**Introduction**

F-actin (fibrillar actin) is one of the cytoskeletal proteins which forms microfilaments. Cytoskeletal proteins regulate many crucial cell functions, such as growth, proliferation, differentiation, movement or transport [1]. Despite indisputable F-actin occurrence in the cytoplasm, localization of polymerized form of actin within the area of nucleus still raises many controversies. G-actin (globular actin), which is the precursor for F-actin, is present in the nucleus and it's responsible for transcription factors and RNA polymerases regulation. Some data indicate that the pool of nuclear G-actin is insufficient for polymerization to F-actin. On the other hand, F-actin was found in SWI/SNF chromatin remodeling complexes [2,3] and in Xenopus oocytes, during the meiotic maturity [4]. It is probable that nuclear actin regulates the activity of histone acetyltransferase, ATPase present in chromatin remodeling complexes and that it controls functions of transcription factors from the myocardin/MAL family, with filaments polymerization and depolymerization being directly involved in these processes [4-6].

Arsenic trioxide (As$_2$O$_3$, ATO) is well known chemical compound and despite its high toxicity, as early as in fifteenth century it was known that in low doses it has therapeutic properties [7]. In the beginning, medications based on arsenic trioxide, were applied mostly superficially, as ointments for skin cancers, however in the '70s of twentieth ATO was started to use in intravenous infusions [8-9]. Nowadays, As$_2$O$_3$ is known to be efficient in the treatment of acute promyelocytic leukemia and myelodysplastic syndromes (MDS) [10-11]. It was also started to be used in cases of acute myeloid leukemia, multiple myeloma and solid carcinoma [9]. In last years studies over the effect of ATO has mainly included experiments on cell lines, i.a. glioma, prostate, ovarian, gallbladder, intestinal and cervical cancers [12-16]. There are ongoing clinical trials to

### Abstract:
Actin is one of the cytoskeletal proteins that take part in many cellular processes. The aim of this study was to show the influence of Trisenox (arsenic trioxide), on the cytoplasmic and nuclear F-actin organization. Arsenic trioxide is the proapoptotic factor. Together with increasing doses, it caused the increase in the number of cells undergoing apoptosis. Under arsenic trioxide treatment, cytoplasmic and nuclear F-actin (polymerized form of G-actin) was found reorganized. It was transformed into granulated structures. In cytometer studies fluorescence intensity of cytoplasmic F-actin after ATO treatment decreasing urgently in comparison to control. The obtained results may suggest the involvement of F-actin in apoptosis, especially in chromatin reorganization.

**Key words:** F-actin; K562; arsenic trioxide (Trisenox); apoptosis, fluorescence; flow cytometry

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**Effect of arsenic trioxide (Trisenox) on actin organization in K-562 erythroleukemia cells**

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study the application of this compound in the treatment of circulatory and lymphatic system proliferative diseases. It is used not only in the monotherapy but also in combination with other compounds, i.a. retinoid acid, ascorbic acid and GM-CSF [17-19]. The impact of ATO bases mainly on the inhibition of proliferation and introduction of cancer cells on the route of programmed cell death. Its apoptotic effect has been proved for many years, however scientists all over the world are still proposing its new mechanisms. It is suggested that arsenic trioxide may be involved in the effect on the activity of JNK kinases, NFkB transcription factor, glutathione, caspases as well as pro- (Bax, Bak, Bid) and antiapoptotic proteins (Bcl-2, Bcl-XL) [20-24]. It was demonstrated by Chen et al. [25] that ATO may have an apoptotic effect, by degradation of the PML/RARα fusion protein and downregulation of bcl-2 expression. On the contrary, a suggestion exists that ATO can modulate the glutathione redox system and it is independent of PML/RAR? and it does not require downregulation of bcl-2 expression [8]. The aim of this work was to study F-actin organization in whole cells and nuclei isolated from cells undergoing apoptosis, induced by Trisenox which contains arsenic trioxide as active substance, and it is used in leukemia treatment.

Materials and methods

Cell Culture. Study material was K-562 cell line (human erythroleukemic cell line ATCC CCL 243). Cells were grown in RPMI 1640 medium supplemented with 10% (m/v) fetal bovine serum (FBS) and antibiotics, at 37°C in a 5% CO2 atmosphere. Apoptosis was induced by Trisenox (Cephalon, UK) – drug used in cases of acute promyelocytic leukemia, which contains As2O3 (1 mg/ml). Trisenox is water-soluble form of ATO, which includes additional factors adjusting pH. There were used ATO doses of 0.6, 1.2 and 2.4 µg/ml of medium. Cells were incubated with the cytotastic for 24 hours. Control cells were grown in the same conditions, without the addition of Trisenox to the medium.

Nuclei isolation. Cellular precipitation was suspended in homogenizing environment of: 0.5 M Tris-HCl (pH 7.5); 1 M saccharose; 0.5 M CaCl2; 0.5 M MgCl2; 2-mercaptoethanol; Nonidet. The environment-suspension ratio was 5:1. Homogenate was crashed with Teflon piston in glass homogenizer on ice and than poured to centrifugal tubes and centrifuged at 700g (10 min, 2°C). Precipitation was suspended in 1ml of homogenizing environment, without Nonidet and slowly overlaid on present in centrifugal tube cooled in liquid nitrogen. The PI/RNase solution used to detect stage of cell cycle was added succeeding TUNEL-FITC labeling. Further analysis was performed on a Becton Dickinson FACSScan.

Flow cytometry

Cell cycle analysis. Apoptosis was detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) technique using the APO-DIRECT kit (BD Bioscience, Pharmingen) according to the instruction of the supplier with following modifications in fixation. Cells were fixed on ice for 15 min with 1% formaldehyde and permeabilized in 70% ethanol for 30 min on ice. The PI/RNase solution used to detect stage of cell cycle was added succeeding TUNEL-FITC labeling. Further analysis was performed on a Becton Dickinson FACSScan. For cell cycle analysis doublets were excepted from the final analysis using linear plots FL2-A towards, to FL2-W. Cells were identified as G0/G1, S, G2/M and cells with a DNA content of more than 4n and less than 2n (apoptotic DNA). By using FlowJo cell cycle analysis software (Tree Star, USA) were determined the percentages of cells in G1, S, G2/M cell cycle phases.

Apoptosis. Staining with Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, San Diego, CA) was used to determine the apoptosis, employed according to manufacturer's instructions. Nuclei were counterstained with propidium iodide. The supernatant was removed and annexin V FITC were added. The cell were incubated for 15 min and then centrifuged. Further supernatant removal, binding buffer and PI (propidium iodide), were added to the cell. Then, the cells were incubated for 5 min. Further analysis was performed with the use of Becton Dickinson FACSScan.

F-actin content. Cells were fixed with 4% paraformaldehyde (20 min at 4°C) and afterwards rinsed with PBS and permeabilized with 0.1 % Triton X-100 (3 min). After washing in PBS, Alexa

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Fluor 488-phalloidin (Molecular Probes, Eugene, Oregon, USA) was used to stain the material (20 min, RT). Becton Dickinson FACScan was used to subsequent analysis.

Statistical analysis. STATISTICA 6.0 for Windows (StatSoft software, Tulsa, OK, USA) was used for the statistical analysis. All comparisons based on ANOVA Repeated Measures Designs and were presented as Duncan’s test results.

Results
Arsenic trioxide induces apoptosis, even with the lowest dose. Number of apoptotic K-562 cells increases together with the dose of used compound. In control cells the level of apoptosis is minimal and it does not exceed 2.5%. However the population of apoptotic cells rises to 24.7% with 0.6 μg/ml, 33% with 1.2 μg/ml and 42% with 2.4 μg/ml dose of ATO used (Fig.1). Under the influence of ATO F-actin undergoes reorganization, both in whole cells and in isolated cellular nuclei. In control cells F-actin is localized mostly at peripheries, but is also present in cytoplasm, in the form of network and granulated structures (Fig. 2A). Nuclei does not show apoptotic features (Fig. 2A DAPI). After the treatment with the lowest arsenic trioxide dose (0.6 µg/ml) the number of cells significantly decreases and F-actin – besides the localization in cytoplasm – is present mainly at cells peripheries, where it forms granulates. DAPI staining reveals apoptotic features within the nucleus (Fig. 2B). The use of medium dose (1.2 µg/ml) causes rapid decrease in cell number and F-actin placement at cells peripheries and the highest dose of 2.4 µg/ml induces evident granulation of F-actin present at cells edges (Fig. 2 C,D).

Quantitative studies, performed with flow cytometer, show that the level of F-actin in whole cells is changing after the application of ATO (Fig. 3, 4). In control cells fluorescence intensity is order of 2500, however after application of the lowest ATO dose in about 60% of cells population decreasing to value near 200. Remaining 40% of cells is also demonstrating lower fluorescence level. In dose 1.2 μg/ml is increasing cell number with lower level of F-actin fluorescence, however in comparison to the lowest dose (0.6 μg/ml) fluorescence intensity is growing slightly. Analysis of 2.4 μg/ml dose also display not significantly grow of F-actin fluorescence in comparison to previous dose, but cell number with fluorescence on very high level achieve only 6%. There were statistically significant differences (Duncan’s test, p<0.05). Isolated nuclei also show F-actin reorganization (Fig.5). In comparison to control cells nuclei, where it is present as a network, mainly at nucleus peripheries, the lowest dose forces it to form granulates and with the medium dose used, this granulation is evident. Nuclei were not isolated during highest ATO dose treatment, as there was a large percentage of apoptotic cells, showing their disintegration. Besides studies, showing the percentage of apoptotic cells, it was also tested the cell division cycle. Results points out the increased number of cells in G1 phase and decreased in G2 phase, after ATO treatment, in comparison to control (Fig. 6).

Discussion
The apoptotic effect of arsenic trioxide has been known for years. This cytostatic is used in the treatment of acute promyelocytic leukemia (Trisenox drug) and it also induces programmed cell death in non-leukemic cell lines, i.a. breast, ovarian, prostate and liver cancers, multiple myeloma, glioma and gallbladder [11-16,20,26-29]. Results of present work, are similar to those obtained by Wang et al. [30] and Shim et al. [31], which show apoptotic effect of ATO on K-562 cell line. During the apoptosis, cytoskeleton undergoes reorganization. Previous works, with the use of other
proapoptotic factors points changes in all of these structure proteins. The aim of present work was to show arsenic trioxide influence on one of the elements of cytoskeleton – actin filaments. Results indicate on F-actin reorganization. In control cells actin filaments form network in the cytoplasm and they are localized at cells peripheries, whereas with the addition of cytostatic they transform into granulated structures also placed in the peripheries. This effect is getting stronger together with the increase of ATO dose. Previous studies, described by Shen et al. also point reorganization of F-actin structure under influence of ATO, in SHEE (embryonic esophageal epithelium) cells [32]. They show changes in F-actin organization, from fibrillar structures within whole cytoplasm of control cells to punctual structures in SHEE cells, shrinked after arsenic trioxide. Similar results of F-actin reorganization due to the application of other cytostatics i.a. taxol, doxorubicin and growth factors (ATRA, G-CSF) are described [34-35]. F-actin accumulation on cells

Fig. 3.4. Flow cytometric analysis of F-actin content in K-562 cell line after ATO treatment. Cells were treated with ATO for 24h at concentrations: 0.6, 1.2 and 2.4 µg/ml. Fluorescence intensity analysis of F-actin in K-562 showed changes of fluorescence intensity.
edges is probably related to its participation in the formation of apoptotic blebs [36]. Those structures are characteristic for apoptosis and after cell disintegration they form so-called apoptotic bodies, containing cellular organelles and remains of chromatin and cytoplasm. Many researchers, from all over the world, point this protein participation in the formation of apoptotic structures. Keller et al. [37] reveal local reorganization of F-actin, while the creation of apoptotic blebs, whereas Cotter et al. [38] point necessary actin polymerization during the formation of apoptotic blebs. F-actin rich labeling in places of formation was observed also after the use of doxorubicin and taxol, in CHOAA8, HL-60 and K-562 cell lines [36,39]. Described results indicate the indisputable participation of skeleton structures in apoptosis, despite the fact, that some researchers, e.g. Olins [40], suggest F-actin involvement only in differentiation process. Cytometric studies of F-actin level shows its decrease, after the use of proapoptotic factor, in comparison to control. Lower level of studied protein was also observed by Shen et al. [32], after ATO application in SHEE cells and by Veselska et al. [34], who used ATRA in HL-60 cell line. The presence of F-actin in the cytoplasm is indisputable, but there has been a dispute for years, on this protein localization in nucleus. F-actin presence in nuclei was described by Grzanka et al. [33,39]. They suggested its role in chromatin reor-

Fig. 5. Fluorescence microscopy studies of F-actin localization changes in isolated nuclei after ATO treatment in K-562 cells. In isolated nuclei from control cells F-actin was formed network localized mainly in nucleus periphery. After ATO treatment F-actin was formed granular structures. A – control (F-actin, DAPI); B – 0.6 µg/ml (F-actin, DAPI); C – 1.2 µg/ml (F-actin, DAPI).

Fig. 6. Flow cytometric cell cycle analysis of K-562 cells after ATO treatment. Cells were treated with ATO for 24h at concentrations: 0.6, 1.2 and 2.4 µg/ml. Cell cycle analysis showed the increase in percentage of G1 and decrease in G2 phase cells.
organization during the creation of morphological features, characteristic for apoptotic cells. During the present study it was revealed that the use of proapoptotic factor caused chromatin condensation, thus F-actin formed aggregated structures. Rapid changes, taking place in the nucleus area and F-actin participation in the reorganization of chromatin, are probably responsible for F-actin intensity level within this organelle. It was pointed by Zhao et al. [2], that F-actin is directly involved in chromatin reorganization, by presence in BAF complex, where it acts as a nucleotides shifting factor [2,41,42]. It was also told that ANC-1 (domain, binding actin with the nucleus) and UNC-84 protein are responsible of connecting actin with nucleus [43]. Widlak et al. point actin influence on translocation of transcriptional RNA and in determination of the chromosomal area, which limits the mobility of chromatin fragments [42]. Other scientists, like Hoffman et al., describe F-actin presence in the process of retroviral RNA and protein kinase inhibitor (PKI) transportation, due to nuclear actin association with nucleoplasmatic filaments in nuclear pore complex. Presented in present work, nuclear reorganization of F-actin under influence of ATO is probably connected with above-cited processes. Flow cytometry, besides experiments pointing F-actin level and cells percentage after ATO treatment, was also used to study cell cycle. The use of ATO causes the increased number of cells on G1 phase in compare to control, and decreased in G2. Similar results were obtained by Zhang et al. [45] who studied HL-60 and Shao et al. [29] with human gastric carcinoma cells. Other researchers, e.g. Liu [28] and Park [46] point cells arrest in G2/M phase, after the use of ATO, however they emphasize that it depend on used cell line, dose of cytostatic and time of incubation. Studies conducted on seven myeloma cell lines indicate that it is also dependent on whether the cell line is of wild type or it has genes mutation, i.e. p53 or p21.

In conclusion, results, obtained in this work, indicate on reorganization of F-actin, both cytoplasmic and nuclear, after ATO treatment, in comparison to control cells. ATO is also a proapoptotic factor and in doses given in the article, it causes K-562 cells stoppage in G1 phase of cell cycle.

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References


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