DOI: http://dx.doi.org/10.12775/MBS.2014.010

Medical and Biological Sciences, 2014, 28/2, 11-17

ORIGINAL ARTICLE / PRACA ORYGINALNA

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THE INFLUENCE OF DOXORUBICIN ON NUCLEAR AND CYTOPLASMIC POOL OF F-ACTIN IN THE A549 CELL LINE

WPŁYW DOKSORUBICYNY NA JĄDROWĄ I CYTOPLAZMATYCZNĄ PULĘ F-AKTYNY W LINII KOMÓRKOWEJ A549

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Summary

The cytoskeleton as an intracellular system plays an important role in the proper functioning of the cell. F-actin is one of the components, which built this structure. Microfilaments are involved in cell shape maintenance, polarity and cell motility. The aim of the present study was to determine the effect of doxorubicin on the actin reorganization and type of induced cell death in A549 cell line. In order to examine F-actin, the material was evaluated by the confocal and classical fluorescence microscope. Furthermore, changes in morphology and ultrastructure were analyzed by a light and transmission electron microscopy. The obtained data showed that doxorubicin causes a dose-dependent decrease in A549 cell viability. Moreover, the treatment with doxorubicin resulted in the reorganization of F-actin as well as the induction of apoptosis and mitotic catastrophe in the non-small lung cancer cells. In addition, the existence of actin in the nucleus was confirmed.

Streszczenie

Cytoszkielet stanowi międzykomórkowy system, odgrywający istotną rolę w prawidłowym funkcjonowaniu każdej komórki. Jednym z jego komponentów jest F-aktyna, zaangażowana w zmiany kształtu, polarność, a także ruch komórek. Celem przedstawionej pracy było określenie wpływu doksorubicyny na reorganizację cytoszkieletu aktynowego oraz rodzaj indukowanej śmierci w komórkach linii A549. Wyniki badań oceniano przy użyciu klasycznego oraz konfokalnego mikroskopu fluorescencyjnego, a także z wykorzystaniem mikroskopii świetlnej oraz transmisyjnej mikroskopii elektronowej. W toku badań wykazano dawkozależną wrażliwość komórek linii A549 na doksorubicynę. Wraz ze wzrostem cytostatyku, wzrastał odsetek komórek martwych. Ponadto stwierdzono, że doksorubicyna powoduje zmiany w reorganizacji F-aktyny w komórkach niedrobnokomórkowego raka płuca, a także może indukować apoptozę oraz katastrofę mitotyczną. Dodatkowo potwierdzono występowanie aktyny na terenie jądra komórkowego.

Key words: A549 cell line, doxorubicin, cell death, apoptosis, mitotic catastrophe, F-actin
Słowa kluczowe: linia komórkowa A549, doksorubicyna, śmierć komórki, apoptoza, katastrofa mitotyczna, F-aktyna

INTRODUCTION

The cytoskeleton as an intracellular system plays an important role in the proper functioning of the cell [1]. It is involved in numerous cellular processes and activities, including cell motility, intracellular transport, cell division and cell death [1,6,9]. The main cytoskeletal structures are actin filaments (microfilaments), intermediate filaments and microtubules [1]. The actin cytoskeleton occurs as a G-actin (globular actin, molecular weight 42kDa) and the F-actin (filamentous actin). F- actin is a polarized structure, consisting of two chains helically coiled around each other, which are built by G-actin subunits [1,6,9]. Actin filaments create mainly two types of networks within the cell. The first type is flat or two-dimensional and the other one is three-dimensional. Additionally, microfilaments can form structures such as: microvilli lining the intestine, epithelial cells, shrinkable rings. Microfilaments are also a part of sarcomere. The actin cytoskeleton is present in all eukaryotic cells [1,5,20]. The existence of actin inside the nucleus has been highly controversial for many years [4,9]. In 1978 Clark and Merrium described a protein with characteristics of actin in the nuclear fraction of Xenopus laevius [21]. Currently, in addition to widely accepted actin presence in the nucleus, it has been established that nuclear actin plays an important role in chromatin remodeling, transcription regulation and nuclear structure organization [10, 23].

Doxorubicin is a cytostatic which was isolated from cultures of Streptomyces peuceticus s. Caesius [3, 24, 26]. Mechanism of the drug's activity is various. Inhibition of topoisomerase II and DNA intercalation are considered one of the most common effects of doxorubicin action, which lead to strand break-related DNA damage, blocking of DNA replication and ultimately inhibition of protein synthesis. Furthermore, the reactive oxygen species (ROS), the certain products of doxorubicin metabolism may also contribute to DOX-induced DNA damage [17, 24]. The cytostatic is used in chemotherapy of breast cancer, lung cancer, soft tissue sarcoma, Hodgkin's lymphoma, multiple myeloma or acute lymphoblastic leukemia [26]. Doxorubicin at low concentrations may cause the senescence or mitotic catastrophe, while high concentrations can induce apoptosis [14].

Apoptosis is one of the types of programmed cell death. It was first observed in the nineteenth century, while the term 'apoptosis' was introduced in 1972 by Kerr et al. [8, 16]. This is an active process, requiring the activation of multiple gene and an input of energy. During apoptosis, many morphological, biochemical and molecular changes can be seen. The main features are: changes on the nuclear level (chromatin condensation, shrinkage and fragmentation of nucleus), condensation of cytoplasm and the formation of blebs and apoptotic bodies [3, 7, 16, 18, 27]. Apoptosis is a physiological process which is essential during embryogenesis and in the postnatal period. The dysregulation of apoptosis may lead to Alzheimer's, Parkinson's and Huntington's disease, stroke or coronary (heart) disease. Defects in apoptotic pathways are also frequently observed in human cancers [8].

The aim of our study was to determine the effect of doxorubicin on the reorganization of the nuclear and cytoplasmic actin cytoskeleton and the type of induced cell death in A549 cell line.

MATERIALS AND METHODS

Cell culture

The A549 cell line was kindly provided by Ph.D. P. Kopiński (Department of Gene Therapy, Nicolaus Copernicus University Collegium Medicum in Bydgoszcz, Poland). The cells were grown in DMEM supplemented with 10% fetal bovine serum and 50 mg/ml gentamycin. The non-small cell lung cancer cells were grown at 37°C in a humidified 5% CO_2 atmosphere. After 24 h the cells were treated with doxorubicin at concentration: 0.2 μM, 1 μM and 2.5 μM for 24h. Control cells were cultured under the same conditions except of the drug treatment.

Cell viability

For the cell viability analysis a commercial Tali®Viability Kit (Invitrogen) was used according to the attached protocol. The cells were analyzed using the imaging cytometer Tali® (Invitrogen). For statistical analysis GraphPad Prism 5.00 (GraphPad Software) was used (the non-parametric Mann-Whitney U test; $p<0.05$).

Light microscopy

For the morphological analysis the cell were grown on the slides. Next, the cells were fixed for 20 min in 4% paraformaldehyde (pH 7.4, RT) and rinsed three times in PBS for 5 min. The cells were stained with Mayer's hematoxylin (3min, RT), rinsed under tap water and washed with PBS (10 min, RT). The final

step was to close the preparations using Aqua PolyMount. The cell morphology was observed using Eclipse E800 light microscope (Nikon), computer image analysis system (NIS-Elements software 3.30) and a CCD camera (DS-5MC-U, Nikon).

Fluorescence microscopy

In order to label F-actin, A549 cells were fixed in 4% paraformaldehyde for 20 minutes (pH 7.4, RT). Afterwards the cells were rinsed three times with PBS for 5 minutes (RT), treated with Triton X-100 (RT) and 1% BSA in PBS for 20 minutes (RT). Next, the cells were incubated with Alexa Fluor 488 conjugated phalloidin for 20 minutes in the dark (dilution 1:40; RT). The cells were rinsed three times with PBS for 3 minutes (RT) and then the nuclei were labeled with DAPI (dilution 1:100) for 10 minutes in the dark (RT). The slides were closed with Aqua PolyMount and analyzed using the Eclipse E800 fluorescence microscope (Nikon). The preparations were also analyzed using a Nikon C1 confocal microscope and computer image analysis system based on a digital version of EZ- C1 Ver.2.20 program (Nikon).

Electron microscopy

In order to perform ultrastructural analysis, the cells were fixed with 3.6% glutaraldehyde for 30 minutes (RT) and washed in cacodylic buffer (0.1M pH = 7.4). Then, for 1h the cells were preserved with 1% osmium tetroxide in cacodylic buffer and dehydrated with increasing concentrations of alcohols and acetone. Next, the cells were saturated with the mixture of epoxy resin and acetone and ultimately embedded in epoxy resin with the addition of accelerator. The polymerization of the resin proceeded for 24h at 37°C and then for 120h at 65°C. The material was cut into ultra-thin sections by using a Reichert OmU3 ultramicrotome and then counterstained with uranyl acetate. Preparations were analyzed using the transmission electron microscope JEM 100 CX (JEOL, Tokyo, Japan).

Table I. *The effects of low Dox doses on the survival C6 cell line*

Tabela I. *Wpływ niskich dawek Dox na przeżycie komórek linii C6*

Dawka/ Dose (nM)	Średnia/Mean (%)	Odchylenie standardowe/Stand. Deviation	Mediana/ Median
Kontrola/Control	97.27	1.668	97.00
50 nM Dox	93.53	1.727	94.00
100 nM Dox	9080	2.336	91.00
200 nM Dox	8847	3.091	88.00

Table II. *The influence of Dox on fluorescence F-actin involved in intercellular interaction C6 cell line*

Tabela II. *Wpływ doksorubicyny na fluorescencję F-aktyny zaangażowanej w relacje międzykomórkowe linii C6*

Dawka/Dose (nM)	Średnia/Mean (%)	Odchylenie standardowe/Stand. Deviation	Mediana/ Median
Kontrola/Control	97.27	1.668	97.00
50 nM Dox	93.53	1.727	94.00
100 nM Dox	90.80	2.336	91.00
200 nM Dox	8847	3.091	88.00

RESULTS

The influence of doxorubicin on the viability of A549 cells

The image-based cytometry analysis revealed a dose-dependent decrease in the viability of A549 cells treated with doxorubicin. As shown in Figure 1, the mean viability rate was 95% in control groups, 91% after the treatment with 0.2 μM of DOX, 82% and 75% following exposure to 1 and 2.5 μM doxorubicin, respectively. All the concentrations of DOX used in this study induced statistically significant differences ($p<0.05$) in the mean percentage of surviving cells in comparison to control (Fig. 1).

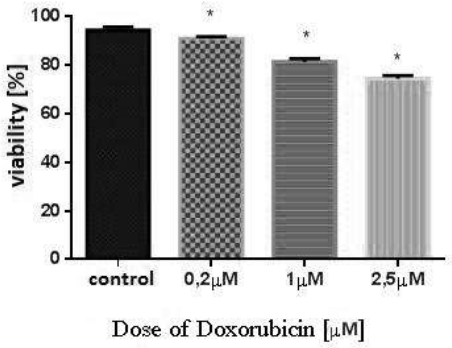


Fig. 1. *The influence of low dose doxorubicin on percentage of surviving C6 cell line. Statistical differences were marked with asterisk*

Ryc. 1. *Wpływ niskich stężeń doksorubicyny na przeżycie komórek linii C6. Różnice istotne statystycznie oznaczono gwiazdką*

The effect of doxorubicin on the morphological and ultrastructure alterations in the A549 cells

The changes in cell viability were accompanied by morphological and ultrastructural alterations, which progressed gradually with increasing concentrations of DOX. The A549 control cells exhibited a typical epithelial-like morphology and maintained their adherence to each other (Fig. 2A). Many of DOX-treated cells lost cell-cell and cell-substrate contacts becoming round up in shape. In these cells the

chromatin condensation was observed (Fig. 2C). Following DOX treatment, the population of enlarged cells with fragmented nuclei was also noticed (Fig. 2C,D). Moreover, at the electron microscopic level, the shrunken cells with fragmentized nucleus and membrane blebbing were seen in the cell populations exposed to doxorubicin (Fig. 3C,D). Visible ultrastructural changes also included the swollen mitochondria as well as electron-dense and electron-lucent vacuole-like structures (Fig. 3C,E).

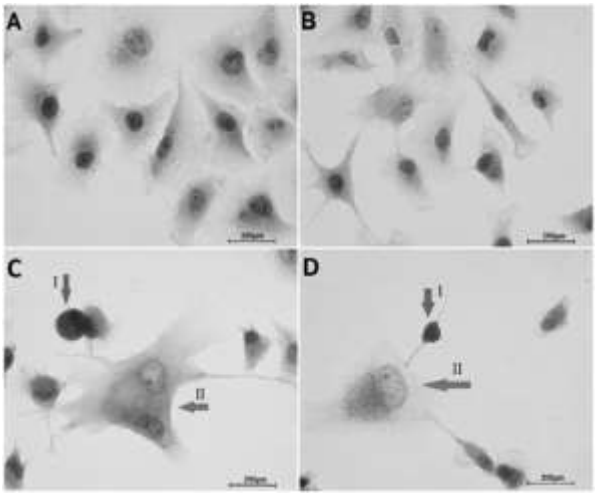


Fig. 2. *The light microscopy studies of Dox-treated C6 cell line. A,A' – cell control, cell created tight clusters, and had spindle-shaped; B, B'-50nM Dox, larger intercellular spaces than contol cells and more number of tabs (arrows), C,C'- 100nM Dox, cell created loose clusters, irregular nuclei, condensation of chromatin (arrows), D,D'-200nM Dox, weakness cell-cell interaction, fragmentation of nuclei (arrows). A,B,C,D – significations x10, A',B',C',D' – significations x10*

Ryc. 2. *Ocena komórek linii C6 traktowanych Dox w mikroskopie świetlnym. A, A'- Kontrola, komórki tworzą ścisłe skupiska, wrzecionowaty kształt; B, B'-50nM Dox - większe przestrzenie międzykomórkowe w porównaniu do kontroli i większa liczba wypustek (strzałki), C, C'-100nM Dox, komórki tworzą luźne skupiska, nieregularne jądra, kondensacja chromatyny (strzałki) D,D' – 200nM Dox, osłabienie kontaktu komórka-komórka, fragmentacja jądra (strzałki). A,B,C,D – powiększenie x10, A',B',C',D' – powiększenie x100*

Fluorescence microscopic analysis of actin filaments revealed some of their characteristic architectural changes after the treatment of A549 cells with doxorubicin. Control A549 cells exhibited well-developed cortical actin as well stress fibers, which traversed the cell (Fig. 4A). After doxorubicin treatment, the enlarged cells with F-actin in the form of aggregates accumulated in the cytoplasm were seen (Fig. 4B). Furthermore, the shrunken cells with ring-like structures around the nuclei were observed (Fig. 4C). The number of cells that these changes concerned increased with the increase in dose of doxorubicin (Fig. 4).

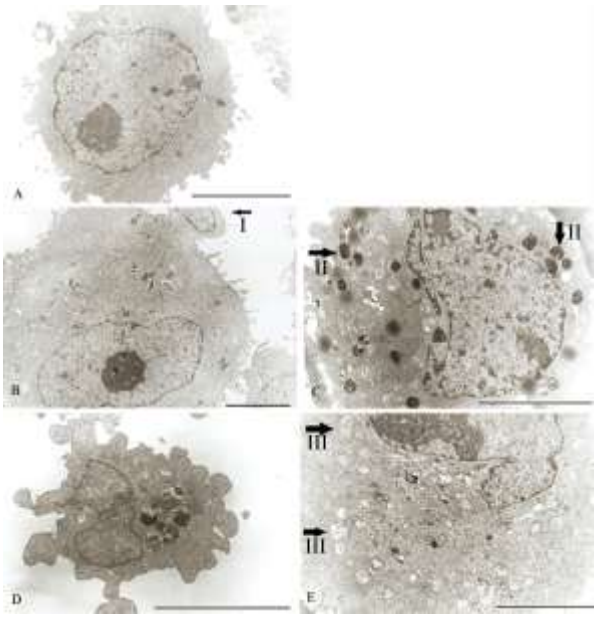


Fig. 3. *The electron microscopy studies of Dox-treatment C6 cell line. A-control cell x11333, B- 50nM, condensation of heterochromatin (arrows) x11047, C-100nM, condensation and marginalization of chromatin, shrunk nuclei (arrows) x15085, D-200nM, condensation of chromatin, vacuolization of cytoplasm, cell processes (arrows) x 11667*

Ryc. 3. *Analiza komórek linii C6 traktowanych Dox w transmisyjnym mikroskopie elektronowym. A-kontrola x11333, B- 50nM, skupiska heterochromatyny (strzałki) x11047, C-100nM, kondensacja i marginalizacja chromatyny, nieregularne jądra komórkowe (strzałki) x 15085, D-200nM, kondensacja chromatyny, wakuolizacja cytoplazmy, wypustki cytoplazmatyczne (strzałki) x 11667*

In addition, the confocal microscopy observations showed that F-actin was present not only in the cytoplasm of A549 cells but also within their nucleus. In the DOX-treated cell populations, the nuclear pool of F-actin seemed to be increased in comparison to the control groups. Furthermore, the nuclear organization of F-actin changed from short fibers observed in the control cells to large clusters revealed in the cells exposed to doxorubicin (Fig. 5).

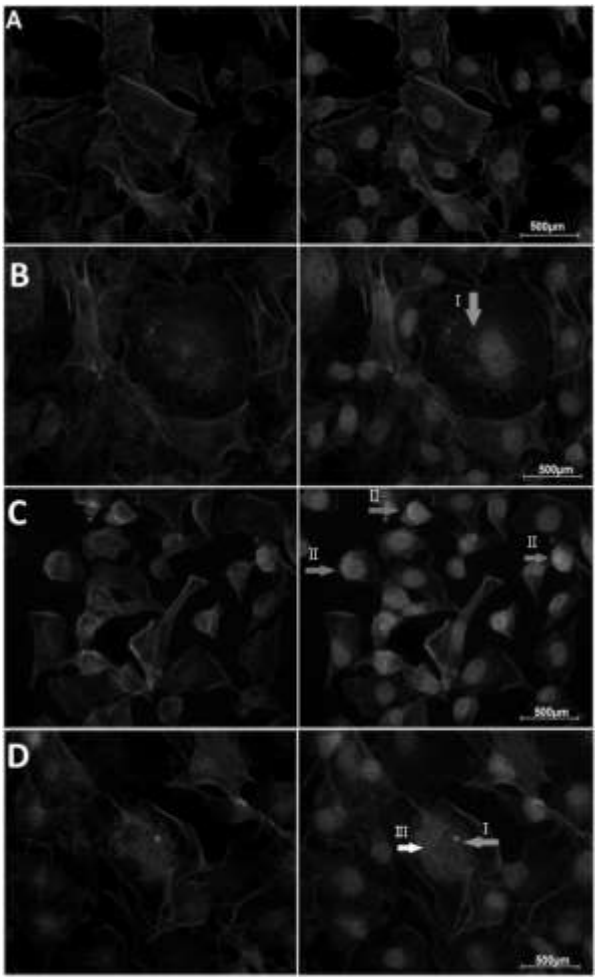
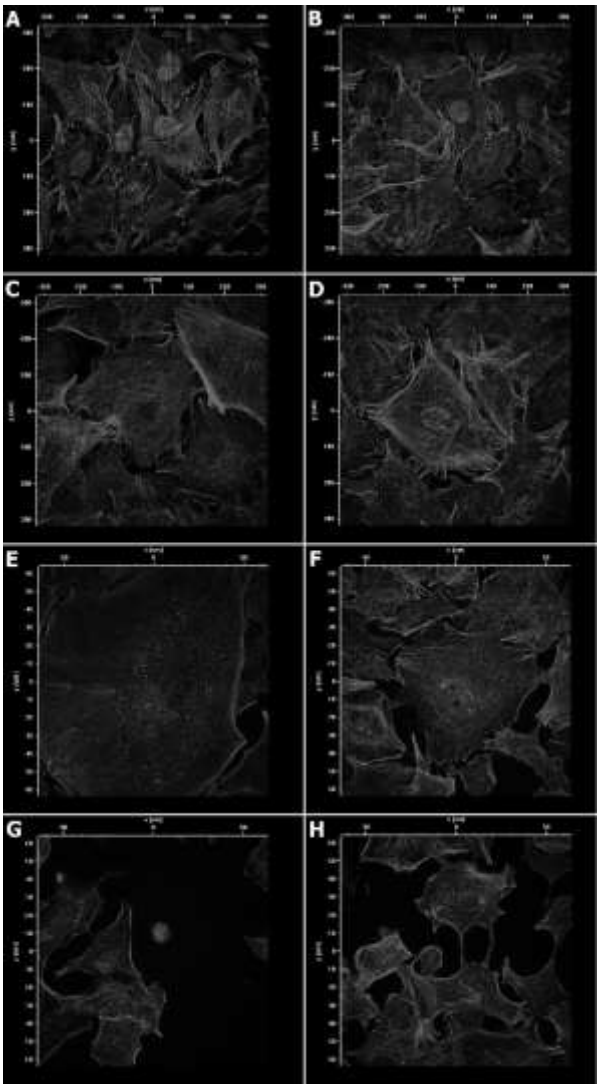


Fig. 4. *Fluorescence of F-actin involved in interactions between C6 cell line. A- cell control, B-50 nM Dox, C-100 nM Dox, D-200 nM Dox. The intercellular spaces and cell processes were shown by arrows. Bar= 50 µm*

Ryc. 4. *Fluorescencja F-aktyny zaangażowanej w interakcje międzykomórkowe linii C6. A-kontrola, B-50 nM Dox, C-100 nM Dox, D-200 nM Dox. Strzałki oznaczają przestrzenie międzykomórkowe i wypustki cytoplazmatyczne. Bar=50 µm*



Ryc. 5. *Wpływ dokosrubicyny na fluorescencje F-aktyny zaangażowanej w relacje międzykomórkowe linii C6. Różnice istotne statystyczne oznaczone strzałką*

Fig. 5. *The influence of Dox on fluorescence F-actin involved in intercellular interaction C6 cell line. Statistical differences were marked with asterisk*

The assessment of F-actin organization in the doxorubicin-treated A549 cells

DISCUSSION

The lung cancer is the most common type of cancer and it remains the main cause of cancer-related mortality mostly among Polish men. Despite the fact that doxorubicin is widely used in cancer chemotherapy, the precise mechanisms of DOX action are still not fully understood. The non-small cell lung cancer cell line A549 has been used as model in cancer research. In the present study the effect of doxorubicin

on A549 cell was investigated. The cells were treated with cytostatic at concentrations of 0.2 μM, 1 μM and 2.5 μM. The results were evaluated using light, electron, confocal and fluorescence microscopy. We have shown that A549 cells exhibit sensitivity to doxorubicin in a dose-dependent manner. Similar results were presented by Litwiniec et al., who showed that the treatment of A549 cells with doxorubicin caused the increase in the number of necrotic, early- and late apoptotic cells. They have also revealed that the increased concentration of the cytostatic resulted in DNA fragmentation and the occurrence of polyploid cells [19]. In our data, the analysis of the cells at the level of light and transmission electron microscopy has showed changes in cell shape and size as well as the structure of the nucleus. Here, the cells with characteristic apoptotic features e.g. cell shrinkage, membrane blebbing and the condensation of chromatin were noticed. The giant cells with mitotic catastrophe-like phenotype were also observed. Furthermore, Litwiniec et al. also revealed the autophagic vacuoles in the cytoplasm of DOX-treated A549 cells [19]. These results are in agreement with our observations since we have also observed the formation of electron-dense and electron-lucent vacuole-like structures in the A549 cells exposed to doxorubicin.

In the present study the changes in the organization of F-actin by the confocal and classic fluorescence microscope were also investigated. The apoptotic cells with F-actin on the cell periphery were observed. Besides, in the cells with mitotic catastrophe-like phenotype, the F-actin in the form of clusters in the central part of the cell was also noticed. Similar results have been obtained by Grzanka et al. who treated the CHO AA8 and K-562 cell lines with doxorubicin [12,13,14]. The literature has documented that depolymerization and cleavage of F-actin is necessary to the realization of apoptosis [28]. Moreover, the functions and existence of actin in the nucleus area have been disputed for many years. In 2002, Olave et al. suggested that nuclear actin may be involved in transcription and cross-linking DNA [29]. Litwiniec et al. analyzed the organization and nuclear level of G-actin in doxorubicin-treated non-small lung cancer cells. They revealed that G-actin was more concentrated in the perinuclear/nuclear region than in the cytoplasm of the A549 cells [19]. In turn, in this study the confocal fluorescence microscopy has enabled to evaluate the presence of F-actin in the nucleus of doxorubicin-treated A549 cells. It is now becoming more and clearer that nuclear actin can exist in the form of both monomers and filaments. Grzanka et al. also reported the presence of F-actin in the nucleus of human leukemia HL-60 cells. The cited author hypothesized that the existence of F-actin in the nucleus can be associated with chromatin remodeling during cell death [11,12].

In conclusion, the obtained data have demonstrated that non-small lung cancer cells exhibit sensitivity to doxorubicin in a dose-dependent manner. Doxorubicin induced morphological and ultrastructural changes associated with apoptosis and mitotic catastrophe. In addition, the treatment with DOX resulted in the reorganization of the actin cytoskeleton. It has also been shown that F- actin is present not only in the cytoplasm but also in the nucleus.

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Received: 26.02.2013
Accepted for publication: 17.03.2014