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Apoptosis of murine L1210 leukemia cells induced by 2-acetyl-3-(6-methoxybenzothiazo)-2-ylamino-acrylonitril involves ROS-mitochondrial mediated death signalling and activation of p38 MAPK

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Abstract

Benzothiazoles are multitarget agents with a broad spectrum of biological activity. Some of them are now in cancer clinical testing. 2-acetyl-3-(6-methoxybenzothiazo)-2-ylaminoacrylonitril (AMBAN) is a new synthetically prepared derivative which showed cytotoxic effects on cancer cells in our previous study. The aim of the present study was to examine the cytotoxic/antiproliferative effect of AMBAN and induction of apoptosis on murine leukemia L1210 cells. Further, the molecular mechanism involved in AMBAN-induced apoptosis was investigated. Benzothiazole acted cytotoxically on leukemia L1210 cells and induced apoptotic cell death. AMBAN elevated the level of ROS in time-dependent manner, decreased the mitochondrial membrane potential, activated caspases 9 and 3, induced the cytochrome c release, PARP (poly (ADP-ribose) polymerase) cleavage and led to intranucleosomal DNA fragmentation. It can be concluded that AMBAN induced apoptosis in L1210 cells through mitochondrial/caspase 9/caspase 3-dependent pathway. p38 MAPK and not JNK or ERK was associated with the proapoptotic activity of AMBAN.

Key words: benzothiazole derivative, apoptosis, caspase, reactive oxygen species, leukemia, p38 MAPK

Introduction

Benzothiazole core is a highly important scaffold for drug development because of wide spectrum of its biological activities: antimicrobial, antituberculosis, antileishmanial, antiinflammatory, anticonvulsant, antihistamine, immunomodulatory and neuroprotective (Ballel et al. 2005, Delmas et al. 2004, Russo et al. 2004, Holcombe et al. 2006, Labiche and Grotta 2004). Benzothiazole derivatives have been shown to be useful for treating various diseases including neurodegenerative disorders, local brain ischemia and cancer (Choi et al. 2007). Among the antitumour agents discovered in recent years, the identification of various 2-(4-aminophenyl)benzothiazoles as potent and selective antitumour drugs against breast, ovarian, colon and renal cancer cell lines has stimulated remarkable interest (Trapani et al. 2001). Some of the 2-amino derivatives have been reported to possess cytotoxicity on cancer cells which is comparable to that of cisplatin (Hutchinson et al. 2003). The interesting anticancer properties of these compounds take the initiative of synthesis and anticancer studies of many new benzothiazoles (Yoshida et al. 2005, Sharma and Ghoshal 2006, Kamal et al. 2008, Prasad et al. 2008).

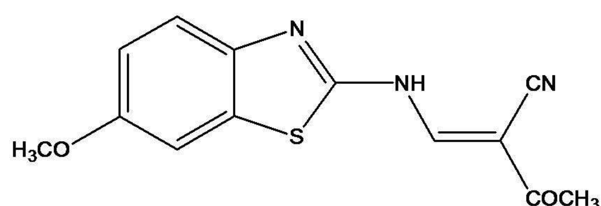
Benzothiazole derivatives are known as inhibitors of topoisomerase II, tyrosine kinase, ubiquitin proteasome system and mitochondrial respiration (Pinar et al. 2004, Yates 1991, Guedat and Colland 2007, Gribkoff and Bozik. 2008). It has been reported that benzothiazoles effectively block glutamate neurotransmission, inhibit huntingtin aggregation in the cell culture, increase intracellular level of Ca^{2+} (Choi et al. 2007). Some benzothiazoles induce cell cycle arrest, activate caspases, target CYP1 and interact with DNA molecule (Trapani et al. 2003, Bradshaw and Westwell 2004, Stojkovic et al. 2006).

To obtain new biologically active agents, a series of substituted nitrogen atom containing heterocyclic compounds was prepared. Some of these compounds showed biological activities towards bacterial, yeast and fungi strains (Černuchová et al. 2005). To evaluate the biological properties of AMBAN in this study, we investigated its effects on cell proliferation and on induction of apoptosis in murine leukemia L1210 cells. In addition, the molecular mechanisms of AMBAN induced apoptosis were investigated with the aim of monitoring the activation of mitogen-activated protein kinases.

Materials and Methods

Materials

2-acetyl-3-(6-methoxybenzothiazo)-2-ylamino-acrylonitril (AMBAN, Scheme 1) was prepared by Černuchová et al. (2005). Chromatographically pure benzothiazole was dissolved in 100% dimethyl sulfoxide (DMSO). Final concentration of DMSO never exceeded 0.1% (v/v) in either control or treated cells. Comparison of the control cells treated with 0.1% DMSO and control cells without DMSO did not show any differences in all experiments. Cell culture media, fetal calf serum (FCS) and antibiotics (penicillin, streptomycin) were obtained from Biocom (Slovakia). All other chemicals used were purchased from Sigma (St Louis, MO, USA).



Chemical structure of the tested derivative:
2-acetyl-3-(6-methoxybenzothiazo)-2-ylamino acrylonitril (AMBAN).

Cells

The murine L1210 leukemia cells (obtained from ATCC, Rockville, MD, USA) were grown in RPMI-1640 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 $\mu g.ml^{-1}$ streptomycin at 37°C in a 5% CO_2 incubator. All experiments were performed in Petri dishes (Ø 60 mm). The cells were plated at the density of $8 \cdot 10^4$ L1210 cells per 1 mL and incubated for 24 h prior to the experiments. Cell viability was determined by 0.4% Trypan blue staining.

The growth inhibition assay

A starting inoculum of $8 \cdot 10^4$ of L1210 cells per 1 mL in the exponential phase of growth was used. After 24 h incubation, AMBAN was applied in final concentrations of 0.37, 3.66, 18.29, 36.59, 91.47 and 182.94 $\mu mol.L^{-1}$. After 24, 48 and 72 h exposure, the number of cells was determined by a direct counting using hemocytometer.

Eletrophoretic determination of apoptosis

Cells treated with 3.66-91.47 μmol.L^{-1} of AMBAN for 24 and 48 h were harvested, washed in PBS and lysed in 100 μL of lysis solution (10 mmol.L^{-1} Tris, 10 mmol.L^{-1} EDTA, 0.5 % (v/v) Triton X-100), supplemented with proteinase K (1 mg.ml^{-1}). Samples were then incubated at 37 °C for 1 h and heated at 70 °C for 10 min. Following lysis, RNAse (200 μg.mL^{-1}) was added and repeated incubation at 37 °C for 1 h followed. The samples were subjected to electrophoresis at 40 V for 3 h in 1.3 % (w/v) agarose gel complemented with ethidium bromide (EtBr).

Determination of apoptosis by fluorescence microscopy

Cells treated with 36.59 and 91.47 μmol.L^{-1} of AMBAN for 24 and 48 h were resuspended in 1 mL of fresh medium and 40 μL of Hoechst 33258 (1 μg.mL^{-1}) and 15 μL of propidium iodide (PI) (5 μg.mL^{-1}) were added to cell suspension and incubated for 30 min at laboratory temperature. Cells were separated by centrifugation, resuspended in 40 μL of fresh medium and monitored by fluorescence microscopy (Zeiss Jenalumar, Jena, Germany).

Caspase 3 activity assay

Cell lysates (from L1210 cells treated with 36.59 and 91.47 μmol.L^{-1} of AMBAN for 24 and 48 h) were prepared and caspase 3 activity was measured sphectrophotometrically according to the manufacturer´s protocol (CaspACE™ Assay System Colorimetric, Promega Corporation, Madison, WI, USA). Briefly, 28 μg of total protein was added to the reaction mixtures containing colorimetric substrate peptides specific for caspase 3 (Ac-DEVD-pNA) at 37°C for 12 h. Absorbance was determined at 405 nm using microplate reader (Humareader, Wiesbaden, SRN). Protein concentration was determined by Lowry et al. (1951).

Detection of caspase 8 and caspase 9 activity

Caspase 8 and caspase 9 activities were measured by means of luminescence assay according to the manufacturer´s protocol (Caspase-Glo® 8/9 Assay, Promega Corporation, USA). Briefly, 100 μL of Caspase - Glo™ 8 Reagent for measuring caspase 8 activity (with specific substrate Z-LETD-aminoluciferin) or 100 μL of Caspase - Glo™ 9 Reagent for measuring caspase 9 activity (with the specific substrate Z-LEHD-aminoluciferin) respectively, were added to the test tube with 100 μL of cell suspension containing 50 000 cells (treated with

36.59 and 91.47 μmol.L^{-1} of AMBAN for 24 and 48 h), mixed and the luminescence signal was measured immediately for 2 h (in 30 min intervals).

Detection of reactive oxygen species (ROS)

The level of ROS was examined using 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA). Cells were treated with 36.59 and 91.47 μmol.L^{-1} of AMBAN for 30-180 min to detect the changes in the levels of ROS. The cells were harvested, washed and resuspended in PBS and 1 μL of DCFH-DA (10 mmol.L^{-1}) was added. The cells were incubated at 37°C for 30 min and analyzed by fluorescence microscopy.

Detection of mitochondrial membrane potential

The changes in the mitochondrial membrane potential were determined using 3, 3´-dihexyloxacarbocyanine iodide ($DiOC_6$). Cells were treated with 36.59 and 91.47 μmol.L^{-1} of AMBAN for 24 and 48 h to detect the changes of mitochondrial membrane potential. The cells were harvested, washed and resuspended in PBS and 4 μL of $DiOC_6$ (40 μmol.L^{-1}) was added. The cells were incubated at 37 °C for 30 min and analysed by fluorescence microscopy.

Western blot analyses

The cells were washed twice with PBS, lysed in lysis buffer (25 mmol.L^{-1} Tris-HCl (pH 7.2), 0.1% SDS, 0.1% Triton X-100, 150 mmol.L^{-1} NaCl, 1 mmol.L^{-1} EDTA, 10 mg.mL^{-1} aprotinin, and 5 mg.mL^{-1} leupeptin) for 30 min on ice, separated by centrifugation and supernatant was collected. Equal amounts of cell lysate proteins were separated on 12 % SDS–polyacrylamide gel and transferred to nitrocellulose membrane. Anti-cytochrome *c*, anti-PARP, as well as anti-ERK1/2, anti-p38 MAPK, anti-JNK1/2, anti-phospho-ERK1/2, anti-phospho-p38 MAPK and anti-phospho-JNK1/2 antibodies were used as primary antibodies. Horseradish-peroxidase-conjugated goat anti-rabbit and anti-mouse IgG were used as the secondary antibodies. The signal was visualized using an enhanced chemiluminescence detection reagents and membrane was exposed to film.

Statistical analysis

Results are shown as the arithmetic means ± s.d. of the mean of three separate experiments. Statistical analysis was performed by the Kruskal-Wallis one-way ANOVA test for nonparametric measurements ($H > 3.86$, $P < 0.05$ were considered statistically significant). The antiproliferative effect was analysed by Friedman´s nonparametric test ($P < 0.0037$ was considered statistically significant).

Results

To evaluate the growth inhibition effect of AMBAN (Scheme 1) towards L1210 leukemia cells, the cells were treated with 0.37-182.94 μmol.L^{-1} of AMBAN for 24-72 h. As shown in Fig. 1, AMBAN induced concentration- and time-dependent inhibition of proliferation. The highest tested concentration of AMBAN (182.94 μmol.L^{-1}) had an acute cytotoxic effect manifested by immediate degeneration of L1210 cells. AMBAN concentrations 91.47 and 36.59 μmol.L^{-1} induced delayed cytotoxic effects. AMBAN inhibited cell growth with an IC_{50} within the range of 5.6-11.89 μmol.L^{-1} (Table 1).

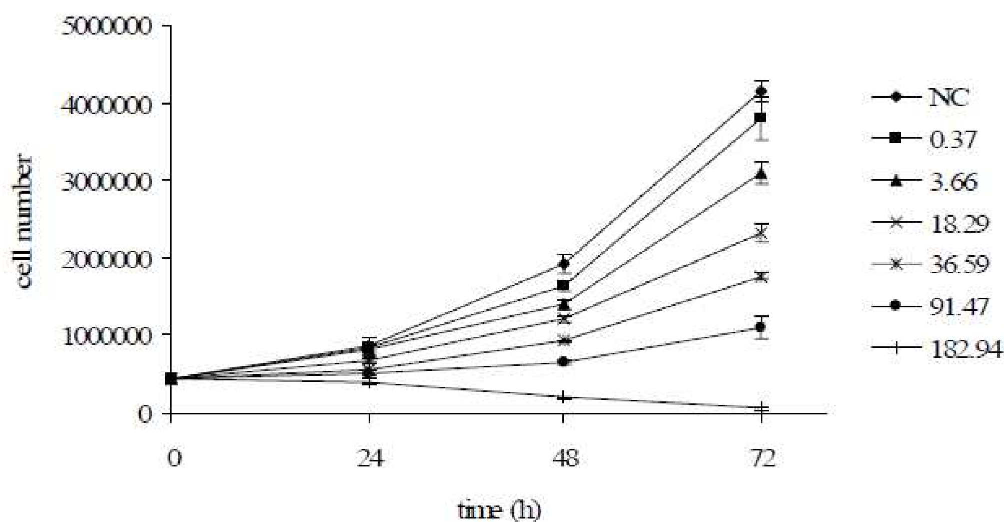


Fig.1. Growth curve of AMBAN-treated L1210 cells in the course of 72 h. NC – control cells treated with 0.1% DMSO. Concentrations of AMBAN: 0.37-182.94 μmol.L^{-1}. Each point presents the mean ± SD of five experiments.

Table 1. Cytotoxic activity of AMBAN on L1210 cells. The values of IC_{50} and IC_{100} are in µmol.L^{-1} and represent means ± SD of three independent experiments

Time (h)	24	48	72
IC_{50}	11.89 ± 1.23	8.20 ± 0.94	5.60 ± 0.89
IC_{100}	182.94 ± 9.61	182.94 ± 8.75	182.94 ± 10.11

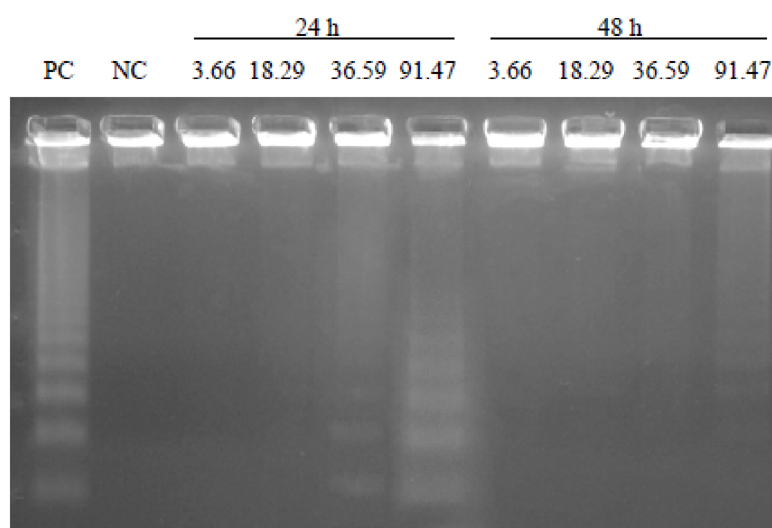


Fig.2. Electrophoretic detection of intranucleosomal fragmentation of cellular DNA after 24, 48 and 72 h treatment of L1210 cells with AMBAN. PC - positive control (6 µmol.L^{-1} cisplatine), NC - control cells treated with 0.1 % DMSO. Concentrations of derivative: 3.66 – 91.47 µmol.L^{-1}.

Cells treated with different concentrations of AMBAN (3.66-91.47 µmol.L^{-1}) for 24 and 48 h were subjected to agarose gel electrophoresis to assess apoptotic DNA fragmentation. The fragmentation observed was concentration-dependent (Fig. 2). Apoptosis of cells treated with AMBAN was further confirmed by Hoechst / PI double staining and western blot analysis by detection of fragments of PARP (3.66-91.47 µmol.L^{-1}) (Fig. 3 and Fig. 7).

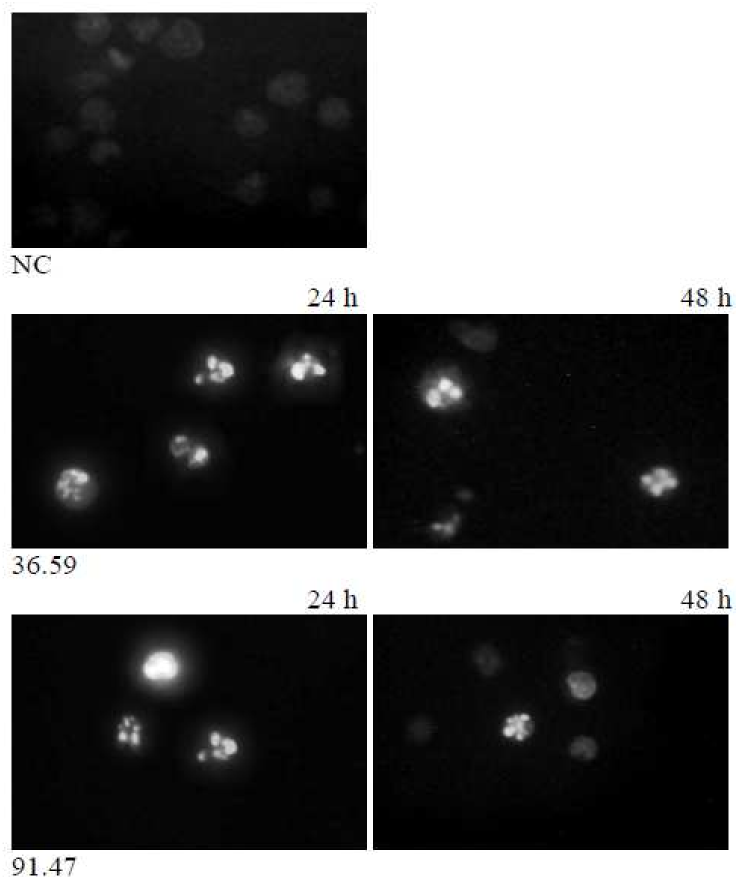


Fig.3. Detection of apoptosis in L1210 cells treated for 24, 48 and 72 h with AMBAN by fluorescent microscopy. NC - control cells treated with 0.1% DMSO. Concentrations of derivative: 36.59 and 91.47 μmol.L^{-1}. Magnification of microscope: 600.

To characterise the molecular events involved in AMBAN-induced apoptosis, we examined the AMBAN-induced activation of caspase 3 and caspases 8 and 9 by monitoring of increasing spectrophotometric or luminescence signals, respectively. As presented in Fig. 4, AMBAN induced concentration- and time-dependent activation of caspase 3 and caspase 9, with slight increase in caspase 8 activity.

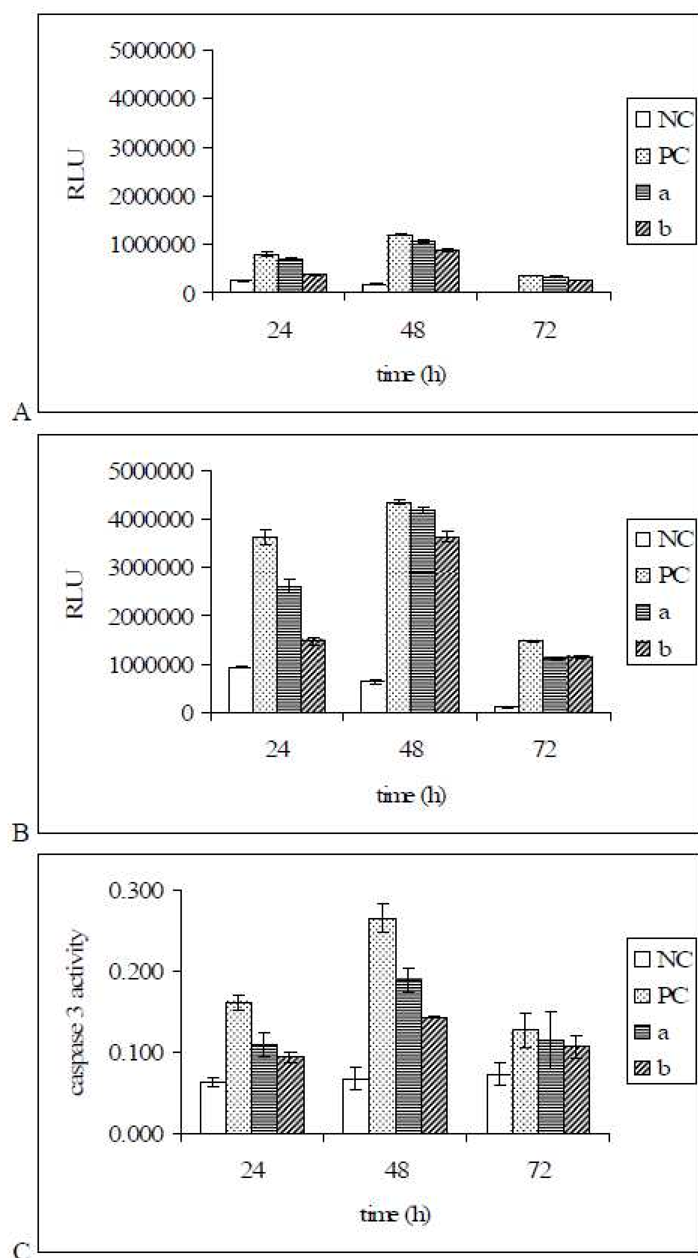


Fig.4. Activity of caspase 8 (A), caspase 9 (B) and caspase 3 (C) after 24, 48 and 72 h treatment L1210 cells with AMBAN.
NC – control cells treated with 0.1% DMSO, PC - positive control with 6 μmol.L^{-1} cisplatine. Concentrations of AMBAN: 91.47 (a) and 36.59 μmol.L^{-1} (b).

To evaluate the ROS production in AMBAN-treated cells, the intracellular ROS level was monitored using intracellular peroxide-dependent oxidation of DCFH-DA to form fluorescent DCF. DCF fluorescence was detected in cells treated with 36.59 and 91.47 μmol.L^{-1} of AMBAN for 2 h (Fig. 5). Increased level of ROS is connected with reduced mitochondrial membrane potential.

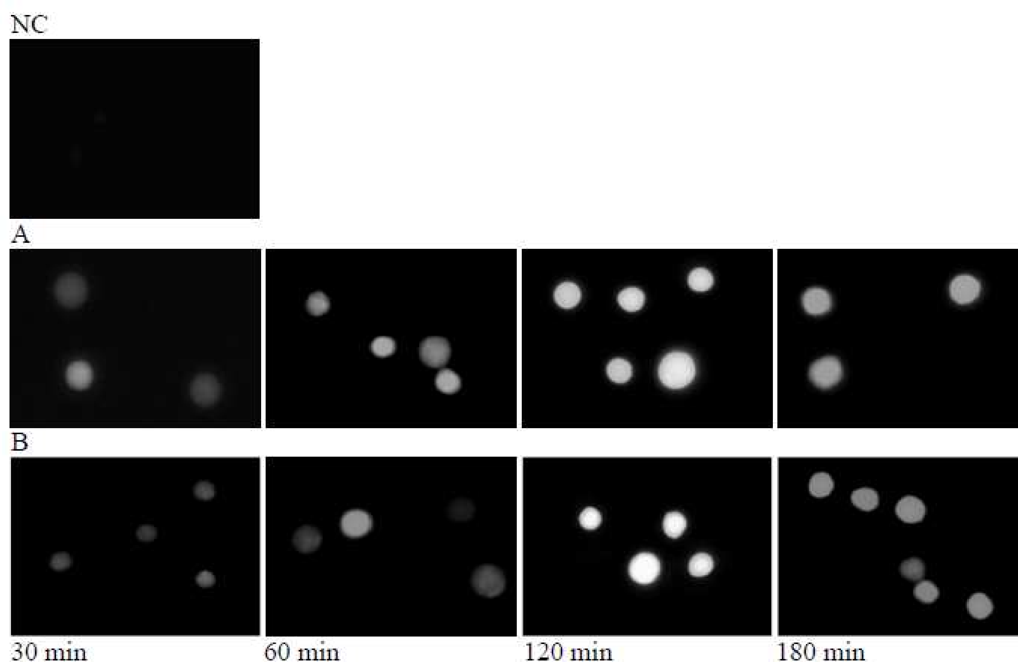


Fig.5. Effect of AMBAN concentrations of 91.47 (A) and 36.59 (B) $\mu mol.L^{-1}$ on ROS production in L1210 cells analysed by fluorescent microscopy using H_2DCFA. Cells were treated with derivative for 30 – 180 min. NC - control cell treated with 0.1% DMSO. Magnification of microscope: 600.

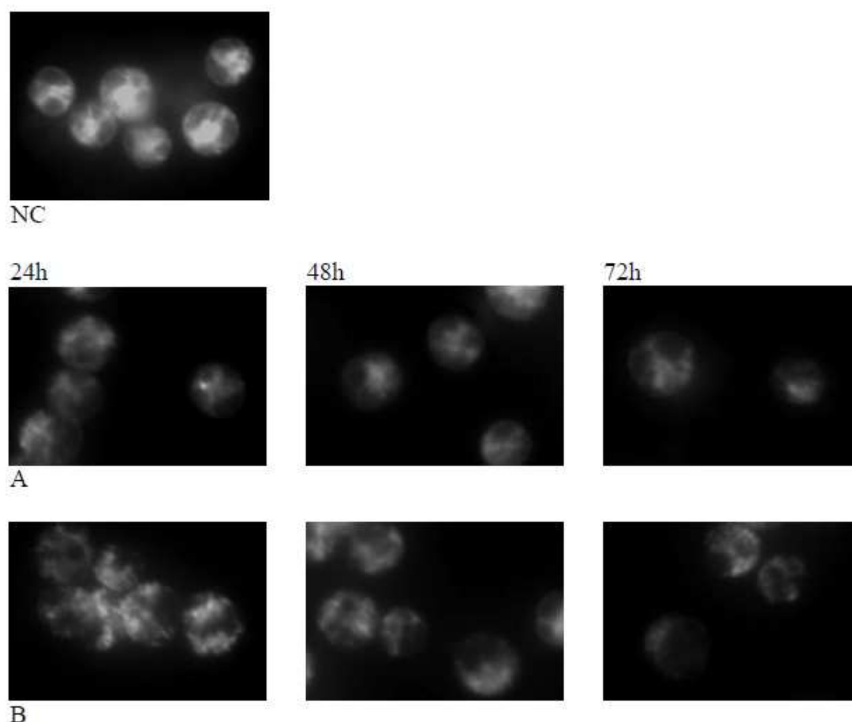


Fig.6. Fluorescent microscopy detection of mitochondrial membrane potential changes in L1210 cells treated with AMBAN using $DiOC_6$. Cells were treated for 24, 48 and 72 h with concentrations of 91.47 (A) and 36.59 (B) $\mu mol.L^{-1}$. NC - control cells treated with 0.1% DMSO. Magnification of microscope: 800.

Thus, in the next experiments we examined the AMBAN-treated cells for changes in mitochondrial membrane potential using fluorescence sensitive probe $DiOC_6$. As shown in the Fig. 6, AMBAN decreased mitochondrial membrane potential of treated cells. Western blot analysis confirmed the increased level of cytochrome *c* in cytosolic fractions of AMBAN-treated cells (Fig. 7).

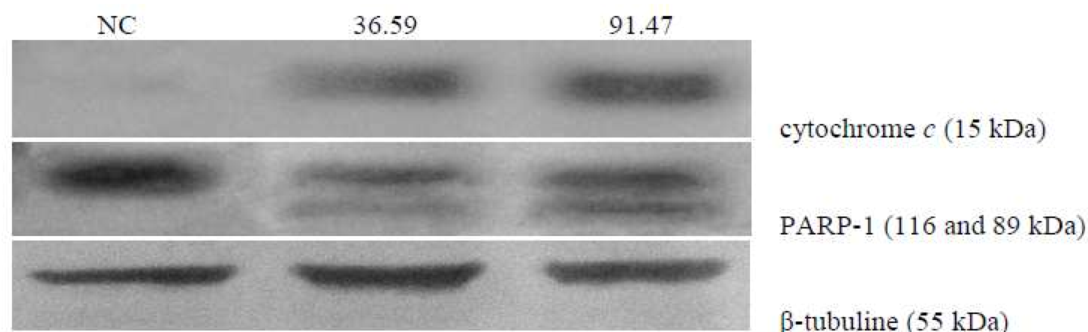


Fig.7. Detection of cytochrome *c* release and PARP-1 degradation in L1210 cells treated for 24 h with AMBAN concentrations of 36.59 (A) and 91.47 (B) $\mu mol.L^{-1}$. NC - control cells treated with 0.1% DMSO. β-tubuline- control for protein measurement.

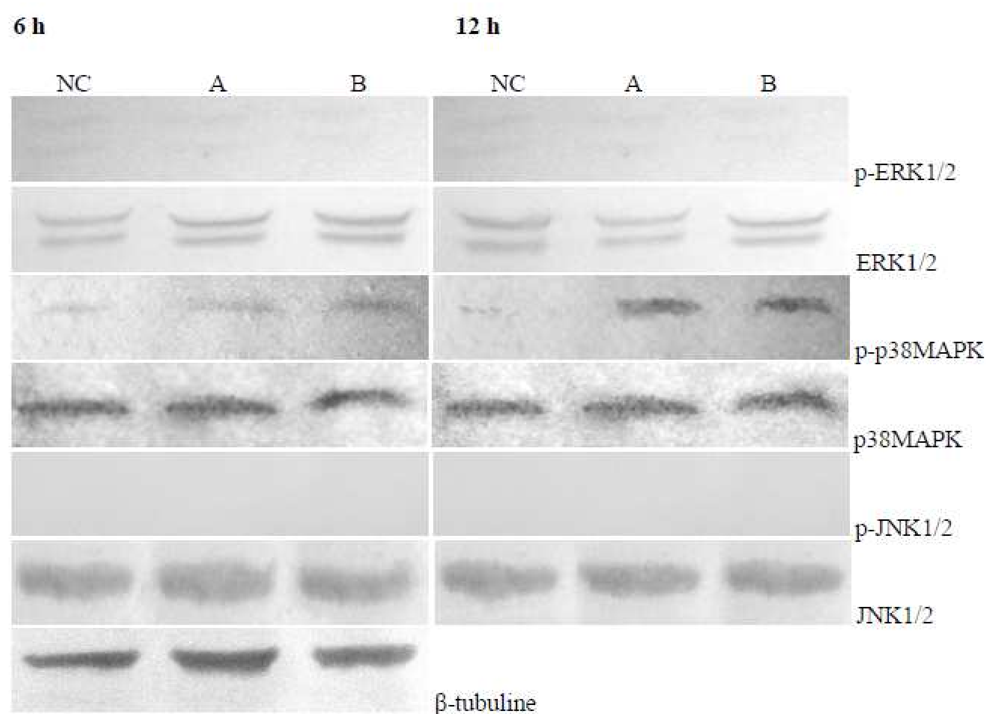


Fig.8. Detection of activation of ERK1/2 (44 and 42 kDa), p38MAPK (38 kDA) and JNK1/2 (46 kDa) in L1210 cells after treatment with AMBAN concentrations of 36.59 (A) and 91.47 (B) $\mu mol.L^{-1}$. NC - control cells treated with 0.1% DMSO. β-tubuline (55 kDa) - control for protein measurement.

To assess the role of MAPK in AMBAN-induced apoptosis, we treated the cells with different concentrations of AMBAN for various times and the phosphorylation of extracellular-regulated ERK1/2, stress activated p38 MAPK and JNK1/2 kinases were examined by western blot analysis. As shown in Fig. 8, activation of p38 MAPK was evident after 6 h and was maximal after 12 h. No significant increase in phosphorylation of ERK1/2 or JNK1/2 was observed.

Discussion

With the aim of obtaining new anticancer agents, a series of substituted nitrogen atom containing heterocyclic compounds was prepared. Our previous experiments revealed that some of these agents are potent drugs towards bacterial, yeast and fungi strains. Interestingly, 2-acetyl-3-(6-methoxybenzothiazo)-2-ylamino-acrylonitril (AMBAN) did not possess antimicrobial activity. Instead, this agent manifested a significant antiproliferative activity towards leukemia cells (Černuchová et al. 2005). This finding prompted us to study the effect of AMBAN on both cell growth and induction of apoptosis in murine leukemia L1210 cells, and to elucidate the molecular mechanisms involved in AMBAN-induced apoptosis in more details, with the emphasis on activation of caspases and protein kinases cascades.

We found out that AMBAN caused different cytotoxic effects depending on concentration and time of exposure (Fig. 1, Table 1). This derivative induced apoptotic cell death of L1210 cells at concentrations of 36.59 and 91.47 $\mu mol.L^{-1}$, as it is obvious from monitoring of internucleosomal DNA fragmentation of AMBAN-treated cells (Fig. 2). In additition, the apoptotic bodies formation was observed in drug treated cells (double staining with Hoechst 33258 and PI) confirming the potential of AMBAN to induce apoptosis in L1210 cells (Fig. 3). To reveal the molecular mechanisms involved in AMBAN-induced apoptosis in L1210 cells, in the next experiments we studied its effect on caspases activities. As shown in Fig. 4, cells treated with AMBAN had activated caspase 9 and caspase 3 due to released cytochrome *c* from mitochondria (Fig. 7) when mitochondrial membrane potential decreased (Fig. 6). The programmed cell death was finalized by PARP degradation (Fig. 7) and internucleosomal DNA fragmentation (Fig. 2). Our findings are in agreement with previous studies showing that different benzothiazole derivatives can induce apoptosis of cancer cells (Zhu et al. 2002, Pietrancosta et al. 2006).

Recently, it has been demonstrated that not only signals from membrane surface receptors but also oxidative stimuli occurring in cytoplasm may be transmitted through cellular signal transduction pathways to the nucleus for the control of cell division and survival (Kurata 2000, Park and Kim 2005). To reveal if AMBAN influenced the level of ROS, we stained drug treated cells with DCFH-DA. As presented in the Fig. 5, this derivative significantly increased the levels of ROS. Furthermore, the activation of stress activated protein kinase p38 MAPK was observed (Fig. 8). These findings emphasised that AMBAN induced apoptosis in L1210 cells through p38 MAPK activation, the crucial mitogen activated protein kinase that is involved in signal transduction machinery for regulation of cell growth, differentiation and apoptosis (Park and Kim 2005).

In summary, we can conclude that newly prepared 2-acetyl-3-(6-methoxybenzothiazo)-2-ylaminoacrylonitril (AMBAN) possesses a significant antiproliferative activity and is a potent inducer of programmed cell death in leukemia cells through mitochondrial/caspase 9/caspase 3-dependent pathway.

Acknowledgements

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