Estimation of taxol influence on changes in tubulin and vimentin systems in K-562 and HL-60 cell lines by immunofluorescence microscopy

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The cytoskeletal system may be considered as an additional pathway involved in process of apoptosis and can be promising target for development of new chemotherapies. The study describes alterations in the distribution of vimentin and tubulin in taxol treated K-562 and HL-60 cells in relation to apoptotic changes. K-562 and HL-60 cells were treated with taxol in a range of concentrations (0.02–10 μM) for 72 hours. Significant changes in distribution of studied proteins occurred in the range 2–10 μM of taxol. K-562 cells showed thin network of vimentin distributed throughout cells or collapsed on nucleus. There were also cells with bright aggregates remembering apoptotic bodies. HL-60 cells showed strong labeling of vimentin in the cytoplasm as well as at the site of apoptotic bodies. Vimentin collapsed on the nucleus, labeling at poles and along the major axis of the cell were also seen. K-562 and HL-60 cells showed radial labeling of tubulin from the centre, aggregates at the surface and bundled microtubules. These findings indicate that alterations in expression of studied cytoskeletal proteins after treatment with taxol were dose-dependent and related with characteristic features of apoptosis.

Key words: taxol, K-562 cells, HL-60 cells, immunofluorescence microscopy, vimentin, tubulin, apoptosis

Taxol is an important drug, which exhibits antitumor activity for treatment of various cancers, as ovarian, breast, lung, malignant melanoma as well as leukemias [4, 13, 20, 28, 35, 37, 47]. It is a diterpenoid plant product and it can also be obtained by chemical synthesis [21, 32]. The taxol family is unique among the many microtubule directed drugs; taxol enhances tubulin polymerization, and prevents microtubule depolymerization induced by calcium or low temperature [40, 43]. It acts as a mitotic inhibitor, blocking cells in the G2/M phase of the cell cycle. The cells may escape from mitosis by pass cytokinesis, and reenter the next round of DNA synthesis to form polyploid cells, which eventually die; this is the proposed mechanism of taxol cytotoxicity [26]. The cytoskeleton is formed by fibrillar systems such as microtubules, microfilaments and intermediate filaments. They form a structural network, which connects the plasma membrane to cytoplasmic organelles and the nucleus. In conjunction with associated proteins, these filamentous networks are considered to establish and maintain cellular organization and perform vital functions in differentiation and development [22]. Microtubules have been shown to be responsible for determining the polarity of cells and the intermediated filaments appear to be involved in physically organizing the interior of the cell [46]. It has been suggested that vimentin filaments and microtubules are closely associated, and disturbance of microtubules may also affect the organization of intermediate filaments [8, 12, 22, 42, 44, 46]. The present study investigated the alteration of tubulin and vimentin in K-562 and HL-60 cells treated with various concentrations of taxol by fluorescence microscopy.

Material and methods

The human erythroleukemic cell line K-562 (ATCC, CCL 243) and HL-60 promyelocytic cell line (ATCC CCL 240) were studied. For experimental studies cells were diluted to $5x10^5$ cells/ml and were grown in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum and 20 μl gentamycin in fully humidified atmosphere of 5% CO_2 at 37 °C. The cells were incubated with 5 different concentrations of taxol (Sigma) 0.02; 0.2; 2; 5 and 10 μM/l for 72 hours. Control flasks without taxol were treated identically.

Cell viability was assessed by the trypan blue dye exclusion method. Mayer hematoxylin (Serva) staining was used for morphological analysis. Vimentin and tubulin were labeled by indirect immunofluorescence method. To reduce the lose of soluble proteins during permeabilization, cells were prefixed with bifunctional protein crosslinking reagent DSP (dithiobis-succinimidylpropionate) (Sigma) in HBSS (Hanks' balanced salt solutions) for 10 min at 37 °C. Cells were first extracted in 0.5% Triton X-100 containing DSP for 10 min at 37 °C and then in 0.5% Triton X-100 in microtubule stabilizing buffer (MTSB) for 5 min at 37 °C. For estimation in fluorescence microscope, cells were collected by using a cytocentrifuge directly onto microscope slides, fixed with 4% paraformaldehyde in MTSB for 15 min at 37 °C, and blocked with glycine. Then they were incubated in BSA-TBS (1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.12% sodium azide, pH 7.6) for 5 min and with anti-tubulin (Sigma) or with monoclonal anti-vimentin IgG (Sigma) in BSA-TBS for 45 min at 37 °C in moist chamber. Cells were rinsed three times and incubated with TRITC-labeled goat anti rabbit IgG (Sigma) for tubulin and FITC-labeled goat antimouse IgG (Sigma) for vimentin for 45 min at 37 °C in moist chamber. They were rinsed twice in BSA-TBS, then in PBS and mounted in gelvatol. Immunolabeling of tubulin and vimentin used in this study has been previously described by BELL and SAFIEJKO-MROCZKA [2]. The organization of tubulin and vimentin was examined with an Eclipse E600 microscope equipped with Y-FL epifluorescence device (Nikon, Tokyo, Japan). Image analysis was performed using Laboratory Universal Computer Image Analysis for fluorescence microscopy, Lucia DI. Statistical analysis was performed by using the Levene and Kruskal-Wallis tests. Calculations were performed using the STATISTICA for Windows 5.1 computer program [39].

Table 1. The influence of taxol doses on mean percentage of cells with apoptotic morphology

Dose (μM)	K-562			HL-60		
	Mean %	Std. deviation	Median	Mean %	Std. deviation	Median
0.0	2.9	1.87	3	5.2	2.04	6
0.02	5.0	1.60	5	6.1	1.33	6
0.2	10.8	3.36	11	15.9	3.18	16
2.0	16.9	1.91	17	30.7	5.16	29
5.0	26.3	7.06	27	34.9	3.41	36
10.0	30.5	5.15	29	46.4	6.29	49

Table 2. The influence of taxol doses on mean percentage of viable cells

Dose (μM)	K-562			HL-60		
	Mean %	Std. deviation	Median	Mean %	Std. deviation	Median
0.0	96.0	2.26	96	94.0	2.14	94
0.02	92.0	3.25	92	88.2	2.63	88
0.2	85.1	3.76	85	76.0	3.46	76
2.0	67.8	8.25	68	33.1	7.20	33
5.0	67.0	3.84	67	34.6	7.63	34
10.0	65.0	3.28	65	29.6	6.99	28

Results

Taxol caused significant changes in morphology and distribution of the studied cytoskeletal proteins, especially in HL-60 cells. There were 46.4% of HL-60 cells with blebs and buds treated with highest dose of taxol and 30.5% of K-562 cells (Tab. 1). Table 2 shows the influence of taxol on the number of viable cells. The mean percentage of apoptotic cells, mean cell area and the number of viable cells were significantly correlated with the dose of taxol ($p<0.001$). Figure 1 shows the cells stained by Mayer hematoxylin. The morphological criteria used to define apoptotic cells were nuclear fragmentation, and cell shrinkage with the formation of membrane blebs and buds. The reorganization of vimentin and tubulin in K-562 and HL-60 leukemia cells after treatment with taxol was dose-dependent. The distribution of both cytoskeletal proteins with 0.02 μM of taxol was close to control cells (Fig. 2a–d, Fig. 3a–d). Exposure of K-562 and HL-60 cells to 0.2 μM of taxol showed vimentin organized in meshwork in cytoplasm and in some of cells around the nucleus. In HL-60 line cells with bright aggregates at their surface and at one pole were also present (Fig. 4). Tubulin in both cell lines treated with 0.2 μM of taxol was rather translocated to the centre where bright labeling was often

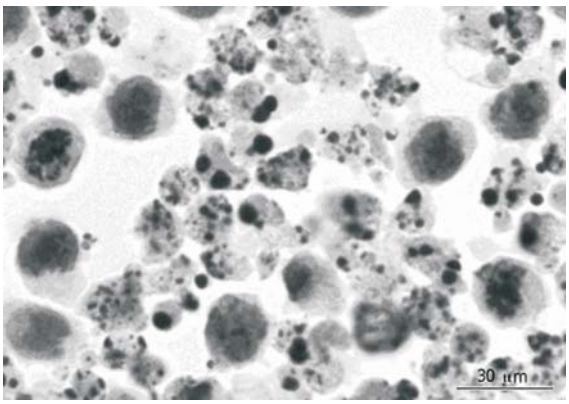

Figure 1. HL-60 cells cultured with 10 μM of taxol and stained by Mayer hematoxylin. Cells with shrinkage, blebs, buds and nuclear fragmentation are seen. Bar: 30 μm.

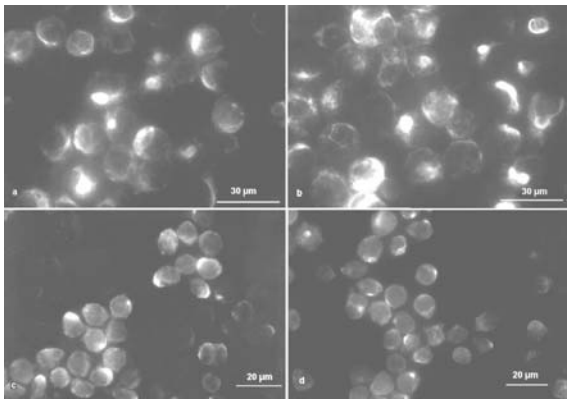

Figure 2. Immunofluorescence of vimentin. K-562 cells cultured without taxol (a) and in the presence of 0.02 μM (b). HL-60 cells without taxol (c) and treated with 0.02 μM of taxol (d). Bar: 30 μm. (a, b). Bar: 20 μm (c, d).

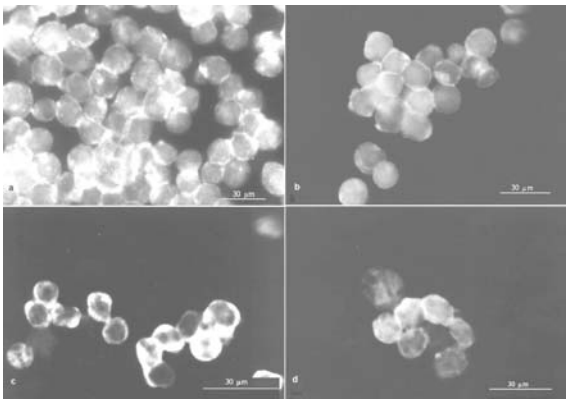

Figure 3. Labeling of tubulin in K-562 (a, b) and HL-60 cells (c, d). Cells without drug (a, c) and treated with 0.02 μM of taxol (b, d). Bar: 30 μm.

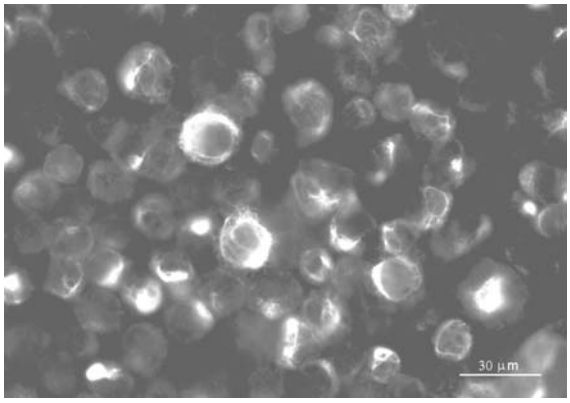

Figure 4. K-562 cells treated with 0.2 μM of taxol. Labeling of vimentin is seen as meshwork in cytoplasm and around the nucleus.

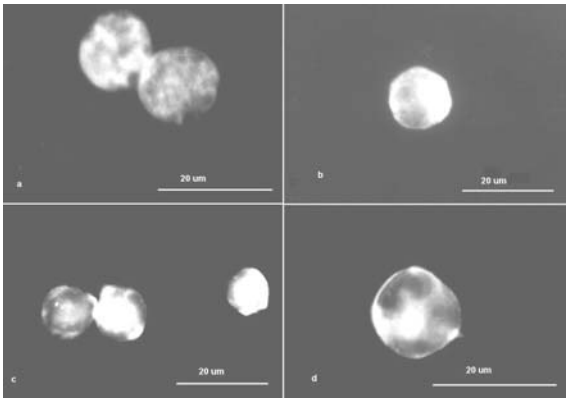

Figure 5. HL-60 cells cultured in the presence of 0.2 μM of taxol. The bright labeling of tubulin is seen throughout cells (a, b) in the centre (c, d) and as radial concentrations running from the center of the cell (d) as well as aggregates at the surface (c). Bar: 20 μm.

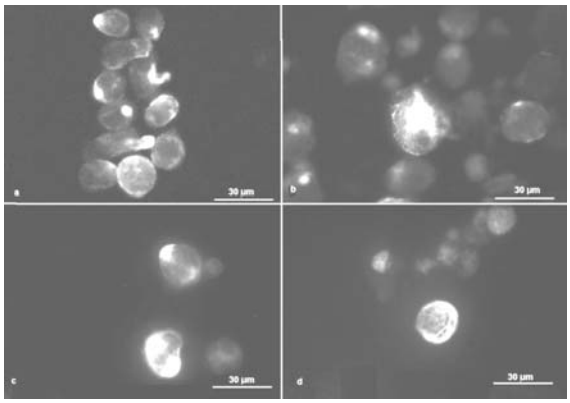

Figure 6. HL60-cells treated with 2 μM (a); 5 μM (b, c) and 10 μM (d) of taxol. Immunofluorescence for vimentin is seen in the structures remembering apoptotic bodies (a), the cell with clustered network of vimentin on nucleus is also seen (b). There are cells with vimentin labeling at the opposite poles of the cells and vimentin filaments along the major axis (c), the cell with thin network of filaments (d). Bar: 30 μm.

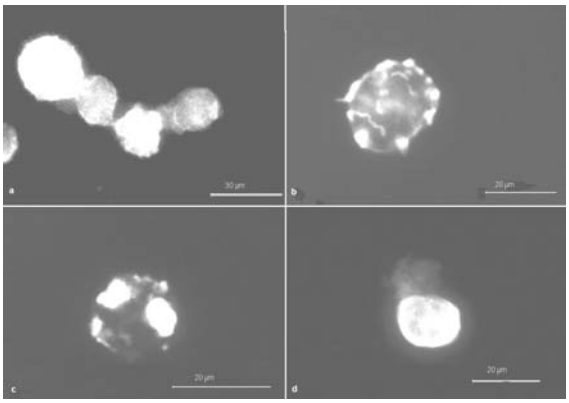

Figure 7. HL-60 cells cultured in the presence of 5 μM (a, b) and 10 μM (c, d) of taxol. The strong labeling of tubulin is in the center of the cell (a) aggregates at the surface (b, c) and thin bundles (b) also radial concentration of tubulin running from the center of the cell are seen (d). Bar: 20 μm (b, c, d). Bar: 30 μm (a).

seen. Radial labeling and aggregates at the surface were also observed (Fig. 5a–d). Significant changes in distribution of vimentin and tubulin occurred in the range 2–10 μM of taxol. In K-562 cells vimentin was often visible as thin network distributed throughout cells and in some cases vimentin was collapsed on the nucleus. There were also cells with bright aggregates at the surface remembering apoptotic body formation. HL-60 cells showed strong labeling of vimentin network in the cytoplasm as well as high concentration of staining as aggregates at the periphery, at the site of apoptotic bodies. There were cells with vimentin filaments collapsed in their centre and cells with labeling at their poles and also with intermediate microfilaments concentrated along the major axis (Fig. 6a–d).

Discussion

The knowledge on the cytoskeleton is still not complete, especially in functions so important for cells as proliferation, differentiation and apoptosis. It is known that reorganization of cytoskeletal proteins is connected with process of apoptosis [1, 3, 6, 7, 10, 14]; HASSAN et al [19], LEUNG et al [23, 24], LEVEE et al [25], MASHIMA et al [27], MULLER et al [30], SHANG et al [41], GRZANKA et al [15–17], and OLINS et al [33] suggested that reorganization of cytoskeletal proteins are involved also in cell differentiation. There are other studies on cytoskeleton with process of differentiation [5, 11, 18, 24, 29, 31, 34, 45]. However, there are still a lot of questions remaining to be answered. To study the reorganization of cytoskeletal proteins in cancer cells in relation with morphological features of apoptosis induced by cytostatics seems to be promising for the development of new cancer therapy. Cytostatic drugs are strong poisons, which damage a lot of important cellular processes (e.g. mitosis, synthesis of DNA, RNA and proteins) and these changes can induce programmed cell death (apoptosis) in which cytoskeletal proteins are involved. In this report we chose taxol (a compound obtained from bark of *Taxus brevifolia*) which is a well known stabilizer of microtubules. It is characterized by strong affinity for tubulin and remarkable antitumor activity *in vitro* and *in vivo* [38]. Apart from stabilizing the microtubule system taxol binds to Bcl-2 protein changing its conformation and activity it may be a signal to apoptosis [36]. Taxol stabilizes microtubules but still is not much known about influence of that poison on others constituents of cytoskeleton. The present studies showed that taxol affected not only microtubules but also the organization of vimentin filaments. There are studies with fluorescence methods confirming ours. CHU et al [9] studied influence of taxol on reorganization of vimentin in 9L rat brain tumor cells and showed that treatment with taxol affected cytoskeletal systems which resulted in the collapse of intermediate filament network, leading to increase of cell contractility. They found not only reorganization of vimentin filaments but also changes in microtubules. In their study vimentin filaments were totally collapsed and clustered around the nucleus, whereas the microtubules were less affected. In the present study reorganization of vimentin and tubulin leaded to changes in cell morphology and seemed to be connected with changes related to apoptosis. Especially in HL-60 cells these proteins seem to be related with the reorganization of cytoskeleton during the process of apoptotic body formation. GANGEMI et al [14] in HL-60 cells treated with taxol also observed appearance of morphological features consistent with the process of apoptosis but not in K-562 cells. They concluded that the antineoplastic effect of taxol is mediated in susceptible cell lines by induction of the apoptotic machinery and that K-562 partial resistance may depend upon the intrinsic inability of these tumor cells to undergo apoptosis. This conclusion might be supported by present studies. Here taxol exhibited cytotoxic effect on both lines but HL-60 was more sensitive to the concentration range 2–10 μM of taxol. It was also showed that the antiproliferative activity of taxol was associated with marked reorganization of cytoskeletal structures. BROWN et al [5] and HASS et al [18] reported also changes in microtubules and alterations in vimentin of HL-60 cells. Our results demonstrate that cytotoxicity of taxol in HL-60 and K-562 cell lines are associated with the reorganization of not only tubulin but also vimentin filaments. These structural changes in cytoskeletal proteins were associated with characteristic morphological features of apoptosis. The present studies and ours previous with other cytostatic drugs also on tubulin and vimentin suggest that these cytoskeletal proteins could be promising targets for development of new chemotherapies.

References

[1] ASUMENDI A, ANDOLLO N, BOYANO MD, HILARIO E, PEREZ-YARZA G et al. The role of cleavage of cell structures during apoptosis. Cell Mol Biol 2000; 46: 1–11.

[2] BELL PB, SAFIEJKO-MROCZKA B. Improved methods for preserving macromolecular structures and visualizing them by fluorescence and scanning electron microscopy. Scanning Microsc 1995; 9: 843–860.

[3] BONANNO E, RUZITTU M, CARLA EC, MONTINARI MR, PAGLIARA P et al. Cell shape and organelle modification in apoptotic U937 cells. Eur J Histochem 2000; 44: 237–246.

[4] BONNETERRE J, TUBIANA-HULIN M, CATIMEL G. Paclitaxel, epirubicin and cyclophosphamide combination in first-line treatment of metastatic breast cancer: a dose escalation study. Oncology 2004; 66: 185–191.

[5] BROWN WJ, NORWOOD CF, SMITH RG, SNELL WJ et al. Development of capping ability during differentiation of HL-60 human promyelocytic leukemia cells. J Cell Physiol 1981; 106**:** 127–136.

[6] BURSCH W, HOCHEGGER K, TOROK L, MARIAN B, ELLINGER A et al. Autophagic and apoptotic types of programmed cell death exhibit different fates of cytoskeletal filaments. J Cell Sci 2000; 113: 1189–1198.

[7] CHEN Z, NAITO M, MASHIMA T, TSURURO T. Activation of actin-cleavable interleukin 1beta-converting enzyme (ICE) family protease CPP-32 during chemotherapeutic agent induced apoptosis in ovarian carcinoma cells. Cancer Res 1996; 56: 5224–5229.

[8] CHANG L, GOLDMAN RD. Intermediate filaments mediate cytoskeletal crosstalk. Nat Rev Mol Cell Biol 2004; 5: 601–613.

[9] CHU JJ, CHEN KD, LIN YL, FEI CY, CHIANG AS et al. Taxol induces concomitant hyperphosphorylation and reorganization of vimentin intermediate filaments in 9L rat brain tumor cells. J Cell Biochem 1998; 68: 472–483.

[10] COTTER TG, LENNON SV, GLYNN JM, GREEN DR. Microfilament-disrupting agents prevent the formation of apoptotic bodies in tumor cells undergoing apoptosis. Cancer Res 1992; 52: 997–1005.

[11] DELLAGI K, BROUET JC. Alteration of vimentin intermediate filaments expression during differentiation of HL60 and U937 human leukemic cell lines. Leuk Res 1984; 8: 611–616.

[12] DRABEROVA E, DRABER P. A microtubule-interacting protein involved in coalignment of vimentin intermediate filaments with microtubules. J Cell Sci 1993; 106: 1263–1273.

[13] EINZIG AI, HOCHSTER H, WIERNIK PH, TRUMP DL, DUTCHER JP et al. A phase II study of taxol in patients with malignant melanoma. Invest New Drugs 1991; 9: 59–64.

[14] GANGEMI RM, TISO M, MARCHETTI C, SEVERI AB, FABBI M. Taxol cytotoxicity on human leukemia cell lines is a function of their susceptibility to programmed cell death. Cancer Chemother Pharmacol 1995; 36: 385–392.

[15] GRZANKA A. Actin distribution patterns in HL-60 leukemia cells treated with etoposide. Acta Histochem 2000; 103: 453–464.

[16] GRZANKA A, GRZANKA D. The influence of etoposide on the distribution of tubulin in human leukemia cell line HL-60. Med Sci Monit 2003; 9: BR66–BR69.

[17] GRZANKA A, GRZANKA D, ORLIKOWSKA M. Cytoskeletal reorganization during process of apoptosis induced by cytostatic drugs in K-562 and HL-60 leukemia cell lines. Biochem Pharmacol 2003; 66: 1611–1617.

[18] HASS R, BARTELS H, TOPLEY N, HADAM M, KOHLER L et al. TPA-induced differentiation and adhesion of U937 cells: changes in ultrastructure, cytoskeletal organization and expression of cell surface antigens. Eur J Cell Biol 1989; 48: 282–293.

[19] HASSAN Z, FADEEL B, ZHIVOTOVSKY B, HELLSTROM-LINDBERG E. Two pathways of apoptosis induced with all trans retinoic acid and etoposide in the myeloid cell line P39. Exp Hematol 1999; 27: 1322–1329.

[20] HOLMES FA, WALTERS RS, THERIAULT RL, FORMAN AD, NEWTON LK et al. Phase II trial of taxol, an active drug in the treatment of metastatic breast cancer. J Natl Cancer Inst 1991; 83: 1797–1805.

[21] HOLTON RA, SOMOZA C, KIM HB, LIANG F, BIEDIGER RJ et al. First total synthesis of taxol. J Am Chem Soc 1994; 116: 1597–1600.

[22] KELLY RB. Microtubules, membrane traffic, and cell organization. Cell 1990; 61: 5–7.

[23] LEUNG MF, LIN TS, SARTORELLI AC. Changes in actin and actin-binding proteins during the differentiation of HL-60 leukemia cells. Cancer Res 1992; 52: 3063–3066.

[24] LEUNG MF, SOKOLOSKI JA, SARTORELLI AC. Changes in microtubules, microtubule-associated proteins, and intermediate filaments during the differentiation of HL-60 leukemia cells. Cancer Res 1992; 52: 949–954.

[25] LEVEE MG, DABROWSKA MI, LELLI JL, HINSHAW DB. Actin polymerization and depolymerization during apoptosis in HL-60 cells. Am J Physiol 1996; 271: C1981–1992.

[26] LONG BH, FAIRCHILD CR. Paclitaxel inhibits progression of mitotic cells to G1 phase by interference with spindle formation without affecting other microtubule functions during anaphase and telephase. Cancer Res 1994; 54: 4355–4361.

[27] MASHIMA T, NAITO M, FUJITA N, NOGUCHI K, TSURUO T. Identification of actin as a substrate of ICE and an ICE-like protease and involvement of an ICE-like protease but not ICE in VP-16 induced U937 apoptosis. Biochem Biophys Res Commun 1995; 217: 1185–1192.

[28] MCGUIRE WP, ROWINSKY EK, ROSENSHEIN NB, GRUMBINE FC, ETTINGER DS et al. Taxol: A unique antineoplastic agent with significant activity in advanced ovarian epithelial neoplasms. Ann Intern Med 1989; 111: 273–279.

[29] MEYER WH, HOWARD TH. Actin polymerization and its relationship to locomotion and chemokinetic response in maturing human promyelocytic leukemia cells. Blood 1987; 70: 363–367.

[30] MULLER K, DULKU S, HARDWICK SJ, SKEPPER JN, MITCHINSON MJ. Changes in vimentin in human macrophages during apoptosis induced by oxidised low density lipoprotein. Atherosclerosis 2001; 156: 133–147.

[31] NGAI J, CAPETANAKI YG, LAZARIDES E. Differentiation of murine erythroleukemia cells results in the rapid repression of vimentin gene expression. J Cell Biol 1984; 99: 306–314.

[32] NICOLAOU KC, YANG Z, LIU JJ, UENO H, NANTERMET PG et al. Total synthesis of taxol. Nature 1994; 367: 630–634.

[33] OLINS AL, HERRMANN H, LICHTER P, OLINS DE. Retinoic acid differentiation of HL-60 cells promotes cytoskeletal polarization. Exp Cell Res 2000; 254: 130–142.

[34] OLINS AL, OLINS DE. Cytoskeletal influences on nuclear shape in granulocytic HL-60 cells. BMC Cell Biol 2004; 5: 30.

[35] REED E, KOHN EC, SAROSY G, DABHOLKAR M, DAVIS P et al. Paclitaxel, cisplatin, and cyclophosphamide in human ovarian cancer: molecular rationale and early clinical results. Semin Oncol 1995; 22: 90–96.

[36] RODI DJ, JANES RW, SANGANEE HJ, HOLTON RA, WALLACE BA et al. Screening of a library of phage-displayed peptides identifies human bcl-2 as a taxol-binding protein. J Mol Biol 1999; 285: 197–203.

[37] ROWINSKY EK, BURKE PJ, KARP JE, TUCKER RW, ETTINGER DS et al. Phase I and pharmacodynamic study of taxol in refractory acute leukemias. Cancer Res 1989; 49: 4640–4647.

[38] ROWINSKY EK, CAZENAVE LA, DONEHOWER RC. Taxol: a novel investigational antimicrotubule agent. J Natl Cancer Inst 1990; 82: 1247–1259.

[39] SAWICKI S. Elementary statistics for physicians. Warsaw: Medical Publishers PZWL, 1982 [in Polish].

[40] SCHIFF PB, FANT J, HORWITZ SB. Promotion of microtubule assembly in vitro by taxol. Nature 1979; 277: 665–667.

[41] SHANG X, SHIONO Y, FUJITA Y, OKA S, YAMAZAKI Y. Synergistic enhancement of apoptosis by DNA and cytoskeleton--damaging agents: a basis for combination chemotherapy of cancer. Anticancer Res 2001; 21: 2585–2589.

[42] SVITKINA TM, VERKHOVSKY AB, BORISY GG. Plectin sidearms mediate interaction of intermediate filaments with microtubules and other components of the cytoskeleton. J Cell Biol 1996; 135: 991–1007.

[43] THOMPSON WC, WILSON L, PURICH DL. Taxol induces microtubule assembly at low temperature. Cell Motil 1981; 1: 445–454.

[44] TREVOR KT, McGUIRE JG, LEONOVA EV. Association of vimentin intermediate filaments with the centrosome. J Cell Sci 1995; 108: 343–356.

[45] UEDA N, WALKER PD, HSU SM, SHAH S. Activation of a 15-kDa endonuclease in hypoxia/reoxygenation injury without morphologic features of apoptosis. Proc Natl Acad Sci USA 1995; 92: 7202–7206.

[46] VASILIEV JM. Polarization of pseudopodial activities: cytoskeletal mechanisms. J Cell Sci 1991; 98: 1–4.

[47] WIERNIK PH, SCHWARTZ EL, EINZIG A, STRAUMAN JJ, LIPTON RB et al. Phase I trial of taxol given as a 24 hour infusion every 21 days: responses observed in metastatic melanoma. J Clin Oncol 1987; 5: 1232–1239.