Inhibition of the Pentose-phosphate Pathway Selectively Sensitizes Leukemia Lymphocytes to Chemotherapeutics by ROS-independent Mechanism

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Abstract. *The aim of the present study was to investigate: (i) the possibility of sensitizing leukemia lymphocytes to anticancer drugs by inhibiting pentose-phosphate pathway using 6-aminonicotinamide (6-ANA); (ii) to find combinations with synergistic cytotoxic effect on leukemia lymphocytes and to investigate their cytotoxicity towards normal lymphocytes; (iii) and to clarify the role of reactive oxygen species (ROS) in the induction of apoptosis by those combinations. The study covers 15 anticancer drugs – conventional and new-generation. The experiments were performed on Jurkat leukemia cell line and normal lymphocytes, isolated from clinically healthy blood donors. Four parameters were analyzed simultaneously in both cell suspensions treated by drug or 6-ANA (separately, and in combination): cell viability, induction of apoptosis, level of ROS, and level of protein–carbonyl products. Most combinations of drug plus 6-ANA were characterized by synergistic cytotoxic effects on Jurkat cells. The synergism increased with increasing incubation time. Upon combination of 6-ANA with conventional chemotherapeutic (e.g. doxorubicin), synergistic cytotoxic effects were also detected in normal lymphocytes. In both cell types, the cytotoxicity of the combination of doxorubicin plus 6-ANA was accompanied by increased induction of apoptosis, but by a slight reduction of ROS and protein–carbonyl products compared to cells treated with doxorubicin only. Upon combination of 6-ANA with new-generation anticancer drugs (e.g. everolimus or barasertib), the synergistic cytotoxic effect on leukemia lymphocytes was also accompanied by very strong induction of apoptosis through ROS-independent mechanism(s). Neither of these combinations exhibited any cytotoxicity towards normal lymphocytes. The data suggest that 6-ANA could be used as a supplementary component in anticancer chemotherapy, and would allows therapeutic doses of anticancer drugs to be reduced, thereby minimizing their side-effects.*

Many studies have demonstrated that anthracyclines, widely used to treat various malignant tumors, increase intracellular generation of reactive oxygen species (ROS) and alter cellular redox homeostasis (1-10). For example, the antitumor activity of one of the most effective anthracycline antibiotics, doxorubicin, is due to DNA damage, mainly through inhibition of DNA topoisomerase II enzyme after induction of double-strand DNA breaks (11). However, doxorubicin is localized in mitochondria and is highly susceptible to enzymatic reactions with generation of ROS and products of free radical oxidation, which can cause mitochondrial swelling, ultrastructural changes and alteration of mitochondrial function (12, 13). Doxorubicin-induced oxidative stress causes depolarization of the mitochondrial membrane, which leads to apoptosis. Another widely used anthracycline, cisplatin, interacts with nucleophilic N7 sites of purine bases in DNA, leading to formation of DNA–protein and DNA–DNA inter- and intra-strand cross-links (14). It has been reported that cisplatin also induces production of ROS and can bind to mitochondrial DNA (15-18). Bleomycin, a conventional antitumor drug for treatment of lymphomas, squamous cell carcinomas and testicular

tumors, is well-known to cause single- and double-strand breaks in cellular DNA *in vivo* and *in vitro* leading finally to genomic instability of damaged cells (19). The mechanism of antineoplastic effect of bleomycin includes formation of bleomycin–iron complex which can reduce molecular oxygen to superoxide and hydroxyl radicals that can attack DNA and cause strand cleavage (1). Many experimental and clinical studies have considered that the antitumor activity of conventional anticancer drugs are usually due to induction of oxidative stress in cancer cells (1, 5, 6, 8, 11), but unfortunately side-effects of these anticancer drugs are due to an increased level of ROS in normal cells and disruption of their redox homeostasis (1-12, 20). Some of the most effective chemotherapeutics which are used for treatment of variety of cancer types have shown toxic side-effects on non-cancer tissues. Efforts are being directed to achieve therapeutic selectivity and to prevent development of drug resistance because it is important to design new strategies that exploit the difference in redox homeostasis between normal cells and cancer cells (21-23).

It is generally accepted that cancer cells are characterized by a higher level of ROS due to mitochondrial dysfunction and up-regulation of the NADPH-dependent oxidase complex (NOX), as well as by a higher level of reducing equivalents due to the abnormal production of NADP(H) and thiols (*e.g.* glutathione) as a result of accelerated glycolysis (the Warburg effect) and the pentose-phosphate cycle in comparison with normal cells (21-25). However, these reducers are rapidly consumed in cancer cells, in order to maintain accelerated anabolism, which is necessary for their proliferation and survival (24, 25).

A promising strategy for achieving therapeutic selectivity and efficiency in cancer is to take advantage of the fundamental difference between cancer cells and normal cells in their biochemical metabolism. 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 undesired side-effects (26-29).

It is assumed that the pentose-phosphate pathway, one of the major branches of the glycolytic pathway, promotes cancer progression, as well as drug resistance, due to the high biosynthetic demands of a rapidly growing cancer cells and their need to adapt to stressful environments (30, 31). The pentose-phosphate pathway is essential for the survival of cancer cells because it generates pentose-5-phosphate for the synthesis of nucleotides and reducing equivalent NADPH, which is needed for cell proliferation and regulation of ROS. It has been reported that several oxidative (*e.g.* glucose-6-phosphate dehydrogenase and 6-phospho-gluconate dehydrogenase) and non-oxidative (*e.g.* transketolase-like-1 and transaldolases) branches of the pentose-phosphate pathway are activated during carcinogenesis (30-34).

An inhibitor of the pentose-phosphate pathway, 6-aminonicotine-amide (6-ANA), is in preclinical investigation as a modulator of cytotoxicity of antineoplastic drugs, particularly DNA-damaging chemotherapeutics (35). Data indicate that 6-ANA sensitizes a number of cancer cell lines to doxorubicin, cisplatin, paclitaxel, *etc*. (36-38). The anticancer activity of 6-ANA is associated with dysregulation of the pentose-phosphate pathway (39, 40). 6-ANA is transformed in cells into 6-amino-$NADP^+$ by NAD^+ glycohydrolase. This NADP analog inhibits 6-phosphogluconate dehydrogenase much more efficiently than other NADP(H)-requiring enzymes, such as glutathione reductase and glucose-6-phosphate dehydrogenase (41, 42).

The aim of the present study was to investigate: (i) the possibility of sensitizing leukemia lymphocytes to anticancer drugs using 6-ANA; (ii) to find combinations with synergistic cytotoxic effect on leukemia lymphocytes and to investigate their cytotoxicity towards normal lymphocytes; (iii) and to clarify the role of ROS in the induction of apoptosis and cytotoxicity by those combinations. The study covers 15 anticancer drugs, both conventional and new-generation.

Materials and Methods

Cells and treatment protocol. The experiments were performed on Jurkat leukemia lymphocytes derived from patients with acute lymphoblastic leukemia, as well as on normal lymphocytes isolated from healthy blood donors using Lymphosepar-I (Immuno-Biological Laboratories Co., Fujioka, Japan). The cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), in a humidified atmosphere at 37°C with 5% CO_2. All cells were collected by centrifugation (1000 × *g* for 10 min) and replaced in fresh medium without antibiotics, before treatment with anticancer drugs.

The drugs were dissolved in dimethyl sulfoxide (DMSO; suitable for cell culture) 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 cells (1×10^6 cells/ml) at the concentrations mentioned below (single dose) and cells were then incubated for different lengths of times intervals in cell incubator. At the end of each time, aliquots were used for cell viability assay.

The cells were incubated with 6-ANA, drug, or drug plus 6-ANA at the following concentrations: 50 μM 6-ANA (Sigma-Aldrich, Steinheim, Germany); 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-Aldrich); 0.5 μM bleomycin (Nippon Kayaku Co., Tokyo, Japan); 0.1 μM AZD-7762 (Sigma-Aldrich); 0.01 μM MLN-2238 (Selleckchem); 0.025 μM MG-132 (Wako, Tokyo, Japan); 10 μM lomustine (Sigma-Aldrich); 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 6-ANA (applied separately) induced about 20% inhibition of cell growth.

Cell viability assay. Cell viability was analyzed using trypan blue staining and Countess™ Automated Cell Counter (Invitrogen, OR, USA) at very precise standardization of measurements. Three independent experiments (with two repetitive measurements) for each experiment were performed for each sample. Non-treated cells were used as controls. The 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 Flourescence (Cell Biolabs., Inc., San Diego, CA, USA). The method is based on the use of fluorogenic probe, 2',7'-dichlorodihydrofluorescein diacetate-DiOxyQ. In the cytosol, the probe is deacetylated to the non-fluorescent form. This in turn reacts with ROS and reactive nitrogen species (RNS) (predominantly H_2O_2, $ROO^{\bullet}$, NO, $ONOO^-$) with formation of fluorescent 2',7'-dichlorodihydrofluorescein (DCF). The intensity of DCF fluorescence (λ_{ex}=480 nm, λ_{em}=530 nm) is proportional of the amount of ROS/RNS in the biological sample.

The amount of ROS/RNS was calculated by calibration curve, based on standard solution of DCF in PBS. The measurements were performed on Tecan Infinite F200 PRO microplate reader (Tecan Austria GmbH, Mannedorf, Austria).

Briefly, the cells (1×10^6 cells/ml) were collected by centrifugation ($1,000 \times g$ for 10 min) and lysed by 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 instruction.

Protein-carbonyl assay. The amount of protein–carbonyl products was analyzed using OxiSelect™ Protein Carbonyl Spectrophotometric Assay Kit (Cell Biolabs, Inc.). 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 with formation of protein–hydrazone. The amount of protein–hydrazone is then determined spectrophotometrically at 375 nm.

Briefly, cells (1×10^6 cells/ml) were collected by centrifugation ($1,000 \times g$ for 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 (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 instruction. Oxidized bovine serum albumin was used as a standard.

Apoptosis assay. The induction of apoptosis was analyzed by the expression of phosphatidylserine (PSer) on the cell surface using FITC-Annexin V Apoptosis Detection Kit (BioVision, Milpitas, CA, USA).

Briefly, cells (1×10^6 cells/ml) were incubated with drug, 6-ANA or their combination, under the conditions mentioned above. At each time-point, the cells were collected by centrifugation ($1,000 \times g$ for 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 suspension were incubated with 5 μl of fluorescein isothiocyanate (FITC)–annexin V for 10 min at room temperature in the dark. The cells were then washed three times

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_{ex}=488 nm and λ_{em}=535 nm using Tecan Infinite F200 PRO microplate reader (Tecan Austria GmbH).

Statistical analysis. The results are expressed as the mean±SD. Statistical analysis was performed with Student's *t*-test. A value of $p<0.05$ was considered significant.

Results and Discussion

The data in Figure 1 demonstrate the viability of leukemia lymphocytes (Jurkat) and normal lymphocytes after treatment by 6-ANA at different concentrations and for different times. Applied alone in concentrations in the range of 5 to 50 μM, 6-ANA exhibited cytotoxicity only towards leukemia lymphocytes not to normal lymphocytes. At a concentration of 50 μM, 6-ANA caused about 20% inhibition of cell proliferation (IC_{20}) in leukemia lymphocytes (Figure 1A) without cytotoxic effect on normal lymphocytes after 48-h incubation (Figure 1B). This concentration was selected for further experiments.

Several groups have also described a relatively high anticancer activity of 6-ANA (over 25 μM) against cultured cell lines (36-45) and low cytotoxicity towards non-cancerous cells (45). In these studies, the inhibition of pentose-phosphate pathway was recognized as a major mechanism for induction of apoptosis in cancer cells (36-45). Other studies demonstrated that 6-ANA inhibits poly(ADP-ribose) polymerase (PARP), with the result that PARP cannot participate in DNA repair after induction of oxidative damage (46-48). However, it is still unclear whether the inhibition of PARP by 6-ANA is responsible for induction of apoptosis in cancer cells.

In the second part of our study, Jurkat cells were treated with: (i) drug only; (ii) 6-ANA only; and (iii) combination of drug and 6-ANA. The aim of this step of the study was to find combinations with synergistic cytotoxic effects on leukemia cells. Figure 2 shows the proliferation of Jurkat cells treated with 6-ANA in the absence and presence of drug for 24-72 hours. In order to distinguish synergistic effects from antagonistic/additive effects in combinations, we calculated the effect of each combination on cell proliferation as a percentage of the effect of the respective drug applied alone and compared it with the effect of 6-ANA applied alone, as described previously (29). The red line in Figure 2B indicates the effect of 6-ANA alone on cancer cell proliferation. In the case of drug plus 6-ANA, data located to the left of the red line reflect synergistic cytotoxic effects, while the data located to the right of the red line represent antagonistic effects. All data matching the red line reflect an additive affect. The cytotoxicity of each drug (applied separately at the selected concentration) ranged from about
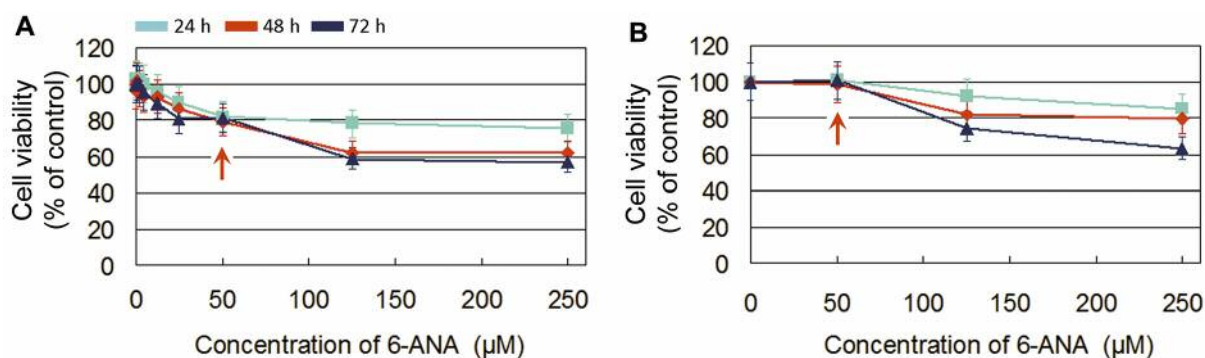
Figure 1. *Effect of 6-amino-nicotinamide (6-ANA) on viability of leukemia lymphocytes Jurkat (A) and normal lymphocytes (B) at different incubation times – concentration dependent curves. The data are the mean±SD from six independent experiments. The red arrow indicates the concentration of 6-ANA (50 μM), selected for further experiments in combination with anticancer drugs.*

10-20% after 24-h incubation to about 20-30% after 48-h and 72-h incubation.

Most of the combinations (drug plus 6-ANA) were characterized by synergistic cytotoxic effects on Jurkat cells compared with cells treated with drug only (Figure 2B). The synergism increased with incubation time. The greatest synergistic cytotoxic effects were observed after treatment of Jurkat cells with 6-ANA in combination with bortezomib, barasertib, everolimus, lonafarnib, or palbociclib.

Two of these synergistic combinations were selected for the next step of this study, namely everolimus plus 6-ANA and barasertib plus 6-ANA. In our recent study, we observed that everolimus and barasertib (applied alone) did not increase the level of ROS in leukemia and normal lymphocytes (49). In addition, we selected a combination of 6-ANA with the conventional anticancer drug doxorubicin, which is known to increase ROS in cancer and normal cells (11-13). We investigated the effect of these combinations on viability of normal lymphocytes and clarified whether their synergistic cytotoxicity was accompanied by enhancement of ROS and induction of apoptosis in both cell types (leukemia and normal).

Doxorubicin, at a concentration of 0.1 μM, was equally cytotoxic towards leukemia and normal lymphocytes (Figure 3). Cytotoxicity increased with incubation time, from ~5-10% after 24 h up to ~25% after 48 h. This was accompanied by increased levels of ROS and protein–carbonyl products and induction of apoptosis, especially at 48 h. 6-ANA increased the cytotoxicity of doxorubicin by up to 40% for both cell types, which was accompanied by increased induction of apoptosis *via* ROS-independent mechanism(s): the levels of ROS and protein–carbonyl products were actually slightly or significantly lower than in cells treated with doxorubicin only. The synergistic cytotoxicity of doxorubicin plus 6-ANA might be attributed to effect of both compounds on several onco-targets, but not to overproduction of ROS. It was

reported that 6-ANA increases the sensitivity of doxorubicin-resistant cell lines to tumor necrosis factor alpha *via* down-regulation of human epidermal growth factor receptor (50). 6-ANA regulates the level of glutathione and inhibits multidrug resistance-associated proteins in doxorubicin-resistant and doxorubicin-sensitive cell lines (38).

Everolimus is an inhibitor of the mammalian target of rapamycin (mTOR) pathway, which plays a fundamental role in the 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 (51-59). It sensitizes cancer cells to other anticancer drugs, as well as preventing the development of multidrug resistance through altering the balance between pro-apoptotic and anti-apoptotic factors (56, 59). Schwarz *et al.* showed that the anticancer effect of everolimus was not mediated by increased production of ROS (60), but Pignochino *et al.* suggested the involvement of ROS in this process (61).

Our study shows that everolimus, at 5 μM, exhibited cytotoxicity (~10-15% after 48-h incubation) towards Jurkat cells but not towards normal lymphocytes (Figure 4). In Jurkat cells, the cytotoxicity of everolimus applied alone was accompanied by strong induction of apoptosis but a relatively slight (insignificant) increase of the level of ROS and without changes in the level of protein–carbonyl products. 6-ANA increased the cytotoxicity of everolimus towards leukemia lymphocytes by up to ~60% after 48-h incubation, but the combination did not affect the viability of normal lymphocytes. In Jurkat cells, 6-ANA significantly increased (up to 2.5 times) the induction of apoptosis by everolimus, but reduced the level of ROS below that even of the control (Figure 4A). In normal lymphocytes, the combination did not affect the level of ROS nor the level of protein–carbonyl products and apoptosis (Figure 4B).
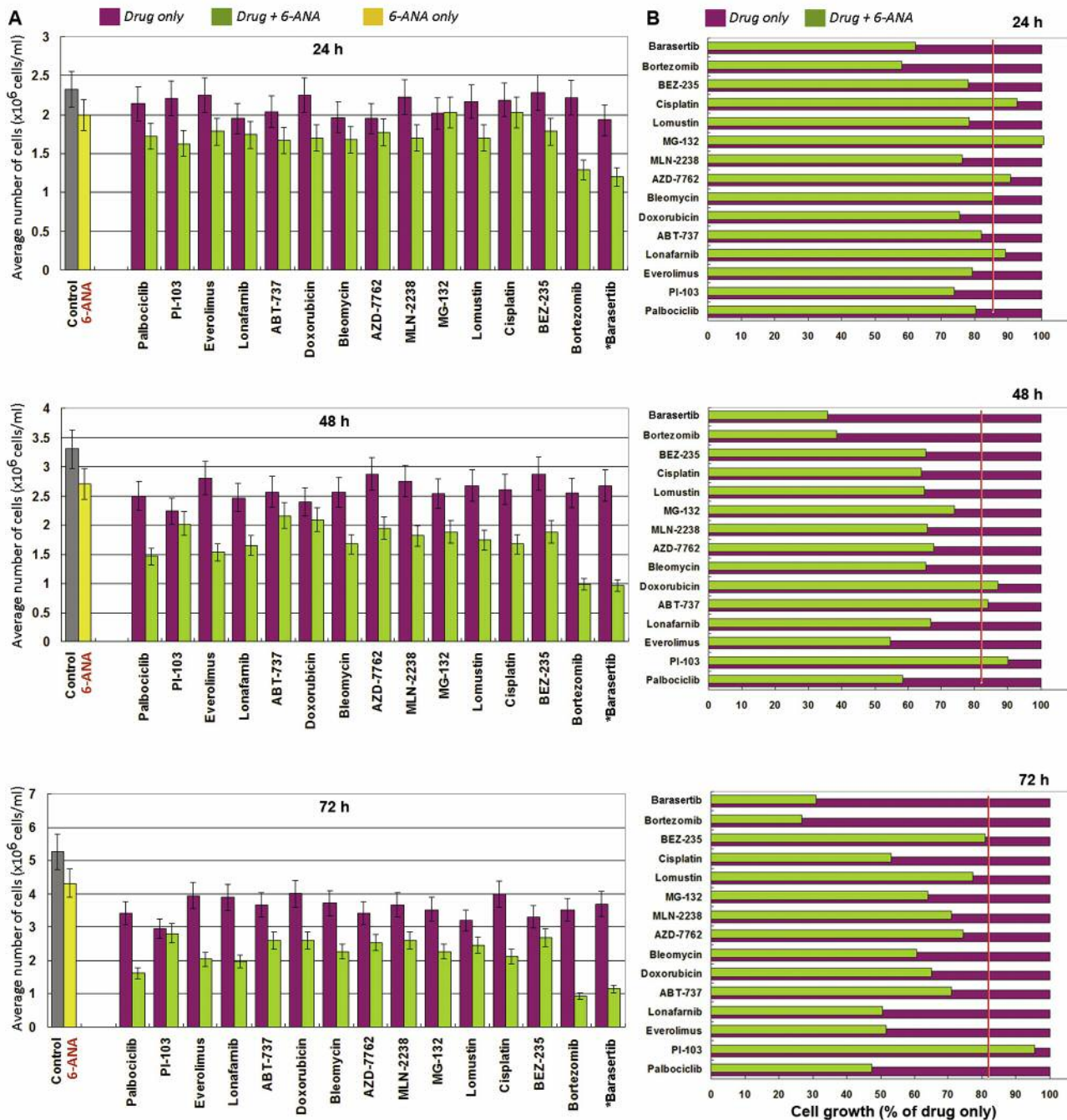
Figure 2. *A: Effect of 6-amino-nicotinamide (6-ANA; 50 μM) and anticancer drugs (at concentrations given in the Materials and Methods) on proliferation of Jurkat cells after incubation for 24, 48 and 72 h. The data are the mean±SD from six independent experiments. B: Effect of each combination (drug plus 6-ANA) on cell proliferation as a percentage of the effect of that when the drug was applied alone. The red line indicates the effect of 6-ANA on cell proliferation of cancer cells as a percentage of the control (untreated cells). *In this experiment, the concentration of barasertib was 50 nM.*

Similar tendencies were observed in cells treated with barasertib and its combination with 6-ANA (Figure 5). Both cell lines were treated with a very low concentration of barasertib (10 nM). At this concentration, barasertib applied alone did not affect the viability of either cell type. However, its combination with 6-ANA induced a very strong cytotoxic effect on Jurkat cells (~75%), which was accompanied by strong induction of apoptosis and relatively moderate
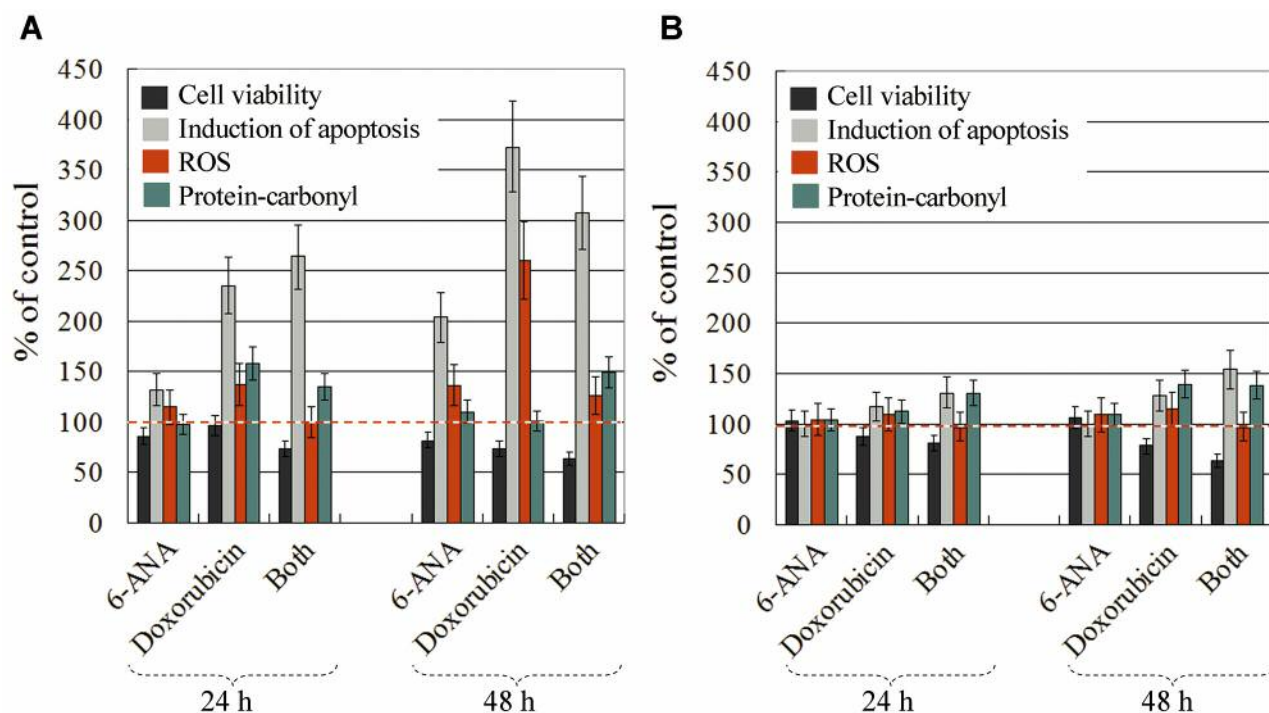
Figure 3. *Effects of 6-amino-nicotinamide (6-ANA; 50 μM), doxorubicin (0.1 μM) and their combination on cell viability, induction of apoptosis, reactive oxygen species (ROS) and protein–carbonyl products in Jurkat leukemia cells (A) and normal lymphocytes (B) after 24- and 48-h incubation at 37°C in a humidified atmosphere. The data are the mean±SD from three independent experiments.*

Figure 4. *Effects of 6-amino-nicotinamide (6-ANA; 50 μM), everolimus (5 μM) and their combination on cell viability, induction of apoptosis, reactive oxygen species (ROS) and protein–carbonyl products in Jurkat leukemia cells (A) and normal lymphocytes (B) after 24- and 48-h incubation at 37°C in a humidified atmosphere. The data are the mean±SD from three independent experiments.*
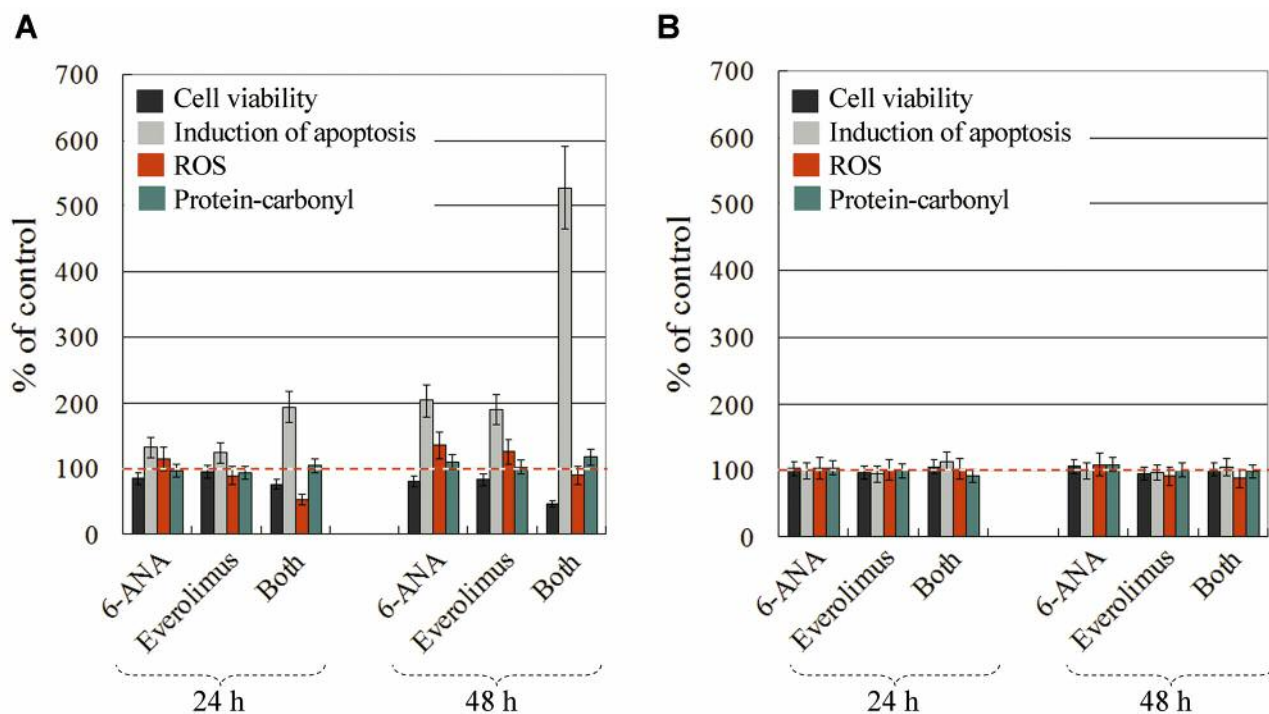
Figure 5. *Effects of 6-amino-nicotinamide (6-ANA; 50 μM), barasertib (0.01 μM) and their combination on cell viability, induction of apoptosis, reactive oxygen species (ROS) and protein–carbonyl products in Jurkat leukemia cells (A) and normal lymphocytes (B) after 24- and 48-h incubation at 37°C in a humidified atmosphere. The data are the mean±SD from three independent experiments.*
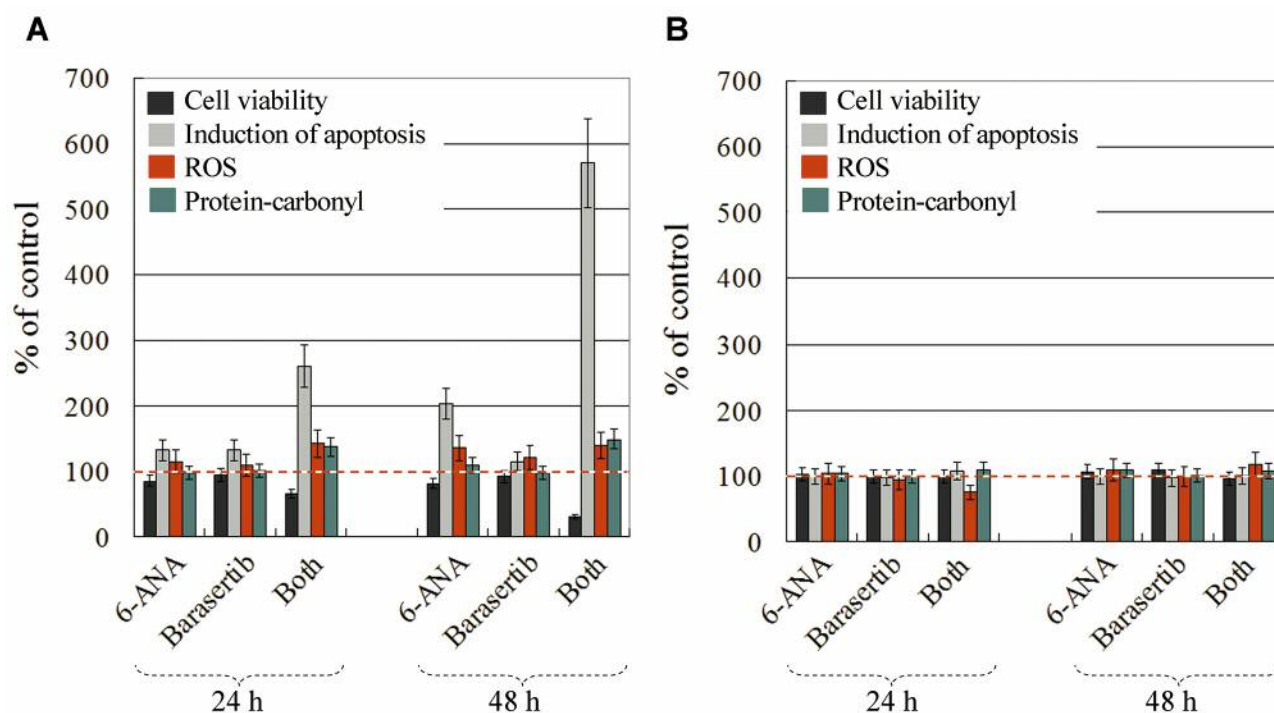
enhancement of ROS and protein–carbonyl levels, as detected after 48-h incubation (Figure 5A). In normal lymphocytes, the combination did not affect the level of ROS and protein–carbonyl products, nor did it induce apoptosis (Figure 5B).

Barasertib belongs to a new class of pyrazoloquinazolines, selective inhibitors of aurora B kinase, discovered and described in 2007 (62). The aurora kinases have been the subject of considerable interest as targets for the development of new anticancer agents. Little is known about the molecular mechanisms of action of barasertib, except that it inhibits aurora B kinase, and provokes cell-cycle arrest and apoptosis, as well as enhancing the response to chemotherapy (63-65).

In the literature, there are many data regarding sensitizing cancer cells to conventional anticancer drugs (such as doxorubicin, bleomycin, and cisplatin) by inhibitors of the pentose-phosphate cycle (31, 32, 66, 67). Most of them suggest ROS-dependent mechanisms. A limited number of studies describe the effect of 6-ANA on the cytotoxicity of new-generation anticancer drugs (recently approved for clinical use or still in clinical trials), such as the proteasome inhibitor MG-132 (68). As far as we are aware, there are no data about the effect of 6-ANA on the efficiency of new-generation anticancer drugs such as AZD7762 (selective inhibitor of checkpoint kinases), ABT-737 (selective inhibitor

of B-cell lymphoma 2 proteins), barasertib (selective inhibitor of aurora B kinase), everolimus (mTOR inhibitor), lonafarnib (farnesyltrasferase inhibitor), MLN-2238 and bortezomib (proteasome inhibitors), and palbociclib (selective inhibitor of cyclin-dependent kinases). To our knowledge, our study is the first to report synergistic cytotoxicity between 6-ANA (as a redox modulator) and both everolimus and barasertib (as anticancer drugs) which is ROS-independent and specific for cancer cells (particularly for Jurkat acute lymphoblastic leukemia cells). These combinations are harmless to normal lymphocytes and do not induce production of ROS in those cells. The data suggest that 6-ANA could be used as a supplementary component in anticancer chemotherapy, which would allow reduction of the therapeutic doses of barasertib and everolimus, minimizing their side-effects. This statement can also be made for all the anticancer drugs investigated here, whose cytotoxicity increased by combining them with 6-ANA.

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