**FOLIA HISTOCHEMICA ET CYTOBIOLOGICA**
Vol. 42, No. 4, 2004
pp. 221-227

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

**Ewa Czeczuga-Semeniuk[1], Sławomir Wołczyński[1], Milena Dąbrowska[2], Janusz Dzięcioł[3] and Tomasz Anchim[1]**

[1]Department of Gynaecological Endocrinology, [2]Department of Hematological Diagnostics and [3]Department of Anatomy, Medical University, Białystok, Poland.

**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 [$^3$H] 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 ($E_2$), 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 $E_2$ - 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 particulary effective. The inhibitory effect was obtained when 20 nM of DOX and $E_2$, TAM or ATRA were used simultaneously. The use of $E_2$ 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].

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

**Correspondence:** E. Czeczuga-Semeniuk, Dept. Gynaecological Endocrinology, Medical University, M. Skłodowskiej-Curie 24 A, 15-276 Białystok, Poland; e-mail: czeczuga@wp.pl

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-3,17β-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 μg/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% $CO_2$/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 $5 \times 10^4$ 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.

**[$^3$H]thymidine incorporation.** Cell proliferation in the culture was assessed by measuring incorporation of [$^3$H]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, [$^3$H]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 (Cytofix, 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, $5 \times 10^4$ 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

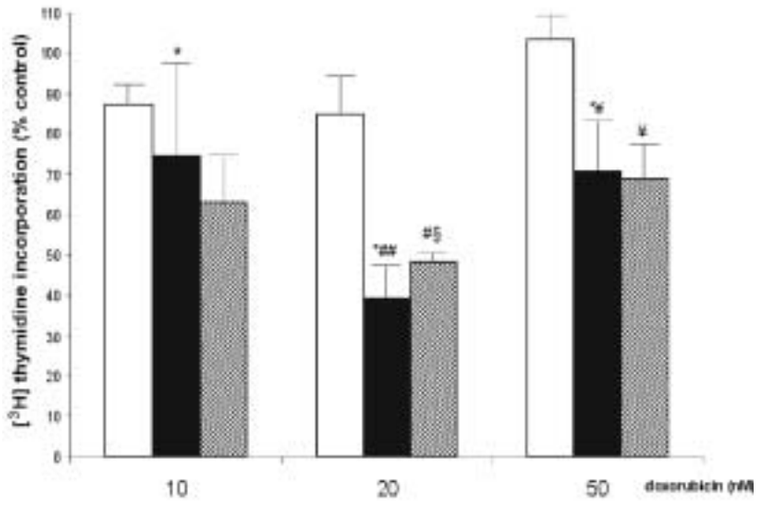


**Fig. 1.** The influence of doxorubicin (DOX) and doxorubicin combined with 17β-estradiol ($E_2$) or with tamoxifen (TAM) on [$^3$H] thymidine incorporation into MCF-7 breast cancer cells. Open bars - DOX; filled bars - DOX + $E_2$ (2 nM); hatched bars - DOX + TAM (10 μM). Exposure time 24 hrs. Control = 100%. Data presented as mean values ± 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 μM group.

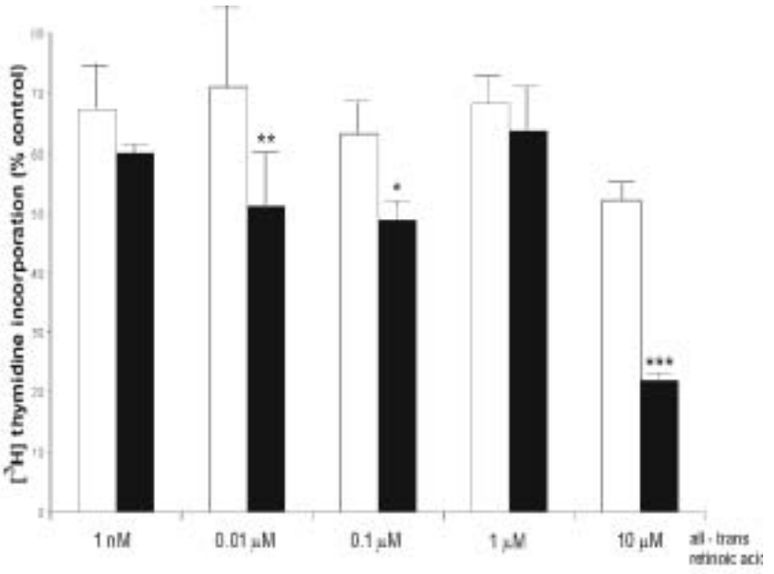


**Fig. 2.** The influence of various concentrations of all-*trans* retinoic acid (ATRA) alone and in combination with doxorubicin (DOX) on [$^3$H] thymidine incorporation into MCF-7 breast cancer cells. Open bars - ATRA; filled bars - ATRA + DOX (20 nM).Exposure time 24 hrs. Control = 100%. Data presented as mean values ± 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 ± 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±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 effectivnes of 20 nM doxorubicin was significantly enhanced by its combination with 10 μM 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 μM 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%, $E_2$ -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 subtoxic 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 $D_3$ [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 $D_3$ 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 RARα 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 conected 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-$\beta_1$ [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 μM doxorubicin. Addition of all-*trans* retinoic acid did not markedly increase that percentage. Despite high antiproliferative efficacy of 20 nM doxorubicin and 10 μM all-*trans* retinoic acid combination, the percentage of apoptotic cells was only 25%±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.

## References


[ 1] Barranco SC, Gerner EW, Burk KH, Humphrey RM (1973) Survival and cell kinetics effects of adriamycin on mammalian cells. Cancer Res 33: 11-16

[ 2] Benchekroun MN, Robert J (1992) Measurement of a doxorubicin-induced lipid peroxidation under the conditions that determine cytotoxicity in cultured tumor cells. Anal Biochem 201: 326-330

[ 3] Bontenbal M, Sieuwerts AM, Peters HA, Sonneveld P, Foekens JA, Klijn JGM (1990) Manipulation of cell cycle kinetics: influence of the cytotoxicity of doxorubicin in human breast cancer cells. J Steroid Biochem Molec Biol 37: 1097-1101

[ 4] Bontenbal M, Sonneveld P, Foekens JA, Klijn JGM (1988) Oestradiol enhances doxorubicin uptake and cytotoxicity in human breast cancer cells, Eur J Cancer Clin Oncol 24: 1409-1414

[ 5] Brenner DE, Galloway S, Cooper J, Noone R, Hande KR (1985) Improved high-performance liquid chromatography assay of doxorubicin: Detection of circulating aglycones in human plasma and comparison with thin-layer chromatography. Cancer Chemother Pharmacol 14: 139-145

[6] Chambon P (1994) The retinoid signaling pathway: molecular and genetic analyses. Semin Cell Biol 5: 115-125

[ 7] Chambon P (1995) The molecular and genetic dissection of the retinoid signalling pathway. Rec Prog Horm Res 50: 317-332

[ 8] Ciftci K, Su J, Trovitch PB (2003) Growth factors and chemotherapeutic modulation of breast cancer cells. J Pharm Pharmacol 55: 1135-1141

[ 9] Crawford KW, Bowen WD (2002) Sigma-2 receptor agonists activate a novel apoptotic pathway and potentiate antineoplastic drugs in breast tumor cell lines. Cancer Res 62: 313-322

[10] Czeczuga-Semeniuk E, Anchim T, Dzięcioł J, Dąbrowska M, Wołczyński S (2004) Can transforming growth factor-β1 and retinoids modify the activity of estradiol and antiestrogens in MCF-7 breast cancer cells? Acta Biochim Pol (in press)

[11] Czeczuga-Semeniuk E, Wołczyński S, Anchim T, Dzięcioł J, Dąbrowska M, Pietruczuk M (2002) Effect of melatonin and all-*trans* retinoic acid on the proliferation and induction of the apoptotic pathway in the culture of human breast cancer cell line MCF-7. Pol J Pathol 53: 59-65

[12] Czeczuga-Semeniuk E, Wołczyński S, Dzięcioł J, Dąbrowska M, Anchim T, Tomaszewska I (2001) 13-*cis* retinoic acid and all-*trans* retinoic acid in the regulation of the proliferation and survival of human breast cancer cell line MCF-7. Cell Biol Mol Lett 6: 925-939

[13] De Gregorio MW, Ford JM, Benz CC, Wiebe VJ (1989) Toremifene: pharmacologic and pharmacokinetic basis of reversing multidrug resistance. J Clin Oncol 7: 1359-1364

[14] Elmore LW, Rehder CW, Di X, McChesney PA, Jackson-Cook CK, Gewirtz DA, Holt SE (2002) Adriamycin-induced senescence in breast tumor cells involves functional p53 and telomere dysfunction. J Biol Chem 277: 35509-35515

[15] Evans RM (1988) The steroid and thyroid hormone receptor superfamily. Science 240: 889-895

[16] Falkson G, Falkson CI, Falkson H (1992) Combination chemotherapy. In: Medical management of breast cancer. Powles TJ, Smith IE [Eds], Martin Dunitz, London, pp 139-151

[17] Fornari FA, Randolph JK, Yalowich JC, Ritke MK, Gewirtz DA (1994) Interference with DNA unwinding by doxorubicin in MCF-7 breast tumor cells. Mol Pharmacol 45: 649-656

[18] Gooch JL, Yee D (1999) Strain-specific differences in formation of apoptotic DNA ladders in MCF-7 breast cancer cells. Cancer Lett 144: 31-37

[19] Gosalvez M, Van Rossum GD, Bianco MF (1979) Inhibition of sodium-potassium-activated adenosine 5'-triphosphatase and ion transport by adriamycin. Cancer Res 39: 257-261

[20] Gudas LJ (1994) Retinoids and vertebrate development. J Biol Chem 269: 15399-15402

[21] Hug V, Johnston DT, Finders M, Hortobagyi G (1986) Use of growth-stimulatory hormones to improve the *in vitro* therapeutic index of doxorubicin for human breast tumors. Cancer Res 46: 147-152

[22] Kaleagasioglu F, Doepner G, Biesalski HK, Berger MR (1993) Antiproliferative activity of retinoic acid and some novel retinoid derivatives in breast and colorectal cancer cell lines of human origin. Arzneim Forsch 43: 487-490

[23] Krinsky NI, Wang X-D, Tang G, Russell RM (1993) Mechanism of carotenoid cleavage to retinoids. In: Carotenoids in Human Health. Canfield LM, Krinsky NI, Olson JA [Eds], New York, The New York Academy of Sciences, New York, pp 167-176

[24] Kurbacher ChM, Wagner U, Kolster B, Andreotti PE, Krebs D, Bruckner HW (1996) Ascorbic acid (vitamin C) improves the antineoplastic activity of doxorubicin, cisplatin, and paclitaxel in human breast carcinoma cells *in vitro*. Cancer Lett 103: 183-189

[25] Lacroix A, Lippman ME (1980) Binding of retinoids to human breast cancer cell lines and their effects on cell growth. J Clin Invest 65:586-591

[26] Lang Y-H, Priebe W, Perez-Soler R (1993) Apoptosis induced by anthracycline antibiotics in P388 parent and multidrug-resistant cells. Cancer Res 53: 1845-1852

[27] Leid M, Kastner P, Chambon P (1992) Multiplicity generates diversity in the retinoic acid signaling pathways. Trends Biochem Sci 17: 427-433

[28] Lotan R (1979) Different susceptibilities of human melanoma and breast carcinoma cell lines to retinoic acid-induced growth inhibition. Cancer Res 39: 1014-1019
[29] McCormick AM, Napoli JL (1982) Identification of 5,6-epoxy retinoic acid as an endogenous retinol metabolite. J Biol Chem 257: 1730-1735
[30] Momparler RL, Karon M, Siegel SE, Avila F (1976) Effect of adriamycin on DNA, RNA and protein synthesis in cell-free systems and intact cells. Cancer Res 36: 2891-2895
[31] Myers C (1997) Anthracyclines and DNA intercalators. In: Cancer medicine. Holland JF, Frei E, Bast RC Jr [Eds], Williams & Wilkins, Philadelphia, pp 977-988
[32] Napoli JL (1993) Biosynthesis and metabolism of retinoic acid: roles of CRBP and CRABP in retinoic acid homeostasis. J Nutr 123: 362-366
[33] Nass SJ, Li M, Amundadottir LT, Furth PA, Dickson RB (1996) Role for BCL-xL in the regulation of apoptosis by EGF and TGFbeta1 in c-MYC overexpressing mammary epithelial cells. Biochem Biophys Res Commun 227: 248-256
[34] Panasci L, Jean-Claude BJ, Vasilescu D, Mustafa A, Damian S, Damian Z, Georges E, Liu Z, Batist G, Leyland-Jones B (1996) Sensitization to doxorubicin resistance in breast cancer cell lines by tamoxifen and megestrol acetate. Biochem Pharmacol 52: 1097-1102
[35] Raghunand N, He X, van Sluis R, Mahoney B, Baggett B, Taylor CW, Paine-Murrieta G, Roe D, Bhujwalla ZM, Gillies RJ (1999) Enhancement of chemotherapy by manipulation of tumour pH. Br J Cancer 80: 1005-1011
[36] Rasbridge SA, Gillett CE, Seymour A-M, Patel K, Richards MA, Rubens RD, Millis RR (1994) The effects of chemotherapy on morphology, cellular proliferation, apoptosis and oncoprotein expression in primary breast carcinoma. Br J Cancer 70: 335-341
[37] Robert J, Illiadis A, Hoerni B, Cano J, Durand M, Lagarde C (1982) Pharmacokinetics of adriamycin in patients with breast cancer: correlation between pharmacokinetic parameters and clinical short-term response. Eur J Cancer Clin Oncol 18: 739-745
[38] Rubens RD (1992) Single-agent chemotherapy. In: Medical management of breast cancer. Powles TJ, Smith IE [Eds], Martin Dunitz, London, pp 131-137
[39] Shaikh NA, Owen AM, Ghilchik M, Braunsberg H (1989) Adriamycin action on human breast cancer cells: enhancement by medroxyprogesterone acetate. Int J Cancer 43: 733-736
[40] Siegfried JA, Kennedy KA, Sartorelli AC, Tritton TR (1983) The role of membranes in the mechanism of action of the antineoplastic agent adriamycin. J Biol Chem 258: 339-343
[41] Sinha BK, Katki AG, Batist G, Cowan KH, Myers ChE (1987) Differential formation of hydroxyl radicals by adriamycin in sensitive and resistant MCF-7 human breast tumor cells: implications for the mechanism of action. Biochemistry 26: 3776-3781
[42] Skladanowski A, Konopa J (1993) Adriamycin and daunomycin induce programmed cell death (apoptosis) in tumor cells. Biochem Pharmacol 46: 375-382
[43] Sporn M, Roberts AB (1983) Role of retinoids in differentiation and carcinogenesis. Cancer Res 43: 3034-3040
[44] Takatsuka J, Takahashi N, De Luca LM (1996) Retinoic acid metabolism and inhibition of cell proliferation: an unexpected liaison. Cancer Res 56: 675-678
[45] Tewey KM, Rowe TC, Yang L, Halligan BD, Liu LF (1984) Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science 226: 466-468
[46] Toma S, Maselli G, Dastoli G, De Francisci E, Raffo P (1997) Synergistic effect between doxorubicin and a low dose of all-*trans*-retinoic acid in MCF-7 breast cancer cell line. Cancer Lett 116: 103-110
[47] Van der Leede B-J, Folkers GE, van den Brink CE, Van den Saag PT, Van der Burg B (1995) Retinoic acid receptor α isoform is induced by estradiol and confers retinoic acid sensitivity in human breast cancer cells. Mol Cell Endocrinol 109: 77-86
[48] Vichi P, Tritton TR (1989) Stimulation of growth in human and murine cells by adriamycin. Cancer Res 49: 2679-2682
[49] Wang Q, Yang W, Uytingco MS, Christakos S, Wieder R (2000) 1,25-Dihydroxyvitamin $D_3$ and all-*trans*-retinoic acid sensitize breast cancer cells to chemotherapy-induced cell death. Cancer Res 60: 2040-2048
[50] Woods K, Randolph JK, Gewirtz DA (1994) Antagonism between tamoxifen and doxorubicin in the MCF-7 human breast tumor cell line. Biochem Pharmacol 47: 1449-1452
[51] Zhu W-Y, Jones CS, Kiss A, Matsukuma K, Amin S, De Luca LM (1997) Retinoic acid inhibition of cell cycle progression in MCF-7 human breast cancer cells. Exp Cell Res 234: 293-299
[52] Zoli W, Ricotti L, Barzanti F, Dal Susino M, Frassineti GL, Milandri C, Casadei Giunchi D, Amadori D (1999) Schedule-dependent interaction of doxorubicin, paclitaxel and gemcitabine in human breast cancer cell lines. Int J Cancer 80: 413-416
[53] Zunino F, Gambetta R, DiMarco A (1975) The inhibition *in vitro* of DNA polymerase and RNA polymerases by daunomycin and adriamycin. Biochem Pharmacol 24: 309-311

*Accepted June 29, 2004*