

-1-

METHOD FOR TREATING IDH1 INHIBITOR-RESISTANT SUBJECTS

The present invention relates to the treatment of cancer in subjects with a mutant isocitrate dehydrogenase 1 (IDH1) inhibitor disclosed herein.

5 IDH1 is an enzyme that catalyzes the conversion of isocitrate to α -ketoglutarate (α -KG) and reduces nicotinamide adenine dinucleotide phosphate (NADP⁺) to NADPH (Megias-Vericat J, *et al.*, *Blood Lymph. Cancer: Targets and Therapy* 2019; 9: 19-32).

Neomorphic (*de novo*) mutations in IDH1, *e.g.*, at IDH1 amino acid residue R132, contribute to tumorigenesis in several types of cancer, including solid tumors and
10 hematologic malignancies (Badur MG, *et al.*, *Cell Reports* 2018; 25: 1680). IDH1 mutations can result in high levels of 2-hydroxyglutarate (2-HG), which inhibits cellular differentiation, and inhibitors of mutant IDH1 can reduce 2-HG levels, which promotes cellular differentiation (Molenaar RJ, *et al.*, *Oncogene* 2018; 37: 1949-1960).

For example, acute myeloid leukemia (AML) is characterized by a diverse
15 spectrum of mutated genes and a multi-clonal genomic architecture comprising preleukemic and leukemic clones that evolve dynamically over time and under the selective pressure of therapy (Bloomfield CD, *et al.*, *Blood Revs.* 2018; 32: 416-425).

Induction chemotherapy with cytarabine and an anthracycline ("7 + 3") has been the standard of care for more than 4 decades for subjects with newly diagnosed AML. In
20 recent years, five drugs have been approved by the U.S. Food and Drug Administration for treating AML: midostaurin, enasidenib, CPX-351, gemtuzumab ozogamicin (Bloomfield CD, *et al.*, *Blood Revs.* 2018; 32: 416-425), and ivosidenib (Megias-Vericat J, *et al.*, *Blood Lymph. Cancer: Targets and Therapy* 2019; 9: 19-32).

Approximately 60% to 70% of adults with AML can be expected to attain
25 complete remission (CR) status following appropriate induction therapy, and more than 25% of adults with AML (about 45% of those who attain CR) can be expected to survive 3 or more years and may be cured.

However, IDH1 resistance mutations are observed in 7–14% of AML subjects, and the associated high 2-HG level can result in an epigenetic hyper-methylation
30 phenotype and a block in differentiation, resulting in leukemogenesis (Megias-Vericat J, *et al.*, *Blood Lymph. Cancer: Targets and Therapy* 2019; 9: 19-3). In addition, mutations

-2-

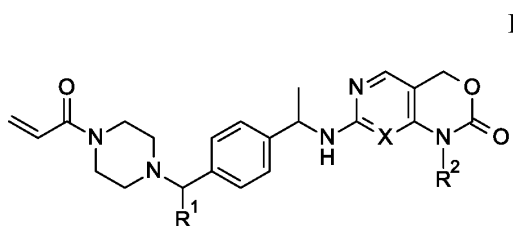
in the Flt3 kinase are observed in approximately one third of AML subjects (Lee HJ, *et al.*, *Oncotarget* 2018; 9: 924-936).

So called “secondary” IDH1 mutations, as defined herein, may contribute to relapse after treatment with a mutant IDH1 inhibitor. For example, to date, six post-ivosidenib treatment secondary IDH1 mutations have been reported: R119P, G131A, D279N, S280F, G289D or H315D (Choe S, *et al.*, “Molecular mechanisms mediating relates following ivosidenib monotherapy in subjects with *IDH1*-mutant relapsed or refractory acute myeloid leukemia,” 61st Am. Soc. Hematol. (ASH) Annual Meeting poster, Dec. 7-10, 2019, Orlando, FL, USA; Choe, S., *et al.*, *Blood Adv.* 2020; 4(9): 1894-1905).

Thus, there remains a need for alternative mutant IDH1-related cancer therapies, particularly for subjects in whom secondary IDH1 mutations occur after initial mutant IDH1 inhibitor therapy.

Certain mutant IDH1 inhibitors are disclosed in WO 2018/111707 A1, including a compound defined herein as “Compound A,” which is a covalent inhibitor of mutant IDH1 that modifies a single cysteine (Cys269) in an allosteric binding pocket, rapidly inactivates the enzyme, and selectively inhibits 2-HG production, without affecting α -KG levels (WO 2018/111707 A1).

The present invention provides a method for treating cancer, comprising administering to a human cancer subject having an IDH1 R132 mutation and one or more secondary IDH1 mutations a therapeutically effective amount of a compound of the Formula I:



wherein:

R¹ is -CH₂CH(CH₃)₂, -CH₂CH₃, -CH₂CH₂OCH₃, or -CH₂-cyclopropyl;

R² is -CH₃ or -CH₂CH₃; and

X is N or CH;

-3-

or a pharmaceutically acceptable salt thereof.

In one embodiment of the method of the invention, in the compound of Formula I, X is N, or a pharmaceutically acceptable salt thereof. In another embodiment, X is N, R¹ is -CH₂-cyclopropyl, and R² is -CH₂CH₃, or a pharmaceutically acceptable salt thereof.

5 In another embodiment, X is N, R¹ is -CH₂-cyclopropyl, and R² is -CH₂CH₃.

In another embodiment, the compound of Formula I is:

7-[[[(1S)-1-[4-[(1R)-2-Cyclopropyl-1-(4-prop-2-enoylpiperazin-1-yl)ethyl]phenyl]ethyl]amino]-1-ethyl-4H-pyrimido[4,5-d][1,3]oxazin-2-one;

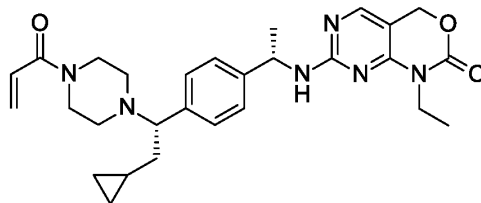
7-[[[(1S)-1-[4-[(1S)-2-cyclopropyl-1-(4-prop-2-enoylpiperazin-1-yl)ethyl]phenyl]ethyl]amino]-1-ethyl-4H-pyrimido[4,5-d][1,3]oxazin-2-one; or

1-Ethyl-7-[[[(1S)-1-[4-[1-(4-prop-2-enoylpiperazin-1-yl)propyl]phenyl]ethyl]amino]-4H-pyrimido[4,5-d][1,3]oxazin-2-one;

or a pharmaceutically acceptable salt of any one thereof.

In another embodiment, the compound of Formula I is 7-[[[(1S)-1-[4-[(1S)-2-cyclopropyl-1-(4-prop-2-enoylpiperazin-1-yl)ethyl]phenyl]ethyl]amino]-1-ethyl-4H-pyrimido[4,5-d][1,3]oxazin-2-one.

In another embodiment, the compound of Formula I is:



(referred to herein as “Compound A”), or a pharmaceutically acceptable salt thereof.

20 In another embodiment, the compound is Compound A.

In one embodiment of the method of the invention, the R132 mutation is R132H.

In another embodiment, the IDH1 mutation is R132C. In another embodiment, the IDH1 mutation is R132G. In another embodiment, the IDH1 mutation is R132L. In another embodiment, the IDH1 mutation is R132S.

25 In one embodiment of the method of the invention, the one or more secondary IDH1 mutations is one or more of R119P, G131A, D279N, S280F, G289D or H315D. In

-4-

another embodiment, the secondary IDH1 mutation is two or more of R119P, G131A, D279N, S280F, G289D or H315D.

In another embodiment, the subject is identified as having an R132 IDH1 mutation. In another embodiment, the subject is identified as having an R132 IDH1
5 mutation in tissue.

In another embodiment, the subject is identified as having one or more secondary IDH1 mutations.

In another embodiment, the cancer is a hematologic malignancy, and the subject is identified as having an R132 IDH1 mutation in blood, bone marrow, lymph node or
10 lymphatic fluid. In another embodiment, the subject is identified as having an R132 IDH1 mutation in blood cells, bone marrow cells, or blood cells, or lymph node cells, or lymphatic fluid cells. In another embodiment, the subject is identified as having one or more secondary IDH1 mutations.

In another embodiment, the cancer is a solid tumor cancer, and the subject is identified as having an R132 IDH1 mutation in solid tumor tissue. In another
15 embodiment, the solid tumor tissue is cholangiocarcinoma tissue. In another embodiment, the subject is identified as having an R132 IDH1 mutation in solid tumor tissue cells. In another embodiment, the subject is identified as having one or more secondary IDH1 mutations.

In one embodiment of the method of the invention, the cancer is a solid tumor. In another embodiment, the solid tumor is cholangiocarcinoma, head & neck cancer, chondrosarcoma, hepatocellular carcinoma, melanoma, pancreatic cancer, astrocytoma, oligodendroglioma, glioma, glioblastoma, bladder carcinoma, colorectal cancer, lung
20 cancer, or sinonasal undifferentiated carcinoma. In another embodiment, the lung cancer is non-small cell lung cancer. In another embodiment, the solid tumor is cholangiocarcinoma.
25

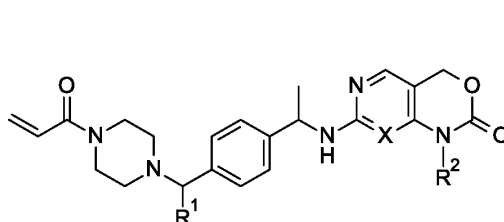
In another embodiment, the cancer is a hematologic malignancy. In another embodiment, the hematologic malignancy is acute myeloid leukemia, myelodysplastic syndrome myeloproliferative neoplasm, angioimmunoblastic T-cell
30 lymphoma, T-cell acute lymphoblastic leukemia, polycythemia vera, essential

-5-

thrombocythemia, primary myelofibrosis or chronic myelogenous leukemia. In another embodiment, the hematologic malignancy is acute myeloid leukemia.

In another embodiment, the subject had been treated with a mutant IDH1 inhibitor other than a compound of Formula I. In another embodiment, the mutant IDH1 inhibitor other than a compound of Formula I is vorasidenib, BAY-1436032, AGI-5198, IDH305, or ivosidenib. In another embodiment, the mutant IDH1 inhibitor other than a compound of Formula I is ivosidenib. In another embodiment, the subject had been treated with a mutant IDH1 inhibitor other than a compound of Formula I prior to treatment with a compound of Formula I.

The present invention also provides a compound of Formula I:



wherein:

R¹ is -CH₂CH(CH₃)₂, -CH₂CH₃, -CH₂CH₂OCH₃, or -CH₂-cyclopropyl;

R² is -CH₃ or -CH₂CH₃;

X is N or CH; or a pharmaceutically acceptable salt thereof;

for use in treating cancer in a human subject having an IDH1 R132 mutation and one or more secondary IDH1 mutations.

For the compound of Formula I, it is preferred that X is N, or a pharmaceutically acceptable salt thereof; it is preferred that R¹ is -CH₂-cyclopropyl, or a pharmaceutically acceptable salt thereof; it is preferred that R² is -CH₂CH₃, or a pharmaceutically acceptable salt thereof; it is more preferred that X is N, R¹ is -CH₂-cyclopropyl, and R² is -CH₂CH₃, or a pharmaceutically acceptable salt thereof; it is most preferred that X is N, R¹ is -CH₂-cyclopropyl, and R² is -CH₂CH₃.

Preferred compounds of Formula I are:

7-[[[(1S)-1-[4-[(1R)-2-Cyclopropyl-1-(4-prop-2-enoylpiperazin-1-yl)ethyl]phenyl]ethyl]amino]-1-ethyl-4H-pyrimido[4,5-d][1,3]oxazin-2-one;

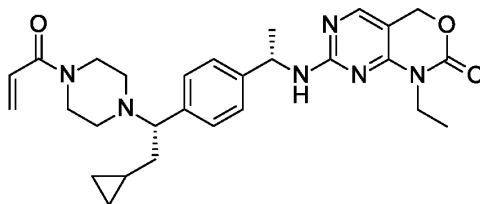
-6-

7-[[[(1S)-1-[4-[(1S)-2-cyclopropyl-1-(4-prop-2-enoylpiperazin-1-yl)ethyl]phenyl]ethyl]amino]-1-ethyl-4H-pyrimido[4,5-d][1,3]oxazin-2-one; or

1-Ethyl-7-[[[(1S)-1-[4-[1-(4-prop-2-enoylpiperazin-1-yl)propyl]phenyl]ethyl]amino]-4H-pyrimido[4,5-d][1,3]oxazin-2-one;

5 or a pharmaceutically acceptable salt of any one thereof.

A more preferred compound of Formula I is:



(Compound A), or a pharmaceutically acceptable salt thereof.

In another embodiment of the present invention, the cancer is relapsed cancer. In
10 another embodiment, the relapsed cancer is a solid tumor cancer. In another embodiment,
the relapsed solid tumor cancer is cholangiocarcinoma. In another embodiment, the
relapsed cancer is hematologic malignancy. In another embodiment, the relapsed
hematologic malignancy is relapsed AML.

In another embodiment of the present invention, the cancer is refractory cancer.
15 In another embodiment, the refractory cancer is a solid tumor cancer. In another
embodiment, the refractory solid tumor cancer is cholangiocarcinoma. In another
embodiment, the refractory cancer is hematologic malignancy. In another embodiment,
the refractory hematologic malignancy is refractory AML.

In another embodiment of the present invention, the cancer is advanced cancer. In
20 another embodiment, the advanced cancer is an advanced solid tumor cancer. In another
embodiment, the advanced solid tumor cancer is cholangiocarcinoma. In another
embodiment, the advanced cancer is an advanced hematologic malignancy. In another
embodiment, the advanced hematologic malignancy is advanced AML.

In another embodiment, the AML is acute promyelocytic leukemia.

25 As used above, and throughout the description of the invention, the following
terms, unless otherwise indicated, shall be understood to have the following meanings:

-7-

The term “hematologic tissue” refers to blood, bone marrow, spleen, lymph node, or lymphatic fluid.

The term “solid tumor tissue” refers to tissue that is not hematologic tissue. Non-limiting examples of solid tissue are cholangial tissue, pancreatic tissue, head tissue, neck
5 tissue, hepatic tissue, skin tissue, astrocytoma tissue, oligodendroglial tissue, glial tissue, brain tissue, bladder tissue, colorectal tissue, lung tissue, and sinonasal undifferentiated carcinoma.

The term “solid tumor cancer” means that the cancer originated in a tissue that is not blood, bone marrow, lymph node or lymphatic fluid.

10 The term “hematologic malignancy” relates to cancer that in the blood, the bone marrow, the lymph node or the lymphatic fluid.

The term “advanced hematological malignancy” refers to malignancy that has spread to lymph nodes or to other tissues outside of the blood or the bone marrow.

The term “cancer subject” means a subject who has been diagnosed with cancer.

15 The term “refractory cancer” refers to cancer that has been treated, but the human cancer subject did not respond to treatment.

The term “relapsed cancer” means that the human cancer subject responded to treatment for a period of time, but that the cancer has reoccurred.

The term “advanced cancer” refers to cancer that has spread to lymph nodes or to
20 other tissues outside of the cancer’s point of origin. For example, advanced acute myeloid leukemia is acute myeloid leukemia that has spread to a tissue outside of the blood or the bone marrow.

The term “solid tumor subject” means a subject who has been diagnosed with a solid tumor cancer. In one embodiment, the solid tumor cancer is cholangiocarcinoma.

25 The term “hematologic malignancy subject” means a subject who has been diagnosed with a hematologic malignancy. In one embodiment, the hematologic malignancy subject is an AML subject. The term “AML subject” means a subject who has been diagnosed with AML. Methods for diagnosing AML are known to those of ordinary skill in the art, *e.g.*, in Dohner H, *et al.*, *Blood* 2017; 129: 424-447.

30 The terms “acute myeloid leukemia,” “acute myelogenous leukemia,” and “acute nonlymphocytic leukemia” are synonymous.

“Responsiveness to hematologic malignancy (*e.g.*, AML) treatment” includes improvement in overall survival, partial response, long-term stable disease, or improvement in long-term survival characterized as complete remission (determined by less than 5% myeloblasts in bone marrow, the absence of circulating blasts, hematologic recovery (as evidenced by a peripheral blood absolute neutrophil count greater than 1,000 cells/ μ L and a platelet count greater than 100,000/ μ L, without the need for red blood cell transfusion, and the absence of extramedullary disease) (Bloomfield CD, *et al.*, *Blood Revs.* 2018; 32: 416-425).

The term “IDH1 R132 mutation” refers to an IDH1 mutation at amino acid residue 132 in a subject’s IDH1 gene, as determined, *e.g.*, in the subject’s nucleic acid (*e.g.*, DNA). As used herein, an “IDH1 R132 mutation” is not a “secondary IDH1 mutation.”

The term “secondary IDH1 mutation” refers to an IDH1 mutation that occurs in the IDH1 enzyme in a human subject after treatment with a mutant IDH1 inhibitor other than a compound of Formula I herein. In one embodiment of the method of the invention, the one or more secondary IDH1 mutations is one or more of R119P, G131A, D279N, S280F, G289D or H315D in IDH1. However, other secondary IDH1 mutations may be reported in the future. As used herein, a “secondary IDH1 mutation” is not an “IDH1 R132 mutation.”

The term “mutant IDH1 inhibitor” refers to a compound that inhibits the enzyme activity of and/or the production of 2-HG by a mutant IDH1 enzyme. Methods for assaying mutant IDH1 enzyme activity are known to those of ordinary skill in the art, *e.g.*, in WO 2018/111707 A1. In the term “mutant IDH1 inhibitor, the word “mutant” refers to the IDH1 gene, not the inhibitor.

The term “identified as having an IDH1 R132 mutation” means that nucleic acid (*e.g.*, DNA) from a human subject’s tissue or cells has been analyzed to determine if the human subject has an IDH1 R132 mutation. In one embodiment, one or more of the human subject’s blood cells, bone marrow cells, lymph node, lymph node cells, lymphatic fluid or lymphatic fluid cells has been analyzed for an IDH1 R132 mutation. In another embodiment, the human subject’s solid tissue has been analyzed for an IDH1 R132 mutation.

In the method of the present invention, the party who identifies the human subject as having an IDH1 R132 mutation can be different than the party that administers the compound. In one embodiment, the party who identifies the human subject as having an IDH1 R132 mutation is different than the party that administers the compound.

5 The term "identified as having one or more secondary IDH1 mutation(s)" means that nucleic acid (*e.g.*, DNA) from one or more of the human subject's blood cells, bone marrow cells, lymph node, lymph node cells, lymphatic fluid or lymphatic fluid cells has been analyzed to determine if a human subject has one or more secondary IDH1 mutation(s).

10 Analytical methods for identifying genetic mutations are known to those of ordinary skill in the art (Clark, O., *et al.*, *Clin. Cancer. Res.* 2016; 22: 1837-42), including, but not limited to, karyotyping (Guller JL, *et al.*, *J. Mol. Diagn.* 2010; 12: 3-16), fluorescence *in situ* hybridization (Yeung DT, *et al.*, *Pathology* 2011; 43: 566-579), Sanger sequencing (Lutha, R *et al.*, *Haematologica* 2014; 99: 465-473), metabolic
15 profiling (Miyata S, *et al.*, *Scientific Reports* 2019; 9: 9787), polymerase chain reaction (Ziai, JM and AJ Siddon, *Am. J. Clin. Pathol* 2015; 144: 539-554), and next-generation sequencing (*e.g.*, whole transcriptome sequencing) (Lutha, R *et al.*, *Haematologica* 2014; 99: 465-473; Wang H-Y, *et al.*, *J. Exp. Clin. Cancer Res.* 2016; 35: 86).

 The terms "treatment," "treat," "treating," and the like, are meant to include
20 slowing, stopping, or reversing the progression of cancer. These terms also include alleviating, ameliorating, attenuating, eliminating, or reducing one or more symptoms of a disorder or condition, even if the cancer is not actually eliminated and even if progression of the cancer is not itself slowed, stopped or reversed.

 "Therapeutically effective amount" means the amount of a compound, or
25 pharmaceutically acceptable salt thereof, administered to the subject that will elicit the biological or medical response of or desired therapeutic effect on a subject. A therapeutically effective amount can be readily determined by the attending clinician, as one skilled in the art, by the use of known techniques and by observing results obtained under analogous circumstances. In determining the effective amount for a subject, a
30 number of factors are considered by the attending clinician, including, but not limited to: size, age, and general health of the subject; the specific disease or disorder involved; the

degree of or involvement or the severity of the disease or disorder; the response of the individual subject; the particular compound administered; the mode of administration; the bioavailability characteristics of the preparation administered; the dose regimen selected; the use of concomitant medication; and other relevant circumstances.

5 A compound of Formula I herein can optionally be formulated as a pharmaceutical composition administered by any route which makes the compound bioavailable, including oral, intravenous, and transdermal routes. It is preferred that such compositions are formulated for oral administration. Such pharmaceutical compositions and processes for preparing same are well known in the art. (See, e.g., Remington: The
10 Science and Practice of Pharmacy (D.B. Troy, Editor, 21st Edition, Lippincott, Williams & Wilkins, 2006).

A "pharmaceutically acceptable carrier, diluent, or excipient" is a medium generally accepted in the art for the delivery of biologically active agents to mammals, e.g., humans.

15 It will be understood by one of ordinary skill in the art that compounds administered in the method of the invention are capable of forming salts. The compounds react with any of a number of inorganic and organic acids to form pharmaceutically acceptable acid addition salts. Such pharmaceutically acceptable acid addition salts and common methodology for preparing them are well known in the art. *See, e.g., P. Stahl, et*
20 *al., HANDBOOK OF PHARMACEUTICAL SALTS: PROPERTIES, SELECTION AND USE, (VCHA/Wiley-VCH, 2008).*

"Pharmaceutically acceptable salts" or "a pharmaceutically acceptable salt" refers to the relatively non-toxic, inorganic and organic salt or salts of the compounds of the present invention (S.M. Berge, *et al.*, "Pharmaceutical Salts", *Journal of Pharmaceutical*
25 *Sciences*, Vol 66, No. 1, January 1977).

Materials, Methods and Results

Compounds and Formulation. Ivosidenib and Compound A are prepared as a 20 mM stock in 100% DMSO (dimethyl sulfoxide) (Sigma, D2438) and diluted serially in
30 100% DMSO to achieve the desired concentrations. DMSO preps are further diluted with cell culture media prior to addition in the assay.

-11-

Cell lines. U-87 MG cells (ATCC, HTB-14) are cultured and assayed in MEM (Gibco, 11095) with 2 mM GlutaMAX (Gibco, 35050), 1 mM Pyruvate (Gibco, 11360), 0.1 mM NEAA ((Non-essential amino acid) Gibco, 11140) and 10% dialyzed FBS (Fetal bovine serum) (Gibco, 26400). Ba/F3 cells (DSMZ, ACC 300) are cultured and assayed in RPMI 1640 (Gibco, 22400) with 10% heat inactivated FBS (Gibco, 10082-147) and 10 ng/ml mouse IL3 (R&D systems, 403-ML-025).

Cell-based inhibition assays. Cell-based assays are performed by measuring 2-HG in either U-87 MG cells or Ba/F3 cells in which IDH mutations are expressed.

DNA constructs encoding IDH1 mutations are introduced into U-87 MG cells using transfection (Promega FuGENE HD, E2311) or lentiviral transduction, and the IDH-mutant expressing cell lines are selected using blasticidin (5 µg/ml) or puromycin (1 µg/ml). For compound treatment in U-87 MG cells, 20,000-50,000 cells per well are plated in 96 well cell culture plates (Falcon, 353377) 2hrs prior to treatment. Cells are treated with serial dilutions of compound A in standard growth media. Plates are incubated in a mammalian cell culture incubator (humidified, 37°C, 5%CO₂) for 16-72 hrs. Following the incubation period, the media is aspirated and cell lysates are prepared either by addition of 30 µL/well lysis buffer (25 mM Tris-HCl pH7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X 100, 2X Halt protease + phosphatase inhibitor (Pierce, 78441) (for derivatization LC-MS method) or by addition of 100 µL 80% methanol/20% water containing LC-MS internal standards (1 µM ¹³C₄ α-KG/¹³C₅ 2-HG) per well (for ion pairing LC-MS method). 96-well sample plates are then sealed, shaken at 450 rpm for 10 min, and then placed at -20°C and stored until LC-MS analysis.

Ba/F3 cells are transfected with DNA constructs encoding IDH1R132H-myc, IDH1R132H_S280F-myc, or IDH1R132C_S280F-myc constructs using NEON Transfection system (Life Technologies, MPK10025) and isolated using Puromycin (2 µg/mL) or Blasticidin (10 µg/mL). Stably transfected lines are used for inhibitor assays. 15,000 Ba/F3 cells per well are plated in 96 well cell culture plates (Falcon, 353377) 2 hours prior to treatment. Cells are treated with serial dilutions of the desired compounds in standard growth media. Plates are incubated in a mammalian cell culture incubator (humidified, 37 °C, 5% CO₂) for the desired time (72 or 96 hours). Following the

incubation period, conditioned media from each well is collected for 2-HG analysis by LC-MS.

LC-MS metabolite analysis of conditioned media and cell lysates. The effect of inhibitors on the concentrations of 2-HG are determined by liquid chromatography-mass spectrometry (LC-MS) analysis of cell lysates or conditioned media using either a derivatization method or an ion-pairing method as described below.

For the derivatization LC-MS method, calibration curves are prepared by spiking 2-HG and α -KG into cell culture media and cell lysis buffer respectively. The method utilizes derivatization with O-benzylhydroxylamine prior to analysis by LC-MS. 10 μ L of each standard or sample (media or cell extract) is placed into a deep-well 96-well plate and combined with 100 μ L of internal standard solution containing 10 μ M d5-3-hydroxyglutarate and 10 μ M d6- α -KG. 50 μ L of 1M O-benzylhydroxylamine in pyridine buffer (8.6% pyridine, pH 5) and 50 μ L of 1 M N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) in pyridine buffer is added to each sample. The derivatization reaction proceeds at room temperature for one hour. Using a Beckman Biomek FX liquid handler, 300 μ L of ethyl acetate is added to each sample. Plates are sealed and vortexed for 5 minutes, followed by centrifugation for 5 minutes at 4000 rpm in Eppendorf 5810R centrifuge. 220 μ L of the upper layer is transferred to a new 96-well plate. Samples are dried under heated nitrogen at 50 °C and reconstituted with 100 μ L of methanol/water (1:1). 1 μ L of derivatized sample is injected onto an LC-MS system consisting of a Shimadzu Prominence 20A HPLC system and a Thermo Quantum Ultra™ triple quadrupole mass spectrometer. Analytes are separated on a Water XBridge™ C18 column (2.1 x 50 mm, 3.5 μ m) with a flow rate of 0.6 mL/minute. Mobile phase A is 0.1% formic acid in water and mobile phase B is methanol. The gradient profile is: 0 minutes, 5% B; 2 minutes, 100% B; 4.00 minutes, 100% B; 4.1 minutes, 5% B; 5.50 minutes, stop. The mass spectrometer utilizes a HESI-II probe operated in positive ion selected reaction monitoring mode. Calibration curves are constructed by plotting analyte concentrations vs. analyte/internal standard peak area ratios and performing a quadratic fit of the data using a 1/concentration weighting with Xcalibur™ software. Analyte concentrations for the unknowns are back calculated from the calibration curves.

For the ion-pairing LC-MS method, calibration curves are prepared by spiking 2-HG and α -KG into 80% methanol/20% water containing LC-MS internal standards (1 μ M $^{13}\text{C}_4$ α -KG/ $^{13}\text{C}_5$ 2-HG). The quantitation of 2-HG and α -KG is accomplished using an AB Sciex 6500 mass spectrometer with an ESI probe and interfaced with an UHPLC system in the negative multiple-reaction monitoring (MRM) mode. The UHPLC system consists of an Agilent 1290 binary pump, thermostatted column compartment (TCC), and sampler. The injection volume is 1 μ L for cell culture extracts. The extracts are chromatographically resolved using a Hypercarb column, 2.1x20 mm, 5.0 mm Javelin HTS (Thermo Scientific, PN: 35005-022135). Mobile phase A is water/10mM tributylamine/15mM acetic acid. Mobile phase B is acetonitrile/20mM tributylamine/30mM acetic acid. The solvent flow rate is 1.0 mL/min. The isocratic condition is kept at 26% mobile phase B. The valve, sample loop, and needle are washed with 50% acetonitrile: 50% methanol for 20 seconds. The column temperature is kept at 55°C. Calibration curves are calculated by least-square linear regression with 1/x weighting. 2-HG and α -KG are quantified using standard curve and ratio of the peak area of analytes to internal standard. Data analysis is performed using MultiQuant 3.0 (AB Sciex). The raw data are exported to Excel spreadsheets.

Determination of IC₅₀ Curves. IC₅₀ Curves for each compound are obtained using four parameter data fitting analysis in GraphPad/Prism software.

In experiments performed essentially as described above, the IC₅₀ results set forth in Table 1 are obtained.

Table 1. IC₅₀ Results		
Cell Line Construct	Ivosidenib IC₅₀ (nM)	Compound A IC₅₀ (nM)
U87MG R132H	27	0.32
U87MG R132H_S280F	>1000	2.58
U87MG R132C_S280F	>1000	12.48
Ba/F3 R132H	15.92	0.11
Ba/F3 R132H_S280F	>1000	1.13
Ba/F3 R132C_S280F	>1000	1.53

-14-

The results in Table 1 indicate that each of ivosidenib and Compound A is effective in inhibiting R132H mutant IDH1 in each R132H construct cell line. However, while Compound A is effective in inhibiting R132H_S280F mutant IDH1 and R132C_S280F in each cell line construct, ivosidenib is not effective in inhibiting

5 R132H_S280F mutant IDH1 in either cell line construct and ivosidenib is not effective in inhibiting R132C_S280F mutant IDH1 in either cell line construct.

In experiments performed essentially as described above, the IC₅₀ results set forth in Table 2 are obtained.

Table 2. IC₅₀ Results		
Cell Line Construct	Compound A IC ₅₀ (nM)	Std Dev
U87MG IDH1R132H_H315D cis	1.49	0.2
U87MG IDH1R132H_R119P cis	2.52	0.46
U87MG IDH1R132H_G131A cis	5.89	0.88
U87MG IDH1R132H_D279N cis	13.8	2.5
U87MG IDH1R132C_H315D cis	1.33	0.42
U87MG IDH1R132C_R119P cis	10.5	3.8
U87MG IDH1R132C_G289D cis	23.5	1.6
U87MG IDH1R132C_G131A cis	25.6	6.5
U87MG IDH1R132C_D279N cis	101.7	13.9
U87MG IDH1R132L_H315D cis	5.07	0.5
U87MG IDH1R132L_R119P cis	89.7	31.3
U87MG IDH1R132L_G289D cis	29.4	4.1
U87MG IDH1R132L_G131A cis	64.5	65.2
U87MG IDH1R132L_S280F cis	179	62
U87MG IDH1R132L_D279N cis	645	142

10

The results in Table 2 indicate that Compound A is effective in inhibiting IDH1 second site resistant mutants in the context of IDH1 R132H, R132C or R132L driver mutations.

15