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The effect of doxorubicin and retinoids on proliferation, necrosis and apoptosis in MCF-7 breast cancer cells

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Abstract: Doxorubicin (Adriamycin) is the most active drug in the treatment of breast cancer. The aim of this study was to investigate the interaction of doxorubicin and retinoids in the inhibition of proliferation of hormone sensitive (ER+) human breast cancer cell line MCF-7 and to find out whether this combination can result in the enhancement of its therapeutic effect. As a comparison we also used estradiol and tamoxifen. We also made an attempt to elucidate the effect of these compounds on the stimulation of the apoptotic pathway in breast cancer cells. Cell proliferation in a 24-hour culture was assessed by [³H] thymidine incorporation into cancer cells and by immunocytochemical analysis of cellular cycle-related PCNA and Ki-67 antigens expression, after the incubation of the cell culture with 10, 20 and 50 nM doxorubicin (DOX), 2 nM estradiol (E₂), 10 μM tamoxifen (TAM) and 1 nM, 0.01, 0.1, 1 and 10 μM of all-*trans* retinoid acid (ATRA). The assessment of cell viability and analysis of apoptotic and necrotic cells were performed after the 72-hour incubation of the culture with the examined substances and following apoptosis induction using acridine orange and ethidine bromide. Of the doxorubicin concentrations used in the study, 20 nM inhibited thymidine incorporation to 84.83±10.00% (control=100%). In the same culture conditions, 2 nM E₂ stimulated cancer cells to 157.09±8.84%. Concentrations of 10 μM TAM and 10 μM ATRA inhibited the proliferation to 63.16±7.85% and 52.19±3.21%, respectively. A statistically significant reduction of these values was observed when 20 nM DOX was added to medium with E₂ - 39.24±7.6%, TAM - 48.34±2.05% and ATRA - 21.98±1.69%, respectively; the percentage of PCNA- and Ki-67-positive cells was also reduced. Despite high antiproliferative efficacy of 20 nM DOX and 10 μM ATRA combination, the percentage of apoptotic cells was only 25±0.81%, being similar to that obtained in the culture with 20 nM DOX. The concentrations of 10, 20 and 50 nM DOX that were used to inhibit the proliferation of MCF-7 cell line were not particularly effective. The inhibitory effect was obtained when 20 nM of DOX and E₂, TAM or ATRA were used simultaneously. The use of E₂ caused a two-fold decrease in the percentage of proliferating cells. It was also shown that the effectiveness of DOX in combination with ATRA is significantly higher than that of DOX combined with TAM, which might suggest a valuable approach to the treatment of breast cancer.

Key words: Doxorubicin - All-trans retinoic acid - Estradiol - Tamoxifen - MCF-7 - Proliferation - Apoptosis

Introduction

The encouraging outcomes of chemotherapy achieved in the treatment of carcinomas in the 70s (advantage over hormonal therapy) have made it the major palliative mode of treatment of metastases as well as an adjuvant method to treat limited neoplasms. Of several preparations that cause regression of breast cancer metastases, doxorubicin (Adriamycin) is the most active first-line monotherapeutic [38]. However, used as the second-line drug it is less effective in evoking response of proliferating neoplastic tissues. It is, however, still a golden standard in the combination of CAF (Cyclophosphamide, Adriamycin, 5-Fluorouracil) [16].

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Doxorubicin is the first generation anthracycline antibiotic of wide spectrum of action. At the cellular level, it is incorporated inbetween two nitric bases of double DNA helix, thus causing the inhibition of DNA-dependent DNA and RNA polymerases [53]. This results in the suppression of DNA and RNA synthesis and damage to DNA repair mechanisms [30]. Doxorubicin also has been shown to alter topoisomerase II activity [45]. Anthracyclines cause rupture of DNA strands, which is associated with generation of free radicals in the process of biotransformation of *e.g.* doxorubicin [41]. Free radicals are also responsible for the inhibition

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of the respiratory chain enzymes in mitochondria and membrane lipid oxidation [2]. A destructive effect of anthracyclines on the function and morphology of cells may be also due to disturbances in Ca^{2+} turnover and inhibition of sodium-potassium ATPase activity [19, 40]. These drugs affect each phase of cell development - doxorubicin has a stronger effect on the S phase of the cell cycle [1].

Whether the enhancement of the therapeutic effect of doxorubicin can be achieved by its combination with compounds other than hormones or growth factors, has been considered for years. In this context, retinoids as compounds of different mechanism of action at the cellular level seem to be an interesting alternative.

Retinoids - a group of natural and synthetic analogues of vitamin A - affect the growth and differentiation of many normal and neoplastic cells [43]. All-*trans* retinoic acid is an endogenous metabolite of retinol [23, 29] and shows an antiproliferative activity in cell cultures of such lines as NIH 3T3 (fibroblasts), K-308 (keratinocytes), HL-60 (myeloid leukemia), BXPC-3 (pancreatic adenocarcinoma) [44], A 375 and Hs 939 (melanoma) [28], SW 948 (colorectal cancer) [22] and in many human breast cancer cell lines (MCF-7, T-47D, BT-474, ZR 75.1, 734 B, Hs 578T) [12, 28, 44]. Zhu *et al.* [51] revealed that retinoic acid-induced inhibition of MCF-7 cell growth occurs through induction of G1 arrest with a concomitant reduction in the proportion of cells in S and G2 + M phases. The effect of retinoids on the process of transcription occurs *via* interactions with RAR and RXR receptors that belong to the family of steroid-thyroid hormonal nuclear receptors [7, 15]. These receptors acts as ligand-inducible transcription factors that regulate the transcription of target genes by binding to specific DNA sequences (retinoic acid response elements, RAREs and RXREs) [6, 20]. In addition, retinoid-binding proteins CRAB-I and CRAB-II regulate accessibility of retinoic acid to nuclear receptors [32] and are responsible for transportation of retinoids from cytosol to the cell nucleus [27].

The aim of this study was to investigate the interaction of doxorubicin and retinoids in the inhibition of proliferation of human breast cancer cells and to find out whether this combination can result in the enhancement of its therapeutic effect. We also made an attempt to elucidate the effect of these compounds on the stimulation of the apoptotic pathway in breast cancer cells. Two standard substances used for MCF-7 culture experiments, one stimulating - estradiol - and the other inhibiting - tamoxifen - were also applied in order to compare their effect when combined with doxorubicin.

Materials and methods

Chemicals. All-*trans* retinoic acid (Tretinoin), tamoxifen (Citrate Salt Tamoxifen), 17β -estradiol (1,3,5[10]-estratriene-3,17 β -diol)

and doxorubicin hydrochloride (Hydroxydaunorubicin) were obtained from Sigma (St. Louis, MO, USA). The following antibodies: PCNA - Proliferating Cell Nuclear Antigen: monoclonal mouse antibody (clone PC 10) and Ki 67: monoclonal mouse antibody (clone Ki 67) were obtained from Dako (Glostrup, Denmark). Tretinoin was diluted in ethyl alcohol and then in the culture medium, to final concentrations of 1 nM, 0.01, 0.1, 1 and 10 μM . Tamoxifen was added to the culture at a concentration of 10 μM . 17β -estradiol was added to the culture at a concentration of 2 nM. Doxorubicin was diluted in the culture medium, to final concentrations 10, 20 and 50 nM.

Culture of MCF-7 cell line. The hormone sensitive (ER+) MCF-7 human breast cancer cell line (American Type Culture Collection, Rockville, MD) was cultured in DMEM medium (Sigma, St. Louis, MO, USA) supplemented with 10% FBS (Sigma, St. Louis, MO, USA), 50 $\mu\text{g}/\text{ml}$ streptomycin, and 100 U/ml penicillin in 75 cm^2 plastic flasks (Nunc, Roskilde, Denmark), at 37°C, in a humid incubator with 5% $\text{CO}_2/95\%$ air. The cell line was passaged once a week. The cells for the experiment were obtained from passages 3-7, inoculated in 24-well plates (Nunc, Roskilde, Denmark) at 5×10^4 cells/well and grown to 85% confluence in Dulbecco's modified Eagle's medium (DME/F12, Sigma, St. Louis, MO, USA) supplemented as above. During the experiments, cells were detached with 0.05% trypsin/0.02% EDTA (Sigma, St. Louis, MO, USA).

Experiments aimed at assessment of cell proliferation in culture were carried out in DME/F12 Ham medium (Sigma, St. Louis, MO, USA), supplemented with a synthetic CPSR-1 serum substitute (Sigma, St. Louis, MO, USA). Incubation of the MCF-7 cells with the examined substances was performed for 24 hours.

[^3H]thymidine incorporation. Cell proliferation in the culture was assessed by measuring incorporation of [^3H]thymidine (Amersham, United Kingdom, specific activity 925 GBq/mmol), after incubation of the cell culture in the medium with or without the examined substances. Two hours prior to the termination of the experiment, [^3H]thymidine was added to the culture at 18.8 KBq/well. After 2-3 washings of the culture with cold phosphate buffer, trypsinisation and precipitation (3 washings with 10% trichloroacetic acid), the precipitate was flooded with Instagel scintillation fluid (Packard, Groningen, The Netherlands). Radioactivity was expressed in dpm per well.

Immunocytochemical examinations. Immunocytochemical examinations were carried out in chambers for histochemical examinations (Lab-tek 4 well chamber slide, Nunc, Naperville, IL, USA). Cell material was fixed with cytofix (Cytifix, Merck, Darmstadt, Germany). A 2-step streptavidin-biotin LSAB kit + HRP kit (with horseradish peroxidase) was used for detection of primary antibodies: PCNA in dilution 1:100 and Ki 67 in dilution 1:25, incubation time - 15 min at room temperature. The incubation time with secondary antibodies was 15 min at room temperature. The antigen-antibody reaction was visualized with DAB (diaminobenzidine) chromogen. The cells were counted with the use of Olympus Micro-Image ImCD UDF morphometric program. The individual microscopic fields were photographed and then the cells were counted on the monitor and the percentage of immunopositive cells in comparison to all cells was established.

Determination of apoptotic index. Determination of cell viability and analysis of apoptotic and necrotic cells were based on 72-hour cultures. Staining was performed with the method of Wright-Giemsa, using a Fisher Leuko Stat kit. MCF-7 cells, cultured in 6-well plates (Nunc, 5×10^4 cells/well), were also stained to reveal apoptosis and necrosis with 10 mM acridine orange and 10 nM ethidium bromide. After removal of the medium the cells were detached with 0.05% trypsin and 0.02% EDTA for 1 min and rinsed. Cell suspension (250 μl) was mixed with 10 μl of acridine orange-ethidium mixture, and

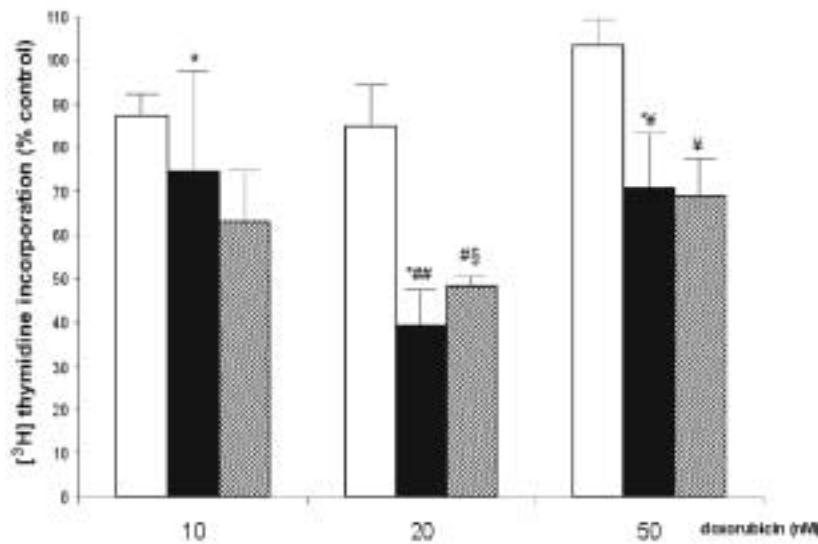


Fig. 1. The influence of doxorubicin (DOX) and doxorubicin combined with 17 β -estradiol (E₂) or with tamoxifen (TAM) on [³H] thymidine incorporation into MCF-7 breast cancer cells. Open bars - DOX; filled bars - DOX + E₂ (2 nM); hatched bars - DOX + TAM (10 μ M). Exposure time 24 hrs. Control = 100%. Data presented as mean values \pm SD (n=4). *p<0.0001 relative to the estradiol group; ^ p<0.01 relative to the DOX 10 nM group; # p<0.01, ## p<0.0001 relative to the DOX 20 nM group; ¥ p<0.005 relative to the DOX 50 nM group; § p<0.01 relative to the TAM 10 μ M group.

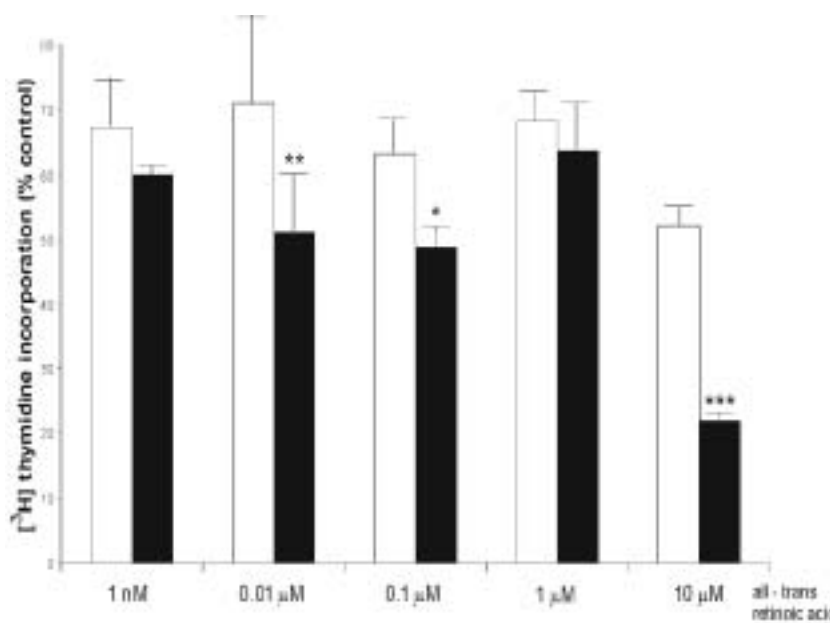


Fig. 2. The influence of various concentrations of all-*trans* retinoic acid (ATRA) alone and in combination with doxorubicin (DOX) on [³H] thymidine incorporation into MCF-7 breast cancer cells. Open bars - ATRA; filled bars - ATRA + DOX (20 nM). Exposure time 24 hrs. Control = 100%. Data presented as mean values \pm SD (n=4). *p<0.01, **p<0.005, ***p<0.0001 relative to the ATRA group.

200 cells/sample were examined in a fluorescence microscope (Nikon) to count living cells with normal nuclei, living cells with apoptotic nuclei, necrotic cells with normal nuclei, and necrotic cells with apoptotic nuclei (cells which entered apoptosis but then developed necrosis).

Statistical analysis. The results were obtained from 3 separate experiments in 4 replicates. In all experiments, mean values \pm standard deviation (SD) for 4 measurements of each parameter were calculated. The Mann-Whitney test was used to perform statistical analysis.

Results

Of the doxorubicin concentrations used in the study, 20 nM inhibited thymidine incorporation to 84.83 \pm 10.00%. It was the most effective concentration and we used it in further experiments. Concentration of 50 nM had no

effect on MCF-7 cells. In the same culture conditions, 2 nM estradiol alone stimulated cancer cells. It was very interesting that despite of the used concentrations of doxorubicin, their combination with 2 nM estradiol caused statistically significant decrease in the percentage of proliferating cells. Also the effectiveness of 20 nM doxorubicin was significantly enhanced by its combination with 10 μ M tamoxifen (Fig. 1). Among substances used in the experiments, combinations of 20 nM doxorubicin with increasing concentrations of all-*trans* retinoic acid revealed a statistically significant decrease in the percentage of proliferating cells and the most effective was the combination with 10 μ M all-*trans* retinoic acid (Fig. 2). The same effect was also noted in the culture - the percentage of PCNA- and Ki-67-positive cells was significantly reduced. The lowest percentage

Table 1. The influence of the studied compounds and their combinations on the percentage of PCNA- and Ki 67- positive MCF-7 breast cancer cells

Group	PCNA	Ki 67
Control	91.75±2.75	97.25±2.5
E ₂ 2 nM	84.50±1.29	84.00±3.26
TAM 10 µM	46.75±2.21**###	49.25±4.42****###
ATRA 10 µM	42.75±1.89****###	43.75±3.77****###
DOX 10 nM	76.50±3.51*	80.00±2.94*
DOX 20 nM	71.25±4.34*	60.50±5.19****#
DOX 50 nM	86.25±3.30	81.00±1.82*
DOX 20 nM + E ₂ 2 nM	38.75±1.70****###	34.00±4.24****###
DOX 20 nM+ TAM 10 µM	42.25±1.70****###	49.75±4.99****###
DOX 20 nM+ ATRA 10 µM	25.75±1.50****###	50.00±2.58****###

Exposure time 24 hrs; Control=100%, E₂ -17β-estradiol, TAM - tamoxifen, DOX -doxorubicin, ATRA - all-*trans* retinoic acid. Data presented as mean values ± SD (n=4). *p<0.01, **p<0.005, ***p<0.001, ****p<0.0001 relative to the control group. # p<0.01, ## p<0.005, ### p<0.001, #### p<0.0001 relative to the estradiol group.

Table 2. Influence of doxorubicin, tamoxifen and all-*trans* retinoic acid on apoptosis in MCF-7 breast cancer cells

%	Control	DOX 10 nM	DOX 20 nM	DOX 50 nM
Viable	95.00±1.77	45.25±3.30**	69.87±1.65**	55.50±3.34**
Apoptotic	2.02±0.40	43.25±2.50**	25.00±1.25**	37.50±2.34**
Necrotic	3.01±0.27	12.25±1.70*	6.87±1.10*	8.00±1.58*
	TAM 10 µM	+TAM 10 µM		
Viable	64.75±1.93	74.50±3.24	61.62±2.49	57.00±2.48
Apoptotic	30.37±2.05	20.75±2.21	37.50±1.87	35.50±2.48
Necrotic	6.06±1.23	6.12±1.25	2.07±0.29¥	8.12±2.49
	ATRA 10 µM	+ATRA 10 µM		
Viable	73.50±1.95	42.12±2.86#	71.12±3.47	50.25±1.84#
Apoptotic	20.50±2.48	48.00±3.53#	25.00±0.81	45.18±2.96#
Necrotic	7.37±1.79	10.75±1.93	4.62±1.37	5.50±0.54

Exposure time 76 hrs. 100% = viable (%) + apoptotic (%) + apoptotic/necrotic (%) + necrotic (%). Data presented as mean values ± SD (n=4). DOX - doxorubicin, TAM - tamoxifen, ATRA - all-*trans* retinoic acid. *p<0.02, **p<0.0001 relative to the control group; ¥ p<0.02 relative to the TAM group, # p<0.0003 relative to the ATRA group.

of PCNA-positive cells was observed after simultaneous addition of 20 nM doxorubicin and all-*trans* retinoic acid, while the lowest percentage of Ki-67-positive cells was observed after the addition of 20 nM doxorubicin with estradiol (Tab. 1). The highest percentage of apoptotic cells was obtained in media containing 10 nM doxorubicin and the combination of 10 nM doxorubicin and 10 µM all-*trans* retinoic acid (Tab. 2).

Discussion

Numerous reports have shown that doxorubicin inhibits the growth of breast cancer cell lines [17, 18, 52], including MCF-7 in a dose and time-dependent manner

[8, 18, 46]. The cytotoxic activity of doxorubicin was somewhat higher in the ER+ than in the ER- cell line [52]. The results of a 24-hour incubation of cell line MCF-7 using 0.1, 1 and 10 µM doxorubicin presented by Ciftci *et al.* [8] have demonstrated that this drug inhibits proliferation to approximately 70-80% irrespective of concentration, and prolongation of culture time up to 48 hours reduces this value to 45% but only for 10 µM (MTT method). In the 24-hour culture using lower doxorubicin concentrations, we obtained similar percentage values. The concentration of 50 nM, that is typically sustained in the peripheral blood for up to 12 h following i.v. administration [5, 37] showed no effect in our experiments. Kurbacher *et al.* [24] also revealed that

MCF-7 and MDA-MB-231 lines were not particularly sensitive to doxorubicin, and a significant reduction of survival fraction of MCF-7 cells was observed only after high concentration of doxorubicin (1 μM and higher). The concentrations of 10^{-3} and 10^{-4} μM had even a slight stimulatory effect (microplate ATP bioluminescence assay). Also Vichi and Tritton [48] reported an increasingly stimulatory effect of doxorubicin, albeit at sub-toxic concentrations.

Estrogen is a promoting factor in mammary carcinogenesis. Estradiol stimulates the growth of breast neoplasms [33], but molecular mechanisms of this stimulation still remain unclear. Our previous results confirmed the stimulatory effect of estradiol on the growth of MCF-7 cells even in the presence of some concentrations of retinoids [12]. It is also known that combination of growth stimulation and cytotoxic therapy leads to an enhanced cell kill in breast cancer [4, 21]. In our study, simultaneous addition of estradiol and doxorubicin to the cell culture increased the sensitivity of MCF-7 cell line to doxorubicin. Although 20 nM doxorubicin inhibited the growth of cancer cells to 84.83%, a two-fold decrease in cell proliferation was observed in the presence of estradiol and 20 nM doxorubicin. Apart from estradiol, this effect is also exerted by EGF, insulin and hydrocortisone [21]. Craford and Bowen [9] revealed that selective sigma-2 receptor agonists in subtoxic doses can potentiate the action of doxorubicin in breast tumor cells. It has been also demonstrated that even pretreatment of MCF-7 breast cancer cells with 1 nM of estradiol enhances the cytotoxic effect of doxorubicin [3].

A therapeutic effect of doxorubicin is observed in approximately half of the patients treated for breast cancer. However, growing resistance to this chemotherapeutic drug is a common problem. Since this resistance is multidirectional (to chemotherapeutic agents belonging to various chemical groups), it can be avoided by potentiating the cytotoxic effect of doxorubicin. Raghunand *et al.* [35] reported that growing pH values increased this cytotoxic effect approximately 2.5 times *in vitro* and *in vivo*. The effect of doxorubicin can be also enhanced by pre-treatment with: medroxyprogesterone [39], megestrol [34], vitamin D₃ [49] vitamin C [24] or simultaneous administration of these compounds with the SERM's [34] or retinoids [49]. Panasci *et al.* [34] reported that the combination of tamoxifen and megestrol caused a 2.5-fold increase in cell sensitivity to doxorubicin. Moreover, tamoxifen (3-8 μM) is able to "reverse" the resistance to doxorubicin in doxorubicin-resistant MCF-7/DOX cell line [13].

Our results suggest that the effect of tamoxifen is dependent on doxorubicin concentration. After simultaneous addition of 20 nM doxorubicin and 10 μM tamoxifen to the culture media, we observed decrease in the percentage of proliferating cells to 48.34%, *i.e.* to value

obtained in the experiments performed by Woods *et al.* [50] after application of over twice as high doxorubicin concentration in 72-hour culture. These authors also reported that combination of doxorubicin and tamoxifen had no additive effect (MTT method).

The treatment of breast cancer cell lines (MCF-7, T-47D, MDA-MB-231, MCF-10 and MCF-12) with 1.25-Dihydroxyvitamin D₃ and all-*trans* retinoic acid either individually or in combination, sensitizes cells to the effects of doxorubicin and this effect is greater also in ER(+) lines [49]. The ER(+) breast cancer cell lines are generally more sensitive to retinoids than ER(-) [25] and it is associated with expression of high levels of RAR α mRNA [47].

In our experiments we observed the potentiating effect of increasing concentrations of all-*trans* retinoic acid on doxorubicin-induced cytotoxicity and the most effective combination was 20 nM doxorubicin with 10 μM all-*trans* retinoic acid. It was also confirmed by assessment of PCNA- and Ki67-positive cells. In the study of Toma *et al.* [46], irrespective of the application procedure (cells were treated with 10^{-7} M all-*trans* retinoic acid before, during and after doxorubicin incubation), all-*trans* retinoic acid was able to potentiate the antiproliferative action of doxorubicin and this effect was mainly marked at low doses of doxorubicin. The authors also observed a synergistic effect when all-*trans* retinoic acid was present before and during exposure to doxorubicin.

In our study it was shown that low concentration of doxorubicin in combination with estradiol, tamoxifen and all-*trans* retinoic acid results in the most effective and statistically significant decrease in the percentage of proliferating MCF-7 cells. The comparison of the substances used in combination with doxorubicin revealed all-*trans* retinoic acid to be the most effective compound, even more effective than tamoxifen, commonly used in breast cancer treatment.

Apoptosis, *i.e.* programmed cell death plays an essential role in both physiological and pathological processes in many human tissues. Defects in the regulation of this pathway lead to many diseases, including breast cancer. As concluded above, all-*trans* retinoic acid enhance the sensitivity of MCF-7 breast cancer cells to doxorubicin. We also tried to answer the question, if these events are connected with the apoptosis pathway. In our previous studies, we observed apoptotic cells after administration to the culture media of 13-*cis* retinoic acid, all-*trans* retinoic acid [12] and combination of retinoids with melatonin or with TGF- β_1 [10, 11].

Doxorubicin has been reported to induce apoptosis [26, 36, 42], and its effect is concentration-dependent [31]. In experiments conducted by Ciftci *et al.* [8], the number of apoptotic cells was minimal during a 12-hour period. Only few apoptotic cells were detected after 24 and 48 h exposure to doxorubicin (0.1 μM - 4.1%; 1 μM

- 4.8%). In 72-hour experiments we observed the greatest number of apoptotic cells after application of 10 μ M doxorubicin. Addition of all-*trans* retinoic acid did not markedly increase that percentage. Despite high antiproliferative efficacy of 20 nM doxorubicin and 10 μ M all-*trans* retinoic acid combination, the percentage of apoptotic cells was only 25% \pm 0.81, being similar to that obtained in the culture with 20 nM doxorubicin. This needs further explanation.

Elmore *et al.* [14] revealed that doxorubicin can induce programmed cell death in MCF-7 cell line when there is no functional caspase 3, and even after elimination of p53 function. Apoptosis has been described in detail and consists of a number of sequential events. DNA degradation occurs at the late stages of this process. Gooch *et al.* [18] demonstrated that MCF-7 cells appeared to have strain-specific differences in their ability to undergo DNA fragmentation following doxorubicin treatment. The lack of DNA laddering observed in MCF-7 was not related to the ability of these cells to undergo apoptosis.

In our study, doxorubicin did not inhibit proliferation as actively as tamoxifen or retinoids; however, its combination with these compounds considerably reduced the percentage of proliferating MCF-7 breast cancer cells. All-*trans* retinoid acid in combination with doxorubicin effectively inhibits cell proliferation, induces apoptotic pathway and may be considered an essential therapeutic element in breast cancer treatment.

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