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Evaluation of the combined effects of doxorubicin and bortezomib on the human acute lymphoblastic leukemia cell line

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ABSTRACT

Increasing numbers of oncological patients and growing drug resistance ensure that new methods of cancer treatment are intensively sought. Combining drugs for a synergistic effect is one of several possible ways to mitigate this problem. This leads to reducing the effective drug dose and the occurrence of side effects. Doxorubicin (DOX) is an antineoplastic agent that has several mechanisms of action. DOX intercalates between base pairs of DNA helix, inhibits topoisomerase II and also forms reactive oxygen species. Bortezomib (BZT) is an antitumor agent belonging to the group of proteasome inhibitors. It has been observed that BZT triggers an oxidative stress response *in vitro* and *in vivo*. Accumulation of oxidatively damaged proteins and the simultaneously blocking of the proteasome can be very damaging to the tumour cell. For this reason, the aim of the study was to assess the potentially synergistic effect of DOX and BZT on human acute lymphoblastic leukemia (ALL). In the work, the cells were treated with both agents and their combinations and the effect was evaluated on the basis of morphological assessment, MTT assay and level of reduced glutathione measurement.

The study has shown that on acute lymphoblastic leukemia cells, synergistic effects came about in the combination of 1nM BZT with a wide range of concentrations of DOX. Herein, the visible, coactive effect of DOX and BZT was observed on oxidative stress levels. This phenomenon can be essential in blunting the possibility of rapid manifestation of resistance seen in BZT monotherapy. In addition, the needed very low concentrations of DOX reduce the risk of therapy side effect.

INTRODUCTION

Because of the increased incidence of cancer and the growing resistance to current drugs, new ways of treatment are sought. One of the strategies is to apply the phenomenon of synergy so as to utilize a combination of drugs already in use for cancer treatment. Moreover, the benefit in synergy to reduce concentrations of drugs can minimise potentially harmful side effects.

Bortezomib (BZT) is a unique and specific inhibitor of the proteasome pathway. This anticancer agent quickly and reversibly inhibits the 26S proteasome that is an enzyme complex responsible for the degradation of cellular proteins. Proteasome is present in all eukaryotic cells, but cancer cells are more sensitive to their inhibition than are normal cells. Cancer cells cannot process the excess of contradictory cellular regulatory signals and undergo apoptosis. In contrast, normal cells can recover. Therefore, bortezomib has potential as a chemotherapeutic agent in many different types of tumours [1,2].

Doxorubicin (DOX) is one of the most frequently used medications in cancer chemotherapy. It is an anthracycline antitumor antibiotic that has a multidirectional mechanism of action. DOX intercalates into DNA between base pairs and inhibits topoisomerase II. It also generates oxygen free radicals via oxidation-reduction reactions. This causes accumulation of oxidatively changed proteins [3].

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Generation and accumulation of oxygen free radicals in the process of one-electron reduction of DOX brings about oxidative modification of cellular proteins. Simultaneously blocking the proteasome 26S by BZT and establishing an excess of oxidative modified proteins can be very damaging to the tumour cell. Hence co-administration of BZT and DOX may induce a synergistic effect.

Similarly to DOX, BZT used alone induces oxidative stress in multiple myeloma, mantle-cell-lymphoma, head and neck squamous cell carcinoma and non-small cell lung cancers [4-7]. Currently, FDA has approved the administration of BZT and pegylated liposomal DOX together and also in combination with dexamethasone (PAD) for the treatment for relapsed or refractory multiple myeloma [8,9]. What is more, the combination of BZT and DOX has undergone several different phases of clinical trials in a few types of cancers [10].

Acute lymphoblastic leukemia (ALL) originates from B and T cell precursors. After acute myelogenous leukemia, it is the second most malignant hematopoietic neoplasm with a very rapid course. ALL mainly affects young people below 30 years of age. This type of leukemia is the most common cancer among children and accounts for about 80% of all diagnosed childhood leukemias [11]. DOX is commonly used in the treatment of ALL. There are clinical reports on the effectiveness of bortezomib therapy compared to ALL, but only in combination with other known chemotherapeutics [11,12]. For this reason the aim of the study was the evaluation of the combined effects of doxorubicin and bortezomib on viability and redox balance in a human acute lymphoblastic leukemia cell line.

METHODS

Cell culture

In the study, the acute lymphoblastic leukaemia CCRF-HSB-2 (ATCC, USA) cell line was used. The cells were cultured under standard conditions: at 37°C, in 5% CO_2 and at pH= 7.0-7.6 in IMDM medium (USA, ATCC) supplemented with 10% foetal bovine serum (USA, ATCC).

Morphological assessment

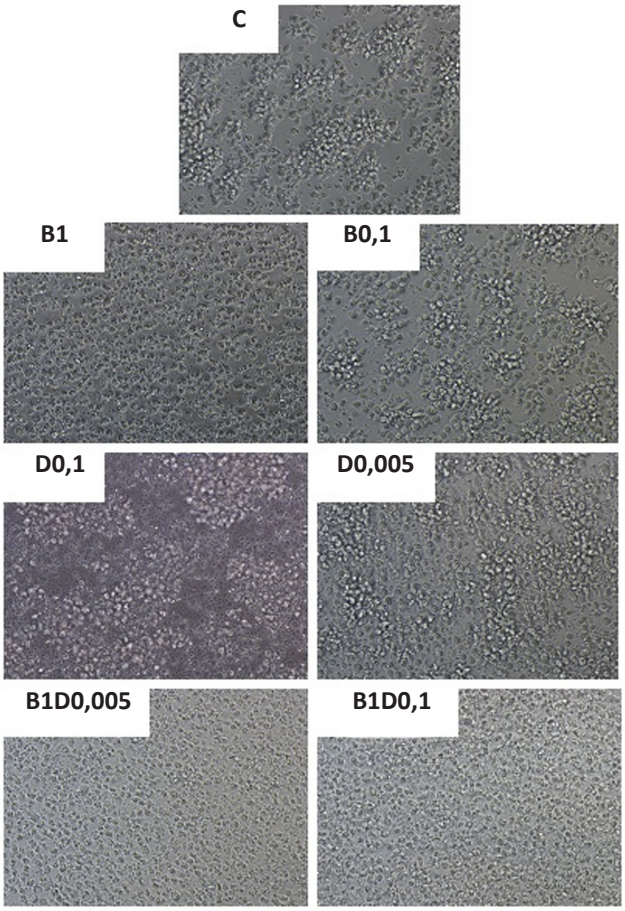
Morphological assessment was performed using a Nikon Eclipse Ti phase-contrast microscope and NIS-Elements Imaging Processing Software (Nikon, Tokyo, Japan).

Cell treatment

Cells were incubated with bortezomib (USA, Selleckchem) and doxorubicin (Austria, EBEWE Pharma) for 24 hours. The compounds were applied in a series of concentrations corresponding to a lower level of clinical concentrations and below: BZT – 1 and 0.1 nM and DOX – 0.1, 0.05, 0.01 and 0.005 μM.

MTT test

The viability of the cells was examined by standard MTT assay, using the MTT Cell Proliferation Assay Kit (Invitrogen, US). In this test, the activity of mitochondrial enzyme-succinate dehydrogenase is used. This enzyme, in living cells, is responsible for the transformation of soluble tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to insoluble formazan, which has a purple colour. MTT assay was based upon spectrophotophotometric measurement of the intensity of the resulting colour at 540 nm, using a PowerWave™ microplate spectrophotometer (Bio-Tek Instruments, USA).



C – control, **B1** – bortezomib 1 nM; **B0,1** – bortezomib 0,1 nM; **D0,1** – doxorubicin 0,1 μM; **D0,005** – doxorubicin 0,005 μM; **B1D0,005** – bortezomib 1 nM and doxorubicin 0,005 μM; **B1D0,1** – bortezomib 1 nM and doxorubicin 0,1 μM

Figure 1. ALL cells treated with BZT and DOX

Assessment of oxidative stress by the GSH level

Oxidative stress was assessed by measuring changes in the GSH level via the use of a NucleoCounter® NC-3000™ instrument, according to the manufacturer's recommended procedure.

Statistical analysis

The results were analysed statistically in the STATISTICA v. 12.0 application (StaftSoft, Cracow, Poland), using mean and standard deviation values. To compare more than two groups, one-way analysis of variance ANOVA and post hoc multiple comparisons on a basis of Tukey's HSD test were applied.

RESULTS

Assessment of cell morphology

In treatment with BZT alone, a significant increase of dead cells was observed at concentration 1nM (B1), but no morphological changes were seen in cells treated with

lower amounts. Among the samples in which DOX alone was added, an increased amount of dead cells was observed only at 0,1 μM. In the case of co-administration, a significant increase of dead cells was observed in combinations of 1 nM BZT (B1) with each concentration of DOX. These cells are small, irregular and contain a lot of grain. In other drug combinations, such morphological changes were not evident.

MTT assay

A Synergistic effect was evident in cultures treated with 1nM BZT and each concentration of DOX (B1D0,1; B1D0,05; B1D0,01 and B1D0,005). Combinations of lower BZT concentrations with DOX induced attenuation of the DOX effect.

GSH level

BZT in the form of B1 had a significant effect on GSH level whether alone or in combination with each concentration of DOX. In addition, the GSH decrease was seen in D0,1; D0,1B0,5; D0,1B0,2 and D0,05B0,5.

DISCUSSION

Usage of single agents *in vivo* treatment of leukemia has generated a rapid development of resistance in leukemia cells to cytotoxic chemotherapies. To prevent the development of drug resistance, most treatment regimens combine several chemotherapy agents [14]. We tested the BZT, DOX and their combination on human acute lymphoblastic leukemia cell line. Synergistic application of drugs is now a common means of treating cancer. Prevention of increasing drug resistance and reduced incidence of side effects are positive aspects of this development.

In the study, bortezomib and doxorubicin were used. Bortezomib (BZT) inhibits the 26S proteasome complex and hence disrupts the function of the ubiquitin-proteasome pathway. This leads to blockage of protein, as well as transcription factor degradation and causes an accumulation of damage proteins. Additionally, there are reports that BZT triggers generation of the reactive oxygen species in cancer cells. Doxorubicin (DOX) is an commonly used antitumor agent. It inhibits topoisomerase II, intercalates into double-stranded DNA and generates oxygen-free radicals. Reactive Oxygen Species (ROS) such as that generated by doxorubicin induce oxidative change of proteins. Blockage of the 26S proteasome and the accumulation of oxidatively modified proteins harm the tumor cell. Thus, co-administration of bortezomib and doxorubicin creates a synergistic effect [13].

We observed that DOX brings about a decrease in viability of ALL cells in concentrations which are lower than that used in clinical treatment. Approximate IC_{50} value estimated in our MTT assay is about 0,01 μM. The impact of doxorubicin on an acute lymphoblastic leukemia cell

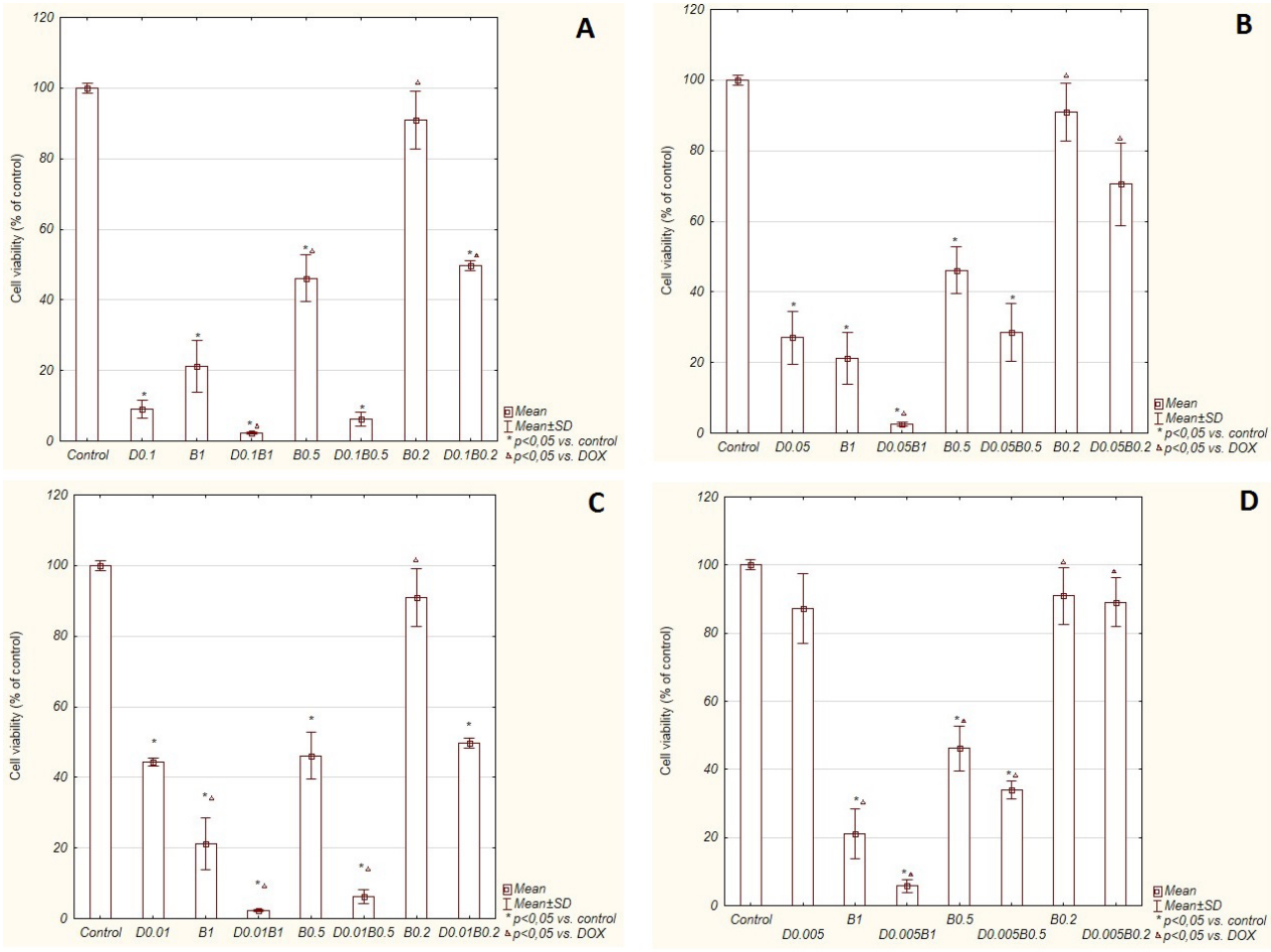


Figure 2. ALL cells viability based on MTT assay results (expressed as % of control culture viability) (B – bortezomib as nM; D – doxorubicin as μM)

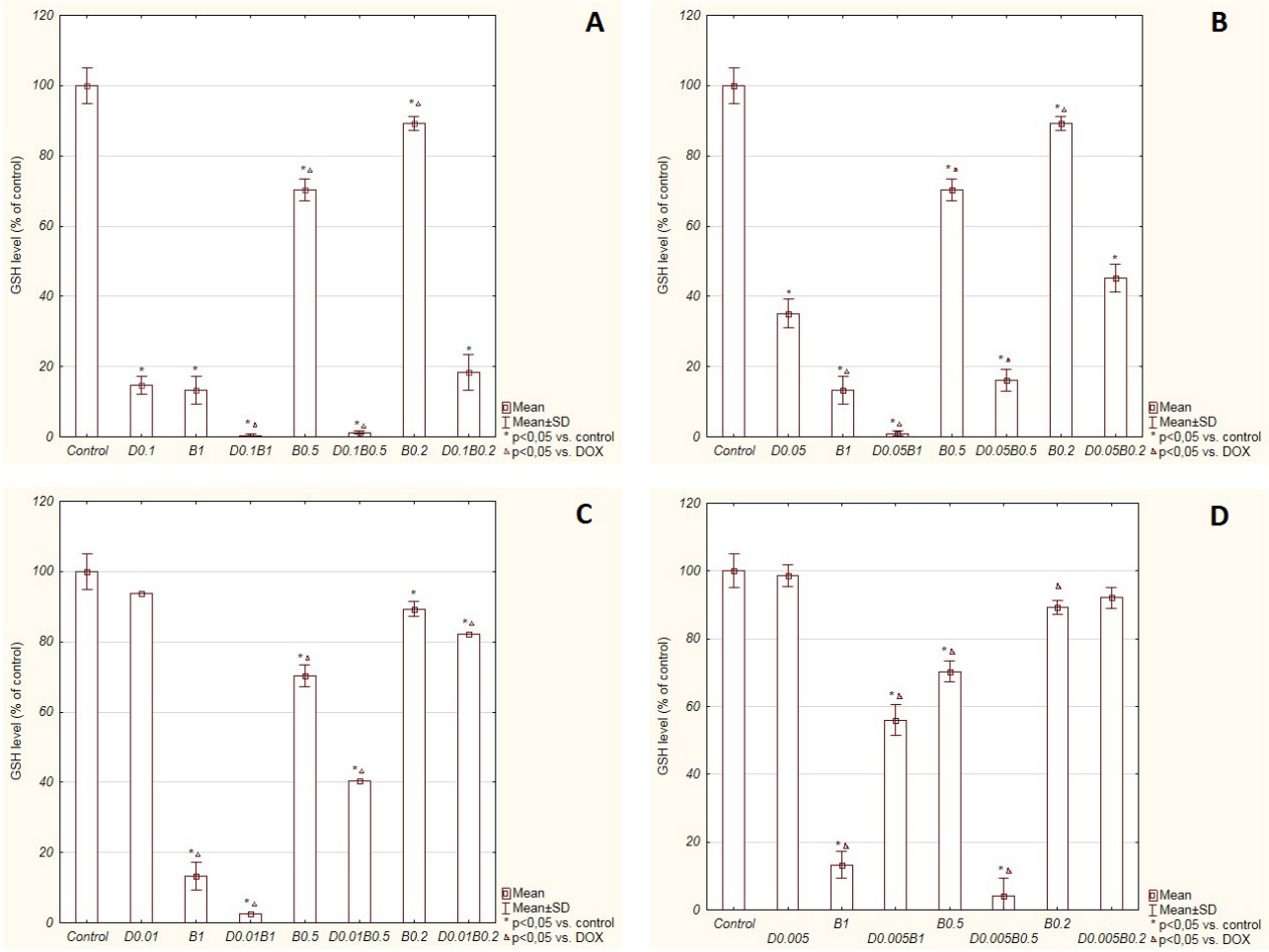


Figure 3. GSH level (expressed as % of control culture results) (B – bortezomib as nM, D – doxorubicin as μM)

line was also tested by Irimia *et al*. [15]. In the study, the Jurkat ALL cells were treated by DOX for 18 h. Here, the IC_{50} value was 0,57 μM. The difference between our results is significant. Unfortunately, Irimia *et al*. do not describe the method in which this value was specified. In our work, so as to observe the synergistic effect of DOX and BZT, lower concentrations of the compounds were used. This was due to the high cytotoxicity of greater concentrations of BZT and DOX. Accordingly, such big differences between the IC_{50} values are difficult to explain. It is possible that this effect is associated with the variety of the cell lines used in the study. However, in another study carried out by Horton *et al*. [14], the IC_{50} for the acute lymphoblastic leukemia Jurkat cell line is about 0,08 μM. This was determined on the base of MTT assay. This result is similar to that obtained in our tests.

It should be noted that the MTT assay was performed after 72h of incubation with the compound. Still, Horton *et al*. also determined DOX IC_{50} for other leukemic cell lines (T cell ALL HSB-2 and two pre-B ALL cell lines, Reh and RS4). In these lines, IC_{50} is lower compared to that of the Jurkat cell line and it is very similar or even equal with the 0,01μM obtained in our research.

As seen in the MTT assay, IC_{50} for bortezomib in our study in ALL cells is about 0,5 nM. Horton *et al*. used a several lines of ALL and other leukemia lines in their study in which they incubated cells with BZT for 72h and then carried out an MTT assay. The values of IC_{50} for the Jurkat cell line were 6,3 nM, for Molt3 – 2,3 nM, for Molt4 – 4 nM. There is a big difference in IC_{50} values between cell lines. Moreover, they are higher than that obtained in our study [14].

Horton *et al*. also investigated the interactions between BZT and other chemotherapeutic agents. They noticed a synergistic effect between BZT and DOX in ALL cells. We also examined the occurrence of the synergistic effect in combinations of BZT and DOX in ALL cells. Surprisingly, synergism is notable in the combination of 1 nM BZT with each (and even the lowest) concentrations of DOX. We observed this effect in morphological assessment, in MTT assay and in GSH level assessment in ALL cells [14].

On the basis of the results mentioned above, we can suspect that the synergistic action of BZT and DOX is related to oxidative stress and accumulation of damaged proteins. In the assay in which the level of GSH was measured, we investigated the severity of oxidative stress. In the samples where combinations of 1 nM BZT with DOX were used, we saw intensification of oxidative stress as against the individually applied drugs. Moreover, looking further on in this test, we noticed a similar effect in combinations of lower concentrations of BZT and DOX. For example, increased oxidative stress occurs in D0,1B0,5; D0,05B0,5 and D0,01B0,5. For this reason, we hold that the cytotoxic activity of this combination results from oxidative stress.

The high level of oxidative stress may be due to endoplasmic reticulum (ER) stress. ER stress in our experiment is the

result of the accumulation of unfolded proteins engendered by BZT proteasome inhibition. ER stress leads inter alia to overproduction of ROS. In ER stress-dependent ROS production, several enzymatic components are involved. Some of these enzymes catalyse protein folding. ROS are by-products of this reaction. The ER stress conditions bring about increased activity of these enzymes because of the large number of unfolded proteins. This results in increased production of oxygen free radicals. Additionally, the system protein folding is highly dependent on ATP. Maintaining this system requires a large amount of ATP, which, in turn, causes the increased mitochondrial respiration in which ROS are generated [4].

Searching for the synergistic action of drugs on cancers should be continued. Reduction of the concentrations of used drugs gives the opportunity to avoid or reduce harmful side effects and prevent increasing drug resistance.

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