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(54) Title: PHARMACEUTICAL COMBINATIONS FOR USE IN THE TREATMENT OF CANCER

(57) Abstract: Combinations of a CDK 4/6 inhibitor and a glutaminase inhibitor are useful for the treatment of cancer, particularly: colon cancer, breast cancer, melanoma, glioblastoma, osteosarcoma, cervical cancer, lymphoma, squamous cell skin cancer, and glioma. A preferred CDK 4/6 inhibitor is 6-acetyl-8-cyclopentyl-5-methyl-2-((5-(piperazin-1-yl)pyridin-2-yl)amino)pyrido[2,3-d]pyrimidin-7(8H)-one hydrochloride. Preferred glutaminase inhibitors are bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide and 2-(pyridin-2-yl)-N-(5-(4-(6-(2-(3-(trifluoromethoxy)phenyl)acetamido)pyridazin-3-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide. The combinations present a synergistic effect in the treatment of mammals, including humans. The administration may be simultaneous, separated or sequential.



Pharmaceutical combinations for use in the treatment of cancer

The invention relates to the field of human therapy, particularly of cancer treatment.

5 BACKGROUND ART

Genes that have a role in the cell cycle control (checkpoints, regulation of transcription or cell cycle progression) are frequently altered in cancer. Cyclin-dependent kinases CDK4 and CDK6 are promising targets for inhibiting cell cycle progression since their
10 overexpression is implicated in a wide range of human cancers but little is known of the metabolic consequences associated to this deregulation. The deregulation of the cell cycle regulatory network through CDK4/6 inhibition should produce a large list of vulnerabilities associated to metabolic and gene expression modifications.

15 The use of CDK 4/6 inhibitors such as palbociclib (PD0332991), LEE011 and LY2835219 are currently under clinical trials (S. Diaz-Moralli et al. "Targeting cell cycle regulation in cancer therapy", *Pharmacol. Ther.* 2013, vol. 138, pp. 255-271; and M.C. Casimiro et al., "Overview of cyclins D1 function in cancer and the CDK inhibitor landscape: Past and present", *Expert Opin. Investig. Drugs* 2014, vol. 23, pp. 295-304). PD0332991 has been
20 extensively studied for its efficacy in tissue culture model systems as well as in mouse xenograft models of colorectal cancer among others (S. J. Baker et al., "CDK4: A key player in the cell cycle, development, and cancer", *Genes Cancer* 2012, vol. 3, pp. 658-669). However, the efficacy profile of PD0332991 has been somewhat disappointing.

25 Glutaminase is the first essential enzyme for mitochondrial glutamine metabolism and catalyses the conversion of glutamine to glutamate, an amino acid required by cells for several crucial functions. Most cancer cells need glutamine to thrive while non-tumor cells do not show this pronounced dependence on glutamine. The dependence on glutamine metabolism probably results from an alternate use of the tricarboxylic acid (TCA) cycle to
30 generate energy and building blocks in tumor cells, requiring a higher supply of glutamate into the TCA cycle through α -ketoglutarate (α -KG) to replenish the TCA cycle intermediates.

It is obviously desirable to provide new drugs for improving the treatment of cancer.

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SUMMARY OF INVENTION

An aspect of the present invention relates to a pharmaceutical combination comprising a therapeutically effective amount of a CDK 4/6 inhibitor or a pharmaceutically acceptable

salt thereof, and a therapeutically effective amount of a glutaminase inhibitor or a pharmaceutically acceptable salt thereof. The term "combination" as used herein refers to the combined administration of the CDK 4/6 inhibitor and the glutaminase inhibitor, which may be administered together in the same composition (i.e. through a single pharmaceutical product) or as separate compositions (i.e. through separate pharmaceutical products).

A "CDK 4/6 inhibitor" refers to a dual cyclin-dependent kinase inhibitor of cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 6 (CDK6), which inhibits the CDK4 and the CDK6. In particular embodiments of the present invention, the CDK 4/6 inhibitor is 6-acetyl-8-cyclopentyl-5-methyl-2-((5-(piperazin-1-yl)pyridin-2-yl)amino)pyrido[2,3-d]pyrimidin-7(8H)-one hydrochloride (PD-0332991), 7-cyclopentyl-N,N-dimethyl-2-((5-(piperazin-1-yl)pyridin-2-yl)amino)-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide (LEE011), N,1,4,4-tetramethyl-8-((4-(4-methylpiperazin-1-yl)phenyl)amino)-4,5-dihydro-1H-pyrazolo[4,3-h]quinazoline-3-carboxamide (PHA848125), N-(5-((4-ethylpiperazin-1-yl)methyl)pyridin-2-yl)-5-fluoro-4-(4-fluoro-1-isopropyl-2-methyl-1H-benzo[d]imidazol-6-yl)pyrimidin-2-amine (LY2835219), capridine beta, FLX925, GIT28, GIT30, GIT38, MMD37K, or P276.

In particular embodiments of the present invention the glutaminase inhibitor is O-(2-diazoacetyl)-L-serine (azaserine), 6-diazo-5-oxo-L-norleucine (DON), glutamine, ibotenic acid, L-[aS,5S]-a-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin), bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES), or 2-(pyridin-2-yl)-N-(5-(4-(6-(2-(3-(trifluoromethoxy)phenyl)acetamido)pyridazin-3-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (CB-839).

In particular embodiments, the CDK 4/6 inhibitor is 6-acetyl-8-cyclopentyl-5-methyl-2-((5-(piperazin-1-yl)pyridin-2-yl)amino)pyrido[2,3-d]pyrimidin-7(8H)-one hydrochloride (PD-0332991), and its combinations with the above-mentioned glutaminase inhibitors are particular embodiments of the invention.

In particular embodiments, the glutaminase inhibitor is bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES), and its combinations with the above-mentioned CDK 4/6 inhibitors are particular embodiments of the invention.

In particular embodiments, the glutaminase inhibitor is 2-(pyridin-2-yl)-N-(5-(4-(6-(2-(3-(trifluoromethoxy)phenyl)acetamido)pyridazin-3-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (CB-839), and its combinations with the above mentioned CDK 4/6 inhibitors are particular embodiments of the invention.

In particular embodiments, in the above-mentioned combinations the molar ratio of CDK 4/6 inhibitor to glutaminase inhibitor is from 10:1 to 1:10; preferably of 1:4.

- 5 Other aspect of the present invention relates to pharmaceutical compositions comprising therapeutically effective amounts of any of the above-mentioned combinations, plus pharmaceutically acceptable excipients or carriers.

10 Other aspect of the present invention relates to the above-mentioned combinations for use as active pharmaceutical ingredients. In particular embodiments, they are for use in the treatment of cancer in mammals, including humans. In more particular embodiments, they are for use in colon cancer, breast cancer, melanoma, glioblastoma, osteosarcoma, cervical cancer, lymphoma, squamous cell skin cancer, or glioma. In preferred
15 embodiments, they are for use in the treatment of colon cancer or breast cancer. In other words, the present invention relates to methods of treatment of mammals (humans included) suffering from cancer, comprising the administration of a therapeutically effective amount of any of the above-mentioned combinations. In particular embodiments, the cancer is colon cancer, breast cancer, melanoma, glioblastoma, osteosarcoma, cervical cancer, lymphoma, squamous cell skin cancer, or glioma. In particular
20 embodiments the cancer is colon cancer or breast cancer.

The pharmaceutical combinations of the present invention, as well as their corresponding pharmaceutical compositions, may be administered simultaneously, separately or sequentially. The term "simultaneously" means that the two therapeutic products of the
25 combination are administered concurrently under a single administration. The term "separately" means administration of the components of the combination independently of each other at different time points. The term "sequentially" means that the two therapeutic products of the combination are available to act therapeutically within the same time-frame

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Inventors have found a surprisingly synergistic effect of the combinations of the present invention. The term synergistic effect in the present invention means that the effect that results from using the combination of the CDK 4/6 inhibitor and the glutaminase inhibitor, is greater than the sum of the effects that result from using either the CDK 4/6 inhibitor or
35 the glutaminase inhibitor as a monotherapy.

Throughout the present specification, by the term "treatment" it is meant eliminating, reducing or ameliorating the cause or the effects of a disease. For purposes of this invention treatment includes -but is not limited to- the following: alleviation, amelioration or

elimination of one or more symptoms of the disease; diminishment of the extent of the disease; stabilized (i.e. not worsening) state of disease; delay or slowing of disease progression; amelioration or palliation of the disease state; and remission of the disease (whether partial or total).

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The pharmaceutical compositions of the invention are useful to treat diseases in which CDK 4/6 and glutaminase play a role in mammals, including human beings. These diseases include -but are not limited to- proliferative disorders, particularly colon cancer, breast cancer, melanoma, glioblastoma, osteosarcoma, cervical cancer, lymphoma, squamous cell skin cancer, and glioma (cf. e.g. S. Lapenna et al., *Nat. Rev. Drug. Discov.* 10 2009, vol. 8, pp. 547-566).

Throughout the description and claims the word "comprise" and its variations are not intended to exclude other technical features, additives, components, or steps.

15 Furthermore, the word "comprise" encompasses the case of "consisting of". Additional objects, advantages and features of the invention will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the invention. The following examples and drawings are provided by way of illustration and are not intended to be limiting of the present invention.

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BRIEF DESCRIPTION OF DRAWINGS

FIG. 1A. Synergistic antiproliferative effect of PD0332991 and BPTES combined treatment in HCT116 cells. HCT116 cells were cultured at the indicated concentrations of inhibitors for 96 h and cell proliferation was determined by Hoechst staining. Cell viability 25 (% Viab) was assessed after incubation with PD0332991, BPTES and a combination of the two inhibitors at the indicated concentrations (μM). Results are shown as percentage of proliferation relative to untreated cells (mean $\pm$ SD of $n = 6$). In FIG. 1B-4C, cell viabilities were assessed by analogous procedures.

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FIG. 1B. Synergistic antiproliferative effect of PD0332991 and CB-839 combined treatment in HCT116 cells. HCT116 cells were cultured at the indicated concentrations of inhibitors for 96 h and cell proliferation was determined by Hoechst staining.

35 FIG. 2A. Synergistic antiproliferative effect of PD0332991 and BPTES combined treatment in MCF7 cells. MCF7 cells were cultured at the indicated concentrations of inhibitors for 96 h and cell proliferation was determined by Hoechst staining.

FIG. 2B. Synergistic antiproliferative effect of PD0332991 and CB-839 combined treatment in MCF7 cells. MCF7 cells were cultured at the indicated concentrations of inhibitors for 96 h and cell proliferation was determined by Hoechst staining.

- 5 FIG. 3A. Synergistic antiproliferative effect of PD0332991 and BPTES combined treatment in SKBR3 cells. SKBR3 cells were incubated with PD0332991 and BPTES at the indicated concentrations for 96 h and cell proliferation was determined by Hoechst staining.
- 10 FIG. 3B. Synergistic antiproliferative effect of PD0332991 and CB-839 combined treatment in SKBR3 cells. SKBR3 cells were incubated with PD0332991 and CB-839 at the indicated concentrations for 96 h and cell proliferation was determined by Hoechst staining.
- 15 FIG. 4A. Selective cytotoxicity of PD0332991 and BPTES combined treatment for cancer cells. BJ and HCT116 cells were treated with PD0332991 and BPTES at the indicated concentrations for 96 h and cell proliferation was determined by Hoechst staining.
- FIG. 4B. Selective cytotoxicity of PD0332991 and BPTES combined treatment for cancer
20 cells (2). BJ and HCT116 cells were treated with PD0332991 and BPTES at the indicated concentrations for 96 h and cell proliferation was determined by Hoechst staining.
- FIG. 4C. Selective cytotoxicity of PD0332991 and CB-839 combined treatment for cancer
25 cells. BJ, HCT116, MCF7 and SKBR3 cells were treated with PD0332991 and CB-839 at the indicated concentrations for 96 h and cell proliferation was determined by Hoechst staining.

- FIG. 5A. PD0332991 and BPTES combined treatment decreased the formation of
spheroids. Images of HTC116 spheroids after 10 days of treatment. HTC116 spheroids
30 were grown and treated for 10 days with PD0332991 (2 μ M), BPTES (10 μ M) and the combination of the two inhibitors (PD0332991 at 2 μ M and BPTES at 10 μ M). C, Control; CM, Complete medium without inhibitors.

- FIG. 5B. PD0332991 and BPTES combined treatment decreased the formation of
35 spheroids. Quantification of the total spheroid volume after 10 days of treatment. Spheroids were scored by image acquisition and spheroid volume quantification with ImageJ software. Results are shown as percentage of total spheroid volume relative to untreated cells. Data are represented as mean $\pm$ SD of n = 4. Statistically significant differences between CDK4/6-inhibited and control cells were indicated at p < 0,001 (***),

while differences between treatment (BPTES) and the corresponding control (PD0332991-treated cells or untreated cells in complete medium) were shown at $p < 0,001$ (###). C, Control; CM, Complete medium without inhibitors; Sph V, Spheroid volume.

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FIG. 5C. PD0332991 and glutaminase inhibitor (BPTES or CB-839) combined treatment decreased the formation of spheroids. Images of MCF7 and SKBR3 spheroids after 10 days of treatment. MCF7 and SKBR3 spheroids were grown and treated for 10 days with PD0332991 (2 μ M), BPTES (10 μ M), CB-839 (10 μ M) and each combination of
10 PD03329921 with the two glutaminase inhibitors (PD0332991 at 2 μ M and BPTES at 10 μ M, or PD0332991 at 2 μ M and CB-839 at 10 μ M). C, Control; CM, Complete medium without inhibitors.

FIG. 5D. PD0332991 and glutaminase inhibitor (BPTES or CB-839) combined treatment
15 decreased the formation of spheroids in MCF7 cells. Quantification of the total spheroid volume after 10 days of treatment. Spheroids were scored by image acquisition and spheroid volume quantification with ImageJ software. Results are shown as percentage of total spheroid volume relative to untreated MCF7 cells. Data are represented as mean $\pm$ SD of $n=4$. Statistically significant differences between CDK4/6-inhibited and control cells
20 were indicated at $p < 0,001$ (***), while differences between treatment (BPTES or CB-839) and the corresponding control (PD0332991-treated cells or untreated cells in complete medium) were shown at $p < 0,001$ (###) for CDK4/6-inhibited cells and at $p < 0,001$ (###) for control cells. C, Control; CM, Complete medium without inhibitors; Sph V, Spheroid volume.

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FIG. 5E. PD0332991 and glutaminase inhibitor (BPTES or CB-839) combined treatment decreased the formation of spheroids in SKBR3 cells. Quantification of the total spheroid volume after 10 days of treatment. Spheroids were scored by image acquisition and spheroid volume quantification with ImageJ software. Results are shown as percentage of
30 total spheroid volume relative to untreated SKBR3 cells. Data are represented as mean $\pm$ SD of $n = 4$. Statistically significant differences between CDK4/6-inhibited and control cells were indicated at $p < 0.001$ (***), while differences between treatment (BPTES or CB-839) and the corresponding control (PD0332991-treated cells or untreated cells in complete medium) were shown at $p < 0.001$ (###) for CDK4/6-inhibited cells and at $p < 0.01$ (##) for control cells. C, Control; CM, Complete medium without inhibitors; Sph V, Spheroid
35 volume.

DESCRIPTION OF EMBODIMENTS

Chemicals. All products were purchased from Sigma-Aldrich Co (St Louis, MO, USA), unless otherwise specified. The specific CDK4/6 inhibitor PD0332991 was purchased from Sigma-Aldrich. Stock solutions of 5 mM were prepared with culture water. The glutaminase inhibitor bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) was obtained from Sigma-Aldrich. The selective glutaminase inhibitor CB-839 was obtained from Selleckchem.com. Antibiotic (10 000 U mL⁻¹ penicillin, 10 mg mL⁻¹ streptomycin), Dulbecco's Phosphate Buffer Saline (PBS), Trypsin EDTA solution C (0.05% trypsin-0.02% EDTA) were purchased from Biological Industries (Kibbutz Beit Haemet, Israel) and Fetal Bovine Serum (FBS) from Invitrogen (Carlsbad, CA, USA).

Cell culture. Human colorectal carcinoma HCT116 cells (ATCC, Manassas, VA, USA) and human breast adenocarcinoma SKBR3 cells (ATCC) were cultured in Dulbecco's modified Eagle Medium (DMEM): Nutrient mixture HAM F12 (1:1) with L-glutamine and 12,5 mM D-glucose. Human skin BJ fibroblasts (ATCC) were grown in DMEM with L-glutamine and 25 mM D-glucose. Human breast adenocarcinoma MCF7 cells (ATCC) were cultured in MEM medium without phenol red (Gibco) containing 10 mM D-glucose, 2 mM L-glutamine, 1 mM pyruvate (Biological Industries), 0.01 mg mL⁻¹ insulin and 1% non-essential aminoacids (Biological Industries). Media were supplemented with 10% heat-inactivated FBS, penicillin (50 U mL⁻¹) and streptomycin (50 µg mL⁻¹). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂.

Cell proliferation assays. Cell proliferation and viability were assessed using Hoechst stain (HO33342; 2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate), a cell-permeable blue fluorescent dye that stains DNA. 24 h after seeding 2×10³ cells/well for HCT116 cells, 5×10³ cells/well for MCF7 and SKBR3 cells, and 10×10³ cells/well for BJ fibroblasts in 96-well plates, media were replaced with complete fresh media (control condition, 100 % viability) and media containing the drug or the combination of drugs under study (PD0332991 and BPTES or CB-839). At the end of the experiment, cells were washed with PBS before adding 100 µL of 0.01% SDS per well. Plates were then stored frozen at -20°C. To analyze the samples, plates were thawed at 37°C until fully liquid and 100 µL of 4 µg mL⁻¹ HO33342 in stain solution buffer (1 M NaCl, 1 mM EDTA, 10 mM Tris, pH 7.4) were added to each well, minimizing light exposure. Tinfoil-covered plates were placed on a shaker and incubated at 37 °C for 1 h. Finally, fluorescence was measured in a fluorescence plate reader at 355 nm excitation and 460 nm emission. Cell viability was assessed and represented as a percentage of viability relative to untreated control cells.

Spheroid formation assay. 10^4 cells were seeded in 24-well Ultra-Low Attachment culture plates (Corning) in presence of the specified inhibitor(s) in serum-free media supplemented with 20 ng mL^{-1} EGF, 20 ng mL^{-1} bFGF, $10 \text{ } \mu\text{g mL}^{-1}$ heparin, B27 (1:50), $5 \text{ } \mu\text{g mL}^{-1}$ insulin and $0,5 \text{ } \mu\text{g mL}^{-1}$ hydrocortisone, and allowed to grow for 10 days. At the end of the experiment, spheroids were photographed and incubated with $0,5 \text{ mg mL}^{-1}$ 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 2-3 h until fully stained. Finally, plates were scanned and spheroids were scored by image acquisition and spheroid volume quantification with ImageJ software (public domain National Institutes of Health, USA, <http://rsbweb.nih.gov/ij/>).

Data analysis and statistical methods. Experiments were carried out at least in triplicate and repeated three times. To evaluate the effects of combined drug treatments the multiple drug-effect analysis of Chou-Talalay (cf. T.C. Chou and P. Talalay, "Quantitative analysis of dose-effect relationships: The combined effects of multiple drugs or enzyme inhibitors", *Adv. Enzyme Regul.* 1984, vol. 22, pp. 27-55) was used with the CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA). PD0332991 and BPTES or CB-839 interactions were quantified by determining the Combination Index (CI), where $\text{CI} < 1$, $\text{CI} = 1$, and $\text{CI} > 1$ indicate synergism, additivity, and antagonism, respectively. All data are expressed as mean $\pm$ standard deviation (SD). CI values are interpreted as follows.

CI value	Agonistic effect
$< 0,10$	Very strong synergism
$0,10-0,30$	Strong synergism
$0,30-0,70$	Synergism
$0,70-0,90$	Moderate to slight synergism
$0,90-1,10$	Nearly additive
$1,10-1,45$	Slight to moderate antagonism
$1,45-3,30$	Antagonism
$> 3,30$	Strong to very strong antagonism

Statistical analyses were performed using Statgraphics statistical package (Statgraphics Centurion XVI, StatPoint technologies Inc., Warrenton, VA, USA). Control and treatment measurements were compared using ANOVA and two-tailed independent sample Student's t tests. Differences were considered to be significant at $p < 0.001$.

Example 1A: Effect of PD0332991 and BPTES on cell viability in HCT116 cells (colon cancer). It has been studied the effect of the two specific inhibitors of PD0332991 and BPTES alone or in constant ratio (1:4) combination on HCT116 cell viability (FIG. 1A). HCT116 cells were grown in 96-well plates and treated with PD0332991 and BPTES at the specified concentrations for 96 h (Table 1). Then, cell proliferation was assessed by

Hoechst staining. The combination of PD0332991 and BPTES treatments in a wide dose range showed a strong synergism in the antiproliferative effect with a $CI < 0.3$, what suggests that PD0332991 and BPTES co-treatment is an efficient strategy to decrease the chemotherapeutic dose required for therapy and consequently, the overall toxicity.

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PD0332991 (μ M)	BPTES (μ M)	Viability (%)	CI Value
0.5	2	40.3 ± 3.9	<i>0.195</i>
1	4	31.3 ± 2.5	<i>0.227</i>
1.5	6	17.7 ± 2.3	<i>0.122</i>
2	8	16.5 ± 0.9	<i>0.146</i>
2.5	10	11.8 ± 1.9	<i>0.107</i>
3	12	9.2 ± 1.7	<i>0.088</i>
3.5	14	7.9 ± 1.0	<i>0.082</i>
4	16	5.1 ± 1.3	<i>0.049</i>
4.5	18	5.0 ± 1.7	<i>0.055</i>
5	20	5.0 ± 0.7	<i>0.060</i>

Table 1A. Synergistic antiproliferative effect of PD0332991 and BPTES combination treatment.

HCT116 cells were treated for 96 h at the indicated concentrations of PD0332991 and BPTES in a constant ratio (1:4) combination. The CI results revealed a strong synergy ($CI < 0.3$) in the antiproliferative effects of PD0332991 and BPTES at each dose combination tested.

Example 1B: Effect of PD0332991 and CB-839 on cell viability in HCT116 cells (colon cancer). It has been studied the effect of the two specific inhibitors of PD0332991 and CB-839 alone or in constant ratio (10:1) combination on HCT116 cell viability (FIG. 1B). HCT116 cells were grown in 96-well plates and treated with PD0332991 and CB-839 at the specified concentrations for 96 h (Table 1B). The combination of PD0332991 and CB-839 treatments in a wide dose range showed a strong synergism in the antiproliferative effect with a $CI < 0.3$, what suggests that PD0332991 and CB-839 co-treatment is an efficient strategy to decrease the chemotherapeutic dose required for therapy and consequently, the overall toxicity.

[PD0332991] (μM)	[CB-839] (μM)	Viability (%)	CI Value
0.01	0.001	82.7 ± 1.1	0.112
0.05	0.005	54.1 ± 2.5	0.090
0.1	0.01	42.8 ± 0.7	0.097
0.25	0.025	29.9 ± 0.2	0.113
0.5	0.05	25.1 ± 0.6	0.164
0.75	0.075	22.2 ± 1.0	0.197
1	0.1	22.7 ± 1.6	0.274
1.5	0.15	16.0 ± 0.6	0.214
2	0.2	12.0 ± 0.1	0.194
3	0.3	5.1 ± 0.1	0.082

Table 1B. Synergistic antiproliferative effect of PD0332991 and CB-839 combination treatment. HCT116 cells were treated for 96 h at the indicated concentrations of PD0332991 and CB-839 in a constant ratio (10:1) combination. The CI results revealed a strong synergy ($\text{CI} < 0.3$) in the antiproliferative effects of PD0332991 and CB-839 at each dose combination tested.

Example 2A: Effect of PD0332991 and BPTES on cell viability in MCF7 cells (breast cancer ER positive and HER2 negative). To determine if this combination treatment obtained similar growth inhibitory results in other cancer cell lines, the effect of PD0332991 and BPTES was measured alone or in constant ratio (1:4) combination on cell viability in MCF7 cells (breast cancer ER positive and HER2 negative). MCF7 cells were seeded in 96-well plates, incubated for 96 h at the indicated concentrations of PD0332991 and BPTES (Table 2A, FIG. 2A). In agreement with the effects on HCT116 cells, PD0332991 and BPTES combination exhibited a strong synergism ($\text{CI} < 0.3$) at low drug concs. and a synergistic antiproliferative effect ($\text{CI} < 0.7$) at a higher dose range.

PD0332991 (μM)	BPTES (μM)	Viability (%)	CI Value
0.05	0.2	63.0 ± 4.6	0.163
0.1	0.4	56.8 ± 0.8	0.196
0.25	1	38.5 ± 2.6	0.130
0.5	2	31.1 ± 2.3	0.161
0.75	3	29.8 ± 2.3	0.222
1	4	28.7 ± 2.4	0.277
1.5	6	27.1 ± 2.0	0.377
2	8	24.9 ± 2.6	0.438
3	12	24.3 ± 1.9	0.636

Table 2A: Synergistic antiproliferative effect of PD0332991 and BPTES combined treatment in MCF7 cells. MCF7 cells were treated for 96 h at the specified concentrations of PD0332991 and BPTES in a constant ratio (1:4) combination. The CI results revealed a strong synergism ($CI < 0.3$) at low drug concentrations and a synergistic antiproliferative effect ($CI < 0.7$) at the high-dose range of PD0332991 and BPTES.

Example 2B: Effect of PD0332991 and CB-839 on cell viability in MCF7 cells (breast cancer ER positive and HER2 negative). To determine if this combination treatment obtained similar growth inhibitory results in other cancer cell lines, the effect of PD0332991 and CB-839 alone or in constant ratio (10:1) combination on cell viability in MCF7 cells (breast cancer ER positive and HER2 negative) was measured. MCF7 cells were seeded in 96-well plates, incubated for 96 h at the indicated concentrations of PD0332991 and CB-839 (Table 2B, FIG. 2B). In agreement with the effects on HCT116 cells, PD0332991 and CB-839 combination exhibited a very strong synergism ($CI < 0.1$).

PD0332991 (μ M)	CB-839 (μ M)	Viability (%)	CI Value
0.01	0.001	64.3 ± 5.6	0.036
0.05	0.005	55.5 ± 6.7	0.069
0.1	0.01	40.3 ± 5.3	0.030
0.25	0.025	27.4 ± 2.2	0.021
0.5	0.05	27.7 ± 4.8	0.043
0.75	0.075	28.8 ± 3.6	0.072
1	0.1	23.4 ± 2.1	0.055
1.5	0.15	21.3 ± 2.1	0.066
2	0.2	21.2 ± 2.7	0.087

Table 2B: Synergistic antiproliferative effect of PD0332991 and CB-839 combined treatment in MCF7 cells. MCF7 cells were treated for 96 h at the specified concentrations of PD0332991 and CB-839 in a constant ratio (10:1) combination. The CI results revealed a very strong synergism ($CI < 0.1$) of PD0332991 and CB-839 at each dose combination.

Example 3A: Effect of PD0332991 and BPTES on cell viability in SKBR3 cells (breast cancer ER negative and HER2 positive). The effect of PD0332991 and BPTES alone and in constant ratio (1:4) combination on cell viability in SKBR3 cells (breast cancer ER negative and HER2 positive) was also measured. SKBR3 cells were grown in 96-well plates and incubated for 96 h at the indicated concentrations of PD0332991 and BPTES (Table 3A, FIG. 3A). In agreement with the effects on HCT116 and MCF7 cells, the

simultaneous treatment of PD0332991 and BPTES exhibited a strong synergistic antiproliferative effect with a $CI < 0.3$ at a wide dose range.

PD0332991 (μ M)	BPTES (μ M)	Viability (%)	CI Value
0.5	2	67 ± 2.8	0.222
1	4	59 ± 2.8	0.223
1.5	6	52 ± 1.3	0.197
2	8	45 ± 2.1	0.145
2.5	10	39 ± 0.1	0.112
3	12	39 ± 1.9	0.138
4	16	38 ± 1.0	0.169
5	20	35 ± 1.7	0.160
6	24	27 ± 0.9	0.094
7	28	23 ± 1.4	0.077

- 5 Table 3A: Synergistic antiproliferative effect of PD0332991 and BPTES combined treatment in SKBR3 cells. SKBR3 cells were treated for 96 h at the specified concentrations of PD0332991 and BPTES in a constant ratio (1:4) combination. The CI results revealed a strong synergistic antiproliferative activity ($CI < 0.3$) of PD0332991 and BPTES combination at a wide dose range.

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- Example 3B: Effect of PD0332991 and CB-839 on cell viability in SKBR3 cells (breast cancer ER negative and HER2 positive). The effect of PD0332991 and CB-839 alone and in constant ratio (10:1) combination on cell viability in SKBR3 cells (breast cancer ER negative and HER2 positive) was also measured. SKBR3 cells were grown in 96-well plates and incubated for 96 h at the indicated concentrations of PD0332991 and CB-839 (Table 3B, FIG. 3B). In agreement with the effects on HCT116 and MCF7 cells, the simultaneous treatment of PD0332991 and CB-839 exhibited a strong synergistic antiproliferative effect with a $CI < 0.2$ at a wide dose range.

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PD0332991 (μM)	CB-839 (μM)	Viability (%)	CI Value
0.01	0.001	86.7 ± 3.9	0.070
0.05	0.005	72.1 ± 4.0	0.049
0.1	0.01	66.9 ± 3.5	0.059
0.25	0.025	58.5 ± 1.2	0.070
0.5	0.05	57.9 ± 3.6	0.133
0.75	0.075	53.2 ± 1.7	0.136
1	0.1	53.2 ± 3.0	0.180
1.5	0.15	45.4 ± 4.3	0.142
2	0.2	35.9 ± 2.6	0.085
3	0.3	23.3 ± 2.7	0.036

Table 3B: Synergistic antiproliferative effect of PD0332991 and CB-839 combined treatment in SKBR3 cells. SKBR3 cells were treated for 96 h at the specified concentrations of PD0332991 and CB-839 in a constant ratio (10:1) combination. The CI results revealed a strong synergistic antiproliferative activity (CI<0.2) of PD0332991 and CB-839 combination at a wide dose range.

Example 4A: cell viability dose-response curve of the non-tumor BJ human foreskin fibroblast cell line using the Hoechst stain system. To test the selective cytotoxicity of PD0332991 and BPTES combination treatment for cancer cells, the cell viability dose-response curve of the non-tumor BJ human foreskin fibroblast cell line was determined using the Hoechst stain assay. HCT116 and BJ cells were grown in 96-well plates and incubated for 96 h at the indicated concentrations with PD0332991 alone or with addition of 10 μM BPTES (FIG. 4A) and at the indicated concentrations of the combination of PD0332991 and BPTES (FIG. 4B), and the effect on cell proliferation was determined. As seen in FIG. 4A and 4B, the simultaneous incubation with PD0332991 and BPTES at all concentrations tested had little or no effect on non-tumor BJ cells viability but greatly impaired proliferation of HCT116 cells. For example, combination treatment with 1 μM PD0332991 and 10 μM BPTES caused no cytotoxic effects on non-tumor BJ cells while producing an approximate 80% reduction in HCT116 cell viability. All together, these results support the concomitant use of PD0332991 with BPTES as a promising chemotherapeutic therapy with selective antiproliferative effects on cancer cells.

Example 4B: cell viability dose-response curve of the non-tumor BJ human foreskin fibroblast cell line using the Hoechst stain system. To test the selective cytotoxicity of PD0332991 and CB-839 combination treatment for cancer cells, the cell viability dose-response curve of the non-tumor BJ human foreskin fibroblast cell line was (FIG. 4C). BJ

cells were grown in 96-well plates and incubated for 96 h at the indicated concentrations with PD0332991 and CB-839, and the effect on cell proliferation was determined. As seen in FIG. 4C, the simultaneous incubation with PD0332991 and CB-839 at all concentrations tested had little or no effect on non-tumor BJ cells viability but greatly impaired

proliferation of HCT116, MCF7 and SKBR3 cells. E.g. combination treatment with 3 μ M PD0332991 and 0.3 μ M CB-839 caused no cytotoxic effects on non-tumor BJ cells while producing an approximate 80% reduction in HCT116, MCF7 and SKBR3 cell viability. All together, these results support the concomitant use of PD0332991 with CB-839 as a promising chemotherapeutic therapy with selective antiproliferative effects on cancer cells.

Example 5: Combination therapy validation in 3D *in vitro* culture system. To test whether the combined therapies studied here presented similar results in a more physiologically relevant system, we generated spheroids of HCT116, MCF7 and SKBR3 cells as an *in vitro* tumor model system of intermediate complexity between standard monolayer cultures and tumors *in vivo*. Indeed, the spheroid model has been increasingly recognized as a primary tool for positive selection in innovative drug development therapies since it can remarkably reflect the 3D heterogeneous microenvironments and the therapeutically relevant pathophysiological gradients of *in vivo* tumor (F. Hirschhaeuser et al., "Multicellular tumor spheroids: an underestimated tool is catching up again", *J. Biotechnol.* 2010, vol. 148, pp. 3-15.). Toward this end, HCT116, MCF7 and SKBR3 cells were grown in ultra low attachment plates with 2 μ M PD0332991, 10 μ M BPTES, 10 μ M CB-839 and the combination of the two inhibitors (PD0332991 at 2 μ M and BPTES at 10 μ M or PD0332991 at 2 μ M and CB-839 at 10 μ M), and were scored for spheroids after 10 days. Inhibition of CDK4/6 alone decreased the anchorage-independent colony-forming growth of HCT116, MCF7 and SKBR3 cells (FIG. 5A, 5B, 5C, 5D and 5E), while the combination of PD0332991 with the glutaminase inhibitor BPTES reduced the formation of spheroids to a greater extent than either therapy alone (FIG. 5A, 5B, 5C, 5D and 5E). Consistent with our results in monolayer cells, BPTES treatment alone produced no effects on total spheroid volume while, when combined with CDK4/6 inhibition, dramatically abrogated the formation of spheroids, diminishing the overall spheroid volume to less than 15% (FIG. 5B, 5D and 5E). The combination of PD0332991 with the glutaminase inhibitor CB-839 reduced also the formation of spheroids to a greater extent either therapy alone (FIG. 5C, 5D and 5E). These results reinforce the synergistic antiproliferative effect of PD0332991 and BPTES combined therapy in tumor cells, as well as the synergistic antiproliferative effect of PD0332991 and CB-839 combined therapy in tumor cells.

CLAIMS

1. A pharmaceutical combination comprising a therapeutically effective amount of a CDK 4/6 inhibitor or a pharmaceutically acceptable salt thereof, and a therapeutically effective
5 amount of a glutaminase inhibitor or a pharmaceutically acceptable salt thereof.
2. The combination according to claim 1, wherein the CDK 4/6 inhibitor is selected from the group consisting of: 6-acetyl-8-cyclopentyl-5-methyl-2-((5-(piperazin-1-yl)pyridin-2-yl)amino)pyrido[2,3-d]pyrimidin-7(8H)-one hydrochloride, 7-cyclopentyl-N,N-dimethyl-2-
10 ((5-(piperazin-1-yl)pyridin-2-yl)amino)-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide, N,1,4,4-tetramethyl-8-((4-(4-methylpiperazin-1-yl)phenyl)amino)-4,5-dihydro-1H-pyrazolo[4,3-h]quinazoline-3-carboxamide, N-(5-((4-ethylpiperazin-1-yl)methyl)pyridin-2-yl)-5-fluoro-4-(4-fluoro-1-isopropyl-2-methyl-1H-benzo[d]imidazol-6-yl)pyrimidin-2-amine, capridine beta, FLX925, GIT28, GIT30, GIT38, MMD37K, and P276.
- 15 3. The combination according to any of the claims 1-2, wherein the glutaminase inhibitor is selected from the group consisting of: O-(2-diazoacetyl)-L-serine, 6-diazo-5-oxo-L-norleucine, glutamine, ibotenic acid, L-[aS,5S]-a-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, bis-2-(5-phenylacetamido-1,2,4- thiadiazol-2-yl)ethyl sulfide, and
20 2-(pyridin-2-yl)-N-(5-(4-(6-(2-(3-(trifluoromethoxy)phenyl)acetamido)pyridazin-3-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide.
4. The combination according to any of the claims 2-3, wherein the CDK 4/6 inhibitor is 6-acetyl-8-cyclopentyl-5-methyl-2-((5-(piperazin-1-yl)pyridin-2-yl)amino)pyrido[2,3-
25 d]pyrimidin-7(8H)-one hydrochloride.
5. The combination according to any of the claims 3-4, wherein the glutaminase inhibitor is bis-2-(5-phenylacetamido-1,2,4- thiadiazol-2-yl)ethyl sulfide.
- 30 6. The combination according to any of the claims 3-4, wherein the glutaminase inhibitor is 2-(pyridin-2-yl)-N-(5-(4-(6-(2-(3-(trifluoromethoxy)phenyl)acetamido)pyridazin-3-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide.
7. The combination according to any of the claims 1-6, wherein the molar ratio of CDK 4/6
35 inhibitor to glutaminase inhibitor is from 10:1 to 1:10.
8. The combination according to claim 7, wherein the molar ratio is 1:4.

9. A pharmaceutical composition comprising a therapeutically effective amount of a combination as defined in any of the claims 1-8, plus pharmaceutically acceptable excipients or carriers.
- 5 10. A combination as defined in any of the claims 1-8, for use as active pharmaceutical ingredient.
11. A combination as defined in any of the claims 1-8, for use in the treatment of cancer in mammals, including humans.
- 10 12. The combination for use according to claim 11, wherein the cancer is selected from the group consisting of: colon cancer, breast cancer, melanoma, glioblastoma, osteosarcoma, cervical cancer, lymphoma, squamous cell skin cancer, and glioma.
- 15 13. The combination for use according to claim 12, wherein the cancer is selected from the group consisting of colon cancer and breast cancer.
14. The combination for use according to any of the claims 10-13, that is administered simultaneously, separately or sequentially.
- 20 15. A method of treatment of a mammal that is suffering from cancer, comprising the administration of a therapeutically effective amount of a combination as defined in any of the claims 1-8, together with adequate amounts of pharmaceutically acceptable excipients or carriers.
- 25 16. The method according to claim 15, wherein the cancer is selected from the group consisting of: colon cancer, breast cancer, melanoma, glioblastoma, osteosarcoma, cervical cancer, lymphoma, squamous cell skin cancer and glioma.
- 30 17. The method according to claim 16, wherein the cancer is selected from the group consisting of colon cancer and breast cancer.
18. The method according to any of the claims 15-17, wherein the administration is simultaneous, separated or sequential.

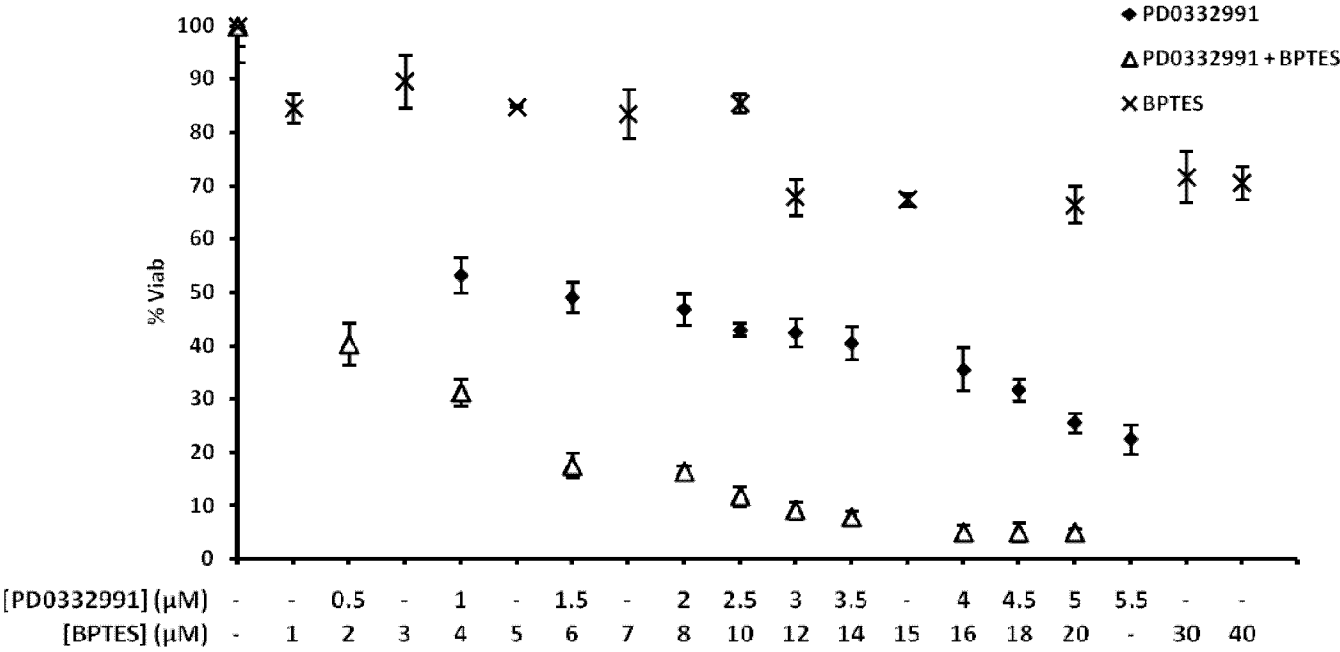


FIG 1A

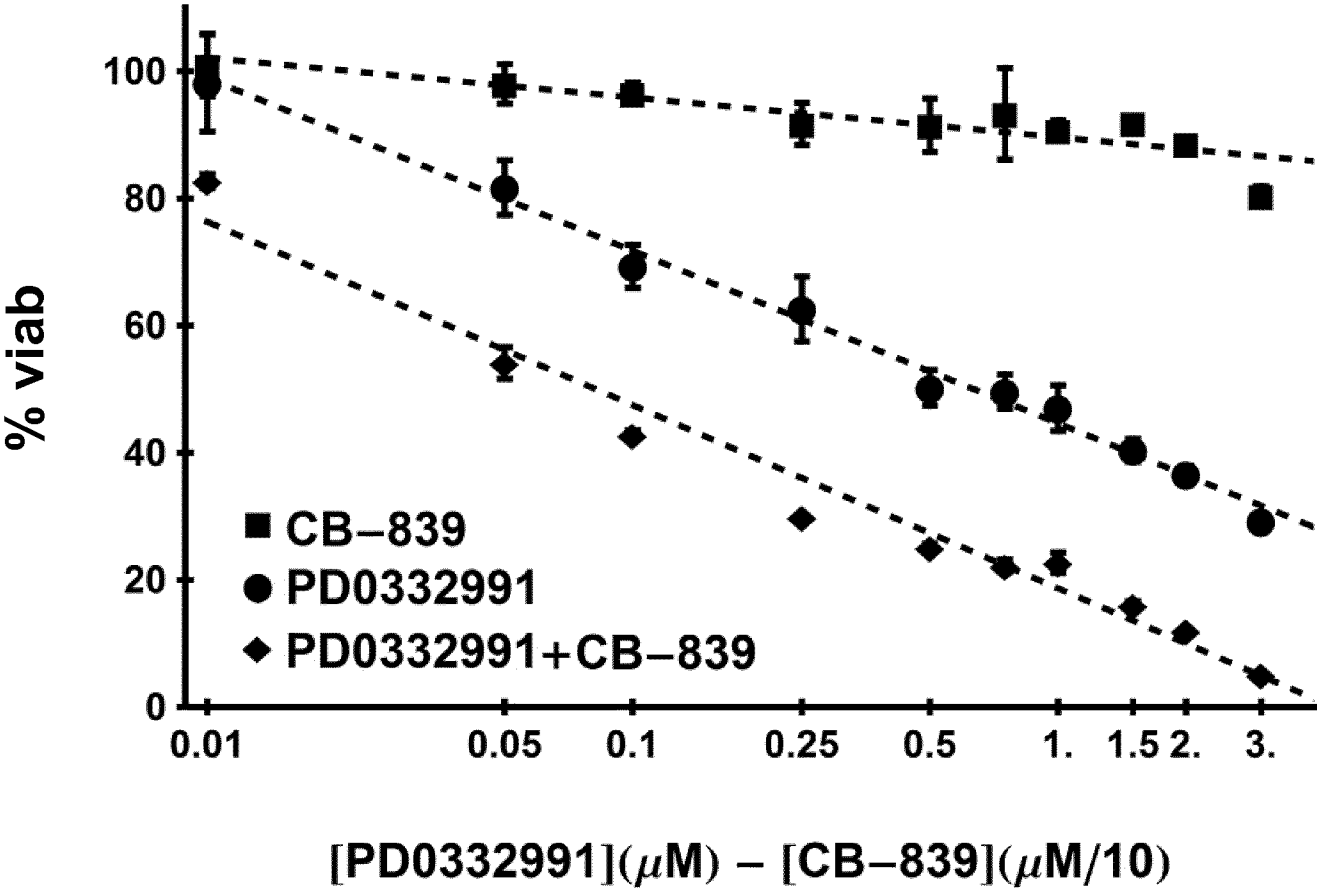


FIG 1B

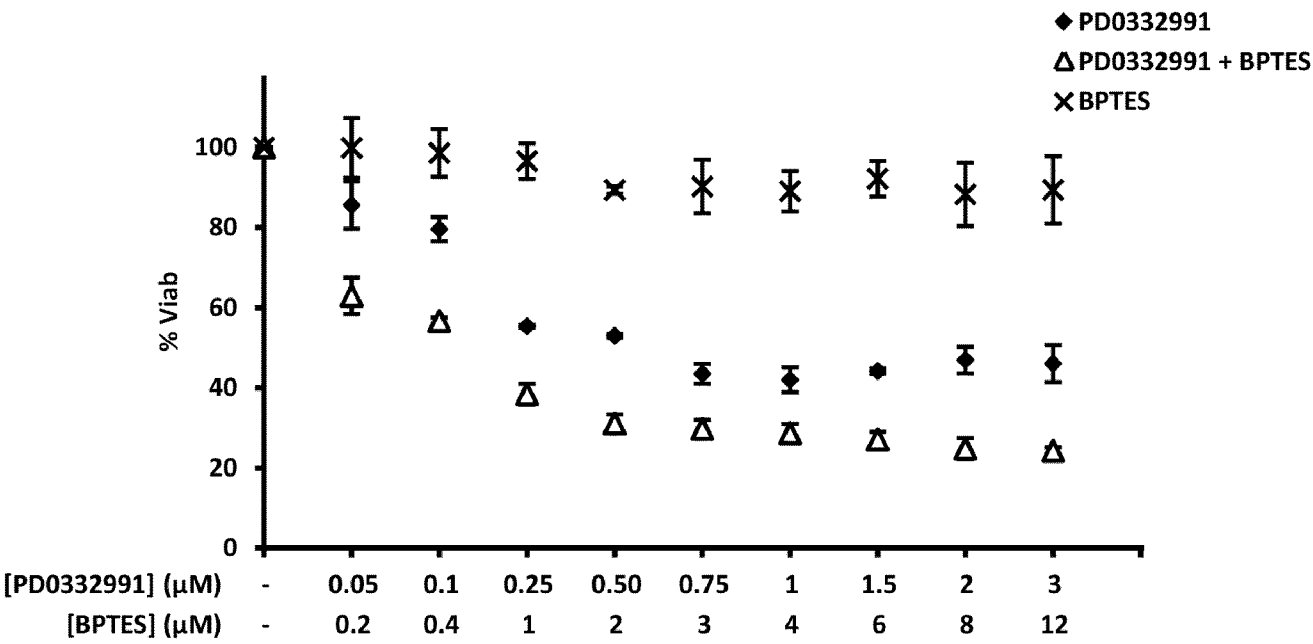


FIG 2A

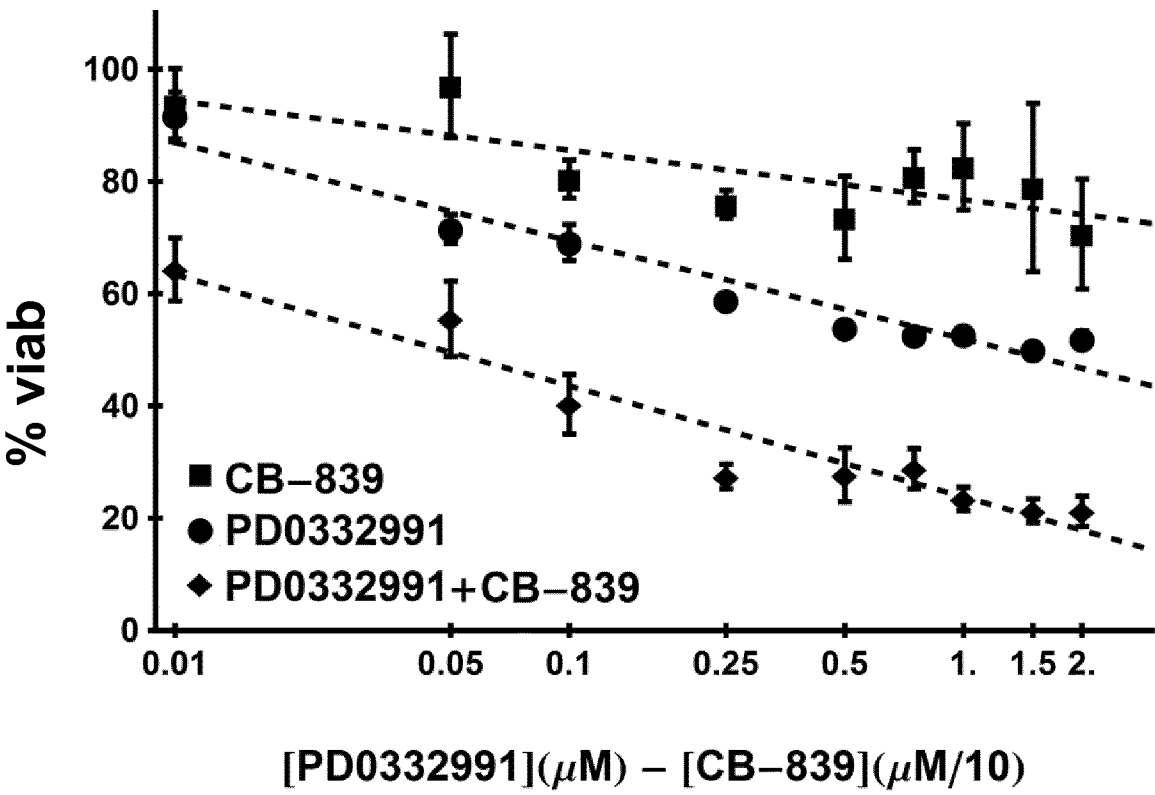


FIG 2B

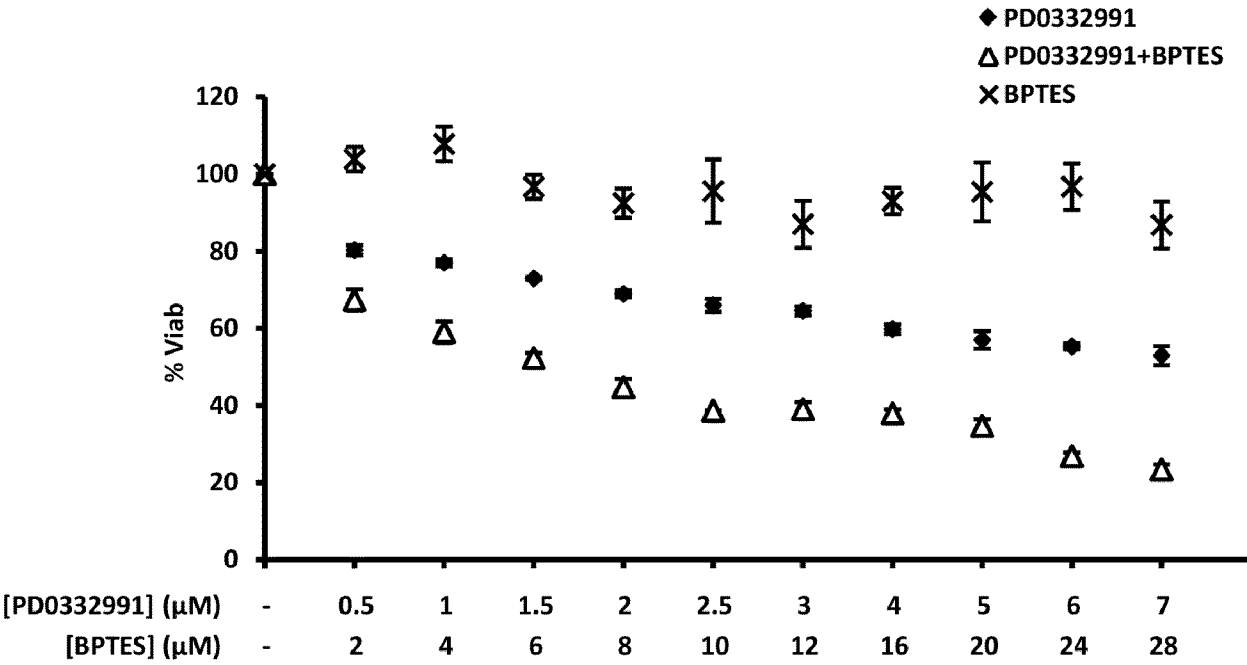


FIG 3A

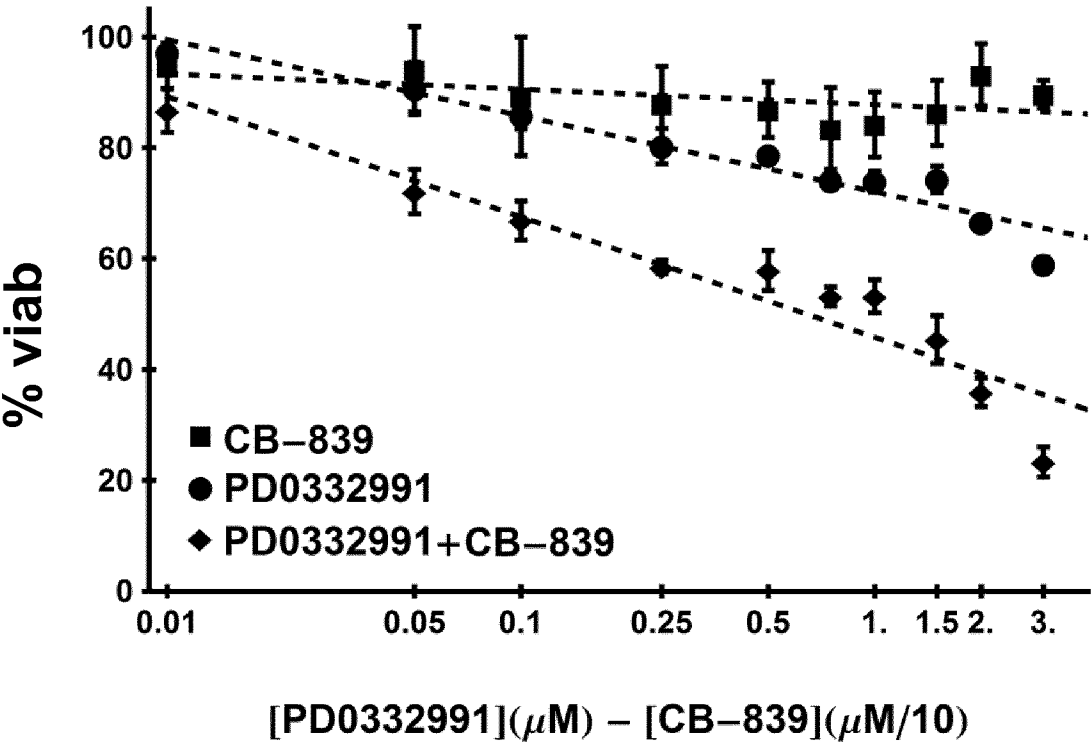


FIG 3B

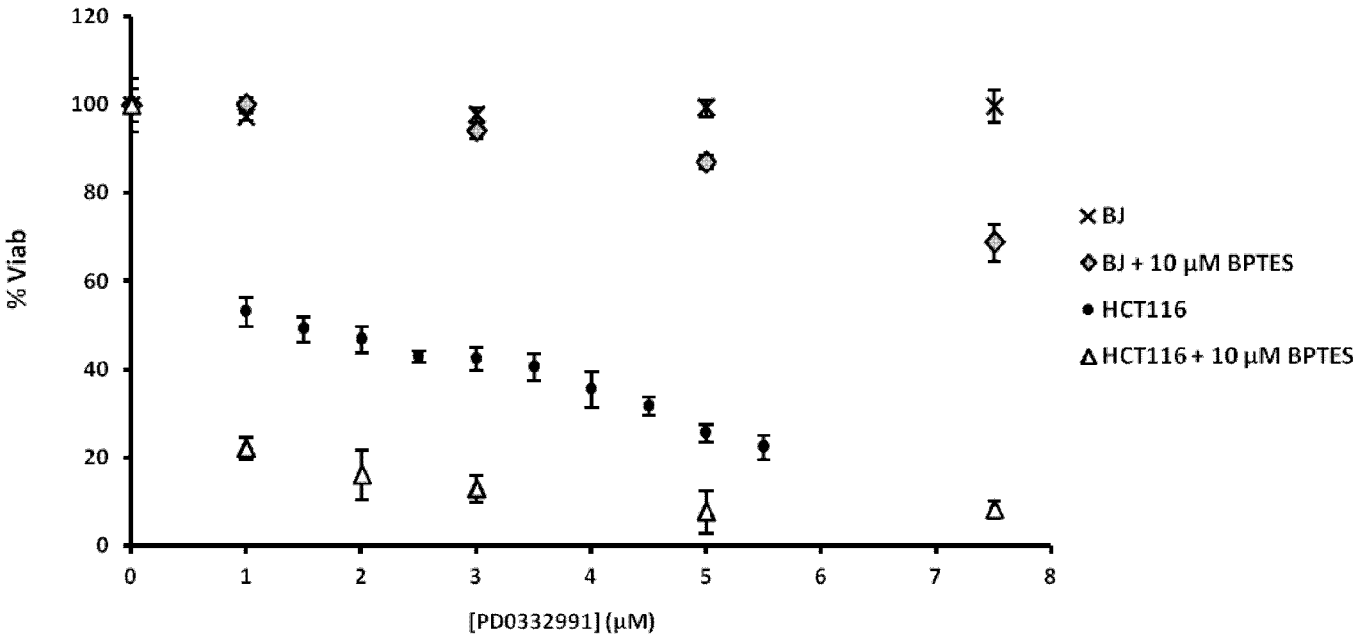


FIG 4A

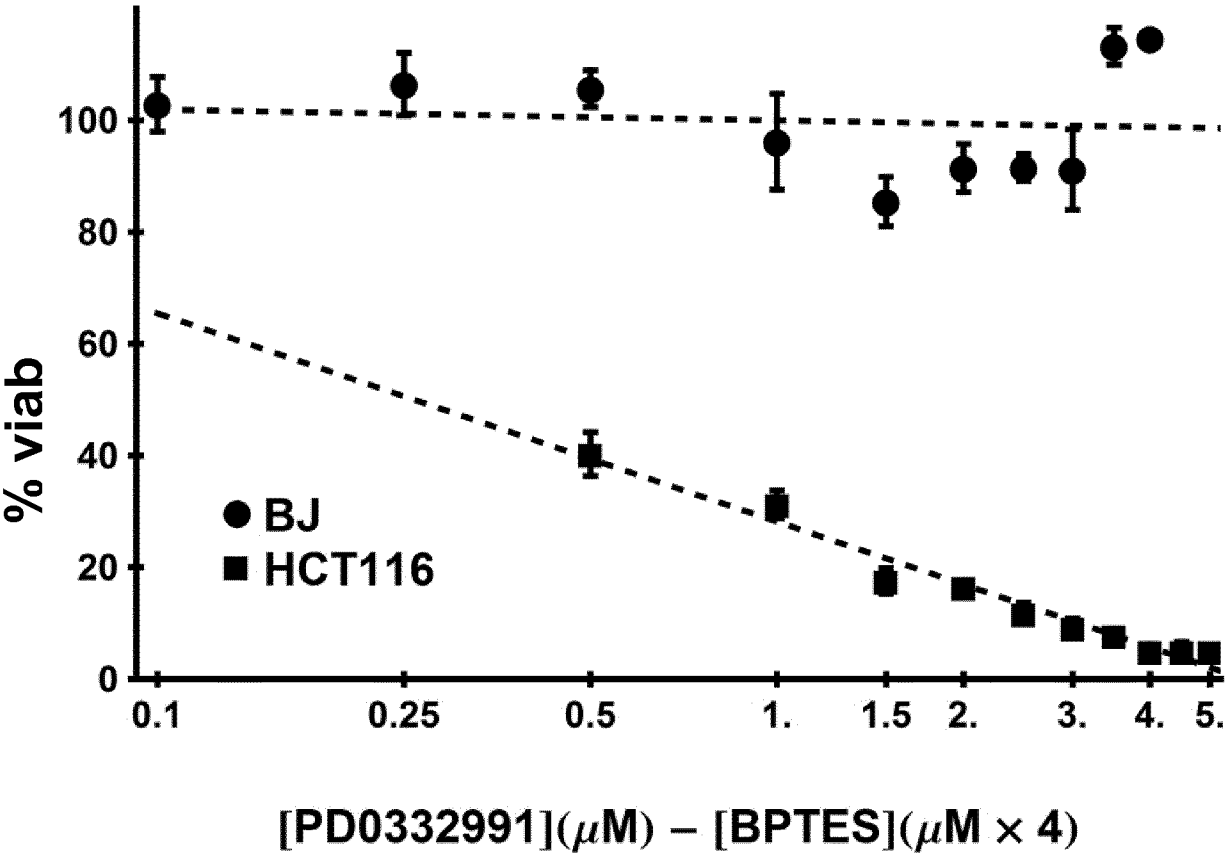


FIG 4B

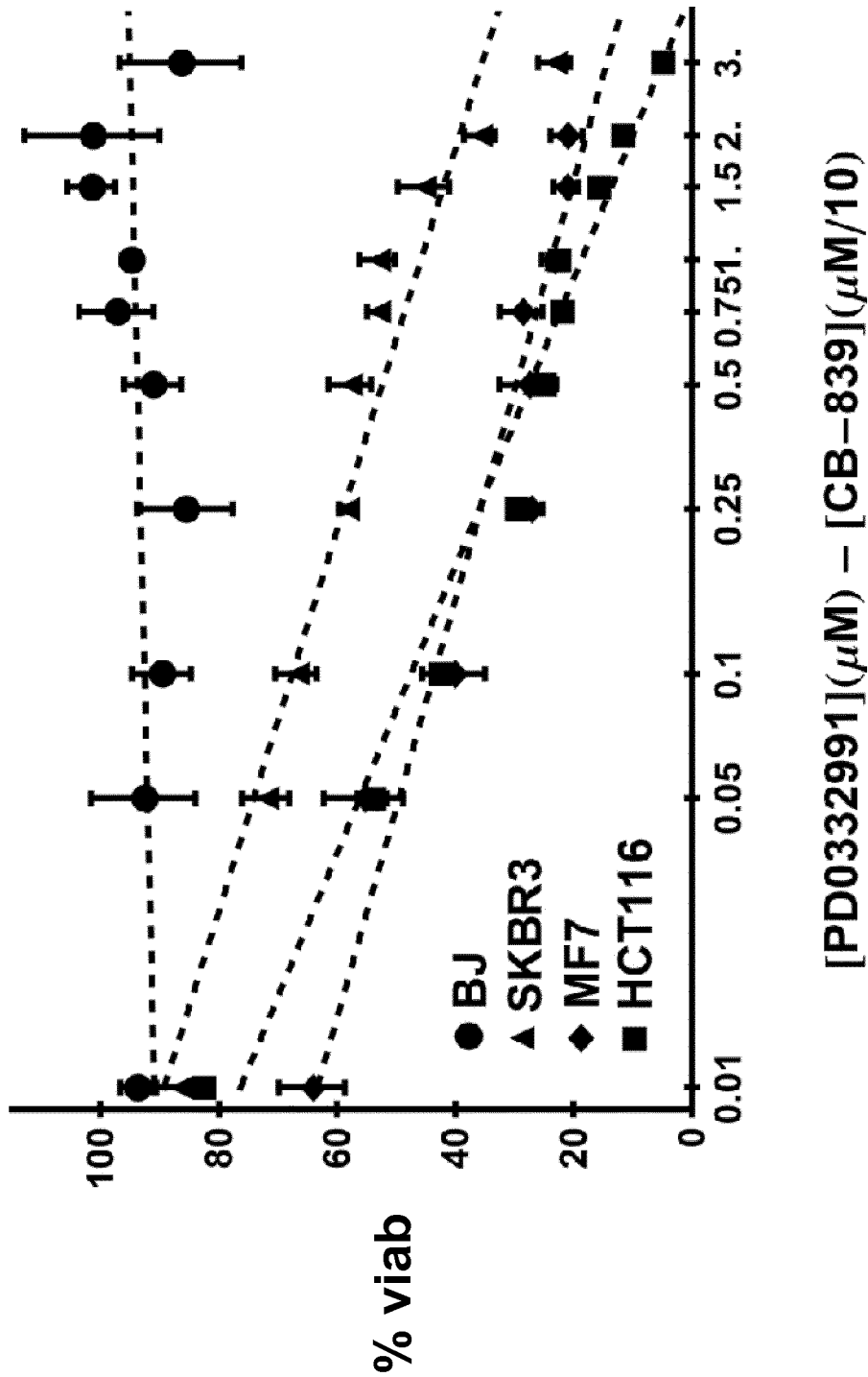


FIG 4C

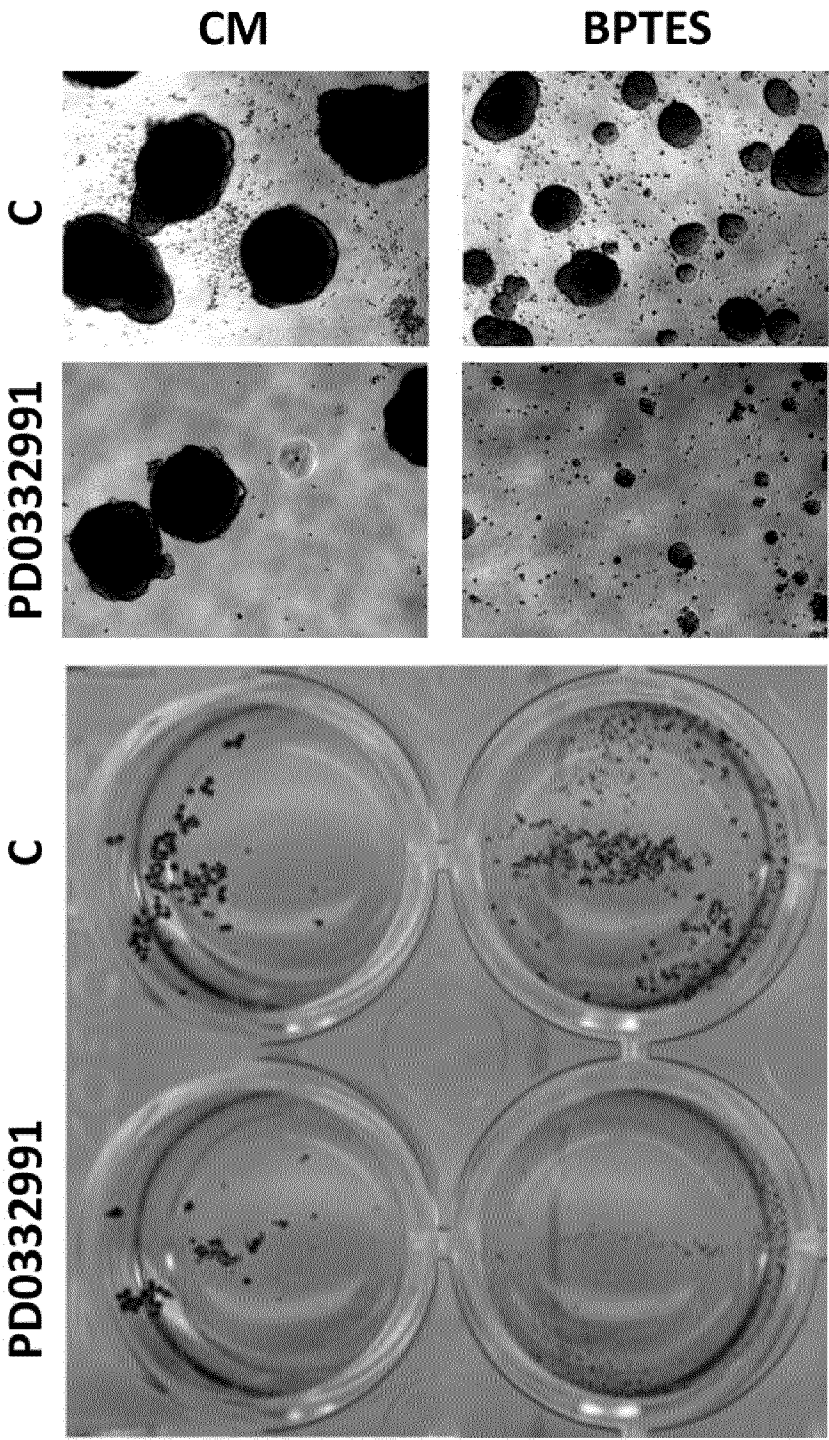


FIG 5A

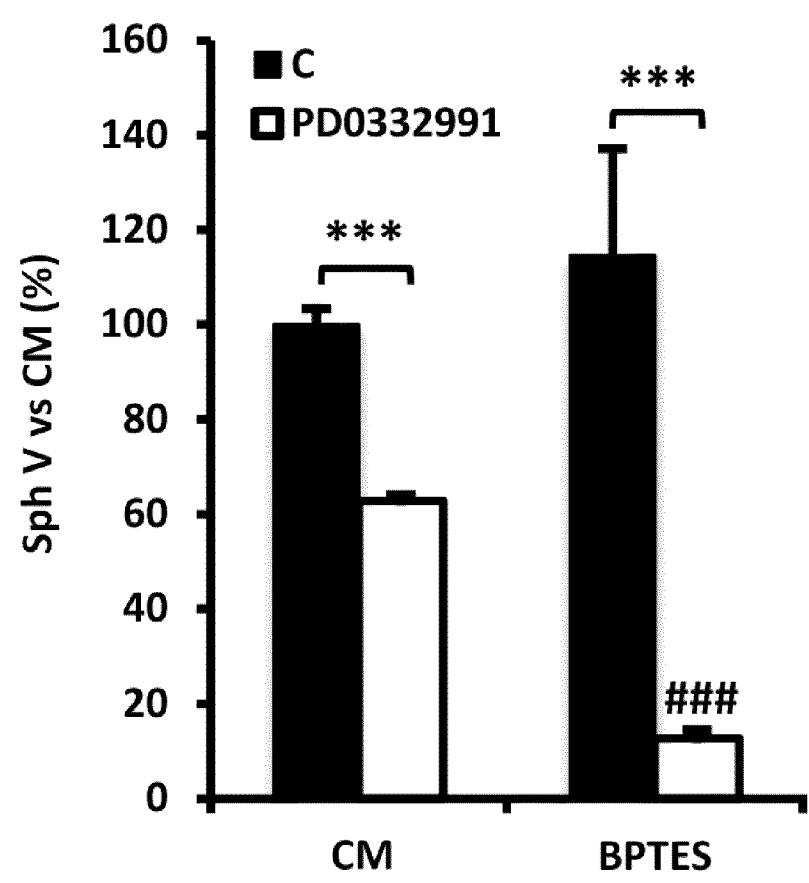


FIG 5B

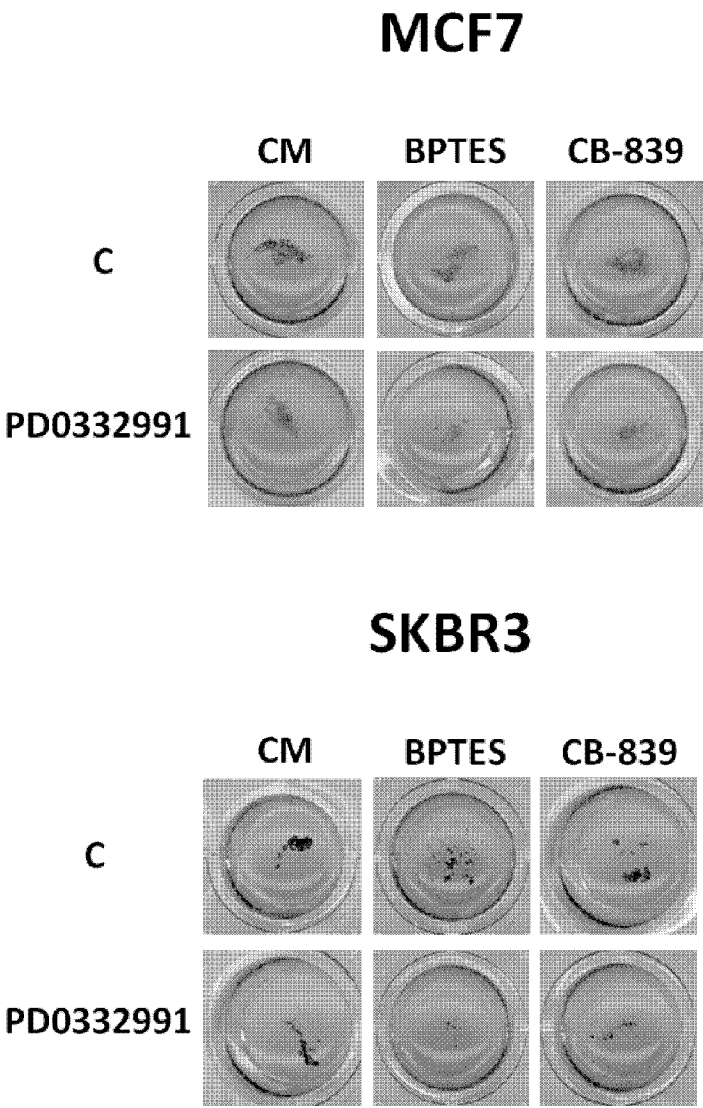


FIG 5C

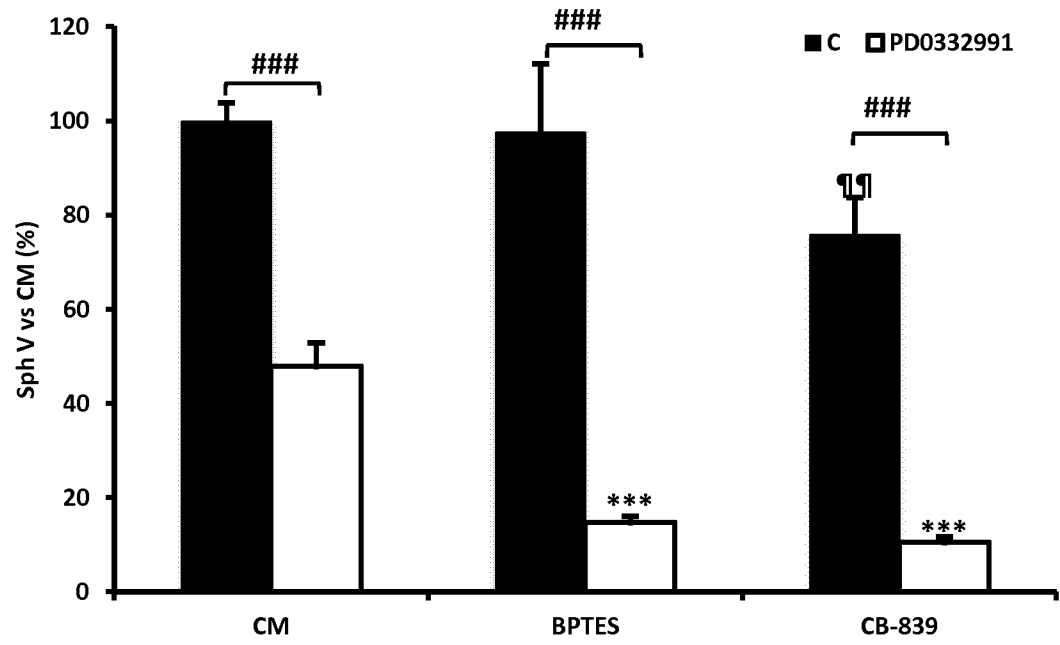


FIG 5D

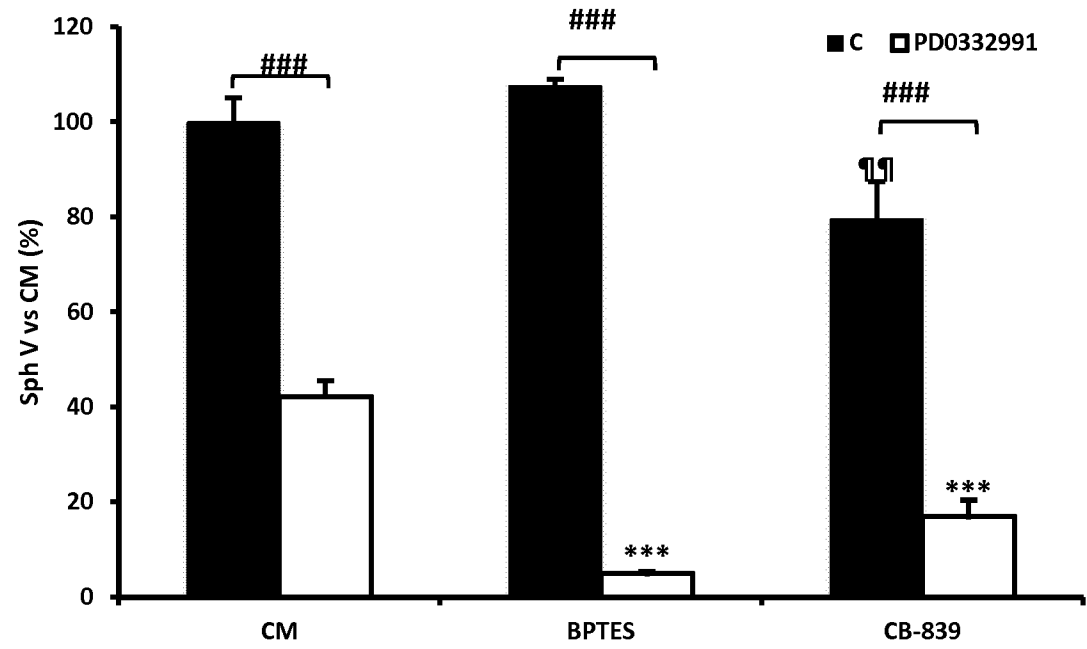


FIG 5E

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/067524

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K45/06 A61K31/42 A61K31/433 A61K31/501 A61K31/517
A61K31/519 A61K31/655 A61P35/00

ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K A61P

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

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

EPO-Internal, WPI Data, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2011/143160 A2 (UNIV JOHNS HOPKINS [US]; RIGGINS GREGORY JOSEPH [US]; SELTZER MEGHAN J) 17 November 2011 (2011-11-17) claims 1,2 -----	1-18
Y	WO 2015/095840 A1 (BIOMED VALLEY DISCOVERIES INC [US]) 25 June 2015 (2015-06-25) paragraph [0135]; claim 1; table 8 -----	1-18
Y	WO 2015/022609 A1 (NOVARTIS AG [CH]; CHEN YAN [US]; HUANG XIZHONG [US]; KIM SUNKYU [US]) 19 February 2015 (2015-02-19) page 40; claims 1,7; example 2 -----	1-18
Y	US 2005/261260 A1 (GIANELLA-BORRADORI ATHOS [DE]) 24 November 2005 (2005-11-24) the whole document ----- -/-	1-18

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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

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

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

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

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"&" document member of the same patent family

Date of the actual completion of the international search

6 September 2016

Date of mailing of the international search report

13/09/2016

Name and mailing address of the ISA/

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Allnutt, Sarah

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/067524

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	M. I. GROSS ET AL: "Antitumor Activity of the Glutaminase Inhibitor CB-839 in Triple-Negative Breast Cancer", MOLECULAR CANCER THERAPEUTICS, vol. 13, no. 4, 12 February 2014 (2014-02-12), pages 890-901, XP055243589, US ISSN: 1535-7163, DOI: 10.1158/1535-7163.MCT-13-0870 abstract -----	1-18

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Information on patent family members

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