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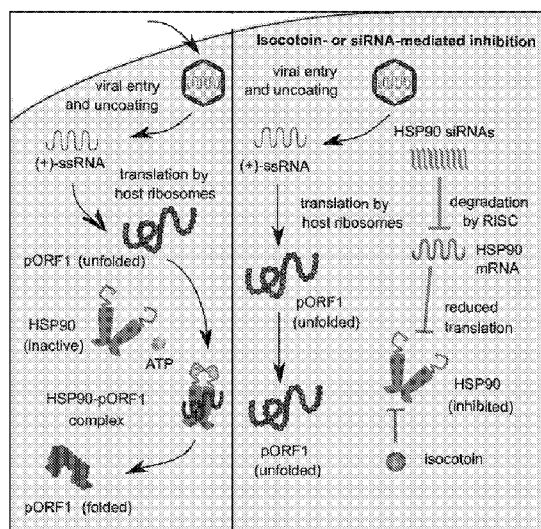
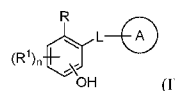


FIG. 11G

(57) Abstract: Provided herein are methods involving a compound of the following structural formula: (I) or a pharmaceutically acceptable salt thereof, wherein values for the variables are as described herein. For example, methods for inhibiting replication of a virus, treating a viral infection, inhibiting heat shock protein 90 and treating a heat shock protein 90-mediated disease or condition using a compound of Structural Formula I are provided.

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

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Small Molecule Inhibitors of Viral Replication

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/911,040, filed on October 4, 2019, and U.S. Provisional Application No. 62/846,843, filed on May 13, 2019. The entire teachings of the above applications are incorporated herein by reference.

INCORPORATION BY REFERENCE OF MATERIAL IN ASCII TEXT FILE

[0002] This application incorporates by reference the Sequence Listing contained in the following ASCII text file being submitted concurrently herewith:

a) File name: 53911022002_SEQUENCELISTINGFINAL.txt; created May 4, 2020, 4.1 KB in size.

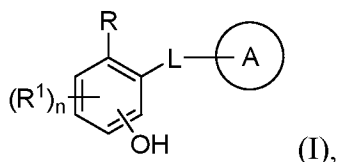
BACKGROUND

[0003] There is no approved direct-acting antiviral treatment for hepatitis E virus (HEV), which causes approximately 14 million symptomatic infections and approximately 300,000 deaths per year globally. Following infection, immunocompromised persons and pregnant women experience particularly severe clinical manifestations including liver cirrhosis and acute liver failure, respectively. Ribavirin monotherapy can be used to treat chronic hepatitis E in solid-organ transplant recipients; however, ribavirin is not safe for pregnant women and, furthermore, ribavirin-resistant HEV strains are emerging.

[0004] Accordingly, there is a need for therapies for HEV and other viral infections.

SUMMARY

[0005] Provided herein is a method of inhibiting replication of a virus (*e.g.*, a hepatitis E virus (HEV), an HEV in a cell), comprising contacting a cell infected with the virus (*e.g.*, an HEV, one or more HEV particles) with a compound represented by the following structural formula:



or a pharmaceutically acceptable salt thereof, wherein:

Ring A is aryl (*e.g.*, phenyl) or heteroaryl (*e.g.*, oxazolyl, pyridinyl, benzothiazolyl, thiazolyl, pyrazolyl or benzofuranyl), and is optionally substituted with one or more substituents independently selected from halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, $-(CH_2)_{0-2}$ -aryl, $-(CH_2)_{0-2}$ -heteroaryl, $-(CH_2)_{0-2}$ -cycloalkyl, or $-(CH_2)_{0-2}$ -heterocyclyl, carboxy or $-O(CH_2)_mO-$;

m is 1, 2, 3, 4 or 5 (*e.g.*, 1, 2 or 3);

L is $-C(O)(CH_2)_p-$, $-C(O)(CH_2)_p-O-$ or heteroarylene (*e.g.*, oxazolylen, pyrimidinylene or pyrazolylen), wherein p is 0, 1 or 2 (*e.g.*, 0 or 1), and R is hydrogen, halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenoxy, alkynoxy, $-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl; or

L is $-C(O)(CH_2)_p-$, wherein p is 1 or 2, and R and a methylene carbon of $-C(O)(CH_2)_p-$, together with their intervening carbon atoms, form a fused ring (*e.g.*, containing five, six, seven or eight members independently selected from carbon, oxygen, nitrogen and sulfur);

R¹ is halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenoxy, alkynoxy, $-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl; and

n is 0, 1, 2 or 3,

wherein the aryl and heteroaryl of R and R¹, and the heteroarylene of L are each optionally and independently substituted with one or more substituents selected from halo, alkyl, haloalkyl, amino, alkylamino, dialkylamino or carboxamido.

[0006] Also provided herein is a method of inhibiting replication of a virus (*e.g.*, an HEV) comprising contacting a cell infected with the virus with a compound of Appendix 1, 1', 2, 2', 3 or 4, or a pharmaceutically acceptable salt thereof, or isocotoin, or a pharmaceutically acceptable salt thereof.

[0007] Also provided herein is a method of treating a viral infection (*e.g.*, an HEV infection) in a subject in need thereof, comprising administering to the subject an effective amount of a compound represented by Structural Formula I, or a pharmaceutically acceptable salt thereof, wherein values for the variables in Structural Formula I are as described herein.

[0008] Also provided herein is a method of treating a viral infection (*e.g.*, an HEV infection) in a subject in need thereof, comprising administering to the subject an effective amount of a compound of Appendix 1, 1', 2, 2', 3 or 4, or a pharmaceutically acceptable salt thereof, or isocotoin, or a pharmaceutically acceptable salt thereof.

[0009] Also provided herein is a method of inhibiting heat shock protein 90 in a cell, comprising contacting the cell with a compound represented by Structural Formula I, or a pharmaceutically acceptable salt thereof, wherein values for the variables in Structural Formula I are as described herein.

[0010] Also provided herein is a method of inhibiting heat shock protein 90 in a cell, comprising contacting the cell with a compound of Appendix 1, 1', 2, 2', 3 or 4, or a pharmaceutically acceptable salt thereof, or isocotoin, or a pharmaceutically acceptable salt thereof.

[0011] Also provided herein is a method of treating a heat shock protein 90-mediated disease or condition (*e.g.*, an HEV infection) in a subject in need thereof, comprising administering to the subject an effective amount of a compound represented by Structural Formula I, or a pharmaceutically acceptable salt thereof, wherein values for the variable in Structural Formula I are as described herein.

[0012] Also provided herein is a method of treating a heat shock protein 90-mediated disease or condition (*e.g.*, an HEV infection) in a subject in need thereof, comprising administering to the subject an effective amount of a compound of Appendix 1, 1', 2, 2', 3 or 4, or a pharmaceutically acceptable salt thereof, or isocotoin, or a pharmaceutically acceptable salt thereof.

[0013] Also provided herein is a compound for use in inhibiting replication of a virus (*e.g.*, an HEV), treating a viral infection (*e.g.*, an HEV infection), inhibiting heat shock protein 90 or treating a heat shock protein 90-mediated disease or condition, wherein the compound is described herein (*e.g.*, a compound of Structural Formula I; a compound of Appendix 1, 1', 2, 2', 3 or 4; isocotoin, or a pharmaceutically acceptable salt of any of the foregoing). Also provided herein is use of a compound described herein (*e.g.*, a compound of Structural Formula I; a compound of Appendix 1, 1', 2, 2', 3 or 4; isocotoin, or a pharmaceutically acceptable salt of any of the foregoing) for the manufacture of a medicament for inhibiting replication of a virus (*e.g.*, an HEV), treating a viral infection (*e.g.*, an HEV infection), inhibiting heat shock protein 90 or treating a heat shock protein 90-mediated disease or condition.

[0014] Compounds of Structural Formula I are effective inhibitors of viral replication *in vitro*. In functional assays of HEV using hepatoma cells, certain representative compounds of Structural Formula I exhibited higher potency than ribavirin, low host cytotoxicity, and pan-genotypic efficacy, suggesting that the compounds of Structural Formula I may be promising candidates for novel, direct-acting therapies against viruses, such as HEV.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be provided by the Office upon request and payment of the necessary fee.

[0016] The foregoing will be apparent from the following more particular description of example embodiments, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating embodiments.

[0017] FIG. 1. Genomic organization of HEV, a single-stranded positive sense RNA virus.

[0018] FIGs. 2A-2C. Characterization of Huh7 Kernow C1 p6-BSR/ZsGreen. FIG. 2A: The p6/BSR-2A-ZsGreen replicon genome was derived from KernowC1p6, with ORFs 2 and 3 replaced by a blasticidin resistance-conferring gene (BSR) and a ZsGreen fluorescence reporter (ZsG), with a 2A self-cleaving peptide in between. FIG. 2B: GFP channel image of Huh7 cells transfected with p6/BSR-2A-ZsGreen and selected under blasticidin pressure to generate a population highly expressing ZsGreen. FIG. 2C: Flow cytometric analysis of p6/BSR-2A-ZsGreen-transfected Huh7 cells (right; 90.2% ZsGreen positive) versus naïve Huh7 cells (left; 3.58% ZsGreen positive).

[0019] FIGs. 3A-3E. Schematic of replicon-based compound screening assay. FIG. 3A: Huh7 p6/BSR-2A-ZsGreen cells were seeded into 384-well plates containing one distinct compound/well from the Princeton University Small Molecule Screening Facility. 60,536 compounds in total were screened (188 plates). Cells were seeded at a density of 8000 cells/well, with each compound diluted to a concentration of 50µM. Four columns of each plate were used for positive (untreated Huh7 p6/BSR-2A-ZsGreen cells) and negative (naïve Huh7 cells) controls. FIG. 3B: A GFP channel image (top) and brightfield image (bottom) were taken of each well on day 4 using the Perkin Elmer Operetta High-Content Imaging System. Fluorescence in the GFP channel images were quantified using a custom Python

script, and decreased fluorescence was used as a metric to select hits with putative antiviral activity. Approximately 800 hits were manually screened for cytotoxicity using the corresponding brightfield images. 37 hits were ultimately chosen for further characterization. FIG. 3C: The 37 selected hits were tested in the same 384-well format against Huh7 p6/BSR-2A-ZsGreen cells at doses from 0.78-100 μ M. Fluorescence in the wells was quantified and used to generate dose titration curves for each compound. Seven compounds had IC₅₀ values lower than that of ribavirin, for example the compound abbreviated G11. FIG. 3D: The seven compounds were tested at doses ranging from 1.5625-100 μ M against the p6/Gluc replicon, in order to provide a more direct readout of activity. Two compounds, isocotoin and gitoxin, showed dose-dependent inhibition of p6/Gluc. FIG 3E: In an ATP live-cell viability assay, gitoxin demonstrated high cytotoxicity. RLU=relative light units. Error bars indicate 1 standard deviation (SD) from mean.

[0020] FIGs. 4A-4G. Isocotoin inhibits replication of p6/Gluc. FIG. 4A: Structure of isocotoin. FIG. 4B: Schematic of KernowC1p6 replicon and p6/Gluc replicon. FIG. 4C: Isocotoin inhibits p6/Gluc replication more efficiently than ribavirin *in vitro*. FIG. 4D: Isocotoin inhibits replication of full-length KernowC1-p6 *in vitro*. FIG. 4E: Schematic of T7-tagBFP-Gluc. FIG. 4F: Isocotoin and ribavirin do not inhibit translation of T7-tagBFP-Gluc. FIG. 4G: Isocotoin is non-cytotoxic to Huh7 cells up to 12.5 μ M. RLU = relative light units. Error bars indicate 1 SD from mean.

[0021] FIGs. 5A-5G. Isocotoin inhibits replication of genetically diverse strains of HEV. FIG. 5A: Of the 8 HEV genotypes categorized in *Orthohepevirus A*, genotypes 1-4 are human-tropic. FIG. 5B: Gluc-expressing replicon genomes were generated for HEV strains derived from genotypes 1, 3, and 4. Three of the original strains were isolated from human patients, and one was isolated from a swine host. FIGs. 5C-5F: Isocotoin inhibits replication of p6/Gluc (GT3), Sar55/Gluc (GT1), SHEV-3/Gluc(GT3), and TW6196E/Gluc(GT4) replicon strains. FIG. 5G: Corresponding IC₅₀ values for dose titration data with standard error indicated. RLU = relative light units. Error bars indicate 1 SD from mean.

[0022] FIGs. 6A-6D. Isocotoin inhibits replication of other (+)-sense RNA viruses. FIGs. 6A-6B: Isocotoin inhibits replication of Gluc-expressing HEV, yellow fever virus 17D (YFV), and hepatitis C virus (HCV) genomes to a greater extent than ribavirin. RLU values are normalized to untreated conditions. FIG. 6C: Schematic of pACNR-FLYF-17D-Gluc-BSD-Ires construct, which is derived from pACNR/FLYF-17D (GenBank ID: AY640589). FIG. 6D: 2'C-Methyladenosine (2'CMA), a potent and specific inhibitor of HCV replication,

was tested against the Gluc-expressing HEV-, YFV-, and HCV-derived viruses.

RLU=relative light units. All data points are mean of triplicate wells (n=3). Error bars indicate 1 SD from mean.

[0023] FIGs. 7A-7G. Isocotoin inhibits strains exhibiting higher replicative capacity *in vitro*. FIG. 7A: Schematic of suboptimal dosing experiment. FIG. 7B: p6/BSR-2A-ZsGreen]-expressing Huh7 cells serially passaged in medium containing 30 μ M isocotoin showed a decrease in ZsGreen expression up to passage 5, and a subsequent increase in ZsGreen expression between passages 5-10. FIG. 7C: At passage 10, the F470S point mutation was found in 2/10 colonies sequenced. An additional similar mutation F473S was found in 1/10 colonies. FIG. 7D: Replication kinetics for p6/Gluc[F470S], p6/Gluc[G1634R], p6/Gluc[Y1320H], and p6/Gluc-WT over 4 days. FIG. 7E: Schematics of viral genome and Gluc-expressing replicons. The p6/Gluc[F470S], p6/Gluc[Y1320H], and p6/Gluc[G1634R] strains are derived from p6/Gluc and contain point mutations in the PCP, RdRp, and RdRp and regions respectively. FIGs. 7F-7G: Dose titration of isocotoin and ribavirin against mutant strains. BSR = blasticidin resistance gene; BSD = blasticidin; ZsG = ZsGreen; RLU = relative light units. Error bars indicate 1 SD from mean.

[0024] FIGs. 8A-8B. Structure-activity relationship (SAR) analysis is used to correlate functional groups with biological activity and identify compounds exhibiting higher potency against p6/Gluc. FIG. 8A: First round of SAR analysis. Structurally related compounds to isocotoin show greater inhibition of p6/Gluc at the 25 μ M dose. FIG. 8B: Second round of SAR analysis. Structurally related compounds to isocotoin show greater inhibition of p6/Gluc at the 1.5625 μ M dose. RLU = relative light units. Error bars indicate 1 SD from mean.

[0025] FIG 9. Structure-activity relationship (SAR) analysis Round 1. A subset of structurally related compounds to isocotoin showed greater inhibition of p6/Gluc. Another subset showed no effect, and finally one subset is associated with a slight increase in replication levels. RLU = relative light units. Error bars indicate 1 SD from mean.

[0026] FIGs. 10A-10B. SAR analysis Round 2. FIG. 10A: Second round of SAR analysis. A subset of structurally related compounds to isocotoin showed greater inhibition of p6/Gluc. One subset of compounds showed high potency but was associated with high cytotoxicity. Another subset was less effective than isocotoin and ribavirin. Data is compiled from multiple batches of experiments hence variation in control curves from isocotoin and ribavirin. FIG. 10B: Titration of highly effective compounds at lower doses. RLU = relative light units. Error bars indicate 1 SD from mean.

[0027] FIGs. 11A-11G. Isocotoin inhibits HEV replication through interference with HSP90. FIG. 11A: Schematic of cellular thermal shift assay (CETSA) workflow. FIG. 11B: Heatmaps showing CETSA data for HSP90AA1 and HSP90AB1. Numbers in heatmaps indicate foldchange in soluble protein at indicated isocotoin concentration and heating temperature. FIG. 11C: AUY-922, STA-9090, VER-50589, and 17-AAG are potent inhibitors of HEV [p6/Gluc strain] replication. FIG. 11D: Treatment with HSP90-specific siRNA results in a 69% reduction in HSP90 protein levels at 72h post-transfection. HSP90 bands are normalized to β -actin band intensity for each lane. FIG. 11E: Treatment with HSP90-specific siRNA results in 54% reduction in HSP90 α and 74% reduction in HSP90 β mRNA levels. Data are combined from two repeat experiments and normalized to cellular GAPDH levels. FIG. 11F: Treatment with HSP90-specific siRNA results in reduced viral replication of HEV Δ ORF2/3[Gluc] as measured via *Gaussia luciferase* secretion 3 days post transfection. *Gaussia luciferase* levels were decreased 51% as compared to mock-transfected cells and 36% as compared to cells treated with negative control, seed sequence-matched siRNA. Data are combined from two repeat experiments and normalized to viral replication levels in cells treated with transfection reagent. FIG. 11G: Hypothesized general mechanism for isocotoin-mediated inhibition of pORF1 folding.

[0028] FIGs. 12A-12B. SAR Analysis Data. FIG. 12A: Approximately 75 commercially available compounds with structural similarities to isocotoin were screened in successive rounds to correlate functional groups with biological activity, and to identify compounds with higher potency than isocotoin. Dose titration assays were conducted in 96-well format for all 75 compounds against the p6/Gluc replicon. Six selected compounds are shown above, with columns indicating luciferase activity at the approximate 15 μ M dose. The most potent analogs identified were AUY-922 and VER-50589, which are known HSP90 inhibitors. FIG. 12B: ATP-based live cell assay shows that known HSP90 inhibitors 17-AAG, VER-50589, AUY-922, and STA-9090 are inhibitors of cell growth.

[0029] FIG. 13. Cellular Thermal Shift Assay data. Cellular thermal shift data for ABHD10, STOML2, OTC, HSP90AA1, HSP90AB1, and HSPA14. Numbers in heatmaps indicate foldchange in soluble protein at indicated isocotoin concentration and heating temperature. ABHD10, STOML2, OTC, and HSP90AA1 were among hits showing the greatest increase in soluble protein fraction upon addition of drug. HSP90AB1 showed a relatively modest, but identifiable increase. HSPA14 is shown as an example of a protein that did not produce a thermal shift.

[0030] FIGs. 14A-14D. HSP90 knockdown assays. FIG. 14A: Western blot indicating expression of HSP90 α/β 72h post-treatment with transfection reagents only (Mock), seed sequence-matched negative control siRNA ((-)siRNA), or HSP90 siRNA. FIG. 14B: 8-bit image of Western blot used for band quantification. FIG. 14C: Profile plot showing density peaks and raw quantification values for bands in FIG. 14B. FIG. 14D: Treatment with HSP90-specific siRNA results in reduced viral replication of HEV Δ ORF2/3[Gluc] as measured via Gaussia luciferase secretion on days 1-4 post-transfection. RLU=relative light units. n=12 wells per data point. Error bars indicate 1 SD from mean.

[0031] FIGs. 15A-15E. *In vivo* testing of isocotoin in human liver chimeric mice. FIG. 15A: Human albumin levels were measured in serum to indicate engraftment levels. FIG. 15B: Eight mice were injected with stool filtrate from HEV-infected rhesus macaques to establish chronic infection. Weight of the mice post-infection as percentage of baseline. FIG. 15C: Viral titers in stool pellets collected from the mice. L.O.D. = limit of detection. FIG. 15D: Six mice were treated with isocotoin for seven days at a 50 mg/kg dose injected daily intraperitoneally. FIG. 15E: Analysis of viral RNA extracted from stool pellets before (blue) and after (red) treatment. RT-qPCR was performed in duplicate.

DETAILED DESCRIPTION

[0032] A description of example embodiments follows.

Definitions

[0033] Compounds described herein include those described generally, and are further illustrated by the classes, subclasses, and species disclosed herein. As used herein, the following definitions shall apply unless otherwise indicated. For purposes of this invention, the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, Handbook of Chemistry and Physics, 75th Ed. Additionally, general principles of organic chemistry are described in “Organic Chemistry”, Thomas Sorrell, University Science Books, Sausalito: 1999, and “March’s Advanced Organic Chemistry”, 5th Ed., Ed.: Smith, M.B. and March, J., John Wiley & Sons, New York: 2001, the relevant contents of which are incorporated herein by reference.

[0034] Unless specified otherwise within this specification, the nomenclature used in this specification generally follows the examples and rules stated in Nomenclature of Organic Chemistry, Sections A, B, C, D, E, F, and H, Pergamon Press, Oxford, 1979, which is incorporated by reference herein for its chemical structure names and rules on naming

chemical structures. Optionally, a name of a compound may be generated using a chemical naming program (*e.g.*, CHEMDRAW®, version 17.0.0.206, PerkinElmer Informatics, Inc.).

[0035] “Alkyl” refers to a saturated, aliphatic, branched or straight-chain, monovalent, hydrocarbon radical having from one to 25 (*e.g.*, from one to 20, from one to 15, from one to 10, from one to five) carbon atoms. When there is an indication of the number of carbon atoms in an alkyl group, the alkyl group has the indicated number of carbon atoms. Thus, “(C₁-C₅)alkyl” means a radical having from 1-5 carbon atoms in a linear or branched arrangement. Examples of alkyl groups include methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, t-butyl, n-pentyl, isopentyl, neopentyl, 2-methylpentyl, n-hexyl, and the like.

[0036] “Alkoxy” refers to an alkyl radical attached through an oxygen linking atom, wherein alkyl is as described herein. Examples of alkoxy include, but are not limited to, methoxy, ethoxy, propoxy, isopropoxy, and the like.

[0037] “Alkenyl” refers to an aliphatic, branched or straight-chain, monovalent, hydrocarbon radical having at least one carbon-carbon double bond and from two to 25 (*e.g.*, from two to 20, from two to 15, from two to 10, from two to five) carbon atoms. When there is an indication of the number of carbon atoms in an alkenyl group, the alkenyl group has the indicated number of carbon atoms. Thus, “(C₂-C₅)alkenyl” means a radical having at least one carbon-carbon double bond and from two to five carbon atoms in a linear or branched arrangement. Examples of alkenyl groups include ethenyl, 2-propenyl, 1-propenyl, 2-methyl-1-propenyl, 1-butenyl, 2-butenyl, 1-pentenyl, 2-pentenyl, 3-pentenyl, allyl, 1, 3-butadienyl, 1, 3-dipentenyl, 1,4-dipentenyl, 1-hexenyl, 1,3-hexenyl, 1,4-hexenyl, 1,3,5-trihexenyl, 2,4-dihexenyl, and the like.

[0038] “Alkenoxy” refers to an alkenyl radical attached through an oxygen linking atom, wherein alkenyl is as described herein. Examples of alkenoxy include, but are not limited to, ethenoxy, propenoxy, and the like.

[0039] “Alkynyl” refers to an aliphatic, branched or straight-chain, monovalent, hydrocarbon radical having at least one carbon-carbon triple bond and from two to 25 (*e.g.*, from two to 20, from two to 15, from two to 10, from two to five) carbon atoms. When there is an indication of the number of carbon atoms in an alkynyl group, the alkynyl group has the indicated number of carbon atoms. Thus, “(C₂-C₅)alkynyl” means a radical having at least one carbon-carbon triple bond and from two to five carbon atoms in a linear or branched arrangement. Examples of alkynyl groups include ethynyl, 1-propynyl, 2-propynyl, 1-

butynyl, 2-butynyl, 2-methyl-1-butynyl, 1-pentynyl, 2-pentynyl, 3-pentynyl, 3-methyl-1-pentynyl, 2-methyl-1-pentynyl, 1-hexynyl, 2-hexynyl, 3-hexynyl, and the like.

[0040] “Alkynoxy” refers to an alkynyl radical attached through an oxygen linking atom, wherein alkynyl is as described herein. Examples of alkynoxy include, but are not limited to, ethynoxy, propynoxy, and the like.

[0041] “Amino” means -NH_2 .

[0042] “Alkylamino” refers to -N(H)(alkyl) , wherein alkyl is as described herein. Alkylamino includes, but is not limited to, methylamino and ethylamino.

[0043] “Aryl” refers to a monocyclic or polycyclic (*e.g.*, bicyclic, tricyclic), carbocyclic, aromatic ring system having from six to 25 (*e.g.*, from six to 20, from six to 15, from six to 10) ring atoms. When there is an indication of the number of ring atoms in an aryl group, the aryl group has the indicated number of ring atoms. Thus, “(C₆-C₁₅)aryl” means an aromatic ring system having from six to 15 ring atoms. Examples of aryl include phenyl and naphthyl.

[0044] “Carboxy” refers to -COOH .

[0045] “Carboxamido” refers to $\text{-C(O)NR}^\circ\text{R}^\circ$, wherein R[°] and R[°] are each independently hydrogen or alkyl, wherein alkyl is as described herein. When R[°] and R[°] are both alkyl, the alkyls can be the same or different. Carboxamido includes, but is not limited to, -C(O)NH_2 , $\text{-C(O)N(H)(CH}_2\text{CH}_3)$, $\text{-C(O)N(CH}_3)_2$ and $\text{-C(O)N(CH}_3)(\text{CH}_2\text{CH}_3)$.

[0046] “Cycloalkyl” refers to a saturated, aliphatic, monovalent, monocyclic or polycyclic, hydrocarbon ring radical having from three to 25 (*e.g.*, from three to 20, from three to 15, from three to 10, from three to eight) ring atoms. When there is an indication of the number of ring atoms in a cycloalkyl group, the cycloalkyl group has the indicated number of ring atoms. Thus, “(C₃-C₈)cycloalkyl” means a ring radical having from three to eight ring atoms. Cycloalkyl includes, but is not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and the like.

[0047] “Dialkylamino” refers to -N(alkyl)_2 , wherein alkyl is as described herein. Each alkyl in a dialkylamino group can be the same as, for example, in $\text{-N(CH}_3)_2$, or different as, for example, in $\text{-N(CH}_3)(\text{CH}_2\text{CH}_3)$.

[0048] “Heteroaryl” refers to a monocyclic or polycyclic (*e.g.*, bicyclic, tricyclic), aromatic, hydrocarbon ring system having from five to 25 (*e.g.*, from five to 20, from five to 15, from five to 10, 5 or 6) ring atoms, wherein at least one carbon atom (*e.g.*, one, two, three, four or five) in the ring system has been replaced with a heteroatom selected from nitrogen, sulfur and oxygen. When there is an indication of the number of ring atoms in a heteroaryl

group, the heteroaryl group has the indicated number of ring atoms. Thus, “(C₅-C₁₅)heteroaryl” means a heterocyclic aromatic ring system having from five to 15 ring atoms consisting of carbon, nitrogen, sulfur and oxygen. In some embodiments, a heteroaryl contains 1, 2, 3 or 4 (*e.g.*, 1, 2 or 3) heteroatoms independently selected from nitrogen, sulfur and oxygen. Monocyclic heteroaryls include, but are not limited to, furan, oxazole, thiophene, triazole, triazolone, triazene, thiadiazole, oxadiazole, imidazole, isothiazole, isoxazole, pyrazole, pyridazine, pyridine, pyrazine, pyrimidine, pyrrole, tetrazole and thiazole. Bicyclic heteroaryls include, but are not limited to, indolizine, indole, isoindole, indazole, benzimidazole, benzofuran, benzothiazole, purine, quinoline, isoquinoline, cinnoline, phthalazine, quinazoline, quinoxaline, naphthyridine and pteridine.

[0049] “Heterocyclyl” refers to a saturated, aliphatic, monocyclic or polycyclic (*e.g.*, bicyclic, tricyclic), monovalent, hydrocarbon ring system having from three to 25 (*e.g.*, from three to 20, from three to 15, from three to 10, from three to eight) ring atoms, wherein at least one carbon atom in the ring system (*e.g.*, one, two, three, four or five) has been replaced with a heteroatom selected from nitrogen, sulfur and oxygen. When there is an indication of the number of ring atoms in a heterocyclyl group, the heterocyclyl group has the indicated number of ring atoms. Thus, “(C₃-C₈)heterocyclyl” means a heterocyclic ring system having from three to eight ring atoms consisting of carbon, nitrogen, sulfur and oxygen. A heterocyclyl can be monocyclic, fused bicyclic, bridged bicyclic or polycyclic, but is typically monocyclic. In some embodiments, a heterocyclyl contains 1, 2, 3 or 4 (*e.g.*, 1, 2 or 3) heteroatoms independently selected from nitrogen, sulfur and oxygen. When one heteroatom is sulfur, it can be optionally mono- or di-oxygenated (*i.e.*, -S(O)- or -S(O)₂, respectively). Examples of monocyclic heterocyclyls include, but are not limited to, aziridine, azetidine, pyrrolidine, piperidine, piperazine, azepane, tetrahydrofuran, tetrahydropyran, morpholine, thiomorpholine, dioxide and oxirane.

[0050] “Halogen” and “halo” are used interchangeably herein, and each refers to fluorine, chlorine, bromine, or iodine. In some embodiments, halogen is selected from fluoro, chloro or bromo.

[0051] “Haloalkyl” refers to an alkyl radical wherein at least one hydrogen of the alkyl has been replaced by a halogen, and alkyl is as described herein. “Haloalkyl” includes mono, poly, and perhaloalkyl groups, wherein each halogen is independently selected from fluorine, chlorine, bromine and iodine (*e.g.*, fluorine, chlorine and bromine). In one aspect, haloalkyl

is perhaloalkyl (*e.g.*, perfluoroalkyl). Haloalkyl includes, but is not limited to, trifluoromethyl and pentafluoroethyl.

[0052] “Haloalkoxy” refers to a haloalkyl radical attached through an oxygen linking atom, wherein haloalkyl is as described herein. Haloalkoxy includes, but is not limited to, trifluoromethoxy.

[0053] “Hydroxy” means -OH.

[0054] Groups described herein having two or more points of attachment (*e.g.*, divalent, trivalent, or polyvalent; typically, divalent as, for example, in the heteroarylene group designated variable L) are designated by use of the suffix, “ene.” For example, divalent heteroaryl groups are heteroarylene groups, and so forth.

[0055] It is understood that substituents on the compounds of the invention can be selected by one of ordinary skill in the art to provide compounds that are chemically stable and that can be readily synthesized by techniques known in the art, as well as those methods set forth below. The term “stable,” as used herein, refers to compounds that are not substantially altered when subjected to conditions to allow for their production, detection and, in certain embodiments, recovery, purification and use for one or more of the purposes disclosed herein. Combinations of substituents envisioned by this invention are preferably those that result in the formation of stable or chemically feasible compounds.

[0056] A designated group is unsubstituted, unless otherwise indicated. When the term “substituted” precedes a designated group, it means that one or more hydrogens of the designated group are replaced with a suitable substituent. Unless otherwise indicated, an “optionally substituted” group or “substituted or unsubstituted” group can have a suitable substituent at each substitutable position of the group and, when more than one position in any given structure may be substituted with more than one substituent selected from a specified group, the substituent can be the same or different at every position. Alternatively, an “optionally substituted” group or “substituted or unsubstituted” group can be unsubstituted.

[0057] Suitable substituents for an optionally substituted or substituted or unsubstituted group include, but are not limited to, halo, hydroxy, cyano, nitro, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenyl, alkenoxy, alkynyl, alkynoxy, $-(CH_2)_{0-2}$ aryl, $-(CH_2)_{0-2}$ heteroaryl, $-(CH_2)_{0-2}$ cycloalkyl, $-(CH_2)_{0-2}$ heterocyclyl, carboxy, $-(CH_2)_n-$ wherein n is 1, 2, 3, 4, or 5 (*e.g.*, 1, 2 or 3), $-O(CH_2)_mO-$ wherein m is 1, 2, 3, 4 or 5 (*e.g.*, 1, 2 or 3), amino, alkylamino, dialkylamino or carboxamido, or oxo. In some embodiments, suitable substituents are

selected from halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, $-(CH_2)_{0-2}aryl$, $-(CH_2)_{0-2}heteroaryl$, $-(CH_2)_{0-2}cycloalkyl$, $-(CH_2)_{0-2}heterocyclyl$, carboxy or $-O(CH_2)_mO-$ wherein m is 1, 2, 3, 4 or 5 (e.g., 1, 2 or 3). In some embodiments, suitable substituents are selected from halo, alkyl, haloalkyl, amino, alkylamino, dialkylamino or carboxamido. In some embodiments, suitable substituents are selected from halo, hydroxy, cyano, nitro, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenyl, alkenoxy, alkynyl, alkynoxy, carboxy, $-(CH_2)_n-$ wherein n is 1, 2, 3, 4, or 5 (e.g., 1, 2 or 3), $-O(CH_2)_mO-$ wherein m is 1, 2, 3, 4 or 5 (e.g., 1, 2 or 3), amino, alkylamino, dialkylamino or carboxamido, or oxo. In embodiments, suitable substituents are selected from halo, hydroxy, cyano, nitro, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenyl, alkenoxy, alkynyl, alkynoxy, $-(CH_2)_{0-2}aryl$, $-(CH_2)_{0-2}heteroaryl$, $-(CH_2)_{0-2}cycloalkyl$, $-(CH_2)_{0-2}heterocyclyl$, $-(CH_2)_n-$ wherein n is 1, 2, 3, 4, or 5 (e.g., 1, 2 or 3), $-O(CH_2)_mO-$ wherein m is 1, 2, 3, 4 or 5 (e.g., 1, 2 or 3) or carboxamido, or oxo.

[0058] As used herein, the term “pharmaceutically acceptable salt” refers to those salts which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of mammals without undue toxicity, irritation, allergic response and the like, and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well known in the art. For example, S. M. Berge *et al.* describe pharmaceutically acceptable salts in detail in J. Pharmaceutical Sciences, 1977, 66, 1–19, the relevant teachings of which are incorporated herein by reference in their entirety. Pharmaceutically acceptable salts of the compounds described herein include salts derived from suitable inorganic and organic acids, and suitable inorganic and organic bases.

[0059] Examples of pharmaceutically acceptable acid addition salts are salts of an amino group formed with inorganic acids such as hydrochloric acid, hydrobromic acid, phosphoric acid, sulfuric acid and perchloric acid, or with organic acids such as acetic acid, oxalic acid, maleic acid, tartaric acid, citric acid, succinic acid or malonic acid, or by using other methods used in the art, such as ion exchange. Other pharmaceutically acceptable acid addition salts include adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, cinnamate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, formate, fumarate, glucoheptonate, glycerophosphate, gluconate, glutarate, glycolate, hemisulfate, heptanoate, hexanoate, hydroiodide, hydroxybenzoate, 2-hydroxy-ethanesulfonate, hydroxymaleate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate,

persulfate, 2-phenoxybenzoate, phenylacetate, 3-phenylpropionate, phosphate, pivalate, propionate, pyruvate, salicylate, stearate, succinate, sulfate, tartrate, thiocyanate, p-toluenesulfonate, undecanoate, valerate salts, and the like. Either the mono-, di- or tri-acid salts can be formed, and such salts can exist in either a hydrated, solvated or substantially anhydrous form.

[0060] Salts derived from appropriate bases include salts derived from inorganic bases, such as alkali metal, alkaline earth metal, and ammonium bases, and salts derived from aliphatic, alicyclic or aromatic organic amines, such as methylamine, trimethylamine and picoline, or $N^+((C_1-C_4)alkyl)_4$ salts. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, barium and the like. Further pharmaceutically acceptable salts include, when appropriate, nontoxic ammonium, quaternary ammonium, and amine cations formed using counterions such as halide, hydroxide, carboxylate, sulfate, phosphate, nitrate, lower alkyl sulfonate and aryl sulfonate.

[0061] Compounds described herein can also exist as various “solvates” or “hydrates.” A “hydrate” is a compound that exists in a composition with one or more water molecules. The composition can include water in stoichiometric quantities, such as a monohydrate or a dihydrate, or can include water in random amounts. A “solvate” is similar to a hydrate, except that a solvent other than water, such as methanol, ethanol, dimethylformamide, diethyl ether, or the like replaces water. Mixtures of such solvates or hydrates can also be prepared. The source of such solvate or hydrate can be from the solvent of crystallization, inherent in the solvent of preparation or crystallization, or adventitious to such solvent.

[0062] Any formula given herein is also intended to represent unlabeled forms as well as isotopically labeled forms of the compounds. Isotopically labeled compounds have structures depicted by the formulas given herein except that one or more atoms are replaced by an atom having a selected atomic mass or mass number. Examples of isotopes that can be incorporated into compounds of the present disclosure include isotopes of hydrogen, carbon, nitrogen and oxygen, such as 2H , 3H , ^{11}C , ^{13}C , ^{14}C and ^{15}N , respectively. The present disclosure includes various isotopically labeled compounds as defined herein, for example, those into which radioactive isotopes, such as 3H and ^{14}C , or those into which non-radioactive isotopes, such as 2H and ^{13}C , have been incorporated. Such isotopically labelled compounds are useful in metabolic studies (with ^{14}C), reaction kinetic studies (with, for example, 2H or 3H), detection or imaging techniques, such as positron emission tomography (PET) or single-photon emission computed tomography (SPECT), including drug or substrate tissue

distribution assays, or in radioactive treatment of patients. Further, substitution with heavier isotopes, particularly deuterium (*i.e.*, ^2H or D) may afford certain therapeutic advantages resulting from greater metabolic stability, for example, increased *in vivo* half-life or reduced dosage requirements or an improvement in therapeutic index.

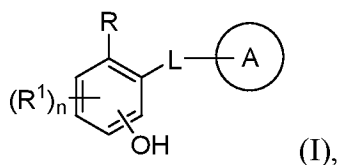
[0063] Isotopically labeled compounds of the present disclosure can generally be prepared by conventional techniques known to those skilled in the art by substituting an appropriate or readily available isotopically labeled reagent for a non-isotopically labeled reagent otherwise employed. Such compounds have a variety of potential uses, *e.g.*, as standards and reagents in determining the ability of a potential pharmaceutical compound to bind to target proteins or receptors, or for imaging compounds of this disclosure bound to biological receptors *in vivo* or *in vitro*.

[0064] Compounds disclosed herein may have asymmetric centers, chiral axes, and chiral planes (*e.g.*, as described in: E. L. Eliel and S. H. Wilen, Stereo-chemistry of Carbon Compounds, John Wiley & Sons, New York, 1994, pages 1119-1190), and occur as racemates, racemic mixtures, or as individual diastereomers or enantiomers, with all possible isomers and mixtures thereof, including optical isomers, being included in the present invention. When a disclosed compound is depicted by structure without indicating the stereochemistry, and the compound has one chiral center, it is to be understood that the structure encompasses one enantiomer or diastereomer of the compound separated or substantially separated from the corresponding optical isomer(s), a racemic mixture of the compound and mixtures enriched in one enantiomer or diastereomer relative to its corresponding optical isomer(s).

[0065] When introducing elements disclosed herein, the articles “a,” “an,” “the,” and “said” are intended to mean that there are one or more of the elements. The terms “comprising,” “having” and “including” are intended to be open-ended, and mean that there may be additional elements other than the listed elements.

Compounds

[0066] In a first embodiment, the compound is represented by the following structural formula:



or a pharmaceutically acceptable salt thereof, wherein:

Ring A is aryl (*e.g.*, phenyl) or heteroaryl (*e.g.*, oxazolyl, pyridinyl, benzothiazolyl, thiazolyl, pyrazolyl or benzofuranyl), and is optionally substituted with one or more substituents independently selected from halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, $-(CH_2)_{0-2}$ -aryl, $-(CH_2)_{0-2}$ -heteroaryl, $-(CH_2)_{0-2}$ -cycloalkyl, $-(CH_2)_{0-2}$ -heterocyclyl, carboxy or $-O(CH_2)_mO-$ (*e.g.*, halo, hydroxy, alkyl, alkoxy, $-(CH_2)_{0-2}$ -aryl, $-(CH_2)_{0-2}$ -heteroaryl, $-(CH_2)_{0-2}$ -cycloalkyl, $-(CH_2)_{0-2}$ -heterocyclyl, carboxy or $-O(CH_2)_mO-$);
 m is 1, 2, 3, 4 or 5;

L is $-C(O)(CH_2)_p-$, $-C(O)(CH_2)_pO-$ or heteroarylene (*e.g.*, oxazolylen, pyrimidinylene or pyrazolylen), wherein p is 0, 1 or 2 (*e.g.*, 0 or 1), and R is hydrogen, halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenoxy, alkynoxy, $-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl (*e.g.*, hydrogen, halo, hydroxy, alkyl, alkoxy, alkenoxy, alkynoxy, $-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl); or
 L is $-C(O)(CH_2)_p-$, wherein p is 1 or 2, and R and a methylene carbon of $-C(O)(CH_2)_p-$, together with their intervening carbon atoms, form a fused ring (*e.g.*, containing five, six, seven or eight members independently selected from carbon, oxygen, nitrogen and sulfur);

R¹ is halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenoxy, alkynoxy, $-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl (*e.g.*, halo, hydroxy, alkyl, alkoxy, alkenoxy, alkynoxy, $-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl); and
 n is 0, 1, 2 or 3,

wherein the aryl and heteroaryl of R and R¹, and the heteroarylene of L are each optionally and independently substituted with one or more substituents (*e.g.*, one, two or three substituents) selected from halo, alkyl, haloalkyl, amino, alkylamino, dialkylamino or carboxamido.

[0067] In a first aspect of the first embodiment, the compound is not AUY-922, VER-50589 or STA-9090, or a pharmaceutically acceptable salt of any of the foregoing. Values for the variables are as described in the first embodiment.

[0068] In a second aspect of the first embodiment, L is heteroarylene (*e.g.*, (C₅-C₆)heteroarylene. Values for the remaining variables are as described in the first embodiment, or first aspect thereof.

[0069] In a third aspect of the first embodiment, L is oxazolylen, pyrazolylen, pyrimidinylene or triazolonylene. Values for the remaining variables are as described in the first embodiment, or first or second aspect thereof.

[0070] In a fourth aspect of the first embodiment, the heteroarylene of L is optionally substituted with one substituent selected from halo, alkyl, haloalkyl, amino, alkylamino, dialkylamino or carboxamido. Values for the variables are as described in the first embodiment, or first through third aspects thereof.

[0071] In a fifth aspect of the first embodiment, Ring A is phenyl. Values for the remaining variables are as described in the first embodiment, or first through fourth aspects thereof.

[0072] In a sixth aspect of the first embodiment, Ring A is heteroaryl (*e.g.*, oxazolyl, pyridinyl, benzothiazolyl, thiazolyl, pyrazolyl or benzofuranyl). Values for the remaining variables are as described in the first embodiment, or first through fifth aspects thereof.

[0073] In a seventh aspect of the first embodiment, Ring A is indolyl, pyrazolyl, benzofuranyl, benzothiazolyl, or thiazolyl. Values for the remaining variables are as described in the first embodiment, or first through sixth aspects thereof.

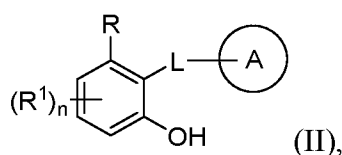
[0074] In an eighth aspect of the first embodiment, m is 1, 2 or 3. Values for the remaining variables are as described in the first embodiment, or first through seventh aspects thereof.

[0075] In a ninth aspect of the first embodiment, n is 0, 1 or 2. Values for the remaining variables are as described in the first embodiment, or first through eighth aspects thereof.

[0076] In a tenth aspect of the first embodiment, R is hydrogen. Values for the remaining variables are as described in the first embodiment, or first through ninth aspects thereof.

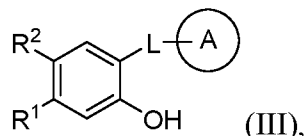
[0077] In an eleventh aspect of the first embodiment, R¹ is halo, hydroxy, alkyl, alkoxy, alkenoxy, alkynoxy, -(CH₂)₀₋₂-aryl, or -(CH₂)₀₋₂-heteroaryl. Values for the remaining variables are as described in the first embodiment, or first through tenth aspects thereof.

[0078] A second embodiment is a compound represented by the following structural formula:



or a pharmaceutically acceptable salt thereof, wherein values for the variables are as described in the first embodiment, or any aspect thereof, or the fourth embodiment.

[0079] A third embodiment is a compound represented by the following structural formula:



or a pharmaceutically acceptable salt thereof, wherein R^2 is hydrogen, halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenoxy, alkynoxy, $-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl. Values for the remaining variables are as described in the first embodiment, or any aspect thereof, or the fourth embodiment.

[0080] In a first aspect of the third embodiment, R^1 is hydroxy, alkoxy, haloalkoxy, alkenoxy or alkynoxy. Values for the remaining variables are as described in the first embodiment, or any aspect thereof, or the third or fourth embodiment.

[0081] In a second aspect of the third embodiment, R^2 is hydrogen, halo, alkyl or haloalkyl. Values for the remaining variables are as described in the first embodiment, or any aspect thereof, or the third embodiment, or the first aspect thereof, or the fourth embodiment.

[0082] In a fourth embodiment, the compound is represented by Structural Formula I, or a pharmaceutically acceptable salt thereof, wherein:

Ring A is substituted or unsubstituted aryl (*e.g.*, phenyl) or substituted or unsubstituted heteroaryl (*e.g.*, oxazolyl, pyridinyl, benzothiazolyl, thiazolyl, pyrazolyl or benzofuranyl);

L is $-C(O)(CH_2)_p-$, $-C(O)(CH_2)_p-O-$ or substituted or unsubstituted heteroarylene (*e.g.*, oxazolylene, pyrimidinylene or pyrazolylene), wherein p is 0, 1 or 2 (*e.g.*, 0 or 1), and R is hydrogen, halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenoxy, alkynoxy, substituted or unsubstituted $-(CH_2)_{0-2}$ -aryl, or substituted or unsubstituted $-(CH_2)_{0-2}$ -heteroaryl; or

L is $-C(O)(CH_2)_p-$, wherein p is 1 or 2, and R and a methylene carbon of $-C(O)(CH_2)_p-$, together with their intervening carbon atoms, form a substituted or unsubstituted fused ring (*e.g.*, containing five, six, seven or eight members independently selected from carbon, oxygen, nitrogen and sulfur);

R¹ is halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenoxy, alkynoxy, substituted or unsubstituted -(CH₂)₀₋₂-aryl, or substituted or unsubstituted -(CH₂)₀₋₂-heteroaryl; and

n is 0, 1, 2 or 3. Alternative values and optional substituents for the variables are as described in the first, second or third embodiment, or any aspect thereof. Optional substituents for the variables further include those substituents described as suitable substituents herein.

[0083] Specific examples of compounds useful in the methods of the invention include any of the compounds of Appendices 1, 1', 2, 2', 3 and 4, or a pharmaceutically acceptable salt thereof, such as isocotoin, or a pharmaceutically acceptable salt thereof.

[0084] Compounds of Structural Formula I are readily obtainable by a person of ordinary skill in the art, as by chemical synthesis or through a commercial source. For example, many of the compounds of Structural Formula I and Appendices 1, 1', 2, 2', 3 and 4 are commercially available, for example, from the vendors indicated in Appendices 1, 1', 2, 2', 3 and 4.

Compositions

[0085] Also provided herein is a pharmaceutical composition comprising a compound disclosed herein (*e.g.*, a compound of any one of Structural Formulas I, II and III; a compound of Appendix 1, 1', 2, 2', 3 or 4; isocotoin), or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier. The compositions can be used in the methods described herein, *e.g.*, to supply a compound described herein, or a pharmaceutically acceptable salt thereof.

[0086] “Pharmaceutically acceptable carrier” refers to a non-toxic carrier or excipient that does not destroy the pharmacological activity of the agent with which it is formulated and is nontoxic when administered in doses sufficient to deliver an effective amount of the agent. Pharmaceutically acceptable carriers that may be used in the compositions described herein include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium

carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.

[0087] Compositions described herein may be administered orally, parenterally (including subcutaneously, intramuscularly, intravenously, intradermally, by inhalation, topically, rectally, nasally and vaginally) or buccally, or via an implanted reservoir. The term “parenteral,” as used herein, includes subcutaneous, intracutaneous, intravenous, intramuscular, intraocular, intravitreal, intra-articular, intra-arterial, intra-synovial, intrasternal, intrathecal, intralesional, intrahepatic, intraperitoneal intralesional and intracranial injection or infusion techniques. In some embodiments, provided compounds or compositions are administrable orally.

[0088] Compositions provided herein can be orally administered in any orally acceptable dosage form including, but not limited to, capsules, tablets, aqueous suspensions, dispersions and solutions. In the case of tablets for oral use, carriers commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried cornstarch. When aqueous suspensions and/or emulsions are required for oral use, the active ingredient can be suspended or dissolved in an oily phase and combined with emulsifying and/or suspending agents. If desired, certain sweetening, flavoring or coloring agents may also be added.

[0089] In some embodiments, an oral formulation is formulated for immediate release or sustained/delayed release.

[0090] Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound is mixed with at least one inert, pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and/or (a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, (b) binders, such as carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidone, sucrose, and acacia, (c) humectants such as glycerol, (d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, (e) solution retarding agents such as paraffin, (f) absorption accelerators such as quaternary ammonium salts, (g) wetting agents, such as acetyl alcohol and glycerol monostearate, (h) absorbents such as kaolin and bentonite clay, and (i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols,

sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets and pills, the dosage form may also comprise buffering agents.

[0091] Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and elixirs. Liquid dosage forms may contain inert diluents commonly used in the art, such as water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol (ethanol), isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, or mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming, and preservative agents.

[0092] Compositions suitable for buccal or sublingual administration include tablets, lozenges and pastilles, wherein the active ingredient is formulated with a carrier such as sugar and acacia, tragacanth, or gelatin and glycerin.

[0093] Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using excipients such as lactose or milk sugar, as well as high molecular weight polyethylene glycols and the like. The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions that can be used include polymeric substances and waxes.

[0094] A compound described herein, or a pharmaceutically acceptable salt thereof, can also be in micro-encapsulated form with one or more excipients, as noted above. In such solid dosage forms, the compound or pharmaceutically acceptable salt can be admixed with at least one inert diluent such as sucrose, lactose or starch. Such dosage forms can also comprise, as is normal practice, additional substances other than inert diluents, *e.g.*, tableting lubricants and other tableting aids such as magnesium stearate and microcrystalline cellulose.

[0095] Compositions for oral administration may be designed to protect the active ingredient against degradation as it passes through the alimentary tract, for example, by an outer coating of the formulation on a tablet or capsule.

[0096] In another embodiment, a compound or pharmaceutically acceptable salt described herein can be provided in an extended (or “delayed” or “sustained”) release composition. This delayed-release composition comprises the compound or pharmaceutically acceptable salt in combination with a delayed-release component. Such a composition allows targeted release of a provided agent into the lower gastrointestinal tract, for example, into the small intestine, the large intestine, the colon and/or the rectum. In certain embodiments, a delayed-release composition further comprises an enteric or pH-dependent coating, such as cellulose acetate phthalates and other phthalates (*e.g.*, polyvinyl acetate phthalate, methacrylates (Eudragits)). Alternatively, the delayed-release composition provides controlled release to the small intestine and/or colon by the provision of pH-sensitive methacrylate coatings, pH-sensitive polymeric microspheres, or polymers which undergo degradation by hydrolysis. The delayed-release composition can be formulated with hydrophobic or gelling excipients or coatings. Colonic delivery can further be provided by coatings which are digested by bacterial enzymes such as amylose or pectin, by pH-dependent polymers, by hydrogel plugs swelling with time (Pulsincap), by time-dependent hydrogel coatings and/or by acrylic acid-linked-to-azoaromatic bonds coatings.

[0097] Compositions described herein can also be administered subcutaneously, intraperitoneally or intravenously. Compositions described herein for intravenous, subcutaneous, or intraperitoneal injection may contain an isotonic vehicle such as sodium chloride injection, Ringer’s injection, dextrose injection, dextrose and sodium chloride injection, lactated Ringer’s injection, or other vehicles known in the art.

[0098] Compositions described herein can also be administered in the form of suppositories for rectal administration. These can be prepared by mixing a compound or pharmaceutically acceptable salt described herein with a suitable non-irritating excipient that is solid at room temperature but liquid at rectal temperature and, therefore, will melt in the rectum to release the drug. Such materials include cocoa butter, beeswax and polyethylene glycols.

[0099] Compositions described herein can also be administered topically, especially when the target of treatment includes areas or organs readily accessible by topical application, including diseases of the eye, the skin, or the lower intestinal tract. Suitable topical formulations are readily prepared for each of these areas or organs.

[00100] Topical application for the lower intestinal tract can be effected in a rectal suppository formulation (see above) or in a suitable enema formulation. Topically-transdermal patches can also be used.

[00101] For other topical applications, the compositions can be formulated in a suitable ointment containing the active component suspended or dissolved in one or more carriers. Carriers for topical administration include, but are not limited to, mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene, polyoxypropylene compound, emulsifying wax and water and penetration enhancers. Alternatively, compositions can be formulated in a suitable lotion or cream containing the active agent suspended or dissolved in one or more pharmaceutically acceptable carriers. Alternatively, the composition can be formulated with a suitable lotion or cream containing the active agent suspended or dissolved in a carrier with suitable emulsifying agents. In some embodiments, suitable carriers include, but are not limited to, mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water. In other embodiments, suitable carriers include, but are not limited to, penetration enhancers.

[00102] For ophthalmic use, compositions can be formulated as micronized suspensions in isotonic, pH adjusted sterile saline, or, preferably, as solutions in isotonic, pH adjusted sterile saline, either with or without a preservative such as benzylalkonium chloride. Alternatively, for ophthalmic use, the compositions can be formulated in an ointment such as petrolatum.

[00103] Compositions can also be administered by nasal aerosol or inhalation, for example, for the treatment of asthma. Such compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and can be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other conventional solubilizing or dispersing agents. Without wishing to be bound by any particular theory, it is believed that local delivery of a composition described herein, as can be achieved by nasal aerosol or inhalation, for example, can reduce the risk of systemic consequences of the composition.

[00104] The amount of a compound described herein, or a pharmaceutically acceptable salt thereof, that can be combined with the carrier materials to produce a composition in a single dosage form will vary depending upon the host treated, the particular mode of administration and the activity of the agent employed. Preferably, compositions should be formulated so that a dosage of from about 0.01 mg/kg to about 100 mg/kg body weight/day

of the compound, or pharmaceutically acceptable salt thereof, can be administered to a subject receiving the composition.

[00105] The desired dose may conveniently be administered in a single dose or as multiple doses administered at appropriate intervals such that, for example, the agent is administered 1, 2, 3, 4, 5, 6 or more times per day. The daily dose can be divided, especially when relatively large amounts are administered, or as deemed appropriate, into several, for example 2, 3, 4, 5, 6 or more, administrations.

[00106] It should also be understood that a specific dosage and treatment regimen for any particular patient will depend upon a variety of factors, including the activity of the specific agent employed, the age, body weight, general health, sex, diet, time of administration, rate of excretion, drug combination, the judgment of the treating physician and the severity of the particular disease being treated. The amount of a compound or pharmaceutically acceptable salt in the composition will also depend upon the particular compound or pharmaceutically acceptable salt in the composition.

[00107] Other pharmaceutically acceptable carriers, adjuvants and vehicles that can be used in the compositions of this invention include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, self-emulsifying drug delivery systems (SEDDS) such as d- α -tocopherol polyethylene glycol 1000 succinate, surfactants used in pharmaceutical dosage forms such as Tweens or other similar polymeric delivery matrices, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat. Cyclodextrins such as α -, β -, and γ -cyclodextrin, or chemically modified derivatives such as hydroxyalkylcyclodextrins, including 2- and 3-hydroxypropyl- β -cyclodextrins, or other solubilized derivatives can also be advantageously used to enhance delivery of agents described herein.

[00108] The compositions can be in the form of a sterile injectable preparation, for example, as a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to techniques known in the art using suitable dispersing or wetting agents (such as, for example, Tween 80) and suspending agents. The sterile injectable

preparation can also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are mannitol, water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be employed including synthetic mono- or diglycerides. Fatty acids, such as oleic acid and its glyceride derivatives, are useful in the preparation of injectables, as are natural pharmaceutically acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions can also contain a long-chain alcohol diluent or dispersant, or carboxymethyl cellulose or similar dispersing agents which are commonly used in the formulation of pharmaceutically acceptable dosage forms such as emulsions and or suspensions. Other commonly used surfactants such as Tweens or Spans and/or other similar emulsifying agents or bioavailability enhancers which are commonly used in the manufacture of pharmaceutically acceptable solid, liquid, or other dosage forms can also be used for the purposes of formulation.

[00109] In some embodiments, compositions comprising a compound described herein, or a pharmaceutically acceptable salt thereof, can also include one or more other therapeutic agents (*e.g.*, one or more other anti-viral agents, such as ribavirin), *e.g.*, in combination.

[00110] Also provided herein is a kit comprising a compound described herein, or a pharmaceutically acceptable salt thereof, and an additional therapeutic agent(s) (*e.g.*, an additional anti-viral agent, such as ribavirin). In one embodiment, the kit comprises an effective amount of a compound described herein, or a pharmaceutically acceptable salt thereof, to treat a disease, disorder or condition described herein, and an effective amount of an additional therapeutic agent(s) to treat the disease, disorder or condition. In some embodiments, the kit further comprises written instructions for administering the compound, or a pharmaceutically acceptable salt thereof, and the additional agent(s) to a subject to treat a disease, disorder or condition described herein.

[00111] The compositions described herein can, for example, be administered by injection, intravenously, intraarterially, intraocularly, intravitreally, subdermally, orally, buccally, nasally, transmucosally, topically, in an ophthalmic preparation, or by inhalation, with a dosage ranging from about 0.5 mg/kg to about 100 mg/kg of body weight or, alternatively, in a dosage ranging from about 1 mg/dose to about 1000 mg/dose, every 4 to 120 hours, or according to the requirements of the particular agent.

[00112] Typically, the compositions will be administered from about 1 to about 6 times per day or, alternatively, as a continuous infusion. The amount of active ingredient that can be combined with a carrier material to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. A typical preparation will contain from about 1% to about 99%, *e.g.*, from about 5% to about 95%, from about 1% to about 75%, from about 1% to about 50%, from about 1% to about 40%, from about 1% to about 30%, from about 1% to about 25%, from about 1% to about 20%, from about 5% to about 75%, from about 5% to about 50%, from about 5% to about 40%, from about 5% to about 30%, from about 5% to about 25%, from about 10% to about 75%, from about 10% to about 50%, from about 10% to about 40%, from about 10% to about 30% or from about 10% to about 25%, active compound (w/w). Alternatively, a preparation can contain from about 20% to about 80% active compound (w/w).

[00113] Doses lower or higher than those recited above may be required. Specific dosage and treatment regimens for any particular patient will depend upon a variety of factors, including the activity of the specific agent employed, the age, body weight, general health status, sex, diet, time of administration, rate of excretion, drug combination, the severity and course of the disease, condition or symptoms, the patient's disposition to the disease, condition or symptoms, and the judgment of the treating physician.

Methods

[00114] One embodiment provides a method of inhibiting replication of a virus (*e.g.*, a positive-sense, single-stranded RNA virus; a flavivirus; a HEV), comprising contacting a cell infected with the virus (*e.g.*, one or more viral particles) with a compound disclosed herein (*e.g.*, a compound represented by Structural Formula (I), (II) or (III); a compound of Appendix 1, 1', 2, 2', 3 or 4; isocotoin), or a pharmaceutically acceptable salt thereof. In some aspects, the virus is a positive-sense, single-stranded RNA virus. In some aspects, the virus is an HEV. In some aspects, the virus is an HCV. In some aspects, the virus is a yellow fever virus. In some aspects, the method is performed *in vitro*. In some aspects, the method is performed *ex vivo*. In some aspects, the method is performed *in vivo* as, for example, when the cell is in a subject (*e.g.*, a patient). In some aspects, the cell is a hepatocyte (*e.g.*, a Huh7 cell), a gut epithelial cell or a central nervous system (CNS) cell. It has been shown that gut epithelial cells can be infected *in vitro* with HEV. *See* Marion, O., *et al.* "Hepatitis E virus replication in human intestinal cells," *Gut* 2020 May; 69(5):901-910, the entire contents of which are incorporated herein by reference. However, HEV's cellular tropism may also span

into CNS cells. *See*, for example, Shi, R., *et al.*, “Evidence of Hepatitis E virus breaking through the blood-brain barrier and replicating in the central nervous system,” *J. Viral. Hepat.* 2016; 23(11):930-939, the entire contents of which are incorporated herein by reference.

[00115] A positive-sense, single-stranded RNA virus is a virus whose genetic material consists of positive-sense, single-stranded RNA. Positive-sense, single-stranded RNA viruses include the hepatitis (*e.g.*, hepatitis C, hepatitis E) virus, flaviviruses (*e.g.*, yellow fever virus, West Nile virus, Dengue virus, Zika virus,), rhinoviruses and coronaviruses (*e.g.*, severe acute respiratory syndrome (SARS) coronavirus, Middle East respiratory syndrome-related (MERS) coronavirus).

[00116] HEV is a positive-sense single-stranded RNA virus of the *Hepeviridae* family measuring approximately 7.2kB in length. The virus contains three open reading frames (ORFs), of which ORF1 is the largest (approximately 5,100 base pairs), and encodes a number of viral proteins including a methyltransferase, putative cysteine protease (PCP) region, RNA helicase, and RNA-dependent RNA polymerase (RdRp) (FIG. 1). These proteins play critical roles in viral gene expression, transcription, and interactions with host proteins. ORF2 of HEV encodes the viral capsid protein, and ORF3 encodes a small protein essential for viral egress. During viral genomic replication, an intermediate, negative-sense RNA template is used to transcribe several-fold greater amounts of positive-sense RNA strands.

[00117] The strains of HEV affecting humans fall under the genus *Orthohepeviridae* and are primarily transmitted through contaminated drinking water or the consumption of infected pork, venison, or wild boar meat. HEV infection most commonly manifests as self-limiting, acute hepatitis in healthy individuals, typically lasting for a month. However, pregnant women and immunocompromised individuals experience more severe symptoms. Pregnant women have up to a 30% mortality rate in the third trimester from HEV infection, particularly from genotype 1 strains of the virus. Immunocompromised individuals exposed to HEV can develop chronic infection, leading to the rapid progression of liver cirrhosis in as little as two years.

[00118] Another embodiment provides a method of treating a viral infection (*e.g.*, an HEV infection) in a subject in need thereof, comprising administering to the subject an effective amount of a compound disclosed herein (*e.g.*, a compound represented by Structural Formula (I), (II) or (III); a compound of Appendix 1, 1', 2, 2', 3 or 4; isocotoin), or a pharmaceutically

acceptable salt thereof. In some aspects, the viral infection is caused by a positive-sense, single-stranded RNA virus (*e.g.*, a flavivirus). In some aspects, the viral infection is caused by an HEV. In some aspects, the viral infection is caused by an HCV. In some aspects, the viral infection is caused by a yellow fever virus. In some aspects, the viral infection is an acute viral infection (*e.g.*, acute HEV infection). In some aspects, the viral infection is a chronic viral infection (*e.g.*, chronic HEV infection).

[00119] “Treating,” as used herein, refers to taking steps to deliver a therapy to a subject, such as a human, in need thereof. “Treating” includes inhibiting the disease or condition (*e.g.*, as by slowing or stopping its progression or causing regression of the disease or condition), and/or relieving the symptoms resulting from the disease or condition.

[00120] As used herein, “subject” includes humans, domestic animals, such as laboratory animals (*e.g.*, dogs, monkeys, pigs, rats, mice, *etc.*), household pets (*e.g.*, cats, dogs, rabbits, *etc.*) and livestock (*e.g.*, pigs, cattle, sheep, goats, horses, *etc.*), and non-domestic animals. In some embodiments, a subject is a human.

[00121] A subject is “in need of” a treatment if such subject would benefit from such treatment (*e.g.*, biologically, medically or in quality of life).

[00122] “Patient” refers to a human subject.

[00123] In some embodiments, a subject is immunocompromised. In some embodiments, a subject is pregnant.

[00124] “An effective amount” is an amount effective, at dosages and for periods of time necessary, to achieve a desired therapeutic result (*e.g.*, treatment, healing, inhibition or amelioration of physiological response or condition, *etc.*). The full therapeutic effect does not necessarily occur by administration of one dose, and may occur only after administration of a series of doses. Thus, an effective amount may be administered in one or more administrations. An effective amount may vary according to factors such as disease state, age, sex, and weight of a subject, mode of administration and the ability of a therapeutic, or combination of therapeutics, to elicit a desired response in a subject. An effective amount of an agent to be administered can be determined by a clinician of ordinary skill using the guidance provided herein and other methods known in the art.

[00125] Suitable dosages can be from about 0.001 mg/kg to about 100 mg/kg, from about 0.01 mg/kg to about 100 mg/kg, from about 0.01 mg/kg to about 10 mg/kg, from about 0.01 mg/kg to about 1 mg/kg body weight per treatment, for example, administered one, two, three, four, five or six, preferably, one, two or three, times per day. Determining the dosage

for a particular agent, subject and disease is well within the abilities of one of skill in the art. Preferably, the dosage does not cause or produces minimal adverse side effects.

[00126] A compound described herein, or a pharmaceutically acceptable salt thereof, or a composition described herein can be administered via a variety of routes of administration, including, for example, oral, dietary, topical, transdermal, rectal, parenteral (*e.g.*, intra-arterial, intravenous, intramuscular, subcutaneous injection, intradermal injection), intravenous infusion and inhalation (*e.g.*, intrabronchial, intranasal or oral inhalation, intranasal drops) routes of administration, depending on the compound and the particular disease to be treated. Administration can be local or systemic as indicated. The preferred mode of administration can vary depending on the particular compound, pharmaceutically acceptable salt or composition chosen.

[00127] Also provided herein is a method of inhibiting heat shock protein 90 in a cell, comprising contacting the cell with a compound disclosed herein (*e.g.*, a compound represented by Structural Formula (I), (II) or (III); a compound of Appendix 1, 1', 2, 2', 3 or 4; isocotoin), or a pharmaceutically acceptable salt thereof. In some aspects, the method is performed *in vitro*. In some aspects, the method is performed *ex vivo*. In some aspects, the method is performed *in vivo* as, for example, when the cell is in a subject (*e.g.*, a patient). In some aspects, the cell is a hepatocyte (*e.g.*, a Huh7 cell).

[00128] Also provided herein is a method of treating a heat shock protein 90-mediated disease or condition in a subject in need thereof, comprising administering to the subject an effective amount of a compound disclosed herein (*e.g.*, a compound represented by Structural Formula (I), (II) or (III); a compound of Appendix 1, 1', 2, 2', 3 or 4; isocotoin), or a pharmaceutically acceptable salt thereof. In some aspects, the heat shock protein 90-mediated disease or condition is a viral infection, such as any of the viral infections disclosed herein.

[00129] A “heat shock protein 90-mediated disease or condition” is any disease or condition directly or indirectly regulated by heat shock protein 90. Examples of heat shock protein 90-mediated diseases or conditions include cancer (*e.g.*, solid tumors, hematological cancers), neurodegenerative diseases, inflammatory diseases or conditions and viral infections (such as the viral infections disclosed herein).

[00130] Examples of cancer treatable according to the methods described herein include Acute Lymphoblastic Leukemia (ALL); Acute Myeloid Leukemia (AML); Adrenocortical Carcinoma; Adrenocortical Carcinoma, Childhood; AIDS-Related Cancer (*e.g.*, Kaposi

Sarcoma, AIDS-Related Lymphoma, Primary CNS Lymphoma); Anal Cancer; Appendix Cancer; Astrocytomas, Childhood; Atypical Teratoid/Rhabdoid Tumor, Childhood, Central Nervous System; Basal Cell Carcinoma of the Skin; Bile Duct Cancer; Bladder Cancer; Bladder Cancer, Childhood; Bone Cancer (including Ewing Sarcoma, Osteosarcoma and Malignant Fibrous Histiocytoma); Brain Tumors/Cancer; Breast Cancer; Burkitt Lymphoma; Carcinoid Tumor (Gastrointestinal); Carcinoid Tumor, Childhood; Cardiac (Heart) Tumors, Childhood; Embryonal Tumors, Childhood; Germ Cell Tumor, Childhood; Primary CNS Lymphoma; Cervical Cancer; Childhood Cervical Cancer; Cholangiocarcinoma; Chordoma, Childhood; Chronic Lymphocytic Leukemia (CLL); Chronic Myelogenous Leukemia (CML); Chronic Myeloproliferative Neoplasms; Colorectal Cancer; Childhood Colorectal Cancer; Craniopharyngioma, Childhood; Cutaneous T-Cell Lymphoma (*e.g.*, Mycosis Fungoides and Sézary Syndrome); Ductal Carcinoma In Situ (DCIS); Embryonal Tumors, Central Nervous System, Childhood; Endometrial Cancer (Uterine Cancer); Ependymoma, Childhood; Esophageal Cancer; Childhood Esophageal Cancer; Esthesioneuroblastoma; Ewing Sarcoma; Extracranial Germ Cell Tumor, Childhood; Extragenital Germ Cell Tumor; Eye Cancer; Childhood Intraocular Melanoma; Intraocular Melanoma; Retinoblastoma; Fallopian Tube Cancer; Fibrous Histiocytoma of Bone, Malignant, and Osteosarcoma; Gallbladder Cancer; Gastric (Stomach) Cancer; Childhood Gastric (Stomach) Cancer; Gastrointestinal Carcinoid Tumor; Gastrointestinal Stromal Tumors (GIST); Childhood Gastrointestinal Stromal Tumors; Germ Cell Tumors; Childhood Central Nervous System Germ Cell Tumors (*e.g.*, Childhood Extracranial Germ Cell Tumors, Extragenital Germ Cell Tumors, Ovarian Germ Cell Tumors, Testicular Cancer); Gestational Trophoblastic Disease; Hairy Cell Leukemia; Head and Neck Cancer; Heart Tumors, Childhood; Hepatocellular (Liver) Cancer; Histiocytosis, Langerhans Cell; Hodgkin Lymphoma; Hypopharyngeal Cancer; Intraocular Melanoma; Childhood Intraocular Melanoma; Islet Cell Tumors, Pancreatic Neuroendocrine Tumors; Kaposi Sarcoma; Kidney (Renal Cell) Cancer; Langerhans Cell Histiocytosis; Laryngeal Cancer; Leukemia; Lip and Oral Cavity Cancer; Liver Cancer; Lung Cancer (Non-Small Cell and Small Cell); Childhood Lung Cancer; Lymphoma; Male Breast Cancer; Malignant Fibrous Histiocytoma of Bone and Osteosarcoma; Melanoma; Childhood Melanoma; Melanoma, Intraocular (Eye); Childhood Intraocular Melanoma; Merkel Cell Carcinoma; Mesothelioma, Malignant; Childhood Mesothelioma; Metastatic Cancer; Metastatic Squamous Neck Cancer with Occult Primary; Midline Tract Carcinoma With NUT Gene Changes; Mouth Cancer; Multiple Endocrine

Neoplasia Syndromes; Multiple Myeloma/Plasma Cell Neoplasms; Mycosis Fungoides; Myelodysplastic Syndromes, Myelodysplastic/Myeloproliferative Neoplasms; Myelogenous Leukemia, Chronic (CML); Myeloid Leukemia, Acute (AML); Myeloproliferative Neoplasms, Chronic; Nasal Cavity and Paranasal Sinus Cancer; Nasopharyngeal Cancer; Neuroblastoma; Non-Hodgkin Lymphoma; Non-Small Cell Lung Cancer; Oral Cancer, Lip and Oral Cavity Cancer and Oropharyngeal Cancer; Osteosarcoma and Malignant Fibrous Histiocytoma of Bone; Ovarian Cancer; Childhood Ovarian Cancer; Pancreatic Cancer; Childhood Pancreatic Cancer; Pancreatic Neuroendocrine Tumors; Papillomatosis (Childhood Laryngeal); Paraganglioma; Childhood Paraganglioma; Paranasal Sinus and Nasal Cavity Cancer; Parathyroid Cancer; Penile Cancer; Pharyngeal Cancer; Pheochromocytoma; Childhood Pheochromocytoma; Pituitary Tumor; Plasma Cell Neoplasm/Multiple Myeloma; Pleuropulmonary Blastoma; Pregnancy and Breast Cancer; Primary Central Nervous System (CNS) Lymphoma; Primary Peritoneal Cancer; Prostate Cancer; Rectal Cancer; Recurrent Cancer; Renal Cell (Kidney) Cancer; Retinoblastoma; Rhabdomyosarcoma, Childhood; Salivary Gland Cancer; Sarcoma (*e.g.*, Childhood Rhabdomyosarcoma, Childhood Vascular Tumors, Ewing Sarcoma, Kaposi Sarcoma, Osteosarcoma (Bone Cancer), Soft Tissue Sarcoma, Uterine Sarcoma); Sézary Syndrome; Skin Cancer; Childhood Skin Cancer; Small Cell Lung Cancer; Small Intestine Cancer; Soft Tissue Sarcoma; Squamous Cell Carcinoma of the Skin; Squamous Neck Cancer with Occult Primary, Metastatic; Stomach (Gastric) Cancer; Childhood Stomach (Gastric) Cancer; T-Cell Lymphoma, Cutaneous (*e.g.*, Mycosis Fungoides and Sézary Syndrome); Testicular Cancer; Childhood Testicular Cancer; Throat Cancer (*e.g.*, Nasopharyngeal Cancer, Oropharyngeal Cancer, Hypopharyngeal Cancer); Thymoma and Thymic Carcinoma; Thyroid Cancer; Transitional Cell Cancer of the Renal Pelvis and Ureter; Ureter and Renal Pelvis, Transitional Cell Cancer; Urethral Cancer; Uterine Cancer, Endometrial; Uterine Sarcoma; Vaginal Cancer; Childhood Vaginal Cancer; Vascular Tumors; Vulvar Cancer; and Wilms Tumor and Other Childhood Kidney Tumors.

[00131] Metastases of the aforementioned cancers can also be treated in accordance with the methods described herein. In some embodiments, the cancer is a metastatic cancer.

[00132] Examples of neurodegenerative diseases treatable according to the methods described herein include Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, prion disease, spinocerebellar ataxia, spinal muscular atrophy and motor neuron disease.

[00133] Examples of inflammatory diseases or conditions treatable according to the methods described herein include multiple sclerosis, Goodpasture syndrome, psoriasis, ankylosing spondylitis, antiphospholipid antibody syndrome, gout, arthritis (*e.g.*, rheumatoid arthritis), myositis, scleroderma, Sjogren's syndrome, systemic lupus erythematosus and vasculitis.

[00134] In an aspect of any of the methods disclosed herein, the compound is not AUY-922, VER-50589, STA-9090 or 17-AAG (*e.g.*, AUY-922, VER-50589 or STA-9090), or a pharmaceutically acceptable salt of any of the foregoing.

[00135] A compound described herein, or a pharmaceutically acceptable salt thereof, can also be administered in combination with one or more other therapies (*e.g.*, additional anti-viral agent(s), such as ribavirin). When administered in a combination therapy, the compound, or pharmaceutically acceptable salt thereof, can be administered before, after or concurrently with the other therapy (*e.g.*, additional anti-viral agent(s)). When co-administered simultaneously (*e.g.*, concurrently), the compound, or pharmaceutically acceptable salt thereof, and other therapy can be in separate formulations or the same formulation. Alternatively, the compound, or pharmaceutically acceptable salt thereof, and other therapy can be administered sequentially, *e.g.*, as separate compositions, within an appropriate time frame as determined by a skilled clinician (*e.g.*, a time sufficient to allow an overlap of the pharmaceutical effects of the therapies).

[00136] In some embodiments, a method described herein further comprises contacting the cell with an additional therapeutic agent (*e.g.*, an additional anti-viral agent, such as ribavirin).

[00137] In some embodiments, a method described herein further comprises administering to the subject an effective amount of an additional therapeutic agent (*e.g.*, an additional anti-viral agent, such as ribavirin).

EXEMPLIFICATION

[00138] Treatment options for HEV infection are limited. In patients who develop chronic hepatitis E while taking immunosuppressive drugs (*e.g.*, after receiving organ transplantation), a reduction in the immunosuppressive regimen is first attempted. This results in clearance of the virus in one-third of patients. When this is not successful, patients are typically treated with ribavirin, a nucleoside analog and broad-spectrum antiviral, and/or pegylated IFN- α (pegIFN- α). pegIFN- α is less commonly used, since it is associated with severe side effects and can lead to transplant rejection in organ transplant recipients.

Ribavirin monotherapy is 78% effective in clearing chronic HEV infection; however, it is highly teratogenic and cannot be used in pregnant patients. Furthermore, HEV strains with “fitness-enhancing” mutations have been identified in patients showing clinical resistance to ribavirin treatment. There are currently no other clinically approved drugs for hepatitis E.

[00139] Methods and compositions for inhibiting HEV are disclosed in International Publication No. WO 2018/057773, the entire contents of which are incorporated herein by reference.

Replicon-based high-throughput screening assay to identify compounds inhibiting HEV gene expression, transcription, (proteolytic processing), genomic replication

[00140] To identify small molecules with antiviral activity against HEV, proteins in ORF1 were targeted¹⁴⁻¹⁷. A replicon-based screening assay was used in which ORFs 2 and 3 of HEV, which encode the capsid protein and an ion channel required for viral egress respectively, were replaced in the viral genome with blasticidin resistance-conferring and ZsGreen fluorescent reporters (FIGs. 2A-2C)^{18,19}. Approximately 60,000 small molecules from the Princeton University Small Molecule Screening Center were tested against the replicon genome, with decreased fluorescence used as a readout to indicate inhibition of genomic replication (FIGs. 3A-3E).

[00141] Briefly, replicon-expressing cells were seeded in 384-well format and treated with a 50 μ M dose of each compound for four days. A GFP channel image and bright-field image were taken of each well on day 4 using a Perkin Elmer Operetta High-Content Imaging System. Fluorescence levels in the GFP channel images were quantified using a custom Python script (publicly sourced at: <https://github.com/aploss/PLOCUS>), and decreased fluorescence was used as a metric to select hits with putative antiviral activity. The bright-field images were used to remove false positive hits that showed decreased fluorescence due to cytotoxicity, resulting in 37 non-cytotoxic hits from the high-throughput assay. Through dose titration assays on the hits, isocotoin was identified as a promising therapeutic candidate against HEV replication.

Isocotoin inhibits genetically diverse HEV genotypes and other (+)-sense RNA viruses.

[00142] Through successive rounds of screening, isocotoin was identified as a promising therapeutic candidate against HEV (FIG. 4A). *In vitro* functional assays were performed using a secreted Gaussia luciferase (Gluc)-expressing HEV replicon derived from the KernowC1p6 HEV strain, abbreviated p6/Gluc (HEV Δ ORF2/3[Gluc]) (FIG. 4B), and supernatant Gluc levels were measured as a proxy for viral replication²⁰. Dose titration

assays, in which Huh7 hepatoma cells were transfected with p6/Gluc (HEV Δ ORF2/3[Gluc]) *in vitro* transcribed RNA and treated with varying doses of ribavirin or isocotoin for 4 days, revealed that isocotoin exhibits an IC₅₀ value of 6.1 μ M as compared to 12.8 μ M for ribavirin, the only currently available treatment for HEV infection in immunocompromised patients (FIG. 4C). RT-qPCR assays confirmed that isocotoin also inhibits the full-length KernowC1p6 in a dose-dependent manner (FIG. 4D).

[00143] To test whether isocotoin was simply inhibiting generalized protein translation, a reporter construct was created. The reporter construct, T7-tagBFP-Gluc, contained Gluc and the fluorescent protein tagBFP, and could be *in vitro*-transcribed from a T7 promoter, but was devoid of any HEV-derived viral proteins (FIG. 4E). Capped tagBFP-Gluc RNA was transfected into cells that were then treated with varying doses of isocotoin for 4 days. Known translation inhibitors cycloheximide and roclagamide, an analogue of silvestrol²¹, and ribavirin (RBV), which is a nucleoside analog that does not affect translation²², were included as controls. The results indicated that whereas cycloheximide and roclagamide suppress Gluc levels ~10-fold, isocotoin did not lead to a significant decrease in Gluc levels as compared to ribavirin (FIG. 4F).

[00144] To ensure that cytotoxicity did not account for the observed reductions in replication, an ATP-based cell viability assay was performed on Huh7 cells after treatment with isocotoin or ribavirin. The results indicated that isocotoin displayed cytotoxicity at doses 25 μ M and above, but not at doses up to 12.5 μ M (FIG. 4G). Since isocotoin exhibits antiviral effects at doses lower than 12.5 μ M (its IC₅₀ value is 6.1 μ M), the antiviral effects at these lower doses cannot be attributed to cytotoxicity. Collectively, these data suggest that isocotoin specifically interferes with HEV replication in contrast with silvestrol, which was previously shown to inhibit HEV through general suppression of protein translation²¹.

Testing isocotoin against diverse HEV genotypes

[00145] Next, it was determined whether isocotoin exhibited activity against genetically diverse HEV genotypes. At least eight genotypes of HEV (genotypes 1-8) have been identified in mammals, with genotypes 1-4 accounting for the majority of reported infections in humans (FIG. 5A)²³. cDNAs are only currently available for genotypes 1, 3, and 4, and, very recently, 5. Of these, the KernowC1p6 (genotype 3) and Sar55 (genotype 1) strains are known to replicate robustly in cell culture systems, and are frequently used for *in vitro* studies. To evaluate its pan-genotypic antiviral efficacy, isocotoin was tested against Gluc-expressing replicons from the Sar55 (genotype 1), SHEV-3 (genotype 3, swine-derived), and

TW6196E (genotype 4) HEV strains in addition to p6/Gluc (KernowC1p6/Gluc) (named Sar55/Gluc, SHEV-3/Gluc, and TW6196E/Gluc, respectively) (FIG. 5B)²¹. Dose titration experiments against Sar55/Gluc and p6/Gluc (KernowC1p6/Gluc) demonstrated that isocotoin was effective against both strains and had a lower IC₅₀ than ribavirin (FIGs. 5C, 5D, 5G). For SHEV-3 and TW6196E, replicative capacity in cell culture is much lower, but a modest inhibitory effect was observed with isocotoin (these assays display large error due to poor replicative capacity) (FIGs. 5E, 5F).

Testing isocotoin against other positive-sense RNA viruses

[00146] To examine whether the antiviral properties of isocotoin were specific to HEV or broadly effective against other viruses, isocotoin was tested against other positive-sense, single-stranded RNA viruses, including Gluc-expressing genomes from hepatitis C virus (Jc1(p7nsGluc2A), abbreviated 'HCV') and yellow fever virus 17-D vaccine strain (YFV17D-Gluc-BSD-Ires, abbreviated 'YFV17D')²⁴ (FIGs. 6A, 6C, 6D). Ribavirin, a drug that used to be the standard of care for treating HCV and is still commonly used for treating HEV, and 2'-C-methyladenosine, a specific inhibitor of the HCV NS5B protein, were used as controls (FIG. 6B). While RBV had only minor inhibitory effects on any of the viruses under the experimental conditions used here, isocotoin showed dose-dependent inhibitory effects against all three viruses. Collectively, these data suggest that isocotoin operates through a common inhibitory mechanism against all three viruses tested.

Suboptimal Dosing Experiments To Identify Adaptive Mutations

[00147] To determine isocotoin's mechanism of action, suboptimal dosing experiments were performed in which HEV was passaged in the presence of sublethal doses of isocotoin for a prolonged time. The virus was expected to evolve resistance mutations to isocotoin that could be sequenced and used to identify the drug's target (FIG. 7A). For these experiments, a replicon genome derived from KernowC1p6, in which ORFs 2 and 3 were replaced with a blasticidin resistance-conferring gene (BSR) and a ZsGreen fluorescence reporter, abbreviated p6/BSR-2A-ZsGreen (FIG. 7A), was used. Cells transfected with p6/BSR-2A-ZsGreen were maintained in 30µM isocotoin, 10 µg/mL blasticidin, or 0.15% DMSO (concentration of vehicle for isocotoin), and fluorescence levels were measured at each passage with flow cytometry. Though some toxicity was observed at the 30 µM isocotoin dose, cells were still able to grow at a reduced rate and recover from repeated passaging. Naïve Huh7 cells were included as a negative control for fluorescence.

[00148] Isocotoin treatment led to a sharp decrease in ZsGreen levels during the first few passages, with a subsequent increase in ZsGreen levels starting at passage 6 (FIG. 7B). By passage 9, ZsGreen levels approached the levels seen in the positive control (cells under selection with blasticidin) (FIG. 7B). Intracellular RNA was extracted from both passage 1 and passage 10 cell lysates and reverse transcribed to produce cDNA. Overlapping segments covering ORF1 were PCR-amplified, sequenced, and aligned to identify any regions that had mutated from passage 1 to passage 10. Although no dominant mutation was found, a highly conserved palindromic 'FCCF' peptide sequence located at residues 470-473 in the putative cysteine protease region was found to contain a phenylalanine to serine point mutation in 30% of the clones (FIG. 7C). The F470S and F473S single point mutations were inserted into the wild-type p6/Gluc replicon (HEVΔORF2/3[Gluc] genome), and it was observed that p6/Gluc[F470S] (HEVΔORF2/3[Gluc][F470S]) exhibited a higher replicative capacity *in vitro* than either the wild-type p6/Gluc strain (parental HEVΔORF2/3[Gluc]) or the p6/Gluc[F473S] (HEVΔORF2/3[Gluc][F473S]) strain, which was replication-inhibited (data not shown). These data suggested that the emergence of the F470S point mutation in passage 10 cells may have been due to the enhanced replicative capacity of this strain, and not isocotoin resistance.

Isocotoin exhibits inhibitory activity against HEV strains harboring mutations associated with clinical resistance to ribavirin.

[00149] Emergence of ribavirin-resistant strain poses a significant problem for the treatment of hepatitis E. Previous studies characterizing clinically ribavirin-resistant strains *in vitro* had identified that G1634R and Y1320H point mutations in the RdRp region of ORF1 similarly led to enhanced replicative capacity *in vitro*^{9,25}. p6/Gluc[Y1320H] (HEVΔORF2/3[Gluc][Y1320H]) and p6/Gluc[G1634R] (HEVΔORF2/3[Gluc][G1634R]) mutant replicons were generated to compare their replicative capacities to that of the newly identified p6/Gluc[F470S] (HEVΔORF2/3[Gluc][F470S]) (FIG. 7E). Measurement of supernatant Gluc over 4 days post-transfection in Huh7 cells revealed that p6/Gluc[F470S] (HEVΔORF2/3[Gluc][F470S]) exhibited a higher replicative capacity than both p6/Gluc[Y1320H] (HEVΔORF2/3[Gluc][Y1320H]) and p6/Gluc[G1634R] (HEVΔORF2/3[Gluc][G1634R]) (FIG. 7D). These results suggest that the F470S mutation could potentially lead to clinical ribavirin resistance through a similar mechanism as the previously identified mutant strains. All three higher-replicating strains exhibited a higher sensitivity to isocotoin than to ribavirin (FIGs. 7F, 7G).

Structure Activity Relationship Analysis reveals compounds structurally analogous to isocotoin with higher potency against HEV replication

[00150] To identify the specific functional groups within isocotoin mediating its biological activity, two rounds of structure-activity relationship (SAR) analysis were performed in which structural analogs to isocotoin were evaluated for efficacy against p6/Gluc. In these experiments, dose titration assays were performed against Huh7 cells transfected with p6/Gluc *in vitro* transcribed RNA. In the first round of SAR, a chemical space was identified that led to greater inhibition of HEV replication than the parent compound isocotoin (Appendices 1, 1'). In the second round of SAR, a set of compounds were chosen that explored further modifications within this chemical space that could further improve potency (Appendices 2, 2').

[00151] During the first round of SAR analysis, a compound was identified – IBS102 – that led to an approximately 10-fold greater decrease in RLU than isocotoin at the 25 μ M dose (FIG. 8A). During the second round of SAR analysis, the compound IBS672 was found to lead to an approximately 40-fold greater decrease in RLU than isocotoin at 1.5625 μ M (FIG. 8B).

Isocotoin directly binds heat shock protein 90 (HSP90)

[00152] Thermal proteome profiling was performed to identify direct binding targets of isocotoin (FIG. 11A). Briefly, this assay identifies proteins whose stability changes in the presence of the drug due to binding interactions with the drug.

[00153] Thirty-one targets were identified exhibiting thermal stabilization in the presence of isocotoin. The hits included Hsp90 α 1 and Hsp90 β , two Hsp90 isoforms known to play broad pro-viral roles (FIG. 11B)²⁶. HSP90 are a family of conserved, abundant, and constitutively expressed molecular chaperones assisting in the maturation and localization of hundreds of cellular proteins. A diverse array of viruses, including herpes simplex viruses, simian virus 40, and HCV, rely on Hsp90 for critical functions including virus internalization, localization, and complex assembly^{28–31}. Furthermore, many viruses are hyper-dependent on Hsp90 function, such that Hsp90 inhibition disproportionately impairs the viral life cycle as compared to regular host functions²⁶. Hsp90 is thought to bind the HEV capsid protein for intracellular trafficking of the virus during the early stages of infection. However, since the assay described herein employed a reporter genome lacking the capsid protein gene (ORF2), it was hypothesized that Hsp90 may play an additional, yet uncharacterized role specific to HEV genomic replication³².

Hsp90 is an essential host factor for HEV replication

[00154] Hsp90 inhibitors, AUY-922 and VER-50589, STA-9090 and 17-AAG, were tested against HEV Δ ORF2/3[Gluc] replication (FIG. 11C). All four compounds inhibited HEV replication, confirming the essential role of HSP90 in viral replication (FIG. 11C). Unlike isocotoin however, the four compounds exhibited cytostatic properties inhibiting cell growth, and therefore may not be ideal therapeutic candidates for the treatment of hepatitis E (FIGs. 12A, 12B).

[00155] To further corroborate these data, an siRNA-mediated knockdown of Hsp90AA1 and Hsp90AB1 was performed to examine the effect on viral replication. Hsp90 protein levels were reduced 69%, and mRNA levels were reduced 54% for Hsp90AA1 and 74% for Hsp90AB1 post-siRNA transfection (FIGs. 11D, 11E). Seed sequence-matched negative control siRNAs did not demonstrate knockdown of Hsp90 protein or mRNA (FIGs. 11D, 11E). Hsp90 knockdown reduced viral replication by 51% compared to cells treated with transfection reagent only ("mock"), and by 36% compared to cells treated with seed sequence-matched negative control siRNA (FIG. 11F). The incomplete inhibition of viral replication is likely due to incomplete knockdown of HSP90, as demonstrated by Western blot.

In vivo functional validation of isocotoin

[00156] HEV can infect a wide range of hosts including primates, swine, deer, and rabbits. Mice are a small and tractable animal model; however, they are not naturally susceptible to HEV infection. Therefore, to determine whether isocotoin could exert an inhibitory effect against HEV *in vivo*, a human liver chimeric mouse model, which has previously been shown to be susceptible to HEV, was used. Given that most compounds identified in primary screening assays require extensive pharmacokinetic characterization and chemical modification before translation to an *in vivo* setting, immediate success of the compound in its current form when tested in mice was not expected.

[00157] To generate human liver chimeric mice, female fumaryl acetoacetate hydrolase knockout (*fah*^{-/-}), non-obese diabetic (NOD) *recombinase activating gene 1* deficient (*rag1*^{-/-}) *interleukin 2 receptor gamma chain deficient* (*il2rg*^{NULL}) (FNRG) mice were injected intrasplenically with commercially obtained human adult hepatocytes. Highly engrafted animals (human albumin concentration in the serum >2.5 mg/ml) were injected intravenously with stool filtrate from HEV-infected rhesus macaques, and viral titers were monitored in the stool and serum. Consistent with previous reports, the animals did not show

any symptoms of illness during the course of infection, maintaining stable weight throughout (FIG. 15B).

[00158] To test whether isocotoin could lower viral levels in the mice, six mice were drug-treated for 7 days, at a 50mg/kg dose, injected daily intraperitoneally. No symptoms of drug toxicity were observed in the mice at any point during the treatment. Two infected mice were maintained as untreated controls and were injected with vehicle only. Stool pellets were collected from the mice daily, and blood was drawn pre- and post-treatment. At the end of the treatment period, the mice were euthanized, and their liver tissue was harvested.

[00159] Based on analysis of viral RNA levels in stool and serum pre- and post-treatment, there was not a clear decrease in viral levels from isocotoin treatment (FIGs. 15D and 15E). However, this work is preliminary, and follow-up studies are needed to understand whether the drug is actually reaching the liver in its active form, the pharmacokinetic profile of the drug, and the optimal duration and dose of treatment.

Discussion

[00160] This study provides an important step forward in developing therapeutics against HEV infection, where there is a dire need for new treatments, especially for vulnerable patient populations such as immunocompromised individuals and pregnant women. A robust high-throughput screening platform was developed, and used to assess small molecules for inhibitory activity against HEV replication, and isocotoin was identified as a candidate using this assay. Isocotoin was shown to directly bind to two HSP90 proteins, and targeted inhibition or depletion of HSP90 proteins was shown to severely impair HEV replication. Collectively, these data provide the first evidence that HSP90 proteins play an essential role in HEV genomic replication, and suggest that HSP90 inhibitors may provide a novel therapeutic approach for treating hepatitis E. Furthermore, since HSP90 inhibitors employ a distinct mechanism of action from ribavirin, they may be effective in cases of ribavirin resistance, or as combination therapy with ribavirin.

[00161] Isocotoin was identified using the p6/BSR-2A-zsGreen replicon, a reporter genome that can be used for future high-throughput studies. A publicly sourced custom script was also developed to rapidly quantify fluorescence in large image datasets and to select promising candidate wells (<https://github.com/aploss/PLOCUS>). In the screening assay, only one dose (50 μ M) per compound was tested. This dose was chosen based on the *in vitro* efficacy of ribavirin, but as a result, compounds that were effective at lower doses but cytotoxic at the 50 μ M dose would not have been selected in the screening assay. However,

the assay is adaptable to testing compounds at lower doses, or at multiple doses to calculate dose titrations. Therefore, this platform is a tractable tool for future screening assays against HEV replication.

[00162] Two-dimensional thermal proteome profiling (2D-TPP) was used to identify binding targets of isocotoin, towards determining its mechanism of action. The hits identified did not include any HEV viral proteins, which was consistent with the data demonstrating that isocotoin was effective against several different viruses (HCV and YFV-17D) and, therefore, unlikely to be specifically targeting an HEV protein. Though the 2D-TPP measured a relatively modest shift in stability for HSP90 proteins upon addition of isocotoin, protein complexes such as HSP90 typically show smaller thermal shifts from binding interactions due to the initial presence of many pre-existing stabilizing interactions. The assays testing other HSP90 inhibitors against HEV and measuring HEV replication in the presence of HSP90 knockdown confirm the importance of HSP90 for the viral life cycle.

[00163] Using the CellTiterGlo assay, which measures ATP levels as a proxy for viable cells, it was found that isocotoin has a similar cytotoxicity profile *in vitro* to ribavirin. A caveat is that the cytotoxicity assays were performed in hepatoma cell lines, which are more susceptible to HSP90 inhibition than non-tumor derived cell lines. Therefore, cytotoxicity in non-tumor liver cells may actually be lower the results reported herein. Indeed, when mice were treated for seven days with 50mg/kg doses of isocotoin, no obvious toxicity was observed.

[00164] This study also resulted in the discovery of the F470S fitness-enhancing mutation in the putative cysteine protease (PCP) region of p6/Gluc. This mutant strain replicates more robustly *in vitro* than the wild-type p6/Gluc strain and two previously discovered mutant strains with fitness-enhancing mutations (G1634R and Y1320H, both in the RNA-dependent RNA polymerase region of ORF1). The PCP region of HEV, so-named due to sequence similarity with the rubella virus protease, is poorly understood, and its protease activity remains highly controversial. The discovery of a replication-enhancing mutation in this region provides a look into the important functional sites of this enigmatic domain. Due to its robust replicative capacity, the F470S strain may also prove useful for *in vitro* studies that require higher replication levels, such as viral production assays.

[00165] Preliminary *in vivo* studies, in which HEV-infected liver chimeric mice were treated with an arbitrary dose of isocotoin for seven days, did not result in a reduction in viral titers. However, as a compound that has never been used in animal studies, extensive *in vivo*

follow-up studies are necessary to understand isocotoin's pharmacokinetic and pharmacodynamic properties, dosing, and potential adverse effects.

[00166] In conclusion, a tractable screening assay for the identification of molecules inhibiting HEV replication is disclosed, as is the first evidence that HSP90 proteins play an essential role in HEV genomic replication and may be viable therapeutic targets for treating hepatitis E.

Materials and Methods

[00167] *Study design.* The objective of this study was to identify small molecules with antiviral activity against proteins encoded by HEV ORF1. A high-throughput screening platform was developed to test a library of ~60,000 compounds from the Princeton University Small Molecule Screening Center against a fluorescent HEV replicon. The HEV replicon was derived from the pKernowC1p6 construct, kindly provided by Dr. Suzanne Emerson (NIAID). Images were acquired using the Operetta CLS High Content Screening System (PerkinElmer, Waltham, MA) and analyzed using a custom Python script (<https://github.com/aploss/PLOCUS>).

[00168] All follow-up *in vitro* experiments to characterize hits were conducted using Huh7 hepatoma cells, with each condition tested in at least three biological replicate wells. No data were excluded from analyses.

[00169] *Cell lines and culture conditions.* Huh7 cells were obtained from the American Tissue Culture Collection (ATCC). These cells were authenticated and were clear of mycoplasma contamination. All cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher) supplemented with 10% (v/v) fetal bovine serum (FBS) (Omega Scientific), 100 U/mL penicillin, and 100 mg/mL streptomycin (P/S). To generate the replicon cell line used in the high-throughput screening assay, Huh7 cells were transfected with p6/BSR-2A-ZsGreen in vitro transcribed RNA and maintained in DMEM 10% FBS, P/S supplemented with 10 µg/mL blasticidin.

[00170] *Generation of T7-tagBFP-Gluc.* To make the T7-TagBFP-GLuc construct, SP6-TagBFP-2A-FLuc was used as a template and the primers PU-O-6099 and PU-O-6100 were designed to partially anneal immediately upstream and downstream of the SP6 promoter. The unbound portions of PU-O-6099 and PU-O-6100 contained the T7 promoter sequence. Subsequent amplification with the Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) resulted in production of a linear TagBFP-FLuc intermediate construct with T7 overhangs, and ligation was then accomplished using the In-Fusion® HD

Cloning Kit (Takara Bio, Mountain View, CA). Next, PCR amplification of the T7-TagBFP-2A backbone was completed using the Q5® High-Fidelity DNA Polymerase and PU-O-6101 and PU-O-6102 as the primers. Separately, amplification of the Gluc gene was accomplished in the same manner, using KernowC1-p6/Gluc as the backbone and PU-O-6103 and PU-O-2783 as the primers. The Gluc insert was then ligated to the T7-TagBFP-2A backbone using the In-Fusion® HD Cloning Kit.

Table 1. Primers

PU-O-6099	ATACGTAATACGACTCACTATAGAATACAAGCTTATGAGCG	SEQ ID NO: 1
PU-O-6100	AGTCGTATTACGTATGTGTATGATACATAAGGTTATGT	SEQ ID NO: 2
PU-O-6101	CAGAACTTTGACTCCCATCGGTCCAGGATTCTC	SEQ ID NO: 3
PU-O-6102	GCCGGTGGTGACTAAATGGAAGACGCCAAA	SEQ ID NO: 4
PU-O-6103	TTAGTCACCACCGGCC	SEQ ID NO: 5
PU-O-2783	ATGGGAGTCAAAGTTCTGTTTGC	SEQ ID NO: 6

[00171] *Generation of p6/Gluc[G1634R] (KernowC1-p6/Gluc[G1634R]), p6/Gluc[Y1320H] (KernowC1-p6/Gluc[Y1320H]), and p6/Gluc[F470S] (KernowC1-p6/Gluc[F470S]) strains.* To make the KernowC1-p6/Gluc[Y1320H] construct, QuikChange II XL (Agilent Technologies, Santa Clara, CA) site-directed mutagenesis was performed using KernowC1-p6/GLuc as the template and PU-O-5963 and PU-O-5964 as the primers. Successful incorporation of the mutation was confirmed with Sanger sequencing.

[00172] To make the KernowC1-p6/Gluc[G1634R] (KernowC1-p6[G1634R]) construct, QuikChange II XL (Agilent Technologies, Santa Clara, CA) site-directed mutagenesis was performed using KernowC1-p6/GLuc as the template and PU-O-5965 and PU-O-5966 as the primers. Successful incorporation of the mutation was confirmed with Sanger sequencing.

[00173] To make the KernowC1-p6/Gluc[F470S] construct, QuikChange (Stratagene) site-directed mutagenesis was performed using KernowC1-p6/Gluc as the template and PU-O-6433 and PU-O-6434 as the primers. Successful incorporation of the mutation was confirmed with Sanger sequencing.

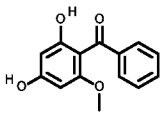
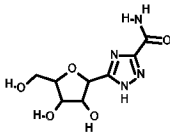
Table 2. Primers

PU-O-5963	CGTAGGACGAAGTTACATGAGGCAGCACATTCAGATGTCC	SEQ ID NO: 7
PU-O-5964	GGACATCTGAATGTGCTGCCTCATGTAACCTTCGTCCTACG	SEQ ID NO: 8
PU-O-5965	CTGTTTGTGATTTCTTCGAAGGTTGACGAACGTTGCGC	SEQ ID NO: 9

PU-O-5966	GCGCAACGTTTCGTCAACCTTCGAAGGAAATCACAAACAG	SEQ ID NO: 10
PU-O-6433	GAAAGTCGCGGGTAAATCCTGCTGTTTTATGCGG	SEQ ID NO: 11
PU-O-6434	CCGCATAAAACAGCAGGATTTACCCGCGACTTTC	SEQ ID NO: 12

[00174] *In Vitro Transcription Assay and Viral RNA Transfection.* HEV KernowC1-p6, KernowC1-p6/Gluc, KernowC1-p6/Gluc[G1634R], KernowC1-p6/Gluc[Y1320H], and KernowC1-p6/Gluc[F470S] were linearized by MluI. pSAR55-Gluc was linearized by BglII, pGEM-9Zf-pSHEV3-Gluc was linearized by XbaI, and pGEM-7Zf(-)-TW6196E and pGEM-7Zf(-)-TW6196E-Gluc were linearized by SpeI. T7-tagBFP-Gluc was linearized by EcoRI. Viral capped RNAs were transcribed in vitro from linearized plasmid using HiScribe T7 antireverse cap analog (ARCA) mRNA kit (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. The *in vitro* transcription (IVT) reaction mixture of 20 μ l was assembled by adding DNA template (1 μ g), 10 μ l of 2 $\times$ ARCA/nucleotide triphosphate (NTP) mix and 2 μ l of T7 RNA polymerase Mix. The reaction mixture was incubated at 37°C for 3 h, and 2 μ l of DNase was added to the IVT reaction mixture and incubated for 15 min at 37°C to remove the template DNA. Then, the viral RNA was purified by LiCl precipitation. Viral RNA was transfected into Huh7 cells using TransIT-mRNA transfection reagent (Mirus Bio LLC, Madison, WI) according to the manufacturer's instructions.

[00175] *Dose titration assays.* Huh7 cells were seeded in a 96-well plate at a seeding density of 6,250 cells/well. The next day, the cells were transfected with 50ng/well HEV Δ ORF2/3[Gluc] IVT RNA using TransIT-mRNA transfection reagent (Mirus Bio LLC, Madison, WI) according to the instructions. Five hours post-transfection, the cells were incubated in DMEM 10%FBS 0.1%Pen/Strep medium containing isocotoin or ribavirin at the following doses: 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 μ M. Three wells were used per dose for triplicate data. Isocotoin and ribavirin compound information is shown below. Most compounds were dissolved in DMSO, and DMSO concentration was kept constant at 0.1% in all wells.

Structure	IUPAC Name	Vendor Information	Abbreviation
	4-benzoyl-5-methoxybenzene-1,3-diol	MicroSource Discovery 201519	isocotoin
	1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1,2,4-triazole-3-carboxamide	Sigma R9644	ribavirin

[00176] *Gaussia luciferase assays.* Gaussia luciferase activity was determined using Luc-Pair Renilla luciferase HS assay kit (GeneCopoeia, Rockville, MD). Specifically, 10 µl of harvested cell culture medium was added per well of a 96-well solid white, flat-bottom polystyrene microplate (Corning, NY, USA), followed by the addition of Renilla luciferase assay substrate and the detection of luminescence was performed using a Berthold luminometer.

[00177] *Quantification of HEV RNA by RT-qPCR Assay.* Huh7 cells were seeded 42,000 cells/well in 24-well plates. One day post-seeding, the cells were transfected with 10ng/well KernowC1p6 or KernowC1p6-GAD *in vitro* transcribed RNA. The next day, the medium on the cells transfected with KernowC1p6 was changed to 0.1% DMSO medium containing 25µM, 12.5µM, 6.25µM, or 0µM isocotoin. The negative control cells transfected with KernowC1p6-GAD were maintained in medium with 0.1% DMSO. The media on the cells was changed every 2 days to fresh drug-containing or DMSO-containing media. On day 6, cells were harvested and lysed in RLT buffer using 350µL per well. Cell lysates were homogenized by pipetting 6-7 times with a needle syringe. Total RNA was extracted using BioBasic RNA MiniPreps SuperKit (Amherst, NY). RT-qPCR was performed on the samples using qScript XLT 1-Step RT-qPCR ToughMix (Quanta Bio) according to the manufacturer's instructions. *In vitro* transcribed KernowC1p6 RNA of known concentration was serially diluted 1:10 and included as controls to generate a standard curve to calculate absolute RNA copies/µL in the samples.

Table 3. Primers for HEV RNA quantification

Forward	GGTGGTTTCTGGGGTGAC	SEQ ID NO: 13
Reverse	AGGGGTTGGTTGGATGAA	SEQ ID NO: 14
FAM-BHQ Probe	TGATTCTCAGCCCTTCGC	SEQ ID NO: 15

[00178] *Measurement of Cytotoxicity by CellTiterGlo Assay.* Huh7 cells were seeded in a 96-well plate at a seeding density of 6,250 cells/well. The next day, the cells were incubated in DMEM, 10% FBS, 0.1%Pen/Strep medium containing isocotoin or ribavirin at the following doses: 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56μM. Three wells were used per dose for triplicate data. On day 4, ATP quantity was determined using CellTiter-Glo 2.0 Assay (Promega, Madison, WI) according to the manufacturer's instructions. Readout was performed using a Berthold luminometer.

[00179] *Translation Inhibition Assay.* Huh7 cells were seeded at 6,250 cells per well in 96-well format. The cells were then transfected with 50ng T7-TagBFP-Gluc *in vitro* transcribed RNA per well. Five hours post-transfection, transfection medium was changed to medium containing isocotoin, ribavirin, cycloheximide, or roclagamide at 6 or 7 different concentrations. DMSO concentration was kept constant in all the wells at 0.125%. For each drug dose, triplicate wells were tested. On day four post-transfection, a luciferase assay was performed on supernatant media to measure Gluc expression.

[00180] *Statistics – calculation of IC₅₀.* Half maximal inhibitory concentrations were calculated by graphing dose-titration data as nonlinear curve fits with three parameters using GraphPad Prism (v8.1.2).

[00181] *Replication kinetics assay.* Huh7 cells were seeded in 6-well plates at 2x10⁵ cells/wells. One day post-seeding, the cells were transfected with 1ug/well *in vitro* transcribed RNA of p6/Gluc (HEVΔORF2/3[Gluc]), HEVΔORF2/3[Gluc][F398S], p6/Gluc[F470S], p6/Gluc[G1634R] (HEVΔORF2/3[Gluc][G1634R]), or p6/Gluc[Y1320H] (HEVΔORF2/3[Gluc][Y1320H]). 50μL supernatant was collected on each day up to day 4 and stored at -20°C. On day 4 post-transfection, a luciferase assay was performed on the supernatant collections to measure Gluc expression.

[00182] *Gluc-expressing HCV and YFV-17D strains.* The pACNR-FLYF-17D-Gluc-BSD-Ires construct was derived from pACNR/FLYF-17D (GenBank ID: AY640589). Briefly, a Gaussia luciferase (Gluc)-P2A-Blasticidin resistance gene (BSD)-EMCV Ires cassette (Gluc-P2A-BSD-Ires) was introduced downstream of the capsid protein, thereby replacing most of the PrM and E protein coding sequence. The Gluc-P2A-BSD-Ires cassette was flanked by the

first six amino acids of Pr and by the 26 last amino acids of E to allow for correct Gluc and NS1 protein processing. The Jc1(p7nsGluc2A) is a J6/JFH genome that includes a Gaussia luciferase gene inserted between p7 and NS2.

[00183] *Suboptimal Dosing Assay to Identify Resistance Mutations.* Huh7 cells were transfected with p6/BSR-2A-ZsGreen *in vitro* transcribed RNA and passaged in the presence of 10ug/mL blasticidin to generate a population that was 85-95% positive for ZsGreen expression, measured using flow cytometry. The Huh7[p6/BSR-2A-ZsGreen] cells were then seeded in 6-well format and passaged in the presence of isocotoin, 10ug/mL blasticidin, or 0.15% DMSO. At each passage, 1/3 of cells were analyzed with flow cytometry, 1/3 of cells were lysed in RLT buffer and stored at -80°C, and 1/3 cells were seeded in a fresh 6-well plate to continue serial passaging.

[00184] *Viral Sequencing.* RNA was extracted from cell lysates using the BioBasic kit according to the manufacturer's instructions. cDNA was generated using the iScript Reverse Transcriptase kit according to manufacturer's instructions. PCR fragments were amplified using Bullseye Taq polymerase in order to generate TA overhangs for TOPO cloning. Subsequently the Takara TOPO-TA cloning kit was used according to manufacturer's instructions. Sequence alignment and analysis was performed using SnapGene software.

[00185] *Flow Cytometry Analysis.* Expression of p6/BSR-2A-ZsGreen was analyzed by flow cytometry on a BD LSRII flow cytometer (five-laser SORP LSRII with high-throughput sampler). Huh7 cells were transfected with p6/BSR-2A-ZsGreen and passaged in DMEM 10%FBS 1%PS containing 10µg/mL blasticidin to select for successfully transfected cells. After 3 days of transduction, cells were fixed in 4% (wt/vol) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15 min, followed by one wash with PBS. The efficiencies of transduction of transgenes were determined by simultaneous expression of ZsGreen. All samples were analyzed on a BD LSRII flow cytometer using FlowJo software.

[00186] *Two-dimensional thermal proteome profiling (2D-TPP) in crude cell extract.* Two-dimensional thermal proteome profiling was performed as previously described (PMID: 30858367). Briefly, Huh7 cells transfected with KernowC1-p6/BSR-2A-ZsGreen were selected under blasticidin pressure (10µg/mL) to generate a cell population highly expressing ORF1 proteins. The cells were lysed in cold PBS with three freeze/thaw cycles in liquid nitrogen followed by mechanical shearing. Aliquots of this crude lysate were incubated with vehicle (DMSO) or isocotoin at 0.5 µM, 2 µM, 7.5 µM or 30 µM for 10 min at 25 °C. The samples were aliquoted to a PCR plate, and heated for 3 min to ten different temperatures (37

°C – 66.3 °C) in a PCR machine (Agilent SureCycler 8800). The crude lysates were further treated with NP-40 and benzonase (final concentration: 0.8% NP40, 1.5 mM MgCl₂, 1× cOmplete protease inhibitor (Roche), 1× PhosSTOP (Roche), 250 U/ml benzonase in PBS) for 1 h at 4 °C. Removal of protein aggregates was performed as previously described (PMID: 29980614), and the remaining soluble proteins were digested overnight according to a modified SP3 protocol (PMID: 25358341; PMID: 30464214). Peptides were labeled with TMT10plex (ThermoFisher Scientific), fractionated into twelve fractions under high pH conditions and analyzed with liquid chromatography coupled to tandem mass spectrometry, as previously described (PMID: 29706546). Protein identification and quantification was performed using IsobarQuant (PMID: 26379230) and Mascot 2.4 (Matrix Science) against a FASTA file with homo sapiens (Proteome ID: UP000005640) and the protein sequence of ORF1 polyprotein from Hepatitis E virus (Uniprot ID: H9E9C7_HEV). Data was analyzed with the TPP package for R (PMID: 26379230).

[00187] *Data availability:* The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://www.proteomexchange.org>) via the PRIDE partner repository with the dataset identifier PXD014485.

[00188] *Plotting – heat maps.* Heatmaps were plotted using Xcode (v10.2.1).

[00189] *Hsp90 knockdown experiments.* Pooled siRNAs targeting HSP 90α/β were purchased from Santa Cruz Biotechnology (sc-35608, Cruz Biotechnology, Dallas, TX), and seed sequence-matched siRNAs were custom designed to use as negative controls for knockdown assays (Sigma, Madison, WI)³⁸. For knockdown experiments, Huh7 cells were reverse transfected with 50nM HSP 90α/β siRNA or negative control siRNA using Dharmafect 4 Transfection Reagent (Dharmacon, Lafayette, CO) according to the manufacturer's instructions.

[00190] *Transfection of HEVΔORF2/3[Gluc] following HSP90 knockdown.* One day after reverse transfection with siRNA, Huh7 cells were transfected with 6.25ng HEVΔORF2/3[Gluc] in vitro transcribed RNA per well of a 96-well plate using TransIT-mRNA transfection reagent (Mirus Bio LLC, Madison, WI) according to the instructions. The medium on the cells was changed 5 hours post transfection. Supernatant was collected on day 3 for Gaussia luciferase measurement.

[00191] *RTqPCR analysis.* Cells were lysed on day 2 post siRNA reverse transfection using RLT buffer, and total RNA was extracted using BioBasic RNA MiniPreps SuperKit (Amherst, NY). RTqPCR analysis was performed using SYBR® Green PCR Master Mix

(Thermo Fisher Scientific) and MultiScribe™ Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer's instructions. Each sample was measured in quadruplicate wells. Results were normalized to average GAPDH housekeeping gene levels.

Table 4. Primers used for RTqPCR

HSP90AA1-F	CATAACGATGATGAGCAGTACGC	SEQ ID NO: 16
HSP90AA1-R	GACCCATAGGTTTACCTGTGT	SEQ ID NO: 17
HSP90AB1-F	AGAAATTGCCCAACTCATGTCC	SEQ ID NO: 18
HSP90AB1-R	ATCAACTCCCGAAGGAAAATCTC	SEQ ID NO: 19
GAPDH-F	GAAGGTGAAGGTCGGAGTC	SEQ ID NO: 20
GAPDH-R	GAAGATGGTGATGGGATTTC	SEQ ID NO: 21

[00192] $\Delta\Delta C_t$ values were calculated to determine fold difference in HSP90 α and β RNA levels between siRNA-transfected and negative control-transfected cells.

[00193] *Western blotting.* Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblotting was performed as follows: After trypsinization and cell pelleting at $2,000 \times g$ for 10 min, whole-cell lysates were harvested in RIPA lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitor cocktail (ThermoFisher Scientific, Waltham, MA). Lysates were electrophoresed in 10% polyacrylamide gels and transferred onto nitrocellulose membrane. The blots were blocked at room temperature for 30 min using 5% nonfat milk in $1 \times$ phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20. The blots were exposed to primary antibodies (anti HSP90 α/β (sc-13119, Santa Cruz Biotechnology, Dallas, TX) and anti- β -actin (13E5, Cell Signaling Technology, Danvers, MA) in 5% nonfat milk in $1 \times$ PBS containing 0.1% Tween 20 overnight. The blots were then washed in $1 \times$ PBS containing 0.1% Tween 20. Afterwards, 30 min exposure to DyLight800 and DyLight680-conjugated secondary antibodies and subsequent washes were performed as described for the primary antibodies. Membranes were visualized using the Odyssey CLx Imaging System (LI-COR Biotechnology, Lincoln, NE) and images were processed using ImageJ Version 2.0.0-rc-43/1.50e³⁹.

Screening assay

[00194] *Small Molecule Library.* The Princeton University Small Molecule Screening Center currently has 75,000 singleton compounds as 10mM DMSO solutions. This collection was assembled from evaluation of over 10 million commercially available compounds, with consideration to include tractable, drug-like chemical entities while maintaining maximum

internal chemical diversity and remaining differentiated from compound collections at typical academic screening centers. Compounds were sourced from (including, but not limited to) Chemdiv, Chembridge, Asinex, Aldrich, Molecular Spectrum and Selleckchem. Bulk liquid and reagent handling is accomplished using the Bravo Automated Liquid Handling Platform (Agilent Technologies). Dispensing of small molecule solutions is achieved using the Echo® 550 Liquid Handler (Labcyte, San Jose, CA), a non-contact acoustic dispenser.

[00195] *Screening Assay Setup.* 384-well plastic tissue culture plates were seeded with 150 nL of compounds dissolved as 10 mM DMSO stock solutions using the Echo® 550 Liquid Handler. 30 µL Huh7 cells transfected with p6/BSR-2A-ZsGreen (“replicon cells”) were then seeded in the wells at a density of 8,000 cells per well, using a MultiDrop Combi Reagent Dispenser (Thermo Fischer Scientific). In each plate, 64 wells were reserved for positive control untreated cells (32 wells) and for negative control, non-fluorescent naïve Huh7 cells (32 wells). After four days, transmitted light and GFP fluorescence images were taken of the wells (in total, two images per well) using the Operetta CLS High Content Screening System (PerkinElmer, Waltham, MA).

[00196] *Image Analysis.* A custom Python script was developed to analyze the effectiveness of different substances for inhibition of viral replication with a high throughput. This involved analyzing images of wells from 188 plates, each containing 384 wells, 32 of which were dedicated to negative controls and 32 to positive controls. Using the tail differentials in the color distribution of fluorescence images, wells similar to the negative controls were tagged. The algorithm was tuned to maximize Z-factors and thus, confidence, in determining potential matches. Finally, the program listed candidate wells in importance order, and this list was used to manually verify the visual image of the wells to eliminate cytotoxic wells.

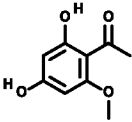
[00197] *Second-Round Screening.* 37 compounds selected for second-round screening were seeded at eight concentrations in duplicate using the Echo® 550 Liquid Handler (Labcyte). Huh7 replicon cells were then seeded in the wells at 8000 cells per well using the MultiDrop Combi Reagent Dispenser (Thermo Fischer Scientific). Image acquisition was performed using the Operetta CLS High Content Screening System (PerkinElmer), and analysis was performed with the previously mentioned custom Python script. GraphPad Prism was used to plot dose titration curves and calculate IC₅₀ values for each compound.

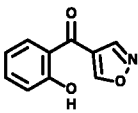
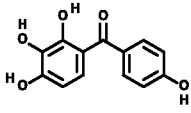
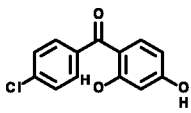
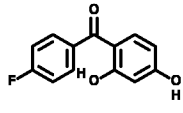
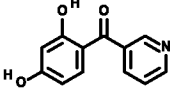
Structure-Activity Relationship Analysis

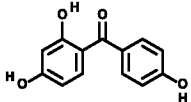
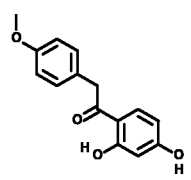
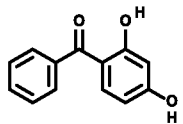
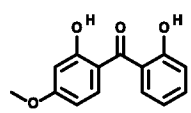
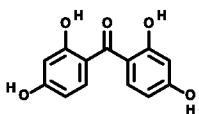
[00198] *Compound preparation.* All SAR analysis compounds (listed in Appendices 1, 1', 2, 2', 3) were ordered in powder form and dissolved in DMSO to a 10mM concentration. 10μL aliquots were stored at -80 °C. When conducting the dose titration experiments, the compounds to be tested were thawed fresh from -80 °C and used immediately.

[00199] *Dose titration assays.* Huh7 cells were seeded 6,250 cells/well in a 96-well plate one day prior to treatment. The outer wells of each plate were not used as experimental wells due to increased evaporation and were instead filled with 200μL PBS to keep the plates humidified. On the day of treatment, the cells were transfected with 50ng/well p6/Gluc *in vitro* transcribed RNA using TrasIT mRNA transfection reagent (Mirus Bio LLC, Madison, WI) according to the manufacturer's instructions. Five hours later, the medium on the cells was changed to medium containing compounds to be tested at doses ranging from 0.39μM to 100μM. Each dose was tested in triplicate, and isocotoin and ribavirin were included as controls in all experiments. To prepare the compound-containing media, the 10mM aliquots of compound in DMSO were thawed and dissolved in DMEM 10%FBS 0.1%Pen/Strep to make 100μM solutions. The 100μM solutions were then serially diluted 1:2 in DMEM 10%FBS 0.1%Pen/Strep to generate 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125, and 0.39μM solutions. The cells were left in 166μL/well compound-containing media for 4 days, with a luciferase readout performed on day 4.

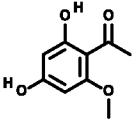
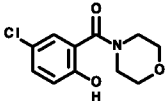
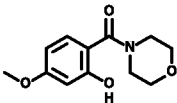
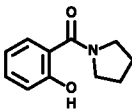
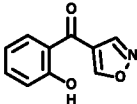
Appendix 1. SAR Analysis Round 1 Molecules

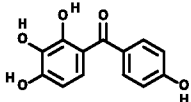
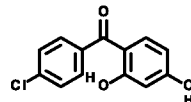
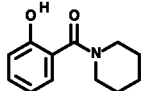
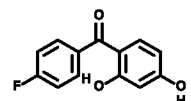
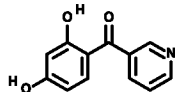
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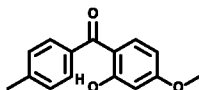
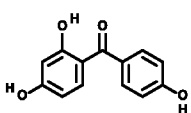
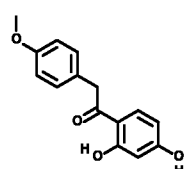
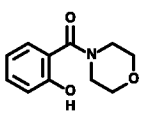
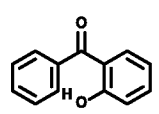
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	(4-hydroxyphenyl)-(2,3,4-trihydroxyphenyl)methanone	InterBioScreen Ltd STOCK2S-11493	IBS493
	(4-chlorophenyl)-(2,4-dihydroxyphenyl)methanone	InterBioScreen Ltd STOCK7S-53102	IBS102
	(2,4-dihydroxyphenyl)-(4-fluorophenyl)methanone	InterBioScreen Ltd STOCK7S-53659	IBS659
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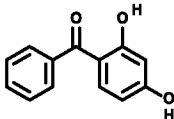
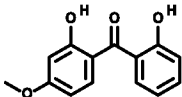
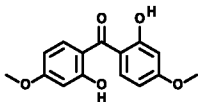
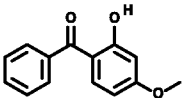
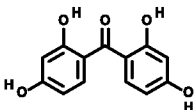
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	1-(2,4-dihydroxyphenyl)-2-(4-methoxyphenyl)ethanone	InterBioScreen Ltd STOCK5S-54694	IBS694
	(2,4-dihydroxyphenyl)-phenylmethanone	Specs AE- 641/01968047	SPE047
	(2-hydroxy-4-methoxyphenyl)-(2-hydroxyphenyl)methanone	AK Scientific K820	K820
	bis(2,4-dihydroxyphenyl)methanone	AK Scientific I955	I955

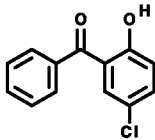
Appendix 1'. SAR Analysis Round 1 Molecules

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	(2-hydroxy-4-methoxyphenyl)-morpholin-4-ylmethanone	Enamine Z68159307	ENA307
	(2-hydroxyphenyl)-pyrrolidin-1-ylmethanone	Enamine Z235583000	ENA000
	(2-hydroxyphenyl)-(1,2-oxazol-4-yl)methanone	Enamine Z57983002	ENA002

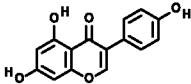
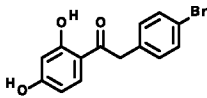
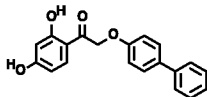
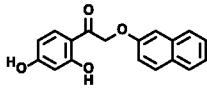
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	(4-chlorophenyl)-(2,4-dihydroxyphenyl)methanone	InterBioScreen Ltd STOCK7S-53102	IBS102
	(2-hydroxyphenyl)-piperidin-1-ylmethanone	InterBioScreen Ltd STOCK5S-49711	IBS711
	(2,4-dihydroxyphenyl)-(4-fluorophenyl)methanone	InterBioScreen Ltd STOCK7S-53659	IBS659
	(2,4-dihydroxyphenyl)-pyridin-3-ylmethanone	InterBioScreen Ltd STOCK6S-83914	IBS914

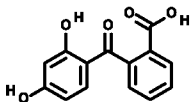
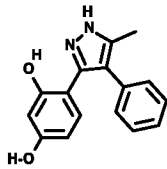
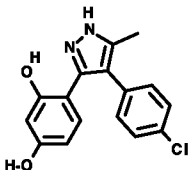
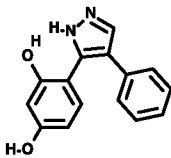
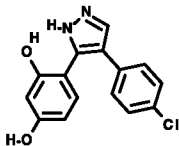
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	(2,4-dihydroxyphenyl)-(4-hydroxyphenyl)methanone	InterBioScreen Ltd STOCK2S-15237	IBS237
	1-(2,4-dihydroxyphenyl)-2-(4-methoxyphenyl)ethanone	InterBioScreen Ltd STOCK5S-54694	IBS694
	(2-hydroxyphenyl)-morpholin-4-ylmethanone	InterBioScreen Ltd STOCK1S-59117	IBS117
	(2-hydroxyphenyl)-phenylmethanone	Specs AE- 562/43458976	SPE976

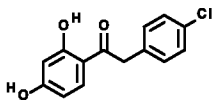
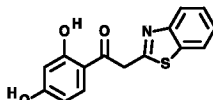
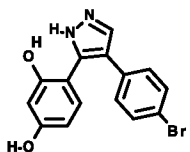
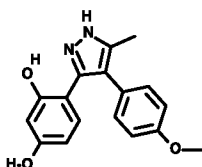
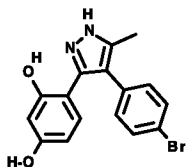
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	bis(2-hydroxy-4-methoxyphenyl)methanone	AK Scientific L008	L008
	(2-hydroxy-4-methoxyphenyl)-phenylmethanone	AK Scientific I956	I956
	bis(2,4-dihydroxyphenyl)methanone	AK Scientific I955	I955

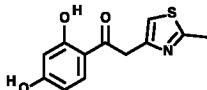
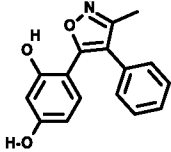
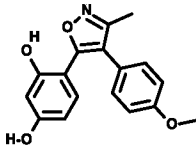
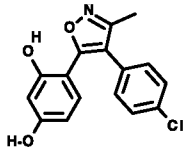
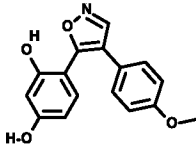
Structure	IUPAC Name	Vendor Information	Abbreviation
	(5-chloro-2-hydroxyphenyl)-phenylmethanone	AK Scientific O344	O344

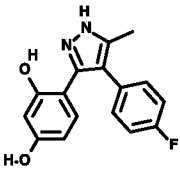
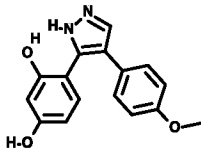
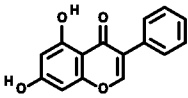
Appendix 2. SAR Analysis Round 2 Molecules

Structure	IUPAC Name	Vendor Information	Abbreviation
	5,7-dihydroxy-3-(4-hydroxyphenyl)chromen-4-one	AK Scientific J10015	AK015
	2-(4-bromophenyl)-1-(2,4-dihydroxyphenyl)ethanone	ChemBridge Corporation 7113606	CB606
	1-(2,4-dihydroxyphenyl)-2-(4-phenylphenoxy)ethanone	ChemBridge Corporation 6688097	CB097
	1-(2,4-dihydroxyphenyl)-2-naphthalen-2-yloxyethanone	ChemBridge Corporation 6686070	CB070

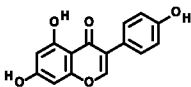
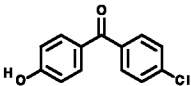
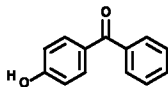
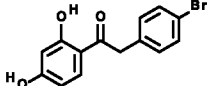
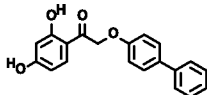
Structure	IUPAC Name	Vendor Information	Abbreviation
	2-(2,4-dihydroxybenzoyl)benzoic acid	ChemBridge Corporation 5629492	CB492
	4-(5-methyl-4-phenyl-1H-pyrazol-3-yl)benzene-1,3-diol	InterBioScreen Ltd. STOCK4S-19358	IBS358
	4-[4-(4-chlorophenyl)-5-methyl-1H-pyrazol-3-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK4S-18140	IBS140
	4-(4-phenyl-1H-pyrazol-5-yl)benzene-1,3-diol	InterBioScreen Ltd. STOCK4S-09309	IBS309
	4-[4-(4-chlorophenyl)-1H-pyrazol-5-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK4S-25057	IBS057

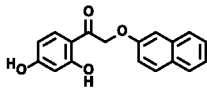
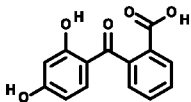
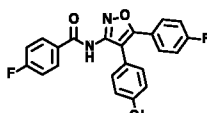
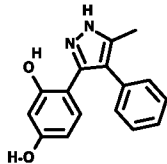
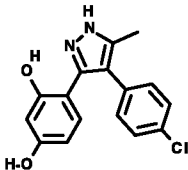
Structure	IUPAC Name	Vendor Information	Abbreviation
	2-(4-chlorophenyl)-1-(2,4-dihydroxyphenyl)ethanone	InterBioScreen Ltd. STOCK1S-14547	IBS547
	2-(1,3-benzothiazol-2-yl)-1-(2,4-dihydroxyphenyl)ethanone	InterBioScreen Ltd. STOCK4S-42707	IBS707
	4-[4-(4-bromophenyl)-1H-pyrazol-5-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK3S-05374	IBS374
	4-[4-(4-methoxyphenyl)-5-methyl-1H-pyrazol-3-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK3S-49162	IBS162
	4-[4-(4-bromophenyl)-5-methyl-1H-pyrazol-3-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK3S-45674	IBS674

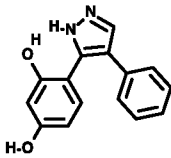
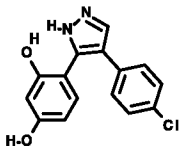
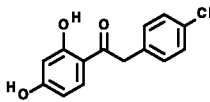
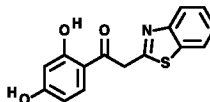
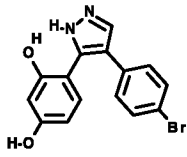
Structure	IUPAC Name	Vendor Information	Abbreviation
	1-(2,4-dihydroxyphenyl)-2-(2-methyl-1,3-thiazol-4-yl)ethanone	InterBioScreen Ltd. STOCK1S-18526	IBS526
	4-(3-methyl-4-phenyl-1,2-oxazol-5-yl)benzene-1,3-diol	InterBioScreen Ltd. STOCK6S-00308	IBS308
	4-[4-(4-methoxyphenyl)-3-methyl-1,2-oxazol-5-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK5S-98672	IBS672
	4-[4-(4-chlorophenyl)-3-methyl-1,2-oxazol-5-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK5S-97287	IBS287
	4-[4-(4-methoxyphenyl)-1,2-oxazol-5-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK5S-97821	IBS821

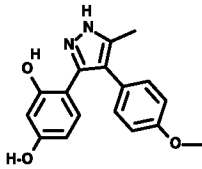
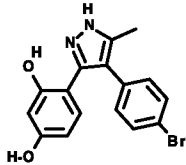
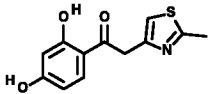
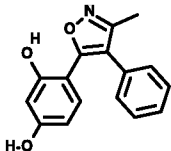
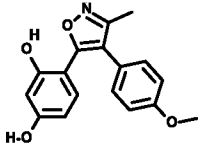
Structure	IUPAC Name	Vendor Information	Abbreviation
	4-[4-(4-fluorophenyl)-5-methyl-1 <i>H</i> -pyrazol-3-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK4S-69521	IBS521
	4-[4-(4-methoxyphenyl)-1 <i>H</i> -pyrazol-5-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK4S-82525	IBS525
	5,7-dihydroxy-3-phenylchromen-4-one	InterBioScreen Ltd. STOCK1N-88582	IBS582

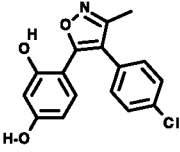
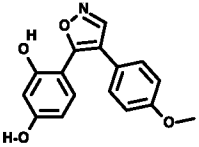
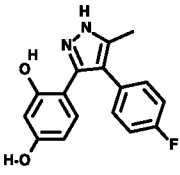
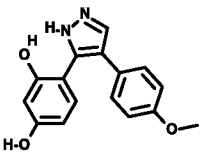
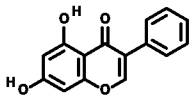
Appendix 2'. SAR Analysis Round 2 Molecules

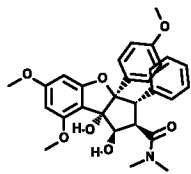
Structure	IUPAC Name	Vendor Information	Abbreviation
	5,7-dihydroxy-3-(4-hydroxyphenyl)chromen-4-one	AK Scientific J10015	AK015
	(4-chlorophenyl)-(4-hydroxyphenyl)methanone	AK Scientific A347	AK347
	(4-hydroxyphenyl)-phenylmethanone	AK Scientific J91321	AK321
	2-(4-bromophenyl)-1-(2,4-dihydroxyphenyl)ethanone	ChemBridge Corporation 7113606	CB606
	1-(2,4-dihydroxyphenyl)-2-(4-phenylphenoxy)ethanone	ChemBridge Corporation 6688097	CB097

Structure	IUPAC Name	Vendor Information	Abbreviation
	1-(2,4-dihydroxyphenyl)-2-naphthalen-2-yloxyethanone	ChemBridge Corporation 6686070	CB070
	2-(2,4-dihydroxybenzoyl)benzoic acid	ChemBridge Corporation 5629492	CB492
	<i>N</i> -[4-(4-chlorophenyl)-5-(4-fluorophenyl)-1,2-oxazol-3-yl]-4-fluorobenzamide	ChemDiv, Inc. C301-7780	CD780
	4-(5-methyl-4-phenyl-1 <i>H</i> -pyrazol-3-yl)benzene-1,3-diol	InterBioScreen Ltd. STOCK4S-19358	IBS358
	4-[4-(4-chlorophenyl)-5-methyl-1 <i>H</i> -pyrazol-3-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK4S-18140	IBS140

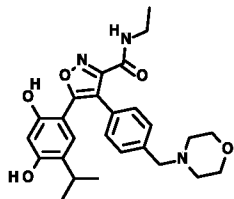
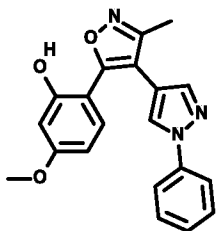
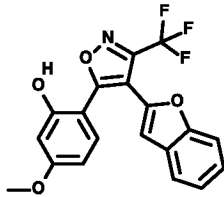
Structure	IUPAC Name	Vendor Information	Abbreviation
	4-(4-phenyl-1 <i>H</i> -pyrazol-5-yl)benzene-1,3-diol	InterBioScreen Ltd. STOCK4S-09309	IBS309
	4-[4-(4-chlorophenyl)-1 <i>H</i> -pyrazol-5-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK4S-25057	IBS057
	2-(4-chlorophenyl)-1-(2,4-dihydroxyphenyl)ethanone	InterBioScreen Ltd. STOCK1S-14547	IBS547
	2-(1,3-benzothiazol-2-yl)-1-(2,4-dihydroxyphenyl)ethanone	InterBioScreen Ltd. STOCK4S-42707	IBS707
	4-[4-(4-bromophenyl)-1 <i>H</i> -pyrazol-5-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK3S-05374	IBS374

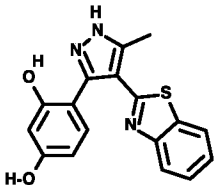
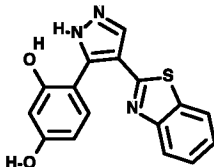
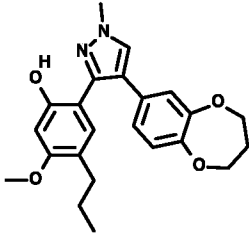
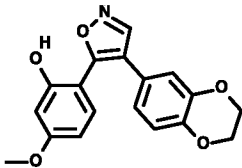
Structure	IUPAC Name	Vendor Information	Abbreviation
	4-[4-(4-methoxyphenyl)-5-methyl-1 <i>H</i> -pyrazol-3-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK3S-49162	IBS162
	4-[4-(4-bromophenyl)-5-methyl-1 <i>H</i> -pyrazol-3-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK3S-45674	IBS674
	1-(2,4-dihydroxyphenyl)-2-(2-methyl-1,3-thiazol-4-yl)ethanone	InterBioScreen Ltd. STOCK1S-18526	IBS526
	4-(3-methyl-4-phenyl-1,2-oxazol-5-yl)benzene-1,3-diol	InterBioScreen Ltd. STOCK6S-00308	IBS308
	4-[4-(4-methoxyphenyl)-3-methyl-1,2-oxazol-5-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK5S-98672	IBS672

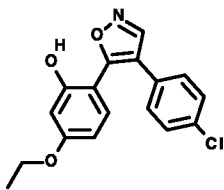
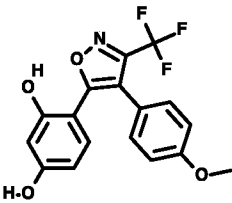
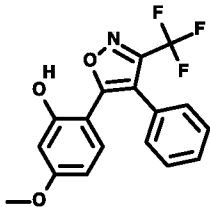
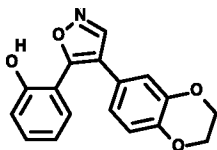
Structure	IUPAC Name	Vendor Information	Abbreviation
	4-[4-(4-chlorophenyl)-3-methyl-1,2-oxazol-5-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK5S-97287	IBS287
	4-[4-(4-methoxyphenyl)-1,2-oxazol-5-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK5S-97821	IBS821
	4-[4-(4-fluorophenyl)-5-methyl-1H-pyrazol-3-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK4S-69521	IBS521
	4-[4-(4-methoxyphenyl)-1H-pyrazol-5-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK4S-82525	IBS525
	5,7-dihydroxy-3-phenylchromen-4-one	InterBioScreen Ltd. STOCK1N-88582	IBS582

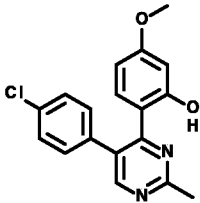
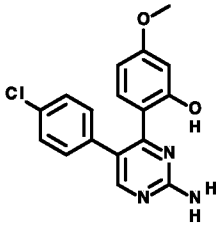
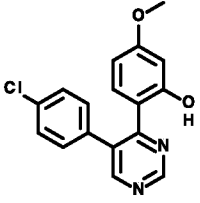
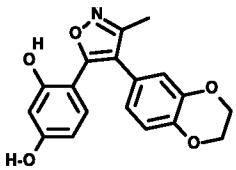
Structure	IUPAC Name	Vendor Information	Abbreviation
	(1 <i>R</i> ,2 <i>R</i> ,3 <i>S</i> ,3 <i>aR</i> ,8 <i>bS</i>)-1,8 <i>b</i> -dihydroxy-6,8-dimethoxy-3 <i>a</i> -(4-methoxyphenyl)- <i>N,N</i> -dimethyl-3-phenyl-2,3-dihydro-1 <i>H</i> -cyclopenta[<i>b</i>][1]benzofuran-2-carboxamide	MedChemExpress Europe HY-19356	MCE356, roclagamide

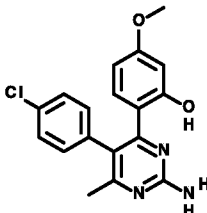
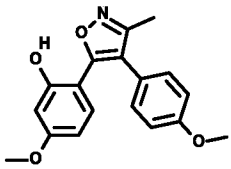
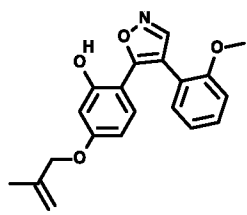
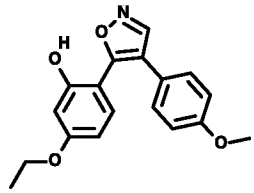
Appendix 3. SAR Analysis Round 3 Molecules

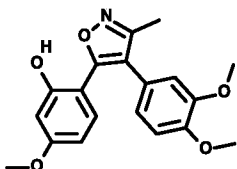
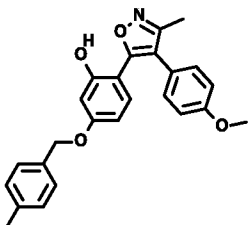
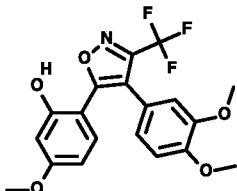
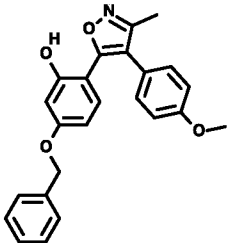
Structure	IUPAC Name	Vendor Information	Abbreviation
	5-(2,4-dihydroxy-5-propan-2-ylphenyl)- <i>N</i> -ethyl-4-[4-(morpholin-4-ylmethyl)phenyl]-1,2-oxazole-3-carboxamide	Axon Medchem 1542	AM542 (AUY-922)
	5-methoxy-2-[3-methyl-4-(1-phenylpyrazol-4-yl)-1,2-oxazol-5-yl]phenol	ChemBridge Corporation 6873595	CB595
	2-[4-(1-benzofuran-2-yl)-3-(trifluoromethyl)-1,2-oxazol-5-yl]-5-methoxyphenol	ENAMINE Ltd. Z56793271	ENA271

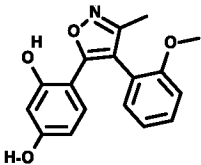
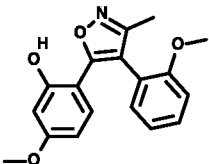
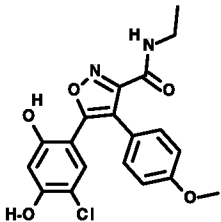
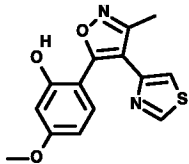
Structure	IUPAC Name	Vendor Information	Abbreviation
	4-[4-(1,3-benzothiazol-2-yl)-5-methyl-1 <i>H</i> -pyrazol-3-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK2S-77259	IBS259
	4-[4-(1,3-benzothiazol-2-yl)-1 <i>H</i> -pyrazol-5-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK1S-68454	IBS454
	2-[4-(3,4-dihydro-2 <i>H</i> -1,5-benzodioxepin-7-yl)-1-methylpyrazol-3-yl]-5-methoxy-4-propylphenol	InterBioScreen Ltd. STOCK1N-06154	IBS154
	2-[4-(2,3-dihydro-1,4-benzodioxin-6-yl)-1,2-oxazol-5-yl]-5-methoxyphenol	InterBioScreen Ltd. STOCK1N-07078	IBS078

Structure	IUPAC Name	Vendor Information	Abbreviation
	2-[4-(4-chlorophenyl)-1,2-oxazol-5-yl]-5-ethoxyphenol	InterBioScreen Ltd. STOCK6S-00206	IBS206
	4-[4-(4-methoxyphenyl)-3-(trifluoromethyl)-1,2-oxazol-5-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK5S-98901	IBS901
	5-methoxy-2-[4-phenyl-3-(trifluoromethyl)-1,2-oxazol-5-yl]phenol	InterBioScreen Ltd. STOCK5S-99550	IBS550
	2-[4-(2,3-dihydro-1,4-benzodioxin-6-yl)-1,2-oxazol-5-yl]phenol	InterBioScreen Ltd. STOCK1N-01831	IBS831

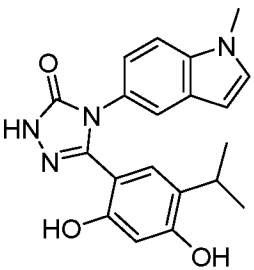
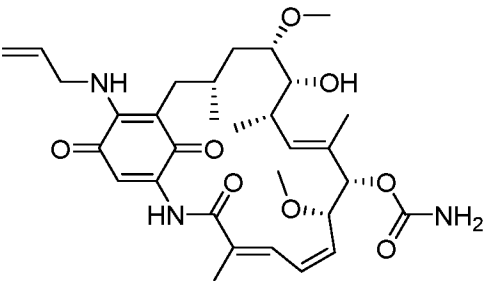
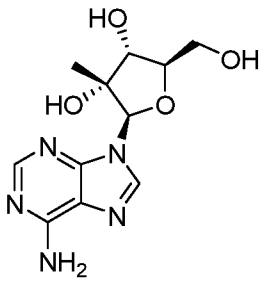
Structure	IUPAC Name	Vendor Information	Abbreviation
	2-[5-(4-chlorophenyl)-2-methylpyrimidin-4-yl]-5-methoxyphenol	InterBioScreen Ltd. STOCK5S-56524	IBS524
	2-[2-amino-5-(4-chlorophenyl)pyrimidin-4-yl]-5-methoxyphenol	InterBioScreen Ltd. STOCK5S-16629	IBS629
	2-[5-(4-chlorophenyl)pyrimidin-4-yl]-5-methoxyphenol	InterBioScreen Ltd. STOCK5S-09547	IBS547
	4-[4-(2,3-dihydro-1,4-benzodioxin-6-yl)-3-methyl-1,2-oxazol-5-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK1N-10332	IBS332

Structure	IUPAC Name	Vendor Information	Abbreviation
	2-[2-amino-5-(4-chlorophenyl)-6-methylpyrimidin-4-yl]-5-methoxyphenol	InterBioScreen Ltd. STOCK5S-21266	IBS266
	5-methoxy-2-[4-(4-methoxyphenyl)-3-methyl-1,2-oxazol-5-yl]phenol	InterBioScreen Ltd. STOCK5S-99516	IBS516
	2-[4-(2-methoxyphenyl)-1,2-oxazol-5-yl]-5-(2-methylprop-2-enoxy)phenol	InterBioScreen Ltd. STOCK6S-01466	IBS466
	5-ethoxy-2-[4-(4-methoxyphenyl)-1,2-oxazol-5-yl]phenol	InterBioScreen Ltd. STOCK6S-02983	IBS983

Structure	IUPAC Name	Vendor Information	Abbreviation
	2-[4-(3,4-dimethoxyphenyl)-3-methyl-1,2-oxazol-5-yl]-5-methoxyphenol	InterBioScreen Ltd. STOCK6S-04304	IBS304
	2-[4-(4-methoxyphenyl)-3-methyl-1,2-oxazol-5-yl]-5-[(4-methylphenyl)methoxy]phenol	InterBioScreen Ltd. STOCK6S-04330	IBS330
	2-[4-(3,4-dimethoxyphenyl)-3-(trifluoromethyl)-1,2-oxazol-5-yl]-5-methoxyphenol	InterBioScreen Ltd. STOCK6S-07597	IBS597
	2-[4-(4-methoxyphenyl)-3-methyl-1,2-oxazol-5-yl]-5-phenylmethoxyphenol	InterBioScreen Ltd. STOCK6S-09992	IBS992

Structure	IUPAC Name	Vendor Information	Abbreviation
	4-[4-(2-methoxyphenyl)-3-methyl-1,2-oxazol-5-yl]benzene-1,3-diol	InterBioScreen Ltd. STOCK6S-10451	IBS451
	5-methoxy-2-[4-(2-methoxyphenyl)-3-methyl-1,2-oxazol-5-yl]phenol	InterBioScreen Ltd. STOCK6S-11060	IBS060
	5-(5-chloro-2,4-dihydroxyphenyl)-N-ethyl-4-(4-methoxyphenyl)-1,2-oxazole-3-carboxamide	TargetMol T2258	TM258 (VER-50589)
	5-methoxy-2-[3-methyl-4-(1,3-thiazol-4-yl)-1,2-oxazol-5-yl]phenol	UkrOrgSynthesis Ltd. Stock PB56845823	UOS823

Appendix 4. Additional Molecules

Structure	IUPAC Name	Vendor Information	Abbreviation
	3-(2,4-dihydroxy-5-propan-2-ylphenyl)-4-(1-methylindol-5-yl)-1H-1,2,4-triazol-5-one	Selleck Chem S1159	STA-9090
	[(4E,6Z,8S,9S,10E,12S,13R,14S,16R)-13-hydroxy-8,14-dimethoxy-4,10,12,16-tetramethyl-3,20,22-trioxo-19-(prop-2-enylamino)-2-azabicyclo[16.3.1]docosa-1(21),4,6,10,18-pentaen-9-yl] carbamate	Selleck Chem S1141	17-AAG
	(2R,3R,4R,5R)-2-(6-aminopurin-9-yl)-5-(hydroxymethyl)-3-methyloxolane-3,4-diol	Carbosynth 15397-12-3	2'CMA

[00200] *Generation of liver chimeric mice.* Cryopreserved human adult primary hepatocytes were obtained from Bioreclamation (Westbury, NY) and washed with high glucose DMEM. Using isoflurane anesthesia, human cell suspensions were injected intrasplenically into female *fah*^{-/-}*NOD rag1*^{-/-}*il2rgnull* (FNRG) mice that were generated by backcrossing of the *fah*^{-/-} allele to *NOD rag1*^{-/-}*il2rgnull* (NRG) animals obtained from Jackson Labs as described previously. Approximately 1×10^6 human adult hepatocytes were transplanted per mouse using protocols previously established in the Ploss lab. Starting on the

day of transplantation mice were cycled off the drug NTBC (Yecuris Inc., Tualatin, OR). All mice were maintained at the Laboratory Animal Resource Center at Princeton University.

[00201] All animal experiments described in this study were performed in accordance with protocols (number 1930-19) that were reviewed and approved by the Institutional Animal Care and Use and Committee of Princeton University.

[00202] *Assessment of engraftment by human albumin ELISA.* Levels of human albumin in mouse serum were quantified by ELISA; 96-well flat-bottomed plates (Nunc, Thermo Fischer Scientific, Witham, MA) were coated with goat anti-human albumin antibody (1:500, Bethel) in coating buffer (1.59g Na₂CO₃, 2.93g NaHCO₃, 1L dH₂O, pH = 9.6) for 1 hour at 37 °C. The plates were washed four times with wash buffer (0.05% Tween 20 (Sigma Aldrich, St. Louis, MO) in 1x PBS) then incubated with superbloc buffer (Fisher Scientific, Hampton NH) for 1 hour at 37 °C. Plates were washed twice. Human serum albumin (Sigma Aldrich, St. Louis MO) was diluted to 1 µg/ml in sample diluent (10% Superblock, 90% wash buffer), then serially diluted 1:2 in 135 µl sample diluent to establish an albumin standard. Mouse serum (5 µl) was used for a 1:10 serial dilution in 135 µl sample diluent. The coated plates were incubated for 1 hour at 37 °C, then washed three times. Mouse anti-human albumin (50 µl, 1:2000 in sample diluent, Abcam, Cambridge, UK) was added and plates were incubated for 2 hours at 37 °C. Plates were washed four times, and 50 µl of goat anti-mouse-HRP (1:10,000 in sample diluent, LifeTechnologies, Carlsbad, CA) was added and incubated for 1 hour at 37 °C. Plates were washed six times. TMB (100 µl) substrate (Sigma Aldrich, St. Louis, MO) was added and the reaction was stopped with 12.5µl of 2N H₂SO₄. Absorbance was read at 450λ on the BertholdTech TriStar (Bad Wildbad, Germany).

[00203] *Measurement of HEV titers in stool.* The mice were separated into individual cages for approximately 15-30min. Stool pellets were collected from the cages using sterile forceps. The mice were returned to their original cages. Stool samples were either kept on ice and analyzed immediately or kept on ice and transferred to -80 °C until analysis. The Nucleospin RNA Stool kit (TakaraBio USA, Mountain View, CA) was used to extract RNA from the samples. A spiked sample with 1.301E9 copies of Kc1p6 RNA was included as a control for extraction efficiency.

[00204] *RT-qPCR:* The QuantaQ ToughMix RTqPCR kit (QuantaBio, Beverly, MA) was used, with 10µL reaction volumes.

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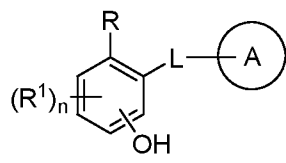
[00205] The teachings of all patents, published applications and references cited herein are incorporated by reference in their entirety.

[00206] While example embodiments have been particularly shown and described, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the embodiments encompassed by the appended claims.

CLAIMS

What is claimed is:

1. A method of inhibiting replication of a virus, comprising contacting a cell infected with the virus with a compound represented by the following structural formula:



or a pharmaceutically acceptable salt thereof, wherein:

Ring A is aryl or heteroaryl, and is optionally substituted with one or more substituents independently selected from halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, $-(CH_2)_{0-2}$ -aryl, $-(CH_2)_{0-2}$ -heteroaryl, $-(CH_2)_{0-2}$ -cycloalkyl, or $-(CH_2)_{0-2}$ -heterocyclyl, carboxy or $-O(CH_2)_mO-$;

m is 1, 2, 3, 4 or 5;

L is $-C(O)(CH_2)_p-$, $-C(O)(CH_2)_pO-$ or heteroarylene, wherein p is 0, 1 or 2, and R is hydrogen, halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenoxy, alkynoxy, $-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl; or

L is $-C(O)(CH_2)_p-$, wherein p is 1 or 2, and R and a methylene carbon of $-C(O)(CH_2)_p-$, together with their intervening carbon atoms, form a fused ring;

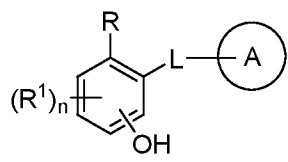
R¹ is halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenoxy, alkynoxy, $-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl; and

n is 0, 1, 2 or 3,

wherein the aryl and heteroaryl of R and R¹, and the heteroarylene of L are each optionally and independently substituted with one or more substituents selected from halo, alkyl, haloalkyl, amino, alkylamino, dialkylamino or carboxamido.

2. A method of inhibiting replication of a virus, comprising contacting a cell infected with the virus with a compound of Appendix 1, 1', 2, 2', 3 or 4, or a pharmaceutically acceptable salt thereof.
3. The method of claim 1 or 2, wherein the virus is a positive-sense, single-stranded RNA virus.
4. The method of claim 3, wherein the virus is a flavivirus.

5. The method of any one of claims 1-4, wherein the virus is a hepatitis E virus (HEV).
6. The method of any one of claims 1-4, wherein the virus is a hepatitis C virus (HCV).
7. The method of any one of claims 1-4, wherein the virus is a yellow fever virus (YFV).
8. A method of treating a viral infection in a subject in need thereof, comprising administering to the subject an effective amount of a compound represented by the following structural formula:



or a pharmaceutically acceptable salt thereof, wherein:

Ring A is aryl or heteroaryl, and is optionally substituted with one or more substituents independently selected from halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, $-(CH_2)_{0-2}$ -aryl, $-(CH_2)_{0-2}$ -heteroaryl, $-(CH_2)_{0-2}$ -cycloalkyl, or $-(CH_2)_{0-2}$ -heterocyclyl, carboxy or $-O(CH_2)_mO-$;

m is 1, 2, 3, 4 or 5;

L is $-C(O)(CH_2)_p-$, $-C(O)(CH_2)_pO-$ or heteroarylene, wherein p is 0, 1 or 2, and R is hydrogen, halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenoxy, alkynoxy, $-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl; or

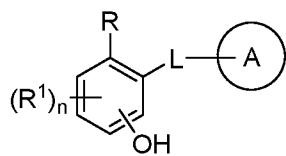
L is $-C(O)(CH_2)_p-$, wherein p is 1 or 2, and R and a methylene carbon of $-C(O)(CH_2)_p-$, together with their intervening carbon atoms, form a fused ring; R¹ is halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenoxy, alkynoxy, $-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl; and

n is 0, 1, 2 or 3,

wherein the aryl and heteroaryl of R and R¹, and the heteroarylene of L are each optionally and independently substituted with one or more substituents selected from halo, alkyl, haloalkyl, amino, alkylamino, dialkylamino or carboxamido.

9. A method of treating a viral infection in a subject in need thereof, comprising administering to the subject an effective amount of a compound of Appendix 1, 1', 2, 2', 3 or 4, or a pharmaceutically acceptable salt thereof.

10. The method of claim 8 or 9, wherein the viral infection is caused by a positive-sense, single-stranded RNA virus.
11. The method of claim 10, wherein the viral infection is caused by a flavivirus.
12. The method of any one of claims 8-11, wherein the viral infection is caused by a hepatitis E virus (HEV).
13. The method of any one of claims 8-11, wherein the viral infection is caused by a hepatitis C virus (HCV).
14. The method of any one of claims 8-11, wherein the viral infection is caused by a yellow fever virus (YFV).
15. A method of inhibiting heat shock protein 90 in a cell, comprising contacting the cell with a compound represented by the following structural formula:



or a pharmaceutically acceptable salt thereof, wherein:

Ring A is aryl or heteroaryl, and is optionally substituted with one or more substituents independently selected from halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, $-(CH_2)_{0-2}$ -aryl, $-(CH_2)_{0-2}$ -heteroaryl, $-(CH_2)_{0-2}$ -cycloalkyl, or $-(CH_2)_{0-2}$ -heterocyclyl, carboxy or $-O(CH_2)_mO-$;

m is 1, 2, 3, 4 or 5;

L is $-C(O)(CH_2)_p-$, $-C(O)(CH_2)_pO-$ or heteroarylene, wherein p is 0, 1 or 2, and R is hydrogen, halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenoxy, alkynoxy,

$-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl; or

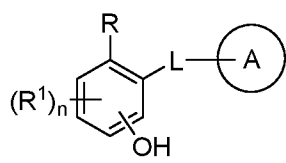
L is $-C(O)(CH_2)_p-$, wherein p is 1 or 2, and R and a methylene carbon of $-C(O)(CH_2)_p-$, together with their intervening carbon atoms, form a fused ring;

R¹ is halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenoxy, alkynoxy, $-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl; and

n is 0, 1, 2 or 3,

wherein the aryl and heteroaryl of R and R¹, and the heteroarylene of L are each optionally and independently substituted with one or more substituents selected from halo, alkyl, haloalkyl, amino, alkylamino, dialkylamino or carboxamido, provided that the compound is not AUY-922, VER-50589 or STA-9090, or a pharmaceutically acceptable salt of any of the foregoing.

16. A method of inhibiting heat shock protein 90 in a cell, comprising contacting the cell with a compound of Appendix 1, 1', 2, 2', 3 or 4, or a pharmaceutically acceptable salt thereof, provided that the compound is not AUY-922, VER-50589, STA-9090 or 17-AAG, or a pharmaceutically acceptable salt of any of the foregoing.
17. A method of treating a heat shock protein 90-mediated disease or condition in a subject in need thereof, comprising administering to the subject an effective amount of a compound represented by the following structural formula:



or a pharmaceutically acceptable salt thereof, wherein:

Ring A is aryl or heteroaryl, and is optionally substituted with one or more substituents independently selected from halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, $-(CH_2)_{0-2}$ -aryl, $-(CH_2)_{0-2}$ -heteroaryl, $-(CH_2)_{0-2}$ -cycloalkyl, or $-(CH_2)_{0-2}$ -heterocyclyl, carboxy or $-O(CH_2)_mO-$;

m is 1, 2, 3, 4 or 5;

L is $-C(O)(CH_2)_p-$, $-C(O)(CH_2)_p-O-$ or heteroarylene, wherein p is 0, 1 or 2, and R is hydrogen, halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenoxy, alkynoxy, $-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl; or

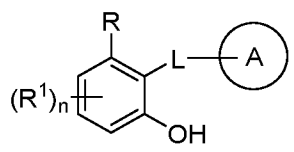
L is $-C(O)(CH_2)_p-$, wherein p is 1 or 2, and R and a methylene carbon of $-C(O)(CH_2)_p-$, together with their intervening carbon atoms, form a fused ring;

R¹ is halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenoxy, alkynoxy, $-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl; and

n is 0, 1, 2 or 3,

wherein the aryl and heteroaryl of R and R¹, and the heteroarylene of L are each optionally and independently substituted with one or more substituents selected from halo, alkyl, haloalkyl, amino, alkylamino, dialkylamino or carboxamido, provided that the compound is not AUY-922, VER-50589 or STA-9090, or a pharmaceutically acceptable salt of any of the foregoing.

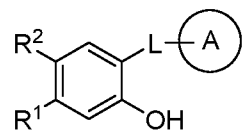
18. A method of treating a heat shock protein 90-mediated disease or condition in a subject in need thereof, comprising administering to the subject an effective amount of a compound of Appendix 1, 1', 2, 2', 3 or 4, or a pharmaceutically acceptable salt thereof, provided that the compound is not AUY-922, VER-50589, STA-9090 or 17-AAG, or a pharmaceutically acceptable salt of any of the foregoing.
19. The method of any one of claims 1-18, wherein the compound is isocotoin, or a pharmaceutically acceptable salt thereof.
20. The method of any one of claims 1, 3-8, 10-15 and 17, wherein the compound is represented by the following structural formula:



or a pharmaceutically acceptable salt thereof.

21. The method of any one of claims 1, 3-8, 10-15, 17 and 20, wherein L is heteroarylene.
22. The method of claim 21, wherein L is (C₅-C₆)heteroarylene.
23. The method of claim 22, wherein L is oxazolylene, pyrazolylene, pyrimidinylene or triazolylene.
24. The method of any one of claims 1, 3-8, 10-15, 17 and 20-23, wherein the heteroarylene of L is optionally substituted with one substituent selected from halo, alkyl, haloalkyl, amino, alkylamino, dialkylamino or carboxamido.
25. The method of any one of claims 1, 3-8, 10-15, 17 and 20-24, wherein Ring A is phenyl.

26. The method of any one of claims 1, 3-8, 10-15, 17 and 20-24, wherein Ring A is heteroaryl.
27. The method of claim 26, wherein Ring A is indolyl, pyrazolyl, benzofuranyl, benzothiazolyl, or thiazolyl.
28. The method of any one of claims 1, 3-8, 10-15, 17 and 20-27, wherein Ring A is optionally substituted with one or more substituents independently selected from halo, hydroxy, alkyl, alkoxy, $-(CH_2)_{0-2}$ -aryl, $-(CH_2)_{0-2}$ -heteroaryl, $-(CH_2)_{0-2}$ -cycloalkyl, or $-(CH_2)_{0-2}$ -heterocyclyl, carboxy or $-O(CH_2)_mO-$.
29. The method of any one of claims 1, 3-8, 10-15, 17 and 20-28, wherein m is 1, 2 or 3.
30. The method of any one of claims 1, 3-8, 10-15, 17 and 20-29, wherein n is 0, 1 or 2.
31. The method of any one of claims 1, 3-8, 10-15, 17 and 20-30, wherein R is hydrogen.
32. The method of any one of claims 1, 3-8, 10-15, 17 and 20-31, wherein R^1 is halo, hydroxy, alkyl, alkoxy, alkenoxy, alkynoxy, $-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl.
33. The method of any one of claims 1, 3-8, 10-15, 17 and 20-31, wherein the compound is represented by the following structural formula:



or a pharmaceutically acceptable salt thereof, wherein:

R^2 is hydrogen, halo, hydroxy, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenoxy, alkynoxy, $-(CH_2)_{0-2}$ -aryl, or $-(CH_2)_{0-2}$ -heteroaryl.

34. The method of claim 33, wherein R^1 is hydroxy, alkoxy, haloalkoxy, alkenoxy or alkynoxy.
35. The method of claim 33 or 34, wherein R^2 is hydrogen, halo, alkyl or haloalkyl.

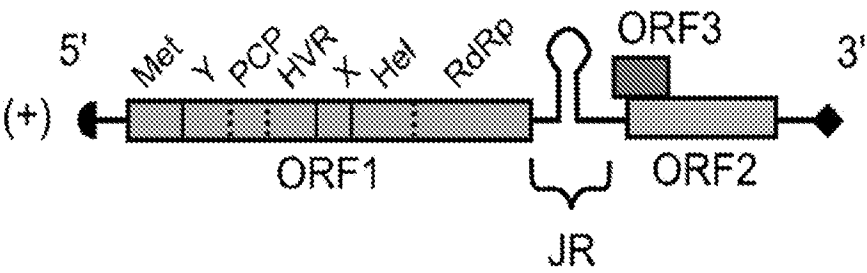


FIG. 1

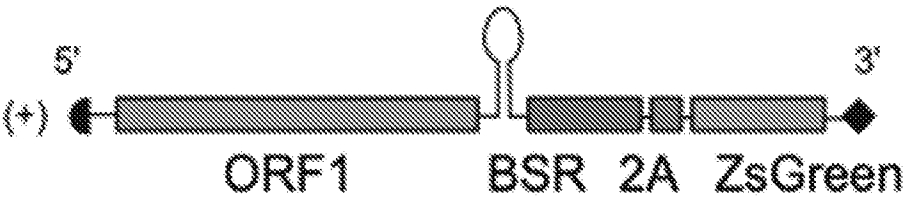


FIG. 2A

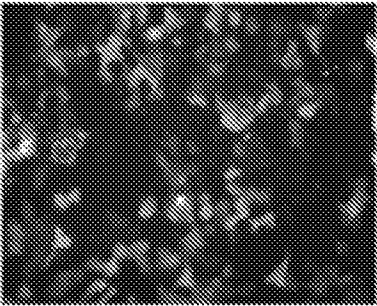


FIG. 2B

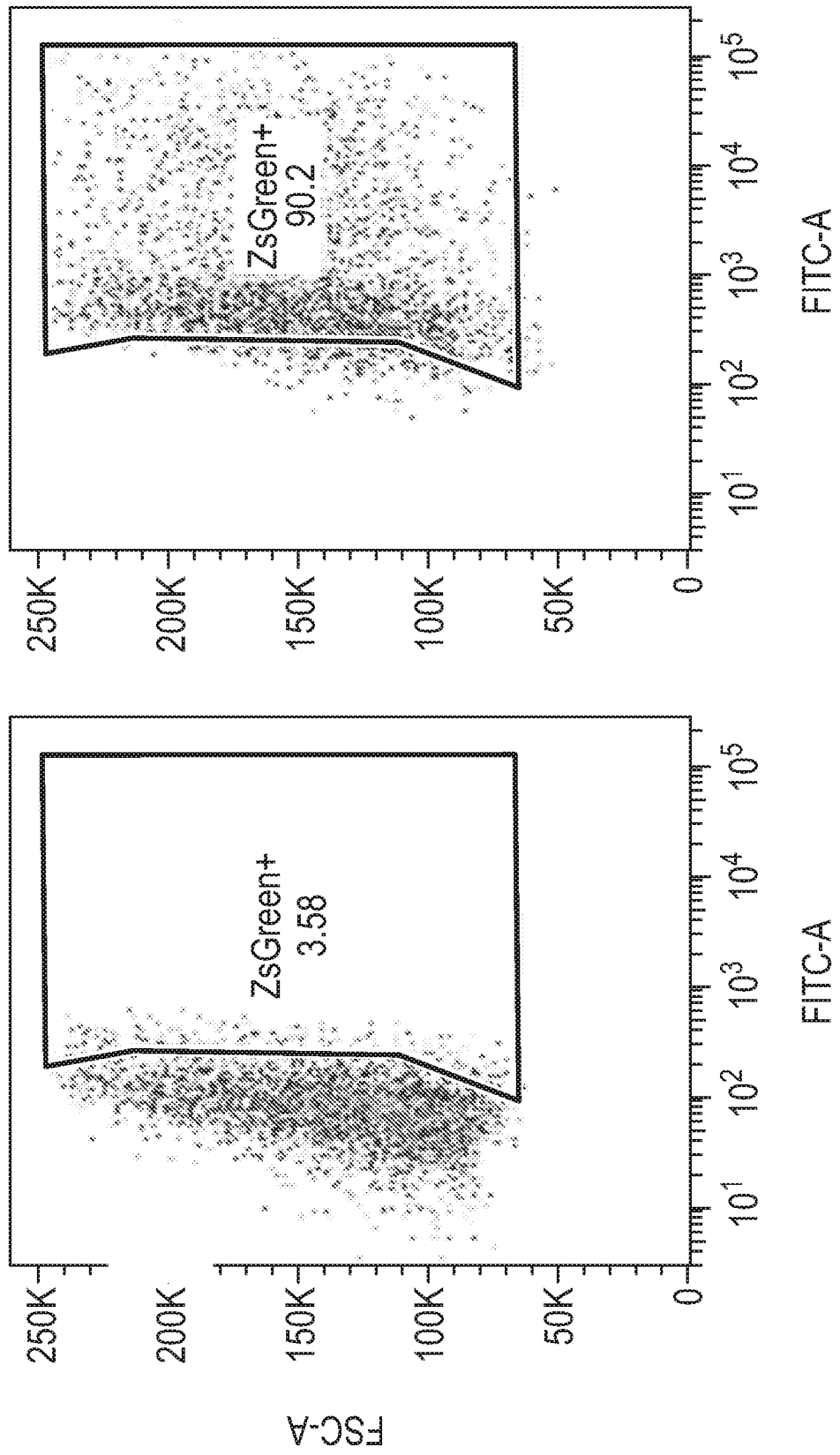


FIG. 2C

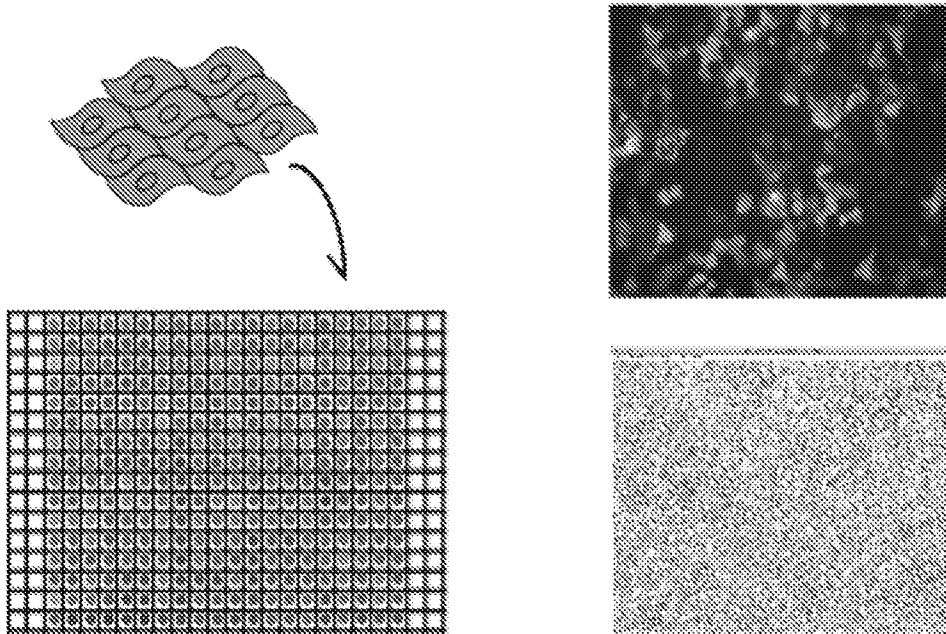


FIG. 3A

FIG. 3B

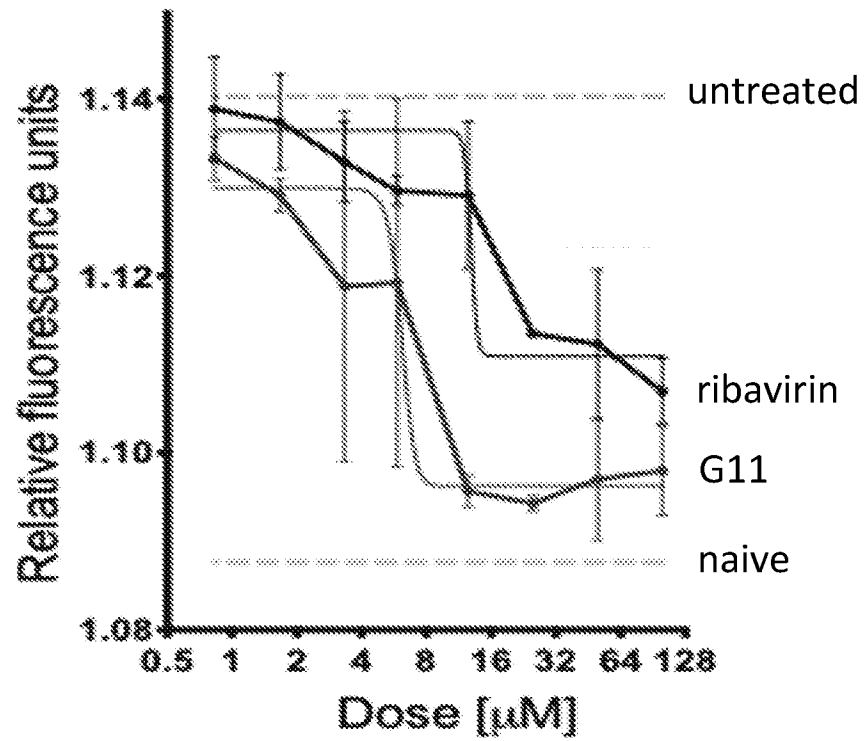


FIG. 3C

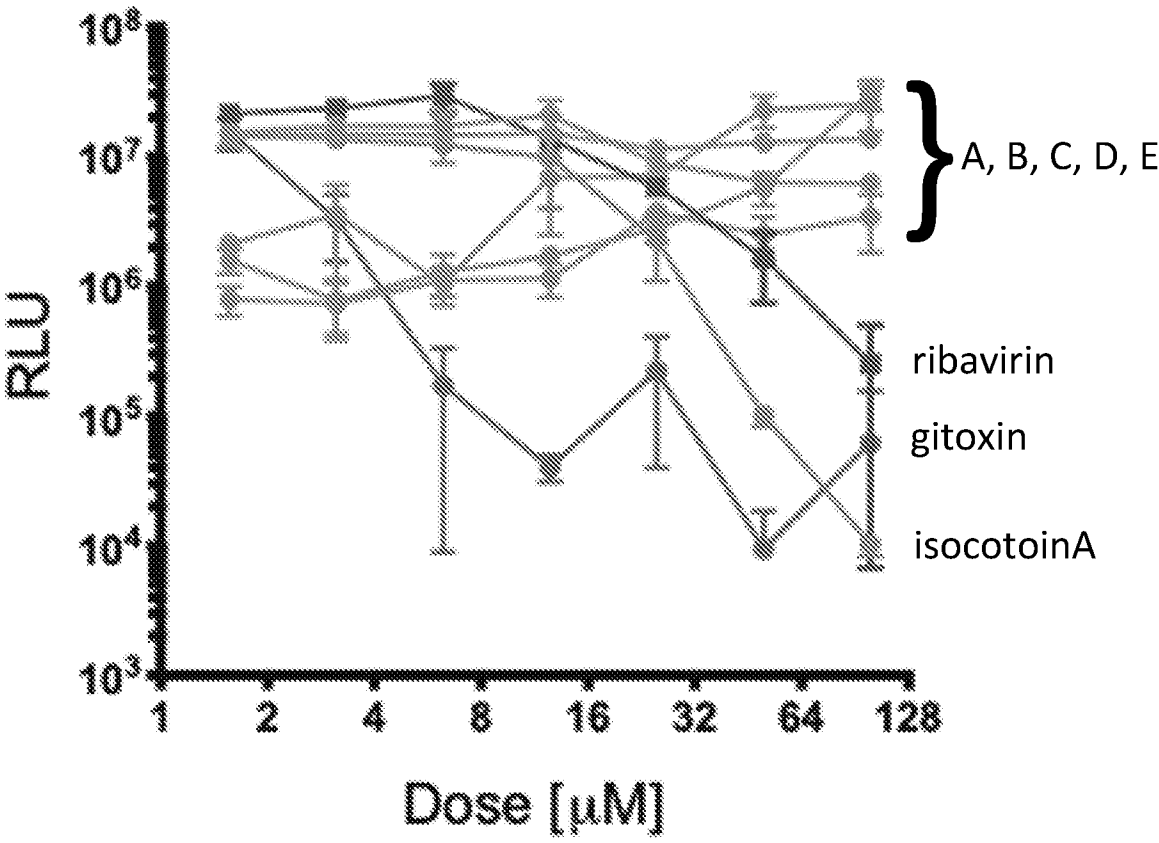


FIG. 3D

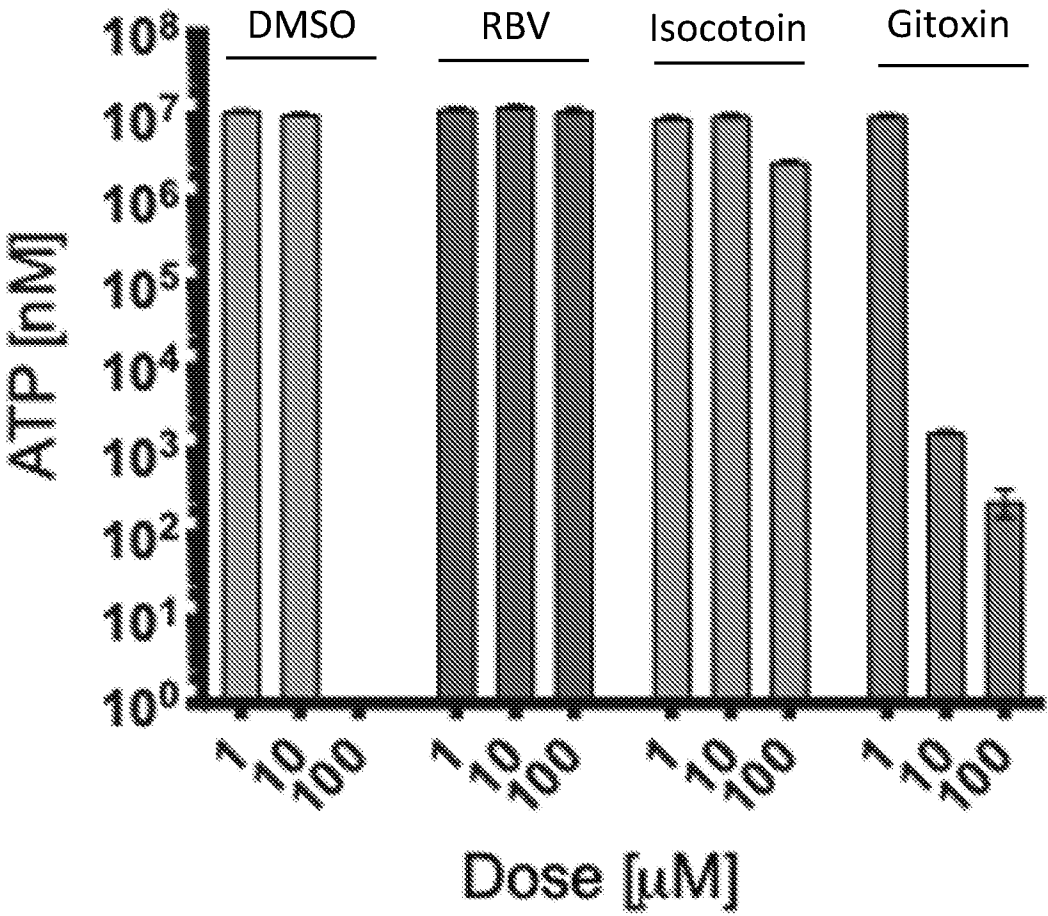


FIG. 3E

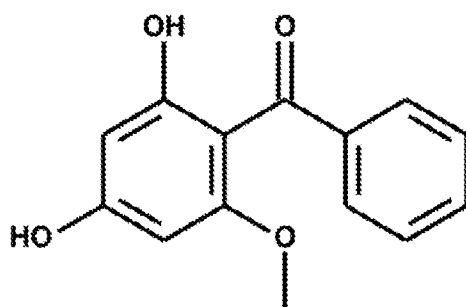


FIG. 4A

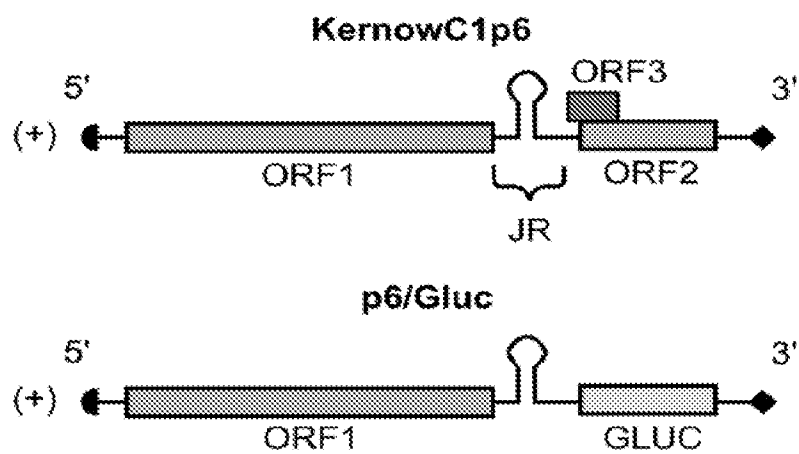


FIG. 4B

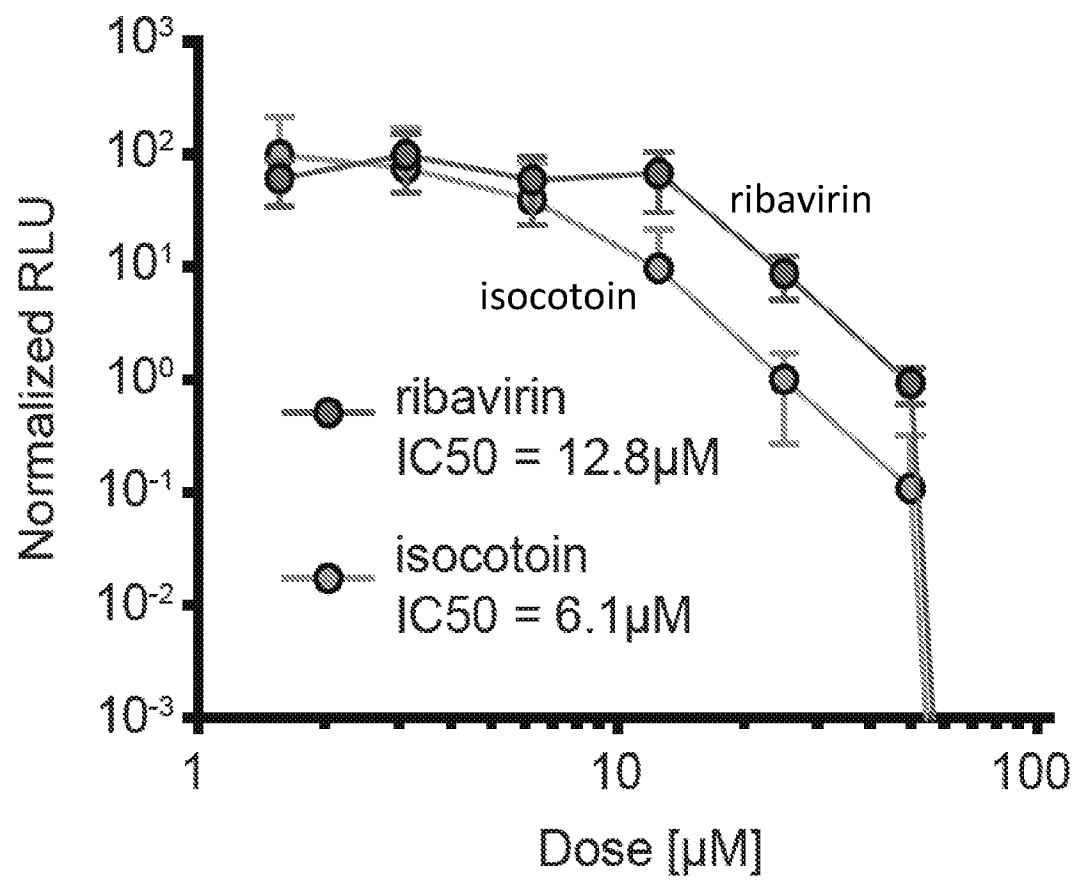


FIG. 4C

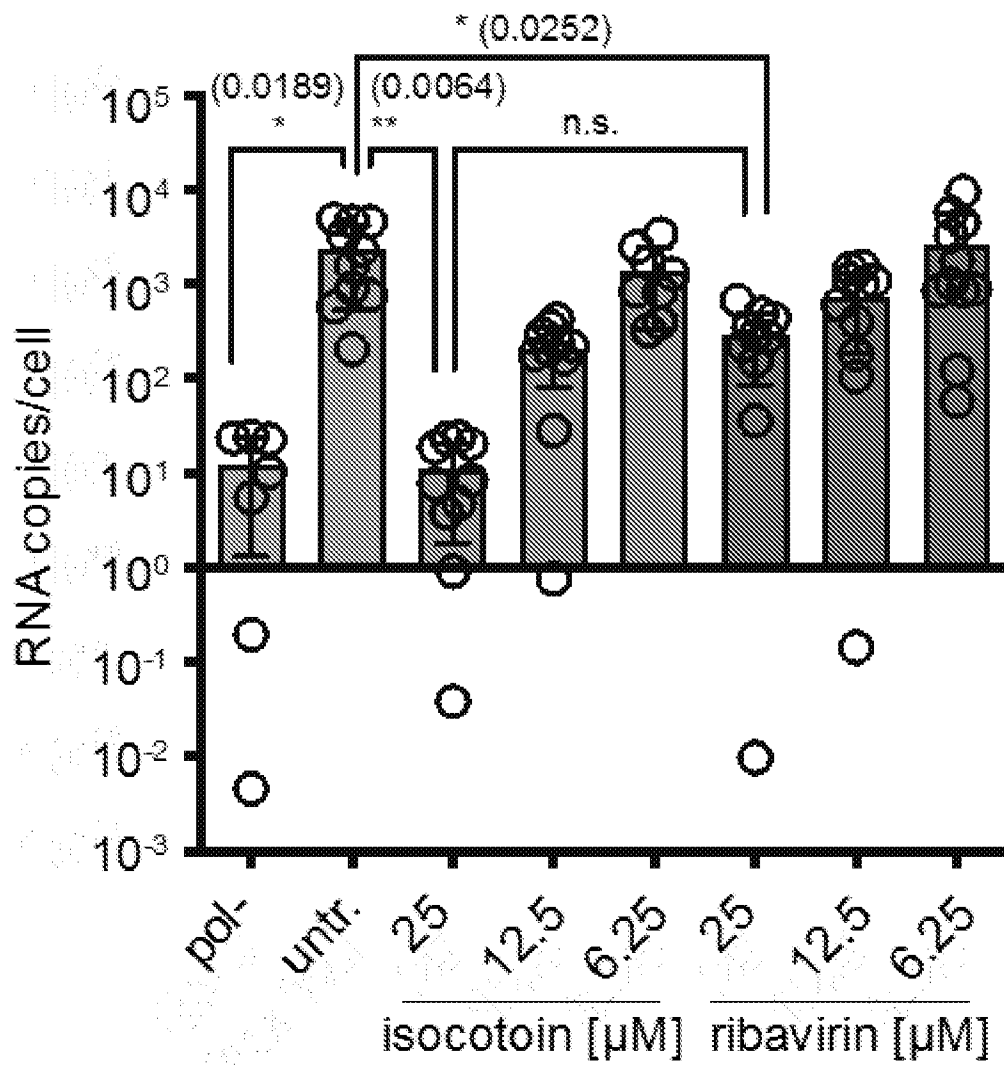


FIG. 4D



FIG. 4E

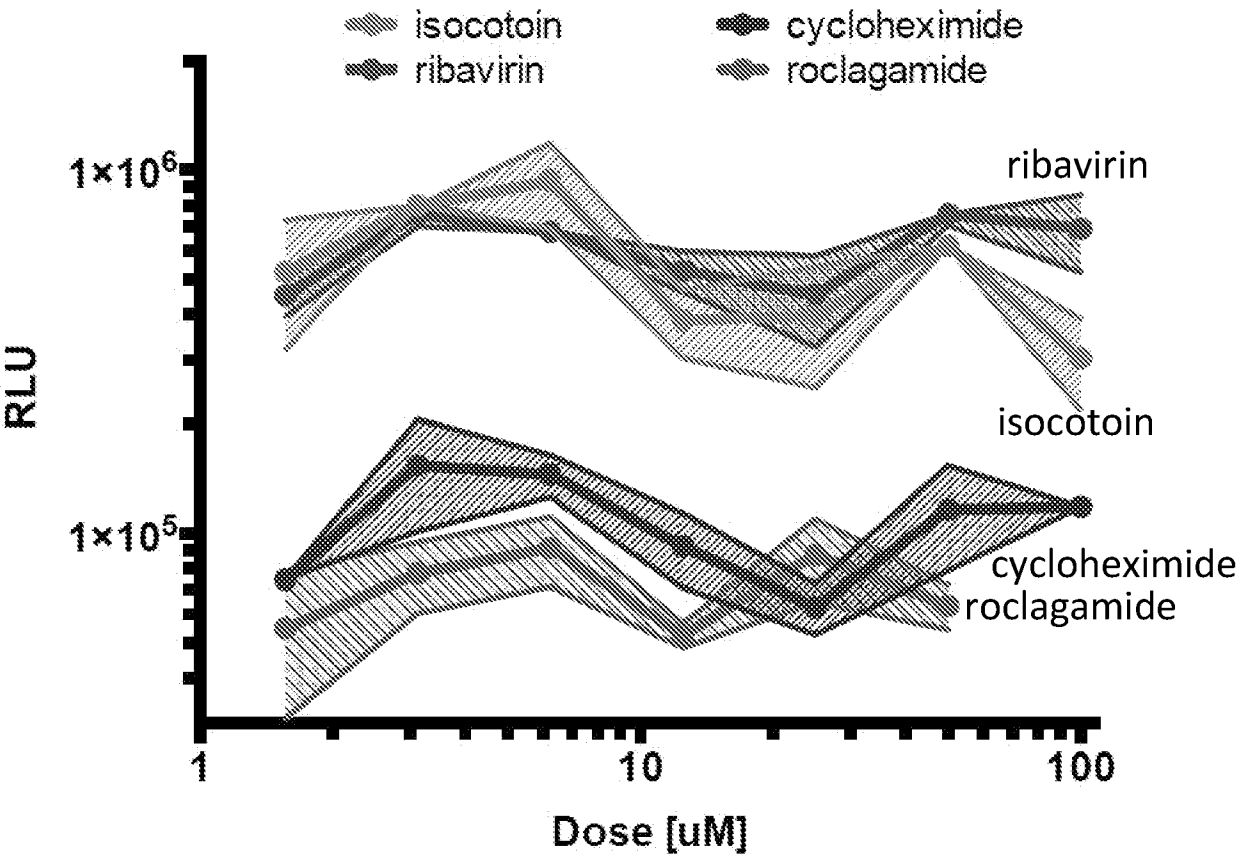


FIG. 4F

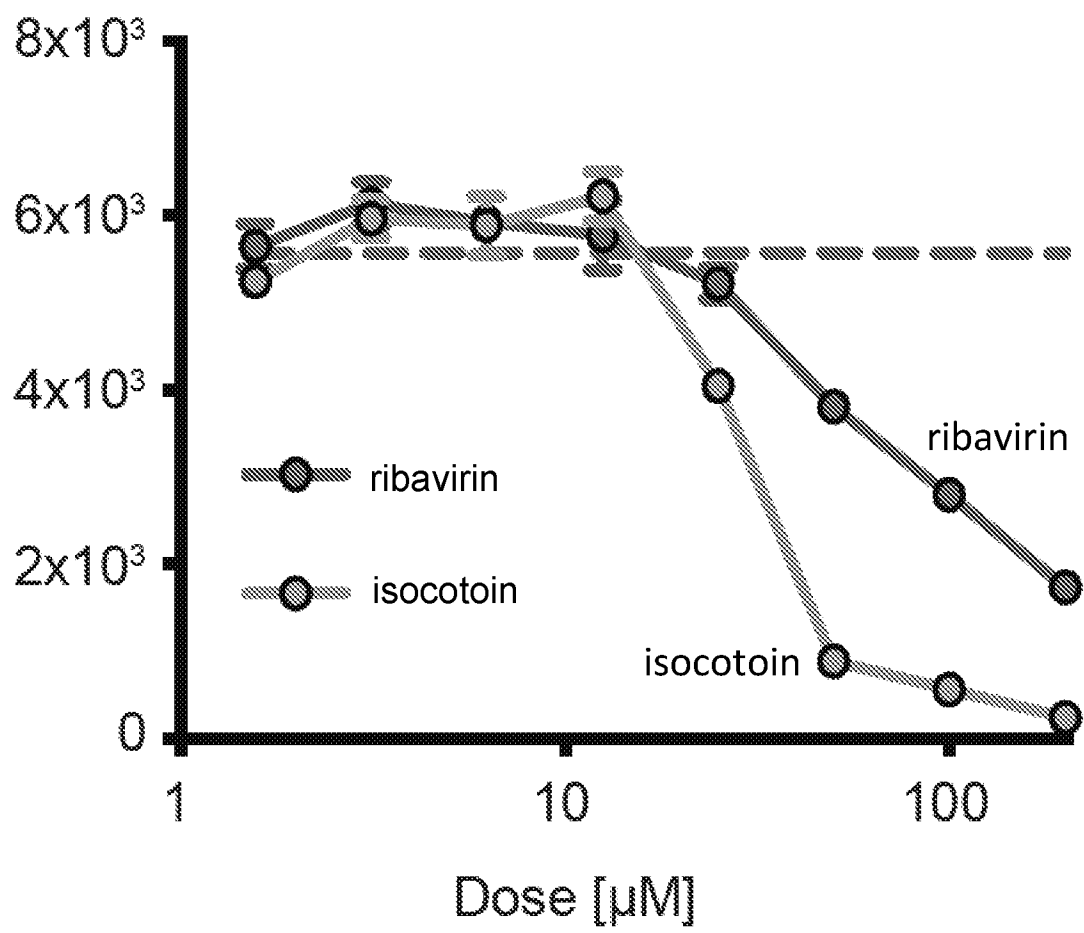


FIG. 4G

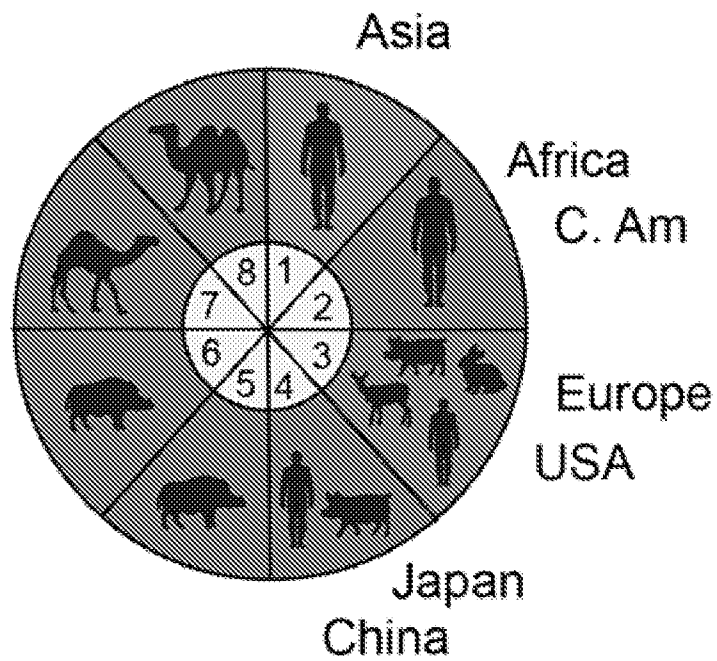


FIG. 5A

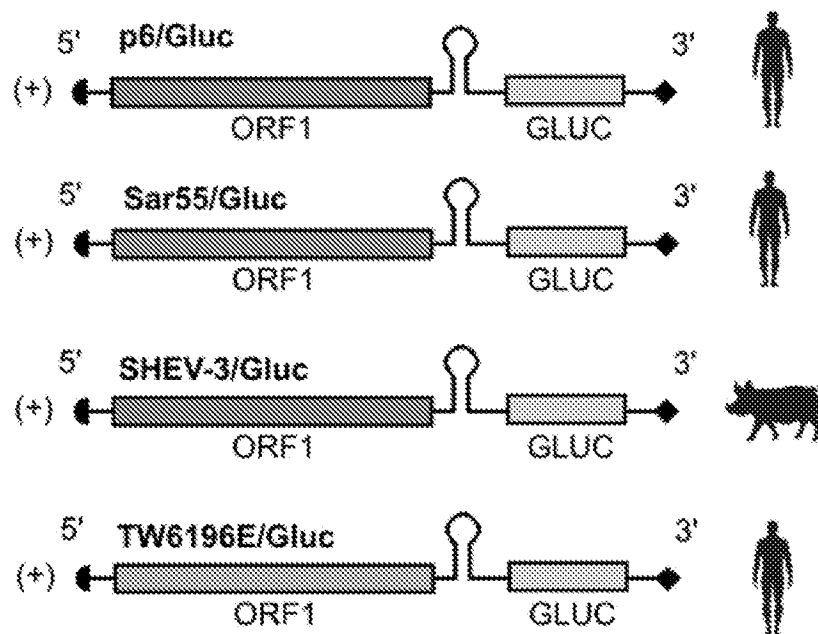


FIG. 5B

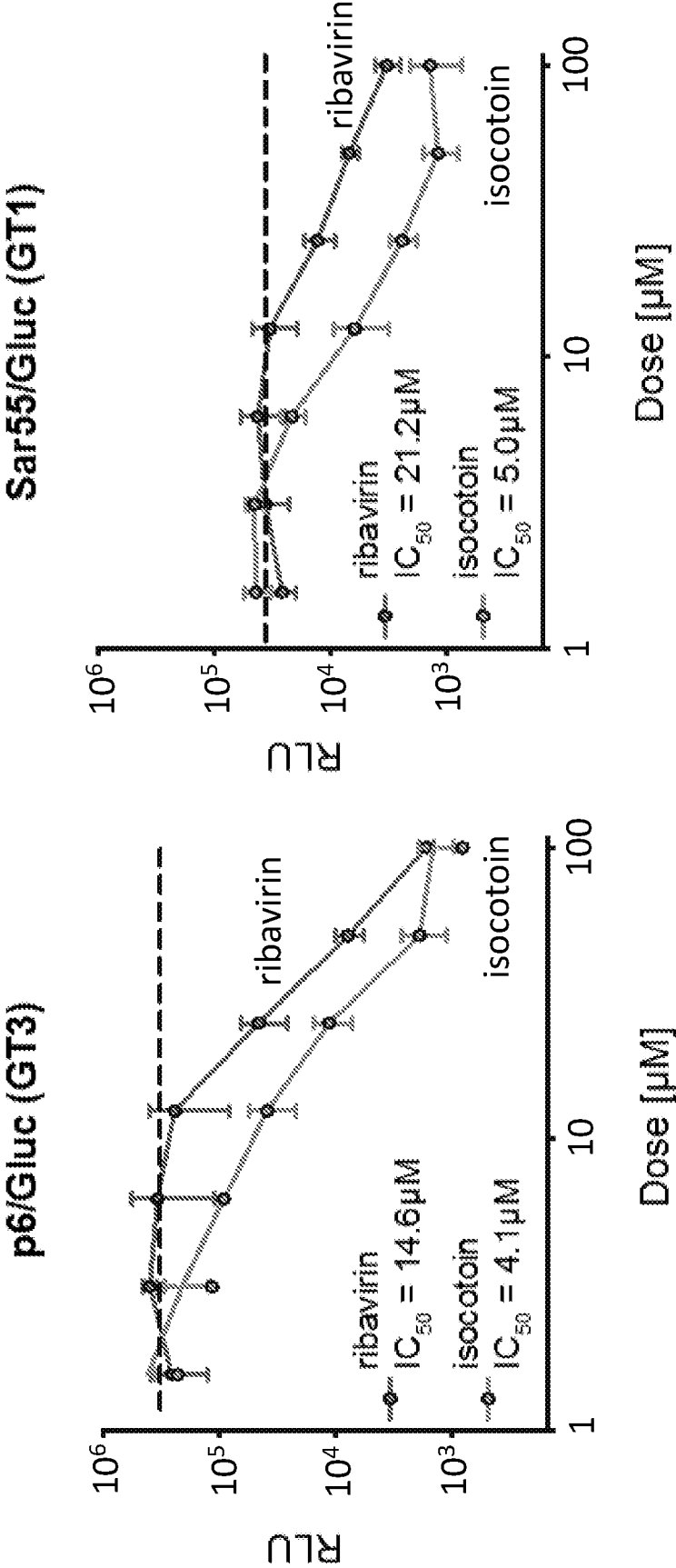


FIG. 5C

FIG. 5D

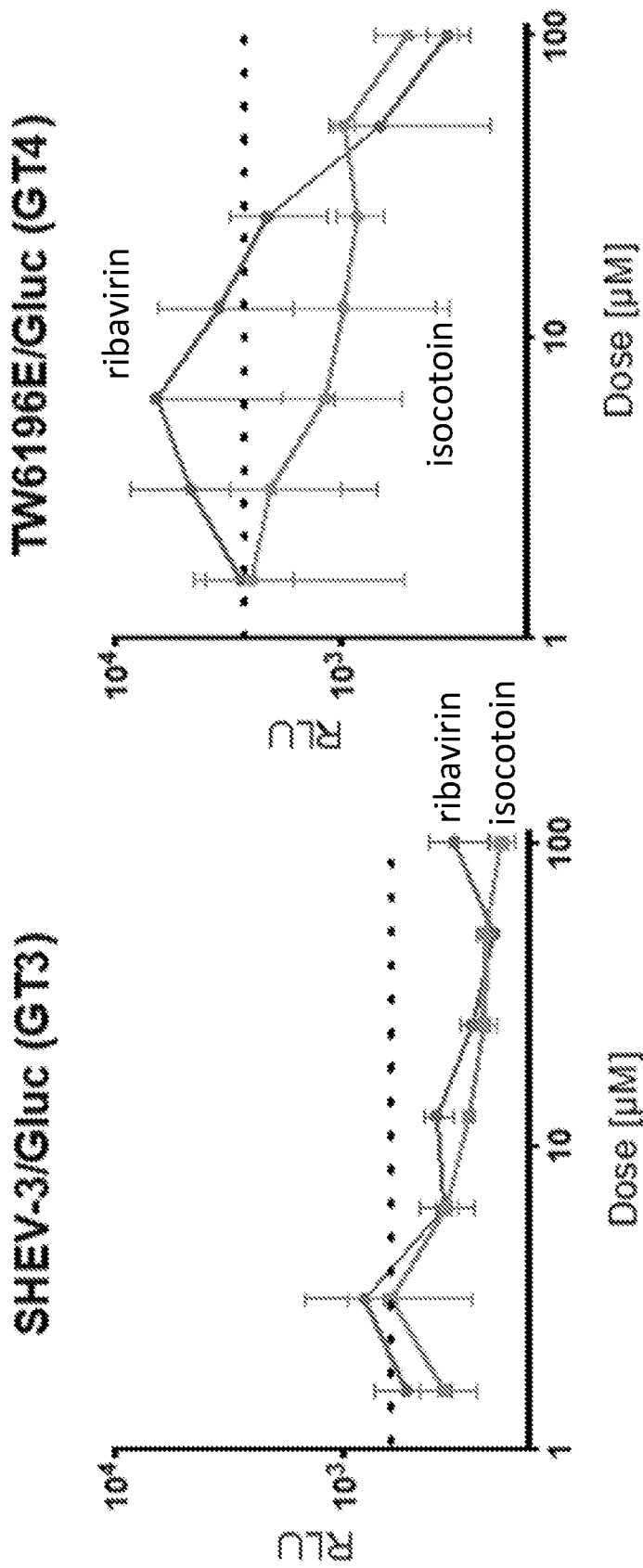


FIG. 5F

FIG. 5E

IC50 [μM]	Kc1p6/Gluc	Sar55/Gluc	SHEV-3/Gluc	TW6196E/Gluc
isocotoin	4.134±1.213	4.952±1.232	6.130 ± [v wide]	3.056±3.844
ribavirin	14.63±1.400	21.26±1.358	5.933±[v wide]	24.45±1.658

FIG. 5G

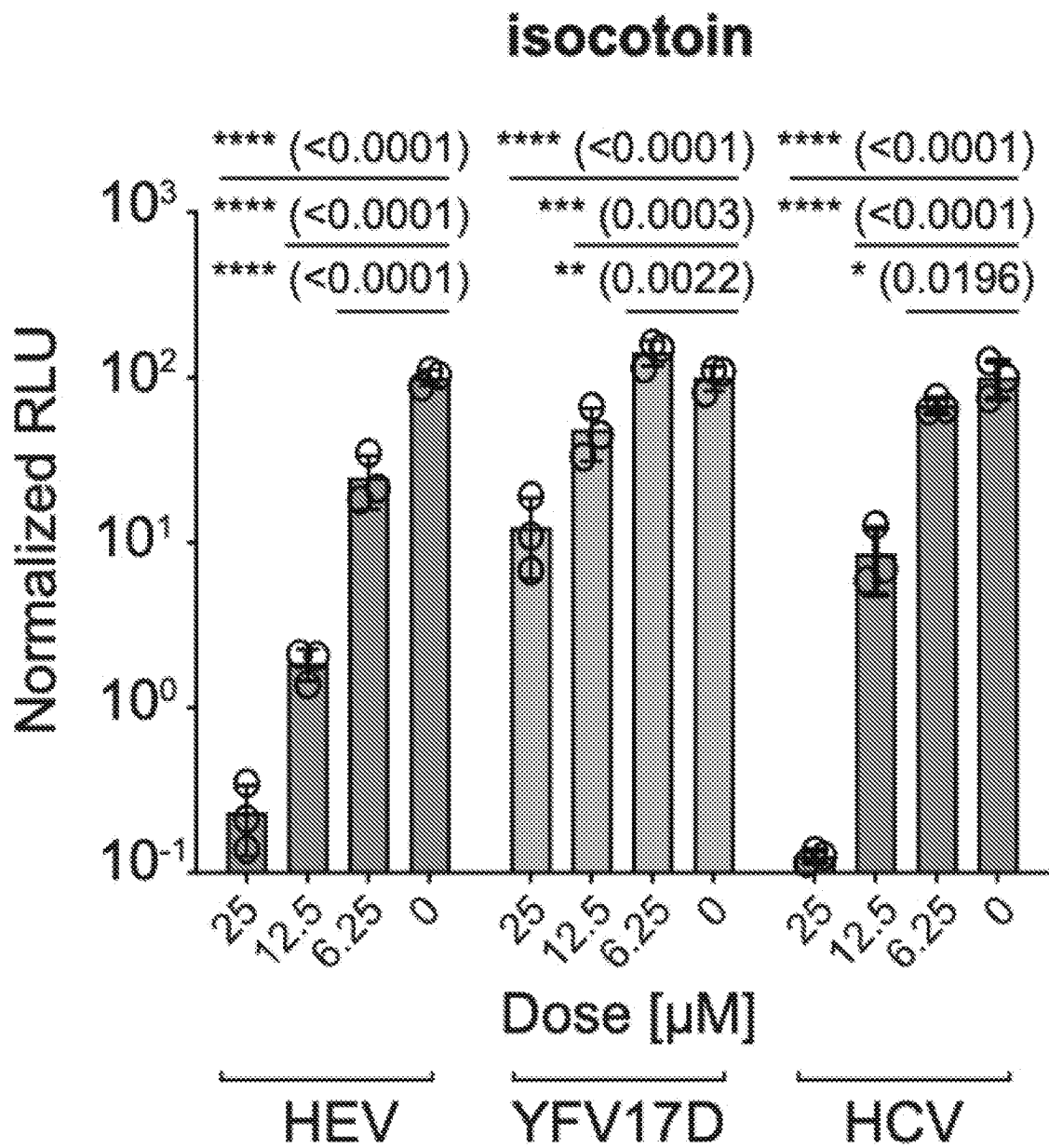


FIG. 6A

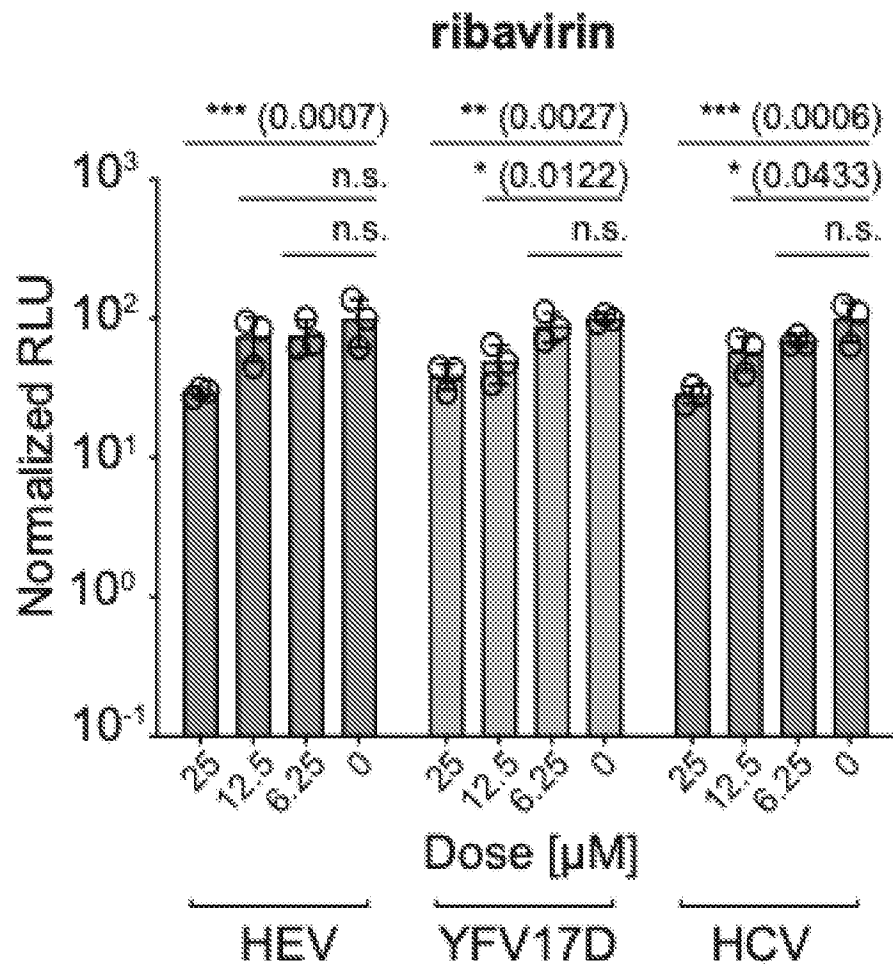


FIG. 6B

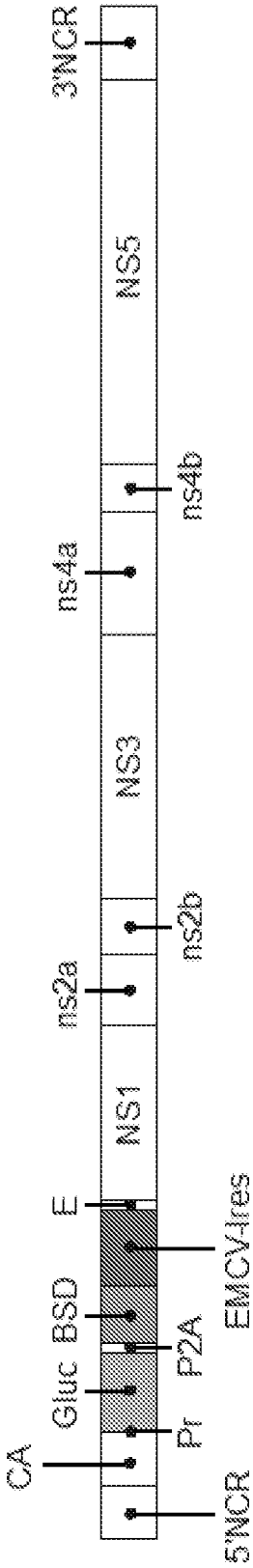


FIG. 6C

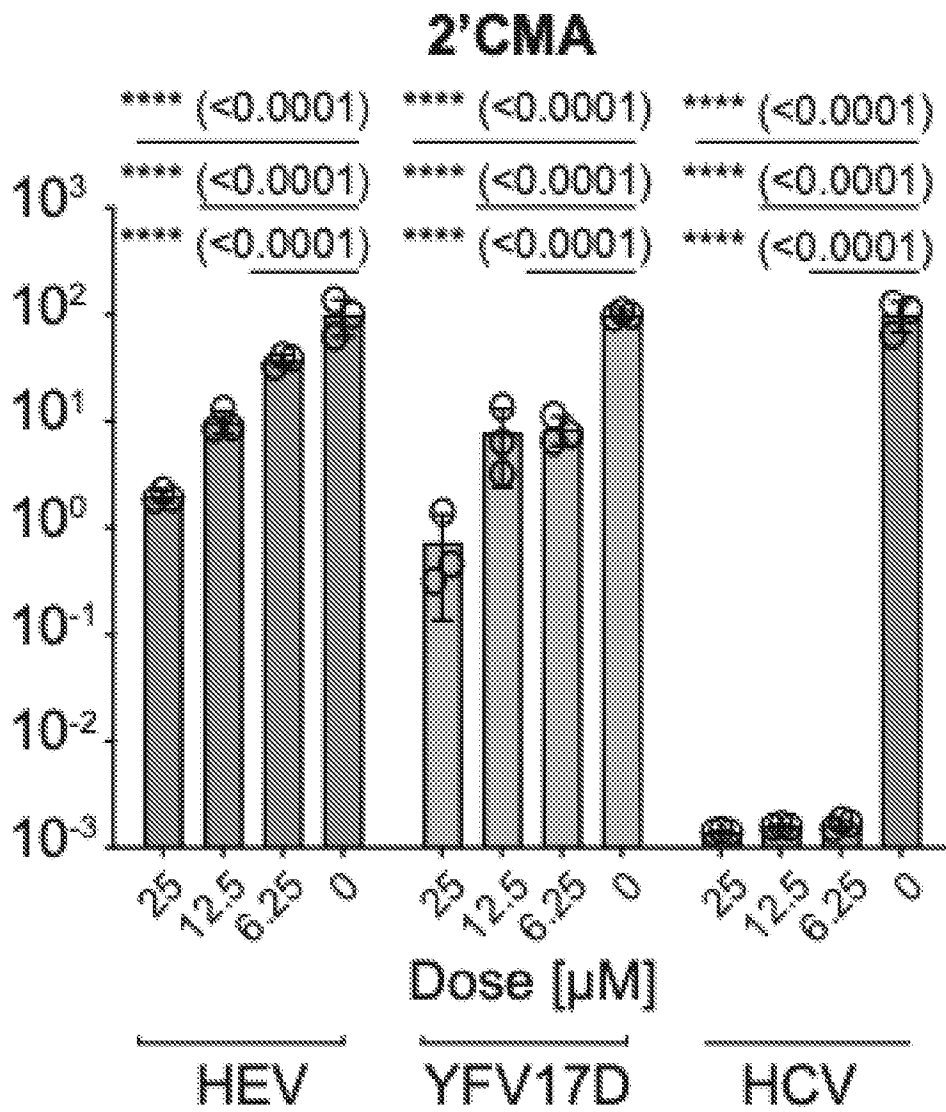


FIG. 6D

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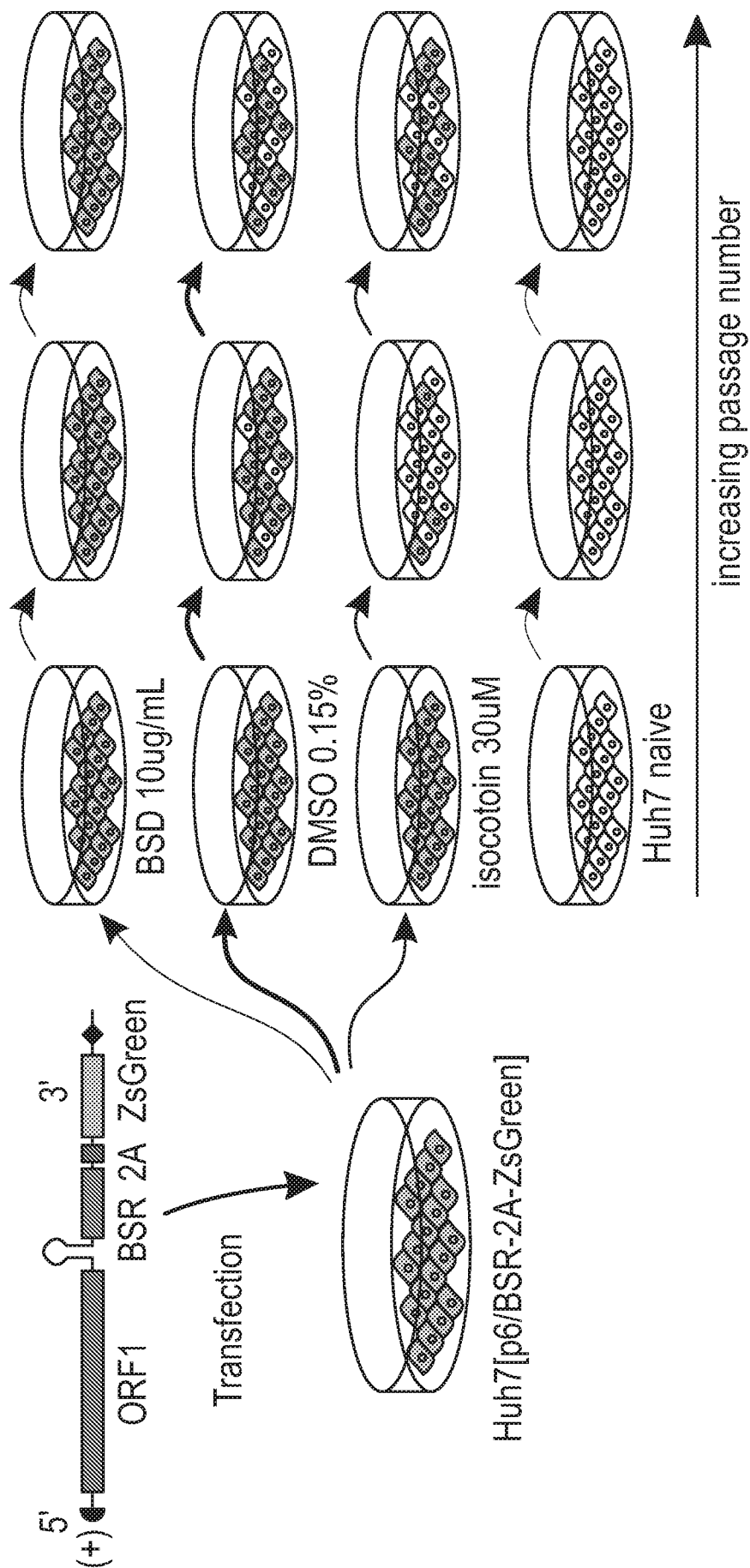


FIG. 7A

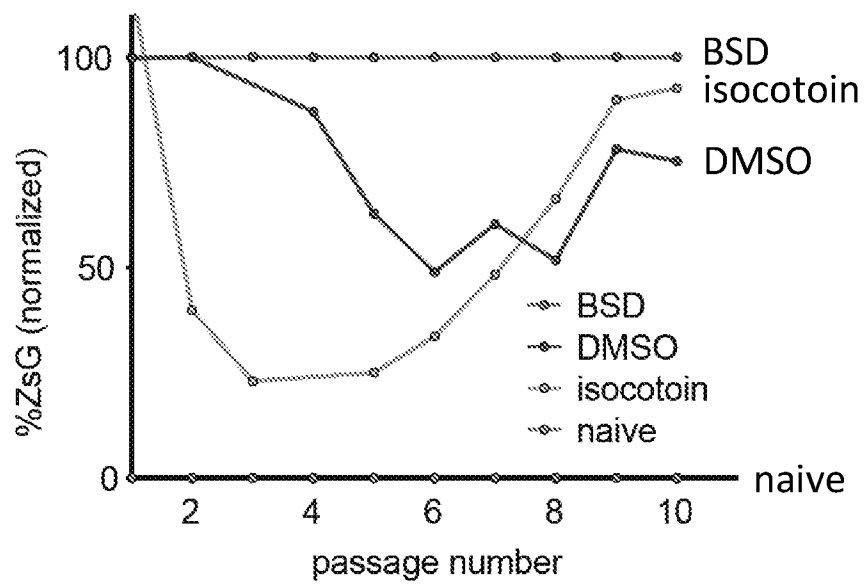


FIG. 7B

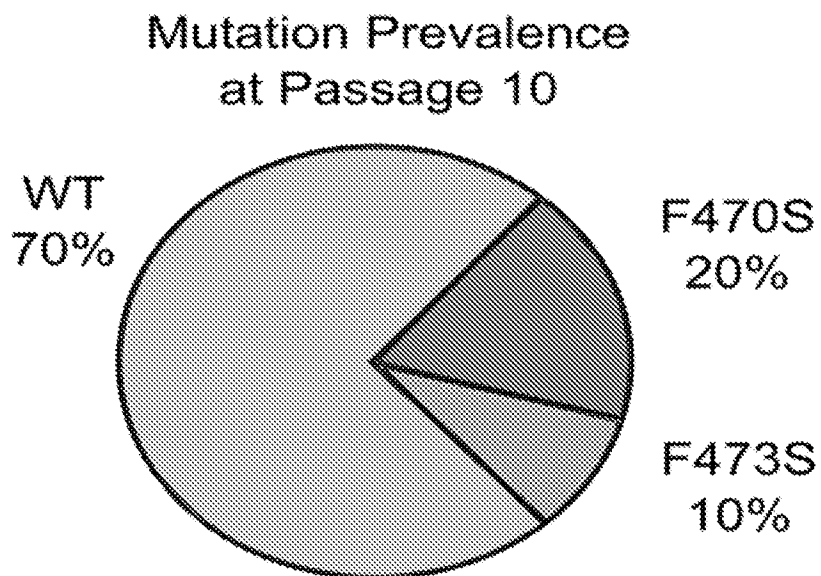


FIG. 7C

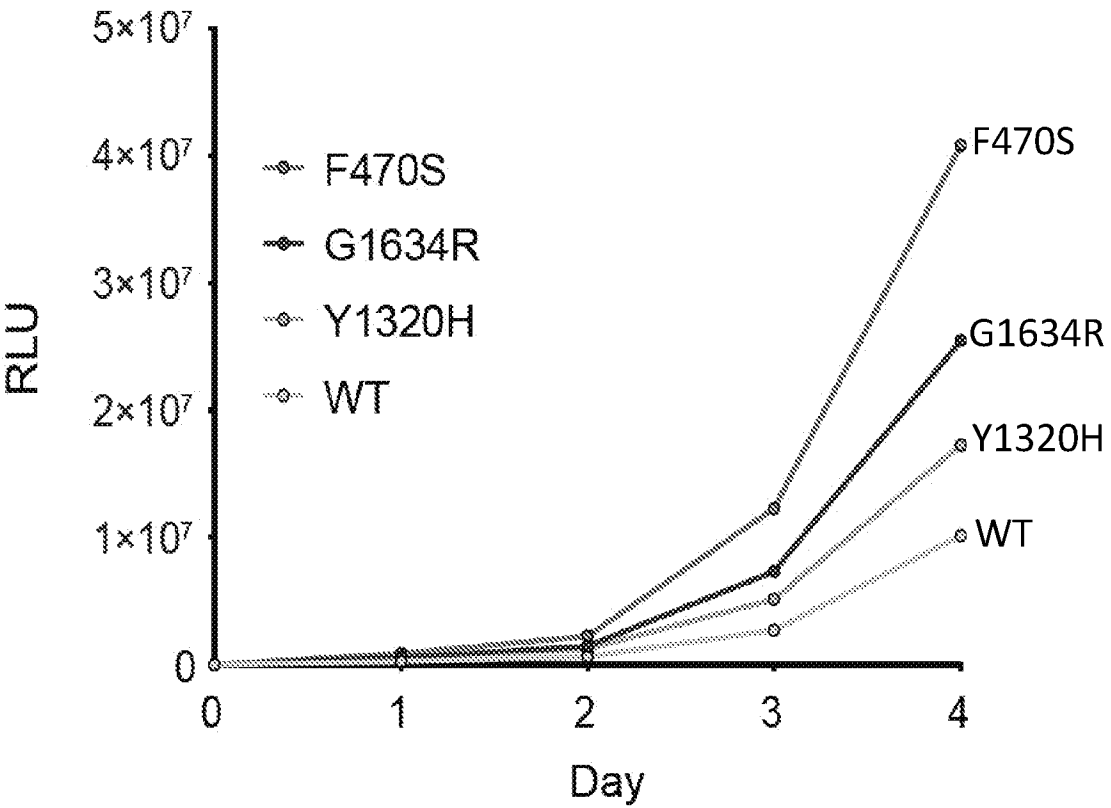


FIG. 7D

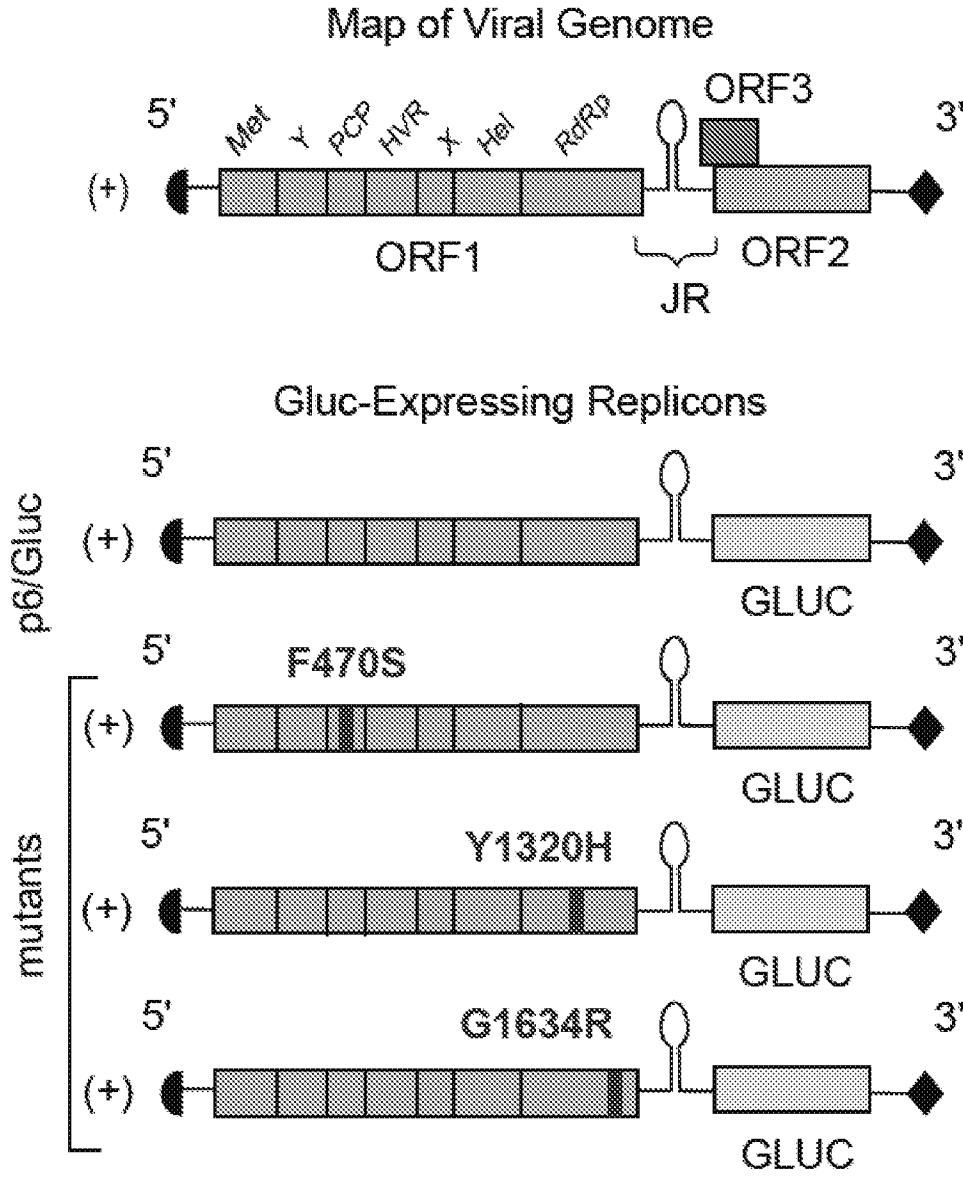


FIG. 7E

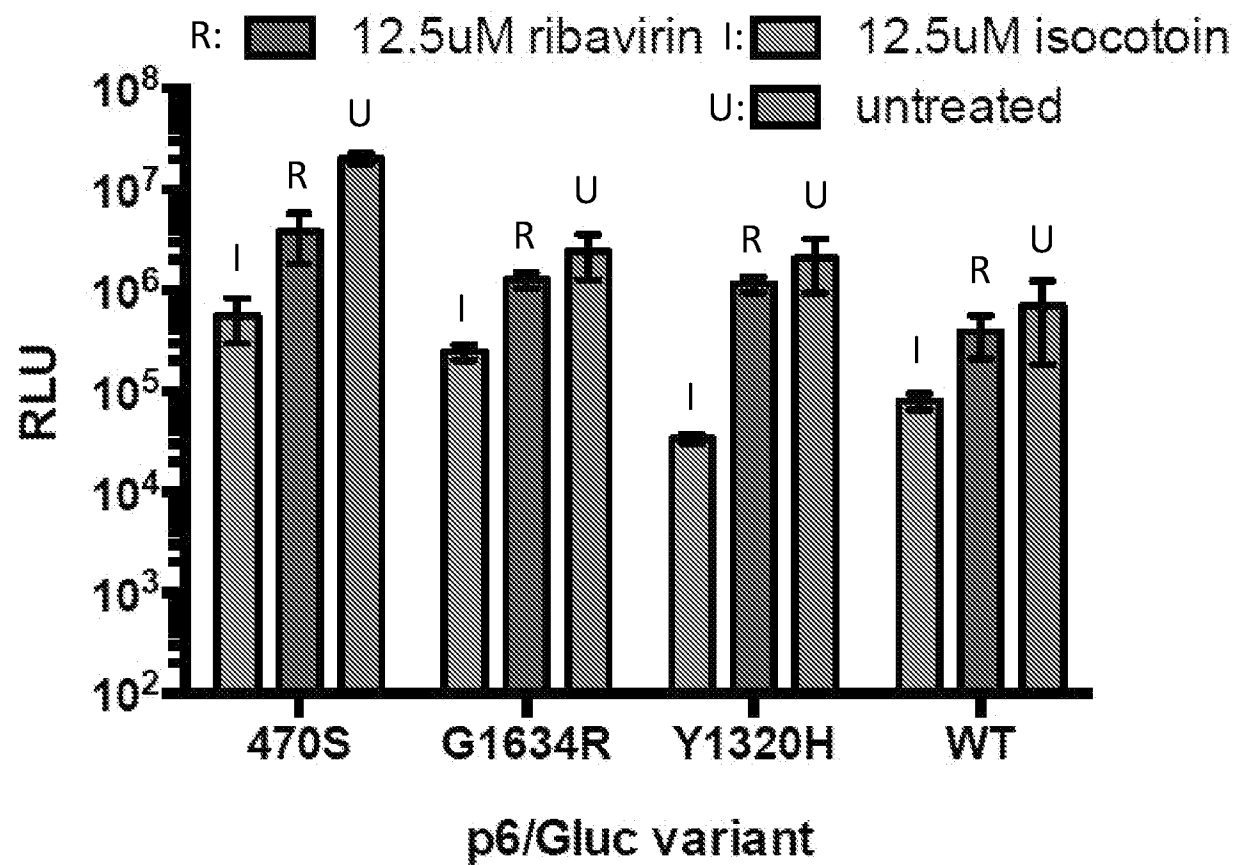


FIG. 7F

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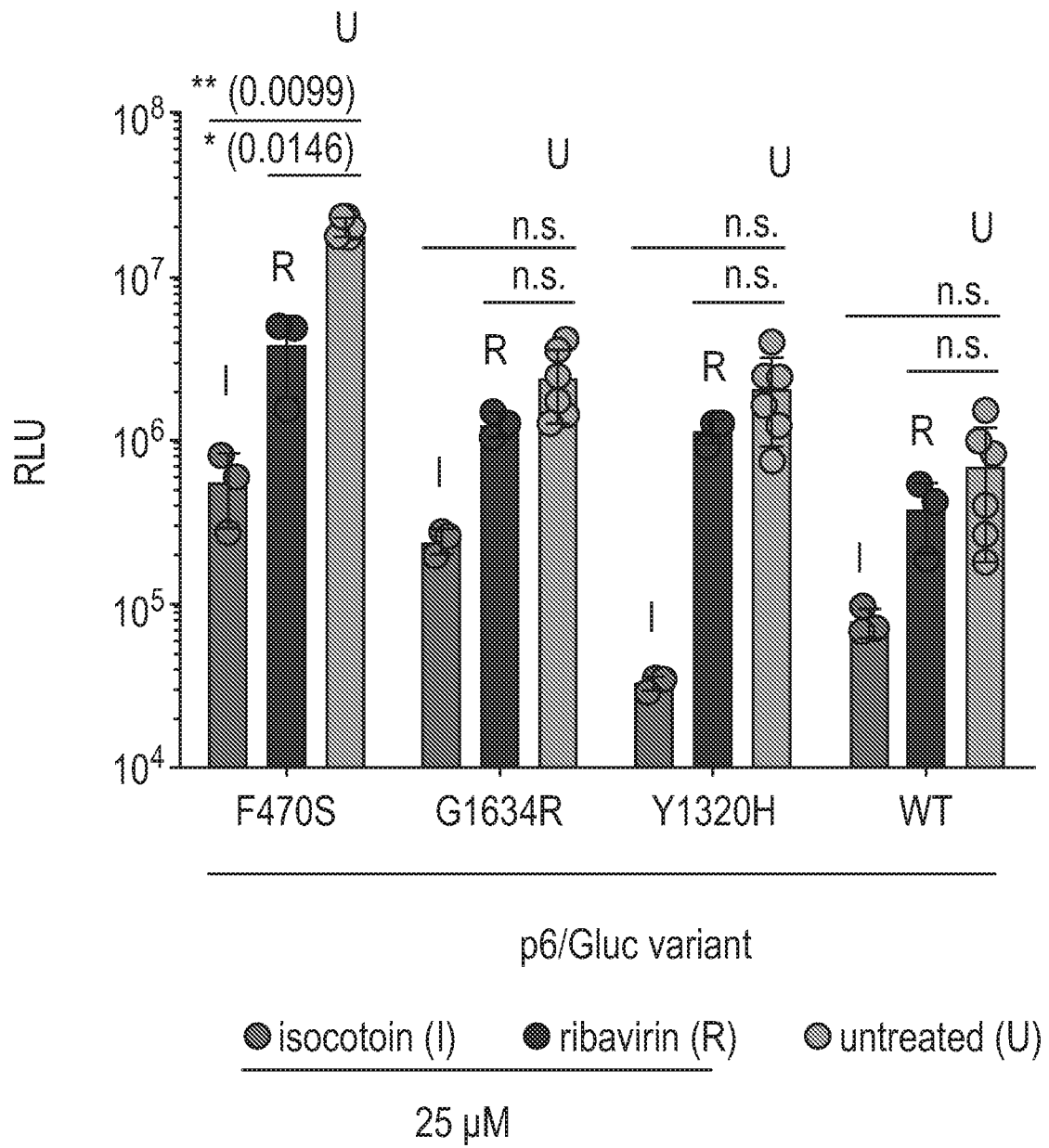


FIG. 7G

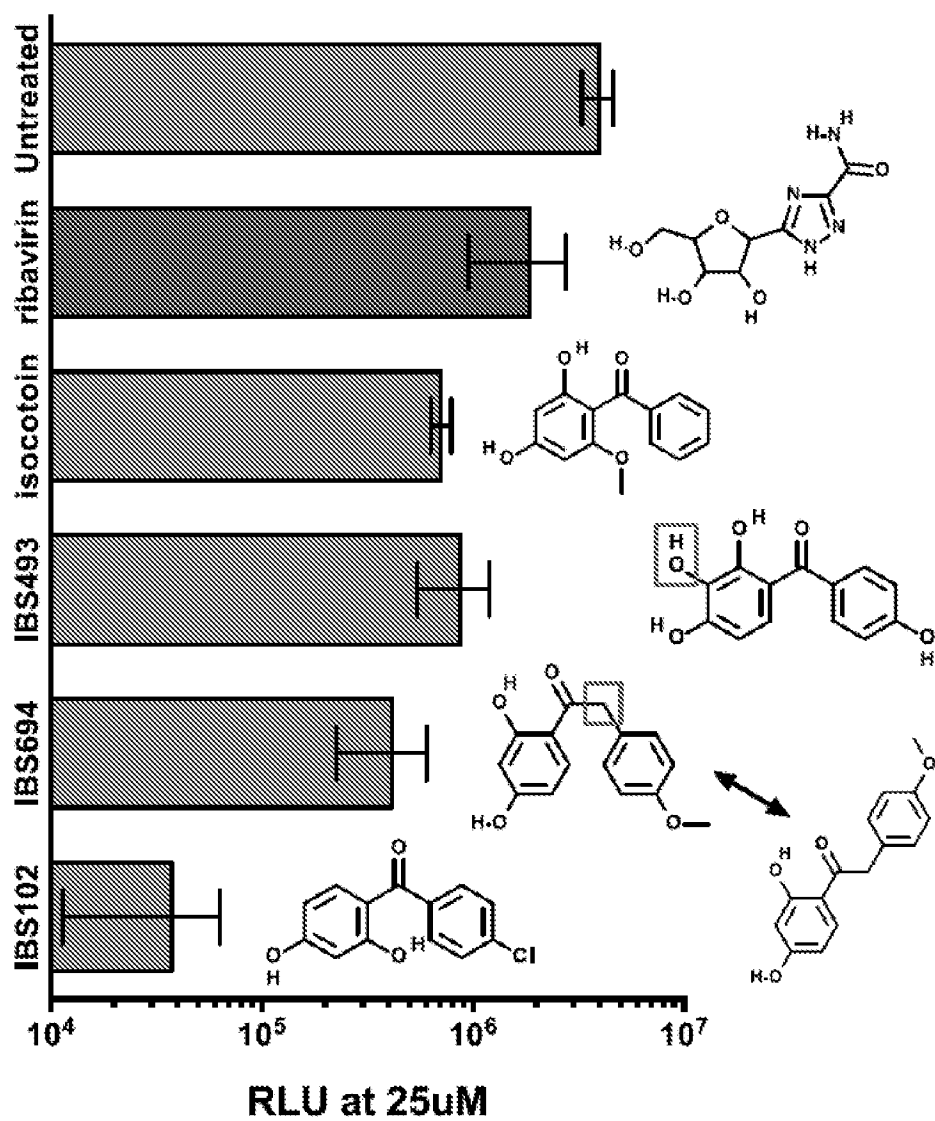


FIG. 8A

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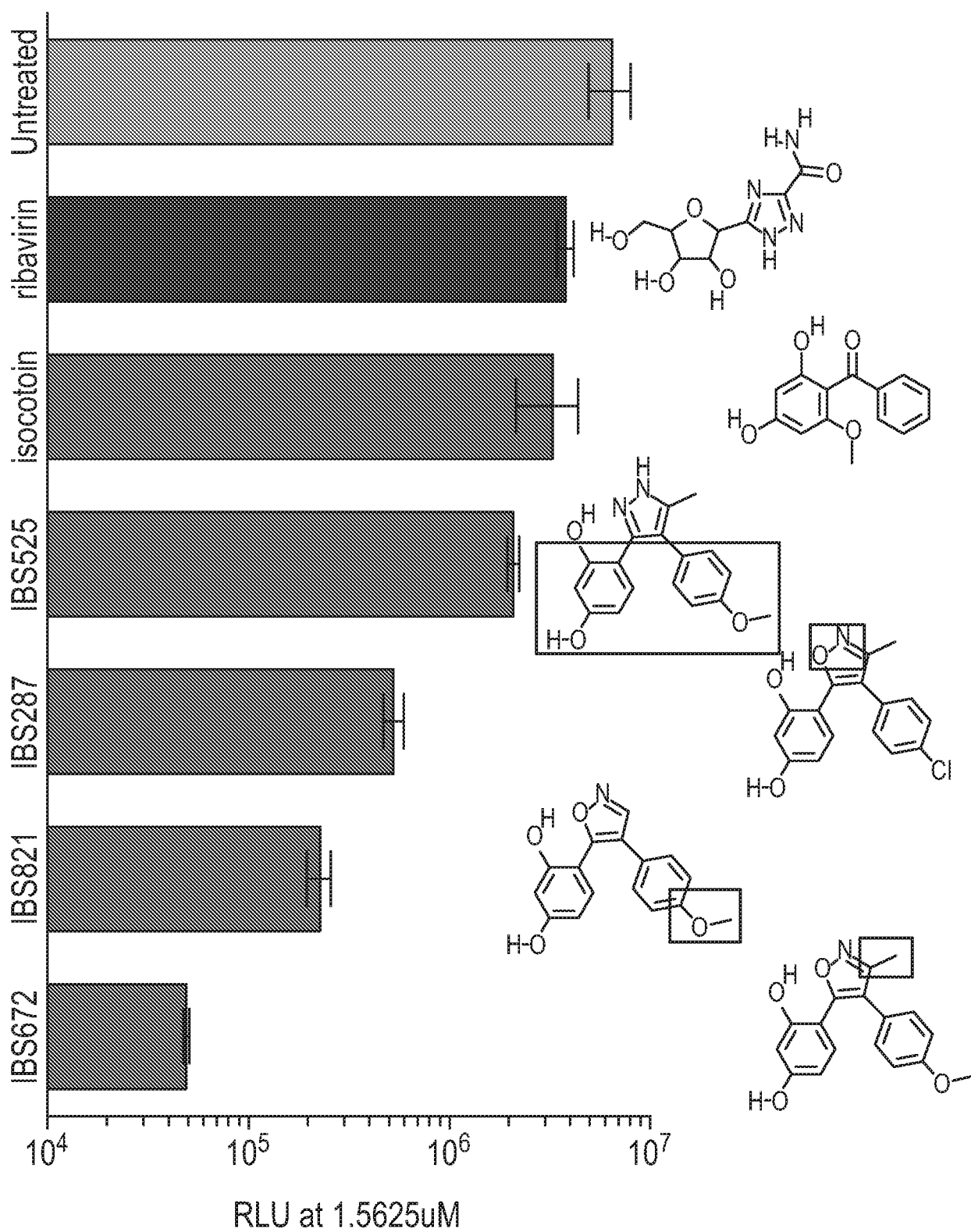


FIG. 8B

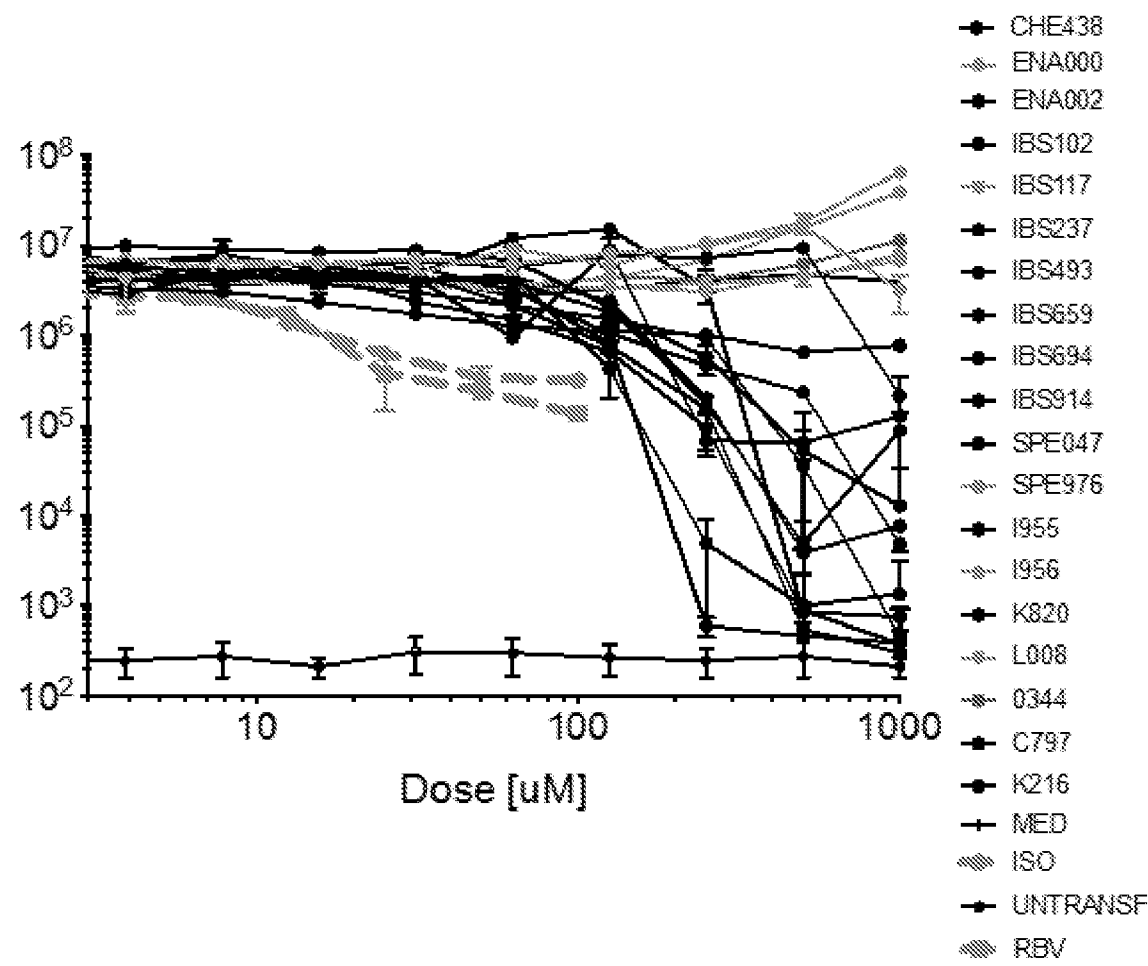


FIG. 9

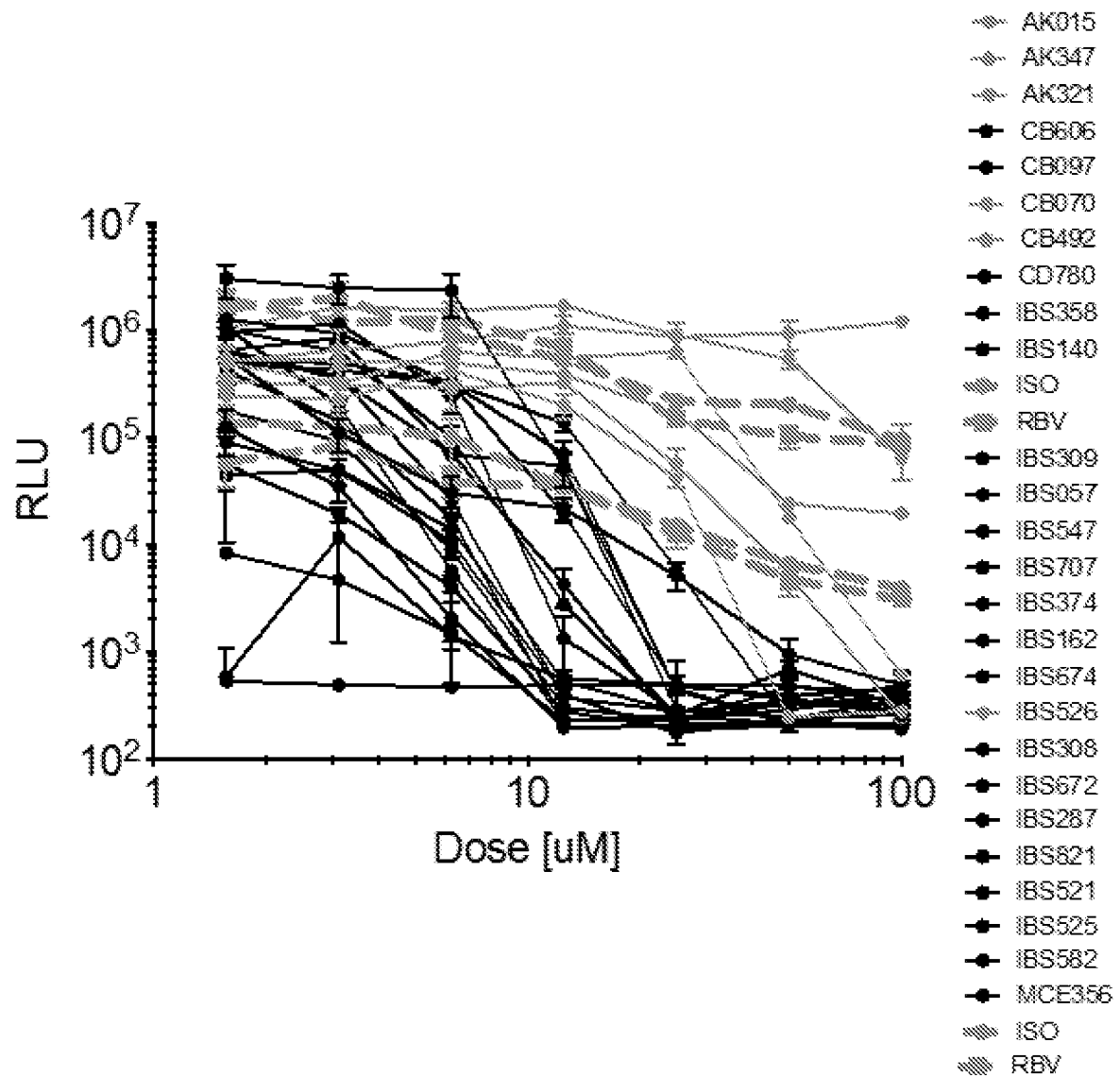


FIG. 10A

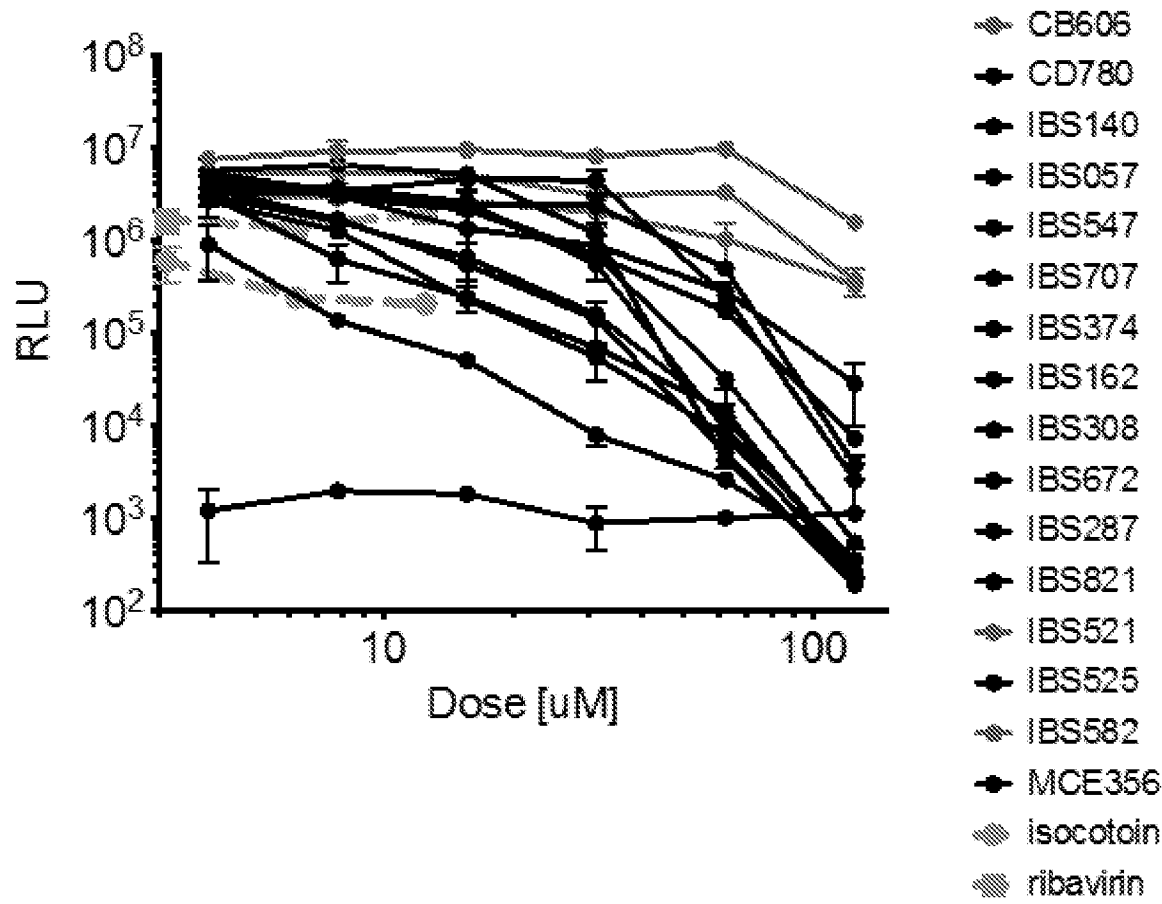


FIG. 10B

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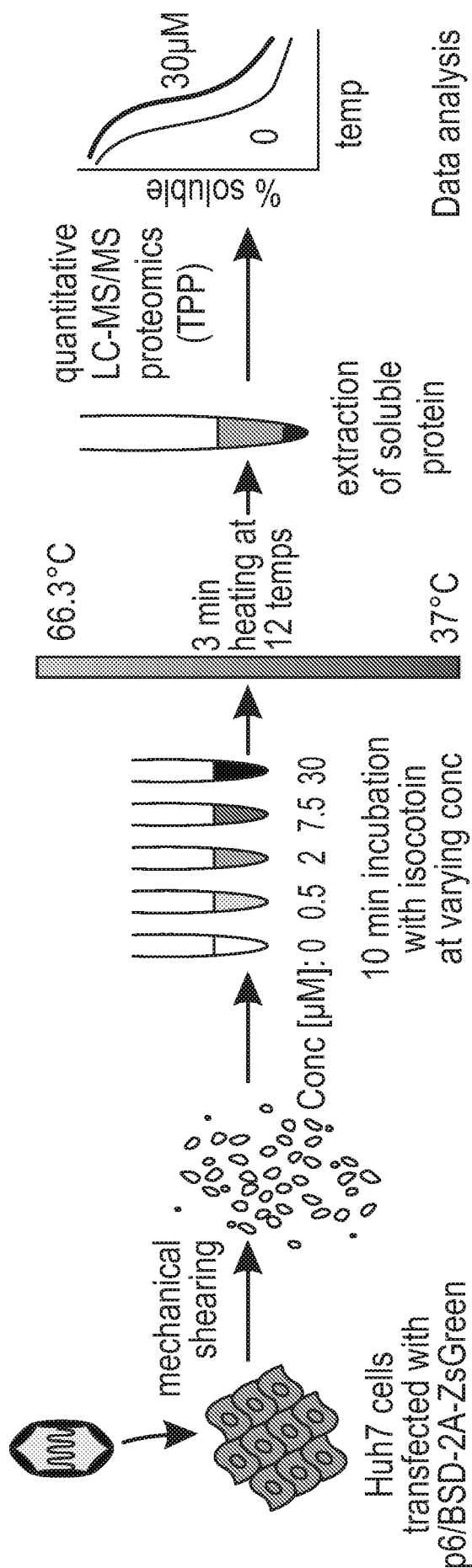


FIG. 11A

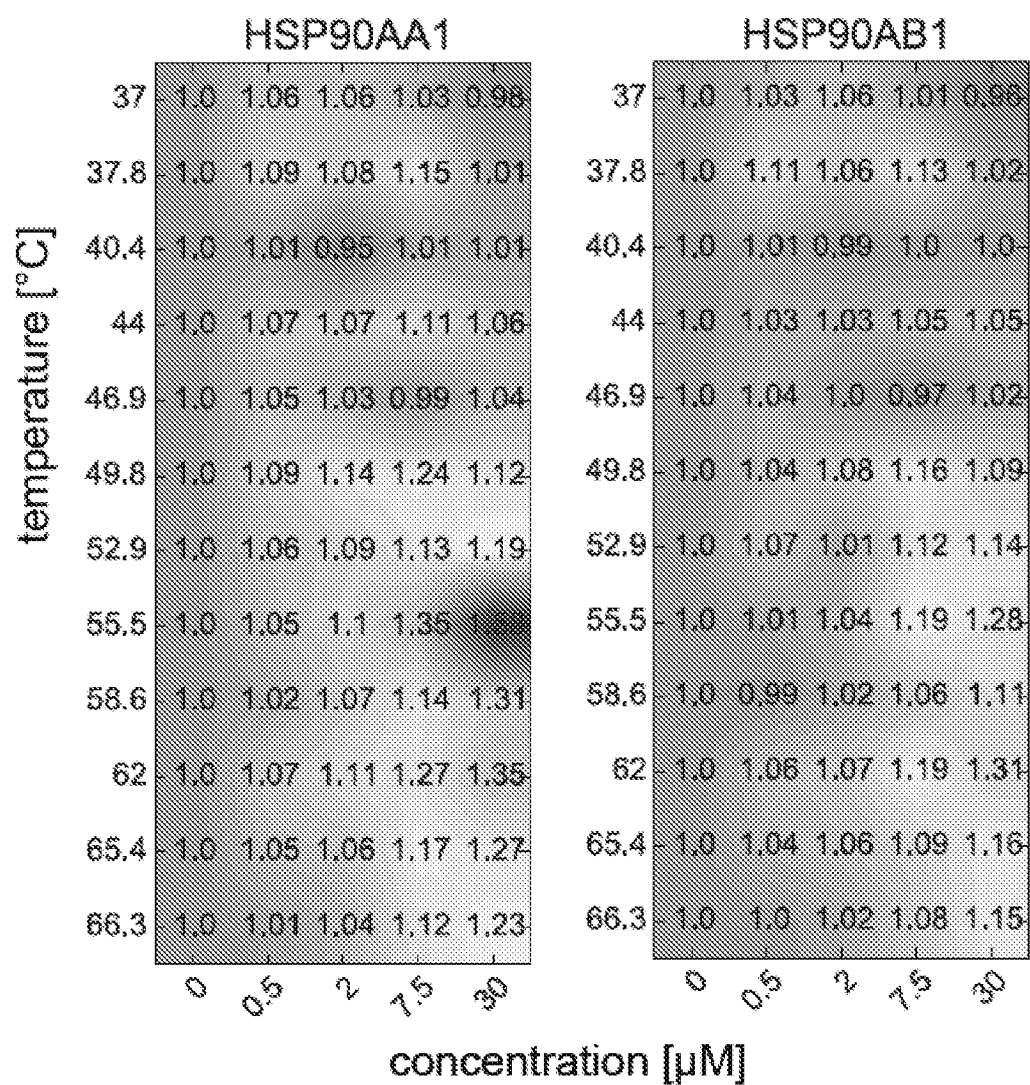


FIG. 11B

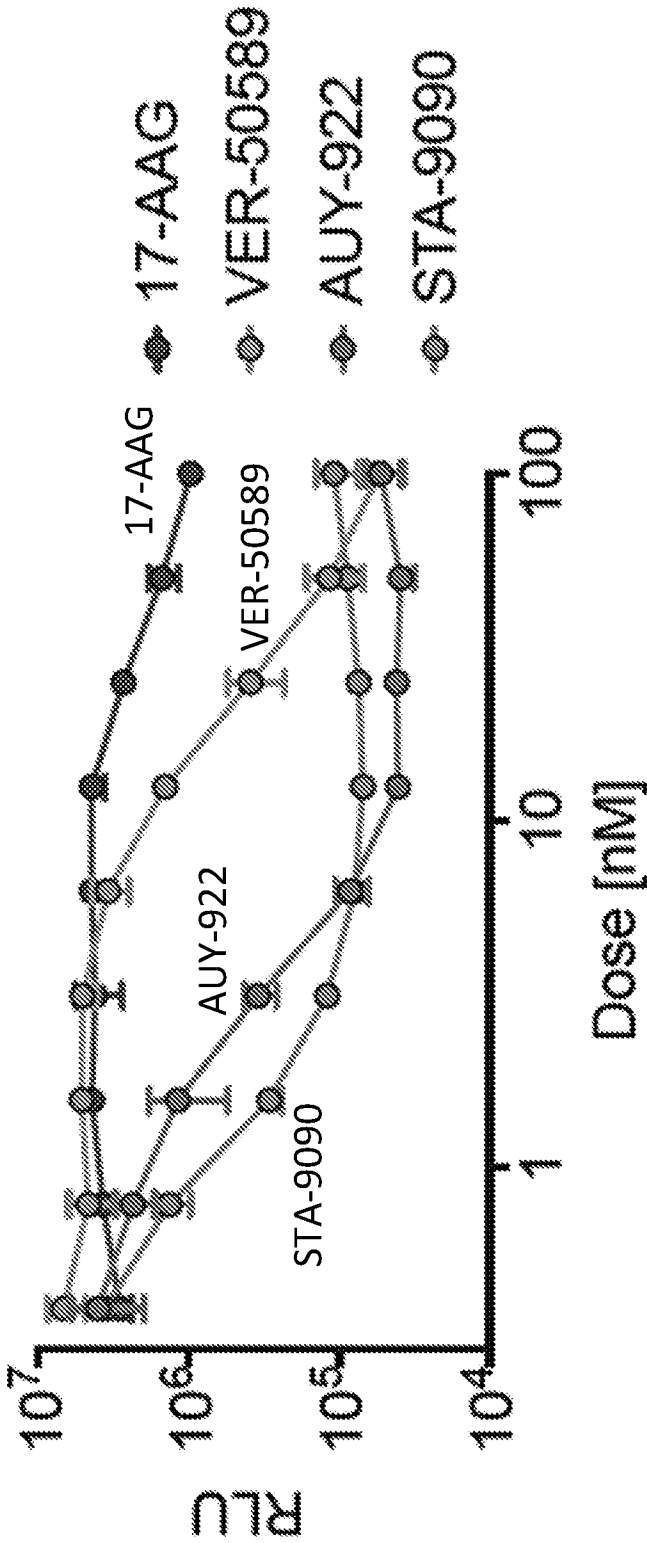


FIG. 11C

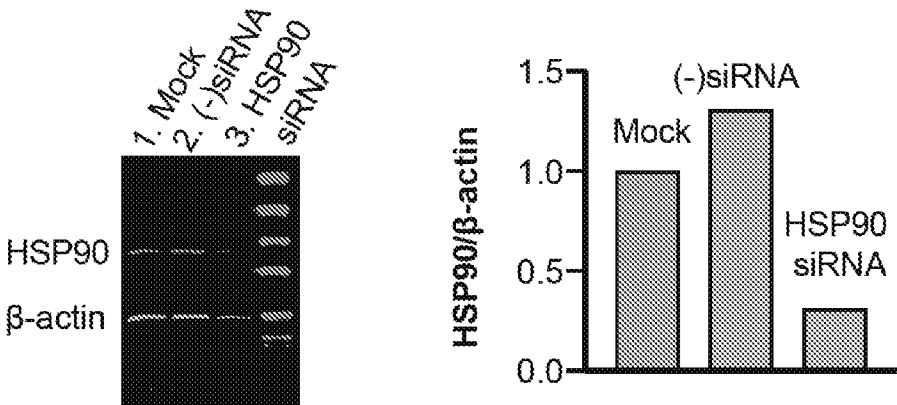


FIG. 11D

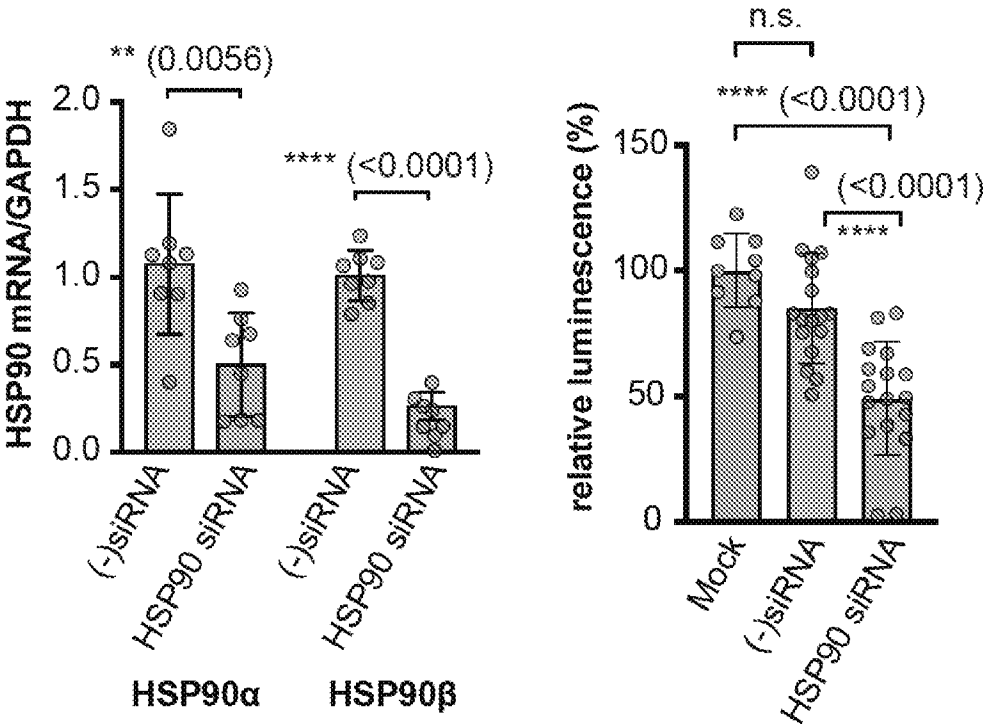


FIG. 11E

FIG. 11F

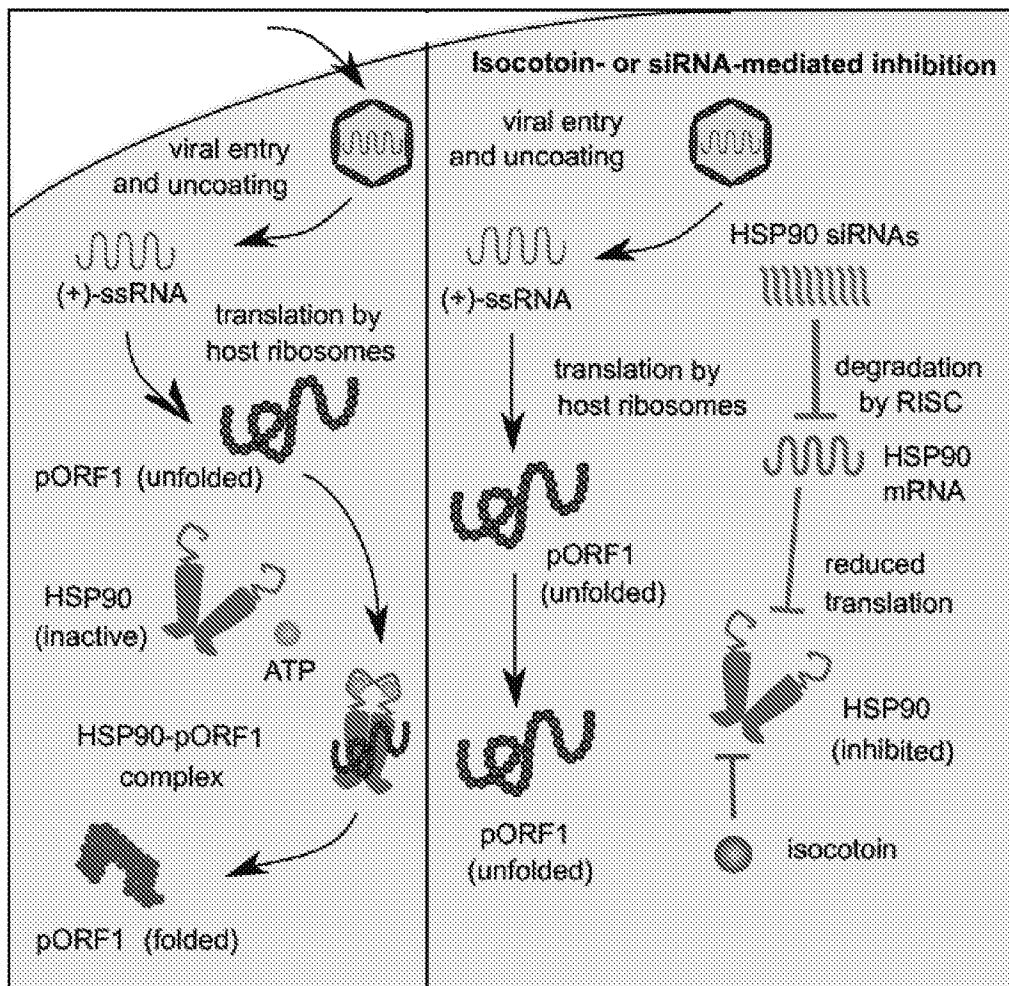


FIG. 11G

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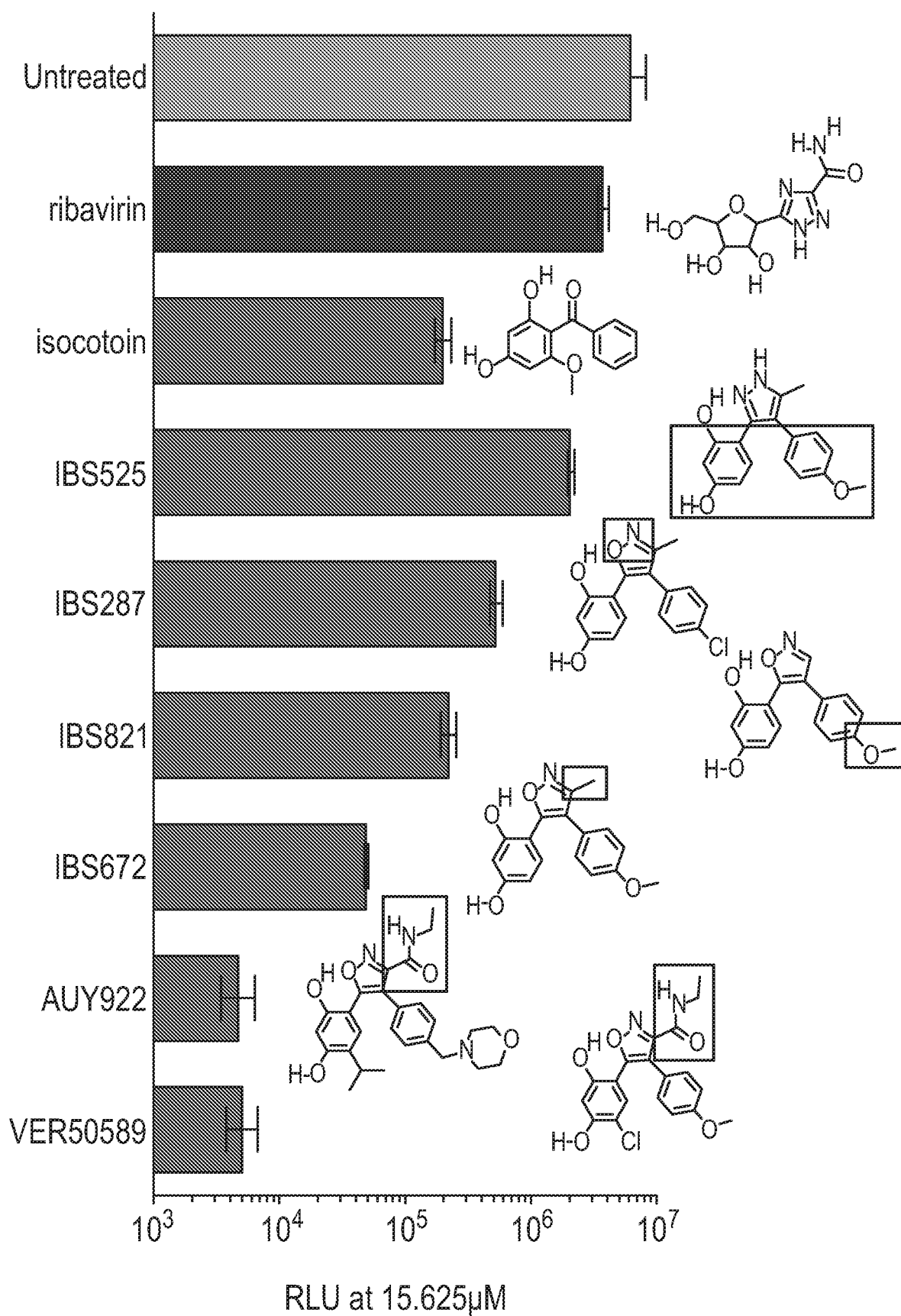


FIG. 12A

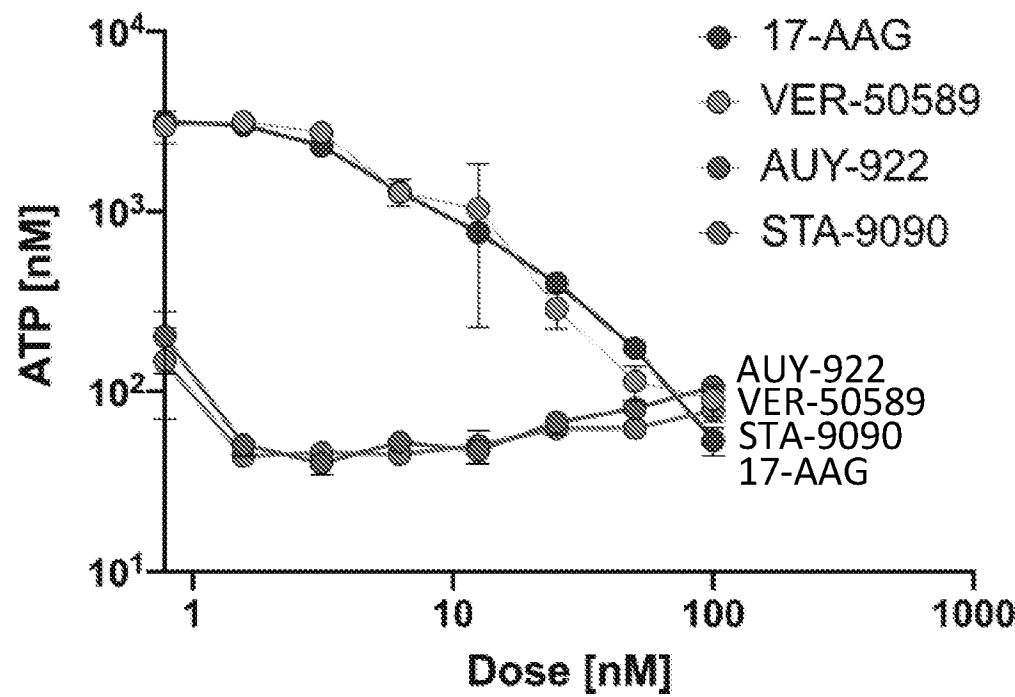


FIG. 12B

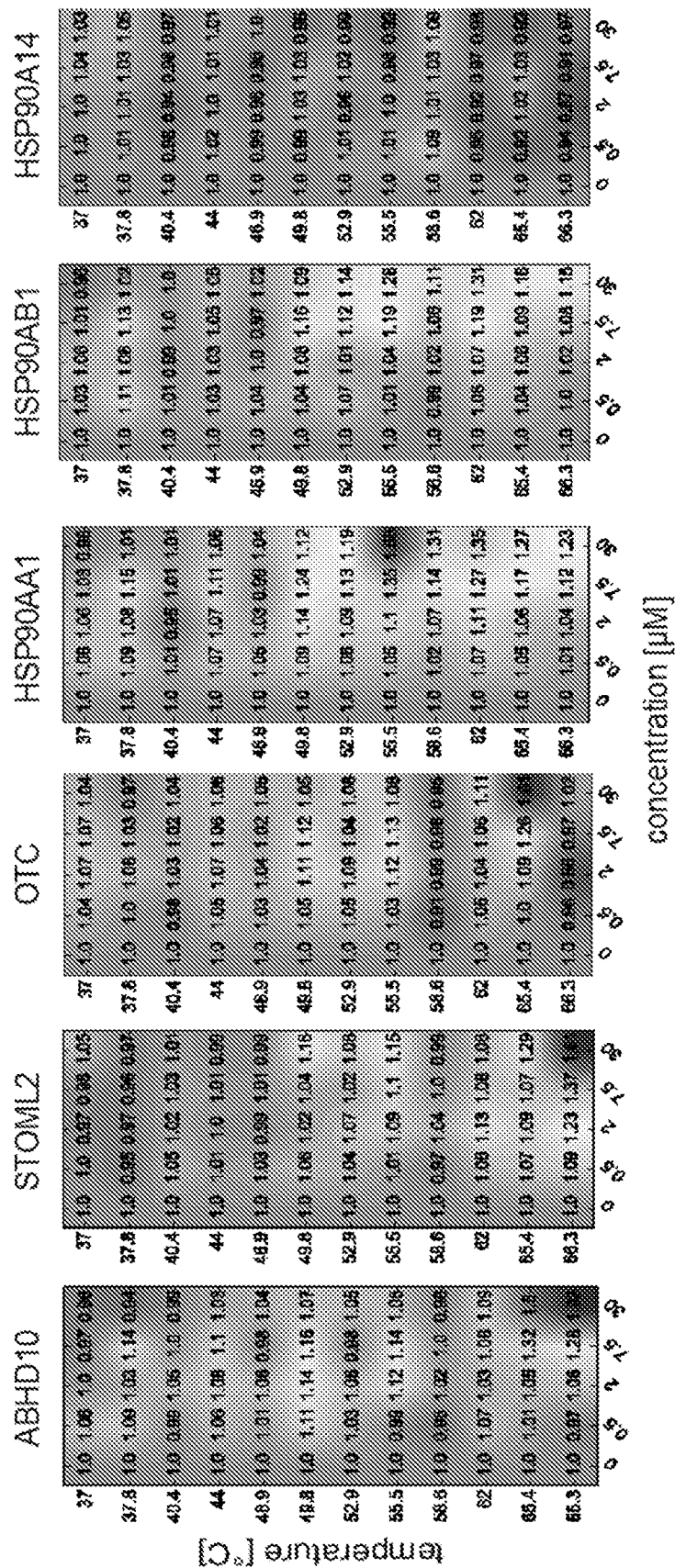


FIG. 13

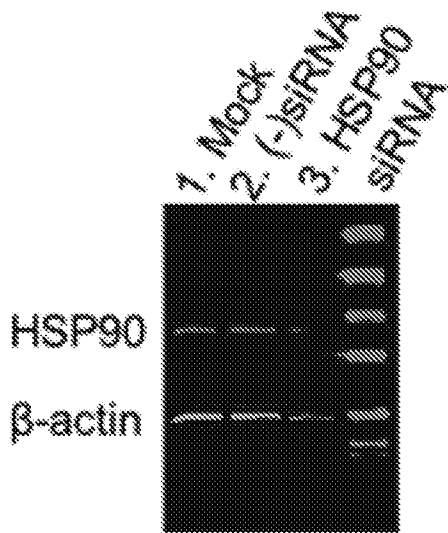


FIG. 14A

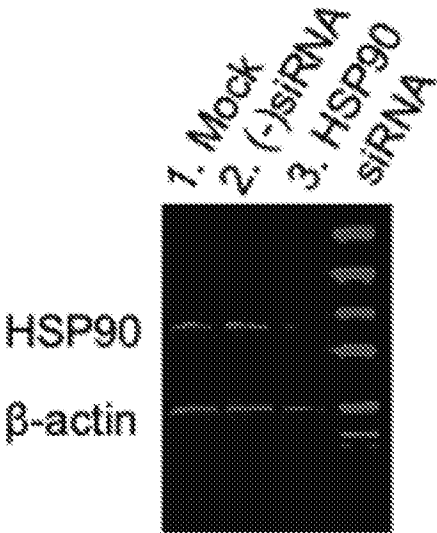


FIG. 14B

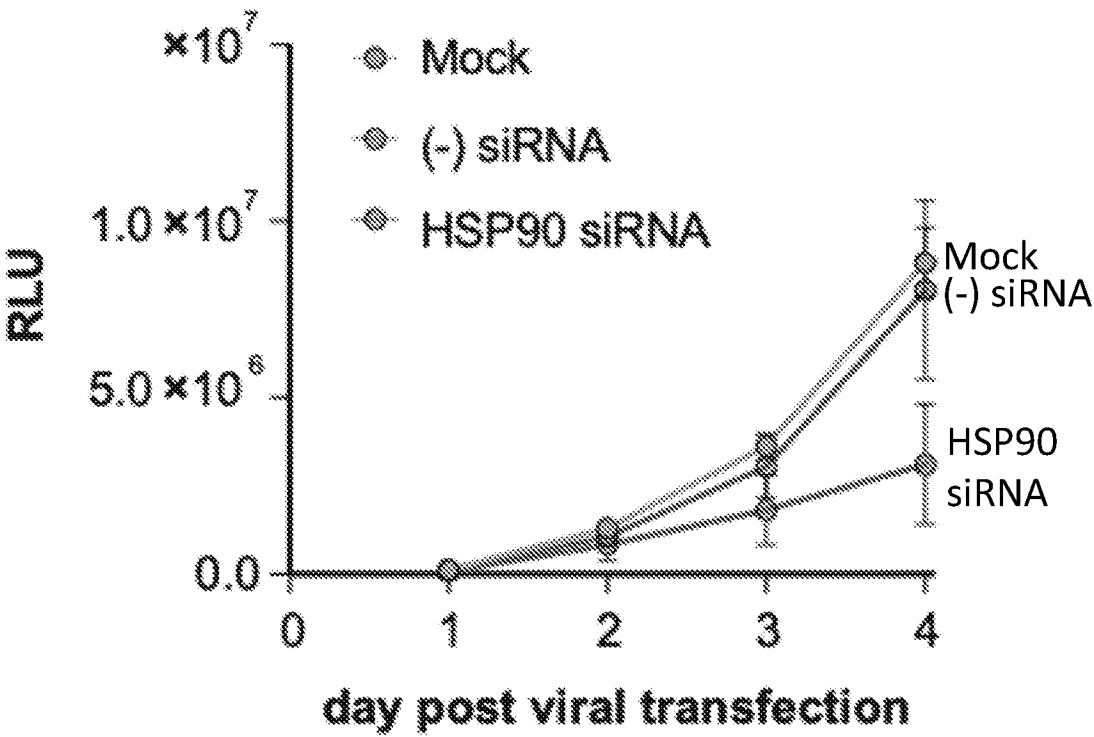


FIG. 14C

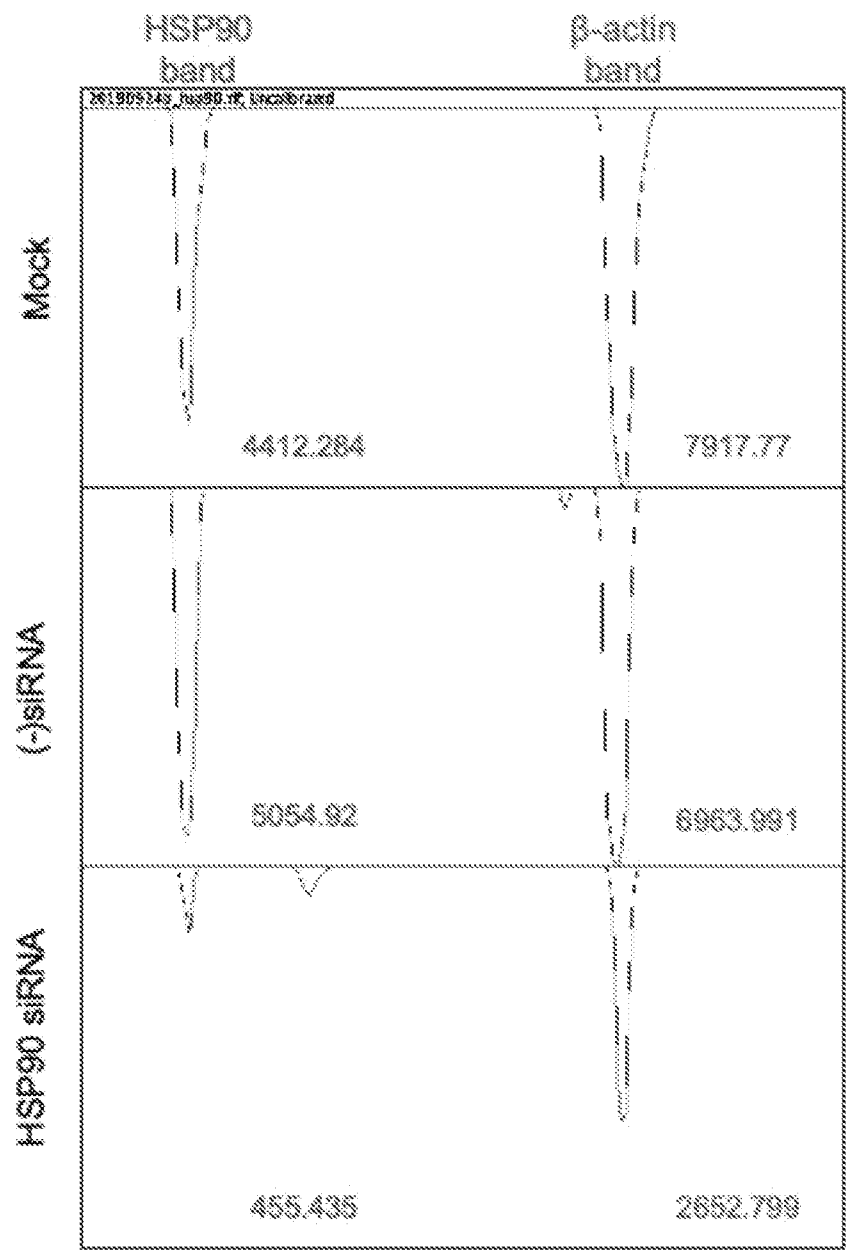


FIG. 14D

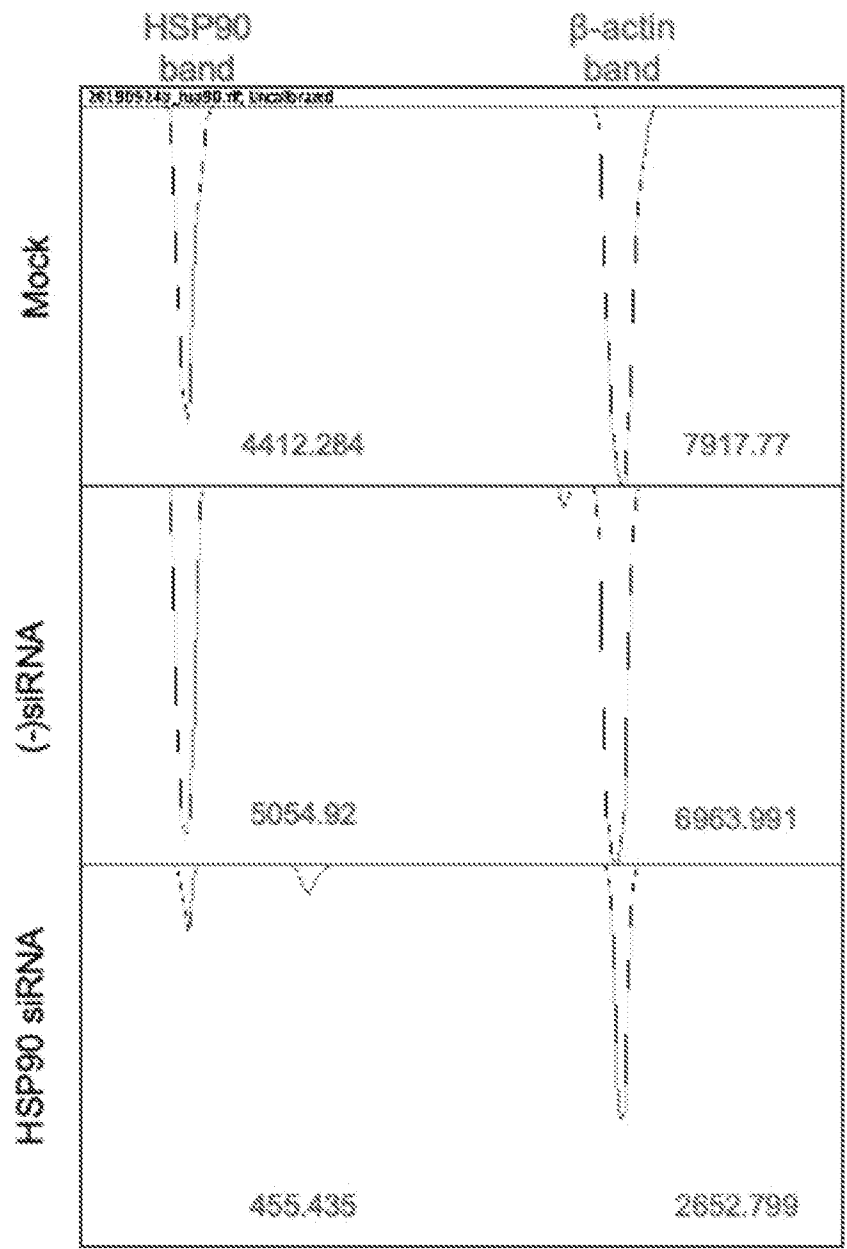


FIG. 14D

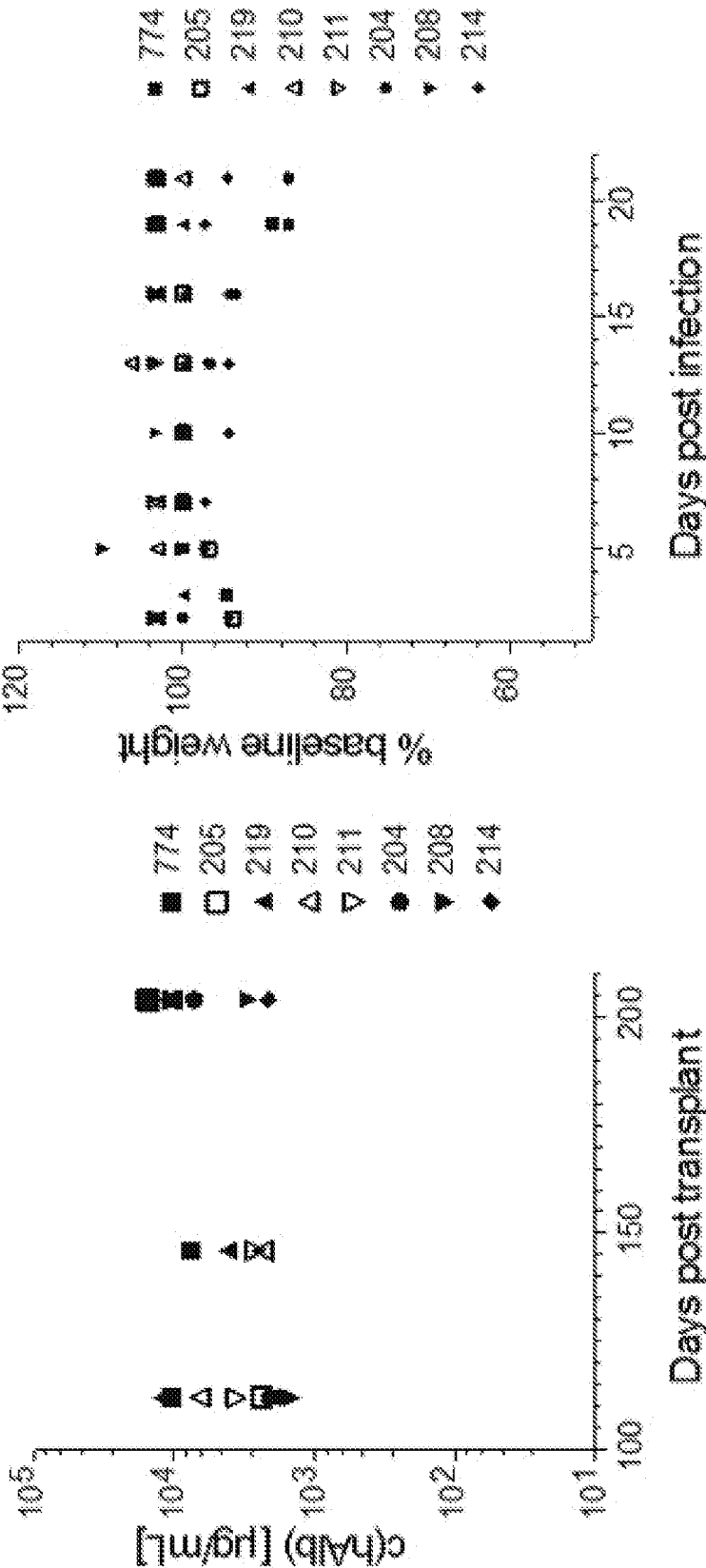


FIG. 15A

FIG. 15B

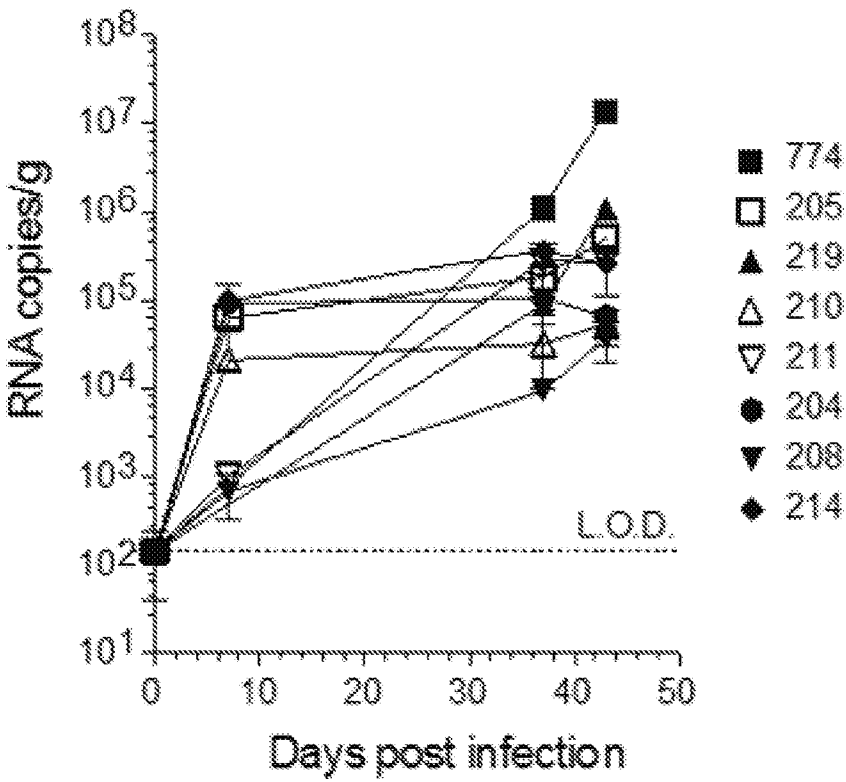


FIG. 15C

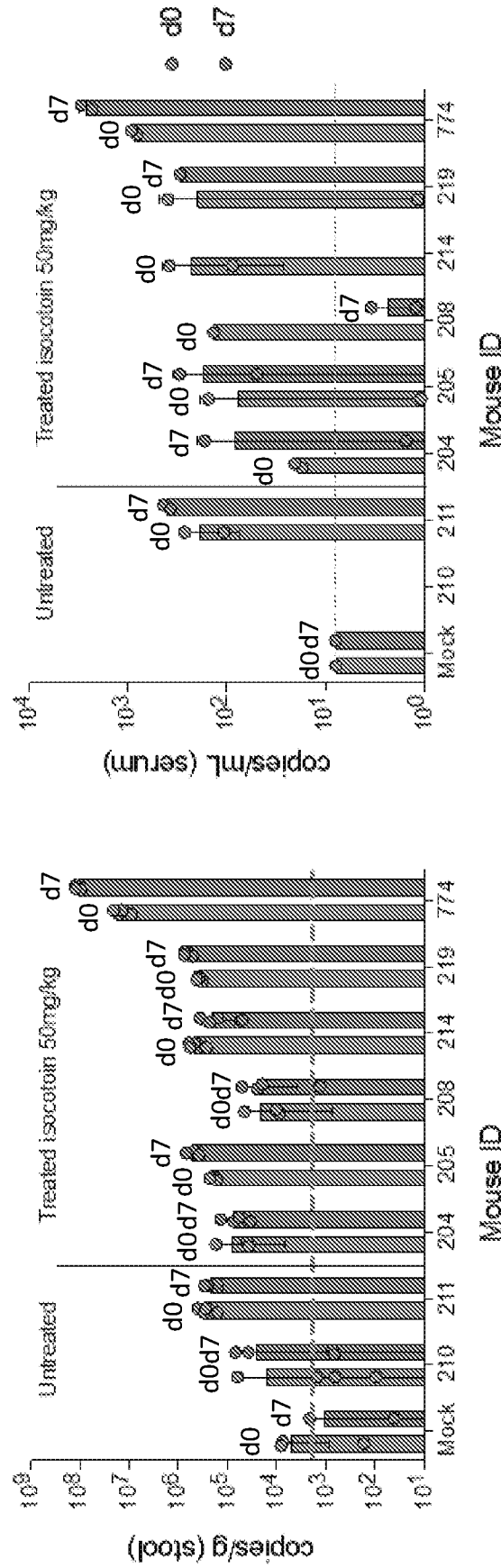


FIG. 15E

FIG. 15D

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/32449

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 31/16; A61K 31/351; A61K 31/4965 (2020.01)

CPC - A61K 31/16; A61K 31/215; A61K 31/351; A61K 31/437

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)

See Search History document

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

See Search History document

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 8,450,310 B2 (DRYSDALE et.al.) 28 May 2013 (28.05.2013) Col 4, in 9-25; Col 12, in 48-52; Col 15, in 24-40; Col 162, in 7-24	15-18 ----- 1-4; 8-11
Y	NAKAGAWA et.al. Hsp90 Inhibitors Suppress HCV Replication in Replicon Cells and Humanized Liver Mice in Biochemical and Biophysical Research Communications, 2007, Vol. 353, pp. 882-888. abstract, pg. 882, Col 2, para 1; 886, Col 1, para 3, Col 2, para 2	1-4; 8-11
A	LI et.al. Plant Phenolic Compounds as Potential Lead Compounds in Functional Foods for Antiviral Drug Discovery in Current Organic Chemistry, 2017, Vol. 21, pp. 1841-1860. ENTIRE DOCUMENT	1-4; 8-11; 15-18
A	CHAN et.al. Novel Benzophenones as Non-nucleoside Reverse Transcriptase Inhibitors of HIV-1 in J. Med. Chem. 2004, Vol. 47, pp. 1175-1182. ENTIRE DOCUMENT	1-4; 8-11; 15-18



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

30 JULY 2020

Date of mailing of the international search report

01 SEP 2020

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
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Facsimile No. 571-273-8300

Authorized officer

Lee Young

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/32449

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.:
because they relate to subject matter not required to be searched by this Authority, namely:
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.: 5-7;12-14;19-35
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 additional fees, this Authority did not invite payment of 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.