

## Synergistic Anti-leukemic Effects of CK2 Inhibitors and Pentabromobenzylisothiourreas *In Vitro*

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**Abstract.** Background: Casein kinase-2 (CK2) inhibitors and pentabromobenzylisothiourreas are promising anti-leukemic agents for treatment, both alone and in combination. In this study, we examined pro-apoptotic and cytostatic effects of three CK2 inhibitors: one known, 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) and two new: 2-(4-methylpiperazin-1-yl)-4,5,6,7-tetrabromo-1H-benzimidazole (MPT) and 2-aminoethyleamino-4,5,6,7-tetrabromo-1H-benzimidazole (AEAT), as well as of certain S-2,3,4,5,6-pentabromobenzylisothiuronium bromides: ZKK-3, ZKK-9, ZKK-13, against the human acute myelogenous leukemia cell line (KG-1). Cells were treated with CK2 inhibitors alone and in combination with the pentabromobenzylisothiourreas. Materials and Methods: Evaluation of synergistic and pro-apoptotic effects, mitochondrial membrane potential ( $\Delta\Psi_m$ ) assay, poly(ADP-ribose) polymerase (PARP) cleavage assay, and cell-cycle progression of KG-1 cells were carried out using the flow cytometric technique and fluorescent microscopic analysis. Western blots were used for analysis of B-cell lymphoma-2 (BCL-2) family proteins in whole-cell extracts. Results: The tested CK2 inhibitors DMAT, MPT, AEAT exhibited synergistic proapoptotic effect in combination with ZKK-3, ZKK-9 and ZKK-13. The agents revealed different pro-apoptotic efficacies against leukemia cell line KG-1. The highest apoptotic activity of the tested compounds was exhibited by AEAT. Conclusion: Combination of CK2 inhibitors and pentabromobenzylisothiourreas-induced synergistic anti-leukemic effects against KG-1 acute myelogenous leukemia cells *in vitro*.

Owing to the pioneering work of Fisher and Krebs (1), and the research that followed it is now widely-recognized that

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reversible phosphorylation of proteins is central to the regulation of many aspects of cell physiology and function. Inhibition of protein kinases by small molecules is a powerful way to control signal transduction cascades and a fruitful approach to drug discovery. Protein kinase inhibitors are good candidate drugs for the treatment of many signal transduction disorders. The search for selective kinase inhibitors with potential to treat cancer, inflammation and apoptosis-related disorders has greatly intensified. A landmark event in this quest became the approval in 2001 of Gleevec (imatinib mesylate), the first drug rationally-developed as a specific Abelson murine leukemia viral oncogene homolog (ABL) tyrosine protein kinase inhibitor, for the treatment of chronic myeloid leukemia (2). Since then, a great number of protein kinase inhibitors have been introduced as new drugs into clinical practice, e.g. sunitinib, gefitinib or sorafenib (3-5).

Abnormally high levels of casein kinase-2 (CK2) activity have been documented in a number of human malignancies including, but not limited to, kidney (6), mammary (7), prostate (8), head and neck (9) and colorectal (10) cancer. Experimental data support the notion that CK2 promotes cell survival through oncogene regulation and plays a global anti-apoptotic role (11). Hence, CK2 inhibitors have been suggested as promising drugs for anticancer therapy, and CX-4945, a rationally-designed CK2 inhibitor, is currently under investigation in a phase I clinical trial in patients with advanced solid tumors or Castleman's disease and in multiple myeloma (12).

Combining drugs that work through different mechanisms allows for maximization of synergistic effects and increased clinical efficacy while reducing drug dosages, treatment times and unwanted side-effects. Hence, multidrug therapy is often used in medicinal praxis. Several reports have been published on promising *in vitro* synergistic effects of protein kinase inhibitors and other biologically-active substances against selected malignancies, e.g. lung cancer (13, 16) pancreatic cancer (2, 14) and acute lymphoblastic leukemia (15, 17), including CK2 inhibitor – 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) and Gleevec combination (18).

Another interesting class of compounds with a potential for cancer treatment are isothioureas. They are amphiphilic compounds carrying a highly basic isothiourea group of  $pK_a \approx 10$ ; hence, at physiological pH they exist in the protonated (cationic) form, which is of key importance for molecular recognition of specific receptors. Our interest in this compound class is driven mostly by the multi-faceted biological properties of *N*-substituted *S*-benzylisothioureas. These derivatives, similarly to other isothioureas and aliphatic guanidines, are inhibitors of nitric oxide synthases (NOSs) (19, 20). Because of the essential role of NOSs in a plethora of physiological and pathological phenomena, there is a continuing search for more specific and stronger inhibitors of these enzymes (21-23) and some isothioureas with considerable NOS inhibitory activity showed promise as *in vitro* chemopreventive agents in rat tracheal epithelial cells treated with the carcinogen benzo[a]pyrene (24).

Modification of a molecule of a biologically-active compound by introducing a substituent may occasionally result in an unexpected result. Interestingly, despite being polybrominated compounds like a number of strong CK2 inhibitors 4,5,6,7-tetrabromo-1*H*-benzotriazole (TBB or TBBt), 4,5,6,7-tetrabromo-1*H*-benzimidazole (TBI or TBBz), 2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole (DMAT), (E)-3-(2,3,4,5-tetrabromophenyl)acrylic acid (TBCA), *S*-pentabromobenzylisothioureas have distinct protein kinase inhibition profile (25). For instance, *N,N'*-dimethyl-*S*-2,3,4,5,6-pentabromobenzylisothiourea (ZKK-3) has moderate inhibitory activity against seven protein kinases that play important roles in the metabolism of normal and cancer cells [Extracellular signal-regulated kinase 8 (ERK8), protein kinase D1 (PKD1), NIMA-related kinases 2A (NEK2A), proviral integration site for Moloney murine leukemia virus (PIM1, PIM3) kinases, the type 1 insulin-like growth factor receptor, a receptor tyrosine kinase (IGF-1R), and insulin receptor (IR)], but not against CK2. Results of our earlier study on the activity of *S*-pentabromobenzylisothioureas (ZKKs) and their combination with CK2 inhibitors against prostate and glioblastoma cancer cell lines *in vitro* (25, 26) encouraged us to extend testing to leukemia cells. In this study we compared anti-leukemic activity of CK2 inhibitors and three *N*-substituted ZKKs (Figure 1) most effective against human prostate adenocarcinoma cell line PC3 (25) alone and in combination.

## Materials and Methods

**Agents.** Three CK2 inhibitors: one known, 2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole (DMAT) and two new: 4,5,6,7-tetrabromo-2-(4-methylpiperazin-1-yl)-1*H*-benzimidazole (MPT) and 2-aminoethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole (AEAT) were obtained as previously described (27, 28). Three ZKKs: *N,N'*-dimethyl-*S*- (ZKK-3), *N,N*-dimethyl-*S*- (ZKK-9) and *N,N'*-diisopropyl-*S*- (ZKK-13) 2,3,4,5,6-pentabromobenzylisothiourenium

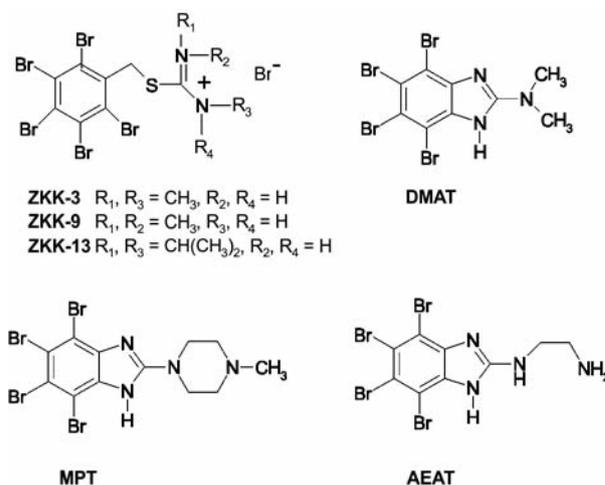


Figure 1. The chemical structures of the tested compounds. ZKK-3: *N,N'*-dimethyl-*S*-2,3,4,5,6-pentabromobenzylisothiourenium bromide; ZKK-9: *N,N*-dimethyl-*S*-(2,3,4,5,6-pentabromobenzyl)isothiourenium bromide; ZKK-13: *N,N'*-diisopropyl-*S*-(2,3,4,5,6-pentabromobenzyl) isothiourenium bromide; DMAT: 2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole; MPT: 4,5,6,7-tetrabromo-2-(4-methylpiperazin-1-yl)-1*H*-benzimidazole; AEAT: 2-aminoethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole.

bromides were synthesized according to published procedures (25, 26, 29). The compounds were dissolved in dimethylsulfoxide (DMSO) and then diluted in the medium for the experiment. The concentration of the studied drugs ranged from 5 to 20  $\mu\text{M}$ . In all experiments, control cells were incubated in DMSO alone. The final concentration of DMSO was maintained to 0.1%.

**Cell culture and agent treatment.** KG-1 (human acute myelogenous leukemia) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) (CytoGen, Sinn, Germany) supplemented with 20% (v/v) of heat-inactivated fetal bovine serum (CytoGen) and 1% (v/v) of antibiotic-antimycotic solution (CytoGen), at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$  in air. All experiments were performed on exponentially-growing cultures. The compounds studied were added to the cultures as solutions in DMSO (Sigma); control cultures were treated with the same volume of the solvent alone. After culturing the cells with the studied compounds for 24 or 48 h, the cells were collected and used for labeling.

**Apoptosis assay by annexin V/propidium iodide (PI) labeling.** Apoptosis was measured using the fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I (BD Biosciences, Pharmingen, San Diego, CA, USA). After 24 or 48 h incubation with the tested agents, cells were collected by centrifugation, rinsed twice with cold phosphate-buffered saline (PBS) and suspended in binding buffer at  $1 \times 10^6$  cells/ml. One-hundred microliter aliquots of the cell suspensions were labeled according to manufacturer's instructions. Briefly, annexin V-FITC and PI were added to the cell suspension and the mixture was vortexed and incubated for 15 min at room temperature in the dark. Then 400  $\mu\text{l}$  of cold binding buffer was added and the cells were vortexed again and kept on ice. Flow cytometry measurements were performed within 1 h after labeling.

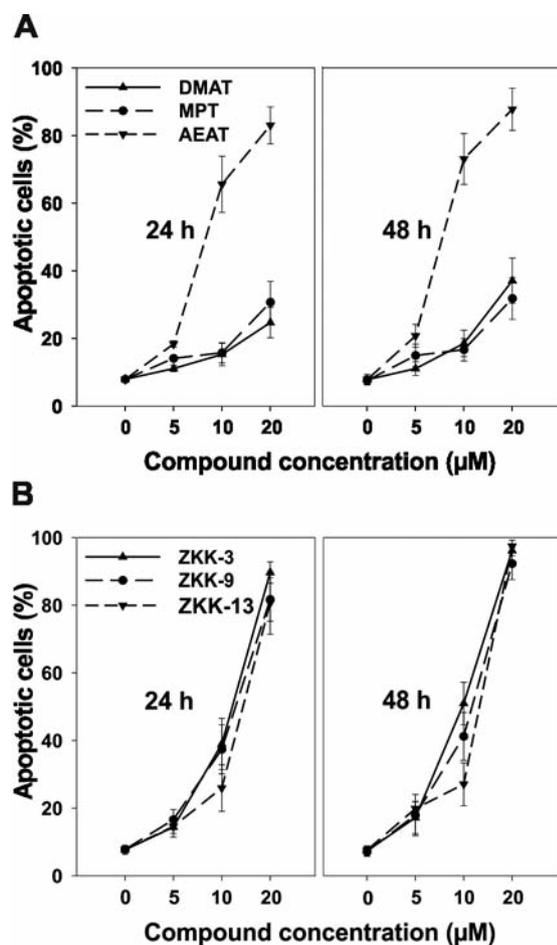


Figure 2. Induction of apoptosis by DMAT, MPT, AEAT, ZKK3, ZKK9 and ZKK13 in KG-1 cells. The data were determined by FACS cytometer after 24 and 48 h treatment. Cells were stained with annexin V-FITC and PI. Each point represents the mean  $\pm$  S.D. ( $n \geq 4$ ).

**Mitochondrial membrane potential ( $\Delta\Psi_m$ ) assay.** Mitochondrial membrane potential was assessed by flow cytometry using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1; Sigma). JC-1 undergoes potential-dependent accumulation in mitochondria. In healthy cells, the dye accumulates in mitochondria, forming aggregates with red fluorescence (FL-2 channel), whereas in dead and apoptotic cells the dye remains in the cytoplasm in a monomeric form and emits green fluorescence (FL-1 channel). Cells were harvested by centrifugation 48 h post-treatment, suspended in 1 ml of complete culture medium at approximately  $1 \times 10^6$  cells/ml and incubated with 2.5  $\mu$ l of JC-1 solution in DMSO (1 mg/ml) for 15 min at 37°C in the dark. The stained cells were then washed with cold PBS, suspended in 400  $\mu$ l of PBS and then examined with a FACSCalibur flow cytometer equipped with the CellQuest software (BD Biosciences, San Jose, CA, USA).

**PARP cleavage assay.** Caspase-3 and caspase-7 cleave PARP. PARP cleavage was detected by flow cytometry using Anti-PARP CSSA FITC Apoptosis Detection Kit (Invitrogen, MD, USA)

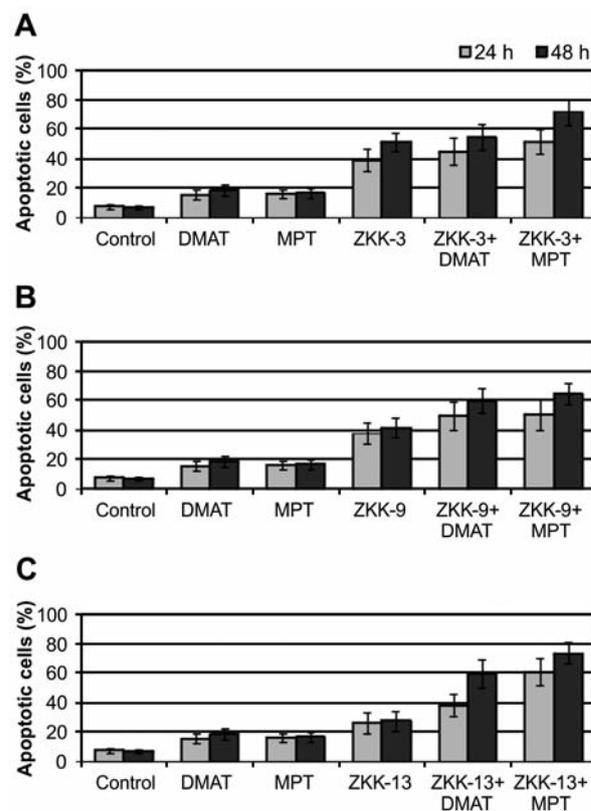


Figure 3. Pro-apoptotic and anti-leukemic activity of two selected CK2 inhibitors (DMAT, MPT) and N-substituted pentabromobenzylisothioureas alone and in combination on KG-1 cells (Panel A, B, C). The concentration of the studied compounds was 10  $\mu$ M. The data were determined by FACS cytometer after 24 and 48 h treatment. Cells were stained with annexin V-FITC and PI. Each bar represents the mean  $\pm$  S.D. ( $n \geq 4$ ).

according to the manufacturer's protocol. The FITC-conjugated anti-PARP antibody employed in the kit specifically recognizes the 85-kDa fragment of cleaved PARP. The cells for the assay were harvested after 48 h culturing with the tested compounds and were washed twice with PBS immediately prior to use. The level of cleaved PARP protein was expressed as fluorescence intensity assessed using CellQuest and the free WinMDI software package written by Joe Trotter of the Scripps Institute (La Jolla, CA, USA).

**Morphological evaluation.** After exposure to drugs, the cells were collected, washed with cold PBS and fixed with 70% ethanol at  $-20^\circ\text{C}$  for at least 24 h. Ethanol was then washed out and the cells were stained with 1.0  $\mu\text{g/ml}$  4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) and 20  $\mu\text{g/ml}$  sulforhodamine 101. Cell morphology was evaluated using a BX60 fluorescence microscope equipped with a DP50 digital camera (Olympus, Japan).

**Western blot analyses.** The cells were washed with cold PBS buffer and then the whole cell extractions were prepared using M-PER reagent (Pierce, Rockford, IL, USA). Protein concentration in the samples was measured using BCA protein assay kit (Pierce). Samples containing 30  $\mu\text{g}$  of protein were denatured and

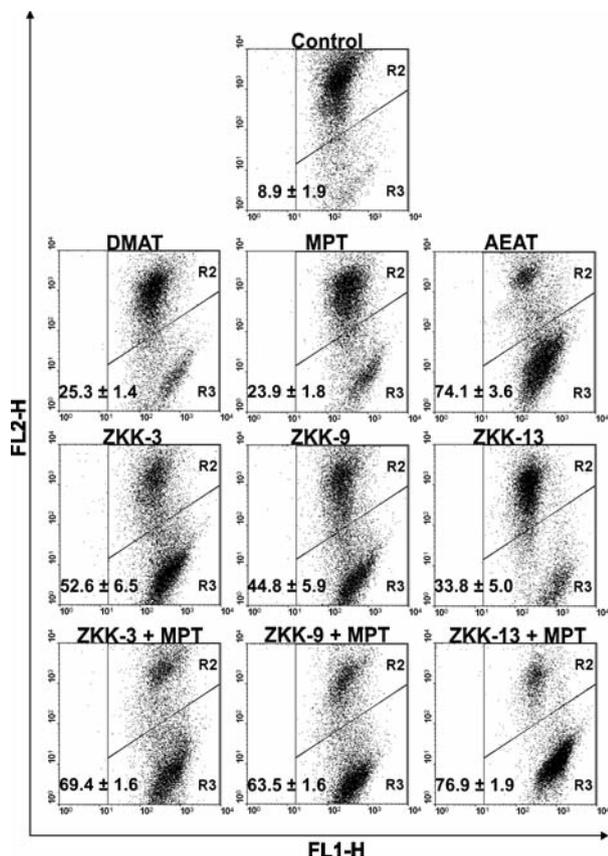


Figure 4. Representative flow cytograms demonstrating changes in mitochondrial membrane potential ( $\Delta\Psi_m$ ) of KG-1 cells induced by 48 h culture with compounds alone and in combination. The concentration of the studied compounds was 10  $\mu\text{M}$ . The cells were stained with JC-1 dye. The cells in the lower right (R3) quadrant exhibit increased red-to-green fluorescence ratio (apoptotic cells).

fractionated by 12 or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred to a nitrocellulose membrane and probed with primary anti-human antibodies specific to: BAD, BAX, BCL-2 and NIP1 (Apoptosis I Sampler Kit; BD Biosciences). Secondary antibodies conjugated with horseradish peroxidase (HRP) were used. Protein bands were visualized by using a CN/DAB substrate kit (Thermo Scientific, Meridian Rd, Rockford, IL, USA).

**Inhibition of protein kinases by ZKK-3.** Protein kinase inhibitory activity of 10  $\mu\text{M}$  solution of ZKK-3 was tested using a panel of 130 selected protein kinases at the Division of Signal Transduction Therapy, International Centre for Kinase Profiling, at the University of Dundee, Scotland.

**Cell-cycle analysis.** After exposure to the tested compounds, the cells were washed with cold PBS and fixed at  $-20^\circ\text{C}$  in 70% ethanol for at least 24 h. The cells were then washed in PBS and stained with 50  $\mu\text{g}/\text{ml}$  PI and 100  $\mu\text{g}/\text{ml}$  RNase solution in PBS supplemented with 0.1% v/v Triton X-100 by 30-min incubation in the dark at room temperature. Cell DNA content and the distribution

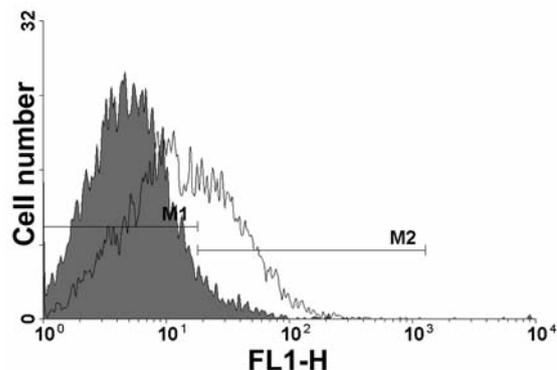


Figure 5. Proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) protein in KG-1 cells exposed for 48 h to ZKK-3. Representative histograms showing increased levels of 85 kDa fragment of PARP protein, indicating induction of apoptosis after ZKK-3 treatment. Histogram of KG-1 control cells (darkened) and overlay histogram of treated cells (empty) at 10  $\mu\text{M}$  ZKK-3. Marker M1 designates negative cell populations whereas M2 designates positive cell populations (i.e. apoptosis).

of the cells in different phases of the cell cycle were determined by flow cytometry employing MacCycle (Phoenix Flow Systems, San Diego, CA, USA) and CellQuest software packages.

**Flow cytometry.** Flow cytometric analyses were run on a FACSCalibur flow cytometer (BD Biosciences), and analyzed using the CellQuest and WinMDI 2.9 softwares. The DNA histograms obtained were analyzed using the MacCycle software.

## Results

**Induction of apoptosis of KG-1 leukemia cell line.** Induction of apoptosis by the tested compounds was determined using annexin V/PI labeling 24 and 48 h after the treatment. The examined CK2 inhibitors and pentabromobenzylisothioureas (Figure 1) used alone induced apoptotic death of this cell line. The observed apoptotic effects were dose- and time-dependent. Among the tested compounds, the most effective was AEAT, which at 10  $\mu\text{M}$  concentration and for 24 h incubation induced a significant apoptotic effect (Figure 2). All the CK2 inhibitors examined, when applied in combination with the ZKKs exhibited synergistic proapoptotic effects, the results for AEAT are not shown. The average percentage of apoptotic cells increased significantly after exposure to agents applied in combination, as compared with those applied alone. This synergistic effect was time-dependent. The most potent synergistic effect was induced by the combination of CK2 inhibitors with ZKK-13 after 48-h incubation (Figure 3).

**Changes in mitochondrial membrane potential ( $\Delta\Psi_m$ ).** Analysis of the respective flow cytograms (Figure 4) showed that the tested agents used alone and in combination

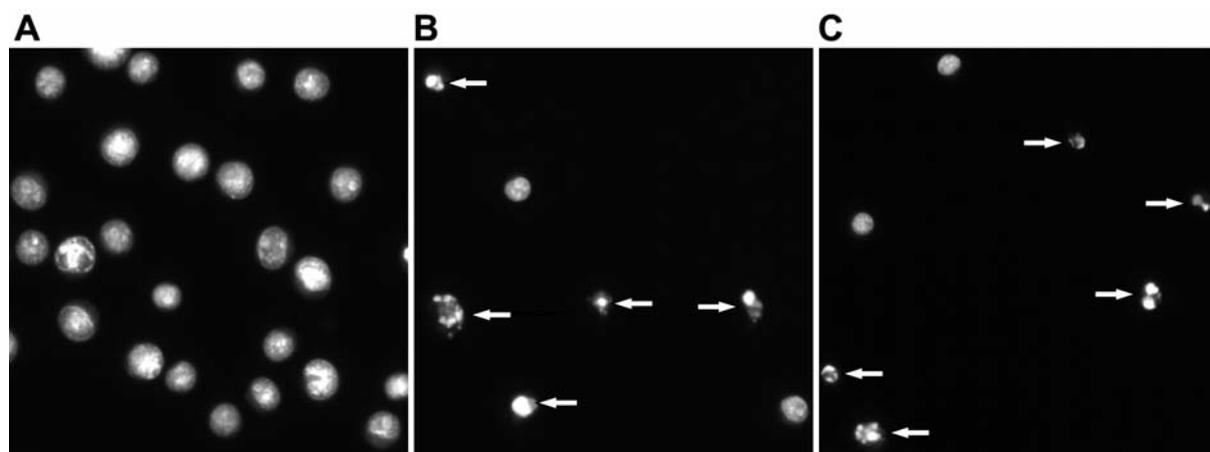


Figure 6. Morphology (fluorescence microscopy employing DAPI/sulforhodamine 101 staining) of KG-1 cells cultured for 48 h in the absence (A) and presence of ZKK-3 (10  $\mu$ M) (B), and with presence of the combination MPT (10  $\mu$ M) with ZKK-3 (10  $\mu$ M) (C). Arrows indicate apoptotic bodies.

increased mitochondrial membrane de-polarization (as evidenced by the shift in green-to-red fluorescence ratio) in the KG-1 cell line.

**Cleavage of PARP protein.** Apoptosis was confirmed by detecting PARP cleavage after 48-h incubation with the tested compounds used alone and in combination. During compound - induced apoptosis, 85 kDa PARP fragments were detected with the use of specific antibody (Figure 5 shows exemplary cytograms).

**Morphological changes in cells.** The effects of the compounds on the morphology of cells were recorded at various times by microscopy. No changes were observed for untreated cells. Characteristic apoptotic changes in the morphology of cells (changes in chromatin concentration and apoptotic bodies formation) were observed by fluorescence microscopy in the treated cells (Figure 6).

**Effect of compounds on the levels of BCL-2 family proteins.** To determine the levels of pro- and antiapoptotic proteins, we performed western blot analysis of whole-cell extracts obtained from KG-1 cells cultured in the presence of ZKK-3 (concentration 10  $\mu$ M, 20  $\mu$ M) after 48 h of incubation. We found that levels of pro-apoptotic (BAX, BAD, NIP1) and anti-apoptotic (BCL-2) proteins decreased after treatment with ZKK-3 (Figure 7).

**Kinase inhibition profile of ZKK-3.** The profile of kinase inhibition of ZKK-3 indicates no specificity of this compound (Figure 8). At 10  $\mu$ M ZKK-3 was not a good inhibitor of any protein kinase tested. However, seven protein kinases that play an important role in cell metabolism of normal and cancer cells (ERK8, PKD1,

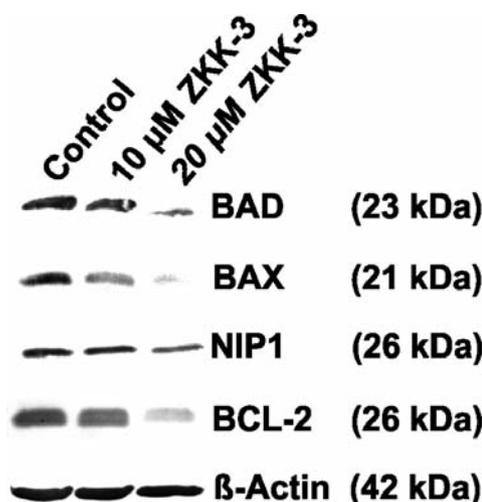


Figure 7. Western blot analysis of B-cell lymphoma 2 (BCL-2) family proteins (pro- and antiapoptotic) in the whole-cell extracts obtained from KG-1 cell line cultured in the presence of ZKK-3 (concentration 10  $\mu$ M, 20  $\mu$ M) after 48 h of incubation. Preparation of cell extracts and protein detection is described in the Materials and Methods.

NEK2A, PIM1, PIM3, IGF-1R and IR) were moderately-inhibited (*i.e.* less than 30% residual activity) at this inhibitor concentration.

**Effect of compounds on cell-cycle progression.** Figure 9 demonstrates changes in cell-cycle progression of KG-1 cells after 48 h incubation with the tested compounds. The compounds exerting cytostatic effect also caused a concentration-dependent accumulation of cells in the G<sub>1</sub> and S phases and at the border of these phases, reducing the number of cells in the G<sub>2</sub>M phase of the cell cycle. The

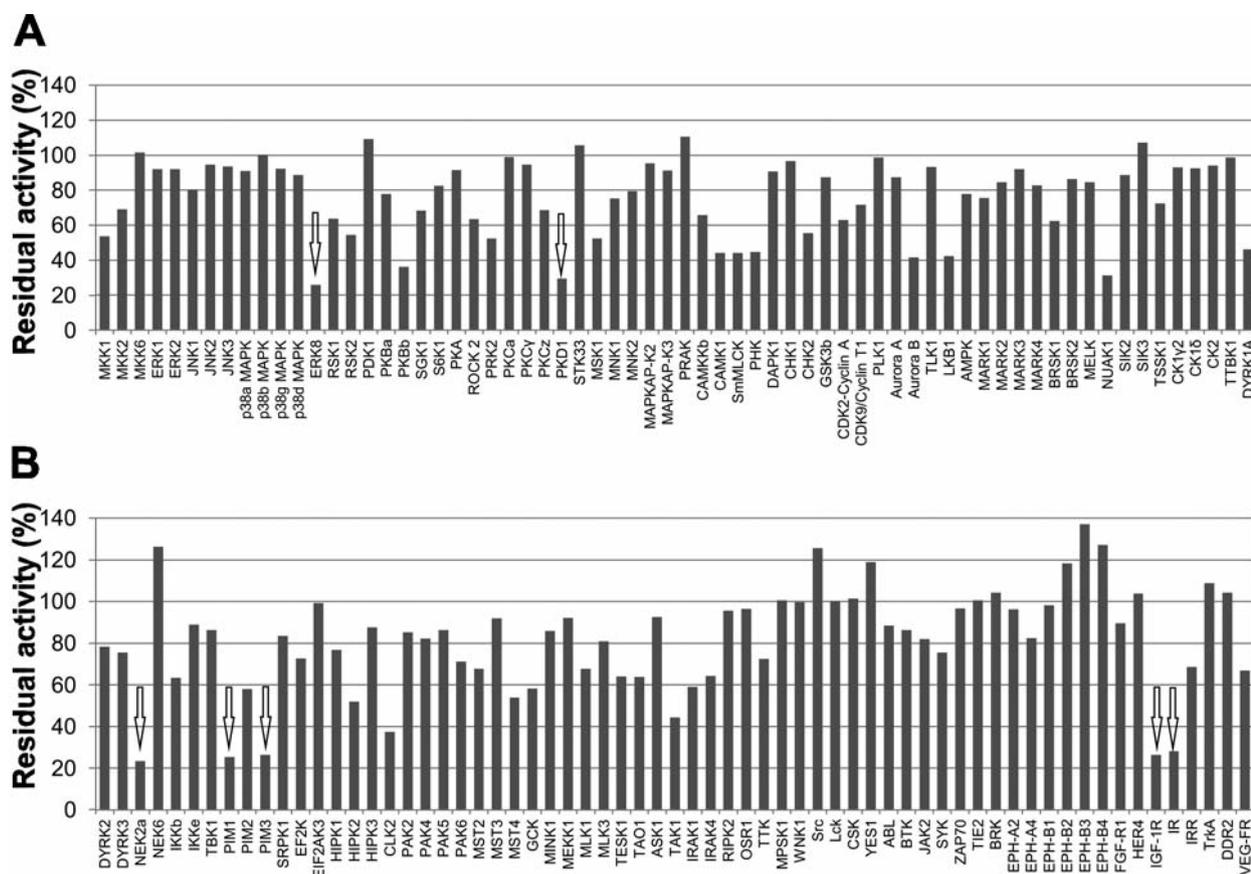


Figure 8. The selectivity profile of ZKK-3 on a large panel of kinases. Residual activity was determined in the presence of 10  $\mu$ M inhibitor and expressed as a percentage of the control without inhibitor.

tested compounds at 20  $\mu$ M induced large changes in the cell cycle distribution (Figure 10C), therefore, precise statistical analysis of DNA histograms by the MacCycle computer program was often impossible.

**Discussion**

In the present study anti-leukemic effects of CK2 inhibitors and pentabromobenzylisothioureas (Figure 1), used alone and in combination, against the human acute myelogenous leukemia cell line KG-1 were examined. It was found that all the examined CK2 inhibitors and pentabromobenzylisothioureas induced apoptotic cell death and evoked characteristic apoptotic changes: increased mitochondrial membrane depolarization, cleavage of PARP protein, changes in the morphology and down-regulation of BCL-2 family proteins (Figures 2-7). These changes were accompanied by changes in cell-cycle progression (Figures 9 and 10). The most effective compound at apoptosis induction was AEAT, a known CK2 inhibitor ( $IC_{50}$ =0.28  $\mu$ M for CK2) (Pinna and Cozza, personal comm.).

It should be noted that AEAT is more effective than the very potent CK2 inhibitor DMAT ( $IC_{50}$ =0.14  $\mu$ M for CK2). DMAT, MPT and AEAT applied in combination with the pentabromobenzylisothiourenium bromides had synergistic pro-apoptotic effects; the most potent synergistic effect was induced particularly in combination with ZKK-13 after 48 h incubation (Figure 3).

For ZKK-3, its influence on the level of BCL-2 family proteins playing a control role in the mitochondrial apoptotic pathway was determined. The results showed a reduction of BCL-2 family proteins (Figure 7) in KG-1 cells treated with ZKK-3 (10 and 20  $\mu$ M, as compared to control cells). These results may be related to the partial degradation of the proteins during apoptosis. It is known that following a death signal, BAX is translocated to the outer mitochondrial membrane, where it promotes permeabilization that favors the release of cytochrome *c* and other apoptogenic factors (30). In healthy cells, BAX is a monomeric cytosolic protein which upon apoptotic stimuli changes its conformation and translocates to the mitochondria (30, 31). In the case of

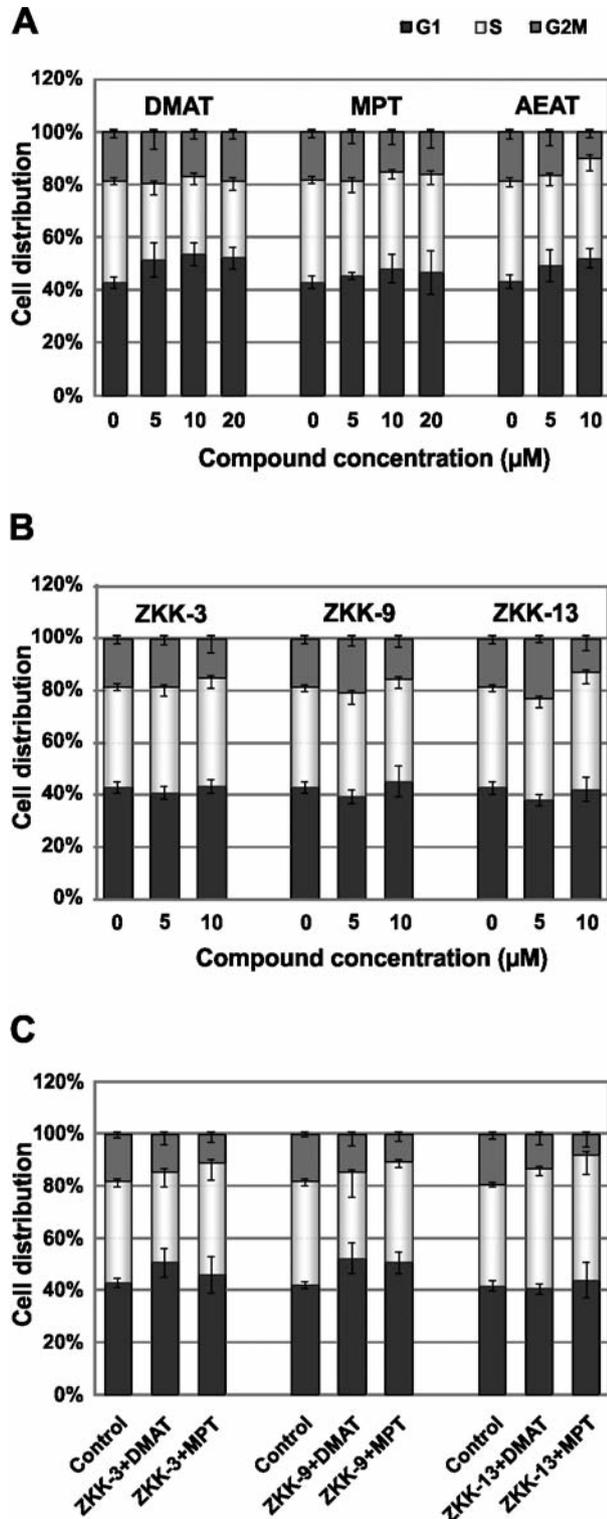


Figure 9. Changes in cell-cycle progression in KG-1 cells after 48 h treatment with compounds alone (A, B) and in combination (C). Each bar represents the mean $\pm$ S.D. ( $n\geq 4$ ). The data obtained from FACSCalibur flow cytometer were analyzed using MacCycle software to determine the percentage of cells in each phase of the cell cycle.

BAD, in healthy cells it is phosphorylated by kinases such as protein kinase A and B, and as a consequence of this, is bound and sequestered by 14-3-3 scaffold proteins. De-phosphorylation of BAD by  $Ca^{2+}$ -activated calcineurin correlates with its dissociation from 14-3-3 in the cytosol and its subsequent translocation to the mitochondria where BAD presumably inactivates BCL-XL, leading to apoptosis (32). Therefore, a reduced level of BAX and BAD proteins may suggest their translocation to the mitochondria. Western blot analysis also revealed a decrease in the level of NIP1 (pro-apoptotic) protein involved in apoptosis (33, 34), and a reduction of BCL-2 (antiapoptotic) protein at 20  $\mu$ M ZKK-3 (Figure 7).

ZKK-3 was found to be a relatively potent inhibitor of seven protein kinases PIM1, PIM3, ERK8, PKD1, NEK2A, IGF-1R and IR (Figure 8), important from the point of view of targeted cancer therapy. PIMs are overexpressed in many types of tumors and promote cell growth and resistance to apoptosis (35). Therefore, these kinases are attractive targets for drug development (36-39). ERK8 has been implicated in cell transformation and in the protection of genomic integrity and, therefore, proposed as a novel potential therapeutic target for cancer (40, 41). Altered expression and dysregulation of a PKD1 was observed in many types of tumor. Particularly it was observed that PKD1 overexpression increases the aggressiveness of MCF-7 breast cancer cells through enhancing their oncogenic properties (42). NEK2A is a serine/threonine protein kinase that locates at the centrosome and is essential for centrosome and exact chromosome segregation. It is purported to be related to the development and progression of breast carcinoma and may be considered as a novel potential biomarker for diagnosis and a possible therapeutic target for human breast cancer (43). IGF-1R, a receptor tyrosine kinase, is well-established as a key regulator of tumor cell growth and survival. The IGF-1R is implicated in several types of cancer, including breast, prostate, and lung cancer. Overexpression and activation of IGF-1R and elevated IGF ligand levels have been observed in a number of human cancers and down-regulation of IGF-1R leads to massive apoptosis of cancer cells (44-47). There is also a growing body of data to support a role for the structurally- and functionally-related IR in human cancer (45).

In conclusion, our results showed that the tested compounds, alone and in combination, induced apoptosis of acute myelogenous leukemia cells *in vitro*. At the same time synergistic anti-leukemic effects were observed between CK2 inhibitors and ZKKs. Therefore ZKK-3, ZKK-9 and ZKK-13 can be considered as valuable potential anti-neoplastic agents. Although ZKK-3, ZKK-9 and ZKK-13, contrary to DMAT, MPT and AEAT are not CK2 kinase inhibitors they exhibit relatively potent activity against several other prosurvival kinases. Their pro-apoptotic effects may be related to the intrinsic apoptotic pathway, since they

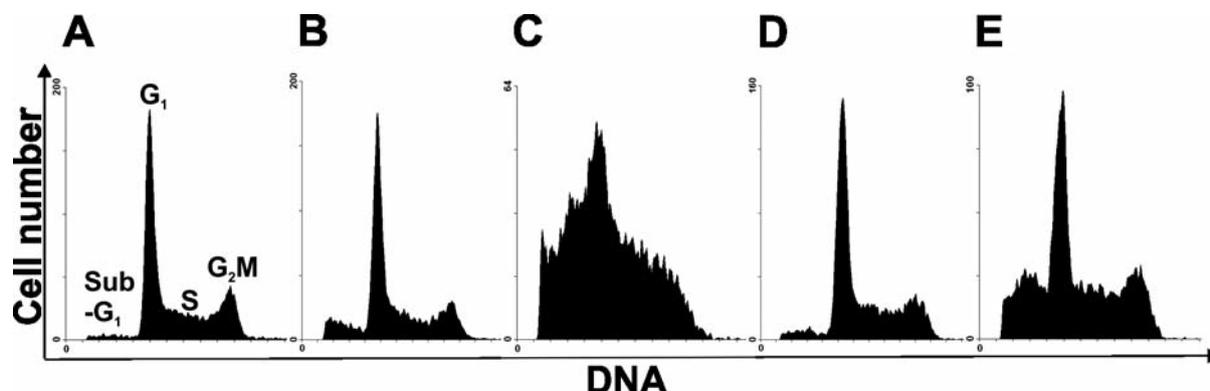


Figure 10. Exemplary DNA histograms of KG-1 cells treated for 48 h with compounds. The data were obtained from FACSCalibur flow cytometer. A: Control (no ZKK-3 added); B: 10  $\mu$ M ZKK-3; C: 20  $\mu$ M ZKK-3; D: 10  $\mu$ M MPT; E: combination 10  $\mu$ M ZKK-3 and 10  $\mu$ M MPT.

evoke increased mitochondrial outer membrane depolarization and changes in pro- and anti-apoptotic protein levels. Since CK2 is an anti-apoptotic enzyme, the combination of its inhibitor with a pro-apoptotic drug seems to be a rational means for the development of new multidrug anticancer therapies.

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