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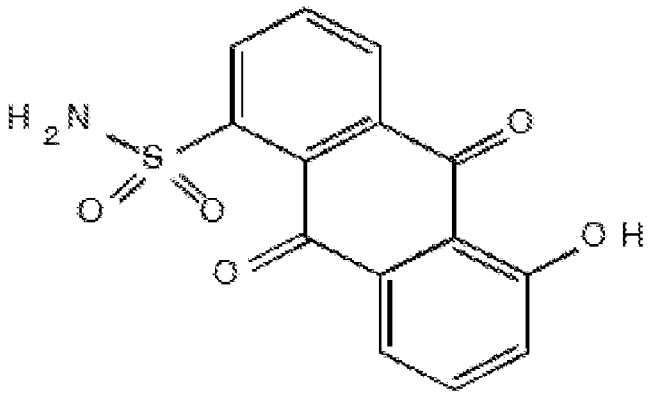
[Continued on next page]

(54) Title: MATERIALS AND METHODS TO INHIBIT MULTIPLE MYELOMA CANCER CELLS

(57) Abstract: Described herein are molecules useful to affect cancer cells, along with related methods. The present compounds, formulations, kits and methods are useful for diagnostic, therapeutic and research. STAT3 inhibitors, particularly LLL12, are disclosed. The STAT3 inhibitors are useful to treat breast cancer in general and breast cancer initiating cells in particular.



LLL12

Figure 6

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— *with sequence listing part of description (Rule 5.2(a))*

TITLE

MATERIALS AND METHODS TO INHIBIT MULTIPLE MYELOMA CANCER CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of United States Provisional Application No. 61/432,826 filed January 14, 2011, the disclosures of which is incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with Government support under grant R21CA133652-01 awarded by the National Institutes for Health (NIHR21). The government has certain rights in the invention.

SEQUENCE LISTING

[0002.1] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on January 13, 2012, is named 604_52589_SEQ_LIST_OSURF-11099.txt and is 3,055 bytes in size.

TECHNICAL FIELD OF THE INVENTION

[0003] The present invention is based on the observation that a certain class of small molecules inhibits multiple myeloma cancer cells. Therefore, the present invention is in the fields of biochemistry, molecular biology and medicine.

BACKGROUND OF THE INVENTION

[0004] Multiple Myeloma (MM) is the second most common hematologic malignancy and accounts for about 20,000 new diagnoses annually in the United States. The incidence of the disease is rising and, despite the advent of agents such as lenalidomide and bortezomib, the disease remains incurable and new therapies are desperately needed.

[0005] Signal Transducers and Activators of Transcription (STAT) proteins are transcription factors which normally mediate orderly and tightly regulated signaling processes initiated through extracellular cytokines and growth factors. On the other hand, constitutive activation of STATs has been demonstrated to contribute to oncogenesis and STAT3 in particular, is considered to be an oncogene due to its ability to promote malignancy. STAT3 activation occurs through phosphorylation of the tyrosine 705 (Tyr_{705}) residue, leading to dimerization and translocation from the cytoplasm to the nucleus. In the nucleus, STAT3 binding to target genes induces the transcription and up regulation of proliferation and anti-apoptotic associated proteins.

[0006] Constitutively active STAT3 may be sufficient for inducing cellular transformation and resistance to transformation was observed in STAT3-deficient cells. STAT3 is frequently activated in many types of human solid and blood cancer and contribute to cancer progression. The STAT3 signaling pathway is especially important in the proliferation, chemoresistance, and survival of MM cells through constitutive phosphorylation of STAT3 or in response to interleukin (IL)-6 produced by cells in the bone marrow microenvironment or by MM cells, per se. Inhibition of constitutive STAT3 signaling by a dominant-negative mutant, a JAK2 inhibitor (AG490), and other strategies leads to apoptosis in MM cells.

[0007] While STAT3 may be important for normal embryologic development, it appears to be less important for the function of differentiated tissues. For example, no discernable deleterious effects were observed when STAT3 antisense therapy was used to deplete protein from normal cells in mice. Furthermore, fibroblasts deficient in STAT3 exhibited similar proliferative capacities compared to their wild-type counterparts, similar survival *in vitro*, and responded appropriately to several growth factors.

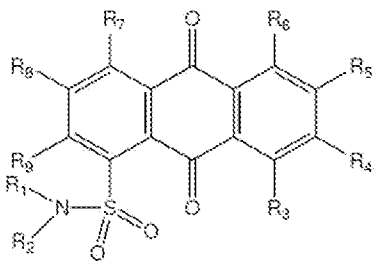
[0008] Using structure-based drug design, the present inventors developed a STAT3 inhibitor, named LLL12 (Figure 6). The inventors herein have now shown, inter alia, that LLL12 and similar molecules inhibit multiple myeloma cancer cells.

[0009] This invention therefore contributes effective therapeutic, diagnostic and prophylactic agents having increased positive results and fewer side effects. The invention also provides methods for making related compounds, formulations, compositions, kits, etc.

SUMMARY OF THE INVENTION

[0010] Herein, the inventors characterize the effects of LLL12 on MM tumor cells. The inventors demonstrate that LLL12 exhibits high specificity for inhibiting STAT3 phosphorylation and leads to down regulation of STAT3-modulated proliferation and survival genes. LLL12 inhibits proliferation and induces apoptosis of primary, human MM cells *in vitro*, even in samples procured from patients with MM clinically resistant to lenalidomide and bortezomib. LLL12 suppresses *in vivo* MM tumor growth in a mouse xenograft model.

[0011] The present invention therefore provides compounds of the Formula I:



wherein

i. R1, R2 are independently hydrogen or alkyl M, wherein M is 1, 2, 3, 4, 5 or 6 carbons;

ii. R3, R5, R6, R8, R9 are independently hydrogen, alkyl, alkoxy, halogen, NO_2, NH_2, or hydroxyl; and

iii. R4, R7 are independently alkyl, alkoxy, O-alkyl, N-alkyl, aromatic, heteroaromatic, cyclic, or heterocyclic.

Also provided is a pharmaceutically-formulation of LLL12, a compound of Formula I, wherein R1, R2, R3, R4, R5, R7, R8 and R9 are each hydrogen and R6 is hydroxyl.

[0012] The present invention also includes methods to inhibit at least one multiple myeloma cancer cell, comprising introducing a compound of Formula I to at least one multiple myeloma cancer cell, and inhibiting at least one multiple myeloma cancer cell.

[0013] Preferred are those methods wherein the compound of Formula I is LLL12, or a pharmaceutically-acceptable formulation thereof. Preferred are those methods wherein the inhibition is measured by observing cell apoptosis. Preferred are those methods wherein the inhibition is measured by observing prevention of STAT3 SH2 dimerization. Preferred are those methods wherein the inhibition is measured by observing a decrease in the levels of expression of STAT3 phosphorylation. Preferred are those methods wherein the inhibition is measured by observing inhibition of downstream targets of STAT3, in particular those wherein the downstream targets are selected from the group comprising: cyclin; Bcl-2; and surviving, or those wherein the inhibition is measured by observing induction of cleaved PARP and caspase-3.

[0014] Also preferred are those methods wherein the cells are resistant to a chemotherapy drug. Particularly, preferred are those wherein the cells are resistant to lenalidomide, and/or those wherein the cells are resistant to bortezomib.

[0015] Also provided are methods herein wherein LLL12 inhibition is measured by determining an IC50 value from between 0.26μM and 1.96μM. Also provided are methods herein wherein LLL12 inhibition is measured by reduced proliferation of cells. Also provided are methods herein wherein LLL12 inhibition is measured by reduced resistance to lenalidomide.

[0016] Also provided are methods to decrease the ability of multiple myeloma cells to proliferate, comprising a administering a compound of Formula I to a multiple myeloma cell-containing medium. Also provided are such methods wherein the multiple myeloma cell-containing medium is a mammalian cell culture. Also provided are such methods wherein the multiple myeloma cell-containing medium is a mammal. In particular, there are provided methods wherein the mammal is selected from the group comprising: human; livestock; companion animal; research mammal; and zoo animal. Particularly preferred are such methods wherein the mammal is a mouse.

[0017] Also provided are methods to treat multiple myeloma cancer in a patient in need of such treatment, comprising administering a therapeutically-effective pharmaceutically-acceptable formulation of at least one compound of Formula I. Preferred are such methods wherein the compound of Formula I is LLL12. Preferred are such methods which further comprise administering to the patient at least one additional chemotherapeutic drug. Preferred are such

methods wherein the additional chemotherapeutic drug is lenalidomide. Preferred are such methods wherein the additional chemotherapeutic drug is bortezomib. Preferred are such methods wherein the additional chemotherapeutic drugs are lenalidomide and bortezomib.

[0018] Definitions

[0019] "Observing" means ascertaining physical (including chemical, biological, crystallographical) attributes, via scientifically-reliable assay, including optional use of any scientifically-reliable assay(s) described herein, and optional use of computer generation and/or analysis of the results of any assay(s).

[0020] "MM.1S" and "MM1.S" are interchangeable names for a human-source multiple myeloma cell line known in the art, and available from ATCC, Lonza or other non-commercial sources.

[0021] All other terms herein have the meaning as understood in the global scientific art (in the case of a scientific term) and/or in general U.S. English usage (in the case of non-scientific terms).

[0022] Various objects and advantages of this invention will become apparent to those skilled in the art from the following detailed description, when read in light of the accompanying drawings.

BRIEF DESCRIPTION OF THE FIGURES

[0023] The application contains one or more figures executed in color and/or one or more photographs. Copies of color figures(s) and/or photograph(s) will be provided upon request and payment of the necessary fee.

[0024] **Figures 1A-1E**. LLL12 inhibited constitutive and cytokine-induced STAT3 phosphorylation, reduced expression of downstream STAT3 targets, and exhibited high specificity for STAT3.

[0025] **Figure 1A, Figure 1B:** The human MM cell lines U266 (**Figure 1A**) and ARH-77 (**Figure 1B**) exhibit constitutively phosphorylated STAT3. LLL12 (2.5μM or 5μM) inhibited STAT3 phosphorylation at Tyr705, resulting in induction of apoptosis as shown by cleaved PARP, caspase-3 and caspase 8. Other signaling pathways were not affected by LLL12 (mTOR, Src, and ERK1/2). GAPDH is shown as a loading control.

[0026] **Figure 1C:** LLL12 blocked STAT3 phosphorylation in purified, freshly isolated primary, human MM tumor cells from multiple myeloma patient (M.M.P., representative result in n=3 independent patient samples) leading to PARP and caspase-3 cleavage.

[0027] **Figure 1D:** LLL12 was more potent than Stattic (2 hours pre-treatment) in inhibiting IL-6-induction of STAT3 phosphorylation in the MM.1S cell line.

[0028] **Figure 1E:** LLL12 inhibited STAT3 but not STAT1 and STAT2 phosphorylation induced by interferon-α (25ng/ml) in the MM.1S cell line. Cells were pre-treated with LLL12 and/or Stattic for 2 hours, then were stimulated with IL-6 or interferon-α for 30-minutes in the presence or absence of LLL12.

[0029] **Figures 2A-2D.** LLL12 inhibited STAT3 DNA binding activity and expression of downstream targets associated with proliferation and survival of MM tumor cells.

[0030] **Figure 2A:** U266 (left) and ARH-77 (right) MM cells were treated in LLL12 (2.5 - 10μM) or DMSO for 24 hours and nuclear extracts were examined for DNA binding activity. LLL12 induced statistically significant (*) reductions in STAT3 DNA binding activity, results are representative of two independent experiments in each cell line.

[0031] **Figure 2B:** U266 (left) and ARH-77 (right) human MM cell lines were cultured in LLL12 (5 μM) or DMSO for 24 hours. Reverse transcriptase PCR reveals decreased expression of STAT3 target genes in LLL12-treated cells as compared to DMSO control following treatment with LLL12.

[0032] **Figure 2C:** U266 (left) and ARH-77 (right) - Downstream STAT3 target proteins, Cyclin D1, Survivin, Bcl-2, and DNMT1 were downregulated by LLL12 as shown by western blot analysis.

[0033] **Figure 2D:** Downstream STAT3 target proteins, Survivin, Bcl-2 and Bcl-XL were downregulated by LLL12 as shown by western blot analysis.

[0034] **Figures 3A-3B.** LLL12 induced apoptosis in U266 and ARH-77 multiple myeloma cells.

[0035] **Figure 3A:** Representative histograms of U266 and ARH-77 MM cells cultured for 24 hours in DMSO or LLL12 (2.5μM or 5μM) following annexin V/ PI staining and flow cytometric analysis.

[0036] **Figure 3B:** U266 (left) and ARH-77 (right) LLL12 led to dose-dependent, statistically significant (*) increases in apoptosis of MM cells, $p < 0.05$ in all instances, results are representative of at least 3 independent experiments.

[0037] **Figures 4A-4G.** LLL12 inhibited cell proliferation induced by IL-6 and blocked IL-6 mediated drug resistance in human MM cells.

[0038] **Figure 4A:** MM.1S cells were treated with IL-6 (25ng/ml) with or without LLL12. IL-6 increases cell proliferation, which could be blocked by LLL12.

[0039] **Figure 4B:** Treatments of MM.1S cells with lenalidomide caused a dose-dependent reduction of cell viability. IL-6 reversed lenalidomide induced inhibition of cell viability. Pre-treatment of MM.1S cells with LLL12, further reversed the rescue of lenalidomide-mediated inhibition of cell viability by IL-6.

[0040] **Figure 4C:** U266 cells secreted higher levels of IL-6 than MM.1S cells.

[0041] **Figure 4D:** U266 cells expressed higher levels of IL-6 than MM.1S cells. Both MM cell lines expressed similar levels of IL-6R and Gp130.

[0042] **Figure 4E:** U266 cells expressed higher levels of STAT3 phosphorylation than MM.1S cells.

[0043] **Figure 4F:** U266 cells were more resistance to lenalidomide than MM.1S cells.

[0044] **Figure 4G:** LLL12 enhanced lenalidomide-mediated inhibition of cell proliferation in U266 MM cells.

[0045] **Figures 5A-5E.** LLL12 abrogated *in vivo* MM tumor cell growth. ARH-77 MM flank tumors were established in 12 NOD/SCID mice, then randomized to receive intraperitoneal injection with 5mg/kg of LLL12 or DMSO. Tumor volume was measured every other day and tumor mass was determined after 26 days.

[0046] **Figure 5A, Figure 5B, Figure 5C:** LLL12 statistically significantly (*, all comparisons shown $p < 0.05$) impaired MM tumor cell growth as assessed by serial volume measurements (**Figure 5A**), serial tumor weight measurements (**Figure 5B**), and tumor mass (**Figure 5C**).

[0047] **Figure 5D:** Western blotting demonstrated inhibition of STAT3 phosphorylation in response to LLL12 therapy.

[0048] **Figure 5E:** The bodyweights of LLL12-treated mice were not decreased and similar to that of the vehicle-treated mice over 15-day of treatments.

[0049] **Figure 6.** LLL12 structure.

[0050] **Figure 7.** LLL12 (2 hours exposure at 10μM) inhibited cytokine-induction of STAT3 phosphorylation in the IM-9 MM cell line (which does not express constitutive STAT3 phosphorylation). However, LLL12 did not inhibit phosphorylation of STAT1, STAT2, STAT4, and STAT6 induced by 30-min stimulation with 25ng/ml of interferon-α interferon-γ, and IL-4.

[0051] **Figure 8**. LLL12 inhibited nuclear localization of STAT3. Left panels show nuclear localization of STAT3 (red) in DMSO treated U266 human MM cells whereas right panels show that LLL12 treatment leaded to STAT3 being retained in cytoplasm. The nuclei were stained with DAPI (blue).

[0052] **Figures 9A-9C.** LLL12 inhibited the cell viability of CD138 (+) primary cells, but had no effect on cell viability of peripheral blood mononuclear cell (PBMC) and CD138 (-) marrow fraction.

[0053] **Figure 9A:** PBMC were cultured for 7 days in RPMI media with 10% FBS and conditions as shown above. LLL12 had no effect on cell viability (representative result from n=3 donors).

[0054] **Figure 9B:** Bone marrow aspirate was obtained from a patient with MM. CD138 (+) cells (most of them are *myeloma* cells) were isolated by passive selection technique. The CD138 (-) marrow fraction and the CD138 (+) cells were cultured in DMSO or LLL12 (10μM) for 24h and viability was measured by MTS assay (5 replicates per condition). LLL12 suppressed viability of CD138 (+) cells by 44% ($p < 0.01$) but did not affect viability of CD138 (-) cells.

[0055] **Figure 9C:** IL-6 independent MM cell line RPMI-8226 were treated in LLL12 (2.5μM) or DMSO for 24 hour. LLL12 did not induce cleaved caspase-3 and cleaved PARP in this cell line.

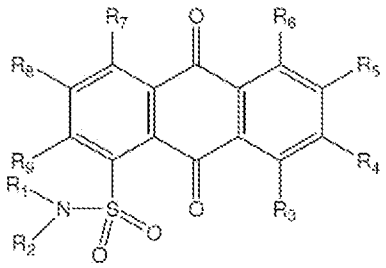
[0056] **Figure 10.** Primers used (**SEQ ID NOS: 1-12**, respectively, in order of appearance).

DETAILED DESCRIPTION

[0057] MM remains incurable despite the advent of therapies and, with the incidence of the disease rising, new treatments are urgently needed. STAT3 plays a crucial role in MM, proliferation, resistance to apoptosis, and survival and represents an important molecular target for the development of therapies. However, to date, the translation of anti-STAT3 therapies into clinical trials has been difficult. Previous methods aimed at blocking STAT3 have included the use of RNA interference, STAT3 antisense oligonucleotides, and dominant negative STAT3. Although the stated approaches have been successful limitations apply to the RNA or antisense oligonucleotides delivery and stability. No direct STAT3 inhibitors have begun assessment in clinical studies to date.

[0058] The inventors herein describe the effects of compounds of the Formula I on multiple myeloma (MM) cells.

[0059] The compounds of the Formula I are:



wherein

i. R1, R2 are independently hydrogen or alkyl M, wherein M is 1, 2, 3, 4, 5 or 6 carbons;

ii. R3, R5, R6, R8, R9 are independently hydrogen, alkyl, alkoxy, halogen, NO_2, NH_2, or hydroxyl; and

iii. R4, R7 are independently alkyl, alkoxy, O-alkyl, N-alkyl, aromatic, heteroaromatic, cyclic, or heterocyclic.

[0060] LLL12 is a compound of Formula I wherein R1, R2, R3, R4, R5, R7, R8 and R9 are each hydrogen and R6 is hydroxyl. LLL12 specifically inhibited STAT3 phosphorylation, nuclear localization, DNA binding activity down-regulated STAT3 downstream genes, and induced apoptosis in MM cells.

[0061] LLL12 significantly inhibited STAT3 phosphorylation, induced apoptosis in primary MM cells which came from patients that were clinically resistant to lenalidomide and bortezomib.

[0062] LLL12 is a potent inhibitor of cell proliferation with IC_{50} values ranging between 0.26μM and 1.96μM in MM and primary MM cells. LLL12 also inhibited STAT3 phosphorylation induced by interleukin-6 (IL-6) and interferon-α, but not STAT1, STAT2, STAT4, and STAT6 phosphorylation induced by interferon-α, interferon-γ, and interleukin-4 indicating the selectivity of LLL12 for STAT3. The selectively of LLL12 on STAT3 was further demonstrated on 21 protein kinases, which LLL12 had IC_{50} values ≥73.92μM. In addition, IL-6 promoted the cell proliferation

and resistance to lenalidomide in MM cells.

[0063] However, the pre-treatment of LLL12 blocked the promotion of the cell proliferation and resistance to lenalidomide by IL-6.

[0064] Furthermore, LLL12 significantly blocked tumor growth of MM cells in mouse model. The results show that LLL12 blocks constitutive STAT3 and IL-6 induced STAT3 signaling.

[0065] LLL12 can be synthesized using more than one method. In one embodiment, LL12 can be synthesized by reacting an unsubstituted or substituted naphthalene sulfonyl chloride compound with a nitrogen containing compound to form an unsubstituted or substituted naphthalene sulfonyl amine; oxidizing the unsubstituted or substituted naphthalene sulfonyl amine of step i) to yield an unsubstituted or substituted naphthoquinone compound; and, catalyzing via a Diels-Alder reaction of 3-hydroxy-2-pyrone with the unsubstituted or substituted naphthoquinone compound of step ii) to yield a compound of Formula I. In certain syntheses, the nitrogen containing compound of step i) comprises ammonium hydroxide and the naphthalene sulfonyl chloride is unsubstituted.

[0066] LLL12 is a small molecule inhibitor of STAT3 with high specificity to the Tyr_{705} residue mediating activation of STAT3 signaling via phosphorylation. Herein, the inventors present the data characterizing LLL12 as a therapy for MM. For example, LLL12 prevents phosphorylation of STAT3 and this effect is associated with caspase-mediated apoptosis in MM cells. In addition to the constitutive activity of STAT3 in MM cell lines and primary tumor samples, the data show that LLL12 prevents STAT3 activation induced through cytokine stimulation, including IFN-α and IL-6. These effects appear STAT3-specific, in that no inhibitory effects of LLL12 on other STAT-family members such as STAT1, STAT2, STAT4, and STAT6 upon IFN-α, IFN-γ, and IL-4 stimulation.

[0067] In blocking STAT3 phosphorylation, the inventors demonstrate that LLL12 prevents STAT3 nuclear localization as well as DNA binding activity. Additionally, LLL12 leads to down regulation of expression of downstream targets of STAT3 that are involved in proliferation and survival of MM cells, providing further mechanistic detail regarding the pro-apoptotic effects of LLL12 in MM. These effects culminate in statistically significant, deleterious effects on MM cell *in vitro* viability as well as impaired *in vivo* MM tumor cell growth in a murine model.

[0068] IL-6 is a key cytokine mainly produced by myeloma cells and a variety of other cells in the marrow microenvironment. A high IL-6 serum level is often associated to worse progression-free survival and overall survival in myeloma. IL-6 exerts its biological effects through binding to two signal transducing receptor subunits gp130 and IL-6R. IL-6 binding results in gp130 and IL-6R dimerization and in the subsequent activation of Janus kinases/STAT3 pathway.

[0069] The inventors observed that IL-6 increases cell proliferation/viability in MM.1S cells and this is inhibited by small molecule, LLL12. This is consistent with the stimulation of STAT3 phosphorylation by IL-6 and inhibited by LLL12 in MM.1S cells.

[0070] Treatments of MM.1S cells with lenalidomide also caused a dose-dependent reduction of cell viability and were reversed with IL-6 treatment. However, pre-treatment with LLL12

reversed the rescue of lenalidomide-mediated inhibition of cell viability by IL-6.

[0071] The inventors also observed U266 cells express higher levels of IL-6 and STAT3 phosphorylation compared to the MM.1S cells. Interestingly, U266 cells are also more resistant to lenalidomide than MM.1S cells. Treatment of LLL12 blocks lenalidomide-resistant in U266 cells showing that LLL12 is likely to enhance the effects of lenalidomide through the inhibition of IL-6/P-STAT3 pathway in U266 cells. Therefore, these results show that IL-6 conferred resistance to lenalidomide and this promoting activity was blocked by STAT3 inhibitor LLL12.

[0072] Constitutive STAT3 activity has also been implicated in chemoresistance against a number of effective anti-MM therapies. While not wishing to be bound by theory, it is also now believed that STAT3 is also implicated in emergent resistance to lenalidomide and bortezomib. For example, curcumin, a natural product derived from *Curcuma longa*, may enhance the effects of anti-cancer agents against MM and other cancers through STAT3 inhibition. However, curcumin also has a number of off-target effects in addition to STAT3 inhibition and doses associated with biologic activity are associated with clinical toxicities. In contrast, LLL12 is not a derivative from curcumin and the inhibitory effects of LLL12 appear STAT3-selective, in that no effects of LLL12 on twelve other human protein kinases were observed.

[0073] Interestingly, in patient samples procured from patients with disease clinically-resistant to lenalidomide and bortezomib, the inventors still observed potent anti-STAT3 effects with LLL12 and related induction of apoptosis, showing that, by inhibiting STAT3 survival pathway, LLL12 is able to overcome drug resistance in MM.

[0074] *Compound assessments for drug-likeness:*

[0075] Drug-likeness characteristics of LLL12 were evaluated using QikProp (Schrodinger LLC). The absorption, distribution, metabolism, excretion, and toxicity (ADME/Tox) of LLL12 were computed. Fifty "drug-likeness" parameters were evaluated, including molecular weight, polarity, solubility, cell permeability, blood brain barrier, HERG K+ blockage, HSA binding, metabolic stability, and more. LLL12 showed desirable "druglike" properties. Selected highlights are listed here: (1) possible *in vivo* metabolic reactions range only from 1 to 3; (2) composite logP values range from -2 to 2; (3) predicted IC50 values for HERG K+ channels are around -3, well above -5 for any concern; (4) predicted Caco-2 and MCDK cell permeability values are acceptable; (5) predicted brain/blood partition coefficients are above -3; (6) predicted index of binding to human serum albumin ranges from -0.5 to -0.8, well within recommended range of - 1.5 – 1.5; (7) predicted human oral absorption percentage is around 60%. Compared to existing drugs, LLL12 is 90% similar to Sulfacytine and Chlorthalidone. Overall, LLL12 is worthy of medicinal chemistry research effort for further optimization.

[0076] *In Vitro Methods:*

[0077] Also provided herein are *in vitro* methods for selectively inhibiting multiple myeloma cancer cells, cell growth, proliferation and migration arrest and/or apoptosis of multiple myeloma

cancer cells, by contacting the cells with an effective amount of a composition containing a compound herein, or a pharmaceutically acceptable salt or hydrate thereof. Competitive assays using the present compounds, tissue localization assays, toxicology screens, etc. using the presently-invented compounds, compositions, formulations, etc. are within the scope of the present invention.

[0078] Although the methods can be practiced *in vitro*, it is contemplated that, in certain embodiments, the preferred embodiments for the methods comprise contacting the cells *in vivo*, i.e., by administering the compounds to a subject harboring cancer cells in need of treatment.

[0079] The present invention, at least in part, is further defined in the following Examples, in which all parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. All publications, including patents and non-patent literature, referred to in this specification are expressly incorporated by reference. The following examples are intended to illustrate certain preferred embodiments of the invention and should not be interpreted to limit the scope of the invention as defined in the claims, unless so specified. Data are presented as Mean ± SEM and compared using Student's t-test. Significance was accepted at $p<0.05$.

[0080] *EXAMPLES*

[0081] *Example 1*

[0082] *Cell lines and primary MM tumor cells*

[0083] Human MM cell lines (U266, ARH-77, IM-9, and MM.1S) were purchased from the American Type Culture Collection (Manassas, VA). MM cell lines were maintained in RPMI1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 4.5 g/L L-glutamine, sodium pyruvate, and 1% penicillin/streptomycin and maintained in a humidified 37°C incubator with 5% CO_2. Primary MM tumor cells were obtained with written informed consent from patients with MM under an IRB-approved procurement protocol and isolated by positive selection utilizing EasySep CD138(+) magnetic nanoparticles per manufacturer's instructions (StemCell Technologies, Vancouver, BC).

[0084] *Small molecular JAK2, STAT3 inhibitors and Lenalidomide*

[0085] LLL12, a STAT3 inhibitor , and WP1066, a JAK2 inhibitor, were synthesized at The Ohio State University (P-K Li, College of Pharmacy). AG490, a JAK2 inhibitor, Stattic and S3I-201, two STAT3 SH2 inhibitors, were purchased from Calbiochem (Darmstadt, Germany). Lenalidomide was purchased from LC Laboratories (Woburn, MA). Drugs were dissolved in sterile dimethyl sulfoxide (DMSO) to make 20mM stock solution, stored at -20°C until use.

[0086] *Protein kinase activity assay*

[0087] The effects of LLL12 on twenty one purified human protein kinases were performed at

Millipore UK Limited (Dundee, UK) using a validated kinase profiler assay with appropriate kinase inhibitors, which gave half-maximal inhibitory concentrations (IC_{50}) values at nM ranges as positive controls. The IC_{50} inhibitory values of LLL12 on kinase activity were determined using 10 different concentrations of LLL12 (up to 100 μM).

[0088] Cell Viability Assay

[0089] U266, ARH-77, and primary MM cells (3,000-5,000/well in 96-well plates) incubated with indicated concentrations of compounds in triplicate at 37°C for 72 hours. 3-(4, 5-Dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) viability assay was performed according to manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). The absorbance was recorded at 595 nm. IC_{50} values were determined using Sigma Plot 9.0 Software (Systat Software Inc., San Jose, CA). The primary MM tumor cells (CD138+) and CD138-marrow fraction were from a patient with MM and were cultured in DMSO or LLL12 (10μM) for 24h. Cell viability was measured by MTS assay (5 replicates per condition).

[0090] *Nuclear staining*

[0091] To examine whether LLL12 inhibits STAT3 nuclear localization, the immunofluorescence staining was performed according to the protocol from the Cell Signaling Technology. Briefly, after wash once with Phosphate Buffered Saline (PBS) buffer, U266 MM cells were blocked with 5% normal goat serum for 1 hour and incubated with rabbit polyclonal anti-human STAT3 antibody (Cell Signaling Technology, Beverly, MA, 1/100 dilution) overnight. Then, cells were washed and incubated with goat anti-rabbit IgG Alexa Fluor 594 (1/100) for 1 hour and counterstained for nuclei with Hoechst (50 ng/ml) for 5 min. Stained slides were mounted with mounting medium (Vector Laboratories, Burlingame, CA) and analyzed under a fluorescence microscope (Zeiss Axioskop microscope, Carl Zeiss, Gottingen, Germany). Pictures were captured using an AxioCam HRc camera and Axiovision 3.1 software (Carl Zeiss, Gottingen, Germany).

[0092] *Western Blot analysis*

[0093] MM cells were treated with LLL12 (5μM or 10μM) or DMSO for 24 hours before cells were collected for Western blot analysis. For interferon-α (IFN-α), interferon- γ (IFN-γ), IL-4, and IL-6 stimulation experiments, IM-9 and MM.1S MM cells were serum-starved for 24 hours and left untreated or pre-treated with LLL12 (2.5-10 μM), Stattic (2.5-20 μM) or DMSO for 2 hours. Then, 25ng/ml IFN-α, IFN-γ, or IL-4 was added, the cells were harvested for Western Blot analysis 30 minutes later. MM cells were lysed in cold RIPA lysis buffer containing protease inhibitors and subjected to SDS-PAGE. Proteins were transferred on to PVDF membrane and probed with antibodies. Antibodies (Cell Signaling Technology, Beverly, MA) against phospho-specific STAT1 (Tyrosine 701), phospho-specific STAT2 (Tyrosine 690), phospho-specific STAT3 (Tyrosine 705), phospho-specific STAT4 (Tyrosine 693), phospho-specific STAT6 (Tyrosine 641), phospho-specific ERK1/2 (Threonine 202/Tyrosine 204), phospho-specific Src (Tyrosine 416), phospho-specific mTOR (Serine 2448), cleaved Poly (ADP-ribose) polymerase (PARP), cleaved caspase-3,

cleaved caspase-8, phospho-independent STAT3 cyclin D, Bcl-2, Twist1, DNMT1, survivin, and GAPDH were used for western blots. Membranes were analyzed using enhanced chemiluminescence Plus reagents and scanned with a Storm Scanner (Amersham Pharmacia Biotech Inc, Piscataway, NJ).

[0094] *STAT3 DNA binding activity*

[0095] U266 and ARH-77 MM cells were treated with LLL12 (2.5μM and 5μM) or DMSO for 24 hours. The nuclear extracts were analyzed for STAT3 DNA binding activity using a STAT3 Transcription Factor Kit (Clontech Inc, Mountain View, CA). Statistical significance ($p < 0.05$) relative to DMSO (vehicle control) is designated by an asterisk in figures.

[0096] *Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)*

[0097] U266 and ARH-77 MM cells were treated with LLL12 (2.5 and/or 5 μM) or DMSO for 24 hours. RNAs of ARH77, U266 and MM.1S cells were collected using RNeasy Kits (Qiagen, Valencia, CA). Primer sequences and source information of STAT3 downstream target genes can be found in **Figure 10**. PCR amplification was done under the following conditions: 5 min at 94°C followed by 25 cycles of 30 seconds at 94°C, 30 sec at 55°C, and 30 seconds at 72°C with a final extension of 5 min at 72°C. Primer sequences **[SEQ ID NOs:1-12]** are shown in **Figure 10**.

[0098] *Flow Cytometry*

[0099] Apoptotic cell death induced by LLL12 was quantified by flow cytometry with Annexin-V / propidium iodide (PI) double staining (BD Pharmingen, San Jose, CA). After treatment with LLL12 or DMSO for 24 hours, U266 and ARH-77 MM cells were harvested and washed with cold PBS. The cell pellet was re-suspended in 1× binding buffer at a concentration of 1 × 106 cells/ml. 100 μl of cell suspension was transferred into another tube. 5 μl of Annexin V-FITC and 5 μl of PI were added for 15 minutes at room temperature (RT) in darkness, and then analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ) within 1 hour.

[00100] *Mouse Xenograft Model*

[00101] $2x10^7$ ARH-77 MM tumor cells were injected subcutaneously into the right flank of 4- to 5- week-old female NOD/SCID mice (Harlan Laboratories, Indianapolis, IN, USA). After 10 days, mice were divided into two treatment groups (n=6 each): (a) control vehicle (100% DMSO) and (b) 5mg/kg of LLL12. LLL12 or DMSO was administered via intraperitoneal injection. Tumor growth was determined by caliper-measured the length (L) and width (W) every other day, and tumor volume was calculated on the basis of the following formula: volume = (π/6) LW2. Bodyweights of mice were measured daily during 14 days period treatments.

[00102] *Statistical Analysis*

[00103] The Student's t test was used for the evaluation of differences of STAT3 DNA binding activity, percentage of apoptosis, and tumor volume between DMSO- and LLL12-treated experiments. IC_{50} values were determined using Sigma Plot 9.0 Software (Systat Software Inc., San Jose, CA). P values of <0.05 were considered significant and all tests were two-sided.

[00104] *Example 2*

[00105] *LLL12 specifically inhibits STAT3 phosphorylation and induces apoptosis in MM cells*

[00106] In the human MM cell lines U266 and ARH-77, which express elevated levels of STAT3 phosphorylation, LLL12 inhibited STAT3 phosphorylation at tyrosine residue 705 (Tyr 705) (**Figure 1A** and **Figure 1B**). Inhibition of STAT3 phosphorylation was associated with induction of apoptosis, as evidenced by PARP, caspase-3, and caspase-8 cleavage (**Figure 1A** and **Figure 1B**). LLL12 also inhibited STAT3 phosphorylation and induced apoptosis in primary, human MM cells from three different patients (**Figure 1C**). Importantly, patients M.M.P. 2 and M.M.P. 3 were clinically resistant to prior therapy with lenalidomide and bortezomib. These primary MM cells were all found to have constitutively active STAT3. Thus, therapy targeting STAT3 inhibition is an effective treatment for patients with lenalidomide and bortezomib resistant disease.

[00107] The inventors also detected the effects of LLL12 on the phosphorylation of mTOR, Src and ERK(1/2). They were not reduced significantly (**Figure 1A** and **Figure 1B**) which showed the selective inhibition of LLL12 to STAT3. In order to characterize the specificity of these effects of LLL12 in MM cells further, the inventors examined whether LLL12 exhibited inhibitory effects on 21 other human protein kinase activities using a standardized, validated kinase profile. LLL12 does not inhibit BMX, BRK, CSK, Fgr, Fyn, JAK3, LcK, Lyn, or ZAP-70 which contain an SH2 domain (Table 1) at achievable, relevant *in vivo* concentrations (IC_{50} are at least 57.19 μM to > 100 μM.

[00108] Furthermore, LLL12 exhibits no relevant inhibition against other protein kinases, including AKT2, CDK1/cyclin B, FAK, FGFR2, IKKβ, JNK1, JNK2, PAK1, PAK2, PDGFR-α, PKC-α, and PKC-δ (IC50 are 91.76 μM to > 100 μM, **Table 1**). These results support the selectivity of LLL12 to inhibit STAT3 and thus induce pro-apoptotic effects in MM specifically through STAT3 inhibition.

Table 1. The effect of LLL12 on kinase activity of 21 human protein kinases

	Protein Kinases	IC50 (μM)	Protein Kinases	IC50 (μM)	Protein Kinases	IC50 (μM)
Tyrosine kinases containing SH2 Domain	BMX	>100	Fgr	57.19	LcK	>100
	BRK	>100	Fyn	>100	Lyn	>100
	CSK	73.92	JAK3	89.70	ZAP-70	>100
Other human protein kinases	AKT2	91.76	IKKb	>100	PAK2	>100
	CDK1/CyclinB	>100	JNK1	>100	PDGFR-a	>100
	FAK	>100	JNK2	>100	PKC-a	>100
	FGFR2	>100	PAK1	>100	PKC-d	>100

[00109] *Example 3*

[00110] *LLL12 inhibits STAT3 but not other STATs phosphorylation induced by interleukin-6, interferon-α, interferon-γ, and IL-4.*

[00111] The relationship between the ambient microenvironment and MM tumor cells is important to maintaining and perpetuating the tumor cell clone via cytokine-mediated signaling in particular, IL-6. The MM.1S human MM cell line, which expresses very lower constitutively phosphorylated STAT3, was utilized to determine whether LLL12 is capable of inhibiting cytokine-induced STAT3 phosphorylation. Pretreatment of MM.1S multiple myeloma cells stimulated by IL-6 and IFN-α respectively with LLL12 prevented phosphorylation of STAT3, but not STAT1 or STAT2 (**Figure 1D** and **Figure 1E**). Another reported STAT3 inhibitor, Stattic, was found to be much less potent than LLL12 in inhibiting IL-6-or IFN-α mediated STAT3 phosphorylation (**Figure 1D** and **Figure 1E**). LLL12 also inhibited STAT3 phosphorylation induced by IFN-α but did not or much less to inhibit the stimulation STAT1 and/or STAT2 phosphorylation by IFN-α or IFN-γ in IM-9 multiple myeloma cells (**Figure 7**). Similarly, LLL12 did not prevent phosphorylation of STAT4 or STAT6 by IL-4 in IM-9 human MM cells. These results further highlight the specificity of LLL12 for STAT3 inhibition without associated effects on other members of the STAT signaling protein family.

[00112] *Example 4*

[00113] *LLL12 inhibits STAT3 nuclear localization, STAT3 DNA binding activity and the expression of STAT3 downstream target genes*

[00114] To confirm the inhibition of STAT3 signaling by LLL12, the inventors examined whether or not LLL12 inhibits STAT3 nuclear localization, because the main function of STAT3 is to function as a transcription factor in nucleus. In U266 MM cells treated with DMSO, STAT3 was mainly observed localized in nuclei (Left panels, **Figure 8**). However, in U266 cells treated with LLL12, most STAT3 was retained in cytoplasm and absent in nuclei (Right panels, **Figure 8**). The inventors also examined the effect of LLL12 on STAT3 DNA binding activity in U266 and ARH-77 MM cells. LLL12 caused a statistically significant inhibition (approximately 30% reduction with 2.5μM and 55% reduction, $p < 0.05$) of STAT3 DNA binding activity in U266 and ARH-77 MM cells (**Figure 2A**). These findings demonstrate that inhibition of STAT3 phosphorylation by LLL12 consequently impairs STAT3 function in MM cells.

[00115] To characterize further the functional effects of LLL12-induced STAT3 inhibition, the inventors examined the transcription of STAT3 downstream target genes involved in proliferation and survival of MM cells by reverse transcriptase PCR, including cyclin D1, survivin, Twist1, DNMT1, and Bcl-2. U266 and ARH-77 MM cell lines were treated with LLL12 or DMSO for 24-h. Reverse Transcriptase PCR was run for cyclin D1, survivin, Bcl-2, Bcl-XL, and DNMT1. LLL12 treatment resulted in an inhibition of the transcription of all five of these STAT3-regulated genes in

both MM cell lines (**Figure 2B**). These downstream target proteins of STAT3 were also down regulated by LLL12 (**Figure 2C**), which confirm the results of the inhibition at mRNA levels (**Figure 2D**).

[00116] *Example 5*

[00117] *LLL12 impairs MM cell proliferation and viability*

[00118] Cell viability assays were conducted to examine the inhibitory affect of LLL12 on U266 ARH-77 human MM cell lines and primary MM cells from two patients. IC50 values after 72 hours of treatment were calculated for LLL12 and compared to other previously characterized compounds: JAK2 inhibitors: WP1066 and AG490, and STAT3 inhibitors: Stattic, LLL3, and S3I-201. Results summarized in Table 2 show that IC_{50} values for LLL12 are 0.26 μM and 1.96 μM in U266 and ARH-77 MM cells respectively, indicating greater or comparable inhibitory potency of LLL12 than other inhibitors tested and comparable to the activity of Stattic (**Table 2**). LLL12 is also potent in primary multiple myeloma cells (M.M.P.4 and M.M.P.5) isolated from two patients that are clinically resistant to lenalidomide and bortezomib (**Table 2**).

Table 2. IC_{50} (mM) of LLL12 and other JAK2 or STAT3 SH2 inhibitors in human multiple myeloma cells

	LLL12	LLL3	WP1066	Stattic	S3I-201	AG490
U266	0.49	2.03	1.38	0.99	8.87	9.50
ARH-77	1.96	11.48	4.97	2.58	35.25	28.20
M.M.P. 4	0.26	6.08	5.03	0.32	>10.0	>10.0
M.M.P. 5	0.45	4.80	0.82	0.49	>5.0	>5.0

[00119] In contrast, LLL12 has little effects on normal human peripheral blood lymphocytes (**Figure 9A**) and normal human marrow [CD138(-)] cells (**Figure 9B**). The inventors also examined the effect of LLL12 on survival of IL-6 independent MM cell lines RPMI-8226. LLL12 did not induce cleaved caspase-3 and cleaved PARP in this cell line (**Figure 9C**). The phosphorylation of STAT3 at tyrosine residue 705 (Tyr 705) of this cell line was too low to be detected (**Figure 9C**).

[00120] To quantify the apoptotic effects of LLL12, U266 and ARH-77 MM cell lines were cultured in LLL12 or DMSO for 24 hours and stained with Annexin V and PI. Representative findings are shown in **Figure 3A**. LLL12 led to a dose-dependent increase in apoptosis, as shown in **Figure 3A** and **Figure 3B**. Apoptosis was observed in 38.2% (+/- 7.5%, $p < 0.05$ compared to DMSO) in U266 MM cells at LLL12 (2.5μM) and 53.3% (+/- 7.1%, $p < 0$, 05 compared to DMSO) at 5μM. Apoptosis of 70.8% (+/- 1.7%, $p < 0.05$ compared to DMSO) of ARH-77 cells was seen at LLL12 (2.5μM) and 73.5% (+/- 4.1, $p < 0.05$ compared to DMSO) at 5μM (**Figure 3B**).

[00121] *Example 6*

[00122] *IL-6 promotes cell viability, confers resistance to growth inhibition induced by lenalidomide and is suppressed by LLL12 in MM.1S human MM cells.*

[00123] To explore the proliferation/cell viability promoting role of IL-6 in MM cells, MM.1S MM cells were stimulated with IL-6 for 48 hours and cells viability was detected by MTT assay. IL-6 increases cell proliferation/viability by two-fold which could be blocked by small molecular STAT3 inhibitor, LLL12 (**Figure 4A**). This is consistent with the stimulation of STAT3 phosphorylation by IL-6 and inhibited by LLL12 in MM.1S cells (see **Figure 1D**).

[00124] Treatments of MM.1S cells with lenalidomide caused a dose-dependent reduction of cell viability (**Figure 4B**). This inhibition of cell viability by was reversed with IL-6 treatment. However, pre-treatment of MM.1S cells with LLL12, further reversed the rescue of lenalidomide-mediated inhibition of cell viability by IL-6 (**Figure 4B**). Thus, these results show that IL-6 conferred resistance to growth inhibition by lenalidomide and this activity was blocked by small molecular STAT3 inhibitor, LLL12.

[00125] *Example 7*

[00126] *LLL12 enhanced the activity of lenalidomide in U266 human MM cells with higher levels of IL-6/P-STAT3*

[00127] The inventors also examined the effects of lenalidomide on U266 human MM cells. The inventors compared the level of IL-6 /P-STAT3 pathway in U266 and MM.1S cell lines. ELISA assay showed that the level of IL-6 in the medium of U266 cells is higher than MM.1S cells (**Figure 4C**). U266 cell line also has higher level of IL-6 gene expression as detected by RT-PCR (**Figure 4D**). Western blot results also showed the phosphorylation of STAT3 (Tyr 705) in U266 is higher than MM.1S cell line (**Figure 4E**). Since U266 cells express higher levels of IL-6 and STAT3 phosphorylation than MM.1S cells, the inventors determined that U266 cells may be more resistant to certain drugs. The results demonstrated that U266 cells were indeed more resistant to lenalidomide compared to MM.1S cells (**Figure 4F**). Furthermore, LLL12 could increase the sensitive of U266 cells to lenalidomide treatment (**Figure 4G**). The Combinatorial Index (CI) value for all the combinations of treatments were less than one (1), indicating synergism between LLL12 and lenalidomide. Thus, while not wishing to be bound by theory, the inventors herein now believe that LLL12 enhances the effects of lenalidomide through the inhibition of IL-6/STAT3 pathway in U266 multiple myeloma cells.

[00128] *Example 8*

[00129] *LLL12 suppresses MM tumor growth in vivo*

[00130] A murine xenograft model was utilized to determine the *in vivo* anti-MM effects of LLL12. 10 days after subcutaneous implantation of $5x10^6$ ARH-77 MM tumor cells in NOD/SCID mice, subjects were randomized to receive daily via intraperitoneal administration of 5 mg/kg LLL12 (6 mice each group) ***or*** DMSO vehicle control (6 mice each group). As shown in **Figures**

5A-5C, LLL12 significantly suppressed tumor volume (**Figure 5A** and **Figure 5C**, $p < 0.05$ for all comparisons indicated), tumor weight (**Figure 5B**, $p < 0.05$), and inhibited STAT3 phosphorylation (**Figure 5D**) compared with DMSO-treated controls. The bodyweights of LLL12-treated mice were not reduced and similar to that of the vehicle-treated mice showing that LLL12 toxicity was minimal (**Figure 5E**). These results demonstrate that LLL12 is potent in suppressing tumor growth of human MM cells *in vivo*.

[00131] *Example 9*

[00132] *Uses of LLL12 and Related Compounds*

[00133] The present invention provides options that are advantageous over previously-known compounds, compositions, formulations, research tools, diagnostics, and therapies. With regard to therapeutic superiority, because the present compounds are selective for STAT3 inhibition, the present compounds do not have the potential toxic side effects of previously-known treatment methods. In other words, in certain embodiments, the present compounds and methods have little or no impact non-cancerous cells. Moreover, the selective nature and potentency of the present compounds allow synergy with conventional anti-cancer agents, thereby reducing the overall toxic load of any given treatment. In effect, the present compounds allow conventional anti-cancer treatments to exert greater effect at lower dosage. In certain non-limiting examples, an effective dose (ED50) for an anti-cancer agent or combination of conventional anti-cancer agents when used in combination with the present compounds can be less than the ED50 for the anti-cancer agent alone. Also, in certain non-limiting embodiments, the therapeutic index (TI) for such anti-cancer agent or combination of such anti-cancer agent when used in combination with a compound herein is greater than the TI for conventional anti-cancer agent regimen alone.

[00134] In yet other embodiments, the method combines the present compounds with other therapies such as chemotherapies and/or radiation therapies, including ionizing radiation, gamma radiation, or particle beams.

[00135] *Dosages and Dosage Schedules*

[00136] The dosage regimen can be selected in accordance with a variety of factors including type, species, age, weight, sex and the type of cancer being treated; the severity (i.e., stage) of the cancer to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound or salt thereof employed. An ordinarily skilled physician or veterinarian can readily determine and prescribe the effective amount of the drug required to treat, for example, to prevent, inhibit (fully or partially) or arrest the progress of the disease.

[00137] Non-limiting examples of suitable dosages can include total daily dosage of between about 25-4000 mg/m^2 administered orally once-daily, twice-daily or three times-daily, continuous (every day) or intermittently (e.g., 3-5 days a week). For example, the compositions can be administered in a total daily dose, or divided into multiple daily doses such as twice daily, and three times daily.

[00138] Other non-limiting examples of suitable dosages and methods of administration can include the intravenous administration directly to the tumor site via a catheter.

[00139] In addition, the administration can be continuous, i.e., every day, or intermittently. The terms "intermittent" or "intermittently" as used herein means stopping and starting at either regular or irregular intervals. For example, intermittent administration may be administration one to six days per week or it may mean administration in cycles (e.g., daily administration for two to eight consecutive weeks, then a rest period with no administration for up to one week) or it may mean administration on alternate days.

[00140] In addition, the compositions may be administered according to any of prescribed schedules, consecutively for a few weeks, followed by a rest period. For example, the composition may be administered according to any one of the prescribed schedules from two to eight weeks, followed by a rest period of one week, or twice daily at a dose for three to five days a week.

[00141] It should be apparent to a person skilled in the art that the various dosages and dosing schedules described herein merely set forth specific embodiments and should not be construed as limiting the broad scope of the invention. Any permutations, variations and combinations of the dosages and dosing schedules are included within the scope of the present invention.

[00142] *Pharmaceutical Compositions*

[00143] The compounds of the invention, and derivatives, fragments, analogs, homologs pharmaceutically acceptable salts or hydrate thereof, can be incorporated into pharmaceutical compositions suitable for oral administration, together with a pharmaceutically acceptable carrier or excipient. Such compositions typically comprise a therapeutically effective amount of any of the compounds described herein, and a pharmaceutically acceptable carrier. Preferably, the effective amount is an amount effective to selectively induce terminal differentiation of suitable neoplastic cells and less than an amount which causes toxicity in a patient.

[00144] Any inert excipient that is commonly used as a carrier or diluent may be used in the formulations of the present invention, such as for example, a gum, a starch, a sugar, a cellulosic material, an acrylate, or mixtures thereof. The compositions may further comprise a disintegrating agent (e.g., croscarmellose sodium) and a lubricant (e.g., magnesium stearate), and in addition may comprise one or more additives selected from a binder, a buffer, a protease inhibitor, a surfactant, a solubilizing agent, a plasticizer, an emulsifier, a stabilizing agent, a viscosity increasing agent, a sweetener, a film forming agent, or any combination thereof. Furthermore, the compositions of the present invention may be in the form of controlled release or immediate release formulations.

[00145] The pharmaceutical compositions can be administered orally, and are thus formulated in a form suitable for oral administration, i.e., as a solid or a liquid preparation. Suitable solid oral formulations include tablets, capsules, pills, granules, pellets and the like. Suitable liquid oral formulations include solutions, suspensions, dispersions, emulsions, oils and the like.

[00146] As used herein, "pharmaceutically acceptable carrier" is intended to include any and all

solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration, such as sterile pyrogen-free water. Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field, which is incorporated herein by reference. Preferred examples of such carriers or diluents include, but are not limited to, water, saline, finger's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

[00147] Non-limiting examples of solid carriers/diluents include, but are not limited to, a gum, a starch (e.g., corn starch, pregelatinized starch), a sugar (e.g., lactose, mannitol, sucrose, dextrose), a cellulosic material (e.g., microcrystalline cellulose), an acrylate (e.g., polymethylacrylate), calcium carbonate, magnesium oxide, talc, or mixtures thereof.

[00148] Non-limiting examples of liquid formulations, pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, emulsions or oils. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Examples of oils are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, mineral oil, olive oil, sunflower oil, and fish-liver oil. Solutions or suspensions can also include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide.

[00149] In addition, the compositions may further comprise binders (e.g., acacia, cornstarch, gelatin, carbomer, ethyl cellulose, guar gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, povidone), disintegrating agents (e.g., cornstarch, potato starch, alginic acid, silicon dioxide, croscarmellose sodium, crospovidone, guar gum, sodium starch glycolate, Primogel), buffers (e.g., tris-HCI, acetate, phosphate) of various pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), protease inhibitors, surfactants (e.g., sodium lauryl sulfate), permeation enhancers, solubilizing agents (e.g., glycerol, polyethylene glycerol), a glidant (e.g., colloidal silicon dioxide), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite, butylated hydroxyanisole), stabilizers (e.g., hydroxypropyl cellulose, hyroxypropylmethyl cellulose), viscosity increasing

agents (e.g., carbomer, colloidal silicon dioxide, ethyl cellulose, guar gum), sweeteners (e.g., sucrose, aspartame, citric acid), flavoring agents (e.g., peppermint, methyl salicylate, or orange flavoring), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), lubricants (e.g., stearic acid, magnesium stearate, polyethylene glycol, sodium lauryl sulfate), flow-aids (e.g., colloidal silicon dioxide), plasticizers (e.g., diethyl phthalate, triethyl citrate), emulsifiers (e.g., carbomer, hydroxypropyl cellulose, sodium lauryl sulfate), polymer coatings (e.g., poloxamers or poloxamines), coating and film forming agents (e.g., ethyl cellulose, acrylates, polymethacrylates) and/or adjuvants.

[00150] In certain embodiments, the active compounds can be prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art.

[00151] It is especially advantageous to formulate oral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

[00152] The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration. For example, the compounds may be administered intravenously on the first day of treatment, with oral administration on the second day and all consecutive days thereafter. The compounds of the present invention may be administered for the purpose of preventing disease progression or stabilizing tumor growth.

[00153] The preparation of pharmaceutical compositions that contain an active component is well understood in the art, for example, by mixing, granulating, or tablet-forming processes. The active therapeutic ingredient is often mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient. For oral administration, the active agents are mixed with additives customary for this purpose, such as vehicles, stabilizers, or inert diluents, and converted by customary methods into suitable forms for administration, such as tablets, coated tablets, hard or soft

gelatin capsules, aqueous, alcoholic or oily solutions and the like as detailed above.

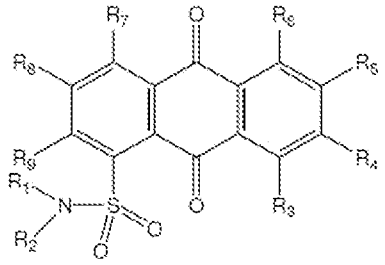
[00154] The amount of the compound or formulation administered to the patient is less than an amount that would cause toxicity in the patient. In the certain embodiments, the amount of the compound that is administered to the patient is less than the amount that causes a concentration of the compound in the patient's plasma to equal or exceed the toxic level of the compound. Preferably, the concentration of the compound in the patient's plasma is maintained at about 10 nM. In another embodiment, the concentration of the compound in the patient's plasma is maintained at about 25 nM. In another embodiment, the concentration of the compound in the patient's plasma is maintained at about 50 nM.

[00155] In another embodiment, the concentration of the compound in the patient's plasma is maintained at ranges between about 10 to about 50 nM. The optimal amount of the compound that should be administered to the patient in the practice of the present invention will depend on the particular compound used and the type of cancer being treated.

CLAIMS

What is claimed is:

1. A compound of the Formula I:



wherein

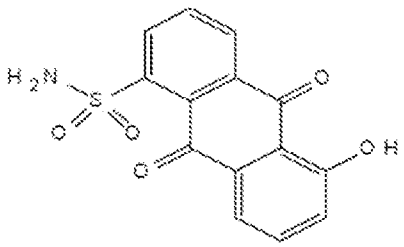
i. R1, R2 are independently hydrogen or alkyl M, wherein M is 1, 2, 3, 4, 5 or 6 carbons;

ii. R3, R5, R6, R8, R9 are independently hydrogen, alkyl, alkoxy, halogen, NO_2, NH_2, or hydroxyl; and

iii. R4, R7 are independently alkyl, alkoxy, O-alkyl, N-alkyl, aromatic, heteroaromatic, cyclic, or heterocyclic, and pharmaceutically-acceptable formulations thereof.

2. A compound of claim 1, wherein R1, R2, R3, R4, R5, R7, R8 and R9 are each hydrogen and R6 is hydroxyl.

3. A compound of claim 1, which is the compound of Formula II:



Formula II

and pharmaceutically-acceptable formulations thereof.

4. A method to inhibit at least one multiple myeloma cancer cell, comprising introducing a compound of Claim 1 to at least one multiple myeloma cancer cell, and inhibiting at least one multiple myeloma cancer cell.

5. A method of claim 4, wherein the compound of Formula I is LLL12, or a pharmaceutically-acceptable formulation thereof.

6. A method of claim 4, wherein the inhibition is measured by observing cell apoptosis.

7. A method of claim 4, wherein the inhibition is measured by observing prevention of STAT3 SH2 dimerization.

8. A method of claim 4, wherein the inhibition is measured by observing a decrease in the levels of expression of STAT3 phosphorylation.

9. A method of claim 4, wherein the inhibition is measured by observing inhibition of downstream targets of STAT3.

10. A method of claim 9, wherein the downstream targets are selected from the group comprising: cyclin; Bcl-2; and survivin.

11. A method of claim 9, wherein the inhibition is measured by observing induction of cleaved PARP and caspase-3.

12. A method of claim 4, wherein the cells are resistant to a chemotherapy drug.

13. A method of claim 12, wherein the cells are resistant to lenalidomide.

14. A method of claim 12, wherein the cells are resistant to bortezomib.

15. A method of claim 5, wherein inhibition is measured by determining an IC_{50} value from between 0.26μM and 1.96μM.

16. A method of claim 5, wherein inhibition is measured by reduced proliferation of cells.

17. A method of claim 5, wherein inhibition is measured by reduced resistance to lenalidomide.

18. A method to decrease the ability of multiple myeloma cells to proliferate, comprising a administering a compound of Claim 1 to a multiple myeloma cell-containing medium.

19. A method of claim 18, wherein the multiple myeloma cell-containing medium is a mammalian cell culture.

20. A method of claim 18, wherein the multiple myeloma cell-containing medium is a mammal.

21. A method of claim 20, wherein the mammal is selected from the group comprising: human; livestock; companion animal; research mammal; and zoo animal.

22. A method of claim 20, wherein the mammal is a mouse.

23. A method to treat multiple myeloma cancer in a patient in need of such treatment, comprising administering a therapeutically-effective pharmaceutically-acceptable formulation of at least one compound of Claim 1.

24. A method of claim 23, wherein the compound of Formula I is LLL12.

25. A method of claim 23, which further comprises administering to the patient at least one additional chemotherapeutic drug.

26. A method of claim 25, wherein the additional chemotherapeutic drug is lenalidomide.

27. A method of claim 25, wherein the additional chemotherapeutic drug is bortezomib.

28. A method of claim 25, wherein the additional chemotherapeutic drugs are lenalidomide and bortezomib.

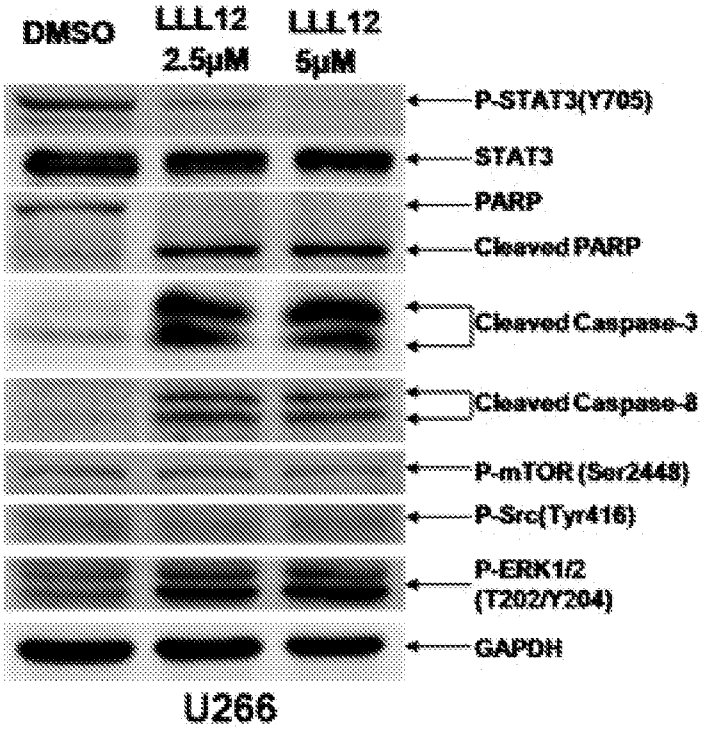


Figure 1A

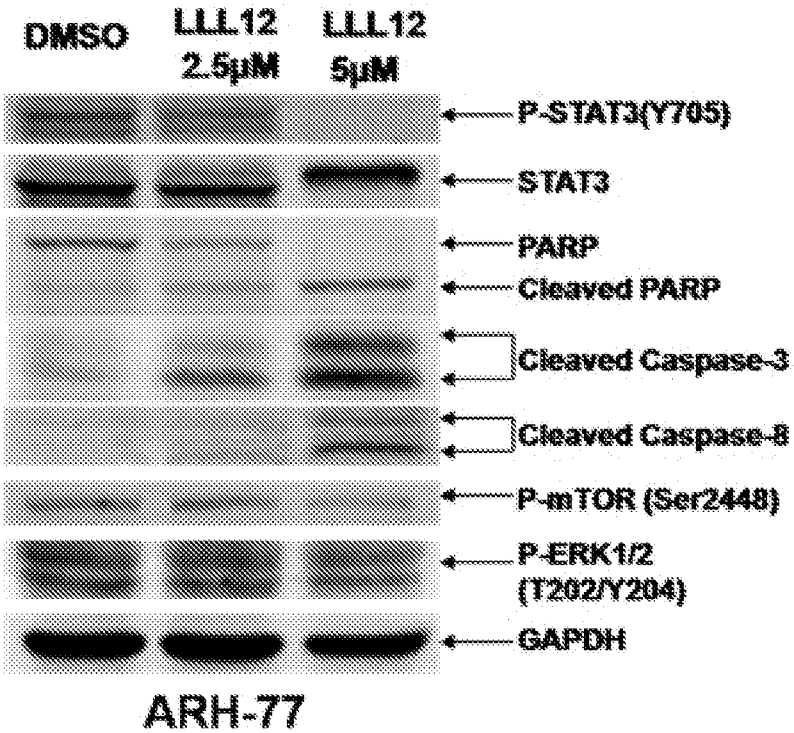


Figure 1B

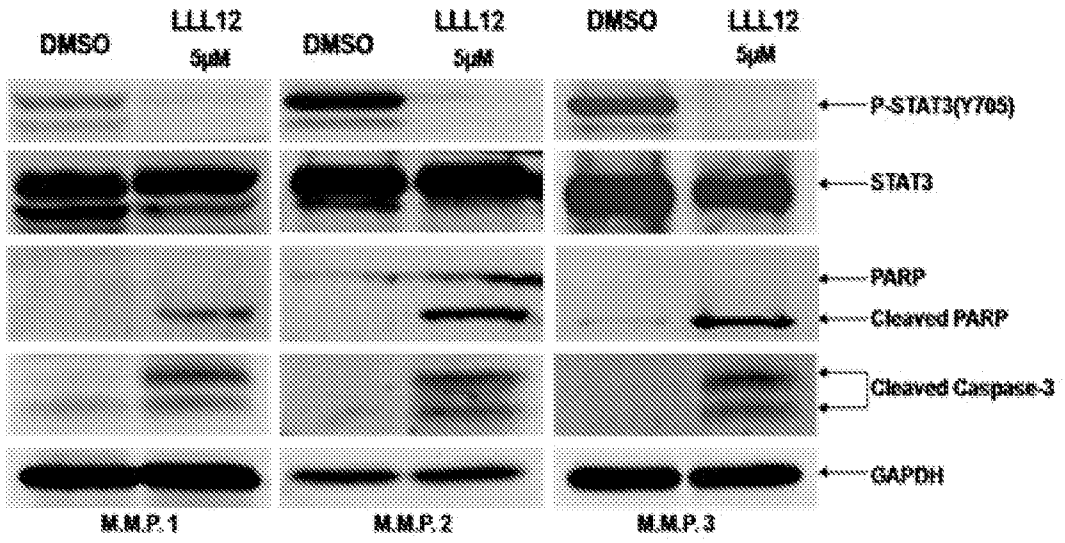


Figure 1C

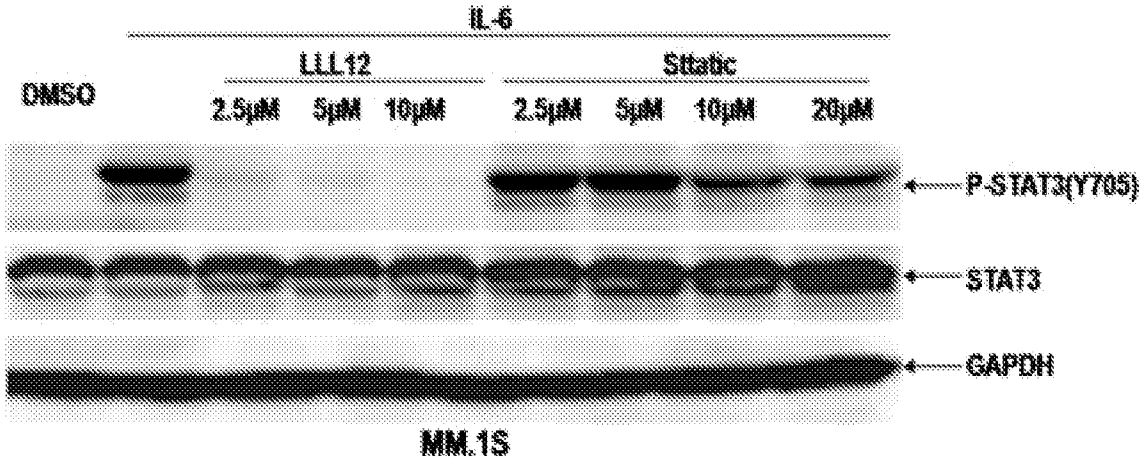


Figure 1D

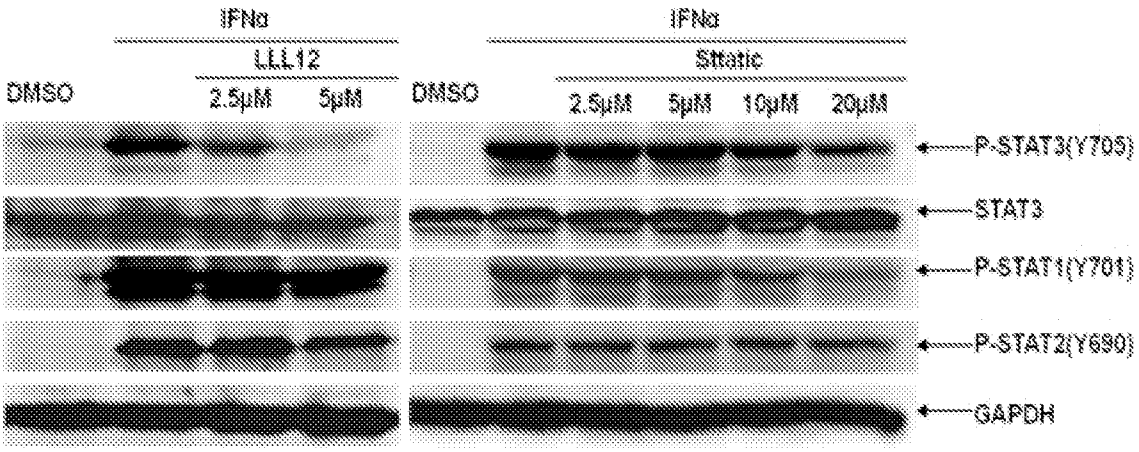


Figure 1E

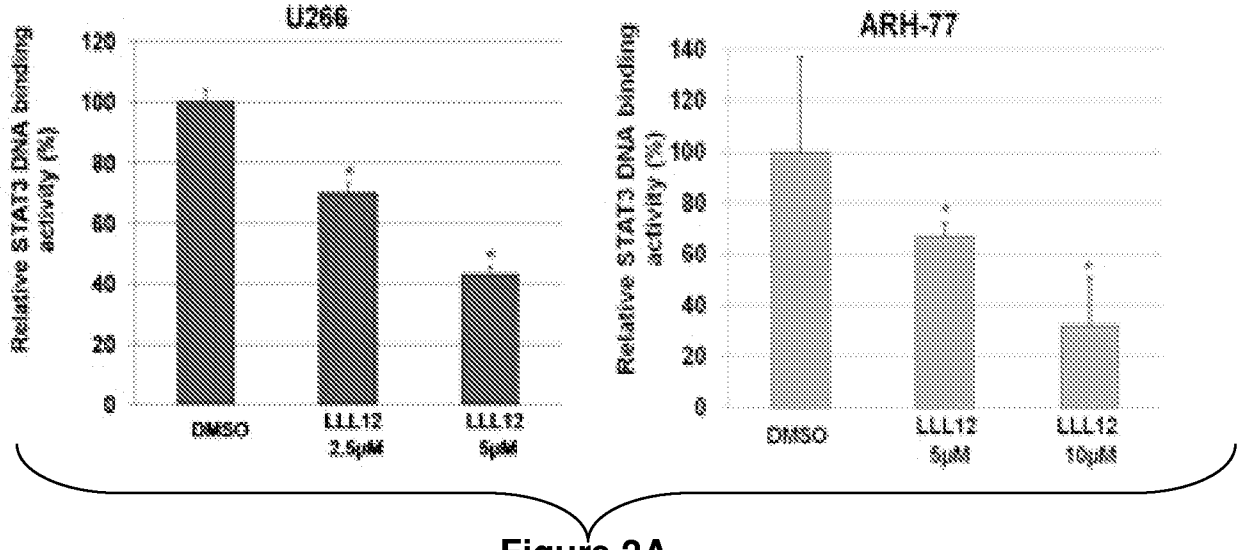


Figure 2A

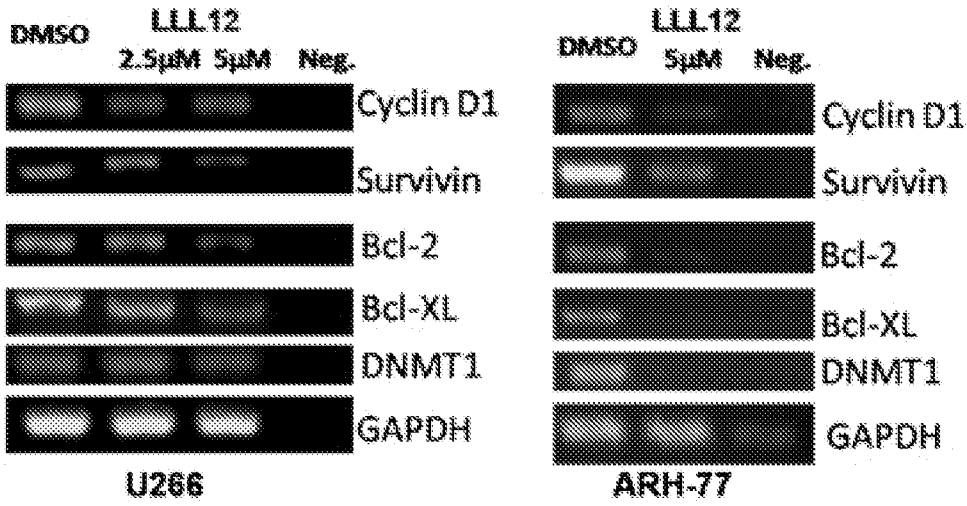


Figure 2B

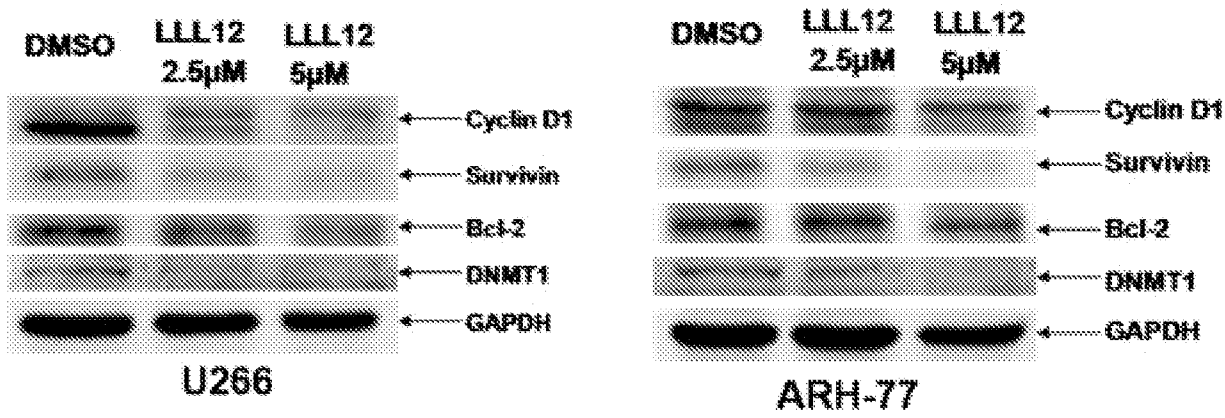


Figure 2C

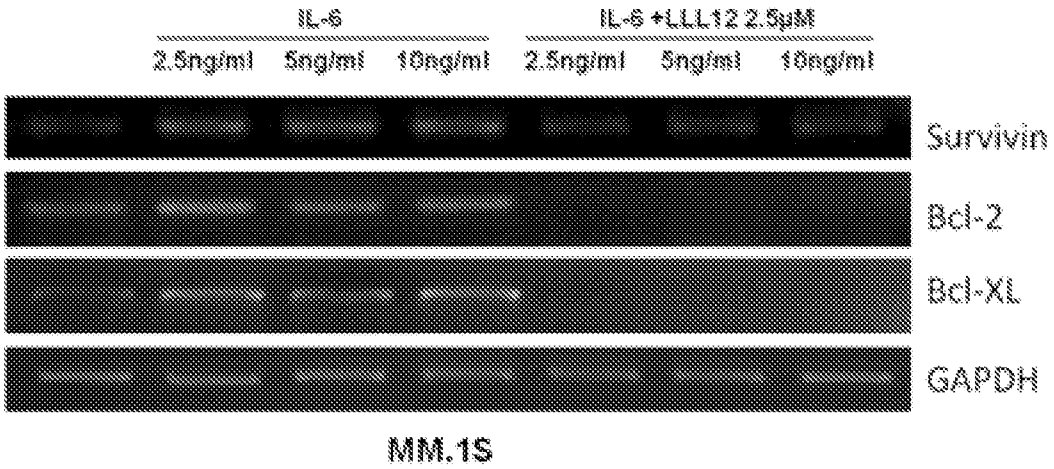


Figure 2D

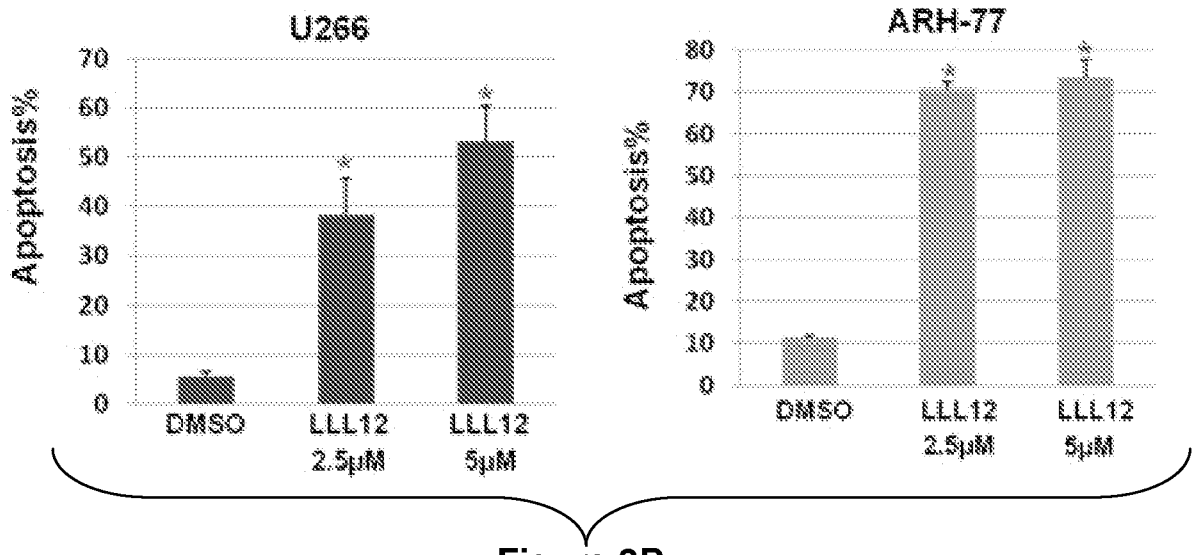


Figure 3B

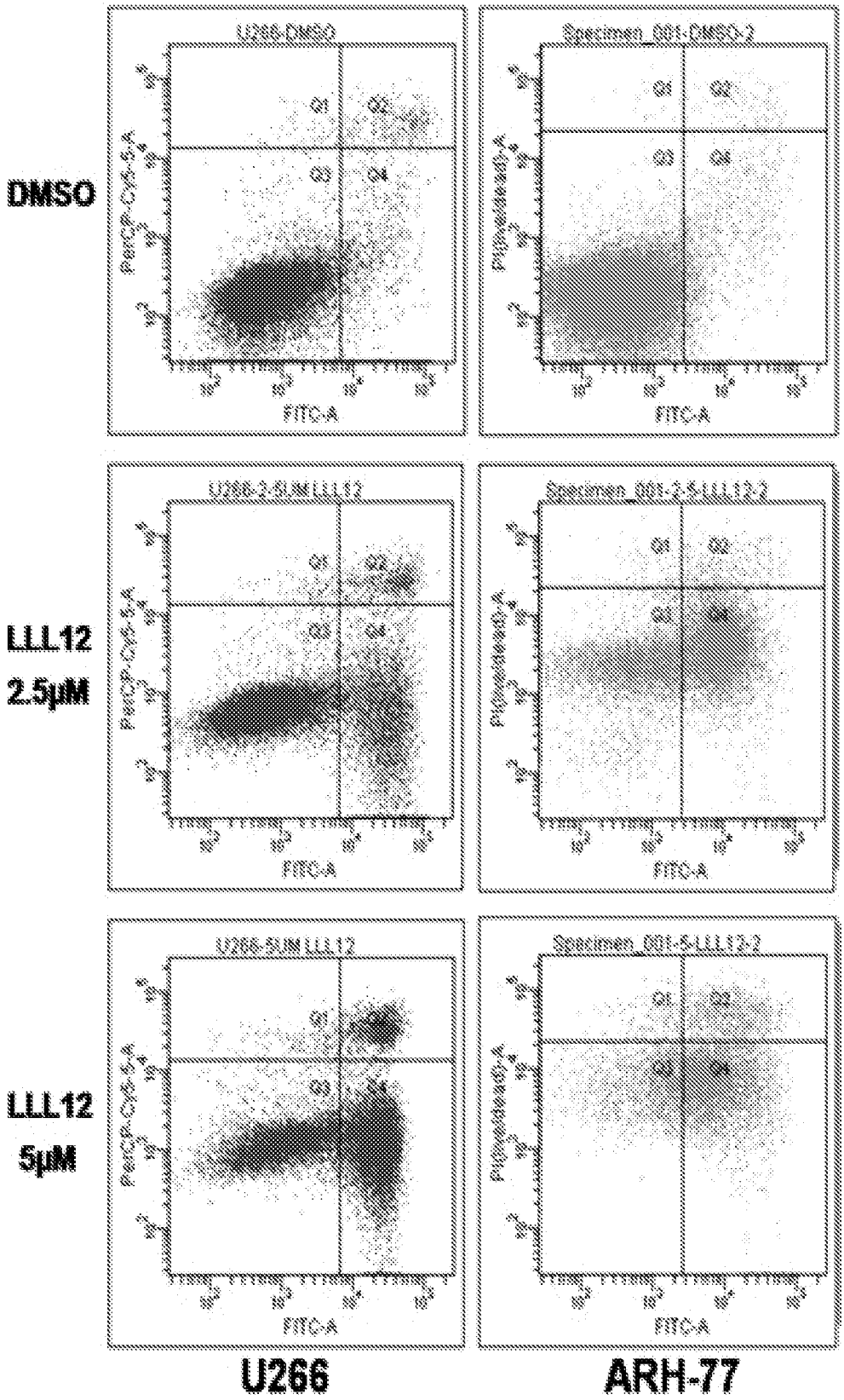


Figure 3A

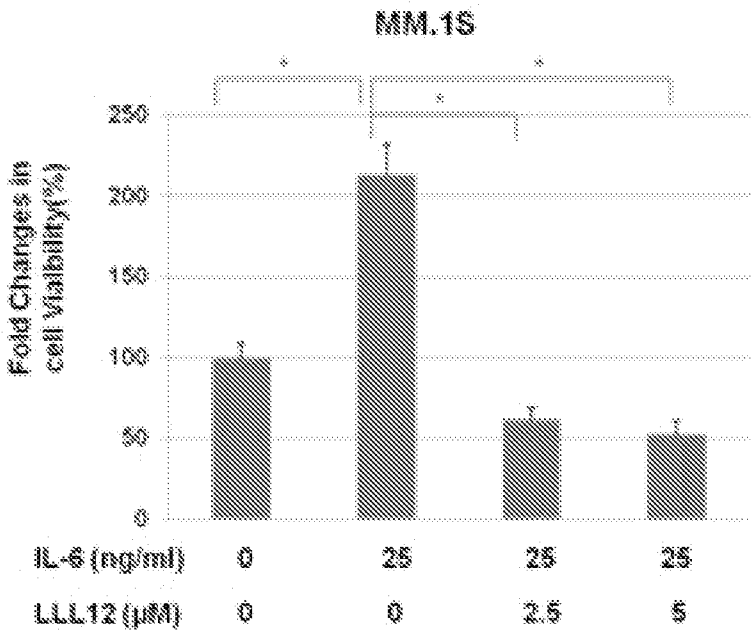


Figure 4A

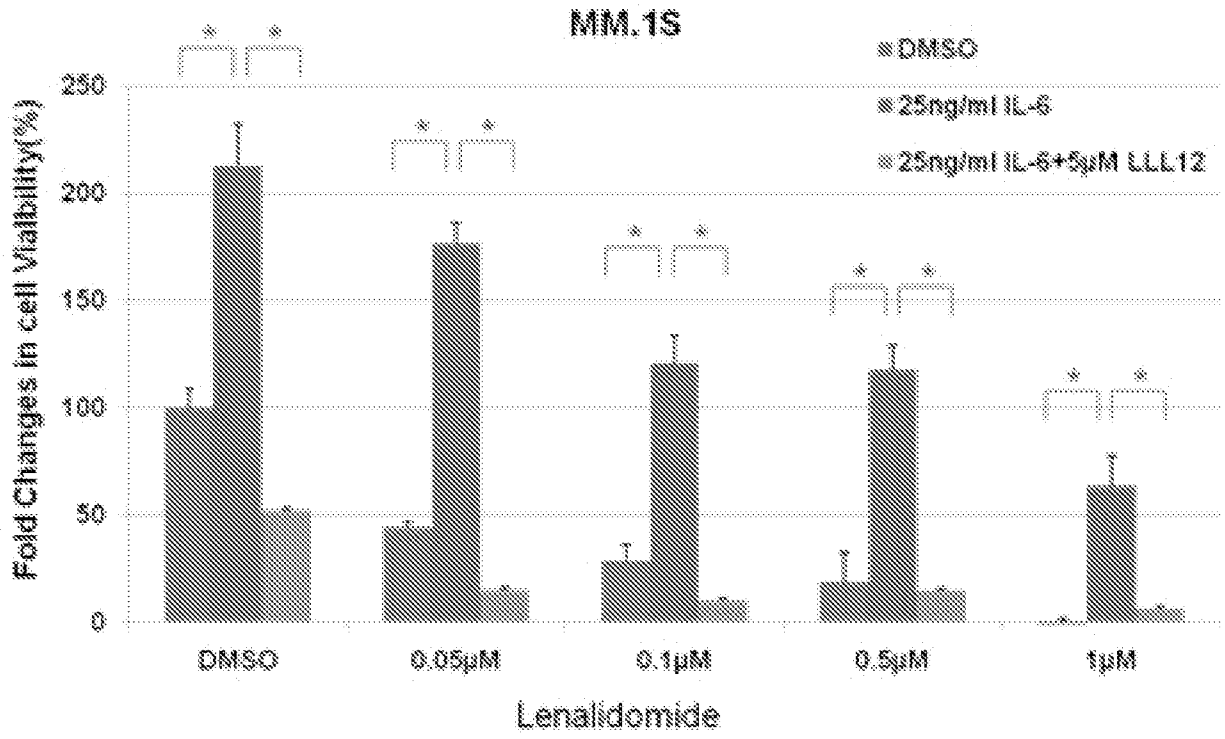


Figure 4B

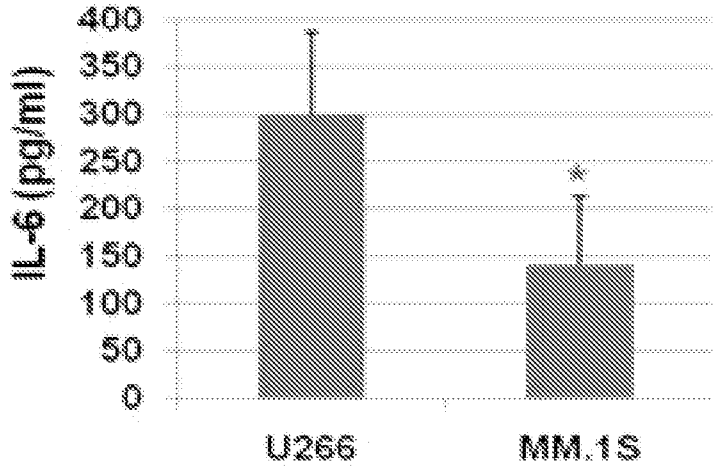


Figure 4C

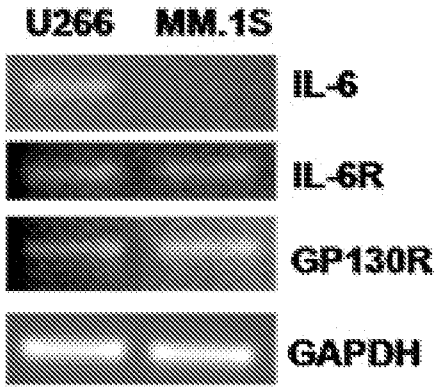


Figure 4D

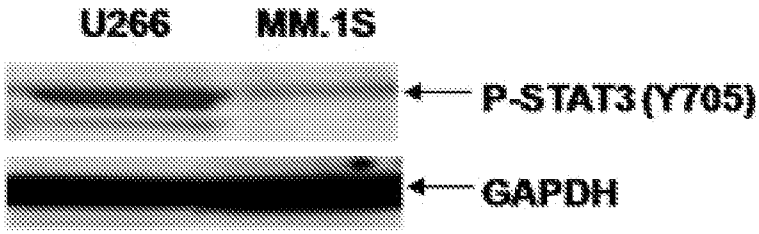


Figure 4E

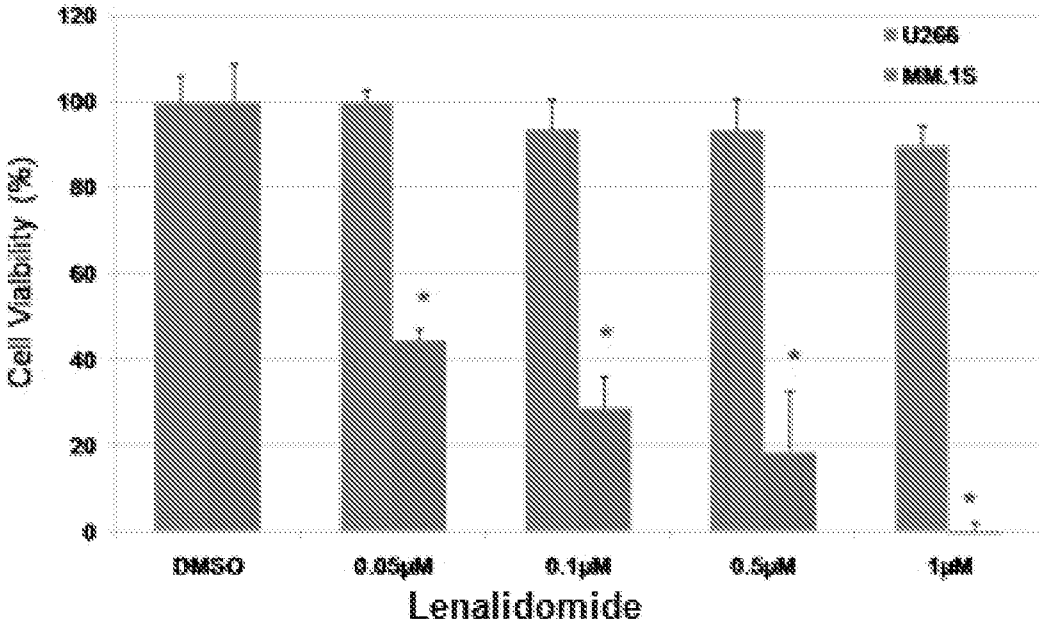


Figure 4F

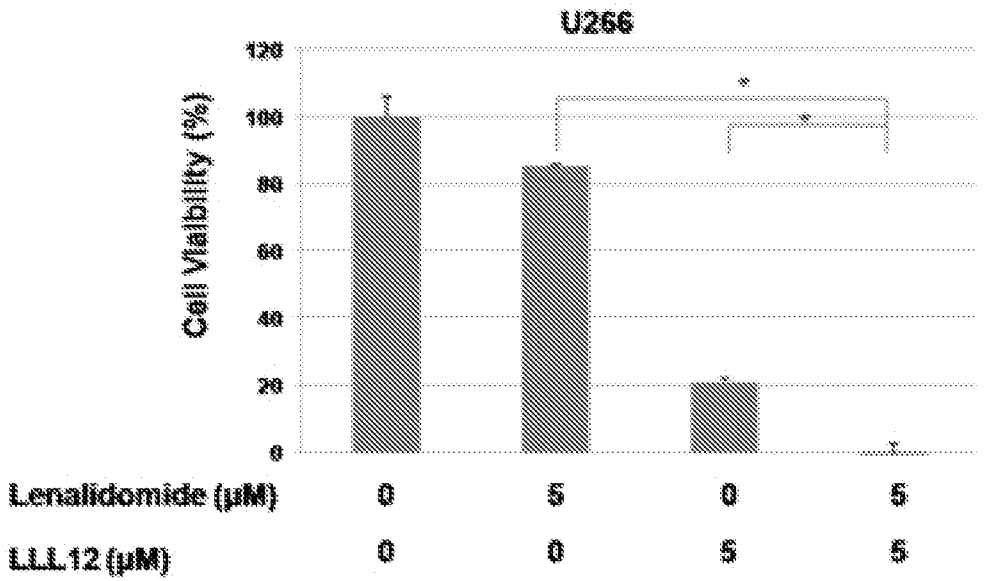


Figure 4G

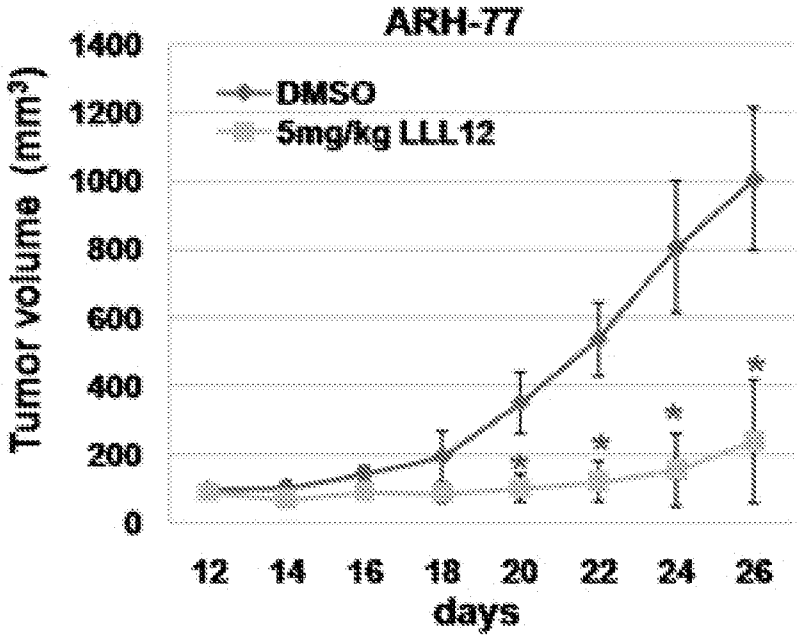


Figure 5A

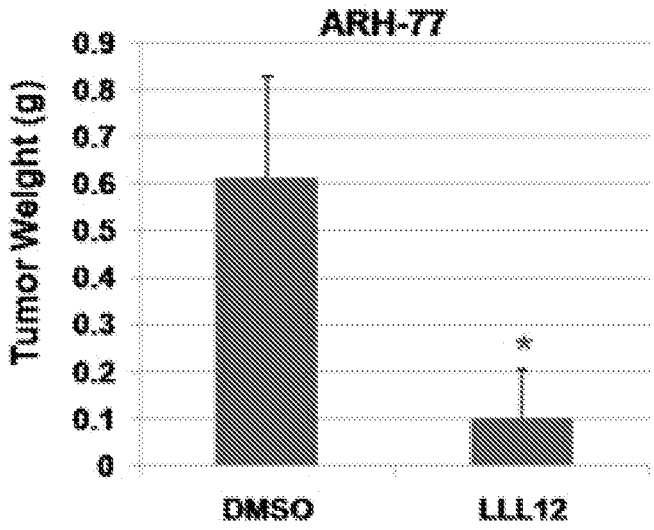


Figure 5B

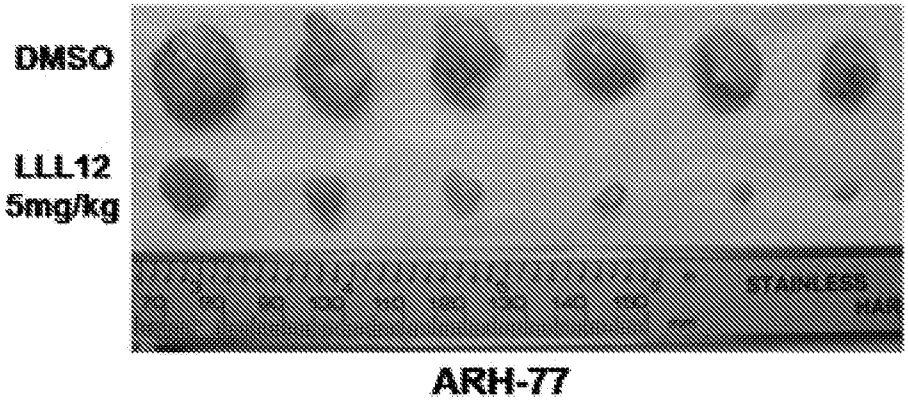


Figure 5C

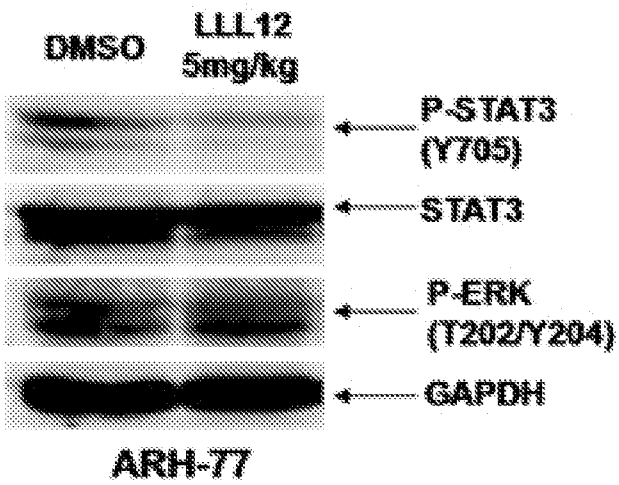


Figure 5D

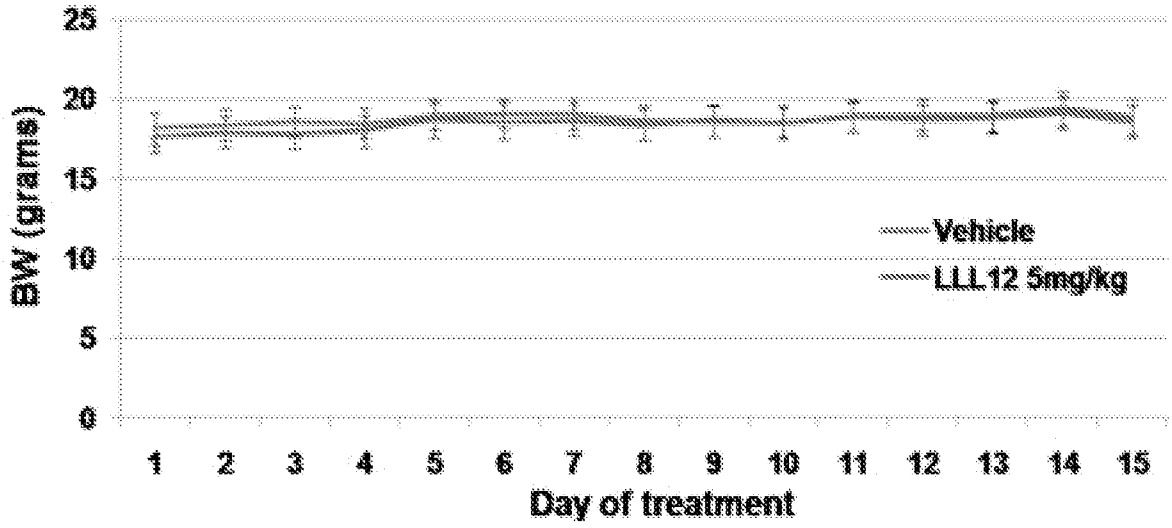
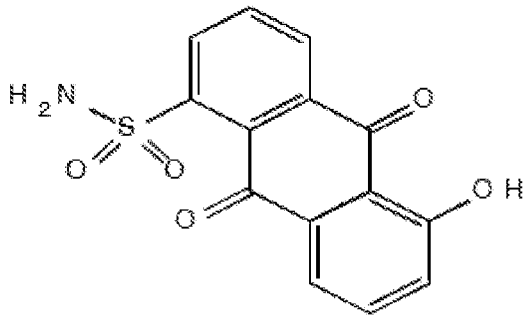


Figure 5E



LLL12

Figure 6

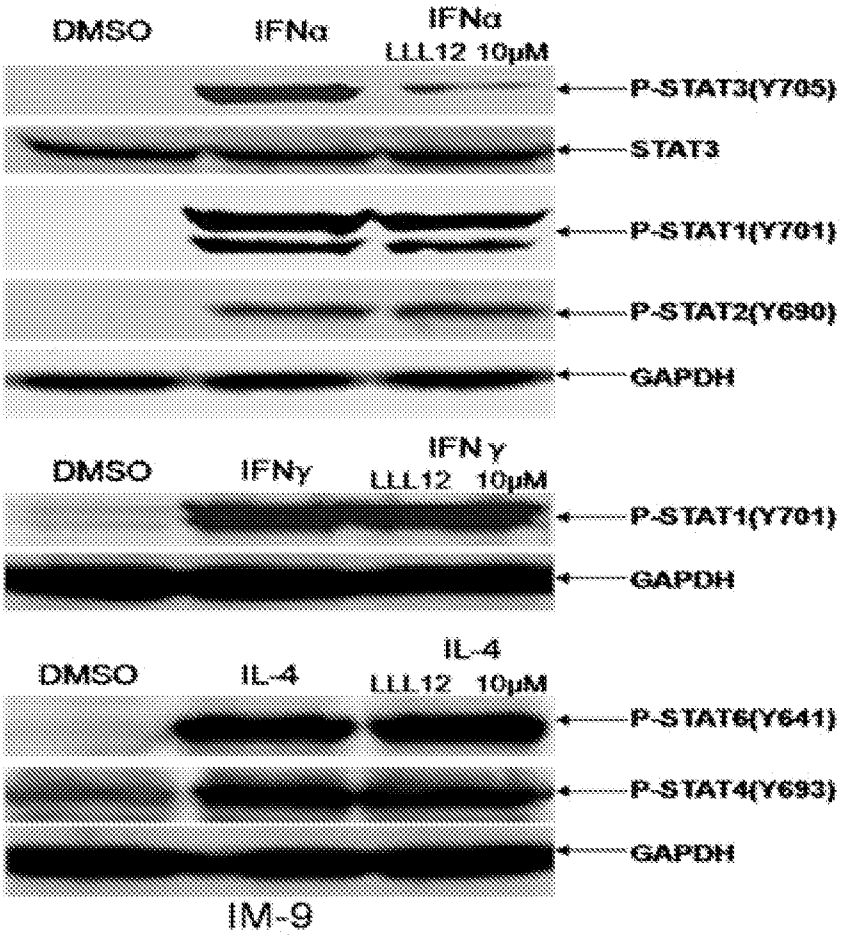


Figure 7

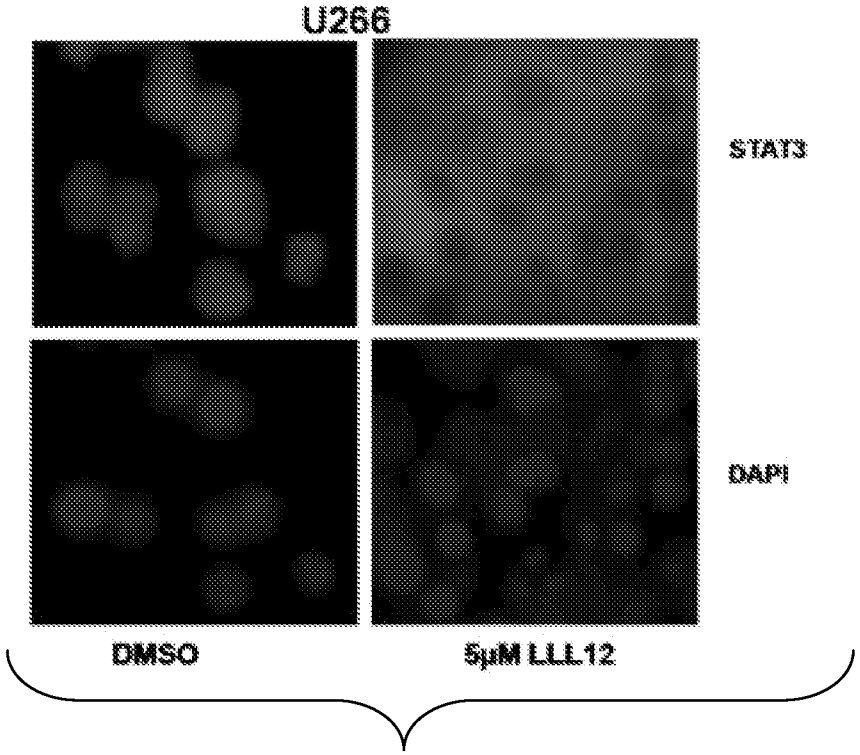


Figure 8

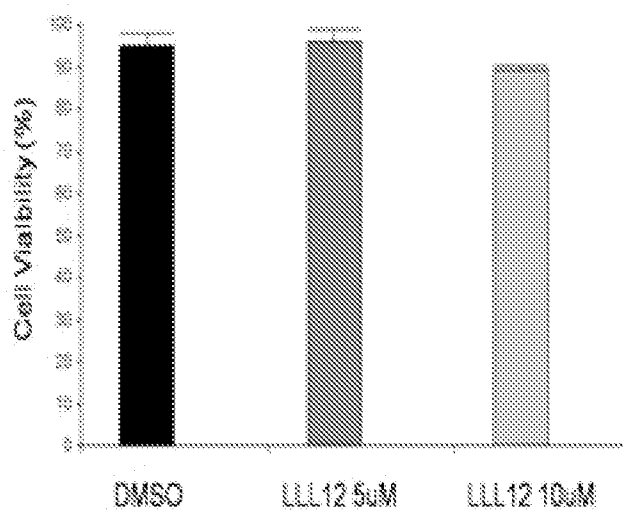


Figure 9A

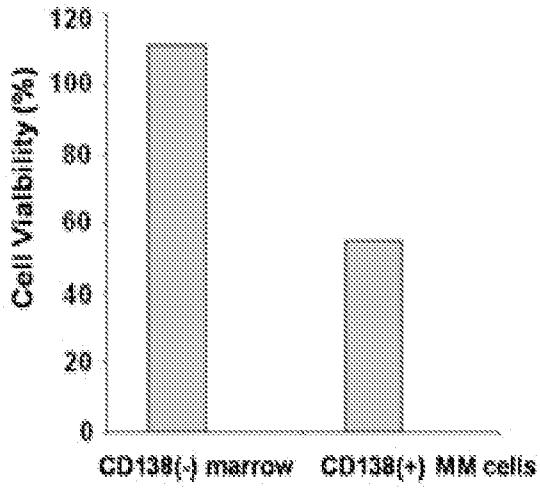


Figure 9B

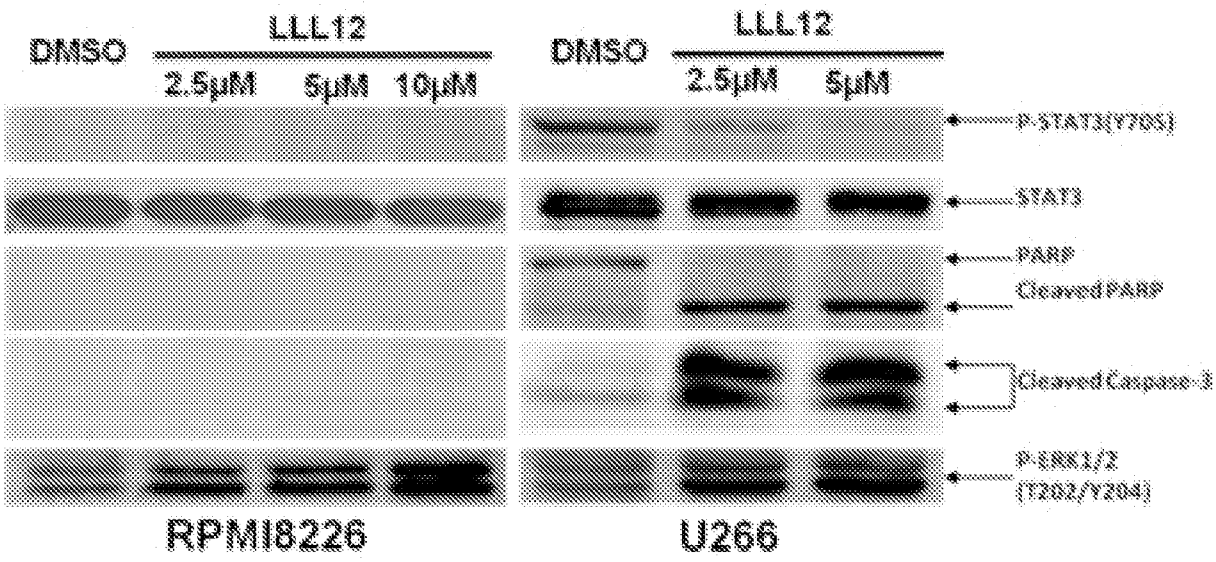


Figure 9C

Gene	Primers	Size
Cyclin D1	Forward: 5'-GCTGGAGCCCGTGAAAAAGA-3' Reverse: 5'-CTCCGCCTCTGGCATTTTG-3'	247
Survivin	Forward: 5'-ACCAGGTGAGAAGTGAGGGA-3' Reverse: 5'-AACAGTAGAGGAGCCAGGGA-3'	309
Bcl-2	Forward: 5'-TCTTTGAGTTCGGTGGGGTC-3' Reverse: 5'-TGCATATTTGTTTGGGGCAGG-3'	304
Bcl-Xl	Forward: 5'-TTGGACAATGGACTGGTTGA-3' Reverse: 5'-GTAGAGTGGATGGTCAGTG-3'	765
DNMT1	Forward: 5'- GTGGGGGACTGTGTCTCTGT -3' Reverse: 5'- TGAAAGCTGCATGTCCTCAC -3'	204
IL-6	Forward: 5'- TACCCCCAGGAGAAGATTCC-3' Reverse: 5'- TTTCAGCCATCTTTGGAAGG-3'	193
IL-6R		150
GP130		103
GAPDH	Forward: 5'- TGATGACATCAAGAAGGTGGTGAAG-3' Reverse: 5'-TCCTTGGAGGCCATGTGGGCAT-3'	240

Figure 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/21393

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07C 303/00; A01N 41/06; A01N 43/04; C12N 5/00 (2012.01)
USPC - 564/87
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(8): C07C 303/00; A01N 41/06; A01N 43/04; C12N 5/00 (2012.01)
USPC: 564/87

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC(8): IPC(8)-C07C 303/00; A01N 41/06; A01N 43/04; C12N 5/00 (2012.01)
USPC: 514/602, 514/49, 435/375

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST, GoogleScholar, Dialog
STAT3 inhibitors, LLL12, Multiple Myeloma, ST A T3 SH2 dimerization, apoptosis, ST AT3 phosphorylation, cyclin, Bcl-2, survivin, PARP and caspase-3, lenalidomide, bortezomib, mouse

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Li et al. A Novel Small Molecule, LLL12, Inhibits STAT3 Phosphorylation and Activities and Exhibits Potent Growth-Suppressive Activity in Human Cancer Cells. Neoplasia. 2010 January; 12(1): 39-50. page 42, Fig. 1A, page 43, Fig. 2, page 44, col 1, page 46, col 2, page 46, Table 1, page 47, col 2, page 49, col 1	1-28
Y	US 6,875,746 B1 (Nair et al.) 05 April 2005 (05.04.2005) col 2, ln 18-49, col 7, ln 54 to col 8, ln 14, col 11, ln 34-52	1-28
Y	US 2007/0060521 A1 (JOVE et al.) 15 March 2007 (15.03.2007) para[0011, para[0116]	4-28
Y	Real et al. Resistance to chemotherapy via Stat3-dependent overexpression of Bcl-2 in metastatic breast cancer cells. Oncogene (2002) 21, 7611-7618. Abstract	12-14 and 17
Y	Matsui et al. Clonogenic Multiple Myeloma Progenitors, Stem Cell Properties, and Drug Resistance. Cancer Res 2008;68:190-197. Published online January 2, 2008. Abstract, page 191, col 2	13-14, 17 and 26-28
A,P	US 2011/0212911 A1 (LI et al.) 1 September 2011 (01.09.2011) para[0011]	1-28

☐ Further documents are listed in the continuation of Box C. ☐

* 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
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"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
14 April 2012 (14.04.2012)	30 APR 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)