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# SYNTHESIS AND PROAPOPTOTIC PROPERTIES OF NEW CASEIN KINASE II INHIBITORS

MIROSŁAWA KORONKIEWICZ[1], MAGDA ŻUKOWSKA[1], ZDZISŁAW CHILMONCZYK[1], ANDRZEJ ORZESZKO[2,3] and ZYGMUNT KAZIMIERCZUK[2]*

[1]National Medicines Institute, 30/34 Chełmska St., 00-750 Warszawa, Poland
[2]Institute of Chemistry, Warsaw University of Life Sciences, 159C Nowoursynowska St., 02-787 Warszawa, Poland
[3]Military University of Technology, 2 Kaliskiego St., 00-908 Warszawa, Poland

**Abstract**: Casein kinase II (CK2) is the most pleiotropic of all protein kinases with more than 300 substrates implicated in a wide variety of cellular functions as signal transduction, proliferation and cell survival. Increased levels of CK2 has been demonstrated in a number of cancers, where it regulates the activity of various oncoproteins and tumor suppressor proteins. Therefore, CK2 inhibitors could be considered as potential anticancer drugs in monotherapy or in combination with known cytostatics. In this study, we examined proapoptotic activity of new strong CK2 inhibitor – 4,5,6,7-tetraiodobenzimidazole (TIBI) ($IC_{50}$ = 38 nM) as well as new derivatives of 4,5,6,7-tetrabromobenzimidazole and 4,5,6,7-tetraiodobenzimidazole. All the tested compounds induced apoptosis and cytostatic effects in the promyelocytic leukemia cell line (HL-60). The proapoptotic effect was concentration and time dependent. The changes of the mitochondrial membrane potential and cell cycle progression were also observed.

**Keywords**: casein kinase II, CK2, apoptosis, CK2 inhibitors, tetraiodobenzimidazoles, tetrabromobenzimidazoles

Casein kinase II (CK2) is the most pleiotropic of all protein kinases with more than 300 substrates implicated in a wide variety of cellular functions as signal transduction, proliferation and cell survival (1–3). CK2 level is consistently increased in various examined human cancers and experimental tumors, such as kidney, mammary gland, lung, prostate, adenocarcinoma, head and neck cancers (3–8). Overexpression of CK2 is also correlated with tumor aggression (5, 9) and is considered an unfavorable prognostic marker in prostate and lung cancers as well as in acute myeloid leukemia (10–12). Development of CK2 inhibitors is ongoing in preclinical studies, resulting in the generation of a number of CK2-directed compounds [reviews: (3, 13, 14)]. CK2-inhibitors were used alone or in combination with other drugs enhanced their efficiency as it was shown studying the growth inhibition of malignant lymphoblastic leukemia cells by the mixtures of 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) and imatinib (Gleevec) (15).

In the late 1980s, polyhalogenated benzimidazoles were found to be valuable scaffolds effectively competing with ATP binding site of CK2 (14). Their structural analogue 4,5,6,7-tetrabromo-1*H*-benzotriazole (TBB) was found later to be one of the most poweful inhibitors of CK2. The structural studies proved that presence of four bromine atoms in the benzene part of heterocycles is critical to fill the CK2 hydrophobic pocket to the ATP-binding site (16, 17).

Recently, we have found that 4,5,6,7-tetraiodo-1*H*-benzimidazole (TIBI) ($K_i$ = 0.023 μM) is a several times more potent inhibitor of CK2 than TBB ($K_i$ = 0.4 μM) or 4,5,6,7-tetrabromo-1*H*-benzimidazole (TBI) ($K_i$ = 0.05 μM) (18). In the present paper, we describe the synthesis and physicochemical properties of new derivatives of 4,5,6,7-tetrabromobenzimidazole and 4,5,6,7-tetraiodobenzimidazole. Two obtained compounds **1c** (TBIPIP) and **2c** (TIBIPIP) as well as new strong CK2 inhibitor – 4,5,6,7-tetraiodobenzimidazole (TIBI) – were chosen for examination of their ability to induce apop-

* Corresponding author: e-mail: ZKazimierczuk@gmail.com

totic and cytostatic effects in the promyelocytic leukemia cell line (HL-60).

## EXPERIMENTAL

All chemicals and solvents were purchased from Sigma-Aldrich or POCh. Melting points (uncorr.) were measured in open capillary tubes on a Gallenkamp-5 melting point apparatus.

$^1$H-NMR spectra (in ppm) were measured with a Varian Gemini 200 MHz (or a Varian UNITYplus 500 MHz) spectrometers at 298 K in DMSO-$d_6$ using tetramethylsilane as an internal standard. UV absorption spectra were recorded on a UV8500 (Techcomp, China) spectrophotometer. Mass spectra (70 eV) were obtained with a model AMD-604 Intectra spectrometer. Elemental analyses of the newly obtained compounds were within ± 0.4% of the respective theoretical values.

### Syntheses

The synthesis of TBI and TIBI as well as their 2-bromoderivatives were performed according the previously described procedures (18, 19).

### General procedure for the preparation of tetrabromobenzimidazoles 1a-1c

A mixture of 2,4,5,6,7-pentabromo-1H-benzimidazole (**1**) (650 mg, 1.27 mmol) and respective amine (41 mmol) in 25 mL of ethanol was refluxed for 24 h. The resulted solution was evaporated to dryness and the residue was crystallized twice from ethanol to yield the desired product.

4,5,6,7-Tetrabromo-2-piperidin-1-yl-1H-benzimidazole (**1a**)

Amine: piperidine (3.5g, 41 mmol)). Yield 420 mg (64%), white powder: m.p. 271–273°C (decomp.). $^1$H-NMR (DMSO-$d_6$, δ, ppm): 1.60 (m, 6H, $CH_2CH_2CH_2$), 3.62 (t, 4H, $CH_2$-N, $J$ = 5.5 Hz), 11.6 (s, 1H, H-N). UV (MeOH): 239 (45200), 273 (12900), 316.5 (12600). MS (m/z): 521 (17, $M^+$ + 4,), 519 ($M^+$ + 2), 517 (100, $M^+$), 515 (75, $M^+$ – 2), 513 (18, $M^+$ – 4), 489 (63, $M^+$ – 24). Analysis: calcd. for $C_{12}H_{11}Br_4N_3$ ( 516.86): C, 27.89, H, 2.15, N, 8.13%; found: C, 27.72, H, 2.19, N, 8.04%.

4,5,6,7-Tetrabromo-2-morpholin-4-yl-1H-benzimidazole (**1b**)

Amine: morpholine (3.6 g, 41 mmol). Crystalline product, yield 73%, m.p. 280–282°C (decomp.). $^1$H-NMR (DMSO-$d_6$, δ, ppm): 3.62 (t, 4H, $CH_2$-O, $J$ = 4.2 Hz), 3.71 (t, 4H, $CH_2$-N, $J$ = 5.1 Hz), 11.8 (s, 1H, H-N). UV (MeOH): 237 (38100), 271.5 (12000), 315 (10950). MS (m/z): 523 (12, $M^+$ + 4), 521 (48, $M^+$ + 2), 519 (74, $M^+$), 517 (47, $M^+$ – 2), 515 ($M^+$ – 4), 461 (100, $M^+$ – 58). Analysis: calcd. for $C_{11}H_9Br_4N_3O$ (518.83): C, 25.47, H, 1.75, N, 8.10%; found: C, 25.40, H, 1.84, N, 7.99%.

4,5,6,7-Tetrabromo-2-(4-methylpiperazin-1-yl)-1H-benzimidazole (**1c**, TBIPIP)

Amine: N-methylpiperazine (3.5 g, 35 mmol). Yield 71%, m p. 283°C (decomp.). $^1$H-NMR (DMSO-$d_6$, δ, ppm): 2.22 (s, 3H, $CH_3$), 2.41(t, 4H, $H_2C$-NMe, $J$ = 7.8 Hz), 3.63 (t, 4H, $CH_2$-N, $J$ = 5.1 Hz), 11.8. UV (MeOH): 237 (44100), 272 (13700), 315 (13050). MS (m/z): 534 (4, $M^+$ + 2), 532 (7, $M^+$), 530 (4, $M^+$ – 2), 462 (25, ($M^+$ – 70), 83 (100, $M^+$ – 429). Analysis: calcd. for $C_{12}H_{12}Br_4N_4$ (531.87): C, 27.10; H, 2.27; N, 10.53%; found: C, 27.15; H, 2.36; N, 10.40%.

### General procedure for the preparation of tetraiodobenzimidazoles 2a-2c

A mixture of 2-bromo-,4,5,6,7-tetraiodo-1H-benzimidazole (**2**) (650 mg, 0.85 mmol) and respective amine (35 mmol) in 25 mL of ethanol was refluxed for 12 h. The resulted solution was evaporated to dryness and the residue was crystallized from ethanol to yield the desired product.

4,5,6,7-Tetraiodo-2-piperidin-1-yl-1H-benzimidazole **(2a)**

Amine: piperidine (3.0 g, 35 mmol)). Yield 430 mg (72%) of yellowish powder, m.p. 231–233°C (with $I_2$ evol.). $^1$H-NMR (DMSO-$d_6$, δ, ppm): 1.58 (m, 6H, $CH_2CH_2CH_2$), 3.58 (t, 4H, $CH_2$-N, $J$ = 5.5 Hz), 10.4 (s, 1H, H-N). UV (MeOH): 247 (45200), 277 (sh, 13900), 318 (13500). MS (m/z): 705 (100, $M^+$), 675 (25, $M^+$ – 30), 649 (14, $M^+$ – 56). Analysis: calcd. for $C_{12}H_{11}I_4N_3$ (704.86): C, 20.45; H, 1.57; N, 5.96%; found: C, 20.54; H 1.68; N, 5.87%.

4,5,6,7-Tetraiodo-2-morpholin-4-yl-1H-benzimidazole **(2b)**

Amine: morpholine (3.1 g, 35 mmol). Yield 65%, m.p. 234–236°C (with $I_2$ evol.). $^1$H-NMR (DMSO-$d_6$, δ, ppm): 3.59 (t, 4H, $CH_2$-O, $J$ = 5.1 Hz), 3.70 (t, 4H, $CH_2$-N, $J$ = 5.2 Hz), 10.9 (s, 1H, H-N). UV (MeOH): 247 (23800), 277 (sh, 13800), 317 (13600). MS (m/z): 707 (100, $M^+$), 663 (9, $M^+$ – 44), 649 (21, $M^+$ – 58). Analysis: calcd. for $C_{11}H_9I_4N_3$) (706.83): C, 18.69; H, 1.28; N, 5.94%; found: C, 18.58; H, 1.35; N, 5.84%.

4,5,6,7-Tetraiodo-2-(4-methylpiperazin-1-yl)-1H-benzimidazole **(2c**, TIBIPIP**)**

Amine: N-methylpiperazine (3.2 g, 32 mmol). Yield 62%, m.p. 244–246°C (with $I_2$ evol.). $^1$H-NMR (DMSO-$d_6$, δ, ppm): 2.22 (s, 3H, $CH_3$), 2.41 (t, 4H, $H_2C$-NMe, $J$ = 4.9 Hz), 3.60 (t, 4H, $CH_2$-N, $J$ = 5.0 Hz), 10.4 (bs, 1H, H-N). UV (MeOH): 248 (43700), 277 (sh, 13600), 317 (13800). MS (m/z): 720 (27, $M^+$), 650 (58, $M^+ - 70$), 636 (36, $M^+ - 84$), 83 (100, $M^+ - 437$). Analysis: calcd. for $C_{12}H_{12}I_4N_4$) (719.87): C, 20.02; H, 1.68; N, 7.78%; found: C, 19.90; H, 1.78; N, 7.72%.

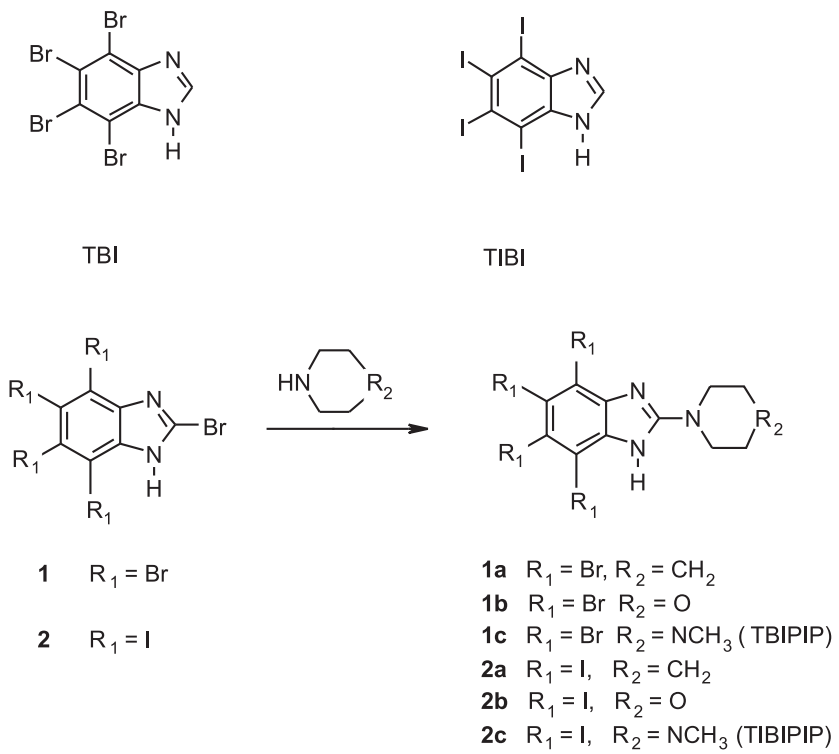
**Antileukemic evaluations**

**Cell culture and compounds treatments**

The HL-60 (human promyelocytic leukemia cells) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in RPMI-1640 with L-glutamine medium (Gibco, Grand Island, NY, USA) with 10% (v/v) heat inactivated fetal bovine serum (FBS; Gibco) and antibiotic–antimycotic solution (Gibco). Cells were grown at 37°C in a humidified atmosphere including 5% $CO_2$. For experiments $2.5\times10^5$ cells/mL in 3 mL medium were seeded in a 6-well plate (Nunc, Denmark). All experiments were performed on cultures in the exponential phase of growth. Cells were incubated with compounds for 24 and 48 h. The compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma) and then diluted in the media for experiments. In all experiments, control cells were incubated with DMSO alone. The concentrations of the studied compounds were in the range from 10 to 50 μM. Cells were subsequently collected, rinsed with cold PBS and prepared for labeling.

**Apoptosis assay by Annexin V/PI labeling**

Apoptosis was measured using an Annexin-V FITC Apoptosis Kit (Invitrogen). The cells were harvested at 24 h and 48 h post-treatment. Cells were subsequently collected by centrifugation, twice rinsed with cold PBS and prepared for labeling. After washing with PBS, $2\times10^6$ cells per mL were suspended in binding buffer on ice. Aliquots of 100 μL of the cell suspension were labeled according to manufacturer's procedure. The Annexin V-FITC (fluorescein isothiocyanate) and PI (propidium iodide) were added to the cell suspension, mixed and incubated for 15 min at room temperature in the




Scheme 1. Synthesis of compounds **1a**-**1c** and **2a**-**2c.**

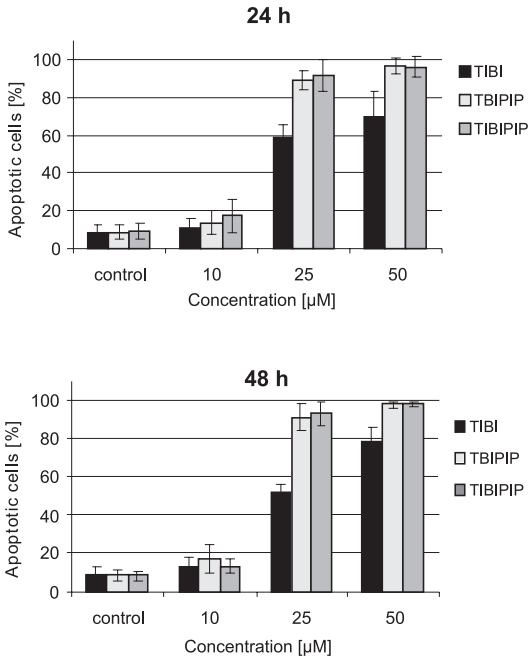



Figure 1. Induction of apoptosis by TIBI, TBIPIP ((**1c**) and TIBIPIP (**2c**) in HL-60 cells. The data were determined by FACS cytometer after 24 h and 48 h treatment. Cells were stained with Annexin V-FITC and PI. The data are expressed as the mean ± SD ($n \geq 3$)

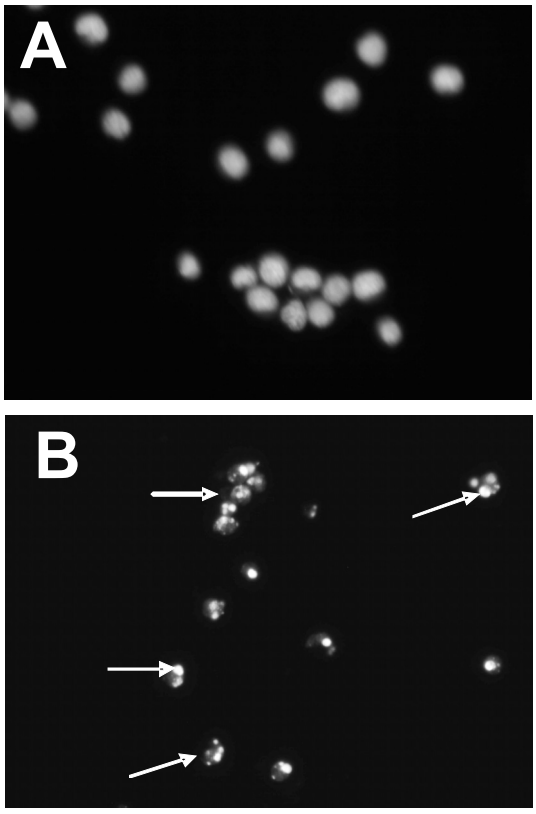



Figure 2. Morphological examination of HL-60 cells stained with DAPI/sulforhodamine 101.
A. Control population. B. Cells treated with 25μM TIBIPIP (**2c**), 48 h. Apoptotic bodies depicted with arrows

dark. Then 400 μL of cold binding buffer was added and cells were vortexed and kept on ice. Flow cytometry measurements were performed within 1 h after staining.

**Morphological evaluation**

Morphology of cells was assessed by fluorescence microscopy. Cells were collected after exposure to the compounds, washed in cold PBS and fixed in ice cold 70% ethanol for at least 24 h, then washed out from alcohol and stained with 1.0 μg/mL DAPI (4',6-diamidino-2-phenylindole) and 10 μg/mL sulforhodamine 101. Cell morphology was evaluated using BX60 fluorescence microscope equipped with digital camera DP50 (Olympus, Japan).

**Mitochondrial membrane potential ($\Delta\Psi_m$) measurement**

The mitochondrial membrane potential was measured by flow cytometry using JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide, SIGMA). Cells were harvested at 48 h post-treatment, suspended in 1 mL of complete culture medium at approximately of $1\times10^6$ cells/mL and incubated with 2.5 μL (1 mg/mL DMSO) JC-1 for 15 min at 37°C in the dark. Stained cells were washed in cold PBS, suspended in 300 μL of PBS and then examined by FACSCalibur flow cytometer.

**Cell cycle analysis**

Cells were collected after exposure to the compounds, washed in cold PBS and fixed in ice cold 70% ethanol (–20°C) for at least 24 h, then washed out from alcohol and stained with 50 μg/mL propidium iodide (PI) and 100 μg/mL RNase in 0.1% PBST solution (PBS supplemented with 0.1% Triton®100) for 30 min in the dark at room temperature. Flow cytometric determinations of DNA content in the cells were performed using flow cytometer.

**Flow cytometry**

Cytometric data were measured using a BD FACSCalibur flow cytometer, analyzed by CellQuest software (BD Biosciences, San Jose, CA, USA) and WinMIDI 2.9 (Joseph Trotter). The DNA

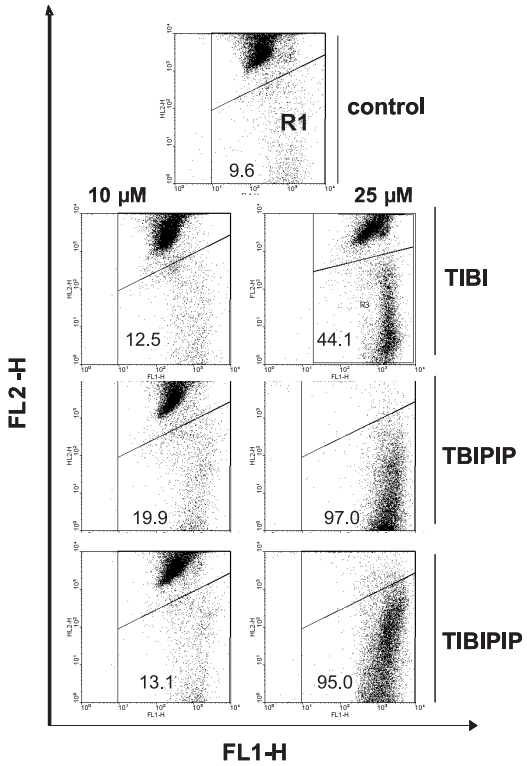



Figure 3. Exemplary changes in mitochondrial membrane potential with the percentage of apoptotic cells (shown in R1 region) in HL-60 cell line after 48 h treatment with TIBI, TBIPIP and TIBIPIP. Stained with JC-1 fluorochrome

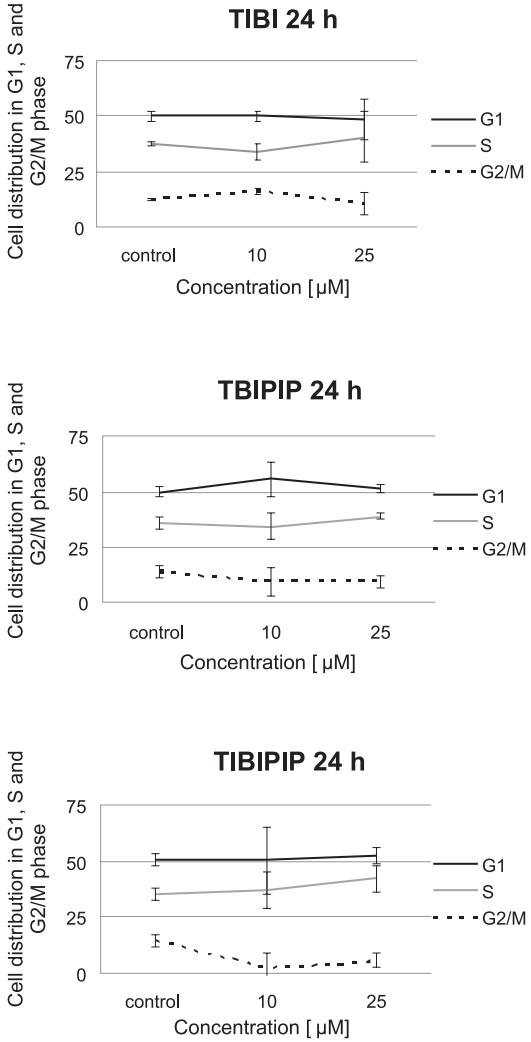



Figure 4. Changes in cell progression in HL-60 after 24 h treatment with TIBI, TBIPIP (**1c**) and TIBIPIP (**2c**). Each point represents the mean ± SD (n ≥ 3). The data obtained from FACSCalibur flow cytometer, were analyzed using MacCycle software to determine the percentage of cells in each phase of the cell cycle

histograms were analyzed using MacCycle (Phoenix Flow Systems, San Diego, CA, USA)

**Data analysis**

Data were presented as the mean values ± SD (standard deviation) (Microsoft Excel, Microsoft, Redmond, WA, USA).

## RESULTS AND DISCUSSION

The synthesis of 2-substituted derivatives of tetrahalogenobenzimidazoles was performed from respective 2-bromoderivatives of 4,5,6,7-tetrabromobenzimidazole (**1**) (19) and 4,5,6,7-tetraiodobenzimidazole (**2**) (18). The prolonged heating of **1** and **2** with piperidine, morpholine and N-methylpiperazine in etanolic solution provided 2-aminoderivatives **1a**-**1c** and **2a**-**2c**, respectively (Scheme 1). The yields after crystallization were between 62% and 74%. The substances structure was determined by $^{1}$H-NMR, UV-spectroscopy, MS spectra and elemental analyses.

**Induction of apoptosis by tested compounds in HL-60 cell line**

Induction of apoptosis by new benzimidazoles were determined using Annexin V/PI labeling 24 and 48 h after the treatment. The examined compounds (TIBI, TIBIPIP, TBIPIP) strongly induced apoptotic death in cell line. The observed apoptotic effects were dose and time dependent (Fig. 1). Among tested compounds, the most effective were new derivatives TIBIPIP and TBIPIP, which at 25 μM concentration and 24 h incubation time induced large apoptotic effect. The higher concentration (50 μM) do not show distinct enhancement of apoptosis.

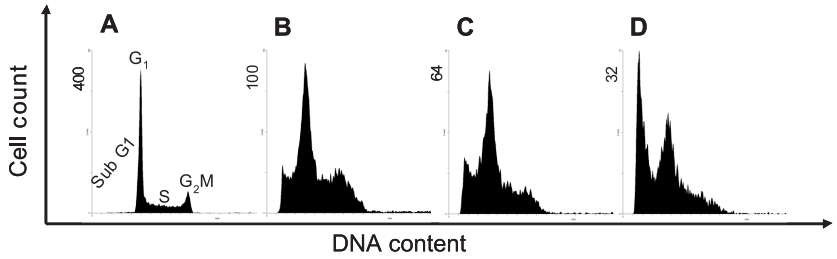



Figure 5. Exemplary DNA histograms HL-60 cells treated for 48 h with TIBI, TBIPIP (**1c**) and TIBIPIP (**2c**). **A.** Control; **B.** 50 μM TIBI; **C**.50 μM TIBIPIP; **D.** 50 μM TBIPIP. The data obtained from FACSCalibur flow cytometer

**Morphological changes in cells**

The effects of the compounds on the morphology of cells were observed 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 appereance of apoptotic bodies) were observed by fluorescence microscopy in the treated cells (Fig. 2).

**The changes in mitochondrial membrane potential ($\Delta\Psi_m$)**

The $\Delta\Psi_m$ was evaluated using JC-1 dye. This dye exhibits potential-dependent accumulation in mitochondria, indicated by red and green fluorescence. In healthy cells, the dye accumulates in mitochondria, forming aggregates that emit red fluorescence (FL-2 channel), in apoptotic cells the dye remains in the cytoplasm as monomers form and emit green fluorescence (FL-1 channel).

Cells, previously treated for 48 h with the series of benzimidazoles, were stained with the dye and then examined by flow cytometer. The analysis of cytograms (Fig. 3) showed that the tested compounds in HL-60 cell line increased mitochondrial membrane depolarization (green fluorescence).

**Effect of tested agents on cell cycle progression**

The examined compounds also influenced the cell cycle progresion of HL-60 leukemia cells after 24 and 48 h incubation time increasing the number of cells mainly in G1, S and at the border of G1/S phases. The accumulation of cells depends on the concentration of tested compounds as shown for TIBI, TBIPIP and TIBIPIP (Fig. 4). The tested compounds at 50 μM concentration and 24 h incubation time as well at 25, 50 μM concentration and 48 h incubation time induced large changes of cell cycle (Fig. 5), therefore, precise statistical analysis DNA histograms by MacCycle computer program was frequently impossible.

## CONCLUSIONS

All three examined compounds (TIBI, TBIPIP, TIBIPIP) exhibited strong proapoptotic and cytostatic effects on promyelocytic leukemia cell line (HL-60). The results with Annexine V/FITC showed that the compounds induced apoptosis (Fig. 1) and apoptotic bodies formation (Fig. 2), evoked mitochondrial outer membrane depolarization and permeabilization (Fig. 3) and influenced cell cycle progression increasing the number of cells mainly in G1, S and at the border of G1/S phases (Figs. 4, 5). Those observations indicated also that the tested compounds are cell permeable drugs. TIBI – the new CK2 inhibitor – as well as two modified benzimidazole derivatives (TBIPIP – **1c,** $IC_{50}$ = 0.33 μM and TIBIPIP – **2c**, $IC_{50}$ = 0.075 μM, F. Meggio, unpublished results) exhibited cytotoxic properties, however, **1c** and **2c** were slightly more potent than TIBI. That may suggests that CK2 activity is important for proliferation of HL-60 cells and CK2 inhibitors are potential antileukemic drugs alone or in combination with other cytostatics.

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