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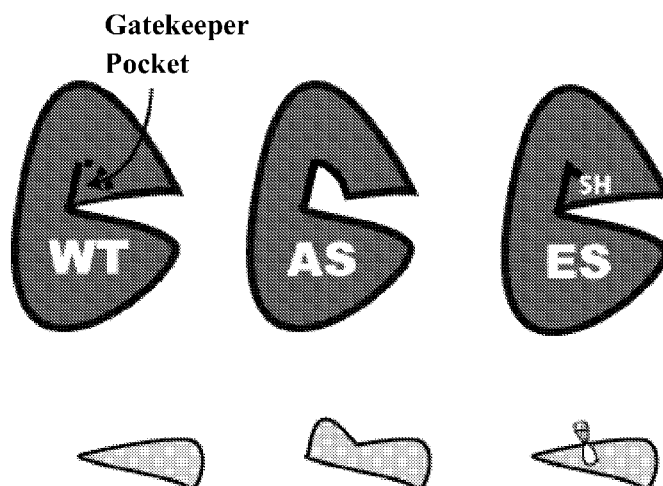
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(54) Title: METHODS AND COMPOSITIONS FOR KINASE INHIBITION

FIG. 1



(57) Abstract: The present invention sets forth a new chemical genetic approach for engineering kinase enzymes with a cysteine gatekeeper residue as well as for developing electrophilic inhibitors thereto. The present invention also provides a Src proto-oncogenic tyrosine kinase with a cysteine gatekeeper that recapitulates wild type activity and can be irreversibly inhibited both in vitro and in cells. The present invention also provides methods and compositions for modulating kinases and for treating kinase-associated diseases.



METHODS AND COMPOSITIONS FOR KINASE INHIBITION

CROSS-REFERENCES TO RELATED APPLICATIONS

- 5 [0001] This application claims the benefit of U.S. Provisional Patent Application 61/351,663, filed June 04, 2010, which is hereby incorporated by reference in its entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER 10 FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with Government support under Grant Nos. 5F32CA138103-2 and 1R01EB001987-16, awarded by the National Institutes of Health. The Government has certain rights in this invention.

15 BACKGROUND OF THE INVENTION

- [0003] Kinases, which constitute a large family of enzymes (>500 in humans), catalyze the transfer of the γ -phosphate of ATP to protein substrates. Reversible phosphorylation plays a paramount role in cell signaling processes and is regulated by kinases and phosphatases. Accordingly, kinases are critical mediators of a myriad of signal transduction processes.
- 20 Aberrant kinase activity is linked to cancer as well as metabolic, immunological, and nervous system disorders. As a result, kinases have emerged as an important class of drug targets for human disease. However, due to the conserved nature of the active sites of the protein kinase family, it is difficult to obtain selective inhibitors for any one kinase.
- [0004] There are at least 518 kinases, such as those which catalyze the transfer of the
25 gamma phosphate of ATP to protein and small molecule substrates and are involved in cell signaling processes. Small molecules provide a means for delineating kinase signaling because they are fast acting and dosable. However, because all kinase active sites recognize ATP, it is difficult to develop selective ATP- competitive inhibitors. Several years ago, a chemical genetic strategy for selective kinase inhibition was developed with reversible
30 inhibitors (U.S. Patent Publication No. 2009/0221614). The chemical genetic strategy involves the engineered mutation of a conserved bulky residue in the kinase active site known

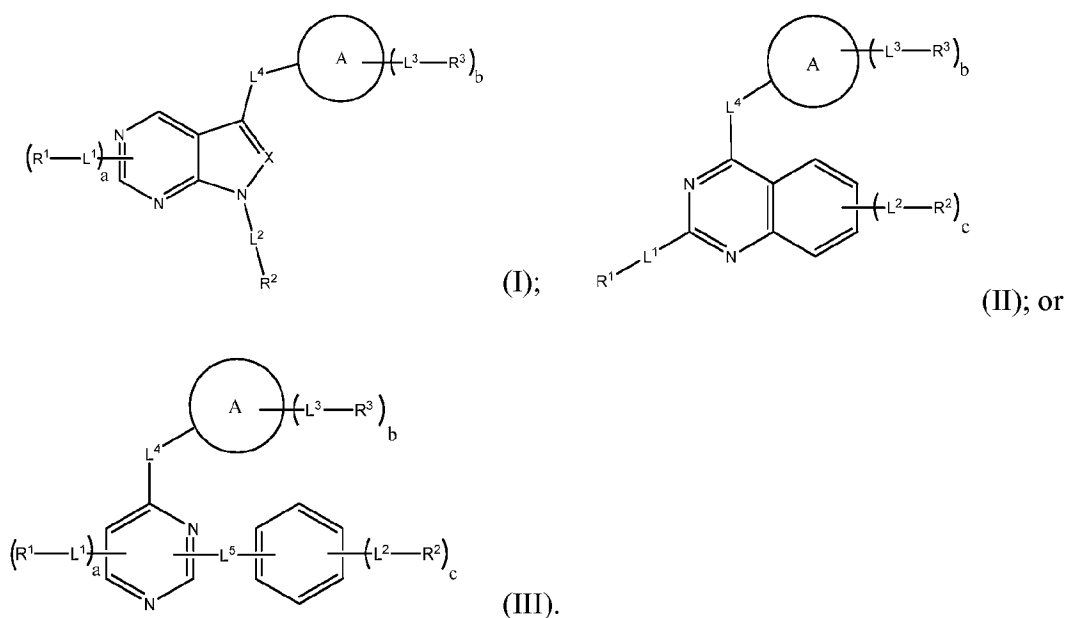
as "the gatekeeper" to a small residue such as glycine or alanine (See Bishop AC, et al. (1998) Design of allele-specific inhibitors to probe protein kinase signaling. *Curr Biol* 8(5):257-266; and Bishop AC, et al. (2000) A chemical switch for inhibitor-sensitive alleles of any protein kinase. *Nature* 407(6802):395-401). The engineered active site can then
 5 accommodate an inhibitor capable of occupying the newly formed binding pocket. While this strategy has utility, mutation of the gatekeeper residue to a small amino acid may impair the activity of the kinase and the selective inhibition can only be applied to one kinase at a time. In addition, it is sometimes not possible to achieve the desired potency.

[0005] It is known in the field that mutations in Leucine-Rich Repeat Kinase 2 (Lrrk-2) can
 10 lead to Parkinson's Disease. Also, it is thought that Parkinson's Disease (PD) is caused by uncontrolled apoptosis of dopaminergic neurons. Because inhibition of Lrrk-2 kinase activity can inhibit the apoptotic effects, there is a need to develop inhibitors for Lrrk-2 to provide treatments for Parkinson's Disease.

[0006] As such, there is a need in the field to develop kinase gatekeeper residue mutations
 15 which do not diminish kinase activity or ATP affinity as well as small molecules which inhibit these kinases. There is also a need to develop effective Lrrk-2 inhig Surprisingly, the present invention solves these as well as other problems in the field.

BRIEF SUMMARY OF THE INVENTION

[0007] In one aspect, the present invention provides a compound having the formula:



20 X is =N- or =C(L⁶-R⁶)-. Ring A is, in each instance, independently selected from cycloalkyl, heterocycloalkyl, aryl, or heteroaryl; L¹, L², L³, L⁴, L⁵, and L⁶ are, in each instance,

independently selected from a bond, -C(O)-, -C(O)N(R⁷)-, -C(O)O-, -S(O)_g-, -S(O)₂N(R⁷)-, -O-, -N(R⁷)-, -N(R⁷)C(O)N(R⁸)-, substituted or unsubstituted alkylene, substituted or unsubstituted heteroalkylene, substituted or unsubstituted cycloalkylene, substituted or unsubstituted heterocycloalkylene, substituted or unsubstituted arylene, or substituted or unsubstituted heteroarylene, wherein g is an integer from 0 to 2; R¹, R², R³, R⁴, R⁵, R⁶, R⁷, and R⁸ are, in each instance, independently selected from hydrogen, halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, or substituted or unsubstituted heteroaryl; a is an integer from 0 to 2; b is an integer from 0 to 5; and c is an integer from 0 to 4.

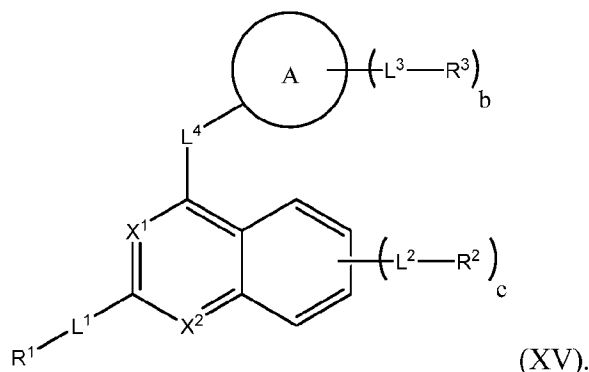
[0008] In a second aspect, the present invention provides a recombinant kinase comprising a cysteine substitution at a gatekeeper amino acid position.

[0009] In a third aspect, the present invention provides a co-crystal comprising a recombinant kinase and a compound of provided herein (*e.g.* formula I, II, or III).

[0010] In a fourth aspect, the present invention provides an isolated nucleic acid comprising a polynucleotide sequence encoding a recombinant kinase provided herein.

[0011] In a fifth aspect, the present invention provides a method of inhibiting a recombinant kinase provided herein, comprising contacting the recombinant kinase with an effective amount of an inhibitor provided herein, thereby inhibiting the recombinant kinase.

[0012] In a sixth aspect, the present invention provides a compound having the formula:



X¹ and X² are, in each instance, independently =N- or =C(-L⁶-R⁶)-. Ring A is as defined above. R¹, R², and R³ are as defined above. L¹, L², and L³ are as defined above. The variables b and c are as defined above.

[0013] In a seventh aspect, the present invention provides a method of inhibiting a Lrrk-2 kinase, the method comprising contacting the Lrrk-2 kinase with an effective amount of a Lrrk-2 inhibitor, thereby inhibiting the Lrrk-2 kinase.

5 [0014] In an eighth aspect, the present invention provides a method of forming a recombinant kinase, comprising transforming a cell with a nucleic acid as set forth herein, thereby forming a recombinant kinase as set forth herein.

[0015] In a ninth aspect, the present invention provides a method of treating a kinase-associated disease or condition, in a patient in need thereof, said method comprising administering to said patient a therapeutically effective amount of a compound of the present
10 invention, thereby treating a kinase-associated disease or condition.

[0016] In a tenth aspect, the present invention provides a method of treating a Lrrk-2-associated disease or condition, in a patient in need thereof, said method comprising administering to said patient a therapeutically effective amount of a compound of the present invention, thereby treating a Lrrk-2-associated disease or condition.

15 [0017] In an eleventh aspect, the present invention provides a kit comprising, a recombinant kinase or a nucleic acid provided herein; and instructions for using the kit.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] FIG. 1 shows a schematic of the chemical genetic strategies for inhibiting protein kinases. Kinases are depicted on top, e.g. *WT*, *AS*, and *ES*, and inhibitors types are
20 represented on the bottom. Wild type (*WT*) kinases generally harbor hydrophobic gatekeeper residues and may not be inhibited selectively. An analog-sensitive (*AS*) protein kinase has an engineered glycine or alanine gatekeeper and may be selectively inhibited by a bulky inhibitor. An electrophile-sensitive (*ES*) protein kinase contains an engineered cysteine gatekeeper and may be selectively inhibited by an electrophilic inhibitor.

25 [0019] FIG. 2 shows the crystal structure of compound **9** bound covalently to c-Src-*ES1*. The experimental electron density of c-Src-*ES1* at 2.20 Å resolution is shown ($2F_o - F_c$ map at 1σ). (A) The pyrazolopyrimidine portion of compound **9** (green) interacts with the hinge region of c-Src (Met-341 and Glu-339), while the sulfonamide group makes a hydrogen
30 bonds with Glu-310 of the αC helix (B) Electron density reveals a covalent linkage between Cys-338 and compound **9**. The oxygen atoms of the sulfonamide interact with the backbone of Asp-404 and via a water molecule with Phe-405, both of which are part of the DFG-motif of the kinase (C) Comparison of structural features of compound **9** bound to c-Src-*ES1* and a

known pyrazolopyrimidine compound bound to WT c-Src. Both compounds engage the hinge region in a similar fashion and bind the α C helix in the “in” conformation.

Furthermore, both compounds participate in hydrogen bonding interactions with Glu-310 and backbone amides of the DFG-motif. However while the known pyrazolopyrimidine compound binds in the “DFG-out” conformation, compound **9** engages the “DFG-in” orientation. The sulfhydryl of the Cys-338 points in the opposite direction relative to the hydroxyl group of Thr-338 in order to facilitate a covalent bond with compound **9**.

[0020] FIG. 3 shows an assay for MOK inhibition by cysteine gatekeeper-targeting compounds. (top) FLAG-MOK expressed in COS7 cells was immunoprecipitated and assayed *in vitro* with a myelin basic protein (MBP) substrate and inhibitors at a concentration of 1 μ M. Autoradiography is shown. (center) Quantification of the percent MBP phosphorylated from three independent experiments with associated standard errors. All values are normalized relative to the MOK + DMSO lane. (bottom) Western blot of loading controls for FLAG-MOK are shown.

[0021] FIG. 4 shows a cellular dose response analysis for inhibition of v-Src-ES1 (I338C) with electrophilic inhibitors. Cells transfected NIH-3T3 with either v-Src-ES1 or I338T v-Src were treated with electrophilic inhibitors or non-reactive analogs for one hour (see the far right column of each run, e.g. 10 μ M 11; 10 μ M 11; 10 μ M 14; 10 μ M 14). Kinase activity was monitored by blotting for global phosphotyrosine levels. Actin blots were included to control for protein content.

[0022] FIG. 5 shows relative rates of wild type c-Src and T338C c-Src following treatment with PP1, **13** or **9** and purification by gel filtration. Assay was done in triplicate, and average values with standard errors are given.

[0023] FIG. 6 shows ESI-*oa*-TOF mass spectral analysis of covalent labeling of T338C c-Src and WT c-Src with compound **9**. T338C c-Src (a) or WT c-Src (b) (15 μ M) was incubated with two equivalents of compound **9** and analyzed by full-protein mass spectrometry after 5 minutes of reaction. A 372 Da mass change occurs upon covalent labeling. Deconvoluted mass spectra are shown.

[0024] FIG. 7 shows analysis of the activity of v-Src gatekeeper variants in cells by Western blot. NIH-3T3 cells lines were infected with several v-Src gatekeeper variants. The kinase activity of the variants was analyzed by blotting for global phosphotyrosine levels (pTyr). The Src and actin blots account for Src expression levels and total protein content, respectively.

[0025] FIG. 8 shows the amino acid sequence of Src and also the nucleic acid sequence encoding therefore.

[0026] FIG. 9 shows inhibition of Lrrk-2 kinase activity.

[0027] FIG. 10 shows inhibition of Lrrk-2 kinase activity.

5 [0028] FIG. 11 shows selectivity of compound 19 (3-vs-Q) in the Invitrogen SelectScreen Kinase Assay.

[0029] FIG. 12 shows SAR analysis and inhibition as dependent on a vinylsulfonamide in the 3 position.

10 [0030] FIG. 13 shows SAR analysis and inhibition as dependent on a vinylsulfonamide in the 3 position.

[0031] FIG. 14 shows SAR analysis and inhibition as dependent on a vinylsulfonamide in the 3 position.

[0032] FIG. 15 shows the synthesis of compounds suitable for use with the present invention.

15 [0033] FIG. 16 shows *in vitro* kinase assay data for wild type and G2019S Lrrk-2.

[0034] FIG. 17 shows toxicity profiles. LD50 of compound 19 (3-vs-Q) LD50 = 3.92 μM; All other compounds ≥ 50 μM

[0035] FIG. 18 shows toxicity profiles. LD50 of compound 19 (3-vs-Q) LD50 = 3.92 μM; All other compounds ≥ 50 μM

20 [0036] FIG. 19 shows toxicity profiles. LD50 of compound 19 (3-vs-Q) LD50 = 3.92 μM; All other compounds ≥ 50 μM

[0037] FIG. 20 shows immunocytochemistry. Top left: Staruasporeine, TUNEL stain; Top Right: G2019S mutant, -drug; Bottom: G2019S mutant, +Th.

[0038] FIG. 21 shows assay data.

25 [0039] FIG. 22 shows a synthesis of compounds suitable for use with the present invention.

DETAILED DESCRIPTION OF THE INVENTION

I. General

Provided herein, *inter alia*, are methods and compositions for imparting to a kinase the capability of being inhibited by a heterocyclic compound *e.g.*, a cysteine substituted kinase having a gatekeeper amino acid residue within an ATP binding site of a kinase replaced with a cysteine residue. Also provided are methods and compositions for inhibiting a kinase with a heterocyclic compound. Furthermore, methods and compositions are provided for determining a biological activity of a kinase and treating kinase-associate diseases. In addition, methods and compositions are provided for inhibiting a Lrrk-2 kinase.

II. Definitions

[0040] The term “alkyl,” by itself or as part of another substituent, means, unless otherwise stated, a straight (i.e. unbranched) or branched chain, , or combination thereof, which may be fully saturated, mono- or polyunsaturated and can include di- and multivalent radicals, having the number of carbon atoms designated (*i.e.* C₁-C₁₀ means one to ten carbons). Examples of saturated hydrocarbon radicals include, but are not limited to, groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, homologs and isomers of, for example, n-pentyl, n-hexyl, n-heptyl, n-octyl, and the like. An unsaturated alkyl group is one having one or more double bonds or triple bonds. Examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butylnyl, and the higher homologs and isomers.

[0041] The term “alkylene” by itself or as part of another substituent means a divalent radical derived from an alkyl, as exemplified, but not limited, by $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-$, $-\text{CH}_2\text{CH}=\text{CHCH}_2-$, $-\text{CH}_2\text{C}\equiv\text{CCH}_2-$, $-\text{CH}_2\text{CH}_2\text{CH}(\text{CH}_2\text{CH}_2\text{CH}_3)\text{CH}_2-$. Typically, an alkyl (or alkylene) group will have from 1 to 24 carbon atoms, with those groups having 10 or fewer carbon atoms being preferred in the present invention. A “lower alkyl” or “lower alkylene” is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms.

[0042] The term “heteroalkyl,” by itself or in combination with another term, means, unless otherwise stated, a stable straight or branched chain, or combinations thereof, consisting of at least one carbon atoms and at least one heteroatom selected from the group consisting of O, N, P, Si and S, and wherein the nitrogen, phosphorus, and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O,

N, P and S and Si may be placed at any interior position of the heteroalkyl group or at the position at which alkyl group is attached to the remainder of the molecule. Examples include, but are not limited to,

- 5 -CH₂-CH₂-O-CH₃, -CH₂-CH₂-NH-CH₃, -CH₂-CH₂-N(CH₃)-CH₃, -CH₂-S-CH₂-CH₃, -CH₂-CH₂-,
 -S(O)-CH₃, -CH₂-CH₂-S(O)₂-CH₃, -CH=CH-O-CH₃, -Si(CH₃)₃, -CH₂-CH=N-OCH₃, -CH=CH-N(CH₃)-CH₃, O-CH₃, -O-CH₂-CH₃, and -CN. Up to two or three heteroatoms may be consecutive, such as, for example, -CH₂-NH-OCH₃ and -CH₂-O-Si(CH₃)₃. Similarly, the term “heteroalkylene” by itself or as part of another substituent means a divalent radical
 10 derived from heteroalkyl, as exemplified, but not limited by, -CH₂-CH₂-S-CH₂-CH₂- and -CH₂-S-CH₂-CH₂-NH-CH₂-. For heteroalkylene groups, heteroatoms can also occupy either or both of the chain termini (*e.g.*, alkyleneoxo, alkylenedioxo, alkyleneamino, alkylenediamino, and the like). Still further, for alkylene and heteroalkylene linking groups, no orientation of the linking group is implied by the direction in which the formula of the
 15 linking group is written. For example, the formula -C(O)OR'- represents both -C(O)OR'- and -R'OC(O)-. As described above, heteroalkyl groups, as used herein, include those groups that are attached to the remainder of the molecule through a heteroatom, such as -C(O)R', -C(O)NR', -NR'R", -OR', -SR', and/or -SO₂R'. Where “heteroalkyl” is recited, followed by recitations of specific heteroalkyl groups, such as -NR'R" or the like, it will be
 20 understood that the terms heteroalkyl and -NR'R" are not redundant or mutually exclusive. Rather, the specific heteroalkyl groups are recited to add clarity. Thus, the term “heteroalkyl” should not be interpreted herein as excluding specific heteroalkyl groups, such as -NR'R" or the like.

- [0043] The terms “cycloalkyl” and “heterocycloalkyl”, by themselves or in combination
 25 with other terms, represent, unless otherwise stated, cyclic versions of “alkyl” and “heteroalkyl”, respectively. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. Examples of cycloalkyl include, but are not limited to, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Examples of heterocycloalkyl include, but are not
 30 limited to, 1-(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1-piperazinyl, 2-piperazinyl, and the like. The terms “cycloalkylene” and “heterocycloalkylene” refer to the divalent derivatives of cycloalkyl and heterocycloalkyl, respectively.

[0044] The term “aryl” means, unless otherwise stated, a polyunsaturated, aromatic, hydrocarbon substituent which can be a single ring or multiple rings (preferably from 1 to 3 rings) which are fused together (e.g. naphthyl) or linked covalently. The term “heteroaryl” refers to aryl groups (or rings) that contain heteroatoms (in at least one ring in the case of multiple rings) selected from N, O, and S, wherein the nitrogen and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a carbon or heteroatom. Non-limiting examples of aryl and heteroaryl groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 6-isoquinolyl, 2-quinoxaliny, 5-quinoxaliny, 3-quinolyl, and 6-quinolyl. Thus, the term “heteroaryl” include fused ring structures in which at least one ring includes at least two double bonds. Substituents for each of above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described below. The terms “arylene” and “heteroarylene” refer to the divalent radicals of aryl and heteroaryl, respectively.

[0045] For brevity, the term “aryl” when used in combination with other terms (e.g., aryloxo, arylthioxo, arylalkyl) includes both aryl and heteroaryl rings as defined above. Thus, the term “arylalkyl” is meant to include those radicals in which an aryl group is attached to an alkyl group (e.g., benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (e.g., a methylene group) has been replaced by, for example, an oxygen atom (e.g., phenoxymethyl, 2-pyridyloxymethyl, 3-(1-naphthyloxy)propyl, and the like). However, the term “haloaryl,” as used herein is meant to cover only aryls substituted with one or more halogens.

[0046] Where a heteroalkyl, heterocycloalkyl, or heteroaryl includes a specific number of members (e.g. “3 to 7 membered”), the term “member” refers to a carbon or heteroatom.

[0047] The term “oxo” as used herein means an oxygen that is double bonded to a carbon atom.

[0048] Each of above terms (e.g., “alkyl,” “heteroalkyl,” “cycloalkyl, and “heterocycloalkyl”, “aryl,” “heteroaryl” as well as their divalent radical derivatives) are

meant to include both substituted and unsubstituted forms of the indicated radical. Preferred substituents for each type of radical are provided below.

[0049] Substituents for alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl monovalent and divalent derivative radicals (including those groups often referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) can be one or more of a variety of groups selected from, but not limited to: -OR', =O, =NR', =N-OR', -NR'R'', -SR', -halogen, -SiR'R''R''', -OC(O)R', -C(O)R', -CO₂R', -C(O)NR'R'', -OC(O)NR'R'', -NR''C(O)R', -NR'-C(O)NR''R'', -NR''C(O)OR', -NR-C(NR'R'')=NR'', -S(O)R', -S(O)₂R', -S(O)₂NR'R'', -NRSO₂R', -CN and -NO₂ in a number ranging from zero to (2m'+1), where m' is the total number of carbon atoms in such radical. R', R'', R''' and R'''' each preferably independently refer to hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl (e.g., aryl substituted with 1-3 halogens), substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or arylalkyl groups. As used herein, an "alkoxy" group is an alkyl attached to the remainder of the molecule through a divalent oxygen radical. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R'', R''' and R'''' groups when more than one of these groups is present. When R' and R'' are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 4-, 5-, 6-, or 7-membered ring. For example, -NR'R'' is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents, one of skill in the art will understand that the term "alkyl" is meant to include groups including carbon atoms bound to groups other than hydrogen groups, such as haloalkyl (e.g., -CF₃ and -CH₂CF₃) and acyl (e.g., -C(O)CH₃, -C(O)CF₃, -C(O)CH₂OCH₃, and the like).

[0050] Similar to the substituents described for alkyl radicals above, exemplary substituents for aryl and heteroaryl groups (as well as their divalent derivatives) are varied and are selected from, for example: halogen, -OR', -NR'R'', -SR', -halogen, -SiR'R''R''', -OC(O)R', -C(O)R', -CO₂R', -C(O)NR'R'', -OC(O)NR'R'', -NR''C(O)R', -NR'-C(O)NR''R'', -NR''C(O)OR', -NR-C(NR'R'')=NR'', -NR-C(NR'R'')=NR'', -S(O)R', -S(O)₂R', -S(O)₂NR'R'', -NRSO₂R', -CN and -NO₂, -R', -N₃, -CH(Ph)₂, fluoro(C₁-C₄)alkoxo, and fluoro(C₁-C₄)alkyl, in a number ranging from zero to the total number of open valences on aromatic ring system; and where R', R'', R''' and R'''' are preferably independently selected from hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl,

substituted or unsubstituted aryl and substituted or unsubstituted heteroaryl. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R'', R''' and R'''' groups when more than one of these groups is present.

- 5 [0051] Two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally form a ring of the formula -T-C(O)-(CRR')_q-U-, wherein T and U are independently -NR-, -O-, -CRR'- or a single bond, and q is an integer of from 0 to 3. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula -A-(CH₂)_r-B-, wherein A and B are
- 10 independently -CRR'-, -O-, -NR-, -S-, -S(O)-, -S(O)₂-, -S(O)₂NR'- or a single bond, and r is an integer of from 1 to 4. One of the single bonds of the new ring so formed may optionally be replaced with a double bond. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula
- 15 -(CRR')_s-X'-(C''R''')_d-, where s and d are independently integers of from 0 to 3, and X' is -O-, -NR'-, -S-, -S(O)-, -S(O)₂-, or -S(O)₂NR'-. The substituents R, R', R'' and R''' are preferably independently selected from hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, and substituted or unsubstituted heteroaryl.

- [0052] As used herein, the term "heteroatom" or "ring heteroatom" is meant to include
- 20 oxygen (O), nitrogen (N), sulfur (S), phosphorus (P), and silicon (Si).

- [0053] The terms "halo" or "halogen," by themselves or as part of another substituent, mean, unless otherwise stated, a fluorine, chlorine, bromine, or iodine atom. Additionally, terms such as "haloalkyl," are meant to include monohaloalkyl and polyhaloalkyl. For example, the term "halo(C₁-C₄)alkyl" is mean to include, but not be limited to,
- 25 trifluoromethyl, 2,2,2-trifluoroethyl, 4-chlorobutyl, 3-bromopropyl, and the like.

- [0054] A "size-limited substituent" or "size-limited substituent group," as used herein means a group selected from all of the substituents described above for a "substituent group," wherein each substituted or unsubstituted alkyl is a substituted or unsubstituted C₁-C₂₀ alkyl, each substituted or unsubstituted heteroalkyl is a substituted or unsubstituted 2 to 20
- 30 membered heteroalkyl, each substituted or unsubstituted cycloalkyl is a substituted or unsubstituted C₄-C₈ cycloalkyl, and each substituted or unsubstituted heterocycloalkyl is a substituted or unsubstituted 4 to 8 membered heterocycloalkyl.

[0055] A “lower substituent” or “ lower substituent group,” as used herein means a group selected from all of the substituents described above for a “substituent group,” wherein each substituted or unsubstituted alkyl is a substituted or unsubstituted C₁-C₈ alkyl, each substituted or unsubstituted heteroalkyl is a substituted or unsubstituted 2 to 8 membered heteroalkyl, each substituted or unsubstituted cycloalkyl is a substituted or unsubstituted C₅-C₇ cycloalkyl, and each substituted or unsubstituted heterocycloalkyl is a substituted or unsubstituted 5 to 7 membered heterocycloalkyl.

[0056] In some embodiments, each substituted group described in the compounds herein is substituted with at least one substituent group. More specifically, in some embodiments, each substituted alkyl, substituted heteroalkyl, substituted cycloalkyl, substituted heterocycloalkyl, substituted aryl, substituted heteroaryl, substituted alkylene, substituted heteroalkylene, substituted cycloalkylene, substituted heterocycloalkylene, substituted arylene, and/or substituted heteroarylene described in the compounds herein are substituted with at least one substituent group. In other embodiments, at least one or all of these groups are substituted with at least one size-limited substituent group. Alternatively, at least one or all of these groups are substituted with at least one lower substituent group.

[0057] In other embodiments of the compounds herein, each substituted or unsubstituted alkyl is a substituted or unsubstituted C₁-C₂₀ alkyl, each substituted or unsubstituted heteroalkyl is a substituted or unsubstituted 2 to 20 membered heteroalkyl, each substituted or unsubstituted cycloalkyl is a substituted or unsubstituted C₃-C₈ cycloalkyl, and/or each substituted or unsubstituted heterocycloalkyl is a substituted or unsubstituted 3 to 8 membered heterocycloalkyl. In some embodiments of the compounds herein, each substituted or unsubstituted alkylene is a substituted or unsubstituted C₁-C₂₀ alkylene, each substituted or unsubstituted heteroalkylene is a substituted or unsubstituted 2 to 20 membered heteroalkylene, each substituted or unsubstituted cycloalkylene is a substituted or unsubstituted C₃-C₈ cycloalkylene, and/or each substituted or unsubstituted heterocycloalkylene is a substituted or unsubstituted 3 to 8 membered heterocycloalkylene.

[0058] In some embodiments, each substituted or unsubstituted alkyl is a substituted or unsubstituted C₁-C₈ alkyl, each substituted or unsubstituted heteroalkyl is a substituted or unsubstituted 2 to 8 membered heteroalkyl, each substituted or unsubstituted cycloalkyl is a substituted or unsubstituted C₅-C₇ cycloalkyl, and/or each substituted or unsubstituted heterocycloalkyl is a substituted or unsubstituted 5 to 7 membered heterocycloalkyl. In some embodiments, each substituted or unsubstituted alkylene is a substituted or unsubstituted C₁-C₈ alkylene, each substituted or unsubstituted heteroalkylene is a substituted or unsubstituted

2 to 8 membered heteroalkylene, each substituted or unsubstituted cycloalkylene is a substituted or unsubstituted C₅-C₇ cycloalkylene, and/or each substituted or unsubstituted heterocycloalkylene is a substituted or unsubstituted 5 to 7 membered heterocycloalkylene. In some embodiments, the compound is a chemical species set forth in the Examples section below.

[0059] A “bulky residue” in an amino acid residue having a side chain group that is larger (i.e. having more atoms and tending to fill more space) than glycine and alanine, and optionally larger than cysteine. A bulky residue may be methionine, leucine, phenylalanine and threonine. In some embodiments, the bulky residue may be larger than leucine, isoleucine and threonine. In some embodiments, the bulky residue includes a cyclic moiety.

[0060] “Electrophilic” is used herein in accordance with its plain ordinary meaning and refers to a chemical group having a tendency to attract, acquire or accept electrons or react at electron-rich sites.

[0061] “Nucleophilic” is used herein in accordance with its plain ordinary meaning and refers to a chemical group having a tendency to donate electrons (e.g. lower electron density) or react at electron poor sites.

[0062] “Electrophilic moiety” as used herein refers to a functional group or chemical substituent that is electrophilic. Example electrophilic moieties include, but are not limited to vinylsulfonamides, acrylamides, epoxides, and fluoromethylketones.

[0063] As defined herein, the term “electrophilic substituent” is a substituent that is electrophilic. An electrophilic substituent, electrophilic moieties and electrophilic chemical groups are typically electron-poor functional groups and can react with an electron-donating group, such as a nucleophile, by accepting an electron pair. In some embodiments, the electrophilic substituent, moiety or chemical group of a compound is capable of reacting with a cysteine residue. In some embodiments, the electrophilic substituent, moiety or chemical group is capable of forming a covalent bond with a cysteine residue within the ATP binding site of the kinase. The covalent bond is usually formed between the electrophilic substituent, moiety or chemical group and the sulfhydryl group of the cysteine and may be a reversible or irreversible bond. In some embodiments, the covalent bond is irreversible.

[0064] As used herein, the terms “protein kinase” or “kinase” are used in accordance with its plain ordinary meaning and refer to an enzyme that is capable of phosphorylating an amino acid residue, e.g. an amino acid residue on a protein. Typically specific serine,

threonine, or tyrosine residues are phosphorylated. Thus, protein kinase encompasses serine protein kinases, threonine protein kinases, and tyrosine protein kinases. An "inhibitor of a protein kinase" is a compound or agent that reduces the activity of a protein kinase. In some embodiments, a "protein kinase inhibitor" is a compound that reduces the activity of the protein kinase by binding to the protein kinase. Thus, a "protein kinase inhibitor" can inhibit activity of the enzyme in a competitive, or a noncompetitive manner.

[0065] As defined herein, the term "cysteine substituted kinase" refers to a recombinant kinase where a gatekeeper amino acid residue (e.g. within an ATP binding site of the kinase) is replaced with a cysteine residue. Similarly, a "glycine substituted kinase" refers to a recombinant kinase where a gatekeeper amino acid residue (e.g. within an ATP binding site of the kinase) is replaced with a glycine residue, and a "alanine substituted kinase" refers to a kinase where a gatekeeper amino acid residue (e.g. within an ATP binding site of the kinase) is replaced with an alanine residue.

[0066] As defined herein, the term "fused rings" refers to a ring system with two or more rings having at least one bond and two atoms in common.

[0067] The terms "nucleic acid," "oligonucleotide," "polynucleotide," and like terms typically refer to polymers of deoxyribonucleotides or ribonucleotides in either single- or double-stranded form, and complements thereof. The term "nucleotide" typically refers to a monomer. The terms encompass nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs).

[0068] Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)). The

term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

[0069] Nucleic acids "hybridize" when they associate, typically in solution. Nucleic acids hybridize due to a variety of well-characterized physico-chemical forces, such as hydrogen bonding, solvent exclusion, base stacking and the like. As used herein, the term "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments, such as Southern and Northern hybridizations, are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen, 1993, "Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes," Part I, Chapter 2 (Elsevier, New York), which is incorporated herein by reference.

[0070] The terms "peptide," "polypeptide," and "protein" are used interchangeably herein to refer to a polymer of amino acid residues.

[0071] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α -carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid.

[0072] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0073] An amino acid or nucleotide base "position" is denoted by a number that sequentially identifies each amino acid (or nucleotide base) in the reference sequence based on its position relative to the N-terminus (or 5'-end). Due to deletions, insertions, truncations, fusions, and the like that must be taken into account when determining an optimal alignment, in general the amino acid residue number in a test sequence determined by simply counting from the N-terminus will not necessarily be the same as the number of its corresponding position in the reference sequence. For example, in a case where a variant has a deletion

relative to an aligned reference sequence, there will be no amino acid in the variant that corresponds to a position in the reference sequence at the site of deletion. Where there is an insertion in an aligned reference sequence, that insertion will not correspond to a numbered amino acid position in the reference sequence. In the case of truncations or fusions there can be stretches of amino acids in either the reference or aligned sequence that do not correspond to any amino acid in the corresponding sequence.

[0074] The terms "numbered with reference to" or "corresponding to," when used in the context of the numbering of a given amino acid or polynucleotide sequence, refers to the numbering of the residues of a specified reference sequence when the given amino acid or polynucleotide sequence is compared to the reference sequence.

[0075] A "conservative substitution" as used with respect to amino acids, refers to the substitution of an amino acid with a chemically similar amino acid. Amino acid substitutions which often preserve the structural and/or functional properties of the polypeptide in which the substitution is made are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, in "The Proteins," Academic Press, New York. The most commonly occurring exchanges are isoleucine/valine, tyrosine/phenylalanine, aspartic acid/glutamic acid, lysine/arginine, methionine/leucine, aspartic acid/asparagine, glutamic acid/glutamine, leucine/isoleucine, methionine/isoleucine, threonine/serine, tryptophan/phenylalanine, tyrosine/histidine, tyrosine/tryptophan, glutamine/arginine, histidine/asparagine, histidine/glutamine, lysine/asparagine, lysine/glutamine, lysine/glutamic acid, phenylalanine/leucine, phenylalanine/methionine, serine/alanine, serine/asparagine, valine/leucine, and valine/methionine. In some embodiments, there may be at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, or at least 40 conservative substitutions.

[0076] The term "amino acid substitution set" or "substitution set" refers to a group of amino acid substitutions. A substitution set can have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more amino acid substitutions.

[0077] The term "isolated" refers to a nucleic acid, polynucleotide, polypeptide, protein, or other component that is partially or completely separated from components with which it is normally associated (other proteins, nucleic acids, cells, etc.). In some embodiments, an isolated polypeptide or protein is a recombinant polypeptide or protein.

[0078] A nucleic acid (such as a polynucleotide), a polypeptide, or a cell is "recombinant" when it is artificial or engineered, or derived from or contains an artificial or engineered

protein or nucleic acid (*e.g.* non-natural or not wild type). For example, a polynucleotide that is inserted into a vector or any other heterologous location, *e.g.*, in a genome of a recombinant organism, such that it is not associated with nucleotide sequences that normally flank the polynucleotide as it is found in nature is a recombinant polynucleotide. A protein
5 expressed *in vitro* or *in vivo* from a recombinant polynucleotide is an example of a recombinant polypeptide. Likewise, a polynucleotide sequence that does not appear in nature, for example a variant of a naturally occurring gene, is recombinant.

[0079] "Identity" or "percent identity," in the context of two or more polypeptide sequences, refers to two or more sequences or subsequences that are the same or have a
10 specified percentage of amino acid residues that are the same (*e.g.*, share at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 88% identity, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity) over a specified region to a reference sequence, when
15 compared and aligned for maximum correspondence over a comparison window, or designated region as measured using a sequence comparison algorithms or by manual alignment and visual inspection.

[0080] Optimal alignment of sequences for comparison and determination of sequence identity can be determined by a sequence comparison algorithm or by visual inspection (see,
20 generally, Ausubel *et al.*, *infra*). When optimally aligning sequences and determining sequence identity by visual inspection, percent sequence identity is calculated as the number of residues of the test sequence that are identical to the reference sequence divided by the number of non-gap positions and multiplied by 100. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates
25 and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters as known in the art, for example BLAST or BLAST 2.0. For example, comparison can be conducted, *e.g.*, by the local
homology algorithm of Smith & Waterman, 1981, *Adv. Appl. Math.* 2:482, by the homology
30 alignment algorithm of Needleman & Wunsch, 1970, *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson & Lipman, 1988, *Proc. Nat'l. Acad. Sci. USA* 85:2444, or by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr.,
Madison, Wis.). Thus alignment can be carried out for sequences that have deletions and/or

additions, as well as those that have substitutions, as well as naturally occurring, e.g., polymorphic or allelic variants, and man-made variants.

[0081] The phrase "substantial sequence identity" or "substantial identity," in the context of two nucleic acid or polypeptide sequences, refers to a sequence that has at least 70% identity to a reference sequence. Percent identity can be any integer from 70% to 100%. Two nucleic acid or polypeptide sequences that have 100% sequence identity are said to be "identical." A nucleic acid or polypeptide sequence are said to have "substantial sequence identity" to a reference sequence when the sequences have at least about 70%, at least about 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% or greater sequence identity as determined using the methods described herein, such as BLAST using standard parameters as described above.

[0082] The term "pre-protein" refers to a protein including an amino-terminal signal peptide (or leader sequence) region attached. The signal peptide is cleaved from the pre-protein by a signal peptidase prior to secretion to result in the "mature" or "secreted" protein.

[0083] A "vector" is a DNA construct for introducing a DNA sequence into a cell. A vector may be an expression vector that is operably linked to a suitable control sequence capable of effecting the expression in a suitable host of the polypeptide encoded in the DNA sequence. An "expression vector" has a promoter sequence operably linked to the DNA sequence (*e.g.*, transgene) to drive expression in a host cell, and in some embodiments a transcription terminator sequence.

[0084] The term "expression" includes any step involved in the production of the polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

[0085] The term "operably linked" refers to a configuration in which a control sequence is appropriately placed at a position relative to the coding sequence of the DNA sequence such that the control sequence influences the expression of a polypeptide.

[0086] An amino acid or nucleotide sequence (*e.g.*, a promoter sequence, signal peptide, terminator sequence, etc.) is "heterologous" to another sequence with which it is operably linked if the two sequences are not associated in nature.

[0087] The terms "transform" or "transformation," as used in reference to a cell, means a cell has a non-native nucleic acid sequence integrated into its genome or as an episome (*e.g.*, plasmid) that is maintained through multiple generations.

[0088] The term "culturing" refers to growing a population of microbial cells under suitable conditions in a liquid or solid medium.

[0089] The term "introduced," as used in the context of inserting a nucleic acid sequence into a cell, means conjugated, transfected, transduced or transformed (collectively "transformed") or otherwise incorporated into the genome of, or maintained as an episome in, the cell.

[0090] As defined herein, the term "gatekeeper amino acid residue" or "gatekeeper residue" refers to a residue (*e.g.* within the ATP binding site of a kinase) that is capable of controlling or modulating the ability of a kinase substrate to bind to the kinase. For example, in some embodiments, the accessibility of a protein kinase substrate to the ATP binding site is controlled by the gatekeeper residue. In certain embodiments, the gatekeeper residue controls the ability of the substrate to access or bind a hydrophobic pocket adjacent to the ATP binding site. (Elphick *et al.* *ACS Chemical Biology*, 2:299-314, 2007). As defined herein, a natural gatekeeper residue refers to a gatekeeper residue identified in a wild-type kinase. Examples of gatekeeper residues include, *e.g.*, Thr338 of c-Src (v-Src numbering, see Liu *et al.*, *Chemistry & Biology*, 6:671-678, 1999), and Thr 493 of rsk2 (see US Application No. 2009/0221614). Gatekeeper residues in other kinases, *e.g.*, gatekeeper residues corresponding to Thr338 of c-Src can be readily identified by structure-based sequence alignment of kinase domain of various src or non-src kinases. The following is a structure-based sequence alignment of several kinase domains (see U.S. Patent Publication No. 2009/0221614). The gatekeeper residues referred to herein are highlighted in bold italics:

Name	Sequence	SEQ ID NO:
src	---PEAFLQEAQVMK--KLRHEKLVQLYAVVSEEP---IYIV TEYM	52
rsk2	---KRDPTTEEIEILLR-YGQHPNIITLKDVYDDGKY--VYVV TELM	53
nek2	-EVEKQMLVSEVNLLR--ELKHPNIVRYDRIIDRTNTTLYIV MEYC	54
mekk1	QEEVVEALREEIRMMS--HLNHPNIIRMLGATCEKSN--YNLF IEWM	55
msk1	---MEANTQKEITALK-LCEGHPNIVKLHEVFHDQLH--TFLV MELL	56
plk1	-PHQREKMSMEISIHR--SLAHQHVVGFGHGFEDNDF--VFVV LELC	57

[0091] Additional gatekeeper residues in various kinases can be identified by sequence alignment (see Liu et al., Chemistry & Biology, 6:671-678, 1999). For example, gatekeeper residues in various kinases corresponding to Thr338 of v-Src are highlighted in bold underlined in the sequence alignment below:

5

Name	Start	Sequence	SEQ ID NO:
		338 ↓	
v-Src	(318)	RHEKLVQLYAMVSE-----EPIYIV <u>I</u> EYMSK--GSLLDFLKGEMGKY	58
c-Src	(318)	RHEKLVQLYAVVSE-----EPIYIV <u>I</u> EYMSK--GSLLDFLKGETGKY	59
Lck	(296)	QHQRVLRLYAVVTQ-----EPIYII <u>I</u> EYMEN--GSLVDFLKTPSGIK	60
Fyn	(319)	KHDKLVQLYAVVSE-----EPIYIV <u>I</u> EYMNK--GSLLDFLKDGEGR	61
c-Yes	(325)	RHDKLVPLYAVVSE-----EPIYIV <u>I</u> EFMSK--GSLLDFLKEGDGKY	62
Yrk	(318)	RHDKLVQLYAVVSE-----EPIYIV <u>I</u> EFMSQ--GSLLDFLKDGDGRY	63
c-Fgr	(311)	RHDKLVQLYAVVSE-----EPIYIV <u>I</u> EFMCH--GSLLDFLKNPEGQD	64
Lyn	(295)	QHDKLVRRLYAVVTRE-----EPIYII <u>I</u> EYMAK--GSLLDFLKSDEGGK	65
Hck	(318)	QHDKLVLKLHAVVTK-----EPIYII <u>I</u> EFMAK--GSLLDFLKSDEGSK	66
Blk	(287)	QHERLVRRLYAVVTR-----EPIYIV <u>I</u> EYMAR--GCLLDFLKTDEGSR	67
Abl	(313)	KHPNLVQLLGVCVCTRE-----PPFYII <u>I</u> EFMTY--GNLLDYLRECNRQE	68
Btk	(473)	SHEKLVQLYGVCTKQ-----RPIFII <u>I</u> EYMAN--GCLLNLYLREMRHR	69
Csk	(244)	RHSNLVQLLGIVVEEK-----GGLYIV <u>I</u> EYMAK--GSLVDYLRSRGRSV	70
PDGFR	(660)	PHLVNVNLLGACTKG-----GPIYII <u>I</u> EYCRY--GDLVDYLHRNKHTF	71
p38	(85)	GLLDVFTPARSLEEF-----NDVVLV <u>I</u> HLMGA--DLNNIVKCQKLTD	72
ZAP-70	(394)	DNPYIVRLIGVCQA-----EALMLV <u>I</u> EMAGG--GPLHKFL-VGKREE	73
JAK2	(906)	QHDNIVKYKVCYSAGR-----RNLRLI <u>I</u> EYLPY--GSLRDYLQKHKE	74
PKA	(99)	NFPFLVKLEFSFKDN-----SNLYMV <u>I</u> EYVPG--GEMFSLHRRIGR	75
CamK II	(68)	KHPNIVRLHDSISEE-----GHHYLI <u>I</u> DLVTG--GELFEDIVAREY	76
Cdk2	(59)	NHPNIVKLLDVIHTE-----NKLYLV <u>I</u> EFLHQ--DLKKFMDASALTG	77

[0092] “Control” or “control experiment” is used in accordance with its plain ordinary meaning and refers to an experiment in which the subjects or reagents of the experiment are treated as in a parallel experiment except for omission of a procedure, reagent, or variable of the experiment. In some instances, the control is used as a standard of comparison in evaluating experimental effects.

[0093] “Contacting” is used in accordance with its plain ordinary meaning and refers to the process of allowing at least two distinct species (e.g. chemical compounds including biomolecules, or cells) to become sufficiently proximal to react, interact or physically touch. It should be appreciated, however, the resulting reaction product can be produced directly from a reaction between the added reagents or from an intermediate from one or more of the

added reagents which can be produced in the reaction mixture. The term “contacting” includes incubating an inhibitor with the kinase.

[0094] As defined herein, the term “inhibition”, “inhibit”, “inhibiting” and the like in reference to a kinase-inhibitor interaction means negatively affecting (e.g. decreasing) the activity of the kinase relative to the activity of the kinase in the absence of the inhibitor. Thus, inhibition includes, at least in part, partially or totally blocking stimulation, decreasing, preventing, or delaying activation, or inactivating, desensitizing, or down-regulating signal transduction. Similarly an “inhibitor” is a compound that inhibits kinase activity, e.g., by binding, partially or totally block stimulation, decrease, prevent, or delay activation, or inactivate, desensitize, or down-regulate signal transduction.

[0095] “Disease” or “condition” refer a state of being or health status of a patient or subject capable of being treated with the compounds provided herein. Examples of disorders or conditions include, but are not limited to, cancer, cardiovascular disease, hypertension, Syndrome X, depression, anxiety, glaucoma, human immunodeficiency virus (HIV) or acquired immunodeficiency syndrome (AIDS), neurodegeneration, Alzheimer's disease, Parkinson's disease, cognition enhancement, Cushing's Syndrome, Addison's Disease, osteoporosis, frailty, muscle frailty, inflammatory diseases, osteoarthritis, rheumatoid arthritis, asthma and rhinitis, adrenal function-related ailments, viral infection, immunodeficiency, immunomodulation, autoimmune diseases, allergies, wound healing, compulsive behavior, multi-drug resistance, addiction, psychosis, anorexia, cachexia, post-traumatic stress syndrome, post-surgical bone fracture, medical catabolism, major psychotic depression, mild cognitive impairment, psychosis, dementia, hyperglycemia, stress disorders, antipsychotic induced weight gain, delirium, cognitive impairment in depressed patients, cognitive deterioration in individuals with Down's syndrome, psychosis associated with interferon-alpha therapy, chronic pain, pain associated with gastroesophageal reflux disease, postpartum psychosis, postpartum depression, neurological disorders in premature infants, and migraine headaches. In some instances, “disease” or “condition” refer to cancer. In some further instances, “cancer” refers to human cancers and carcinomas, sarcomas, adenocarcinomas, lymphomas, leukemias, etc., including solid and lymphoid cancers, kidney, breast, lung, bladder, colon, ovarian, prostate, pancreas, stomach, brain, head and neck, skin, uterine, testicular, glioma, esophagus, and liver cancer, including hepatocarcinoma, lymphoma, including B-acute lymphoblastic lymphoma, non-Hodgkin's lymphomas (e.g., Burkitt's, Small Cell, and Large Cell lymphomas) and Hodgkin's lymphoma, leukemia (including AML, ALL, and CML), and multiple myeloma.

[0096] “Patient” or “subject in need thereof” refers to a living organism suffering from or prone to a condition that can be treated by administration of a pharmaceutical composition as provided herein. Non-limiting examples include humans, other mammals and other non-mammalian animals.

5 [0097] Abbreviations used herein have their conventional meaning within the chemical and biological arts.

[0098] Where substituent groups are specified by their conventional chemical formulae, written from left to right, they equally encompass the chemically identical substituents that would result from writing the structure from right to left, e.g., $-\text{CH}_2\text{O}-$ is equivalent to

10 $-\text{OCH}_2-$.

[0099] As used herein, the symbol, , indicates the point of attachment of a substituent to the remainder of a molecule.

[0100] A “substituent group,” as used herein, means a group selected from the following moieties:

15 [0101] (A) $-\text{OH}$, $-\text{NH}_2$, $-\text{SH}$, $-\text{CN}$, $-\text{CF}_3$, $-\text{NO}_2$, oxo, halogen, unsubstituted alkyl, unsubstituted heteroalkyl, unsubstituted cycloalkyl, unsubstituted heterocycloalkyl, unsubstituted aryl, unsubstituted heteroaryl, and

[0102] (B) alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl, substituted with at least one substituent selected from:

20 [0103] (i) oxo, $-\text{OH}$, $-\text{NH}_2$, $-\text{SH}$, $-\text{CN}$, $-\text{CF}_3$, $-\text{NO}_2$, halogen, unsubstituted alkyl, unsubstituted heteroalkyl, unsubstituted cycloalkyl, unsubstituted heterocycloalkyl, unsubstituted aryl, unsubstituted heteroaryl, and

[0104] (ii) alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl, substituted with at least one substituent selected from:

25 [0105] (a) oxo, $-\text{OH}$, $-\text{NH}_2$, $-\text{SH}$, $-\text{CN}$, $-\text{CF}_3$, $-\text{NO}_2$, halogen, unsubstituted alkyl, unsubstituted heteroalkyl, unsubstituted cycloalkyl, unsubstituted heterocycloalkyl, unsubstituted aryl, unsubstituted heteroaryl, and

[0106] (b) alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, or heteroaryl, substituted with at least one substituent selected from oxo, -OH, -NH₂, -SH, -CN, -CF₃, -NO₂, halogen, unsubstituted alkyl, unsubstituted heteroalkyl, unsubstituted cycloalkyl, unsubstituted heterocycloalkyl, unsubstituted aryl, and unsubstituted heteroaryl.

5 [0107] A “size-limited substituent” or “ size-limited substituent group,” as used herein means a group selected from all of the substituents described above for a “substituent group,” wherein each substituted or unsubstituted alkyl is a substituted or unsubstituted C₁-C₂₀ alkyl, each substituted or unsubstituted heteroalkyl is a substituted or unsubstituted 2 to 20 membered heteroalkyl, each substituted or unsubstituted cycloalkyl is a substituted or
10 unsubstituted C₄-C₈ cycloalkyl, and each substituted or unsubstituted heterocycloalkyl is a substituted or unsubstituted 4 to 8 membered heterocycloalkyl.

[0108] A “lower substituent” or “ lower substituent group,” as used herein means a group selected from all of the substituents described above for a “substituent group,” wherein each substituted or unsubstituted alkyl is a substituted or unsubstituted C₁-C₈ alkyl, each
15 substituted or unsubstituted heteroalkyl is a substituted or unsubstituted 2 to 8 membered heteroalkyl, each substituted or unsubstituted cycloalkyl is a substituted or unsubstituted C₅-C₇ cycloalkyl, and each substituted or unsubstituted heterocycloalkyl is a substituted or unsubstituted 5 to 7 membered heterocycloalkyl.

[0109] The compounds of the present invention may exist as salts. The present invention
20 includes such salts. Examples of applicable salt forms include hydrochlorides, hydrobromides, sulfates, methanesulfonates, nitrates, maleates, acetates, citrates, fumarates, tartrates (eg (+)-tartrates, (-)-tartrates or mixtures thereof including racemic mixtures, succinates, benzoates and salts with amino acids such as glutamic acid. These salts may be prepared by methods known to those skilled in art. Also included are base addition salts such
25 as sodium, potassium, calcium, ammonium, organic amino, or magnesium salt, or a similar salt. When compounds of the present invention contain relatively basic functionalities, acid addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired acid, either neat or in a suitable inert solvent. Examples of acceptable acid addition salts include those derived from inorganic acids like hydrochloric,
30 hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic, or phosphorous acids and the like, as well as the salts derived organic acids like acetic, propionic, isobutyric, maleic, malonic, benzoic, succinic, suberic, fumaric, lactic, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric, methanesulfonic, and the

like. Also included are salts of amino acids such as arginate and the like, and salts of organic acids like glucuronic or galactunoric acids and the like. Certain specific compounds of the present invention contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.

5 [0110] The neutral forms of the compounds are preferably regenerated by contacting the salt with a base or acid and isolating the parent compound in the conventional manner. The parent form of the compound differs from the various salt forms in certain physical properties, such as solubility in polar solvents.

[0111] Certain compounds of the present invention can exist in unsolvated forms as well as
10 solvated forms, including hydrated forms. In general, the solvated forms are equivalent to unsolvated forms and are encompassed within the scope of the present invention. Certain compounds of the present invention may exist in multiple crystalline or amorphous forms. In general, all physical forms are equivalent for the uses contemplated by the present invention and are intended to be within the scope of the present invention.

15 [0112] Certain compounds of the present invention possess asymmetric carbon atoms (optical or chiral centers) or double bonds; the enantiomers, racemates, diastereomers, tautomers, geometric isomers, stereoisomeric forms that may be defined, in terms of absolute stereochemistry, as (R)- or (S)- or, as (D)- or (L)- for amino acids, and individual isomers are encompassed within the scope of the present invention. The compounds of the present
20 invention do not include those which are known in art to be too unstable to synthesize and/or isolate. The present invention is meant to include compounds in racemic and optically pure forms. Optically active (R)- and (S)-, or (D)- and (L)-isomers may be prepared using chiral synthons or chiral reagents, or resolved using conventional techniques. When the compounds described herein contain olefinic bonds or other centers of geometric asymmetry, and unless
25 specified otherwise, it is intended that the compounds include both E and Z geometric isomers.

[0113] The term "tautomer," as used herein, refers to one of two or more structural isomers which exist in equilibrium and which are readily converted from one isomeric form to another.

30 [0114] It will be apparent to one skilled in the art that certain compounds of this invention may exist in tautomeric forms, all such tautomeric forms of the compounds being within the scope of the invention.

[0115] Unless otherwise stated, structures depicted herein are also meant to include all stereochemical forms of the structure; i.e., the R and S configurations for each asymmetric center. Therefore, single stereochemical isomers as well as enantiomeric and diastereomeric mixtures of the present compounds are within the scope of the invention.

5 [0116] Unless otherwise stated, structures depicted herein are also meant to include compounds which differ only in the presence of one or more isotopically enriched atoms. For example, compounds having the present structures except for the replacement of a hydrogen by a deuterium or tritium, or the replacement of a carbon by ^{13}C - or ^{14}C -enriched carbon are within the scope of this invention.

10 [0117] The compounds of the present invention may also contain unnatural proportions of atomic isotopes at one or more of atoms that constitute such compounds. For example, the compounds may be radiolabeled with radioactive isotopes, such as for example tritium (^3H), iodine-125 (^{125}I) or carbon-14 (^{14}C). All isotopic variations of the compounds of the present invention, whether radioactive or not, are encompassed within the scope of the present
15 invention.

[0118] The term "pharmaceutically acceptable salts" is meant to include salts of active compounds which are prepared with relatively nontoxic acids or bases, depending on the particular substituent moieties found on the compounds described herein. When compounds of the present invention contain relatively acidic functionalities, base addition salts can be
20 obtained by contacting the neutral form of such compounds with a sufficient amount of the desired base, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable base addition salts include sodium, potassium, calcium, ammonium, organic amino, or magnesium salt, or a similar salt. When compounds of the present invention contain relatively basic functionalities, acid addition salts can be obtained by contacting the
25 neutral form of such compounds with a sufficient amount of the desired acid, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable acid addition salts include those derived from inorganic acids like hydrochloric, hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic, or phosphorous acids and the like, as well as the
30 salts derived from relatively nontoxic organic acids like acetic, propionic, isobutyric, maleic, malonic, benzoic, succinic, suberic, fumaric, lactic, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric, methanesulfonic, and the like. Also included are salts of amino acids such as arginate and the like, and salts of organic acids like glucuronic or galactunoric acids and the like (*see*, for example, Berge *et al.*, "Pharmaceutical Salts", *Journal of*

Pharmaceutical Science, 1977, 66, 1-19). Certain specific compounds of the present invention contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.

[0119] In addition to salt forms, the present invention provides compounds, which are in a prodrug form. Prodrugs of the compounds described herein are those compounds that readily undergo chemical changes under physiological conditions to provide the compounds of the present invention. Additionally, prodrugs can be converted to the compounds of the present invention by chemical or biochemical methods in an *ex vivo* environment. For example, prodrugs can be slowly converted to the compounds of the present invention when placed in a transdermal patch reservoir with a suitable enzyme or chemical reagent.

[0120] The terms “a,” “an,” or “a(n),” when used in reference to a group of substituents herein, mean at least one. For example, where a compound is substituted with “an” alkyl or aryl, the compound is optionally substituted with at least one alkyl and/or at least one aryl. Moreover, where a moiety is substituted with an R substituent, the group may be referred to as “R-substituted.” Where a moiety is R-substituted, the moiety is substituted with at least one R substituent and each R substituent is optionally different.

[0121] Description of compounds of the present invention are limited by principles of chemical bonding known to those skilled in the art. Accordingly, where a group may be substituted by one or more of a number of substituents, such substitutions are selected so as to comply with principles of chemical bonding and to give compounds which are not inherently unstable and/or would be known to one of ordinary skill in the art as likely to be unstable under ambient conditions, such as aqueous, neutral, and several known physiological conditions. For example, a heterocycloalkyl or heteroaryl is attached to the remainder of the molecule via a ring heteroatom in compliance with principles of chemical bonding known to those skilled in the art thereby avoiding inherently unstable compounds.

[0122] The terms “treating” or “treatment” refers to any indicia of success in the treatment or amelioration of an injury, pathology or condition, including any objective or subjective parameter such as abatement; remission; diminishing of symptoms or making the injury, pathology or condition more tolerable to the patient; slowing in the rate of degeneration or decline; making the final point of degeneration less debilitating; improving a patient’s physical or mental well-being. The treatment or amelioration of symptoms can be based on objective or subjective parameters; including the results of a physical examination, neuropsychiatric exams, and/or a psychiatric evaluation. For example, the certain methods

presented herein successfully treat cancer by decreasing the incidence of cancer and or causing remission of cancer.

[0123] An “effective amount” is an amount sufficient to contribute to the treatment, prevention, or reduction of a symptom or symptoms of a disease. An “effective amount” may also be referred to as a “therapeutically effective amount.” A “reduction” of a symptom or symptoms (and grammatical equivalents of this phrase) means decreasing of the severity or frequency of the symptom(s), or elimination of the symptom(s). A “prophylactically effective amount” of a drug is an amount of a drug that, when administered to a subject, will have the intended prophylactic effect, e.g., preventing or delaying the onset (or reoccurrence) of a disease, or reducing the likelihood of the onset (or reoccurrence) of a disease or its symptoms. The full prophylactic effect does not necessarily occur by administration of one dose, and may occur only after administration of a series of doses. Thus, a prophylactically effective amount may be administered in one or more administrations. An “activity decreasing amount,” as used herein, refers to an amount of antagonist required to decrease the activity of an enzyme relative to the absence of the antagonist. A “function disrupting amount,” as used herein, refers to the amount of antagonist required to disrupt the function of an osteoclast or leukocyte relative to the absence of the antagonist.

[0124] As used herein, the phrase “ATP-binding pocket” refers to the active site of a kinase that binds ATP. The active site of the kinase where ATP binds is the set of amino acid residues that are able to interact with and or bind to an ATP molecule or an ATP competitive inhibitor.

[0125] As used herein, the term “mutated” refers to a kinase with a non-natural (e.g. non-wild type) amino acid sequence. A mutated kinase is typically recombinant (e.g. engineered). In some embodiments as described below, the mutated kinase has a cysteine residue substitution at the gatekeeper amino acid position. As used herein, the term “unmutated” refers to the corresponding kinase wherein the mutation (e.g. a cysteine residue is substituted for a gatekeeper amino acid position) is not present (e.g. the natural or wild-type sequence). Thus, in some instances, unmutated refers to the wild-type or natural kinase. In some other instances, the corresponding kinase is another recombinant kinase having similar but distinct substitutions.

[0126] As used herein the term “not substantially lower” when referring to k_{cat} means that the k_{cat} is not less than a thousandth, *i.e.* 1/1000, of the corresponding k_{cat} used for comparison. In some embodiments, the k_{cat} is not less than a hundredth, *i.e.* 1/100, of the

corresponding k_{cat} used for comparison. In some instances, the k_{cat} is not less than a tenth, *i.e.* 1/10, of the corresponding k_{cat} used for comparison. In some instances, the k_{cat} is not less than a quarter, *i.e.* 1/4, of the corresponding k_{cat} used for comparison. In some instances, the k_{cat} is not less than half, *i.e.* 1/2, of the corresponding k_{cat} used for comparison. For example, an engineered or mutated kinase may have a k_{cat} that is not substantially lower than the corresponding k_{cat} of the corresponding wild-type or natural or unmutated kinase.

[0127] As used herein the terms “not substantially lower” when referring to K_m means that the K_m is not less than a thousandth, *i.e.* 1/1000, of the corresponding K_m used for comparison. In some embodiments, the K_m is not less than a hundredth, *i.e.* 1/100, of the corresponding K_m used for comparison. In some instances, the K_m is not less than a tenth, *i.e.* 1/10, of the corresponding K_m used for comparison. In some instances, the K_m is not less than a quarter, *i.e.* 1/4, of the corresponding K_m used for comparison. In some instances, the K_m is not less than half, *i.e.* 1/2, of the corresponding K_m used for comparison. For example, an engineered or mutated kinase may have a K_m that is not substantially lower than the corresponding K_m of the corresponding wild-type or natural or unmutated kinase.

[0128] “Disease” or “condition” refer a state of being or health status of a patient or subject capable of being treated with the compounds provided herein. Examples of disorders or conditions include, but are not limited to, cancer, cardiovascular disease, hypertension, Syndrome X, depression, anxiety, glaucoma, human immunodeficiency virus (HIV) or acquired immunodeficiency syndrome (AIDS), neurodegeneration, Alzheimer's disease, Parkinson's disease, cognition enhancement, Cushing's Syndrome, Addison's Disease, osteoporosis, frailty, muscle frailty, inflammatory diseases, osteoarthritis, rheumatoid arthritis, asthma and rhinitis, adrenal function-related ailments, viral infection, immunodeficiency, immunomodulation, autoimmune diseases, allergies, wound healing, compulsive behavior, multi-drug resistance, addiction, psychosis, anorexia, cachexia, post-traumatic stress syndrome, post-surgical bone fracture, medical catabolism, major psychotic depression, mild cognitive impairment, psychosis, dementia, hyperglycemia, stress disorders, antipsychotic induced weight gain, delirium, cognitive impairment in depressed patients, cognitive deterioration in individuals with Down's syndrome, psychosis associated with interferon-alpha therapy, chronic pain, pain associated with gastroesophageal reflux disease, postpartum psychosis, postpartum depression, neurological disorders in premature infants, and migraine headaches. In some instances, “disease” or “condition” refer to cancer. In some further instances, “cancer” refers to human cancers and carcinomas, sarcomas, adenocarcinomas, lymphomas, leukemias, etc., including solid and lymphoid cancers, kidney,

breast, lung, bladder, colon, ovarian, prostate, pancreas, stomach, brain, head and neck, skin, uterine, testicular, glioma, esophagus, and liver cancer, including hepatocarcinoma, lymphoma, including B-acute lymphoblastic lymphoma, non-Hodgkin's lymphomas (*e.g.*, Burkitt's, Small Cell, and Large Cell lymphomas) and Hodgkin's lymphoma, leukemia
5 (including AML, ALL, and CML), and multiple myeloma.

[0129] As used herein, the term "kinase-associated disease" refers to a disease or condition that is mediated, at least in part, by a kinase.

[0130] As used herein, the term "Lrrk-2-associated disease" refers to a disease or condition that is mediated, at least in part, by a Lrrk-2 kinase.

- 10 [0131] As used herein, the term "cancer" refers to all types of cancer, neoplasm or malignant tumors found in mammals, including leukemia, carcinomas and sarcomas. Exemplary cancers include cancer of the brain, breast, cervix, colon, head & neck, liver, kidney, lung, non-small cell lung, melanoma, mesothelioma, ovary, sarcoma, stomach, uterus and Medulloblastoma. Additional examples include, Hodgkin's Disease, Non-Hodgkin's
15 Lymphoma, multiple myeloma, neuroblastoma, ovarian cancer, rhabdomyosarcoma, primary thrombocytosis, primary macroglobulinemia, primary brain tumors, cancer, malignant pancreatic insulanoma, malignant carcinoid, urinary bladder cancer, premalignant skin lesions, testicular cancer, lymphomas, thyroid cancer, neuroblastoma, esophageal cancer, genitourinary tract cancer, malignant hypercalcemia, endometrial cancer, adrenal cortical
20 cancer, neoplasms of the endocrine and exocrine pancreas, and prostate cancer.

- [0132] The term "leukemia" refers broadly to progressive, malignant diseases of the blood-forming organs and is generally characterized by a distorted proliferation and development of leukocytes and their precursors in the blood and bone marrow. Leukemia is generally clinically classified on the basis of (1) the duration and character of the disease-acute or
25 chronic; (2) the type of cell involved; myeloid (myelogenous), lymphoid (lymphogenous), or monocytic; and (3) the increase or non-increase in the number abnormal cells in the blood-leukemic or aleukemic (subleukemic). The P₃₈₈ leukemia model is widely accepted as being predictive of *in vivo* anti-leukemic activity. It is believed that a compound that tests positive in the P₃₈₈ assay will generally exhibit some level of anti-leukemic activity *in vivo* regardless
30 of the type of leukemia being treated. Accordingly, the present invention includes a method of treating leukemia, and, preferably, a method of treating acute nonlymphocytic leukemia, chronic lymphocytic leukemia, acute granulocytic leukemia, chronic granulocytic leukemia, acute promyelocytic leukemia, adult T-cell leukemia, aleukemic leukemia, a leukocythemic

leukemia, basophylic leukemia, blast cell leukemia, bovine leukemia, chronic myelocytic leukemia, leukemia cutis, embryonal leukemia, eosinophilic leukemia, Gross' leukemia, hairy-cell leukemia, hemoblastic leukemia, hemocytoblastic leukemia, histiocytic leukemia, stem cell leukemia, acute monocytic leukemia, leukopenic leukemia, lymphatic leukemia, 5 lymphoblastic leukemia, lymphocytic leukemia, lymphogenous leukemia, lymphoid leukemia, lymphosarcoma cell leukemia, mast cell leukemia, megakaryocytic leukemia, micromyeloblastic leukemia, monocytic leukemia, myeloblastic leukemia, myelocytic leukemia, myeloid granulocytic leukemia, myelomonocytic leukemia, Naegeli leukemia, plasma cell leukemia, multiple myeloma, plasmacytic leukemia, promyelocytic leukemia, 10 Rieder cell leukemia, Schilling's leukemia, stem cell leukemia, subleukemic leukemia, and undifferentiated cell leukemia.

[0133] The term "sarcoma" generally refers to a tumor which is made up of a substance like the embryonic connective tissue and is generally composed of closely packed cells embedded in a fibrillar or homogeneous substance. Sarcomas which can be treated with a combination 15 of antineoplastic thiol-binding mitochondrial oxidant and an anticancer agent include a chondrosarcoma, fibrosarcoma, lymphosarcoma, melanosarcoma, myxosarcoma, osteosarcoma, Abemethy's sarcoma, adipose sarcoma, liposarcoma, alveolar soft part sarcoma, ameloblastic sarcoma, botryoid sarcoma, chloroma sarcoma, chorio carcinoma, embryonal sarcoma, Wilms' tumor sarcoma, endometrial sarcoma, stromal sarcoma, Ewing's 20 sarcoma, fascial sarcoma, fibroblastic sarcoma, giant cell sarcoma, granulocytic sarcoma, Hodgkin's sarcoma, idiopathic multiple pigmented hemorrhagic sarcoma, immunoblastic sarcoma of B cells, lymphoma, immunoblastic sarcoma of T-cells, Jensen's sarcoma, Kaposi's sarcoma, Kupffer cell sarcoma, angiosarcoma, leukosarcoma, malignant mesenchymoma sarcoma, parosteal sarcoma, reticulocytic sarcoma, Rous sarcoma, serocystic sarcoma, 25 synovial sarcoma, and telangiectaltic sarcoma.

[0134] The term "melanoma" is taken to mean a tumor arising from the melanocytic system of the skin and other organs. Melanomas which can be treated with a combination of antineoplastic thiol-binding mitochondrial oxidant and an anticancer agent include, for example, acral-lentiginous melanoma, amelanotic melanoma, benign juvenile melanoma, 30 Cloudman's melanoma, S91 melanoma, Harding-Passey melanoma, juvenile melanoma, lentigo maligna melanoma, malignant melanoma, nodular melanoma, subungal melanoma, and superficial spreading melanoma.

[0135] The term "carcinoma" refers to a malignant new growth made up of epithelial cells tending to infiltrate the surrounding tissues and give rise to metastases. Exemplary

carcinomas which can be treated with a combination of antineoplastic thiol-binding mitochondrial oxidant and an anticancer agent include, for example, acinar carcinoma, acinous carcinoma, adenocystic carcinoma, adenoid cystic carcinoma, carcinoma adenomatosum, carcinoma of adrenal cortex, alveolar carcinoma, alveolar cell carcinoma, basal cell carcinoma, carcinoma basocellulare, basaloid carcinoma, basosquamous cell carcinoma, bronchioalveolar carcinoma, bronchiolar carcinoma, bronchogenic carcinoma, cerebriiform carcinoma, cholangiocellular carcinoma, chorionic carcinoma, colloid carcinoma, comedo carcinoma, corpus carcinoma, cribriform carcinoma, carcinoma en cuirasse, carcinoma cutaneum, cylindrical carcinoma, cylindrical cell carcinoma, duct carcinoma, carcinoma durum, embryonal carcinoma, encephaloid carcinoma, epiermoid carcinoma, carcinoma epitheliale adenoides, exophytic carcinoma, carcinoma ex ulcere, carcinoma fibrosum, gelatiniformi carcinoma, gelatinous carcinoma, giant cell carcinoma, carcinoma gigantocellulare, glandular carcinoma, granulosa cell carcinoma, hair-matrix carcinoma, hematoid carcinoma, hepatocellular carcinoma, Hurthle cell carcinoma, hyaline carcinoma, hypemephroid carcinoma, infantile embryonal carcinoma, carcinoma in situ, intraepidermal carcinoma, intraepithelial carcinoma, Krompecher's carcinoma, Kulchitzky-cell carcinoma, large-cell carcinoma, lenticular carcinoma, carcinoma lenticulare, lipomatous carcinoma, lymphoepithelial carcinoma, carcinoma medullare, medullary carcinoma, melanotic carcinoma, carcinoma molle, mucinous carcinoma, carcinoma muciparum, carcinoma mucocellulare, mucoepidermoid carcinoma, carcinoma mucosum, mucous carcinoma, carcinoma myxomatodes, nasopharyngeal carcinoma, oat cell carcinoma, carcinoma ossificans, osteoid carcinoma, papillary carcinoma, periportal carcinoma, preinvasive carcinoma, prickle cell carcinoma, pultaceous carcinoma, renal cell carcinoma of kidney, reserve cell carcinoma, carcinoma sarcomatodes, schneiderian carcinoma, scirrhus carcinoma, carcinoma scroti, signet-ring cell carcinoma, carcinoma simplex, small-cell carcinoma, solanoid carcinoma, spheroidal cell carcinoma, spindle cell carcinoma, carcinoma spongiosum, squamous carcinoma, squamous cell carcinoma, string carcinoma, carcinoma telangiectaticum, carcinoma telangiectodes, transitional cell carcinoma, carcinoma tuberosum, tuberous carcinoma, verrucous carcinoma, and carcinoma villosum.

[0136] The term "pharmaceutically acceptable salts" is meant to include salts of the active compounds which are prepared with relatively nontoxic acids or bases, depending on the particular substituents found on the compounds described herein. When compounds of the present invention contain relatively acidic functionalities, base addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired

base, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable base addition salts include sodium, potassium, calcium, ammonium, organic amino, or magnesium salt, or a similar salt. When compounds of the present invention contain relatively basic functionalities, acid addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired acid, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable acid addition salts include those derived from inorganic acids like hydrochloric, hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic, or phosphorous acids and the like, as well as the salts derived from relatively nontoxic organic acids like acetic, propionic, isobutyric, maleic, malonic, benzoic, succinic, suberic, fumaric, lactic, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric, methanesulfonic, and the like. Also included are salts of amino acids such as arginate and the like, and salts of organic acids like glucuronic or galactunoric acids and the like (*see*, for example, Berge *et al.*, "Pharmaceutical Salts", *Journal of Pharmaceutical Science*, **1977**, 66, 1-19). Certain specific compounds of the present invention contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.

[0137] The neutral forms of the compounds are preferably regenerated by contacting the salt with a base or acid and isolating the parent compound in the conventional manner. The parent form of the compound differs from the various salt forms in certain physical properties, such as solubility in polar solvents.

[0138] In addition to salt forms, the present invention provides compounds, which are in a prodrug form. Prodrugs of the compounds described herein are those compounds that readily undergo chemical changes under physiological conditions to provide the compounds of the present invention. Additionally, prodrugs can be converted to the compounds of the present invention by chemical or biochemical methods in an *ex vivo* environment. For example, prodrugs can be slowly converted to the compounds of the present invention when placed in a transdermal patch reservoir with a suitable enzyme or chemical reagent.

[0139] Certain compounds of the present invention can exist in unsolvated forms as well as solvated forms, including hydrated forms. In general, the solvated forms are equivalent to unsolvated forms and are encompassed within the scope of the present invention. Certain compounds of the present invention may exist in multiple crystalline or amorphous forms. In general, all physical forms are equivalent for the uses contemplated by the present invention and are intended to be within the scope of the present invention.

[0140] Certain compounds of the present invention possess asymmetric carbon atoms (optical centers) or double bonds; the racemates, diastereomers, geometric isomers and individual isomers are encompassed within the scope of the present invention.

5 [0141] The compounds of the present invention may also contain unnatural proportions of atomic isotopes at one or more of the atoms that constitute such compounds. For example, the compounds may be radiolabeled with radioactive isotopes, such as for example tritium (^3H), iodine-125 (^{125}I) or carbon-14 (^{14}C). All isotopic variations of the compounds of the present invention, whether radioactive or not, are encompassed within the scope of the present invention.

10 [0142] As used herein, the term "salt" refers to acid or base salts of the compounds used in the methods of the present invention. Illustrative examples of acceptable salts are mineral acid (hydrochloric acid, hydrobromic acid, phosphoric acid, and the like) salts, organic acid (acetic acid, propionic acid, glutamic acid, citric acid and the like) salts, quaternary ammonium (methyl iodide, ethyl iodide, and the like) salts.

15 [0143] As used herein, the term "isomers" refers to compounds having the same number and kind of atoms, and hence the same molecular weight, but differing in respect to the structural arrangement or configuration of the atoms.

[0144] As used herein, the term "tautomer," refers to one of two or more structural isomers which exist in equilibrium and which are readily converted from one isomeric form to
20 another.

III. Introduction

[0145] Provided herein are, *inter alia*, novel methods and compositions for inhibiting a protein kinase, *e.g.*, a cysteine substituted kinase, determining the function of a protein kinase in a cell, and treating kinase-associated diseases and conditions. Certain heterocyclic
25 compounds having an electrophilic substituent provided herein that specifically, and optionally irreversibly, inhibit cysteine substituted kinases. In some embodiments, the heterocyclic compound comprises two or more fused rings and an electrophilic substituent. In some embodiments, at least one of the two or more fused rings comprises a nitrogen atom. In some embodiments, the heterocyclic compounds inhibit a cysteine substituted kinase, *i.e.*,
30 a kinase having a cysteine residue in the gatekeeper position of the ATP binding site. In some embodiments, the heterocyclic compounds also inhibit a kinase not having a cysteine residue in the gatekeeper position (*e.g.* of the ATP binding site).

IV. Compounds

[0146] The present invention provides compounds suitable for use with the methods and assays described herein.

[0147] In some other embodiments, the heterocyclic compounds useful for inhibiting a kinase include two or more fused rings which include at least one heteroatom selected from N, O, or S. In some embodiments, the fused rings are substituted with a ring selected from substituted or unsubstituted cycloalkyl, substituted or unsubstituted aryl, or substituted or unsubstituted heteroaryl. In some other embodiments, the cycloalkyl, heterocycloalkyl, aryl, or heteroaryl is substituted with a substituent selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl. In other embodiments, the ring which substitutes the fused rings is an aryl or heteroaryl. In some embodiments, the ring which substitutes the fused rings is an aryl. In some other embodiments, the ring which substitutes the fused rings is an aryl which is substituted with an electrophilic substituent that is capable of accepting electron density from a cysteine gatekeeper residue of a protein kinase. In some embodiments, the electrophilic substituent is capable of forming a covalent bond to the sulfhydryl group of the cysteine gate keeper residue.

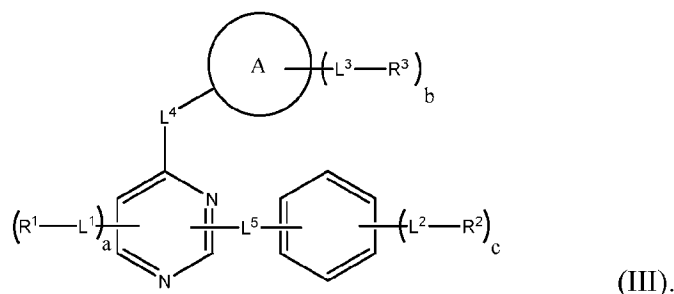
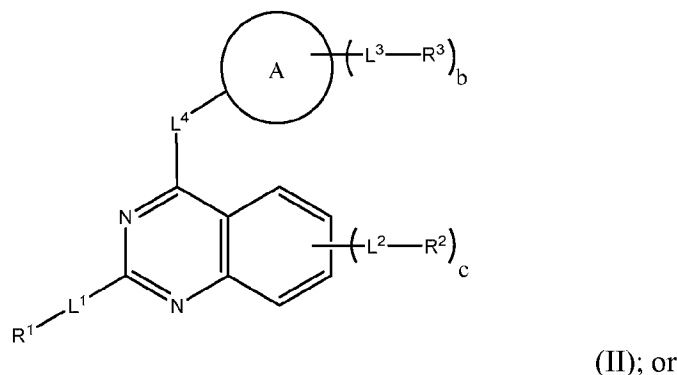
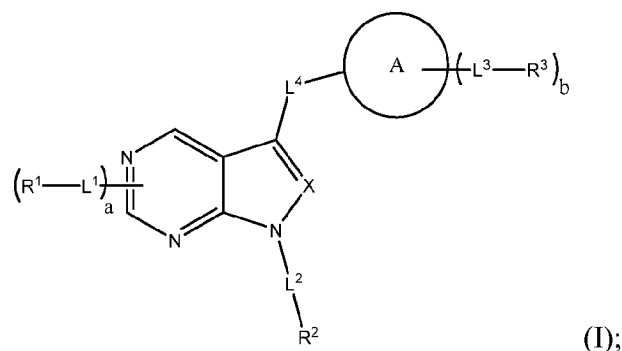
[0148] In some embodiments, the compound is a substituted or unsubstituted phenyl-derivatized pyrazolopyrimidine, *e.g.* 3-phenyl-substituted pyrazolopyrimidines, having an electrophilic substituent. In some other embodiments, the present invention provides 3-phenyl-substituted pyrazolopyrimidines which are synthesized with an electrophilic groups at positions expected to be in close proximity to the gatekeeper residue. In some embodiments, compound is a substituted or unsubstituted quinazoline having an electrophilic substituent. In some embodiments, compound is a substituted or unsubstituted 4-anilinoquinazoline, *e.g.* Michael acceptor-derivatized 4-anilinoquinazolines, having an electrophilic substituent. In some embodiments, the compound is a substituted or unsubstituted benzyl-derivatized pyrazolopyrimidine having an electrophilic substituent. In some embodiments, the compound is a substituted or unsubstituted pyrazolopyrimidine having an electrophilic substituent pyrazolopyrimidine.

[0149] In some other embodiments, the electrophilic substituent is an electrophilic ATP-binding pocket moiety (*i.e.* a chemical moiety that interacts with amino acids that form part of the ATP-binding pocket). In other embodiments, the electrophilic substituent is a

vinylsulfonamide, a vinylsulfone, an acrylamide, a chloroacetamide, an α -chloroacetamide, an epoxide, or a fluoromethylketones.

[0150] In some embodiments the compounds described herein are inhibitors of kinases (“kinase inhibitors”) such as an inhibitor of a recombinant cysteine gatekeeper kinase (“cysteine gatekeeper kinase inhibitor”). In some other embodiments, the cysteine gatekeeper kinase inhibitor includes an ATP-binding pocket moiety (e.g. an ATP-binding pocket moiety including a heterocyclic moiety) covalently bound to an electrophilic moiety capable of binding the thiol of the gatekeeper cysteine residue of the cysteine gatekeeper kinase. In some embodiments, the inhibitor is one or more of the compounds set forth in Table 1 of Formulas (I) to (XXIX) (e.g. Formula (I) to (XIV)).

[0151] In some embodiments, the compounds has the formula:



In these formula, X is =N- or =C(-L⁶-R⁶)-; Ring A is, in each instance, independently cycloalkyl, heterocycloalkyl, aryl, or heteroaryl; L¹, L², L³, L⁴, L⁵, and L⁶ are, in each

instance, independently selected from a bond, -C(O)-, -C(O)N(R⁷)-, -C(O)O-, -S(O)_g- (*i.e.* -S-, -S(O)- or -S(O)₂-), -S(O)₂N(R⁷)-, -O-, -N(R⁷)-, -N(R⁷)C(O)N(R⁸)-, substituted or unsubstituted alkylene, substituted or unsubstituted heteroalkylene, substituted or unsubstituted cycloalkylene, substituted or unsubstituted heterocycloalkylene, substituted or unsubstituted arylene, or substituted or unsubstituted heteroarylene, wherein g is independently an integer from 0 to 2; R¹, R², R³, R⁴, R⁵, R⁶, R⁷, and R⁸ are, in each instance, independently selected from hydrogen, halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, or substituted or unsubstituted heteroaryl; a is an integer from 0 to 2; b is an integer from 0 to 5; and c is an integer from 0 to 4. In some embodiments, A is aryl (e.g. phenyl). In some embodiments, R⁷ is hydrogen. In some embodiments, R⁷ and R⁸ are hydrogen. R¹, R², R³, R⁴, R⁵, R⁶, R⁷, and R⁸ may also independently be hydrogen, halogen (e.g. -Cl or -F), -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, or substituted or unsubstituted heteroaryl.

[0152] In some embodiments, R¹ is hydrogen, halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R⁹-substituted or unsubstituted alkyl, R⁹-substituted or unsubstituted heteroalkyl, R⁹-substituted or unsubstituted cycloalkyl, R⁹-substituted or unsubstituted heterocycloalkyl, R⁹-substituted or unsubstituted aryl, or R⁹-substituted or unsubstituted heteroaryl.

[0153] R⁹ is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R¹⁰-substituted or unsubstituted alkyl, R¹⁰-substituted or unsubstituted heteroalkyl, R¹⁰-substituted or unsubstituted cycloalkyl, R¹⁰-substituted or unsubstituted heterocycloalkyl, R¹⁰-substituted or unsubstituted aryl, or R¹⁰-substituted or unsubstituted heteroaryl.

[0154] R¹⁰ is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R¹¹-substituted or unsubstituted alkyl, R¹¹-substituted or unsubstituted heteroalkyl, R¹¹-substituted or unsubstituted cycloalkyl, R¹¹-substituted or unsubstituted heterocycloalkyl, R¹¹-substituted or unsubstituted aryl, or R¹¹-substituted or unsubstituted heteroaryl.

[0155] In some embodiments, R² is hydrogen, halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R¹²-substituted or unsubstituted

alkyl, R¹²-substituted or unsubstituted heteroalkyl, R¹²-substituted or unsubstituted cycloalkyl, R¹²-substituted or unsubstituted heterocycloalkyl, R¹²-substituted or unsubstituted aryl, or R¹²-substituted or unsubstituted heteroaryl.

[0156] R¹² is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R¹³-substituted or unsubstituted alkyl, R¹³-substituted or unsubstituted heteroalkyl, R¹³-substituted or unsubstituted cycloalkyl, R¹³-substituted or unsubstituted heterocycloalkyl, R¹³-substituted or unsubstituted aryl, or R¹³-substituted or unsubstituted heteroaryl.

[0157] R¹³ independently is halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R¹⁴-substituted or unsubstituted alkyl, R¹⁴-substituted or unsubstituted heteroalkyl, R¹⁴-substituted or unsubstituted cycloalkyl, R¹⁴-substituted or unsubstituted heterocycloalkyl, R¹⁴-substituted or unsubstituted aryl, or R¹⁴-substituted or unsubstituted heteroaryl.

[0158] In some embodiments, R³ hydrogen, is halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R¹⁵-substituted or unsubstituted alkyl, R¹⁵-substituted or unsubstituted heteroalkyl, R¹⁵-substituted or unsubstituted cycloalkyl, R¹⁵-substituted or unsubstituted heterocycloalkyl, R¹⁵-substituted or unsubstituted aryl, or R¹⁵-substituted or unsubstituted heteroaryl.

[0159] R¹⁵ is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R¹⁶-substituted or unsubstituted alkyl, R¹⁶-substituted or unsubstituted heteroalkyl, R¹⁶-substituted or unsubstituted cycloalkyl, R¹⁶-substituted or unsubstituted heterocycloalkyl, R¹⁶-substituted or unsubstituted aryl, or R¹⁶-substituted or unsubstituted heteroaryl.

[0160] R¹⁶ is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R¹⁷-substituted or unsubstituted alkyl, R¹⁷-substituted or unsubstituted heteroalkyl, R¹⁷-substituted or unsubstituted cycloalkyl, R¹⁷-substituted or unsubstituted heterocycloalkyl, R¹⁷-substituted or unsubstituted aryl, or R¹⁷-substituted or unsubstituted heteroaryl.

[0161] In some embodiments, R⁴ is hydrogen, halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R¹⁸-substituted or unsubstituted alkyl, R¹⁸-substituted or unsubstituted heteroalkyl, R¹⁸-substituted or unsubstituted

cycloalkyl, R¹⁸-substituted or unsubstituted heterocycloalkyl, R¹⁸-substituted or unsubstituted aryl, or R¹⁸-substituted or unsubstituted heteroaryl.

[0162] R¹⁸ independently is halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R¹⁹-substituted or unsubstituted alkyl, R¹⁹-substituted or unsubstituted heteroalkyl, R¹⁹-substituted or unsubstituted cycloalkyl, R¹⁹-substituted or unsubstituted heterocycloalkyl, R¹⁹-substituted or unsubstituted aryl, or R¹⁹-substituted or unsubstituted heteroaryl.

[0163] R¹⁹ is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R²⁰-substituted or unsubstituted alkyl, R²⁰-substituted or unsubstituted heteroalkyl, R²⁰-substituted or unsubstituted cycloalkyl, R²⁰-substituted or unsubstituted heterocycloalkyl, R²⁰-substituted or unsubstituted aryl, or R²⁰-substituted or unsubstituted heteroaryl.

[0164] In some embodiments, R⁵ is hydrogen, halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R²¹-substituted or unsubstituted alkyl, R²¹-substituted or unsubstituted heteroalkyl, R²¹-substituted or unsubstituted cycloalkyl, R²¹-substituted or unsubstituted heterocycloalkyl, R²¹-substituted or unsubstituted aryl, or R²¹-substituted or unsubstituted heteroaryl.

[0165] R²¹ is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R²²-substituted or unsubstituted alkyl, R²²-substituted or unsubstituted heteroalkyl, R²²-substituted or unsubstituted cycloalkyl, R²²-substituted or unsubstituted heterocycloalkyl, R²²-substituted or unsubstituted aryl, or R²²-substituted or unsubstituted heteroaryl.

[0166] R²² is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R²³-substituted or unsubstituted alkyl, R²³-substituted or unsubstituted heteroalkyl, R²³-substituted or unsubstituted cycloalkyl, R²³-substituted or unsubstituted heterocycloalkyl, R²³-substituted or unsubstituted aryl, or R²³-substituted or unsubstituted heteroaryl.

[0167] In some embodiments, R⁶ is hydrogen, halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R²⁴-substituted or unsubstituted alkyl, R²⁴-substituted or unsubstituted heteroalkyl, R²⁴-substituted or unsubstituted cycloalkyl, R²⁴-substituted or unsubstituted heterocycloalkyl, R²⁴-substituted or unsubstituted aryl, or R²⁴-substituted or unsubstituted heteroaryl.

[0168] R^{24} is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R^{25} -substituted or unsubstituted alkyl, R^{25} -substituted or unsubstituted heteroalkyl, R^{25} -substituted or unsubstituted cycloalkyl, R^{25} -substituted or unsubstituted heterocycloalkyl, R^{25} -substituted or unsubstituted aryl, or R^{25} -substituted or unsubstituted heteroaryl.

[0169] R^{25} is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R^{26} -substituted or unsubstituted alkyl, R^{26} -substituted or unsubstituted heteroalkyl, R^{26} -substituted or unsubstituted cycloalkyl, R^{26} -substituted or unsubstituted heterocycloalkyl, R^{26} -substituted or unsubstituted aryl, or R^{26} -substituted or unsubstituted heteroaryl.

[0170] In some embodiments, R^7 is hydrogen, halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R^{27} -substituted or unsubstituted alkyl, R^{27} -substituted or unsubstituted heteroalkyl, R^{27} -substituted or unsubstituted cycloalkyl, R^{27} -substituted or unsubstituted heterocycloalkyl, R^{27} -substituted or unsubstituted aryl, or R^{27} -substituted or unsubstituted heteroaryl.

[0171] R^{27} is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R^{28} -substituted or unsubstituted alkyl, R^{28} -substituted or unsubstituted heteroalkyl, R^{28} -substituted or unsubstituted cycloalkyl, R^{28} -substituted or unsubstituted heterocycloalkyl, R^{28} -substituted or unsubstituted aryl, or R^{28} -substituted or unsubstituted heteroaryl.

[0172] R^{28} is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R^{29} -substituted or unsubstituted alkyl, R^{29} -substituted or unsubstituted heteroalkyl, R^{29} -substituted or unsubstituted cycloalkyl, R^{29} -substituted or unsubstituted heterocycloalkyl, R^{29} -substituted or unsubstituted aryl, or R^{29} -substituted or unsubstituted heteroaryl.

[0173] In some embodiments, R^8 is hydrogen, halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R^{30} -substituted or unsubstituted alkyl, R^{30} -substituted or unsubstituted heteroalkyl, R^{30} -substituted or unsubstituted cycloalkyl, R^{30} -substituted or unsubstituted heterocycloalkyl, R^{30} -substituted or unsubstituted aryl, or R^{30} -substituted or unsubstituted heteroaryl.

[0174] R^{30} is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R^{31} -substituted or unsubstituted alkyl, R^{31} -substituted or

unsubstituted heteroalkyl, R^{31} -substituted or unsubstituted cycloalkyl, R^{31} substituted or unsubstituted heterocycloalkyl, R^{31} -substituted or unsubstituted aryl, or R^{31} -substituted or unsubstituted heteroaryl.

[0175] R^{31} is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R^{32} -substituted or unsubstituted alkyl, R^{32} -substituted or unsubstituted heteroalkyl, R^{32} -substituted or unsubstituted cycloalkyl, R^{32} -substituted or unsubstituted heterocycloalkyl, R^{32} -substituted or unsubstituted aryl, or R^{32} -substituted or unsubstituted heteroaryl.

[0176] In some embodiments, R^{11} , R^{14} , R^{17} , R^{20} , R^{23} , R^{26} , R^{29} , and R^{32} are independently hydrogen, halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, unsubstituted alkyl, unsubstituted heteroalkyl, unsubstituted cycloalkyl, unsubstituted heterocycloalkyl, unsubstituted aryl, or unsubstituted heteroaryl.

[0177] In some embodiments, L^1 is in each instance, independently selected from a bond, -C(O)-, -C(O)N(R^7)-, -C(O)O-, -S(O)_g-, -S(O)₂N(R^7)-, -O-, -N(R^7)-, -N(R^7)C(O)N(R^8)-, R^{33} -substituted or unsubstituted alkylene, R^{33} -substituted or unsubstituted heteroalkylene, R^{33} -substituted or unsubstituted cycloalkylene, R^{33} -substituted or unsubstituted heterocycloalkylene, R^{33} -substituted or unsubstituted arylene, or R^{33} -substituted or unsubstituted heteroarylene.

[0178] R^{33} is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R^{34} -substituted or unsubstituted alkyl, R^{34} -substituted or unsubstituted heteroalkyl, R^{34} -substituted or unsubstituted cycloalkyl, R^{34} -substituted or unsubstituted heterocycloalkyl, R^{34} -substituted or unsubstituted aryl, or R^{34} -substituted or unsubstituted heteroaryl.

[0179] R^{34} is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R^{35} -substituted or unsubstituted alkyl, R^{35} -substituted or unsubstituted heteroalkyl, R^{35} -substituted or unsubstituted cycloalkyl, R^{35} -substituted or unsubstituted heterocycloalkyl, R^{35} -substituted or unsubstituted aryl, or R^{35} -substituted or unsubstituted heteroaryl.

[0180] In some embodiments, L^2 is in each instance, independently selected from a bond, -C(O)-, -C(O)N(R^7)-, -C(O)O-, -S(O)_g- (i.e. -S-, -S(O)- or -S(O)₂-), -S(O)₂N(R^7)-, -O-, -N(R^7)-, -N(R^7)C(O)N(R^8)-, R^{36} -substituted or unsubstituted alkylene, R^{36} -substituted or unsubstituted heteroalkylene, R^{36} -substituted or unsubstituted cycloalkylene, R^{36} -substituted

or unsubstituted heterocycloalkylene, R³⁶-substituted or unsubstituted arylene, or R³⁶-substituted or unsubstituted heteroarylene.

[0181] R³⁶ is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R³⁷-substituted or unsubstituted alkyl, R³⁷-substituted or unsubstituted heteroalkyl, R³⁷-substituted or unsubstituted cycloalkyl, R³⁷-substituted or unsubstituted heterocycloalkyl, R³⁷-substituted or unsubstituted aryl, or R³⁷-substituted or unsubstituted heteroaryl.

[0182] R³⁷ is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R³⁸-substituted or unsubstituted alkyl, R³⁸-substituted or unsubstituted heteroalkyl, R³⁸-substituted or unsubstituted cycloalkyl, R³⁸-substituted or unsubstituted heterocycloalkyl, R³⁸-substituted or unsubstituted aryl, or R³⁸-substituted or unsubstituted heteroaryl.

[0183] In some embodiments, L³ is in each instance, independently selected from a bond, -C(O)-, -C(O)N(R⁷)-, -C(O)O-, -S(O)_g- (i.e. -S-, -S(O)- or -S(O)₂-), -S(O)₂N(R⁷)-, -O-, -N(R⁷)-, -N(R⁷)C(O)N(R⁸)-, R³⁹-substituted or unsubstituted alkylene, R³⁹-substituted or unsubstituted heteroalkylene, R³⁹-substituted or unsubstituted cycloalkylene, R³⁹-substituted or unsubstituted heterocycloalkylene, R³⁹-substituted or unsubstituted arylene, or R³⁹-substituted or unsubstituted heteroarylene.

[0184] R³⁹ is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R⁴⁰-substituted or unsubstituted alkyl, R⁴⁰-substituted or unsubstituted heteroalkyl, R⁴⁰-substituted or unsubstituted cycloalkyl, R⁴⁰-substituted or unsubstituted heterocycloalkyl, R⁴⁰-substituted or unsubstituted aryl, or R⁴⁰-substituted or unsubstituted heteroaryl.

[0185] R⁴⁰ is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R⁴¹-substituted or unsubstituted alkyl, R⁴¹-substituted or unsubstituted heteroalkyl, R⁴¹-substituted or unsubstituted cycloalkyl, R⁴¹-substituted or unsubstituted heterocycloalkyl, R⁴¹-substituted or unsubstituted aryl, or R⁴¹-substituted or unsubstituted heteroaryl.

[0186] In some embodiments, L⁴ is in each instance, independently selected from a bond, -C(O)-, -C(O)N(R⁷)-, -C(O)O-, -S(O)_g- (i.e. -S-, -S(O)- or -S(O)₂-), -S(O)₂N(R⁷)-, -O-, -N(R⁷)-, -N(R⁷)C(O)N(R⁸)-, R³⁹-substituted or unsubstituted alkylene, R⁴²-substituted or unsubstituted heteroalkylene, R⁴²-substituted or unsubstituted cycloalkylene, R⁴²-substituted

or unsubstituted heterocycloalkylene, R⁴²-substituted or unsubstituted arylene, or R⁴²-substituted or unsubstituted heteroarylene.

[0187] R⁴² is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R⁴³-substituted or unsubstituted alkyl, R⁴³-substituted or unsubstituted heteroalkyl, R⁴³-substituted or unsubstituted cycloalkyl, R⁴³-substituted or unsubstituted heterocycloalkyl, R⁴³-substituted or unsubstituted aryl, or R⁴³-substituted or unsubstituted heteroaryl.

[0188] R⁴³ is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R⁴⁴-substituted or unsubstituted alkyl, R⁴⁴-substituted or unsubstituted heteroalkyl, R⁴⁴-substituted or unsubstituted cycloalkyl, R⁴⁴-substituted or unsubstituted heterocycloalkyl, R⁴⁴-substituted or unsubstituted aryl, or R⁴⁴-substituted or unsubstituted heteroaryl.

[0189] In some embodiments, L⁵ is in each instance, independently selected from a bond, -C(O)-, -C(O)N(R⁷)-, -C(O)O-, -S(O)_g- (i.e. -S-, -S(O)- or -S(O)₂), -S(O)₂N(R⁷)-, -O-, -N(R⁷)-, -N(R⁷)C(O)N(R⁸)-, R⁴⁵-substituted or unsubstituted alkylene, R⁴⁵-substituted or unsubstituted heteroalkylene, R⁴⁵-substituted or unsubstituted cycloalkylene, R⁴⁵-substituted or unsubstituted heterocycloalkylene, R⁴⁵-substituted or unsubstituted arylene, or R⁴⁵-substituted or unsubstituted heteroarylene.

[0190] R⁴⁵ is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R⁴⁶-substituted or unsubstituted alkyl, R⁴⁶-substituted or unsubstituted heteroalkyl, R⁴⁶-substituted or unsubstituted cycloalkyl, R⁴⁶-substituted or unsubstituted heterocycloalkyl, R⁴⁶-substituted or unsubstituted aryl, or R⁴⁶-substituted or unsubstituted heteroaryl.

[0191] R⁴⁶ is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R⁴⁷-substituted or unsubstituted alkyl, R⁴⁷-substituted or unsubstituted heteroalkyl, R⁴⁷-substituted or unsubstituted cycloalkyl, R⁴⁷-substituted or unsubstituted heterocycloalkyl, R⁴⁷-substituted or unsubstituted aryl, or R⁴⁷-substituted or unsubstituted heteroaryl.

[0192] In some embodiments, L⁶ is in each instance, independently selected from a bond, -C(O)-, -C(O)N(R⁷)-, -C(O)O-, -S(O)_g- (i.e. -S-, -S(O)- or -S(O)₂), -S(O)₂N(R⁷)-, -O-, -N(R⁷)-, -N(R⁷)C(O)N(R⁸)-, R⁴⁸-substituted or unsubstituted alkylene, R⁴⁸-substituted or unsubstituted heteroalkylene, R⁴⁸-substituted or unsubstituted cycloalkylene, R⁴⁸-substituted

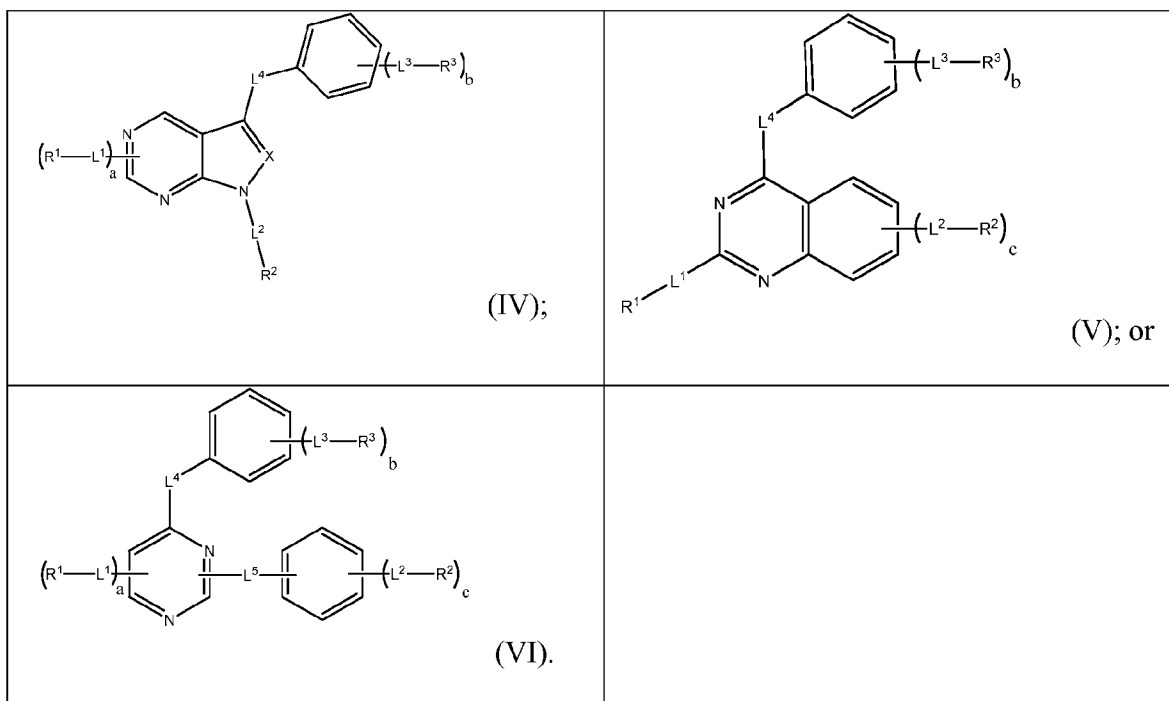
or unsubstituted heterocycloalkylene, R⁴⁸-substituted or unsubstituted arylene, or R⁴⁸-substituted or unsubstituted heteroarylene.

[0193] R⁴⁸ is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R⁴⁹-substituted or unsubstituted alkyl, R⁴⁹-substituted or unsubstituted heteroalkyl, R⁴⁹-substituted or unsubstituted cycloalkyl, R⁴⁹-substituted or unsubstituted heterocycloalkyl, R⁴⁹-substituted or unsubstituted aryl, or R⁴⁹-substituted or unsubstituted heteroaryl.

[0194] R⁴⁹ is independently halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, R⁵⁰-substituted or unsubstituted alkyl, R⁵⁰-substituted or unsubstituted heteroalkyl, R⁵⁰-substituted or unsubstituted cycloalkyl, R⁵⁰-substituted or unsubstituted heterocycloalkyl, R⁵⁰-substituted or unsubstituted aryl, or R⁵⁰-substituted or unsubstituted heteroaryl.

[0195] In some embodiments, R³⁵, R³⁸, R⁴¹, R⁴⁴, R⁴⁷ and R⁵⁰ are independently hydrogen, unsubstituted alkyl, unsubstituted heteroalkyl, unsubstituted cycloalkyl, unsubstituted heterocycloalkyl, unsubstituted aryl, or unsubstituted heteroaryl.

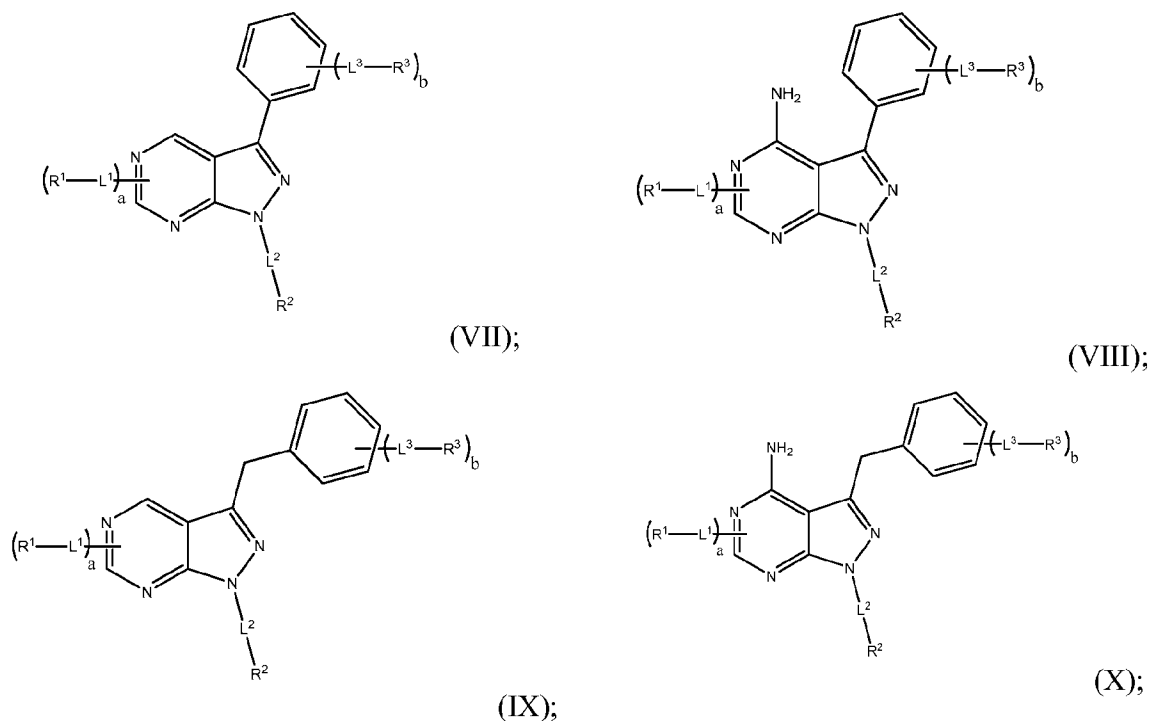
[0196] In some other embodiments, the compounds have the formula (where the variables are as described above):

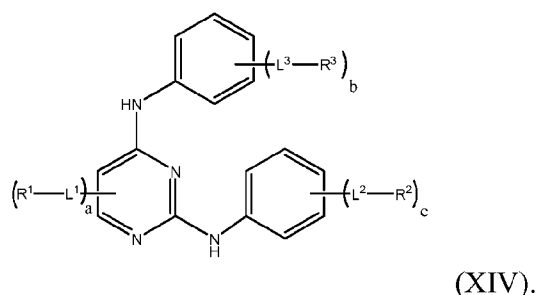
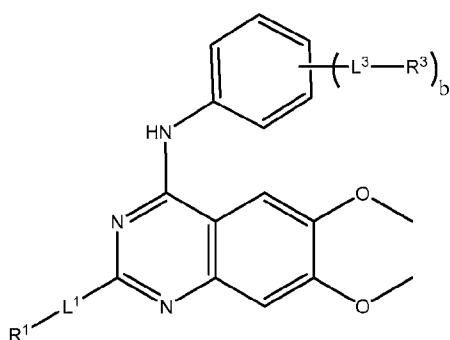
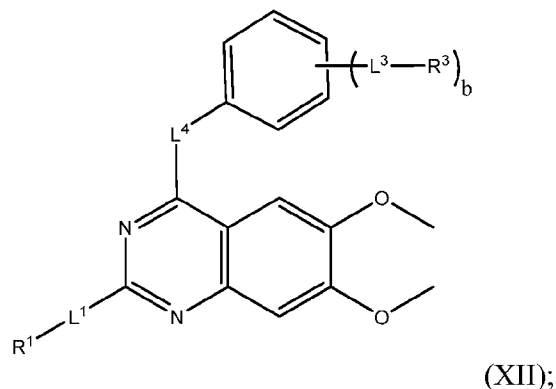
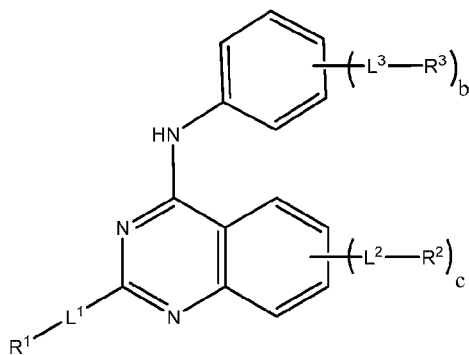


[0197] In certain embodiments, L^1 , L^2 , L^3 , L^4 , L^5 , and L^6 are, in each instance, independently a bond, -NH-, or substituted or unsubstituted C_1 - C_5 alkylene. In certain other embodiments, L^6 is a bond, -NH-, or unsubstituted C_1 - C_5 alkylene.

[0198] In some other embodiments, the compounds have the formula (where the variables are as described above):

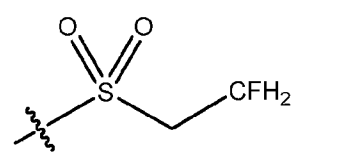
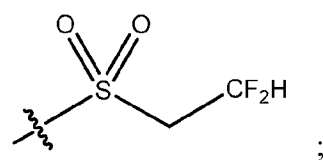
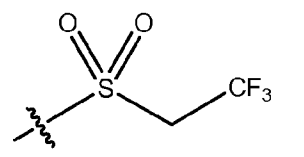
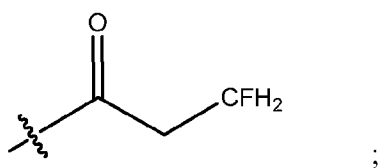
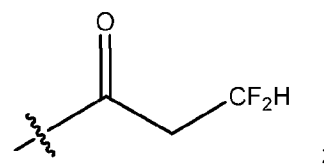
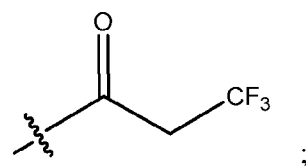
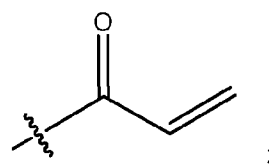
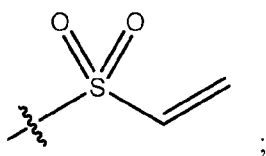
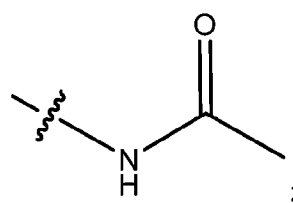
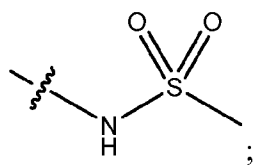
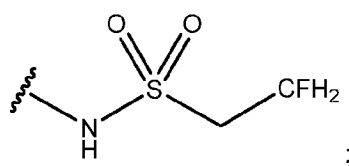
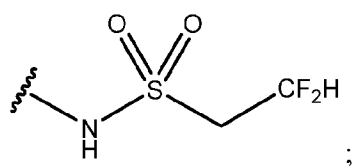
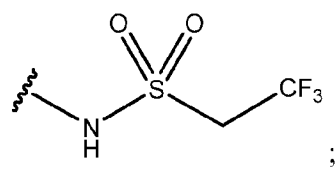
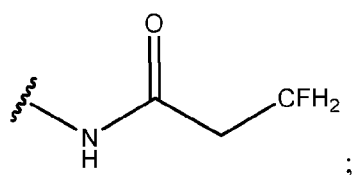
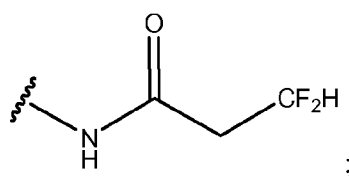
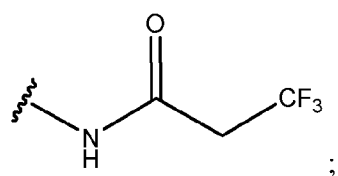
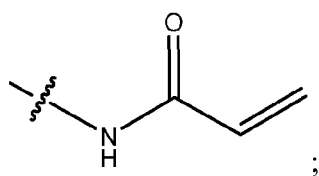
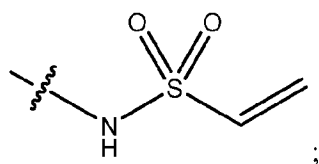
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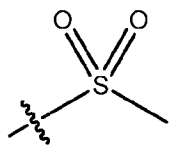




- In certain embodiments, at least one $-L^3-R^3$ is an electrophilic moiety (e.g. $-L^3-R^3$ is or includes an electrophilic moiety). For example, in some embodiments, $-L^3-R^3$ forms an electrophilic moiety. In other embodiments, one of L^3 or R^3 is an electrophilic moiety (e.g. one of L^3 or R^3 is or includes an electrophilic moiety). In some embodiments, L^3 forms an electrophilic moiety. In other embodiments, R^3 forms an electrophilic moiety. In certain other embodiments, L^3 is a bond, $-NH-$, substituted or unsubstituted alkylene, or substituted or unsubstituted heteroalkylene; and R^3 is a substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, or halogen. In some of the embodiments, L^3 is $-C(O)-$, $-S(O)_2-$, $-NHC(O)-$, or $-NHS(O)_2-$. R^3 may be a substituted or unsubstituted alkyl (e.g. substituted or unsubstituted C_1 to C_5 alkyl). For example, R^3 may be an unsubstituted alkyl or alkyl substituted with chloro, fluoro, methyl, difluoromethyl, or trifluoromethyl. In other embodiments, R^3 is ethenyl, ethyl, 2,2,2-trichloroethyl, 2,2-dichloroethyl, 2-chloroethyl, 2,2,2-trifluoroethyl, 2,2-difluoroethyl, or 2-fluoroethyl, propyl, isopropyl, 1-propenyl, or 2-propenyl.

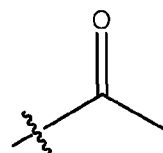
[0199] In some embodiments, the compounds suitable for use with the present invention have the structure of one of the formula cited herein wherein $-L^3-R^3$ is:





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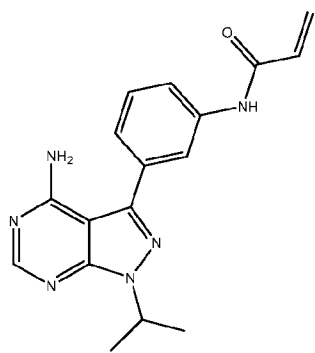
or



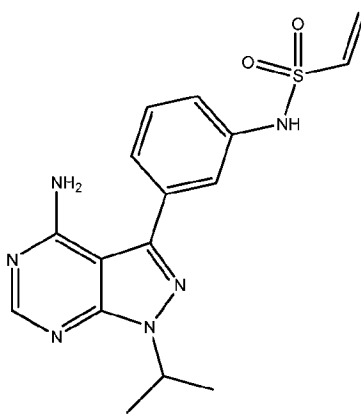
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[0200] In some other embodiments, the compounds suitable for use with the present invention include those wherein L^1 is a bond. R^1 may be hydrogen or NH_2 . In certain embodiments, L^1 is a bond; and R^1 is hydrogen. In certain other embodiments, L^1 is a bond; and R^1 is NH_2 . In some embodiments, the present invention provides a compound where L^2 is a bond; and R^2 is methyl, ethyl, propyl, isopropyl, butyl, tert-butyl, pentyl, cyclopentyl, hexyl, cyclohexyl, methoxy, ethoxy, propoxy, or butoxy. In certain embodiments, R^2 is isopropyl or cyclopentyl. In other embodiments, R^2 is isopropyl. In other embodiments, R^2 is cyclopentyl. In some other embodiments, R^2 is methoxy. In certain embodiments, c is 2; L^2 is a bond, and R^2 is methoxy, ethoxy, propoxy, or butoxy. In other embodiments, R^2 is methoxy.

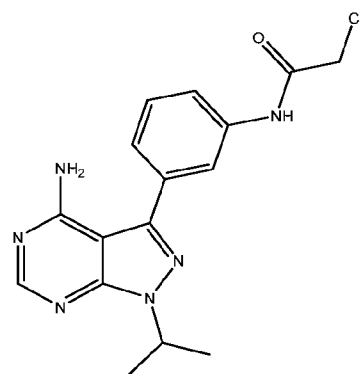
[0201] In some embodiments, the present invention provides compounds that have:



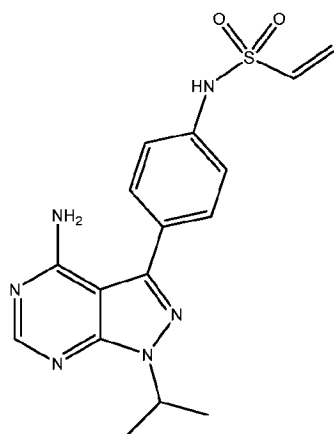
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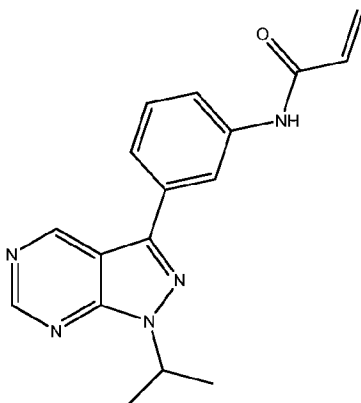
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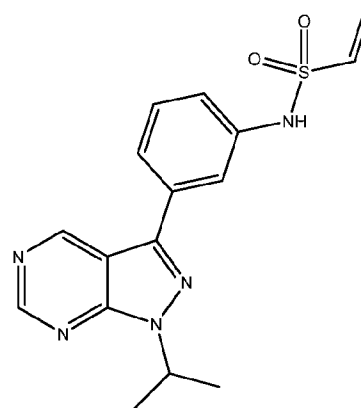
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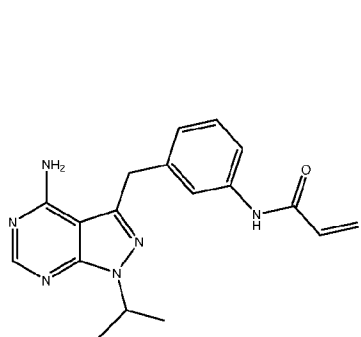
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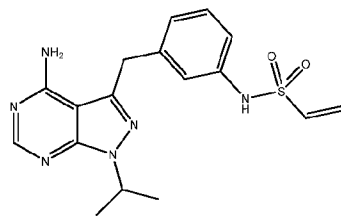
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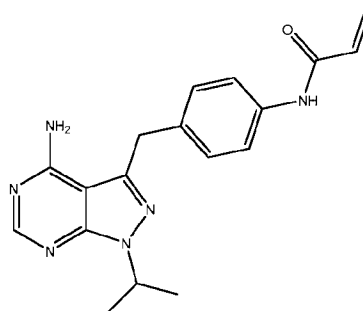
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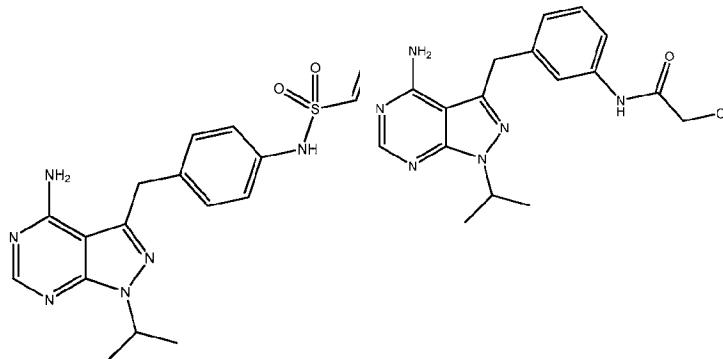
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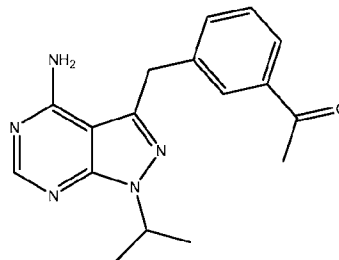
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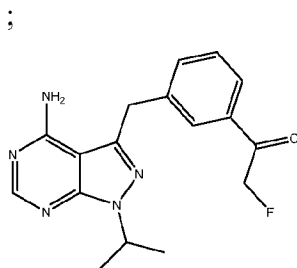
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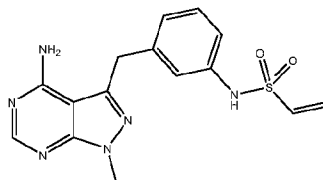
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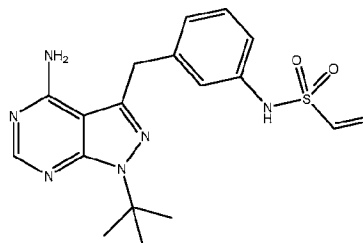
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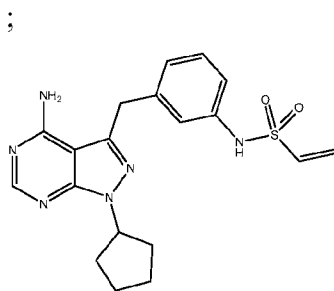
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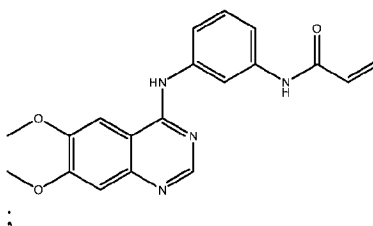
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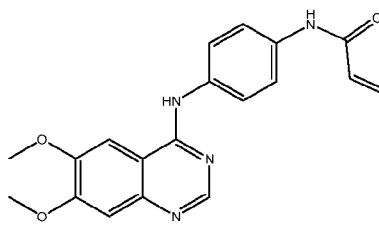
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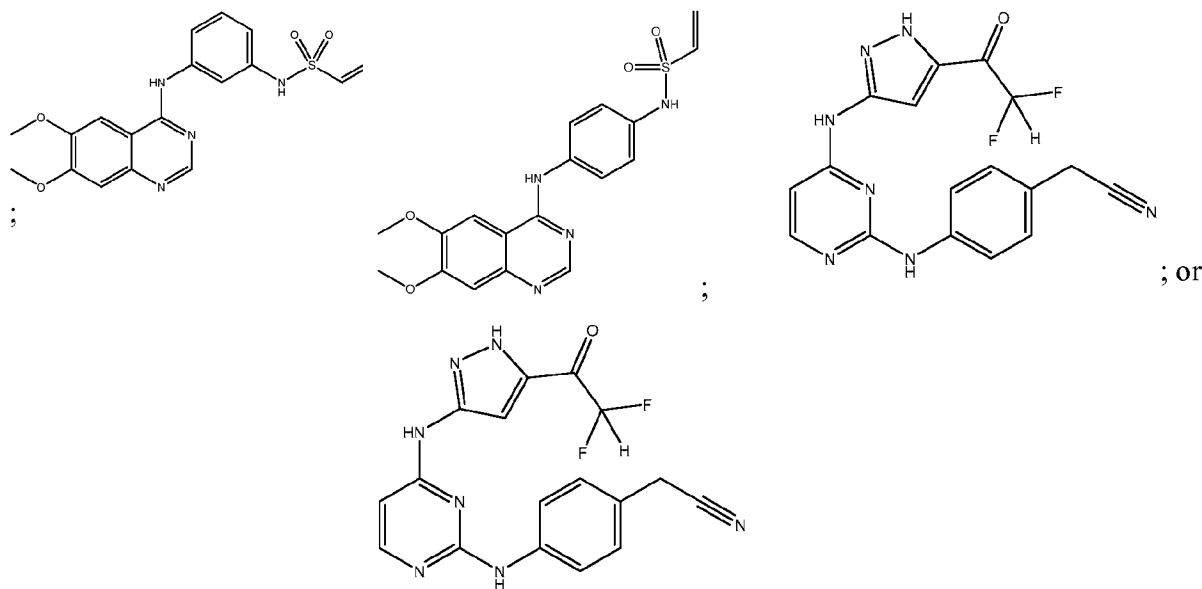
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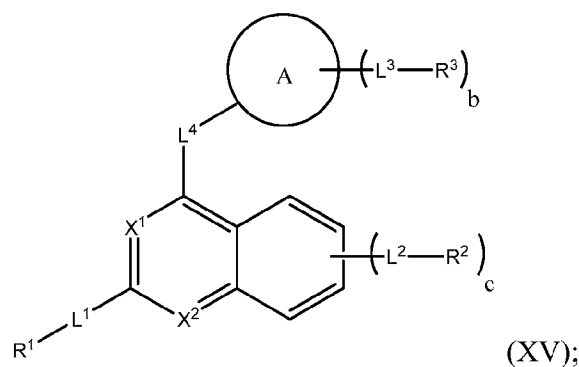
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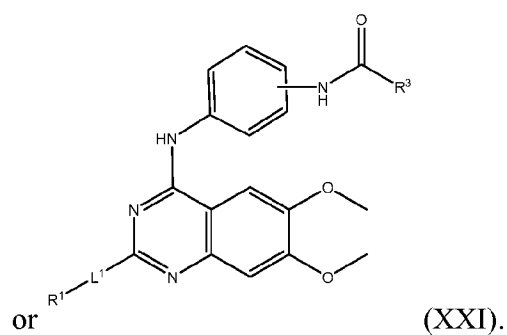
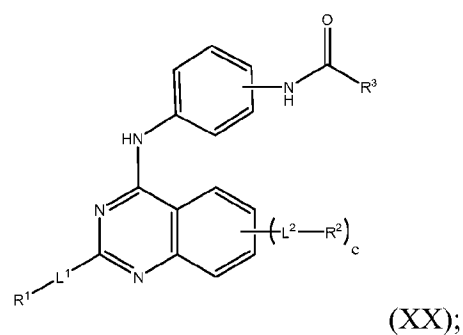
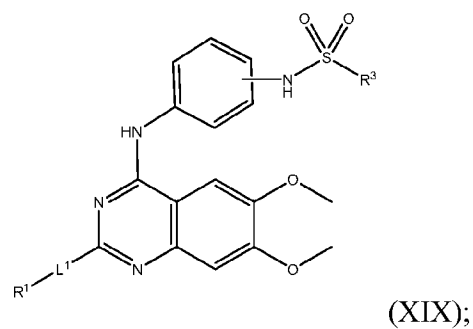
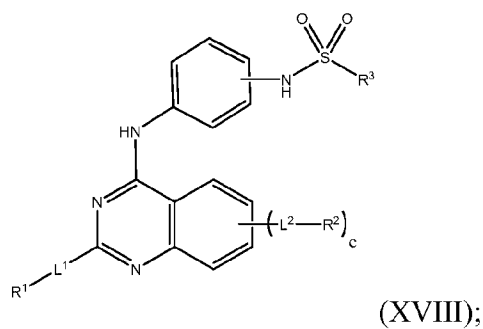
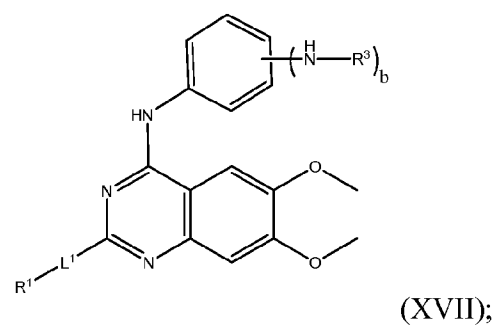
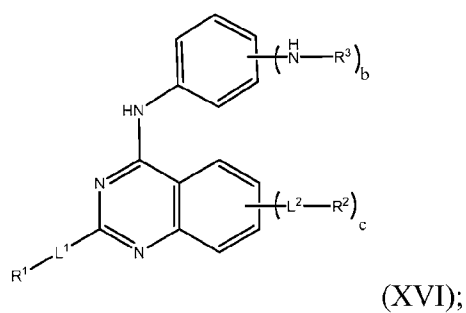
[0202] In some other embodiments, the present invention provides a compound having the below formula (which are useful *inter alia*, as inhibitors of Lrrk-2 kinases):



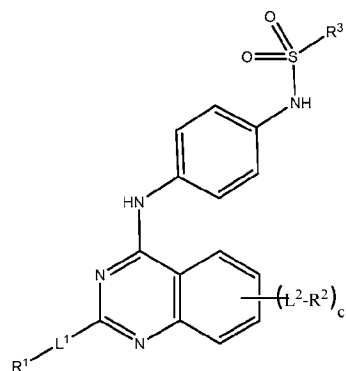
- X^1 and X^2 are, in each instance, independently =N- or =C(- L^6 - R^6)-. Ring A is, in each instance, independently selected from cycloalkyl, heterocycloalkyl, aryl, or heteroaryl. L^1 , L^2 , L^3 , and L^4 are as defined above (e.g., in each instance, independently selected from a bond, -C(O)-, -C(O)N(R^7)-, -C(O)O-, -S(O)_g-, -S(O)₂N(R^7)-, -O-, -N(R^7)-, -N(R^7)C(O)N(R^8)-), substituted or unsubstituted alkylene, substituted or unsubstituted heteroalkylene, substituted or unsubstituted cycloalkylene, substituted or unsubstituted heterocycloalkylene, substituted or unsubstituted arylene, or substituted or unsubstituted heteroarylene, wherein g is an integer from 0 to 2). R^1 , R^2 , R^3 , R^6 , R^7 , and R^8 are as defined above (e.g., in each instance, independently selected from hydrogen, halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or

unsubstituted aryl, or substituted or unsubstituted heteroaryl). The variable b is as defined above (e.g. an integer from 0 to 5; and c is as defined above (e.g. an integer from 0 to 4).

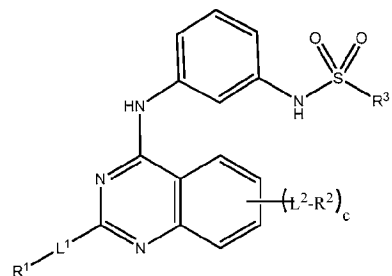
[0203] In some embodiments, the present invention provides a compound having the formula:



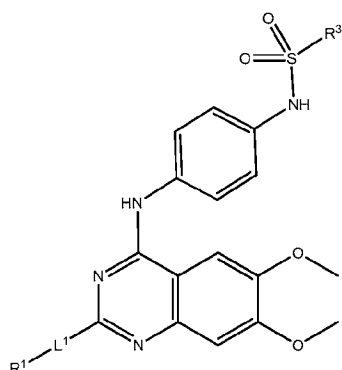
- 5 [0204] In some other embodiments, the compound provided herein has the formula (with the variables as defined above):



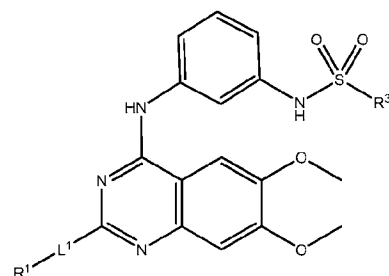
(XXII);



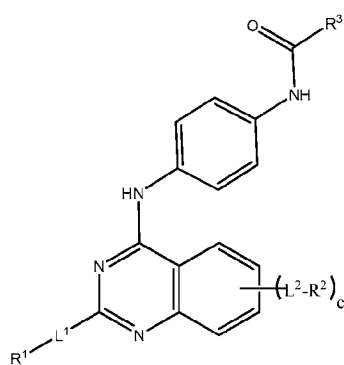
(XXIII);



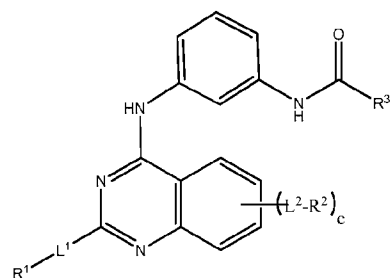
(XXIV);



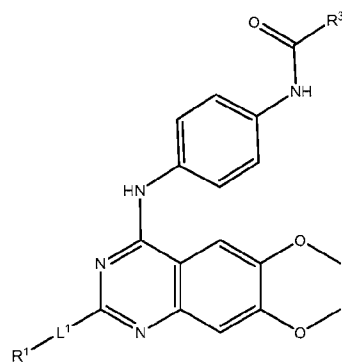
(XXV);



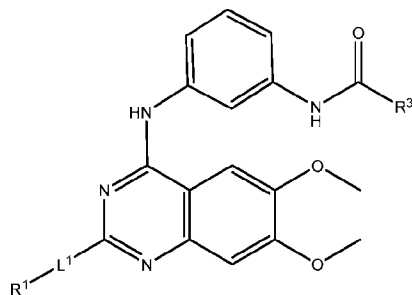
(XXVI);



(XXVII)

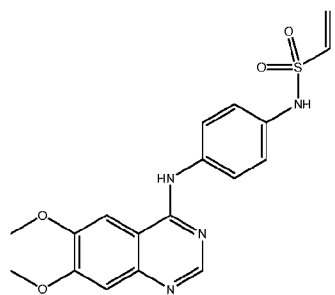


(XXVIII); or

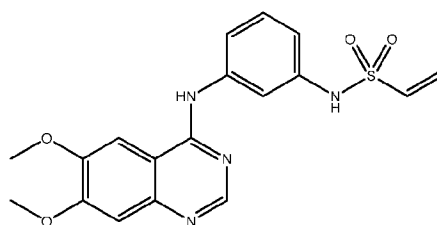


(XXIX).

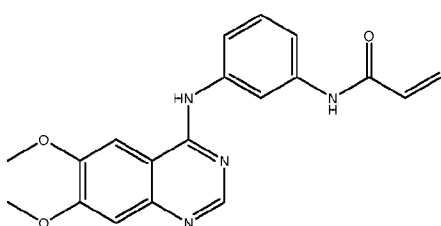
[0205] In other embodiments, the present invention provides compounds having the formula in the table below ("Table 1"):



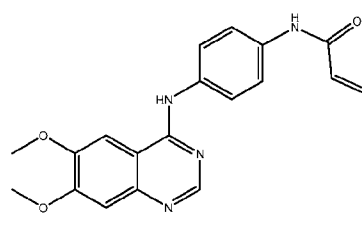
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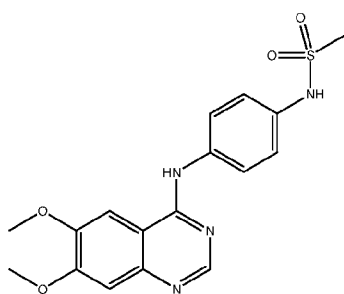
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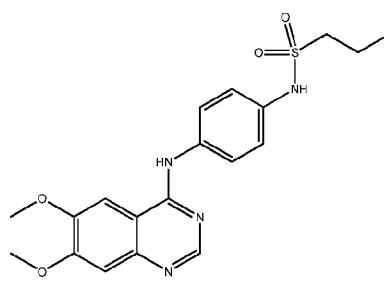
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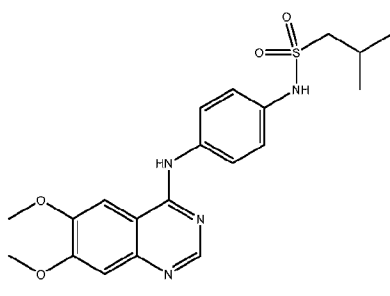
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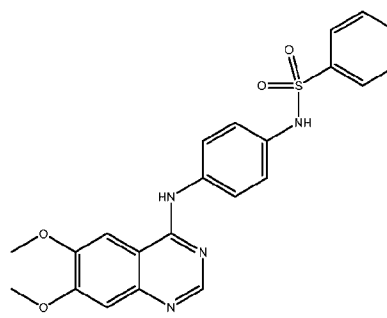
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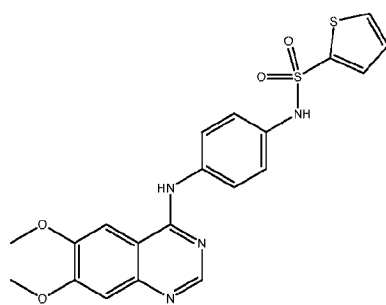
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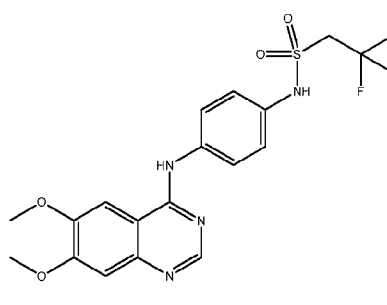
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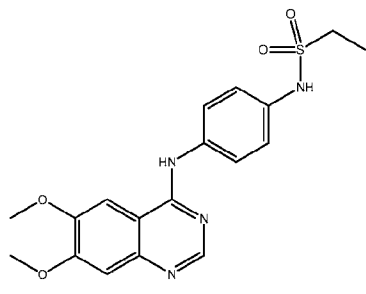
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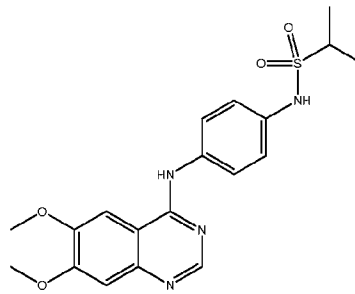
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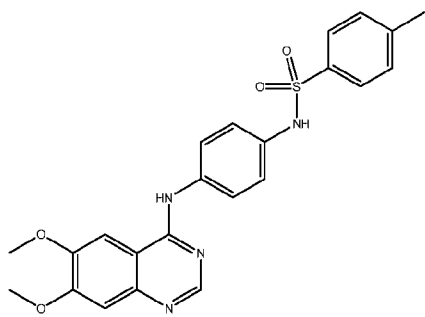
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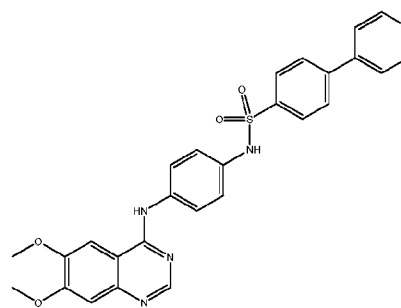
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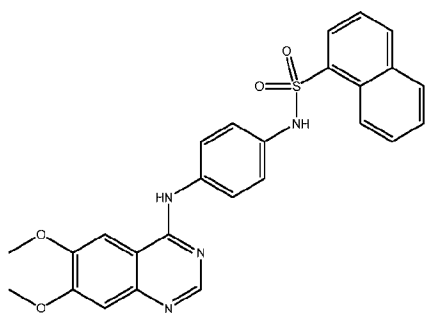
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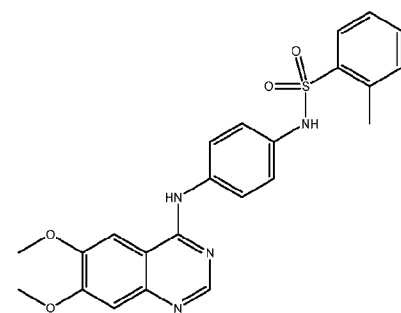
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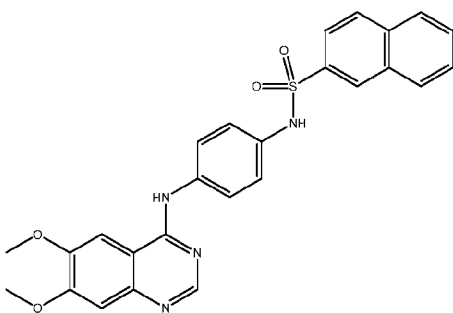
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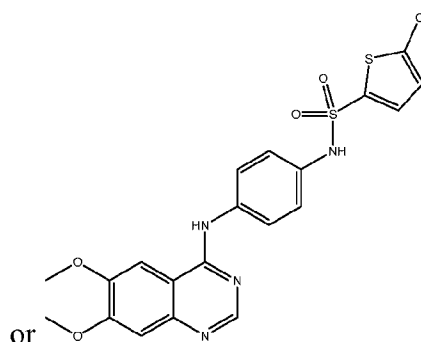
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or

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[0206] In certain embodiments, L^3 is selected from a bond, -NH-, -C(O)-, -S(O₂)-, substituted or unsubstituted alkylene, or substituted or unsubstituted heteroalkylene. R^3 may be selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, or halogen. In other embodiments, R^3 is methyl; difluoromethyl; trifluoromethyl; ethenyl; ethyl; 2,2,2-trichloroethyl; 2,2-dichloroethyl; 2-chloroethyl; 2,2,2-trifluoroethyl; 2,2-difluoroethyl; or 2-fluoroethyl; propyl; isopropyl; 1-propenyl; 2-propenyl; butyl; tert-butyl; naphthyl; thiophene; 2-chloro-thiophene; phenyl; 2-methyl-phenyl; 3-methyl-phenyl; 4-methyl-phenyl; 2-phenyl-phenyl; 3-phenyl-phenyl; 4-phenyl-phenyl 2-chloro-thiophene; or 3-chloro-thiophene.

[0207] As described above, the term “inhibitor” may refer to an inhibitor of a recombinant kinase comprising a cysteine substitution at a gatekeeper amino acid position (i.e. a cysteine gatekeeper kinase inhibitor) and includes a compound described herein such as the compound of Formulae (I) to (XIV). In some embodiments, the inhibitors are able to covalently bind to cysteine. In some other embodiments, the inhibitors inhibit the kinase by bonding to the sulphydryl group of the cysteine residue at the gatekeeper amino acid position. In some

embodiments, a compound provided herein may be a Lrrk-2 kinase inhibitor. In some embodiments, the Lrrk-2 kinase inhibitor is one or more of the compounds set forth in Table Z and/or a compound of Formula (XV) to (XXIX).

[0208] A person having ordinary skill in the art would immediately take into account the widely known principles of chemical when considering the description of compounds provided herein. Accordingly, where a group may be substituted by one or more of a number of substituents, such substitutions are selected so as to comply with principles of chemical bonding and to give compounds which are not inherently unstable and/or would be known to one of ordinary skill in the art as likely to be unstable under ambient conditions, such as aqueous, or neutral conditions.

[0209] It will be apparent to one skilled in the art that certain compounds of this invention may exist in tautomeric forms, all such tautomeric forms of the compounds being within the scope of the invention.

[0210] Unless otherwise stated, structures depicted herein are also meant to include compounds which differ only in the presence of one or more isotopically enriched atoms. For example, compounds having the present structures except for the replacement of a hydrogen by a deuterium or tritium, the replacement of a carbon by ^{13}C - or ^{14}C -enriched carbon, or the replacement of an iodine by ^{125}I , are within the scope of this invention. All isotopic variations of the compounds of the present invention, whether radioactive or not, are encompassed within the scope of the present invention.

[0211] The compounds of the present invention also include the salts, hydrates, solvates and prodrug forms. The compounds of the present invention also include the isomers and metabolites of those described in Formula (I) – (XXIX).

[0212] Salts include, but are not limited, to sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, phosphonic acid, isonicotinate, lactate, salicylate, citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucaronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, and pamoate (i.e., 1,1'-methylene-bis-(2-hydroxy-3-naphthoate)) salts. Other salts include, but are not limited to, salts with inorganic bases including alkali metal salts such as sodium salts, and potassium salts; alkaline earth metal salts such as calcium salts, and magnesium salts; aluminum salts; and ammonium salts. Other salts with organic

bases include salts with diethylamine, diethanolamine, meglumine, and N,N'-dibenzylethylenediamine.

[0213] The neutral forms of the compounds can be regenerated by contacting the salt with a base or acid and isolating the parent compound in the conventional manner. The parent form of the compound differs from the various salt forms in certain physical properties, such as solubility in polar solvents, but otherwise the salts are equivalent to the parent form of the compound for the purposes of the present invention.

[0214] Certain compounds of the present invention can exist in unsolvated forms as well as solvated forms, including hydrated forms. In general, the solvated forms are equivalent to unsolvated forms and are encompassed within the scope of the present invention. Certain compounds of the present invention may exist in multiple crystalline or amorphous forms. In general, all physical forms are equivalent for the uses contemplated by the present invention and are intended to be within the scope of the present invention.

[0215] Certain compounds of the present invention possess asymmetric carbon atoms (optical centers) or double bonds; the enantiomers, racemates, diastereomers, tautomers, geometric isomers, stereoisomeric forms that may be defined, in terms of absolute stereochemistry, as (R)- or (S)- or, as (D)- or (L)- for amino acids, and individual isomers are encompassed within the scope of the present invention. The compounds of the present invention do not include those which are known in art to be too unstable to synthesize and/or isolate. The present invention is meant to include compounds in racemic and optically pure forms. Optically active (R)- and (S)-, or (D)- and (L)-isomers may be prepared using chiral synthons or chiral reagents, or resolved using conventional techniques.

[0216] The present invention also provides compounds which are in a prodrug form. Prodrugs of the compounds described herein are those compounds that readily undergo chemical changes under physiological conditions to provide the compounds of the present invention. Additionally, prodrugs can be converted to the compounds of the present invention by chemical or biochemical methods in an *ex vivo* environment. For example, prodrugs can be slowly converted to the compounds of the present invention when placed in a transdermal patch reservoir with a suitable enzyme or chemical reagent.

[0217] In some embodiments, each substituted group described above for the compounds of the present invention is substituted with at least one substituent group. More specifically, in some embodiments, each substituted alkyl, substituted heteroalkyl, substituted cycloalkyl, substituted heterocycloalkyl, substituted aryl, substituted heteroaryl, substituted or

unsubstituted alkylene, substituted or unsubstituted heteroalkylene, substituted or unsubstituted cycloalkylene, substituted or unsubstituted heterocycloalkylene, substituted or unsubstituted arylene or substituted or unsubstituted heteroarylene described above is substituted with at least one substituent group. In other embodiments, at least one or all of these groups are substituted with at least one size-limited substituent group. Alternatively, at least one or all of these groups are substituted with at least one lower substituent group.

[0218] In other embodiments of the compounds described above, each substituted or unsubstituted alkyl is a substituted or unsubstituted C₁-C₂₀ alkyl, each substituted or unsubstituted alkylene is a substituted or unsubstituted C₁-C₂₀ alkylene, each substituted or unsubstituted heteroalkyl is a substituted or unsubstituted 2 to 20 membered heteroalkyl, each substituted or unsubstituted heteroalkylene is a substituted or unsubstituted 2 to 20 membered heteroalkylene, each substituted or unsubstituted cycloalkyl is a substituted or unsubstituted C₃-C₈ cycloalkyl, each substituted or unsubstituted cycloalkylene is a substituted or unsubstituted C₃-C₈ cycloalkylene, each substituted or unsubstituted heterocycloalkyl is a substituted or unsubstituted 4 to 8 membered heterocycloalkyl, each substituted or unsubstituted heterocycloalkylene is a substituted or unsubstituted 4 to 8 membered heterocycloalkylene, each substituted or unsubstituted aryl is a substituted or unsubstituted C₆ or C₈ aryl, each substituted or unsubstituted arylene is a substituted or unsubstituted C₆ or C₈ arylene, each substituted or unsubstituted heteroaryl is a substituted or unsubstituted C₅ or C₆ heteroaryl, and each substituted or unsubstituted heteroarylene is a substituted or unsubstituted C₅ or C₆ heteroarylene.

[0219] Alternatively, each substituted or unsubstituted alkyl is a substituted or unsubstituted C₁-C₈ alkyl, each substituted or unsubstituted alkylene is a substituted or unsubstituted C₁-C₈ alkylene, each substituted or unsubstituted heteroalkyl is a substituted or unsubstituted 2 to 8 membered heteroalkyl, each substituted or unsubstituted heteroalkylene is a substituted or unsubstituted 2 to 8 membered heteroalkylene, each substituted or unsubstituted cycloalkyl is a substituted or unsubstituted C₃-C₆ cycloalkyl, each substituted or unsubstituted cycloalkylene is a substituted or unsubstituted C₃-C₆ cycloalkylene, each substituted or unsubstituted heterocycloalkyl is a substituted or unsubstituted 3 to 6 membered heterocycloalkyl, and each substituted or unsubstituted heterocycloalkylene is a substituted or unsubstituted 3 to 6 membered heterocycloalkylene.

V. Kinases

[0220] In some embodiments, the present invention provides a recombinant kinase comprising a cysteine substitution at a gatekeeper amino acid position (also referred to as a “cysteine gatekeeper kinase”, a “recombinant kinase of the present invention” or a “recombinant kinase set forth herein”). For example, the recombinant kinase can comprise a sequence having a cysteine substitution at the position corresponding to Thr338 of c-Src, such as the positions shown for SEQ ID NOs:58-77, and sequences having substantial identity thereto. That is, the recombinant kinase can comprise a sequence having at least about 85, 90, 92, 93, 94, 95, 96, 97, 98, or 99% identity to a sequence of any one of SEQ ID NOs:58-77, with a cysteine substitution at the position corresponding to Thr338 of c-Src.

[0221] In some embodiments, the recombinant kinase can have a sequence of SEQ ID NO:2 (T338C c-Src), or a sequence having substantial identity thereto. In some embodiments the recombinant kinase can comprise a sequence having at least about 85, 90, 92, 93, 94, 95, 96, 97, 98, or 99% identity to the sequence of SEQ ID NO:2 with a cysteine at the position corresponding to 338 (with reference to the full length sequence of SEQ ID NO:3). In some embodiments, the recombinant kinase comprises less than the full length of SEQ ID NO:2 or 3 (and substantially identical variants thereof), but retains the cysteine substitution at the position corresponding to amino acid 338 of SEQ ID NO:3. In some embodiments, the recombinant kinase comprises at least 8, 10, 12, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, 175, 200 or more contiguous amino acids of SEQ ID NO:2 or a substantially identical sequence over that span retaining a cysteine at the amino acid position corresponding to 338 of SEQ ID NO:3.

[0222] In some embodiments, the recombinant kinase can have a sequence of any one of SEQ ID NOs:24-45 or a sequence having substantial identity thereto. These kinase sequences have a naturally occurring gatekeeper cysteine, *i.e.* a cysteine at the position corresponding to amino acid 338 in c-Src (SEQ ID NO:2 shows the T338C c-Src, while SEQ ID NO:4 shows the wild type T338 c-Src sequence). In some embodiments the recombinant kinase can comprise a sequence having at least about 85, 90, 92, 93, 94, 95, 96, 97, 98, or 99% identity to the sequence of any one of SEQ ID NOs:24-45 with a cysteine at the position corresponding to amino acid 338 of c-Src. In some embodiments, the recombinant kinase comprises less than the full length of any one of SEQ ID NOs:24-45 (and substantially identical variants thereof), but retains the cysteine at the position corresponding to amino acid 338 of c-Src. In some embodiments, the recombinant kinase comprises at least 8, 10, 12, 20,

25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, 175, 200 or more contiguous amino acids of any one of SEQ ID NOs:24-45 or a substantially identical sequence over that span retaining a cysteine at the position corresponding to 338 of SEQ ID NO:2.

[0223] In some other embodiments, the recombinant kinase has a k_{cat} activity that is not substantially lower than the k_{cat} activity of the corresponding wild-type kinase. In some embodiments, the k_{cat} activity that is not substantially lower than the k_{cat} activity of the corresponding wild-type kinase. In some embodiments, the recombinant kinase has a K_{m} binding affinity for ATP of the recombinant kinase is not substantially lower than the K_{m} binding affinity for ATP of the corresponding wild type kinase. In some embodiments, the K_{m} binding affinity for ATP of the recombinant kinase is not substantially lower than the K_{m} binding affinity for ATP of the corresponding wild type kinase. The activity is considered “not substantially lower” when the activity is not less than 5-fold less, e.g., 4-fold, 3-fold, or 2-fold less than the reference kinase. In some cases, the term “not substantially lower” is determined in terms of percentage, and a not substantially lower activity is at least 50% of the reference kinase, e.g. higher than 50% of the activity of a wild type kinase. In some embodiments, the activity is 60, 70, 75, 80, 85, 90, 95% or higher of the activity of the reference kinase.

[0224] The present invention provides methods for evaluating the use of a cysteine gatekeeper kinase.

[0225] In some embodiments, the recombinant kinase includes a recombinant kinase is selected from Src (e.g., c-Src (SEQ ID NOs:2-23 or 59) or v-Src (SEQ ID NO: 46-50 or 58); MOK; Sgk494; Yak/Yrk; SRPK1; CDK; DICTY-I; PAK/STE20; or Ctrl/DPYK1 with a cysteine at the gatekeeper position (at the amino acid position corresponding to 338 of the c-Src protein of SEQ ID NO:3. In some embodiments, the recombinant kinase is a recombinant Src and the gatekeeper amino acid position is T338.

[0226] In some embodiments, the recombinant kinase has a greater catalytic efficiency than the corresponding wild type kinase. For example, the kinase activity is greater than 100% of the reference kinase (e.g., wild type c-Src of SEQ ID NO:3). In some embodiments, the activity is 1.2-fold, 1.5-fold, 1.8-fold, 2-fold, 2.5-fold, 3-fold, 4-fold, 5-fold, 10-fold or higher than the reference. In some embodiments, the catalytic efficiency is measured as the ratio of $k_{\text{cat}}/K_{\text{m}}$.

[0227] In some embodiments, the recombinant kinase further comprises an additional amino acid substitution corresponding to position V323 of c-Src (see, e.g., SEQ ID NOs:10-

23). That is, the recombinant kinase can be a cysteine gatekeeper kinase, i.e., comprising a sequence having substantial identity to any one of SEQ ID NOs:2-77 with a cysteine at the position corresponding to amino acid 338 of SEQ ID NO:3, and additionally include a substitution at the position corresponding to amino acid 323 of SEQ ID NO:3. One of skill will understand that the positions corresponding to those of SEQ ID NO:3 can be ascertained for other kinase sequences.

[0228] In some embodiments, the recombinant kinase further comprises an additional amino acid substitution corresponding to the position M314 of c-Src, e.g. to gly (G) or ala (A). For example, the recombinant kinase can be a cysteine gatekeeper kinase, i.e., comprising a sequence having substantial identity to any one of SEQ ID NOs:2-77 with a cysteine at the position corresponding to amino acid 338 of SEQ ID NO:3, and additionally include a substitution at the position corresponding to amino acid 314 of SEQ ID NO:3. One of skill will understand that the positions corresponding to those of SEQ ID NO:3 can be ascertained for other kinase sequences.

[0229] In some embodiments, the recombinant kinase includes substitutions at two or all three positions corresponding to positions 338, 314 and 323 of c-Src (SEQ ID NO:3). In some embodiments, the recombinant kinase comprises a sequence having substantial identity to SEQ ID NO:2 with a C at the position corresponding to amino acid 338 of c-Src (the full length sequence of SEQ ID NO:3), and also has a substitution at the position corresponding to amino acid 314 of c-Src. In some embodiments, the recombinant kinase comprises a sequence having substantial identity to SEQ ID NO:2 with a C at the position corresponding to amino acid 338 (of the full length sequence of SEQ ID NO:3), and also has a substitution at the position corresponding to amino acid 323 of c-Src. In some embodiments, the recombinant kinase comprises a sequence having substantial identity to SEQ ID NO:2 with a C at the position corresponding to amino acid 338 of c-Src (the full length sequence of SEQ ID NO:3), and also has a substitution at the positions corresponding to amino acids 314 and 323 of c-Src. Again, one of skill will be able to determine the corresponding amino acid positions for kinases with sequences that are not perfectly aligned with c-Src.

[0230] In some embodiments, the cysteine gatekeeper kinase has an additional amino acid substitution of alanine (A) or serine (S) at the position corresponding to V323 of c-Src (V323A(c-Src-ES2)] or V323S (c-Src-ES3). In some other embodiments, the recombinant kinase having an additional amino acid substitution at VAL323 has a greater catalytic efficiency of the corresponding recombinant kinase that does not have an additional amino

acid substitution at VAL323. In some embodiments, the catalytic efficiency is measured as the ratio of k_{cat}/K_m .

[0231] In some embodiments, the corresponding substitutions can be performed in other kinases. A person having ordinary skill in the art would understand which amino acids correspond to VAL 323 in other kinases.

[0232] In some embodiments, the present invention provides methods and compositions for modifying the microenvironment around the cysteine gatekeeper by alteration of one nearby residue (e.g. Val323) in order to impact inhibitor potency. For example, liberating additional space with a V323A mutation resulted in a 5-fold increase in potency for **13**, while the V3232S mutation had a 12-fold effect. In some embodiments, the present invention provides methods of boosting potency which may allow dosing levels sufficient to substantially minimize off-target effects with MOK kinase (the effects due to MOK inhibition can be taken into account by comparing effects in *WT* vs. *ES* expressing cells).

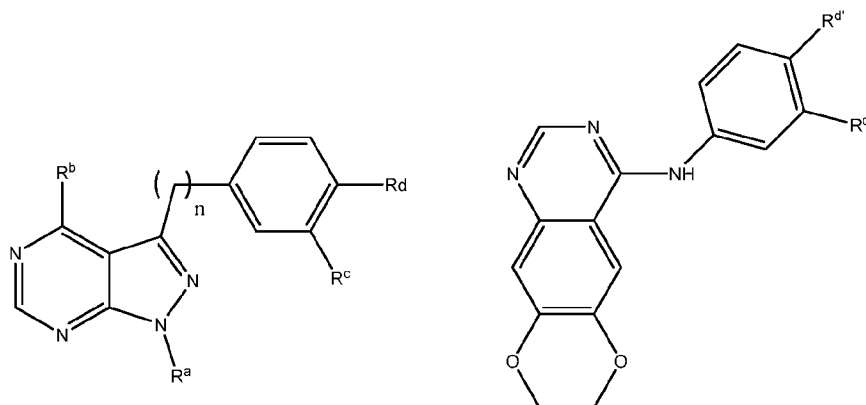
A. Forming a Kinase

[0233] In some other embodiments, the present invention provides a method of forming a recombinant kinase described herein, wherein the method includes transforming a cell with a nucleic acid encoding a recombinant kinase described herein, thereby forming a recombinant kinase described herein. In some embodiments, the recombinant kinase is selected from Src; MOK; Sgk494; Lrrk-2; Yak/Yrk; SRPK1; CDK; DICTY-I; PAK/STE20; or Ctrl/DPYK1 as described herein.

B. Structure Activity Relationship Studies - Inhibition of Src

[0234] In some embodiment, the present invention provides a series of 3-phenyl-substituted pyrazolopyrimidines with electrophilic groups at positions expected to be in close proximity to the gatekeeper residue and as set forth in Table 1. In some other embodiments, the electrophiles include meta and para substituents of the 3-phenyl ring and vinylsulfonamides as well as acrylamides and chloroacetamides. A meta-substituted vinylsulfonamide, **3** inhibited T338C relative to WT c-Src (>9-fold increase), while a para-substituted version, **5**, elicited a ~6-fold improvement (Table 1). Acrylamides (**1**) and chloroacetamides (**6**) were also shown to be inhibitors. Under the assay conditions used (10 min preincubation with inhibitor prior to addition of ATP) IC_{50} values under 5 μM for either **2** or **4** for WT or T338C c-Src were not shown.

[0235] **Table 1.** IC₅₀ values for electrophile derivatized pyrazolopyrimidines and 4-anilinoquinazolines against WT c-Src and T338C c-Src. Scaffolds are depicted such that the hinge-binding element is located on the left. Note that for covalent inhibitors IC₅₀ values are time-dependent. In these assays, the inhibitors were preincubated with the Src for ten minutes prior to assay initialization by addition of ATP.



Compound	n	R ^a	R ^b	R ^c	R ^d	WT c-Src IC ₅₀ (nM)	T338C c-Src IC ₅₀ (nM)
1	0	<i>i</i> Pr	NH ₂	NHCOCHCH ₂	H	2319	419
2	0	<i>i</i> Pr	H	NHCOCHCH ₂	H	>5000	>5000
3	0	<i>i</i> Pr	NH ₂	NHSO ₂ CHCH ₂	H	1004	111
4	0	<i>i</i> Pr	H	NHSO ₂ CHCH ₂	H	>5000	>5000
5	0	<i>i</i> Pr	NH ₂	H	NHSO ₂ CHCH ₂	899	145
6	0	<i>i</i> Pr	NH ₂	NHCOCH ₂ Cl	H	>5000	817
7	1	<i>i</i> Pr	NH ₂	NHCOCHCH ₂	H	>5000	2762
8	1	<i>i</i> Pr	NH ₂	H	NHCOCHCH ₂	>5000	>5000
9	1	<i>i</i> Pr	NH ₂	NHSO ₂ CHCH ₂	H	>5000	150
10	1	<i>i</i> Pr	NH ₂	H	NHSO ₂ CHCH ₂	3083	1759
11	1	<i>i</i> Pr	NH ₂	NHSO ₂ CH ₂ CH ₃	H	>5000	3497
12	1	<i>i</i> Pr	NH ₂	NHCOCH ₂ Cl	H	>5000	>5000
13	1	<i>i</i> Pr	NH ₂	COCH ₂ F	H	>5000	338
14	1	<i>i</i> Pr	NH ₂	COCH ₃	H	>5000	4520
15	1	Me	NH ₂	NHSO ₂ CHCH ₂	H	>5000	3161
16	1	<i>t</i> Bu	NH ₂	NHSO ₂ CHCH ₂	H	>5000	618
17	1	Cp	NH ₂	NHSO ₂ CHCH ₂	H	>5000	196
Compound				R ^{c'}	R ^{d'}		
18				NHCOCHCH ₂	H	>5000	1661
19				NHSO ₂ CHCH ₂	H	>5000	1004
20				H	NHSO ₂ CHCH ₂	2170	560

[0236] An array of 3-benzyl-substituted pyrazolopyrimidines modified with electrophiles or isosteric and unreactive negative control groups at the meta and para positions were

synthesized and screened against WT and T338C c-Src (compounds 7-17, Table 1). The benzyl functionalized compounds inhibited wild type c-Src (IC₅₀ values >5 μM).

Compound, 9, which is functionalized with a vinylsulfonamide, exhibited an IC₅₀ value of 150 nM. An unreactive control compound 11 resulted in a 23-fold drop in potency. A

5 fluoromethylketone bearing compound, 13, yielded an IC₅₀ value of 338 nM, which was >13-fold more potent than the corresponding ketone, 14.

[0237] The present invention also provides methods of determining the activity effects of modifying the N1 position of pyrazolopyrimidines by a structure activity relationship (SAR).

10 In some embodiments, this includes using the pyrazolopyrimidine scaffold with a benzyl-linked *m*-vinylsulfonamide, see compounds 9, 15-17; Table 1. This analysis revealed that secondary alkyl groups such as isopropyl (9) and cyclopentyl (17) moieties inhibit T338C c-Src. These results indicate that substitution at N1 can be used to modulate potency against T338C c-Src. Accordingly, the present invention provides methods of modulating the potency against kinases, such as c-Src.

15 [0238] The present invention provides methods and compositions that are suitable for use with a variety of kinases, *e.g.* recombinant, wild type, natural, mutant, and unmutated. In some embodiments, these kinases include c-Src, Src; MOK; Sgk494; Yak/Yrk; SRPK1; CDK; DICTY-I; PAK/STE20; or Ctrl/DPYK1.

[0239] In some embodiments, the recombinant kinases described herein include an
20 approximate 15 residue His tag in addition to the sequence for the actual protein, *e.g.* linker and heptamer for specific TEV protein cutting. In some instances TEV may be cut at residue 248, 249, or 250. It is understood by those in the art that the DNA sequence can be optimized with respect to the code or sequence without affecting the primary protein encoded thereby.

[0240] The following sets forth gatekeeper residues. In some embodiments, the
25 gatekeeper residue is cysteine. In some embodiments the kinase is natural, wild type, or recombinant.

[0241] As described herein, a Cys gatekeeper is an attractive target for the inhibitory compounds described herein. Representative kinases having a naturally occurring Cys at the gatekeeper position include the entries of Table 2 following. As customary in the art, the
30 terms "GI: number," "GI: No." and the like refer to a unique sequence identifier (*i.e.*, "GenBank Identifier") for a sequence.

Table 2.

SEQ ID NO:	GI: No.	Species
24	4587987	<i>Arabidopsis thaliana</i>
25	194240495	<i>Arabidopsis</i>
26	1785621	<i>Arabidopsis</i>
27	4678270	<i>Arabidopsis</i>
28	4678272	<i>Arabidopsis</i>
29	4678273	<i>Arabidopsis</i>
30	4678277	<i>Arabidopsis</i>
31	4886274	<i>Arabidopsis</i>
32	3047095	<i>Arabidopsis</i>
33	334188021	<i>Arabidopsis</i>
34	9294588	<i>Arabidopsis</i>
35	11120792	<i>Arabidopsis</i>
36	11120796	<i>Arabidopsis</i>
37	8777331	<i>Arabidopsis</i>
38	7106391	<i>Mus musculus</i>
39	1705720	<i>Carassius auratus</i>
40	6648996	<i>Capsicum annuum</i>
41	7630151	<i>Leishmania major</i>
42	5139689	<i>Homo sapiens</i>
43	486948	<i>Trichomonas vaginalis</i>
44	254688446	<i>Plasmodium falciparum</i>
45	13509297	<i>Dictyostelium discoideum</i>

VI. Co-Crystals of kinase and a compound

[0242] The present invention provides co-crystals of a kinase and a compound, *e.g.* co-crystal structure of T338C c-Src with a vinylsulfonamide-derivatized pyrazolopyrimidine inhibitor is provided, see Example 34.

[0243] In the 9-c-Src-ES1 co-crystal structure, the pyrazolopyrimidine pharmacophore interacts with the backbone amides of Glu339 and Met341 of the hinge region (**Fig. 2A**). The oxygen atoms of the sulfonamide hydrogen bond directly to the backbone amide of Asp404 and to that of Phe405 via a water molecule (**Fig. 2B, C**). Additionally, the nitrogen of the sulfonamide makes a direct hydrogen bond to the side chain Glu310 (**Figs. 2A, C**). In crystal structures of wild type Src, the hydroxyl of the gatekeeper threonine is often directed towards the C4-exocyclic amine of the adenine portion of ATP mimetics.

[0244] In the 9 c-Src-ES1 co-crystal structure, the sulfhydryl of Cys338 adopts a distinct rotamer to accommodate the bulky C-3 benzyl group and facilitate a covalent bond (**Fig. 2C**). The flexible ethylsulfonamide moiety is situated to allow the covalent linkage with Cys338 (**Fig. 2B**). The side chain of Met314, a critical component of the hydrophobic spine, is dramatically shifted relative to its position in other c-Src structures (**Figs. 2B, C**). Movement of Met314 may prevent a steric clash with the ethylsulfonamide moiety of 9.

VII. Pharmaceutical Compositions

[0245] In some embodiments, the present invention provides a pharmaceutical composition comprising a compound as set forth herein (e.g. a compound of Formula (I) – (XXIX)) and a pharmaceutically acceptable excipient.

5 [0246] “Pharmaceutically acceptable excipient” and “pharmaceutically acceptable carrier” refer to a substance that aids the administration of an active agent to and absorption by a subject and can be included in the compositions of the present invention without causing a significant adverse toxicological effect on the patient. Non-limiting examples of pharmaceutically acceptable excipients include water, NaCl, normal saline solutions, lactated
10 Ringer’s, normal sucrose, normal glucose, binders, fillers, disintegrants, lubricants, coatings, sweeteners, flavors and colors, and the like. One of skill in the art will recognize that other pharmaceutical excipients are useful in the present invention.

[0247] The compounds and compositions of the present invention can be prepared and administered in a wide variety of oral, parenteral and topical dosage forms. Oral preparations
15 include tablets, pills, powder, dragees, capsules, liquids, lozenges, cachets, gels, syrups, slurries, suspensions, etc., suitable for ingestion by the patient. The compounds of the present invention can also be administered by injection, that is, intravenously, intramuscularly, intracutaneously, subcutaneously, intraduodenally, or intraperitoneally. Also, the compounds described herein can be administered by inhalation, for example,
20 intranasally. Additionally, the compounds and compositions of the present invention can be administered transdermally. The GR modulators of this invention can also be administered by intraocular, intravaginal, and intrarectal routes including suppositories, insufflation, powders and aerosol formulations (for examples of steroid inhalants, see Rohatagi, *J. Clin. Pharmacol.* 35:1187-1193, 1995; Tjwa, *Ann. Allergy Asthma Immunol.* 75:107-111, 1995).
25 Accordingly, the present invention also provides pharmaceutical compositions including a pharmaceutically acceptable carrier or excipient and either a compound of Formula I, or a pharmaceutically acceptable salt of a compound of Formula I.

[0248] For preparing pharmaceutical compositions from the compounds of the present invention, pharmaceutically acceptable carriers can be either solid or liquid. Solid form
30 preparations include powders, tablets, pills, capsules, cachets, suppositories, and dispersible granules. A solid carrier can be one or more substances, which may also act as diluents, flavoring agents, binders, preservatives, tablet disintegrating agents, or an encapsulating material. Details on techniques for formulation and administration are well described in the

scientific and patent literature, see, e.g., the latest edition of Remington's Pharmaceutical Sciences, Maack Publishing Co, Easton PA ("Remington's").

[0249] In powders, the carrier is a finely divided solid, which is in a mixture with the finely divided active component. In tablets, the active component is mixed with the carrier having the necessary binding properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain from 5% or 10% to 70% of the active compound.

[0250] Suitable solid excipients include, but are not limited to, magnesium carbonate; magnesium stearate; talc; pectin; dextrin; starch; tragacanth; a low melting wax; cocoa butter; carbohydrates; sugars including, but not limited to, lactose, sucrose, mannitol, or sorbitol, starch from corn, wheat, rice, potato, or other plants; cellulose such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; and gums including arabic and tragacanth; as well as proteins including, but not limited to, gelatin and collagen. If desired, disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

[0251] Dragee cores are provided with suitable coatings such as concentrated sugar solutions, which may also contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound (i.e., dosage). Pharmaceutical preparations of the invention can also be used orally using, for example, push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating such as glycerol or sorbitol. Push-fit capsules can contain GR modulator mixed with a filler or binders such as lactose or starches, lubricants such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the GR modulator compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycol with or without stabilizers.

[0252] For preparing suppositories, a low melting wax, such as a mixture of fatty acid glycerides or cocoa butter, is first melted and the active component is dispersed homogeneously therein, as by stirring. The molten homogeneous mixture is then poured into convenient sized molds, allowed to cool, and thereby to solidify.

[0253] Liquid form preparations include solutions, suspensions, and emulsions, for example, water or water/propylene glycol solutions. For parenteral injection, liquid preparations can be formulated in solution in aqueous polyethylene glycol solution.

5 [0254] Aqueous solutions suitable for oral use can be prepared by dissolving the active component in water and adding suitable colorants, flavors, stabilizers, and thickening agents as desired. Aqueous suspensions suitable for oral use can be made by dispersing the finely divided active component in water with viscous material, such as natural or synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia, and dispersing or
10 wetting agents such as a naturally occurring phosphatide (e.g., lecithin), a condensation product of an alkylene oxide with a fatty acid (e.g., polyoxyethylene stearate), a condensation product of ethylene oxide with a long chain aliphatic alcohol (e.g., heptadecaethylene oxycetanol), a condensation product of ethylene oxide with a partial ester derived from a fatty acid and a hexitol (e.g., polyoxyethylene sorbitol mono-oleate), or a condensation product of
15 ethylene oxide with a partial ester derived from fatty acid and a hexitol anhydride (e.g., polyoxyethylene sorbitan mono-oleate). The aqueous suspension can also contain one or more preservatives such as ethyl or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents and one or more sweetening agents, such as sucrose, aspartame or saccharin. Formulations can be adjusted for osmolarity.

20 [0255] Also included are solid form preparations, which are intended to be converted, shortly before use, to liquid form preparations for oral administration. Such liquid forms include solutions, suspensions, and emulsions. These preparations may contain, in addition to the active component, colorants, flavors, stabilizers, buffers, artificial and natural sweeteners, dispersants, thickeners, solubilizing agents, and the like.

25 [0256] Oil suspensions can be formulated by suspending a GR modulator in a vegetable oil, such as arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin; or a mixture of these. The oil suspensions can contain a thickening agent, such as beeswax, hard paraffin or cetyl alcohol. Sweetening agents can be added to provide a palatable oral preparation, such as glycerol, sorbitol or sucrose. These formulations can be
30 preserved by the addition of an antioxidant such as ascorbic acid. As an example of an injectable oil vehicle, see Minto, *J. Pharmacol. Exp. Ther.* 281:93-102, 1997. The pharmaceutical formulations of the invention can also be in the form of oil-in-water emulsions. The oily phase can be a vegetable oil or a mineral oil, described above, or a mixture of these. Suitable emulsifying agents include naturally-occurring gums, such as gum

acacia and gum tragacanth, naturally occurring phosphatides, such as soybean lecithin, esters or partial esters derived from fatty acids and hexitol anhydrides, such as sorbitan mono-oleate, and condensation products of these partial esters with ethylene oxide, such as polyoxyethylene sorbitan mono-oleate. The emulsion can also contain sweetening agents and
5 flavoring agents, as in the formulation of syrups and elixirs. Such formulations can also contain a demulcent, a preservative, or a coloring agent.

VIII. Administration

[0257] The compositions of the present invention can be delivered by transdermally, by a topical route, formulated as applicator sticks, solutions, suspensions, emulsions, gels, creams,
10 ointments, pastes, jellies, paints, powders, and aerosols.

[0258] The compositions of the present invention can also be delivered as microspheres for slow release in the body. For example, microspheres can be administered via intradermal injection of drug-containing microspheres, which slowly release subcutaneously (see Rao, *J. Biomater Sci. Polym. Ed.* 7:623-645, 1995; as biodegradable and injectable gel formulations
15 (see, e.g., Gao *Pharm. Res.* 12:857-863, 1995); or, as microspheres for oral administration (see, e.g., Eyles, *J. Pharm. Pharmacol.* 49:669-674, 1997). Both transdermal and intradermal routes afford constant delivery for weeks or months.

[0259] The pharmaceutical compositions of the present invention can be provided as a salt and can be formed with many acids, including but not limited to hydrochloric, sulfuric,
20 acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms. In other cases, the preparation may be a lyophilized powder in 1 mM-50 mM histidine, 0.1%-2% sucrose, 2%-7% mannitol at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

[0260] In another embodiment, the compositions of the present invention are useful for
25 parenteral administration, such as intravenous (IV) administration or administration into a body cavity or lumen of an organ. The formulations for administration will commonly comprise a solution of the compositions of the present invention dissolved in a pharmaceutically acceptable carrier. Among the acceptable vehicles and solvents that can be employed are water and Ringer's solution, an isotonic sodium chloride. In addition, sterile
30 fixed oils can conventionally be employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid can likewise be used in the preparation of injectables. These solutions are sterile and generally free of undesirable matter. These formulations may

be sterilized by conventional, well known sterilization techniques. The formulations may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents, e.g., sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of the compositions of the present invention in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight, and the like, in accordance with the particular mode of administration selected and the patient's needs. For IV administration, the formulation can be a sterile injectable preparation, such as a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic parenterally-acceptable diluent or solvent, such as a solution of 1,3-butanediol.

[0261] In another embodiment, the formulations of the compositions of the present invention can be delivered by the use of liposomes which fuse with the cellular membrane or are endocytosed, *i.e.*, by employing ligands attached to the liposome, or attached directly to the oligonucleotide, that bind to surface membrane protein receptors of the cell resulting in endocytosis. By using liposomes, particularly where the liposome surface carries ligands specific for target cells, or are otherwise preferentially directed to a specific organ, one can focus the delivery of the compositions of the present invention into the target cells *in vivo*. (See, *e.g.*, Al-Muhammed, *J. Microencapsul.* 13:293-306, 1996; Chonn, *Curr. Opin. Biotechnol.* 6:698-708, 1995; Ostro, *Am. J. Hosp. Pharm.* 46:1576-1587, 1989).

[0262] The pharmaceutical preparation is preferably in unit dosage form. In such form the preparation is subdivided into unit doses containing appropriate quantities of the active component. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, such as packeted tablets, capsules, and powders in vials or ampoules. Also, the unit dosage form can be a capsule, tablet, cachet, or lozenge itself, or it can be the appropriate number of any of these in packaged form.

[0263] The quantity of active component in a unit dose preparation may be varied or adjusted from 0.1 mg to 10000 mg, more typically 1.0 mg to 1000 mg, most typically 10 mg to 500 mg, according to the particular application and the potency of the active component. The composition can, if desired, also contain other compatible therapeutic agents.

[0264] The compounds described herein can be used in combination with one another, with other active agents known to be useful in modulating a protein kinase, or with adjunctive agents that may not be effective alone, but may contribute to the efficacy of the active agent.

[0265] In some embodiments, co-administration includes administering one active agent within 0.5, 1, 2, 4, 6, 8, 10, 12, 16, 20, or 24 hours of a second active agent. Co-administration includes administering two active agents simultaneously, approximately simultaneously (e.g., within about 1, 5, 10, 15, 20, or 30 minutes of each other), or sequentially in any order. In some embodiments, co-administration can be accomplished by co-formulation, i.e., preparing a single pharmaceutical composition including both active agents. In other embodiments, the active agents can be formulated separately. In another embodiment, the active and/or adjunctive agents may be linked or conjugated to one another.

IX. Nucleic Acids

[0266] In some embodiments, the present invention provides an isolated nucleic acid comprising a nucleic acid sequence encoding a recombinant kinase provided herein (i.e. a recombinant kinase comprising a cysteine substitution at a gatekeeper amino acid position). This is also referred to herein as a “nucleic acid of the present invention.” Thus, provided herein are nucleic acids that encode the cysteine gatekeeper kinases described herein, e.g., recombinant kinases having a cysteine in the position corresponding to amino acid 338 of c-Src (SEQ ID NO:3).

In some embodiments, the nucleic acid sequence encodes a sequence or an enzymatically functional fragment thereof, set forth in SEQ ID NOs 2-77. The enzymatically functional fragment may be 50, 100, 150, or 200 bases in length. In some embodiments, the nucleic acid encodes a polypeptide having substantial identity to any one of SEQ ID NOs:2-77 wherein the polypeptide has a cysteine at the position corresponding to amino acid 338 of c-Src. One of skill will understand that a number of nucleic acid sequences will encode the same polypeptide, due to the degeneracy of the nucleic acid code. In some embodiments, the nucleic acid encodes a polypeptide encoding any one of SEQ ID NOs:2-77, wherein the polypeptide has a cysteine at the position corresponding to 338 of c-Src, or a sequence having at least 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% identity thereto. In some embodiments, the polypeptide is shorter than the full length of any one of SEQ ID NOs:2-77, but retains enzymatic (kinase) activity. In some embodiments, the polypeptide is at least 25, 30, 40, 50, 75, 80, 100, 120, 150, 200 or more amino acids in length, and has substantial identity over the corresponding length of the selected sequence (selected from the sequences

consisting of SEQ ID NOs:2-77, having a C at the position corresponding to 338 of c-Src). For the non-identical amino acids, one of skill will understand that conservative amino acid substitutions can be included.

5 In some embodiments, the nucleic acid encodes a polypeptide having substantial identity to any one of SEQ ID NOs:2-77 wherein the polypeptide has a cysteine at the position corresponding to amino acid 338 of c-Src, and an additional amino acid substitution at the position corresponding to amino acid 323 of c-Src and/or the position corresponding to amino acid 314 of c-Src. One of skill will understand that a number of nucleic acid sequences will encode the same polypeptide, due to the degeneracy of the nucleic acid code. In some
10 embodiments, the nucleic acid encodes a polypeptide encoding any one of SEQ ID NOs:2-77, wherein the polypeptide has a cysteine at the position corresponding to 338 of c-Src, and optionally one or both of the substitutions at positions corresponding to amino acids 314 or 323 of c-Src, or a sequence having at least 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% identity thereto. In some embodiments, the polypeptide is shorter than the full length of
15 any one of SEQ ID NOs:2-77, but retains enzymatic (kinase) activity. In some embodiments, the polypeptide is at least 25, 30, 40, 50, 75, 80, 100, 120, 150, 200 or more amino acids in length, and has substantial identity over the corresponding length of the selected sequence (selected from the sequences consisting of SEQ ID NOs:2-77, having a C at the position corresponding to 338 of c-Src, and optionally one or both of the substitutions at positions
20 corresponding to amino acids 314 or 323 of c-Src).

In some other embodiments, the present invention provides an expression cassette comprising a nucleic acid of the present invention. In yet other embodiments, the expression cassette is a recombinant viral vector. In some other embodiments, the expression cassette is inside of a host cell. In other embodiments, the expression cassette is selected from mammalian, non-
25 mammalian, mouse, rat, or human. In some embodiments, the recombinant kinase is inside a cell. In some other embodiments, the cell is selected from mammalian, non-mammalian, mouse, rat, or human. Thus, in some embodiments, a transgenic mouse or rat is provided, wherein the transgenic mouse or rat expresses a recombinant kinase comprising a cysteine substitution at a gatekeeper amino acid position as described above. Methods of producing a
30 transgenic mouse or rat that expresses recombinant proteins and enzymes are well-known in the art. A detailed description for such procedures may be found elsewhere, for example at U.S. Patent No. 4,736,866, the contents of which are incorporated by reference in their entirety for all purposes.

A. Descriptions of SEQ ID NOs (1-51) follows.

[0267] The following sets forth SEQ ID NOs:1-51 that are suitable for use with the compositions, methods, and kits herein:

SEQ ID NO:	Description
1	DNA construct for T338C c-src (251-533)
2	Protein encoded by SEQ ID NO:1
3	Gallus gallus proto-oncogene (c-Src)
4	c-Src (251-533)
5	c-Src (251-533) with GHM at N-terminal
6	[T338X]c-Src (251-533)
7	GHM-[T338X]c-Src (251-533) (GHM at N-terminal)
8	[T338C]c-Src (251-533) (c-Src "ES1")
9	GHM-[T338C]c-Src (251-533) (GHM at N-terminal) (c-Src "ES1")
10	[T338X, V323X]c-Src (251-533)
11	GHM-[T338X, V323X]c-Src (251-533) (GHM at N-terminal)
12	[T338C, V323X]c-Src (251-533)
13	GHM-[T338C, V323X]c-Src (251-533) (GHM at N-terminal)
14	[T338C, V323A]c-Src (251-533) (c-Src "ES2")
15	GHM-[T338C, V323A]c-Src (251-533) (GHM at N-terminal) (c-Src "ES2")
16	[T338C, V323S]c-Src (251-533) (c-Src "ES3")
17	GHM-[T338C, V323S]c-Src (251-533) (GHM at N-terminal) (c-Src "ES3")
18	[T338C, V323D]c-Src (251-533) (c-Src "ES4")
19	GHM-[T338C, V323D]c-Src (251-533) (GHM at N-terminal) (c-Src "ES4")
20	[T338C, V323E]c-Src (251-533) (c-Src "ES5")
21	GHM-[T338C, V323E]c-Src (251-533) (GHM at N-terminal) (c-Src "ES5")
22	[T338C, V323H]c-Src (251-533) (c-Src "ES6")
23	GHM-[T338C, V323H]c-Src (251-533) (GHM at N-terminal) (c-Src "ES6")
24	Kinases with gatekeeper Cys (Arabidopsis thaliana) 4587987
25	Kinases with gatekeeper Cys (Arabidopsis thaliana) 194240495
26	Kinases with gatekeeper Cys (Arabidopsis thaliana) 1785621
27	Kinases with gatekeeper Cys (Arabidopsis thaliana) 4678270
28	Kinases with gatekeeper Cys (Arabidopsis thaliana) 4678272
29	Kinases with gatekeeper Cys (Arabidopsis thaliana) 4678273
30	Kinases with gatekeeper Cys (Arabidopsis thaliana) 4678277
31	Kinases with gatekeeper Cys (Arabidopsis thaliana) 4886274
32	Kinases with gatekeeper Cys (Arabidopsis thaliana) 3047095
33	Kinases with gatekeeper Cys (Arabidopsis thaliana) 334188021/15238494
34	Kinases with gatekeeper Cys (Arabidopsis thaliana) 9294588
35	Kinases with gatekeeper Cys (Arabidopsis thaliana) 11120792
36	Kinases with gatekeeper Cys (Arabidopsis thaliana) 11120796
37	Kinases with gatekeeper Cys (Arabidopsis thaliana) 8777331
38	Kinases with gatekeeper Cys (Mus musculus) 7106391
39	Kinases with gatekeeper Cys (Carassius auratus) 1705720
40	Kinases with gatekeeper Cys (Capsicum annuum) 6648996
41	Kinases with gatekeeper Cys (Leishmania major) 7630151
42	Kinases with gatekeeper Cys (Homo sapiens) 5139689
43	Kinases with gatekeeper Cys (Trichomonas vaginalis) 486948
44	Kinases with gatekeeper Cys (Plasmodium falciparum) 254688446/3845109
45	Kinases with gatekeeper Cys (Dictyostelium discoideum) 13509297
46	v-Src (Rous sarcoma virus)
47	[I338X]v-Src

48	[I338C]v-Src
49	[I338T]v-Src
50	[I338G]v-Src
51	Artificial sequence (substrate for methods of testing)

[0268] An exemplary DNA construct useful for the methods described herein was synthesized (SEQ ID NO:1). This construct encodes the expressed protein set forth in SEQ ID NO:2. The expressed protein includes a His⁶ tag sequence at the N-terminal, useful for purification of recombinantly expressed protein as known in the art. The expressed protein further includes a spacer sequence (i.e., DYDIPTT, SEQ ID NO:2 residues 7-13) and a tobacco etch viral (TEV) protease site (i.e., ENLYFQG, SEQ ID NO:2, residues 14-20) as known in the art. An additional spacer (e.g., SEQ ID NO:2, residues 21-22) may be present in expressed proteins, which spacers residues may occupy the N-terminal position(s) of the expressed protein after protease cleavage (e.g., TEV protease cleavage). Thus, it is understood that reference to “c-Src (251-533)” and variants thereof herein contemplates expressed proteins having one or more amino acids at the N-terminal which may result from the process of recombinant protein production. For example, after the action of the TEV protease on the protein of SEQ ID NO:2, the expressed c-Src (251-533) protein may include the N-terminal tripeptide “GHM.” It is understood that absent indication otherwise, the numbering of c-Src proteins and variants as discussed herein follows the numbering of the full c-Src protein (SEQ ID NO:3). For example, full length c-Src (SEQ ID NO:3) contains 533 residues. Accordingly, residues 23-305 of SEQ ID NO:2 correspond to residues 251-533 of SEQ ID NO:3. c-Src (251-533) is expressly set forth in SEQ ID NO:4. A recombinantly expressed and processed protein of c-Src (251-533), as described above, having the N-terminal tripeptide “GHM” is set forth in SEQ ID NO:5.

[0269] In some embodiments, a c-Src variant is provided wherein the residue at the position equivalent to Thr³³⁸ of c-Src (SEQ ID NO:3) is substituted with another amino acid. In some embodiments, the substituted amino acid is a naturally occurring amino acid, as known in the art. Exemplary recombinantly expressed proteins having this substitution are set forth in SEQ ID NO:6 and SEQ ID NO:7, wherein SEQ ID NO:7 further includes the N-terminal tripeptide “GHM” as described above. Similarly, in some embodiments, a v-Src variant is provided wherein the residue at the position equivalent to Thr³³⁸ of v-Src (SEQ ID NO:46) is substituted with another amino acid. An exemplary recombinantly expressed protein having this substitution is set forth in SEQ ID NO:47. Specific exemplary recombinantly expressed proteins having a C, T or G substitution at position 338 of v-Src

(i.e., [I338C]v-Src, [I338T]v-Src, [I338G]v-Src), are set forth in SEQ ID NO: 48, SEQ ID NO:49 and SEQ ID NO:50, respectively.

[0270] In some embodiments, a protein kinase is provided having a Thr to Cys substitution at the position corresponding to residue 338 of c-Src (i.e., T338C substitution). The protein may be a fragment of full length c-Src. Recombinantly expressed protein variants of c-Src (251-533) having a T338C substitution (i.e., [T338C]c-Src(251-533)) are set forth in SEQ ID NO:8 and SEQ ID NO:9. It is understood that within the context of protein descriptive names, bracketed (i.e., “[]”) entries denote substitution(s), and that parenthetical entries after the protein name denote the corresponding residues of the fragment. For example, “[T338C]c-Src(251-533)” refers to the fragment of c-Src from residue 251 to residue 533, additionally having a Thr to Cys substitution at position 338 (c-Src numbering). These proteins are also known as “c-Src ES1” proteins. It is further understood that, as customary in the art, the term “XNNNY” refers to substitution of residue “X” at position “NNN” with residue “Y.”

[0271] In some embodiments, a plurality of substitutions of c-Src, or fragment thereof, are provided. For example, in some embodiments, a protein having double substitutions at residues T³³⁸ and V³²³ of c-Src is provided. In some embodiments, a protein having double substitutions at residues T³³⁸ and V³²³ of a fragment of c-Src (e.g., c-Src(251-533)) is provided. See SEQ ID NO:10. In some embodiments, the fragment of c-Src includes an N-terminal oligopeptide sequence resulting from processing of the recombinant protein as described above. See SEQ ID NO:11.

[0272] In some embodiments, there is provided a T338C substitution of c-Src, or fragment thereof (e.g., c-Src(251-533)), in combination with a substitution at position 323. See SEQ ID NO:12. In some embodiments, such doubly substituted fragment of c-Src includes an N-terminal oligopeptide sequence resulting from processing of the recombinant protein as described above. See SEQ ID NO:13.

[0273] In some embodiments, a T338C substitution of c-Src, or fragment thereof (e.g., c-Src(251-533)), in combination with a specific substitution at position 323 is provided. For example, [T³³⁸C, V³²³A]c-Src(251-533) is set forth in SEQ ID NO:14, and the corresponding protein having an N-terminal oligopeptide sequence resulting from processing of the recombinant protein is set forth in SEQ ID NO:15. These doubly substituted proteins are also known as the so-called c-Src “ES2” variant.

[0274] Further exemplary of this embodiment, there is provided [$T^{338}C$, $V^{323}S$]c-Src(251-533) (SEQ ID NO:16), and the corresponding protein having an N-terminal oligopeptide sequence resulting from processing of the recombinant protein (SEQ ID NO:17). These doubly substituted proteins are also known as the so-called c-Src “ES3” variant.

5 [0275] Further exemplary of this embodiment, there is provided [$T^{338}C$, $V^{323}D$]c-Src(251-533) (SEQ ID NO:18), and the corresponding protein having an N-terminal oligopeptide sequence resulting from processing of the recombinant protein (SEQ ID NO:19). These doubly substituted proteins are also known as the so-called c-Src “ES4” variant.

[0276] Further exemplary of this embodiment, there is provided [$T^{338}C$, $V^{323}E$]c-Src(251-10 533) (SEQ ID NO:20), and the corresponding protein having an N-terminal oligopeptide sequence resulting from processing of the recombinant protein (SEQ ID NO:21). These doubly substituted proteins are also known as the so-called c-Src “ES5” variant.

[0277] Further exemplary of this embodiment, there is provided [$T^{338}C$, $V^{323}H$]c-Src(251-15 533) (SEQ ID NO:22), and the corresponding protein having an N-terminal oligopeptide sequence resulting from processing of the recombinant protein (SEQ ID NO:23). These doubly substituted proteins are also known as the so-called c-Src “ES6” variant.

X. Methods

A. General

[0278] In some embodiments, the present invention provides methods of determining the 20 role of a kinase in a cell. In certain embodiments, the methods include determining the dependence of transformed cells on aberrant oncogenic signaling by the EGFR kinase. In other embodiments, the determining includes assaying inhibitor-induced conformational changes of kinases. In other embodiments, the methods include elucidating the mechanisms of inhibitor-induced Akt hyperphosphorylation. In some embodiments, the methods include 25 transactivation of RAF dimmers.

[0279] In some other embodiments, the present invention provides a chemical genetic approach based on engineered shape complementarity between the kinase active site and a small molecule inhibitor, which allows systematic discovery of an inhibitor for a particular kinase. In some embodiments, a conserved hydrophobic residue in the kinase active site 30 known as the “gatekeeper” is mutated to a small residue such as glycine or alanine to generate a uniquely targetable mutant kinase termed an analog-sensitive (*AS*) allele.

[0280] In certain other embodiments, the present invention provides methods of making engineered kinase which can be targeted with sterically bulky analogs of natural kinase inhibitors, which are capable of occupying the enlarged engineered kinase pocket (**Fig. 1**). In some embodiments, the methods include wild type kinases which may be resistant to inhibition by the bulky analog as the result of a steric clash with naturally occurring gatekeeper residues (*e.g.* Met, Leu, Phe, Thr, Gln and others). In yet other instances, the wild type kinases may not be resistant to inhibition by the bulky analog as the result of a steric clash with naturally occurring gatekeeper residues (*e.g.* Met, Leu, Phe, Thr, Gln and others).

B. Structure activity relationship (SAR) analysis

[0281] In order to determine the effects of modifying the group at this position, a structure activity relationship (SAR) analysis was performed on the pyrazolopyrimidine scaffold with a benzyl-linked *m*-vinylsulfonamide (compounds **9**, **15-17**; **Table 2**). This analysis revealed that secondary alkyl groups such as isopropyl (**9**) and cyclopentyl (**17**) moieties elicited optimal activity against T338C c-Src. Relative to isopropyl substitution, *tert*-butyl (**16**) and methyl (**15**) derivatization resulted in 4- and 21-fold drops in potency, respectively. Collectively, these results indicate that substitution at N1 can be used to modulate potency against T338C c-Src. In some instances, Michael acceptor-derivatized 4-anilinoquinazolines were synthesized and evaluated as inhibitors (compounds **18-20**; **Table 2**).

[0282] In some embodiments, the *ES* kinase alleles should be useful for a host of other applications. For example, fluorescently labeled versions of the inhibitors could be used to quantitatively probe the occupancy of kinase active sites to determine the percent activity required for signaling events. In some other embodiments, the present invention provides a method for determining the properties of pseudokinases, for which there is no good readout of active site occupancy. In certain embodiments, the present invention sets forth the use of irreversible inhibitors and allows for the validation of target specificity.

[0283] In some embodiments, the present invention provides methods of evaluating the reversibility of inhibition of a kinase as set forth herein. In some embodiments, an electrophilic inhibitors covalently interact with the cysteine gatekeeper. In one instance, two compounds, **9** and **13**, were assayed accordingly. Both compounds inhibited T338C c-Src in a time-dependent manner (**Table 3**).

[0284] In addition, when T338C c-Src was treated with either inhibitor and purified by gel filtration and the inhibitory activity against the kinase was retained. See Fig. 5. In contrast, in the case of WT c-Src, inhibitory activity was lost after gel filtration. Importantly, inhibition

by PP1, a reversible Src inhibitor, was abrogated in the cases of both WT and T338C c-Src following gel filtration (FIG. 5). Full protein mass spectrometry suggested specific labeling of T338C relative to WT c-Src for **9** (FIG. 2). However, under similar conditions, an adduct formation with **13** was not observed, possibly due to a reversible covalent interaction. The results suggest that covalent binding of the electrophilic inhibitors depend on the presence of a cysteine gatekeeper, *i.e.* T338C c-Src as electrophile-sensitive c-Src1 (c-Src-ES1).

[0285] Table 3 - Compounds **9** and **13** exhibit time-dependent inhibition against T338C c-Src. Compounds were preincubated with the enzyme prior to initializing the reaction with ATP.

Compound	Preincubation Time (min)		
	2	20	40
	T338C c-Src IC ₅₀ (nM)		
9	981	309	138
13	1281	318	136

[0286] Table 3 shows compounds **9** and **13** exhibit time-dependent inhibition against T338C c-Src. Compounds were preincubated with the enzyme prior to initializing the reaction with ATP.

XI. Methods of Inhibiting a Kinase

[0287] In some other embodiments, the present invention provides a method of imparting to a kinase the capability of being inhibited by a heterocyclic compound, comprising replacing a gatekeeper amino acid residue within an ATP binding site of a kinase with a cysteine residue thereby forming a cysteine substituted kinase.

[0288] In some embodiments, the present invention provides a method of inhibiting a recombinant kinase as set forth herein, comprising contacting the recombinant kinase with an effective amount of an inhibitor, thereby inhibiting the recombinant kinase. In some embodiments, the inhibitor is capable of forming a covalent bond to the cysteine at the gatekeeper amino acid position of the recombinant kinase. In some embodiments, the inhibitor is a compound as set forth herein, *e.g.* a compound of formulas I-XXIX. In some other embodiments, the method further comprises determining a level of inhibition for the recombinant kinase. In some embodiments, the determining of said level of inhibition for the recombinant kinase comprises: determining an amount of enzymatic activity of the recombinant kinase in the presence of the inhibitor; determining an amount of enzymatic activity of the recombinant kinase in the absence of the inhibitor; and comparing the amount

of enzymatic activity of the recombinant kinase in the presence of the inhibitor with the amount of enzymatic activity of the recombinant kinase in the absence of the inhibitor, thereby determining a level of inhibition for the recombinant kinase. In some embodiments, the enzymatic activity is selected from phosphorylation of a non-specific protein target, phosphorylation of a specific protein target, consumption of ATP, or cell growth.

[0289] In other embodiments, the present invention provides a method as set forth herein wherein the recombinant kinase is in a cell. In some embodiments, the methods set forth herein further comprise determining a function of the recombinant kinase in the cell, by: determining an amount of enzymatic activity of the recombinant kinase in the presence of the inhibitor in the cell; determining an amount of enzymatic activity of the recombinant kinase in the absence of the inhibitor in the cell; and comparing the amount of enzymatic activity of the recombinant kinase in the presence of the inhibitor with the amount of enzymatic activity of the recombinant kinase in the absence of the inhibitor, thereby determining a function of the recombinant kinase in the cell. In some embodiments, the enzymatic activity is selected from phosphorylation of a specific protein target.

[0290] In other embodiments, the methods as set forth herein include a recombinant kinase is selected from Src; MOK; Sgk494; Lrrk-2; Yak/Yrk; SRPK1; CDK; DICTY-I; PAK/STE20; or Ctrl/DPYK1. In some embodiments, the recombinant kinase is Src.

[0291] In some embodiments, the present invention provides a method of inhibiting a Lrrk-2 kinase, comprising contacting the Lrrk-2 kinase with an effective amount of a Lrrk-2 inhibitor, thereby inhibiting the recombinant Lrrk-2 kinase. A Lrrk-2 inhibitor is compound of Formula (XV) to (XXIX) including embodiments thereof.

XII. Methods of Treating

[0292] In some embodiments, the present invention provides a method of treating a kinase-associated disease or condition, in a patient in need thereof. The method includes administering to the patient a therapeutically effective amount of a compound provided herein, thereby treating a kinase-associated disease or condition. In some embodiments, the compound is a kinase inhibitor capable of forming a covalent bond to the cysteine at the gatekeeper amino acid position of the recombinant kinase. In some embodiments, the compound is a compound as set forth herein, *e.g.* a compound of formulas (I) – (XXIX) including embodiments thereof. In certain embodiments, the kinase-associated disease or condition is selective from cancer, immunological disorders, neurological disorders, neurodegenerative disorders, infections, metabolic diseases, *Leishmania major*, zoonotic

cutaneous leishmaniasis, *Plasmodium falciparum*, malaria, *Trichomonas vaginalis*, and trichomiasis. In certain other embodiments, the cancer is selected from neoplasm or malignant tumors found in mammals; leukemia; carcinomas and sarcomas; cancer of the brain, breast, cervix, colon, head and neck, liver, kidney, lung, non-small cell lung, ovary, testicle, stomach, uterus; melanoma; mesothelioma; Medulloblastoma; Hodgkin's Disease, Non-Hodgkin's Lymphoma; multiple myeloma; neuroblastoma; rhabdomyosarcoma; primary thrombocytosis; primary macroglobulinemia; primary brain tumors; malignant pancreatic insulanoma; malignant carcinoid; urinary bladder cancer; premalignant skin lesions; lymphomas; thyroid cancer; neuroblastoma; esophageal cancer; genitourinary tract cancer; malignant hypercalcemia; endometrial cancer; adrenal cortical cancer; neoplasms of the endocrine and exocrine pancreas; or prostate cancer. In yet other embodiments, the disease or condition is a neurodegenerative disease selective from Parkinson's disease.

[0293] In some other embodiments, the present invention also provides a method of treating a Lrrk-2-associated disease or condition, in a patient in need thereof, said method comprising administering to said patient a therapeutically effective amount of a Lrrk-2 inhibitor, thereby treating a Lrrk-2-associated disease or condition. In some embodiments, the disease or condition is a neurodegenerative disease selected from Parkinson's Disease.

[0294] In some embodiments, the methods further include the step of allowing the cell to express the recombinant kinase.

20 XIII. Tables Relevant to the Methods and Assays Herein

[0295] Table 4 – Relative k_{cat}/K_m for a series of c-Src variants

c-Src Variant	rel. k_{cat}/K_m
ES1	1.00 ± 0.09
ES2	0.39 ± 0.03
ES3	0.21 ± 0.04
ES4	N.D.
ES5	N.D.
ES6	N.D.

[0296] Table 5a-5c show relative catalytic efficiency for T338C c-Src with second-site mutations (ES1 = T338C; ES2 = T338C/V323A; ES3 = T338C/V323S, ES4 = T338C/V323D; ES5 = T338C/V323E; ES6 = T338C/V323H). Data were fitted to the Michaelis-Menten equation and standard errors of the fits are reported. Data are unitless.

[0297] Table 5 shows kinome-wide screening of a panel of inhibitors. Compounds were screened using the **SelectScreen**TM platform developed by Life Technologies. Z'lyte (a, measures kinase activity), Adapta (b, measures kinase activity) and Lantha assays (c, measures ATP binding) were performed. Inhibition data are represented in a heat map format.

[0298] Table 6 shows comparison of selectivity of **13**, 1NA-PP1 and 1NM-PP1. All kinases for which > 40 % inhibition was observed in a kinome wide Z'lyte screen (Life Technologies) are shown. Legend for Tables 5-6: <40% inhibition (gray); 40%-80% inhibition (white); ≥ 80% inhibition (diagonal stripes).

Table 5a.

<u>Conc</u>			1000 nM	1000 nM	1000 nM	1000 nM	1000 nM
<u>Compound</u>			3	4	9	13	20
ABL1	Activity	Km app	7	5	4	2	1
ABL1 E255K	Activity	Km app	14	8	5	3	8
ABL1 G250E	Activity	Km app	3	1	0	-3	1
ABL1 T315I	Activity	Km app	-1	3	4	-2	4
ABL1 Y253F	Activity	Km app	17	16	12	10	12
ABL2 (Arg)	Activity	Km app	17	15	11	5	8
ACVR1B (ALK4)	Activity	Km app	13	8	5	2	-1
ADRBK1 (GRK2)	Activity	Km app	23	22	21	18	13
ADRBK2 (GRK3)	Activity	Km app	0	0	1	-1	0
AKT1 (PKB alpha)	Activity	Km app	-4	-1	0	0	-4
AKT2 (PKB beta)	Activity	Km app	5	3	4	5	3
AKT3 (PKB gamma)	Activity	Km app	-1	2	5	5	1
ALK	Activity	Km app	5	7	2	0	1
AMPK A1/B1/G1	Activity	Km app	14	20	29	28	14
AMPK A2/B1/G1	Activity	Km app	12	14	20	19	7
AURKA (Aurora A)	Activity	Km app	7	4	9	3	12
AURKB (Aurora B)	Activity	Km app	9	9	9	5	27
AURKC (Aurora C)	Activity	Km app	8	8	7	6	25
AXL	Activity	Km app	9	6	1	1	9
BLK	Activity	Km app	48	34	20	10	42
BMX	Activity	Km app	82	85	68	9	20
BRAF	Activity	100	35	5	7	4	6
BRAF V599E	Activity	100	81	13	17	21	9
BRSK1 (SAD1)	Activity	Km app	11	14	13	9	17
BTK	Activity	Km app	44	36	42	1	17
CAMK1D (CaMKI delta)	Activity	Km app	21	22	18	20	16
CAMK2A (CaMKII alpha)	Activity	Km app	-2	1	2	5	1
CAMK2B (CaMKII beta)	Activity	Km app	6	6	11	1	8
CAMK2D (CaMKII delta)	Activity	Km app	16	15	8	10	8
CAMK4 (CaMKIV)	Activity	Km app	9	8	10	11	7
CDC42 BPA (MRCKA)	Activity	Km app	12	16	22	24	20
CDC42 BPB (MRCKB)	Activity	Km app	1	-1	-3	-7	-1
CDK1/cyclin B	Activity	Km app	10	2	7	4	1
CDK2/cyclin A	Activity	Km app	9	5	16	11	0
CDK5/p25	Activity	Km app	13	9	21	12	9
CDK5/p35	Activity	Km app	18	8	24	7	5

<u>Conc</u>			1000 nM	1000 nM	1000 nM	1000 nM	1000 nM
<u>Compound</u>			3	4	9	13	20
CHEK1 (CHK1)	Activity	Km app	11	-3	-5	-7	-11
CHEK2 (CHK2)	Activity	Km app	0	-3	2	2	17
CLK1	Activity	Km app	8	7	12	7	7
CLK2	Activity	Km app	0	-1	0	-1	-3
CLK3	Activity	Km app	6	8	7	6	7
CSF1R (FMS)	Activity	Km app	7	10	7	4	5
CSK	Activity	Km app	22	9	9	8	12
CSNK1A1 (CK1 alpha 1)	Activity	Km app	20	12	6	14	0
CSNK1D (CK1 delta)	Activity	Km app	9	5	8	15	4
CSNK1E (CK1 epsilon)	Activity	Km app	18	6	10	40	7
CSNK1G1 (CK1 gamma 1)	Activity	Km app	3	4	4	1	15
CSNK1G2 (CK1 gamma 2)	Activity	Km app	7	7	8	3	36
CSNK1G3 (CK1 gamma 3)	Activity	Km app	6	11	10	9	31
CSNK2A1 (CK2 alpha 1)	Activity	Km app	17	18	5	14	12
CSNK2A2 (CK2 alpha 2)	Activity	Km app	6	-2	-1	1	-5
DAPK3 (ZIPK)	Activity	Km app	1	2	3	0	3
DCAMKL2 (DCK2)	Activity	Km app	7	5	6	2	8
DNA-PK	Activity	Km app	68	27	15	12	12
DYRK1A	Activity	Km app	-1	-1	1	-5	-3
DYRK1B	Activity	Km app	-1	2	2	-1	2
DYRK3	Activity	Km app	3	2	23	0	4
DYRK4	Activity	Km app	1	3	3	3	2
EEF2K	Activity	Km app	5	7	8	7	5
EGFR (ErbB1)	Activity	Km app	50	26	8	-5	90
EGFR (ErbB1) L858R	Activity	Km app	61	36	23	1	71
EGFR (ErbB1) L861Q	Activity	Km app	87	46	18	1	90
EGFR (ErbB1) T790M	Activity	Km app	36	27	12	4	12
EGFR (ErbB1) T790M L858R	Activity	Km app	64	39	19	10	23
EPHA1	Activity	Km app	33	14	14	15	11
EPHA2	Activity	Km app	7	5	6	4	5
EPHA4	Activity	Km app	19	8	8	7	9
EPHA5	Activity	Km app	22	11	11	5	7
EPHA8	Activity	Km app	23	7	9	9	6
EPHB1	Activity	Km app	12	6	6	5	7
EPHB2	Activity	Km app	21	12	15	14	12
EPHB3	Activity	Km app	40	10	18	6	8
EPHB4	Activity	Km app	14	11	11	6	9
ERBB2 (HER2)	Activity	Km app	40	23	19	16	55
ERBB4 (HER4)	Activity	Km app	70	92	15	5	66
FER	Activity	Km app	13	9	7	7	10
FES (FPS)	Activity	Km app	8	10	2	12	11
FGFR1	Activity	Km app	24	53	22	15	1
FGFR2	Activity	Km app	18	3	4	3	8
FGFR3	Activity	Km app	20	10	4	6	9
FGFR3 K650E	Activity	Km app	28	2	6	-2	9
FGFR4	Activity	Km app	17	8	5	6	-1
FGR	Activity	Km app	79	16	20	6	30
FLT1 (VEGFR1)	Activity	Km app	3	1	2	-2	2
FLT3	Activity	Km app	29	0	6	-7	31
FLT3 D835Y	Activity	Km app	34	16	42	8	15
FLT4 (VEGFR3)	Activity	Km app	28	33	13	9	41
FRAP1 (mTOR)	Activity	Km app	24	-2	-8	-8	-10
FRK (PTK5)	Activity	Km app	25	9	8	5	9
FYN	Activity	Km app	17	9	10	6	-3
GRK4	Activity	Km app	10	10	5	-2	0
GRK5	Activity	Km app	1	-3	-3	-3	2
GRK6	Activity	Km app	15	16	10	7	5

<u>Conc</u>			1000 nM	1000 nM	1000 nM	1000 nM	1000 nM
<u>Compound</u>			3	4	9	13	20
GRK7	Activity	Km app	-1	0	-1	-3	0
GSK3A (GSK3 alpha)	Activity	Km app	3	5	5	-3	-3
GSK3B (GSK3 beta)	Activity	Km app	3	0	3	-5	-3
HCK	Activity	Km app	17	4	6	5	11
HIPK1 (Myak)	Activity	Km app	8	5	4	4	4
HIPK2	Activity	Km app	11	6	4	4	4
HIPK3 (YAK1)	Activity	Km app	5	6	4	3	5
HIPK4	Activity	Km app	6	6	9	4	7
IGF1R	Activity	Km app	5	4	0	-7	2
IKBKB (IKK beta)	Activity	Km app	16	17	18	16	12
IKBKE (IKK epsilon)	Activity	Km app	16	20	15	14	10
INSR	Activity	Km app	-2	2	3	2	0
INSRR (IRR)	Activity	Km app	9	10	9	9	11
IRAK4	Activity	Km app	4	1	2	-6	-2
ITK	Activity	Km app	3	-6	-4	-4	-7
JAK1	Activity	Km app	4	8	14	16	11
JAK2	Activity	Km app	7	5	5	4	2
JAK2 JH1 JH2	Activity	Km app	1	-1	-6	-4	-1
JAK2 JH1 JH2 V617F	Activity	Km app	2	1	-3	-4	-2
JAK3	Activity	Km app	23	72	14	10	12
KDR (VEGFR2)	Activity	Km app	6	-14	-17	-18	16
KIT	Activity	Km app	17	19	12	7	6
KIT T670I	Activity	Km app	10	7	8	7	3
LCK	Activity	Km app	48	-1	-13	-18	11
LTK (TYK1)	Activity	Km app	1	0	-4	-5	1
LYN A	Activity	Km app	34	9	12	8	28
LYN B	Activity	Km app	39	16	15	14	30
MAP2K1 (MEK1)	Activity	100	31	7	3	5	0
MAP2K2 (MEK2)	Activity	100	49	12	11	10	7
MAP2K6 (MKK6)	Activity	100	5	8	11	13	16
MAP3K8 (COT)	Activity	100	33	2	0	0	0
MAP3K9 (MLK1)	Activity	Km app	8	5	4	1	21
MAP4K2 (GCK)	Activity	Km app	-16	2	7	11	3
MAP4K4 (HGK)	Activity	Km app	15	14	16	18	21
MAP4K5 (KHS1)	Activity	Km app	22	17	48	20	36
MAPK1 (ERK2)	Activity	Km app	3	6	3	2	2
MAPK10 (JNK3)	Activity	100	6	13	2	7	10
MAPK11 (p38 beta)	Activity	Km app	9	11	9	10	9
MAPK12 (p38 gamma)	Activity	Km app	9	13	10	9	14
MAPK13 (p38 delta)	Activity	Km app	2	6	6	5	6
MAPK14 (p38 alpha)	Activity	100	21	20	22	24	22
MAPK14 (p38 alpha) Direct	Activity	Km app	-1	4	9	12	10
MAPK3 (ERK1)	Activity	Km app	15	31	13	10	14
MAPK8 (JNK1)	Activity	100	17	21	24	21	19
MAPK9 (JNK2)	Activity	100	5	7	8	8	7
MAPKAPK2	Activity	Km app	1	2	4	6	5
MAPKAPK3	Activity	Km app	5	6	5	3	5
MAPKAPK5 (PRAK)	Activity	Km app	-4	-1	4	7	7
MARK1 (MARK)	Activity	Km app	-1	0	2	2	-4
MARK2	Activity	Km app	1	2	4	5	1
MARK3	Activity	Km app	6	8	6	2	5
MARK4	Activity	Km app	2	4	3	-1	-1
MATK (HYL)	Activity	Km app	3	5	6	5	4
MELK	Activity	Km app	16	28	29	15	32
MERTK (cMER)	Activity	Km app	9	5	2	4	8
MET (cMet)	Activity	Km app	-4	27	10	8	0
MET M1250T	Activity	Km app	7	7	4	2	6

<u>Conc</u>			1000 nM	1000 nM	1000 nM	1000 nM	1000 nM
<u>Compound</u>			3	4	9	13	20
MINK1	Activity	Km app	31	17	23	19	22
MKNK1 (MKNK1)	Activity	Km app	5	0	-4	-5	16
MST1R (RON)	Activity	Km app	13	11	9	5	9
MST4	Activity	Km app	8	13	21	7	30
MUSK	Activity	Km app	15	12	11	18	1
MYLK2 (skMLCK)	Activity	Km app	2	2	5	0	2
NEK1	Activity	Km app	14	-5	7	4	10
NEK2	Activity	Km app	-1	-4	-10	0	-8
NEK4	Activity	Km app	8	10	17	19	15
NEK6	Activity	Km app	4	5	10	12	4
NEK7	Activity	Km app	7	8	8	8	6
NEK9	Activity	Km app	15	12	13	12	12
NTRK1 (TRKA)	Activity	Km app	37	-3	21	17	48
NTRK2 (TRKB)	Activity	Km app	16	4	23	12	-1
NTRK3 (TRKC)	Activity	Km app	16	-5	18	-6	-8
PAK1	Activity	Km app	12	12	10	13	16
PAK2 (PAK65)	Activity	Km app	18	12	12	13	10
PAK3	Activity	Km app	4	3	6	6	2
PAK4	Activity	Km app	-5	-8	-7	-6	-5
PAK6	Activity	Km app	7	10	10	8	5
PAK7 (KIAA1264)	Activity	Km app	9	13	13	0	11
PASK	Activity	Km app	11	9	8	7	7
PDGFRA (PDGFR alpha)	Activity	Km app	13	6	5	4	16
PDGFRA D842V	Activity	Km app	5	3	0	3	9
PDGFRA T674I	Activity	Km app	11	11	-2	4	11
PDGFRA V561D	Activity	Km app	19	12	5	1	27
PDGFRB (PDGFR beta)	Activity	Km app	16	10	5	5	11
PDK1	Activity	100	5	11	11	11	11
PDK1 Direct	Activity	Km app	1	-1	3	0	-5
PHKG1	Activity	Km app	8	8	8	10	5
PHKG2	Activity	Km app	1	6	4	-1	7
PIM1	Activity	Km app	16	12	11	12	10
PIM2	Activity	Km app	3	1	2	-1	-3
PKN1 (PRK1)	Activity	Km app	15	17	18	12	9
PLK1	Activity	Km app	-7	-3	-6	-3	4
PLK2	Activity	Km app	13	8	7	1	7
PLK3	Activity	Km app	2	4	0	-2	3
PRKACA (PKA)	Activity	Km app	-1	0	-1	-4	-2
PRKCA (PKC alpha)	Activity	Km app	8	15	19	17	18
PRKCB1 (PKC beta I)	Activity	Km app	-1	11	10	8	5
PRKCB2 (PKC beta II)	Activity	Km app	9	13	7	4	4
PRKCD (PKC delta)	Activity	Km app	14	18	20	16	17
PRKCE (PKC epsilon)	Activity	Km app	12	16	21	19	10
PRKCG (PKC gamma)	Activity	Km app	18	13	22	19	14
PRKCH (PKC eta)	Activity	Km app	28	6	25	24	10
PRKCI (PKC iota)	Activity	Km app	10	13	13	13	10
PRKCN (PKD3)	Activity	Km app	8	7	12	16	11
PRKCQ (PKC theta)	Activity	Km app	9	11	13	13	12
PRKCZ (PKC zeta)	Activity	Km app	0	5	5	8	7
PRKD1 (PKC mu)	Activity	Km app	8	10	17	18	10
PRKD2 (PKD2)	Activity	Km app	11	11	12	19	16
PRKG1	Activity	Km app	1	2	3	-1	4
PRKG2 (PKG2)	Activity	Km app	0	0	-1	0	0
PRKX	Activity	Km app	3	5	3	1	5
PTK2 (FAK)	Activity	Km app	7	7	8	7	6
PTK2B (FAK2)	Activity	Km app	5	3	2	1	1
PTK6 (Brk)	Activity	Km app	68	-3	30	27	74

<u>Conc</u>			1000 nM	1000 nM	1000 nM	1000 nM	1000 nM
<u>Compound</u>			3	4	9	13	20
RAF1 (cRAF) Y340D Y341D	Activity	100	39	7	7	7	18
RET	Activity	Km app	41	11	12	8	40
RET V804L	Activity	Km app	4	3	6	2	13
RET Y791F	Activity	Km app	44	9	10	8	41
ROCK1	Activity	Km app	2	0	0	-3	-1
ROCK2	Activity	Km app	18	21	19	14	24
ROS1	Activity	Km app	7	23	5	2	5
RPS6KA1 (RSK1)	Activity	Km app	1	1	1	-2	5
RPS6KA2 (RSK3)	Activity	Km app	2	8	3	0	12
RPS6KA3 (RSK2)	Activity	Km app	4	4	4	4	4
RPS6KA4 (MSK2)	Activity	Km app	11	7	8	6	6
RPS6KA5 (MSK1)	Activity	Km app	7	8	6	3	7
RPS6KA6 (RSK4)	Activity	Km app	24	21	19	5	53
RPS6KB1 (p70S6K)	Activity	Km app	8	8	14	13	10
SGK (SGK1)	Activity	Km app	10	4	6	-1	2
SGK2	Activity	Km app	9	10	7	5	11
SGKL (SGK3)	Activity	Km app	5	5	5	3	6
SNF1LK2	Activity	Km app	3	5	2	1	3
SRC	Activity	Km app	40	3	1	-6	20
SRC N1	Activity	Km app	53	8	13	4	19
SRMS (Srm)	Activity	Km app	94	19	11	10	13
SRPK1	Activity	Km app	2	3	4	4	2
SRPK2	Activity	Km app	13	13	11	12	9
STK22B (TSSK2)	Activity	Km app	5	3	0	0	6
STK22D (TSSK1)	Activity	Km app	3	6	10	12	11
STK23 (MSSK1)	Activity	Km app	7	7	9	6	8
STK24 (MST3)	Activity	Km app	13	13	9	10	13
STK25 (YSK1)	Activity	Km app	10	8	10	0	9
STK3 (MST2)	Activity	Km app	-7	-9	-8	-9	-13
STK4 (MST1)	Activity	Km app	12	5	3	2	3
SYK	Activity	Km app	-7	-6	-6	-8	-2
TAOK2 (TAO1)	Activity	Km app	6	4	6	2	2
TBK1	Activity	Km app	5	-2	0	-11	-5
TEK (Tie2)	Activity	Km app	-3	-9	-8	-7	-10
TXK	Activity	Km app	80	66	93	7	62
TYK2	Activity	Km app	-2	-2	-2	-5	-5
TYRO3 (RSE)	Activity	Km app	20	13	10	8	14
YES1	Activity	Km app	64	15	17	8	43
ZAP70	Activity	Km app	12	13	10	11	10

Table 5b

<u>Conc</u>			1000 nM	1000 nM	1000 nM	1000 nM	1000 nM
<u>Compound</u>			3	4	9	13	20
CAMK1 (CaMK1)	Activity	100	6	11	4	8	-20
CDK7/cyclin H/MNAT1	Activity	Km app	-7	-1	20	-10	10
CDK9/cyclin T1	Activity	Km app	23	14	-2	9	13
CHUK (IKK alpha)	Activity	Km app	1	5	12	6	2
DAPK1	Activity	Km app	-1	5	9	5	5
GSG2 (Haspin)	Activity	Km app	21	6	13	12	9
IRAK1	Activity	Km app	0	8	15	-14	13
LRRK2	Activity	Km app	7	7	0	-2	21

LRRK2 G2019S	Activity	Km app	-4	-13	-17	-7	-5
NUAK1 (ARK5)	Activity	Km app	9	15	22	14	12
PI4KA (PI4K alpha)	Activity	10	-12	-6	0	2	9
PI4KB (PI4K beta)	Activity	Km app	37	13	29	13	9
PIK3C2A (PI3K-C2 alpha)	Activity	Km app	-3	9	3	-4	8
PIK3C2B (PI3K-C2 beta)	Activity	100	26	2	3	-1	13
PIK3C3 (hVPS34)	Activity	Km app	-6	3	-1	-5	0
PIK3CA/PIK3R1 (p110 alpha/p85 alpha)	Activity	Km app	51	-1	7	2	9
PIK3CD/PIK3R1 (p110 delta/p85 alpha)	Activity	Km app	66	7	18	-1	4
PIK3CG (p110 gamma)	Activity	Km app	48	11	-3	-2	5
SPHK1	Activity	Km app	-16	-2	3	8	7
SPHK2	Activity	100	9	-4	-7	-14	4

Table 5c.

<u>Conc</u>		1000 nM	1000 nM	1000 nM	1000 nM	1000 nM
<u>Compound</u>		3	4	9	13	20
ACVR1 (ALK2)	Binding	83	10	12	3	5
ACVR2B	Binding	20	19	4	8	-11
BMPR1A (ALK3)	Binding	37	5	4	4	12
CAMKK1 (CAMKKA)	Binding	1	2	1	-10	0
CAMKK2 (CaMKK beta)	Binding	8	8	5	-1	5
CDK8/cyclin C	Binding	23	27	20	9	4
CDK9/cyclin K	Binding	11	3	10	6	6
CLK4	Binding	35	7	19	7	5
DDR1	Binding	1	1	-3	0	2
DDR2	Binding	7	2	-1	4	5
DMPK	Binding	5	3	3	1	10
EPHA3	Binding	2	8	-3	5	-3
EPHA7	Binding	-3	-5	-1	1	10
KIT V654A	Binding	12	5	1	1	8
LIMK1	Binding	21	4	2	-6	-4
LIMK2	Binding	14	12	8	9	10
MAP2K1 (MEK1) S218D S222D	Binding	98	4	5	1	2
MAP2K3 (MEK3)	Binding	12	18	9	0	24
MAP2K6 (MKK6) S207E T211E	Binding	64	3	4	6	1
MAP3K10 (MLK2)	Binding	20	3	1	2	9
MAP3K11 (MLK3)	Binding	5	2	4	-2	9
MAP3K14 (NIK)	Binding	-6	-13	-9	-12	-9
MAP3K2 (MEKK2)	Binding	13	0	8	1	6
MAP3K3 (MEKK3)	Binding	13	7	10	8	10
MAP3K5 (ASK1)	Binding	-15	-11	-4	-8	4
MAP3K7/MAP3K7IP1 (TAK1-TAB1)	Binding	2	2	2	-2	6
MKNK2 (MNK2)	Binding	19	2	2	2	13
MLCK (MLCK2)	Binding	1	9	10	6	14
MYLK (MLCK)	Binding	3	1	0	0	0
NLK	Binding	56	3	-1	-4	-10
RIPK2	Binding	77	6	4	2	8
SLK	Binding	4	4	4	5	5
STK16 (PKL12)	Binding	2	-3	6	1	3
STK17A (DRAK1)	Binding	1	0	-2	7	2
STK33	Binding	3	7	-5	-6	5

TAOK3 (JIK)	Binding	1	-5	-2	-3	4
TEC	Binding	15	3	14	14	5
TGFBR1 (ALK5)	Binding	65	7	10	7	3
TNK2 (ACK)	Binding	11	3	5	4	10
TTK	Binding	30	12	22	16	25
WEE1	Binding	20	1	1	-2	-14
WNK2	Binding	55	9	7	9	15
ZAK	Binding	75	27	33	4	8

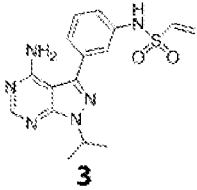
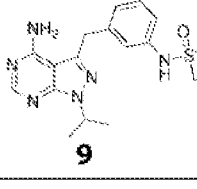
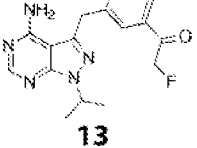
[0299] Table 6 Comparison of selectivity of 13, 1NA-PP1 and 1NM-PP1. All kinases for which > 40 % inhibition was observed in a kinome wide Z'lyte screen (Life Technologies) are shown.

Table 6.

Kinase tested	13	1NA-PP1	1NM-PP1
BMX	9	26	52
BTK	1	49	61
CSF1R (FMS)	4	40	28
CSNK1E (CK1 epsilon)	40	91	96
EGFR (ErbB1) L858R	1	40	29
EGFR (ErbB1) T790M	4	48	38
EGFR (ErbB1) T790M L858R	10	65	53
EPHA1	15	93	83
EPHA2	4	75	43
EPHA4	7	83	43
EPHA5	5	86	56
EPHA8	9	67	51
EPHB1	5	63	44
EPHB2	14	78	58
EPHB3	6	48	35
EPHB4	6	73	62
FGR	6	71	42
FRK (PTK5)	5	89	47
FYN	6	45	37
HCK	5	59	31
LCK	-18	52	35
LYN A	8	82	55
LYN B	14	81	55
MAP4K4 (HGK)	18	92	56
MAP4K5 (KHS1)	20	69	31
MINK!	19	89	52
PRKACA (PKA)	-4	12	41
PRKCN (PKD3)	16	69	65
PRKD1 (PKC mu)	18	1	56
PRKD2 (PKD2)	19	59	83
PRKGI	-1	75	0
PTK6 (Brk)	27	98	55
RET	8	79	65
RET Y791F	8	84	70
SRC	-6	65	27
SRMS (Srm)	10	61	7

YES1	8	54	42
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[0300] **Table 7.** IC₅₀ values of a panel of electrophilic inhibitors against c-Src-ES variants with second-site mutations (ES1 = T338C; ES2 = T338C/V323A; ES3 = T338C/V323S).

Compound	c-Src variant IC ₅₀ (nM)		
	ES1	ES2	ES3
 3	111	63	131
 9	150	207	424
 13	338	67	29

5

XIV. Kit

[0301] In some other embodiments, the present invention provides a kit comprising, a recombinant kinase described herein (see section (V) above) or a nucleic acid described herein (See section IX) and instructions for using the kit. The instructions for using the kit describe the steps set forth in a method provided herein (see section X, XI and XII).

10

[0302] In some embodiments, the present invention provides a kit for testing for inhibition of kinase activity comprising a heterocyclic compound, wherein the heterocyclic compound comprises two or more fused rings and an electrophilic substituent, wherein at least one of the two or more fused rings comprises a nitrogen atom, and a cysteine substituted kinase wherein a gatekeeper amino acid residue within an ATP binding site of the kinase is replaced with a cysteine residue.

15

XV. Examples

[0303] **Chemical synthesis.** Reactions were performed in flame dried flasks under argon with magnetic stirring. All ¹H and ¹³C NMR spectra were recorded on a Varian Innova 400 spectrometer and referenced to solvent peaks. ¹H chemical shifts are reported in δ (ppm) as

20

s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) or br (broad). Low resolution mass spectra (LC/ESI-MS) were recorded on a Waters Micromass ZQ equipped with a Waters 2695 Separations Module and a XTerra MS C18 3.5 mm column (Waters). RP-HPLC was performed on a Varian ProStar solvent delivery system equipped with a Zorbax 300-SB C18 column using CH₃CN/H₂O/0.1%TFA (1-100% gradient) and monitoring at 260 nm.

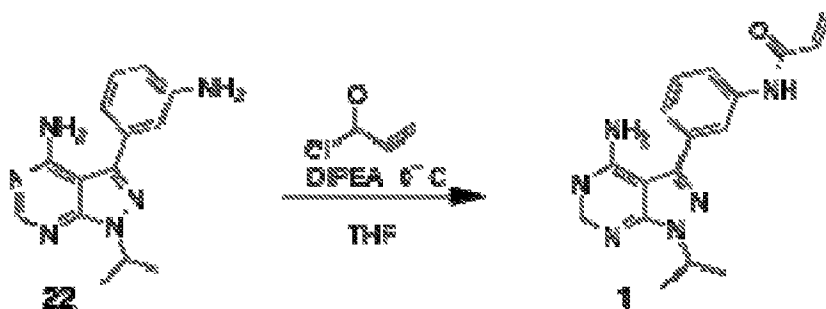
Example 1- Preparation of 3-(3-aminophenyl)-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (22).

[0304] This compound was prepared in a similar procedure to that used for (18).

Example 2 – Preparation of 3-(4-aminophenyl)-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (23).

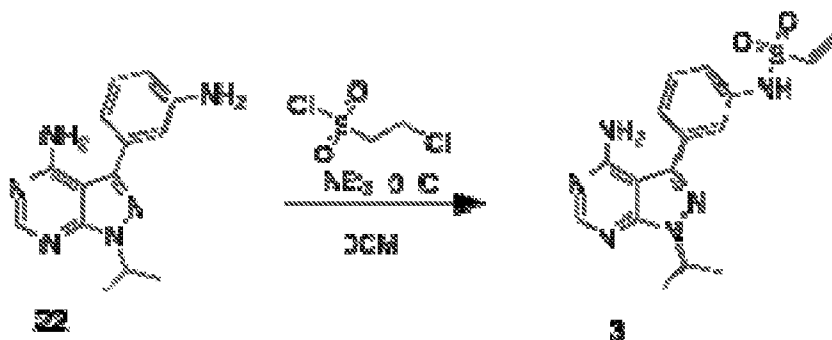
[0305] This compound was prepared in a similar procedure to that used for (18).

Example 3 – Preparation of N-(3-(4-amino-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)phenyl)acrylamide (1)



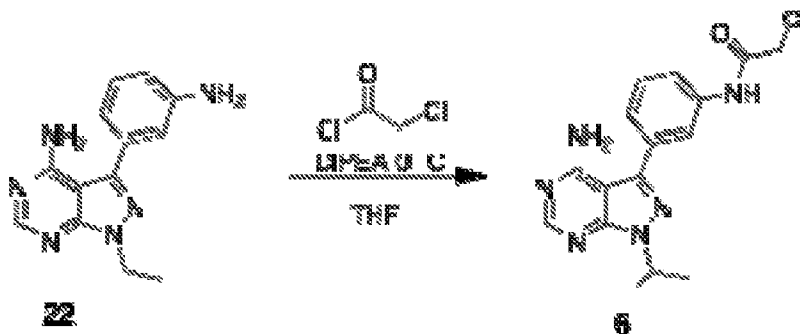
[0306] A solution of tetrahydrofuran (20 mL), compound **22** (219 mg, 0.817 mmol) and diisopropylethylamine (156 μ L, 0.895 mmol) was cooled to 0° C. Acryloyl chloride (67 μ L, 0.828 mmol) was added and the reaction was allowed to proceed for 1 hour and afterwards concentrated *in vacuo*. The residue was dissolved in dichloromethane (20 mL) and washed with saturated sodium bicarbonate (20 mL). The aqueous layer was extracted with dichloromethane (2 x 20 mL). The combined organic layers were dried with MgSO₄, filtered and concentrated *in vacuo*. The product was purified by preparative RP-HPLC and lyophilized (70 mg, 26 % yield): ¹H NMR (400 MHz, DMSO) δ 10.40 (s, 1H), 8.38 (s, 1H), 8.04 (s, 1H), 7.73 (d, *J* = 8.0, 1H), 7.52 (t, *J* = 7.9, 1H), 7.39 (d, *J* = 7.7, 1H), 6.47 (dd, *J* = 17.0, 10.1, 1H), 6.29 (dd, *J* = 17.0, 2.0, 1H), 5.80 (dd, *J* = 10.1, 2.0, 1H), 5.11 (hept, *J* = 6.6, 1H), 1.51 (d, *J* = 6.7, 6H); ¹³C NMR (100 MHz, DMSO) δ 163.47, 154.87, 151.90, 150.94, 144.73, 139.53, 132.54, 131.68, 129.88, 127.31, 123.42, 119.96, 119.22, 96.91, 48.80, 21.74; [M+H]⁺ calculated for C₁₇H₁₈N₆O 323.1, found 323.5.

Example 4 – Preparation of N-(3-(4-amino-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)phenyl)ethanesulfonamide (3)



- 5 [0307] A solution of dichloromethane (2 mL), compound **22** (20 mg, 0.075 mmol) and triethylamine (11 μ L, 0.079 mmol) was cooled to 0° C. 2-chloro-1-ethane sulfonyl chloride (7 μ L, 0.067 mmol) was added and the reaction was allowed to proceed for 1 hour prior to addition of saturated sodium bicarbonate (10 mL) and extraction with dichloromethane (3 x 10 mL). The combined organic layers were dried with MgSO_4 , filtered and concentrated *in vacuo*. The product was purified by preparative RP-HPLC and lyophilized (5.6 mg, 23 %
- 10 yield): ^1H NMR (400 MHz, DMSO) δ 10.22 (s, 1H), 8.31 (s, 1H), 7.49 (t, $J = 7.9$, 1H), 7.45 (s, 1H), 7.38 (d, $J = 7.8$, 1H), 7.27 (d, $J = 7.3$, 1H), 6.86 (dd, $J = 16.4$, 10.0, 1H), 6.17 (d, $J = 16.4$, 1H), 6.08 (d, $J = 9.9$, 1H), 5.08 (hept, $J = 6.7$, 1H), 1.50 (d, $J = 6.7$, 6H).; ^{13}C NMR (100 MHz, DMSO) δ 155.01, 151.96, 151.19, 144.29, 138.50, 136.32, 133.09, 130.22,
- 15 127.79, 123.73, 120.09, 119.20, 96.94, 48.85, 21.73; $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{16}\text{H}_{18}\text{N}_6\text{O}_2\text{S}$ 359.1, found 359.4.

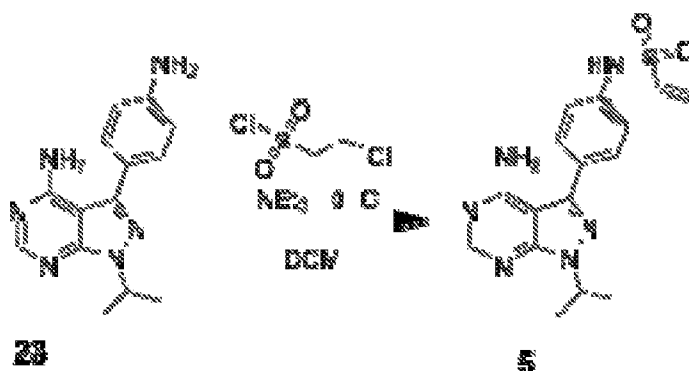
Example 5 – Preparation of N-(3-(4-amino-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)phenyl)-2-chloroacetamide (6)



- 20 [0308] A solution of THF (20 mL), compound **22** (200 mg, 0.75 mmol) and DIPEA (143 μ L, 0.821 mmol) was cooled to 0° C. Chloroacetyl chloride (54 μ L, 0.67 mmol) was added and the reaction was allowed to proceed for 1 hour and afterwards concentrated *in vacuo*. The residue was dissolved in dichloromethane (20 mL) and washed with saturated sodium

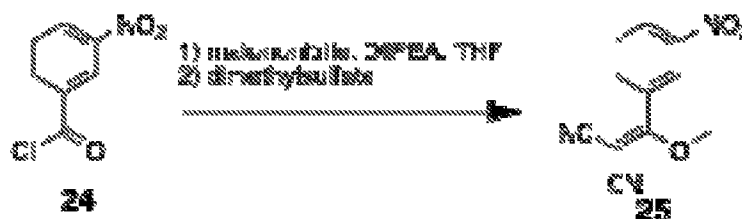
bicarbonate (20 mL). The aqueous layer was extracted with dichloromethane (2 x 20 mL). The combined organic layers were dried with MgSO_4 , filtered and concentrated *in vacuo*. The product was purified by preparative RP-HPLC and lyophilized (30.4 mg, 13 % yield): ^1H NMR (400 MHz, DMSO) δ 10.57 (s, 1H), 8.41 (s, 1H), 7.95 (s, 1H), 7.67 (d, $J = 7.6$, 1H), 7.53 (t, $J = 7.9$, 1H), 7.41 (d, $J = 7.7$, 1H), 5.11 (hept, $J = 6.7$, 1H), 4.30 (s, 2H), 1.51 (d, $J = 6.7$, 6H); ^{13}C NMR (100 MHz, DMSO) δ 165.06, 154.82, 151.89, 150.90, 144.68, 139.03, 132.62, 128.28, 123.81, 120.02, 119.24, 96.92, 48.84, 43.55, 21.77; $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{16}\text{H}_{17}\text{ClN}_6\text{O}$ 345.1, found 345.4.

Example 6 – Preparation of N-(4-(4-amino-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)phenyl)ethanesulfonamide (5)



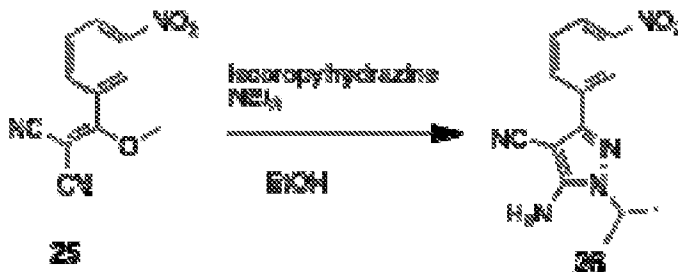
[0309] A solution of dichloromethane (5 mL), compound 23 (45 mg, 0.168 mmol) and triethylamine (71 μL , 0.509 mmol) was cooled to 0°C . 2-chloro-1-ethanesulfonyl chloride (16 μL , 0.148 mmol) was added and the reaction was allowed to proceed for 1 hour prior to addition of saturated sodium bicarbonate (10 mL) and extraction with dichloromethane (2 x 10 mL). The combined organic layers were dried with MgSO_4 , filtered and concentrated *in vacuo*. The product was purified by preparative RP-HPLC and lyophilized (8.7 mg, 16 % yield): ^1H NMR (400 MHz, DMSO) δ 10.30 (s, 1H), 8.36 (s, 1H), 7.60 (d, $J = 8.5$, 2H), 7.31 (d, $J = 8.6$, 2H), 6.86 (dd, $J = 16.4$, 9.9, 1H), 6.21 (d, $J = 16.4$, 1H), 6.09 (d, $J = 9.9$, 1H), 5.08 (hept, $J = 6.7$, 2H), 1.49 (d, $J = 6.7$, 6H); ^{13}C NMR (100 MHz, DMSO) δ 155.44, 152.03, 151.64, 144.22, 138.54, 136.17, 129.25, 127.98, 127.41, 119.52, 96.95, 48.66, 21.75; $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{16}\text{H}_{18}\text{N}_6\text{O}_2\text{S}$ 359.1, found 359.5.

Example 7 – Preparation of 2-(methoxy(3-nitrophenyl)methylene)malononitrile (25)



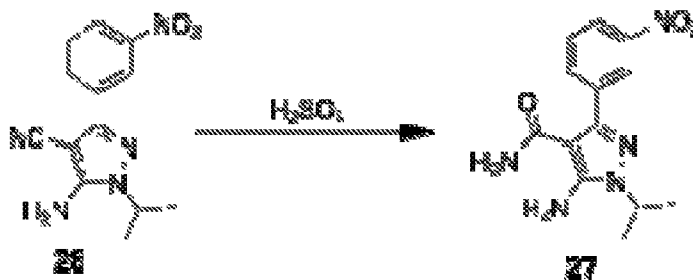
[0310] A solution of 3-nitrobenzoylchloride (25 g, 134 mmol), malononitrile (9.74 g, 147 mmol) and THF (140 mL) was cooled to 0° C. DIPEA (59 mL, 335 mmol) was added dropwise and the reaction was allowed to warm to room temperature and proceed for 2 hours. Afterwards, dimethylsulfate (38 mL, 399 mmol) was added and the temperature was raised to 70° C for 4 hours. Next, the reaction mixture was brought to room temperature and allowed to proceed for an additional 12 hours. EtOAc (200 mL) was added to the reaction mixture in addition to brine (200 mL). The organic and aqueous layers were separated and the aqueous layer was extracted with EtOAc (4 x 25 mL). The combined organic layers were dried with MgSO₄ and concentrated *in vacuo*. The material was purified over a silica column using chloroform: hexane (90:10) initially and eluted with pure chloroform. After concentrating the fractions containing the product, a yellow oil was triturated with diethyl ether to yield a solid (11.3 g, 37 % yield): ¹H NMR (400 MHz, DMSO) δ 8.63 (s, 1H), 8.51 (d, *J* = 7.7, 1H), 8.16 (d, *J* = 7.7, 1H), 7.94 (t, *J* = 7.8, 1H), 3.93 (s, 3H); ¹³C NMR (100 MHz, DMSO) δ 183.66, 147.82, 135.23, 131.00, 129.55, 127.15, 124.08, 113.31, 111.87, 66.71, 61.87; [M+H]⁺ calculated for C₁₁H₇N₃O₃ 228.0, found 214.10 (product appears to hydrolyze during LC/MS analysis).

Example 8 – 5-amino-1-isopropyl-3-(3-nitrophenyl)-1H-pyrazole-4-carbonitrile (26)



[0311] Compound **25** (5g, 21.8 mmol), isopropylhydrazine hydrochloride (2.41 g, 21.8 mmol) (purchased from Ryan Scientific) and triethylamine (6.40 mL, 46.0 mmol) were allowed to react in ethanol (145 mL) at room temperature for 1 hr. After concentrating the reaction mixture, it was purified by silica chromatography using a chloroform/ methanol solvent system (methanol gradient increased with time from 0- 10%). The relevant fractions were concentrated *in vacuo* to yield a yellow powder (4.65 g, 79 % yield): ¹H NMR (400 MHz, DMSO) δ 8.59 (s, 1H), 8.24 (m, 2H), 7.78 (t, *J* = 8.2, 1H), 6.82 (br, 2H), 4.53 (hept, *J* = 6.5, 1H), 1.36 (d, *J* = 6.5, 6H); ¹³C NMR (100 MHz, DMSO) δ 153.04, 148.76, 146.98, 133.97, 132.22, 131.19, 123.80, 120.37, 116.28, 70.74, 48.64, 22.02; [M+H]⁺ calculated for C₁₃H₁₃N₅O₂ 272.1, found 272.3.

Example 9 – Preparation of 5-amino-1-isopropyl-3-(3-nitrophenyl)-1H-pyrazole-4-carboxamide (27)



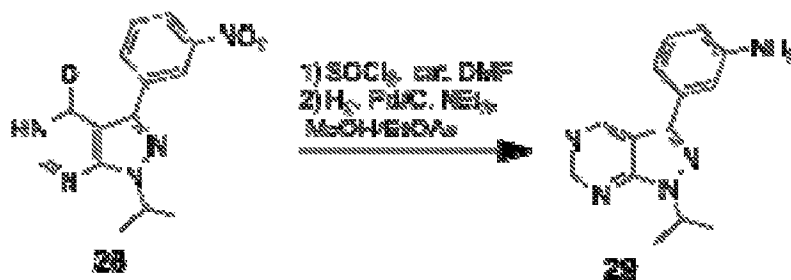
[0312] Compound 26 (100 mg, 0.369 mmol) was added to concentrated sulfuric acid (1 mL) and heated to 65° C for 3 hours. Afterwards, the reaction mixture was poured into ice water and the pH was brought to 14 with 10 M NaOH. The aqueous material was extracted several times with dichloromethane. The combined organic layers were dried over MgSO_4 , filtered and concentrated to a solid (91 mg, 85 % yield): ^1H NMR (400 MHz, DMSO) δ 8.32 (s, 1H), 8.21 (d, $J = 8.2$, 1H), 7.98 (d, $J = 7.7$, 1H), 7.69 (t, $J = 8.0$, 1H), 6.20 (br, 2H), 4.51 (hept, 1H), 1.35 (d, $J = 6.5$, 6H); ^{13}C NMR (100 MHz, DMSO) δ 166.17, 149.37, 147.65, 145.50, 135.63, 134.92, 129.69, 122.79, 122.43, 95.43, 47.11, 21.50; $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{13}\text{H}_{15}\text{N}_5\text{O}_3$ 290.1, found 290.0.

Example 10 – Preparation of 1-isopropyl-3-(3-nitrophenyl)-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (28)



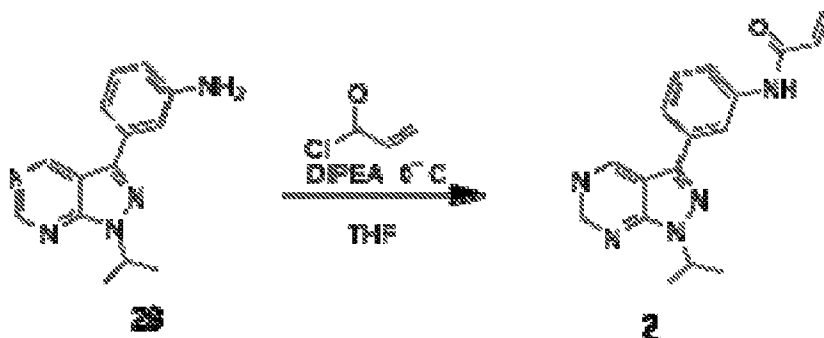
[0313] Compound 27 (1 g, 3.46 mmol) was added to formamide (1.167 mL, 29.3 mmol) and heated to 160° C for 40 hours. Afterwards, the reaction mixture was allowed to cool to room temperature and diluted into ice cold water. The mixture was filtered and a solid was collected (943 mg, 91 % yield): ^1H NMR (400 MHz, DMSO) δ 12.37 (s, 1H), 9.32 (s, 1H), 8.81 (d, $J = 7.8$, 1H), 8.23 (d, $J = 8.1$, 1H), 8.13 (s, 1H), 7.75 (t, $J = 8.0$, 1H), 5.06 (hept, $J = 6.6$, 1H), 1.52 (d, $J = 6.7$, 6H); ^{13}C NMR (100 MHz, DMSO) δ 157.77, 152.48, 148.16, 148.06, 143.85, 133.58, 129.95, 123.05, 122.07, 103.02, 49.07, 21.74; $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{14}\text{H}_{13}\text{N}_5\text{O}_3$ 300.1, found 300.0.

Example 11 – Preparation of 3-(1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)aniline (28)



[0314] Compound **28** (2 g, 6.68 mmol) was mixed with thionyl chloride (12.5 mL, 171 mmol) and ten drops of DMF and heated to 80° C for forty minutes. Afterwards, the reaction mixture was poured onto 300 mL of ice and the pH was adjusted to 8 with saturated sodium carbonate. The solution was extracted with dichloromethane (3 x 150 mL). The combined organic layers were dried over MgSO₄ and concentrated *in vacuo* (2.04 g, yield 96 %). The resulting solid (1 g, 3.34 mmol) was dissolved in MeOH/EtOAc (30 mL/ 20 mL) and reacted with 10 % Pd/C (540 mg) and triethylamine (466 µL, 3.34 mmol) under a H₂ atmosphere for 24 hours. The reaction mixture was filtered over celite and concentrated. The material was resuspended in dichloromethane (100 mL), which was washed with saturated sodium bicarbonate (2 x 100 mL) prior to drying with MgSO₄ and concentrating *in vacuo*. The resulting solid was purified over silica using a dichloromethane/methanol (0 -5%) solvent system (487 mg, 58 % yield): ¹H NMR (400 MHz, DMSO) δ 9.59 (s, 1H), 9.02 (s, 1H), 7.34 (s, 1H), 7.23 (d, *J* = 7.6, 1H), 7.18 (t, *J* = 7.7, 1H), 6.66 (d, *J* = 7.7, 1H), 5.30 (br, 2H), 5.21 (hept, *J* = 6.7, 1H), 1.56 (d, *J* = 6.7, 6H); ¹³C NMR (100 MHz, DMSO) δ 154.60, 153.03, 151.87, 149.28, 143.17, 132.14, 129.59, 114.65, 114.32, 112.06, 111.85, 48.53, 21.72; [M+H]⁺ calculated for C₁₄H₁₅N₅ 254.1, found 254.0.

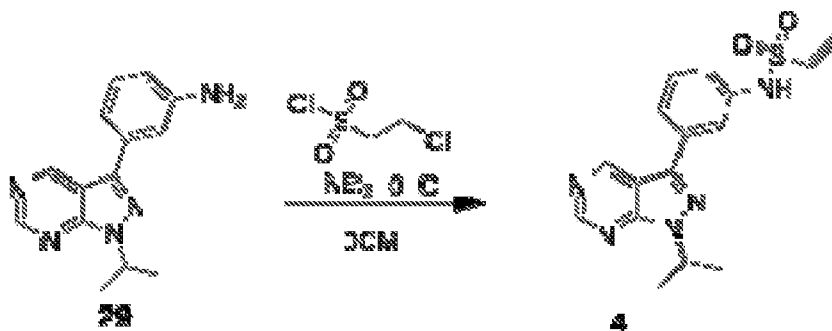
Example 12 – Preparation of N-(3-(1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)phenyl)acrylamide (2)



[0315] Compound **2** was prepared by the same procedure that was used for compound **1** (49 % yield): ¹H NMR (400 MHz, DMSO) δ 10.36 (s, 1H), 9.66 (s, 1H), 9.08 (s, 1H), 8.41 (t, *J* = 1.8, 1H), 7.84 (m, 2H), 7.52 (t, *J* = 7.9, 1H), 6.48 (dd, *J* = 17.0, 10.0, 1H), 6.32 (dd, *J* =

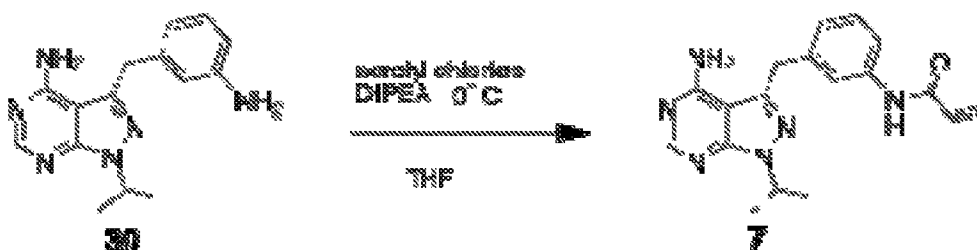
17.0, 2.0, 1H), 5.81 (dd, $J = 10.0, 2.0$, 1H), 5.24 (hept, $J = 6.6$, 1H), 1.58 (d, $J = 6.7$, 6H); ^{13}C NMR (100 MHz, DMSO) δ 163.39, 154.53, 152.74, 151.97, 142.36, 139.75, 132.03, 131.77, 129.75, 127.24, 121.84, 119.79, 117.31, 111.98, 48.81, 21.73; $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{17}\text{H}_{17}\text{N}_5\text{O}$ 308.1, found 308.6.

5 **Example 13 – Preparation of N-(3-(1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)phenyl)ethanesulfonamide (4)**



[0316] Compound 4 was prepared by the same procedure that was used for compound 3 (18 % yield): ^1H NMR (400 MHz, DMSO) δ 10.17 (s, 1H), 9.61 (s, 1H), 9.08 (s, 1H), 7.90 (t, $J = 1.9$, 1H), 7.82 (d, $J = 7.7$, 1H), 7.49 (t, $J = 7.9$, 1H), 7.29 (ddd, $J = 8.2, 2.2, 0.9$, 1H), 6.87 (dd, $J = 16.4, 9.9$, 1H), 6.17 (d, $J = 16.4$, 1H), 6.07 (d, $J = 9.9$, 1H), 5.23 (hept, $J = 6.7$, 1H), 1.57 (d, $J = 6.7$, 6H); ^{13}C NMR (100 MHz, DMSO) δ 154.56, 152.69, 151.97, 141.98, 138.59, 136.20, 132.46, 130.16, 127.98, 122.09, 119.78, 117.64, 111.94, 48.89, 21.71; $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{16}\text{H}_{17}\text{N}_5\text{O}_2\text{S}$ 344.11, found 344.2.

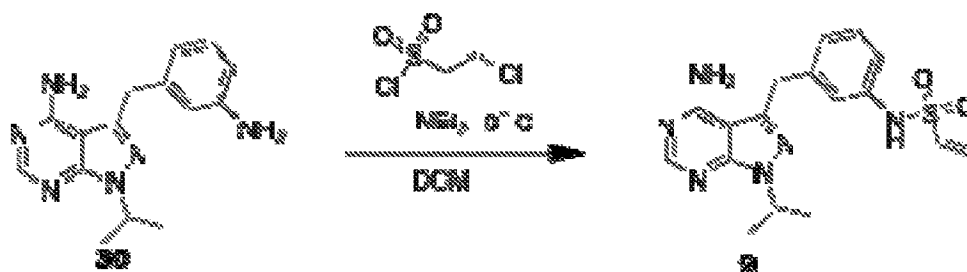
15 **Example 14 – Preparation of N-(3-((4-amino-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)methyl)phenyl)acrylamide (7)**



[0317] A solution of THF (20 mL), compound 30 (200 mg, 0.708 mmol) (prepared as in Dar et al. {Dar, 2008 #18}) and N,N -diisopropylethylamine (136 μL , 0.781 mmol) was cooled 0°C , at which point freshly distilled acryloyl chloride (52 μL , 0.642 mmol) was added. After one hour, the reaction mixture was concentrated *in vacuo*. The material was resuspended in dichloromethane (20 mL), which was washed with saturated sodium bicarbonate (20 mL). The aqueous layer was extracted with dichloromethane (3 x 20 mL) and the organic layers were subsequently combined, dried over MgSO_4 , filtered and concentrated to a solid. The material was purified by RP-HPLC and lyophilized to a white

powder (113 mg, 47 % yield): ^1H NMR (400 MHz, DMSO) δ 10.06 (s, 1H), 8.33 (s, 1H), 7.54 (s, 1H), 7.50 (d, $J = 8.1$, 1H), 7.24 (t, $J = 7.9$, 1H), 6.96 (d, $J = 7.7$, 1H), 6.40 (dd, $J = 17.0$, 10.1, 1H), 6.22 (dd, $J = 17.0$, 2.0, 1H), 5.72 (dd, $J = 10.1$, 1.9, 1H), 4.99 (hept, $J = 6.5$, 1H), 4.40 (s, 2H), 1.46 (d, $J = 6.7$, 6 H); ^{13}C NMR (100 MHz, DMSO) δ 163.06, 153.94, 151.30, 149.68, 144.69, 139.16, 139.08, 131.86, 128.82, 126.78, 123.59, 119.34, 117.51, 97.90, 48.74, 33.03, 21.70; $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{18}\text{H}_{20}\text{N}_6\text{O}$ 337.2, found 337.4.

Example 15 – Preparation of N-(3-((4-amino-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)methyl)phenyl)ethanesulfonamide (9)

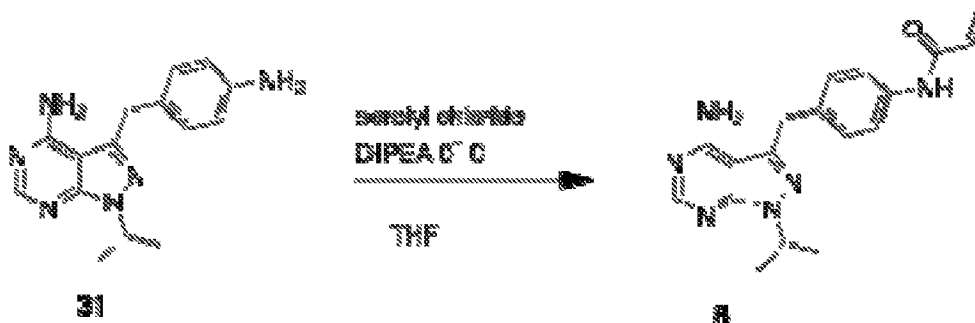


10 [0318] A solution of dichloromethane (20 mL), compound **30** (190 mg, 0.673 mmol) and triethylamine (188 μL , 1.35 mmol) was cooled to 0°C . 2-chloro-1-ethanesulfonyl chloride (70 μL , 0.670 mmol) was added and the reaction was allowed to proceed for 1 hour prior to addition of saturated sodium bicarbonate (20 mL) and extraction with dichloromethane (3 x 20 mL). The combined organic layers were dried with MgSO_4 , filtered and concentrated *in vacuo*. The product was purified by preparative RP-HPLC and lyophilized (14 mg, 6 %

15 yield): ^1H NMR (400 MHz, DMSO) δ 9.95 (s, 1H), 8.31 (s, 1H), 7.21 (t, $J = 8.1$, 1H), 6.97 (m, 3H), 6.69 (dd, $J = 16.4$, 9.9, 1H), 5.97 (d, $J = 16.5$, 1H), 5.93 (d, $J = 9.9$, 1H), 5.00 (hept, $J = 6.7$, 1H), 4.38 (s, 2H), 1.46 (d, $J = 6.7$, 6H); ^{13}C NMR (100 MHz, DMSO) δ 154.31, 151.46, 150.23, 144.31, 137.91, 136.26, 136.16, 129.25, 127.42, 123.79, 119.26, 117.52,

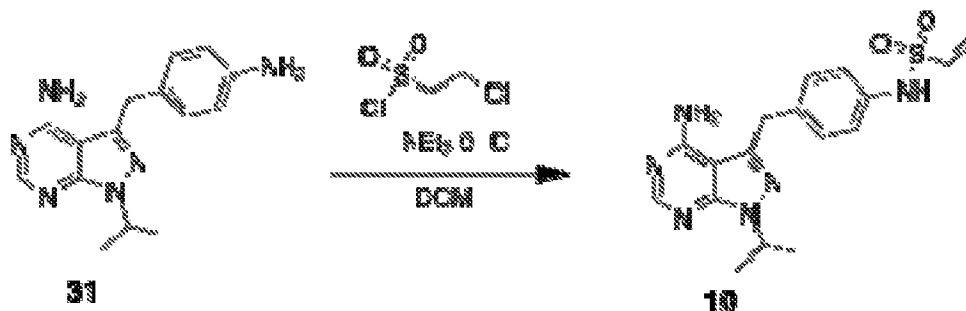
20 97.91, 48.59, 32.81, 21.70; $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{17}\text{H}_{20}\text{N}_6\text{O}_2\text{S}$ 373.1, found 373.4.

Example 16 – Preparation of N-(4-((4-amino-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)methyl)phenyl)acrylamide (8)



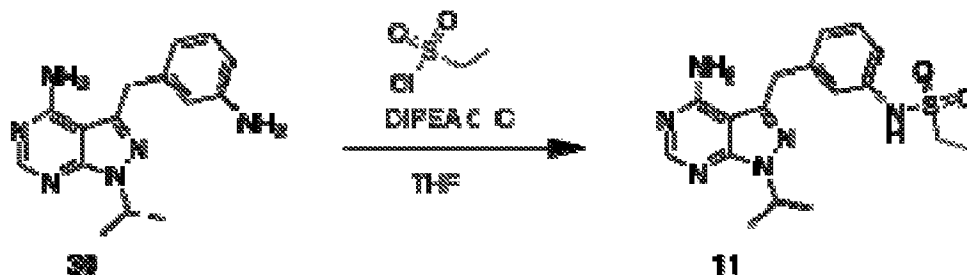
[0319] A solution of THF (20 mL), compound **31** (200 mg, 0.708 mmol) (prepared as in Dar et al. {Dar, 2008 #18}) and *N,N*-diisopropylethylamine (136 μ L, 0.781 mmol) was cooled 0° C, at which point freshly distilled acryloyl chloride (52 μ L, 0.642 mmol) was added. After one hour, the reaction mixture was concentrated *in vacuo*. The material was resuspended in dichloromethane (20 mL), which was washed with saturated sodium bicarbonate (20 mL). The aqueous layer was extracted with dichloromethane (3 x 20 mL) and the organic layers were subsequently combined, dried over MgSO₄, filtered and concentrated to a solid. The material was purified by RP-HPLC and lyophilized to a white powder (78 mg, 30 % yield): ¹H NMR (400 MHz, DMSO) δ 10.09 (s, 1H), 8.31 (s, 1H), 7.57 (d, *J* = 8.5, 2H), 7.18 (d, *J* = 8.5, 2H), 6.41 (dd, *J* = 17.0, 10.1, 1H), 6.22 (dd, *J* = 17.0, 2.1, 1H), 5.73 (dd, *J* = 10.1, 2.1, 1H), 5.00 (hept, *J* = 6.7, 1H), 4.36 (s, 3H), 1.44 (d, *J* = 6.7, 6H); ¹³C NMR (100 MHz, DMSO) δ 163.0, 153.9, 151.3, 149.6, 145.1, 137.4, 133.4, 131.9, 128.7, 126.7, 119.5, 97.8, 48.7, 32.5, 21.6; [M+H]⁺ calculated for C₁₈H₂₀N₆O 337.2, found 337.4.

Example 17 – Preparation of N-(4-((4-amino-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)methyl)phenyl)ethanesulfonamide (10)



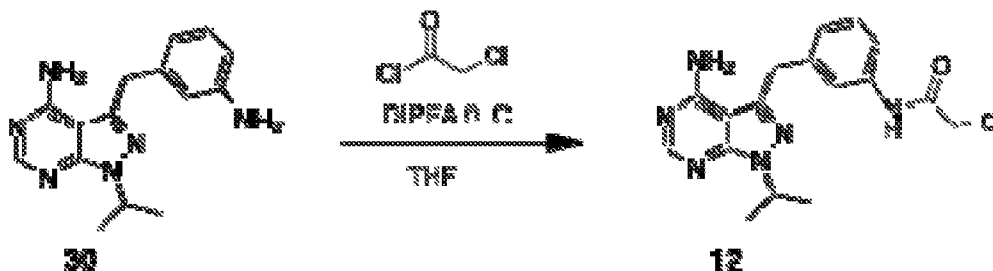
[0320] A solution of dichloromethane (20 mL), compound **31** (200 mg, 0.708 mmol) and triethylamine (200 μ L, 1.43 mmol) was cooled to 0° C. 2-chloro-1-ethanesulfonyl chloride (70 μ L, 0.670 mmol) was added and the reaction was allowed to proceed for 1 hour prior to addition of saturated sodium bicarbonate (20 mL) and extraction with dichloromethane (3 x 20 mL). The combined organic layers were dried with MgSO₄, filtered and concentrated *in vacuo*. The product was purified by preparative RP-HPLC and lyophilized (14 mg, 6 % yield): ¹H NMR (400 MHz, DMSO) δ 9.91 (s, 1H), 8.30 (s, 1H), 7.16 (d, *J* = 8.3, 2H), 7.05 (d, *J* = 8.4, 2H), 6.74 (dd, *J* = 16.4, 9.9, 1H), 6.07 (d, *J* = 16.4, 1H), 6.00 (d, *J* = 10.0, 1H), 5.03 – 4.94 (m, 1H), 4.34 (s, 2H), 1.44 (d, *J* = 6.6, 6H); ¹³C NMR (100 MHz, DMSO) δ 154.0, 151.4, 149.8, 144.7, 136.3, 136.0, 133.9, 129.3, 127.5, 120.0, 97.9, 48.6, 32.3, 21.7; [M+H]⁺ calculated for C₁₇H₂₀N₆O₂S 373.1, found 373.4.

Example 18 – Preparation of N-(3-((4-amino-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)methyl)phenyl)ethanesulfonamide (11)



- 5 [0321] A solution of tetrahydrofuran (5 mL), compound **30** (50 mg, 0.177 mmol) and diisopropylethylamine (34 μ L, 0.195 mmol) was cooled to 0° C. Ethanesulfonylchloride (15 μ L, 0.159 mmol) was added and the reaction was allowed to proceed for one hour. After one hour, the reaction mixture was concentrated *in vacuo*. The material was resuspended in dichloromethane (10 mL), which was washed with saturated sodium bicarbonate (10 mL).
- 10 The aqueous layer was extracted with dichloromethane (3 x 10 mL) and the organic layers were subsequently combined, dried over MgSO₄, filtered and concentrated to a solid. The combined organic layers were dried with MgSO₄, filtered and concentrated *in vacuo*. The product was purified by preparative RP-HPLC and lyophilized (25 mg, 38 % yield): ¹H NMR (400 MHz, DMSO) δ 9.72 (s, 1H), 8.34 (s, 1H), 7.23 (t, *J* = 7.8, 1H), 7.09 (s, 1H), 7.03 (d, *J* = 7.9, 1H), 6.98 (d, *J* = 7.6, 1H), 5.01 (hept, *J* = 6.7, 1H), 4.40 (s, 2H), 3.01 (q, *J* = 7.3, 2H),
- 15 1.46 (d, *J* = 6.7, 6H), 1.13 (t, *J* = 7.3, 3H); ¹³C NMR (100 MHz, DMSO) δ 154.11, 151.38, 150.04, 144.45, 139.67, 138.54, 129.28, 123.66, 119.19, 117.45, 97.89, 48.57, 44.89, 32.84, 21.68, 7.90; [M+H]⁺ calculated for C₁₇H₂₂N₆O₂S 375.1, found 375.6.

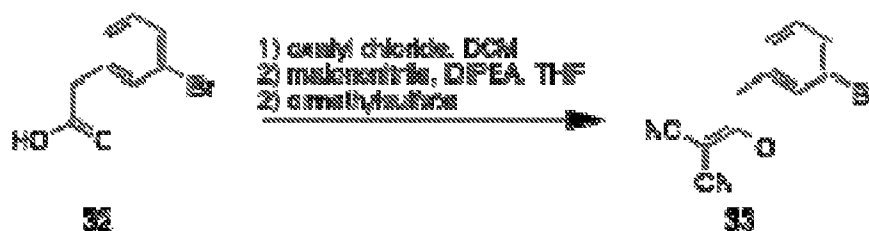
Example 19 – Preparation of N-(3-((4-amino-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)methyl)phenyl)-2-chloroacetamide (12)



- 20 [0322] A solution of tetrahydrofuran (10 mL), compound **30** (100 mg, 0.354 mmol) and diisopropylethylamine (68 μ L, 0.390 mmol) was cooled to 0° C. Chloroacetylchloride (25.4 μ L, 0.313 mmol) was added and the reaction was allowed to proceed for one hour. After one
- 25 hour, the reaction mixture was concentrated *in vacuo*. The material was resuspended in dichloromethane (10 mL), which was washed with saturated sodium bicarbonate (10 mL).

The aqueous layer was extracted with dichloromethane (3 x 10 mL) and the organic layers were subsequently combined, dried over MgSO₄, filtered and concentrated to a solid. The combined organic layers were dried with MgSO₄, filtered and concentrated *in vacuo*. The product was purified by preparative RP-HPLC and lyophilized (35 mg, 31 % yield): ¹H NMR (400 MHz, DMSO) δ 10.22 (s, 1H), 8.32 (s, 1H), 7.46 (s, 1H), 7.41 (d, *J* = 8.2, 1H), 7.25 (t, *J* = 7.8, 1H), 6.99 (d, *J* = 7.6, 1H), 5.00 (hept, *J* = 6.7, 1H), 4.40 (s, 2H), 4.20 (s, 2H), 1.46 (d, *J* = 6.7, 6H); ¹³C NMR (100 MHz, DMSO) δ 164.5, 154.0, 151.3, 150.0, 144.6, 139.2, 138.6, 128.9, 124.0, 119.3, 117.5, 97.9, 48.7, 43.6, 33.0, 21.7; [M+H]⁺ calculated for C₁₇H₁₉ClN₆O 359.1, found 359.2.

10 Example 20 – Preparation of 2-(methoxy(3-bromobenzyl)methylene)malononitrile (33)



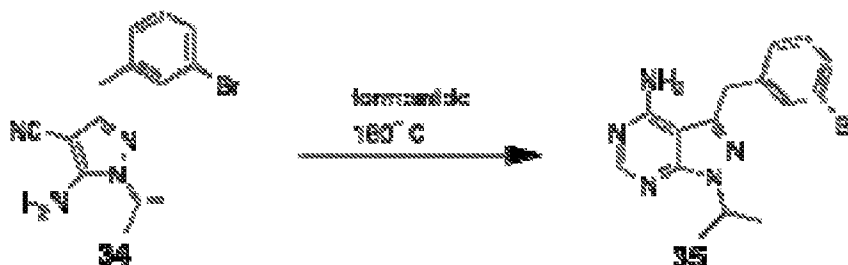
[0323] A solution of 3-bromoacetyl acetic acid (5 g, 23.3 mmol) was mixed with oxalyl chloride (10 mL, 121 mmol) in dichloromethane (75 mL) at room temperature for 30 minutes and then concentrated *in vacuo*. To the resulting solid was added malononitrile (1.69 g, 25.6 mmol) and THF (25 mL). After cooling to 0° C, DIPEA (10.1 mL, 58.1 mmol) was added dropwise and the reaction was allowed to warm to room temperature and proceed for 2 hours. Afterwards, dimethylsulfate (6.60 mL, 69.3 mmol) was added and the temperature was raised to 70° C for 4 hours. Next, the reaction mixture was brought to room temperature and allowed to proceed for an additional 12 hours. EtOAc (50 mL) was added to the reaction mixture in addition to brine (50 mL). The organic and aqueous layers were separated and the aqueous layer was extracted with EtOAc (4 x 25 mL). The combined organic layers were dried with MgSO₄ and concentrated *in vacuo*. The material was purified over a silica column using chloroform: hexane (90:10) initially and eluted with pure chloroform. After concentrating the fractions containing the product, an amber oil was triturated with diethyl ether to yield a solid (2.057 g, 32 % yield): ¹H NMR (400 MHz, DMSO) δ 7.56 (m, 2H), 7.38 (t, *J* = 7.7, 1H), 7.31 (d, *J* = 7.7, 1H), 4.19 (s, 2H), 4.01 (s, 3H); ¹³C NMR (100 MHz, DMSO) δ 187.96, 135.22, 131.33, 131.18, 130.77, 127.18, 122.32, 113.75, 112.03, 65.62, 60.00, 35.60; [M+H]⁺ calculated for C₁₂H₉BrN₂O 274.9, 276.9 (~50:50), found 274.8, 276.5.

Example 21 – Preparation of 5-amino-3-(3-bromobenzyl)-1-isopropyl-1H-pyrazole-4-carbonitrile (34)



[0324] Compound **33** (3.124 g, 11.3 mmol), isopropylhydrazine hydrochloride (1.27 g, 11.5 mmol) (purchased from Ryan Scientific) and triethylamine (6.40 mL, 46.0 mmol) were allowed to react in ethanol (75 mL) at room temperature for 1 hr. After concentrating the reaction mixture, it was purified by silica chromatography using a chloroform/ methanol solvent system (methanol gradient increased with time from 0- 10%). The relevant fractions were concentrated *in vacuo* to yield a yellow powder (3.15 g, 87 % yield): ¹H NMR (400 MHz, DMSO) δ 7.41 (m, 2H), 7.26 (td, *J* = 7.7, 1.2, 1H), 7.21 (dt, *J* = 7.7, 1.3, 1H), 6.50 (s, 2H), 4.38 (hept, *J* = 6.5, 1H), 3.81 (s, 2H), 1.27 (d, *J* = 6.5, 6H); ¹³C NMR (100 MHz, DMSO) δ 151.01, 150.15, 141.24, 131.05, 130.53, 129.16, 127.45, 121.55, 115.16, 71.77, 47.24, 32.88, 21.35; [M+H]⁺ calculated for C₁₄H₁₅BrN₄ 319.0, 321.0 found 318.9: 321.0.

Example 22 – Preparation of 3-(3-bromobenzyl)-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (35)



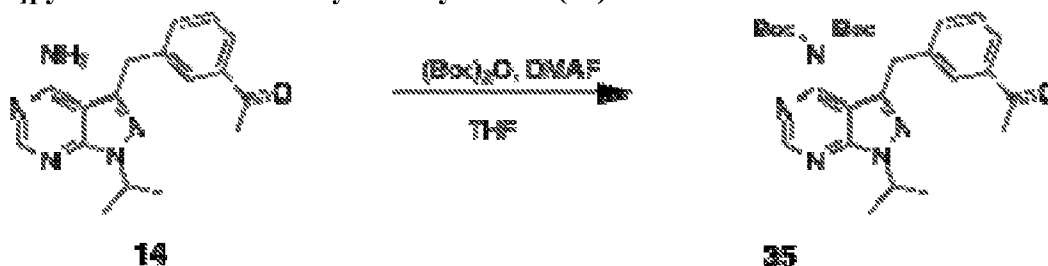
[0325] Compound **34** (6.09 g, 19.1 mmol) was added to formamide (26.6 mL, 668 mmol) and heated to 160° C for 27 hours. Afterwards, the reaction mixture was allowed to cool to room temperature and diluted into ice cold water (50 mL). A viscous material was filtered and dissolved in EtOAc. This solution was washed with brine and concentrated *in vacuo* (6.23 g, 91 % yield): ¹H NMR (400 MHz, DMSO) δ 8.13 (s, 1H), 7.49 (s, 1H), 7.37 (dt, *J* = 7.5, 1.7, 1H), 7.22 (m, 2H), 4.95 (hept, *J* = 6.6, 1H), 4.38 (s, 2H), 1.43 (d, *J* = 6.7, 6H); ¹³C NMR (100 MHz, DMSO) δ 157.90, 155.39, 153.22, 142.01, 141.86, 131.16, 130.52, 129.00, 127.40, 121.56, 98.39, 47.76, 32.66, 21.71; [M+H]⁺ calculated for C₁₅H₁₆BrN₅ 346.0, 348.0 found 346.0: 348.0.

Example 23 – Preparation of 3-(3-acetylbenzyl)-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (14)



[0326] Anhydrous toluene (10 mL) was degassed prior to addition of **34** (3.5 g, 10.1 mmol)
 5 Tributyl(1-ethoxyvinyl)tin (4.081 mL, 12.1 mmol), tetrakis(triphenylphosphine) palladium
 (1.169 g, 10 mol %) and heating to 120° C. After 16 hours, the reaction mixture was
 concentrated *in vacuo*. Next, a THF/ 1M HCl solution (33 mL/ 10mL) was added to the
 brownish material and the reaction was allowed to proceed at room temperature for 12 hours.
 Afterwards, EtOAc (175 mL) was added to the mixture, which was washed with saturated
 10 sodium bicarbonate (700 mL) and extracted with 1 M HCl (2 x 525 mL). The pH was
 adjusted to a value of 13 and the mixture was extracted with EtOAc (2 x 525 mL). The
 organic layers were dried with sodium sulfate and concentrated *in vacuo*. The material was
 purified by silica chromatography using a chloroform/ methanol solvent system (methanol
 gradient increased with time from 0- 8 %). The relevant fractions were concentrated *in vacuo*
 15 to yield a solid (2.035 g, 65 % yield): ¹H NMR (400 MHz, DMSO) δ 8.13 (s, 1H), 7.91 (s,
 1H), 7.79 (d, *J* = 7.5, 1H), 7.48 (d, *J* = 7.7, 1H), 7.43 (t, *J* = 7.6, 1H), 4.97 (hept, *J* = 6.7, 1H),
 4.46 (s, 2H), 2.53 (s, 3H), 1.44 (d, *J* = 6.7, 6H); ¹³C NMR (100 MHz, DMSO) δ 197.80,
 157.96, 155.40, 153.24, 142.22, 139.80, 136.84, 133.15, 128.75, 128.10, 126.32, 98.40,
 47.71, 32.94, 26.70, 21.77; [M+H]⁺ calculated for C₁₇H₁₉N₅O 310.1, found 310.0.

Example 24 – Preparation of 3-(3-acetylbenzyl)-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-4- di-*t*-butoxycarbonyl amine (14)

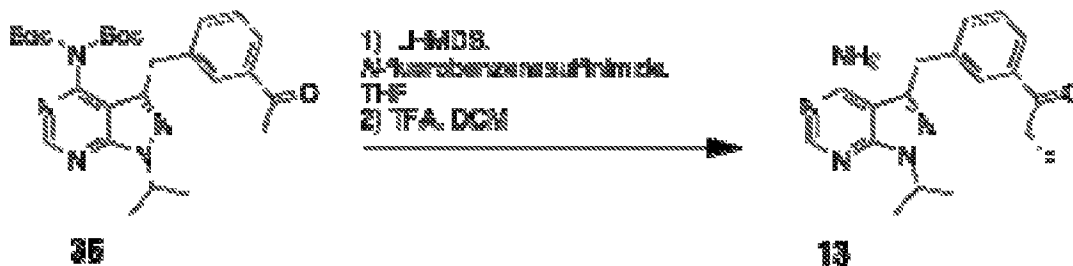


[0327] Compound **14**, di-*tert*-butyldicarbonate and dimethylaminopyridine were mixed and
 allowed to react for 3 hours at room temperature. Afterwards, the reaction mixture was
 25 diluted with EtOAc and washed with 1 M HCl and brine. The organic solution was dried with
 MgSO₄ and concentrated *in vacuo*. The material was purified over a silica column using a
 hexane/ ethyl acetate solvent system (1.834 g, 55 % yield): ¹H NMR (400 MHz, DMSO) δ

8.92 (s, 1H), 7.81 (d, $J = 7.9$, 1H), 7.77 (s, 1H), 7.41 (t, $J = 7.7$, 1H), 7.29 (d, $J = 7.7$, 1H), 5.20 (hept, $J = 6.7$, 1H), 4.25 (s, 2H), 2.53 (s, 3H), 1.55 (d, $J = 6.7$, 6H), 1.28 (s, 18H). ^{13}C NMR (100 MHz, DMSO) δ 197.42, 154.90, 154.28, 153.19, 149.83, 141.77, 138.25, 136.88, 132.65, 128.85, 127.74, 126.52, 109.01, 83.67, 49.08, 33.51, 27.19, 26.58, 21.63; $[\text{M}+\text{H}]^+$

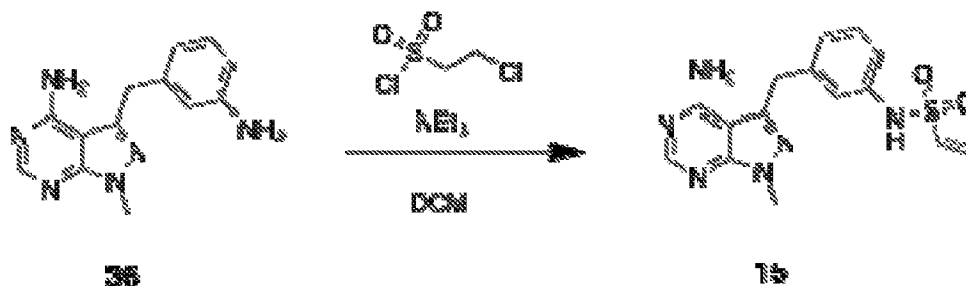
5 calculated for $\text{C}_{27}\text{H}_{35}\text{N}_5\text{O}_5$ 510.2, found 510.1.

Example 25 – Preparation of 1-(3-((4-amino-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)methyl)phenyl)-2-fluoroethanone (13)



[0328] Compound **35** (253 mg, 0.496 mmol) was mixed with anhydrous THF (2 mL) and cooled to -78°C , whereupon 1.0 M LHMDs in THF (0.645 mL, 0.645 mmol) was added dropwise via syringe and allowed to react for 15 minutes. *N*-fluorobenzenesulfinamide (250 mg, 0.794 mmol) in THF (2 mL) was then added dropwise and the reaction mixture was allowed to come to room temperature over 30 minutes. The reaction mixture was cooled to -78°C and saturated ammonium chloride (100 mL) was added dropwise. The reaction mixture was extracted with EtOAc (70 mL), and the resulting organic layer was washed with saturated sodium bicarbonate (1 x 70 mL) and brine (1 x 70 mL). The organic layer was concentrated *in vacuo* to give a yellow oil. The resulting product was purified over a silica column (hexane/ethyl acetate solvent system) and fractions containing the monofluorinated product and unreacted material (**35**) were pooled (they were inseparable). This combined mixture (93 mg) was reacted with TFA (1.5 mL) in DCM for 5 hours at room temperature and then concentrated *in vacuo*. The Boc-protected material was then resuspended in EtOAc (30 mL) and washed with saturated sodium bicarbonate (1 x 30 mL) and brine (1 x 30 mL). After concentrating the organic layer, the monofluorinated product was purified by preparative TLC using a 8 % MeOH/ CHCl_3 solvent system (22 mg, 14 % yield): ^1H NMR (400 MHz, DMSO) δ 9.91 (s, 1H), 8.30 (s, 1H), 7.16 (d, $J = 8.3$, 2H), 7.05 (d, $J = 8.4$, 2H), 6.74 (dd, $J = 16.4$, 9.9, 1H), 6.07 (d, $J = 16.4$, 1H), 6.00 (d, $J = 10.0$, 1H), 5.03 – 4.94 (m, 1H), 4.34 (s, 2H), 1.44 (d, $J = 6.6$, 6H); ^{13}C NMR (100 MHz, DMSO) δ 154.0, 151.4, 149.8, 144.7, 136.3, 136.0, 133.9, 129.3, 127.5, 120.0, 97.9, 48.6, 32.3, 21.7; $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{17}\text{H}_{18}\text{FN}_5\text{O}$ 328.1, found 328.1.

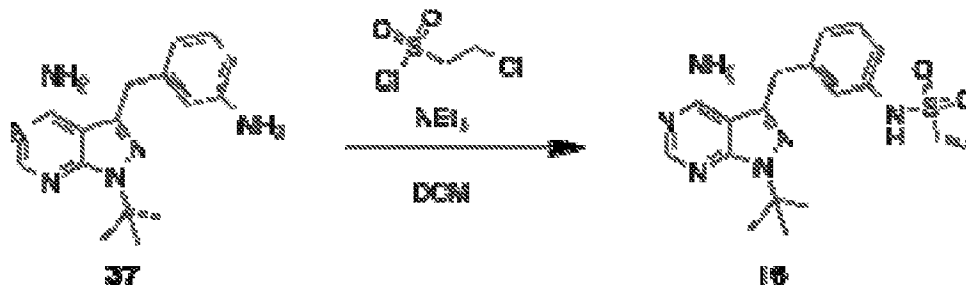
Example 26 – Preparation of N-(3-((4-amino-1-methyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)methyl)phenyl)ethanesulfonamide (15)



5 [0329] Compound **36** was prepared by the method of Dar et al. {Dar, 2008 #18}.

Compound **15** was prepared by the same method used for compound **9** (22 % yield): ¹H NMR (400 MHz, DMSO) δ 9.94 (s, 1H), 8.36 (s, 1H), 7.21 (t, *J* = 7.8, 1H), 6.98 (m, 3H), 6.71 (dd, *J* = 16.4, 9.9, 1H), 6.03 (d, *J* = 16.4, 1H), 5.97 (d, *J* = 9.9, 1H), 4.36 (s, 2H), 3.91 (s, 3H); ¹³C NMR (100 MHz, DMSO) δ 154.2, 152.3, 150.4, 144.6, 139.4, 137.9, 136.2, 129.2, 127.6, 124.0, 119.4, 117.5, 97.8, 33.7, 32.8; [M+H]⁺ calculated for C₁₅H₁₆N₆O₂S 345.1, found 345.4.

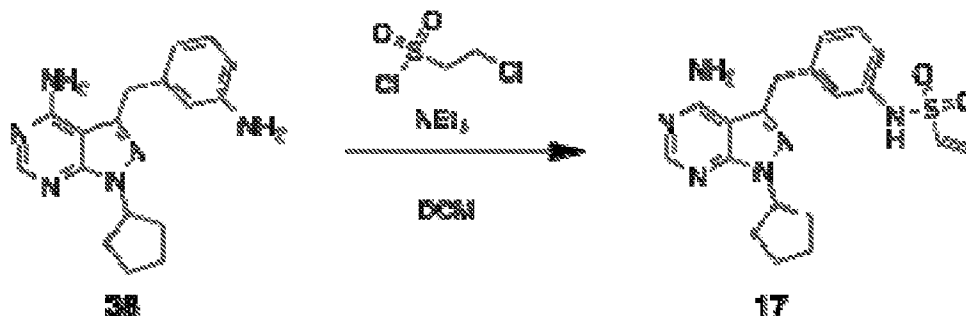
Example 27 – Preparation of N-(3-((4-amino-1-tert-butyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)methyl)phenyl)ethanesulfonamide (16)



15 [0330] Compound **37** was prepared by the method of Dar et al. {Dar, 2008 #18}.

Compound **16** was prepared by the same method used for compound **9** (10 % yield): ¹H NMR (400 MHz, DMSO) δ 9.95 (s, 1H), 8.30 (s, 1H), 7.21 (t, *J* = 8.1, 1H), 6.97 (m, 3H), 6.69 (dd, *J* = 16.4, 9.9, 1H), 5.94 (m, 2H), 4.36 (s, 2H), 1.71 (s, 9H); ¹³C NMR (100 MHz, DMSO) δ 154.5, 152.3, 149.6, 142.5, 139.6, 137.9, 136.2, 129.1, 127.4, 123.8, 119.1, 117.4, 99.1, 60.2, 32.8, 28.8; [M+H]⁺ calculated for C₁₈H₂₂N₆O₂S 387.2, found 387.5.

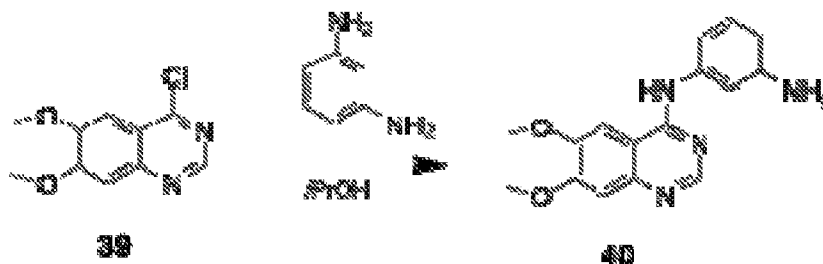
Example 28 – Preparation of N-(3-((4-amino-1-cyclopentyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)methyl)phenyl)ethanesulfonamide (17)



5 [0331] Compound **38** was prepared by the method of Dar et al. {Dar, 2008 #18}.

Compound **17** was prepared by the same method used for compound **9** (18 % yield): ^1H NMR (400 MHz, DMSO) δ 9.95 (s, 1H), 8.37 (d, J = 6.8, 1H), 7.21 (t, J = 8.0, 1H), 6.98 (m, 3H), 6.69 (dd, J = 16.4, 9.9, 1H), 5.98 (d, J = 16.4, 1H), 5.93 (d, J = 9.9, 1H), 5.18 (t, J = 7.2, 1H), 4.38 (s, 2H), 2.08 (m, 2H), 1.98 (m, 2H), 1.88 (m, 2H), 1.68 (m, 2H); ^{13}C NMR (100 MHz, DMSO) δ 153.7, 151.7, 149.6, 144.7, 139.5, 137.9, 136.2, 129.1, 127.4, 123.8, 119.2, 117.5, 97.9, 57.2, 32.8, 31.9, 24.3; $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{19}\text{H}_{22}\text{N}_6\text{O}_2\text{S}$ 399.2, found 399.4.

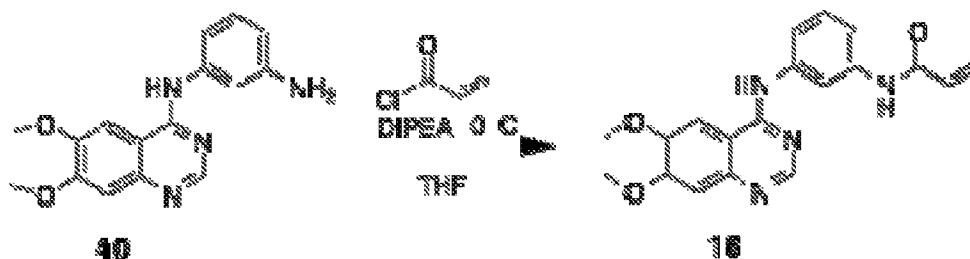
Example 29 – Preparation of N1-(6,7-dimethoxyquinazolin-4-yl)benzene-1,3-diamine



[0332] Compound **39** was prepared by a previously described method {Perera, 2008 #110}.

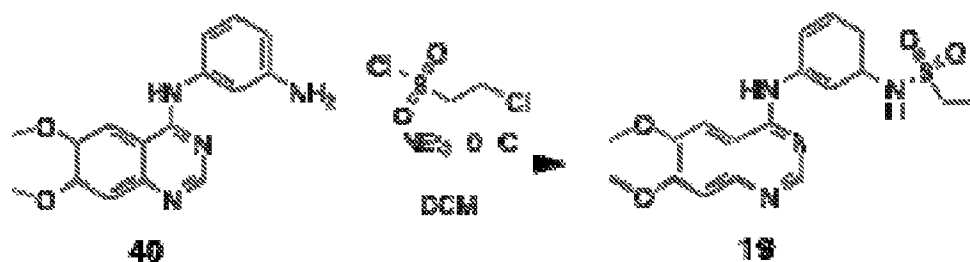
15 Compound **39** (300 mg, 1.34 mmol) and 1,3- phenylenediamine (1.78 g, 16.5 mmol) were heated to 90° C in isopropanol and allowed to react for 1.5 hours, after which the reaction was brought to room temperature. The resulting green, solid product was collected by filtration and washed with cold isopropanol (173 mg, 44 % yield): ^1H NMR (400 MHz, DMSO) δ 11.01 (br, 1H), 8.72 (s, 1H), 8.24 (s, 1H), 7.36 (s, 1H), 7.15 (t, J = 8.0, 1H), 7.00 (s, 1H), 6.93 (d, J = 7.9, 1H), 6.62 (d, J = 8.0, 1H), 3.99 (s, 3H), 3.97 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ 157.85, 155.85, 149.92, 149.21, 137.73, 129.08, 113.52, 112.92, 110.99, 107.35, 103.60, 100.86, 56.73, 56.33; $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{16}\text{H}_{16}\text{N}_4\text{O}_2$ 297.1, found 297.4.

Example 30 – Preparation of N-(3-(6,7-dimethoxyquinazolin-4-ylamino)phenyl)acrylamide (18)



- 5 [0333] A solution of THF (7 mL), compound **40** (75 mg, 0.253 mmol) and *N,N*-diisopropylethylamine (96 μ L, 0.551 mmol) was cooled 0° C, at which point freshly distilled acryloyl chloride (19 μ L, 0.230 mmol) was added. After one hour, the reaction mixture was concentrated *in vacuo*. The material was resuspended in dichloromethane (10 mL), which was washed with saturated sodium bicarbonate (10 mL). The aqueous layer was extracted
- 10 with dichloromethane (2 x 10 mL) and the organic layers were subsequently combined, dried over MgSO_4 , filtered and concentrated to a solid. The material was purified by RP-HPLC and lyophilized to a powder (40 mg, 50 % yield): ^1H NMR (400 MHz, DMSO) δ 10.81 (br, 1H), 10.31 (s, 1H), 8.78 (s, 1H), 8.17 (s, 1H), 8.06 (s, 1H), 7.41 (m, 3H), 7.25 (s, 1H), 6.47 (dd, $J = 17.0, 10.1$, 1H), 6.28 (dd, $J = 17.0, 1.9$, 1H), 5.79 (dd, $J = 10.1, 1.9$, 1H), 4.00 (s,
- 15 6H); ^{13}C NMR (100 MHz, DMSO) δ 163.32, 158.05, 156.18, 150.11, 149.32, 139.48, 137.32, 131.73, 129.02, 127.18, 119.88, 117.10, 115.49, 107.30, 103.23, 100.68, 56.57, 56.45; $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{19}\text{H}_{18}\text{N}_4\text{O}_3$ 351.1, found 351.4.

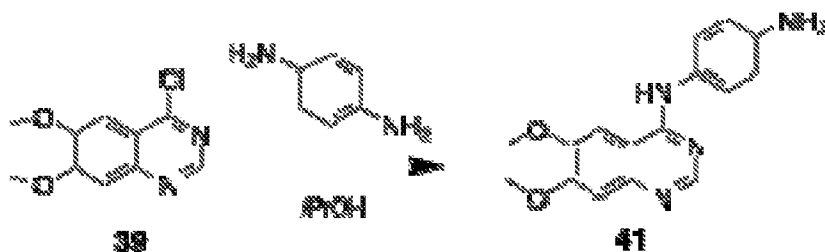
Example 31 – Preparation of N-(3-(6,7-dimethoxyquinazolin-4-ylamino)phenyl)ethanesulfonamide (19)



- 20 [0334] A solution of dichloromethane (10 mL), compound **40** (97 mg, 0.327 mmol) and triethylamine (138 μ L, 0.989 mmol) was cooled to 0° C. 2-chloro-1-ethane sulfonyl chloride (30 μ L, 0.287 mmol) was added and the reaction was allowed to proceed for 1 hour prior to
- 25 removal of a greenish precipitate and addition of saturated sodium bicarbonate (10 mL) and extraction with dichloromethane (2 x 10 mL). The combined organic layers were dried with MgSO_4 , filtered and concentrated *in vacuo*. The product (organic layers and green precipitate

combined) was purified by preparative RP-HPLC and lyophilized (40 mg, 36 % yield): ^1H NMR (400 MHz, DMSO) δ 10.71 (br, 1H), 10.20 (s, 1H), 8.76 (s, 1H), 8.02 (s, 1H), 7.52 (s, 1H), 7.39 (m, 2H), 7.25 (s, 1H), 7.06 (m, 1H), 6.82 (dd, $J = 16.4, 9.9$, 1H), 6.18 (d, $J = 16.4$, 1H), 6.10 (d, $J = 9.9$, 1H), 3.99 (s, 6H); ^{13}C NMR (100 MHz, DMSO) δ 157.94, 156.12, 150.06, 149.43, 138.29, 137.85, 136.06, 129.40, 128.11, 119.92, 117.13, 115.32, 107.41, 103.15, 100.96, 56.55, 56.42; $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{18}\text{H}_{18}\text{N}_4\text{O}_4\text{S}$ 387.1, found 387.5.

Example 32 – Preparation of N1-(6,7-dimethoxyquinazolin-4-yl)benzene-1,4-diamine (41)

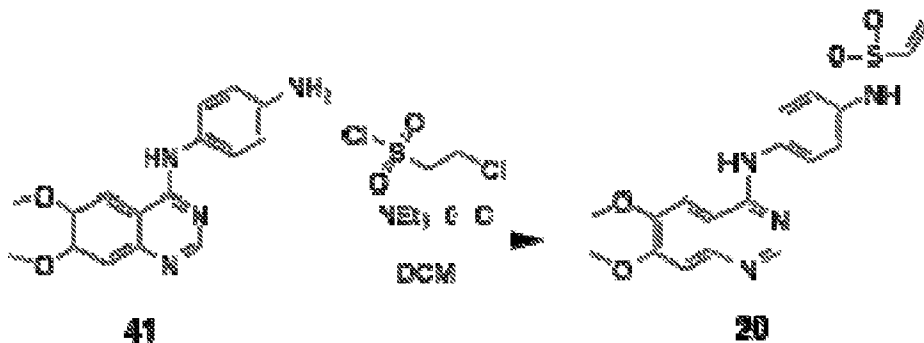


10

[0335] Compound **39** was prepared by a previously described method {Perera, 2008 #110}. Compound **39** (600 mg, 2.68 mmol) and 1,3-phenylenediamine (3.57 g, 33 mmol) were heated to 90° C in isopropanol and allowed to react for 1.5 hours, after which the reaction was brought to room temperature. The resulting solid product was collected by filtration and washed with cold isopropanol (775 mg, 98 % yield): ^1H NMR (400 MHz, DMSO) δ 10.38 (br, 1H), 8.59 (s, 1H), 8.00 (s, 1H), 7.28 (d, $J = 8.6$, 2H), 7.20 (s, 1H), 6.79 (s, 1H), 6.66 (d, $J = 8.6$, 2H), 3.96 (s, 6H); ^{13}C NMR (100 MHz, DMSO) δ 157.31, 155.07, 150.47, 149.39, 145.88, 126.53, 125.38, 113.97, 107.62, 103.05, 102.79, 56.49, 56.10; $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{16}\text{H}_{16}\text{N}_4\text{O}_2$ 297.1, found 297.4.

20

Example 33 – Preparation of N-(4-(6,7-dimethoxyquinazolin-4-ylamino)phenyl)ethanesulfonamide (20)



[0336] A solution of dichloromethane (10 mL), compound **41** (100 mg, 0.337 mmol) and triethylamine (141 μL , 1.02 mmol) was cooled to 0° C. 2-chloro-1-ethanesulfonyl chloride (32 μL , 0.304 mmol) was added and the reaction was allowed to proceed for 1 hour prior to

25

removal of a precipitate and addition of saturated sodium bicarbonate (10 mL) and extraction with dichloromethane (2 x 10 mL). The combined organic layers were dried with MgSO₄, filtered and concentrated *in vacuo*. The product was purified by preparative RP-HPLC and lyophilized (24 mg, 20 % yield): ¹H NMR (400 MHz, DMSO) δ 10.81 (br, 1H), 10.13 (s, 1H), 8.75 (s, 1H), 8.02 (s, 1H), 7.58 (d, *J* = 8.9, 2H), 7.25 (m, 3H), 6.83 (dd, *J* = 16.4, 10.0, 1H), 6.15 (d, *J* = 16.5, 1H), 6.07 (d, *J* = 10.0, 1H), 3.99 (s, 3H), 3.98 (s, 3H); ¹³C NMR (100 MHz, DMSO) δ 157.93, 156.16, 150.10, 149.26, 136.23, 135.75, 132.76, 127.82, 125.54, 120.00, 107.16, 103.16, 100.51, 56.55, 56.45; [M+H]⁺ calculated for C₁₈H₁₈N₄O₄S 387.1, found 387.4.

10

Example 34 – Gel filtration and kinetic assays

[0337] Inhibitors (final concentration 23 μM) were incubated with c-Src variants (final concentration 11.5 μM) in kinase reaction buffer (50 mM Tris pH 8, 100 mM NaCl, 1 mM DTT, 5 % glycerol, 5 % DMSO) for 25 minutes at room temperature. The solutions (2.6 mL total) were then passed over PD10 desalting columns (GE Healthcare) using the kinase reaction buffer for elution. Src concentrations were calculated using the extinction coefficient for c-Src (51.140 mM⁻¹cm⁻¹) {Seeliger, 2005 #100}. Kinase assays were performed as described in the experimental section at a final enzyme concentration of 10 nM.

15

[0338] Protein labeling conditions: Kinase labeling reactions were performed by incubating 30 μL quantities of c-Src variants (in 50 mM Tris pH 8, 100 mM NaCl, 1 mM DTT, 5 % glycerol) with two equivalents of inhibitor in DMSO (final DMSO concentration = 2.4 %). The covalent labeling reaction was quenched by removing 4 μL and adding it to 31 μL of 0.1 % formic acid. The sample was then analyzed by ESI-MS/MS.

20

Example 35 –Crystallization and data collection for c-Src-ES- 9

[0339] The c-Src-ES variant was prepared and purified as described above, run over a 0.22 μm PVDF centrifugal filter and diluted to 1.5-3 mg/ml in 50 mM Tris (pH 8.0), 100 mM NaCl, 5% (v/v) glycerol, 1 mM DTT. Compound 9 was freshly dissolved in DMSO and added to the protein solutions (1.5- 3 equivalents). After 1.5 hours of incubation at room temperature, the reaction mixtures were spun at 10,000 rpm and the supernatants were collected. Hanging drop crystallization conditions were set up by mixing 1:1 protein and precipitation solutions (100 mM MES (pH 6.5), 50 mM NaOAc, 4-8 % PEG 4000). After 24- 48 hours at room temperature, thin plate-like crystals were observed. Crystals were cryoprotected in the crystallization solution supplemented with 25 % glycerol and stored in liquid nitrogen prior to obtaining diffraction data at beamline 8.2.2 (wavelength of 1.0088

25

30

nm, nitrogen gas stream at 100 K) at the Berkeley Lab Advanced Light Source. Data was processed with HKL2000 (HKL Research, Inc.) and Phenix software{Adams, #108}.

- [0340] Crystal structure of c-Src-ES1 with **9** - In order to elucidate the binding mode for a kinase with a cysteine gatekeeper and an irreversible inhibitor, an X-ray crystal structure of the catalytic domain of c-Src-ES1 (residues 251-533) bound to **9** was solved (**Fig. 2**). Co-crystallization through incubation of c-Src-ES1 with **9** was performed using hanging-drop vapor diffusion. The complex was solved by molecular replacement and, contained two molecules in the crystallographic asymmetric unit of the *P1* space group. The structure was refined to 2.2 Å and exhibited electron density for **9** covalently bound to Cys338. Poor electron density was observed near the N-terminus (residues 251-256) and in flexible regions of the kinase such as the glycine-rich loop (residues 275-278) and the activation segment (residues 407-424). However, the DFG motif at the beginning of the activation segment (residues 404-406) was clearly resolved and was in the conformation associated with an active kinase (DFG-in).
- [0341] The binding mode of **9** (a vinylsulfonamide functionalized compound) with c-Src-ES1 is related to Type I½ kinase inhibition. Like Type I inhibitors, Type I½ inhibitors bind the active conformation of the kinase (DFG-in) and engage in a series of hydrogen bonds in the hinge region. Type I½ are similar to Type II inhibitors in that they occupy the pocket situated behind the gatekeeper and hydrogen bond to the carboxylate of the conserved glutamate on the αC-helix and backbone amide of the DFG aspartate (**Fig. 2C**). The hydrogen bonds afforded by the tetrahedral arrangement of the sulfone may contribute to the increased potency of **9** relative to **7**, which contains an acrylamide.

Example 36 –Kinome- wide profiling of inhibitors.

- [0342] The percent inhibition results in FIG. were generated with biochemical enzymatic kinase assays using the SelectScreen® Kinase Profiling Service (Life Technologies Corporation, Madison, WI). Compounds were assayed at 1 μM at an ATP concentration equal to the ATP *K_m*,app for the assay following the detailed procedures described in the SelectScreen® Customer Protocol and Assay Conditions documents located at www.invitrogen.com/kinaseprofiling.
- [0343] In order to identify potential off-targets, a panel of the electrophilic inhibitors that showed inhibition of c-Src-ES1 against 307 kinases was screened (**Table 5**). Compounds that were profiled include, but are not limited to, **3**, **4**, **9**, **13**, and **20**. Excluding **3**, all of the compounds had relatively few off-target effects. The exocyclic amine mimics N6 of ATP

and plays an important role in a hydrogen bonding interaction with the hinge region of kinases. Several of the kinases for which >80 % inhibition was achieved with vinylsulfonamide-based inhibitors was observed are those with exposed cysteines near the active site (*e.g.* EGFR, HER4, BTK, BMX, TXK). The fluoromethylketone-type compound, 13, had a clean profile against kinases in the panel. The present invention provides one of the most selective chemical genetic kinase inhibitor reported to date.

Example 37 – Site Directed Mutagenesis

[0344] The T338C mutation was introduced to a pET-28 vector containing a hexahistidine - tagged Src construct using standard site directed mutagenesis methods. The protein was produced in *E. coli* BL21DE3 cells containing YopH phosphatase and GroEL. The cells were grown in Terrific Broth containing (kanamycin, 50 mg/mL/streptomycin, 50 mg/mL). Cells were grown to an OD_{600nm} of 1.2 at 37° C, and cooled for 1 hour with shaking at 18° C. Afterwards, the cells were induced for 16 h at 18° C with 0.2 mM IPTG. Cells were harvested and resuspended in 50 mM Tris (pH 8.0), 500 mM NaCl, 5% glycerol, 25 mM imidazole for purification over Ni-NTA resin.

Example 38 – Expression and Purification of c-Src variants

[0345] Hexahistidine-tagged recombinant chicken c-Src (residues 251-533) was prepared in a similar manner to that described in Seeliger MA, *et al. Protein Sci.* 14 (12):3135-3139 with the modifications used by Blair JA, *et al.* (2007) Structure-guided development of affinity probes for tyrosine kinases using chemical genetics. *Nat Chem Biol* 3(4):229-238. The hexahistidine tag was removed with AcTev protease (Invitrogen) and concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient of 52,370 M⁻¹cm⁻¹. All mutations were introduced using the site-directed mutagenesis protocol of Zheng L, Baumann U, Reymond JL (2004) An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Res* 32(14):e115. Protein aliquots were stored at -80 °C in 50 mM Tris (pH 8), 100 mM NaCl, 1 mM DTT and 5 % glycerol.

Example 39 – In vitro kinase assays

[0346] *In vitro* kinase assays for c-Src variants were performed in 50 mM Tris (pH 8.0), 10 mM MgCl₂ and 1 mg/mL BSA. When obtaining kinetic parameters (*k*_{cat}, *K*_m) kinase and peptide substrate (IYGEFKKK) (SEQ ID NO:51) concentrations were 2 nM and 500 μM, respectively, while ATP concentrations ranged from 2000 – 0.655 μM. Addition of nonradioactive ATP supplemented with ³²P ATP (3,000 Ci/mmol, NEN) was used to initiate kinase reactions. Time points were selected such that product formation never exceeded

10%. Reactions were quenched by spotting 3 μ L quantities onto phosphocellulose sheets (P81, Whatman). Afterwards, the sheets were washed 3 x 5 minutes in 0.5 % phosphoric acid and dried. Radioactivity was measured by phosphorimaging and recorded on a Typhoon fluorescence imager (Molecular Dynamics). Data were plotted as rate (min^{-1}) versus ATP concentration and fitted to the Michaelis–Menten equation, $v = [(k_{\text{cat}})[S]]/(K_m + [S])$, using Kaleidagraph software (Synergy) to extract kinetic parameters. When obtaining IC_{50} values for the inhibitors, 2% (v/v) DMSO was included in kinase reactions. In these cases ATP, peptide, and enzyme concentrations were 15 nM, 100 μ M and 5 nM, respectively, while inhibitor concentrations ranged from 10,000- 0.610 nM. In all cases, a ten-minute preincubation step between the kinase and the inhibitor preceded addition of ATP and a fifteen-minute reaction. The data was fitted to a sigmoidal dose-response curve using Prism 4.0c (GraphPad Software) to obtain IC_{50} values.

Example 40 – Crystallization and data collection for c-Src-ES1-9

[0347] The c-Src-ES1 variant was prepared and purified as described above, run over a 0.22 μ m PVDF centrifugal filter and diluted to 1.5-3 mg/ml in 50 mM Tris (pH 8.0), 100 mM NaCl, 5% (v/v) glycerol, 1 mM DTT. Compound 9 was freshly dissolved in DMSO and added to the protein solutions (1.5- 3 equivalents). After 1.5 hours of incubation at room temperature, the reaction mixtures were spun at 10,000 rpm and the supernatants were collected. Hanging drop crystallization conditions were set up by mixing 1:1 protein and precipitation solutions (100 mM MES (pH 6.5), 50 mM NaOAc, 4-8 % PEG 4000). After 24- 48 hours at room temperature, thin plate-like crystals were observed. Crystals were cryoprotected in the crystallization solution supplemented with 25 % glycerol and stored in liquid nitrogen prior to obtaining diffraction data at beamline 8.2.2 (wavelength of 1.0088 nm, nitrogen gas stream at 100 K) at the Berkeley Lab Advanced Light Source. Data was processed with HKL2000 (HKL Research, Inc.) and Phenix software.

Example 41 –Immunoprecipitation and assay of MOK

[0348] A plasmid encoding full-length mouse MOK with a FLAG-tag for expression in mammalian cells was used. Immunoprecipitation from Cos7 cells was performed using a procedure similar to Miyata Y, Akashi M, Nishida E (1999). Molecular cloning and characterization of a novel member of the MAP kinase superfamily. *Genes Cells* 4(5):299-309, with the following modification: MOK was directly immunoprecipitated on ANTI-FLAG M2 magnetic beads (Sigma-Aldrich). Kinase assays were performed directly on-bead for 60 minutes in 30 μ L quantities of 50 mM Tris-HCl, 16 mM MOPS, 150 mM NaCl, 10

mM MgCl₂, 20 mM β-glycerophosphate, 2 mM EGTA, 0.8 mM sodium orthovanadate, 0.4 mM dithiothreitol, 0.1 mM ATP (supplemented with ³²P ATP (3,000 Ci/mmol, NEN)), and 20 μg of a protein substrate (myelin basic protein) at a pH of 8. Inhibitors were used at a concentration of 1 μM (final DMSO concentration, 2 %). Myelin basic protein

5 phosphorylation was analyzed by SDS-PAGE and autoradiography. MOK levels were evaluated by Western blot using HRP conjugated ANTI-FLAG M2 antibody at a 1000:1 dilution.

Example 42 – Inhibition assays with v-Src transformed NIH-3T3 cells

[0349] NIH-3T3 cell lines transformed with v-Src gatekeeper variants were prepared using
10 a procedure similar to that in Bishop AC, *et al.* (1998) Design of allele-specific inhibitors to probe protein kinase signaling. *Curr Biol* 8(5):257-266. Cells were grown to 60-90 % confluence in DMEM supplemented with fetal bovine serum (10 %), penicillin ‘G’ (100 units/ ml) and streptomycin sulfate (100 μg/ml) (PenStrep, UCSF Cell Culture Facility) prior to treatment with kinase inhibitors dissolved in DMSO (final DMSO concentration, 0.5 %).
15 Following 1 hour of incubation with inhibitors at 37 °C, cells were harvested in lysis buffer (50 mM Tris (pH 7.4), 300 mM NaCl, 5 mM EDTA, 1 % triton, 0.02 % NaN₃, 1 X complete mini protease inhibitor (Roche), 1 mM PMSF, 1X PHOS-stop (Roche), 0.02 μM microcystin, 2 mM sodium orthovanadate), normalized for concentration and analyzed by Western blot for global phosphotyrosine levels (4G10, Millipore, 1:1000). Levels of β-actin (β-actin
20 Antibody, Cell Signaling, 1:1000) and v-Src (Src 32G6 rabbit mAb, Cell Signaling, 1:1000) were ascertained by Western blot.

Example 43 - Blockade of v-Src-ES1 activity in cells with electrophilic inhibitors

[0350] I338C (v-Src-ES1, SEQ ID NO:48), I338T (SEQ ID NO:49), I338G (v-Src-AS1, SEQ ID NO:50) and WT v-Src-transformed NIH-3T3 cell lines were generated. Unlike c-
25 Src, v-Src is constitutively active and harbors an isoleucine gatekeeper. The I338T v-Src variant was generated for consistency with the *in vitro* c-Src studies. For each cell line, global levels of phosphotyrosine were analyzed (Fig. 7). Importantly, the v-Src-ES1 variant was an excellent mimic of WT v-Src, while the activity of v-Src-AS1 (the mutant used in previous chemical genetic studies) was markedly diminished as judged by whole cell
30 phosphotyrosine levels. To determine whether the electrophilic inhibitors function in cells, the v-Src-ES1 and I338T v-Src-transformed cell lines were treated with **9** and **13**. Both **9** and **13** inhibited v-Src-ES1 in a dose-dependent manner, while isosteric control compounds (**11** and **14**) showed no activity (Fig. 4). Furthermore, neither **9** nor **13** inhibited I338T v-Src

even at levels as high as 10 μM . Collectively, these results suggest that a kinase with a cysteine gatekeeper can be selectively targeted in cells.

Example 44 - Second-site mutations to modulate inhibitor potency

The design strategy, in order to determine whether further kinase engineering could enhance potency, was to either enhance the reactivity of the cysteine by installing nearby hydrophilic/basic residues or to slightly enlarge the area around the cysteine to allow for additional rotational freedom to facilitate optimized thiol-electrophile attack geometry. Accordingly, mutations at Val323—a residue within 4 Å of the gatekeeper (**Fig. 2C**) in c-Src—were introduced in combination with T338C. Of the double mutants, V323A/T338C (c-Src-ES2) and V323S/T338C (c-Src-ES3) had substantial activity, while V323D/T338C (c-Src-ES4), V323E/T338C (c-Src-ES5), and V323H/T338C (c-Src-ES6) were inactive (**Table 4**). Enhanced inhibitor potency was observed for both c-Src-ES2 and c-Src-ES3 when treated with **13** (**Table 3**). In the latter case, a 12-fold improvement was noted relative to c-Src-ES1. Interestingly, the potencies of **3** and **9** were not modulated appreciably upon introduction of the additional mutations. Taken together, these results indicate that the judicious placement of a secondary mutation can be an effective means for modulating inhibitor potency for an ES allele, but that this strategy needs to be evaluated on a case-by-case basis.

Example 45 - Evaluating the use of a cysteine gatekeeper kinase.

[0351] A recombinant wild type (WT) and T338C c-Src was generated. The recombinant wild type (WT) and the T338C c-Src were assayed for kinase activity, see Table 8. The k_{cat} value for T338C c-Src (183 min^{-1}) closely approximated that of WT (159 min^{-1}) and was ~3.5-fold greater than that of c-Src-AS1 (51.9 min^{-1}). The T338C c-Src variant also recapitulated WT in affinity for ATP as determined by the Michaelis constant (K_m) values ($21.9 \mu\text{M}$ vs. $31.9 \mu\text{M}$), while c-Src-AS1 ($87.5 \mu\text{M}$) exhibited ~4-fold loss relative to T338C c-Src. These effects translate to a 14-fold improvement in catalytic efficiency (k_{cat}/K_m) for T338C c-Src in relation to c-Src-AS1.

Table 8. Kinetic parameters for c-Src variants. Values were determined by fitting data to the Michaelis-Menten equation. Standard errors associated with the fits are reported.

c-Src Variant	$k_{\text{cat}} (\text{min}^{-1})$	$K_{m, \text{ATP}} (\mu\text{M})$	$k_{\text{cat}}/K_m (\text{min}^{-1} \mu\text{M}^{-1})$
WT	159 ± 4	31.9 ± 3.0	4.99 ± 0.40
T338C	183 ± 3	21.9 ± 1.7	8.34 ± 0.57
AS1	51.9 ± 1.9	87.5 ± 12.6	0.592 ± 0.072

[0352] Kinetic measurements reveal that in the case of c-Src, the ES1 variant is a mimic of wild type activity. Furthermore, the ES1 variant of v-Src is also a mimic of the wild type,

- which contains a particularly hydrophobic (isoleucine) gatekeeper. See Tables 9a-9c following. Results on the selectivity of **compound 19** in the Invitrogen SelectScreen® Kinase Assay are provided in Tables 9a-9c following. Table 9a is the LanthaScreen™ heat map., Table 9b is the Adapta® heat map, and Table 9c is the Z'-lyte™ heat map. Legend for
- 5 Tables 9a-9c: <40% inhibition (gray); 40%-80% inhibition (white); ≥ 80% inhibition (diagonal stripes). Selected results in the assays are further provided in Fig. 11. In the table below, Cmpd 3-vs-Q refers to compound 19.

Table 9a.

Kinase			Cmpd 3-vs-Q 1000 nM
ACVR1 (ALK2)	Binding		4
ACVR2B	Binding		19
BMPR1A (ALK3)	Binding		10
CAMKK1 (CAMKKA)	Binding		-5
CAMKK2 (CaMKK beta)	Binding		6
CDK8/cyclin C	Binding		4
CDK9/cyclin K	Binding		0
CLK4	Binding		24
DDR1	Binding		0
DDR2	Binding		5
DMPK	Binding		10
EPHA3	Binding		1
EPHA7	Binding		-2
KIT V654A	Binding		6
LIMK1	Binding		1
LIMK2	Binding		6
MAP2K1 (MEK1) S218D S222D	Binding		20
MAP2K3 (MEK3)	Binding		6
MAP2K6 (MKK6) S207E T211E	Binding		3
MAP3K10 (MLK2)	Binding		6
MAP3K11 (MLK3)	Binding		1
MAP3K14 (NIK)	Binding		-12
MAP3K2 (MEKK2)	Binding		2
MAP3K3 (MEKK3)	Binding		9
MAP3K5 (ASK1)	Binding		3
MAP3K7/MAP3K7IP1 (TAK1-TAB1)	Binding		2
MKNK2 (MNK2)	Binding		36
MLCK (MLCK2)	Binding		36

Kinase			Cmpd 3-vs-Q 1000 nM
MYLK (MLCK)	Binding		3
NLK	Binding		5
RIPK2	Binding		52
SLK	Binding		7
STK16 (PKL12)	Binding		-5
STK17A (DRAK1)	Binding		21
STK33	Binding		-7
TAOK3 (JIK)	Binding		-3
TEC	Binding		3
TGFBR1 (ALK5)	Binding		10
TNK2 (ACK)	Binding		10
TTK	Binding		16
WEE1	Binding		14
WNK2	Binding		10
ZAK	Binding		6

Table 9b.

Kinase			Cmpd 3-vs-Q 1000 nM
CAMK1 (CaMK1)	Activity	100	-21
CDK7/cyclin H/MNAT1	Activity	Km app	0
CDK9/cyclin T1	Activity	Km app	-24
CHUK (IKK alpha)	Activity	Km app	3
DAPK1	Activity	Km app	6
GSG2 (Haspin)	Activity	Km app	-8
IRAK1	Activity	Km app	6
LRRK2	Activity	Km app	75
LRRK2 G2019S	Activity	Km app	91
NUAK1 (ARK5)	Activity	Km app	24
PI4KA (PI4K alpha)	Activity	10	7
PI4KB (PI4K beta)	Activity	Km app	14
PIK3C2A (PI3K-C2 alpha)	Activity	Km app	-1
PIK3C2B (PI3K-C2 beta)	Activity	100	65
PIK3C3 (hVPS34)	Activity	Km app	1
PIK3CA/PIK3R1 (p110 alpha/p85 alpha)	Activity	Km app	13
PIK3CD/PIK3R1 (p110 delta/p85 alpha)	Activity	Km app	10
PIK3CG (p110 gamma)	Activity	Km app	27
SPHK1	Activity	Km app	3
SPHK2	Activity	100	-11

Table 9c.

Kinase			Cmpd 3-vs-Q 1000 nM
ABL1	Activity	Km app	4
ABL1 E255K	Activity	Km app	7
ABL1 G250E	Activity	Km app	2
ABL1 T315I	Activity	Km app	11
ABL1 Y253F	Activity	Km app	12
ABL2 (Arg)	Activity	Km app	10
ACVR1B (ALK4)	Activity	Km app	-1
ADRBK1 (GRK2)	Activity	Km app	13
ADRBK2 (GRK3)	Activity	Km app	-1
AKT1 (PKB alpha)	Activity	Km app	0
AKT2 (PKB beta)	Activity	Km app	4
AKT3 (PKB gamma)	Activity	Km app	4
ALK	Activity	Km app	3
AMPK A1/B1/G1	Activity	Km app	18
AMPK A2/B1/G1	Activity	Km app	15
AURKA (Aurora A)	Activity	Km app	12
AURKB (Aurora B)	Activity	Km app	26
AURKC (Aurora C)	Activity	Km app	1
AXL	Activity	Km app	19
BLK	Activity	Km app	24
BMX	Activity	Km app	24
BRAF	Activity	100	4
BRAF V599E	Activity	100	13
BRSK1 (SAD1)	Activity	Km app	35
BTX	Activity	Km app	59
CAMK1D (CaMKI delta)	Activity	Km app	15
CAMK2A (CaMKII alpha)	Activity	Km app	4
CAMK2B (CaMKII beta)	Activity	Km app	5
CAMK2D (CaMKII delta)	Activity	Km app	10
CAMK4 (CaMKIV)	Activity	Km app	10
CDC42 BPA (MRCKA)	Activity	Km app	23
CDC42 BPB (MRCKB)	Activity	Km app	-4
CDK1/cyclin B	Activity	Km app	3
CDK2/cyclin A	Activity	Km app	4
CDK5/p25	Activity	Km app	10
CDK5/p35	Activity	Km app	5
CHEK1 (CHK1)	Activity	Km app	-6
CHEK2 (CHK2)	Activity	Km app	53
CLK1	Activity	Km app	11
CLK2	Activity	Km app	-3

Kinase			Cmpd 3-vs-Q 1000 nM
CLK3	Activity	Km app	6
CSF1R (FMS)	Activity	Km app	3
CSK	Activity	Km app	12
CSNK1A1 (CK1 alpha 1)	Activity	Km app	0
CSNK1D (CK1 delta)	Activity	Km app	5
CSNK1E (CK1 epsilon)	Activity	Km app	6
CSNK1G1 (CK1 gamma 1)	Activity	Km app	12
CSNK1G2 (CK1 gamma 2)	Activity	Km app	31
CSNK1G3 (CK1 gamma 3)	Activity	Km app	31
CSNK2A1 (CK2 alpha 1)	Activity	Km app	13
CSNK2A2 (CK2 alpha 2)	Activity	Km app	-1
DAPK3 (ZIPK)	Activity	Km app	0
DCAMKL2 (DCK2)	Activity	Km app	5
DNA-PK	Activity	Km app	25
DYRK1A	Activity	Km app	-2
DYRK1B	Activity	Km app	3
DYRK3	Activity	Km app	5
DYRK4	Activity	Km app	3
EEF2K	Activity	Km app	5
EGFR (ErbB1)	Activity	Km app	84
EGFR (ErbB1) L858R	Activity	Km app	69
EGFR (ErbB1) L861Q	Activity	Km app	76
EGFR (ErbB1) T790M	Activity	Km app	27
EGFR (ErbB1) T790M L858R	Activity	Km app	41
EPHA1	Activity	Km app	19
EPHA2	Activity	Km app	4
EPHA4	Activity	Km app	8
EPHA5	Activity	Km app	7
EPHA8	Activity	Km app	11
EPHB1	Activity	Km app	8
EPHB2	Activity	Km app	15
EPHB3	Activity	Km app	8
EPHB4	Activity	Km app	7
ERBB2 (HER2)	Activity	Km app	66
ERBB4 (HER4)	Activity	Km app	80
FER	Activity	Km app	6
FES (FPS)	Activity	Km app	16
FGFR1	Activity	Km app	8

Kinase			Cmpd 3-vs-Q 1000 nM
FGFR2	Activity	Km app	14
FGFR3	Activity	Km app	11
FGFR3 K650E	Activity	Km app	18
FGFR4	Activity	Km app	14
FGR	Activity	Km app	33
FLT1 (VEGFR1)	Activity	Km app	2
FLT3	Activity	Km app	32
FLT3 D835Y	Activity	Km app	99
FLT4 (VEGFR3)	Activity	Km app	34
FRAP1 (mTOR)	Activity	Km app	-7
FRK (PTK5)	Activity	Km app	11
FYN	Activity	Km app	3
GRK4	Activity	Km app	15
GRK5	Activity	Km app	49
GRK6	Activity	Km app	27
GRK7	Activity	Km app	-2
GSK3A (GSK3 alpha)	Activity	Km app	-2
GSK3B (GSK3 beta)	Activity	Km app	-4
HCK	Activity	Km app	13
HIPK1 (Myak)	Activity	Km app	3
HIPK2	Activity	Km app	4
HIPK3 (YAK1)	Activity	Km app	3
HIPK4	Activity	Km app	17
IGF1R	Activity	Km app	5
IKBKB (IKK beta)	Activity	Km app	13
IKBKE (IKK epsilon)	Activity	Km app	10
INSR	Activity	Km app	0
INSRR (IRR)	Activity	Km app	11
IRAK4	Activity	Km app	3
ITK	Activity	Km app	-3
JAK1	Activity	Km app	14
JAK2	Activity	Km app	0
JAK2 JH1 JH2	Activity	Km app	-5
JAK2 JH1 JH2 V617F	Activity	Km app	-4
JAK3	Activity	Km app	37
KDR (VEGFR2)	Activity	Km app	6
KIT	Activity	Km app	7
KIT T670I	Activity	Km app	5
LCK	Activity	Km app	0
LTK (TYK1)	Activity	Km app	4
LYN A	Activity	Km app	21

Kinase			Cmpd 3-vs-Q 1000 nM
LYN B	Activity	Km app	25
MAP2K1 (MEK1)	Activity	100	3
MAP2K2 (MEK2)	Activity	100	13
MAP2K6 (MKK6)	Activity	100	17
MAP3K8 (COT)	Activity	100	-1
MAP3K9 (MLK1)	Activity	Km app	3
MAP4K2 (GCK)	Activity	Km app	-13
MAP4K4 (HGK)	Activity	Km app	23
MAP4K5 (KHS1)	Activity	Km app	23
MAPK1 (ERK2)	Activity	Km app	2
MAPK10 (JNK3)	Activity	100	2
MAPK11 (p38 beta)	Activity	Km app	14
MAPK12 (p38 gamma)	Activity	Km app	10
MAPK13 (p38 delta)	Activity	Km app	5
MAPK14 (p38 alpha)	Activity	100	24
MAPK14 (p38 alpha) Direct	Activity	Km app	11
MAPK3 (ERK1)	Activity	Km app	10
MAPK8 (JNK1)	Activity	100	19
MAPK9 (JNK2)	Activity	100	8
MAPKAPK2	Activity	Km app	6
MAPKAPK3	Activity	Km app	5
MAPKAPK5 (PRAK)	Activity	Km app	7
MARK1 (MARK)	Activity	Km app	-1
MARK2	Activity	Km app	2
MARK3	Activity	Km app	5
MARK4	Activity	Km app	1
MATK (HYL)	Activity	Km app	5
MELK	Activity	Km app	20
MERTK (cMER)	Activity	Km app	12
MET (cMet)	Activity	Km app	3
MET M1250T	Activity	Km app	8
MINK1	Activity	Km app	17
MKNK1 (MNK1)	Activity	Km app	40
MST1R (RON)	Activity	Km app	11
MST4	Activity	Km app	15
MUSK	Activity	Km app	14
MYLK2 (skMLCK)	Activity	Km app	13
NEK1	Activity	Km app	-2
NEK2	Activity	Km app	-1
NEK4	Activity	Km app	22
NEK6	Activity	Km app	6

Kinase			Cmpd 3-vs-Q 1000 nM
NEK7	Activity	Km app	6
NEK9	Activity	Km app	10
NTRK1 (TRKA)	Activity	Km app	18
NTRK2 (TRKB)	Activity	Km app	8
NTRK3 (TRKC)	Activity	Km app	3
PAK1	Activity	Km app	14
PAK2 (PAK65)	Activity	Km app	12
PAK3	Activity	Km app	3
PAK4	Activity	Km app	-4
PAK6	Activity	Km app	8
PAK7 (KIAA1264)	Activity	Km app	9
PASK	Activity	Km app	8
PDGFRA (PDGFR alpha)	Activity	Km app	18
PDGFRA D842V	Activity	Km app	22
PDGFRA T674I	Activity	Km app	25
PDGFRA V561D	Activity	Km app	45
PDGFRB (PDGFR beta)	Activity	Km app	8
PDK1	Activity	100	16
PDK1 Direct	Activity	Km app	0
PHKG1	Activity	Km app	10
PHKG2	Activity	Km app	4
PIM1	Activity	Km app	12
PIM2	Activity	Km app	0
PKN1 (PRK1)	Activity	Km app	19
PLK1	Activity	Km app	-1
PLK2	Activity	Km app	10
PLK3	Activity	Km app	-2
PRKACA (PKA)	Activity	Km app	0
PRKCA (PKC alpha)	Activity	Km app	14
PRKCB1 (PKC beta I)	Activity	Km app	10
PRKCB2 (PKC beta II)	Activity	Km app	5
PRKCD (PKC delta)	Activity	Km app	15
PRKCE (PKC epsilon)	Activity	Km app	17
PRKCG (PKC gamma)	Activity	Km app	14
PRKCH (PKC eta)	Activity	Km app	15
PRKCI (PKC iota)	Activity	Km app	10
PRKCN (PKD3)	Activity	Km app	10
PRKCQ (PKC theta)	Activity	Km app	13
PRKCZ (PKC zeta)	Activity	Km app	8
PRKD1 (PKC mu)	Activity	Km app	12

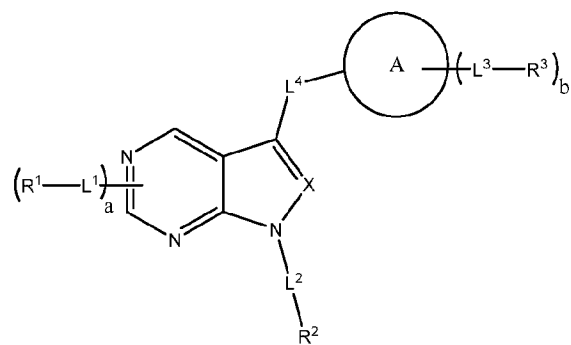
Kinase			Cmpd 3-vs-Q 1000 nM
PRKD2 (PKD2)	Activity	Km app	12
PRKG1	Activity	Km app	2
PRKG2 (PKG2)	Activity	Km app	-1
PRKX	Activity	Km app	3
PTK2 (FAK)	Activity	Km app	9
PTK2B (FAK2)	Activity	Km app	4
PTK6 (Brk)	Activity	Km app	9
RAF1 (cRAF) Y340D Y341D	Activity	100	21
RET	Activity	Km app	19
RET V804L	Activity	Km app	12
RET Y791F	Activity	Km app	25
ROCK1	Activity	Km app	-3
ROCK2	Activity	Km app	13
ROS1	Activity	Km app	40
RPS6KA1 (RSK1)	Activity	Km app	6
RPS6KA2 (RSK3)	Activity	Km app	26
RPS6KA3 (RSK2)	Activity	Km app	9
RPS6KA4 (MSK2)	Activity	Km app	6
RPS6KA5 (MSK1)	Activity	Km app	0
RPS6KA6 (RSK4)	Activity	Km app	49
RPS6KB1 (p70S6K)	Activity	Km app	10
SGK (SGK1)	Activity	Km app	6
SGK2	Activity	Km app	8
SGKL (SGK3)	Activity	Km app	4
SNF1LK2	Activity	Km app	4
SRC	Activity	Km app	4
SRC N1	Activity	Km app	15
SRMS (Srm)	Activity	Km app	71
SRPK1	Activity	Km app	2
SRPK2	Activity	Km app	10
STK22B (TSSK2)	Activity	Km app	2
STK22D (TSSK1)	Activity	Km app	13
STK23 (MSSK1)	Activity	Km app	14
STK24 (MST3)	Activity	Km app	11
STK25 (YSK1)	Activity	Km app	5
STK3 (MST2)	Activity	Km app	-9
STK4 (MST1)	Activity	Km app	2
SYK	Activity	Km app	-2
TAOK2 (TAO1)	Activity	Km app	2
TBK1	Activity	Km app	-2

Kinase			Cmpd 3-vs-Q 1000 nM
TEK (Tie2)	Activity	Km app	-7
TXK	Activity	Km app	78
TYK2	Activity	Km app	-4
TYRO3 (RSE)	Activity	Km app	22
YES1	Activity	Km app	30
ZAP70	Activity	Km app	10

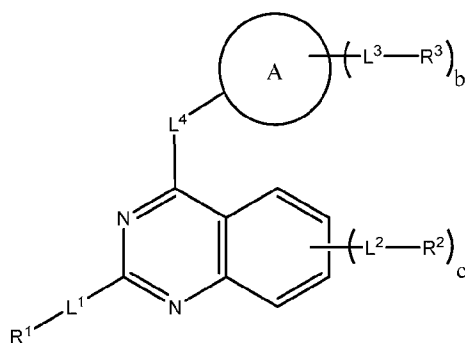
[0353] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, one of skill in the art will appreciate that certain changes and modifications may be practiced within the scope of the appended claims. In addition, each reference provided herein is incorporated by reference in its entirety to the same extent as if each reference was individually incorporated by reference. Where a conflict exists between the instant application and a reference provided herein, the instant application shall dominate.

WHAT IS CLAIMED IS:

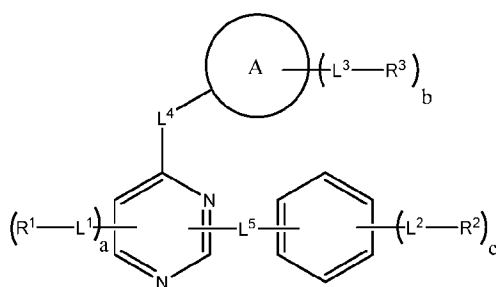
1. A compound having the formula:



(I);



(II); or



(III);

wherein X is =N- or =C(-L⁶-R⁶)-;

Ring A is, in each instance, independently selected from cycloalkyl,

heterocycloalkyl, aryl, or heteroaryl;

L¹, L², L³, L⁴, L⁵, and L⁶ are, in each instance, independently selected from a

bond, -C(O)-, -C(O)N(R⁷)-, -C(O)O-, -S(O)_g-, -S(O)₂N(R⁷)-, -O-,

-N(R⁷)-, -N(R⁷)C(O)N(R⁸)-, substituted or unsubstituted alkylene,

substituted or unsubstituted heteroalkylene, substituted or

unsubstituted cycloalkylene, substituted or unsubstituted

heterocycloalkylene, substituted or unsubstituted arylene, or

substituted or unsubstituted heteroarylene, wherein g is an integer from

0 to 2;

$R^1, R^2, R^3, R^4, R^5, R^6, R^7$, and R^8 are, in each instance, independently selected from hydrogen, halogen, -CN, -OH, -NH₂, -COOH, -CONH₂, -NO₂, -SH, -SO₂Cl, -SO₃H, -SO₄H, -SO₂NH₂, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted aryl, or substituted or unsubstituted heteroaryl;

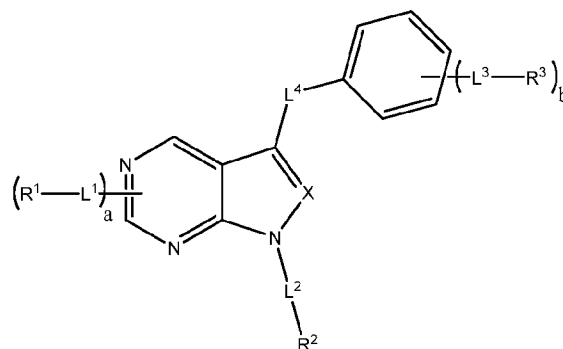
a is an integer from 0 to 2;

b is an integer from 0 to 5;

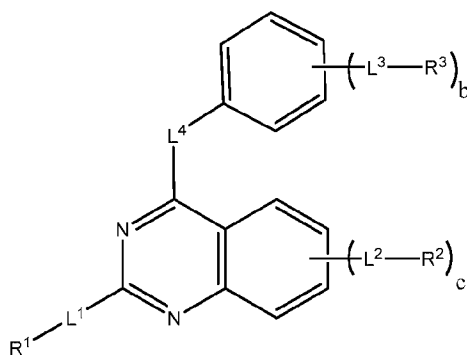
c is an integer from 0 to 4, and

the salts and isomers thereof.

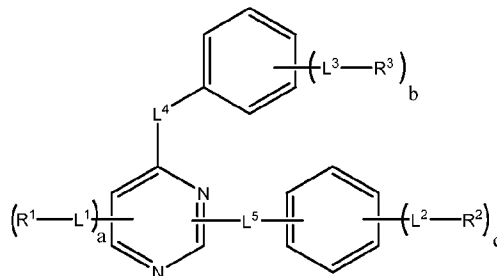
2. The compound of claim 1, having the formula:



(IV);



(V); or



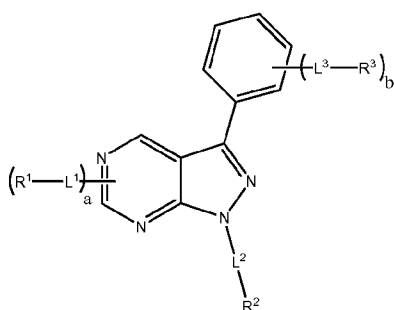
(VI).

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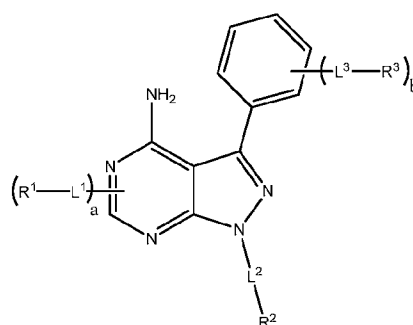
1 3. The compound of claim 2, wherein L^1 , L^2 , L^3 , L^4 , L^5 , and L^6 are, in
 2 each instance, independently a bond, -NH-, or substituted or unsubstituted C_1 - C_5 alkylene.

1 4. The compound of claim 2, wherein L^6 is a bond, -NH-, or
 2 unsubstituted C_1 - C_5 alkylene.

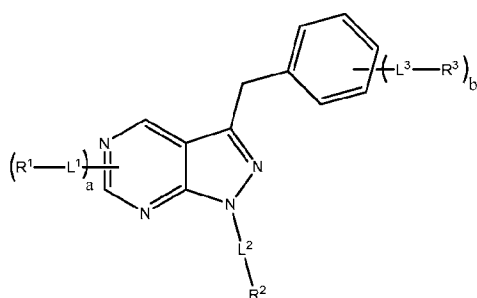
1 5. The compound of claim 1, having the formula:



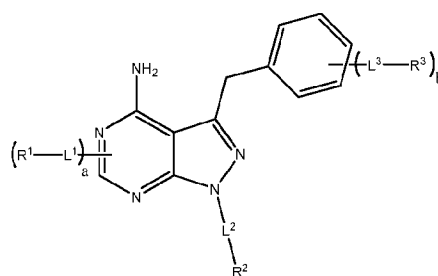
(VII);



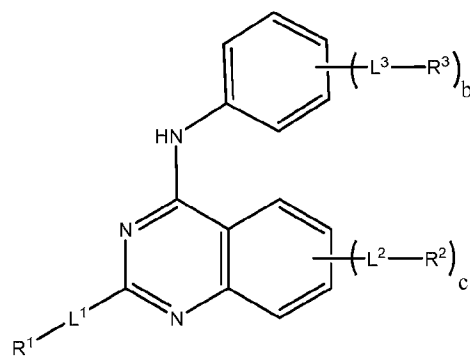
(VIII);



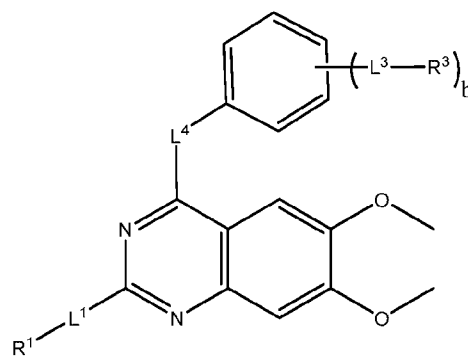
(IX);



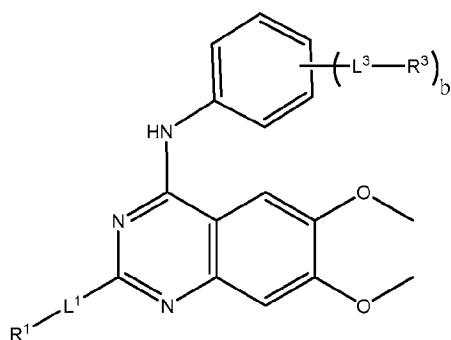
(X);



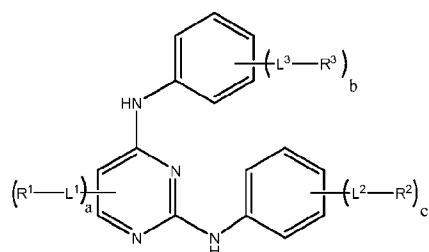
(XI);



(XII);

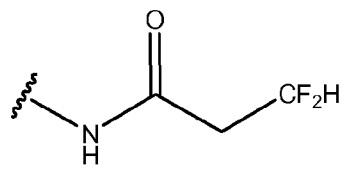
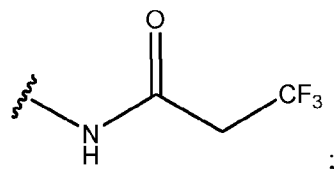
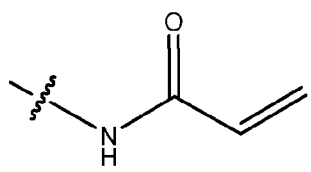
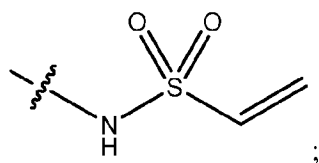


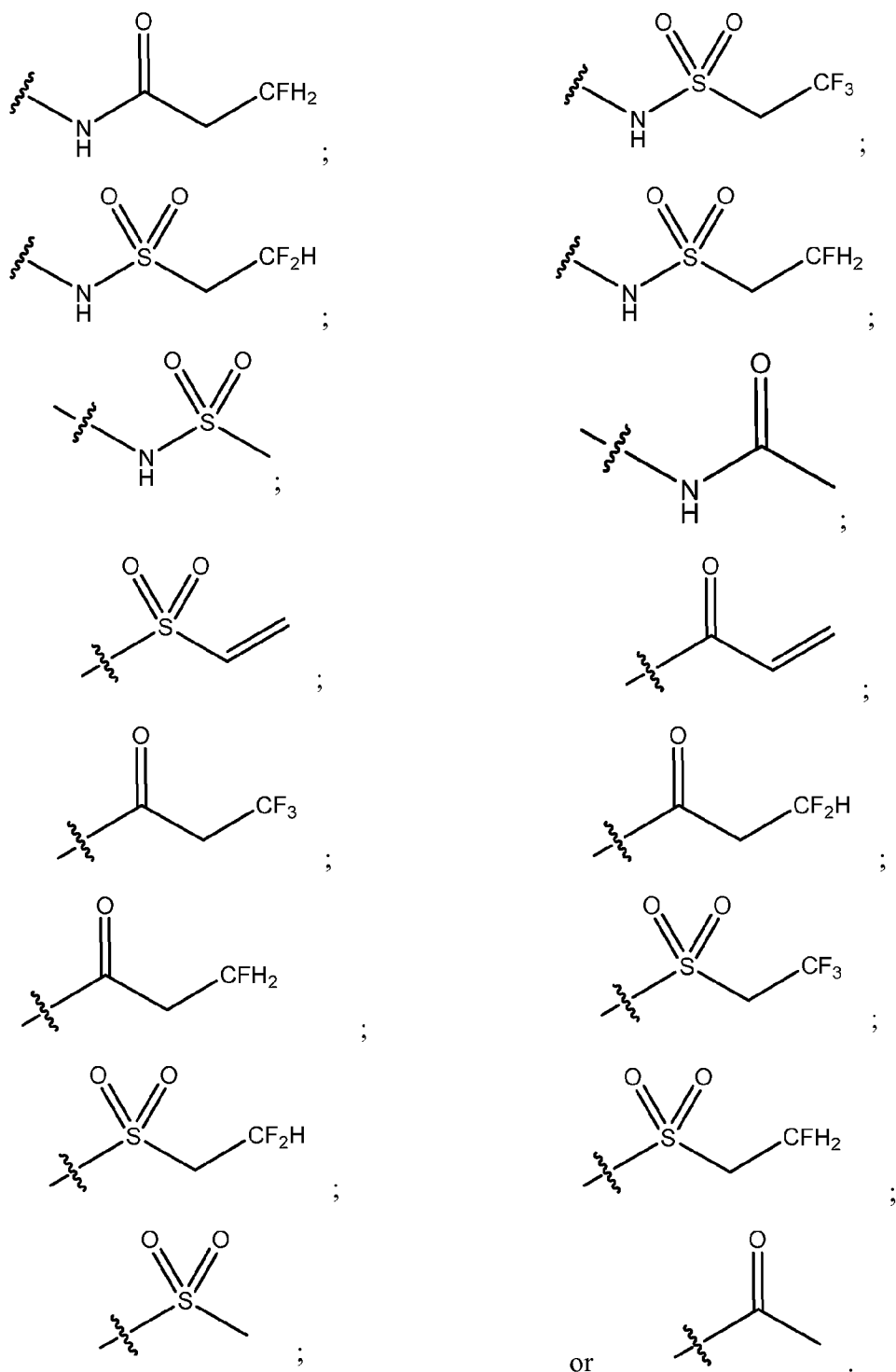
(XIII); or



(XIV).

- 1 6. The compound of claim 5, wherein at least one -L³-R³ is an
2 electrophilic moiety.
- 1 7. The compound of claim 6, wherein
2 L³ is a bond, -NH-, substituted or unsubstituted alkylene, or substituted or
3 unsubstituted heteroalkylene; and
4 R³ is a substituted or unsubstituted alkyl, substituted or unsubstituted
5 heteroalkyl, or halogen.
- 1 8. The compound of claim 6, wherein
2 L³ is -C(O)-, -S(O)₂-, -NHC(O)-, or -NHS(O)₂-; and
3 R³ is unsubstituted alkyl or alkyl substituted with chloro, fluoro, methyl,
4 difluoromethyl, or trifluoromethyl.
- 1 9. The compound of claim 8, wherein R³ is ethenyl, ethyl, 2,2,2-
2 trichloroethyl, 2,2-dichloroethyl, 2-chloroethyl, 2,2,2-trifluoroethyl, 2,2-difluoroethyl, or 2-
3 fluoroethyl, propyl, isopropyl, 1-propenyl, or 2-propenyl.
- 1 10. The compound of claim 9, wherein -L³-R³ is:





- 1 11. The compound of one of claims 1 to 10, wherein,
 2 L¹ is a bond; and
 3 R¹ is hydrogen or NH₂.

- 1 12. The compound of claim 11, wherein,
 2 L¹ is a bond; and

3 R^1 is hydrogen.

1 13. The compound of claim 11, wherein,

2 L^1 is a bond; and

3 R^1 is NH_2 .

1 14. The compound of one of claims 1 to 10, wherein

2 L^2 is a bond; and

3 R^2 is methyl, ethyl, propyl, isopropyl, butyl, tert-butyl, pentyl, cyclopentyl,
4 hexyl, cyclohexyl, methoxy, ethoxy, propoxy, or butoxy.

1 15. The compound of claim 14, wherein R^2 is isopropyl or cyclopentyl.

1 16. The compound of claim 15, wherein R^2 is isopropyl.

1 17. The compound of claim 15, wherein R^2 is cyclopentyl.

1 18. The compound of claim 14, wherein R^2 is methoxy.

1 19. The compound of one of claims 1 to 10, wherein

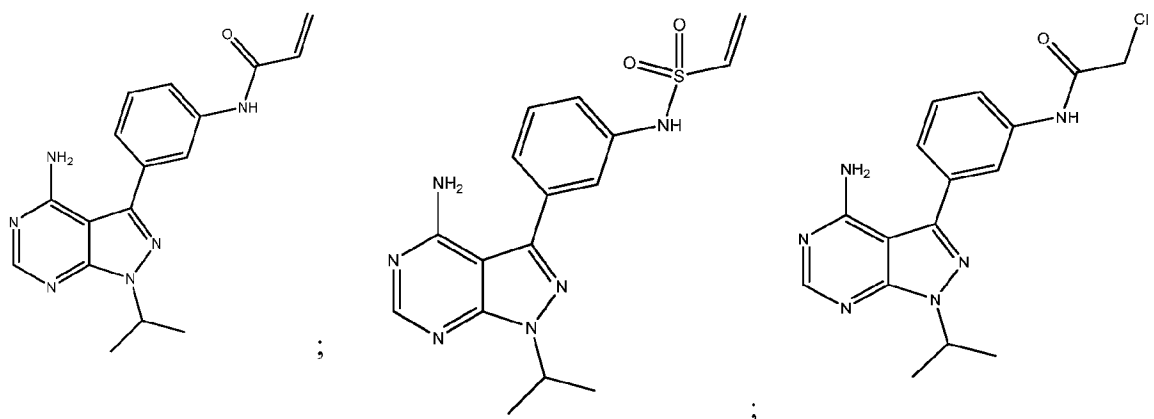
2 c is 2;

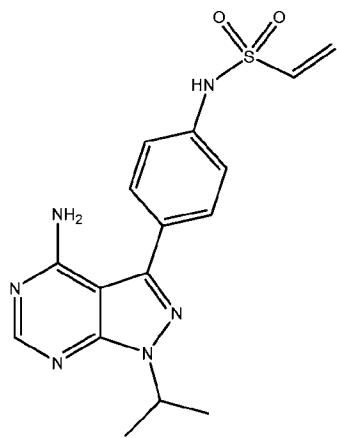
3 L^2 is a bond, and

4 R^2 is selected from methoxy, ethoxy, propoxy, or butoxy.

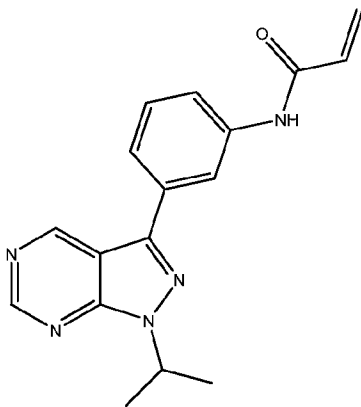
1 20. The compound of claim 19, wherein R^2 is methoxy.

1 21. The compound of claim 1, having the formula:

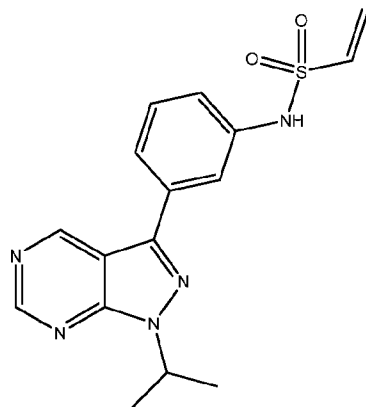




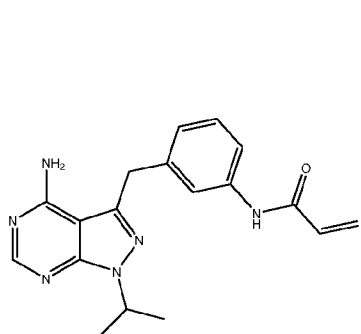
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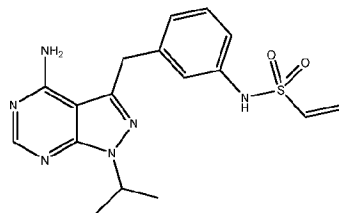
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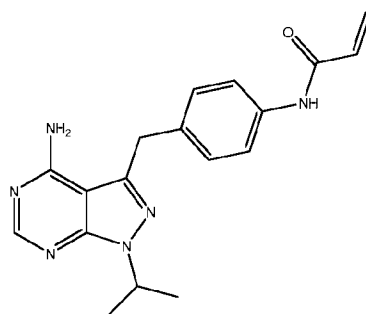
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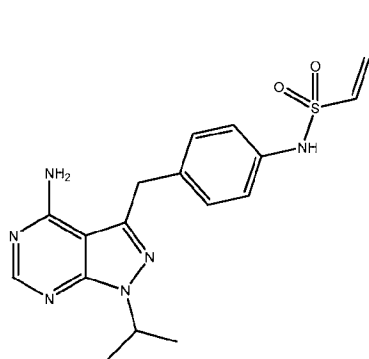
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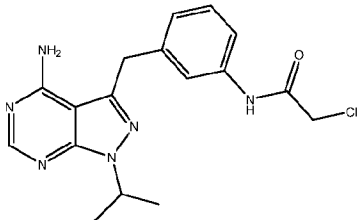
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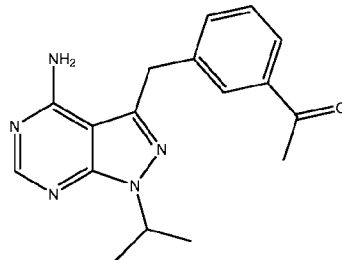
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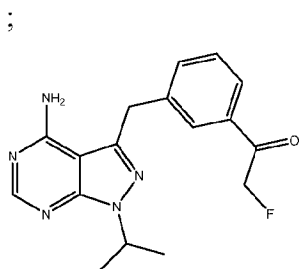
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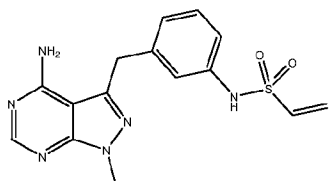
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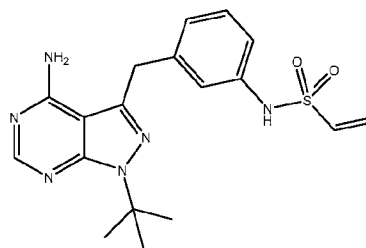
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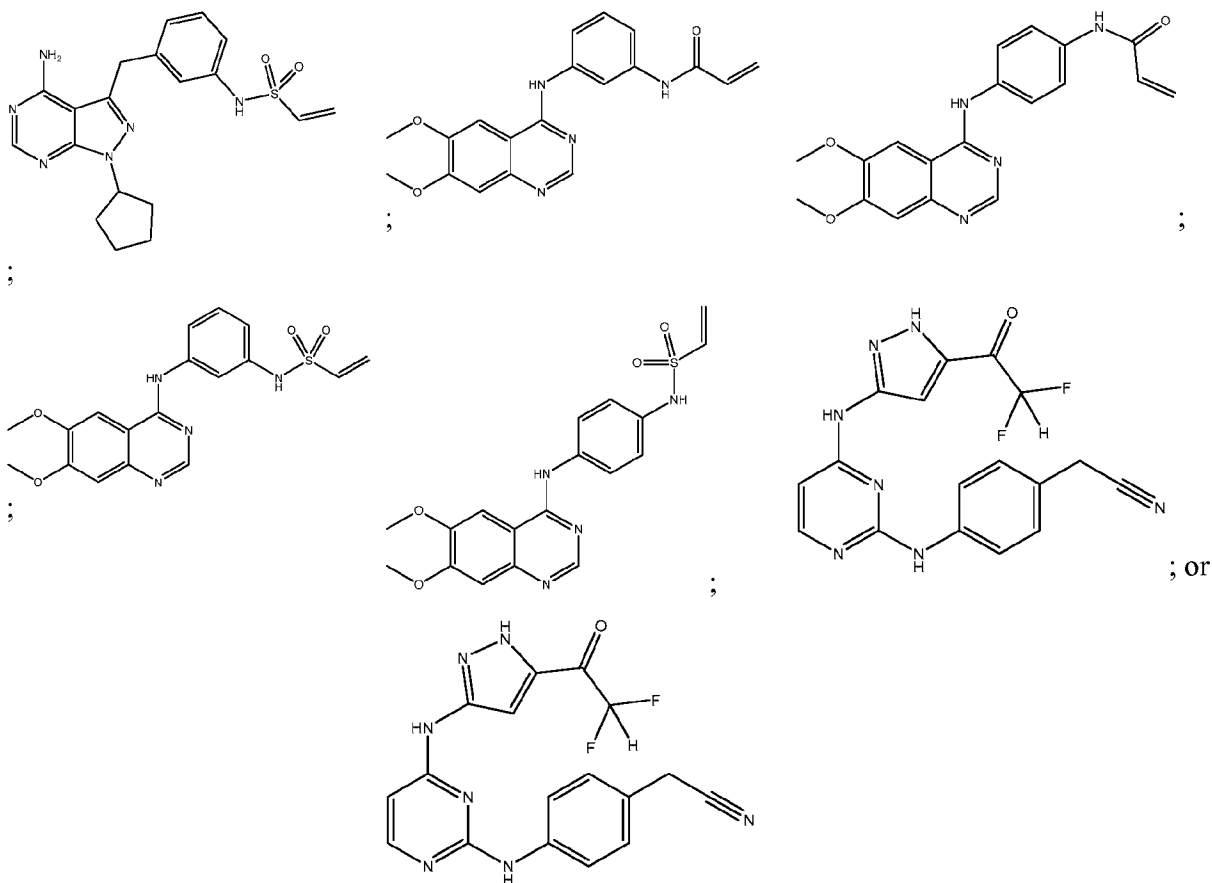
;



;



;



1 22. A pharmaceutical composition comprising a compound of one of
2 claims 1 to 10 or 21 and a pharmaceutically acceptable excipient.

1 23. A recombinant kinase comprising a cysteine substitution at a
2 gatekeeper amino acid position.

1 24. The recombinant kinase of claim 23, wherein the k_{cat} activity of the
2 recombinant kinase is not substantially lower than the k_{cat} activity of the corresponding wild-
3 type kinase.

1 25. The recombinant kinase of claim 23, wherein the K_m binding affinity
2 for ATP of the recombinant kinase is not substantially lower than the K_m binding affinity for
3 ATP of the corresponding wild type kinase.

1 26. The recombinant kinase of claim 23, wherein the recombinant kinase is
2 selected from Src; MOK; Sgk494; Yak/Yrk; SRPK1; CDK; DICTY-I; PAK/STE20; or
3 Ctrl/DPYK1.

1 27. The recombinant kinase of claim 23, wherein the recombinant kinase is
2 a recombinant Src and the gatekeeper amino acid position is T338.

1 28. A co-crystal comprising the recombinant kinase of claim 23 and a
2 compound of claims 1 to 10 and 21.

1 29. The recombinant kinase of claim 23, having a greater catalytic
2 efficiency than the corresponding wild type kinase.

1 30. The recombinant kinase of claim 23, further comprising an additional
2 amino acid substitution at VAL323.

1 31. The recombinant kinase of claim 30, wherein the catalytic efficiency of
2 the recombinant kinase having an additional amino acid substitution at VAL323 is greater
3 than the catalytic efficiency of the corresponding recombinant kinase that does not have an
4 additional amino acid substitution at VAL323.

1 32. The recombinant kinase of claims 29 or 31, wherein the catalytic
2 efficiency is measured as the ratio of k_{cat}/K_m .

1 33. An isolated nucleic acid comprising a polynucleotide sequence
2 encoding a recombinant kinase of claim 23.

1 34. An expression cassette comprising the nucleic acid of claim 33.

1 35. The expression cassette of claim 34, wherein the expression cassette is
2 a recombinant viral vector.

1 36. The expression cassette of claim 34, wherein the expression cassette is
2 inside of a host cell.

1 37. The expression cassette of claim 36, wherein the cell is selected from
2 mammalian, non-mammalian, mouse, rat, or human.

1 38. The recombinant kinase of claim 23, wherein the recombinant kinase is
2 inside a cell.

1 39. The recombinant kinase of claim 38, wherein the cell is selected from
2 mammalian, non-mammalian, mouse, rat, or human.

1 40. A method of inhibiting a recombinant kinase of claim 23, comprising
2 contacting the recombinant kinase with an effective amount of an inhibitor, thereby inhibiting
3 the recombinant kinase.

1 41. The method of claim 40, wherein the inhibitor is capable of forming a
2 covalent bond to the cysteine at the gatekeeper amino acid position of the recombinant
3 kinase.

1 42. The method of claim 40, wherein the inhibitor is a compound of one of
2 claims 1 to 10 or 21.

1 43. The method of claim 40, further comprising determining a level of
2 inhibition for the recombinant kinase.

1 44. The method of claim 43, wherein the determining of said level of
2 inhibition for the recombinant kinase comprises
3 determining an amount of enzymatic activity of the recombinant kinase in the
4 presence of the inhibitor;
5 determining an amount of enzymatic activity of the recombinant kinase in the
6 absence of the inhibitor; and
7 comparing the amount of enzymatic activity of the recombinant kinase in the
8 presence of the inhibitor with the amount of enzymatic activity of the
9 recombinant kinase in the absence of the inhibitor, thereby determining
10 a level of inhibition for the recombinant kinase.

1 45. The method of claim 44, wherein the enzymatic activity is selected
2 from phosphorylation of a non-specific protein target, phosphorylation of a specific protein
3 target, consumption of ATP, or cell growth.

1 46. The method of claim 40, wherein said recombinant kinase is in a cell.

1 47. The method of claim 46, further comprising determining a function of
2 the recombinant kinase in the cell, by:
3 determining an amount of enzymatic activity of the recombinant kinase in the
4 presence of the inhibitor in the cell;
5 determining an amount of enzymatic activity of the recombinant kinase in the
6 absence of the inhibitor in the cell; and

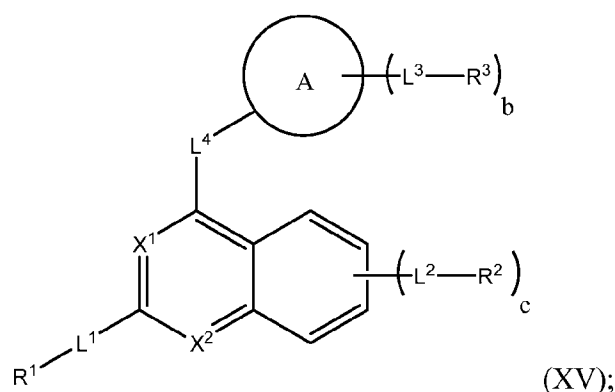
7 comparing the amount of enzymatic activity of the recombinant kinase in the
 8 presence of the inhibitor with the amount of enzymatic activity of the
 9 recombinant kinase in the absence of the inhibitor, thereby determining
 10 a function of the recombinant kinase in the cell.

1 48. The method of claim 47, wherein the enzymatic activity is selected
 2 from phosphorylation of a specific protein target.

1 49. The method of claims 40, 41, and 43-47, wherein the recombinant
 2 kinase is selected from Src; MOK; Sgk494; Lrrk-2; Yak/Yrk; SRPK1; CDK; DICTY-I;
 3 PAK/STE20; or Ctrl/DPYK1.

1 50. The method of claim 49, wherein the recombinant kinase is Src.

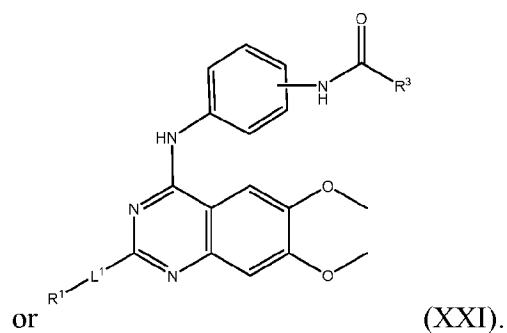
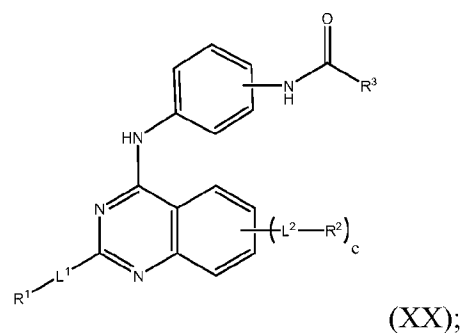
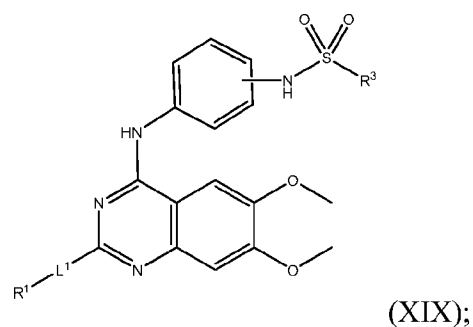
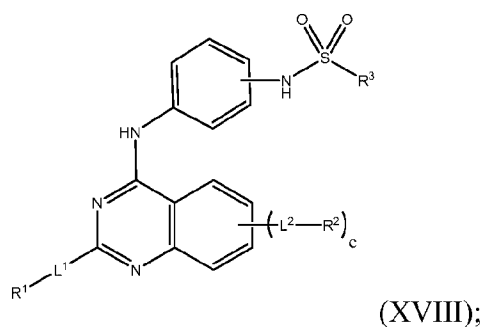
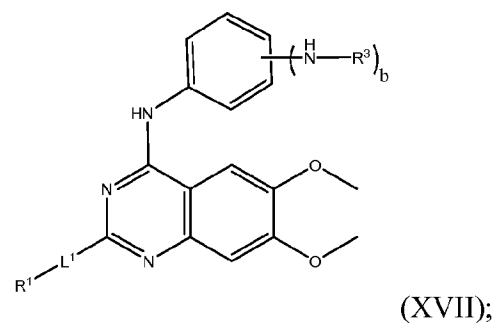
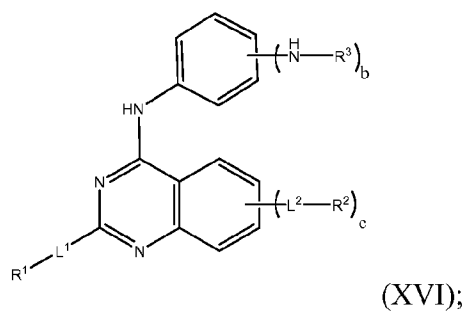
1 51. A compound having the formula:



2 wherein X^1 and X^2 are, in each instance, independently $=N-$ or $=C(-L^6-R^6)-$;
 3 Ring A is, in each instance, independently selected from cycloalkyl,
 4 heterocycloalkyl, aryl, or heteroaryl;
 5 L^1 , L^2 , L^3 , and L^4 are, in each instance, independently selected from a bond,
 6 $-C(O)-$, $-C(O)N(R^7)-$, $-C(O)O-$, $-S(O)_g-$, $-S(O)_2N(R^7)-$, $-O-$,
 7 $-N(R^7)-$, $-N(R^7)C(O)N(R^8)-$, substituted or unsubstituted alkylene,
 8 substituted or unsubstituted heteroalkylene, substituted or
 9 unsubstituted cycloalkylene, substituted or unsubstituted
 10 heterocycloalkylene, substituted or unsubstituted arylene, or
 11 substituted or unsubstituted heteroarylene, wherein g is an integer from
 12 0 to 2;
 13 R^1 , R^2 , R^3 , R^6 , R^7 , and R^8 are, in each instance, independently selected from
 14 hydrogen, halogen, $-CN$, $-OH$, $-NH_2$, $-COOH$, $-CONH_2$, $-NO_2$, $-SH$,
 15 $-SO_2Cl$, $-SO_3H$, $-SO_4H$, $-SO_2NH_2$, substituted or unsubstituted alkyl,

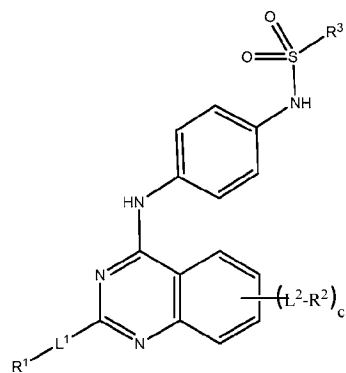
- 16 substituted or unsubstituted heteroalkyl, substituted or unsubstituted
 17 cycloalkyl, substituted or unsubstituted heterocycloalkyl, substituted or
 18 unsubstituted aryl, or substituted or unsubstituted heteroaryl;
 19 b is an integer from 0 to 5; and
 20 c is an integer from 0 to 4.

1 52. The compound of claim 51, having the formula:

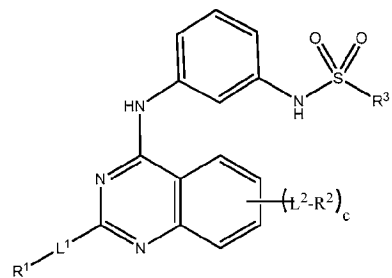


1 53. The compound of claim 51, having the formula:

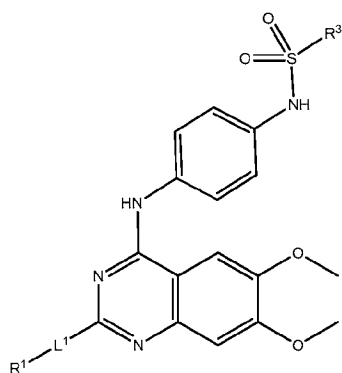
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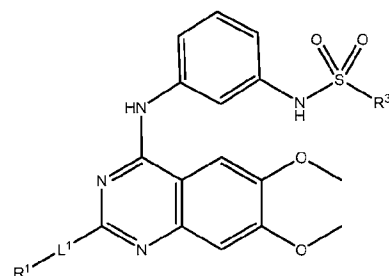
(XXII);



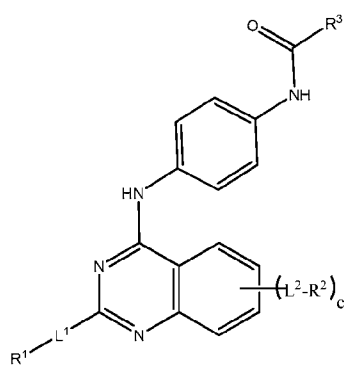
(XXIII);



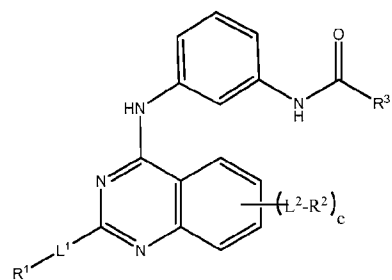
(XXIV);



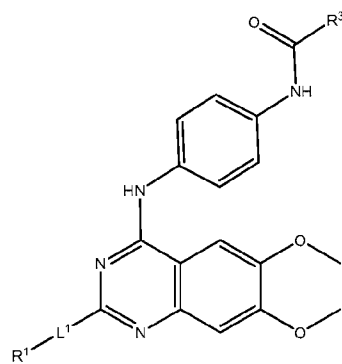
(XXV);



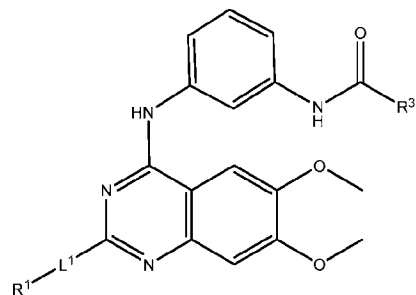
(XXVI);



(XXVII)



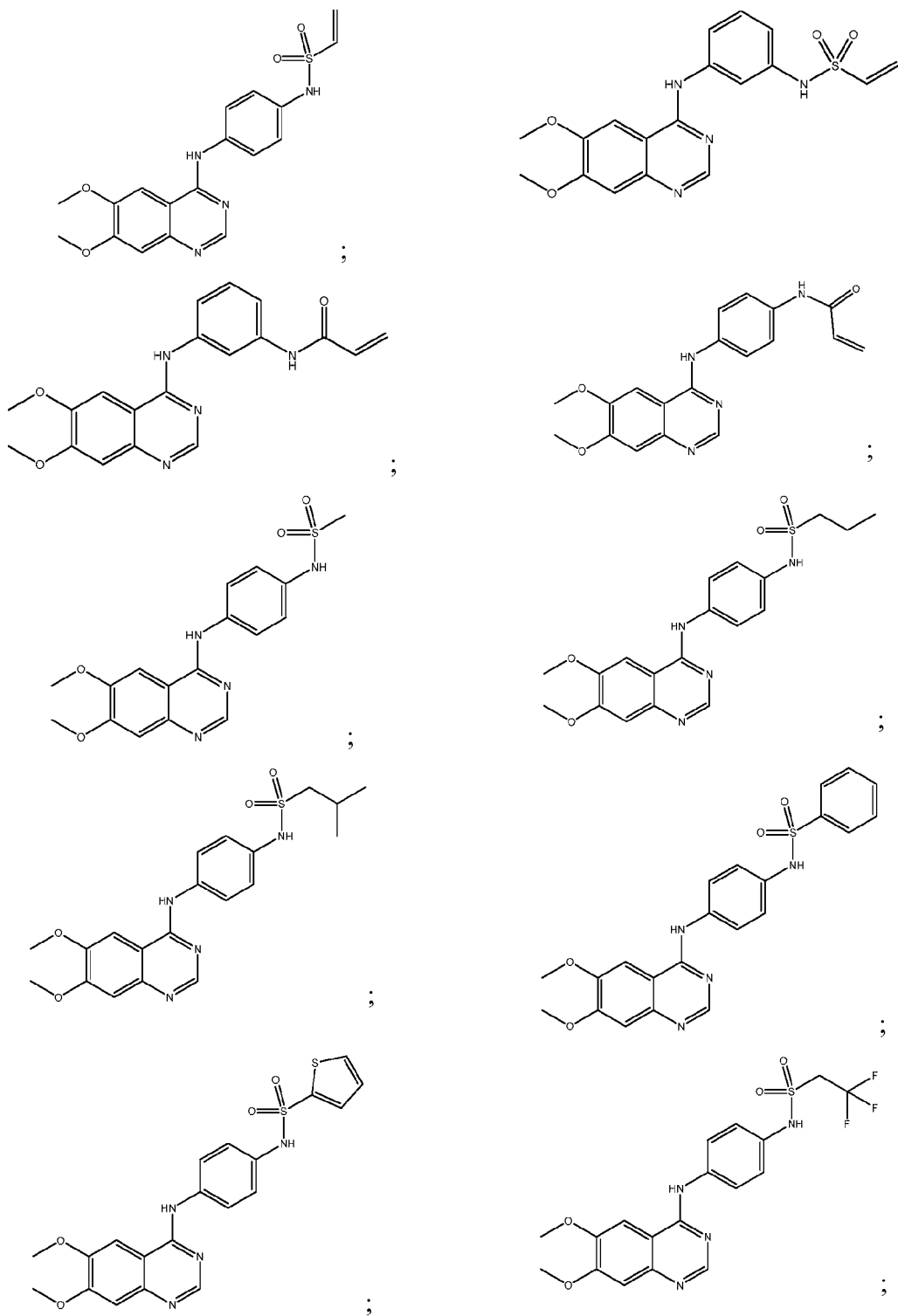
(XXVIII); or

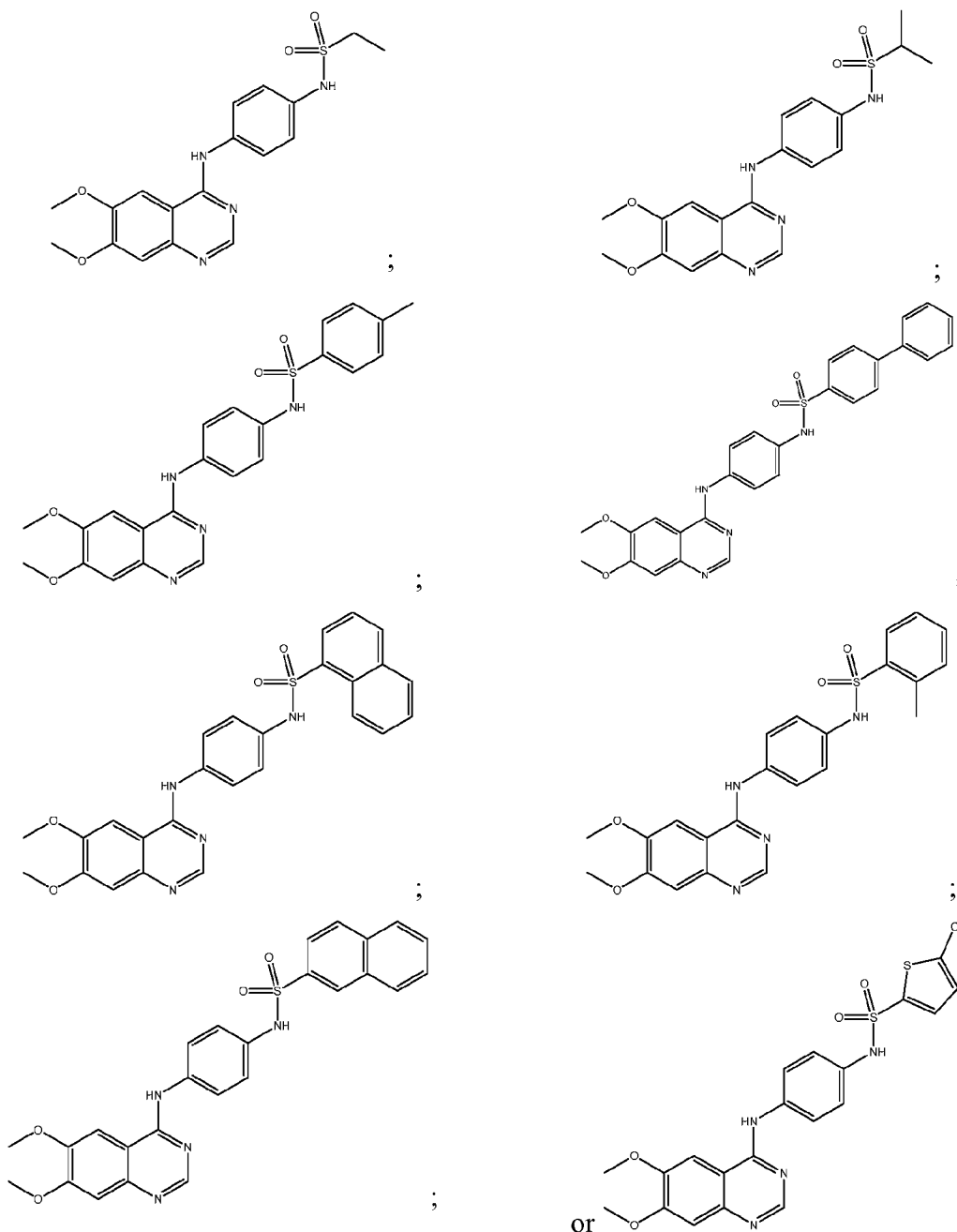


(XXIX).

1

54. The compound of claim 51, having the formula:





1 55 The compound of claim 53, wherein
 2 L^3 selected from a bond, -NH-, -C(O)-, -S(O₂)-, substituted or unsubstituted
 3 alkylene, or substituted or unsubstituted heteroalkylene; and
 4 R^3 selected from substituted or unsubstituted alkyl, substituted or
 5 unsubstituted heteroalkyl, or halogen.

1 56. The compound of claim 55, wherein R^3 is methyl; difluoromethyl;
 2 trifluoromethyl; ethenyl; ethyl; 2,2,2-trichloroethyl; 2,2-dichloroethyl; 2-chloroethyl; 2,2,2-
 3 trifluoroethyl; 2,2-difluoroethyl; or 2-fluoroethyl; propyl; isopropyl; 1-propenyl; 2-propenyl;

4 butyl; tert-butyl; naphthyl; thiophene; 2-chloro-thiophene; phenyl; 2-methyl-phenyl; 3-methyl-
5 phenyl; 4-methyl-phenyl; 2-phenyl-phenyl; 3-phenyl-phenyl; 4-phenyl-phenyl 2-chloro-
6 thiophene; or 3-chloro-thiophene.

1 57. A pharmaceutical composition comprising a compound of any one of
2 claims 51 to 56 and a pharmaceutically acceptable excipient.

1 58. A method of inhibiting a Lrrk-2 kinase, comprising contacting the
2 Lrrk-2 kinase with an effective amount of a Lrrk-2 inhibitor, thereby inhibiting the
3 recombinant Lrrk-2 kinase.

1 59. The method of inhibiting a LrrK-2 kinase, wherein the inhibitor is a
2 compound of one of claims 51 to 56.

1 60. A method of forming the recombinant kinase of claim 23, comprising
2 transforming a cell with the nucleic acid of claim 33, thereby forming the recombinant kinase
3 of claim 23.

1 61. A method of treating a kinase-associated disease or condition, in a
2 patient in need thereof, said method comprising administering to said patient a therapeutically
3 effective amount of a compound of one of claims 1 to 10 or 21, thereby treating a kinase-
4 associated disease or condition.

1 62. The method of claim 61, wherein the kinase-associated disease or
2 condition is selective from cancer, immunological disorders, neurological disorders,
3 neurodegenerative disorders, infections, metabolic diseases, Leishmania major, zoonotic
4 cutaneous leishmaniasis, Plasmodium falciparum, malaria, Trichomonas vaginalis, and
5 trichomiasis.

1 63. The method of claim 62, wherein the cancer is selected from neoplasm
2 or malignant tumors found in mammals; leukemia; carcinomas and sarcomas; cancer of the
3 brain, breast, cervix, colon, head and neck, liver, kidney, lung, non-small cell lung, ovary,
4 testicle, stomach, uterus; melanoma; mesothelioma; Medulloblastoma; Hodgkin's Disease,
5 Non-Hodgkin's Lymphoma; multiple myeloma; neuroblastoma; rhabdomyosarcoma; primary
6 thrombocytosis; primary macroglobulinemia; primary brain tumors; malignant pancreatic
7 insulanoma; malignant carcinoid; urinary bladder cancer; premalignant skin lesions;
8 lymphomas; thyroid cancer; neuroblastoma; esophageal cancer; genitourinary tract cancer;

9 malignant hypercalcemia; endometrial cancer; adrenal cortical cancer; neoplasms of the
10 endocrine and exocrine pancreas; or prostate cancer.

1 64. The method of claim 61, wherein the disease or condition is a
2 neurodegenerative disease selective from Parkinson's disease.

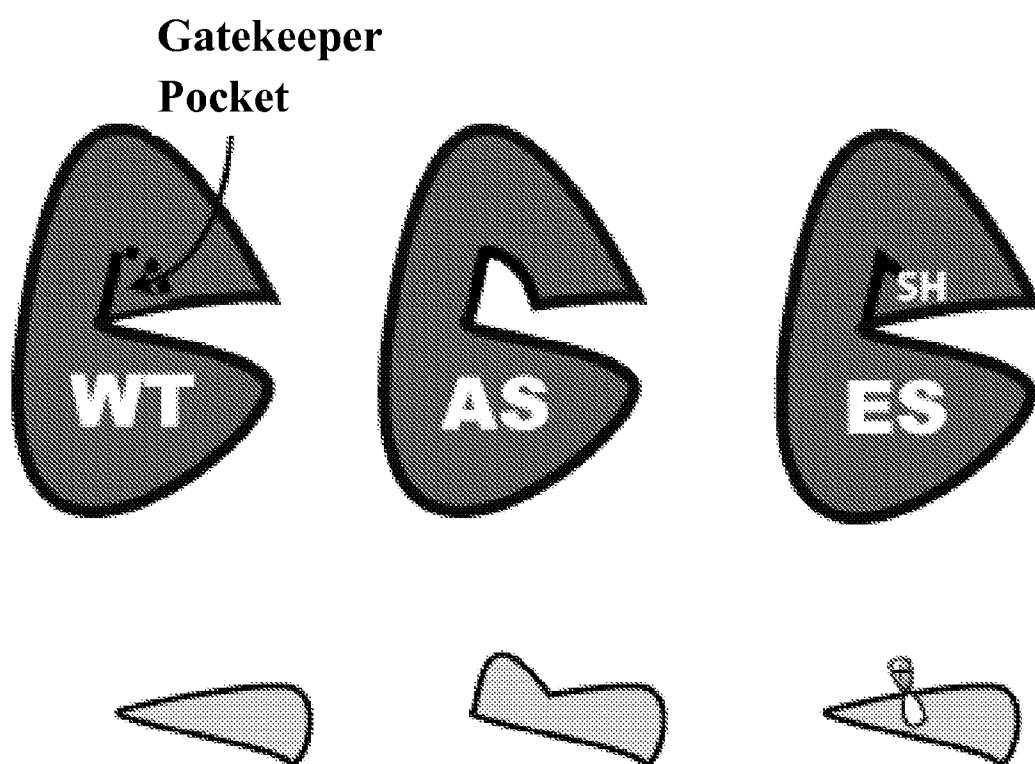
1 65. A method of treating a Lrrk-2-associated disease or condition, in a
2 patient in need thereof, said method comprising administering to said patient a therapeutically
3 effective amount of a compound of one of claims 51 to 56, thereby treating a Lrrk-2-
4 associated disease or condition.

1 66. The method of claim 65, wherein the disease or condition is a
2 neurodegenerative disease selected from Parkinson's Disease.

1 67. A kit comprising,
2 a recombinant kinase of claim 23 or a nucleic acid of claim 33;
3 and instructions for using the kit.

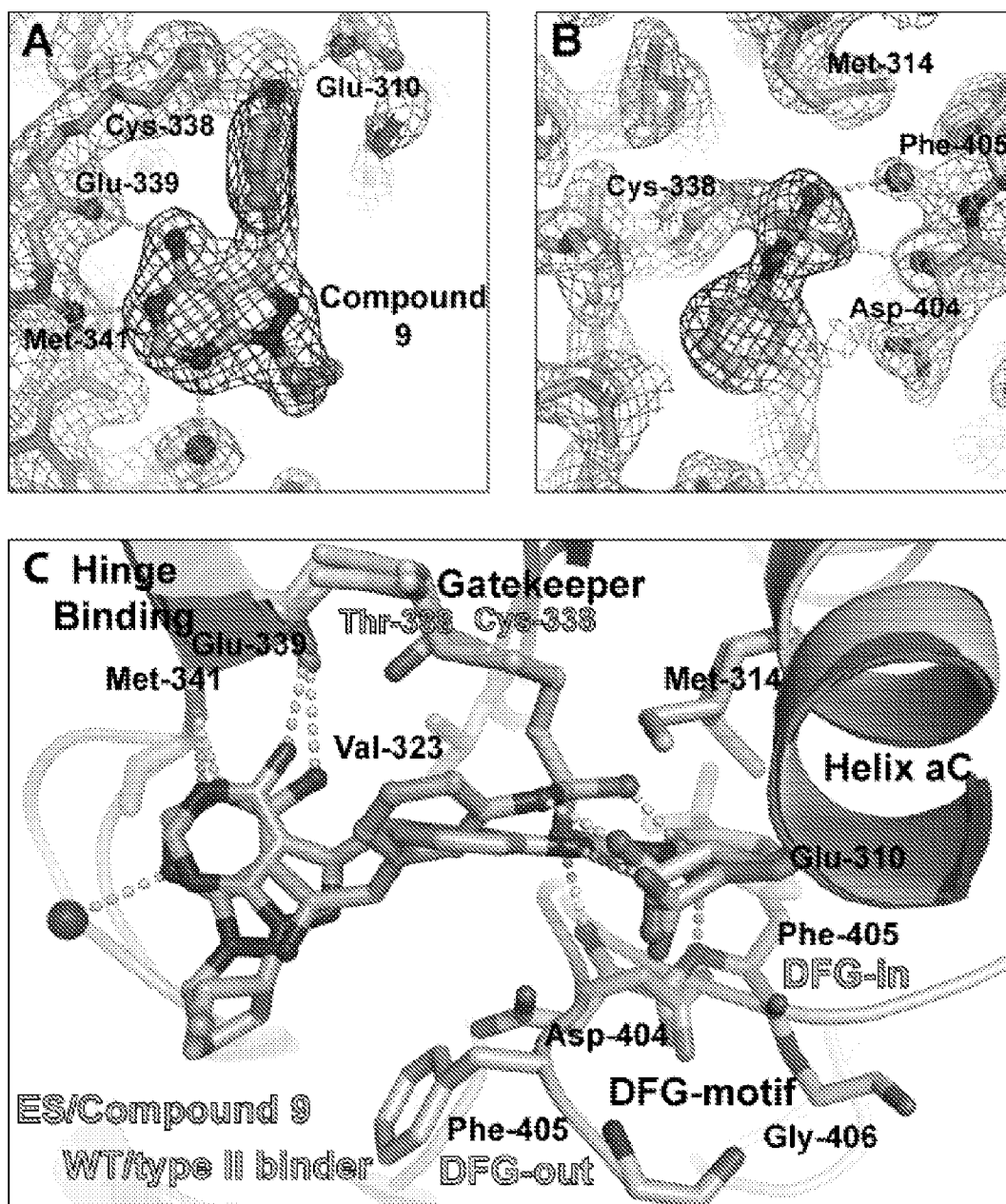
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FIG. 1



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FIG. 2



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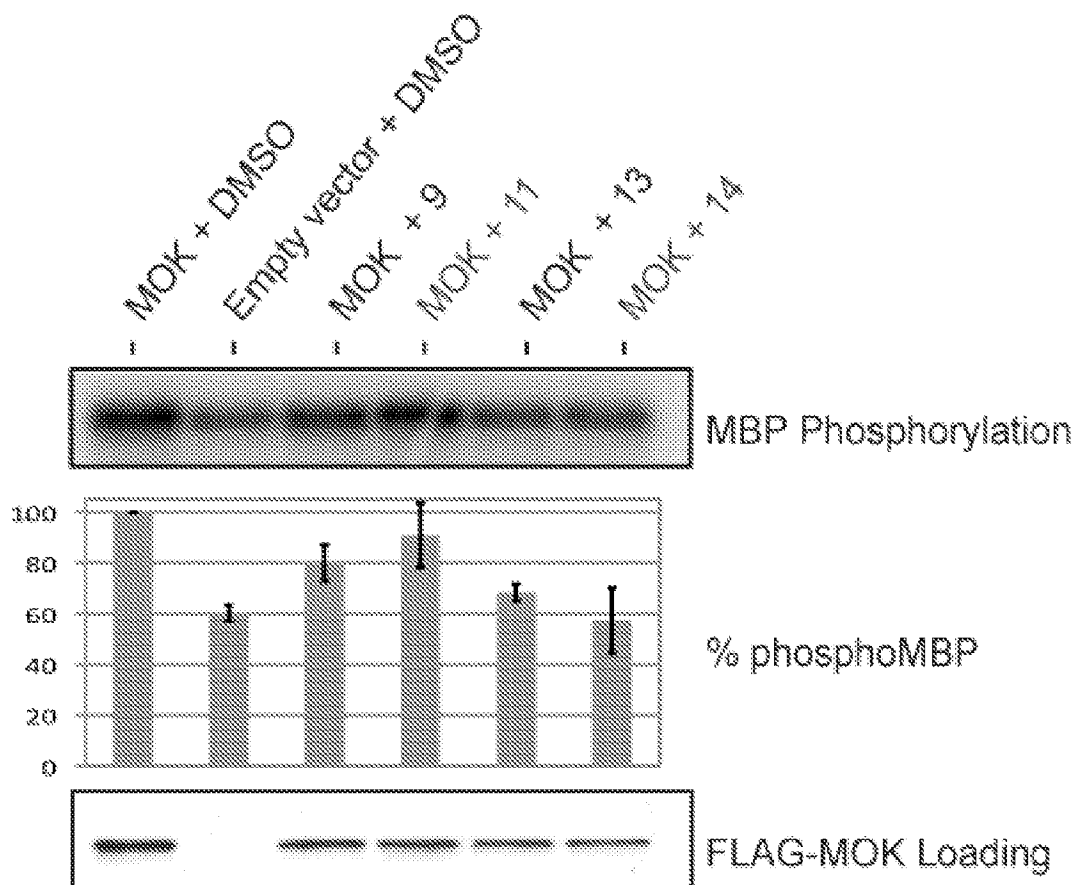
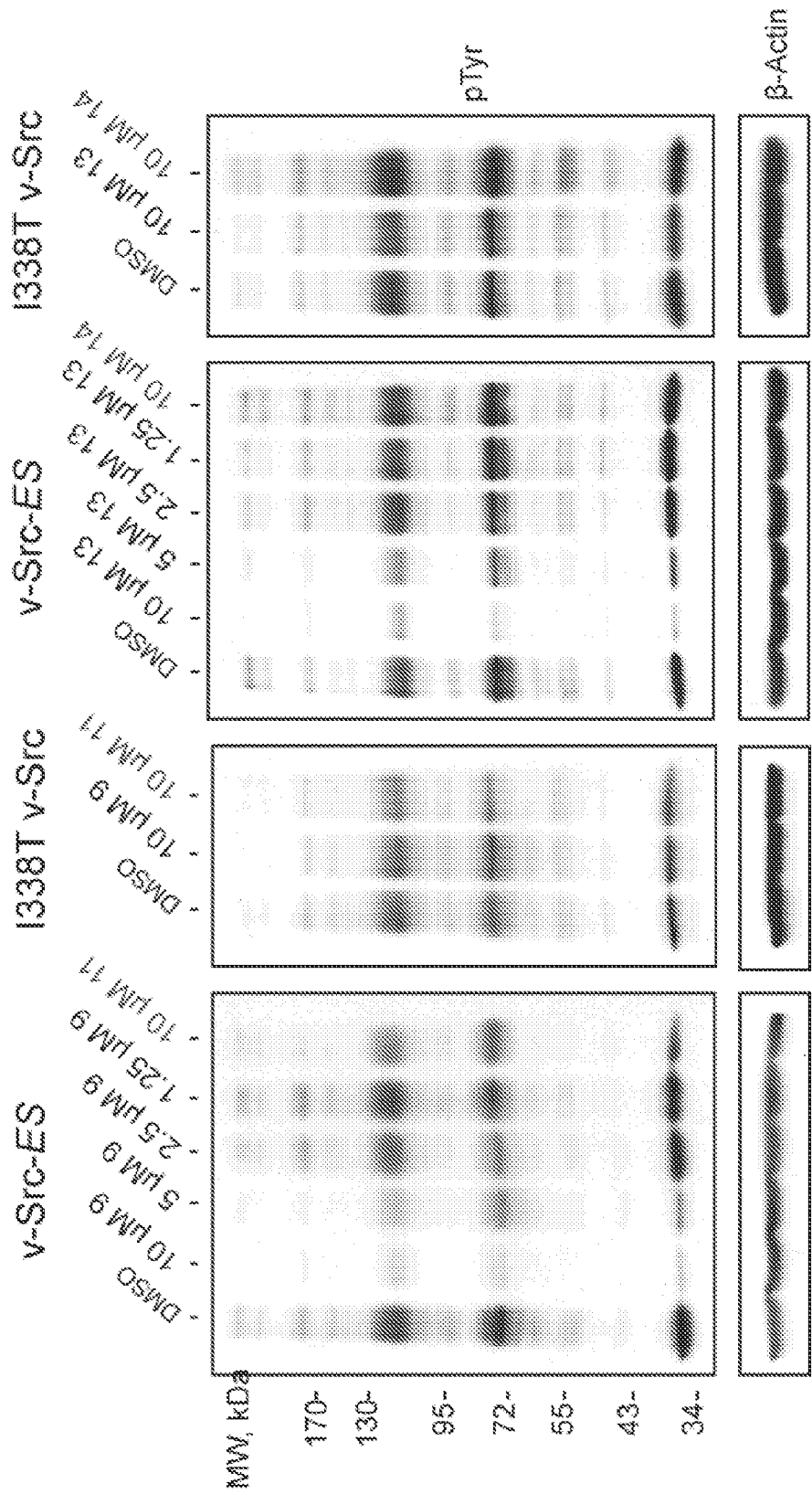
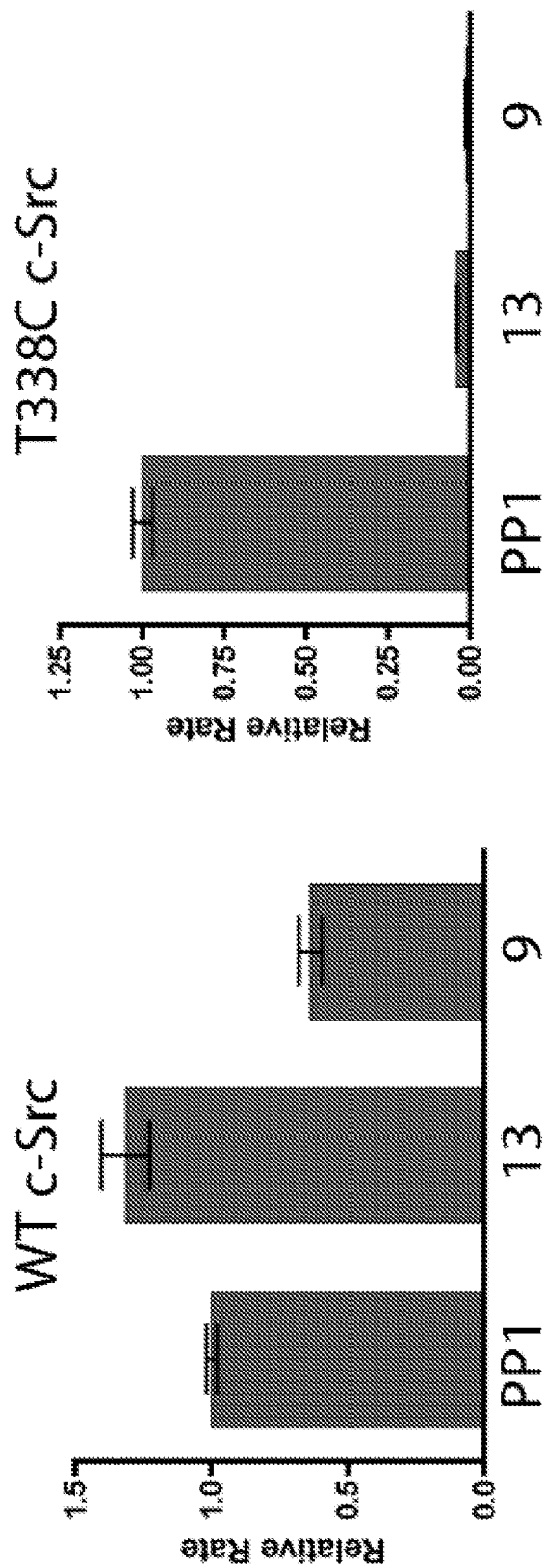
FIG. 3

FIG. 4

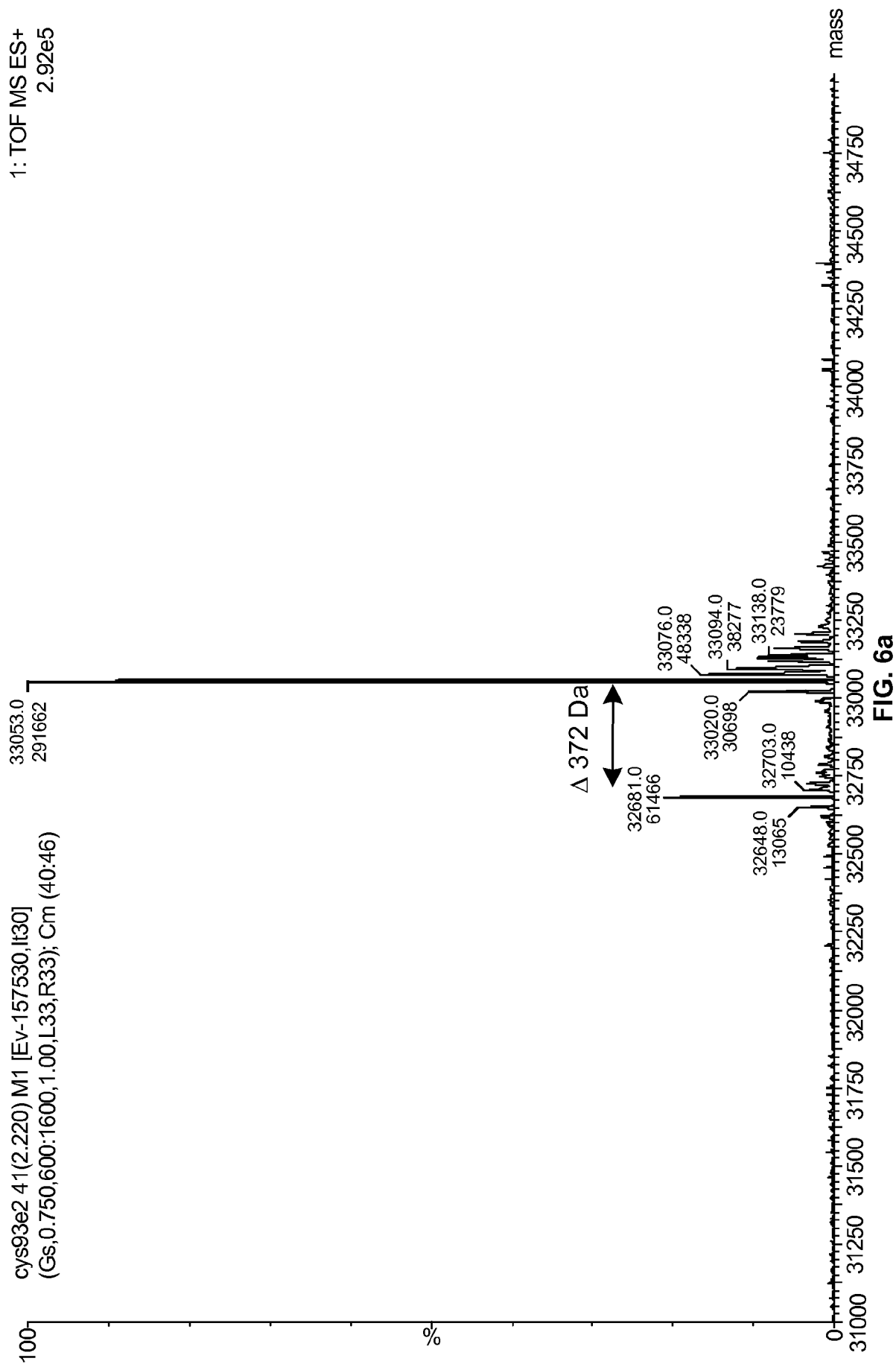


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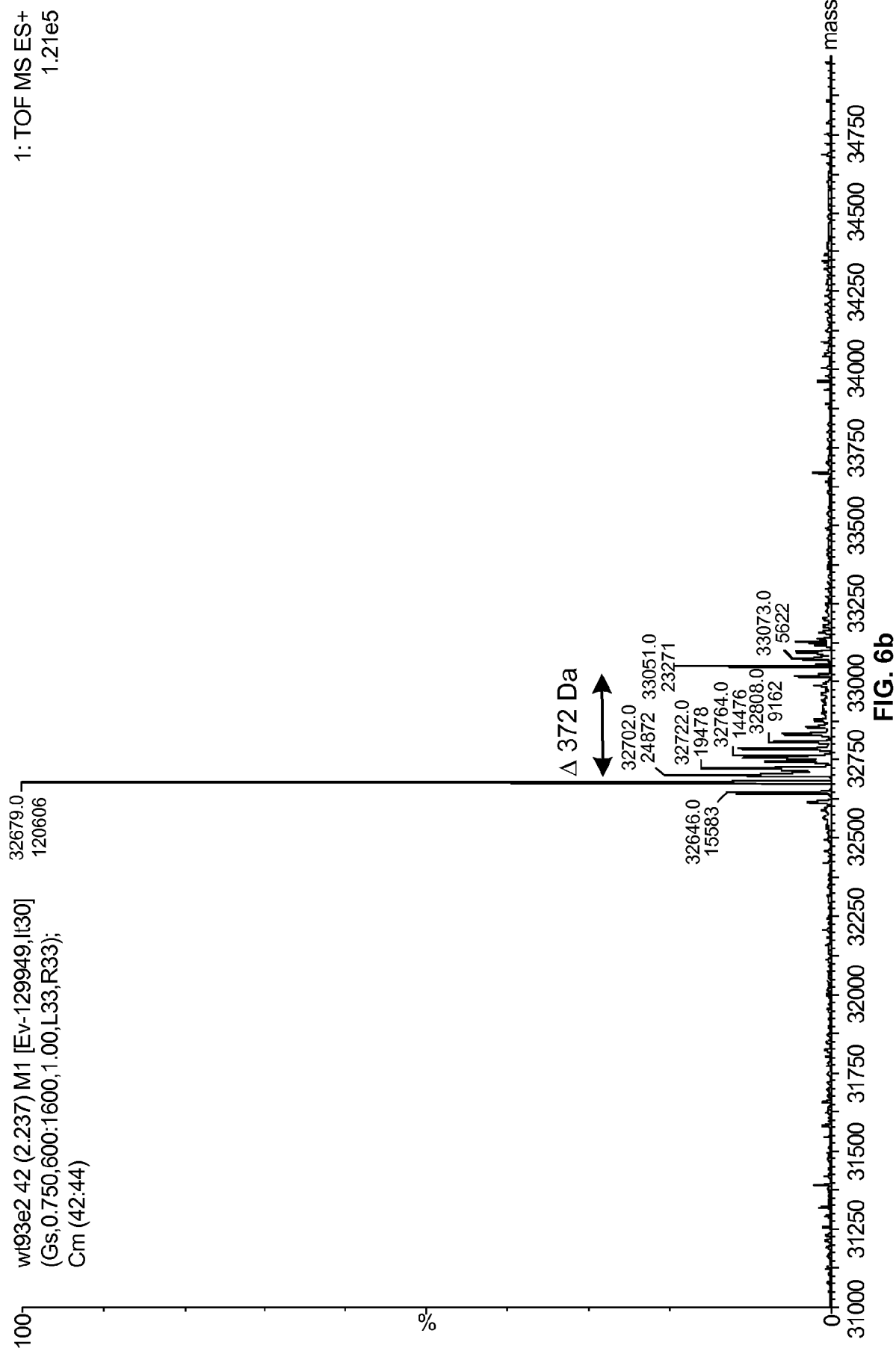
FIG. 5



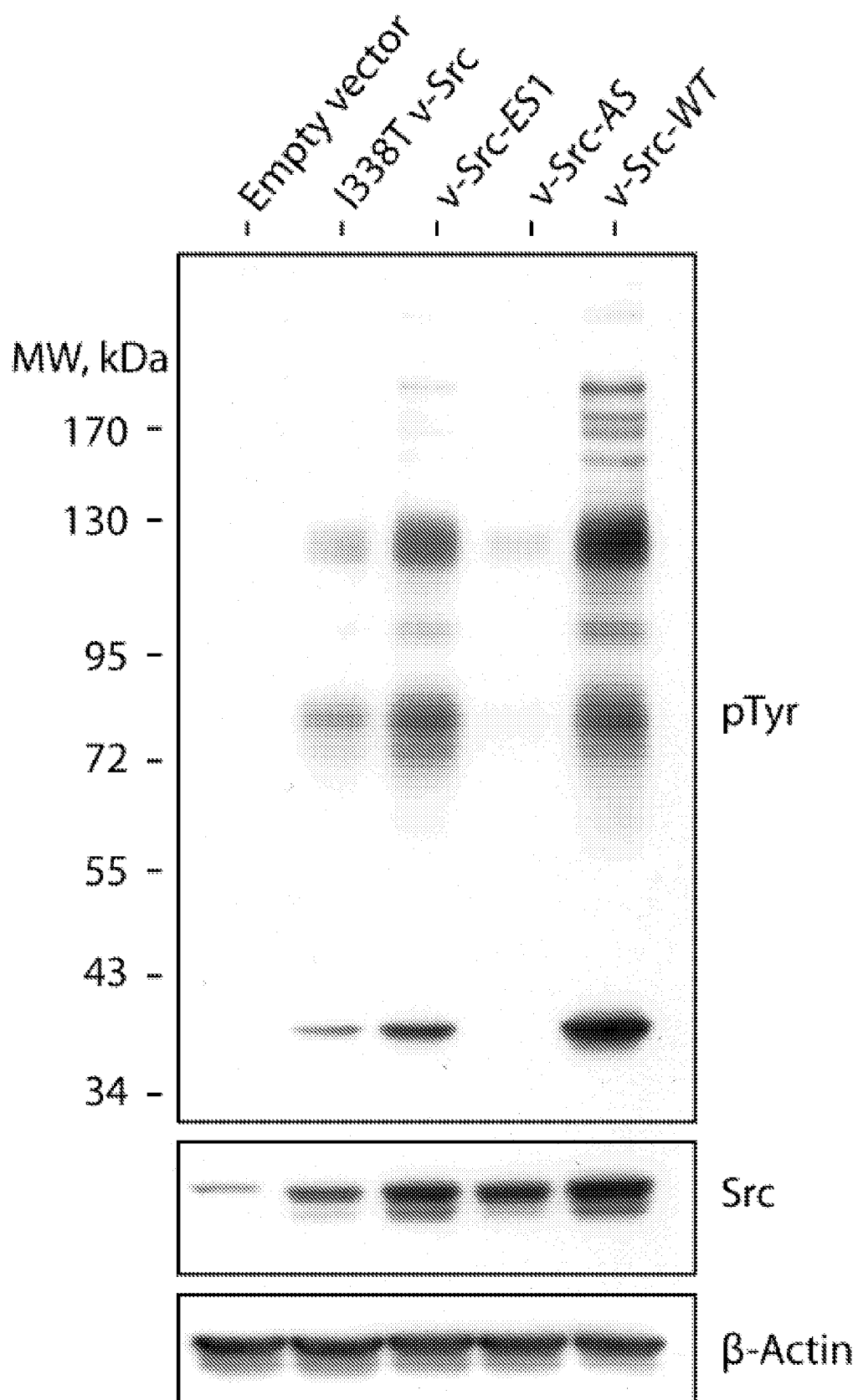
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FIG. 7

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Thu May 26, 2011 14:21 PDT

AG_144492-2_cSRCf-T338C-A_t7promoterB06.seq

/Users/algarske/Documents/Sequencing/cSRC/AG_144492-2_cSRCf-T338C-A_t7promoterB06.seq

From 1 to 918.

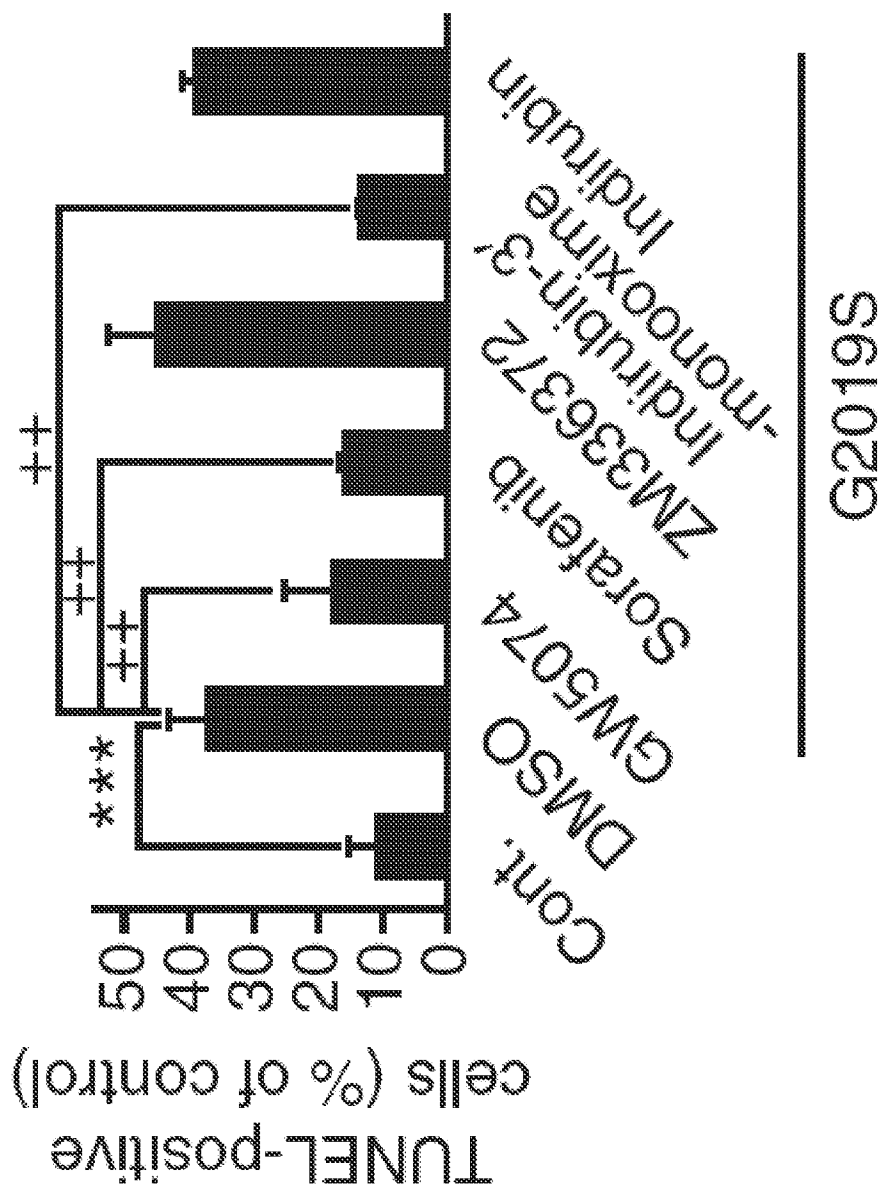
Translation 305 a.a. MW=35116.20999999997

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CATCATCATCATCATCACGATTACGATATCCCAACGACCGAAAACCTTTACTTCCAGGGC
H M Q T Q G L A K D A W E I P R E S L R
CATATGCAGACCCAGGGACTCGCCAAGGACGCGTGGGAAATCCCCGGGAGTCGCTGCGG
L E V K L G Q G C F G E V W M G T W N G
CTGGAGGTGAAGCTGGGGCAGGGCTGCTTTGGAGAGGTCTGGATGGGGACCTGGAACGGC
T T R V A I K T L K P G T M S P E A F L
ACCACCAGAGTGGCCATAAAGACTCTGAAGCCCGGCACCATGTCCCCGGAGGCCTTCCTG
Q E A Q V M K K L R H E K L V Q L Y A V
CAGGAAGCCCCAAGTGATGAAGAAGCTCCGGCATGAGAAGCTGGTTCAGCTGTACGCAGTG
V S E E P I Y I V C E Y M S K G S L L D
GTGTCCGAAGAGCCCATCTACATCGTCTGTGAGTACATGAGCAAGGGGAGCCTCCTGGAT
F L K G E M G K Y L R L P Q L V D M A A
TTCCTGAAGGGAGAGATGGGCAAGTACCTGCGGCTGCCACAGCTCGTCGATATGGCTGCT
Q I A S G M A Y V E R M N Y V H R D L R
CAGATTGCATCCGGCATGGCCTATGTGGAGAGGATGAACTACGTGCACCGAGACCTGCGG
A A N I L V G E N L V C K V A D F G L A
GCGGCCAACATCCTGGTGGGGGAGAACCTGGTGTGCAAGGTGGCTGACTTTGGGCTGGCA
R L I E D N E Y T A R Q G A K F P I K W
CGCCTCATCGAGGACAACGAGTACACAGCACGGCAAGGTGCCAAGTTCCCCATCAAGTGG
T A P E A A L Y G R F T I K S D V W S F
ACAGCCCCCGAGGCAGCCCTCTATGGCCGGTTCACCATCAAGTCGGATGTCTGGTCCTTC
G I L L T E L T T K G R V P Y P G M V N
GGCATCCTGCTGACTGAGCTGACCACCAAGGGCCGGGTGCCATACCCAGGGATGGTCAAC
R E V L D Q V E R G Y R M P C P P E C P
AGGGAGGTGCTGGACCAGGTGGAGAGGGGCTACCGCATGCCCTGCCCCGCCGAGTGCCCC
E S L H D L M C Q C W R K D P E E R P T
GAGTCGCTGCATGACCTCATGTGCCAGTGCTGGCGGAAGGACCCTGAGGAGCGGCCCCACT
F E Y L Q A F L E D Y F T S T E P Q Y Q
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P G E N L *
CCTGGAGAGAACCTATAG

FIG. 8

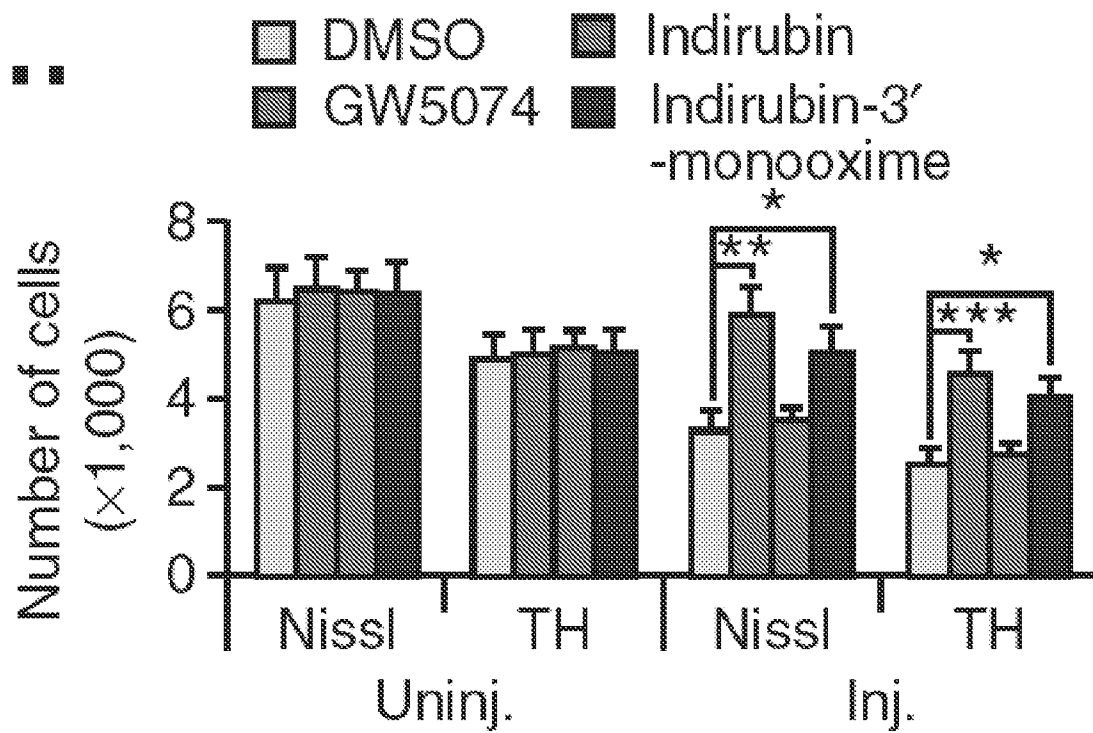
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FIG. 9



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FIG. 10



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FIG. 11

3-vs-Q

BTK	59
CHEK2 (CHK2)	53
EGFR (ErbB1)	84
EGFR (ErbB1) L858R	69
EGFR (ErbB1) L861Q	76
EGFR (ErbB1) T790M L858R	41
ERBB2 (HER2)	66
ERBB4 (HER4)	80
FLT3 D835Y	99
GRK5	49
LRRK2	75
LRRK2 G2019S	91
PDGFRA V561D	45
PIK3C2B (PI3K-C2 beta)	65
RPS6KA6 (RSK4)	49
SRMS (Srm)	71
TXK	78

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FIG. 12

WT LRRK2	G2019S LRRK2
156 nM	33 nM
>1000 nM	>1000 nM
>1000 nM	>1000 nM
>1000 nM	>1000 nM

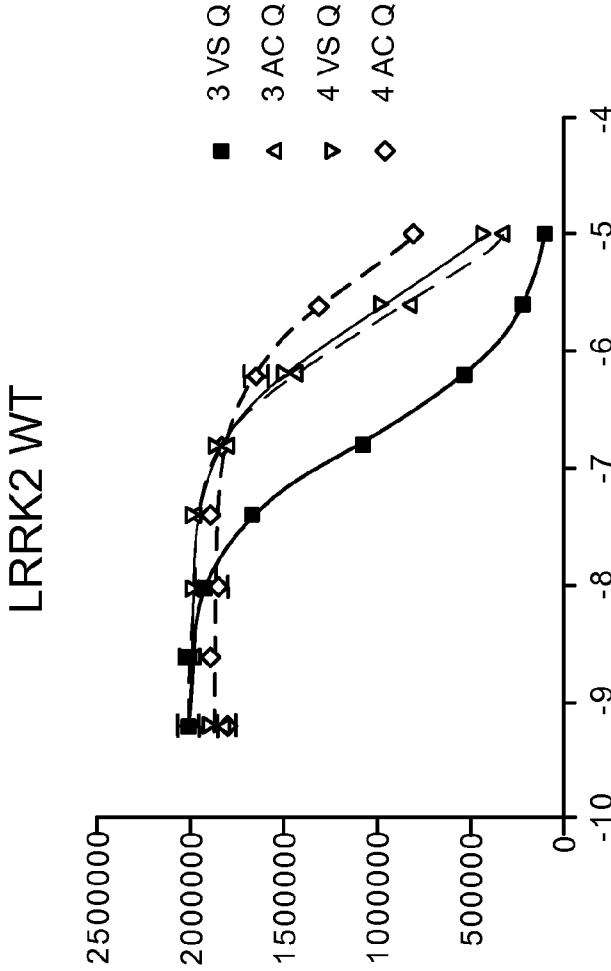


FIG. 13

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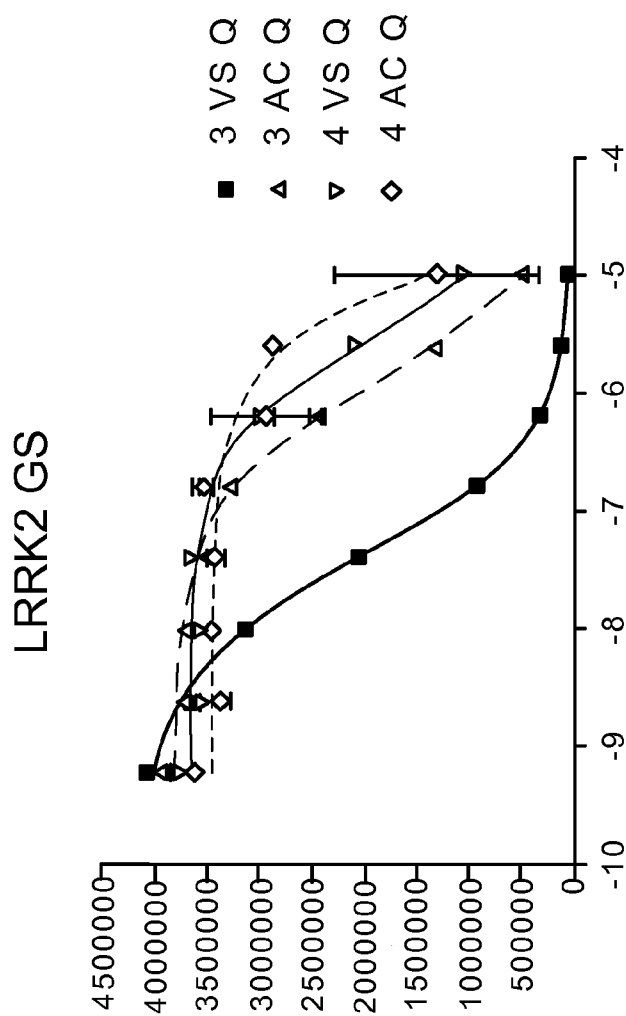
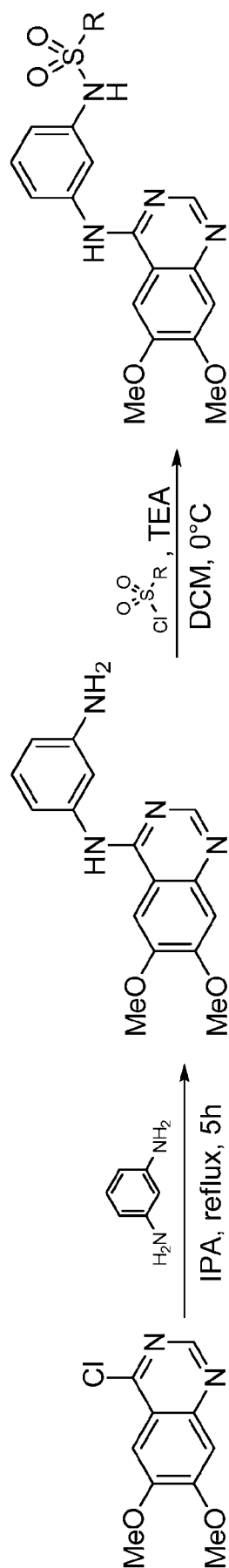


FIG. 14

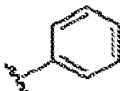
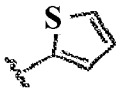
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FIG. 15

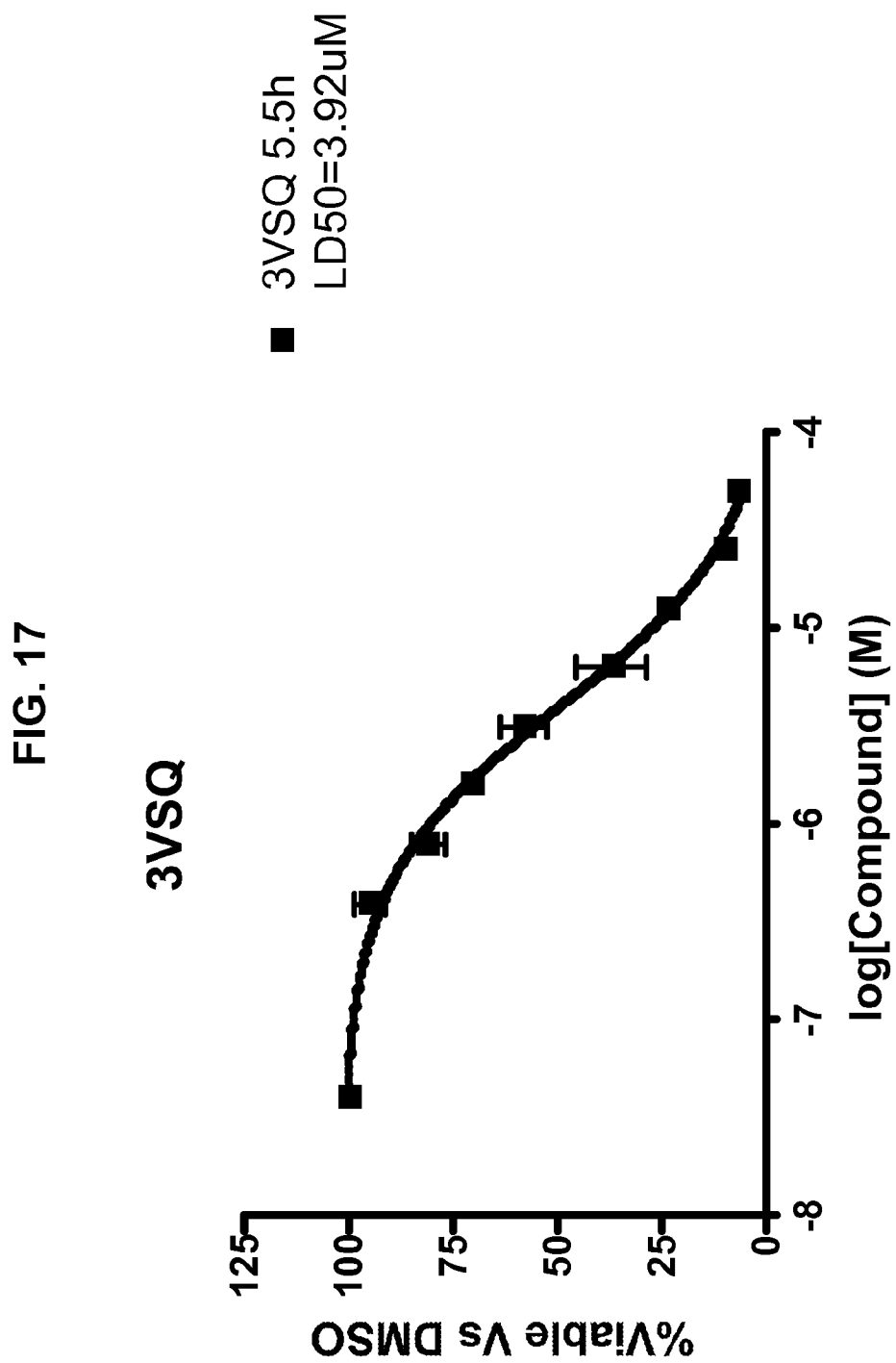


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FIG. 16

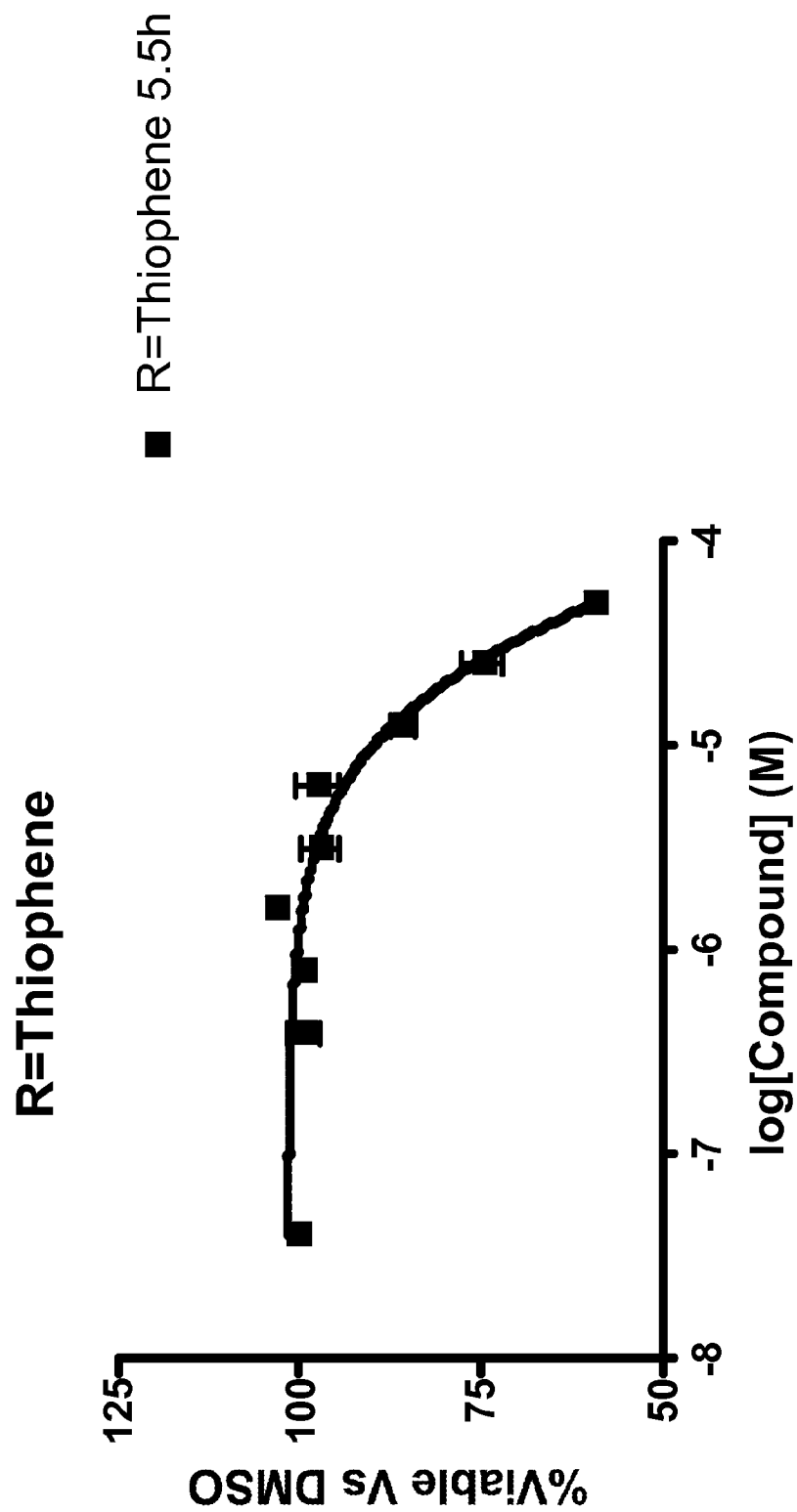
R=	WT LRRK2 IC ₅₀	G2019SLRRK2 IC ₅₀
 (3-vs-Q)	213 nM	45.5 nM
 Me	353 nM	100 nM
	334 nM	121 nM
	241 nM	90.1 nM
	186 nM	46.9 nM
	329 nM	57.7nM
	380 nM	274 nM
	334 nM	121 nM

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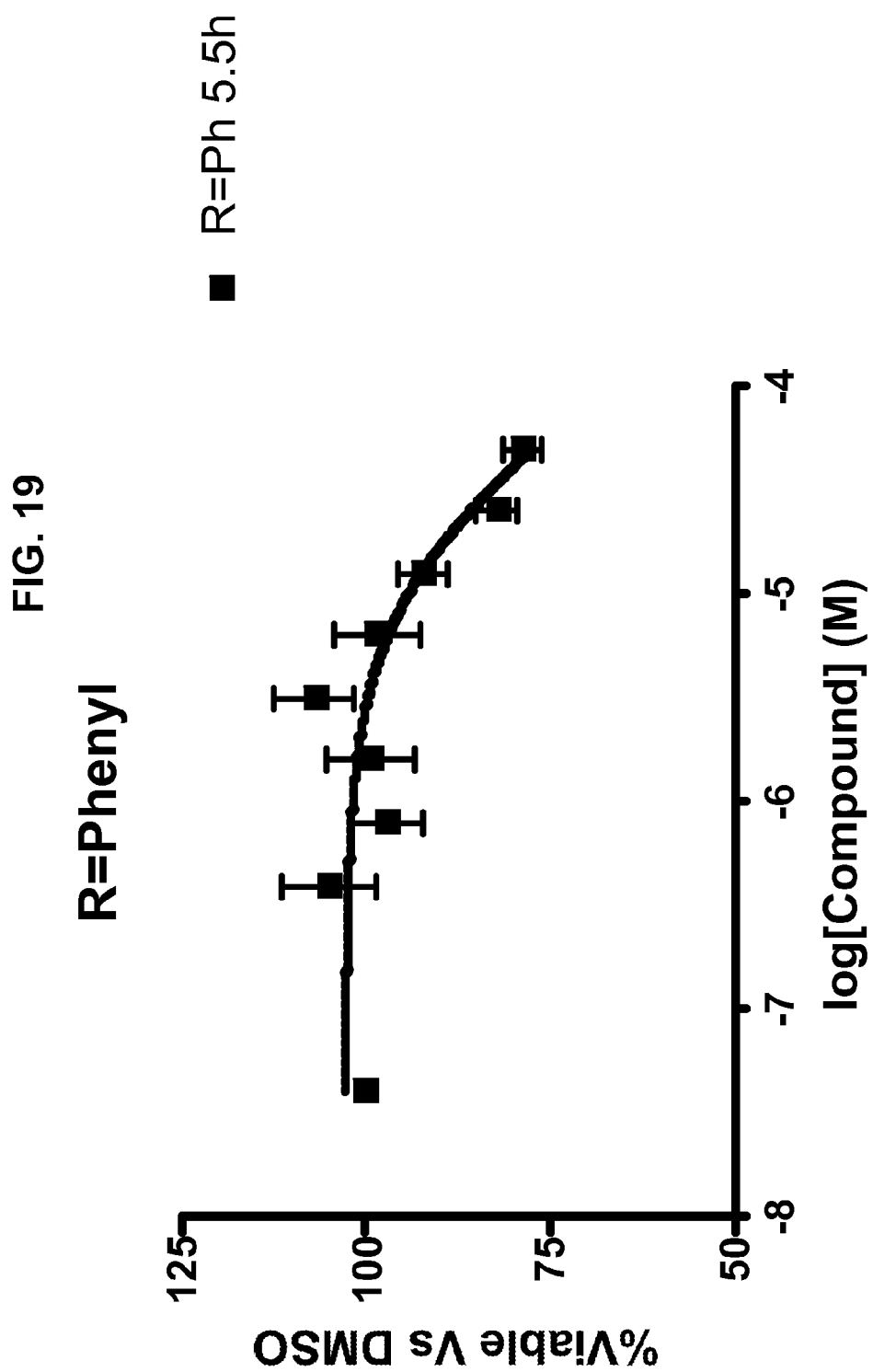


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FIG. 18

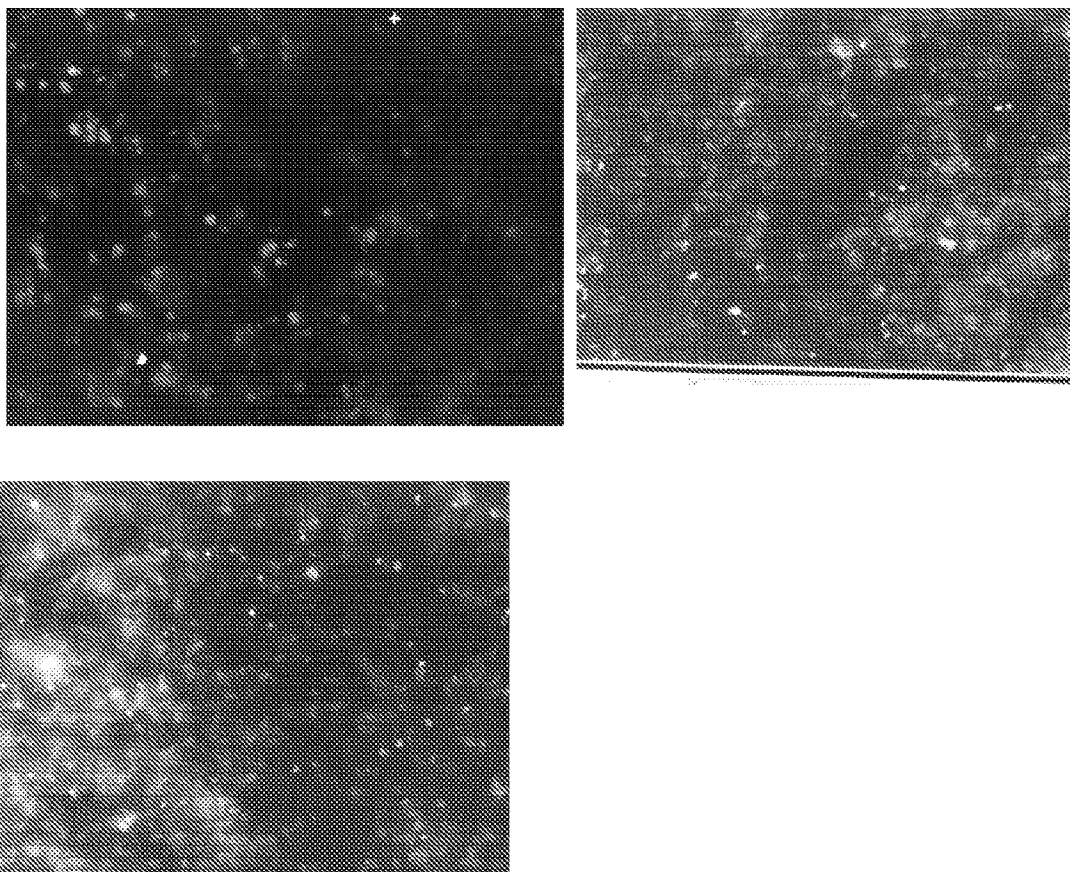


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FIG. 20



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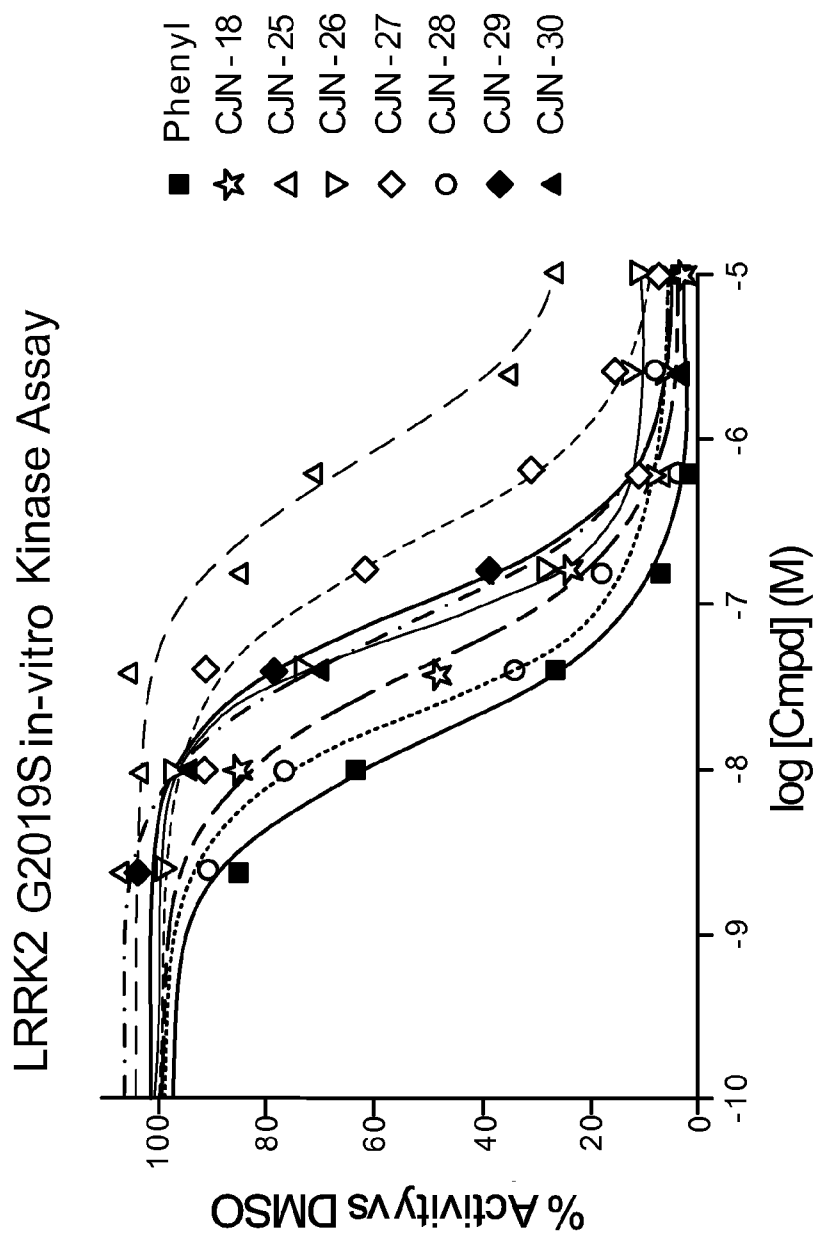


FIG. 21

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FIG. 22

