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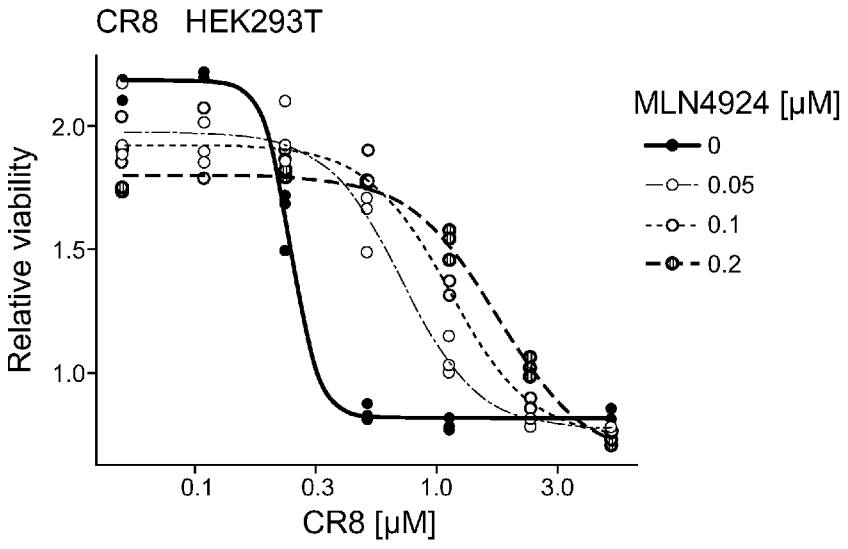
(54) Title: PHENOTYPIC ASSAY TO IDENTIFY PROTEIN DEGRADERS

FIG. 2H



(57) Abstract: Disclosed is a method for identifying cullin dependent small molecule degraders via phenotypic screening.

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[Continued on next page]

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PHENOTYPIC ASSAY TO IDENTIFY PROTEIN DEGRADERS

RELATED APPLICATIONS

[0001] This application claims the benefit of priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No: 63/140,605, filed on January 22, 2021, which is incorporated herein by reference in its entirety.

GOVERNMENT LICENSE RIGHTS

[0002] This invention was made with government support under grant number R35 CA253125 awarded by The National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] Molecular glue compounds induce protein–protein interactions that, in the context of a ubiquitin ligase, lead to protein degradation (Stanton *et al.*, Science *359*:eaao5902 (2018)). Unlike proteolysis-targeting chimeric molecules (PROTAC®s), molecular glue compounds are small molecules (also known as small molecule degraders) that induce an interaction between a substrate receptor of an E3 ubiquitin ligase and a target protein leading to proteolysis of the target. Examples of molecular glues that induce proteolysis of targets include IMiDs (immune modulatory drugs; *e.g.*, thalidomide), which generate a novel interaction between a substrate *(e.g.*, IKZF1/3) and cereblon, a substrate receptor (also known as DCAF) for Cullin-RING ubiquitin ligase 4 (CRL4) den Besten and Lipford, Nat. Chem. Biol. 16(11):1157-1158 (2020). Unlike traditional enzyme inhibitors, these molecular glue degraders act substoichiometrically to catalyze the rapid depletion of previously inaccessible targets (Chopra *et al.*, Drug Discov. Today. Technol. *31*:5–13 (2019)). Although highly desirable, molecular glue degraders have only been found serendipitously. Strategies available for identifying or designing these compounds are limited (Slabicki et al., Nature DOI: 10.1038/s41586-020-2374-x (2020)).

SUMMARY OF THE INVENTION

[0004] The present invention is directed to a method for identifying a cullin dependent small molecule (*e.g.*, monofunctional or multi-functional) degrader, comprising:

incubating mammalian cells with a small molecule test compound in an amount sufficient to cause a change in phenotype in the cells, and a neddylation inhibitor at concentration nontoxic or of low toxicity to the cells; and

measuring a difference in phenotype in the cells following incubation relative to a control that comprises the cells incubated with the test compound but not the neddylation inhibitor, wherein a difference in the phenotype in the cells relative to the control indicates that the test compound causes cullin dependent degradation of a target protein.

[0005] In some embodiments, the test compound is a natural product. In some embodiments, the test compound is a synthetic compound. In some embodiments, the test compound is a semi-synthetic compound.

[0006] In some embodiments, the test compound is a small molecule kinase inhibitor. In some embodiments, the test compound is a small molecule tyrosine kinase inhibitor.

[0007] In some embodiments, the test compound is a small molecule therapeutic agent.

[0008] In some embodiments, the test compound is a small molecule anticancer therapeutic agent.

[0009] In some embodiments, the small molecule inhibitor is CR8, CC-885, or indisulam.

[0010] In some embodiments, the neddylation inhibitor is MLN4924.

[0011] In some embodiments, the concentration of the test compound is between about 0.001 μM to about 100 μM.

[0012] In some embodiments, the concentration of the neddylation inhibitor is less than 1μM.

[0013] In some embodiments, the concentration of the neddylation inhibitor is between about 10 nM to about 500 nM.

[0014] In some embodiments, the concentration of the neddylation inhibitor is about 100 nM.

[0015] In some embodiments, the cells are pretreated with the neddylation inhibitor prior to treatment with the test compound.

[0016] In some embodiments, the mammalian cells are human cells. In some embodiments, the cells are primary cells. In some embodiments, the primary cells are isolated from a tissue or a biopsy sample.

[0017] In some embodiments, the human cells are myeloid cells, lymphoid cells, neural cells, epithelial cells, endothelial cells, stem or progenitor cells, hepatocytes, myoblasts, osteoblasts, osteoclasts, lymphocytes, keratinocytes, melanocytes, mesothelial cells, germ cells, muscle cells, fibroblasts, transformed cells, non-transformed cells, or cancer cells.

[0018] In some embodiments, the transformed cells are Cas9 stable cells.

[0019] In some embodiments, the cells are SNGM, P31FU, OVK18, PFEIFFER, ES-2, OCIM1, K562, HEK293, or HEK293T cells.

[0020] In some embodiments, the difference in phenotype in the cells is increased protein abundance, decreased protein abundance, increased protein activity, decreased protein activity, increased gene expression, decreased gene expression, changed cellular proliferation, changed protein localization, increased or decreased mRNA transcript abundance of predetermined genes, or cell viability.

[0021] In some embodiments, the difference in phenotype in the cells is measured by flow cytometry, fluorescence-activated cell sorting (FACS), mass cytometry, and/or magnetic sorting, protein localization, cellular morphology, gene expression by RNA sequencing (RNAseq), Luminex® multiplex bead assay, quantitative polymerase chain reaction (qPCR), next generation sequencing (NGS), or immunoblotting. In some embodiments, the Luminex® multiplex bead assay is the L1000 assay.

[0022] In some aspects, the invention is directed to high throughput screening (HTS) methods for identifying cullin dependent small molecule degraders.

[0023] Without intending to be bound by any particular theory of operation, the method of the present invention identifies small molecule (*e.g.*, monofunctional and multiple-functional) degraders by observing a change phenotype in cells, *e.g.*, cell viability or cell death, that is rescuable by co-treatment with the neddylation inhibitor. The rescue identifies compounds that cause protein degradation mediated by E3 ligase machinery which produces a toxic effect on the cell. The presence of the neddylation inhibitor prevents the degradation, thus rescuing the cell. This approach is generalizable to any phenotype, and presents a new way of identifying small molecule degrader compounds.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] FIG. 1A-FIG. 1B are a set of graphs showing inhibition of neddylation as a readout for functional degradation (Slabicki *et al.*, Nature DOI: 10.1038/s41586-020-2374-x (2020)). FIG. 1A shows that neddylation inhibitor MLN4924 is not toxic to cells <1 μM. FIG. 1B shows that CR8 cell killing is rescued by low dose neddylation inhibitor MLN4924.

[0025] FIG. 2A-FIG. 2H are a set of graphs showing that MLN4924 rescues CR8 (cyclin-dependent kinase (CDK) inhibitor) induced toxicity in SNGM (FIG. 2A), P31FUJ (FIG. 2B),

OVK18 (FIG. 2C), PFEIFFER (FIG.2D), ES-2 (FIG. 2E), OCIM1 (FIG. 2F), K562 (FIG. 2G), and HEK293T (FIG. 2H) cell lines.

[0026] FIG. 3A-FIG. 3H are a set of graphs showing that MLN4924 rescues CC885 (cereblon (CRBN) modulator) induced toxicity in SNGM (FIG. 3A), P31FUJ (FIG. 3B), OVK18 (FIG. 3C), PFEIFFER (FIG. 3D), ES-2 (FIG. 3E), OCIM1 (FIG. 3F), K562 (FIG. 3G), and HEK293T (FIG. 3H) cell lines.

[0027] FIG. 4A-FIG. 4H are a set of graphs showing that MLN4924 rescues indisulam (CDK) induced toxicity in SNGM (FIG. 4A), P31FUJ (FIG. 4B), OVK18 (FIG. 4C), PFEIFFER (FIG. 4D), ES-2 (FIG. 4E), OCIM1 (FIG. 4F), K562 (FIG. 4G), and HEK293T (FIG. 4H) cell lines.

DETAILED DESCRIPTION OF THE INVENTION

[0028] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the subject matter herein belongs. As used in the specification and the appended claims, unless specified to the contrary, the following terms have the meaning indicated in order to facilitate the understanding of the present invention.

[0029] As used in the description and the appended claims, the singular forms “a”, “an”, and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a composition” includes mixtures of two or more such compositions, reference to “an inhibitor” includes mixtures of two or more such inhibitors, and the like.

[0030] Unless stated otherwise, the term “about” means within 10% (*e.g.*, within 5%, 2% or 1%) of the particular value modified by the term “about.”

[0031] The transitional term “comprising,” which is synonymous with “including,” “containing,” or “characterized by,” is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. By contrast, the transitional phrase “consisting of” excludes any element, step, or ingredient not specified in the claim. The transitional phrase “consisting essentially of” limits the scope of a claim to the specified materials or steps “and those that do not materially affect the basic and novel characteristic(s)” of the claimed invention.

[0032] The terms "determining", "measuring", "evaluating", "assessing" and "assaying" as used herein generally refer to any form of measurement, and include determining if an element

is present or not. These terms include both quantitative and/or qualitative determinations. Assessing may be relative or absolute. "Assessing the presence of" can include determining the amount of something present, as well as determining whether it is present or absent.

[0033] The term "small molecule" as used herein refers to an organic molecule or compound that is monofunctional and that ranges in size from about 50 to about 10,000 daltons, usually from about 50 to about 5,000 daltons and more usually from about 100 to about 1000 daltons.

[0034] The term "small molecule degrader," also known as "molecular glue," refers to a small molecule that induces or stabilizes interactions between proteins. Known small molecule degraders, *e.g.*, thalidomide analogs, bind to the substrate receptors of E3 ubiquitin ligases and recruit target proteins for their ubiquitination and subsequent degradation by proteasomal degradation. Small molecule degraders also are referred to herein as cullin dependent degraders.

[0035] The term "concentration of low toxicity" as used herein refers to a concentration that kills less than about 50% of untreated control cells. For example, a low toxicity concentration of neddylation inhibitor MLN4924 is between about 10 nM to about 999 nM, or between about 10 nM to about 500 nM. For example, a low toxicity concentration of neddylation inhibitor MLN4924 is about 200 nM.

[0036] The term "natural product" as used herein refers to a small molecule organic compound produced by an organism, including a primary and secondary metabolite.

[0037] The term "synthetic compound" refers to a small molecule organic compound made by chemical synthesis, especially to imitate a natural product.

[0038] The term "semi-synthetic compound" refers to a small molecule compound made by synthesis from a naturally occurring compound.

[0039] The present invention is directed to a method for identifying a cullin dependent small molecule (*e.g.*, monofunctional or multi-functional) degrader, comprising:

incubating mammalian cells with a small molecule test compound in an amount sufficient to cause a change in phenotype in the cells, and a neddylation inhibitor at a concentration that is nontoxic or of low toxicity to the cells; and

measuring a difference in phenotype in the cells following incubation relative to a control that comprises the cells incubated with the test compound but not the neddylation inhibitor, wherein a difference in the phenotype in the cells relative to the control indicates that the test compound causes cullin dependent degradation of a target protein. As persons skilled in the

art would appreciate, the control could be performed simultaneously or not, and with the same amount of the small molecule and for the same amount of incubation time.

[0040] In some embodiments, the test compound is a natural product. In some embodiments, the test compound is a synthetic compound. In some embodiments, the test compound is a semi-synthetic compound.

[0041] In some embodiments, the test compound is a small molecule kinase inhibitor. In some embodiments, the test compound is a small molecule tyrosine kinase inhibitor.

[0042] Tyrosine kinase inhibitors (TKIs) are a class of chemotherapy medications that inhibit, or block, one or more of the enzyme tyrosine kinases. Over 25 TKIs have been approved by the U.S. Food and Drug Administration for use in humans (Roskoski, Pharmacol Res. *144*:19-50 (2019)). Exemplary TKIs include Ruxolitinib, Tofacitinib, Lapatinib, Vandetanib, Sorafenib, Sunitinib, Axitinib, Nintedanib, Regorafenib, Pazopanib, Lenvatinib, Crizotinib, Ceritinib, Cabozantinib, DWF, Afatinib, Ibrutinib, B43, KU004, Foretinib, KRCA-0008, PF-06439015, PF-06463922, Canertinib, GSA-10, GW2974, GW583340, WZ4002, CP-380736, D2667, Mubritinib, PD153035, PD168393, Pelitinib, PF-06459988, PF-06672131, PF-6422899, PKI-166, Reveromycin A, Tyrphostin 1, Tyrphostin 23, Tyrphostin 51, Tyrphostin AG 528, Tyrphostin AG 658, Tyrphostin AG 825, Tyrphostin AG 835, Tyrphostin AG 1478, Tyrphostin RG 13022, Tyrphostin RG 14620, B178, GSK1838705A, PD-161570, PD 173074, SU-5402, Roslin 2, Picropodophyllotoxin, PQ401, I-OMe-Tyrphostin AG 538, GNF 5837, GW441756, Tyrphostin AG 879, DMPQ, JNJ-10198409, PLX647, Trapidil, Tyrphostin A9, Tyrphostin AG 370, Lestaurtinib, DMH4, Geldanamycin, Genistein, GW2580, Herbimycin A, Lavendustin C, Midostaurin, NVP-BHG712, PD158780, PD-166866, PF-06273340, PP2, RPI, SU 11274, SU5614, Symadex, Tyrphostin AG 34, Tyrphostin AG 974, Tyrphostin AG 1007, UNC2881, Honokiol, SU1498, SKLB1002, CP-547632, JK-P3, KRN633, SC-1, ST638, SU 5416, Sulochrin, Tyrphostin SU 1498, S8567, rociletinib, Dacomitinib, Tivantinib, Neratinib, Masitinib, Vatalanib, Icotinib, XL-184, OSI-930, AB 1010, Quizartinib, AZD9291, Tandutinib, HM61713, Brigantinib, Vemurafenib (PLX-4032), Semaxanib, AZD2171, Crenolanib, Damnacanthal, Fostamatinib, Motesanib, Radotinib, OSI-027, Linsitinib, BIX02189, PF-431396, PND-1186, PF-03814735, PF-431396, sirolimus, temsirolimus, everolimus, deforolimus, zotarolimus, BEZ235, INK128, Omipalisib, AZD8055, MHY1485, PI-103, KU-0063794, ETP-46464, GDC-0349, XL388, WYE-354, WYE-132, GSK1059615, WAY-600, PF-04691502, WYE-687, PP121, BGT226, AZD2014,

PP242, CH5132799, P529, GDC-0980, GDC-0994, XMD8-92, Ulixertinib, FR180204, SCH772984, Trametinib, PD184352, PD98059, Selumetinib, PD325901, U0126, Pimasertinib, TAK-733, AZD8330, Binimetinib, PD318088, SL-327, Refametinib, GDC-0623, Cobimetinib, BI-847325, Adaphostin, GNF 2, PPY A, AIM-100, ASP 3026, LFM A13, PF 06465469, (-)-Terreic acid, AG-490, BIBU 1361, BIBX 1382, BMS 599626, CGP 52411, GW 583340, HDS 029, HKI 357, JNJ 28871063, WHI-P 154, PF 431396, PF 573228, FIIN 1, PD 166285, SUN 11602, SR 140333, TCS 359, BMS 536924, NVP ADW 742, PQ 401, BMS 509744, CP 690550, NSC 33994, WHI-P 154, KB SRC 4, DDR1-IN-1, PF 04217903, PHA 665752, SU 16f, A 419259, AZM 475271, PP 1, PP 2, 1-Naphthyl PP1, Src I1, ANA 12, PD 90780, Ki 8751, Ki 20227, ZM 306416, ZM 323881, AEE 788, GTP 14564, PD 180970, R 1530, SU 6668, and Toceranib.

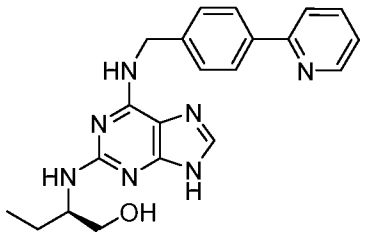
[0043] In some embodiments, the test compound is a member of a library or collection of compounds or agents of interest (*e.g.*, TKIs). The library of compounds may be composed of a plurality of chemical compounds and agents that may be assembled from a single or multiple sources. A library may include chemical compounds and agents such as chemically synthesized substances, products of biotechnological processes, naturally occurring substances or products resulting from combinatorial chemistry techniques or combinations thereof. In some embodiments, the library includes a plurality of clinical or preclinical small molecule drugs.

[0044] In some embodiments, the test compound is a small molecule therapeutic agent.

[0045] In some embodiments, the test compound is a small molecule anticancer therapeutic agent.

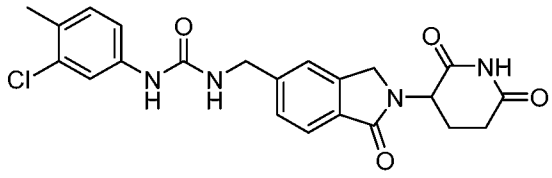
[0046] In some embodiments, the test compound is CR8, CC-885, or indisulam.

[0047] CR8, also known as (*R*)-CR8 and (2R)-2-[[9-(Methylethyl)-6-[[[4-(2-pyridinyl)phenyl]methyl]amino]-9H-purin-2-yl]amino]-1-butanol, has the following structure:



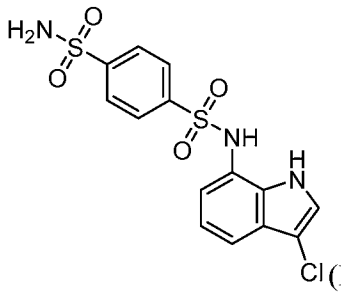
(CR8).

[0048] CC-885, also known as N-(3-chloro-4-methylphenyl)-N'-[[2-(2,6-dioxo-3-piperidinyl)-2,3-dihydro-1-oxo-1H-isoindol-5-yl]methyl]-urea, has the following structure:



(CC-885).

[0049] Indisulam, also known as E7070 and N-(3-chloro-1H-indol-7-yl)benzene-1,4-disulfonamide, has the following structure:



(Indisulam).

[0050] The amount of the test compound is sufficient to cause a measurable change in phenotype in the cells. In some embodiments, the concentration of the test compound is between about 0.001 μM to about 100 μM. In some embodiments, the concentration of the test compound is between about 0.001 μM to about 30 μM. In some embodiments, the concentration of the test compound is between about 0.03 μM to about 30 μM. In some embodiments, the concentration of the test compound is between about 0.1 μM to about 3 μM. In some embodiments, the concentration of the test compound is between about 0.001 μM to about 0.1 μM.

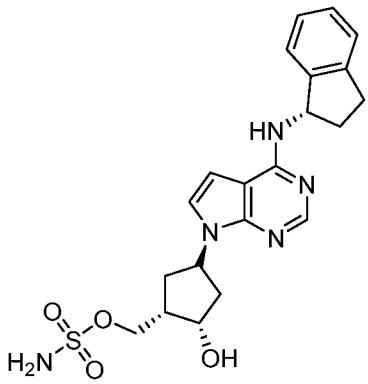
Neddylation inhibitors

[0051] Neddylation is a post-translational protein modification that conjugates ubiquitin-like protein neuronal precursor cell-expressed developmentally down-regulated protein 8 (NEDD8). Similar to ubiquitination, neddylation is mediated by a cascade of three NEDD8 specific enzymes, an E1 activating enzyme, an E2 conjugating enzyme and one of the several E3 ligases. By altering its substrates' conformation, stability, subcellular localization or binding affinity to DNA or proteins, neddylation regulates diverse cellular processes including the ubiquitin-proteasome system-mediated protein degradation, protein transcription, cell signaling, etc. (Kandala et al., Am J Cardiovasc Dis. *4(4):*140-158 (2014)). The best-characterized substrates of neddylation are cullin family members that function as indispensable components of multiunit cullin-RING E3 ubiquitin ligases (CRLs), whose activity requires cullin neddylation (Zhou et al., Molecular cancer *18*:77(2019)). The small molecule MLN4924 (Pevonedistat), an adenosine sulfamate analog, potently and selectively

inhibits NEDD8 activating enzyme (NAE), the E1 enzyme (Soucy *et al.*, Nature *458*:732–736 (2009).

[0052] Neddylation inhibitors effectively block cullin neddylation and inactivate CRLs. This in turn leads to accumulation of various CRL substrates, thus triggering multiple cellular responses, including cell cycle arrest, apoptosis, senescence and autophagy in a cell-type dependent manner (Zhou et al., Molecular cancer 18:77(2019)).

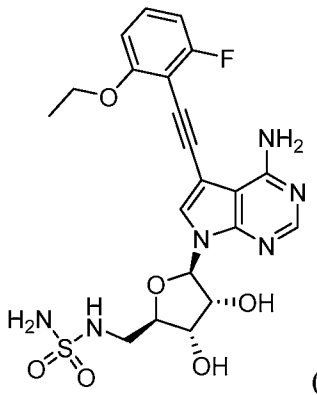
[0053] In some embodiments, the neddylation inhibitor is MLN4924, also known as ((1S,2S,4R)-4-(4-((1S)-2,3-Dihydro-1H-inden-1-ylamino)-7H-pyrrolo(2,3-d)pyrimidin-7-yl)-2-hydroxycyclopentyl)methyl sulphamate, has the following structure:



(MLN4924).

[0054] In some embodiments, the neddylation inhibitor is a derivative or analog of MLN492. Analogs of MLN492 that may be useful as neddylation inhibitors are described in U.S. Patent 7,951,810 B2 and U.S. Patent Application Publication 2018/0086785 A1.

[0055] Another exemplary NAE or neddylation inhibitor, which may be used in the present invention, is TAS4464, which is also known as 4-amino-7-[(2R,3R,4S,5R)-3,4-dihydroxy-5-[(sulfamoylamino)methyl]oxolan-2-yl]-5-[2-(2-ethoxy-6-fluorophenyl)ethynyl]pyrrolo[2,3-d]pyrimidine and has the following structure:



(TAS4464).

[0056] In some embodiments, the neddylation inhibitor is a derivative or analog of TAS4464. Analogs of TAS4464 that may be useful as neddylation inhibitors are described in U.S. Patent 9,963,456 B2.

[0057] In some embodiments, the concentration of the neddylation inhibitor is below 1μM. In some embodiments, the concentration of the neddylation inhibitor is between about 10 nM to about 999 nM.

[0058] In some embodiments, the concentration of the neddylation inhibitor is between about 10 nM to about 500 nM.

[0059] In some embodiments, the concentration of the neddylation inhibitor is about 100 nM.

[0060] The cells may be pretreated with different concentrations of the neddylation inhibitor, typically for about 10 minutes to about 24 hours prior to treatment with the test compound in dose response assays.

[0061] The cells are incubated in the presence of the test compound and the neddylation inhibitor for an amount of time sufficient to allow for a measurable change in phenotype to occur. In some embodiments, the cells are incubated with neddylation inhibitor and test compound for at least 3 to 5 days. In some embodiments, the cells are incubated with neddylation inhibitor and test compound for at least 3 days. In some embodiments, incubation time may be shortened to 10 minutes – 24 hours to investigate protein levels or other more general readout, *e.g.*, cellular differentiation and/or gene expression by RNA sequencing (RNAseq) or quantitative polymerase chain reaction (qPCR).

Cells

[0062] Mammalian cells suitable for use in the present methods are those that can be maintained or propagated *in vitro* and that naturally display, or that may be genetically modified to display, a phenotype (such as one described herein) the alteration of which is desired to be monitored for modulation. For example, cells may display a phenotype whose inhibition within the assay is to be determined. Mammalian cells which display the appropriate phenotypic characteristics (phenotype) may be identified or obtained by any convenient technique or source, including those known to the person of ordinary skill in the art.

[0063] Such cells may be stable cell lines, such as those obtainable from American Type Culture Collection (ATCC) or other cell repositories. Alternatively, the mammalian cells employed may be primary cells derived from a tissue or organ of an individual organism. In certain embodiments, the mammalian cells may be transiently transfected with genetic

constructs, such as those involved in the (desired) phenotype, for example a reporter gene. In other embodiments, the mammalian cells may be infected with a virus or bacterium which leads to an alteration in the phenotype. Of particular utility are human cells, murine cells, hamster cells, rate cells, primate cells, and cells from domestic mammals (such as ovine, bovine, equine, canine or feline cells). Yet other cells for employment in the present methods include mammalian cells derived from a cancer or tumor, or those associated with some other disease or abnormality of a mammal.

[0064] In some embodiments, the mammalian cells are primary cells. In some embodiments, the primary cells are isolated from a tissue or a biopsy sample.

[0065] In some embodiments, the mammalian cells are human cells.

[0066] In some embodiments, the human cells are myeloid cells, lymphoid cells, neural cells, epithelial cells, endothelial cells, stem or progenitor cells, hepatocytes, myoblasts, osteoblasts, osteoclasts, lymphocytes, keratinocytes, melanocytes, mesothelial cells, germ cells, muscle cells, fibroblasts, transformed cells, non-transformed cells, or cancer cells.

[0067] In some embodiments, the transformed cells are Cas9 stable cells.

[0068] In some embodiments, the cells are SNGM, P31FU, OVK18, PFEIFFER, ES-2, OCIM1, K562, HEK293, or HEK293T cells.

Difference in phenotype in cells

[0069] In some embodiments, the phenotype to be monitored for change may be one having been specifically engineered for a given cell type. For example, a mammalian cell type may be genetically modified to result in a phenotype that is convenient or suitable for detection, such as a fluorescent protein/marker (such as GFP; eGFP), a luminescent protein/marker (such as luciferase) or a cell-surface marker than is detectable, *e.g.*, with a labelled antibody, or intracellular proteins stained with labeled antibodies.

[0070] In some embodiments, the phenotype of the mammalian cell is any one of the following, namely: one associated with a cell signaling pathway, preferably an activated cell signaling pathway; and/or one selected from the list consisting of: luminescence, fluorescence, viability, senescence, differentiation, migration, invasion, chemotaxis, apoptosis, immunological anergy, surface marker expression, progress through the cell cycle, transcriptional activity, protein expression, glycosylation, resistance to infection, permeability and reporter-gene activity. In some embodiments, the phenotype is not death and/or reduced (or decreased) growth, such as a phenotype that is not rescue of a cell from cytokine

dependence, is not rescue from apoptosis (including neutrophil apoptosis/cell-death), is not induction of colony formation, or is not a rescue screen. For example, a screen for such a phenotype may be designed to detect a trait or characteristic of the cell that is not a rescue from cell death or an increase in growth of the mammalian cell.

[0071] In some embodiments, the phenotypic difference in the cells is increased protein abundance, decreased protein abundance, increased protein activity, decreased protein activity, increased gene expression, decreased gene expression, changed cellular proliferation, changed protein localization, or cell viability. In some embodiments, the phenotype difference is an increased or decreased mRNA transcript abundance of predetermined genes.

Analytical methods

[0072] Methods to assess phenotypic difference in cells are known in the art. One exemplary method is an immunoassay. In their simplest and most direct sense, immunoassays are binding assays involving binding between antibodies and antigen. Representative examples of immunoassays include enzyme linked immunosorbent assays (ELISAs), enzyme linked immunospot assay (ELISPOT), radioimmunoassays (RIA), radioimmune precipitation assays (RIPA), immunobead capture assays, Western blotting, dot blotting, gel-shift assays, Flow cytometry, immunohistochemistry, fluorescence microscopy, protein arrays, multiplexed bead arrays, magnetic capture, in vivo imaging, fluorescence resonance energy transfer (FRET), and fluorescence recovery/localization after photobleaching (FRAP/FLAP).The steps of various useful immunodetection methods have been described in the scientific literature, such as, *e.g.*, Maggio *et al.*, ed. (1980), *Enzyme Immunoassay*, CRC, Boca Raton, FL and Nakamura, et al., Enzyme Immunoassays: *Heterogeneous and Homogeneous Systems in Handbook of Experimental Immunology 4th Edition, Vol. 1: Immunochemistry*, Chapter 27, edited by D. M. Weir, Blackwell Scientific Publications, Oxford, (1986), each of which is incorporated herein by reference in its entirety and specifically for its teaching regarding immunodetection methods.

[0073] In some embodiments, the phenotypic difference in the cells is measured by flow cytometry, fluorescence-activated cell sorting (FACS), mass cytometry, and/or magnetic sorting, protein localization, cellular morphology, gene expression by RNA sequencing (RNAseq), Luminex® multiplex bead assay, quantitative polymerase chain reaction (qPCR), next generation sequencing (NGS), or immunoblotting.

[0074] In some embodiments, an increase in abundance of mRNA transcripts of predetermined genes is determined via a Luminex® multiplex bead assay, e.g., the L1000 assay. L1000 is a high-throughput gene expression assay that measures the mRNA transcript abundance of 978 "landmark" genes from human cells. Landmark genes were chosen to be expressed across lineage which would enable the prediction of expression of other genes not directly measured. The "L" in L1000 refers to the Landmark genes measured in the assay. Measurements are made using the 500 colors of Luminex® beads such that two transcripts are identified by a single bead color. Expression of 80 control transcripts, chosen for their invariant expression across cell states, as well as the 978 Landmark genes are also measured. L1000 is highly reproducible, comparable to RNA sequencing, and suitable for computational inference of the expression levels of 81% of non-measured transcripts. Subramanian *et al.*, Cell *171(6)*:1437–1452.e17 (2017).

High throughput screenings

[0075] In some embodiments, the disclosed methods entail high throughput screening (HTS) for identifying cullin dependent small molecule degraders. The term "high-throughput" relates to an assay system for the rapid testing of a plurality of compounds in a short time. Thus, the assaying time per tested compound is minimized. The assay is preferably carried out in multiwell plates, *e.g.*, 6-well plates, 12-well plates, 24-well plates, 96-well plates or 384-well plates, or 1536 plates, even more preferably in 96-well plates, 384-well or 1536 plates. Thus, the term "high-throughput" encompasses screening activity in which human intervention is minimized, and automation is maximized. For example, high-throughput screening involves automated pipetting, mixing, and heating. Alternatively, a high-throughput method is one in which for example hundreds of compounds can be screened per 24-hour period by a single individual operating a single suitable apparatus. "Automation" refers to a method or any one or more steps thereof that is fully or partly controlled and/or carried out by one or more technical devices, preferably pipetting robots. Methods for conducting a variety of different HTS assays, in particular HTS screening assays are known in the art. The skilled person knows how to conduct HTS methods by the methods used in the context of the present invention.

[0076] These and other aspects of the present invention will be further appreciated upon consideration of the following Examples, which are intended to illustrate certain particular embodiments of the invention but are not intended to limit its scope, as defined by the claims.

EXAMPLES

[0077] Example 1: Inhibition of neddylation as a readout for functional degradation.

[0078] HEK293T cells were treated with various concentration of MG132 (proteasome inhibitor), MLN7243 (E1 ubiquitin-activating enzyme inhibitor) or MLN4924 (cullin neddylation inhibitor) for three days. Cell viability was assessed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega®, G7572) on CLARIOstar® Plus Multi-mode Microplate Reader, MARS 3.4 (BMG LabTech). Cell viabilities were calculated relative to DMSO controls. Standard four-parameter log-logistic curves are fitted with the 'dr4pl' R package. The results are shown in FIG. 1A.

[0079] HEK293T-Cas9 cells that were also treated with 100 nM MLN4924 or DMSO in combination with compound CR8 for three days (n = 3). The results are shown in FIG. 2B.

[0080] The results illustrated in FIG. 1A-FIG. 1B show that titration of MLN4924 up to 1 μM did not have a significant effect on cell viability (FIG. 1A), but a 100 nM dose of MLN4924 was able to rescue CR8 induced toxicity (FIG. 1B). These results confirmed that addition of low dose of MLN4924 can achieve a functional effect of reduced toxicity, when the toxicity is caused by neddylation dependent mechanism, such as degradation of Cyclin K and CDK13 by CR8.

[0081] Ubiquitin proteasome system (UPS) compounds, including the neddylation inhibitor MLN4924, are known to be toxic at high to mid concentrations (>1 μM). It was believed that low doses would not inhibit neddylation. Applicant surprisingly found that low dose MLN4924 (*e.g.*, ~100 nM) was nontoxic to cells and it potently blocked neddylation generating a sufficient window to observe phenotypic difference in the cells.

[0082] Example 2: MLN4924 rescue of CR8 (cyclin-dependent kinase (CDK) inhibitor) induced toxicity in multiple cell lines.

[0083] HEK293T-Cas9 cells were resuspended at 0.15×10^6 per ml and plated on a 384-well plate with 50 μL per well and MLN4924, MLN7243 or MG132 with or without CR8 serially diluted with D300e Digital Dispenser (Tecan).

[0084] A panel of SNGM, P31FUJ, OVK18, PFEIFFER, ES-2, OCIM1 or HEK293T cells was resuspended at ~0.15×10^6 per ml and plated on a 384-well plate with 50 μL per well. Cells were pretreated with MLN4924 at 0, 0.05, 0.1 or 0.2 μM concentration and treated with CR8 in dose response.

[0085] After three days of drug exposure, cell viability was assessed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega®, G7572) on CLARIOstar® Plus Multi-mode Microplate Reader, MARS 3.4 (BMG LabTech). Cell viabilities were calculated relative to DMSO controls. The half-maximum effective concentration (EC_{50}) values were derived from standard four-parameter log-logistic curves fitted with the 'dr4pl' R package.

[0086] The results illustrated in FIG.2A-FIG. 2H show that MLN4924 rescued CR8 induced toxicity in tested cell lines.

[0087] Example 3: MLN4924 rescue of CC885 (cereblon (CRBN) modulator) induced toxicity in multiple cell lines.

[0088] HEK293T-Cas9 cells were resuspended at 0.15×10^6 per ml and plated on a 384-well plate with 50 µL per well and MLN4924, MLN7243 or MG132 with or without CC-885 serially diluted with D300e Digital Dispenser (Tecan).

[0089] A panel of SNGM, P31FUJ, OVK18, PFEIFFER, ES-2, OCIM1 or HEK293T cells was resuspended at $\sim 0.15 \times 10^6$ per ml and plated on a 384-well plate with 50 µL per well. Cells were pretreated with MLN4924 at 0, 0.05, 0.1 or 0.2 µM concentration and treated with CC-885 in dose response.

[0090] After three days of drug exposure, cell viability was assessed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega®, G7572) on CLARIOstar® Plus Multi-mode Microplate Reader, MARS 3.4 (BMG LabTech). Cell viabilities were calculated relative to DMSO controls. The half-maximum effective concentration (EC50) values were derived from standard four-parameter log-logistic curves fitted with the 'dr4pl' R package.

[0091] The results illustrated in FIG.3A-FIG. 3H show that MLN4924 rescues CC-885 induced toxicity in multiple cell lines.

[0092] Example 4: MLN4924 rescue of indisulam (CDK) induced toxicity in multiple cell lines.

[0093] HEK293T-Cas9 cells were resuspended at 0.15×10^6 per ml and plated on a 384-well plate with 50 µL per well and MLN4924, MLN7243 or MG132 with or without indisulam serially diluted with D300e Digital Dispenser (Tecan).

[0094] A panel of SNGM, P31FUJ, OVK18, PFEIFFER, ES-2, OCIM1 or HEK293T cells was resuspended at $\sim 0.15 \times 10^6$ per ml and plated on a 384-well plate with 50 µL per well. Cells were pretreated with MLN4924 at 0, 0.05, 0.1 or 0.2 µM concentration and treated with indisulam in dose response.

[0095] After three days of drug exposure, cell viability was assessed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega®, G7572) on CLARIOstar® Plus Multi-mode Microplate Reader, MARS 3.4 (BMG LabTech). Cell viabilities were calculated relative to DMSO controls. The half-maximum effective concentration (EC50) values were derived from standard four-parameter log-logistic curves fitted with the 'dr4pl' R package.

[0096] The results illustrated in FIG.4A-FIG. 4H show that MLN4924 reduced indisulam induced toxicity in limited number of cell lines.

[0097] Established molecules CR8, CC-885, and indisulam, were confirmed as cullin dependent small molecule degraders using the method described herein. CR8 induces the degradation of Cyclin K via direct binding of CR8-CDK12-Cyclin K to damage specific DNA binding protein 1 (DDB1). CC-885 is a CRBN based degrader of G1 to S phase transition 1 (GSPT1). Indisulam is a DDB1 and CUL4 associated factor 15 (DCAF15) based degrader of splicing factor RNA binding motif protein 39 (RBM39). All of the three compounds caused dose dependent cell killing via proteasomal degradation. Treatment of CR8, CC-885 and indisulam induced dose dependent toxicity in a panel of cell lines, which was rescued by co-treatment with neddylation inhibitor MLN4924. The rescue of CR8 and CC-885 induced toxicity was near universal. However, co-treatment of neddylation inhibitor MLN4924 and indisulam of ES-2 or HEK293T cells failed to reduce the cytotoxic effect. This may have been due to differential expression of the DCAF15 E3 ligase or reduced sensitivity to RBM39 degradation.

[0098] FIG. 2A-FIG. 4H illustrate co-treatments of CC-885, CR8 and Indisulam with three doses of MLN4924 (50, 100, 200 nM). Increased doses of neddylation inhibitor decreased CellTiter-Glo® levels for different cell lines. Optimal concentration of MLN4924 may be characterized with increased toxicity.

[0099] All patent publications and non-patent publications are indicative of the level of skill of those skilled in the art to which this invention pertains. All these publications are herein incorporated by reference to the same extent as if each individual publication were specifically and individually indicated as being incorporated by reference.

[00100] Although the invention herein has been described with reference to particular embodiments, it is to be understood that these embodiments are merely illustrative of the principles and applications of the present invention. It is therefore to be understood that numerous modifications may be made to the illustrative embodiments and that other

arrangements may be devised without departing from the spirit and scope of the present invention as defined by the appended claims.

What is claimed is:

1. A method for identifying a cullin dependent small molecule degrader of a target protein in a cellular screen, comprising:

incubating mammalian cells with a small molecule test compound in an amount sufficient to cause a change in phenotype in the cells, and a neddylation inhibitor at concentration that is nontoxic or of low toxicity to the cells; and

measuring a difference in phenotype in the cells following incubation relative to a control that comprises the cells incubated with the test compound but not the neddylation inhibitor, wherein a difference in the phenotype in the cells relative to the control indicates that the test compound causes cullin dependent degradation of the target protein.

2. The method of claim 1, wherein the test compound is a natural product, a synthetic compound, or semi-synthetic compound.

3. The method of any one of claims 1-2, wherein the test compound is a small molecule kinase inhibitor.

4. The method of any one of claims 1-3, wherein the test compound is a small molecule tyrosine kinase inhibitor.

5. The method of any one of claims 1-2, wherein the test compound is a clinical or preclinical small molecule drug.

6. The method of any one of claims 1-2, wherein the test compound is a small molecule therapeutic agent.

7. The method of claim 6, wherein the test compound is a small molecule anticancer therapeutic agent.

8. The method of claim 1, wherein the test compound is CR8, CC-885, or indisulam.

9. The method of any one of claims 1-8, wherein the concentration of the test compound is between about 0.001 μM to about 100 μM.

10. The method of any one of claims 1-9, wherein the neddylation inhibitor is MLN4924.

11. The method of any one of claims 1-9, wherein the neddylation inhibitor is a MLN4924 analog.

12. The method of any one of claims 1-9, wherein the neddylation inhibitor is TAS4464.

13. The method of any one of claims 1-9, wherein the neddylation inhibitor is a TAS4464 analog.

14. The method of any one of claims 1-13, wherein the concentration of the neddylation inhibitor is below 1μM.

15. The method of any one of claims 1-14, wherein the concentration of the neddylation inhibitor is between about 10 nM to about 500 nM.

16. The method of any one of claims 1-15, wherein the concentration of the neddylation inhibitor is about 100 nM.

17. The method of any one of claims 1-16, wherein the cells are pretreated with the neddylation inhibitor prior to incubation with the test compound.

18. The method of any one of claims 1-17, wherein the mammalian cells are human cells.

19. The method of any one of claims 1-18, wherein the cells are myeloid cells, lymphoid cells, neural cells, epithelial cells, endothelial cells, stem or progenitor cells, hepatocytes, myoblasts, osteoblasts, osteoclasts, lymphocytes, keratinocytes, melanocytes, mesothelial cells, germ cells, muscle cells, fibroblasts, transformed cells, non-transformed cells, or cancer cells.

20. The method of any one of claims 1-19, wherein the cells are primary cells.

21. The method of claim 20, wherein the primary cells are isolated from a tissue or a biopsy sample.

22. The method of any one of claims 1-19, wherein the cells are transformed cells.

23. The method of claim 22, wherein the transformed cells are Cas9 stable cells.

24. The method of any one of claims 1-19, wherein the cells are SNGM, P31FU, OVK18, PFEIFFER, ES-2, OCIM1, K562, HEK293, or HEK293T cells.

25. The method of claim 1-24, wherein the difference in the phenotype in the cells is cell viability.

26. The method of any one of claims 1-24, wherein the difference in the phenotype in the cells is increased protein abundance, decreased protein abundance, increased protein activity, decreased protein activity, increased gene expression, decreased gene expression, changed cellular proliferation, increased or decreased abundance of mRNA transcripts of landmark genes, or changed protein localization.

27. The method of any one of claims 1-26, wherein the difference in the phenotype in the cells is measured by flow cytometry, fluorescence-activated cell sorting (FACS), mass cytometry, and/or magnetic sorting, protein localization, cellular morphology, gene expression by RNA sequencing (RNAseq), Luminex multiplex bead assay, quantitative polymerase chain reaction (qPCR), next generation sequencing (NGS), or immunoblotting.

28. The method of claim 27, wherein the Luminex multiplex bead assay is L1000 assay.

FIG. 1A

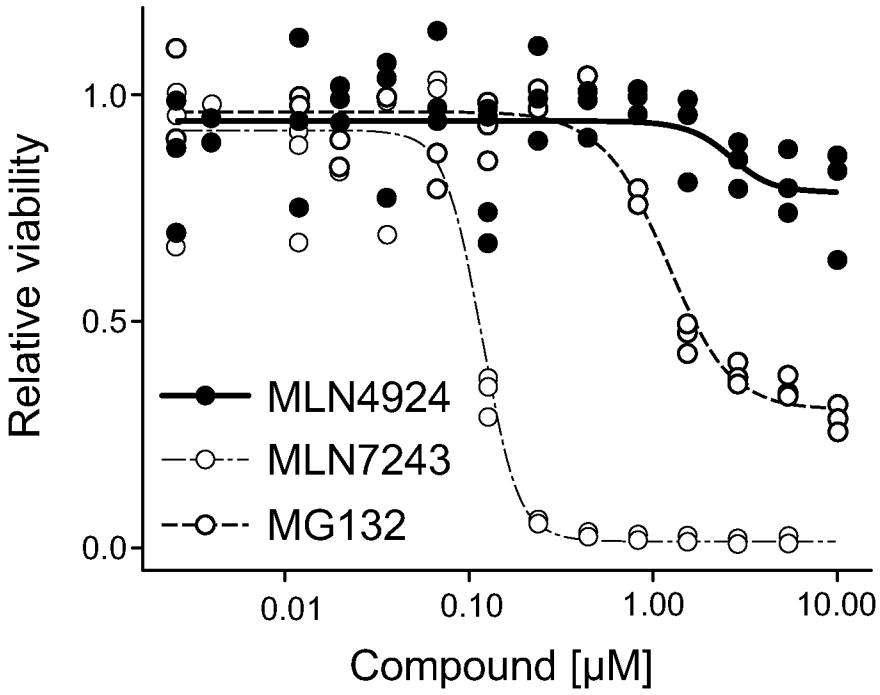

Relative viability
1.0
0.5
0.0
MLN4924
MLN7243
MG132
0.01
0.10
1.00
10.00
Compound [μM]

FIG. 1B

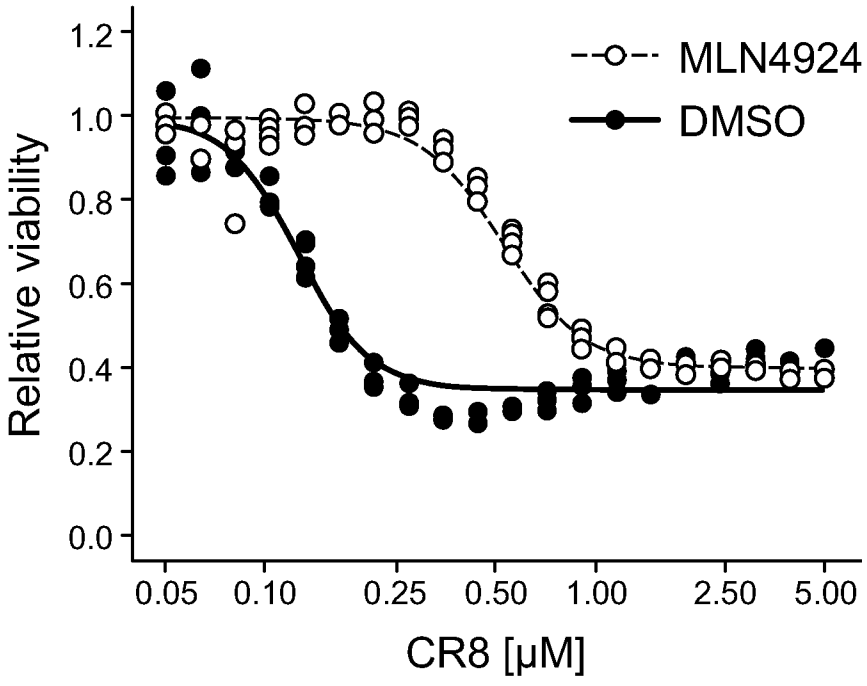

Relative viability
1.2
1.0
0.8
0.6
0.4
0.2
0.0
MLN4924
DMSO
0.05
0.10
0.25
0.50
1.00
2.50
5.00
CR8 [μM]

FIG. 2A

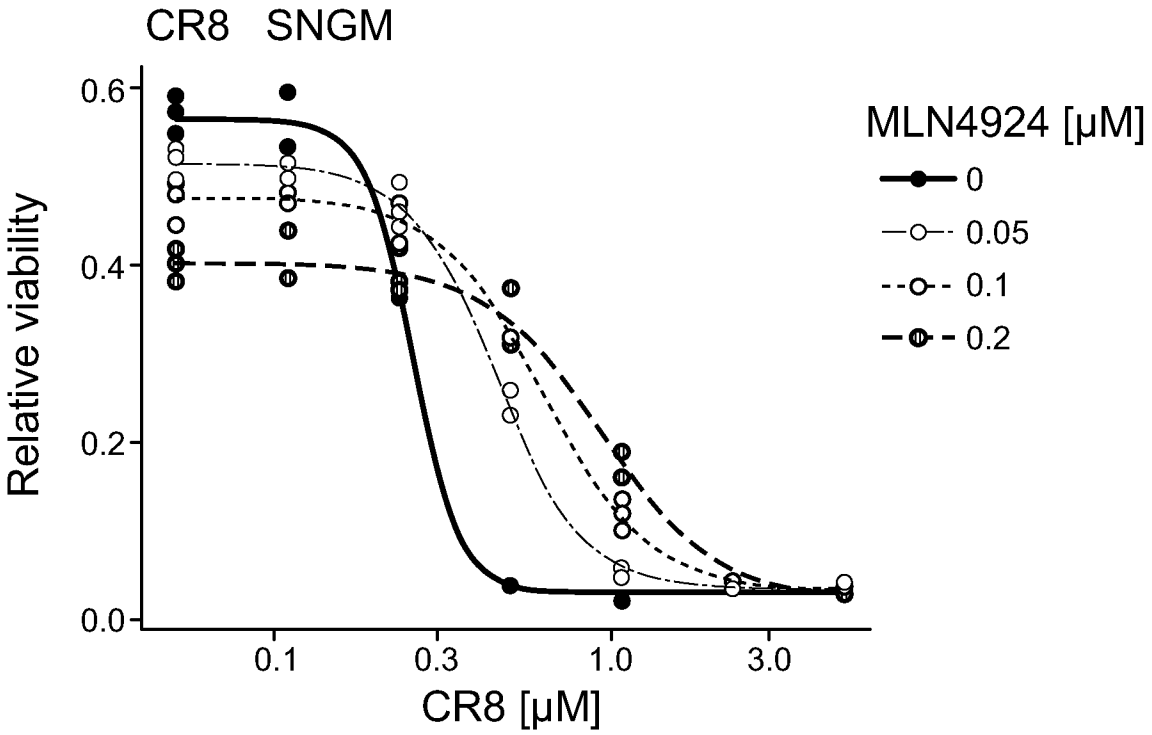

CR8 SNGM
Relative viability
0.6
0.4
0.2
0.0
0.1
0.3
1.0
3.0
CR8 [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 2B

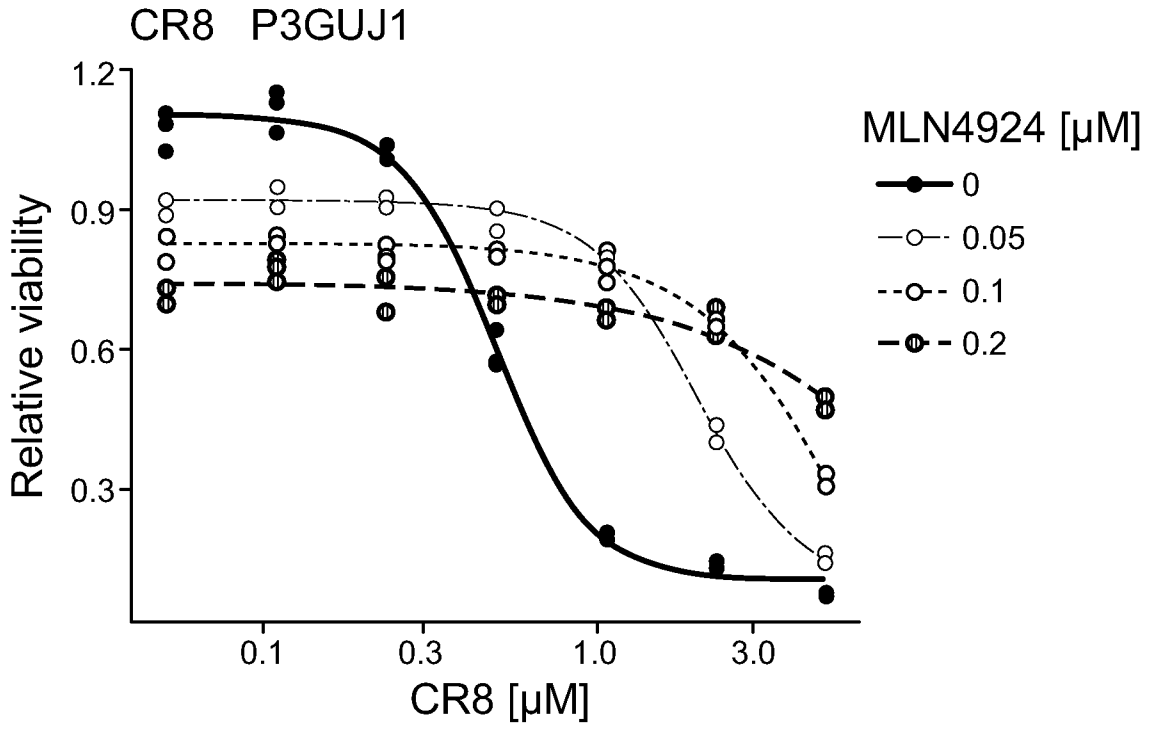

CR8 P3GUJ1
Relative viability
1.2
0.9
0.6
0.3
0.1
0.3
1.0
3.0
CR8 [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 2C

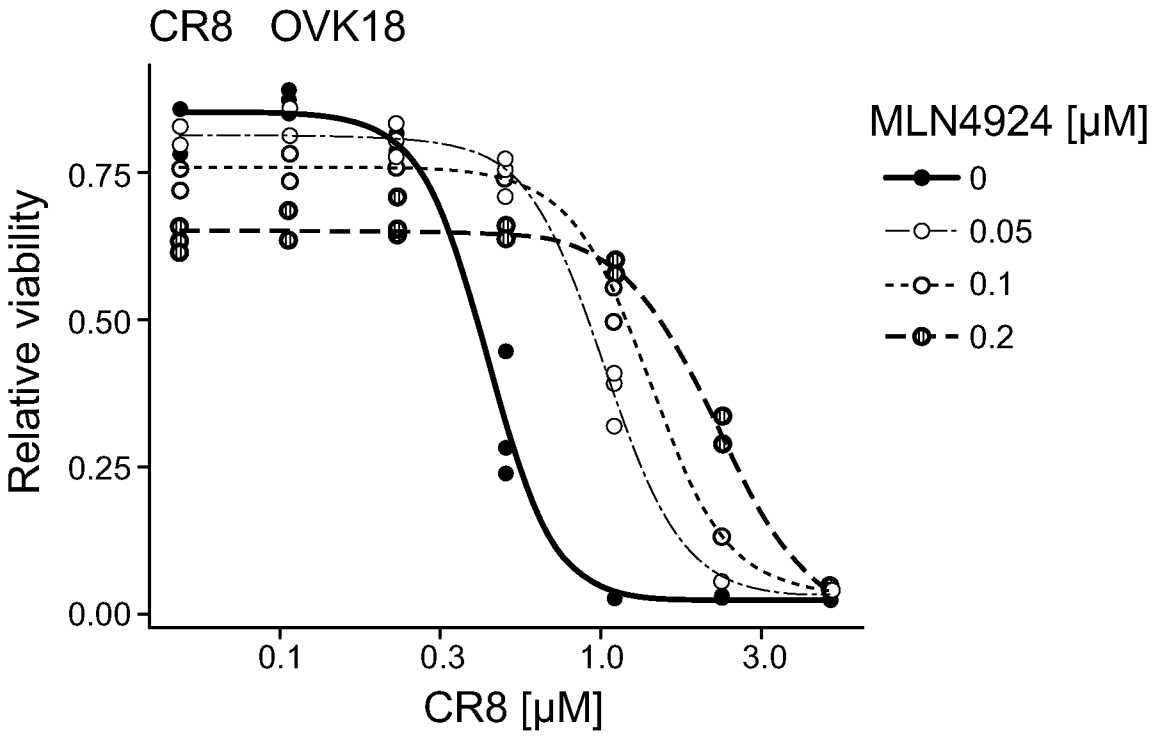

CR8 OVK18
Relative viability
0.00
0.25
0.50
0.75
0.1
0.3
1.0
3.0
CR8 [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 2D

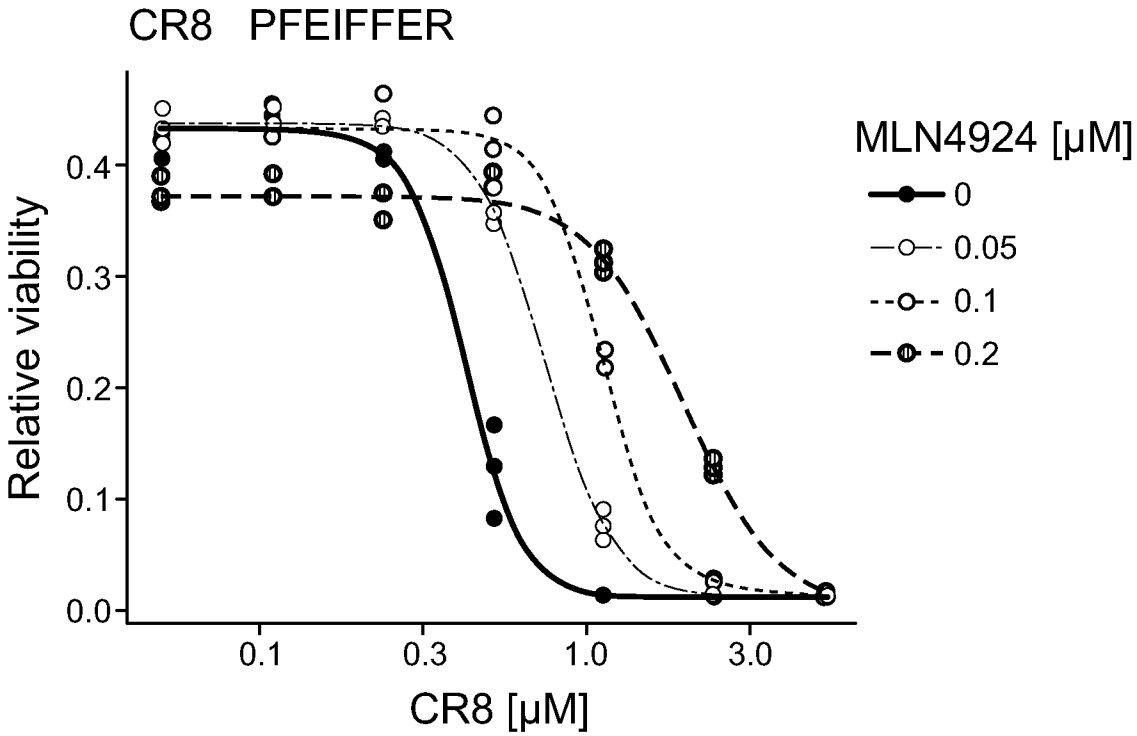

CR8 PFEIFFER
Relative viability
0.0
0.1
0.2
0.3
0.4
0.1
0.3
1.0
3.0
CR8 [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 2E

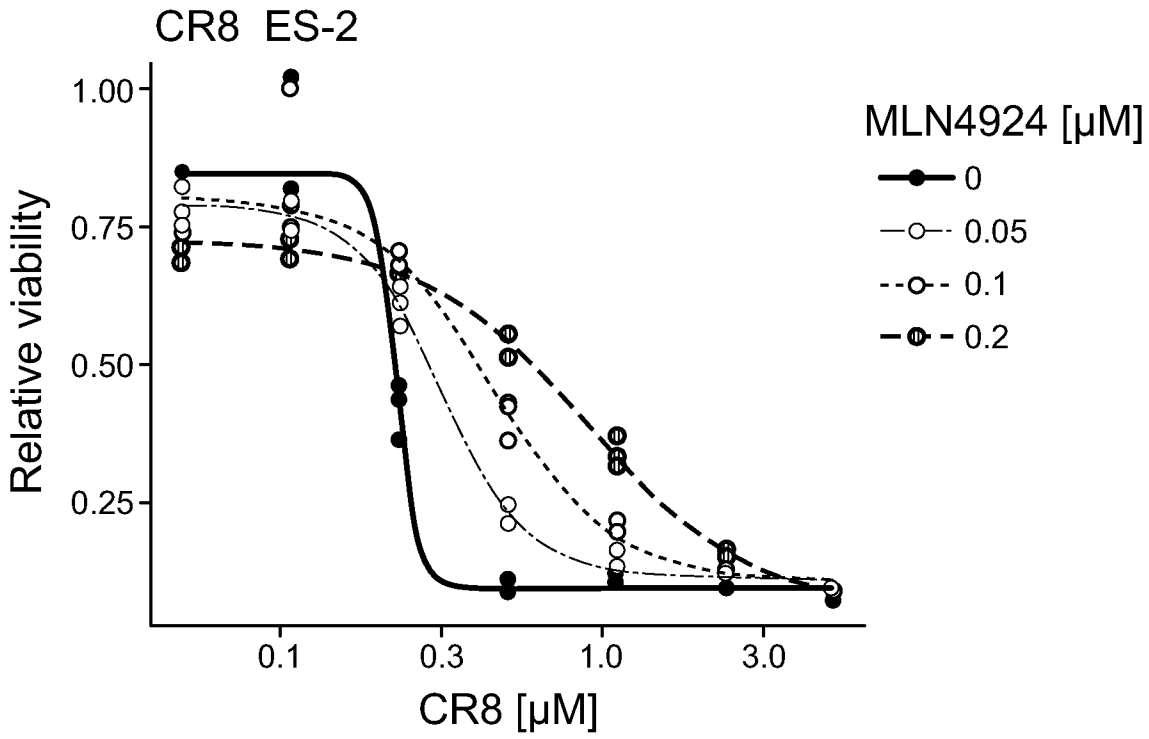

CR8 ES-2
Relative viability
1.00
0.75
0.50
0.25
0.1
0.3
1.0
3.0
CR8 [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 2F

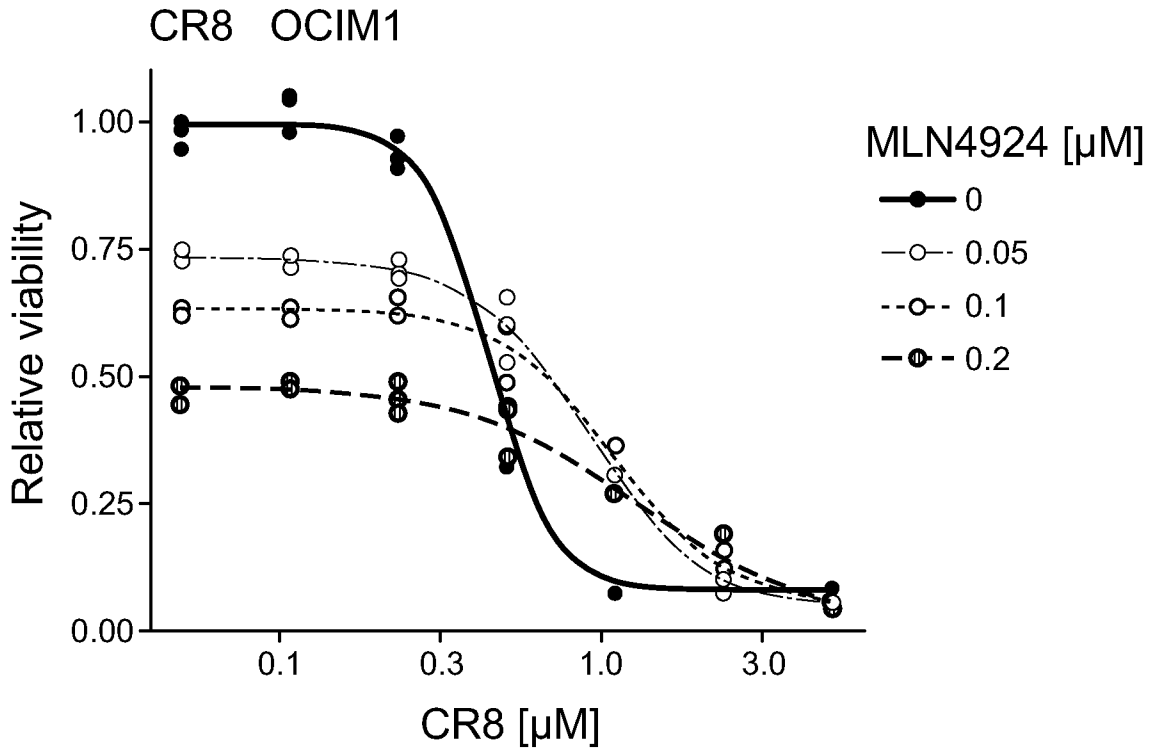

CR8 OCIM1
Relative viability
1.00
0.75
0.50
0.25
0.00
0.1
0.3
1.0
3.0
CR8 [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 2G

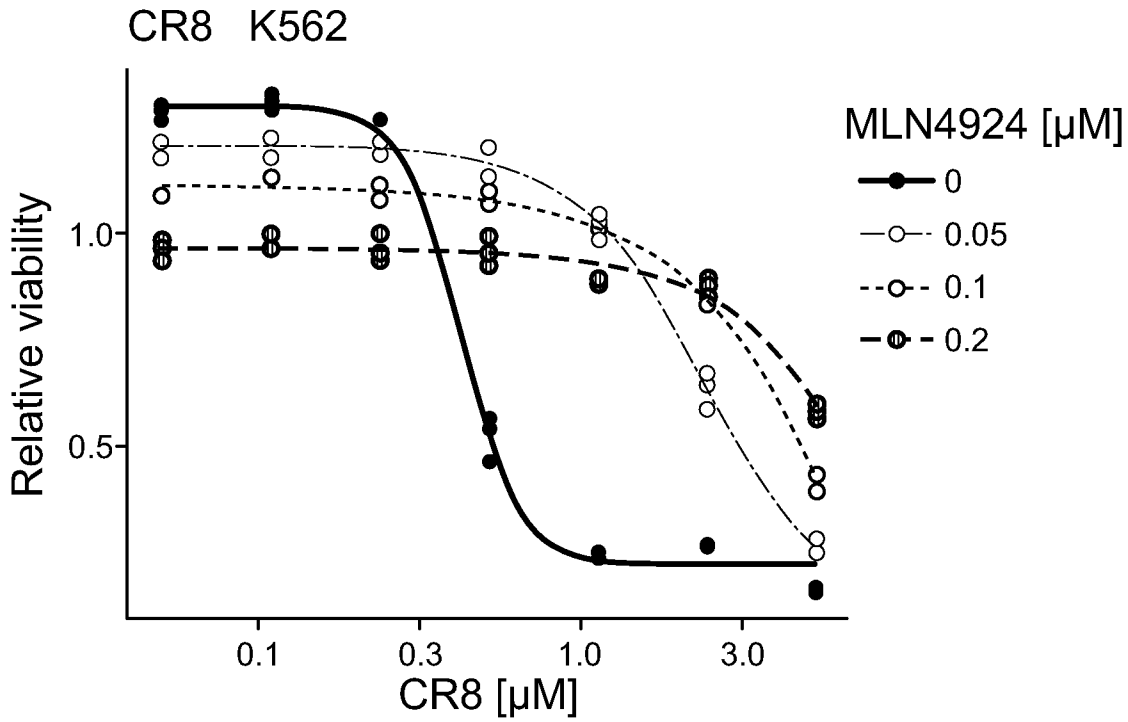

CR8 K562
Relative viability
1.0
0.5
0.1
0.3
1.0
3.0
CR8 [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 2H

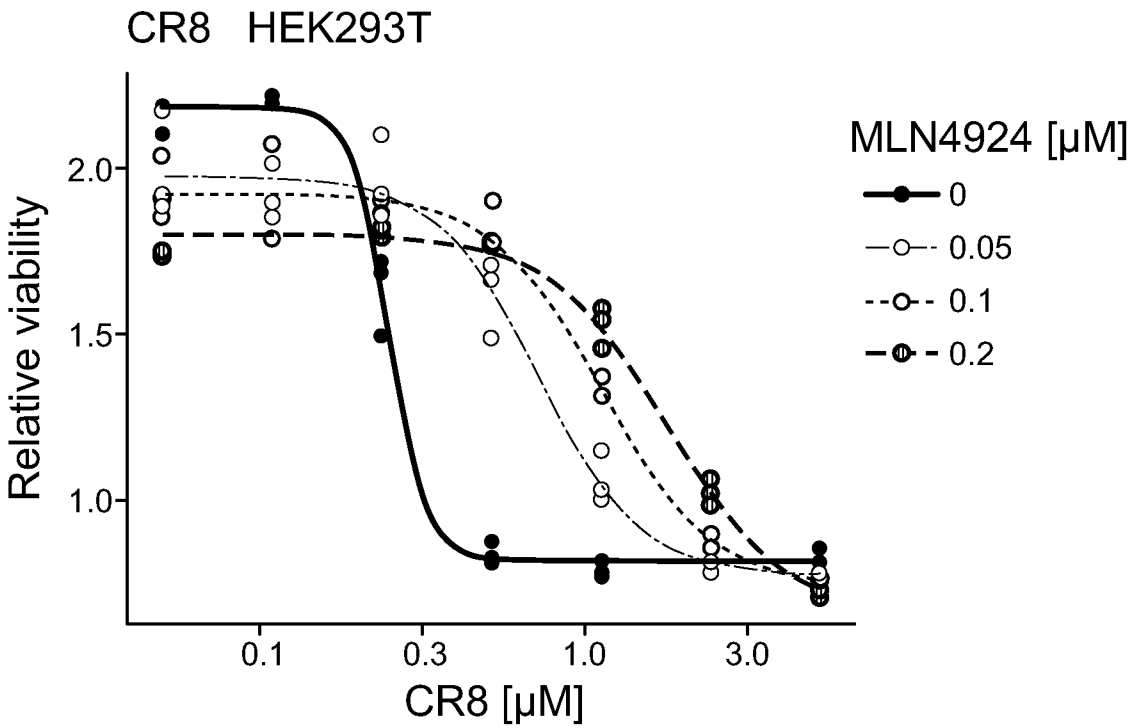

CR8 HEK293T
Relative viability
2.0
1.5
1.0
0.1
0.3
1.0
3.0
CR8 [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 3A

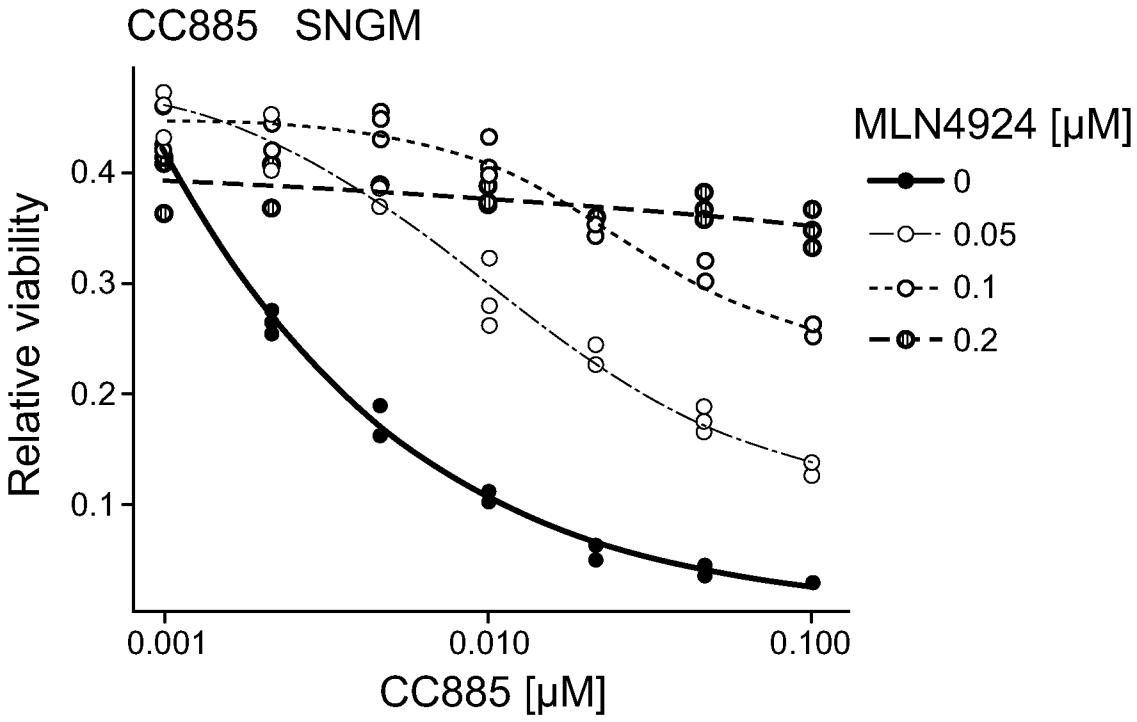

CC885 SNGM
Relative viability
0.4
0.3
0.2
0.1
0.001
0.010
0.100
CC885 [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 3B

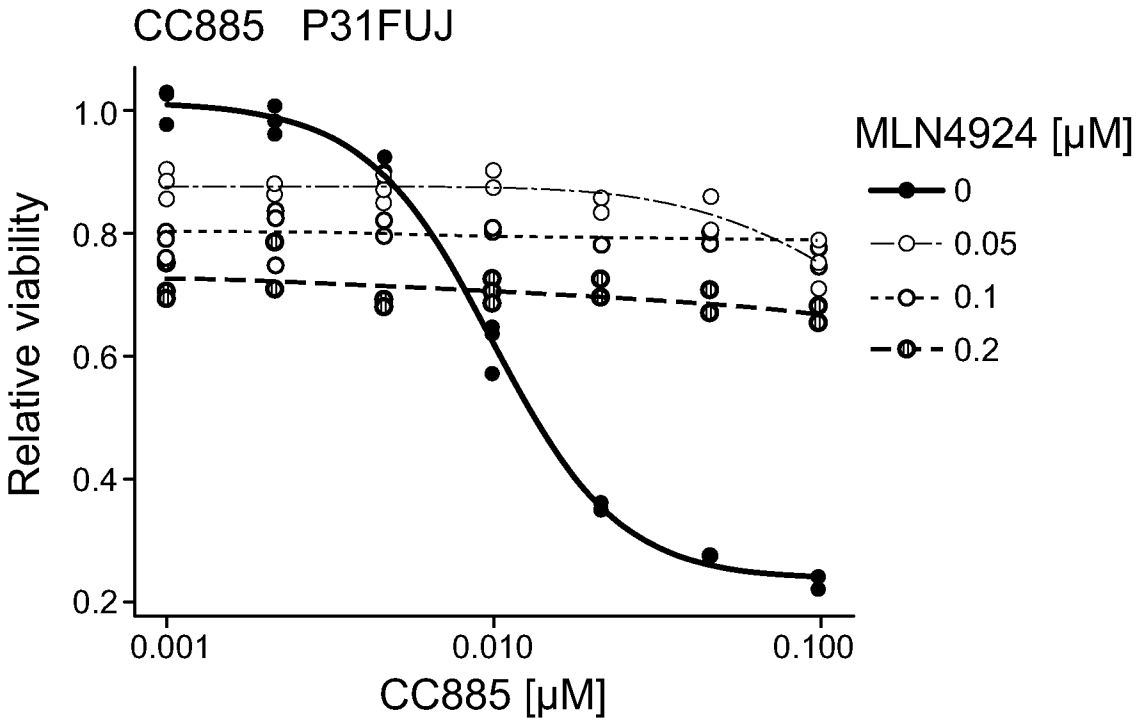

CC885 P31FUJ
Relative viability
1.0
0.8
0.6
0.4
0.2
0.001
0.010
0.100
CC885 [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 3C

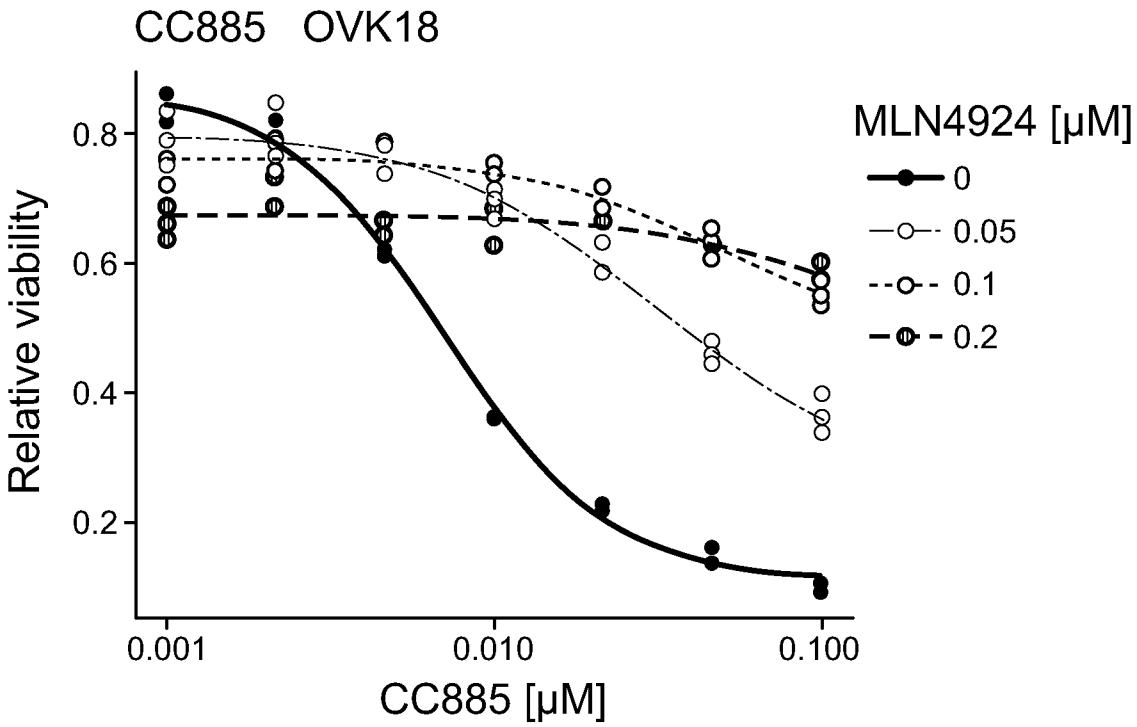

CC885 OVK18
Relative viability
0.8
0.6
0.4
0.2
0.001
0.010
0.100
CC885 [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 3D

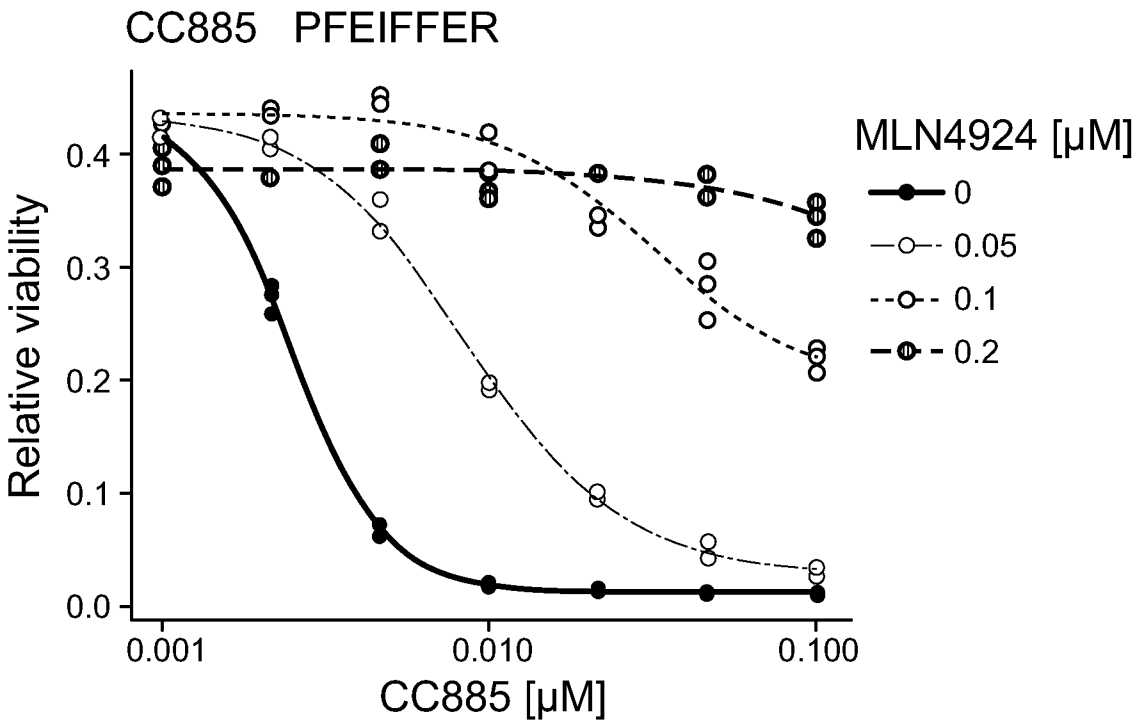

CC885 PFEIFFER
Relative viability
0.4
0.3
0.2
0.1
0.0
0.001
0.010
0.100
CC885 [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 3E

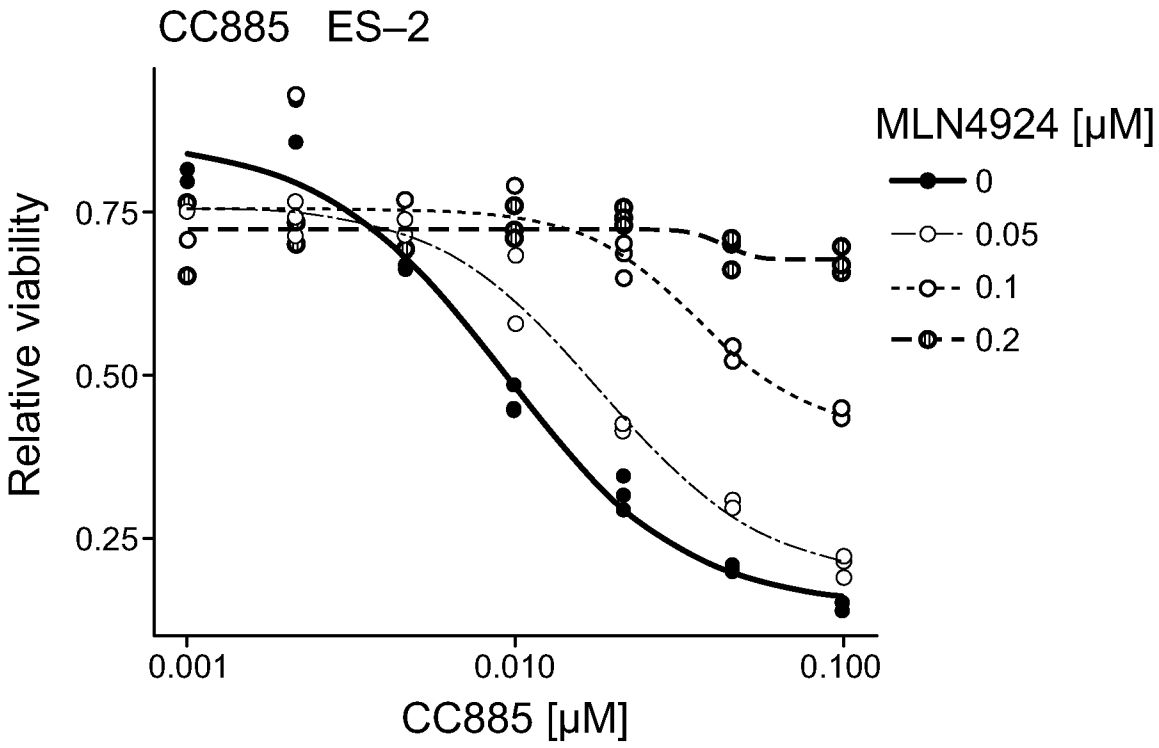

CC885 ES–2
Relative viability
0.75
0.50
0.25
0.001
0.010
0.100
CC885 [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 3F

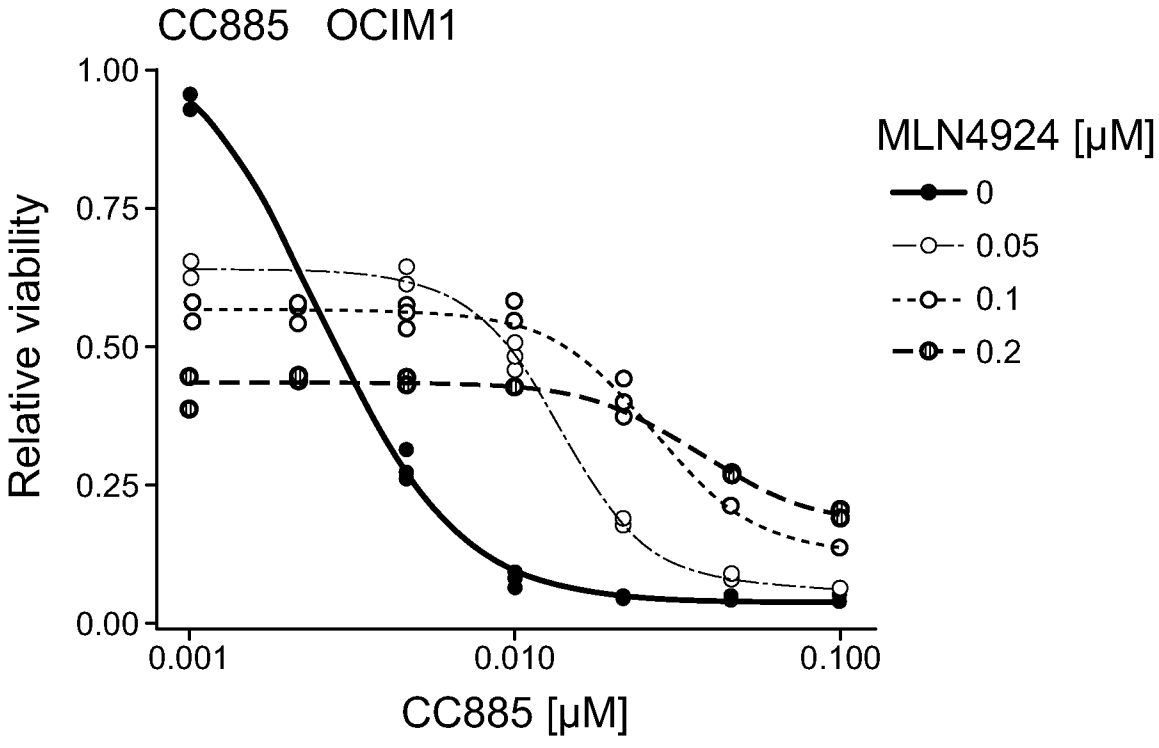

CC885 OCIM1
Relative viability
1.00
0.75
0.50
0.25
0.00
0.001
0.010
0.100
CC885 [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 3G

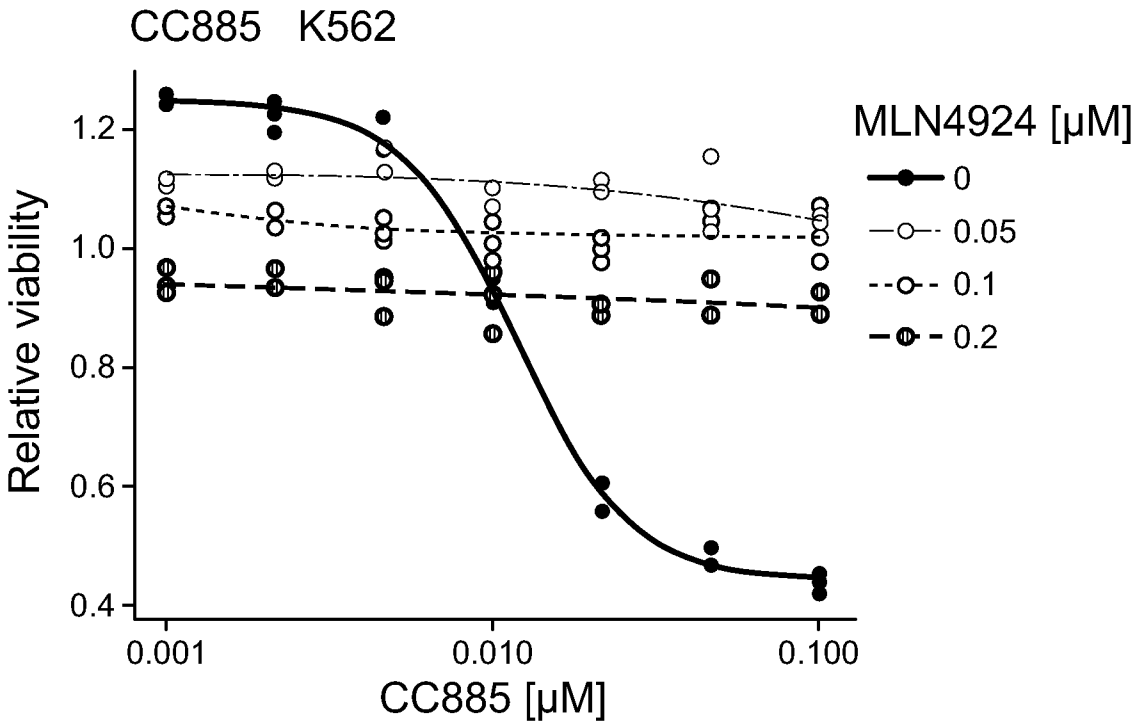

CC885 K562
Relative viability
1.2
1.0
0.8
0.6
0.4
0.001
0.010
0.100
CC885 [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 3H

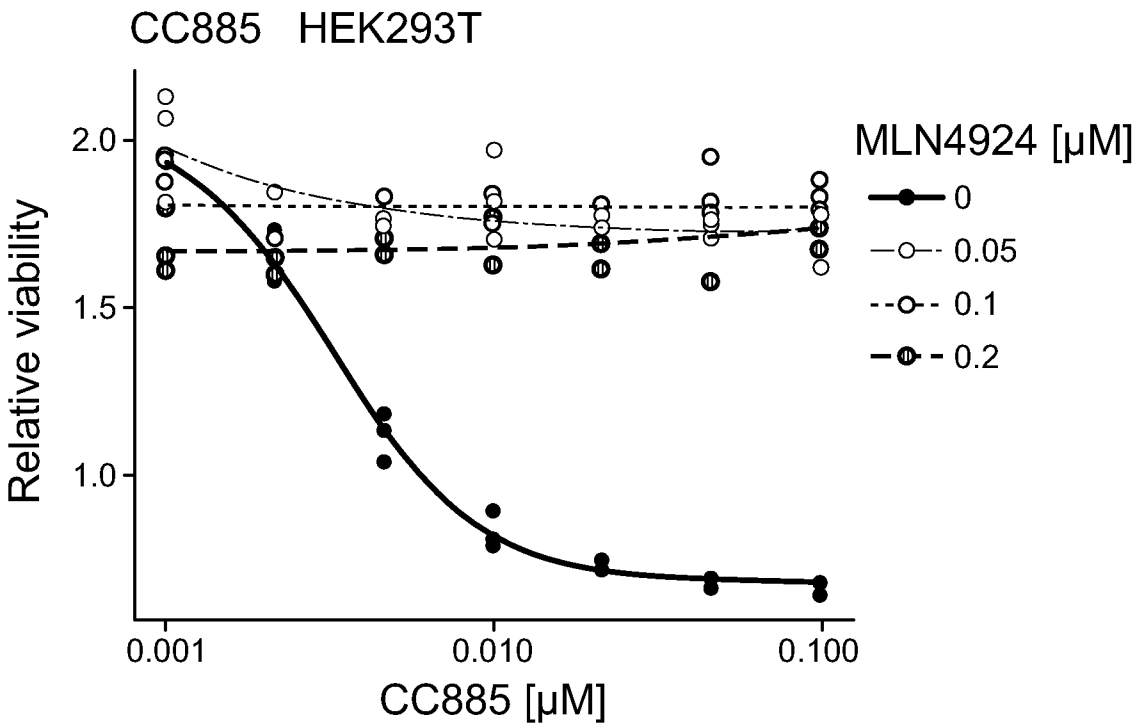

CC885 HEK293T
Relative viability
2.0
1.5
1.0
0.001
0.010
0.100
CC885 [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 4A

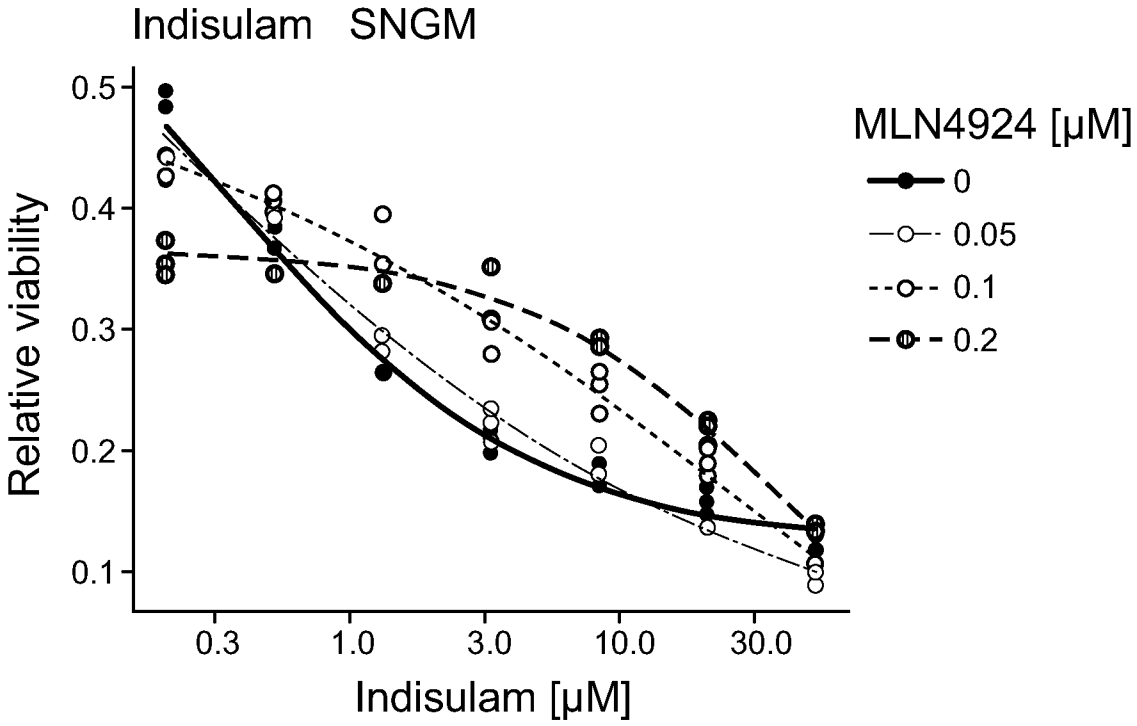

Indisulam SNGM
Relative viability
0.5
0.4
0.3
0.2
0.1
0.3
1.0
3.0
10.0
30.0
Indisulam [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 4B

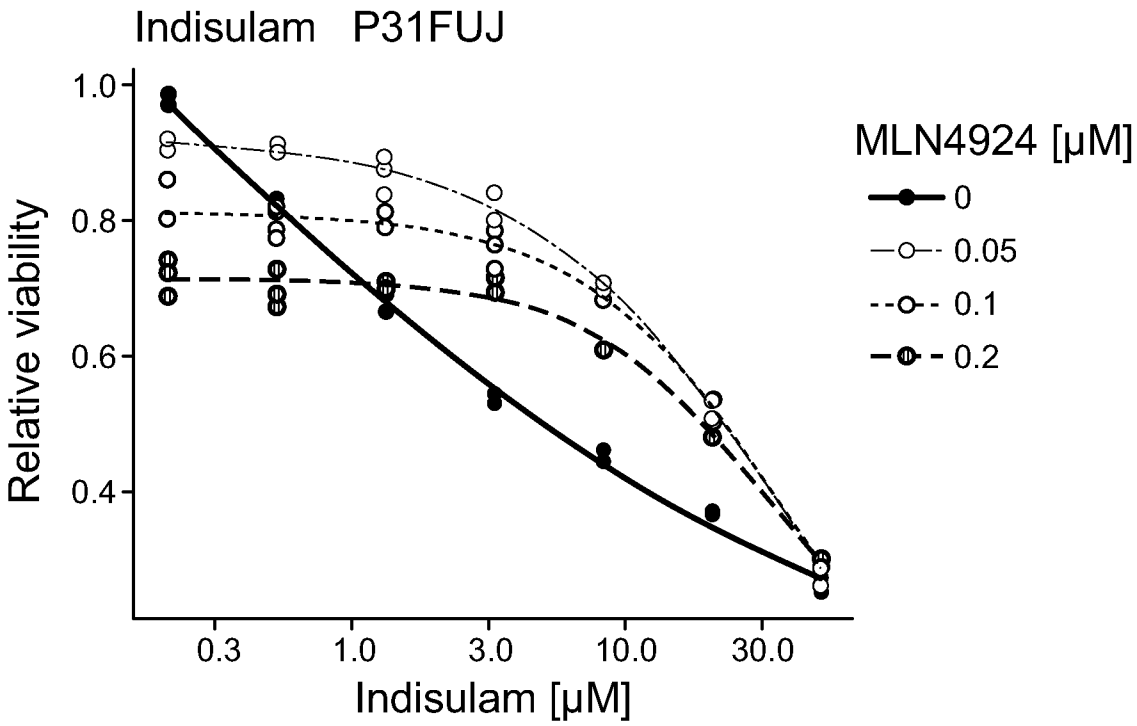

Indisulam P31FUJ
Relative viability
1.0
0.8
0.6
0.4
0.3
1.0
3.0
10.0
30.0
Indisulam [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 4C

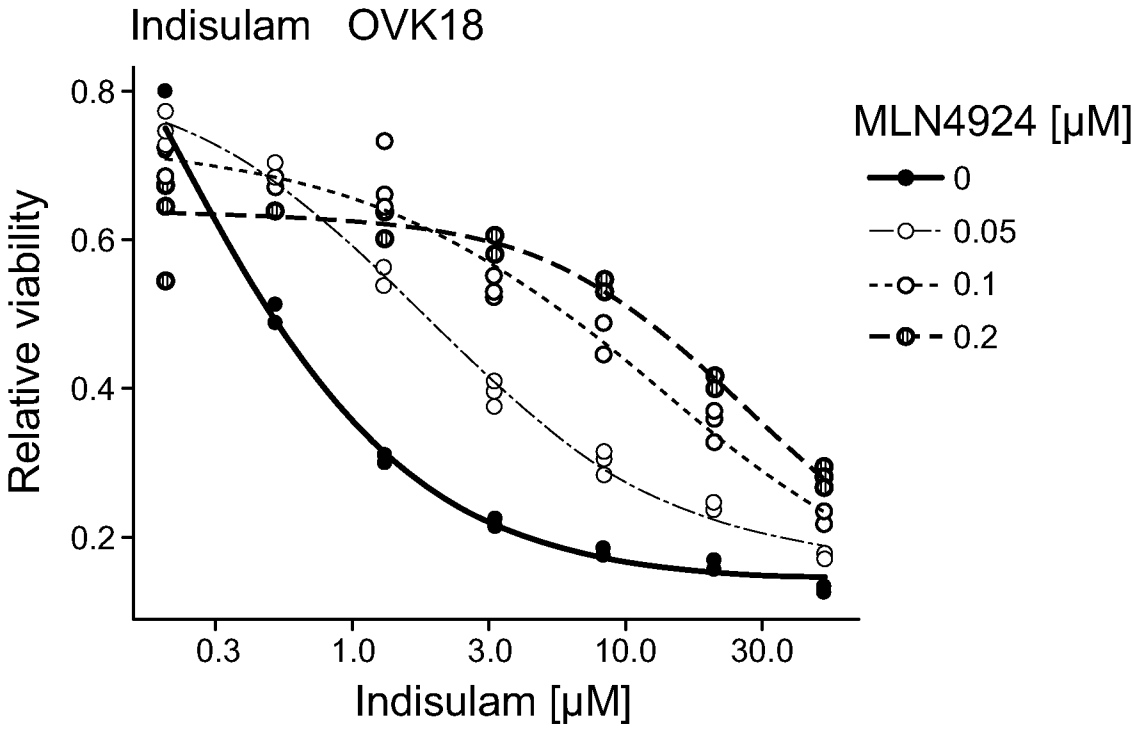

Indisulam OVK18
Relative viability
0.8
0.6
0.4
0.2
0.3
1.0
3.0
10.0
30.0
Indisulam [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 4D

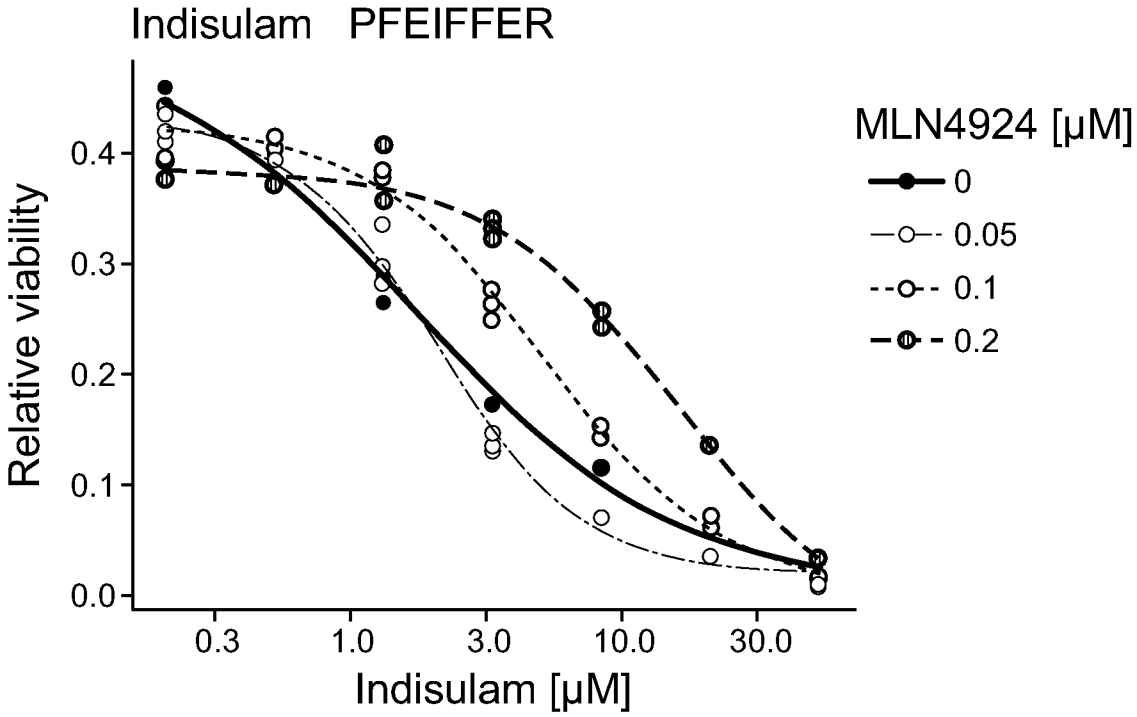

Indisulam PFEIFFER
Relative viability
0.4
0.3
0.2
0.1
0.0
0.3
1.0
3.0
10.0
30.0
Indisulam [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 4E

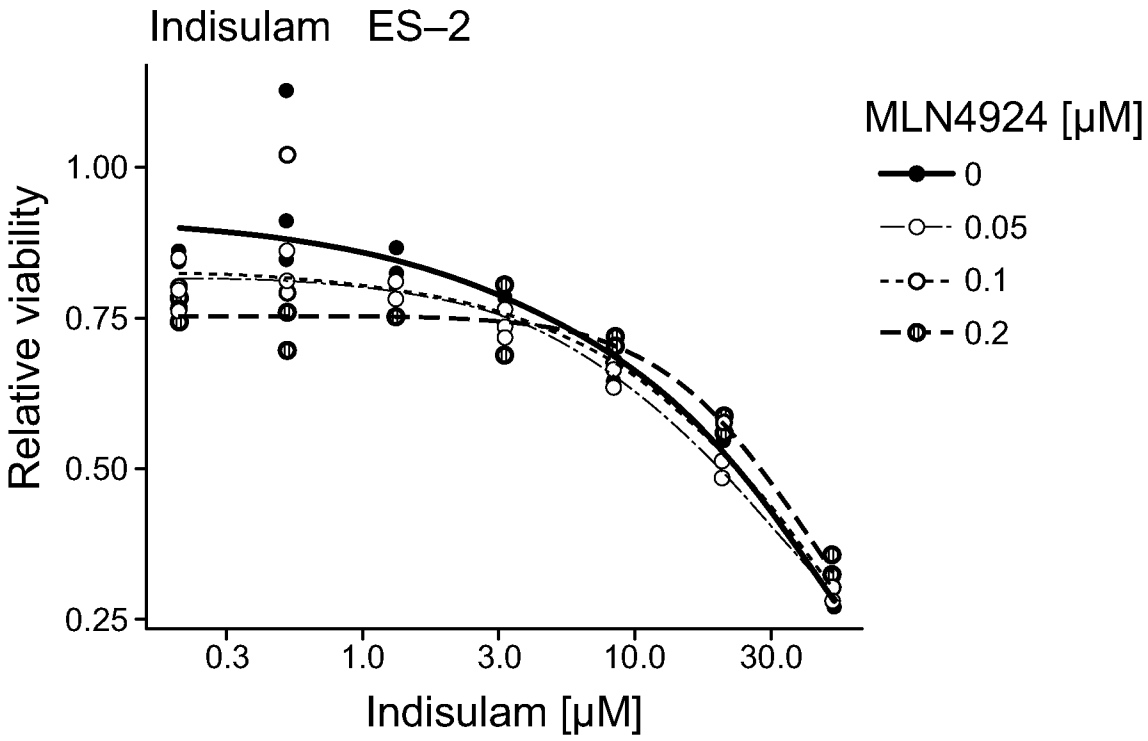

Indisulam ES–2
Relative viability
1.00
0.75
0.50
0.25
0.3
1.0
3.0
10.0
30.0
Indisulam [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 4F

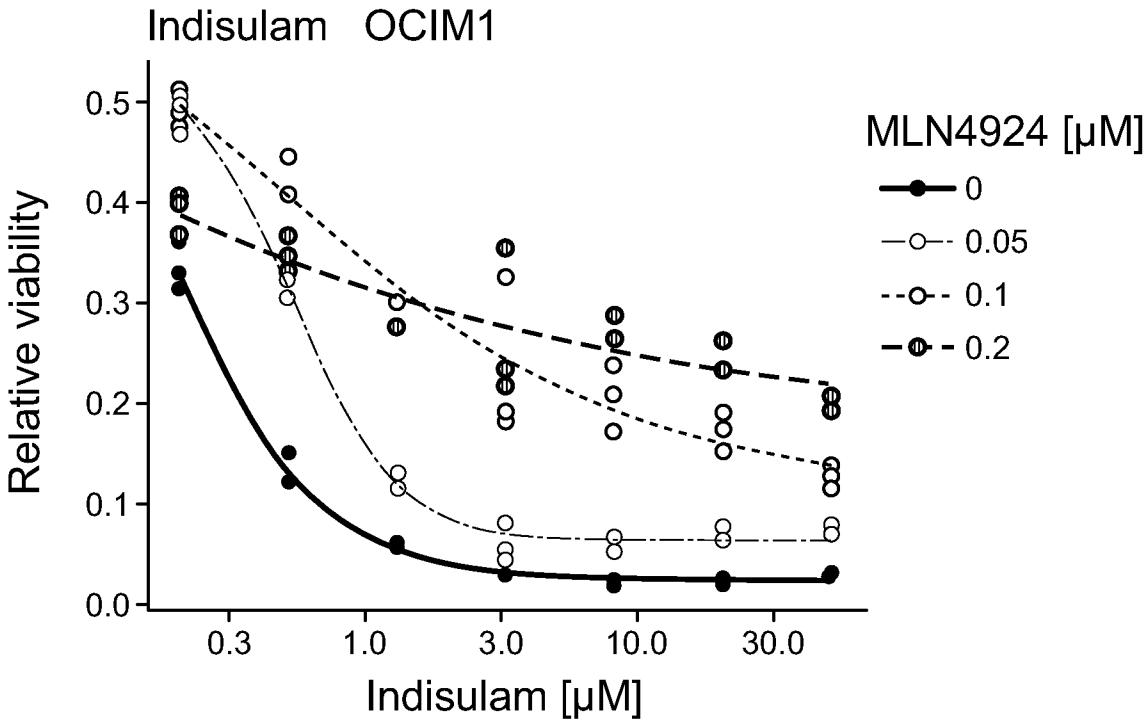

Indisulam OCIM1
Relative viability
0.5
0.4
0.3
0.2
0.1
0.0
0.3
1.0
3.0
10.0
30.0
Indisulam [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 4G

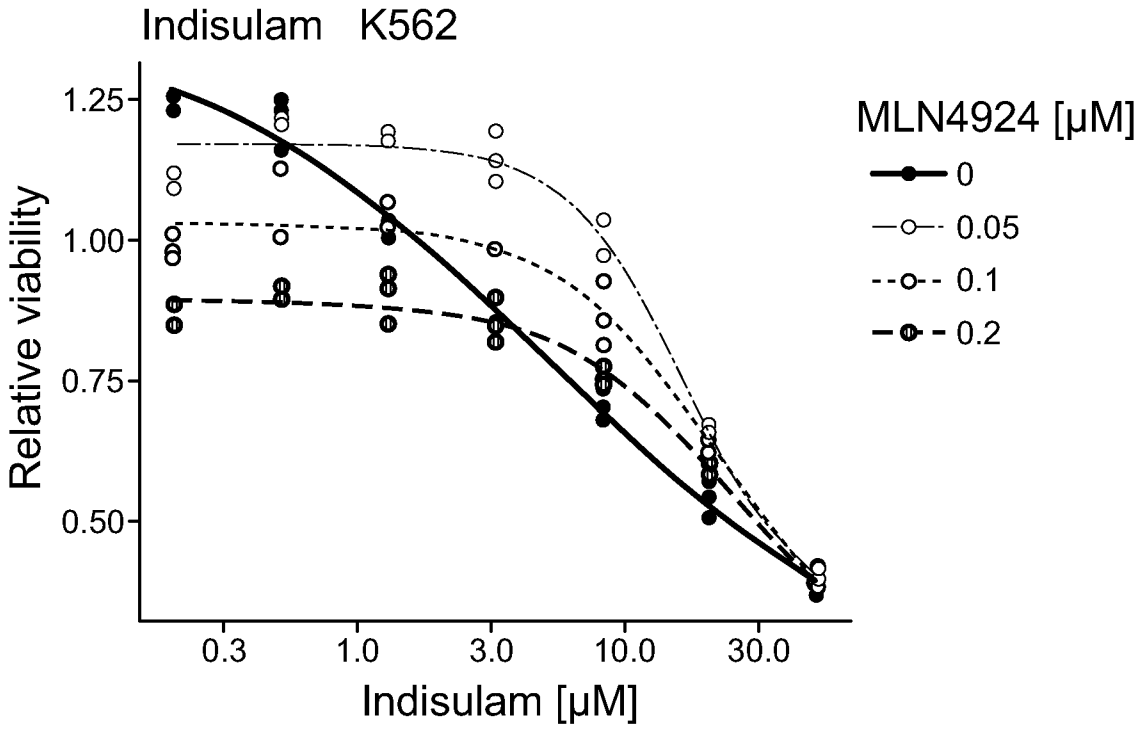
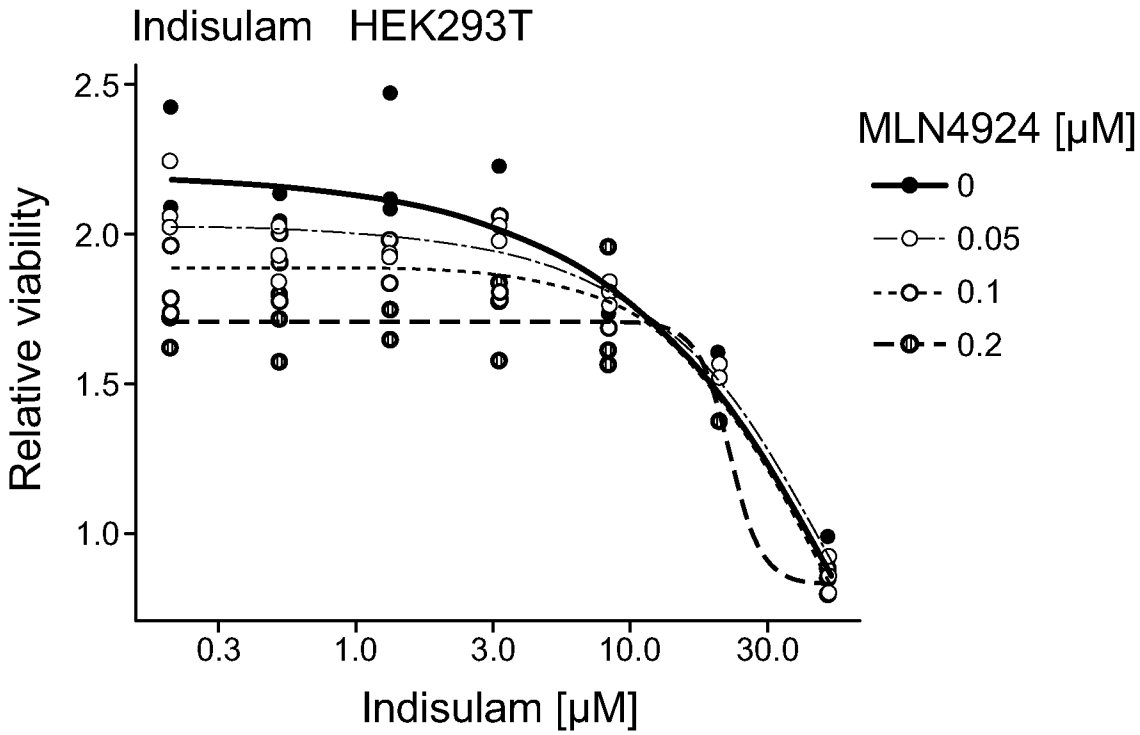

Indisulam K562
Relative viability
1.25
1.00
0.75
0.50
0.3
1.0
3.0
10.0
30.0
Indisulam [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

FIG. 4H


Indisulam HEK293T
Relative viability
2.5
2.0
1.5
1.0
0.3
1.0
3.0
10.0
30.0
Indisulam [μM]
MLN4924 [μM]
0
0.05
0.1
0.2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 22/13294

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A01K 67/033; A61K 49/00; C12N 15/85 (2022.01)

CPC - A01K 67/0336; A61K 49/0008; C12N 15/8509

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	Slabicki et al., "The CDK inhibitor CR8 acts as a molecular glue degrader thatdepletes cyclin K" 03 June 2020 (03.06.2020) entire document especially Page 1, Para 1; Page 21, Para 1; Figure 2d	1-3 and 5-8
A	US 2019/0374657 A1 (DANA - FARBER CANCER INSTITUTE , INC) 12 December 2019 (12.12.2019) entire document	1-3 and 5-8
A	US 2018 / 0140578 A1 (Board of Regents of the University of Texas System) 24 May 2018 (24.05.2018) entire document	1-3 and 5-8
A	US 2020/0348285 A1 (DANA - FARBER CANCER INSTITUTE , INC .) 05 November 2020 (05.11.2020) entire document	1-3 and 5-8

☐ 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	Date of mailing of the international search report
24 March 2022	APR 18 2022
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Kari Rodriquez Telephone No. PCT Helpdesk: 571-272-4300

Form PCT/ISA/210 (second sheet) (July 2019)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 22/13294

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.: 4,9-28
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.