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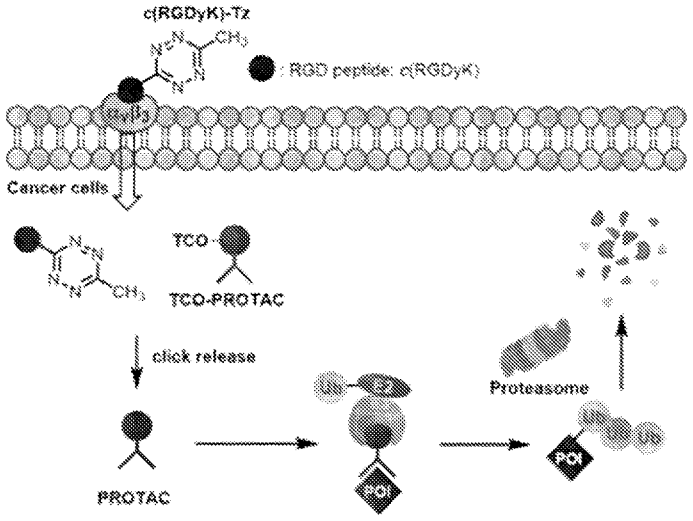
(54) Title: BIOORTHOGONAL ACTIVATION OF PROTAC PRODRUGS

FIG. 1A



(57) Abstract: Described herein is an anticancer combination therapy comprising a PROTAC pro-drug having a transcyclooctene (TCO) moiety and one or more compound having a tetrazine. In one embodiment, the PROTAC pro-drug is not active in the absence of the one or more compound having a tetrazine. In another embodiment, the tetrazine is administered prior to the PROTAC pro-drug. Also described herein is an anticancer combination therapy comprising a PROTAC pro-drug having a galactose, or derivative thereof, and one or more antineoplastic agent. In one embodiment, the PROTAC pro-drug is not active in the absence of the one or more antineoplastic agent. In another embodiment, the antineoplastic agent is administered prior to the PROTAC pro-drug.

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[Continued on next page]

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BIOORTHOGONAL ACTIVATION OF PROTAC PRODRUGS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application No. 63/489,178, filed on March 8, 2023, and U.S. Provisional Patent Application No. 63/620,051, filed on January 11, 2024, each of which is incorporated by reference herein in its entirety.

FEDERALLY SPONSORED RESEARCH

This invention was made with government support under grant number GM130772 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

Emerging proteolysis-targeting chimaeras (PROTACs) exploit cellular quality control machinery to selectively degrade target proteins including the undruggable targets such as transcriptional factors and scaffold proteins. PTOTACs, which do not rely on occupancy-driven pharmacology, offers unparalleled power over traditional small-molecule inhibitors in that they can degrade their protein targets in a sub-stoichiometric, catalytic fashion and overcome drug resistance. However, potential toxicity from systemic degradation of proteins in healthy cells and undesirable ligase-mediated off-target effects may limit their application in clinical practice. To address these issues, PROTAC prodrugs have been developed to selectively deliver and/or activate at the tumor sites, mainly relying on tumor biomarkers, such as the tumor microenvironment-activation, folate, and aptamers. However, tumor microenvironment may cause premature drug activation before entering cancerous cells and protein receptors are rarely tumor cell specific and in many cases the expression level between cancer and normal cells is not significant. Additionally, the therapeutic resistance mutation of protein receptors makes the targeting approach difficult and vulnerable in cancer treatment. So far, no small molecule-based targeting strategies for cancer therapy have made it to the clinic. Highly selective antibody-drug conjugates (ADCs) have also been applied for PROTACs. Nonetheless, the use of antibodies suffers from drawbacks including high production cost, receptor saturation, poor solid tumor penetration and severe immunogenicity. In addition, each mAb developed is effective for only certain types of cancer because the targeted protein receptors vary from cancer to cancer. Abiotic light controllable PROTACs enable achieving spatiotemporal regulation of the activity of the PROTAC molecules. Nevertheless, these methods are limited cancer types with light accessibility and in some cases, UV light activation is used and often induces undesired tissue damage.

Bioorthogonal-activatable prodrugs are a promising strategy that allows precise and abiotic activation of drugs. The fast reaction kinetics and the excellent bioorthogonality and biocompatibility enable the tetrazine (Tz) and transcyclooctene (TCO)-engaged inverse-electron-demand Diels-Alder (IEDDA) proceeded efficiently in complex biological context. The TCO-caged pro-drugs have attracted great attention and produced the first clinical anticancer drug delivery studies in human. However, the prodrug strategy has been mainly focused on caging cytotoxic agents. The application for PROTACs has not been reported. Moreover, due to the lack of targeted delivery capacity of the Tz activation component, an excessive amount of the Tz is generally used. This may cause additional side effects.

The αvβ3 integrin is one of the most well-studied targets for drug delivery into cancer cells because it is highly expressed in certain cancer cell surface and plays a key role in cell invasion and proliferation during tumor vascular remodeling and angiogenesis. As such, a αvβ3 integrin-targeting strategy has been used for decades in both tumor imaging, diagnosis, and cancer-targeting drug delivery.

Cellular senescence, a stable and stagnant terminal state that occurs after stress-induced cellular damage, has been recognized as a hallmark of aging and cancer. The accumulation of cellular senescence when aging promotes the release of pro-inflammatory, pro-apoptotic and pro-fibrotic senescence-associated secretory phenotype (SASP) factors. These factors can impair the ability of tissue regeneration and drives chronic low-grade inflammation, which exacerbates the aging process and promote neoplastic growth, therapy resistance, immunosuppression, metastasis and angiogenesis in tumor. Methods for selective manipulation of senescent cells contribute to the understanding of the underlying mechanism of action and offer new therapeutic strategies for the selective removal of senescent cells (e.g., 'senolysis') that improves age-related diseases and cancer therapy. However, the lack of methods for selective targeting these cells and the heterogeneity and dynamics of senescent cells result in notable limitations of senolytics in terms of accuracy (on-site efficacy and off-site toxicity) and broad-spectrum activity (effectiveness against different types of senescent cells). Therefore, there is a demand for developing new technologies to enhance onsite efficacy and off-site toxicity.

What is needed are bioorthogonal-activatable pro-drugs that can target senescent cells.

SUMMARY

One embodiment described herein is a combination therapeutic comprising: a therapeutically effective amount of a PROTAC pro-drug, or pharmaceutically acceptable salts or esters thereof, wherein the PROTAC pro-drug comprises a ligand targeting a protein of interest,

a PROTAC linker, an E3 ligase targeting ligand, and a transcyclooctene (TCO) moiety; and a therapeutically effective amount of one or more compounds comprising a tetrazine (Tz), or pharmaceutically acceptable salts or esters thereof. In one aspect, the transcyclooctene (TCO) moiety is covalently attached to the E3 ligase target ligand. In another aspect, the transcyclooxtene (TCO) moiety is covalently attached to the E3 ligase target ligand via a pro-drug linker. In another aspect, the PROTAC pro-drug comprises a structure:

$$X–L^1–Y,$$

wherein

X is the ligand targeting a protein of interest;

L^1 is the PROTAC linker; and

Y is the E3 ligase targeting ligand.

In another aspect, the PROTAC pro-drug comprises a structure:

$$X–L^1–Y–L^2–Q,$$

wherein

X is the ligand targeting a protein of interest;

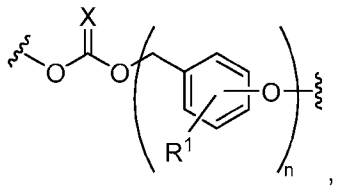
L^1 is the PROTAC linker;

Y is the E3 ligase targeting ligand;

L^2 is a pro-drug linker; and

Q is the transcyclooctene (TCO) moiety.

In another aspect, the prodrug linker comprises a linker:



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wherein

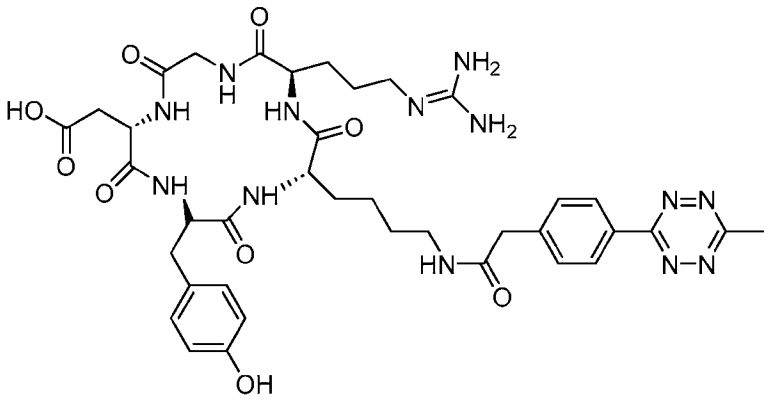
X is O, S, or N;

R^1 is hydrogen, halogen, C_1–$_6$alkyl, C_1–$_6$alkylene, C_1–$_6$haloalkyl, cyano, $–OR^{1a}$, $–SR^{1a}$, $–CO_2R^{1a}$, $–C(O)R^{1a}$, $–SO_2R^{1b}$, $–N(R^{1b})_2$, $–CO_2N(R^{1b})_2$, $–NO_2$, or $–N(R\text{-}R^{1b})–OR^{1a}$; and

n = 0–1.

In another aspect, the E3 ligase target ligand is a von Hippel-Lindau (VHL) protein targeting ligand. In another aspect, the von Hippel-Lindau (VHL) protein targeting ligand comprises VH032, VH032-OH, (S,R,S)-AHPC-Me, pharmaceutically acceptable salts thereof, or derivatives thereof.

In another aspect, the PROTAC pro-drug comprises MS-99, ARV-771, DT2216, TD-004, ARCC-4, VZ-185, ERD-308, BI-0319, SJFα, UNC-6852, DAT8, pharmaceutically acceptable salts thereof, or derivatives thereof. In another aspect, the one or more compounds comprises a tetrazine (Tz) selected from:



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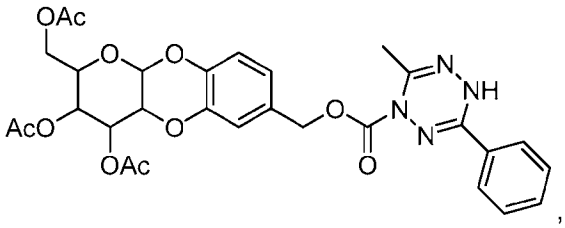

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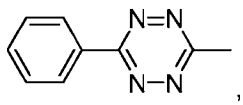

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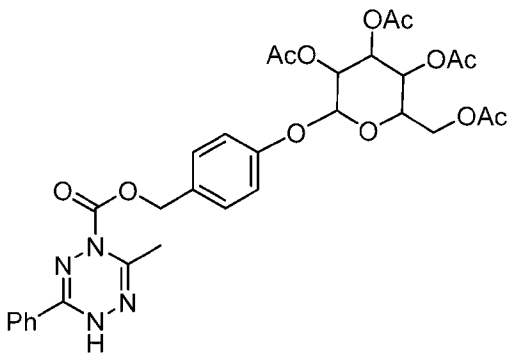

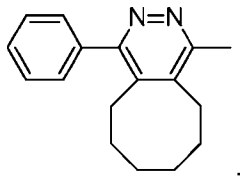

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, or

In another aspect, the therapeutically effective amount of the PROTAC pro-drug is 1–200 mg/kg. In another aspect, the therapeutically effective amount of the one or more compound comprising a tetrazine (Tz) is 1–200 mg/kg.

Another embodiment described herein is a method for treating a disease or disorder, the method comprising: sequentially or simultaneously administering to a subject in need thereof: a therapeutically effective amount of a PROTAC pro-drug, or pharmaceutically acceptable salts or esters thereof, wherein the PROTAC pro-drug comprises a ligand targeting a protein of interest, a PROTAC linker, an E3 ligase targeting ligand, and a transcyclooctene (TCO) moiety; and a

therapeutically effective amount of one or more compounds comprising a tetrazine (Tz), or pharmaceutically acceptable salts or esters thereof; and repeating administration until the disease or disorder is treated, ameliorated, or symptoms are reduced. In one aspect, the disease or disorder is a cancer, neurodegenerative disease, aging, autoimmune diseases, or viral infections. In another aspect, the cancer comprises a breast cancer, melanoma, glioblastoma multiforme, colon cancer, prostate cancer, acute myeloid leukemia, or acute lymphoblastic leukemia and their corresponding senescent cells. In another aspect, administering comprises intraperitoneal injection, intramuscular injection, subcutaneous injection, intravenous injection, intrathecal infusion, oral administration, or a combination thereof. In another aspect, an amount of a PROTAC increases and an amount of PROTAC pro-drug decreases. In another aspect, the PROTAC pro-drug and one or more antineoplastic agents reduce an amount of a target protein. In another aspect, the therapeutically effective amount of the PROTAC pro-drug is added at a period of time prior to addition of the therapeutically effective amount of the one or more compounds comprising a tetrazine (Tz). In another aspect, the period of time is 0–168 hours.

Another embodiment described herein is use of the PROTAC pro-drug and the one or more compounds comprising a tetrazine (Tz), or pharmaceutically acceptable salts or esters thereof, as a medicament for the treatment of cancer in subject in need thereof.

Another embodiment described herein is a kit comprising: one or more PROTAC pro drugs, or pharmaceutically acceptable salts or esters thereof; one or more compounds comprising a tetrazine (Tz), or pharmaceutically acceptable salts or esters thereof; optionally a device or means for administering the PROTAC pro-drug and one or more compounds comprising a tetrazine (Tz); optionally tamper resistant packaging; and optionally, a label or instructions for use thereof.

One embodiment described wherein is a combination therapeutic comprising: a therapeutically effective amount of a PROTAC pro-drug, or pharmaceutically acceptable salts or esters thereof, wherein the PROTAC pro-drug comprises a ligand targeting a protein of interest, a PROTAC linker, an E3 ligase targeting ligand, and a galactose, or derivative thereof; and a therapeutically effective amount of one or more antineoplastic agents, or pharmaceutically acceptable salts or esters thereof. In one aspect, the galactose, or derivative thereof, is covalently attached to the E3 ligase target ligand. In another aspect, the galactose, or derivative thereof is covalently attached to the E3 ligase target ligand via a pro-drug linker. In another aspect, the PROTAC pro-drug comprises a structure:

$$X–L^1–Y,$$

wherein

X is the ligand targeting a protein of interest;

L^1 is the PROTAC linker; and

Y is the E3 ligase targeting ligand.

In another aspect, the PROTAC pro-drug comprises a structure:

$$X–L^1–Y–L^2–Q,$$

wherein

X is the ligand targeting a protein of interest;

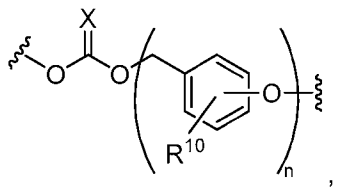
L^1 is the PROTAC linker;

Y is the E3 ligase targeting ligand;

L^2 is a pro-drug linker; and

Q is galactose or a derivative thereof.

In another aspect, the pro-drug linker has a structure:



,

wherein

X is O, S, or N;

R^{10} is hydrogen, halogen, $C_{1–6}$alkyl, $C_{1–6}$alkylene, $C_{1–6}$haloalkyl, cyano, $–OR^{1a}$, $–SR^{1a}$, $–CO_2R^{1a}$, $–C(O)R^{1a}$, $–SO2R^{1b}$, $–N(R^{1b})_2$, $–CO_2N(R^{1b})_2$, $–NO_2$, or $–N(R\text{-}R^{1b})–OR^{1a}$; and

n = 0–1.

In another aspect, the E3 ligase target ligand is a von Hippel-Lindau (VHL) protein targeting ligand. In another aspect, the von Hippel-Lindau (VHL) protein targeting ligand comprises VH032, VH032-OH, (S,R,S)-AHPC-Me, pharmaceutically acceptable salts thereof, or derivatives thereof. In another aspect, the PROTAC pro-drug comprises MS-99, ARV-771, DT2216, TD-004, ARCC-4, VZ-185, ERD-308, BI-0319, SJFα, UNC-6852, DAT8, pharmaceutically acceptable salts thereof, or derivatives thereof. In another aspect, the one or more antineoplastic agents comprise DNA damaging agents, chemical toxic agents, or radiation. In another aspect, the DNA damaging agents comprise etoposide, camptothecin, doxorubicin, pharmaceutically acceptable salts thereof, or derivatives thereof. In another aspect, the chemical toxic agents comprise taxol, pharmaceutically acceptable salts thereof, or derivatives thereof. In another aspect, the galactose is a β-galactose. In another aspect, the therapeutically effective amount of the PROTAC pro-drug

is 1–200 mg/kg. In another aspect, the therapeutically effective amount of the one or more antineoplastic agent is 1–200 mg/kg.

Another embodiment described herein is a method for treating a disease or disorder, the method comprising: sequentially or simultaneously administering to a subject in need thereof a therapeutically effective amount of a PROTAC pro-drug, or pharmaceutically acceptable salts or esters thereof, wherein the PROTAC pro-drug comprises a ligand targeting a protein of interest, a PROTAC linker, an E3 ligase targeting ligand, and galactose, or a derivative thereof; and a therapeutically effective amount of one or more antineoplastic agents, or pharmaceutically acceptable salts or esters thereof; and repeating administration until the disease or disorder is treated, ameliorated, or symptoms are reduced. In one aspect, the disease or disorder is a cancer, neurodegenerative disease, aging, or viral infections. In another aspect, the cancer comprises a breast cancer, melanoma, glioblastoma multiforme, colon cancer, prostate cancer, acute myeloid leukemia, or acute lymphoblastic leukemia and their corresponding senescent cells. In another aspect, administering comprises intraperitoneal injection, intramuscular injection, subcutaneous injection, intravenous injection, intrathecal infusion, oral administration, or a combination thereof. In another aspect, an amount of a PROTAC increases and an amount of PROTAC pro-drug decreases. In another aspect, the PROTAC pro-drug and the one or more antineoplastic agents reduce an amount of a target protein. In another aspect, the PROTAC pro-drug and the one or more antineoplastic agents target senescent cells. In another aspect, the therapeutically effective amount of the antineoplastic agents is added to induce cellular senesce. In another aspect, the therapeutically effective amount of the antineoplastic agents is added at a period of time prior to addition of the therapeutically effective amount of the PROTAC pro-drug. In another aspect, the period of time is 0–168 hours.

Another embodiment described herein is the use of the PROTAC pro-drug and the one or more antineoplastic agents, or pharmaceutically acceptable salts or esters thereof, as a medicament for the treatment of cancer in subject in need thereof.

Another embodiment described herein a kit comprising: one or more PROTAC pro-drugs, or pharmaceutically acceptable salts or esters thereof; one or more antineoplastic agents, or pharmaceutically acceptable salts or esters thereof; optionally a device or means for administering the PROTAC pro-drug and the one or more antineoplastic agents; optionally tamper resistant packaging; and optionally, a label or instructions for use thereof.

DESCRIPTION OF THE DRAWINGS

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

FIG. 1A–C show the bioorthogonal activation and release of TCO-PROTAC prodrugs. FIG. 1A shows the bioorthogonal activation and release of TCO-PROTAC prodrugs by αvβ3 integrin targeted c(RGDyK)-Tz in tumor cells. FIG. 1B shows structures of TCO-ARV-771 and c(RGDyK)-Tz TCO-Tz and the 'click and release' reaction between TCO-ARV-771 and c(RGDyK)-Tz releases the activated ARV-771. FIG. 1C shows molecular docking data between VHL protein and VHL ligand. FIG. 1D shows Hela cells imaged with c(RGDyK) and c(RGDyK)-Tz. After the 3 h incubation of Hela cells with DMSO, c(RGDyK) (500 nM) or c(RGDyK)-Tz (500 nM), the cells were washed three times with PBS and then treated with TCO-Cy5 for another 15 min. The images were captured under a 60× camera.

FIG. 2A–B show the docking TCO-ARV-771 and ARV-771 into VHL protein. FIG. 2A shows TCO caged ligand and VHL protein. FIG. 2B shows the ligand and VHL protein.

FIG. 3A–B show the reaction of TCO-ARV-771 with c(RGDyK)-Tz and stability analysis of c(RGDyK)-Tz. FIG. 3A shows TCO-ARV-771 (green), c(RGDyK)-Tz (purple), ARV-771 (blue), and the reaction mixture after 15 min (red), 30 min (black). FIG. 3B shows the stability analysis of c(RGDyK)-Tz by using UFLC method.

FIG. 4A–E show the degradation ability of TCO-ARV-771 with c(RGDyK)-Tz. FIG. 4A shows an immunoblot analysis of BRD4 levels from HeLa cells treated with TCO-ARV-771 for 16 h. FIG. 4B shows an immunoblot analysis of BRD4 levels from Hela cells treated with 0.4 μM TCO-ARV-771 and various concentration of c(RGDyK)-Tz. Hela cells were treated with different concentrations of c(RGDyK)-Tz for 3 h followed by treatment with TCO-ARV-771 (0.4 μM) for 16 h. FIG. 4C shows an immunoblot analysis of BRD4 levels from Hela cells treated with positive control ARV-771. FIG. 4D shows an immunoblot analysis of BRD4 lev-els from Hela cells treated with various concentrations of TCO-ARV-771 and 1.0 μM c(RGDyK)-Tz. Hela cells were treated with of 1 μM c(RGDyK)-Tz for 3 h followed by treatment with different concentrations of TCO-ARV-771 for 16 h. FIG. 4E shows the cell viability of Hela cells with ARV-771, TCO-ARV-771, c(RGDyK)-Tz or TCO-ARV-771 and c(RGDyK)-Tz (2.5 equivalent, 3 h pretreatment) for 72 h. F) Immunoblot analysis of BRD4 levels from Hela cells treated with c(RGDyK) for 16 h.

FIG. 5A–D shows the degradation ability of TCO-ARV771 with c(RGDyK)-Tz. FIG. 5A shows a Western blot analysis of BRD4 protein levels from HeLa cells treated with c(RGDyK)-Tz for 16 h. FIG. 5B shows a Western blot analysis of BRD4 and GAPDH levels in Hela cells after

the co-treatment with 5 μM MG-132 and crPROTAC (TCO-ARV-771 + c(RGDyK)-Tz) for 16 h. FIG. 5C shows a Western blot analysis of BRD4 and GAPDH levels in Hela cells after the co-treatment with VHL ligand and crPROTAC (TCO-ARV-771 + c(RGDyK)-Tz) for 16 h. FIG. 5D shows a Western blot analysis of BRD4 and GAPDH levels in Hela cells after the co-treatment with c(RGDyK) and TCO-ARV-771 for 16 h.

FIG. 6A–D show the degradation ability of TCO-ARV-771 with c(RGDyK)-Tz. FIG. 6A shows a Western blot analysis of BRD4 protein levels from U87 cells treated with the indicated doses of treatments for 16 h. FIG. 6B shows a Western blot analysis of BRD4 and GAPDH levels in HS27 cells with different concentrations of TCO-ARV-771 for 16 h. FIG. 6C–D show cell viability of U87 or HS27 cells treatment with ARV-771, TCO-ARV-771, c(RGDyK)-Tz (FIG. 6C) or crPROTAC [TCO-ARV-771 + c(RGDyK)-Tz (2.5 equivalent, 3 h pretreatment)] (FIG. 6D) for 72 h.

FIG. 7A–B show flow cytometry of apoptosis assays. FIG. 7A shows the effects of compounds TCO-ARV-771, crPROTAC (TCO-ARV-771 + c(RGDyK)-Tz) and ARV-771 on the induction of apoptosis in Hela cells. Hela cells were cultured with or without TCO-ARV-771 and c(RGDyK)-Tz and ARV-771 for 16 h, and Annexin V and 7-AAD staining for flow cytometry was performed. Representative dot-plot graphs of each group. FIG. 7B shows apoptosis comparison.

FIG. 8A–B show proteomic analyses of TCO-ARV-771 and crPROTAC. Hela cells were treated with either compound 0.4 μM TCO-ARV-771, or 1 μM c(RGDyK)-Tz (3 h, pretreatment) + 0.4 μM TCO-ARV-771 for 16 h. Lysates were subjected to mass spec-based proteomics analysis. FIG. 8A shows a volcano plot shows protein abundance (log2) as a function of significance level (−log10). FIG. 8B shows a heat map analysis to screen between TCO-ARV-771 and crPROTAC groups (TCO-ARV-771 + c(RGDyK)-Tz).

FIG. 9 shows the level of BRD4 expression in different tumor types from the TCGA database in TIMER.

FIG. 10 shows Heat map results. A total of 2159 proteins were identified, and only the ones with at least one uniquely identified peptide are displayed.

FIG. 11A–D show the degradation assay of TCO-DT2216 with c(RGDyK)-Tz. FIG. 11A shows the 'click' reaction between TCO-DT2216 and c(RGDyK)-Tz releases the activated DT2216. FIG. 11B shows a Western blot analysis of Bcl-XL protein levels from HeLa cells treated with the indicated doses of treatments for 16 h. FIG. 11C shows a Western blot analysis of Bcl-XL and GAPDH levels in Hela cells after the co-treatment with 10 μM VHL ligand and crPROTAC (TCO-DT2216 + c(RGDyK)-Tz) for 16 h. FIG. 11D shows a Western blot analysis of BRD4 and GAPDH levels in Hela cells after the co-treatment with 5 μM MG-132 and crPROTAC (TCO-DT2216 + c(RGDyK)-Tz) for 16 h.

FIG. 12A–D show a proposed mechanism and data supporting the proposed mechanism. FIG. 12A shows a proposed mechanism where SA-β-gal activated Gal-Tz may lead to the formation of tetrazine. FIG. 12B shows UFLC monitoring the stability of Gal-Tz (1 mM) in pH 7.4, phosphate buffered saline (PBS) at 37 °C. FIG. 12C shows the cell viability of Gal-Tz and Tz in senescent HeLa cells. FIG. 12D shows UFLC monitoring the course of porcine liver esterase (E) and β-gal co-catalyzed hydrolysis of Gal-Tz (1 mM) in phosphate buffer (pH 7.4) at 37 °C for 2 h.

FIG. 13 shows SA-β-gal staining of control and etoposide (5 μM) induced senescent HeLa cells.

FIG. 14A–B show proposed schematics for the release of a TCO in the presence of Tz. FIG. 14A shows the release of TCO from HCA in response to Gal-Tz to produce liberated HCA that appears red. FIG. 14B shows the release of TCA from HCA in response to Tz to produce liberated HCA.

FIG. 15A–C show control and Tz treated cell imaging studies. FIG. 15A shows- n-HeLa (non-senescent) and s-HeLa (senescent) cells imaging with Gal-Tz and TCO-HCA. After the 3 h incubation Gal-Tz (3 μM), the cells were washed three times with PBS and then treated with TCO-HCA (1 μM) for another 30 min. After the cells were washed by PBS three times, the images were taken under the 60 × camera. The Hoechst stain was used to locate the nucleus. FIG. 15B shows etoposide-induced senescent HeLa cells imaging with HCA and tHCA. After the 0.5 h incubation with HCA or tHCA (1 μM), the cells were washed three times with PBS and captured under the 60X camera. FIG. 15C HeLa cells imaging with HCA and tHCA. After the 0.5 h incubation with HCA or tHCA (1 μM), the cells were washed three times with PBS and captured under the 60 × camera.

FIG. 16 shows a schematic showing Gal-Tz treatment in senescent calls to produce Tz and react with TCO-Dox (pro-drug) to produce liberated Dox (doxorubicin).

FIG. 17 shows representative curves from UFLC analysis show cumulative release of Dox after mixing Tz (3 μM) with TCO-Dox (1 μM) in PBS (pH 7.4) buffer for indicated times.

FIG. 18 shows the absorbance spectra of the reaction between TCO-Dox and Tz (final concentrations were 5 μM for the TCO-Dox and 50 μM for the Tz).

FIG. 19A–B show percent viability curves in response to Dox, TCO-Dox, and TCO-Dox + Gal-Tz treatment (3 equivalent, 3 h pretreatment) for 72 h. FIG. 19A shows cell viability in non-senescent HeLa cells. FIG. 19B shows cell viability in senescent HeLa cells.

FIG. 20A–B show bar graphs of cell viability in non-senescent and senescent HeLa cells in response to Gal-Tz (6 μM), TCO-Dox (2 μM), Dox (2 μM) and TCO-Dox (2 μM) with Gal-Tz (6 μM, 3 h pretreatment), followed by incubation at 37 °C for 24 h. FIG. 20A shows non-senescent

HeLa cells in response to treatment. FIG. 20B shows senescent HeLa cells in response to treatment.

FIG. 21A–B show cell viability of A549 and etoposide-induced senescent A549 cells treated with Dox or [TCO-Dox + Gal-Tz (3 equivalent, 3 h pretreatment)] for 72 h. FIG. 21A shows cell viability of non-senescent A549 cells in response to treatment. FIG. 21B shows cell viability of etoposide-induced senescent A549 cells in response to treatment.

FIG. 22 shows a schematic showing Gal-Tz treatment in senescent calls to produce Tz and react with TCO-ARV-771 (PROTAC pro-drug) to produce liberated ARV-771 (PROTAC).

FIG. 23A–B show dose-response cell viability curves of n-HeLa and s-HeLa cells with Dox, TCO-Dox, and TCO-Dox with Gal-Tz (3 equivalent, 3 h pretreatment) for 72 h. FIG. 23A shows cell viability of senescent HeLa in response to treatment. FIG. 23B shows cell viability of non-senescent HeLa cells in response to treatment.

FIG. 24A–B show dose-response cell viability curves of A549 and etoposide-induced senescent A549 cells treated with TCO-ARV-771, ARV-771, TCO-ARV-771 + Gal-Tz (3 equivalent, 3 h pretreatment)] for 72 h. Fig. 24A shows the cell viability of etoposide-induced senescent A549 cells in response to treatment. FIG. 24B shows the cell viability of non-senescent A549 cells in response to treatment.

FIG. 25A–C show immunoblot analysis of BRD4 levels in response to ARV-771 or TCO-ARV-771 with Gal-Tz for 16 h. FIG. 25A shows the immunoblot analysis for non-senescent HeLa cells and senescent HeLa cells in response to treatment. FIG. 25B shows the immunoblot for senescent HeLa cells in response to treatment. FIG. 25C shows the immunoblot for non-senescent and senescent A549 cells.

FIG. 26 shows flow cytometry apoptosis analysis in non-senescent HeLa cells (n-HeLa) in response to treatment with TCO-ARV-771, Gal-Tz, (TCO-ARV-771 + Gal-Tz, 3 h pretreatment), and ARV-771 after 24 hours.

FIG. 27 shows flow cytometry apoptosis analysis in senescent HeLa (s-HeLa) cells in response to treatment with TCO-ARV-771, Gal-Tz, (TCO-ARV-771 + Gal-Tz, 3 h pretreatment), and ARV-771 after 24 hours.

FIG. 28 shows the volcano plot protein abundance (log2) as a function of the significance level (−log10). Fold change is s-HeLa/n-HeLa.

FIG. 29 shows a schematic for the in vivo processing of a galactose PROTAC pro-drug.

FIG. 30A–C show docking results and scores of von Hippel-Lindau (E3 ligase) targeting ligands. FIG. 30A shows the docking of a first-generation von Hippel-Lindau ligand. FIG. 30B

shows the docking of a second generation von Hippel-Lindau ligand. FIG. 30C shows the docking of a Gal- von Hippel-Lindau ligand.

FIG. 31A–B shows stability analysis of Gal-ARV-771. FIG. 31A shows high performance liquid chromatography (HPLC) analysis of Gal-ARV-771 in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal bovine serum (FBS) over for 1-, 2-, and 24 hours. FIG. 31B shows UFLC monitoring the course of porcine liver esterase (E) and SA-β-gal co-catalyzed hydrolysis of Gal-ARV-771 (100 μM) in pH 7.4 phosphate buffer at 37 °C for 16 h.

FIG. 32 shows SA-β-gal staining of control and etoposide (5 μM) induced senescent A549 cells (left).

FIG. 33A–B immunoblot analysis of BRD4 levels in n-A549 and s-A549 cells treated with ARV-771 or Gal-ARV-771 for 16 h. FIG. 33A shows BRD4 levels in n-A549 and s-A549 cells treated with 0-, 50-, and 100 nM of ARV-771. FIG. 33B shows BRD4 levels in n-A549 and s-A549 cells treated with 0-, 10-, 20-, 50-, and 100 nM Gal-ARV-771.

FIG. 34A–C show protein expression and cell viability changes in response to Gal-ARV-771 in n-HeLa and s-HeLa. FIG. 34A shows Immunoblot analysis of BRD4 levels from n-HeLa and s-HeLa cells treated with ARV-771 and Gal-ARV-771 for 16 h. FIG. 34B shows dose-response cell viability curves of n-HeLa and s-HeLa in response to Gal-ARV-771. FIG. 34C shows dose-response cell viability curves of n-HeLa and s-HeLa in response to ARV-771.

FIG. 35A–C show protein expression and cell viability changes in response to Gal-ARV-771 in n-U87 and s-U87. FIG. 34A shows Immunoblot analysis of BRD4 levels from n-U87and s-U87 cells treated with ARV-771 and Gal-ARV-771 for 16 h. FIG. 34B shows dose-response cell viability curves of n-U87 and s-U87 in response to Gal-ARV-771. FIG. 34C shows dose-response cell viability curves of n-U87 and s-U87 in response to ARV-771.

FIG. 36A–B show microscopic images and staining of control (non-senescent) and senescent cells in response to 5 μM etoposide for three days. FIG. 36A shows HeLa cells. FIG. 36B shows U87 cells.

FIG. 37 shows immunoblot analysis of BRD4 levels in s-A549 cells co-treated with 5 μM MG-132 or 10 μM VHL ligand for 16 h.

FIG. 38A–B show dose-response cell viability curves of n-A549 and s-A549 treated with ARV-771 or Gal-ARV-771 for 72 h. FIG. 38A shows the cell viability of n-A549 and s-A549 in response to Gal-ARV-771. FIG. 38B shows the cell viability of n-A549 and s-A549 in response to ARV-771.

FIG. 39 shows flow cytometry apoptosis analysis in non-senescent HeLa cells (n-HeLa) in response to treatment with 1 μM of ARV-771 or Gal-ARV-771 for 24 h.

FIG. 40A–C shows crystal violet assay results in response to ARV-771 (0.5 μM) and Gal-ARV-771 (0.5 μM) in normal A549 and etoposide-induced senescent A549 cells for 2 weeks.

FIG. 41 displays the chemical structure of Gal-MS99.

FIG. 42 shows immunoblot analysis of NPM-ALK levels in n-Karpas 299 treated with MS99 and Gal-MS99 for 16 h.

FIG. 43 shows immunoblot analysis of NPM-ALK levels in s-Karpas 299 cells treated with MS99 and Gal-MS99 for 16 h.

FIG. 44A–B show dose-response cell viability curves of n-Karpas 299 and s-Karpas 299 cells treated with Gal-MS99 and MS99. FIG. 44A shows the cell viability of n-Karpas 299 and s-Karpas 299 with Gal-MS99. FIG. 44B shows the cell viability of s-Karpas 299 with MS99.

FIG. 45A–C show concomitant treatment with prodrug Gal-ARV-771 and etoposide significantly inhibits tumor growth in a human lung cancer xenograft mouse model. FIG. 45A shows a schematic of the regime for concomitant treatment on A549 xenograft-bearing mice. FIG. 45B shows changes in tumor volume in mice treated with etoposide (5 mg/kg) and ARV-771 (20 mg/kg) or Gal-ARV-771 (Gal, 20 mg/kg). FIG. 45C shows changes in mice weight in mice treated with etoposide (5 mg/kg) and ARV-771 (20 mg/kg) or Gal-ARV-771 (Gal, 20 mg/kg).

FIG. 46A–C shows a visual representation of a tumor in response to control, Gal-ARV-771 alone and drug treatment (Gal-ARV-771 and etoposide) therapy. FIG. 46A shows control mice. FIG. 46B shows Gal-ARV-771 only treatment . FIG. 46C shows Gal-ARV-771 and etoposide treatment.

FIG. 47 shows representative histological images of tumors at the end of concomitant treatment regimes, stained for BRD4, Ki-67 and cleaved caspase 3.

FIG. 48A–C graphically display quantification of BRD4, Ki-67 and cleaved caspase-3-positive cells in tumors from animals treated with vehicle, Gal-ARV-771, or etoposide and Gal-ARV-771 (n = 3 tumors per group). For quantification, a total of 3 fields per tumor was analyzed. *p < .05; **p < .01; ***p < .001; ****p < .0001.

FIG. 49A–B show Proteomic analysis of Gal-ARV-771 in A549 cells. Normal A549 cells and etoposide-induced senescent A549 cells were treated 50 nM Gal-ARV-771 for 16 h. Lysates were subject-ed to mass spec-based proteomic analysis. FIG. 49A shows a volcano plot depicting the distribution of all proteins (n = 4889) with relative protein abundance (log2FC) as a function of significance level (-log10P-Value). FIG. 49B shows a heat map of 6 individual sample abundances for the top five up- and down- regulated proteins from proteomics data.

DETAILED DESCRIPTION

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. For example, any nomenclatures used in connection with, and techniques of biochemistry, molecular biology, immunology, microbiology, genetics, cell and tissue culture, and protein and nucleic acid chemistry described herein are well known and commonly used in the art. In case of conflict, the present disclosure, including definitions, will control. Exemplary methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in practice or testing of the embodiments and aspects described herein.

As used herein, the terms "amino acid," "nucleotide," "polynucleotide," "vector," "polypeptide," and "protein" have their common meanings as would be understood by a biochemist of ordinary skill in the art. Standard single letter nucleotides (A, C, G, T, U) and standard single letter amino acids (A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y) are used herein.

As used herein, terms such as "include," "including," "contain," "containing," "having," and the like mean "comprising." The present disclosure also contemplates other embodiments "comprising," "consisting essentially of," and "consisting of" the embodiments or elements presented herein, whether explicitly set forth or not. As used herein, "comprising," is an "open-ended" term that does not exclude additional, unrecited elements or method steps. As used herein, "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 characteristics of the claimed invention. As used herein, "consisting of" excludes any element, step, or ingredient not specified in the claim.

As used herein, the term "a," "an," "the" and similar terms used in the context of the disclosure (especially in the context of the claims) are to be construed to cover both the singular and plural unless otherwise indicated herein or clearly contradicted by the context. In addition, "a," "an," or "the" means "one or more" unless otherwise specified.

As used herein, the term "or" can be conjunctive or disjunctive.

As used herein, the term "and/or" refers to both the conjuctive and disjunctive.

As used herein, the term "substantially" means to a great or significant extent, but not completely.

As used herein, the term "about" or "approximately" as applied to one or more values of interest, refers to a value that is similar to a stated reference value, or within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, such as the limitations of the measurement system. In one aspect, the term "about" refers to any values, including both integers and fractional

components that are within a variation of up to ± 10% of the value modified by the term "about." Alternatively, "about" can mean within 3 or more standard deviations, per the practice in the art. Alternatively, such as with respect to biological systems or processes, the term "about" can mean within an order of magnitude, in some embodiments within 5-fold, and in some embodiments within 2-fold, of a value. As used herein, the symbol "~" means "about" or "approximately."

All ranges disclosed herein include both end points as discrete values as well as all integers and fractions specified within the range. For example, a range of 0.1–2.0 includes 0.1, 0.2, 0.3, 0.4 . . . 2.0. If the end points are modified by the term "about," the range specified is expanded by a variation of up to ±10% of any value within the range or within 3 or more standard deviations, including the end points, or as described above in the definition of "about."

As used herein, the terms "active ingredient" or "active pharmaceutical ingredient" refer to a pharmaceutical agent, active ingredient, compound, or substance, compositions, or mixtures thereof, that provide a pharmacological, often beneficial, effect.

As used herein, the terms "control," or "reference" are used herein interchangeably. A "reference" or "control" level may be a predetermined value or range, which is employed as a baseline or benchmark against which to assess a measured result. "Control" also refers to control experiments or control cells.

As used herein, the term "dose" denotes any form of an active ingredient formulation or composition, including cells, that contains an amount sufficient to initiate or produce a therapeutic effect with at least one or more administrations. "Formulation" and "composition" are used interchangeably herein.

As used herein, the term "prophylaxis" refers to preventing or reducing the progression of a disorder, either to a statistically significant degree or to a degree detectable by a person of ordinary skill in the art.

As used herein, the terms "effective amount" or "therapeutically effective amount," refers to a substantially non-toxic, but sufficient amount of an action, agent, composition, or cell(s) being administered to a subject that will prevent, treat, or ameliorate to some extent one or more of the symptoms of the disease or condition being experienced or that the subject is susceptible to contracting. The result can be the reduction or alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. An effective amount may be based on factors individual to each subject, including, but not limited to, the subject's age, size, type or extent of disease, stage of the disease, route of administration, the type or extent of supplemental therapy used, ongoing disease process, and type of treatment desired.

As used herein, the term "subject" refers to an animal. Typically, the subject is a mammal. A subject also refers to primates (e.g., humans, male or female; infant, adolescent, or adult), non-human primates, rats, mice, rabbits, pigs, cows, sheep, goats, horses, dogs, cats, fish, birds, and the like. In one embodiment, the subject is a primate. In one embodiment, the subject is a human.

As used herein, a subject is "in need of treatment" if such subject would benefit biologically, medically, or in quality of life from such treatment. A subject in need of treatment does not necessarily present symptoms, particular in the case of preventative or prophylaxis treatments.

As used herein, the terms "inhibit," "inhibition," or "inhibiting" refer to the reduction or suppression of a given biological process, condition, symptom, disorder, or disease, or a significant decrease in the baseline activity of a biological activity or process.

As used herein, "treatment" or "treating" refers to prophylaxis of, preventing, suppressing, repressing, reversing, alleviating, ameliorating, or inhibiting the progress of biological process including a disorder or disease, or completely eliminating a disease. A treatment may be either performed in an acute or chronic way. The term "treatment" also refers to reducing the severity of a disease or symptoms associated with such disease prior to affliction with the disease. "Repressing" or "ameliorating" a disease, disorder, or the symptoms thereof involves administering a cell, composition, or compound described herein to a subject after clinical appearance of such disease, disorder, or its symptoms. "Prophylaxis of" or "preventing" a disease, disorder, or the symptoms thereof involves administering a cell, composition, or compound described herein to a subject prior to onset of the disease, disorder, or the symptoms thereof. "Suppressing" a disease or disorder involves administering a cell, composition, or compound described herein to a subject after induction of the disease or disorder thereof but before its clinical appearance or symptoms thereof have manifest.

"Administration" or "administering," as used herein, refers to providing, contacting, and/or delivery of a compound or compounds by any appropriate route to achieve the desired effect. Administration may include, but is not limited to, oral, sublingual, parenteral (e.g., intravenous, subcutaneous, intracutaneous, intramuscular, intraarticular, intraarterial, intrasynovial, intrasternal, intrathecal, intralesional or intracranial injection), transdermal, topical, buccal, rectal, vaginal, nasal, ophthalmic, via inhalation, and implants.

"Antineoplastic agent," as used herein, refers to compounds capable of ceasing cell growth. An antineoplastic agent is any compound with therapeutic utility in the treatment of diseases characterized by abnormal cell growth. Non-limiting examples of disease include neoplasms and cancer. This term as used herein may also refer to chemotherapeutic agents. An antineoplastic agent may exist as a pharmaceutically acceptable salt, prodrug, or prodrug of a

prodrug. Non-limiting examples of antineoplastic agents and chemotherapeutics include alkylating agents, such as nitrogen mustards (for example, chlorambucil, chlormethine, cyclophosphamide, ifosfamide, and melphalan), nitrosoureas (for example, carmustine, fotemustine, lomustine, and streptozocin), platinum compounds (for example, carboplatin, cisplatin, oxaliplatin, and BBR3464), busulfan, dacarbazine, mechlorethamine, procarbazine, temozolomide, thiotepa, and uramustine; antimetabolites, such as folic acid (for example, methotrexate, pemetrexed, and raltitrexed), purines (for example, cladribine, clofarabine, fludarabine, mercaptopurine, and thioguanine), pyrimidines (for example, capecitabine), cytarabine, fluorouracil (e.g., 5–FU), and gemcitabine; plant alkaloids, such as podophyllum (for example, etoposide, and teniposide), taxane (for example, docetaxel and paclitaxel), vinca (for example, vinblastine, vincristine, vindesine, and vinorelbine); cytotoxic / antitumor antibiotics, such as anthracycline family members (for example, daunorubicin, doxorubicin, epirubicin, idarubicin, mitoxantrone, pirarubicin, vosaroxin, valrubicin, and mitoxantrone), bleomycin, hydroxyurea, geldan amycin, 17-N-allylamino-17-demethoxygeldanamycin(17 AAG), 17-dimethylaminoethylamino-17 demethoxygeldanamycin (17-DMAG), and mitomycin; topoisomerase inhibitors, such as camptothecin, 10-hydroxycamptothecin, irinotecan, SN-38, topotecan, rebeccamycin, Adriamycin, camptothecin, doxorubicin, pharmaceutically acceptable salts thereof, or derivatives thereof. In some embodiment, the antineoplastic agents comprise DNA damaging agents, chemical toxic agents, or radiation. Examples of chemical toxic agents include, but are not limited to, taxol, pharmaceutically acceptable salts, thereof, or derivatives thereof. In some embodiments, the antineoplastic agents induce senescence in cancer cells.

"Pro-drug" refers to a derivative of an active agent that requires a transformation within the body or cell to release the active agent or active pharmaceutical ingredient. In certain embodiments, the transformation is an enzymatic transformation. In certain embodiments, the transformation is activated by a tumor-specific environment. Pro-drugs are frequently, although not necessarily, pharmacologically inactive until converted to the active agent.

Definitions of specific functional groups and chemical terms are described in more detail herein. The chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, *Handbook of Chemistry and Physics*, 75th ed., inside cover, and specific functional groups are generally defined as described therein. Additionally, general principles of organic chemistry, as well as specific functional moieties and reactivity, are described in Thomas Sorrell, *Organic Chemistry*, University Science Books, Sausalito, 1999; Smith and March, *March's Advanced Organic Chemistry*, 5th ed, John Wiley & Sons, Inc., New York, 2001; Larock, *Comprehensive Organic Transformations*, VCH Publishers, Inc., New York, 1989; and

Carruthers, *Some Modern Methods of Organic Synthesis*, 3rd ed, Cambridge University Press, Cambridge, 1987.

As used herein, the term "alkyl" refers to a straight or branched hydrocarbon radical having from 1 to 12 (e.g., C_1–C_{12}) carbon atoms and includes, for example, methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, *sec*-butyl, isobutyl, *tert*-butyl, *n*-pentyl, iso-pentyl, *n*-hexyl, and the like.

As used herein, the term "alkenyl" refers to straight and branched hydrocarbon radicals having from 2 to 12 carbon atoms (e.g., C_2–C_{12}) and at least one double bond and includes, but is not limited to, ethenyl, 3-buten-1-yl, 2-ethenylbutyl, 3-hexen-1-yl, and the like. The term "alkenyl" includes cycloalkenyl, and heteroalkenyl in which 1 to 3 heteroatoms selected from O, S, N, or substituted nitrogen may replace carbon atoms.

As used herein, the term "alkynyl" refers to straight and branched hydrocarbon radicals having from 2 to 12 carbon atoms (e.g., C_2–C_{12}) and at least one triple bond and includes, but is not limited to, ethynyl, 3-butyn-1-yl, propynyl, 2-butyn-1-yl, 3-pentyn-1-yl, and the like.

As used herein, the term "cycloalkyl" refers to a monocyclic or polycyclic hydrocarbyl group having from 3 to 8 carbon atoms (e.g., C_3–C_8), for instance, cyclopropyl, cycloheptyl, cyclooctyl, cyclodecyl, cyclobutyl, adamantyl, norpinanyl, decalinyl, norbornyl, cyclohexyl, and cyclopentyl. Such groups can be substituted with groups such as hydroxy, keto, amino, alkyl, and dialkylamino, and the like. Also included are rings in which 1 to 3 heteroatoms replace carbons. Such groups are termed "heterocyclyl," which means a cycloalkyl group also bearing at least one heteroatom selected from O, S, N, or substituted nitrogen. Examples of such groups include, but are not limited to, oxiranyl, pyrrolidinyl, piperidyl, tetrahydropyran, and morpholine.

As used herein, the term "alkoxy" refers to a straight or branched chain alkyl groups having 1–10 carbon atoms (e.g., C_2–C_{10}) and linked through oxygen. Examples of such groups include, but are not limited to, methoxy, ethoxy, propoxy, isopropoxy, *n*-butoxy, sec-butoxy, tert-butoxy, pentoxy, 2-pentyloxy, isopentoxy, neopentoxy, hexoxy, 2-hexoxy, 3-hexoxy, and 3-methylpentoxy. In addition, alkoxy refers to polyethers such as –O– $(CH_2)_2$–O–CH_3, and the like.

The alkyl, alkenyl, alkoxy, and alkynyl groups described herein are optionally substituted (i.e., may be substituted, but are not necessarily substituted), preferably by 1 to 3 groups selected from NR_4R_5, phenyl, substituted phenyl, thio C_1–C_6 alkyl, C_1–C_6 alkoxy, hydroxy, carboxy, C_1–C_6 alkoxycarbonyl, halo, nitrile, cycloalkyl, and a 5- or 6-membered carbocyclic ring or heterocyclic ring having 1 or 2 heteroatoms selected from nitrogen, substituted nitrogen, oxygen, and sulfur. "Substituted nitrogen" means nitrogen bearing C_1–C_6 alkyl or $(CH_2)_pPh$ where p is 1, 2, or 3. Perhalo and polyhalo substitution is also included.

Examples of substituted alkyl groups include, but are not limited to, 2-aminoethyl, 2-hydroxyethyl, pentachloroethyl, trifluoromethyl, 2-diethylaminoethyl, 2-dimethylaminopropyl, ethoxycarbonylmethyl, 3-phenylbutyl, methanylsulfanylmethyl, methoxymethyl, 3-hydroxypentyl, 2-carboxybutyl, 4-chlorobutyl, 3-cyclopropylpropyl, pentafluoroethyl, 3-morpholinopropyl, piperazinylmethyl, and 2-(4-methylpiperazinyl)ethyl.

Examples of substituted alkynyl groups include, but are not limited to, 2-methoxyethynyl, 2-ethylsulfanylethynyl, 4-(1-piperazinyl)-3-(butynyl), 3-phenyl-5-hexynyl, 3-diethylamino-3-butynyl, 4-chloro-3-butynyl, 4-cyclobutyl-4-hexenyl, and the like.

Typical substituted alkoxy groups include aminomethoxy, trifluoromethoxy, 2-diethylaminoethoxy, 2-ethoxycarbonylethoxy, 3-hydroxypropoxy, 6-carboxhexyloxy, and the like.

Further, examples of substituted alkyl, alkenyl, and alkynyl groups include, but are not limited to, dimethylaminomethyl, carboxymethyl, 4-dimethylamino-3-buten-1-yl, 5-ethylmethylamino-3-pentyn-1-yl, 4-morpholinobutyl, 4-tetrahydropyrinidylbutyl, 3-imidazolidin-1-ylpropyl, 4-tetrahydrothiazol-3-yl-butyl, phenylmethyl, 3-chlorophenylmethyl, and the like.

As used herein, the term “anion” means a negatively charged species such as chloride, bromide, trifluoroacetate, or triethylammonium. The term “cation” refers to a positively charged species, such as sodium, potassium, or ammonium.

As used herein, the term “acyl” refers to alkyl or aryl (Ar) group having from 1–10 carbon atoms bonded through a carbonyl group, i.e., R–C(O)–. For example, acyl includes, but is not limited to, a C_1–C_6 alkanoyl, including substituted alkanoyl, wherein the alkyl portion can be substituted by an amine, amide, carboxylic, or heterocyclic group. Typical acyl groups include acetyl, benzoyl, and the like.

As used herein, the term “aryl” refers to an aromatic monocyclic hydrocarbon ring system or a polycyclic ring system where at least one of the rings in the ring system is an aromatic hydrocarbon ring and any other aromatic rings in the ring system include only hydrocarbons. In some embodiments, a monocyclic aryl group can have from 6 to 14 carbon atoms and a polycyclic aryl group can have from 8 to 14 carbon atoms (e.g., C_8–C_{14}). The aryl group can be covalently attached to the defined chemical structure at any carbon atom(s) that result in a stable structure. In some embodiments, an aryl group can have only aromatic carbocyclic rings, e.g., phenyl, 1-naphthyl, 2-naphthyl, anthracenyl, phenanthrenyl groups, and the like. In other embodiments, an aryl group can be a polycyclic ring system in which at least one aromatic carbocyclic ring is fused (i.e., having a bond in common with) to one or more cycloalkyl or cycloheteroalkyl rings. Examples of such aryl groups include, among others, benzo derivatives of cyclopentane (i.e., an indanyl group, which is a 5,6-bicyclic cycloalkyl/aromatic ring system), cyclohexane (i.e., a

tetrahydronaphthyl group, which is a 6,6-bicyclic cycloalkyl/aromatic ring system), imidazoline (i.e., a benzimidazolinyl group, which is a 5,6-bicyclic cycloheteroalkyl/aromatic ring system), and pyran (i.e., a chromenyl group, which is a 6,6-bicyclic cycloheteroalkyl/aromatic ring system). Other examples of aryl groups include benzodioxanyl, benzodioxolyl, chromanyl, indolinyl groups, and the like.

As used herein, the terms "halogen" or "halo" refer to fluorine, bromine, chlorine, or iodine.

As used herein, the term "haloalkyl" refers to an alkyl group having one or more halogen substituents. In some embodiments, a haloalkyl group can have 1 to 10 carbon atoms (e.g., C_1–C_8). Examples of haloalkyl groups include CF_3, C_2F_5, CHF_2, CH_2F, CCl_3, $CHCl_2$, CH_2Cl, C_2Cl_5, and the like. Perhaloalkyl groups, i.e., alkyl groups wherein all the hydrogen atoms are replaced with halogen atoms (e.g., CF_3 and C_2F_5), are included within the definition of "haloalkyl." For example, a C_{1-10} haloalkyl group can have the formula $-C_iH_{2i+1-j}X_j$, wherein X is F, Cl, Br, or I, i is an integer in the range of 1 to 10, and j is an integer in the range of 0 to 21, provided that j is less than or equal to 2_i+1.

As used herein, the term "heteroaryl" refers to an aromatic monocyclic ring system containing at least one ring heteroatom selected from O, N, and S or a polycyclic ring system where at least one of the rings in the ring system is aromatic and contains at least one ring heteroatom. A heteroaryl group can have from 5 to 14 ring atoms (e.g., C_5–C_{14}), and contains 1–6 ring heteroatoms (e.g., N, O, S, P, or the like). In some embodiments, heteroaryl groups can include monocyclic heteroaryl rings fused to one or more aromatic carbocyclic rings, non-aromatic carbocyclic rings, or non-aromatic cycloheteroalkyl rings. The heteroaryl group can be covalently attached to the defined chemical structure at any heteroatom or carbon atom that results in a stable structure. Generally, heteroaryl rings do not contain O–O, S–S, or S–O bonds. However, one or more N or S atoms in a heteroaryl group can be oxidized (e.g., pyridine N-oxide, thiophene S-oxide, thiophene S,S-dioxide). Examples of such heteroaryl rings include pyrrolyl, furyl, thienyl, pyridyl, pyrimidyl, pyridazinyl, pyrazinyl, triazolyl, tetrazolyl, pyrazolyl, imidazolyl, isothiazolyl, thiazolyl, thiadiazolyl, isoxazolyl, oxazolyl, oxadiazolyl, indolyl, isoindolyl, benzofuryl, benzothienyl, quinolyl, 2-methylquinolyl, isoquinolyl, quinoxalyl, quinazolyl, benzotriazolyl, benzimidazolyl, benzothiazolyl, benzisothiazolyl, benzisoxazolyl, benzoxadiazolyl, benzoxazolyl, cinnolinyl, 1*H*-indazolyl, 2*H*-indazolyl, indolizinyl, isobenzofuyl, naphthyridinyl, phthalazinyl, pteridinyl, purinyl, oxazolopyridinyl, thiazolopyridinyl, imidazopyridinyl, furopyridinyl, thienopyridinyl, pyridopyrimidinyl, pyridopyrazinyl, pyridopyrdazinyl, thienothiazolyl, thienooxazolyl, thienoimidazolyl groups, and the like. Further examples of heteroaryl groups

include 4,5,6,7-tetrahydroindolyl, tetrahydroquinolinyl, benzothienopyridinyl, benzofuropyridinyl groups, and the like.

As used herein, the term "lower alkenyl" refers to alkenyl groups which contains 2 to 6 carbon atoms (e.g., C_2–C_6). An alkenyl group is a hydrocarbyl group containing at least one carbon-carbon double bond. As defined herein, it may be unsubstituted or substituted with the substituents described herein. The carbon-carbon double bonds may be between any two carbon atoms of the alkenyl group. It is preferred that it contains 1 or 2 carbon-carbon double bonds and more preferably one carbon-carbon double bond. The alkenyl group may be straight chained or branched. Examples include but are not limited to ethenyl, 1-propenyl, 2-propenyl, 1-butenyl, 2-butenyl, 2-methyl-1-propenyl, 1,3-butadienyl, and the like.

As used herein, the term "lower alkynyl" refers to an alkynyl group containing 2–6 carbon atoms (e.g., C_2–C_6). An alkynyl group is a hydrocarbyl group containing at least one carbon-carbon triple bond. The carbon-carbon triple bond may be between any two-carbon atom of the alkynyl group. In an embodiment, the alkynyl group contains 1 or 2 carbon-carbon triple bonds and more preferably one carbon-carbon triple bond. The alkynyl group may be straight chained or branched. Examples include but are not limited to ethynyl, 1-propynyl, 2-propynyl, 1-butynyl, 2-butynyl and the like.

As used herein, the term "carbalkoxy" refers to an alkoxycarbonyl group, where the attachment to the main chain is through the carbonyl group, e.g., –C(O)–. Examples include but are not limited to methoxy carbonyl, ethoxy carbonyl, and the like.

As used herein, the term "oxo" refers to a double-bonded oxygen (i.e., =O). It is also to be understood that the terminology C(O) refers to a –C=O group, whether it be ketone, aldehyde or acid or acid derivative. Similarly, S(O) refers to a –S=O group.

As used herein, the term "cycloalkyl" refers to a non-aromatic carbocyclic group including cyclized alkyl, alkenyl, and alkynyl groups. A cycloalkyl group can be monocyclic (e.g., cyclohexyl) or polycyclic (e.g., containing fused, bridged, and/or spiro ring systems), wherein the carbon atoms are located inside or outside of the ring system. A cycloalkyl group can have from 3 to 14 ring atoms (e.g., from 3 to 8 carbon atoms for a monocyclic cycloalkyl group and from 7 to 14 carbon atoms for a polycyclic cycloalkyl group). Any suitable ring position of the cycloalkyl group can be covalently linked to the defined chemical structure. Examples of cycloalkyl groups include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclopentenyl, cyclohexenyl, cyclohexadienyl, cycloheptatrienyl, norbornyl, norpinyl, norcaryl, adamantyl, and spiro[4.5]decanyl groups, as well as their homologs, isomers, and the like.

As used herein, the term "heteroatom" refers to an atom of any element other than carbon or hydrogen and includes, for example, nitrogen, oxygen, sulfur, phosphorus, and selenium.

As used herein, the term "cycloheteroalkyl" refers to a non-aromatic cycloalkyl group that contains at least one (e.g., one, two, three, four, or five) ring heteroatom selected from O, N, and S, and optionally contains one or more (e.g., one, two, or three) double or triple bonds. A cycloheteroalkyl group can have from 3 to 14 ring atoms and contains from 1 to 5 ring heteroatoms (e.g., from 3–6 ring atoms for a monocyclic cycloheteroalkyl group and from 7 to 14 ring atoms for a polycyclic cycloheteroalkyl group). The cycloheteroalkyl group can be covalently attached to the defined chemical structure at any heteroatom(s) or carbon atom(s) that results in a stable structure. One or more N or S atoms in a cycloheteroalkyl ring may be oxidized (e.g., morpholine N-oxide, thiomorpholine S-oxide, thiomorpholine S,S-dioxide). Cycloheteroalkyl groups can also contain one or more oxo groups, such as phthalimidyl, piperidinyl, oxazolidinoxyl, 2,4(1*H*,3*H*)-dioxo-pyrimidinyl, pyridin-2(1*H*)-onyl, and the like. Examples of cycloheteroalkyl groups include, among others, morpholinyl, thiomorpholinyl, pyranyl, imidazolidinyl, imidazolinyl, oxazolidinyl, pyrazolidinyl, pyrazolinyl, pyrrolidinyl, pyrrolinyl, tetrahydrofuranyl, tetrahydrothienyl, piperidinyl, piperazinyl, azetidine, and the like.

With the advancement of proteolysis targeting chimeras (PROTACs) as a promising therapeutic modality into the clinical studies, their potential toxicity from uncontrolled degradation of proteins and undesirable ligase-mediated off-target effects has become an important concern. Precision manipulation of degradation activity of PROTACs could minimize potential toxicity and side effects. Extensive efforts have been devoted to developing cancer biomarker activatable prodrugs of PROTACs. Described herein, is a bioorthogonal on-demand prodrug strategy (termed click-release "crPROTACs") that enabled on-target activation of PROTAC prodrugs and release of PROTACs in cancer cells. A PROTAC as described herein can be any PROTAC. Examples of PROTACs include, but are not limited to, MS-99, ARV-771, DT2216, TD-004, ARCC-4, VZ-185, ERD-308, BI-0319, SJFα, UNC-6852, DAT8, pharmaceutically acceptable salts thereof, or derivatives thereof.

Bioorthogonal-activatable prodrugs offer an unrivaled selectivity for activation and delivery of drugs without interacting or interfering with biological systems. Amongst the bioorthogonal reactions developed to date, the inverse electron demand Diels–Alder (IEDDA) reaction of 1,2,4,5-tetrazines with strained dienophiles stands out for unmatchable kinetics, non-requirement for catalysts, and excellent orthogonality and biocompatibility. In particular, allylic trans-cyclooctene (TCO) dienophiles serve as versatile masking groups in prodrug design, which can be efficiently decaged via click-release reaction with tetrazine. Notably, the chemistry has led to

the first tetrazine activatable TCO-caged doxorubicin prodrug into clinical studies. As applications of the tetrazine ligation rapidly expand, there is a growing demand for strategies that can precisely control the tetrazine activity in biological systems. Along this line, elegant light-mediated oxidation and decaging of 1,4-dihydrotetrazines have been devised to achieve spatiotemporal regulation of tetrazine activity. More recently, the control of tetrazine activity has also been nicely realized by a host-guest pair between synthetic macrocyclic naphthotubes and phenyltetrazine. However, these functionalized tetrazine and 1,4-dihydrotetrazines cannot response to disease related biomarkers. Therefore, they fall short of site-selective activation capacity at the desired disease site, which is crucial for minimizing unwanted off-site adverse effects.

Inactive PROTAC prodrugs TCO-ARV-771 and TCO-DT2216 were rationally designed by conjugating a bioorthogonal transcyclooctenes (TCO) group into the ligand of the VHL E3 ubiquitin ligase. A tetrazine (Tz) modified cancer biomarker integrin targeted ligand RGD peptide c(RGDyK)-Tz as an activation component is developed for on-target click-release of the PROTAC prodrugs to achieve targeted degradation of proteins of interest (POIs) in cancer cells versus noncancerous normal cells. The studies show that the PROTAC prodrugs can be selectively activated and exert on their degradation activity in cancer cell biomarker integrin αvβ3-dependent manner. The crPROTAC strategy provides a general, abiotic approach to achieve selective degradation of POIs in cancer cells.

In one embodiment described herein is the design of a αvβ3 integrin binding ligand c(RGDyK) with tetrazine to create c(RGDyK)-Tz for the selective activation of TCO-caged PROTAC prodrugs in cancer cells to achieve controllable targeted degradation of a protein of interest (POI), thus minimizing potential unwanted toxicity to normal tissues (FIG. 1A). The TCO-PROTACs, TCO-ARV-771 and TCO-DT2216, were developed by incorporating the TCO group onto the hydroxyl group of a well-studied VHL-based PROATCs, ARV-7717 and DT2216 (FIG. 1B). These prodrugs are stable and bioorthogonal to biological systems while they can be selectively activated by c(RGDyK)-Tz via IEDDA reaction in cancer cells to release active ARV-771 and DT2216, which then recruit endogenous VHL E3 ubiquitin ligase to ubiquitinate the POI for subsequent degradation in proteasome (FIG. 1A).

Cellular senescence is a stable and stagnant state that in which cells remain viable and metabolically active, but are incapable of undergoing additional cycles of division. The development and accumulation of cellular senescence promote the release of pro-inflammatory, proapoptotic and pro-fibrotic the senescence-associated secretory phenotype (SASP) factors. These factors can promote neo-plastic growth, therapy resistance, immunosuppression, metastasis and angiogenesis. Moreover, senescent cancer cells can remain dormant and viable

for long periods of time, which poses a risk for tumor relapse. Therefore, understanding and exploiting senescence is a new approach to improve long-term outcomes from cancer treatment.

Senolytic drugs that selectively destroy senescent cells have emerged as viable modes for treatment of disorders related to senescence. Senolytic agents, initially developed because of their ability to transiently switch off senescence-associated antiapoptotic pathways, have been derived for the most part from anticancer drugs such as inhibitors of Bcl-2 family members, HSP90 and MDM2. Despite the encouraging results that have come from clinical studies, these efforts have shown that senolytics suffer from toxicity issues owing to off-target effects associated with non-significant differences in the expression levels of their targets between senescent cells and normal cells.

Senescence-associated β-galactosidase (SA-β-gal) is a widely used biomarker for senescent cells as the enhanced activity of this lysosomal enzyme is a common feature of these types of cells. As such, SA-β-gal has been widely used for the imaging and detection of senescent cells. A particular challenge in the design of senescent cell-targeting pro-drugs is that many require high doses of the pro-drug conjugates to fully suppress a single senescent cell antiapoptotic pathway (SCAP). The high dosage requirement creates a particular challenge related to therapeutic selectivity because a significant percentage of senescent cells are located in normal tissues. Thus, senescent cell-targeting pro-drugs can disrupt the structural integrities of tissue or vascular endothelial cells, leading to fibrosis and the collapse of liver and perivascular tissue. These off-target activity of current senescent cell-targeting pro-drug significantly limit the use of these agents especially in elderly individuals or those with comorbidities.

In one embodiment described herein, galactose and dihydrotetrazine conjugates (e.g., Gal-Tz) were designed and used in combination with a PROTAC to selectively target senescent cancer cells. These combined the galactose and dihydrotetrazine conjugates selectively eliminate senescent over non-senescent cells using doxorubicin (Dox) and PROTAC ARV-771 as senolytics.

In another embodiment described herein, Gal-PROTACs were designed based upon the ARV-77129 and MS99 PROTAC scaffolds to selectively activated by SA-β-gal in etoposide-induced senescent cancer cells. Both agents catalyze ubiquitin-proteasome-mediated degradation of their respective target proteins, BRD4 and NPM-ALK and effectively induce apoptosis in senescent cells and have higher senolytic indexes than the PROTAC itself.

Cancer Combination Therapy

The disclosed compounds can be used as single agents or in combination with one or more other drugs in the treatment, prevention, control, amelioration or reduction of risk of the aforementioned diseases, disorders and conditions for which the compound or the other drugs have utility, where the combination of drugs together are safer or more effective than either drug alone. The other drug(s) can be administered by a route and in an amount commonly used therefor, contemporaneously or sequentially with a disclosed compound. When a disclosed compound is used contemporaneously with one or more other drugs, a pharmaceutical composition in unit dosage form containing such drugs and the disclosed compound may be used. However, the combination therapy can also be administered on overlapping schedules. It is also envisioned that the combination of one or more active ingredients and a disclosed compound can be more efficacious than either as a single agent. Thus, when used in combination with one or more other active ingredients, the disclosed compounds and the other active ingredients can be used in lower doses than when each is used singly.

The pharmaceutical compositions and methods of the present invention can further comprise other therapeutically active compounds as noted herein which are usually applied in the treatment of the above mentioned pathological conditions.

The above combinations include combinations of a disclosed compound not only with one other active compound, but also with two or more other active compounds. Likewise, disclosed compounds can be used in combination with other drugs that are used in the prevention, treatment, control, amelioration, or reduction of risk of the diseases or conditions for which disclosed compounds are useful. Such other drugs can be administered, by a route and in an amount commonly used therefor, contemporaneously, or sequentially with a compound of the present invention. When a compound of the present invention is used contemporaneously with one or more other drugs, a pharmaceutical composition containing such other drugs in addition to a disclosed compound is preferred. Accordingly, the pharmaceutical compositions include those that also contain one or more other active ingredients, in addition to a compound of the present invention.

The weight ratio of a disclosed compound to the second active ingredient can be varied and will depend upon the effective dose of each ingredient. Generally, an effective dose of each will be used. Combinations of a compound of the present invention and other active ingredients will generally also be within the aforementioned range, but in each case, an effective dose of each active ingredient should be used.

Accordingly, the disclosed compounds can be used alone or in combination with other agents which are known to be beneficial in the subject indications or other drugs that affect

receptors or enzymes that either increase the efficacy, safety, convenience, or reduce unwanted side effects or toxicity of the disclosed compounds. The subject compound and the other agent can be co-administered, either in concomitant therapy or in a fixed combination.

A compound or composition described herein may be used in combination with other known therapies. Administered "in combination," as used herein, means that two (or more) different treatments are delivered to the subject during the course of the subject's affliction with the disease or disorder, e.g., the two or more treatments are delivered after the subject has been diagnosed with the disorder and before the disorder has been cured or eliminated or treatment has ceased for other reasons. In some embodiments, the delivery of one treatment is still occurring when the delivery of the second begins, so that there is overlap in terms of administration. This is sometimes referred to herein as "simultaneous" or "concurrent delivery." In other embodiments, the delivery of one treatment ends before the delivery of the other treatment begins. In some embodiments of either case, the treatment is more effective because of combined administration. For example, the second treatment is more effective, e.g., an equivalent effect is seen with less of the second treatment, or the second treatment reduces symptoms to a greater extent, than would be seen if the second treatment were administered in the absence of the first treatment, or the analogous situation is seen with the first treatment. In some embodiments, delivery is such that the reduction in a symptom, or other parameter related to the disease or disorder is greater than what would be observed with one treatment delivered in the absence of the other. The effect of the two treatments can be partially additive, wholly additive, or greater than additive. The delivery can be such that an effect of the first treatment delivered is still detectable when the second is delivered.

A compound described herein and at least one additional therapeutic agent can be administered simultaneously, in the same or in separate compositions, or sequentially. For sequential administration, a compound or composition described herein can be administered first, and the additional agent can be administered subsequently, or the order of administration can be reversed.

In some embodiments, a compound or composition described herein can be administered in combination with other therapeutic treatment modalities, including surgery, radiation, cryosurgery, and/or thermotherapy. Such combination therapies may advantageously utilize lower dosages of the administered agent and/or other chemotherapeutic agent, thus avoiding possible toxicities or complications associated with the various therapies. The phrase "radiation" includes, but is not limited to, external-beam therapy which involves three-dimensional, conformal radiation therapy where the field of radiation is designed to conform to the volume of tissue

treated; interstitial-radiation therapy where seeds of radioactive compounds are implanted using ultrasound guidance; and a combination of external-beam therapy and interstitial-radiation therapy.

In some embodiments, a compound or composition described herein are administered with at least one additional therapeutic agent, such as a chemotherapeutic agent. In certain embodiments, a compound or composition described herein are administered in combination with one or more additional chemotherapeutic agents, e.g., with one or more chemotherapeutic agents described herein.

In some embodiments, a compound or composition described herein is administered in combination with one or more additional chemotherapeutic agents. Any chemotherapeutic agent can be used; exemplary chemotherapeutic agents are well-known to those skilled in the art. A compound or composition may also be administered in combination with other agents that may be administered to a subject in need thereof, such as hormones, steroids, antimicrobial agents, an agent or procedure to mitigate potential side effects from the agent compositions such as diarrhea, nausea and vomiting, an immunosuppressive agent, a CYP3A4 inhibitor, an antiemetic, and the like.

When formulating the pharmaceutical compositions described herein, the clinician may utilize preferred dosages as warranted by the condition of the subject being treated. For example, in one embodiment, a compound or composition described herein may be administered at a dosing schedule described herein, e.g., once every one, two, three, four, five or six weeks.

Also, in general, a compound or composition described herein, and an optional additional chemotherapeutic agent(s) do not have to be administered in the same pharmaceutical composition, and may, because of different physical and chemical characteristics, have to be administered by different routes. The determination of the mode of administration and the advisability of administration, where possible, in the same pharmaceutical composition, is well within the knowledge of the skilled clinician. The initial administration can be made according to established protocols known in the art, and then, based upon the observed effects, the dosage, modes of administration and times of administration can be modified by the skilled clinician.

The actual dosage of a compound or composition described herein and/or any additional chemotherapeutic agent employed may be varied depending upon the requirements of the subject and the severity of the condition being treated. Determination of the proper dosage for a particular situation is within the skill of the art. Generally, treatment is initiated with smaller dosages which are less than the optimum dose of the compound. Thereafter, the dosage is increased by small amounts until the optimum effect under the circumstances is reached.

In some embodiments, when a compound or composition described herein is administered in combination with one or more additional chemotherapeutic agents, the additional chemotherapeutic agent (or agents) is administered at a standard dose.

The particular choice of additional anti-proliferative cytotoxic agent(s) or radiation will depend upon the diagnosis of the attending physicians and their judgment of the condition of the subject and the appropriate treatment protocol.

If a compound or composition described herein and the additional chemotherapeutic agent(s) and/or radiation are not administered simultaneously or essentially simultaneously, then the initial order of administration of a compound or composition described herein, and the additional chemotherapeutic agent(s) and/or radiation, may be varied. Thus, for example a compound or composition described herein may be administered first followed by the administration of the additional chemotherapeutic agent(s) and/or radiation; or the additional chemotherapeutic agent(s) and/or radiation may be administered first followed by the administration of a compound or composition described herein. This alternate administration may be repeated during a single treatment protocol. The determination of the order of administration, and the number of repetitions of administration of each therapeutic agent during a treatment protocol, is well within the knowledge of the skilled physician after evaluation of the disease being treated and the condition of the subject.

Thus, in accordance with experience and knowledge, the practicing physician can modify each protocol for the administration of a component (a compound or composition described herein, anti-neoplastic agent(s), or radiation) of the treatment according to the individual subject's needs, as the treatment proceeds.

The attending clinician, in judging whether treatment is effective at the dosage administered, will consider the general well-being of the subject as well as more definite signs such as relief of disease-related symptoms, inhibition of tumor growth, actual shrinkage of the tumor, or inhibition of metastasis. Size of the tumor can be measured by standard methods such as radiological studies, e.g., CAT or MRI scan, and successive measurements can be used to judge whether or not growth of the tumor has been retarded or even reversed. Relief of disease related symptoms such as pain, and improvement in overall condition can also be used to help judge effectiveness of treatment.

Pharmaceutical Salts

The disclosed compounds may exist as pharmaceutically acceptable salts. The term "pharmaceutically acceptable salt" refers to salts or zwitterions of the compounds which are water

or oil-soluble or dispersible, suitable for treatment of disorders without undue toxicity, irritation, and allergic response, commensurate with a reasonable benefit/risk ratio and effective for their intended use. The salts may be prepared during the final isolation and purification of the compounds or separately by reacting an amino group of the compounds with a suitable acid. For example, a compound may be dissolved in a suitable solvent, such as but not limited to methanol and water and treated with at least one equivalent of an acid, like hydrochloric acid. The resulting salt may precipitate out and be isolated by filtration and dried under reduced pressure. Alternatively, the solvent and excess acid may be removed under reduced pressure to provide a salt. Representative salts include acetate, adipate, alginate, citrate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, camphorate, camphorsulfonate, digluconate, glycerophosphate, hemisulfate, heptanoate, hexanoate, formate, isethionate, fumarate, lactate, maleate, methanesulfonate, naphthylenesulfonate, nicotinate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, picrate, oxalate, maleate, pivalate, propionate, succinate, tartrate, thrichloroacetate, trifluoroacetate, glutamate, para-toluenesulfonate, undecanoate, hydrochloric, hydrobromic, sulfuric, phosphoric and the like. The amino groups of the compounds may also be quaternized with alkyl chlorides, bromides, or iodides such as methyl, ethyl, propyl, isopropyl, butyl, lauryl, myristyl, stearyl and the like.

Basic addition salts may be prepared during the final isolation and purification of the disclosed compounds by reaction of a carboxyl group with a suitable base such as the hydroxide, carbonate, or bicarbonate of a metal cation such as lithium, sodium, potassium, calcium, magnesium, or aluminum, or an organic primary, secondary, or tertiary amine. Quaternary amine salts can be prepared, such as those derived from methylamine, dimethylamine, trimethylamine, triethylamine, diethylamine, ethylamine, tributylamine, pyridine, *N*,*N*-dimethylaniline, *N*-methylpiperidine, *N*-methylmorpholine, dicyclohexylamine, procaine, dibenzylamine, *N*,*N*-dibenzylphenethylamine, 1-ephenamine and *N*,*N*′-dibenzylethylenediamine, ethylenediamine, ethanolamine, diethanolamine, piperidine, piperazine, and the like.

Formulations

The disclosed compounds may be incorporated into pharmaceutical compositions suitable for administration to a subject (such as a patient, which may be a human or non-human). The disclosed compounds may also be provided as formulations.

The pharmaceutical compositions and formulations may include a “therapeutically effective amount” or a “prophylactically effective amount” of the agent. A “therapeutically effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve

the desired therapeutic result. A therapeutically effective amount of the composition may be determined by a person skilled in the art and may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the composition to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of a compound of the invention (e.g., a compound of formula (I)) are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

While a compound described herein may be administered alone in the methods described herein, it may also be presented as one or more pharmaceutical compositions (e.g., formulations). A compound described herein may be formulated with one or more pharmaceutically acceptable carriers, adjuvants, excipients, diluents, fillers, buffers, stabilizers, preservatives, lubricants, or other materials well known to those skilled in the art and optionally other therapeutic or prophylactic agents.

Accordingly, the methods described herein include administration of one or more pharmaceutical compositions, as discussed herein, in which a compound described herein is admixed together with one or more pharmaceutically acceptable carriers, excipients, buffers, adjuvants, stabilizers, or other materials, as described herein.

Suitable carriers, excipients, etc. can be found in standard pharmaceutical texts, for example, Remington's Pharmaceutical Sciences, 18th edition, Mack Publishing Company, Easton, Pa., 1990.

The formulations may conveniently be presented in unit dosage form and may be prepared by any methods known in the art of pharmacy. Such methods include the step of bringing into association the active compound(s) with the carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the active compound with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

Formulations may be in the form of liquids, solutions, suspensions, emulsions, elixirs, syrups, tablets, lozenges, granules, powders, capsules, cachets, pills, ampoules, suppositories, pessaries, ointments, gels, pastes, creams, sprays, mists, foams, lotions, oils, boluses, electuaries, or aerosols.

Formulations suitable for oral administration (e.g., by ingestion) may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active compound; as a powder or granules; as a solution or suspension in an aqueous or nonaqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion; as a bolus; as an electuary; or as a paste.

A tablet may be made by conventional means, e.g., compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active compound in a free-flowing form such as a powder or granules, optionally mixed with one or more binders (e.g., povidone, gelatin, acacia, sorbitol, tragacanth, hydroxypropylmethyl cellulose); fillers or diluents (e.g., lactose, microcrystalline cellulose, calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc, silica); disintegrants (e.g., sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose); surface-active or dispersing or wetting agents (e.g., sodium lauryl sulfate); and preservatives (e.g., methyl p-hydroxybenzoate, propyl p-hydroxybenzoate, sorbic acid). Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active compound therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

Formulations suitable for parenteral administration (e.g., by injection, including cutaneous, subcutaneous, intramuscular, intravenous and intradermal), include aqueous and nonaqueous isotonic, pyrogen-free, sterile injection solutions which may contain anti-oxidants, buffers, preservatives, stabilizers, bacteriostats, and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents, and liposomes or other microparticulate systems which are designed to target the compound to blood components or one or more organs. Examples of suitable isotonic vehicles for use in such formulations include Sodium Chloride Injection, Ringer's Solution, or Lactated Ringer's Injection. The formulations may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use.

Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, and tablets. Formulations may be in the form of liposomes or other

microparticulate systems which are designed to target the active compound to blood components or one or more organs.

Formulations suitable for topical administration (e.g., transdermal, intranasal, ocular, buccal, and sublingual) may be formulated as an ointment, cream, suspension, lotion, powder, solution, past, gel, spray, aerosol, or oil. Alternatively, a formulation may comprise a patch or a dressing such as a bandage or adhesive plaster impregnated with active compounds and optionally one or more excipients or diluents.

Formulations suitable for topical administration in the mouth include lozenges comprising the active compound in a flavored basis, usually sucrose and acacia or tragacanth; pastilles comprising the active compound in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the active compound in a suitable liquid carrier.

Formulations suitable for topical administration to the eye also include eye drops wherein the active compound is dissolved or suspended in a suitable carrier, especially an aqueous solvent for the active compound.

Formulations suitable for nasal administration, wherein the carrier is a solid, include a coarse powder having a particle size, for example, in the range of about 20 to about 500 microns which is administered in the manner in which snuff is taken, i.e., by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations wherein the carrier is a liquid for administration as, for example, nasal spray, nasal drops, or by aerosol administration by nebulizer, include aqueous or oily solutions of the active compound.

Formulations suitable for administration by inhalation include those presented as an aerosol spray from a pressurized pack, with the use of a suitable propellant, such as dichlorodifluoromethane, trichlorofluoromethane, dichoro-tetrafluoroethane, carbon dioxide, or other suitable gases. Further formulations suitable for inhalation include those presented as a nebulizer.

Formulations suitable for topical administration via the skin include ointments, creams, and emulsions. When formulated in an ointment, the active compound may optionally be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the active compounds may be formulated in a cream with an oil-in-water cream base. If desired, the aqueous phase of the cream base may include, for example, at least about 30% w/w of a polyhydric alcohol, i.e., an alcohol having two or more hydroxyl groups such as propylene glycol, butane-1,3-diol, mannitol, sorbitol, glycerol and polyethylene glycol and mixtures thereof. The topical formulations may desirably include a compound which enhances absorption or penetration

of the active compound through the skin or other affected areas. Examples of such dermal penetration enhancers include dimethylsulfoxide and related analogues.

When formulated as a topical emulsion, the oily phase may optionally comprise merely an emulsifier (otherwise known as an emulgent), or it may comprises a mixture of at least one emulsifier with a fat or an oil or with both a fat and an oil. Preferably, a hydrophilic emulsifier is included together with a lipophilic emulsifier which acts as a stabilizer. It is also preferred to include both an oil and a fat.

Together, the emulsifier(s) with or without stabilizer(s) make up the so-called emulsifying wax, and the wax together with the oil and/or fat make up the so-called emulsifying ointment base which forms the oily dispersed phase of the cream formulations.

Suitable emulgents and emulsion stabilizers include Tween 60, Span 80, cetostearyl alcohol, myristyl alcohol, glyceryl monostearate and sodium lauryl sulfate. The choice of suitable oils or fats for the formulation is based on achieving the desired cosmetic properties, since the solubility of the active compound in most oils likely to be used in pharmaceutical emulsion formulations may be very low. Thus, the cream should preferably be a non-greasy, non-staining and washable product with suitable consistency to avoid leakage from tubes or other containers. Straight or branched chain, mono- or dibasic alkyl esters such as diisoadipate, isocetyl stearate, propylene glycol diester of coconut fatty acids, isopropyl myristate, decyl oleate, isopropyl palmitate, butyl stearate, 2-ethylhexyl palmitate or a blend of branched chain esters known as Crodamol CAP may be used, the last three being preferred esters.

These may be used alone or in combination depending on the properties required. Alternatively, high melting point lipids such as white soft paraffin and/or liquid paraffin or other mineral oils can be used.

Formulations suitable for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams, or spray formulations containing in addition to the active compound, such carriers as are known in the art to be appropriate.

Pharmaceutical Compositions and Formulations

Thus, the compounds and their pharmaceutically acceptable salts may be formulated for administration by, for example, solid dosing, eye drop, in a topical oil-based formulation, injection, inhalation (either through the mouth or the nose), implants, or oral, buccal, parenteral, or rectal administration. Techniques and formulations may generally be found in “Remington’s

Pharmaceutical Sciences," (Meade Publishing Co., Easton, Pa.). Therapeutic compositions must typically be sterile and stable under the conditions of manufacture and storage.

The route by which the disclosed compounds are administered, and the form of the composition will dictate the type of carrier to be used. The composition may be in a variety of forms, suitable, for example, for systemic administration (e.g., oral, rectal, nasal, sublingual, buccal, implants, or parenteral) or topical administration (e.g., dermal, pulmonary, nasal, aural, ocular, liposome delivery systems, or iontophoresis).

Carriers for systemic administration typically include at least one of diluents, lubricants, binders, disintegrants, colorants, flavors, sweeteners, antioxidants, preservatives, glidants, solvents, suspending agents, wetting agents, surfactants, combinations thereof, and others. All carriers are optional in the compositions.

Suitable diluents include sugars such as glucose, lactose, dextrose, and sucrose; diols such as propylene glycol; calcium carbonate; sodium carbonate; sugar alcohols, such as glycerin; mannitol; and sorbitol. The amount of diluent(s) in a systemic or topical composition is typically about 50 to about 90%.

Suitable lubricants include silica, talc, stearic acid and its magnesium salts and calcium salts, calcium sulfate; and liquid lubricants such as polyethylene glycol and vegetable oils such as peanut oil, cottonseed oil, sesame oil, olive oil, corn oil and oil of theobroma. The amount of lubricant(s) in a systemic or topical composition is typically about 5 to about 10%.

Suitable binders include polyvinyl pyrrolidone; magnesium aluminum silicate; starches such as corn starch and potato starch; gelatin; tragacanth; and cellulose and its derivatives, such as sodium carboxymethylcellulose, ethyl cellulose, methylcellulose, microcrystalline cellulose, and sodium carboxymethylcellulose. The amount of binder(s) in a systemic composition is typically about 5 to about 50%.

Suitable disintegrants include agar, alginic acid and the sodium salt thereof, effervescent mixtures, croscarmellose, crospovidone, sodium carboxymethyl starch, sodium starch glycolate, clays, and ion exchange resins. The amount of disintegrant(s) in a systemic or topical composition is typically about 0.1 to about 10%.

Suitable colorants include a colorant such as an FD&C dye. When used, the amount of colorant in a systemic or topical composition is typically about 0.005 to about 0.1%.

Suitable flavors include menthol, peppermint, and fruit flavors. The amount of flavor(s), when used, in a systemic or topical composition is typically about 0.1 to about 1.0%.

Suitable sweeteners include aspartame and saccharin. The amount of sweetener(s) in a systemic or topical composition is typically about 0.001 to about 1%.

Suitable antioxidants include butylated hydroxyanisole ("BHA"), butylated hydroxytoluene ("BHT"), and vitamin E. The amount of antioxidant(s) in a systemic or topical composition is typically about 0.1 to about 5%.

Suitable preservatives include benzalkonium chloride, methyl paraben and sodium benzoate. The amount of preservative(s) in a systemic or topical composition is typically about 0.01 to about 5%.

Suitable glidants include silicon dioxide. The amount of glidant(s) in a systemic or topical composition is typically about 1 to about 5%.

Suitable solvents include water, isotonic saline, ethyl oleate, glycerine, hydroxylated castor oils, alcohols such as ethanol, and phosphate buffer solutions. The amount of solvent(s) in a systemic or topical composition is typically from about 0 to about 100%.

Suitable suspending agents include AVICEL RC-591 (from FMC Corporation of Philadelphia, PA) and sodium alginate. The amount of suspending agent(s) in a systemic or topical composition is typically about 1 to about 8%.

Suitable surfactants include lecithin, Polysorbate 80, and sodium lauryl sulfate, and the TWEENS from Atlas Powder Company of Wilmington, Delaware. Suitable surfactants include those disclosed in the C.T.F.A. Cosmetic Ingredient Handbook, 1992, pp.587-592; Remington's Pharmaceutical Sciences, 15th Ed. 1975, pp. 335-337; and McCutcheon's Volume 1, Emulsifiers & Detergents, 1994, North American Edition, pp. 236-239. The amount of surfactant(s) in the systemic or topical composition is typically about 0.1% to about 5%.

Although the amounts of components in the systemic compositions may vary depending on the type of systemic composition prepared, in general, systemic compositions include 0.01% to 50% of an active compound (e.g., a compound of formula (I)) and 50% to 99.99% of one or more carriers. Compositions for parenteral administration typically include 0.1% to 10% of actives and 90% to 99.9% of a carrier including a diluent and a solvent.

Compositions for oral administration can have various dosage forms. For example, solid forms include tablets, capsules, granules, and bulk powders. These oral dosage forms include a safe and effective amount, usually at least about 5%, and more particularly from about 25% to about 50% of actives. The oral dosage compositions include about 50% to about 95% of carriers, and more particularly, from about 50% to about 75%.

Tablets can be compressed, tablet triturates, enteric-coated, sugar-coated, film-coated, or multiple-compressed. Tablets typically include an active component, and a carrier comprising ingredients selected from diluents, lubricants, binders, disintegrants, colorants, flavors, sweeteners, glidants, and combinations thereof. Specific diluents include calcium carbonate,

sodium carbonate, mannitol, lactose, and cellulose. Specific binders include starch, gelatin, and sucrose. Specific disintegrants include alginic acid and croscarmellose. Specific lubricants include magnesium stearate, stearic acid, and talc. Specific colorants are the FD&C dyes, which can be added for appearance. Chewable tablets preferably contain sweeteners such as aspartame and saccharin, or flavors such as menthol, peppermint, fruit flavors, or a combination thereof.

Capsules (including implants, time release and sustained release formulations) typically include an active compound (e.g., a compound of formula (I)), and a carrier including one or more diluents disclosed above in a capsule comprising gelatin. Granules typically comprise a disclosed compound, and preferably glidants such as silicon dioxide to improve flow characteristics. Implants can be of the biodegradable or the non-biodegradable type.

The selection of ingredients in the carrier for oral compositions depends on secondary considerations like taste, cost, and shelf stability, which are not critical for the purposes of this invention.

Solid compositions may be coated by conventional methods, typically with pH or time-dependent coatings, such that a disclosed compound is released in the gastrointestinal tract in the vicinity of the desired application, or at various points and times to extend the desired action. The coatings typically include one or more components selected from the group consisting of cellulose acetate phthalate, polyvinyl acetate phthalate, hydroxypropyl methyl cellulose phthalate, ethyl cellulose, EUDRAGIT® coatings (available from Evonik Industries of Essen, Germany), waxes and shellac.

Compositions for oral administration can have liquid forms. For example, suitable liquid forms include aqueous solutions, emulsions, suspensions, solutions reconstituted from non-effervescent granules, suspensions reconstituted from non-effervescent granules, effervescent preparations reconstituted from effervescent granules, elixirs, tinctures, syrups, and the like. Liquid orally administered compositions typically include a disclosed compound and a carrier, namely, a carrier selected from diluents, colorants, flavors, sweeteners, preservatives, solvents, suspending agents, and surfactants. Peroral liquid compositions preferably include one or more ingredients selected from colorants, flavors, and sweeteners.

Other compositions useful for attaining systemic delivery of the subject compounds include sublingual, buccal and nasal dosage forms. Such compositions typically include one or more of soluble filler substances such as diluents including sucrose, sorbitol and mannitol; and binders such as acacia, microcrystalline cellulose, carboxymethyl cellulose, and hydroxypropyl

methylcellulose. Such compositions may further include lubricants, colorants, flavors, sweeteners, antioxidants, and glidants.

The disclosed compounds can be topically administered. Topical compositions that can be applied locally to the skin may be in any form including solids, solutions, oils, creams, ointments, gels, lotions, shampoos, leave-on and rinse-out hair conditioners, milks, cleansers, moisturizers, sprays, skin patches, and the like. Topical compositions include: a disclosed compound (e.g., a compound of formula (I)), and a carrier. The carrier of the topical composition preferably aids penetration of the compounds into the skin. The carrier may further include one or more optional components.

The amount of the carrier employed in conjunction with a disclosed compound is sufficient to provide a practical quantity of composition for administration per unit dose of the compound. Techniques and compositions for making dosage forms useful in the methods of this invention are described in the following references: Modern Pharmaceutics, Chapters 9 and 10, Banker & Rhodes, eds. (1979); Lieberman et al., Pharmaceutical Dosage Forms: Tablets (1981); and Ansel, Introduction to Pharmaceutical Dosage Forms, 2nd Ed., (1976).

A carrier may include a single ingredient or a combination of two or more ingredients. In the topical compositions, the carrier includes a topical carrier. Suitable topical carriers include one or more ingredients selected from phosphate buffered saline, isotonic water, deionized water, monofunctional alcohols, symmetrical alcohols, aloe vera gel, allantoin, glycerin, vitamin A and E oils, mineral oil, propylene glycol, PPG-2 myristyl propionate, dimethyl isosorbide, castor oil, combinations thereof, and the like. More particularly, carriers for skin applications include propylene glycol, dimethyl isosorbide, and water, and even more particularly, phosphate buffered saline, isotonic water, deionized water, monofunctional alcohols, and symmetrical alcohols.

The carrier of a topical composition may further include one or more ingredients selected from emollients, propellants, solvents, humectants, thickeners, powders, fragrances, pigments, and preservatives, all of which are optional.

Suitable emollients include stearyl alcohol, glyceryl monoricinoleate, glyceryl monostearate, propane-1,2-diol, butane-1,3-diol, mink oil, cetyl alcohol, isopropyl isostearate, stearic acid, isobutyl palmitate, isocetyl stearate, oleyl alcohol, isopropyl laurate, hexyl laurate, decyl oleate, octadecan-2-ol, isocetyl alcohol, cetyl palmitate, di-n-butyl sebacate, isopropyl myristate, isopropyl palmitate, isopropyl stearate, butyl stearate, polyethylene glycol, triethylene glycol, lanolin, sesame oil, coconut oil, arachis oil, castor oil, acetylated lanolin alcohols, petroleum, mineral oil, butyl myristate, isostearic acid, palmitic acid, isopropyl linoleate, lauryl lactate, myristyl lactate, decyl oleate, myristyl myristate, and combinations thereof. Specific

emollients for skin include stearyl alcohol and polydimethylsiloxane. The amount of emollient(s) in a skin-based topical composition is typically about 5% to about 95%.

Suitable propellants include propane, butane, isobutane, dimethyl ether, carbon dioxide, nitrous oxide, and combinations thereof. The amount of propellant(s) in a topical composition is typically about 0% to about 95%.

Suitable solvents include water, ethyl alcohol, methylene chloride, isopropanol, castor oil, ethylene glycol monoethyl ether, diethylene glycol monobutyl ether, diethylene glycol monoethyl ether, dimethylsulfoxide, dimethyl formamide, tetrahydrofuran, and combinations thereof. Specific solvents include ethyl alcohol and homotopic alcohols. The amount of solvent(s) in a topical composition is typically about 0% to about 95%.

Suitable humectants include glycerin, sorbitol, sodium 2-pyrrolidone-5-carboxylate, soluble collagen, dibutyl phthalate, gelatin, and combinations thereof. Specific humectants include glycerin. The amount of humectant(s) in a topical composition is typically 0% to 95%.

The amount of thickener(s) in a topical composition is typically about 0% to about 95%.

Suitable powders include beta-cyclodextrins, hydroxypropyl cyclodextrins, chalk, talc, fullers earth, kaolin, starch, gums, colloidal silicon dioxide, sodium polyacrylate, tetra alkyl ammonium smectites, trialkyl aryl ammonium smectites, chemically-modified magnesium aluminum silicate, organically-modified montmorillonite clay, hydrated aluminum silicate, fumed silica, carboxyvinyl polymer, sodium carboxymethyl cellulose, ethylene glycol monostearate, and combinations thereof. The amount of powder(s) in a topical composition is typically 0% to 95%.

The amount of fragrance in a topical composition is typically about 0% to about 0.5%, particularly, about 0.001% to about 0.1%.

Suitable pH adjusting additives include HCl or NaOH in amounts sufficient to adjust the pH of a topical pharmaceutical composition.

Dosages

It will be appreciated that appropriate dosages of the active compounds and compositions comprising the active compounds, can vary from patient to patient. Determining the optimal dosage will generally involve the balancing of the level of therapeutic benefit against any risk or deleterious side effects of the treatments described herein. The selected dosage level will depend on a variety of factors including, but not limited to, the activity of the particular compound, the route of administration, the time of administration, the rate of excretion of the compound, the duration of the treatment, other drugs, compounds, and/or materials used in combination, and the age, sex, weight, condition, general health, and prior medical history of the patient. The amount

of compound and route of administration will ultimately be at the discretion of the physician, although generally the dosage will be to achieve local concentrations at the site of action which achieve the desired effect without causing substantial harmful or deleterious side-effects.

Administration in vivo can be effected in one dose, continuously or intermittently (e.g., in divided doses at appropriate intervals) throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the formulation used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated. Single or multiple administrations can be carried out with the dose level and pattern being selected by the treating physician.

In general, a suitable dose of the active compound may be in the range of about 100 g to about 250 mg per kilogram body weight of the subject per day.

In one embodiment the dose of a PROTAC pro-drug, salt thereof, or derivative thereof, comprises from about 0.001 to about 200 mg/kg. In one aspect, the dose comprises about 0.001 mg/kg, 0.002 mg/kg, 0.003 mg/kg, 0.004 mg/kg, 0.005 mg/kg, 0.006 mg/kg, 0.007 mg/kg, 0.008 mg/kg, 0.009 mg/kg, 0.01 to about 20 mg/kg, 0.01 mg/kg, 0.02 mg/kg, 0.03 mg/kg, 0.04 mg/kg, 0.05 mg/kg, 0.06 mg/kg, 0.07 mg/kg, 0.08 mg/kg, 0.09 mg/kg, 0.1 mg/kg, 0.11 mg/kg, 0.12 mg/kg, 0.13 mg/kg, 0.14 mg/kg, 0.15 mg/kg, 0.16 mg/kg, 0.17 mg/kg, 0.18 mg/kg, 0.19 mg/kg, 0.2 mg/kg, 0.21 mg/kg, 0.22 mg/kg, 0.23 mg/kg, 0.24 mg/kg, 0.25 mg/kg, 0.26 mg/kg, 0.27 mg/kg, 0.28 mg/kg, 0.29 mg/kg, 0.3 mg/kg, 0.31 mg/kg, 0.32 mg/kg, 0.33 mg/kg, 0.34 mg/kg, 0.35 mg/kg, 0.36 mg/kg, 0.37 mg/kg, 0.38 mg/kg, 0.39 mg/kg, 0.4 mg/kg, 0.41 mg/kg, 0.42 mg/kg, 0.43 mg/kg, 0.44 mg/kg, 0.45 mg/kg, 0.46 mg/kg, 0.47 mg/kg, 0.48 mg/kg, 0.49 mg/kg, 0.5 mg/kg, 0.6 mg/kg, 0.7 mg/kg, 0.8 mg/kg, 0.9 mg/kg, 1 mg/kg, 1.1 mg/kg, 1.2 mg/kg, 1.3 mg/kg, 1.4 mg/kg, 1.5 mg/kg, 1.6 mg/kg, 1.7 mg/kg, 1.8 mg/kg, 1.9 mg/kg, 2 mg/kg, 2.1 mg/kg, 2.2 mg/kg, 2.3 mg/kg, 2.4 mg/kg, 2.5 mg/kg, 2.6 mg/kg, 2.7 mg/kg, 2.8 mg/kg, 2.9 mg/kg, 3 mg/kg, 3.1 mg/kg, 3.2 mg/kg, 3.3 mg/kg, 3.4 mg/kg, 3.5 mg/kg, 3.6 mg/kg, 3.7 mg/kg, 3.8 mg/kg, 3.9 mg/kg, 4 mg/kg, 4.1 mg/kg, 4.2 mg/kg, 4.3 mg/kg, 4.4 mg/kg, 4.5 mg/kg, 4.6 mg/kg, 4.7 mg/kg, 4.8 mg/kg, 4.9 mg/kg, 5 mg/kg, 5.5 mg/kg, 6 mg/kg, 5.3 mg/kg, 5.4 mg/kg, 5.5 mg/kg, 5.6 mg/kg, 5.7 mg/kg, 5.8 mg/kg, 5.9 mg/kg, 6 mg/kg, 6.1 mg/kg, 6.2 mg/kg, 6.3 mg/kg, 6.4 mg/kg, 6.5 mg/kg, 6.6 mg/kg, 6.7 mg/kg, 6.8 mg/kg, 6.9 mg/kg, 7 mg/kg, 7.1 mg/kg, 7.2 mg/kg, 7.3 mg/kg, 7.4 mg/kg, 7.5 mg/kg, 7.6 mg/kg, 7.7 mg/kg, 7.8 mg/kg, 7.9 mg/kg, 8 mg/kg, 8.1 mg/kg, 8.2 mg/kg, 8.3 mg/kg, 8.4 mg/kg, 8.5 mg/kg, 8.6 mg/kg, 8.7 mg/kg, 8.8 mg/kg, 8.9 mg/kg, 9 mg/kg, 9.1 mg/kg, 9.2 mg/kg, 9.3 mg/kg, 9.4 mg/kg, 9.5 mg/kg, 9.6 mg/kg, 9.7 mg/kg, 9.8 mg/kg, 9.9 mg/kg, 10 mg/kg, 10.5 mg/kg, 11 mg/kg, 11.5 mg/kg, 12 mg/kg, 12.5 mg/kg, 13 mg/kg, 13.5 mg/kg, 14 mg/kg, 14.5 mg/kg, 15 mg/kg, 15.5 mg/kg, 16 mg/kg, 16.5 mg/kg, 17 mg/kg, 17.5 mg/kg, 18 mg/kg, 18.5 mg/kg, 19 mg/kg, 19.5 mg/kg, 20

mg/kg, 40 mg/kg, 60 mg/kg, 80 mg/kg, 100 mg/kg, 120 mg/kg, 140 mg/kg, 160 mg/kg, 180 mg/kg, 200 mg/kg or greater.

In another aspect, the dose of a PROTAC pro-drug, salt thereof, or derivative thereof, comprises about 0.001–0.1 mg/kg, 0.002–0.1 mg/kg, 0.003–0.1 mg/kg, 0.004–0.1 mg/kg, 0.005–0.1 mg/kg, 0.006–0.1 mg/kg, 0.007–0.1 mg/kg, 0.008–0.1 mg/kg, 0.009–0.1 mg/kg, 0.001–0.5 mg/kg, 0.001–1 mg/kg, 0.001–1.5 mg/kg, 0.001–2 mg/kg, 0.001–2.5 mg/kg, 0.001–3 mg/kg, 0.001–3.5 mg/kg, 0.001–4 mg/kg, 0.001–4.5 mg/kg, 0.001–5 mg/kg, 0.001–10 mg/kg, 0.001–15 mg/kg, 0.001–20 mg/kg, 0.001–25 mg/kg, 0.001–50 mg/kg, 0.001–75 mg/kg, 0.001–100 mg/kg, 0.001–125 mg/kg, 0.001–150 mg/kg, 0.001–175 mg/kg, 0.001–200 mg/kg, 0.01–10 mg/kg, 0.01–15 mg/kg, 0.01–20 mg/kg, 0.01–0.1 mg/kg, 0.01–0.2 mg/kg, 0.01–0.3 mg/kg, 0.01–0.4 mg/kg, 0.01–0.5 mg/kg, 0.01–0.6 mg/kg, 0.01–0.7 mg/kg, 0.01–0.8 mg/kg, 0.01–0.9 mg/kg, 0.01–1 mg/kg, 0.01–5 mg/kg, 0.01–10 mg/kg, 0.01–15 mg/kg, 0.01–20 mg/kg, 0.01–25 mg/kg, 0.01–50 mg/kg, 0.01–75 mg/kg, 0.01–100 mg/kg, 0.01–125 mg/kg, 0.01–150 mg/kg, 0.01–175 mg/kg, 0.01–200 mg/kg, 0.1–1 mg/kg, 0.1–1.5 mg/kg, 0.1–2 mg/kg, 0.1–2.5 mg/kg, 0.1–3 mg/kg, 0.1–3.5 mg/kg, 0.1–4 mg/kg, 0.1–4.5 mg/kg, 0.1–5 mg/kg, 0.1–10 mg/kg, 0.1–15 mg/kg, 0.1–20 mg/kg, 0.1–25 mg/kg, 0.1–50 mg/kg, 0.1–75 mg/kg, 0.1–100 mg/kg, 0.1–125 mg/kg, 0.1–150 mg/kg, 0.1–175 mg/kg, 0.1–200 mg/kg, 1–5 mg/kg, 1–5.5 mg/kg, 1–6 mg/kg, 1–6.5 mg/kg, 1–7 mg/kg, 1–7.5 mg/kg, 1–8 mg/kg, 1–8.5 mg/kg, 1–9 mg/kg, 1–9.5 mg/kg, 1–10 mg/kg, 1–10.5 mg/kg, 1–11 mg/kg, 1–11.5 mg/kg, 1–12 mg/kg, 1–12.5 mg/kg, 1–13 mg/kg, 1–13.5 mg/kg, 1–14 mg/kg, 1–14.5 mg/kg, 1–15 mg/kg, 1–15.5 mg/kg, 1–16 mg/kg, 1–16.5 mg/kg, 1–17 mg/kg, 1–17.5 mg/kg, 1–18 mg/kg, 1–18.5 mg/kg, 1–19 mg/kg, 1–19.5 mg/kg, 1–20 mg/kg, 1–25 mg/kg, 1–50 mg/kg, 1–75 mg/kg, 1–100 mg/kg, 1–125 mg/kg, 1–150 mg/kg, 1–175 mg/kg, 1–200 mg/kg, 5–10 mg/kg, 5–10.5 mg/kg, 5–11 mg/kg, 5–11.5 mg/kg, 5–12 mg/kg, 5–12.5 mg/kg, 5–13 mg/kg, 5–13.5 mg/kg, 5–14 mg/kg, 5–14.5 mg/kg, 5–15 mg/kg, 5–15.5 mg/kg, 5–16 mg/kg, 5–16.5 mg/kg, 5–17 mg/kg, 5–17.5 mg/kg, 5–18 mg/kg, 5–18.5 mg/kg, 5–19 mg/kg, 5–19.5 mg/kg, or 5–20 mg/kg, 5–25 mg/kg, 5–50 mg/kg, 5–75 mg/kg, 5–100 mg/kg, 5–125 mg/kg, 5–150 mg/kg, 5–175 mg/kg, 5–200 mg/kg, 10–25 mg/kg, 10–50 mg/kg, 10–75 mg/kg, 10–100 mg/kg, 10–125 mg/kg, 10–150 mg/kg, 10–175 mg/kg, 10–200 mg/kg, including all endpoints, integers and subranges within the disclosed ranges.

In one embodiment the dose of the one or more tetrazine compound, salt thereof, or derivative thereof, comprises from about 0.001 to about 200 mg/kg. In one aspect, the dose comprises about 0.001 mg/kg, 0.002 mg/kg, 0.003 mg/kg, 0.004 mg/kg, 0.005 mg/kg, 0.006 mg/kg, 0.007 mg/kg, 0.008 mg/kg, 0.009 mg/kg, 0.01 to about 20 mg/kg, 0.01 mg/kg, 0.02 mg/kg, 0.03 mg/kg, 0.04 mg/kg, 0.05 mg/kg, 0.06 mg/kg, 0.07 mg/kg, 0.08 mg/kg, 0.09 mg/kg, 0.1 mg/kg,

0.11 mg/kg, 0.12 mg/kg, 0.13 mg/kg, 0.14 mg/kg, 0.15 mg/kg, 0.16 mg/kg, 0.17 mg/kg, 0.18 mg/kg, 0.19 mg/kg, 0.2 mg/kg, 0.21 mg/kg, 0.22 mg/kg, 0.23 mg/kg, 0.24 mg/kg, 0.25 mg/kg, 0.26 mg/kg, 0.27 mg/kg, 0.28 mg/kg, 0.29 mg/kg, 0.3 mg/kg, 0.31 mg/kg, 0.32 mg/kg, 0.33 mg/kg, 0.34 mg/kg, 0.35 mg/kg, 0.36 mg/kg, 0.37 mg/kg, 0.38 mg/kg, 0.39 mg/kg, 0.4 mg/kg, 0.41 mg/kg, 0.42 mg/kg, 0.43 mg/kg, 0.44 mg/kg, 0.45 mg/kg, 0.46 mg/kg, 0.47 mg/kg, 0.48 mg/kg, 0.49 mg/kg, 0.5 mg/kg, 0.6 mg/kg, 0.7 mg/kg, 0.8 mg/kg, 0.9 mg/kg, 1 mg/kg, 1.1 mg/kg, 1.2 mg/kg, 1.3 mg/kg, 1.4 mg/kg, 1.5 mg/kg, 1.6 mg/kg, 1.7 mg/kg, 1.8 mg/kg, 1.9 mg/kg, 2 mg/kg, 2.1 mg/kg, 2.2 mg/kg, 2.3 mg/kg, 2.4 mg/kg, 2.5 mg/kg, 2.6 mg/kg, 2.7 mg/kg, 2.8 mg/kg, 2.9 mg/kg, 3 mg/kg, 3.1 mg/kg, 3.2 mg/kg, 3.3 mg/kg, 3.4 mg/kg, 3.5 mg/kg, 3.6 mg/kg, 3.7 mg/kg, 3.8 mg/kg, 3.9 mg/kg, 4 mg/kg, 4.1 mg/kg, 4.2 mg/kg, 4.3 mg/kg, 4.4 mg/kg, 4.5 mg/kg, 4.6 mg/kg, 4.7 mg/kg, 4.8 mg/kg, 4.9 mg/kg, 5 mg/kg, 5.5 mg/kg, 6 mg/kg, 5.3 mg/kg, 5.4 mg/kg, 5.5 mg/kg, 5.6 mg/kg, 5.7 mg/kg, 5.8 mg/kg, 5.9 mg/kg, 6 mg/kg, 6.1 mg/kg, 6.2 mg/kg, 6.3 mg/kg, 6.4 mg/kg, 6.5 mg/kg, 6.6 mg/kg, 6.7 mg/kg, 6.8 mg/kg, 6.9 mg/kg, 7 mg/kg, 7.1 mg/kg, 7.2 mg/kg, 7.3 mg/kg, 7.4 mg/kg, 7.5 mg/kg, 7.6 mg/kg, 7.7 mg/kg, 7.8 mg/kg, 7.9 mg/kg, 8 mg/kg, 8.1 mg/kg, 8.2 mg/kg, 8.3 mg/kg, 8.4 mg/kg, 8.5 mg/kg, 8.6 mg/kg, 8.7 mg/kg, 8.8 mg/kg, 8.9 mg/kg, 9 mg/kg, 9.1 mg/kg, 9.2 mg/kg, 9.3 mg/kg, 9.4 mg/kg, 9.5 mg/kg, 9.6 mg/kg, 9.7 mg/kg, 9.8 mg/kg, 9.9 mg/kg, 10 mg/kg, 10.5 mg/kg, 11 mg/kg, 11.5 mg/kg, 12 mg/kg, 12.5 mg/kg, 13 mg/kg, 13.5 mg/kg, 14 mg/kg, 14.5 mg/kg, 15 mg/kg, 15.5 mg/kg, 16 mg/kg, 16.5 mg/kg, 17 mg/kg, 17.5 mg/kg, 18 mg/kg, 18.5 mg/kg, 19 mg/kg, 19.5 mg/kg, 20 mg/kg, 40 mg/kg, 60 mg/kg, 80 mg/kg, 100 mg/kg, 120 mg/kg, 140 mg/kg, 160 mg/kg, 180 mg/kg, 200 mg/kg or greater.

In another aspect, the dose of one or more tetrazine, salt thereof, or derivative thereof, comprises about 0.001–0.1 mg/kg, 0.002–0.1 mg/kg, 0.003–0.1 mg/kg, 0.004–0.1 mg/kg, 0.005–0.1 mg/kg, 0.006–0.1 mg/kg, 0.007–0.1 mg/kg, 0.008–0.1 mg/kg, 0.009–0.1 mg/kg, 0.001–0.5 mg/kg, 0.001–1 mg/kg, 0.001–1.5 mg/kg, 0.001–2 mg/kg, 0.001–2.5 mg/kg, 0.001–3 mg/kg, 0.001–3.5 mg/kg, 0.001–4 mg/kg, 0.001–4.5 mg/kg, 0.001–5 mg/kg, 0.001–10 mg/kg, 0.001–15 mg/kg, 0.001–20 mg/kg, 0.001–25 mg/kg, 0.001–50 mg/kg, 0.001–75 mg/kg, 0.001–100 mg/kg, 0.001–125 mg/kg, 0.001–150 mg/kg, 0.001–175 mg/kg, 0.001–200 mg/kg, 0.01–10 mg/kg, 0.01–15 mg/kg, 0.01–20 mg/kg, 0.01–0.1 mg/kg, 0.01–0.2 mg/kg, 0.01–0.3 mg/kg, 0.01–0.4 mg/kg, 0.01–0.5 mg/kg, 0.01–0.6 mg/kg, 0.01–0.7 mg/kg, 0.01–0.8 mg/kg, 0.01–0.9 mg/kg, 0.01–1 mg/kg, 0.01–5 mg/kg, 0.01–10 mg/kg, 0.01–15 mg/kg, 0.01–20 mg/kg, 0.01–25 mg/kg, 0.01–50 mg/kg, 0.01–75 mg/kg, 0.01–100 mg/kg, 0.01–125 mg/kg, 0.01–150 mg/kg, 0.01–175 mg/kg, 0.01–200 mg/kg, 0.1–1 mg/kg, 0.1–1.5 mg/kg, 0.1–2 mg/kg, 0.1–2.5 mg/kg, 0.1–3 mg/kg, 0.1–3.5 mg/kg, 0.1–4 mg/kg, 0.1–4.5 mg/kg, 0.1–5 mg/kg, 0.1–10 mg/kg, 0.1–15 mg/kg, 0.1–20 mg/kg, 0.1–25 mg/kg, 0.1–50 mg/kg, 0.1–75 mg/kg, 0.1–100 mg/kg, 0.1–125 mg/kg, 0.1–150

mg/kg, 0.1–175 mg/kg, 0.1–200 mg/kg, 1–5 mg/kg, 1–5.5 mg/kg, 1–6 mg/kg, 1–6.5 mg/kg, 1–7 mg/kg, 1–7.5 mg/kg, 1–8 mg/kg, 1–8.5 mg/kg, 1–9 mg/kg, 1–9.5 mg/kg, 1–10 mg/kg, 1–10.5 mg/kg, 1–11 mg/kg, 1–11.5 mg/kg, 1–12 mg/kg, 1–12.5 mg/kg, 1–13 mg/kg, 1–13.5 mg/kg, 1–14 mg/kg, 1–14.5 mg/kg, 1–15 mg/kg, 1–15.5 mg/kg, 1–16 mg/kg, 1–16.5 mg/kg, 1–17 mg/kg, 1–17.5 mg/kg, 1–18 mg/kg, 1–18.5 mg/kg, 1–19 mg/kg, 1–19.5 mg/kg, 1–20 mg/kg, 1–25 mg/kg, 1–50 mg/kg, 1–75 mg/kg, 1–100 mg/kg, 1–125 mg/kg, 1–150 mg/kg, 1–175 mg/kg, 1–200 mg/kg, 5–10 mg/kg, 5–10.5 mg/kg, 5–11 mg/kg, 5–11.5 mg/kg, 5–12 mg/kg, 5–12.5 mg/kg, 5–13 mg/kg, 5–13.5 mg/kg, 5–14 mg/kg, 5–14.5 mg/kg, 5–15 mg/kg, 5–15.5 mg/kg, 5–16 mg/kg, 5–16.5 mg/kg, 5–17 mg/kg, 5–17.5 mg/kg, 5–18 mg/kg, 5–18.5 mg/kg, 5–19 mg/kg, 5–19.5 mg/kg, or 5–20 mg/kg, 5–25 mg/kg, 5–50 mg/kg, 5–75 mg/kg, 5–100 mg/kg, 5–125 mg/kg, 5–150 mg/kg, 5–175 mg/kg, 5–200 mg/kg, 10–25 mg/kg, 10–50 mg/kg, 10–75 mg/kg, 10–100 mg/kg, 10–125 mg/kg, 10–150 mg/kg, 10–175 mg/kg, 10–200 mg/kg, including all endpoints, integers and subranges within the disclosed ranges.

In one embodiment the dose of one or more antineoplastic agent, salt thereof, or derivative thereof, comprises from about 0.001 to about 200 mg/kg. In one aspect, the dose comprises about 0.001 mg/kg, 0.002 mg/kg, 0.003 mg/kg, 0.004 mg/kg, 0.005 mg/kg, 0.006 mg/kg, 0.007 mg/kg, 0.008 mg/kg, 0.009 mg/kg, 0.01 to about 20 mg/kg, 0.01 mg/kg, 0.02 mg/kg, 0.03 mg/kg, 0.04 mg/kg, 0.05 mg/kg, 0.06 mg/kg, 0.07 mg/kg, 0.08 mg/kg, 0.09 mg/kg, 0.1 mg/kg, 0.11 mg/kg, 0.12 mg/kg, 0.13 mg/kg, 0.14 mg/kg, 0.15 mg/kg, 0.16 mg/kg, 0.17 mg/kg, 0.18 mg/kg, 0.19 mg/kg, 0.2 mg/kg, 0.21 mg/kg, 0.22 mg/kg, 0.23 mg/kg, 0.24 mg/kg, 0.25 mg/kg, 0.26 mg/kg, 0.27 mg/kg, 0.28 mg/kg, 0.29 mg/kg, 0.3 mg/kg, 0.31 mg/kg, 0.32 mg/kg, 0.33 mg/kg, 0.34 mg/kg, 0.35 mg/kg, 0.36 mg/kg, 0.37 mg/kg, 0.38 mg/kg, 0.39 mg/kg, 0.4 mg/kg, 0.41 mg/kg, 0.42 mg/kg, 0.43 mg/kg, 0.44 mg/kg, 0.45 mg/kg, 0.46 mg/kg, 0.47 mg/kg, 0.48 mg/kg, 0.49 mg/kg, 0.5 mg/kg, 0.6 mg/kg, 0.7 mg/kg, 0.8 mg/kg, 0.9 mg/kg, 1 mg/kg, 1.1 mg/kg, 1.2 mg/kg, 1.3 mg/kg, 1.4 mg/kg, 1.5 mg/kg, 1.6 mg/kg, 1.7 mg/kg, 1.8 mg/kg, 1.9 mg/kg, 2 mg/kg, 2.1 mg/kg, 2.2 mg/kg, 2.3 mg/kg, 2.4 mg/kg, 2.5 mg/kg, 2.6 mg/kg, 2.7 mg/kg, 2.8 mg/kg, 2.9 mg/kg, 3 mg/kg, 3.1 mg/kg, 3.2 mg/kg, 3.3 mg/kg, 3.4 mg/kg, 3.5 mg/kg, 3.6 mg/kg, 3.7 mg/kg, 3.8 mg/kg, 3.9 mg/kg, 4 mg/kg, 4.1 mg/kg, 4.2 mg/kg, 4.3 mg/kg, 4.4 mg/kg, 4.5 mg/kg, 4.6 mg/kg, 4.7 mg/kg, 4.8 mg/kg, 4.9 mg/kg, 5 mg/kg, 5.5 mg/kg, 6 mg/kg, 5.3 mg/kg, 5.4 mg/kg, 5.5 mg/kg, 5.6 mg/kg, 5.7 mg/kg, 5.8 mg/kg, 5.9 mg/kg, 6 mg/kg, 6.1 mg/kg, 6.2 mg/kg, 6.3 mg/kg, 6.4 mg/kg, 6.5 mg/kg, 6.6 mg/kg, 6.7 mg/kg, 6.8 mg/kg, 6.9 mg/kg, 7 mg/kg, 7.1 mg/kg, 7.2 mg/kg, 7.3 mg/kg, 7.4 mg/kg, 7.5 mg/kg, 7.6 mg/kg, 7.7 mg/kg, 7.8 mg/kg, 7.9 mg/kg, 8 mg/kg, 8.1 mg/kg, 8.2 mg/kg, 8.3 mg/kg, 8.4 mg/kg, 8.5 mg/kg, 8.6 mg/kg, 8.7 mg/kg, 8.8 mg/kg, 8.9 mg/kg, 9 mg/kg, 9.1 mg/kg, 9.2 mg/kg, 9.3 mg/kg, 9.4 mg/kg, 9.5 mg/kg, 9.6 mg/kg, 9.7 mg/kg, 9.8 mg/kg, 9.9 mg/kg, 10 mg/kg, 10.5 mg/kg, 11 mg/kg, 11.5 mg/kg, 12

mg/kg, 12.5 mg/kg, 13 mg/kg, 13.5 mg/kg, 14 mg/kg, 14.5 mg/kg, 15 mg/kg, 15.5 mg/kg, 16 mg/kg, 16.5 mg/kg, 17 mg/kg, 17.5 mg/kg, 18 mg/kg, 18.5 mg/kg, 19 mg/kg, 19.5 mg/kg, 20 mg/kg, 40 mg/kg, 60 mg/kg, 80 mg/kg, 100 mg/kg, 120 mg/kg, 140 mg/kg, 160 mg/kg, 180 mg/kg, 200 mg/kg or greater.

In another aspect, the dose of one or more antineoplastic agent, salt thereof, or derivative thereof, comprises about 0.001–0.1 mg/kg, 0.002–0.1 mg/kg, 0.003–0.1 mg/kg, 0.004–0.1 mg/kg, 0.005–0.1 mg/kg, 0.006–0.1 mg/kg, 0.007–0.1 mg/kg, 0.008–0.1 mg/kg, 0.009–0.1 mg/kg, 0.001–0.5 mg/kg, 0.001–1 mg/kg, 0.001–1.5 mg/kg, 0.001–2 mg/kg, 0.001–2.5 mg/kg, 0.001–3 mg/kg, 0.001–3.5 mg/kg, 0.001–4 mg/kg, 0.001–4.5 mg/kg, 0.001–5 mg/kg, 0.001–10 mg/kg, 0.001–15 mg/kg, 0.001–20 mg/kg, 0.001–25 mg/kg, 0.001–50 mg/kg, 0.001–75 mg/kg, 0.001–100 mg/kg, 0.001–125 mg/kg, 0.001–150 mg/kg, 0.001–175 mg/kg, 0.001–200 mg/kg, 0.01–10 mg/kg, 0.01–15 mg/kg, 0.01–20 mg/kg, 0.01–0.1 mg/kg, 0.01–0.2 mg/kg, 0.01–0.3 mg/kg, 0.01–0.4 mg/kg, 0.01–0.5 mg/kg, 0.01–0.6 mg/kg, 0.01–0.7 mg/kg, 0.01–0.8 mg/kg, 0.01–0.9 mg/kg, 0.01–1 mg/kg, 0.01–5 mg/kg, 0.01–10 mg/kg, 0.01–15 mg/kg, 0.01–20 mg/kg, 0.01–25 mg/kg, 0.01–50 mg/kg, 0.01–75 mg/kg, 0.01–100 mg/kg, 0.01–125 mg/kg, 0.01–150 mg/kg, 0.01–175 mg/kg, 0.01–200 mg/kg, 0.1–1 mg/kg, 0.1–1.5 mg/kg, 0.1–2 mg/kg, 0.1–2.5 mg/kg, 0.1–3 mg/kg, 0.1–3.5 mg/kg, 0.1–4 mg/kg, 0.1–4.5 mg/kg, 0.1–5 mg/kg, 0.1–10 mg/kg, 0.1–15 mg/kg, 0.1–20 mg/kg, 0.1–25 mg/kg, 0.1–50 mg/kg, 0.1–75 mg/kg, 0.1–100 mg/kg, 0.1–125 mg/kg, 0.1–150 mg/kg, 0.1–175 mg/kg, 0.1–200 mg/kg, 1–5 mg/kg, 1–5.5 mg/kg, 1–6 mg/kg, 1–6.5 mg/kg, 1–7 mg/kg, 1–7.5 mg/kg, 1–8 mg/kg, 1–8.5 mg/kg, 1–9 mg/kg, 1–9.5 mg/kg, 1–10 mg/kg, 1–10.5 mg/kg, 1–11 mg/kg, 1–11.5 mg/kg, 1–12 mg/kg, 1–12.5 mg/kg, 1–13 mg/kg, 1–13.5 mg/kg, 1–14 mg/kg, 1–14.5 mg/kg, 1–15 mg/kg, 1–15.5 mg/kg, 1–16 mg/kg, 1–16.5 mg/kg, 1–17 mg/kg, 1–17.5 mg/kg, 1–18 mg/kg, 1–18.5 mg/kg, 1–19 mg/kg, 1–19.5 mg/kg, 1–20 mg/kg, 1–25 mg/kg, 1–50 mg/kg, 1–75 mg/kg, 1–100 mg/kg, 1–125 mg/kg, 1–150 mg/kg, 1–175 mg/kg, 1–200 mg/kg, 5–10 mg/kg, 5–10.5 mg/kg, 5–11 mg/kg, 5–11.5 mg/kg, 5–12 mg/kg, 5–12.5 mg/kg, 5–13 mg/kg, 5–13.5 mg/kg, 5–14 mg/kg, 5–14.5 mg/kg, 5–15 mg/kg, 5–15.5 mg/kg, 5–16 mg/kg, 5–16.5 mg/kg, 5–17 mg/kg, 5–17.5 mg/kg, 5–18 mg/kg, 5–18.5 mg/kg, 5–19 mg/kg, 5–19.5 mg/kg, or 5–20 mg/kg, 5–25 mg/kg, 5–50 mg/kg, 5–75 mg/kg, 5–100 mg/kg, 5–125 mg/kg, 5–150 mg/kg, 5–175 mg/kg, 5–200 mg/kg, 10–25 mg/kg, 10–50 mg/kg, 10–75 mg/kg, 10–100 mg/kg, 10–125 mg/kg, 10–150 mg/kg, 10–175 mg/kg, 10–200 mg/kg, including all endpoints, integers and subranges within the disclosed ranges.

Cancer

The methods described herein can be used with any cancer, for example those described by the National Cancer Institute. The cancer can be a carcinoma, a sarcoma, a myeloma, a leukemia, a lymphoma or a mixed type. Exemplary cancers described by the National Cancer Institute include:

Digestive/gastrointestinal cancers such as anal cancer; bile duct cancer; extrahepatic bile duct cancer; appendix cancer; carcinoid tumor, gastrointestinal cancer; colon cancer; colorectal cancer including childhood colorectal cancer; esophageal cancer including childhood esophageal cancer; gallbladder cancer; gastric (stomach) cancer including childhood gastric (stomach) cancer; hepatocellular (liver) cancer including adult (primary) hepatocellular (liver) cancer and childhood (primary) hepatocellular (liver) cancer; pancreatic cancer including childhood pancreatic cancer; sarcoma, rhabdomyosarcoma; islet cell pancreatic cancer; rectal cancer; and small intestine cancer;

Endocrine cancers such as islet cell carcinoma (endocrine pancreas); adrenocortical carcinoma including childhood adrenocortical carcinoma; gastrointestinal carcinoid tumor; parathyroid cancer; pheochromocytoma; pituitary tumor; thyroid cancer including childhood thyroid cancer; childhood multiple endocrine neoplasia syndrome; and childhood carcinoid tumor;

Eye cancers such as intraocular melanoma; and retinoblastoma;

Musculoskeletal cancers such as Ewing's family of tumors; osteosarcoma/malignant fibrous histiocytoma of the bone; childhood rhabdomyosarcoma; soft tissue sarcoma including adult and childhood soft tissue sarcoma; clear cell sarcoma of tendon sheaths; and uterine sarcoma;

Breast cancer such as breast cancer including childhood and male breast cancer and breast cancer in pregnancy;

Neurologic cancers such as childhood brain stemglioma; brain tumor; childhood cerebellar astrocytoma; childhood cerebral astrocytoma/malignant glioma; childhood ependymoma; childhood medulloblastoma; childhood pineal and supratentorial primitive neuroectodermal tumors; childhood visual pathway and hypothalamic glioma; other childhood brain cancers; adrenocortical carcinoma; central nervous system lymphoma, primary; childhood cerebellar astrocytoma; neuroblastoma; craniopharyngioma; spinal cord tumors; central nervous system atypical teratoid/rhabdoid tumor; central nervous system embryonal tumors; and childhood supratentorial primitive neuroectodermal tumors and pituitary tumor;

Genitourinary cancers such as bladder cancer including childhood bladder cancer; renal cell (kidney) cancer; ovarian cancer including childhood ovarian cancer; ovarian epithelial cancer; ovarian low malignant potential tumor; penile cancer; prostate cancer; renal cell cancer including

childhood renal cell cancer; renal pelvis and ureter, transitional cell cancer; testicular cancer; urethral cancer; vaginal cancer; vulvar cancer; cervical cancer; Wilms tumor and other childhood kidney tumors; endometrial cancer; and gestational trophoblastic tumor; Germ cell cancers such as childhood extracranial germ cell tumor; extragonadal germ cell tumor; ovarian germ cell tumor;

Head and neck cancers such as lip and oral cavity cancer; oral cancer including childhood oral cancer; hypopharyngeal cancer; laryngeal cancer including childhood laryngeal cancer; metastatic squamous neck cancer with occult primary; mouth cancer; nasal cavity and paranasal sinus cancer; nasopharyngeal cancer including childhood nasopharyngeal cancer; oropharyngeal cancer; parathyroid cancer; pharyngeal cancer; salivary gland cancer including childhood salivary gland cancer; throat cancer; and thyroid cancer;

Hematologic/blood cell cancers such as a leukemia (e.g., acute lymphoblastic leukemia including adult and childhood acute lymphoblastic leukemia; acute myeloid leukemia including adult and childhood acute myeloid leukemia; chronic lymphocytic leukemia; chronic myelogenous leukemia; and hairy cell leukemia); a lymphoma (e.g., AIDS-related lymphoma; cutaneous T-cell lymphoma; Hodgkin's lymphoma including adult and childhood Hodgkin's lymphoma and Hodgkin's lymphoma during pregnancy; non-Hodgkin's lymphoma including adult and childhood non-Hodgkin's lymphoma and non-Hodgkin's lymphoma during pregnancy; mycosis fungoides; Sezary syndrome; Waldenstrom's macroglobulinemia; and primary central nervous system lymphoma); and other hematologic cancers (e.g., chronic myeloproliferative disorders; multiple myeloma/plasma cell neoplasm; myelodysplastic syndromes; and myelodysplastic/myeloproliferative disorders);

Lung cancer such as non-small cell lung cancer; and small cell lung cancer;

Respiratory cancers such as adult malignant mesothelioma; childhood malignant mesothelioma; malignant thymoma; childhood thymoma; thymic carcinoma; bronchial adenomas/carcinoids including childhood bronchial adenomas/carcinoids; pleuropulmonary blastoma; non-small cell lung cancer; and small cell lung cancer;

Skin cancers such as Kaposi's sarcoma; Merkel cell carcinoma; melanoma; and childhood skin cancer; AIDS-related malignancies;

Other childhood cancers, unusual cancers of childhood and cancers of unknown primary site; and metastases of the aforementioned cancers can also be treated or prevented in accordance with the methods described herein.

The methods described herein may be suited to treat bladder, testicular, ovarian, head and neck, cervical, lung, mesothelioma, esophageal, melanoma, brain tumor, neuroblastoma, colorectal, Wilms' tumor, retinoblastoma, breast, endometrial, adrenocortical, anal, biliary tract,

carcinoid tumors, choriocarcinoma, gastric, liver cancer, non-Hodgkin's lymphoma, osteosarcoma, soft-tissue sarcomas, penile, malignant thymoma, anaplastic thyroid cancer, rhabdoid tumor of the kidney, advanced medullary thyroid cancer, carcinoid, mesothelioma, bone, gliomas or prostate cancers. In embodiments, the methods suitably treat bladder cancer (e.g., muscle-invasive bladder carcinoma, advanced or metastatic bladder carcinoma), testicular cancer (e.g., nonseminomatous testicular carcinoma, disseminated seminoma testis or extragonadal germ-cell tumors), ovarian cancer (e.g., ovarian epithelial cancer or ovarian germcell tumors), head and neck cancer (e.g., squamous cell carcinoma), cervical cancer (e.g., invasive, metastatic or recurrent cervical cancer), lung cancer (e.g., small cell lung cancer or non-small cell lung cancer), Wilms' tumor, brain tumors (e.g., gliomas, medulloblastoma or germ cell tumors), neuroblastoma, retinoblastoma, mesothelioma (e.g., malignant pleural mesothelioma), esophageal cancer (e.g., localized or advanced esophageal cancer), and colorectal cancer.

Modes of Administration

Methods of treatment may include any number of modes of administering a disclosed composition. Modes of administration may include tablets, pills, dragees, hard and soft gel capsules, granules, pellets, aqueous, lipid, oily or other solutions, emulsions such as oil-in-water emulsions, liposomes, aqueous or oily suspensions, syrups, elixirs, solid emulsions, solid dispersions or dispersible powders. For the preparation of pharmaceutical compositions for oral administration, the agent may be admixed with commonly known and used adjuvants and excipients such as for example, gum arabic, talcum, starch, sugars (such as, e.g., mannitose, methyl cellulose, lactose), gelatin, surface-active agents, magnesium stearate, aqueous or non-aqueous solvents, paraffin derivatives, cross-linking agents, dispersants, emulsifiers, lubricants, conserving agents, flavoring agents (e.g., ethereal oils), solubility enhancers (e.g., benzyl benzoate or benzyl alcohol) or bioavailability enhancers (e.g., Gelucire™). In the pharmaceutical composition, the agent may also be dispersed in a microparticle, e.g., a nanoparticulate composition.

For parenteral administration, the agent can be dissolved or suspended in a physiologically acceptable diluent, such as, e.g., water, buffer, oils with or without solubilizers, surface-active agents, dispersants or emulsifiers. As oils for example and without limitation, olive oil, peanut oil, cottonseed oil, soybean oil, castor oil and sesame oil may be used. More generally spoken, for parenteral administration, the agent can be in the form of an aqueous, lipid, oily or other kind of solution or suspension or even administered in the form of liposomes or nano-suspensions.

The term "parenterally," as used herein, refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

It will be apparent to one of ordinary skill in the relevant art that suitable modifications and adaptations to the compositions, formulations, methods, processes, and applications described herein can be made without departing from the scope of any embodiments or aspects thereof. The compositions and methods provided are exemplary and are not intended to limit the scope of any of the specified embodiments. All of the various embodiments, aspects, and options disclosed herein can be combined in any variations or iterations. The scope of the compositions, formulations, methods, and processes described herein include all actual or potential combinations of embodiments, aspects, options, examples, and preferences herein described. The exemplary compositions and formulations described herein may omit any component, substitute any component disclosed herein, or include any component disclosed elsewhere herein. The ratios of the mass of any component of any of the compositions or formulations disclosed herein to the mass of any other component in the formulation or to the total mass of the other components in the formulation are hereby disclosed as if they were expressly disclosed. Should the meaning of any terms in any of the patents or publications incorporated by reference conflict with the meaning of the terms used in this disclosure, the meanings of the terms or phrases in this disclosure are controlling. Furthermore, the foregoing discussion discloses and describes merely exemplary embodiments. All patents and publications cited herein are incorporated by reference herein for the specific teachings thereof.

Various embodiments and aspects of the inventions described herein are summarized by the following clauses:

Clause 1. A combination therapeutic comprising:

a therapeutically effective amount of a PROTAC pro-drug, or pharmaceutically acceptable salts or esters thereof,

wherein the PROTAC pro-drug comprises a ligand targeting a protein of interest, a PROTAC linker, an E3 ligase targeting ligand, and a transcyclooctene (TCO) moiety; and

a therapeutically effective amount of one or more compounds comprising a tetrazine (Tz), or pharmaceutically acceptable salts or esters thereof.

Clause 2. The combination therapeutic of clause 1, wherein the transcyclooctene (TCO) moiety is covalently attached to the E3 ligase target ligand.

Clause 3. The combination therapeutic of clauses 1 or 2, wherein the transcyclooxtene (TCO) moiety is covalently attached to the E3 ligase target ligand via a pro-drug linker.

Clause 4. The combination therapeutic of any one of clauses 1–3, wherein the PROTAC pro-drug comprises a structure:

$$X–L^1–Y,$$

wherein

X is the ligand targeting a protein of interest;

L^1 is the PROTAC linker; and

Y is the E3 ligase targeting ligand.

Clause 5. The combination therapeutic of any one of clauses 1–4, wherein the PROTAC pro-drug comprises a structure:

$$X–L^1–Y–L^2–Q,$$

wherein

X is the ligand targeting a protein of interest;

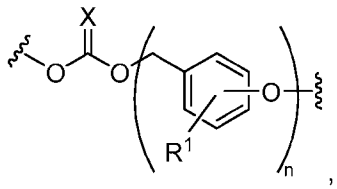
L^1 is the PROTAC linker;

Y is the E3 ligase targeting ligand;

L^2 is a pro-drug linker; and

Q is the transcyclooctene (TCO) moiety.

Clause 6. The combination therapeutic of any one of clauses 1–5, wherein the prodrug linker comprises a linker:



,

wherein

X is O, S, or N;

R^1 is hydrogen, halogen, $C_{1–6}$alkyl, $C_{1–6}$alkylene, $C_{1–6}$haloalkyl, cyano, $–OR^{1a}$, $–SR^{1a}$, $–CO_2R^{1a}$, $–C(O)R^{1a}$, $–SO_2R^{1b}$, $–N(R^{1b})_2$, $–CO_2N(R^{1b})_2$, $–NO_2$, or $–N(R\text{-}R^{1b})–OR^{1a}$; and

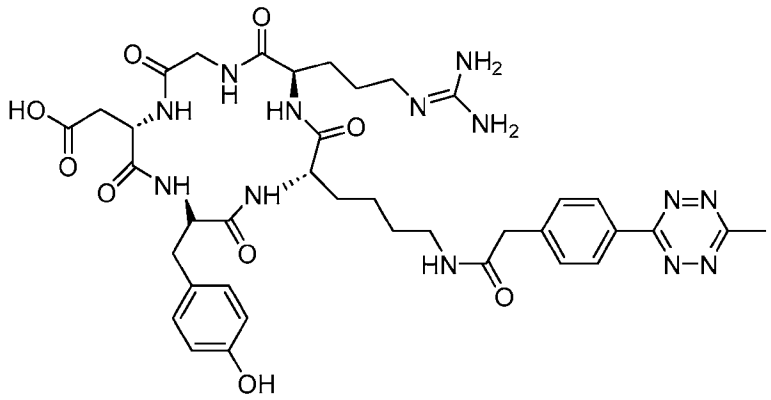
n = 0–1.

Clause 7. The combination therapeutic of any one of clauses 1–6, wherein the E3 ligase target ligand is a von Hippel-Lindau (VHL) protein targeting ligand.

Clause 8. The combination therapeutic of any one of clauses 1–7, wherein the von Hippel-Lindau (VHL) protein targeting ligand comprises VH032, VH032-OH, (S,R,S)-AHPC-Me, pharmaceutically acceptable salts thereof, or derivatives thereof.

Clause 9. The combination therapeutic of any one of clauses 1–8, wherein the PROTAC pro-drug comprises MS-99, ARV-771, DT2216, TD-004, ARCC-4, VZ-185, ERD-308, BI-0319, SJFα, UNC-6852, DAT8, pharmaceutically acceptable salts thereof, or derivatives thereof.

Clause 10. The combination therapeutic of any one of clauses 1–9, wherein the one or more compounds comprises a tetrazine (Tz) selected from:



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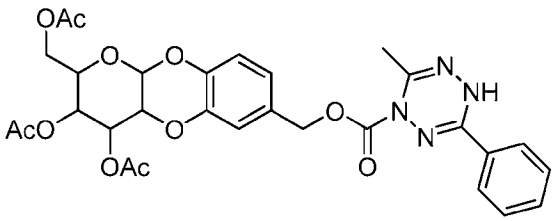

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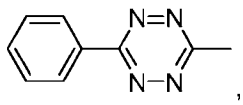

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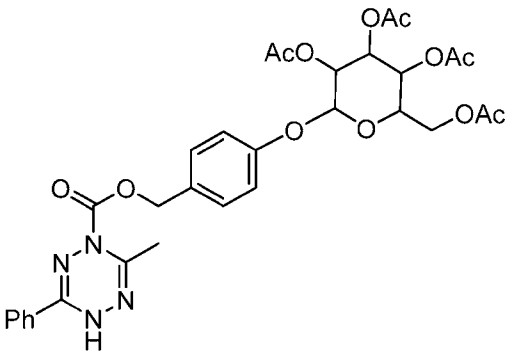

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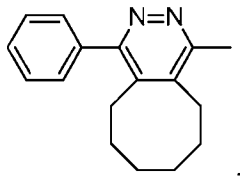

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Clause 11. The combination therapeutic of any one of clauses 1–10, wherein the therapeutically effective amount of the PROTAC pro-drug is 1–200 mg/kg.

Clause 12. The combination therapeutic of any one of clauses 1–11, wherein the therapeutically effective amount of the one or more compound comprising a tetrazine (Tz) is 1–200 mg/kg.

Clause 13. A method for treating a disease or disorder, the method comprising:

sequentially or simultaneously administering to a subject in need thereof:

a therapeutically effective amount of a PROTAC pro-drug, or pharmaceutically acceptable salts or esters thereof,

wherein the PROTAC pro-drug comprises a ligand targeting a protein of interest, a PROTAC linker, an E3 ligase targeting ligand, and a transcyclooctene (TCO) moiety; and

a therapeutically effective amount of one or more compounds comprising a tetrazine (Tz), or pharmaceutically acceptable salts or esters thereof; and

repeating administration until the disease or disorder is treated, ameliorated, or symptoms are reduced.

Clause 14. The method of clause 13, wherein the disease or disorder is a cancer, neurodegenerative disease, aging, autoimmune diseases, or viral infections.

Clause 15. The method of clauses 13 or 14, wherein the cancer comprises a breast cancer, melanoma, glioblastoma multiforme, colon cancer, prostate cancer, acute myeloid leukemia, or acute lymphoblastic leukemia and their corresponding senescent cells.

Clause 16. The method of any one of clauses 13–15, administering comprises intraperitoneal injection, intramuscular injection, subcutaneous injection, intravenous injection, intrathecal infusion, oral administration, or a combination thereof.

Clause 17. The method of any one of clauses 13–16, wherein an amount of a PROTAC increases and an amount of PROTAC pro-drug decreases.

Clause 18. The method of any one of clauses 13–17, wherein the PROTAC pro-drug and one or more antineoplastic agents reduce an amount of a target protein.

Clause 19. The method of any one of clauses 13–18, wherein the therapeutically effective amount of the PROTAC pro-drug is added at a period of time prior to addition of the therapeutically effective amount of the one or more compounds comprising a tetrazine (Tz).

Clause 20. The method of any one of clauses 13–19, wherein the period of time is 0–168 hours.

Clause 21. Use of the PROTAC pro-drug and the one or more compounds comprising a tetrazine (Tz), or pharmaceutically acceptable salts or esters thereof, of any one of clauses 1–20 as a medicament for the treatment of cancer in subject in need thereof.

Clause 22. A kit comprising:

one or more PROTAC pro-drugs, or pharmaceutically acceptable salts or esters thereof, of any one of clauses 1–20;

one or more compounds comprising a tetrazine (Tz), or pharmaceutically acceptable salts or esters thereof, of any one of clauses1–20;

optionally a device or means for administering the PROTAC pro-drug and one or more compounds comprising a tetrazine (Tz);

optionally tamper resistant packaging; and

optionally, a label or instructions for use thereof.

Clause 23. A combination therapeutic comprising:

a therapeutically effective amount of a PROTAC pro-drug, or pharmaceutically acceptable salts or esters thereof,

wherein the PROTAC pro-drug comprises a ligand targeting a protein of interest, a PROTAC linker, an E3 ligase targeting ligand, and a galactose, or derivative thereof; and

a therapeutically effective amount of one or more antineoplastic agents, or pharmaceutically acceptable salts or esters thereof.

Clause 24. The combination therapeutic of clauses 23, wherein the galactose, or derivative thereof, is covalently attached to the E3 ligase target ligand.

Clause 25. The combination therapeutic of any one of clauses 23 or 24, wherein the galactose, or derivative thereof is covalently attached to the E3 ligase target ligand via a pro-drug linker.

Clause 26. The combination therapeutic of any one of clauses 23–25, wherein the PROTAC pro-drug comprises a structure:

$$X–L^1–Y,$$

wherein

X is the ligand targeting a protein of interest;

L^1 is the PROTAC linker; and

Y is the E3 ligase targeting ligand.

Clause 27. The combination therapeutic of any one of clauses 23–26, wherein the PROTAC pro-drug comprises a structure:

$$X–L^1–Y–L^2–Q,$$

wherein

X is the ligand targeting a protein of interest;

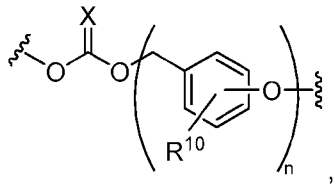
L^1 is the PROTAC linker;

Y is the E3 ligase targeting ligand;

L^2 is a pro-drug linker; and

Q is galactose or a derivative thereof.

Clause 28. The combination therapeutic of any one of clauses 23–27, wherein the pro-drug linker has a structure:



,

wherein

X is O, S, or N;

R^{10} is hydrogen, halogen, C_1–$_6$alkyl, C_1–$_6$alkylene, C_1–$_6$haloalkyl, cyano, $–OR^{1a}$, $–SR^{1a}$, $–CO_2R^{1a}$, $–C(O)R^{1a}$, $–SO2R^{1b}$, $–N(R^{1b})_2$, $–CO_2N(R^{1b})_2$, $–NO_2$, or $–N(R\text{-}R^{1b})–OR^{1a}$; and

n = 0–1.

Clause 29. The combination therapeutic of any one of clauses 23–28, wherein the E3 ligase target ligand is a von Hippel-Lindau (VHL) protein targeting ligand.

Clause 30. The combination therapeutic of any one of clauses 23–29, wherein the von Hippel-Lindau (VHL) protein targeting ligand comprises VH032, VH032-OH, (S,R,S)-AHPC-Me, pharmaceutically acceptable salts thereof, or derivatives thereof.

Clause 31. The combination therapeutic of any one of clauses 23–30, wherein the PROTAC pro-drug comprises MS-99, ARV-771, DT2216, TD-004, ARCC-4, VZ-185, ERD-308, BI-0319, SJFα, UNC-6852, DAT8, pharmaceutically acceptable salts thereof, or derivatives thereof.

Clause 32. The combination therapeutic of any one of clauses 23–31, wherein the one or more antineoplastic agents comprise DNA damaging agents, chemical toxic agents, or radiation.

Clause 33. The combination therapeutic of any one of clauses 23–32, wherein the DNA damaging agents comprise etoposide, camptothecin, doxorubicin, pharmaceutically acceptable salts thereof, or derivatives thereof.

Clause 34. The combination therapeutic of any one of clauses 23–33, wherein the chemical toxic agents comprise taxol, pharmaceutically acceptable salts thereof, or derivatives thereof.

Clause 35. The combination therapeutic of any one of clauses 23–34, wherein the galactose is a β-galactose.

Clause 36. The combination therapeutic of any one of clauses 23–35, wherein the therapeutically effective amount of the PROTAC pro-drug is 1–200 mg/kg.

Clause 37. The combination therapeutic of any one of clauses 23–36, wherein the therapeutically effective amount of the one or more antineoplastic agent is 1–200 mg/kg.

Clause 38. A method for treating a disease or disorder, the method comprising:

sequentially or simultaneously administering to a subject in need thereof

a therapeutically effective amount of a PROTAC pro-drug, or pharmaceutically acceptable salts or esters thereof,

wherein the PROTAC pro-drug comprises a ligand targeting a protein of interest, a PROTAC linker, an E3 ligase targeting ligand, and galactose, or a derivative thereof; and

a therapeutically effective amount of one or more antineoplastic agents, or pharmaceutically acceptable salts or esters thereof; and

repeating administration until the disease or disorder is treated, ameliorated, or symptoms are reduced.

Clause 39. The method of clause 38, wherein the disease or disorder is a cancer, neurodegenerative disease, aging, or viral infections.

Clause 40. The method of clauses 38 or 39, wherein the cancer comprises a breast cancer, melanoma, glioblastoma multiforme, colon cancer, prostate cancer, acute myeloid leukemia, or acute lymphoblastic leukemia and their corresponding senescent cells.

Clause 41. The method of any one of clauses 38–40, wherein administering comprises intraperitoneal injection, intramuscular injection, subcutaneous injection, intravenous injection, intrathecal infusion, oral administration, or a combination thereof.

Clause 42. The method of any one of clauses 38–41, wherein an amount of a PROTAC increases and an amount of PROTAC pro-drug decreases.

Clause 43. The method of any one of clauses 38–42, wherein the PROTAC pro-drug and the one or more antineoplastic agents reduce an amount of a target protein.

Clause 44. The method of any one of clauses 38–43, wherein the PROTAC pro-drug and the one or more antineoplastic agents target senescent cells.

Clause 45. The method of any one of clauses 38–44, wherein the therapeutically effective amount of the antineoplastic agents is added to induce cellular senesce.

Clause 46. The method of any one of clauses 38–45, wherein the therapeutically effective amount of the antineoplastic agents is added at a period of time prior to addition of the therapeutically effective amount of the PROTAC pro-drug.

Clause 47. The method of any one of clauses 38–46, wherein the period of time is 0–168 hours.

Clause 48. Use of the PROTAC pro-drug and the one or more antineoplastic agents, or pharmaceutically acceptable salts or esters thereof, of any one of clauses 23–47 as a medicament for the treatment of cancer in subject in need thereof.

Clause 49. A kit comprising:

one or more PROTAC pro-drugs, or pharmaceutically acceptable salts or esters thereof, of claim of any one of clauses 23–47;

one or more antineoplastic agents, or pharmaceutically acceptable salts or esters thereof, of any one of clauses 23–47;

optionally a device or means for administering the PROTAC pro-drug and the one or more antineoplastic agents;

optionally tamper resistant packaging; and

optionally, a label or instructions for use thereof.

EXAMPLES

Example 1

Materials

Common materials or chemical reagents were purchased from commercial sources and used without further purification. The solvents were used by dry solvents system. All reactions were monitored by TLC or LC-MS. Purification was conducted on preparative flash column chromatography and preparative reversed-phase high performance liquid chromatography (RP-HPLC) with solvent systems specified. Nuclear magnetic resonance (NMR) spectra were recorded on automated Bruker AVIII-500 instruments. High-resolution mass spectra (HRMS) were recorded on a Bruker microTOF II instrument in positive ion mode using an Agilent G1969 API-TOF with an electrospray ionization (ESI) source. Ultraperformance liquid chromatography (UPLC) spectra for compounds were acquired using a Shimadzu LabSolutions system. Anti-BRD4 antibody (catalog no. 13440S) was purchased from Cell Signaling Technology. Anti-BRD4 antibody (catalog no. PA5-41550) was purchased from Thermo Fisher. Anti-GAPDH (catalog no.

M7815), and Anti-Rabbit antibodies (catalog no. MFCD00162788) were purchased from Millipore Sigma. Anti-mouse IgG antibody (catalog no. A28177), and Anti-Bcl-XL antibody (catalog no. 66020-1-IG) were purchased from the Thermo Fisher. All primary antibodies were used at a 1:1000 dilution in 5% non-fat milk in Phosphate-buffered saline with 0.1% Tween-20 (PBST) buffer for western blot assay. All secondary antibodies were used at a 1:2000 dilution in 5% non-fat milk in Phosphate-buffered saline with 0.1% Tween-20 (PBST) buffer for western blot assay.

Materials

Common materials or chemical reagents were purchased from commercial sources and used without further purification. The solvents were used by dry solvents system. All reactions were monitored by TLC or LC-MS. Purification was conducted on preparative flash column chromatography and preparative reversed-phase high performance liquid chromatography (RP-HPLC) with solvent systems specified. Nuclear magnetic resonance (NMR) spectra were recorded on automated Bruker AVIII-500 instruments. High-resolution mass spectra (HRMS) were recorded on a Bruker microTOF II instrument in positive ion mode using an Agilent G1969 API-TOF with an electrospray ionization (ESI) source. Analytical HPLC chromatography was performed on a Shimadzu LC-20AD UFLC equipped with a 4.6 mm C18 reverse-phase column. All separations involved mobile phase of 0.1% TFA (v/v) in water (solvent A) and 0.1% trifluoroacetic acid (TFA) (v/v) in acetonitrile (ACN) (solvent B).

Anti-BRD4 antibody (catalog no. 13440S) was purchased from Cell Signaling Technology. Anti-GAPDH (catalog no. M7815), and Anti-Rabbit antibodies (catalog no. MFCD00162788) were purchased from Millipore Sigma. Anti-mouse IgG antibody (catalog no. A28177) was purchased from the Thermo Fisher. All primary antibodies were used at a 1:1000 dilution in 5% non-fat milk in Phosphate-buffered saline with 0.1% Tween-20 (PBST) buffer for western blot assay. All secondary antibodies were used at a 1:2000 dilution in 5% non-fat milk in phosphate-buffered saline with 0.1% Tween-20 (PBST) buffer for western blot assay. β-Galactosidase (β-Gal) from Escherichia coli (catalog no. 9031-11-2) and Esterase from porcine liver (catalog no. 9016-18-6) were purchased from Millipore Sigma.

Materials

Common materials or chemical reagents were purchased from commercial sources and used without further purification. The solvents were used by dry solvents system. All reactions were monitored by thin layer chromatography (TLC) or liquid chromatography-mass spectrometry (LC-MS). Purification was conducted on preparative flash column chromatography and

preparative reversed-phase high performance liquid chromatography (RP-HPLC) with solvent systems specified. Nuclear magnetic resonance (NMR) spectra were recorded on automated Bruker AVIII-500 instruments. High-resolution mass spectra (HRMS) were recorded on a Bruker microTOF II instrument in positive ion mode using an Agilent G1969 API-TOF with an electrospray ionization (ESI) source. Ultraperformance liquid chromatography (UPLC) spectra for compounds were acquired using a Shimadzu LabSolutions system. Anti-BRD4 antibody (catalog no. 13440S) and anti-ALK antibody (catalog no. 3633T) were purchased from Cell Signaling Technology. Anti-GAPDH (catalog no. M7815), and Anti-Rabbit antibodies (catalog no. MFCD00162788) were purchased from Millipore Sigma. Anti-mouse IgG antibody (catalog no. A28177) was purchased from the Thermo Fisher. All primary antibodies were used at a 1:1000 dilution in 5% non-fat milk in Phosphate-buffered saline with 0.1% Tween-20 (PBST) buffer for western blot assay. All secondary antibodies were used at a 1:2000 dilution in 5% non-fat milk in Phosphate-buffered saline with 0.1% Tween-20 (PBST) buffer for western blot assay. β-Galactosidase from Escherichia coli (catalog no. 9031-11-2) and Esterase from porcine liver (catalog no. 9016-18-6) were purchased from Millipore Sigma.

Stability Studies

The stability assay of c(RGDyK)-Tz, TCO-ARV-771 and ARV-771 were performed using UFLC on a Shimadzu after incubating in PBS at 37 °C for 16 h. Gradient elution was performed from 10% acetonitrile to 100% acetonitrile in 10 mM ammonium bicarbonate pH 9.4 over 30 min.

Cell Culture

Human fibroblast (Hs-27), Hela, U87-MG, and A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (GIBCO, catalog no. 10437) and 1% penicillin/streptomycin (Thermo Fisher, catalog no.30-002-CI). The cells were grown at 37 °C with 5% CO_2.

Cell Imaging

The cells were seeded in the 6-well plate and allowed to growth overnight. After the treatments, the cells were fixed with 4% formaldehyde solution for 30 mins at room temperature and washed 3 times with PBS. Then the cells were treated with 1× DAPI and 5 μM TCO-Cy5 (Click chemistry tools, catalog no. 1089-5) for 10 mins at room temperature. After the 3 times PBS washing, the cells were observed and imaged by using the ImageXpress Micro 4 High-Content Imaging System (Molecular Devices).

Cell Imaging in Response to Gal-Tz

The cells were seeded in the 6-well plate and allowed to growth overnight. After the pre-treatments (etoposide-induced senescence), the cells were treated with 3 μM Gal-Tz. After the 3 h treatment, the media were removed and washed. Then, the cells were treated with 1 μM tHCA for 30 mins. After the treatment, the cells were fixed with 4% formaldehyde solution for 30 min at room temperature and washed 3 times with PBS. Then the cells were treated with 1X Hoechst 33342 for 15 min at room temperature. After another 3 times PBS washing, the cells were observed and imaged by using the ImageXpress Micro 4 High-Content Imaging System (Molecular Devices).

Cell Viability Assay

Tumor cells were plated in 96-well plates with 6.0×10^3 cells in each well and subsequently incubated for 24 h in a moist atmosphere of 5% CO_2 and 37 °C. Then, different concentrations of test compounds or vehicles were added to triplicate wells. After incubation for an additional 72 h, 10 μL CCK-8 solution (Dojindo Molecular Technologies, catalog no. CK04-11) was added to each well, then the plates were incubated for 2–5 h at 37 °C.The absorbance was read at 450 nm on a Microplate Reader. The values of IC_{50} were calculated by the Logit method with the GraphPad software.

Immunoblot Assay

Cells were lysed in RIPA buffer supplemented with protease inhibitors. The lysates (40-60 μg protein) were then resolved by 4–12% Mini Protein Gel (Thermo Fisher) at 70 V for 10 mins and 200 V for 25 mins. Then the proteins were transferred from the gel to PVDF membrane (Bio-Rad) at 20 V for 120 mins. The membrane was incubated with primary antibody at 4 °C overnight, washed 3 times with PBST, incubated secondary antibody in 5% nonfat milk for 60 mins at room temperature and then washed 3 times with PBST. The membranes were detected under the SuperSignal West Atto Ultimate Sensitivity Chemiluminescent Substrate (catalog no. A38555).

Apoptosis Assay

Hela cells or senescent HeLa cells were placed in 6-well transparent plates and then treated with test compounds and vehicle in a moist atmosphere of 5% CO_2 at 37 °C for 24–48 h. After that, the cells were washed twice with cold Cell staining Buffer (BioLegend, catalog no. 420201-BL) twice, and then were resuspended in Binding buffer (BioLegend, catalog no. 640914).

5 µL FITC Annexin V (BioLegend, catalog no. 640914) and 10 µL Propidium Iodide Solution (BioLegend, catalog no. 640914) were added to the resuspended cell solution, which was then incubated for 15 min at room temperature in the dark. The analysis of stained cells was performed by a flow cytometer (BD FACS Canto II).

Proteomics Analyses

In-gel Digestion

Boiled clarified whole cell lysate (35 µg) was separated by 10% SDS-PAGE and stained with Bio-Safe Coomassie G-250 Stain (#1610786; Biorad, Hercules, CA). Each lane of the SDS-PAGE gel was cut into a single slice corresponding to the size BRD4 migrates on an SDS-PAGE gel. The gel slices were subjected to trypsin digestion and the resulting peptides were purified by C18-based desalting exactly as previously described.

Mass Spectrometry and Database Search

HPLC-ESI-MS/MS was performed in positive ion mode on a Thermo Scientific Orbitrap Fusion Lumos tribrid mass spectrometer fitted with an EASY-Spray Source (Thermo Scientific, San Jose, CA). NanoLC was performed as previously described. Tandem mass spectra were extracted from Xcalibur 'RAW' files and charge states were assigned using the ProteoWizard 2.1.x msConvert script using the default parameters. The fragment mass spectra were searched against the SwissProt_2022 database (Homo sapiens, 20402 entries) using Mascot (Matrix Science, London, UK; version 2.8.0.1) using the default probability cut-off score. The search variables that were used were: 10 ppm mass tolerance for precursor ion masses and 0.5 Da for product ion masses; digestion with trypsin; a maximum of two missed tryptic cleavages; variable modifications of oxidation of methionine and phosphorylation of serine, threonine, and tyrosine. Cross-correlation of Mascot search results with X! Tandem was accomplished with Scaffold (version Scaffold_5.1.2; Proteome Software, Portland, OR, USA). Probability assessment of peptide assignments and protein identifications were made using Scaffold. Only peptides with ≥ 95% probability were considered.

Label-free Peptide/Protein Quantification and Identification

Progenesis QI for proteomics software (version 2.4, Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK) was used to perform ion-intensity based label-free quantification as previously described. In brief, in an automated format, raw files were imported and converted into two-dimensional maps (*y*-axis = time, *x*-axis = *m/z*) followed by selection of a reference run for

alignment purposes. An aggregate data set containing all peak information from all samples was created from the aligned runs, which was then further narrowed down by selecting only +2, +3, and +4 charged ions for further analysis. The samples were then grouped and a peak list of fragment ion spectra from only the top eight most intense precursors of a feature was exported in Mascot generic file (.mgf) format and searched against the SwissProt_2022 database (*Homo sapiens*, 20402 entries) using Mascot (Matrix Science, London, UK; version 2.8.0.1). The search variables that were used were: 10 ppm mass tolerance for precursor ion masses and 0.5 Da for product ion masses; digestion with trypsin; a maximum of two missed tryptic cleavages; variable modifications of oxidation of methionine and phosphorylation of serine, threonine, and tyrosine; 13C=1. The resulting Mascot .xml file was then imported into Progenesis, allowing for peptide/protein assignment, while peptides with a Mascot Ion Score of <25 were not considered for further analysis. Protein quantification was performed using only non-conflicting peptides and precursor ion-abundance values were normalized in a run to those in a reference run (not necessarily the same as the alignment reference run). Unbiased hierarchal clustering analysis (heat map) was performed in Perseus.

UFLC analysis of release ability of Gal-Tz

Gal-Tz (1 mM) was incubated in PBS (pH = 7.4, 37 °C), with (10 U/mL) β-gal (Millipore Sigma, catalog no. 9031-11-2), and (20 U/mL) esterase (Millipore Sigma, catalog no. 9016-18-6) monitored by UFLC at 2 h. After preparation, the samples were collected and passed through a 0.22 μm filter, and 10 μL of each sample was loaded onto UFLC. An UV/vis detector was used to monitor the products at 254 nm.

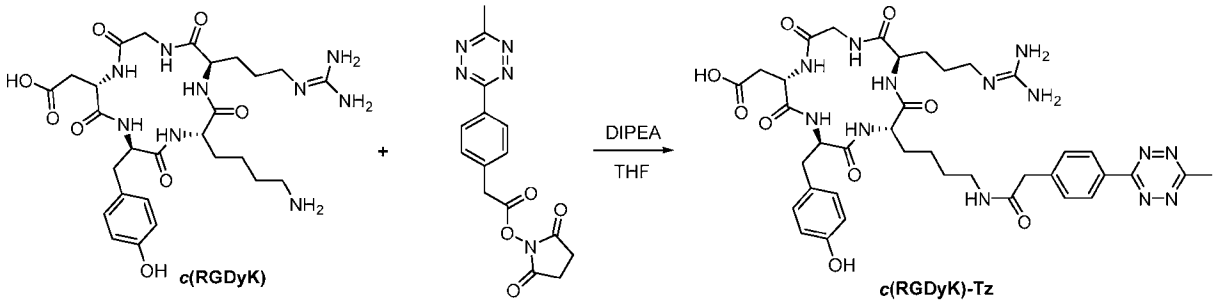
Senescence-associated β-galactosidase (SA- β-Gal) staining

Briefly, A549 or HeLa were treated with 5 μM etoposide for 3 days. Then the cells were incubated with fresh medium for another day. After the treatment, the cells were washed with PBS twice, fixed with 4% paraformaldehyde for 15 min, and then incubated for overnight at 37 °C with the SA-β-Gal staining solution of the Senescence β-Galactosidase Staining Kit (Cell signaling Technology, catalog no. 9860S) according to the manufacturer's instructions. The cell images were taken by the ECHO Microscopes.

Example 2

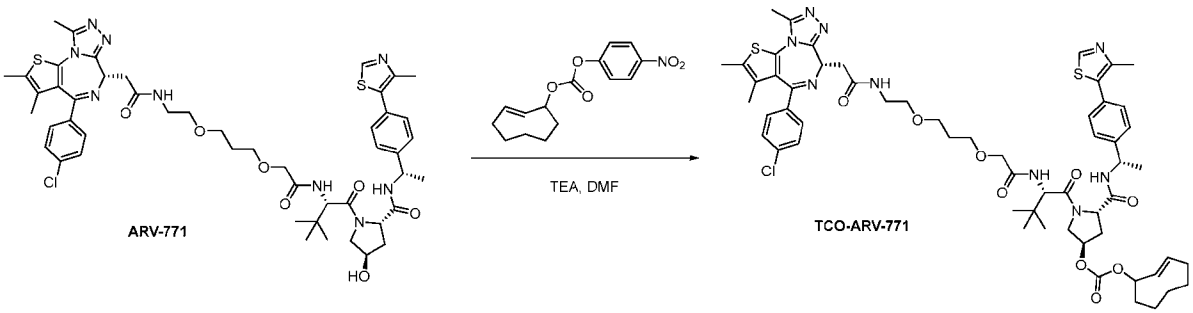
Synthesis and Characterization of c(RGDyK)-Tz, TCO-ARV-771 and TCO-DT2216

Scheme S1. Example Synthesis of Example c(RGDyK)-Tz



c(RGDyK)-Tz. (35.00 mg, 0.0565 mmol), which was synthesized by solid-phase synthesis, was added to 4-methyltetrazine-NHS ester (27.00 mg, 0.0825 mmol) as a solution in anhydrous dimethylformamide (800mL). DIPEA (32 µL) was added, and the reaction was allowed to stir at room temperature for 24 h. The solution was concentrated by rotary evaporation and purified by RP HPLC to yield 14.3 mg (0.017 mmol, 30%) as a pink solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.29 (s, 1H), 9.17 (s, 1H), 8.42 (d, J = 7.9 Hz, 2H), 8.18 (t, J = 5.7 Hz, 1H), 8.10 (d, J = 8.5 Hz, 1H), 8.03 (d, J = 7.2 Hz, 1H), 7.94 (d, J = 7.2 Hz, 1H), 7.59 (d, J = 7.9 Hz, 1H), 7.55 (d, J = 8.1 Hz, 2H), 7.46 (t, J = 6.0 Hz, 1H), 6.92 (d, J = 8.1 Hz, 2H), 6.63 (d, J = 8.1 Hz, 2H), 4.68–4.57 (m, 1H), 4.40–4.31 (m, 1H), 4.19–4.10 (m, 1H), 4.07–4.00 (m, 1H), 3.95–3.87 (m, 1H), 3.56 (s, 2H), 3.24 (dd, J = 14.9, 3.8 Hz, 2H), 3.10–3.05 (m, 2H), 3.00 (s, 3H), 2.81–2.66 (m, 4H), 2.42–2.34 (m, 2H), 1.76–1.66 (m, 2H), 1.61–1.48 (m, 2H), 1.39–1.29 (m, J = 6.4, 5.9 Hz, 4H), 1.13–1.04 (m, 2H).

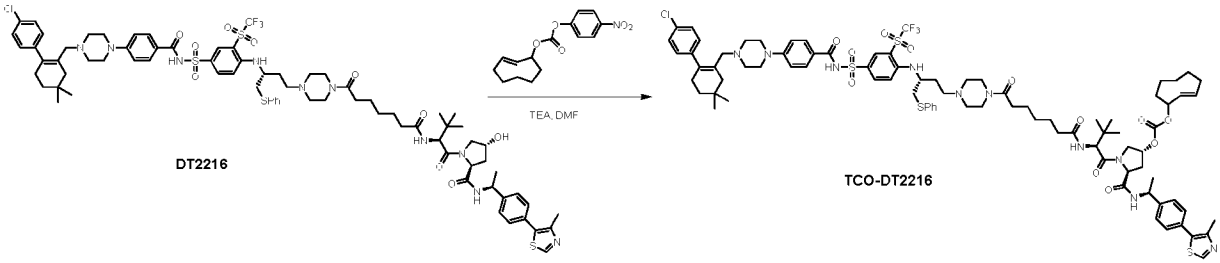
Scheme S2. Example Synthesis of Example PROTAC Pro-drug TCO-ARV-771



TCO-ARV-771. TCO-Ns (12 mg, 0.04 mmol) was dissolved in DMF (5 mL). Triethylamine (5.6 µL, 0.04 mmol) was added, followed by ARV-771 hydrochloride (20 mg, 0.02 mmol). The mixture was stirred in the dark at room temperature for 3 d. The solvent was removed under high vacuum and the residue was by prep-RP-HPLC using methanol and water as the eluent. The product fraction was lyophilized to yield TCO-ARV771 (4.5 mg, 19 % yield). ^{1}H NMR (500 MHz, methanol-

d_4) δ 8.99 (s, 1H), 7.50–7.37 (m, 8H), 5.83–5.71 (m, 1H), 5.58–5.47 (m, 1H), 5.29–5.21 (m, 2H), 5.04–4.96 (m, 1H), 4.70–4.55 (m, 3H), 4.23–4.11 (m, 1H), 4.04–3.85 (m, 3H), 3.69–3.56 (m, 7H), 3.51–3.42 (m, 3H), 2.72 (s, 3H), 2.50–2.40 (m, 8H), 2.18–1.79 (m, 7H), 1.77–1.67 (m, 4H), 1.66–1.55 (m, 2H), 1.53–1.43 (m, 4H), 1.08–1.03 (m, 10H), 0.92–0.82 (m, 2H).

Scheme S3. Example Synthesis of Example PROTAC Pro-drug TCO-DT2216



TCO-DT2216. TCO-Ns (18 mg, 0.06 mmol) was dissolved in DMF (5 mL). Triethylamine (14 μL, 0.1 mmol) was added, followed by addition of DT2216 (46 mg, 0.03 mmol), which was synthesized by following literature procedure. The mixture was stirred in the dark at room temperature for 3 d. The solvent was removed under high vacuum and the residue was by prep-RP-HPLC using methanol and water as the eluent. The product fraction was lyophilized to yield TCO-DT2216 (13 mg, 26 % yield). ^{1}H NMR (800 MHz, Chloroform-*d*) δ 8.73 (s, 1H), 8.35 (d, J = 2.0 Hz, 1H), 8.12 (dd, J = 9.1, 2.2 Hz, 1H), 7.71 (d, J = 8.7 Hz, 2H), 7.42–7.31 (m, 11H), 7.31–7.27 (m, 2H), 7.25–7.22 (m, 1H), 7.01–6.98 (m, 2H), 6.78–6.74 (m, 2H), 6.55 (dd, J = 9.7, 1.6 Hz, 1H), 6.18 (dd, J = 9.3, 3.2 Hz, 1H), 5.85–5.76 (m, 1H), 5.52–5.46 (m, 1H), 5.28–5.24 (m, 1H), 5.08–5.04 (m, 1H), 4.72–4.68 (m, 1H), 4.64–4.61 (m, 1H), 4.17–4.12 (m, 1H), 4.07 (t, J = 6.1 Hz, 2H), 3.92–3.88 (m, 1H), 3.87–3.81 (m, 2H), 3.13–3.09 (m, 2H), 2.99–2.95 (m, 4H), 2.70–2.64 (m, 3H), 2.52–2.51 (m, 3H), 2.47–2.42 (m, 2H), 2.35–2.31 (m, 3H), 2.29 (t, J = 7.5 Hz, 2H), 2.26–2.21 (m, 2H), 2.19–2.15 (m, 3H), 2.12–2.08 (m, 3H), 2.03–1.97 (m, 2H), 1.96–1.93 (m, 1H), 1.87–1.82 (m, 2H), 1.79 (t, J = 8.2 Hz, 2H), 1.70–1.66 (m, 3H), 1.62–1.58 (m, 4H), 1.51–1.48 (m, 3H), 1.48–1.46 (m, 3H), 1.35–1.28 (m, 6H), 1.03–1.00 (m, 9H), 0.99 (s, 6H).

Design and Synthesis of TCO-ARV-771 Prodrugs and αvβ3 Integrin Targeted c(RGDyK)-Tz and Imaging Study of c(RGDyK)-Tz

Although TCO-tetrazine click release strategy has been used for on-demand activation and delivery of TCO caging cytotoxic agents, the application for PROTACs has not been documented. It was thought that incorporation of the TCO moiety into PROTACs could generate

the first bioorthogonal PROATC prodrugs. Critically, the caging should abolish the degradation activity of the PROTAC. Moreover, to ensure that the TCO-PROTAC design is generally applicable, a TCO group was added onto a widely used E3 ubiquitin ligase VHL ligand. For VHL-derived PROTACs, the hydroxyl group in the VHL ligand is critical for the recruitment of VHL E3 ubiquitin ligase. Accordingly, a TCO caged PROTAC molecule was developed by incorporating TCO via carbonate bond onto the hydroxyl group of a well-studied VHL-based bromodomain (BRD) degrader, ARV-771 (FIG. 1B). Molecular docking studies of the TCO-ARV-771 PROTAC revealed that such modification dramatically decreased the interaction ability between the VHL ligand and the binding pocket in VHL (FIG. 1C and FIG. 2). It is expected that the TCO-ARV-771 should be inactive and stable in biological media, whereas it can be bioorthogonally activated by Tz to trigger release of ARV-771 degrader. In the reported Tz activated TCO-prodrugs, the Tz component is non-cancer cell selective. To achieve cancer cell selective activation, a new Tz was designed and conjugated with a αvβ3 integrin binding ligand c(RGDyK) (FIG. 1A–B). The newly designed c(RGDyK)-Tz and TCO-ARV-771 were efficiently synthesized, characterized, and used for the following biological studies (Scheme S1 and S2).

Click Release Study in Chemical System and Fluorescence Imaging of c(RGDyK)-Tz in αvβ3 Integrin Ex-Pressed Hela Cells

To demonstrate the click-release capability of the newly designed TCO-ARV-771 reaction with c(RGDyK)-Tz via IEDDA, the reaction was performed in pH 7.4 phosphate buffer. By real-time monitoring the process with LC-MS, TCO-ARV-771 was shown to be facilely converted to ARV-771 completely within 30 min upon the treatment of c(RGDyK)-Tz (FIG. 3A). The stability of the c(RGDyK)-Tz was also determined by the ultra-fast liquid chromatography (UFLC) and it was stable over 96 h (FIG. 3B).

The c(RGDyK)-Tz was designed to selectively deliver to cancer cell via cancer cell surface αvβ3 integrin. Fluorescence imaging studies were performed with Hela cells, which overexpresses αvβ3 integrin. c(RGDyK)-Tz (500 nM) was pre-incubated for 3 h in Hela cells, then fluorescence imaging probe TCO-Cy5 was added and incubated for 15 min before being subjected to high-content fluorescent imaging microscopy. The results revealed the c(RGDyK)-Tz group exhibited red color generated by Cy5 (FIG. 1D). In contrast, treatment with DMSO or the control c(RGDyK) ligand without Tz moiety did not exhibit fluorescence inside cells. These studies indicated that the c(RGDyK)-Tz could bind to αvβ3 integrin and got inside the cells via possible passive diffusion.

Degradation of BRD4 in Hela Cells by Bioorthogonal Activation of TCO-ARV-771 with c(RGDyK)-Tz

Having demonstrated c(RGDyK)-Tz capable of entering into HeLa cells, the click-release capacity of TCO-ARV-771 was tested in the cells. The TCO-ARV-771 did not exhibit the degradation ability for BRD4 in Hela cells from the concentration of 40–400 nM (FIG. 4A). However, when the cells was treated with c(RGDyK)-Tz, the degradation was observed (FIG. 4B). At a concentration of 0.4 μM TCO-ARV-771 used, c(RGDyK)-Tz elicited concentration-dependent degradation of BRD4 with complete degradation at 1.0 μM c(RGDyK)-Tz (FIG. 4B). Notably, comparable degradation activity was observed at the same concentration of TCO-ARV-771 prodrug (0.4 μM) and parent drug ARV-771 (FIG. 4B–C). This implies that the Tz-mediated click-releasing drug strategy was able to deliver and activate the active drug ARV-771 highly efficiently. Experiments were performed by varying the concentration of TCO-ARV-771 with 1.0 μM c(RGDyK)-Tz. Again, complete degradation could be achieved at 0.4 μM TCO-ARV-771 (FIG. 6D). These studies validated that that BRD4 degradation activity was dependent on the both TCO-ARV-771 and c(RGDyK)-Tz. Finally, cell viability was determined (FIG. 4E). TCO-ARV-771 prodrug and c(RGDyK)-Tz have much lower cell killing effects with IC_{50}: 4.45 μM and >10 μM, respectively in Hela cells. However, the crPROTAC treatment showed the similar cytotoxicity to the active ARV-771 group (IC_{50}: 389 nM vs 466 nM). When c(RGDyK) was used without the tetrazine activation moiety, this group did not show any degradation (FIG. 4F). Taken together, these results support that the crPROTAC efficiently activated and released the PROTAC in the αvβ3 integrin highly expressed Hela cells.

Degradation of BRD4 by ARV-771 Produced from Bioorthogonal Reaction of TCO-ARV-771 with c(RGDyK)-Tz via the Ubiquitin-Proteasome Pathway

It was thought that ARV-771 generated from the bioorthogonal reaction of TCO-ARV-771 with c(RGDyK)-Tz can induce degradation of BRD4 via ubiquitination and then proteasome degradation. To validate the degradation of the target protein depending on the ubiquitin pathway, experiments were conducted by incubation of 1 μM c(RGDyK)-Tz in Hela cells for 3 h, washed with PBS, and then treated with TCO-ARV-771 at various concentrations and ARV-771 and DMSO as control for 16 h. The degradation activity between TCO-ARV-771, ARV-771 and DMSO was compared. The results revealed that when the concentration of TCO-ARV-771 was 0.4 μM, the BRD4 protein was degraded efficiently at a similar level to that with ARV-771, while no degradation was observed with DMSO (FIG. 5A). When the proteasome inhibitor MG-132 was added, the BRD4 protein was not degraded due to the inhibition of proteasome (FIG. 5B). Further-

more, the Hela cells was treated with the free VHL ligand and TCO-ARV-771, degradation activity was reduced significantly because the free VHL ligand competed with the generated ARV-771 for ubiquitination (FIG. 5C). In the control, co-treatment c(RGDyK) peptide without tetrazine functional group with TCO-ARV-771 did not induce BRD4 degradation (FIG. 5D). The studies supported that the resulting ARV-771 exhibited the same degradation mechanism as ARV-771 via the ubiquitin-proteasome pathway.

Selective Degradation of BRD4 in αvβ3 Integrin Highly Expressed Cancer Cells

The bioorthogonal on-demand and on-target strategy is designed to selectively activate TCO-ARV-771 prodrugs in the αvβ3 integrin overexpressed cancer cells to generate active PROATC ARV-771 for degradation of BRD4 proteins. To demonstrate the selectivity, PROATC ARV-771 was applied to U87 cancer cell lines, which overexpress αvβ3 integrin. In parallel, low αvβ3 expressing level of normal fibroblast HS-27 cells was used as the control group. A similar protocol used in Hela cells was em-ployed in U87 and HS-27. When the U87 cell was incubated with 0.4 μM TCO-ARV-771 and 1.0 μM c(RGDyK)-Tz, a comparable degradation activity for BRD4 was observed to the cell treated with 0.4 μM ARV-771 (FIG. 6A). The results are also consistent with the studies in Hela cells. Without TCO-ARV-771 or 1.0 μM c(RGDyK)-Tz, no degradation was found. However, the normal HS27 cells did not show degradation activity when they were treated with 0.4 μM TCO-ARV-771 and 1.0 μM c(RGDyK)-Tz. This suggests that c(RGDyK)-Tz was not able to selectively de-liver into HS27 cells due to the lack of αvβ3 integrin, which is believed to play the roles in targeting and drug transport (FIG. 6B). The effective targeted delivery and activation of ARV-771 from TCO-ARV-771 in U87 cells enabled inducing potent toxicity with IC_{50} 667 nM, which is compa-rable to the cells, which were treated with ARV-771 (IC_{50} 345 nM, FIG. 6C). The activity is similar to that observed in Hela cells as well. In contrast, significant reduced cyto-toxicity (IC_{50}: 7.58 μM) was observed in the HS27 cells (FIG. 6D). Taken together, these results revealed that the TCO-tetrazine prodrug strategy can bioorthogonally and selectively target and activate prodrugs in the αvβ3 integrin-positive cancer cells.

Bioorthogonal Reaction of TCO-ARV-771 and c(RGDyK)-Tz Induces Apoptosis In Hela Cells

The bioorthogonal trigger release of ARV-771 from the reaction of TCO-ARV-771 and c(RGDyK)-Tz induced BRD4 degradation and subsequent cell apoptosis. Flow cytometric analyses were performed to evaluate the anti-tumor ability of the bioorthogonal activation strategy. Treatment only with 0.4 μM TCO-ARV-771 showed low toxic effect on Hela cells (FIG. 7A). However, 13.58% apoptosis was induced by the reaction of TCO-ARV-771 (0.4 μM) with 1

μM c(RGDyK)-Tz group. A similar apoptosis level was also observed with ARV-771 control group (FIG. 7A–B).

Proteomic Analysis Showing crPROTAC Selectively Degrades BRD4 Protein

To determine the selective degradation effect on BRD4 protein by the bioorthogonal activation and drug release strategy, the proteome of Hela cells was examined. A quantitative multiplexed approach was employed to measure the cellular protein levels in Hela cells, which were treated with TCO-ARV-771 or crPROTAC (TCO-ARV-771 + c(RGDyK)-Tz) and (FIG. 8). Compared to the TCO-ARV-771 groups, the crPROTAC groups resulted in an increase of 120 proteins and a decrease of 328 proteins (FIG. 9). The results (FIG. 8A) showed that the level of target protein, BRD4, decreased significantly in the crPROTAC groups. Moreover, changes of up/down-stream proteins were detected that may be associated with BRD4. For example, the expression level of BCOR protein decreased. BCOR is an interacting corepressor of BCL-6 that enhances BCL-6-mediated transcriptional repression in cancers. Furthermore, the thioredoxin-interacting protein (TXNIP) increased under the treatment of CRPTC. TXNIP, a member of the alpha-arrestin family, prevents proliferation by inducing the apoptosis signal-regulating kinase 1 enzyme (ASK1). The changes of these tumor-related proteins suggest that there is a potential correlation between BRD4 and BCOR or TXNIP proteins. The significant changes of the protein level are listed in FIG. 8B and FIG. 10. This information can be used to understand the degradation mechanism of BRD4.

crPROTACT of CO-DT2216 and c(RGDyK)-Tz Induces Bcl-XL Degradation In Hela Cells

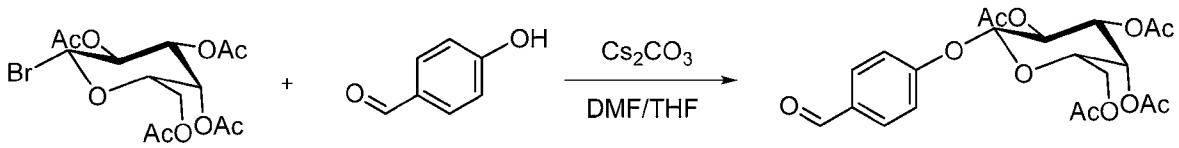
To examine whether the TCO-Tz mediated bioorthogonal strategy can also be applied to other VHL-based PROTACs, the TCO-DT2216 prodrug was designed and synthesized (FIG. 11A). It is known that DT2216 can degrade the Bcl-XL protein in Hela cells. TCO-DT2216 was synthesized in a similar man-ner to TCO-ARV-771 (Scheme S3). The degradation effect of TCO-DT2216 on Bcl-XL was tested in Hela cells. The resulted DT2216 from the reaction of TCO-DT2216 with c(RGDyK)-Tz degraded Bcl-XL as efficiently as DT2216 when the cells were pretreated with c(RGDyK)-Tz (FIG. 11B). However, no degradation activity was observed with c(RGDyK) group, which cannot trigger release of DT2216. Experiments confirmed that the produced DT2216 degraded the Bcl-XL protein via the ubiquitin-proteasome pathway. The presence of the VHL ligand (FIG. 11C) and proteasome inhibitor MG-132 (FIG. 11D) blocked the degradation activity significantly.

A bioorthogonal on-demand prodrug strategy was developed for the precise control of PROTAC's on-target degradation activity in a cancer cell selective manner. Different from conventional prodrugs for PROTACs using cancer biomarkers or tumor microenvironment for drug delivery and activation, the bioorthogonal on-demand approach enables on-target activation and release of PROTACs at a tumor site and thus minimize premature drug activation. Inactive PROTAC prodrugs TCO-ARV-771 and TCO-DT2216 were selectively activated in cancer cells ver-sus noncancerous normal cells by the cancer cell targeted RGD peptide c(RGDyK)-Tz. The PROTAC ARV-771 and DT2216 were selectively delivered into cancers and exhibited their degradation activities. Further studies of crPROTACs will be pursued to evaluate their efficiency in vivo. This strategy can serve as a general platform by conjugating different target ligands such as folate and applying to all VHL-recruiting PROTACs to achieve selectively degrade POIs in cancer cells and to minimize.

Example 2

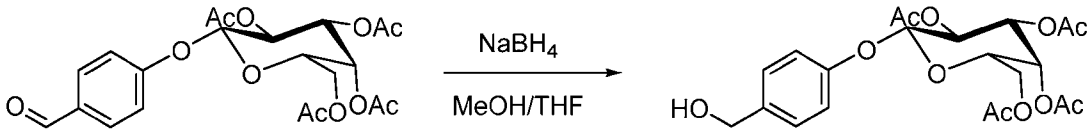
Example Synthesis and Characterization of Gal-Tz.

Scheme S4. Example Synthesis of Galactose Derivatized Benzaldehyde



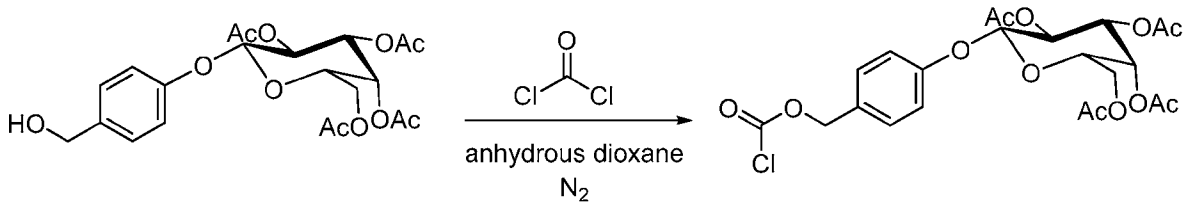
(2*R*,3*S*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-(4-formylphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate. Cesium carbonate (2.70 g, 8.29 mmol) was added to DMF/THF (1/9, 10 mL). To the mixture, DMF/THF (1/9, 7 mL) containing compound 4-hydroxybenzaldehyde (250 mg, 2.04 mmol) was dropped for 10 minutes. In addition, compound (2*R*,3*S*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-bromotetrahydro-2H-pyran-3,4,5-triyl triacetate (999 mg, 2.44 mmol) dissolved in DMF/THF (1/9, 7 mL) was dropped to the mixture for 10 minutes. After stirring at room temperature for 24 hours, the mixture was washed with saturated NaHCO3 aqueous solution (3x) and brine (1x), and the organic phase was dried with sodium sulfate and evaporated. The residue was purified by flash chromatography (n-hexane/EtOAc = 4/6) to obtain product as a thick white gel (283 mg, yield 43%). ^{1}H NMR (400 MHz, $CDCl_3$): δ. 9.93 (s, 1H), 7.86 (d, 2H, J = 8.7 Hz), 7.12 (d, 2H, J = 8.7 Hz), 5.52 (dd, 1H, J = 7.8, 10.5 Hz), 5.50 (d, 1H, J = 3.2 Hz), 5.16 (d, 1H, J = 7.8 Hz), 5.14 (dd, 1H, J = 3.2, 10.5 Hz), 4.11-4.26 (m, 3H), 2.20 (s, 3H), 2.08 (s, 6H), 2.01 (s, 3H).

Scheme S5. Example Synthesis of Aldehyde Reduction to Hydroxyl



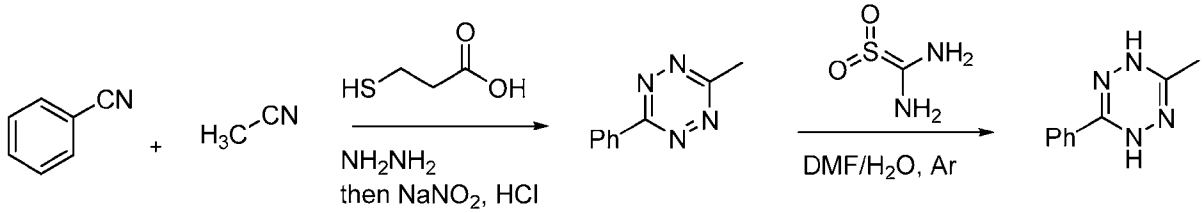
(2*R*,3*S*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-(4-(hydroxymethyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate. (2*R*,3*S*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-(4-formylphenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (226 mg, 0.5 mmol) was added to MeOH/THF (1/1, 10 mL). To the mixture, NaBH4 (28.4 mg, 0.75mmol) was added in 3 batches at 0 °C. After stirring at 0 °C for 3 hours, water (10 mL) and EtOAc (10 mL) were added to the mixture. And crude products were extracted with EtOAc (10 mL) for 3 times. The organic phase was dried with sodium sulfate and evaporated. The residue was purified by flash chromatography (n-hexane/EtOAc = 2/8) to obtain compound 4 as a white solid (204 mg, yield 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20 (s, 2H), 6.91 (d, J = 7.2 Hz, 2H), 5.38 (d, J = 9.9 Hz, 2H), 5.07–4.90 (m, 2H), 4.53 (s, 2H), 4.21–3.94 (m, 3H), 2.14 (d, J = 5.6 Hz, 3H), 2.03–1.85 (m, 10H).

Scheme S6. Example Synthesis of Chloroformate



(2*R*,3*S*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-(4 (((chlorocarbonyl)oxy)methyl)phenoxy) tetrahydro-2H-pyran-3,4,5-triyl triacetate. A solution of (2*R*,3*S*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-(4-(hydroxymethyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (180 mg, 0.4 mmol) in anhydrous dioxane (2 mL) was treated with phosgene (20% in toluene, 1.4 mL, 2 mmol), and the reaction was stirred for 20 h under an N_2 atmosphere at 21 °C. The mixture was concentrated in vacuo, redissolved, and concentrated with toluene (2×) to afford the crude compound as a clear oil. The crude product was used directly for next step without purification.

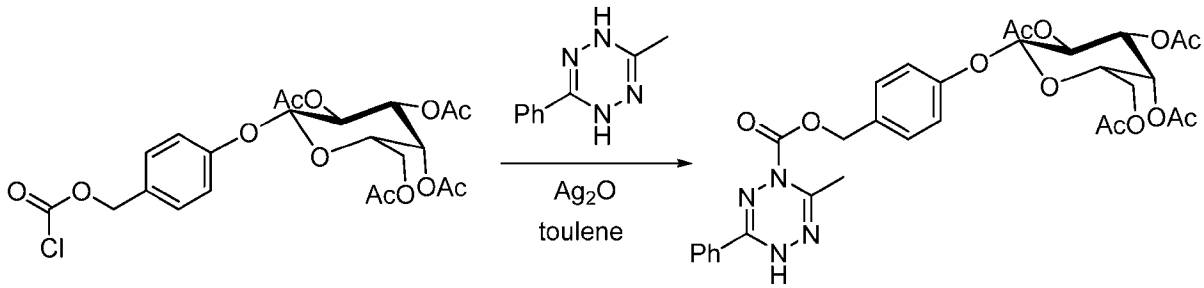
Scheme S7. Example Synthesis of Example Tetrazine



3-methyl-6-phenyl-1,2,4,5-tetrazine. A mixture of benzonitrile (6 mmol), acetonitrile (24 mmol), and 3-mercaptopropionic acid (264 µL, 3 mmol) was cooled to 0 °C under argon. Anhydrous hydrazine (4.6 mL, 96 mmol) was added dropwise to the mixture. The reaction mixture was stirred in an oil bath at 50 °C for 24 hours. Upon completion, the reaction solution was cooled with ice water. A solution of sodium nitrite in ice water was slowly added into the reaction mixture, followed by slow addition of 1M HCl. Addition of 1M HCl continued until gas evolution ceased. Then, the reaction mixture was extracted with CH_2Cl_2 and washed with a saturated solution of NaCl in water. The extract was combined, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using 30%–50% CH_2Cl_2/Hexane as the eluents yielding the compound tetrazine as a pink solid.

3-methyl-6-phenyl-1,4-dihydro-1,2,4,5-tetrazine. In a sealed flask, the reductant thiourea dioxide (1.5 mmol, 1.5 eq) was added to a solution of tetrazine (1.0 mmol) in 7.5 mL of DMF/H_2O (v/v = 10/1) at room temperature under argon. The reaction mixture was stirred in an oil bath at 95 °C for 1–2 hours. Upon completion, the color of the reaction mixture changed from pink to light yellow. Under argon, 20 mL of EtOAc was added the reaction mixture, which was washed by 3 mL of H_2O. The extract was combined and concentrated by reduced pressure. The residue was purified by column chromatography on silica gel under argon using CH_2Cl_2 to 4% MeOH/CH_2Cl_2 as the eluents yielding the desired dihydrotetrazine. ^{1}H NMR (400 MHz, chloroform-D) δ 8.02 (d, J = 6.9 Hz, 2H), 7.49 (d, J = 6.3 Hz, 3H), 2.61 (s, 3H).

Scheme S8. Example Synthesis of Example Gal-Tz



(2*R*,3*S*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-(4-(((6-methyl-3-phenyl-1,4-dihydro-1,2,4,5-tetrazine-1-carbonyl)oxy)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate. (2*R*,3*S*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-(4 (((chlorocarbonyl)oxy)methyl)phenoxy) tetrahydro-2H-pyran-3,4,5-triyl triacetate (0.4 mmol) and 3-methyl-6-phenyl-1,4-dihydro-1,2,4,5-tetrazine (0.6 mmol) were mixed with 7 mL anhydrous toluene in a over-heated flask with stirring bar, followed by addition of Ag_2O (0.7 mmol). The reaction mixture was stirred under reflux for 1h. Upon

completion, the reaction mixture was filtered and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using 60%-80% Hexane/EtOAc as the eluents yielding Gal-Tz (0.12 mmol, 30%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.71–7.63 (m, 2H), 7.53–7.48 (m, 1H), 7.45–7.42 (m, 2H), 7.39–7.36 (m, 3H), 6.99–6.96 (m, 2H), 5.49–5.42 (m, 3H), 5.26 (s, 2H), 5.09 (dd, J = 10.4, 3.4 Hz, 1H), 5.04–5.01 (m, 1H), 4.20 (dd, J = 11.3, 7.0 Hz, 1H), 4.16–4.11 (m, 1H), 4.10 (d, J = 7.1 Hz, 1H), 4.06–4.02 (m, 1H), 2.29 (s, 3H), 2.05–2.01 (m, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.2, 170.4, 170.2, 170.1, 169.4, 157.0, 154.9, 151.0, 131.7, 130.8, 130.1 (2C), 129.0 (2C), 128.9, 126.5 (2C), 117.0, 99.6, 71.1, 70.8, 68.6, 67.6, 66.9, 61.4, 60.4, 20.74, 20.67, 20.6, 18.2, 14.2.

Design, Synthesis, and Trigger Release Studies of Gal-Tz

To design a SA-β-gal sensitive tetrazine derived molecule, the incorporation of the galactose (Gal) moiety into dihydrotetrazine via a 4-(hydroxymethyl)phenol linker would form a stable Gal caged Gal-Tz conjugate was investigated. Such a chemical modification could prevent the oxidation of dihydrotetrazine to tetrazine and thus abolishing the ligation activity (FIG.12A). Moreover, the Gal modified structure can serve as a recognition moiety for SA-β-gal. The 2-in-1 Gal-Tz can sense senescent biomarker SA-β-gal and control tetrazine activity. In SA-β-gal overexpressed senescent cells, the acetyl and the glycosidic bond can be cleaved to trigger generation of tetrazine. The resulting tetrazine then reacts with TCO-caged prodrugs to click and release drugs. The designed conjugate Gal-Tz was synthesized in 4 steps from 1-bromo-2, 3, 4, 6-tetraacyl-D-galactoside. The stability of Gal-Tz in pH 7.4 PBS solution was tested. It was stable for over 24 h (FIG. 12B). The cell viability of both Gal-Tz and Tz in senescent HeLa cells was evaluated. The results indicated that Gal-Tz and Tz exhibit low toxicity up to 10 mM (FIG. 12C). Due to the high stability and low toxicity of Gal-Tz the following biological studies were performed. Gal-Tz was designed to respond to SA-β-gal and esterase to generate PhTz. The critical click-release experiments with Gal-Tz were conducted accordingly. The compound was co-incubated with porcine liver esterase (E) and β-galactosidase (β-Gal) in pH 7.4 PBS at 37 °C for 2 h. UFLC (ultra-fast liquid chromatography) analysis revealed that Gal-Tz was smoothly converted to Tz (FIG. 12D).

Fluorescence Imaging of SA-β-Gal Activated Tetrazine Release in Senescent Cells

Gal-Tz was designed to be selectively activated in senescent cells to release active tetrazine. Fluorescence imaging studies were conducted in senescent HeLa cells to examine the ability of Gal-Tz to selectively activate senescent cells. Senescent HeLa cells were generated by

low-dose treatment with etoposide (eto). Quantification of senescence activity revealed that etoposide induced an enhancement in the activity of the broadly used biomarker for senescent cells, SA-β-gal (FIG.13). A TCO caged fluorophore HCA probe TCO-HCA (tHCA) was designed and synthesized for monitoring the release of Tz (FIG.14A). tHCA alone was non-fluorescent; howevever, when the released Tz reacted with tHCA in pH 7.4 PBS a IR (red) fluorescence was produced (FIG. 14B). Cellular imaging studies were subsequently performed without and with pre-incubation of Gal-Tz (3 μM) for 3 h. In these studies, the senescent HeLa (s-HeLa) and non-senescent n-HeLa cells were washed three times with PBS and then treated with TCO-HCA (1 μM) for another 30 min. The PBS washed cells were imaged by fluorescence microscope. The results revealed that the Gal-Tz treated s-HeLa cells exhibited a red color associated with HCA (FIG.15), but no fluorescence was seen in the absence of Gal-Tz (FIG. 15B). In contrast, n-HeLa cells did not exhibit fluorescence (FIG. 15A). In control, red fluorescence was observed with s- and n-HeLa cells when they were treated with HCA (FIG. 15B–C) while no fluorescence was showed for n-HeLa cells with tHCA (FIG. 15C). These studies showed that designed Gal-Tz can cross cell membrane and respond to senescent HeLa cells selectively to control release of the tetrazine.

Click and Release Studies of Doxorubicin in Senescent Cancer Cells

Having demonstrated that the Tz can be released from Gal-Tz in senescent cells, the capacity of targeted activation and delivery of senolytics for the treatment of senescent cells was tested. In the first study, the bioorthogonal activation and release system using TCO-caged doxorubicin (Dox) as a model was tested. SA-β-gal sensitive Gal-Tz in senescent cells appears to trigger click-release of chemotherapeutic Dox from TCO-Dox to induce apoptosis (FIG. 16). At first, the drug release system between free tetrazine (Tz) and TCO-Dox was tested. The UFLC was applied to monitor the release process in which Dox was formed from the incubation of TCO-Dox with Tz in pH 7.4 PBS buffer. The results suggested the release reaction finished within 30 min (FIG. 17). The inverse electron demand Diels-Alder (IEDDA) reaction takes place rapidly in association with k_{obs} = 2.8 × 10^{-3} (FIG. 18). Senolytic effect studies in etoposide-induced senescent HeLa (s-HeLa) cells and non-senescent HeLa (n-HeLa) as control were conducted. Treatment of both n-HeLa and s-Hela cells with Dox led to similar killing effects with IC_{50} = 463 and 255 nM, respectively (FIG.19A–B). However, significantly reduced activity (IC_{50} = 5.72 μM) was observed with TCO-Dox + Gal-Tz group in n-HeLa cells (FIG. 20A) and TCO-Dox in s-HeLa cells (IC_{50} = 4.88 μM), whereas the same group exhibited comparable senolytic activity with IC50 = 348 nM to that of Dox (IC_{50} = 255 nM) in s-HeLa cells (FIG. 20B). The senolytic index between

n-HeLa and s-HeLa cells for the TCO-Dox + Gal-Tz group was determined to be 16.44. Similar levels of cell viability in co-treatment groups were also observed in non-senescent and senescent A549 cells (FIG. 21A–B). Consistent with the results observed in the A549 cells, selective killing effect only occurred in senescent cells. These studies suggest that the bioorthogonal activation strategy as a general approach can be applicable to multiple tumor types. Furthermore, the studies also demonstrate that the Dox senolytic effect can be highly selectively controlled by the Gal-Tz mediated bioorthogonal chemistry.

Click and Release Studies of PROTAC ARV-771 in Senescent Cancer Cells

In recent years, proteolysis-targeting chimeras (PROTACs) have emerged as a new modality for the treatment of cancer and other diseases. Distinctly different from traditional small-molecule inhibitors that function by occupancy-driven pharmacology, PROTACs instead degrade their protein targets by hijacking the cellular ubiquitin-proteasome protein degradation pathway. Importantly, PROTACs offer the unique ability to catalytically and selectively induce protein degradation in a sub-stoichiometric fashion. These features would remove the need for high dosages, thus mitigating related toxicities and drug resistance associated with inhibitor based senolytics. The promising therapeutic modality stimulates provided motivation to explore it as new senolytics for the eradication of senescent cells.

To test the feasibility, a TCO group caged PROTAC ARV-771 (TCO-ARV-771), which can degrade BRD4 protein, was designed. The TCO group was conjugated with the hydroxyl group of the VHL ligand in ARV-771 since such modification can abolish its degradation activity. It is believed that ARV-771 can be generated to restore its BRD4 degradation activity by reacting Gal-Tz with TCO-ARV-771 in senescent cells to induce cell death (FIG. 22). Indeed, comparable cytotoxicities between co-treatment Gal-Tz + TCO-ARV-771 group (IC_{50} = 722 nM) and group ARV-771 (IC_{50} = 540 nM) were observed in s-HeLa cells (FIG. 23A), while TCO-ARV-771 in the absence of Gal-Tz had significantly reduced activity (IC_{50} = 4.26 μM) (FIG. 23B). In comparison, Gal-Tz + TCO-ARV-771 co-treatment group did not induce significant cytotoxicity (IC_{50} = 3.15 μM) in the n-HeLa cells (FIG. 23B). In contrast, ARV-771 alone treatment led to the significant loss of cell viability (IC_{50} = 368 nM). Similar studies with non-senescent A549 (n-A539) and etoposide-induced senescent A549 cells (s-A549) were also conducted. Similar activities of co-treatment (Gal-Tz + TCO-ARV-771) (IC_{50} = 399 nM) and ARV-771 (IC_{50} = 419 nM) were observed in s-A549 cells (FIG. 24A). However, in n-A549 cells the co-treatment group induced toxicity was significantly lower than that of ARV-771 (IC_{50} = 629 nM) (FIG. 24B). The differences in cell viability between normal and senescent cancer cells revealed the selective treatment of senescent cells

could be achieved by bioorthogonal chemistry. These results also demonstrate that TCO-ARV-771 and ARV-771 induced apoptosis is independent of cell type. This property is in contrast to other types of senolytic drugs which have different activities in various senescent cell types due to the dynamic and highly heterogeneous nature of the senescence levels.

Furthermore, the degradation ability of Gal-Tz + TCO-ARV-771 in both n-HeLa and s-HeLa cells were tested. In the control experiments, ARV-771 (100 nM) degraded the BRD4 protein in both n-HeLa and s-HeLa cells (FIG. 25A). However, in n-HeLa cells, TCO-ARV-771 (100 nM) or TCO-ARV-771 (100 nM) with Gal-Tz (100 nM and 300 nM, 3 h pretreatment) treatments did not induce the degradation of BRD4 protein (FIG.25A). Only in s-HeLa cells, the co-treatment displayed the degradation activity, indicating that ARV-771 was generated from TCO-ARV-771 selectively in the senescent cells (FIG. 25A). Next, to validate the observed BRD4 degradation is dependent on the concentration and ubiquitin pathway, experiments were conducted with various concentrations of Gal-Tz and TCO-ARV-771 and in the presence of MG-132, a proteasome inhibitor. The results showed that MG-132 inhibits the degradation ability of Gal-Tz + TCO-ARV-771 (FIG.25B), indicating that the activity of TCO-ARV-771 + Gal-Tz was operated through the ubiquitin-proteasome pathway. Moreover,studies with s-A549 cells were also conducted. Similar degradation activities in s-A549 cells (FIG. 25C) suggested that designed TCO-PROTAC with Gal-Tz could degrade the target protein in different senescent cancer cells.

SA-β-gal activated click-release reaction induces degradation and apoptosis. To further understand the biological effects induced by Gal-Tz + TCO-ARV-771, flow cytometry assays were used to determine the apoptosis. In n-HeLa cells, ARV-771 (1 μM, FIG. 26E) induced a higher degree of apoptosis (19.16%) than control (2.48%, FIG. 26A), Gal-Tz (4.69%, FIG. 26B), TCO-ARV-771 (5.21%, FIG. 26C) and the Gal-Tz + TCO-ARV-771 co-treatment group (7.91%, FIG. 26D).

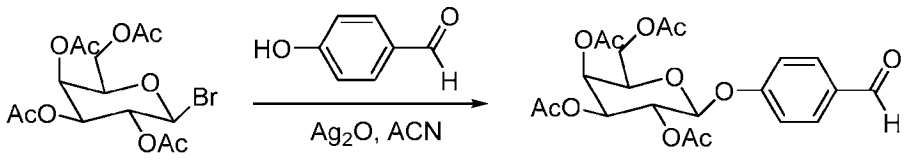
HeLa cells, both ARV-771 (FIG. 27E) and the co-treatment (FIG. 27D) groups exhibited similar higher apoptosis activities (53% and 49.7%). Moreover, proteomics assays were used to determine the BRD4 expression level between normal and senescent HeLa cells. A quantitative multiplexed approach was employed to measure cellular protein levels in both n-HeLa and s-HeLa cells, that were treated with TCO-ARV-771 (100 nM) and Gal-Tz (300 nM, 3 h pretreatment) overnight. The data shown in FIG. 28 indicated that the expressional level of BRD4 in s-HeLa is significantly lower than n-HeLa cells. Taken together, the studies further validate that the control release of ARV-771 in senescent cancer cells to degrade BRD4 is the mechanism for inducing senescent cell apoptosis.

In the described research above, a bioorthogonal senolytic strategy for selective elimination of senescent cells was developed. Engineering a galactose moiety into dihydrotetrazine creates a unique 2-in-1 Gal-Tz structure, which serves both as recognition moiety for SA-β-gal and a caging group for control of tetrazine activity. Unlike the free tetrazines, Gal-Tz enables control of tetrazine activity and can be selectively activated by SA-β-gal to in situ produce tetrazine for subsequent click-to-release reaction in senescent cells. This demonstrates that Gal-Tz can selectively activate TCO-caged fluorophore HCA for senescent cell imaging. Moreover, Gal-Tz may enable efficient click-to-release of Dox from TCO-Dox prodrug to eradicate senescent HeLa and A549 cells over non-senescent counterparts with 16.44 senolytic index. Furthermore, the strategy for selective activation and delivery of PROTACs as senolytics has been successfully leveraged and validated. PROTAC prodrug TCO-ARV-771 appears to be selectively activated by Gal-Tz and delivered into senescent HeLa and A549 cells to induce BRD4 degradation. Further studies of this methodology will be pursued to evaluate their in vivo efficiency of this protocol. This novel bioorthogonal approach will be generally applicable to the senescent treatment.

Example 3

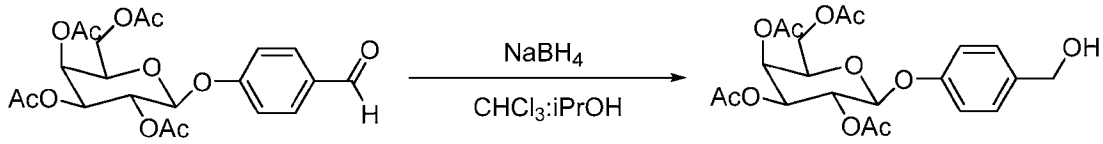
Example Synthesis of Galactose Modified PROTAC

Scheme S9.



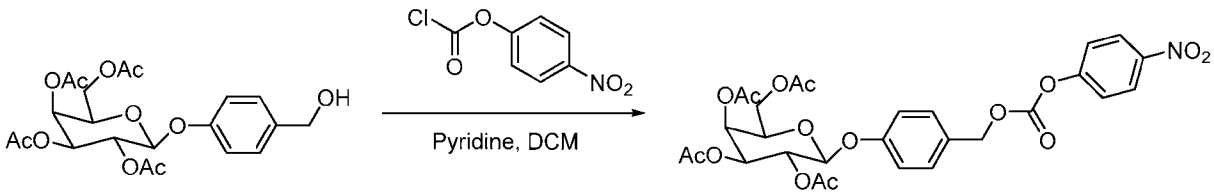
4-O-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl)-4-oxybenzaldehyde. To the mixture of α-D-galactopyranosyl Bromide (1.9 g, 4.63 mmol) and p-hydroxy benzaldehyde (622 mg, 5.09 mmol) in acetonitrile (49 ml) Ag2O (2.14 g, 9.26 mmol) was added at room temperature and stirred for 16 hours. After completion of the reaction, the reaction mixture was filtered through a celite pad and washed with ethyl acetate. The solvent was dried, and pure 1 (1.6 g, 76% was obtained after flash chromatography (SiO_2, 30 % EtOAc in Hexane). ^1H NMR (400 MHz, $CDCl_3$) δ 9.91 (s, 1H), 7.88–7.80 (m, 2H), 7.14–7.06 (m, 2H), 5.56–5.46 (m, 1H), 5.46 (s, 1H), 5.19–5.08 (m, 2H), 4.27–4.14 (m, 2H), 4.14–4.08 (m, 1H), 2.17–1.96 (m, 12H); 13C NMR (101 MHz, $CDCl_3$) δ 190.8, 170.4, 170.3, 170.2, 169.4, 161.4, 131.9, 116.8, 98.7, 71.4, 70.8, 68.5, 66.8, 61.4, 20.8, 20.8, 20.7, 20.7; LCMS (ESI-MH+) calcd for $C_{21}H_{27}O_{11}$: 455, found 455.

Scheme S10. Example Synthesis of Aldehyde to Hydroxyl Reduction



4-(hydroxymethyl)phenyl-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside). To the solution of 4-O-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl)-4-oxybenzaldehyde (500 mg, 1.1 mmol) in CHCl3/iPrOH (33 ml, 3:1) in ice-bath condition, was added $NaBH_4$ (88 mg, 2.32 mmol) in two portions. The reaction mixture was brought to room temperature and stirred for 3 hours, washed with 10% citric acid solution, saturated $NaHCO_3$, water, and brine. The mixture was filtered and dried over $MgSO_4$ and concentrated. Pure product (313 mg, 63% was obtained by flash chromatography (SiO_2, 50% EtOAc in Hexane) as a white solid. ^1H NMR (500 MHz, $CDCl_3$) δ 7.57–7.48 (m, 2H), 7.26–7.20 (m, 2H), 5.76–5.67 (m, 2H), 5.35 (dd, J = 10.4, 3.4 Hz, 1H), 5.27 (d, J = 7.9 Hz, 1H), 4.88 (s, 2H), 4.47 (dd, J = 11.3, 6.9 Hz, 1H), 4.40 (dd, J = 11.3, 6.4 Hz, 1H), 4.29 (td, J = 6.6, 1.2 Hz, 1H), 2.42 (s, 3H), 2.30 (d, J = 2.8 Hz, 6H), 2.25 (s, 3H); 13C NMR (126 MHz, CDCl3) δ 170.4, 170.3, 170.2, 169.4, 156.5, 135.9, 128.5, 117.1, 99.8, 71.0, 70.8, 68.7, 66.9, 64.8, 61.4, 20.7, 20.7, 20.6; LCMS (ESI-MH+) calcd for $C_{21}H_{27}O_{11}$: 455, found 455.

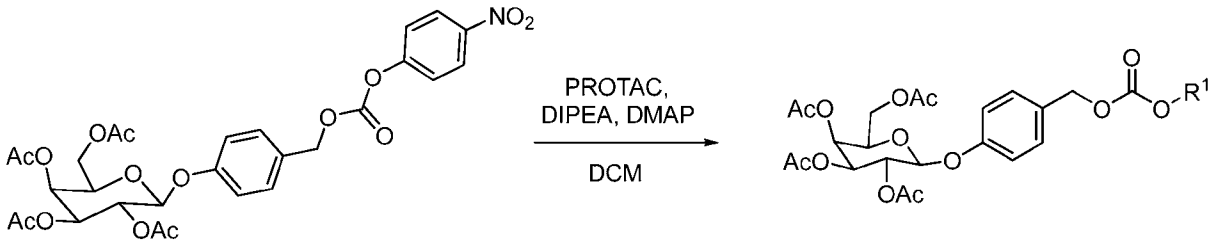
Scheme S11. Example Synthesis of Example Pro-drug Intermediate



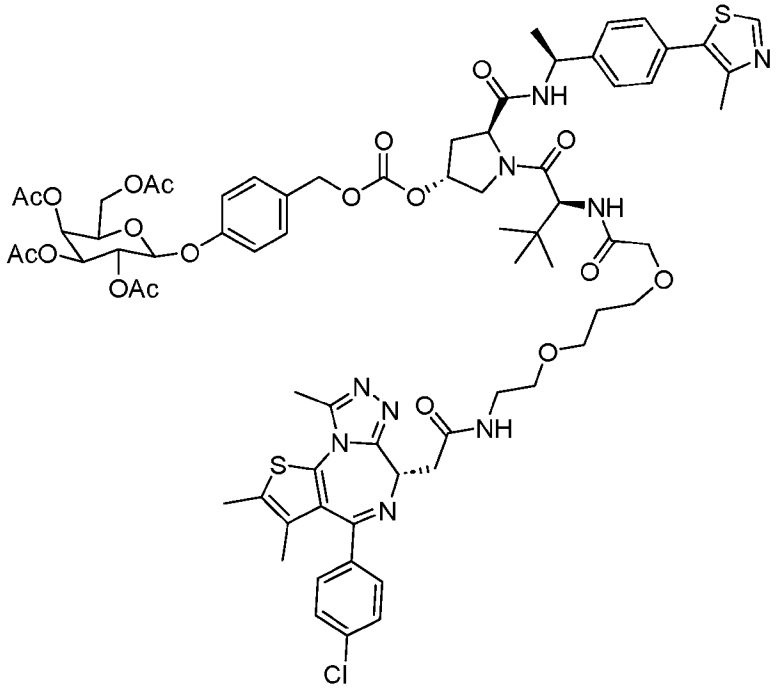
4-(4-nitro-phenoxycarbonyloxymethyl)phenyl]-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside). The mixture of 2 (193 mg, 0.424 mmol), p-nitro-phenyl chloroformate (257 mg, 1.27 mmol), and pyridine (103 µl) in DCM (10 mL) was stirred at room temperature under N_2 gas. Completion of the reaction was checked by TLC. After completion of the reaction, it was washed with water and extracted with ethyl acetate. The organic portion was dried and concentrated. Pure product (199 mg, 76%) was obtained by flash chromatography (SiO_2, 30-50 % EtOAc in Hexane) as a white solid. ^1H NMR (500 MHz, $CDCl_3$) δ 8.26 (d, J = 2.2 Hz, 1H), 8.25 (d, J = 2.2 Hz, 1H), 7.38 (d, J = 2.0 Hz, 1H), 7.37–7.35 (m, 2H), 7.34 (d, J = 2.2 Hz, 1H), 7.02 (d, J = 2.1 Hz, 1H), 7.01 (d, J = 2.1 Hz, 1H), 5.48 (dd, J = 10.5, 7.9 Hz, 1H), 5.45 (dd, J = 3.4, 1.1 Hz, 1H), 5.23 (s, 2H), 5.10 (dd, J = 10.4, 3.4 Hz, 1H), 5.05 (d, J = 7.9 Hz, 1H), 4.22 (dd, J = 11.3, 6.9 Hz, 1H), 4.14 (dd, J = 11.3, 6.4 Hz, 1H), 4.05 (td, J = 6.6, 1.2 Hz, 1H), 2.17 (s, 3H), 2.05 (d, J =

1.6 Hz, 6H), 2.00 (s, 3H); 13C NMR (101 MHz, CDCl3) δ 170.5, 170.3, 170.2, 169.5, 157.5, 155.6, 152.5, 130.7, 129.2, 125.4, 121.8, 117.2, 99.5, 71.2, 70.8, 70.6, 68.6, 66.9, 61.4, 20.8, 20.8, 20.7. HRMS (ESI-MNa+) calcd for $C_{28}H_{29}NO_{15}Na$: 642.1434, found 642.1429.

Scheme S12. Example Synthesis of Example PROTAC Galactose Pro-drug

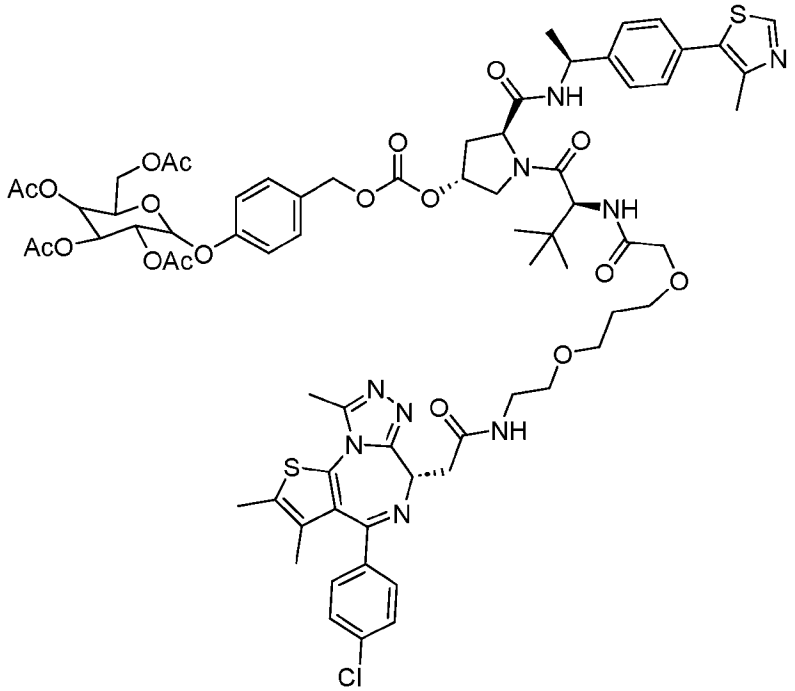


The solution of a PROTAC (25 mg, 0.0227 mmol) and 4-(4-nitro-phenoxycarbonyloxymethyl)phenyl]-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside) (17 mg, 0.0273 mmol) in DCM (10 mL) was stirred at room temperature in the presence of DIPEA (8 µl) and catalytic DMAP for 48 hours till completion. After completion, the solvent was dried and purified using prep HPLC to get off-white product 6 (22 mg, 63%).



(2*R*,3*S*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-(4-((((((3R,5S)-1-((*S*)-2-(5-(4-(4-((5-chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-isopropoxy-2-methylphenyl)piperidin-1-yl)-5-oxopentanamido)-3,3-dimethylbutanoyl)-5-(((S)-1-(4-(4-

methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-3-yl)oxy)carbonyl)oxy)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (Gal-MS99). ^{1}H NMR (500 MHz, MeOD) δ 8.85 (s, 1H), 8.42 (t, J = 8.7 Hz, 1H), 8.14 (d, J = 2.1 Hz, 1H), 7.91 (dt, J = 8.0, 2.0 Hz, 1H), 7.80 (d, J = 8.0 Hz, 1H), 7.65 (ddd, J = 8.6, 7.4, 1.6 Hz, 1H), 7.43–7.24 (m, 7H), 6.99 (t, J = 8.3 Hz, 2H), 6.81–6.72 (m, 1H), 5.42 (dd, J = 8.5, 3.5 Hz, 1H), 5.37–5.29 (m, 1H), 5.26–5.18 (m, 2H), 5.17–5.09 (m, 1H), 5.08–5.02 (m, 1H), 4.97 (dq, J = 9.9, 7.0 Hz, 1H), 4.68 (d, J = 13.3 Hz, 1H), 4.61–4.54 (m, 1H), 4.54–4.44 (m, 2H), 4.39–4.26 (m, 1H), 4.26–4.20 (m, 1H), 4.19–4.09 (m, 2H), 4.01 (d, J = 12.5 Hz, 1H), 3.85 (dt, J = 12.2, 4.4 Hz, 1H), 3.36–3.33 (m, 1H), 3.14 (dtd, J = 16.0, 13.2, 2.5 Hz, 1H), 3.00–2.89 (m, 1H), 2.74–2.64 (m, 1H), 2.45 (d, J = 2.0 Hz, 3H), 2.41–2.31 (m, 4H), 2.19–2.10 (m, 10H), 2.06–1.94 (m, 9H), 1.90 (q, J = 7.3 Hz, 2H), 1.84–1.70 (m, 2H), 1.46 (dd, J = 9.8, 7.0 Hz, 3H), 1.32–1.28 (m, 3H), 1.28–1.20 (m, 9H), 1.06 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.0, 171.9, 170.6, 170.4, 170.3, 169.6, 169.4, 160.5, 157.3, 157.2, 154.3, 152.4, 149.3, 144.6, 141.8, 136.2, 134.7, 131.7, 130.4, 129.8, 129.8, 128.3, 127.1, 126.7, 125.9, 125.7, 123.9, 117.1, 111.6, 105.7, 99.6, 76.2, 71.8, 71.2, 70.8, 69.7, 68.7, 66.9, 61.4, 58.9, 56.6, 53.9, 49.3, 38.4, 35.7, 35.2, 33.5, 33.0, 32.3, 29.7, 26.5, 22.1, 21.9, 20.8, 20.7, 20.7, 20.7, 18.8, 15.4, 14.0; HRMS (ESI-MH+) calcd for $C_{78}H_{97}ClN_9O_{20}S_2$: 1578.5979, found 1578.5972.



(2*R*,5*R*)-2-(acetoxymethyl)-6-(4-((((((3*R*,5*S*)-1-((S)-2-(tert-butyl)-15-((*S*)-4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)-4,14-dioxo-6,10-

dioxa-3,13-diazapentadecanoyl)-5-(((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-3-yl)oxy)carbonyl)oxy)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (Gal-ARV-771). ^{1}H NMR (500 MHz, DMSO) δ 8.99 (s, 1H), 8.46 (d, J = 7.6 Hz, 1H), 8.27 (t, J = 5.7 Hz, 1H), 7.48 (d, J = 8.7 Hz, 2H), 7.45–7.40 (m, 4H), 7.36 (dt, J = 7.9, 5.5 Hz, 5H), 7.02–6.95 (m, 2H), 5.48 (d, J = 7.8 Hz, 1H), 5.34 (d, J = 3.2 Hz, 1H), 5.28 (dd, J = 10.4, 3.5 Hz, 1H), 5.21 (dd, J = 10.3, 7.9 Hz, 1H), 5.17 (s, 1H), 5.09 (s, 2H), 4.92–4.87 (m, 1H), 4.55–4.49 (m, 1H), 4.44 (dt, J = 13.8, 8.4 Hz, 3H), 4.09 (d, J = 6.4 Hz, 2H), 3.99–3.93 (m, 1H), 3.91 (d, J = 2.9 Hz, 2H), 3.78 (dd, J = 11.9, 3.6 Hz, 1H), 3.53 (t, J = 6.5 Hz, 2H), 3.48 (t, J = 6.5 Hz, 2H), 3.42 (t, J = 5.8 Hz, 2H), 3.32–3.20 (m, 4H), 2.60 (s, 3H), 2.45 (s, 3H), 2.40 (s, 3H), 2.32 (dd, J = 13.8, 7.8 Hz, 1H), 2.14 (s, 3H), 2.03 (s, 3H), 2.00 (s, 4H), 1.94 (s, 3H), 1.78 (p, J = 5.5 Hz, 2H), 1.61 (s, 3H), 1.36 (d, J = 7.0 Hz, 3H), 0.94 (s, 9H). ^{13}C NMR (126 MHz, DMSO) δ 184.9, 170.0, 169.8, 169.6, 169.2, 169.1, 168.7, 163.1, 158.4, 158.1, 156.6, 155.1, 153.7, 151.5, 149.9, 147.7, 144.6, 136.7, 135.3, 132.2, 131.1, 130.3, 130.2, 129.9, 129.7, 129.6, 128.9, 128.5, 126.3, 116.3, 97.6, 76.8, 70.4, 70.1, 69.4, 68.9, 68.7, 68.3, 68.0, 67.2, 67.0, 61.3, 58.0, 55.9, 53.8, 47.9, 40.1, 38.6, 37.4, 35.3, 34.6, 29.4, 26.1, 22.5, 20.5, 20.4, 20.4, 20.3, 15.9, 14.0, 12.7, 11.3.

Design, Synthesis and Trigger Release Studies of Gal-ARV-771

The fundamental design strategy was based upon on the premise that incorporation of the SA-β-gal sensitive galactose (Gal) moiety into PROTACs would form Gal-PROTAC prodrugs, in which proper caging of a key moiety in the PROTAC would abolish its degradation activity. In addition, proper placement of the Gal group into the E3 ubiquitin ligase VHL ligand may give the Gal-PROTAC general applicability. It is known that the hydroxyl group in the VHL ligand plays a critical role in recruitment of VHL E3 ubiquitin ligase54-55 and that caging the –OH group with folate,56 aptamers57 and a bioorthogonal TCO group58-60 blocks its degradation activity. These observations led to the design of a Gal caged PROTAC, in which the Gal moiety is connected via a carbonate linked 4-hydroxyl-benzyl alcohol group to the hydroxyl group of the VHL-based bromodomain (BRD) degrader, ARV-77129 (FIG. 29). The results of docking studies provided support for the expectation that the generated PROTAC, Gal-ARV-771, would possess decreased interactions between the VHL ligand and residues in the VHL protein binding site (FIG. 30A–C). It is expected that the Gal-ARV-771 would be inactive in media and that it would be selectively activated by SA-β-gal and an esterase present in senescent cells to release the protein degrader ARV-771. To demonstrate the feasibility of the prodrug design strategy, Gal-ARV-771 was synthesized, characterized, and employed in the following biological studies.

Gal-ARV-771 was synthesized and characterized, and then tested in biological studies. First, the stability of Gal-ARV-771 was studied by incubating it (100 μM) in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal bovine serum (FBS). Real-time monitoring using UFLC (Ultra-Fast Liquid Chromatography) showed that Gal-ARV-771 is stable over a 24 h period (FIG. 31A). In contrast, in the presence of both esterase [porcine liver (catalog no. 9016-18-6), hydrolysis of the acyl group] and the human lysosomal β-galactosidase GLB1,38, 61 Gal-ARV-771 is nearly completely consumed within 16 h to cleanly form ARV-771 (FIG. 31B). Notably, in the absence of the esterase, GLB1 does not promote hydrolysis of the glycosidic bond in Gal-ARV-771 because the acylated Gal moiety in the prodrug is not recognized by GLB1. These studies suggest that Gal-ARV-771 releases ARV-771 through the combined action of a esterase and β-galactosidase.

Gal-ARV-771 Triggers Ubiquitin-Proteasome-Dependent Degradation of BRD4 in Senescent A549 Cells

The PROTAC BET degrader ARV-771, developed previously for degradation of BRD4 protein in cancer cells, was employed to demonstrate the efficacy of Gal-ARV-771 as a prodrug for inducing selective degradation of BRD4 protein in senescent cells. For this purpose, senescent A549 cells were generated by low-dose treatment with etoposide. Quantification of senescence activity revealed that etoposide induced an enhancement in the activity of the broadly used biomarker for senescent cells, SA-β-gal (FIG.32). In a control experiment, treatment of both normal A549 (n-A549) cells and etoposide-induced senescent A549 (s-A549) cells with ARV-771 (50 and 100 nM) induced degradation of BRD4 (FIG. 33A). However, Gal-ARV-771 (50 nM) displayed degradation activity only in the s-A549 cells (FIG. 33B), indicating that this prodrug is converted to ARV-771 by a combination of esterases and SA-β-gal selectively ex-pressed in the senescent cells. HeLa (FIG. 34A–C) and U87 (FIG. 35A–C) cancer cells lines, and their respective etoposide induced senescent counterparts were utilized to demonstrate that the approach of inducing BRD4 degradation using a pro-drug may be applicable to multiple tumor types (FIG. 36A–B). Consistent with the results observed in the A549 cells, selective degradation of BRD4 only occurred in senescent cells. To validate that BRD4 degradation is dependent on the ubiquitin proteasome pathway, experiments were conducted with the proteasome inhibitor MG-132 or free VHL ligand. The results demonstrate that both MG-132 and the VHL ligand inhibit the degradation abilities of both Gal-ARV-771 and ARV-771 (FIG. 37), showing that Gal-ARV-771 and ARV-771 both depend on the ubiquitin-proteasome pathway.

Gal-ARV-771 Expresses Selective Activity Against Etoposide-Enduced Senescent A549 Cells

Gal-ARV-771 appears to have a protein degradation ability in s-A549 cells that is similar to that of ARV-771. Cell viability assays were performed to substantiate the expectation that Gal-ARV-771 is selectively activated to form ARV-771 only in senescent cells. s-A549 and n-A549 cells were treat-ed with different doses of ARV-771 and Gal-ARV-771 for 72 h. Cell viability assays determined that the IC_{50} of Gal-ARV-771 was 3.29 μM for n-A549 and 640 nM for s-A549 cells (FIG. 38A). Moreover, the senolytic index of Gal-ARV-771 between n-A549 and s-A549 cells is 5.17. In contrast, the IC_{50} values for ARV-771 (FIG. 38B) show that it is highly active against both n-A549 (354 nM) and s-A549 (603 nM) cells. In addition, the IC_{50} of Gal-ARV-771 and ARV-771 in HeLa (FIG. 34B) and U87 cells (FIG. 34C) show that Gal-ARV-771 has a higher senolytic index than ARV-771. These results also demonstrate that Gal-ARV-771 induced apoptosis is independent of cell type. This property is in contrast to other types of senolytic drugs which have different activities in various senescent cell types due to the dynamic and highly heterogeneous nature of the senescence levels. To validate these findings, flow cytometry assays were carried out to determine the antitumor activity of Gal-ARV-771 in senescent cancer cells. In normal A549 cells (FIG. 39A–C), ARV-771 (1.0 μM) induced a higher degree of apoptosis (26.86%) than does Gal-ARV-771 (12.79%). However, in the senescent counterparts (FIG. 40A–C), both ARV-771 and Gal-ARV-771 (1 μM) have similar anticancer activity (31.2% and 31.5%). Furthermore, the results of crystal violet assays also indicate that the toxicity of Gal-ARV-771 is higher against etoposide-induced senescent A549 cells (FIG. 40A–C). The results presented here clearly demonstrate that Gal-ARV-771 selectively promotes apoptosis of senescent cancer cells.

Degradation of NPM-ALK Protein by Gal-MS9 in Senescent Karpas 299 Cells

To demonstrate the potential broad applications of the pro-drug strategy for selective protein degradation in senescent cells, a second VHL-based PROTAC, Gal-MS99, directed against a totally independent protein target was designed (FIG. 41). It was previously reported that MS99 degrades the nucleophosmin (NPM)–anaplastic lymphoma kinase (ALK) fusion protein. Because Karpas 299 cells overexpress NPM-ALK,65 normal Karpas 299 (n-Karpas 299) and senescent Karpas 299 (s-Karpas 299) cells were employed to evaluate the degradation ability of Gal-MS99. The results demonstrate that Gal-MS99 does not promote degradation of NPM-ALK in n-Karpas 299 cells even at a concentration of 1 μM (FIG. 42), while in s-Karpas 299 cells this protein is degraded by both MS99 and Gal-MS99 (FIG. 43). Specifically, the IC_{50} values for NPM-ALK degradation by Gal-MS99 in n-Karpas 299 and s-Karpas 299 cells are 2.162 μM and

454.8 nM, respectively (FIG. 44A). The senolytic index between n-A549 and s-A549 cells is 4.75. However, the senolytic index of MS99 between n-A549 and s-A549 cells is only 1.59 (IC_{50} = 368.8 nM in n-Karpas 299 and 232.4 nM in s-Karpas 299) (FIG. 44B). Taken together, these investigations demonstrate that the prodrug design approach serves as a general strategy for selective degradation of a target protein in senescent cancer cells and for minimizing toxicity to non-senescent cells.

Gal-ARV-771 is well tolerated and has potent in vivo anti-senescent activity.

In the next phase of this investigation, the tolerability and therapeutic benefit of Gal-ARV-771 utilizing the A549 xenograft mouse model was examined. In this experiment, A549 cells were transplanted subcutaneously into the flanks of severe combined immunodeficient (SCID) mice and the tumor bearing mice were treated with etoposide to induce senescence. Specifically, when tumors reached an average volume of 80-100 mm^3, the mice were then treated with etoposide (5 mg/kg) and daily doses of Gal-ARV-771 (20 mg/kg) or ARV-771 (20 mg/kg) alone or in combination for 18 d (FIG. 45A). Both ARV-771 and Gal-ARV-771 significantly inhibited tumor growth in etoposide treated mice (TGI% = 80% for ARV-771 and 74% for Gal-ARV-771) (FIG. 45B). Notably, body weight measurements and daily observations showed that these agents were very well tolerated as no significant toxicity was observed (FIG. 45C). In contrast, treatment of the mice with Gal-ARV-771 alone did not have a comparable effect on tumor growth inhibition, indicating that its therapeutic effect in the A549 mouse model requires induction of senescence by etoposide (FIG. 46A–C). Moreover, immunohistochemistry (IHC) assays revealed that treatment with etoposide and Gal-ARV-771 significantly reduced tumor expression levels of BRD4 (FIG. 47 and FIG. 48A). Importantly, combination treatment also significantly reduced tumor cell proliferation (Ki-67 IHC) (FIG. 47 and FIG. 48B), and concomitantly increased apoptosis (cleaved caspase-3) (FIG. 47 and FIG. 48C). These findings suggest that the anti-tumor effect of the prodrug is caused by apoptosis of senescent cells. Taken together, the results described here demonstrate that combined use of senescence-inducing therapy with senotherapy is highly effective in inhibiting tumor growth in vivo. The data provide a preclinical proof-of-principle of the therapeutic benefits of Gal-ARV-771 as a potent PROTAC prodrug.

Proteomic Analysis Demonstrates that Gal-ARV-771 Selectively Degrades BRD4 in s-A549 Cells

To evaluate the effects of Gal-ARV-771 on BRD4 in the senescent cells, global proteomic analyses of n-A549 and s-A549 cells was conducted. A quantitative multiplexed approach was employed to measure cellular protein levels in both normal and etoposide-induced senescent

A549 cells treated with Gal-ARV-771 (50 nM, FIG. 48A–B). Compared to that in n-A549 cells, the expression level of BRD4 was significantly lower in the s-A549 counterparts. Changes in the senescent-related biomarker L1CAM,66 which previous studies have shown to be induced in premature forms of chemically triggered senescence were also conducted (FIG. 49A).67 L1CAM might represent a new potential senescence biomarker, especially since it is a cell membrane protein. The notable changes of the top five protein levels also included ARGAL and BTBD8 (FIG. 49B). The information could be used to better understand the degradation mechanism of Gal-ARV-771 in s-A549 cells. Taken together, the proteomics data further demonstrate the degradation abil-ity of Gal-ARV-771 in chemically induced senescent cells.

In summary, a unique and effective SA-β-Gal responsive prodrug strategy for selective degradation of senescent cancer cells was designed and developed. The PROTACs Gal-ARV-771 and Gal-MS99 may be selectively activated in SA-β-Gal expressed cancer senescent cells to release protein degrading ARV-771 and MS99. Furthermore, these pro-drugs decrease the cell viability of multiple types of senescent cancer cells suggesting broad therapeutic applicability. Importantly, the results clearly show that concomitant treatment with the promising senolytic prodrug Gal-ARV-771 and etoposide leads to effective inhibition of tumor growth in the A549 lung cancer mouse model. Perhaps the most significant feature of this investigation is that it demonstrates that Gal-PROTAC prodrugs have the unique ability to degrade target proteins in a sub-stoichiometric and catalytic fashion, which should minimize the development of drug resistance. These collective findings define Gal-PROTAC prodrugs as a novel and powerful tool for the selective eradication of relapse-driving senescent cancer cells as well as mitigation of other human senescence-related disorders.

CLAIMS

What is claimed:

1. A combination therapeutic comprising:

a therapeutically effective amount of a PROTAC pro-drug, or pharmaceutically acceptable salts or esters thereof,

wherein the PROTAC pro-drug comprises a ligand targeting a protein of interest, a PROTAC linker, an E3 ligase targeting ligand, and a transcyclooctene (TCO) moiety; and

a therapeutically effective amount of one or more compounds comprising a tetrazine (Tz), or pharmaceutically acceptable salts or esters thereof.

2. The combination therapeutic of claim 1, wherein the transcyclooctene (TCO) moiety is covalently attached to the E3 ligase target ligand.

3. The combination therapeutic of claim 1, wherein the transcyclooxtene (TCO) moiety is covalently attached to the E3 ligase target ligand via a pro-drug linker.

4. The combination therapeutic of claim 1, wherein the PROTAC pro-drug comprises a structure:

$$X–L^1–Y,$$

wherein

X is the ligand targeting a protein of interest;

L^1 is the PROTAC linker; and

Y is the E3 ligase targeting ligand.

5. The combination therapeutic of claim 1, wherein the PROTAC pro-drug comprises a structure:

$$X–L^1–Y–L^2–Q,$$

wherein

X is the ligand targeting a protein of interest;

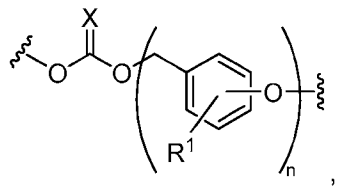
L^1 is the PROTAC linker;

Y is the E3 ligase targeting ligand;

L^2 is a pro-drug linker; and

Q is the transcyclooctene (TCO) moiety.

6. The combination therapeutic of claim 3 or 5, wherein the prodrug linker comprises a linker:



wherein

X is O, S, or N;

R^1 is hydrogen, halogen, C_1–$_6$alkyl, C_1–$_6$alkylene, C_1–$_6$haloalkyl, cyano, –OR^{1a}, –SR^{1a}, –CO_2R^{1a}, –$C(O)R^{1a}$, –SO_2R^{1b}, –$N(R^{1b})_2$, –$CO_2N(R^{1b})_2$, –NO_2, or –$N(R\text{-}R^{1b})$–OR^{1a}; and

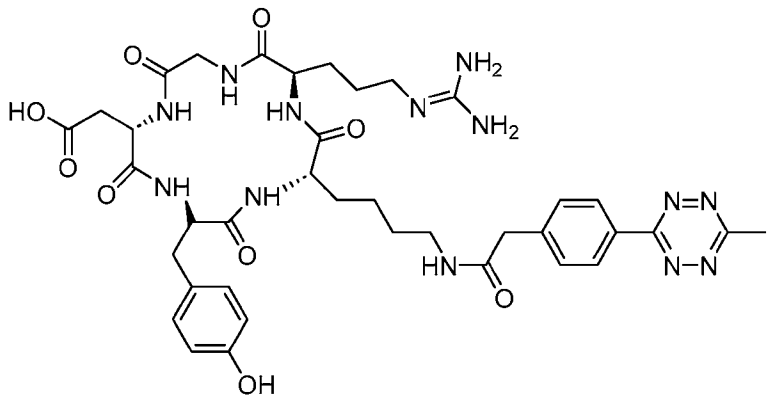
n = 0–1.

7. The combination therapeutic of claim 1, wherein the E3 ligase target ligand is a von Hippel-Lindau (VHL) protein targeting ligand.

8. The combination therapeutic of claim 1, wherein the von Hippel-Lindau (VHL) protein targeting ligand comprises VH032, VH032-OH, (S,R,S)-AHPC-Me, pharmaceutically acceptable salts thereof, or derivatives thereof.

9. The combination therapeutic of 1, wherein the PROTAC pro-drug comprises MS-99, ARV-771, DT2216, TD-004, ARCC-4, VZ-185, ERD-308, BI-0319, SJFα, UNC-6852, DAT8, pharmaceutically acceptable salts thereof, or derivatives thereof.

10. The combination therapeutic of claim 1, wherein the one or more compounds comprises a tetrazine (Tz) selected from:



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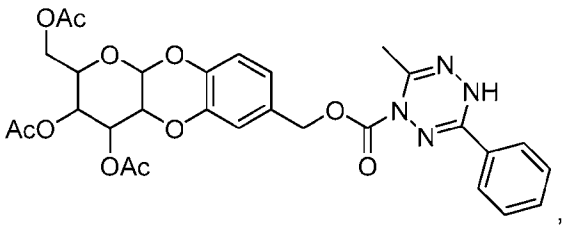

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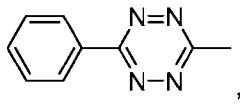

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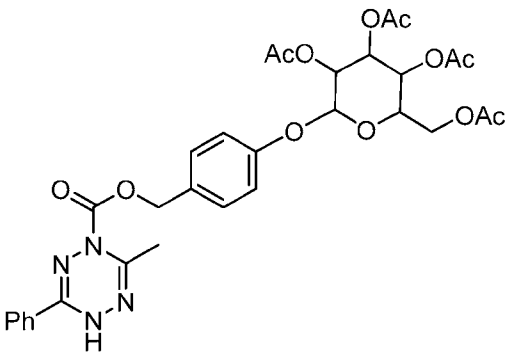

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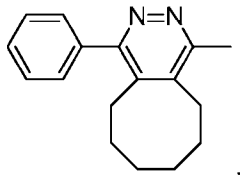

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11. The combination therapeutic of claim 1, wherein the therapeutically effective amount of the PROTAC pro-drug is 1–200 mg/kg.

12. The combination therapeutic of claim 1, wherein the therapeutically effective amount of the one or more compound comprising a tetrazine (Tz) is 1–200 mg/kg.

13. A method for treating a disease or disorder, the method comprising:
sequentially or simultaneously administering to a subject in need thereof:
a therapeutically effective amount of a PROTAC pro-drug, or pharmaceutically acceptable salts or esters thereof,

wherein the PROTAC pro-drug comprises a ligand targeting a protein of interest, a PROTAC linker, an E3 ligase targeting ligand, and a transcyclooctene (TCO) moiety; and

a therapeutically effective amount of one or more compounds comprising a tetrazine (Tz), or pharmaceutically acceptable salts or esters thereof; and

repeating administration until the disease or disorder is treated, ameliorated, or symptoms are reduced.

14. The method of claim 13, wherein the disease or disorder is a cancer, neurodegenerative disease, aging, autoimmune diseases, or viral infections.

15. The method of claim 14, wherein the cancer comprises a breast cancer, melanoma, glioblastoma multiforme, colon cancer, prostate cancer, acute myeloid leukemia, or acute lymphoblastic leukemia and their corresponding senescent cells.

16. The method of claim 13, wherein administering comprises intraperitoneal injection, intramuscular injection, subcutaneous injection, intravenous injection, intrathecal infusion, oral administration, or a combination thereof.

17. The method of claim 13, wherein an amount of a PROTAC increases and an amount of PROTAC pro-drug decreases.

18. The method of claim 13, wherein the PROTAC pro-drug and one or more antineoplastic agents reduce an amount of a target protein.

19. The method of claim 13, wherein the therapeutically effective amount of the PROTAC pro-drug is added at a period of time prior to addition of the therapeutically effective amount of the one or more compounds comprising a tetrazine (Tz).

20. The method of claim 19, wherein the period of time is 0–168 hours.

21. Use of the PROTAC pro-drug and the one or more compounds comprising a tetrazine (Tz), or pharmaceutically acceptable salts or esters thereof, as a medicament for the treatment of cancer in subject in need thereof.

22. A kit comprising:

one or more PROTAC pro-drugs, or pharmaceutically acceptable salts or esters thereof, of claim 1;

one or more compounds comprising a tetrazine (Tz), or pharmaceutically acceptable salts or esters thereof;

optionally a device or means for administering the PROTAC pro-drug and one or more compounds comprising a tetrazine (Tz);

optionally tamper resistant packaging; and

optionally, a label or instructions for use thereof.

23. A combination therapeutic comprising:

a therapeutically effective amount of a PROTAC pro-drug, or pharmaceutically acceptable salts or esters thereof,

wherein the PROTAC pro-drug comprises a ligand targeting a protein of interest, a PROTAC linker, an E3 ligase targeting ligand, and a galactose, or derivative thereof; and

a therapeutically effective amount of one or more antineoplastic agents, or pharmaceutically acceptable salts or esters thereof.

24. The combination therapeutic of claim 23, wherein the galactose, or derivative thereof, is covalently attached to the E3 ligase target ligand.

25. The combination therapeutic of claim 23, wherein the galactose, or derivative thereof is covalently attached to the E3 ligase target ligand via a pro-drug linker.

26. The combination therapeutic of claim 23, wherein the PROTAC pro-drug comprises a structure:

$$X–L^1–Y,$$

wherein

X is the ligand targeting a protein of interest;

L^1 is the PROTAC linker; and

Y is the E3 ligase targeting ligand.

27. The combination therapeutic of claim 23, wherein the PROTAC pro-drug comprises a structure:

$$X–L^1–Y–L^2–Q,$$

wherein

X is the ligand targeting a protein of interest;

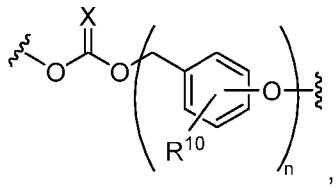
L^1 is the PROTAC linker;

Y is the E3 ligase targeting ligand;

L^2 is a pro-drug linker; and

Q is galactose or a derivative thereof.

28. The combination therapeutic of claim 25 or 27, wherein the pro-drug linker has a structure:



,

wherein

X is O, S, or N;

R^{10} is hydrogen, halogen, C_1–$_6$alkyl, C_1–$_6$alkylene, C_1–$_6$haloalkyl, cyano, $–OR^{1a}$, $–SR^{1a}$, $–CO_2R^{1a}$, $–C(O)R^{1a}$, $–SO2R^{1b}$, $–N(R^{1b})_2$, $–CO_2N(R^{1b})_2$, $–NO_2$, or $–N(R\text{-}R^{1b})–OR^{1a}$; and

n = 0–1.

29. The combination therapeutic of claim 23, wherein the E3 ligase target ligand is a von Hippel-Lindau (VHL) protein targeting ligand.

30. The combination therapeutic of claim 23, wherein the von Hippel-Lindau (VHL) protein targeting ligand comprises VH032, VH032-OH, (S,R,S)-AHPC-Me, pharmaceutically acceptable salts thereof, or derivatives thereof.

31. The combination therapeutic of 23, wherein the PROTAC pro-drug comprises MS-99, ARV-771, DT2216, TD-004, ARCC-4, VZ-185, ERD-308, BI-0319, SJFα, UNC-6852, DAT8, pharmaceutically acceptable salts thereof, or derivatives thereof.

32. The combination therapeutic of claim 23, wherein the one or more antineoplastic agents comprise DNA damaging agents, chemical toxic agents, or radiation.

33. The combination therapeutic of claim 32, wherein the DNA damaging agents comprise etoposide, camptothecin, doxorubicin, pharmaceutically acceptable salts thereof, or derivatives thereof.

34. The combination therapeutic of claim 32, wherein the chemical toxic agents comprise taxol, pharmaceutically acceptable salts thereof, or derivatives thereof.

35. The combination therapeutic of claim 23, wherein the galactose is a β-galactose.

36. The combination therapeutic of claim 23, wherein the therapeutically effective amount of the PROTAC pro-drug is 1–200 mg/kg.

37. The combination therapeutic of claim 23, wherein the therapeutically effective amount of the one or more antineoplastic agent is 1–200 mg/kg.

38. A method for treating a disease or disorder, the method comprising:
sequentially or simultaneously administering to a subject in need thereof
a therapeutically effective amount of a PROTAC pro-drug, or pharmaceutically acceptable salts or esters thereof,
wherein the PROTAC pro-drug comprises a ligand targeting a protein of interest, a PROTAC linker, an E3 ligase targeting ligand, and galactose, or a derivative thereof; and
a therapeutically effective amount of one or more antineoplastic agents, or pharmaceutically acceptable salts or esters thereof; and
repeating administration until the disease or disorder is treated, ameliorated, or symptoms are reduced.

39. The method of claim 38, wherein the disease or disorder is a cancer, neurodegenerative disease, aging, or viral infections.

40. The method of claim 39, wherein the cancer comprises a breast cancer, melanoma, glioblastoma multiforme, colon cancer, prostate cancer, acute myeloid leukemia, or acute lymphoblastic leukemia and their corresponding senescent cells.

41. The method of claim 38, wherein administering comprises intraperitoneal injection, intramuscular injection, subcutaneous injection, intravenous injection, intrathecal infusion, oral administration, or a combination thereof.

42. The method of claim 38, wherein an amount of a PROTAC increases and an amount of PROTAC pro-drug decreases.

43. The method of claim 38, wherein the PROTAC pro-drug and the one or more antineoplastic agents reduce an amount of a target protein.

44. The method of claim 38, wherein the PROTAC pro-drug and the one or more antineoplastic agents target senescent cells.

45. The method of claim 38, wherein the therapeutically effective amount of the antineoplastic agents is added to induce cellular senesce.

46. The method of claim 38, wherein the therapeutically effective amount of the antineoplastic agents is added at a period of time prior to addition of the therapeutically effective amount of the PROTAC pro-drug.

47. The method of claim 46, wherein the period of time is 0–168 hours.

48. Use of the PROTAC pro-drug and the one or more antineoplastic agents, or pharmaceutically acceptable salts or esters thereof, as a medicament for the treatment of cancer in subject in need thereof.

49. A kit comprising:

one or more PROTAC pro-drugs, or pharmaceutically acceptable salts or esters thereof, of claim 38;

one or more antineoplastic agents, or pharmaceutically acceptable salts or esters thereof;

optionally a device or means for administering the PROTAC pro-drug and the one or more antineoplastic agents;

optionally tamper resistant packaging; and

optionally, a label or instructions for use thereof.

FIG. 1A

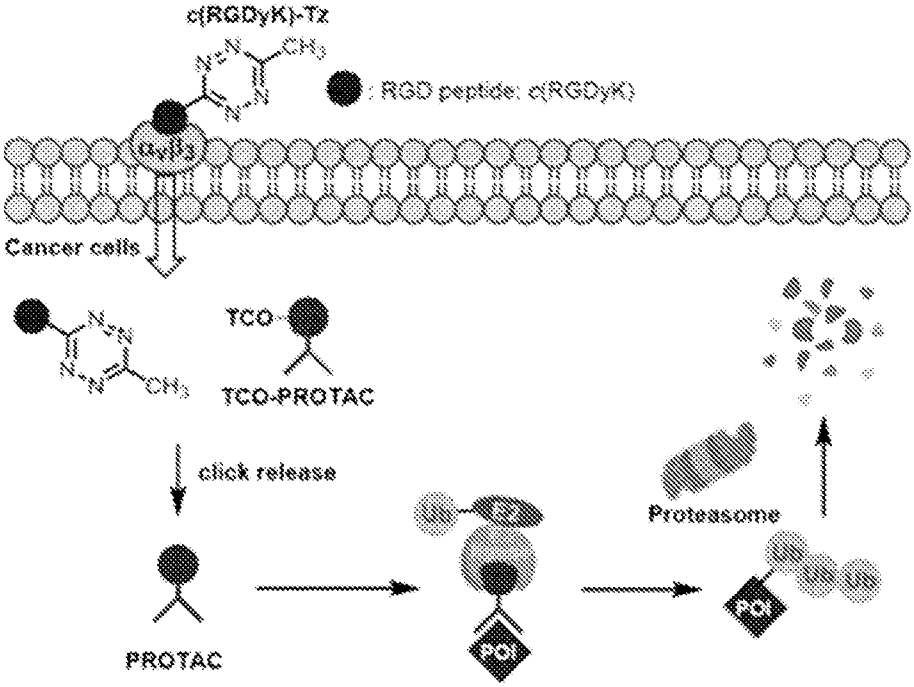

c(RGDyK)-Tz
: RGD peptide: c(RGDyK)
αvβ3
Cancer cells
TCO
TCO-PROTAC
click release
PROTAC
E3
POI
Proteasome
Ub

FIG. 1B

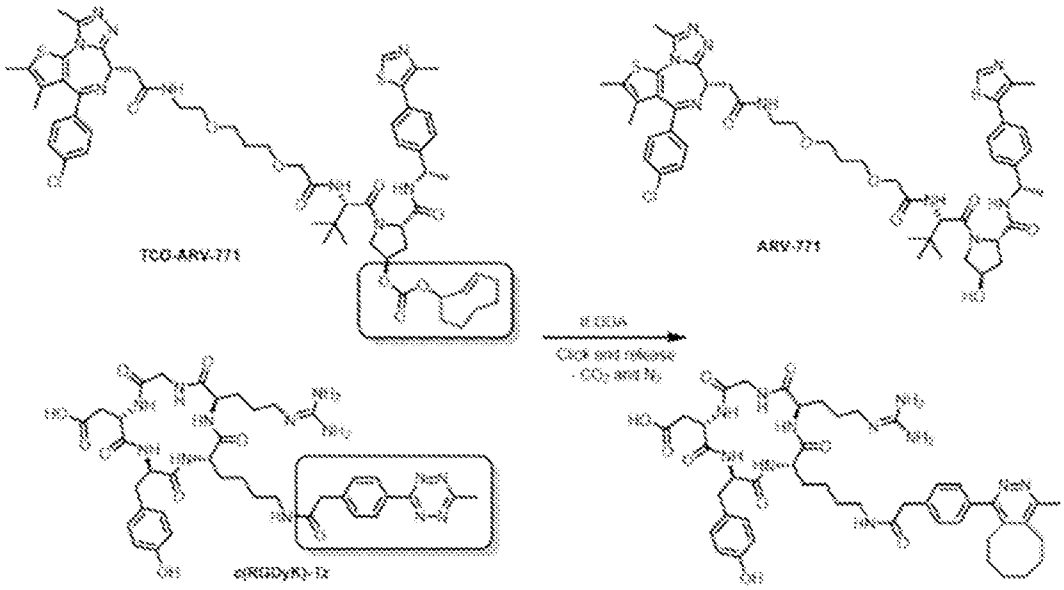

TCO-ARV-771
ARV-771
c(RGDyK)-Tz

FIG. 1 C

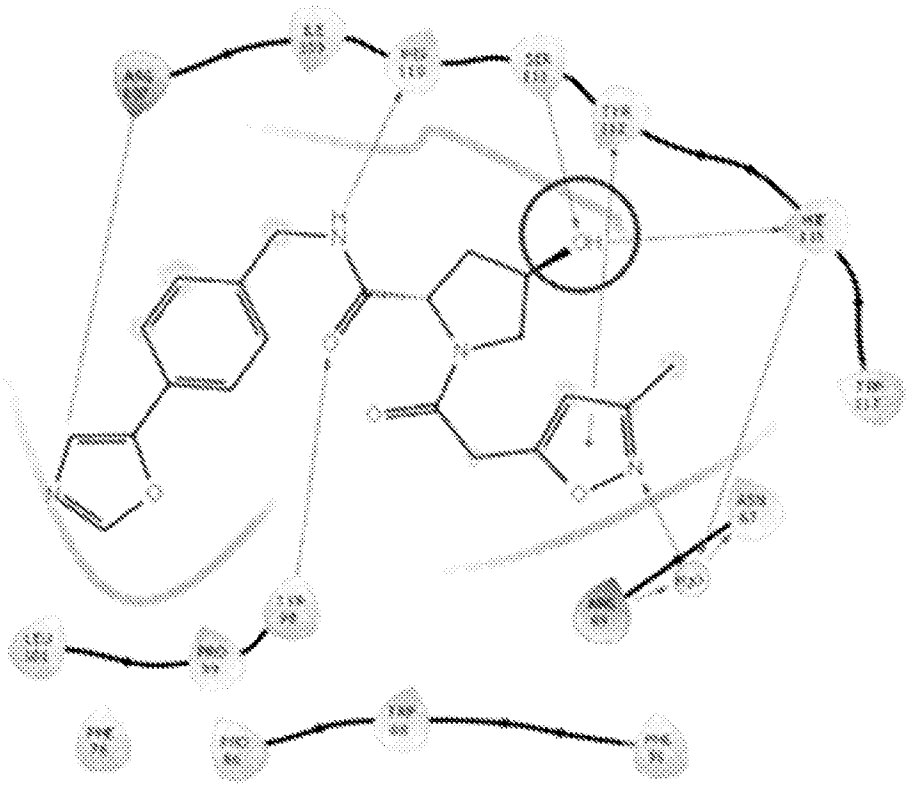


FIG. 1D

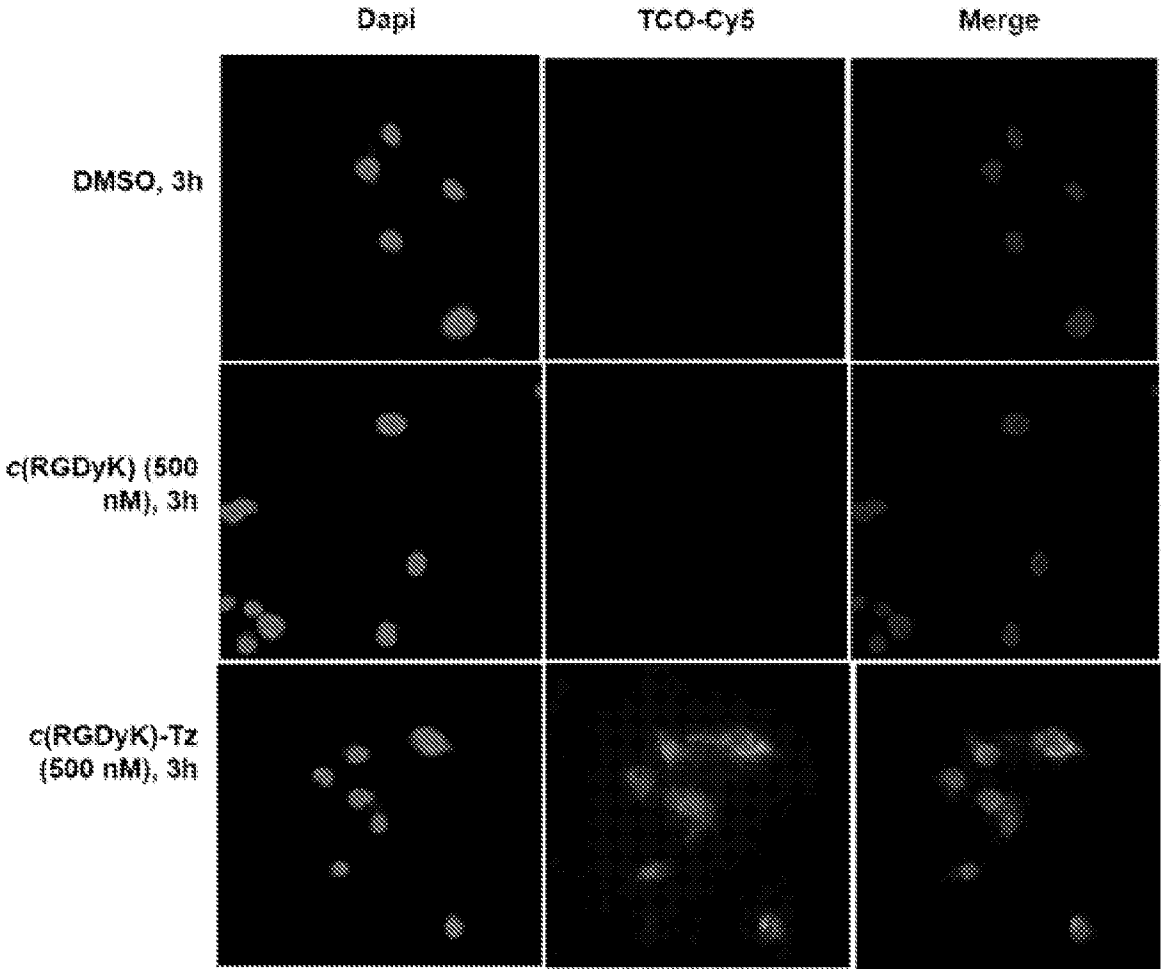
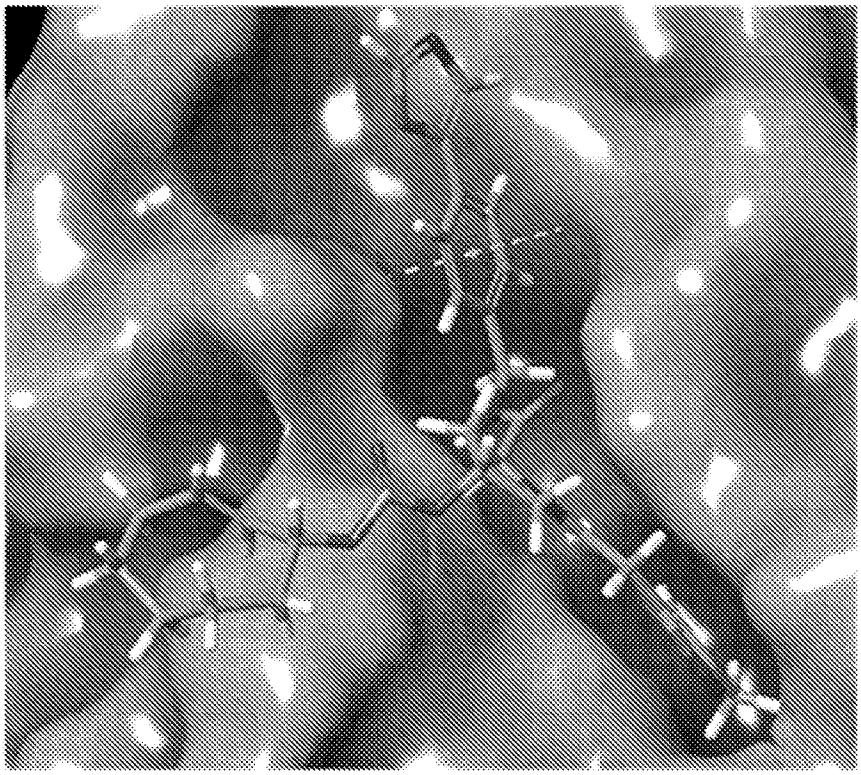
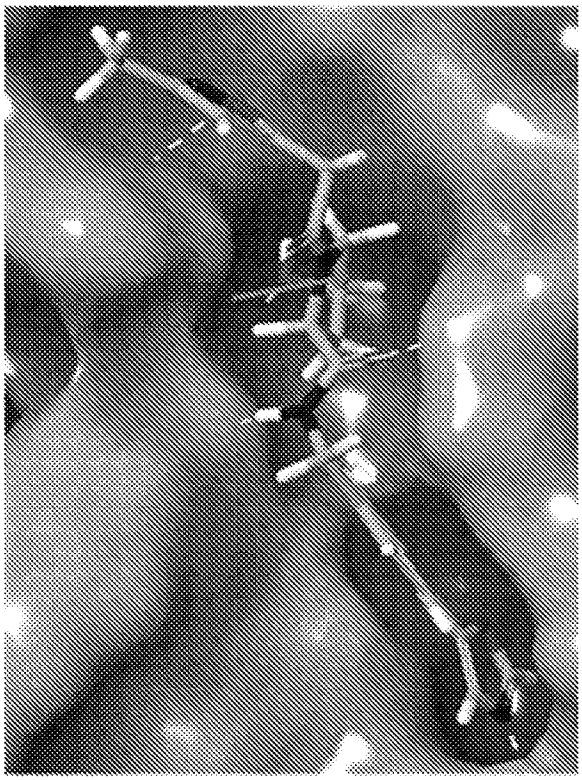


FIG. 2A



Docking score:
-4.688

FIG. 2B



Docking score:
-9.170

FIG. 3A

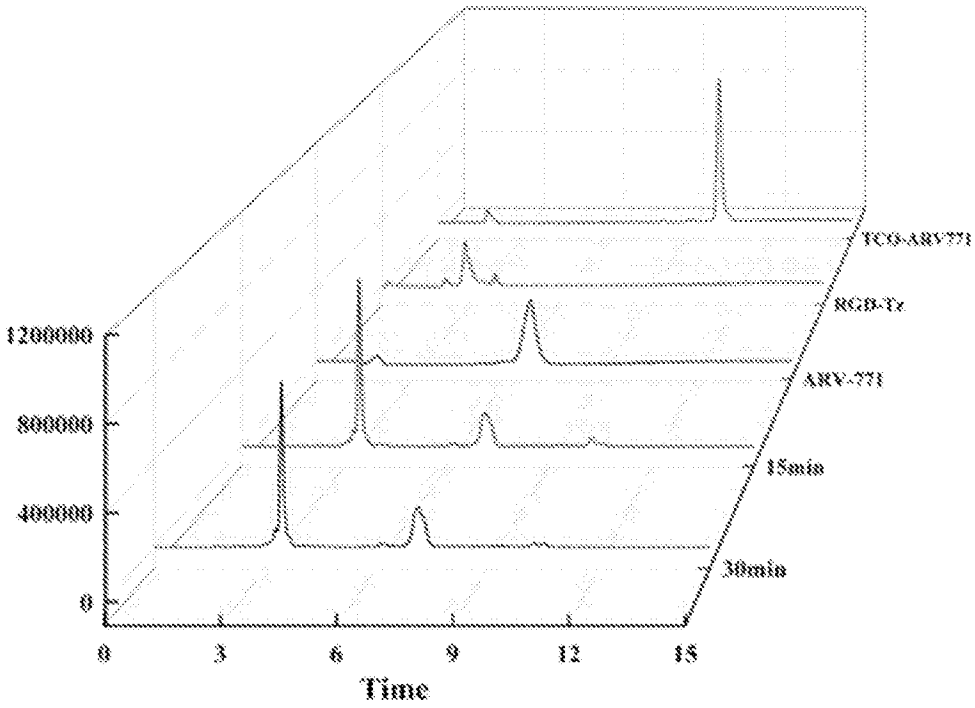

1200000
800000
400000
0
0
3
6
9
12
15
Time
30min
15min
ARV-771
RGD-Yz
TCO-ARV771

FIG. 3B

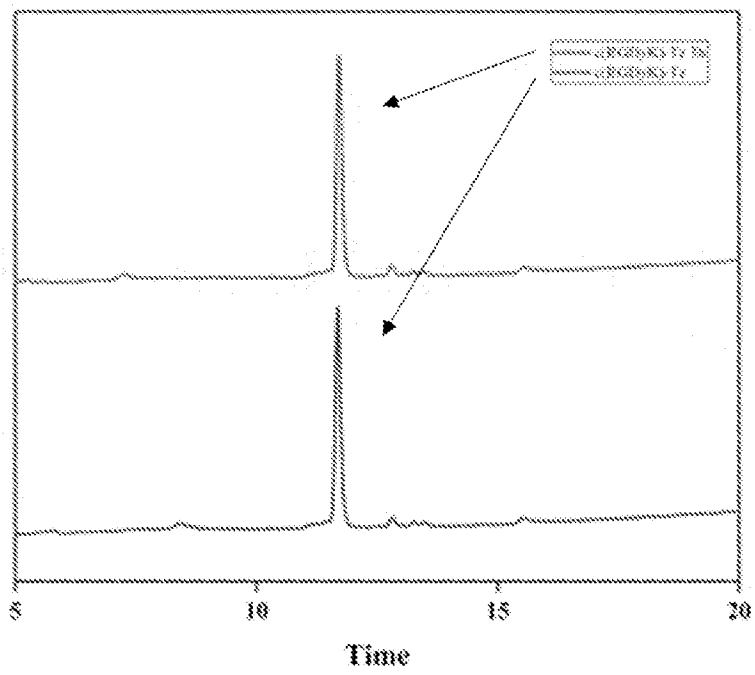

5
10
15
20
Time

FIG. 4A

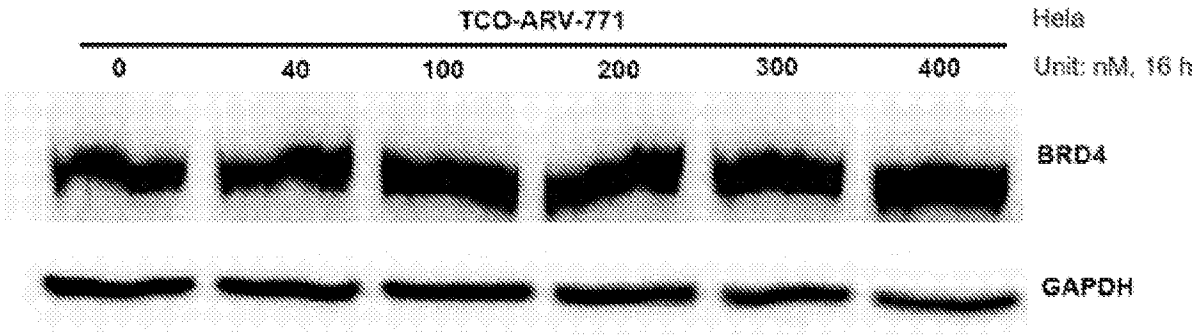


FIG. 4B

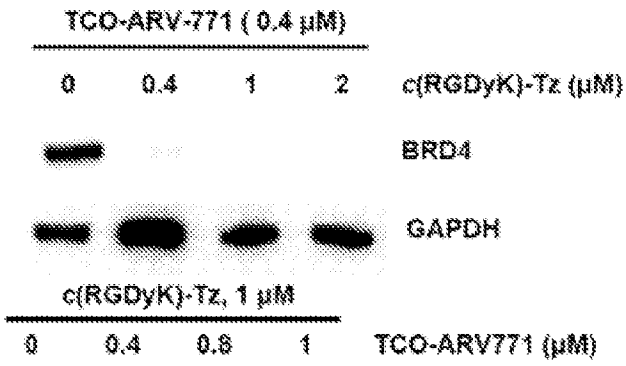


FIG. 4C

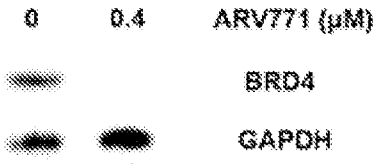


FIG. 4D

FIG. 4E

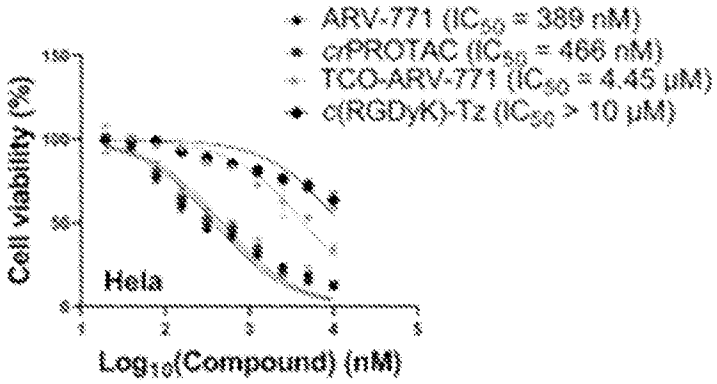

ARV-771 (IC_{50} = 389 nM)
cPROTAC (IC_{50} = 466 nM)
TCO-ARV-771 (IC_{50} = 4.45 μM)
c(RGDyK)-Tz (IC_{50} > 10 μM)
Cell viability (%)
Hela
Log_{10}(Compound) (nM)

FIG. 4F

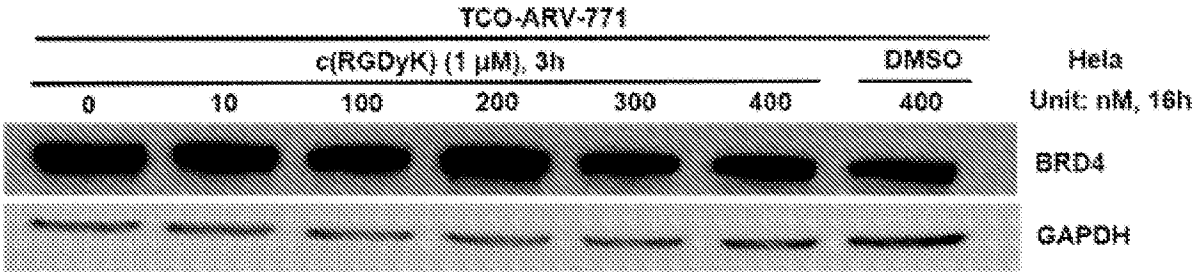

TCO-ARV-771
c(RGDyK) (1 μM), 3h
DMSO
Hela
0
10
100
200
300
400
400
Unit: nM, 16h
BRD4
GAPDH

FIG. 5A

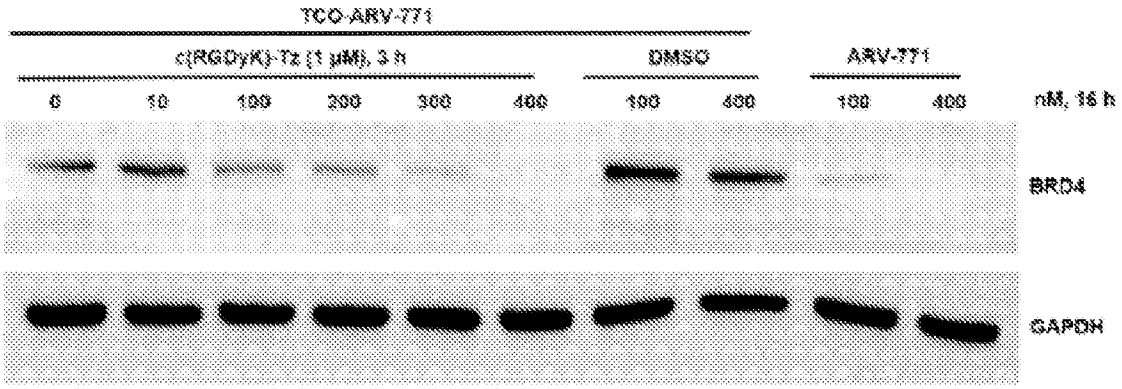

TCO-ARV-771
c(RGDyK)-Tz (1 μM), 3 h
DMSO
ARV-771
0
10
100
200
300
400
100
400
100
400
nM, 16 h
BRD4
GAPDH

FIG. 5B

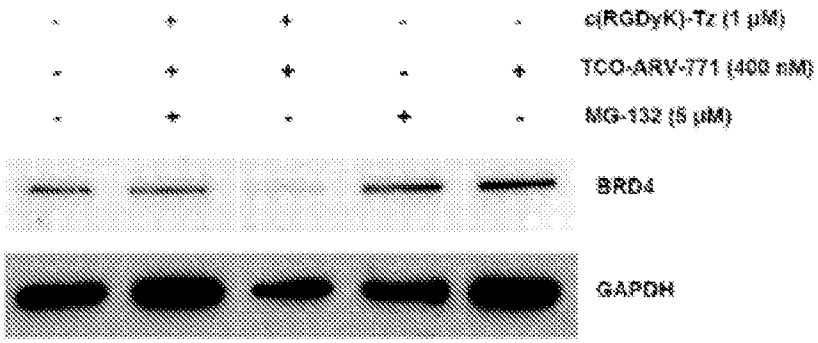

c(RGDyK)-Tz (1 μM)
TCO-ARV-771 (400 nM)
MG-132 (5 μM)
BRD4
GAPDH

FIG. 5C

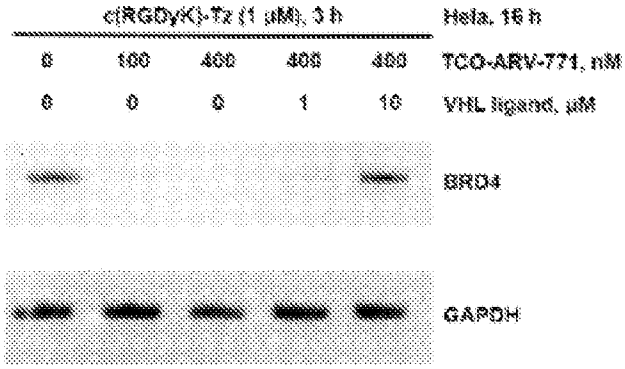

c(RGDyK)-Tz (1 μM), 3 h
Hela, 16 h
0
100
400
400
400
TCO-ARV-771, nM
0
0
0
1
10
VHL ligand, μM
BRD4
GAPDH

FIG. 5D

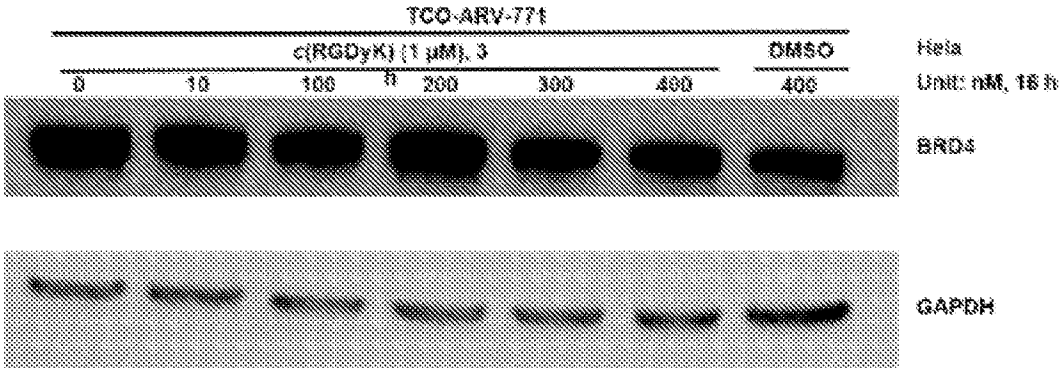

TCO-ARV-771
c(RGDyK) (1 μM), 3
DMSO
Hela
0
10
100
200
300
400
400
Unit: nM, 16 h
BRD4
GAPDH

FIG. 6A

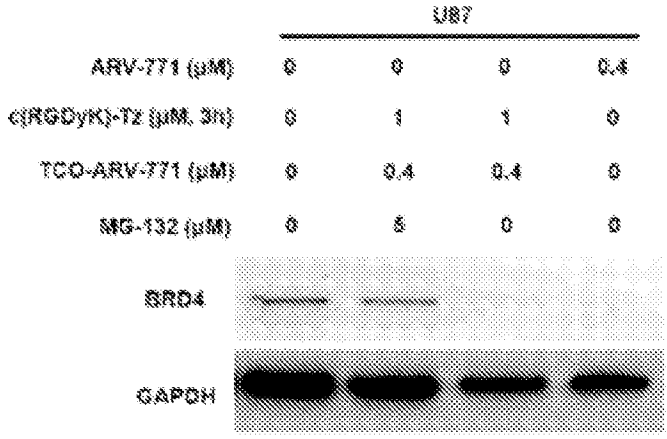


FIG. 6B

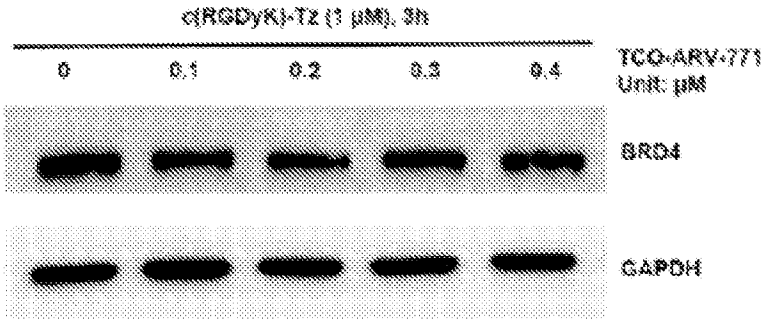


FIG. 6C

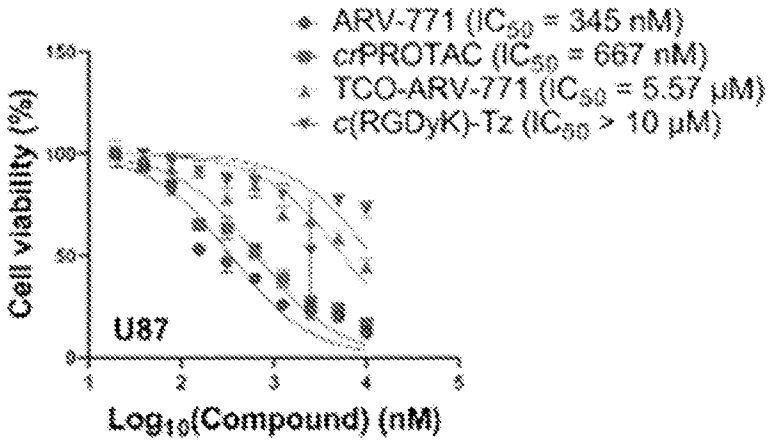


FIG. 6D

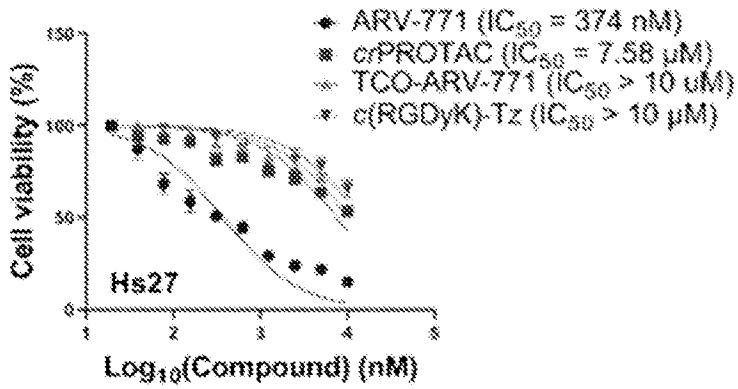

ARV-771 (IC_{50} = 374 nM)
c/PROTAC (IC_{50} = 7.58 μM)
TCO-ARV-771 (IC_{50} > 10 uM)
c(RGDyK)-Tz (IC_{50} > 10 μM)
Cell viability (%)
150
100
50
0
Hs27
1
2
3
4
5
Log_{10}(Compound) (nM)

FIG. 7A

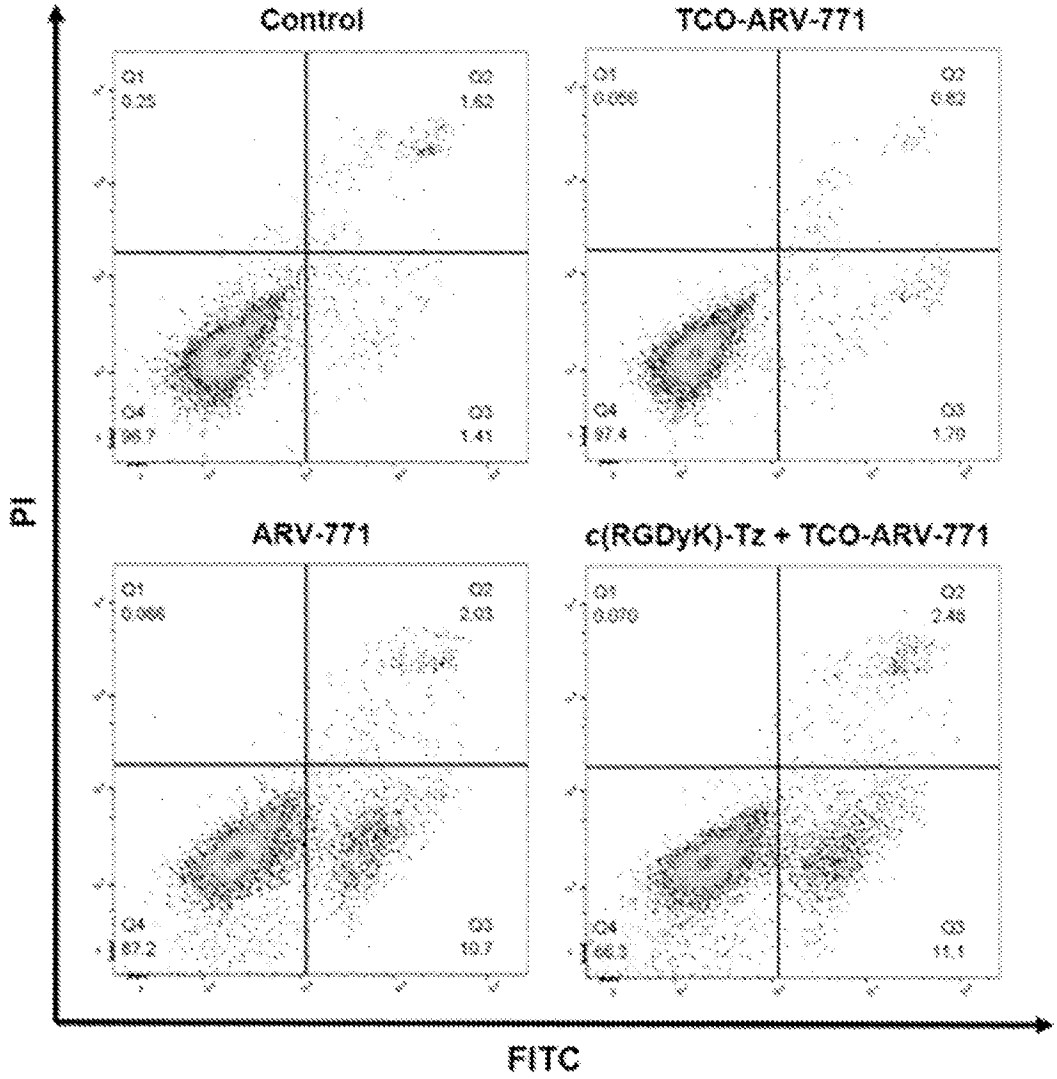


FIG. 7B

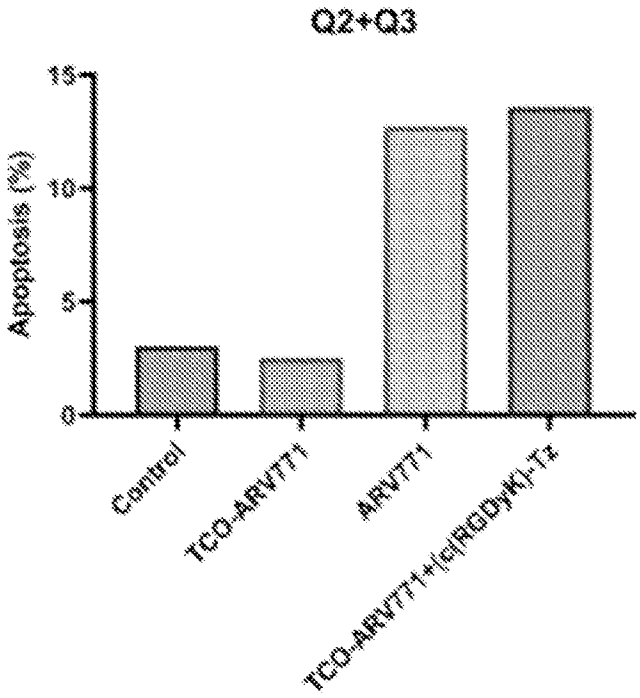

Q2+Q3
Apoptosis (%)
15
10
5
0
Control
TCO-ARV771
ARV771
TCO-ARV771+[c(RGDyK)]-Tz

FIG. 8A

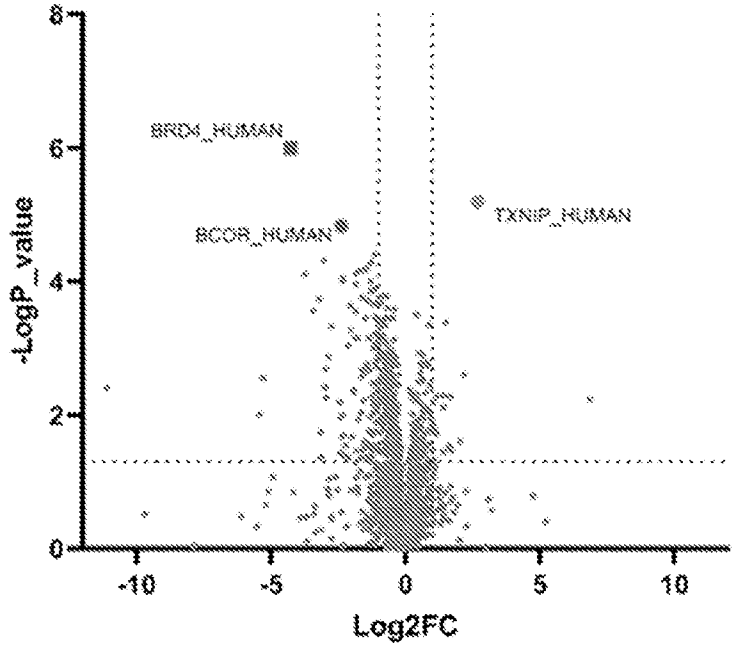

BRD4_HUMAN
BCOR_HUMAN
TXNIP_HUMAN
-LogP_value
Log2FC
8
6
4
2
0
-10
-5
0
5
10

FIG. 8B

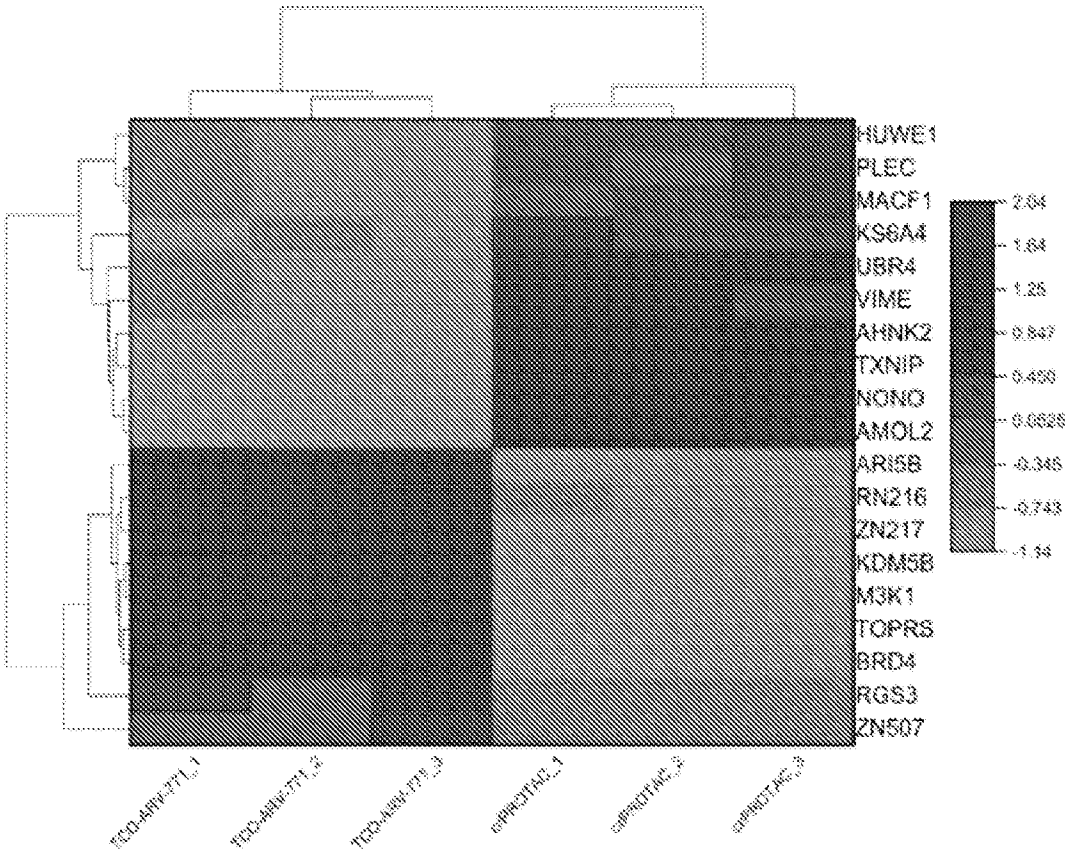

HUWE1
PLEC
MACF1
KS6A4
UBR4
VIME
AHNK2
TXNIP
NONO
AMOL2
ARI5B
RN216
ZN217
KDM5B
M3K1
TOPRS
BRD4
RGS3
ZN507
2.04
1.64
1.25
0.847
0.450
0.0528
-0.345
-0.743
-1.14
TCO-ARV-771_1
TCO-ARV-771_2
TCO-ARV-771_3
cPROTAC_1
cPROTAC_2
cPROTAC_3

FIG. 9

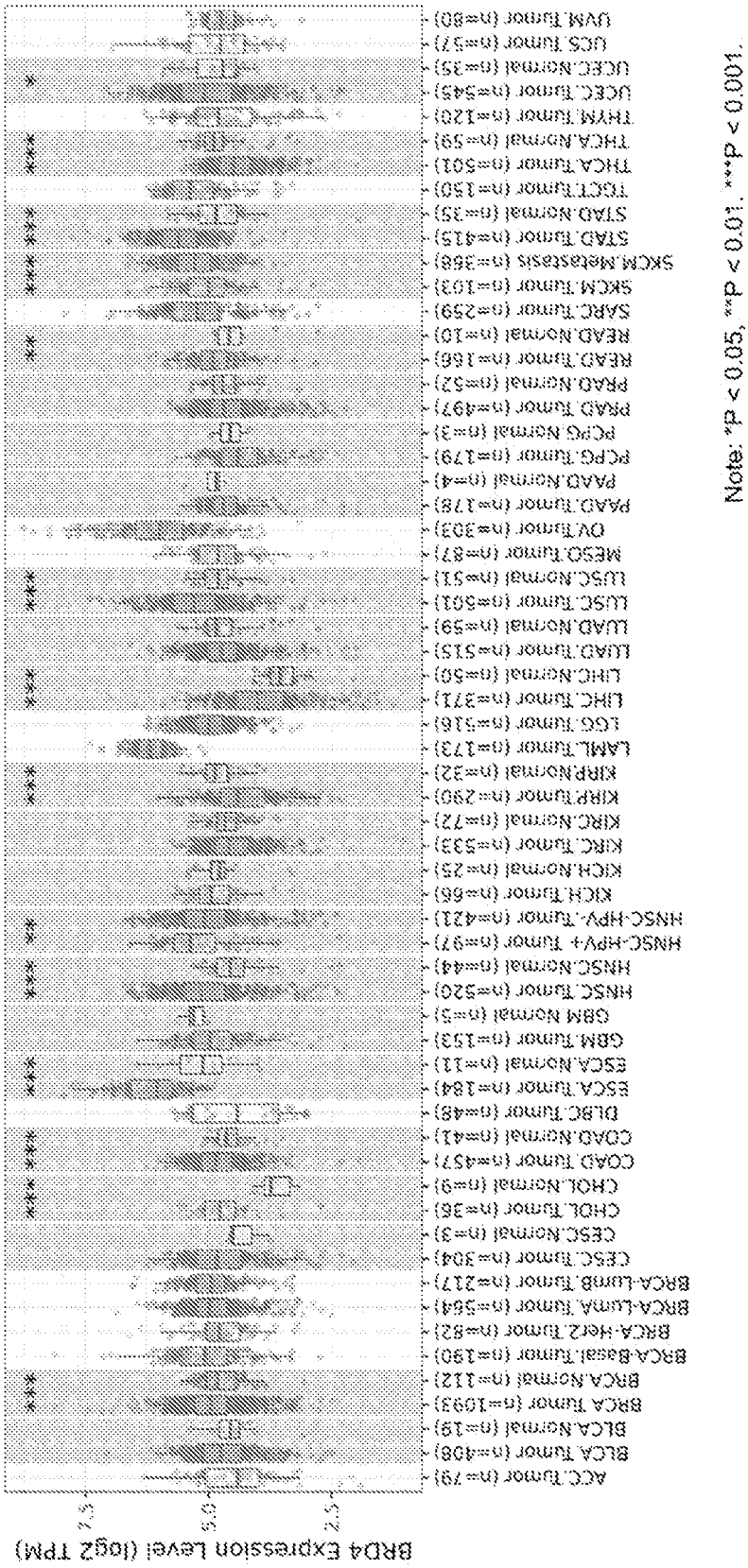

ACC.Tumor (n=79)
BLCA.Tumor (n=408)
BLCA.Normal (n=19)
BRCA.Tumor (n=1093)
BRCA.Normal (n=112)
BRCA.Basal.Tumor (n=190)
BRCA.Her2.Tumor (n=82)
BRCA.LumA.Tumor (n=564)
BRCA.LumB.Tumor (n=217)
CESC.Tumor (n=304)
CESC.Normal (n=3)
CHOL.Tumor (n=36)
CHOL.Normal (n=9)
COAD.Tumor (n=457)
COAD.Normal (n=41)
DLBC.Tumor (n=48)
ESCA.Tumor (n=184)
ESCA.Normal (n=11)
GBM.Tumor (n=153)
GBM Normal (n=5)
HNSC.Tumor (n=520)
HNSC.Normal (n=44)
HNSC-HPV+ Tumor (n=97)
HNSC-HPV- Tumor (n=421)
KICH.Tumor (n=66)
KICH.Normal (n=25)
KIRC.Tumor (n=533)
KIRC.Normal (n=72)
KIRP.Tumor (n=290)
KIRP.Normal (n=32)
LAML.Tumor (n=173)
LGG.Tumor (n=516)
LIHC.Tumor (n=371)
LIHC.Normal (n=50)
LUAD.Tumor (n=515)
LUAD.Normal (n=59)
LUSC.Tumor (n=501)
LUSC.Normal (n=51)
MESO.Tumor (n=87)
OV.Tumor (n=303)
PAAD.Tumor (n=178)
PAAD.Normal (n=4)
PCPG.Tumor (n=179)
PCPG.Normal (n=3)
PRAD.Tumor (n=497)
PRAD.Normal (n=52)
READ.Tumor (n=166)
READ.Normal (n=10)
SARC.Tumor (n=259)
SKCM.Tumor (n=103)
SKCM.Metastasis (n=358)
STAD.Tumor (n=415)
STAD.Normal (n=35)
TGCT.Tumor (n=150)
THCA.Tumor (n=501)
THCA.Normal (n=59)
THYM.Tumor (n=120)
UCEC.Tumor (n=545)
UCEC.Normal (n=35)
UCS.Tumor (n=57)
UVM.Tumor (n=80)
7.5
5.0
2.5
BRD4 Expression Level (log2 TPM)
Note: *P < 0.05, **P < 0.01, ***P < 0.001.

FIG. 10

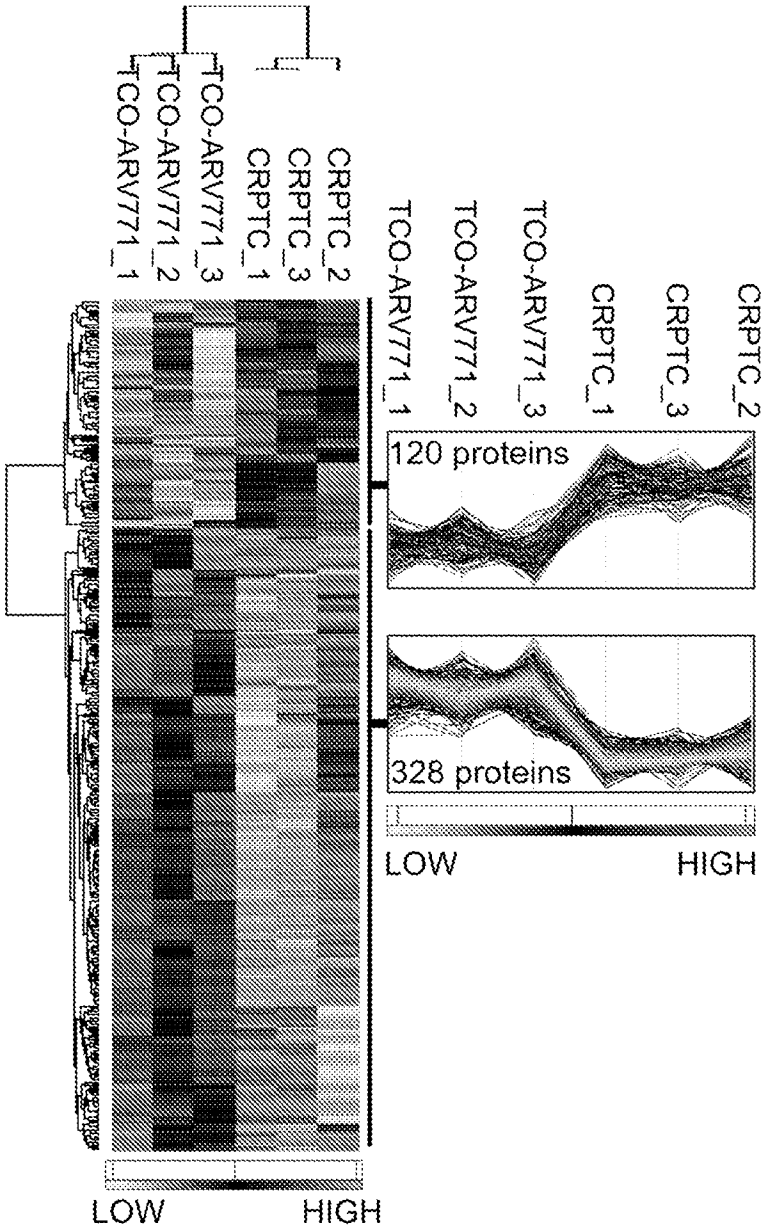

TCO-ARV771_1
TCO-ARV771_2
TCO-ARV771_3
CRPTC_1
CRPTC_3
CRPTC_2
LOW
HIGH
120 proteins
328 proteins
LOW
HIGH

FIG. 11A

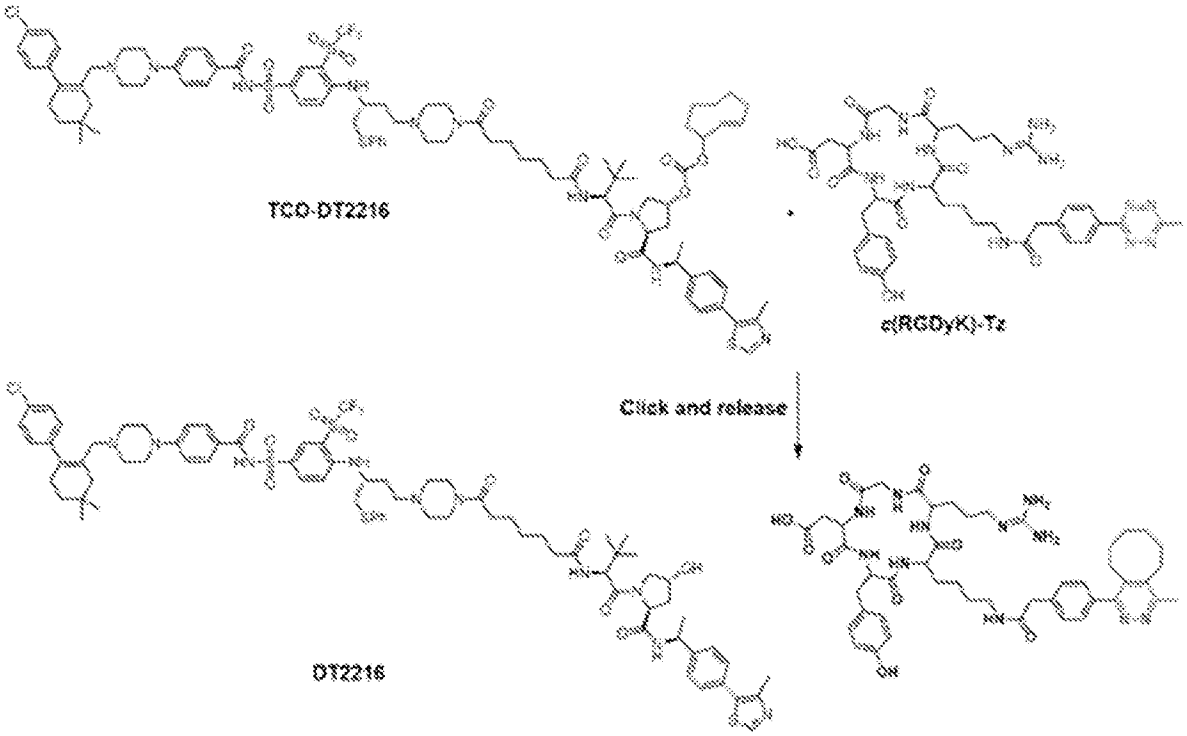


FIG. 11B

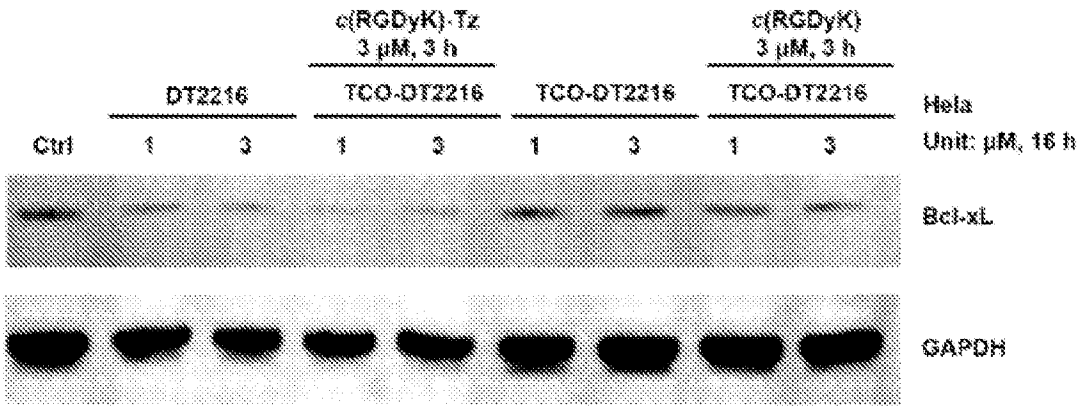


FIG. 11C

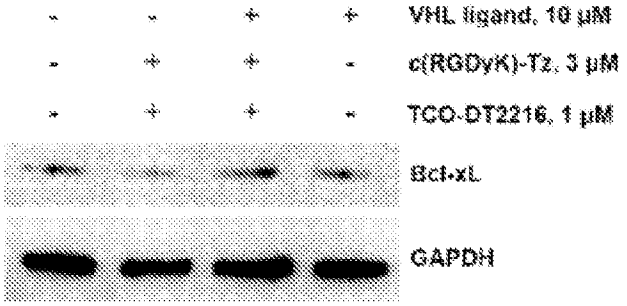


FIG. 11D

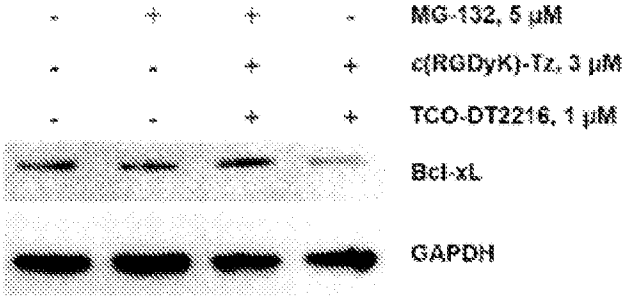


FIG. 12A

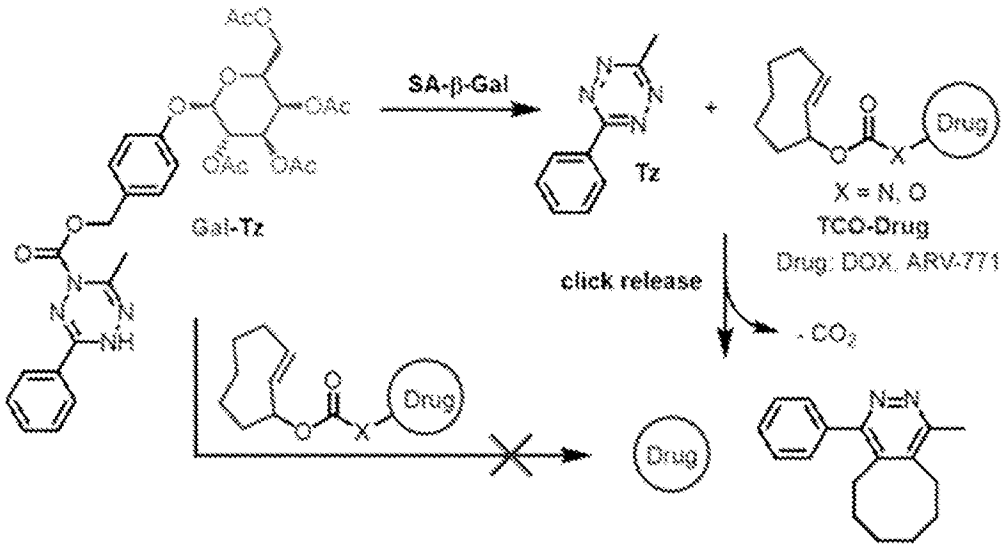


FIG. 12B

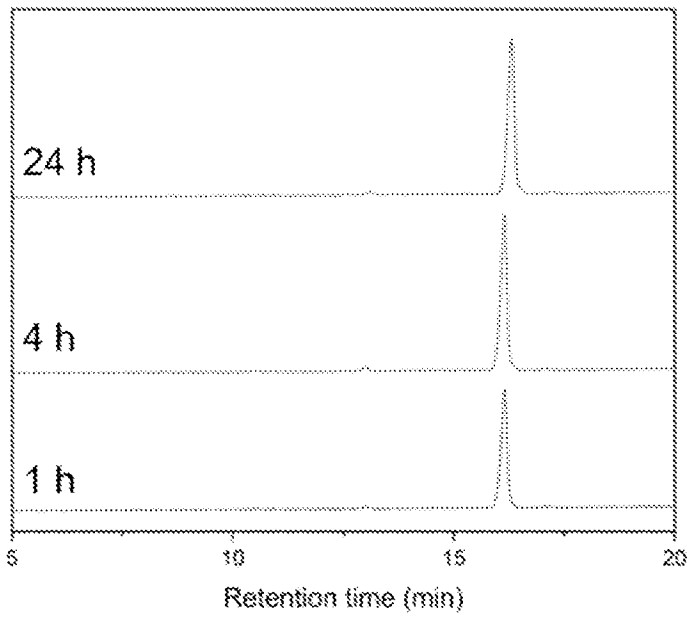


FIG. 12C

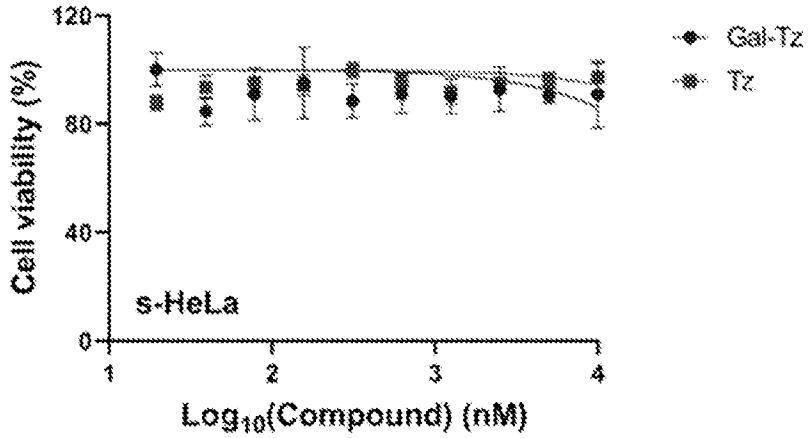


FIG. 12D

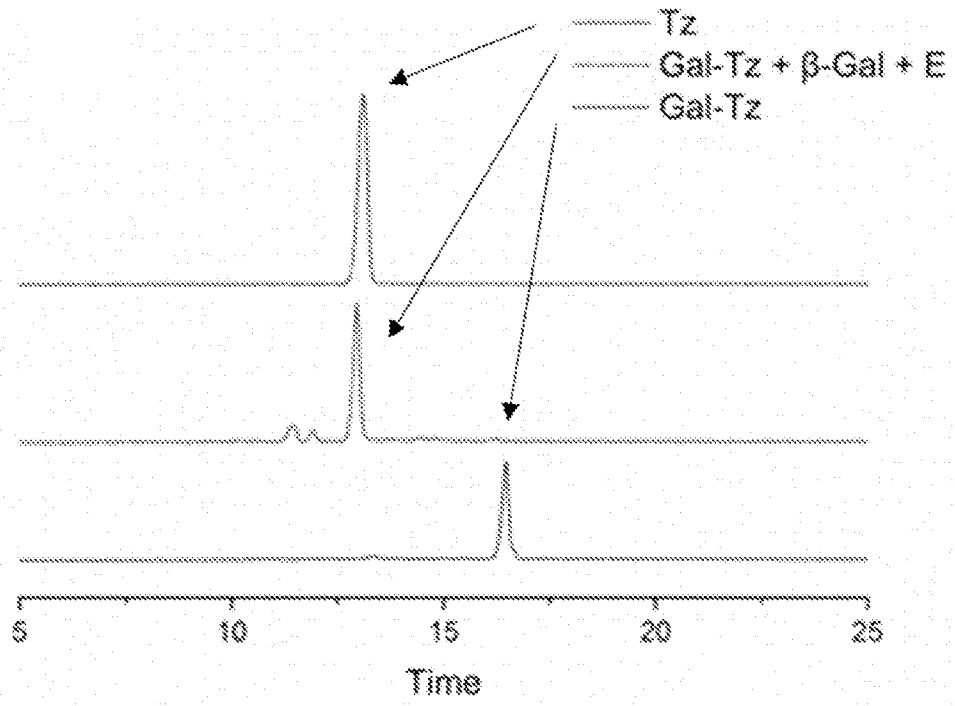


FIG. 13

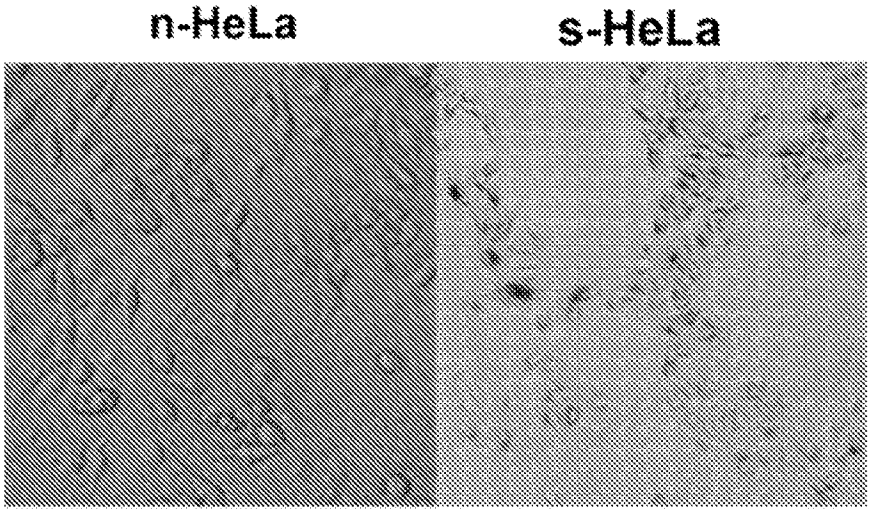


FIG. 14A

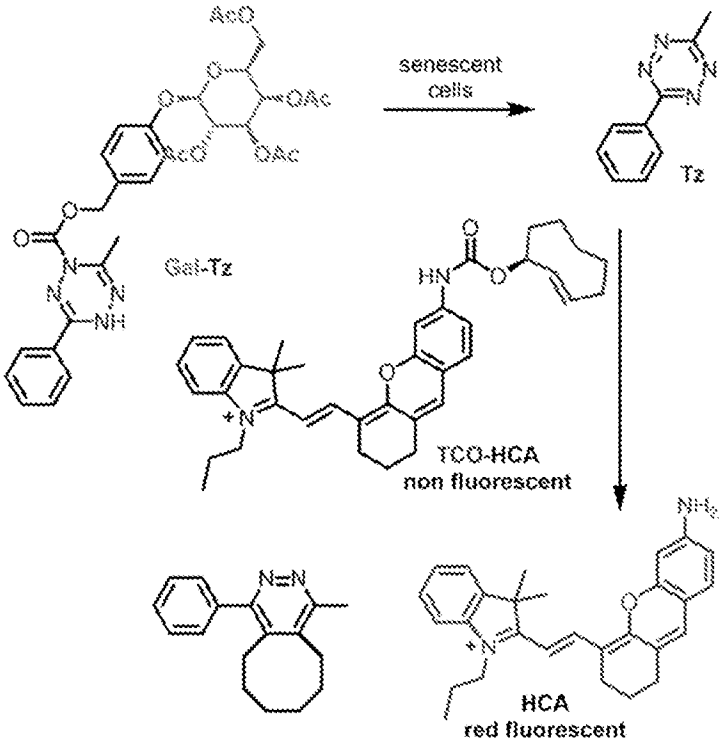


FIG. 14B

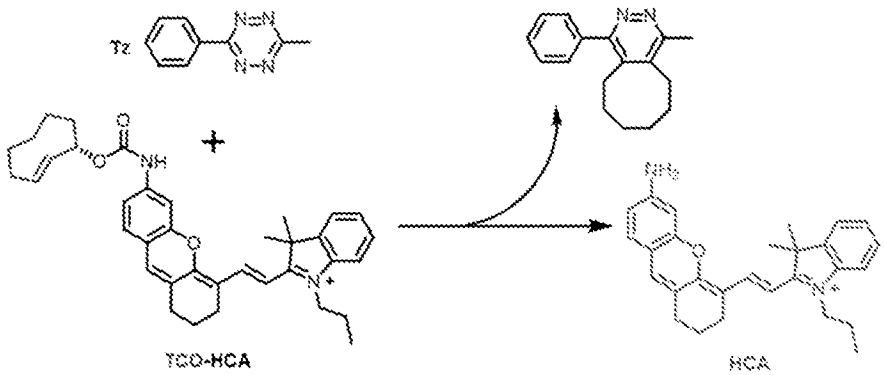


FIG. 15A

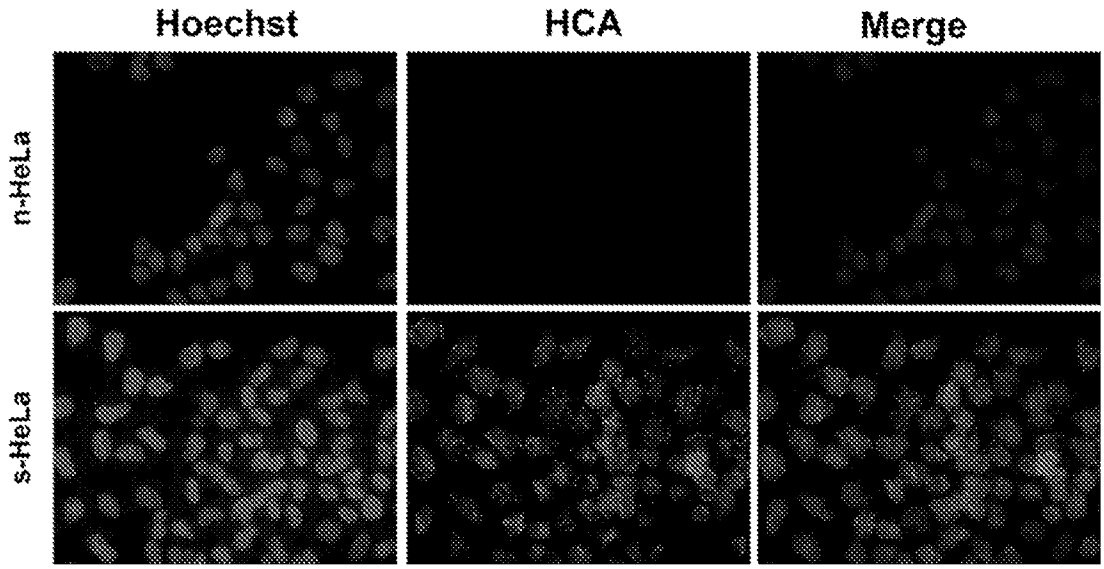


FIG. 15B

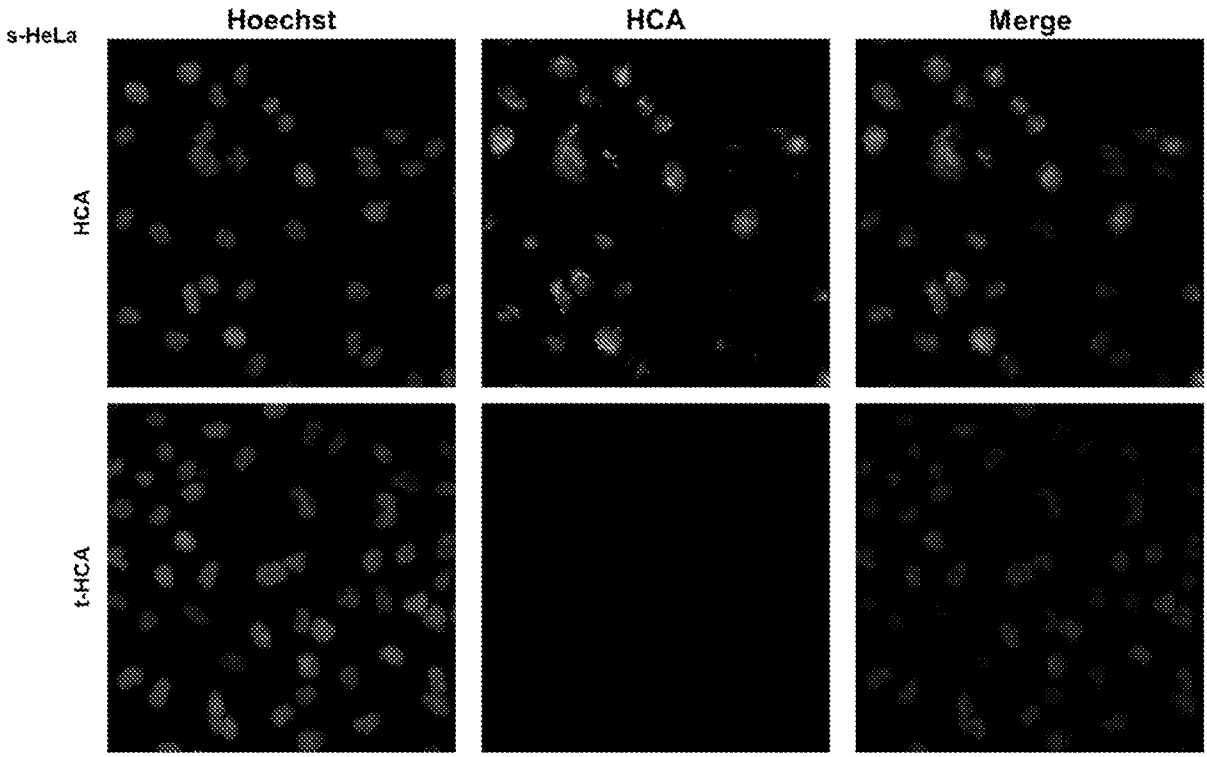


FIG. 15C

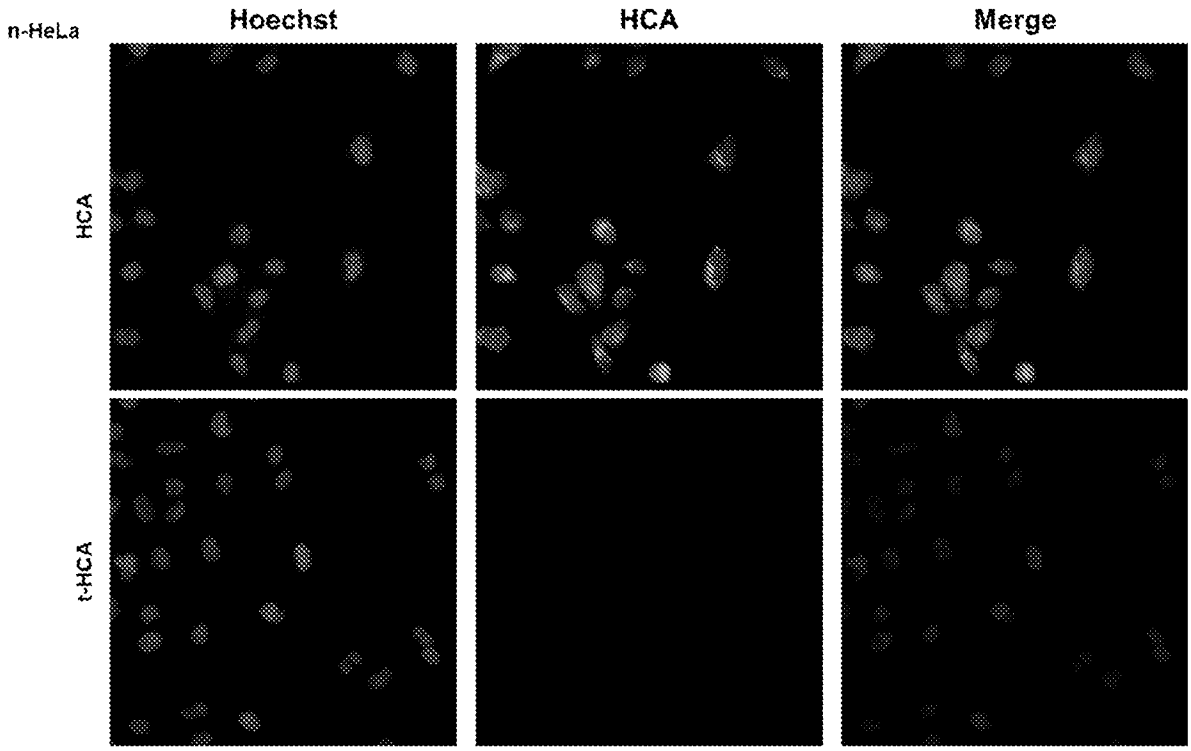


FIG. 16

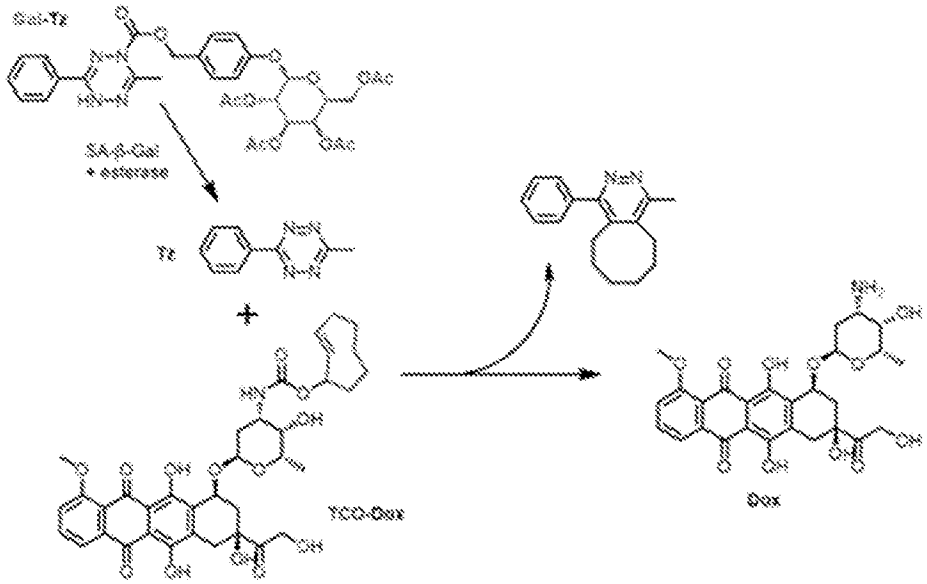


FIG. 17

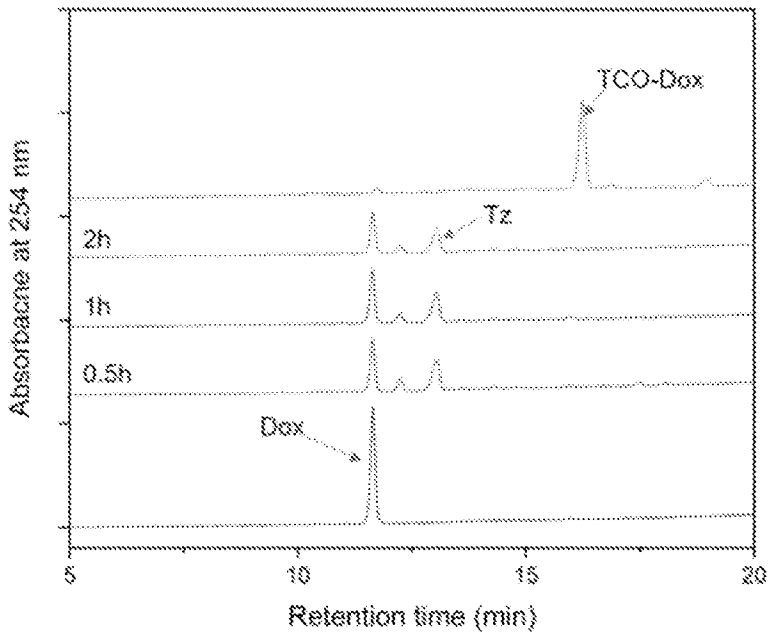

TCO-Dox
Tz
2h
1h
0.5h
Dox
Absorbacne at 254 nm
5
10
15
20
Retention time (min)

FIG. 18

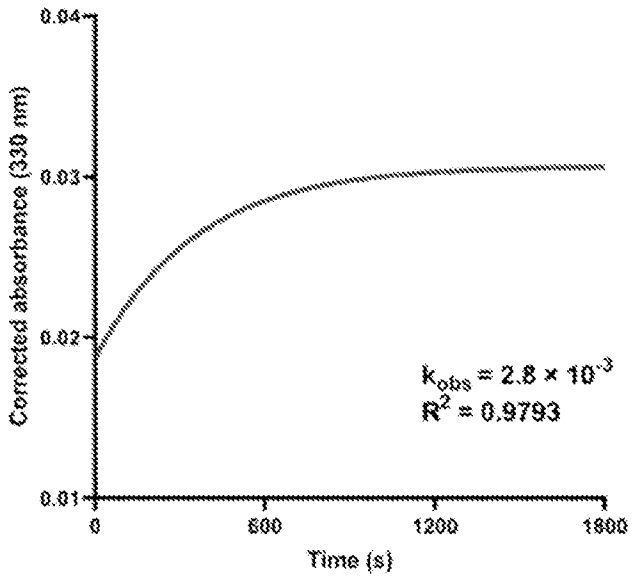

0.04
0.03
0.02
0.01
Corrected absorbance (330 nm)
k_{obs} = 2.8 × 10^{-3}
R^2 = 0.9793
0
600
1200
1800
Time (s)

FIG. 19A

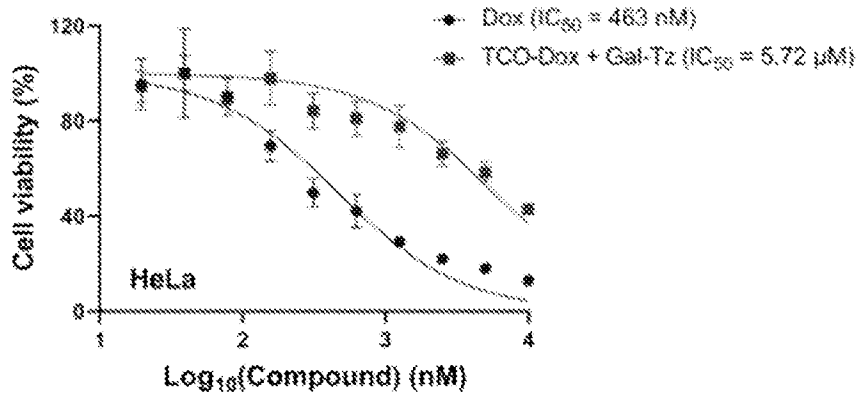


FIG. 19B

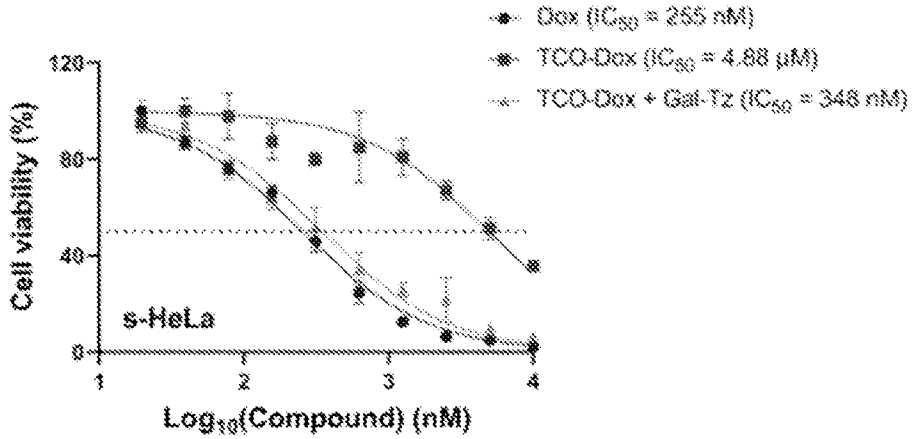


FIG. 20A

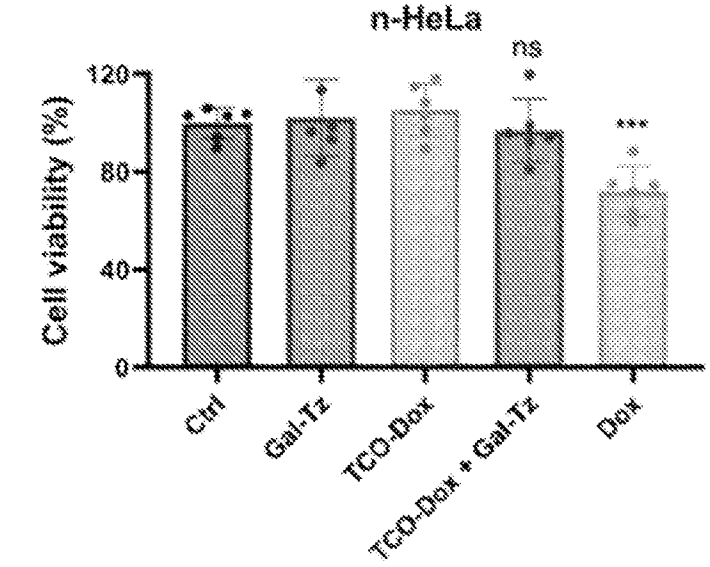


FIG. 20B

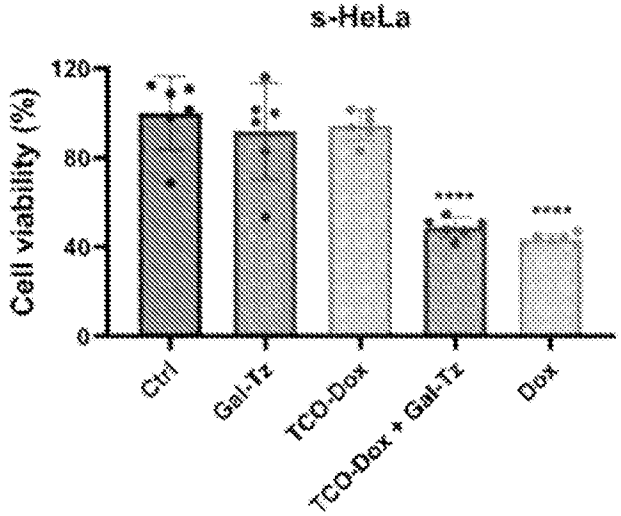


FIG. 21A

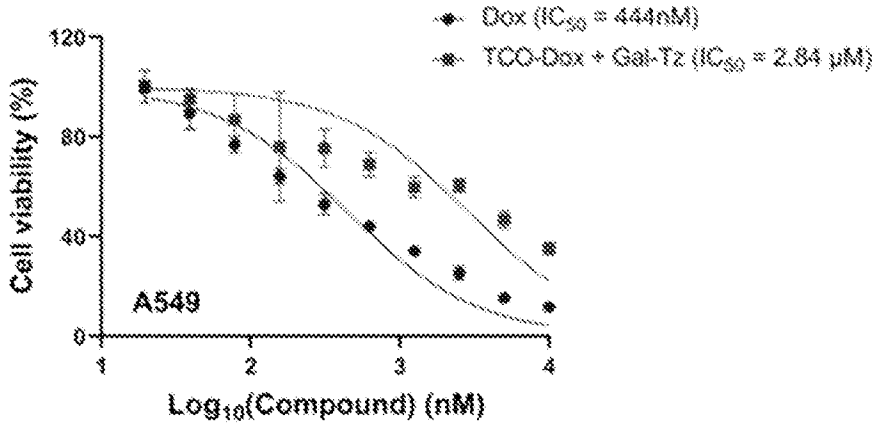


FIG. 21B

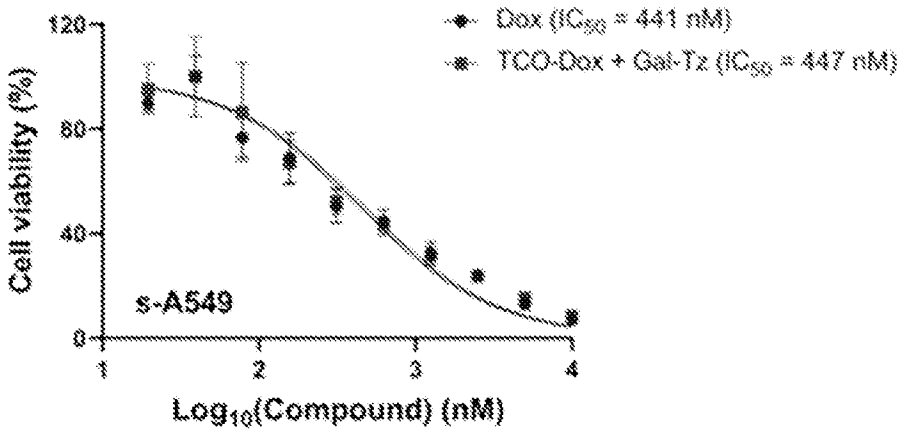


FIG. 22

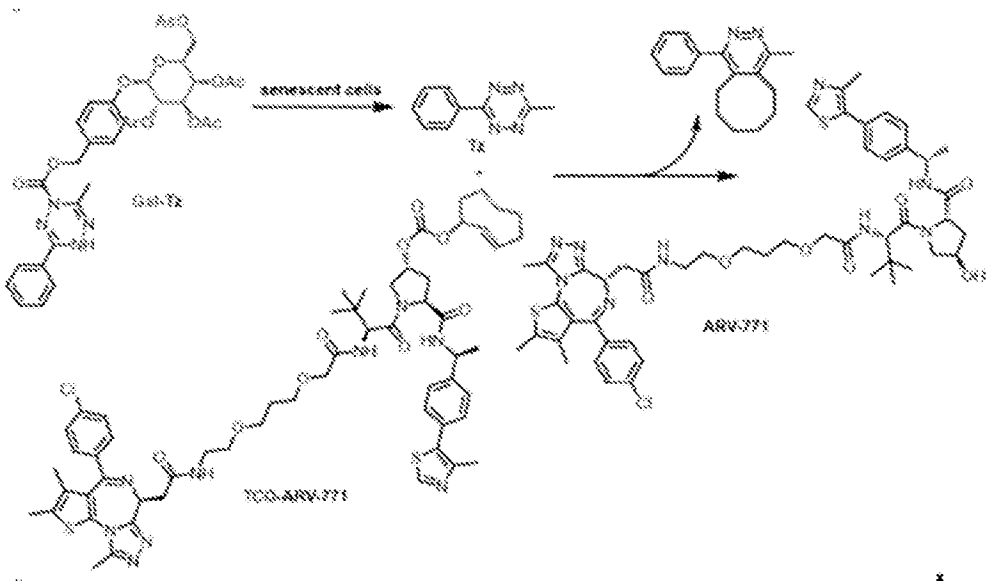


FIG. 23A

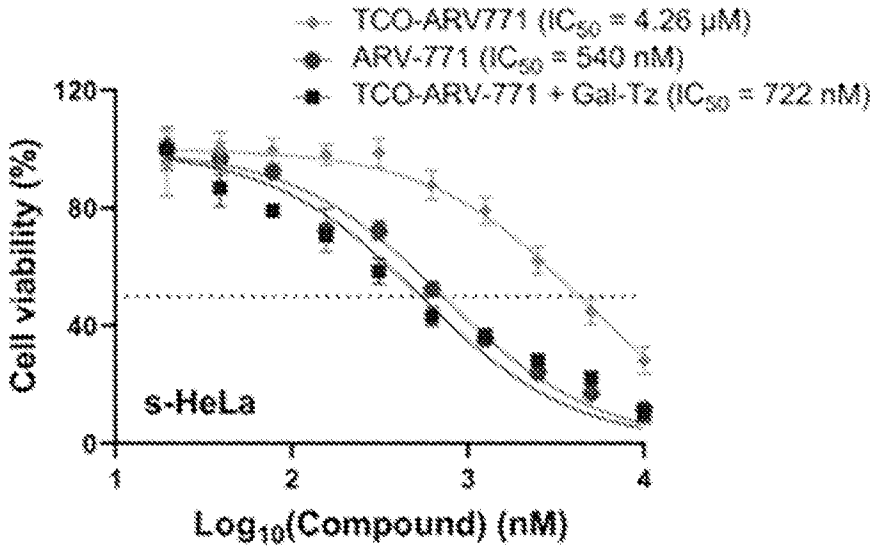


FIG. 23B

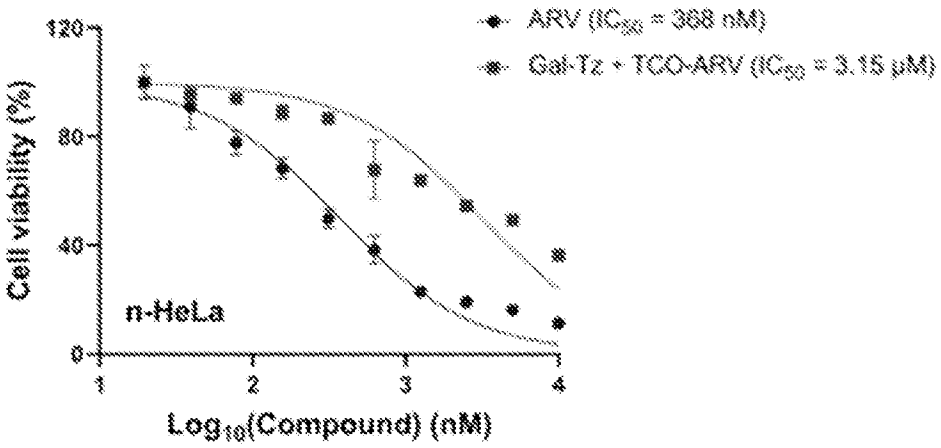


FIG. 24A

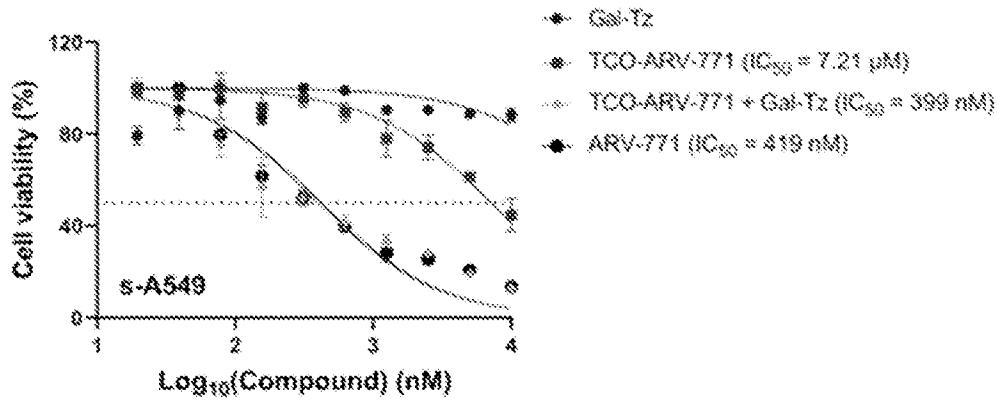


FIG. 24B

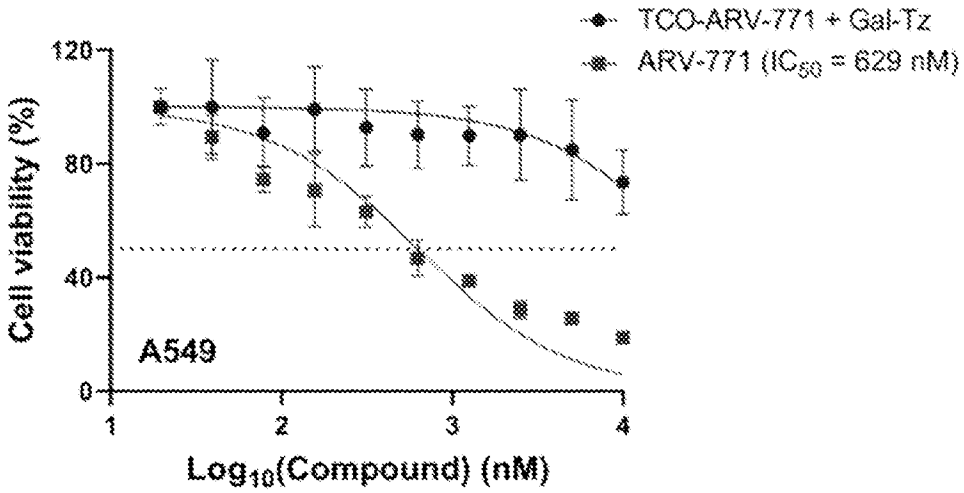


FIG. 25A

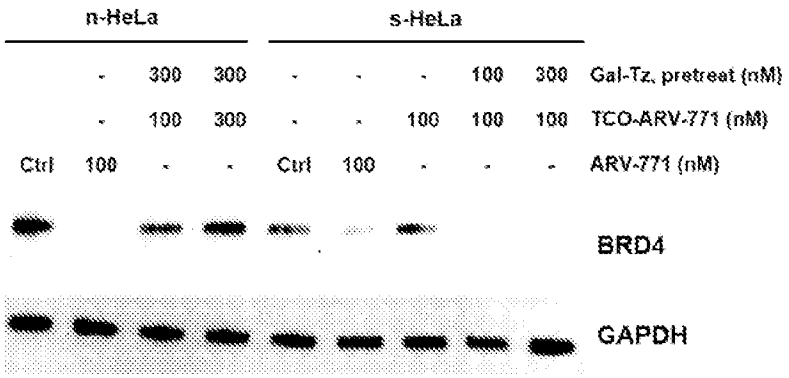


FIG. 25B

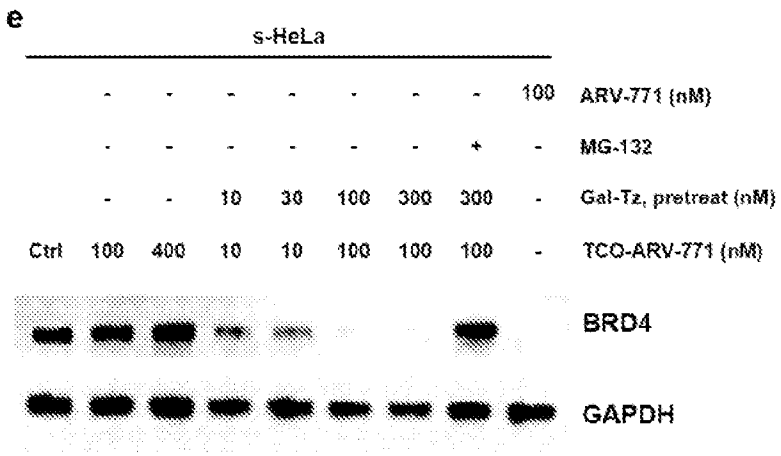


FIG. 25C

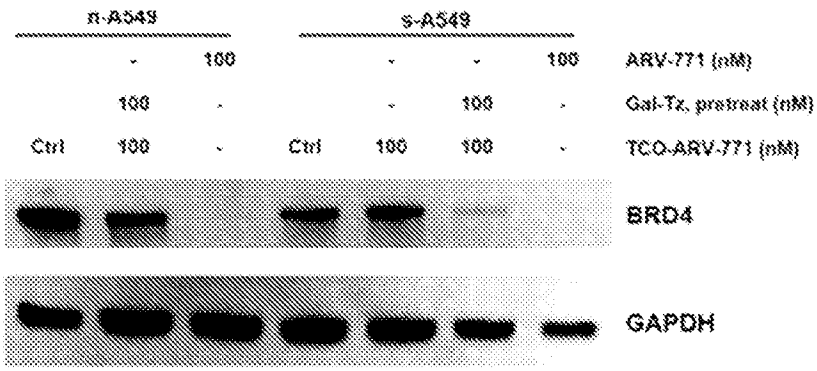


FIG. 26

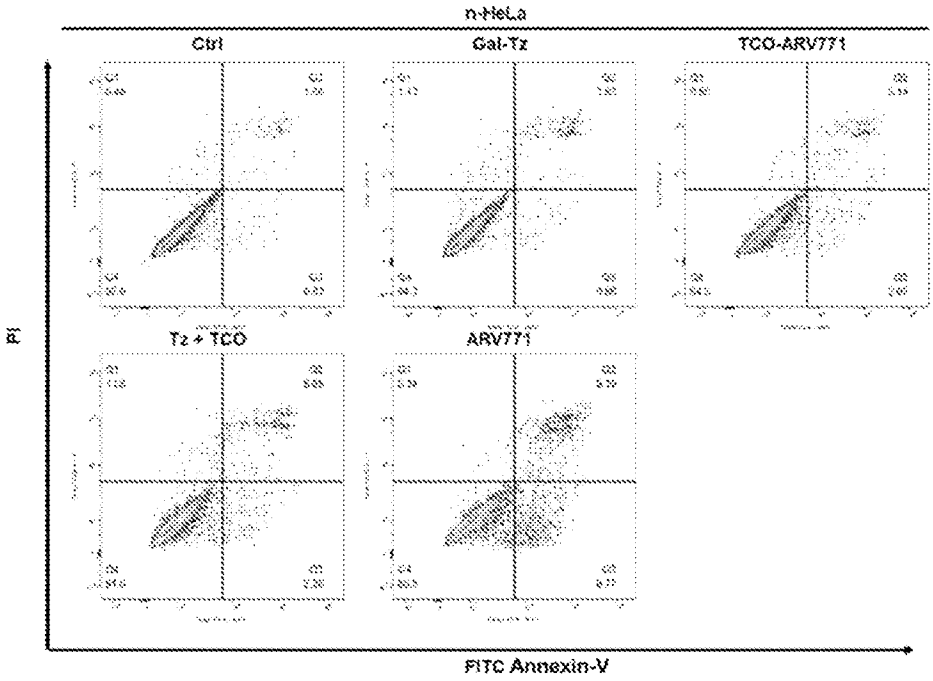

n-HeLa
Ctrl
Gal-Tz
TCO-ARV771
Tz + TCO
ARV771
PI
FITC Annexin-V

FIG. 27

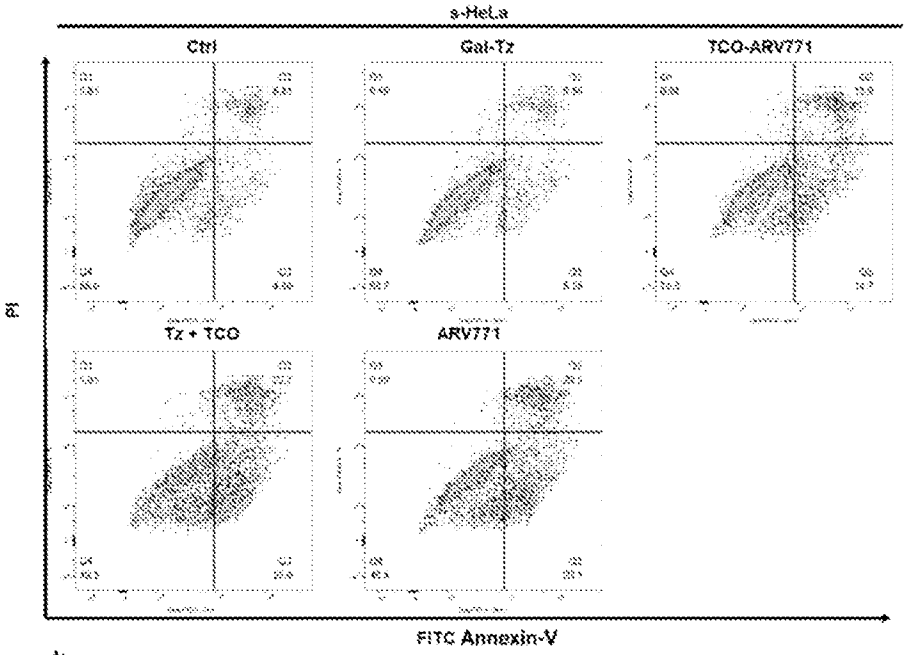

s-HeLa
Ctrl
Gal-Tz
TCO-ARV771
Tz + TCO
ARV771
PI
FITC Annexin-V

FIG. 28

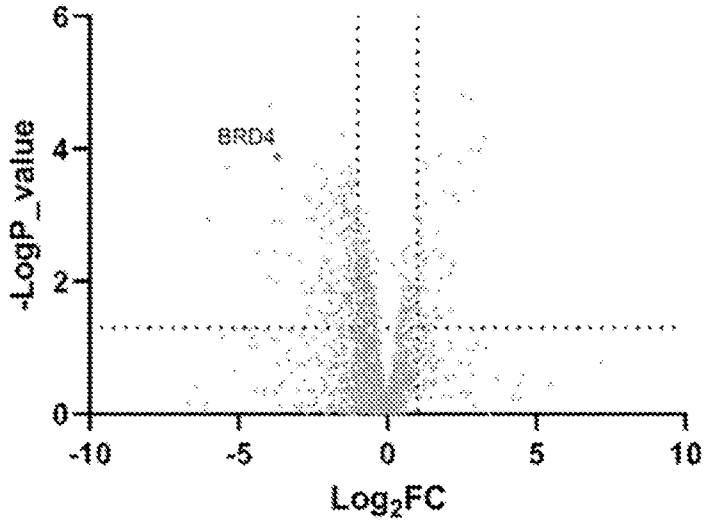

6
4
2
0
-LogP_value
BRD4
-10
-5
0
5
10
Log2FC

FIG. 29

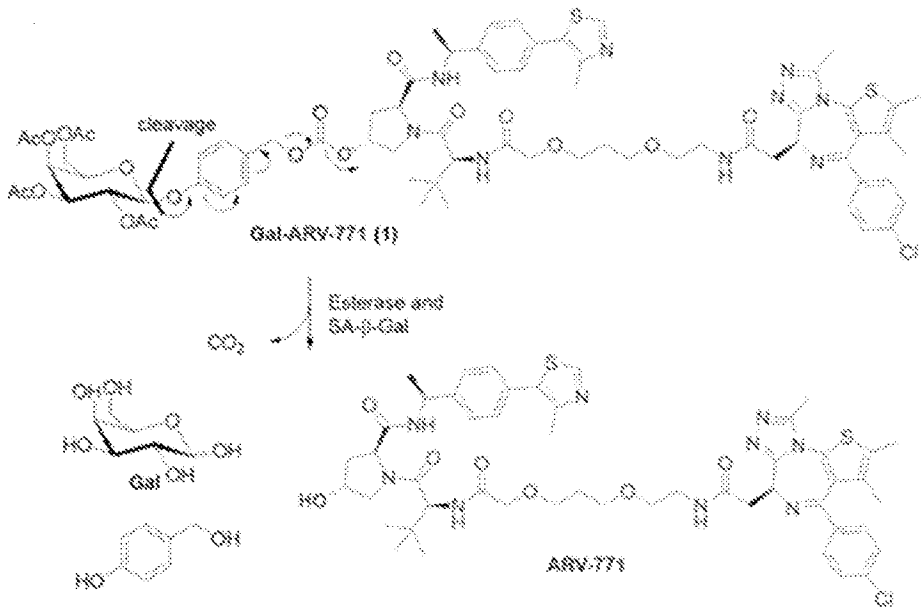
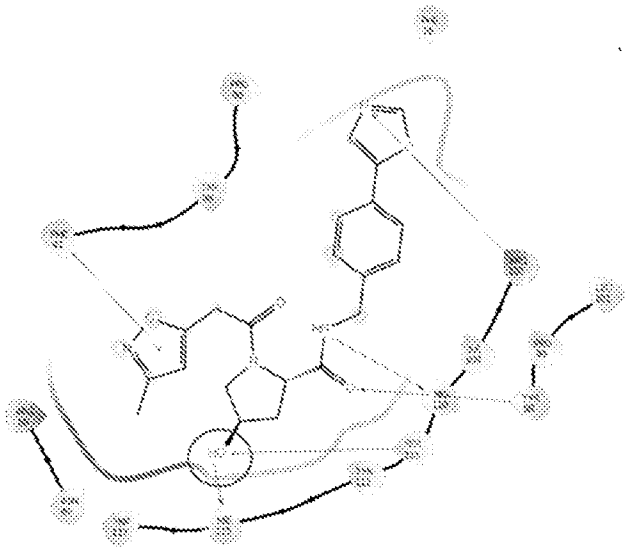
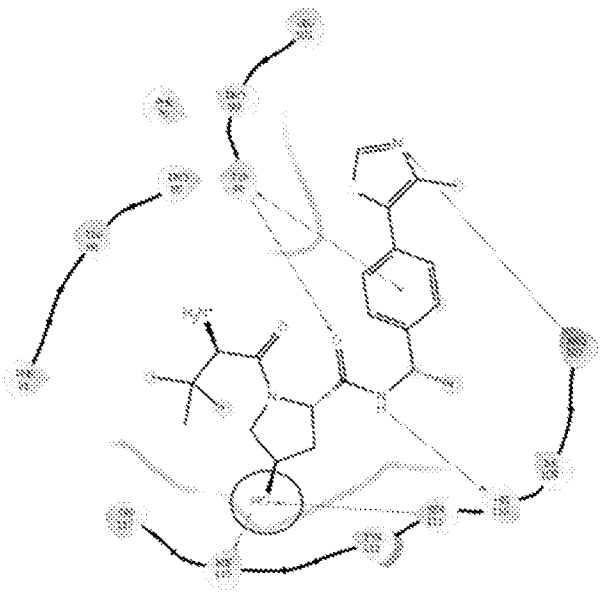

cleavage
Gal-ARV-771 (1)
Esterase and
SA-β-Gal
Gal
ARV-771

FIG. 30A



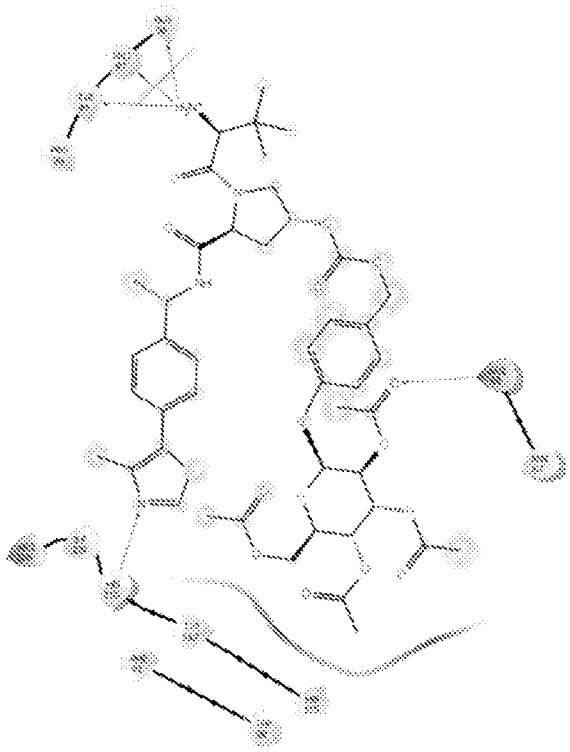
Docking score: -9.277

FIG. 30B



Docking score: -8.760

FIG. 30C



Docking score: -3.314

FIG. 31A

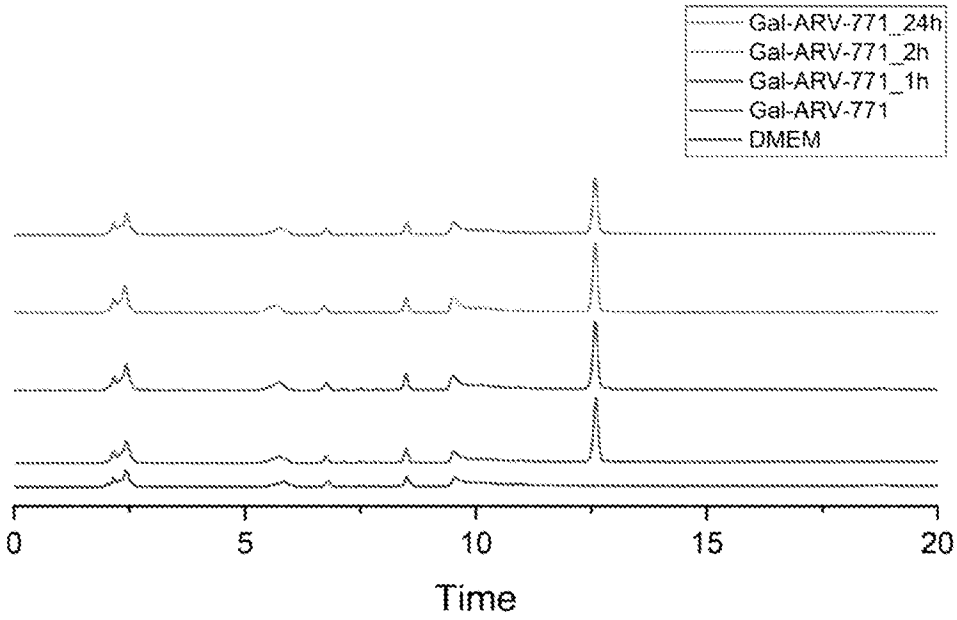


FIG. 31B

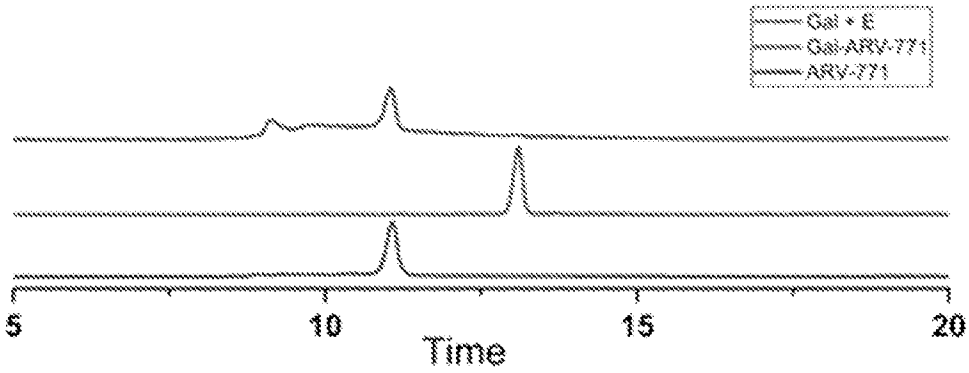


FIG. 32

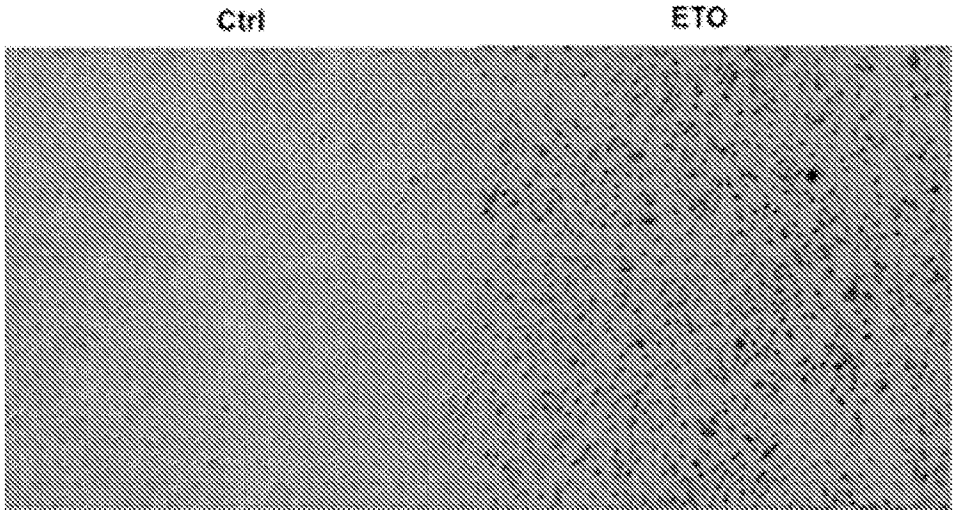


FIG. 33A

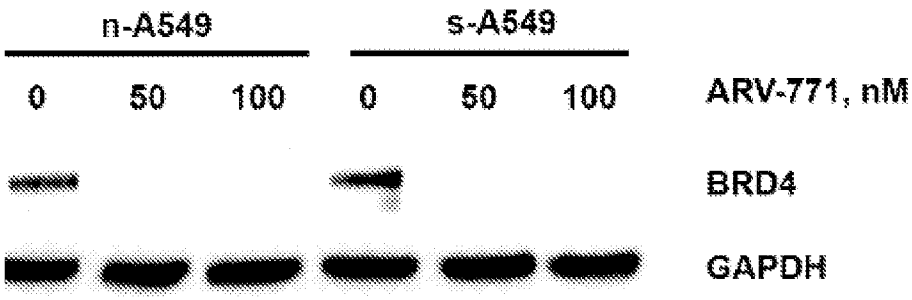


FIG. 33B

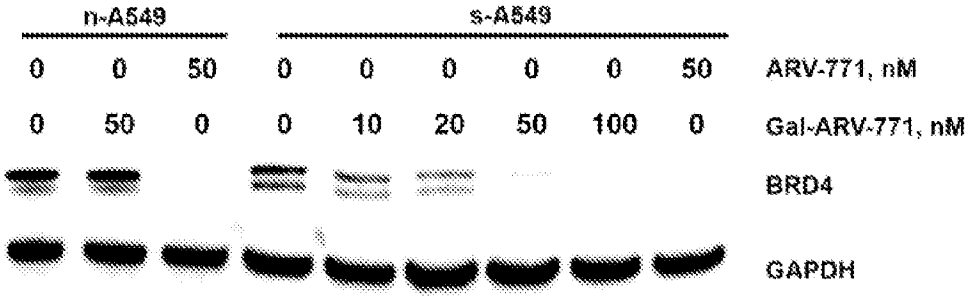


FIG. 34A

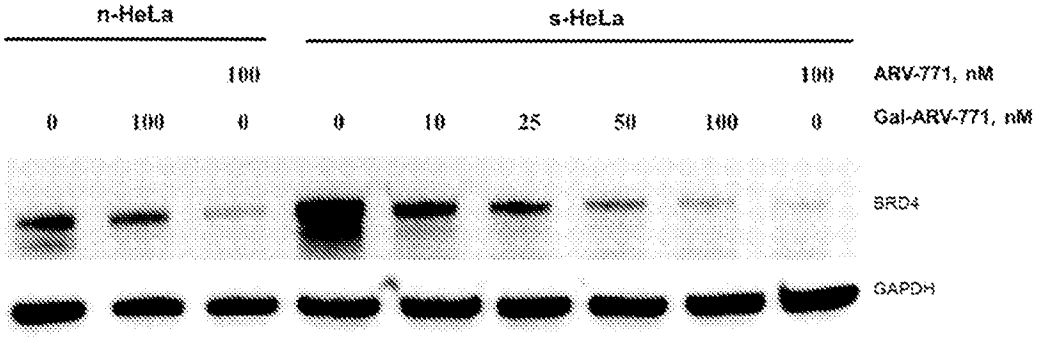


FIG. 34B

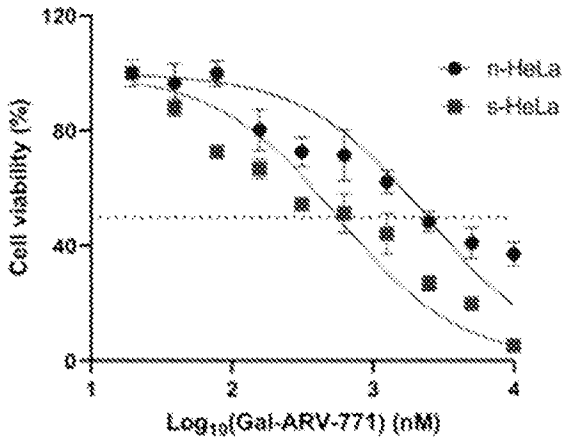


FIG. 34C

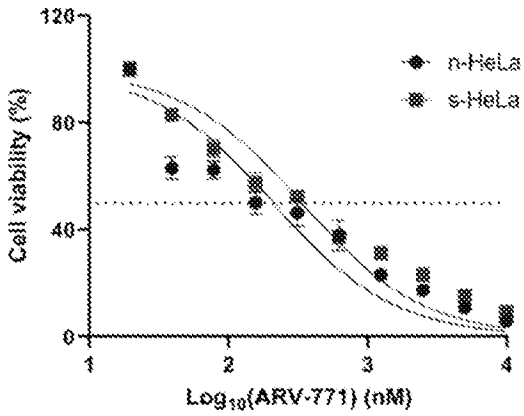


FIG. 35A

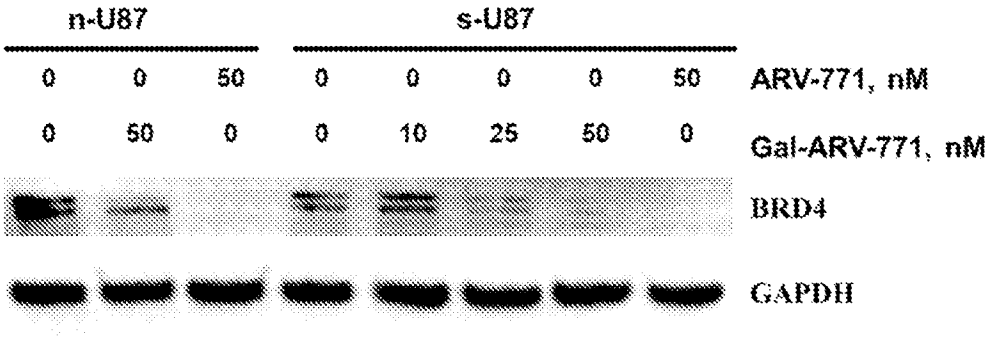


FIG. 35B

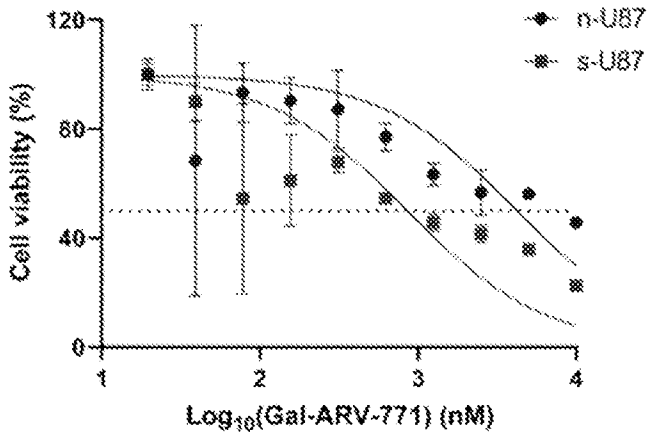


FIG. 35C

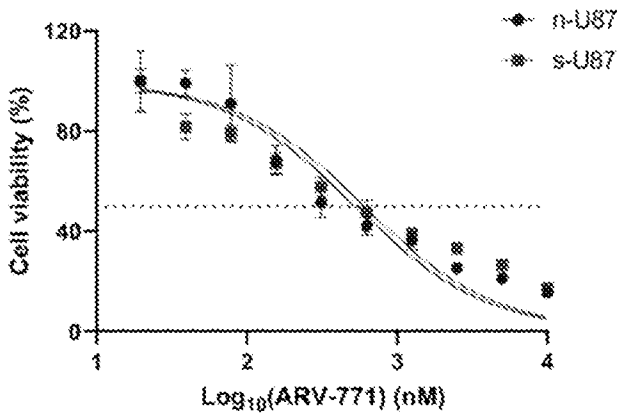


FIG. 36A

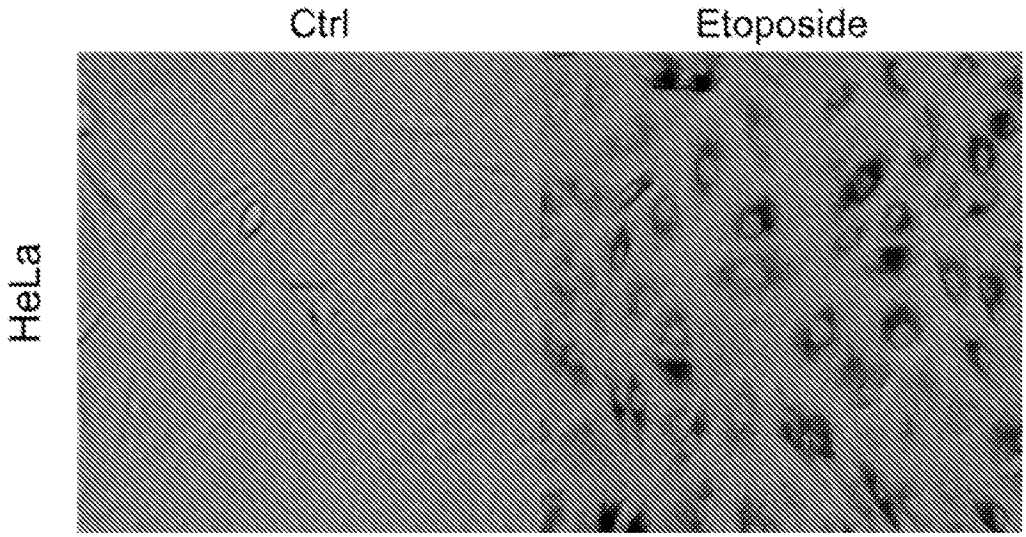


FIG. 36B

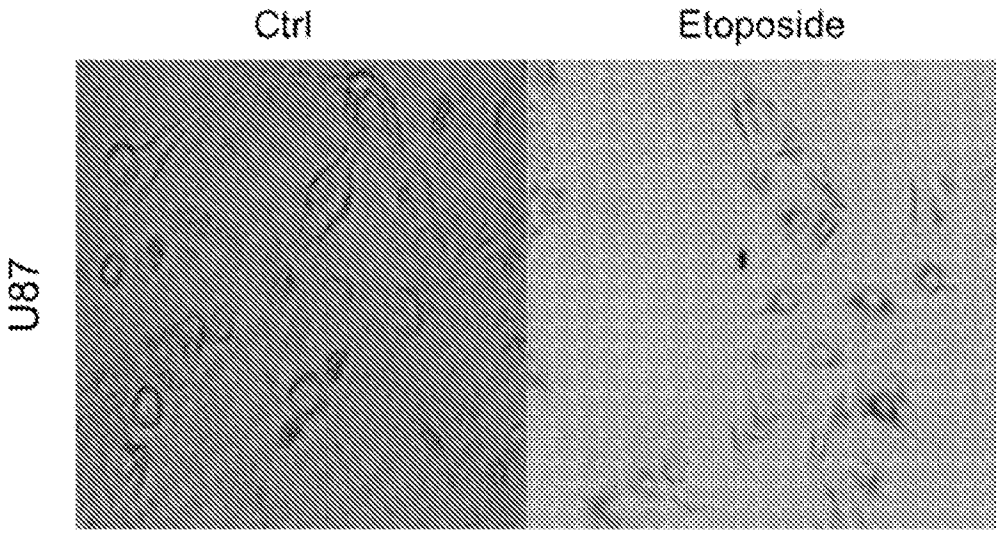


FIG. 37

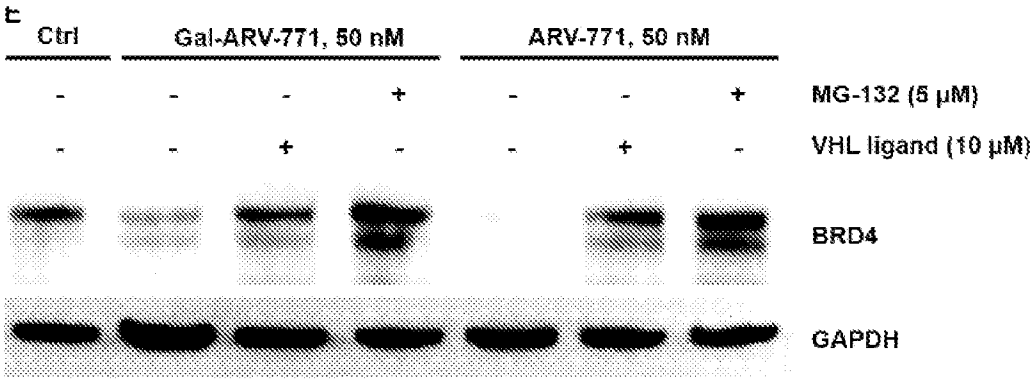


FIG. 38A

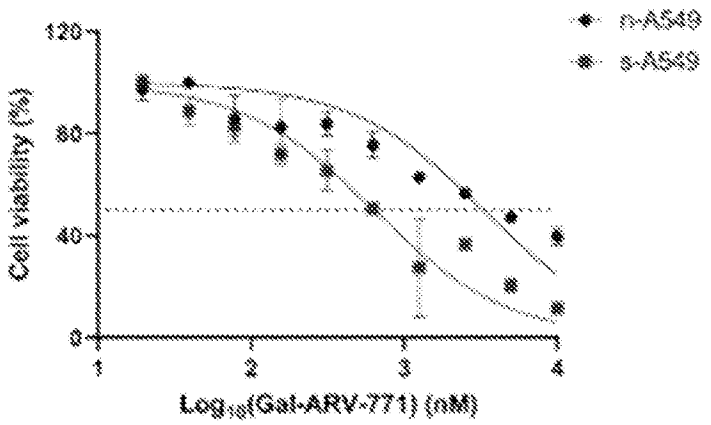


FIG. 38B

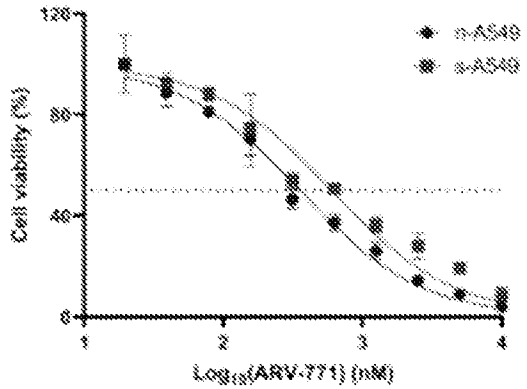


FIG. 39

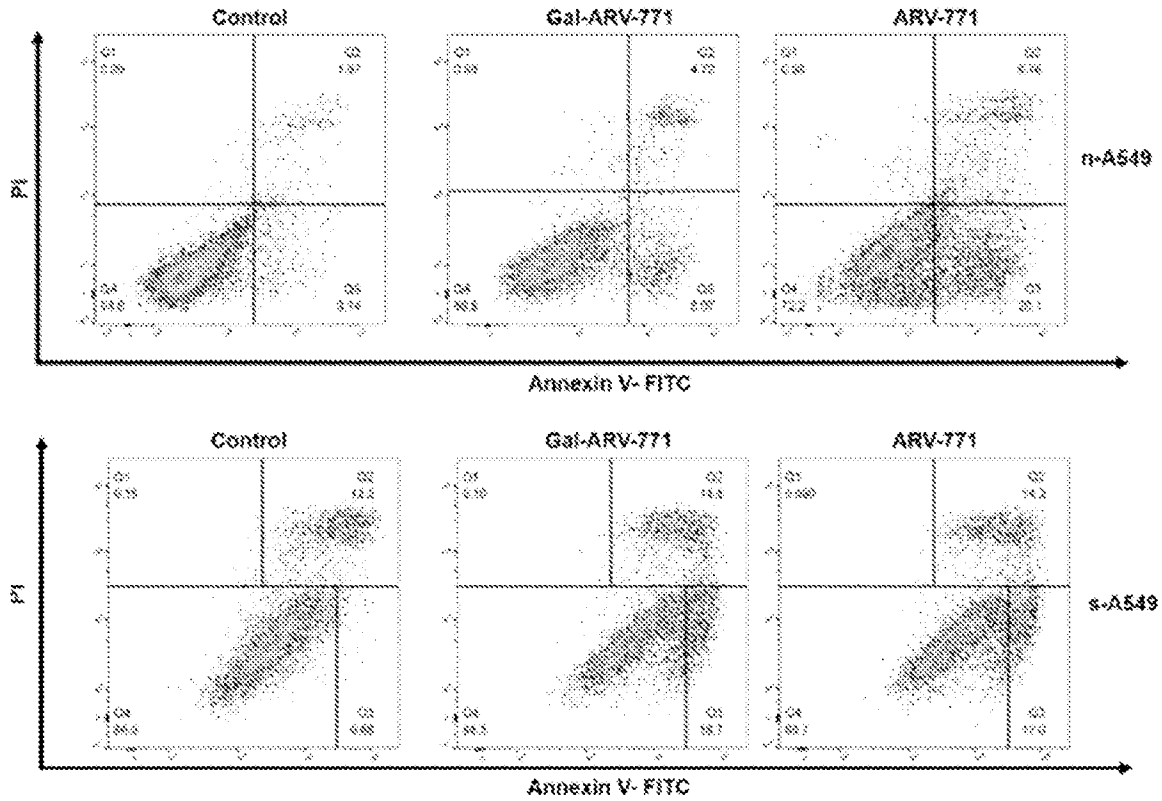

Control
Gal-ARV-771
ARV-771
n-A549
PI
Annexin V- FITC
Control
Gal-ARV-771
ARV-771
s-A549
PI
Annexin V- FITC

FIG. 40A

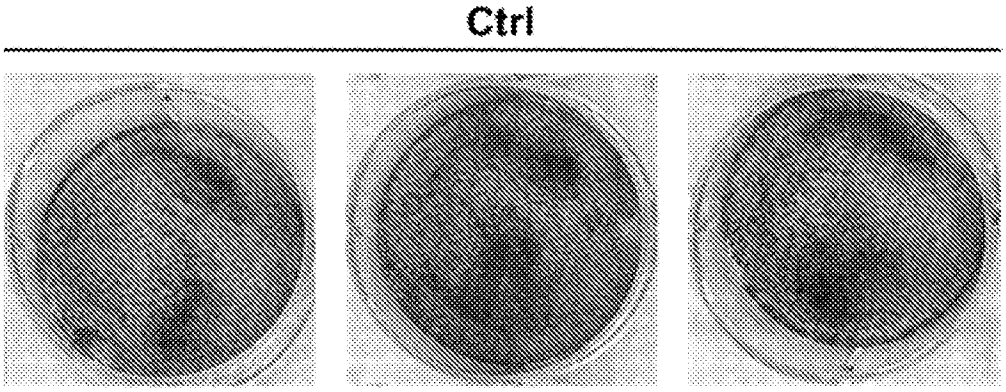


FIG. 40B

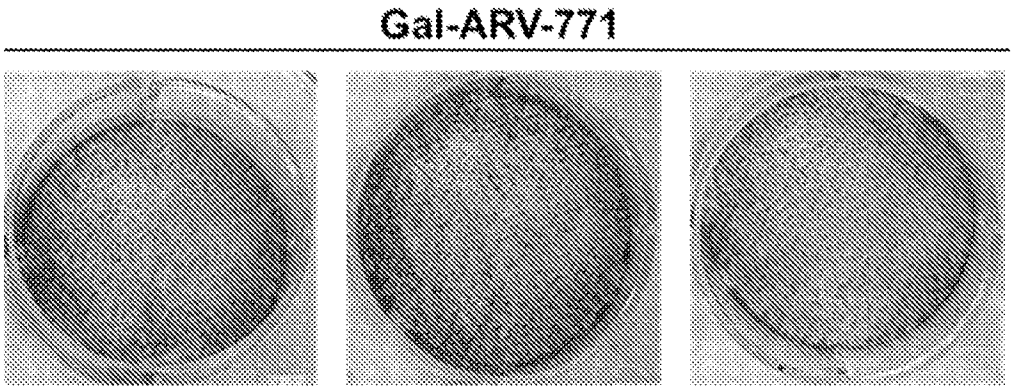


FIG. 40C

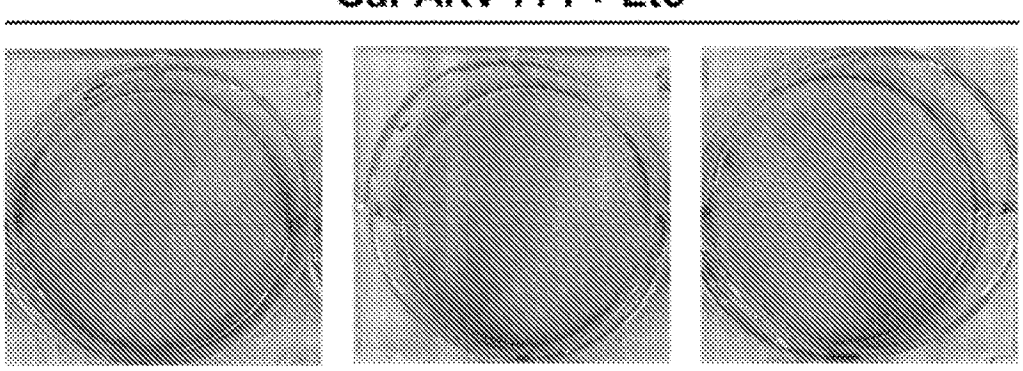


FIG. 40D

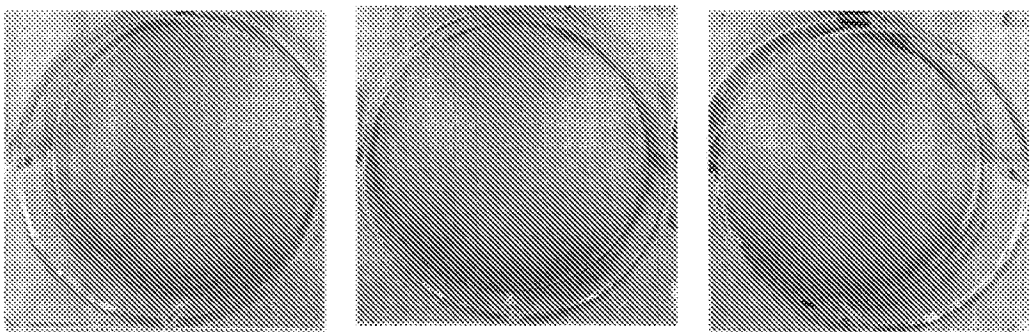
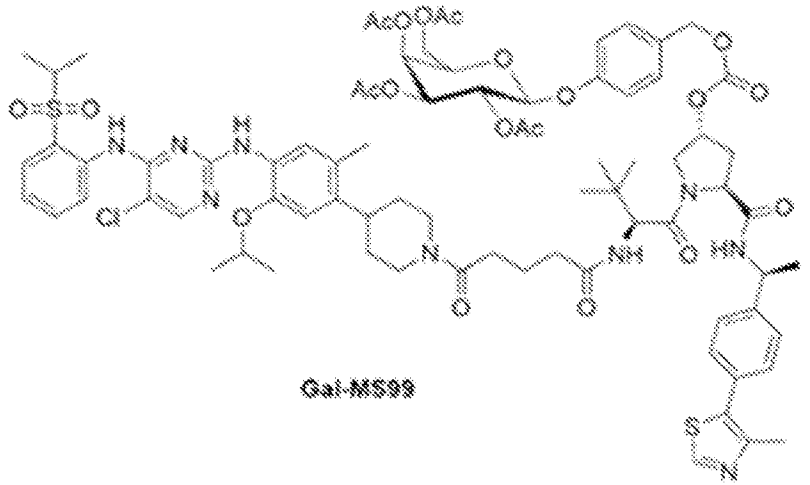


FIG. 41



Gal-MS99

FIG. 42

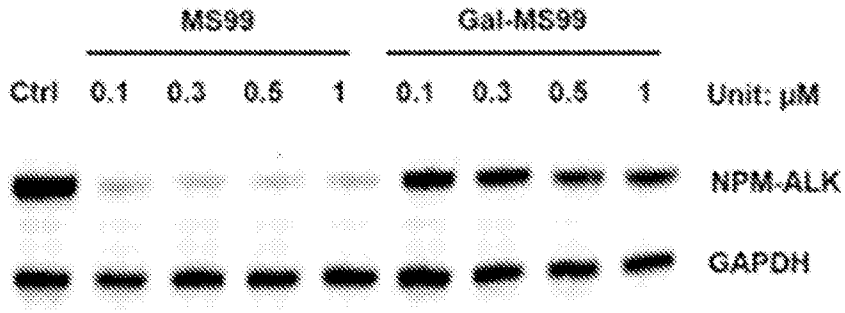


FIG.43

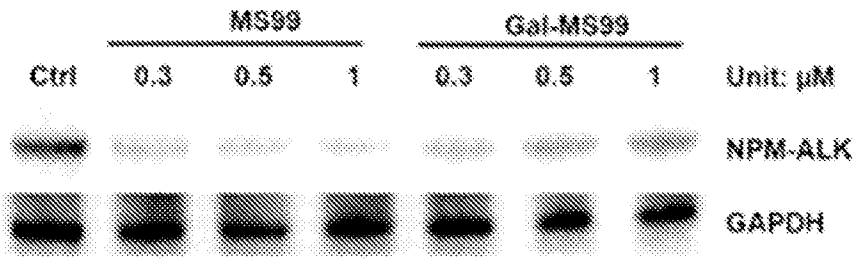


FIG. 44A

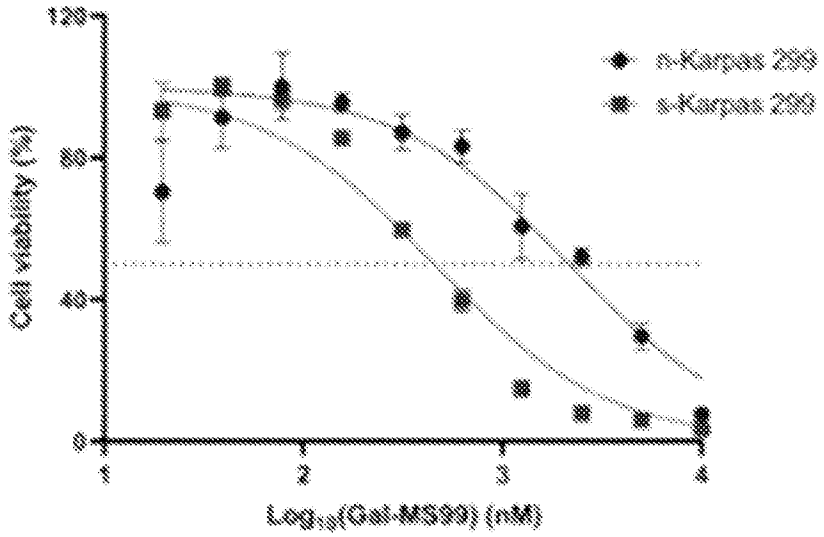


FIG. 44B

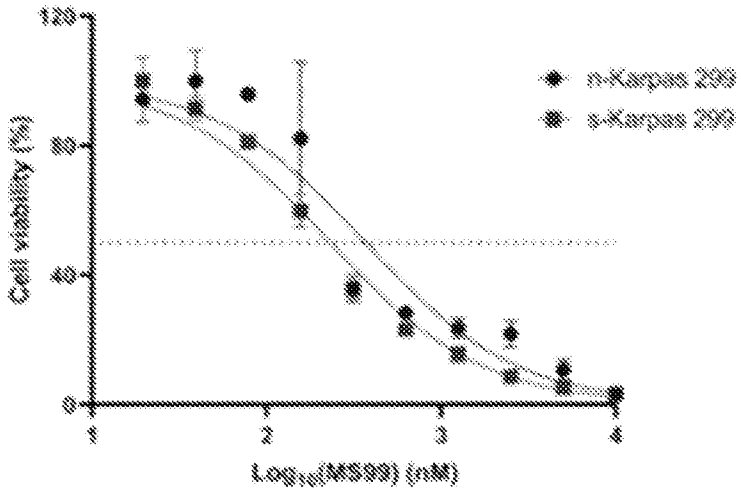


FIG. 45A

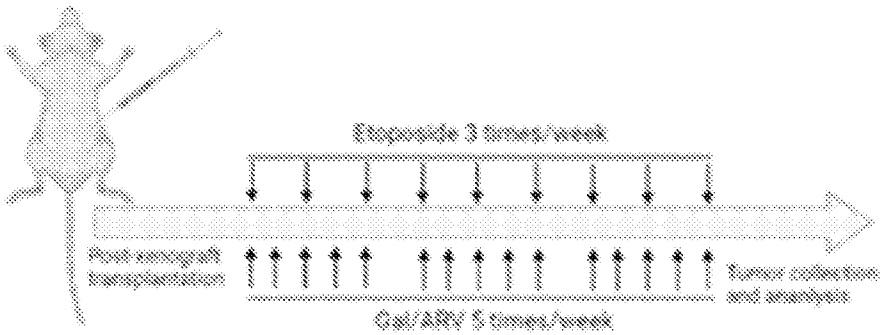


FIG. 45B

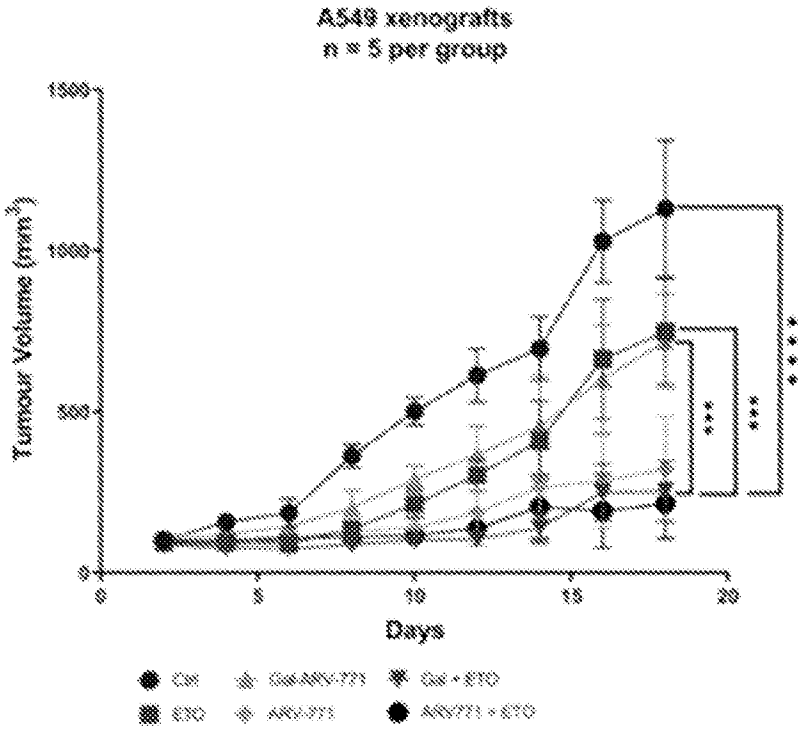


FIG. 45C

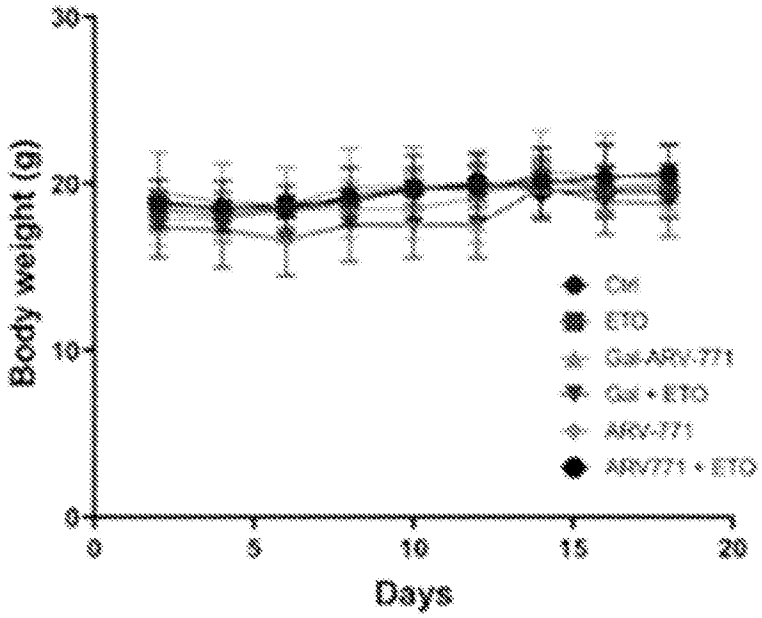

Body weight (g)
30
20
10
0
0
5
10
15
20
Days
Ctrl
ETO
Ctrl-ARV-771
Ctrl + ETO
ARV-771
ARV771 + ETO

FIG. 46A

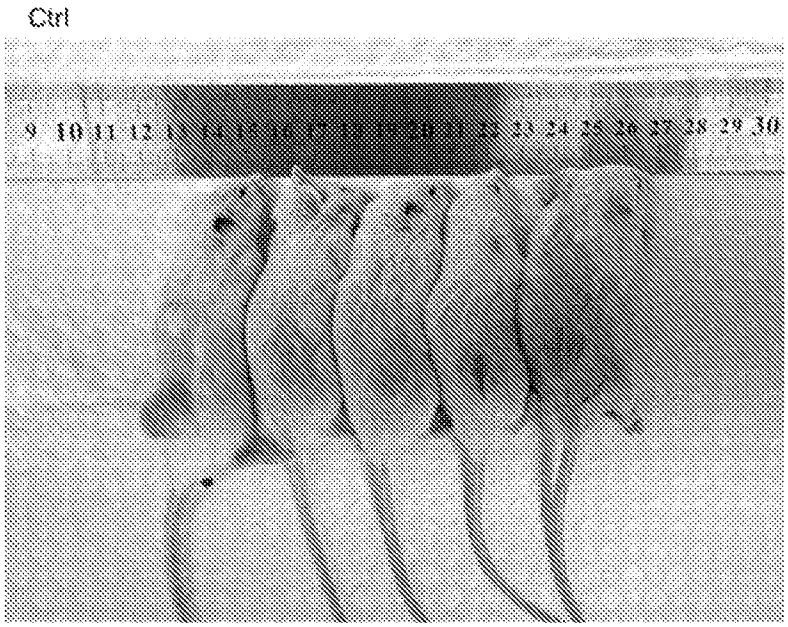


FIG. 46B

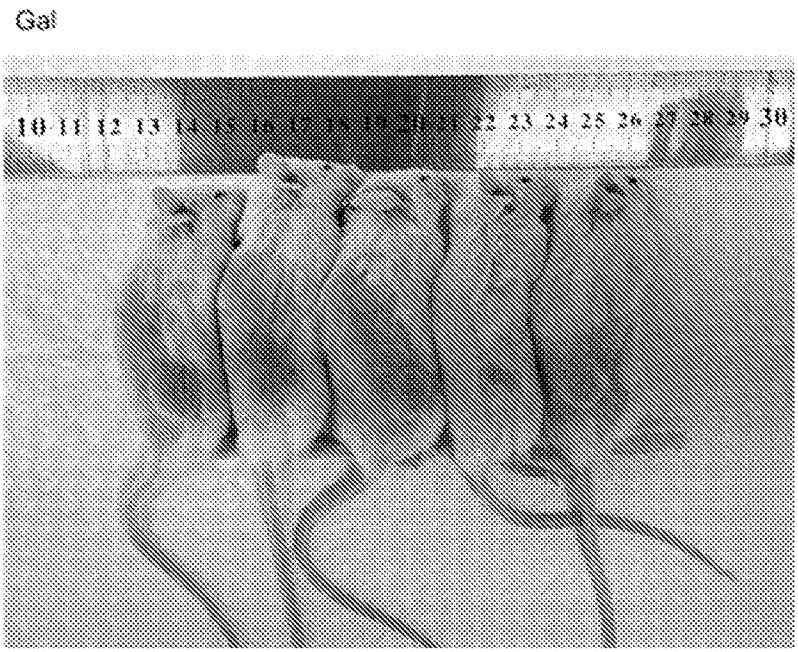


FIG. 46C

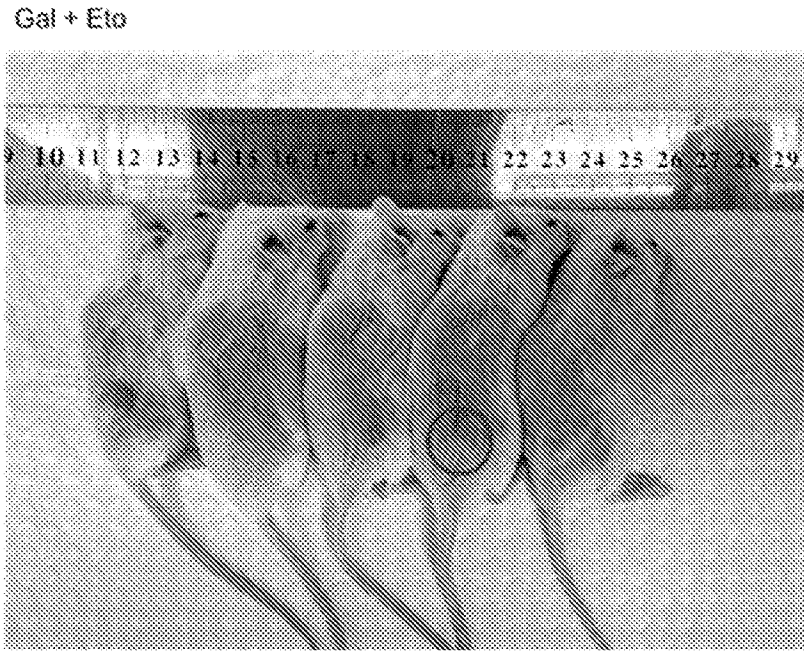


FIG. 47

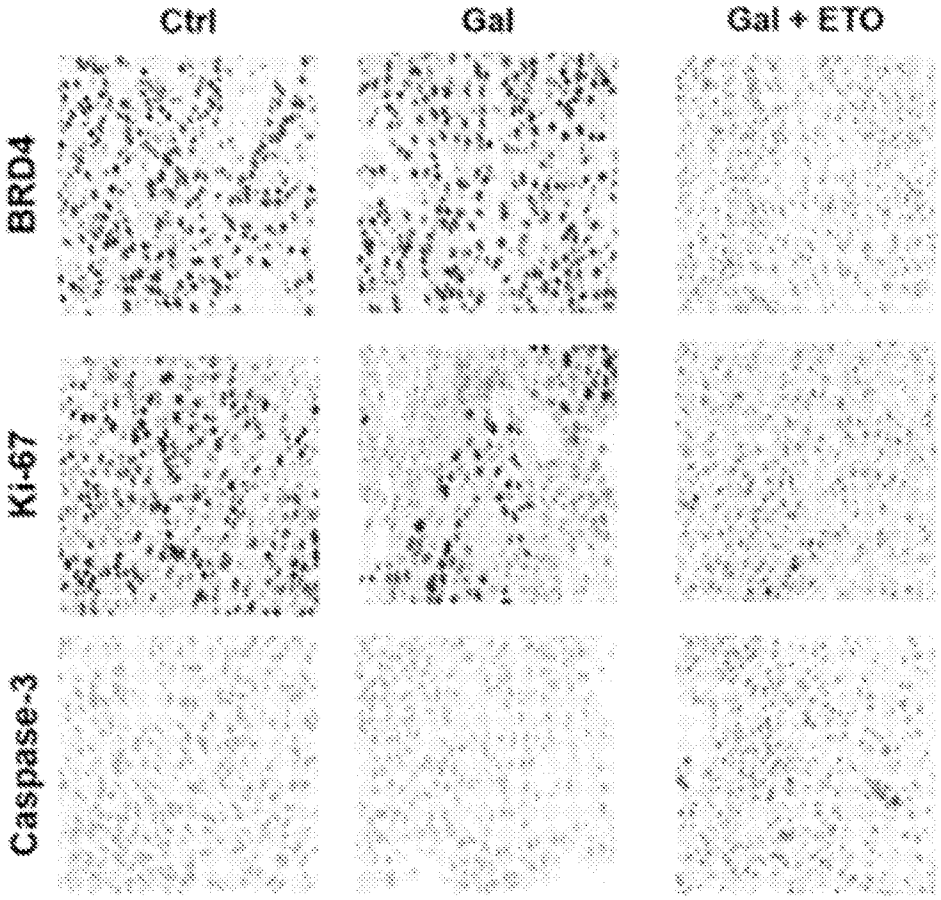


FIG. 47B

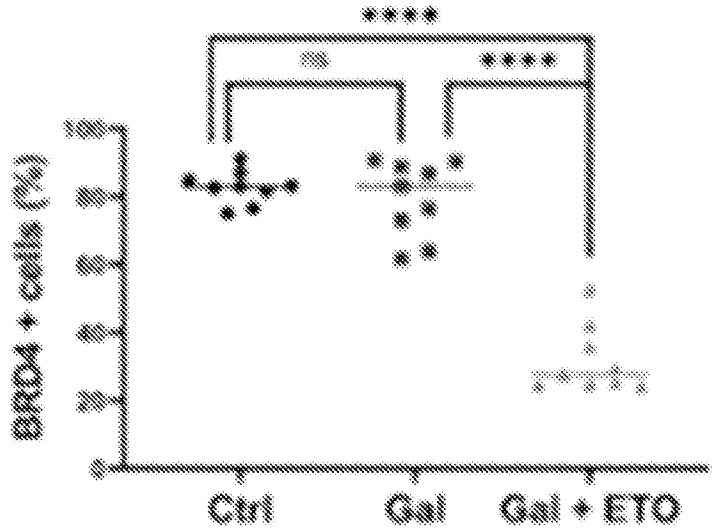


FIG. 47C

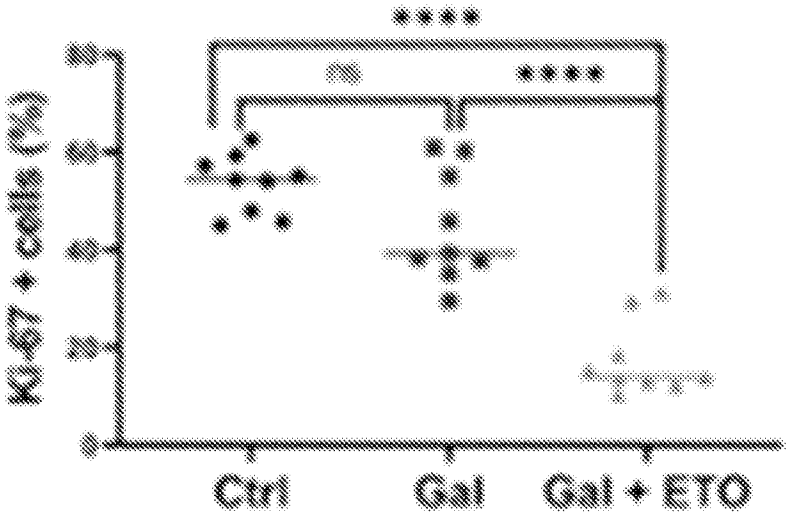


FIG. 47D

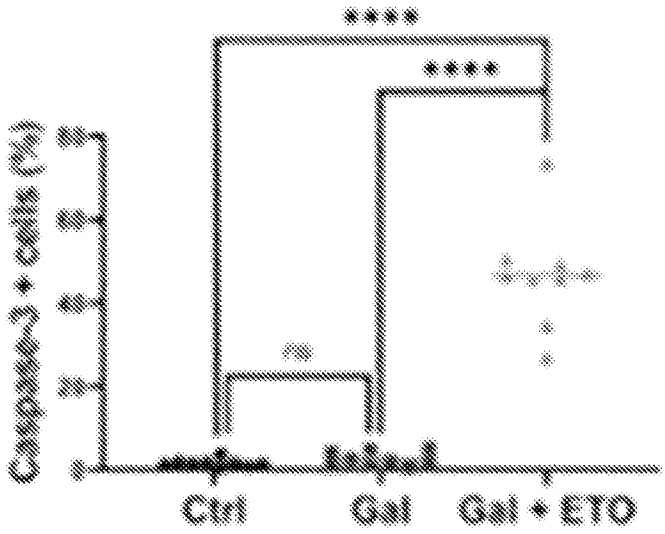


FIG. 49A

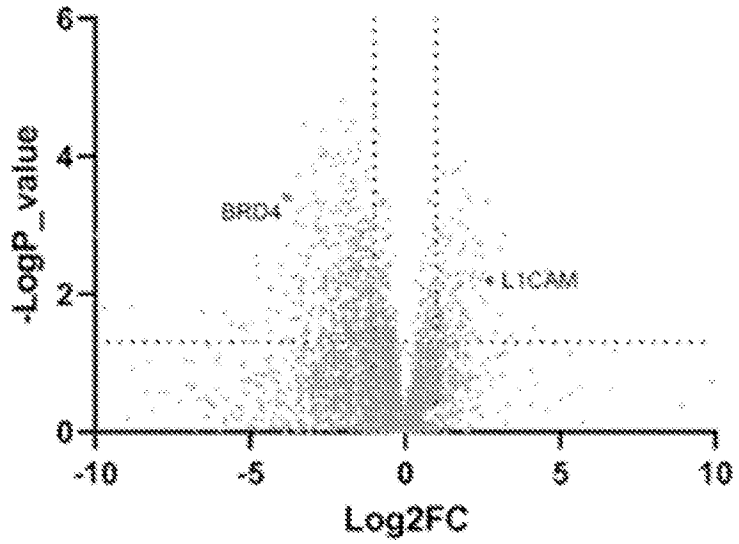
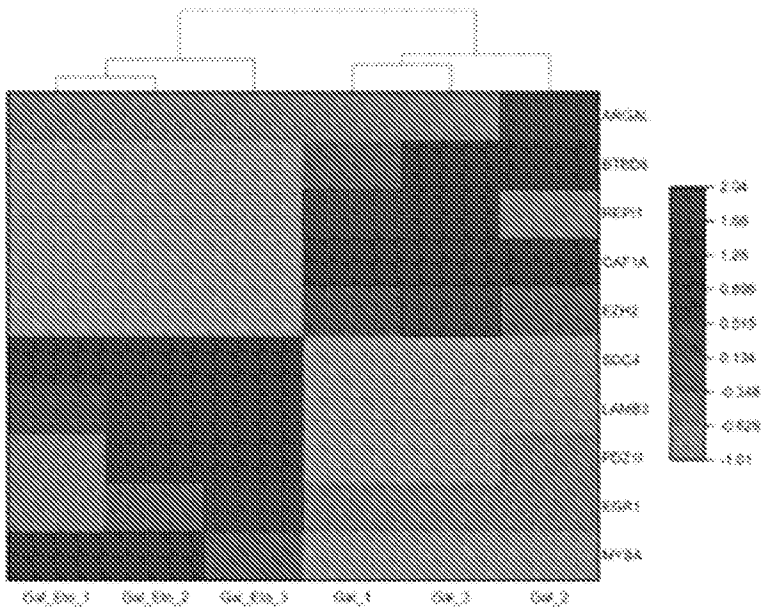


FIG. 49B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 24/18886

A. CLASSIFICATION OF SUBJECT MATTER

IPC - INV. A61K 31/395, A61K 31/4965, A61K 31/495, A61K 31/13 (2024.01)
ADD. A61K 31/33 (2024.01)

CPC - INV. A61K 31/395, A61K 31/4965, A61K 31/495, A61K 31/13
ADD. A61K 31/33

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 --- A	US 2018/0256586 A1 (Arvinas Inc) 13 September 2018 (13.09.2018); para[0017] para[0033] para[0056] para[1361] para[1404]	48 --- 21 --- 1-20, 22-47, 49
Y	US 2021/0030900 A1 (The University Of Melbourne) 04 February 2021 (04.02.2021); para[0022] para[0023]	21
A	Lebraud et al. 'Protein Degradation by In-Cell Self-Assembly of Proteolysis Targeting Chimeras', ACS Central Science, 05 December 2016 (05.12.2016), Vol.2, pages927-934; p928	1-20, 22
A	US 2019/0275161 A1 (Otsuka Pharmaceutical Co Ltd.) 12 September 2019 (12.09.2019); Abstract para[0013] para[0040] para[0057] para[0233]	1-20, 22-37, 38-47, 49
A	Tsai et al. 'Single-Domain Antibodies as Crystallization Chaperones to Enable Structure-Based Inhibitor Development for RBR E3 Ubiquitin Ligases', Cell Chemical Biology, 05 December 20	1-20, 22
A	WO 2021/198965 A1 (Orum Therapeutics, Inc.) 07 October 2021 (07.10.2021); p101 para[0133] para[0413] para[0472]	23-37, 38-47, 49
E/A	Chang et al. 'Selective Elimination of Senescent Cancer Cells by Galacto-Modified PROTACs', Journal of Medicinal Chemistry, 18 April 2024 (18.04.2024), Vol.67, pages7301-7311; entire document	1-49

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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"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
21 May 2024	JUL 01 2024
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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 24/18886

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P/A	Chang et al. 'Senolysis Enabled by Senescent Cell-Sensitive Bioorthogonal Tetrazine Ligation', Angewandte Chemie International Edition, 17 January 2024 (17.01.2024), Vol.6, pages1-8; entire document	1-49