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(54) **Title:** MULTIVALENT RAS BINDING COMPOUNDS

(57) **Abstract:** The present invention provides, inter alia, compounds that selectively bind a RAS protein at two or more sites and methods for their synthesis. Compositions and kits containing the compounds, as well as methods of using the compounds and compositions for ameliorating or treating the effects of a disease associated with altered RAS signaling, such as a cancer, in a subject and methods for effecting cancer cell death are also provided herein. Methods of identifying a multivalent compound which binds selectively to a target protein also are provided herein.



WO 2015/184349 A2

MULTIVALENT RAS BINDING COMPOUNDS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of U.S. Patent Application Serial No. 62/005,831, filed on May 30, 2014 which application is incorporated by reference herein in its entirety.

GOVERNMENT FUNDING

[0002] This invention was made with government support under grants 5R01CA097061, 5R01GM085081, R01CA161061, and 1S10RR025431-01A1 awarded by the National Institutes of Health and grant CHE 0840451 awarded by the National Science Foundation. The government has certain rights in the invention.

FIELD OF INVENTION

[0003] The present invention provides, *inter alia*, compounds that selectively bind a RAS protein at two or more sites. Compositions and kits containing the compounds, as well as methods of using the compounds and compositions for ameliorating or treating the effects of a disease associated with altered RAS signaling in a subject and methods for effecting cancer cell death are also provided herein.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

[0004] This application contains references to amino acids and/or nucleic acid sequences that have been filed concurrently herewith as

sequence listing text file 0365302_sequences.txt, file size of 132 KB, created on May 29, 2015. The aforementioned sequence listing is hereby incorporated by reference in its entirety pursuant to 37 C.F.R. § 1.52(e)(5).

BACKGROUND OF THE INVENTION

[0005] At least 85% of human proteins are considered to be challenging targets for small molecule drugs using conventional discovery approaches, such as high-throughput screening of existing chemical libraries (Hopkins *et al.*, 2002). A particularly important, but historically intractable, subset of these proteins are those that elicit their biological effects through protein-protein interactions (Nero *et al.*, 2014); while some protein-protein interactions consisting of short alpha helical domains inserted into a deep hydrophobic pocket in an interacting protein have been amenable to disruption with small molecules (*e.g.*, the p53-Mdm2 interaction (Vassilev *et al.*, 2004)), many protein-protein interactions have been largely resistant to small molecule inhibition using high-throughput screening of standard chemical libraries. Within this category are the RAS GTPases, which are proposed to be among the most tantalizing and thoroughly validated targets in cancer biology due to their high prevalence and frequent essentiality in lethal malignancies (Downward *et al.*, 2003). RAS gene mutations are found at high rates in three of the top four lethal malignancies in the United States—pancreatic (90%), colon (45%), and lung cancers (35%) (*Id.*). Many tumors have been shown to be dependent on continued expression of oncogenic RAS proteins in cell and animal models (Weinstein *et al.*, 2008). However, RAS proteins have been viewed as challenging targets, primarily due to the lack of a sufficiently large and deep hydrophobic site for small molecule

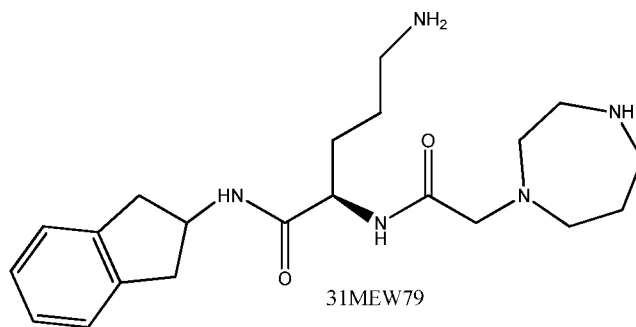
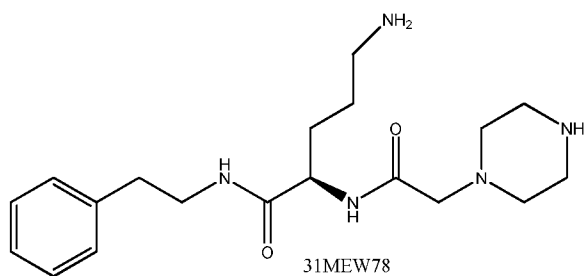
binding, aside from the GTP-binding site. The picomolar affinity of GTP (John *et al.*, 1990) makes competitive inhibition impractical, in contrast to the ATP-binding site on kinases. For these reasons, traditional high-throughput screening has been unable to provide high affinity small molecule RAS ligands.

[0006] The RAS proteins play a central role in a number of signal transduction pathways controlling cell growth and differentiation. They function as a binary switch, transitioning from an inactive GDP-bound state to an active GTP-bound state (Downward *et al.*, 2003). GTP binding enables several residues, primarily in the switch I region (residues 30-40) and the switch II region (residues 60-70) to adopt a conformation that permits RAS effector proteins to bind; this transition is reciprocally regulated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). A mutation resulting in the impairment of the intrinsic GTPase activity of RAS proteins, or preventing GAP binding, constitutively activates downstream signaling pathways and contributes to the malignant phenotype. Thus, there exists an unmet need for compounds that selectively bind a RAS protein, particularly an oncogenic mutant of a RAS protein.

SUMMARY OF THE INVENTION

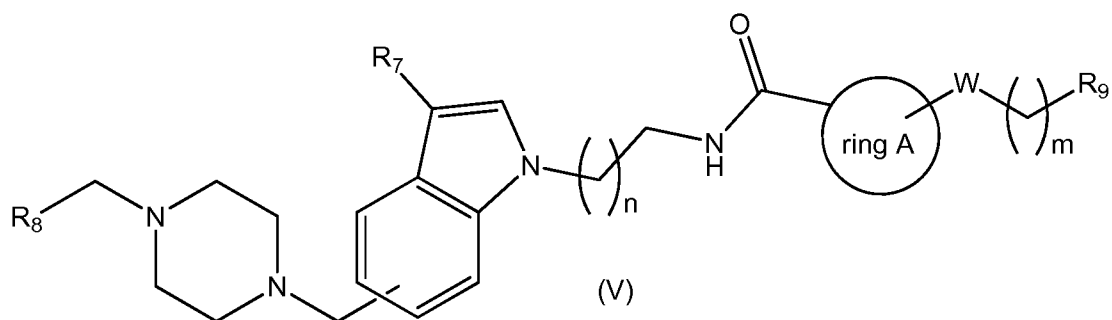
[0007] One embodiment of the present invention is a compound that selectively binds a RAS protein at two or more sites.

[0008] Another embodiment of the present invention is a compound selected from the group consisting of



and crystalline forms, hydrates, or pharmaceutically acceptable salts thereof.

[0009] A further embodiment of the present invention is a compound having the structure of formula (V):



wherein:

R_7 is selected from the group consisting of H, halide, C_{1-4} aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, $-O-C_{1-4}$ alkyl and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R_8 is selected from the group consisting of no atom, H, alkyl, aryl, and C_{1-4} alkyl-O-aryl wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, and a combination thereof;

R_9 is selected from the group consisting of no atom, H, C_{1-4} alkyl, and aryl optionally substituted with the group consisting of ether, halide, and a combination thereof;

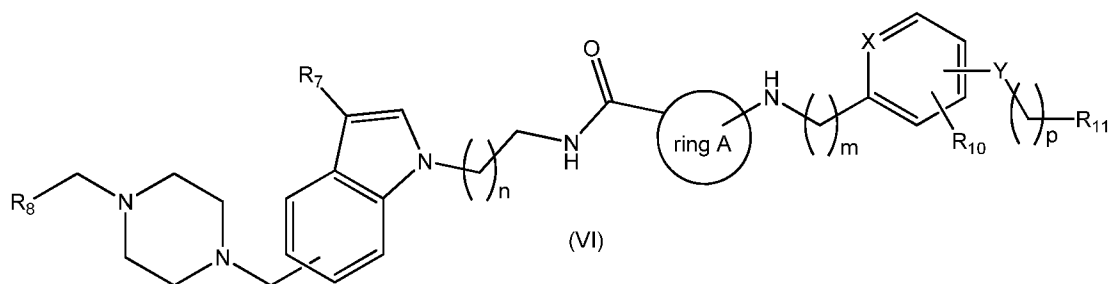
W is selected from the group consisting of no atom and NH;

m and n are independently selected from the group consisting of an integer between 0-5; and

ring A is a heterocycle with at least 1 ring nitrogen and optionally substituted with C_{1-4} alkyl or a halide,

or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

[0010] An additional embodiment of the present invention is a compound having the structure of formula (VI):



wherein:

R_7 is selected from the group consisting of H, halide, C_{1-4} aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, $-O-C_{1-4}$ alkyl, and a

combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R₈ and R₁₁ are independently selected from the group consisting of no atom, H, alkyl, aryl and C₁₋₄alkyl-O-aryl, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with one or more groups consisting of halide, ether, C₁₋₄alkyl, -O-C₁₋₄alkyl, and a combination thereof, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof;

R₁₀ is selected from the group consisting of no atom, H, halide, C₁₋₄ aliphatic, and -O-C₁₋₄alkyl;

X is selected from the group consisting of CH and N;

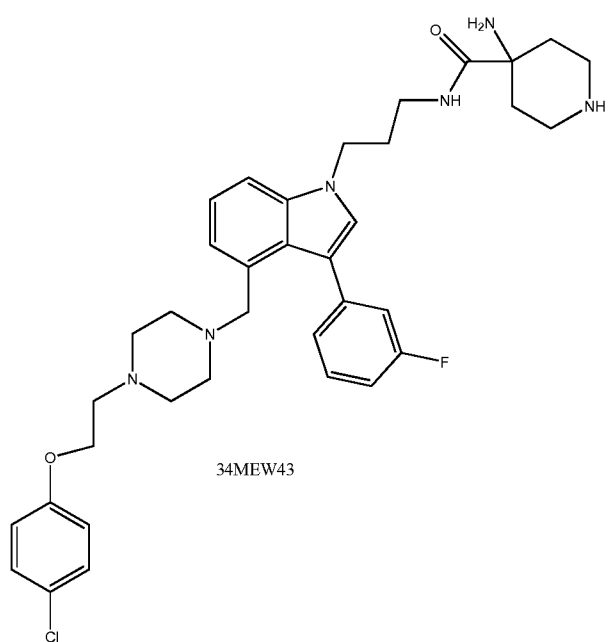
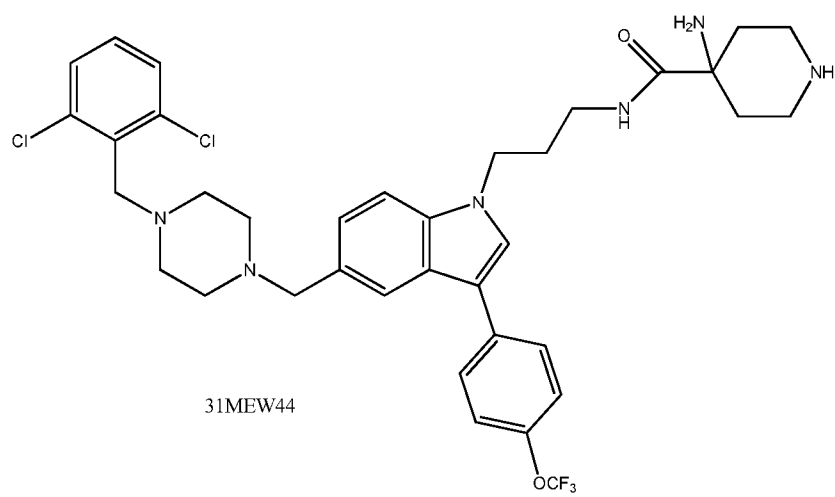
Y is selected from the group consisting of no atom and O;

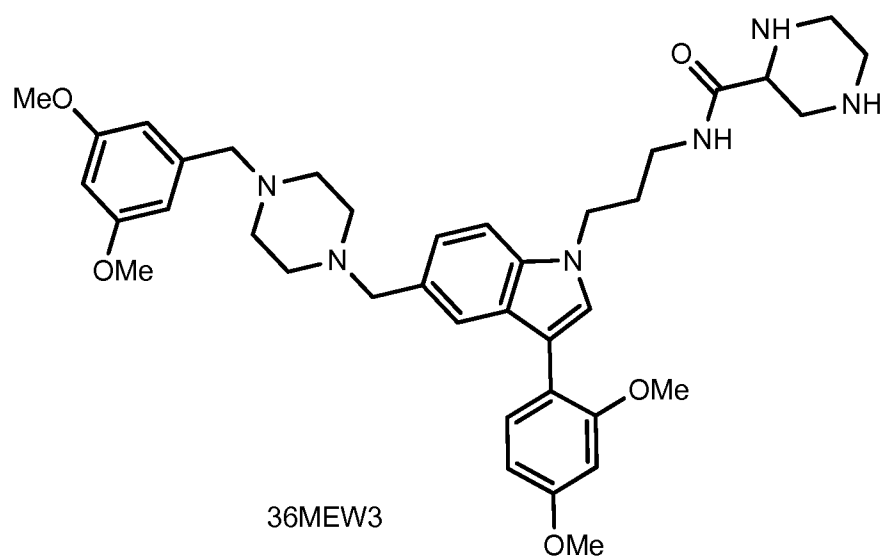
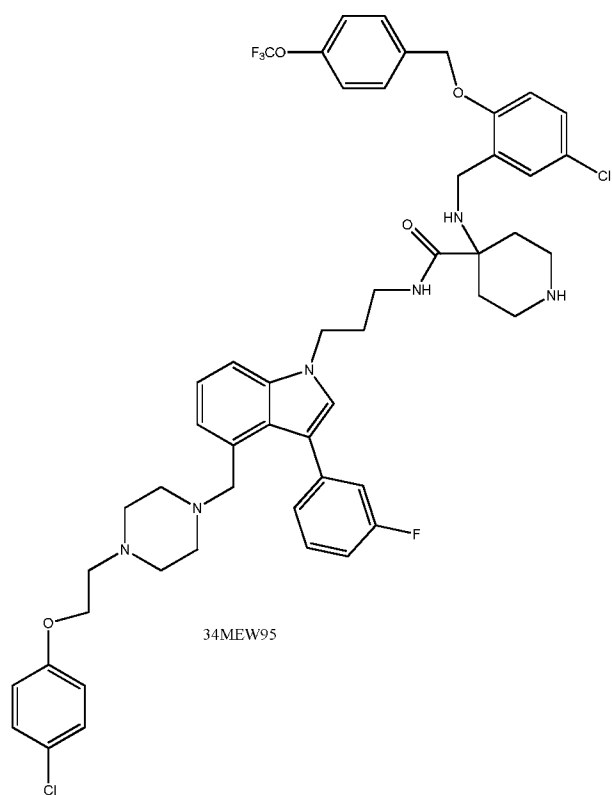
m, n, and p are independently selected from the group consisting of an integer between 0-5; and

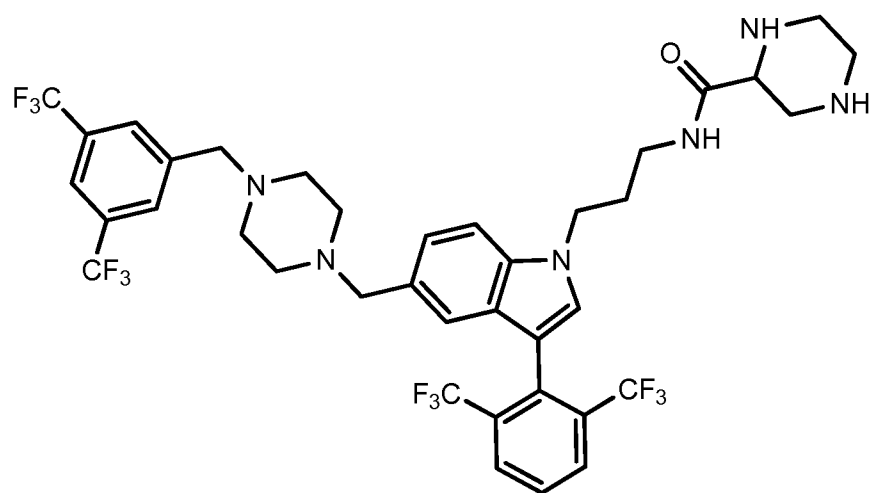
ring A is a heterocycle with at least 1 ring nitrogen and optionally substituted with C₁₋₄alkyl or a halide,

or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

[0011] Another embodiment of the present invention is a compound selected from the group consisting of

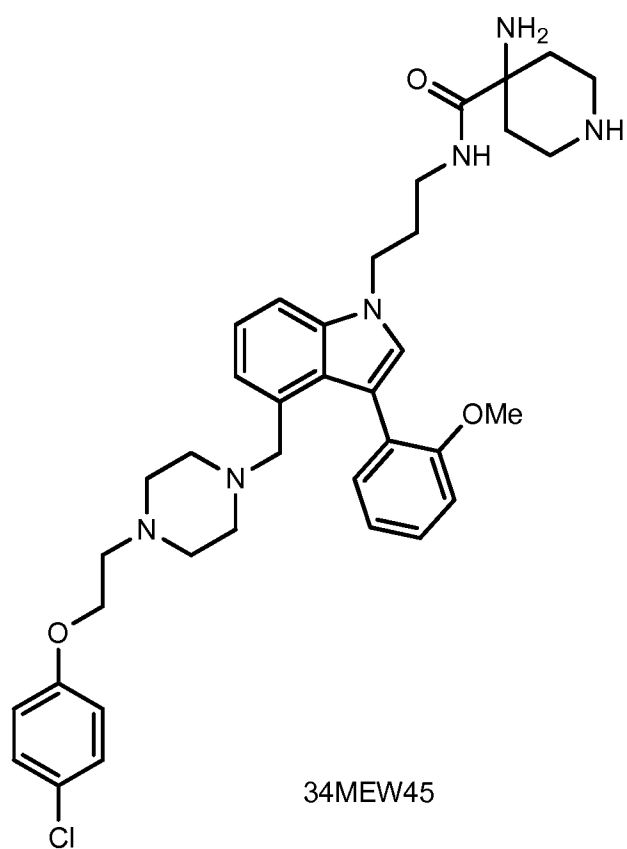






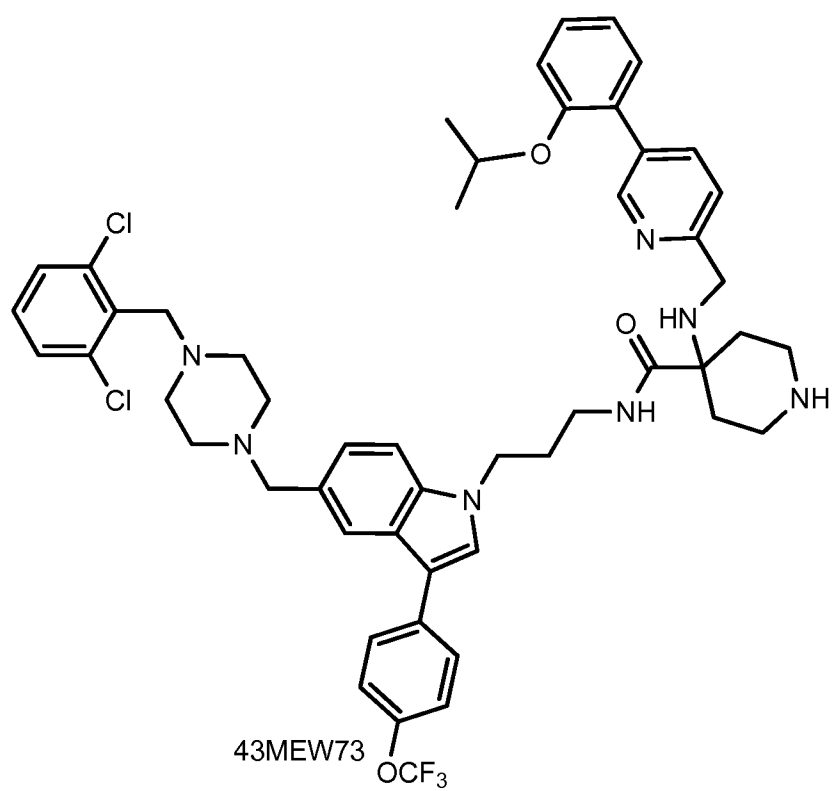
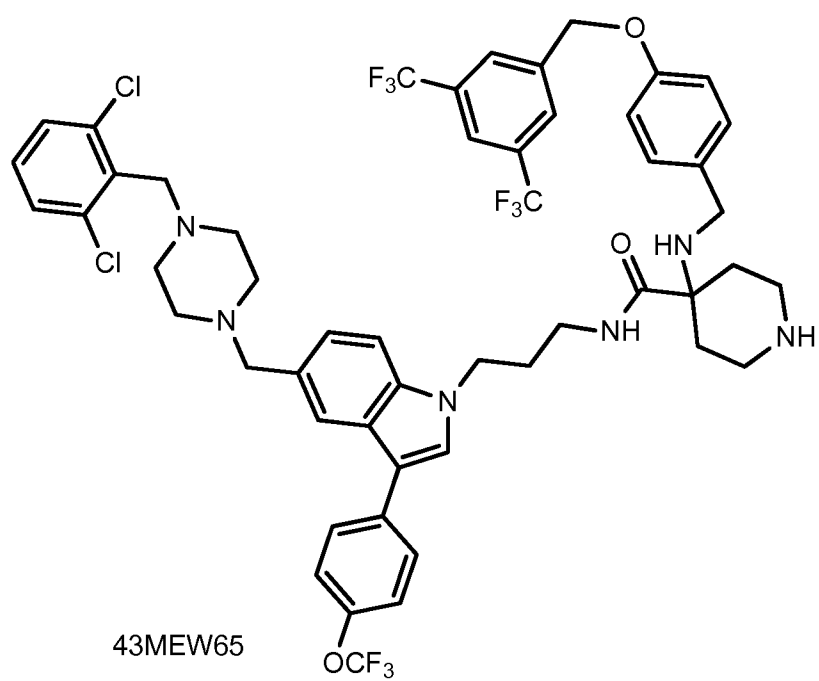
32MEW56

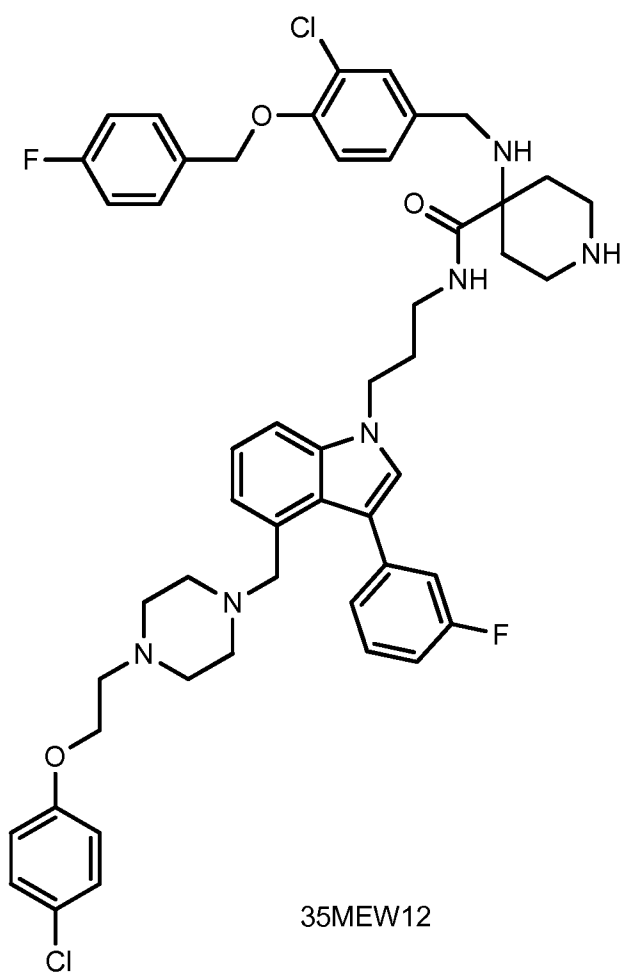
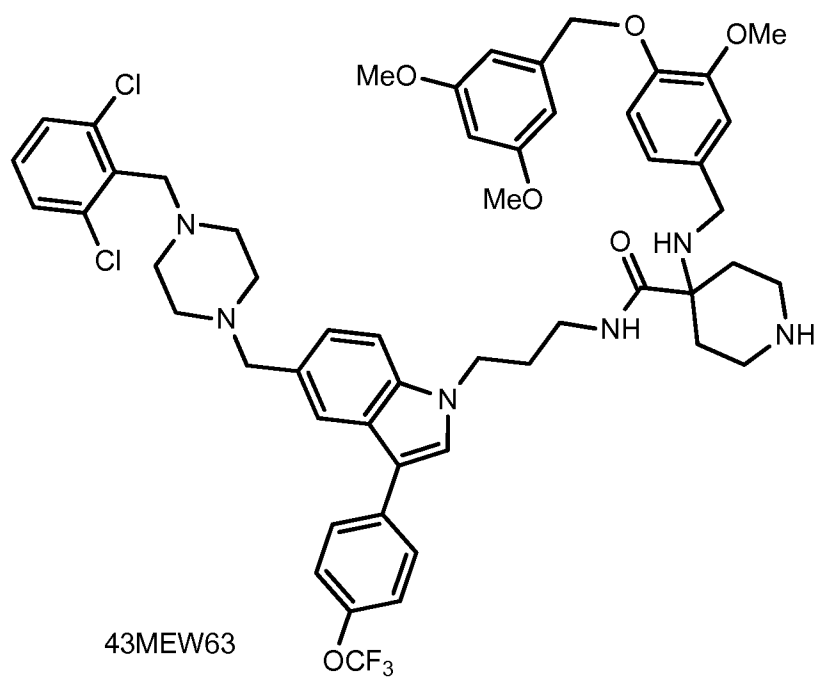
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34MEW45

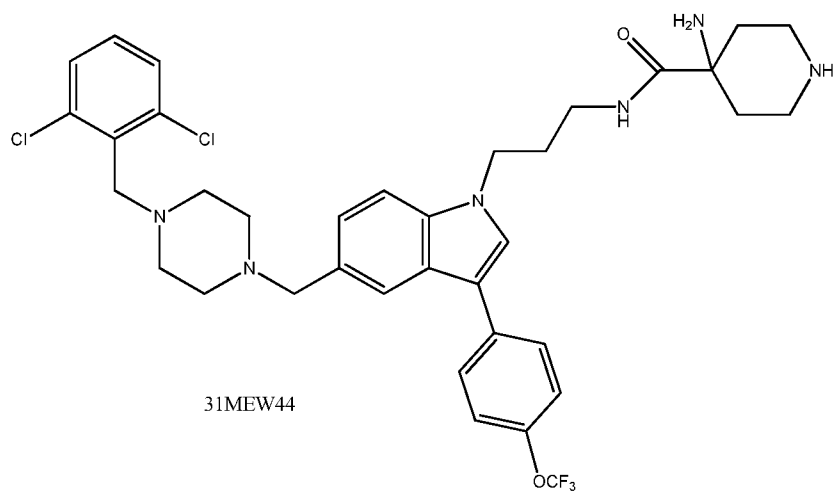
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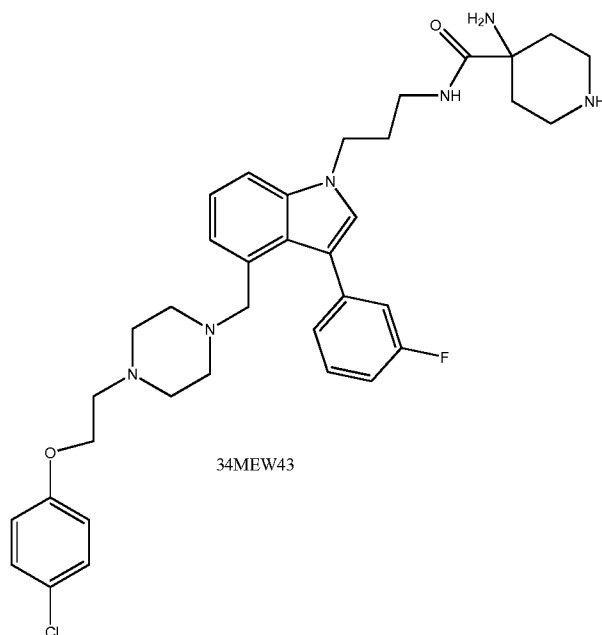
and crystalline forms, hydrates, or pharmaceutically acceptable salts thereof.

[0012] A further embodiment of the present invention is a compound having the structure:



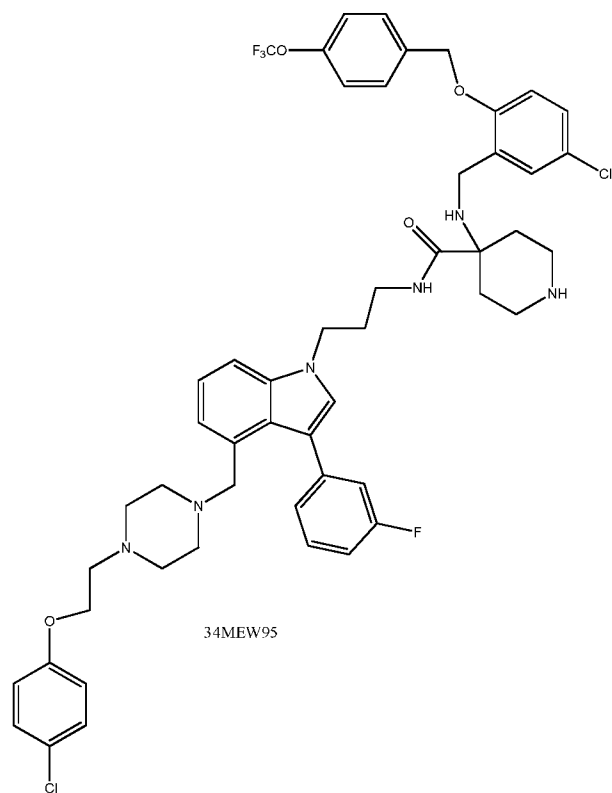
or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

[0013] An additional embodiment of the present invention is a compound having the structure:



or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

[0014] Another embodiment of the present invention is a compound having the structure:



or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

[0015] A further embodiment of the present invention is a pharmaceutical composition that comprises a pharmaceutically acceptable carrier and any compound disclosed herein.

[0016] An additional embodiment of the present invention is a method for ameliorating or treating the effects of a disease associated with altered RAS signaling in a subject. The method comprises administering to the subject an effective amount of any compound disclosed herein.

[0017] Another embodiment of the present invention is a method for ameliorating or treating the effects of a disease associated with altered RAS signaling in a subject. The method comprises administering to the subject an effective amount of any pharmaceutical composition disclosed herein.

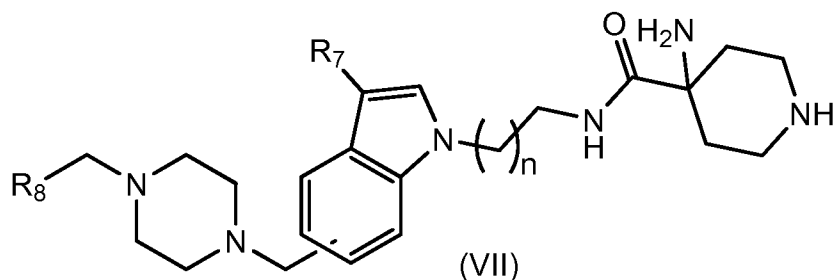
[0018] A further embodiment of the present invention is a method for effecting cancer cell death. The method comprises contacting a cancer cell with an effective amount of any compound disclosed herein.

[0019] Another embodiment of the present invention is a kit for treating or ameliorating the effects of a disease related to altered RAS signaling in a subject in need thereof. The kit comprises an effective amount of any compound or pharmaceutical composition disclosed herein, packaged together with instructions for its use.

[0020] A further embodiment of the present invention is a kit for treating or ameliorating the effects of a cancer in a subject in need thereof. The kit comprises an effective amount of any compound or pharmaceutical composition disclosed herein, packaged together with instructions for its use.

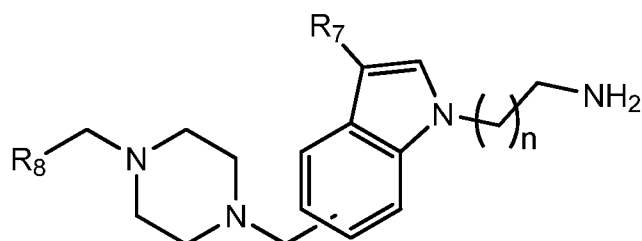
[0021] An additional embodiment of the present invention is a composition that comprises any compound disclosed herein.

[0022] An additional embodiment of the present invention is a method of preparing a compound having the structure of formula (VII):

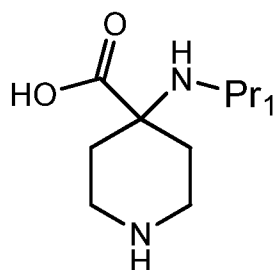


The method comprises the steps of

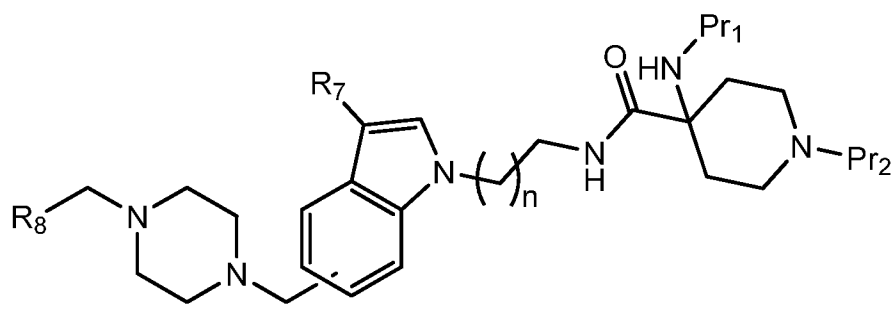
- i) reacting a compound having the structure:



with a compound having the structure:



under conditions sufficient to form a compound having the structure:



wherein:

R_7 is selected from the group consisting of H, halide, C_{1-4} aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, $-O-C_{1-4}$ alkyl, and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R_8 is selected from the group consisting of no atom, H, alkyl, aryl and C_{1-4} alkyl-O-aryl, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the

aryl is optionally substituted with one or more groups consisting of halide, ether, C₁₋₄alkyl, and a combination thereof;

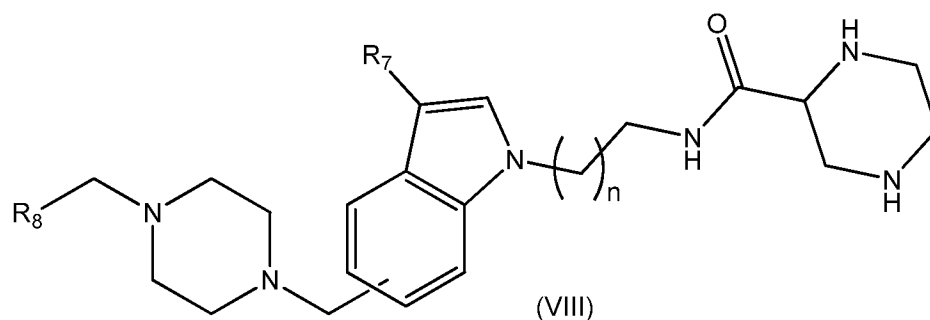
n is selected from the group consisting of an integer between 0-5; and

Pr₁ and Pr₂ are independently selected from the group consisting of nitrogen protecting groups;

ii) removing the Pr₁ protecting group; and

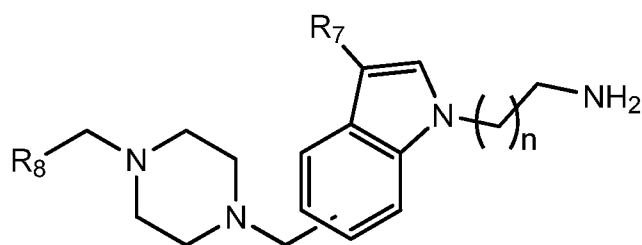
iii) removing the Pr₂ protecting group.

[0023] An additional embodiment of the present invention is a method of preparing a compound having the structure of formula (VIII):

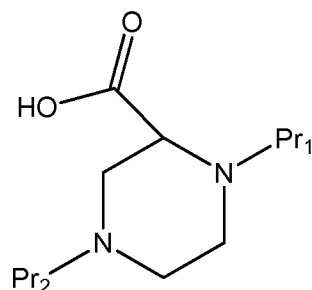


The method comprises the steps of

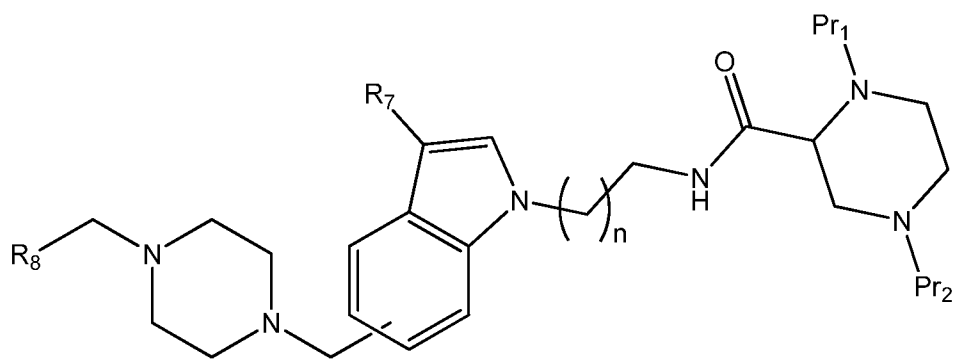
i) reacting a compound having the structure:



with a compound having the structure:



under conditions sufficient to form a compound having the structure:



wherein:

R₇ is selected from the group consisting of H, halide, C₁₋₄ aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C₁₋₄alkyl, -O-C₁₋₄alkyl, and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

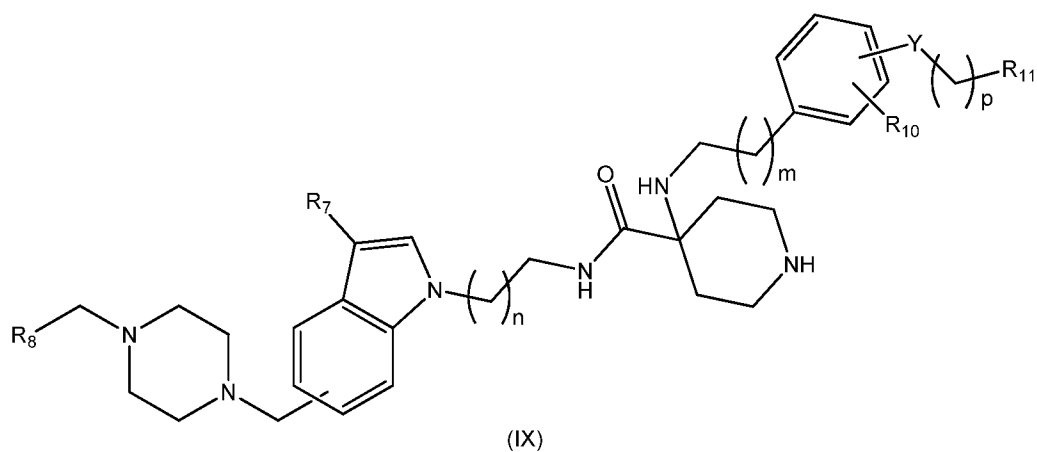
R₈ is selected from the group consisting of no atom, H, alkyl, aryl and C₁₋₄alkyl-O-aryl, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with one or more groups consisting of halide, ether, C₁₋₄alkyl, and a combination thereof;

n is selected from the group consisting of an integer between 0-5; and

Pr₁ and Pr₂ are independently selected from the group consisting of nitrogen protecting groups;

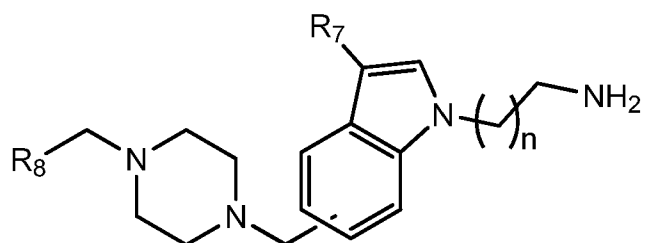
- ii) removing the Pr₁ protecting group; and
- iii) removing the Pr₂ protecting group.

[0024] An additional embodiment of the present invention is a method of preparing a compound having the structure of formula (IX):

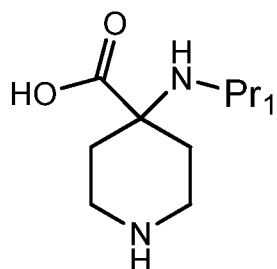


The method comprises the steps of

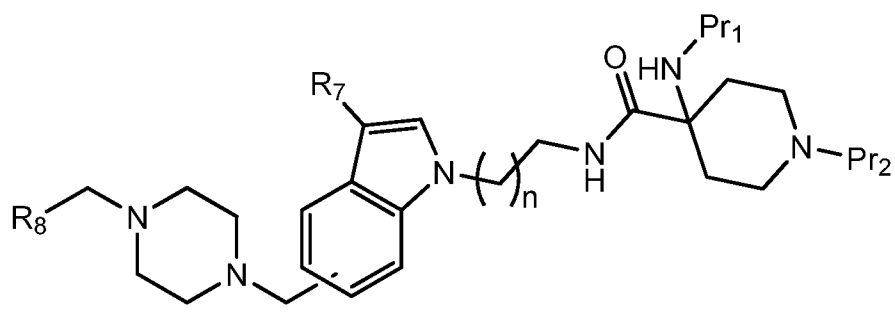
- i) reacting a compound having the structure:



with a compound having the structure:



under conditions sufficient to form a compound having the structure:



wherein:

R₇ is selected from the group consisting of H, halide, C₁₋₄ aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C₁₋₄alkyl, -O-C₁₋₄alkyl, and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R₈ and R₁₁ are independently selected from the group consisting of no atom, H, alkyl, aryl and C₁₋₄alkyl-O-aryl, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with the group consisting of halide, ether, C₁₋₄alkyl, -O-C₁₋₄alkyl, and a combination thereof, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof;

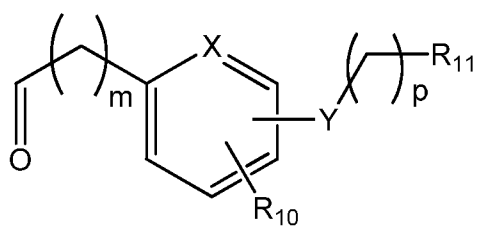
R₁₀ is selected from the group consisting of no atom, H, halide, C₁₋₄ aliphatic and -O-C₁₋₄alkyl;

Y is selected from the group consisting of no atom and O;

m, n, and p are independently selected from the group consisting of an integer between 0-5; and;

Pr₁ and Pr₂ are independently selected from the group consisting of nitrogen protecting groups;

- ii) removing the Pr₁ protecting group
- iii) reacting the product of step ii) with a compound having the structure:



wherein X is selected from the group consisting of CH and N; and

iv) removing the Pr_2 protecting group.

[0025] An additional embodiment of the present invention is a method of identifying a multivalent compound which binds selectively to a target protein. The method comprises the steps of

i) identifying a first and second target site on the target protein, wherein the first and second target sites are adjacent to each other;

ii) identifying a first compound fragment that selectively binds to the target protein at the first target site and a second compound fragment that selectively binds to the target protein at the second site; and

iii) creating a structure of the multivalent compound comprising the first compound fragment linked to the second compound fragment, thereby identifying the multivalent compound.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] FIGS. 1A-C show design and biochemical evaluation of inhibitors derived from a RAS-effector-inspired pharmacophore model. FIG. 1A shows co-crystal structures of HRAS with PI3Ky (grey, PDB: 1HE8), the RAS-binding domain of CRAF (yellow, PDB: 3KUD), and RALGDS (red, PDB: 1LFD) aligned. FIG. 1B shows an example of the conserved interactions across the D38 site with the effectors. Each effector has either an arginine or lysine capable of making an electrostatic interaction with E37 on HRAS. FIG. 1C shows the selected effector residues that interact with residues 36-39 on HRAS. FIGS. 1D-L show analysis of the interaction between HRAS with three effector proteins and creation of a RAS-effector-derived pharmacophore

model. FIG. 1D shows co-crystal structures of HRAS with PI3Ky (grey, PDB: 1HE8), the RAS binding domain of CRAF (yellow, PDB: 3KUD), RALGDS (red, PDB: 1LFD) aligned. FIG. 1E shows the same view as FIG. 1D, with the individual residues shown. FIG. 1F shows the residues on the effector proteins interacting with I36. Each effector has a complementary hydrophobic residue. FIG. 1G shows the conformational change undergone by I36 going from the GDP to GTP form. HRAS^{A59G} bound to GDP in yellow (PDB: 1LF5) and HRAS^{A59G} bound to GTP in blue (PDB: 1LF0). FIG. 1H shows a view of I36 transition, with a surface representation for the GDP form. In the GTP-bound form, I36 adopts a solvent exposed state. FIG. 1I shows basic residues of the effector proteins in close proximity to D38. FIG. 1J shows effector residues with hydrogen bonding properties close to S39. FIG. 1K shows that each effector has either a arginine or lysine capable of making an electrostatic interaction with E37. FIG. 1L shows the selected effector residues interacting with residues 36-39 on HRAS.

[0027] FIG. 2 shows conversion of the selected effector residues into a searchable pharmacophore model.

[0028] FIG. 3 shows retrosynthetic analysis and a schematic of one of the in silico libraries designed to match the properties in the pharmacophore model.

[0029] FIG. 4A shows structures of the two top pharmacophore hits. FIG. 4B shows binding of 31MEW79 to KRAS and measurement of dissociation constants. Dissociation constants were $K_D = 3.8 \text{ mM} \pm 0.13 \text{ mM}$ for GppNHp-bound KRAS^{G12D} and $K_D = 7.1 \text{ mM} \pm 0.68 \text{ mM}$ for GDP-bound KRAS^{G12D}. FIG. 4C shows binding of 31MEW78 to KRAS and

measurement of dissociation constants. Dissociation constants were $K_D = 11$ mM $\pm$ 0.50 mM for GppNHp-bound KRAS^{G12D} and $K_D = 12$ mM $\pm$ 0.43 mM for GDP-bound KRAS^{G12D}.

[0030] FIG. 5 shows differential scanning fluorimetry of 31MEW79 and 31MEW78 with 5 μ M KRAS^{G12D} in the presence of increasing concentration of compound. The ΔT_m was calculated by subtracting the T_m of liganded KRAS^{G12D} protein from unliganded KRAS^{G12D} and is expressed as absolute value of the mean $\pm$ standard error of the mean (sem).

[0031] FIG. 6 shows that small-molecule inhibitors do not bind to the GTP-binding pocket on KRAS. A nucleotide displacement assay was performed in duplicate with 2.5 μ M KRAS^{G12D} •BODIPY-GTP in the presence of increasing concentration of inhibitors or unlabeled GTP or GDP. Free unlabeled GTP and GDP can displace bound BODIPY-GTP from KRAS but the inhibitors cannot because they bind to a different site of KRAS^{G12D}.

[0032] FIG. 7 shows results from microscale thermophoresis performed with 31MEW79 on GTP-loaded KRAS^{G12D} in triplicate.

[0033] FIG. 8 shows pulldown of GTP-loaded KRAS^{G12D} with the CRAF RBD in the presence of 31MEW79 and 31MEW78

[0034] FIGS. 9A-B shows NMR assignments of KRAS G12D loaded with a non-hydrolyzable GTP analog, GppNHp. FIG. 9A shows ¹H-¹⁵N HSQC spectrum of KRAS G12D bound to GppNHp. FIG. 9B shows 3D-1H-15N-1H-NOESY-HSQC and 3D-1H-15N-1H-TOCSY-HSQC experiments were performed to confirm assignments. FIG. 9B shows representative strips for residues T35-E37 in KRAS^{G12D} bound to GppNHp from ¹⁵N NOESY-HSQC

spectrum (blue) and ^{15}N TOCSY-HSQC spectrum (purple). ^{15}N TOCSY-HSQC spectrum helped identify the spin system and ^{15}N NOESY-HSQC spectrum was then used for sequential assignments. The path in red shows the sequential NOEs of H_N - H_N or H_N - H_alpha .

[0035] FIG. 10 shows ^1H - ^{15}N HSQC spectrum of 50 μM KRAS^{G12D} bound-to GppNHp in the absence (blue) and presence (red) of 250 μM inhibitor 31MEW79. Magnification of I36, E37 and S39 in the top left corner. These residues are shrinking (I36 and E37), or shifting (S39)-upon compound treatment.

[0036] FIGS. 11A-B show design of multivalent inhibitors. FIG. 11A shows KRAS^{G12D} (PDB: 4DSN) with the switch 1 region shown in green and the switch 2 region in purple. FIG. 11B shows the location of three sites on KRAS targeted: D38 site in yellow, A59 site in blue, Y32 site in red. FIGS. 11C-E show conformational change of Y32 going from the inactive to the active form of HRAS. FIG. 11C shows flip of Y32 from the GDP-bound to GTP-bound forms. HRAS^{A59G} bound to GDP in yellow (PDB: 1LF5) and HRAS^{A59G} bound to GTP in blue (PDB: 1LF0). FIG. 11D shows surface representation of the GDP bound form. FIG. 11E shows surface representation of the GTP-bound form, revealing a larger pocket that is otherwise blocked by Y32 in the GDP bound form.

[0037] FIGS. 12A-D show design of multivalent inhibitors. FIG. 12A shows two of the top-scoring existing fragments docked into the D38 and A59 sites. FIG. 12B shows two top-scoring designed D38 fragments, docked as methylamine amides to represent the type of linkage that would occur to the adjacent A59 fragment. FIG. 12C shows examples of the indole scaffolds and

substituent arrangements attempted. The highest-scoring set was the substitution pattern in panel 4. FIG. 12D shows the structures and predicted docking poses of two of the two-site, D38-A59-binding compounds.

[0038] FIGS. 13A-C show design of multivalent inhibitors. FIG. 13A shows a view of the D38-binding moiety of 34MEW43, which shows the interaction of the amine groups with the side chains of D38 and D33. The amine indicated was optimally positioned to extend into the adjacent Y32 site. FIG. 13B shows the structure and predicted docking pose of the three-site inhibitor 34MEW95, based on the two-site compound 34MEW43. FIG. 13C shows a schematic of the scaffold for the three-site compounds with the points of diversity indicated and the library size of a single three-site compound vs. the sequential fragment docking approach implemented.

[0039] FIGS. 14A-L show biochemical evaluation of D38-A59 two-site inhibitors. FIG. 14A shows (left panel) the docking pose of 31MEW44 in KRAS^{G12D} (PDB: 4DSN) with residues shifting upon compound treatment colored in red, and (right panel) ¹H-¹⁵N HSQC spectrum of 50 μ M KRAS^{G12D} bound-to GppNHp in the absence (blue) and presence (red) of 250 μ M inhibitor 31MEW44. FIGS. 14B-C show biochemical evaluation of 34MEW43. FIG. 14B shows ¹H-¹⁵N HSQC spectrum of 50 μ M KRAS^{G12D} bound to GppNHp in the absence (blue) and presence (red) of 250 μ M inhibitor 34MEW43. Zoom in on the residues of D38 pocket are shown in the top left corner. These residues are growing (I36), shrinking (E37), or shifting (S39) upon compound treatment. FIG. 14C shows differential scanning fluorimetry of 5 μ M KRAS^{G12D} bound to GTP in the presence of increasing concentration of compound. The ΔT_m was calculated by subtracting the T_m of liganded

KRAS^{G12D} protein from unliganded KRAS^{G12D} and are expressed as absolute value of the mean $\pm$ sem. FIG. 14D shows in vitro RAS pulldown in the presence of 31MEW44 and 34MEW43 with GTP-loaded KRAS^{G12D} using CRAF-RBD agarose beads. FIG. 14E shows in vitro RAS pulldown in the presence of 31MEW44 and 34MEW43 using GTP-loaded KRAS^{G12D} and GST-tagged RALGDS using glutathione beads. FIG. 14F shows MST of the three-site compound 34MEW95 and the compound from which it was derived, 34MEW43, using KRAS^{G12D}-GppNHp. FIG. 14G shows MST of the two-site compound 31MEW44 and its differential selectivity towards the active (GppNHp bound) form vs. inactive (GDP bound) form of KRAS^{G12D}. FIG. 14H shows the effect of mutating residues within the docking site on KRAS^{G12D} on the affinity of 31MEW44 by MST. FIG. 14I shows isothermal calorimetry titration (top left panel) of KRAS^{G12D}-GppNHp into 31MEW44 and the thermodynamic parameters (right panel) of 31MEW44 binding. FIG. 14J shows normalized thermophoretic traces of a dilution series of 31MEW44 with GppNHp bound KRAS^{G12D}, RHEB, RHOA, and RALA. FIGS. 14K-L shows the effect of 31MEW44 binding to KRAS^{G12D}. FIG. 14 K (left panel) shows 31MEW44 bound to KRAS^{G12D} with shifted residues highlighted. FIG. 14K (right panel) shows the reverse view of KRAS^{G12D} with shifted residues highlighted. FIG. 14L shows chemical shift perturbations for each residue in KRAS^{G12D} upon 31MEW44 binding.

[0040] FIGS. 15A-C show the effect of multivalent RAS inhibitors on cell viability and modulation of RAS signaling pathways. Measured inhibitor EC₅₀ values for a panel of cancer cell lines (across an 8-point dilution series for 24 hours) is shown as a function of cell viability after RAS knockdown

using siRNA (FIG. 15A shows 31MEW44, FIG. 15B shows 34MEW43, and FIG. 15C shows 34MEW95). In each cell line, the mutant RAS isoform was knocked down, or the KRAS isoform if they consisted of wild-type RAS. EC₅₀ values were determined from three independent measurements performed on different days.

[0041] FIGS. 16A-B show the effect of multivalent RAS inhibitors on cell viability and modulation of RAS signaling pathways, and the ability of 31MEW44 to prevent anchorage independent growth. FIG. 16A shows (top panel) images of MDA-MB-231 cells after 72 hours in low adherence plates forming three dimensional multicellular spheroids when untreated, and killed by 31MEW44 at 20 μ M, and (bottom panel) dose-response curves of the effect of 31MEW44 on viability in MDA-MB-231 and SW480 cells grown in low adherence plates, expressed as growth inhibition. FIG. 16B shows dose-response curves with 31MEW44, 34MEW43 and 34MEW95.

[0042] FIGS. 17A-B show the effect of multivalent RAS inhibitors on cell viability and modulation of RAS signaling pathways. HT-1080 cells were retrovirally transfected with a pBABE-puro vector containing KRAS^{G12V} or the empty vector. Following selection with puromycin (1 μ g/mL treatment for 10 days) the RNA was extracted from the two cell lines and the expression of KRAS was quantified by qPCR (FIG. 17A). Transfected cells were then treated with 31MEW44, 34MEW43 and 34MEW95 at 5 μ M for 24 hours (FIG. 17B). All measurements were performed in triplicate.

[0043] FIG. 18 shows HT-1080 cells treated with 31MEW44 (5 μ M), 34MEW43 (10 μ M), 34MEW95 (5 μ M) and staurosporin for 24 hours. Cells were then lysed and treated with a pro-fluorescent caspase 3/7 substrate

(rhodamine 110 bis-N-CBZ-L-aspartyl-L-glutamyl-L-valyl-aspartic acid amide) for 16 hours.

[0044] FIGS. 19A-B show BJeLR cells were treated with DMSO, RAS inhibitor at 2 μ M and 8 μ M, U0126 at 8 μ M, or BEZ-235 at 0.2 μ M for 24 hours under serum free conditions. Cells were then lysed and the lysate was subjected to detection of phosphorylated Erk and total Erk by western blotting (FIG. 19A). Three independent experiments yielded essentially equivalent results. The quantification is shown in FIG. 19B.

[0045] FIGS. 20A-B show BJeLR cells were treated as described in FIGS. 19A-B and the lysate was incubated with CRAF-RBD bound agarose beads. The beads were then washed three times with PBS to remove any unbound RAS and the bound fraction was then denatured and subjected to detection by western blotting using a pan-RAS antibody (FIG. 20A). The quantification is shown in FIG. 20B.

[0046] FIGS. 21A-B show BJeLR cells were treated as described in FIGS. 19A-B and the lysate was subjected to detection of phosphorylated AKT (ser 437) and total AKT by western blotting (FIG. 21A). The quantification is shown in FIG. 21B).

[0047] FIGS. 22A-D show BJeLR cells were treated with DMSO, 31 MEW44 at 5 μ M and/or 25 μ M alongside BJHLT treated with DMSO for one hour. Cells were lysed and the RNA was extracted, converted to cDNA and quantified by qPCR. FIG. 22A shows relative quantity of uPA RNA, FIG. 22B shows relative quantity of MMP9 RNA, FIG. 22C shows relative quantity of cMYC RNA, and FIG. 22D shows relative quantity of lactate dehydrogenase

RNA. FIG. 22E shows relative quantity of DUSP6 RNA. FIG. 22F shows relative quantity of ID2 RNA.

[0048] FIGS. 23A-F shows efficacy of 31MEW44 in patient-derived T-ALL samples cultured *in vitro*. PDTALL22 (FIG. 23A) has *NRAS*^{G13V} and PDTALL26 (FIG. 23B) has *NRAS*^{G13D}, while PDTALL6, 9, 13 and 19 (FIGS. 23C-F, respectively) all have wild-type *NRAS*. Mutation status was verified by sequencing.

[0049] FIG. 24 shows stability of 31MEW44 and 7-ethoxycoumarin incubated with mouse liver microsomes.

[0050] FIG. 25 shows a total of 42 male C57 adult mice were dosed with 31MEW44 in 10% NMP/90% PEG-400 at 30 mg/kg. Shown is the concentration of 31MEW44 measured in the plasma over 12 hours after dosing intravenously or orally. All measurements were performed in triplicate.

[0051] FIG. 26 shows tumor sizes of the vehicle and 31MEW44 treatment groups at day 0 after 8 week old female nude mice were injected with 8 million MD-MB-231 cells to generate tumor xenografts.

[0052] FIG. 27 shows average tumor size in the tumor xenograft of FIG. 26 that were treated with 31MEW44 (8 mg/mL, 5% DMSO in HBSS at pH 4) dosed at 20 mg/kg once per day or vehicle.

[0053] FIG. 28 shows tumor weight of dissected xenografts from the mice of FIG. 26 on day 7.

[0054] FIG. 29 shows the effect of 31MEW44 on MDA-MB-231 xenografts. FIG. 29A shows 31MEW44-treated and vehicle-treated tumors from the tumor xenograft mice of FIG. 26 after dissection. FIG. 29B shows a

quantitative graph of average tumor size. Eight week old nude female mice were injected with 7 million MDA-MB-231 cells. After tumors reached an average size of 58 cubic millimeters they were treated with vehicle orally (10 doses), 31MEW44 orally (180 mg/kg, 10 doses), or by a combination of intravenous and intraperitoneal injections (30 mg/kg, 4 IV doses, 6 IP doses) over two weeks.

[0055] FIGS. 30A-B show a western blot of phosphorylated ERK performed on xenograft samples (FIG. 30A) with the quantification (FIG. 30B).

[0056] FIGS. 31A-B show that 31MEW44 is selectively lethal to cell lines dependent on mutant RAS, induced caspase activation and prevention of anchorage independent growth. FIG. 31A shows the effect of 31MEW44 on the viability of a panel of cancer cell lines. Cell lines were treated in 6-well format for 24 hours with 5 μ M 31MEW44. FIG. 31B shows the correlation of cell line sensitivity of mutant RAS knockdown to 2.5 μ M 31MEW44 treatment. The viability was measured 72 hours after reverse transfection with siRNA when cell death control siRNA resulted in complete loss of viability. Knockdown was confirmed by qPCR of the mutated isoform.

[0057] FIGS. 32A-B show the sensitivity of a cell line panel to mutant RAS knockdown and the correlation of the sensitivity to the knockdown with the sensitivity to 31MEW44. FIG. 32A shows the measured viability 72 hours post-transfection of cell lines that were reverse transfected with siRNA against the mutated RAS isoform. FIG. 32B shows the percent viability after 24 hours of treatment with the inhibitor at 5 μ M plotted against the percent viability measured 72 hours after reverse transfection.

[0058] FIG. 33A shows the effect of 31MEW44 on the growth inhibition of MEFs containing a floxed allele of Kras compared to induction of CRE recombinase by 4OHT. Cells were treated in 6-well format with 31MEW44 at 2.5 μ M, 5 μ M, or DMSO for 24 hours. In parallel, cells were treated with DMSO or 4OHT at 600 nM for 6 days. FIG. 33B shows the induction of caspase 3/7 activation by 31MEW44. HT-1080 cells treated were with 31MEW44 or staurosporin for 24 hours. Cells were then lysed and treated with a pro-fluorescent caspase 3/7 substrate (rhodamine 110 bis-N-CBZ-L-aspartyl-L-glutamyl-L-valyl-aspartic acid amide) for 16 hours and measured.

[0059] FIG. 34A shows the effect of KRAS and effector overexpression on 31MEW44 sensitivity. HT-1080 cells were retrovirally transfected with a pBABE-puro empty vector or vector containing KRAS^{G12V}, PI3K^{E545K}, or BRAF^{V600E}. Following selection with puromycin, a population of the PI3K^{E545K} transfected cells were transfected a second time with a pBABE-neo-BRAF^{V600E} vector and selected a second time with geneticin. Stable cell lines were then treated with 31MEW44 for 24 hours in 6-well format. Cell lysates were analyzed by western blotting for levels of downstream phosphorylated ERK and AKT. FIG. 34B shows a dose-response curve of 34MEW43 and the 3-site compound 34MEW95 in 3D cell culture of MDA-MB-231 cells.

[0060] FIGS. 35A-B show validation of the efficacy of the transfection of mutant KRAS, PI3K, and BRAF. FIG. 35A shows qPCR of KRAS, PI3K, and BRAF. Each transfected population was analyzed for expression of each targeted gene. FIG. 35B shows a western blot of downstream phosphorylated ERK1/2 and AKT (S473) compared to total ERK1/2 and AKT in transfected

cell lines. Immediately preceeding the evaluation of 31MEW44, a sample of cells from each transfection condition were taken, lysed and analyzed.

[0061] FIGS. 36A-B show the effect of 31MEW44 on the RAS-RALGDS pathway. In FIG. 36A, BJeLR cells were seeded in 2% FBS in DMEM 18 hours prior to treatment with 31MEW44 and U0126 in 2% FBS in DMEM for 3 hours. Cells were then lysed and the lysate was incubated with RalBP1 agarose beads for 2 hours before being washed twice with PBS, denatured and subsequently detected by western blotting. FIG. 36B shows cRAF, PI3Ky, and panRAS pulldowns from BJeLR cells seeded in 10% FBS in DMEM 18 hours prior to treatment with 31MEW44 in 10% FBS in DMEM for 3 hours.

[0062] FIG. 37 shows expression levels of mutant RAS with 31MEW44 and doxorubicin treatment. Each cell line indicated was treated with 10 μ M 31MEW44 for 4 hours or 24 hours before being lysed and analyzed for expression of the mutant RAS isoform by qPCR. Samples were normalized to DMSO treatment.

[0063] FIGS. 38A-D show 31MEW44 activity in a patient derived T-ALL xenograft. FIG. 38A shows the effect of 31MEW44 on a patient-derived T-ALL sample PDTALL22 as a luciferase expressing primograft. Mice were randomized into two treatment groups of 5 mice with equal loads of luciferase. Mice were treated with 31MEW44 (30 mg/kg) or vehicle by intraperitoneal injection once daily on days: 0, 1, 4, 5, and 7. FIG. 38B shows representative images of mice from each treatment group at days 0, 4, and 8 (final day) of the study. FIG. 38C is a bar graph representing spleen weight in grams. FIG.

38D shows the percentage of human CD45+ cells in the spleen of vehicle and 31MEW44-treated mice.

[0064] FIGS. 39A-F show that 31MEW44 displays genotype selective toxicity. FIG. 39A is a survival curve of $KP^{ff}C$ and wild-type mice that received 30 mg/kg 31MEW44 once daily for five days. FIG. 39B is a graph showing the measurement of wild-type mouse weight each day of the five day study. FIG. 39C shows an analysis of tissue samples taken from $KP^{ff}C$ mice by western blotting for phosphorylated ERK1/2, total ERK1/2, phosphorylated AKT (S473), and total AKT. Samples were taken prior to treatment (biopsy, bx) and post-treatment (necropsy, nx) from each of three mice receiving either 31MEW44 (30 mg/kg) or vehicle dosed once daily by intraperitoneal injection. FIG. 39D shows images from representative sections of the biopsy and necropsy samples from 31MEW44 and vehicle treated mice. Hematoxylin and eosin, phosphorylated ERK1/2, total ERK1/2, and cleaved caspase-3 were detected by immunohistochemistry. FIGS. 39E-F show an analysis of cleaved caspase-3 in $KP^{ff}C$ mice tumor sections. FIG. 39E shows immunohistochemistry of tumor sections taken before and after treatment using an anti-cleaved caspase-3 antibody. FIG. 39F is a graph showing the quantification of the levels of cleaved caspase-3. Levels are expressed as number of cleaved caspase-3 positive cells per 40X field. Shown is an average of 3 sections taken from 3 separate mice +/- the standard deviation.

DETAILED DESCRIPTION OF THE INVENTION

[0065] One embodiment of the present invention is a compound that selectively binds a RAS protein at two or more sites.

[0066] As used herein, “selectively binds”, and grammatical variations thereof, means a binding reaction between two molecules that is at least two times the background and more typically more than 10 to 100 times background molecular associations under physiological conditions. Likewise, compounds “selective” for a given form of a RAS protein may exhibit molecular associations under physiological conditions at least two times the background and more typically more than 10 to 100 times background.

[0067] As used herein, RAS proteins include all RAS isoforms, which are members of a family of GTPase proteins frequently mutated in numerous cancers. The terms, “isoform” and grammatical variations thereof, refer to functionally similar proteins that have a similar, but not identical amino acid sequence, and may also be differentially post-translationally modified. RAS isoforms include, but are not limited to HRAS, KRAS, and NRAS. The HRAS, KRAS, and NRAS proteins are highly homologous to one another and have similar mechanisms of action. However, these proteins are distinct in their post-translational modifications, resulting in disparate cell trafficking routes and subcellular localization. Hence, HRAS, KRAS, and NRAS affect cellular processes in distinct ways. For example, HRAS is the most effective RAS protein at transforming fibroblasts. Furthermore, NRAS transforms hematopoietic cells most efficiently. Likewise, KRAS-deficient mice are embryonic lethal whereas NRAS or HRAS knock outs are essentially phenotypically normal (Parikh, et al., 2007). Representative HRAS, KRAS, and NRAS nucleic acid and polypeptide sequences are shown in Tables 1, 2, and 3, respectively, below.

Table 1
HRAS Sequences

SEQ ID NO.	Nucleotide/Polypeptide	Organism	Gene Name	Additional Information
1	Nucleotide	Homo sapiens	HRAS	Variant 1
2	Nucleotide	Homo sapiens	HRAS	Variant 2
3	Nucleotide	Homo sapiens	HRAS	Variant 3
4	Polypeptide	Homo sapiens	HRAS	Isoform 1
5	Polypeptide	Homo sapiens	HRAS	Isoform 2
6	Nucleotide	Mus musculus	HRAS	Variant 1
7	Nucleotide	Mus musculus	HRAS	Variant 2
8	Nucleotide	Mus musculus	HRAS	Variant 3
9	Polypeptide	Mus musculus	HRAS	Isoform 1
10	Polypeptide	Mus musculus	HRAS	Isoform 2
11	Nucleotide	Rattus norvegicus	HRAS	Variant 1
12	Nucleotide	Rattus norvegicus	HRAS	Variant 2
13	Polypeptide	Rattus norvegicus	HRAS	
14	Nucleotide	Canis lupus familiaris	HRAS	Variant 1
15	Nucleotide	Canis lupus familiaris	HRAS	Variant 2
16	Polypeptide	Canis lupus familiaris	HRAS	Isoform 1
17	Polypeptide	Canis lupus familiaris	HRAS	Isoform 2
18	Nucleotide	Gallus gallus	HRAS	
19	Polypeptide	Gallus gallus	HRAS	
20	Nucleotide	Bos taurus	HRAS	Variant 1
21	Nucleotide	Bos taurus	HRAS	Variant 2
22	Polypeptide	Bos taurus	HRAS	Isoform 1
23	Polypeptide	Bos taurus	HRAS	Isoform 2

Table 2
KRAS Sequences

SEQ ID NO.	Nucleotide/Polypeptide	Organism	Gene Name	Additional Information
24	Nucleotide	Homo sapiens	KRAS	Variant a
25	Nucleotide	Homo sapiens	KRAS	Variant b

SEQ ID NO.	Nucleotide/Polypeptide	Organism	Gene Name	Additional Information
26	Polypeptide	Homo sapiens	KRAS	Isoform a
27	Polypeptide	Homo sapiens	KRAS	Isoform b
28	Nucleotide	Mus musculus	KRAS	
29	Polypeptide	Mus musculus	KRAS	
30	Nucleotide	Rattus norvegicus	KRAS	
31	Polypeptide	Rattus norvegicus	KRAS	
32	Nucleotide	Canis lupus familiaris	KRAS	Predicted variant 1
33	Nucleotide	Canis lupus familiaris	KRAS	Predicted variant 2
34	Polypeptide	Canis lupus familiaris	KRAS	Predicted isoform 1
35	Polypeptide	Canis lupus familiaris	KRAS	Predicted isoform 2
36	Nucleotide	Gallus gallus	KRAS	
37	Polypeptide	Gallus gallus	KRAS	
38	Nucleotide	Bos taurus	KRAS	
39	Polypeptide	Bos taurus	KRAS	

Table 3
NRAS Sequences

SEQ ID NO.	Nucleotide/Polypeptide	Organism	Gene Name
40	Nucleotide	Homo sapiens	NRAS
41	Polypeptide	Homo sapiens	NRAS
42	Nucleotide	Mus musculus	NRAS
43	Polypeptide	Mus musculus	NRAS
44	Nucleotide	Rattus norvegicus	NRAS
45	Polypeptide	Rattus norvegicus	NRAS
46	Nucleotide	Canis lupus familiaris	NRAS
47	Polypeptide	Canis lupus familiaris	NRAS
48	Nucleotide	Gallus gallus	NRAS
49	Polypeptide	Gallus gallus	NRAS
50	Nucleotide	Bos taurus	NRAS
51	Polypeptide	Bos taurus	NRAS

[0068] The term “sites”, and grammatical variations thereof, means any region of a protein, including those regions comprising the exterior, solvent-

exposed portion of a protein. Such a site may be a pocket where other protein species or compounds interact with the RAS protein. Sites also may become available for binding upon conformation change. For example, RAS has a pocket present only in the active form, when Y32 undergoes a conformational change in which it flips over to the other end of the nucleotide-binding site and forms a hydrogen bond with the gamma phosphate of GTP. This change unveils a pocket (termed the Y32 site) that is not present in the GDP-bound form. Compounds of the present invention may bind a RAS protein at two or more sites, including 2, 3, 4, 5, 6, 7, 8, 9, 10, or more sites on the RAS protein.

[0069] In one aspect of this embodiment, the compound selectively binds to a first site on the RAS protein that comprises at least one amino acid from the switch 1 region (near D38). As used herein, “near”, as it relates to distances from certain residues, such as D38, A59, or I21, means within about 9 angstroms of the residue, including, but not limited to, within 1, 2, 3, 4, 5, 6, 7, or 8 angstroms of the residue on the RAS protein that corresponds to the amino acid number (such as 38, 59, or 21) of the human HRAS protein (SEQ ID NO. 4 or 5). The corresponding regions of HRAS from other animal, as well as NRAS, KRAS, or other RAS proteins from human and other animals, are also within the scope of the present invention and are readily determined by one skilled in the art. See, *e.g.*, Valencia et al., 1991. “Corresponds,” with reference to amino acid numbers on RAS, means consistent with, as done by sequence alignment. Multiple sequence alignment methods including pairwise sequence alignment methods, may be used to determine the position in a RAS protein that corresponds to the positions listed above. Methods of

sequence alignment are well-known. Many sequence alignment softwares are available. These programs include, *e.g.*, BLAST, ClustalW, SEQALN, DNA Baser, MEME/MAST, BLOCKS, and eMOTIF. Preferably, the sequence alignment software is BLAST.

[0070] Preferably, the compound selectively binds to a second site on the RAS protein that comprises at least one amino acid located between the switch 1 and switch 2 regions (near A59). In another preferred aspect, the compound also selectively binds to at least one amino acid near I21 (Y32 site) of the RAS protein.

[0071] In the present invention, the switch 1 region, located near the D38 site, includes residues 30-40 corresponding to the human RAS protein (*e.g.*, SEQ ID NOs: 4, 5, 26, 27, or 41). The D38 site is one region of conserved interaction between RAS proteins and RAF, RALGDS, and PI3K. The switch 2 region is near A59 and comprises residues 60-70 corresponding to the human RAS protein (*e.g.*, SEQ ID NOs: 4, 5, 26, 27, or 41). The A59 site is located between the switch 1 and switch 2 regions and is adjacent to the D38 site.

[0072] As used herein, “at least one amino acid” from any of the regions or locations of a RAS protein disclosed herein include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more amino acids, up to, and including, the number of amino acids comprising the entire designated region or location of RAS.

[0073] In another aspect of this embodiment, the compound is selective for a GTP-bound RAS protein. As disclosed further in the Examples, the GTP-bound, active form of a RAS protein contains a pocket, termed the “Y32

site”, that is not present in the GDP-bound, inactive form of RAS protein. The Y32 site is located near residue I21 of the human RAS protein (e.g., SEQ ID NOs: 4, 5, 26, 27, or 41). Residue Y32 undergoes a conformation change in the active, GTP-bound form of RAS protein, revealing the aforementioned pocket. Therefore, oncogenic RAS isoforms, and the cancer cells that express them, may be targeted by compounds selective for GTP-bound RAS at, for example, the Y32 site. Preferably, compounds of the present invention may also be selective for a non-GDP-bound form of the RAS protein. A non-GDP-bound form of a RAS protein may be, for example, a GTP-bound form of a RAS protein or a RAS protein not bound to any nucleotides.

[0074] In a further aspect of this embodiment, the RAS protein is an isoform selected from the group consisting of HRAS, KRAS, NRAS, and combinations thereof.

[0075] In an additional aspect of this embodiment, the RAS protein is an oncogenic mutant. As used herein, an “oncogenic mutant” is a RAS variant that contains an alteration in the amino acid sequence and has the potential to cause a cell to become cancerous. In the context of RAS protein, an oncogenic mutant may be a constitutively active, continually GTP-bound isoform of RAS protein. Preferably, the RAS protein is an oncogenic mutant selected from the group consisting of HRAS^{G12D}, KRAS^{G12D}, NRAS^{Q61K}, NRAS^{G13V}, and NRAS^{G13D}, the mutations based on the human isoform for the respective protein, e.g., SEQ ID NO: 4, 5, 26, 27, or 41. In terms of oncogenicity, mutations at residues 12-13 of a RAS protein render RAS’s GTPase portion insensitive to activation by GAPs, while mutations at residue

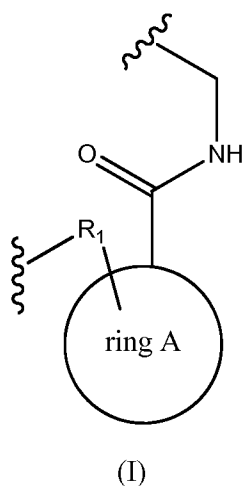
61 affect the enzymatic active site of a RAS protein directly, thereby essentially inactivating the GTPase activity of a RAS protein.

[0076] In another aspect of this embodiment, the compound selectively binds to at least one amino acid near D38, A59, and optionally I21 (Y32 site) in a RAS protein. Preferably, the compound comprises a region A that binds to at least one amino acid near D38 on a RAS protein and comprises a heterocycle with at least one ring nitrogen.

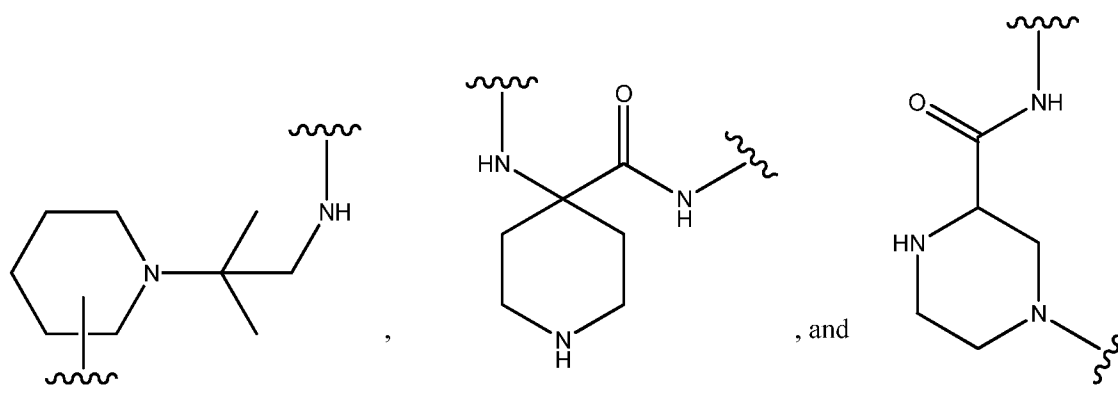
[0077] As used herein, the term “heterocycle” means substituted or unsubstituted non aromatic ring structures. Preferably the heterocycle comprises 3 to 8 membered rings, and at least one heteroatom, preferably one to four heteroatoms, more preferably one or two heteroatoms. Such heterocycles may include at least one ring nitrogen. The term “heterocycle” also includes polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings wherein at least one of the rings is heterocyclic, *e.g.*, the other cyclic ring(s) can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryls, and/or heterocyclyls. Heterocycle groups of the present invention include, for example, piperidine, piperazine, pyrrolidine, morpholine, lactones, lactams, and the like.

[0078] The term “heteroatom” as used herein means an atom of any element other than carbon or hydrogen. Preferred heteroatoms are nitrogen, oxygen, and sulfur; more preferably, nitrogen and oxygen.

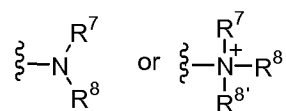
[0079] In one preferred embodiment, the A region of the compound comprises a fragment having formula (I):



wherein ring A is a heterocycle with at least one ring nitrogen, and R₁ is selected from the group consisting of no atom, amine, and C₁₋₄ aliphatic. More preferably, the A region of the compound is selected from the group consisting of:



[0080] The terms “amine” and “amino” are art-recognized and refer to both unsubstituted and substituted amines and salts thereof, *e.g.*, a moiety that can be represented by



wherein R⁷, R⁸, and R^{8'} each independently represent a hydrogen or a hydrocarbonyl group, or R⁷ and R⁸ taken together with the N atom to which they

are attached complete a heterocycle having from 4 to 8 atoms in the ring structure. The term "primary" amine means only one of R^7 and R^8 or one of R^7 , R^8 , and $R^{8'}$ is a hydrocarbyl group. Secondary amines have two hydrocarbyl groups bound to N. In tertiary amines, all three groups, R^7 , R^8 , and $R^{8'}$, are replaced by hydrocarbyl groups.

[0081] The term " C_{x-y} " when used in conjunction with a chemical moiety, such as, alkyl, alkenyl, or alkoxy is meant to include groups that contain from x to y carbons in the chain. For example, the term " C_{x-y} alkyl" means substituted or unsubstituted saturated hydrocarbon groups, including straight-chain alkyl and branched-chain alkyl groups that contain from x to y carbons in the chain, including haloalkyl groups such as trifluoromethyl and 2,2,2-trifluoroethyl, etc. The terms " C_{2-y} alkenyl" and " C_{2-y} alkynyl" refer to substituted or unsubstituted unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double or triple bond respectively.

[0082] The term "aliphatic", as used herein, means a group composed of carbon and hydrogen atoms that does not contain aromatic rings. Accordingly, aliphatic groups include alkyl, alkenyl, alkynyl, and carbocyclyl groups. A preferred C_{1-4} aliphatic is a vinyl moiety.

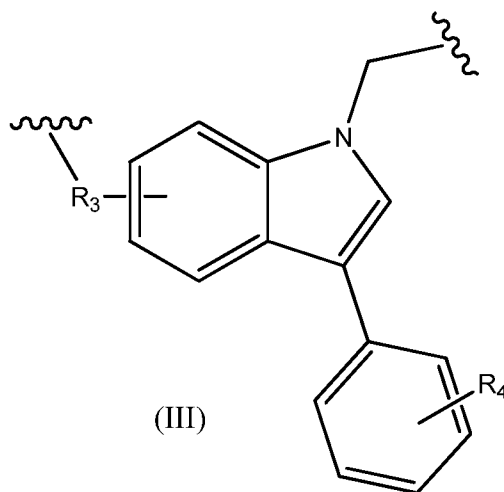
[0083] The term "alkyl" means the radical of saturated aliphatic groups that does not have a ring structure, including straight-chain alkyl groups, and branched-chain alkyl groups. In certain embodiments, a straight chain or branched chain alkyl has 4 or fewer carbon atoms in its backbone (e.g., C_1 - C_4 for straight chains, C_3 - C_4 for branched chains).

[0084] The term “alkenyl”, as used herein, means an aliphatic group containing at least one double bond.

[0085] The term “alkynyl”, as used herein, means an aliphatic group containing at least one triple bond.

[0086] In another preferred embodiment, the compound comprises a region B that binds to at least one amino acid near A59 on the RAS protein and comprises an indole. The term “indole” is art-recognized and means any compound containing a benzene ring fused to a pyrrole ring.

[0087] More preferably, the B region of the compound comprises a structure of formula (III):



wherein

R₃ is selected from the group consisting of heterocycle, aryl, and amine, which heterocycle, aryl, and amine may be optionally substituted with the group selected from halide, C₁₋₄ aliphatic, and combinations thereof; and

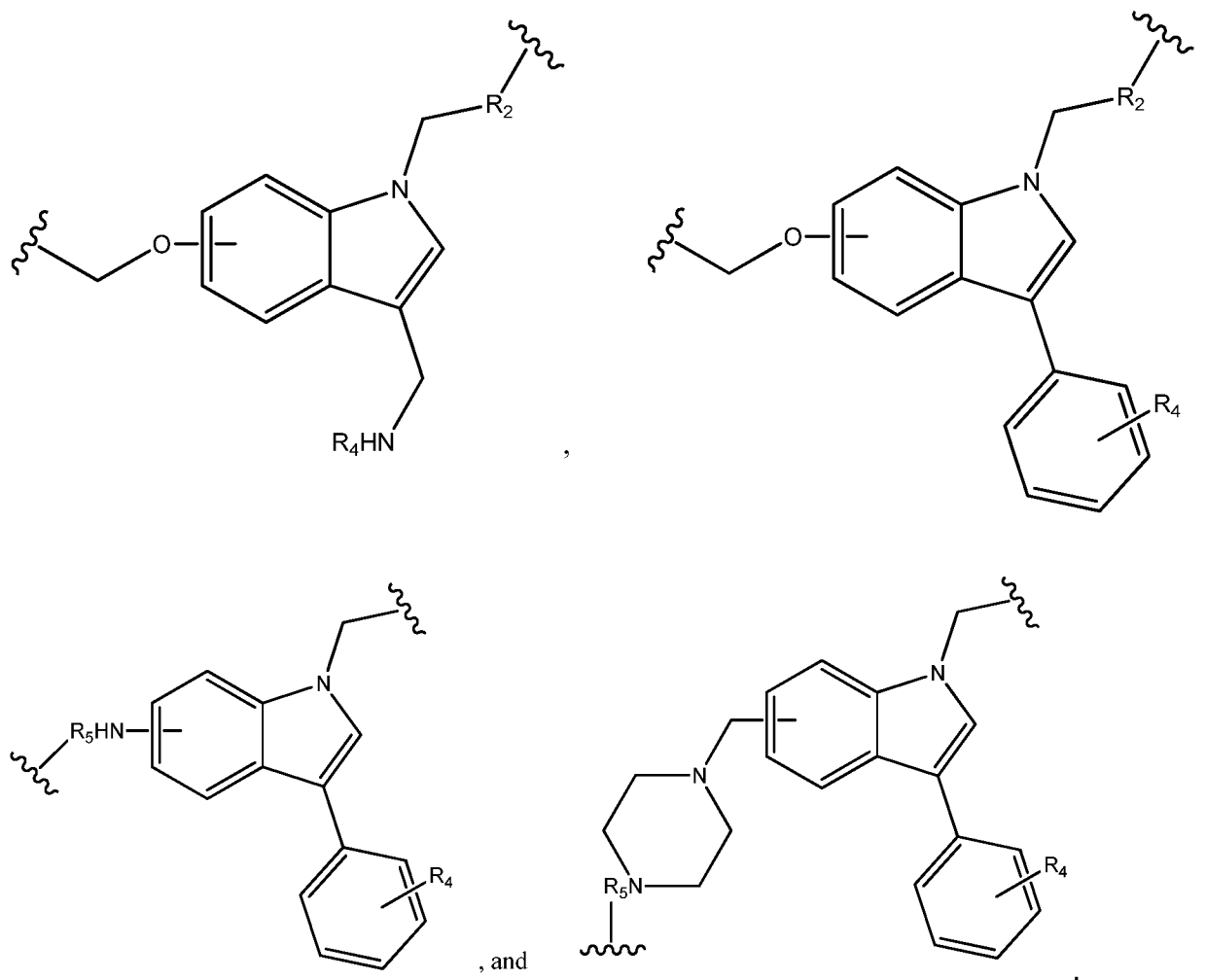
R₄ is selected from the group consisting of no atom, H, aryl, halide, C₁₋₄ aliphatic -O-C₁₋₄alkyl wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof.

[0088] The term “aryl” as used herein includes substituted or unsubstituted single-ring aromatic groups in which each atom of the ring is carbon. Preferably the ring is a 3- to 8-membered ring, more preferably a 6-membered ring. The term “aryl” also includes polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings wherein at least one of the rings is aromatic, *e.g.*, the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryls, and/or heterocyclyls. Aryl groups include benzene, naphthalene, phenanthrene, phenol, aniline, and the like.

[0089] The term “substituted” means moieties having substituents replacing a hydrogen on one or more carbons of the backbone. It will be understood that “substitution” or “substituted with” includes the implicit proviso that such substitution is in accordance with the permitted valence of the substituted atom and the substituent, and that the substitution results in a stable compound, *e.g.*, which does not spontaneously undergo transformation such as by rearrangement, cyclization, elimination, etc. The permissible substituents can be one or more and the same or different for appropriate organic compounds.

[0090] As used herein, a “halide” means a halogen atom such as fluorine, chlorine, bromine, iodine, or astatine.

[0091] In another preferred embodiment, the B region of the compound is selected from the group consisting of

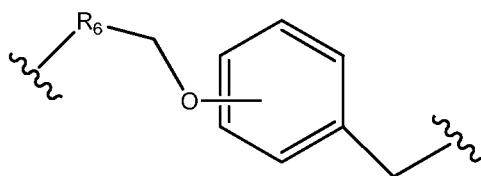


wherein

R₂ and R₅ are independently selected from the group consisting of no atom, aryl, and C₁₋₄ aliphatic; and

R₄ is selected from the group consisting of no atom, H, aryl, halide, C₁₋₄ aliphatic, -O-C₁₋₄alkyl wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof.

[0092] In another preferred embodiment, the compound comprises a region C that binds to at least one amino acid near I21 (Y32 site) of the RAS protein and comprises an aromatic ring. More preferably, the C region of the compound comprises a structure of formula (IV):



(IV)

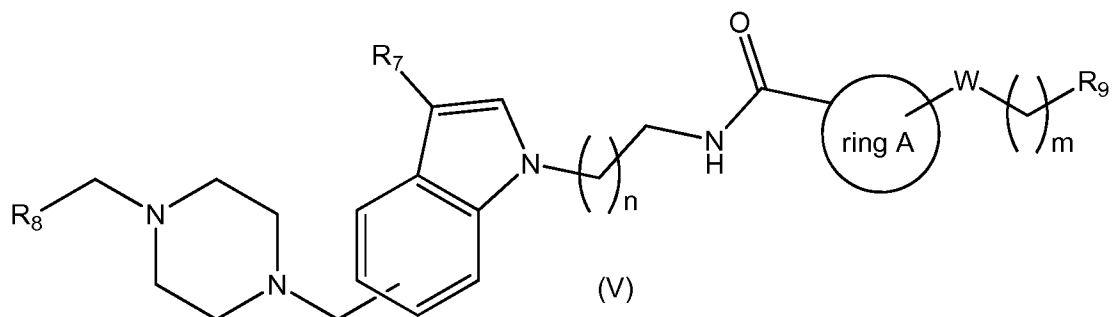
wherein R_6 is selected from the group consisting of no atom, H, alkyl, and aryl, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with the group consisting of halide, ether, C_{1-4} alkyl, and a combination thereof.

[0093] As used herein, an “aromatic ring” is an aryl or a heteroaryl. The term “heteroaryl” includes substituted or unsubstituted aromatic single ring structures, preferably 3- to 8-membered rings, more preferably 5- to 7-membered rings, even more preferably 5- to 6-membered rings, whose ring structures include at least one heteroatom, preferably one to four heteroatoms, more preferably one or two heteroatoms. The term “heteroaryl” also includes polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings wherein at least one of the rings is heteroaromatic, *e.g.*, the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls, heteroaryls, and/or heterocyclyls. Heteroaryl groups include, for example, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, pyrazole, pyridine, pyrazine, pyridazine, and pyrimidine, and the like.

[0094] The term “ether”, as used herein, means a hydrocarbyl group linked through an oxygen to another hydrocarbyl group. Accordingly, an ether substituent of a hydrocarbyl group may be hydrocarbyl-O-. Ethers may be either symmetrical or unsymmetrical. Examples of ethers include, but are not

limited to, heterocycle-O-heterocycle and aryl-O-heterocycle. Ethers include “alkoxyalkyl” groups, which may be represented by the general formula alkyl-O-alkyl.

[0095] In a further aspect of this embodiment, the compound has a structure of formula (V):



wherein:

R_7 is selected from the group consisting of H, halide, C_{1-4} aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, $-O-C_{1-4}$ alkyl and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R_8 is selected from the group consisting of no atom, H, alkyl, aryl, and C_{1-4} alkyl-O-aryl wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, and a combination thereof;

R_9 is selected from the group consisting of no atom, H, C_{1-4} alkyl, and aryl optionally substituted with the group consisting of ether, halide, and a combination thereof;

W is selected from the group consisting of no atom and NH;

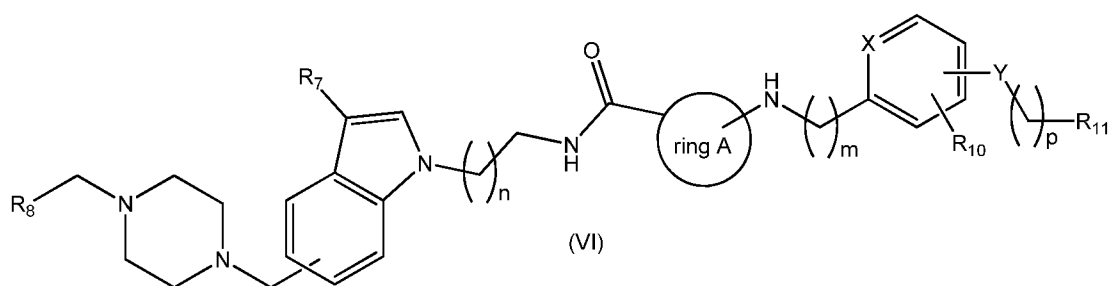
m and n are independently selected from the group consisting of an integer between 0-5; and

ring A is a heterocycle with at least 1 ring nitrogen and optionally substituted with C₁₋₄alkyl or a halide,

or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

[0096] As used herein, an “integer between 0-5” means 0, 1, 2, 3, 4, or 5.

[0097] In an additional aspect of this embodiment, the compound has a structure of formula (VI):



wherein:

R₇ is selected from the group consisting of H, halide, C₁₋₄ aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C₁₋₄alkyl, -O-C₁₋₄alkyl, and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R₈ and R₁₁ are independently selected from the group consisting of no atom, H, alkyl, aryl and C₁₋₄alkyl-O-aryl, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with one or

more groups consisting of halide, ether, C₁₋₄alkyl, -O-C₁₋₄alkyl, and a combination thereof, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof;

R₁₀ is selected from the group consisting of no atom, H, halide, C₁₋₄ aliphatic, and -O-C₁₋₄alkyl;

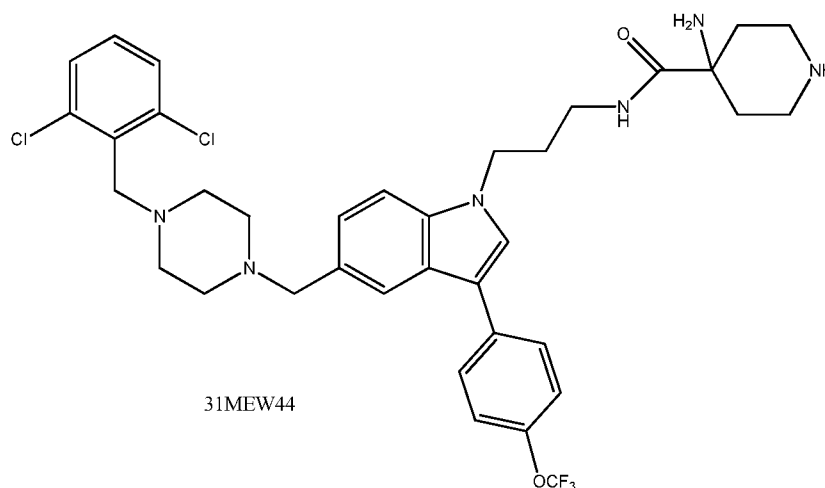
X is selected from the group consisting of CH and N;

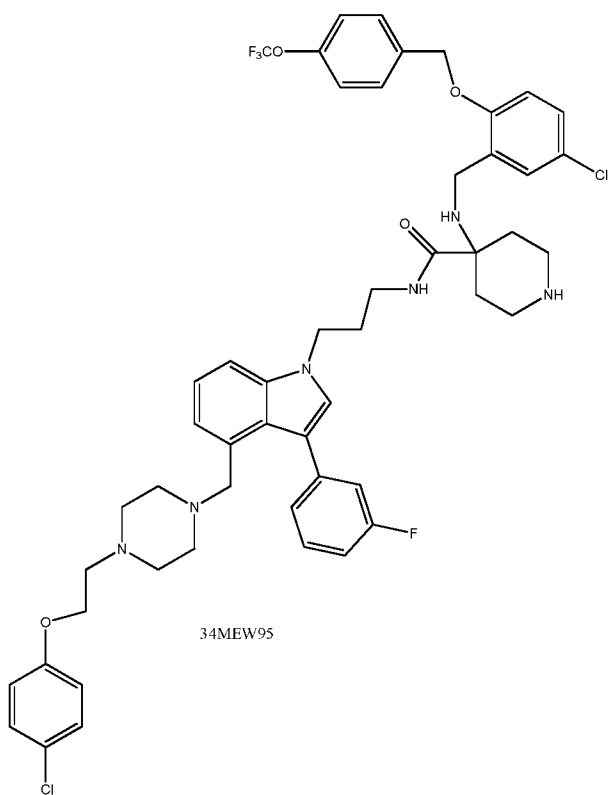
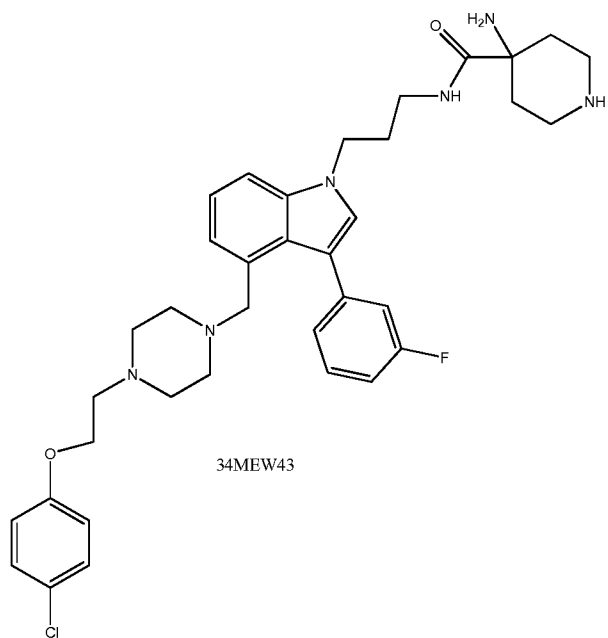
Y is selected from the group consisting of no atom and O;

m, n, and p are independently selected from the group consisting of an integer between 0-5; and ring A is a heterocycle with at least 1 ring nitrogen and optionally substituted with C₁₋₄alkyl or a halide,

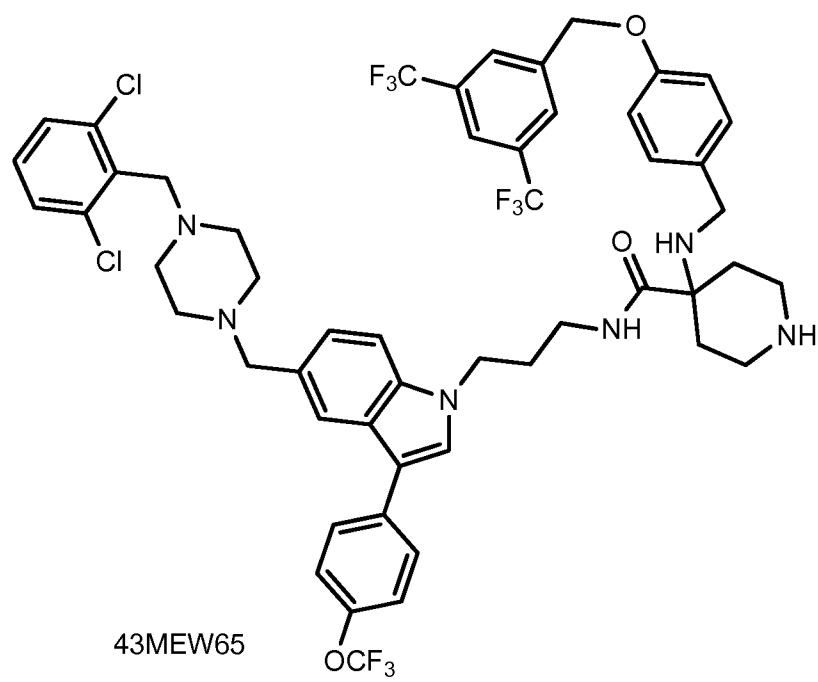
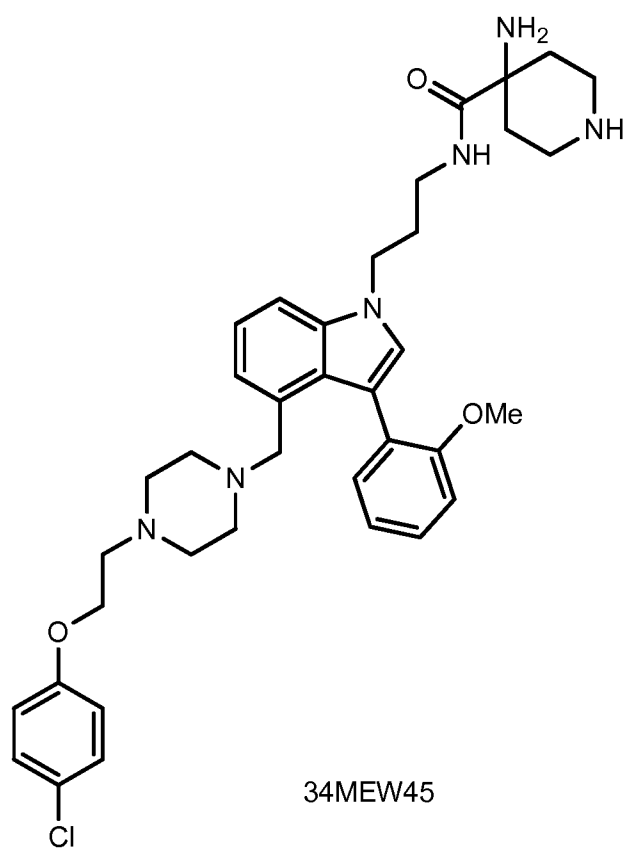
or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

[0098] In another aspect of this embodiment, the compound is selected from the group consisting of

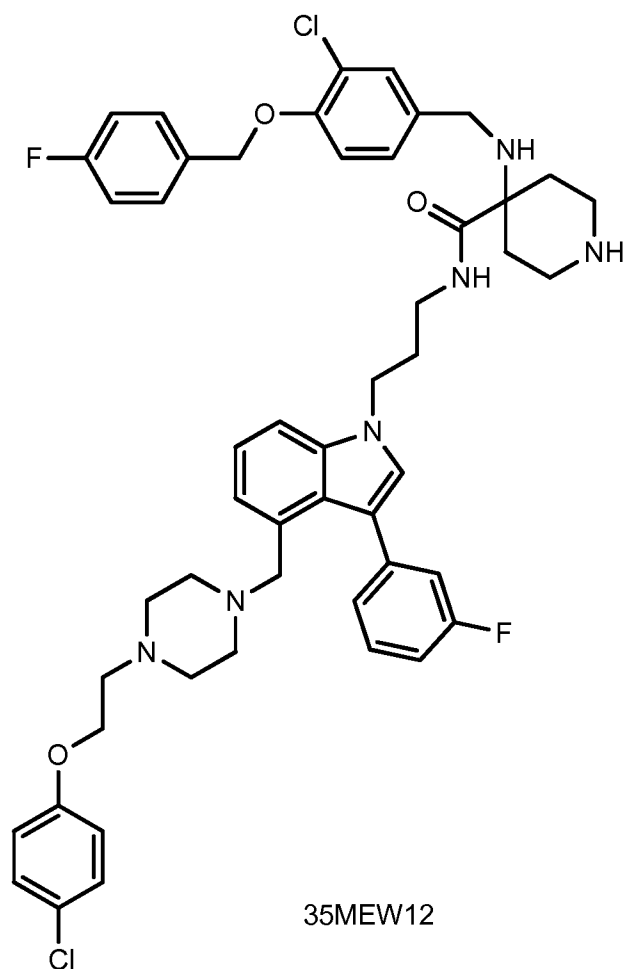






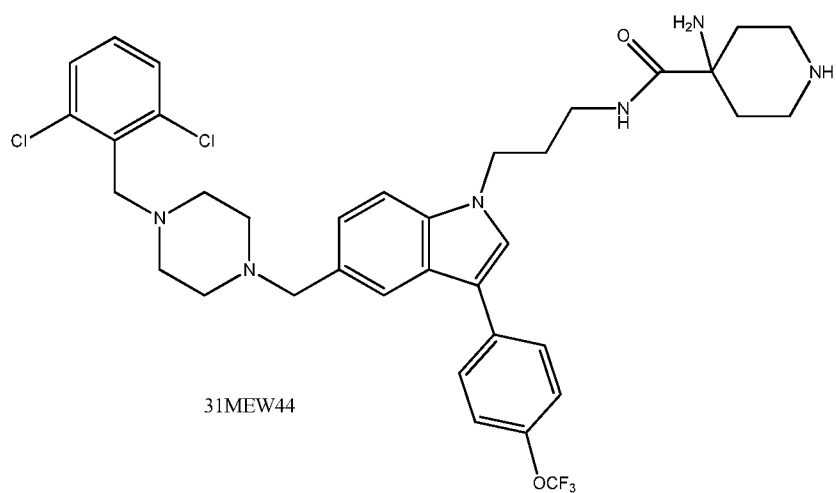






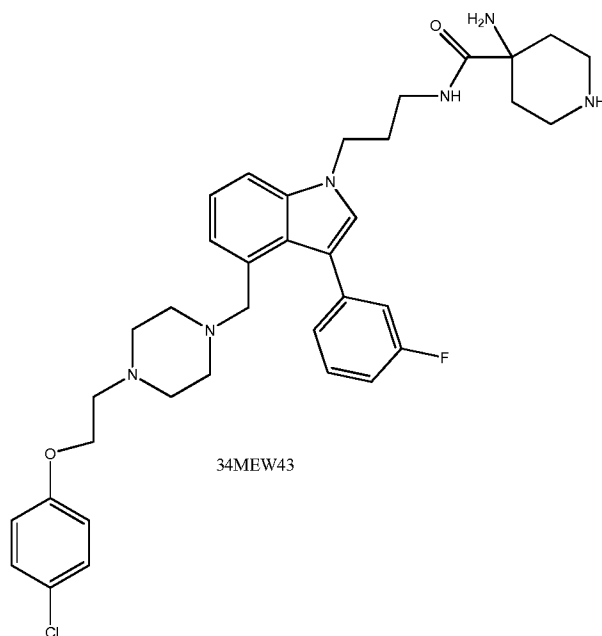
and crystalline forms, hydrates, or pharmaceutically acceptable salts thereof.

[0099] In one preferred embodiment, the compound is



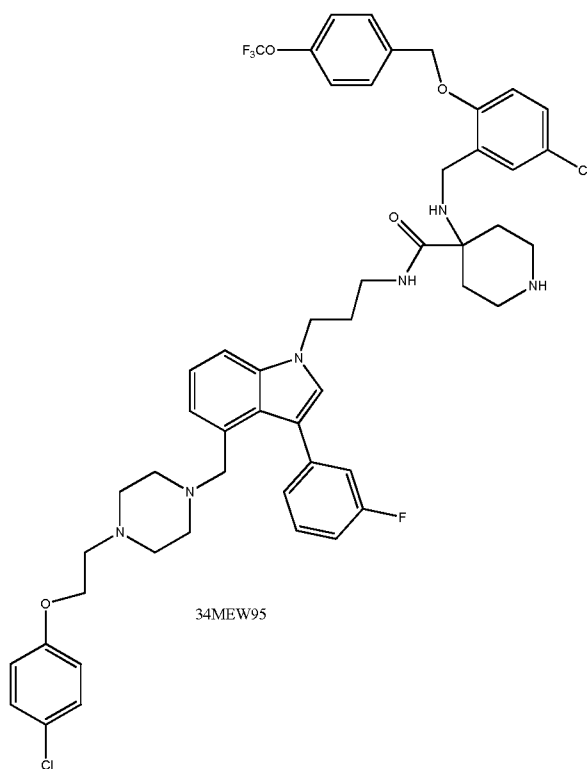
or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

[0100] In another preferred embodiment, the compound is



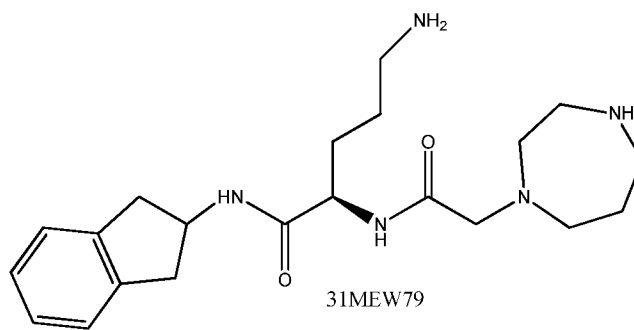
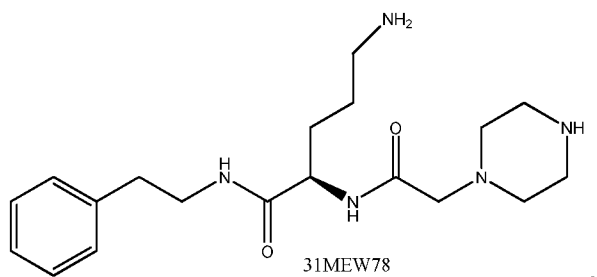
or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

[0101] In a further preferred embodiment, the compound is



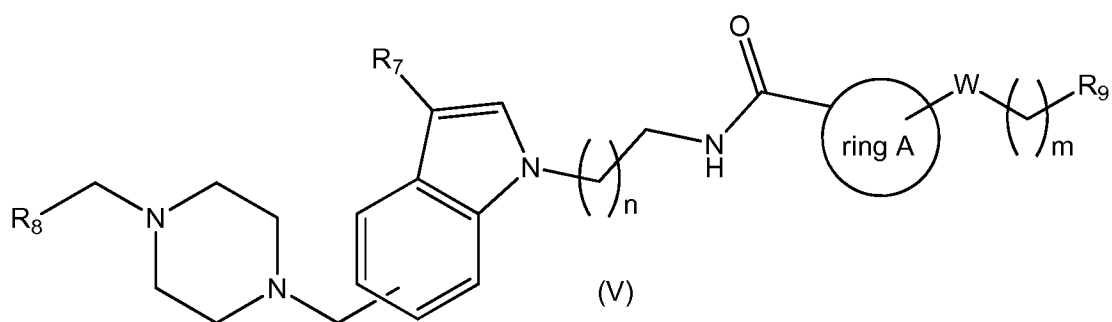
or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

[0102] Another embodiment of the present invention is a compound selected from the group consisting of



and crystalline forms, hydrates, or pharmaceutically acceptable salts thereof.

[0103] A further embodiment of the present invention is a compound having the structure of formula (V):



wherein:

R_7 is selected from the group consisting of H, halide, C_{1-4} aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, $-O-C_{1-4}$ alkyl and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R_8 is selected from the group consisting of no atom, H, alkyl, aryl, and C_{1-4} alkyl-O-aryl wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, and a combination thereof;

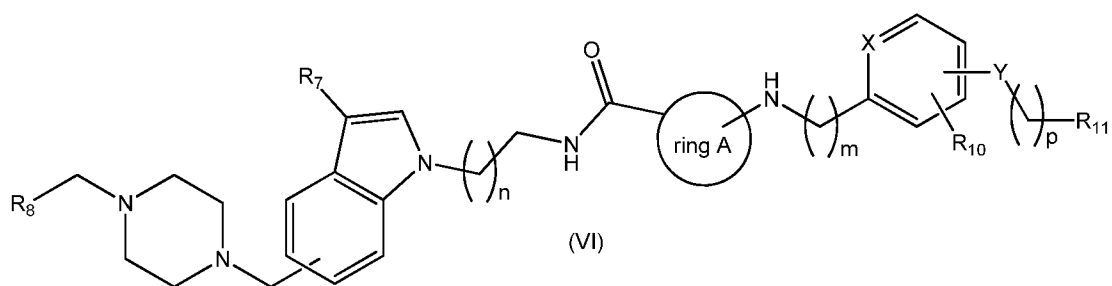
R_9 is selected from the group consisting of no atom, H, C_{1-4} alkyl, and aryl optionally substituted with the group consisting of ether, halide, and a combination thereof;

W is selected from the group consisting of no atom and NH; m and n are independently selected from the group consisting of an integer between 0-5; and

ring A is a heterocycle with at least 1 ring nitrogen and optionally substituted with C_{1-4} alkyl or a halide,

or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

[0104] An additional embodiment of the present invention is a compound having the structure of formula (VI):



wherein:

R_7 is selected from the group consisting of H, halide, C_{1-4} aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of

halide, ether, C₁₋₄alkyl, -O-C₁₋₄alkyl, and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R₈ and R₁₁ are independently selected from the group consisting of no atom, H, alkyl, aryl and C₁₋₄alkyl-O-aryl, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with one or more groups consisting of halide, ether, C₁₋₄alkyl, -O-C₁₋₄alkyl, and a combination thereof, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof;

R₁₀ is selected from the group consisting of no atom, H, halide, C₁₋₄ aliphatic, and -O-C₁₋₄alkyl;

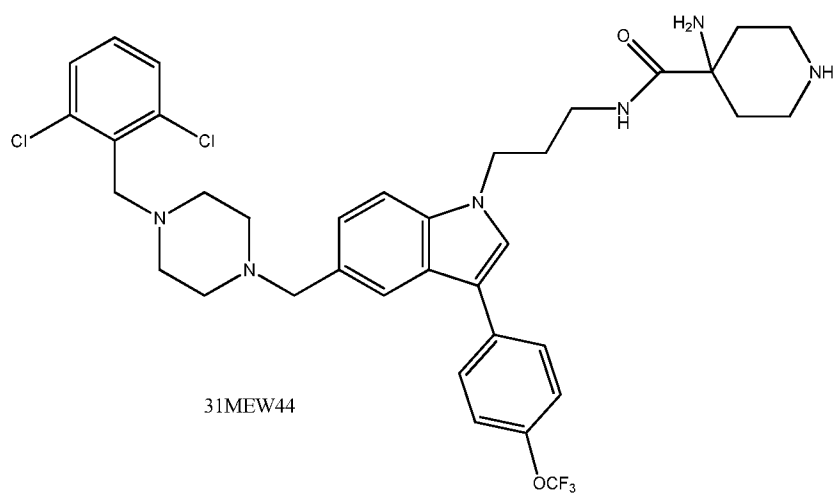
X is selected from the group consisting of CH and N;

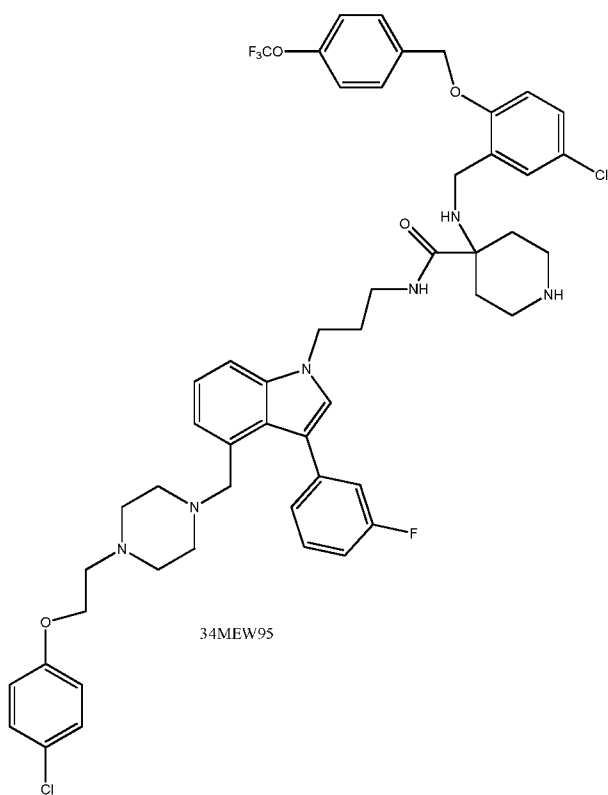
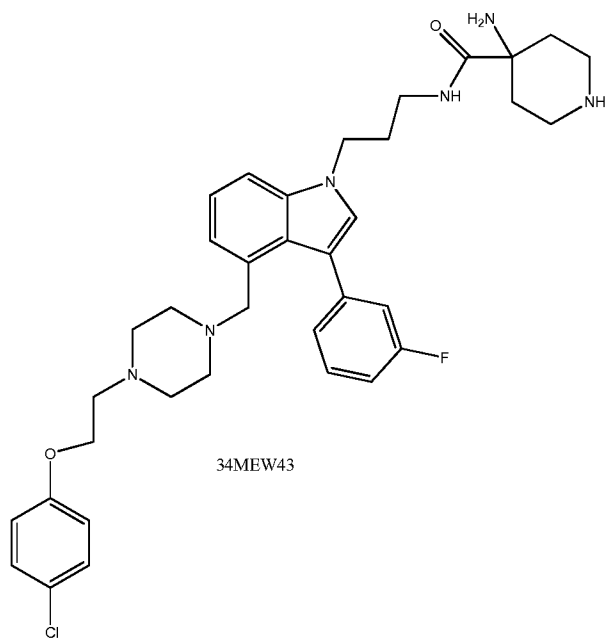
Y is selected from the group consisting of no atom and O;

m, n, and p are independently selected from the group consisting of an integer between 0-5; and ring A is a heterocycle with at least 1 ring nitrogen and optionally substituted with C₁₋₄alkyl or a halide,

or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

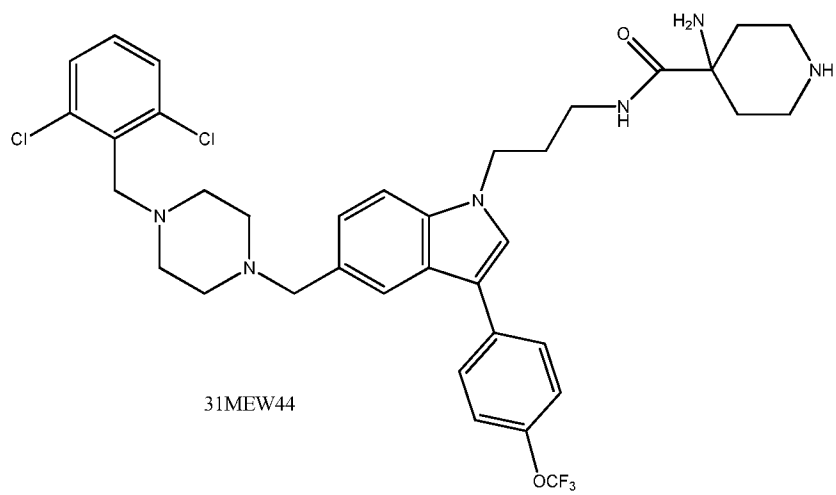
[0105] Another embodiment of the present invention is a compound selected from the group consisting of





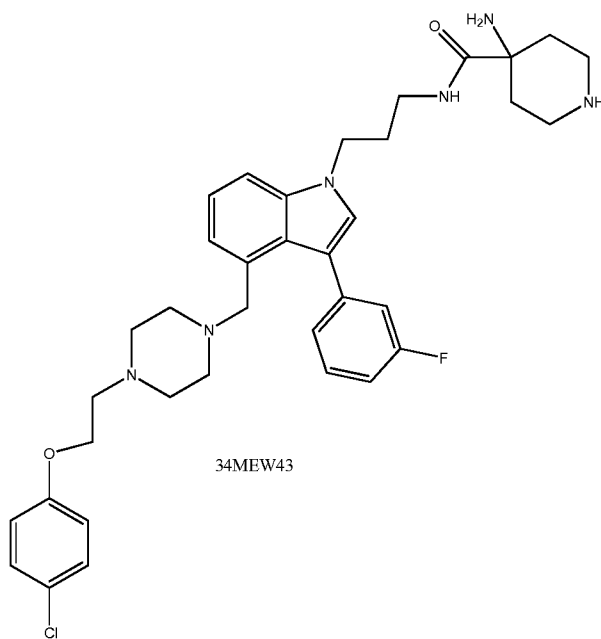
and crystalline forms, hydrates, or pharmaceutically acceptable salts thereof.

[0106] A further embodiment of the present invention is a compound having the structure:



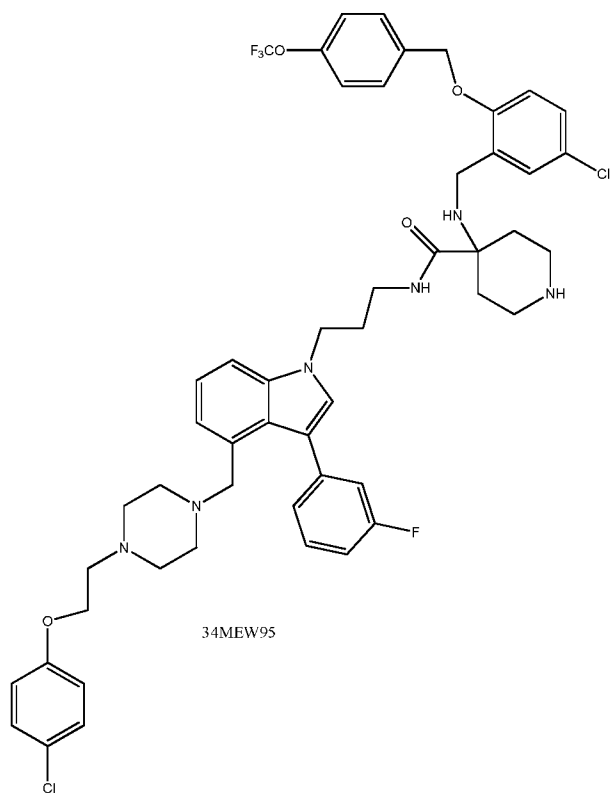
or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

[0107] An additional embodiment of the present invention is a compound having the structure:



or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

[0108] Another embodiment of the present invention is a compound having the structure:



or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

[0109] A further embodiment of the present invention is a pharmaceutical composition. The pharmaceutical composition comprises a pharmaceutically acceptable carrier and any compound disclosed herein. Preferably, the compound has the structure of formula (V), formula (VI), or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof. More preferably, the compound is 34MEW43, 31MEW44, 34MEW95, or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

[0110] An additional embodiment of the present invention is a method for ameliorating or treating the effects of a disease associated with altered RAS signaling in a subject. The method comprises administering to the subject an effective amount of any compound disclosed herein.

[0111] As used herein, the terms “ameliorate”, “ameliorating” and grammatical variations thereof mean to decrease the severity of the symptoms of a disease in a subject.

[0112] As used herein, the terms “treat,” “treating,” “treatment” and grammatical variations thereof mean subjecting an individual subject to a protocol, regimen, process or remedy, in which it is desired to obtain a physiologic response or outcome in that subject, *e.g.*, a patient. In particular, the methods and compositions of the present invention may be used to slow the development of disease symptoms or delay the onset of the disease or condition, or halt the progression of disease development. However, because every treated subject may not respond to a particular treatment protocol, regimen, process or remedy, treating does not require that the desired physiologic response or outcome be achieved in each and every subject or subject population, *e.g.*, patient population. Accordingly, a given subject or subject population, *e.g.*, patient population, may fail to respond or respond inadequately to treatment.

[0113] As used herein, a “subject” is a mammal, preferably, a human. In addition to humans, categories of mammals within the scope of the present invention include, for example, primates, farm animals, domestic animals, laboratory animals, etc. Some examples of agricultural animals include cows, pigs, horses, goats, etc. Some examples of domestic animals include dogs, cats, etc. Some examples of laboratory animals include primates, rats, mice, rabbits, guinea pigs, etc.

[0114] As used herein, the phrase “altered RAS signaling” means any deviation in the activity of a RAS protein from that typically observed from

wild-type RAS protein in a given tissue. Altered RAS signaling may include, for example, increased RAS signaling or decreased RAS signaling. Altered RAS signaling may be caused by one or more mutations in the RAS protein, such as the oncogenic mutations disclosed above. For example, certain RAS protein mutations may enable RAS protein to constitutively exist in its GTP-bound conformation, either by discouraging interaction of RAS protein with various GAP proteins or by disabling the GTPase activity of RAS protein.

[0115] In the present invention, the disease associated with altered RAS signaling may be a cancer, a neurological disorder, a metabolic disorder, an immunological disorder, an inflammatory disorder, and a developmental disorder. Preferably, the disease is selected from the group consisting of autism, rasopathies, neurofibromatosis type 1, Noonan syndrome, Costello syndrome, cardiofaciocutaneous syndrome, hereditary gingival fibromatosis type 1, Legius syndrome, Leopard syndrome, diabetic retinopathy, diabetes, hyperinsulinemia, chronic idiopathic urticarial, autoimmune lymphoproliferative syndrome, and capillary malformation-arteriovenous malformation.

[0116] In the present invention, cancers include both solid and hemotologic cancers. Non-limiting examples of solid cancers include adrenocortical carcinoma, anal cancer, bladder cancer, bone cancer (such as osteosarcoma), brain cancer, breast cancer, carcinoid cancer, carcinoma, cervical cancer, colon cancer, endometrial cancer, esophageal cancer, extrahepatic bile duct cancer, Ewing family of cancers, extracranial germ cell cancer, eye cancer, gallbladder cancer, gastric cancer, germ cell tumor, gestational trophoblastic tumor, head and neck cancer, hypopharyngeal cancer, islet cell carcinoma, kidney cancer, large intestine cancer, laryngeal

cancer, leukemia, lip and oral cavity cancer, liver cancer, lung cancer, lymphoma, malignant mesothelioma, Merkel cell carcinoma, mycosis fungoides, myelodysplastic syndrome, myeloproliferative disorders, nasopharyngeal cancer, neuroblastoma, oral cancer, oropharyngeal cancer, osteosarcoma, ovarian epithelial cancer, ovarian germ cell cancer, pancreatic cancer, paranasal sinus and nasal cavity cancer, parathyroid cancer, penile cancer, pituitary cancer, plasma cell neoplasm, prostate cancer, rhabdomyosarcoma, rectal cancer, renal cell cancer, transitional cell cancer of the renal pelvis and ureter, salivary gland cancer, Sezary syndrome, skin cancers (such as cutaneous t-cell lymphoma, Kaposi's sarcoma, mast cell tumor, and melanoma), small intestine cancer, soft tissue sarcoma, stomach cancer, testicular cancer, thymoma, thyroid cancer, urethral cancer, uterine cancer, vaginal cancer, vulvar cancer, and Wilms' tumor.

[0117] Examples of hematologic cancers include, but are not limited to, leukemias, such as adult/childhood acute lymphoblastic leukemia, adult/childhood acute myeloid leukemia, chronic lymphocytic leukemia, chronic myelogenous leukemia, and hairy cell leukemia, lymphomas, such as AIDS-related lymphoma, cutaneous T-cell lymphoma, adult/childhood Hodgkin lymphoma, mycosis fungoides, adult/childhood non-Hodgkin lymphoma, primary central nervous system lymphoma, Sézary syndrome, cutaneous T-cell lymphoma, and Waldenstrom macroglobulinemia, as well as other proliferative disorders such as chronic myeloproliferative disorders, Langerhans cell histiocytosis, multiple myeloma/plasma cell neoplasm, myelodysplastic syndromes, and myelodysplastic/myeloproliferative neoplasms.

[0118] Preferably, the cancer is selected from the group consisting of pancreatic cancer, colorectal cancer, lung cancer, skin cancer, urinary bladder cancer, thyroid cancer, hematopoietic cancer, prostate cancer, breast cancer, liver cancer, soft tissue cancer, leukemia and bone cancer.

[0119] In a preferred aspect of this embodiment, the cancer is selected from the group consisting of pancreatic cancer, colorectal cancer, fibrosarcoma, breast cancer, lung cancer, skin cancer, leukemia and bone cancer.

[0120] Another embodiment of the present invention is a method for ameliorating or treating the effects of a disease associated with altered RAS signaling in a subject. The method comprises administering to the subject an effective amount of any pharmaceutical composition disclosed herein.

[0121] Suitable and preferred subjects, diseases, and pharmaceutical composition are as disclosed herein.

[0122] A further embodiment of the present invention is a method for effecting cancer cell death. The method comprises contacting a cancer cell with an effective amount of any compound disclosed herein. In this embodiment, "contacting" means bringing the compound into close proximity to the cancer cell. This may be accomplished using conventional techniques of drug delivery to mammals or in the *in vitro* situation by, *e.g.*, providing the compound to a culture media in which the cancer cell is located.

[0123] Suitable and preferred compounds are as disclosed herein. In this embodiment, effecting cancer cell death may be accomplished in cancer cells having various mutational backgrounds as disclosed above.

[0124] The methods of this embodiment, which may be carried out *in vitro* or *in vivo*, may be used to effect cancer cell death by, *e.g.*, killing cancer cells, in cells of the types of cancer disclosed herein.

[0125] In one aspect of this embodiment, the cancer cell is a mammalian cancer cell. Preferably, the mammalian cancer cell is obtained from a mammal selected from the group consisting of humans, primates, farm animals, and domestic animals and laboratory animals. More preferably, the mammalian cancer cell is a human cancer cell.

[0126] Another embodiment of the present invention is a kit for treating or ameliorating the effects of a disease related to altered RAS signaling in a subject in need thereof. The kit comprises an effective amount of any compound or pharmaceutical composition disclosed herein, packaged together with instructions for its use.

[0127] Suitable and preferred subjects, diseases, compounds, and pharmaceutical compositions are as disclosed herein.

[0128] An additional embodiment of the present invention is a kit for treating or ameliorating the effects of a cancer in a subject in need thereof. The kit comprises an effective amount of any compound or pharmaceutical composition disclosed herein, packaged together with instructions for its use.

[0129] Suitable and preferred subjects, diseases, compounds, and pharmaceutical compositions are as disclosed herein.

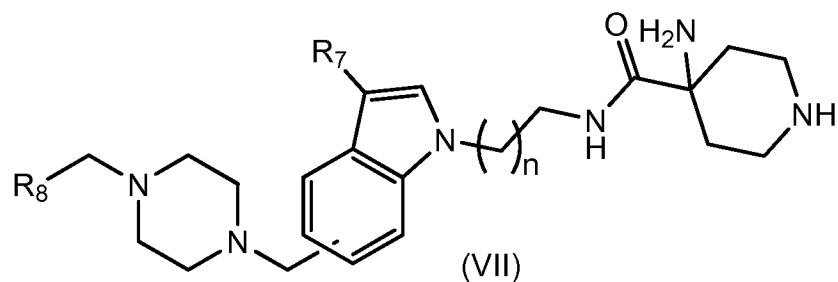
[0130] The kits of the present invention may also include suitable storage containers, *e.g.*, ampules, vials, tubes, etc., for the compounds and compositions of the present invention and other reagents, *e.g.*, buffers, balanced salt solutions, etc., for use in administering the compounds and

compositions to subjects. The compounds and compositions of the present invention may be present in the kits in any convenient form, such as, e.g., in a solution or in a powder form. The kits may further include a packaging container, optionally having one or more partitions for housing the compounds and pharmaceutical compositions and other optional reagents.

[0131] Another embodiment of the present invention is a composition comprising any compound disclosed herein.

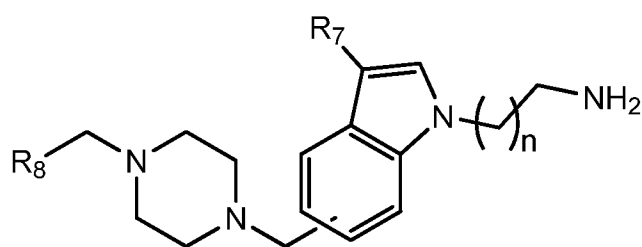
[0132] In one aspect of this embodiment, the composition is a research reagent. As used herein, a “research reagent” is any compound or composition used in the execution of investigational activities.

[0133] An additional embodiment of the present invention is a method of preparing a compound having the structure of formula (VII):

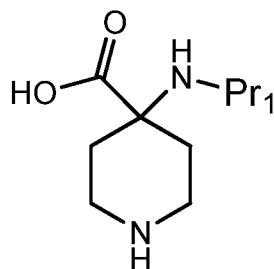


The method comprises the steps of

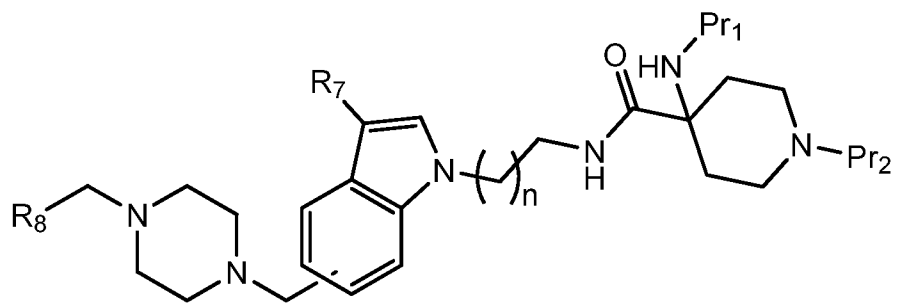
i) reacting a compound having the structure:



with a compound having the structure:



under conditions sufficient to form a compound having the structure:



wherein:

R₇ is selected from the group consisting of H, halide, C₁₋₄ aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C₁₋₄alkyl, -O-C₁₋₄alkyl, and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R₈ is selected from the group consisting of no atom, H, alkyl, aryl and C₁₋₄alkyl-O-aryl, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with one or more groups consisting of halide, ether, C₁₋₄alkyl, and a combination thereof;

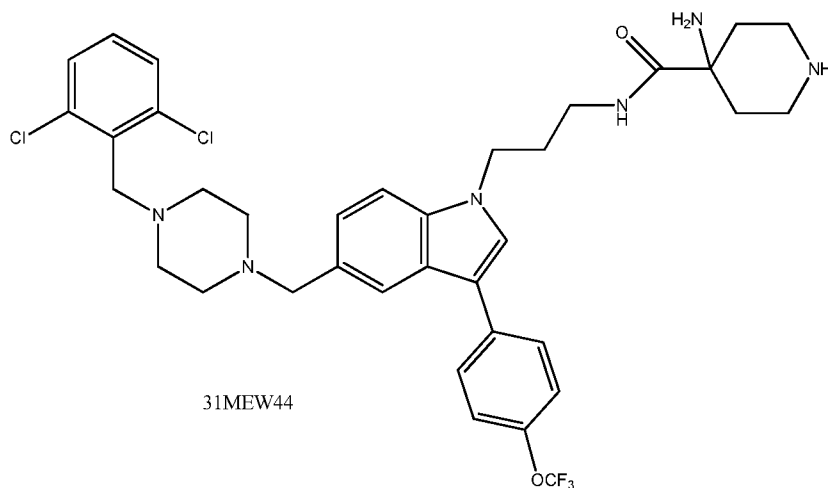
n is selected from the group consisting of an integer between 0-5; and

Pr₁ and Pr₂ are independently selected from the group consisting of nitrogen protecting groups;

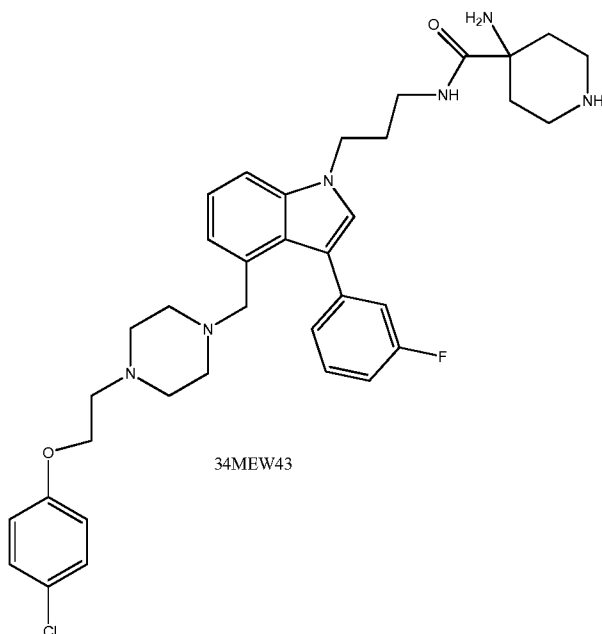
- ii) removing the Pr₁ protecting group; and
- iii) removing the Pr₂ protecting group.

[0134] In one aspect of this embodiment the reaction of step i) is carried out in the presence of dimethylformamide. In another aspect of this embodiment Pr₁ and Pr₂ are F_{moc} or Boc.

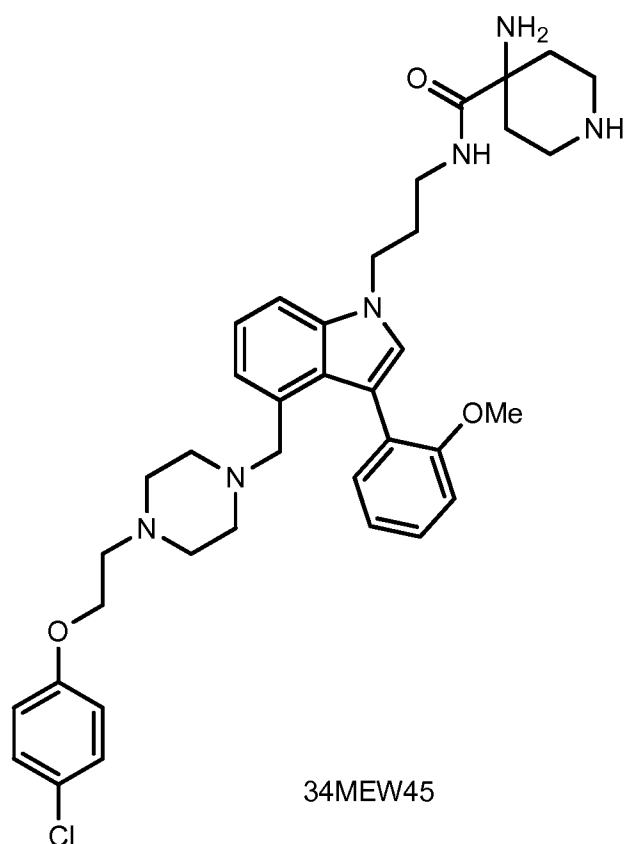
[0135] In one aspect of this embodiment, the compound has the structure



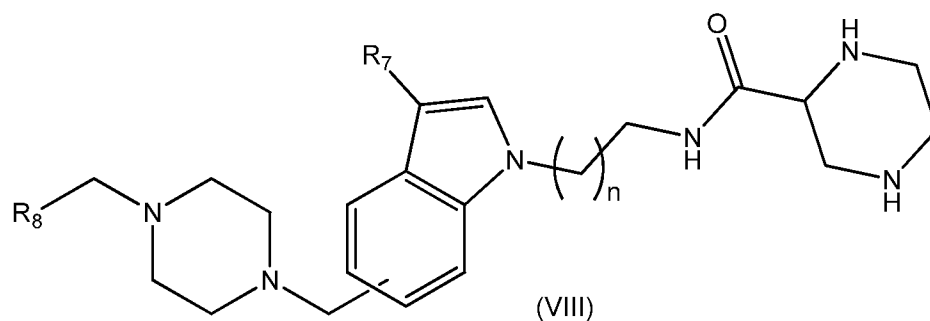
In another aspect of this embodiment, the compound has the structure



In another aspect of this embodiment, the compound has the structure

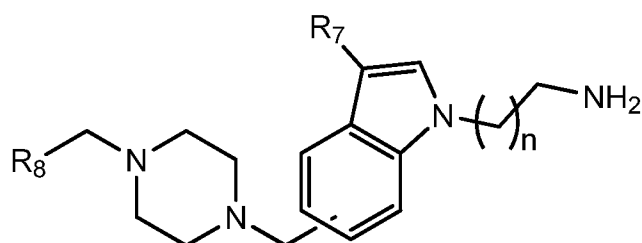


[0136] An additional embodiment of the present invention is a method of preparing a compound having the structure of formula (VIII):

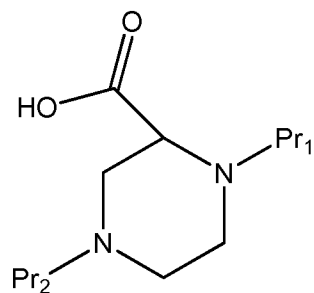


The method comprises the steps of

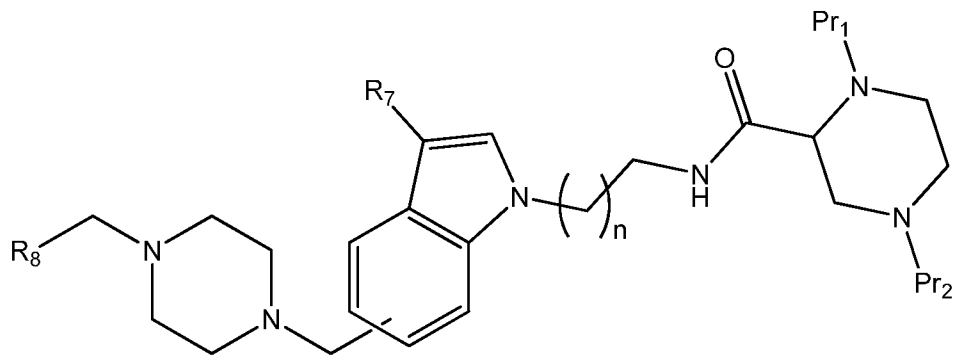
- i) reacting a compound having the structure:



with a compound having the structure:



under conditions sufficient to form a compound having the structure:



wherein:

R_7 is selected from the group consisting of H, halide, C_{1-4} aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, $-O-C_{1-4}$ alkyl, and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R_8 is selected from the group consisting of no atom, H, alkyl, aryl and C_{1-4} alkyl-O-aryl, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, and a combination thereof;

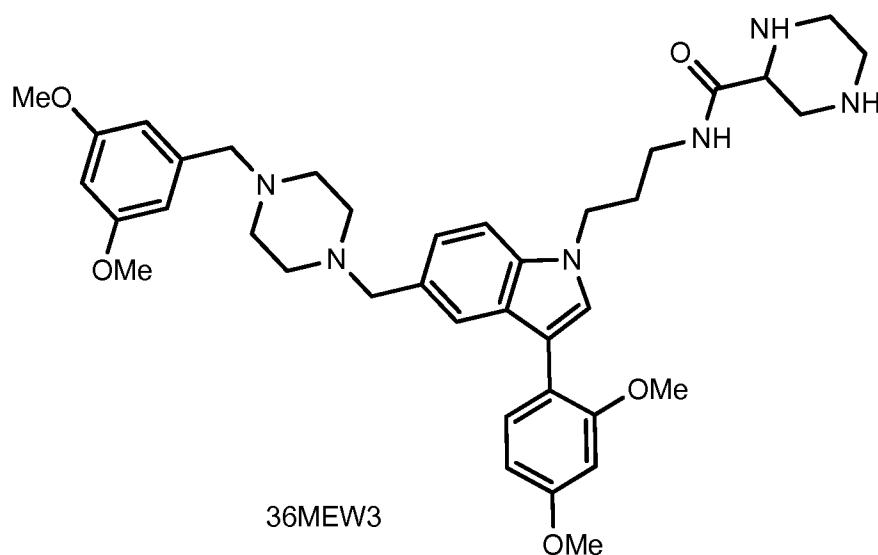
n is selected from the group consisting of an integer between 0-5; and

Pr_1 and Pr_2 are independently selected from the group consisting of nitrogen protecting groups;

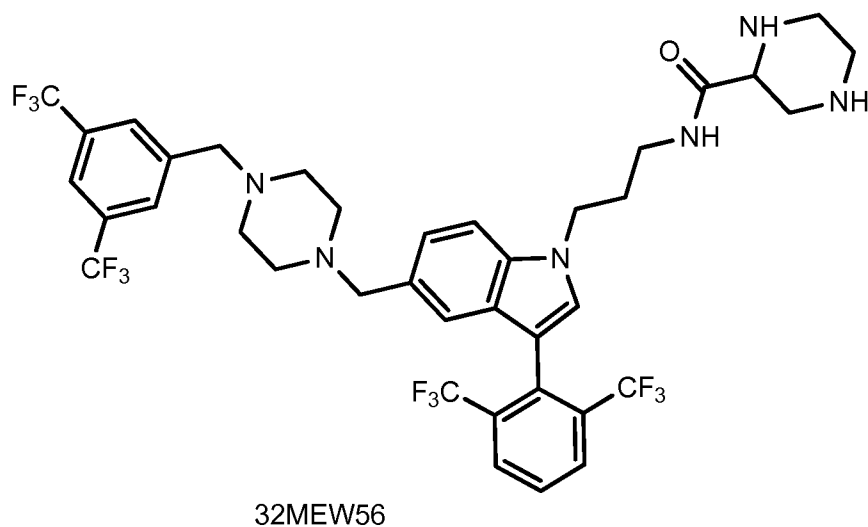
- ii) removing the Pr_1 protecting group; and
- iii) removing the Pr_2 protecting group.

[0137] In one aspect of this embodiment the reaction of step i) is carried out in the presence of dimethylformamide. In another aspect of this embodiment Pr_1 and Pr_2 are F_{moc} or Boc.

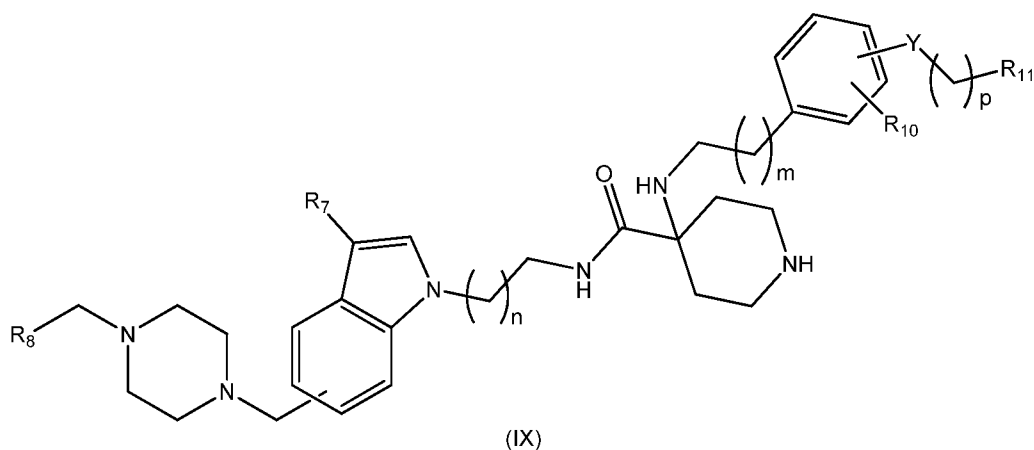
[0138] In one aspect of this embodiment, the compound has the structure



In another aspect of this embodiment, the compound has the structure

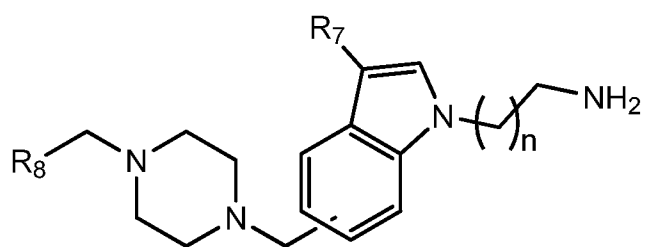


[0139] An additional embodiment of the present invention is a method of preparing a compound having the structure of formula (IX):

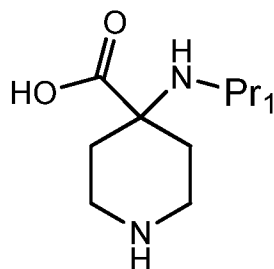


The method comprises the steps of

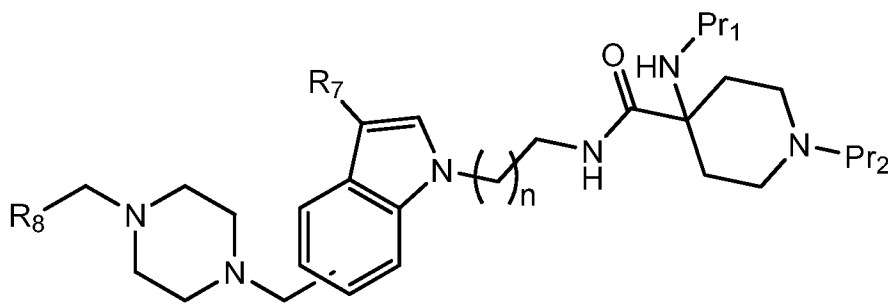
- i) reacting a compound having the structure:



with a compound having the structure:



under conditions sufficient to form a compound having the structure:



wherein:

R_7 is selected from the group consisting of H, halide, C_{1-4} aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, $-O-C_{1-4}$ alkyl, and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R_8 and R_{11} are independently selected from the group consisting of no atom, H, alkyl, aryl and C_{1-4} alkyl-O-aryl, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with the group consisting of halide, ether, C_{1-4} alkyl, $-O-C_{1-4}$ alkyl, and a combination thereof, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof;

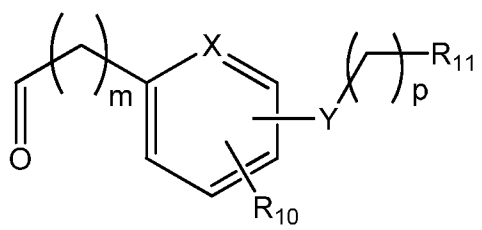
R_{10} is selected from the group consisting of no atom, H, halide, C_{1-4} aliphatic and $-O-C_{1-4}$ alkyl;

Y is selected from the group consisting of no atom and O;

m, n, and p are independently selected from the group consisting of an integer between 0-5; and; Pr_1 and Pr_2 are independently selected from the group consisting of nitrogen protecting groups;

ii) removing the Pr_1 protecting group

iii) reacting the product of step ii) with a compound having the structure:

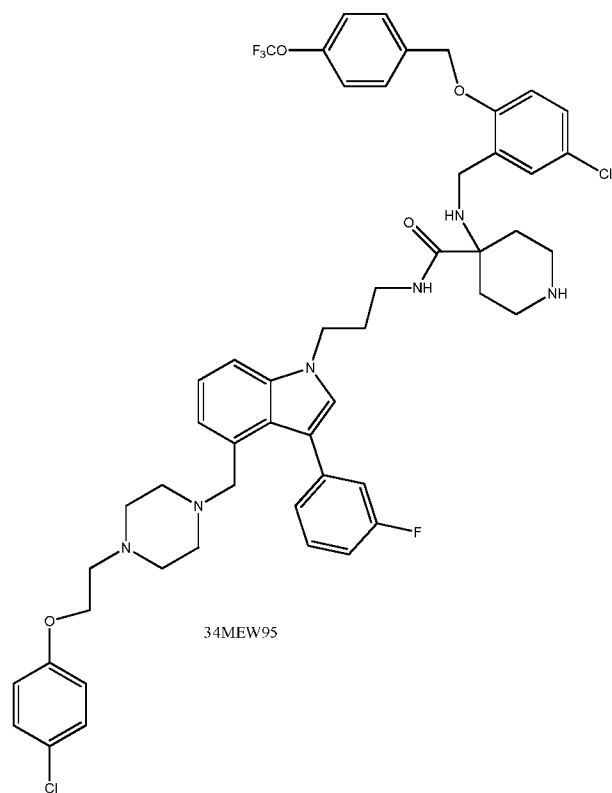


wherein X is selected from the group consisting of CH and N; and

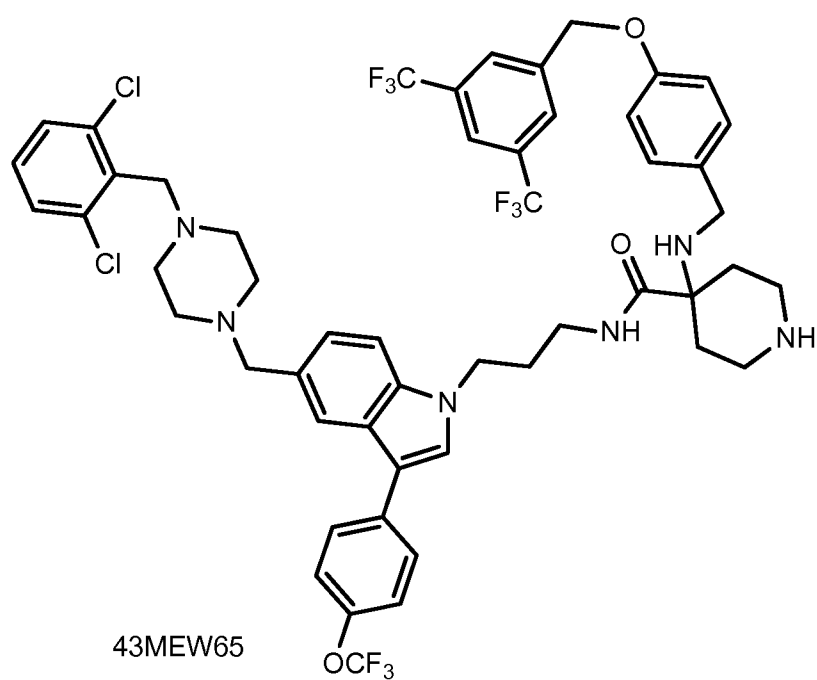
iv) removing the Pr_2 protecting group.

[0140] In one aspect of this embodiment the reaction of step i) is carried out in the presence of dimethylformamide. In one aspect of this embodiment the reaction of step iii) is carried out in the presence of dichloroethane. In another aspect of this embodiment Pr_1 and Pr_2 are F_{moc} or Boc.

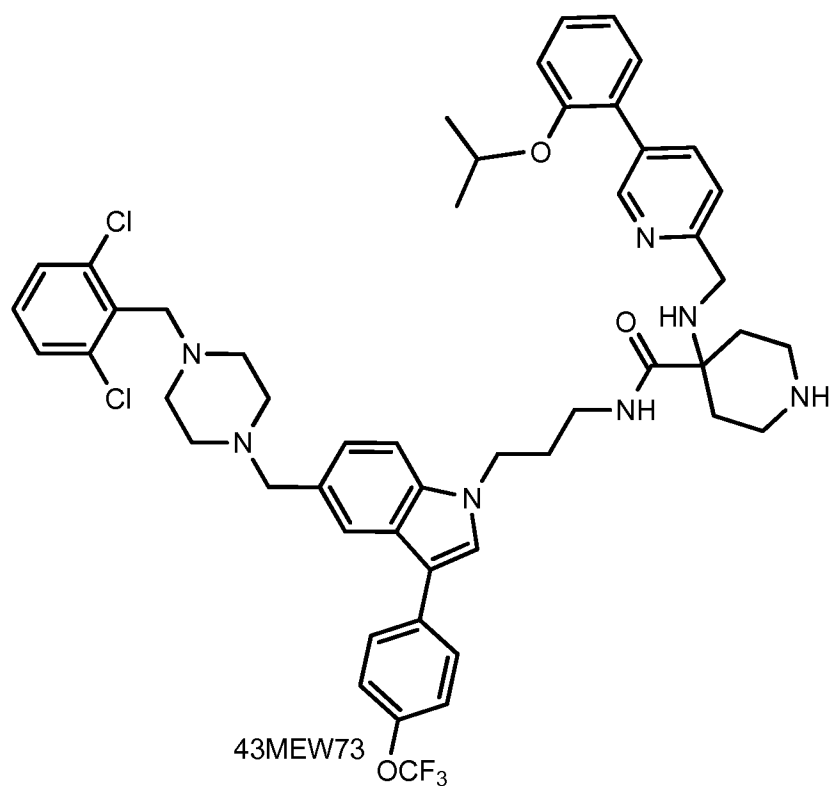
[0141] In one aspect of this embodiment, the compound has the structure



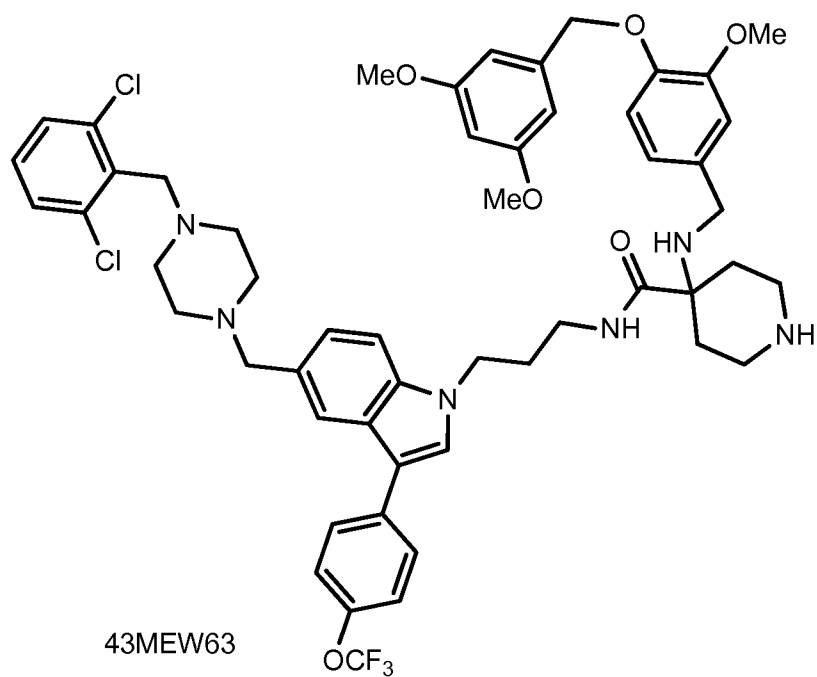
In another aspect of this embodiment, the compound has the structure



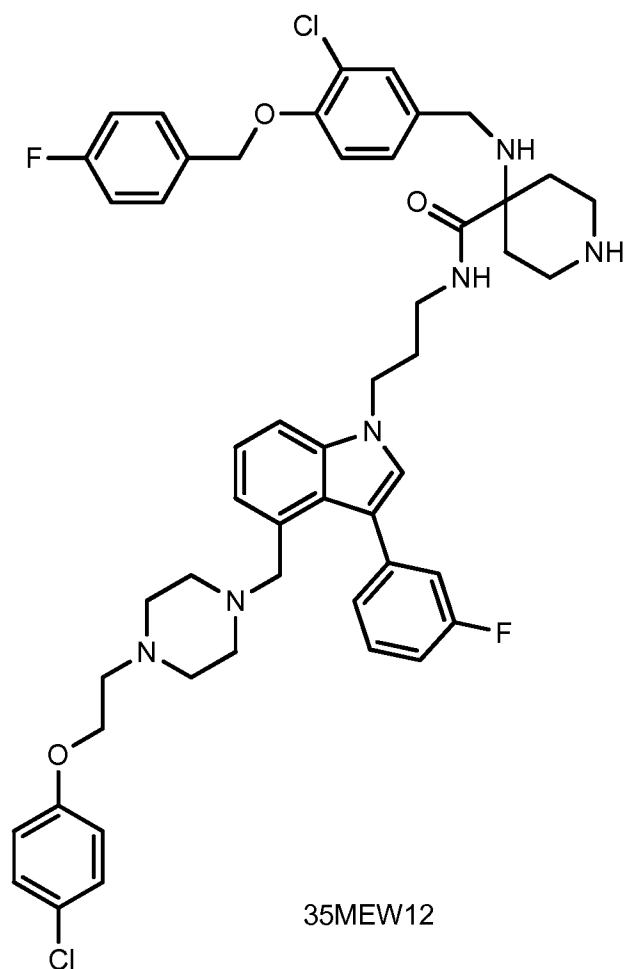
In another aspect of this embodiment, the compound has the structure



In another aspect of this embodiment, the compound has the structure



In another aspect of this embodiment, the compound has the structure



[0142] An additional embodiment of the present invention is a method of identifying a multivalent compound which binds selectively to a target protein. The method comprises the steps of

- i) identifying a first and second target site on the target protein, wherein the first and second target sites are adjacent to each other;
- ii) identifying a first compound fragment that selectively binds to the target protein at the first target site and a second compound fragment that selectively binds to the target protein at the second site; and
- iii) creating a structure of the multivalent compound comprising the first compound fragment linked to the second compound fragment, thereby identifying the multivalent compound.

[0143] In one aspect of this embodiment,

step i) further comprises identifying a third target site on the target protein adjacent to the first and or second target site(s);

step ii) further comprises identifying a third compound fragment that selectively binds to the target protein at the third target site; and

step iii) further comprises creating a structure of the compound comprising the third compound fragment linked to the first and/or the second compound fragment(s).

[0144] In one aspect of this embodiment, step ii) comprises the steps of

a) identifying compounds that bind to the target sites from a chemical library; and

b) creating an in silico library based on a set of structural and functional criteria for the compounds identified in step a) to identify compound fragments that are likely to selectively bind to the target sites. In another aspect of this embodiment the criteria comprise fragment size, hydrophobicity, electrophilicity/nucleophilicity and ability to form hydrogen bonds. In yet another aspect of this embodiment each in silico library consists essentially of synthetically feasible fragments. In yet another aspect of this embodiment compound fragments are identified based on high docking scores.

[0145] In one aspect of this embodiment, the target sites are shallow sites. In another aspect of this embodiment the target sites are present at a position where the target protein binds to a second protein. In another aspect of this embodiment the multivalent compound reduces binding of the target protein to the second protein.

[0146] In one aspect of this embodiment, the target protein is a GTPase. In another aspect of this embodiment, wherein the target protein is a RAS protein, preferably KRAS, more preferably KRAS^{G12D}.

[0147] In one preferred aspect of this embodiment, the first target site is D38. In another preferred aspect of this embodiment the second target site is A59. In yet another preferred aspect of this embodiment the third target site is Y32.

[0148] In the present invention, the term “crystalline form” means the crystal structure of a compound. A compound may exist in one or more crystalline forms, which may have different structural, physical, pharmacological, or chemical characteristics. Different crystalline forms may be obtained using variations in nucleation, growth kinetics, agglomeration, and breakage. Nucleation results when the phase-transition energy barrier is overcome, thereby allowing a particle to form from a supersaturated solution. Crystal growth is the enlargement of crystal particles caused by deposition of the chemical compound on an existing surface of the crystal. The relative rate of nucleation and growth determine the size distribution of the crystals that are formed. The thermodynamic driving force for both nucleation and growth is supersaturation, which is defined as the deviation from thermodynamic equilibrium. Agglomeration is the formation of larger particles through two or more particles (*e.g.*, crystals) sticking together and forming a larger crystalline structure.

[0149] The term “hydrates”, as used herein, means a solid or a semi-solid form of a chemical compound containing water in a molecular complex.

The water is generally in a stoichiometric amount with respect to the chemical compound.

[0150] As used herein, "pharmaceutically acceptable salts" refer to derivatives of the compounds disclosed herein wherein the compounds are modified by making acid or base salts thereof. Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. For example, such salts include salts from ammonia, L-arginine, betaine, benethamine, benzathine, calcium hydroxide, choline, deanol, diethanolamine (2,2'-iminobis(ethanol)), diethylamine, 2-(diethylamino)-ethanol, 2-aminoethanol, ethylenediamine, N-ethyl-glucamine, hydrabamine, 1H-imidazole, lysine, magnesium hydroxide, 4-(2-hydroxyethyl)-morpholine, piperazine, potassium hydroxide, 1-(2-hydroxy-ethyl)-pyrrolidine, sodium hydroxide, triethanolamine (2,2',2''-nitrilotris(ethanol)), trometh-amine, zinc hydroxide, acetic acid, 2,2-dichloro-acetic acid, adipic acid, alginic acid, ascorbic acid, L-aspartic acid, benzenesulfonic acid, benzoic acid, 2,5-dihydroxybenzoic acid, 4-acetamido-benzoic acid, (+)-camphoric acid, (+)-camphor-10-sulfonic acid, carbonic acid, cinnamic acid, citric acid, cyclamic acid, decanoic acid, dodecylsulfuric acid, ethane-1,2-disulfonic acid, ethanesulfonic acid, 2-hydroxy-ethanesulfonic acid, ethylenediamonotetraacetic acid, formic acid, fumaric acid, galacarinic acid, gentisic acid, D-glucoheptonic acid, D-gluconic acid, D-glucuronic acid, glutamic acid, glutaric acid, glutaric acid, 2-oxo-glutaric acid, glycerophosphoric acid, glycine, glycolic acid, hexanoic acid, hippuric acid, hydrobromic acid, hydrochloric acid isobutyric acid, DL-lactic acid, lactobionic

acid, lauric acid, lysine, maleic acid, (-)-L-malic acid, malonic acid, DL-mandelic acid, methanesulfonic acid, galactaric acid, naphthalene-1,5-disulfonic acid, naphthalene-2-sulfonic acid, 1-hydroxy-2-naphthoic acid, nicotinic acid, nitric acid, octanoic acid, oleic acid, orotic acid, oxalic acid, palmitic acid, pamoic acid (embonic acid), phosphoric acid, propionic acid, (-)-L-pyroglutamic acid, salicylic acid, 4-amino-salicylic acid, sebacic acid, stearic acid, succinic acid, sulfuric acid, tannic acid, (+)-L-tartaric acid, thiocyanic acid, p-toluenesulfonic acid and undecylenic acid. Further pharmaceutically acceptable salts can be formed with cations from metals like aluminum, calcium, lithium, magnesium, potassium, sodium, zinc and the like. (also see Pharmaceutical salts, Berge, S.M. *et al.*, J. Pharm. Sci., (1977), 66, 1-19).

[0151] The pharmaceutically acceptable salts of the present invention can be synthesized from a compound disclosed herein which contains a basic or acidic moiety by conventional chemical methods. Generally, such salts can be prepared by reacting the free acid or base forms of these compounds with a sufficient amount of the appropriate base or acid in water or in an organic diluent like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile, or a mixture thereof.

[0152] In the present invention, an "effective amount" or a "therapeutically effective amount" of a compound or composition disclosed herein is an amount of such compound or composition that is sufficient to effect beneficial or desired results as described herein when administered to a subject. Effective dosage forms, modes of administration, and dosage amounts may be determined empirically, and making such determinations is within the skill of the art. It is understood by those skilled in the art that the

dosage amount will vary with the route of administration, the rate of excretion, the duration of the treatment, the identity of any other drugs being administered, the age, size, and species of mammal, *e.g.*, human patient, and like factors well known in the arts of medicine and veterinary medicine. In general, a suitable dose of a compound or composition according to the invention will be that amount of the compound or composition which is the lowest dose effective to produce the desired effect. The effective dose of a compound or composition of the present invention may be administered as two, three, four, five, six or more sub-doses, administered separately at appropriate intervals throughout the day.

[0153] A suitable, non-limiting example of a dosage of any of the compounds or compositions disclosed herein is from about 1 mg/kg to about 2400 mg/kg per day, such as from about 1 mg/kg to about 1200 mg/kg per day, 75 mg/kg per day to about 300 mg/kg per day, including from about 1 mg/kg to about 100 mg/kg per day. Other representative dosages of such agents include about 1 mg/kg, 5 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, 25 mg/kg, 30 mg/kg, 35 mg/kg, 40 mg/kg, 45 mg/kg, 50 mg/kg, 60 mg/kg, 70 mg/kg, 75 mg/kg, 80 mg/kg, 90 mg/kg, 100 mg/kg, 125 mg/kg, 150 mg/kg, 175 mg/kg, 200 mg/kg, 250 mg/kg, 300 mg/kg, 400 mg/kg, 500 mg/kg, 600 mg/kg, 700 mg/kg, 800 mg/kg, 900 mg/kg, 1000 mg/kg, 1100 mg/kg, 1200 mg/kg, 1300 mg/kg, 1400 mg/kg, 1500 mg/kg, 1600 mg/kg, 1700 mg/kg, 1800 mg/kg, 1900 mg/kg, 2000 mg/kg, 2100 mg/kg, 2200 mg/kg, and 2300 mg/kg per day. The effective dose of compounds or compositions disclosed herein, may be administered as two, three, four, five, six or more sub-doses, administered separately at appropriate intervals throughout the day.

[0154] The compounds or compositions of the present invention may be administered in any desired and effective manner: for oral ingestion, or as an ointment or drop for local administration to the eyes, or for parenteral or other administration in any appropriate manner such as intraperitoneal, subcutaneous, topical, intradermal, inhalation, intrapulmonary, rectal, vaginal, sublingual, intramuscular, intravenous, intraarterial, intrathecal, or intralymphatic. Further, compounds or compositions of the present invention may be administered in conjunction with other treatments. Compounds or compositions of the present invention may be encapsulated or otherwise protected against gastric or other secretions, if desired.

[0155] The compositions of the invention comprise one or more active ingredients in admixture with one or more pharmaceutically-acceptable diluents or carriers and, optionally, one or more other compounds, drugs, ingredients and/or materials. Regardless of the route of administration selected, the agents/compounds of the present invention are formulated into pharmaceutically-acceptable dosage forms by conventional methods known to those of skill in the art. See, *e.g.*, Remington, The Science and Practice of Pharmacy (21st Edition, Lippincott Williams and Wilkins, Philadelphia, PA.).

[0156] Pharmaceutically acceptable diluents or carriers are well known in the art (see, *e.g.*, Remington, The Science and Practice of Pharmacy (21st Edition, Lippincott Williams and Wilkins, Philadelphia, PA.) and The National Formulary (American Pharmaceutical Association, Washington, D.C.)) and include sugars (*e.g.*, lactose, sucrose, mannitol, and sorbitol), starches, cellulose preparations, calcium phosphates (*e.g.*, dicalcium phosphate, tricalcium phosphate and calcium hydrogen phosphate), sodium citrate, water,

aqueous solutions (*e.g.*, saline, sodium chloride injection, Ringer's injection, dextrose injection, dextrose and sodium chloride injection, lactated Ringer's injection), alcohols (*e.g.*, ethyl alcohol, propyl alcohol, and benzyl alcohol), polyols (*e.g.*, glycerol, propylene glycol, and polyethylene glycol), organic esters (*e.g.*, ethyl oleate and tryglycerides), biodegradable polymers (*e.g.*, polylactide-polyglycolide, poly(orthoesters), and poly(anhydrides)), elastomeric matrices, liposomes, microspheres, oils (*e.g.*, corn, germ, olive, castor, sesame, cottonseed, and groundnut), cocoa butter, waxes (*e.g.*, suppository waxes), paraffins, silicones, talc, silicylate, etc. Each pharmaceutically acceptable diluent or carrier used in a pharmaceutical composition of the invention must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the subject. Diluents or carriers suitable for a selected dosage form and intended route of administration are well known in the art, and acceptable diluents or carriers for a chosen dosage form and method of administration can be determined using ordinary skill in the art.

[0157] The compositions of the invention may, optionally, contain additional ingredients and/or materials commonly used in pharmaceutical compositions. These ingredients and materials are well known in the art and include (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and silicic acid; (2) binders, such as carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, hydroxypropylmethyl cellulose, sucrose and acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, sodium starch glycolate, cross-linked sodium

carboxymethyl cellulose and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, and sodium lauryl sulfate; (10) suspending agents, such as ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth; (11) buffering agents; (12) excipients, such as lactose, milk sugars, polyethylene glycols, animal and vegetable fats, oils, waxes, paraffins, cocoa butter, starches, tragacanth, cellulose derivatives, polyethylene glycol, silicones, bentonites, silicic acid, talc, salicylate, zinc oxide, aluminum hydroxide, calcium silicates, and polyamide powder; (13) inert diluents, such as water or other solvents; (14) preservatives; (15) surface-active agents; (16) dispersing agents; (17) control-release or absorption-delaying agents, such as hydroxypropylmethyl cellulose, other polymer matrices, biodegradable polymers, liposomes, microspheres, aluminum monostearate, gelatin, and waxes; (18) opacifying agents; (19) adjuvants; (20) wetting agents; (21) emulsifying and suspending agents; (22), solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan; (23) propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as

butane and propane; (24) antioxidants; (25) agents which render the formulation isotonic with the blood of the intended recipient, such as sugars and sodium chloride; (26) thickening agents; (27) coating materials, such as lecithin; and (28) sweetening, flavoring, coloring, perfuming and preservative agents. Each such ingredient or material must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the subject. Ingredients and materials suitable for a selected dosage form and intended route of administration are well known in the art, and acceptable ingredients and materials for a chosen dosage form and method of administration may be determined using ordinary skill in the art.

[0158] The compositions of the present invention suitable for oral administration may be in the form of capsules, cachets, pills, tablets, powders, granules, a solution or a suspension in an aqueous or non-aqueous liquid, an oil-in-water or water-in-oil liquid emulsion, an elixir or syrup, a pastille, a bolus, an electuary or a paste. These formulations may be prepared by methods known in the art, *e.g.*, by means of conventional pan-coating, mixing, granulation or lyophilization processes.

[0159] Solid dosage forms for oral administration (capsules, tablets, pills, dragees, powders, granules and the like) may be prepared, *e.g.*, by mixing the active ingredient(s) with one or more pharmaceutically-acceptable diluents or carriers and, optionally, one or more fillers, extenders, binders, humectants, disintegrating agents, solution retarding agents, absorption accelerators, wetting agents, absorbents, lubricants, and/or coloring agents. Solid compositions of a similar type may be employed as fillers in soft and hard-filled gelatin capsules using a suitable excipient. A tablet may be made

by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using a suitable binder, lubricant, inert diluent, preservative, disintegrant, surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine. The tablets, and other solid dosage forms, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein. They may be sterilized by, for example, filtration through a bacteria-retaining filter. These compositions may also optionally contain opacifying agents and may be of a composition such that they release the active ingredient only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. The active ingredient can also be in microencapsulated form.

[0160] Liquid dosage forms for oral administration include pharmaceutically-acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. The liquid dosage forms may contain suitable inert diluents commonly used in the art. Besides inert diluents, the oral compositions may also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents. Suspensions may contain suspending agents.

[0161] The compositions of the present invention for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more active ingredient(s) with one or more suitable nonirritating diluents or carriers which are solid at room temperature, but liquid at body

temperature and, therefore, will melt in the rectum or vaginal cavity and release the active compound. The pharmaceutical compositions of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such pharmaceutically-acceptable diluents or carriers as are known in the art to be appropriate.

[0162] Dosage forms for the topical or transdermal administration include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches, drops and inhalants. The active agent(s)/compound(s) may be mixed under sterile conditions with a suitable pharmaceutically-acceptable diluent or carrier. The ointments, pastes, creams and gels may contain excipients. Powders and sprays may contain excipients and propellants.

[0163] The compositions of the present invention suitable for parenteral administrations may comprise one or more agent(s)/compound(s) in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or non-aqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain suitable antioxidants, buffers, solutes which render the formulation isotonic with the blood of the intended recipient, or suspending or thickening agents. Proper fluidity can be maintained, for example, by the use of coating materials, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants. These pharmaceutical compositions may also contain suitable adjuvants, such as wetting agents, emulsifying agents and dispersing agents. It may also be desirable to include isotonic agents. In addition, prolonged

absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption.

[0164] In some cases, in order to prolong the effect of a drug (*e.g.*, pharmaceutical formulation), it is desirable to slow its absorption from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility.

[0165] The rate of absorption of the active agent/drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered agent/drug may be accomplished by dissolving or suspending the active agent/drug in an oil vehicle. Injectable depot forms may be made by forming microencapsule matrices of the active ingredient in biodegradable polymers. Depending on the ratio of the active ingredient to polymer, and the nature of the particular polymer employed, the rate of active ingredient release can be controlled. Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue. The injectable materials can be sterilized for example, by filtration through a bacterial-retaining filter.

[0166] Any formulation of the invention may be presented in unit-dose or multi-dose sealed containers, for example, ampules and vials, and may be stored in a lyophilized condition requiring only the addition of the sterile liquid diluent or carrier, for example water for injection, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the type described above.

[0167] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. As used in the specification and the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise.

[0168] For recitation of numeric ranges herein, each intervening number there between with the same degree of precision is explicitly contemplated. For example, for the range of 6-9, the numbers 7 and 8 are contemplated in addition to 6 and 9, and for the range 6.0-7.0, the numbers 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, and 7.0 are explicitly contemplated.

[0169] The following examples are provided to further illustrate the methods of the present invention. These examples are illustrative only and are not intended to limit the scope of the invention in any way.

EXAMPLES

Example 1

Materials and Methods

Software

[0170] Molecular docking was performed using GLIDE (Schrodinger, Inc). Modeling of proteins and ligands were performed using Molecular Operating Environment [MOE] (Chemical Computing Group). All chemical structures were drawn using Chemdraw Ultra version 10.0. (Perkin Elmer). All statistical analyses, EC₅₀ determinations, and viability curves were produced using Prism 5.0c (GraphPad Software).

In silico libraries

[0171] Libraries of commercially available compounds were compiled from the inventories of Asinex, Enamine, Chembridge, ChemDiv, IBS, Life, Maybridge and TimTec.

[0172] The unfiltered commercially available compound library was converted to conformer libraries using OMEGA (Openeye scientific) and screened against the pharamcophore model using ROCS (Openeye scientific).

[0173] A fragment subset of about 60,000 compounds of the unfiltered library was selected using the following filter criteria: LogP < 3, hydrogen bond acceptors $\leq$ 3, hydrogen bond donors $\leq$ 3, molecular weight < 300, aqueous solubility > 0.5 mM. Chemical descriptors were calculated using MOE (Chemical Computing Group)

[0174] Designed libraries of synthetically accessible compounds were compiled using selected commercially available reagents from the inventory of Sigma-Aldrich and Chem-Impex using the Combigen application in MOE (chemical computing group).

Cell viability assays

[0175] All cell culture assays were incubated at 37°C, 5% CO₂ in media containing Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS). 384-well format for EC₅₀ determination: cells were trypsinized, counted, and seeded into 384-well plates at 1,000 cells/well. After 12-16 hours, compounds (as 50 mM stocks in DMSO) were arrayed in an 8- or 16-point dilution series in 384-well polypropylene plates. Compound solutions were transferred at a 1:5 dilution

into the assay plates. After 48 hours, a 50% Alamar blue solution was added to a final concentration of 10% Alamar blue. After 6 hours of incubation, fluorescence intensity was determined using a Victor3 plate reader (Perkin Elmer) with a 535 nm excitation filter and a 590 nm emission filter. All compound measurements were performed in triplicate. For experiments performed in 6-well format, cells were trypsinized, counted, and seeded into 6-well plates at 200,000 cells per well 16 h prior to use. Media was then aspirated and replaced with 2 mL of media containing compounds at the indicated concentrations (from 10 mM stocks in DMSO). After 24 hours, cells were trypsinized and viability was determined using Trypan Blue exclusion assay.

Western blots

[0176] BJELR cells were seeded in 60 mm dishes at 1 million cells/dish in media containing DMEM and 10% FBS with 1% penicillin and streptomycin (PS), 12-16 hours prior to use. The medium was then aspirated and compounds added as solutions in serum free medium (DMEM with 1% PS) to the dishes and treated for 24 hours or at the indicated time points. Following treatment, the medium was aspirated from each dish and cells were washed twice with PBS. Cells were lysed with 60 μ l buffer (50 mM HEPES, 40 mM NaCl, 2 mM EDTA, 0.5% Triton-X, 1.5 mM sodium orthovanadate, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium β -glycerophosphate and protease inhibitor tablet ((Roche), pH 7.4). Unlysed cells and debris were pelleted for 12 minutes at 12,000 rpm at 4°C. Samples were separated using SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Transfer was performed using the iBlot system

(Invitrogen). Membranes were treated with Li-COR odyssey blocking buffer for 1 hour at 25°C, then incubated with primary antibody (1:1000) in a 1:1 solution of PBS-T and Li-COR odyssey blocking buffer overnight at 4°C. Following three 5 minute washes in PBS-T, the membrane was incubated with secondary antibodies (1:2000) in a 1:1 solution of PBS-T and Li-COR Odyssey blocking buffer for 45 minutes at 25°C. Following three 5 minute washes in PBS-T, the membrane was scanned using the Li-COR Odyssey Imaging System. Antibodies for pERK1/2, ERK1/2, pAKT ser473, AKT, pan-RAS, RALA, PI3Kgamma (Cell signaling), and RAF-1 (Santa Cruz) were detected using a goat anti-rabbit or goat anti-mouse IgG antibody conjugated to an IRdye at 800CW and 680CW conjugated, respectively (Li-COR biosciences).

Multicellular tumor spheroids

[0177] Multicellular tumor spheroids (MCTSs) were grown in 96-well Corningware Ultra Low Attachment (ULA) Plates (CLS 3474). 100 μ L of cell suspension containing 2×10^4 cells/ml were added to each well of the ULA plate containing 100 μ L of a 2X solution of the desired concentration of compounds. Cells were incubated at 37°C, 5% CO₂ for 72 hours to allow for MCTS formation. After 72 hours, 50 μ L of a 50% solution of Alamar blue and medium was added and incubated for 12 hours prior to measurements on a Victor3 plate reader as previously described.

Culture conditions and viability measurements in patient derived T-ALL samples

Patient Samples

[0178] T-ALL samples were provided by Columbia Presbyterian Hospital, the Eastern Cooperative Oncology Group (ECOG), University of Padova, and Hospital Central de Asturias with informed consent and analyzed under the supervision of the Columbia University Medical Center Institutional Review Board committee.

Primary cell co-culture and in vitro cell viability assays.

[0179] For the analysis of 31MEW44 on primary T-ALL patient samples, cells were cultured in MEM medium supplemented with 10% FBS, 10% human heat-inactivated serum, 1% penicillin/streptomycin, 1% GlutaMAX, human IL-7 (10 ng/mL), human SCF (50 ng/mL), human FLT3-ligand (20 ng/mL), and insulin (20 nmol/L) on a feeder layer of MS5 stromal cells overexpressing the NOTCH ligand Delta-like 1 as described by Armstrong *et al.* (Armstrong *et al.*, 2009). In these experiments, T-ALL lymphoblasts were cultured in triplicate and treated with either vehicle, DMSO, or 31MEW44 (doses ranging from 1 to 5 μ M). Cells were harvested 72 hours after treatment and analyzed cell viability using the BD cell viability kit with liquid counting beads (BD Bioscience) gating out stroma cells (GFP+), dead cells and particles (PI+). We acquired data using a FACSCanto II flowcytometer (BD Bioscience) and analyzed it using FlowJo software (Tree Star, Inc.). Viability data is represented as % relative to vehicle treatment.

Sequencing

[0180] A region of 124bp from coding exon 1 of the human *NRAS* gene, including G12-G13 mutation hotspot, was amplified from the genomic DNA of six primary T-ALL samples by polymerase chain reaction and was analyzed by direct dideoxynucleotide sequencing using primers FW: 5'-GCTGGTGTGAAATGACT-3' (SEQ ID NO: 52) and RV: 5'-GCTACCACTGGGCCTCACCT-3' (SEQ ID NO: 53).

COSMIC

[0181] Primary T-ALL (PDTALL) 22 cells have a synonymous variant: GCA/GCT (A) and also a variant: GGT/GTT (G13) (G13V). PDTALL 26 cells have a missense variant GGT/GAT (G13) (G13D).

Patient-derived xenograft

[0182] Animals were maintained in the animal facility at the Irving Cancer Center at Columbia University Medical Campus and all animal procedures were approved by the Columbia University IACUC. To generate primary xenografts, cells from T-ALL sample PD22, harboring a mutated allele of *NRAS*, were transplanted via intravenous injection into lethally irradiated primary recipients as previously described (Chiang et al., 2008). Upon detection of human lymphoblasts (human CD45+ cells) in peripheral blood, mice were sacrificed; lymphoblasts isolated from the spleens were transduced with retroviral particles expressing a fusion protein between the red cherry fluorescent protein and luciferase (MigR1 CherryLUC), and cells were re-injected in sublethally irradiated mice (Piovan et al., 2013).

[0183] Mice transplanted with retrovirally transduced cells were imaged regularly until luciferase activity was detected. Tumor cells were harvested

from the spleens of these mice, and injected into secondary recipients. Secondary recipients were randomized into two groups of 5 mice with equal loads of luciferase. Animals were treated I.P. with vehicle or 31MEW44 30 mg/kg in 5% DMSO in HBSS at pH 4, once daily on days 0; 1; 4; 5; 7 and 8, and imaged at day 0 (before treatment), at day 4 and at day 8. Mice were sacrificed at day 8; spleen weight and presence of human CD45⁺ cells in the spleen (lymphoblasts) were documented together with changes in luciferase signal over treatment.

Caspase 3/7 activation assay

[0184] HT-1080 cells were seeded into 384-well plates at 1,000 cells/well. After 12-16 hours, compounds (as 50 mM stocks in DMSO) were arrayed in a dilution series in 384-well polypropylene plates. Compound solutions were transferred at a 1:5 dilution into the assay plates for a total volume of 40 μ L. After 24 hours, 8 μ L of a 1:100 solution of rhodamine 110 bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-aspartic acid amide) to lysis buffer (APO-1, Promega) was added and the plate was wrapped in aluminum foil and incubated at room temperature for 16 hours. Fluorescence intensity was then determined using a Victor3 plate reader (Perkin Elmer) with a 490 nm excitation filter and a 535 nm emission filter. A viability curve was performed in parallel with the same incubation time using the procedure described in the “cell viability assays” section.

Molecular cloning, protein expression, and purification

[0185] Human *KRAS4B* sequence containing the oncogenic Q61H mutation in pENTR221 vector was purchased from Invitrogen (Ultimate ORF Clone IOH9852). To generate the wild-type *KRAS* sequence, a H61Q back

mutation was introduced using QuickChange II site-directed mutagenesis (Agilent Technologies) and confirmed by DNA sequencing (GeneWiz, Inc.). Wild-type *KRAS4B* sequence encoding the catalytic domain (amino acids 1-169 in KRAS) was amplified by PCR and cloned into *Nde I*-*BamHI* sites of pET-15b vector (Novagen) containing the N-terminal His₆ tag. A G12D point mutation was introduced using QuickChange II site-directed mutagenesis (Agilent Technologies). DNA sequencing was performed to confirm the correct amino acid sequence of the construct (GeneWiz, Inc.).

[0186] Mutagenesis of the *KRAS*^{G12D} plasmid was performed using a QuikChange XL site-directed mutagenesis kit from Agilent technologies, according to the manufacturer's protocol. Primers were designed using the Agilent QuikChange Primer Design application and purchased from Integrated DNA Technologies. *KRAS*^{G12D D38A} forward primer 5' ATA TGA TCC AAC AAT AGA GGC TTC CTA CAG GAA GCA AGT AG 3' (SEQ ID NO: 66), *KRAS*^{G12D D38A} reverse primer 5' CTA CTT GCT TCC TGT AGG AAG CCT CTA TTG TTG GAT CAT AT 3' (SEQ ID NO: 67), *KRAS*^{G12D I36N} forward primer 5' CAT TTT GTG GAC GAA TAT GAT CCA ACA AAT GAG GAT TCC TAC AGG 3' (SEQ ID NO: 68), *KRAS*^{G12D I36N} reverse primer 5' CCT GTA GGA ATC CTC ATT TGT TGG ATC ATA TTC GTC CAC AAA ATG 3' (SEQ ID NO: 69). DNA sequencing was performed to confirm the correct amino acid sequence of the construct (GeneWiz, Inc.).

NMR

[0187] For NMR studies, uniformly ¹⁵N-labeled *KRAS*^{G12D} protein with N-terminal His₆ tag was prepared. The *KRAS*^{G12D} construct was expressed in *Escherichia coli* BL21-Gold (DE3) cells (Stratagene) growing at 37°C in M9

minimal medium supplemented with 100 $\mu\text{g/mL}$ ampicillin, metals, 30 mg nicotinic acid, 3 mg *p*-aminobenzoic acid, 0.3 mg biotin, 0.5 mg thiamine hydrochloride, and 0.6 g $^{15}\text{NH}_4\text{Cl}$ as the sole nitrogen source. When the $\text{OD}_{600\text{nm}}$ reached 0.9, protein expression was induced with 1 mM isopropyl β -D-thiogalactoside at 15°C overnight. Cells were pelleted and lysed by sonication in buffer containing 10 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, 5 mM MgCl_2 , pH 7.5, 0.5% CHAPS (w/v), 1 mM PMSF, and 1 mM TCEP. Cell lysate was then centrifuged at 15,000 x g for 45 minutes at 4°C. $\text{KRAS}^{\text{G12D}}$ was first purified from cell lysate using Ni Sepharose 6 Fast Flow beads (GE Life Sciences) and then using gel filtration Superdex 100 column. The fractions containing $\text{KRAS}^{\text{G12D}}$ were pooled together and verified by SDS-PAGE. Protein concentration was determined using absorbance at 280 nm with an extinction coefficient of 11,920 $\text{M}^{-1} \text{cm}^{-1}$ (calculated using MOE). Thrombin was added at 5 U/mg protein to cleave the N-terminal His₆ tag. The reaction was allowed to proceed overnight at 4°C. The next day, the protein solution was passed over Ni-Sepharose 6 Fast Flow beads (GE Life Sciences) and flowthrough containing the ^{15}N -labeled $\text{KRAS}^{\text{G12D}}$ protein without histidine tag was concentrated and flash frozen. Purity was checked by SDS-PAGE gel.

[0188] To load a specific nucleotide onto ^{15}N -labeled $\text{KRAS}^{\text{G12D}}$, the protein was first incubated with 10 molar excess of EDTA for 1 hour at room temperature, then buffer exchanged into HEPES NMR buffer (50 mM HEPES pH 7.4, 50 mM NaCl), and lastly supplemented with 2 mM MgCl_2 , 2 mM TCEP, and 10 molar excess of GDP or GppNHp nucleotide.

[0189] For biochemical studies, the KRAS^{G12D} construct was expressed in *Escherichia coli* BL21-Gold (DE3) cells (Stratagene) growing at 37°C in LB media with 100 µg/mL ampicillin and induced when the OD_{600nm} reached 1.0, with 1 mM isopropyl β-D-thiogalactoside at 15°C overnight. Protein purification was the same as for the ¹⁵N-labeled protein, except the N-terminal His₆ tag was not removed.

In vitro RAS pulldown

[0190] A 20 µM solution of KRAS^{G12D} in 50 mM HEPES, 200 mM sodium chloride, 2 mM TCEP with 1 mM EDTA and 1 mM GTP was gently rotated at 25°C to remove the endogenous nucleotide. Magnesium chloride was then added to 5 mM and the resulting solution was rotated at 4°C for 4 hours. The GTP-loaded KRAS^{G12D} was then diluted to 20 nM and incubated with the inhibitors and 5 µL of CRAF-RBD agarose beads (Millipore), 10 µL of RAF1-RBD agarose beads (EMD Millipore, CN: 14-278), or 100 nM RALGDS (Abcam, CN: ab132590) with 20 µL of glutathione beads for 2 hours. The beads were separated from the lysate via a 0.1 µm filter spin cup and centrifuging at 14,000-15,000 rpm. They were then washed twice with PBS before the addition of 1X SDS. The quantity of RAS in the samples was then analyzed using the previously described Western blotting procedure.

Cell-based RAS pulldown

[0191] BJeLR cells were seeded at one million cells/10 cm dish in 10% FBS and incubated at 37°C overnight. The medium was then aspirated and replaced with serum free media containing the inhibitors (from 10 mM DMSO stocks). The cells were then incubated for 24 hours at 37°C. The medium was removed, washed with cold PBS, lysed and spun down at 13,000 rpm at 4°C

to remove unlysed cells and debris. The lysate was incubated with Raf-1 RBD agarose beads (EMD milipore) for 2 hours with rotation at 4°C. The solution was then spun down at 1500xg and the supernatant removed. The beads were washed twice with PBS, resuspended in 2.5 X SDS, and then analyzed by western blotting procedure.

Determining RAS dependency using siRNAs targeting RAS isoforms

[0192] Small interfering RNAs (siRNAs) targeting each RAS isoform were purchased from Dharmacon Technologies. Reverse transfection was performed by preparing a solution of 1 mL of Opti-MEM (Invitrogen), 6 µL of lipo-RNAiMAX (Invitrogen) and 2-5 µL of RNAi solution (10 µM stock), and by incubating the mixture (1 mL/well) in a 6-well plate for 20-30 minutes at 37°C. While the siRNA complex was forming, 0.2 million cells were suspended in 1 mL of 2X serum-containing media. The cell solution (1 mL) was transferred to each well of the 6-well plate containing siRNA complex (1 mL), and the 6-well plate was returned to the culture incubator. At 24, 48, 72, and 96 hours post-transfection, cells were trypsinized and the number of viable cells was determined using trypan blue exclusion assay.

Confirming RAS knockdown using RT-qPCR experiment

[0193] Cells were detached from the 6-well plate, and 0.5 million cells were collected as a pellet by centrifuging at 1,000 rpm for 5 minutes. Total cellular RNA sample was prepared using RNAeasy extraction kits (QIAGEN) according to manufacturer's instruction. The resulting RNA sample was reverse-transcribed using a High Capacity cDNA Reverse Transcription kit (Life Technologies). The cDNA samples were mixed with TaqMan® probes for each RAS isoform gene, and arrayed on 96-well plates in triplicate. Each

plate was loaded onto a ViiA7 Real-Time PCR system (Life Technologies) for qPCR reaction. Comparative analysis ($\Delta\Delta C_t$ analysis) was performed with ACTB (human actin b), an internal reference gene.

Immunoprecipitations

[0194] BJeLR cells were seeded 16 hours prior to use in 10% FBS in DMEM. Media was aspirated and replaced with media containing 31MEW44 (from a 10 mM DMSO stock). After 6 hours cells were washed twice with ice cold buffer (25 mM tris, 100 mM NaCl, 1 mM TCEP, 5 mM MgCl₂, 0.1% tween-20 and 1 protease inhibitor/25 mL). Cells were scrapped, pelleted at 13,000 rpm for 10 minutes at 4°C, then passed through a 26 gauge needle several times. The solution was spun down a second time at 13,000 rpm for 15 minutes at 4°C, to remove unlysed cells and debris. HRAS antibody (Santa cruz, SC-520) was then added to the lysate (1:100) and the solution was rocked at 4°C for 16 hours. Protein A agrose beads were then added and the solution was rotated at 4°C for an additional 6 hours. The solutions were spun down at 1500xg for 2 minutes and the supernatant was removed by syringe. The beads were washed twice by this process with buffer, then resuspended in 2.5X SDS.

RALA activation assay

[0195] BJeLR cells were seeded 16 hours prior to use in 2% FBS in DMEM. Media was aspirated and replaced with media containing 31MEW44 (from a 10 mM DMSO stock). After 6 hours cells were washed twice with ice cold buffer (25 mM tris, 100 mM NaCl, 1 mM TCEP, 5 mM MgCl₂, 0.1% tween-20 and 1 protease inhibitor/25 mL). Cells were scrapped, pelleted at 13,000 rpm for 10 minutes at 4°C, then passed through a 26 gauge needle

several times. The solution was spun down a second time at 13,000 rpm for 15 minutes at 4°C, to remove unlysed cells and debris. RALBP1 agarose beads (EMD Millipore) were then added to the lysate and the solution was rotated at 4°C for 2 hours. The solutions were spun down at 1500xg for 2 minutes and the supernatant was removed by syringe. The beads were washed twice by this process with buffer, then resuspended in 2.5X SDS.

Differential Scanning Fluorimetry

[0196] A fluorescent thermal shift assay was used to investigate the binding of synthesized ligands to K-Ras G12D protein. The assay was carried out in triplicate in 384-well optical plates containing 5 µM protein, varying concentration of ligand from 500 µM to 1 µM, and 5X SYPRO Orange dye (Invitrogen). Samples were heated at 3°C/minute from 25°C to 95°C and protein unfolding was observed by monitoring the fluorescence of SYPRO orange dye (Invitrogen) at an excitation of 470 nm and an emission of 623 nm using a ViiA7 real-time PCR machine (Applied Biosystems). K-Ras G12D protein preloaded with the specified nucleotide was incubated with ligand for 30 minutes at room temperature before the addition of SYPRO Orange dye. All experiments were performed in triplicate. Data were analyzed using Protein Thermal Shift™ Software (Applied Biosystems) to determine the unfolding transition temperature, T_m , of each well. The ΔT_m was calculated by subtracting the T_m of liganded K-Ras G12D protein from unliganded K-Ras G12D and are expressed as absolute value of the mean $\pm$ sem.

Protein NMR Spectroscopy

[0197] The ^1H - ^{15}N HSQC experiments were performed on Bruker Avance III 500 (500 MHz) and Avance III 500 Ascend (500 MHz)

spectrometers at 298K. The buffer consisted of 50 mM HEPES pH 7.4, 50 mM NaCl, 2 mM MgCl₂, 2 mM TCEP, and 10% D₂O. Assignments of wild-type KRAS loaded with GDP were previously published by Vo *et al.* (2013). The conditions reported in Vo *et al.*, 2013 were used to efficiently transfer the assignments to the peaks of ¹H-¹⁵N HSQC spectrum of the KRAS^{G12D} GDP protein and then to KRAS^{G12D} GppNHp loaded protein. To verify the assignments, 3D-¹H-¹⁵N-¹H-NOESY-HSQC and 3D-¹H-¹⁵N-¹H-TOCSY-HSQC experiments were performed on KRAS^{G12D} protein loaded with either GDP or GppNHp nucleotide. The 3D NMR experiments were performed on Bruker Avance US² 800 (800 MHz) and Bruker Avance III 600 (600 MHz) spectrometers equipped with cryogenic probes. The ¹⁵N-NOESY-HSQC data set were recorded using a mixing time of 75 ms. The ¹⁵N-TOCSY-HSQC data were recorded using a mixing time of 60 ms. All data were processed and analyzed using TopSpin 3.1 (Bruker). The assignments were performed using Sparky (T. D. Goddard and D. G. Kneller, UCSF).

Nucleotide Displacement Assay

[0198] To investigate whether compounds were binding to the same site as GTP, a fluorescent polarization assay was implemented using fluorescently-labeled BODIPY-GTP as a probe. When BODIPY-GTP is free in solution, it has a low polarization. However, when BODIPY-GTP is bound to K-Ras protein, the polarization intensity is high. K-Ras G12D bound to BODIPY-GTP was incubated with different concentrations of ligand or unlabeled GTP or GDP in buffer containing 25 mM Tris-HCl, pH 8, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, and 1 mM TCEP. The final concentration of K-Ras G12D bound to BODIPY-GTP in each well was 2.5 μM. The samples

were incubated at 25°C for 12 hours with gentle shaking to allow for the slow intrinsic nucleotide release reaction to take place. The change in fluorescence was measured on a Victor3 plate reader (Perkin Elmer) in 384-well black low-volume plates (Corning, Inc.). The BODIPY-GTP fluorophore was excited at 485 nm and emission was monitored at 535 nm.

Gene expression analysis by RT-qPCR

[0199] Cells from six-well plates were trypsinized and centrifuged at 3,000 rpm for 3 minutes. The cell pellet was then lysed and the RNA was extracted using QIAshredder and RNeasy extraction kits (QIAGEN) according to the manufacturer's protocol. 2 micrograms of RNA from each sample was then converted to cDNA using the TaqMan RT Kit (Applied Biosystems). Primers for Quantitative PCR (qPCR) were designed with Primer Express. qPCR was performed using Power SYBR Green Master Mix (Applied Biosystems) in a 96-well format, in triplicate, using an Applied Biosystems 7300 Cyclor set to absolute quantification. Expression changes were computed using the $\Delta\Delta Ct$ method with GAPDH as an internal reference gene. The primers used were as follows: urokinase-type plasminogen activator (uPA) Forward 5' GGATGTGCCCTGAAGGACAA 3' (SEQ ID NO: 54), reverse 5' TGCGGATCCAGGGTAAGAAG 3' (SEQ ID NO: 55); matrix metalloprotease 9 (MMP9) forward 5' GAGTGGCAGGGGGAAGATGC 3' (SEQ ID NO: 56), reverse 5' CCTCAGGGCACTGCAGGATG 3' (SEQ ID NO: 57); lactate dehydrogenase (LDH) forward 5' GCCCGACGTGCATTCCCGATTCTT 3' (SEQ ID NO: 58), reverse 5' GACGGCTTTCTCCCTCTTGCTGACG 3' (SEQ ID NO: 59); CMYC forward 5'

TCAAGAGGTGCCACGTCTCC 3' (SEQ ID NO: 60), reverse 5'
TCTTGGCAGCAGGATAGTCCTT 3' (SEQ ID NO: 61).

RAS, PI3K and BRAF overexpression.

[0200] Phoenix-AMPHO (ATCC: CRL-3213) cells were seeded in a 6-well plate at 600,000 cells/well 24 hours prior to use in 10% FBS and 1% PS in DMEM. A solution of lipofectamine 2000 (6 μ L) in 100 μ L opti-mem media (reduced serum media) and the plasmid (2.5 μ g) in 100 μ L opti-mem media were combined and incubated 5 minutes at 25 °C, then added to 1.8 mL of opti-mem in each well. After 12 hours, the media was replaced with 10% FBS and 1% PS in DMEM. The next day the supernatant was collected three times spaced 4 hours apart and polybrene was added (1/1000). The supernatant was filtered (0.45 μ m) and added to HT1080 cells seeded at 100,000 cells per well (6-well dish) in 2 mL portions spaced 4 hours apart. After 48 hours, the cells were trypsinized and re-seeded in medium containing puromycin (2 μ g/mL). After 6 days of selection the cells were analyzed for expression using the aforementioned qPCR procedure. 2 mL solutions of the inhibitors were added to a 6-well plate of the transfected HT-1080 cells (100,000 cells/well) and treated for 24 hours. The cells were then trypsinized, re-suspended in 1 mL of medium and viability was measured by VI-CELL (Beckman Coulter) through mixing with trypan blue.

Microscale thermophoresis

[0201] KRAS^{G12D} (250 μ M) in 25 mM HEPES, 100 mM sodium chloride, 2 mM TCEP with 20 mM EDTA and 1 mM GppNHp was shaken at 220 rpm at 30°C to remove the endogenous nucleotide. The solution was placed on ice for 2 minutes prior to the addition of 65 mM MgCl₂. After an additional 10

minutes incubation on ice, 200 μ L of a 10 μ M solution of KRAS^{G12D} was combined with 200 μ L of a 20 μ M solution of NT-647-NHS-ester dye (from a 652.4 μ M stock). The protein/dye mixture was rotated at 4°C for 30 minutes, before being separated from the excess dye and buffer exchanged into 25 mM Tris, 100 mM sodium chloride, 2 mM TCEP, and 5 mM MgCl₂ via a nap-5 column. The test compounds were arrayed across a 16-point dilution series consisting of 1.5% DMSO with 25 mM Tris, 100 mM NaCl, 2 mM TCEP, 5 mM MgCl₂ and 0.05% tween-20. Thermophoretic movement of the fluorescently labeled protein with the inhibitors was performed using a Monolith NT.115 (Nanotemper Technologies). RALA and RHOA were purchased commercially from Abcam (RALA CN:ab102555, RHOA CN:ab101594).

Isothermal titration calorimetry (ITC)

[0202] All ITC experiments were carried out at 25°C on a MicoCal Auto-ITC₂₀₀ system (GE Healthcare). Due to low solubility of 31MEW44 in aqueous buffers, for all ITC experiments the compound was loaded into the cell and the KRAS^{G12D}•GppNHp protein was loaded into the syringe.

[0203] Working stocks of compound 31MEW44 were prepared in 100% DMSO at 20 mM. 450 μ L of the compound solution was loaded into the cell at 250 μ M in ITC buffer (25 mM Tris pH 7.4, 1 mM TCEP, 100 mM NaCl, 5 mM MgCl₂, 0.05% Tween-20) with a final DMSO concentration at 1.25% (v/v). KRAS^{G12D}•GppNHp was buffer exchanged into same ITC buffer using Amicon Ultra 10 kDa size exclusion filter spin columns (buffer replaced with ITC buffer four times). Prior to loading 130 μ L of KRAS^{G12D}•GppNHp into the syringe at 2.5 mM, DMSO was added to match the amount DMSO present in the cell. ITC titration experiments were carried out at 25°C with 19 injections, 2 μ L per

injection, and 180 seconds between each injection. Reference cell power was set to 5 μ cal/sec. A control experiment was performed where ITC buffer was titrated into compound 31MEW44 alone to account for heat released due to dilution. This background was subtracted from test data before the final dissociation constant was obtained. Data were analyzed using the one-site binding model in Origin 7.1 software. The dissociation constant, K_d , was calculated according to equation $K_d = 1/K_a$. Gibbs free energy, ΔG , was calculated from the equation $\Delta G = -RT \ln K_a$. $-T\Delta S$ was calculated from the equation $\Delta G = \Delta H - T\Delta S$. All other parameters, K_a , n , ΔH , were determined directly from the titration data.

Mutagenesis

[0204] Mutagenesis of the $KRAS^{G12D}$ plasmid was performed using a QuikChange XL site-directed mutagenesis kit from Agilent Technologies, according to the manufacturer's protocol. Primers were designed using the Agilent QuikChange Primer Design application and purchased from Integrated DNA Technologies: $KRAS^{G12D D38A}$ forward primer 5' ATA TGA TCC AAC AAT AGA GGC TTC CTA CAG GAA GCA AGT AG 3' (SEQ ID NO: 62), $KRAS^{G12D D38A}$ reverse primer 5' CTA CTT GCT TCC TGT AGG AAG CCT CTA TTG TTG GAT CAT AT 3' (SEQ ID NO: 63), $KRAS^{G12D I36N}$ forward primer 5' CAT TTT GTG GAC GAA TAT GAT CCA ACA AAT GAG GAT TCC TAC AGG 3' (SEQ ID NO: 64), $KRAS^{G12D I36N}$ reverse primer 5' CCT GTA GGA ATC CTC ATT TGT TGG ATC ATA TTC GTC CAC AAA ATG 3' (SEQ ID NO: 65).

Microsomal stability

[0205] Test compounds (0.5 μ M) were incubated at 37°C for up to 45 minutes in 50 mM of potassium phosphate buffer (pH 7.4) containing microsomal protein (0.5 mg/mL) and an NADPH generating system (0.34 mg/mL β -nicotinamide adenine dinucleotide phosphate (NADP), 1.56 mg/mL glucose-6-phosphate, 1.2 units/mL glucose-6-phosphate dehydrogenase). At 0, 5, 15, 30 and 45 minute intervals an aliquot was taken and quenched with acetonitrile (ACN) containing an internal standard. No-cofactor controls at 45 minutes were prepared. Following completion of the experimentation, the samples were analyzed by LC-MS/MS using a Shimadzu HPLC and an Applied Biosystem API4000.

In vivo pharmacokinetic analysis

[0206] 27 mg of 31MEW44, was dissolved in 5.4 mL of 10% NMP/90% PEG-400 to yield a dosing solution with a final concentration of 5 mg/mL. The dose formulation was prepared freshly in the morning of dosing day. A total of 42 male C57 adult mice, each approximately 25 grams in body weight, were administered at 20 mg/kg dose via a single IV bolus injection or a single oral gavage. Blood samples (approximately 400 μ L) were collected from three mice per time point via terminal brachial bleed at pre-dose and 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours and 12 hours post-dose. Blood samples were placed into tubes containing K2 EDTA anti-coagulant, and centrifuged at about 2,100 g (rcf) for 10 minutes at 4°C to separate plasma. Following centrifugation, the resulting plasma was transferred to clean tubes and stored frozen at -80°C. The mouse plasma samples (50 μ L) were aliquoted, spiked with internal standard (250 ng/mL tolbutamide), and then extracted with

protein precipitation. The supernatant of each sample was diluted and injected into a LC-MS/MS system. The data acquisition and processing were performed using a Sciex API 5500 mass spectrometer with Analyst 1.6.2 software. The standard curve range for the plasma samples was analyzed using a calibration curve of 0.5-5000 ng/mL. The pharmacokinetic (PK) analysis and interpretation of the results were conducted using Winnonlin Phoenix Software.

Mouse xenograft

[0207] For the therapeutic study, athymic nude mice (eight weeks; Charles River Laboratories) were injected with 7 million MDA-MB-231 cells subcutaneously. After 3 days, mice were separated into treatment groups of roughly equal tumor size (58 mm^3) and dosed with 180 mg/kg 31MEW44 orally (12 mg/mL, 10% DMSO, pH 4), vehicle orally, or by a combination of intraperitoneal and intravenous injections at 30 mg/kg (4 mg/mL, 5% DMSO in HBSS at pH 4). Over 14 days mice received a total of 10 doses of 31MEW44 or vehicle orally, or six intraperitoneal injections and 4 intravenous injections. Tumor size was measured by electronic caliper every 2 days and calculated using the formula: $0.523 \times \text{Length} \times \text{width}^2$.

[0208] For the pharmacodynamic study, athymic nude mice (eight weeks; Charles River Laboratories) were injected with 8 million MDA-MB-231 cells subcutaneously. After four days, mice were separated into treatment groups of roughly equal tumor average size and population, and dosed with 30 mg/kg 31MEW44 in 5% DMSO HBSS at pH 4 intraperitoneal or vehicle (5% DMSO HBSS at pH 4) once per day for six days. Tumor size was

measured by electronic caliper every two days and calculated using the formula: $0.523 \times \text{Length} \times \text{width}^2$.

[0209] Mice were euthanized using a CO₂ gas chamber before xenograft dissection. Tumors were then weighed, frozen and stored at -80°C. Segments of the tumor were taken (about 60 mg) and suspended in 120 µL lysis buffer. Xenografts were then lysed by sonication (40 amp for 10-15 seconds) and samples were centrifuged at 14,000 rpm at 4°C for 30 minutes to remove unlysed cells and debris. The supernatant was then analyzed by Western blotting using the aforementioned protocol.

KP^{fl/fl}C mouse study

[0210] KRAS^{LSLG12D}; p53^{fl/fl}; Pdx1-Cre (KP^{F/F}C) mice have been previously described (Bardeesy *et al.*, 2006). Animals were housed in a barrier facility and monitored daily prior to enrollment on studies. All experiments were carried out in compliance with established IACUC guidelines of Columbia University.

Animal surgery

[0211] KP^{F/F}C were palpated twice weekly to assess for tumor formation. Upon discovery of a palpable mass deemed amenable to surgery, tumors were accessed by abdominal laparotomy as previously described for biopsy procedures (Sastru *et al.*, 2014). In short, mice were anesthetized with isoflurane, prepared for aseptic surgery, and injected with buprenorphine intra-operatively to initiate post-operative analgesia. Following visual identification, tumors were held in place with a pair of biopsy forceps while a small-diameter biopsy punch (2-mm diameter, Zivic Instruments PUN2000) was used to cleanly remove a tissue sample. The resultant wound was filled

with an absorbable, gelatin compressed sponge to staunch possible bleeding. Incisions were sutured closed and the mouse was allowed to recover from 24-48 hours prior to study initiation.

Sample processing and storage

[0212] Biopsy samples derived from small animal surgery were divided in two specimens. The first was stored in 10% buffered formalin phosphate overnight at 4°C and then placed in 70% ethanol for extended storage prior to processing and embedding in paraffin wax blocks. The second was embedded in O.C.T. compound and subsequently frozen atop a bath of liquid nitrogen prior to long-term storage at -80°C. Tumor samples taken at necropsy were processed and stored identically to those described above.

Immunohistochemistry

[0213] Paraffin embedded samples were sectioned at 5µM thickness and mounted on positively charged sample slides. These slides were heated at 60°C for 15-30 minutes and subsequently rehydrated by standard protocols. Unmasking was performed in 10 mM citrate buffer, pH 6 for 5 minutes in a pressure cooker at high temperature, followed by a peroxidase quench in 3% hydrogen peroxide for 20 minutes. Blocking was carried out using 1.5% horse serum and 2% animal free blocker (Vector Labs) in TBS-T for 1 hour at room temperature. Slides were incubated with primary antibody (cleaved caspase-3, catalog no. 9664; ERK, catalog no. 4695; pERK, catalog no. 4376. All antibodies from Cell Signaling) overnight at 4°C. Slides were then allowed to equilibrate to room temperature prior to washing with TBS-T and incubation with secondary antibody (ImmPress polymer reagent, Vector Labs). Signal was developed with ImmPACT DAB Peroxidase Substrate

(Vector Labs). Slides were counterstained with hematoxylin for 30 seconds. For quantification of cleaved-caspase 3 staining, all available 40x fields on three separate sections of biopsy samples and twenty total 40x fields from two separate sections of necropsy samples were analyzed for each study mouse.

Drug studies

[0214] Mice were dosed once daily with 30mg/kg of 31MEW44 by way of intraperitoneal injection. Mice were monitored closely for changes in health status and were sacrificed after 5 days on study or once they met endpoint criteria in keeping with IACUC standards.

Example 2

Synthesis of Chemical Materials

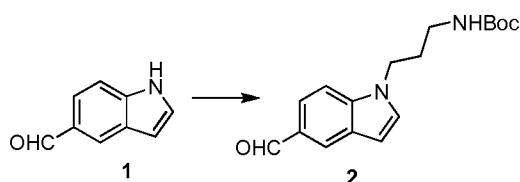
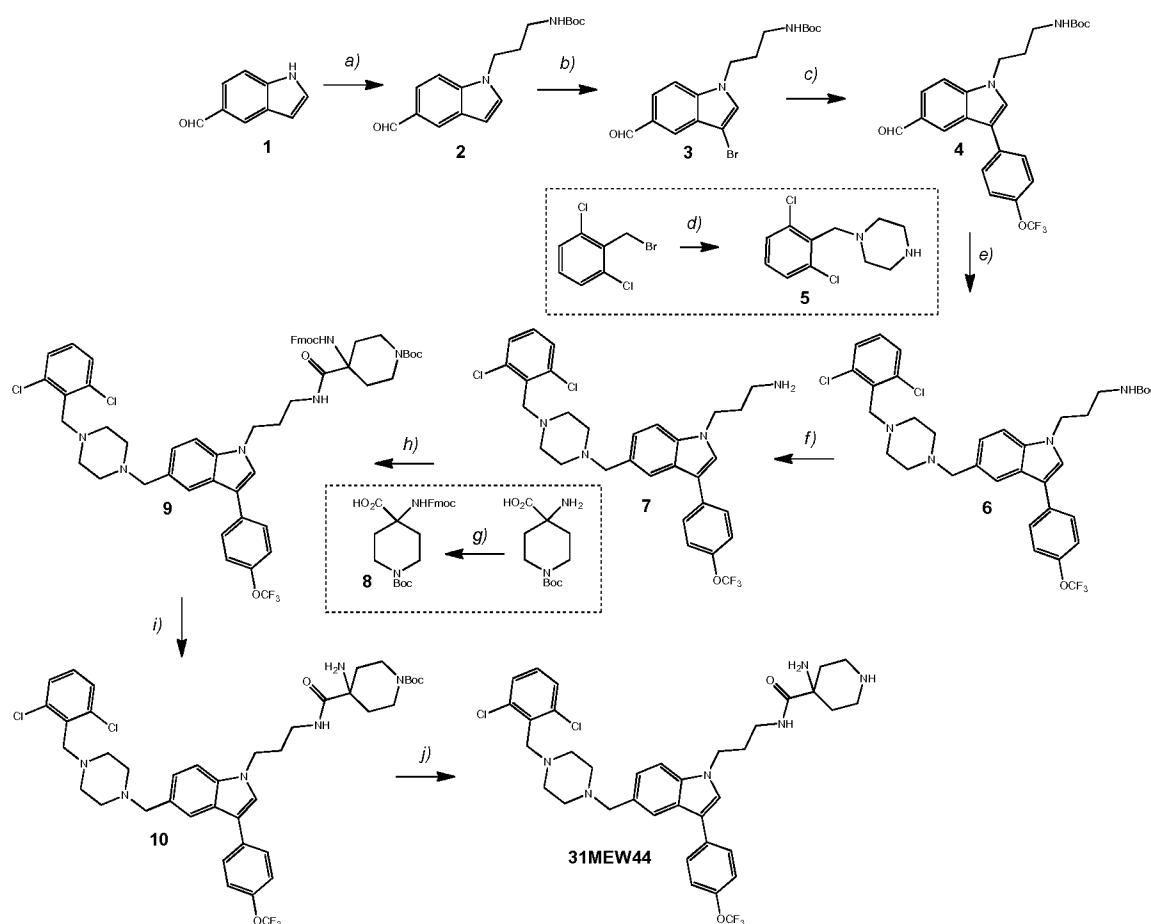
General information

[0215] All reactions were carried out under a nitrogen atmosphere under anhydrous conditions unless indicated otherwise. Anhydrous methylene chloride (DCM), tetrahydrofuran (THF) and N,N-dimethylformamide (DMF) were purchased from Sigma-Aldrich. Reactions were magnetically stirred and monitored by thin layer chromatography carried out by Merck pre-coated 0.25 mm silica plates containing a 254 nm fluorescence indicator. Flash chromatography was performed on a Teledyne combiflash companion automatic flash chromatography system. Preparative thin layer chromatography was performed on 1 mm plates. Proton nuclear magnetic resonance spectra (^1H NMR, 300 MHz, 400 MHz, 500 MHz) and proton decoupled carbon nuclear magnetic resonance spectra (^{13}C NMR, 100 MHz, 125 MHz) were obtained on a Bruker DPX 300, 400, or 500 MHz instruments

in deuteriochloroform (CDCl_3) with residual chloroform as internal standard. Other deuterated solvents that were used include d_4 -MeOD and d_6 -DMSO.

Abbreviations

[0216] DIPEA = diisopropylethyl amine, EtOAc = ethyl acetate, MeOH = methanol, DCE = 1,2-dichloroethane, $\text{Pd}(\text{PPh}_3)_4$ = Tetrakis(triphenylphosphine)palladium(0), Na_2SO_4 = sodium sulfate, MgSO_4 = magnesium sulfate, NaHCO_3 = sodium bicarbonate, NH_4Cl = ammonium chloride, TFA = trifluoroacetic acid, HBTU = O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate, HCl = hydrochloric acid, THF = tetrahydrofuran, rt = room temperature.

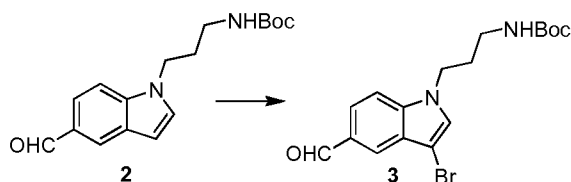
Synthesis of two- and three-site compounds

tert-butyl 3-(5-formyl-1H-indol-1-yl)propylcarbamate (Scheme 1, compound 2)

[0217] To a solution of 1H-indole-5-carbaldehyde (Scheme 1, compound 1) (3.5 g, 24 mmol) in DMF (100 mL) at 0°C, sodium hydride (60% in mineral oil) (1.1 g, 28.8 mmol, 1.2 eq) was added in several portions over about 5 minutes. The mixture was stirred for 45 minutes at 0°C before the sequential addition of 3-(Boc-amino)propyl bromide (8 g, 33.6 mmol, 1.4 eq)

and sodium iodide (3.6 g, 24 mmol, 1.0 eq). The solution was warmed to 80°C and stirred for 48 hours. Upon completion, the reaction was diluted with saturated aqueous NaHCO₃ and extracted 3 times with EtOAc. The combined organic layers were washed with brine, dried (Na₂SO₄), concentrated, and the crude material was purified by combi flash 0 to 50% EtOAc in hexanes (3.4 g, 47% yield). ¹H NMR (400 MHz, Chloroform-*d*) δ 10.05 (s, 1H), 8.18 (d, *J* = 1.5 Hz, 1H), 7.81 (dd, *J* = 8.7, 1.6 Hz, 1H), 7.26 (d, *J* = 3.1 Hz, 1H), 6.75-6.60 (m, 1H), 4.54 (s, 1H), 4.25 (t, *J* = 6.9 Hz, 2H), 3.17 (d, *J* = 7.2 Hz, 2H), 2.08 (p, *J* = 6.9 Hz, 2H), 1.47 (s, 8H). ¹³C NMR (101 MHz, CDCl₃) 192.44, 156.08, 139.19, 129.79, 129.34, 128.41, 126.56, 121.80, 109.80, 103.60, 44.05, 38.04, 30.63, 28.38.

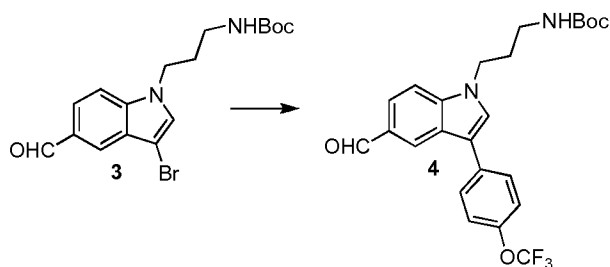
[0218] HRMS (*m/z*): [M⁺] calcd for C₁₇H₂₂N₂O₃, 302.37, found 302.16.



tert-butyl 3-(3-bromo-5-formyl-1H-indol-1-yl)propylcarbamate (Scheme 1, compound 3)

[0219] To a solution of *tert*-butyl 3-(5-formyl-1H-indol-1-yl)propylcarbamate (Scheme 1, compound 2) (1.8 g, 5.95 mmol) in THF (120 mL) at -78°C, Br₂ (0.367 mL, 7.1 mmol, 1.2 eq) was added dropwise over about 5 minutes. The resulting mixture was stirred at -78°C for 2 hours. Upon completion, the reaction contents were poured onto a solution of ice (about 300 g), water (200 mL), ammonium hydroxide (1 mL, 12 M), sodium

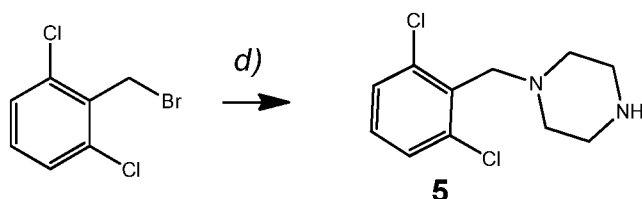
thiosulfate pentahydrate (1 mL, saturated solution in water). The crude material was extracted 3 times with EtOAc, the combined organic layers were washed with brine, dried (Na_2SO_4), concentrated, and the crude material was purified by combiflash 0 to 50% EtOAc in hexanes to yield *tert*-butyl 3-(3-bromo-5-formyl-1H-indol-1-yl)propylcarbamate (Scheme 1, compound 3) (1.2 g, 53% yield). ^1H NMR (400 MHz, Chloroform-*d*) δ 10.09 (s, 1H), 8.12 (d, J = 1.5 Hz, 1H), 7.86 (dd, J = 8.7, 1.5 Hz, 1H), 7.43 (d, J = 8.6 Hz, 1H), 7.30 (s, 1H), 4.57 (s, 1H), 4.23 (t, J = 6.9 Hz, 2H), 3.18 (d, J = 6.8 Hz, 2H), 2.08 (q, J = 6.7 Hz, 2H), 1.47 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) 191.19, 155.31, 137.89, 128.98, 127.87, 126.53, 124.05, 121.63, 109.40, 91.20, 78.67, 76.65, 76.33, 76.01, 43.54, 37.09, 29.82, 27.57, 27.54. HRMS (m/z): $[\text{M}^+]$ calcd for $\text{C}_{17}\text{H}_{21}\text{BrN}_2\text{O}_3$, 381.26, found 380.07



tert-butyl 3-(5-formyl-3-(4-(trifluoromethoxy)phenyl)-1H-indol-1-yl)propylcarbamate (Scheme 1, compound 4)

[0220] To a solution of *tert*-butyl 3-(3-bromo-5-formyl-1H-indol-1-yl)propylcarbamate (Scheme 1, compound 3) (1.49 g, 3.91 mmol) in dioxane (30 mL), 4-(trifluoromethoxy)phenylboronic acid (1.20 g, 5.87 mmol, 1.5 eq), $\text{Pd}(\text{PPh}_3)_4$ (0.225 g, 0.195 mmol, 0.05 eq), and a solution of potassium carbonate (1.08 g, 7.82 mmol, 2.0 eq) were added sequentially. The resulting mixture was heated to 80°C and stirred for 48 hours. Upon completion, the reaction was diluted with saturated aqueous NaHCO_3 and extracted 3 times

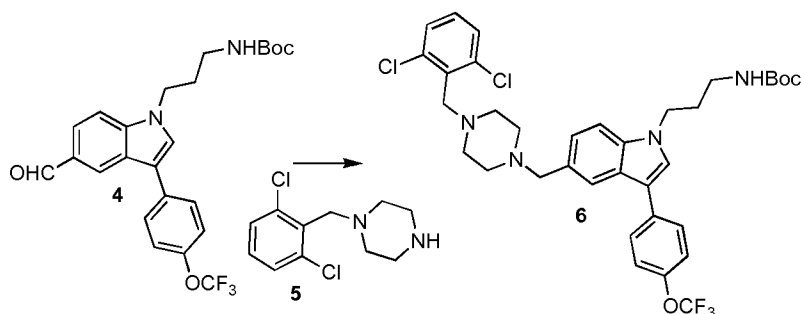
with EtOAc. The combined organic layers were dried (Na_2SO_4), concentrated, and the crude material was purified by combiflash 0 to 40% EtOAc in hexanes to yield *tert*-butyl 3-(5-formyl-3-(4-(trifluoromethoxy)phenyl)-1H-indol-1-yl)propylcarbamate (Scheme 1, compound 4) (1.3 g, 72%). ^1H NMR (400 MHz, Chloroform-*d*) δ 10.00 (s, 1H), 8.33 (d, J = 1.6 Hz, 1H), 7.78 (dd, J = 8.7, 1.6 Hz, 1H), 7.66-7.59 (m, 2H), 7.41 (d, J = 5.6 Hz, 2H), 7.29 (d, J = 8.3 Hz, 2H), 5.01 (s, 1H), 4.24 (t, J = 6.9 Hz, 2H), 3.30 ? 3.09 (m, 2H), 2.16-1.97 (m, 3H), 1.45 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) 191.45, 155.36, 139.01, 132.36, 128.96, 127.71, 126.66, 125.19, 124.10, 121.66, 120.61, 116.82, 109.40, 76.58, 76.26, 75.94, 43.21, 29.63, 27.43. HRMS (m/z): $[\text{M}^+]$ calcd for $\text{C}_{24}\text{H}_{25}\text{F}_3\text{N}_2\text{O}_4$, 462.46, found 462.18



1-(2,6-dichlorobenzyl)piperazine (5)

[0221] To a solution of piperazine (112 mmol, 6.0 eq) in THF (180 mL) at 0°C , a solution of 2,6-dichlorobenzyl bromide (4.5 g, 18.8 mmol) in THF (20 mL) was added dropwise over 10 minutes. The resulting mixture was slowly allowed to warm to room temperature and stirred for 24 hours. Upon completion, the THF was removed and the crude material was re-suspended in DCM and water, and extracted 2 additional times with DCM. The combined organic layers were dried (Na_2SO_4), concentrated, and the crude material was purified by combiflash 0 to 20% MeOH in DCM to provide 1-(2,6-dichlorobenzyl)piperazine (Scheme 1, compound 5) (2.3 g, 50% yield). ^1H

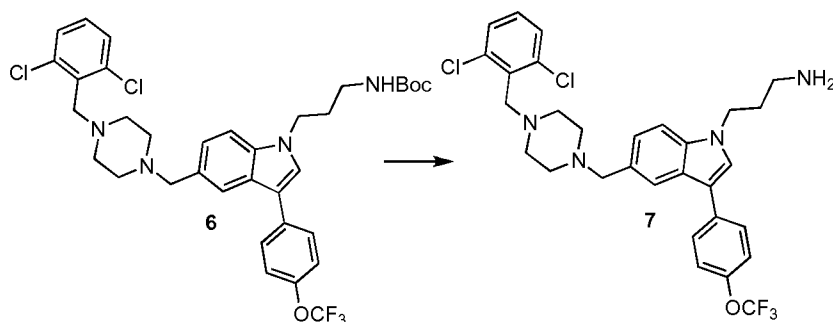
NMR (400 MHz, Methanol- d_4) δ 7.62 -7.30 (m, 2H), 7.23 (dd, J = 8.7, 7.4 Hz, 1H), 3.74 (s, 2H), 2.92-2.69 (m, 4H), 2.56 (t, J = 4.9 Hz, 4H). ^{13}C NMR (101 MHz, MeOD) 136.76, 133.67, 129.18, 128.24, 56.55, 53.41, 44.95. HRMS (m/z): $[M]^+$ calcd for $\text{C}_{11}\text{H}_{14}\text{Cl}_2\text{N}_2$, 245.15, found 245.06



tert-butyl 3-(5-((4-(2,6-dichlorobenzyl)piperazin-1-yl)methyl)-3-(4-(trifluoromethoxy)phenyl)-1H-indol-1-yl)propylcarbamate (Scheme 1, compound 6)

[0222] To a solution of *tert*-butyl 3-(5-formyl-3-(4-(trifluoromethoxy)phenyl)-1H-indol-1-yl)propylcarbamate (Scheme 1, compound 4) (1.1 g, 2.38 mmol) in DCE (15 mL), 1-(2,6-dichlorobenzyl)piperazine (Scheme 1, compound 5) (1.75 g, 7.14 mmol, 3.0 eq) and zinc chloride (65 mg, 0.476 mmol, 0.2 eq) were added. The resulting mixture was stirred at 60°C for 2 hours before the addition of a solution of sodium cyanoborohydride (309 mg, 4.76 mmol, 2.0 eq) in methanol (3 mL). The mixture was stirred for an additional 6 hours at 60°C. Upon completion, the reaction was concentrated and purified directly by combiflash 0 to 5% MeOH in DCM to yield *tert*-butyl 3-(5-((4-(2,6-dichlorobenzyl)piperazin-1-yl)methyl)-3-(4-(trifluoromethoxy)phenyl)-1H-indol-1-yl)propylcarbamate (6) (0.7 g, 42% yield). ^1H NMR (400 MHz, Methanol- d_4) δ 8.01 (d, J = 1.6 Hz, 1H), 7.82-7.74 (m, 2H), 7.65 (s, 1H), 7.58 (d, J = 8.5 Hz, 1H), 7.43-7.33 (m, 5H), 7.32-7.22 (m, 2H), 4.37-4.26 (m, 4H), 3.87 (s, 2H), 3.33 (p, J = 1.6 Hz, 4H),

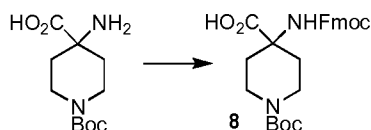
3.22-3.03 (m, 6H), 2.82 (s, 4H), 2.05 (t, J = 6.8 Hz, 2H), 1.45 (s, 8H). ^{13}C NMR (101 MHz, MeOD) 137.27, 136.67, 134.52, 132.90, 129.55, 128.34, 128.23, 127.60, 126.30, 124.15, 122.22, 121.12, 115.36, 110.27, 61.40, 55.10, 51.60, 49.79, 43.43, 29.96, 27.36. HRMS (m/z): $[\text{M}^+]$ calcd for $\text{C}_{35}\text{H}_{39}\text{Cl}_2\text{F}_3\text{N}_4\text{O}_3$, 691.61, found 691.24



3-(5-((4-(2,6-dichlorobenzyl)piperazin-1-yl)methyl)-3-(4-(trifluoromethoxy)phenyl)-1H-indol-1-yl)propan-1-amine (Scheme 1, compound 7)

[0223] To a solution of *tert*-butyl 3-(5-((4-(2,6-dichlorobenzyl)piperazin-1-yl)methyl)-3-(4-(trifluoromethoxy)phenyl)-1H-indol-1-yl)propan-1-amine (Scheme 1, compound 6) (2.8 g, 4.05 mmol) in dioxane (80 mL) a solution of HCl (4 M in dioxane) was added (30 mL, 7.50 mmol) and the resulting solution was stirred for 24 hours. Upon completion, the dioxane was removed, and the crude material was re-suspended in methanol and an excess of potassium carbonate was added (about 6 g). The slurry was stirred at room temperature for 1 hour to ensure basification. The potassium carbonate was filtered off, the solution was concentrated and purified by preparative TLC (20% MeOH in DCM) to provide 3-(5-((4-(2,6-dichlorobenzyl)piperazin-1-yl)methyl)-3-(4-(trifluoromethoxy)phenyl)-1H-indol-1-yl)propan-1-amine (Scheme 1, compound 7) (1.5 g, 63% yield).

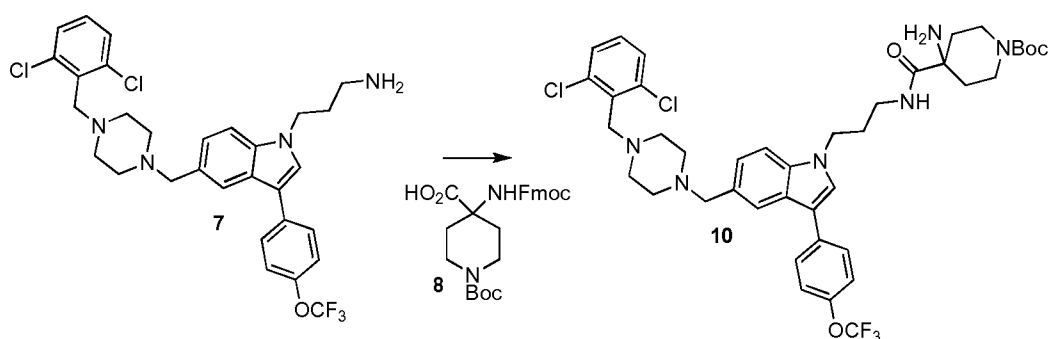
[0224] ^1H NMR (400 MHz, DMSO- d_6) δ 8.21-7.97 (m, 2H), 7.90 (s, 1H), 7.83 (d, J = 8.3 Hz, 2H), 7.66 (d, J = 8.5 Hz, 1H), 7.49-7.29 (m, 6H), 4.37 (t, J = 6.9 Hz, 2H), 3.72 (s, 2H), 2.80 (t, J = 7.5 Hz, 3H), 2.75-2.58 (m, 4H), 2.51 (p, J = 1.9 Hz, 2H), 2.19-2.04 (m, 2H). ^{13}C NMR (101 MHz, DMSO) 146.47, 136.74, 136.36, 134.79, 133.44, 130.38, 128.94, 128.42, 128.18, 125.46, 121.83, 121.76, 114.42, 110.76, 55.68, 51.36, 43.24, 40.40, 40.19, 40.12, 39.98, 39.77, 39.69, 39.56, 39.48, 39.35, 39.27, 39.15, 36.76, 28.12. HRMS (m/z): $[\text{M}^+]$ calcd for $\text{C}_{30}\text{H}_{31}\text{Cl}_2\text{F}_3\text{N}_4\text{O}$, 591.49, found 591.18



4-(((9H-fluoren-9-yl)methoxy)carbonylamino)-1-(tert-butoxycarbonyl)piperidine-4-carboxylic acid (Scheme 1, compound 8)

[0225] A solution of 4-amino-1-(tert-butoxycarbonyl)piperidine-4-carboxylic acid (5 g, 20.5 mmol) in THF (300 mL) and Na_2CO_3 (6.45 g, 61.5 mmol, 3.0 eq in 64.5 mL of water) was cooled to 0°C before the dropwise addition of a solution of Fmoc chloride (5.3 g, 30.7 mmol, 1.5 eq) in THF (30 mL). The resulting mixture was slowly warmed to 25°C and stirred for an additional 12 hours. Upon completion, the reaction contents were carefully acidified with HCl (1 M), and the crude material was extracted with EtOAc (three times). The combined organic layers were dried (Na_2SO_4), concentrated, and the crude material was purified by combiflash 0 to 10% MeOH in DCM to provide 4-(((9H-fluoren-9-yl)methoxy)carbonylamino)-1-(tert-butoxycarbonyl)piperidine-4-carboxylic acid (Scheme 1, compound 8) (4.02 g, 42% yield).

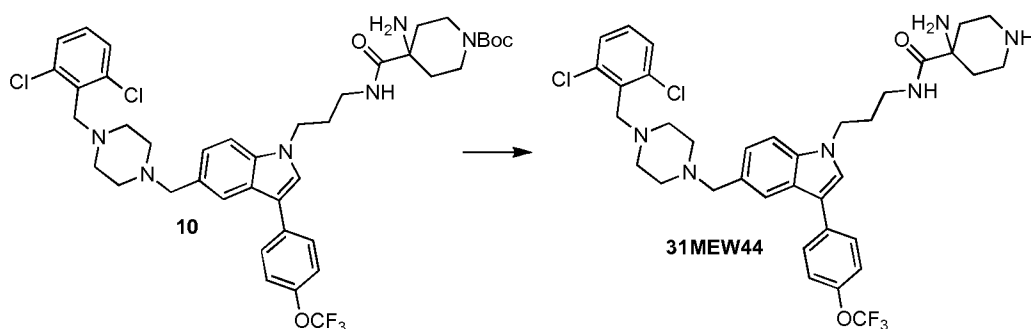
[0226] ^1H NMR (400 MHz, Chloroform- d) δ 7.75 (d, J = 7.5 Hz, 2H), 7.57 (d, J = 7.5 Hz, 2H), 7.45-7.34 (m, 2H), 7.30 (td, J = 6.9, 6.3, 1.4 Hz, 2H), 4.68-4.26 (m, 2H), 4.19 (t, J = 6.5 Hz, 1H), 3.96-3.65 (m, 3H), 3.08 (s, 2H), 1.91-1.77 (m, 2H), 1.48 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) 177.19, 154.73, 143.67, 141.32, 127.72, 127.08, 124.95, 119.97, 80.06, 67.90, 66.86, 57.49, 47.19, 31.98, 28.42, 25.57. HRMS (m/z): $[\text{M}^+]$ calcd for $\text{C}_{26}\text{H}_{30}\text{N}_2\text{O}_6$, 466.53, found 466.2



tert-butyl 4-amino-4-(3-(5-((4-(2,6-dichlorobenzyl)piperazin-1-yl)methyl)-3-(4-(trifluoromethoxy)phenyl)-1H-indol-1-yl)propylcarbamoyl)piperidine-1-carboxylate (Scheme 1, compound 10)

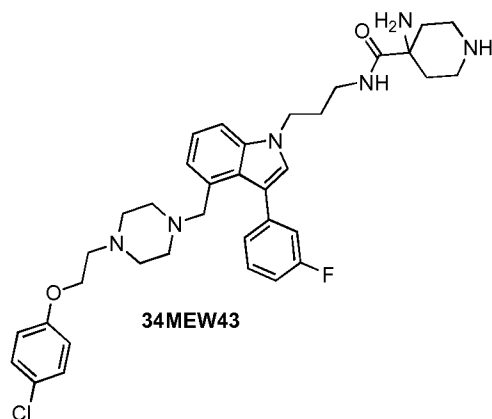
[0227] HBTU (1.16 g, 3.05 mmol, 1.2 eq) was added to a solution of 4-(((9H-fluoren-9-yl)methoxy)carbonylamino)-1-(tert-butoxycarbonyl)piperidine-4-carboxylic acid (Scheme 1, compound 8) (1.42 g, 3.05 mmol, 1.2 eq) and EDIPA (530 μL , 3.05 mmol, 1.2 eq) in DMF (20 mL) at 0°C and stirred for 30 minutes. A solution of 3-(5-((4-(2,6-dichlorobenzyl)piperazin-1-yl)methyl)-3-(4-(trifluoromethoxy)phenyl)-1H-indol-1-yl)propan-1-amine (Scheme 1, compound 7) (1.5 g, 2.54 mmol) in DMF (3 mL) was added and stirred for an additional 6 hours. Upon completion, the reaction was quenched with saturated aqueous NaHCO_3 and extracted 3 times with EtOAc. The combined organic layers were washed with brine, dried (Na_2SO_4), concentrated, and the crude material was purified by combiflash 0 to 5% MeOH in DCM. The slightly

impure material (2.1 g) was suspended in DCM (15 mL) and piperidine (1.2 mL, 12.1 mmol, 6.0 eq) was added and stirred for 24 hours. Upon completion, the reaction was concentrated and purified directly by combiflash 0 to 5% MeOH in DCM, to yield *tert*-butyl 4-amino-4-(3-(5-((4-(2,6-dichlorobenzyl)piperazin-1-yl)methyl)-3-(4-(trifluoromethoxy)phenyl)-1H-indol-1-yl)propylcarbamoyl)piperidine-1-carboxylate (Scheme 1, compound 10) (0.89 g, 43% yield). ^1H NMR (400 MHz, Methanol- d_4) δ 7.84 (d, J = 1.6 Hz, 1H), 7.78-7.69 (m, 2H), 7.55 (s, 1H), 7.42 (d, J = 8.5 Hz, 1H), 7.35-7.24 (m, 4H), 7.23-7.13 (m, 2H), 4.24 (t, J = 6.7 Hz, 2H), 3.78 (dt, J = 13.7, 4.2 Hz, 2H), 3.72 (d, J = 6.3 Hz, 4H), 3.66 (s, 2H), 3.58-3.41 (m, 3H), 3.33 (p, J = 1.7 Hz, 1H), 3.24 (t, J = 6.6 Hz, 2H), 3.14 (s, 2H), 2.69-2.39 (m, 9H), 2.07 (dd, J = 8.2, 5.2 Hz, 3H), 1.97-1.79 (m, 2H), 1.66 (d, J = 5.4 Hz, 1H), 1.55 (dtd, J = 11.3, 5.8, 3.5 Hz, 4H), 1.46 (d, J = 3.4 Hz, 9H), 1.32-1.21 (m, 2H). ^{13}C NMR (101 MHz, MeOD) 177.86, 173.27, 154.90, 154.76, 146.75, 136.51, 136.32, 134.73, 133.47, 128.98, 128.03, 127.82, 127.38, 126.62, 125.83, 123.84, 120.91, 120.53, 114.77, 109.36, 79.48, 62.74, 56.08, 55.57, 55.08, 52.23, 51.84, 48.10, 47.88, 47.67, 47.46, 47.25, 47.05, 47.03, 46.84, 43.69, 36.77, 35.75, 33.61, 29.21, 27.16, 25.76, 24.05. HRMS (m/z): $[\text{M}^+]$ calcd for $\text{C}_{41}\text{H}_{49}\text{Cl}_2\text{F}_3\text{N}_6\text{O}_4$, 817.77, found 817.38



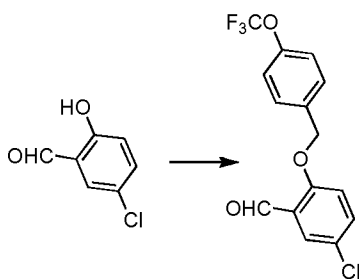
4-amino-N-(3-(5-((4-(2,6-dichlorobenzyl)piperazin-1-yl)methyl)-3-(4-(trifluoromethoxy)phenyl)-1H-indol-1-yl)propyl)piperidine-4-carboxamide:
31MEW44

[0228] *Tert*-butyl 4-amino-4-(3-(5-((4-(2,6-dichlorobenzyl)piperazin-1-yl)methyl)-3-(4-(trifluoromethoxy)phenyl)-1H-indol-1-yl)propylcarbamoyl)piperidine-1-carboxylate (Scheme 1, compound 10) (40 mg, 0.049 mmol) was dissolved in 1,4-dioxane (0.5 mL) before the addition of HCl in 1,4-dioxane (0.1 mL of a 4 M solution). The resulting mixture was stirred for 6 hours at 25°C. Upon completion, the 1,4-dioxane was removed, and the residue was resuspended in MeOH and solid K₂CO₃ (100 mg, XS) was added. The crude material was purified by preparative TLC (15% MeOH in DCM) to provide 31MEW44 (18 mg, 51%). ¹H NMR (400 MHz, *d*₄-MeOD) δ 7.82 (s, 1H), 7.74 (d, *J* = 8.7 Hz, 2H), 7.55 (s, 1H), 7.42 (d, *J* = 8.4 Hz, 1H), 7.34 (d, *J* = 8.0 Hz, 2H), 7.31 (d, *J* = 8.2 Hz, 2H), 7.21 (dd, *J* = 8.5, 7.5 Hz, 2H), 4.26 (t, *J* = 6.7 Hz, 2H), 3.75 (s, 2H), 3.60 (s, 2H), 3.24 (t, *J* = 6.6 Hz, 2H), 2.99-2.89 (m, 4H), 2.60 (brs, 4H), 2.49 (brs, 4H), 2.11-1.97 (m, 4H), 1.35 (brd, *J* = 13.9 Hz, 2H). ¹³C NMR (100 MHz, *d*₄-MeOD) δ 179.4, 148.3, 138.1, 137.8, 136.4, 135.1, 130.5, 129.7, 129.6, 128.1, 127.4, 125.4, 122.5, 121.9, 116.3, 110.8, 64.5, 57.2, 56.2, 53.9, 53.7, 49.8, 45.2, 41.8, 38.3, 34.8, 30.8. HRMS (*m/z*): [M+H]⁺ calcd for C₃₆H₄₂Cl₂F₃N₆O₂, 717.2698, found 717.2675



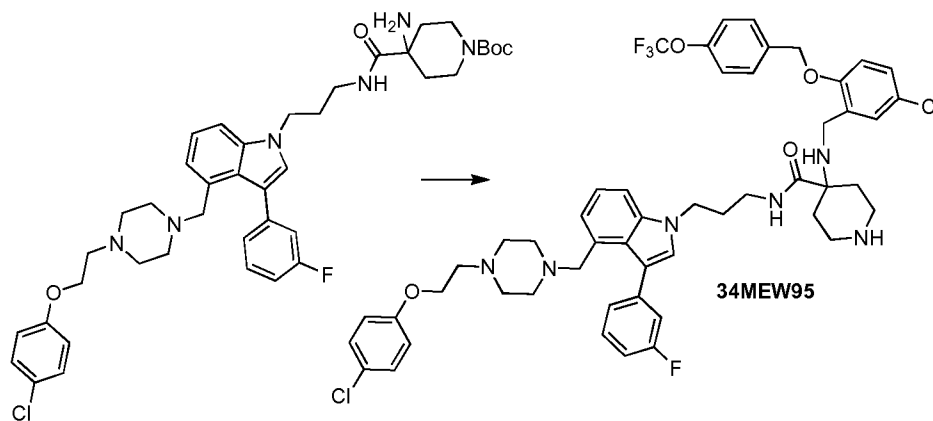
4-amino-N-(3-(4-((4-(2-(4-chlorophenoxy)ethyl)piperazin-1-yl)methyl)-3-(3-fluorophenyl)-1H-indol-1-yl)propyl)piperidine-4-carboxamide: 34MEW43

[0229] The compound was prepared according to the protocols for 31MEW44. 1H-indole-4-carbaldehyde was used in place of compound 1, step a in scheme 1. For step c, 3-fluorophenyl boronic acid was used in place of 4-trifluoromethoxy phenyl boronic acid. For step d, 4-chlorophenyl 2-bromo ether was used in place of 2,6-dichlorobenzylbromide, the resulting product was then used in step e. The subsequent steps are identical to the synthesis of 31MEW44. ¹H NMR (400 MHz, Methanol-*d*₄) δ 7.44 (dd, *J* = 8.4, 1.0 Hz, 1H), 7.38 (td, *J* = 8.0, 6.1 Hz, 1H), 7.34-7.22 (m, 5H), 7.22-7.15 (m, 1H), 7.08-6.98 (m, 2H), 6.94-6.87 (m, 2H), 4.34-4.21 (m, 2H), 4.06 (t, *J* = 5.5 Hz, 2H), 3.56 (s, 2H), 2.73 (t, *J* = 5.5 Hz, 2H), 2.43 (s, 3H), 2.25-2.08 (m, 7H). ¹³C NMR (101 MHz, MeOD) 177.31, 163.55, 161.13, 136.91, 128.94, 127.83, 126.24, 125.39, 122.16, 121.08, 116.98, 116.77, 115.65, 112.55, 109.28, 59.49, 56.42, 53.71, 52.66, 51.31, 43.53, 39.68, 36.94, 31.10, 29.33. HRMS (*m/z*): [M⁺] calcd for C₃₆H₄₄ClFN₆O₂, 647.22, found 647.34



5-chloro-2-(4-(trifluoromethoxy)benzyloxy)benzaldehyde

[0230] To a solution of 5-chlorosalicylaldehyde (commercially available from Sigma, St. Louis, MO) (82 mg, 0.56 mmol) in DMF (1 mL), K_2CO_3 (87 mg, 0.63 mmol, 1.2 eq) was added and stirred for 10 minutes at 25°C before the addition of 4-(trifluoromethoxy)benzyl bromide (commercially available from Sigma) (126 μ L, 0.788 mmol, 1.4 eq). The resulting mixture was stirred at 25°C for 12 hours. Upon completion, the reaction contents were diluted with saturated $NaHCO_3$ and extracted with EtOAc (three times). The combined organic layers were washed once with brine, dried (Na_2SO_4), concentrated and purified by combiflash 0 to 30% EtOAc to provide 5-chloro-2-(4-(trifluoromethoxy)benzyloxy)benzaldehyde (99 mg, 53% yield). ^1H NMR (400 MHz, Chloroform- d) δ 10.49 (s, 1H), 7.84 (d, J = 2.8 Hz, 1H), 7.55-7.45 (m, 3H), 7.34-7.23 (m, 3H), 7.01 (d, J = 8.9 Hz, 1H), 5.20 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) 188.13, 135.38, 128.78, 128.31, 121.32, 114.54, 70.03. HRMS (m/z): $[M^+]$ calcd for $C_{15}H_{10}ClF_3O_3$, 330.69, found 329.02



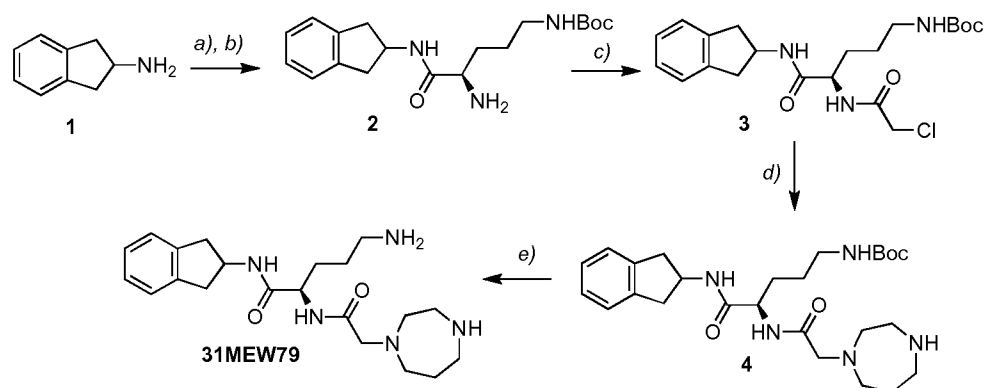
4-(5-chloro-2-(4-(trifluoromethoxy)benzyloxy)benzylamino)-N-(3-(4-((4-(2-(4-chlorophenoxy)ethyl)piperazin-1-yl)methyl)-3-(3-fluorophenyl)-1H-indol-1-yl)propyl)piperidine-4-carboxamide: 34MEW95

[0231] 4-amino-4-(3-(4-((4-(2-(4-chlorophenoxy)ethyl)piperazin-1-yl)methyl)-3-(3-fluorophenyl)-1H-indol-1-yl)propylcarbamoyl)piperidine-1-carboxylate was synthesized using the methods described for 31MEW44 with modifications. 1H-indole-4-carbaldehyde was used in place of compound 1, step a in scheme 1. For step c, 3-fluorophenyl boronic acid was used in place of 4-trifluoromethoxy phenyl boronic acid. For step d, 4-chlorophenyl 2-bromo ether was used in place of 2,6-dichlorobenzylbromide, the resulting product was then used in step e. The subsequent steps are identical to the synthesis of 31MEW44.

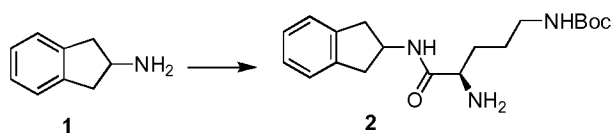
[0232] To a solution of *tert*-butyl 4-amino-4-(3-(4-((4-(2-(4-chlorophenoxy)ethyl)piperazin-1-yl)methyl)-3-(3-fluorophenyl)-1H-indol-1-yl)propylcarbamoyl)piperidine-1-carboxylate (29 mg, 0.0443 mmol) in DCE (1 mL), 5-chloro-2-(4-(trifluoromethoxy)benzyloxy)benzaldehyde (44 mg, 0.133 mmol, 3.0 eq), and MgSO₄ (10 mg) were added and stirred at 40°C for 1 hour prior to the addition of sodium triacetoxyborohydride (19 mg, 0.0886 mmol, 2.0 eq). The resulting mixture was stirred for an additional 8 hours at 40°C before being concentrated and purified directly by preparative TLC (2% MeOH

in DCM). The Boc group of the product was then removed (using the protocol for 31MEW44) to provide 34MEW95 (14 mg, 33% yield over two steps). ^1H NMR (400 MHz, Methanol- d_4) δ 7.41-7.29 (m, 5H), 7.29-7.13 (m, 8H), 7.12-6.97 (m, 4H), 6.92-6.86 (m, 2H), 5.71 (s, 1H), 4.95 (q, J = 11.1 Hz, 2H), 4.14 (q, J = 7.1 Hz, 2H), 4.05 (t, J = 5.5 Hz, 2H), 3.53 (s, 2H), 3.44 (ddd, J = 14.7, 9.1, 6.3 Hz, 1H), 3.16 (dtd, J = 27.2, 13.1, 11.7, 4.3 Hz, 3H), 3.00-2.80 (m, 2H), 2.72 (t, J = 5.5 Hz, 2H), 2.09-1.79 (m, 5H), 1.71 (d, J = 14.1 Hz, 1H). ^{13}C NMR (101 MHz, MeOD) 175.98, 157.45, 155.76, 136.79, 135.21, 130.37, 130.19, 129.73, 129.32, 128.89, 128.77, 128.68, 127.46, 126.16, 126.14, 126.01, 125.37, 125.29, 122.16, 121.13, 120.82, 116.95, 116.91, 116.74, 115.62, 113.99, 112.43, 112.22, 108.87, 69.47, 65.27, 59.72, 58.80, 56.68, 52.97, 51.76, 43.26, 40.50, 40.39, 38.38, 31.20, 29.71, 27.33. HRMS (m/z): $[M]^+$ calcd for $\text{C}_{51}\text{H}_{54}\text{Cl}_2\text{F}_4\text{N}_6\text{O}_4$, 961.61, found 961.3

Synthesis of pharmacophore compounds

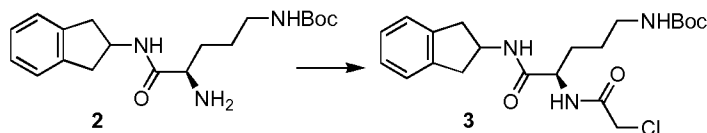


Scheme 2 - synthesis of 31MEW79: a) (R)-2-amino-5-(tert-butoxycarbonylamino)pentanoic acid (1.0 eq), EDIPA (1.2 eq), HBTU (1.2 eq), 0°C THF, 30 min, then 2-aminoindan (1.5 eq), 25°C, 12 hr; b) piperidine (4.0 eq), 25°C, THF, 24 hr; c) EDIPA (1.1 eq), chloroacetyl chloride (1.1 eq), 0°C, THF, 6 hr; d) homopiperazine (6.0 eq), 0°C to 25°C, THF, 12hr; e) TFA (XS)



(R)-*tert*-butyl 4-amino-5-(2,3-dihydro-1*H*-inden-2-ylamino)-5-oxopentylcarbamate (Scheme 2, compound 2)

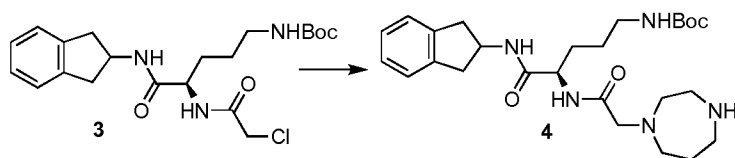
[0233] To a solution of *(R)*-2-amino-5-(*tert*-butoxycarbonylamino)pentanoic acid (1.1 g, 2.42 mmol) and EDIPA (0.51 mL, 2.9 mmol, 1.2 eq) at 0°C in THF (30 mL), HBTU (1.1 g, 2.9 mmol, 1.2 eq) was added. After 30 minutes of stirring 2-aminoindan (0.48 mL, 3.63 mmol, 1.5 eq) was added and the resulting mixture was slowly warmed to 25°C and stirred for an additional 12 hours. After consumption of *(R)*-2-amino-5-(*tert*-butoxycarbonylamino) pentanoic acid, piperidine (0.95 mL, 9.68 mmol, 4.0 eq) was added to the crude reaction mixture and stirred for an additional 12 hours. Upon completion, the contents of the reaction were concentrated and purified by combiflash 0 to 20% MeOH in DCM to provide *(R)*-*tert*-butyl 4-amino-5-(2,3-dihydro-1*H*-inden-2-ylamino)-5-oxopentylcarbamate (Scheme 2, compound 2) (0.5 g, 59% yield). ¹H NMR (400 MHz, Methanol-d₄) δ 7.35-7.13 (m, 4H), 4.66 (s, 1H), 3.46-2.67 (m, 6H), 1.89-1.51 (m, 2H), 1.44 (s, 9H). ¹³C NMR (101 MHz, MeOD) 157.16, 140.71, 140.68, 127.04, 126.41, 124.52, 124.24, 78.61, 39.59, 39.12, 38.97, 37.88, 27.47. HRMS (m/z): [M⁺] calcd for C₁₉H₂₉N₃O₃, 347.45, found 348.2



(R)-*tert*-butyl 4-(2-chloroethanamido)-5-(2,3-dihydro-1*H*-inden-2-ylamino)-5-oxopentylcarbamate (Scheme 2, compound 3)

[0234] To a solution of *(R)*-*tert*-butyl 4-amino-5-(2,3-dihydro-1*H*-inden-2-ylamino)-5-oxopentylcarbamate (Scheme 2, compound 2) (0.5 g, 1.43 mmol) and EDIPA (0.25 mL, 1.58 mmol, 1.1 eq) at 0°C in THF (30 mL) a solution of chloroacetyl chloride (0.126 mL, 1.58 mmol, 1.1 eq) in THF (3 mL)

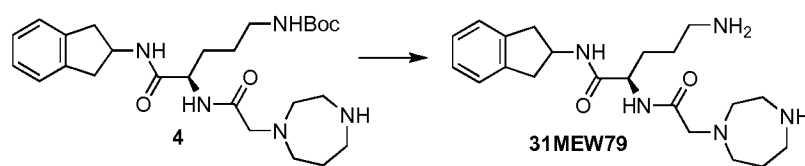
was added slowly dropwise. The resulting mixture was slowly warmed to 25°C and stirred for an additional 6 hours. Upon completion, the reaction was diluted with saturated aqueous NaHCO₃ and extracted 3 times with ethyl acetate. The combined organic layers were dried (Na₂SO₄), concentrated, and the crude material was purified by combiflash 0 to 10% MeOH in DCM to provide (*R*)-*tert*-butyl 4-(2-chloroethanamido)-5-(2,3-dihydro-1*H*-inden-2-ylamino)-5-oxopentylcarbamate (Scheme 2, compound 3) (0.13 g, 0.306 mmol, 21% yield). ¹H NMR (400 MHz, chloroform-*d*) δ 7.46 (d, *J* = 8.3 Hz, 1H), 7.27-7.11 (m, 5H), 4.89 (t, *J* = 6.2 Hz, 1H), 4.69 (dt, *J* = 7.7, 5.4 Hz, 1H), 4.64-4.52 (m, 1H), 4.06-3.78 (m, 2H), 3.29 (tt, *J* = 12.2, 6.8 Hz, 3H), 3.06 (dd, *J* = 13.5, 6.4 Hz, 1H), 2.83 (dt, *J* = 16.0, 6.3 Hz, 2H), 1.90-1.75 (m, 1H), 1.67 (dd, *J* = 14.1, 7.1 Hz, 1H), 1.50 (p, *J* = 7.0 Hz, 2H), 1.40 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) 170.89, 166.25, 156.48, 140.73, 140.68, 126.79, 126.76, 124.71, 124.64, 79.29, 52.23, 50.55, 42.38, 39.85, 39.77, 39.36, 30.56, 28.41, 26.27. HRMS (*m/z*): [*M*+]⁺ calcd for C₂₁H₃₀ClN₃O₄, 423.93, found 424.20



(*R*)-*tert*-butyl 4-(2-(1,4-diazepan-1-yl)ethanamido)-5-(2,3-dihydro-1*H*-inden-2-ylamino)-5-oxopentylcarbamate (Scheme 2, compound 4)

[0235] To a solution of homopiperazine (92.1 mg, 0.920 mmol, 6.0 eq) in THF at 0°C, a solution of (*R*)-*tert*-butyl 4-(2-chloroethanamido)-5-(2,3-dihydro-1*H*-inden-2-ylamino)-5-oxopentylcarbamate (Scheme 2, compound 3) (0.153 mmol) in THF (3 mL) was added. The resulting mixture was slowly warmed to 25°C and stirred for 24 hours. Upon completion, the reaction contents were concentrated and purified by combiflash 0 to 30% MeOH to

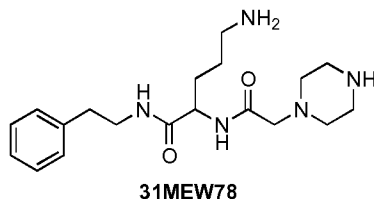
provide (*R*)-*tert*-butyl 4-(2-(1,4-diazepan-1-yl)ethanamido)-5-(2,3-dihydro-1*H*-inden-2-ylamino)-5-oxopentylcarbamate (Scheme 2, compound 4) (70 mg, 93% yield). ¹H NMR (400 MHz, Methanol-*d*₄) δ 7.22 (dd, *J* = 5.4, 3.4 Hz, 2H), 7.16 (dt, *J* = 5.1, 3.7 Hz, 2H), 4.60 (tt, *J* = 7.4, 5.6 Hz, 1H), 4.40 (dd, *J* = 8.2, 5.6 Hz, 1H), 3.60-3.47 (m, 1H), 3.33 (q, *J* = 1.6 Hz, 3H), 3.31-3.18 (m, 4H), 3.06 (td, *J* = 6.9, 2.1 Hz, 2H), 3.03-2.92 (m, 8H), 2.92-2.82 (m, 3H), 2.78 (dt, *J* = 10.0, 5.6 Hz, 4H), 1.95-1.73 (m, 4H), 1.73-1.62 (m, 1H), 1.56-1.47 (m, 2H), 1.44 (s, 9H). ¹³C NMR (101 MHz, MeOD) 172.28, 171.92, 140.70, 140.67, 126.36, 124.19, 61.52, 56.63, 55.08, 52.33, 50.64, 48.94, 39.03, 38.83, 30.62, 29.96, 29.13, 27.40, 25.95. HRMS (*m/z*): [*M*+]⁺ calcd for C₂₆H₄₁N₅O₄, 487.63, found 488.32



(*R*)-2-(2-(1,4-diazepan-1-yl)ethanamido)-5-amino-*N*-(2,3-dihydro-1*H*-inden-2-yl)pentanamide: 31MEW79

[0236] (*R*)-*tert*-butyl 4-(2-(1,4-diazepan-1-yl)ethanamido)-5-(2,3-dihydro-1*H*-inden-2-ylamino)-5-oxopentylcarbamate (Scheme 2, compound 4) (50 mg, 0.103 mmol) was dissolved in TFA (0.5 mL) and stirred for 12 hours at 25°C. Upon completion, the TFA was removed and the residue was re-suspended in MeOH and solid K₂CO₃ (100 mg, XS) was added. The crude material was purified by preparative TLC (15% MeOH in DCM) to provide 31MEW79 (28 mg, 70% yield). ¹H NMR (400 MHz, Methanol-*d*₄) δ 7.22 (dt, *J* = 7.6, 3.6 Hz, 2H), 7.19-7.09 (m, 2H), 4.59 (tt, *J* = 7.3, 5.6 Hz, 1H), 4.43 (dd, *J* = 8.1, 5.3 Hz, 1H), 3.31-3.17 (m, 4H), 2.97 (dt, *J* = 8.4, 6.4 Hz, 4H), 2.93-2.72 (m, 4H), 2.04 (p, *J* = 5.9 Hz, 2H), 1.92-1.81 (m, 1H), 1.74 (tdd, *J* = 15.0, 8.8,

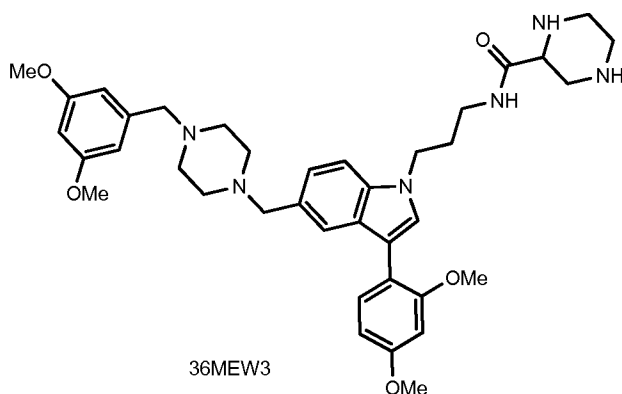
5.5 Hz, 3H). ^{13}C NMR (101 MHz, MeOD) 126.39, 124.18, 61.00, 54.63, 52.05, 50.75, 45.88, 44.56, 38.92, 38.81, 29.38, 25.46, 23.57. HRMS (m/z): $[\text{M}^+]$ calcd for $\text{C}_{21}\text{H}_{33}\text{N}_5\text{O}_2$, 387.52, found 388.27



4-amino-N-(3-(4-((4-(2-(4-chlorophenoxy)ethyl)piperazin-1-yl)methyl)-3-(3-fluorophenyl)-1H-indol-1-yl)propyl)piperidine-4-carboxamide: 31MEW78

[0237] This compound was prepared using the protocols for 31MEW79.

^1H NMR (400 MHz, Methanol- d_4) δ 7.34-7.15 (m, 4H), 4.40 (dd, J = 8.2, 5.0 Hz, 1H), 3.54-3.37 (m, 2H), 3.31 (dd, J = 10.3, 5.2 Hz, 4H), 3.19 (q, J = 16.0 Hz, 2H), 2.94 (td, J = 6.9, 3.2 Hz, 2H), 2.80 (q, J = 6.7, 6.1 Hz, 5H), 1.92-1.78 (m, 1H), 1.69 (dq, J = 19.4, 8.1, 6.5 Hz, 2H). ^{13}C NMR (101 MHz, MeOD) 138.91, 128.47, 128.11, 126.01, 60.06, 52.30, 49.46, 43.21, 40.51, 38.78, 34.94, 29.02, 23.51. HRMS (m/z): $[\text{M}^+]$ calcd for $\text{C}_{19}\text{H}_{31}\text{N}_5\text{O}_2$, 361.48, found 362.26.



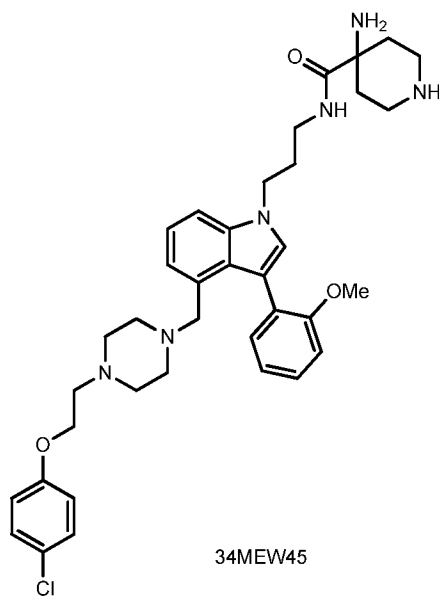
N-(3-(5-((4-(3,5-dimethoxybenzyl)piperazin-1-yl)methyl)-3-(2,4-dimethoxyphenyl)-1H-indol-1-yl)propyl)piperazine-2-carboxamide: 36MEW3

[0238] 36MEW3 was synthesized according to the same procedure as

31MEW44 substituting the appropriate commercially available reagents.

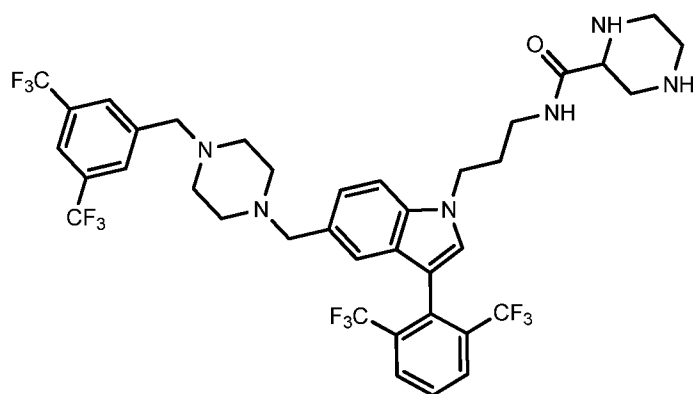
Nitrogen protected carboxy piperizne was substituted for compound 8. ^1H

NMR (400 MHz, Methanol- d_4) δ 7.62 (s, 1H), 7.50 – 7.40 (m, 3H), 7.20 (d, J = 9.8 Hz, 1H), 6.68 (d, J = 2.5 Hz, 1H), 6.64 (dd, J = 8.3, 2.5 Hz, 1H), 6.52 (d, J = 2.3 Hz, 2H), 6.40 (t, J = 2.3 Hz, 1H), 4.55 (s, 3H), 4.29 (t, J = 6.8 Hz, 2H), 3.87 (s, 3H), 3.82 (s, 3H), 3.77 (s, 6H), 3.73 (s, 2H), 3.53 – 3.48 (m, 3H), 3.16 (p, J = 1.7 Hz, 1H), 2.77 – 2.48 (m, 11H), 2.36 (d, J = 7.5 Hz, 1H), 2.11 (t, J = 6.7 Hz, 2H), 0.95 – 0.84 (m, 4H).



4-amino-N-(3-(4-((4-(2-(4-chlorophenoxy)ethyl)piperazin-1-yl)methyl)-3-(2-methoxyphenyl)-1H-indol-1-yl)propyl)piperidine-4-carboxamide: 34MEW45
[0239] 34MEW45 was synthesized according to the same procedure as

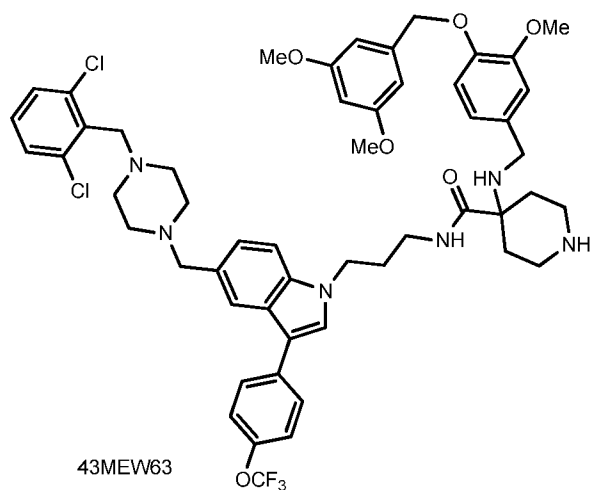
34MEW95 substituting the appropriate commercially available reagents. ^1H NMR (400 MHz, Methanol- d_4) δ 7.66 (d, J = 1.5 Hz, 1H), 7.58 (dd, J = 7.5, 1.7 Hz, 1H), 7.54 (s, 1H), 7.46 (d, J = 8.4 Hz, 1H), 7.32 – 7.20 (m, 4H), 7.11 (dd, J = 8.3, 1.1 Hz, 1H), 7.05 (td, J = 7.5, 1.2 Hz, 1H), 6.96 – 6.88 (m, 2H), 4.32 (t, J = 6.6 Hz, 2H), 4.12 (t, J = 5.5 Hz, 2H), 3.86 (s, 3H), 3.71 (s, 2H), 3.25 – 3.17 (m, 4H), 2.83 (t, J = 5.5 Hz, 2H), 2.66 (s, 7H), 2.20 – 2.07 (m, 4H), 1.49 (d, J = 14.4 Hz, 2H).



32MEW56

N-(3-(5-((4-(3,5-bis(trifluoromethyl)benzyl)piperazin-1-yl)methyl)-3-(2,6-bis(trifluoromethyl)phenyl)-1H-indol-1-yl)propyl)piperazine-2-carboxamide:
32MEW56

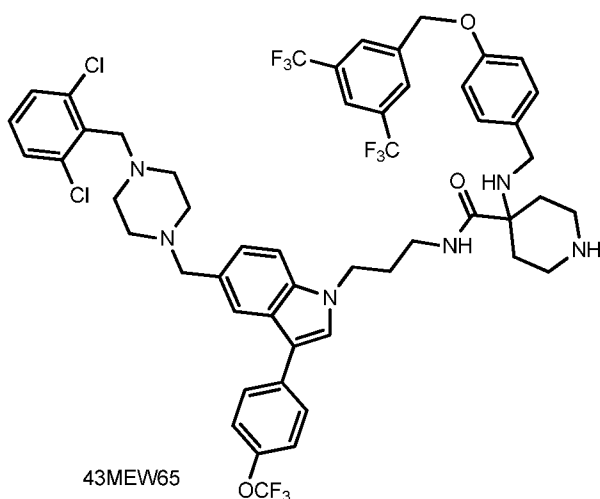
[0240] 32MEW56 was synthesized according to the same procedure as 31MEW44 substituting the appropriate commercially available reagents. Nitrogen protected carboxy piperizne was substituted for compound 8. ^1H NMR (400 MHz, Methanol- d_4) δ 8.23 (d, J = 1.6 Hz, 1H), 7.97 (s, 1H), 7.93 – 7.83 (m, 2H), 7.56 (d, J = 8.5 Hz, 1H), 7.32 (dd, J = 8.5, 1.5 Hz, 1H), 4.35 (t, J = 6.8 Hz, 1H), 3.86 (s, 1H), 3.73 (s, 1H), 3.61 – 3.46 (m, 1H), 3.16 – 2.83 (m, 3H), 2.67 (d, J = 56.9 Hz, 4H), 2.15 (t, J = 6.8 Hz, 1H).



43MEW63

N-(3-(5-((4-(2,6-dichlorobenzyl)piperazin-1-yl)methyl)-3-(4-(trifluoromethoxy)phenyl)-1*H*-indol-1-yl)propyl)-4-((4-((3,5-dimethoxybenzyl)oxy)-3-methoxybenzyl)amino)piperidine-4-carboxamide: 43MEW63

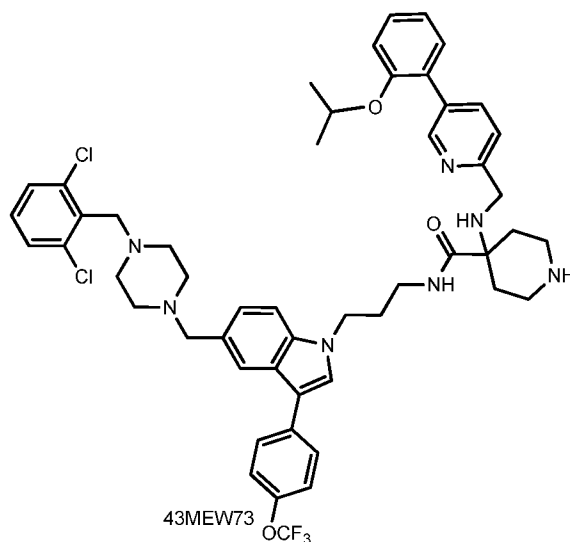
[0241] 43MEW63 was synthesized using the same procedures as 34MEW95 substituting the appropriate commercially available reagents. ¹H NMR (400 MHz, Methanol-*d*₄) δ 7.84 (s, 1H), 7.74 (d, *J* = 8.7 Hz, 2H), 7.49 (s, 1H), 7.43 – 7.29 (m, 5H), 7.29 – 7.17 (m, 2H), 6.96 (s, 1H), 6.90 – 6.80 (m, 2H), 6.59 (d, *J* = 2.3 Hz, 2H), 6.40 (d, *J* = 2.4 Hz, 1H), 5.00 (s, 2H), 4.25 (t, *J* = 6.9 Hz, 2H), 3.81 (s, 3H), 3.79 (s, 2H), 3.75 (s, 6 H), 3.62 (s, 2H), 3.53 – 3.47 (m, 3H), 3.19 – 2.91 (m, 6H), 2.63 (s, 4H), 2.50 (s, 4H), 2.16 – 1.96 (m, 5H), 1.75 (d, *J* = 14.2 Hz, 2H).



4-((4-((3,5-bis(trifluoromethyl)benzyl)oxy)benzyl)amino)-*N*-(3-(5-((4-(2,6-dichlorobenzyl)piperazin-1-yl)methyl)-3-(4-(trifluoromethoxy)phenyl)-1*H*-indol-1-yl)propyl)piperidine-4-carboxamide: 43MEW65

[0242] 43MEW65 was synthesized using the same procedures as 34MEW95 substituting the appropriate commercially available reagents. ¹H NMR (400 MHz, Methanol-*d*₄) δ 8.03 (s, 2H), 7.93 (s, 1H), 7.86 – 7.81 (m, 1H), 7.77 – 7.70 (m, 2H), 7.51 (s, 1H), 7.43 (d, *J* = 8.5 Hz, 1H), 7.40 – 7.34 (m, 2H), 7.26 (dddd, *J* = 23.7, 16.0, 8.2, 1.3 Hz, 7H), 7.00 – 6.93 (m, 2H), 5.20 (s, 2H), 4.27 (t, *J* = 6.8 Hz, 2H), 3.78 (s, 2H), 3.62 (s, 2H), 3.48 (s, 3H), 3.11 –

2.99 (m, 2H), 2.99 – 2.89 (m, 2H), 2.63 (s, 4H), 2.50 (s, 4H), 2.11 (t, $J = 6.7$ Hz, 2H), 1.99 (ddd, $J = 13.9, 9.7, 4.0$ Hz, 2H), 1.70 (d, $J = 14.5$ Hz, 2H).



N-(3-(5-((4-(2,6-dichlorobenzyl)piperazin-1-yl)methyl)-3-(4-(trifluoromethoxy)phenyl)-1H-indol-1-yl)propyl)-4-(((5-(2-isopropoxyphenyl)pyridin-2-yl)methyl)amino)piperidine-4-carboxamide:
43MEW73

[0243] 43MEW73 was synthesized using the same procedures as 34MEW95 substituting the appropriate commercially available reagents. ^1H NMR (400 MHz, Methanol- d_4) δ 8.65 (d, $J = 2.2$ Hz, 1H), 7.91 (dd, $J = 8.1, 2.2$ Hz, 1H), 7.85 (s, 1H), 7.76 – 7.69 (m, 2H), 7.53 (s, 1H), 7.47 (d, $J = 8.0$ Hz, 1H), 7.42 – 7.33 (m, 4H), 7.33 – 7.22 (m, 4H), 7.21 – 6.99 (m, 4H), 4.59 (p, $J = 6.3$ Hz, 1H), 4.30 (t, $J = 6.9$ Hz, 2H), 3.79 (d, $J = 7.6$ Hz, 4H), 3.69 (s, 2H), 2.61 (d, $J = 29.0$ Hz, 8H), 2.28 – 2.08 (m, 4H), 1.94 (d, $J = 15.8$ Hz, 3H), 1.31 – 1.21 (m, 3H), 1.20 (d, $J = 6.0$ Hz, 5H).

4-((3-chloro-4-((4-(2-fluorobenzyl)oxy)benzyl)amino)-N-(3-(4-((4-(2-(4-chlorophenoxy)ethyl)piperazin-1-yl)methyl)-3-(3-fluorophenyl)-1H-indol-1-yl)propyl)piperidine-4-carboxamide: 35MEW12

[0244] 35MEW12 was synthesized according to the same procedures as 34MEW95 substituting the appropriate commercially available reagents. ^1H NMR (400 MHz, Methanol- d_4) δ 7.51 – 7.41 (m, 3H), 7.41 – 7.28 (m, 3H), 7.28 – 7.20 (m, 4H), 7.20 – 7.13 (m, 2H), 7.13 – 7.06 (m, 2H), 7.06 – 6.98 (m,

3H), 6.93 – 6.86 (m, 2H), 5.11 (s, 2H), 4.26 (t, $J = 6.9$ Hz, 2H), 4.06 (t, $J = 5.5$ Hz, 2H), 3.56 – 3.49 (m, 4H), 3.33 – 3.08 (m, 10H), 2.72 (t, $J = 5.5$ Hz, 2H), 2.41 (s, 3H), 2.12 (td, $J = 15.9, 13.5, 7.6$ Hz, 8H), 1.89 (d, $J = 14.7$ Hz, 2H).

Example 3

Results

[0245] The majority of cellular responses that oncogenic RAS proteins elicit are transduced by the effectors RAF, RALGDS, and PI3K (Downward *et al.*, 2003). The analysis of the co-crystal structures of HRAS with PI3K γ (PDB: 1HE8), with the C-RAF RAS-binding domain (RBD, PDB: 3KUD), and with RALGDS (PDB: 1LFD) revealed conserved interactions between these effector proteins and a short stretch of amino acids on the switch 1 region (residues 36-39) of the RAS proteins, which are referred to as the D38 site (FIGS. 1A-L). For example, I36 on RAS undergoes a conformational change when transitioning from the GDP-bound to the GTP-bound form (FIGS. 1G-H) in which it adopts a solvent-exposed conformation (Hall *et al.*, 2002). In this active form, each of the effector proteins has a complementary hydrophobic residue for interacting with I36, providing a favorable entropic gain. Mutation of D38A on HRAS completely prevents HRAS-mediated activation of these three effectors (Pacold *et al.*, 2000). Similarly, mutation of the residue in the effector domain interacting with D38 on HRAS proteins results in the affinity being greatly diminished or completely eliminated (Pacold *et al.*, 2000, Huang *et al.*, 1998, Block *et al.*, 1996). The sensitivity of this region to alterations in residues involved in effector binding suggests that it would be an ideal site to target, from a functional perspective, with small molecules. Additionally, the conformational changes adopted by this region may allow one to target RAS

proteins selectively in their active state; this may be relevant as some small GTPases have been shown to transmit different signals in the GDP-bound state (Ho *et al.*, 2008).

[0246] This goal was pursued using a *pharmacophore* strategy to test the notion that this site represents a viable pharmacological target; a pharmacophore is the spatial orientation of chemical features (hydrophobic regions, hydrogen bond donors and acceptors, cations, anions) that confer upon a small molecule its biological activity (Sun *et al.*, 2008). Pharmacophore screening is an approach used primarily for lead optimization of small molecules; this involves construction of a model based on bioactive ligands and subsequent screening of virtual collections of molecules for their ability to match the pharmacophore features (Id.). Extending this strategy to lead discovery for protein-protein interactions (PPIs) was attempted by mapping key interactions at the interface of the RAS-effector complexes and building a pharmacophore model that captures these interactions (FIGS. 1A-L, FIG. 2). Recognizing that the potential affinity of ligands to this site might ultimately be limited by its shallow nature, it was then sought to improve affinity of ligands to RAS proteins by designing compounds that extend into two adjacent shallow pockets near the D38 site, creating multivalent small molecule ligands.

Example 4

Pharmacophore virtual screening yields compounds that bind to RAS proteins

[0247] A pharmacophore model was constructed (using R.O.C.S., Openeye, Inc.) based on the residues on effector proteins that interact with

the side chains and amide backbones of S39, D38, E37 and I36 on the human HRAS protein in the co-crystal structures (FIGS. 1A-C, FIG. 2). The notion that a compound from readily available sources could meet the requirements of this pharmacophore model was then tested by screening a compound database of 4.7 million small molecules from the inventory of seven different chemical vendors; this yielded only relatively modest matches based on Tanimoto coefficient (about 0.8 maximum), confirming the suspicion that pre-assembled libraries of compounds would be insufficient for inhibiting RAS proteins. In order to identify compounds with a closer resemblance to the pharmacophore model, custom in silico libraries of synthetically accessible compounds (using Molecular Operating Environment (MOE) software, Chemical Computing Group, Inc.) specifically designed to match the model were created (FIG. 3).

[0248] A set of 45 of the closest matches (ranging from 0.85-0.97 Tanimoto coefficient) were synthesized (based on Scheme 2 with appropriate modifications) and tested for their ability to bind to KRAS^{G12D} by differential scanning fluorimetry. Compounds 31MEW78 and 31MEW79 (FIG. 4) both elicited a dose-dependent thermal shift in KRAS^{G12D} stability (FIG. 5). In order to quantify the binding of the pharmacophore-derived compounds, GTP-loaded KRAS G12D was labeled with NT-647-maleimide and the binding to 31MEW79 was measured using microscale thermophoresis, which analyzes changes in the migration of macromolecules across a temperature gradient in the presence of small molecule ligands (Zheng *et al.*, 2013). To account for the possibility of binding in the GTP pocket, 31MEW79 and 31MEW78 were tested in a nucleotide displacement assay with BODIPY-GTP. No

displacement of the BODIPY nucleotide in the presence of either inhibitor was observed (FIG. 6). Microscale thermophoresis measurements on 31MEW79 yielded a calculated dissociation constant K_D of $33 \mu\text{M} \pm 6 \mu\text{M}$ (FIG. 7). To support the binding of 31MEW79 to the D38 site on KRAS, mutations in the predicted binding region were generated - KRAS^{G12D D38A} and KRAS^{G12D I36N}. This yielded a 1.5 to 3.0 fold loss in affinity: KRAS^{G12D D38A} $K_D = 83 \mu\text{M} \pm 11 \mu\text{M}$ and KRAS^{G12D I36N} $K_D = 47 \mu\text{M} \pm 9 \mu\text{M}$. In order to test the ability of the compounds to prevent the interaction of KRAS^{G12D} protein with its effector CRAF, an *in vitro* pull-down with GTP-loaded KRAS^{G12D} was performed using CRAF RBD-GST, with glutathione beads (FIG. 8). A dose-dependent decrease in CRAF-bound KRAS^{G12D} was observed for 31MEW79 and 31MEW78, indicating abrogation of this interaction.

[0249] To further investigate the binding site of these compounds with KRAS, 2D ¹H-¹⁵N Heteronuclear Single Quantum Coherence (2D ¹H-¹⁵N HSQC) experiments were performed on uniformly ¹⁵N-labeled KRAS^{G12D} protein, with and without test compounds. The ¹H-¹⁵N HSQC spectra of the KRAS^{G12D} GDP-loaded protein and GppNHp-loaded protein were assigned using the previously published wild-type KRAS GDP assignments (Vo *et al.*, 2013). To verify these assignments, 3D-¹H-¹⁵N-¹H-NOESY-HSQC and 3D-¹H-¹⁵N-¹H-TOCSY-HSQC experiments were performed on KRAS^{G12D} protein loaded with either GDP or GppNHp (FIGS. 9A-B). When ¹⁵N-labeled KRAS^{G12D} protein was treated with 31MEW79, significant chemical shifts corresponding to the side chains of S39, E37, and I36 were observed, supporting the notion that binding occurs in the region that the compounds had been designed to interact with (FIG. 10).

Example 5

Computational design of three-site compounds

[0250] While this pharmacophore strategy yielded compounds capable of binding to RAS proteins and inhibiting their interaction with the C-RAF RBD *in vitro*, they did so with only moderate affinity, similar to previous attempts to identify small molecule ligands for RAS proteins (Maurer *et al.*, 2012, Shima *et al.*, 2013, Ostrem *et al.*, 2013), highlighting the limits of single-site binding compounds. A strategy was thus sought for increasing small molecule ligand affinity. Analysis of the KRAS^{G12D} (PDB: 4DSN) structure revealed two shallow pockets directly adjacent to the D38 site (FIGS. 11A-B). One site, centered on A59, is located between the switch I and switch II regions (A59 site). On the other side of the D38 site, there exists a pocket present only in the active form of RAS, when Y32 undergoes a conformational change in which it flips over to the other end of the nucleotide-binding site and forms a hydrogen bond with the gamma phosphate of GTP. This change unveils a pocket (termed the Y32 site) that is not present in the GDP-bound form, allowing selective targeting of the active form (FIG. 11C-E).

[0251] In order to produce compounds with improved affinity, a computational fragment-based approach to the design of multivalent ligands was adopted that could span the three shallow pockets described above; it was sought to extend from the D38 site to the adjacent A59 and Y32 sites. A library composed of 60,000 fragments filtered for lead-like properties (see Example 1) was docked (using Glide, Schrodinger Inc.) into each of these sites. Among the top-ranked fragments for the D38 site, a substantial number of aliphatic rings that contained protonated amines making electrostatic

interactions with D38 and D33 was observed (FIG. 12A). Several of the top-scoring fragments in the A59 site contained an indole scaffold (FIG. 12A). These results inspired the design of novel in silico libraries based on these molecular architectures, with the hope of creating high-scoring multivalent ligands.

[0252] A library of compounds that contain an amine or a hydroxyl group for interacting with the aspartic acids in the center of the D38 site was designed, as well as a carboxylic acid moiety that could be used as a linking group for attachment of an adjacent fragment. Two of the fragments scored an order of magnitude better than the rest of the library, and were used to extend the compound to the A59 site (FIG. 12B). In silico libraries of synthetically accessible compounds based on the indole scaffold were then designed, with linkages to the two top-scoring fragments from the D38 site (FIG. 12C). Reliable chemical transformations were then used as the basis for points of diversity in order to accommodate a broad substrate range in the library and to minimize the degree of synthetic route optimization; for example, Suzuki couplings, nucleophilic substitutions, amine couplings, and reductive aminations were used. Extension into the A59 site from the D38 fragments resulted in an additional order of magnitude improvement in docking scores for the highest-ranked compounds (FIG. 12D).

[0253] In order to retain the basic properties of amines within the D38-site-targeting fragments (FIG. 13A), it was sought to link to the Y32-site-targeting fragments via reductive amination. Libraries of synthetically accessible aldehydes were generated and linked to the A59-D38 compounds (FIG. 13B). This fragment design approach to create multivalent ligands was

more computationally efficient compared to fully elaborating all possible compounds and docking them individually (FIG. 13C).

[0254] To support the notion that these compounds would be selective for the GTP-bound form, the potential inhibitors were docked into KRAS in its GDP-bound state (PDB: 4LPK) and a substantial decrease in docking scores of two to three orders of magnitude was observed (Table 4). Since GTPases have significant structural homology, these compounds were docked into a panel of GTPases in the GTP-bound form (RHO A, RHEB, RAC1, RAB3A, RAL A and CDC42) in order to predict the selectivity for RAS GTPases. The closest docking score to GTP-bound RAS was RAL A, which is still a full order of magnitude worse. Computationally, these compounds are predicted to be selective for GTP-bound RAS GTPases.

Table 4
Docking Scores of 31MEW44, 34MEW43, and 34MEW95 Against a Panel of GTPases

		31MEW44	34MEW43	34MEW95
PDB	Protein			
4DSN	KRAS G12D – GTP	-9.33	-9.6	-10
4LPK	KRAS wt – GDP	-6.1	-6.95	-7.62
1AB2	RHO A – GTP	-6.31	-6.98	-6.85
1XTS	RHEB – GTP	-6.31	-7.64	-6.92
3TH5	RAC1 – GTP	-7.24	-7.69	-8.86
3RAB	RAB3A – GTP	-6.66	-6.34	-7.05
1U8Y	RAL A – GTP	-8.65	-8.335	-8.79
2QRZ	CDC42 - GTP	-6.61	-5.57	-7.52

Note: Docking scores were calculated using Glide (Schrodinger, Inc.).

[0255] With the molecular weight of these multivalent ligands deviating from the ideal range for marketed drugs, a computational analysis of the

physical properties (using Qikprop, Schrodinger Inc.) was performed to determine how “drug-like” these molecules are, and if they could be candidates for *in vivo* testing (Table 5). While the number of hydrogen bond acceptors and donors for 31MEW44, 34MEW43 and 34MEW95 are compliant with Lipinski’s rules, the predicted logP values were greater than 5 for 31MEW44 (predicted logP=6) and for 34MEW95 (predicted logP=9.2). However, the predicted human oral absorption (a descriptor that takes into account rotatable bonds, predicted logP, predicted aqueous solubility and predicted cell permeability) was favorable for all three molecules, with values of 57%-86%, suggesting they are viable candidates for *in vivo* studies.

Table 5
Calculated Physiochemical Properties of 31MEW44, 34MEW43, and 34MEW95

	31MEW44	34MEW43	34MEW95
Molecular weight	717.66	647.2	961.93
H-bond donors	3	3	3
H-bond acceptors	6	6	8
log(P)	6	4.8	9.2
polar surface area (A^2)	88.8	91.1	80.7
Caco-2 (nm/sec)	14.6	10.8	53.7
% human oral absorption	57%	61%	86%

Note: Properties were calculated using Qikprop (Schrodinger Inc.).

Example 6

Biochemical evaluation of D38-A59 two-site compounds

[0256] A set of 12 D38-A59 two-site multivalent compounds were synthesized initially (based on Scheme 1 with appropriate modifications) and evaluated by HSQC NMR for binding to KRAS^{G12D}. Two compounds

(31MEW44 and 34MEW43) induced the largest chemical shifts among the ligands tested. The most dramatic shifts were observed in residues S39, D38, E37 and I36, consistent with the predicted docking pose (spectrum for 31MEW44, FIG. 14A; spectrum for 34MEW43, FIG. 14B). Both 31MEW44 and 34MEW43 demonstrated dose-dependent shifts by differential scanning fluorimetry (FIG. 14C). Measuring the affinity of 31MEW44 for the binding-site mutants revealed a 5-10 fold loss in binding affinity by microscale thermophoresis (K_D KRAS^{G12D D38A} = 1900 nM +/- 200 nM, K_D KRAS^{G12D I36N} = 1100 nM +/- 100 nM).

[0257] In another set of experiments, the top D38-A59 two-site multivalent compounds were evaluated by a RAS pulldown using the RAS binding domain of CRAF (the top two hits are shown in FIG. 14D). This abrogation of binding between RAS and its effector protein was also evident in the RAS-RALGDS interaction (FIG. 14E). To quantify the binding of the two-site compounds to RAS, MST was performed again using lysine NT-647-labeled, GppNHp-loaded KRAS^{G12D}. The low aqueous solubility of 34MEW43 prevented obtaining a full dose-response curve; the K_D was estimated to be 73 μ M +/- 3.0 μ M (FIG. 14F). The third (Y32) site was extended into (compound 34MEW95) using this scaffold. This three-site compound exhibited an estimated K_D of 32 μ M +/- 0.85 μ M (FIG. 14F); the low solubility of this larger compound again prevented a full dose-response curve and an accurate estimation of the dissociation constant. The more soluble two-site compound 31MEW44 also exhibited the highest potency among all tested compounds with a measured dissociation constant of 9.0 μ M +/-1.1 μ M (representing three biological replicates with three different synthesized

batches of compound; a representative curve is shown in FIG. 14G); efforts at extending into the Y32 site using 31MEW44 did not yield a compound with a substantial improvement in binding affinity. The results with 31MEW44, which had the most ideal physiochemical properties along with the highest binding affinity, prompted a focus on extensive characterization of this compound.

[0258] To test the hypothesis that 31MEW44 is selective for the GTP-bound form of RAS, we loaded KRAS^{G12D} with GDP, and measured the binding affinity of 31MEW44 using MST: we observed a five-fold loss in affinity ($K_D = 45 \mu\text{M} \pm 3.3 \mu\text{M}$, FIG. 14G). To evaluate whether binding was in the predicted region of RAS, and interacting with the identified effector binding 'hot spot' residues, we performed MST on I36N and D38A mutants and observed a 3.5 to 21-fold loss in binding affinity (FIG. 14H, KRAS^{G12D D38A} $K_D = 33 \pm 2.0 \mu\text{M}$ and KRAS^{G12D I36N} $K_D = 200 \pm 19 \mu\text{M}$).

[0259] The binding site of 31MEW44 was further characterized by HSQC NMR using GppNHp-loaded KRAS^{G12D} (see FIGS. 9A and 9B for full 2D-1H-15N HSQC assignments and representative 3D-1H-15N-1H-NOESY-HSQC and 3D-1H-15N-1H-TOCSY-HSQC assignments on residues 35-37). The most dramatic shifts in amide resonances were observed in residues S39, D38, E37 and I36, consistent with the predicted docking pose (spectrum for 31MEW44, FIG. 14A; spectrum for 34MEW43, FIG. 14B). As a secondary measure of binding, we used isothermal titration calorimetry on GppNHp-loaded KRAS^{G12D}, and observed a dissociation constant of $34 \pm 24 \mu\text{M}$ (a representative trace shown in FIG. 14I).

[0260] To provide evidence that the compound indeed was selective for RAS GTPases, MST binding measurements were performed on GppNHp-

loaded RHEB, RHOA and RALA (FIG. 14J). Consistent with docking results, the compound was selective for RAS proteins; no binding was observed with up to 190 μ M 31MEW44 to any of the other proteins.

[0261] Docking scores and dissociation constants for binding to GppNHp-bound KRAS^{G12D} (PDB:4DSN) were measured as set forth above for additional 2- and 3-site compounds by microscale thermophoresis. The results are shown in Table 6.

Table 6

Docking Scores and K_D values for additional 2- and 3-site compounds

Compound	Docknig Score	K_D (μ M)
36MEW3	-10.02	230 +/- 9
34MEW45	-8.96	1350 +/- 9
32MEW56	-9.33	>10000
43MEW65	-10.0	7.9 +/- 1
43MEW63	-10.6	26 +/- 1
43MEW73	-10.1	24 +/- 1
35MEW12	-9.88	36 +/- 2

Example 7

Evaluation of D38-A59 two-site and Y32-D38-A59 three-site multivalent ligands in cell lines with oncogenic RAS mutations

[0262] In a first set of experiments, the two-site compounds 31MEW44 and 34MEW43 were tested in a panel of cancer cell lines, some with *RAS* gene mutations (Table 7). siRNA knockdown of the mutant RAS isoform or, in the absence of mutation, the KRAS isoform was performed in each of these

cell lines, to examine their degree of dependency on RAS GTPases. Plotting the percent viability of each cell line after knockdown against EC_{50} in each cell line for each compound yielded a high correlation ($R^2 = 0.82-0.86$), supporting the notion that the lethality of these compounds in cells is RAS-mediated within this concentration range (FIGS. 15A-C).

Table 7

Potency of 31MEW44, 34MEW43, and 34MEW95 in a Panel of Cancer Cell Lines with the Viability Measured of Each Lines that was Subjected to a RAS Knockdown

Primary Site	Cell Line (RAS mutation)	Viability after Ras KD	31MEW44 EC50 (μM)	34MEW43 EC50 (μM)	34MEW95 EC50 (μM)
Pancreas	Panc-1 (KRAS G12D)	14%	2.06 +/- 0.17	5.96 +/- 0.97	2.20 +/- 0.42
Colorectal	SW480 (KRAS G12V)	26%	2.18 +/- 0.58	7.06 +/- 1.2	2.49 +/- 0.050
Fibrosarcoma	HT1080 (NRAS Q61K)	28%	2.47 +/- 0.16	5.17 +/- 0.22	3.68 +/- 1.12
Breast	MDA-MB-231 (KRAS G13D)	43%	2.26 +/- 0.44	5.63 +/- 0.46	2.67 +/- 0.43
Lung	Calu-1 (KRAS G12C)	52%	3.60 +/- 0.69	9.42 +/- 1.6	4.73 +/- 0.57
Pancreas	MIA Paca-2 (KRAS G12C)	67%	2.81 +/- 0.29	8.45 +/- 0.80	6.33 +/- 0.79
Skin fibroblast	BJeLR (HRAS G12V)	68%	3.95 +/- 0.85	9.71 +/- 0.36	5.23 +/- 0.50
Pancreas	HPAFII (KRAS G12D)	96%	6.02 +/- 0.064	16.4 +/- 3.6	11.2 +/- 2.6
Osteosarcoma	U2OS (WT)	98%	5.03 +/- 0.51	12.33 +/- 0.50	6.57 +/- 0.37
Pancreas	BxPC-3 (WT)	100%	5.29 +/- 0.36	13.1 +/- 1.6	9.73 +/- 0.25

Note: Cells were treated with compounds for 48 hours across an 8-point dilution series.

[0263] Comparing the lethal potency of these two-site compounds in KRAS-dependent (14% viability after KRAS knockdown) panc-1 cells to non-KRAS dependent (100% viability after KRAS knockdown) BxPC3 cells, a modest two-fold potency difference was observed. Based on these data and the 100% sequence conservation in the Y32-D38-A59 sites among the RAS isoforms, it was speculated that these compounds function as pan-RAS inhibitors. The relatively narrow selectivity window between these RAS mutant and RAS wild-type cell lines may be attributed, in part, to inhibition of the wild-type RAS isoforms, which is likely lethal to transformed cell lines lacking *RAS* mutations, such as BxPC3. Nonetheless, whether a pan-RAS inhibitor would have a therapeutic window is best addressed in primary patient samples and animal studies (see below).

[0264] A series of top-scoring compounds extending into the Y32 site were synthesized based on the 31MEW44 and 34MEW43 structures. 34MEW95, based on the 34MEW43 two-site compound, exhibited a three-fold improvement in potency and selectivity relative to 34MEW43. Thus, extending into a third site can improve compound potency and selectivity for RAS mutant cell lines.

[0265] Next, whether these compounds were able to prevent tumor growth in an anchorage-independent fashion, which is a more physiologically relevant culture condition, was tested. The ability of 31MEW44, 34MEW43 and 34MEW95 to prevent anchorage-independent tumor cell growth was assessed by seeding the breast cancer MDA-MB-231 cell line (with KRAS^{G13D}) in low-adherence plates, resulting in aggregation into tumor-like spheres. Vehicle-treated cells grew into multicellular tumor spheroids (FIGS.

16A-B) that decreased in size in a dose-dependent manner in the presence of each compound. Thus, these compounds are effective at inhibiting tumor cell growth in 3D cultures.

[0266] It was hypothesized that if these compounds were killing cells through RAS inhibition, overexpression of activated RAS would confer a degree of resistance to the inhibitors. HT-1080 cells (NRAS^{Q61K}) were transfected with a pBABE-puro-containing KRAS^{G12V} or empty vector using a retrovirus. A 1.7-fold increase in KRAS expression in the vector containing KRAS^{G12V} was observed relative to the vector alone following selection (FIGS. 17A-B). The two lines were treated with 5 μ M of each inhibitor for 24 hours. A corresponding 1.5-1.8 fold decrease in sensitivity (FIGS. 17A-B) was observed, consistent with the idea that cell death is dependent on RAS, even with only a modest increase in activated RAS expression.

[0267] To test whether these compounds kill cells through caspase-dependent apoptosis, which has been suggested as the mechanism of lethality after loss of RAS expression, HT-1080 (NRAS^{Q61K}) cell lysates were incubated, after treatment of the cells with 31MEW44 (5 μ M), 34MEW43 (10 μ M), 34MEW95 (5 μ M), or staurosporine (5 μ M), with a pro-fluorescent substrate for caspases 3 and 7 (rhodamine 110 bis-N-CBZ-L-aspartyl-L-glutamyl-L-valyl-aspartic acid amide). Increased fluorescence of this substrate was observed, consistent with activation of caspases 3 and/or 7 activation in response to these compounds, similar to what was observed with the known apoptosis-inducing agent staurosporine (FIG. 18).

[0268] In a second set of experiments, 31MEW44 was evaluated in a panel of ten mutant RAS cell lines and four wild-type RAS cell lines (FIG. 31A,

and Table 8 below, EC₅₀ values for 31MEW44, 34MEW43 and 34MEW95). Based on the near identical sequences in the D38 and A59 sites among the RAS isoforms, it was speculated that 31MEW44 would function as a pan-RAS inhibitor. Consistent with the idea that the compound is acting through RAS inhibition, the two most resistant lines, SW982 and SW872, both possess mutant BRAFV600E, which is downstream of RAS and would therefore would be expected to cause resistance to RAS inhibition. A range of sensitivity was observed in the mutant RAS lines, which was hypothesized to be due to their degree of dependency on mutant RAS. To test if this was indeed the case, a knockdown of the mutant isoform was performed and the viability plotted following knockdown against the viability following inhibitor treatment. With 2.5 μ M 31MEW44, a strong correlation in RAS dependency and compound lethality was observed, suggesting this concentration is likely acting on-target ($R^2=0.70$, FIG. 31B). At 5 μ M 31MEW44, the correlation was still present, but lower (FIG. 32B). An interpretation of these data is that 2.5 μ M is more functionally equivalent to the knockdown of a single RAS isoform, whereas the higher concentrations would cause inhibition of the other active isoforms and be more reflective of inhibiting total GTP-bound RAS, revealing a lower correlation with knockdown of one isoform.

Table 8. Measured EC₅₀s of inhibitors in a panel of mutant RAS cell lines.

Cells were treated with inhibitors in 384-well format and viability was measured after 48 hours of treatment using alamar blue.

<i>primary site</i>	<i>cell line (RAS mutation)</i>	31MEW44 EC50 (μM)	34MEW43 EC50 (μM)	34MEW95 EC50 (μM)
pancreas	panc-1 (KRAS ^{G12D})	5.1	6.0	2.2
colorectal	SW480 (KRAS ^{G12V})	2.2	7.1	2.5
fibrosarcoma	HT1080 (NRAS ^{Q61K})	1.1	5.2	3.7
breast	MDA-MB-231 (KRAS ^{G13D})	2.3	5.6	2.7
lung	calu-1 (KRAS ^{G12D})	3.6	9.4	4.7
pancreas	Mia Paca-2 (KRAS ^{G12D})	3.2	8.5	6.3
skin fibroblast	BJeLR (HRAS ^{G12V})	4.0	9.7	5.2
pancreas	HPAFII (KRAS ^{G12D})	5.2	16	11
lung	A549 (KRAS ^{G12S})	4.8		
lung	H441 (KRAS ^{G12V})	4.9		
lung	H358 (KRAS ^{G12C})	2.8		
lung	H23 (KRAS ^{G12C})	2.4		
lung	DLD1 (KRAS ^{Q61H})	8.8		
lung	H460 (KRAS ^{Q61H})	4.9		

[0269] Mouse embryonic fibroblasts (MEFs) have been generated to contain null *Hras* and *Nras* alleles, along with a floxed *Kras* locus that can be excised using a knocked in, inducible Cre recombinase (Drosten *et al.*, 2010). Induction of Cre effectively renders these cells “RASless”, and unable to proliferate. It was demonstrated that complete elimination of K-Ras occurs from 7 to 14 days of treatment with 4-hydroxytamoxifen (4OHT) (Drosten *et al.*, 2010). Experiences with 31MEW44 have shown that cell death by RAS inhibition occurs within 24 hours of compound treatment, indicating faster kinetics than the combination of 4OHT induction and excision of *Kras* by Cre. To see whether 31MEW44 treatment compares with 4OHT treatment, these cells were treated with 31MEW44 (24 hours) or 4OHT (6 days) and compared

the effect on growth inhibition to vehicle (DMSO) treatment (FIG. 33B). The results revealed 91% growth inhibition with 600 nM 4OHT compared to 75% with up to 5 μ M 31MEW44, indicating a similar inhibitory effect on proliferation. Indeed, the fact that a significant number of RASless MEFS remained after treatment indicates that 31MEW44 was not killing all the cells, but was likely inhibiting their growth, similar to 4OHT.

[0270] To test whether 31MEW44 kills sensitive RAS-addicted tumor cells through caspase-dependent apoptosis, which has been suggested as the mechanism of lethality after loss of RAS expression, HT-1080 (NRAS^{Q61K}) cell lysates were incubated, after treatment of the cells with 31MEW44 (2.5 and 5 μ M), or staurosporine (5 μ M), with a pro-fluorescent substrate for caspases 3 and 7 (rhodamine 110 bis-N-CBZ-L-aspartyl-L-glutamyl-L-valyl-aspartic acid amide). Increased fluorescence associated with cleavage of this substrate was observed, consistent with activation of caspases 3 and/or 7 in response to 31MEW44, similar to what was seen with staurosporine, a known apoptosis-inducing agent, at 5 μ M (FIG. 33B, see FIG. 18 for 34MEW43 and 34MEW95 results).

[0271] It was hypothesized that if 31MEW44 was killing these cells through RAS inhibition, overexpression of activated RAS and two of its effectors most commonly associated with the oncogenic phenotype (PI3K and RAF) would confer a degree of resistance to the inhibitors. To test this, HT-1080 cells (NRAS^{Q61K}) were transfected with pBABE-puro-containing KRAS^{G12V}, PI3K^{E545K}, BRAF^{V600E} or empty vector. In the PI3K^{E545K}-transfected line, a second transfection was performed with pBABEneo BRAF^{V600E}. After 5 μ M treatment for 24 h, resistance was indeed observed in all cases, with the

most resistant cells being the those with BRAF^{V600E} and PI3K^{E545K} co-transfected, followed by the BRAF^{V600E} transfected cells (FIG. 34A). Overexpression of these proteins was verified by qPCR and their function activity was measured by western blot of downstream phosphorylated ERK and AKT (FIG. 35).

[0272] The next test determined whether 31MEW44 was able to prevent tumor growth in an anchorage-independent fashion, which is a more physiologically relevant culture condition. The activity of 31MEW44 was assessed by seeding the breast cancer MDA-MB-231 cell line (KRAS^{G13D}) and the colorectal cancer SW480 (KRAS^{G12V}) in low-adherence plates, resulting in aggregation into tumor-like spheres. Vehicle-treated cells grew into multicellular tumor spheroids (FIG. 16A) that decreased in size in a dose-dependent manner in the presence of the inhibitor (34MEW43 and 34MEW95, FIG. 34B). Thus, these compounds are effective at inhibiting tumor cell growth in 3D cultures.

Example 8

Inhibition of RAS signaling by multivalent ligands

[0273] The ability of 31MEW44, 34MEW43, and 34MEW95 to disrupt RAS-RAF-MEK-ERK signaling was examined by measuring phosphorylated ERK abundance upon compound treatment. All three compounds effectively decreased pERK abundance in a dose-dependent manner (FIGS. 19A-B) in BJelR (HRAS^{G12V}), to levels comparable to those caused by the MEK 1/2 inhibitor U0126. This decrease was confirmed to correlate with disruption of the interaction between HRAS and RAF in cells through a RAS pulldown

assay, which yielded a dose-dependent decrease in CRAF-RBD-bound RAS (FIGS. 20A-B). This inhibitory effect was evident as well on the RAS-PI3K-AKT pathway, which exhibited a dose-dependent decrease in phosphorylated AKT (FIGS. 21A-B).

[0274] To test if 31MEW44 was capable of preventing the interaction between RAS and RALGDS (a guanine dissociation stimulator of RALA), a RALA activation assay was performed using RALBP1. Consistent with preventing the RAS-RALGDS interaction, decreased levels of active GTP-bound RALA were observed in a dose-dependent manner (FIG. 36A). To provide further confirmation of direct disruption of RAS-RAF and RAS-PI3K, we performed immunoprecipitation using an HRAS antibody and blotted for cRAF and PI3Kgamma. Compound administration decreased levels of co-immunoprecipitated cRAF and PI3K, indicative of direct inhibition (FIG. 36B).

[0275] Next, the consequences of these RAS inhibitors were investigated at the transcriptional level. Focus was shifted to 31MEW44, which had the most drug-like properties as well as similar potency in 2D cell culture and in the multicellular spheroid assay to the 3-site compound 34MEW95 (based on the 34MEW43 2-site compound). To determine mRNA expression differences manifest upon RAS activation, BJeLR (HRAS^{G12V}) and BJeHLT (wt HRAS) engineered isogenic fibroblasts that differ only by HRAS^{G12V} overexpression in BJeLR cells were utilized. The expression of urokinase-type plasminogen activator (*uPA*) is associated with invasion, metastasis and angiogenesis via breakdown of various components of the extracellular matrix (Pakneshan *et al.*, 2005, Pulayeva-Gupta *et al.*, 2011); *uPA* overexpression is facilitated by RAS activation through the RAS-

RALGDS-RAL pathway (Id.). Inhibition of this cascade was tested for by analyzing *uPA* expression levels, via qPCR, in BJeLR (DMSO treated) versus BJeLR (31MEW44 treated at 5 μ M and 25 μ M) and BJHLT (DMSO treated); a dose-dependent decrease in *uPA* expression upon 31MEW44 treatment was found, similar to the levels found in BJHLT cells (FIG. 22A). Another RAS effector is the GEF TIAM1, which subsequently activates the small GTPase RAC (Kerkhoff *et al.*, 1998). RAC activation leads to overexpression of matrix metalloprotease 9 (*MMP9*) that plays a key role in metastasis and the invasive phenotype (Pulayeva-Gupta *et al.*, 2011, Shin *et al.*, 2005). Downregulation of *MMP9* expression was observed upon 31MEW44 treatment (FIG. 22B). The proliferative status of cells is strongly correlated with the levels of the transcription factor *CMYC* (Pulayeva-Gupta *et al.*, 2011, Kerkhoff *et al.*, 1998). The RAS-RAF signaling cascade is known to be a key regulator of *CMYC* expression (Pakneshan *et al.*, 2005, Kerkhoff *et al.*, 1998). Upon 31MEW44 treatment of BJeLR cells, a dose-dependent reduction in *CMYC* mRNA was observed (FIG. 22C). Finally, the metabolic shift of transformed cells to the aerobic metabolism of glucose is well established (Pulayeva-Gupta *et al.*, 2011, Chiaradonna *et al.*, 2006). Associated with this change is the upregulation of lactate dehydrogenase (*LDH*), which converts the end product of glycolysis (pyruvate) into lactate, a change that has been observed in RAS transformed cells (Chiaradonna *et al.*, 2006). Treatment of 31MEW44, indeed, decreased these elevated levels of LDH, dose-dependently, in BJeLR cells (FIG. 22D). Thus, 31MEW44 reverses the transcriptional changes associated with RAS activation, consistent with the notion that it is a RAS inhibitor. Activation of ERK signaling has been shown to be associated with

the induction of expression of dual specificity-phosphatase (*DUSP6*) (Joseph *et al.*, 2010), while the same study indicated that downregulation of isocitrate dehydrogenase-2 (*IDH2*) is linked to active Erk (Id.). Treatment of BJeLR cells with 31MEW44 at 5 μ M and 25 μ M again showed a profile characteristic of decreased activation of RAS signaling (FIGS. 22E-F).

[0276] In addition to altering genes associated with RAS activation, it was hypothesized that upon inhibitor treatment, cells would attempt to compensate by expressing additional RAS proteins. To examine this, five cell lines were treated with 31MEW44 at 4 and 24 hours, Mia-Paca2 (KRAS^{G12C}), NCI-H441 (KRAS^{G12V}), NCI-H23 (KRAS^{G12C}) BJeLR (HRAS^{G12V}), and HT1080 (NRAS^{Q61K}). In all cell lines, a time-dependent increase of RAS expression was observed (FIG. 37).

Example 9

Effects of multivalent RAS inhibitors on primary patient samples in mouse xenograft tumors

[0277] Although these multivalent pan-RAS compounds do not possess selective inhibitory activity towards mutated RAS proteins, there is ample evidence that tumors with mutated RAS proteins are addicted to these oncogenic isoforms. Thus, it was speculated that pan-RAS inhibitors such as 31MEW44 might have an acceptable therapeutic index in patient cells and *in vivo*. First, 31MEW44 was evaluated in primary patient-derived T-cell acute lymphoblastic leukemia (T-ALL) cells cultured *in vitro* to determine the potency and selectivity in a more clinically relevant model. 31MEW44 was tested in two samples containing mutant *NRAS* (G13V and G13D) as well as four samples possessing wild-type *NRAS* (FIGS. 23A-F). A high degree of

selectivity was observed with the cell lines, with mutant *NRAS* cells retaining only 20-40% viability after 5 μ M 31MEW44 treatment, while no observed decrease in viability was observed in the four cell lines tested with wild-type *NRAS*. This indicated that 31MEW44 could be a viable therapeutic agent in *NRAS* mutated T-ALL.

[0278] It was then sought to determine if 31MEW44 was sufficiently metabolically stable for *in vivo* testing. To see if 31MEW44 was susceptible to metabolism by cytochrome P450 enzymes, the compound was incubated with purified mouse liver microsomes and its degradation was followed by LC-MS. The positive control compound, 7-ethoxycoumarin was determined to have a half-life of 3.9 minutes, while all of 31MEW44 remained after a 45 minute incubation (FIG. 24). Encouraged by the stability of 31MEW44 in the microsome assay, it was sought to determine its *in vivo* pharmacokinetics by analyzing plasma samples of male C57BL6 adult mice with the compound administered both orally (PO) and intravenously (IV). After monitoring the concentration of 31MEW44 over 12 hours, oral delivery yielded a half-life of 3.1 hours, while intravenous delivery showed no elimination, suggesting a half-life > 12 hours (FIG. 25). From these experiments, it was concluded that 31MEW44 is a suitable candidate for *in vivo* testing.

[0279] The *in vivo* efficacy and toxicity of 31MEW44 was assessed in a xenograft mouse tumor model using the aggressive MDA-MB-231 cell line in 8-week-old nude mice. Once tumor xenografts reached an average size of about 58 mm³, mice were separated into treatment groups receiving vehicle, or 31MEW44, either orally or via a combination of intravenous and intraperitoneal injections. Both treatments resulted in an almost complete

elimination of the tumor (FIG. 29B). To see if 31MEW44 was indeed inhibiting RAS signaling *in vivo*, an additional short pharmacodynamic study was performed. In this study, mice were injected in the right flank subcutaneously with 8 million MDA-MB-231 cells. A population of xenograft sizes was visible just three days following injection, indicating a rapidly growing tumor. Mice were separated into treatment groups of equal xenograft population and average size (about 128 cubic millimeters, FIG. 26). Each group was dosed daily by intraperitoneal injection with 31MEW44 at 30 mg/kg or vehicle only (5% DMSO in HBSS at pH 4). No overt toxicity was apparent following daily injections. Following six days of treatment, 31MEW44-treated tumors exhibited a 60% reduction in growth relative to the vehicle-treated group (FIG. 27). The tumors were dissected and weighed (FIGS. 28 and 29A). Segments of the xenografts were lysed by sonication, and analyzed for phosphorylated ERK levels by Western blotting (FIGS. 30A-B). On average, 31MEW44-treated mice exhibited tumor pERK levels about 70% lower than those of the vehicle-treated mouse tumors, indicating the 31MEW44 was able to significantly reduce RAS activation of pERK in these tumors.

[0280] The PDTALL22 patient sample was then used as a luciferase expressing patient-derived xenograft. Mice were imaged after 4 and 8 days of treatment, and a significant decrease in tumor burden was observed (FIG. 38). Consistent with the overall decrease in tumor burden, examination of the spleen revealed a decrease in size with inhibitor treatment, as well as a significant reduction in the percent of human CD45⁺ cells indicating a decrease in the number of human xenografted cells infiltrating into the spleen upon compound treatment (FIG. 38D). Thus, this approach to designing

multivalent ligands yielded a compound with affinity to RAS proteins that can reduce the tumor burden of RAS mutated tumors in mouse xenografts models.

[0281] Mutations in the *RAS* genes are found in 90% of pancreatic cancers (Hopkins *et al.*, 2002). Pancreatic ductal adenocarcinoma is particularly resistant to chemotherapy, as it is known to have a dense, desmoplastic stroma that can limit drug delivery (Oberstein *et al.*, 2013). The most commonly used therapeutic agent, gemcitabine, extends patient survival by only a few weeks (Burris *et al.*, 1997). To see if 31MEW44 could be a potential therapeutic agent for pancreatic cancers, the *Kras*^{LSL.G12D/+}*Tp53*^{fl/fl}*Pdx1-Cre* (KP^{fl/fl}C) mouse model was used (Bardeesy *et al.*, 2006), which allows for both pancreas-specific expression of *Kras*^{G12D} and the deletion of p53. Pre-treatment biopsies were acquired from each mouse by abdominal laparotomy, followed by a day of recovery and treatment with 30 mg/kg 31MEW44, once daily, i.p. Interestingly, toxicity was observed in the KP^{fl/fl}C mice, but not in wild-type mice enrolled in the study (FIGS. 39A-B). The increased sensitivity of these mice was attributed to their lack of one functional *Kras* allele, suggesting due to this artificial condition, RAS inhibitors cannot be fully evaluated for efficacy in this model. Nonetheless, comparison of pre- and post-treatment tumor samples showed a substantial decrease for both phosphorylated AKT (S473) and phosphorylated ERK1/2, indicating that 31MEW44 was infiltrating the tumor and abrogating the RAS-PI3K and RAS-RAF signaling pathways, respectively (FIGS. 39C-D), suggesting that such compounds can indeed be candidate therapeutic agents for pancreatic cancers. A modest increase in cleaved caspase-3 was also observed,

showing that even in this exacting model, 31MEW44 has the capacity to kill RAS-mutant tumor cells (FIGS. 39E-F).

[0282] Over the past two years, compounds that bind to RAS proteins have been identified by several groups, despite the historical challenge of identifying direct ligands for RAS proteins. Maurer *et al.* (Maurer *et al.*, 2012) described compounds that bind to RAS-GDP and prevent SOS-mediated nucleotide exchange. Treatment with an inhibitor of this type would be applicable in cancers that require activation of wild-type RAS through SOS for proliferation; mutated RAS remains unaffected by this class of compounds. Shima *et al.* (Shima *et al.*, 2013) described compounds that inhibit the interaction of RAS with its effector proteins with inhibitor constants (K_i) ranging from 46 to 733 μ M; the modest potency of these compounds likely precludes further development and may impede their use as probes. Ostrem *et al.* (Ostrem *et al.*, 2013) reported covalent inhibitors from a fragment screening approach, tethering, which selectively target KRAS^{G12C} by exploiting the reactive cysteine present in the mutant. While this represents an important advance for addressing some RAS malignancies, it is only applicable to this specific mutant; present in only about one in eight *KRAS* mutated samples (Downward *et al.*, 2014). In the panel of cell lines tested with this compound, the selective inhibition of growth in KRAS^{G12C} cells ranged from three-fold, in the most sensitive cell line, to no selectivity. This narrow window may be attributable to the reactive electrophile present in the inhibitors.

[0283] This longstanding problem of the fundamental challenge of protein druggability was approached using the concept of multivalent ligand design. Analysis of protein structures in the Protein Data Bank suggests only

about 12% of proteins encoded in the genome possess a cavity with the necessary properties for the tight binding of small molecules, based on extrapolation from current drugs; similar analyses suggest that about 3,000 druggable proteins exist, compared to the about 20,000 protein-coding human genes (Hopkins *et al.*, 2002, Verdine *et al.*, 2007). To tap into the vast landscape of challenging, but disease-modifying, therapeutic targets, new strategies may be needed. It is suggested that structure-based design of multivalent ligands for specific proteins may be one such strategy. This strategy has resulted in the creation of pan-RAS inhibitors that have a viable therapeutic index in primary patient samples and in a murine xenograft tumor model; thus, this approach may ultimately be one means of disrupting the oncogenic functions of RAS proteins in human tumors. Moreover, it may be possible to extend this approach to other small GTPases in the RAS superfamily, as well as other challenging protein targets.

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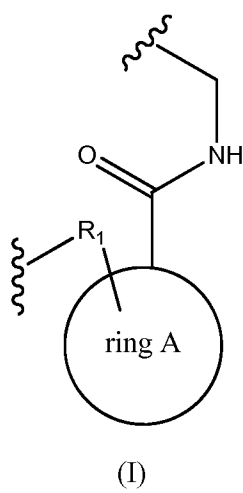
[0284] All documents cited in this application are hereby incorporated by reference as if recited in full herein.

[0285] Although illustrative embodiments of the present invention have been described herein, it should be understood that the invention is not limited to those described, and that various other changes or modifications may be made by one skilled in the art without departing from the scope or spirit of the invention.

WHAT IS CLAIMED IS:

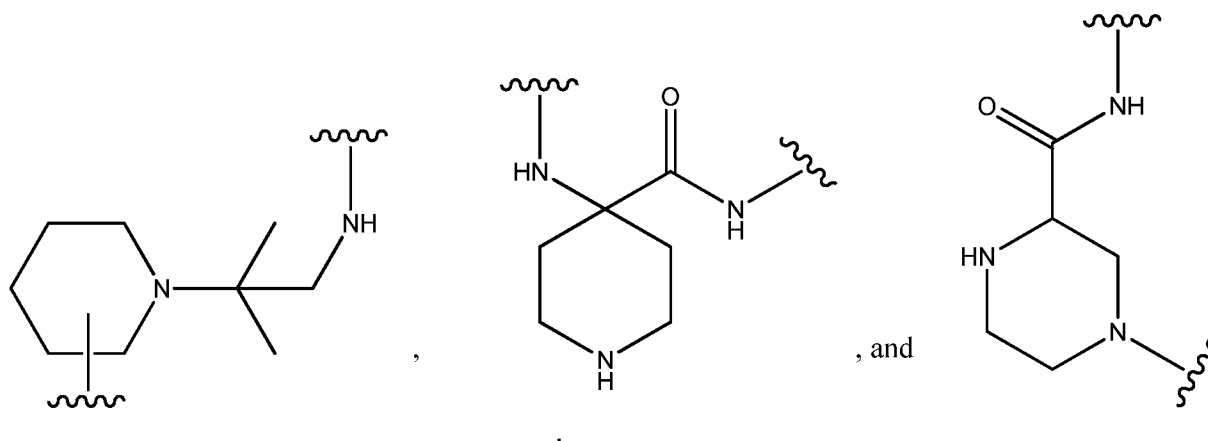
1. A compound that selectively binds a RAS protein at two or more sites.
2. The compound according to claim 1 that selectively binds to a first site on the RAS protein that comprises at least one amino acid from the switch 1 region (near D38).
3. The compound according to claim 2 that selectively binds to a second site on the RAS protein that comprises at least one amino acid located between the switch 1 and switch 2 regions (near A59).
4. The compound according to claim 3 that selectively binds to at least one amino acid near I21 (Y32 site) of the RAS protein.
5. The compound according to claim 1 that is selective for a GTP-bound RAS protein.
6. The compound according to claim 4, that is selective for a non-GDP-bound form of the RAS protein.
7. The compound according to claim 1, wherein the RAS protein is an isoform selected from the group consisting of HRAS, KRAS, NRAS, and combinations thereof.
8. The compound according to claim 1, wherein the RAS protein is an oncogenic mutant.

9. The compound according to claim 8, wherein the RAS protein is an oncogenic mutant selected from the group consisting of HRAS^{G12D}, KRAS^{G12D}, NRAS^{Q61K}, NRAS^{G13V}, and NRAS^{G13D}.
10. The compound according to claim 1 that selectively binds to at least one amino acid near D38, A59, and optionally I21 (Y32 site) in the RAS protein.
11. The compound according to claim 10 that comprises a region A that binds to at least one amino acid near D38 on the RAS protein and comprises a heterocycle with at least one ring nitrogen.
12. The compound according to claim 11, wherein the A region of the compound comprises a fragment having formula (I):



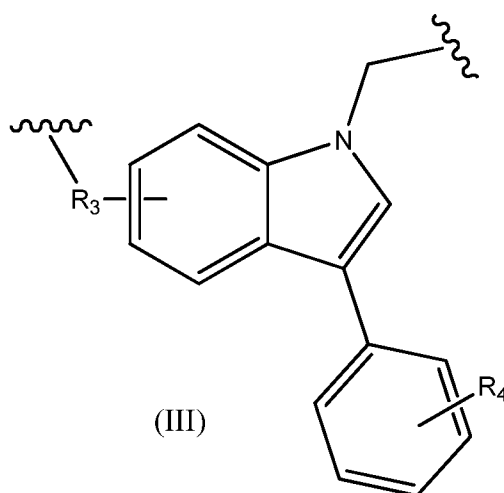
wherein ring A is a heterocycle with at least one ring nitrogen, and R₁ is selected from the group consisting of no atom, amine, and C₁₋₄ aliphatic.

13. The compound according to claim 11, wherein the A region of the compound is selected from the group consisting of:



14. The compound according to claim 10 that comprises a region B that binds to at least one amino acid near A59 on the RAS protein and comprises an indole.

15. The compound according to claim 14, wherein the B region of the compound comprises a structure of formula (III):

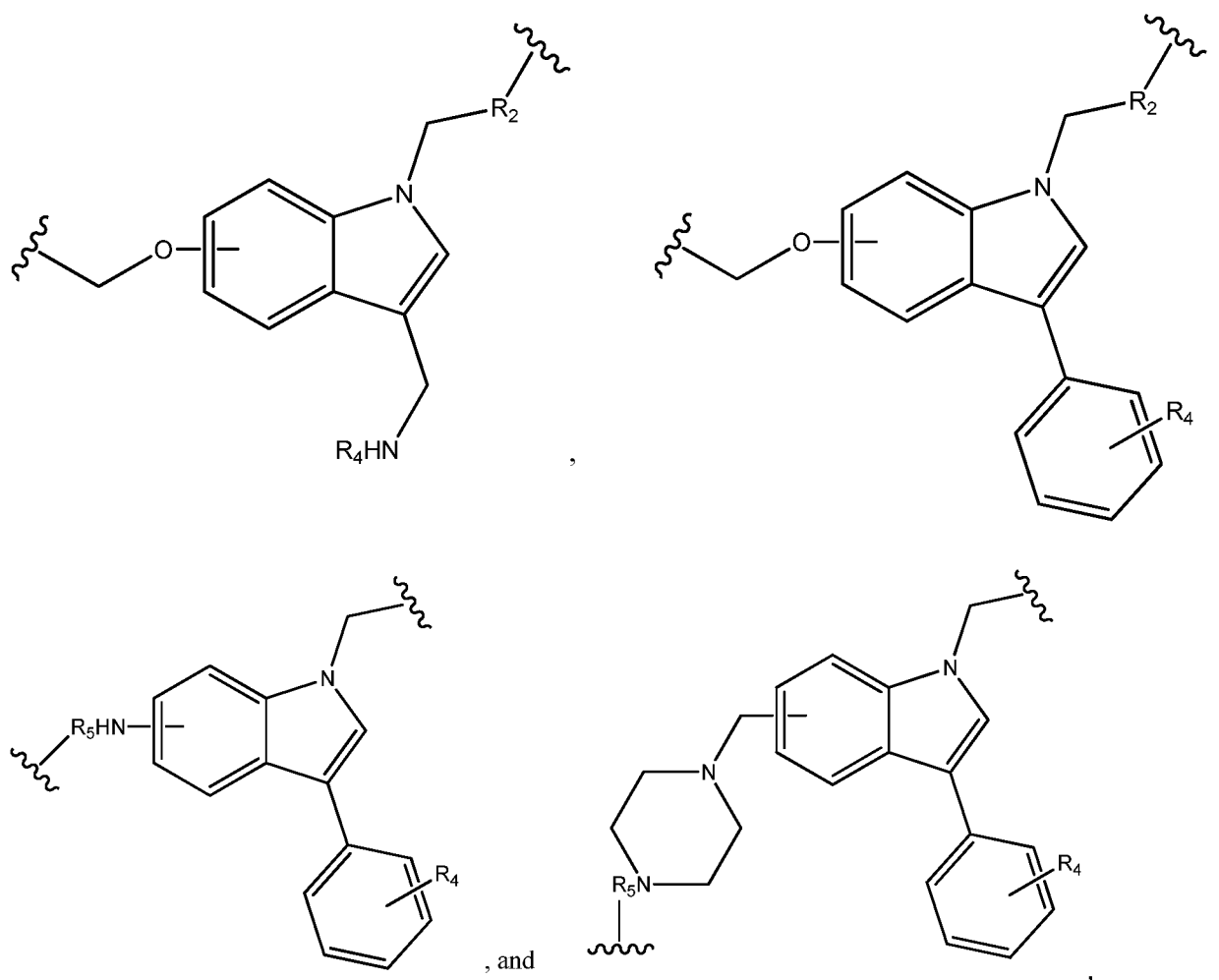


wherein

R₃ is selected from the group consisting of heterocycle, aryl, and amine, which heterocycle, aryl, and amine may be optionally substituted with the group selected from halide, C₁₋₄ aliphatic, and combinations thereof; and

R₄ is selected from the group consisting of no atom, H, aryl, halide, C₁₋₄ aliphatic, -O-C₁₋₄alkyl wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof.

16. The compound according to claim 14, wherein the B region of the compound is selected from the group consisting of



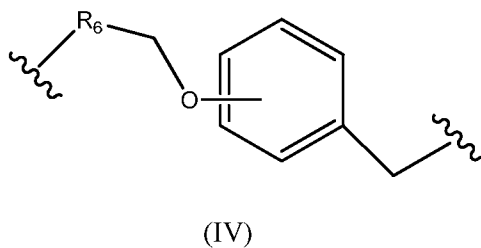
wherein

R₂ and R₅ are independently selected from the group consisting of no atom, aryl, and C₁₋₄ aliphatic; and

R₄ is selected from the group consisting of no atom, H, aryl, halide, C₁₋₄ aliphatic, -O-C₁₋₄alkyl wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof.

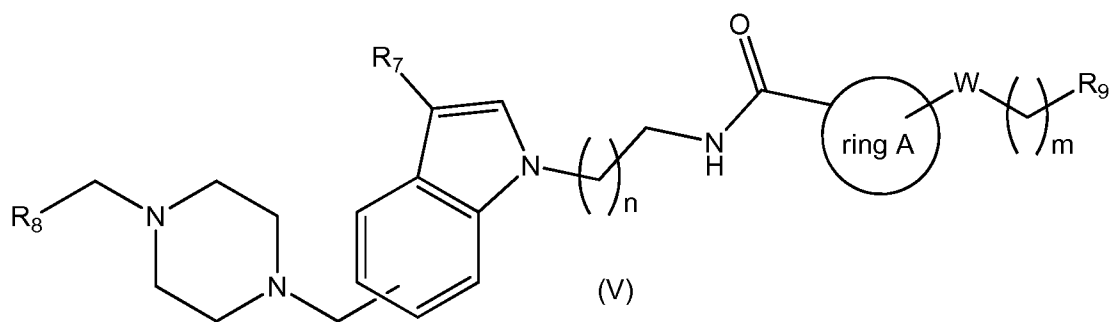
17. The compound according to claim 10, that comprises a region C that binds to at least one amino acid near I21 (Y32 site) of the RAS protein and comprises an aromatic ring.

18. The compound according to claim 17, wherein the C region of the compound comprises a structure of formula (IV):



wherein R₆ is selected from the group consisting of no atom, H, alkyl, and aryl, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with the group consisting of halide, ether, C₁₋₄alkyl, and a combination thereof.

19. The compound according to claim 1, wherein the compound has a structure of formula (V):



wherein:

R_7 is selected from the group consisting of H, halide, C_{1-4} aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, $-O-C_{1-4}$ alkyl and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R_8 is selected from the group consisting of no atom, H, alkyl, aryl, and C_{1-4} alkyl-O-aryl wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, and a combination thereof;

R_9 is selected from the group consisting of no atom, H, C_{1-4} alkyl, and aryl optionally substituted with the group consisting of ether, halide, and a combination thereof;

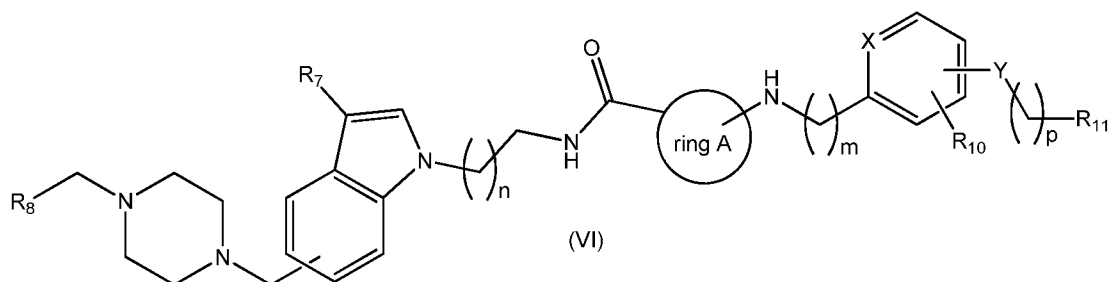
W is selected from the group consisting of no atom and NH;

m and n are independently selected from the group consisting of an integer between 0-5; and

ring A is a heterocycle with at least 1 ring nitrogen and optionally substituted with C_{1-4} alkyl or a halide,

or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

20. The compound according to claim 1, wherein the compound has a structure of formula (VI):



wherein:

R_7 is selected from the group consisting of H, halide, C_{1-4} aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, $-O-C_{1-4}$ alkyl, and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R_8 and R_{11} are independently selected from the group consisting of no atom, H, alkyl, aryl and C_{1-4} alkyl-O-aryl, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, $-O-C_{1-4}$ alkyl, and a combination thereof, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof;

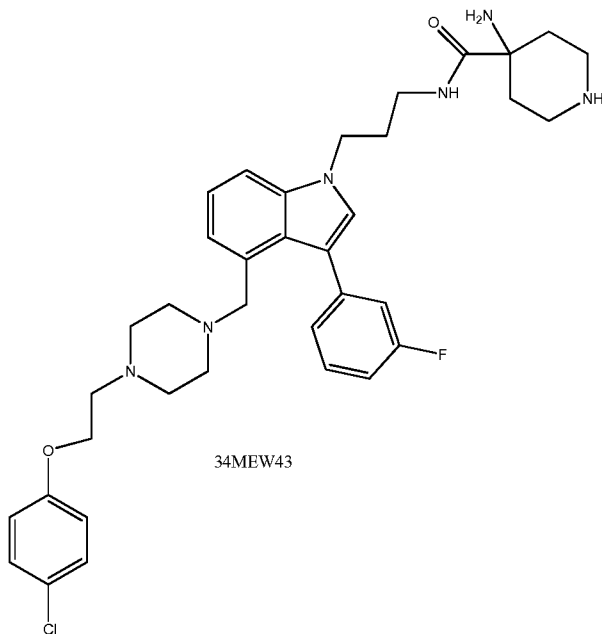
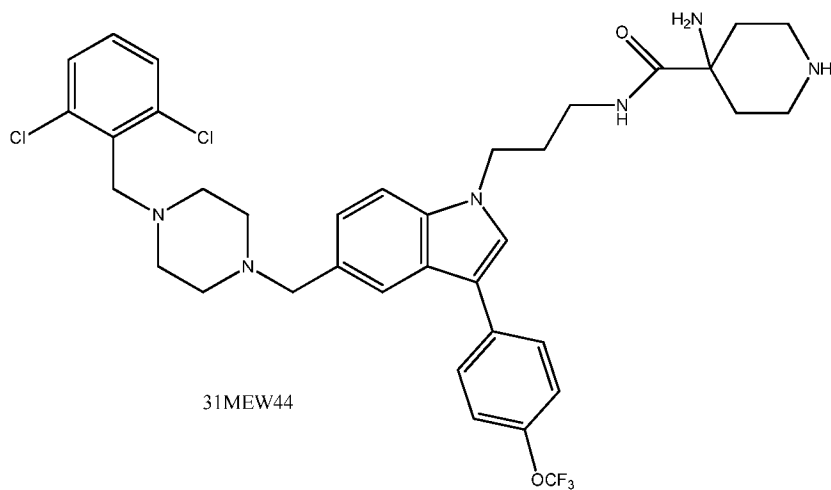
R_{10} is selected from the group consisting of no atom, H, halide, C_{1-4} aliphatic, and $-O-C_{1-4}$ alkyl;

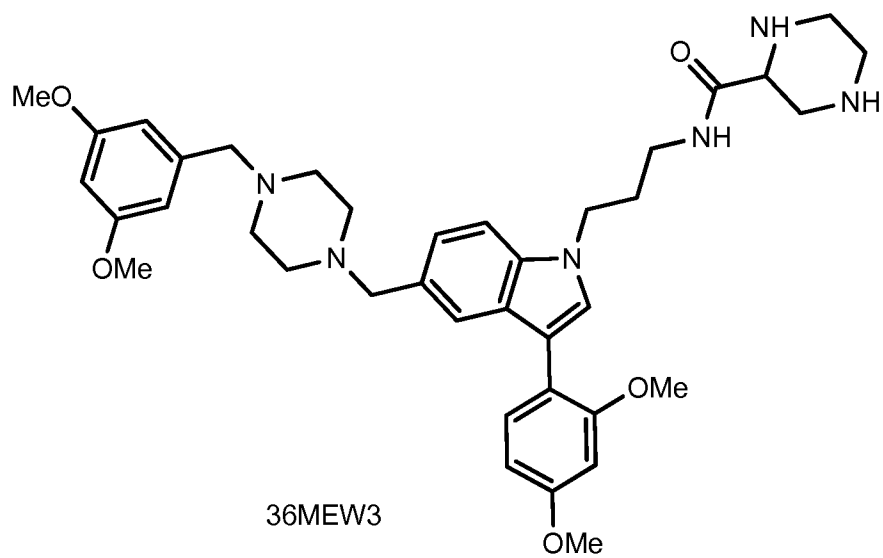
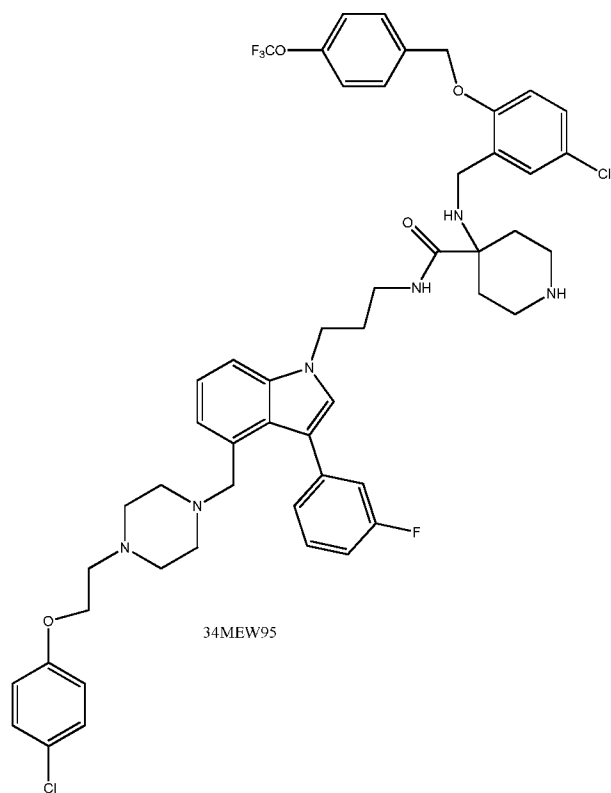
X is selected from the group consisting of CH and N;

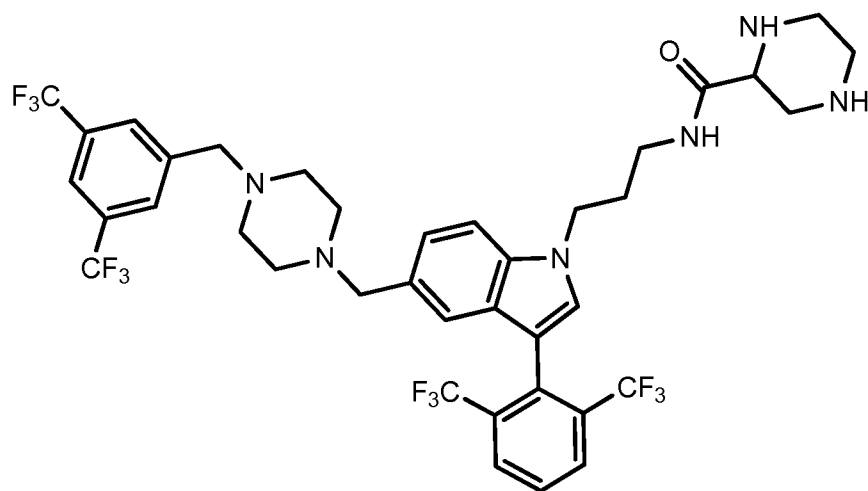
Y is selected from the group consisting of no atom and O;

m, n, and p are independently selected from the group consisting of an integer between 0-5; and ring A is a heterocycle with at least 1 ring nitrogen and optionally substituted with C₁₋₄alkyl or a halide, or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

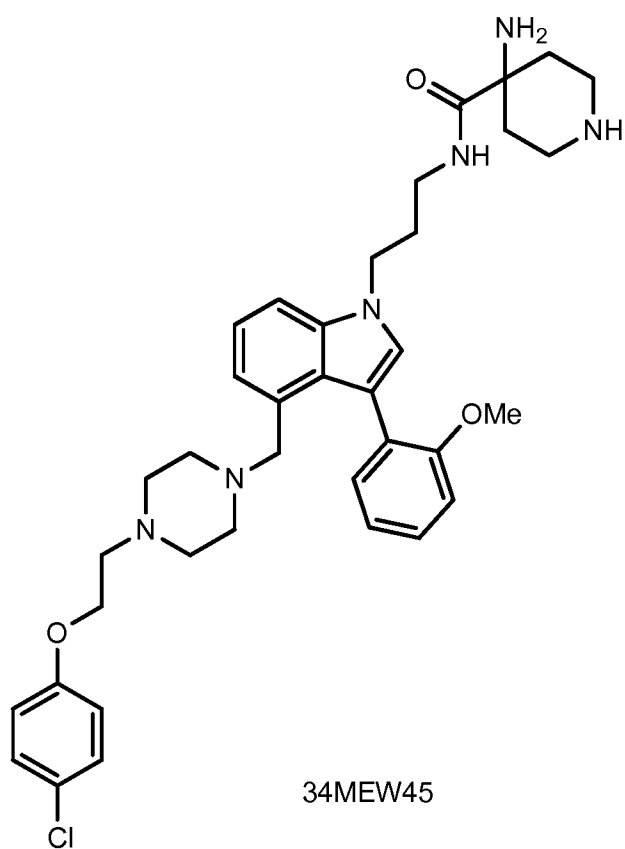
21. The compound according to claim 1, wherein the compound is selected from the group consisting of



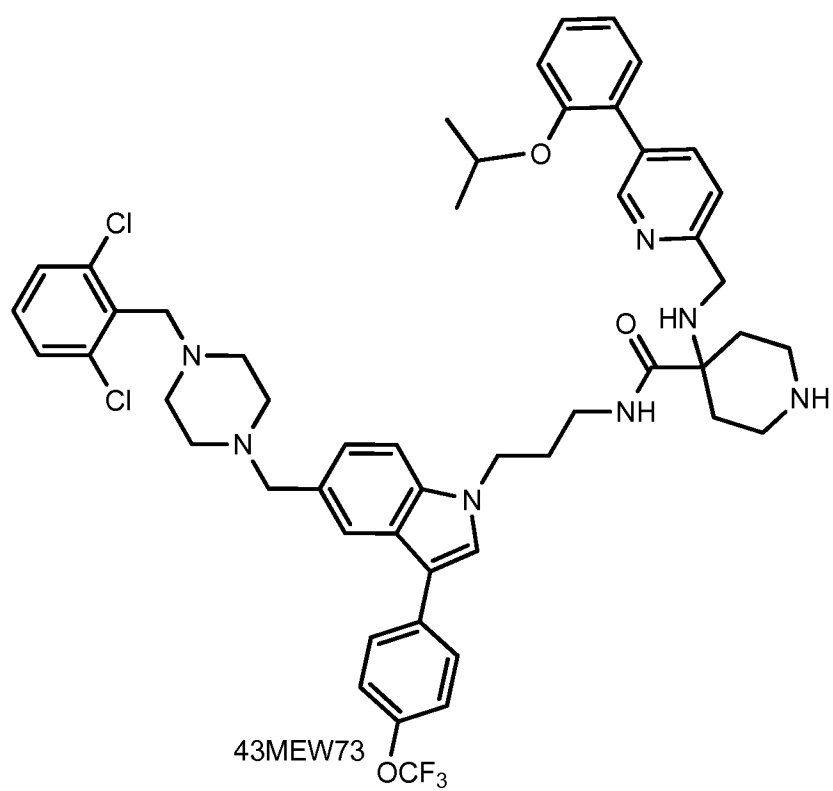
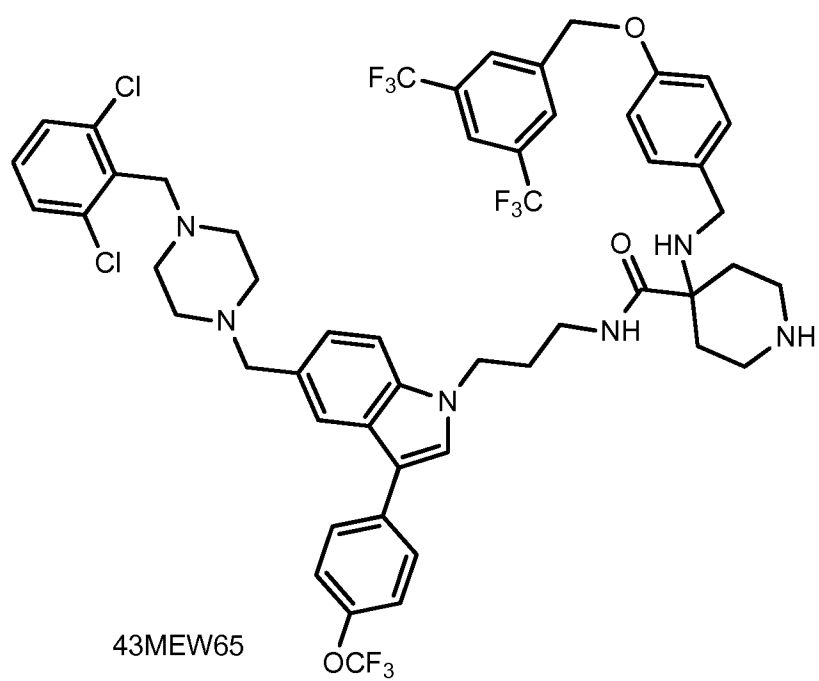




32MEW56



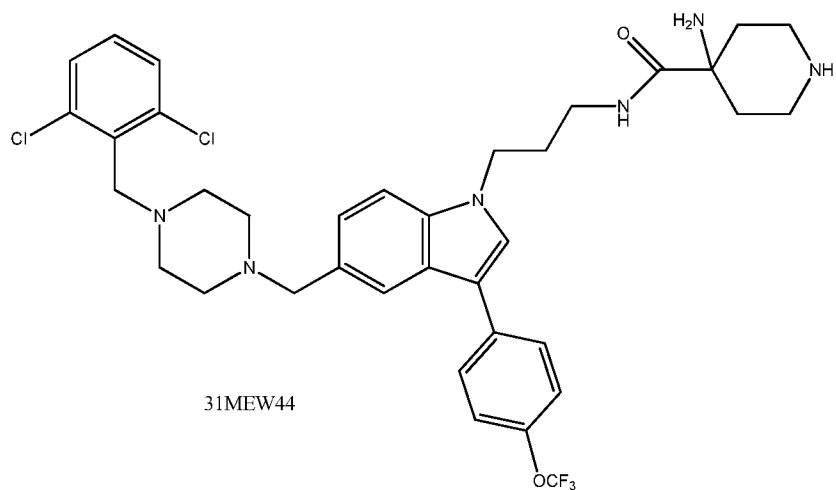
34MEW45





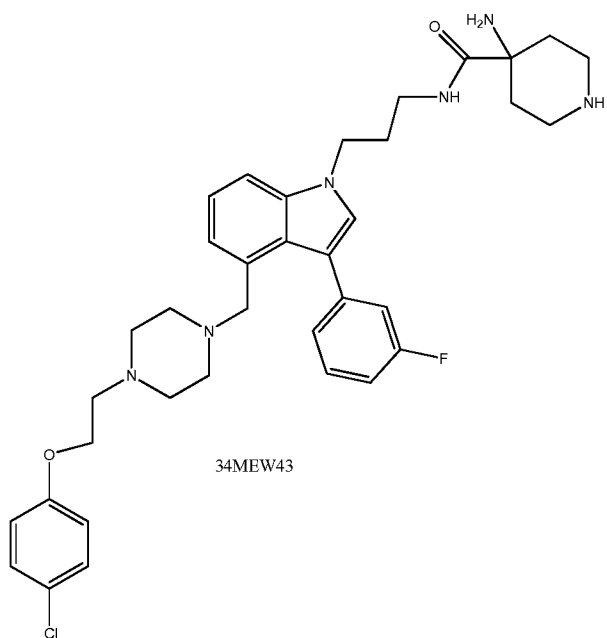
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22. The compound according to claim 21, wherein the compound is



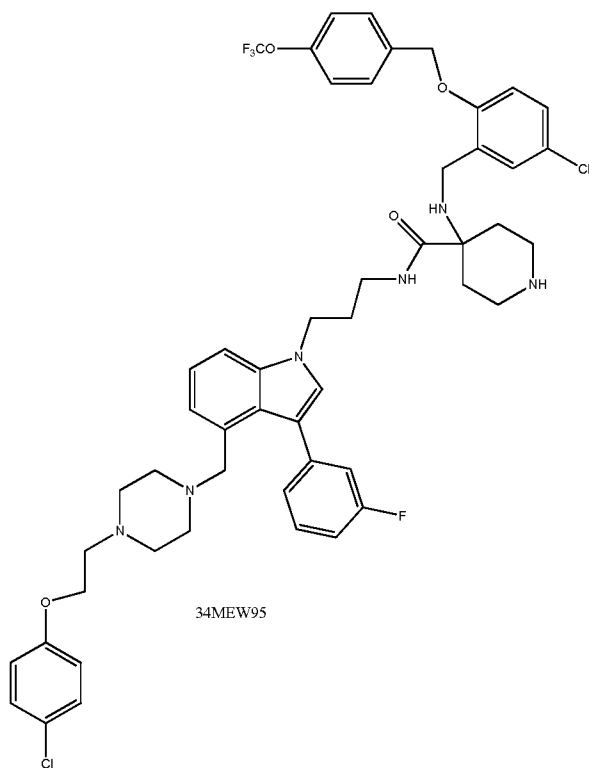
or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

23. The compound according to claim 21, wherein the compound is



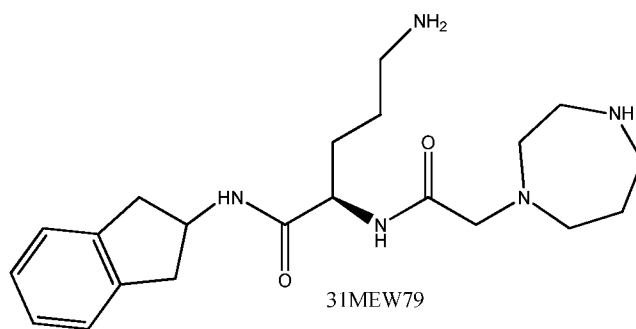
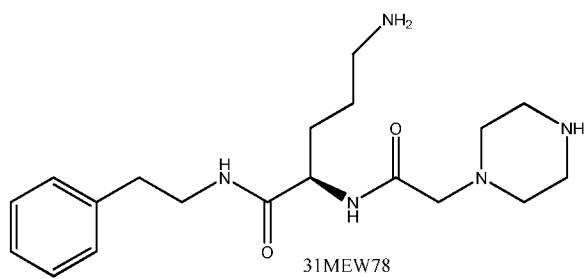
or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

24. The compound according to claim 21, wherein the compound is



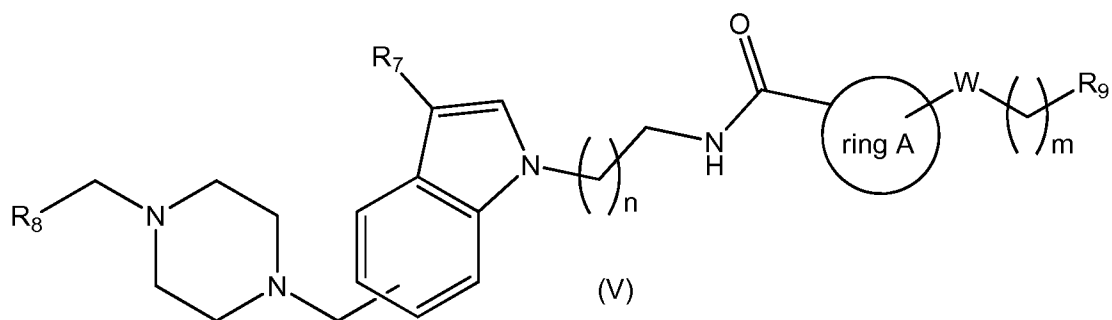
or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

25. A compound selected from the group consisting of



and crystalline forms, hydrates, or pharmaceutically acceptable salts thereof.

26. A compound having the structure of formula (V):



wherein:

R_7 is selected from the group consisting of H, halide, C_{1-4} aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, $-O-C_{1-4}$ alkyl and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R_8 is selected from the group consisting of no atom, H, alkyl, aryl, and C_{1-4} alkyl-O-aryl wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, and a combination thereof;

R_9 is selected from the group consisting of no atom, H, C_{1-4} alkyl, and aryl optionally substituted with the group consisting of ether, halide, and a combination thereof;

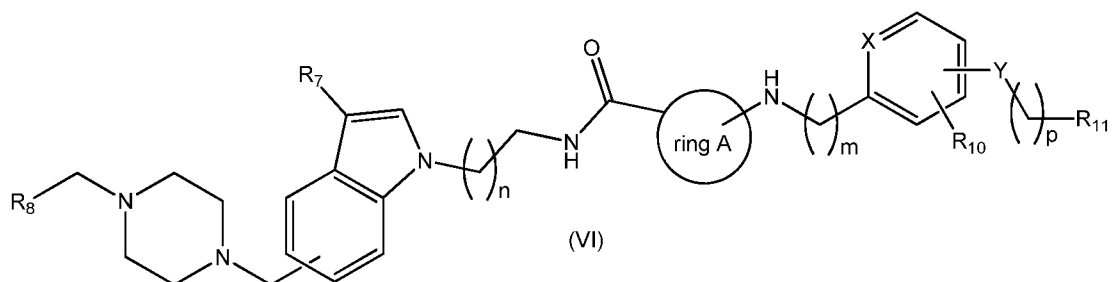
W is selected from the group consisting of no atom and NH;

m and n are independently selected from the group consisting of an integer between 0-5; and

ring A is a heterocycle with at least 1 ring nitrogen and optionally substituted with C_{1-4} alkyl or a halide,

or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

27. A compound having the structure of formula (VI):



wherein:

R_7 is selected from the group consisting of H, halide, C_{1-4} aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, $-O-C_{1-4}$ alkyl, and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R_8 and R_{11} are independently selected from the group consisting of no atom, H, alkyl, aryl and C_{1-4} alkyl-O-aryl, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, $-O-C_{1-4}$ alkyl, and a combination thereof, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof;

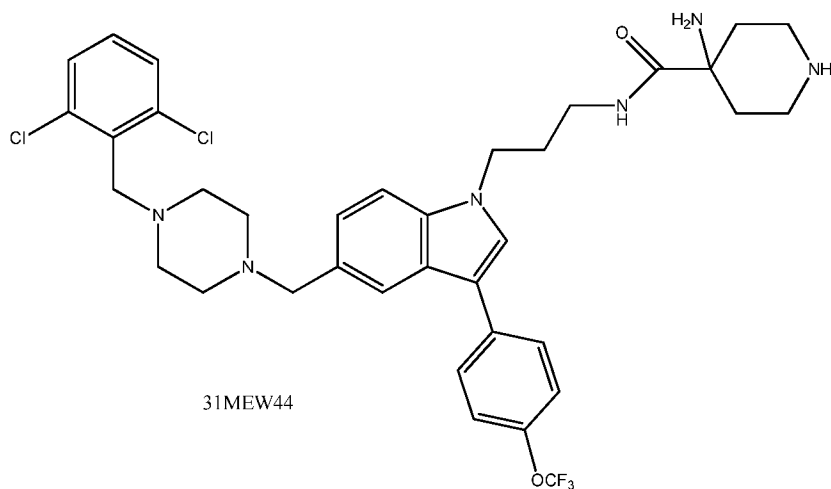
R_{10} is selected from the group consisting of no atom, H, halide, C_{1-4} aliphatic, and $-O-C_{1-4}$ alkyl;

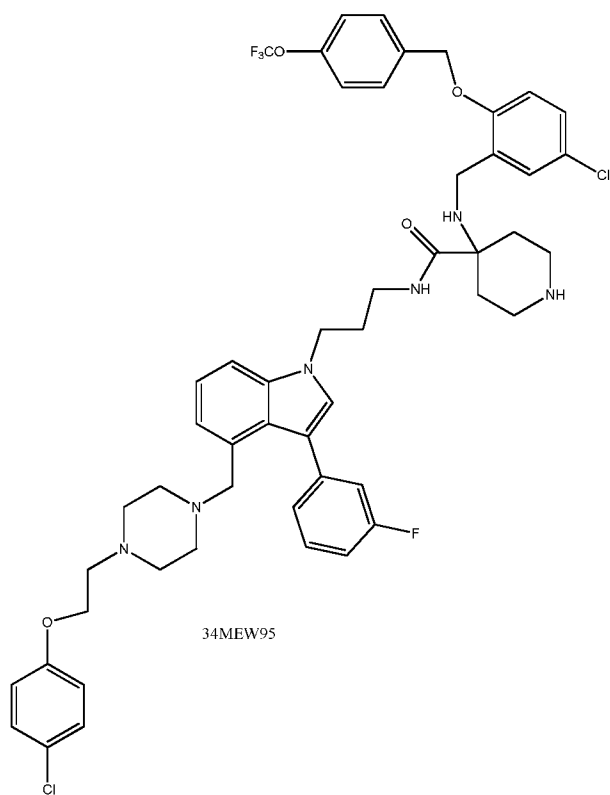
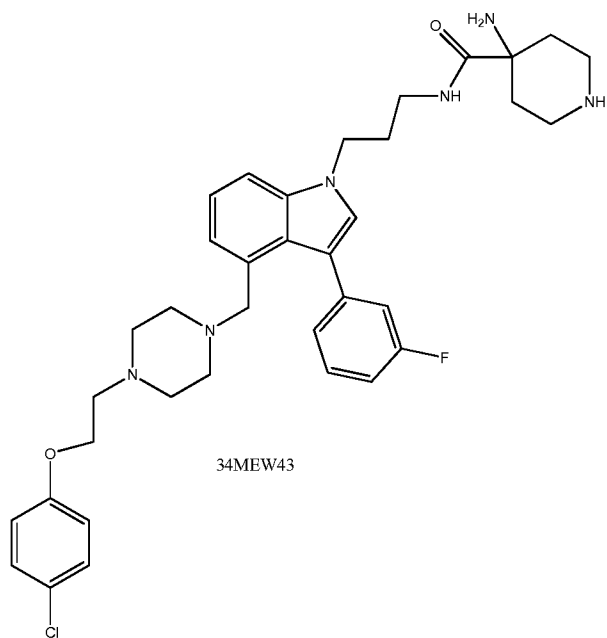
X is selected from the group consisting of CH and N;

Y is selected from the group consisting of no atom and O;

m, n, and p are independently selected from the group consisting of an integer between 0-5; and ring A is a heterocycle with at least 1 ring nitrogen and optionally substituted with C₁₋₄alkyl or a halide, or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

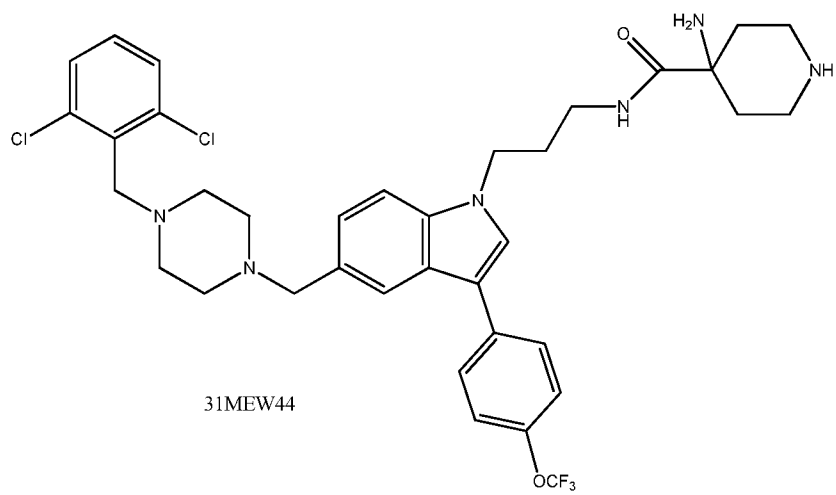
28. A compound selected from the group consisting of





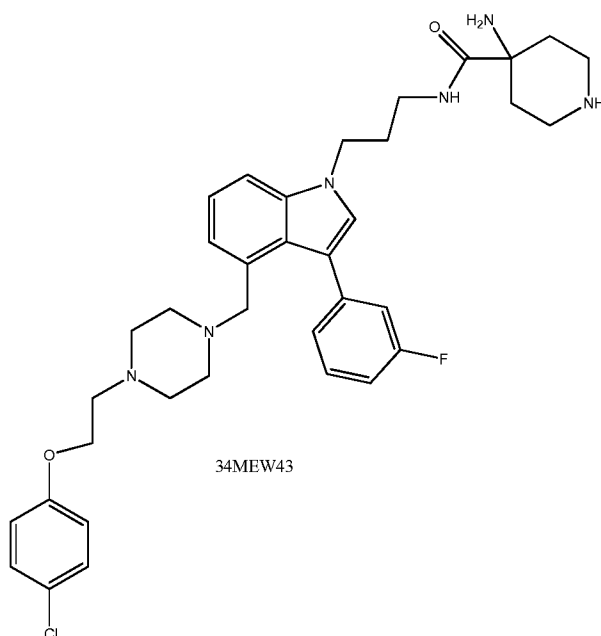
and crystalline forms, hydrates, or pharmaceutically acceptable salts thereof.

29. A compound having the structure:



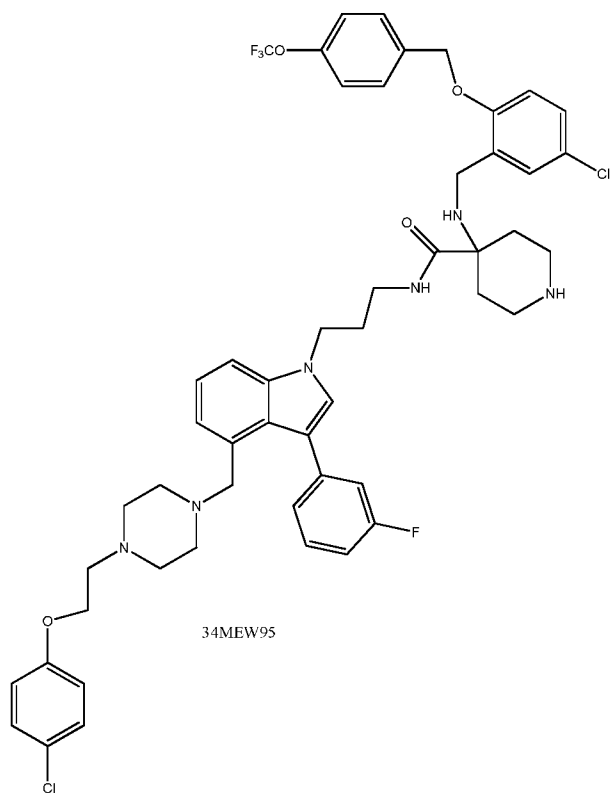
or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

30. A compound having the structure:



or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

31. A compound having the structure:



or a crystalline form, hydrate, or pharmaceutically acceptable salt thereof.

32. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a compound according to any one of claims 1 and 25-28.

33. A method for ameliorating or treating the effects of a disease associated with altered RAS signaling in a subject comprising administering to the subject an effective amount of a compound according to any one of claims 1 and 25-28.

34. The method according to claim 33, wherein the disease is selected from the group consisting of a cancer, a neurological disorder, a metabolic

disorder, an immunological disorder, an inflammatory disorder, and a developmental disorder.

35. The method according to claim 34, wherein the disease is selected from the group consisting of autism, rasopathies, neurofibromatosis type 1, Noonan syndrome, Costello syndrome, cardiofaciocutaneous syndrome, hereditary gingival fibromatosis type 1, Legius syndrome, Leopard syndrome, diabetic retinopathy, diabetes, hyperinsulinemia, chronic idiopathic urticarial, autoimmune lymphoproliferative syndrome, and capillary malformation-arteriovenous malformation.

36. The method according to claim 34, wherein the cancer is selected from the group consisting of pancreatic cancer, colorectal cancer, lung cancer, skin cancer, urinary bladder cancer, thyroid cancer, hematopoietic cancer, prostate cancer, breast cancer, liver cancer, soft tissue cancer, leukemia and bone cancer.

37. The method according to claim 34, wherein the cancer is selected from the group consisting of pancreatic cancer, colorectal cancer, fibrosarcoma, breast cancer, lung cancer, skin cancer, leukemia and bone cancer.

38. The method according to claim 33, wherein the subject is a mammal.

39. The method according to claim 38, wherein the mammal is selected from the group consisting of humans, primates, farm animals, domestic animals and laboratory animals.

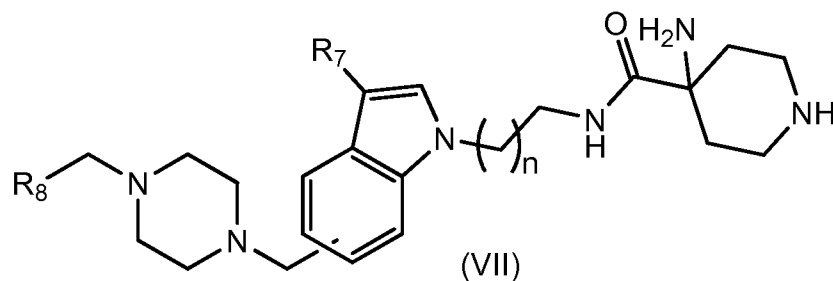
40. The method according to claim 38, wherein the mammal is a human.
41. A method for ameliorating or treating the effects of a disease associated with altered RAS signaling in a subject comprising administering to the subject an effective amount of a pharmaceutical composition according to claim 32.
42. A method for effecting cancer cell death comprising contacting a cancer cell with an effective amount of a compound according to any one of claims 1 and 25-28.
43. A kit for treating or ameliorating the effects of a disease related to altered RAS signaling in a subject in need thereof, the kit comprising an effective amount of a compound according to any one of claims 1 and 25-28, packaged together with instructions for its use.
44. A kit for treating or ameliorating the effects of a disease related to altered RAS signaling in a subject in need thereof, the kit comprising an effective amount of a pharmaceutical composition according to claim 32, packaged together with instructions for its use.
45. A kit for treating or ameliorating the effects of a cancer in a subject in need thereof, the kit comprising an effective amount of a compound according to any one of claims 1 and 25-28, packaged together with instructions for its use.

46. A kit for treating or ameliorating the effects of a cancer in a subject in need thereof, the kit comprising an effective amount of a pharmaceutical composition according to claim 32, packaged together with instructions for its use.

47. A composition comprising a compound according to any one of claims 1 and 25-28.

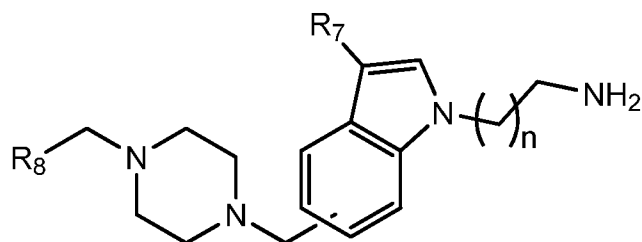
48. The composition according to claim 46, which is a research reagent.

49. A method of preparing a compound having the structure of formula (VII):

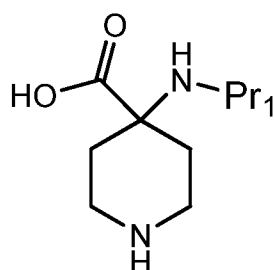


comprising the steps of

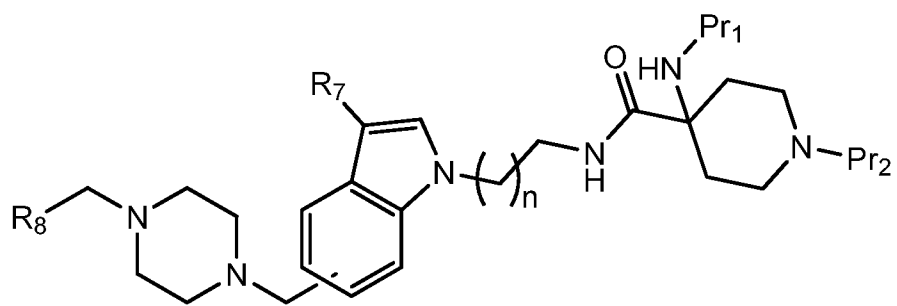
i) reacting a compound having the structure:



with a compound having the structure:



under conditions sufficient to form a compound having the structure:



wherein:

R_7 is selected from the group consisting of H, halide, C_{1-4} aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, $-O-C_{1-4}$ alkyl, and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R_8 is selected from the group consisting of no atom, H, alkyl, aryl and C_{1-4} alkyl-O-aryl, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, and a combination thereof;

n is selected from the group consisting of an integer between 0-5; and

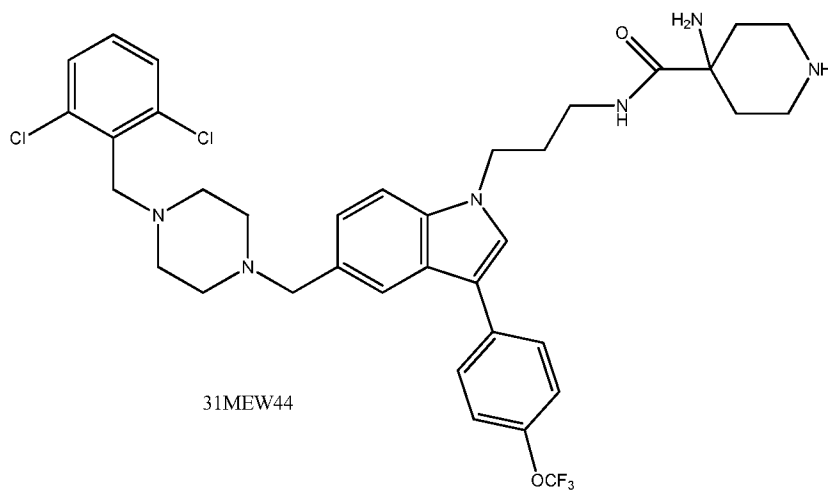
Pr₁ and Pr₂ are independently selected from the group consisting of nitrogen protecting groups;

- ii) removing the Pr₁ protecting group; and
- iii) removing the Pr₂ protecting group.

50. The method of claim 49, wherein the reaction of step i) is carried out in the presence of dimethylformamide.

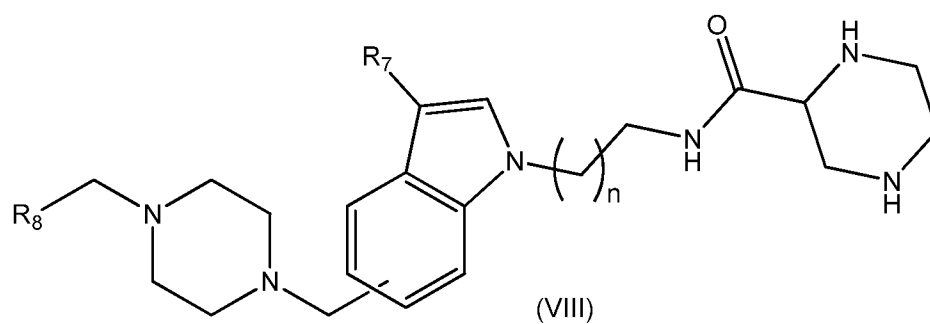
51. The method of claim 49, wherein Pr₁ and Pr₂ are independently selected from from the group consisting of F_{moc} and Boc.

52. The method of claim 49, wherein the compound having the structure of formula (VII) is selected from the group consisting of



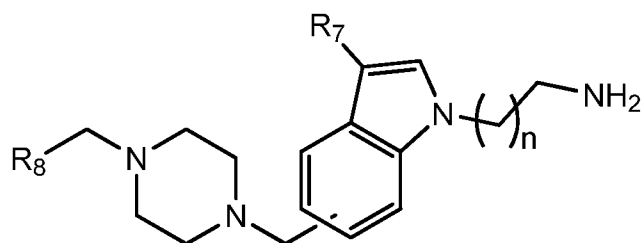


193

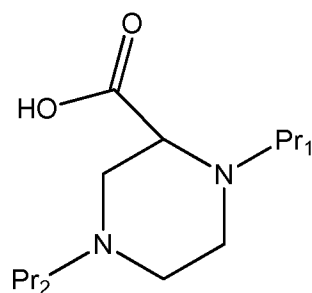


The method comprises the steps of

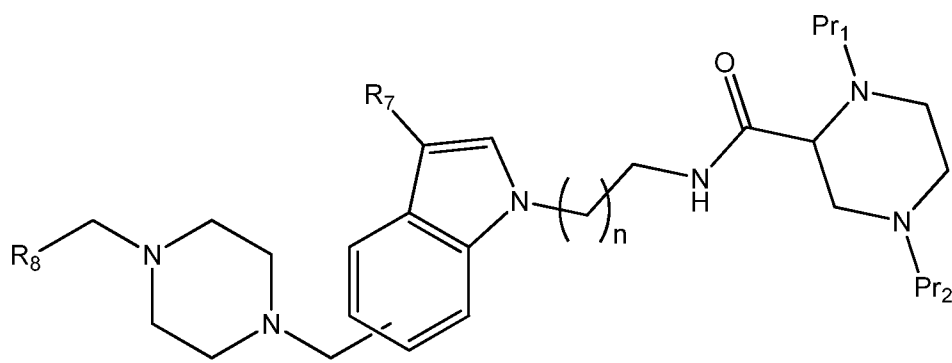
- i) reacting a compound having the structure:



with a compound having the structure:



under conditions sufficient to form a compound having the structure:



wherein:

R_7 is selected from the group consisting of H, halide, C_{1-4} aliphatic, and aryl, wherein the aryl is optionally substituted with one or more

groups consisting of halide, ether, C₁₋₄alkyl, -O-C₁₋₄alkyl, and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R₈ is selected from the group consisting of no atom, H, alkyl, aryl and C₁₋₄alkyl-O-aryl, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with one or more groups consisting of halide, ether, C₁₋₄alkyl, and a combination thereof;

n is selected from the group consisting of an integer between 0-5; and

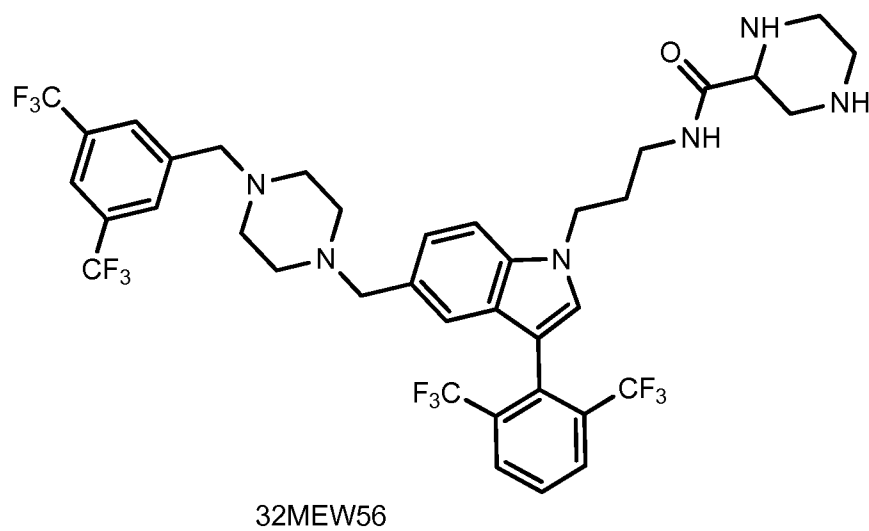
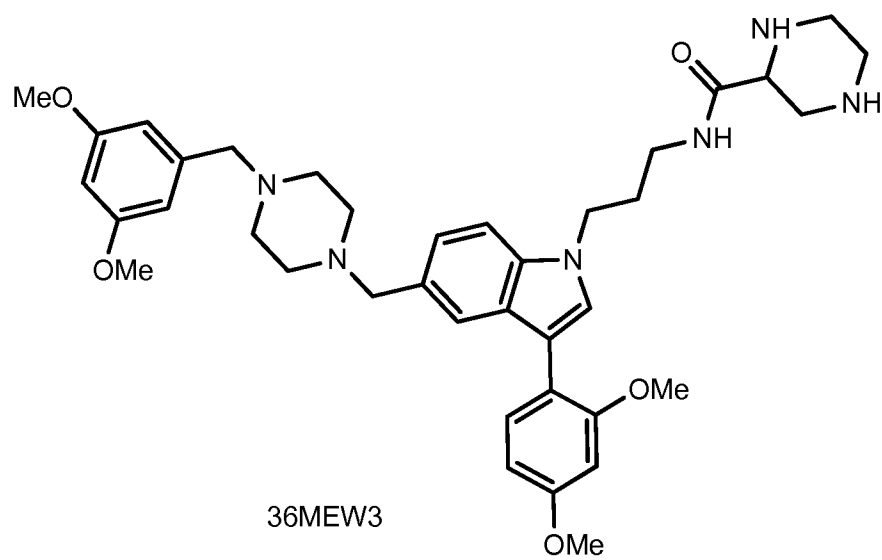
Pr₁ and Pr₂ are independently selected from the group consisting of nitrogen protecting groups;

- ii) removing the Pr₁ protecting group; and
- iii) removing the Pr₂ protecting group.

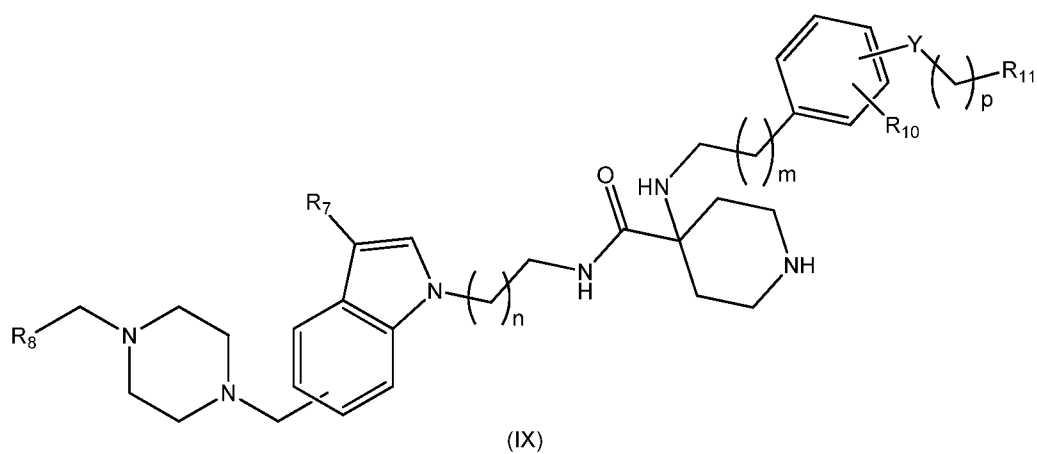
54. The method of claim 49, wherein the reaction of step i) is carried out in the presence of dimethylformamide.

55. The method of claim 49, wherein Pr₁ and Pr₂ are independently selected from the group consisting of F_{moc} and Boc.

56. The method of claim 49, wherein the compound having the structure of formula (VIII) is selected from the group consisting of

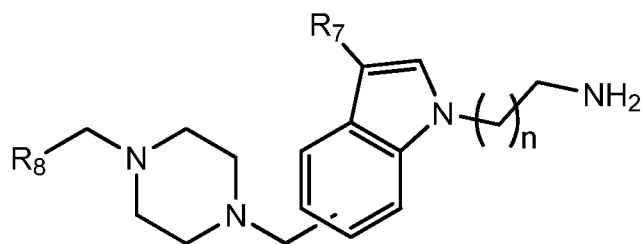


57. A method of preparing a compound having the structure of formula (IX):

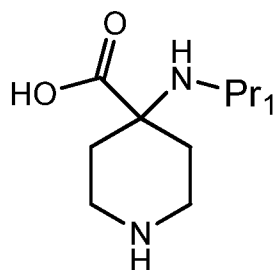


comprising the steps of

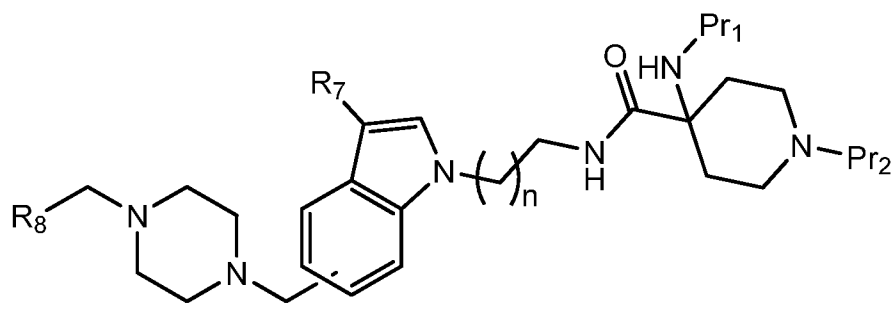
- i) reacting a compound having the structure:



with a compound having the structure:



under conditions sufficient to form a compound having the structure:



wherein:

R_7 is selected from the group consisting of H, halide, C_{1-4} aliphatic, and aryl, wherein the aryl is optionally substituted with one or more groups consisting of halide, ether, C_{1-4} alkyl, $-O-C_{1-4}$ alkyl, and a combination thereof, wherein the alkyl is optionally substituted with one or more groups consisting of halide, ether, and a combination thereof;

R_8 and R_{11} are independently selected from the group consisting of no atom, H, alkyl, aryl and C_{1-4} alkyl-O-aryl, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof, and the aryl is optionally substituted with the group consisting of halide, ether, C_{1-4} alkyl, -O- C_{1-4} alkyl, and a combination thereof, wherein the alkyl is optionally substituted with the group consisting of halide, ether, and a combination thereof;

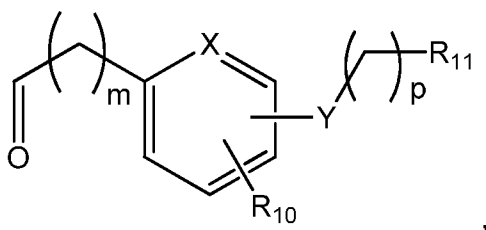
R_{10} is selected from the group consisting of no atom, H, halide, C_{1-4} aliphatic and -O- C_{1-4} alkyl;

Y is selected from the group consisting of no atom and O;

m, n, and p are independently selected from the group consisting of an integer between 0-5; and; Pr_1 and Pr_2 are independently selected from the group consisting of nitrogen protecting groups;

ii) removing the Pr_1 protecting group

iii) reacting the product of step ii) with a compound having the structure:



wherein X is selected from the group consisting of CH and N; and

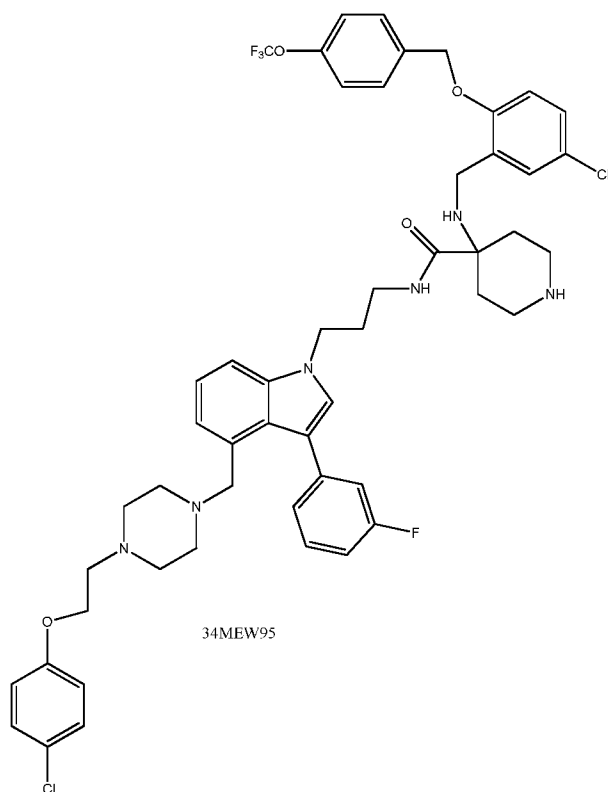
iv) removing the Pr_2 protecting group.

58. The method of claim 57, wherein the reaction of step i) is carried out in the presence of dimethylformamide.

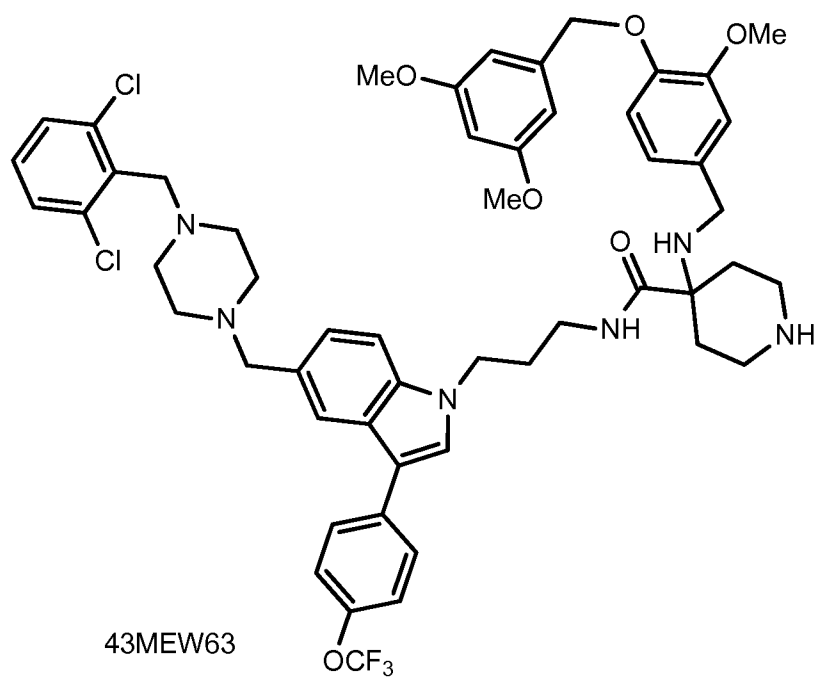
59. The method of claim 57, wherein the reaction of step iii) is carried out in the presence of dichloroethane.

60. The method of claim 57, wherein Pr_1 and Pr_2 are independently selected from the group consisting of F_{moc} and Boc.

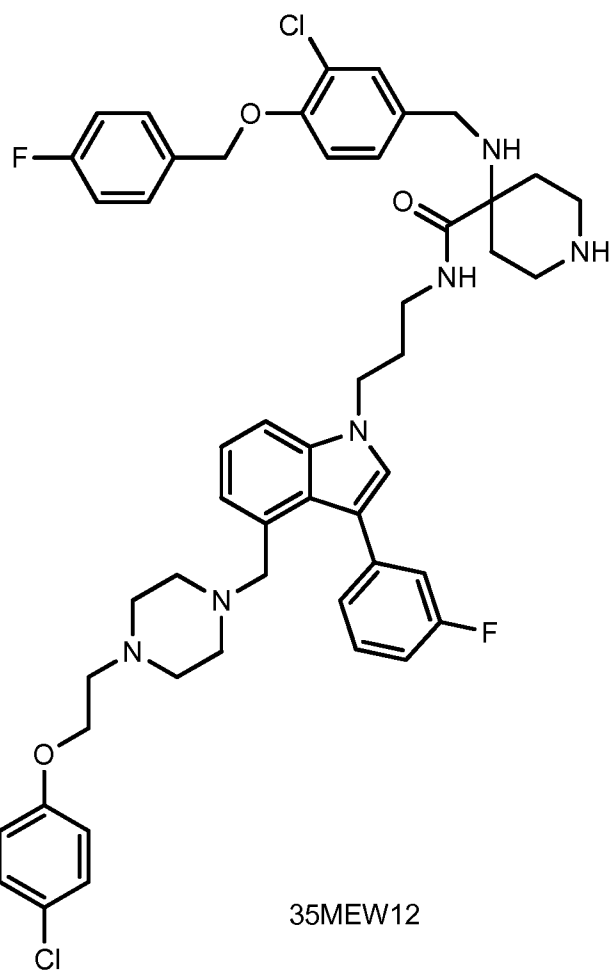
61. The method of claim 57, wherein the compound having the structure of formula (IX) is selected from the group consisting of







, and



62. A method of identifying a multivalent compound which binds selectively to a target protein comprising the steps of

- i) identifying a first and second target site on the target protein, wherein the first and second target sites are adjacent to each other;
- ii) identifying a first compound fragment that selectively binds to the target protein at the first target site and a second compound fragment that selectively binds to the target protein at the second site; and
- iii) creating a structure of the multivalent compound comprising the first compound fragment linked to the second compound fragment, thereby identifying the multivalent compound.

63. The method of claim 62, in which

- step i) further comprises identifying a third target site on the target protein adjacent to the first and or second target site(s);
- step ii) further comprises identifying a third compound fragment that selectively binds to the target protein at the third target site; and
- step iii) further comprises creating a structure of the compound comprising the third compound fragment linked to the first and/or the second compound fragment(s).

64. The method of claim 62 or claim 63, wherein step ii) comprises the steps of

- a) identifying compounds that bind to the target sites from a chemical library; and

b) creating an in silico library based on a set of structural and functional criteria for the compounds identified in step a) to identify compound fragments that are likely to selectively bind to the target sites.

65. The method of claim 64, wherein the criteria comprise fragment size, hydrophobicity, electrophilicity/nucleophilicity and ability to form hydrogen bonds.

66. The method of claim 64, wherein each in silico library consists essentially of synthetically feasible fragments.

67. The method of claim 64, wherein compound fragments are identified based on high docking scores.

68. The method of claim 62 or claim 63, wherein the target sites are shallow sites.

69. The method of claim 62 or claim 63, wherein the target sites are present at a position where the target protein binds to a second protein.

70. The method of claim 69, wherein the multivalent compound reduces binding of the target protein to the second protein.

71. The method of claim 62 or claim 63, wherein the target protein is a GTPase.

72. The method of claim 71, wherein the target protein is a RAS protein.
73. The method of claim 72, wherein the target protein is KRAS.
70. The method of claim 73, wherein the target protein is KRAS^{G12D}.
71. The method of claim 62 or claim 63, wherein the first target site is D38.
72. The method of claim 62 or claim 63, wherein the second target site is A59.
73. The method of claim 63, wherein the third target site is Y32.

FIG. 1

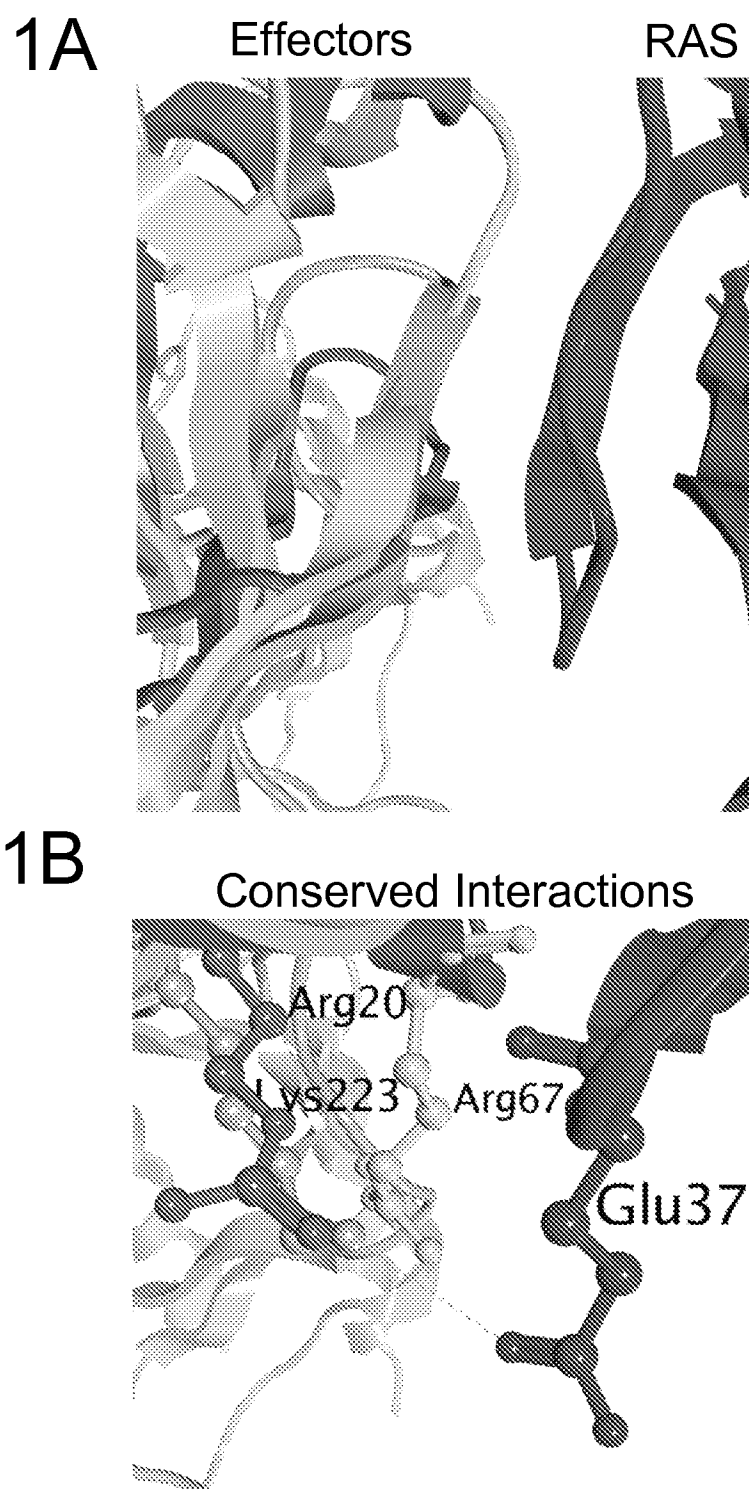
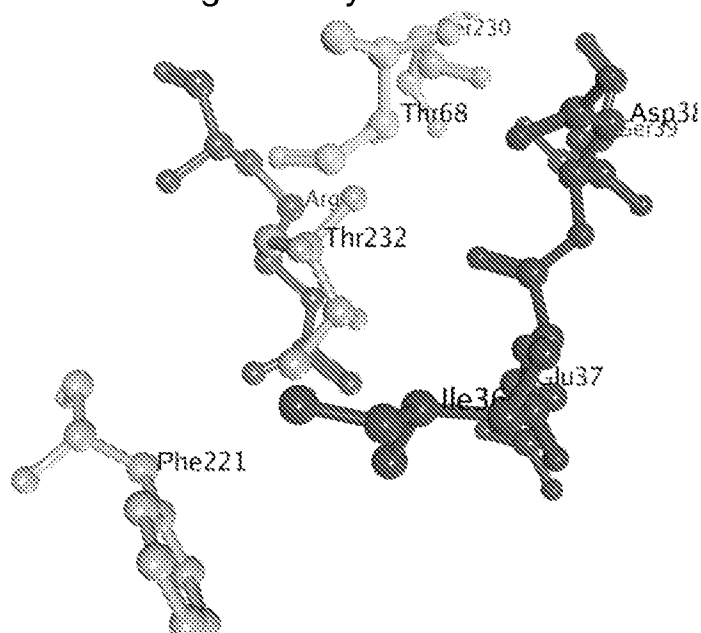


FIG. 1, Con't

1C

Selected effector residues
interacting with key RAS residues



1D

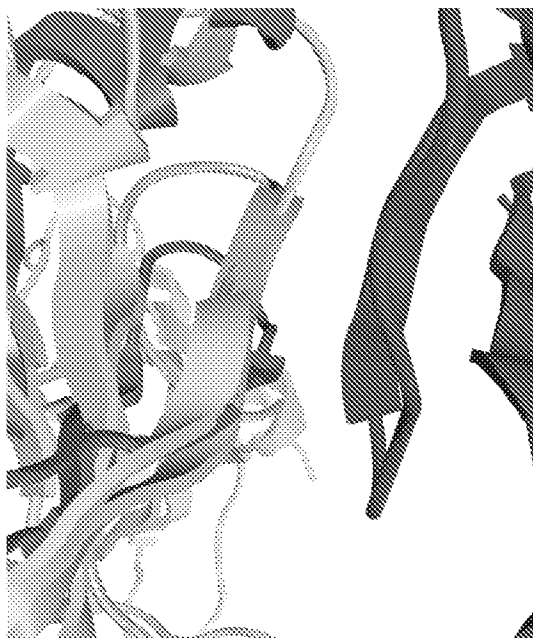


FIG. 1, Con't

1E



1F

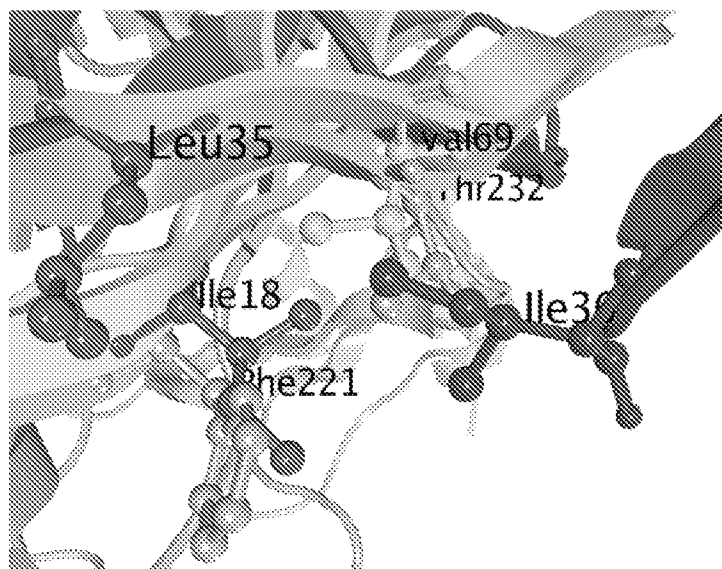
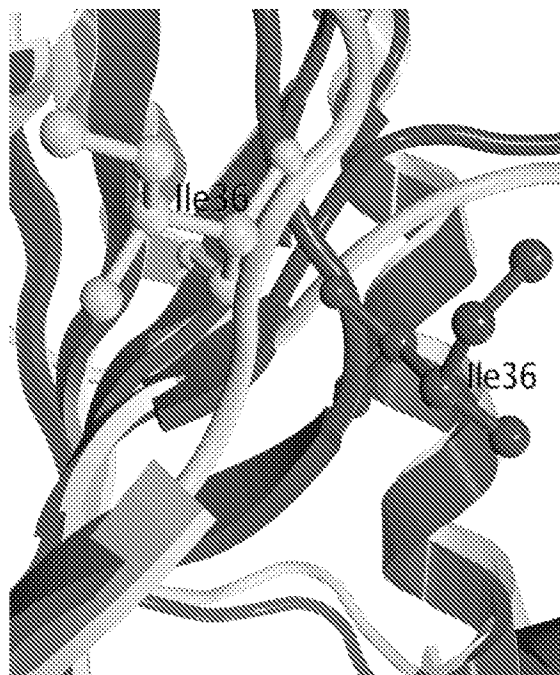


FIG. 1, Con't

1G



1H

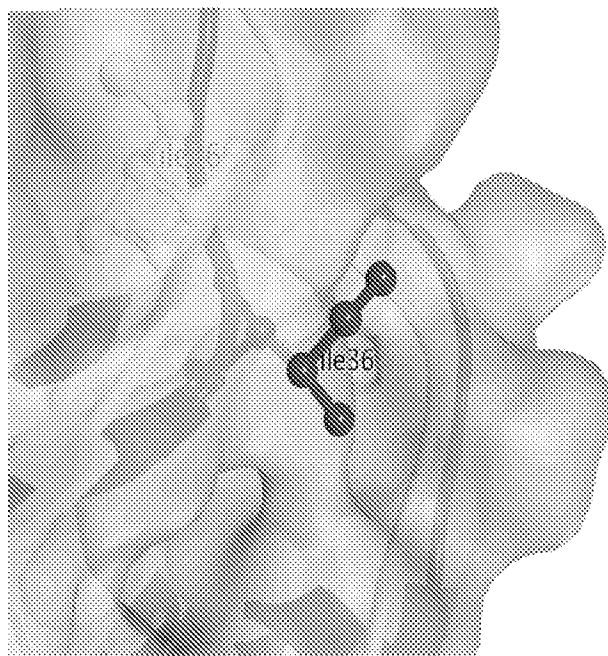
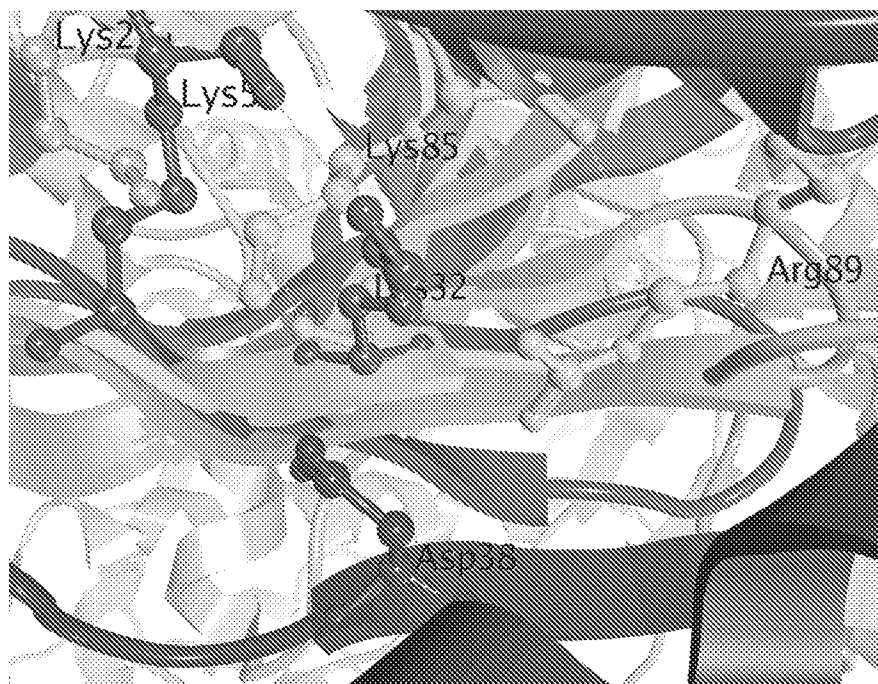


FIG. 1, Con't

1I



1J

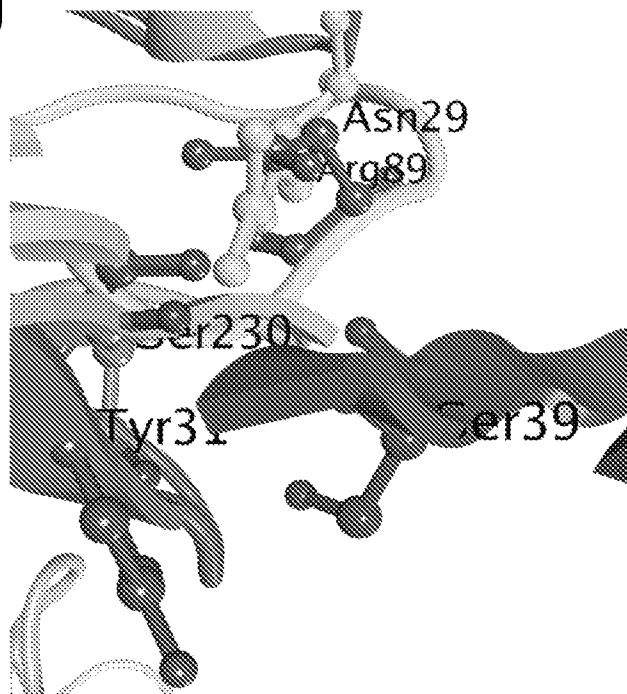
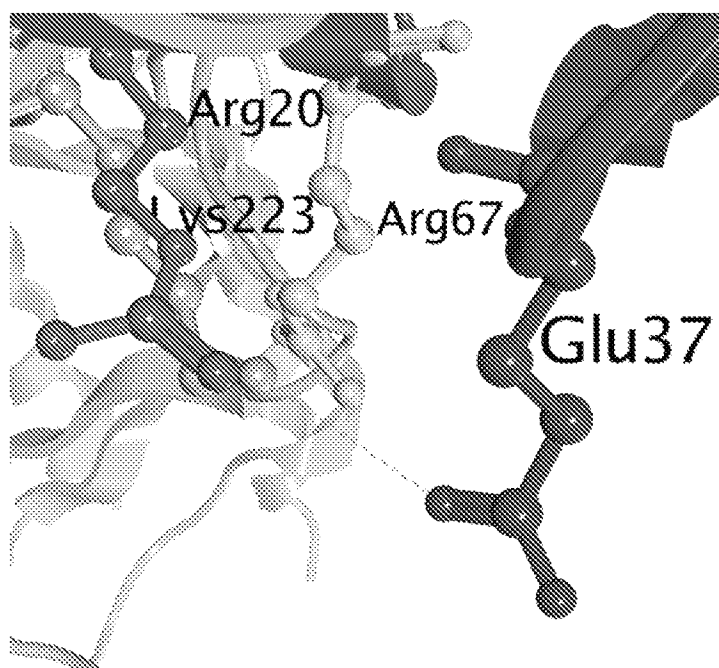


FIG. 1, Con't

1K



1L

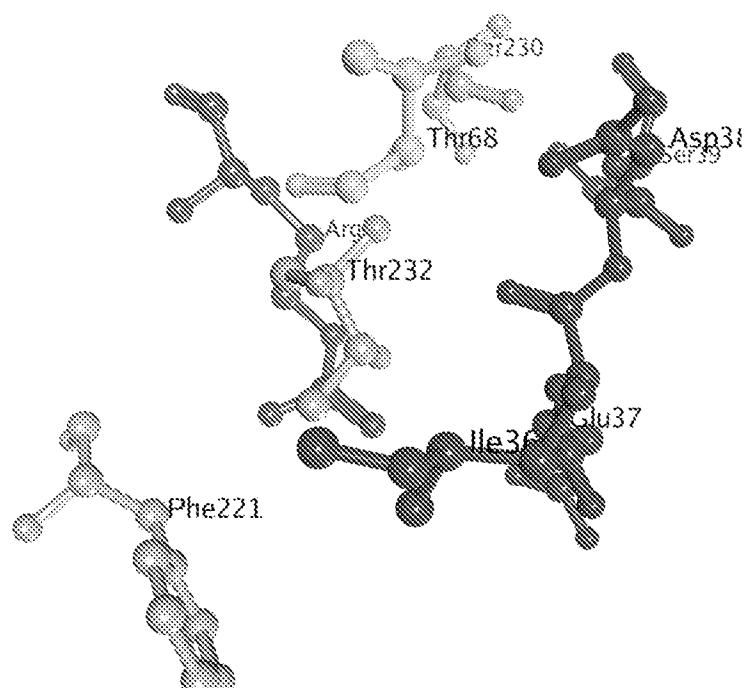
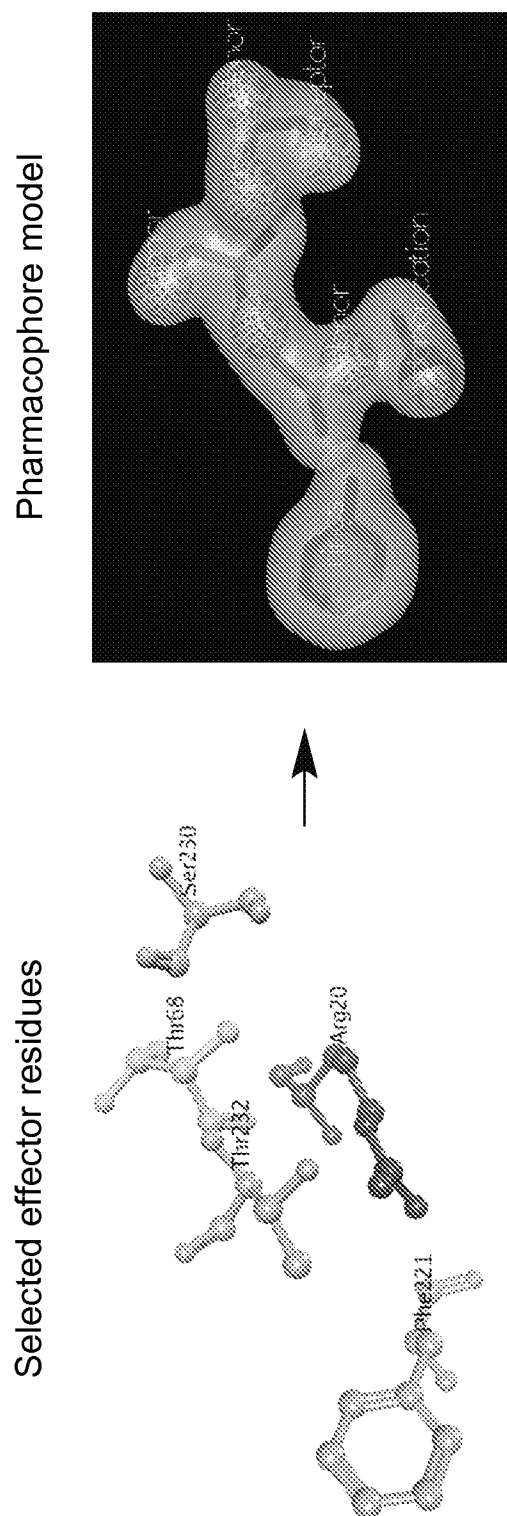


FIG. 2



F/G.3

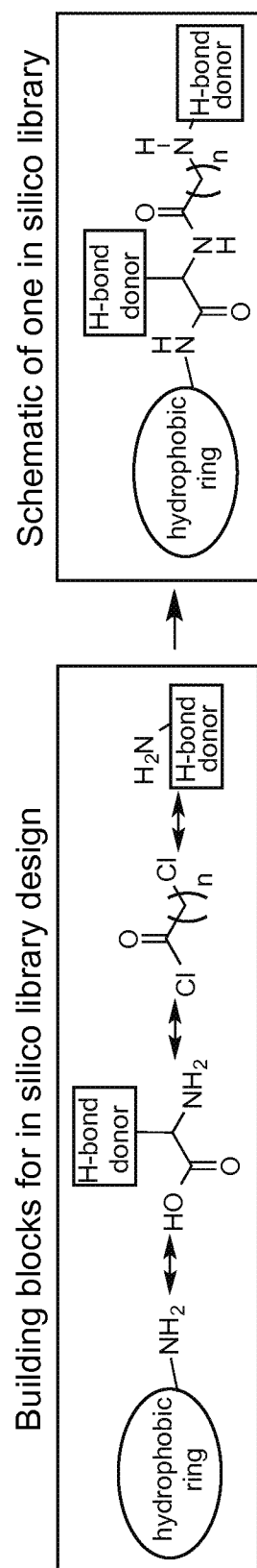


FIG. 4

4A

Top pharmacophore compounds

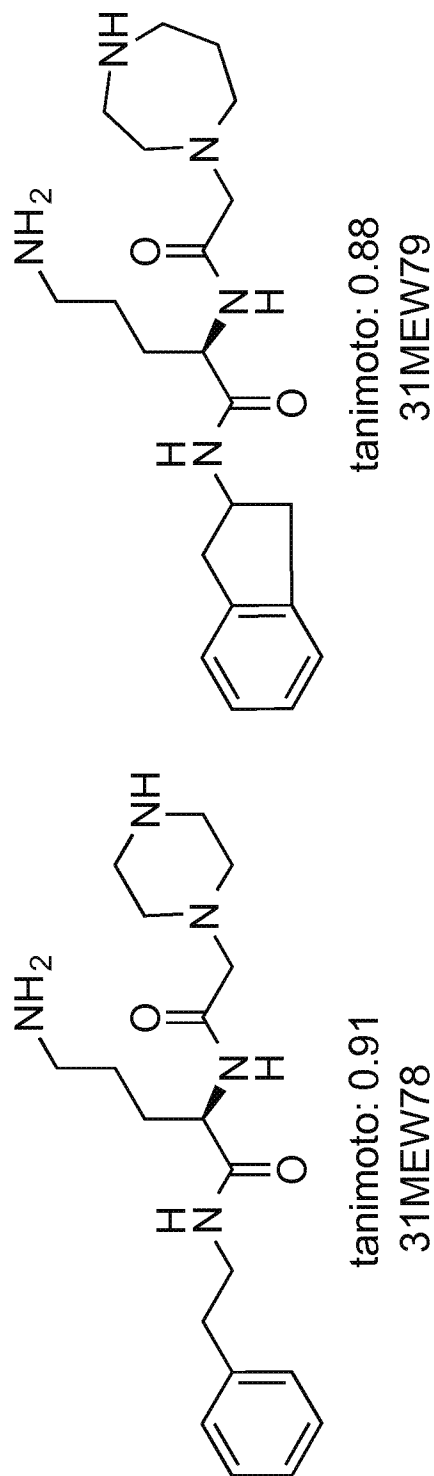


FIG. 4 Con't

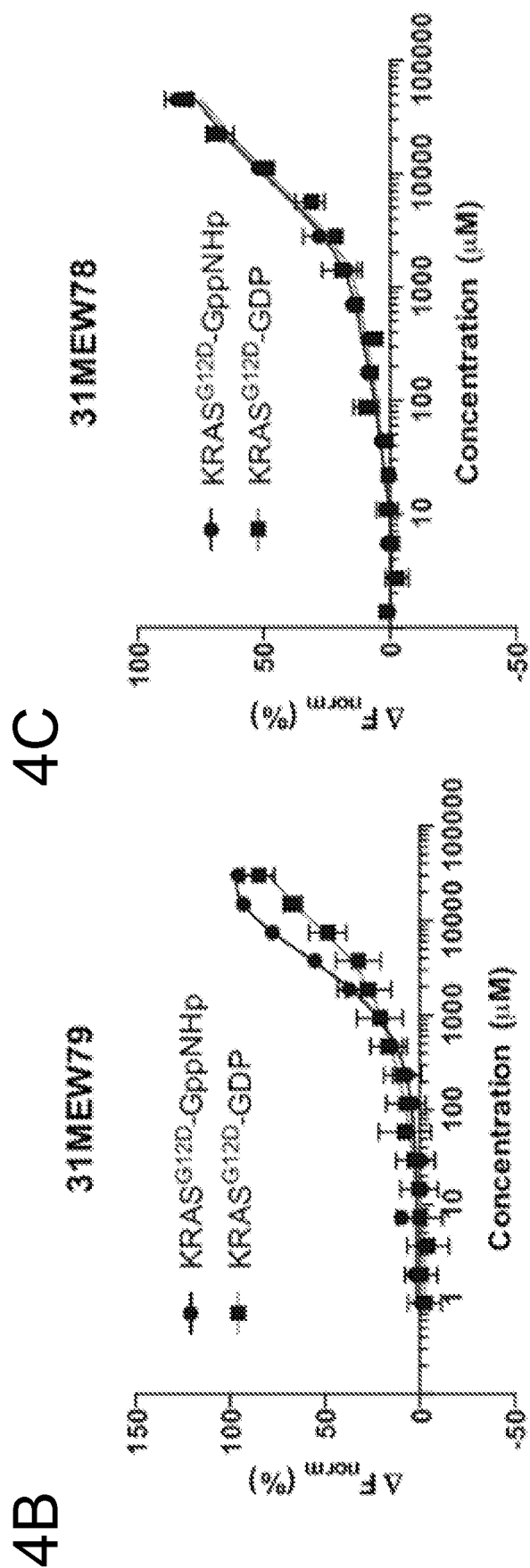


FIG. 5

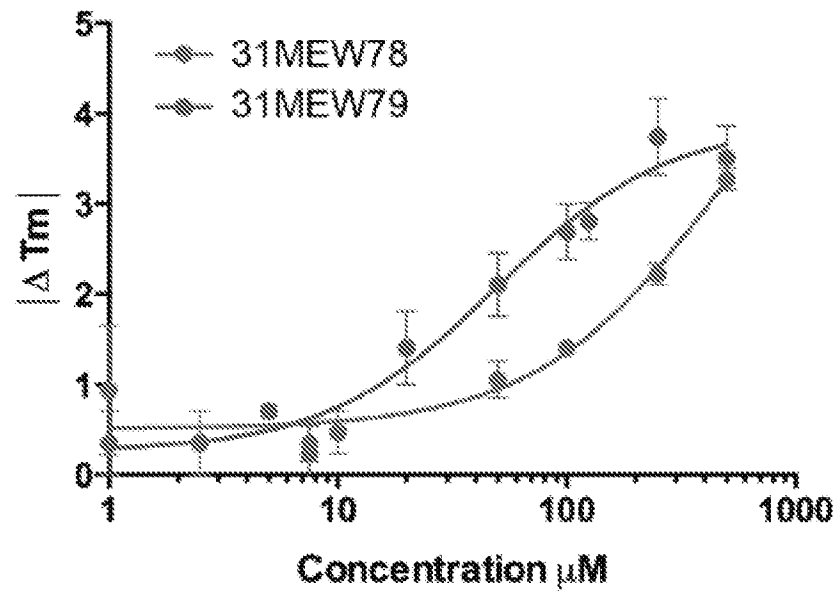


FIG. 6

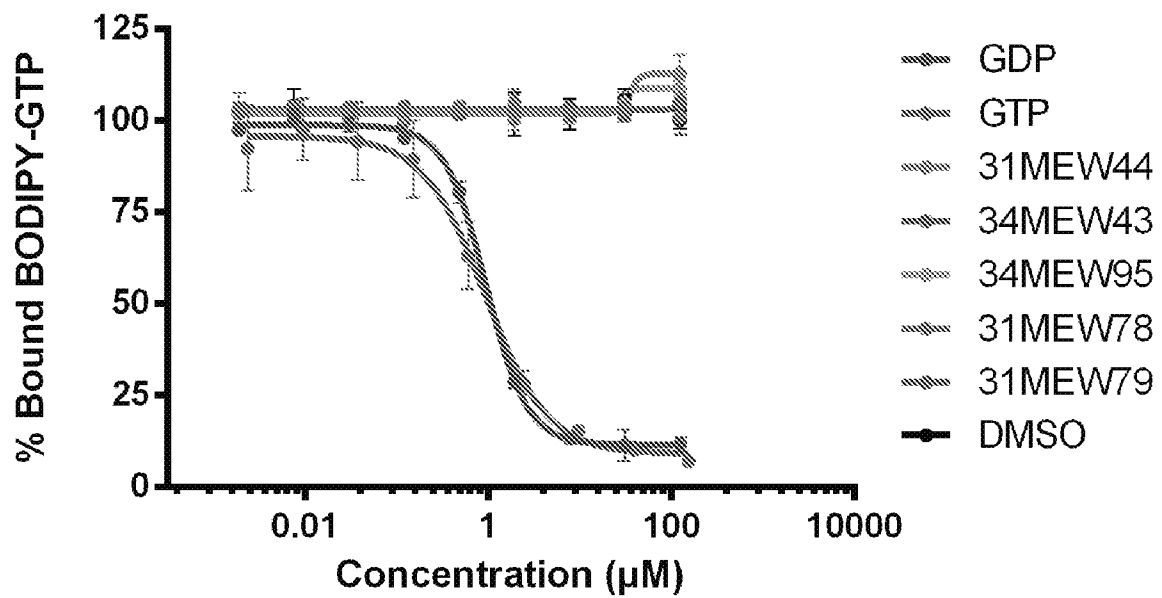


FIG. 7

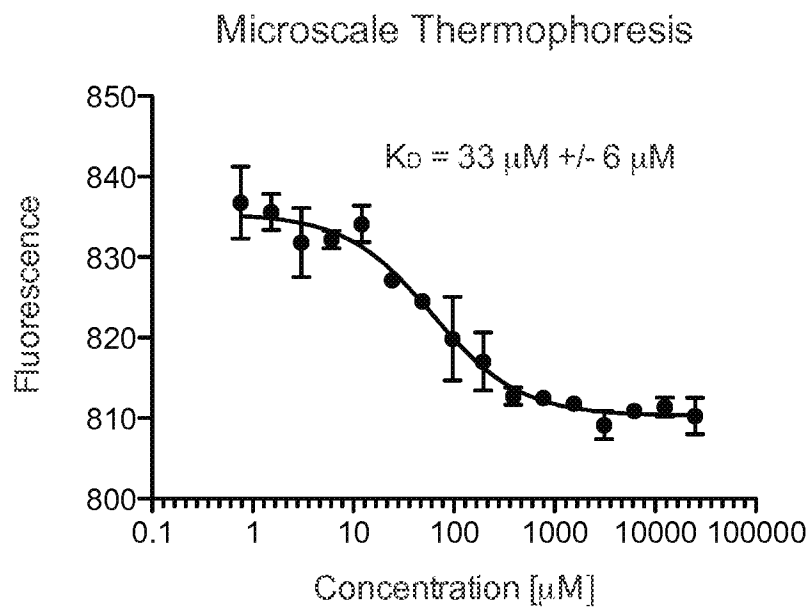
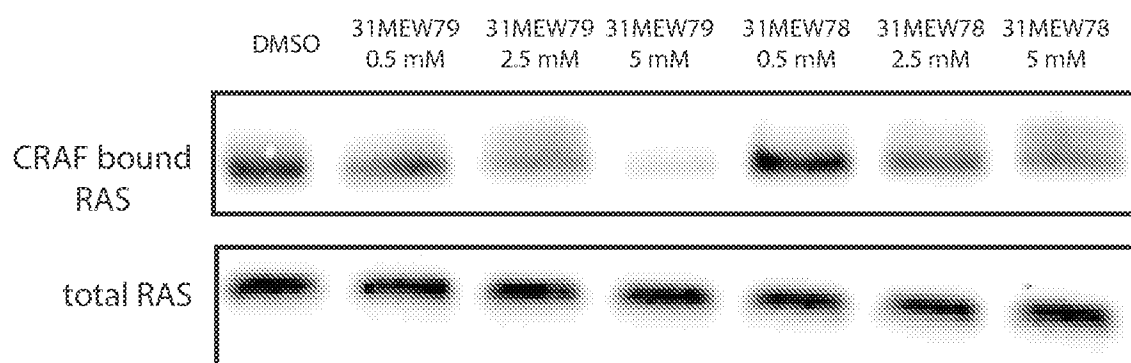


FIG. 8



9A

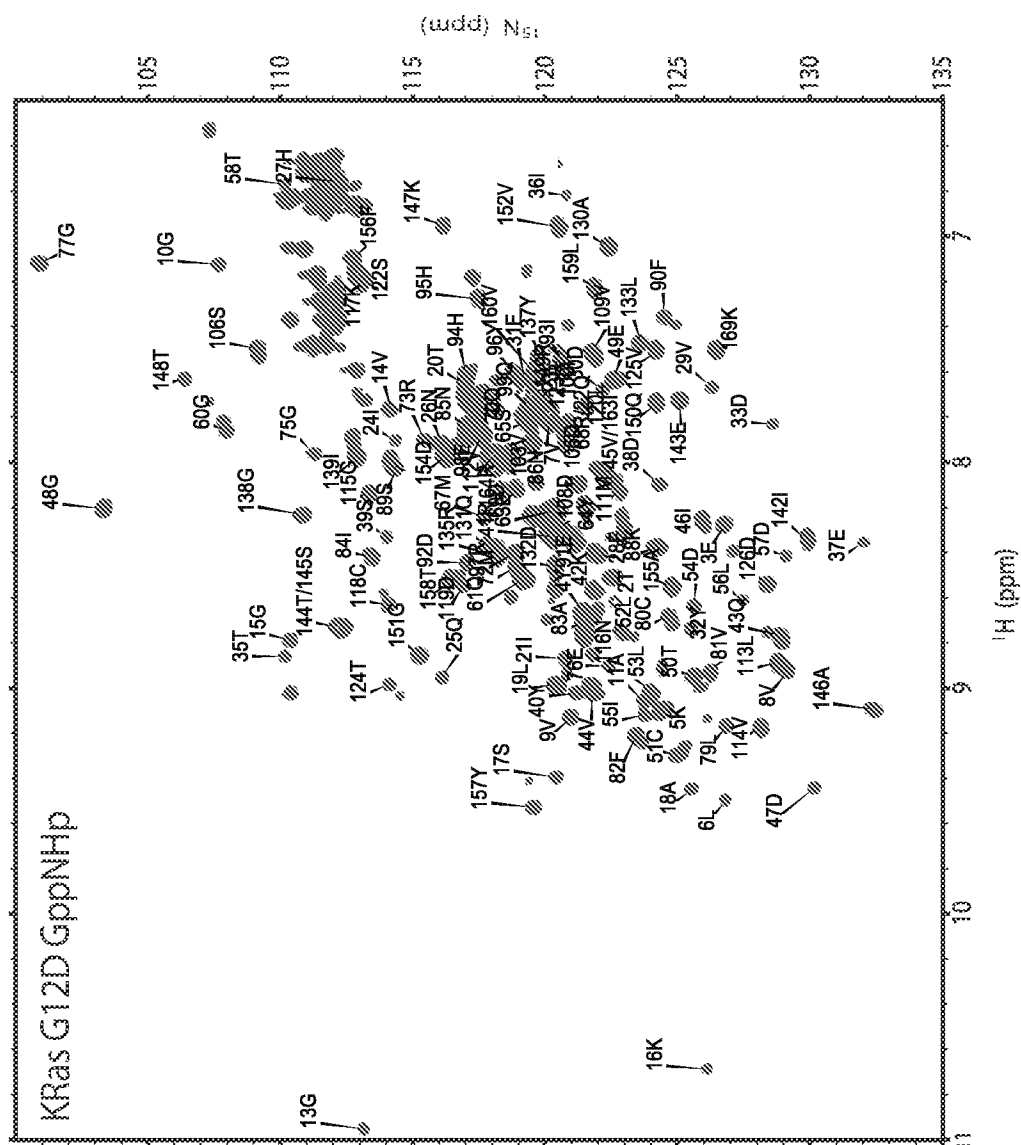


FIG. 9, Con't

9B

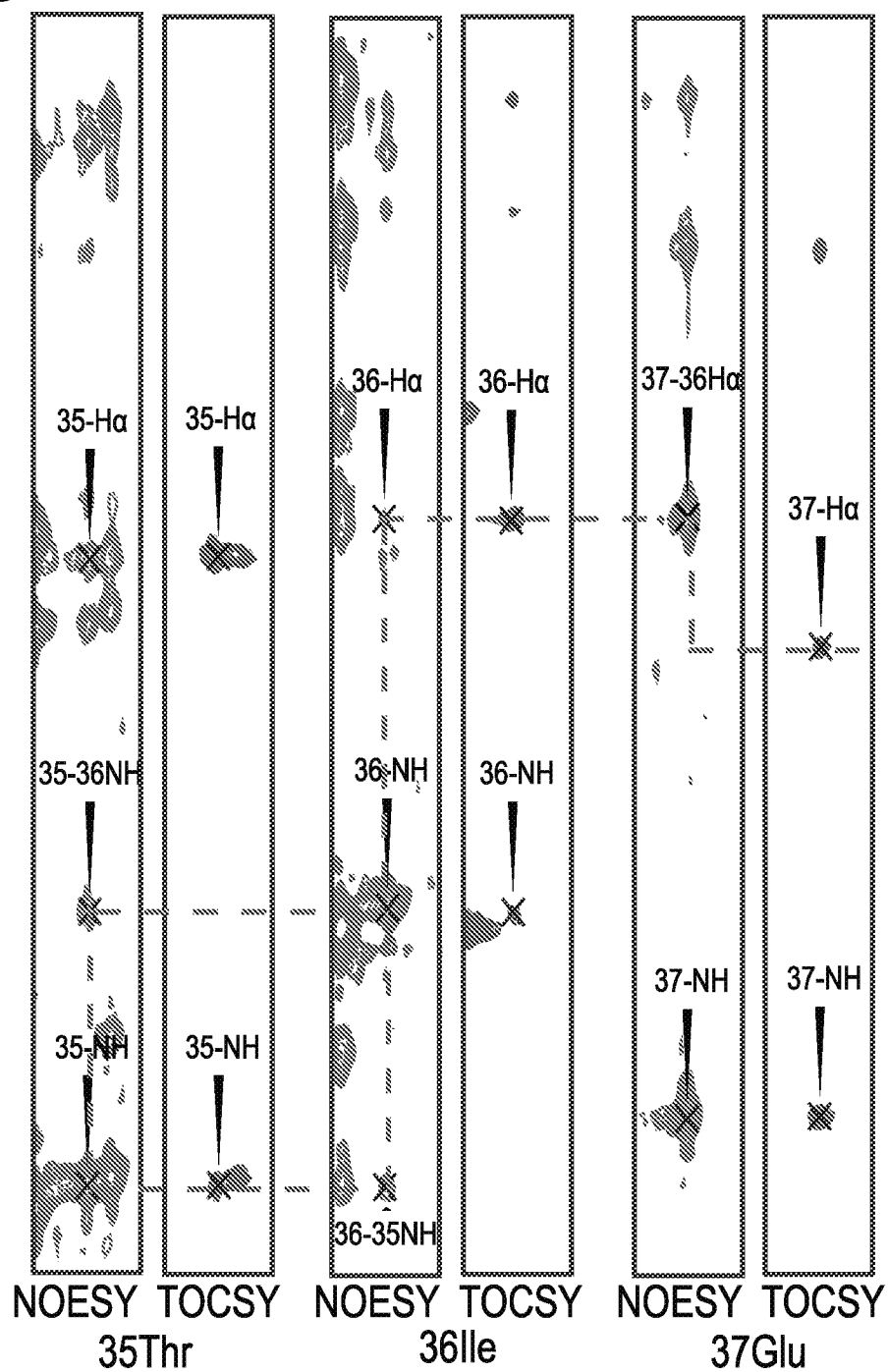


FIG. 10

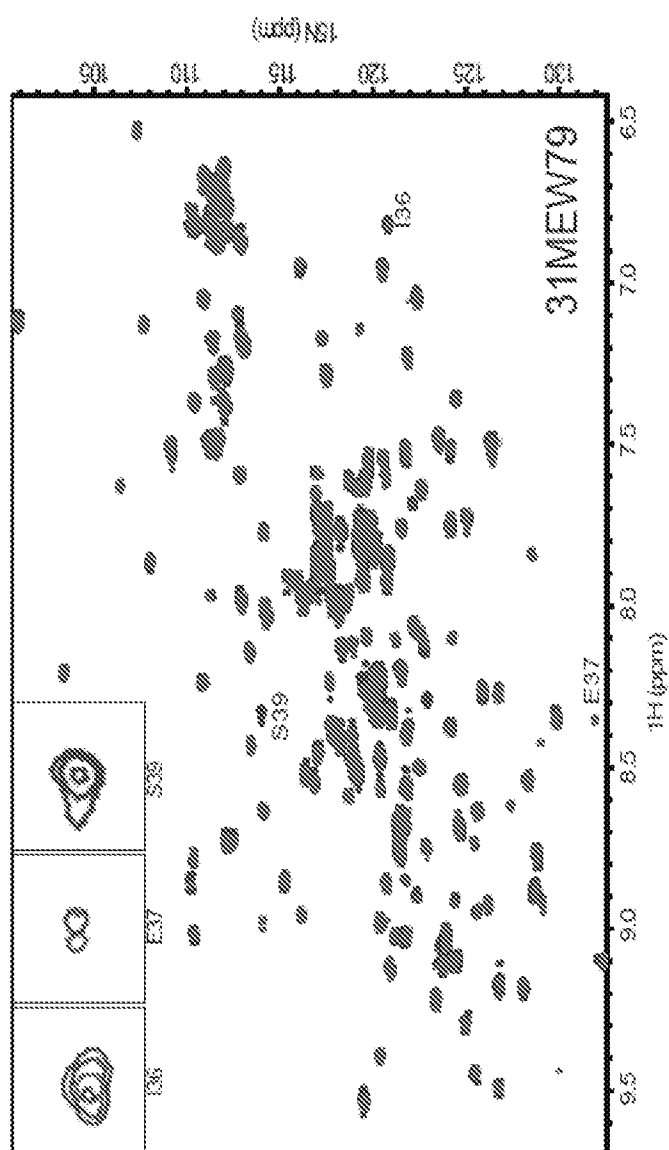


FIG. 11

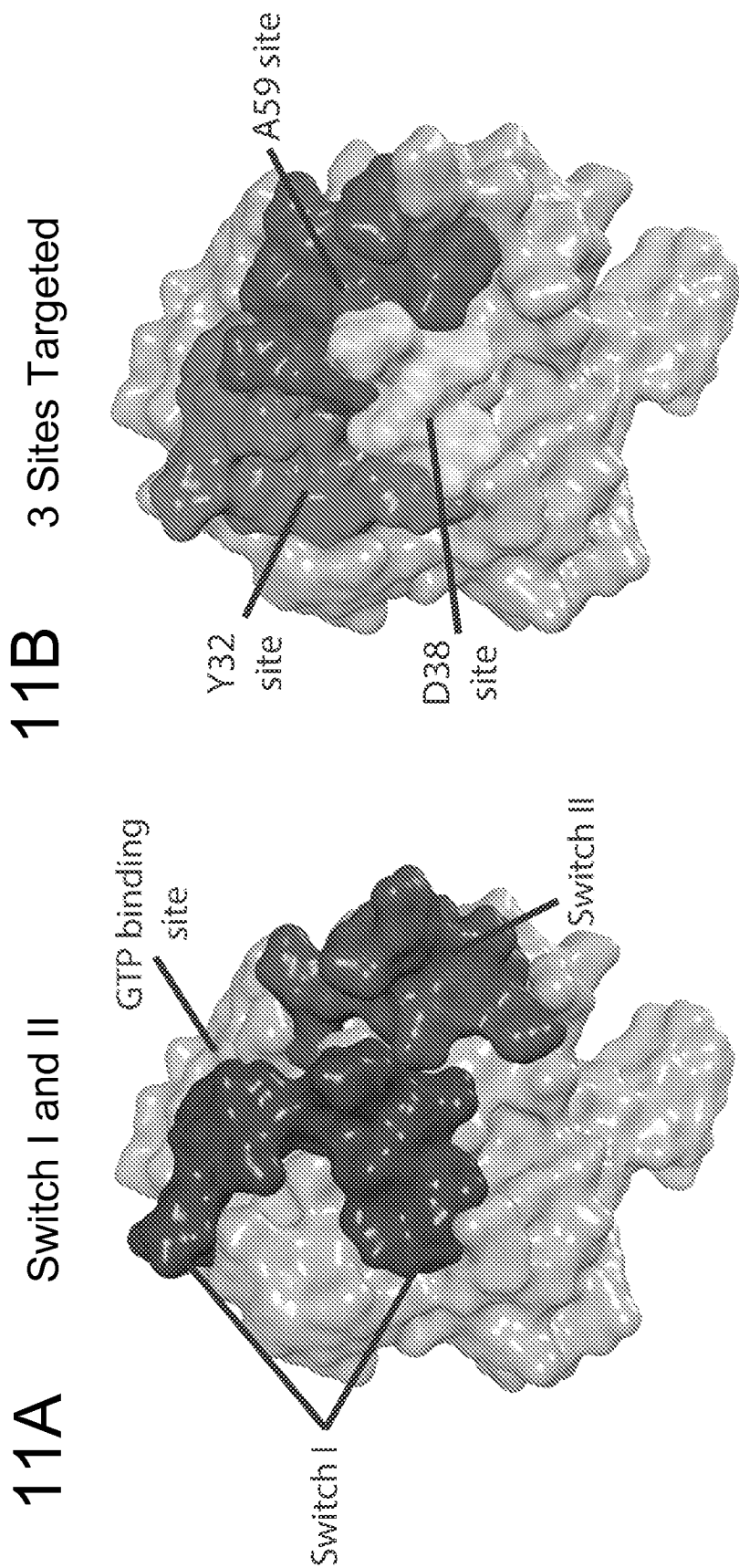
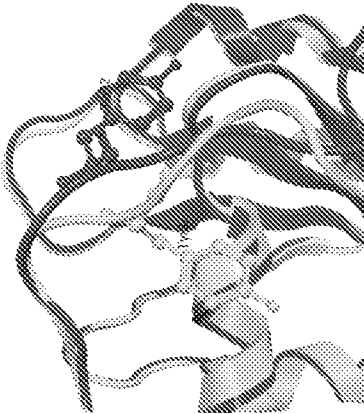
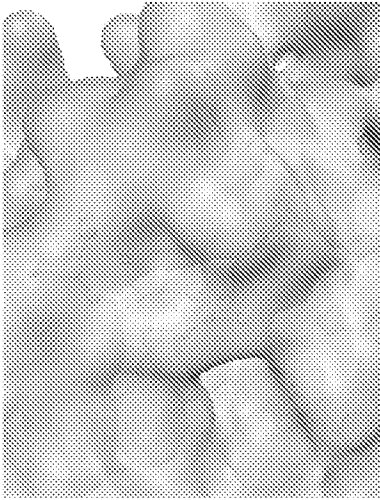


FIG. 11, Con't

11C



11D



11E

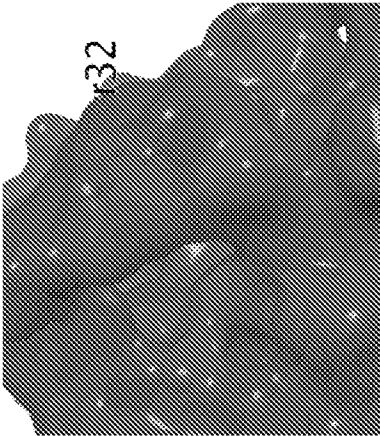
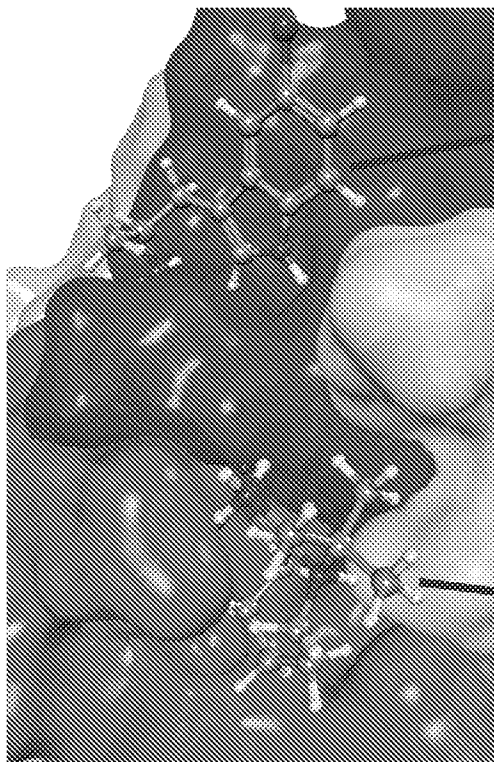


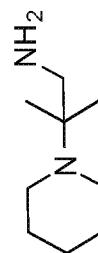
FIG. 12

12A

Top-scoring commercial
fragments in D38 and A59 sites

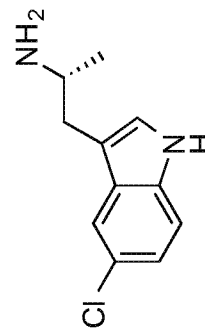
D38 fragment

docking score: -8.33

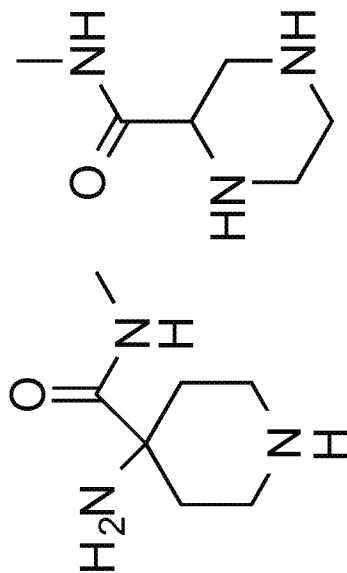


A59 fragment

docking score: -8.50



12B

Top-scoring designed D38
fragments

docking score: -8.39 docking score: -8.38

FIG. 12, Con't

12C

Different indole substitutions designed for A59 site

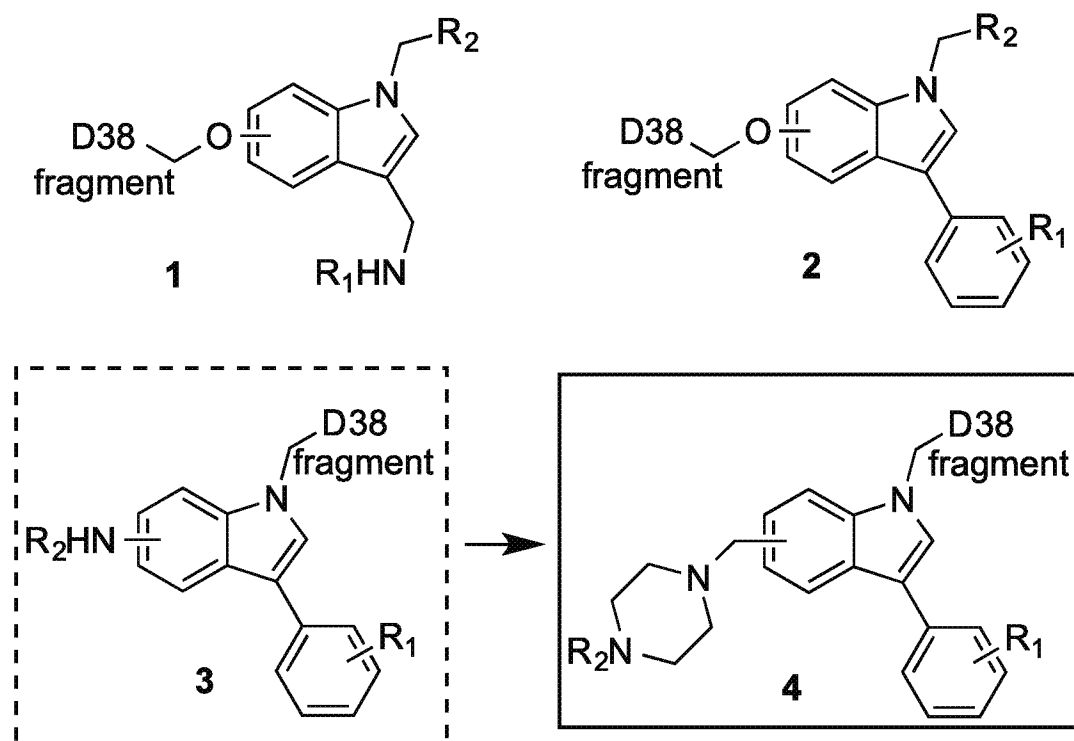
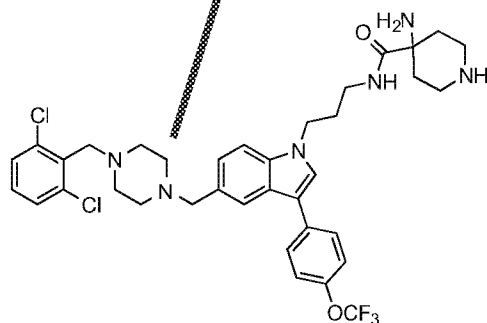
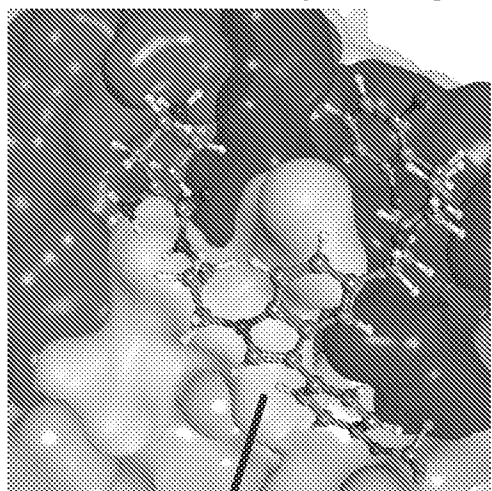


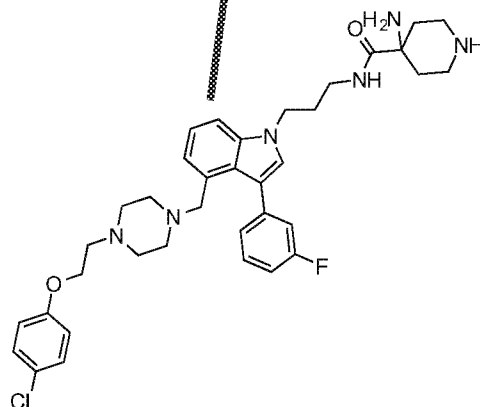
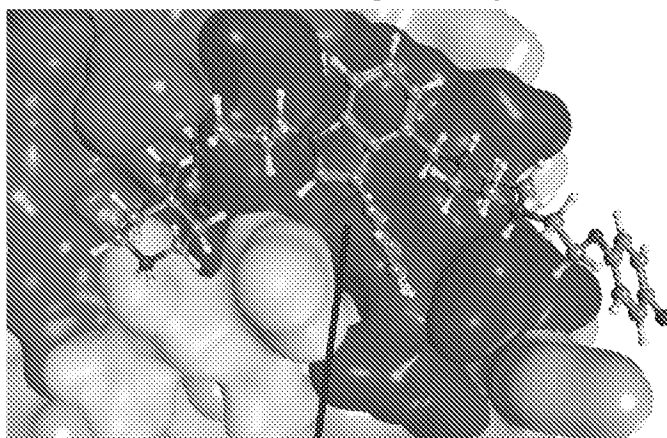
FIG. 12, Con't

12D

Two of the top designed A59-D38 binding compounds



31MEW44
docking score: -9.33

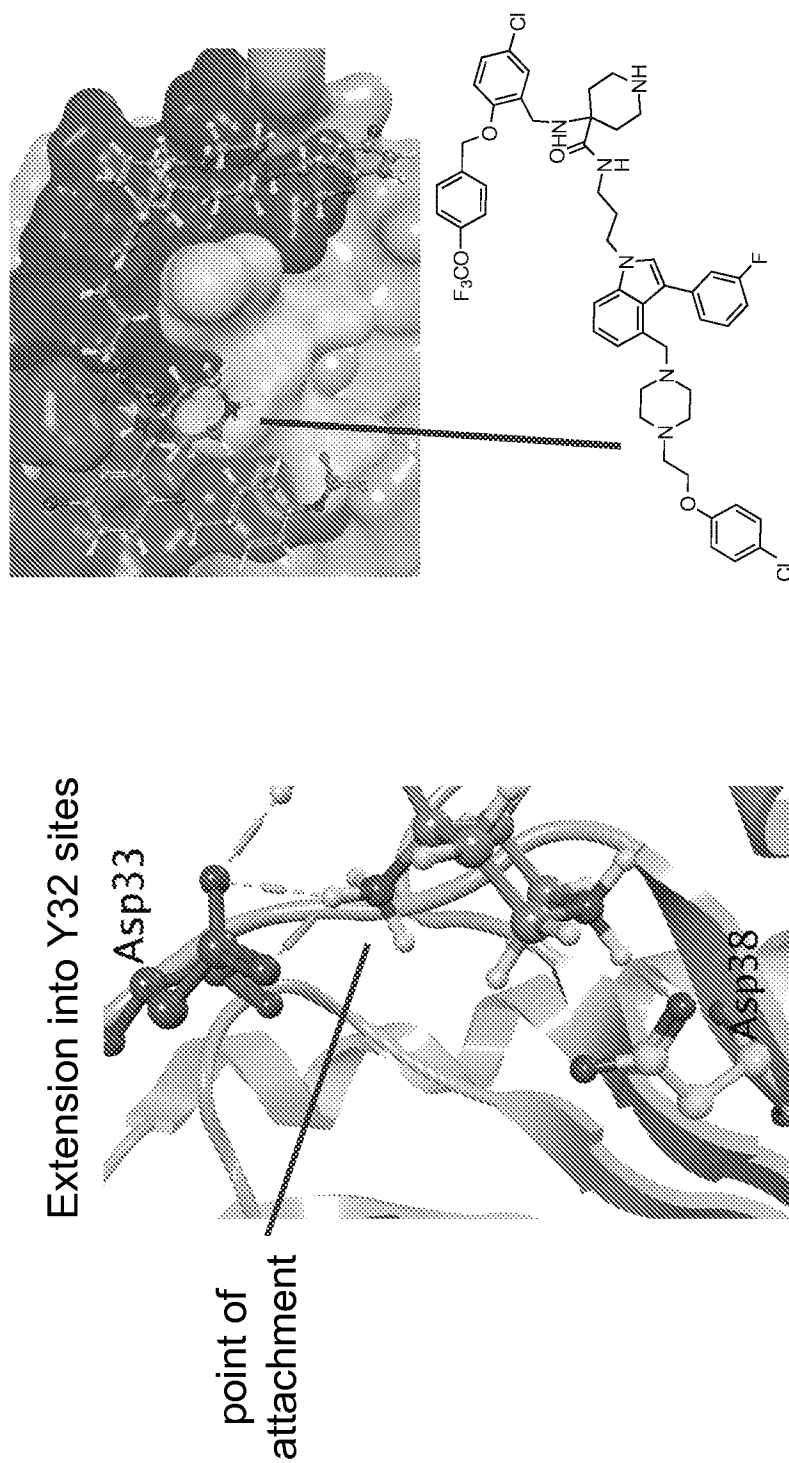


34MEW43
docking score: -9.60

13A

13B

3-site compound based on 34MEW43



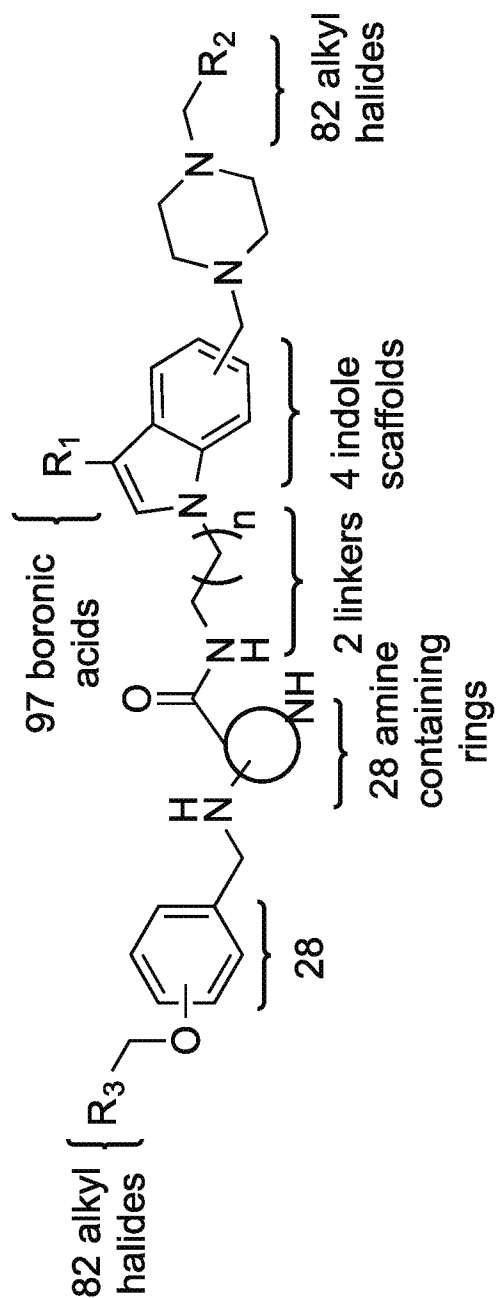
34MEW95

docking score: -10.0

FIG. 13, Con't

13C

Schematic of the 3-site compound design



Single full compound library

fragment growth approach

VS.

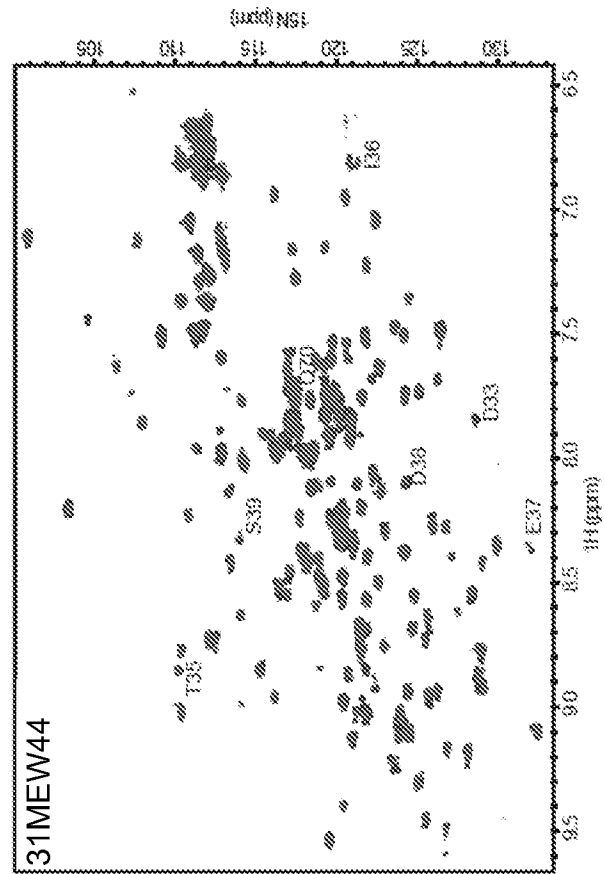
$$82 \times 4 \times 97 \times 2 \times 28 \times 82 \times 25$$

$$3,652,476,800$$

$$28 + 82 \times 4 \times 97 \times 2 \times 2 = 127,264 + \text{active A59-D38 compounds} \times 82 \times 25 = 2,050$$

$$129,342$$

FIG. 14



14A

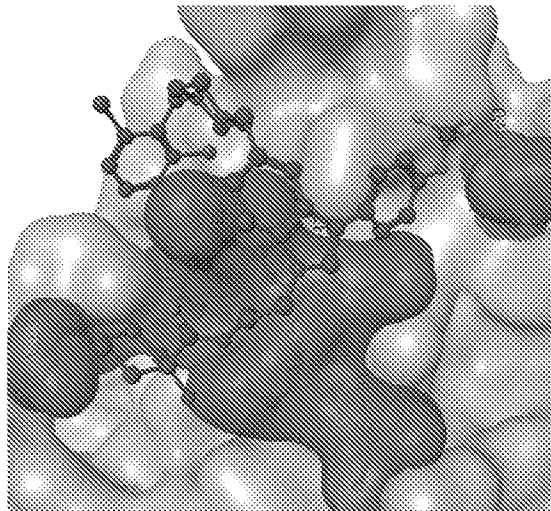


FIG. 14, Con't

14B

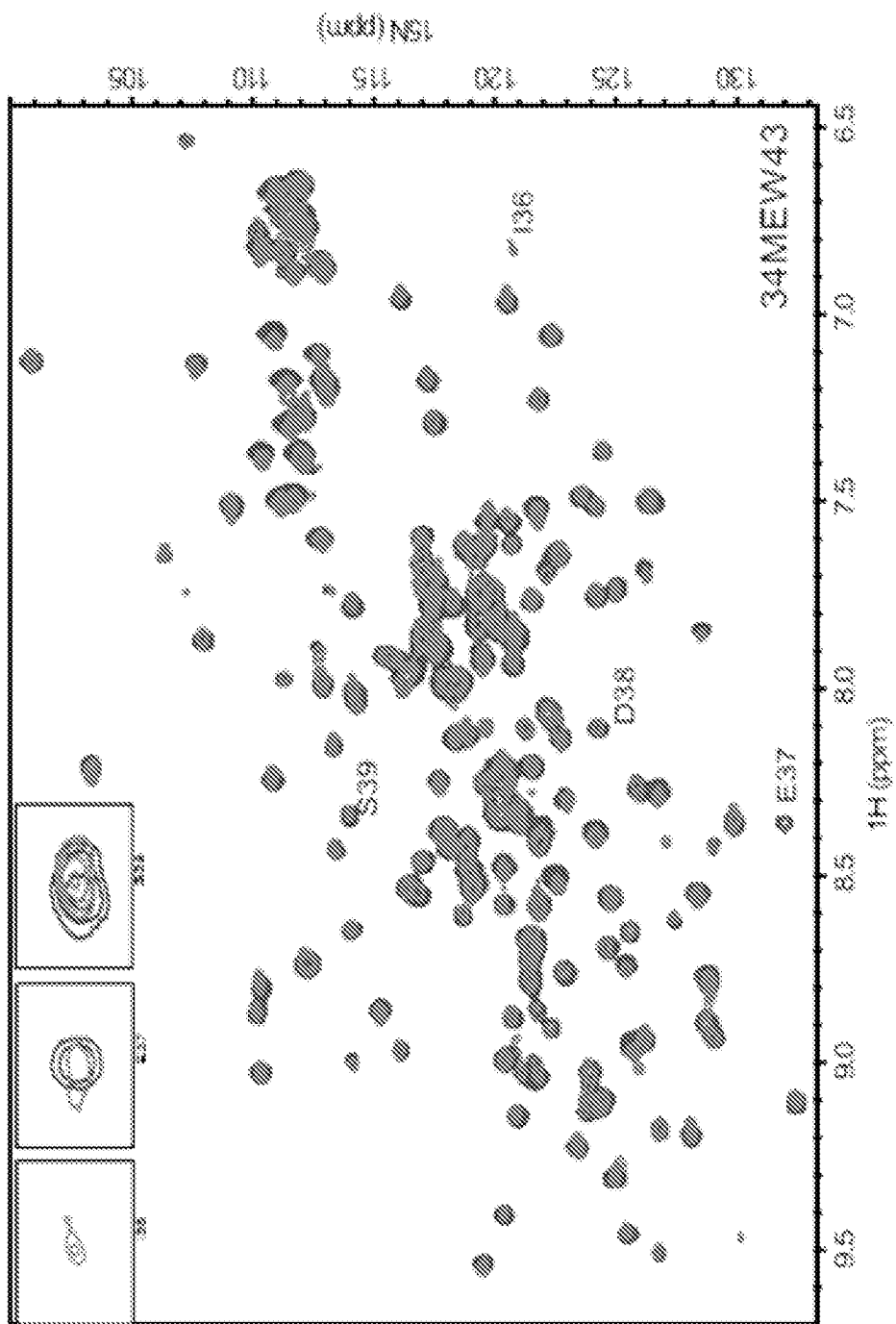
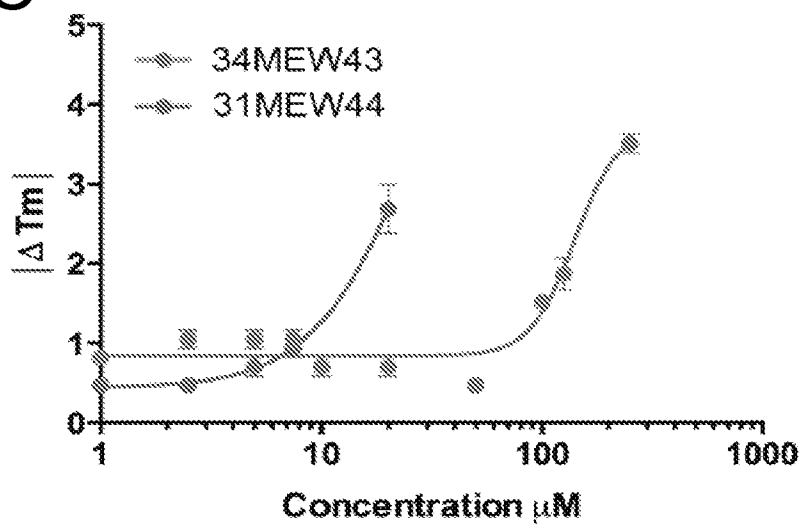
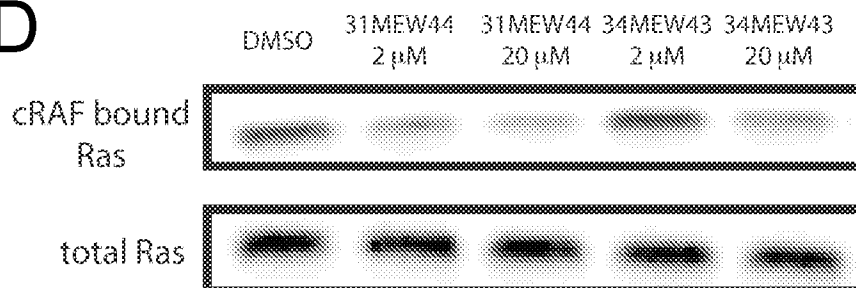


FIG. 14, Con't

14C



14D



14E

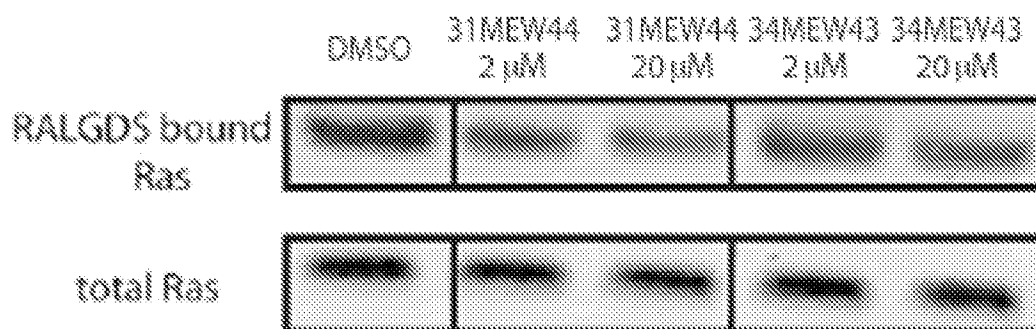
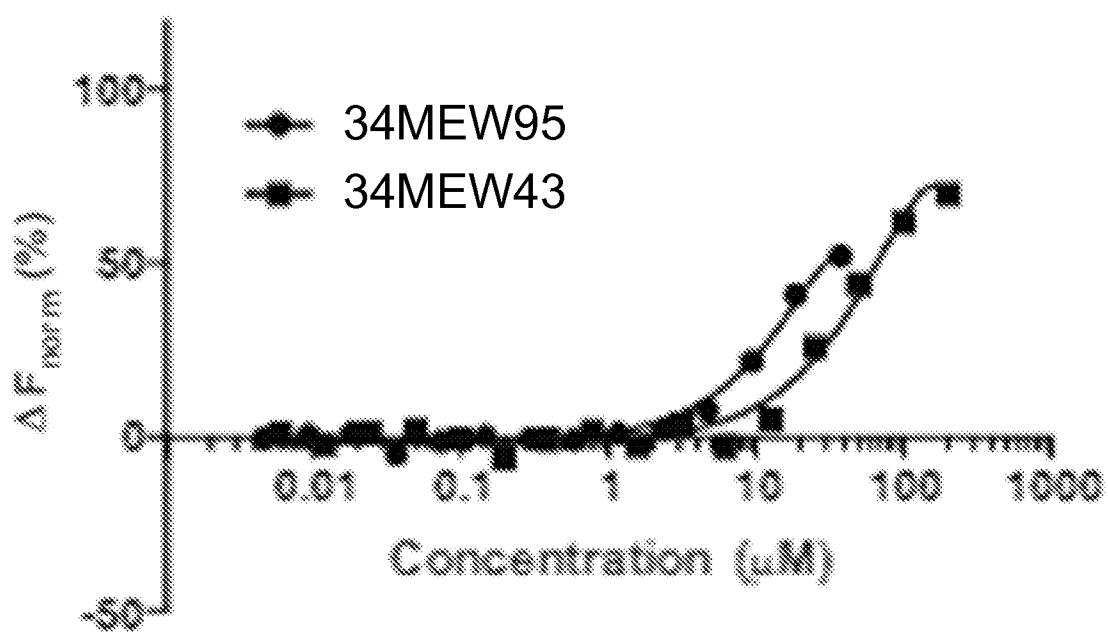


FIG. 14, Con't

14F

Extension from D38 into Y32



14G

2-site compound 31MEW44

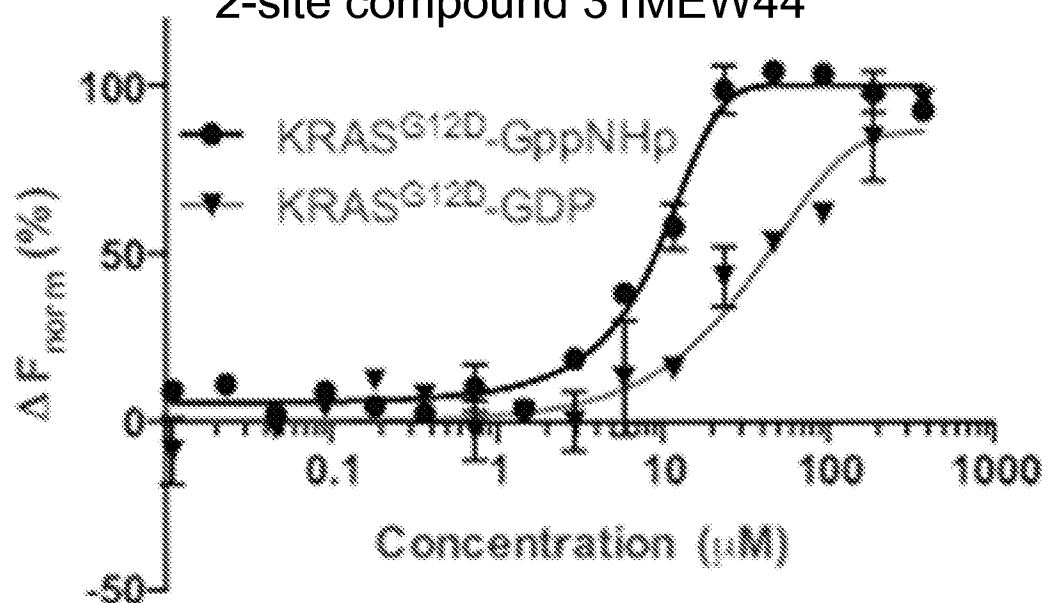
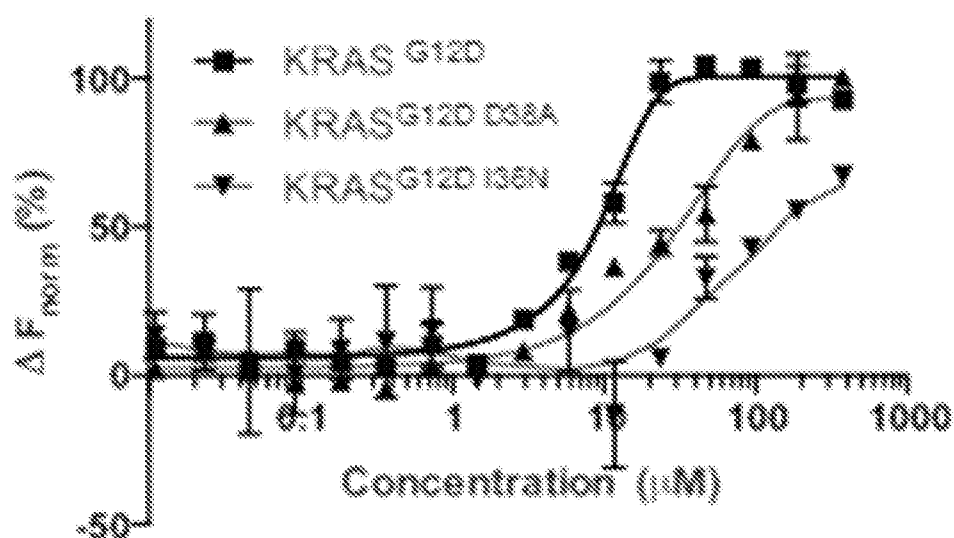


FIG. 14 Con't

14H

Effect of binding site mutations



14I

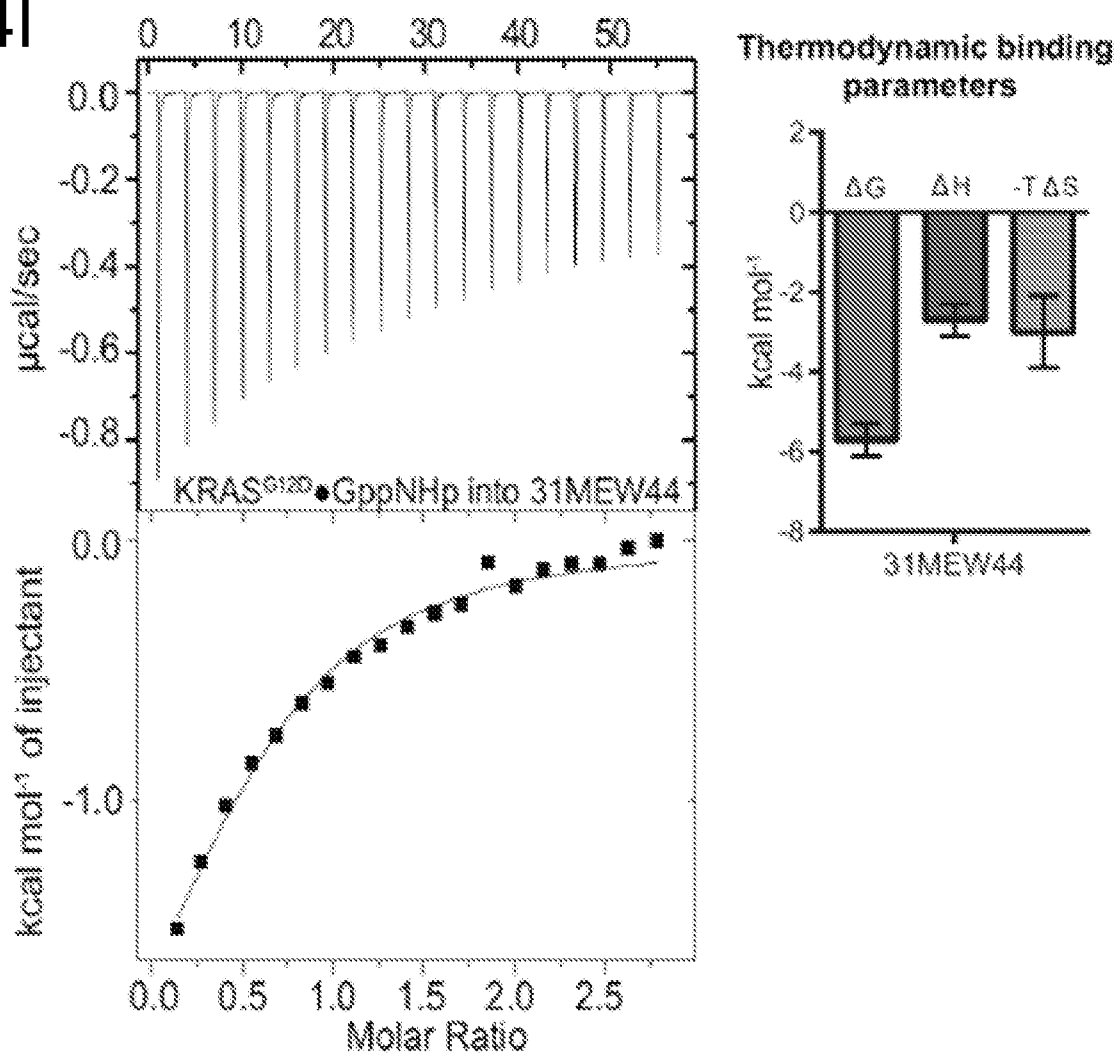


FIG. 14 Con't

14J

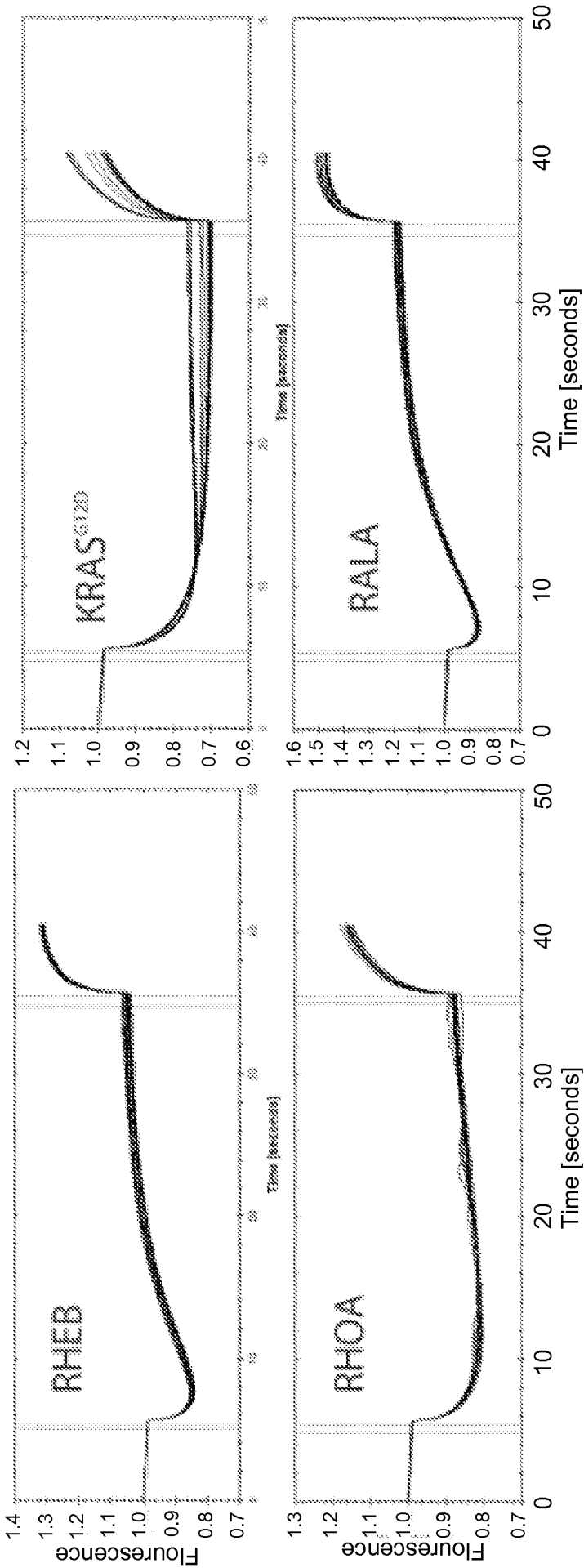
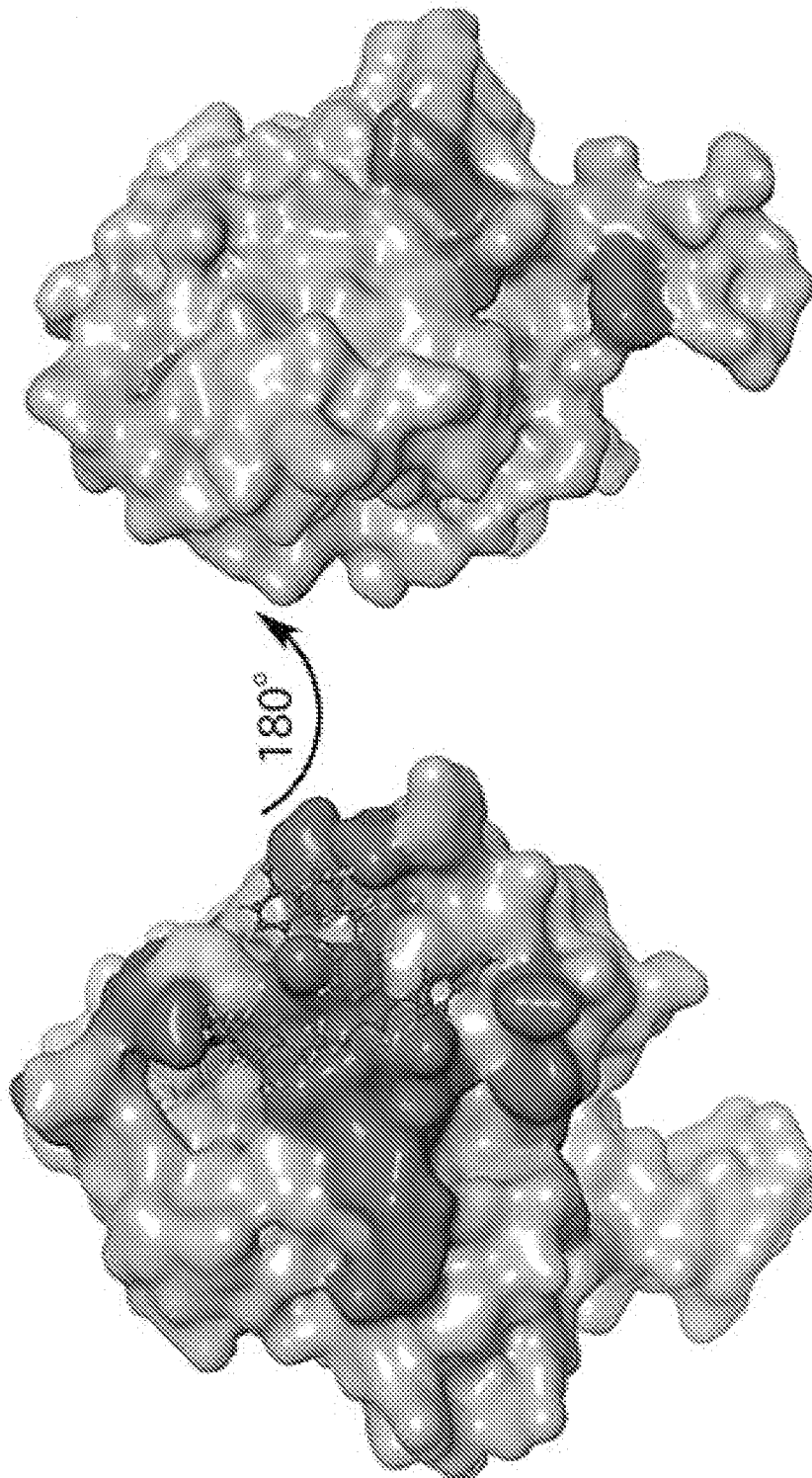


FIG. 14 Con't



14K

FIG. 14 Con't

14L

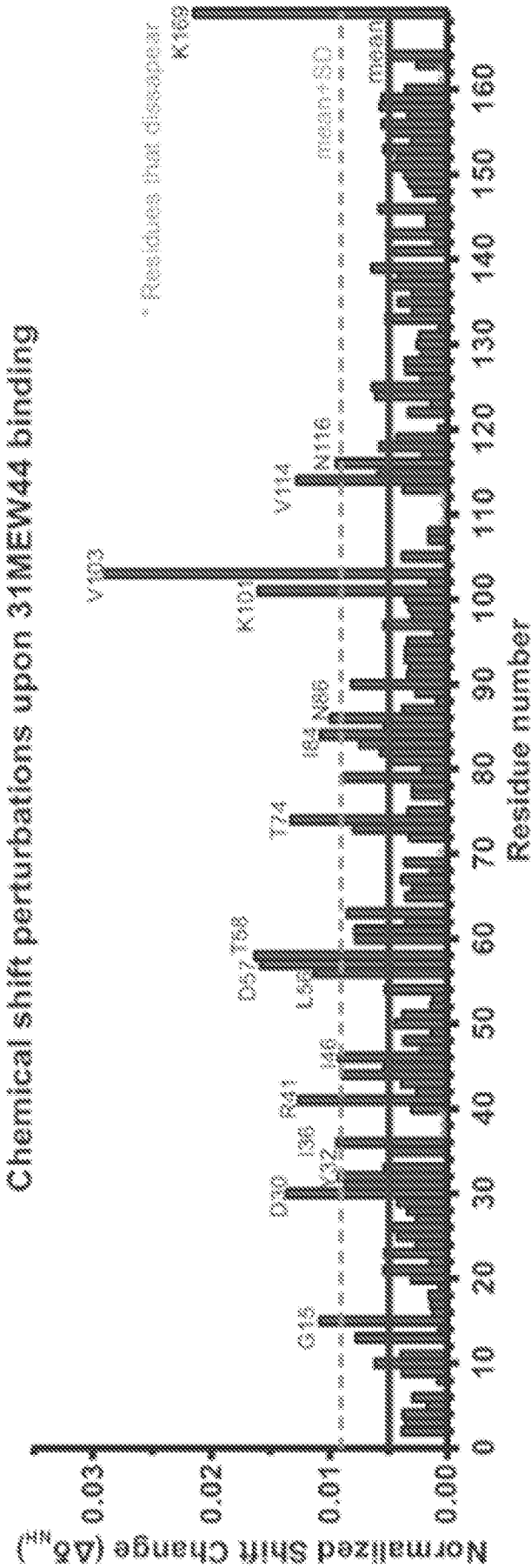
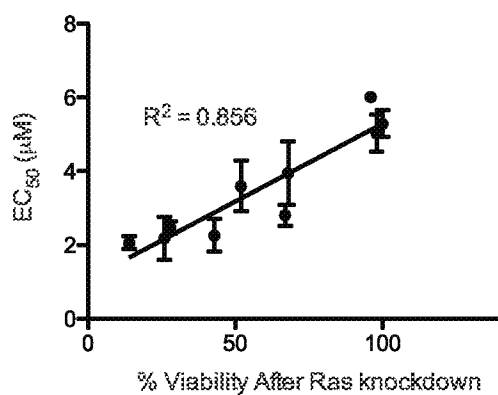


FIG. 15

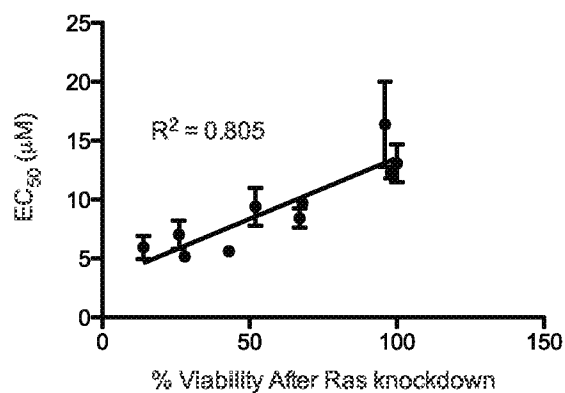
15A

31MEW44



15B

34MEW43



15C

34MEW95

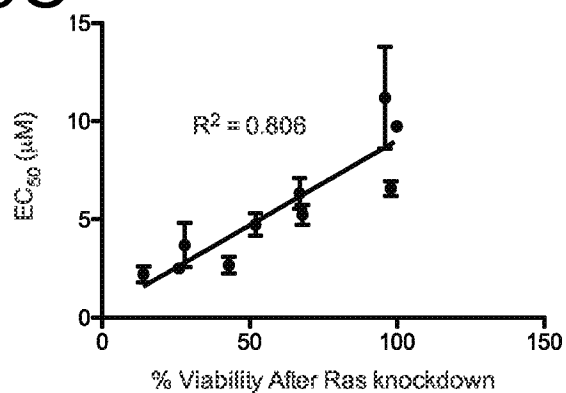


FIG. 16

16A

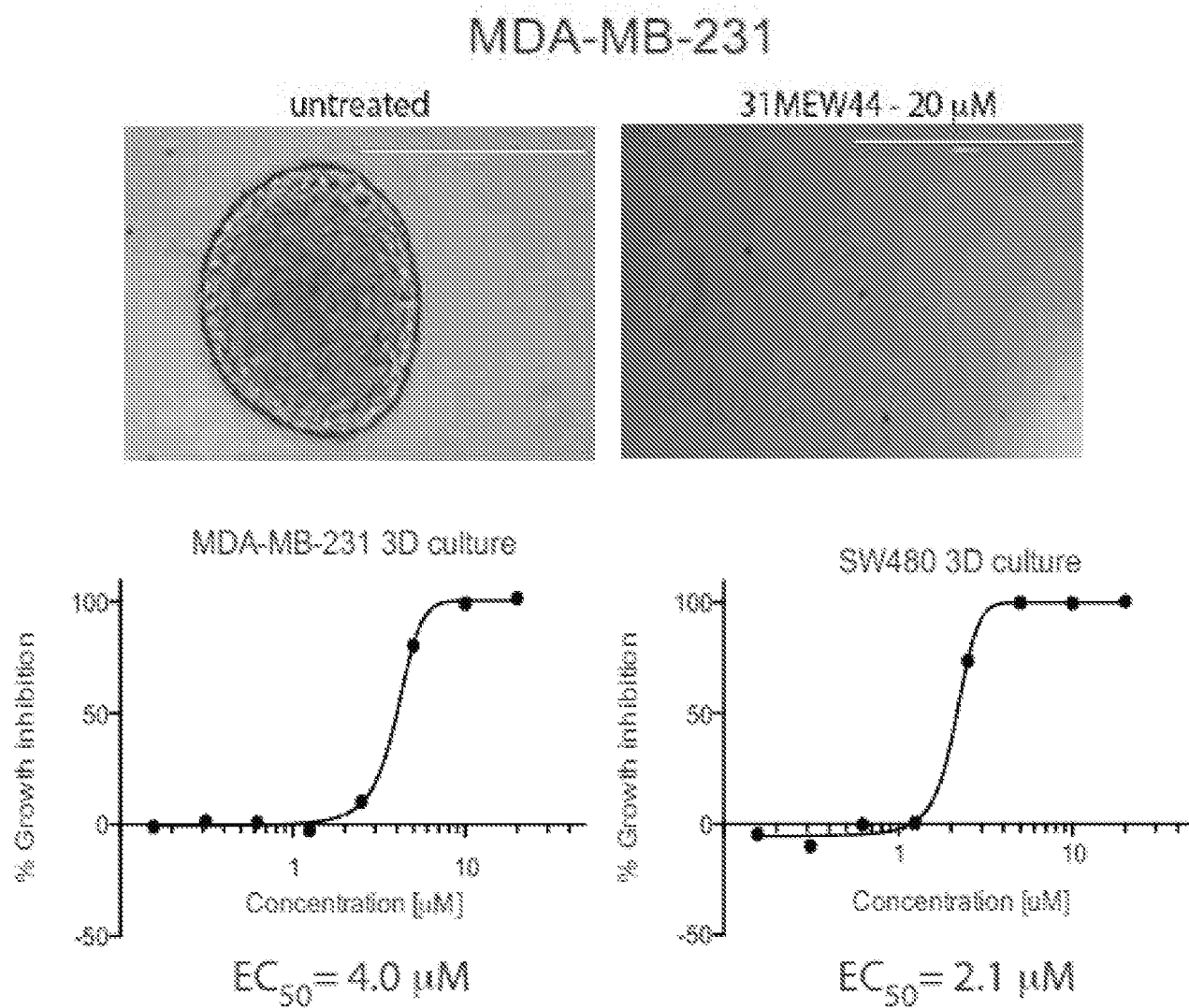


FIG. 16, Con't

16B

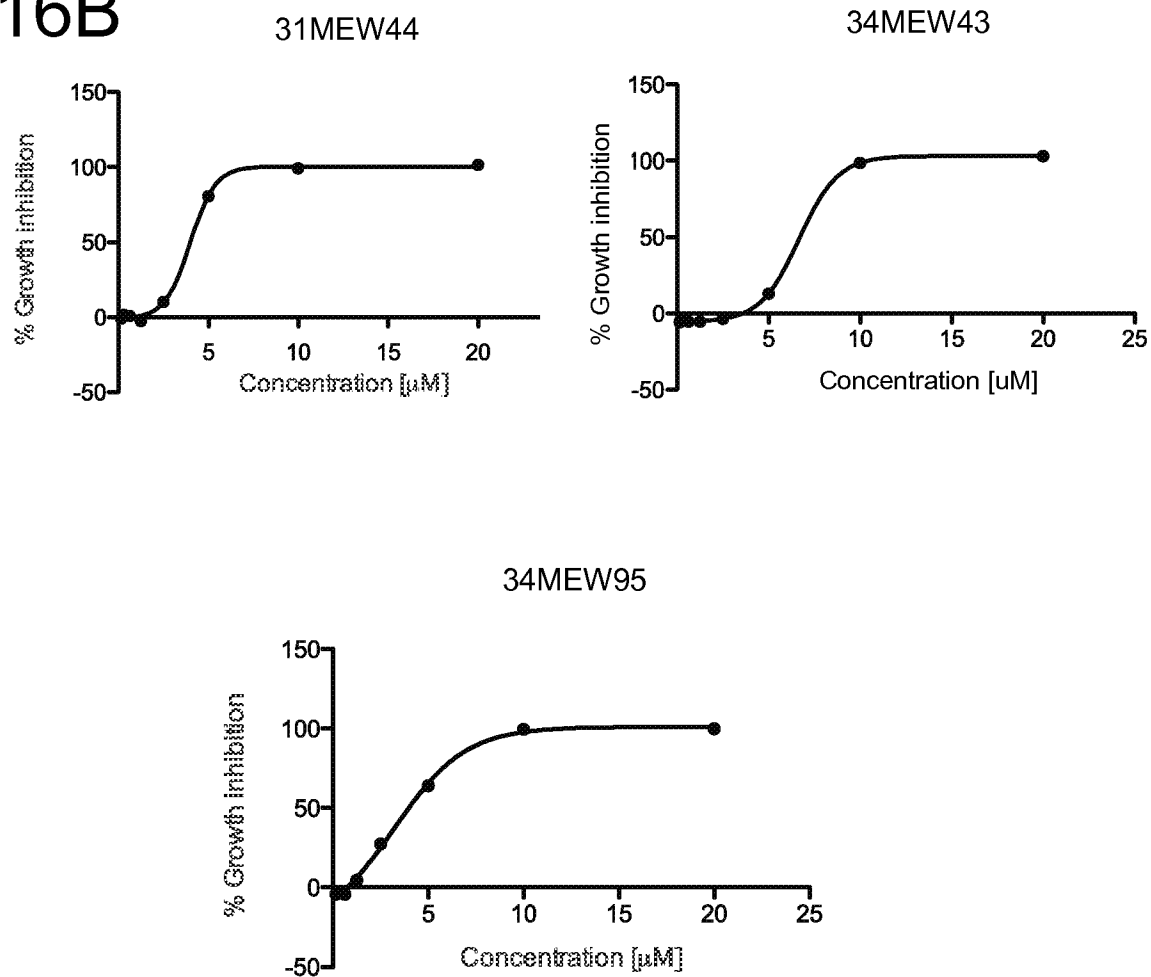
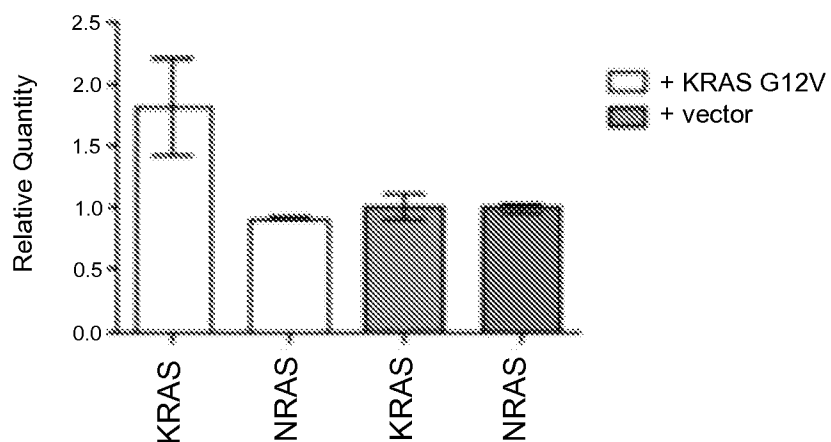


FIG. 17

17A

HT1080 NRAS and KRAS expression



17B

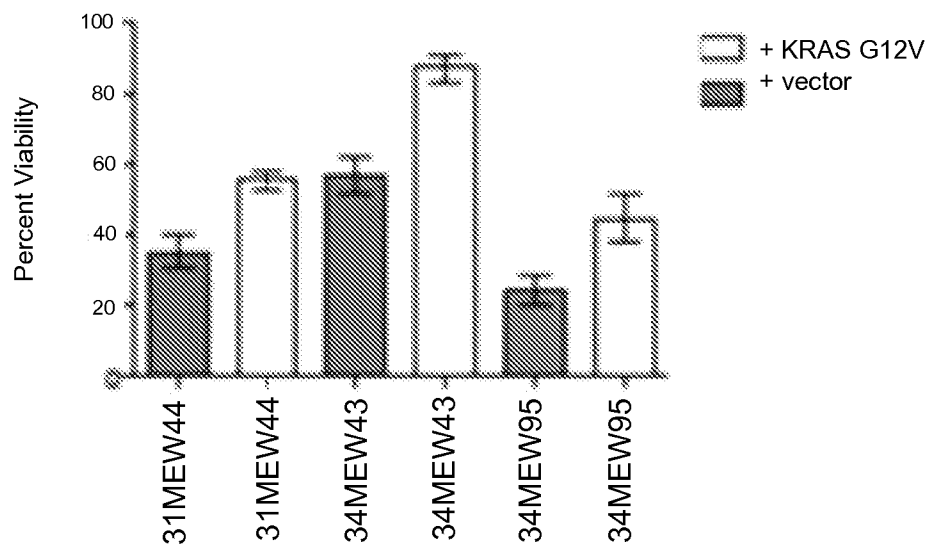


FIG. 18

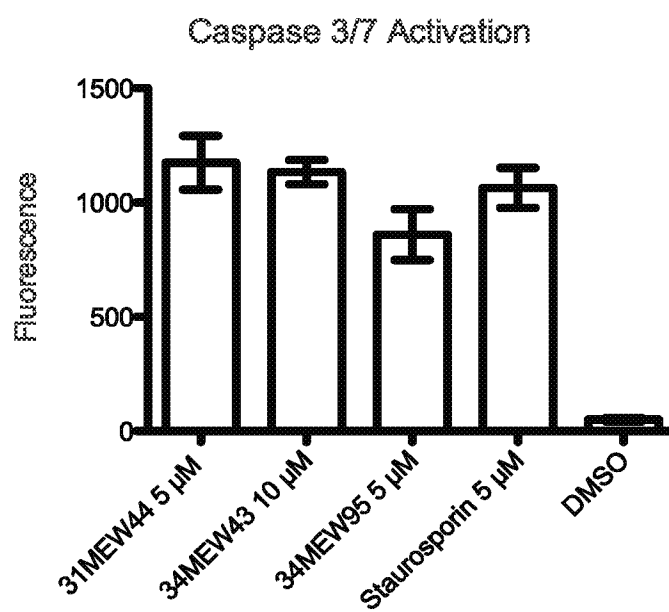


FIG. 19

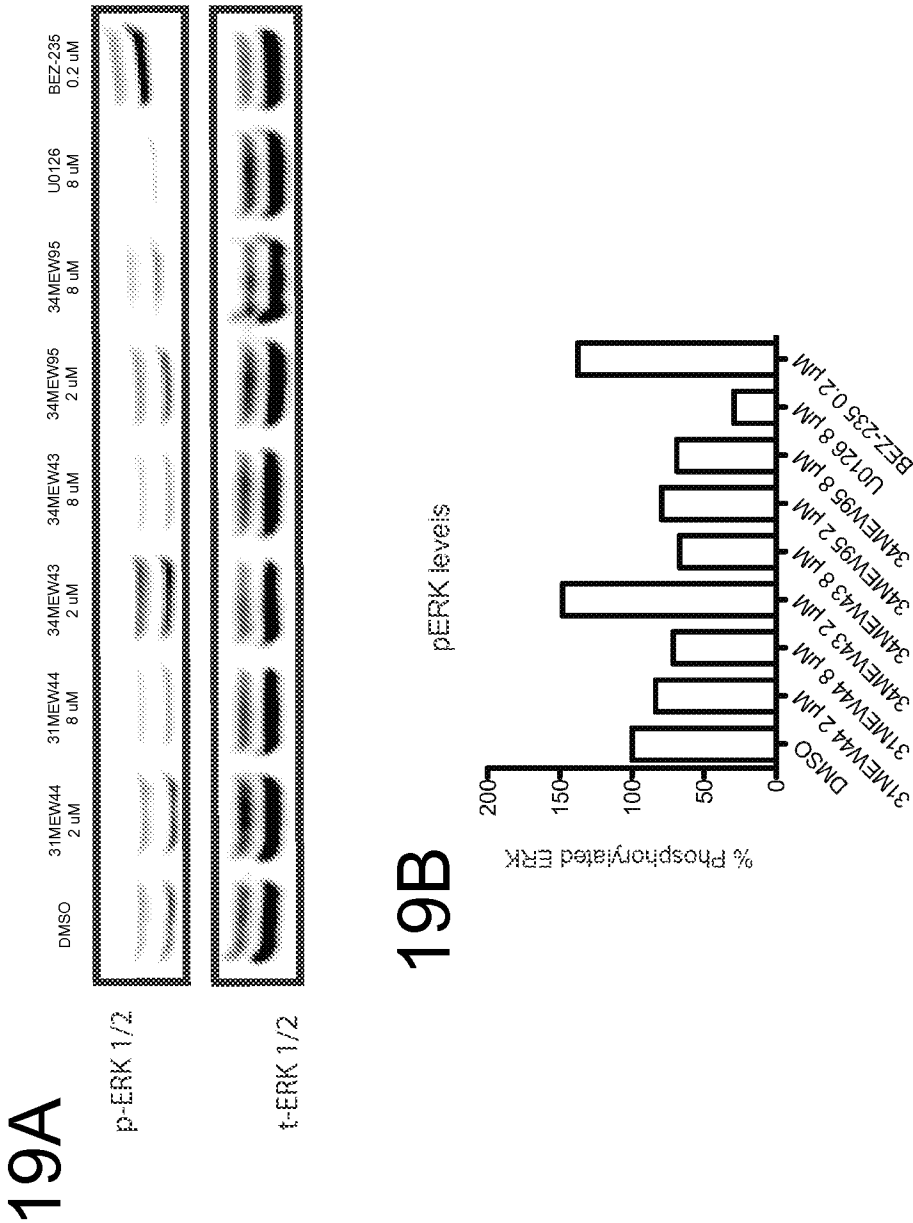


FIG. 20

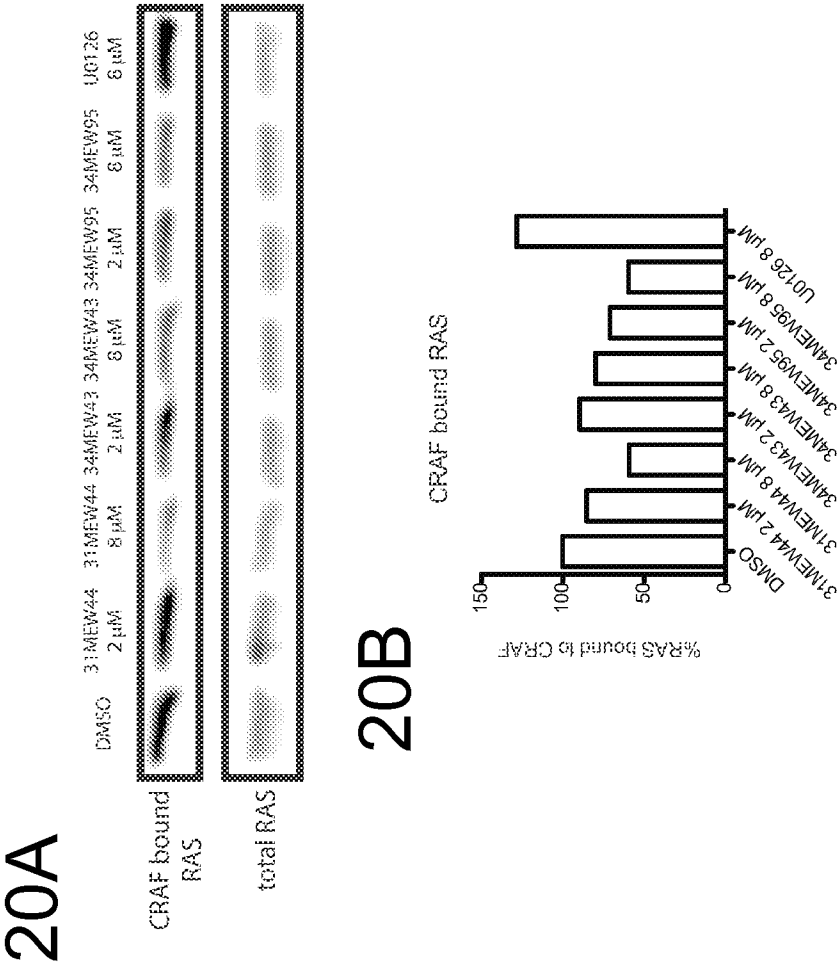
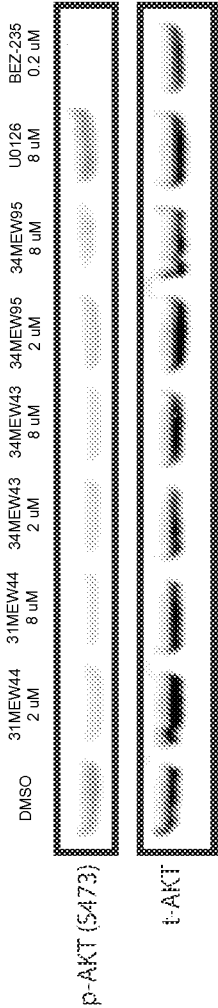


FIG. 21

21A



21B

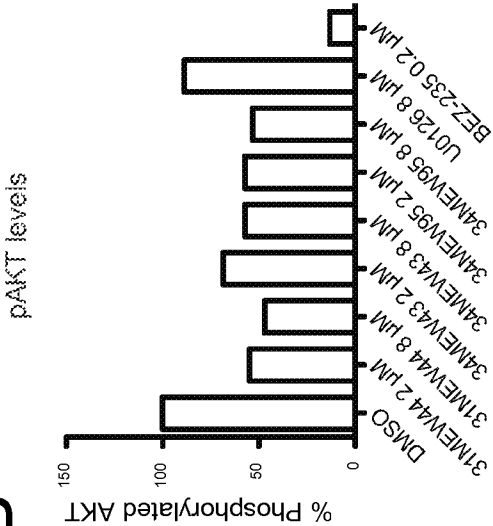


FIG. 22

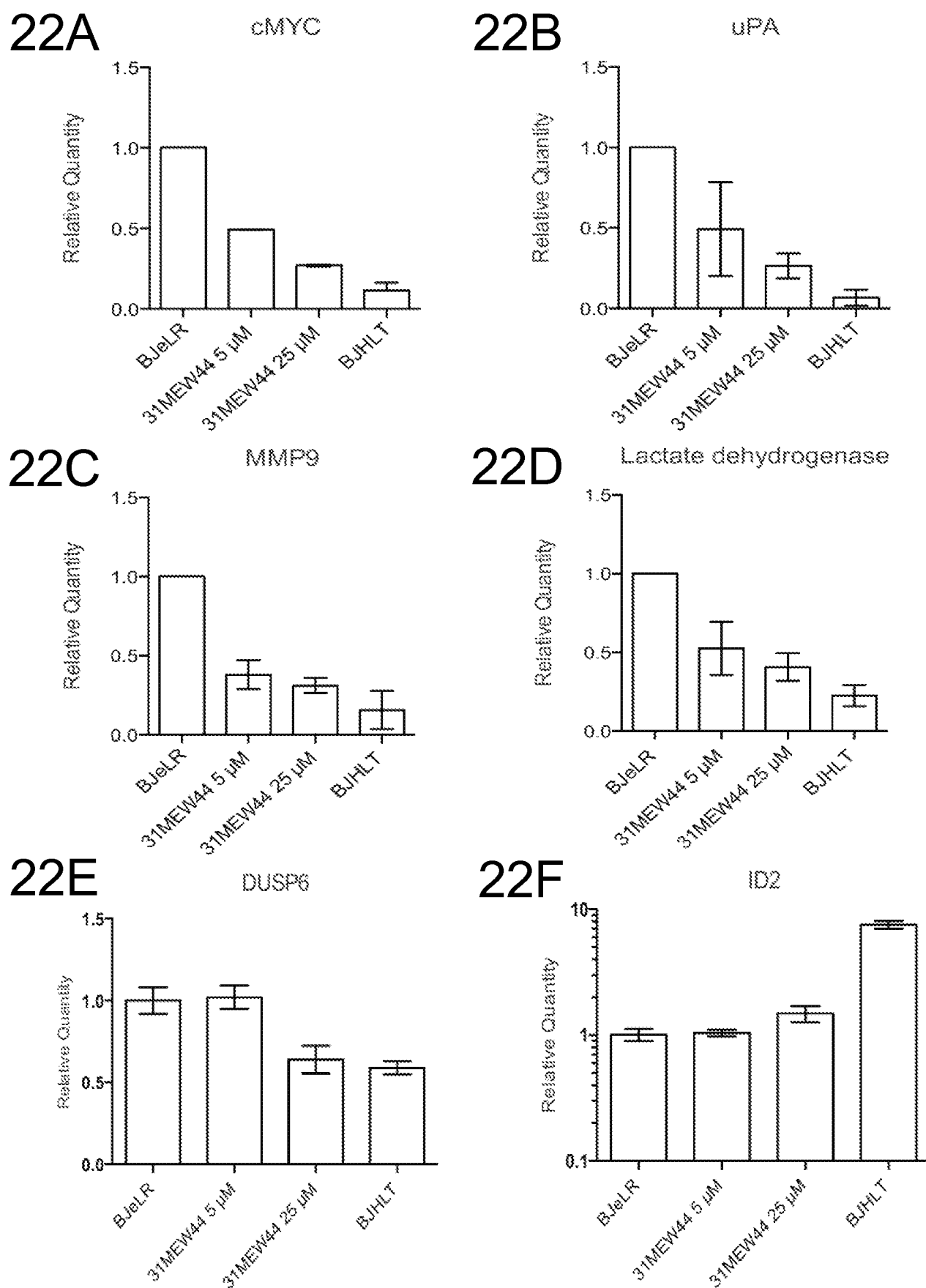
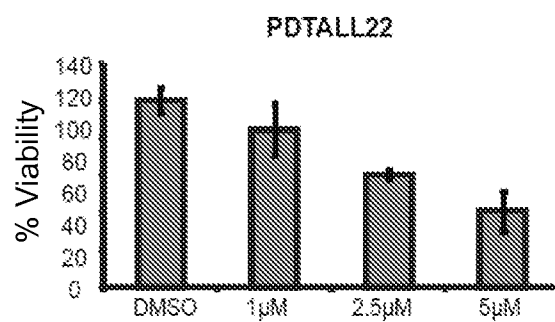
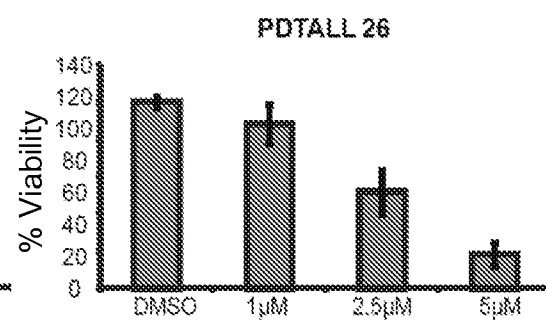


FIG. 23

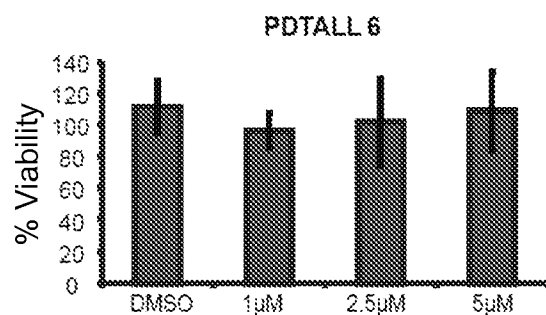
23A



23B



23C



23D

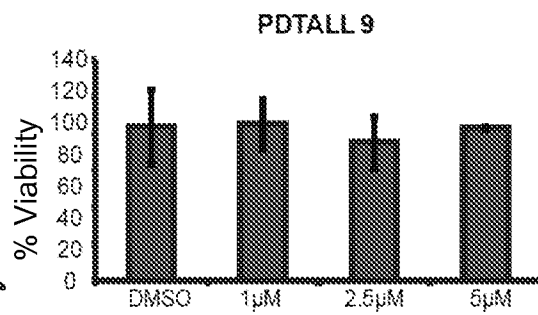
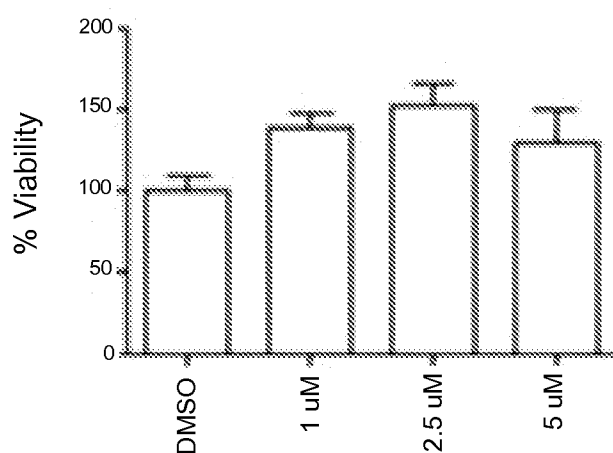


FIG. 23, Con't

23E

PDTALL13



23F

PDTALL19

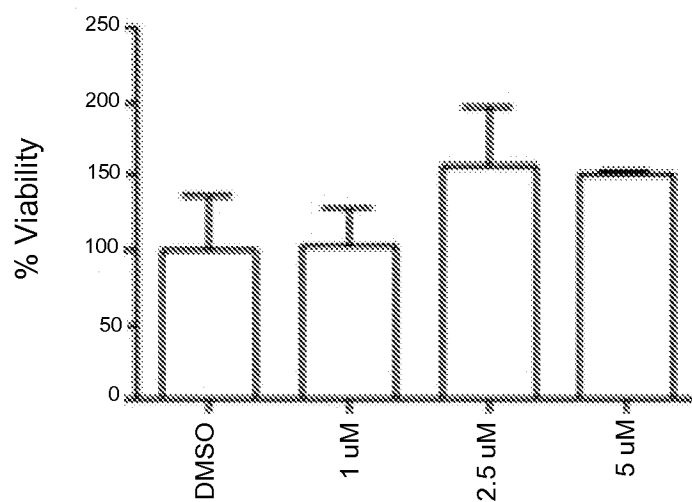


FIG. 24

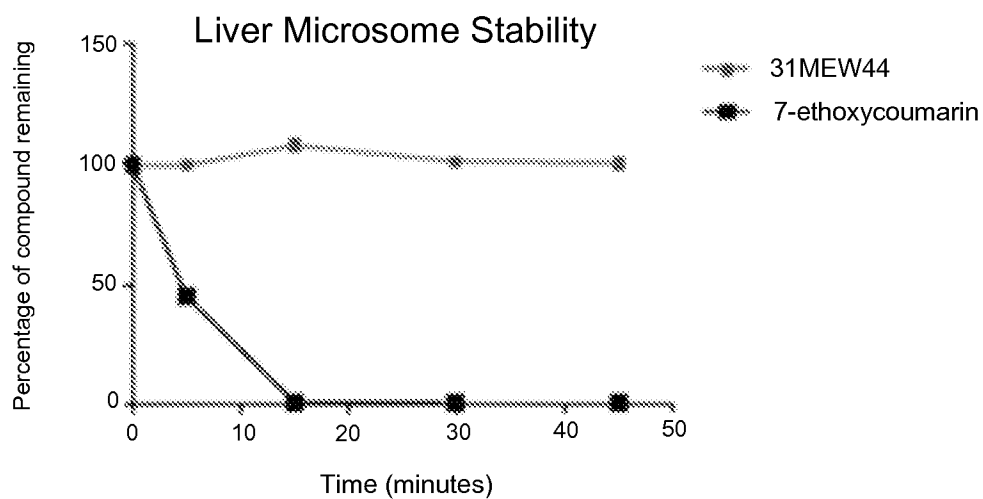


FIG. 25

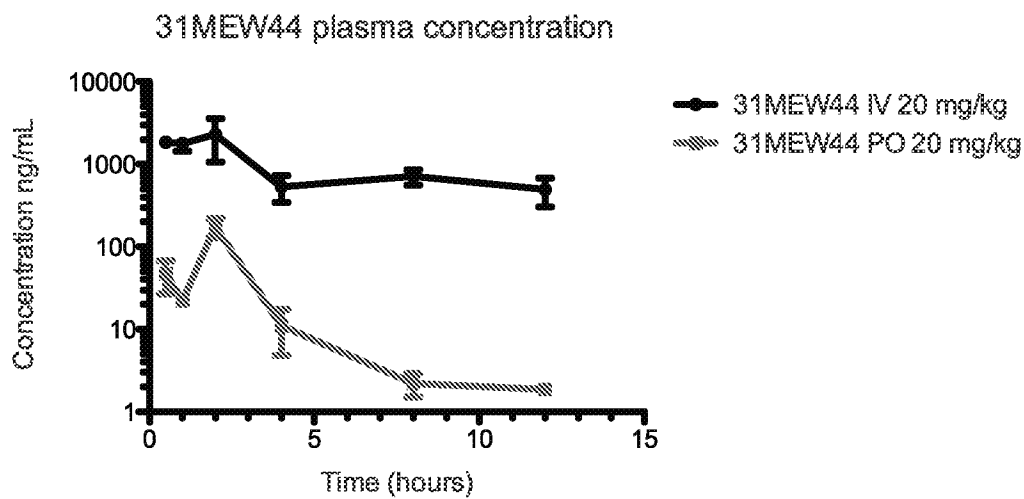


FIG. 26

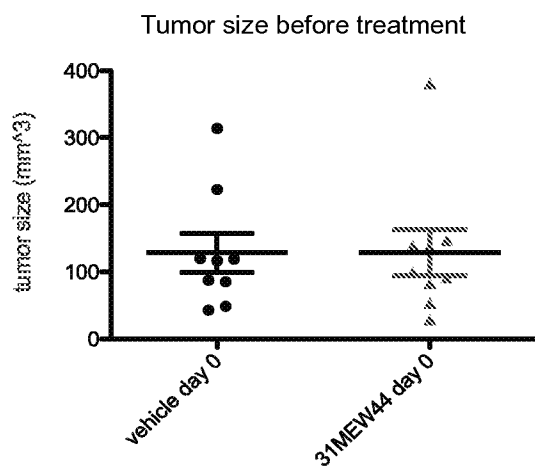


FIG. 27

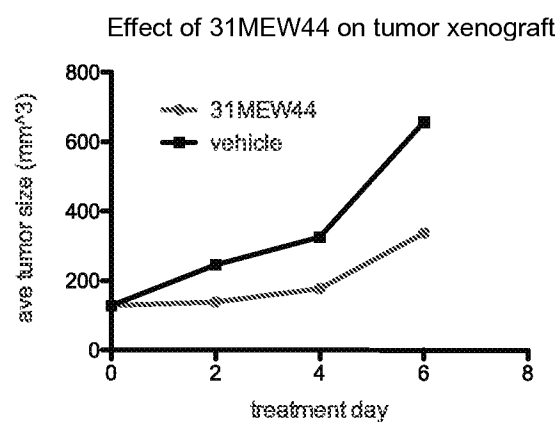


FIG. 28

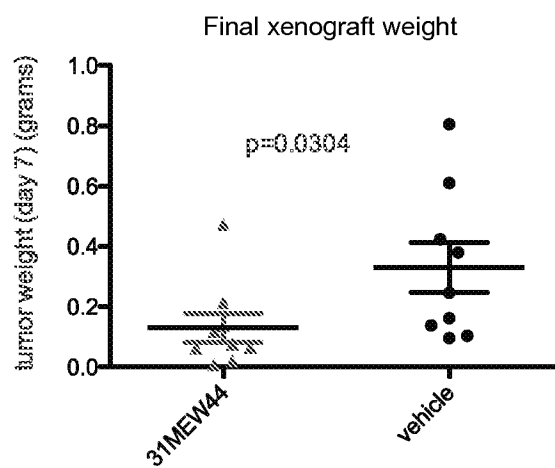
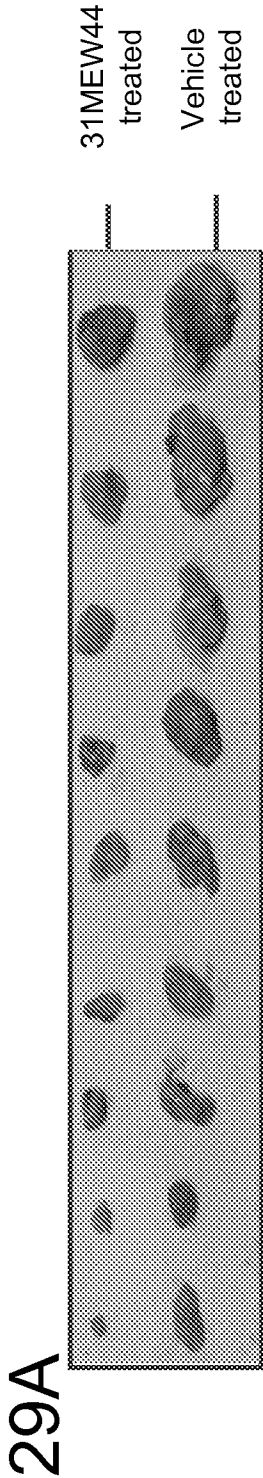
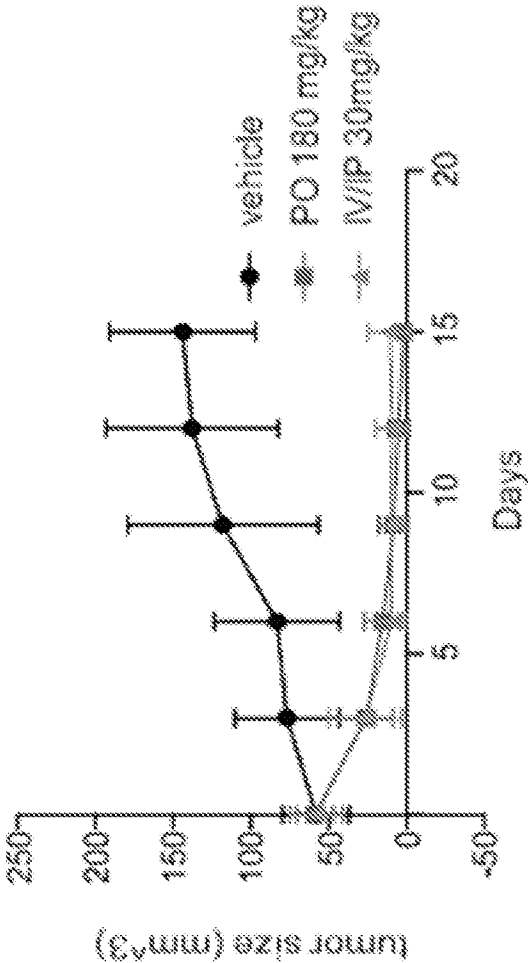


FIG. 29



29B

Effect of 31MEW44 on a tumor xenograft

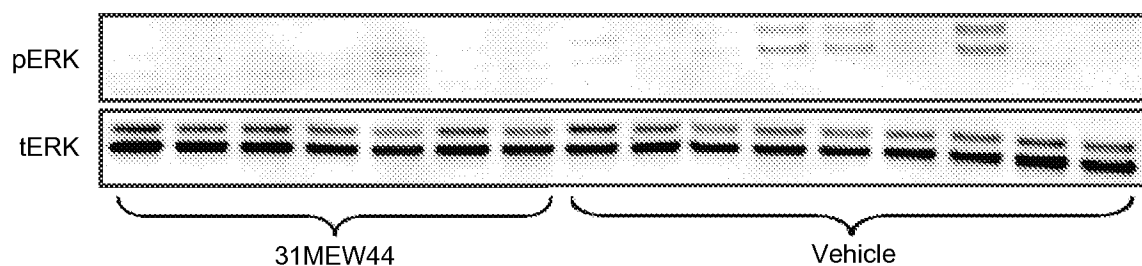


POTALL13

POTALL19

FIG. 30

30A



30B

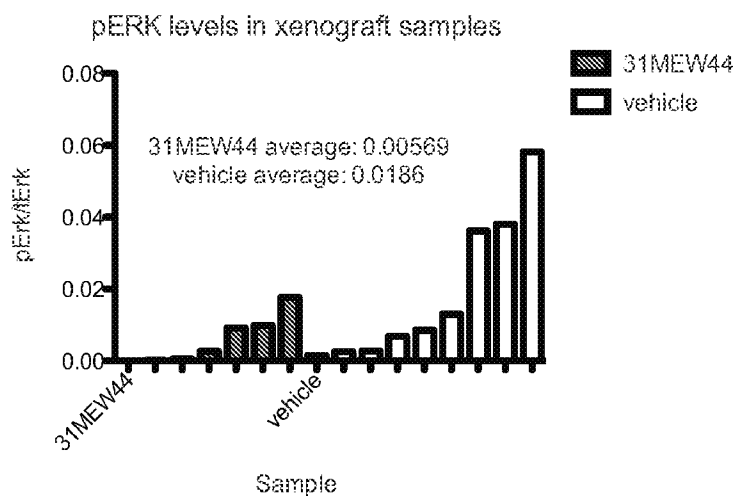
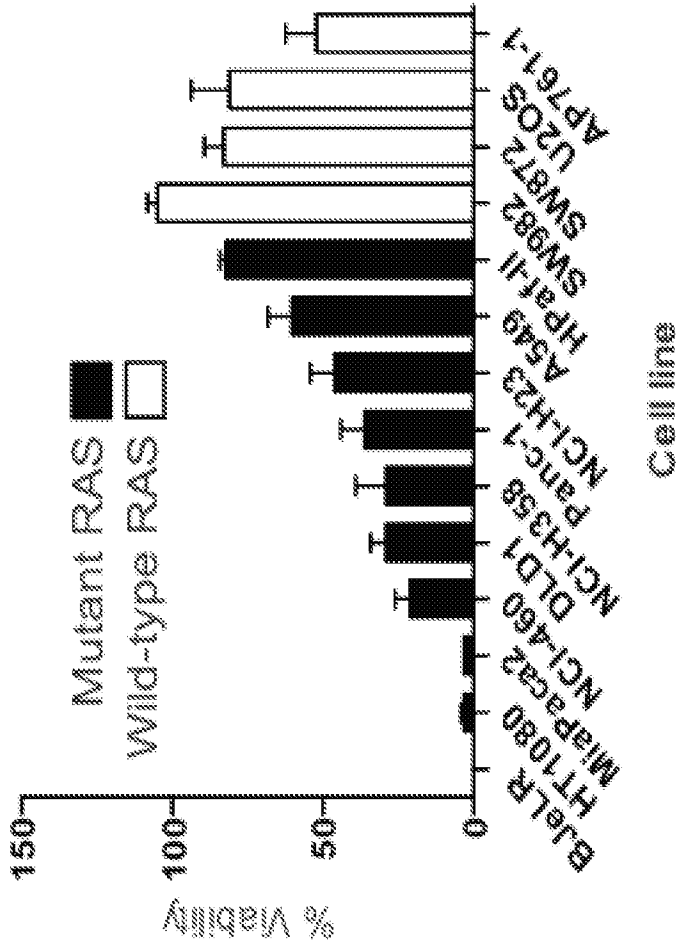


FIG. 31

31A

5 μ M 31MEW44 treatment



31B

2.5 μ M 31MEW44 treatment

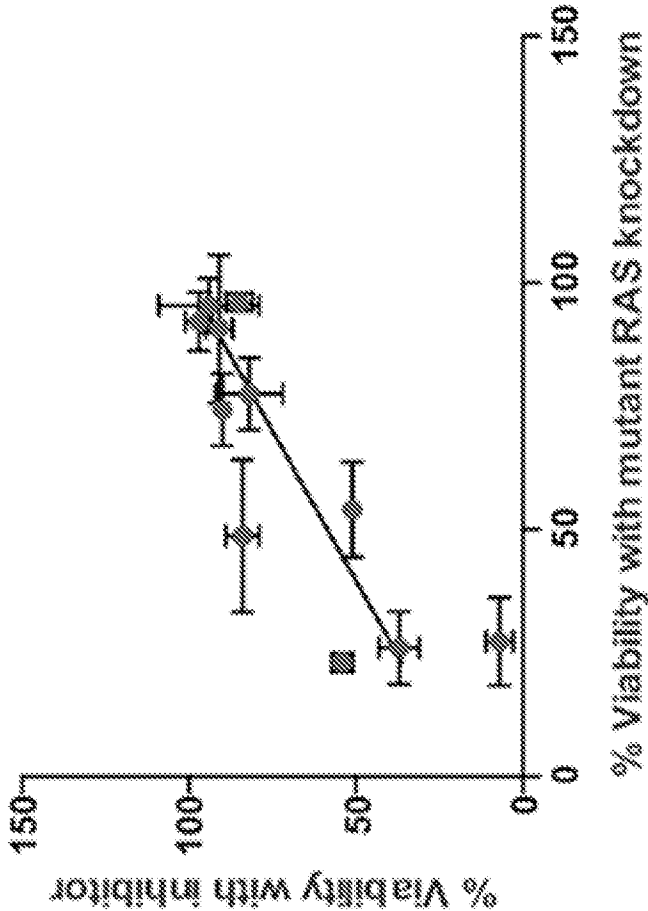
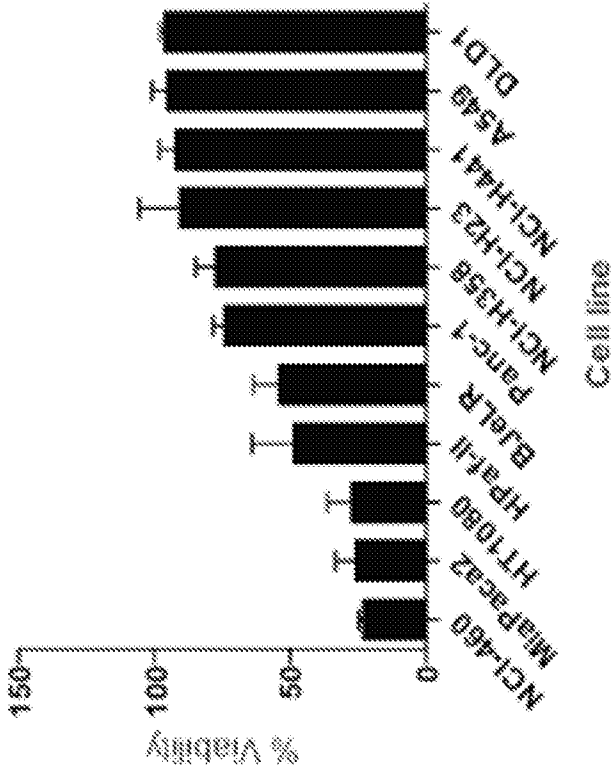


FIG. 32

32A

RAS knockdown at 72 hr



32B

5 μ M 31MEW44 treatment

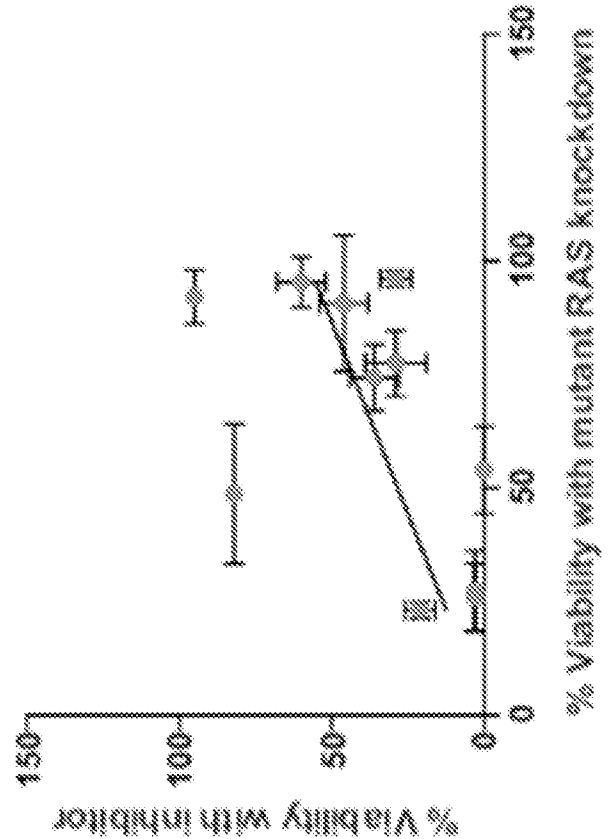


FIG. 33

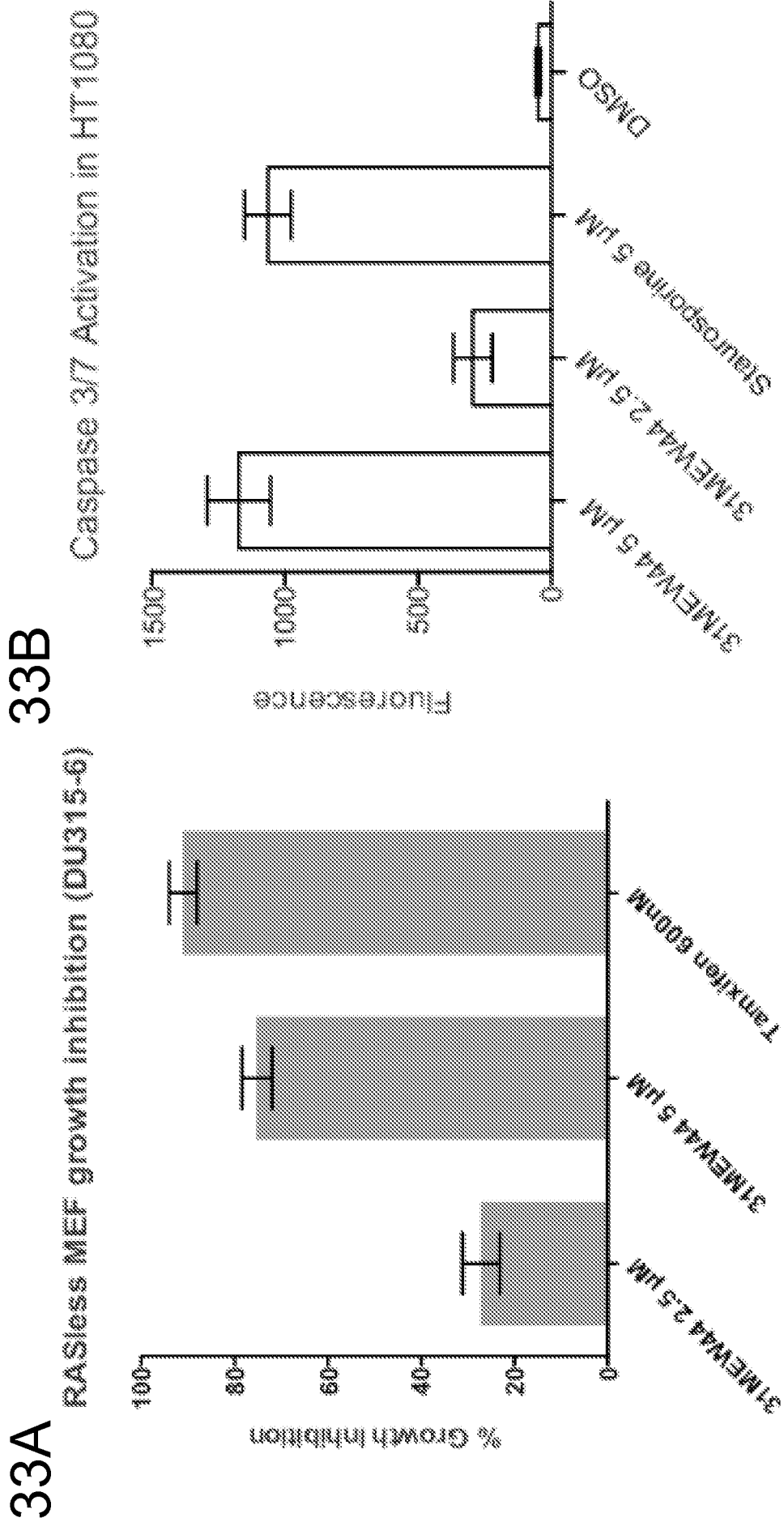
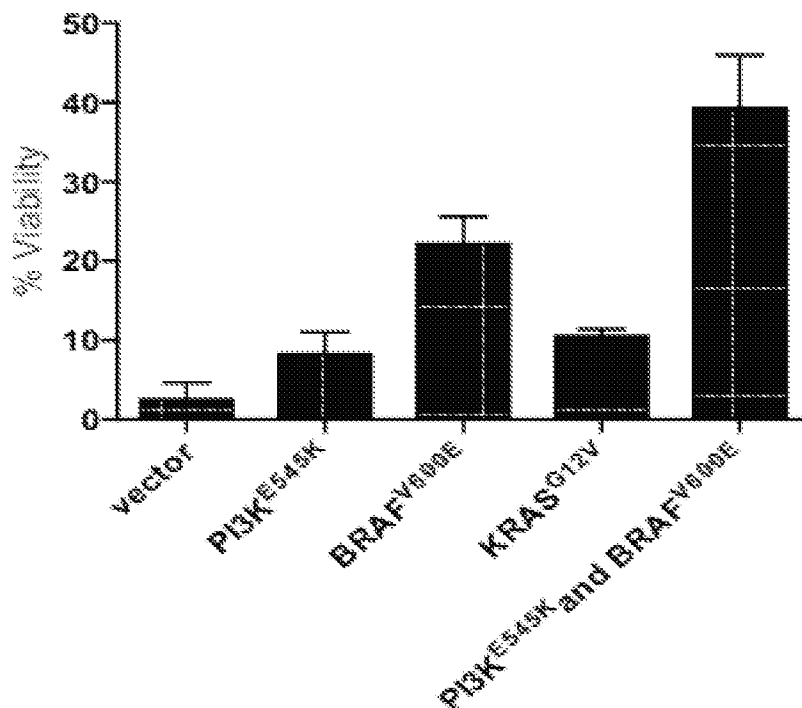


FIG. 34

34A

Effect of mutant KRAS, BRAF and PI3K transfection on HT1080



34B

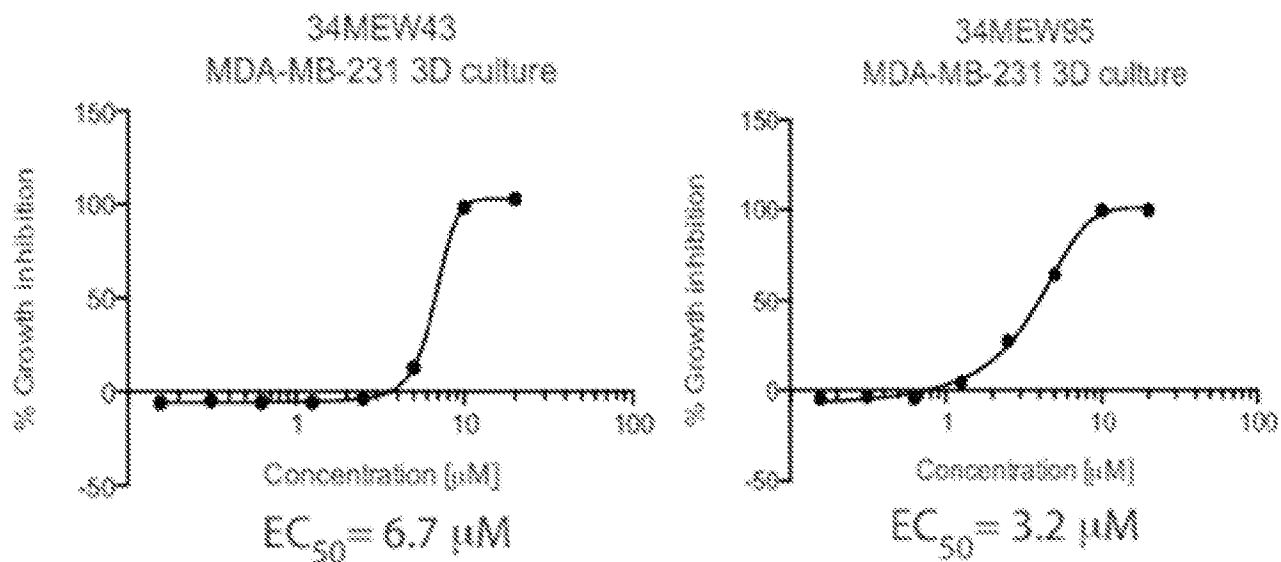
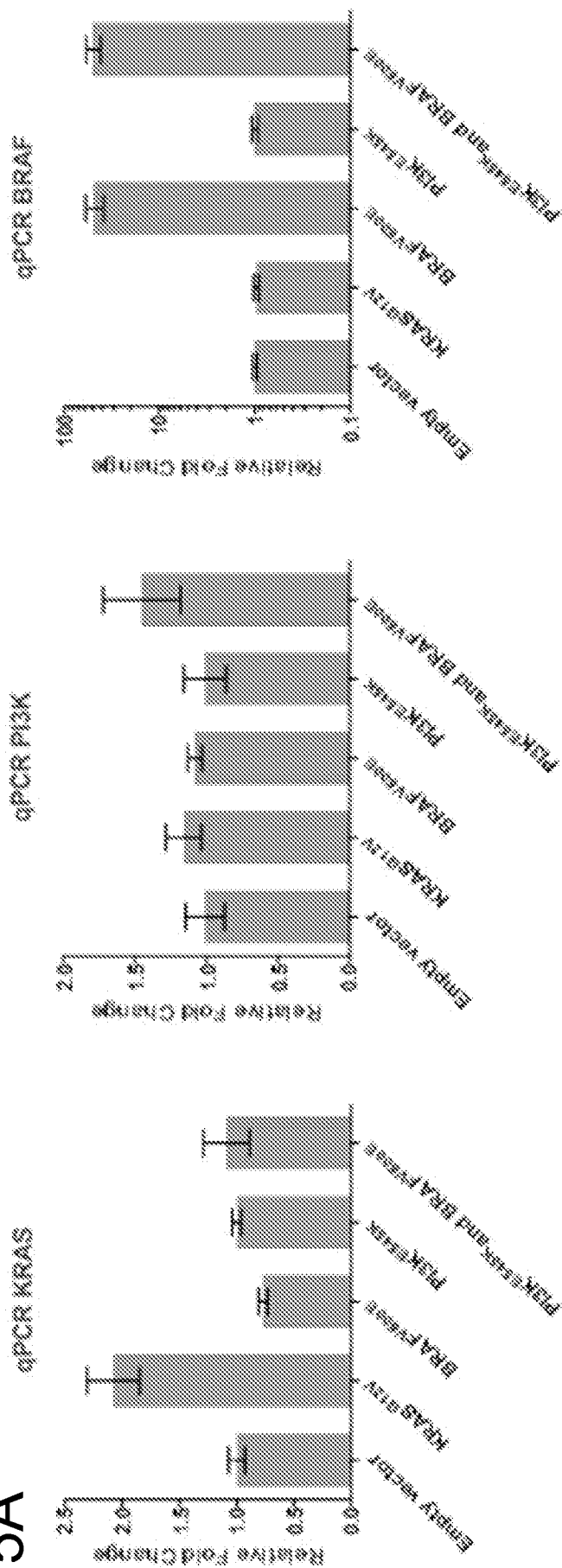


FIG. 35

35A



35B

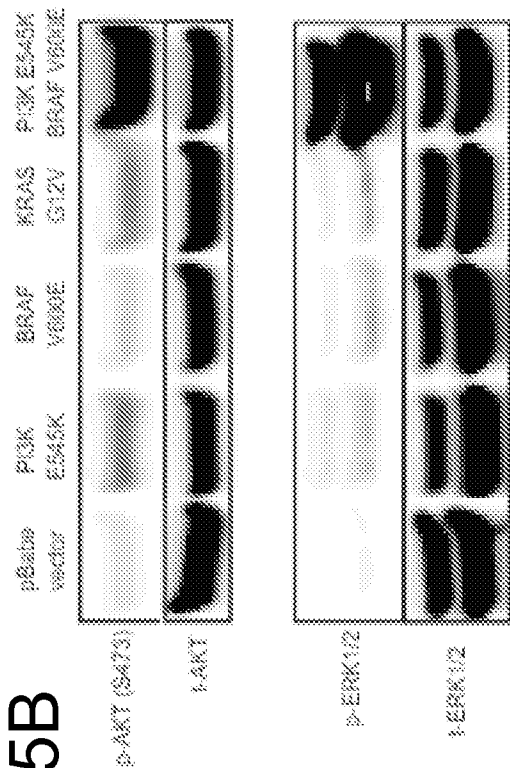
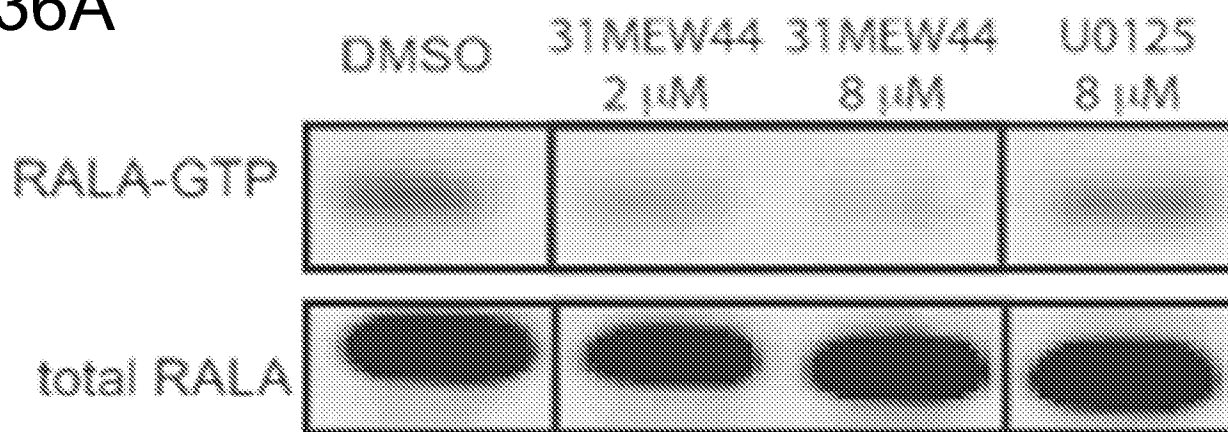


FIG. 36

36A



36B

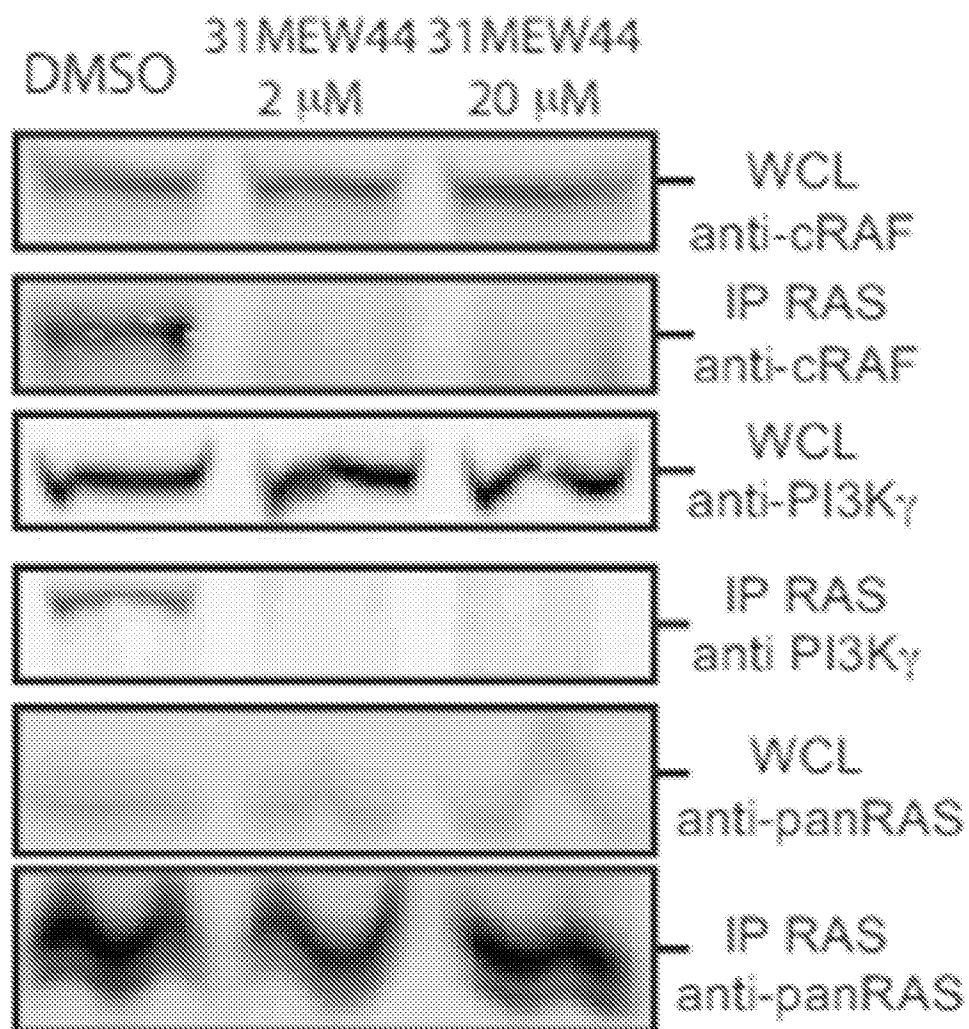


FIG. 37

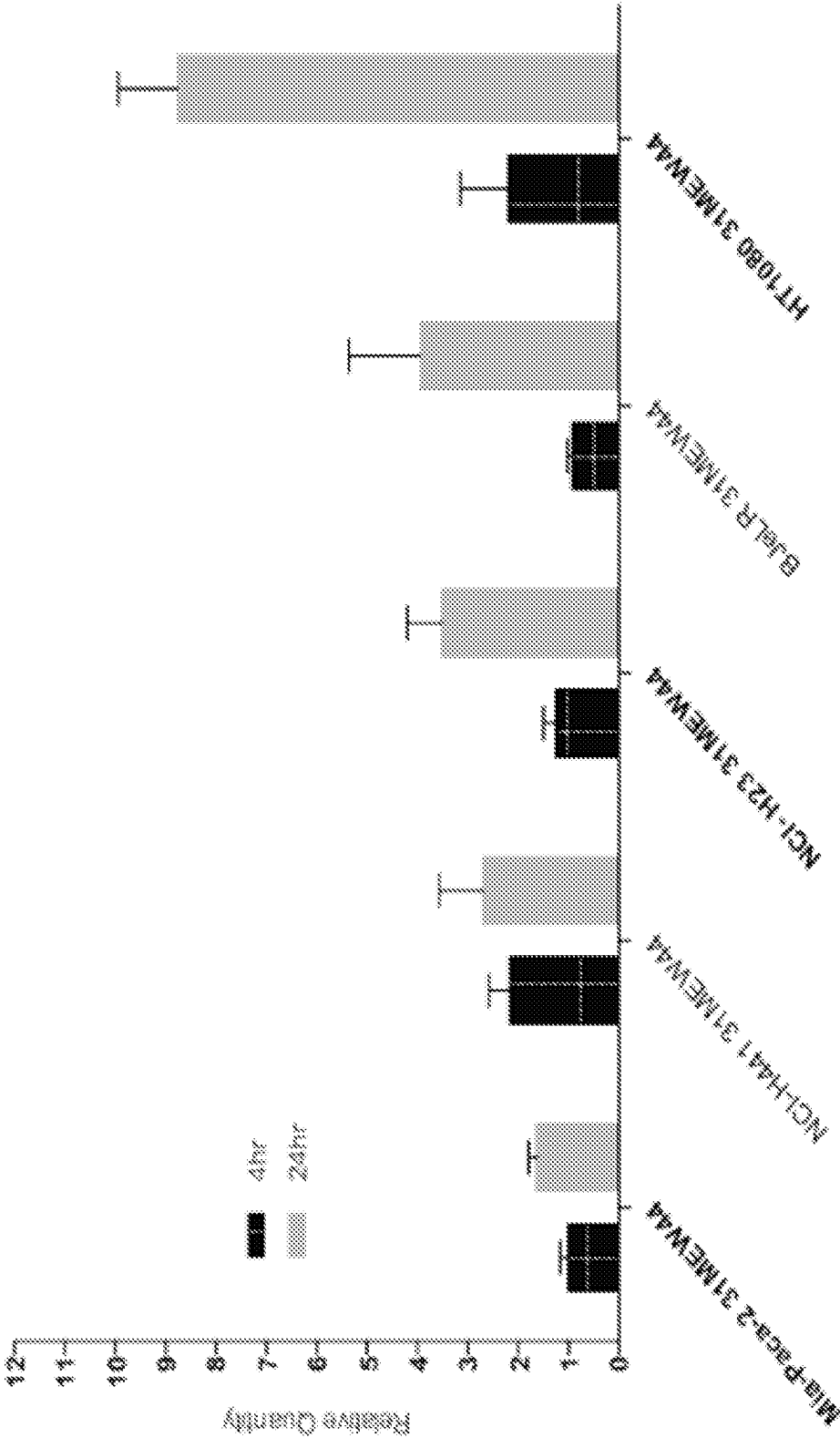
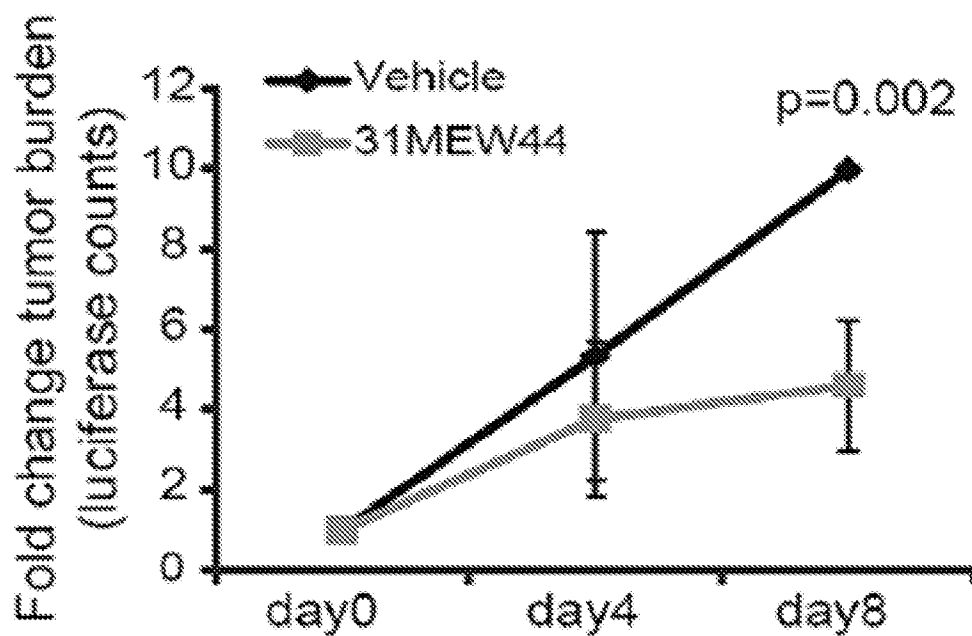


FIG. 38

38A



38B

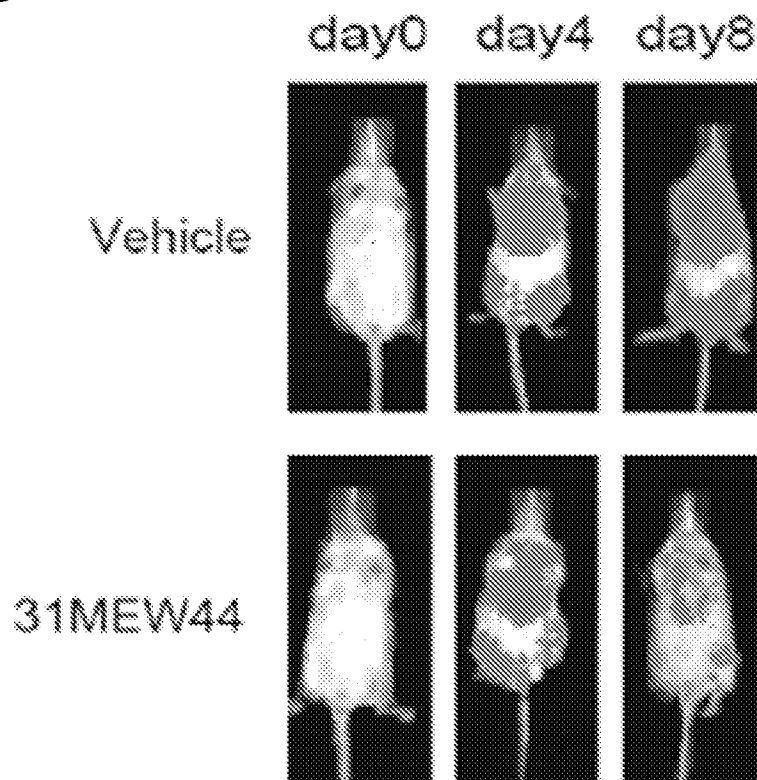
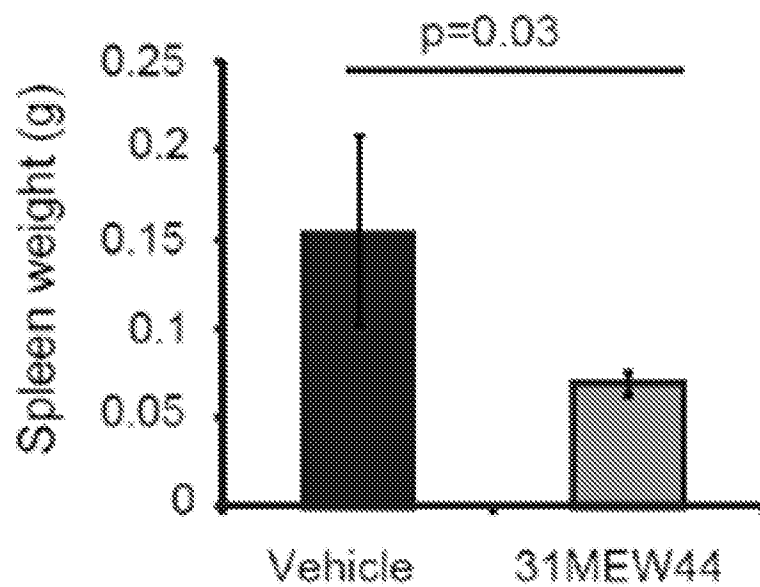


FIG. 38 con't

38C



38D

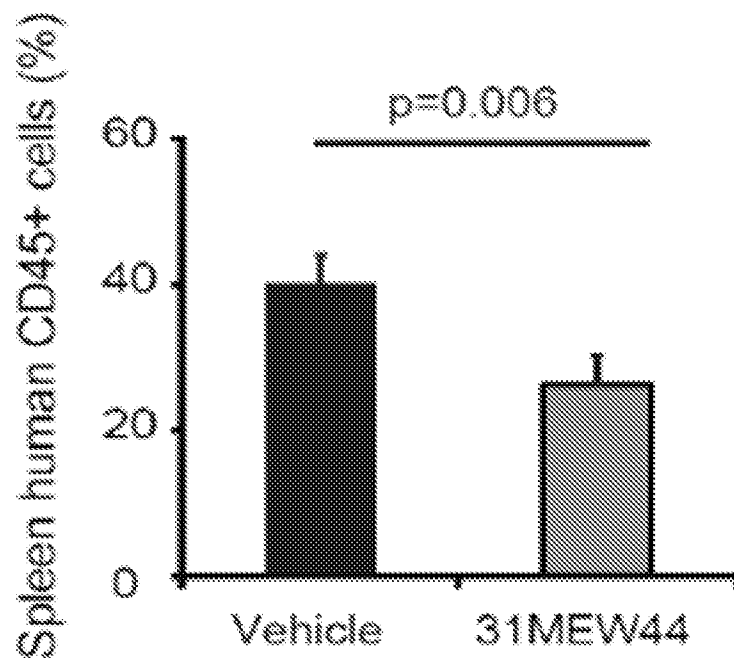
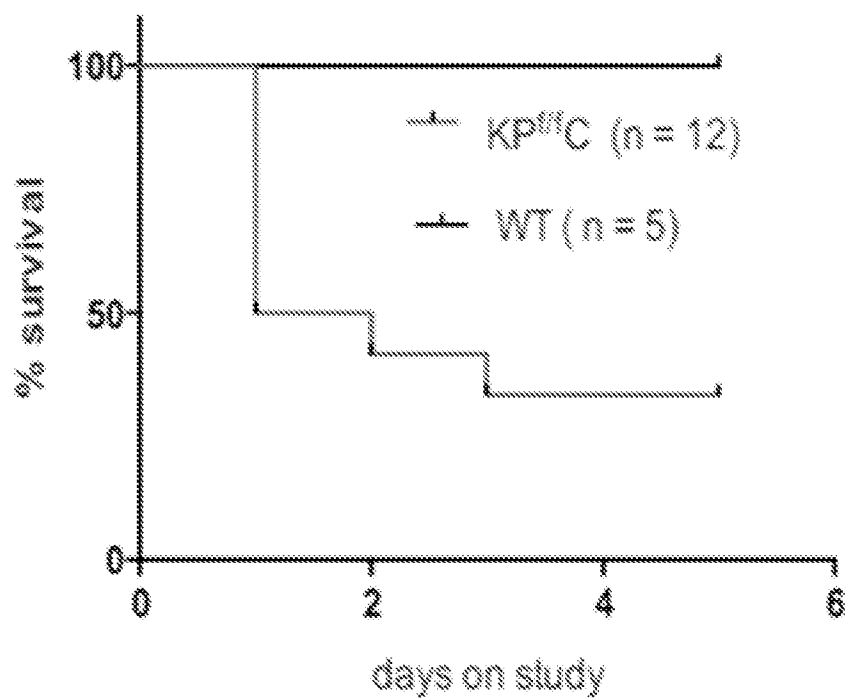


FIG. 39

39A

Genotype selective toxicity of 31MEW44



39B

Weight of wild-type mice in toxicity study

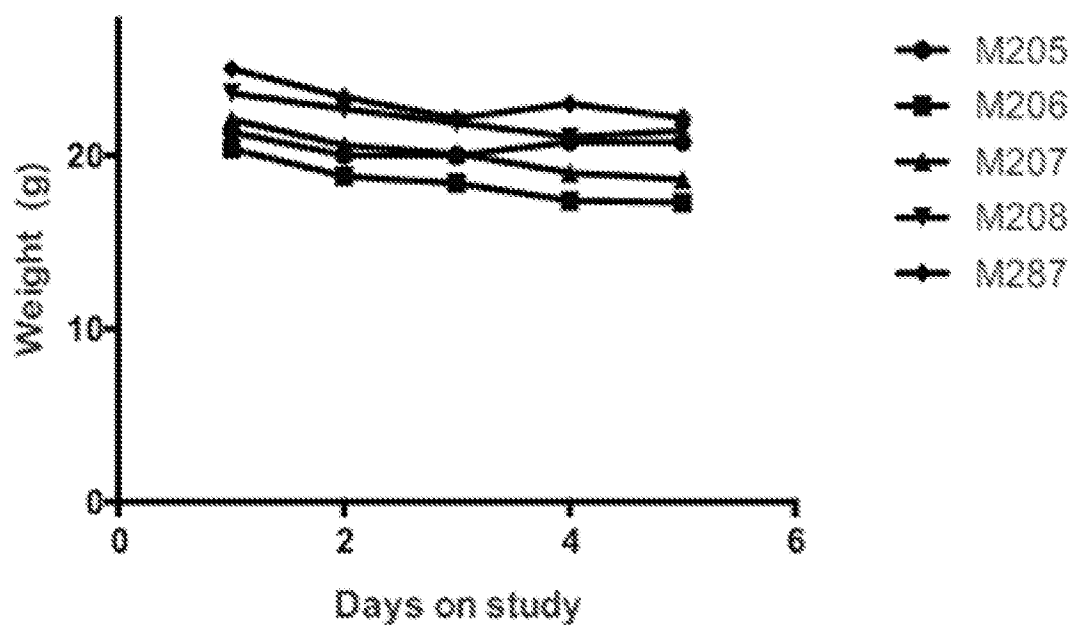


FIG. 39 con't

39C

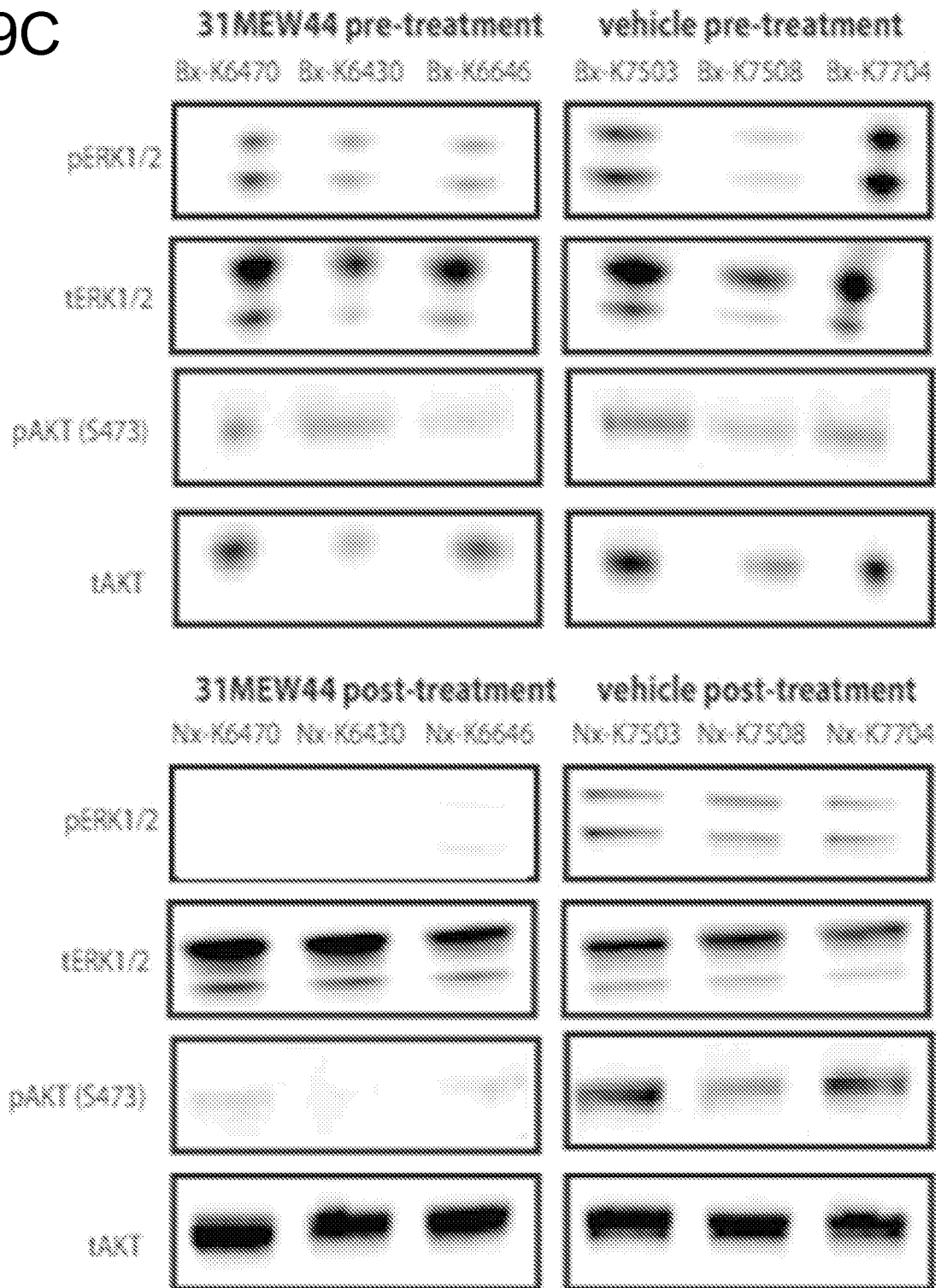


FIG. 39 con't

39D

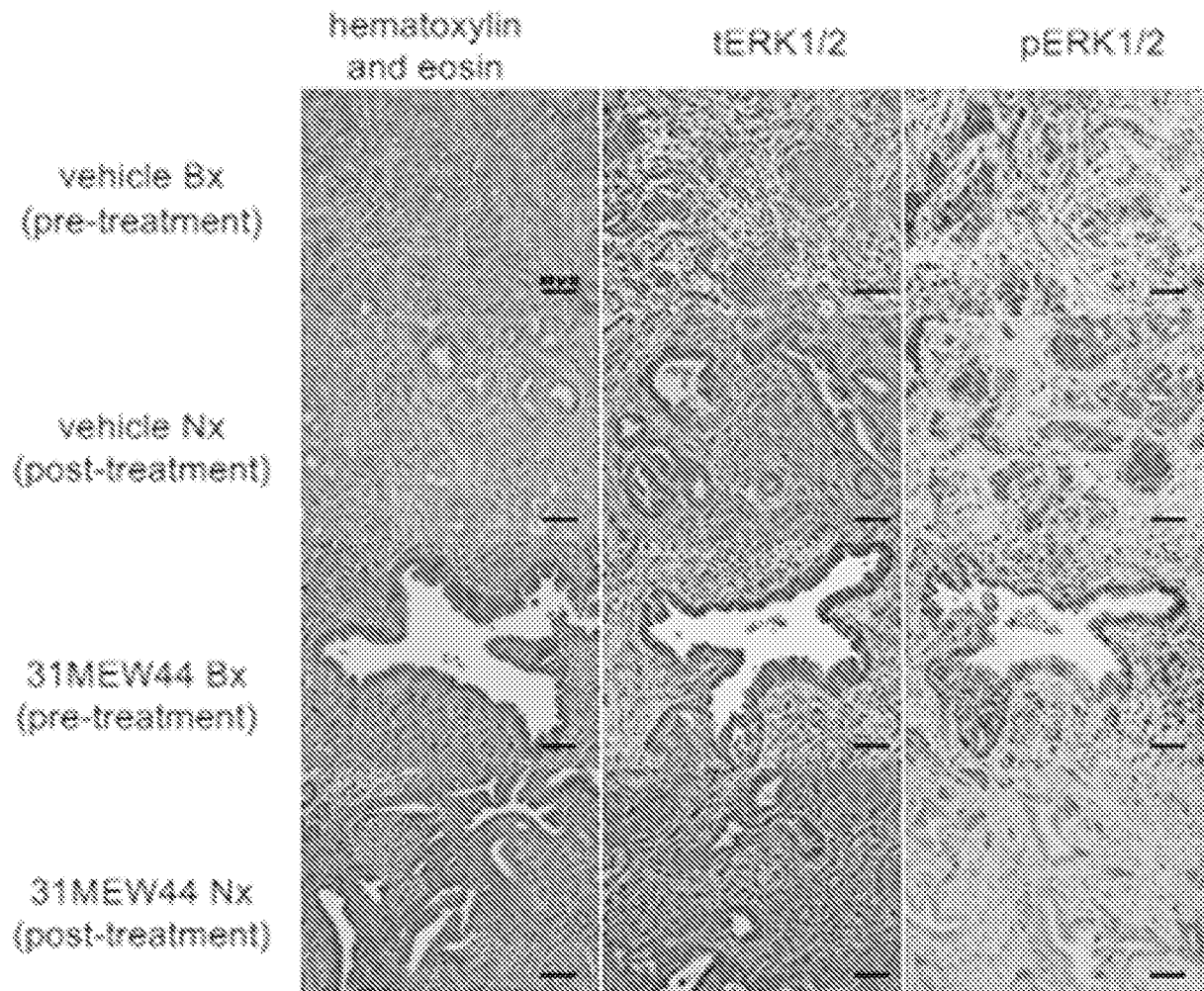
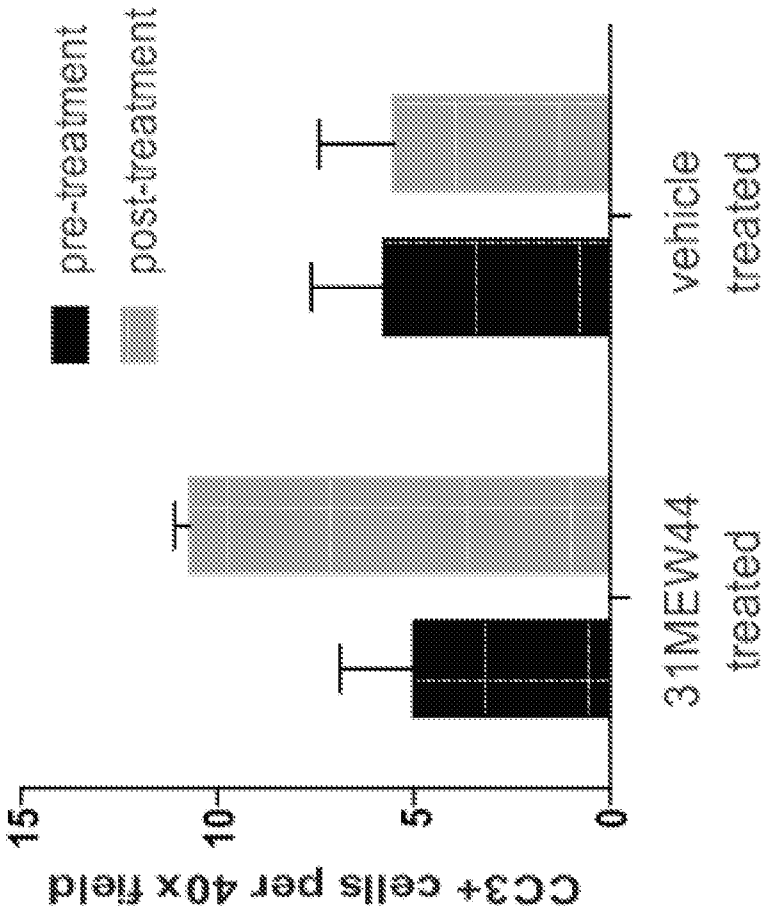


FIG. 39 con't

39F

IHC cleaved caspase-3 quantification



39E

cleaved caspase-3



vehicle Bx
(pre-treatment)

vehicle Nx
(post-treatment)

31MEW44 Bx
(pre-treatment)

31MEW44 Nx
(post-treatment)