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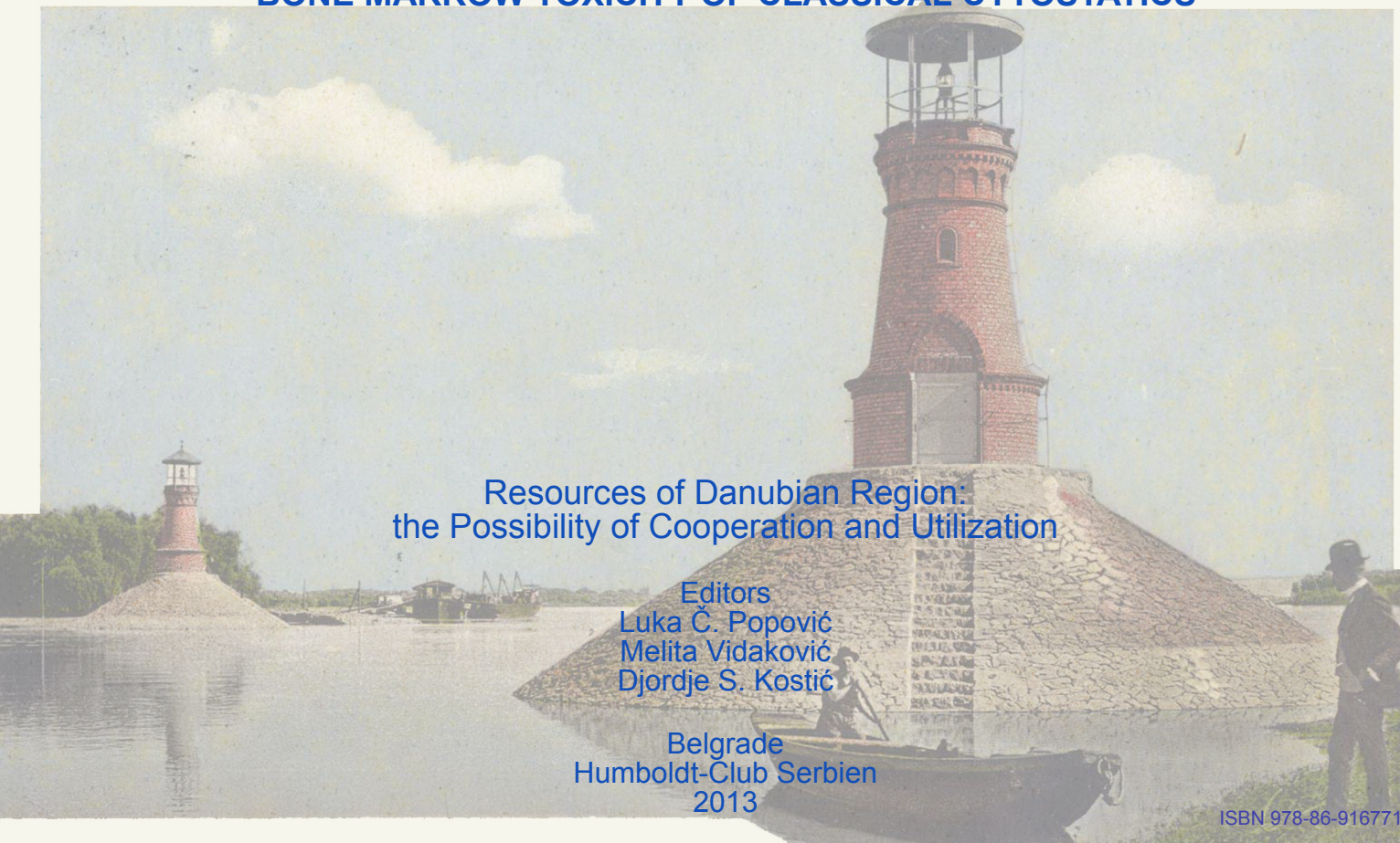
THE NEW ALKYLPHOSPHOCHOLINEERUFOSINE AMELIORATES BONE MARROW TOXICITY OF CLASSICAL CYTOSTATICS



Resources of Danubian Region:
the Possibility of Cooperation and Utilization

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Abstract. Numerous conventional cytostatics run the risk of damaging fast-proliferating tissues. Erufosinepossessesselective activity against tumor cells withoutaffecting the normal haematopoiesis. In the current study we investigated the combination oferufosinewith clinically used cytoreductive agents on leukemic cells and long term bone marrow cell culturesby using theMTT- and CFU (colony forming units)-assays.Erufosine ameliorated the bone marrow toxicity of all cytostatics usedand stimulated the growth ofGM-CFUs. Combinations used showed synergistic drug interactions against malignant cells. In conclusion, erufosine could be used in combinations with classical cytostatics because it lowers their bonemarrow toxicity.
Key words:bone marrow toxicity, erufosine, classical cytostatics, granulocyte/macrophage colonies, synergistic interactions

Introduction

Modern anticancer chemotherapy aims to achieve a high therapeutic effect by minimal risk of damaging normal tissues (Leape et al. 1991; Kohn 2000). A high number of conventional antitumor agents run the risk of damaging fast-proliferating tissues such as the haematopoietic bone marrow, the gastrointestinal tract and other normal tissues via a direct effect on cellular DNA. It is still a great challenge to find new and effective compounds that do not exert such side effects. The development of new combination regimens for cancer chemotherapy is also important because it may prevent or slower the subsequent development of cellular drug resistance and provides a broader range of interactions between drugs and tumor cells, which have various genetic abnormalities within the heterogeneous tumor population. One of the guiding principles for the selection of drugs in combinations is to select the drugs on the basis of toxicity that does not overlap with the toxicity of second drug in the combination because this minimizes the risk of lethal side effect caused by multiple insults to the same organ system by different drugs. This approach allows dose intensity to be maximized.

Erufosine (Erucylphospho-N,N,N-trimethylpropylammonium, EPC3) is a new and promising antineoplastic agent that belongs to the group of alkylphosphocholine (APCs) and exhibits selective activity against tumor cells without affecting the normal bone marrow and is undergoing now phase II clinical trials in leukemia patients *(Yosifov et al. 2010)*. APCs are ether lipid analogues that can be classified in three generations according to their chemical structural and pharmacological properties *(Konstantinov*

et al. 1998; Konstantinov et al. 1998; Konstantinov and Berger 1999). Their mode of action differs completely from that of conventional cytostatics because they do not target DNA but interact primarily with cell membranes, thus promoting clustering of surface lipid rafts, inducing apoptosis and inhibiting cell survival pathways such as the PI3/Akt/mTor Axis and Ras/Raf/MAPK *(Fu et al. 2009; Yosifov et al. 2009; Dineva et al. 2012; Kapoor et al. 2012)*. The first and in most detail investigated alkylphosphocholine is hexadecylphosphocholine (HePC, Miltefosine® Asta Medica). This compound was synthesized from Hirt and Berchold in 1958 and later found to exhibit high antitumor activity.

The pharmacological activity of locally administered Miltefosine was proved in patients with malignant neoplasm of the skin *(Berger et al. 1993; Konstantinov et al. 1998; Konstantinov et al. 1999)*. As a consequence of these trials Miltefosine was approved in 1992 inGermanyfor the treatment of skin metastases in patients with breast cancer. Miltefosine is approved also for the oral treatment of leishmaniasis, a parasitological tropical infectious disease but its oral use in cancer patients was limited by gastrointestinal side effects *(Danhauser-Riedl et al. 1991; Verweij et al. 1992; Sundar et al. 2002)*. Its structural analogue perifosine (Oktadecyl-(1,1-dimethyl-piperidin-4-il)-phosphat, ODPP) is undergoing now phase III clinical trials, for patients with multiple myeloma. Another alkylphosphocholine, edelfosine, is used only for purging bone marrow *ex vivo* prior to bone marrow transplantation because of moderate *in vivo* results *(Koenigsmann et al. 1996)*. Perifosine was selected for clinical development because of its high *in vivo* activity against myeloma cells and superior tolerability by oral administration in clinical tests as compared to miltefosine (Unger et al. 2010).

The unsaturated alkylphosphocholine **erufosine** belongs to the third generation APCs and possesses high cytotoxic activity against leukemic and some solid tumor cell lines. Different from all other APCs, erufosine can be administrated intravenously because it doesn't induce haemolysis and its cholinomimetic properties are less pronounced when compared to miltefosine and perifosine *(Berger et al. 1992; Eibl and Kaufmann-Kolle 1995; Berger et al. 1998; Jendrossek et al. 2002; Fiegl et al. 2007)*. In addition, the intravenous administration of erufosine enhances the drug's efficacy 5 fold as compared to oral administration *(Berger et al. 1992)*.

This is a possibility not only to avoid the dose limiting site effects on the gastro-intestinal system but also to reduce the effective dose. This specific feature and its low toxicity as well as the possibility to stimulate the normal hematopoiesis makes it very attractive either for targeted monotherapy or as a drug partne rwith classical cytostatics in new drug combination regimens *(Georgieva et al. 2002; Konstantinov et al. 2002; Oberle et al. 2005; Martinova et al. 2006; Nyakern et al. 2006; Fiegl et al. 2008; Yosifov et al. 2010; Pitter et al. 2011)*.

In the current study we aimed to investigate whether the combination of erufosine with clinically used cytoreductive agents may reduce bone marrow toxicity without affecting the cytotoxicity of the drugs against malignant cells. For that aim,two classical cytostaticswith high myelotoxicity – cytosine arabinoside (ara C) and epirubicine were chosen for the combination schedule.

The bone marrow toxicity of the combinations was compared with their antitumor activity against the malignant chronic myeloid leukemia cell line K-562in order to test the selective protective effect of erufosine.

Material and methods

Drugs and compounds. The compound erufosine (erucylphospho-N,N,N-trimethylpropanolamine, ErPC3,) was synthesized as previously described (Eibl and Engel, 1992) and was kindly provided at a clinical grade purity from Prof. Eibl, Max Plank-Institut, Goettingen, Germany. Erufosine was dissolved in ethanol/phosphate-buffered saline (PBS) (1:1, v/v) and stored at 4°C. Cytosine arabinoside (Ara C) and epirubicine were supplied at a quality for clinical applications from the respective manufacturer.

Cell line and culture conditions. The leukemic cell line used was the BCR-ABL-expressing line - K-562 (DSMZ No. ACC 10, cell type: human chronic myeloid leukaemia in blast crisis). It was maintained in RPMI-1640 medium (Invitrogen, USA) containing 10 % fetal calf serum (Biochrom, Germany) and 2 mM L-Glutamine (Invitrogen, USA). Cells were grown as suspension cultures (at 37°C in an incubator with humidified atmosphere and 5 % CO_2) and passaged two or three times a week to keep them in log phase.

Long Term Bone Marrow Cell Cultures (LTBMCCs) and Colony Forming Units (CFU) assay. LTBMCCs were derived from murine femurs as previously described (Konstantinov et al. 1998). Briefly, female and male C57BL6 (DKFZ, Heidelberg, Germany) or ICR (National Breeding Center, Slivnica, Bulgaria) mice were used as donors. After euthanising the animal by cervical dislocation, femurs were removed and bone marrow was flushed out aseptically into LTBMCCs medium [10% FCS (Biochrome, Germany), 10% horse serum (Invitrogene, USA), 10^{-6} M hydrocortisone (Sigma), 0.017 mg/ml transferrin, 0.007 µL/ml β-mercaptoethanol, 100 IU/ml penicillin (Invitrogen, USA), 100 µg/ml streptomycin (Invitrogen, USA) and 2.5 µg/ml amphotericin B (Applichem, Germany) in MEM-α (Invitrogen, USA)] using 21-gauge needle fitted to a syringe. The marrow plug was gentle for several times until a single cell suspension was prepared. The primary cells were plated into 25 cm^2 culture flasks (Greiner bio-one, Germany) and cultures were incubated at 37°C in 5% CO_2 in humidified air for two weeks. After an adherent layer has been established LTBMCCs were re-fed by adding fresh bone marrow. Half of the medium was removed weekly from each flask and an aliquot of fresh medium was added. After two weeks further culturing and building of clusters of progenitor hematopoietic cells cultures were treated with erufosine (30 µM), ara C (20 µM) and epirubicine (1,25 µM), and respective combinations as indicated below. Cultures treated with erufosine and cytosine arabinoside were captured for morphological evaluation (x400) as live cultures with an inverted microscope. Afterwards, cells were collected by trypsinization and then 100,000 cells were plated in semisolid medium (0.8% RPMI-methylcellulose, 30% horse serum and 0.1 ng/ml recombinant mouse granulocyte/macrophage-colony stimulating factor) in 24-well plates (three wells/treatment group) and cultivated for 10 days. Colonies (clusters of 20 or more cells) were counted using an inverted microscope (TMS, Nikon, Japan).

MTT-dye Reduction Assay. Cells were seeded in 96-well plates, at cell density 10^3cells/well and incubated for up to 48 h or 96 h with two different concentrations of erufosine and the respective cytostatic in the combination scheme as indicated below. The cell survival fraction was estimated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay as described by Mosmann (Mosmann, 1983) with some modifications. Briefly after incubation with the appropriate drug or combination, MTT-solution (10 mg/ml in PBS) was added (10 µl/well). Plates were further incubated

for 4 h at 37°C and the formazan crystals formed were dissolved by adding 110 µl/well of 0,04 N HCl in 2-propanol.Absorbtion was measured by an ELISA reader (Labexim, Austria) at 540 nm, reference filter 690 nm. For each concentration at least 8 wells were used. One-hundred microlitres RPMI-1640 medium with 10 µl MTT stock-solution and 110 µl 0,04 N HCl in 2-propanol was used as blank solution. Data obtained were presented as percentage of untreated control (untreated control = 100) as shown below.

$$\% \text{ of control} = \frac{E_{\text{(extinction of viable cells in treated samples)}}}{E_{0\text{ (extinction of viable cells in untreated control)}}} \times 100$$

Evaluation of the combination effects. Expected theoretical values were calculated according to the equation:$c = a \times b/100$, where a and b are cell survival values after single agent treatment, counted as part of untreated control. For each concentration expected values were calculated and compared with the experimentally obtained values of the respective combination. If $c_{measured} = c_{calculated}$ the combination effect is additive. If $c_{measured}/c_{calculated} \times 100$is < 70 % the combination effect it synergistic and if $c_{measured}/c_{calculated} \times 100 > 70$ % the combination effect is antagonistic. Cell survival values significantly lower (< 70%) than the expected values indicated the presence of synergistic potentiating (Mosmann 1983).

Statistical analysis. Experiments were performed minimum in triplicate and the results are expressed as means with corresponding standard deviations. The significance of differences in the data was analyzed using Student's *t*-test ($^{*}P<0.05$).

Results

CFU-GM following treatment of murine LTBMCCs with erufosine, epirubicine, ara C or appropriate consecutive combinations

Evident from the experimental data expressed in Fig. 1A, both ara C and epirubucine resulted in significant decline in the bone marrow cells clonogenicity as compared to the control group. No significant reduction in progenitor cell number was found after exposure to erufosine for 24 hours. Erufosine itself even stimulated the growth of GM-CFUs up to 28% of untreated control. The morphological evaluation of LTBMCCs that ara C and epirubicine evoke pronounced changes as compared to the control group or erufosine after alone application (Fig. 1B). The consecutive combination of ara C or epirubicine with erufosine protected the bone marrow cells from the myelotoxic effect of the classical cytostatics and induced significantly (from 20 to 30%) the colony growth of the treated groups. This effect was significantly more potent when erufosine was applied before epirubicine and after ara C (Fig. 4). The percent of the colonies after combination treatment with erufosine as a first compound and epirubicine as a second compound increases the portion of CFU-GM up to 42%, whereas the treatment with epirubicine at the concentration of 1.25 µM alone reduced the colony formation up to 14%.Similarly, the application of erufosine before ara C increased the number of colonies with 25%as compared to ara C treated cells.

The application of erufosine after ara C had more pronounced protective effect upon the progenitor cell population because it resulted in 83% colony formation. As evident from the photomicrographs in Figure 1B the combinations with erufosine induced no distinguishable morphological changes as compared to the control group or treatment with erufosine alone.

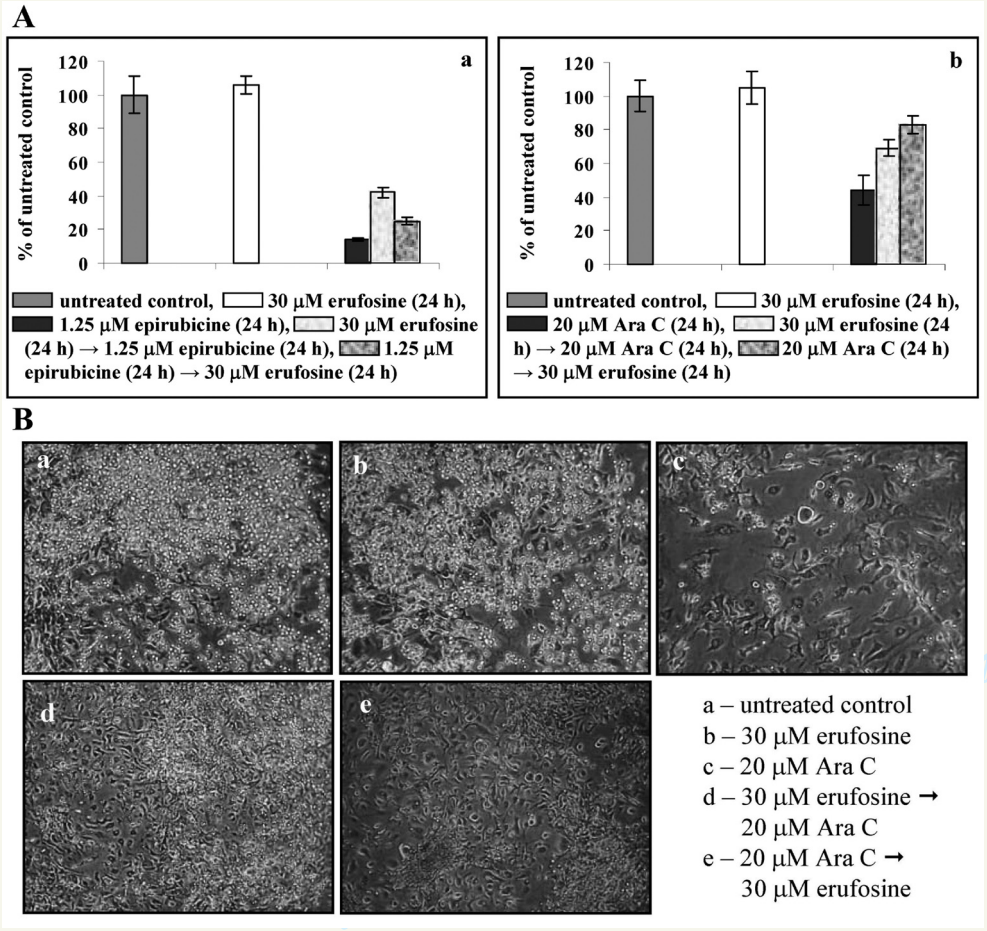

Fig. 1 (A) Clonogenicity (CFU-GM) of normal haematopoietic cells after treatment with erufosine, ara C and epirubicine alone or with the appropriate consecutive combinations between the drugs used. Data are presented as a percent of untreated control. Bars denote standard deviation. (a) Colony formation after treatment of LTBMCCs with erufosine and epirubicine. (b) Colony formation after treatment of LTBMCCs with erufosine and ara C. **(B)** Morphological alterations in long term bone marrow cell cultures (LTBMCCs) after treatment with Ara C and protection of the damaged cells through application of erufosinebefore or after Ara C.

Leukemia cell survival after in vitro combination treatment with erufosine and ara C or epirubicine.

The treatment of the leukemic cell line K-562 with 15 and 30 μM erufosine for 48 and 96 hours didn't lead to a pronounced cytostatic effect. The 15 μM concentration of erufosine even caused stimulation of the cell growth. The antimetabolite ara C significantly reduced by half the viable fraction of K-562 cells after 96 hours incubation with concentrations of 20 and 40 μM, while epirubicine led to the same growth inhibition even at the 48th hour after the treatment. Theanthracycline epirubicine eliminated about 50% of the leukemic cells after incubation with 1.25 μM for 48 hours. The extension of the treatment period up to 96 hours decreased the cell viability with another 13%. The increase of the concentration of epirubicine up to 2.5 μM did not alter the cell survival (Tabl. 1).

Table 1. Cytotoxicity of Erufosine, Epirubicine and Ara C on K-562 cells after 48 h and 96 h single drug treatment.

Cell line	Concentration (μM) of the drugs (name)	Duration of the experiment (h)	T/C %[a]
K-562	15 (ErPC3)	48	101 ± 3,6
	30 (ErPC3)	48	95 ± 2,8
	15 (ErPC3)	96	110 ± 4,3
	30 (ErPC3)	96	103 ± 4,7
	20 (Ara C)	48	89 ± 2,6
	40 (Ara C)	48	91 ± 4,2
	20 (Ara C)	96	49 ± 1,9
	40 (Ara C)	96	49 ± 4,4
	1,25 (Epirubicine)	48	50 ± 2,6
	2,5 (Epirubicine)	48	49 ± 1,8
	1,25 (Epirubicine)	96	37 ± 4,9
	2,5 (Epirubicine)	96	49 ± 8,5

[a]Extinction of viable cells, calculated as percent of the untreated control.

The synergistic combination effects of erufosine and ara C or epirubicine after consecutive treatment of leukemic K-562 cells are summarized in Fig. 1 and 2. First application of ara C on K-562 cells at concentrations 20 and 40 μM followed by treatment with 15 or 30 μM erufosine resulted in clear synergism. The same sequence of the combination of erufosine (15 and 30 μM) with epirubicine at concentrations of 1.25 μM or 2.5 μM led to an additive effect. Noteworthy, there wasn't seen clear increase of the cytotoxic efficacy at the higher drug concentration used.

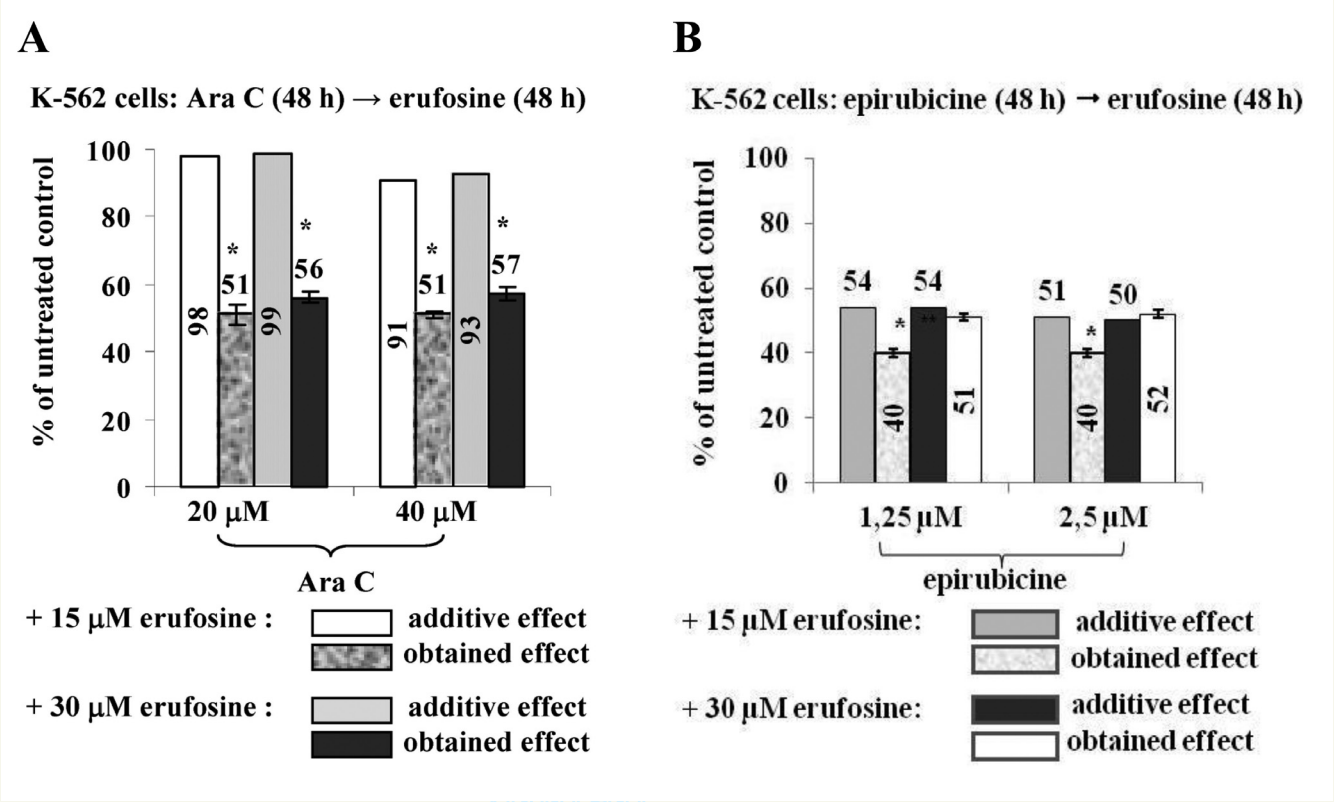

Fig. 2 (A) Vitality of K-562 cells after consecutive treatment with ara C and erufosine (bars with standard deviations) – synergistic effect. **(B)** Viability of K-562 cells after consecutive treatment with epirubicine and erufosine (bars with standard deviations) – additive effect. All values are calculated as a percent of the untreated control and compared to the additive effects (bars without standard deviations). The results are representative of three independent experiments. Significant differences from the respective untreated control were marked with asterisk (Student's t-test; $p < 0.05$).

Discussion

It is known from the literature that alkylphosphocholines have low myelotoxicity (Stekar et al. 1995; Konstantinov et al. 1998; Konstantinov et al. 2002), specifically they spare normal B and Tcells, as well as vascular endothelial cells (Gajate and Mollinedo 2007) and evenstimulate normal haematopoiesis (Konstantinov et al. 1998; Catley et al. 2007).Erufosine itself was found in several studies to be non-toxic for haematopoietic cells (Yosifov et al. 2010) and it has also a mechanism of action that completely differs from that of the classical cytostatics(Zaharieva et al. 2007; Yosifov et al. 2009; Rudner et al. 2010; Dineva et al. 2012; Kapoor et al. 2012). Published in vitro investigations showed synergistic drug interactions of the combinations between erufosine and gleevec, or antisense oligonucleotides directed to the synthesis inhibition of the fusion oncoprotein BCR-ABL. The combination suppressed the proliferation of malignant cells and stimulated the growth of normal haematopoietic cells *(Konstantinov et al. 2002)* which points also to another possible application of erufosine – *ex vivo* purging of bone marrow prior bone marrow transplantations in combination with other cytostatics. In this study, we combined for first time on normal bone marrow cell cultures erufosine with classical cytoreductve drugs such as cytosine arabinoside and epirubicine known for their toxicity on normal haematopoietic cells. In parallel, we tested the cytotoxic activity of the same combinations on the resistant chronic myeloid leukemia derived cell line K-562 in order to find out whether the protective effect of erufosine is specific for normal bone marrow cells. In line with our previous results (Yosifov et al. 2010),we showed that the combinations led to significant protection of the granulocyte/macrophage progenitorsas proved by the CFU-GM colony growth on semisolid media (Fig. 1A). The morphological changesin long term bone marrow cell cultures evoked by exposure to erufosine (Fig. 1B-b) did not differ from the untreated control which is another indication for the lack of myelotoxicity. Evident from the experimental data shown in Fig. 1B-c, ara C treatment resulted in asignificant decline in the bone marrow cells clonogenicity which is in line with the well known myelotoxic potential of the antimetabolite. Erufosine ameliorated the bone marrow toxicity of both, cytosine arabinoside and epirubicine, and this effect was more pronounced in the combination with cytosine arabinoside. Our data constitute also for the first time that erufosine does not reduce the cytotoxic activity of cytosine arabinoside and epirubicine against CML derived leukemia cells. Erufosine even improved the cytotoxic efficacy of cytosine arabinoside as evident from the synergistic drug in teractions against resistant malignant myeloid K-562 cells, which essentially express the oncogene *bcr-abl* (Fig 2A).This opens a new perspective for using erufosine not only as an antineoplastic drug, but also as a protective compound in future therapeutic combination schedules.

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Conslusion

In conclusion, there are preliminary evidences that erufosine could be rationally used in combinations with classical antileukemic cytostatic drugs (constituents of the 7+3 schedule) because it lowers their bone marrow toxicity. Further experiments are needed in order to evaluate this effect in a broader range of leukemic cell lines and other disease models, incl. animal models.

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