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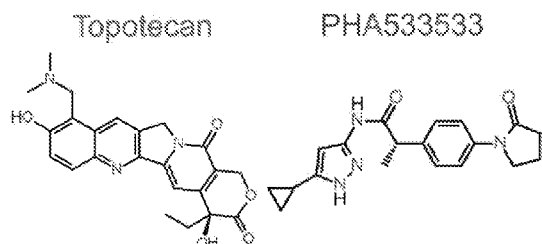
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(54) Title: METHODS AND COMPOSITIONS FOR UNSILENCING IMPRINTED GENES

FIG. 1A



(57) Abstract: The present invention provides methods and compositions for inducing expression of Ube3a in a cell and treating Angelman syndrome in a subject.

METHODS AND COMPOSITIONS FOR UNSILENCING IMPRINTED GENES**STATEMENT OF PRIORITY**

This application claims the benefit, under 35 U.S.C. § 119(e), of U.S. Provisional
5 Application No. 62/874,777, filed on July 16, 2019, the entire contents of which are
incorporated by reference herein.

STATEMENT REGARDING ELECTRONIC FILING OF A SEQUENCE LISTING

A Sequence Listing in ASCII text format, submitted under 37 C.F.R. § 1.821, entitled
10 5470-866WO_ST25.txt, 2,232 bytes in size, generated on July 14, 2020 and filed via EFS-
Web, is provided in lieu of a paper copy. This Sequence Listing is hereby incorporated herein
by reference into the specification for its disclosures.

STATEMENT OF GOVERNMENT SUPPORT

15 This invention was made with government support under Grant Number HD040127
awarded by the National Institutes of Health. The government has certain rights in the
invention.

FIELD OF THE INVENTION

The present invention is directed to methods and compositions for inducing
20 expression of Ube3a in a cell and treating Angelman syndrome in a subject.

BACKGROUND OF THE INVENTION

Angelman syndrome (AS) is a neurodevelopmental disorder affecting ~1:20,000
individuals and characterized by developmental delay, intellectual disability, speech
25 impairment, seizures, and ataxia. No effective therapies currently exist, however the unique
epigenetics of AS offer a potential therapeutic avenue. AS is caused by mutations of the
ubiquitin protein ligase E3A (*UBE3A*) gene, which exhibits parent-of-origin genomic
imprinting. Only the maternal-inherited copy of *UBE3A* is expressed in neurons, while the
paternal-inherited copy is intact but silent. On the other hand, *UBE3A* is biallelically
30 expressed in non-neuronal cells. Although the detailed mechanism of this genomic imprinting
remains unclear, an imprinting center regulates DNA methylation and gene expression in *cis*.
Expression of the paternal *UBE3A* allele is competitively restricted in neurons by expression
of *UBE3A* antisense transcript (*UBE3A-ATS*), which is not fully expressed in non-neuronal
cells due to the presence of a bipartite boundary element.

The present invention provides methods and compositions for unsilencing imprinted genes (e.g., the paternal allele of Ube3a silenced through epigenetic imprinting), thereby providing methods of treatment of genomic imprinting disorders, such as Angelman syndrome.

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SUMMARY OF THE INVENTION

In one aspect, the present invention provides a method of inducing expression of Ube3a in a cell, comprising contacting the cell with an effective amount of one or more inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5, CDK6, CDK8, CDK9,
10 CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2, KMT5C, SETD7, SMYD1, and/or SMYD3, thereby inducing expression of Ube3a in the cell.

An additional aspect of the present invention is a method of treating Angelman Syndrome in a human subject, comprising administering to the subject an effective amount of one or more inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5, CDK6,
15 CDK8, CDK9, CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2, KMT5C, SETD7, SMYD1, and/or SMYD3, thereby treating Angelman syndrome in the subject.

A further aspect of this invention includes a method of treating a disorder associated with an epigenetic modification in a subject, comprising administering to the subject an effective amount of an inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5,
20 CDK6, CDK8, CDK9, CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2, KMT5C, SETD7, SMYD1, and/or SMYD3, thereby treating the disorder associated with the epigenetic modification in the subject.

An additional aspect of the present invention is a method of inducing expression of Ube3a in a cell, comprising contacting the cell with an effective amount of PHA533533 or a
25 salt thereof, (R)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxopyrrolidin-1-yl)phenyl)propanamide, (S)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxooxazolidin-3-yl)phenyl)propanamide, and/or EPZ5676 in any combination, thereby inducing expression of Ube3a in the cell.

Another aspect of the present invention is a method of treating Angelman Syndrome
30 in a human subject, comprising administering to the subject an effective amount of PHA533533 or a salt thereof, (R)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxopyrrolidin-1-yl)phenyl)propanamide, (S)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxooxazolidin-3-yl)phenyl)propanamide, and/or EPZ5676 in any combination, thereby treating Angelman syndrome in the subject.

Another aspect of the present invention is the use of PHA533533 or a salt thereof, (R)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxopyrrolidin-1-yl)phenyl)propenamide, (S)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxooxazolidin-3-yl)phenyl)propenamide, and/or EPZ5676 in any combination in the manufacture of a medicament for the treatment of

5 Angelman Syndrome.

A further aspect of the present invention is the use of PHA533533 or a salt thereof, (R)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxopyrrolidin-1-yl)phenyl)propenamide, (S)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxooxazolidin-3-yl)phenyl)propenamide, and/or EPZ5676 in any combination in the manufacture of a medicament for inducing expression of

10 Ube3a in a cell.

DESCRIPTION OF THE FIGURES

FIGS. 1A-1D show that PHA533533 and Compound X produce unsilencing of paternal *Ube3a-YFP*. **FIG. 1A** shows the chemical structures of topotecan and PHA533533.

15 **FIG. 1B** shows immunofluorescent images of nuclei (Hoechst stain) or paternal UBE3A-YFP (GFP-antibody enhanced) in cultured mouse cortical neurons. Paternal *Ube3a-YFP* was unsilenced by PHA533533 and Compound X, but not by 0.1% DMSO vehicle control (scale bar = 100µm). Topotecan served as a positive control for unsilencing. **FIG. 1C** shows quantitative analysis of UBE3A-YFP. The percentage of neurons expressing paternal

20 UBE3A-YFP was calculated by dividing the number of YFP-positive cells by the total number of nuclei (n=4/group, ** $p < 0.01$, *** $p < 0.001$). **FIG. 1D** shows results from a compound screening of the Pfizer chemogenetic library for paternal *Ube3a* unsilencing agents. Data were normalized to fluorescent intensity and shown as means $\pm$ s.e.m. (n=4/compound). A reliable increase to >1.25 of normalized fluorescent intensity (red line)

25 served as an arbitrary cutoff for hit identification.

FIGS. 2A-2E show pharmacological profiles of PHA533533 and Compound X in unsilencing paternal *Ube3a-YFP* *in vitro*. **FIG. 2A** shows dose-dependence of two novel unsilencers compared to topotecan in unsilencing paternal *Ube3a-YFP* (n=4/group).

Fluorescent intensity of paternal UBE3A-YFP was normalized to vehicle control. **FIG. 2B**

30 shows dose-dependent cytotoxicity of two novel unsilencers compared to topotecan (n=4/group). Luminescence produced by dead-cell protease activity was measured using the Cyto Tox-Glo assay (Promega). **FIG. 2C** shows EC₅₀, **FIG. 2D** shows E_{max}, and **FIG. 2E** shows LC₅₀ of the compounds were estimated (n=4/group, two-way ANOVA with Bonferroni correction for multiple comparison, ** $p < 0.01$).

FIGS. 3A-3G show PHA533533 and Compound X treatments downregulate the *Ube3a-ATS* and produce UBE3A protein from the paternal allele. **FIGS. 3A** and **3B** show Western blot analyses that, like topotecan, PHA533533 and Compound X can produce UBE3A-YFP (**FIG. 3A**) and UBE3A (**FIG. 3B**) protein from the paternal allele, while control Compound Y has no effect. Whole cell lysates from cultured mouse cortical neurons (wildtype, *Ube3a*^{m+/pYFP}, or *Ube3a*^{m-/p+} as indicated) were resolved using SDS-PAGE and immunoblotted with (**FIG. 3A**) anti-GFP, (**FIG. 3B**) anti-Ube3a, or (**FIGS. 3A-3B**) anti- β -actin. Graphs show quantification of normalized signal. DMSO = 0.1% vehicle control; Topotecan = 0.3 μ M; PHA533533 = 1.0 μ M; Compound X = 0.5 μ M; control Compound Y = 0.5 μ M (n=3/group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **FIGS. 3C-3F** show quantitative RT-PCR (qPCR) revealed changes in mRNA of (**FIG. 3C**) *Ube3a-YFP*, (**FIG. 3D**) *Ube3a-antisense (ATS)*, (**FIG. 3E**) *Snord116*, and (**FIG. 3F**) *Snrpn* in cultured mouse cortical neurons after drug treatment. Graphs represent relative mRNA levels normalized to β -actin and shown as means $\pm$ s.e.m. (RQ: Relative quantity, n=3/group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **FIG. 3G** shows the chemical structures of PHA533533 and four analogues thereof.

FIGS. 4A-4C show knockdown of established PHA533533 and Compound X molecular targets is not sufficient to dramatically unsilence paternal *Ube3a-YFP*. **FIG. 4A** shows efficient knockdown of cyclin-dependent kinase 2 (CDK2) did not unsilence paternal *Ube3a-YFP* unless neurons were treated with topotecan or PHA533533. **FIG. 4B** shows efficient knockdown of CDK5 partially unsilenced paternal *Ube3a-YFP*, but unsilencing was further enhanced by treatment of topotecan or PHA533533. **FIG. 4C** shows knockdown of topoisomerase 1 (Top1) did not unsilence paternal *Ube3a-YFP* unless neurons were treated with PHA533533. Whole cell lysates were resolved using SDS-PAGE and immunoblotted with anti-GFP and anti- β -actin. For **FIGS. 4A-4C** each: Left: Knockdown of CDK2, CDK5, and Top1 (n=3/group, ** $p < 0.01$, *** $p < 0.001$). Middle: Immunoblot with anti-GFP and anti- β -actin. Right: Quantitative analysis of UBE3A-YFP unsilencing normalized to β -actin. scr: scrambled shRNA control, WT: wildtype; DMSO, (0.1% vehicle control); Topotecan (0.3 μ M), PHA533533 (1.0 μ M) (n=3/group, * $p < 0.05$, ** $p < 0.01$).

FIGS. 5A-5F show dual inhibition of CDK5 and DOT1L may be required to unsilence paternal *Ube3a-YFP*. **FIG. 5A** shows topoisomerase-based DNA relaxation assay showing that PHA533533 and Compound X partially inhibits Top2a whereas control Compound Y completely inhibits Top2a. ICRF-193, a known Top2a and 2b inhibitor, serves as a positive control. **FIG. 5B** shows Western blot analysis suggesting that dual inhibition of

CDK5 and DOT1L is sufficient for unsilencing of paternal *Ube3a-YFP*. Whole cell lysates from cultured mouse cortical neurons (*Ube3a^{m+/pYFP}*) after drug treatment were resolved using SDS-PAGE and immunoblotted with anti-GFP, anti-H3K79me (di- and tri-), or anti- β -actin. Left: immunoblot with anti-GFP, anti-H3K79me2, anti-H3K79me3, and anti- β -actin.

5 Right: quantitative analysis of H3K79me and unsilenced *Ube3a-YFP*. Levels were normalized to β -actin. WT: wildtype, DMSO (0.1% vehicle control); Topotecan (0.3 μ M); PHA533533 (1.0 μ M); Compound X (0.5 μ M); and control Compound Y (0.5 μ M); EPZ5676 (3 μ M); and EPZ5676 (1 μ M) with P5-A4 (1 μ M) (n=3/group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **FIGS. 5C-5F** show quantitative RT-PCR showing changes in the mRNA levels of
10 **(FIG. 5C) *Ube3a-YFP*, (FIG. 5D) *Ube3a-ATS*, (FIG. 5E) *Snord116*, and (FIG. 5F) *Snrpn*** in cultured mouse cortical neurons after drug treatment. Expression was normalized relative to β -actin and shown as means $\pm$ s.e.m. (RQ: Relative quantity, n=3/groups, * $p < 0.05$, ** $p < 0.01$).

FIG. 6 shows four structural analogues of PHA533533 produced only modest
15 paternal *Ube3a* unsilencing by western blot analysis.

FIGS. 7A-7B show DNA relaxation assays evaluating relative inhibition of Top1 **(FIG. 7A)** and Top2b **(FIG. 7B)** via treatment with PHA533533, PHA793887, Dinaciclib, Compound X, or Topotecan.

FIGS. 8A-8C show PHA533533 unsilencing of paternal *Ube3a-YFP*. **FIG. 8A** shows
20 a schematic of the high content imaging screen. Paternal UBE3A-YFP levels were assessed using GFP antibody-enhanced fluorescence and high-content imaging. **FIG. 8B** shows DAPI (nuclear stain) and paternal UBE3A-YFP immunocytochemistry in cultured neurons treated with 0.1% DMSO (negative control), topotecan (positive control), S-enantiomer of PHA533533, R-enantiomer of PHA533533, or a racemic mixture of S and R enantiomers of
25 PHA533533. **FIG. 8C** shows quantitative analysis of the percentage of cells expressing paternal UBE3A-YFP, calculated by dividing the number of YFP-positive cells by the total number of DAPI-positive nuclei (n=4/group, ** $p < 0.01$, *** $p < 0.001$).

FIGS. 9A-9E show pharmacological profile of PHA533533 in unsilencing paternal *Ube3a-YFP in vitro*. **FIG. 9A** shows dose-dependence of PHA533533 compared to topotecan
30 in unsilencing paternal *Ube3a-YFP* (n=4/group). Fluorescent intensity of paternal UBE3A-YFP was normalized to vehicle control. **FIG. 9B** shows dose-dependent cytotoxicity of PHA533533 compared to topotecan (n=4/group). Luminescence produced by dead-cell protease activity was measured using the Cyto Tox-Glo assay (Promega). **FIG. 9C** shows

EC₅₀, **FIG. 9D** shows E_{max}, and **FIG. 9E** shows LC₅₀ of the compounds shown as mean ± s.e.m.

FIGS. 10A-10E show pharmacological profile of PHA533533 analogs in unsilencing paternal *Ube3a-YFP* *in vitro*. **FIGS. 10A-10C** show dose-dependence of PHA533533 analogs (**FIG. 10A**) PNU0292137, (**FIG. 10B**) PNU0278497, and (**FIG. 10C**) PHA00543738 in unsilencing paternal *Ube3a-YFP* (n=4/group). Fluorescent intensity of paternal UBE3A-YFP was normalized to vehicle control. **FIG. 10D** shows EC₅₀ and **FIG. 10E** shows E_{max} of the PHA533533 and its analogs shown as mean ± s.e.m.

FIGS. 11A-11B show that PHA533533 treatment produces UBE3A protein from the paternal allele. Western blot analyses show UBE3A-YFP (**FIG. 11A**) and UBE3A (**FIG. 11B**) protein from the paternal allele. Whole cell lysates from cultured mouse cortical neurons (wildtype, *Ube3a*^{m+/pYFP}, or *Ube3a*^{m-/p+} as indicated) were resolved using SDS-PAGE and immunoblotted with anti-GFP (**FIG. 11A**), anti-Ube3a (**FIG. 11B**), or anti-β-actin (**FIGS. 11A-11B**) antibodies. Graphs show quantification of normalized signal. DMSO = 0.1% vehicle control; Topotecan = 0.3 μM; PHA533533 = 1.0 μM (n=3/group, * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001).

FIGS. 12A-12D show PHA533533 treatment downregulates *Ube3a-ATS* in neurons. Quantitative RT-PCR (qPCR) changes in mRNA of paternal *Ube3a-YFP* (**FIG. 12A**), *Ube3a-antisense (ATS)* (**FIG. 12B**), *Snord116* (**FIG. 12C**), and *Snrpn* (**FIG. 12D**) in cultured mouse cortical neurons (wildtype or *Ube3a*^{m+/pYFP} as indicated) after drug treatment are shown. Graphs represent relative mRNA levels normalized to β-actin and shown as mean ± s.e.m. DMSO = 0.1% vehicle control; Topotecan = 0.3 μM; PHA533533 = 1.0 μM (RQ: Relative quantity, n=3/group, * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001).

FIGS. 13A-13I show that selective knockdown of established PHA533533 molecular targets is not sufficient to unsilence paternal *Ube3a-YFP* as effectively as PHA533533. **FIG. 13A** shows knockdown of cyclin-dependent kinase 2 (CDK2). **FIG. 13B** shows Western blot analysis and **FIG. 13C** shows quantification of shRNA knockdown. **FIGS. 13D** shows knockdown of cyclin-dependent kinase 5 (CDK5) by shRNA. **FIG. 13E** shows Western blot analysis and **FIG. 13F** shows quantification of CDK5 knockdown. **FIG. 13G** show shRNA knockdown of topoisomerase 1 (Top1). **FIG. 13H** shows Western blot analysis and **FIG. 13I** shows quantification of knockdown of Top1. Whole cell lysates were resolved using SDS-PAGE and immunoblotted with anti-GFP and anti-β-actin. (n=3/group, ** *p* < 0.01, *** *p* < 0.001). For **FIGS. 13B, 13E, 13H** Western immunoblot was performed with anti-GFP and anti-β-actin antibodies. **FIGS. 13C, 13F, 13I**, quantitative analysis of UBE3A-YFP

unsilencing normalized to β -actin. scr: scrambled shRNA control, WT: wildtype; DMSO, (0.1% vehicle control); Topotecan (0.3 μ M), PHA533533 (1.0 μ M) (n=3/group, * $p < 0.05$, ** $p < 0.01$).

FIGS. 14A-14E show that PHA533533 upregulates paternal Ube3a *in vivo*. **FIG. 14A** shows a schematic of the experimental design. **FIGS. 14B-14D** show immunohistochemistry of WT (**FIG. 14B**) or Ube3a^{m+/pYFP} (**FIGS. 14C-14E**) mice GFP-stained sagittal brain sections at postnatal day 13 (P13) following intraperitoneal (IP) injection of saline (**FIGS. 14B-14C**) or 2 mg/kg of PHA533533 (**FIG. 14D**) at P11. **FIG. 14E** show high-magnification confocal micrographs of subcellular localization of paternal UBE3A-YFP in NeuN-positive hippocampal area CA3 neurons in Ube3a^{m+/pYFP} mouse following IP injection of 2 mg/kg of PHA533533. UBE3A-YFP protein shows pronounced nuclear localization, consistent with the predominantly nuclear localization of UBE3A in mature neurons. Scale bars: 1 mm for (**FIGS. 14B-14D**) and 20 μ m for (**FIG. 14E**).

DETAILED DESCRIPTION OF THE INVENTION

Particular aspects of this invention are explained in greater detail below. This description is not intended to be a detailed catalog of all the different ways in which the invention may be implemented, or all the features that may be added to the instant invention. For example, features illustrated with respect to one embodiment may be incorporated into other embodiments, and features illustrated with respect to a particular embodiment may be deleted from that embodiment. In addition, numerous variations and additions to the various embodiments suggested herein will be apparent to those skilled in the art in light of the instant disclosure that do not depart from the instant invention.

Hence, the following specification is intended to illustrate some particular embodiments of the invention, and not to exhaustively specify all permutations, combinations and variations thereof.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. All publications, patent applications, patents, nucleotide sequences, amino acid sequences and other references mentioned herein are incorporated by reference in their entirety.

As used herein, "a," "an," or "the" can mean one or more than one. For example, "a" cell can mean a single cell or a multiplicity of cells.

Also as used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

Furthermore, the term "about," as used herein when referring to a measurable value such as an amount of a compound or agent of this invention, dose, time, temperature, and the like, is meant to encompass variations of $\pm 20\%$, $\pm 10\%$, $\pm 5\%$, $\pm 1\%$, $\pm 0.5\%$, or even $\pm 0.1\%$ of the specified amount.

As used herein, the transitional phrase "consisting essentially of" is to be interpreted as encompassing the recited materials or steps and those that do not materially affect the basic and novel characteristic(s) of the claimed invention. Thus, the term "consisting essentially of" as used herein should not be interpreted as equivalent to "comprising."

All publications, patent applications, patents, nucleotide sequences, amino acid sequences and other references mentioned herein are incorporated by reference in their entirety.

As used herein, an "inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5, CDK6, CDK8, CDK9, CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2, KMT5C, SETD7, SMYD1, and/or SMYD3" is a molecule (e.g., small molecule, e.g., a compound) that interferes with the activity of any of the listed proteins. In some embodiments, an inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5, CDK6, CDK8, CDK9, CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2, KMT5C, SETD7, SMYD1, and/or SMYD3s may be PHA533533 or a derivative (e.g., metabolite thereof, e.g., salt thereof). PHA533533 is a synthetic compound originally designed for inhibiting cyclin-dependent kinase 2 (CDK2), but also inhibits CDK5 (Pevarello et al. *J. Med. Chem.* 2005:48(8):2944-2956). Other inhibitors include, but are not limited to, (R)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxopyrrolidin-1-yl)phenyl)propenamide, (S)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxooxazolidin-3-yl)phenyl)propenamide [also referred to herein as P5-A4], or EPZ5676, as well as any other inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5, CDK6, CDK8, CDK9, CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2, KMT5C, SETD7, SMYD1, and/or SMYD3 now known or later identified. In some embodiments, one or more inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5, CDK6, CDK8, CDK9, CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2,

KMT5C, SETD7, SMYD1, and/or SMYD3 may be blood-brain barrier (BBB) permeable (e.g., a BBB permeant).

As used herein, a "genomic imprinting disorder" means any disorder caused by the mutation or deletion of a gene that is genetically imprinted, any disorder caused by alterations of the normal imprinting pattern, and/or any disorder caused by changes in gene dosage of an imprinted gene. In some embodiments, a genomic imprinting disorder of this invention may be Beckwith-Wiedemann syndrome, Silver-Russell syndrome, Prader-Willi syndrome, and/or Angelman syndrome. In some embodiments, a genomic imprinting disorder of this invention may be Angelman syndrome.

The present invention is based on the development of screening assays to identify substances (e.g., small molecules) that unsilence imprinted genes. This invention is further based on the unexpected discovery that PHA533533 can be used to treat Angelman syndrome.

Thus, in one embodiment, the present invention provides a method of inducing expression of Ube3a in a cell, comprising contacting the cell with an effective amount of one or more inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5, CDK6, CDK8, CDK9, CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2, KMT5C, SETD7, SMYD1, and/or SMYD3, thereby inducing expression of Ube3a in the cell. In some embodiments, the cell can be a central neuron, a peripheral neuron, a neuron differentiated from stem cells, a glial cell, an astrocyte, an oligodendrocyte, a microglial cell, and any combination thereof. In some embodiments, the cell can be in a subject (e.g., a human subject) and the Ube3a that is induced is the paternal allele of Ube3a. Such a subject can be a subject in which function of the maternal allele of Ube3a has been lost and Ube3a protein production is defective in the subject.

In certain embodiments of the present invention, the one or more inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5, CDK6, CDK8, CDK9, CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2, KMT5C, SETD7, SMYD1, and/or SMYD3 has an efficiency, $E_{\max}$, of at least about 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, or 2.5 fold over control, or any value or range therein.

The present invention also provides various compositions. In some embodiments these compositions can be employed, e.g., in the methods described herein. Thus, the present invention provides a composition comprising, consisting essentially of and/or consisting of one or more inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5, CDK6, CDK8, CDK9, CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2, KMT5C, SETD7,

SMYD1, and/or SMYD3 and/or other compound of this invention (e.g., Compound X and/or PHA533533), which can be, for example, in a pharmaceutically acceptable carrier.

"Pharmaceutically acceptable," as used herein, means a material that is not biologically or otherwise undesirable, *i.e.*, the material may be administered to a subject along with the compositions of this invention, without causing substantial deleterious biological effects or interacting in a deleterious manner with any of the other components of the composition in which it is contained. The material would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art (see, *e.g.*, *Remington's Pharmaceutical Science*; latest edition). Exemplary pharmaceutically acceptable carriers for the compositions of this invention include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution, as well as other carriers suitable for injection into and/or delivery to a subject of this invention, particularly a human subject, as would be well known in the art.

It is further contemplated that the present invention provides a kit comprising, consisting essentially of and/or consisting of one or more compositions of this invention. It would be well understood by one of ordinary skill in the art that the kit of this invention can comprise one or more containers and/or receptacles to hold the reagents (e.g., one or more inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5, CDK6, CDK8, CDK9, CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2, KMT5C, SETD7, SMYD1, and/or SMYD3, e.g., Compound X, PHA533533, etc.) of the kit, along with appropriate buffers and/or diluents and/or other solutions and directions for using the kit, as would be well known in the art. The compositions and kits of the present invention can also include other medicinal agents, pharmaceutical agents, carriers and diluents, etc., in any combination. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art.

In the kits of this invention, the compositions can be presented in unit/dose or multi-dose containers, for example, in sealed ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of a sterile liquid carrier, for example, saline or water-for-injection prior to use.

Symptomology of a genomic imprinting disorder and/or a disorder associated with epigenetic modification can include, but is not limited to, severe intellectual disabilities, seizures, EEG abnormalities, gait disturbances, disrupted sleep patterns, somatosensory deficits, profound language impairments, abnormal pain sensitivity, and balance

abnormalities, which can be manifested singly and/or in any combination over time. Thus it is further contemplated that the present invention provides methods of treating one or more of these symptoms in any combination in a subject, comprising administering to the subject an effective amount of one or more inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5, CDK6, CDK8, CDK9, CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2, KMT5C, SETD7, SMYD1, and/or SMYD3.

A subject of this invention can be any animal in which genomic imprinting disorders occur and in particular embodiments, is a human subject, although nonhuman subjects [e.g., animal models of genomic imprinting disorders such as rodents (mice, rats, hamsters, guinea pigs, etc.), pigs, non-human primates] are included within the present invention.

A subject of this invention can be "in need of" the methods of the present invention, e.g., because the subject has, or is believed at risk for, a genomic imprinting disorder including those described herein, such as Angelman syndrome and/or is a subject that would benefit from the methods of this invention. For example, a subject in need of the methods of this invention can be, but is not limited to, a subject diagnosed with, having or suspected to have, or at risk of having or developing a genomic imprinting disorder (e.g., Angelman syndrome).

The term "therapeutically effective amount" or "effective amount," as used herein, refers to that amount of an inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5, CDK6, CDK8, CDK9, CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2, KMT5C, SETD7, SMYD1, and/or SMYD3 and/or other compound and/or composition of this invention that imparts a modulating effect, which, for example, can be a beneficial effect, to a subject afflicted with a condition (e.g., a disorder, disease, syndrome, illness, injury, traumatic and/or surgical wound), including improvement in the condition of the subject (e.g., in one or more symptoms), delay or reduction in the progression of the condition, delay of the onset of the condition, and/or change in clinical parameters, status or classification of a disease or illness, etc., as would be well known in the art.

For example, a therapeutically effective amount or effective amount can refer to the amount of an inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5, CDK6, CDK8, CDK9, CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2, KMT5C, SETD7, SMYD1, and/or SMYD3 and/or other compound and/or composition of this invention that improves a condition in a subject by at least about 5%, e.g., at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at

least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%.

"Treat" or "treating" or "treatment" refers to any type of action that imparts a modulating effect, which, for example, can be a beneficial effect, to a subject afflicted with a condition (e.g., disorder, disease, syndrome, illness, traumatic or surgical wound, injury, etc.), including improvement in the condition of the subject (e.g., in one or more symptoms), delay or reduction in the progression of the condition, delay of the onset of the condition, and/or change in clinical parameters, disease or illness, *etc.*, as would be well known in the art.

By the terms "treat," "treating" or "treatment of" (or grammatically equivalent terms), it is also meant that the severity of the subject's condition is reduced or at least partially improved or ameliorated and/or that some alleviation, mitigation or decrease in at least one clinical symptom is achieved and/or there is a delay in the progression of the condition and/or delay of the onset of a disease or disorder.

By "prevent," "preventing" or "prevention" is meant to avoid or eliminate the development and/or manifestation of a pathological state and/or disease condition or disorder or status in a subject.

Exemplary modes of administration of one or more inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5, CDK6, CDK8, CDK9, CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2, KMT5C, SETD7, SMYD1, and/or SMYD3 (e.g., Compound X, PHA533533, etc.) and/or other compound and/or composition of this invention can include oral, rectal, transmucosal, topical, intranasal, inhalation (e.g., via an aerosol), buccal (e.g., sublingual), vaginal, intrathecal, intracerebroventricular, intraocular, transdermal, *in utero* (or *in ovo*), parenteral (e.g., intravenous, subcutaneous, intradermal, intramuscular [including administration to skeletal, diaphragm and/or cardiac muscle], intradermal, intrapleural, intracerebral, and intraarticular), topical (e.g., to both skin and mucosal surfaces, including airway surfaces, and transdermal administration, and the like, as well as direct tissue or organ injection (e.g., to liver, skeletal muscle, cardiac muscle, diaphragm muscle or brain). The most suitable route in any given case will depend on the nature and severity of the condition being treated and on the nature of the particular therapeutic compound and/or composition that is being used.

For injection, the carrier will typically be a liquid. For other methods of administration, the carrier may be either solid or liquid. For inhalation administration, the carrier will be respirable, and will typically be in solid or liquid particulate form.

Dosages of the one or more inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5, CDK6, CDK8, CDK9, CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2, KMT5C, SETD7, SMYD1, and/or SMYD3 and/or other compound(s) of this invention to be administered to a subject will depend upon the mode of administration, the disease or condition to be treated, the individual subject's condition, the particular therapeutic compound and/or composition, and any other agents being administered to the subject and can be determined in a routine manner according to methods well known in the art. An exemplary dosage range for a human subject is from about 0.001 mg/kg/day to about 500 mg/kg/day. In some embodiments, the dosage range can be from about 0.01 mg/kg/day to about 100 mg/kg/day and in some embodiments, the dosage range can be from about 0.1 mg/kg/day to about 10 mg/kg/day. For example, in some embodiments, the dosage range can be from about 0.1 mg/kg/day to about 2 mg/kg/day, about 1.5 mg/kg/day to about 8 mg/kg/day, about 200 mg/kg/day to about 450 mg/kg/day, or about 1 mg/kg/day to about 5 mg/kg/day or any value or range therein, e.g., about 1 mg/kg/day, about 2 mg/kg/day, about 5 mg/kg/day, about 10 mg/kg/day, about 50 mg/kg/day, about 100 mg/kg/day, about 250 mg/kg/day, or about 450 mg/kg/day.

A nonlimiting example of a method of treating Angelman syndrome in a human subject comprises administering to the subject (e.g., *in utero*, perinatally, postnatally, during infancy, during childhood, during adolescence, during teen years, during early adulthood, during middle adulthood, during late adulthood and any combination thereof), a dose of one or more inhibitor of CDK5, DOT1L, ATXR5, ASH1L, CBP, CDK2, CDK5, CDK6, CDK8, CDK9, CDK16, CDKL1, CDKL3, EHMT1, EHMT2, EZH2, KMT5C, SETD7, SMYD1, and/or SMYD3 of this invention in the range of about 0.01 nmole to about 100 nmole by an intrathecal and/or intracerebroventricular route, or any value or range therein. For example, in some embodiments the dose could be in the range of about 0.1 nmole to about 2 nmole, about 1.5 nmole to about 10 nmole, about 2 nmole to about 5 nmole, about 1 nmole to about 75 nmole, about 50 nmole to about 100 nmole, or about 0.1 nmole, about 0.5 nmole, about 1 nmole, about 2 nmole, about 5 nmole, about 10 nmole, about 25 nmole, about 50 nmole, about 75 nmole, about 100 nmole, about 250 nmole, about 500 nmole, about 750 nmole, about 1 nmole, about 2 nmole, about 5 nmole, about 10 nmole, about 25 nmole, about 50 nmole, about 75 nmole, or about 100 nmole. In some embodiments, the route of administration could be intrathecal and/or intracerebroventricular and the dose could be in the range of about 0.01 mg/kg to about 100 mg/kg or any value or range therein, and in some embodiments could be about 0.1 mg/kg to about 10 mg/kg or any value or range therein. For

example, in some embodiments the dose could be in the range of about 0.1 mg/kg to about 2 mg/kg, about 1.5 mg/kg to about 10 mg/kg, about 2 mg/kg to about 5 mg/kg, about 1 mg/kg to about 75 mg/kg, about 50 mg/kg to about 100 mg/kg, or about 0.1 mg/kg, about 0.5 mg/kg, about 1 mg/kg, about 2 mg/kg, about 5 mg/kg, about 10 mg/kg, about 25 mg/kg, about 50 mg/kg, about 75 mg/kg, about 100 mg/kg.

In some embodiments, a single administration of a therapeutic compound and/or composition of this invention may be effective. In other embodiments, more than one administration (*e.g.*, two, three, four or more administrations) of the therapeutic compound and/or composition may be employed to achieve the desired result over a period of various intervals, *e.g.*, hourly, daily, weekly, monthly, yearly, etc.

The present invention will now be described with reference to the following examples. It should be appreciated that these examples are for the purposes of illustrating aspects of the present invention, and do not limit the scope of the invention as defined by the claims provided herein.

EXAMPLES

Example 1:

There are numerous mechanistic possibilities for reactivating paternal *UBE3A* expression, such as (1) increasing methylation to repress *UBE3A-ATS*, (2) powerfully driving *UBE3A* expression to outcompete *UBE3A-ATS*, or (3) directly repressing *UBE3A-ATS*. Any approach that unsilences the dormant paternal *UBE3A* allele in neurons would offer a therapeutic opportunity for AS.

Because the paternally-inherited copy of *UBE3A* is intact but epigenetically silenced in most mature neurons, maternal allele loss of *UBE3A* largely depletes functional *UBE3A* protein in the brain. This unique epigenetic biology suggests that activating the dormant paternal allele of *UBE3A* could provide a transformative treatment for AS. The feasibility of this approach was previously demonstrated by showing that topoisomerase inhibitors, such as topotecan, can successfully reactivate the paternal *Ube3a* allele in neurons from AS model mice. However, challenges with toxicities and central nervous system (CNS) bioavailability have limited the potential for topoisomerase inhibitors to treat AS, raising a quest to identify novel small molecule *Ube3a* unsilencers. Herein is reported a high content screen, which revealed two small molecules, PHA533533 and Compound X, that effectively reactivate paternal *Ube3a* in neurons from AS model mice. These compounds do not produce *Ube3a* unsilencing through topoisomerase inhibition, suggesting they act through novel mechanisms

of unsilencing that are shown to possibly involve the simultaneous inhibition of cyclin-dependent kinase 5 (CDK5) and disruptor of telomeric silencing 1-like (DOT1L). This study provides novel small molecule approaches and potential mechanisms to unsilence paternal *Ube3a*, and hence, to treat AS. PHA533533 is an inhibitor of cyclin-dependent kinase 2 and 5 (CDK2 and CDK5) (Pevarello et al. *J Med Chem* 2005 48(8):2944-2956). Molecular predictions and subsequent preliminary experiments suggest that these two compounds might be producing their effects through shared action of inhibiting CDK5 and DOT1L [Disruptor of telomeric silencing 1-like; histone H3 lysine 79 (H3K79) specific methyltransferase]. The results of this study provide new therapeutic possibilities for treating AS that could be advanced for preclinical testing of safety and efficacy in mouse models of AS and further vetted using AS patient-derived neuronal cell lines.

Animals: All animal experiments were conducted under the Institutional Animal Care and Use Committee protocol approved by the University of North Carolina School of Medicine. To generate paternal YFP-tagged mice (*Ube3a^{m+/pYFP}*) for high content screen and drug validation, heterozygote *Ube3a-YFP* males were crossed with wildtype (WT) females. *Ube3a^{m+/p-}* females were also crossed with WT males to generate AS model mice (*Ube3a^{m-/p+}*). Mice were housed on a 12 hr:12 hr light/dark cycle and given *ad libitum* access to food and water. Embryonic male and female mice were randomly used for cortical neuronal isolation and culture.

Chemistry and drug preparation: Topotecan hydrochloride and EPZ5676 were purchased from Cayman Chemicals. Dinaciclib and PHA793887 were purchased from SelleckChem. Amsacrine and ICRF-193 were purchased from Sigma Aldrich. Synthesis of PHA533533 and four analogues has been previously described. Individual compounds in the Pfizer chemogenetic library were pre-dissolved in DMSO. The drug plates for screening were prepared at a final concentration of 1 μ M. Cherry-picked compounds listed above were freshly prepared in DMSO as a 10 mM stock and further diluted for the final experimental concentration in culture medium or enzyme assays.

Cell culture and drug treatment: Primary cortical neurons were isolated and cultured by following established protocol. Briefly, cortical neurons were isolated from embryos (E13.5-15.5) carrying paternal *Ube3a-YFP* (*Ube3a^{m+/pYFP}*) or maternal deletion of *Ube3a* (*Ube3a^{m-/p+}*). Isolated cortical neurons were plated onto 384-well plates coated with poly-D-lysine (2.0×10^4 cell/well) for high-content imaging analysis or 6-well plates coated with poly-D-lysine (1.0×10^6 cells/well) for molecular and biochemical assays (western blot analysis, quantitative RT-PCR, and shRNA knockdown). Cultured neurons were maintained for 7

days, replacing medium every 3-4 days. On day 7 (DIV 7), the indicated compounds were added at 0.3-1 μ M to neurons for 72 hr to unsilence paternal *Ube3a* or *Ube3a*-YFP. All drug concentrations are provided in figures.

Fluorescence immunocytochemistry and high content imaging analysis: Fluorescent immunocytochemistry and high content imaging analysis were performed by following previously described protocols with slight modifications. After 72 hr drug treatment, the neurons were fixed with 4% paraformaldehyde in 1x PBS at room temperature for 10 min. After brief washing with 1x PBS three times, neurons were permeabilized with 1% Triton-X 100 in 1x PBS at room temperature for 15 min, followed by blocking with 5% normal goat serum in 0.1% Triton-X 100 in 1x PBS at room temperature. 60 min after blocking, neurons were incubated with primary antibody, rabbit anti-GFP (1:1000, Novus Biologicals), at 4°C overnight. Neurons were then briefly washed with 1x PBS followed by incubation with secondary antibody, goat anti-rabbit Alexa Fluor 488 (1:500, Thermo Fisher/Invitrogen) and Hoechst 33342 (Thermo Fisher), at room temperature for 60 min. Following secondary antibody incubation, neurons were washed with 1x PBS and fluorescent images were acquired using a BD Pathway 855 bioimager. All acquired images were analyzed by CellProfiler.

Cytotoxicity test: Cytotoxicity of drugs was tested in cultured mouse cortical neurons *in vitro*. The manufacturer's protocol was followed to measure luminescence proportional to the number of dead cells using Cyto Tox-Glo assay (Promega). 72 hr after drug treatment, AAF-Glo substrates were directed added to the drug-treated (or 0.1% DMSO vehicle-treated) cortical neurons and incubated them at room temperature for 15 min. Luminescence produced by dead-cell protease activity was then measured.

shRNA knockdown and western blot analysis: Lentiviral shRNAs of Top1 (TRCN0000011884), CDK2 (TRCN0000023146), and CDK5 (TRCN0000009520) were generated using previously described techniques. Cultured cortical neurons were infected at DIV 3 and virus was removed the next day (DIV 4). Neurons were further cultured and treated at DIV 10 with vehicle (0.1% DMSO) or the indicated compounds. To perform western blot analysis, the cultured neurons from 6-well plates were harvested and total protein was extracted with protein extraction buffer 72 hr after drug treatment, Bradford assays were performed to measure protein concentration, and 30 μ g of total protein was loaded for Bis-polyacrylamide gel electrophoresis (Bio-Rad). Electrophoresed proteins were transferred to nitrocellulose membrane (0.45 μ m, Bio-Rad). Membranes were blocked with 5% non-fat milk in 1x TBS-T at room temperature for 30 min followed by overnight 4°C

incubation with primary antibodies [rabbit anti-GFP (1:1000, Novus Biologicals), rabbit anti-Ube3a (1:1000, Bethyl Lab), rabbit anti-H3K79Me2 (1:1000, Abcam), rabbit anti-H3K79Me3 (1:1000, Abcam), and mouse anti- β -actin (1:5000, Sigma)]. The next day, the immunoblot membranes were rinsed with 1x TBS-T three times and incubated with HRP-conjugated secondary antibodies for 1 hr at room temperature (goat anti-rabbit @ 1:1000 or goat anti-mouse @ 1:1000, Vector Labs). Following secondary antibody incubation, the membranes were rinsed (4-5 times) with 1x TBS-T at room temperature for 1 hr and ECL (Bio-Rad) was used to visualize detected proteins using an Amersham Imager 600 (AI600, GE Life Sciences).

Quantitative real-time PCR and topoisomerase-based DNA relaxation assay:

Quantitative real-time PCR (qPCR) was performed using previously reported protocols. Briefly, total RNA was extracted from cultured cortical neurons carrying *Ube3a^{m+/pYFP}* after drug treatment using Direct-zol RNA kit (Zymo Research). cDNA pool was synthesized using 2 μ g of total RNA and qScript cDNA Supermix (Quantabio). qPCR was performed using SsoAdvanced Universal SYBR green Supermix (Bio-rad). The primer sets were *β -actin* (forward, 5'-agagctacgagctgcctgac-3', SEQ ID NO:1; reverse, 5'-agcactgtgtggcgctacag-3, SEQ ID NO:2), *YFP* (forward, 5'-acatgaagcagcagcagcttct-3', SEQ ID NO:3; reverse, 5'-gacgtgtgtgctgtgtgtgtgta-3', SEQ ID NO:4), *Ube3a-ATS* (forward, 5'-acagaacaataggtcaccagggtt-3', SEQ ID NO:5; reverse, 5'-aagcaagactgttcacctcat-3', SEQ ID NO:6), *Snrpn* (forward, 5'-ttggttctgaggagtgtttgc-3', SEQ ID NO:7; reverse, 5'-ccttgaattccaccaccttg-3', SEQ ID NO:8), and *Snord116* (forward, 5'-ggatctatgatgattccag-3', SEQ ID NO:9; reverse, 5'-ggacctcagttccgatga-3', SEQ ID NO:10). qPCR was performed using QuantStudio thermocycler (Thermo Fisher) with the following conditions: 95°C for 5 min and followed by 40 cycles of 95°C 10 sec, 60°C 1 min. Relative quantity (RQ) was calculated after normalized $\Delta\Delta C_t$ was calculated. Topoisomerase (Top)-based DNA relaxation assays were performed to test whether the drugs inhibited Top1, Top2a, and Top2b. Top1 and Top2a assay kits were purchased from TopoGEN, and Top2b enzyme was purchased from Inspiralis. All Top-based assays followed manufacturer protocols.

Statistical analysis: One-way ANOVA with Dunnett's multiple comparison test was performed to determine significant changes in unsilencing of paternal *Ube3a-YFP* or *Ube3a*. Two-way ANOVA with Bonferroni correction for multiple comparisons was performed to determine significant differences in EC_{50} , E_{max} , and LC_{50} .

PHA533533 and Compound X are novel Ube3a unsilencers: A high-content drug screen that was previously developed for primary neurons (Huang et al. *Nature* 2011

481(7380):185-189) was employed to identify novel classes of unsilencers. This screen leverages mice that have a paternal *Ube3a-YFP* knock-in, allowing for fluorescence-based identification of paternal *Ube3a* expression in cultured neurons from these mice. Through screening >2,800 compounds in the Pfizer chemogenetic library, two small molecules,

5 PHA533533 and Compound X, were found that effectively unsilenced paternal *Ube3a-YFP* (**FIG. 1A-1D**). Importantly, PHA533533 and Compound X are not structural analogues, nor are they structural analogues to topotecan (**FIG. 1A**). As a negative control, there was minimal paternal UBE3A-YFP protein expressed in cultured cortical neurons in the presence of 0.1% DMSO (vehicle control) (**FIG. 1B**). As an important positive control, topotecan
10 unsilenced paternal UBE3A-YFP as previously reported (Huang et al) (**FIG. 1B**). In quantifying the results across sample runs (n=4), topotecan ($37.3 \pm 3.7\%$), PHA533533 ($63.7 \pm 14.3\%$), and Compound X ($61.2 \pm 15.7\%$) all significantly increased the percent of neurons expressing UBE3A-YFP above threshold compared to DMSO control ($0.03 \pm 0.004\%$) (**FIG. 1C**). PHA533533 was originally designed for inhibiting cyclin-dependent kinase 2 (CDK2),
15 however it also inhibits CDK5. These data suggest that PHA533533 and Compound X may unsilence paternal *Ube3a* through a previously undescribed mechanism.

To understand the pharmacological properties of these novel unsilencers, their EC_{50} (effective dose producing relative 50% of normalized intensity), E_{max} (maximum effectiveness in normalized intensity), and LC_{50} (lethal dose required to kill 50% of cell
20 population) were measured. Dose response curves indicated that the potency (EC_{50}) of PHA533533 is > 2-fold higher than that of topotecan, whereas Compound X shared a similar potency with topotecan (**FIG. 2A and 2C**). On the other hand, the efficacy (E_{max}) of the three drugs are similar (**FIG. 2A and 2D**). Cytotoxicity tests revealed a comparable LC_{50} for PHA533533 and topotecan, while the LC_{50} of Compound X is ~2-fold less than the other two
25 compounds (**FIG. 2B and 2E**). These data suggest that the novel unsilencers are drug-like small molecules that could be developed as potential AS therapeutics.

PHA533533 and Compound X downregulate Ube3a-antisense transcript and unsilence paternal Ube3a in AS model mice (*Ube3a^{m-/p+}*): To exclude the possibility that
30 image-based results were due to fluorescence artifacts (e.g., intrinsic fluorescence in compounds), western blots were performed to analyze UBE3A-YFP protein expression from drug- and vehicle-treated primary neurons from WT or paternal *Ube3a-YFP* mice. The opportunity was also taken to test the effects of control Compound Y. No UBE3A-YFP signal was detected in neurons from WT mice, demonstrating specificity of our GFP antibody towards YFP (**FIG. 3A**). Paternal UBE3A-YFP was marginally detectable in 0.1% DMSO

treated *Ube3a*^{m+/pYFP} neurons, probably due to incomplete silencing of paternal *Ube3a-YFP* in these immature neurons and/or to small populations of glial cells that normally express *Ube3a* biallelically. These results show that PHA533533 and Compound X increased UBE3A-YFP protein to levels approaching that of topotecan (**FIG. 3A**). However, control
5 Compound Y did not unsilence paternal UBE3A-YFP.

To exclude the possibility that the results were somehow an artifact of the *Ube3a-YFP* knock-in transgene, western blots were performed to analyze UBE3A protein expression from drug- and vehicle-treated primary neurons from WT or AS model mice (*Ube3a*^{m-/p+}). This also provided the opportunity to assess the extent to which the unsilencing agents
10 restored UBE3A protein to WT levels. Topotecan, PHA533533, and Compound X unsilenced paternal UBE3A at levels that approached that of UBE3A levels in DMSO-treated neurons from WT mice (**FIG. 3B**). Little paternal UBE3A was observed when treating neurons from AS model mice with control Compound Y, similar to what was observed in DMSO-treated AS neurons. Two enantiomer pairs of PHA533533 structural analogues were also tested
15 (**FIG. 3G**), and it was found that neither 1 μ M drug treatments (**FIG. 7**), nor dose-response tests, of these compounds could produce appreciable increases in paternal UBE3A-YFP or paternal UBE3A.

Since *Ube3a-ATS* is necessary for epigenetically silencing paternal *Ube3a*, interference of *Ube3a-ATS* provides a mechanistic possibility to produce paternal *Ube3a*
20 unsilencing as previously shown for topotecan. To test this hypothesis, mRNA levels in DMSO- and drug-treated neurons were quantified. As expected, mRNA of *Ube3a-YFP* was upregulated by PHA533533, Compound X, and topotecan, but not by control Compound Y (**FIG. 3C**). Furthermore, *Ube3a-ATS* mRNA was significantly decreased by PHA533533, Compound X, and topotecan (**FIG. 3D**). The observed downregulation of *Ube3a-ATS* led to
25 tests whether the novel unsilencers affected other genes upstream of this locus. *Snord116* and *Snrpn* were also decreased by PHA533533, Compound X, and topotecan, but not by control Compound Y (**FIGS. 3E and 3F**). Taken together, these data indicate that downregulation of *Ube3a-ATS* by PHA533533 and Compound X may mediate unsilencing of paternal *Ube3a*.

Downregulation of cyclin-dependent kinase 5 (CDK5) partially unsilences paternal
30 *Ube3a-YFP*: Next, whether inhibiting potential molecular targets contributes to unsilencing paternal *Ube3a* was investigated. Since PHA533533 can inhibit CDK2 and CDK5, these kinases were individually knocked down by shRNA approaches to test whether direct targeting CDK2 or CDK5 is sufficient for unsilencing of paternal *Ube3a*. Thus, the initial hypothesis that CDK2 and CKD5 are common targets for the novel unsilencing compounds

was tested. CDK2 knockdown did not unsilence paternal *Ube3a-YFP* by itself, nor did it occlude the pharmacological unsilencing of paternal *Ube3a-YFP* (**FIG. 4A**). In contrast, CDK5 knockdown partially unsilenced paternal *Ube3a-YFP* (**FIG. 4B**). These data suggest that off-target effects of PHA533533 may contribute to paternal *Ube3a* unsilencing. Next, whether the observed unsilencing effects might be mediated through off-target effects on Top1 was tested, because it had been previously shown that Top1 is a target of topotecan to unsilence paternal *Ube3a*. In addition, Top1 depletion occludes the ability of topotecan to unsilence paternal *Ube3a*, and a Top1 cleavage complex is required for unsilencing. It was first replicated that Top1 knockdown by itself did not unsilence paternal *Ube3a-YFP* and that it could occlude unsilencing by topotecan (**FIG. 4C**). In contrast, PHA533533 still unsilenced paternal *Ube3a-YFP* when Top1 was knocked down, suggesting that Top1 is not the target for PHA533533 or Compound X and further distinguishing the mechanism of action of these compounds from Top1 inhibitors such as topotecan.

Inhibition of both cyclin-dependent kinase 5 (CDK5) and disruptor of telomeric silencing 1-like (DOT1L; histone H3 lysine 79 specific methyltransferase), unsilences paternal *Ube3a-YFP* in vitro: It was further explored whether PHA533533 and Compound X might act as topoisomerase inhibitors such as indenoisoquinoline- or camptothecin-derived topoisomerase inhibitors. Accordingly, topoisomerase-based DNA relaxation assays were performed to test whether the compounds inhibit topoisomerases. In the presence of Top2a, kinetoplast DNA (kDNA) was unwound and migrated further into the agarose gel (decatenated kDNA), while treatment with ICRF-193, a known Top2a and 2b inhibitor, inhibits the relaxation of catenated kDNA, which remains at the top of the agarose gel. PHA533533 and Compound X partially inhibited Top2a because both catenated and decatenated kDNA were observed. Control Compound Y inhibited Top2a, in a manner that appears comparable to ICRF-193 (**FIG. 5A**). However, these compounds did not inhibit either Top1 (**FIG. 7A**) or Top2b (**FIG. 7B**) as measured in DNA relaxation assays. Thus, the data largely exclude the possibility that the novel *Ube3a* unsilencing compounds act by inhibiting topoisomerases, suggesting that they instead employ novel mechanisms.

The putative molecular target (i.e., DOT1L) of PHA533533 and Compound X prompted testing into whether paternal *Ube3a* could be unsilenced by inhibiting DOT1L solely or in combination with other targets such as CDK5. To this end, EPZ5676 was tested as a highly selective inhibitor of DOT1L (Daigle et al. *Blood* 2013; 122(6):1017-1025). In addition, P5-A4 (**FIG. 6**); was tested as an analogue of PHA533533 that retains known inhibitory activity toward CDK2 and CDK5 but that is not predicted by DockoMatic to target

DOT1L. As shown in **FIG. 5B**, dual treatment of EPZ5676 and P5-A4 unsilenced paternal *Ube3a-YFP*, whereas either EPZ5676 alone (**FIG. 5B**) or P5-A4 alone (**FIG. 6**) did not appreciably unsilence paternal *Ube3a-YFP*. In addition to EPZ5676 (positive control), PHA533533 and Compound X reduced methylation level on histone H3 lysine 79 (H3K79) (**FIG. 5B**), suggesting that PHA533533 and Compound X can inhibit DOT1L in agreement with our molecular prediction. Moreover, mRNAs of target genes were changed by the same treatment (**FIGS. 5C-5F**). In corroboration with the western blot analysis, it was found that mRNA of *Ube3a-YFP* was increased by combined treatment of EPZ5676 and P5-A4 (**FIG. 5C**). This dual pharmacological approach appeared to act by downregulating the *Ube3a-ATS* and its upstream genes *Snord116* and *Snrpn* (**FIGS. 5D-5F**). These data indicate that PHA533533 and Compound X may share molecular targets, CDK5 and DOT1L, that act in concert to unsilence paternal *Ube3a*. As such, these compounds and their molecular targets may provide AS therapeutic avenues.

A high-content screen revealed two novel compounds, PHA533533 and Compound X, that can effectively unsilence the paternal *Ube3a* allele in mouse primary cortical neurons. These compounds do not appear to be acting through off-target effects on topoisomerases (i.e., Top1, Top2a, or Top2b), suggesting they have a novel mechanism of action distinct from previously identified small molecule *Ube3a* unsilencing agents. Instead, these compounds appear to be acting through a mechanism that jointly targets CDK5 and DOT1L.

Pharmacological properties of the novel unsilencers in vitro: The pharmacological profiles (potency, efficacy, and LC₅₀) of the two novel compounds can provide insights into whether they could be drug-like small molecules with clinical potential and how their pharmacological properties compare to another unsilencing agent, topotecan. PHA533533 has a slightly lower potency than topotecan, but has similar efficacy and cytotoxicity (**FIG. 2**). In contrast, Compound X showed comparable potency and efficacy to topotecan, but had slightly increased cytotoxicity than topotecan (**FIG. 2**). Even if PHA533533 and Compound X are not themselves viable options for treating AS, their chemical structures serve as a starting point for chemical modifications to improve potency, efficacy, and pharmacokinetic parameters.

Known and predicted molecular targets: This study sought to gain insights into the mechanism(s) driving paternal *Ube3a* unsilencing by understanding the known and predicted molecular targets of PHA533533 and Compound X. While PHA533533 has well established targets, CDK2 and CDK5, the molecular targets of Compound X are poorly understood. While not wishing to be bound to theory, we hypothesized that PHA533533 and Compound

X may be acting, at least in part, through a previously undescribed mechanism. To address this hypothesis, DockoMatic was used to predict potential shared targets of the three compounds [the two active compounds, PHA533533 and Compound X, as well as the inactive compound, control Compound Y]. The modeling suggested that these compounds share CDK2, CDK5, and Top2a as potential targets. In addition, there is a shared, but unique target, DOT1L, between PHA533533 and Compound X, but not control Compound Y.

For the shared known and predicted targets (CDK2, CDK5, and Top2a), we can exclude the possibility of unsilencing through Top2a inhibition by our compounds because control Compound Y did not unsilence paternal *Ube3a* (**FIG. 3**). Four structural analogues of PHA533533 produced only modest paternal *Ube3a* unsilencing (**FIG. 7**), suggesting that inhibition of CDK2 or CDK5 by itself is unlikely to produce strong unsilencing. Consistent with this idea, shRNA-mediated knockdown of CDK2 failed to produce any observable unsilencing, while knockdown of CDK5 produced only partial unsilencing of paternal *Ube3a-YFP* (**FIG. 4**). These data suggest that PHA533533 and Compound X may act in concert through CDK5 inhibition and another mechanism, such as DOT1L inhibition. To further narrow the focus on DOT1L as a possible contributor to paternal *Ube3a* unsilencing, it was experimentally verified that PHA533533 and Compound X function as inhibitors of DOT1L (**FIG. 5B**), because DOT1L is the only methyltransferase catalyzing H3K79. Moreover, combined treatment to inhibit CDK5 (by P5-A4) and DOT1L (by EPZ5676) unsilenced paternal *Ube3a-YFP* (**FIG. 5B**). Therefore, the results of this study suggest that PHA533533 and Compound X may act in concert through CDK5 and DOT1L inhibition.

Potential mechanism of unsilencing paternal UBE3A: One potential unsilencing mechanism of our novel compounds is that they interfere with long gene transcription, as these compounds downregulated *Ube3a-ATS*, an extremely long transcript. However, there is no evidence proving that CDK5 is directly or indirectly involved in regulating RNA polymerase II (RNAPII) activity to our knowledge. On the other hand, DOT1L is known to be a direct interactor of RNAPII, which is responsible for the transcriptional elongation process and exon definition. Thus, inhibiting DOT1L activity may perturb the elongation process of actively transcribed genes such as *Ube3a-ATS*, supported by reports showing that the interaction of DOT1L with phosphor-Ser2 and/or Ser5 of the c-terminal domain (CTD) in RNAPII facilitates recruitment of DOT1L to actively-transcribed genes. However, data from this study indicate that DOT1L inhibition alone was not sufficient for unsilencing paternal *Ube3a-YFP*. Taken together with the results showing little to no unsilencing by CDK5 perturbation alone (i.e., using shRNA knockdown or using P5-A4), molecular interactions

between CDK5 and DOT1L may mediate unsilencing of paternal *Ube3a*. Additional investigations are required to determine whether 1) changes in RNAPII activity are correlated with drug-mediated CDK5 and DOT1L inhibition, 2) the enrichment levels of histone modifications are correlated with transcriptional activity, 3) molecular interactions between CDK5 and DOT1L are associated with RNAPII activity and genomic imprinting, and/or 4) H3K79 methylation globally impacts chromatin structure in relation to transcription.

Developing AS therapeutics is an unmet need for patients and their families. This study identified PHA533533 and Compound X as novel *Ube3a* unsilencers that may act through their dual inhibition of CDK5 and DOT1L, as selective inhibition of either CDK5 or DOT1L alone through molecular or pharmacological blockade is not sufficient to effectively unsilence paternal *Ube3a*. While not wishing to be bound to theory, it is contemplated that the joint inhibition of CDK5 and DOT1L promotes paternal *Ube3a* unsilencing. This mechanism jointly targeting CDK5 and DOT1L could limit transcriptional elongation, or perhaps more specifically downregulate *Ube3a-ATS*, and as a result unsilence paternal *Ube3a*. This suggests that interference of *UBE3A-ATS* can be achieved by a manner distinct from topoisomerase inhibition. Therefore, the results reported herein provide a novel avenue to develop AS therapies using different classes of small molecules that should be advanced for preclinical testing of safety and CNS efficacy in mouse models of AS.

Example 2:

FIGS. 8A-8C show that PHA533533 produces unsilencing of paternal *Ube3a-YFP*. Small molecules were tested for their ability to unsilence paternal *Ube3a* in cultured cortical neurons derived from 15.5-day old embryos (E15.5) with a paternally inherited *Ube3a-YFP* allele. Paternal UBE3A-YFP levels were assessed using GFP antibody-enhanced fluorescence and high-content imaging. **FIG. 8B** shows DAPI (nuclear stain) and paternal UBE3A-YFP immunocytochemistry in cultured neurons treated with 0.1% DMSO (negative control), topotecan (positive control), S-enantiomer of PHA533533, R-enantiomer of PHA533533, or a racemic mixture of S and R enantiomers of PHA533533. **FIG. 8C** shows quantitative analysis of the percentage of cells expressing paternal UBE3A-YFP, calculated by dividing the number of YFP-positive cells by the total number of DAPI-positive nuclei. These data indicate that PHA533533 can unsilence paternal *Ube3a-YFP*.

The pharmacological profile of PHA533533 in unsilencing paternal *Ube3a-YFP* *in vitro* was also examined. **FIG. 9A** shows dose-dependence of PHA533533 compared to topotecan in unsilencing paternal *Ube3a-YFP*. **FIG. 9B** shows dose-dependent cytotoxicity of

PHA533533 compared to topotecan. EC_{50} (**FIG. 9C**), E_{max} (**FIG. 9D**), and LC_{50} (**FIG. 9E**) of the compounds were also determined.

Similar studies were performed using PHA533533 analogs. Dose-dependence of unsilencing paternal *Ube3a-YFP* by PHA533533 analogs (**FIG. 10A**) PNU0292137, (**FIG. 10B**) PNU0278497, and (**FIG. 10C**) PHA00543738 were determined. **FIG. 10D** shows EC_{50} and **FIG. 10E** shows E_{max} of the PHA533533 analogs compared to PHA533533. These data show that these compounds could not produce appreciable increases in paternal UBE3A-YFP or paternal UBE3A. Therefore, the unsilencing of paternal UBE3A was specific to PHA533533.

FIGS. 11A-11B show that PHA533533 treatment produces UBE3A protein from the paternal allele. Western blot analyses show that, like topotecan, PHA533533 can produce UBE3A-YFP (**FIG. 11A**) and UBE3A (**FIG. 11B**) protein from the paternal allele.

FIGS. 12A-12D show PHA533533 treatment downregulates *Ube3a-ATS* in neurons. Quantitative RT-PCR (qPCR) revealed changes in mRNA of paternal *Ube3a-YFP* (**FIG. 12A**), *Ube3a-antisense (ATS)* (**FIG. 12B**), *Snord116* (**FIG. 12C**), and *Snrpn* (**FIG. 12D**) in cultured mouse cortical neurons (wildtype or *Ube3a^{m+/pYFP}* as indicated) after drug treatment.

FIGS. 13A-13I show that selective knockdown of established PHA533533 molecular targets is not sufficient to unsilence paternal *Ube3a-YFP* as effectively as PHA533533. **FIG. 13A** shows efficient knockdown of cyclin-dependent kinase 2 (CDK2) using shRNA approach. **FIG. 13B** shows Western blot analysis and **FIG. 13C** shows quantification that shRNA knockdown does not unsilence paternal *Ube3a-YFP* unless neurons were treated with topotecan or PHA533533. **FIGS. 13D** shows efficient knockdown of cyclin-dependent kinase 5 (CDK5) by shRNA. **FIG. 13E** shows Western blot analysis and **FIG. 13F** shows quantification that CDK5 knockdown can partially unsilence paternal *Ube3a-YFP*, and this unsilencing was further enhanced by treatment with topotecan or PHA533533. **FIG. 13G** show shRNA knockdown of topoisomerase 1 (Top1). **FIG. 13H** shows Western blot analysis and **FIG. 13I** shows quantification that knockdown of Top1 did not unsilence paternal *Ube3a-YFP*, but Top1 knockdown occluded the ability of topotecan, but not PHA533533, to unsilence paternal *Ube3a-YFP*.

Fluorescence immunocytochemistry and high content imaging analysis: After 72-hour drug treatment, the neurons were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 10 min. After brief washing with PBS three times, neurons were permeabilized with 1% Triton-X 100 in PBS at room temperature for 10 min, followed by blocking with 5% normal goat serum in 0.1% Triton-X 100 in 1x PBS at room

temperature. 30 min after blocking, neurons were incubated with primary antibodies rabbit anti-GFP (1:1000, NB 600308, Novus Biologicals) and mouse anti-NeuN (1:500, MAB 377, Millipore) at 4°C overnight. Neurons were then briefly washed with 1x PBS followed by incubation with secondary antibodies goat anti-rabbit Alexa Fluor 488 (1:500, A11008, Thermo Fisher/Invitrogen) and anti-mouse Alexa Fluor 568 (1:500, A21124, ThermoFisher/Invitrogen) together with DAPI (4', 6-diamidino-2-phenylindole) at room temperature for 60 min. Following secondary antibody incubation, neurons were washed with 1x PBS and fluorescent images were acquired using Nikon Ti2 eclipse fluorescent microscope (Nikon Instruments Inc., Melville, NY, USA). Antibody-enhanced UBE3A-YFP fluorescence intensities were determined from DAPI and NeuN double-positive individual neurons in drug-treated wells and normalized to neurons in untreated wells. All acquired images were analyzed by Nikon NIS-Elements software (Nikon Instruments Inc., Melville, NY, USA). Sigmoidal curve fitting for the dose response analysis was performed using GraphPad Prism software version 8.0 (GraphPad Software, La Jolla, CA, USA). The calculated EC₅₀ values (potencies) and Y value top plateau (estimated efficacies) enables comparative analyses of the relative potency and efficacy of the identified compounds.

Example 3:

The aim of these experiments was to determine if PHA533533 could unsilence the paternal *Ube3a* allele *in vivo*. To this end, PHA533533 (2 mg/kg body weight) or saline vehicle control was administered into *Ube3a*^{m+/pYFP} mice by intraperitoneal injection (IP) at postnatal day 11 (P11) and the mice were sacrificed two days after drug administration at P13 (**Fig. 14A**). Saline treated wild type and *Ube3a*^{m+/pYFP} mice served as immunohistochemistry controls showing the absence of GFP-immunoreactivity in wild type mice (**Fig. 14B**) and expected paternal UBE3A-YFP protein expression in glial cells and in newly generated immature neurons in *Ube3a*^{m+/pYFP} mice (**Fig. 14C**; Judson et al. *J Comp Neurol* 2014 522(8):1874-96), respectively. Peripheral delivery (i.p.) of PHA533533 successfully upregulated paternal *Ube3a-YFP* throughout the brain (**Fig. 14D**). Examining the hippocampal CA3 region more closely, costaining of GFP and the neuronal marker NeuN showed near-complete co-localization (**Fig. 14E**), indicating efficient unsilencing of paternal *Ube3a-YFP* in mature neurons. These data indicate that PHA533533 successfully unsilences the paternal *Ube3a* allele throughout the nervous system.

Intraperitoneal injection and immunohistochemistry: PHA533533 (2 mg/kg body weight) or saline vehicle (0.9% NaCl) was injected intraperitoneally (IP) into unanesthetized postnatal day 11 (P11) mice using 27-gauge needle attached to a 1 ml syringe. PHA533533 was dissolved with 7.5% DMSO in saline. P13 mice were deeply anesthetized with euthasol (100 mg/kg, i.p.) prior to transcardial perfusion with phosphate-buffered saline (PBS), pH 7.4, followed by phosphate-buffered 4% paraformaldehyde, pH 7.4. Perfused brains were postfixed overnight and cryoprotected with 30% sucrose in PBS, pH 7.4, for two days. Cryoprotected brains were frozen on dry ice and cut into 40 µm-thick sections with a sliding microtome (Thermo Scientific, Kalamazoo, MI, USA). Sections were stored in a cryopreservative solution (by volume: 45% PBS, 30% ethylene glycol, 25% glycerol) at -20°C until they were processed for free-floating immunohistochemistry (IHC). For IHC, sections were rinsed several times in PBS before blocking in PBS plus 5% normal goat serum and 0.2% Triton-X-100 (NGST) for 1 hour at room temperature. Sections were incubated with rabbit anti-GFP (1:1000, NB 600308, Novus Biologicals) and mouse anti-NeuN (1:500, MAB 377, Millipore) primary antibodies diluted in NGST for 48 hours at 4°C. Sections were then washed several times in PBS containing 0.2% Triton-X-100 (PBST) before incubation with goat anti-rabbit Alexa Fluor 488 (1:500, A11008, Thermo Fisher/Invitrogen) and anti-mouse Alexa Fluor 568 (1:500, A21124, ThermoFisher/Invitrogen) secondary antibodies together with DAPI (4', 6-diamidino-2-phenylindole) all diluted in NGST for 1 hour at room temperature. Brain sections compared within figure were stained within the same experiment, under identical conditions. Images were acquired using a Zeiss LSM 710 confocal microscope (Carl Zeiss Inc.).

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. Although the invention has been described in detail with reference to preferred embodiments, variations and modifications exist within the scope and spirit of the invention as described and defined in the following claims.

WHAT IS CLAIMED IS:

1. A method of inducing expression of Ube3a in a cell, comprising contacting the cell with an effective amount of PHA533533 or a salt thereof, (R)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxopyrrolidin-1-yl)phenyl)propenamide, (S)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxooxazolidin-3-yl)phenyl)propenamide, and/or EPZ5676 in any combination, thereby inducing expression of Ube3a in the cell.
2. The method of claim 1, wherein the cell is selected from the group consisting of a central neuron, a peripheral neuron, a neuron differentiated from stem cells, a glial cell, an astrocyte, an oligodendrocyte, a microglial cell, and any combination thereof.
3. The method of claim 1 or 2, wherein the cell is in a human subject and the Ube3a is paternal Ube3a.
4. A method of treating Angelman Syndrome in a human subject, comprising administering to the subject an effective amount of PHA533533 or a salt thereof, (R)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxopyrrolidin-1-yl)phenyl)propenamide, (S)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxooxazolidin-3-yl)phenyl)propenamide, and/or EPZ5676 in any combination, thereby treating Angelman syndrome in the subject.
5. The method of any of claims 1-4, wherein the effective amount of PHA533533 or a salt thereof, (R)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxopyrrolidin-1-yl)phenyl)propenamide, (S)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxooxazolidin-3-yl)phenyl)propenamide, and/or EPZ5676 in any combination inhibits a target selected from the group consisting of CDK5, CDK2, DOT1L, ATXR5, CBP, CDK6, CDK8, CDK9, CDK16, CDKL3, EHMT1, EHMT2, KMT5C, SETD7, SMYD1, SMYD3, and any combination thereof.
6. The method of any of claims 1-5, wherein the effective amount of PHA533533 or a salt thereof, (R)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxopyrrolidin-1-yl)phenyl)propenamide, (S)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxooxazolidin-3-yl)phenyl)propenamide, and/or EPZ5676 in any combination has an efficiency E_{max} of at least 1.25 fold over control.

7. The method of any of claims 1-5, wherein the effective amount of PHA533533 or a salt thereof, (R)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxopyrrolidin-1-yl)phenyl)propanamide, (S)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxooxazolidin-3-yl)phenyl)propanamide, and/or EPZ5676 in any combination has an efficiency E_{max} of at least 2.5 fold over control.
8. The method of any one of claims 1-7, comprising an effective amount of PHA533533 and/or derivative thereof.
9. Use of PHA533533 or a salt thereof, (R)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxopyrrolidin-1-yl)phenyl)propanamide, (S)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxooxazolidin-3-yl)phenyl)propanamide, and/or EPZ5676 in any combination in the manufacture of a medicament for the treatment of Angelman Syndrome.
10. Use of PHA533533 or a salt thereof, (R)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxopyrrolidin-1-yl)phenyl)propanamide, (S)-N-(5-cyclopropyl-1H-pyrazol-3-yl)-2-(4-(2-oxooxazolidin-3-yl)phenyl)propanamide, and/or EPZ5676 in any combination in the manufacture of a medicament for inducing expression of Ube3a in a cell.
11. A method of inducing expression of Ube3a in a cell, comprising contacting the cell with an effective amount of one or more inhibitor of CDK5, CDK2, DOT1L, ATXR5, CBP, CDK6, CDK8, CDK9, CDK16, CDKL3, EHMT1, EHMT2, KMT5C, SETD7, SMYD1, and/or SMYD3, thereby inducing expression of Ube3a in the cell.
12. The method of claim 11, wherein the cell is selected from the group consisting of a central neuron, a peripheral neuron, a neuron differentiated from stem cells, a glial cell, an astrocyte, an oligodendrocyte, a microglial cell, and any combination thereof.
13. The method of claim 11 or 12, wherein the cell is in a human subject and the Ube3a is paternal Ube3a.

14. A method of treating Angelman Syndrome in a human subject, comprising administering to the subject an effective amount of one or more inhibitor of CDK5, CDK2, DOT1L, ATXR5, CBP, CDK6, CDK8, CDK9, CDK16, CDKL3, EHMT1, EHMT2, KMT5C, SETD7, SMYD1, and/or SMYD3, thereby treating Angelman syndrome in the subject.

FIG. 1A

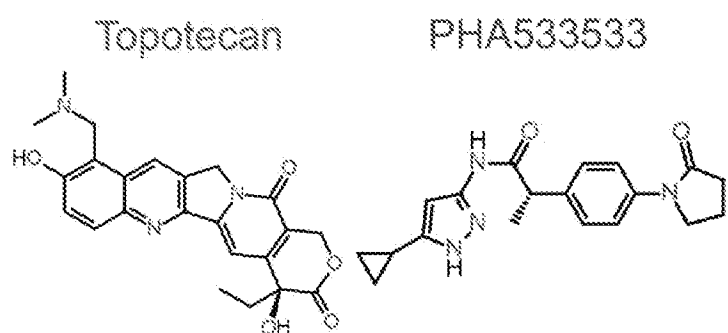


FIG. 1B

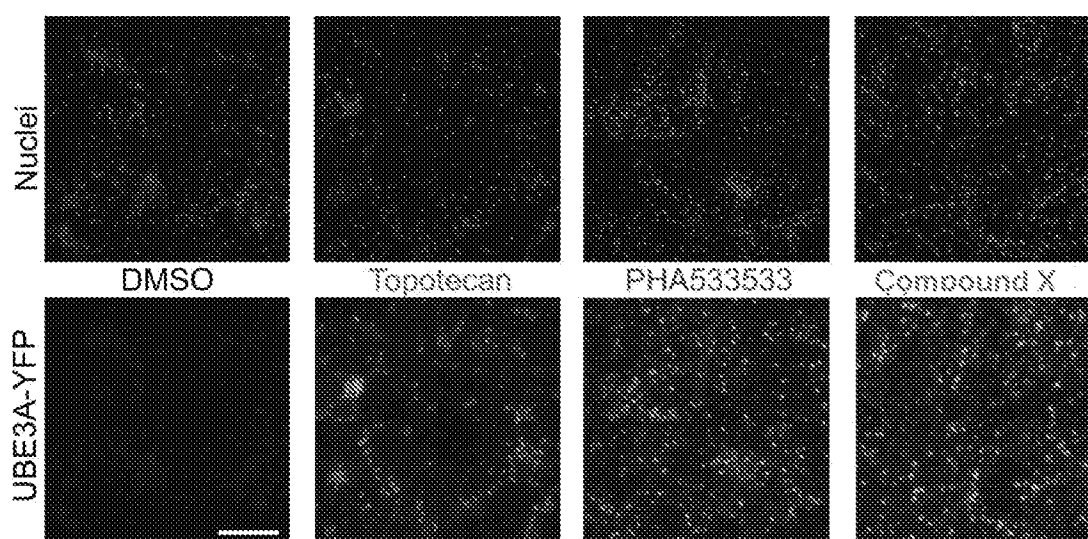


FIG. 1C

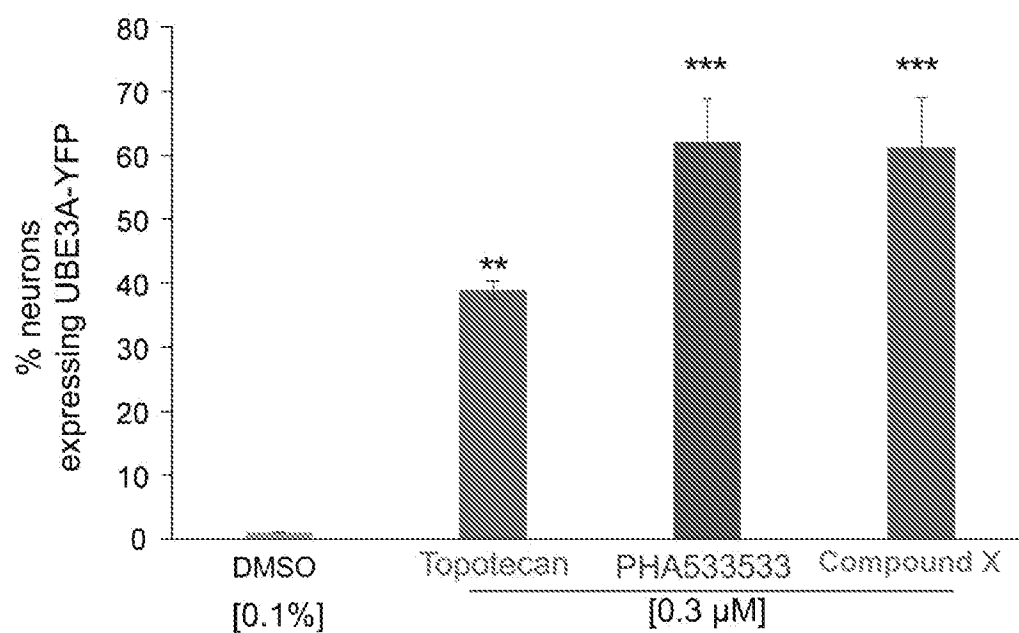


FIG. 1D

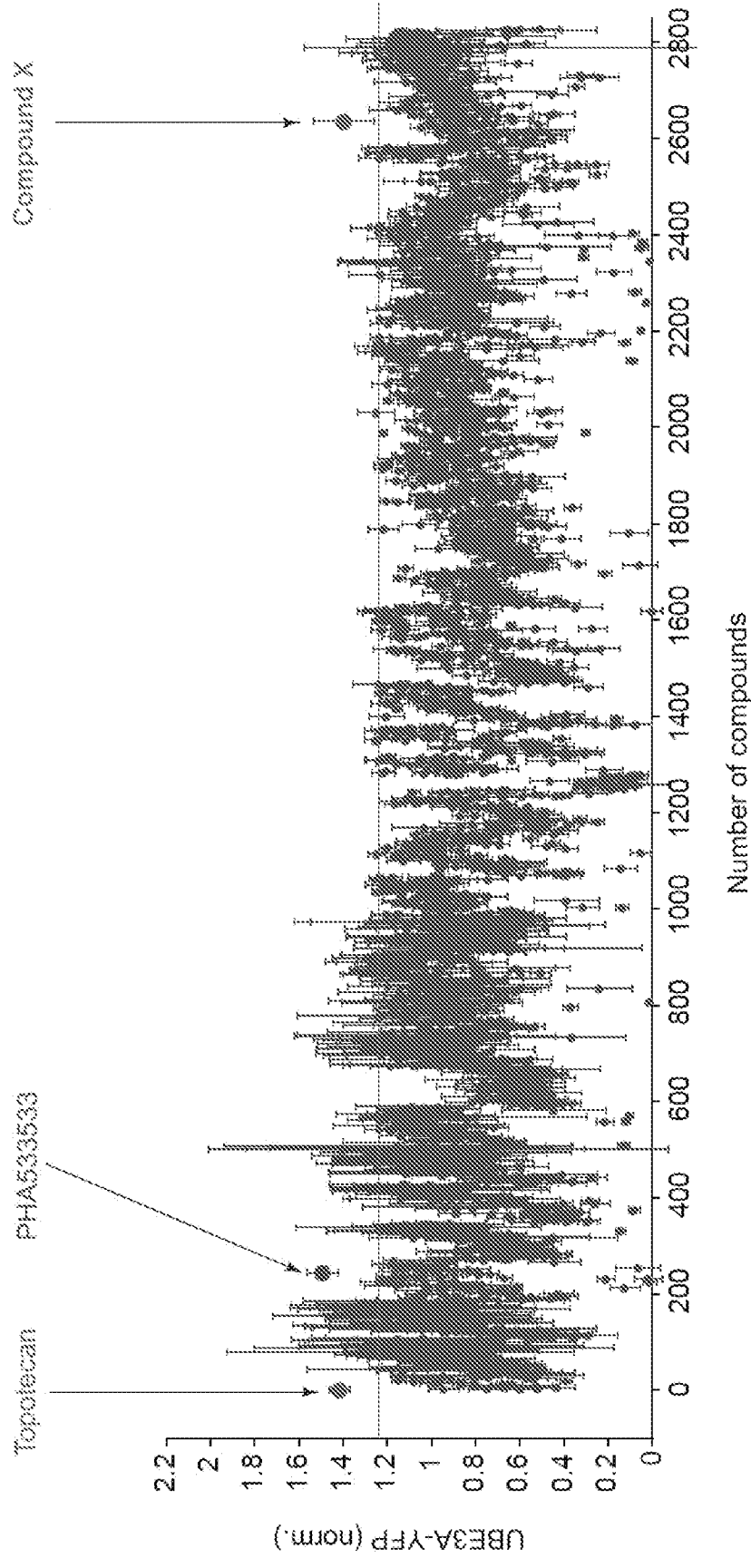


FIG. 2A

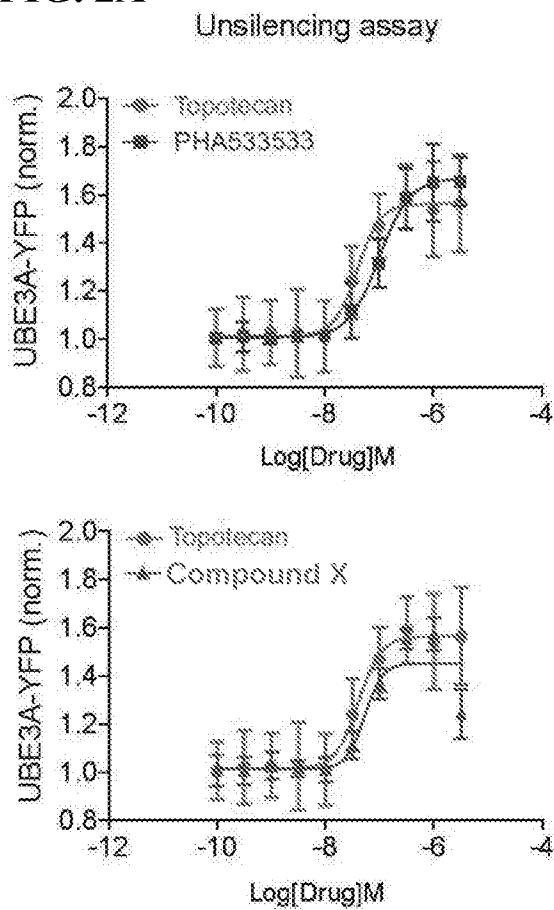


FIG. 2B

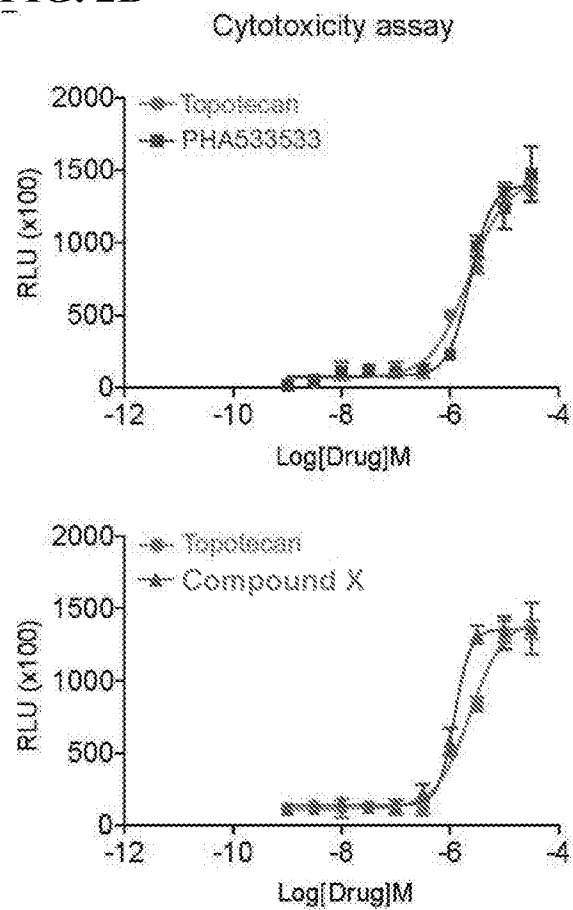


FIG. 2C

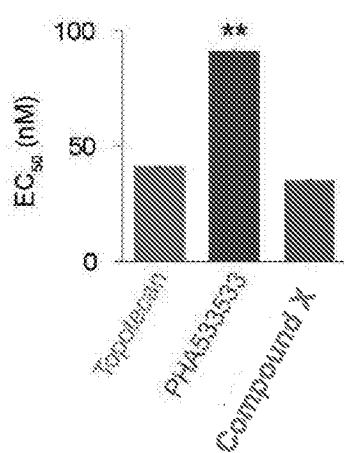


FIG. 2D

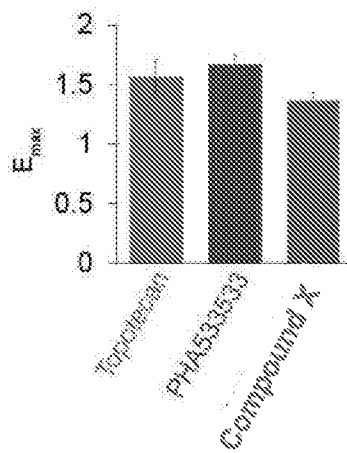


FIG. 2E

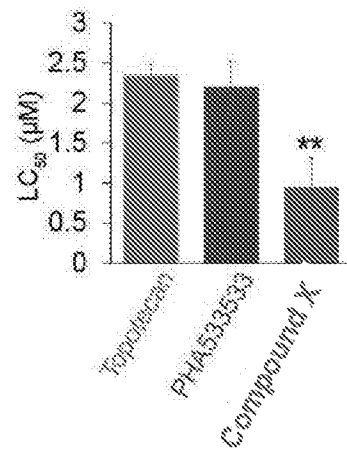


FIG. 3A

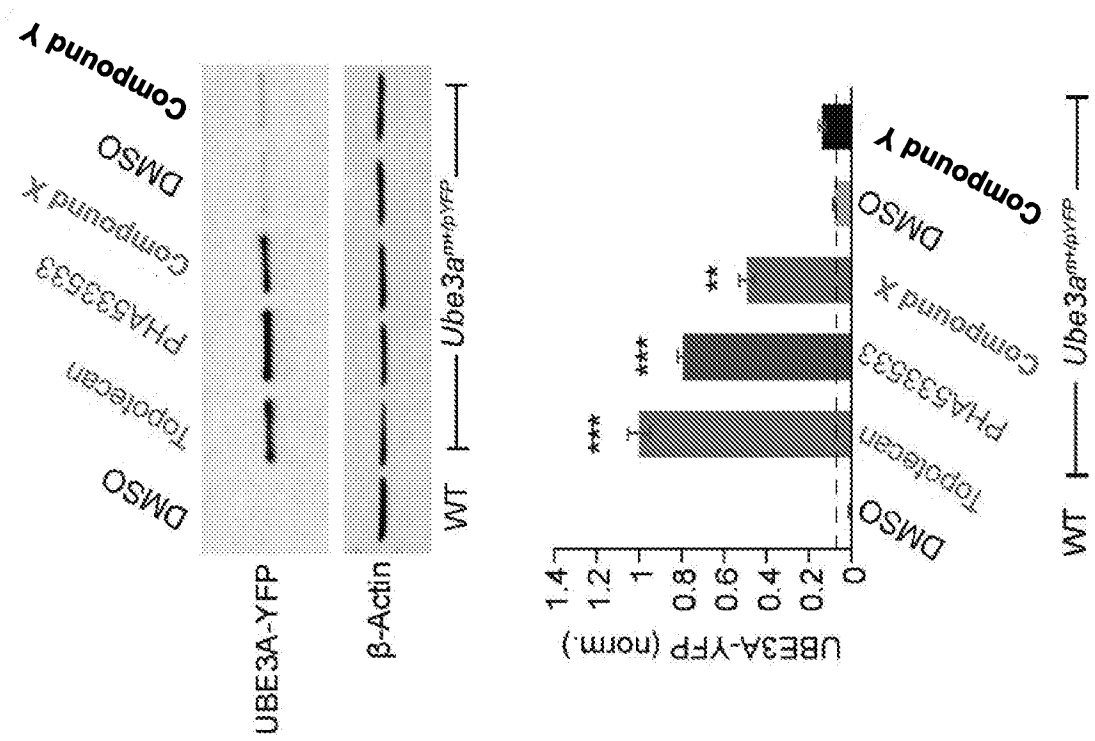


FIG. 3B

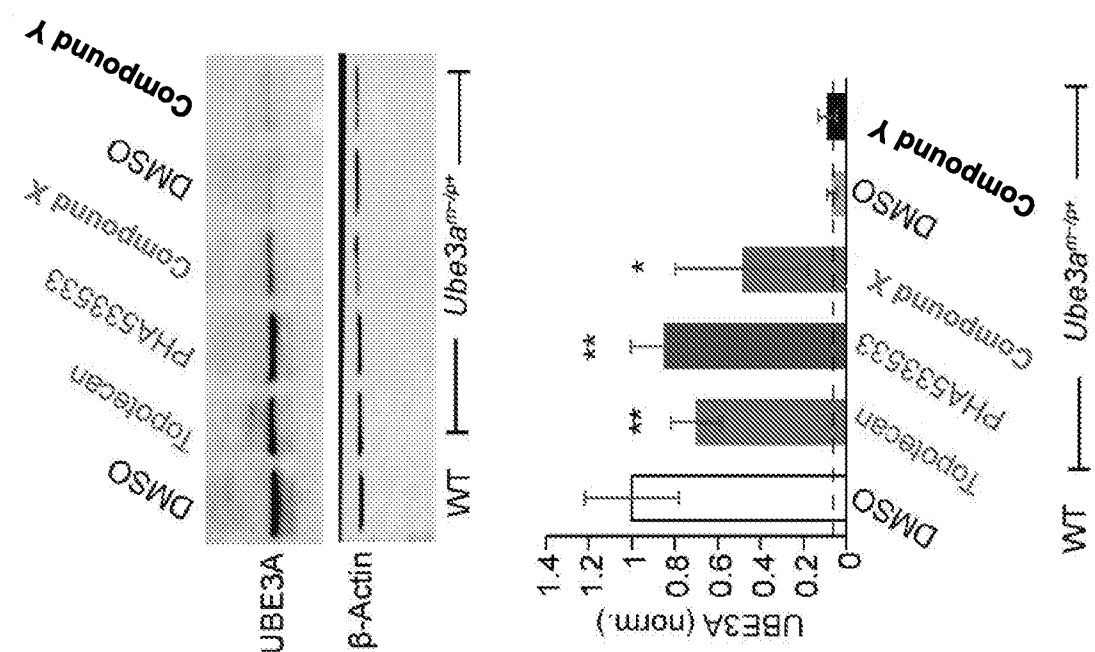


FIG. 3C

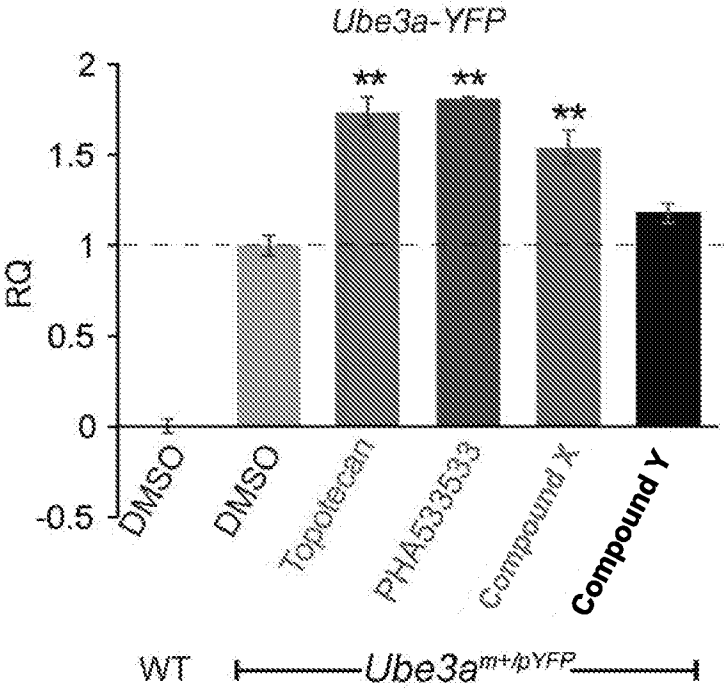


FIG. 3D

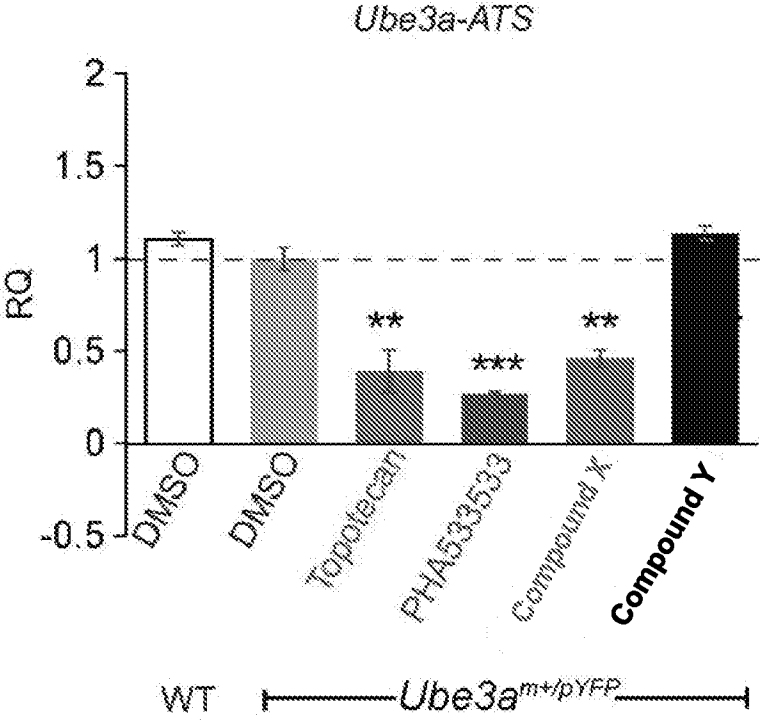


FIG. 3E

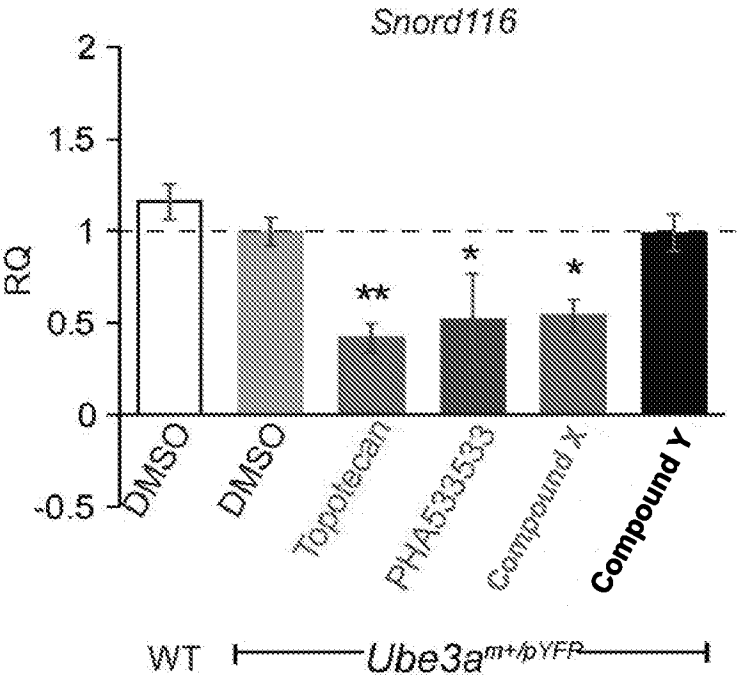


FIG. 3F

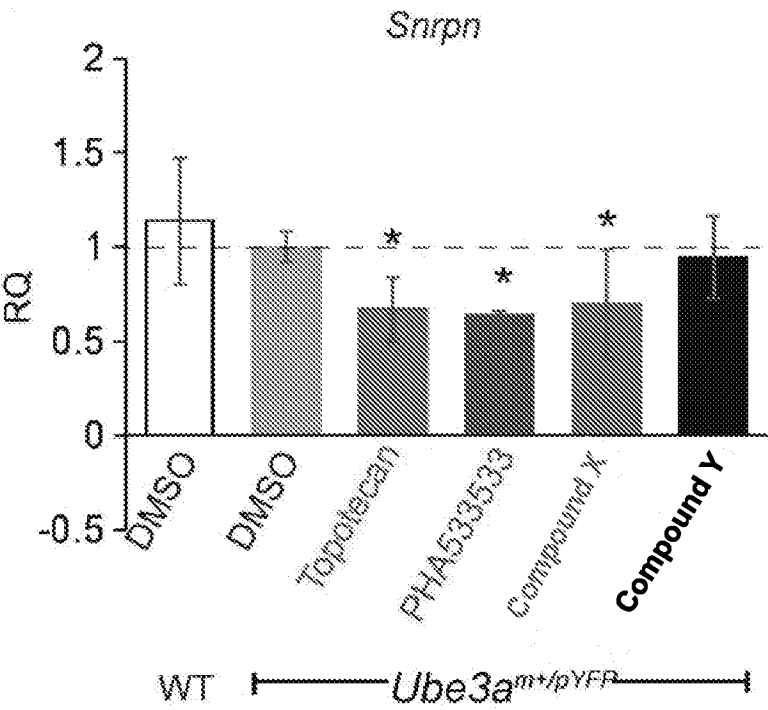


FIG. 3G

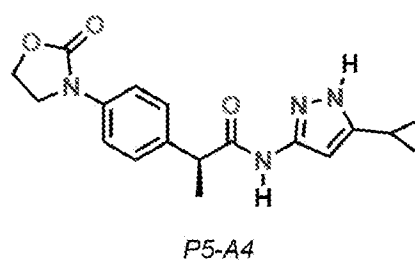
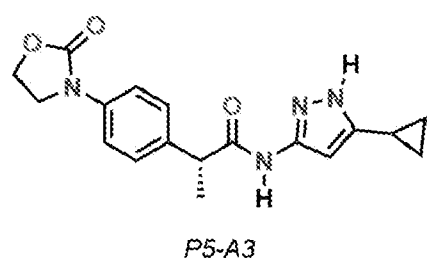
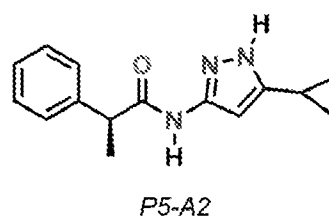
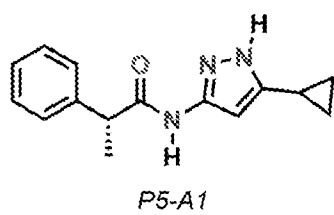
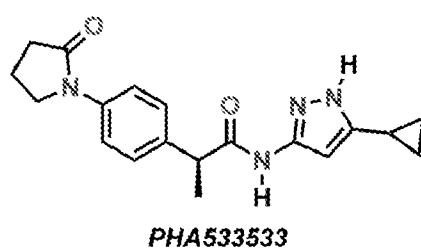


FIG. 4A

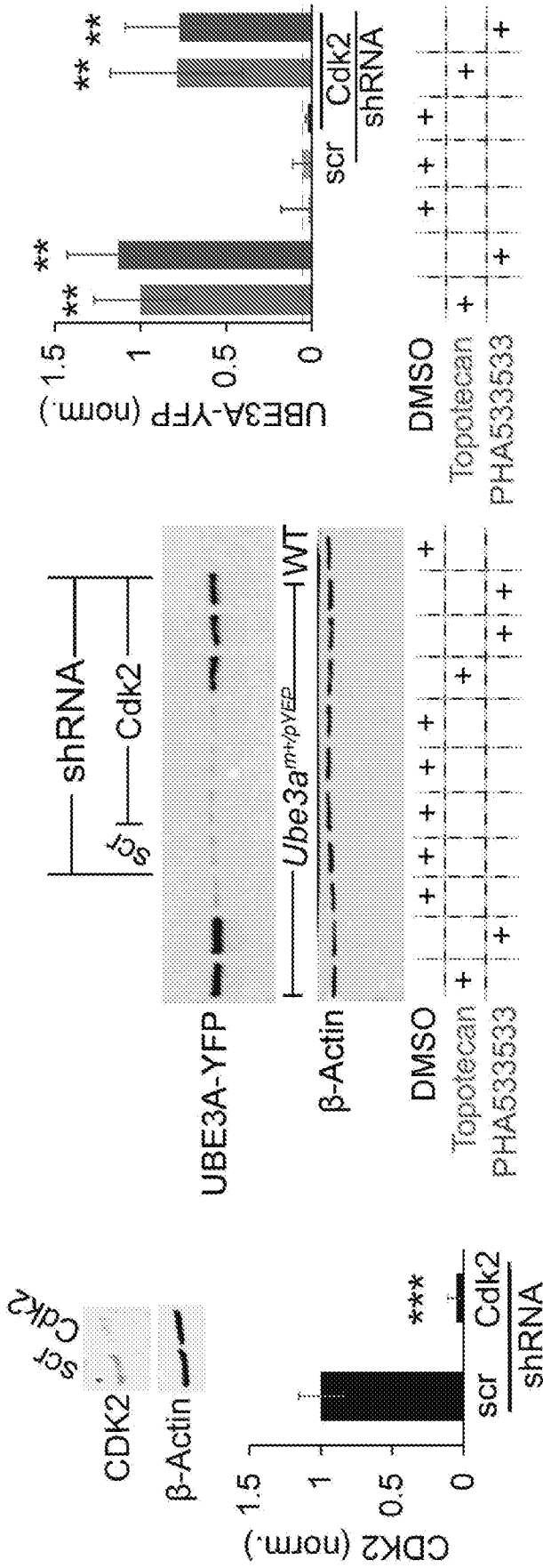


FIG. 4B

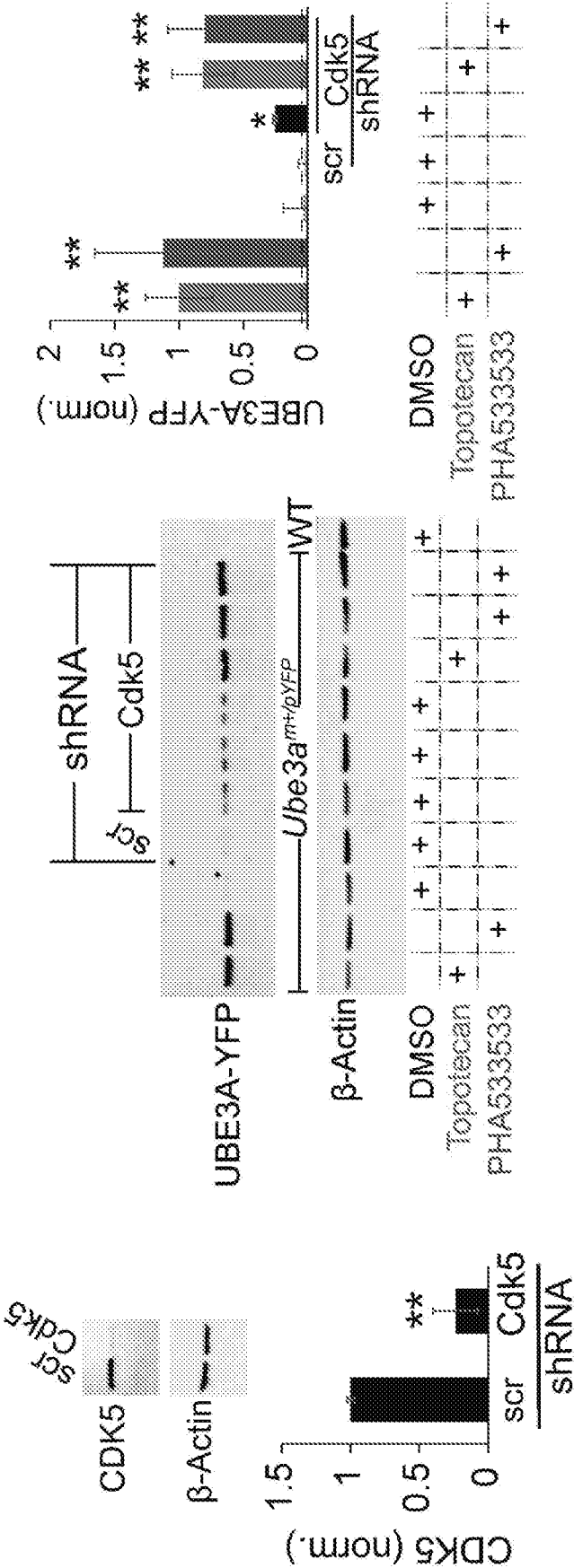
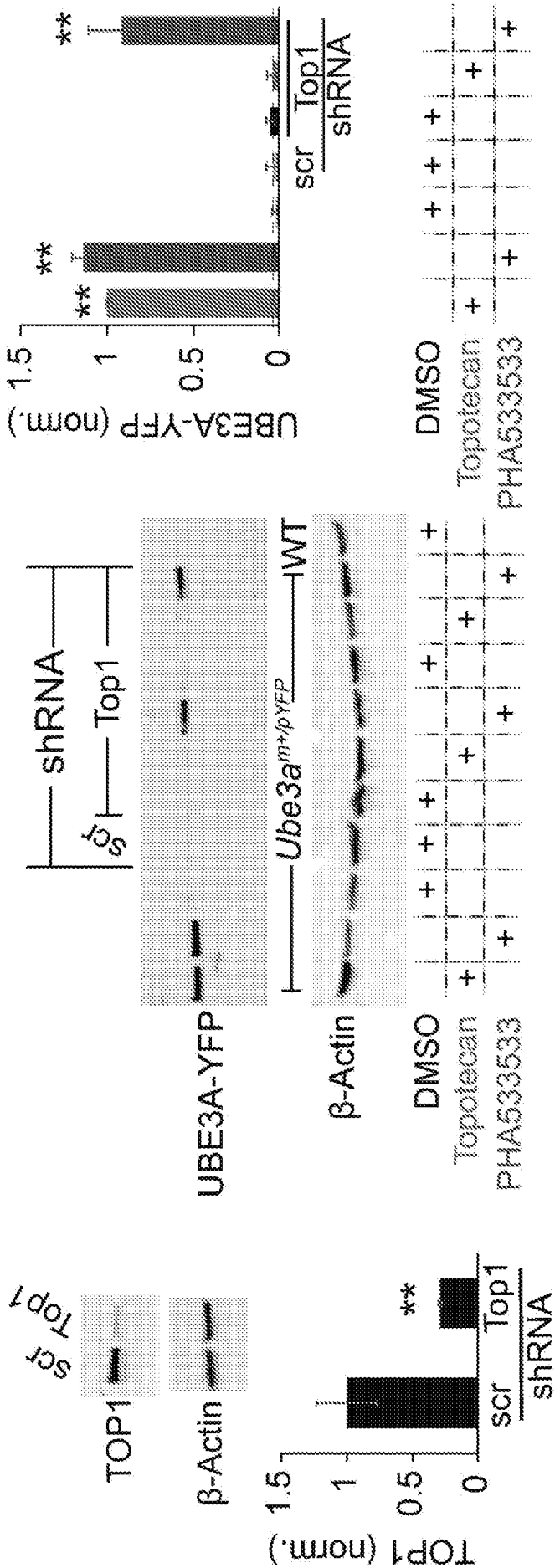


FIG. 4C



12/24

FIG. 5B

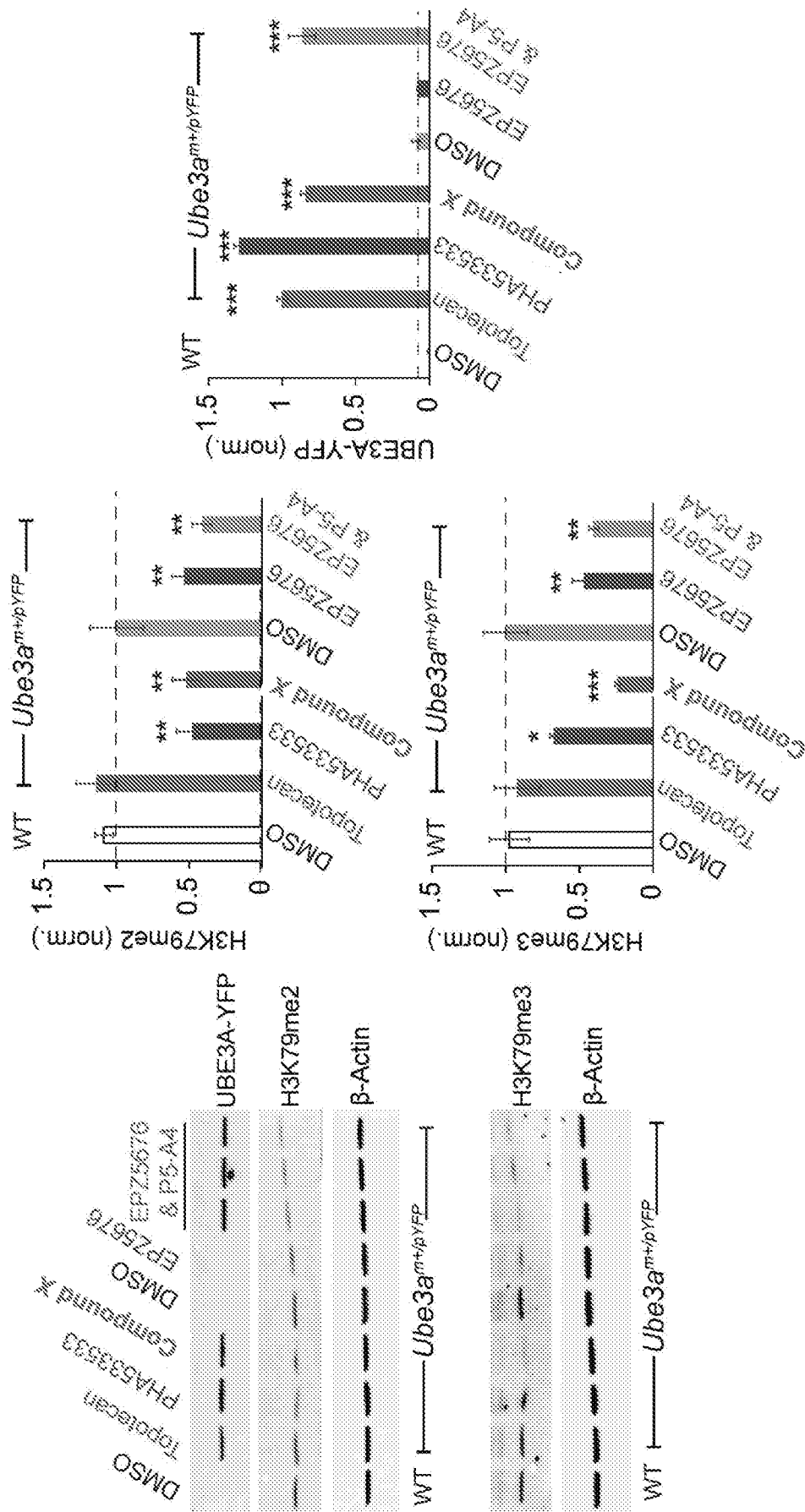


FIG. 5C

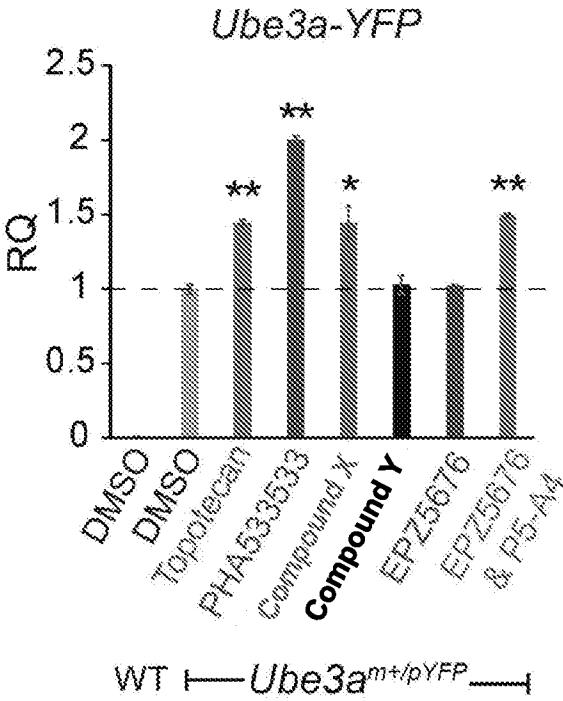


FIG. 5D

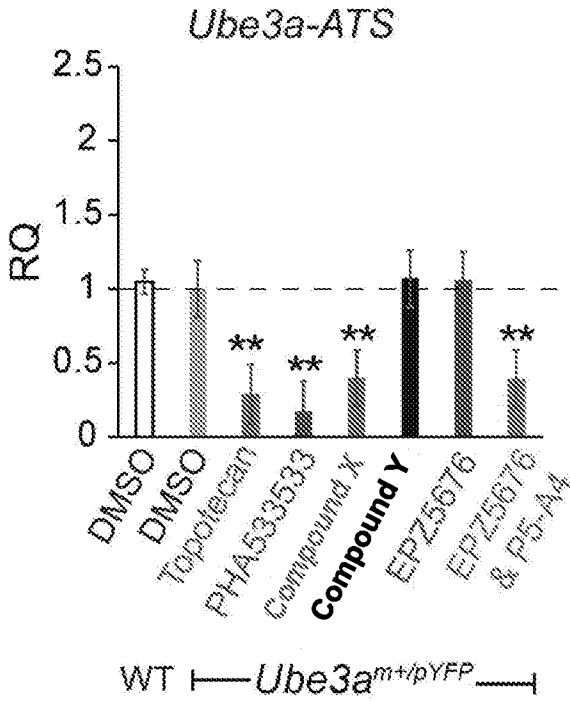


FIG. 5E

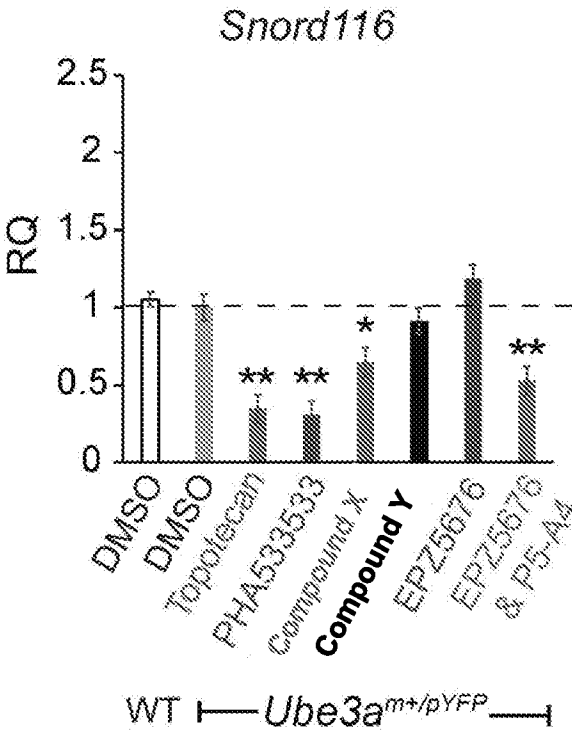


FIG. 5F

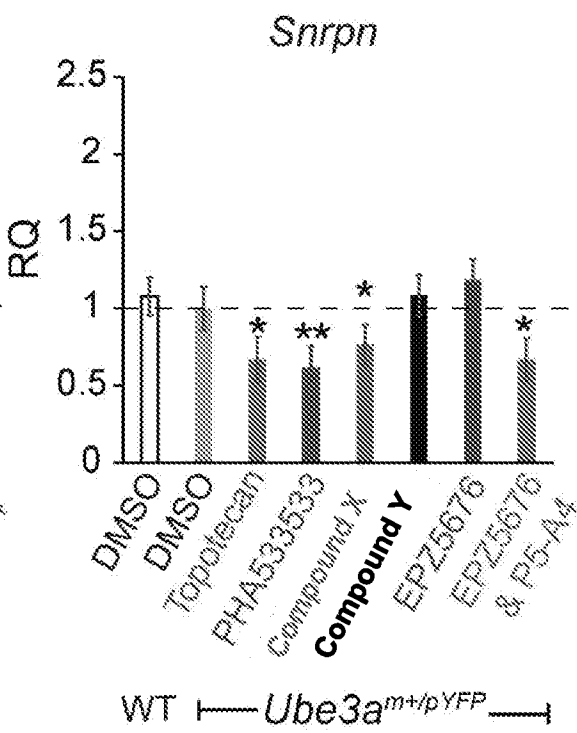


FIG. 6

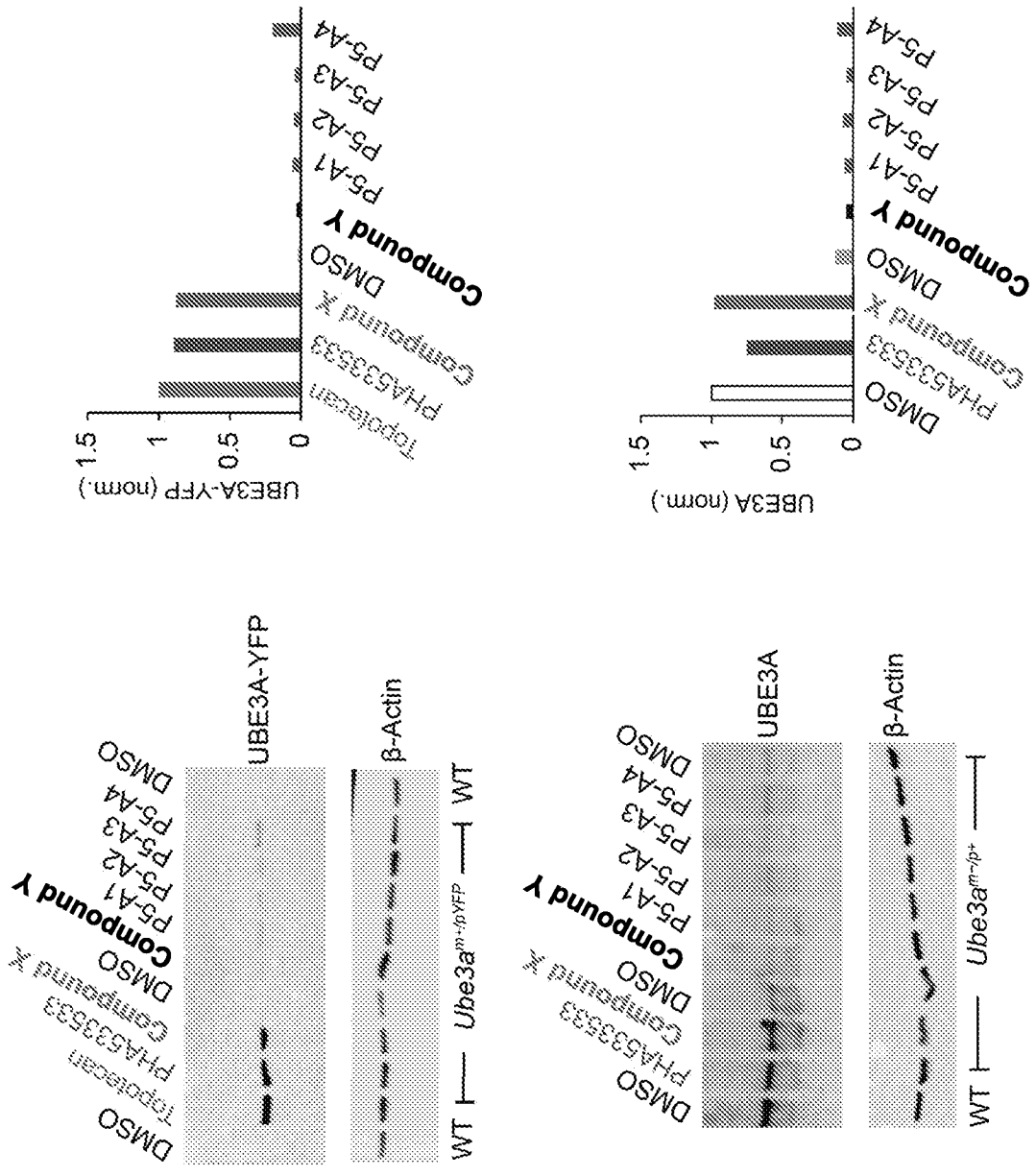


FIG. 8A

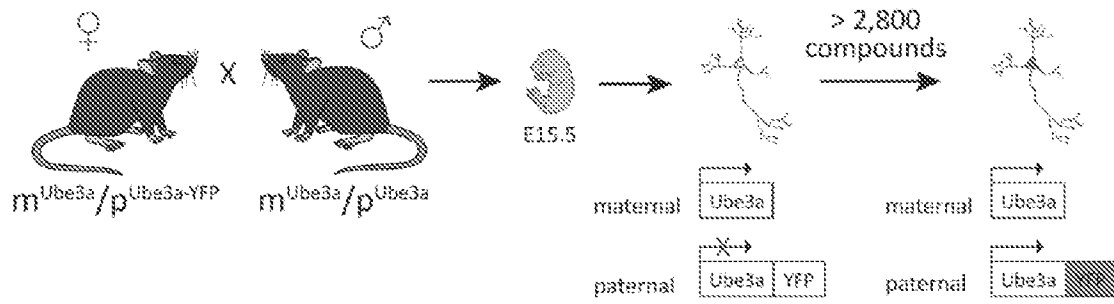


FIG. 8B

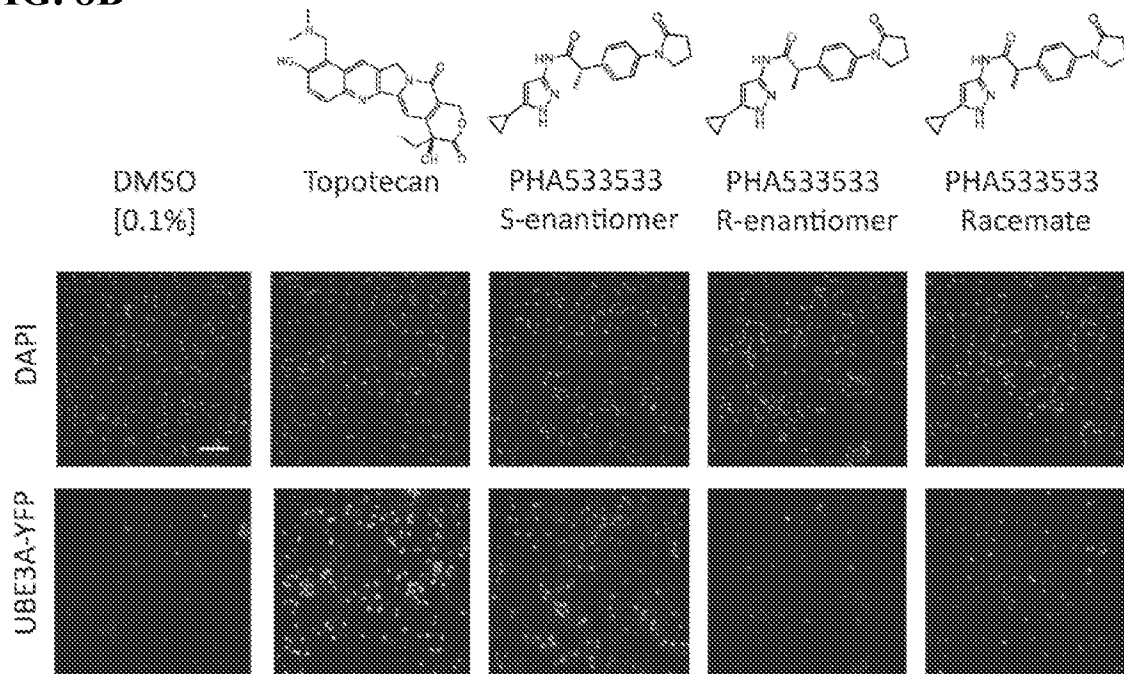


FIG. 8C

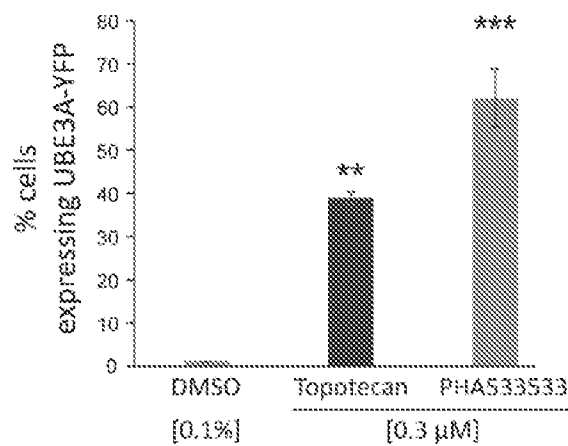


FIG. 9A

Unsilencing assay

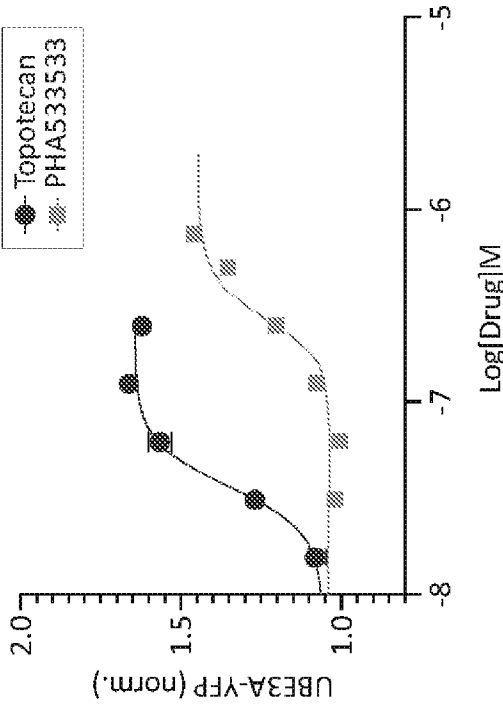


FIG. 9B

Cytotoxicity assay

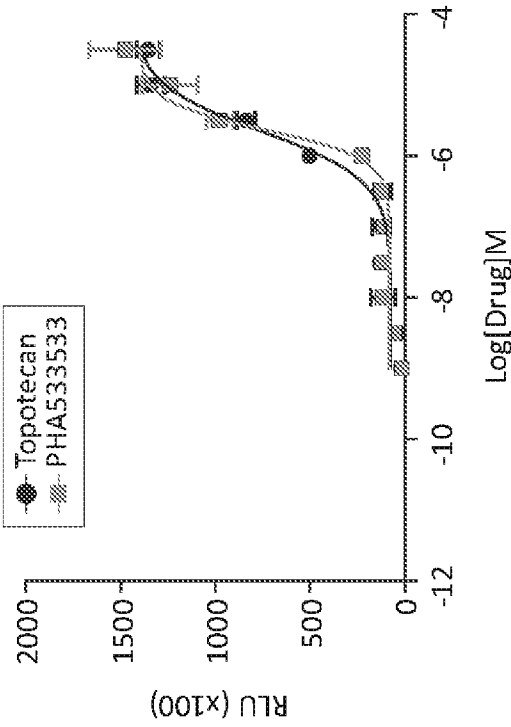


FIG. 9C

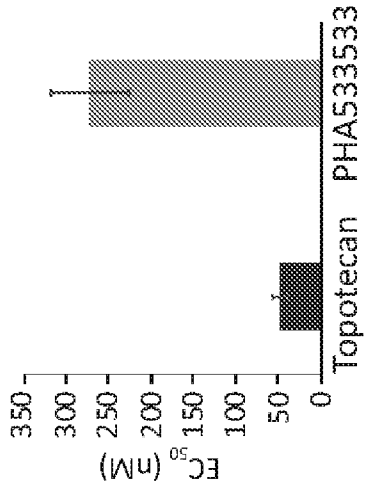


FIG. 9D

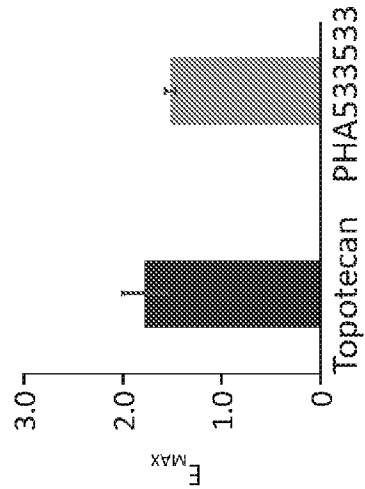


FIG. 9E

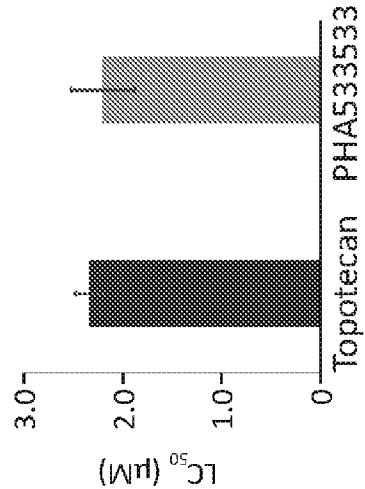


FIG. 10A

PNU0292137

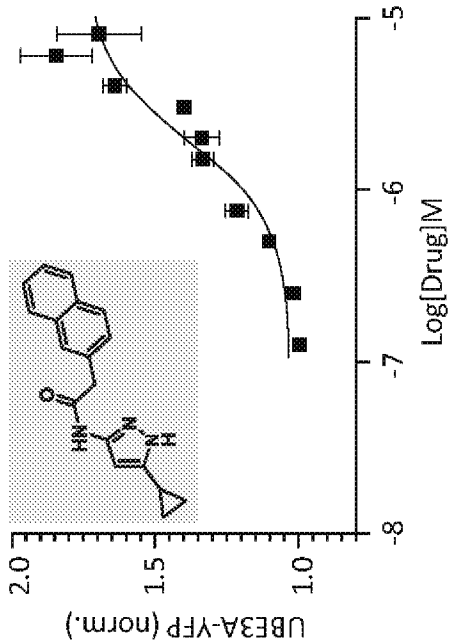


FIG. 10B

PNU0278497

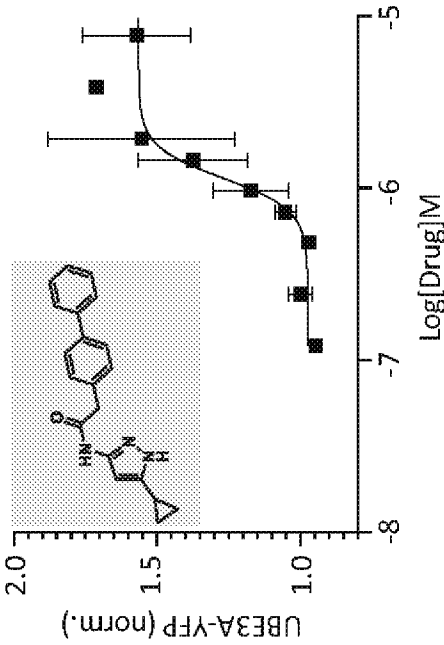


FIG. 10C

PHA00543738

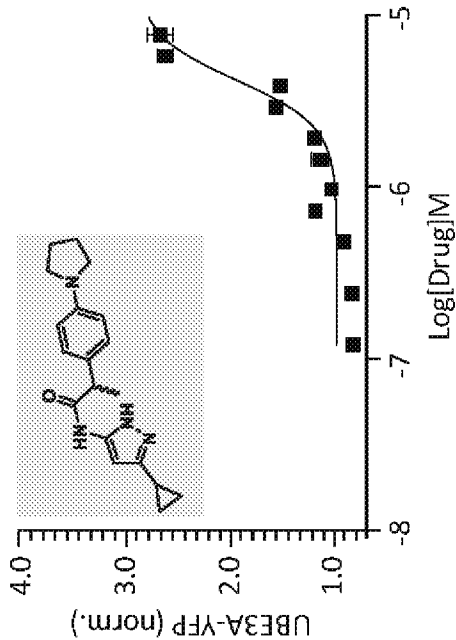


FIG. 10D

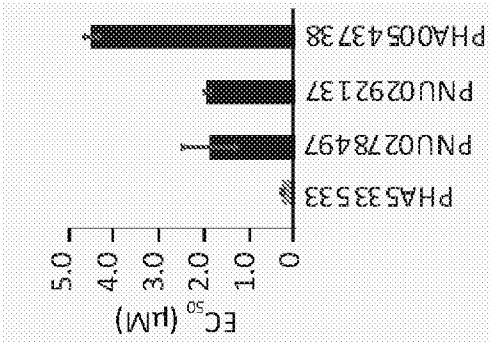
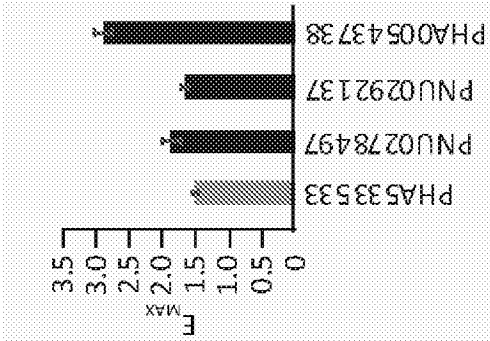


FIG. 10E



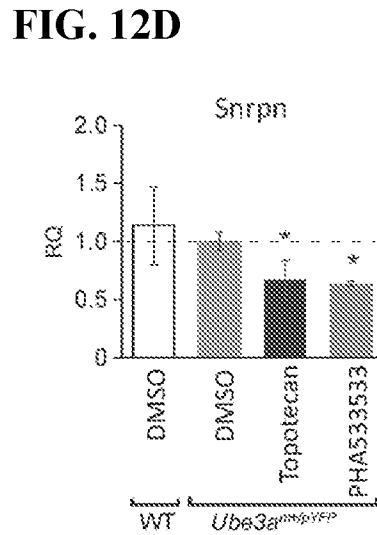
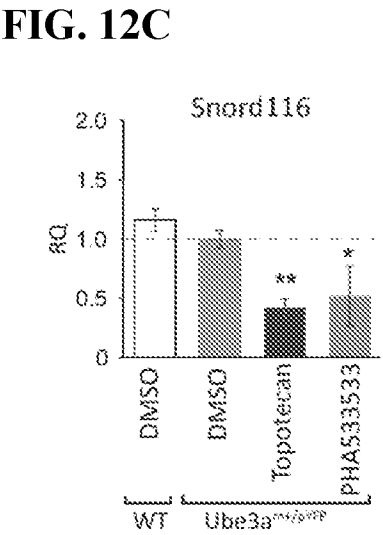
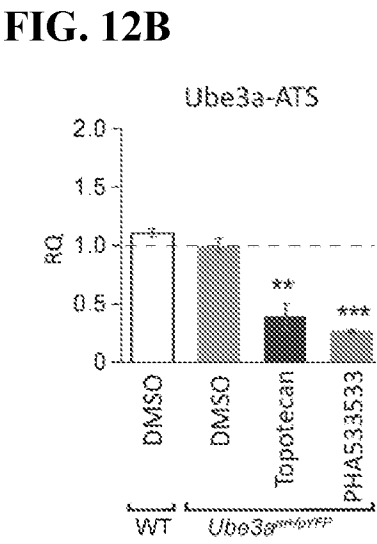
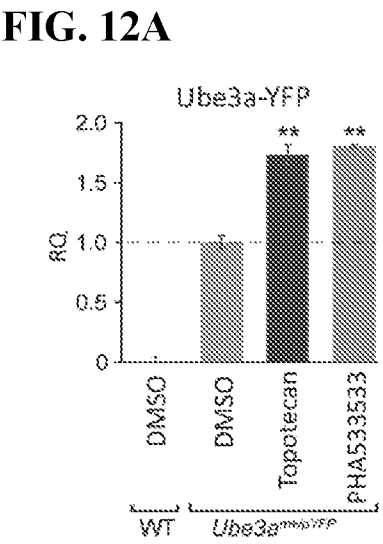
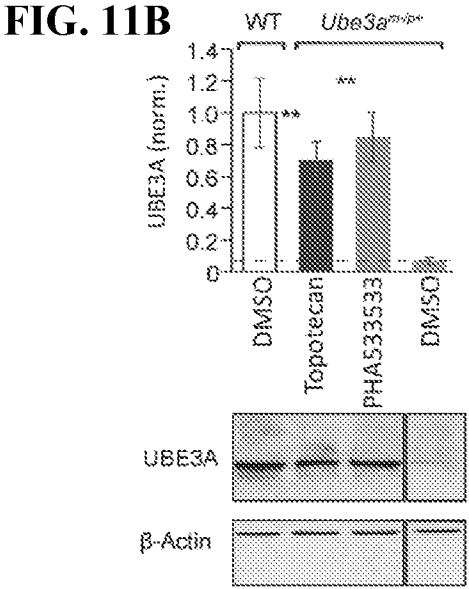
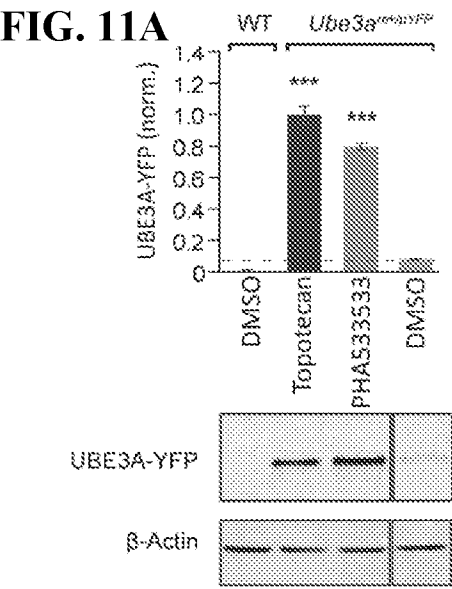


FIG. 13A

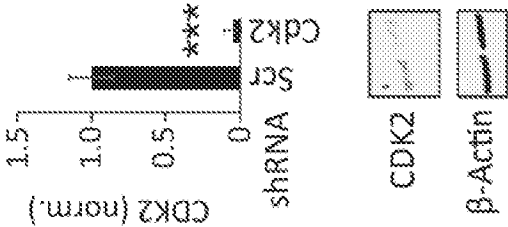


FIG. 13B

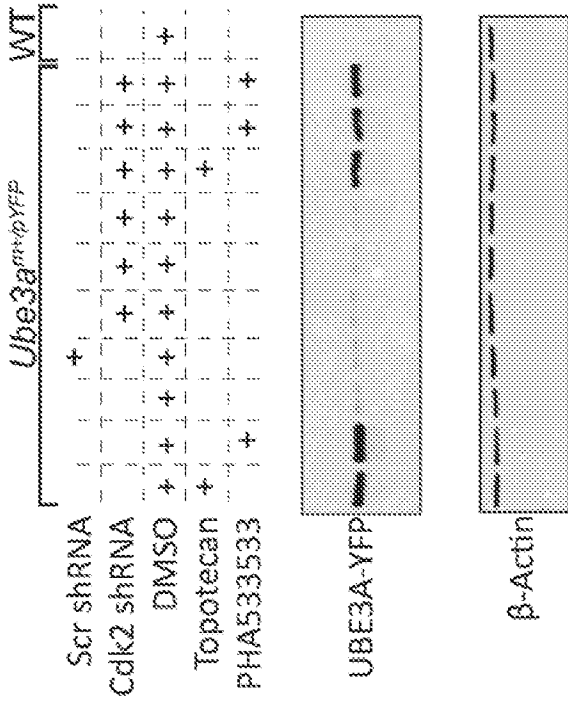


FIG. 13C

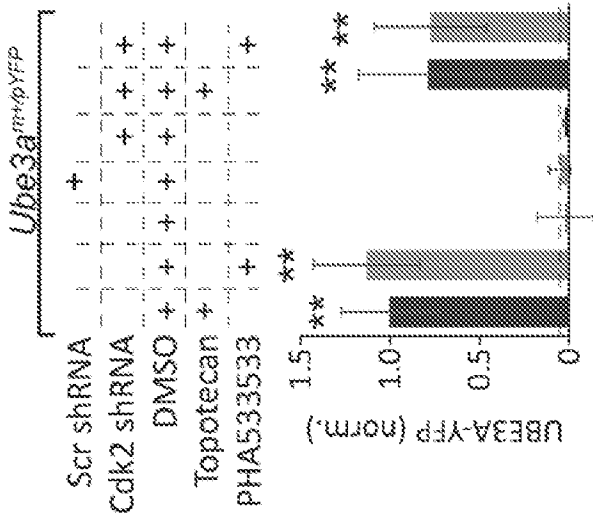


FIG. 13D



FIG. 13E

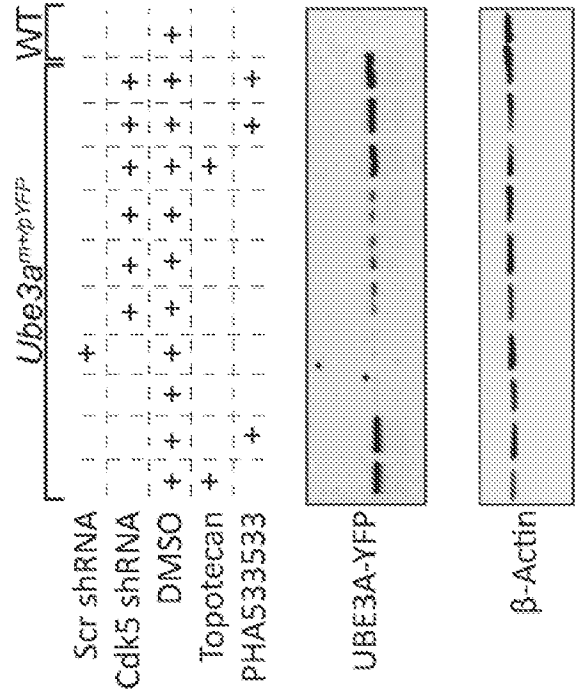


FIG. 13F

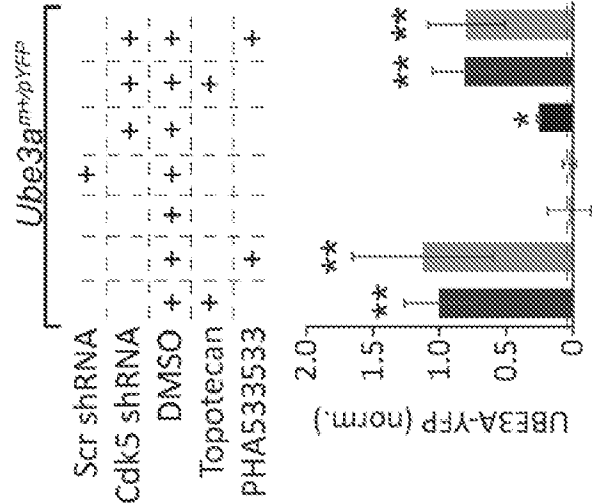


FIG. 13G

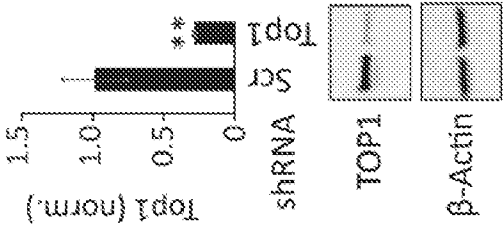


FIG. 13H

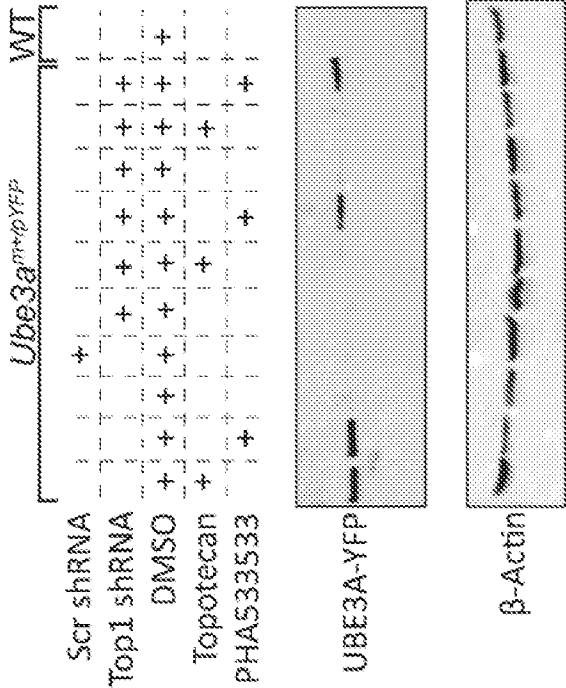


FIG. 13I

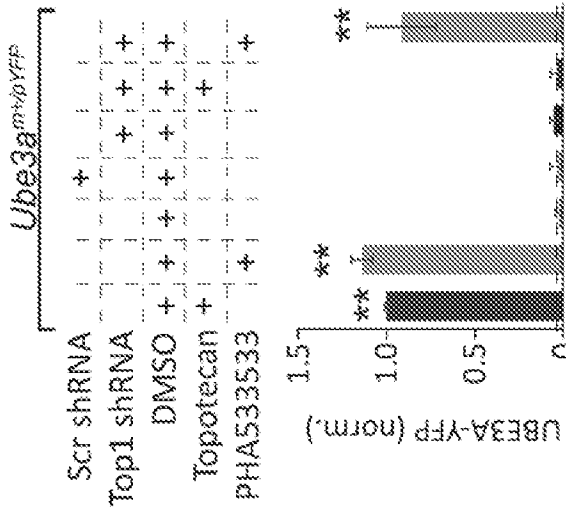


FIG. 14A

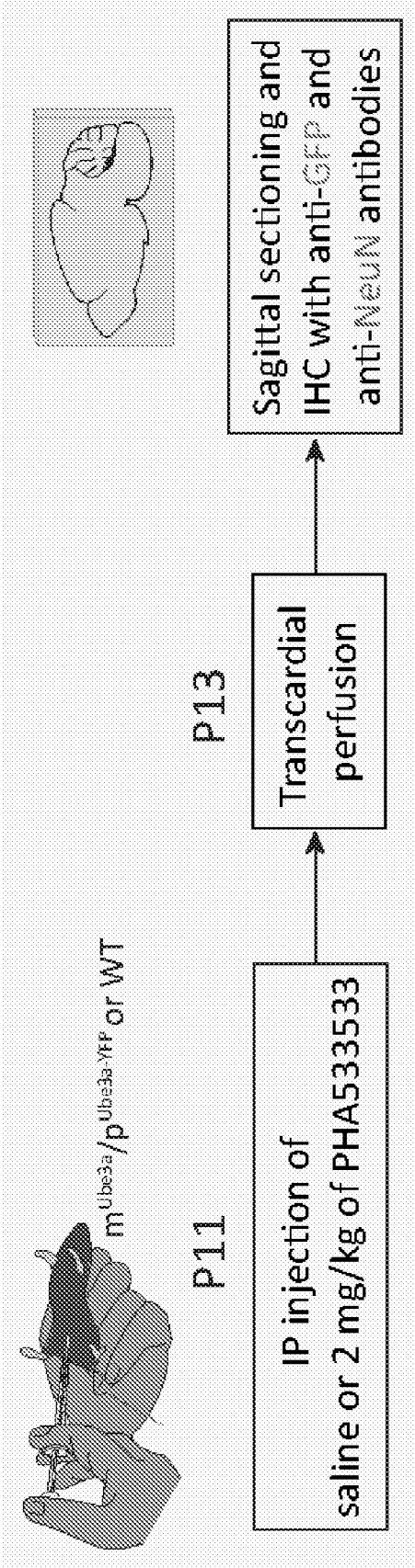


FIG. 14B

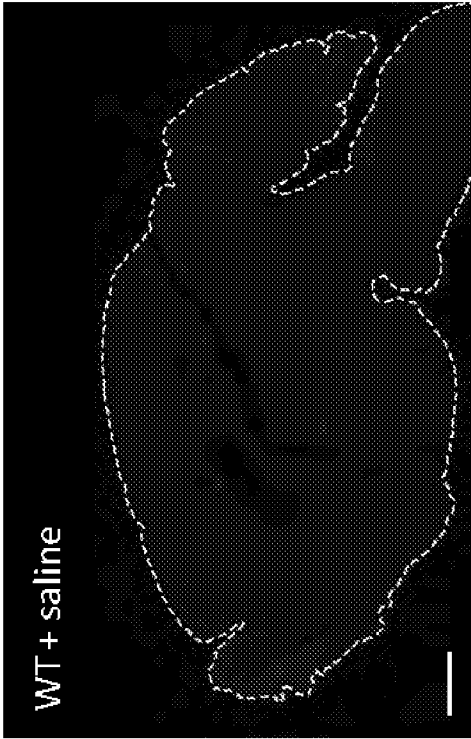


FIG. 14C

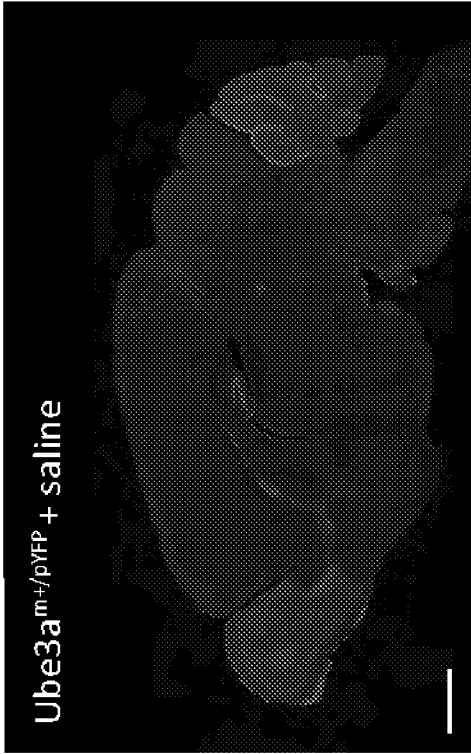


FIG. 14D

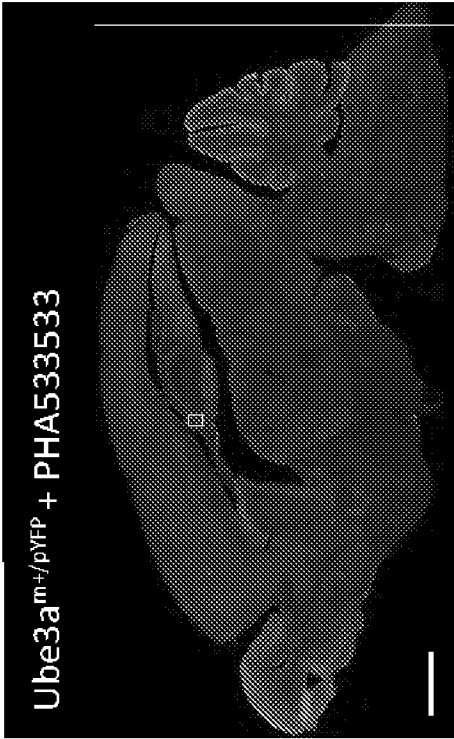


FIG. 14E



A. CLASSIFICATION OF SUBJECT MATTER**A61K 31/4155(2006.01)i, A61K 31/7076(2006.01)i, A61P 25/00(2006.01)i, A61P 43/00(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 31/4155; A61K 31/7105; A61K 48/00; C12N 15/63; A61K 31/7076; A61P 25/00; A61P 43/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: unsilencing imprinted genes, PHA533533, UBE3A, Angelman syndrome, paternal, maternal, neuron

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2018-0016584 A1 (THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL) 18 January 2018 abstract; claims 1-9, 15-23	9-10
A	KANISKAN, H. U. et al., 'Inhibitors of protein methyltransferases and demethylases', Chemical reviews, 2018, vol. 118, pp. 989-1068 abstract; page 1044	9-10
A	MIRABELLA, A. C. et al., 'Chromatin deregulation in disease', Chromosoma, 2016, vol. 125, pp. 75-93 abstract	9-10
A	BOVIO, P. P. et al., 'Differential methylation of H3K79 reveals DOT1L target genes and function in the cerebellum in vivo', Molecular neurobiology, 2019[epub. 10 October 2018], vol. 56, pp. 4273-4287 abstract	9-10
A	WO 2014-004572 A2 (ISIS PHARMACEUTICALS, INC. et al.) 03 January 2014 the whole document	9-10



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

06 November 2020 (06.11.2020)

Date of mailing of the international search report

06 November 2020 (06.11.2020)

Name and mailing address of the ISA/KR

International Application Division

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Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-8, 11-14
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 1-8, 11-14 are directed to a treatment method of the human body by therapy or a method comprising a step of direct medical procedure for the human body, and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and Rule 39.1(iv), to search.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 5-8
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2020/042379

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2018-0016584 A1	18/01/2018	US 10301630 B2 US 2013-0317018 A1 US 9714427 B2 WO 2012-064806 A2 WO 2012-064806 A3	28/05/2019 28/11/2013 25/07/2017 18/05/2012 04/10/2012
WO 2014-004572 A2	03/01/2014	AU 2013-280474 A1 AU 2013-280474 B2 CA 2877905 A1 DK 2864479 T3 DK 3461895 T3 EP 2864479 A2 EP 2864479 B1 EP 3461895 A1 EP 3461895 B1 ES 2688831 T3 JP 2015-529635 A JP 6294876 B2 US 2015-0191723 A1 US 9617539 B2 WO 2014-004572 A3	22/01/2015 03/01/2019 03/01/2014 22/10/2018 20/07/2020 29/04/2015 15/08/2018 03/04/2019 01/07/2020 07/11/2018 08/10/2015 14/03/2018 09/07/2015 11/04/2017 01/05/2014