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(54) Title: COMBINATION TREATMENT OP A G2 CHECKPOINT INHIBITOR (SB-218078) AND A CHEMOTHERAPEUTIC AGENT FOR TUMORIGENIC CELLS IN SOLID TUMORS

(57) Abstract: The present invention is concerned with the use of a G2 checkpoint inhibitor like SB218078, PD0166285 or AZD7762 and a chemotherapeutic agent like cisplatin, paclitaxel gemcitabine in the manufacture of a medicament for the treatment of tumorigenic cells in solid tumours. Also provided is a pharmaceutical pack comprising same, the same for the treatment of tumorigenic cells in solid tumours, and methods of treatment of tumorigenic cells in solid tumours using same.

COMBINATION TREATMENT OF A G2 CHECKPOINT INHIBITOR(SB-218078) AND A CHEMOTHERAPEUTIC AGENT FOR TUMORIGENIC CELLS IN SOLID TUMORS

The present invention is concerned with the use of a G2 checkpoint inhibitor and a chemotherapeutic agent in the manufacture of a medicament for the treatment of tumorigenic cells in solid tumours. Also provided is a pharmaceutical pack comprising same, the same for the treatment of tumorigenic cells in solid tumours, and methods of treatment of tumorigenic cells in solid tumours using same.

Development of resistance to radiation and chemotherapy poses substantial challenges in the treatment of solid malignancies. Recent research demonstrates that tumours arise, spread, acquire resistance to anti-neoplastic drugs and ultimately relapse because they are sustained by a population of undifferentiated cells (1-15; references are detailed at the end of the description, immediately prior to the claims). These cells are endowed with stem cell-like properties of self-renewal and multipotency since they are able to give rise to the differentiated non-tumorigenic bulk tumour population. Although such cells, known as cancer stem cells (CSCs) or tumour initiating cells, represent only a small population in the tumour mass, they are necessary and sufficient for tumour development and maintenance (4). The different sensitivity to anticancer agents of stem and differentiated cancer cell populations might account for the inability of current therapies to eradicate solid tumours (16).

The present inventors have previously discovered lung and colon cancer stem cells by identification and isolation through the CD133 marker (14, 15). Cancer stem cells are slow-dividing cells that have an unlimited proliferative potential. They have a high expression of anti-apoptotic proteins that renders them resistant to conventional therapies (16), thus resulting in the disease relapse that follows the treatment of many common cancers. It is also likely that conventional chemotherapy actually enriches CSCs allowing repopulation of the tumour mass. Bao *et al.* (17) have demonstrated that glioma stem cells identified in CD133 positive cells possess a more functional DNA damage pathway than CD133-negative tumour cells, as a result they are more resistant to radiation therapy.

It is therefore desirable to provide improved treatments for tumorigenic cells, particularly in solid tumours.

Bucher N and Britten CD (21) review the use of G2 checkpoint abrogation and Chk1 (checkpoint kinase-1) targeting in the treatment of cancer. In particular, they state that the use of DNA-damaging agents or antimitotics in combination with a Chk1 inhibitor enhances tumour kill and may also eliminate cell cycle-mediated drug resistance (i.e. drug resistance resulting from the triggering of cellular repair mechanisms at cell cycle checkpoints).

The present inventors have now found that the combination of a G2 checkpoint inhibitor, particularly a G2 checkpoint abrogator, together with a chemotherapeutic agent is surprisingly effective in the treatment of tumorigenic cells (also referred to as cancer stem cells; CSCs), and particularly so in solid tumours. This enhanced efficacy (especially when compared to treatment with chemotherapeutic agents alone) in the treatment of CSCs is neither taught nor suggested by the prior art.

In particular, it is noted that prior art teachings and experiments involving the use of G2 checkpoint abrogators/inhibitors show efficacy against conventional tumour cell lines and not against CSCs. Thus, there is no suggestion in the art of the enhanced efficacy of the combination of G2 abrogators and chemotherapeutic agents. Similarly, there is no teaching of treatment of solid tumours (particularly *in vivo*) with such combinations and as such there is no implicit treatment of tumorigenic cells.

Thus, according to a first aspect of the present invention there is provided the use of a G2 checkpoint inhibitor and a chemotherapeutic agent in the manufacture of a medicament for the treatment of tumorigenic cells in solid tumours.

Also provided according to the present invention is a G2 checkpoint inhibitor and a chemotherapeutic agent for the treatment of tumorigenic cells in solid tumours.

Also provided according to the present invention is a pharmaceutical pack for the treatment of tumorigenic cells in solid tumours, comprising a G2 checkpoint inhibitor and a chemotherapeutic agent.

The G2 checkpoint inhibitor and the chemotherapeutic agent may be provided in a single combined formulation, or may be provided separately for discrete administration either separately or sequentially.

Obviously, further agents for the maintenance and/or restoration of health such as an at least one further chemotherapeutic agent may be provided in any formulation or pharmaceutical pack. Such further agents may include agents designed to cure, alleviate, remove or lessen the symptoms of, or prevent or reduce the possibility of contracting any disorder or malfunction of the human or animal body.

As is shown in the experiments below, the use of a G2 checkpoint inhibitor together with a chemotherapeutic agent can result in a substantially enhanced efficacy in treating tumorigenic stem cells.

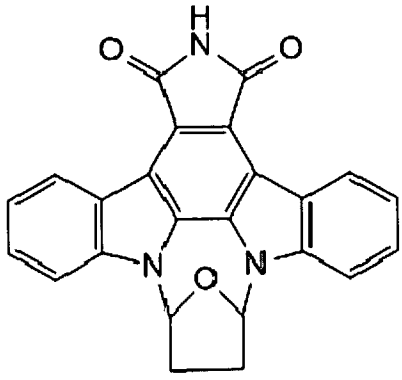
Preferably, the G2 checkpoint inhibitor is a G2 checkpoint abrogator.

Preferably, the G2 checkpoint inhibitor is a Chk1 inhibitor.

Preferably, the G2 checkpoint inhibitor is a Chk1 protein inhibitor.

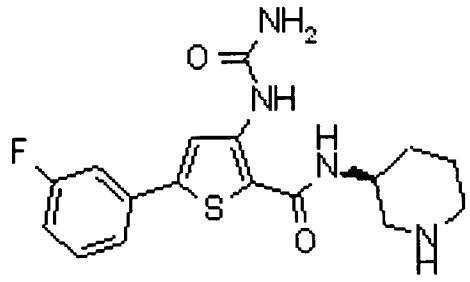
Preferably, the G2 checkpoint inhibitor is a WEE1 inhibitor, more preferably PD0166285 (Pfizer (Ann Arbor, MI, USA)).

Preferably, the G2 checkpoint inhibitor is:



[SB218078; 9,10,11,12,-Tetrahydro-9,12-epoxy-1*H*-diindolo(1,2,3-fg:3',2',1'-kl)pyrrolo(3,4-i)(1,6)benzodiazocine-1,3(2*H*)-dione; Calbiochem, EMD Chemicals Inc., NJ, USA] or a salt thereof.

Preferably, the G2 checkpoint inhibitor is:



[AZD7762, Astra Zeneca, UK; WO 2005/066163] or a salt thereof

Preferably, salts are pharmaceutically acceptable salts.

Results obtained using the above G2 checkpoint inhibitors are detailed below.

A wide range of chemotherapeutic agents are suitable for use in the present invention. Preferably, the chemotherapeutic agent is an antineoplastic agent. Preferably chemotherapeutic agents include Cisplatin, Gemcitabine and Paclitaxel. Other suitable chemotherapeutic agents will be readily apparent to a person of ordinary skill in the art.

Preferably, the tumorigenic cells are selected from the group consisting of: colon cancer stem cells, and lung cancer stem cells. Other suitable tumorigenic cells will be readily apparent to one of ordinary skill in the art.

Also provided according to the present invention is method of treatment of tumorigenic cells in solid tumours, comprising the step of administering a G2 checkpoint inhibitor and a chemotherapeutic agent to a patient in need of same.

As detailed below, the present inventors have isolated cancer stem cells from lung cancer specimens of patients who underwent surgical resection of the tumour. *In vitro* exposure of LCSCs to conventional chemotherapy resulted in a selective G2 phase arrest providing this population with the opportunity to repair the DNA after the drug-induced damage and cell survival. In eukaryotes, the G2 checkpoint effectors are ATM and ATR kinases and the downstream Chk1 kinase (18). During induced G2/M arrest, Chk1 maintains the phosphorylation of Cdc2 protein kinase, whose activity, together with Cyclin B1, is required for entry into mitosis (19, 20). As a consequence, Cdc2 is

inactivated and cells are arrested at checkpoints until damaged DNA is repaired, avoiding propagation of genetic defects to the daughter cells.

In the experiments detailed below, Chk1 inhibitors (SB-218078 and AZD7762) were used either alone or in combination with antineoplastic drugs currently in use in clinical practice. The experimental results show that in lung cancer stem cells, G2 abrogators increased cytotoxicity of DNA damaging agents. Combination therapy studies have also been successfully carried out *in vivo*. Chk1 inhibitors significantly enhance the anti-tumour efficacy of Gemcitabine in mice xenograft tumour model at well tolerated doses.

Naturally, in the various aspects of the present invention an at least one further therapeutic agent may be provided or administered as appropriate. Preferably, an at least one further therapeutic agent is a chemotherapeutic agent.

Further experiments (detailed below) have shown that preferential activation of the DNA damage checkpoint protein Chk1 in response to chemotherapy is likely the main player of drug resistance in lung cancer stem cells (LCSCs) and that its targeting might yield significant therapeutic gains.

Current cancer treatments target the most rapidly dividing differentiated cells, which represent the majority of the tumour bulk, resulting in a remarkable, but transitory remission. As noted above, cancer stem cells (CSCs) are slow-dividing cells that have an unlimited proliferative potential and are resistant to chemotherapy, this understanding being in line with the idea that this population might indeed account for treatment failure.

The further experimental data (below) underscores the fact that the present invention represents an improvement over the prior art clinical use of chemotherapy, with the improvement that Chk1 inhibition could overcome the failure of current treatment on lung cancer by achieving CSC disposal in lung tumors, i.e. prevention of tumour recurrence, metastasis formation and accomplishment of long-term remission and survival of cancer patients.

The further experiments (below) compare cell populations of (i) LCSCs and (ii) their differentiated progeny, and show that both basal and activation levels of Chk1 in

LCSCs are much higher than in their differentiated counterparts, suggesting that Chk1 plays the main role in LCSC chemo-resistance (Figure 8).

The further experiments are focused on the evaluation of the cytotoxic activity of specific Chk1 inhibitors, namely SB218078 and AZD7762 (above), on LCSCs.

The further experiments shows further surprising and unexpected results in that in order to exert the maximum cytotoxic effect, G2 checkpoint inhibitors, particularly Chk1 inhibitors, are best administered after treatment with chemotherapeutic drugs (e.g. anti-neoplastic drugs). The further experiments show that a concomitant (i.e. simultaneous or concurrent) administration of G2 checkpoint inhibitors, particularly Chk1 inhibitors, and chemotherapeutic agents is less effective (Figure 9).

Optimal administration regimes can be readily determined by undertaking further experiments in which administration times are varied, Further optimization of administration regimes (for example according to gender, age, or ethnic origin) can be achieved with appropriate *in vivo* and *in vitro* assays.

Therefore, in the various aspects of the present invention the administration pattern of the medicament (i.e. of the G2 checkpoint inhibitor and the chemotherapeutic agent) is that the G2 checkpoint inhibitor is administered at least 30 minutes after the chemotherapeutic agent is administered. Preferably, it is administered at least 1 hour after the chemotherapeutic agent. Preferably, it is administered at least 2 hours after the chemotherapeutic agent. Preferably, it is administered at least 3 hours after the chemotherapeutic agent. Preferably, it is administered at least 4 hours after the chemotherapeutic agent. Preferably, it is administered at least 5 hours after the chemotherapeutic agent. Preferably, it is administered at least 6 hours after the chemotherapeutic agent. Preferably, it is administered at least 7 hours after the chemotherapeutic agent. Preferably, it is administered at least 8 hours after the chemotherapeutic agent. Preferably it is administered between about one half and 8, 1 and 8, 2 and 8, 3 and 8, 4 and 8, 5 and 8, 6 and 8, or 7 and 8 hours after the chemotherapeutic agent. As appropriate, administration times (above) can be considered to be times for administration or times at which administration is to take place.

The further experiments show that co-administration of AZD7762 and Cisplatin yield the same outcome as co-administration of AZD7762 and Gemcitabine, namely significantly affected growth of human lung carcinoma generated by subcutaneous transplantation of LCSCs into NOD-SCID mice (Figure 10).

The further experiments also show in co-treated mice a marked reduction of the Carcinoembryonic antigen (CEA), whose levels are significantly high in non-small-cell lung cancer patients (Figure 11A).

Immunohistochemical analysis of tumour xenograft tissues has shown that combination treatment with Chk1 inhibitors causes irreparable damage to tumour cells, since expression of p-H2AX is strongly evident in co-treated mice (Figure 11B).

Finally, the further experimental data confirms the exhaustion and/or reduction of CSC compartment *in vivo*, by way of colony forming assay results on cells derived from dissociation of tumour xenografts. The experiments show a significant reduction in cell clonogenic ability in co-treated xenograft-derived cells (Figure 11C), indicating that co-administration of chemotherapy and Chk1 inhibitors could be a potential approach to achieve lung tumour eradication.

Overall, the experimental results demonstrate that Chk1 is indeed a successful target in the treatment of cancer, its chemical inhibition leading to tumorigenic cell population exhaustion in solid tumours. Importantly, to reach the maximum cytotoxic effects, G2 checkpoint inhibitors, particularly Chk1 inhibitors, should be administered shortly after chemotherapy, preferably in a range of 7-8 hours.

The experiments (below) are focused on lung cancer and chemotherapy. However, their findings are more broadly applicable to other cancer types and other treatment modalities where checkpoint activation is an early event, particularly in other cancers. Confirmatory studies for radio-resistance of cancer stem cells in breast cancers and mammary progenitor cells from other groups have been published (22-24) and their teachings may be applied to the performance of confirmatory studies in other cancers.

The invention will be further apparent from the following examples which show by way of example only the use of G2 checkpoint inhibitors and chemotherapeutic agents. Of the figures:

Figure 1A shows cell count after treatment. Y-axis shows count (ranges: top left 0 - 125; centre left 0 - 175; bottom left 0 - 62; top right 0 - 150; centre right 0 - 240; bottom right 0 - 45). X-axis shows PI-A (top and centre) and Pi PE-A (bottom), scale identical on all graphs. X-axis markings are (left to right) 10^2, 10^3, 10^4 and 10^5;

Figure 1B shows Western blot results of treatment of LCSC18, LCSC34 and LCSC36 cells with (left to right) NT, Cisplatin, Gemcitabine, and Paclitaxel, probing for phosphorylated chk1 (pChk1) (top) and beta-actin (bottom). Tabulated results are shown ("+++" = strong positive, "++" = positive, "+" = weak positive, "-" = negative);

Figure 1C shows cell count after treatment. Y-axis shows count (ranges: top left 0 - 8200; bottom left 0 - 680; top right 0 - 3000; bottom right 0 - 350). X-axis shows PI-A (ranges: top 0 - 260; bottom 0 - 150);

Figure 1D shows viability of lung cancer stem cell cloned cells. Y-axis shows cell viability (%) (range: 0 - 100). X-axis columns are (left to right): NT, SB-218078, Gemcitabine, Gemcitabine + SB-218078, Cisplatin, Cisplatin + SB-218078, Paclitaxel, and Paclitaxel + SB-218078. Clones tested are: LCSC18 (top left), LCSC34 (top right), LCSC36 (bottom left), and LCSC136 (bottom right);

Figure 2A shows confocal microscopy results of immunofluorescence staining of cells treated over 6 and 96 hour periods. Columns show (left to right) staining for Phalloidin, staining with DAPI, staining for p-H2AX, and merged results. rows (top to bottom) are control, Paclitaxel, Paclitaxel + SB-218078, Cisplatin, and Cisplatin + SB-218078. Table beneath indicates strength of staining ("+++" = strong positive, "++" = positive, "+" = weak positive, "-" = negative);

Figure 2B shows viability of (left) LCSC34 and (right) LCSC136 differentiated lung cancer stem cells. Axes are as per Figure 1D;

Figure 2C shows gels of (left) LCSC18, (centre) LCSC34 and (right) differentiated LCSC18 for Survivin (top) and beta actin (bottom). Cells were treated with (left to right): control, SB-218078, Cisplatin, and Cisplatin + SB-218078. Tabulated results shown beneath ("+++" = strong positive, "++" = positive, "+" = weak positive, "-" = negative);

Figure 3A shows Caspase-3/7 activation of (left to right) LCSC18, LCSC34, and LCSC136. Y-axes are Caspase-3/7 activation (fold increase vs. control), range 0 - 2.0. X-axes are (left to right): control, SB-218078, Cisplatin, Gemcitabine, Paclitaxel, Cisplatin + SB-218078, Gemcitabine + SB-218078, and Paclitaxel + SB-218078;

Figure 3B shows confocal microscopy of LCSC18 cells. Columns are (left to right) staining with TUNEL, staining with DPI, merged staining, and phase contrast microscopy. Intensity of staining with TUNEL and DAPI is tabulated beneath;

Figure 4A shows confocal microscopy of lung cancer stem cells. Left columns are staining with DAPI, centre columns are staining for Phalloidin, right columns are merge. Rows are (top to bottom): control, SB-218078, Paclitaxel, and Paclitaxel + SB-218-78;

Figure 4B shows bar charts of number of multinucleated cells for (left to right) LCSC18, LCSC34 and LCSC136 clones. Y-axis range is 0 - 100. X-axis columns for each bar chart are (left to right): control, SB-218078, Cisplatin, Cisplatin + SB-218078, Paclitaxel, Paclitaxel + SB-218078, AZD7762, Cisplatin + AZD7762, Paclitaxel + AZD7762;

Figure 5A shows Western blot results of treatment of LCSC18 and LCSC34 with (left) LCSC18 and (right) LCSC34 treated with (left to right): control, SB-218078, Cisplatin, Cisplatin + SB-218-78, Gemcitabine, and Gemcitabine + Sb-218078. probing is for phosphorylated cdc2 (pcdc2) (top), cyclin (middle), and beta-actin (bottom). Table beneath indicates strength of staining ("+++" = strong positive, "++" = positive, "+" = weak positive, "-" = negative);

Figure 5B shows confocal microscopy of LCSC34 cells. Columns are (left to right) staining for cyclin B1, staining with TO-PRO-3, merged staining, and phase contrast microscopy. Results show that the extent of staining is: Cisplatin > Cisplatin + SB-218078 > SB-218078 > control.

Figure 6A shows cells analysed to determine the fractions of nectoric vc. apoptotic cells. Y-axis on all plots is PI A. X-axes are (top row and bottom left) Annexin V Alexa Fluor 647 and (other plots) APC-A.

Figure 6B shows (top) Western bolt results of LCSC36 cells treated with (left to right): control, SB-218078, Cisplatin, and Cisplatin + SB-218078. Staining is for (top to bottom) Caspase-2, Caspase-3, and B-actin. Table beneath indicates strength of staining ("+++" = strong positive, "++" = positive, "+" = weak positive, "-" = negative). Bar chart shows protein expression levels in cells treated with (left four columns) Caspase-2, and (right four columns) Caspase-3. Each set of columns is (left to right) NT, SB-218078, Cisplatin, and Cisplatin + SB-218078. X-axis range is 0 - 50;

Figure 6C shows average numbers of colonies per well for each indicated condition. Mean ± SD of 3 independent experiments performed on 3 different LCSC lines is shown. Y-axis is number of colonies / well, range is (left and

centre) 0 - 100, (right) 0 - 175. Columns on left and centre bar charts are (left to right): Control, SB-218078, Cisplatin, Cisplatin + SB-218078. Columns on right bar chart are (left to right): Control, SB-218078, AZD7762, Cisplatin, Cisplatin + SB-218078, Cisplatin + AZD7762;

Figure 7A shows tumour size (volume cm^3) in mouse xenograft models. Y-axis is tumour size. X-axis is number of days. Lines are (top to bottom): control, Gemcitabine, AZD7762, and AZD7762 + Gemcitabine;

Figure 7B shows a bar chart of tumour masses. Y-axis is tumour mass (g), range 0 - 1.8g. Columns are (left to right): control, Gemcitabine, AZD7762, and AZD7762 + Gemcitabine;

Figure 7C shows FACS analysis of human origin of tumours. Y-axis is murine CD45 (moCD45 PE:Cy5-A). X-axis is HLA (HLA I PE-A);

Figure 8 shows Western blots for p-Chk1 in LCSC#1 (LCSC34) and LCSC#2 (LCSC36) and differentiated progenies left untreated or treated with Gemcitabine for 12 hours;

Figure 9 shows viability of LCSCs after (i) concomitant (simultaneous) administration of Chk1 inhibitor and chemotherapeutic agents, and (ii) administration of Chk1 inhibitor 8 hours after chemotherapeutic agents. X-axis indicates (left to right): NT - control (no treatment); SB (SB-218078 only); Cis (Cisplatin only); Cis+SB (Cisplatin + SB-218078); Paclitaxel (Paclitaxel only); Pacli+SB (Paclitaxel + SB-218078). Y-axis shows cell viability (%);

Figure 10A shows effect of treatment of tumours in NOD-SCID mice with control (squares), AZD7762 (triangles), Cisplatin (circles) and cisplatin + AZD7762 (diamonds). X-axis is number of days. Y-axis is average tumour size (volume in cm^3);

Figure 10B shows average mass of tumours explanted from the mice used for Figure 10B. X-axis is (left to right): control, AZD7762, Cisplatin, and Cisplatin + AZD7762. Y-axis is tumour mass (g);

Figure 11A shows carcinoembryonic antigen (CEA) levels in mice blood serum. X-axis is CEA level (ng/ml). Y-axis is (top to bottom) Gemcitabine + AZD7762; Gemcitabine; Cisplatin + AZD7762; Cisplatin; AZD7762; and control.

Figure 11B shows photographs of immunohistochemistry performed on formalin-fixed paraffin-embedded tissue for phosphorylated H2A.X (pH2A.X) from mice subjected to various treatments. Treatments are (left to right) Cisplatin; Cisplatin + AZD7762, Gemcitabine; and Gemcitabine + AZD7762. Second row of

photographs are enlarged areas indicated in first row of photographs. The table indicates the number of positive p-H2A.X cells visible in the photographs; and

Figure 11C shows colony forming ability of recovered tumour cells. X-axis indicates (left to right): control; AZD7762; Cisplatin; Cisplatin + AZD7762; Gemcitabine; Gemcitabine + AZD7762.

Unless indicated otherwise, error bars shown in the figures indicate ± 1 SD (standard deviation).

General Methods:

Compounds. All compounds were respectively purchased from Axon Medchem (AZD7762), Teval (Cisplatin), Lilly (Gemcitabine), Sigma (Paclitaxel) and Calbiochem (SB-2181078).

Preparation and isolation of lung cancer stem cells (LCSCs). Lung cancer tumour samples were obtained in accordance with consent procedures approved by the Internal Review Board of Department of Laboratory Medicine and Pathology, Sant'Andrea Hospital, University La Sapienza, Rome. Surgical specimens were washed several times and left overnight in DMEM–F12 medium supplemented with high doses of penicillin/ streptomycin and amphotericin B to avoid contamination. Tissue dissociation was carried out by enzymatic digestion (20 mg/ml collagenase II, Gibco-Invitrogen, Carlsbad, CA) for 2 hours at 37° C. Recovered cells were cultured at clonal density in serum-free medium containing 50 mg/ml insulin, 100 mg/ml apo-transferrin, 10 mg/ml putrescine, 0.03 mM sodium selenite, 2 mM progesterone, 0.6% glucose, 5mM HEPES, 0.1% sodium bicarbonate, 0.4% BSA, glutamine and antibiotics, dissolved in DMEM–F12 medium (Gibco-Invitrogen) and supplemented with 20 mg/ml EGF and 10 mg/ml bFGF. Flasks non-treated for tissue culture were used to reduce cell adherence and support growth as undifferentiated tumour spheres. The medium was replaced or supplemented with fresh growth factors twice a week until cells started to grow forming floating aggregates. Cultures were expanded by mechanical dissociation of spheres, followed by re-plating of both single cells and residual small aggregates in complete fresh medium. In these conditions, cancer stem cells are enriched and grow in suspension as spheres. This culture system has been shown to maintain both stemness and tumorigenic properties (14, 15).

Cell cycle analysis. After treatment with different drug combinations, cells were collected, washed with PBS and dissociated with trypsin. Cells were then incubated with a propidium iodide staining solution (trisodium citrate 0.1%, NaCl 9.65mM, NP40 0.3%, PI 50µg/ml, RNase A 200µg/ml) for 30 minutes at room temperature in the dark. Cell cycle profile was analyzed by Fluorescence-activated cell sorting (FACS).

Cell viability assay. Cell viability was measured with colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay or CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to standard protocols. Lung cancer stem cells were seeded at 5000 cells/well in triplicates in a 96-well plate and treated with Cisplatin 5ug/ml, Gemcitabine 250uM, Paclitaxel 30ng/ml and SB-2181078 20nM. Cell viability was analyzed by Victor 2 plate reader equipment (Wallac, Turku, Finland).

PI/Annexin V staining. PI/Annexin V staining was used to determine the fractions of apoptotic versus necrotic cells. A total of 50000 treated cells were washed with Annexin V-binding buffer (2.5mM $CaCl_2$, 140mM NaCl, 10mM HEPES) and thereafter incubated with Annexin V Alexa Fluor 647 1.25 nM (Invitrogen), for 15 minutes at room temperature. Cells were again washed with Annexin V-binding buffer, re-suspended in buffer containing 5 µg/ml propidium iodide (PI) and analysed by FACS.

Caspase Assay. Lung cancer stem cells were seeded at 5000 cells/well in triplicates in a 96-well plate and treated with Cisplatin 5ug/ml (Teval), Gemcitabine 250uM (Lilly), Paclitaxel 30ng/ml (Sigma) and SB-2181078 20nM (Calbiochem). Caspase 3/7 activation was evaluated after 96h by Apo1 Caspase-3/7 Assay kit (Promega). Colorimetric or fluorimetric assays were analyzed by Victor 2 plate reader (Wallac, Turku, Finland).

Apoptosis Assay - TUNEL. The cells were cyto-spun onto glass slides, fixed with 4% buffered paraformaldehyde and then permeabilized with 0.1% Triton X-100. Apoptosis was determined with the In Situ Cell Death Detection kit, Fluorescein (Roche), following the manufacturer's instructions. The cells containing DNA strand breaks were stained with fluorescein-dUTP, detected and counted by fluorescence microscopy.

Western blot. Whole cell-lysates from treated cells were prepared in 1% NP40, 20mM TRIS pH 7.2, 200mM NaCl and Complete Protease Inhibitor Cocktail (Sigma). Western

blot was performed according to standard protocols. Briefly, 20µg of protein extracts were heated to denaturate proteins and were then separated by polyacrylamide gel electrophoresis. Separated proteins were transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham Bioscience). Blocking of non-specific binding was made by incubating the membrane in 5% non-fat dry milk. Membranes were probed with antibodies specific for phosphorylated Chk1 (pchk1) (Cell Signaling Technology, cat. # 2341), cyclin B1 (Santa Cruz Biotechnology, cat #7393), phosphorylated cdc2 (pcdc2) (Cell Signaling Technology, cat. #9111) Survivin (Cell Signaling Technology, cat #2808), Caspase 2 (BD, cat #611022) and Caspase-3 (Upstate, cat #06-735) overnight and an anti-β-actin antibody (Sigma, cat. #A5441) was used to assess equal loading. Membranes were probed with secondary antibodies, incubated with ECL, enhanced chemiluminescence (Thermo Scientific), and then exposed to film.

Immunofluorescence. Treated cells were cyto-spun onto glass slides, fixed with 4% buffered paraformaldehyde and then permeabilized with 0.1% Triton X-100. The slides were incubated over night with anti-phosphoH2A.X 2µg/ml (Upstate), anti-cyclin B1 8µg/ml (Santa Cruz Biotechnology), anti-Tubulin 100µg/ml (Molecular Probes), Phalloidin-Alexa Fluor 488 5 Units/ml (Molecular Probes), anti-mouse Alexa Fluor 488 5ng/ml and anti-mouse 647 secondary antibody 5ng/ml (Invitrogen) for 1h at room temperature. TO-PRO-3 4µM (Invitrogen) or DAPI 3µM (Molecular Probes) were used to visualize nuclei. The slides were analyzed by confocal microscopy using an inverted fluorescence microscope FV1000 (Olympus) with a 60X objective lens.

Soft agar assay. Lung cancer stem cells were treated with Cisplatin (5µg/ml), Paclitaxel (30ng/ml), SB-218078 (20nM), AZD7762 (5nM) or in combinations of drug and inhibitor (SB-218078 and AZD7762 were added after 8 hours). Thereafter, 1000 cells were immediately plated in the top agar layer in each well of a 24-well culture plate with 0.27% top agar layer and 0.4% bottom agar layer (SeaPlaque Agarose, Cambrex). Both layers of the soft agar contained either Cisplatin (5µg/ml), Paclitaxel (30ng/ml), SB-218078 (20nM), AZD7762 (5nM) or combinations of treatment. Cultures were incubated at 37°C for 20 days. Colonies from triplicate wells were stained with crystal violet (0.01% in 10% MetOH), visualized and counted under microscope and photographed with a Nikon D80 camera.

Differentiation of LCSCs. Lung cancer stem cells were plated in DMEM (Gibco) supplemented with 10% FBS (fetal bovine serum) over night for attachment of cells.

DMEM was replaced with BEGM growth medium (Lonza) to facilitate the progress of differentiation and incubated for 72h. Differentiated cells could thereafter be maintained in DMEM supplemented with 10% FBS.

In vivo Studies. Female NOD-SCID mice were purchased from Charles River. All procedures were conducted in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and within the protocols approved by the Istituto Superiore di Sanità. Tumour stem cells were dissociated, counted and resuspended in a mix of sterile PBS and Matrigel (1:1). 50000 lung cancer stem cells (LCSCs) were implanted s.c. (subcutaneously) into the right flank of each mouse in a volume of 0.1 to 0.2 mL using a 25-gauge needle. Tumours were allowed to grow to the designated size of 100 to 200 mm^3 before the administration of compound. Animals were dosed i.v. with 10 mg/kg of AZD7762 and i.p. with 60mg/kg of Gemcitabine every 3 days starting from day 0. Tumour growth was evaluated before every single administration. After 30 days, tumours were removed, weighted and dissociated to analyze the percentage of human cells vs. murine cells using a PE conjugated anti-human HLA1 antibody (eBioscience) and an a PE-Cy5 anti-mouse CD45 antibody (BD Pharmingen) by FACS analysis.

G2 abrogators increase the cytotoxicity of DNA damaging agents in cancer stem cells

In spite of the variety of therapeutic approaches, lung cancer is the most common cause of cancer-related mortality worldwide. Identification of CSCs in lung cancer can therefore contribute to develop innovative and selective therapies to improve the overall survival in patients. The effects on LCSCs of chemotherapeutic agents currently used in clinical practice were investigated. Cisplatin, Gemcitabine and Paclitaxel were used at doses comparable with the higher plasma levels reached in treated lung cancer patients.

<u>SB-218078 increases the cytotoxicity of Cisplatin, Gemcitabine and Paclitaxel on lung cancer stem cells and chemotherapy induces activation of Chk1.</u>

Figure 1A. Lung cancer stem cells were seeded at 250000/ml and treated with Cisplatin (5µg/ml), Gemcitabine (250µM) and Paclitaxel (30ng/ml) for 96h. Control and chemotherapy-treated cells were incubated with 5µg/ml propidium iodide (PI) and analyzed by FACS to determine the cell viability. Results are shown in Figure 1A.

Figure 1B. LCSC18 LCSC34 and LCSC36 were treated with Cisplatin (5µg/ml), Gemcitabine (250µM) and Paclitaxel (30ng/ml) for 96h. Western blot was performed with 20µg of whole cell protein extracts. Membranes were incubated with the antibodies specific for phosphorylated chk1 (pchk1). Anti-β-actin antibody was used to assess equal loading. Results are shown in Figure 1B and the table is indicating the relative expression of proteins analyzed.

Figure 1C. LCSC18 were seeded at 250000/ml and treated with Cisplatin and Paclitaxel as described. After 48 hours of treatment, cells were incubated with a propidium iodide staining solution (trisodium citrate 0.1%, NaCl 9.65mM, NP40 0.3%, PI 50µg/ml, RNase 200µg/ml) for 30 min at room temperature. Cell cycle was analyzed by FACS. Results are shown in Figure 1C.

Figure 1D. Lung cancer stem cell clones 18, 34, 36 and 136 were seeded at 5000 cells/well in triplicates in a 96-well plate and treated with Cisplatin (5µg/ml), Gemcitabine (250µM) or Paclitaxel (30ng/ml) for 96h and treated with or without SB-218078 (20nM) after 8 hours of the total incubation time. Cell viability in control and chemotherapy-treated cells was evaluated after 4 days by MTT assay and cell count. Data are a mean of three independent experiments. Results are shown in Figure 1D.

Results

Neither of the drugs induced remarkable cell death even after a long exposure (Figure 1A). However, it is well known that many of the conventional anticancer treatments at least partly damage the DNA of cells. In response to DNA damage, cells are arrested by multiple cell cycle checkpoints to allow DNA to be repaired before progression into the next phase. There are at least three DNA damage checkpoints, the G1/S, the S and the G2/M, controlled respectively by p53 (25), chk1 and chk2 (26, 27). The activation profiles of these proteins were investigated in the presence and absence of chemotherapy and although it there was no visible change in the phosphorylation status of p53 and chk2 (data not shown), chk1 was highly phosphorylated in the presence of Cisplatin, Gemcitabine and Paclitaxel (Figure 1B). In line with these observations, analysis of cell cycle profile revealed arrest of lung cancer stem cells in G2 phase (Figure 1C).

It was concluded that, following DNA damage, G2 cell cycle arrest allows DNA repair and final cell survival to be achieved. Investigations were undertaken to determine

whether G2 abrogation with the specific chk1 inhibitor SB-218078 (28) could increase the cytotoxicity of these DNA damaging agents. As showed in Figure 1D, although SB-218078 alone has no or little effect, it increases the cytotoxic effect of each drug used. Aligned with the poor therapeutic effects of conventional chemotherapy on lung cancer patients, LCSCs are highly resistant to anti-neoplastic drugs. However, combination therapy with a specific checkpoint inhibitor can overcome G2 phase arrest and induce cell death.

Lung cancer stem cells resistance to chemotherapy relies on over-expression of anti-apoptotic proteins and a functional DNA damage pathway.

The first step in the initiation of activity of DNA damage checkpoint is recognition of DNA damage. One of the earliest modifications of the chromatin structure in the damage response is phosphorylation of H2A.X at Ser 139 (29). To prove the previous assumption, investigations were undertaken to determine whether exposure to Cisplatin or Paclitaxel would induce DNA damage in lung cancer stem cells, thus indicating the presence of DNA double strand breaks (DSBs) and the activation of the DNA damage machinery.

<u>Lung cancer stem cells and differentiated cancer cells posses different repair and survival systems.</u>

Figure 2A. LCSC34 were treated with Cisplatin and Cisplatin + SB-218078 for 6h and 96h. Cells were cyto-spun on glass slides, fixed with 4% buffered paraformaldehyde and then permeabilized with 0.1% Triton X-100. The slides were incubated overnight with anti-phospho-H2A.X, and then with an Alexa Fluor 647 secondary antibody for 1h at room temperature. DAPI was used to visualize nuclei. The slides were analyzed by confocal microscopy using an inverted fluorescence microscope (Olympus). Original magnification 60x.

Figure 2B. Differentiated lung cancer stem cells were seeded at 5000 cells/well in triplicates in a 96-well plate and treated with Cisplatin (5µg/ml), Gemcitabine (250µM) or Paclitaxel (30ng/ml) for 96h and treated with or without SB-218078 (20nM) after 8 hours of the total incubation time. Cell viability in control and chemotherapy-treated cells was evaluated after 4 days by CellTiter-Glo assay.

Figure 2C. LCSC18 and LCSC34 were treated with Cisplatin (5µg/ml), SB-218078 (20nM) and Cisplatin + SB-218078 for 96h. Western blot was performed with 20µg of

whole cell protein extracts. Membranes were incubated with the antibody specific for Survivin. The anti-β-actin antibody was used to assess equal loading. Table is indicating the relative expression of proteins analyzed.

Results

Immunofluorescence staining of phospho-H2A.X after 6h exposure to Cisplatin, Paclitaxel or their combination with SB-218078 clearly showed the presence of nuclear foci of phospho-H2A.X (Figure 2A, left panel). However, after 96h the persistence of p-H2A.X is only evident in the combination with the inhibitor (Figure 2A, right panel), indicating that cells treated with Cisplatin or Paclitaxel alone undergo DNA damage but are also able to repair it, while cells treated with the combination chemotherapeutic agent plus chk1 inhibitor are unable to repair the damage and eventually die.

Differentiated progeny died after 96 hours of exposure to chemotherapy independently of the presence of the chk1 inhibitor, indicating how DNA repair activity is much more efficient in CSCs rather than differentiated cells (Figure 2B). Also of interest is the expression pattern of Survivin. Survivin is a member of the anti-apoptotic proteins family and its function is to inhibit caspases activation leading therefore to negative regulation of apoptosis or programmed cell death. The suppression of apoptosis is a major contributing factor to carcinogenesis. Allowing cells to bypass apoptosis and continue to live and proliferate allows the eventual accumulation of mutations that may render the cell unresponsive to mechanisms that normally regulate cell growth. The anti-apoptotic function of Survivin has been shown to play a significant role in cancer progression. Survivin has been shown to be regulated during the cell cycle as its expression is found to be dominant only in the G2/M phase.

Drug exposure of LCSCs strongly increased Survivin expression confirming the G2 arrest in the presence of antineoplastic drugs and ability of cancer stem cells to escape programmed cell death. However, the combination of Cisplatin with SB-218078 in these cells proved to be effective in overcoming this effect as it reduced Survivin expression rendering cells more susceptible to death (Figure 2C). In differentiated lung cancer cells the protein level of Survivin was not increased in the presence of Cisplatin.

G2 abrogators induce aberrant mitosis in cancer stem cells.

Efficiency of action of DNA damaging agents used in anticancer therapy depends on their ability to induce growth arrest and to activate the cell death machinery. Cell death can be classified according to its morphological appearance (which may be apoptotic, necrotic, autophagic or associated with mitosis), enzymological criteria (with and without the involvement of nucleases or proteases) and functional aspects (programmed or accidental, physiological or pathological) (30). An analysis of the biochemical and morphological features of the cell model in the presence and in the absence of treatments was therefore undertaken.

Combination with SB-218078 does not induce apoptosis in lung cancer stem cells after 96h treatment.

Figure 3A. Lung cancer stem cells were seeded at 5000 cells/well in triplicates in a 96-well plate and treated with Cisplatin (5µg/ml), Gemcitabine (250µM) or Paclitaxel (30ng/ml) for 96h and treated with or without SB-218078 (20nM) after 8 hours of the total incubation time. Caspase-3/7 activation was evaluated after 4 days by Apo1 Caspase-3/7 Assay kit (Promega). Colorimetric or fluorimetric assays were analyzed by Victor 2 plate reader (Wallac, Turku, Finland). Data are a mean of three independent experiments.

Figure 3B. LCSC18 were treated as described for 96h. Cells were cyto-spun on glass slides, fixed with 4% buffered paraformaldehyde and then permeabilized with 0.1% Triton X-100. Occurrence of apoptosis was determined with the In Situ Cell Death Detection kit, Fluorescein (Roche), following the manufacturer's instructions. The cells containing DNA strand breaks were stained with fluorescein-dUTP and detected by fluorescence microscopy. The table is showing the number of apoptotic cells vs non apoptotic cells.

Cisplatin in combination with SB-218078 causes mitotic catastrophe in lung cancer stem cells.

Figure 4A. Lung cancer stem cells were treated as described previously. After 96h, cells were washed, cyto-spun on glass slides and stained with Phalloidin and DAPI to visualize cell membranes and nuclei. The slides were analyzed by confocal microscopy using an inverted fluorescence microscope (Olympus).

Figure 4B. Percentage of multinucleated LCSC18, LCSC34 and LCSC136 was estimated by counting the nuclei in 100 cells on each slide. Data are presented as a mean of three independent experiments.

Results

Apoptosis is recognized as a major barrier that must be circumvented by tumour cells to allow them to survive and proliferate in such stressful conditions (31). Its induction by DNA damage is typically associated with activation of the caspase family of proteases, which eventually leads to biochemical and morphological apoptosis-specific changes such as cellular shrinkage, membrane blebbing and DNA fragmentation (32). The combination of SB-218078 with all the chemotherapeutic drugs indicated that even after prolonged treatment (96h), there was no induction of caspase activation nor were TUNEL positive cells present (Table 1), indicative of DNA fragmentation.

Table 1

	TUNEL positive cells	TUNEL negative cells
Control	2	98
SB-218078	5	95
Cisplatin	3	97
Cisplatin + SB-218078	1	99

In addition to apoptosis, tumours can be effectively eliminated following DNA damage by necrosis, mitotic catastrophe and autophagy. Death by autophagy is characterized by the double-membrane vesicles containing cytosolic organelles (33). Cells dying from mitotic catastrophe are usually large and contain uncondensed chromosomes. The main characteristic of mitotic catastrophe is the formation of multiple micronuclei, and also aberrant mitotic spindle formation can be involved (34). During necrosis, cells swell and loose their membrane integrity (35). Investigation of the changes in cell morphology after drug treatment, showed the presence of a large population of cells with two or more nuclei in the combination Cisplatin and SB-218078, which was indicative of cell death through mitotic catastrophe (Figure 4A and B).

G2 abrogators induce mitotic catastrophe through premature activation of cdc2/cyclin B1 complex.

To gain insight into the molecular mechanisms responsible for the mitotic catastrophe events observed following anti-neoplastic agents and SB-218078 treatment, the expression of cell-cycle regulatory proteins, namely cdc2 and cyclin B1, was analyzed.

Premature activation of cdc2/cyclin B1 complex and translocation of cyclin B1 from the cytoplasm to the nucleus in chemotherapy-SB-218078 treated lung cancer stem cells.

Figure 5A. LCSC18 and LCSC34 were treated with Cisplatin (5μg/ml), Gemcitabine (250μM) for 96h and treated with or without SB-218078 (20nM) after 8 hours of the total incubation time. Western blot was performed with 20μg of whole cell protein extracts. Membranes were incubated with the antibodies specific for phosphorylated cdc2 (pcdc2) and cyclin B1. The anti-β-actin antibody was used to assess equal loading. Table is indicating the relative expression of proteins analyzed.

Figure 5B. LCSC34 were treated with Cisplatin (5μg/ml), SB-218078 (20nM) and Cisplatin + SB-218078 for 96h. Cells were cyto-spun on glass slides, fixed with 4% buffered paraformaldehyde and then permeabilized with 0.1% Triton X-100. The slides were incubated over night with anti-cyclin B1, and then with an Alexa Fluor 488 secondary antibody for 1h at room temperature. TO-PRO-3 was used to visualize nuclei. The slides were analyzed by confocal microscopy using an inverted fluorescence microscope (Olympus). Original magnification 40x.

Results

It is known that Chk1 phosphorylates the family of Cdc25 phosphatases, which in turn inhibit Cdc2 activity and prevent its premature activation, thus maintaining the highly regulated temporal order of cell-cycle progression. In the event of cell-cycle alteration due to DNA damage, Chk1 is activated to phosphorylate Cdc25 leading to its degradation via the proteasomal pathway or sequestration to cytoplasm by binding to 14-3-3 protein (36, 37). As a consequence, Cdc2 is inactivated and cells are arrested at checkpoints until damaged DNA is repaired. It is reasonable to think that under this circumstance, inhibition of Chk1 leads to the improper activation of Cdc2 resulting in checkpoint abrogation and mitotic catastrophe. Cdc2 is therefore the major target of Chk1 and is essential for G1/S and G2/M phase transitions, while cyclin B1 is a regulatory protein involved in mitosis and it translocates from the cytoplasm to the nucleus during M phase.

When cdc2 forms a complex with cyclin B1, a dividing cell is allowed to enter mitosis from G2 phase. The experiments show that addition of SB-218078 after treatment with chemotherapy induced up-regulation of cdc2 activity by de-phosphorylation and decreased cyclin B1 expression (Figure 5A), probably through nuclear translocation and subsequent degradation. These events lead to abrogation of the G2 arrest and aberrant mitotic entry before the completion of DNA repair. The role of cyclin B1 in drug-induced G2 arrest in LCSCs was further investigated. Cyclin B1 accumulates in the cytoplasm through S and G2 phases and translocates to the nucleus during prophase. Results show that in the cells treated with Cisplatin alone, cyclin B1 is clearly located in the cytoplasm, as a sign of cell cycle arrest. In cells treated with Cisplatin and SB-218078, cyclin B1 translocates from the cytoplasm to the nucleus, demonstrating that SB-218078 abrogates the G2 arrest induced by Cisplatin and forces the cells back into the cell cycle (Figure 5B).

Long term affects on survival and colony forming ability.

Unfortunately, universal molecular and mechanistic attributes of mitotic catastrophe are yet to be defined. In some models, mitotic catastrophe is thought to directly cause cell death that is distinct from apoptosis (39) or, alternatively, mitotic catastrophe is inherently associated with activating apoptosis from the M phase (39). Studies with other models, including ionizing radiation and antimitotic drugs, suggested that mitotic catastrophe is not a distinct form of cell death but rather an event that leads to cell death through apoptosis and/or necrosis (38, 39). To distinguish between them and to investigate how severe the damage induced by the combination of anti-neoplastic drugs and SB-218078 was, rescue experiments were performed.

Figure 6A. The combination of Cisplatin and SB-218078 induces irreparable damage in lung cancer stem cells. Lung cancer stem cells were seeded at 250000/ml and treated with Cisplatin (5µg/ml), SB218078 (20ng/ml) and the combination of both for 96h. At the end of the incubation period, cells were washed and plated in serum free medium for a total period of 72h. A total of 50000 cells/condition were double stained for propidium iodide (PI) and Annexin V Alexa Fluor 647 and analysed by FACS to determine the fractions of necrotic vs. apoptotic cells.

Figure 6B. Lung cancer stem cells LCSC36 were treated with Cisplatin (5µg/ml), SB-218078 (20nM) and the combination of both for 96h. At the end of the incubation period, cells were washed and plated in fresh serum free medium for a total period of

72h. Western blot was performed with 20µg of whole cell protein extracts. Membranes were incubated with antibodies specific for the pro-forms of caspase-2 and caspase-3, described in the general methods. The anti-β-actin antibody was used to assess equal loading. The table is indicating the relative expression of inactive pro-forms of caspases 2 and 3.

Figure 6C. Cells were treated with Cisplatin (5µg/ml) and SB-218078 (20nM, added after 8 hours) and then immediately plated in the soft agar top layer. 1000 cells were plated in each well of a 24-well culture plate with 0.4% bottom agar layer and 0.27 % top agar layer. The soft agar contained either SB-218078 (20nM), Cisplatin (5µg/ml) and AZD7762 (5nM), or their combinations. Cultures were incubated at 37°C for 20 days. Colonies were stained with crystal violet 0.01%, visualized and counted under microscope.

Results

Staining with FITC Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) to allow the investigators to identify early apoptotic cells (PI negative, FITC Annexin V positive) from necrotic cells (both FITC Annexin V and PI positive). Results show that the combination of Cisplatin and SB-218078 induced preferentially apoptosis and irreparable damage in LCSCs, as nearly 70% of the cells died even after re-plating them in fresh medium (Figure 6A).

Moreover, to ascertain whether the observed mitotic catastrophe led to apoptosis, the effect of Chk1 inhibition on the major apoptotic pathways was examined. Caspase-3 is the common downstream effector caspase for both the extrinsic/death receptor-induced apoptosis pathway involving caspase-8, and the intrinsic/mitochondria-triggered apoptotic pathway involving caspase-9 (40, 41) and it resulted in strong activation in the combination treatment (Figure 6B). Moreover, cell death occurring during the metaphase/anaphase transition, like in the case of mitotic catastrophe, is characterized by the activation of caspase-2 which can also be activated in response to DNA damage. Caspase-2 appears to be necessary for the onset of apoptosis triggered by several insults, including DNA damage (42, 43). Although the morphological aspect of apoptosis may be incomplete, these alterations constitute the biochemical hallmarks of apoptosis. The combination Cisplatin + SB-218078 is also characterized by the activation of caspase-2, confirming even more that final cell death occurs through apoptosis (Figure 6B).

These results indicated that SB-218078 potentiates the cytotoxicity of chemotherapeutic drugs in short term viability assays. To investigate the long term impact of the combination with SB-218078 in lung cancer stem cells, soft agar assays were performed to evaluate differences in colony forming abilities.

Results show that lung cancer stem cells maintain the ability to form colonies after treatment with Cisplatin, Paclitaxel or SB-218078, but after treatment with the combinations of both Cisplatin and Paclitaxel with SB-218078 the cells lose their ability to grow (Figure 6C).

These results therefore indicate that the combination of chemotherapy with SB-218078 has both short term and long term impact on cell viability in lung cancer stem cells.

Combination of chemotherapy and chk1 inhibitors has strong affects on tumour growth *in vivo*.

Xenotransplantation of LCSCs provides a solid base for the clinic use of therapies which aim to neutralize the pathways promoting tumour survival and growth. To evaluate the ability of chk1 inhibitors to enhance cytotoxicity of chemotherapeutic agents in lung cancer *in vivo*, the effect of AZD7762 on human lung carcinoma generated by subcutaneous transplantation of lung cancer stem cells into NOD-SCID mice was assessed.

<u>AZD7762 potentiates Gemcitabine in mouse xenograft efficacy models.</u>

Figure 7A. NOD-SCID mice bearing tumours obtained by injection of LCSC136 were treated with multiple cycles of therapy every 3d. Each cycle consisted of Gemcitabine alone (60 mg/kg) or Gemcitabine (60 mg/kg) followed by one dose of 10 mg/kg AZD7762 (8 h after Gemcitabine dose). Tumour volume was measured using a callipers on the indicated days. Each Group consisted of 5 animals.

Figure 7B. Tumour masses were evaluated by weighting the tumours right after explantation. The graph shows the median weight value of tumours for each group of mice.

Figure 7C. Evaluation of human origin of the tumours through FACS analysis of human HLA expression vs. murine CD45.

Results

Tumour growth was evaluated in a total period of 30 days. As predicted by *in vitro* studies, AZD7762 potently abrogated tumour growth by enhancing the efficacy of Gemcitabine (Figure. 7A). These data were also confirmed by evaluation of tumour mass (Figure 7B). Finally, analysis of human HLA and murine CD45 in removed samples proved the human origin of the tumours (Figure 7C).

Discussion:

Cell cycle checkpoints are mechanisms that coordinate the progression of cell cycle with intracellular and extracellular events. In response to DNA damage, an elaborate network of signalling pathways, collectively called DNA damage checkpoint, is activated to prevent the damaged DNA from being replicated or transmitted to the next generation. DNA damage checkpoint responses therefore play essential roles in cellular radio and chemosensitivity. Cancer stem cells contribute to tumour repopulation through preferential checkpoint response and DNA repair, and targeting of checkpoint response in cancer stem cells can overcome chemo-resistance and may provide a therapeutic advantage to reduce tumour recurrence. The results obtained so far in this study suggest that SB-218078 and AZD7762 increase the cytotoxic effect of chemotherapeutic drugs in the treatment of lung cancer stem cells by abrogating the chemotherapy-induced G2 arrest. The combination of a G2-abrogator with DNA-damaging drugs might therefore represent a novel approach for lung cancer treatment and lead to final eradication of the tumour.

Further experiments

Materials and methods are the same as above, except where detailed otherwise.

Treatment

For *in vitro* experiments, the following concentrations of chemotherapies and Chk1 inhibitors were used: cisplatin (5 ug/ml), gemcitabine (250 µM), paclitaxel (30 ng/ml), SB218078 (20 nM), AZD7762 (5 nM). For *in vivo* studies we used: gemcitabine (60 mg/kg), cisplatin (3 mg/kg) and AZD7762 (10 mg/kg). Chk1 inhibitors for both, *in vitro* and *in vivo* studies was added 8 hours after chemotherapy.

Western blot.

LCSCs and differentiated counterparts were treated for 12 hours as previously described. Whole cell lysates were prepared in lysis buffer (NP40 1%, 20 mM TRIS (pH 7.2), 200 mM NaCl, Phosphatase Inhibitor Cocktail 1 (used 1:100, P2850, Sigma-Aldrich, St. Louis, MO, USA), Phosphatase Inhibitor Cocktail 2 (used 1:100, P5726, Sigma-Aldrich) and Protease Inhibitor Cocktail (used 1:100, P8340, Sigma-Aldrich)). 20 µg of whole cell extracts were subjected to 8-15% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE), transferred onto Hybond-C paper (Amersham Biosciences), incubated with Chk1, phosphorylated Chk1 (Ser345) from Cell Signaling Technology (Danvers, MA, USA).; β-actin (AC-15) from Sigma-Aldrich was used to asses equal loading. Detection was carried using enhanced chemiluminescence detection kit (Pierce, Rockford, IL, USA).

In vivo studies

Female NOD-SCID mice were purchased from Charles River. All procedures were conducted in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and within the protocols approved by the Istituto Superiore di Sanità. LCSCs were dissociated, counted and resuspended in a mix of PBS and Matrigel (1:1). 50,000 LCSCs were implanted subcutaneously into the right flank of each mouse in a volume of 0.1 to 0.2mL using a 25-gauge needle. Tumours were allowed to grow to the size of 100 to 200 mm^3 before the administration of compounds. Animals were dosed intraperitoneally with gemcitabine or cisplatin and intravenously with AZD7762 every 3 days starting from day 0 and each group consisted of 5 animals. Tumour growth was evaluated with an electronic caliper before every single administration. Levels of carcinoembryonic antigen (CEA) were measured in blood serum obtained by retro-orbital withdraw and evaluated by immunoluminometric technique using Vitros ECI analyzer (Ortho-Clinical Diagnostics Inc. Rochester, NY, USA). After 30 days, tumours were removed. Tumour mass was analyzed by weighting tumours using a PL202-L Precision Balance (Mettler-Toledo, Novate Milanese MI, Italy). Immunohistochemistry was performed on formalin fixed paraffin-embedded tissue or frozen tissue. Paraffin sections (5µm) were de-waxed in xylene and rehydrated with distilled water. The slides were subsequently incubated with phosphorylated H2A.X (Upstate-Millipore). The reaction was performed using Elite Vector Stain ABC systems (Vector Laboratories) and DAB substrate chromogen (DakoCytomation), followed by counterstaining with haematoxylin. The percentage of human cells vs. murine cells was evaluated by FACS analysis using a PE conjugated

anti-human HLA1 antibody (eBioscience) and a PE-Cy5 anti-mouse CD45 antibody (BD Pharmingen).

Soft agar assay

Soft agar colony forming assays were carried out for LCSCs treated with cisplatin, paclitaxel, SB218078, AZD7762 or in combinations of drug and inhibitor for 96 hours. Subsequently, cells were washed and 1,000 cells were plated in the top agar layer in each well of a 24-well culture plate with 0.3% top agar layer and 0.4% bottom agar layer (SeaPlaque Agarose, Cambrex, New Jersey, USA). Cultures were incubated at 37°C for 20 days. Colonies from triplicate wells were stained with crystal violet (0.01% in 10% MetOH), visualized and counted under microscope and photographed with a Nikon D80 camera. For *in vivo* Soft agar colony forming assays was performed on xenograft-derived cells. Briefly tumours were removed and dissociated. Cells recovered were extensively washed and plated in stem cells medium conditions for 3 days. Consequently, 500 cells for each treatment condition were plated as described above.

Figure 8

As shown in teh figure, LCSCs are found to possess a much more efficient activation of Chk1 (i.e. a more efficient DNA repair pathway) than differentiated counterparts.

Figure 9

This is a continuation of the experiments detailed for Figure 1 (above). LCSC#1 (LCSC34) and LCSC#2 (LCSC36) were seeded at 5000 cells/well in triplicates in a 96-well plate and treated with cisplatin (5µg/ml) or paclitaxel (30ng/ml) for 96 hours, and treated (a) with SB-218078 (20nM) or (b) without SB-218078, either (i) simultaneously or (ii) after 8 hours of the total incubation time. Cell viability in control and chemotherapeutic-treated cells was evaluated after 4 days by MTT assay and cell count. Data are a mean of three independent experiments.

Results

Results show that Chk1 inhibitors work more effectively when administered after chemotherapeutic agents, i.e. that Chk1 inhibitors achieve enhanced efficacy when administered after chemotherapeutic agents.

Further experiments were performed as above, with the G2 checkpoint inhibitor (i.e. the Chk1 inhibitor) administered 7 hours after the chemotherapeutic agent, and results (not shown) are generally the same as those shown for 8 hours.

Figure 10

Groups of NOD-SCID mice (five animals per group) were injected with LCSC136 to give tumours. They were then treated with multiple cycles of therapy every 3 days (first treatment is day 0). Each treatment cycle consisted of: Group (i) - control, no treatment; Group (ii) - AZD7762 alone (10 mg/kg); Group (iii) - Cisplatin alone (3mg/kg), or Group (iv) - Cisplatin (3mg/kg) followed by one dose of 10 mg/kg AZD7762 (8 hours after Cisplatin dose). Tumour volume was measured using an electronic caliper on the indicated days. Tumour mass was evaluated by weighing the tumour immediately after explantation at day 30.

Results

Results show that co-administration of Cisplatin with AZD7762 strongly affects tumor growth in terms of growth rate and tumour mass.

Figure 11

A. Carcinoembryonic antigen (CEA) levels in mice blood serum were measured using an immunoluminometric technique. Mean value ± 1 SD of three experiments is reported.

B. Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue for phosphorylated H2A.X (pH2A.X). The number of positive p-H2A.X cells visible in the photographs is represented in the table below the photographs.

C. A colony forming ability assay was performed on recovered tumour cells. The graph shows the average number of colonies/well for each combination of treatment (right panel). Mean value ± 1 SD of three experiments is reported.

Results

The results show that a combination of chemotherapy and Chk1 inhibitors strongly affects tumour growth *in vivo*.

Figure 11A shows that co-administration of Gemcitabine and Cisplatin with AZD7762 strongly reduces expression of CEA.

Figure 11B shows by immunohistochemical analysis of tumour xenograft tissues that combination treatment with Chk1 inhibitors is irreparably damaging tumour cells through an increased expression of pH2A.X.

Figure 11C shows inhibition/reduction of clonogenic ability accounting for disposal of tumorigenic cells (CSCs) in the tumour xenograft.

It will be appreciated that it is not intended to limit the present invention to the above examples only, other embodiments being readily apparent to a person of ordinary skill in the art without departing from the scope of the appended claims.

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CLAIMS

1. The use of a G2 checkpoint inhibitor and a chemotherapeutic agent in the manufacture of a medicament for the treatment of tumorigenic cells in solid tumours.

2. A G2 checkpoint inhibitor and a chemotherapeutic agent for the treatment of tumorigenic cells in solid tumours.

3. A pharmaceutical pack for the treatment of tumorigenic cells in solid tumours, comprising a G2 checkpoint inhibitor and a chemotherapeutic agent.

4. The use, G2 checkpoint inhibitor and a chemotherapeutic agent, or pharmaceutical pack according to any of the preceding claims, the G2 checkpoint inhibitor being a G2 checkpoint abrogator.

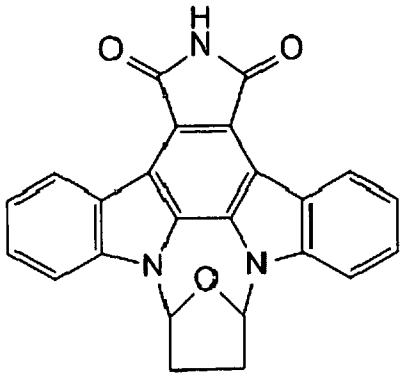
5. The use, G2 checkpoint inhibitor and a chemotherapeutic agent, or pharmaceutical pack according to any of the preceding claims, the G2 checkpoint inhibitor being a Chk1 inhibitor.

6. The use, G2 checkpoint inhibitor and a chemotherapeutic agent, or pharmaceutical pack according to any of the preceding claims, the G2 checkpoint inhibitor being a Chk1 protein inhibitor.

7. The use, G2 checkpoint inhibitor and a chemotherapeutic agent, or pharmaceutical pack according to any of the preceding claims, the G2 checkpoint inhibitor being a WEE1 inhibitor.

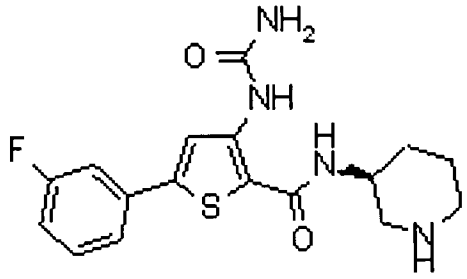
8. The use, G2 checkpoint inhibitor and a chemotherapeutic agent, or pharmaceutical pack according to claim 7, the WEE1 inhibitor being PD0166285.

9. The use, G2 checkpoint inhibitor and a chemotherapeutic agent, or pharmaceutical pack according to any of the preceding claims, the G2 checkpoint inhibitor being:



(9,10,11,12,-Tetrahydro-9,12-epoxy-1*H*-diindolo(1,2,3-fg:3',2',1'-kl)pyrrolo(3,4-i)(1,6)benzodiazocine-1,3(2*H*)-dione) or a salt thereof.

10. The use, G2 checkpoint inhibitor and a chemotherapeutic agent, or pharmaceutical pack according to any of the preceding claims, the G2 checkpoint inhibitor being:



or a salt thereof.

11. The use, G2 checkpoint inhibitor and a chemotherapeutic agent, or pharmaceutical pack according to any of the preceding claims, the chemotherapeutic agent being an antineoplastic agent.

12. The use, G2 checkpoint inhibitor and a chemotherapeutic agent, or pharmaceutical pack according to any of the preceding claims, the chemotherapeutic agent being selected from the group consisting of: Cisplatin, Paclitaxel and Gemcitabine.

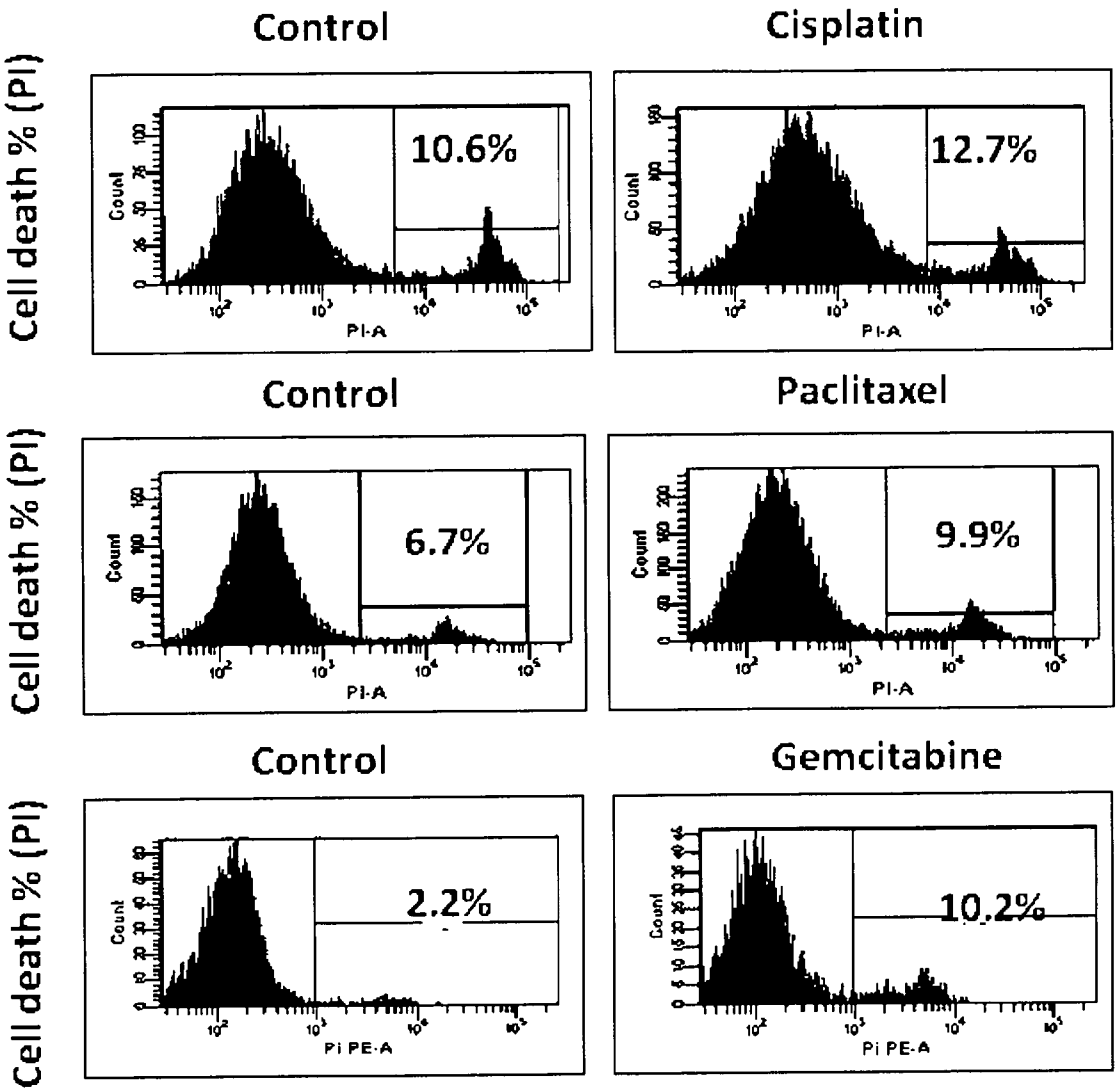
13. The use, G2 checkpoint inhibitor and a chemotherapeutic agent, or pharmaceutical pack according to any of the preceding claims, the tumorigenic cells being selected from the group consisting of: colon cancer stem cells, and lung cancer stem cells.

14. A method of treatment of tumorigenic cells in solid tumours, comprising the step of administering a G2 checkpoint inhibitor and a chemotherapeutic agent to a patient in need of same.

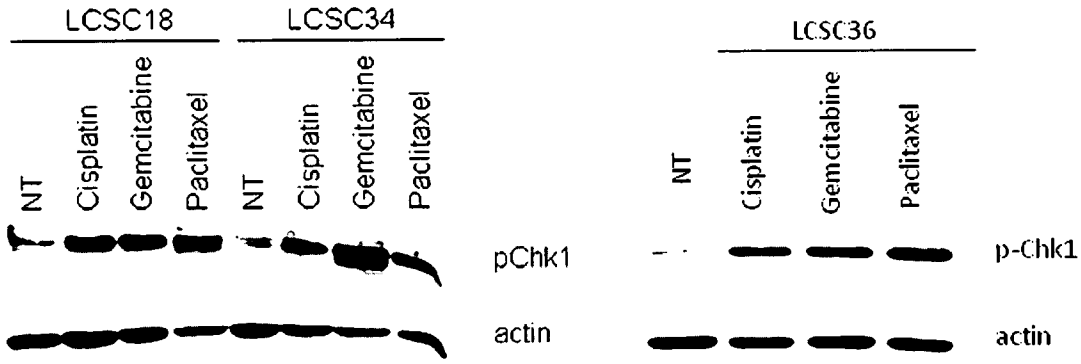
15. The use, G2 checkpoint inhibitor and a chemotherapeutic agent, pharmaceutical pack, or method of treatment according to any of the preceding claims, wherein the administrative pattern of the medicament comprises the administration of the G2 checkpoint inhibitor at least 30 minutes after administration of the chemotherapeutic agent.

Figure 1

A

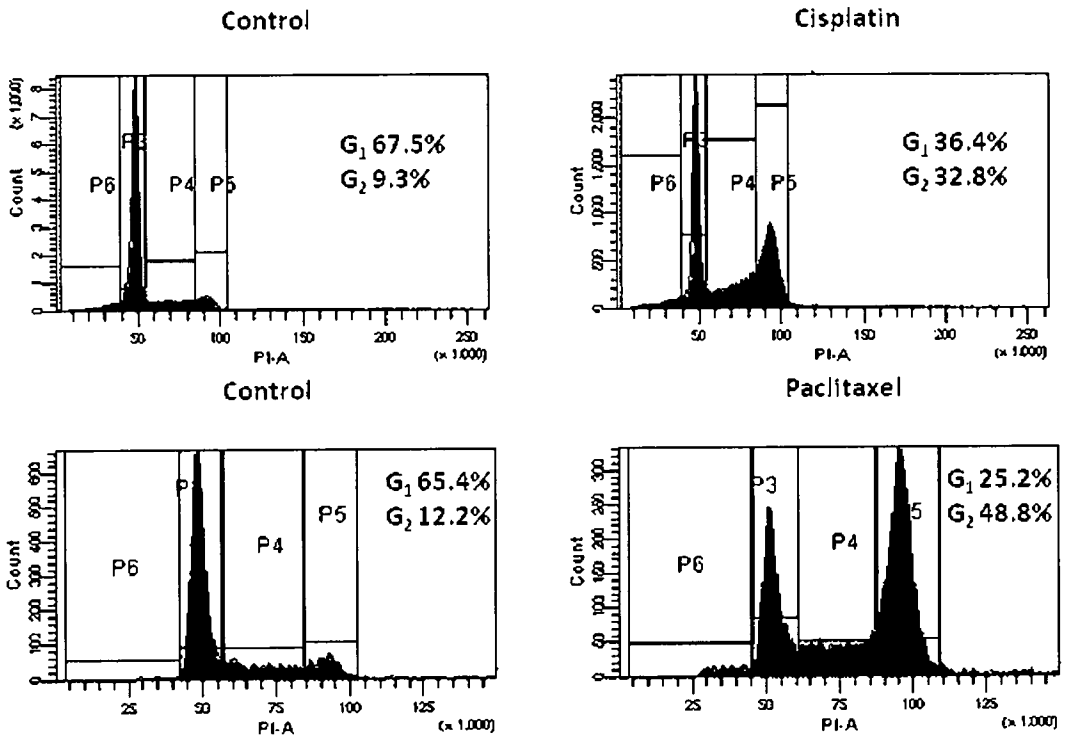

Control
Cisplatin
Cell death % (PI)
10.6%
12.7%
Count
PI-A
Control
Paclitaxel
Cell death % (PI)
6.7%
9.9%
Count
PI-A
Control
Gemcitabine
Cell death % (PI)
2.2%
10.2%
Count
Pi PE-A

B



		NT	Cisplatin	Paclitaxel	Gemcitabine
LCSC18	pChk1	+	+++	+++	+++
	Actin	+++	+++	+++	+++
LCSC34	pChk1	-	+++	+++	+++
	Actin	+++	+++	+++	+++
LCSC36	pChk1	-	+++	+++	+++
	Actin	+++	+++	+++	+++

C


Control
Cisplatin
Control
Paclitaxel
P6
P4
P5
P3
G1 67.5%
G2 9.3%
G1 36.4%
G2 32.8%
G1 65.4%
G2 12.2%
G1 25.2%
G2 48.8%
Count
PI-A

D

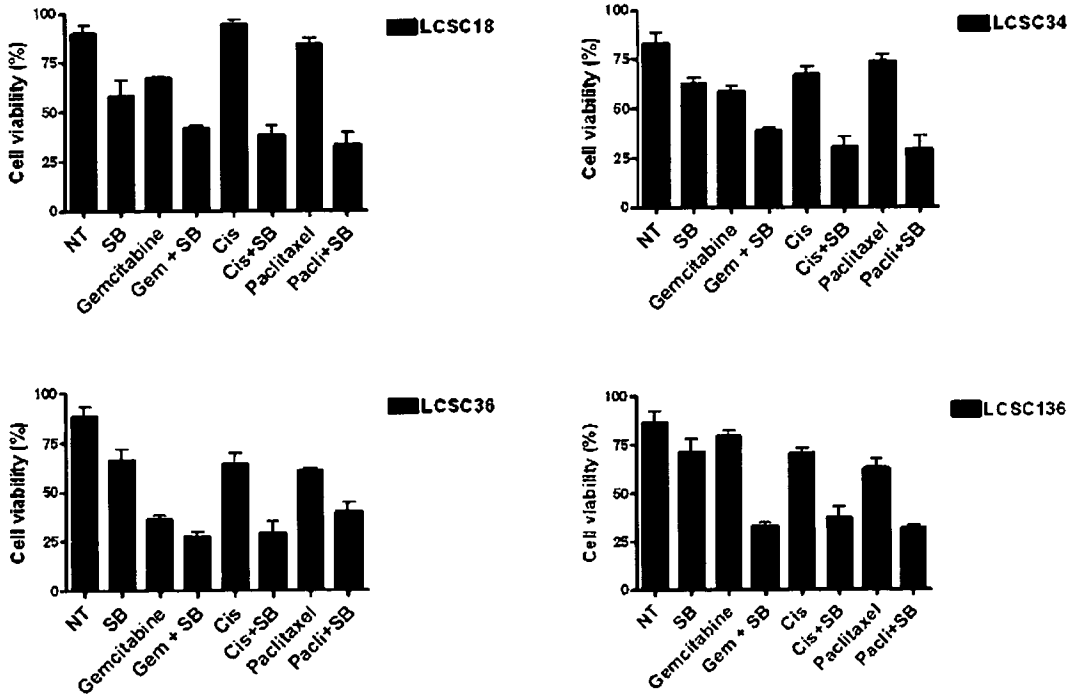
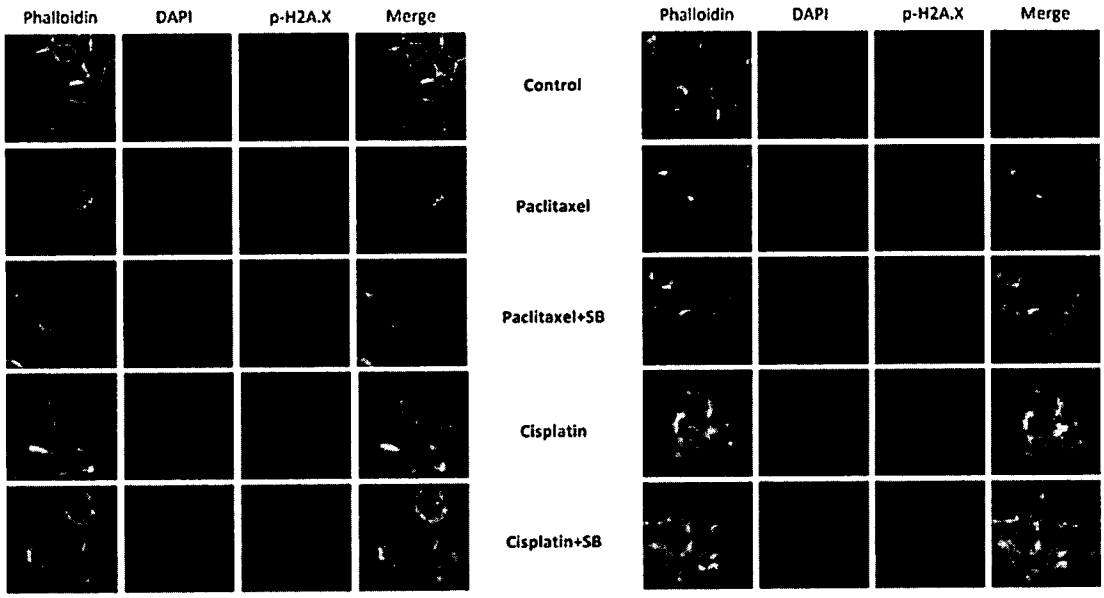

LCSC18
LCSC34
LCSC36
LCSC136
Cell viability (%)
NT
SB
Gemcitabine
Gem + SB
Cis
Cis+SB
Paclitaxel
Pacli+SB

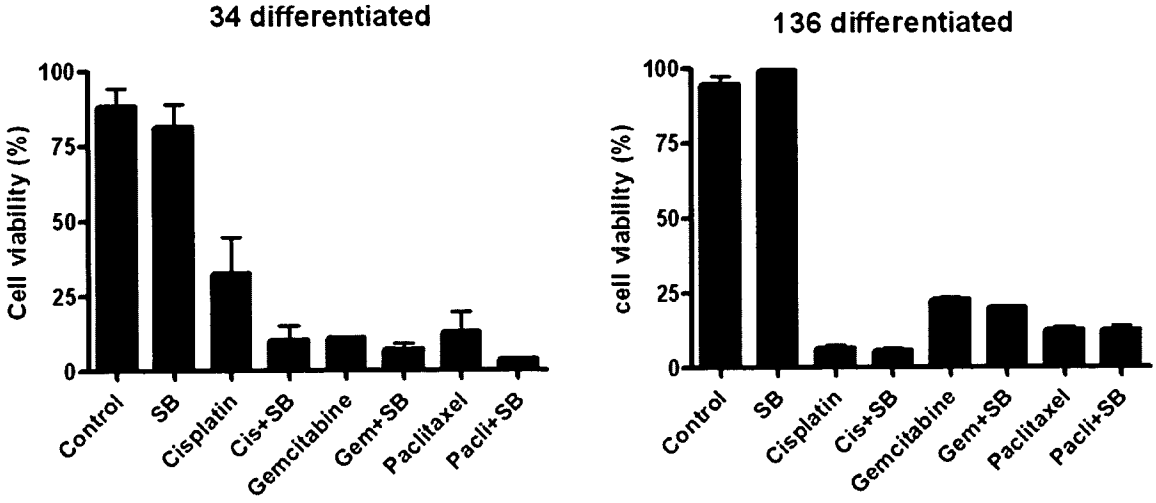
Figure 2

A

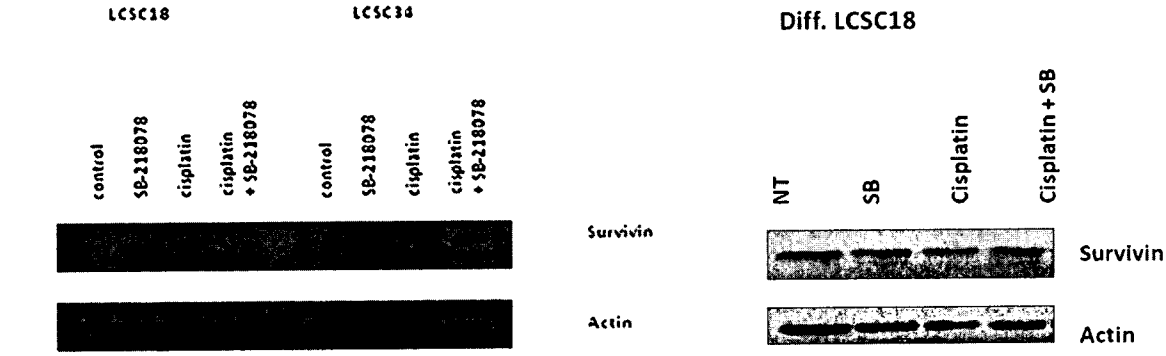


6h				96h		
Phaloidin	DAPI	p-H2AX		Phaloidin	DAPI	p-H2AX
+++	+++	-	Control	+++	+++	-
+++	+++	++	Paclitaxel	+++	+++	+
+++	+++	++	Paclitaxel + SB	+++	+++	+++
+++	+++	++	Cisplatin	+++	+++	-
+++	+++	++	Cisplatin + SB	+++	+++	++

B



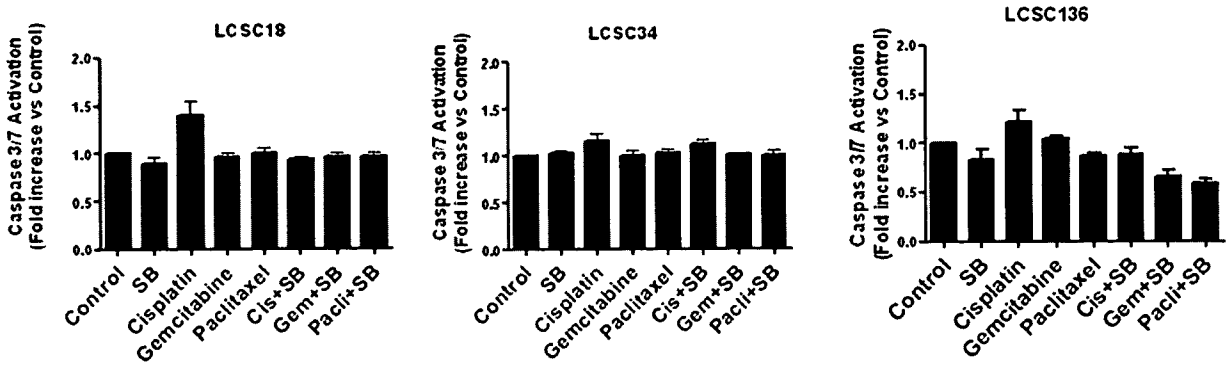
C

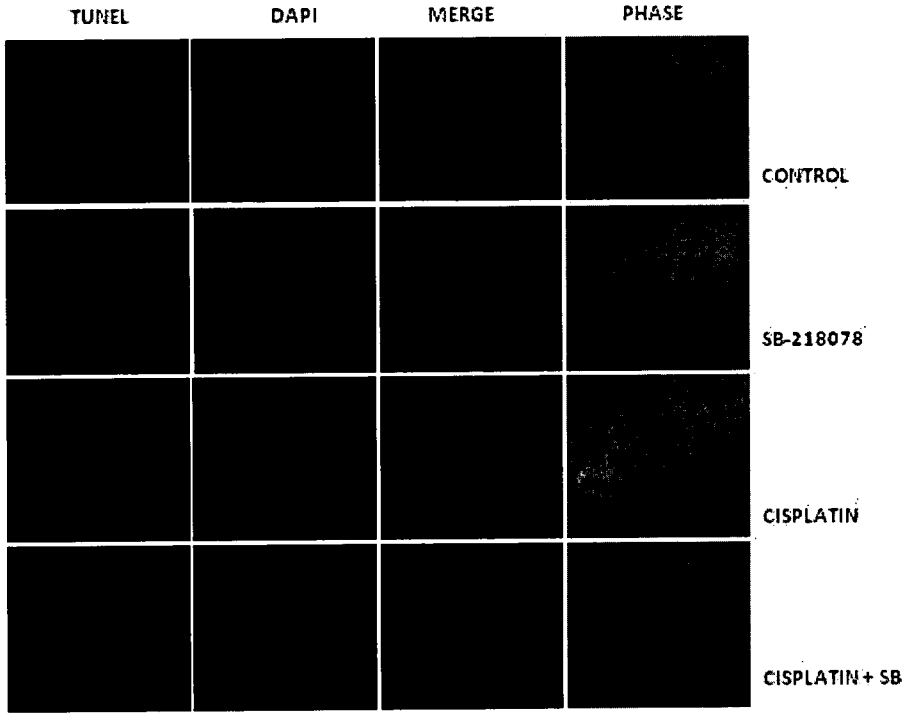


		NT	SB-218078	Cisplatin	Cisplatin + SB-218078
LCSC18	Survivin	-	-	+++	-
	Actin	+++	+++	+++	+++
LCSC34	Survivin	-	-	+++	-
	Actin	+++	+++	+++	+++
Diff. LCSC18	Survivin	++	++	++	++
	Actin	+++	+++	+++	++

Figure 3

A





B

TUNEL	DAPI	
+	+	Control
+	+	SB-218078
-	++	Cisplatin
-	++	Cisplatin + SB-218078

A

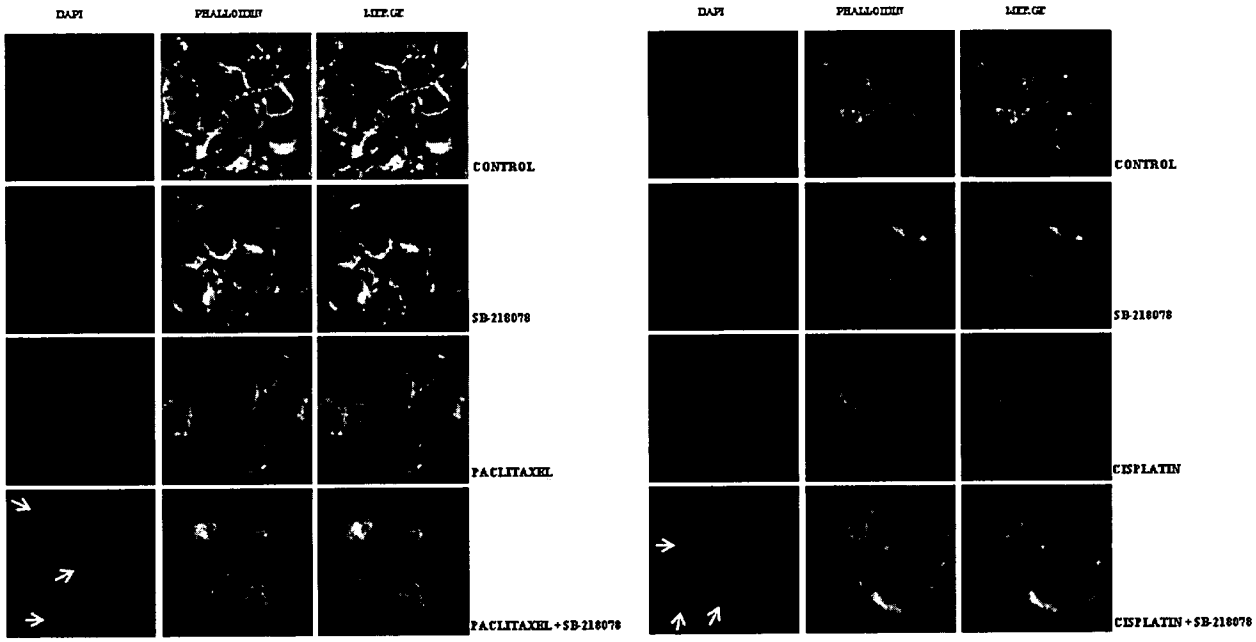

DAPI
PHALLOIDIN
MERGE
CONTROL
SB-218078
PACLITAXEL
PACLITAXEL + SB-218078
DAPI
PHALLOIDIN
MERGE
CONTROL
SB-218078
CISPLATIN
CISPLATIN + SB-218078

Figure 4

B

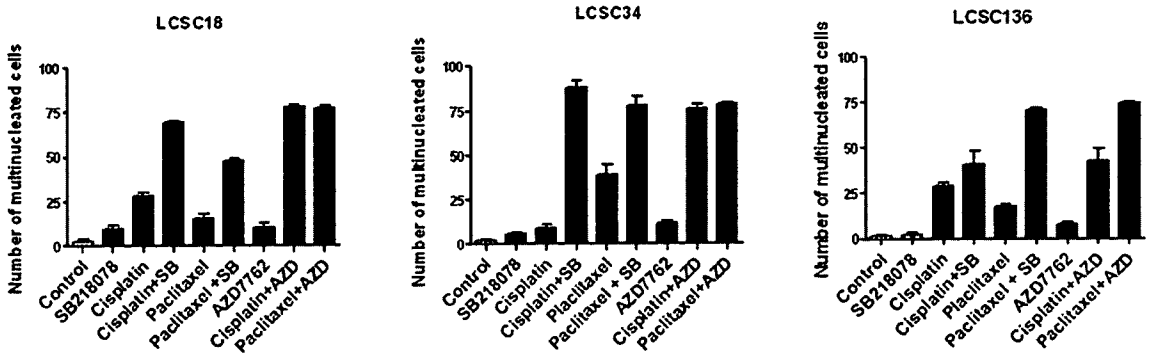
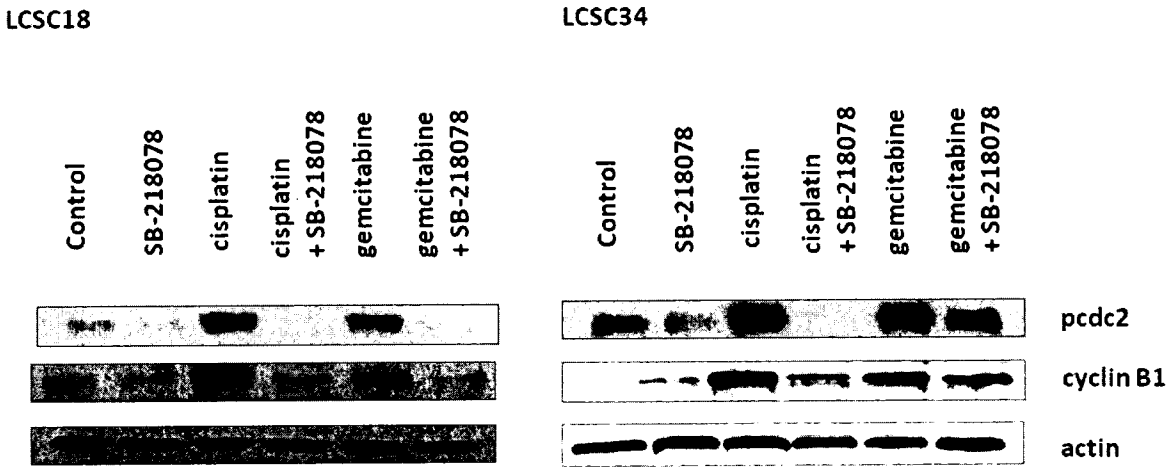

LCSC18
LCSC34
LCSC136
Number of multinucleated cells
100
75
50
25
0
Control
SB218078
Cisplatin
Cisplatin+SB
Placlitaxel
Paclitaxel + SB
AZD7762
Cisplatin+AZD
Paclitaxel+AZD

Figure 5



A

		Control	SB-218078	Cisplatin	Cisplatin + SB-218078	Gemcitabine	Gemcitabine + SB-218078
LCSC18	pcdc2	-	-	+++	-	+++	-
	cyclin B1	-	-	+++	-	+++	-
	Actin	+++	+++	+++	+++	+++	+++
LCSC34	pcdc2	+	-	+++	-	+++	++
	cyclin B1	-	-	+++	+	+++	+
	Actin	+++	+++	+++	+++	+++	+++

B

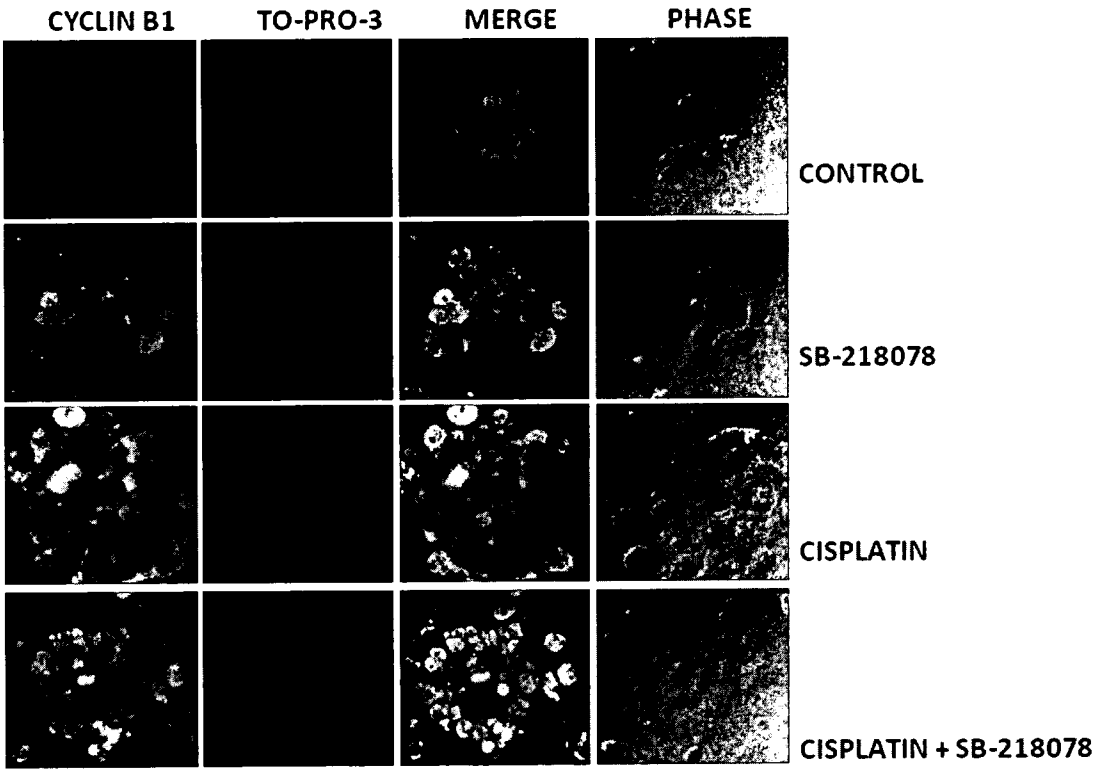


Figure 6

A

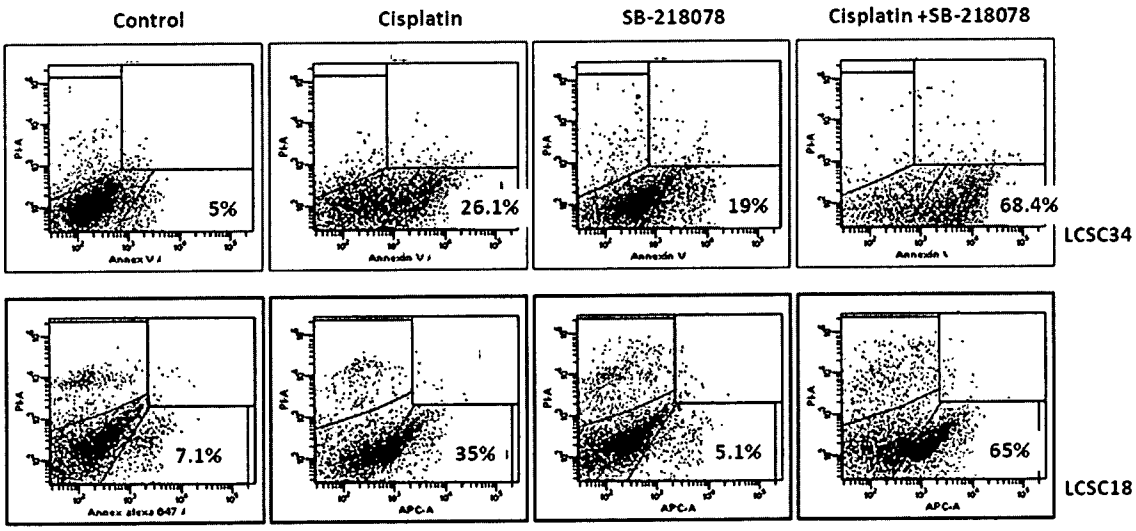
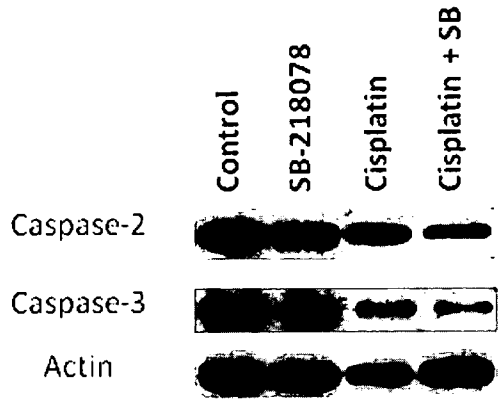


Figure 6

B



	Control	SB-218078	Cisplatin	Cisplatin + SB-218078
Caspase-2	+++	+++	+++	++
Caspase-3	+++	+++	++	+
Actin	+++	+++	+++	+++

C

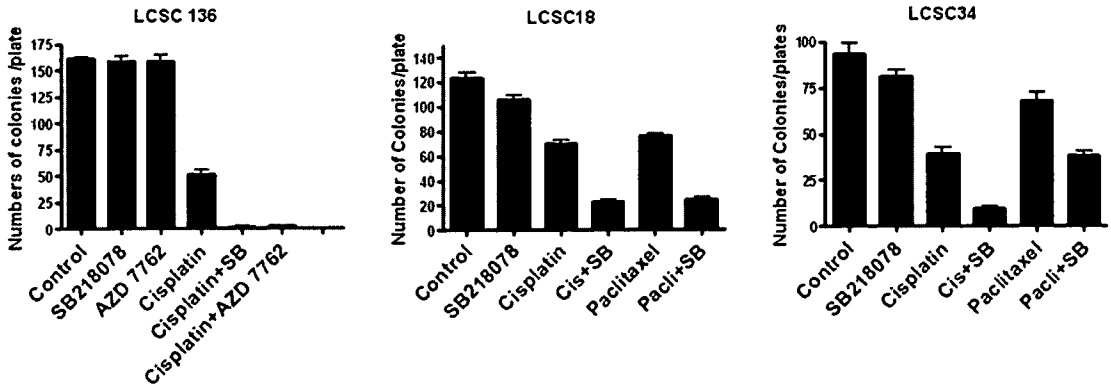


Figure 7

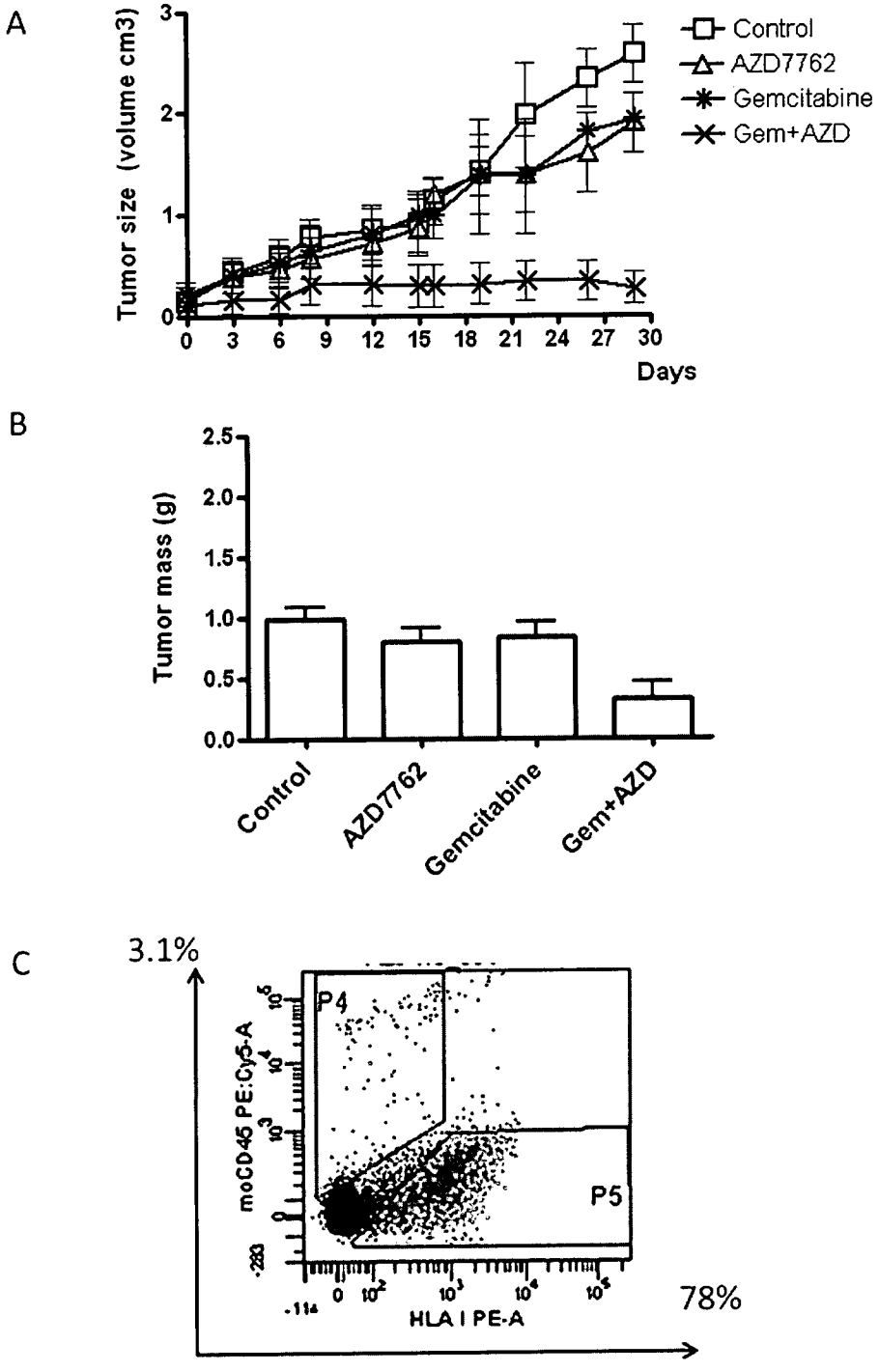
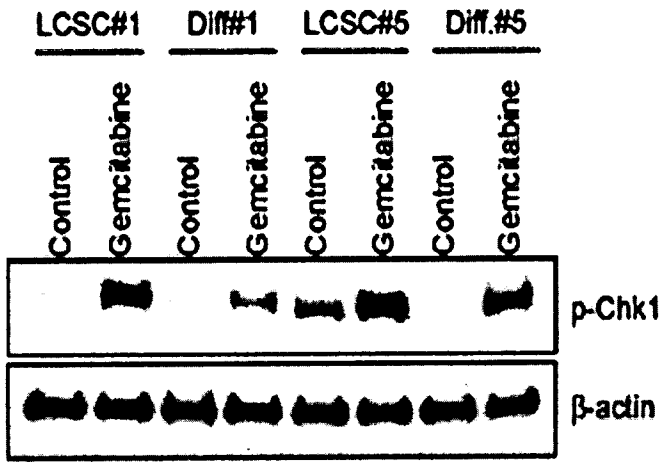

A
Tumor size (volume cm3)
Days
Control
AZD7762
Gemcitabine
Gem+AZD
B
Tumor mass (g)
Control
AZD7762
Gemcitabine
Gem+AZD
C
3.1%
P4
P5
78%
HLA I PE-A

Figure 8



LCSC#1	Control	Gemcitabine
pChk1	-	+++++
Actin	+++	+++

Diff.LCSC#1	Control	Gemcitabine
pChk1	-	++
Actin	+++	+++

LCSC#5	Control	Gemcitabine
pChk1	++	+++++
Actin	+++	+++

Diff.LCSC#5	Control	Gemcitabine
pChk1	-	++
Actin	+++	+++

Figure 9

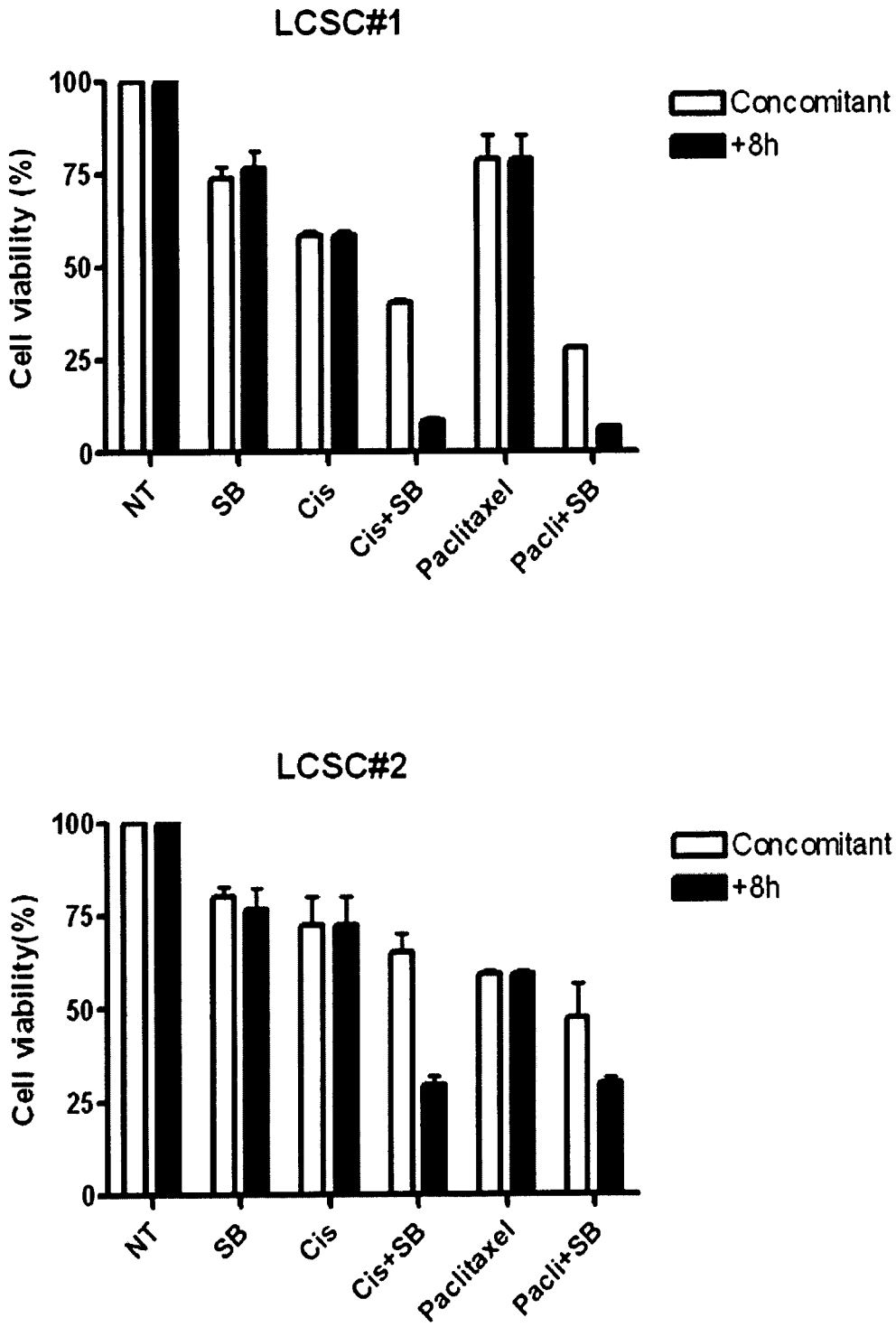
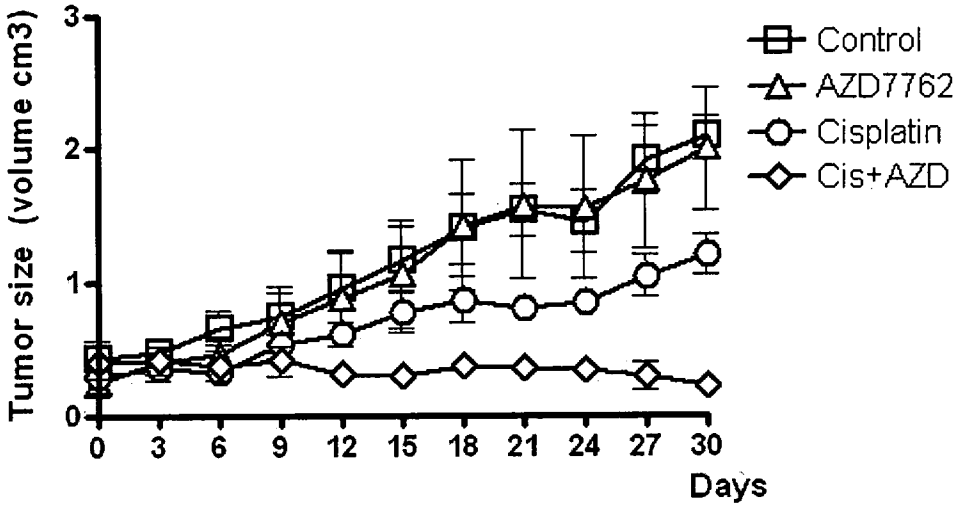

LCSC#1
Cell viability (%)
100
75
50
25
0
NT
SB
Cis
Cis+SB
Paclitaxel
Pacli+SB
Concomitant
+8h
LCSC#2
Cell viability(%)
100
75
50
25
0
NT
SB
Cis
Cis+SB
Paclitaxel
Pacli+SB
Concomitant
+8h

Figure 10

A


Tumor size (volume cm3)
3
2
1
0
0 3 6 9 12 15 18 21 24 27 30
Days
Control
AZD7762
Cisplatin
Cis+AZD

B

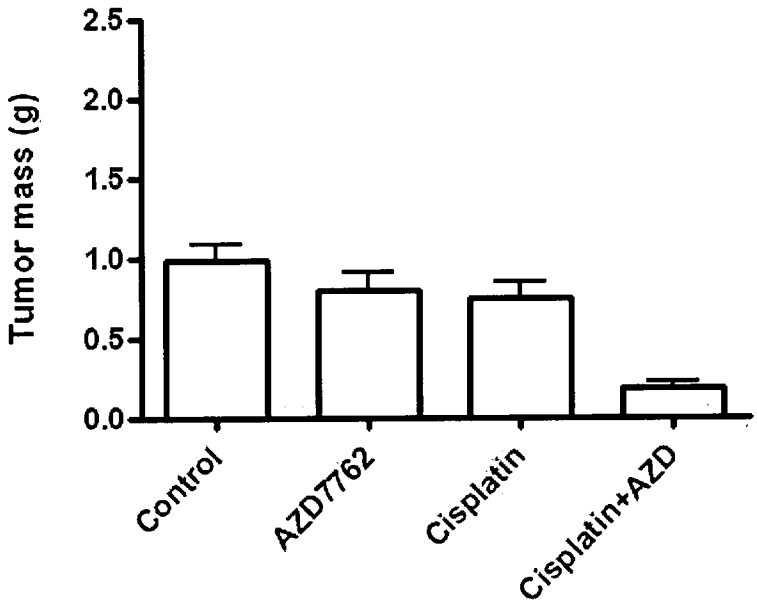
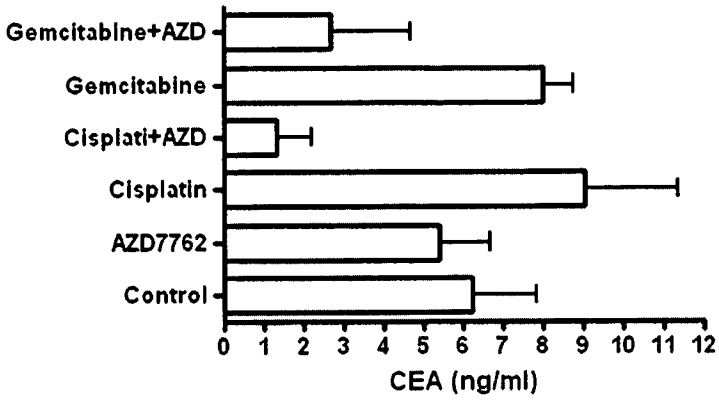
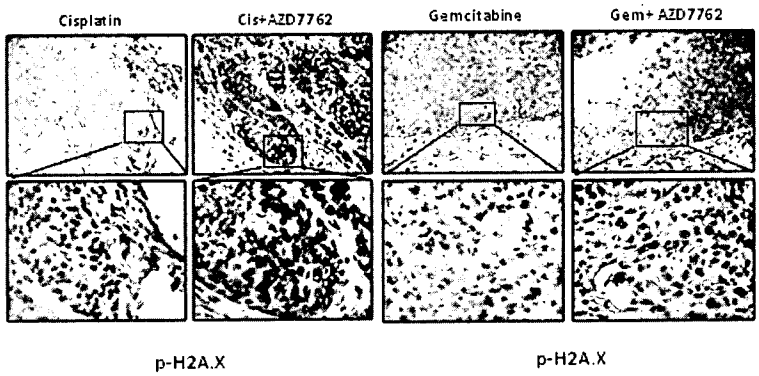

Tumor mass (g)
2.5
2.0
1.5
1.0
0.5
0.0
Control
AZD7762
Cisplatin
Cisplatin+AZD

Figure 11

A



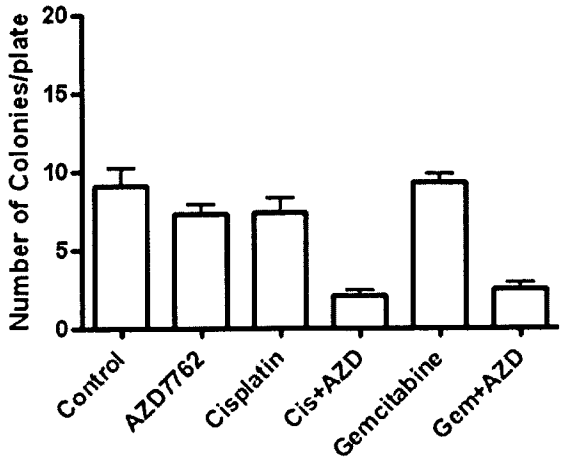
B



Number of positive p-H2A.X cells

	Cisplatin	Cisp+AZD	Gemcitabine	Gem+AZD
P-H2A.X	+++	++++++++	++	+++++

C



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/004119

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/4535 A61K31/522 A61K31/553 A61P35/00 A61K45/06
A61K31/282 A61K31/337 A61K31/7068

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 practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data, 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.
X	WO 2005/066163 A2 (ASTRAZENECA AB [SE]; ASTRAZENECA UK LTD [GB]; ASHWELL SUSAN [US]; GERO) 21 July 2005 (2005-07-21) page 33, lines 5-13	1-15
Y	page 67, lines 6-17 compound 166 page 97 page 35, lines 1-4	1-15

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 October 2010	30/11/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Baumgärtner, Heike

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	KAZUNO H ET AL: "1-(3-C-Ethynyl-[beta]-D-ribo-pentofuranosyl)cytosine (ECyd, TAS-106), a novel potent inhibitor of RNA polymerase, potentiates the cytotoxicity of CDDP in human cancer cells both in vitro and in vivo" INTERNATIONAL JOURNAL OF ONCOLOGY, vol. 34, no. 5, 2009, pages 1373-1380, XP002605648 SPANDIDOS PUBLICATIONS GRC ISSN: 1019-6439 DOI: 10.3892/IJO_00000264 page 1380, last paragraph	1-10, 13-15
Y		1-15

X	VITALE ILIO ET AL: "Inhibition of Chk1 kills tetraploid tumor cells through a p53-dependent pathway." PLOS ONE, vol. 2, no. 12, E1337, 2007, pages 1-14, XP002605649 ISSN: 1932-6203 line 7 to 5 from the bottom page 5, paragraph 2	1-5,7-15
Y		1-15

X	ZHU XUEMING ET AL: "In vitro and in vivo characterizations of tetrandrine on the reversal of P-glycoprotein-mediated drug resistance to paclitaxel" ANTICANCER RESEARCH, vol. 25, no. 3B, May 2005 (2005-05), pages 1953-1962, XP009140222 ISSN: 0250-7005 the whole document	1-15

Y	WO 00/16781 A1 (SMITHKLINE BEECHAM CORP [US]; GILMARTIN AIDAN G [US]; HO MAUREEN L [US) 30 March 2000 (2000-03-30) sentence bridging page 9 and page 10 claim 1	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2010/004119

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2005066163 A2	21-07-2005	AR 047366 A1	18-01-2006
		AU 2004312193 A1	21-07-2005
		BR PI0418351 A	08-05-2007
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