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PROAPOPTOTIC EFFECTS OF NEW PENTABROMOBENZYLISOTHIOURONIUM SALTS IN A HUMAN PROSTATE ADENOCARCINOMA CELL LINE

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Abstract: Prostate cancer is the second most common cancer in elderly men worldwide and its incidence rate is rising continuously. Agents capable of inducing apoptosis in prostate cancer cells seem a promising approach to treat this malignancy. In this study we describe the synthesis of a number of novel N- and N,N'-substituted S-2,3,4,5,6-pentabromobenzylisothiouronium bromides and their activity against the human prostate adenocarcinoma PC3 cell line. All the compounds produced changes in mitochondrial transmembrane potential and cell cycle progression, showed a cytostatic effect and induced apoptosis in the tested cancer line in a concentration- and time-dependent manner. The most effective compounds **ZKK-3**, **ZKK-9** and **ZKK-13** produced, at 20 μM concentration, apoptosis in 42, 46, and 66% of the cells, respectively, after 48 h incubation. Two selected S-2,3,4,5,6-pentabromobenzylisothiouronium bromides (**ZKK-3**, **ZKK-9**) showed also a synergic proapoptotic effect with the new casein kinase II inhibitor 2-(4-methylpiperazin-1-yl)-4,5,6,7-tetrabromo-1*H*-benzimidazole (TBIPIP) in the PC3 cell line.

Keywords: pentabromobenzylisothioureas, CK2 inhibitor, apoptosis, kinases, prostate cancer, flow cytometry

Prostate cancer is one of the most common malignancies in elderly men in the Western world (1). Growth of most prostate cancers is initially dependent on androgens and androgen receptor signalling. Therefore, androgen ablation by orchiectomy and/or treatment with LHRH-analogs or anti-androgens are the most common therapy strategies. However, both surgical and chemical castration eventually leads to a selection of hormone-refractory cells, which show activation of anti-apoptotic signalling pathways (2). The agents capable of inducing apoptosis in prostate cancer cells seem another promising approach to treat this malignancy, particularly in hormone-independent prostate cancer. In this study, we describe the synthesis of modified S-pentabromobenzyl-isothiouronium bromides and their activity against the prostate cancer cell line PC3. Looking for possible mechanism of action of these isothioureas we have tested inhibitory properties of N,N'-dimethyl-S-pentabromobenzylisothiouronium bromide (ZKK-3) as representative in a large panel of protein kinases.The inhibitors of protein kinases were studied as possible anticancer agents in prostate cancer cell lines. Beside CK2 inhibitors (3, 4), also inhibitors of other protein kinases, e.g., of PIM, PKD or IGF-1R, are considered as prospective drugs for the treatment of prostate cancer (5–7).

Isothioureas make a class of amphiphilic compounds carrying a highly basic isothiourea group of pK_a » 10; hence, at physiological pH they exist in protonated (cation) form. Our interest in this compound class is mostly driven by multifaceted biological properties of substituted S-benzylisothioureas. These derivatives, similarly to other isothioureas and aliphatic guanidines, are inhibitors of nitric oxide synthases (NOSs) (8, 9). Because of an essential role of NOSs in a plethora of physiological and pathological phenomena, there is an ongoing search for better (i.e., more specific and stronger) inhibitors of these enzymes (8–11). Some isothioureas with considerable NOS inhibitory activity showed promise as chemopreventive agents in rat tracheal epithelial cells treated with the carcinogen benzo[a]pyrene (12). More recently, a number of S-pentabromobenzylisothiourea derivatives have been found to show

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substantial cytotoxicity against human glioblastoma cells (13). Some modified S-benzylisotioureas show also a considerable antimicrobial activity (14, 15). Lately, it has been reported that modified S-benzylisothioureas are effective inhibitors of indoleamine-2,3-dioxogenase (IDO) at submicromolar concentrations (16). It is known that IDO is overexpressed in variety of diseases including cancer, and play an important role in the process of immune escape of tumors (17, 18). Below, we show our preliminary study of cytotoxic activity against the human prostate cancer cell line PC3 of a number of novel N-substituted S-pentabromobenzylisothiouronium bromides (**2a**–**2g**) with regard to their structure and apoptosis induction. Additionally we examined compound **ZKK-3** exhibiting high proapoptotic activity in human promyelocytic leukemia (HL-60) cell line (19).

EXPERIMENTAL

Chemistry

Melting points were determined on a Gallenkamp melting point apparatus, Mod. MFB 595 030G, in open capillary tubes. The ^{1}H-NMR spectra were recorded on a Bruker AMX instrument (400 MHz ^{1}H frequency) at 25°C. Chemical shifts given in δ-units are reported in ppm from internal tetramethylsilane standard. The solvent used for NMR spectra was deuteriodimethylsulfoxide. Elemental analyses were performed at the Faculty of Chemistry, Warsaw Technical University using a Heraeus CHN Rapid Analyzer. N,N'-dimethyl-S-(2,3,4,5,6-pentabromobenzyl)isothiouronium bromide (**ZKK-3**) and 4,5,6,7-tetrabromo-2-(4-methylpiperazin-1-yl)-1H-benzimidazole (**TBIPIP**) were obtained according to previously described procedures (19, 20).

General procedure for the preparation of N-substituted S-(2,3,4,5,6-pentabromobenzyl)-isothiouronium bromides (ZKK-9 through ZKK-15)

To a hot solution of thiourea derivative (**1a**–**1g**) (2.1 mmol) in anhydrous ethanol (12 mL) 2,3,4,5,6-pentabromobenzyl bromide (2 mmol) was added. The mixture was refluxed for 20 min and then the solvent was partially evaporated to a final volume of about 6 mL. This was left refrigerated overnight. The chromatographically pure crystals that formed were filtered off and washed with a small volume of cold ethanol/diethyl ether mixture (1:1, v/v). For elemental analysis, a small amount of the product was recrystallized from ethanol. Synthesis scheme and chemical structure of **ZKKs** are shown in Scheme 1.

N,N-Dimethyl-S-(2,3,4,5,6-pentabromobenzyl)isothiouronium bromide (ZKK-9)

Yield 62%, m.p. 273–274°C. ^{1}H-NMR (DMSO-d_6, δ, ppm): 3.32 (s, 6H, 2 × CH_3), 4.90 (s, 2H, -CH_2-), 9.20 (bs, 2H, H_2N). Analysis: calcd. for $C_{10}H_{10}N_2SBr_6$ (669.69): C, 17.94; H, 1.51; N, 4.18%; found: C, 17.82; H, 1.61; N, 4.10%.

N,N,N'-Trimethyl-S-(2,3,4,5,6-pentabromobenzyl)isothiouronium bromide (ZKK-10)

Yield 66%, m.p. 234–236°C. ^{1}H-NMR (DMSO-d_6, δ, ppm): 3.12 (s, 3H, CH_3), 3.32 (s, 6H, 2 × CH_3), 4.72 (s, 2H, -CH_2-), 9.37 (bs, 1H, H-N). Analysis: calcd. for $C_{11}H_{12}N_2SBr_6$ (683.71): C, 19.32; H, 1.77; N, 4.10%; found: C, 19.25; H, 1.84; N, 4.03%.

N,N,N',N'-Tetramethyl-S-(2,3,4,5,6-pentabromobenzyl)isothiouronium bromide (ZKK-11)

Yield 73%, m.p. 235–236°C. ^{1}H-NMR (DMSO-d_6, δ, ppm): 3.32 (s, 12H, 4 × CH_3), 4.72 (s, 2H, -CH_2-). Analysis: calcd. for $C_{12}H_{14}N_2SBr_6$ (697.74): C, 20.66; H, 2.02; N, 4.01%; found: C, 20.55; H, 2.10; N, 3.92%.

N-(Isopropyl)-S-(2,3,4,5,6-pentabromobenzyl)isothiouronium bromide (ZKK-12)

Yield 69%, m.p. 276–278°C. ^{1}H-NMR (DMSO-d_6, δ, ppm): 1.19 (d, 6H, J = 6.4 Hz, 2 × CH_3), 3.92 (m, 1H, H-C), 4.89 (s, 2H, -CH_2-), 9.47 and 9.72 (2bs, 3H, H-N and H_2N). Analysis: calcd. for $C_{11}H_{12}N_2SBr_6$ (683.71): C, 19.32; H, 1.77; N, 4.10%; found C, 19.28; H, 1.81; N, 4.04%.

N,N'-Diisopropyl-S-(2,3,4,5,6-pentabromobenzyl)isothiouronium bromide (ZKK-13)

Yield 75%, m.p. 239–241°C. ^{1}H-NMR (DMSO-d_6, δ, ppm): 1.24 (d, 12H, J = 6.2 Hz, 4 × CH_3), 4.08 (m, 2H, H-C), 4.86 (s, 2H, -CH_2-), 9.10 and 9.43 (2bs, 2H, 2 × H-N). Analysis: calcd. for $C_{14}H_{18}N_2SBr_6$ (25.79): C: 23.17; H, 2.50; N, 3.86%; found: C, 23.24; H, 2.61; N, 3.75%.

N-(*tert*-Butyl)-S-(2,3,4,5,6-pentabromobenzyl)isothiouronium bromide (ZKK-14)

Yield 73%, m.p. 266–268°C. ^{1}H-NMR (DMSO-d_6, δ, ppm): 1.39 (s, 9H, 3 × CH_3), 4.87 (s, 2H, -CH_2-), 8.79 and 9.52 (2bs, 3H, H-N and H_2N). Analysis: calcd. for $C_{12}H_{14}N_2SBr_6$ (697.74): C, 20.66; H, 2.02; N, 4.01%; found: C, 20.56; H, 2.07; N, 3.94%.

N-Benzyl-S-(2,3,4,5,6-pentabromobenzyl)isothiouronium bromide (ZKK-15)

Yield 71%, m.p. 253–255°C. ^{1}H-NMR (DMSO-d_6, δ, ppm): 4.56 and 4.89 (2s, 4H, 2 × -CH_2-), 7.30–7.45 (m, 5H, H-arom.), 9.6 (bs, 3H, H-N and H_2N). Analysis: calcd. for $C_{15}H_{12}N_2SBr_6$ (731.76): C, 24.62; H, 1.65, N, 3.83%; found: C, 24.55; H, 1.71; N, 3.74%.

Evaluation of proapoptotic potency

Cell culture and compounds treatments

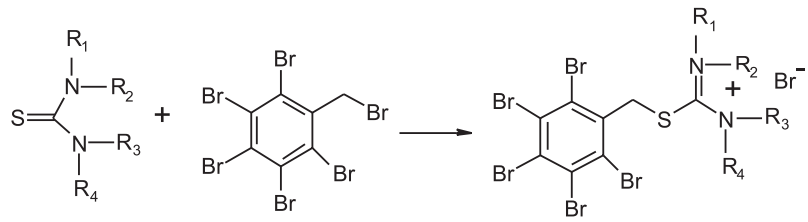
PC3 (human prostate adenocarcinoma) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown as monolayer culture in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (Gibco) and 1% (v/v) of antibiotic–antimycotic solution (Gibco), at 37°C in a humidified atmosphere of 5% CO_2 in air. All experiments were performed in exponentially growing cultures. The compounds studied were added to the cultures as solutions in dimethyl sulfoxide (DMSO; Sigma); control cultures were treated with the same volume of the solvent. 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 Annexin-V FITC Apoptosis Kit (Invitrogen). After 24- or 48-hour incubation with the tested compounds, the cells were collected by centrifugation, rinsed twice with cold phosphate-buffered saline (PBS) and suspended in binding buffer at 2 × 10^6 cells/mL. One-hundred µL aliquots of the cell suspension were labeled according to the kit 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 µL 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.

Morphological evaluation

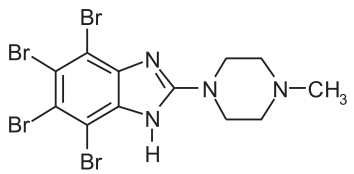
After exposure to drugs, the cells were collected, washed with cold PBS and fixed with 70% ethanol at -20°C for at least 24 h. Next, ethanol was washed out and the cells were stained with 1.0 µg/mL DAPI and 20 µg/mL sulforhodamine 101. Cell morphology was evaluated using a BX60 fluo-



ZKK-3 R_1, R_3 = CH_3, R_2, R_4 = H [19]

1a R_1, R_2 = CH_3, R_3, R_4 = H
1b R_1, R_2, R_3 = CH_3, R_4 =H
1c R_1, R_2, R_3, R_4 = CH_3
1d R_1 = $CH(CH_3)_2$, R_2, R_3, R_4 = H
1e R_1, R_3 = $CH(CH_3)_2$, R_2, R_4 = H
1f R_1 = $C(CH_3)_3$, R_2, R_3, R_4 = H
1g R_1 = benzyl, R_2, R_3, R_4 = H

2a ZKK-9 R_1, R_2 = CH_3, R_3, R4 = H
2b ZKK-10 R_1, R_2, R_3 = CH_3, R_4 = H
2c ZKK-11 R_1, R_2, R_3, R_4 = CH_3
2d ZKK-12 R_1 = $CH(CH_3)_2$, R_2, R_3, R_4 = H
2e ZKK-13 R_1, R_3 = $CH(CH_3)_2$, R_2, R_4 = H
2f ZKK-14 R_1 = $C(CH_3)_3$, R_2, R_3, R_4 = H
2g ZKK-15 R_1 = benzyl, R_2, R_3, R_4 = H



TBIPIP [20]

Scheme 1. Synthesis of isothiouronium salts **2a**–**2g** (**ZKK's**) and structure of the CK2 inhibitor TBIPIP

rescence microscope equipped with a DP50 digital camera (Olympus, Japan).

Mitochondrial membrane potential ($\Delta\Psi_m$) assay

Mitochondrial membrane potential was assessed by flow cytometry using JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide; 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 1×10^6 cells/mL and incubated with 2.5 µL 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 µL 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 poly(ADP-ribose) polymerase (PARP). PARP cleavage was detected by flow cytometry using Anti-PARP CSSA FITC Apoptosis Detection Kit (Invitrogen) according to 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 just prior to use. The level of cleaved PARP protein was expressed as fluorescence intensity that was assessed using CellQuest and the free WinMDI software package written by Joseph Trotter of the Scripps Institute (La Jolla, CA, USA).

Cell cycle analysis

After exposure to the tested compounds, the cells were washed with cold PBS and fixed at -20°C in 70% ethanol for at least 24 h. Next, the cells were washed in PBS and stained with 50 µg/mL PI and 100 µg/mL RNase solution in PBST (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 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 cytometry analyses were run on a FACSCalibur flow cytometer and analyzed using the CellQuest and WinMDI 2.9 softwares. The DNA histograms obtained were analyzed using the MacCycle software.

Inhibition of protein kinases by ZKK-3

Protein kinase inhibitory activity of 10 mM solution of **ZKK-3** was tested using a panel of 130 selected protein kinases in the Division of Signal Transduction Therapy, International Centre for Kinase Profiling, at the University of Dundee, Scotland.

RESULTS AND DISCUSSION

The N-substituted pentabromobenzylisothioureas (**ZKK9–ZKK15**) were obtained from 2,3,4,5,6-pentabromobenzyl bromide and respective thiourea derivatives **1a–1g** (Scheme 1). The products – isothiouronium bromides (**2a–2g**, **ZKK-9–ZKK-15**) crystallized from the reaction mixture after concentration. The compounds were characterized using ^{1}H-NMR and elemental analyses.

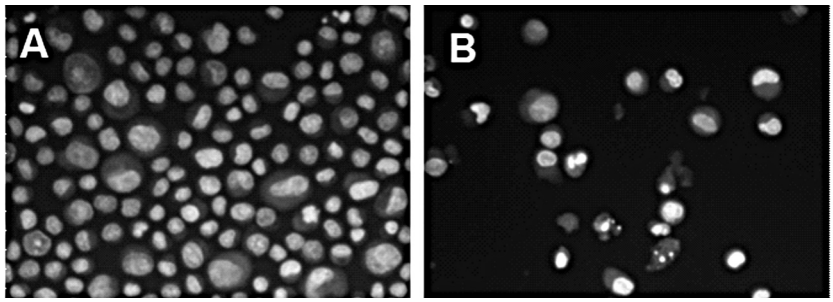


Figure 1. Morphology (fluorescence microscopy employing DAPI/sulforhodamine 101 staining) of PC3 cells cultured for 48 h in the absence (control, panel **A**) and presence of **ZKK-3** (20 µM, panel **B**)

Induction of apoptosis by ZKKs in PC3 cell line

All the examined ZKKs compounds evoked characteristic apoptotic changes in the morphology of PC3 cells (chromatin concentration and apoptotic bodies formation, see Fig. 1). The observed apoptotic effect was dose- and time-dependent (Fig. 2). N,N'-Diisopropyl-S-(2,3,4,5,6-pentabromobenzyl)isothiouronium bromide (**ZKK-13**) was the most effective, and induced apoptosis in 66% of the cells at 20 μM concentration and 48 h incubation time. At the highest tested concentration (50 μM) and 48 h incubation time significant apoptosis of PC-3 cells was noted also for **ZKK-9**, **ZKK-10**, **ZKK-13** and **ZKK-15**, whereas **ZKK-11**, **ZKK-12** and **ZKK-14** exhibited only minor proapoptotic activity. Two selected S-2,3,4,5,6-pentabromobenzylisothiouronium bromides (**ZKK-3** and **ZKK-9**) showed also a synergic proapoptotic effect with the

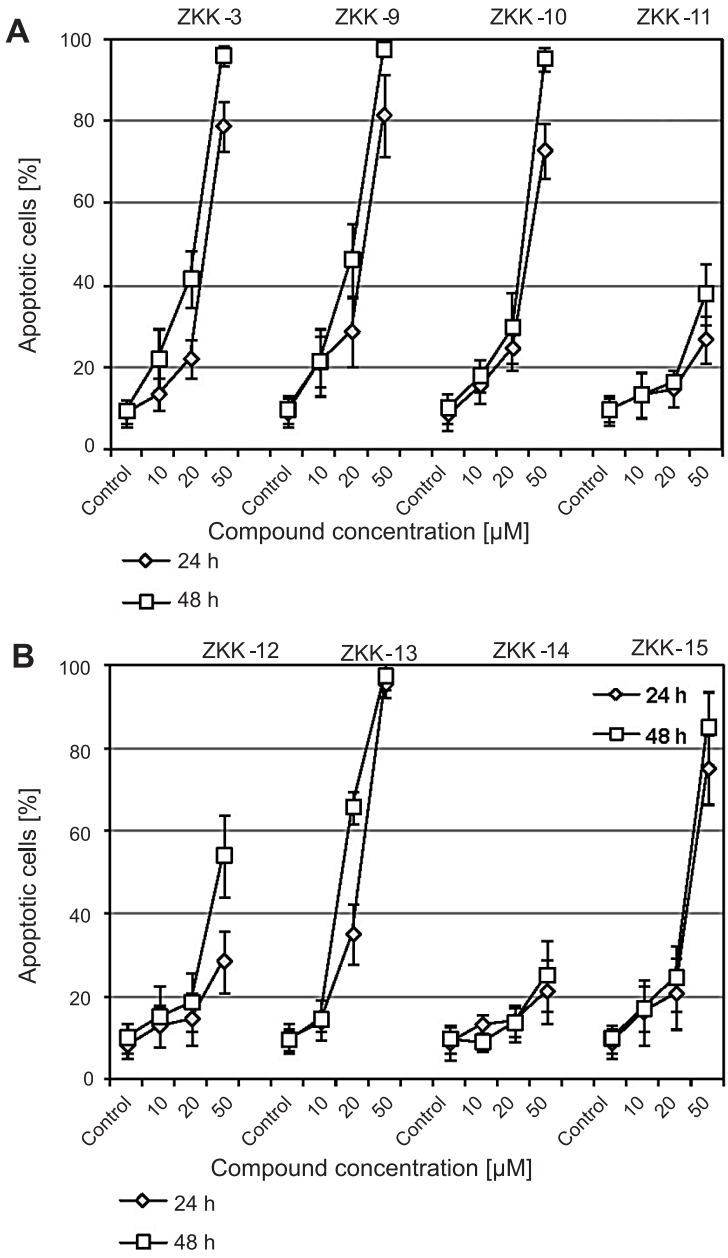


Figure 2**.** (panel: **A**, **B**) Induction of apoptosis by **ZKKs** in PC3 cells. The data were determined by FACS cytometer after 24 and 48 h treatment. Cells were stained with annexin V-FITC and PI. Each point represents the mean ± S.D. (n = 4)

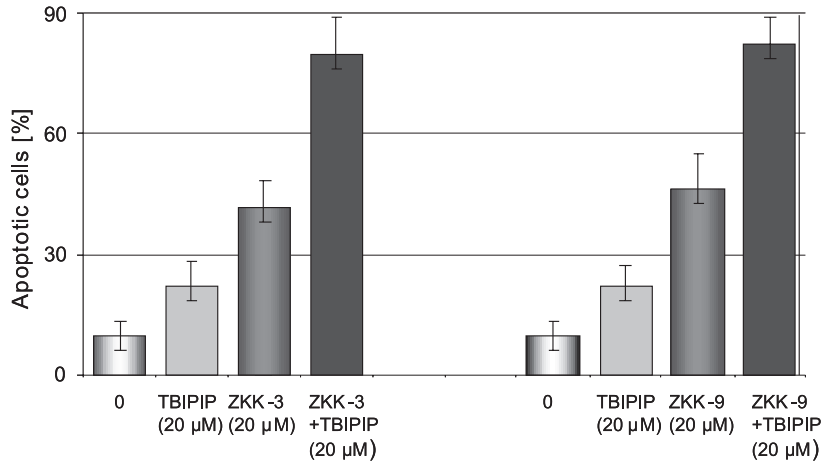


Figure 3. Induction of apoptosis in PC3 cells after 48 h treatment with **ZKK-3**, **ZKK-9** alone or in combination with TBIPIP. Each bar represents the mean ± S.D. (n = 4)

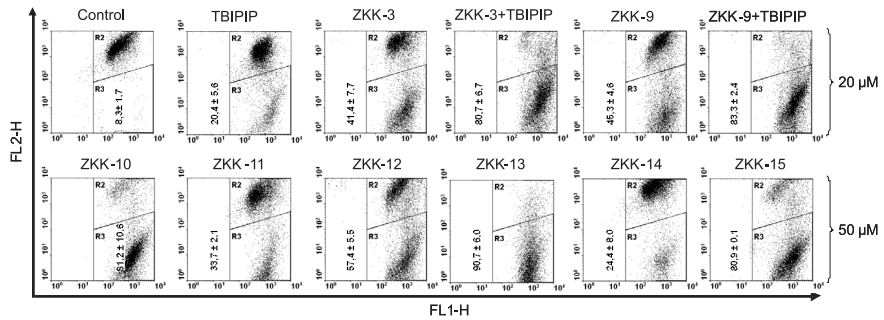


Figure 4. Representative flow cytograms demonstrating changes in mitochondrial membrane potential ($\Delta\Psi_m$) of PC3 cells induced by 48 h culturing with: **ZKK-3**, **ZKK-9** alone or in combination with **TBIPIP** (upper panel) and various **ZKKs** compounds (lower panel). The cells were stained with JC-1 dye. The cells in the lower right (R3) quadrant showed increased read-to-green fluorescence ratio (apoptotic cells)

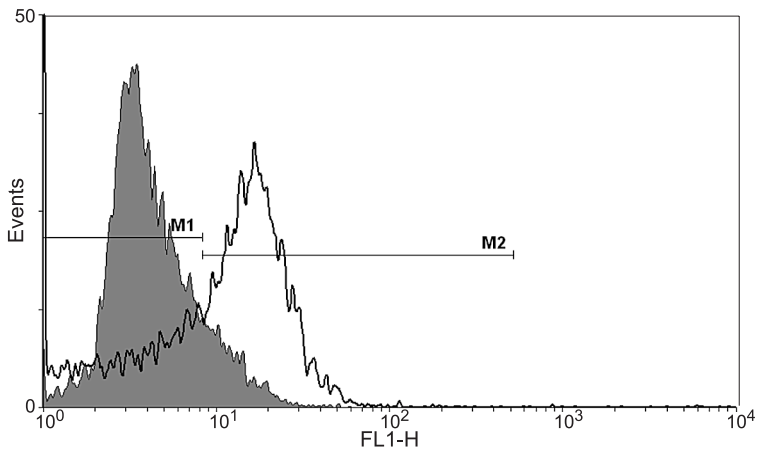


Figure 5. Effect of **ZKK-3** on proteolytic cleavage of PARP protein in PC3 cells was exposed for 48 h to **ZKK**-3. Representative histograms showing increased level of 85 kDa fragment of PARP protein indicating induction of apoptosis after **ZKK-3** treatment. Histogram of PC3 control cells and overlay histogram of treated cells at 50 μM **ZKK-3**. Marker M1 designates negative cell populations whereas M2 designates positive cell populations (indicate apoptosis)

previously described (19) casein kinase II inhibitor 2-(4-methylpiperazin-1-yl)-4,5,6,7-tetrabromo-1*H*-benzimidazole (**TBIPIP**) in the PC-3 cell line (Fig. 3).

The data obtained allow formulating no clear structure-function relationship for the new isothiouronium salts. In compound **ZKK-11** the positive charge is sheltered by methyl groups whereas compounds **ZKK-12** and **ZKK-14** have bulky *tert*-butyl and isopropyl substituents on a nitrogen atom of the isothiourea residue. However, **ZKK-13** with two N,N'-isopropyl groups was the most potent in terms of apoptosis induction. This characteristic probably depends on the specific conformations of the substituted isothiourea group and additional structural studies are needed to for a detailed explanation of this phenomenon.

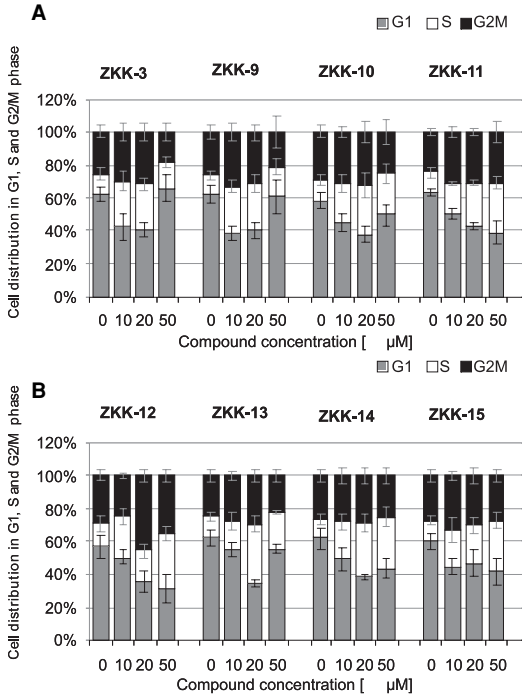


Figure 6. (panel: **A**, **B**) Changes in cell cycle progression in PC3 cells after 48 h treatment with **ZKK**s. Each bar represents the mean ± S.D. (n = 4). The data obtained from FACSCalibur flow cytometer were analyzed using MacCycle software to determine the percentage of cells in each phase of the cell cycle

Changes in mitochondrial membrane potential ($\Delta\Psi_m$)

Analysis of the respective flow cytograms (Fig. 4) showed that the tested compounds increased mitochondrial membrane depolarization (as evidenced by the shift in green-to-red fluorescence ratio) in the PC3 line.

ZKKs-induced cleavage of PARP protein

Apoptosis induced in PC3 cells by incubation with **ZKK-**3 was associated with an increase in the level of 85 kDa fragments of PARP protein. The presence of 85 kDa PARP fragments was revealed using specific antibody (Fig. 5).

Effect of ZKKs on cell cycle progression

Figures 6 and 7 demonstrate changes in the cell cycle progression of PC3 cells after 48 h incubation with the tested compounds. The compounds exerting cytostatic effect also caused a concentration-dependent accumulation of cells in of S, G_2M phases and at the border of this phases, decreasing the number of cells in the G_1 phase of the cell cycle.

Kinase inhibition profile of ZKK-3

The profile of kinase inhibition of **ZKK-3** indicates no specificity of this compound (Fig. 8). At 10

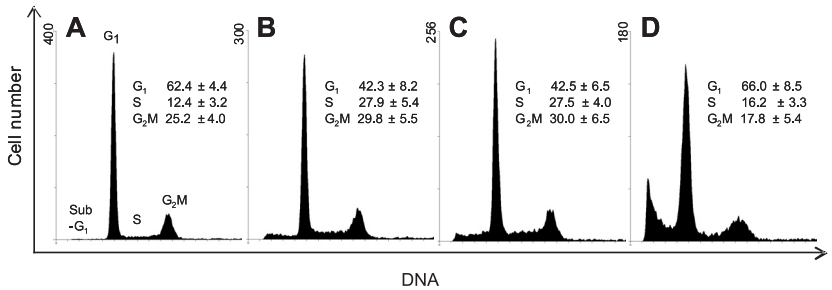


Figure 7. Exemplary DNA histograms of PC3 cells treated for 48h with **ZKK-3** The data obtained from FACSCalibur flow cytometer and analyzed using MacCycle software to determine the percentage of cells in each phase of the cell cycle. Panel **A**: Control (no **ZKK-3** added); panel **B**: 10 μM **ZKK-**3; panel **C**: 20 μM ZKK-3; panel **D**: 50 μM ZKK-3

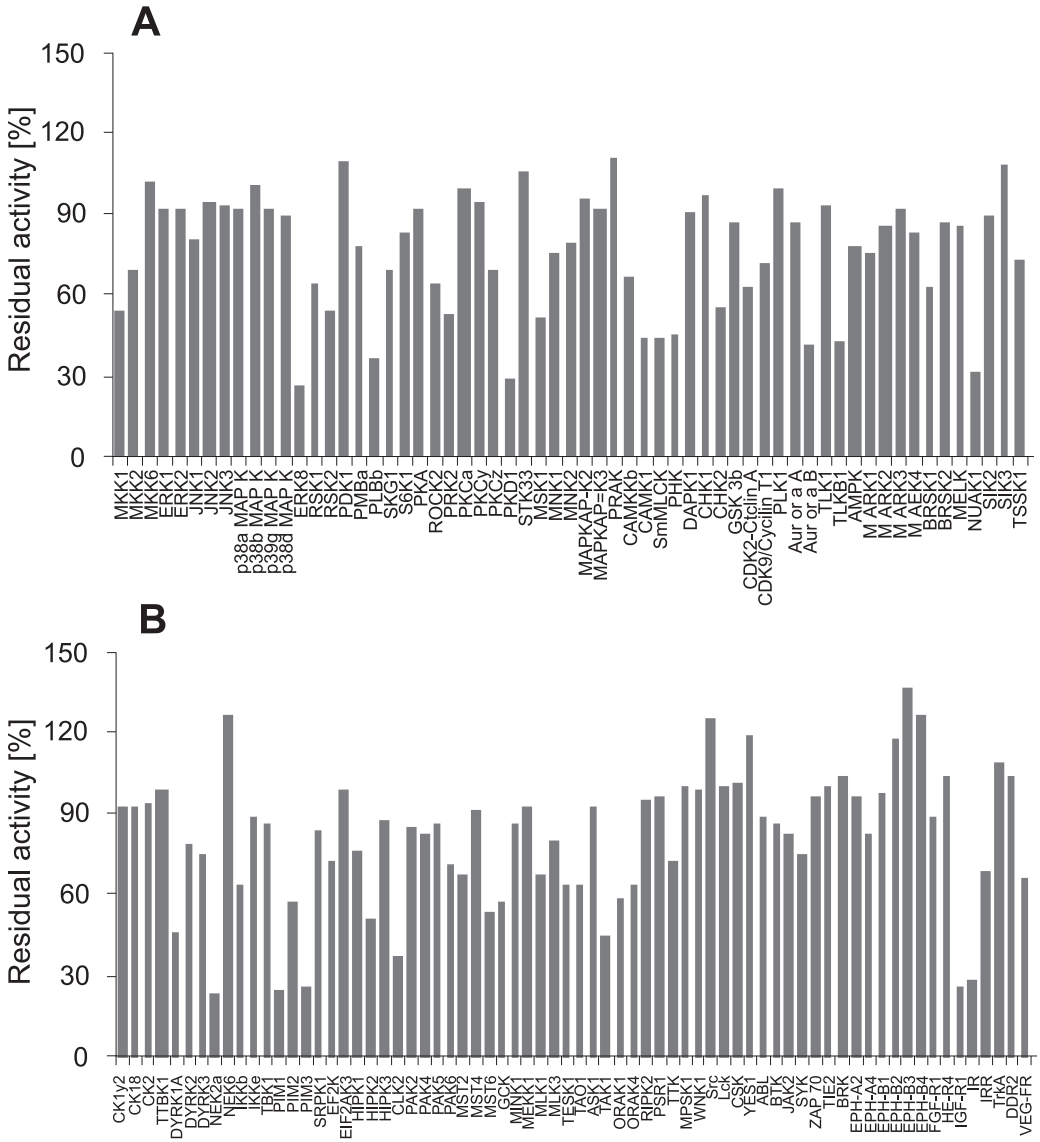


Figure 8. (panel: **A**, **B**) The selectivity profile on large kinase panel of **ZKK-3**. Residual activity was determined in the presence of 10 μM inhibitor and expressed as a percentage of the control without inhibitor.

mM concentration, **ZKK-3** was not a good inhibitor of any protein kinase tested. Only 7 protein kinases that play an important role in cell metabolism of normal and cancer cells (ERK8, PDK1, NEK2a, PIM1, PIM3 IGF-1R and IR) showed a moderate inhibition (i.e., less than 30% residual activity) at this inhibitor concentration. Of these, IGF-1R (IGF-1 receptor) mediates the action of IGF-1 that is known to contribute to the development of prostate cancer by blocking apoptosis and promoting proliferation (21, 22), and there is also evidence linking PIM1 with the development and progression of prostate cancer (23). These findings do not exclude other mechanisms of **ZKKs**' cytotoxicity in prostate cancer cells. For instance, a number of other isothiouronium compounds have been found to exert a considerable anti-cancer action due to their inhibiting nitric oxide synthase(s) [24, 25]. Hence, more studies are needed to solve this problem.

CONCLUSIONS

The obtained results indicate that some of the novel N- and N,N'-substituted S-2,3,4,5,6-pentabro-

mobenzylisothiouronium bromides show promise for utility, alone or in combination with casein kinase II inhibitors, against prostate cancer.

Acknowledgments

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