5 µM
ASNá4 1 µM

FIG. 7E

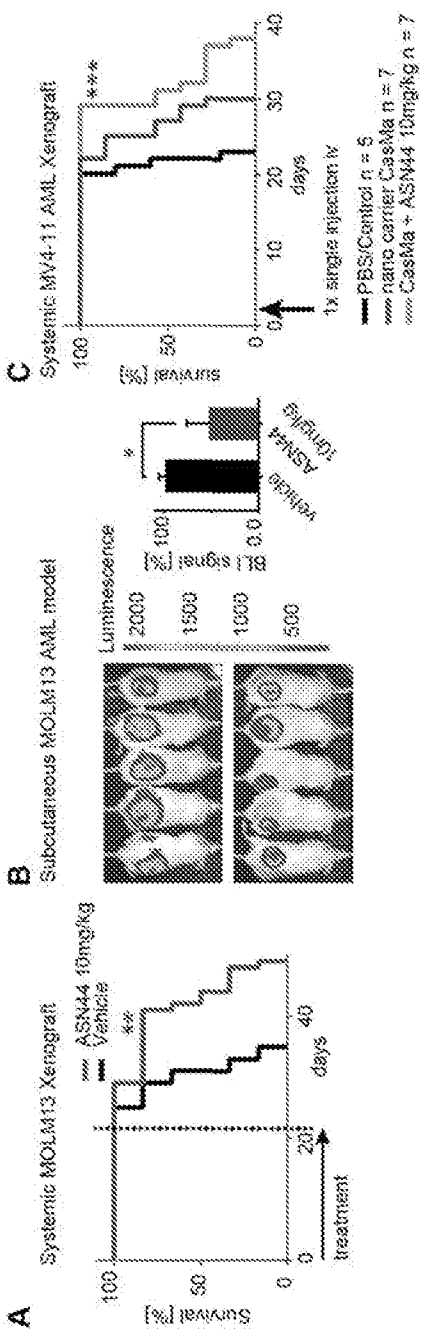


FIG. 8A

FIG. 8B

FIG. 8C

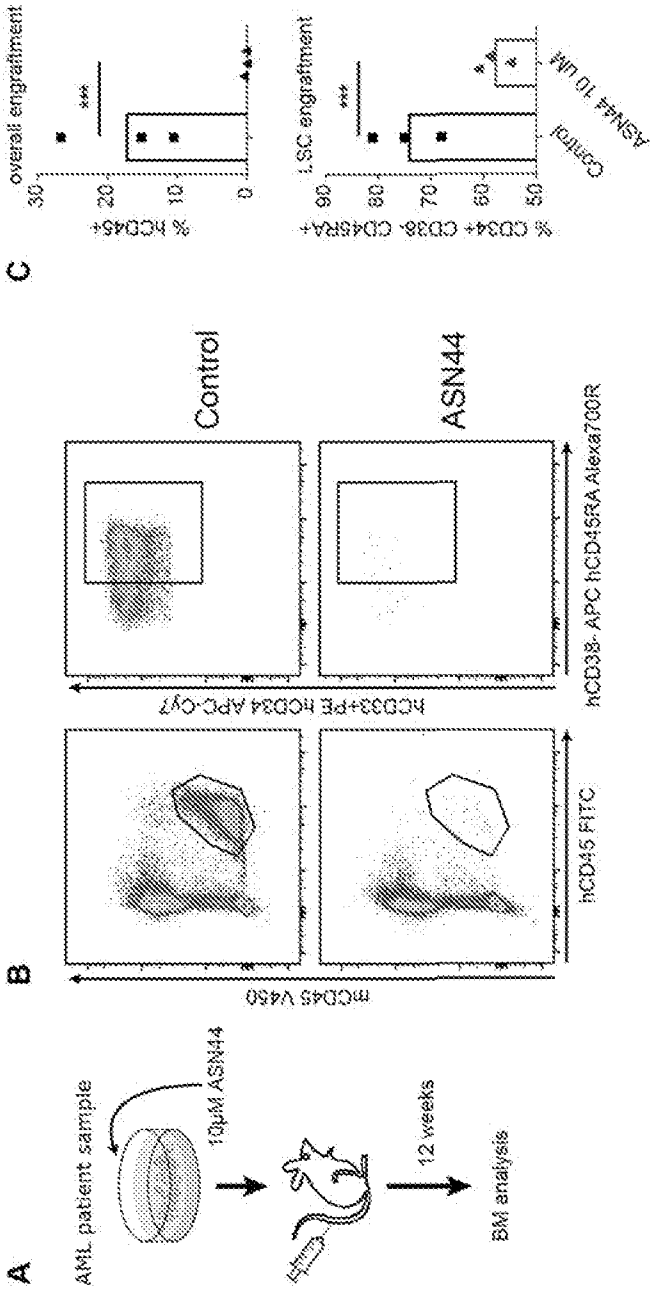


FIG. 9A

FIG. 9B

FIG. 9C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/053511

A. CLASSIFICATION OF SUBJECT MATTER
IPC (2019.01) A61K 31/474100, A61P 35/00, A61P 35/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (2019.01) A61K 31/474100, A61P 35/00, A61P 35/02, C07D 491/056

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2015/0140071 A1 (Ayyappan K. Rajasekaran [US]) 21 May 2015 (2015/05/21) Paragraphs [0003], [0026], [0027], [0148] and [0153]. The compound of Formula 2, and compound ALD-6.	1-9
X	WO 2014/207213 A1 (MEDIZINISCHE UNIVERSITAT INNSBRUCK [AT]) 31 Dec 2014 (2014/12/31) Abstract, page 1, page 14, page 15 lines 15 and 23, and pages 40, 41 and 43. Compounds PKCe2020, PKCe2045, PKCe2046, PKCe2047, PKCe2088 and PKCe2089.	1,4,7-9
Y	Zipeto, M. A. et al. ADAR1 activation drives leukemia stem cell self-renewal by impairing Let-7 biogenesis. Cell Stem Cell (04 August 2016), 19(2), 177-191. Retrieved from URL: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4975616/>. 04 Aug 2016 (2016/08/04) The whole document. Specifically see the results titled: "JAK2 and BCR-ABL1 Regulate Let-7 Biogenesis and LSC Self-renewal".	4-9

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 Jan 2019	24 Jan 2019
Name and mailing address of the ISA: Israel Patent Office Technology Park, Bldg.5, Malcha, Jerusalem, 9695101, Israel Facsimile No. 972-2-5651616	Authorized officer SOMECH Erez Telephone No. 972-2-5651762

B. FIELDS SEARCHED:

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

Databases consulted: Google Patents, CAPLUS, BIOSIS, EMBASE, MEDLINE, MARPAT, REGISTRY, PubMed, Google Scholar, DWPI
Search terms used: let-7, let7, let*7, micro RNA, miRNA, micro*RNA, RNA silencing, LIN28b, LIN28*, transcription*, expression, phosphodiesterase, PDE, PDE10, PDE10a, cAMP, FLT3, HMGA2, HMGA1, HMGA*, C/EBP, enhancer binding proteins, IL6, MYC, cMyc, HSP90, CREB, UPR, PI3K, cancer, leukemia, leukem*, AML, CML, leukemia stem cells, LSC, LSCs, *prolifer*, *hyperprolif*, *carcinom*, *neoplas*, *tumor, glioma, sarcoma, *blastoma, squamous cell carcinoma, lung cancer, SCLC, NSCLC, apoptosis.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/053511

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Cinkornpumin, J. et al. A small molecule screen to identify regulators of let-7 targets. Scientific reports (21 November 2017), 7(1), 15973. Retrieved from URL: <https://www.nature.com/articles/s41598-017-16258-9 >. 21 Nov 2017 (2017/11/21) The whole document.	1-9
A	PubChem BioAssay AID 940, "Modulators of the EP2 prostaglandin E2 receptor - Primary Screening". PubChem BioAssay Database, National Center for Biotechnology Information. Deposit Date: 21 December 2007. Retrieved from URL: <https://pubchem.ncbi.nlm.nih.gov/bioassay/940>. 21 Dec 2007 (2007/12/21) See PubChem CIDs 3207829 and 1450666.	1-9
A	Li, Y. Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. Cancer research (15 August 2009), 69(16), 6704-6712. Retrieved from URL: <http://cancerres.aacrjournals.org/content/canres/69/16/6704.full.pdf>. 15 Aug 2009 (2009/08/15)	1-9
A	PubChem BioAssay AID 449703, "NOVARTIS: Inhibition of Plasmodium falciparum 3D7 (drug-susceptible) proliferation in erythrocyte-based infection assay". PubChem BioAssay Database, National Center for Biotechnology Information. Deposit Date: 08 July 2010. Retrieved from URL: <https://pubchem.ncbi.nlm.nih.gov/bioassay/449703>. 08 Jul 2010 (2010/07/08) See PubChem CID 1450647.	1-9

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US2018/053511

Patent document cited search report	Publication date	Patent family member(s)	Publication Date
US 2015/0140071 A1	21 May 2015	US 2015140071 A1	21 May 2015
		US 9636340 B2	02 May 2017
		US 2017231975 A1	17 Aug 2017
WO 2014/207213 A1	31 Dec 2014	WO 2014207213 A1	31 Dec 2014