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(54) **Title**: XZH-5 INHIBITS CONSTITUTIVE AND INTERLEUKIN-6-INDUCED STAT3 PHOSPHORYLATION IN HUMAN HEPATOCELLULAR CARCINOMA CELLS

(57) **Abstract**: The present disclosure provides compounds, compositions of matter and methods related to XZH-5 and related chemical therapeutics. The inhibitory effects of a series of small molecules structurally related to XZH-5 on STAT3 phosphorylation is disclosed, as are the results of that inhibition, including induction of apoptosis, reduction of colony forming ability, inhibition of IL-6- induced STAT3 phosphorylation, and nuclear translocation and STAT3 DNA binding activity.

TITLE

XZH-5 INHIBITS CONSTITUTIVE AND INTERLEUKIN-6-INDUCED STAT3 PHOSPHORYLATION IN HUMAN HEPATOCELLULAR CARCINOMA CELLS

Inventor: Chenglong Li, Jiayuh Lin, Hong Wang

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of United States Provisional Application Number 61/421, 341 filed December 9, 2010, the entire disclosure of which is expressly incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was not made with any government support and the government has no rights in the invention.

TECHNICAL FIELD OF THE INVENTION

[0003] This invention is in the field of chemistry, particularly pharmacology. The invention is useful for cancer research and treating cancers.

BACKGROUND OF THE INVENTION

[0004] Each year 16,200 cases of liver and intrahepatic bile duct cancer are expected in the U.S. Hepatocellular carcinoma (HCC) is the most common malignance of all primary liver cancers with only 9% of patients surviving five years. The median survival time is less than one year. The main risk factors of HCC include Hepatitis B virus, Hepatitis C virus, alcohol and tobacco. However, like other human malignancies, the molecular mechanisms of HCC are not fully understood.

[0005] Signal transducer and activator of transcription 3 (STAT3) is frequently detected in a variety of human cancers and may induce tumor formation in nude mice. STAT3 is involved in oncogenesis, proliferation, angiogenesis, immune evasion and drug resistance through regulating genes, such as Bcl-2, Bcl-XL, CyclinD1, IL-6, Mcl-1, p53, Survivin, HIF-1α, and VEGF. Blockade of constitutively activated STAT3 can cause apoptosis in vitro, inhibit tumor growth in vivo, and enhance the sensitivity to radiotherapy and chemotherapy.

[0006] Growing evidence demonstrates that STAT3 plays an important role in HCC development, progression, and prognosis. STAT3 promotes invasion and migration of HCC cells. Antisense oligonucleotide or siRNA targeting STAT3 inhibits the growth and metastasis of HCC cells. Targeting STAT3 with small molecules can also decrease proliferation of HCC cells and increase sensitivity to anticancer drugs.

[0007] Interleukin-6 is an inflammatory cytokine, which activates STAT3 through binding to one IL-6 receptor and two gp130 molecules. Then STAT3 molecules are recruited to gp130 and are

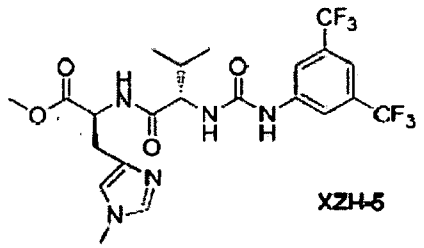
phosphorylated by Janus kinases (JAK). Phosphorylated STAT3 molecules dimerize via Src homology-2 (SH2) domain and translocate to the nucleus, where the STAT3 dimer binds to a specific DNA element to regulate downstream genes, which are involved in cell proliferation, angiogenesis, and anti-apoptosis.

[0008] Patients with HCC have higher levels of serum IL-6 than those with liver cirrhosis and healthy controls. In addition, IL-6 level is much higher in HCC stage III patients than in other stage patients. STAT3, the major transducer to relay the IL-6 signal to the nucleus, is found significantly correlated with the prognosis of patients with HCC.

SUMMARY OF THE INVENTION

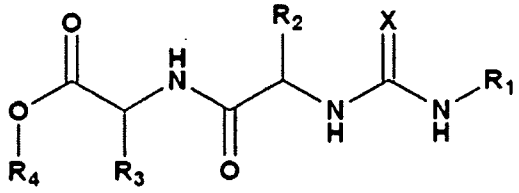
[0009] The inventors characterize herein the inhibitory effects of a novel small molecule XZH-5 on STAT3 phosphorylation in HCC cell lines. XZH-5 reduced constitutive STAT3 phosphorylation at Tyr705 and the expression of STAT3 downstream genes. The inhibition of STAT3 in HCC cells resulted in the induction of apoptosis and reduction of colony forming ability. In addition, XZH-5 also inhibited IL-6-induced STAT3 phosphorylation, nuclear translocation and STAT3 DNA binding activity. In contrast, it had no effect on IFN-γ-induced STAT1 phosphorylation, indicating the more selective effects on STAT3.

[0010] In a first broad aspect, there are provided compounds having the general Formula I, or pharmaceutically acceptable salts, formulations, or excipients thereof:



[Formula I]

[0011] In a second broad aspect, there are provided compounds having the general Formula II, or pharmaceutically acceptable salts, formulations, excipients thereof:



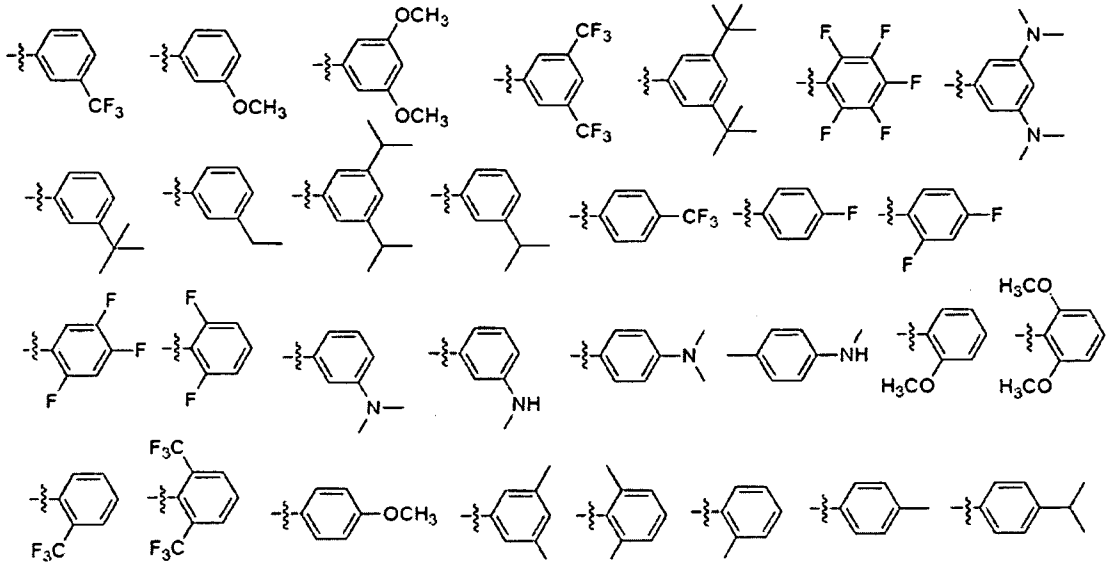
Formula II

wherein X= O, S,

R4 = H, CH_3, CH_2CH_3, iPr, t-Bu, n-Bu, n-pentyl

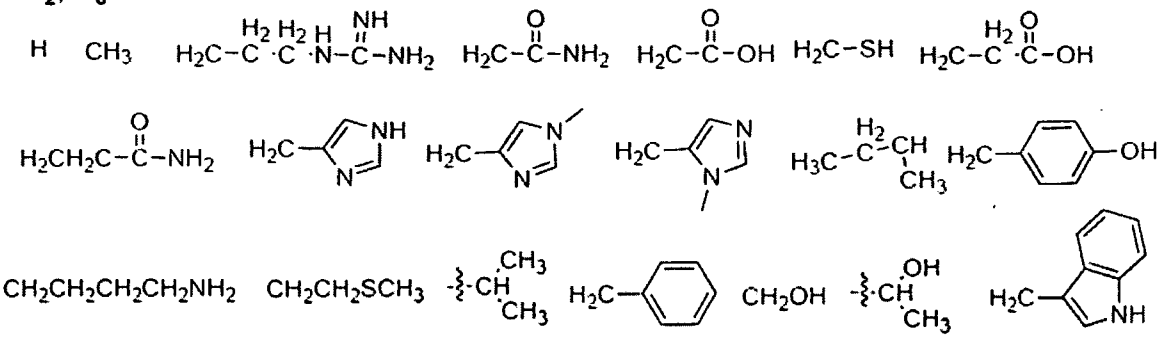
[0012]

R_1 =

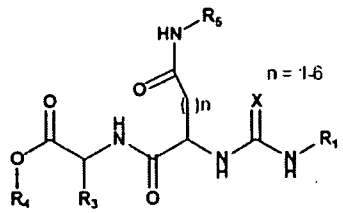


[0013] And

R_2, R_3 =



[0014] In a third broad aspect, there are provided compounds having the general Formula III or pharmaceutically acceptable salts, formulations, excipients thereof:

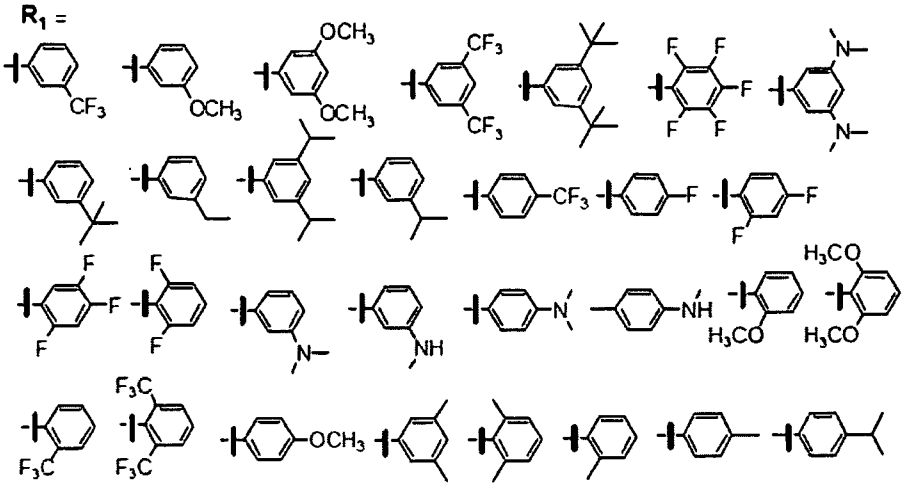


Formula III

Wherein X= O, S;

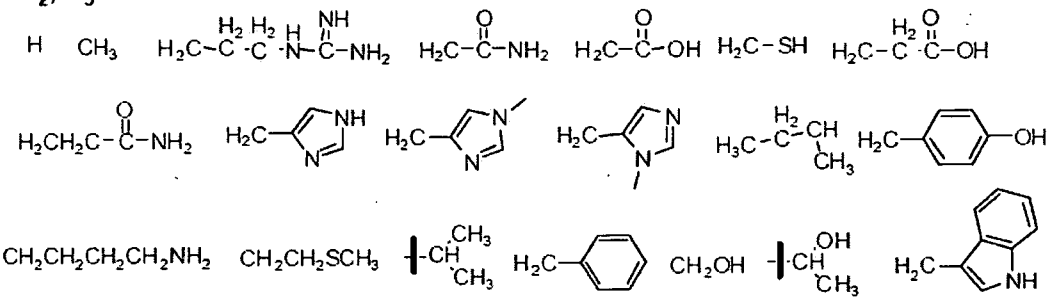
R_4 = H, CH_3, CH_2CH_3,

R_1 =



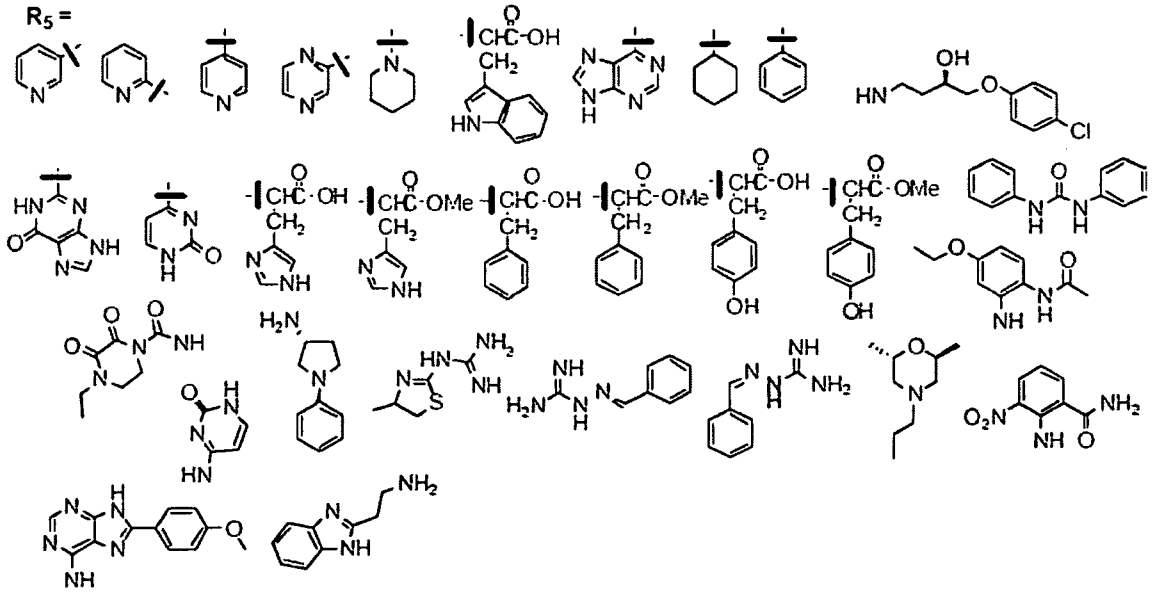
[0015] And

R_2, R_3 =

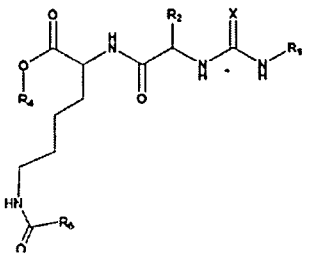


[0016] And

R_5 =



[0017] In a fourth broad aspect, there are provided compounds having the general Formula IV or pharmaceutically acceptable salts, formulations, excipients thereof:



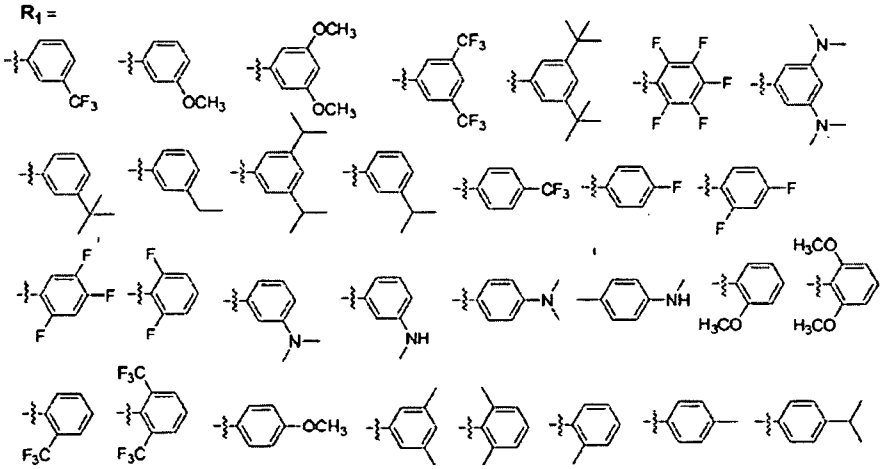
Formula IV

wherein X= O, S;

R4 = H, CH_3, CH_2CH_3,

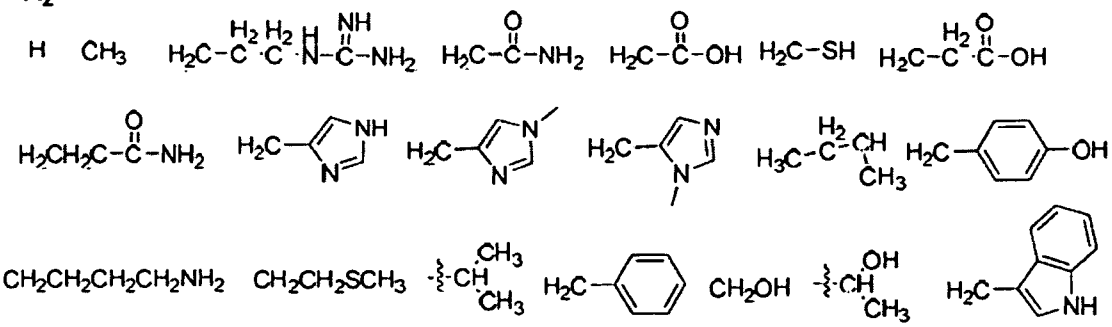
[0018] And

R_1 =



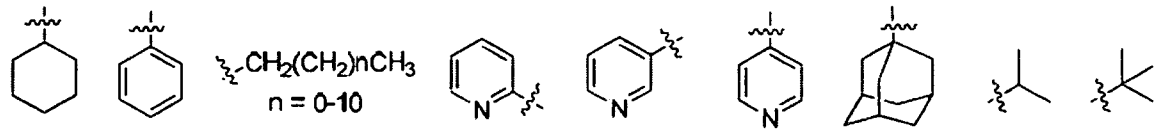
[0019] And

R_2 =

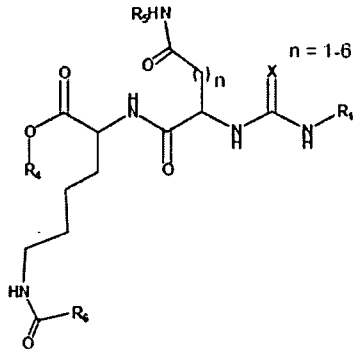


[0020] And

R_6 =



[0021] In a fifth broad aspect, there are provided compounds having the general Formula V or pharmaceutically acceptable salts, formulations, excipients thereof:

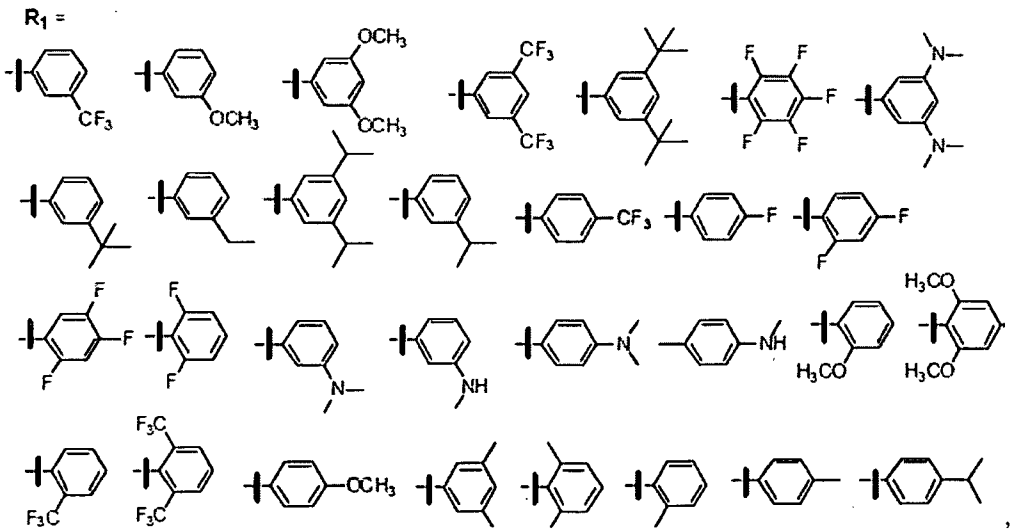


Formula V

wherein X= O, S;

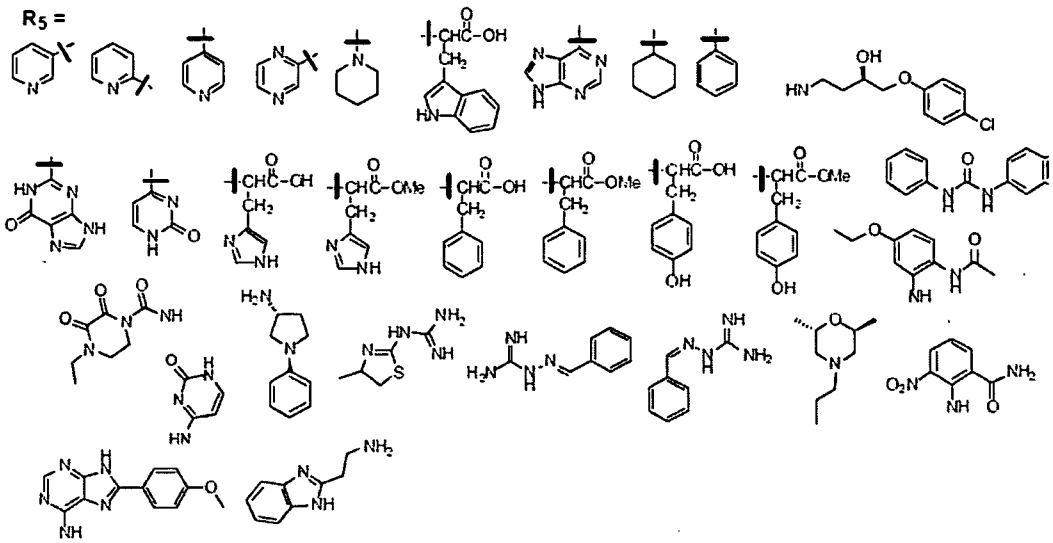
R_4 = H, CH_3, CH_2CH_3,

R_1 =



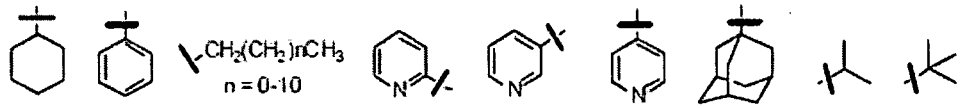
[0022] And

R_5 =



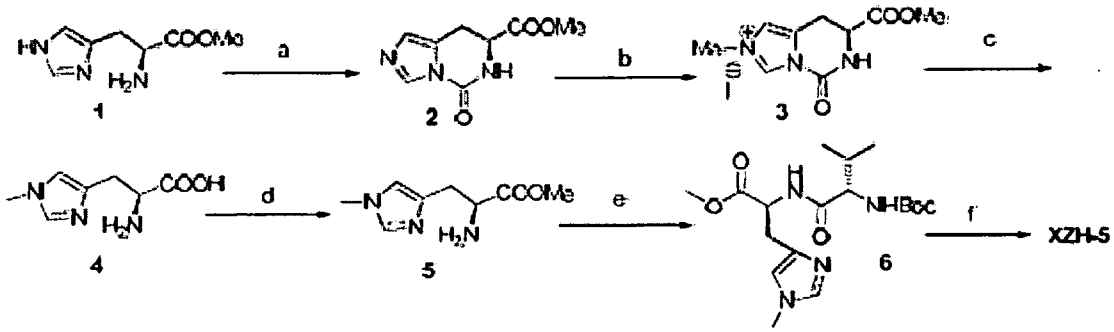
[0023] And

R_6 =



[0024] Also provided are compositions of matter, comprising a compound herein and a pharmaceutically-acceptable excipient, carrier, diluent or salt. Individual enantiomers, combinations, prodrugs, intermediates, racemates and metabolites related to the present compounds are also provided.

[0025] In another there is provided herein a method to synthesize a compound of Formula I which includes:



[0026] Wherein a) N,N'-Carbonydiimidazol, DMF, 60°C, 60%. b) MeI, CH_3CN, 80°C, 90%. c) 6N HCl, reflux, 90%. d) $SOCl_2$, MeOH, reflux, 95%. e) (L)-Boc-Valine, DCC, HOBT, TEA, DCM, rt, 60%. f) TFA, DCM rt, then TEA, 3,5-Bis (trifluoromethyl) phenyl isocyanate ($(CF_3)_2C_6H_3NCO$), DCM, 85% (2 steps).

[0027] In certain embodiments, the compound of Formula I is a particular enantiomer or is further purified to one particular enantiomer.

[0028] In another aspect of the present invention, there are provided methods to inhibit STAT3 activation in a cell, comprising introducing a compound of Formula I herein to a STAT3-expressing cell, and measuring STAT3 activation inhibition. Preferred are those methods wherein inhibition is measured by observing a STAT3-related effect, such as: cell apoptosis; prevention of STAT3 SH2 dimerization; a decrease in the levels of expression of STAT3 phosphorylation; inhibition of downstream targets of STAT3, especially cyclin; Bcl-2; and surviving and/or induction of cleaved PARP and caspase-3; a reduction in STAT3 phosphorylation after inducing IL-6 in MDA-MD-453 breast cancer cells; reduction of STAT3 DNA binding activity after compound introduction; reduction of STAT3–dependent transcriptional activity after compound introduction.

[0029] In another aspect of the present invention, there are provided methods to inhibit transcription of STAT3 regulated genes, comprising administering a compound of Formula I herein. Preferred are those methods wherein said transcription inhibition is measured via reverse transcriptase PCR.

[0030] In another aspect of the present invention, there are provided methods to decrease the ability of tumor cells to form colonies, comprising a administering a compound of Formula I herein to a tumor cell-containing medium. Preferred are those methods wherein the tumor cell-containing medium is a mammalian cell culture, although those methods wherein said tumor cell-containing medium is a mammal are also preferred. More preferred methods are those wherein said mammal is selected from the group consisting essentially of: human; livestock; companion animal; and zoo animal.

[0031] In another aspect of the present invention, there are provided methods to inhibit tumor cell migration, comprising administering a compound of Formula I herein to a tumor cell-containing medium. Preferred are those methods wherein the tumor cell-containing medium is a mammalian cell culture, although those methods wherein said tumor cell-containing medium is a mammal are also preferred. More preferred methods are those wherein said mammal is selected from the group consisting essentially of: human; livestock; companion animal; and zoo animal.

[0032] In another aspect of the present invention, there are provided methods to inhibit tumor cell proliferation, comprising administering a compound of Formula I herein to a tumor cell-containing medium. Preferred are those methods wherein the tumor cell-containing medium is a mammalian cell culture, although those methods wherein said tumor cell-containing medium is a mammal are also preferred. More preferred methods are those wherein said mammal is selected from the group consisting essentially of: human; livestock; companion animal; and zoo animal.

[0033] In another aspect of the present invention, there are provided methods to treat cancer in a patient in need of such treatment, comprising administering a pharmaceutically-acceptable

formulation of at least one compound of Formula I herein. Preferred are those methods wherein the cancer treated is selected from the group consisting essentially of: breast cancer; glioblastoma; and pancreatic cancer. More preferred are those methods which further comprises administering to the patient at least one additional chemotherapeutic drug. Most preferred are those methods wherein said additional chemotherapeutic drug is doxorubicin, gemcitabine or a combination of the two.

[0034] Also provided by the present invention are methods to determine the presence of tumor cells in a sample, comprising introducing a compound of Formula I to a cell sample, and measuring STAT3 inhibition.

[0035] Also provided are methods to identify compounds useful to inhibit STAT3 activation, comprising comparing the ability of a compound of Formula I to inhibit STAT3 activation to the ability of a test compound to inhibit STAT3 activation.

[0036] Also provided are kits comprising a compound of Formula I. Those kits which comprise a compound of Formula I and also comprise nucleic acid molecules useful to identify STAT3 transcription are preferred.

[0037] *Definitions*

[0038] "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).

[0039] 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).

[0040] 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.

[0041] Other systems, methods, features, and advantages of the present invention will be or will become apparent to one with skill in the art upon examination of the following drawings and detailed description. It is intended that all such additional systems, methods, features, and advantages be included within this description, be within the scope of the present invention, and be protected by the accompanying claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0042] The patent or application file contains one or more drawings executed in color and/or one or more photographs. Copies of this patent or patent application publication with color drawing(s) and/or photograph(s) will be provided by the Patent Office upon request and payment of the necessary fee.

[0043] **Figs. 1A-B**. SD-1029 and Stattic inhibit STAT3 activation.

[0044] **Fig. 1A**. SNU387 and SNU398 cells were treated with 10 μM of SD-1029 or 20 μM of

Stattic. Phosphorylated STAT3, STAT3 and cleaved caspase-3 were examined by western blotting.

[0045] **Fig. 1B**. SNU387 and SNU398 cells were treated with 10 μM of SD-1029 or 20 μM of Stattic. After 24 hours, cell viability was measured. The data represented three independent results.

[0046] **Figs. 2A-2C**. XZH-5 synthesis.

[0047] **Fig. 2A**. The structure of XZH-5.

[0048] **Fig. 2B**. Reagents and conditions of XZH-5 synthesis: a) N,N'-Carbonydiimidazol, DMF, 60°C, 60%. b) MeI, CH_3CN, 80°C, 90%. c) 6N HCl, reflux, 90%. d) $SOCl_2$, MeOH, reflux, 95%. e) (L)-Boc-Valine, DCC, HOBT, TEA, DCM, rt, 60%. f) TFA, DCM rt, then TEA, 3,5-Bis (trifluoromethyl) phenyl isocyanate ($(CF_3)_2C_6H_3NCO$), DCM, 85% (2 steps).

[0049] **Fig. 2C**. The computer model of STAT3 and XZH-5 interaction.

[0050] **Figs. 3A-3C**. XZH-5 inhibits STAT3 phosphorylation.

[0051] **Fig. 3A**. SNU-387, SNU-398, HepG2 and Huh-7 cells were treated with different concentrations of XZH-5. Phosphorylated STAT3 and total STAT3 were analyzed by western blotting.

[0052] **Fig. 3B**. Phosphorylated p65 and total p65 were analyzed in XZH-5 treated cells by western blotting.

[0053] **Fig. 3C**. SNU-387, SNU-398, HepG2 and Huh-7 cells were treated with XZH-5. The mRNA expression of Bcl-XL, CyclinD1, HIF-1α, Survivin, and VEGF was analyzed by RT-PCR.

[0054] **Figs. 4A-4C**. XZH-5 induces apoptosis and reduces cell viability.

[0055] **Fig. 4A**. SNU-387, SNU-398, HepG2 and Huh-7 cells were treated with XZH-5. Cleaved PARP and cleaved caspase-3 were analyzed by western blotting.

[0056] **Fig. 4B**. Caspase-3/7 activity was measured in XZH-5 treated SNU387 and SNU398 cells. The data represented three independent results.

[0057] **Fig. 4C**. Cell viability was measured in XZH-5 treated SNU-387, SNU-398, HepG2 and Huh-7 cells. The data represented three independent results.

[0058] **Figs. 5A-5D**. XZH-5 inhibits IL-6-induced STAT3 phosphorylation.

[0059] **Fig. 5A**. Hep3B cells were cultured in serum free medium overnight and then were pre-treated with XZH-5 for 2 hours, followed by 25 ng/ml of IL-6 or IFN-γ. Phosphorylated STAT3 and phosphorylated STAT1 were analyzed by western blot.

[0060] **Fig.5B**. Hep3B cells were treated as described in Fig. 5A. The distribution of phosphorylated STAT3 (Y705) was analyzed by immunofluorescence.

[0061] **Fig. 5C**. STAT3 DNA binding ability was analyzed by DNA binding assay. The data represented three independent results.

[0062] **Fig. 5D**. JAK1, JAK2 and phosphorylated JAK2 were analyzed by western blotting in IL-6 and/or XZH-5 treated Hep3B cells.

[0063] **Fig. 6**. XZH-5 suppresses IL-6-induced STAT3 nuclear translocation. Hep3B cells were

treated as described in Fig. 5A. The distribution of total STAT3 was analyzed by immunofluorescence.

[0064] **Fig. 7.** XZH-5 reduces colony forming ability. Huh-7 and SNU-398 cells were treated with 30 μM of XZH-5 for 2 hours. After the treatment, living cells were counted and the same number of cells were re-seeded and cultured for two week. Colonies were fixed by ice-cold methanol and stained by 1% crystal violet.

[0065] **Fig.8.** XZH-5 causes less cell death in cells expressing low pSTAT3. HH, Hep3B, SNU-398, SNU-387, HepG2, and Huh-7 cells were treated with 30 μM and 40 μM of XZH-5 for 24 h. After the treatment, cell viability was measured.

DETAILED DESCRIPTION OF THE INVENTION

[0066] STAT3, a STAT family member, is a transcription factor, which may regulate genes involved in cell growth, cell cycle regulation, angiogenesis, migration, invasion and anti-apoptosis. STAT3 is persistently activated in a variety of primary human cancers. In cell culture, STAT3 activation is necessary either for cell transformation or anti-apoptosis. Moreover, STAT3 activation plays an important role in tumor initiation and development in many types of human cancer, such as breast carcinoma, cervical cancer, hepatocellular carcinoma, lung cancer, ovarian cancer, and prostate cancer. Cell transformation always accompanies STAT3 phosphorylation at tyrosine residue 705, which may promote proliferation through CyclinD1, Myc and Pim-1, block apoptosis through Bcl-XL and Survivin, and increase angiogenesis through VEGF. Down-modulation of constitutively active STAT3 not only induces apoptosis in cancer cells but also overcomes chemo-resistance and radio-resistance.

[0067] In the present invention, the inventors demonstrate that a novel small molecule XZH-5 inhibited constitutively activated STAT3 in a dose dependent manner in HCC cells. RT-PCR results also showed that the expression of STAT3 downstream genes, such as Bcl-XL, CyclinD1, HIF-1α, Survivin, and VEGF, were down-regulated, which further induced apoptosis and decreased cell viability.

[0068] STAT3 can be rapidly activated in response to diverse stimuli, including hormones, growth factors, cytokines and various stresses. It is well documented that autocrine or paracrine loop of interleukine-6 secretion is involved in STAT3 activation. Malignant pleural effusion (MPE) is a poor prognostic marker for non-small lung cancer patients. Elevated autocrine IL-6/STAT3/VEGF signaling is found in MPE patients. IL-6/STAT3 is also a critical promoter for early colitis-associated cancer and further development. Moreover, the inventors previously reported that IL-6 induces anti-apoptosis upon drug treatment in HCC cells through STAT3 pathway.

[0069] Here the inventors investigated the inhibitory effect of XZH-5 on IL-6-induced STAT3 phosphorylation in a HCC cell line. The data showed that XZH-5 suppressed IL-6-enhanced STAT3

phosphorylation at Tyr705, blocked IL-6-induced STAT3 nuclear translocation, and decreased IL-6-elevated STAT3 DNA binding ability. In contrast to the inhibition of IL-6-induced STAT3 phosphorylation, IFN-γ-induced STAT1 phosphorylation was not affected by the treatment of XZH-5, showing that XZH-5 more selectively inhibited STAT3 activation.

[0070] In summary, the present invention results indicated that XZH-5 is useful as a platform for further development of STAT3 selective small-molecule inhibitors for HCC therapy.

[0071] XZH-5 is based on natural amino acids with potentially less side effects than Stattic. Another advantage is ease of design and synthesis of the new analogs.

[0072] The inhibitors described in this invention are useful to treat most types of adult and childhood cancers including sarcomas, brain tumors, breast, prostate, colon, ovarian, pancreatic, lung cancers, multiple myeloma, and leukemias that express constantly activated STAT3.

[0073] XZH-5 and its analogs are also useful to solve the problem of the cancer-promoting function of STAT3 by directly inhibit STAT3.

[0074] The invention herein is also useful for developing cancer drugs that target STAT3 in cancer patients as personalized medicine.

[0075] The invention herein is also useful to product XZH-5 and its analogs to provide reagents to inhibit STAT3 in cancer cells.

[0076] The inventors herein synthesized new analogs of XZH-5, where such XZH-5 and XZH-5 analogs are useful to inhibit STAT3, induce apoptosis, inhibit tumor growth in mouse model in a variety of human cancer cells (childhood sarcomas, liver, breast, and pancreatic cancers).

[0077] *In Vitro Methods*:

[0078] The present invention also provides in vitro methods for selectively inhibiting STAT3 activation, cell growth, proliferation and migration arrest and/or apoptosis of 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.

[0079] Although the methods of the present invention can be practiced in vitro, it is contemplated that 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.

[0080] The present invention 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 $p<0.05$.

[0081] Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

[0082] In these examples, the inventors demonstrate that a novel small molecule XZH-5 inhibited constitutive and IL-6-induced STAT3 phosphorylation in human hepatocellular carcinoma cells. Although many STAT3 small molecule inhibitors have been described, none of them are currently selected for clinical trials. XZH-5 may be a platform for further development of STAT3 specific small molecule inhibitors for HCC therapy.

[0083] ***Example 1.***

[0084] *Materials and Methods*

[0085] *Cell Culture.*

[0086] Human hepatoma cell lines, Hep3B, HepG2, Huh-7, SNU-387, and SNU-398 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Hep3B and HepG2 cells were cultured in Minimum Essential Medium, Eagle (MEM) (ATCC) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. SNU-387 and SNU-398 cells were cultured in RPMI 1640 medium (ATCC) supplemented with 10% FBS and 1% penicillin/streptomycin. Huh-7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. Interleukin-6 was purchased from Cell Sciences (Canton, MA).

[0087] *Western blotting.*

[0088] Total protein was prepared by lysing the cells in ice-cold RIPA buffer containing 1× proteasome inhibitor cocktail and 1× phosphatase inhibitor cocktail. The lysates were then centrifuged at 13,000 rpm at 4°C for 15 min and the supernatant was collected. Total protein was separated by SDS-PAGE gel, transferred to PVDF member and immunoblotted with appropriate antibodies. Antibodies to phospho-STAT3 (Y705), STAT3, cleaved caspase-3, cleaved PARP, phospho-STAT1 (Y701), STAT1, phospho-JAK2 (Y1007/1008), JAK2, phospho-JAK1 (Y1022/1023), JAK1, phosphor-JNK (Y183/Y185), phosphor-ERK (T202/Y204), GAPDH and HRP conjugated secondary antibodies were from Cell Signaling Technology (Beverly, MA). Target proteins were examined by chemiluminescence (Cell Signaling Technology).

[0089] *RT-PCR.*

[0090] Total RNA was extracted from cultured cell lines with RNeasy kit (Qiagen, Valencia,

CA). Reverse transcription was performed with Omniscript reverse transcription kit (Qiagen). RT conditions: 37°C for 1 hour. PCR conditions: 94°C for 5 minutes followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute with a final extension of 72°C for 10 minutes.

[0091] *Viability*.

[0092] Cells were seeded in a 96-well plate and treated as indicated in the text. After the treatment, medium was discarded and 50 µl of 1×HBSS with CyQUANT (Molecule Probe, Invitrogen, Carlsbad, CA) was added to each well. After one hour incubation at 37°C, fluorescence was read at 485 nm.

[0093] *Immunofluorescence*.

[0094] Cells were seeded on a glass slide and were treated as indicated in the text. After the treatment, the cells were washed with ice-cold phosphate-buffered saline (PBS) buffer, and were fixed with ice-cold methanol at -20°C for 15 minutes. After two washing with ice-cold PBS buffer, the slide was blocked with PBS buffer containing 5% normal goat serum and 0.3% Triton X-100 at room temperature for at least 1 hour. Then the slide was incubated with specific primary antibody at 4°C overnight. After the overnight incubation, the slide was washed with PBS buffer containing 0.1% Tween-20. Then the cells were incubated with FITC-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) or Alexa Fluor 594 anti-rabbit secondary antibody (Molecule Probe, Invitrogen) at room temperature for 1 hour. The cells were mounted with Vectashield HardSet mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Pictures were captured by Leica Microsystems (Bannockburn, IL).

[0095] *DNA Binding Assay*.

[0096] DNA binding assay was performed to explore STAT3 DNA binding ability after a specific treatment. According to the manual of STAT3 DNA binding ELISA Kit (Active Motif, Carlsbad, CA), cells were seeded in a 10 cm plate. After the treatment as indicated in the text, nuclear protein was extracted and was mixed with a STAT3 specific DNA probe. The protein and DNA complex was then transferred into ELISA assay plate. After the incubation of primary and secondary antibodies, the developing solution was added to each well. The stop solution (1% SDS) was added to each well after the color was well developed. The absorbance was read at 450 nm.

[0097] *Apoptotic Assay*.

[0098] Apoptosis was measured with caspase3/7 assay (Promega, Madison, WI) according to the manufacture's protocol. Briefly, 20,000 cells were seeded in 96-well plates. After the treatment as indicated in the text, 100 µl of Apo-One Caspase3/7 reagent was added to each well and was incubated at 37°C for at least 30 minutes. The fluorescence was read at an excitation wavelength range of 485 nm and an emission wavelength range of 530 nm.

[0099] *Colony formation*.

[00100] Cells were treated as indicated. The treated cells were then trypsinized, stained with

trypan blue, and counted. The same number of cells was re-seeded on 10 cm plates and the cells were allowed to grow for 2 weeks without any treatments. The cells were then fixed by ice-cold methanol for 15 minutes and stained with 1% crystal violet in methanol.

[00101] ***Example 2.***

[00102] *Blockade of STAT3 induces apoptosis in HCC cells.*

[00103] To investigate whether blocking STAT3 activation would cause apoptosis, the inventors treated HepG2, Huh-7, SNU-387, and SNU-398 cells, which showed constitutively activated STAT3, with JAK/STAT3 inhibitor SD-1029 or STAT3 dimerization inhibitor Stattic. As shown in **Fig. 1A**, the treatment decreased STAT3 phosphorylation at Tyr 705 and induced the levels of cleaved caspase-3. Cell viability assay was further performed and the results demonstrated that the treatment reduced cell viability, indicating that STAT3 activation was required for cell survival in some HCC cells (**Fig. 1B**).

[00104] ***Example 3.***

[00105] *Small molecule XZH-5 inhibits STAT3 activation.*

[00106] XZH-5 was synthesized as illustrated in **Fig. 2A** and **Fig. 2B**. The computer model predicted that XZH-5 bound to STAT3 SH2 domain (**Fig. 2C**).

[00107] XZH-5 molecule (larger sticks) formed four hydrogen bonds with SH2 domain (red dotted lines): two with Arg609, one with Ser636 and one with Lys591. In addition, the triflurobenzyl ring had hydrophobic interaction with a side pocket of SH2 domain. To access the effects of XZH-5 on inhibition of STAT3 activation, HepG2, Huh-7, SNU-387, and SNU-398 cells were treated with different concentrations of XZH-5 overnight, and then phosphorylated STAT3 at Tyr705 was analyzed by western blot.

[00108] The results clearly showed that XZH-5 decreased constitutively activated STAT3 in a dose-dependent manner, whereas the expression of total STAT3 was not affected by the treatment (**Fig. 3A**).

[00109] Additionally, the treatment with XZH-5 did not change the levels of phosphorylated ERK and phosphorylated JNK (**Fig. 3B**).

[00110] To further determine whether XZH-5 would affect STAT3 downstream genes, the inventors performed RT-PCR to examine the mRNA expression of Bcl-XL, CyclinD1, HIF-1α, Survivin, and VEGF. As shown in **Fig. 3C**, the treatment of XZH-5 down-regulated the mRNA expression of these STAT3 targeted genes.

[00111] ***Example 4.***

[00112] *XZH-5 induces apoptosis in HCC cells.*

[00113] To investigate whether blocking STAT3 phosphorylation with XZH-5 would cause apoptosis, HepG2, Huh-7, SNU-387, and SNU-398 cells were treated with XZH-5, as indicated (30 or 40 μM). Results demonstrated that cleaved PARP and cleaved caspase-3 were induced by XZH-5

treatment (**Fig. 4A**).

[00114] The inventors further measured caspase-3/7 activity in XZH-5 treated cells. As shown in **Fig.4B**, caspase-3/7 activity was induced by the treatment. In addition, the treatment decreased cell viability of all cell lines (**Fig. 4C**), which was consistent with the apoptotic results.

[00115] ***Example 5.***

[00116] *XZH-5 inhibits exogenous IL-6-induced STAT3 phosphorylation.*

[00117] To investigate whether XZH-5 would inhibit exogenous IL-6-induced STAT3 activation, Hep3B cells with low levels of phosphorylated STAT3 were cultured in serum free medium overnight. Then the cells were pre-treated with 20 μM and 30 μM of XZH-5 for 2 hours, followed by 25 ng/ml of IL-6 for 30 minutes. After the treatment, phosphorylated STAT3 and total STAT3 were analyzed by western blot.

[00118] As illustrated in **Fig. 5A**, the pre-treatment with 20 μM of XZH-5 affected IL-6-caused STAT3 phosphorylation at Tyr705, and 30 μM of XZH-5 effectively inhibited STAT3 activation. In contrast to the inhibitory effects on IL-6-induced STAT3 phosphorylation, XZH-5 did not affect IFN-γ-induced STAT1 phosphorylation (**Fig. 5A**), indicating that XZH-5 more selectively targeted STAT3 phosphorylation.

[00119] In addition, immunofluorescent results revealed that IL-6 induced phosphorylated STAT3 nuclear accumulation, whereas XZH-5 pre-treatment blocked this process (**Fig. 5B**).

[00120] To further examine whether XZH-5 pre-treatment may decrease STAT3 DNA binding ability, the inventors treated Hep3B cells as described in Figure 5B and performed DNA binding ELISA. The results demonstrated that IL-6 induced STAT3 DNA binding ability, whereas XZH-5 pre-treatment decreased this ability (**Fig. 5C**).

[00121] The inventors also observed that XZH-5 decreased JAK2 phosphorylation and total JAK2 expression. Phosphorylated JAK1 was not detectable and JAK1 expression was not affected (**Fig. 5D**).

[00122] To confirm whether the phosphorylation induced by IL-6 may translocate STAT3 to the nucleus and whether XZH-5 pre-treatment may block this process, the inventors cultured Hep3B cells in serum free medium overnight. Then the cells were pre-treated with 30 μM of XZH-5 for 2 hours, followed by 25 ng/ml of IL-6 for 30 minutes. The treated cells were stained with anti-STAT3 primary antibody and Alexa Fluor 594 secondary antibody.

[00123] **Fig. 6** demonstrated that STAT3 was in the cytoplasm when cultured in serum free medium. IL-6 treatment induced STAT3 nuclear translocation, whereas XZH-5 pre-treatment blocked IL-6-induced STAT3 nuclear translocation.

[00124] ***Example 6.***

[00125] *XZH-5 reduces colony forming ability.*

[00126] To investigate whether XZH-5 treatment may disrupt the colony forming capability, Huh-

7 and SNU-398 cells were treated with XZH-5 for 2 hours. After the treatment, the same number of living cells were seeded and cultured in medium without XZH-5 for two weeks. Then cells were fixed by cold methanol and stained by1% of crystal violet. As shown in **Fig. 7**, XZH-5 treatment remarkably reduced colony forming ability.

[00127] The inventors determined whether XZH-5 would cause less cell death in cells expressing low levels of phosphorylated STAT3. Normal liver cells and Hep3B cells expressing low levels of pSTAT3 were compared with other liver cancer cell lines expressing constitutively activated STAT3. The results in **Fig. 8** showed that 30 and 40 μM of XZH-5 significantly led to more cell death in Huh-7, SNU-398, SNU-387, and HepG2 cells), indicating that the major target of XZH-5 is STAT3.

[00128] ***Example 7.***

[00129] *Uses of XZH-5 and Related Compounds.*

[00130] 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, there present invention provides compounds and methods with little or not 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.

[00131] 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.

[00132] *Dosages and Dosage Schedules.*

[00133] 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.

[00134] Non-limiting examples of suitable dosages can include total daily dosage of between

about 25-4000 mg/m2 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.

[00135] Other non-limiting examples of suitable dosages and methods of administration can include the intravenous administration directly to the tumor site via a catheter.

[00136] 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.

[00137] 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.

[00138] 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.

[00139] Pharmaceutical Compositions: 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.

[00140] 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.

[00141] 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.

[00142] 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.

[00143] 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.

[00144] 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.

[00145] 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-HCl, 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.

[00146] 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.

[00147] 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.

[00148] 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.

[00149] 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.

[00150] 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.

[00151] 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.

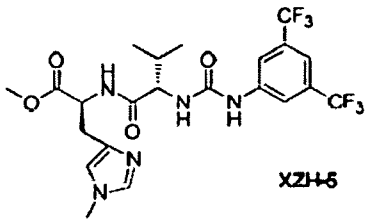
[00152] While the invention has been described with reference to various and preferred embodiments, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted for elements thereof without departing from the essential scope of the invention. In addition, many modifications may be made to adapt a particular situation or material to the teachings of the invention without departing from the essential scope thereof.

[00153] Therefore, it is intended that the invention not be limited to the particular embodiment disclosed herein contemplated for carrying out this invention, but that the invention will include all embodiments falling within the scope of the claims.

CLAIMS

What is claimed is:

1.) A compound having the general formula I:

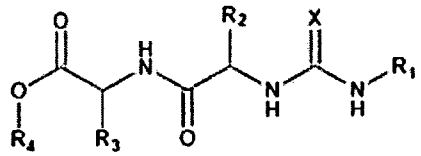


Formula I

2.) A compound of claim 1, which comprises activity-retaining substitutions.

3.) A composition of matter, comprising a compound of claim 1 and a pharmaceutically-acceptable excipient, carrier, diluent or salt.

4.) A compound having the general formula II, or pharmaceutically acceptable salts, formulations, excipients thereof:

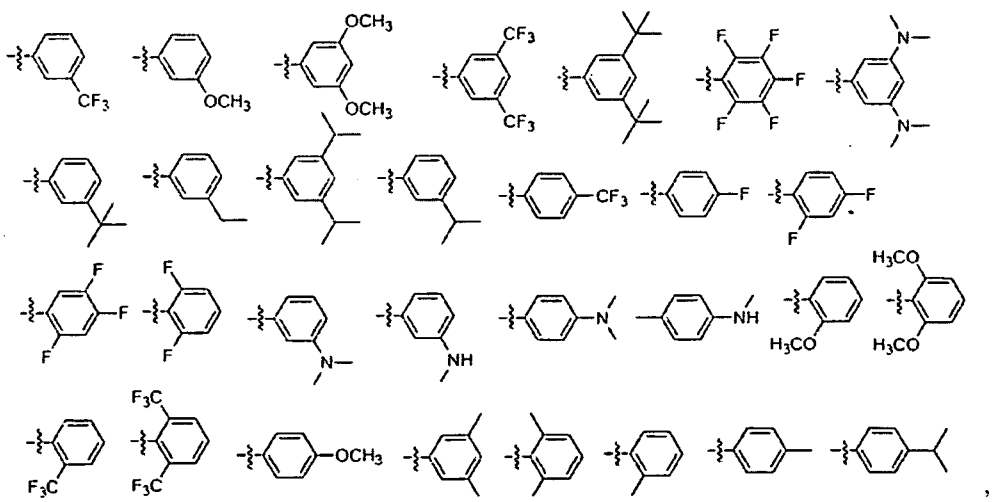


Formula II

wherein X= O, S,

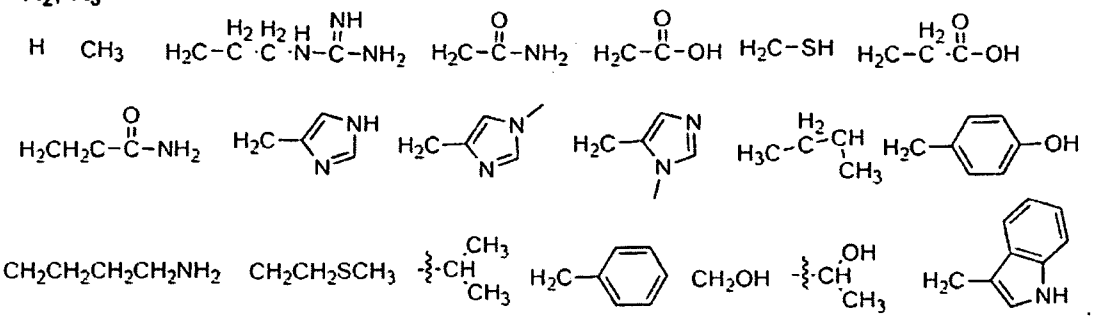
R4 = H, CH_3, CH_2CH_3, iPr, t-Bu, n-Bu, n-pentyl,

R_1 =

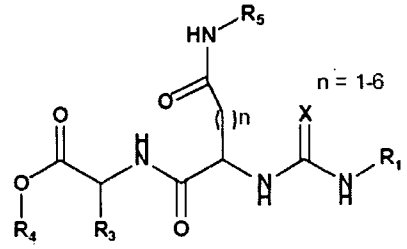


and

R_2, R_3 =



5.) A compound having the general formula III or pharmaceutically acceptable salts, formulations, excipients thereof:

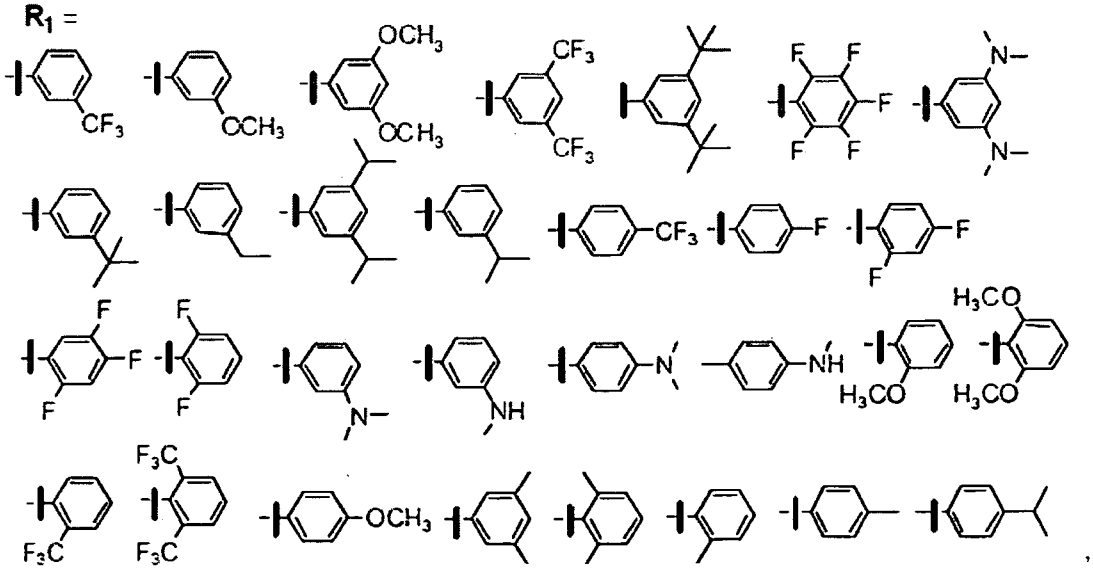


Formula III

wherein X= O, S;

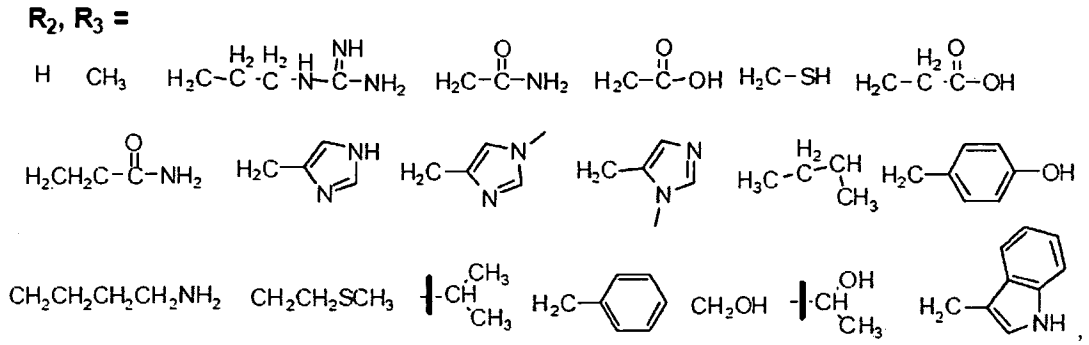
R_4 = H, CH_3, CH_2CH_3,

R_1 =



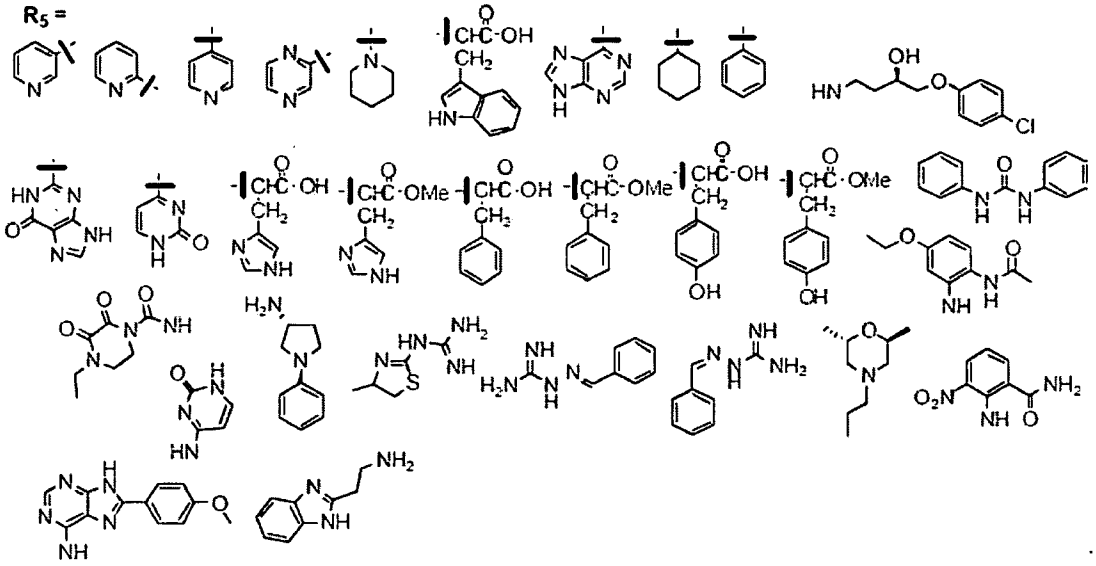
and

R_2, R_3 =

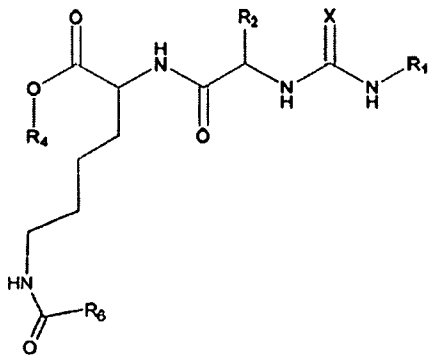


and

R_5 =



6.) A compound having the general formula IV or pharmaceutically acceptable salts, formulations, excipients thereof:

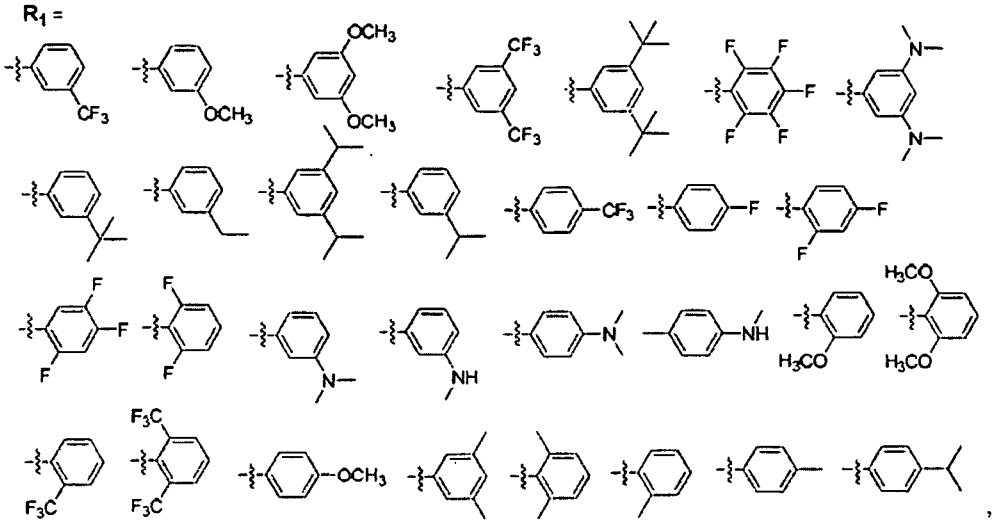


Formula IV

wherein X= O, S;

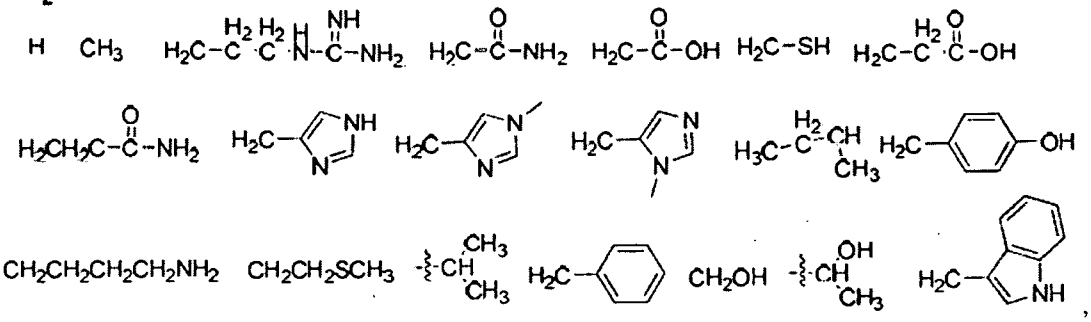
R4 = H, CH3, CH_2CH_3,

R_1 =



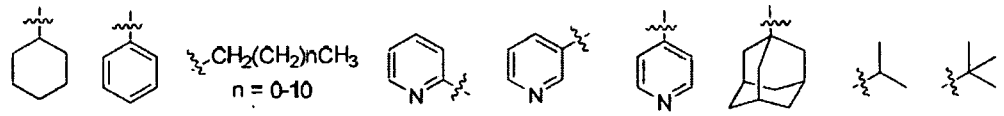
and

R_2 =

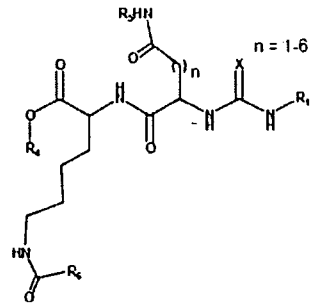


and

R_0 =



7.) A compound having the general formula V or pharmaceutically acceptable salts, formulations, excipients thereof:

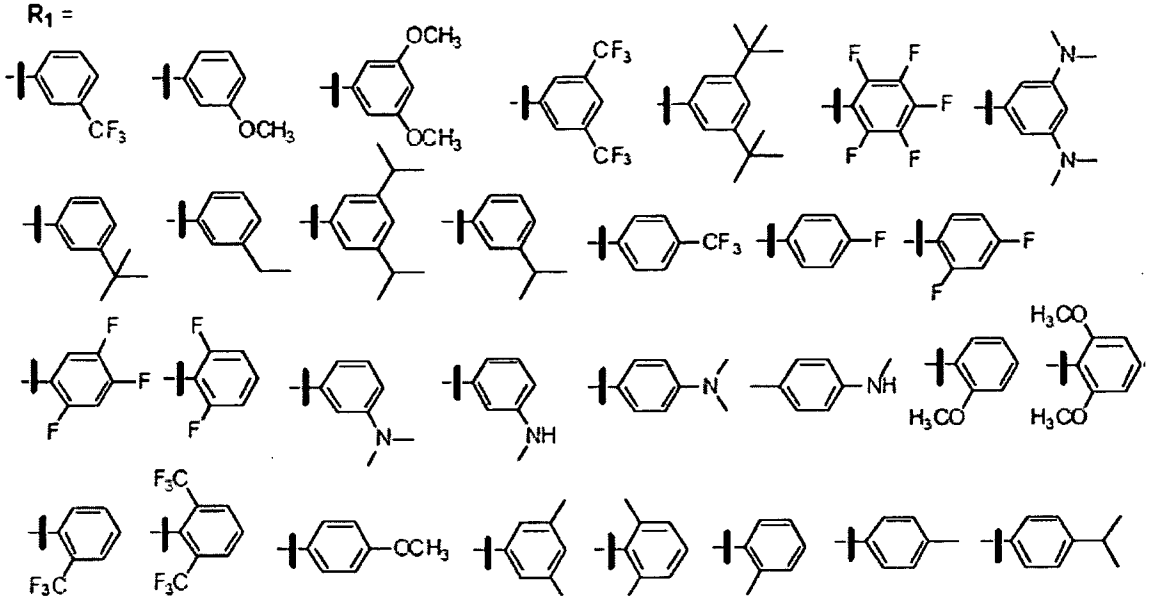


Formula V

wherein X= O, S;

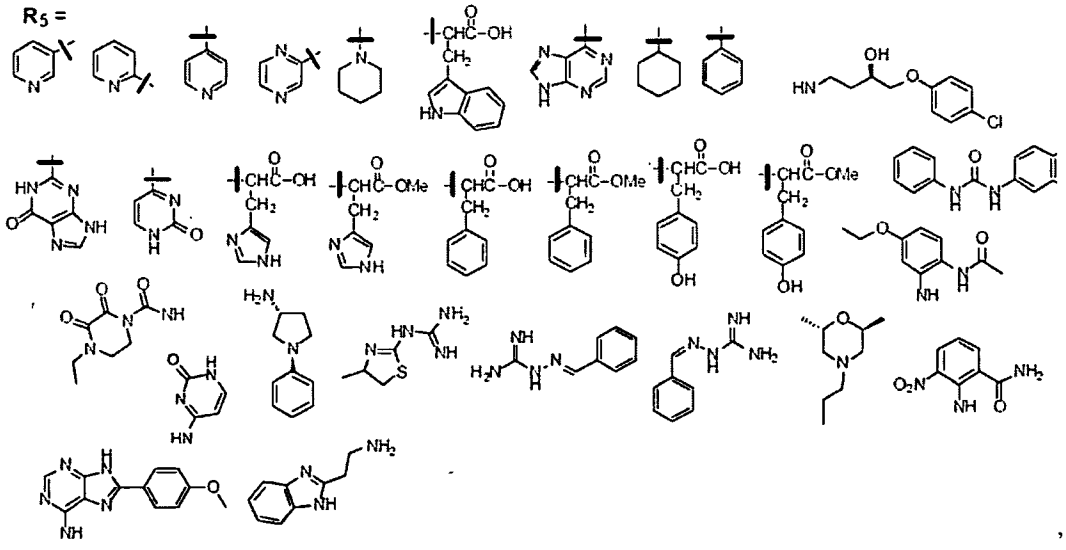
R_4 = H, CH_3, CH_2CH_3,

R_1 =



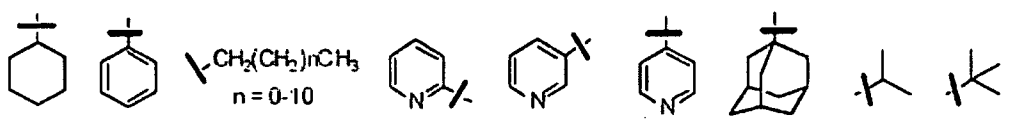
and

R_5 =

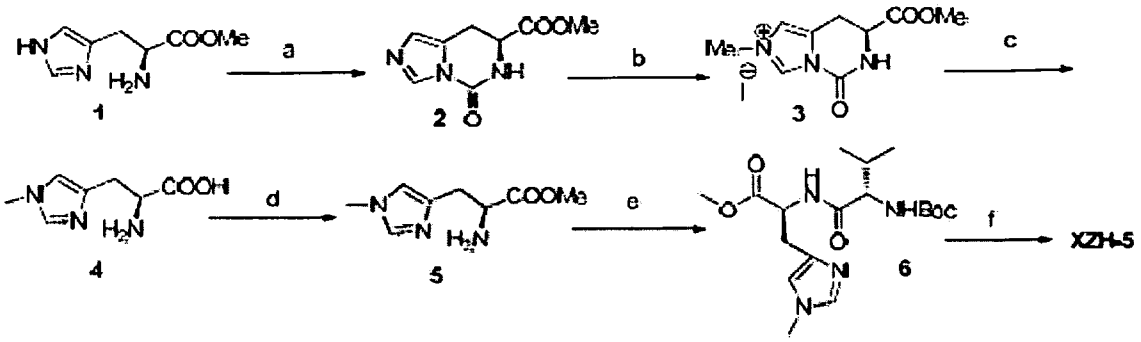


and

R_6 =



8.) A method to synthesize a compound of claim 1, comprising:



wherein a) N,N'-Carbonydiimidazol;. b) MeI, CH_3CN; c) 6*N* HCl, reflux; d) $SOCl_2$, MeOH; e) (L)-Boc-Valine, DCC, HOBT, TEA, DCM; f) TFA, DCM , then TEA, 3,5-Bis (trifluoromethyl) phenyl isocyanate ($(CF_3)_2C_6H_3NCO$), DCM, 85% (2 steps).

9.) A method of claim 8, wherein the compound of Formula I is further purified to one particular enantiomer.

10.) A method to inhibit STAT3 activation in a cell, comprising introducing a compound of claim 1 through 7 to a STAT3-expressing cell, and measuring STAT3 activation inhibition.

11.) A method of claim 10, wherein said inhibition is measured by observing cell apoptosis.

12.) A method of claim 10, wherein said inhibition is measured by observing prevention of STAT3 SH2 dimerization.

13.) A method of claim 10, wherein said inhibition is measured by observing a decrease in the levels of expression of STAT3 phosphorylation.

14.) A method of claim 10, wherein said inhibition is measured by observing inhibition of downstream targets of STAT3.

15.) A method of claim 14, wherein said downstream targets are selected from the group consisting of: cyclin; Bcl-2; and survivin.

16.) A method of claim 10, wherein said inhibition is measured by observing induction of cleaved PARP and caspase-3.

17.) A method of claim 10, wherein said inhibition is measured by inducing IL-6 in MDA-MD-453 breast cancer cells and observing a reduction in phosphorylation after induction.

18.) A method of claim 10, wherein said inhibition is measured by observing reduction of STAT3 DNA binding activity after said compound introduction.

19.) A method of claim 10, wherein said inhibition is measured by observing reduction of STAT3–dependent transcriptional activity after said compound introduction.

20.) A method to inhibit transcription of STAT3 regulated genes, comprising administering a compound of claim 1 through 7.

21.) A method of claim 20, wherein said transcription inhibition is measured via reverse transcriptase PCR.

22.) A method to decrease the ability of tumor cells to form colonies, comprising a administering a compound of claim 1 through 7 to a tumor cell-containing medium.

23.) A method of claim 22, wherein said tumor cell-containing medium is a mammalian cell culture.

24.) A method of claim 22, wherein said tumor cell-containing medium is a mammal.

25.) A method of claim 24, wherein said mammal is selected from the group consisting essentially of: human; livestock; companion animal; and zoo animal.

26.) A method to inhibit tumor cell migration, comprising administering a compound of claim 1 through 7 to a tumor cell-containing medium.

27.) A method of claim 26, wherein said tumor cell-containing medium is a mammalian cell culture.

28.) A method of claim 26, wherein said tumor cell-containing medium is a mammal.

29.) A method of claim 28, wherein said mammal is selected from the group consisting essentially of: human; livestock; companion animal; and zoo animal.

30.) A method to inhibit tumor cell proliferation, comprising administering a compound of claim 1 through 7 to a tumor cell-containing medium.

31.) A method of claim 30, wherein said tumor cell-containing medium is a mammalian cell culture.

32.) A method of claim 30, wherein said tumor cell-containing medium is a mammal.

33.) A method of claim 32, wherein said mammal is selected from the group consisting essentially of: human; livestock; companion animal; and zoo animal.

34.) A method to treat 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 through 7.

35.) A method of claim 34, wherein said cancer treated is selected from the group consisting essentially of: breast cancer; glioblastoma; and pancreatic cancer.

36.) A method of claim 34, which further comprises administering to the patient at least one additional chemotherapeutic drug.

37.) A method of claim 36, wherein said additional chemotherapeutic drug is doxorubicin.

38.) A method of claim 36, wherein said additional chemotherapeutic drug is gemcitabine.

39.) A method of claim 36, wherein said additional chemotherapeutic drugs are doxorubicin and gemcitabine.

40.) A method to determine the presence of cancer cells in a sample, comprising introducing a compound of claim 1 through 7 to a cell sample, and identifying whether STAT3 activation is inhibited.

41.) A method to identify compounds useful to inhibit STAT3 activation, comprising comparing the ability of a compound of claim 1 through 7 to inhibit STAT3 activation to the ability of a test compound to inhibit STAT3 activation.

42.) A kit comprising a compound of claim 1 through 7.

43.) A kit of claim 42, which further comprises nucleic acid molecules useful to identify STAT3 transcription.

44.) A pharmaceutically-acceptable formulation useful for treating cancer, comprising a compound of claim 1 through 7 and at least one adjuvant.

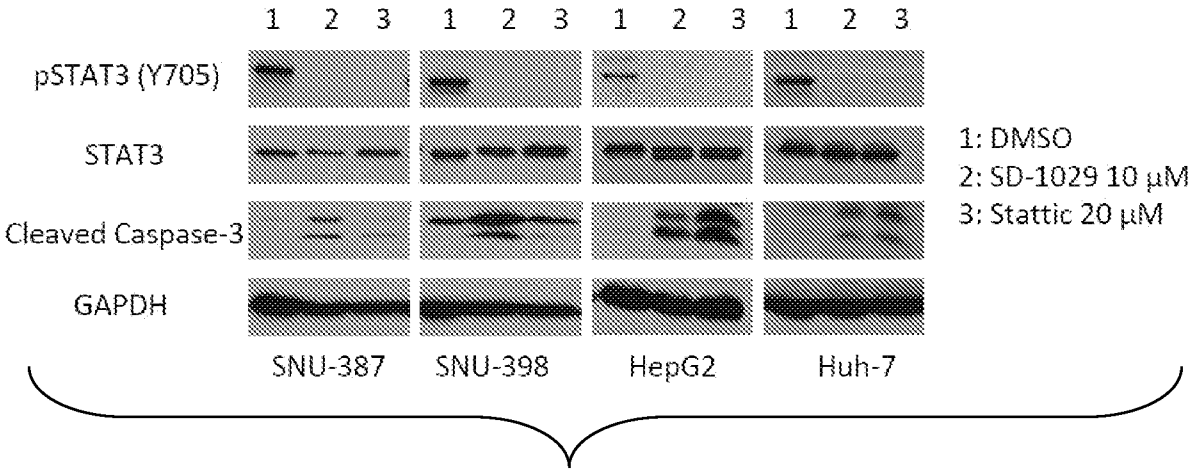


Figure 1A

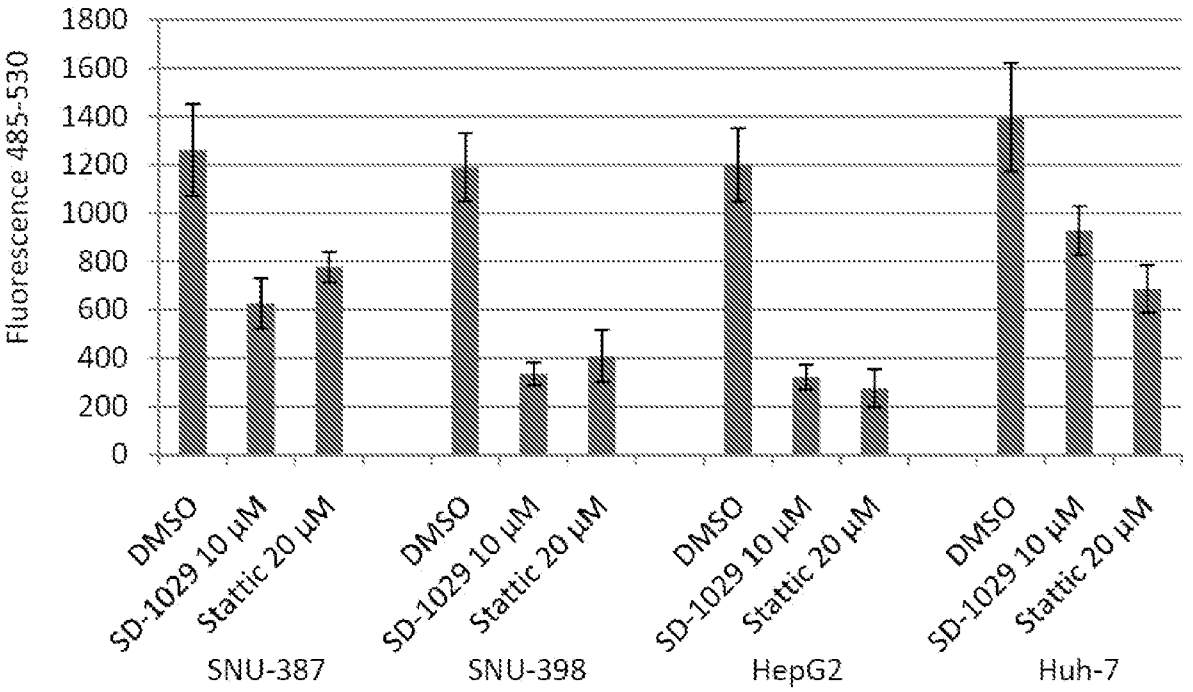


Figure 1B

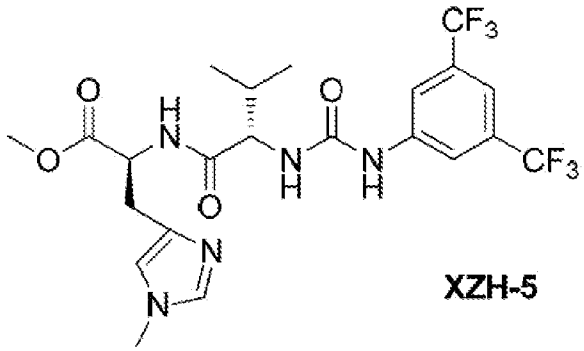


Figure 2A

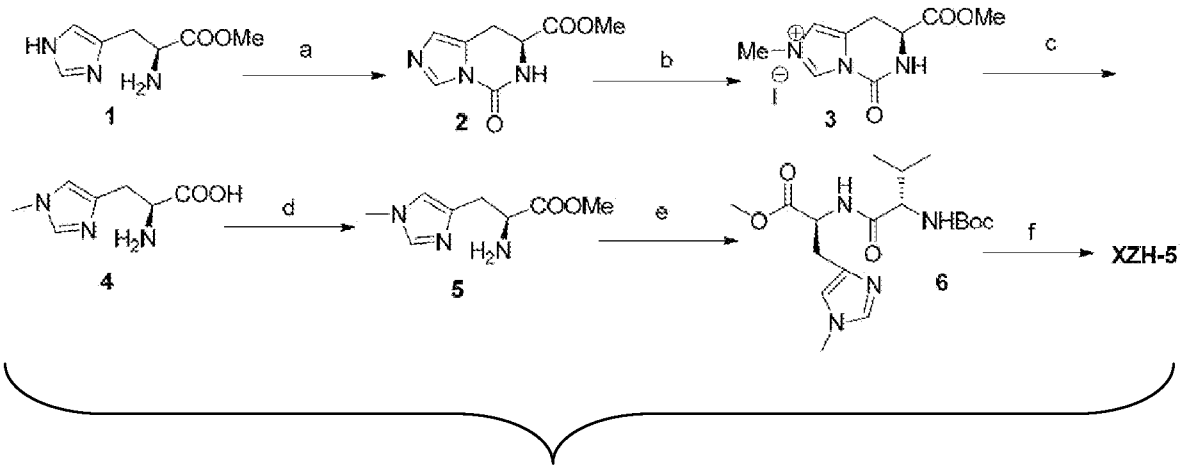


Figure 2B

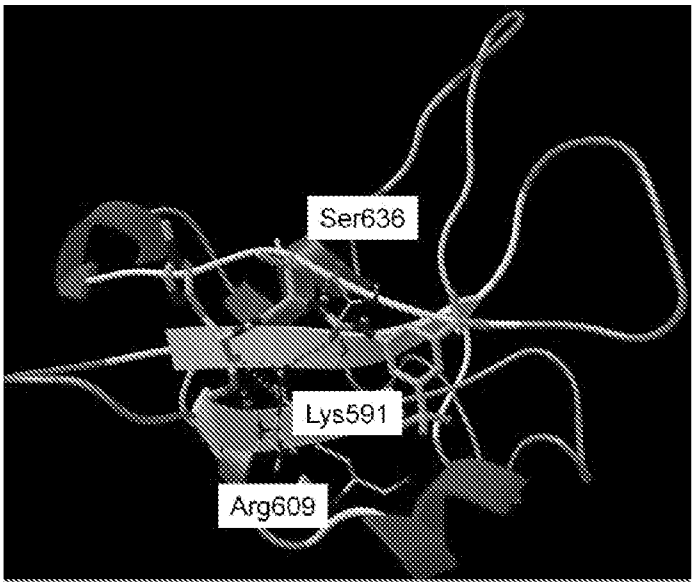


Figure 2C

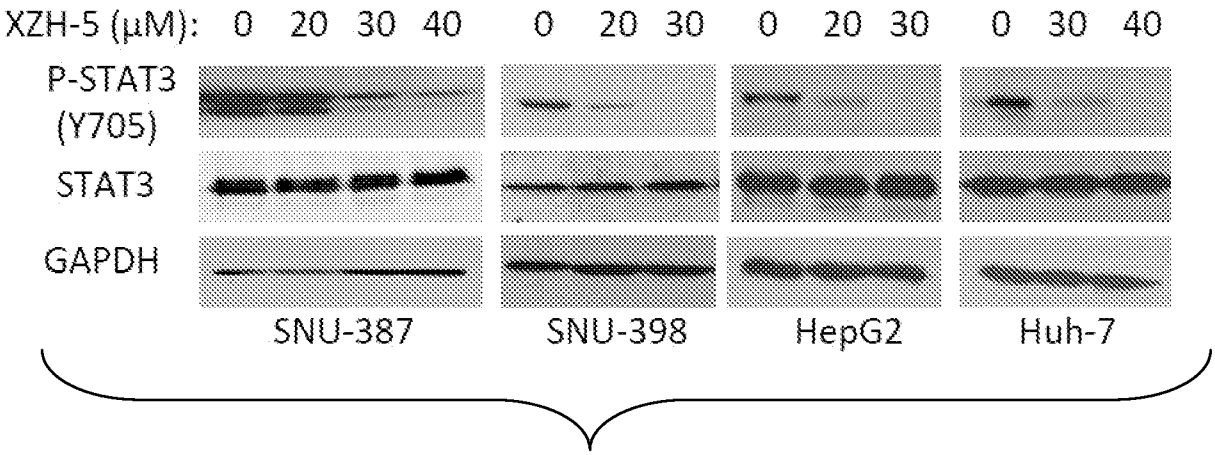


Figure 3A

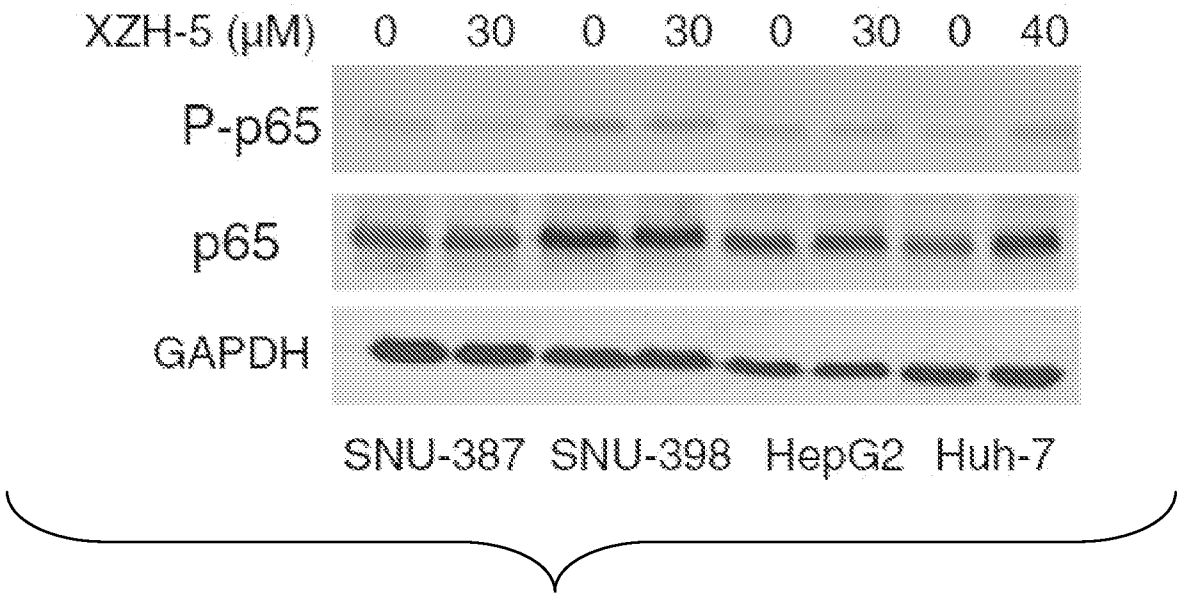


Figure 3B

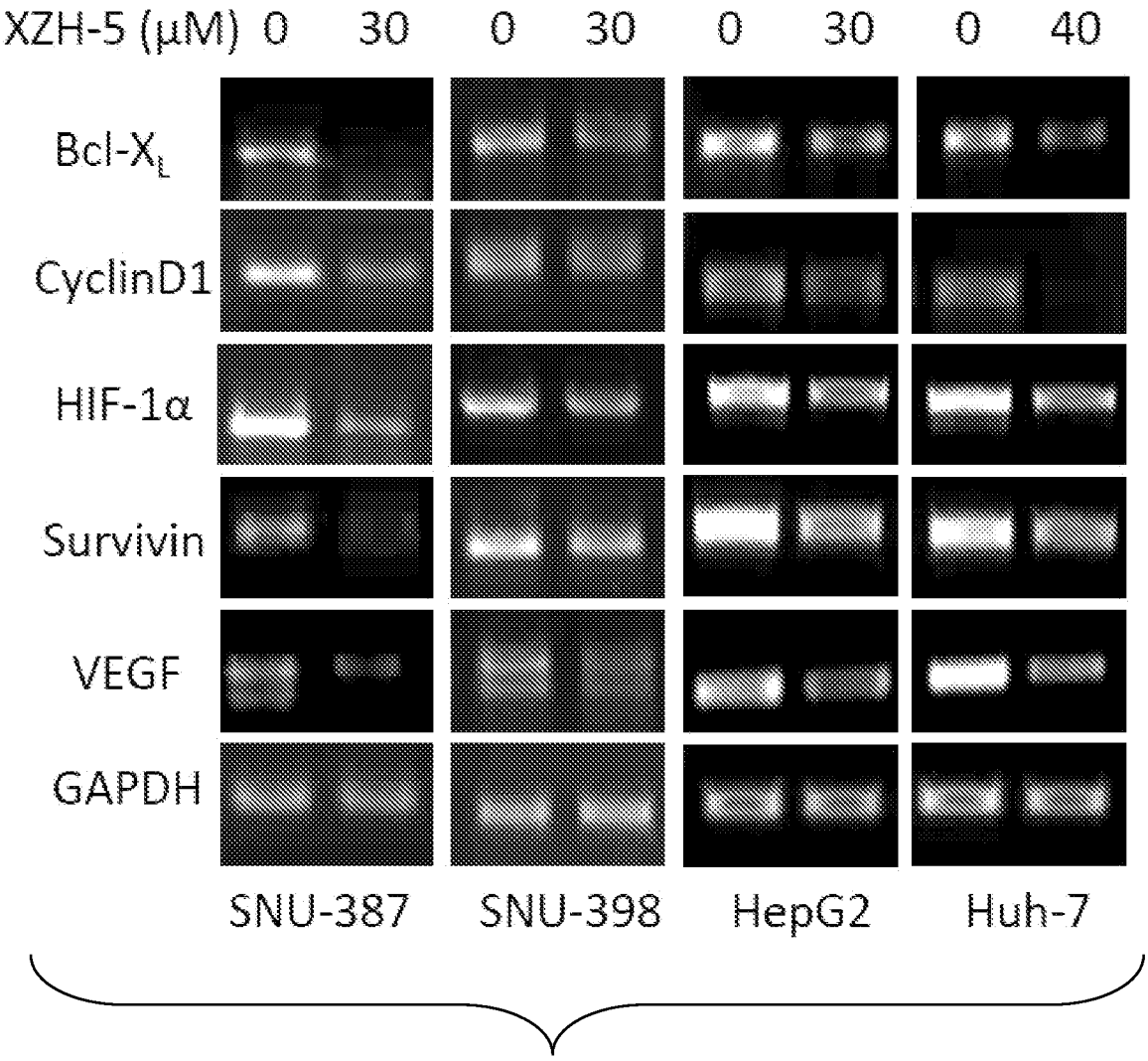


Figure 3C

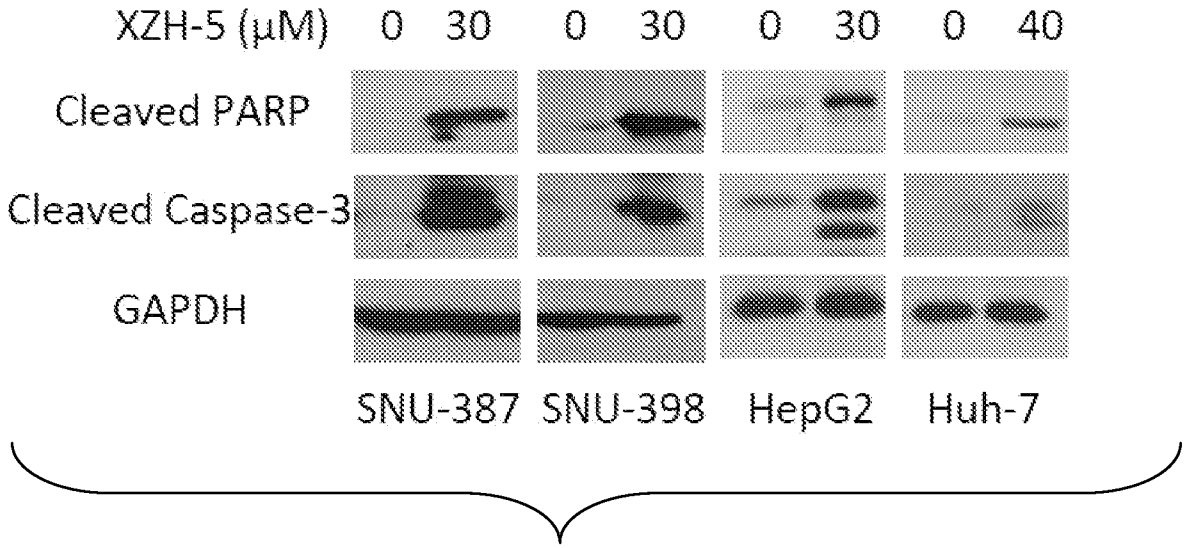


Figure 4A

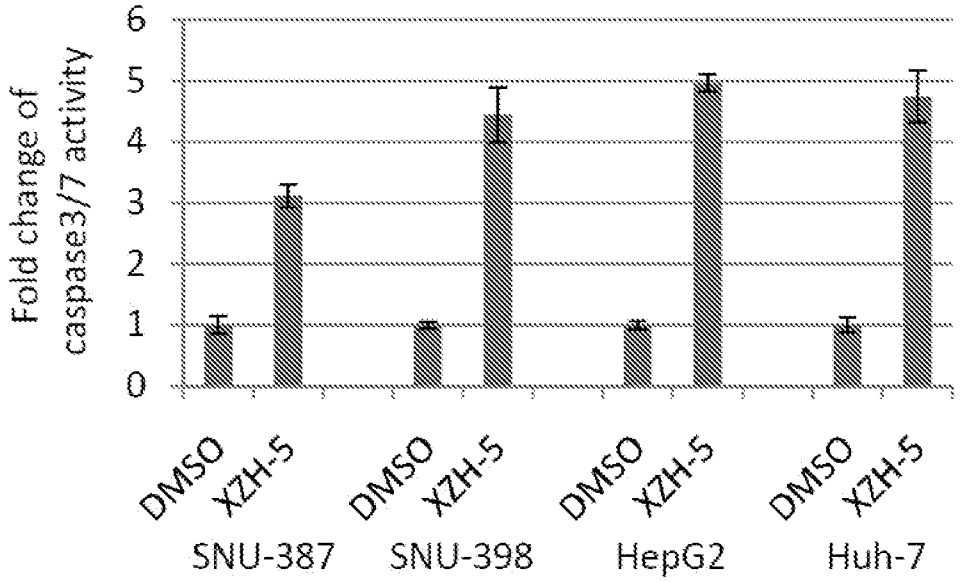


Figure 4B

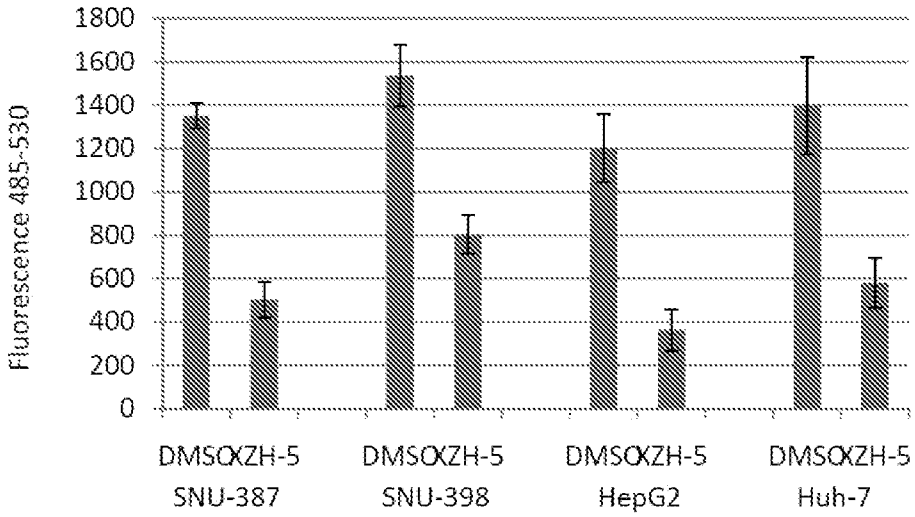


Figure 4C

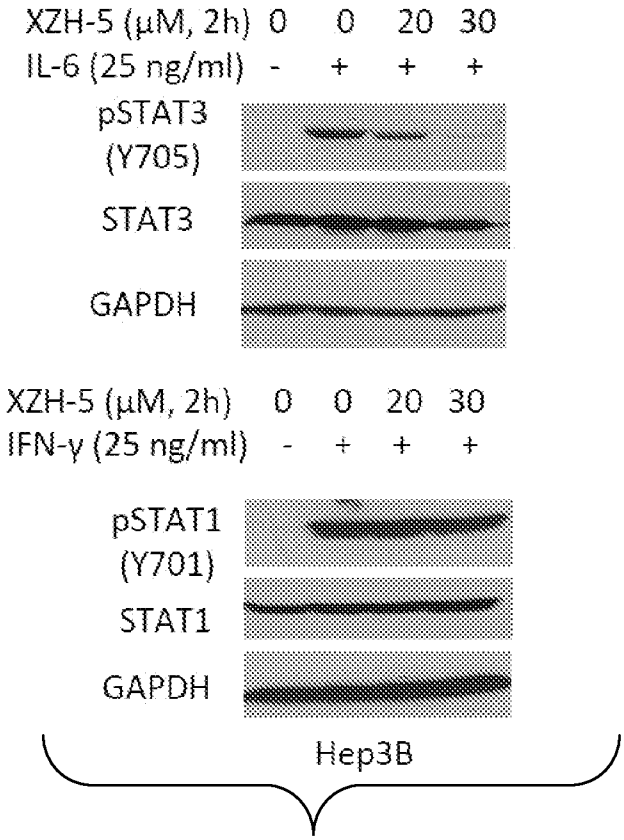


Figure 5A

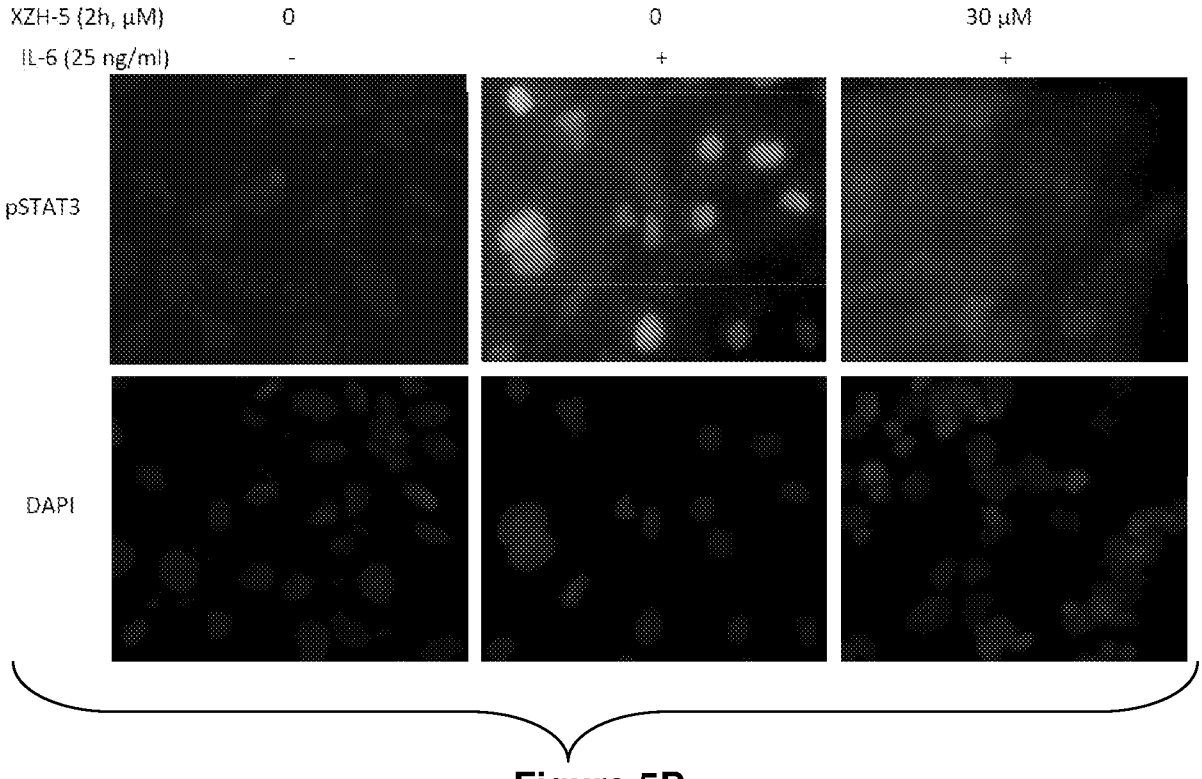


Figure 5B

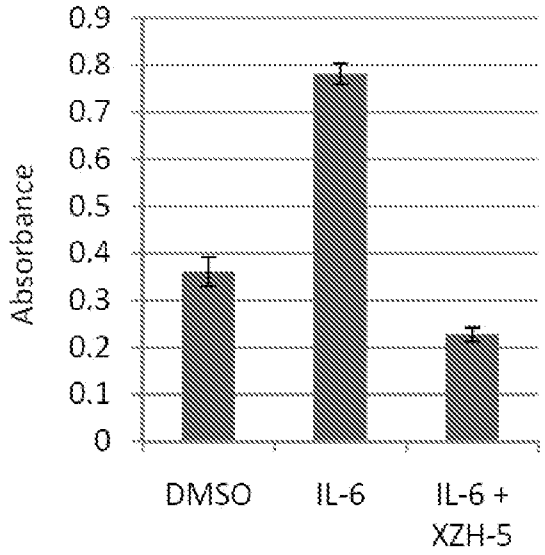


Figure 5C

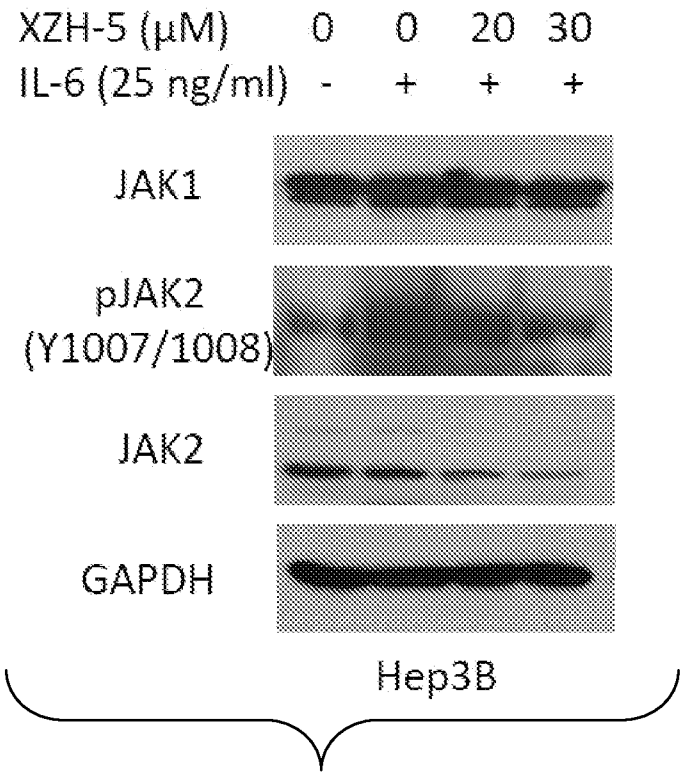


Figure 5D

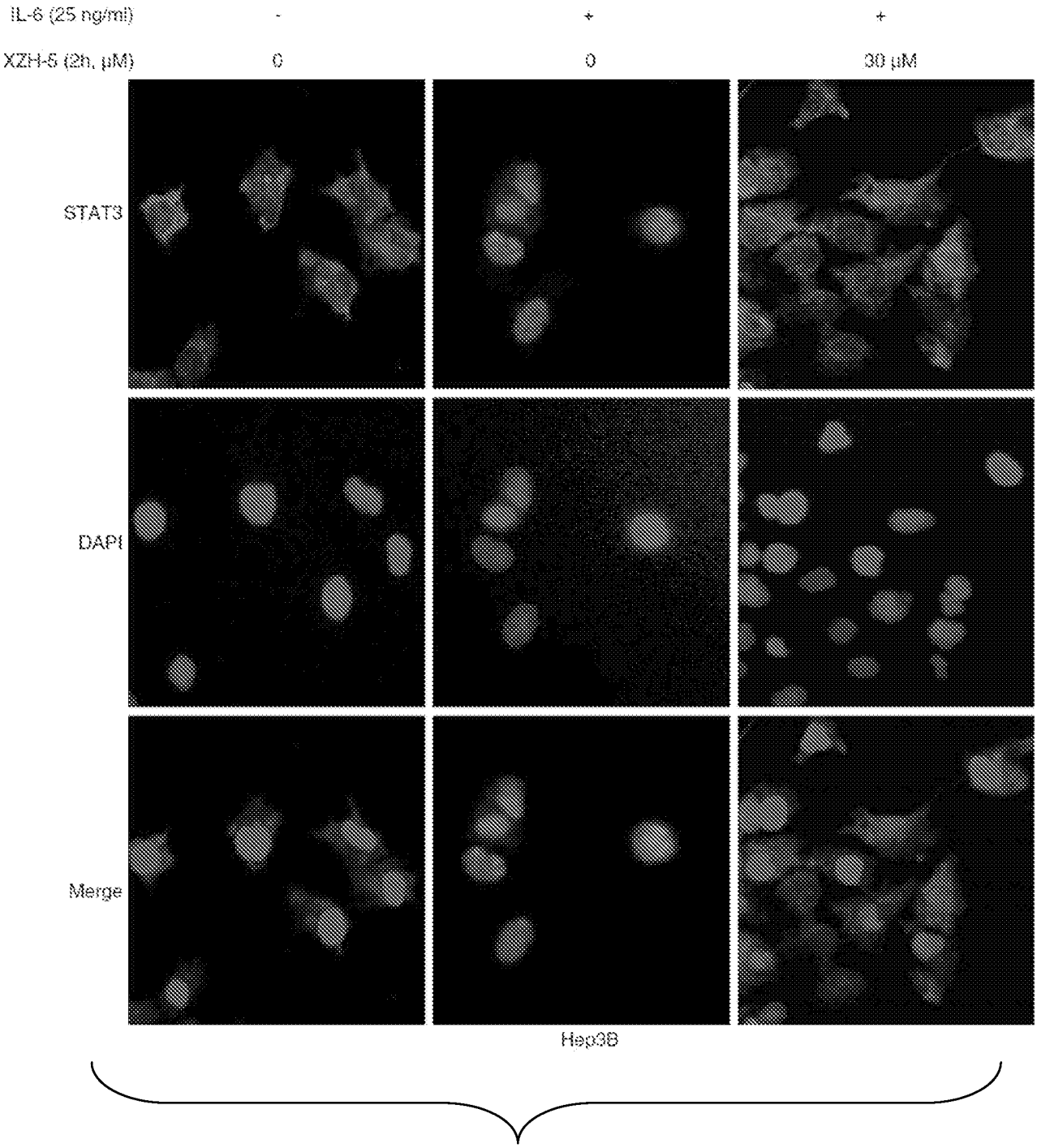


Figure 6

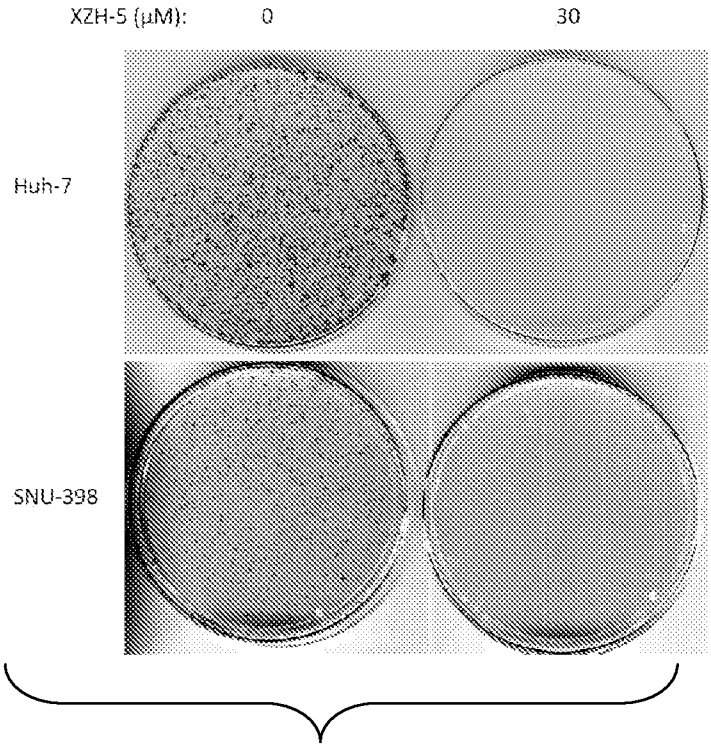


Figure 7

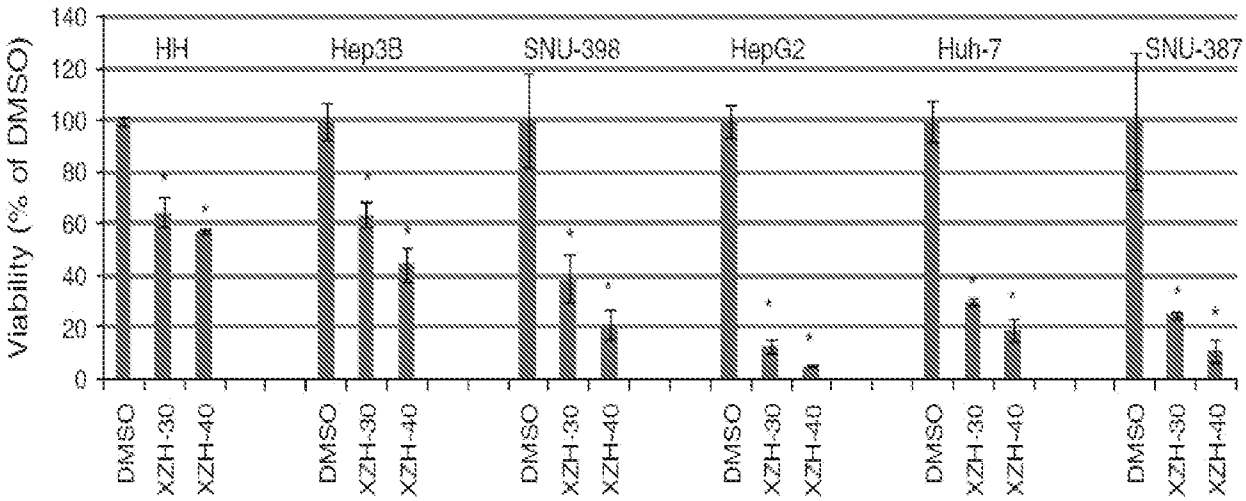


Figure 8