2-Deoxy-D-glucose Sensitizes Cancer Cells to Barasertib and Everolimus by ROS-independent Mechanism(s)

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Abstract. *The aim of the present study was to investigate: (i) the possibility of sensitizing cancer cells to anticancer drugs using the redox modulator 2-deoxy-D-glucose (2-DDG); (ii) to find such combinations with synergistic cytotoxic effect; (iii) and to clarify the role of reactive oxygen species (ROS) for induction of apoptosis and cytotoxicity through these combinations. The study covers 15 anticancer drugs – both conventional and new-generation. Four parameters were analyzed simultaneously in Jurkat leukemia cells, treated by drugs or 2-DDG (separately or in combination): cell viability, induction of apoptosis, levels of ROS, and level of protein-carbonyl products. Very well-expressed synergistic cytotoxic effects were found after 48-h treatment of Jurkat cells with 2-DDG in combination with: palbociclib, everolimus, lonafarnib, bortezomib, and barasertib. The synergistic cytotoxic effect of everolimus with 2-DDG was accompanied by very strong induction of apoptosis in cells, but a very strong reduction of ROS level. Changes in the levels of protein-carbonyl products were not detected. The synergistic cytotoxic effect of barasertib with 2-DDG was accompanied by very strong induction of apoptosis in cells, without any increase of ROS levels, but with an enhancement of protein-carbonyl products.*

Over 50 years' experience in free radical biology and medicine has shown the crucial role of redox signaling in carcinogenesis (1-9). The cells and tissues of healthy mammals are characterized by a low steady-state level of reactive oxygen species (ROS) and a constant level of reducing equivalents, while cancer cells are characterized by increased levels of ROS and reducing equivalents (7). Cancer cells are also characterized by an abnormal production of NADP(H) and thiols (*e.g.* glutathione) as a result of accelerated glycolysis (the Warburg effect) and the pentose-phosphate cycle. However, these reducers are rapidly consumed to maintain accelerated anabolism, which is necessary for cell proliferation and immortalization.

A moderate increase in ROS can promote cell proliferation and differentiation (10). However, extremely excessive amounts of ROS can cause irreversible oxidative damage to bio-macromolecules, and leads to apoptosis and cell death (5). Therefore, maintaining ROS homeostasis at low levels is crucial for normal cell survival, while moderate enhancement of ROS is associated with abnormal cancer cell growth and disruption of redox homeostasis (5).

Prolonged function of cells at abnormal steady-state levels of ROS provokes genetic mutations, which makes such cells well-adapted to oxidative stress. This process is the basis of malignant transformation. Cancer cells are usually characterized by an increased antioxidant capacity (11). The cells that survive intrinsic oxidative stress mobilize a set of adaptive mechanisms that not only activate ROS-scavenging systems to cope with the stress, but also inhibit apoptosis. Such adaptation contributes to malignant transformation, metastasis and resistance to anticancer drugs (12).

Harris *et al.* reported that normal epithelial cells exposed to low, but continuous levels of exogenous oxidants become resistant to subsequent oxidative stress, (13). This observation suggests that cells can adapt and survive under certain levels of oxidative stress. Those cells that survive oxidative stress are likely to have acquired adaptive mechanisms to counteract the potential toxic effects of elevated ROS and promote cell-survival pathways (10). Nonn *et al.* reported that Harvey rat sarcoma viral (*HRAS*) oncogene-transformed cells, which exhibit increased

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superoxide and hydrogen peroxide levels, are also characterized by increased levels of antioxidants (*e.g.* peroxiredoxin-3 and thioredoxin peroxidase) in comparison to their non-cancerous parental cells (14). Their enhanced antioxidant capability is likely to serve as a compensatory mechanism to evade ROS-induced apoptosis. As such, the abrogation of this adaptation mechanism could be an attractive strategy for preferentially affecting cancer cells and may therefore have promising therapeutic implications (5).

Our recent data on experimental animals showed that reductive processes dominate over oxidative processes in the tissues of healthy organism, while oxidation dominates over reduction in the tissues (cancerous and non-cancerous) of cancer-bearing organism (7-9). Moreover, the tissue redox status is very sensitive to cancer progression and anticancer therapy (9). These data suggest that tissue redox status could be a diagnostic marker, a therapeutic target, and a hallmark for evaluation and planning of therapeutic strategy against cancer.

It is widely accepted that conventional anticancer drugs increase intracellular levels of ROS and induce cytotoxic effects in both cancer and normal cells (15-19). The harmful side-effects of chemotherapy are usually due to ROS-mediated mechanism(s) and disturbance of redox homeostasis of non-cancer cells and tissues. Studies demonstrated that using natural or synthetic substances affecting cellular redox homeostasis (redox-modulators) in combination with chemotherapy can protect normal cells against oxidative stress (16, 18-20). However, it was found that conventional antioxidants may, in fact, reduce the therapeutic effect of the anticancer drug.

A promising strategy to achieve therapeutic selectivity and efficiency in cancer is to take advantage of the fundamental difference between cancer cells and normal cells in their biochemical metabolism (21). The targeting of unique biochemical alterations in cancer cells might be a feasible approach to achieve therapeutic activity and selectivity and perhaps to prevent the development of drug resistance and side-effects (5).

One of the most prominent metabolic alterations in cancer cells is the increase in aerobic glycolysis and the dependency on glycolytic pathway for ATP synthesis, known as the Warburg effect. Targeting the glycolytic pathway may preferentially kill malignant cells or at least sensitize these cells to conventional chemotherapy or radiotherapy.

One of the most widely used inhibitors of glycolysis is 2-deoxy-D-glucose (2-DDG), a structural analog of glucose, differing at the second carbon atom by the substitution of hydroxyl group with hydrogen (21-28). 2-DDG undergoes facilitated diffusion into cells *via* glucose transporters. It selectively accumulates in cancer cells by metabolic trapping because of increased uptake and high intracellular levels of hexokinase or phosphorylating activity due to accelerated glycolysis. Once inside the cells, 2-DDG is phosphorylated by hexokinase, with formation of 2-DDG-6-phosphate, which is not metabolized and blocks the glycolytic pathway (21-28).

Many studies have shown that 2-DDG affects energy metabolism, cell proliferation kinetics, radiation-induced DNA repair, and micronuclei formation in cancer cells (29-32). The treatment of cancer cells with 2-DDG limited the synthesis of NADPH by 50% in comparison with non-treated cells (22). 2-DDG induces intracellular ATP depletion (23, 24), disruption of thiol metabolism (23, 25-28, 33, 34) and eventually induction of oxidative stress, mainly in cancer cells. All these alterations result in disturbance of redox homeostasis of cancer cells by 2-DDG without any significant influence on the viability of normal cells.

The aim of the present study was to investigate: (i) the possibility of sensitizing cancer cells to anticancer drugs using redox-modulator 2-DDG; (ii) to find combinations with a synergistic cytotoxic effect; (iii) and to clarify the role of ROS in the induction of apoptosis and cytotoxicity by those combinations. The study covers 15 anticancer drugs – conventional and new-generation.

Materials and Methods

Cells and treatment protocol. The experiments were performed on the cancer cell line Jurkat (Hayashibara Chem. Lab., Okayama, Japan), derived from patients with acute lymphoblastic leukemia. The cells were cultured in RPMI-1640 medium (Sigma-Aldrich, Steinheim, Germany), supplemented with 10% heat-inactivated fetal bovine serum (FBS)(Gibco, Auckland, New Zealand) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin)(Gibco), in a humidified atmosphere at 37˚C with 5% CO_2. All cells were collected by centrifugation (1,000 ×*g*, 10 min) and replaced in a fresh medium without antibiotics before treatment with anticancer drugs.

The drugs were dissolved in dimethyl sulfoxide (DMSO; suitable for cell culture)(Sigma-Aldrich) or phosphate-buffered saline (PBS; 10 mM, pH 7.4). The final concentration of DMSO in the cell suspension did not exceed 1%. At this concentration, DMSO did not influence cell viability.

The drugs were applied to the cells (1×10^6 cell/ml) at the concentrations below (single dose) and incubated at different time intervals in a cell incubator. At each time interval, aliquots were used for cell viability assay.

The cells were incubated with redox-modulator, drug, or drug plus redox modulator at the following concentrations: 250 μM 2-DDG (Sigma), 0.25 μM palbociclib (Selleckchem, Huston, TX, USA), 0.5 μM PI-103 (Selleckchem), 5 μM Everolimus (Selleckchem), 0.5 μM lonafarnib (Selleckchem), 0.1 μM ABT-737 (Selleckchem), 0.1 μM doxorubicin (Sigma), 0.5 μM bleomycin (Noppon Kayaku Co., Tokyo, Japan), 0.1 μM AZD-7762 (Sigma), 0.01 μM MLN-2238 (Selleckchem), 0.025 μM MG-132 (Wako, Tokyo, Japan), 10 μM lomustine (Sigma), 2.5 μM cisplatin (Selleckchem), 0.025 μM BEZ-235 (Selleckchem), 0.01 μM bortezomib (Selleckchem), 0.05 μM or 0.01 μM barasertib (Selleckchem). The selected concentrations of drugs and 2-DDG induce about 20% inhibition of Jurkat cell growth.

Cell viability assays. Cell viability was analyzed using trypan blue staining and Countess™ Automated Cell Counter (Invitrogen, Oregon, USA) at very precise standardization of the measurements. Three independent experiments (with two repetitive measurements for each experiment) were performed for each sample. Non-treated cells were used as controls. Data are presented as the mean±SD.

Intracellular ROS assay. The amount of ROS was analyzed using OxiSelect™ *In vitro* ROS/RNS Assay Kit – Green Fluorescence (Cell Biolabs, Inc., San Diego, CA, USA). The method is based on the use of the fluorogenic probe 2',7'-dichlorodihydrofluorescein DiOxyQ (DCFH-DiOxyQ). In the cytosol, the probe is deacetylated to the non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH). DCFH reacts rapidly with ROS and reactive nitrogen species (RNS)(predominantly H_2O_2, $ROO^{\bullet}$, NO, $ONOO^-$) with formation of the fluorescent product 2',7'-dichlorodihydrofluorescein (DCF). The intensity of DCF fluorescence (λ_{ex}=480 nm, λ_{em}=530 nm) is proportional to the amount of ROS/RNS in the biological sample.

The amount of ROS/RNS was calculated by calibration curve based on DCF standard solutions in PBS. The measurements were performed on a Tecan Infinite F200 PRO (Tecan Austria GmbH, Mannedorf, Austria) microplate reader. Briefly, the cells (1×10^6 cells/ml) were collected by centrifugation (1000× *g*, 10 min) and lysed using 300 μl of 0.1% sodium dodecylsulfate (SDS; dissolved in PBS) within 30 min on ice. The lysates were adjusted to equal protein concentration (range 1-10 mg/ml) using PBS. Protein concentration was analyzed by Bradford assay. Each sample was subjected to ROS/RNS assay, according to the manufacturer's instructions.

Protein-carbonyl assay. The amount of protein-carbonyl products was analyzed using OxiSelect™ Protein Carbonyl Spectrophotometric Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA). The most common products of protein oxidation in biological samples are the protein-carbonyl derivatives of proline, arginine, lysine and threonine. These derivatives are chemically stable and serve as markers of oxidative stress. The analysis of these products is based on derivatization of the carbonyl groups with dinitrophenylhydrazine (DNFH) with formation of protein-hydrazone. The amount of protein-hydrazone was detected spectrophotometrically at 375 nm.

Briefly, the cells (1×10^6 cells/ml) were collected by centrifugation (1,000 ×*g*, 10 min) and lysed using 300 μl of 0.1% SDS (dissolved in PBS) within 30 min on ice. The lysates were adjusted to equal protein concentration (in the range 1-10 mg/ml) using PBS. Protein concentration was analyzed by Bradford assay. Each sample was subjected to protein-carbonyl assay, according to the manufacturer's instructions. Oxidized bovine serum albumin (Cell Biolabs, Inc.) was used as a standard.

Apoptosis assay. The induction of apoptosis was analyzed by the expression of phosphatidylserine (PSer) on the cell surface, using fluorescein isothiocyanate (FITC)-Annexin V Apoptosis Detection Kit (BioVision, Milpitas, CA, USA). Briefly, the cells (1.0×10^6 cells/ml) were incubated with drug, redox modulator or their combination, under the conditions mentioned above. At each time-point the cells were collected by centrifugation (1,000 ×*g*, 10 min), washed twice with PBS containing 2.5 mM $CaCl_2$ (annexin V-binding buffer), and re-suspended in the same buffer. One hundred microliters of the cell suspension were incubated with 5 μl of FITC-annexin V for 10 min at room temperature in a dark place. The cells were washed three time with annexin V-binding buffer and finally were re-suspended in 500 μl of the same buffer. FITC-annexin V, bound to PSer exposed on the cell surface, was detected spectrofluorimetrically at λ_{em}=535 nm and λ_{ex}=488 nm, using a Tecan Infinite F200 PRO microplate reader (Tecan Austria GmbH).

Results and Discussion

The cells were treated with: (i) drug only; (ii) 2-DDG only; and (iii) combination of drug and 2-DDG. To distinguish synergistic cytotoxic effects from antagonistic/additive effects in the tested combinations, we calculated the effect of each combination on cell proliferation as a percentage of the effect of the respective drug applied alone. The general idea is illustrated by Figure 1. Each sample treated with drug only (in the absence of 2-DDG) was considered as a respective control (100% proliferative activity; Figure 1 – columns in grey). The effect of each combination (drug + 2-DDG) was calculated as a percentage of this control (Figure 1 – columns in black). The effect of 2-DDG was calculated as a percentage of other control untreated cells (the proliferative activity in this sample was considered 100%). The grey line in Figure 1 indicates the effect of 2-DDG (applied separately) on cell proliferation. In the case of drug plus 2-DDG, the data located to the left of the grey line reflect synergistic cytotoxic effects, while the data located to the right of the grey line represent antagonistic effects. All data matching the grey line reflect an additive affect.

Data in Figure 2 demonstrate the proliferation of Jurkat cells treated with 2-DDG and drugs, alone and in combination, within 24 and 48 h. At the selected concentrations, the cytotoxicity of each drug (applied separately) varied from ~10-20% after 24-h incubation to ~20-30% after 48-h incubation. The cytotoxicity of 2-DDG ranged from 7% to 15%, depending on incubation time.

At 24 h of treatment, most combinations were characterized by enhanced inhibition of cell proliferation compared with cells treated with drug only, but the cytotoxic effects were mostly additive (Figure 2A1 and B1).

The major molecular targets of the drugs investigated in this study are shown in Table I. Most of them are key enzymes in the regulation of cell signaling. It takes time for detection of the effects on cell proliferation as a result of down-regulation or up-regulation of these enzymes – *i.e.* at least one complete cell cycle. Since the doubling time of Jurkat cells is 25-35 h, the data obtained at the 24th h are not indicative enough of the real effect of the drugs and their combinations with 2-DDG on cell proliferation. The data obtained at the 48th h are more representative.

Very well-expressed synergistic cytotoxic effects were observed after 48-h treatment of Jurkat cells with 2-DDG in combination with: palbociclib, everolimus, lonafarnib, bortezomib, and barasertib (Figure 2 A2 and B2). This synergism significantly increased after 72-h incubation (data are not shown).

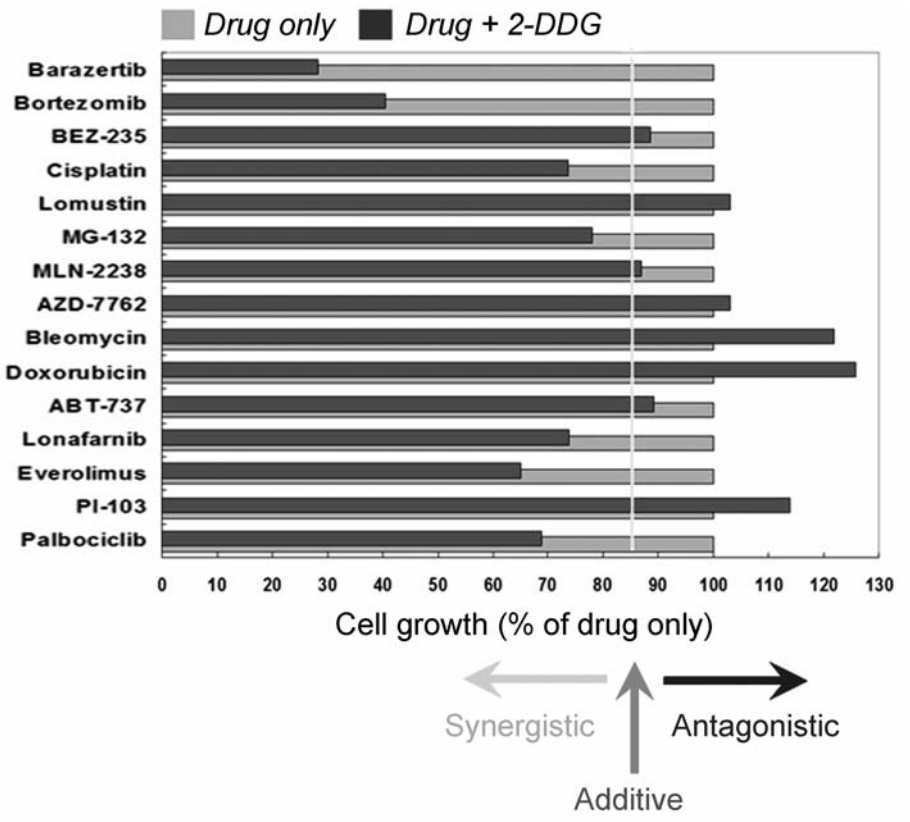

Figure 1. *Effect of each combination (drug + redox modulator) on cell proliferation, expressed as a percentage of the effect of the respective drug applied alone (considered as 100% proliferative activity). The grey line shows the effect of redox modulator 2-deoxy-D-glucose (2-DDG) (alone) on cell proliferation, expressed as a percentage of the control (untreated cells).*

The next step of this study was to clarify whether the cytotoxicity of the combinations was accompanied by enhancement of ROS in cell suspensions and induction of apoptosis. Three combinations were selected: one including conventional anticancer drug (doxorubicin), and two others including new-generation drugs, barasertib and everolimus. Cells were treated with the selected drugs and their combinations, and four parameters were investigated simultaneously: (i) cell viability; (ii) expression of PSer on the cell surface as a marker for induction of apoptosis; (iii) level of ROS, detected by cell-penetrating and ROS-sensitive fluorescent marker; (iv) level of protein-carbonyl products as end-products of oxidative stress.

Figure 3 shows that the combination of doxorubicin and 2-DDG was characterized by slightly synergistic cytotoxic effect after 24-h incubation, and antagonistic cytotoxic effect after 48 h, compared to administration of doxorubicin and 2-DDG alone. This was accompanied by antagonistic effects on the level of ROS and protein-carbonyl products, as well as on the induction of apoptosis in the cells. 2-DDG reduced doxorubicin-mediated cytotoxicity by reducing the level of ROS and suppressing apoptosis.

It is widely accepted that the anticancer effect of doxorubicin is mediated by abnormal production of ROS, which also causes toxic side-effects on non-cancer cells and tissues (35-37). The data in the literature describe different (even opposite) effects of 2-DDG with regard to the cytotoxicity and anticancer activity of doxorubicin (21, 38-40). For example, Thakkar *et al*. found that 2-DDG reduced the incidence of doxorubicin-induced apoptosis *in vivo* (in mouse small intestine) (38). Ahmad *et al*. observed that 2-DDG sensitized rapidly dividing T47D breast cancer cells to doxorubicin, but did not sensitize slowly-growing MCF-7 breast cancer cells (39). In T47D cells, synergistic cytotoxicity was detected within 24-h treatment with doxorubicin and 2-DDG, which was accompanied by a decrease of total intracellular glutathione and disruption of cellular redox status (39).

Simons *et al*. reported a sensitizing effect of human head and neck cancer cells to cisplatin (also a conventional anticancer drug) using 2-DDG (40). However, the concentration of 2-DDG used in that study was rather high (20 mM) and a very strong cytotoxic effect was found even

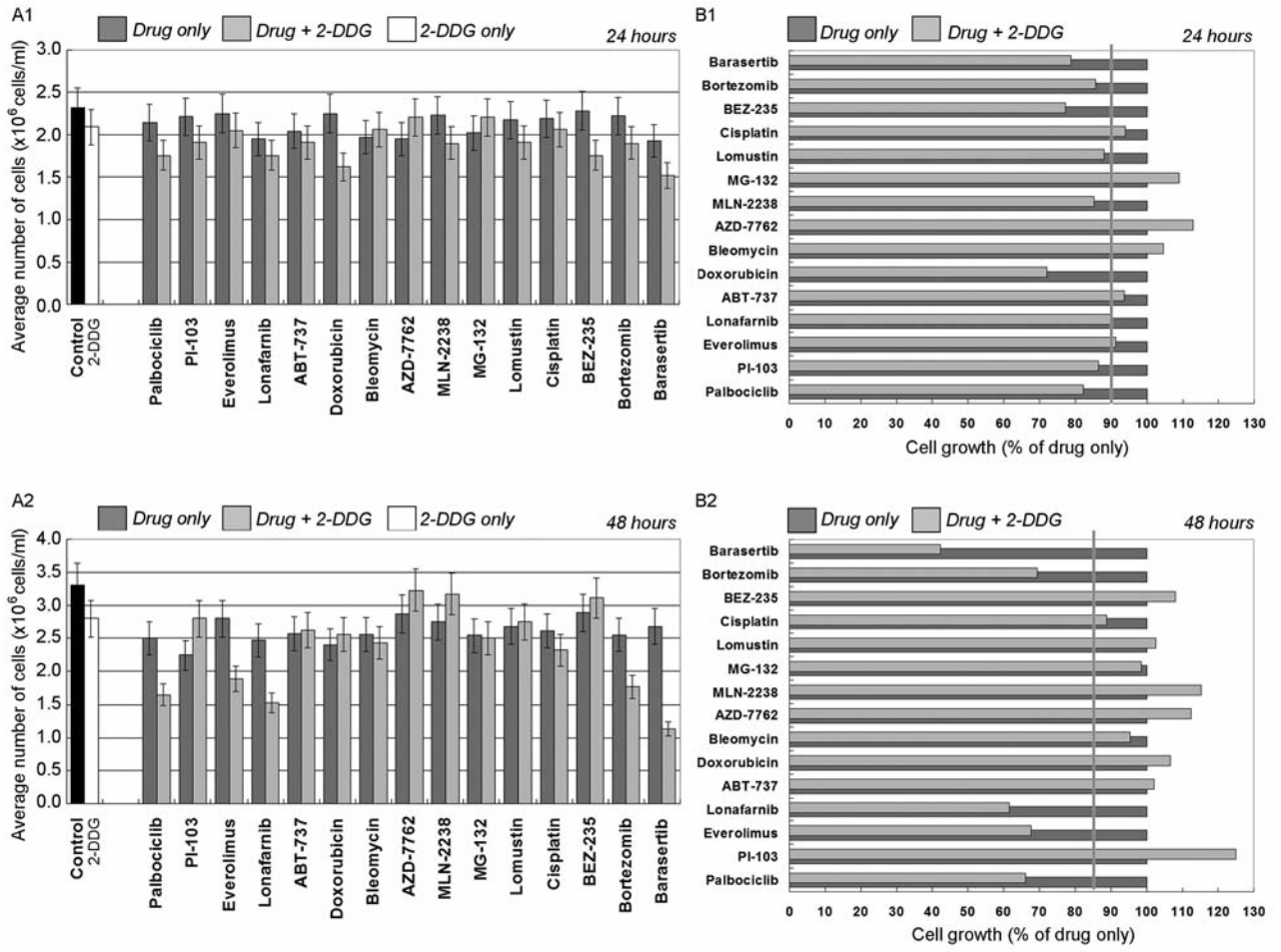

Figure 2. *A: Effect of 2-deoxy-D-glucose (2-DDG) and anticancer drugs on cell proliferation activity of Jurkat cells after incubation for 24 h (A1) and 48 h (A2). Incubation conditions: 1×10^6 cells/ml, 2-DDG with/without drug (in concentrations described in the Materials and Methods), at 37˚C in a humidified atmosphere. Data are the mean±SD from six independent experiments. B: Effect of each combination (drug + 2-DDG) on cell proliferation as a percentage of the effect of chemotherapeutic applied alone after incubation for 24 h (B1) and 48 h (B2). The grey line indicates the effect of 2-DDG on cell proliferation of cancer cells as a percentage of the control (untreated cells). *In this experiment, the concentration of barasertib was 50 nM.*

Table I. *Major molecular targets of the investigated drugs.*

Drug	Molecular targets
Doxorubicin	Anthracycline anticancer antibiotic, interacting with DNA; topoisomerase II inhibitor
Bleomycin	Glycopeptide anticancer antibiotic, breakage of DNA
Cisplatin	Platinum-containing anticancer drug, causing cross-linking of DNA
Lomustin	Alkylating anticancer drug (nitrosourea), interacting with DNA
AZD-7762	Selective inhibitor of checkpoint kinases (CHK1, CHK2)
MLN-2238	Proteasome inhibitor
MG-132	Proteasome inhibitor
BEZ-235	Phosphoinositide 3-kinase/mammalian target of rapamycin (PI3K/mTOR) inhibitor
Bortezomib	Proteasome inhibitor
Barasertib	Selective inhibitor of aurora B kinase
ABT-737	Selective inhibitor of B cell lymphoma 2 (BCL2) proteins
Palbociclib	Selective inhibitor of cyclin-dependent kinases (CDK4, CDK6)
PI-103	Selective (ATP-competitive) PI3K/mTOR inhibitor
Everolimus	mTOR inhibitor
Lonafarnib	Farnesyltransferase inhibitor

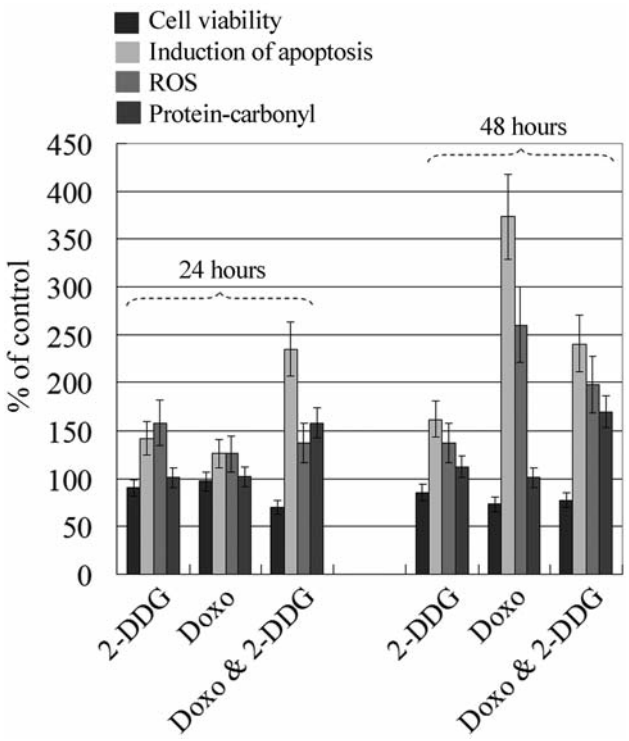

Figure 3. *Effects of doxorubicin (Doxo; 0.1 μM) in the absence and presence of 2-deoxy-D-glucose (2-DDG; 250 μM) on cell viability, induction of apoptosis, level of reactive oxygen species (ROS) and level of protein-carbonyl products in Jurkat cells after 24 and 48 h incubation at 37˚C in a humidified atmosphere. Data are the mean±SD from three independent experiments.*

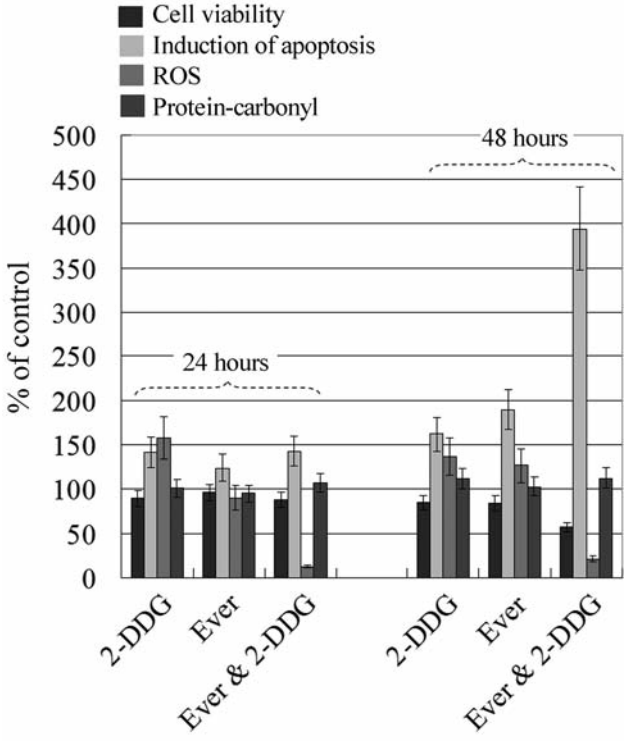

Figure 4. *Effects of everolimus (Ever; 5 μM) in the absence and presence of 2-deoxy-D-glucose (2-DDG; 250 μM) on cell viability, induction of apoptosis, level of reactive oxygen species (ROS) and level of protein-carbonyl products in Jurkat cells after 24 and 48 h incubation at 37˚C in a humidified atmosphere. Data are the mean±SD from three independent experiments.*

in the absence of cisplatin. In this case, it is difficult to estimate whether the effect of the combination of cisplatin and 2-DDG is additive, synergistic or antagonistic.

Wartenberg *et al*. showed that the inhibition of glycolysis by 2-DDG for 24 h in DU-145 and Gli36 tumor spheroids is accompanied by ROS generation (41). 2-DDG suppressed the delivery of doxorubicin into the spheroids and reduced the cytotoxic effect of the drug.

Recently, Wang *et al*. reported that the treatment of papillary thyroid carcinoma cell lines (PTCC) by doxorubicin with 2-DDG for 48 h resulted in higher cytotoxicity compared to cells treated by doxorubicin alone (42). The concentrations of doxorubicin and 2-DDG used were similar to those in our study. However, their conclusion is somewhat confusing depending on the published data. They claim that the half-maximal inhibitory concentration (IC_{50}) for doxorubicin in combination with 2-DDG was significantly lower than that for doxorubicin alone, but the cytotoxicity of 2-DDG applied alone was also very high (~50% at 250 μM 2-DDG). Thus, the final cytotoxic effect of the combination on PTCC was antagonistic. Their data from the apoptosis assay show that the induction of apoptosis in doxorubicin-treated cells was in fact higher than in cells treated with the combination of doxorubicin and 2-DDG.

Aghaee *et al*. analyzed data from several studies and indicated that the combined treatment of cancer cells with doxorubicin and 2-DDG for 24 h results in a synergistic cytotoxic effect (43).

We also observed such paradox – on 24-h incubation there was a synergistic cytotoxic effect of doxorubicin and 2-DDG on Jurkat cells, but on 48-h incubation, the effect was antagonistic (Figure 2B1 and B2). Obviously, the cytotoxicity of the combination depends on the incubation time and has to be considered as a dynamic process. Our data also demonstrate that 2-DDG reduces doxorubicin-mediated generation of ROS and induction of apoptosis after 48-hour incubation, which results in diminishing of the cytotoxicity of doxorubicin towards Jurkat cells (Figure 3).

In the case of combination of everolimus and 2-DDG, we observed a very clear synergistic cytotoxic effect after 48-h treatment compared to administration of everolimus and of 2-DDG alone. This was accompanied by a very strong

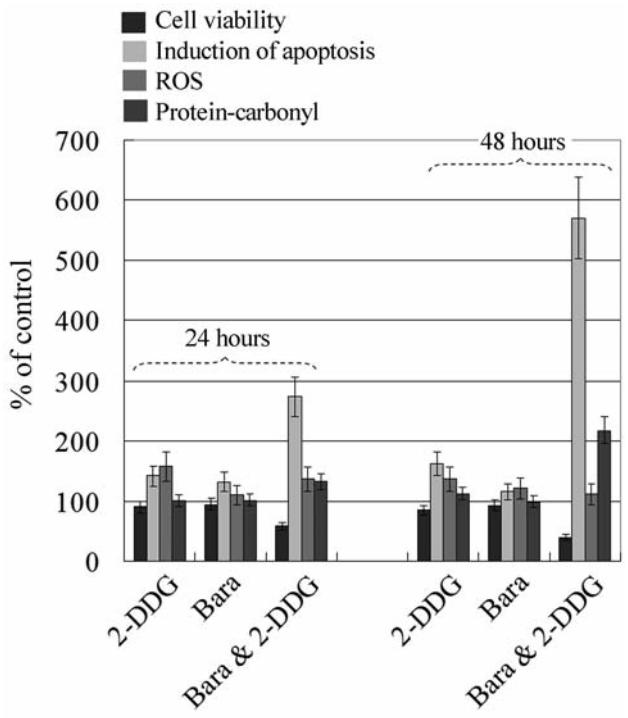

Figure 5. *Effects of barasertib (Bara; 0.01 μM) in the absence and presence of 2-deoxy-D-glucose (2-DDG; 250 μM) on cell viability, induction of apoptosis, level of reactive oxygen species (ROS) and level of protein-carbonyl products in Jurkat cells after 24 and 48 h incubation at 37˚C in a humidified atmosphere. Data are the mean±SD from three independent experiments.*

decrease of ROS, but a very strong increase of apoptosis of the cells (Figure 4). Changes in the level of protein-carbonyl products were not detected – they were at the level of the control in all samples. In the literature, there are no data on the cytotoxicity of everolimus in combination with 2-DDG, nor of the effect of this combination on the cross-talk between ROS and induction of apoptosis in the cells.

Everolimus is an inhibitor of mammalian target of rapamycin (mTOR) pathway that plays a fundamental role in regulation of cell viability, translational initiation, and cell-cycle progression. This drug is usually used in the clinic as an immunosuppressant to prevent rejection of organ transplants. In the past 10 years, it was found that everolimus also possesses anticancer activity (44-49). It sensitizes cancer cells to other anticancer drugs, as well as preventing the development of multidrug resistance through altering the balance between apoptotic and antiapoptotic factors (50-55). Several studies have shown that the anticancer effect of everolimus was not mediated by increased production of ROS (56, 57). For example, Pignochino *et al*. reported that everolimus reduced ROS production, but increased apoptosis in osteosarcoma cell lines after 48-h treatment (57). Klawitter *et al*. observed that everolimus enhanced ROS formation in C6 glioma cells, but had only minor effects on normal rat brain tissues (58). In some cells, everolimus increased, but in others it did not alter or even reduced ROS production induced by other drugs (*e.g*. sorafenib and cyclosporin) (57, 58). In these studies, the ROS analysis was carried out using DCF probes – specific predominantly for hydrogen peroxide or other hydroperoxides.

Recently, Pignochino *et al*. reported that everolimus potentiates the anticancer activity and induction of apoptosis of sorafenib in malignant pleural mesothelioma cells by a ROS-mediated mechanism (59). In this case, the ROS analysis was carried out using MitoSOX™ – a fluorescent probe specific for mitochondrial superoxide.

In our study, we found that the combination of everolimus and 2-DDG was characterized by a very low level of ROS (below that of the control; as analyzed by DCF probe), but by a very strong induction of apoptosis and cytotoxicity compared to the drug and redox-modulator applied alone (Figure 4). This could be explained with a synergistic inhibition of glycolysis by the combination in addition to the mTOR-dependent regulation of apoptosis by everolimus. Klawitter *et al*. showed that everolimus inhibited cytosolic glycolysis but did not cause changes in mitochondrial energy production (58). The described experimental data show that everolimus would be a very promising anticancer agent in combination with redox modulators.

Barasertib belongs to a new class of pyrazoloquinazolines, selective inhibitors of aurora B kinase, discovered and described in 2007 (60, 61). The aurora kinases have been the subject of considerable interest as targets for the development of new anticancer agents. The inhibition of aurora B kinase gives rise to the more pronounced antiproliferative phenotype and the most clinically advanced agents reported to date that typically inhibit both aurora A and B kinases. Little is known on the molecular mechanisms of action of barasertib, except that it inhibits aurora B kinase, provokes cell-cycle arrest and apoptosis, as well as enhancing the response to chemotherapy (62-64).

In our study, we used barasertib at a very low concentration – 10 nM only. At this concentration, the drug practically did not influence cell viability, ROS or the protein-carbonyl level, nor did it induce apoptosis (Figure 5). However, in combination with 2-DDG, a very strong synergistic cytotoxic effect was detected, accompanied by a strong induction of apoptosis, without increase of ROS level, but with an enhancement of the protein-carbonyl products in the treated cells.

In conclusion, the present study shows that combining new-generation anticancer drugs, such as barasertib and everolimus, with a redox-modulator such as 2-DDG markedly enhances the anticancer effect at a very low concentration of the drug, and potentially strongly

minimizing the side-effects. In some cases, using a redox-modulator, it is possible to influence the cellular redox status in such a way as to reduce the production of ROS, but induce apoptosis of cancer cells by ROS-independent mechanism(s).

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