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(54) Title: PYRROLOPYRIMIDINE CARBOXAMIDES

(57) Abstract: Pyrrolopyrimidine carboxamides and pharmaceutically acceptable salts thereof; pharmaceutical compositions comprising such compounds and salts; use of such compounds and salts to treat or prevent cancers, including PKMYT1-dependent cancers; kits comprising such compounds and salts; and methods for manufacturing such compounds and salts.



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PYRROLOPYRIMIDINE CARBOXAMIDES

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit under 35 U.S.C. §119(e) of U.S. Provisional Application No. 63/506,165, filed June 5, 2023, and U.S. Provisional Application No. 63/594,968, filed November 1, 2023, each of which is incorporated by reference in its entirety for all purposes.

FIELD

[002] The present disclosure relates generally to pyrrolopyrimidine carboxamide compounds and pharmaceutically acceptable salts thereof. The disclosure further relates to pharmaceutical compositions comprising such compounds and salts; use of such compounds and salts to treat or prevent cancers, including cancers having a PKMYT1-dependency because of elevated basal levels of replication stress; kits comprising such compounds and salts; and methods for manufacturing such compounds and salts.

BACKGROUND

[003] The activity of protein kinase CDK1 (also called cell division cycle 2 protein, or CDC2) transitions a cell from the G2 phase of the cell cycle into mitosis (M) where cell division occurs. In response to DNA damage, WEE1 kinase family members WEE1 and PKMYT1 inhibit CDK1 to prevent the cell from dividing until the damaged DNA is repaired (G2/M DNA damage cell cycle checkpoint arrest).

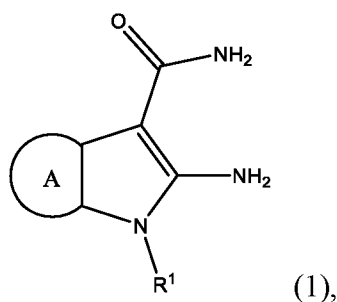
[004] During the replication or synthesis (S-) phase of the cell cycle, in which the genome is duplicated in preparation for cell division, events can occur that result in the stalling of the DNA polymerase and the replication fork. This situation is referred to as replication stress and can result in the generation of DNA damage that leads to a dependency on replication stress response proteins as well as G2/M cell cycle checkpoint proteins such as ATR, WEE1, and PKMYT1. Forment, J. V. and M. J. O'Connor, "Targeting the replication stress response in cancer," *Pharmacol Ther* 188: 155-167 (2018). In such cancers with DNA damage resulting from replication stress, whether endogenous or induced by DNA damaging agents or other targeted agents, there is a greater dependency on PKMYT1. Inhibition of PKMYT1 can lead to premature entry into mitosis with unrepaired DNA damage resulting in the induction of cancer

cell death in mitosis. Chow, J. P. and R. Y. Poon, "The CDK1 inhibitory kinase MYT1 in DNA damage checkpoint recovery," *Oncogene* 32(40): 4778-4788(2013). Consequently, PKMYT1 inhibitors have the potential to induce cancer cell death, either as a monotherapy in cancers with elevated levels of endogenous or basal replication stress, or in combination with other agents that induce greater levels of replication stress in cancers.

[005] Targeted pharmacological inhibition of PKMYT1 activity is a presently unexploited therapeutic approach for treating cancers with elevated basal levels of replication stress and there currently are no approved pharmacological agents that inhibit PKMYT1. Accordingly, there is a need for PKMYT1 inhibitors, particularly PKMYT1 inhibitors having pharmacologically appropriate properties (such as selectivity against WEE1, bioavailability, *etc.*) required for suitable administration to a subject in need of such treatment. The present disclosure addresses this unmet need by providing such compounds together with corresponding pharmaceutical compositions and methods for the treatment or prevention of cancers, including cancers where there is a PKMYT1-dependency because of elevated basal levels of replication stress, either as monotherapy or in combination therapies where such a PKMYT1 dependency can be induced with DNA damaging agents or other targeted therapies such as PARP inhibitors or ATR inhibitors.

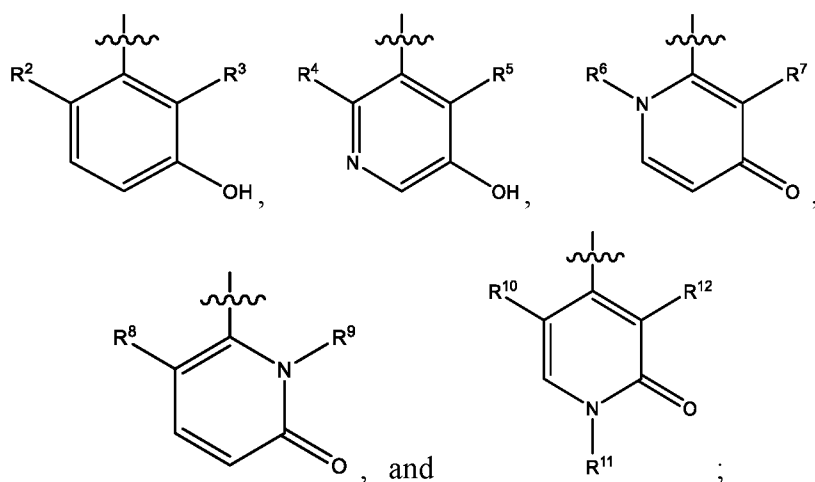
SUMMARY

[006] In one aspect, the present disclosure provides compounds having the structure of Formula (1):



or a pharmaceutically acceptable salt thereof, wherein:

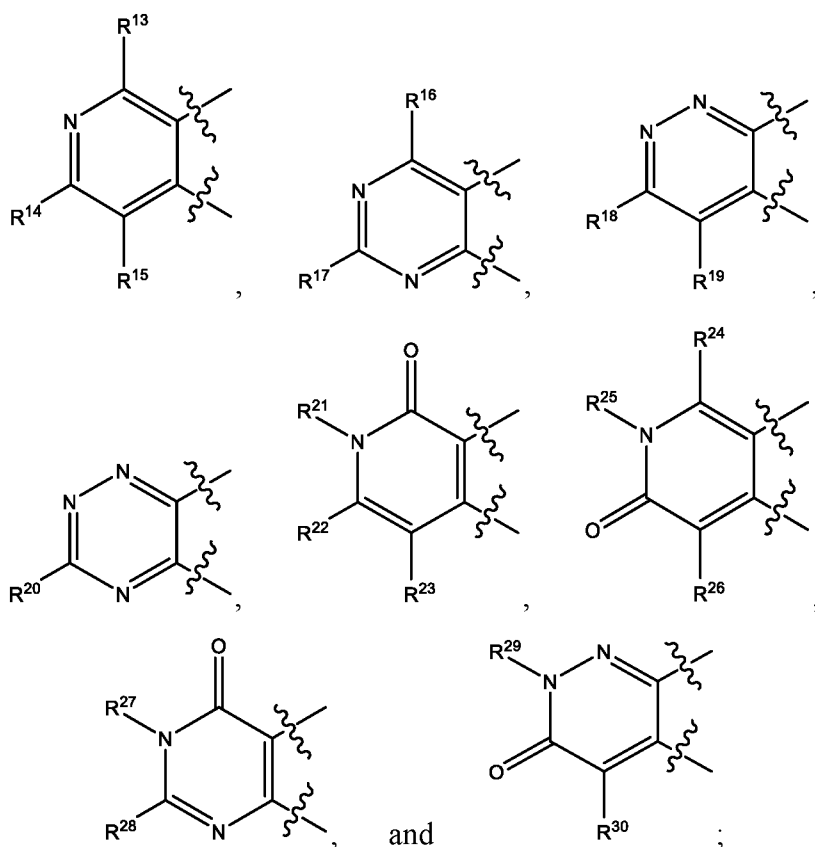
R¹ is selected from the group consisting of:



R^2 , R^3 , R^4 , R^5 , R^7 , R^8 , R^9 , R^{10} , and R^{12} are independently selected from the group consisting of halogen and C_{1-3} -alkyl;

R^6 and R^{11} are independently selected from the group consisting of hydrogen and C_{1-3} -alkyl;

A is selected from the group consisting of:



R^{13} and R^{24} are independently selected from the group consisting of hydrogen, C_{1-3} -alkyl, and C_{1-3} -alkoxy;

R^{14} , R^{17} , R^{18} , R^{20} , R^{22} , and R^{28} are independently selected the group consisting of:

- (a) hydrogen;
- (b) halogen;
- (c) cyano;
- (d) C_{1-6} -alkyl, wherein the C_{1-6} -alkyl is optionally substituted with one or more substituents independently selected from halogen;
- (e) $-C_{1-6}$ -alkenyl, wherein the C_{1-6} -alkenyl is optionally substituted with one or more substituents independently selected from halogen;
- (f) C_{1-6} -alkynyl, wherein the C_{1-6} -alkynyl is optionally substituted with one or more substituents independently selected from halogen;
- (g) C_{3-6} -cycloalkyl, wherein the C_{3-6} -cycloalkyl is optionally substituted with one or more substituents independently selected from the group consisting of halogen, C_{1-6} -alkyl, halo- C_{1-6} -alkyl, C_{1-6} -alkoxy, and halo- C_{1-6} -alkoxy;
- (h) C_{1-6} -alkoxy; wherein the C_{1-6} -alkoxy is optionally substituted with one or more substituents independently selected from halogen;
- (i) $-NR^{31}R^{32}$;
- (j) $-S(O)_2-C_{1-6}$ -alkyl, wherein the $-S(O)_2-C_{1-6}$ -alkyl is optionally substituted with one or more substituents independently selected from halogen;
- (k) phenyl, wherein the phenyl is optionally substituted with one or more substituents independently selected from the group consisting of halogen, C_{1-6} -alkyl, halo- C_{1-6} -alkyl, C_{1-6} -alkoxy, and halo- C_{1-6} -alkoxy; and
- (l) heterocyclyl containing a total of 4 to 10 ring atoms, wherein the heterocyclyl ring: (i) is a saturated, partially saturated, or completely unsaturated monocyclic or fused bicyclic ring, (ii) has one or two ring heteroatoms independently selected from nitrogen, oxygen, and sulfur with the remaining ring atoms being carbon, and (iii) is optionally substituted with one or more substituents independently selected from the group consisting of halogen, oxo, C_{1-6} -alkyl, halo- C_{1-6} -alkyl, and C_{3-6} -cycloalkyl;

R^{15} , R^{19} , R^{23} , R^{26} , and R^{30} are independently selected from hydrogen, halogen, cyano, C_{1-3} -alkyl, and C_{1-3} -alkoxy;

R^{16} is selected from the group consisting of hydrogen, halogen, C_{1-3} -alkyl, halo- C_{1-3} -alkyl, C_{1-3} -alkoxy, and halo- C_{1-3} -alkoxy;

R^{21} , R^{25} , R^{27} , and R^{29} are independently selected from hydrogen and $-X-R^{33}$;

X is a bond or C₁₋₃-alkyl;

R³¹ and R³² are independently selected from hydrogen and C₁₋₆-alkyl; and

R³³ is selected from the group consisting of C₁₋₃-alkyl, C₃₋₆-cycloalkyl, phenyl, and 4- to 6-membered ring heteroaryl having one or two nitrogen ring atoms; wherein the C₁₋₃-alkyl, C₃₋₆-cycloalkyl, phenyl, and 4- to 6-membered ring heteroaryl are optionally substituted with one or more substituents independently selected from the group consisting of halogen and C₁₋₆-alkyl.

[007] In another aspect, the present disclosure provides compounds wherein the structure of Formula (1) is selected from the group consisting of Formulae (2), (3), (4), (5), (6), (7), (8), and (9) as further defined in this disclosure, and pharmaceutically acceptable salts thereof.

[008] In another aspect, the present disclosure provides compounds wherein the structure of Formula (1) is selected from the group consisting of Formulae (2-A), (2-B), (2-C), (2-D), and (2-E) as further defined in this disclosure, and pharmaceutically acceptable salts thereof.

[009] In another aspect, the present disclosure provides compounds wherein the structure of Formula (1) is selected from the group consisting of Formulae (3-A), (3-B), (3-C), (3-D), and (3-E) as further defined in this disclosure, and pharmaceutically acceptable salts thereof.

[0010] In another aspect, the present disclosure provides compounds wherein the structure of Formula (1) is selected from the group consisting of Formulae (4-A), (4-B), (4-C), (4-D), and (4-E) as further defined in this disclosure, and pharmaceutically acceptable salts thereof.

[0011] In another aspect, the present disclosure provides compounds wherein the structure of Formula (1) is selected from the group consisting of Formulae (5-A), (5-B), (5-C), (5-D), and (5-E) as further defined in this disclosure, and pharmaceutically acceptable salts thereof.

[0012] In another aspect, the present disclosure provides compounds wherein the structure of Formula (1) is selected from the group consisting of Formulae (6-A), (6-B), (6-C), (6-D), and (6-E) as further defined in this disclosure, and pharmaceutically acceptable salts thereof.

[0013] In another aspect, the present disclosure provides compounds wherein the structure of Formula (1) is selected from the group consisting of Formulae (7-A), (7-B), (7-C), (7-D), and (7-E) as further defined in this disclosure, and pharmaceutically acceptable salts thereof.

[0014] In another aspect, the present disclosure provides compounds wherein the structure of Formula (1) is selected from the group consisting of Formulae (8-A), (8-B), (8-C), (8-D), and (8-E) as further defined in this disclosure, and pharmaceutically acceptable salts thereof.

[0015] In another aspect, the present disclosure provides compounds wherein the structure of Formula (1) is selected from the group consisting of Formulae (9-A), (9-B), (9-C), (9-D), and (9-E) as further defined in this disclosure, and pharmaceutically acceptable salts thereof.

[0016] In another aspect, the present disclosure provides pharmaceutical compositions comprising a therapeutically-effective amount of a compound having the structure of Formula (1), or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

[0017] In another aspect, the present disclosure provides pharmaceutical compositions comprising therapeutically-effective amounts of a compound having the structure of Formula (1), or a pharmaceutically acceptable salt thereof; a second pharmacological agent; and a pharmaceutically acceptable carrier.

[0018] In another aspect, the present disclosure provides methods for treating or preventing cancers by administering a therapeutically effective amount of a compound having the structure of Formula (1), or pharmaceutically acceptable salt thereof, to a subject in need thereof. In a further aspect, the cancer is a solid tumor cancer. In a still further aspect, the cancer is a hematological cancer. In a still further aspect, the cancer has a PKMYT1-dependency because of elevated levels of replication stress.

[0019] In another aspect, the present disclosure provides compounds having the structure of Formula (1), or pharmaceutically acceptable salts thereof, for use as a medicament for treating or preventing cancers. In a further aspect, the cancer has a PKMYT1-dependency because of elevated levels of replication stress.

[0020] In another aspect, the present disclosure provides use of compounds having the structure of Formula (1), or pharmaceutically acceptable salts thereof, to prepare a medicament for treating or preventing cancers. In a further aspect, the cancer has a PKMYT1-dependency because of elevated levels of replication stress.

[0021] In another aspect, the present disclosure provides kits comprising a compound having the structure of Formula (1), or pharmaceutically acceptable salt thereof.

[0022] In another aspect, the present disclosure provides methods for preparing compounds having the structure of Formula (1), or pharmaceutically acceptable salts thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] Fig. 1 illustrates WEE1 and PKMYT1 regulation of the mitotic cell.

[0024] Fig. 2 illustrates the effect of treatment with a PKMYT1 inhibitor tool compound (GI_{50}) on 16 cancer cell line models having either elevated or low levels of basal replication stress.

[0025] Fig. 3 illustrates the replication fork velocities determined for the uterine and breast cancer cell line models of Fig. 2 treated with the PKMYT1 inhibitor tool compound.

[0026] Fig. 4 illustrates the replication fork velocities determined for the same uterine and breast cancer cell lines of Fig. 2 that were treated instead with Example B-1, Isomer 1 (i.e., the eutomer of Compound 1).

[0027] Figure 5 illustrates the dose response curves obtained for Compound 1, Isomer 1 and Compound 137, Isomer 2 in an *in vitro* hematopoietic stem and progenitor cell assay assessing bone marrow toxicity.

[0028] Fig. 6 illustrates the differential expression of phosphorylated proteins in OVCAR3 cells after 24-hour treatment with Compound 137, Isomer 2 or control (adavosertib).

[0029] Fig. 7 illustrates (i) the effect of PKMYT1 deletion from SKOV3 cells on CDK1 T14 phosphorylation (left and middle panels) after treatment with Compound 1, Isomer 1, and (ii) the effect of treatment (GI_{50}) with Compound 137, Isomer 2 on PKMYT1 knock out SKOV3 cells (i.e., cells that do not express the drug target PKMYT1) (right panel).

[0030] Fig. 8-A illustrates the tolerability in mice of a 300 mg/kg BID dosing regimen with Compound 137, Isomer 2 over a 28-day period.

[0031] Fig. 8-B illustrates the tolerability in mice of a 100 mg/kg BID dosing regimen with Compound 137, Isomer 2 in combination with either irinotecan hydrochloride (left panel) or gemcitabine (right panel) over a 28-day period.

[0032] Fig. 9 illustrates PKMYT1 substrate (CDK1) phosphorylation (on Thr14) in OVCAR3 tumor bearing SCID mice following dose scheduling with Compound 137, Isomer 2 (twice-daily dosing for 21 days).

[0033] Fig. 10 illustrates the *in vivo* efficacy of Compound 137, Isomer 2 in combination with gemcitabine in a human ovarian OVCAR3 xenograft model.

[0034] Fig. 11 illustrates *in vivo* efficacy of Compound 137, Isomer 2 in combination with irinotecan hydrochloride in a human colorectal SW620 xenograft model.

[0035] Fig. 12 illustrates *in vitro* efficacy of Compound 137, Isomer 2, alone or in combination with 1 nM exatecan, in PKMYT1 wildtype and PKMYT1 knockout SKOV3 cells.

[0036] Fig. 13 illustrates the effect on *in vivo* biomarker expression (phospho-CDK1 Thr14, gH2AX, and phospho-histone H3 Ser10) in a human colorectal SW620 xenograft model after treatment with irinotecan hydrochloride, either alone or in combination with Compound 137, Isomer 2 (100 mg/kg BID).

[0037] Figure 14 is a metabolism scheme showing the dominant routes of biotransformations (oxidation and glucuronidation) observed *in vitro* of Compound 137, Isomer 2.

[0038] Figure 15 shows the pharmacokinetic profiles in CD-1 mice (total blood plasma concentration) of Compound 137, Isomer 2 following administration either I.V. (0.5 mg/kg, solid line) or P.O. (1 mg/kg, dashed line).

[0039] Figure 16 shows the pharmacokinetic profiles in SCID mice (total blood plasma concentration) of Compound 137, Isomer 2 following administration P.O. at three dose levels: 10 mg/kg (solid line), 30 mg/kg (dashed line), and 100 mg/kg (dotted line).

[0040] Figure 17 shows the pharmacokinetic profiles in Han Wistar rats (total blood plasma concentration) of Compound 137, Isomer 2 following administration I.V. (0.5 mg/kg) to either intact (solid line) or bile-duct cannulated (dashed line) animals.

[0041] Figure 18 shows the pharmacokinetic profiles in Han Wistar rats (total blood plasma concentration) of Compound 137, Isomer 2 following administration P.O. at three dose levels: 10 mg/kg (solid line), 30 mg/kg (dashed line), and 100 mg/kg (dotted line).

[0042] Figure 19 shows a comparison of the pharmacokinetic profiles in SCID mice (total blood plasma concentration) of Compound 137, Isomer 2 following repeat BID oral administration to male SCID mice (n=2) at 100 mg/kg on day 1 (solid line) and day 28 (dashed line).

[0043] Figure 20 shows the pharmacokinetic profiles in SCID mice (total blood plasma concentration) of Compound 137, Isomer 2 following administration P.O. at 300 mg/kg in 5% DMSO, 50% of 20% Captisol, and 45% WFI (pH 3-3.2) (solid line) and in 0.5% HPMC/0.1% Tween (dashed line).

[0044] For convenience, Compound 1, Isomer 1 is identified in the Figures as AZ1-1 and Compound 137, Isomer 2 is identified in the Figures as AZ137-2.

DETAILED DESCRIPTION

[0045] Many embodiments are detailed throughout this disclosure and will be apparent to a reader skilled in the art. The disclosure is not to be interpreted as being limited to any particular embodiment(s) described herein.

I. Definitions

[0046] With respect to the embodiments disclosed in this disclosure, the following terms have the meanings set forth below:

[0047] Reference to “a” or “an” means “one or more.” Throughout, the plural and singular should be treated as interchangeable, other than the indication of number.

[0048] Unless the context requires otherwise, the words "comprise" or "comprises" or "comprising" are used on the basis and clear understanding that they are to be interpreted inclusively, rather than exclusively, and that Applicant intends each of those words to be so interpreted in construing this patent, including the claims below.

[0049] The term “cyano” (alone or in combination with another term(s)) means CN.

[0050] The term “halogen” (alone or in combination with another term(s)) means a fluorine radical (which may be depicted as F), chlorine radical (which may be depicted as Cl), bromine radical (which may be depicted as Br), or iodine radical (which may be depicted as I).

[0051] The term “oxo” (alone or in combination with another term(s)) means an oxo radical, and may be depicted as =O.

[0052] The term “alkyl” (alone or in combination with another term(s)) means a straight or branched chain saturated hydrocarbyl substituent (*i.e.*, a substituent containing only carbon and hydrogen). Alkyl typically contains from 1 to about 20 carbon atoms, more typically from 1 to about 12 carbon atoms, even more typically from 1 to about 8 carbon atoms, and still even more typically from 1 to about 6 carbon atoms. Examples of such substituents include methyl, ethyl, propyl (including n-propyl and isopropyl), butyl (including n-butyl, isobutyl, sec-butyl, and tert-butyl), pentyl (including n-pentyl, isoamyl, and 2,2-dimethylpropyl), and hexyl.

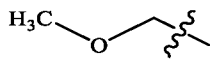
[0053] The term “alkenyl” (alone or in combination with another term(s)) means a straight or branched chain saturated hydrocarbyl substituent (*i.e.*, a substituent containing only carbon and hydrogen) containing one or more double bonds in the alkyl chain. Alkenyl typically contains from 2 to about 20 carbon atoms, more typically from 2 to about 12 carbon atoms, even more typically from 2 to about 8 carbon atoms, and still even more typically from 2 to about 6 carbon

atoms. Examples of such substituents include ethenyl, propenyl (including 1-propenyl and 2-propenyl), butyl (including 2-butenyl and 3-butenyl), pentenyl, and hexenyl.

[0054] The term “alkynyl” (alone or in combination with another term(s)) means a straight or branched chain saturated hydrocarbyl substituent (*i.e.*, a substituent containing only carbon and hydrogen) containing one or more triple bonds in the alkyl chain. Alkynyl typically contains from 2 to about 20 carbon atoms, more typically from 2 to about 12 carbon atoms, even more typically from 2 to about 8 carbon atoms, and still even more typically from 2 to about 6 carbon atoms. Examples of such substituents include ethynyl, propynyl (including 1-propynyl and 2-propynyl), butynyl (including 1butynyl and 2butynyl), pentynyl (including penta-1,3-diene, 2-methyl-1,3-butadiene, and penta-1,2-diene), and hexynyl.

[0055] The term “cycloalkyl” (alone or in combination with another term(s)) means a saturated carbocyclyl substituent containing from 3 to about 14 carbon ring atoms, more typically from 3 to about 12 carbon ring atoms, and even more typically from 3 to about 8 carbon ring atoms. A cycloalkyl includes a single carbon ring, which typically contains from 3 to 6 carbon ring atoms. Examples of singlering cycloalkyls include cyclopropyl, cyclobutyl, cyclopentyl, and cyclohexyl.

[0056] The term “alkoxy” (alone or in combination with another term(s)) means an alkylether substituent, *i.e.*, alkyl-O-. Examples of alkoxy include methoxy (CH₃-O-), ethoxy, n-propoxy, iso-propoxy, n-butoxy, iso-butoxy, sec-butoxy, and tert-butoxy. Thus, for example, the term “alkoxyalkyl” (alone or in combination with another term(s)) means alkyl substituted with alkoxy such as “methoxymethyl” which may be depicted as:



[0057] The term “heterocyclyl” (alone or in combination with another term(s)) means a saturated, partially saturated, or completely unsaturated (*i.e.*, “heteroaryl”) ring structure containing a total of 3 to 14 ring atoms. At least one of the ring atoms is a heteroatom (*i.e.*, oxygen, nitrogen, or sulfur), with the remaining ring atoms being independently selected from the group consisting of carbon, oxygen, nitrogen, and sulfur in stable combinations known to those of skill in the art.

[0058] In some instances, the number of carbon atoms in a substituent (e.g., alkyl, cycloalkyl, *etc.*) is indicated by the prefix “C_{x-y}”, wherein x is the minimum and y is the maximum number of carbon atoms in the substituent. Thus, for example, “C₁₋₆alkyl” refers to an alkyl substituent

containing from 1 to 6 carbon atoms. Illustrating further, C₃₋₆cycloalkyl refers to a cycloalkyl substituent containing from 3 to 6 carbon ring atoms.

[0059] The prefix “halo” indicates that the substituent to which the prefix is attached is substituted with one or more independently selected halogen radicals. For example, haloalkyl means an alkyl substituent wherein at least one hydrogen radical is replaced with a halogen radical. Where there is more than one hydrogen replaced with halogens, the halogens may be the identical or different. Examples of haloalkyls include fluoromethyl, difluoromethyl, trifluoromethyl, difluoroethyl, 1,1,1trifluoroethyl, pentafluoroethyl, difluoropropyl, heptafluoropropyl, chloromethyl, dichloromethyl, trichloromethyl, difluorochloromethyl, dichlorofluoromethyl, and dichloropropyl.

[0060] A substituent is “substitutable” if it comprises at least one carbon or nitrogen atom that is bonded to one or more hydrogen atoms. Thus, for example, hydrogen, halogen, and cyano do not fall within this definition.

[0061] If a substituent is described as being “substituted,” a non-hydrogen radical is in the place of a hydrogen radical on a carbon or nitrogen of the substituent. Thus, for example, a substituted alkyl substituent is an alkyl substituent wherein at least one non-hydrogen radical is in the place of a hydrogen radical on the alkyl substituent. To illustrate, monofluoroalkyl is alkyl substituted with a fluoro radical, and difluoroalkyl is alkyl substituted with two fluoro radicals. It should be recognized that if there is more than one substitution on a substituent, each nonhydrogen radical may be identical or different (unless otherwise stated).

[0062] If a substituent is described as “optionally substituted”, the substituent may be either (1) not substituted, or (2) substituted. If a carbon of a substituent is described as optionally substituted with one or more of a list of substituents, one or more of the hydrogens on the carbon (to the extent there are any) may separately and/or together be replaced with an independently selected optional substituent. If a nitrogen of a substituent is described as optionally substituted with one or more of a list of substituents, one or more of the hydrogens on the nitrogen (to the extent there are any) may each be replaced with an independently selected optional substituent.

[0063] If substituents are described as being “independently selected” from a group, each substituent is selected independently of the other. Each substituent therefore may be identical to or different from the other substituent(s).

[0064] The term “atropisomers” refers to the stereoisomers resulting from hindered rotation about one or more single bonds, where the energy barrier to rotation is high enough to allow for

the isolation of the conformers. As used in this disclosure, the term “eutomer” refers to the more pharmacologically active conformer of an atropisomer and the term “distomer” refers to the less pharmacologically active conformer of an atropisomer.

[0065] The term “pharmaceutically acceptable” is used adjectivally in this disclosure to mean that the modified noun is appropriate for use as a pharmaceutical product or as a part of a pharmaceutical product. For example, “pharmaceutically acceptable salts” are salts that are suitable for use in mammals, particularly humans, and include salts with an inorganic base, organic base, inorganic acid, organic acid, or basic or acidic amino acid that are suitable for use in mammals, particularly humans.

[0066] A “therapeutically effective amount” of a pharmacological agent is an amount that is sufficient to effect beneficial or desired results, including clinical results, and, as such, will depend upon the situation in which it is being administered. Where the pharmacological agent is being administered to treat cancer, for example, a therapeutically effective amount of the agent is an amount of the agent that is sufficient, either alone or in combination with additional therapies, to provide an anti-cancer effect in a subject as compared to the response obtained without administration of the agent.

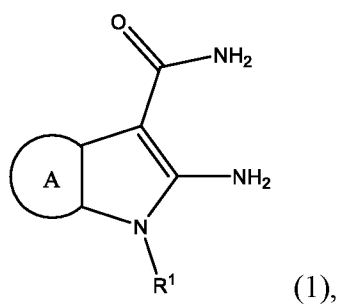
[0067] The term “preventing” is readily understood by an ordinarily skilled physician and, with respect to treatment of a particular condition, is intended to have its normal meaning and includes primary prophylaxis to prevent the development of the condition and secondary prophylaxis whereby the condition has already developed and the patient is temporarily or permanently protected against exacerbation or worsening of the disease or the development of new symptoms associated with the condition.

[0068] The term “treating” is readily understood by an ordinarily skilled physician and, with respect to treatment of a particular condition, can include (1) diminishing the extent or cause of the condition being treated, and/or (2) alleviating or ameliorating one or more symptoms associated with that condition. Treatment of cancer, for example, can include stabilizing (*i.e.*, not worsening), delaying, or slowing the spread or progression of the cancer; prolonging survival as compared to expected survival if not receiving treatment; and/or otherwise ameliorating or palliating the cancer or the severity of the cancer, in whole or in part.

II. Compounds

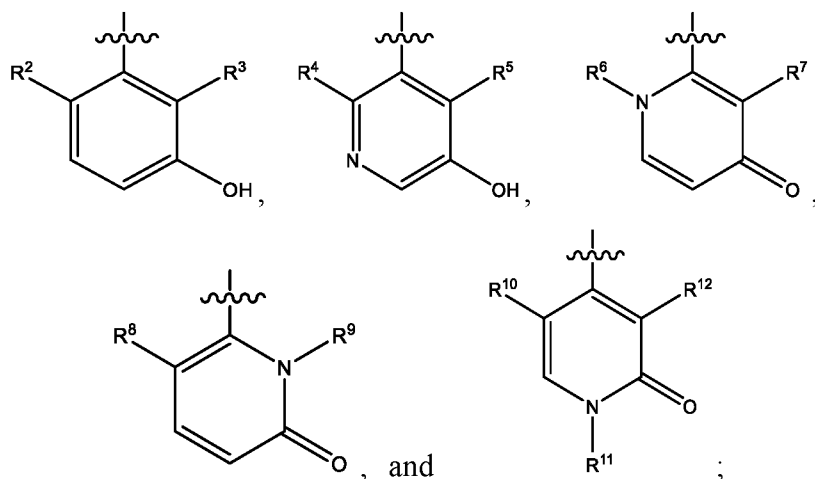
A. Compounds of Formula (1)

[0069] In one embodiment, the present disclosure provides compounds having the structure of Formula (1):



or a pharmaceutically acceptable salt thereof, wherein:

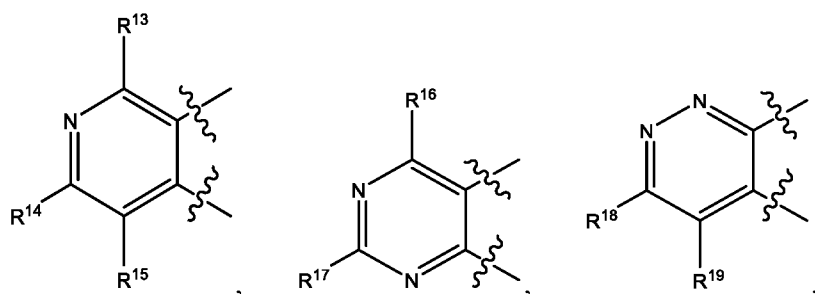
R^1 is selected from the group consisting of:

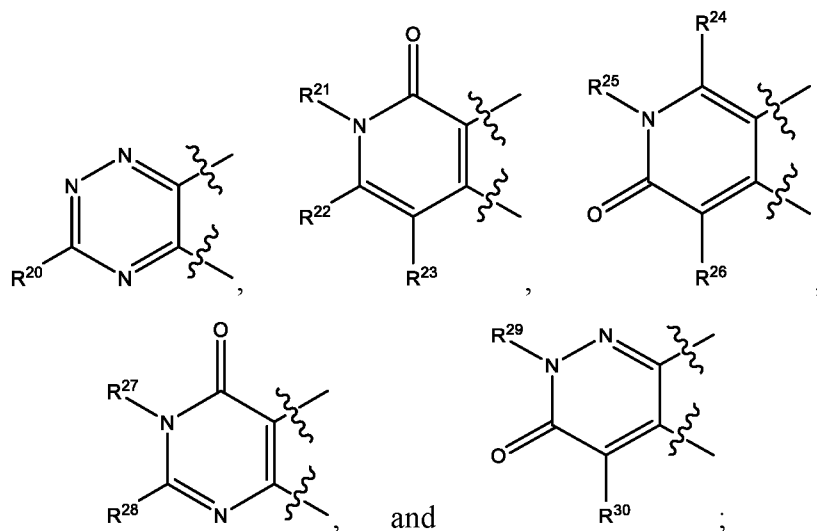


R^2 , R^3 , R^4 , R^5 , R^7 , R^8 , R^9 , R^{10} , and R^{12} are independently selected from the group consisting of halogen and C_{1-3} -alkyl;

R^6 and R^{11} are independently selected from the group consisting of hydrogen and C_{1-3} -alkyl;

A is selected from the group consisting of:





R^{13} and R^{24} are independently selected from the group consisting of hydrogen, C_{1-3} -alkyl, and C_{1-3} -alkoxy;

R^{14} , R^{17} , R^{18} , R^{20} , R^{22} , and R^{28} are independently selected the group consisting of:

- (a) hydrogen;
- (b) halogen;
- (c) cyano;
- (d) C_{1-6} -alkyl, wherein the C_{1-6} -alkyl is optionally substituted with one or more substituents independently selected from halogen;
- (e) $-C_{1-6}$ -alkenyl, wherein the C_{1-6} -alkenyl is optionally substituted with one or more substituents independently selected from halogen;
- (f) C_{1-6} -alkynyl, wherein the C_{1-6} -alkynyl is optionally substituted with one or more substituents independently selected from halogen;
- (g) C_{3-6} -cycloalkyl, wherein the C_{3-6} -cycloalkyl is optionally substituted with one or more substituents independently selected from the group consisting of halogen, C_{1-6} -alkyl, halo- C_{1-6} -alkyl, C_{1-6} -alkoxy, and halo- C_{1-6} -alkoxy;
- (h) C_{1-6} -alkoxy; wherein the C_{1-6} -alkoxy is optionally substituted with one or more substituents independently selected from halogen;
- (i) $-NR^{31}R^{32}$;
- (j) $S(O)_2-C_{1-6}$ -alkyl, wherein the $-S(O)_2-C_{1-6}$ -alkyl is optionally substituted with one or more substituents independently selected from halogen;

(k) phenyl, wherein the phenyl is optionally substituted with one or more substituents independently selected from the group consisting of halogen, C₁₋₆-alkyl, halo-C₁₋₆-alkyl, C₁₋₆-alkoxy, and halo-C₁₋₆-alkoxy; and

(l) heterocyclyl containing a total of 4 to 10 ring atoms, wherein the heterocyclyl ring: (i) is a saturated, partially saturated, or completely unsaturated monocyclic or fused bicyclic ring, (ii) has one or two ring heteroatoms independently selected from nitrogen, oxygen, and sulfur with the remaining ring atoms being carbon, and (iii) is optionally substituted with one or more substituents independently selected from the group consisting of halogen, oxo, C₁₋₆-alkyl, halo-C₁₋₆-alkyl, and C₃₋₆-cycloalkyl;

R¹⁵, R¹⁹, R²³, R²⁶, and R³⁰ are independently selected from hydrogen, halogen, cyano, C₁₋₃-alkyl, and C₁₋₃-alkoxy;

R¹⁶ is selected from the group consisting of hydrogen, halogen, C₁₋₃-alkyl, halo-C₁₋₃-alkyl, C₁₋₃-alkoxy, and halo-C₁₋₃-alkoxy;

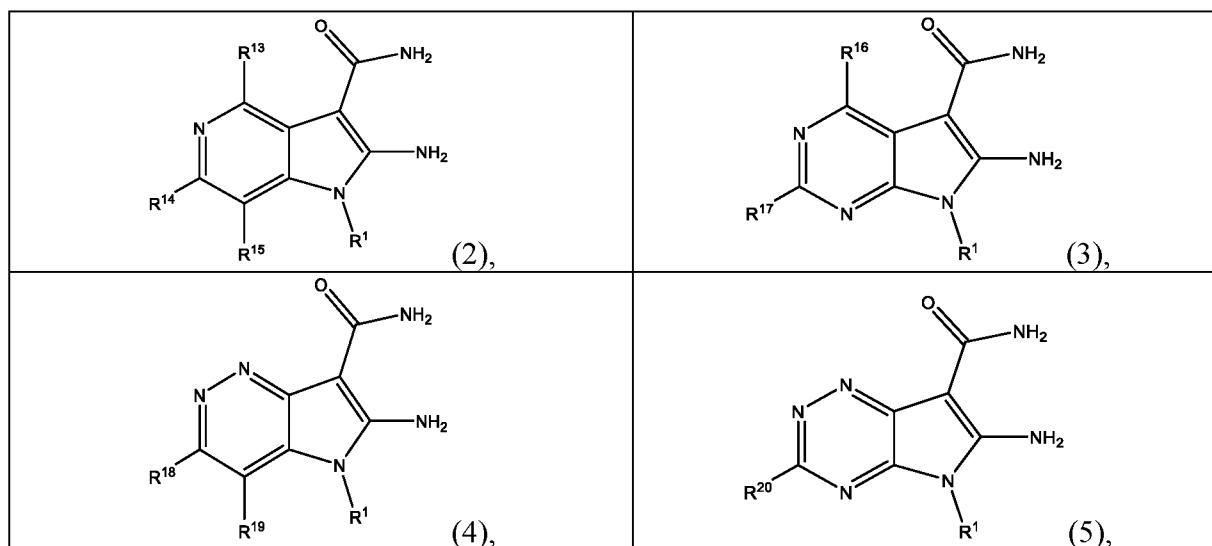
R²¹, R²⁵, R²⁷, and R²⁹ are independently selected from hydrogen and -X-R³³;

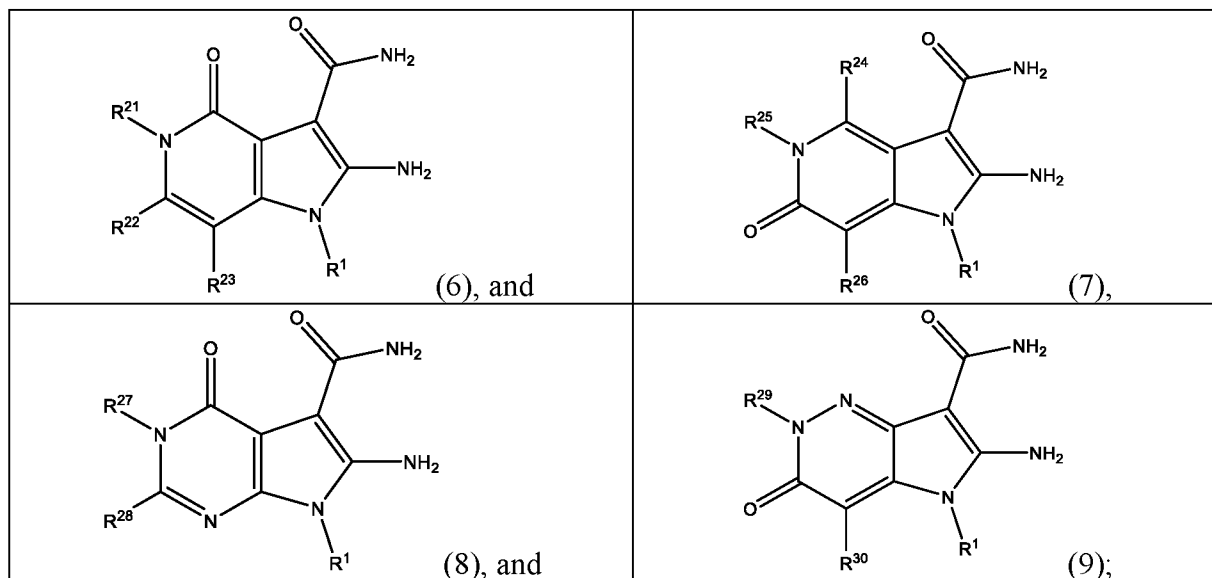
X is a bond or C₁₋₃-alkyl;

R³¹ and R³² are independently selected from hydrogen and C₁₋₆-alkyl; and

R³³ is selected from the group consisting of C₁₋₃-alkyl, C₃₋₆-cycloalkyl, phenyl, and 4- to 6-membered ring heteroaryl having one or two nitrogen ring atoms; wherein the C₁₋₃-alkyl, C₃₋₆-cycloalkyl, phenyl, and 4- to 6-membered ring heteroaryl are optionally substituted with one or more substituents independently selected from the group consisting of halogen and C₁₋₆-alkyl.

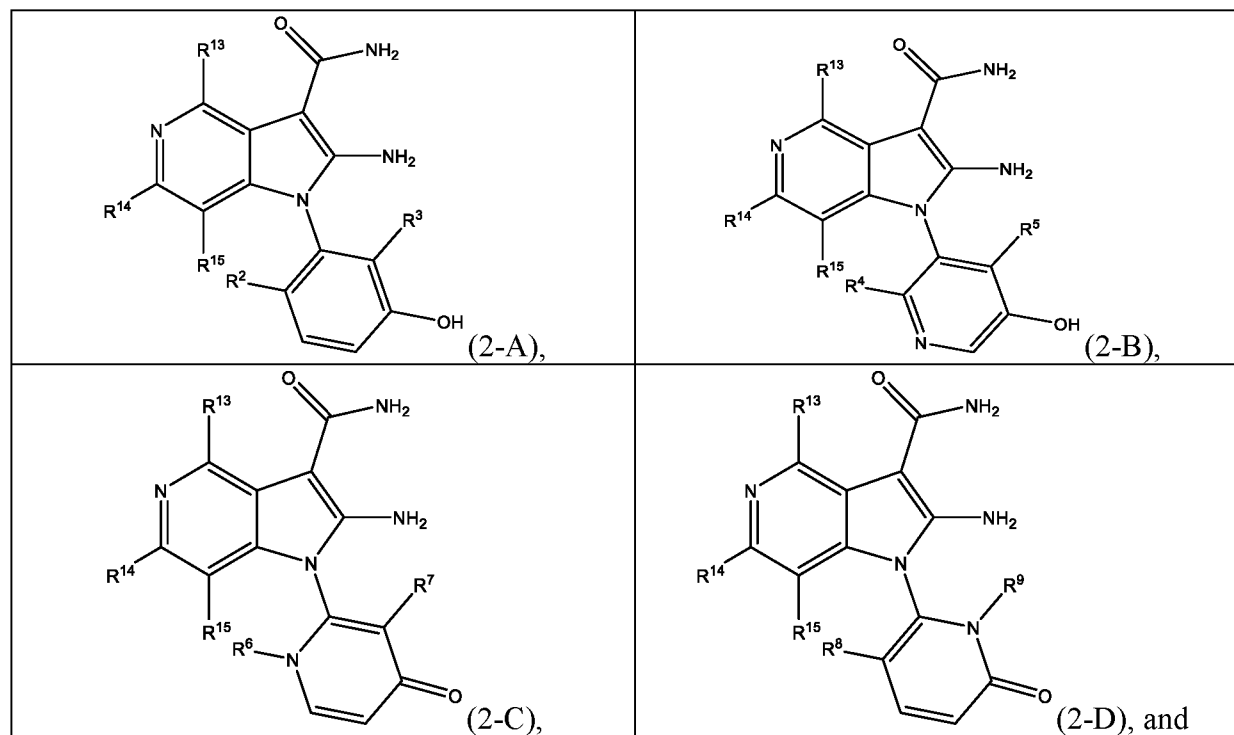
[0070] In some embodiments, the structure of Formula (1) is selected from the group consisting of Formulae (2), (3), (4), (5), (6), (7), (8), and (9):

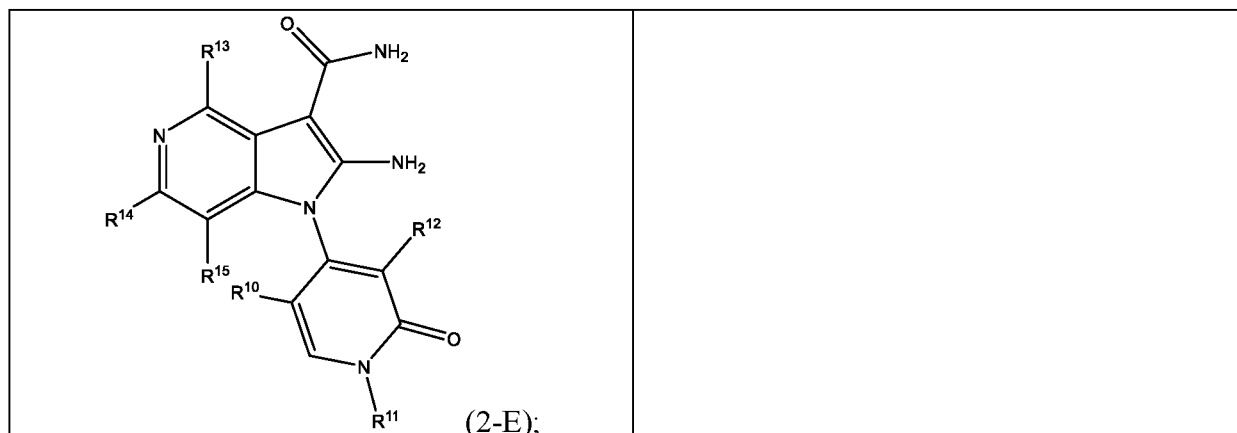




and wherein R^1 through R^{30} are as defined for the compounds having the structure of Formula (1).

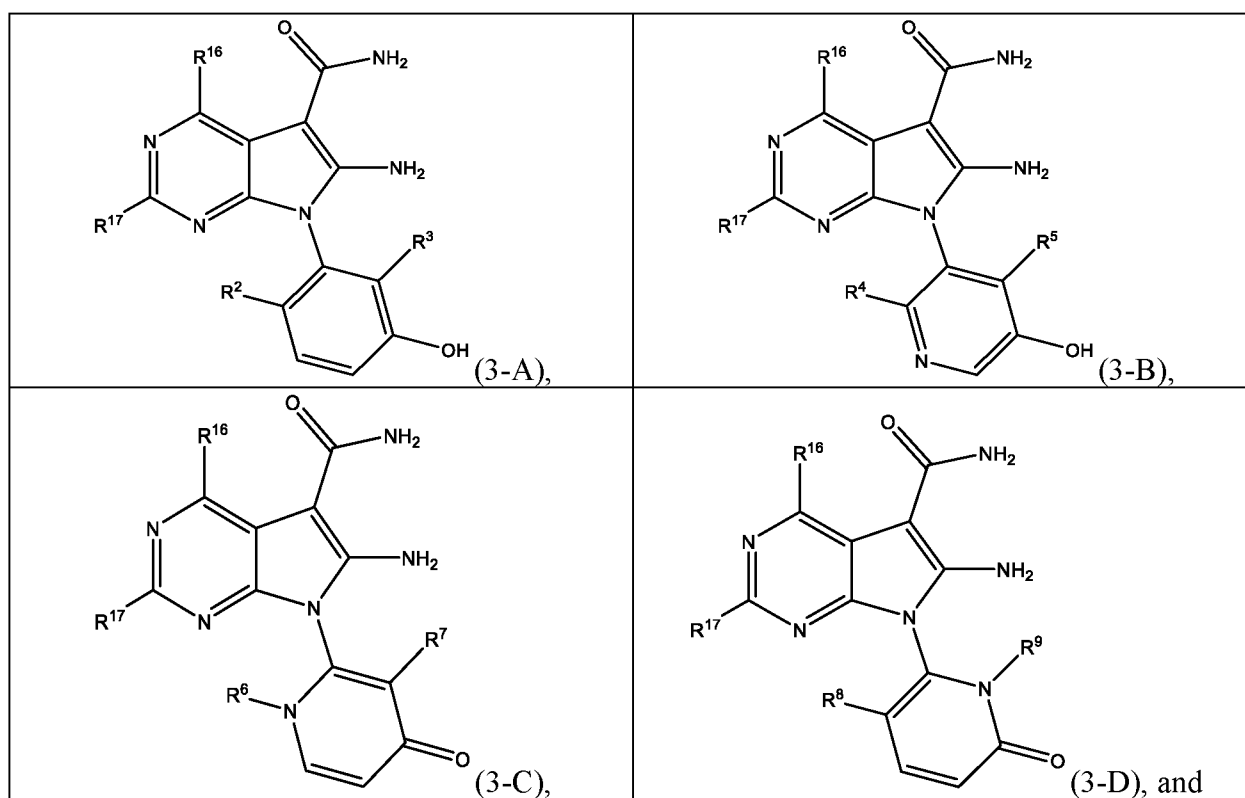
[0071] In some embodiments, the structure of Formula (1) is selected from the group consisting of Formulae (2-A), (2-B), (2-C), (2-D), and (2-E):

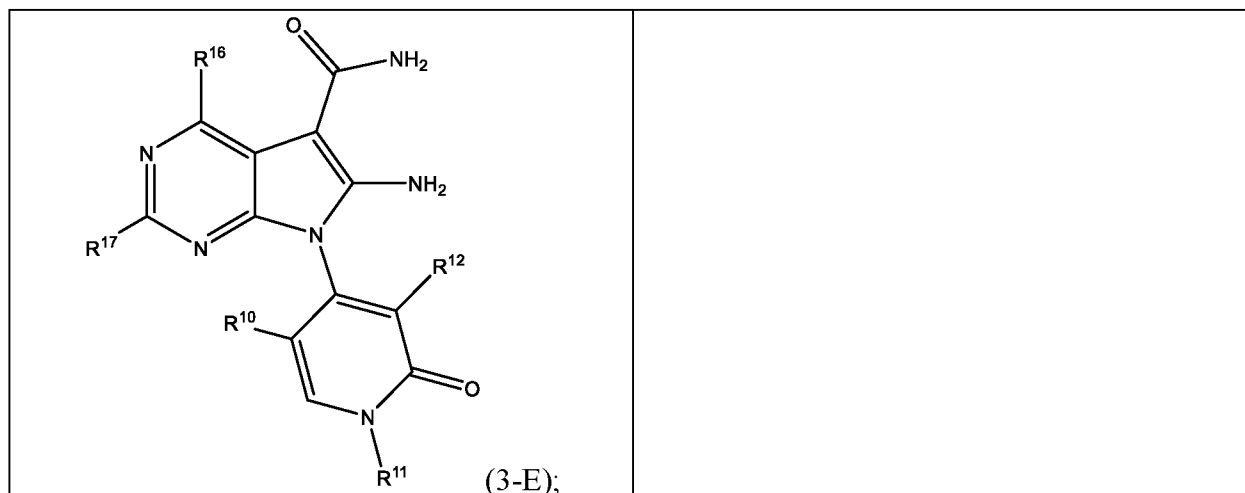




and wherein R^2 through R^{15} are as defined for the compounds having the structure of Formula (1).

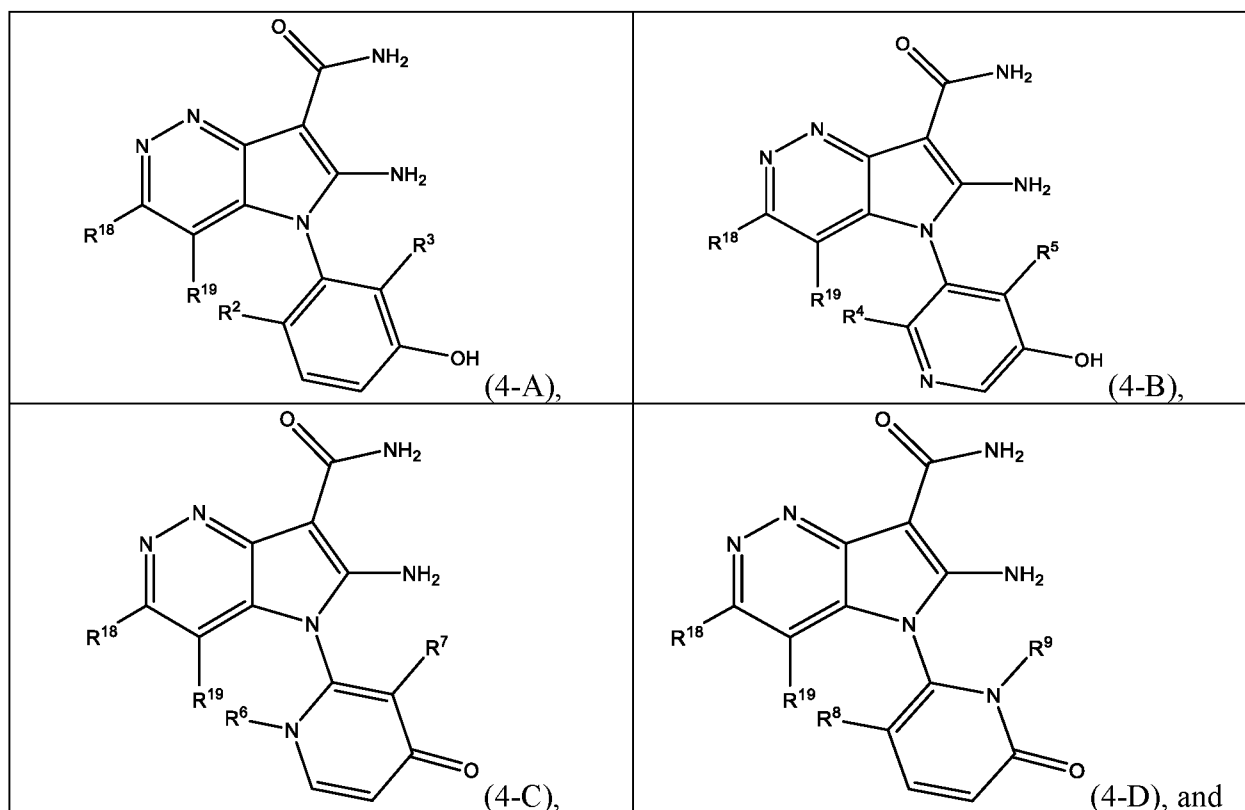
[0072] In some embodiments, the structure of Formula (1) is selected from the group consisting of Formulae (3-A), (3-B), (3-C), (3-D), and (3-E):

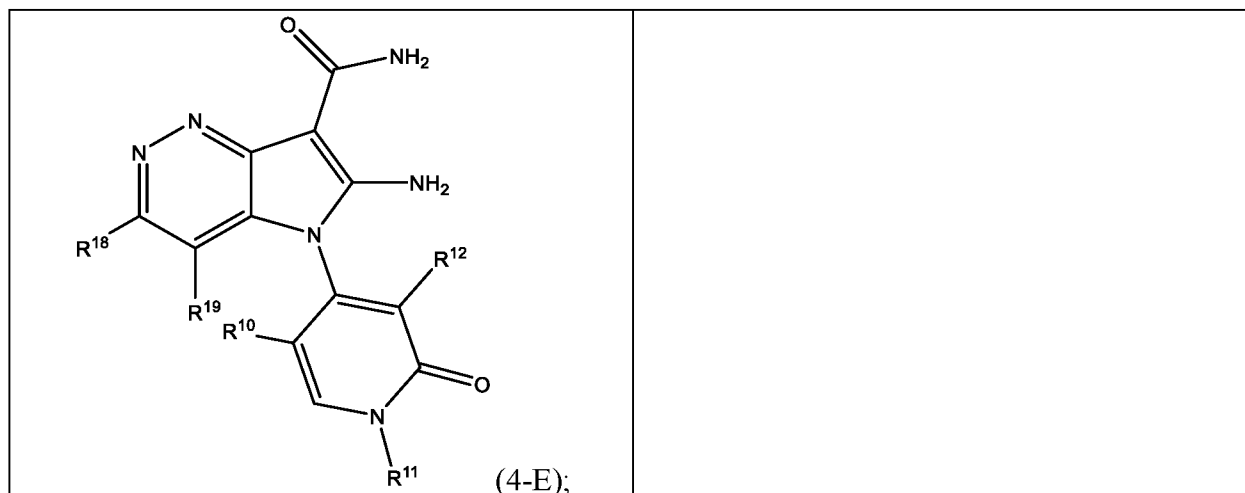




and wherein R^2 through R^{12} , R^{16} , and R^{17} are as defined for the compounds having the structure of Formula (1).

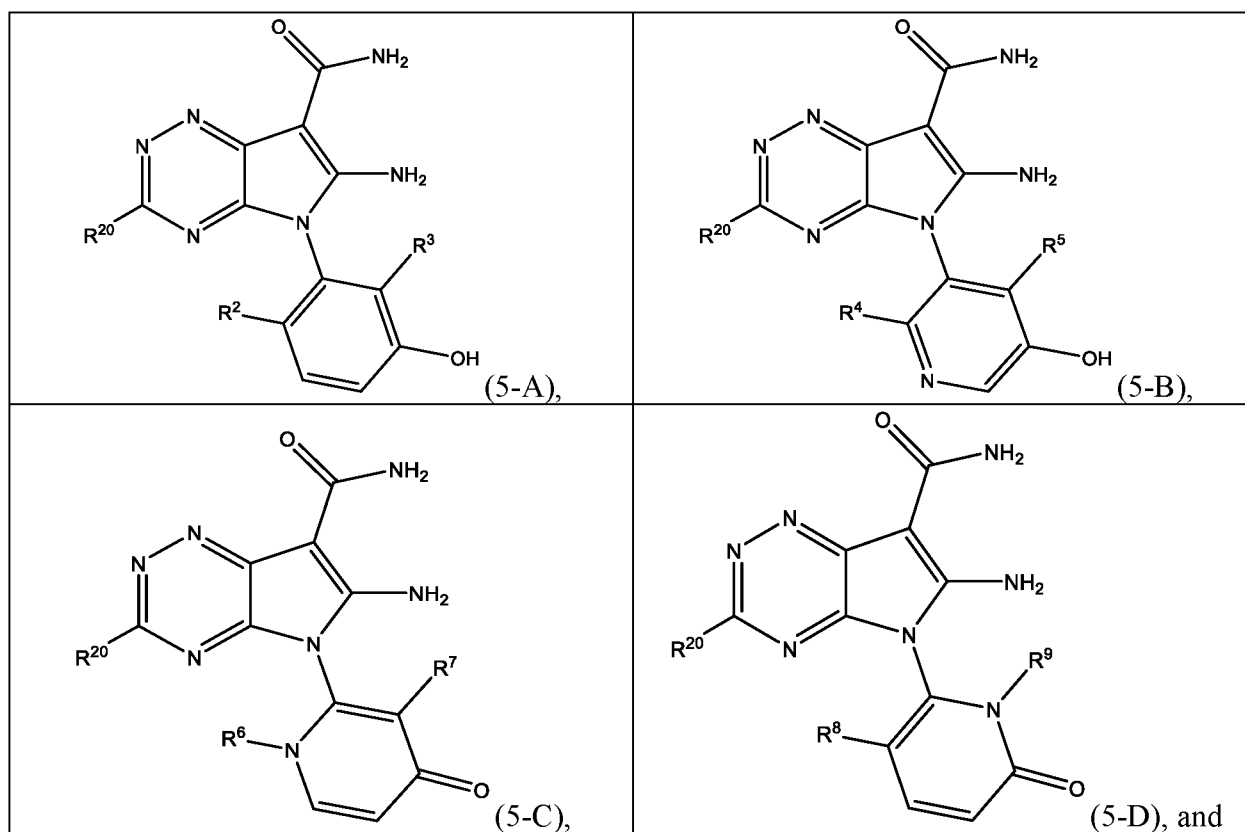
[0073] In some embodiments, the structure of Formula (1) is selected from the group consisting of Formulae (4-A), (4-B), (4-C), (4-D), and (4-E):

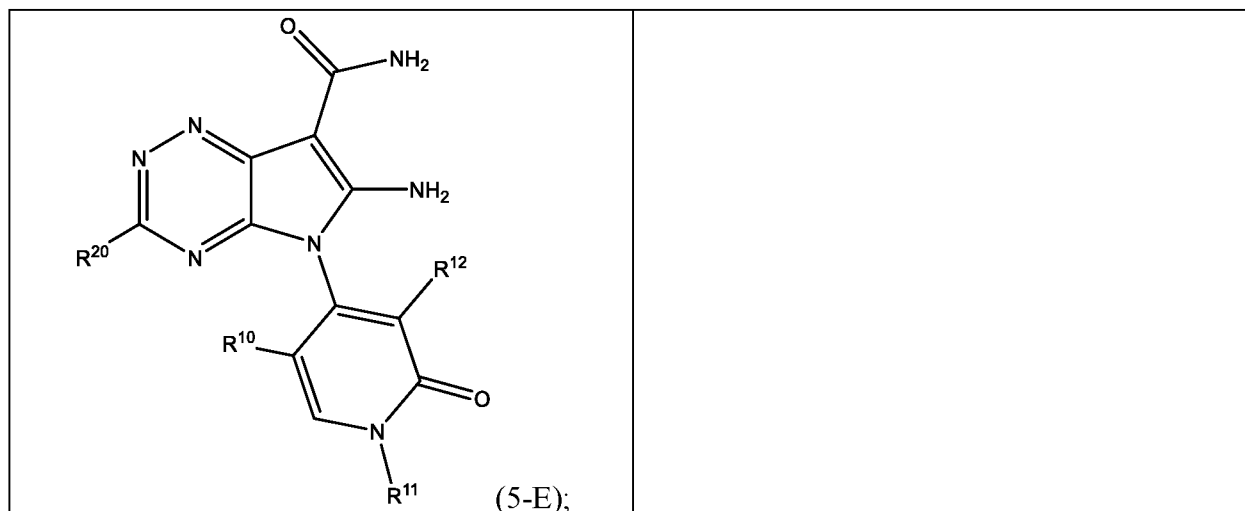




and wherein R^2 through R^{12} , R^{18} , and R^{19} are as defined for the compounds having the structure of Formula (1).

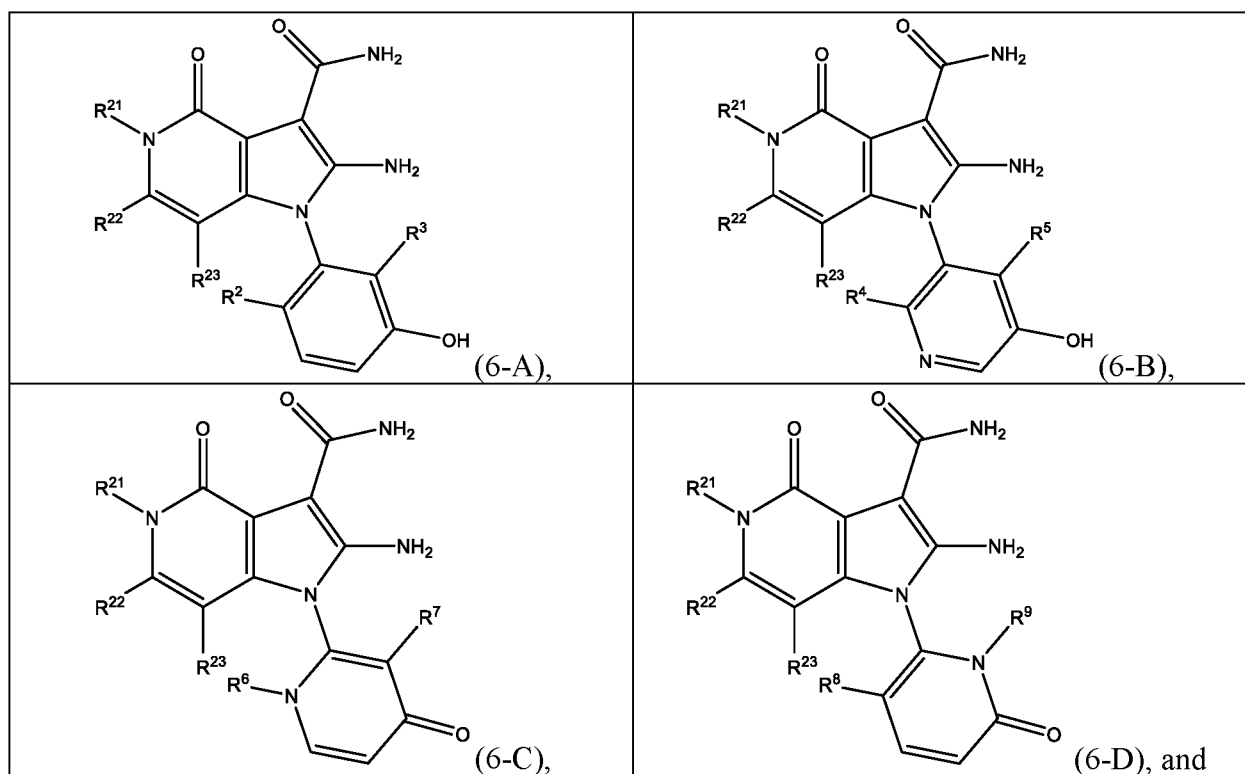
[0074] In some embodiments, the structure of Formula (1) is selected from the group consisting of Formulae (5-A), (5-B), (5-C), (5-D), and (5-E):

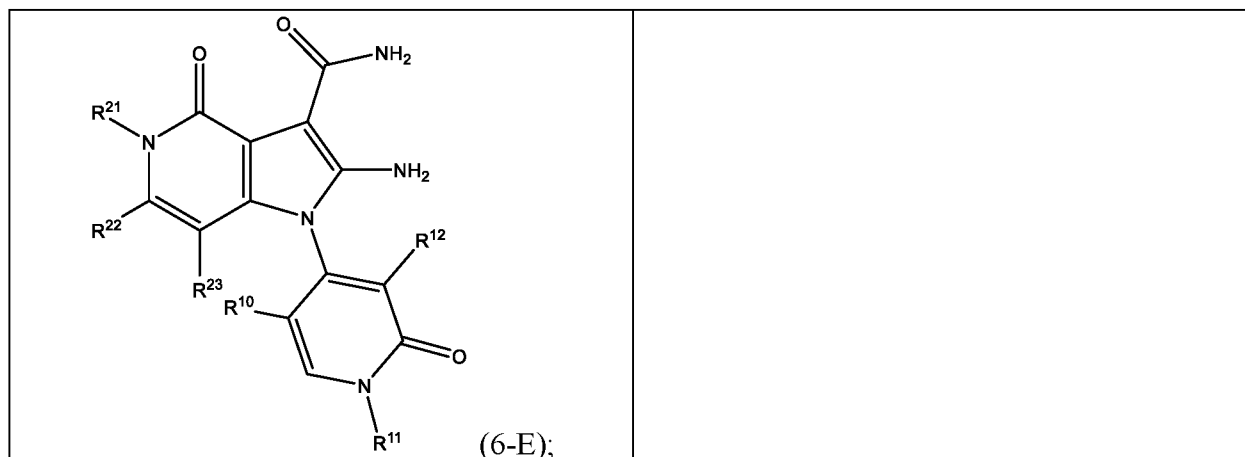




and wherein R^2 through R^{12} and R^{20} are as defined for the compounds having the structure of Formula (1).

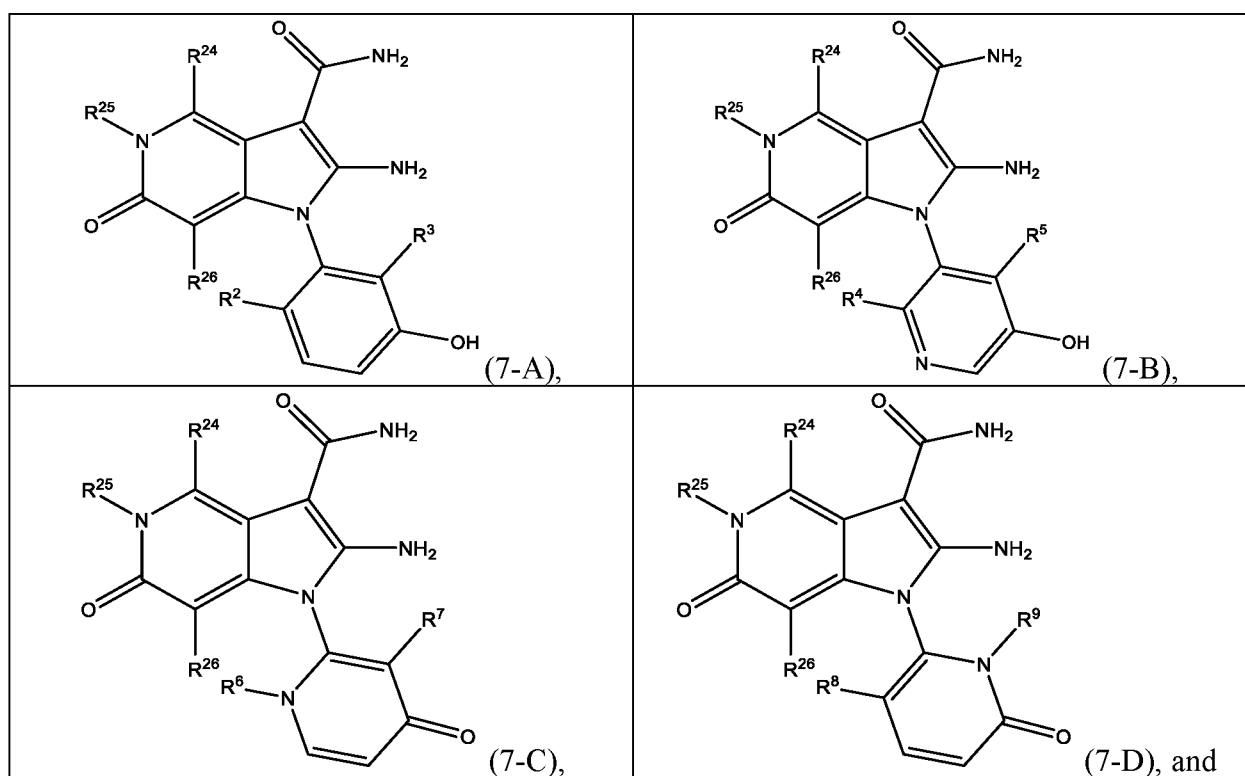
[0075] In some embodiments, the structure of Formula (1) is selected from the group consisting of Formulae (6-A), (6-B), (6-C), (6-D), and (6-E):

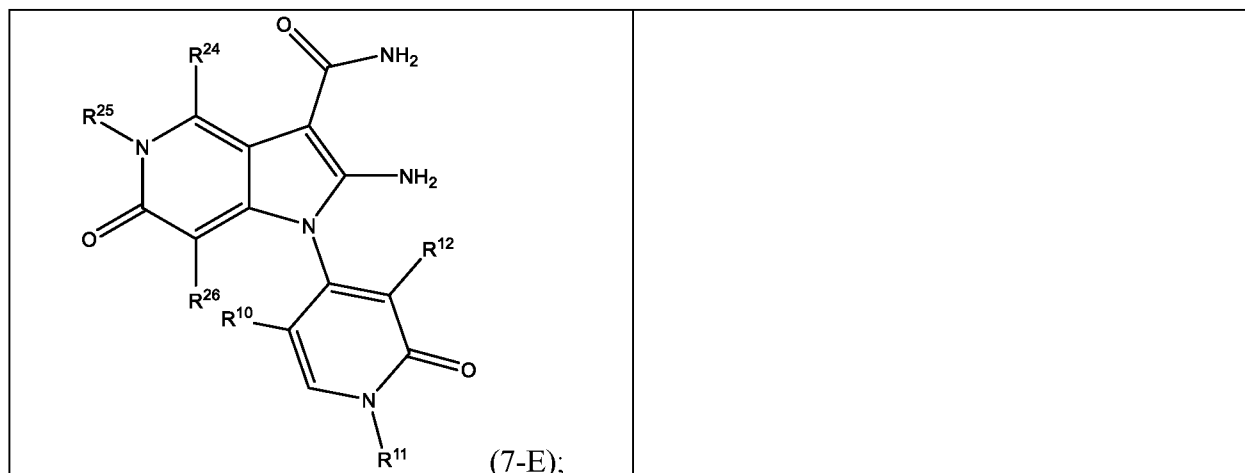




and wherein R^2 through R^{12} , R^{21} , R^{22} , and R^{23} are as defined for the compounds having the structure of Formula (1).

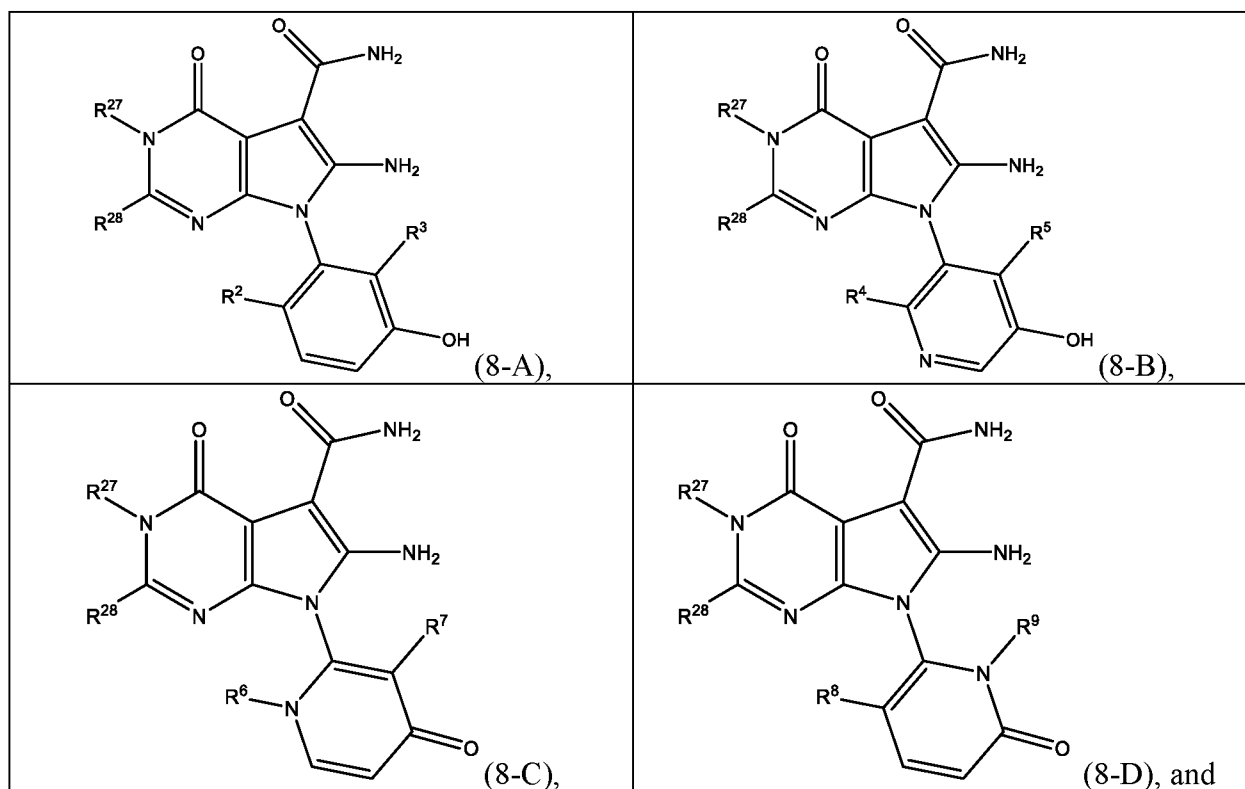
[0076] In some embodiments, the structure of Formula (1) is selected from the group consisting of Formulae (7-A), (7-B), (7-C), (7-D), and (7-E):

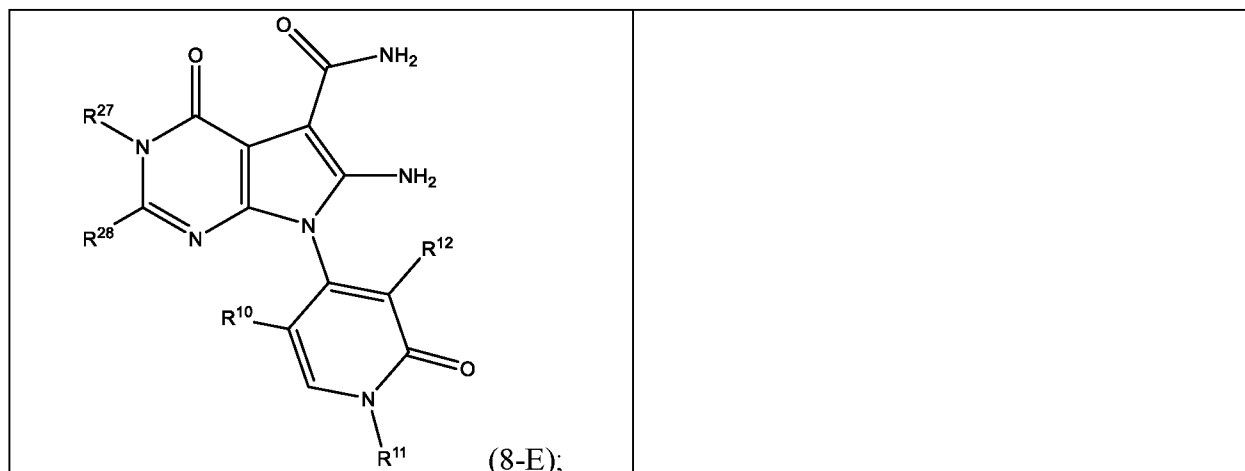




and wherein R^2 through R^{12} , R^{24} , R^{25} , and R^{26} are as defined for the compounds having the structure of Formula (1).

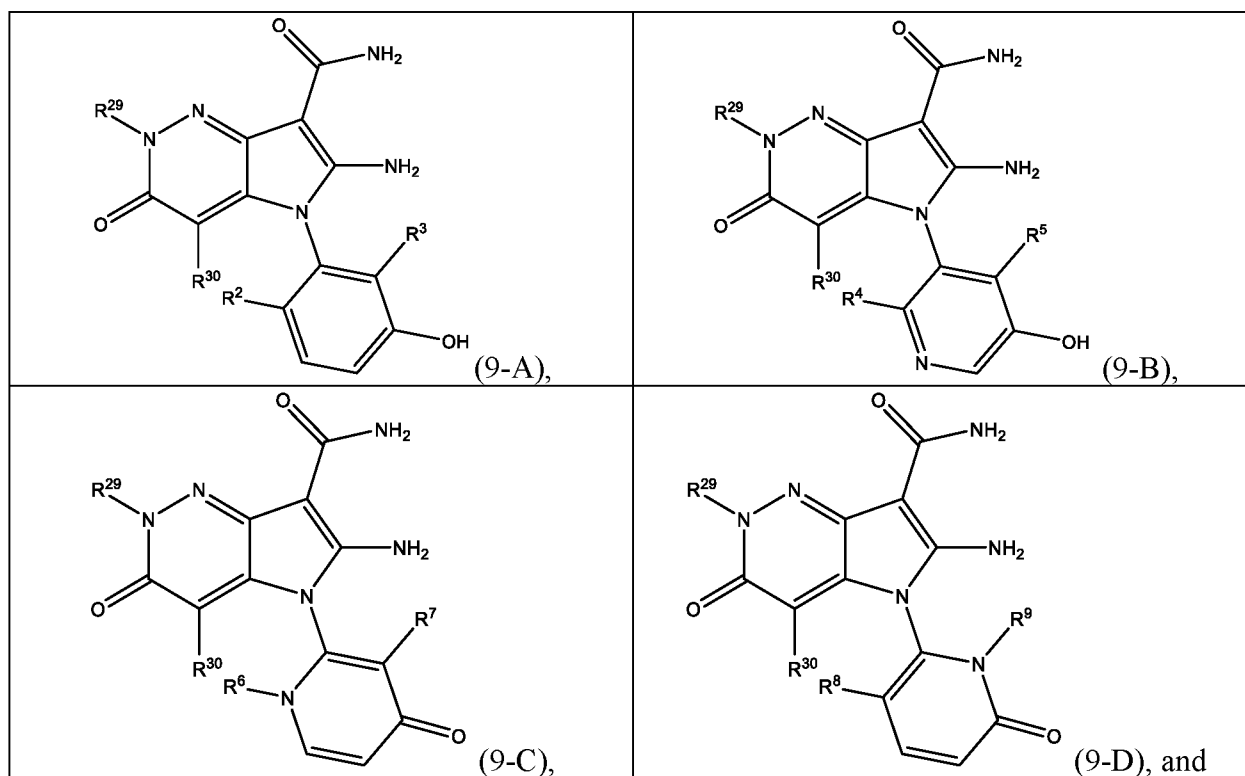
[0077] In some embodiments, the structure of Formula (1) is selected from the group consisting of Formulae (8-A), (8-B), (8-C), (8-D), and (8-E):

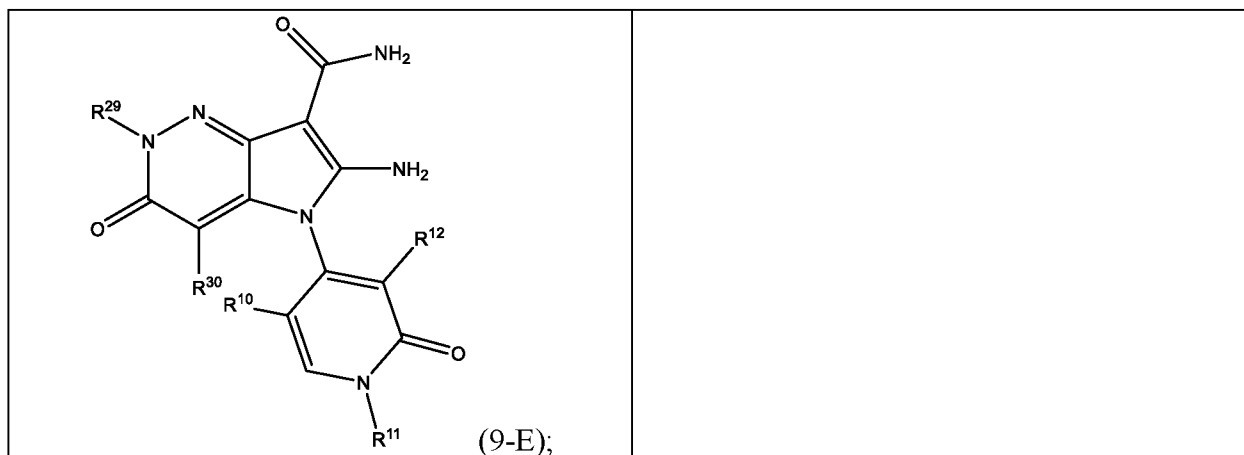




and wherein R² through R¹², R²⁷, and R²⁸ are as defined for the compounds having the structure of Formula (1).

[0078] In some embodiments, the structure of Formula (1) is selected from the group consisting of Formulae (9-A), (9-B), (9-C), (9-D), and (9-E):



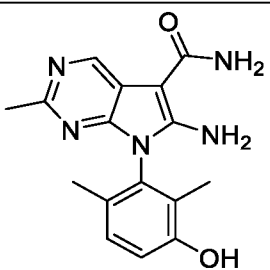
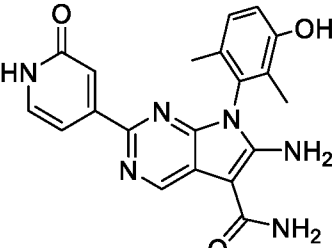


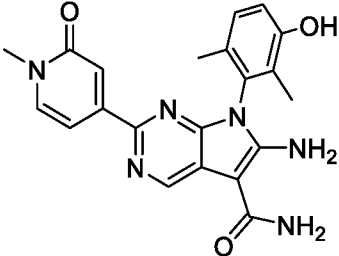
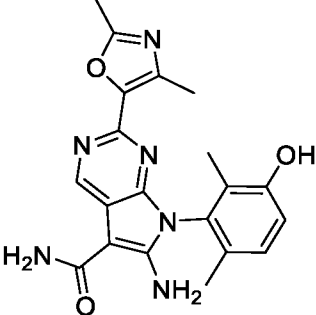
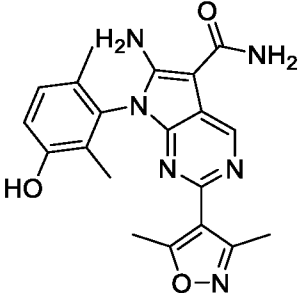
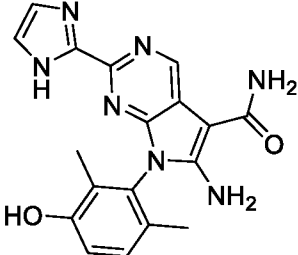
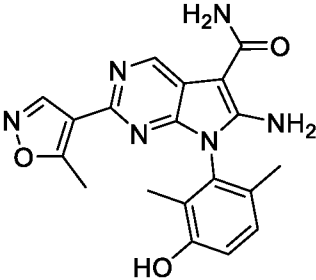
and wherein R^2 through R^{12} , R^{29} , and R^{30} are as defined for the compounds having the structure of Formula (1).

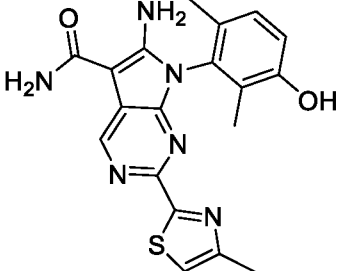
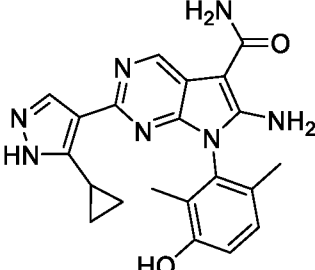
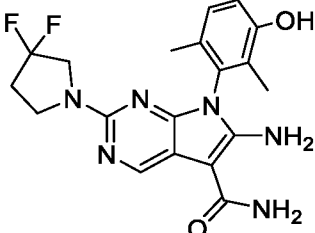
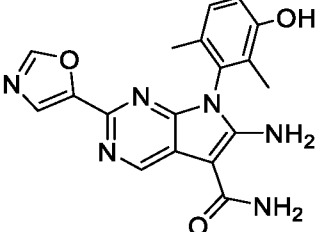
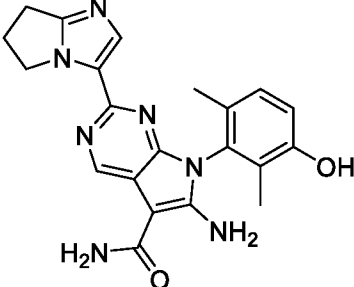
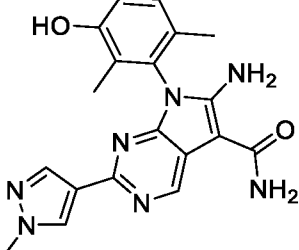
[0079] In some embodiments, the compound having the structure of Formula (1) is an atropisomer. In one aspect, the compound is the eutomer of the atropisomer. In another aspect, the compound is the distomer of the atropisomer.

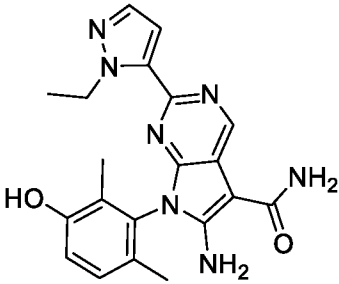
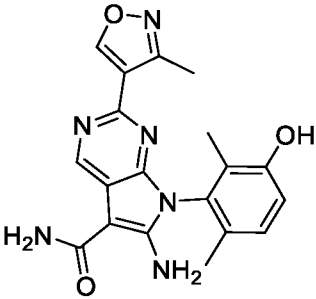
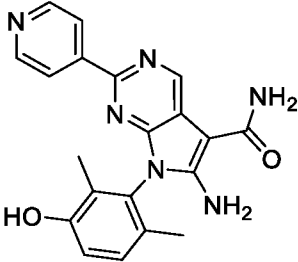
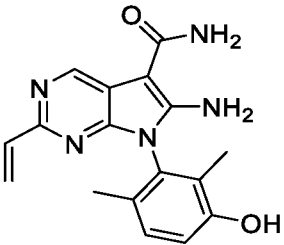
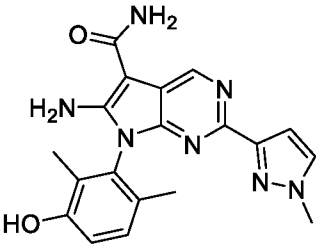
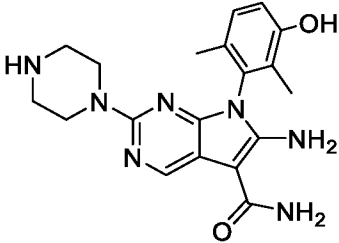
B. Additional Embodiments

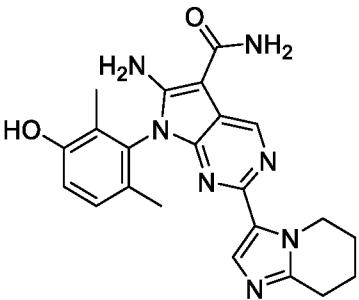
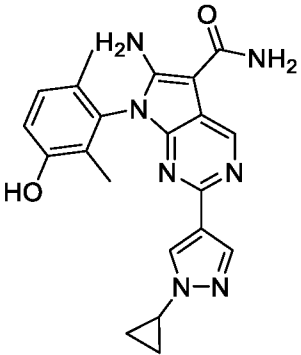
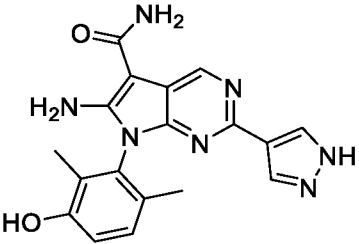
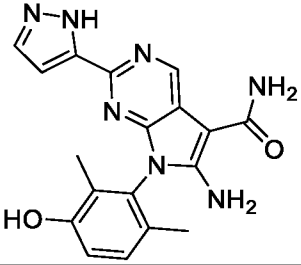
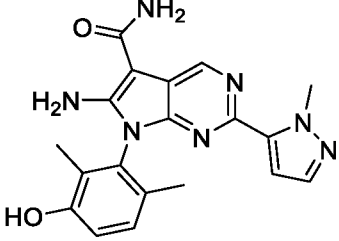
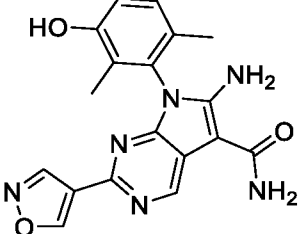
[0080] In some embodiments, the present disclosure provides compounds of Formula (1), and pharmaceutically acceptable salts thereof, wherein the compound is selected from the group consisting of:

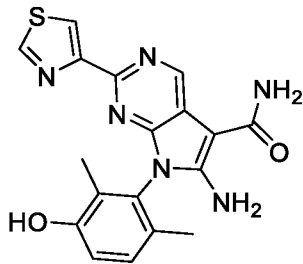
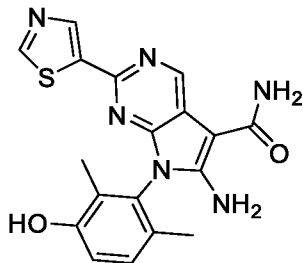
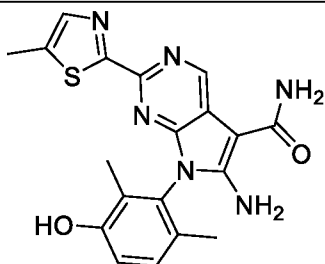
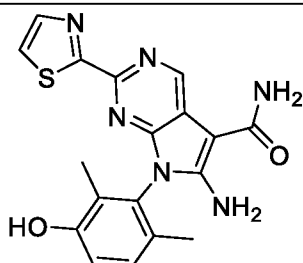
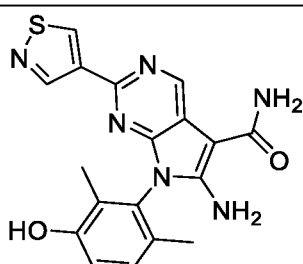
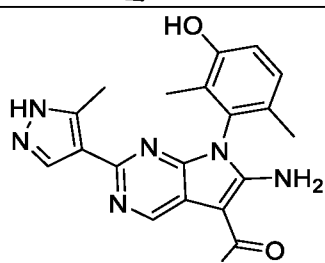
COMPOUND	STRUCTURE
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COMPOUND	STRUCTURE
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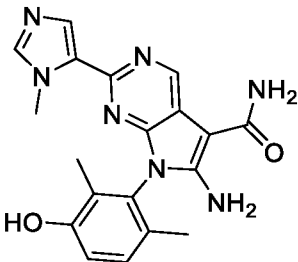
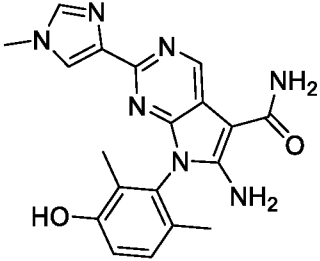
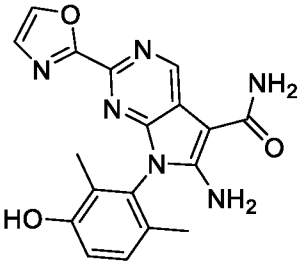
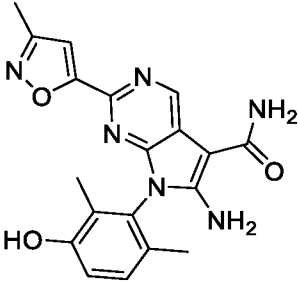
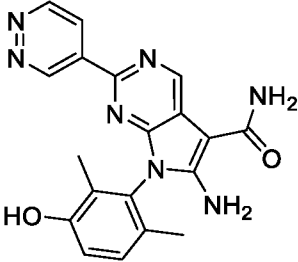
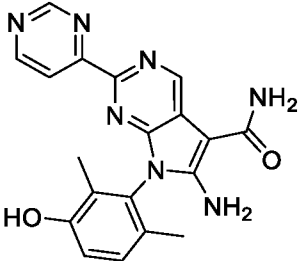
COMPOUND	STRUCTURE
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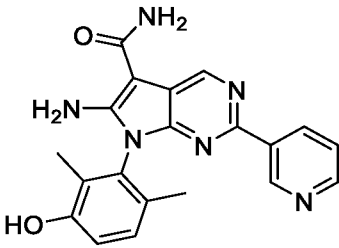
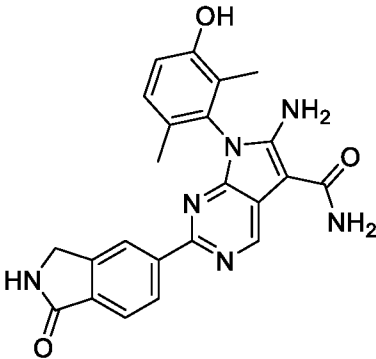
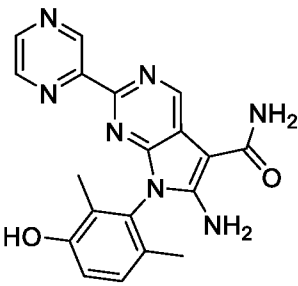
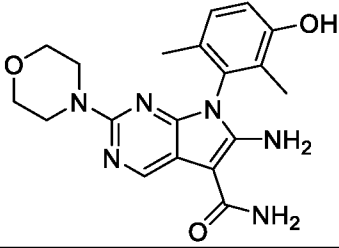
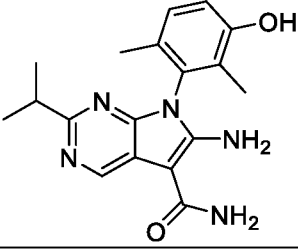
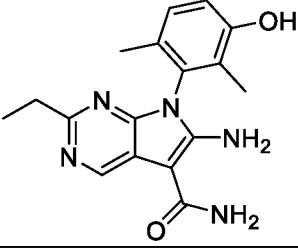
COMPOUND	STRUCTURE
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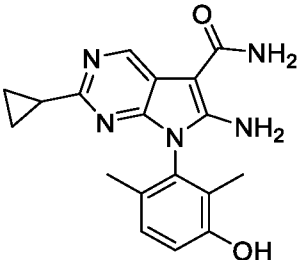
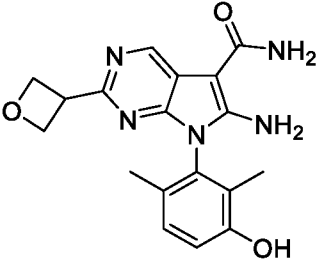
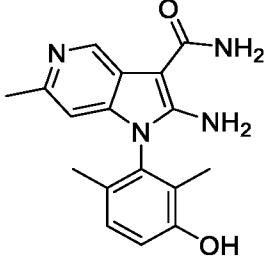
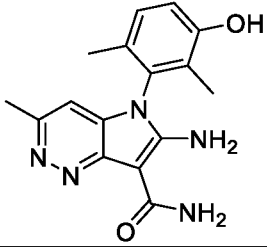
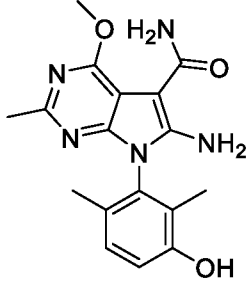
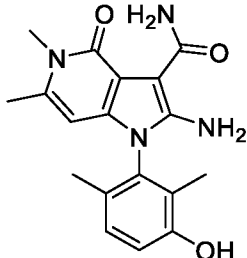
COMPOUND	STRUCTURE
20	
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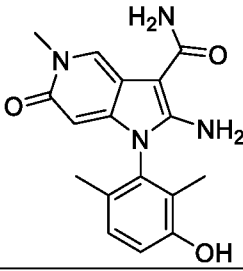
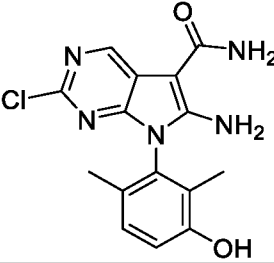
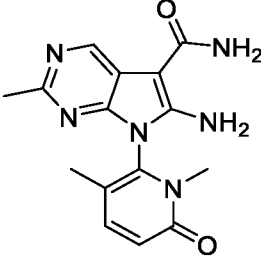
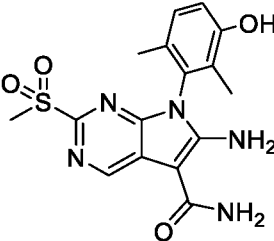
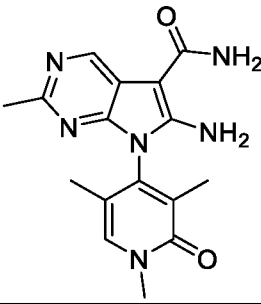
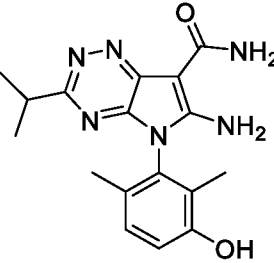
COMPOUND	STRUCTURE
26	
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COMPOUND	STRUCTURE
32	
33	
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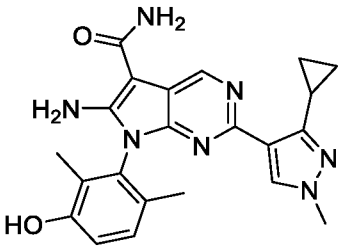
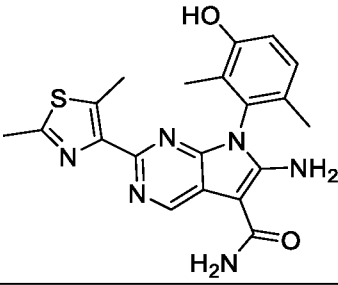
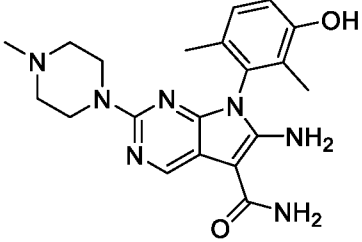
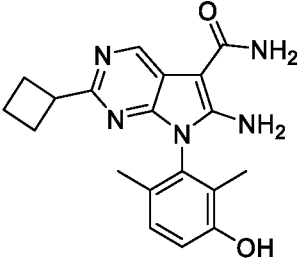
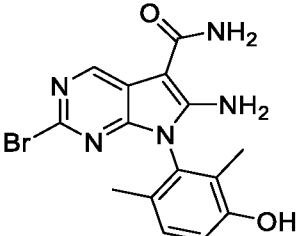
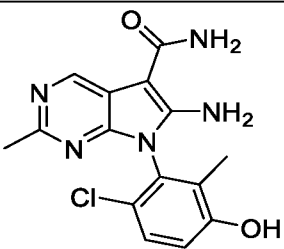
COMPOUND	STRUCTURE
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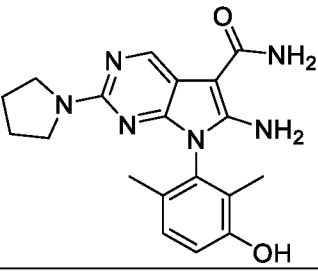
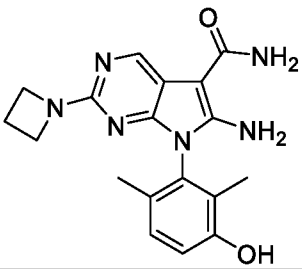
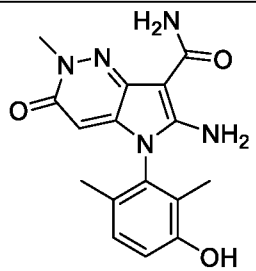
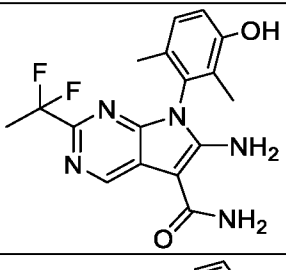
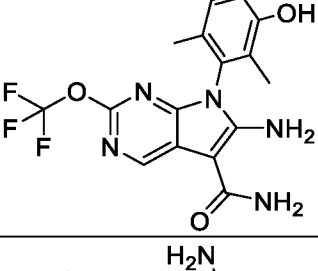
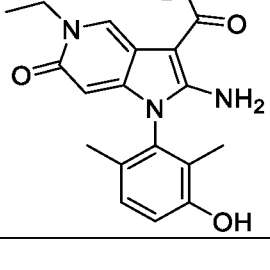
COMPOUND	STRUCTURE
44	
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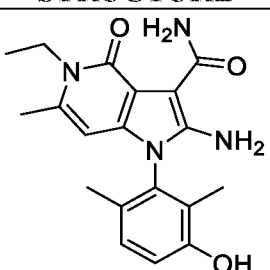
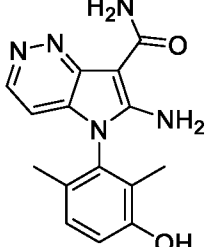
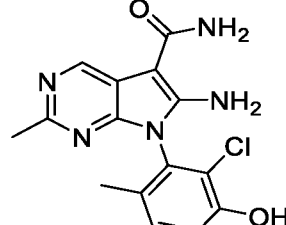
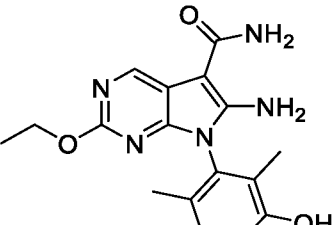
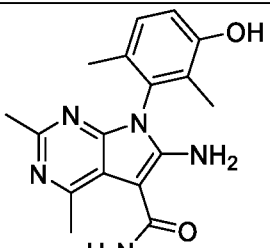
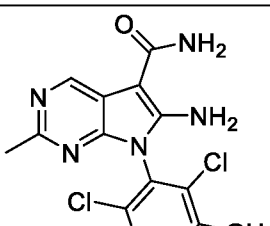
COMPOUND	STRUCTURE
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51	
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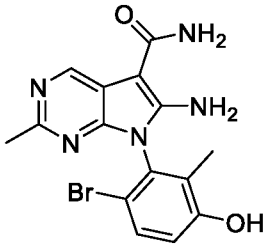
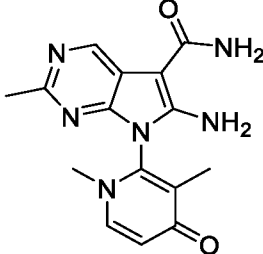
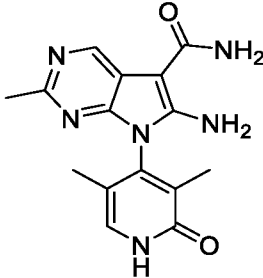
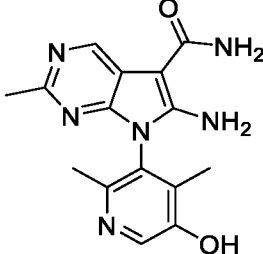
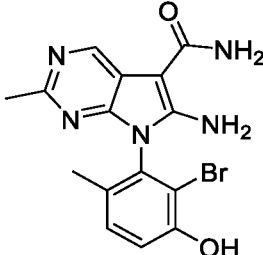
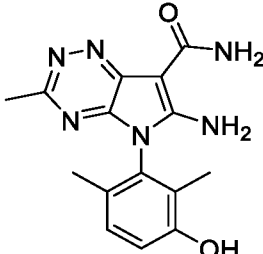
COMPOUND	STRUCTURE
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57	
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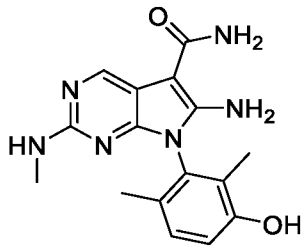
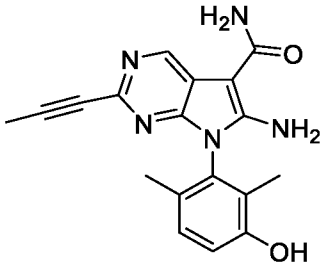
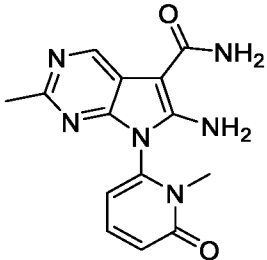
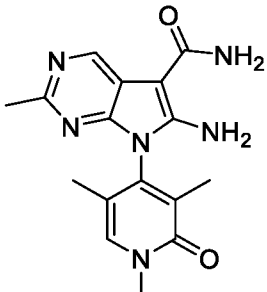
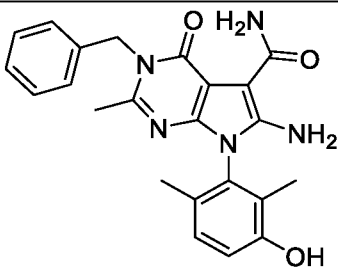
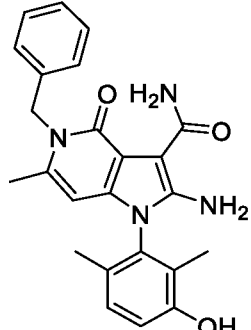
COMPOUND	STRUCTURE
62	
63	
64	
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66	
67	

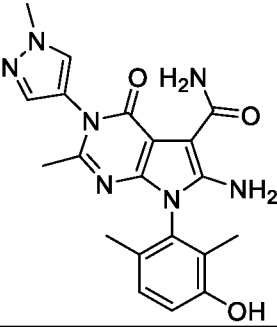
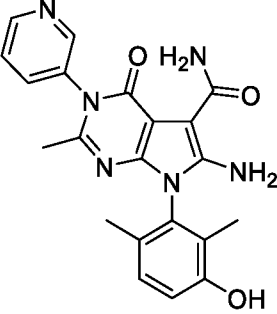
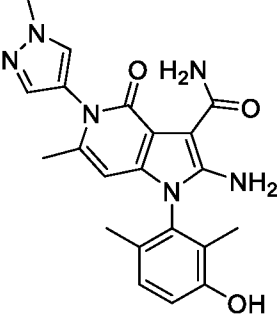
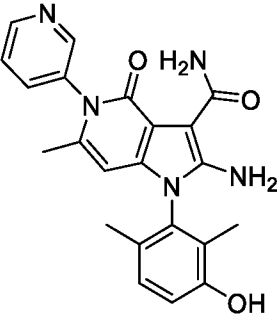
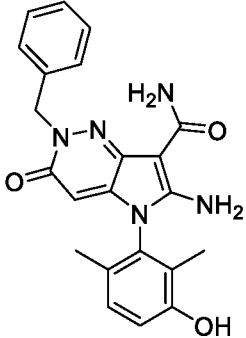
COMPOUND	STRUCTURE
68	
69	
70	
71	
72	
73	

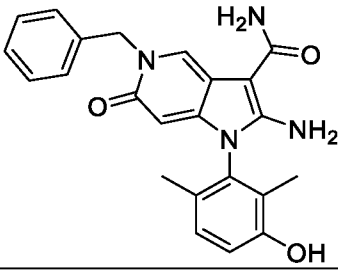
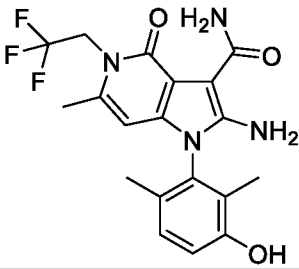
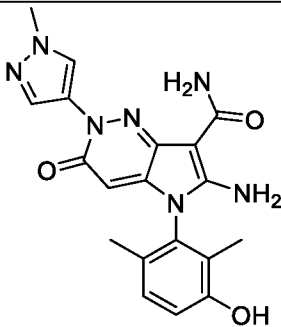
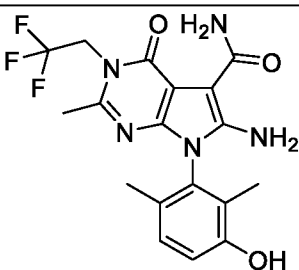
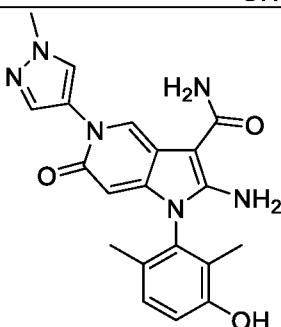
COMPOUND	STRUCTURE
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75	
76	
77	
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79	

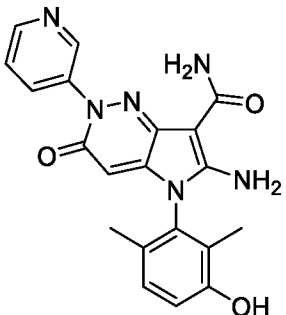
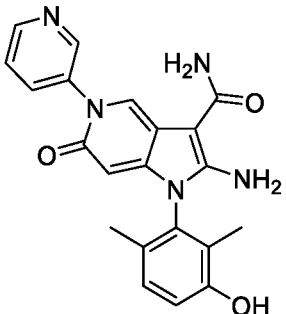
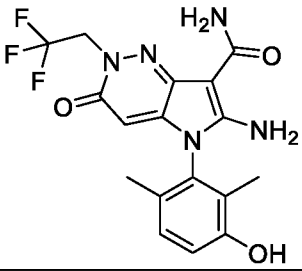
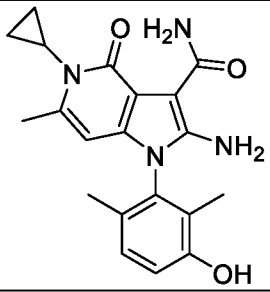
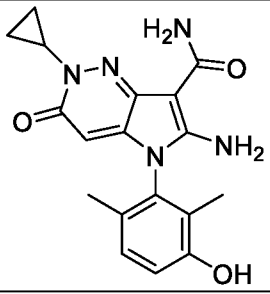
COMPOUND	STRUCTURE
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81	
82	
83	
84	
85	

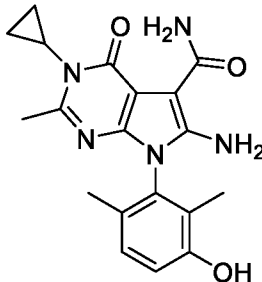
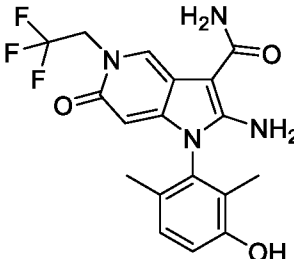
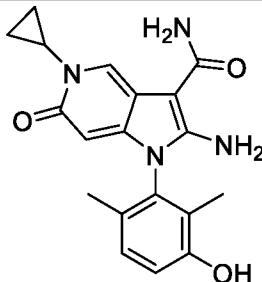
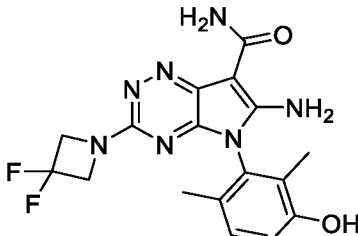
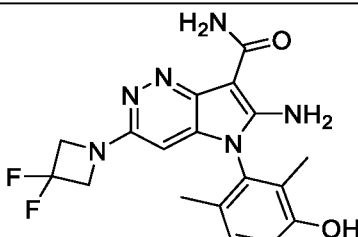
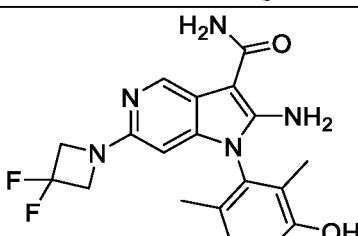
COMPOUND	STRUCTURE
86	
87	
88	
89	
90	
91	

COMPOUND	STRUCTURE
92	
93	
94	
95	
96	
97	

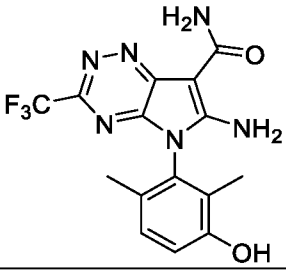
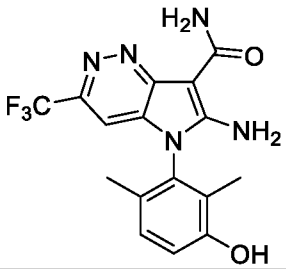
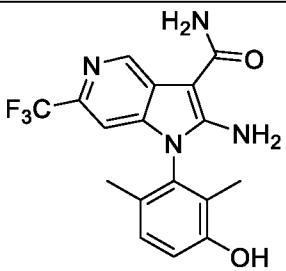
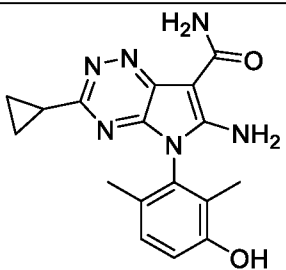
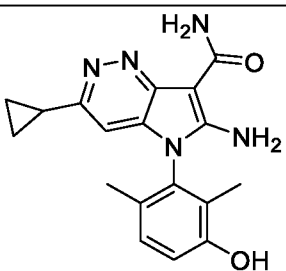
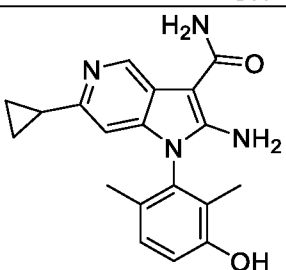
COMPOUND	STRUCTURE
98	
99	
100	
101	
102	

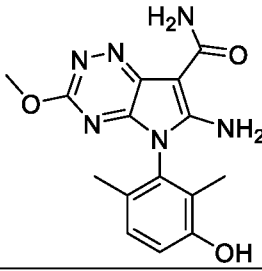
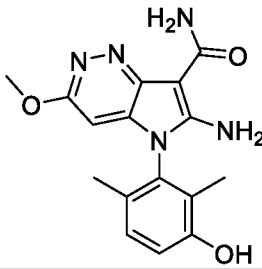
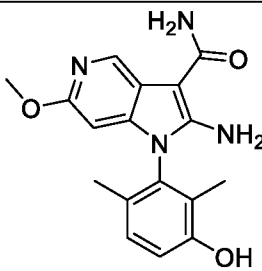
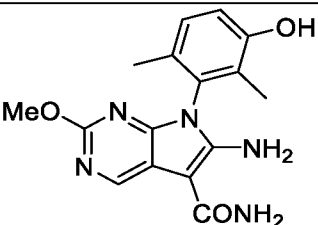
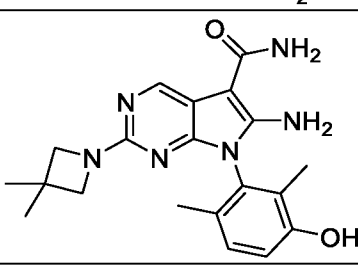
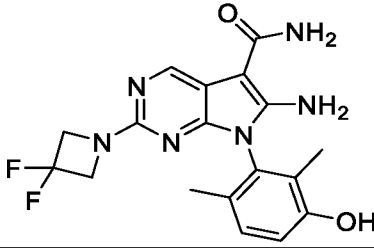
COMPOUND	STRUCTURE
103	
104	
105	
106	
107	

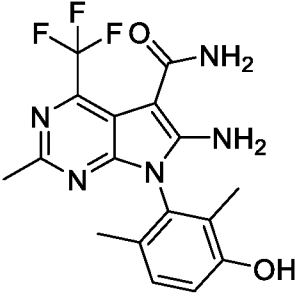
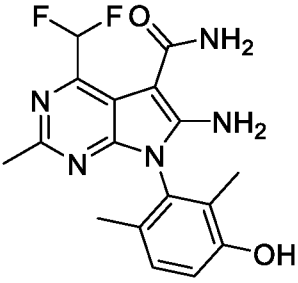
COMPOUND	STRUCTURE
108	
109	
110	
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112	

COMPOUND	STRUCTURE
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118	

COMPOUND	STRUCTURE
119	 <chem>Cc1cc(O)c(C)c(N1C2=C(NC(=O)N)N=NC2=C3C=CC(=C3)N4C=CC=N4C5C=CC=N5)N1</chem>
120	 <chem>Cc1cc(O)c(C)c(N1C2=C(NC(=O)N)N=NC2=C3C=CC(=C3)N4C=CC=N4C5C=CC=N5)N1</chem>
121	 <chem>Cc1cc(O)c(C)c(N1C2=C(NC(=O)N)N=NC2=C3C=CC(=C3)N4C=CC=N4C5C=CC=N5)N1</chem>
122	 <chem>Cc1cc(O)c(C)c(N1C2=C(NC(=O)N)N=NC2=C3C=CC(=C3)N4C=CC=N4C5C=CC=N5)N1</chem>
123	 <chem>Cc1cc(O)c(C)c(N1C2=C(NC(=O)N)N=NC2=C3C=CC(=C3)N4C=CC=N4C5C=CC=N5)N1</chem>
124	 <chem>Cc1cc(O)c(C)c(N1C2=C(NC(=O)N)N=NC2=C3C=CC(=C3)N4C=CC=N4C5C=CC=N5)N1</chem>

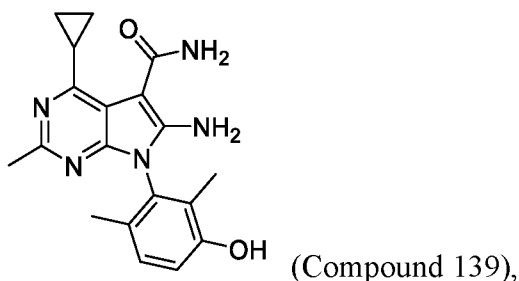
COMPOUND	STRUCTURE
125	
126	
127	
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COMPOUND	STRUCTURE
131	
132	
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134	
135	
136	

COMPOUND	STRUCTURE
137	
138	

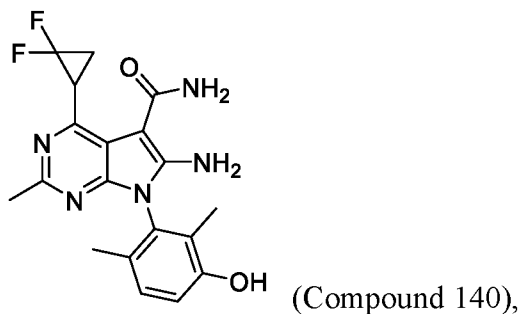
In some embodiments, the compound selected is an atropisomer. In one aspect, the compound selected is the eutomer of the atropisomer.

[0081] In one embodiment, the present disclosure provides a compound that is 6-amino-4-cyclopropyl-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide having the structure:



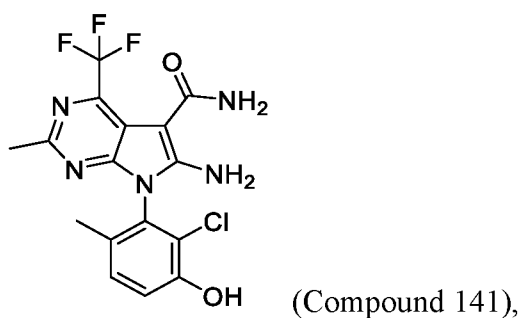
and pharmaceutically acceptable salts thereof. In one aspect, the compound is an atropisomer. In another aspect, the compound selected is the eutomer of the atropisomer.

[0082] In one embodiment, the present disclosure provides a compound that is 6-amino-4-(2,2-difluorocyclopropyl)-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide having the structure:



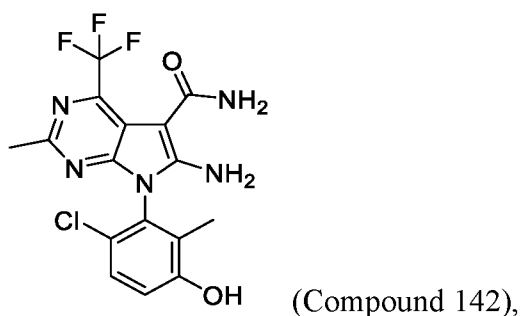
and pharmaceutically acceptable salts thereof. In one aspect, the compound is an atropisomer. In another aspect, the compound selected is the eutomer of the atropisomer.

[0083] In one embodiment, the present disclosure provides a compound that is 6-amino-7-(2-chloro-3-hydroxy-6-methylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide having the structure:



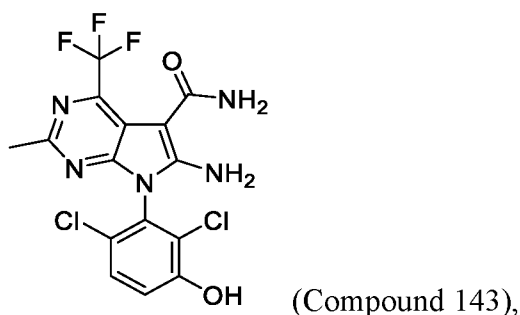
and pharmaceutically acceptable salts thereof. In one aspect, the compound is an atropisomer. In another aspect, the compound selected is the eutomer of the atropisomer.

[0084] In one embodiment, the present disclosure provides a compound that is 6-amino-7-(6-chloro-3-hydroxy-2-methylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide having the structure:



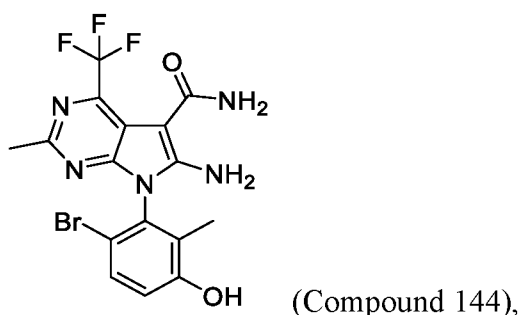
and pharmaceutically acceptable salts thereof. In one aspect, the compound is an atropisomer. In another aspect, the compound selected is the eutomer of the atropisomer.

[0085] In one embodiment, the present disclosure provides a compound that is 6-amino-7-(2,6-dichloro-3-hydroxyphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide having the structure:



and pharmaceutically acceptable salts thereof. In one aspect, the compound is an atropisomer. In another aspect, the compound selected is the eutomer of the atropisomer.

[0086] In one embodiment, the present disclosure provides a compound that is 6-amino-7-(6-bromo-3-hydroxy-2-methylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide having the structure:



and pharmaceutically acceptable salts thereof. In one aspect, the compound is an atropisomer. In another aspect, the compound selected is the eutomer of the atropisomer.

[0087] In one embodiment, the present disclosure provides a compound selected from the group consisting of:

6-amino-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (Compound 137);

6-amino-7-(2-chloro-3-hydroxy-6-methylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (Compound 141);

6-amino-7-(6-chloro-3-hydroxy-2-methylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (Compound 142);

6-amino-7-(2,6-dichloro-3-hydroxyphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (Compound 143); and

6-amino-7-(6-bromo-3-hydroxy-2-methylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (Compound 144);

and pharmaceutically acceptable salts thereof.

In one aspect, the compound is an atropisomer. In another aspect, the compound selected is the eutomer of the atropisomer.

C. Combination of Embodiments

[0088] Any embodiment of the compounds described in the present disclosure can be combined with any other suitable embodiment described herein to provide additional embodiments. For example, where one embodiment of the compounds of Formula (2) individually or collectively describes possible groups for R^{13} , R^{14} , and/or R^{15} and a separate embodiment describes possible groups for R^1 , it is understood that these embodiments can be combined to provide an additional embodiment describing the possible groups described for R^{13} , R^{14} , and/or R^{15} together with the possible groups described for R^1 . In other words, for any of the embodiments of the compounds described in the present disclosure, the R^1 substituent can be as defined in any of the embodiments of R^1 described in this disclosure.

D. Further Embodiments

[0089] In some embodiments, the compounds of the present disclosure have an IC_{50} value for inhibition of PKMYT1 below about 1 μM as measured in the Target Engagement Assay described in Example C-2 below. In one aspect, the IC_{50} value is below about 500 nM. In another aspect, the IC_{50} value is below about 250 nM. In another aspect, the IC_{50} value is below about 100 nM. In another aspect, the compounds have a pharmaceutically acceptable K_d value for PKMYT1 binding affinity as measured in the SPR Direct Binding Assay described in Example C-3 below.

[0090] In some embodiments, the compounds of the present disclosure have a pharmaceutically acceptable selectivity for PKMYT1 relative to WEE1 as measured in the Target

Engagement Assay described in Example C-2 below. In some embodiments, the compounds are at least about 50 times more selective for PKMYT1 relative to WEE1. In another aspect, the compounds are at least about 100 times more selective for PKMYT1 relative to WEE1. In another aspect, the compounds are at least about 250 times more selective for PKMYT1 relative to WEE1. In another aspect, the compounds are at least about 500 times more selective for PKMYT1 relative to WEE1. In another aspect, the compounds of the present disclosure have an IC_{50} value for inhibition of WEE1 greater than about 1 μ M. In another aspect, the IC_{50} value is greater than about 5 μ M. In another aspect, the IC_{50} value is greater than about 10 μ M.

[0091] In some embodiments, the compounds of the present disclosure have an IC_{50} value for inhibition of CDK1 phosphorylation below about 2 μ M as measured in the Functional CDK1 pThr14 AlphaLISA Assay described in Example C-4 below. In one aspect, the IC_{50} value is below about 1 μ M. In another aspect, the IC_{50} value is below about 500 nM. In another aspect, the IC_{50} value is below about 250 nM. In another aspect, the IC_{50} value is below about 100 nM.

[0092] In some embodiments, the compounds of the present disclosure inhibit OVCAR3 cellular proliferation as measured in the Cellular Proliferation Assay described in Example C-5 below. In one aspect, the compounds have a GI_{50} value below about 5 μ M. In another aspect, the GI_{50} value is below about 3 μ M. In another aspect, the GI_{50} value is below about 1.5 μ M.

E. Salts

[0093] The compounds of the present disclosure may exist in salt form or in non-salt form (*i.e.*, as a free base), and the present disclosure covers both salt forms and non-salt forms. The compounds may form acid addition salts or base addition salts. In general, an acid addition salt can be prepared using various inorganic or organic acids. Such salts can typically be formed by, for example, mixing the compound with an acid (e.g., a stoichiometric amount of an acid) using various methods known in the art. This mixing may occur in water, an organic solvent (e.g., ether, ethyl acetate, ethanol, methanol, isopropanol, or acetonitrile), or an aqueous/organic mixture. In another aspect, the acid addition salts are, for example, trifluoroacetate, formate, acetate or hydrochloric. In general, a base addition salt can be prepared using various inorganic or organic bases, for example an alkali or alkaline earth metal salt such as a sodium, calcium or magnesium salt, or other metal salts, such as potassium or zinc, or an ammonium salt, or a salt with an organic base such as methylamine, dimethylamine, trimethylamine, piperidine or morpholine. The skilled person will be aware of the general principles and techniques of

preparing pharmaceutical salts, such as those described in, for example, Berge, S., *et al.*, “Pharmaceutical Salts,” *J. Pharm. Sci.* 66, 1 (1977). Examples of pharmaceutically acceptable salts are also described in “Handbook of Pharmaceutical Salts: Properties, Selection, and Use” by Stahl and Wermuth (Wiley-VCH, Weinheim, Germany, 2002).

F. Isomers

[0094] The compounds and salts of the present disclosure may exist in one or more geometrical, optical, enantiomeric, and diastereomeric forms, including, but not limited to, cis- and trans-forms, E- and Z-forms, and R-, S- and meso-forms. Unless otherwise stated a reference to a particular compound includes all such isomeric forms, including racemic and other mixtures thereof. Where appropriate such isomers can be separated from their mixtures by the application or adaptation of known methods (e.g., chromatographic techniques and recrystallisation techniques). In some embodiments, a single stereoisomer is obtained by isolating it from a mixture of isomers (e.g., a racemate) using, for example, chiral chromatographic separation. In other embodiments, a single stereoisomer is obtained through direct synthesis from, for example, a chiral starting material.

[0095] A particular enantiomer of a compound described herein may be more active than other enantiomers of the same compound. In one embodiment, the compound, or a pharmaceutically acceptable salt thereof, is a single enantiomer being in an enantiomeric excess (%ee) of ≥ 90 , $\geq 95\%$, $\geq 96\%$, ≥ 97 , $\geq 98\%$ or $\geq 99\%$. In one aspect, the single enantiomer is present in an enantiomeric excess (%ee) of $\geq 99\%$.

[0096] In another embodiment, the present disclosure relates to a pharmaceutical composition comprising a compound, or a pharmaceutically acceptable salt thereof, which is a single enantiomer being in an enantiomeric excess (%ee) of ≥ 90 , $\geq 95\%$, $\geq 96\%$, ≥ 97 , $\geq 98\%$ or $\geq 99\%$, or a pharmaceutically acceptable salt thereof, in association with one or more pharmaceutically acceptable excipients. In one aspect, the single enantiomer is present in an enantiomeric excess (%ee) of $\geq 99\%$.

G. Additional Forms

[0097] The compounds and salts of the present disclosure may exist in various tautomeric forms and the disclosure encompasses all such tautomeric forms. “Tautomers” are structural isomers that exist in equilibrium resulting from the migration of a hydrogen atom.

[0098] The compounds of the present disclosure, and pharmaceutically acceptable salts thereof, may exist as solvates (such as a hydrates) as well as unsolvated forms, and the present disclosure covers all such solvates.

[0099] The compounds of the present disclosure, and pharmaceutically acceptable salts thereof, may exist in crystalline or amorphous form, and the present disclosure covers all such forms.

[00100] Compounds and salts of the present disclosure may be isotopically labeled (or “radio-labeled”). In that instance, one or more atoms are replaced by an atom having an atomic mass or mass number different from the atomic mass or mass number typically found in nature. The disclosure encompasses isotopically labelled forms of compounds disclosed herein. Examples of isotopes that may be incorporated include ^2H (also written as “D” for deuterium), ^3H (also written as “T” for tritium), ^{11}C , ^{13}C , ^{14}C , ^{13}N , ^{15}N , ^{15}O , ^{17}O , ^{18}O and ^{36}Cl . The isotope that is used will depend on the specific application of that radio-labeled derivative. For example, for *in vitro* receptor labeling and competition assays, ^3H or ^{14}C are often useful. For radio-imaging applications, ^{11}C is often useful. In some embodiments, the radionuclide is ^3H . In some embodiments, the radionuclide is ^{14}C . In some embodiments, the radionuclide is ^{11}C .

H. Intermediates

[00101] In some embodiments, the present disclosure provides additional compounds that are useful as intermediates for preparing the compounds of the present disclosure, and pharmaceutically acceptable salts thereof.

III. Methods of Use

[00102] One consequence of cancer cell evolution is often the rewiring of the ability to control cell cycle progression which then leads to more rapid oncogenic expansion to aid oncogenesis. More than 50% of all cancers, and over 90% of cancers such as serous-like endometrial, serous ovarian, and basal-like breast cancers, carry a somatic TP53 mutation that can result in inactivation of the tumor suppressor protein p53 and partial or complete inactivation of the G1/S cell cycle checkpoint. Under such circumstances, cells enter the replicative S-phase of the cell cycle prematurely, thereby increasing the likelihood of elevated basal replication stress levels, resulting in a greater dependency on the G2/M cell cycle checkpoint regulated by WEE1 and PKMYT1.

[00103] As previously noted, both WEE1 and PKMYT1 inhibit CDK1 to prevent a cell from dividing until damaged DNA is repaired (G2/M DNA damage cell cycle checkpoint arrest). In contrast to WEE1, however, PKMYT1 is selective for CDK1 relative to CDK2. WEE1 also regulates CDK2 activity that normally prevents aberrant progression of cells through S-phase. Since WEE1 regulates both CDK1 and CDK2, WEE1 inhibition can further increase replication stress (as detected by biomarkers such as phospho-RPA2 (pRPA2) and phospho-Histone H2AX (γ H2AX)) in cancers that already have elevated basal levels of replication stress, creating additional DNA damage during S-phase. Serra, V., et al., "Identification of a Molecularly-Defined Subset of Breast and Ovarian Cancer Models that Respond to WEE1 or ATR Inhibition, Overcoming PARP Inhibitor Resistance," Clin Cancer Res 28(20): 4536-4550. (2022). This increased replication stress can sometimes be sufficient to kill the cancer cells. Lallo, A., et al., "The Combination of the PARP Inhibitor Olaparib and the WEE1 Inhibitor AZD1775 as a New Therapeutic Option for Small Cell Lung Cancer," Clin Cancer Res 24(20): 5153-5164 (2018). See Fig. 1.

[00104] Preclinical and clinical data generated with the WEE1 inhibitor adavosertib have identified cancer backgrounds associated with replication stress that are sensitive to WEE1 inhibition. Such cancer backgrounds include those enriched for p53 mutations and/or one or more aberrations in genes and/or their related protein products including, but are not limited to, cyclin D1, cyclin E1, FBXW7, RB1, INK4a, ARF, CDK4, CDK6, SETD2, KDM4A, LKB1, TSC2, RSP6KA6, MYC, and/or KRAS. These cancer backgrounds are particularly enriched in tumor types such as ovarian cancer, triple negative breast cancer, uterine serous carcinoma, uterine carcinosarcoma, pancreatic cancer, small cell lung cancer, non-small cell lung cancer, esophageal cancer, colorectal cancer, soft tissue sarcoma, skin cancer, bladder cancer, head and neck cancer, glioma, and B-cell lymphoma. Although tumor types harboring these deficiencies can be sensitive to monotherapy with WEE1 inhibitors in the clinic, the fact that WEE1 inhibition also generates S-phase replication stress and DNA damage means that certain normal tissues are also affected resulting in gastrointestinal and hematological toxicities. Consequently, monotherapy with WEE1 inhibitors can have a relatively narrow therapeutic window. Liu, J. F., et al., "Phase II Study of the WEE1 Inhibitor Adavosertib in Recurrent Uterine Serous Carcinoma," Journal of Clinical Oncology 39(14): 1531-1539 (2021).

[00105] Since PKMYT1 regulates CDK1 and not CDK2, PKMYT1 inhibitors have the potential to treat cancers with elevated levels of basal replication stress, either as a single agent or

in combination with DNA damaging agents or targeted agents that increase the levels of replication stress, but with an improved therapeutic window relative to WEE1 inhibitors. As illustrated in Example C-2, cancer backgrounds associated with replication stress that are sensitive to PKMYT1 inhibition include those enriched for one or more aberrations in genes and/or their related protein products including, but are not limited to, cyclin E1, FBXW7, and/or KRAS. Given the dependency of cancers with elevated levels of replication stress on the G2/M cell cycle checkpoint, it is hypothesized that further cancer backgrounds associated with replication stress that are sensitive to PKMYT1 inhibition may include those enriched for TP53 mutations and/or one or more aberrations in genes and/or their related protein products including, but are not limited to, cyclin D1, cyclin E1, FBXW7, RB1, INK4a, ARF, CDK4, CDK6, SETD2, KDM4A, LKB1, TSC2, RSP6KA6, MYC, and/or KRAS. Accordingly, PKMYT1 inhibitors that are selective for CDK1 are desirable and are within the scope of this disclosure.

[00106] The compounds of Formula (1), and pharmaceutically acceptable salts thereof, are inhibitors of PKMYT1 activity. In one aspect, the compounds selectively inhibit PKMYT1 activity.

[00107] In some embodiments, the present disclosure provides a method for treating or preventing cancers in a subject in need thereof by administering to the subject a therapeutically effective amount of a compound of Formula (1), or a pharmaceutically acceptable salt thereof. In one aspect, the cancer is a solid tumor cancer. In another aspect, the solid tumor cancer is selected from the group consisting of ovarian cancer, breast cancer (including triple negative breast cancer), uterine cancer (including uterine serous carcinoma and uterine carcinosarcoma), pancreatic cancer, lung cancer (including small cell lung cancer and non-small cell lung cancer), esophageal cancer, colorectal cancer, soft tissue sarcoma, skin cancer, bladder cancer, head and neck cancer, and glioma. In another aspect, the solid tumor cancer is selected from the group consisting of ovarian cancer, breast cancer (including triple negative breast cancer), uterine cancer (including uterine serous carcinoma and uterine carcinosarcoma), and pancreatic cancer. In another aspect the cancer is a hematological cancer. In another aspect the hematological cancer is B-cell lymphoma.

[00108] In some embodiments, the present disclosure provides a method for treating or preventing cancers in a subject in need thereof by administering to the subject a therapeutically effective amount of a compound of Formula (1), or a pharmaceutically acceptable salt thereof, wherein the cancer is, in whole or in part, a PKMYT1-dependent cancer. In one aspect, the

PKMYT1-dependent cancer is a solid tumor cancer. In another aspect, the PKMYT1-dependent solid tumor cancer is selected from the group consisting of ovarian cancer, breast cancer (including triple negative breast cancer), uterine cancer (including uterine serous carcinoma and uterine carcinosarcoma), pancreatic cancer, lung cancer (including small cell lung cancer and non-small cell lung cancer), esophageal cancer, colorectal cancer, soft tissue sarcoma, skin cancer, bladder cancer, head and neck cancer, and glioma. In another aspect, the PKMYT1-dependent solid tumor cancer is selected from the group consisting of ovarian cancer, breast cancer (including triple negative breast cancer), uterine cancer (including uterine serous carcinoma and uterine carcinosarcoma), and pancreatic cancer. In another aspect the PKMYT1-dependent cancer is a hematological cancer. In another aspect the PKMYT1-dependent hematological cancer is B-cell lymphoma.

[00109] In some embodiments, the present disclosure provides a method for treating or preventing cancers in a subject in need thereof by administering to the subject a therapeutically effective amount of a compound of Formula (1), or a pharmaceutically acceptable salt thereof, wherein the cancer is associated with a higher incidence of genetic or protein aberrations associated with elevated levels of basal replication stress. In one aspect, the cancer is characterized by amplification or overexpression of the gene. In another aspect, the cancer is characterized by one or more deletions and/or mutations in the gene. In another aspect, the cancer is a solid tumor cancer. In another aspect, the solid tumor cancer is selected from the group consisting of ovarian cancer, breast cancer (including triple negative breast cancer), uterine cancer (including uterine serous carcinoma and uterine carcinosarcoma), pancreatic cancer, lung cancer (including small cell lung cancer and non-small cell lung cancer), esophageal cancer, colorectal cancer, soft tissue sarcoma, skin cancer, bladder cancer, head and neck cancer, and glioma. In another aspect, the solid tumor cancer is selected from the group consisting of ovarian cancer, breast cancer (including triple negative breast cancer), uterine cancer (including uterine serous carcinoma and uterine carcinosarcoma), and pancreatic cancer. In another aspect the cancer is a hematological cancer. In another aspect the hematological cancer is B-cell lymphoma.

[00110] In some embodiments, the present disclosure provides a method for treating or preventing cancers in a subject in need thereof by administering to the subject a therapeutically effective amount of a compound of Formula (1), or a pharmaceutically acceptable salt thereof, wherein the cancer is associated with one or more aberrations in a gene, and/or its related protein

product, selected from the group consisting of cyclin E1, FBXW7, and/or KRAS. In one aspect, the cancer is associated with one or more aberrations in the cyclin E1 gene (CCNE1) and/or its related protein product. In another aspect, the cancer is characterized by amplification or overexpression of CCNE1. In another aspect, the cancer is associated with one or more aberrations in the FBXW7 gene and/or its related protein product. In another aspect, the cancer is characterized by one or more deletions and/or mutations in the FBXW7 gene. In another aspect, the cancer is associated with one or more aberrations in the KRAS gene and/or its related protein product. In another aspect, the cancer is characterized by one or more deletions and/or mutations in the KRAS gene.

[00111] In some embodiments, the cancer is ovarian cancer. In one aspect, the ovarian cancer is a PKMYT1-dependent ovarian cancer. In another aspect, the cancer is a platinum-sensitive or platinum-resistant ovarian cancer.

[00112] In some embodiments, the cancer is breast cancer. In one aspect, the breast cancer is a PKMYT1-dependent breast cancer. In another aspect, the breast cancer is selected from the group consisting of hormone receptor positive (HR+) breast cancer, hormone receptor negative (HR-) breast cancer, and triple negative breast cancer. In another aspect, the breast cancer is a chemotherapy-resistant breast cancer. In another aspect, the breast cancer is a radiotherapy-resistant breast cancer. In another aspect, the breast cancer is an advanced or metastatic breast cancer.

[00113] In some embodiments, the cancer is uterine cancer. In one aspect, the uterine cancer is a PKMYT1-dependent uterine cancer. In another aspect, the uterine cancer is uterine serous carcinoma. In another aspect, the uterine cancer is uterine carcinosarcoma.

[00114] In some embodiments, the cancer is pancreatic cancer. In one aspect, the pancreatic cancer is a PKMYT1-dependent pancreatic cancer.

[00115] In some embodiments, the cancer is lung cancer. In one aspect, the lung cancer is a PKMYT1-dependent lung cancer. In another aspect, the lung cancer is small cell lung cancer (SCLC). In another aspect, the lung cancer is non-small cell lung cancer (NSCLC). In another aspect, the non-small cell lung cancer (NSCLC) is squamous cell carcinoma. In another aspect, the non-small cell lung cancer (NSCLC) is adenocarcinoma. In another aspect, the non-small cell lung cancer (NSCLC) is large-cell carcinoma.

[00116] In some embodiments, the cancer is gastric cancer. In one aspect, the gastric cancer is a PKMYT1-dependent gastric cancer.

[00117] In some embodiments, the cancer is esophageal cancer. In one aspect, the esophageal cancer is a PKMYT1-dependent esophageal cancer.

[00118] In some embodiments, the present disclosure provides a method for treating or preventing a hematological cancer in a subject in need thereof by administering to the subject a therapeutically effective amount of a compound of the present disclosure, or a pharmaceutically acceptable salt thereof, wherein the hematological cancer is selected from the group consisting of non-Hodgkin's lymphoma, leukemia, multiple myeloma (MM), and myelodysplastic syndrome (MDS). In one aspect, the hematological cancer is a PKMYT1-dependent hematological cancer. In another aspect, the hematological cancer is non-Hodgkin's lymphoma (NHL). In another aspect, the non-Hodgkin's lymphoma (NHL) is selected from diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, mantle cell lymphoma (MCL), and marginal zone lymphoma. In another aspect, the hematological cancer is leukemia. In another aspect, the leukemia is selected from the group consisting of acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML). In another aspect, the hematological cancer is multiple myeloma (MM). In another aspect, the hematological cancer is myelodysplastic syndrome (MDS). In another aspect, the hematological cancer is diffuse large B-cell lymphoma (DLBCL).

[00119] In some embodiments, the compound of the present disclosure, or a pharmaceutically acceptable salt thereof, is administered as first line therapy.

[00120] In some embodiments, the compound of the present disclosure, or a pharmaceutically acceptable salt thereof, is administered as second line (or later) therapy.

[00121] In some embodiments, the subject to whom a therapeutically effective amount of a compound of the present disclosure, or a pharmaceutically acceptable salt thereof, is administered exhibits a partial response (PR) in response to such treatment.

[00122] In some embodiments, the subject to whom a therapeutically effective amount of a compound of the present disclosure, or a pharmaceutically acceptable salt thereof, is administered exhibits a complete response (CR) in response to such treatment.

[00123] In some embodiments, the subject to whom a therapeutically effective amount of a compound of the present disclosure, or a pharmaceutically acceptable salt thereof, is administered exhibits an improved progression free survival (PFS) in response to such treatment.

[00124] In some embodiments, the subject to whom a therapeutically effective amount of a compound of the present disclosure, or a pharmaceutically acceptable salt thereof, is administered exhibits an improved overall survival (OS) in response to such treatment.

[00125] The subject treated typically will be a human or non-human mammal, particularly a human. Suitable subjects can also include domestic or wild animals; companion animals (including dogs, cats, and the like); livestock (including horses, cows and other ruminants, pigs, poultry, rabbits, and the like); primates (including monkeys such as rhesus monkeys, cynomolgus (also known as crab-eating or long-tailed) monkeys, marmosets, tamarins, chimpanzees, macaques, and the like); and rodents (including rats, mice, gerbils, guinea pigs, and the like).

[00126] In some embodiments, the present disclosure provides the compounds of Formula (1), or pharmaceutically acceptable salts thereof, for use as medicaments.

[00127] In some embodiments, the present disclosure provides for the use of the compounds of the Formula (1), or pharmaceutically acceptable salts thereof, for treating or preventing cancers, including PKMYT1-dependent cancers as discussed above.

[00128] In some embodiments, the present disclosure provides for the use of the compounds of the Formula (1), or pharmaceutically acceptable salts thereof, for the manufacture of medicaments for treating or preventing cancer, including PKMYT1-dependent cancers as discussed above.

IV. Combination Therapies and Fixed-Dose Combinations

[00129] The compounds and pharmaceutically acceptable salts of the present disclosure may be used in the methods described above as either as single pharmacological agents or in combination with other pharmacological agents or techniques including, for example, DNA damaging agents that increase reliance on the G2/M checkpoint and targeted therapies that induce higher levels of replication stress. Such combination therapies may be achieved by way of the simultaneous, sequential, or separate dosing of the individual components of the treatment. These combination therapies (and corresponding combination products) employ the compounds and pharmaceutically acceptable salts of the present disclosure within the dosage ranges described in this application and the other pharmacological agent(s), typically within its approved dosage range(s).

[00130] In some embodiments, the present disclosure provides a combination suitable for use in the treatment or prevention of a PKMYT1-dependent cancer, wherein the combination

comprises a compound of the present disclosure, or a pharmaceutically acceptable salt thereof, and radiotherapy.

[00131] In some embodiments, the present disclosure provides a combination suitable for use in the treatment or prevention of a PKMYT1-dependent cancer, wherein the combination comprises a compound of the present disclosure, or a pharmaceutically acceptable salt thereof, and chemotherapy. In one aspect, the chemotherapy is induction chemotherapy. In another aspect, the chemotherapy is consolidation chemotherapy. In another aspect, the chemotherapy comprises administration of one or more chemotherapeutics selected from the group consisting of an anthracycline, azacytidine, bleomycin, cisplatin, carboplatin, cyclophosphamide, cytarabine, dactinomycin, daunorubicin, docetaxel, doxorubicin, epirubicin, fluorouracil, gemcitabine, idarubicin, mercaptopurine, methotrexate, oxaliplatin, paclitaxel, thioguanine, and/or vincristine.

[00132] In some embodiments, the chemotherapy comprises administration of a nucleoside analog. In one aspect, the nucleoside analog is selected from the group consisting of cytarabine and gemcitabine.

[00133] In some embodiments, the chemotherapy comprises administration of a taxane. In one aspect, the taxane is selected from the group consisting of paclitaxel, docetaxel, and nab-paclitaxel.

[00134] In some embodiments, the chemotherapy comprises administration of a platinum chemotherapeutic. In one aspect, the platinum chemotherapeutic is selected from the group consisting of carboplatin and cisplatin.

[00135] In some embodiments, the chemotherapy further comprises administration of a steroid. In one aspect, the steroid is selected from the group consisting of prednisolone, dexamethasone, and hydrocortisone.

[00136] In some embodiments, the present disclosure provides a combination suitable for use in the treatment or prevention of a PKMYT1-dependent cancer, wherein the combination comprises a compound of the present disclosure, or a pharmaceutically acceptable salt thereof, and a topoisomerase 1 (TOP1) inhibitor, topoisomerase 2 (TOP2) inhibitor, TOP1-antibody drug conjugate (TOP1-ADC), or TOP2-antibody drug conjugate (TOP2-ADC). In one aspect, the TOP1 inhibitor is selected from the group consisting of irinotecan and topotecan. In one aspect, the TOP1-ADC is trastuzumab deruxtecan. In another aspect, the TOP2 inhibitor is selected from the group consisting of etoposide and teniposide.

[00137] In some embodiments, the present disclosure provides a combination suitable for use in the treatment or prevention of a PKMYT1-dependent cancer, wherein the combination comprises a compound of the present disclosure, or a pharmaceutically acceptable salt thereof, and a poly ADP ribose polymerase (PARP) inhibitor. In one aspect, the PARP inhibitor is selected from the group consisting of olaparib, rucaparib, niraparib, talazoparib, AZD5305 (CAS No. 2589531-76-8), and AZD9574 (CAS No. 2756333-39-6). In another aspect, the PARP inhibitor is olaparib. In another aspect, the PARP inhibitor is AZD5305.

[00138] In some embodiments, the present disclosure provides a combination suitable for use in the treatment or prevention of a PKMYT1-dependent cancer, wherein the combination comprises a compound of the present disclosure, or a pharmaceutically acceptable salt thereof, and an ataxia telangiectasia mutated and Rad3-related kinase (ATR) inhibitor. In one aspect, the ATR inhibitor is selected from the group consisting of camonsertib (Repare), tuvusertib (Merck), berzosertib (Merck KGaA), ceralasertib (AstraZeneca), and elimusertib (Bayer). In another aspect, the ATR inhibitor is ceralasertib.

[00139] In some embodiments, the present disclosure provides a combination suitable for use in the treatment or prevention of a PKMYT1-dependent cancer, wherein the combination comprises a compound of the present disclosure, or a pharmaceutically acceptable salt thereof, and a checkpoint kinase 1 (CHK1) inhibitor. In one aspect, the CHK1 inhibitor is selected from the group consisting of bosutinib, crizotinib, dasatinib, erlotinib, gefitinib, lapatinib, pazopanib, ruxolitinib, sunitinib, and vemurafenib.

V. Pharmaceutical Compositions

[00140] The compounds of Formula (1), and pharmaceutically acceptable salts thereof, may be administered as pharmaceutical compositions, comprising one or more pharmaceutically acceptable excipients. Therefore, in some embodiments the present disclosure provides pharmaceutical compositions comprising a compound of Formula (1), or a pharmaceutically acceptable salt thereof, and at least one pharmaceutically acceptable excipient.

[00141] The excipient(s) selected for inclusion in a particular composition will depend on factors such as the mode of administration and the form of the composition provided. Suitable pharmaceutically acceptable excipients are described, for example, in the Handbook of Pharmaceutical Excipients, Sixth Edition, Pharmaceutical Press, edited by Rowe, Ray C; Sheskey, Paul J; Quinn, Marian. Pharmaceutically acceptable excipients may function as, for

example, adjuvants, diluents, carriers, stabilisers, flavourings, colorants, fillers, binders, disintegrants, lubricants, glidants, thickening agents and coating agents. As persons skilled in the art will appreciate, certain pharmaceutically acceptable excipients may serve more than one function and may serve alternative functions depending on how much of the excipient is present in the composition and what other excipients are present in the composition.

[00142] The compositions may be in a form suitable for oral use (for example as tablets, lozenges, hard or soft capsules, aqueous or oily suspensions, emulsions, dispersible powders or granules, syrups or elixirs), for topical use (for example as creams, ointments, gels, or aqueous or oily solutions or suspensions), for administration by inhalation (for example as a finely divided powder or a liquid aerosol), for administration by insufflation (for example as a finely divided powder) or for parenteral administration (for example as a sterile aqueous or oily solution for intravenous, subcutaneous or intramuscular dosing), or as a suppository for rectal dosing. Compositions intended for oral use may contain, for example, one or more coloring, sweetening, flavoring and/or preservative agents.

[00143] The total daily dose will necessarily be varied depending upon the subject treated, the route of administration, any therapies being co-administered, and the severity of the illness being treated, and may include single or multiple doses. Specific dosages can be adjusted, for example, depending upon the condition being treated; the age, body weight, general health condition, sex, and diet of the subject; administration routes; dose intervals; excretion rate; and other drugs being co-administered to the subject. The compound of Formula (1), or a pharmaceutically acceptable salt thereof, typically will be administered to a warm-blooded animal at a unit dose within the range 2.5 to 5000 mg/m² body area of the animal, or approximately 0.05 to 100 mg/kg, and this normally provides a therapeutically effective dose.

[00144] In some embodiments, the present disclosure provides pharmaceutical compositions for use in therapy, comprising a compound of Formula (1), or a pharmaceutically acceptable salt thereof, and at least one pharmaceutically acceptable excipient.

[00145] In some embodiments, the present disclosure provides pharmaceutical compositions for use in the treatment of cancers, comprising a compound of Formula (1), or a pharmaceutically acceptable salt thereof, and at least one pharmaceutically acceptable excipient. In one aspect, the cancer is a PKMYT1-dependent cancer. In another aspect, the PKMYT1-dependent cancer is a solid tumor cancer. In another aspect, PKMYT1-dependent cancer is a hematological cancer.

VI. Kits

[00146] The present disclosure further provides kits comprising a unit dosage form comprising a compound of Formula (1), or a pharmaceutically acceptable salt thereof, contained within a packaging material and a label or package insert which indicates that the unit dosage form can be used for treating one or more of the previously described conditions.

[00147] In some embodiments, the kit comprises a unit dosage form comprising a compound of Formula (1), or a pharmaceutically acceptable salt thereof, contained within a packaging material and a label or package insert which indicates that the pharmaceutical composition can be used for treating a cancer. In one aspect, the cancer is a PKMYT1-dependent cancer. In another aspect, the PKMYT1-dependent cancer is a hematological malignancy. In another aspect, the PKMYT1-dependent cancer is a solid tumor cancer.

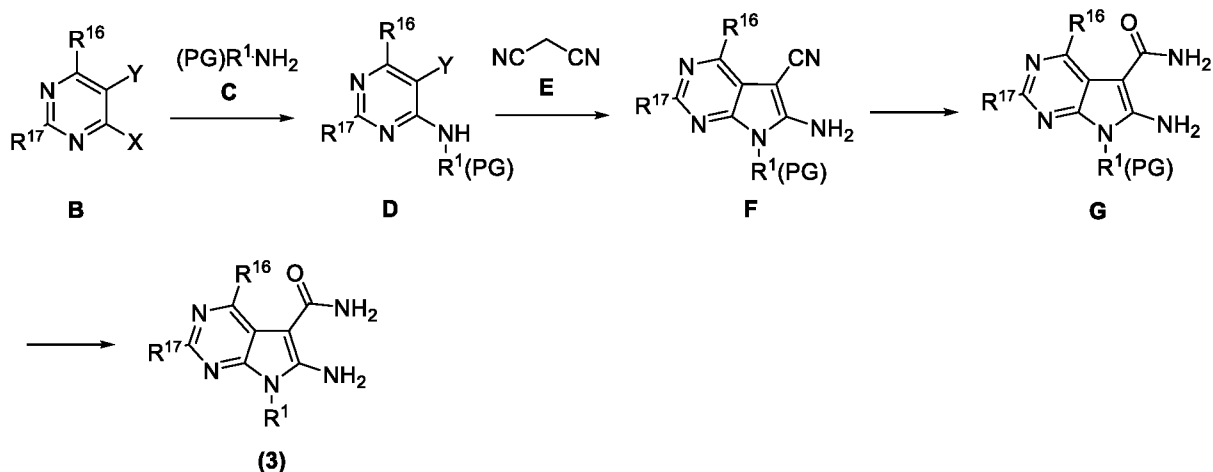
[00148] In some embodiments, kit comprises: (a) a first unit dosage form comprising a compound of Formula (1), or a pharmaceutically acceptable salt thereof; (b) a second unit dosage form comprising a pharmacological agent selected from the group consisting of chemotherapeutics, TOP1 inhibitors, TOP2 inhibitors, TOP1-ADCs, TOP2-ADCs, PARP inhibitors, ATR inhibitors, and CHK1 inhibitors; (c) a container means for containing said first and second dosage forms; and (d) a label or package insert which indicates that the first unit dosage form and second unit dosage form can be used for treating a PKMYT1-dependent cancer.

VII. Methods of Preparation

[00149] The present disclosure further provides processes for the preparation of the compounds of Formula (1) and pharmaceutically acceptable salts thereof.

[00150] Schemes 1 to 11 below illustrate synthetic routes to compounds of Formula (1). One of skill in the art will appreciate that these methods are representative and are not inclusive of all possible methods for preparing the compounds of the present disclosure. The R^X substituents in each Scheme are as defined for the compounds of the present disclosure unless otherwise stated. It is understood that the processes for preparation described in Schemes 1 to 11 can be performed starting from any enantiomer, or a racemic mixture, of intermediate compounds to give compounds of Formula (1) or any stereoisomer of Formula (1). All starting materials are readily available or prepared as described in the Examples.

SCHEME 1

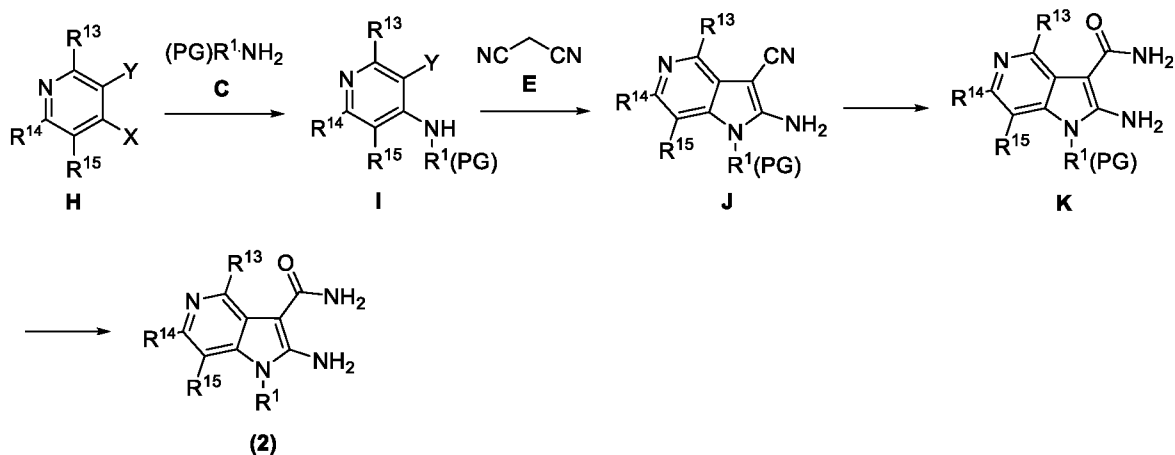


[00151] Scheme 1 illustrates synthetic routes to certain compounds of Formula (1). A compound of formula (B) (X , Y are halogens like fluoro, chloro, bromo, or iodo) may be reacted with an aryl amine of formula (C) (PG are protecting groups such as OMe, OBn, *etc.*) to give a compound of formula (D). The reaction may be performed in the presence of a base (typically an organic base such as DIPEA, *etc.*) or acid (typically a catalytic amount of concentrated HCl) using a solvent (such as *t*-BuOH, *t*-amyl alcohol, DMSO, *etc.*), and at temperatures typically ranging from 80°C to 120°C.

A compound of formula (D) may be reacted with malononitrile of formula (E) to give a compound of formula (F). The reaction may be catalyzed with a suitable Pd-catalyst (such as $PdCl_2(dppf)$, *etc.*) in the presence of a base (such as NaOtBu, *etc.*) using a suitable solvent (such as DME, *etc.*), and at temperatures typically ranging from 80°C to 100°C.

[00152] A compound of formula (F) may be reacted with concentrated sulfuric acid to give a compound of formula (G). The protecting group in the compound of formula (G) may be removed under appropriate reaction conditions to give a compound of Formula (3).

SCHEME 2

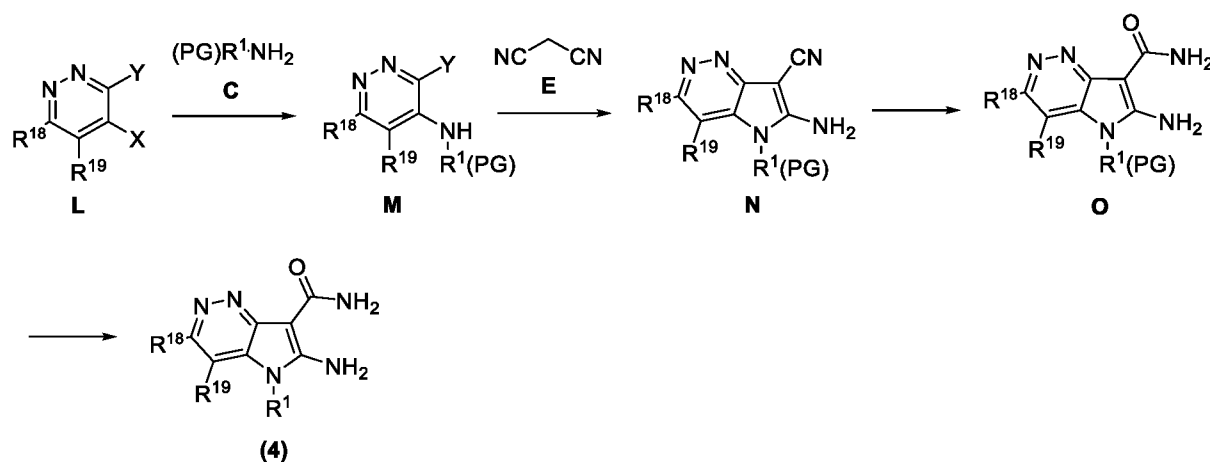


[00153] Scheme 2 illustrates synthetic routes to certain compounds of Formula (1). A compound of formula (H) may be reacted with an aryl amine of formula (C) (PG are protecting groups such as OMe, OBn, *etc.*) to give a compound of formula (I). The reaction may be performed in the presence of a base (typically an organic base such as DIPEA, *etc.*) or acid (typically a catalytic amount of concentrated HCl) using a solvent (such as t-BuOH, t-amyl alcohol, DMSO, *etc.*), and at temperatures typically ranging from 80°C to 120°C.

[00154] A compound of formula (I) may be reacted with malononitrile of formula (E) to give a compound of formula J. The reaction may be catalyzed with a suitable Pd-catalyst (such as PdCl₂(dppf), *etc.*) in the presence of a base (such as NaOtBu, *etc.*) using a suitable solvent (such as DME, *etc.*), and at temperatures typically ranging from 80°C to 100°C.

[00155] A compound of formula J may be reacted with concentrated sulfuric acid to give a compound of formula (K). The protecting group in the compound of formula (K) may be removed under appropriate reaction conditions to give a compound of Formula (2).

SCHEME 3

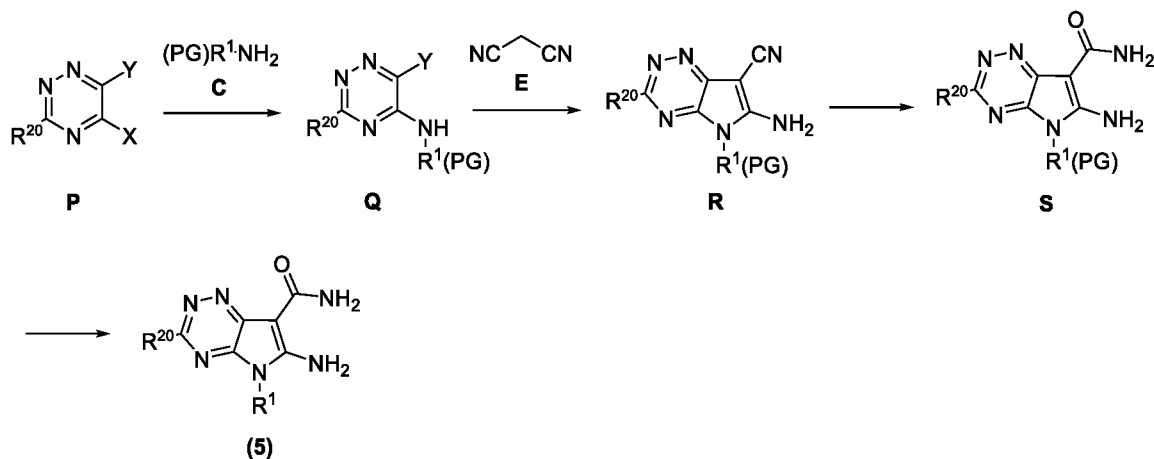


[00156] Scheme 3 illustrates synthetic routes to certain compounds of Formula (1). A compound of formula (L) may be reacted with an aryl amine of formula (C) (PG are protecting groups such as OMe, OBn, *etc.*) to give a compound of formula (M). The reaction may be performed in the presence of a base (typically an organic base such as DIPEA, *etc.*) or acid (typically a catalytic amount of concentrated HCl) using a solvent (such as t-BuOH, t-amyl alcohol, DMSO, *etc.*), and at temperatures typically ranging from 80°C to 120°C.

[00157] A compound of formula (M) may be reacted with malononitrile of formula (E) to give a compound of formula (N). The reaction may be catalyzed with a suitable Pd-catalyst (such as PdCl₂(dppf), *etc.*) in the presence of a base (such as NaOtBu, *etc.*) using a suitable solvent (such as DME, *etc.*), and at temperatures typically ranging from 80°C to 100°C.

[00158] A compound of formula (N) may be reacted with concentrated sulfuric acid to give a compound of formula (O). The protecting group in the compound of formula (O) may be removed under appropriate reaction conditions to give a compound of Formula (4).

SCHEME 4

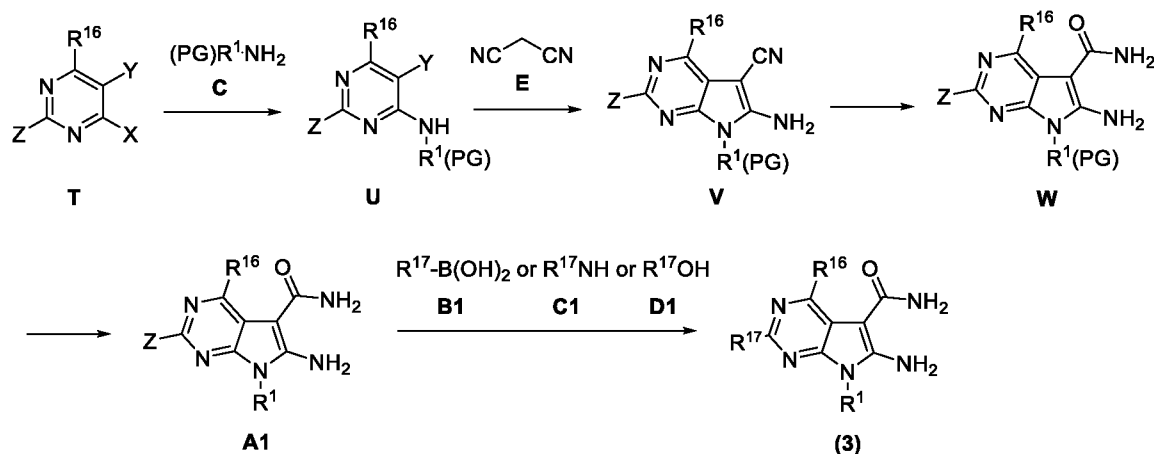


[00159] Scheme 4 illustrates synthetic routes to certain compounds of Formula (1). A compound of formula (P) may be reacted with an aryl amine of formula (C) (PG are protecting groups such as OMe, OBn, *etc.*) to give a compound of formula (Q). The reaction may be performed in the presence of a base (typically an organic base such as DIPEA, *etc.*) or acid (typically a catalytic amount of concentrated HCl) using a solvent (such as t-BuOH, t-amyl alcohol, DMSO, *etc.*), and at temperatures typically ranging from 80°C to 120°C.

[00160] A compound of formula (Q) may be reacted with malononitrile of formula (E) to give a compound of formula (R). The reaction may be catalyzed with a suitable Pd-catalyst (such as PdCl₂(dppf), *etc.*) in the presence of a base (such as NaOtBu, *etc.*) using a suitable solvent (such as DME, *etc.*), and at temperatures typically ranging from 80°C to 100°C.

[00161] A compound of formula (R) may be reacted with concentrated sulfuric acid to give a compound of formula (S). The protecting group in the compound of formula (S) may be removed under appropriate reaction conditions to give a compound of Formula (5).

SCHEME 5



[00162] Scheme 5 illustrates synthetic routes to certain compounds of Formula (1). A compound of formula (T) may be reacted with an aryl amine of formula (C) (PG are protecting groups such as OMe, OBn, *etc.*) to give a compound of formula (U). The reaction may be performed in the presence of a base (typically an organic base such as DIPEA, *etc.*) or acid (typically a catalytic amount of concentrated HCl) using a solvent (such as t-BuOH, t-amyl alcohol, DMSO, *etc.*), and at temperatures typically ranging from 80°C to 120°C.

[00163] A compound of formula (U) may be reacted with malononitrile of formula (E) to give a compound of formula (V). The reaction may be catalyzed with a suitable Pd-catalyst (such as PdCl₂(dppf), *etc.*) in the presence of a base (such as NaOtBu, *etc.*) using a suitable solvent (such as DME, *etc.*), and at temperatures typically ranging from 80°C to 100°C.

[00164] A compound of formula (V) may be reacted with concentrated sulfuric acid to give a compound of formula (W). The protecting group in the compound of formula (W) may be removed under appropriate reaction conditions to give a compound of formula (A1).

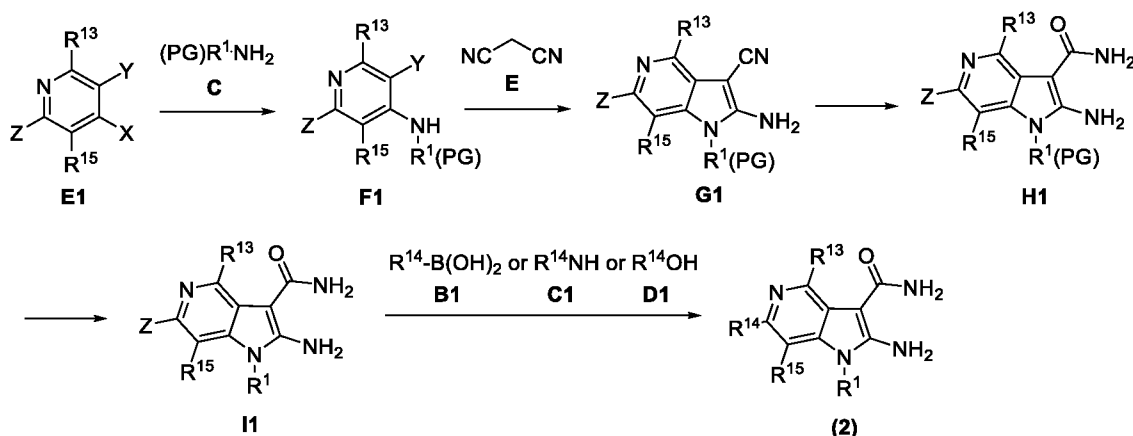
[00165] A compound of formula (A1) may be reacted with boronic acid or ester of formula (B1) to give a compound of Formula (3). The reaction may be catalyzed with a suitable Pd-precatalyst (such as XantPhos Pd G3, XPhos Pd G3, *etc.*) and ligand (such as XPhos, *etc.*) in the presence of a base (such as K₂CO₃, NaOtBu, Cs₂CO₃, *etc.*) using a suitable solvent (1,4-dioxane/water mixture, toluene/water mixture, *etc.*), and at temperatures typically ranging from 60°C to 120°C.

[00166] A compound of formula (A1) may also be reacted with alkyl or aryl amine of formula (C1) to give a compound of Formula (3). The reaction may be catalyzed with a suitable Pd-precatalyst (such as Pd(dba)₂, Pd-PEPPSI-iHeptCl, BrettPhos Pd G3, *etc.*) and ligand (such as

Xphos, Cphos, BrettPhos, *etc.*) in the presence of a base (such as LiHMDS, *etc.*) using a suitable solvent (such as 2-Me THF, 1,4-dioxane, *t*-BuOH, *etc.*) and at temperatures typically ranging from 80°C to 120°C.

[00167] A compound of formula (A1) may also be reacted with aryl alcohol of formula (D1) to give a compound of Formula (3). The reaction may be catalyzed with a suitable Pd-precatalyst (such as RockPhos Pd G3, Cphos Pd G3, *etc.*) and ligand (such as RockPhos, *etc.*) in the presence of a base (such as NaOtBu, Cs₂CO₃, K₃PO₄, *etc.*) using a suitable solvent (such as *t*-BuOH, 1,4-dioxane, *etc.*), and at temperatures typically ranging from 60°C to 120°C.

SCHEME 6



[00168] Scheme 6 illustrates synthetic routes to certain compounds of Formula (1). A compound of formula (E1) may be reacted with an aryl amine of formula (C) (PG are protecting groups such as OMe, OBn, *etc.*) to give a compound of formula (F1). The reaction may be performed in the presence of a base (typically an organic base such as DIPEA, *etc.*) or acid (typically a catalytic amount of concentrated HCl) using a solvent (such as *t*-BuOH, *t*-amyl alcohol, DMSO, *etc.*), and at temperatures typically ranging from 80°C to 120°C.

[00169] A compound of formula (F1) may be reacted with malononitrile of formula (E) to give a compound of formula (G1). The reaction may be catalyzed with a suitable Pd-catalyst (such as PdCl₂(dppf), *etc.*) in the presence of a base (such as NaOtBu, *etc.*) using a suitable solvent (such as DME, *etc.*), and at temperatures typically ranging from 80°C to 100°C.

[00170] A compound of formula (G1) may be reacted with concentrated sulfuric acid to give a compound of formula (H1). The protecting group in the compound of formula (H1) may be removed under appropriate reaction conditions to give a compound of formula (I1).

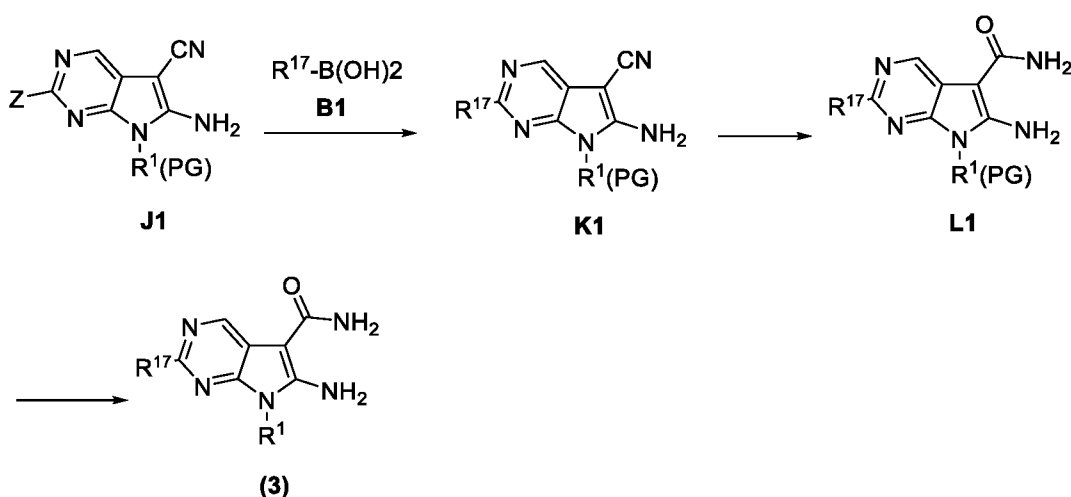
[00171] A compound of formula (I1) may be reacted with boronic acid or ester of formula

(B1) to give a compound of Formula (2). The reaction may be catalyzed with a suitable Pd-precatalyst (such as XantPhos Pd G3, XPhos Pd G3, *etc.*) and ligand (such as XPhos, *etc.*) in the presence of a base (such as K_2CO_3 , NaOtBu, CS_2CO_3 , *etc.*) using a suitable solvent (such as 1,4-dioxane/water mixture, toluene/water mixture, *etc.*), and at temperatures typically ranging from 60°C to 120°C.

[00172] A compound of formula (I1) may also be reacted with alkyl or aryl amine of formula (C1) to give a compound of Formula (2). The reaction may be catalyzed with a suitable Pd-precatalyst (such as $Pd(dba)_2$, Pd-PEPPSI-iHeptCl, BrettPhos Pd G3, *etc.*) and ligand (such as Xphos, Cphos, BrettPhos, *etc.*) in the presence of a base (such as LiHMDS, *etc.*) using a suitable solvent (such as 2-Me THF, 1,4-dioxane, t-BuOH, *etc.*) and at temperatures typically ranging from 80°C to 120°C.

[00173] A compound of formula (I1) may also be reacted with aryl alcohol of formula (D1) to give a compound of Formula (2). The reaction may be catalyzed with a suitable Pd-precatalyst (such as RockPhos Pd G3, CPhos Pd G3, *etc.*) and ligand (such as RockPhos, *etc.*) in the presence of a base (such as NaOtBu, CS_2CO_3 , K_3PO_4 , *etc.*) using a suitable solvent (such as t-BuOH, 1,4-dioxane, *etc.*), and at temperatures typically ranging from 60°C to 120°C.

SCHEME 7

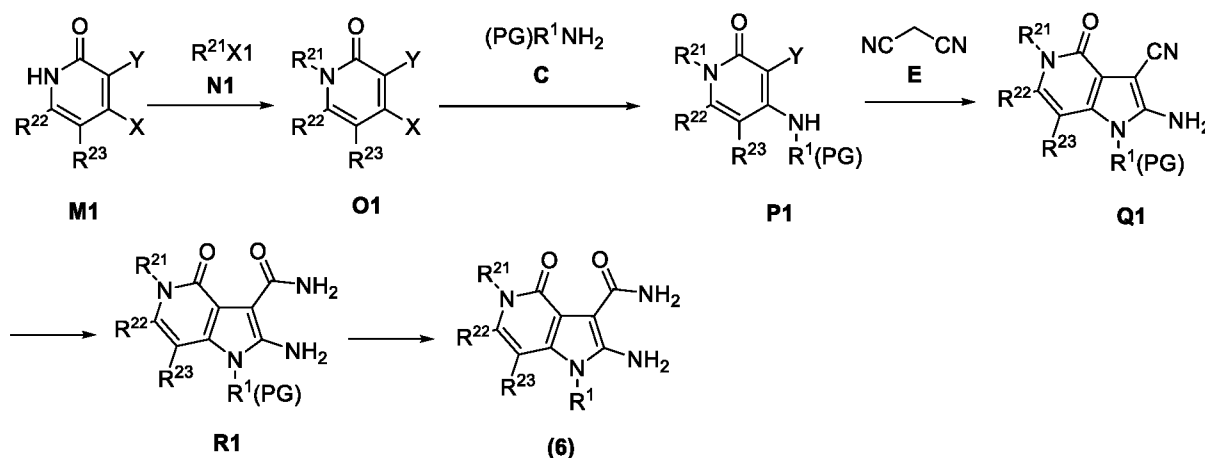


[00174] Scheme 7 illustrates synthetic routes to certain compounds of Formula (1). A compound of formula (J1) may be reacted with boronic acid or ester of formula (B1) to give a compound of formula (K1). The reaction may be catalyzed with a suitable Pd-precatalyst (such as XantPhos Pd G3, XPhos Pd G3, *etc.*) and ligand (such as XPhos, *etc.*) in the presence of a base (such as K_2CO_3 , NaOtBu, CS_2CO_3 , *etc.*) using a suitable solvent (1,4-dioxane/water mixture,

toluene/water mixture, *etc.*), and at temperatures typically ranging from 60°C to 120°C.

[00175] A compound of formula (K1) may be reacted with concentrated sulfuric acid to give a compound of formula (L1). The protecting group in the compound of formula (L1) may be removed under appropriate reaction conditions to give a compound of Formula (3).

SCHEME 8



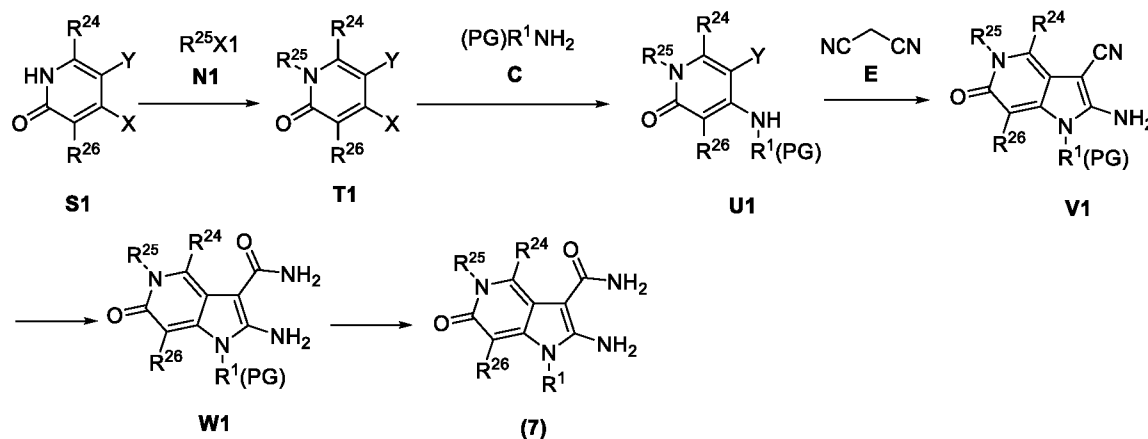
[00176] Scheme 8 illustrates synthetic routes to certain compounds of Formula (1). A compound of formula (M1) may be reacted with a compound of formula (N1) (X1 is typically a leaving group like iodo, bromo, chloro, or triflate) to give a compound of formula (O1). The reaction may be performed in the presence of a base (typically an inorganic base such as K_2CO_3 , *etc.*) or catalyzed with a suitable Pd-catalyst (such as BrettPhos Pd G3, *etc.*) in the presence of a base (such as CS_2CO_3 , *etc.*), using a solvent (such as t-BuOH, DMSO, acetone, *etc.*).

[00177] A compound of formula (O1) may be reacted with an aryl amine of formula (C) (PG are protecting groups such as OMe, OBn, *etc.*) to give a compound of formula (P1). The reaction may be performed in the presence of a base (typically an organic base such as DIPEA, *etc.*) or acid (typically a catalytic amount of concentrated HCl) using a solvent (such as t-BuOH, t-amyl alcohol, DMSO, *etc.*), and at temperatures typically ranging from 80°C to 120°C.

[00178] A compound of formula (P1) may be reacted with malononitrile of formula (E) to give a compound of formula (Q1). The reaction may be catalyzed with a suitable Pd-catalyst (such as $PdCl_2(dppf)$, *etc.*) in the presence of a base (such as NaOtBu, *etc.*) using a suitable solvent (such as DME, *etc.*), and at temperatures typically ranging from 80°C to 100°C.

[00179] A compound of formula (Q1) may be reacted with concentrated sulfuric acid to give a compound of formula (R1). The protecting group in the compound of formula (R1) may be removed under appropriate reaction conditions to give a compound of Formula (6).

SCHEME 9



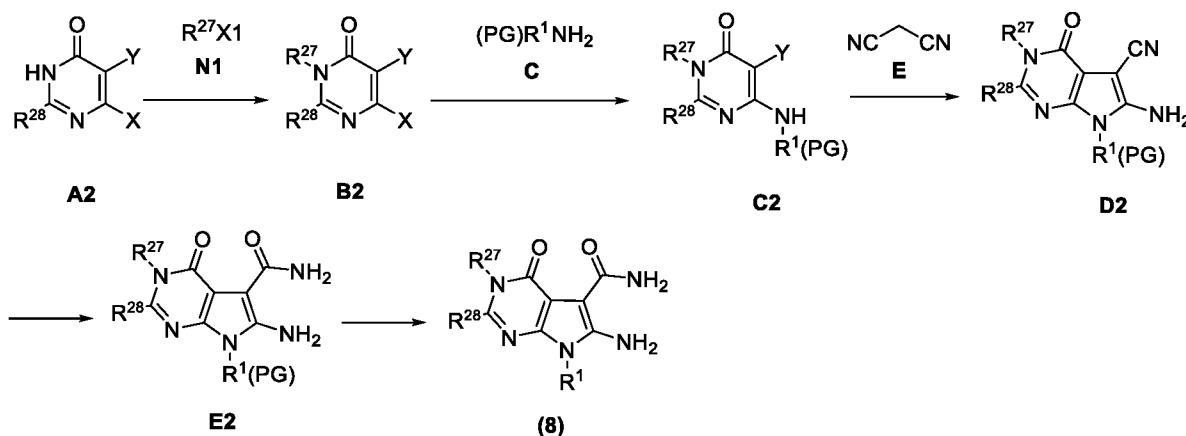
[00180] Scheme 9 illustrates synthetic routes to certain compounds of Formula (1). A compound of formula (S1) may be reacted with a compound of formula (N1) (X1 is typically a leaving group like iodo, bromo, chloro, or triflate) to give a compound of formula (T1). The reaction may be performed in the presence of a base (typically an inorganic base such as K_2CO_3 , *etc.*) or catalyzed with a suitable Pd-catalyst (such as BrettPhos Pd G3, *etc.*) in the presence of a base (such as Cs_2CO_3 , *etc.*), using a solvent (such as t-BuOH, DMSO, acetone, *etc.*).

[00181] A compound of formula (T1) may be reacted with an aryl amine of formula (C) (PG are protecting groups such as OMe, OBn, *etc.*) to give a compound of formula (U1). The reaction may be performed in the presence of a base (typically an organic base such as DIPEA, *etc.*) or acid (typically a catalytic amount of concentrated. HCl) using a solvent (such as t-BuOH, t-amyl alcohol, DMSO, *etc.*), and at temperatures typically ranging from 80°C to 120°C.

[00182] A compound of formula (U1) may be reacted with malononitrile of formula (E) to give a compound of formula (V1). The reaction may be catalyzed with a suitable Pd-catalyst (such as $\text{PdCl}_2(\text{dppf})$, *etc.*) in the presence of a base (such as NaOtBu, *etc.*) using a suitable solvent (such as DME, *etc.*), and at temperatures typically ranging from 80°C to 100°C.

[00183] A compound of formula (V1) may be reacted with concentrated sulfuric acid to give a compound of formula (W1). The protecting group in the compound of formula (W1) may be removed under appropriate reaction conditions to give a compound of Formula (7).

SCHEME 10



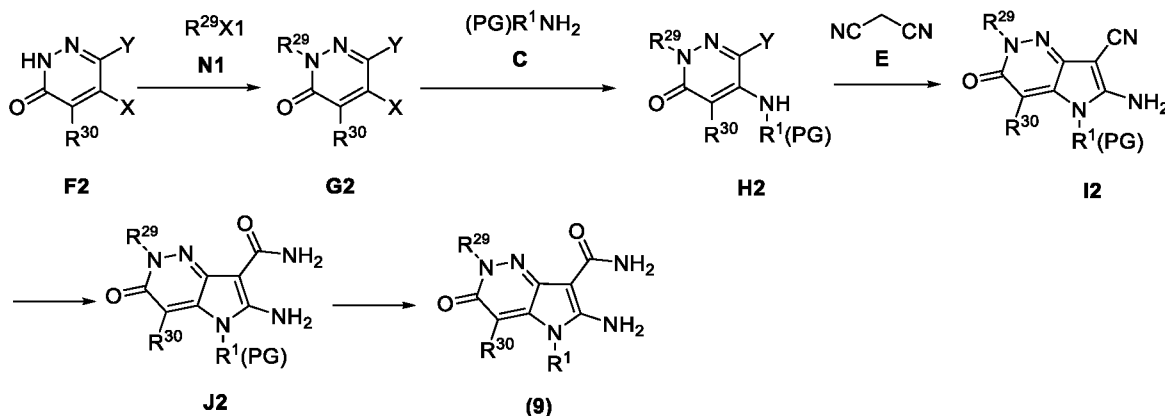
[00184] Scheme 10 illustrates synthetic routes to certain compounds of Formula (1). A compound of formula (A2) may be reacted with a compound of formula (N1) (X1 is typically a leaving group like iodo, bromo, chloro, or triflate) to give a compound of formula (B2). The reaction may be performed in the presence of a base (typically an inorganic base such as K_2CO_3 , *etc.*) or catalyzed with a suitable Pd-catalyst (such as BrettPhos Pd G3, *etc.*) in the presence of a base (such as Cs_2CO_3 , *etc.*), using a solvent (such as *t*-BuOH, DMSO, acetone, *etc.*).

[00185] A compound of formula (B2) may be reacted with an aryl amine of formula (C) (PG are protecting groups such as OMe, OBn, *etc.*) to give a compound of formula (C2). The reaction may be performed in the presence of a base (typically an organic base such as DIPEA, *etc.*) or acid (typically a catalytic amount of concentrated HCl) using a solvent (such as *t*-BuOH, *t*-amyl alcohol, DMSO, *etc.*), and at temperatures typically ranging from 80°C to 120°C.

[00186] A compound of formula (C2) may be reacted with malononitrile of formula (E) to give a compound of formula (D2). The reaction may be catalyzed with a suitable Pd-catalyst (such as $\text{PdCl}_2(\text{dppf})$, *etc.*) in the presence of a base (such as NaOtBu, *etc.*) using a suitable solvent (such as DME, *etc.*), and at temperatures typically ranging from 80°C to 100°C.

[00187] A compound of formula (D2) may be reacted with concentrated sulfuric acid to give a compound of formula (E2). The protecting group in the compound of formula (E2) may be removed under appropriate reaction conditions to give a compound of Formula (8).

SCHEME 11



[00188] Scheme 11 illustrates synthetic routes to certain compounds of Formula (1). A compound of formula (F2) may be reacted with a compound of formula (N1) (X^1 is typically a leaving group like iodo, bromo, chloro, or triflate) to give a compound of formula (G2). The reaction may be performed in the presence of a base (typically an inorganic base such as K_2CO_3 , *etc.*) or catalyzed with a suitable Pd-catalyst (such as BrettPhos Pd G3, *etc.*) in the presence of a base (such as Cs_2CO_3 , *etc.*), using a solvent (such as *t*-BuOH, DMSO, acetone, *etc.*).

[00189] A compound of formula (G2) may be reacted with an aryl amine of formula (C) (PG are protecting groups such as OMe, OBn, *etc.*) to give a compound of formula (H2). The reaction may be performed in the presence of a base (typically an organic base such as DIPEA, *etc.*) or acid (typically a catalytic amount of concentrated HCl) using a solvent (such as *t*-BuOH, *t*-amyl alcohol, DMSO, *etc.*), and at temperatures typically ranging from 80°C to 120°C.

[00190] A compound of formula (H2) may be reacted with malononitrile of formula (E) to give a compound of formula (I2). The reaction may be catalyzed with a suitable Pd-catalyst (such as $PdCl_2(dppf)$, *etc.*) in the presence of a base (such as NaOtBu, *etc.*) using a suitable solvent (such as DME, *etc.*), and at temperatures typically ranging from 80°C to 100°C.

[00191] A compound of formula (I2) may be reacted with concentrated sulfuric acid to give a compound of formula (J2). The protecting group in the compound of formula (J2) may be removed under appropriate reaction conditions to give a compound of Formula (9).

[00192] It should be understood that: (i) the organic reactions described in this disclosure are performed according to laboratory practices known to a person skilled in the art; (ii) some of the reactions described in this disclosure may optionally be performed in different orders than laid out herein; (iii) chiral isomers of compounds in this disclosure can be resolved at any stage in the

synthetic process using chiral resolving agents described in the literature and known to a person skilled in the art, or using chiral chromatography methods described in the literature and known to a person skilled in the art, or as described further in the Examples; (iv) additional and/or other protective groups may optionally be needed in some of the steps described above; and (v) a deprotection step therefore optionally may be performed, using methods described in the literature and known to a person skilled in the art. The protection and deprotection of functional groups are described in “Protective Groups in Organic Synthesis” 3rd Ed, T.W. Greene and P.G.M. Wutz, Wiley-Interscience (1999), which publication is incorporated herein by reference.

VIII. Examples

[00193] The following descriptions of experiments, procedures, examples, and intermediates are intended to exemplify embodiments of the disclosure and are in no way intended to be limiting. Other compounds of this disclosure may be prepared using the methods illustrated in these examples, either alone or in combination with techniques generally known in the art.

A. General Conditions

[00194] Unless otherwise stated:

[00195] ¹H NMR spectra were obtained using a Bruker 300 MHz, 400 MHz or 500 MHz spectrometer at 27 °C unless otherwise noted; chemical shifts are expressed in parts per million (ppm, δ units) and are referenced to the residual mono-¹H isotopologue of the solvent (CHCl₃: 7.24 ppm; CHDCl₂: 5.32 ppm; CD₃S(=O)CD₂H: 2.49 ppm). Coupling constants are given in units of hertz (Hz). Splitting patterns describe apparent multiplicities and are designated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br s (broad singlet). LC-MS was carried out using a Waters UPLC fitted with a Waters SQD mass spectrometer or Shimadzu LC-20AD LC-20XR LC-30AD with a Shimadzu 2020 mass spectrometer. Reported molecular ions correspond to [M+H]⁺ unless otherwise noted; for molecules with multiple isotopic patterns (Br, Cl, *etc.*) the reported value is the one obtained for the lowest isotope mass unless otherwise specified.

[00196] Flash chromatography was performed using straight phase flash chromatography on a SP1TM Purification system from BiotageTM, CombiFlash[®]Rf from ISCO or on Gilson system from Thermo Fisher using normal phase silica FLASH⁺TM (40M, 25M or 12 M) or SNAPTM KP-Sil Cartridges (340, 100, 50 or 10), Flash Column silica-CS columns from Agela, with C18-flash columns or standard flash chromatography. In general, all solvents used were commercially

available and of analytical grade. Anhydrous solvents were routinely used for reactions. Phase Separators used in the examples are ISOLUTE® Phase Separator columns. The starting materials were obtained from commercial sources or made via literature routes.

[00197] The following abbreviations are used:

ACN = Acetonitrile;

BrettPhos = 2-(Dicyclohexylphosphino)3,6-dimethoxy-2',4',6'-triisopropyl-1,1'-biphenyl;

BrettPhos Pd G3 = [(2-Di-cyclohexylphosphino-3,6-dimethoxy-2',4',6'- triisopropyl-1,1'-biphenyl)-2-(2'-amino-1,1' -biphenyl)]palladium(II) methanesulfonate methanesulfonate;

CPhos = 2-Dicyclohexylphosphino-2',6'-bis(N,N-dimethylamino)biphenyl;

CPhos Pd G3 = [(2-Dicyclohexylphosphino-2',6'-bis(N,N-dimethylamino) -1,1'-biphenyl)-2-(2'-amino-1,1'-biphenyl)] palladium(II) methanesulfonate;

BSA = Bovine serum albumin;

Cs₂CO₃ = cesium carbonate;

DCM = dichloromethane;

DEA = diethylamine;

DIPEA = diisopropyl ethylamine;

DME = dimethoxyethane;

DMSO = dimethylsulfoxide;

DMSO-d₆ = deuterated dimethylsulfoxide;

dppf = 1,1'-bis(diphenylphosphino)ferrocene;

EA = ethylacetate;

HBSS = Hanks' Balanced Salt Solution;

HCl = hydrochloric acid;

HP = high pressure;

H₂SO₄ = sulfuric acid;

IPA = isopropylalcohol;

IPTG = Isopropyl β-D-1-thiogalactopyranoside;

K₂HPO₄ = potassium hydrogen phosphate;

K₃PO₄ = potassium phosphate;

LC = liquid chromatography;

LiHMDS = lithium bis(trimethylsilyl)amide;

2-Me THF = 2-methyltetrahydrofuran;

MeCN = acetonitrile;
MeOH = methanol;
MgSO₄ = magnesium sulfate;
MS = mass spectrometry;
NaHCO₃ = sodium bicarbonate;
NaOtBu = sodium tert-butoxide,
NH₄HCO₃ = ammonium bicarbonate;
NMR = nuclear magnetic resonance;
NaOH = sodium hydroxide;
OBn = benzyloxy
OMe = methoxy;
PBS = Phosphate buffered saline;
Pd = palladium;
PdCl₂(dppf) = 1,1'-bis(di-tert-butylphosphino)ferrocene palladium dichloride;
Pd₂dba₃ = Tris(dibenzylideneacetone)dipalladium (0);
Pd-PEPPSI-iHeptCl = dichloro[1,3-bis(2,6-di-4-heptylphenyl)imidazol-2-ylidene](3-chloropyridyl)palladium(II);
PMSF = Phenylmethylsulfonyl fluoride;
PFA = Paraformaldehyde;
RockPhos Pd G3 = [(2-Di-tert-butylphosphino-3-methoxy-6-methyl-2',4',6'-triisopropyl-1,1'-biphenyl)-2-(2-aminobiphenyl)]palladium(II) methanesulfonate;
Rt or RT = retention time;
RU = Response units;
tBuOH = tert-butanol;
TCEP = Tris(2-carboxyethyl)phosphine;
Xantphos = 4,5-bis(diphenylphosphino)-9,9-dimethylxanthene; and
XPhos = 2-Dicyclohexylphosphino-2',4',6'-triisopropylbiphenyl.

[00198] Examples and Intermediate compounds are named using ChemDraw Professional version 21.0.0 from PerkinElmer. ChemDraw Professional version 21.0.0 generates the names of chemical structures using the Cahn-Ingold-Prelog (CIP) rules for stereochemistry and follows IUPAC rules as closely as possible when generating chemical names. Stereoisomers are differentiated from each other by stereodescriptors cited in names and assigned in accordance

with the CIP rules.

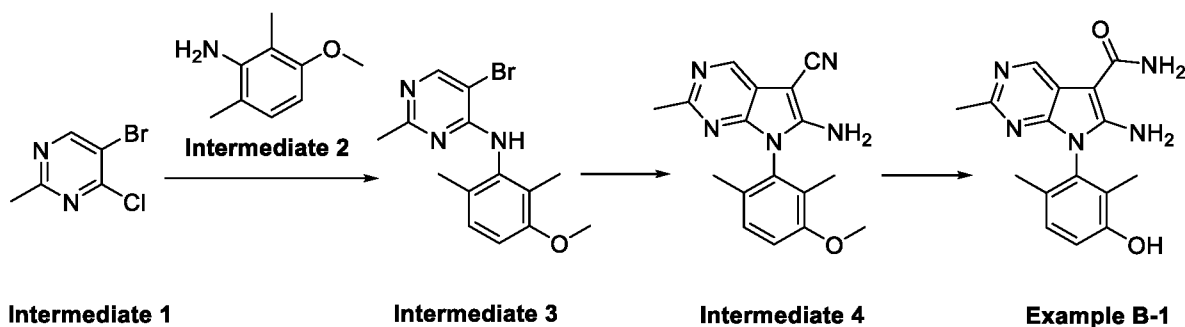
[00199] ChemDraw is optionally using labels in the graphical representation of stereocenters such as ‘&’ and ‘or’ to describe the configuration of the stereochemical centers present in the structure. In general, chemical structures of Examples and Intermediates containing the label ‘&’ at a stereocenter, means the configuration of such Example or Intermediate at that stereocenter is a mixture of both (*R*) and (*S*); and a label ‘or’ means the configuration of such Example or Intermediate at the stereocenter is either (*R*) or (*S*). Absolute, unspecified, ‘&’, and ‘or’ stereocenters can all be present in a single structure.

[00200] In general for structures of Examples and Intermediates where all of the stereocenters are designated as ‘&’, the structure is named with a “rac-” prefix. For structures of Examples and Intermediates where all of the stereocenters are designated as ‘or’, the structure is named with a “rel-” prefix.

[00201] In general Examples and Intermediate compounds are named using the descriptors (*RS*) and (*SR*) to denote general ‘&’ centers for chemical structures with multiple chiral centers where only some are designated as ‘&’. The descriptors (*R**) and (*S**) are used to denote the general ‘or’ centers for chemical structures with multiple chiral centers where only some are designated as ‘or’.

B. Compounds

Example B-1: (*S*)-6-Amino-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-*d*]pyrimidine-5-carboxamide (Compound 1)



Step a) 5-Bromo-N-(3-methoxy-2,6-dimethylphenyl)-2-methylpyrimidin-4-amine (Intermediate 3)

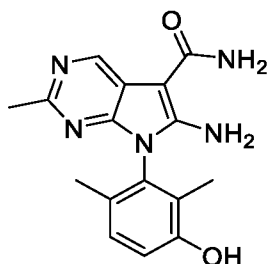
[00202] Concentrated HCl (0.055 mL, 1.81 mmol) was added to 5-bromo-4-chloro-2-methylpyrimidine (**Intermediate 1**, 1.5 g, 7.23 mmol) and 3-methoxy-2,6-dimethylaniline (**Intermediate 2**, 1.093 g, 7.23 mmol) in tBuOH (20 mL). The resulting mixture was stirred at

100 °C for 16 hours. The solvent was removed under reduced pressure. The crude product was purified by flash silica chromatography, elution gradient 0 to 20% MeOH in DCM, further purified by flash C18-flash chromatography, elution gradient 0 to 100% MeCN in water (0.1% NH₄HCO₃). Pure fractions were evaporated to dryness to afford 5-bromo-N-(3-methoxy-2,6-dimethylphenyl)-2-methylpyrimidin-4-amine (**Intermediate 3**, 1.800 g, 77 %) as a yellow solid. ¹H NMR (DMSO-d₆, 300 MHz) δ 1.94 (3H, s), 2.04 (3H, s), 2.38 (3H, s), 3.80 (3H, s), 6.93 (1H, d), 7.13 (1H, d), 8.81 (1H, s), 10.06 (1H, br s); m/z (ES⁺) [M+H]⁺ = 322.

Step b) 6-Amino-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile (Intermediate 4)

[00203] Sodium t-butoxide (0.597 g, 6.21 mmol) was added slowly to a solution of malononitrile (0.431 g, 6.52 mmol) in DME (15 mL). The mixture was stirred at 23 °C for 30 minutes. 5-bromo-N-(3-methoxy-2,6-dimethylphenyl)-2-methylpyrimidin-4-amine (**Intermediate 3**, 1.000 g, 3.10 mmol) and [1,1'-bis(diphenylphosphino)ferrocene]dichloropalladium(II) (0.227 g, 0.31 mmol) were added to the mixture under nitrogen. The resulting mixture was stirred at 90 °C for 16 hours. The solvent was removed under reduced pressure. The crude product was purified by flash silica chromatography, elution gradient 0 to 10% MeOH in DCM. Pure fractions were evaporated to dryness to afford 6-amino-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile (**Intermediate 4**, 0.670 g, 70.2 %) as a red solid. ¹H NMR (DMSO-d₆, 300 MHz) δ 1.71 (3H, s), 1.80 (3H, s), 2.44 (3H, s), 3.85 (3H, s), 7.11 (1H, d), 7.27 (3H, m), 8.49 (1H, s); m/z (ES⁺) [M+H]⁺ = 308.

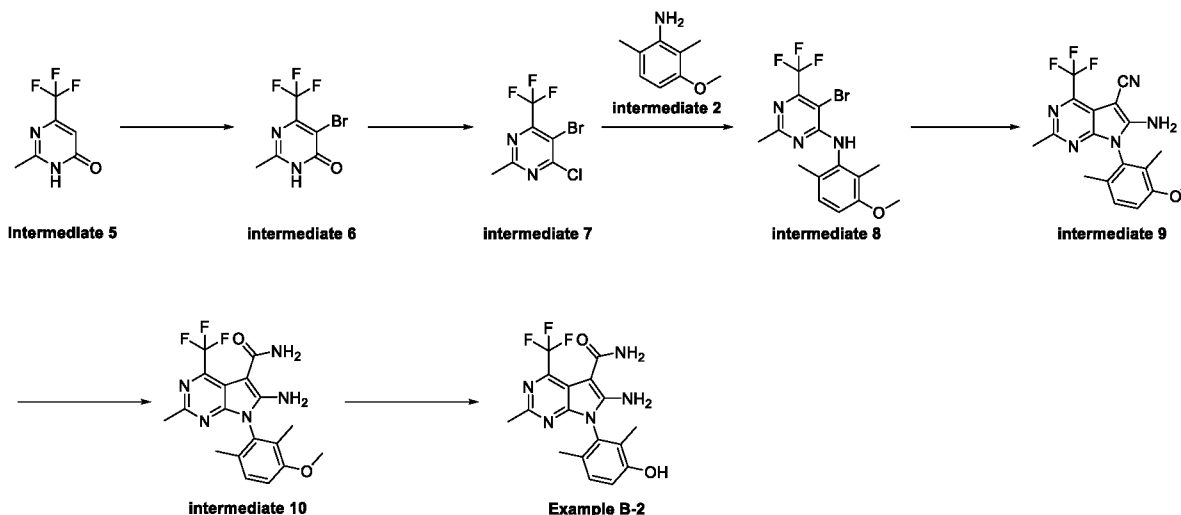
Step c) (S)-6-Amino-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (Compound 1)



[00204] Methanesulfonic acid (4521 mg, 47.05 mmol) was added dropwise to a solution of H₂SO₄ (7.5 mL, 140.71 mmol) in water (0.5 mL). The mixture was stirred at 23°C for 5 minutes. 6-amino-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-

carbonitrile (**Intermediate 4**, 570 mg, 1.85 mmol) was added slowly to the stirred solution below 40°C. The mixture was stirred at room temperature for 90 minutes. DL-methionine (1107 mg, 7.42 mmol) was added slowly to the stirred mixture below 40°C. The mixture was stirred for 15 minutes below 40°C. The resulting mixture was then stirred at 40°C for 16 hours. The mixture was cooled to room temperature and added slowly to an aqueous solution of K₂HPO₄ (0.7 g) and NaOH (3.4 g) in water (30 mL). EA (50 mL) was added and the mixture was stirred for 5 minutes to get a precipitate. The precipitation was collected by filtration. The crude product was purified by preparative HPLC Column: XBridge Prep OBD C18 Column, 30*150 mm, 5µm; Mobile Phase A: Water (10 mmol/L NH₄HCO₃ + 0.05% NH₃H₂O), Mobile Phase B: ACN; Flow rate: 60 mL/min mL/min; Gradient: 5% B to 30% B in 8 min. Fractions containing the desired compound were evaporated to dryness to afford 6-amino-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide as a white solid. The racemic mixture was purified by preparative chiral-HPLC on a column: LuxCellulose-44.6*50mm, 3µm; Mobile Phase A: CO₂, Mobile Phase B: MeOH (0.1% DEA); Flow rate: 4 mL/min; Gradient: isocratic % B. The fractions containing the desired compound were evaporated to dryness to afford (*S*)-6-amino-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (**Example B-1, Isomer 1**, 105 mg, 17.12 %) as a white solid and (*R*)-6-amino-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (**Example B-1, Isomer 2**, 106 mg, 17.47 %) as a white solid. ¹H NMR (400 MHz, DMSO-d₆, 24°C) δ 1.67 (3H, s), 1.76 (3H, s), 2.44 (3H, s), 6.84 (2H, s), 6.94 (1H, d), 7.05 (2H, s), 7.08 (1H, d), 8.88 (1H, s), 9.67 (1H, s); m/z (ES⁺) [M+H]⁺ = 312.

Example B-2: (S)- or (R)-6-amino-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (Compound 137)



Intermediate 6: 5-bromo-2-methyl-6-(trifluoromethyl)pyrimidin-4(3H)-one

[00205] Azobisisobutyronitrile (AIBN) (0.184 g, 1.12 mmol) was added to 2-methyl-6-(trifluoromethyl)pyrimidin-4(3H)-one (**Intermediate 5**, 2.00 g, 11.2 mmol) and NBS (2.398 g, 13.47 mmol) in MeCN (50 mL). The resulting mixture was stirred at 80 °C for 3 hours. The solvent was removed under reduced pressure. The crude product was purified by flash silica chromatography, elution gradient 0 to 20% EtOAc in DCM. Pure fractions were evaporated to dryness to afford 5-bromo-2-methyl-6-(trifluoromethyl)pyrimidin-4(3H)-one (**Intermediate 6**, 2.40 g, 83%) as a white solid. ¹H NMR (300 MHz, DMSO-d₆, 25 °C) δ 2.3 (3H, s), 13.46 (1H, s); m/z (ES⁺) [M+H]⁺ = 257.

Intermediate 7: 5-bromo-4-chloro-2-methyl-6-(trifluoromethyl)pyrimidine

[00206] Phosphorus oxychloride (3.58 g, 23.4 mmol) was added to 5-bromo-2-methyl-6-(trifluoromethyl)-pyrimidin-4(3H)-one (**Intermediate 6**, 2.00 g, 7.78 mmol) in MeCN (20 mL). The resulting solution was stirred at 80 °C for 16 hours. The solvent was removed under reduced pressure. The crude product was purified by flash silica chromatography, elution gradient 0 to 30% EtOAc in DCM. Pure fractions were evaporated to dryness to afford 5-bromo-4-chloro-2-methyl-6-(trifluoromethyl)pyrimidine (**Intermediate 7**, 0.75 g, 35%) as a colourless liquid. ¹H NMR (300 MHz, DMSO-d₆, 23 °C) δ 2.65 (3H, s).

Intermediate 8: 5-bromo-N-(3-methoxy-2,6-dimethylphenyl)-2-methyl-6-(trifluoromethyl)pyrimidin-4-amine

[00207] 3-Methoxy-2,6-dimethylaniline (**Intermediate 2**, 601 mg, 3.98 mmol) was added to 5-bromo-4-chloro-2-methyl-6-(trifluoromethyl)pyrimidine (**Intermediate 7**, 730 mg, 2.65 mmol). The resulting mixture was stirred at 140 °C for 16 hours. The reaction mixture was purified by flash silica chromatography, elution gradient 0 to 30% EtOAc (containing 50% DCM) in petroleum ether. Pure fractions were evaporated to dryness to afford 5-bromo-N-(3-methoxy-2,6-dimethylphenyl)-2-methyl-6-(trifluoromethyl)pyrimidin-4-amine (**Intermediate 8**, 780 mg, 75 %) as a colourless liquid. ¹H NMR (300 MHz, DMSO-d₆, 24 °C) δ 1.99 (6H, d), 2.26 (3H, s), 3.80 (3H, s), 6.89 (1H, d), 7.11 (1H, d), 9.11 (1H, s); m/z (ES⁺) [M+H]⁺ = 390.

Intermediate 9: 6-amino-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile

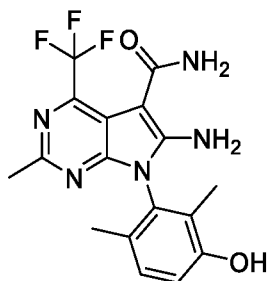
[00208] Sodium t-butoxide (517 mg, 5.38 mmol) was added slowly to a solution of malononitrile (284 mg, 4.31 mmol) in DME (10 mL). The mixture was stirred at r.t. for 30 minutes. 5-Bromo-N-(3-methoxy-2,6-dimethylphenyl)-2-methyl-6-(trifluoromethyl)pyrimidin-4-amine (**Intermediate 8**, 840 mg, 2.15 mmol) and [1,1'-bis(diphenylphosphino)ferrocene] dichloropalladium (II) (158 mg, 0.220 mmol) was added to the mixture. The resulting mixture was stirred at 80 °C for 16 hours. The reaction mixture was filtered. The solvent was removed under the reduced pressure. The crude product was purified by flash silica chromatography, elution gradient 0 to 80% EtOAc (containing 50% DCM) in petroleum ether. Pure fractions were evaporated to dryness to afford 6-amino-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile (**Intermediate 9**, 650 mg, 80%) as a yellow solid. ¹H NMR (400 MHz, DMSO-d₆, 24 °C) δ 1.72 (3H, s), 1.81 (3H, s), 2.52 (3H, s), 3.86 (3H, s), 7.14 (1H, d), 7.28 (1H, d), 7.80 (2H, s); m/z (ES⁺) [M+H]⁺ = 376.

Intermediate 10: 6-amino-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide

[00209] 6-Amino-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile (**Intermediate 9**, 630 mg, 1.68 mmol) was added to sulfuric acid (6 mL). The resulting solution was stirred at r.t. for 1 day. The reaction mixture was poured into water (50 mL), basified with ammonia and then extracted with EtOAc (3 x 50 mL). The organic layer was dried over MgSO₄, filtered and evaporated to afford crude 6-amino-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-

carboxamide (**Intermediate 10**, 650 mg, 98%) as a yellow solid, which was used without further purification. ^1H NMR (300 MHz, DMSO- d_6 , 23°C) δ 1.70 (3H, s), 1.80 (3H, s), 2.47 (3H, s), 3.85 (3H, s), 6.76 (2H, s), 6.94 (2H, s), 7.12 (1H, d), 7.26 (1H, d); m/z (ES^+) $[\text{M}+\text{H}]^+ = 394$.

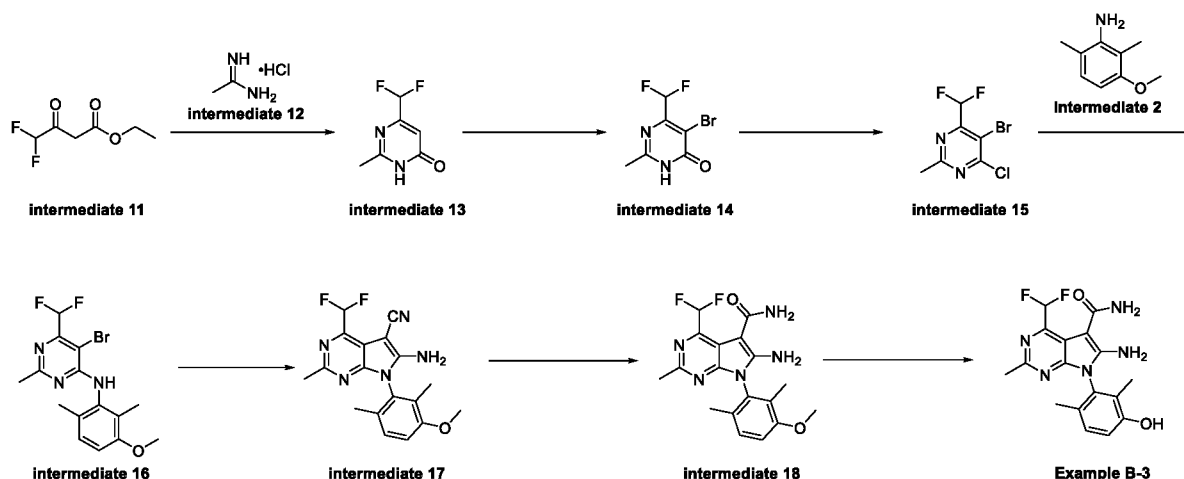
(S)- or (R)-6-amino-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (Compound 137)



[00210] BBR_3 in DCM (3.05 mL, 3.05 mmol) was added dropwise to 6-amino-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (**Intermediate 10**, 300 mg, 0.760 mmol) in DCM (5 mL) at 0 °C. The resulting mixture was stirred at r.t. for 3 hours. The solvent was removed under reduced pressure. The crude product was purified by flash C18-flash chromatography, elution gradient 0 to 60% MeCN in water modified with 0.1% NH_4HCO_3 . Pure fractions were evaporated to dryness to afford (rac)-6-amino-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (210 mg, 73%) as a white solid.

[00211] The racemic mixture was purified by preparative chiral-HPLC on a column: CHIRALPAK IC, 2*25 cm, 5 μm ; Mobile Phase A: HEX (0.5% 2M NH_3 -MeOH), Mobile Phase B: EtOH; Flow rate: 20 mL/min; Gradient: isocratic 7; Wave Length: 220/254 nm; RT1(min): 18.873; RT2(min): 22.707; Sample Solvent: EtOH; Injection Volume: 0.4 mL; Number Of Runs: 22. The fractions containing the desired compound were evaporated to dryness to afford rotational isomer 1 and rotational isomer 2, (S)- or (R)-6-amino-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (**Example B-2**, 167 mg, 34%) as a white solid. ^1H NMR (400 MHz, DMSO- d_6 , 23 °C) δ 1.68 (3H, s), 1.77 (3H, s), 2.49 (3H, s), 6.75 (2H, s), 6.90-7.00 (3H, m), 7.10 (1H, d), 9.64 (1H, s); m/z (ES^+) $[\text{M}+\text{H}]^+ = 380$.

Example B-3: (S)- or (R)-6-amino-4-(difluoromethyl)-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (Compound 138)



Intermediate 13: 6-(difluoromethyl)-2-methylpyrimidin-4(3H)-one

[00212] A mixture of sodium ethoxide (3.07 g, 45.2 mmol) in ethanol (50.0 mL) was added to a stirred mixture of ethyl 4,4-difluoro-3-oxobutanoate (**Intermediate 11**, 5.00 g, 30.1 mmol) and acetimidamide hydrochloride (**Intermediate 12**, 2.85 g, 30.1 mmol) in toluene (100 mL) at 25 °C. The resulting mixture was stirred at 80 °C for 16 hours. The solvent was removed under reduced pressure. The crude product was purified by flash C18-flash chromatography, elution gradient 0 to 40% MeOH in water (0.1% NH_4HCO_3). Pure fractions were evaporated to dryness to afford 6-(difluoromethyl)-2-methylpyrimidin-4(3H)-one (**Intermediate 13**, 1.8 g, 37%) as a white solid. ^1H NMR (300 MHz, $\text{DMSO}-d_6$, 26 °C) δ 2.33 (3H, s), 6.42 (1H, s), 6.67 (1H, t), 12.77 (1H, s); m/z (ES^+) $[\text{M}+\text{H}]^+ = 161$.

Intermediate 14: 5-bromo-6-(difluoromethyl)-2-methylpyrimidin-4(3H)-one

[00213] AIBN (0.185 g, 1.12 mmol) was added to 6-(difluoromethyl)-2-methylpyrimidin-4(3H)-one (**Intermediate 13**, 1.80 g, 11.2 mmol) and NBS (1.20 g, 6.74 mmol) in MeCN (50 mL). The resulting mixture was stirred at 80 °C for 3 hours. The solvent was removed under reduced pressure. The crude product was purified by flash silica chromatography, elution gradient 0 to 100% EtOAc (containing 50% DCM) in petroleum ether. Pure fractions were evaporated to dryness to afford 5-bromo-6-(difluoromethyl)-2-methylpyrimidin-4(3H)-one (**Intermediate 14**, 1.4 g, 52%) as a white solid. ^1H NMR (500 MHz, $\text{DMSO}-d_6$, 25 °C) δ 2.34 (3H, s), 6.99 (1H, t), 13.25 (1H, s); m/z (ES^+) $[\text{M}+\text{H}]^+ = 239$.

Intermediate 15: 5-bromo-4-chloro-6-(difluoromethyl)-2-methylpyrimidine

[00214] Phosphorus oxychloride (8.34 g, 54.4 mmol) was added to 5-bromo-6-(difluoromethyl)-2-methylpyrimidin-4(3H)-one (**Intermediate 14**, 1.30 g, 5.44 mmol) in MeCN (30 mL). The resulting solution was stirred at 80 °C for 16 hours. The solvent was removed under reduced pressure. The crude product was purified by flash silica chromatography, elution gradient 0 to 50% EtOAc in DCM. Pure fractions were evaporated to dryness to afford 5-bromo-4-chloro-6-(difluoromethyl)-2-methylpyrimidine (**Intermediate 15**, 1.1 g, 79%) as a colourless liquid. ¹H NMR (300 MHz, DMSO-d₆, 25 °C) δ 2.66 (3H, s), 7.17 (1H, t); m/z (ES⁺) [M+H]⁺ = 257.

Intermediate 16: 5-bromo-6-(difluoromethyl)-N-(3-methoxy-2,6-dimethylphenyl)-2-methylpyrimidin-4-amine

[00215] 5-Bromo-4-chloro-6-(difluoromethyl)-2-methylpyrimidine (**Intermediate 15**, 1.00 g, 3.88 mmol) was added to 3-methoxy-2,6-dimethylaniline (**Intermediate 2**, 0.705 g, 4.66 mmol). The resulting mixture was stirred at 140 °C for 16 hours. The crude product was purified by flash silica chromatography, elution gradient 0 to 60% EtOAc in petroleum ether. Pure fractions were evaporated to dryness to afford 5-bromo-6-(difluoromethyl)-N-(3-methoxy-2,6-dimethylphenyl)-2-methylpyrimidin-4-amine (**Intermediate 16**, 0.70 g, 48%) as a yellow solid. ¹H NMR (400 MHz, DMSO-d₆, 23 °C) δ 1.93 (3H, s), 2.02 (3H, s), 2.24 (3H, s), 3.78 (3H, s), 6.87 (1H, d), 7.02 (1H, t), 7.09 (1H, d), 8.89 (1H, s); m/z (ES⁺) [M+H]⁺ = 372.

Intermediate 17: 6-amino-4-(difluoromethyl)-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile

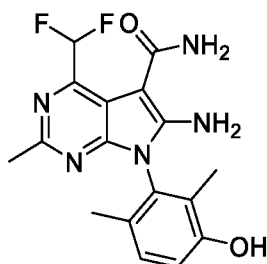
[00216] Sodium t-butoxide (452 mg, 4.70 mmol) was added slowly to a solution of malononitrile (248 mg, 3.76 mmol) in DME (10 mL). The mixture was stirred at r.t. for 30 minutes. 5-Bromo-6-(difluoromethyl)-N-(3-methoxy-2,6-dimethylphenyl)-2-methylpyrimidin-4-amine (**Intermediate 16**, 700 mg, 1.88 mmol) and [1,1'-bis(diphenylphosphino)ferrocene]-dichloropalladium(II) (138 mg, 0.190 mmol) was added to the mixture. The resulting mixture was stirred at 80 °C for 2 hours. The reaction mixture was poured into saturated NaHCO₃ (50 mL), extracted with EtOAc (3 x 50 mL), the organic layer was dried over Na₂SO₄, filtered and evaporated to afford yellow solid. The crude product was purified by flash silica chromatography, elution gradient 0 to 60% EtOAc in petroleum ether. Pure fractions were evaporated to dryness to afford 6-amino-4-(difluoromethyl)-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile (**Intermediate 17**, 570 mg, 85%) as a

yellow solid. ^1H NMR (300 MHz, DMSO- d_6 , 24 °C) δ 1.76 (6H, d), 2.48 (3H, s), 3.85 (3H, s), 7.04 (1H, s), 7.12 (1H, d), 7.26 (1H, d), 7.59 (2H, s); m/z (ES^+) $[\text{M}+\text{H}]^+ = 358$.

Intermediate 18: 6-amino-4-(difluoromethyl)-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide

[00217] 6-Amino-4-(difluoromethyl)-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile (**Intermediate 17**, 520 mg, 1.46 mmol) was added to sulfuric acid (6 mL). The resulting solution was stirred at r.t. for 1 day. The reaction mixture was poured into water (50 mL), basified with ammonia and then extracted with EtOAc (3 x 50 mL). The organic layer was dried over MgSO_4 , filtered and evaporated to afford crude 6-amino-4-(difluoromethyl)-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (**Intermediate 18**, 500 mg, 92%) as a yellow solid, which was used without further purification. ^1H NMR (300 MHz, DMSO- d_6 , 24 °C) δ 1.71 (3H, s), 1.81 (3H, s), 2.48 (3H, s), 3.87 (3H, s), 6.68 (2H, s), 7.05 (2H, s), 7.13 (1H, d), 7.28 (1H, d), 7.73 (1H, s); m/z (ES^+) $[\text{M}+\text{H}]^+ = 376$.

(S)- or (R)-6-amino-4-(difluoromethyl)-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (Compound 138)

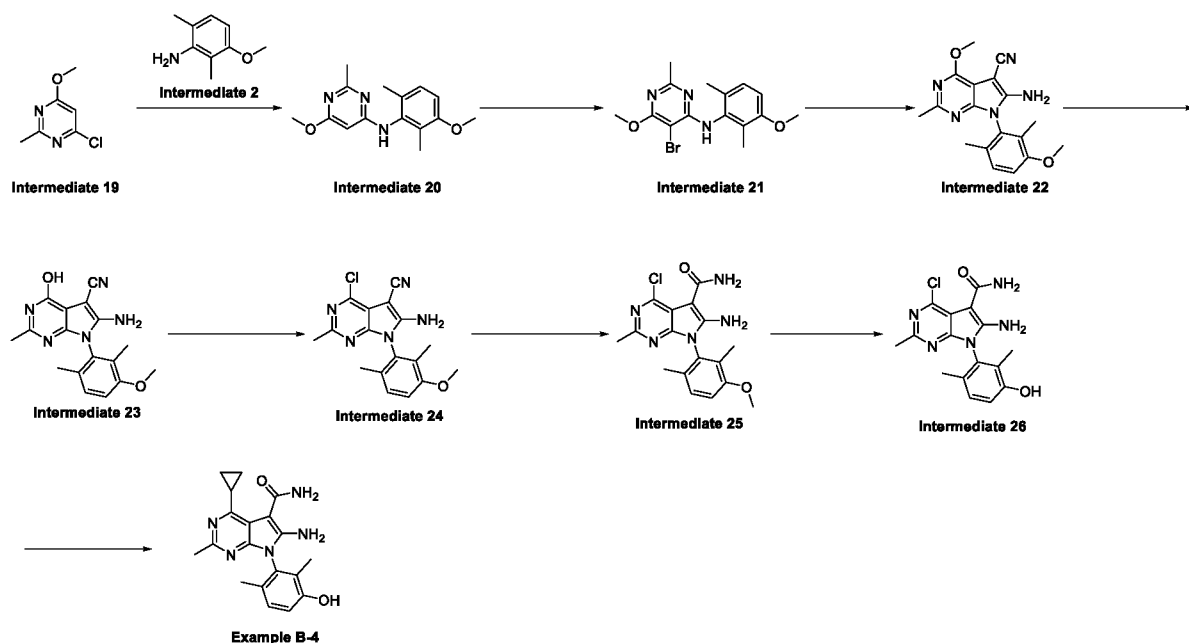


[00218] BBR_3 in DCM (3.20 mL, 3.20 mmol) was added dropwise to 6-amino-4-(difluoromethyl)-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (**Intermediate 18**, 300 mg, 0.80 mmol) in DCM (2 mL) at 0 °C. The resulting mixture was stirred at r.t. for 3 hours. The solvent was removed under reduced pressure. The crude product was purified by flash C18-flash chromatography, elution gradient 0 to 60% MeCN in water modified with 0.1% NH_4HCO_3 . Pure fractions were evaporated to dryness to afford (rac)-6-amino-4-(difluoromethyl)-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (90 mg, 31%) as a white solid.

[00219] The racemic mixture was purified by preparative chiral-HPLC (Column: CHIRALPAK IE, 2*25 cm, 5 μm ; Mobile Phase A: Hex (0.5% 2M NH_3 -MeOH), Mobile Phase B: EtOH; Flow rate: 20 mL/min; Gradient: isocratic 10; Wave Length: 220/254 nm; RT1(min):

17.882; RT2(min): 24.125; Sample Solvent: EtOH; Injection Volume: 0.4 mL; Number Of Runs: 13) to afford rotational isomer 1 and rotational isomer 2, (S) or (R)-6-amino-4-(difluoromethyl)-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (**Example B-3**, 17.40 mg, 17.40 %) as a yellow solid. ^1H NMR (400 MHz, DMSO- d_6 , 23 °C) δ 1.68 (3H, s), 1.77 (3H, s), 2.49 (3H, s), 6.65 (2H, s), 6.96 (1H, d), 7.00–7.14 (3H, m), 7.74 (1H, t), 9.63 (1H, s); m/z (ES^+) $[\text{M}+\text{H}]^+ = 362$.

Example B-4: (S)- or (R)-6-amino-4-cyclopropyl-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (Compound 139)



Intermediate 20: 6-methoxy-N-(3-methoxy-2,6-dimethylphenyl)-2-methylpyrimidin-4-amine

[00220] Xantphos Pd G3 (4.49 g, 4.73 mmol) was added to 4-chloro-6-methoxy-2-methylpyrimidine (**Intermediate 19**, 15.0 g, 94.6 mmol), 3-methoxy-2,6-dimethylaniline (**Intermediate 2**, 15.73 g, 104.1 mmol) and Cs_2CO_3 (92.0 g, 284 mmol) in 1,4-dioxane (300 mL) under nitrogen. The resulting mixture was stirred at 100 °C for 2 hours. The solvent was removed under reduced pressure. The crude product was purified by flash silica chromatography, elution gradient 0 to 40% EtOAc in petroleum ether. Pure fractions were evaporated to dryness to afford 6-methoxy-N-(3-methoxy-2,6-dimethylphenyl)-2-methylpyrimidin-4-amine (**Intermediate 20**, 17 g, 66%) as a pale yellow solid. ^1H NMR (300 MHz, CDCl_3 , 25 °C) δ 2.11 (3H, s), 2.17 (3H, s), 2.45 (3H, s), 3.84 (3H, s), 3.85 (3H, s), 5.08 (1H, s), 6.72 (1H, s), 6.79 (1H, d), 7.09 (1H, d); m/z (ES^+) $[\text{M}+\text{H}]^+ = 274$.

Intermediate 21: 5-bromo-6-methoxy-N-(3-methoxy-2,6-dimethylphenyl)-2-methylpyrimidin-4-amine

[00221] N-Bromosuccinimide (9.77 g, 54.9 mmol) was added to 6-methoxy-N-(3-methoxy-2,6-dimethylphenyl)-2-methylpyrimidin-4-amine (**Intermediate 20**, 15.0 g, 54.9 mmol) in DCM (150 mL) at 25 °C. The resulting solution was stirred at 0 °C for 1 hour. The solvent was removed under reduced pressure. The crude product was purified by flash silica chromatography, elution gradient 0 to 30% EtOAc in petroleum ether. Pure fractions were evaporated to dryness to afford 5-bromo-6-methoxy-N-(3-methoxy-2,6-dimethylphenyl)-2-methylpyrimidin-4-amine (**Intermediate 21**, 15 g, 78%) as a pale yellow solid. ¹H NMR (300 MHz, CDCl₃, 25 °C) δ 2.10 (3H, s), 2.17 (3H, s), 2.34 (3H, s), 3.86 (3H, s), 4.03 (3H, s), 6.54 (1H, s), 6.79 (1H, d), 7.08 (1H, d); m/z (ES⁺) [M+H]⁺ = 352.

Intermediate 22: 6-amino-4-methoxy-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile

[00222] Sodium t-butoxide (8.19 g, 85.2 mmol) was added slowly to a solution of malononitrile (3.38 g, 51.1 mmol) in DME (100 mL). The mixture was stirred at r.t. for 30 minutes. 5-Bromo-6-methoxy-N-(3-methoxy-2,6-dimethylphenyl)-2-methylpyrimidin-4-amine (**Intermediate 21**, 15.0 g, 42.6 mmol) and [1,1'-bis(diphenylphosphino)ferrocene] dichloropalladium(II) (3.12 g, 4.26 mmol) were added to the mixture. The resulting mixture was stirred at 100 °C for 2 hours. The reaction mixture was poured into saturated NaHCO₃ (100 mL), extracted with EtOAc (3 x 150 mL), the organic layer was dried over Na₂SO₄, filtered and evaporated to afford yellow solid. The crude product was purified by flash silica chromatography, elution gradient 0 to 60% MeCN in water (0.1% NH₄HCO₃). Pure fractions were evaporated to dryness to afford 6-amino-4-methoxy-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile (**Intermediate 22**, 12 g, 84 %) as a yellow solid. ¹H NMR (300 MHz, DMSO-d₆, 25 °C) δ 1.71 (3H, s), 1.80 (3H, s), 2.38 (3H, s), 3.85 (3H, s), 4.01 (3H, s), 6.88 (2H, s), 7.10 (1H, d), 7.24 (1H, d); m/z (ES⁺) [M+H]⁺ = 338.

Intermediate 23: 6-amino-4-hydroxy-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile

[00223] Lithium chloride (7.54 g, 178 mmol) was added to 6-amino-4-methoxy-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile (**Intermediate 22**, 12.0 g, 35.6 mmol) and para-toluenesulfonic acid (18.37 g, 106.7 mmol) in DMF (120 mL) at r.t. The resulting mixture was stirred at 100 °C for 3 hours. The reaction

mixture was poured into water (750 mL), extracted with EtOAc (5 x 200 mL), the organic layer was dried over Na₂SO₄, filtered and evaporated to afford grey solid. The grey solid washed with water (2 x 75 mL), filtered and dried to afford clean grey solid 6-amino-4-hydroxy-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile (**Intermediate 23**, 9.00 g, 78%). The product was used in the next step directly without further purification. ¹H NMR (300 MHz, DMSO-d₆, 25 °C) δ 1.73 (3H, s), 1.83 (3H, s), 2.17 (3H, s), 3.83 (3H, s), 6.40 (2H, s), 7.07 (1H, d), 7.21 (1H, d), 12.12 (1H, s); m/z (ES⁺) [M+H]⁺ = 324.

Intermediate 24: 6-amino-4-chloro-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile

[00224] 6-Amino-4-hydroxy-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile (**Intermediate 23**, 12 g, 37mmol) was added slowly to POCl₃ (60 mL) at 0 °C. The resulting solution was stirred at 100 °C for 2 hours. The solvent was removed under reduced pressure. The residue was poured into ice water (200 mL). The resulting mixture was basified with saturated NaHCO₃, extracted with EtOAc (200 mL). The organic layer was washed with water (100 mL X 2), dried over Na₂SO₄, filtered and evaporated to afford crude product. The crude product was purified by flash C18-flash chromatography, elution gradient 0 to 50% MeCN in water (0.1% NH₄HCO₃). Pure fractions were evaporated to dryness to afford 6-amino-4-chloro-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile (**Intermediate 24**, 9.00 g, 71%) as a white solid. ¹H NMR (300 MHz, DMSO-d₆, 25 °C) δ 1.72 (3H, s), 1.81 (3H, s), 2.43 (3H, s), 3.85 (3H, s), 7.12 (1H, d), 7.26 (1H, d), 7.50 (2H, s); m/z (ES⁺) [M+H]⁺ = 342.

Intermediate 25: 6-amino-4-chloro-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide

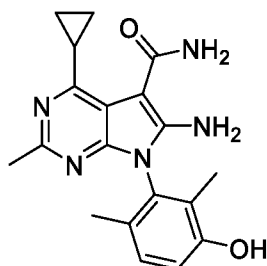
[00225] 6-Amino-4-chloro-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carbonitrile (**Intermediate 24**, 4.00 g, 11.70 mmol) was added in one portion into H₂SO₄ (3 mL) at 25 °C. The resulting mixture was stirred at 25 °C for 2 hours. The reaction mixture was poured into ice water (75 mL). The formed mixture was basified with ammonia, extracted with EtOAc (3 x 100 mL). The organic layer was dried over MgSO₄, filtered and evaporated to afford crude 6-amino-4-chloro-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (**Intermediate 25**, 2.60 g, 62%) as a pale yellow solid. The product was used in the next step directly without further purification. ¹H NMR (300 MHz, DMSO-d₆, 24 °C) δ 1.71 (3H, s), 1.81 (3H, s), 2.42 (3H, s), 3.86 (3H, s), 7.13 (1H, d), 7.28 (3H,

m), two protons exchanged; m/z (ES^+) $[M+H]^+ = 360$.

Intermediate 26: 6-amino-4-chloro-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide

[00226] BBR_3 in DCM (28.9 mL, 28.9 mmol) was added dropwise to 6-amino-4-chloro-7-(3-methoxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (**Intermediate 25**, 2.60 g, 7.23 mmol) in DCM (11 mL) at $-5^\circ C$. The resulting mixture was stirred at $0^\circ C$ for 1 hour. The reaction mixture was poured into ice water (50 mL). The formed mixture was basified with $NaHCO_3$, extracted with EtOAc (5 x 100 mL), the organic layer was dried over $MgSO_4$, filtered and evaporated. The crude product was purified by flash C18-flash chromatography, elution gradient 5 to 60% MeCN in water (0.1% NH_4HCO_3). Pure fractions were evaporated to dryness to afford 6-amino-4-chloro-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (**Intermediate 26**, 1.90 g, 76%) as a white solid. ^1H NMR (300 MHz, $DMSO-d_6$, $24^\circ C$) δ 1.68 (3H, s), 1.76 (3H, s), 2.42 (3H, d), 5.76 (1H, d), 6.95 (1H, d), 7.09 (2H, d), 7.24 (2H, s), 9.64 (1H, s); m/z (ES^+) $[M+H]^+ = 346$.

(S)- or (R)-6-amino-4-cyclopropyl-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (Compound 139)



[00227] 1,1'-Bis(diphenylphosphino)ferrocenedichloropalladium (II) dichloromethane adduct (0.354 g, 0.430 mmol) was added to 6-amino-4-chloro-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (**Intermediate 26**, 1.00 g, 2.89 mmol), cyclopropylboronic acid (0.373 g, 4.34 mmol) and K_2CO_3 (1.20 g, 8.68 mmol) in 1,4-dioxane (20 mL) at r.t. under nitrogen. The resulting mixture was stirred at $100^\circ C$ for 16 hours. The reaction mixture was poured into water (100 mL), extracted with EtOAc (3 x 50 mL), the organic layer was dried over Na_2SO_4 , filtered and evaporated to afford black solid. The crude product was purified by flash C18-flash chromatography, elution gradient 0 to 50% MeCN in water (0.1% NH_4HCO_3). Pure fractions were evaporated to dryness to afford (rac)-6-amino-4-cyclopropyl-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (14 mg, 1.4%) as a white solid.

[00228] The racemic mixture was purified by preparative chiral-HPLC on a column: Lux 5um Cellulose-4, 2.12*25 cm, 5 µm; Mobile Phase A: Hex (0.5% 2M NH₃-MeOH), Mobile Phase B: EtOH; Flow rate: 220 mL/min; Gradient: isocratic 30; Wave Length: 220/254 nm; RT1(min): 12.032; RT2(min): 17.364; Sample Solvent: ETOH; Injection Volume: 1.5 mL; Number Of Runs: 3 to afford rotational isomer 1, (S) or (R)-6-amino-4-cyclopropyl-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide (**Example B-4**), 2.77 mg, 21%) as a white solid and rotational isomer 2. ¹H NMR (400 MHz, DMSO-d₆, 22 °C) δ 1.00 (2H, dq), 1.18 (2H, p), 1.67 (3H, s), 1.76 (3H, s), 2.34 (3H, s), 2.77 (1H, tt), 6.48 (2H, s), 6.80–6.96 (3H, m), 7.07 (1H, d), 9.59 (1H, s); m/z (ES⁺) [M+H]⁺ = 352.

C. Biological Data

Example C-1: Elevated Basal Replication Stress Causes PKMYT1 Inhibitor Sensitivity

A. Methods

[00229] In this study, the PKMYT1 inhibitor concentrations that inhibited cell growth, *in vitro*, by 50% (GI₅₀s) were correlated to basal replication stress levels in several cell line models. To establish the GI₅₀ values, cells were seeded into plates and exposed to a ½ log 9-point dose response of the PKMYT1 inhibitor. After seven days of PKMYT1 inhibitor exposure, cells were treated with Cell-Titer Glo (Promega), allowing for the number of viable cells in culture to be determined by quantification of adenosine triphosphate (ATP), an indicator of metabolically active cells. Sigmoidal standard curves (data not shown) were established and mean GI₅₀ values were interpolated from three (n=3 ±SD) biological replicates.

[00230] GI₅₀ values were then correlated to basal replication stress levels for the 16 cell line models identified in Figs. 2, 3, and 4. Basal replication stress levels for the uterine cancer and breast cancer cell line models used in the study were determined by DNA combing analysis described in Young, L. A., et al. (2019). "Differential Activity of ATR and WEE1 Inhibitors in a Highly Sensitive Subpopulation of DLBCL Linked to Replication Stress." *Cancer Research* 79(14): 3762-3775. The OVCAR3 and TOV112D FBXW7 knock out (KO) ovarian cancer cell lines express elevated levels of the *CCNE1* gene product, cyclin E1 (a biomarker linked to induction of basal replication stress) compared to the SKOV3 and TOV112D ovarian cancer cell lines. See, e.g., Y. P. Kok et al (2020). "Overexpression of Cyclin E1 or Cdc25A leads to replication stress, mitotic aberrancies, and increased sensitivity to replication checkpoint inhibitors." *Oncogenesis* Vol. 9 Issue 10 Pages 88. The Panc0203 pancreatic cancer cell line

expresses elevated levels of phosphorylated RPA2 (a biomarker linked to elevated basal replication stress) while the pancreatic cancer KP4 cell line does not. Serra, V., et al., "Identification of a Molecularly-Defined Subset of Breast and Ovarian Cancer Models that Respond to WEE1 or ATR Inhibition, Overcoming PARP Inhibitor Resistance," Clin Cancer Res 28(20): 4536-4550. (2022).

B. Results

[00231] Figure 2 reports the GI₅₀ values measured in the 16 cell line models (some having elevated basal replication stress) treated with a PKMYT1 inhibitor tool compound. Figure 3 reports the corresponding median replication fork velocities measured by DNA combing analysis for the uterine cancer and breast cancer cell line models treated with the tool compound. The five uterine cancer and breast cancer cell lines of Figure 2 having the lowest GI₅₀ values also had the lowest median replication fork velocities of Figure 3. Figures 2 and 3 collectively show that the cell line models with elevated levels of basal replication stress (KRAS and CCNE1-amplified uterine cancer cell lines, CCNE1-amplified breast cancer cell lines, OVCAR3 (CCNE1-amplified) and TOV112D FBXW7 KO (cyclin E1 high) ovarian cancer cell lines, and panc0203 (pRPA2 high) pancreatic cancer cell line) were the most sensitive cell lines to PKMYT1 inhibition. In contrast, the cell line models with low basal levels of replication stress were more resistant to PKMYT1 inhibition.

[00232] Median replication fork velocities also were measured by DNA combing analysis for the same uterine cancer and breast cancer cell line models treated instead with the PKMYT1 inhibitor of Example B-1, Isomer 1 (i.e., the eutomer of Compound 1). Figure 4 reports the median replication fork velocities determined for the eutomer of Compound 1. The results were analogous to those obtained with the tool compound and confirm that the uterine cancer and breast cancer cell line models with low basal levels of replication stress were more resistant to PKMYT1 inhibition than the cell line models having elevated levels of replication stress.

[00233] Overall, the data show that cancers with elevated levels of basal replication stress have greater sensitivity to PKMYT1 inhibition. The data also provide evidence confirming that replication stress correlates with cyclin E amplification and with other genetic alterations previously linked to replication stress and the related response to WEE1 inhibition.

Example C-2: Target Engagement NanoBRET (Bioluminescence Resonance Energy Transfer) Assay

A. NanoBRET Assay (PKMYT1, WEE1, and EPHB3)

[00234] A whole cell-based target engagement assay was used to investigate target engagement with three targets of interest using the NanoBRET technology. Specifically, compounds were tested to assess inhibition of PKMYT1, WEE1, and EPHB3 in engineered HEK293T cell lines.

[00235] HEK293T cell lines overexpressing PKMYT1, WEE1, and EPHB3 were thawed out from cryopreserved vials at 37°C. The thawed cells were resuspended in 10mL of RPMI-1640 supplemented with 10% Fetal calf serum and 1% GlutaMax. Cell lines were seeded using a Multidrop Combi with a small cassette in 384-well low volume plate (Greiner #784080) at 8000 cells/well in 10µL (for PKMYT1 and EPHB3) and 4000 cells/well in 10µL (for WEE1). The plates were incubated at 37°C, 5% CO₂ for 23 hours.

[00236] After the incubation, media from plates was evacuated using the BlueCat Bluewasher with the GentleSpin step. The tracer probe for each target was dissolved in Opti-MEM at RT and a 10uL volume was dispensed into each well in the plates using the Multidrop Combi with a small cassette. The tracer probes from Promega were dissolved at the concentration of 1µM (K-10 for PKMYT1 #N2640), 0.33µM (K-4 for EPHB3 #N2520) and 0.13µM (K-10 for WEE1 #N2640). At this stage the plates were protected from light by using dark lids.

[00237] Plates were dosed with compounds immediately after probe addition. Compounds dosed (12-point concentration response starting from a top concentration of 10µM to 0.000000248µM) were added to the wells using an Echo 650 Acoustic Dispenser (Beckman) and plates were placed in incubator maintained at 37°C and 5% CO₂ and incubated for two hours.

[00238] After the incubation, plates were equilibrated at room temperature for 10 minutes. 5µL of the 3x NanoBRET assay solution was dispensed in each well of the plate using the Multidrop Combi with a small cassette. The plates were incubated at room temperature for 10 minutes (protected from light) and centrifuged at 300G from 1 minute before reading on the PherSTAR FSX (Optic Module: 460 ± 80nm/610nm-LP; Gain settings: 3600 (A), 2500 (B); Integration time: 0.2 seconds). The acquired raw data for the acceptor and donor channels was loaded into Genedata analyser (Genedata AG) to determine the BRET ratio for the plates.

[00239] IC₅₀ values for the compounds tested are reported in Table 1-A.

TABLE 1-A

COMPOUND	ISOMER	PKMYT1 INHIBITION IC ₅₀ ¹ (μM)	WEE1 INHIBITION IC ₅₀ ¹ (μM)	EPHB3 INHIBITION IC ₅₀ ¹ (μM)
1	Isomer1	0.016	>8.939	1.668
1	Isomer2	0.633	>9.970	>9.970
4	Racemate	0.025	>9.970	1.815
5	Racemate	0.022	>9.970	1.328
10	Isomer1	0.007	5.410	0.036
10	Isomer2	0.869	>9.970	>9.970
10	Racemate	0.009	>9.970	0.091
12	Racemate	0.054	>9.970	1.535
13	Racemate	0.074	>9.970	0.643
17	Isomer1	0.008	>9.970	0.278
17	Isomer2	3.625	>9.970	>9.970
18	Racemate	0.226	>9.970	3.843
22	Racemate	0.031	>9.970	1.854
24	Racemate	0.030	>9.970	0.405
37	Racemate	0.015	5.275	0.310
38	Racemate	0.132	>9.970	0.589
47	Isomer1	0.016	>9.970	0.054
47	Isomer2	1.039	>9.970	6.881
47	Racemate	0.024	>9.970	0.088
48	Isomer1	0.012	>10.000	0.470
48	Isomer2	0.576	>9.970	>9.970
48	Racemate	0.023	>9.970	0.984
49	Isomer1	0.010	>9.960	0.796
49	Isomer2	0.595	>9.970	>9.970
49	Racemate	0.023	>9.970	1.856
50	Isomer1	0.008	>10.000	0.170
50	Isomer2	3.880	>9.970	>9.970
50	Racemate	0.016	>9.970	0.346
52	Isomer1	0.031	>10.000	0.727
52	Isomer2	0.369	>10.000	7.290
53	Isomer1	>9.970	>9.970	>9.970
53	Isomer2	0.080	>9.970	0.677
54	Racemate	0.008	>9.970	0.168
56	Isomer1	0.675	>9.970	2.715
56	Isomer2	>9.970	>9.970	>9.970
57	Isomer1	1.022	>9.970	>9.970
57	Isomer2	0.011	>10.000	1.252
62	Isomer1	>9.970	>9.970	>9.970
62	Isomer2	0.014	>9.970	1.010
70	Isomer1	0.016	>10.000	0.103
70	Isomer2	1.211	>9.970	>9.970
71	Isomer2	1.187	>9.970	>9.970

COMPOUND	ISOMER	PKMYT1 INHIBITION IC ₅₀ ¹ (μM)	WEE1 INHIBITION IC ₅₀ ¹ (μM)	EPHB3 INHIBITION IC ₅₀ ¹ (μM)
71	Isomer1	0.004	>9.970	0.180
72	Racemate	0.018	>9.970	1.756
73	Isomer1	0.011	2.529	2.716
73	Isomer2	1.067	>9.970	>9.970
74	Isomer1	0.006	>9.970	0.026
74	Isomer2	5.225	>9.970	>9.970
75	Isomer1	0.018	>9.970	0.117
75	Isomer2	8.220	>9.970	>9.970
79	Isomer1	0.304	>9.970	0.786
79	Isomer2	>9.970	>9.970	>9.970
82	Racemate	0.023	>9.970	2.423
83	Isomer1	2.170	>9.970	>9.970
83	Isomer2	0.012	>9.970	0.294
84	Racemate	0.063	>9.970	>6.796
85	Isomer1	0.040	>4.516	>9.648
85	Isomer2	2.376	>9.970	>9.970
86	Isomer1	0.562	>9.970	>9.970
86	Isomer2	0.025	7.040	5.793
92	Isomer1	0.018	>9.970	0.191
92	Isomer2	3.062	>9.970	>9.970
93	Isomer1	7.121	>3.155	>9.970
93	Isomer2	0.019	>9.970	1.488
134	Racemate	0.033	>9.970	0.619
135	Isomer1	0.010	>9.970	0.233
135	Isomer2	1.930	>9.970	>9.970
136	Racemate	0.019	>9.970	0.443
137	Isomer2	0.009	>9.970	3.956
138	Isomer2	0.016	>9.970	3.096
139	Isomer1	0.010	>9.970	1.894

¹ IC₅₀ is reported after a single measurement (n=1) or as an average for multiple measurements (n>1).

B. NanoBRET Assay (Kinase Panel)

[00240] Compound 137, Isomer 2 was further profiled (dose response) against selected kinases in a NanoBRET whole-cell target engagement assay conducted at CELLinib GmbH. IC₅₀ values for Compound 137, Isomer 2 are reported in Table 1-B.

TABLE 1-B

KINASE NAME	IC ₅₀ (μM)
EPHB3	1.79
EPHA1	3.17
KIT	4.29
EPHB1	6.32
EPHA2	6.83
EPHA3	8.10
EPHB2	10.90
EPHB4	13.40
EPHA5	15.00
EPHA4	16.10
GSK3b	21.60
ACVR1B	27.10
EPHA8	33.40
PIK3CA	48.50
AurKA	>50
EGFR	>50
FGFR1	>50
FGFR2	>50
KDR	>50
LCK	>50
MAP3K4	>50
PDPK1	>50
SRC	>50
TGFBR1	>50

Example C-3: PKMYT1 Surface Plasmon Resonance (SPR) Direct Binding Assay

A. PKMYT1 Protein Expression and Purification

[00241] Avi-tagged PKMYT1(6His-SSGVDLGT-TEV-AVI-S-PKMYT1(75-362)) was expressed in Escherichia coli BL21(DE3) Tuner cells in Terrific Broth media. Expression was induced at A₆₀₀ = 1.0 with 50 μM IPTG and expression occurred for 20 hours at 18 °C. Cells were collected and stored at −80 °C. Cell pellets were resuspended in 750 ml per 97g pellet of PKMYT1 lysis buffer (50 mM Hepes pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP, DNase (0.1 μg/mL), 0.1 mM PMSF). The solution was homogenized and the clarified lysate loaded into a column for nickel affinity purification preequilibrated in buffer A (50 mM Hepes pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP, 20 mM imidazole)). Protein was eluted in buffer A supplemented with 300 mM imidazole. Fractions containing PKMYT1 were pooled and

buffer exchanged by dialysis into biotinylation buffer (25 mM Hepes pH=7.5, 300 mM potassium glutamate, 0.5 mM TCEP). Biotinylation was achieved using the BirA kit (Avidity) as per manufacturer's instructions and biotinylation confirmed by mass spectrometry. Size exclusion chromatography was used to further purify the PKMYT1 into Storage buffer (25 mM Hepes pH 7.5, 500 mM NaCl, 0.5 mM TCEP), and fractions containing pure PKMYT1 were pooled and concentrated to 10.2 mg/ml and snap frozen on liquid nitrogen.

B. SPR Direct Binding Assay

[00242] All SPR steps were performed on a Biacore 8K Instrument (Cytiva) at a temperature of 25 °C. In the “immobilisation buffer” (50 mM Hepes pH 7.5, 200 mM NaCl, 5 mM MgCl₂, 0.05% Tween-20, 1 mM TCEP) a Series S Sensor Chip SA (Cytiva) was functionalized with ~3000 RU biotinylated Avi-tagged PKMYT1 using a 180s injection at 5 μ l min⁻¹. The surface was conditioned with 10 start-up injections of the “running buffer” (50 mM Hepes pH 7.5, 200 mM NaCl, 5 mM MgCl₂, 0.05% Tween-20, 1 mM TCEP, 1% DMSO). A solvent correction step consisting of a 5-point titration of different DMSO containing running buffer solutions (0-2%) were injected at the beginning and the end of the screening run to correct for DMSO deviations. Three blank cycles of running buffer injections were included preceding each compound injection for blank subtraction. A threefold dilution series for each sample was injected sequentially from low to high concentration over the PKMYT1-coupled surfaces in single-cycle kinetics mode, at a flow rate of 30 μ l min⁻¹ for 120 s, monitoring dissociation for 2500 s.

[00243] All analysis was performed using the Biacore Insight Evaluation Software after applying standard double-referencing and solvent correction. Single-cycle kinetic 1:1 binding model data analysis was applied to determine the on (k_{on}) and off rates (k_{off}) for test compounds. Theoretical R_{max} (the maximal feasible SPR signal generated by an interaction between a ligand–analyte pair; presented in response units (RU)) was determined as the (molecular mass of the compound analyte in solution)/(molecular mass of the immobilized target) $\times$ (amount of immobilized target captured), and the per cent surface activity was determined as the (experimental R_{max})/(theoretical R_{max}).

[00244] Data for the compounds tested are reported in Table 2.

TABLE 2

COMPOUND	ON RATE k_{on} (M ⁻¹ s ⁻¹) ¹	OFF RATE k_{off} (s ⁻¹)	BINDING AFFINITY K_d (M)
1, Isomer 1	5.21e05	7.14e-04	1.37e-09
1, Isomer 2	3.23e03	3.40e-04	1.05e-07

COMPOUND	ON RATE k_{on} ($M^{-1}s^{-1}$) ¹	OFF RATE k_{off} (s^{-1})	BINDING AFFINITY K_D (M)
137, Isomer 2	2.40e05	1.00e-04	4.20e-10

¹ k_{on} and k_{off} are reported after a single measurement ($n=1$) or as an average for multiple measurements ($n>1$).

Example C-4: Functional CDK1 pThr14 AlphaLISA Assay

[00245] Compounds were tested to assess their effect on PKMYT1 activity in a cellular context. Specifically, phosphorylation of CDK1 (a PKMYT1 substrate) on Thr14 was used as a specific marker of PKMYT1 activity. Inhibition of PKMYT1 activity by inhibitors decreases levels of pCDk1(Thr14) in the cell.

[00246] Compounds prepared in DMSO were dispensed into low volume white 384-well plates (Greiner 784075) as concentration-response curves. 5 μ L of FUOV-1 cells in Opti-MEM was seeded on top of the assay ready plate at a density of 3000 cells per well using a multidrop combi. This was incubated for 2 hours at 37°C, 5% CO₂. The rest of the assay was run using the AlphaLISA Surefire Ultra Human Phospho-CDK1 (Thr14) kit available from Perkin Elmer (ALSU-PCDK1-A10K). All reagents were prepared according to the manufacturer's recommendations. 5 μ L of 2 x Lysis Buffer containing added protease inhibitors (Roche, #11836170001) was added to the plate and was mixed thoroughly. Acceptor stock and donor stock was then diluted 1 parts mix to 2 parts HBSS. 4 μ L of acceptor was added using a multidrop Combi and incubated for 1 hour at room temperature. Following this, 4 μ L of donor stock was added using a multidrop Combi and the plate was incubated for 18 hours. Alpha counts were measured using a PheraSTAR FSx (BMG Labtech), and the resulting raw data analysed using Genedata analyser (Genedata AG) to derive compound IC₅₀.

[00247] IC₅₀ values for the compounds tested are reported in Table 3.

TABLE 3

COMPOUND	ISOMER	pCDk1(Thr14) INHIBITION IC ₅₀ ¹ (μ M)
1	Isomer1	0.055
1	Isomer2	2.177
4	Racemate	0.342
5	Racemate	0.431
10	Isomer1	0.105
10	Isomer2	>9.911
10	Racemate	0.244
12	Racemate	0.380
13	Racemate	1.095

COMPOUND	ISOMER	pCDk1(Thr14) INHIBITION IC ₅₀ ¹ (μM)
17	Isomer1	0.106
17	Isomer2	>9.911
18	Racemate	0.519
22	Racemate	0.331
24	Racemate	0.874
37	Racemate	0.243
38	Racemate	2.018
47	Isomer1	0.328
47	Isomer2	>9.911
47	Racemate	0.313
48	Isomer1	0.185
48	Isomer2	6.612
48	Racemate	0.317
49	Isomer1	0.127
49	Isomer2	5.565
49	Racemate	0.187
50	Isomer1	0.110
50	Isomer2	>9.911
50	Racemate	0.273
52	Isomer1	0.641
52	Isomer2	>9.911
53	Isomer1	>9.911
53	Isomer2	2.861
54	Racemate	0.072
56	Isomer1	1.423
56	Isomer2	>9.911
62	Isomer1	>9.911
62	Isomer2	0.421
70	Isomer1	0.115
70	Isomer2	>9.911
71	Isomer2	9.484
71	Isomer1	0.072
72	Racemate	0.347
73	Isomer1	0.068
73	Isomer2	6.009
74	Isomer1	0.139
74	Isomer2	>9.911
75	Isomer1	0.352
75	Isomer2	>9.911
79	Isomer1	0.590
79	Isomer2	>9.911
82	Racemate	0.234
83	Isomer1	>9.911
83	Isomer2	0.158

COMPOUND	ISOMER	pCDk1(Thr14) INHIBITION IC ₅₀ ¹ (μM)
84	Racemate	0.228
85	Isomer1	0.260
85	Isomer2	9.774
86	Isomer1	5.748
86	Isomer2	0.334
92	Isomer1	0.127
92	Isomer2	>9.911
93	Isomer1	>9.911
93	Isomer2	0.150
134	Racemate	0.326
135	Isomer1	0.181
135	Isomer2	>9.911
136	Racemate	0.241
137	Isomer 2	0.040
138	Isomer 2	0.145
139	Isomer 1	0.028

¹ IC₅₀ is reported after a single measurement (n=1) or as an average for multiple measurements (n>1).

Example C-5: Cellular Proliferation in OVCAR3 and SKOV3 Cell Lines (Hoechst Staining)

[00248] An imaging-based assay was used to investigate changes in cell number. Compounds were tested to assess inhibition of cellular proliferation in a CCNE1-amplified ovarian cancer cell line and a CCNE1-nonamplified SKOV3 ovarian cancer cell line.

[00249] OVCAR3 and SKOV3 cells were cultured in RPMI 1640 medium with 4.5 g/ml Glucose, supplemented with 20% heat in-activated Fetal bovine serum and McCoy's modified 5A medium supplemented with 10% heat in-activated Fetal bovine serum along with 1% GlutaMax respectively. Both the cell lines were seeded in complete growth medium using a multidrop into 384-well plates (Greiner, 781090) at the density 4000 cells/well in 40 μL/well. The plates (test compound plates + a day 0 control plate) were placed in incubator maintained at 37°C and 5% CO₂ for about 20 to 22 hours. Day 0 (control plate) was fixed with 4% PFA (v/v), washed 3X in PBS (Biotek 406) and stored at 4°C in 40 ul PBS until further use. A 12-point concentration response starting from a top concentration of 30 μM to 0.00003 uM was added to the test compound plates using an Echo 650 Acoustic Dispenser (Beckman) and then incubated at 37°C and 5% CO₂ for four days. On Day 4, cells were fixed with 4% PFA (v/v) and stained with Hoechst 33342 (Thermofisher H3570) to a final well dilution of 1/10,000 in PBS+1.1% BSA+0.1% TritonX for 1 hour. Cells were washed 3X with PBS and left in 40 ul of PBS. The

stained cells were imaged using Cell Insight cellular imaging and analysis benchtop platform (10X objective). The acquired raw data output for the total cell number was loaded into Genedata analyser (Genedata AG) to determine the rate of proliferation from control plate and test compound dosed plates. The concentration of compound that gives 50% growth inhibition between the day 0 value and the top of the curve of GI₅₀ was calculated for all test compounds.

[00250] GI₅₀ values for the compounds tested are reported in Table 4.

TABLE 4

COMPOUND	ISOMER	OVCAR3 GI ₅₀ ¹ (μM)	SKOV3 GI ₅₀ ¹ (μM)
1	Isomer1	1.313	11.833
1	Isomer2	>30.000	>30.000
4	Racemate	3.827	27.682
5	Racemate	2.206	8.002
12	Racemate	9.821	>30.000
13	Racemate	10.772	>30.000
17	Isomer1	1.396	4.573
17	Isomer2	>30.000	>30.000
18	Racemate	12.211	>30.000
22	Racemate	11.089	>30.000
24	Racemate	8.046	>30.000
37	Racemate	2.074	9.721
38	Racemate	14.227	>30.000
47	Isomer1	3.401	15.685
47	Isomer2	>30.000	>30.000
47	Racemate	8.641	>22.982
48	Isomer1	6.982	15.437
48	Isomer2	7.565	>30.000
48	Racemate	10.370	>21.539
49	Isomer1	3.067	24.101
49	Isomer2	>30.000	>30.000
49	Racemate	13.522	>30.000
50	Isomer1	3.652	6.027
50	Isomer2	>30.000	>30.000
50	Racemate	8.625	10.308
52	Isomer1	28.667	>30.000
52	Isomer2	>30.000	>30.000
56	Isomer1	14.878	>30.000
56	Isomer2	>30.000	>30.000
57	Isomer1	>30.000	22.595
57	Isomer2	3.984	>30.000
62	Isomer1	29.321	>30.000
62	Isomer2	14.153	>30.000
70	Isomer1	5.769	>17.002

COMPOUND	ISOMER	OVCAR3 GI ₅₀ ¹ (μM)	SKOV3 GI ₅₀ ¹ (μM)
70	Isomer2	>30.000	>30.000
73	Isomer1	0.503	4.147
73	Isomer2	>30.000	>30.000
79	Isomer1	4.135	21.173
79	Isomer2	>30.000	>30.000
82	Racemate	1.464	18.159
84	Racemate	6.115	>30.000
86	Isomer1	>30.000	>30.000
86	Isomer2	3.257	17.056
134	Racemate	4.401	15.580
137	Isomer 2	2.207	>20.522
138	Isomer 2	4.896	>29.943
139	Isomer 1	1.779	>10.558

¹ IC₅₀ is reported after a single measurement (n=1) or as an average for multiple measurements (n>1).

Example C-6: Kinase Profiling

A. General Methods

[00251] Test compounds were profiled against a broad panel of kinases at ThermoFisher Scientific using the SelectScreen Kinase Profiling Services. Each kinase assay used one of the following assay technology protocols:

- 1) Z-LYTE™ Screening Protocol and Assay Conditions (Revised 23-Jan-2018 version) available at http://assets.thermofisher.com/TFS-Assets/BID/Methods-&-Protocols/20180123_SSBK_Customer_Protocol_and_Assay_Conditions.pdf;
- 2) Adapta™ Screening Protocol and Assay Conditions (Revised 23-Jan-2018 version) available at http://assets.thermofisher.com/TFS-Assets/BID/Methods-&-Protocols/20180123_SSBK_Adapta_Customer_Protocol_and_Assay_Conditions.pdf ; or
- 3) LanthaScreen™ Eu Kinase Binding Assay Screening Protocol and Assay Conditions (Revised 23-Jan-2018 version) available at http://assets.thermofisher.com/TFS-Assets/BID/Methods-&-Protocols/20180123_SSBK_LanthaScreen_Binding_Customer_Protocol_and_Assay_Conditions.pdf.

B. Kinase Selectivity (Single Point Concentration Mode)

[00252] Compound 137, Isomer 2 was profiled against a broad kinase panel at ThermoFisher Scientific. Kinase selectivity was measured in single point (SP) concentration mode: % inhibition at [Compound 137, Isomer 2] = 1 μ M at K_{mATP} . Data are reported in Table 5-A.

TABLE 5-A

KINASE NAME/ASSAY FORMAT	MEAN INHIBITION (%)
ACVR2B FRET-Lantha SP	102.327
PKMYT1 FRET-Lantha SP	97.172
BMPR1B (ALK6) FRET-Lantha SP	96.641
EPHA1 FRET-Z-Lyte SP	96.280
ICK FRET-Lantha SP	90.275
PTK6 (Brk) *Mn* FRET-Z-Lyte SP	90.004
ACVRL1 (ALK1) FRET-Lantha SP	89.027
TGFB1 (ALK5) FRET-Lantha SP	83.957
ACVR1 (ALK2) FRET-Lantha SP	80.237
bRAF FRET-Lantha SP	78.905
ABL2 (Arg) FRET-Z-Lyte SP	74.616
FGR FRET-Z-Lyte SP	73.456
BMPR1A (ALK3) FRET-Lantha SP	72.857
SRC Hu Phos FRET-Z-Lyte SP	72.168
EPHB3 FRET-Z-Lyte SP	71.690
EPHB2 FRET-Z-Lyte SP	68.536
FRK (PTK5) FRET-Z-Lyte SP	67.392
EPHB1 FRET-Z-Lyte SP	63.359
YES1 FRET-Z-Lyte SP	62.995
ABL1 Hu Phos FRET-Z-Lyte SP	62.791
FYN FRET-Z-Lyte SP	60.587
WEE1 FRET-Lantha SP	60.552
EPHB4 FRET-Z-Lyte SP	59.449
EPHA2 FRET-Z-Lyte SP	59.273
DYRK1B FRET-Z-Lyte SP	54.239
LYN B FRET-Z-Lyte SP	52.127
ACVR2A FRET-Lantha SP	49.464
HCK FRET-Z-Lyte SP	48.744
GSK3B FRET-Z-Lyte SP	46.076
LCK FRET-Z-Lyte SP	45.731
TNK2 (ACK) FRET-Lantha SP	45.685
EPHA5 FRET-Z-Lyte SP	44.210
EPHA3 FRET-Lantha SP	43.400
LYN A Hu Phos FRET-Z-Lyte SP	41.079
bRAF Cascade FRET-Z-Lyte SP	40.753

KINASE NAME/ASSAY FORMAT	MEAN INHIBITION (%)
CDK11 Cyclin C FRET-Lantha SP	35.689
EPHA8 FRET-Z-Lyte SP	35.008
EPHA4 FRET-Z-Lyte SP	34.195
BLK FRET-Z-Lyte SP	34.144
RPS6KA2 (RSK3) FRET-Z-Lyte SP	31.595
SRMS (Srm) FRET-Z-Lyte SP	27.698
LIMK1 FRET-Lantha SP	27.550
GSK3A FRET-Z-Lyte SP	25.607
CSF1R (FMS) FRET-Z-Lyte SP	25.219
PLK3 FRET-Z-Lyte SP	24.992
FGFR1 *Mn* FRET-Z-Lyte SP	23.959
RIPK3 FRET-Lantha SP	23.832
ERN2 FRET-Lantha SP	23.587
DYRK1A FRET-Z-Lyte SP	21.554
AMPK A1/B2/G2 FRET-Z-Lyte SP	21.257
BMX FRET-Z-Lyte SP	19.529
STK39 (STLK3) FRET-Lantha SP	19.173
CDK7/cyclin H/MNAT1 Adapta SP	18.174
RIPK2 FRET-Lantha SP	17.820
CAMK2D FRET-Z-Lyte SP	17.732
SIK1 FRET-Lantha SP	17.289
TLK2 FRET-Lantha SP	16.812
ACVR1B (ALK4) *Mn* Z-Lyte SP	16.559
CDK9/cyclin T1 FRET-Adapta SP	16.235
BTK FRET-Z-Lyte SP	16.116
ITK FRET-Z-Lyte SP	15.820
SIK3 FRET-Lantha SP	15.273
MAP4K4 (HGK) FRET-Z-Lyte SP	15.192
NEK2 FRET-Z-Lyte SP	15.105
IRAK3 FRET-Lantha SP	14.866
RPS6KA6 (RSK4) FRET-Z-Lyte SP	14.718
BRSK2 FRET-Lantha SP	13.846
TAOK1 FRET-Lantha SP	13.755
CDK8/cyclin C FRET-Lantha SP	13.629
RET Hu Phos FRET-Z-Lyte SP	13.595
CDK17/cyclin Y FRET-Z-Lyte SP	13.479
TGFBR2 FRET-Lantha SP	13.412
AMPK A2/B1/G2 FRET-Z-Lyte SP	13.352
PRKCZ (PKC zeta) Z-Lyte SP	13.234
STK17A (DRAK1) FRET-Lantha SP	12.833
FYN A FRET-Lantha SP	12.744

KINASE NAME/ASSAY FORMAT	MEAN INHIBITION (%)
CDK14(PFTK1)/cyclinY Lantha SP	12.615
NTRK1 (TRKA) FRET-Z-Lyte SP	12.557
STK38L (NDR2) FRET-Lantha SP	12.492
NTRK2 (TRKB) *Mn* Z-Lyte SP	11.914
SNF1LK2 FRET-Z-Lyte SP	11.712
HUNK FRET-Lantha SP	11.675
LATS2 FRET-Lantha SP	11.623
MELK FRET-Z-Lyte SP	11.594
CDK2/cyclin A1 FRET-Lantha SP	11.556
CAMK1 (CaMK1) FRET-Adapta SP	11.522
CHUK(IKK alpha) FRET-Adapta SP	11.354
CDK13 Cyclin K FRET-Lantha SP	11.305
MAP2K4 (MEK4) FRET-Lantha SP	11.103
MYO3A (MYO3 alpha) Lantha SP	10.901
PDPK1 Cascade FRET-Z-Lyte SP	10.720
MATK (HYL) FRET-Z-Lyte SP	10.674
RPS6KA1 (RSK1) FRET-Z-Lyte SP	10.570
MAPK8 (JNK1) Cascade Z-Lyte SP	10.253
MYO3B FRET-Lantha SP	9.952
STK17B (DRAK2) FRET-Lantha SP	9.903
MINK1 FRET-Z-Lyte SP	9.318
MAPK12 (p38 gamma) Z-Lyte SP	9.311
TXK FRET-Z-Lyte SP	9.164
CDK6 Cyclin D1 FRET-Adapta SP	9.064
MAPK14 (p38a) Direct Z-Lyte SP	8.973
ULK3 FRET-Lantha SP	8.518
RPS6KA5 (MSK1) FRET-Z-Lyte SP	8.353
ZAP70 *Mn* FRET-Z-Lyte SP	8.344
PRKCN (PKD3) FRET-Z-Lyte SP	8.236
MAP3K3 (MEKK3) FRET-Lantha SP	8.215
STK22D (TSSK1) FRET-Z-Lyte SP	8.038
AMPK (A1/B1/G3) FRET-Lantha SP	7.831
ROS1 FRET-Z-Lyte SP	7.695
CLK4 FRET-Lantha SP	7.663
MAP3K7/MAP3K7IP1 Lantha SP	7.591
DDR1 FRET-Lantha SP	7.439
MARK3 FRET-Z-Lyte SP	7.414
CDK4 Cyclin D1 *Mn* Adapta SP	7.329
MAP2K1 (MEK1) Cascade Z-Lyte SP	7.323
PRKACG FRET-Lantha SP	7.319
EEF2K FRET-Z-Lyte SP	7.251

KINASE NAME/ASSAY FORMAT	MEAN INHIBITION (%)
MAP3K8 (COT) Cascade Z-Lyte SP	7.138
PRKD2 (PKD2) Z-Lyte SP	7.103
RPS6KA3 (RSK2) FRET-Z-Lyte SP	7.084
CLK2 FRET-Z-Lyte SP	7.005
FLT3 Hu Phos FRET-Z-Lyte SP	6.895
CDK2/cyclin A FRET-Z-Lyte SP	6.868
STK24 (MST3) FRET-Z-Lyte SP	6.812
CAMK1G FRET-Z-Lyte SP	6.787
DAPK2 FRET-Lantha SP	6.764
NEK1 FRET-Z-Lyte SP	6.683
MARK1 (MARK) FRET-Z-Lyte SP	6.666
AMPK A1/B2/G3 FRET-Z-Lyte SP	6.628
RPS6KA4 (MSK2) FRET-Z-Lyte SP	6.622
AMPK A1/B1/G1 FRET-Z-lyte SP	6.558
TEK (Tie2) *Mn* FRET-Z-Lyte SP	6.545
GSG2 (Haspin) FRET-Adapta SP	6.522
MAPKAPK5 (PRAK) FRET-Z-Lyte SP	6.462
MST4 FRET-Z-Lyte SP	6.444
AURKB (Aurora B) Z-Lyte SP	6.308
NTRK3 (TRKC) FRET-Z-Lyte SP	6.225
CDK5/p35 FRET-Z-Lyte SP	6.213
CAMK1D FRET-Z-Lyte SP	6.089
ALK FRET-Z-Lyte SP	6.004
PI4KB FRET-Adapta SP	5.927
MAPK10 (JNK3) Cascade Z-Lyte SP	5.848
PASK FRET-Z-Lyte SP	5.814
CDK18/cyclin Y FRET-Z-Lyte SP	5.763
CAMK2G FRET-Lantha SP	5.694
ROCK2 FRET-Z-Lyte SP	5.617
TESK2 FRET-Lantha v2 SP	5.530
NLK FRET-Lantha SP	5.482
PAK2 (PAK65) FRET-Z-Lyte SP	5.449
MAP2K5 (MEK5) FRET-Lantha SP	5.334
SYK FRET-Z-Lyte SP	5.317
CDK4 Cyclin D3 *Mn* Adapta SP	5.195
CLK3 FRET-Z-Lyte SP	5.160
MAPK11 (p38 beta) Z-Lyte SP	5.090
INSR *Mn* FRET-Z-Lyte SP	5.030
PRKCB2 (PKC Beta II) Z-Lyte SP	5.028
AXL FRET-Z-Lyte SP	4.909
TNIK FRET-Lantha SP	4.907

KINASE NAME/ASSAY FORMAT	MEAN INHIBITION (%)
PAK1 FRET-Z-Lyte SP	4.877
STK38 FRET-Lantha SP	4.799
MARK2 FRET-Z-Lyte SP	4.784
PRKCH (PKC eta) Z-Lyte SP	4.761
CSK FRET-Z-Lyte SP	4.754
CSNK1A1 FRET-Z-Lyte SP	4.723
STK4 (MST1) FRET-Z-Lyte SP	4.636
SRPK1 FRET-Z-Lyte SP	4.608
STK22B (TSSK2) FRET-Z-Lyte SP	4.540
DCAMKL2 (DCK2) FRET-Z-Lyte SP	4.498
MERTK (cMER) *Mn* Z-Lyte SP	4.429
CDK1/cyclin B FRET-Z-Lyte SP	4.337
MAPK10 (JNK3) FRET-Lantha SP	4.282
PTK2B (FAK2) *Mn* Z-Lyte SP	4.024
PKN1 (PRK1) FRET-Z-Lyte SP	3.985
CSNK2A2 (CK2alpha2) Z-Lyte SP	3.983
LTK (TYK1) FRET-Z-Lyte SP	3.950
GRK4 FRET-Z-Lyte SP	3.928
INSRR (IRR) *Mn* Z-Lyte SP	3.922
PRKACB FRET-Lantha SP	3.852
AURKA (Aurora A) Z-Lyte SP	3.813
MAPK13 (p38 delta) Z-Lyte SP	3.793
NEK8 FRET-Lantha SP	3.787
PRKD1 (PKC mu) Z-Lyte SP	3.764
PKN2 (PRK2) FRET-lantha SP	3.734
PDGFRB *Mn* FRET-Z-Lyte SP	3.732
FLT4 (VEGFR3) *Mn* Z-Lyte SP	3.700
CHEK1 (CHK1) FRET-Z-Lyte SP	3.644
CDK5/p25 FRET-Z-Lyte SP	3.607
ADRBK2 (GRK3) FRET-Z-Lyte SP	3.532
JAK2 FRET-Z-Lyte SP	3.492
GRK1 FRET-Lantha SP	3.488
CHEK2 (CHK2) FRET-Z-Lyte SP	3.471
PHKG1 FRET-Z-Lyte SP	3.453
IKBKB (IKKbeta) FRET-Z-Lyte SP	3.452
SRPK2 FRET-Z-Lyte SP	3.415
FER FRET-Z-Lyte SP	3.350
KSR2 FRET-Z-Lyte SP	3.346
CSNK1A1L FRET-Z-Lyte SP	3.325
IKBKE (IKK epsilon) Z-Lyte SP	3.320
CDK2/Cyclin E1 FRET-Lantha SP	3.273

KINASE NAME/ASSAY FORMAT	MEAN INHIBITION (%)
PRKCD (PKC delta) Z-Lyte SP	3.258
NEK4 FRET-Z-Lyte SP	3.233
MAPK9 (JNK2) FRET-Lantha SP	3.226
DMPK FRET-Lantha SP	3.222
CDC7/DBF4 FRET-Lantha SP	3.217
MAP3K2 (MEKK2) FRET-Lantha SP	3.169
GRK7 FRET-Z-Lyte SP	2.998
SBK1 FRET-Z-Lyte SP	2.962
AKT3 (PKB gamma) Z-Lyte SP	2.961
AKT2 (PKB beta) FRET-Z-Lyte SP	2.960
FGFR2 *Mn* FRET-Z-Lyte SP	2.906
PRKG2 (PKG2) FRET-Z-Lyte SP	2.847
TNK1 FRET-Z-Lyte SP	2.840
ROCK1 FRET-Z-Lyte SP	2.834
GRK6 FRET-Z-Lyte SP	2.821
CAMKK2 (Beta) FRET-Lantha SP	2.806
MAPK8 (JNK1) FRET-Lantha SP	2.729
JAK3 FRET-Z-Lyte SP	2.710
ERN1 FRET-Lantha SP	2.657
PI4KA FRET-Adapta SP	2.590
PRKG1 FRET-Z-Lyte SP	2.575
JAK1 FRET-Z-Lyte SP	2.549
TYRO3 (RSE) FRET-Z-Lyte SP	2.535
STK16 (PKL12) FRET-Lantha SP	2.533
AMPK A2/B1/G3 FRET-Z-Lyte SP	2.507
CAMK2B FRET-Z-Lyte SP	2.475
PAK3 FRET-Z-Lyte SP	2.463
MAP2K6 (MKK6) FRET-Lantha SP	2.457
SLK Hu Bind FRET-Lantha SP	2.440
DAPK3 (ZIPK) FRET-Z-Lyte SP	2.416
MKNK2 (MNK2) FRET-Lantha SP	2.415
CAMK2A FRET-Z-Lyte SP	2.396
MARK4 FRET-Z-Lyte SP	2.379
PLK1 FRET-Z-Lyte SP	2.360
PIM2 FRET-Z-Lyte SP	2.302
DNA-PK FRET-Z-Lyte SP	2.292
WNK2 FRET-Lantha SP	2.291
EIF2AK2 (PKR) FRET-Lantha SP	2.190
AMPK A2/B2/G3 FRET-Z-Lyte SP	2.185
RPS6KB2 FRET-Z-Lyte SP	2.179
PTK2 (FAK) FRET-Z-Lyte SP	2.177

KINASE NAME/ASSAY FORMAT	MEAN INHIBITION (%)
ADRBK1 (GRK2) FRET-Z-Lyte SP	2.161
MASTL FRET-Lantha SP	2.020
PAK6 FRET-Z-Lyte SP	1.993
HIPK1 (Myak) FRET-Z-Lyte SP	1.992
AMPK A2/B1/G1 FRET-Z-Lyte SP	1.980
MAPK1 (ERK2) FRET-Z-Lyte SP	1.904
STK25 (YSK1) FRET-Z-Lyte SP	1.849
CSNK2A1 (CK2alpha1) Z-Lyte SP	1.840
PRKACA (PKA) FRET-Z-Lyte SP	1.830
MAP3K19 (YSK4) FRET-Z-Lyte SP	1.798
CSNK1D (CK1 delta) Z-Lyte SP	1.788
STK23 (MSSK1) FRET-Z-Lyte SP	1.780
CSNK1G3 (CK1gamma3) Z-Lyte SP	1.739
MST1R (RON) FRET-Z-Lyte SP	1.678
STK3 (MST2) FRET-Z-Lyte SP	1.674
TAOK3 (JIK) FRET-Lantha SP	1.585
SGK (SGK1) FRET-Z-Lyte SP	1.583
NUAK1 (ARK5) FRET-Adapta SP	1.560
EPHA7 FRET-Lantha SP	1.546
FES (FPS) FRET-Z-Lyte SP	1.521
RPS6KB1 (p70S6K) Z-Lyte SP	1.466
HIPK2 FRET-Z-Lyte SP	1.451
AMPK (A2/B2/G2) FRET-Lantha SP	1.418
KDR (VEGFR2) FRET-Z-Lyte SP	1.359
TBK1 FRET-Z-Lyte SP	1.328
MAPKAPK2 FRET-Z-Lyte SP	1.297
MAP3K10 (MLK2) FRET-Lantha SP	1.290
CSNK1G2 (CK1gamma2) Z-Lyte SP	1.270
WNK1 FRET-Lantha SP	1.189
HIPK4 FRET-Z-Lyte SP	1.188
MAP4K3 (GLK) FRET-Lantha SP	1.158
AMPK (A1/B2/G1) FRET-Lantha SP	1.152
PRKCI (PKC iota) Z-Lyte SP	1.145
CDC42 BPG FRET-Z-Lyte SP	1.141
PRKX FRET-Z-Lyte SP	1.119
PLK4 FRET-Lantha SP	1.109
IGF1R FRET-Z-Lyte SP	1.081
AKT1 (PKB alpha) Z-Lyte SP	1.061
NEK6 FRET-Z-Lyte SP	1.053
PAK7 (KIAA1264) FRET-Z-Lyte SP	1.008
CLK1 FRET-Z-Lyte SP	0.999

KINASE NAME/ASSAY FORMAT	MEAN INHIBITION (%)
NIM1K FRET-Z-Lyte SP	0.982
GAK FRET-Lantha SP	0.979
HIPK3 (YAK1) FRET-Z-Lyte SP	0.970
ERBB2 (HER2) *Mn* Z-Lyte SP	0.967
PRKCE (PKC epsilon) Z-Lyte SP	0.959
PIK3CA/PIK3R1 FRET-Adapta SP	0.944
JAK2 JH1 JH2 FRET-Z-Lyte SP	0.927
PHKG2 FRET-Z-Lyte SP	0.872
PIP4K2A FRET-Adapta SP	0.823
ULK2 FRET-Lantha SP	0.726
NEK9 *Mn* FRET-Z-Lyte SP	0.700
PIM3 FRET-Z-Lyte SP	0.696
MKNK1 (MNK1) *Mn* Z-Lyte SP	0.622
CSNK1E (CK1 epsilon) Z-Lyte SP	0.607
TYK2 FRET-Z-Lyte SP	0.594
BRSK1 (SAD1) FRET-Z-Lyte SP	0.568
STK33 FRET-Lantha SP	0.564
DYRK3 FRET-Z-Lyte SP	0.554
MAPK15 (ERK7) FRET-Lantha SP	0.548
PI4K2B *Mn* FRET-Adapta SP	0.544
DCAMKL1 (DCLK1) FRET-Z-Lyte SP	0.527
AMPK (A1/B1/G2) FRET-Lantha SP	0.515
DYRK4 FRET-Z-Lyte SP	0.423
PRKCA (PKC alpha) Z-Lyte SP	0.422
PLK2 FRET-Z-Lyte SP	0.391
MYLK2 FRET-Z-lyte SP	0.373
PRKCB1 (PKC beta I) Z-Lyte SP	0.364
CDK9/cyclin K FRET-Lantha SP	0.354
FGFR4 *Mn* FRET-Z-Lyte SP	0.351
STK32C (YANK3) FRET-Lantha SP	0.307
DYRK2 FRET-Lantha SP	0.289
MAP2K2 (MEK2) Cascade Z-Lyte SP	0.056
ULK1 FRET-Lantha SP	0.045
FRAP1 (mTOR) *Mn* Z-Lyte SP	0.025
MAPK9 (JNK2) Cascade Z-Lyte SP	-0.061
MAPK14 (p38a) Cascade Z-Lyte SP	-0.075
MAPK3 (ERK1) FRET-Z-Lyte SP	-0.113
MAP2K6 (MKK6) Cascade Z-Lyte SP	-0.119
KIT *Mn* FRET-Z-Lyte SP	-0.129
CDK16(PCTK1)/cyclinY Lantha SP	-0.183
AURKC (Aurora C) Z-Lyte SP	-0.353

KINASE NAME/ASSAY FORMAT	MEAN INHIBITION (%)
DDR2 Hu Bind FRET-Lantha SP	-0.431
LRRK2 Hu Phos FRET-Adapta SP	-0.583
MAPKAPK3 FRET-Z-Lyte SP	-0.628
TLK1 FRET-Lantha SP	-0.646
CAMK4 (CaMKIV) FRET-Z-Lyte SP	-0.707
LIMK2 FRET-Lantha SP	-0.783
MAPK7 (ERK5) FRET-Z-Lyte SP	-0.819
MAP4K1 (HPK1) FRET-Lantha SP	-0.865
EPHA6 FRET-Lantha SP	-0.902
MAP2K2 (MEK2) FRET-Lantha SP	-0.931
AMPK (A2/B2/G1) FRET-Lantha SP	-0.942
PIK3C2A FRET-Adapta SP	-0.965
PIK3C2G FRET-Adapta SP	-1.050
MYLK3 Hu Bind FRET-Lantha SP	-1.113
MAP3K11 (MLK3) FRET-Lantha SP	-1.121
MAP4K5 (KHS1) FRET-Z-Lyte SP	-1.141
MAP2K1 (MEK1) FRET-Lantha SP	-1.153
MYLK (MLCK) FRET-Lantha SP	-1.187
PIK3CB/PIK3R2 FRET-Adapta SP	-1.195
PIM1 FRET-Z-Lyte SP	-1.231
CDKL5 FRET-Z-Lyte SP	-1.234
SGKL (SGK3) FRET-Z-Lyte SP	-1.282
TAOK2 (TAO1) FRET-Z-Lyte SP	-1.299
MET (cMet) FRET-Z-Lyte SP	-1.374
GRK5 *Mn* FRET-Z-Lyte SP	-1.432
SGK2 FRET-Z-Lyte SP	-1.482
PIK3CD/PIK3R1 FRET-Adapta SP	-1.504
MYLK4 FRET-Lantha SP	-1.538
CDC42 BPB (MRCKB) Z-Lyte SP	-1.544
PDPK1 (Direct) FRET-Z-lyte SP	-1.600
ZAK FRET-Lantha SP	-1.651
NUAK2 FRET-Lantha SP	-1.795
EGFR (ErbB1) *Mn* Z-Lyte SP	-1.842
DAPK1 FRET-Adapta SP	-1.914
CDK2/Cyclin O FRET-Lantha SP	-2.029
WNK3 FRET-Lantha SP	-2.040
FLT1 (VEGFR1) *Mn* Z-Lyte SP	-2.074
MAP3K14 (NIK) FRET-Lantha SP	-2.232
ANKK1 FRET-Lantha v2 SP	-2.285
CSNK1G1 (CK1gamma1) Z-Lyte SP	-2.320
CDK9 (Inactive) FRET-Lantha SP	-2.367

KINASE NAME/ASSAY FORMAT	MEAN INHIBITION (%)
PIK3CA/PIK3R3 FRET-Adapta SP	-2.464
MAP3K5 (ASK1) FRET-Lantha SP	-2.537
ERBB4 (HER4) *Mn* Z-Lyte SP	-2.799
CDK11(Inactive) FRET-Lantha SP	-2.904
IRAK1 FRET-Adapta SP	-2.983
FGFR3 *Mn* FRET-Z-Lyte SP	-3.160
CDC42 BPA (MRCKA) Z-Lyte SP	-3.181
PIK3C3 (hVPS34) *Mn* Adapta SP	-3.447
AAK1 FRET-Lantha SP	-3.536
TTK FRET-Lantha SP	-3.632
PI4K2A *Mn* FRET-Adapta SP	-3.804
BMPR2 FRET-Lantha SP	-4.214
MUSK *Mn* FRET-Z-Lyte SP	-4.285
CDK3/cyclin E1 FRET-Lantha SP	-4.312
VRK2 FRET-Lantha SP	-4.440
CAMKK1 (Alpha) FRET-Lantha SP	-4.586
SPHK1 FRET-Adapta SP	-4.651
PIP5K1C FRET-Adapta SP	-4.695
TEC FRET-Lantha SP	-5.136
MAP4K2 (GCK) FRET-Z-Lyte SP	-5.172
CASK FRET-Lantha SP	-5.323
CDK5 (Inactive) FRET-Lantha SP	-5.417
IRAK4 *Mn* FRET-Z-Lyte SP	-5.866
PRKCG (PKC gamma) Z-Lyte SP	-5.908
SPHK2 FRET-Adapta SP	-6.141
LRRK2 FL FRET-Adapta SP	-6.193
PIK3CB FRET-Adapta SP	-6.281
MAP3K9 (MLK1) FRET-Z-Lyte SP	-6.777
MLK4 FRET-Lantha SP	-9.807
TESK1 FRET-Lantha SP	-10.686
PIP5K1B FRET-Adapta SP	-11.784
PAK4 FRET-Z-Lyte SP	-12.759
PIP5K1A FRET-Adapta SP	-13.685
STK32B (YANK2) FRET-Lantha SP	-14.522
PIK3CG (p110 gamma) Adapta SP	-15.516
PDGFRA *Mn* FRET-Z-Lyte SP	-15.943

C. Kinase Selectivity (Dose Response)

[00253] Compound 137, Isomer 2 was further profiled for dose response against selected kinases at ThermoFisher Scientific. IC₅₀s were determined from a 10-point concentration response (CR) study with top testing concentration of 10 μ M. Data are reported in Table 5-B.

TABLE 5-B

KINASE NAME/ASSAY FORMAT	IC ₅₀ (μ M)	ATP CONCENTRATION (μ M)
PKMYT1 FRET-Lantha CR	0.003	
ACVR2B FRET-Lantha CR	0.025	
BMPR1B (ALK6) FRET-Lantha CR	0.036	
EPHA1 FRET-Z-Lyte CR	0.037	25.000
RAF1 Y340D Y341D Lantha CR	0.049	
ICK FRET-Lantha CR	0.080	
RAF1 Y340D Y341D Z-Lyte CR	0.083	100.000
TGFBR1 (ALK5) FRET-Lantha CR	0.119	
bRAF FRET-Lantha CR	0.138	
ACVRL1 (ALK1) FRET-Lantha CR	0.167	
ACVR1 (ALK2) FRET-Lantha CR	0.172	
SRC Hu Phos FRET-Z-Lyte CR	0.185	50.000
FGR FRET-Z-Lyte CR	0.201	10.000
ABL2 (Arg) FRET-Z-Lyte CR	0.208	25.000
YES1 FRET-Z-Lyte CR	0.249	25.000
ABL1 Hu Phos FRET-Z-Lyte CR	0.278	10.000
EPHB1 FRET-Z-Lyte CR	0.361	50.000
EPHB2 FRET-Z-Lyte CR	0.374	75.000
LYN B FRET-Z-Lyte CR	0.387	25.000
WEE1 FRET-Lantha CR	0.422	
EPHA2 FRET-Z-Lyte CR	0.451	75.000
EPHA5 FRET-Z-Lyte CR	0.460	150.000
EPHB4 FRET-Z-Lyte CR	0.466	100.000
BMPR1A (ALK3) FRET-Lantha CR	0.478	
DYRK1B FRET-Z-Lyte CR	0.614	75.000
GSK3B FRET-Z-Lyte CR	0.635	10.000
LCK FRET-Z-Lyte CR	0.867	50.000
bRAF Cascade FRET-Z-Lyte CR	0.963	100.000
EPHA3 FRET-Lantha CR	1.090	
EPHA4 FRET-Z-Lyte CR	1.140	100.000
EPHA8 FRET-Z-Lyte CR	1.170	100.000
ACVR1B (ALK4) *Mn* Z-Lyte CR	2.010	5.000
FYN A FRET-Lantha CR	2.410	
PIK3CA E545K/PIK3R1 Adapta CR	10.000	10.000
KIT *Mn* FRET-Z-Lyte CR	10.000	300.000

KINASE NAME/ASSAY FORMAT	IC ₅₀ (μM)	ATP CONCENTRATION (μM)
KDR (VEGFR2) FRET-Z-Lyte CR	10.000	75.000
FGFR2 *Mn* FRET-Z-Lyte CR	10.000	5.000
FGFR1 *Mn* FRET-Z-Lyte CR	10.000	25.000
EGFR (ErbB1) *Mn* Z-Lyte CR	10.000	10.000
AURKA (Aurora A) Z-Lyte CR	10.000	10.000

Example C-7: Metabolic Stability Assays

A. Assessment Using Human Liver Cytosol (HLC)

[00254] The stability of control (zaleplon and 06-benzylguanine) and test compounds was evaluated at 1 μM using 2.5 mg/mL human liver cytosol in the presence and absence of 3 μM raloxifene in 0.1 M phosphate buffer pH 7.4 at 37°C. Aliquots were removed at 0, 10, 30, 60, 90 and 120 min, quenched 1:5 with ACN containing internal standard. Samples were analyzed for loss of parent compound using LC/MS/MS. Potential hydroxylation metabolites were monitored by adding transitions of Parent+16. Extent of inhibition by raloxifene was derived by comparing the formation of metabolite and or the depletion of parent compound in the presence and absence of inhibitor.

B. Assessment Using Human Liver Microsomes (HLM)

[00255] The stability of control (phenacetin, verapamil, diclofenac, imipramine, benzydamine and metoprolol) and test compounds was evaluated using 1 mg/mL HLM (150 mixed gender donor pool) containing 1 mM NADPH, and 1 μM test compound/control. In short, 222.5 μL of HLM mixture containing 1.12 mg/mL HLM in phosphate buffer pH 7.4 was mixed with 25 μL 10 mM NADPH and pre-warmed at 37°C for 8 minutes prior to addition of 2.5 μL of 100 μM test compound/control prepared in DMSO. Aliquots (20 μL) were removed at 0.5, 5, 10, 15, 20 and 30 minutes and quenched with 100 μL of cold stop solution (100% ACN with 100 nM of alprazolam, caffeine, and tolbutamide as internal standards). Samples were centrifuged for 20 minutes at 4,000 rpm and 4°C and a 40 μL aliquot of supernatant was mixed with 160 μL pure water for LC-MS/MS analysis. Each incubation was conducted in singlicate. Peak areas were determined from extracted ion chromatograms and percent (%) parent remaining was calculated from peak area of test compound/control.

C. Assessment Using Human Hepatocytes in Suspension (hHEP)

[00256] The stability of control (phenacetin, verapamil, diclofenac, imipramine, chlorpromazine, and naloxone) and test compounds was evaluated using 1 million viable

cells/mL (10 mixed gender donor pool) and 1 μ M test compound/control. In short, cryopreserved hepatocytes are thawed in a 37° water bath, centrifuged in thawing medium and prepared in Leibovitz's L-15 Medium (pH 7.4) to contain 1 million viable hepatocytes/ml and a final compound concentration of 1 μ M. Cell viability was determined using a Cellometer Vision and greater than 80% cell viability was required to proceed with the compound incubation. The compound/cell solution (250 μ l) was incubated for 2 hours at 37°C and shaken at 900 rpm on an Eppendorf Thermomixer Comfort plate shaker. Samples (20 μ l) were taken at 0.5, 5, 15, 30, 45, 60, 80, 100, and 120 minutes and quenched with 100 μ l of 100% ice-cold acetonitrile. Samples were shaken at 800 rpm for 2 minutes and centrifuged at 4000 rpm for 20 minutes at 4°C to pellet precipitated protein. The supernatant fraction was diluted 1:5 with deionized water, shaken at 1000 rpm for 2 minutes, and further diluted 1:1 with deionized water. Samples were analyzed by LC-MS/MS. Each incubation was conducted in singlicate. Peak areas were determined from extracted ion chromatograms and percent (%) parent remaining was calculated from peak area of test compound/control.

D. Assessment Using Co-Culture Human Hepatocyte Model for Low Clearance

[00257] The stability of the test compound was determined over 72h incubation using human hepatocytes co-cultured with mouse JT3 fibroblast cells (H μ rel). In short, test compound was dissolved in DMSO at 10 mM, then diluted to 2 μ M in dosing medium using Echo dispense. All steps were performed at room temperature, while the 2 μ M test compound was preheated at 37°C before start of incubation. Prior to assay, maintenance medium was removed from the cells, followed by a washing step with 100 μ L of preheated blank dosing medium without serum. Compound incubation was initiated by addition of 50 μ L of dosing medium and 50 μ L of dosing medium containing 2 μ M test compound. The final test concentration was 1 μ M. Plates were kept without shaking in the incubator during the experiments, at 37°C in humidified atmosphere containing 95% air and 5% CO₂. 70 μ L aliquots of sample duplicates were taken at 0, 1, 3, 5, 24, 48 and 72 hours and quenched with 70 μ L of ice-cold acetonitrile stop solution. Quenched samples were diluted with 140 μ L of 0.1% formic acid in water and analyzed on a mass spectrometer.

E. Data Analysis for Metabolic Stability Assessments

[00258] The $t_{1/2}$ and the CL_{int} of the compounds incubated in the HH, HLM, and H μ REL assays as described above were calculated according to the following equations:

$t_{1/2}$ (min) = $\ln(2)/\text{-slope}$, and

Cl_{int} ($\mu\text{l}/\text{min}/10^6$ cells or mg protein) = $\ln(2)*V/(t_{1/2})$,

where V ($\mu\text{l}/\text{x } 10^6$ cells or mg protein) is the incubation volume (μl) divided by the number of cells ($\text{x } 10^6$) or microsomal protein content (mg) in the incubation.

[00259] Data for the metabolic stability assessments are reported in Table 6 below.

TABLE 6

COMPOUND	ISOMER	HLM ($\mu\text{l}/\text{min}/\text{mg}$)	Hhep ($\mu\text{l}/\text{min}/1\text{E}6$)
1	Isomer1	<3.336	<1.000
1	Isomer2	<3.000	
4	Racemate	12.000	
5	Racemate	43.700	
10	Isomer1	49.624	
10	Isomer2	46.045	
10	Racemate	52.407	
12	Racemate	16.900	
13	Racemate	6.070	
17	Isomer1	21.352	
17	Isomer2	13.514	
18	Racemate	4.110	
22	Racemate	16.800	
24	Racemate	14.900	
37	Racemate	43.000	
38	Racemate	10.900	
47	Isomer1	14.288	
47	Isomer2	15.138	
47	Racemate	15.462	
48	Isomer1	74.193	
48	Isomer2	29.010	
48	Racemate	47.123	
49	Isomer1	12.016	<1.000
49	Isomer2	7.555	
49	Racemate	10.055	
50	Isomer1	34.347	
50	Isomer2	16.252	
50	Racemate	24.062	
52	Isomer1	<3.000	
52	Isomer2	<3.000	
53	Isomer1	6.800	
53	Isomer2	9.700	
54	Racemate	16.200	
56	Isomer1	<3.000	
56	Isomer2	<3.000	

COMPOUND	ISOMER	HLM ($\mu\text{L}/\text{min}/\text{mg}$)	Hhep ($\mu\text{L}/\text{min}/1\text{E}6$)
57	Isomer1	8.110	
57	Isomer2	10.481	1.072
62	Isomer1	23.800	
62	Isomer2	33.100	
70	Isomer1	10.024	<1.000
70	Isomer2	17.436	
71	Isomer2	70.948	
71	Isomer1	145.851	
72	Racemate	8.140	
73	Isomer1	<3.000	3.392
73	Isomer2	<3.000	
74	Isomer1	53.282	
74	Isomer2	73.837	
75	Isomer1	13.871	
75	Isomer2	10.395	
79	Isomer1	<3.000	
79	Isomer2	<3.000	
83	Isomer1	8.880	
83	Isomer2	22.200	
84	Racemate	<3.000	
85	Isomer1	<3.000	
85	Isomer2	<3.000	
86	Isomer1	<3.000	
86	Isomer2	4.611	
92	Isomer1	7.183	
92	Isomer2	<3.000	
93	Isomer1	12.494	
93	Isomer2	5.751	
134	Racemate	7.340	
135	Isomer1	42.040	
135	Isomer2	42.608	
136	Racemate	41.253	
137	Isomer 2	<5.834	<1.324
138	Isomer 2	<3.000	<1.000
139	Isomer 1	19.567	4.441

Example C-8: Log D

[00260] The Log D values of test compounds at 10 μM were determined in octanol/PBS pH 7.4 using LC/MS/MS and nicardapine, cyclobenzaprine, and caffeine as control compounds. 10 μL of test compound or control was added to a 96-deep well plate followed by addition of 500 μL of saturated octanol and 500 μL of saturated phosphate buffer. The plate was shaken at room temperature for 2h at 2,000 rpm. The samples were centrifuged at room temperature, at 4,000

rpm for 30 minutes. 100 µL of sample was removed from the octanol and buffer phase. 5 µL of the octanol sample was mixed with 1:1 H₂O:ACN containing internal standard and vortexed at 1,000 rpm for 5 minutes (100-fold octanol sample), 50 µL of 100-fold samples were added to 450 µL of 1:1 H₂O:ACN containing internal standard and vortexed at 1,000 rpm for 5 min (1,000-fold octanol sample). Serial dilution of 1,000-fold octanol samples to 10,000, 100,000 and 1,000,000-fold was conducted. 50 µL of buffer samples were added to 450 µL of 1:1 H₂O:ACN containing internal standard and vortexed at 1,000 rpm for 5 min (10-fold buffer sample), and then further diluted to 100, 1,000 and 10,000 fold with 1:1 H₂O:ACN. Samples were analyzed using LC/MS/MS.

Log D was calculated as follows:

$$\text{Log } D = \frac{1}{n} * \sum \text{Log} \frac{\text{Area ratio octanol} \times \text{dilution factor octanol}}{\text{Area ratio buffer} \times \text{dilution factor buffer}}$$

[00261] Data are reported in Table 7 below.

TABLE 7

COMPOUND	ISOMER	LogD
1	Isomer1	1.349
1	Isomer2	1.384
4	Racemate	2.500
5	Racemate	2.600
10	Isomer1	3.044
10	Isomer2	2.930
10	Racemate	2.776
12	Racemate	2.200
13	Racemate	1.800
17	Isomer1	2.123
17	Isomer2	2.071
18	Racemate	1.400
22	Racemate	1.800
24	Racemate	2.200
37	Racemate	2.600
38	Racemate	1.800
47	Isomer1	1.918
47	Isomer2	1.877
47	Racemate	2.010
48	Isomer1	2.419
48	Isomer2	2.393
48	Racemate	2.487
49	Isomer1	1.791

COMPOUND	ISOMER	LogD
49	Isomer2	1.808
49	Racemate	1.897
50	Isomer1	2.296
50	Isomer2	2.603
50	Racemate	2.353
52	Isomer1	1.780
52	Isomer2	1.693
53	Isomer1	1.900
53	Isomer2	1.800
54	Racemate	2.200
56	Isomer1	1.002
56	Isomer2	0.928
57	Isomer1	1.871
57	Isomer2	1.761
62	Isomer1	2.300
62	Isomer2	2.200
70	Isomer1	1.421
70	Isomer2	1.477
71	Isomer2	2.614
71	Isomer1	2.618
72	Racemate	1.900
73	Isomer1	1.675
73	Isomer2	1.680
74	Isomer1	2.712
74	Isomer2	2.791
75	Isomer1	1.873
75	Isomer2	2.056
79	Isomer1	1.548
79	Isomer2	1.236
83	Isomer1	2.000
83	Isomer2	2.000
84	Racemate	0.800
85	Isomer1	0.963
85	Isomer2	0.973
86	Isomer1	1.688
86	Isomer2	1.708
92	Isomer1	1.493
92	Isomer2	1.351
93	Isomer1	1.870
93	Isomer2	1.759
134	Racemate	1.500
135	Isomer1	2.745
135	Isomer2	2.694
136	Racemate	2.289
137	Isomer 2	1.364

COMPOUND	ISOMER	LogD
138	Isomer 2	1.180
139	Isomer 1	1.867

Example C-9: Caco-2 Cell Permeability

[00262] Test compounds were evaluated for gastrointestinal permeability and potential bioavailability in a Caco-2 cell permeability assay. Detailed description of the methodology was previously published in “Evaluation of the Disconnect between Hepatocyte and Microsome Intrinsic Clearance and *In Vitro In Vivo* Extrapolation Performance”; Williamson Beth, Harlfinger Steffanie, McGinnity F. Dermot; Drug Metab Dispos 48:1137–1146, November 2020. In brief, Caco-2 cells were plated at 6.86×10^5 cells/mL and were cultured for 14 to 18 days with culture medium replaced every other day. Test compound (10 μ M) was added to the donor well and the appearance in the receiver well measured after 2 hours incubation at 37°C. The rate of compound transport in the apical to basolateral (A-B) direction, was determined from the donor well as apical (A) compartment and the receiver as basolateral (B) compartment. Similarly, the rate of compound transport in the basolateral to apical (B-A) direction, the donor well was the basolateral (B) compartment and the receiver was the apical (A) compartment. Samples were analyzed by liquid chromatography (LC)–mass spectrometry (MS)/MS.

[00263] The permeability coefficient (1×10^{-6} cm/s) was calculated using the following equation:

$$P_{app} = (dC_r/dt) \times V_r / (A \times C_0), \text{ and}$$

the efflux ratio (ER) was calculated using the following equation:

$$\text{Efflux ratio} = P_{app}(\text{B to A}) / P_{app}(\text{A to B}),$$

where:

dC_r/dt is the cumulative concentration of the compound in the receiver chamber as a function of time (in μ M/s);

V_r is the solution volume in the receiver chamber (0.1 ml on the apical side and 0.3 ml on the basolateral side);

A is the surface area for the transport (i.e., 0.11 cm² for the area of the monolayer); and

C_0 is the initial concentration in the donor chamber (in μ M).

[00264] Data are reported in Table 8 below.

TABLE 8

COMPOUND	ISOMER	Caco-2 A-B Papp (1E-6.cm/s)	Caco-2 EFFLUX RATIO
1	Isomer1	5.045	5.157
1	Isomer2	2.802	6.579
47	Isomer1	17.626	13.368
47	Isomer2	16.855	14.609
47	Racemate	18.249	23.328
49	Isomer1	8.018	11.874
52	Isomer1	9.952	3.405
56	Isomer1	0.772	9.906
57	Isomer1	11.695	5.516
57	Isomer2	11.238	7.975
70	Isomer1	4.047	5.575
73	Isomer1	4.046	18.480
79	Isomer1	0.859	12.673
84	Racemate	4.366	12.236
137	Isomer 2	30.060	1.842
138	Isomer 2	21.006	3.803
139	Isomer 1	35.644	2.431

*Example C-10: CYP Assays***A. CYP Inhibition**

[00265] The potential for a test compound to inhibit cytochrome P450 (CYP) isoforms was determined using a 150-donor mixed gender human liver microsomes (HLM) and a cocktail of substrates selective for CYPs 1A2, 2C9, 2C19, 2D6 and 3A at incubation concentrations equivalent to their K_m values. Specific inhibitors of these pathways were included as positive controls. Substrates were, Phenacetin (30 μ M), diclofenac (10 μ M), S-mepheytin (35 μ M), bufuralol (5 μ M) and midazolam (3 μ M), for CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4/5, respectively. The final concentration of HLMs was 0.2 mg/mL and the final concentrations of test compound were 0, 0.1, 0.3, 1, 3, 10 and 30 μ M. The incubation was initiated by addition of 20 μ L of 10 mM NADPH in phosphate buffer and quenched 1:1 with ACN containing 3% formic acid and 40 nMol verapamil as internal standard, following five minutes at 37°C. The samples were centrifuged at 4,000 rpm for 30 minutes, placed on ice for 20 min and recentrifuged for an additional 30 min at 4,000 rpm. Supernatant (200 μ L) was transferred for analysis of isoform selective metabolite formation. The extent of inhibition was calculated by comparing the remaining activity in the presence of inhibitor to the DMSO only

control using the following equation. The concentration yielding 50% inhibition, IC₅₀, were calculated using non-linear regression analysis in Xlfit.

$$\text{Area Ratio} = \frac{\text{Peak area}_{\text{analyte}}}{\text{Peak area}_{\text{internal standard}}}$$

$$\text{Remaining Activity (\%)} = \frac{\text{Area Ratio}_{\text{test compound}}}{\text{Area Ratio}_{\text{DMSO control}}} \times 100\%$$

[00266] The IC₅₀ values for Example B-1, Isomer 1 (i.e., the eutomer of Compound 1) and Example B-2, Isomer 2 (i.e., the eutomer of Compound 137) against each of CYPs 1A2, 2C9, 2C19, 2D6, and 3A were greater than 30 μM.

B. CYP Time Dependent Inhibition

[00267] The potential for test compounds to inhibit Cytochrome P450 (CYP) activity in a time dependent manner was determined using 1 mg/mL of a 150-donor mixed gender human liver microsome pool (HLM), 10 μM test compound, a 30 min preincubation time, and a 10-fold dilution step to measure formation of specific substrates towards, CYPs 1A2, 2C9, 2C19, 2D6 and 3A at 3X their respective Km values. Substrates were, Phenacetin (90 μM), diclofenac (30 μM), S-mepheytoin (105 μM), bufuralol (15 μM), and midazolam (9 μM), for CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4/5, respectively. Specific inhibitors of these pathways were included as positive controls. The primary incubation consisted of 1 mg/mL HLM ± 10 mM NADPH with 10 μM of test compound incubated for 30 min at 37°C. At 30 min a 10 μL aliquot of the pre-incubation solution was transferred to the secondary incubation containing 10 mM NADPH and substrate cocktail. Following 15 min incubation at 37°C the secondary incubation was quenched by addition of 150 μL of cold methanol containing 20 nM verapamil as internal standard. A decrease in the formation of isoform specific metabolites was determined by comparing with and with NADPH control and the % time dependent inhibition (TDI) was calculated with the following equation. A % TDI of > 20 was considered positive and warranting further investigation.

$$\% TDI = \left(1 - \frac{\% \text{ control activity}^{NADPH}}{\% \text{ control activity}^{No NADPH}} \right) \times 100$$

where:

$$\% \text{ control activity}^{NADPH} = \frac{(\text{ratio of test compound or inhibitor}^{NADPH})}{(\text{average ratio of vehicle control}^{NADPH})} \times 100$$

$$\% \text{ control activity}^{No NADPH} = \frac{(\text{ratio of test compound or inhibitor}^{No NADPH})}{(\text{average ratio of vehicle control}^{No NADPH})} \times 100$$

The irreversible IC₅₀, representing the concentration where 50% inhibition would be observed, was calculated as follows:

$$\text{projected irreversible IC}_{50} = [I] \times 10^{(50 - \% TDI) \times 0.025}$$

[00268] The % time dependent inhibition (TDI) values calculated for Example B-1, Isomer 1 (i.e., the eutomer of Compound 1) and Example B-2, Isomer 2 (i.e., the eutomer of Compound 137) against each of CYPs 1A2, 2C9, 2C19, 2D6, and 3A were less than 20.

C. CYP Induction

[00269] The potential for test compounds to induce drug metabolizing enzymes was evaluated by assessing the Pregnane X Receptor (PXR) activation potential in DPX2 cells. DPX2 cells were purchased from Puracyp and treated according to the vendor protocol. Test compounds, positive control (rifampicin, 1 and 10 μM) and negative control (propranolol, 10 μM) were prepared in DMSO to a final concentration of 0.1%. Test compounds were evaluated at 0.1, 0.3, 1, 3, 10 and 30 μM to enable derivation of EC₅₀ and E_{max} values, corresponding to the concentration resulting in half maximal induction and the maximal induction observed, respectively. Cells were treated with test compound or control for 24h. After 24h dosing media was removed and 1xCellTiter-Fluor™ was added for 30 minutes at 37°C. The fluorescence was measured using 400 nm excitation and 505 nm emission. The ONE-Glo assay reagent was then added for 5 min and luminescence was determined using a luminometer. The cell viability was determined using the following equation:

$$\% \text{ cell viability} = \left(\frac{RFU_{\text{test}}}{RFU_{\text{vehicle}}} \right) \times 100$$

The normalized luciferase activity was determined by taking the ratios of relative luminescence units (RLU), and relative fluorescence units for three replicates at each concentration and the fold-activation of mRNA levels was determined using the following equation:

$$\text{Fold activation} = \frac{RLU_{\text{test}}}{RFU_{\text{test}}} \bigg/ \frac{RLU_{\text{vehicle}}}{RFU_{\text{vehicle}}}$$

[00270] No induction of PXR was observed for Example B-1, Isomer 1 (i.e., the eutomer of Compound 1).

Example C-11: Aqueous Solubility

[00271] The thermodynamic solubility of the test compounds was measured in a shake-flask approach starting from 10 mM DMSO solutions. DMSO was evaporated and dried compounds were equilibrated in glass vials in aqueous phosphate buffer (0.1 M, pH 7.4) for 24 hours at 25°C under constant stirring. The portion with the dissolved compound was then separated from the remainder through a double centrifugation with a tip wash in between to remove possible interference of residual dried compound. The solutions were diluted with purified water before quantification using UPLC/MS/MS.

[00272] Data are reported in Table 9 below.

TABLE 9

COMPOUND	ISOMER	SOLUBILITY pH 7.4 (μM)
1	Isomer1	1263
1	Isomer2	910
4	Racemate	31
5	Racemate	218
10	Isomer1	18
10	Isomer2	34
10	Racemate	5
12	Racemate	30
13	Racemate	>1000
17	Isomer1	>1000
17	Isomer2	>1000
18	Racemate	62
22	Racemate	>1000
24	Racemate	977
37	Racemate	130
38	Racemate	935

COMPOUND	ISOMER	SOLUBILITY pH 7.4 (μM)
47	Isomer1	>1000
47	Isomer2	>944
47	Racemate	>1000
48	Isomer1	>1000
48	Isomer2	>1000
48	Racemate	>1000
49	Isomer1	>995
49	Isomer2	>1000
49	Racemate	>1000
50	Isomer1	>994
50	Isomer2	>595
50	Racemate	>1000
52	Isomer1	>1000
52	Isomer2	>1000
53	Isomer1	797
53	Isomer2	>1000
56	Isomer1	>1000
56	Isomer2	>1000
57	Isomer1	>918
57	Isomer2	>941
62	Isomer1	360
62	Isomer2	447
70	Isomer1	378
70	Isomer2	236
71	Isomer2	180
71	Isomer1	115
72	Racemate	150
73	Isomer1	118
73	Isomer2	136
74	Isomer1	46
74	Isomer2	11
75	Isomer1	>1000
75	Isomer2	361
79	Isomer1	439
79	Isomer2	>1000
83	Isomer1	871
83	Isomer2	>1000
84	Racemate	395
85	Isomer1	611
85	Isomer2	760
86	Isomer1	84
86	Isomer2	94
92	Isomer1	>1000
92	Isomer2	>1000

COMPOUND	ISOMER	SOLUBILITY pH 7.4 (μM)
93	Isomer1	>1000
93	Isomer2	>1000
134	Racemate	>1000
135	Isomer1	106
135	Isomer2	84
136	Racemate	125
137	Isomer 2	1541
138	Isomer 2	>1000
139	Isomer 1	>958

Example C-12: Kinase Selectivity (Kinase Affinity Tool Assay)

[00273] A Kinase Affinity Tool (“KAT”) chemical proteomics method based on the Kinobead technology¹⁻² was used for *in situ* kinase profiling of test compound. KAT is an affinity matrix made up of promiscuous ATP-competitive kinase inhibitors to enrich for a large subset of the native kinome directly from disease/safety relevant lysate. Proteomic RB50 values indicate the selective affinity of the test compound for the enriched kinases.

[00274] Test compound was incubated with a cell lysate mix of four cell lines (K562, MV4-11, SN-N-BE2, and COLO-205) in a dose-response format for 30 minutes at 4°C. The compound-incubated lysates were then bound to KAT beads for 30 minutes at 4°C. Beads were washed twice with binding buffer followed by two washes with 1X PBS. Proteins bound to beads were reduced, alkylated, and digested overnight with Trypsin/LysC enzyme at 37°C. Digested peptides were eluted with 60% ACN/0.1% formic acid in water and desalted with in-house made C18 StageTips. Peptides were analyzed by label-free Data Independent Acquisition (DIA) quantitative mass spectrometry method on Orbitrap LUMOS. Proteomics data was analyzed with in-house software Doscheda to generate RB50 values³. RB50 values determined for Compound 137, Isomer 2 are reported in Table 10 and confirm that the compound is highly selective for PKMYT1.

[00275] References:

1. Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors Nat. Biotechnol. 2007, 25, 1035-44 <https://www.nature.com/articles/nbt1328>.
2. Optimized chemical proteomics assay for kinase inhibitor profiling J. Proteome Res. 2015, 14, 1574-86 <https://pubs.acs.org/doi/10.1021/pr5012608>.
3. DOSCHEDA: a web application for interactive chemoproteomics data analysis. PeerJ Computer Science 2017 <https://peerj.com/articles/cs-129/>.

TABLE 10

KINASE	RB50 (M)
PKMYT1	8.32E-10
EPHA1	2.40E-09
ACVRL1	1.20E-08
ACVR1B	2.07E-08
BMPR1B	2.07E-08
BMPR1A	4.66E-08
EPHA2	4.85E-08
ACVR1	5.55E-08
ACVR2A	6.27E-08
TGFBR1	8.79E-08
EPHB3	1.14E-07
BCR	1.19E-07
ABL2	1.32E-07
PKN3	1.63E-07
EPHA5	1.68E-07
EPHB4	1.73E-07
EPHB2	1.76E-07
SRC	1.78E-07
EPHB1	1.91E-07
FYN	2.10E-07
TNK2	2.41E-07
FRK	2.45E-07
CDKL5	2.50E-07
PTK6	2.78E-07
CLK1	2.91E-07
DYRK1B	3.08E-07
EPHA4	3.24E-07
WEE1	3.61E-07
DDR1	3.64E-07
FGR	3.87E-07
ABL1	3.89E-07
CLK2	4.17E-07
DYRK1A	4.33E-07
CCNH	1.10E-06
BRAF	2.74E-06
CCNK	4.47E-06
AAK1	>5.00E-05
ADCK1	>5.00E-05
ADCK5	>5.00E-05
ADK	>5.00E-05
ADPGK	>5.00E-05
AGK	>5.00E-05

KINASE	RB50 (M)
AK1	>5.00E-05
AK2	>5.00E-05
AKAP13	>5.00E-05
AKT1	>5.00E-05
AKT2	>5.00E-05
AKT3	>5.00E-05
ALK	>5.00E-05
ARAF	>5.00E-05
ATM	>5.00E-05
ATR	>5.00E-05
AURKA	>5.00E-05
AURKB	>5.00E-05
AXL	>5.00E-05
BMP2K	>5.00E-05
BMPR2	>5.00E-05
BRSK2	>5.00E-05
BTK	>5.00E-05
BUB1	>5.00E-05
BUB1B	>5.00E-05
CAB39	>5.00E-05
CAMK1D	>5.00E-05
CAMK2A	>5.00E-05
CAMK2B	>5.00E-05
CAMK2D	>5.00E-05
CAMK2G	>5.00E-05
CAMK4	>5.00E-05
CAMKK2	>5.00E-05
CASK	>5.00E-05
CCNB1	>5.00E-05
CCNE1	>5.00E-05
CDC42BPA	>5.00E-05
CDC42BPB	>5.00E-05
CDC42BPG	>5.00E-05
CDC7	>5.00E-05
CDK1	>5.00E-05
CDK10	>5.00E-05
CDK12	>5.00E-05
CDK14	>5.00E-05
CDK16	>5.00E-05
CDK17	>5.00E-05
CDK18	>5.00E-05
CDK19	>5.00E-05
CDK2	>5.00E-05

KINASE	RB50 (M)
CDK4	>5.00E-05
CDK5	>5.00E-05
CDK6	>5.00E-05
CDK7	>5.00E-05
CDK8	>5.00E-05
CDK9	>5.00E-05
CHEK1	>5.00E-05
CHEK2	>5.00E-05
CHUK	>5.00E-05
CIT	>5.00E-05
CKB	>5.00E-05
CKMT1A	>5.00E-05
CLK3	>5.00E-05
CLK4	>5.00E-05
CMPK1	>5.00E-05
COASY	>5.00E-05
CPNE3	>5.00E-05
CSK	>5.00E-05
CSNK1A1	>5.00E-05
CSNK1D	>5.00E-05
CSNK1E	>5.00E-05
CSNK1G1	>5.00E-05
CSNK1G2	>5.00E-05
CSNK1G3	>5.00E-05
CSNK2A2	>5.00E-05
CSNK2B	>5.00E-05
DAPK1	>5.00E-05
DAPK2	>5.00E-05
DAPK3	>5.00E-05
DCAKD	>5.00E-05
DCK	>5.00E-05
DDR2	>5.00E-05
DMPK	>5.00E-05
DOLK	>5.00E-05
DTYMK	>5.00E-05
DYRK2	>5.00E-05
EGFR	>5.00E-05
EIF2AK1	>5.00E-05
EPHA6	>5.00E-05
EPHA7	>5.00E-05
EPHB6	>5.00E-05
ERCC2	>5.00E-05
ERN1	>5.00E-05

KINASE	RB50 (M)
ERN2	>5.00E-05
FAM20B	>5.00E-05
FASTKD3	>5.00E-05
FER	>5.00E-05
FES	>5.00E-05
FGFR1	>5.00E-05
FGFR3	>5.00E-05
FGFR4	>5.00E-05
FKBP1A	>5.00E-05
FLT3	>5.00E-05
FN3K	>5.00E-05
FN3KRP	>5.00E-05
GAK	>5.00E-05
GK	>5.00E-05
GPR108	>5.00E-05
GSG2	>5.00E-05
GSK3A	>5.00E-05
GSK3B	>5.00E-05
GTF2H4	>5.00E-05
HCK	>5.00E-05
HIPK1	>5.00E-05
HIPK2	>5.00E-05
HIPK3	>5.00E-05
HK1	>5.00E-05
HTATIP2	>5.00E-05
IGF1R	>5.00E-05
IKBKB	>5.00E-05
IKBKE	>5.00E-05
INSR	>5.00E-05
IPMK	>5.00E-05
IRAK1	>5.00E-05
IRAK3	>5.00E-05
IRAK4	>5.00E-05
ITPK1	>5.00E-05
JAK1	>5.00E-05
JAK2	>5.00E-05
JAK3	>5.00E-05
KHK	>5.00E-05
KIT	>5.00E-05
LATS1	>5.00E-05
LATS2	>5.00E-05
LCK	>5.00E-05
LIMK1	>5.00E-05

KINASE	RB50 (M)
LIMK2	>5.00E-05
LYN	>5.00E-05
MAP2K1	>5.00E-05
MAP2K2	>5.00E-05
MAP2K3	>5.00E-05
MAP2K4	>5.00E-05
MAP2K5	>5.00E-05
MAP2K6	>5.00E-05
MAP3K1	>5.00E-05
MAP3K10	>5.00E-05
MAP3K11	>5.00E-05
MAP3K2	>5.00E-05
MAP3K3	>5.00E-05
MAP3K4	>5.00E-05
MAP3K5	>5.00E-05
MAP3K6	>5.00E-05
MAP4K1	>5.00E-05
MAP4K2	>5.00E-05
MAP4K3	>5.00E-05
MAP4K5	>5.00E-05
MAPK1	>5.00E-05
MAPK10	>5.00E-05
MAPK11	>5.00E-05
MAPK14	>5.00E-05
MAPK15	>5.00E-05
MAPK3	>5.00E-05
MAPK6	>5.00E-05
MAPK7	>5.00E-05
MAPK8	>5.00E-05
MAPK9	>5.00E-05
MAPKAPK2	>5.00E-05
MAPKAPK5	>5.00E-05
MARK2	>5.00E-05
MARK3	>5.00E-05
MARK4	>5.00E-05
MAST3	>5.00E-05
MELK	>5.00E-05
MERTK	>5.00E-05
MET	>5.00E-05
MKNK1	>5.00E-05
MKNK2	>5.00E-05
MNAT1	>5.00E-05
MPP1	>5.00E-05

KINASE	RB50 (M)
MST1R	>5.00E-05
MTOR	>5.00E-05
MYLK	>5.00E-05
MYLK3	>5.00E-05
NEK1	>5.00E-05
NEK2	>5.00E-05
NEK3	>5.00E-05
NEK6	>5.00E-05
NEK7	>5.00E-05
NEK9	>5.00E-05
NLK	>5.00E-05
NME1	>5.00E-05
NME2	>5.00E-05
NME3	>5.00E-05
NTRK1	>5.00E-05
PAK2	>5.00E-05
PAK4	>5.00E-05
PAK6	>5.00E-05
PASK	>5.00E-05
PDGFRB	>5.00E-05
PDIK1L	>5.00E-05
PDPK1	>5.00E-05
PDXK	>5.00E-05
PGK1	>5.00E-05
PHKA2	>5.00E-05
PHKG2	>5.00E-05
PI4K2A	>5.00E-05
PI4KA	>5.00E-05
PIK3C2B	>5.00E-05
PIK3R4	>5.00E-05
PIKFYVE	>5.00E-05
PIM1	>5.00E-05
PIM2	>5.00E-05
PIM3	>5.00E-05
PIP4K2C	>5.00E-05
PKM	>5.00E-05
PKN1	>5.00E-05
PKN2	>5.00E-05
PLK1	>5.00E-05
PLK4	>5.00E-05
PPP4C	>5.00E-05
PRKAA1	>5.00E-05
PRKAB1	>5.00E-05

KINASE	RB50 (M)
PRKAB2	>5.00E-05
PRKACA	>5.00E-05
PRKAG1	>5.00E-05
PRKAG3	>5.00E-05
PRKCA	>5.00E-05
PRKCB	>5.00E-05
PRKCD	>5.00E-05
PRKCE	>5.00E-05
PRKCH	>5.00E-05
PRKCI	>5.00E-05
PRKCQ	>5.00E-05
PRKCZ	>5.00E-05
PRKD2	>5.00E-05
PRKD3	>5.00E-05
PRKDC	>5.00E-05
PRKG1	>5.00E-05
PRKX	>5.00E-05
PTK2	>5.00E-05
PTK2B	>5.00E-05
RAF1	>5.00E-05
RET	>5.00E-05
RIOK1	>5.00E-05
RIPK1	>5.00E-05
RIPK2	>5.00E-05
RIPK3	>5.00E-05
ROCK1	>5.00E-05
ROCK2	>5.00E-05
RPS6KA1	>5.00E-05
RPS6KA2	>5.00E-05
RPS6KA3	>5.00E-05
RPS6KA4	>5.00E-05
RPS6KA5	>5.00E-05
RPS6KA6	>5.00E-05
RPS6KB1	>5.00E-05
SIK2	>5.00E-05
SIK3	>5.00E-05
SLK	>5.00E-05
SMG1	>5.00E-05
STK10	>5.00E-05
STK11	>5.00E-05
STK16	>5.00E-05
STK17A	>5.00E-05
STK17B	>5.00E-05

KINASE	RB50 (M)
STK25	>5.00E-05
STK3	>5.00E-05
STK33	>5.00E-05
STK35	>5.00E-05
STK36	>5.00E-05
STK38L	>5.00E-05
STK4	>5.00E-05
STRADA	>5.00E-05
STRADB	>5.00E-05
SYK	>5.00E-05
TAOK1	>5.00E-05
TAOK2	>5.00E-05
TAOK3	>5.00E-05
TBK1	>5.00E-05
TEC	>5.00E-05
TESK1	>5.00E-05
TGFB2	>5.00E-05
TK1	>5.00E-05
TK2	>5.00E-05
TMEM11	>5.00E-05
TNK1	>5.00E-05
TP53RK	>5.00E-05
TRIM27	>5.00E-05
TTK	>5.00E-05
TYK2	>5.00E-05
TYRO3	>5.00E-05
ULK1	>5.00E-05
ULK3	>5.00E-05
ULK4	>5.00E-05
YES1	>5.00E-05

Example C-13: Radioligand Binding and Cell-Based Functional Assays (Secondary Pharmacology)

[00276] Secondary pharmacology assays were performed at Eurofins using standard experimental techniques against 80 targets across a broad pharmacological space. Assay details along with experimental protocols are described at <https://www.eurofinsdiscoveryservices.com>. Radioligand binding assays were used to assess the ability of Compound 137, Isomer 2 to interact with a panel of G-protein coupled receptors (GPCRs), ion channels, and transmembrane transporters. Assays measuring substrate turnover or phosphorylation by isolated proteins were used for kinase and enzyme targets, allowing direct determination of the mode of action of the

compounds. Where binding activity was observed at a GPCR target, mode of action was determined using cell based functional assays with secondary messenger read outs. Assays were run in eight points concentration response mode with half log dilutions and IC_{50} , EC_{50} or K_i values were determined (Cheng, Y., Prusoff, W.H. 1973 Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction Biochem Pharmacol 22(23):3099-108).

[00277] Table 11 reports the values obtained for Compound 137, Isomer 2.

TABLE 11

TARGET (HUMAN UNLESS OTHERWISE STATED)	BINDING K_i OR ENZYME IC_{50} (μ M)	PHARMACOLOGICAL MODE OF ACTION
v-src sarcoma Kinase	0.15	Inhibitor
ALK4 Kinase	0.19	Inhibitor
Glycogen synthase kinase-3 β	1.1	Inhibitor
c-kit kinase	32	Inhibitor
Fibroblast growth factor receptor 1 kinase (FGFR1 kinase)	40	Inhibitor
Lymphocyte-specific protein tyrosine kinase	53	Inhibitor
Kinase insert domain receptor kinase (KDR)	78	Inhibitor
Thromboxane A2 synthase	90	Inhibitor
TP prostanoid receptor	>30.1	Inactive
Cathepsin S	>10	Inactive
Histone deacetylase 5	>10	Inactive
5-HT _{1A} receptor	>100	Inactive
5-HT _{1B} receptor	>100	Inactive
5-HT _{1D} receptor (Rat)	>100	Inactive
5-HT _{2B} receptor	>100	Inactive
5-HT _{2C} receptor	>100	Inactive
5-HT _{3A} receptor	>100	Inactive
5-HT ₄ receptor	>100	Inactive
5-HT ₇ receptor	>100	Inactive
A ₁ adenosine receptor	>100	Inactive
A _{2A} adenosine receptor	>100	Inactive
AT ₁ angiotensin receptor	>100	Inactive
Acetylcholinesterase	>100	Inactive
Adenosine Transporter (Guinea Pig)	>100	Inactive

TARGET (HUMAN UNLESS OTHERWISE STATED)	BINDING K _i OR ENZYME IC ₅₀ (μM)	PHARMACOLOGICAL MODE OF ACTION
Androgen receptor	>100	Inactive
Aurora A kinase	>100	Inactive
B ₂ bradykinin receptor	>100	Inactive
CB ₁ cannabinoid receptor	>100	Inactive
CCK ₁ cholecystokinin receptor	>100	Inactive
D ₁ dopamine receptor	>100	Inactive
D _{2L} dopamine receptor	>100	Inactive
D ₃ dopamine receptor	>100	Inactive
Dopamine transporter (DAT)	>100	Inactive
ET _A endothelin receptor	>100	Inactive
Epidermal growth factor receptor kinase (EGFR kinase)	>100	Inactive
GABA _A receptor, BNZ site (Rat)	>100	Inactive
GABA _A α1β2γ2 receptor	>100	Inactive
Ghrelin receptor	>100	Inactive
Glucocorticoid Receptor NR3C1	>100	Inactive
Glycine receptor, Strychnine-Sensitive (Rat)	>100	Inactive
H ₁ histamine receptor	>100	Inactive
H ₂ histamine receptor	>100	Inactive
Insulin receptor kinase (IRK)	>100	Inactive
L-Type calcium channel (CaV1.2) (Benzothiazepine) (Rat)	>100	Inactive
L-Type calcium channel (CaV1.2) (Phenylalkylamine) (Rat)	>100	Inactive
M ₁ muscarinic receptor	>100	Inactive
M ₂ muscarinic receptor	>100	Inactive
M ₅ muscarinic receptor	>100	Inactive
MT ₂ melatonin receptor	>100	Inactive
Mitogen activated protein kinase kinase kinase 7 (MAPKKK7)	>100	Inactive
NK ₁ neurokinin receptor	>100	Inactive
NMDA receptor (Agonist site; Rat)	>100	Inactive
NMDA receptor (Phencyclidine site; Rat)	>100	Inactive
Na ⁺ /K ⁺ ATPase (Porcine)	>100	Inactive
Neurotrophic receptor kinase 1 (NTRK1)	>100	Inactive
Noradrenaline Transporter (NET)	>100	Inactive

TARGET (HUMAN UNLESS OTHERWISE STATED)	BINDING K _i OR ENZYME IC ₅₀ (μM)	PHARMACOLOGICAL MODE OF ACTION
Peroxisome proliferator-activated receptor-γ	>100	Inactive
Phosphodiesterase PDE10A2	>100	Inactive
Phosphodiesterase PDE3	>100	Inactive
Phosphodiesterase PDE4D2	>100	Inactive
Phosphodiesterase PDE6 (Bovine)	>100	Inactive
Prostaglandin-endoperoxide synthase 1 (COX1)	>100	Inactive
Prostaglandin-endoperoxide synthase 2 (COX2)	>100	Inactive
Retinoic acid receptor-α	>100	Inactive
Rho Associated, Coiled Coil Containing Protein Kinase 1 (ROCK1)	>100	Inactive
Rho Associated, Coiled Coil Containing Protein Kinase 2 (ROCK2)	>100	Inactive
SST ₄ somatostatin receptor	>100	Inactive
Serotonin Transporter (SERT)	>100	Inactive
Sigma σ ₁ Receptor	>100	Inactive
Translocator Protein (18kDa) (TSPO)	>100	Inactive
V _{1A} vasopressin receptor	>100	Inactive
α ₁ nicotinic receptor	>100	Inactive
α _{1A} adrenoceptor	>100	Inactive
α _{1B} adrenoceptor	>100	Inactive
α _{2A} adrenoceptor	>100	Inactive
α _{2C} adrenoceptor	>100	Inactive
α ₄ nicotinic receptor	>100	Inactive
α ₇ nicotinic receptor	>100	Inactive
β ₁ adrenoceptor	>100	Inactive
β ₂ adrenoceptor	>100	Inactive
δ opioid Receptor	>100	Inactive
κ opioid receptor	>100	Inactive
μ opioid receptor	>100	Inactive

Example C-14: THP-1 Assay (Cytotoxicity)

[00278] Test compounds were evaluated for compound-induced cytotoxicity in a human monocytic cell line (THP-1). The *in vitro* assay used the fluorescent signal generated by the

reduction of non-fluorescent resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) to the fluorescent resorufin (Alamar blue assay) as a measure of cytotoxicity. Because cellular reduction of resazurin is dependent on a pool of reductase or diaphorase enzymes derived from the mitochondria and cytosol, resazurin can be used as an oxidation-reduction indicator in cell viability assays for mammalian cells (McMillian et al, 2002; O'Brien et al, 2000).

[00279] THP-1 cells were subcultured in suspension in a T175cm² tissue culture flask with medium (RPMI, supplemented with 1% L-glutamine and 10% quantified FBS). The sub-cultures were maintained at 37°C in a 95% humidified atmosphere with 5% CO₂ and passaged approximately every 2 to 3 days. Cell density was maintained between 50,000 cells/mL and 1 x10⁶ cells/mL.

[00280] Test compounds (40 nL) were added to a 1536-well low volume plate (Greiner #782077) at 10, half-log concentrations with a top concentration of either 100µM or 250µM depending on the stock concentration selected. For both, the DMSO concentration was 1%.

[00281] Using a Multidrop Combi Reagent dispenser (Type 836), with a Multidrop standard tube plastic tip dispensing cassette, THP-1 cells were seeded into the plates at a density of 2,000 cells per well in 4µl THP-1 media on slow speed. The plates containing THP-1 cells with compounds/solvent were incubated for 48 hours under standard cell culture conditions (37°C, 5% CO₂).

[00282] Following an incubation period of 48 hours, a stock solution of resazurin (113 mg of resazurin in 1000 mL of PBS) was prepared, warmed to 37°C, and vortexed. Using the Multidrop dispenser, with the Multidrop standard cassette, 1µL of stock resazurin solution was added to all wells at high speed to aid mixing. The plates were incubated for two hours under standard cell culture conditions. The plates were then incubated for a further one hour at room temperature, with shaking (700 rpm).

[00283] The plates were then read on a BMG PheraStar reader using an excitation wavelength of 540 nm and an emission wavelength of 580nm. Controls were 100 µM Lapatinib (minimum/inhibitor control) and 1% DMSO (maximum/neutral control). The acquired raw data were loaded into Genedata analyser (Genedata AG) to determine IC₅₀ values. The primary (fluorescent) signal for each concentration-effect curve was normalized to vehicle control (0% inhibition) and inhibitor control (100% inhibition). The obtained inhibition values were fitted using four-parameter logistic equation and the IC₅₀ values calculated.

[00284] Compound 137, Isomer 2 had an IC₅₀ value of 34 μ M indicating that the compound has a reduced risk of cytotoxicity in THP-1 cells relative to earlier compounds tested.

[00285] References:

1. McMillian, MK *et al* (2002). An improved resazurin-based cytotoxicity assay for hepatic cells. *Cell Biol. & Toxicol.*, 18: 157-173.
2. O'Brien, J *et al* (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell toxicity. *Eur J Biochem.*, 267: 5421-5426.

Example C-15: In Vitro Hematopoietic Stem and Progenitor Cell Assay (Bone Marrow Toxicity)

[00286] Test compounds were evaluated to determine their effect on hematopoietic stem and progenitor cell ("HSPC") proliferation and differentiation. Cryopreserved human bone marrow derived CD34⁺ hematopoietic stem and progenitor cells (Lonza) were defrosted and left to recover overnight in maintenance media (StemSpan SFEM II (Stem Cell Technologies) containing 25 ng/ml SCF, 50 ng/ml TPO, and 50 ng/ml Flt3-L human recombinant protein (all Peprotech)), in a humidified incubator at 37°C with 5% CO₂. The next day cells were resuspended in the presence of test compound into media capable of supporting erythroid cell differentiation (Preferred Cell Systems, SEC-BFU1-40H), at a concentration of 10,000 cells/ml. Cells (30 μ l) were plated into black-walled, clear bottomed 384 well tissue culture plates (Perkin Elmer) with the addition of 30, 10, 3.3, 1, 0.3, 0.1, 0.03, 0.01, 0.003, or 0 μ M of test compound. Cells were cultured for 5 days in a humidified incubator at 37°C, with 5% CO₂.

[00287] Viability was determined using CellTiter-Glo 2.0 from Promega (using an optimised volume of 3 μ l/well), with luminescence detected using an Envision plate reader (Perkin Elmer). Relative Luminescence signal was normalised to percentage of control wells (0 μ M compound) in Genedata Screener software (Genedata) or GraphPad Prism 9. The 50% inhibitory concentration (IC₅₀) was determined using a four parameter logistic regression curve fit.

[00288] Results obtained in primary CD34⁺ bone marrow-derived HSPCs induced to differentiate into the erythroid lineage are reported in Table 12 and Figure 5. Table 12 reports the mean IC₅₀ values for Compound 1, Isomer 1 and Compound 137, Isomer 2.

TABLE 12

TEST COMPOUND	MEAN IC ₅₀ VALUE IN PRIMARY HSPC ($\pm$ STANDARD DEVIATION)
Compound 1, Isomer 1	13.9 $\pm$ 4.2 μ M
Compound 137, Isomer 2	30.2 $\pm$ 3.4 μ M

Figure 5 illustrates the dose response curves obtained for those compounds.

Example C-16: Effect on Cell Cycle in Human Ovarian OVCAR3 Cell Line

[00289] An immunoblotting analysis of *CCNE1*-amplified ovarian OVCAR3 cells was performed to determine whether short (24 hours) treatment with Compound 137, Isomer 2 results in DNA damage response (“DDR”) signaling in the replicative phase of the cell cycle. Phospho-CBK1 (Ser345) and phospho-RPA2 (Ser4/Ser8) levels, biomarkers considered to be specifically expressed following DNA damage formation during active replication of cells, were assessed as a measure of effect on cell cycle.

A. Methods

[00290] OVCAR3 cells were treated with Compound 137, Isomer 2 for 24 hours and then lysed in RIPA buffer (Sigma-Aldrich) supplemented with protease inhibitors (Roche), phosphatase inhibitors (Sigma-Aldrich), and benzonase (Merck). After 30 minutes (min) incubation on ice the lysates were cleared by centrifugation at 15,000 rpm 4°C for 20 minutes and the supernatants were kept for sample loading. NuPAGE™ LDS Sample Buffer (ThermoFisher Scientific) and NuPAGE™ Sample Reducing Agent (ThermoFisher Scientific) were added to the samples and heated to 95 °C (5 minutes). Equal amounts of whole cell lysates were separated on 4-12% Bis-Tris NuPAGE gels and analyzed by standard immunoblotting. Primary antibodies: H2AX (Abcam, ab20669), gH2AX (Cell Signaling Technology (CST), 2577), Histone H3 (CST, 3638), phospho-Histone H3 Ser10 (CST, 9701), RPA2 – (Abcam, ab2175), phospho-RPA2 Ser4/Ser8 (Bethyl Labs, A300-245A), CDK1 (CST, 9116), phospho-CDK1 Thr14 (Abcam, ab58509) or BioLegend, 947402), phospho-CDK1 Tyr15 (Abcam, ab275958), CHK1 (CST, #2360), phospho-CHK1 Ser345 (CST, 2348), PKMYT1 (Abcam, ab307146) and vinculin (Sigma, V9131)). Secondary antibody: Anti-rabbit IgG, HRP-linked (CST, #7074) and anti-mouse IgG, HRP-linked (CST, #7076). Adavosertib was used as a treatment control.

B. Results

[00291] The differential expression of the phospho-proteins in response to treatment with Compound 137, Isomer 2 is shown in Figure 6. Treatment of the OVCAR3 cells with Compound 137, Isomer 2 resulted in a concentration-dependent reduction of phospho-CDK1 Thr14 levels. Phospho-CDK1 Thr14 is the direct substrate of PKMYT1. In addition, expression of phospho-Histone H3 Ser10 increased without affecting expression of the WEE1 substrate phospho-CDK1

Tyr15, indicating an increase of the mitotic population due to G2/M DNA damage cell cycle checkpoint override independent of WEE1 inhibition. At the 24-hour treatment time point, minimal to no phosphorylation of biomarkers (phospho-CHK1, phospho-RPA2) linked to DNA damage formation in the actively replicating cell population was observed. This is consistent with PKMYT1 regulating the activity of CDK1, which is not associated with S-phase cell cycle progression. Overall, the data indicate that treatment of the OVCAR3 cells with Compound 137, Isomer 2 resulted in PKMYT1 target engagement, an increased mitotic population, and minimal to no DNA damage signaling of replicating cells.

Example C-17: Cellular Proliferation in PKMYT1 Knock Out SKOV3 Cells

[00292] The potential effect of off-target activity of Compound 137, Isomer 2 and impact on cell viability was evaluated in PKMYT1 knock out (KO) SKOV3 cells. Specifically, loss of PKMYT1 protein expression, reduction of PKMYT1 substrate phosphorylation, and growth inhibition were evaluated in PKMYT1 KO SKOV3 cells.

A. Methods

[00293] CRISPR/Cas9 technology was used to delete *PKMYT1* kinase from SKOV3 cells and prepare two monoclonal PKMYT1 KO clones (B2 and H2). Parental SKOV3 cells and PKMYT1 KO SKOV3 cells were either left untreated or were treated with Compound 1, Isomer 1 for 24 hours. Cells were then lysed in RIPA buffer (Sigma-Aldrich) supplemented with protease inhibitors (Roche, Basel, Switzerland), phosphatase inhibitors (Sigma-Aldrich), and benzonase (Merck). After 30 minutes of incubation on ice the lysates were cleared through centrifugation at 15,000 rpm 4°C for 20 minutes and the supernatants were kept for sample loading. NuPAGE™ LDS Sample Buffer (ThermoFisher Scientific) and NuPAGE™ Sample Reducing Agent (ThermoFisher Scientific) were added to the samples. Equal amounts of whole cell lysates were separated on 4-12% Bis-Tris NuPAGE gels and analyzed by standard immunoblotting. Primary antibody incubation overnight at 4°C: (CDK1 (CST, 9116) pCDK1 Thr14 (Abcam, ab58509), PKMYT1 (Abcam, ab307146) and vinculin (Sigma, V9131). Secondary antibody incubation for two hours at room temperature was performed with anti-rabbit IgG, HRP-linked (CST, 7074) and anti-mouse IgG, HRP-linked (CST, 7076). Quantification was performed using Bio-Rad ChemiDoc, which was also used for visualisation of western blot bands, with quantification manually performed using ImageJ. CDK1 and phospho-CDK1 Thr14 bands were normalised relative to parental bands, and ratios of CDK1 versus phospho-CDK1 Thr14 were calculated.

Graphpad Prism was used to visualise data, plotted as mean with SD (n=2).

[00294] In addition, a CellTiter-Glo (CTG) assay was employed to evaluate the effect of Compound 137, Isomer 2 on growth inhibition of the PKMYT1 KO SKOV3 cells. Parental SKOV3 and OVCAR3 cells were included as controls. Cells were seeded at 500 cells per well in a 96-well plate and exposed to Compound 137, Isomer 2 for seven days. After cell attachment on day 0, Compound 137, Isomer 2 was dispensed from compound stocks dissolved in DMSO, in seven titration dilutions with a top concentration of 30 μ M. DMSO served as a vehicle control. Plates were incubated at 37°C, 5% CO₂. Cell growth was stopped by adding CTG as per manufacturer's instructions (Promega, Madison, WI, USA; G7570) and luminescence read on an Envision plate reader (Perkin Elmer). Data analysis was performed by normalization to vehicle treatment. Data were normalized and plotted to respective vehicle-control using Prism GraphPad software and GI₅₀ values were derived.

B. Results

[00295] The results of the immunoblotting analysis are illustrated in the left and middle panels of Figure 7 and show that PKMYT1 deletion from SKOV3 cells resulted in loss of PKMYT1 protein expression and a significant reduction of phospho-CDK1 Thr14 expression which could not be further reduced by treatment (24 hours) with Compound 1, Isomer 1.

[00296] The GI₅₀ values derived from 7-day end-point CTG measurements for the cell lines treated with Compound 137, Isomer 2 are shown in the right panel of Figure 7 and also reported in Table 13. PKMYT1 deletion from SKOV3 resulted in GI₅₀ values for Compound 137, Isomer 2 greater than 20 μ M.

TABLE 13

CELL LINE	GI ₅₀ VALUES (μ M)			MEAN (N=3, μ M)	SD
SKOV3 Parental	10.12	9.93	9.02	9.69	0.586
SKOV3 PKMYT1 KO (B2)	35.13	39.49	32.64	35.75	3.467
SKOV3 PKMYT1 KO (H2)	26.40	23.82	16.89	22.37	4.918
OVCAR3 (<i>CCNE1</i> amp)	2.88	1.89	1.06	1.94	0.911

[00297] Overall, the data indicate that PKMYT1 knock out (KO) SKOV3 cells are resistant to treatment with Compound 137, Isomer 2.

Example C-18: In Vivo Tolerability (Mouse Model)

[00298] A tolerability study was conducted to evaluate long-term dosing of Compound 137, Isomer 2 in mice as a monotherapy, as a combination therapy with gemcitabine, and as a combination therapy with irinotecan hydrochloride.

A. Monotherapy

[00299] Female SCID mice (18 g+ at time of first procedure) were orally dosed with 300 mg/kg BID of Compound 137, Isomer 2 for 28 days. Compound 137, Isomer 2 was formulated for dosing using a 5% DMSO, 50% of 20% Captisol, 45% water for injection (pH 3-3.2) vehicle. Bodyweights were collected and recorded daily. Clinical observations, which included monitoring of paw and tail swelling, were assessed at least twice daily. 300 mg/kg BID dosing achieved a free $C_{\max}$ for Compound 137, Isomer 2 of approximately 21 μM .

[00300] Figure 8-A illustrates the effect of 300 mg/kg BID monotherapy dosing of Compound 137, Isomer 2 on body weight loss. No significant loss of body weight, paw or tail swelling, or other noticeable adverse events were observed at this monotherapy dose.

B. Combination Therapy (Gemcitabine)

[00301] Female SCID mice (18 g+ at time of first procedure) were orally dosed with 100 mg/kg BID of Compound 137, Isomer 2 for a period of 28 days in combination with four weekly intraperitoneal injections of 20 mg/kg of gemcitabine during that period. Bodyweights were recorded daily. Clinical observations, which included monitoring of paw and tail swelling, were collected and assessed at least twice daily. 100 mg/kg BID dosing achieved a free $C_{\max}$ for Compound 137, Isomer 2 of approximately 5.7 μM .

[00302] Figure 8-B illustrates the effect of such combination dosing of Compound 137, Isomer 2 and gemcitabine on body weight loss. No significant loss of body weight, paw or tail swelling, or other noticeable adverse events were observed at this combination dose.

C. Combination Therapy (Irinotecan)

[00303] Female athymic nude mice (18 g+ at time of first procedure) were orally dosed with 100 mg/kg BID of Compound 137, Isomer 2 for a period of 28 days in combination with four weekly intraperitoneal injections of 50 mg/kg of irinotecan hydrochloride during that period. Bodyweights were collected and recorded daily. Clinical observations, which included monitoring of paw and tail swelling, were assessed at least twice daily. 100 mg/kg BID dosing achieved a free $C_{\max}$ for Compound 137, Isomer 2 of approximately 5.7 μM .

[00304] Figure 8-B illustrates the effect of such combination dosing of Compound 137, Isomer 2 and irinotecan hydrochloride on body weight loss. No significant loss of body weight, paw or tail swelling, or other noticeable adverse events were observed at this combination dose.

Example C-19: In Vivo PKMYT1 Target Engagement

[00305] An *in vivo* study in mice was conducted to evaluate PKMYT1 target engagement by Compound 137, Isomer 2. Target engagement was assessed by analysis of phospho-CDK1 (T14) expression in tumor.

[00306] OVCAR3 tumor-bearing female SCID mice were dosed orally with 10 mg/kg BID, 30 mg/kg BID, or 100 mg/kg BID of Compound 137, Isomer 2. Doses were calculated for individual animals on the day of dosing and administered with a 10 mL/kg dosing volume. After 21 days of dosing, tumors were collected, processed, and analyzed by immunoblotting: Lysis buffer (25 mM Tris/HCl, 3 mM EDTA, 3 mM EGTA, 50 mM NaF, 2 mM orthovanadate, 0.27 M sucrose, 10 mM β -glycerophosphate, 5 mM pyrophosphate, 0.5% Triton X-100), protease inhibitor (Sigma, P8340), phosphatase inhibitors (Sigma, P0044, P5726) and benzonase (Sigma, (E1014 1:2000) was used to lyse and homogenize (3x 30 seconds, 6.0 m/s) tumor pieces in a fast prep tube with lysing matrix. Lysates were sonicated and supernatants collected. Lysates were transferred and chilled in 2ml assay blocks in fast prep tubes and centrifuged again for 15 minutes for 2100 rpm at 4°C to ensure no debris transferred. Supernatants were then boiled for 5 minutes at 95°C and immunoblotted as described in Example C-16, using phospho- CDK1 Thr14 (Biolegend, 947402), and vinculin (Sigma, V9131) primary, and anti-mouse IgG, HRP-linked (CST, 7076) secondary antibodies. Following detection with G-box (Syngene), bands were quantified with Gene Tools software. Phospho-CDK1 Thr14 signals were normalized to vinculin, geometrical means were normalized to the control group and plotted (n= 5-7 mice per condition $\pm$ S.E.M, $P^* < 0.05$ is significant (one-way ANOVA)).

[00307] Figure 9 illustrates the relative *in vivo* expression levels of phospho-CDK1 Thr14 for each dose. The quantified *in vivo* phospho-CDK1 Thr14 signals after 21 days of dosing with Compound 137, Isomer 2 indicated significant PKMTYT1 kinase inhibition versus treatment with vehicle for all three doses tested. All three doses of Compound 137, Isomer 2 tested following 21 days of dosing achieved $\geq$ IC₉₀ inhibition.

Example C-20: Anti-Tumor Effect in Human OVCAR3 and SW620 Xenograft Models

[00308] A study was conducted to evaluate the *in vivo* efficacy of combination therapy with Compound 137, Isomer 2 and the S-phase DNA damaging agents gemcitabine and irinotecan hydrochloride in a human ovarian OVCAR3 model and a human colorectal SW620 xenograft model, respectively.

A. Human Ovarian OVCAR3 Cell Line-Derived Xenograft Model

[00309] OVCAR3 cells were implanted onto the right flank of female SCID mice. When tumors reached approximately 200 mm³, the mice were randomized to treatment groups based on tumor volume and treated according to one of the dosing schedules shown in Table 14.

TABLE 14

GROUP	DOSING SCHEDULE	TGI VS VEHICLE (DAY 21)
1	Vehicle BID administered orally	N/A
2	10 mg/kg BID of Compound 137, Isomer 2 administered orally	25%
3	30 mg/kg BID of Compound 137, Isomer 2 administered orally	12%
4	100 mg/kg BID of Compound 137, Isomer 2 administered orally	77%
5	20 mg/kg Gemcitabine administered intraperitoneal once weekly	63%
6	10 mg/kg BID of Compound 137, Isomer 2 administered orally + 20 mg/kg Gemcitabine administered intraperitoneal once weekly	31% Regression
7	50 mg/kg BID of Compound 137, Isomer 2 administered orally + 20 mg/kg Gemcitabine administered intraperitoneal once weekly	62% Regression

After treatment cessation, the gemcitabine combination arms were left to regrow for an additional 35 days. Tumor sizes were measured twice weekly and relative tumor volumes were plotted (geometrical mean (n= 6) mice per treatment arm $\pm$ S.E.M).

[00310] Figure 10 shows relative tumor volume over time. Tumor volume measurements at the end of treatment (Day 21) with twice-daily dosing of 100 mg/kg of Compound 137, Isomer 2 resulted in significant tumor growth inhibition (TGI, 77% $P < 0.01$) of replication stress high OVCAR3 (*CCNE*-amplified) tumors (see also, Table 14). Twice-daily dosing of 30 mg/kg or 10 mg/kg of Compound 137, Isomer 2 resulted in 12% or 25% tumor growth inhibition, respectively ($P > 0.05$, non-significant). Both combinations tested outperformed Compound 137, Isomer 2

monotherapy and gemcitabine monotherapy. At Day 21, twice-daily dosing of 50 mg/kg of Compound 137, Isomer 2 in combination with 20 mg/kg of gemcitabine administered intraperitoneal once weekly resulted in 62% tumor regression ($P < 0.001$), and twice-daily dosing of 10 mg/kg of Compound 137, Isomer 2 in combination with 20 mg/kg of gemcitabine administered intraperitoneal once weekly resulted in 31% tumor regression ($P < 0.001$). Dose-dependent regrowth to 200 mm³ tumor volumes, following cessation of treatment in the combination arms, was observed after 11 days (10 mg/kg BID of Compound 137, Isomer 2 + gemcitabine) and 35 days (50 mg/kg BID of Compound 137, Isomer 2 + gemcitabine). These results indicate that more durable tumor regressions can be achieved with Compound 137, Isomer 2 in combination with gemcitabine compared to gemcitabine monotherapy, particularly as Compound 137, Isomer 2 exposure increases.

B. Human Colorectal SW620 Cell Line-Derived Xenograft Model

[00311] SW620 cells were implanted onto the right flank of female athymic nude mice. When tumors reached approximately 200 mm³, the mice were randomized to treatment groups based on tumor volume and treated according to one of the dosing schedules shown in Table 15.

TABLE 15

GROUP	DOSING SCHEDULE
1	Vehicle BID administered orally
2	100 mg/kg BID of Compound 137, Isomer 2 administered orally
3	50 mg/kg Irinotecan hydrochloride administered intraperitoneal once weekly
4	10 mg/kg BID of Compound 137, Isomer 2 administered orally + 50 mg/kg Irinotecan hydrochloride administered intraperitoneal once weekly
5	100 mg/kg BID of Compound 137, Isomer 2 administered orally + 50 mg/kg Irinotecan hydrochloride administered intraperitoneal once weekly

Tumor sizes were then measured and relative tumor volumes were plotted (geometrical mean ($n=6$) mice per treatment arm $\pm$ S.E.M).

[00312] Figure 11 shows relative tumor volume over time. Twice-daily dosing of 100 mg/kg of Compound 137, Isomer 2 did not result in tumor growth inhibition of SW620 tumors. Combination benefit and tumor regression (18%, $P<0.01$) was achieved, however, with twice-daily dosing of 100 mg/kg of Compound 137, Isomer 2 in combination with once weekly intraperitoneal dosing of, 50 mg/kg irinotecan hydrochloride, a topoisomerase 1 inhibitor (TOP1i). Combination benefit was observed but less pronounced when Compound 137, Isomer 2 was dosed twice daily at 10 mg/kg in combination with irinotecan hydrochloride. These results appear to indicate that higher Compound 137, Isomer 2 exposure is needed to drive combination

benefit and regression in this SW620 model (Geometrical mean $\pm$ S.E.M., $P < 0.01$ (**), $P < 0.001$ (***) are significant (one-sided t-test with unequal variance).

Example C-21: In Vitro Efficacy in Combination with Exatecan

[00313] An *in vitro* assay was carried out to assess whether treatment of PKMYT1 KO isogenic SKOV3 cells (previously described in Example C-17) with Compound 137, Isomer 2 could further sensitize those cells to exatecan.

[00314] *In vitro* efficacy of a combination of exatecan and Compound 137, Isomer 2 was assessed in a 6x6 concentration compound combination assay. PKMYT1 wildtype SKOV3 cells and PKMYT1 knock out (KO) SKOV3 cells were seeded and left to attach for 24 hours in 384 well plates and then dosed (ECHO acoustic liquid handler) with a range of exatecan and Compound 137, Isomer 2 concentrations, as single agents and in combination. After continuous drug exposure for seven days, cells were treated with CellTiter-Glo® (CTG) to enable measurement of cellular metabolic activity of the treated cells. The metabolic rate, generally considered to correlate with cell survival, was measured as chemiluminescence and raw data was derived for every concentration of both drugs.

[00315] Figure 12 illustrates the effect of treatment with exatecan (1 nM) in combination with a concentration range of Compound 137, Isomer 2 on PKMYT1 wildtype SKOV3 cells and PKMYT1 KO SKOV3 cells (mean (n=3) $\pm$ SD). Treatment of the PKMYT1 KO cells with exatecan (1 nM) resulted in maximum drug activity (E_{max}) that did not improve further with the addition of Compound 137, Isomer 2. Comparison of the combination activity in PKMYT1 wildtype SKOV3 cells to the combination activity in PKMYT1 knockout cells suggests that higher inhibitory concentrations (3-10 μ M) of Compound 137, Isomer 2 can achieve more pronounced *in vitro* efficacy for the combination. Such high inhibitory concentrations of Compound 137, Isomer 2 can be achieved *in vivo* based on comparison with the 100 mg/kg free C_{max} .

Example C-22: Pharmacodynamic Effect in a Human Colorectal SW620 Xenograft Model

[00316] *In vivo* monotherapy with irinotecan generally induces DNA damage in the replicative S-phase of the cell cycle, G2/M checkpoint activation, and PKMYT1 dependence. A study was conducted to evaluate the *in vivo* pharmacodynamic response to combination therapy with irinotecan hydrochloride and Compound 137, Isomer 2 in a human colorectal SW620 xenograft model. The study assessed biomarkers for PKMYT1 target engagement (phospho-CDK1 Thr14),

DNA damage formation (gH2AX), and changes in the relative mitotic population (phospho-histone H3 Ser10) following treatment. These biomarkers were selected to evaluate whether Compound 137, Isomer 2 coverage was sufficient to override the G2/M DNA damage cell cycle checkpoint following administration of irinotecan hydrochloride.

[00317] Human colorectal SW620 cells were implanted onto the right flank of female athymic nude mice. When tumors reached approximately 200 mm³, the mice were randomized to treatment groups based on tumor volume and treated with: (i) vehicle administered orally, (ii) one intraperitoneal dose of irinotecan hydrochloride (50 mg/kg), (iii) twice-daily oral doses (100 mg/kg) of Compound 137, Isomer 2 for three days, or (iv) one intraperitoneal dose of irinotecan hydrochloride (50 mg/kg) plus twice-daily oral doses (100 mg/kg) of Compound 137, Isomer 2 for three days. On the third day at 6 hours after the last dose of Compound 137, Isomer 2, tumors were collected and subsequently processed for immunoblotting as described in Example C-19 using phospho- CDK1 Thr14 (Biolegend, 947402), CDK1 (CST, 9116), Histone H3 (CST, 3638), phospho-Histone H3 Ser10 (CST, 9701), gH2AX (Cell Signalling Technology (CST) and vinculin (Sigma, V9131), and anti-rabbit IgG, HRP-linked (CST, 7074) and anti-mouse IgG, HRP-linked (CST, 7076) secondary antibodies. Following detection with G-box (Syngene), bands were quantified with Gene Tools software. Phospho-CDK1 Thr14 signals were normalized to CDK1 and vinculin, gH2AX was normalized to vinculin, and phospho-histone H3 Ser10 was normalized to histone H3, and vinculin. Geometrical means were normalized to the control group and plotted (n= 3-6 mice per condition $\pm$ S.E.M, $P^* < 0.05$ is significant (one-way ANOVA)).

[00318] Results are shown in Figure 13. Twice-daily oral dosing (100 mg/kg) of Compound 137, Isomer 2 for three days in absence or presence of irinotecan hydrochloride resulted in PKMYT1 target engagement (Phospho-CDK1 Thr14). Intraperitoneal dosing of irinotecan hydrochloride (50 mg/kg) resulted in DNA damage formation (gH2AX) that was not further potentiated by the addition of Compound 137, Isomer 2. Irinotecan hydrochloride monotherapy resulted in a reduced mitotic population (phospho-histone H3 Ser10), a finding consistent with activation of the G2/M DNA damage cell cycle checkpoint following treatment with a TOP1i inhibitor. The combination of irinotecan hydrochloride and Compound 137, Isomer 2, however, did not result in a reduced mitotic population suggesting that Compound 137, Isomer 2 overrides the G2/M cell cycle checkpoint induced by irinotecan hydrochloride treatment resulting in premature mitotic entry. These results show the PKMYT1 dependency of tumors in the presence of exogenous DNA damaging agents (including TOP1 inhibitors) that activate the G2/M cell

cycle checkpoint.

Example C-23: *In Vitro* Biotransformation in Hepatocytes of Multiple Species

[00319] Compound 137, Isomer 2 was incubated (5 μ M) with mouse, rat, dog, minipig, and human hepatocytes (1 million cells/ml) at 37 °C for 180 minutes. Incubations were stopped by addition of organic solvent, and samples centrifuged to separate soluble and insoluble components, with the supernatant stored at -20 °C until required for analysis. A semi-quantitative analysis of the dominant biotransformations occurring to the compound in each of the incubations was performed, with liquid chromatographic separation followed by both UV and MS detection of the eluate. Metabolite abundance was calculated as a percentage of the sum of all compound-related UV peak areas extracted at 330-350 nm in the post-incubation (time = 180 mins) samples of each species. All metabolites with UV abundance >1% observed in human hepatocyte incubations were aligned across species. Table 16 summarizes the *in vitro* human metabolites of Compound 137, Isomer 2 present across species. *M* = Major (>10%), *m* = minor (>1% but $\leq$ 10%), *t* = trace ($\leq$ 1%), *t** = detected by MS response only, ND = Not Detected.

TABLE 16

BIOTRANSFORMATION	SPECIES				
	HUMAN	DOG	MINIPIG	RAT	MOUSE
Glucuronidation	m	M	M	M	M
Oxidation	m	M	m	t*	t*

Figure 14 is a metabolism scheme showing the dominant routes of biotransformations observed *in vitro* of Compound 137, Isomer 2: oxidation and glucuronidation.

Example C-24: *In Vivo* Pharmacokinetic Studies in the CD-1 Mouse

[00320] The pharmacokinetic profile of Compound 137, Isomer 2 was characterised *in vivo* using CD-1 mice following either a single intravenous bolus dose or a single oral bolus dose to allow the calculation of key pharmacokinetic properties.

[00321] Animal experiments were conducted in accordance with the relevant welfare policies and procedures of the organisation conducting the study. Male CD-1 mice, weighing ~20-30 g were housed in polycarbonate animal cages with absorbent rodent bedding, and allowed to acclimatize, with *ad libitum* access to a standard rodent chow food and sterile water. An ambient temperature of 20-25 °C and humidity of 40-70% was maintained, alongside alternating 12-hour light/dark cycles, interrupted only by study-related events (e.g., sampling). The compound was

formulated for administration as an aqueous solution containing 5% dimethylsulfoxide, 95% sulfobutylether- β -cyclodextrin (30% w/v) in purified water for injection (WFI), at a dose level of either 0.5 mg/kg (I.V.) or 1.0 mg/kg (P.O.) animal bodyweight, and dose volume of 2.0 mL/kg (I.V.) or 4.0 mL/kg (P.O.) animal bodyweight. Blood samples (~ 0.02 mL / timepoint) were collected from the dorsal metatarsal vein into tubes containing EDTA-K2 anticoagulant, and placed on wet ice (4 °C) before centrifugation (4000 g, 5 minutes, 4°C) to obtain plasma, and then stored (-75 °C $\pm$ 15 °C) prior to further analysis. Following intravenous administration (time=0), samples were collected at 2, 10, 30, 60, 120, 240, 360, 480, 720, and 1440 minute timepoints. Following oral administration (time=0), samples were collected at 5, 15, 30, 60, 120, 240, 360, 480, 720, and 1440 minute timepoints. The total concentration of the compound was measured in blood plasma samples using a routine liquid chromatography-mass spectrometry method; blank plasma samples and were prepared and analysed in a similar manner. Key pharmacokinetic parameters were calculated using WinNonlin (PhoenixTM). Table 17 provides summary of key pharmacokinetic parameters derived following administration of Compound 137, Isomer 2 to male CD-1 mice (n=2) at 0.5 mg/kg (I.V.) or 1 mg/kg (P.O.).

TABLE 17

PARAMETER	UNITS	I.V.	P.O.
CL	mL/min/Kg	16.7	-
V _{ss}	L/Kg	7.87	-
t _{1/2}	h	10.3	5.37
C _{max}	μ mol/L	1.40	0.3
T _{max}	h	0.03	0.5
AUC	h* μ mol/L	1.34	1.84

Figure 15 shows the pharmacokinetic profiles in male CD-1 mice (total blood plasma concentration) of Compound 137, Isomer 2 following administration either I.V. (0.5 mg/kg, solid line) or P.O. (1 mg/kg, dashed line).

Example C-25: *In Vivo* Pharmacokinetic Studies in the SCID Mouse

[00322] Pharmacokinetic profiles of Compound 137, Isomer 2 in female SCID mice were obtained following oral administration to establish the short-term tolerability, and confirmed the extent and duration of compound exposure. Animal husbandry, dosing, sample collection, and subsequent analytical work to determine total plasma concentrations were conducted in a similar manner as previously described In Example C-24, with minor adjustments. Compound 137,

Isomer 2, formulated in 5% DMSO, 50% of 20% Captisol, and 45% purified water for injection (WFI) (pH 3-3.2), was dosed at three dose levels (10 mg/kg, 30 mg/kg, and 100 mg/kg) and a dose volume of 10 mL/kg. Plasma samples were collected from the lateral tail vein at multiple timepoints (30 / 60 / 120 / 240 / 420 / 1440 minutes) following administration. Table 18 provides a summary of pharmacokinetic parameters derived following oral administration of Compound 137, Isomer 2 to female SCID mice (n=3) at three dose levels: 10 mg/kg, 30 mg/kg, and 100 mg/kg.

TABLE 18

PARAMETER	UNITS	P.O. DOSE (mg/kg)		
		10	30	100
$t_{1/2}$	h	4.4	4.6	4.7
C_{max}	μ M	1.3	3.6	8.7
T_{max}	min	30	30	30
AUC	μ M*h	4.2	9.1	25

Figure 16 shows the pharmacokinetic profiles in female SCID mice (total blood plasma concentration) of Compound 137, Isomer 2 following administration P.O. at three dose levels: 10 mg/kg (solid line), 30 mg/kg (dashed line), and 100 mg/kg (dotted line).

Example C-26: *In Vivo* Pharmacokinetic Studies in The Intact and Bile-Duct-Cannulated Han Wistar Rat

[00323] The pharmacokinetic profile of Compound 137, Isomer 2 was characterized *in vivo* using bile-duct-cannulated Han Wistar rat following an I.V. bolus dose to determine the contribution of enterohepatic circulation to key pharmacokinetic properties.

[00324] Animal experiments were conducted in accordance with the relevant welfare policies and procedures of the organization conducting the study. Male Han Wistar rats – either intact or following routine surgery to cannulate the bile duct – weighing ~200-300 g were housed in polycarbonate animal cages with absorbent rodent bedding, and allowed to acclimatize, with *ad libitum* access to a standard rodent chow food and sterile water. An ambient temperature of 20-25°C and humidity of 40-70% was maintained, alongside alternating 12-hour light/dark cycles, interrupted only by study-related events (e.g., sampling). Compound 137, Isomer 2 was formulated for administration as an aqueous solution containing 5% dimethylsulfoxide, 95% sulfobutylether- β -cyclodextrin (30% w/v) in purified water for injection (WFI), at a dose level of 0.5 mg/kg (I.V.) animal bodyweight, and dose volume of 1.0 ml/kg animal bodyweight. Blood

samples (~0.20 mL / timepoint) were collected from the jugular vein into tubes containing EDTA-K2 anticoagulant, and placed on wet ice (4°C) before centrifugation (4000 g, 5 minutes, 4 °C) to obtain plasma, and then stored (-75 °C +/- 15 °C) prior to further analysis. Following intravenous administration (time=0), samples were collected at multiple timepoints (intact rats: 2, 10, 30, 60, 120, 240, 360, 480, 720, and 1440 minute timepoints; BDC rats: 5, 15, 30, 60, 120, 240, 420, and 1440 minute timepoints) Compound 137, Isomer 2 was measured in blood plasma samples using a routine liquid chromatography-mass spectrometry method; blank plasma samples and were prepared and analysed in a similar manner. Key pharmacokinetic parameters were calculated using WinNonlin (PhoenixTM). Table 19 provides a summary of pharmacokinetic parameters derived following I.V. administration of Compound 137, Isomer 2 to male intact or bile-duct-cannulated (BDC) Han Wistar rats (n=3) at 0.5 mg/ kg.

TABLE 19

PARAMETER	UNITS	ANIMAL CONDITION	
		INTACT	BDC
CL	mL/min/kg	16.7	27.9
V _{ss}	L/kg	7.87	1.19
t _½	h	10.3	2.03
C _{max}	μmol/L	1.4	1.83
T _{max}	h	0.03	0.08
AUC	μM*h	1.34	0.868

Figure 17 shows the pharmacokinetic profiles in male Han Wistar rats (total blood plasma concentration) of Compound 137, Isomer 2 following administration I.V. (0.5 mg/ kg) to either intact (solid line) or bile-duct cannulated (dashed line) animals.

Example C-27: *In Vivo* Pharmacokinetic Studies in the Han Wistar Rat at High Dose

[00325] Pharmacokinetic profiles of Compound 137, Isomer 2 in male Han Wistar rats were obtained following oral administration to establish the short-term tolerability, and confirm the extent and duration of compound exposure at three dose levels (10 mg/kg, 30 mg/kg, and 100 mg/kg) and a dose volume of 10 mL/kg. Animal husbandry, dosing, sample collection, and subsequent analytical work to determine total plasma concentrations were conducted in a similar manner as previously described In Example C-24, with minor adjustments. Plasma samples were collected from the lateral tail vein at multiple timepoints (15 / 30 / 60 / 120 / 240 / 360 / 1440 minutes) following administration. Table 20 provides a summary of the pharmacokinetic parameters derived following oral administration of Compound 137, Isomer 2 to male Han

Wistar rats (n=3) at three dose levels: 10 mg/kg, 30 mg/kg, and 100 mg/kg.

TABLE 20

PARAMETER	UNITS	P.O. DOSE (mg/kg)		
		10	30	300
t _{1/2}	h	6.77	9.10	26.7
C _{max}	μM	1.39	4.52	18.8
T _{max}	min	40.0	20.0	20.0
AUC	μM*h	12.2	41.8	180

Figure 18 shows the pharmacokinetic profiles in male Han Wistar rats (total blood plasma concentration) of Compound 137, Isomer 2 following administration P.O. at three dose levels: 10 mg/kg (solid line), 30 mg/kg (dashed line), and 100 mg/kg (dotted line).

Example C-28: *In Vivo* Pharmacokinetic Studies at High Dose to Assess Substance Tolerability and Effect of Repeat Dosing

[00326] Pharmacokinetic profiles of Compound 137, Isomer 2 in female SCID mice were obtained following repeated daily oral administration to establish tolerability over 28 days, and confirm the extent and duration of compound exposure. Animal husbandry, dosing, sample collection, and subsequent analytical work to determine total plasma concentrations were conducted in a similar manner as previously described in Example C-24, with minor adjustments. Animals were dosed twice daily (8 hours apart) and a dose volume of 10 mL/kg. Plasma samples were collected at multiple timepoints (30 / 12 / 360 / 720 / 1440 minutes) following the first dose on day 1 and day 28. Figure 19 shows a comparison of the pharmacokinetic profiles (total blood plasma concentration) of Compound 137, Isomer 2 following repeat BID oral administration (vertical dotted lines) to female SCID mice (n=2) at 100 mg/kg on day 1 (solid line), and day 28 (dashed line).

Example C-29: *In Vivo* Pharmacokinetic Studies Comparing Substance Formulations in the SCID Mouse at High Dose

[00327] Pharmacokinetic profiles of Compound 137, Isomer 2 in SCID mice were obtained following oral administration to establish the short-term tolerability at high dose, and confirm the extent and duration of compound exposure. Animal husbandry, dosing, sample collection, and subsequent analytical work to determine total plasma concentrations were conducted in a similar manner as previously described in Example C-24, with minor adjustments. Compound 137, Isomer 2 formulated in either 5% DMSO, 50% of 20% Captisol, and 45% purified water for

injection (WFI), pH 3-3.2), or 0.5% HPMC/0.1% Tween was dosed at 300 mg/kg and a dose volume of 10 mL/kg. Plasma samples were collected at multiple timepoints (30 / 12 / 360 / 720 / 1440 minutes) following administration. Table 21 provides a summary of pharmacokinetic parameters derived following oral administration of Compound 137, Isomer 2 formulated in either 5% DMSO, 50% of 20% Captisol, and 45% WFI (pH 3-3.2), or 0.5% HPMC/0.1% Tween 80 to female SCID mice at 300 mg/kg.

TABLE 21

PARAMETER	UNITS	FORMULATION	
		DMSO / CAPTISOL / WFI	HPMC / TWEEN 80
C _{max}	μM	33.9	45.7
T _{max}	min	30	30
AUC	μM*h	141.4	197.8

Figure 20 shows the pharmacokinetic profiles in female SCID mice (total blood plasma concentration) of Compound 137, Isomer 2 following administration P.O. at 300 mg/kg in 5% DMSO, 50% of 20% Captisol, and 45% WFI (pH 3-3.2) (solid line), or 0.5% HPMC/0.1% Tween (dashed line).

* * *

[00328] Although specific embodiments and examples have been described above, these embodiments and examples are only illustrative and do not limit the scope of the disclosure. Changes and modifications can be made in accordance with ordinary skill in the art without departing from the disclosure in its broader aspects as defined in the following claims. For example, any embodiment described herein can be combined with any other suitable embodiment described herein to provide additional embodiments.

[00329] As will be understood by the skilled artisan, all numbers, including those expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth, are approximations and understood as being modified in all instances by the term “about.” These values can vary depending upon the desired properties sought to be obtained by those skilled in the art utilizing the present teachings of the present disclosure. It is also understood that such values inherently contain variability necessarily resulting from the standard deviations found in their respective testing measurements.

[00330] One skilled in the art will also readily recognize that where members are grouped together in a common manner, such as in a Markush group, the present disclosure encompasses

not only the entire group listed as a whole, but each member of the group individually and all possible subgroups of the main group. Additionally, for all purposes, the present disclosure encompasses not only the main group, but also the main group absent one or more of the group members. The present disclosure also envisages the explicit exclusion or disclaimer of one or more of any of the group members in the claimed disclosure.

[00331] As will be understood by one skilled in the art, for any and all purposes, particularly in terms of providing a written description, all ranges disclosed herein also encompass any and all possible subranges and combinations of subranges thereof as well as the individual values making up the range, particularly integer values. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, *etc.* As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, *etc.* For example, the range C₍₁₋₆₎, includes the subranges C₍₂₋₆₎, C₍₃₋₆₎, C₍₃₋₅₎, C₍₄₋₆₎, *etc.*, as well as C₁ (methyl), C₂ (ethyl), C₃ (propyl), C₄ (butyl), C₅ (pentyl) and C₆ (hexyl) individually. As will also be understood by one skilled in the art, all language such as “up to,” “at least,” “greater than,” “less than,” “more than,” “or more” and the like include the number recited and refer to ranges which can be subsequently broken down into subranges as discussed above. In the same manner, all ratios disclosed herein also include all subratios falling within the broader ratio.

[00332] Reference to a “step” in this disclosure is used for convenience purposes only and does not categorize, define, or limit the disclosure as set forth herein.

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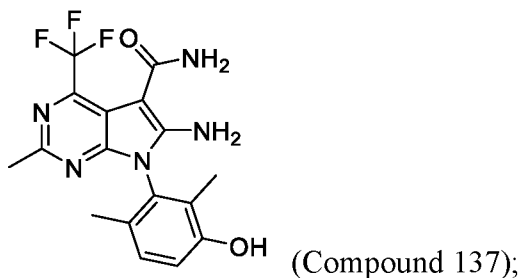
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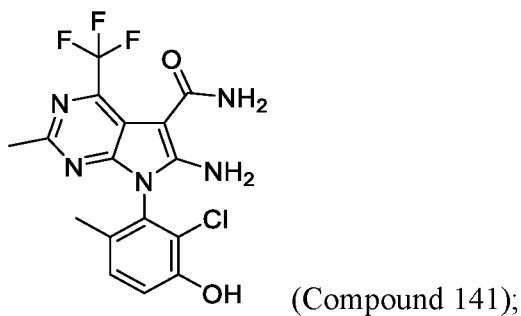
What is claimed is:

1. A compound selected from the group consisting of:

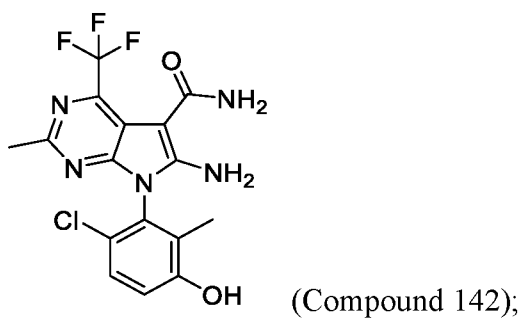
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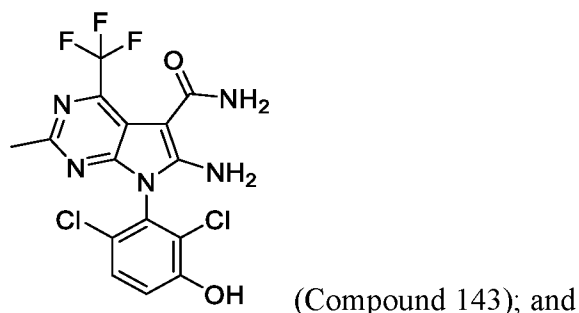
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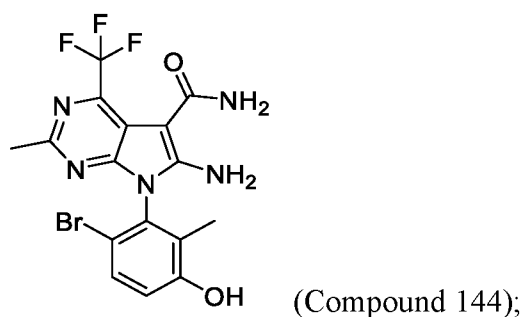
6-amino-7-(6-chloro-3-hydroxy-2-methylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide having the structure:



6-amino-7-(2,6-dichloro-3-hydroxyphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide having the structure:

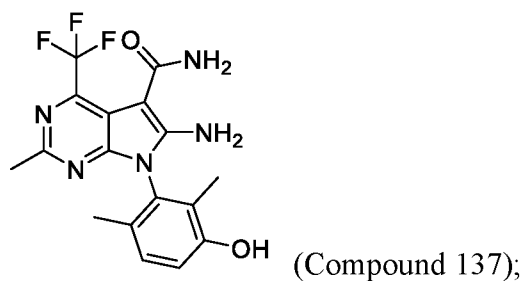


6-amino-7-(6-bromo-3-hydroxy-2-methylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide having the structure:



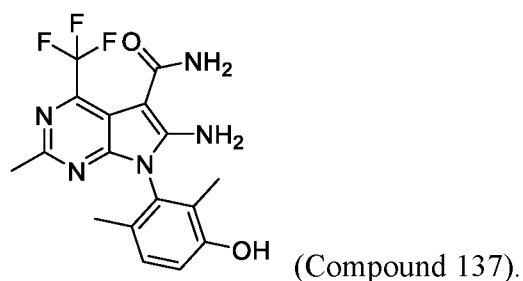
or a pharmaceutically acceptable salt thereof.

2. The compound of claim 1 that is 6-amino-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide having the structure:

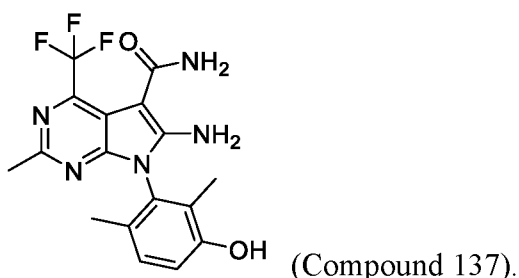


or a pharmaceutically acceptable salt thereof.

3. The compound of claim 1 that is 6-amino-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide having the structure:



4. The salt of claim 1 that is a pharmaceutically acceptable salt of 6-amino-7-(3-hydroxy-2,6-dimethylphenyl)-2-methyl-4-(trifluoromethyl)-7H-pyrrolo[2,3-d]pyrimidine-5-carboxamide having the structure:



5. The compound of any one of claims 1 to 4, or a pharmaceutically acceptable salt thereof, wherein the compound is an atropisomer.

6. The compound of claim 5, or a pharmaceutically acceptable salt thereof, wherein the atropisomer is the eutomer of the atropisomer.

7. A pharmaceutical composition comprising a compound of any one of claims 1 to 6, or a pharmaceutically acceptable salt thereof, and one or more pharmaceutically acceptable excipients.

8. A method of treating or preventing a cancer in a subject suffering from or susceptible to the cancer, the method comprising administering to the subject a therapeutically effective amount of a compound of any one of claims 1 to 6, or a pharmaceutically acceptable salt thereof.

9. The method of claim 8, wherein the cancer is a solid tumor cancer.

10. The method of claim 8, wherein the cancer is a hematological cancer.

11. The method of claim 8, wherein the cancer is selected from the group consisting of ovarian cancer, triple negative breast cancer, small cell lung cancer, non-small cell lung cancer, pancreatic cancer, esophageal cancer, colorectal cancer, uterine serous carcinoma, soft tissue sarcoma, uterine cancer, skin cancer, bladder cancer, head and neck cancer, glioma, and B-cell lymphoma.

12. The method of any of claims 8 to 11, wherein the cancer has a PKMYT1-dependency because of elevated basal levels of replication stress.

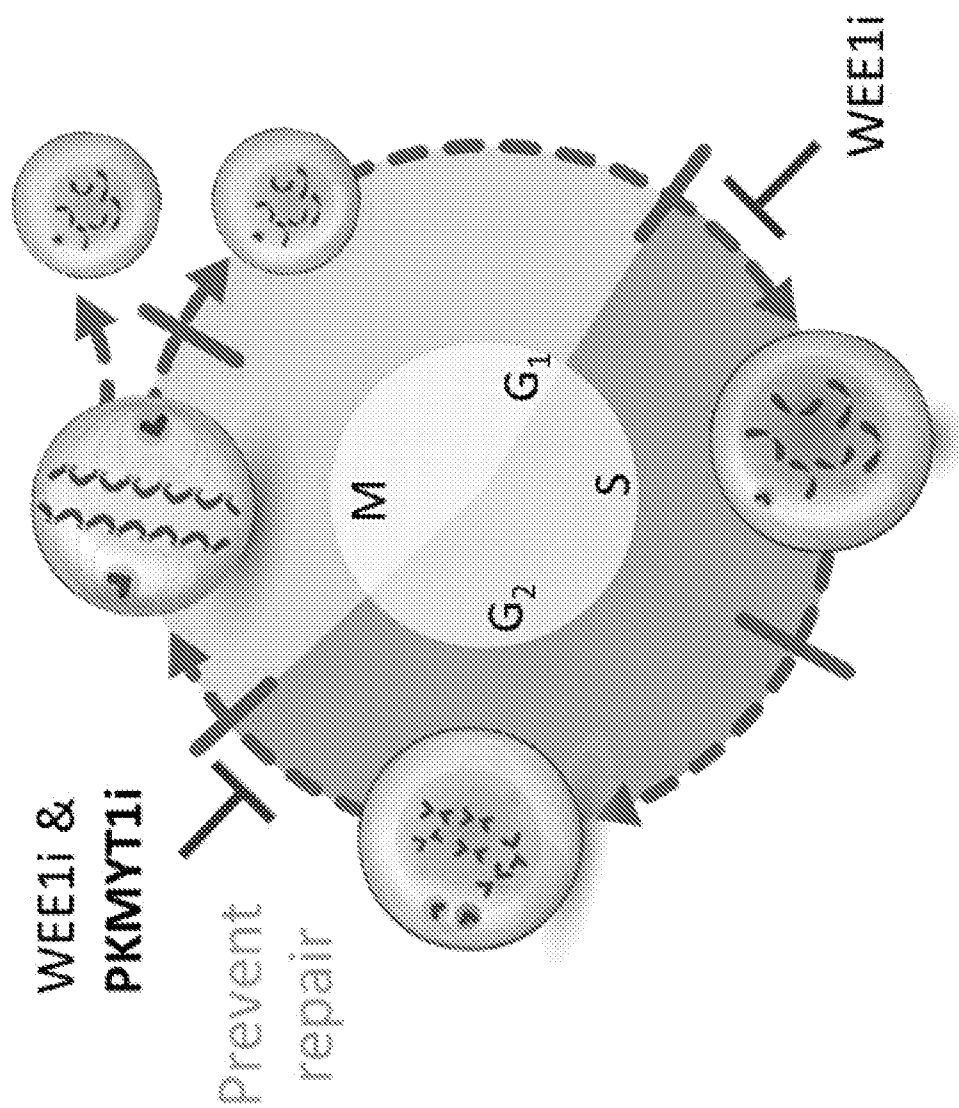


FIG. 1: WEE1 AND PKMYT1 REGULATION OF MITOTIC CELL

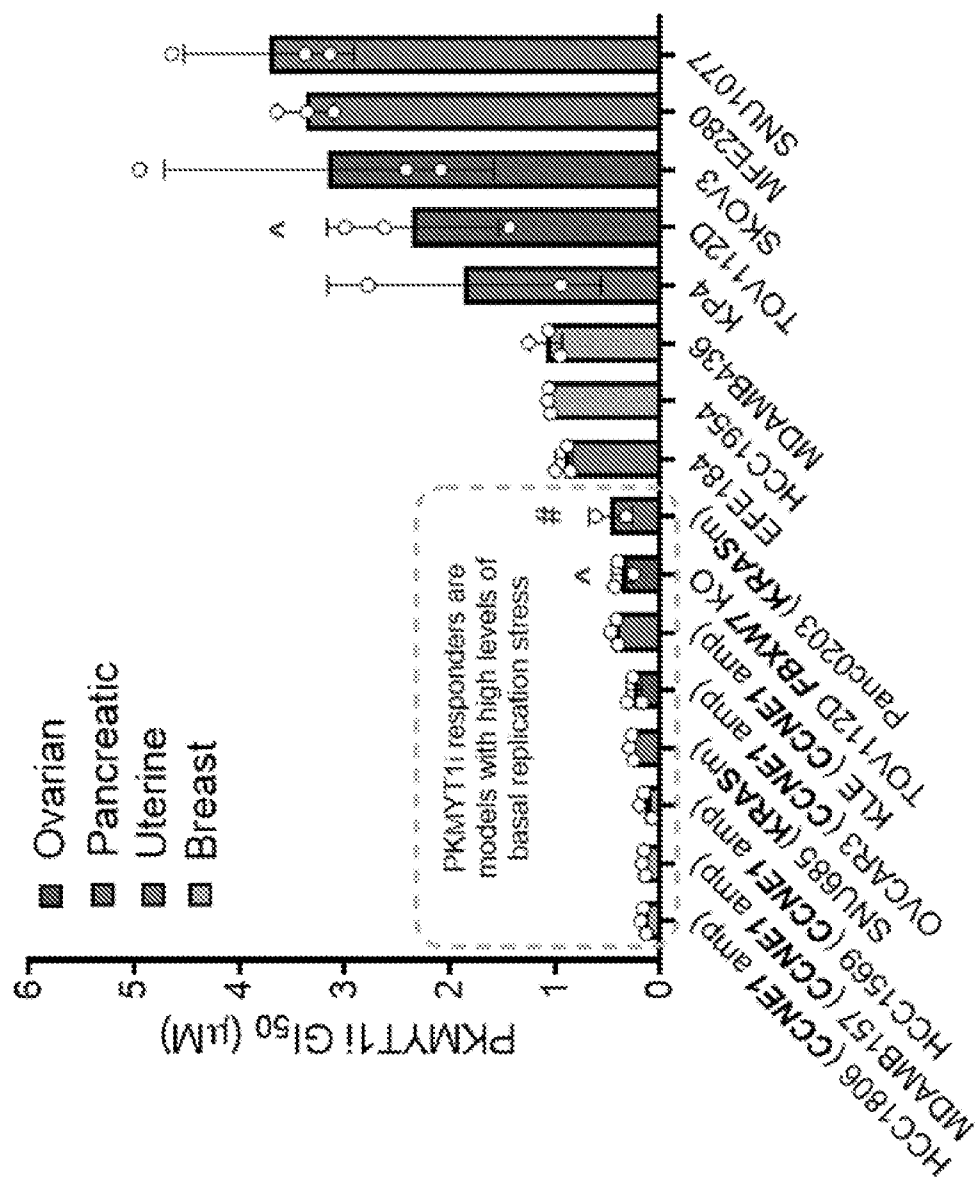


FIG. 2: PKMYT1 INHIBITION (GI₅₀)

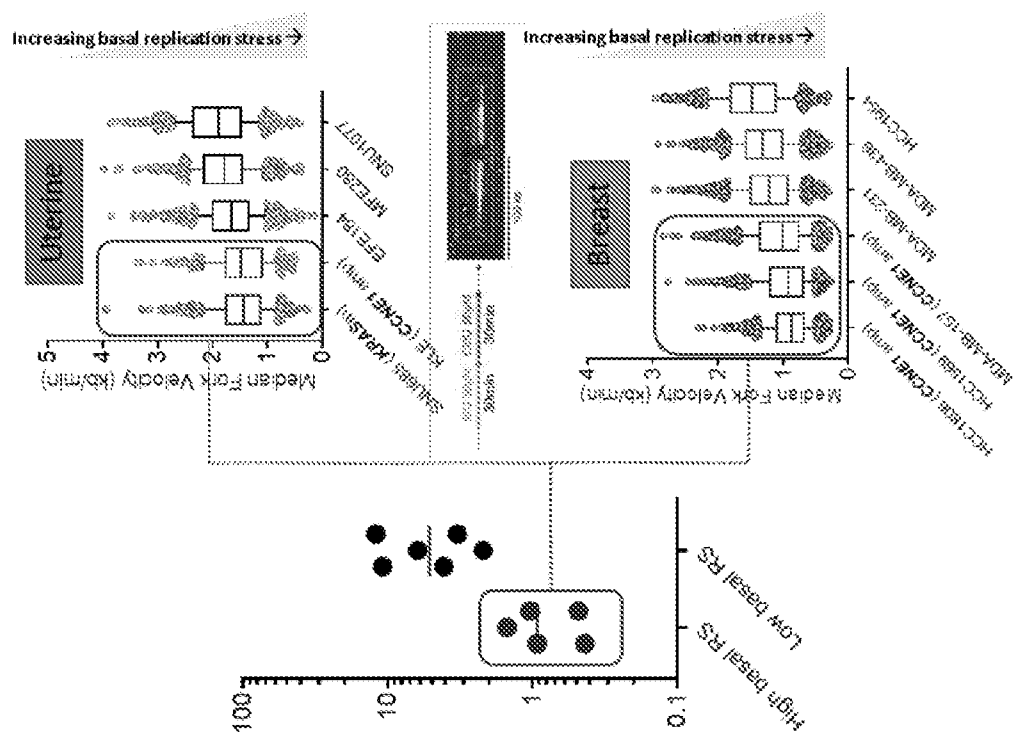


FIG. 4: REPLICATION FORK VELOCITIES WITH AZ1-1

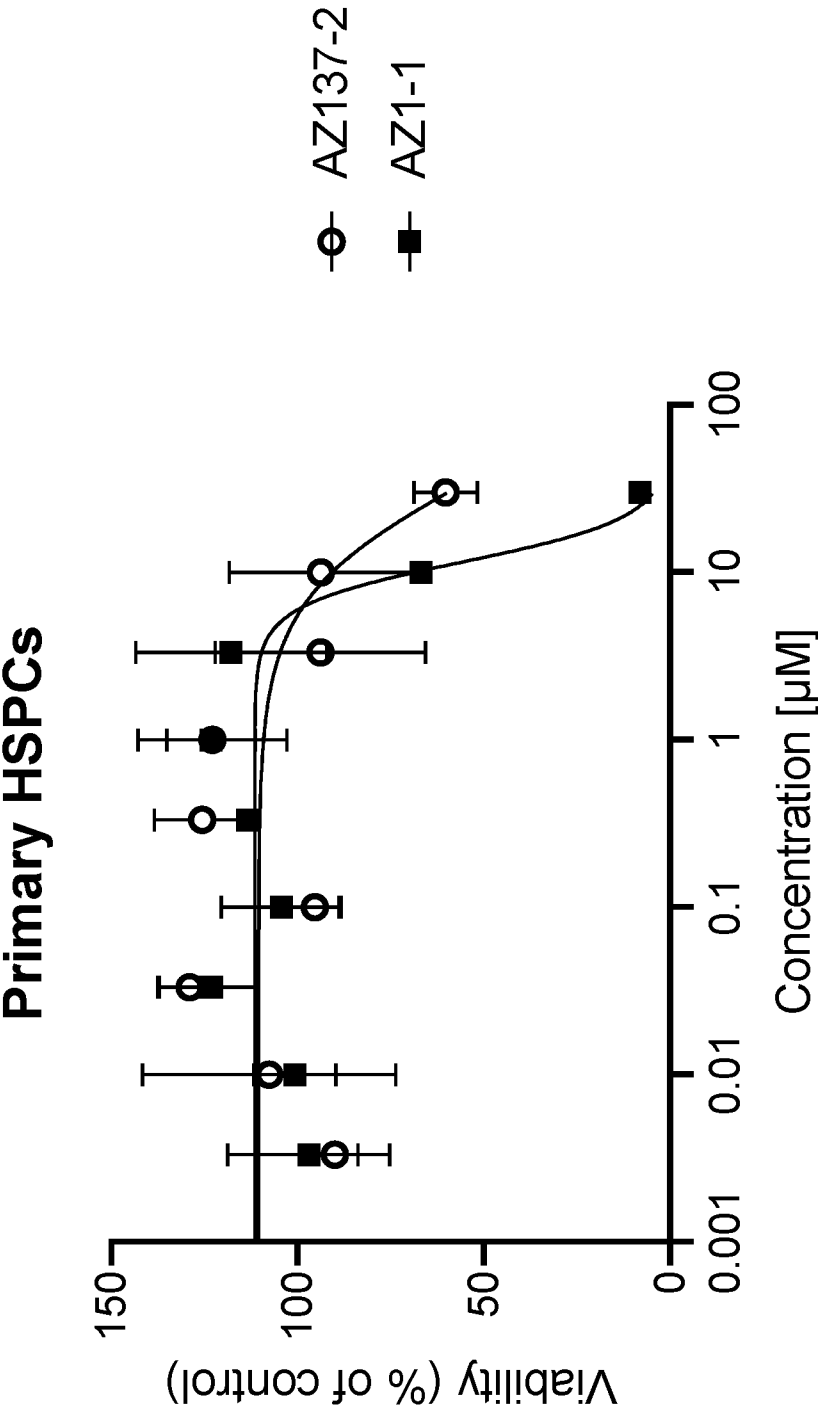


FIG. 5: DOSE RESPONSE CURVES FROM IN VITRO HEMATOPOIETIC STEM AND PROGENITOR CELL ASSAY

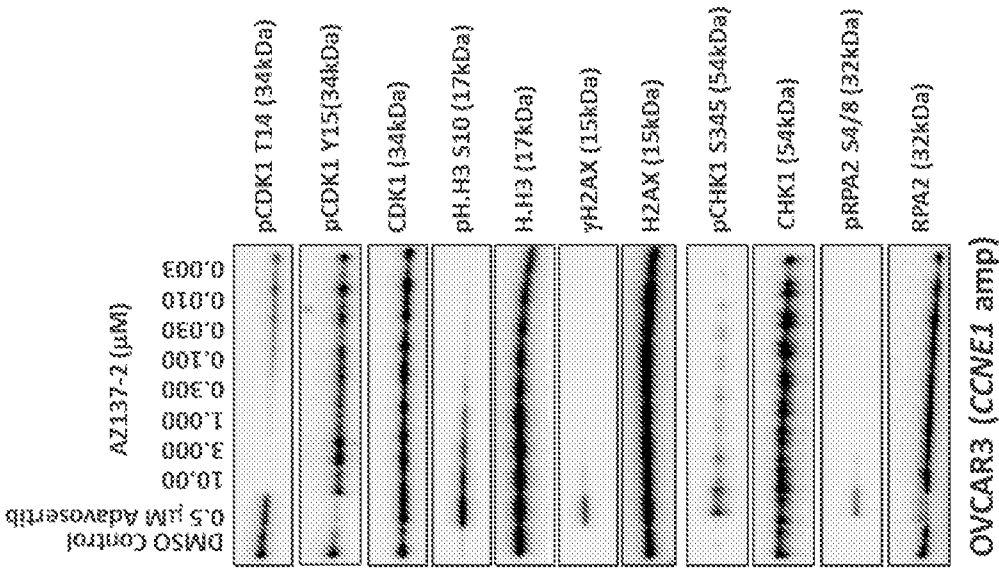


FIG 6: PROTEIN PHOSPHORYLATION IN OVCAR3 CELLS FOLLOWING TREATMENT

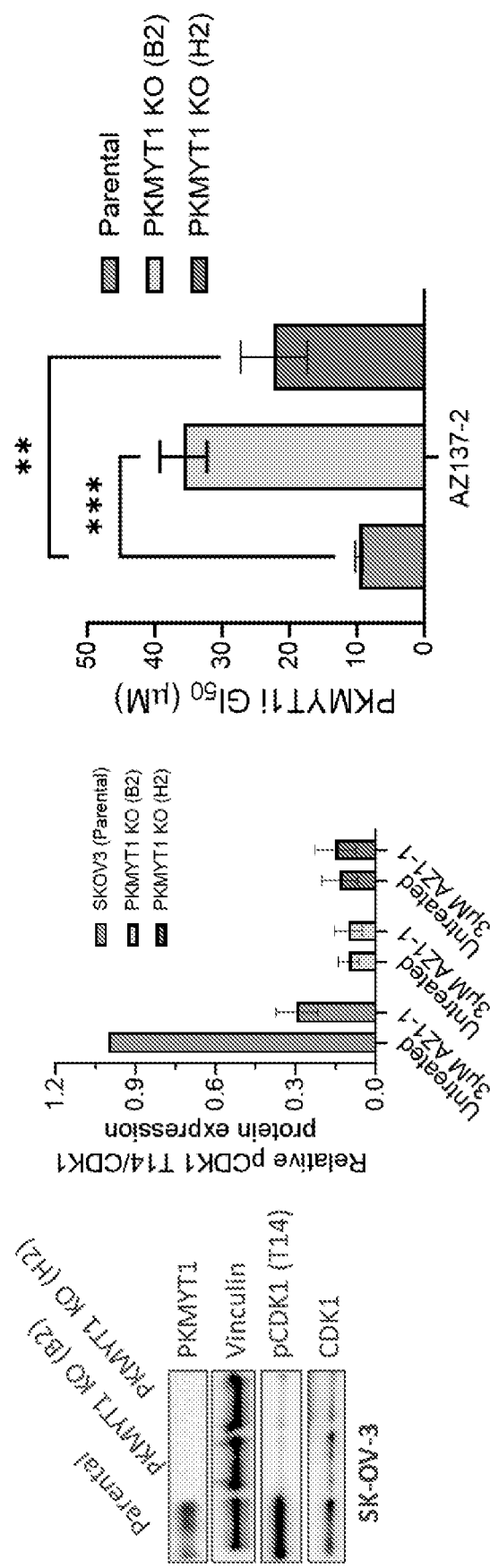


FIG 7: CELLULAR PROLIFERATION IN PKMYT1 KNOCK OUT SKOV3 CELLS

AZ137-2 300 mg/kg BID
Female SCID Mice

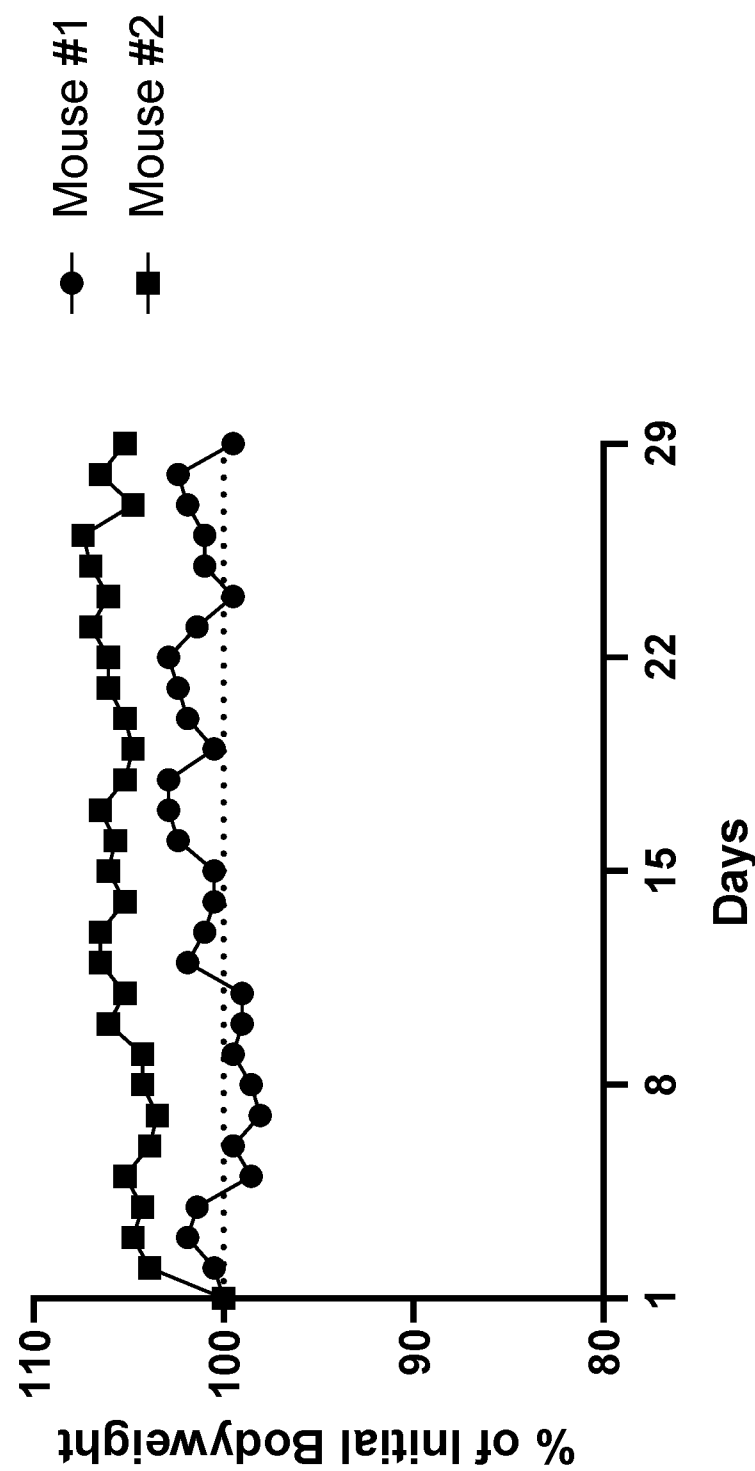


FIG 8-A: TOLERABILITY STUDY IN SCID MICE (MONOTHERAPY)

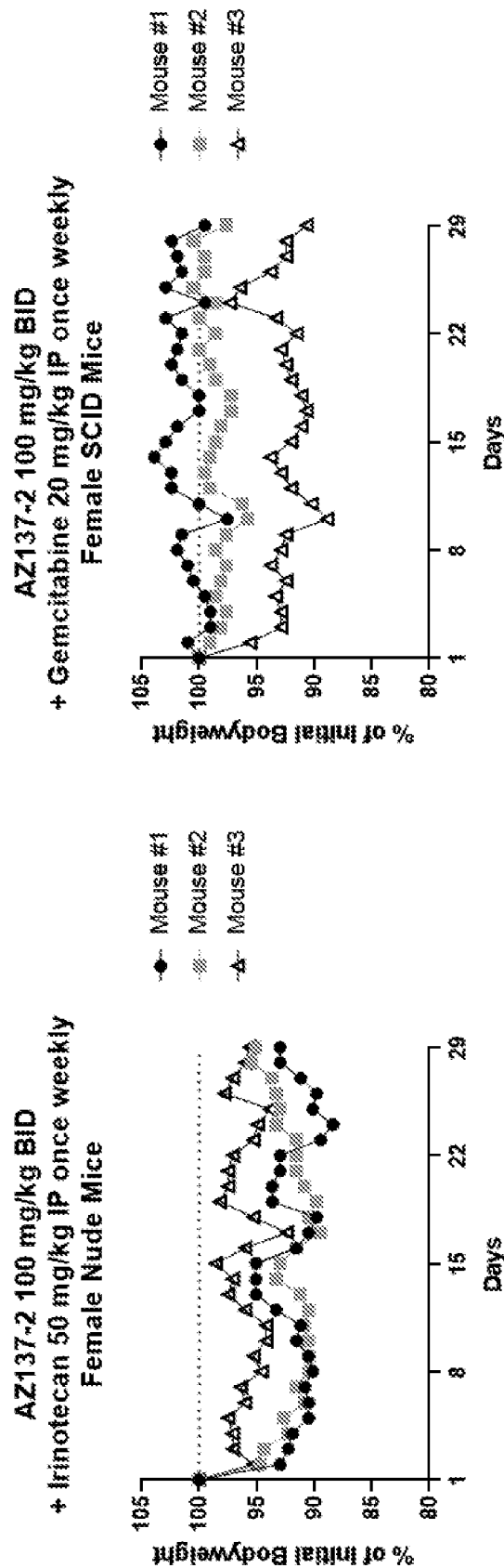


FIG 8-B: TOLERABILITY STUDY IN MICE (GEMCITABINE OR IRINOTECAN COMBINATION)

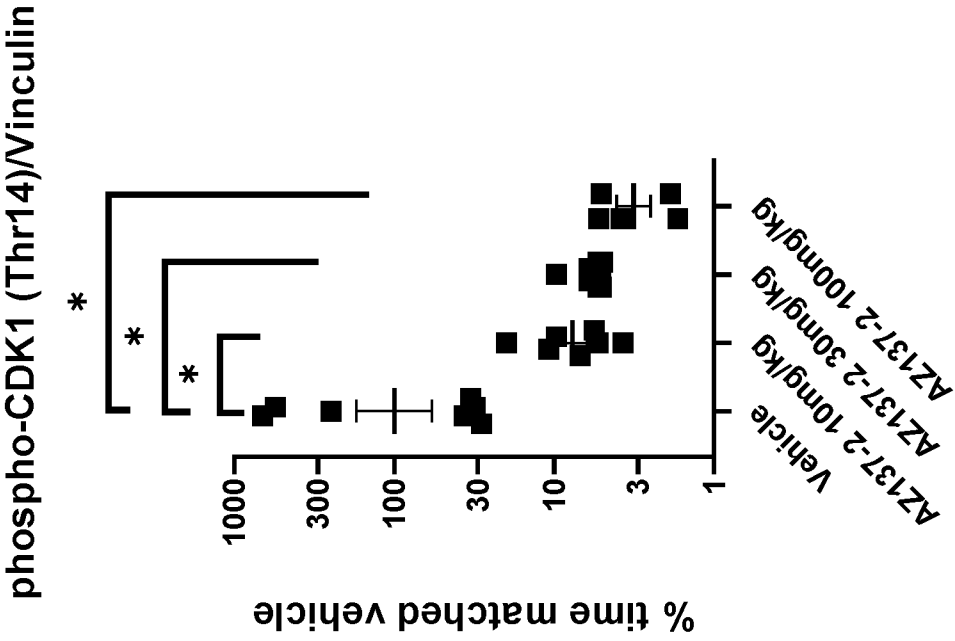


FIG 9: IN VIVO PHOSPHO-CDK1 (Thr14) EXPRESSION FOLLOWING TREATMENT (OVCAR3 MOUSE)

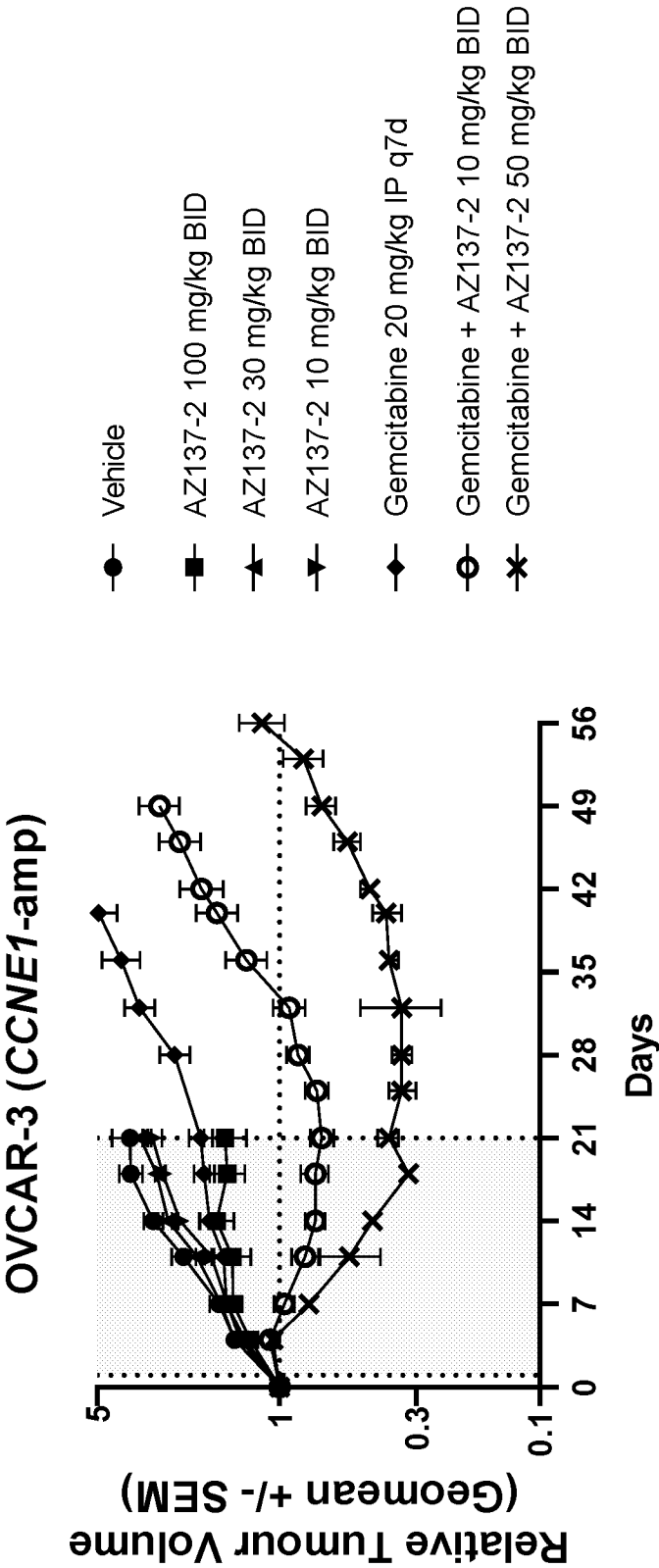


FIG 10: IN VIVO EFFICACY ALONE OR IN COMBINATION WITH GEMCITABINE (OVCAR3)

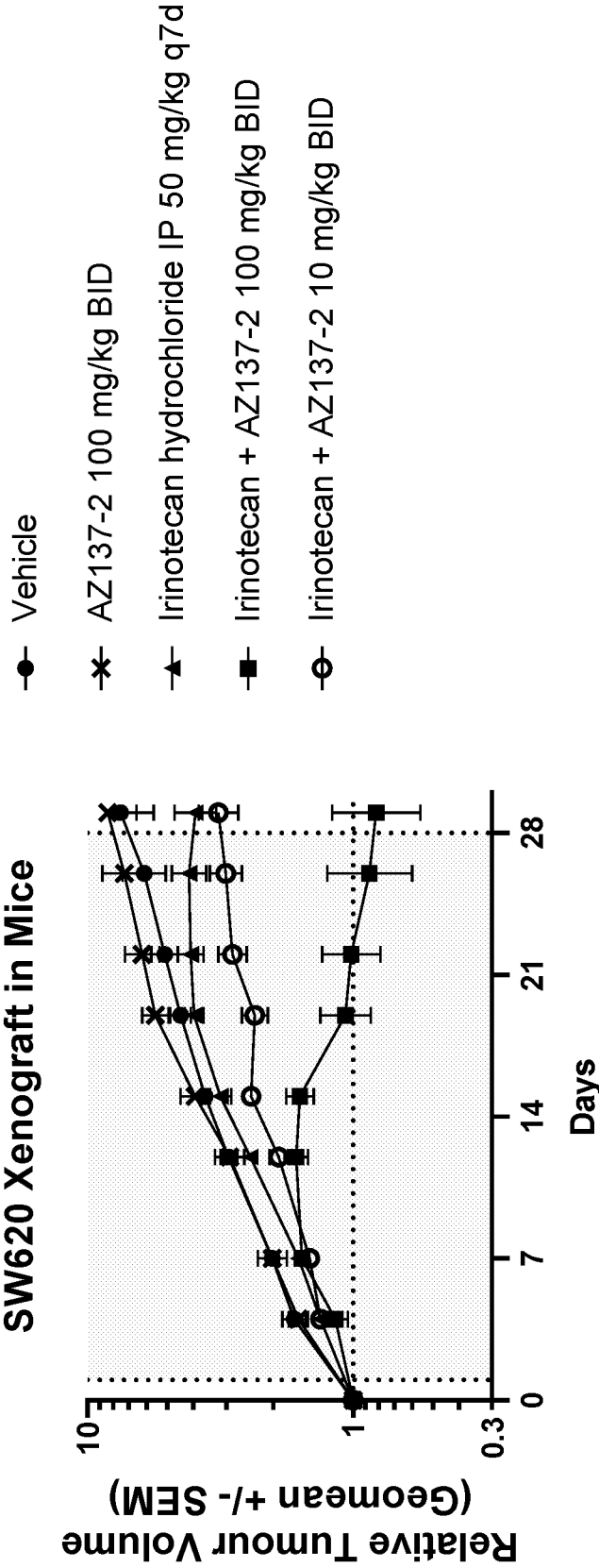


FIG 11: IN VIVO EFFICACY ALONE OR IN COMBINATION WITH IRINOTECAN (SW620)

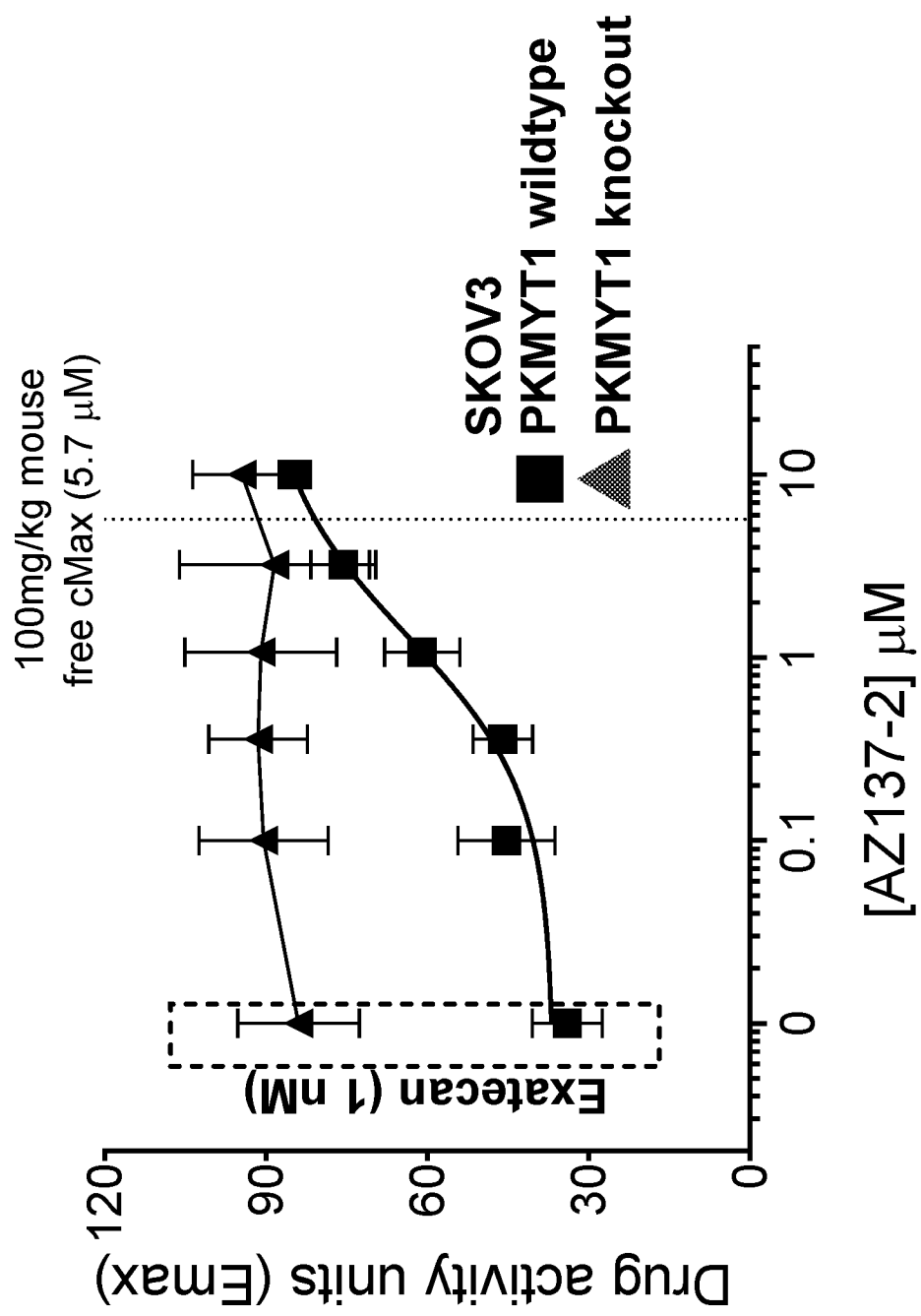


FIG 12: IN VITRO EFFICACY IN WILDTYPE OR PKMYT1 KNOCK OUT CELLS
WITH OR WITHOUT EXATECAN (SKOV3)

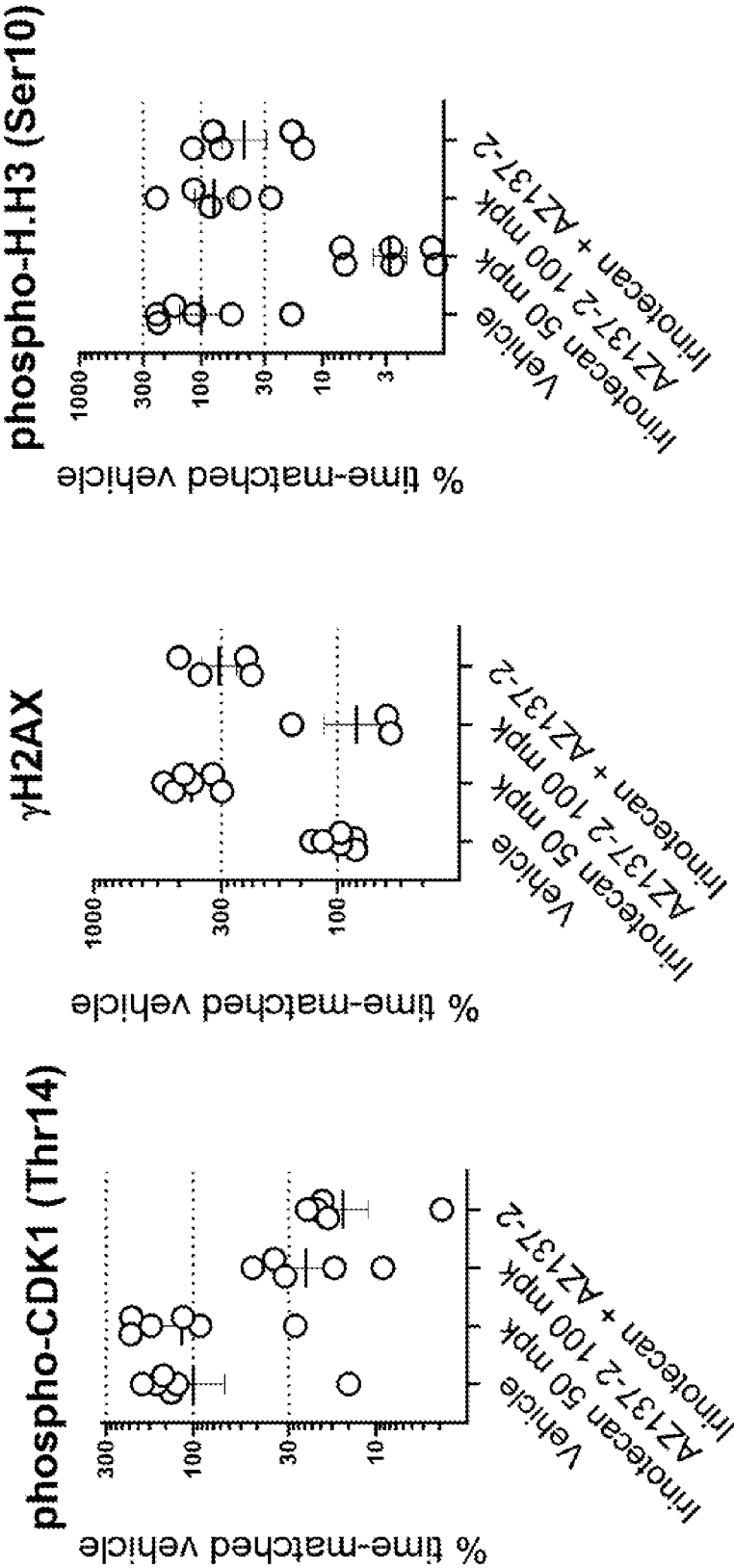


FIG 13: IN VIVO BIOMARKER DETECTION AFTER THREE DAYS OF TREATMENT (SW620)

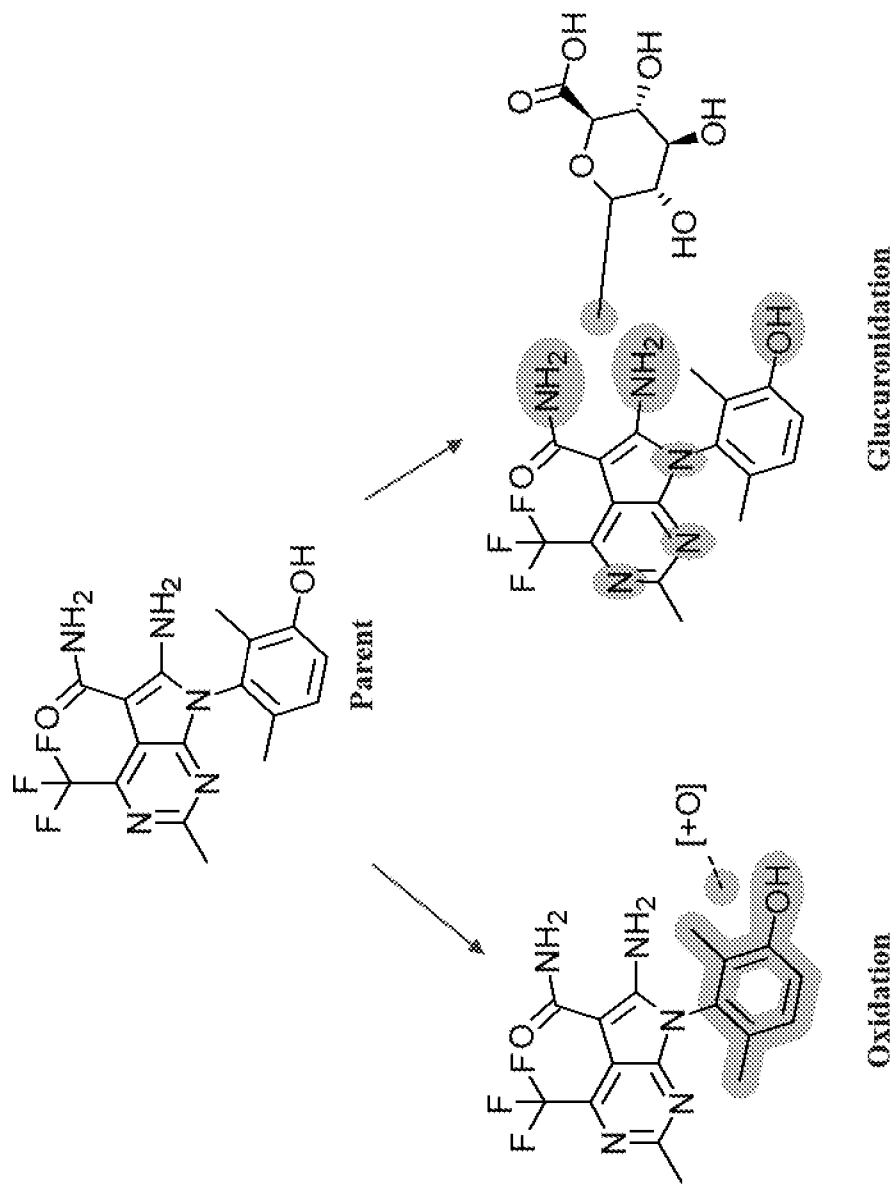


FIGURE 14: *IN VITRO* METABOLISM ROUTES OF AZ137-2

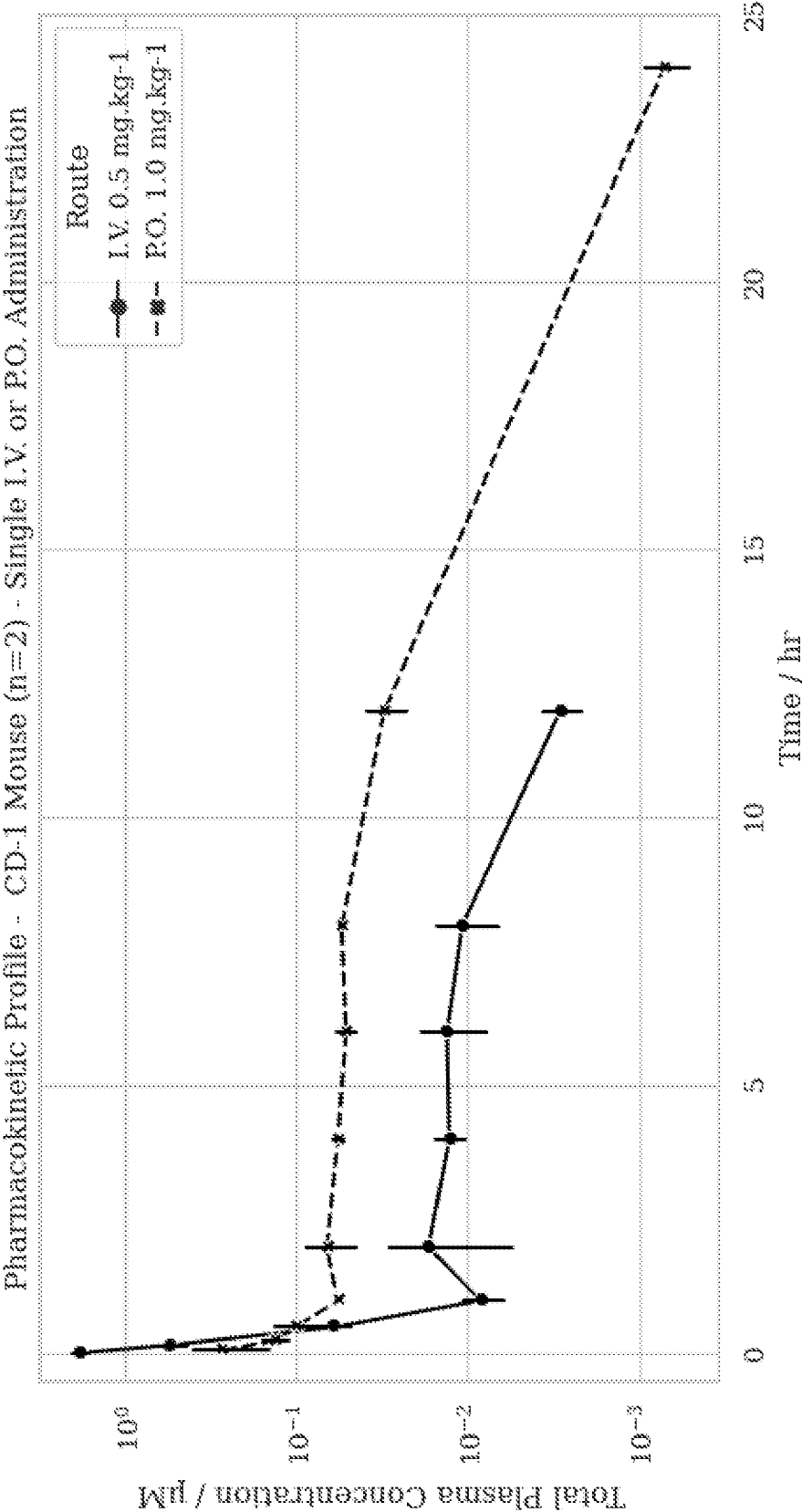


FIGURE 15: PHARMACOKINETIC PROFILES IN CD-1 MICE FOLLOWING ADMINISTRATION OF AZ137-2

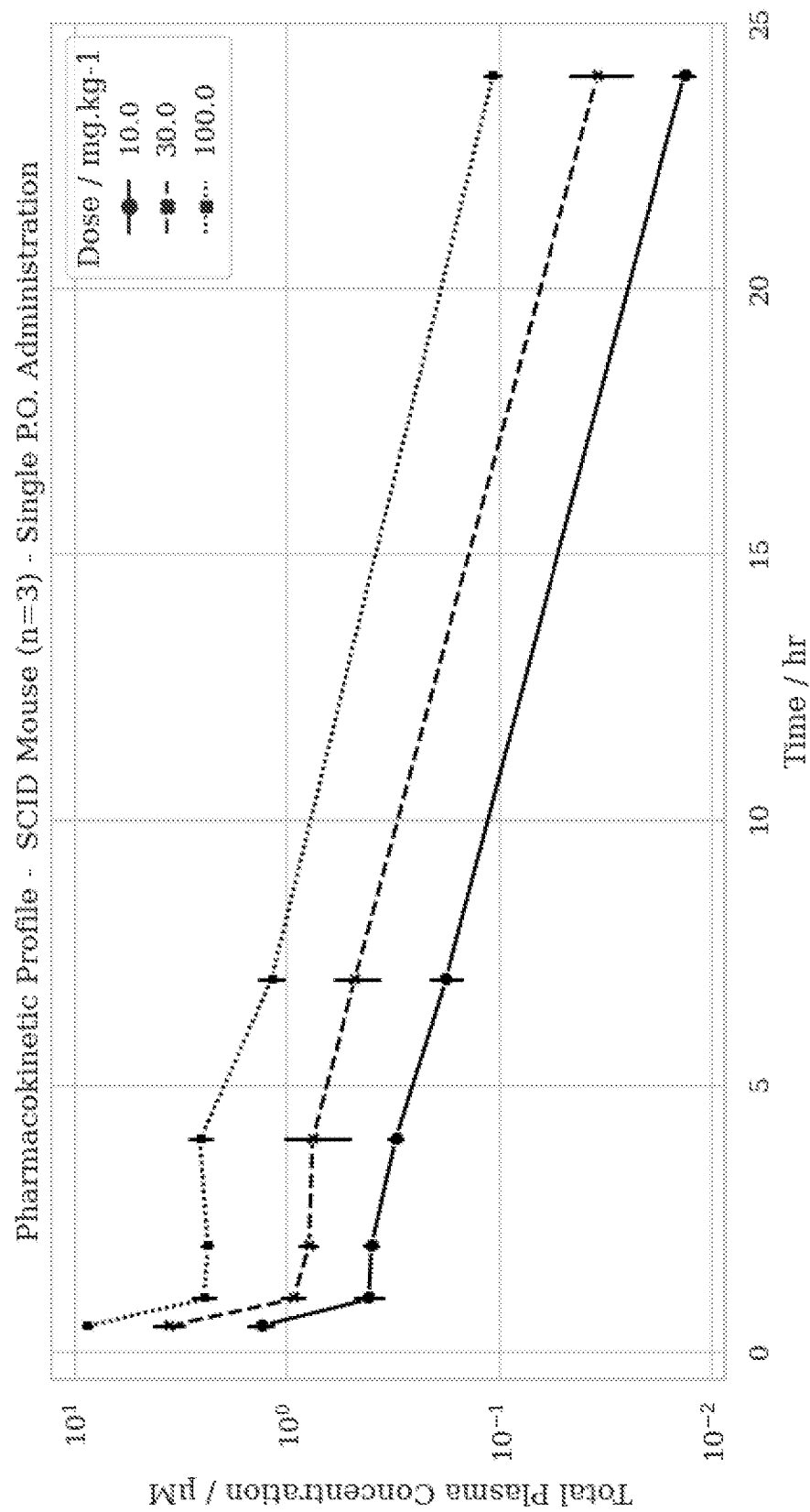


FIGURE 16: PHARMACOKINETIC PROFILES IN SCID MICE FOLLOWING ADMINISTRATION OF AZ137-2

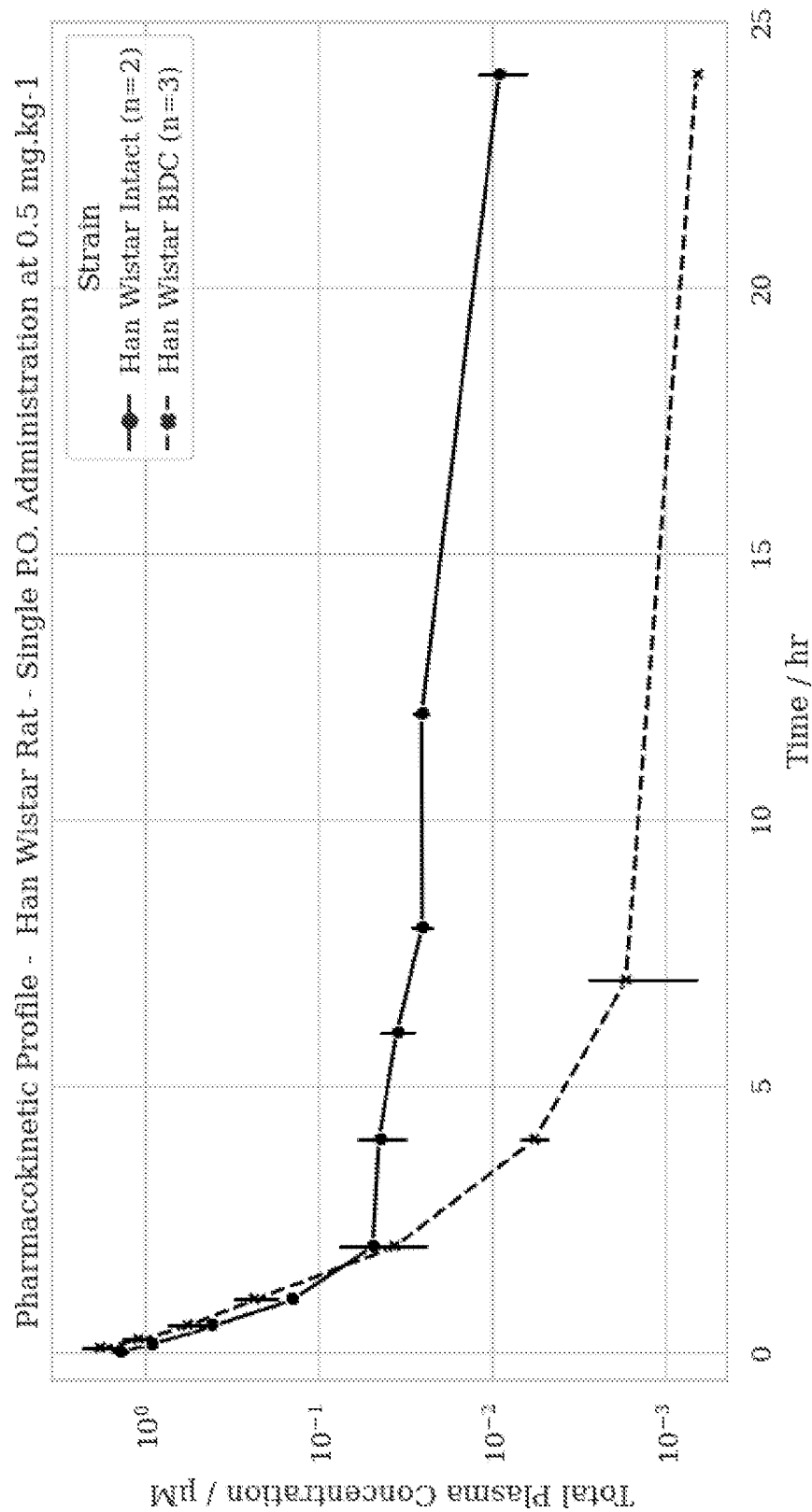


FIGURE 17: PHARMACOKINETIC PROFILES IN HAN WISTAR RATS FOLLOWING ADMINISTRATION OF AZ137-2

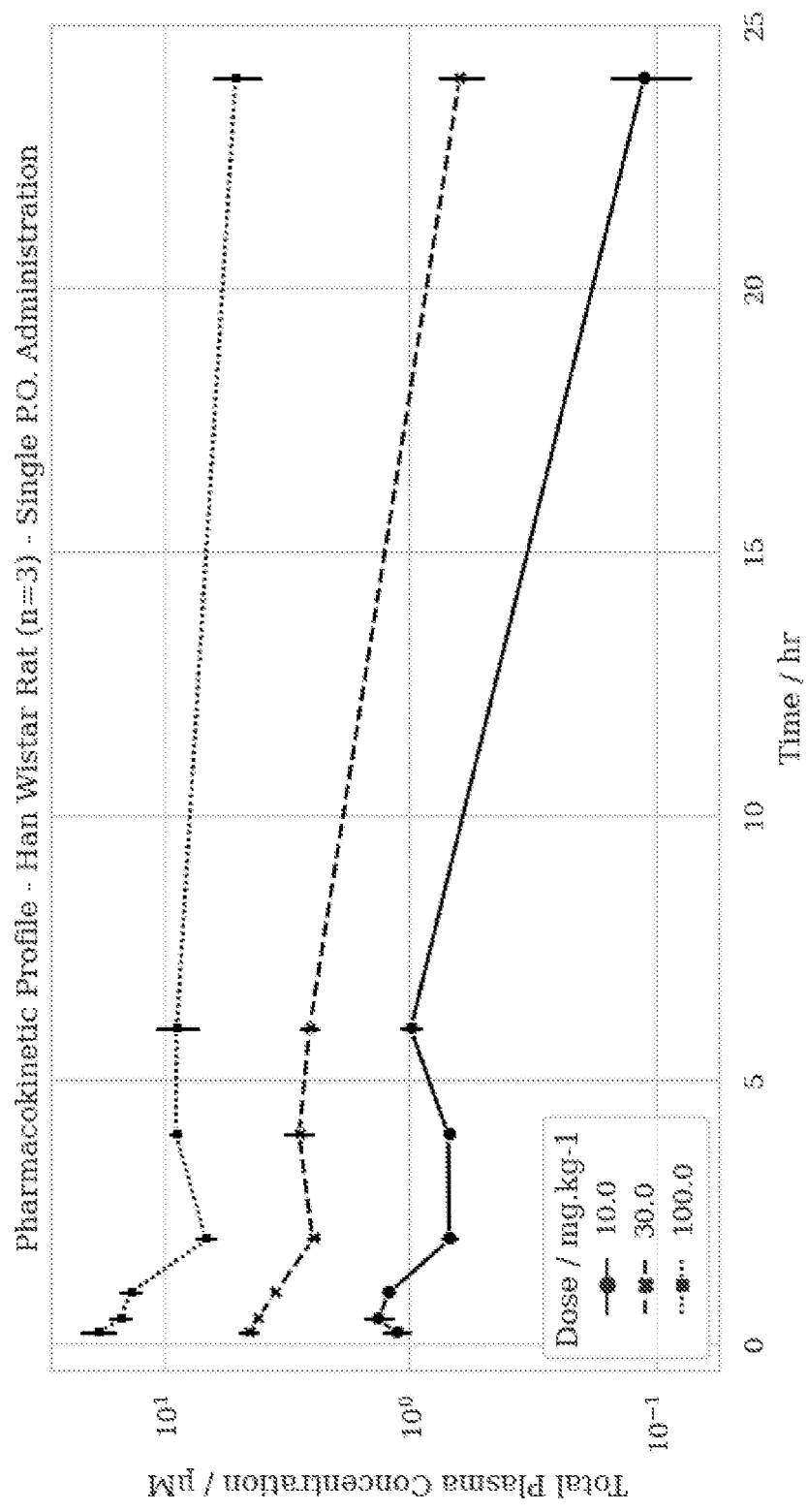


FIGURE 18: PHARMACOKINETIC PROFILES IN HAN WISTAR RATS FOLLOWING ADMINISTRATION OF AZ137-2

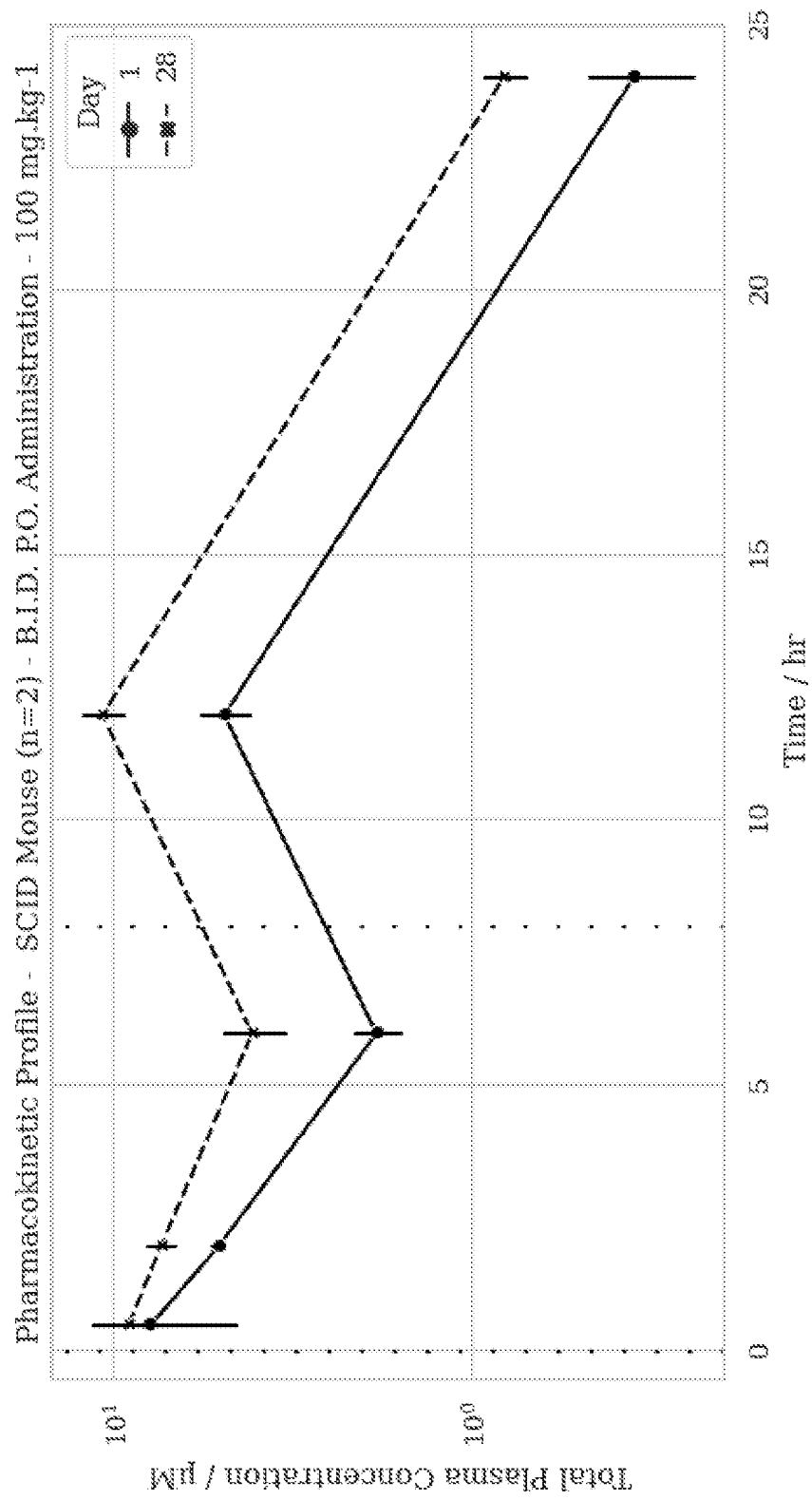


FIGURE 19: PHARMACOKINETIC PROFILES IN SCID MICE FOLLOWING ADMINISTRATION OF AZI37-2

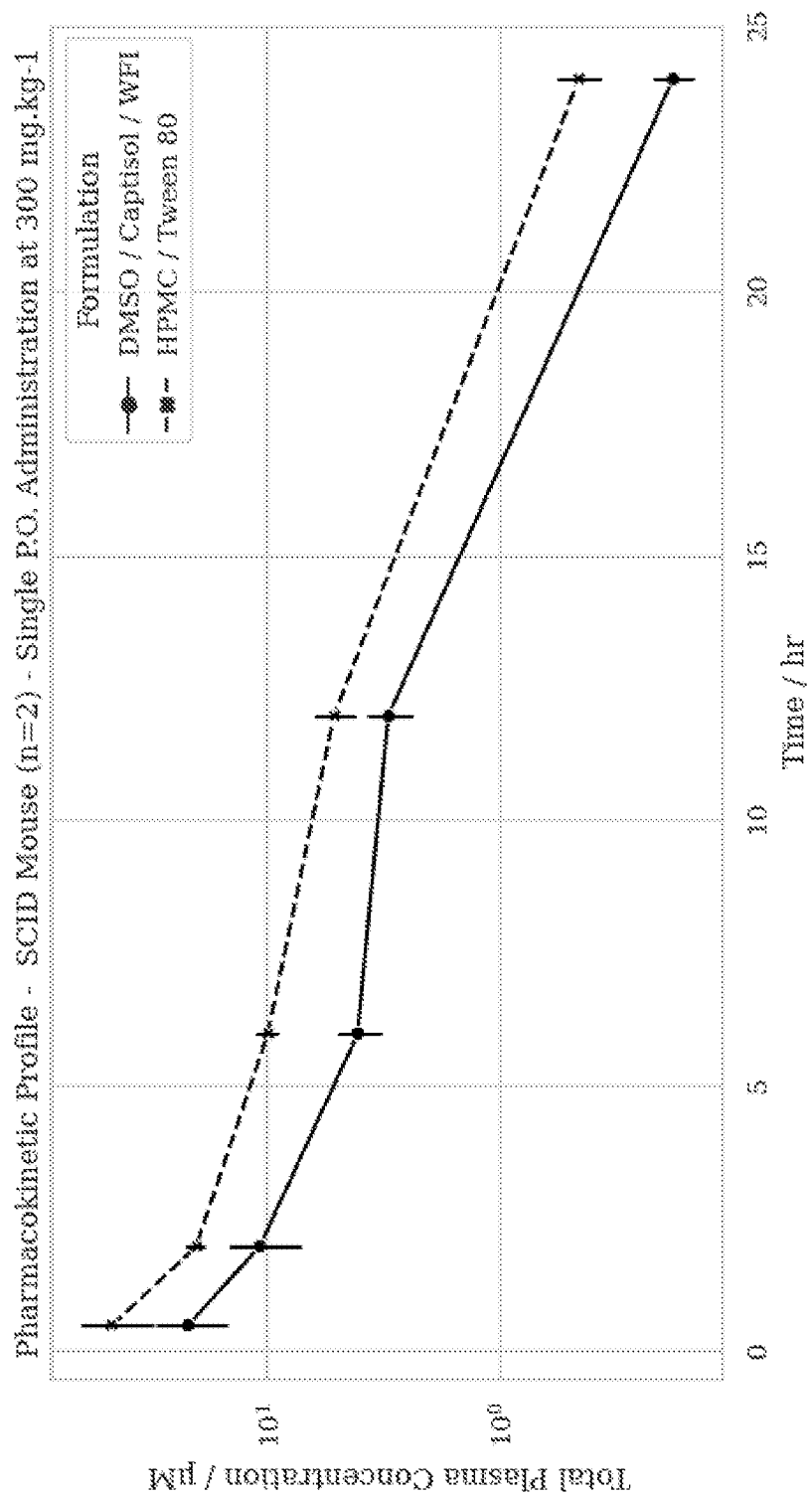


FIGURE 20: PHARMACOKINETIC PROFILES IN SCID MICE FOLLOWING ADMINISTRATION OF AZI37-2

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2024/055444

A. CLASSIFICATION OF SUBJECT MATTER INV. C07D487/04 A61P35/00 A61K31/519 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) A61P C07D		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, CHEM ABS Data, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2021/195781 A1 (REPARE THERAPEUTICS INC [CA]) 7 October 2021 (2021-10-07) the examples; page 5, line 4 - line 5; claim 1 -----	1 - 12
A,P	WO 2023/174329 A1 (INSILICO MEDICINE IP LTD [CN]) 21 September 2023 (2023-09-21) page 1, line 13 - line 15 page 11, line 1 - page 13, line 4 -----	1 - 12
☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 3 September 2024		Date of mailing of the international search report 17/09/2024
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Bakboord, Joan

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IB2024/055444

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