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

EWA CZECZUGA-SEMENIUK¹, SŁAWOMIR WOŁCZYŃSKI¹, JANUSZ DZIECIOL², MILENA DĄBROWSKA³, TOMASZ ANCHIM¹
and IZABELA TOMASZEWSKA¹
¹Department of Gynaecological Endocrinology, Medical Academy of Białystok, 15-276 Białystok, M. Skłodowskiej-Curie 24 A, Poland, ²Department of Pathological Anatomy, Medical Academy of Białystok, 15-269 Białystok, Waszyngtona 13, Poland, ³Department of Hematological Diagnostics, Medical Academy of Białystok, 15-276 Białystok, M. Skłodowskiej-Curie 24 A, Poland

Abstract: Retinoids are a group of compounds which inhibit cell proliferation and induce cellular differentiation. The aim of this study was to compare the antiproliferative activity of various concentrations of 13-cis retinoic acid (isotretinoin) and all-trans retinoic acid (tretinoin) in a culture of the estrogen-sensitive human breast cancer cell line MCF-7. Evaluation was based on [³H]thymidine incorporation into the cancer cells and through immunocytochemical analysis of cell cycle-associated PCNA and Ki-67 protein expression. Both retinoids inhibited [³H]thymidine incorporation into the cancer cells most effectively at a concentration of 3x10⁻⁵M. Two basic substances used for line MCF-7 culture experiments, one stimulating – estradiol – and the other inhibiting – tamoxifen – were applied. Estradiol added to a culture containing decreasing concentrations of isotretinoin (from 3x10⁻³ to 3x10⁻⁸ M) caused a statistically significant reduction in the percentage of [³H]thymidine incorporation into the cancer cell line MCF-7, compared to the 17 β estradiol group (189.25%±62.64, control=100%, p<0.05). In the group of decreasing tretinoin concentrations, statistically significant differences were found only at 3x10⁻³, 3x10⁻⁴ and 3x10⁻⁵ M. Following culture supplementation with tamoxifen (1µM), statistically significant differences were observed only at the highest concentrations of both retinoids (3x10⁻³ and 3x10⁻⁴ M). The evaluation of breast carcinoma cells with a positive immunocytochemical reaction to PCNA and Ki-

* Corresponding author, tel: (48) +85 746-88-18, (48)+85 746-83-43
67 has revealed that isotretinoin reduces their percentage in the most determined and statistically significant way (38.00%±2.58 and 39.25%±3.09), compared to the control group (86.50%±9.20 and 100%±3.87, p<0.001 and p<0.0001) and to the estradiol group (87.00%±6.79 and 86.10%±7.0, p<0.001). Apart from their blocking effect on the cell cycle, retinoids also induce the apoptotic pathway.

**Key Words:** Isotretinoin, Tretinoin, Cell Line MCF-7, Proliferation, Estradiol, Tamoxifen

**INTRODUCTION**

In hormone-dependent breast cancer, apart from the stimulation of estrogenic receptors by estrogens, other metabolic pathways which regulate neoplastic cell functions are also involved. In breast cancer cells, nuclear receptors for retinoids have been found.

It has been demonstrated that in physiological conditions, retinoids play a role in the growth and differentiation of epithelial cells [1, 2] and inhibit neoplastic processes in many organs, including the breast gland [3], inducing differentiation of neoplastic cells both *in vitro* and *in vivo* [4, 5]. Cells contain specific proteins for retinol and retinoic acid, which transport these compounds in the cytoplasm and via the nuclear membrane [6, 7] by binding to nuclear receptors. The nuclear receptors belong to a superfamily of nuclear steroid/thyroid hormone receptors which regulate protein transcription [8-11]. The pathways through which retinoids signal their actions are associated with two groups of retinoid receptors which carry out various tasks:

- retinoic acid receptors (RARs)
- retinoid X receptors – (RXRs), the two groups of which divide into α, β and γ [12]. RARs bind all the isomers of *trans* retinoic acid [9] and the steroisomers of 9-cis and 13-cis retinoic acid with high affinity [13], while RXR receptors bind only the 9-cis retinoic acid steroisomer [14, 15].

The data in the literature published so far inspired us to investigate the effect of retinoids on the proliferation of MCF-7 breast cancer cells and the interaction of retinoids with estrogens and tamoxifen.

**MATERIAL AND METHODS**

**Culture of MCF – 7 line**

The research was carried out on a hormone sensitive cell line (ER +) MCF-7 of human breast cancer (American Type Culture Collection, Rockville, MD) in DMEM medium (Sigma) supplemented with 10% FBS (Sigma) 50µg/ml streptomycin, and 100 U/ml penicillin in 75 cm² plastic flasks (Nunc), at 37 °C, in humid CO₂ incubator (5% CO₂/95% O₂). The cell line was passaged once a week. Cells for the experiment were taken from passages 3 to 7 and inoculated
in 24-well plates (Nunc) - 5 x 10⁴ cells/well to grow as a mono-layer culture in the Eagle medium of the Dulbecco modification (DME/F12, Sigma) supplemented as above.

During the experiments, cells were removed with 0.05% trypsin/0.02% EDTA solutions (Trypsin/EDTA, Sigma).

Experiments connected with cell proliferation in the culture were carried out in 24-well plates (Nunc) in a DME/F12 Ham medium (Sigma), supplemented with a synthetic replacement of CPSR-1 serum (Sigma).

The experimental incubation of cells of the MCF-7 line with the examined substances was performed for 24 hours.

13-cis retinoic acid (Isotretinoin, Sigma) and all-trans retinoic acid (Tretinoin, Sigma) were diluted in ethyl alcohol and then in the culture medium, until final concentrations from 3 x 10⁻³ to 3 x 10⁻⁸ M were reached.

Tamoxifen (Citrate Salt Tamoxifen, Sigma) was added to the culture at a concentration of 1.0 µM, which corresponds to the concentration obtained in the serum of patients treated with a daily dose of 40 mg tamoxifen [16].

17β estradiol (1,3,5 [10]-Estratriene-3,17 β-diol, Sigma) was added to the culture at a concentration of 1 nM.

[^3H]-thymidine incorporation

Cell proliferation in the culture was evaluated via labelled incorporation of[^3H] thymidine (Amersham United Kingdom), (specific activity 925 GBq/mmol), which was performed after the incubation of the cell culture in the medium with or without the examined substances.

Two hours before the termination of the experiment[^3H]-thymidine was added to the culture in the amount of 18.8 KBq/well. Then after several washings (2-3 x) of the culture with a cold solution of phosphate buffer (137mM NaCl, 2.7mM KCl, 6.5mM Na₃HPO₄, 1.5mM KH₂PO₄, pH 7.4), tripsinisation (0.05% trypsin/0.02% EDTA) and precipitation (washing (3x) with 10% trichloroacetic acid), the precipitate was flocculated with scintillation fluid Instagel (Packard). Radioactivity estimated in dpm was expressed in the count per well (dpm/well).

Immunocytochemical examination

Immunocytochemical examinations were performed in chambers for histochemical examinations (Lab-tek chamber slide 4 well, Nunc). Cell material was fixed with cytofix (Cytofix, Merc). As a detecting kit, a 2-degree streptavidine-biotine LSAB kit + HRP kit (with horse-radish peroxidase) was used. In order to visualise the reaction, antigen-antibody chromogen DAB (diaminobenzidine) was applied. Primary detective antibodies were used.

1. PCNA - Proliferating Cell Nuclear Antigen (Dako) - clone PC 10 (1:100, incubation time - 15 minutes)
2. Ki 67 (Dako) - clone Ki 67 (1:25, incubation time - 15 minutes)
The histologic preparation of breast cancer was the positive control. As a negative control, we used the control sugested by the producer: mouse IgG₁ (cat. no. C0931) for Ki67, and mouse IgG₂a (cat. no. X0943) for PCNA. The results were presented as a percentage of immunopositive cells in the culture (taking $10^3$ cells/sample).

**Statistical analysis**

In all the experiments, the mean values and ± standard deviation (SD) for 4 values of each parameter were calculated. Statistical analysis was performed with the Mann-Whitney test.

**RESULTS**

The effect of 24-hour exposure of retinoids in combination with 17β estradiol (1nM) and tamoxifen (1μM) on $^3$H-thymidine incorporation into MCF-7 breast cancer cells

The retinoids examined reduced cell proliferation in a dose-dependent manner (isotretinoin at $3 \times 10^{-3} - 3 \times 10^{-7}$ M, tretinoin at $3 \times 10^{-3} - 3 \times 10^{-6}$ M). A comparison of the effects of isotretinoin and tretinoin on $^3$H thymidine incorporation into MCF-7 breast cancer cells revealed statistically significant differences only at concentrations of $3 \times 10^{-4}$ and $3 \times 10^{-5}$ M (p<0.05). The mean values obtained as control % in the culture at consecutive tretinoin concentrations were slightly higher (Fig.1).

![Graph](image)

**Concentration [M]**

*Fig. 1. The influence of 24-hour exposure to 13-cis retinoic acid (isotretinoin) and all-trans retinoic acid (tretinoin) on the mean $^3$H thymidyne incorporation into breast cancer cells of the MCF-7 line.*
Compared to the basic conditions (cell line MCF-7 culture without the substances examined), estradiol increased the percentage of $[^3]$H thymidine incorporation into the cells examined by a factor of almost two (189.25% ±62.64, control=100%). This significantly decreased following the addition of isotretinoin at concentrations ranging from $3 \times 10^{-3}$ to $3 \times 10^{-8}$ M (3.52% ±0.44 to 112.54% ±51.25, p<0.05), compared to the values obtained in the 17β estradiol group. No statistically significant differences were observed compared to the consecutive isotretinoin concentrations (Fig.2).

![Graph showing the influence of isotretinoin and 17β estradiol on thymidine incorporation](image)

Fig. 2. The influence of 24-hour exposure to 13-cis retinoic acid (isotretinoin) and 13-cis retinoic acid combined with 17β estradiol (1nM) on the mean $[^3]$H thymidine incorporation into breast cancer cells of the MCF-7 line.

The culture containing estradiol and tretinoin caused a decrease in the percentage of $[^3]$H thymidine incorporation into breast cancer cells compared to the 17β estradiol group. Statistically significant differences were observed at tretinoin concentrations of $3 \times 10^{-3}$, $3 \times 10^{-4}$ and $3 \times 10^{-8}$ M in comparison to the estradiol group (Fig.3).

Tamoxifen (1μM) added to the culture with the consecutive concentrations of isotretinoin and tretinoin, significantly reduced the percentage of $[^3]$H thymidine incorporation into breast cancer cells only in the presence of $3 \times 10^{-3}$ and $3 \times 10^{-4}$ M retinoids (p<0.05), as compared to the values obtained in the tamoxifen 1 μM group (51.11% ±4.01) (Figs. 4, 5).
Fig. 3. The influence of 24-hour exposure to all-trans retinoic acid (tretinoin) and all-trans retinoic acid combined with 17βestradiol (1nM) on the mean [³H] thymidine incorporation into breast cancer cells of the MCF-7 line.

Fig. 4. The influence of 24-hour exposure to 13-cis retinoic acid (isotretinoin) and 13-cis retinoic acid combined with tamoxifen (1µM) on the mean [³H] thymidine incorporation into breast cancer cells of the MCF-7 line.
Fig. 5. The influence of 24-hour exposure to all-trans retinoic acid (tretinoin) and all-trans retinoic acid combined with tamoxifen (1μM) on the mean [³H] thymidyne incorporation into breast cancer cells of the MCF-7 line.

**Evaluation of the immunocytochemical reaction of MCF-7 cells to PCNA and Ki 67 antigens**

Following the 24-hour culture of MCF-7 cells in basic conditions, 86.5% ±9.20 of the cells showed a positive immunocytochemical reaction to the PCNA antigen (Tab.1., Fig.6a). The increasing concentrations of tamoxifen used in the culture resulted in a significant decrease in this percentage, in comparison to both the control and estradiol groups. Culture supplementation with isotretinoin reduced the percentage of PCNA-positive cells to 38.0% ±2.58, which was statistically significant (p<0.001) when compared to the control and estradiol groups (Fig. 6b).

Cell culture exposure to isotretinoin also significantly decreased the percentage of cells with positive immunocytochemical reactions to the Ki67 antigen (39.25% ±3.09, p<0.0001), being significantly lower than in the control and estradiol groups (p<0.001) (Fig. 7a, 7b). Statistically significant values in relation to those obtained in the control and estradiol groups were also observed after culture supplementation with increasing concentrations of tamoxifen (Tab. 1).
Fig. 6. MCF-7 breast cancer cells with positive immunocytochemical reactions to the PCNA antigen; (a) control group, magnification 200x (b) isotretinoin 3x10^{-3}M group, magnification 400x
Fig. 7. MCF-7 breast cancer cells with positive immunocytochemical reactions to the Ki 67 antigen; (a) control group, magnification 220x (b) estradiol group, magnification 220x.
Tab.1. Percentage of MCF-7 breast cancer cells with positive immunocytochemical reactions to the PCNA and Ki67 antigens after 24-hour exposure to 17β estradiol, tamoxifen and isotretinoin. Data presented as mean values ± SD (n=4)

<table>
<thead>
<tr>
<th>Group</th>
<th>PCNA</th>
<th>Ki 67</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>86.50 ± 9.20</td>
<td>100.0 ± 3.87</td>
</tr>
<tr>
<td>17 β estradiol 5 μM</td>
<td>87.00 ± 6.79</td>
<td>86.10 ± 7.01*</td>
</tr>
<tr>
<td>tamoxifen 0.1μM</td>
<td>59.71 ± 8.73* *</td>
<td>58.90 ± 8.02**** *</td>
</tr>
<tr>
<td>tamoxifen 1 μM</td>
<td>46.2 ± 7.15** ##</td>
<td>46.40 ± 5.41**** ##</td>
</tr>
<tr>
<td>tamoxifen 10 μM</td>
<td>48.00 ± 11.67** ##</td>
<td>49.11 ± 9.22**** ##</td>
</tr>
<tr>
<td>isotretinoin 3 x 10⁻⁵ M</td>
<td>38.00 ± 2.58*** ###</td>
<td>39.25 ± 3.09**** ###</td>
</tr>
</tbody>
</table>

p<0.01 - relative to the control group, ** p<0.005 - relative to the control group, *** p<0.001 - relative to the control group, **** p<0.0001 - relative to the control group, * p<0.01 - relative to the estradiol group, ** p<0.005 - relative to the estradiol group, *** p<0.001 - relative to the estradiol group.

**DISCUSSION**

According to the data in the literature, retinoids play an essential role in the regulation of tumour growth and disease progression. Retinoic acid, the most active natural metabolite of vitamin A, inhibits the growth of estrogen-dependent breast cancers [17]. Isotretinoin and tretinoin differently inhibit cell proliferation in many cell lines [18, 19]. Bolag et al. found that isotretinoin and tretinoin equally inhibited MCF-7 cell proliferation at high concentrations [18]. Similar observations were made by Frey et al. [19]. The MCF-7 line seems to be more resistant to the action of retinoids than other cell lines. The retinoids used inhibited thymidine incorporation into breast cancer cells. Following the 24-hour incubation, we observed the most significant effect of both retinoids at a concentration of 3x10⁻³ M, although at a concentration of 3x10⁻⁵ M, isotretinoin inhibited thymidine incorporation most effectively. The inhibition with the use of tretinoin required the concentration of 3x10⁻⁴ M. Toma et al. showed that tretinoin effectively inhibited cell proliferation at 10⁻⁹ M, but only after an 8-day incubation of cell culture [20]. In our study the incubation time was never that long and no effect of retinoids on cell proliferation was found in a shorter incubation period at concentrations of 3x10⁻⁷ and 3x10⁻⁷ M.

Estrogens and estrogen-stimulated newly-synthesised growth factors play a decisive role in cell cycle control in hormone-sensitive breast cancer cells [21, 22].

Retinoids, when used at high concentrations, inhibited estradiol-stimulated proliferation. Isotretinoin was more effective. Many authors believe that estrogens stimulate proliferation in MCF-7 cells through increased synthesis.
and secretion of autocrine growth factors, particularly TGF-α [23]. The TGF-α activated pathway is associated with the activation of tyrosine kinase. Active tyrosine kinase induces a cascade of events leading to increased enzymatic activity and protein expression associated with proliferation. Retinoic acid caused the inhibition of TGF-α synthesis in the MCF-7 line cells. It can thus be assumed that growth factor synthesis inhibition by retinoic acid is an important mechanism that inhibits estradiol-stimulated proliferation in MCF-7 lines.

When binding to estrogenic receptors, their selective modulators, including tamoxifen, exert an inhibitory effect on proliferation [24]. Additive effects of tamoxifen and retinoids may have different catch points. In our study, neither isotretinoin nor tretinoin changed thymidine incorporation, thus suggesting that both tamoxifen and retinoids have similar mechanisms. This can be confirmed by the results of other experiments showing that 13-cis retinoic acid in combination with tamoxifen does not show a synergistic effect on estrogen-positive xenographs [25].

The mechanism by which retinoids affect breast cancer cell proliferation has not yet been fully elucidated. It has been observed that retinoic acid causes cell accumulation in the G1 phase of the cell cycle. Teixeira and Pratt have shown that this effect of retinoic acid is related to the inhibition of mRNA kinase for cdk2 and to the reduced activity of cdk2 protein [26].

Immunohistochemical studies have revealed that most MCF-7 cells show a positive reaction to the PCNA and Ki67 antigens, thus indicating that most cells are in the proliferative phase. Cell exposure to estradiol does not increase the percentage of cells with a positive reaction to the PCNA and Ki67 antigens. In the presence of tamoxifen the percentages of PCNA- and Ki67-positive cells are considerably reduced. 13-cis retinoic acid also decreases the percentage of cells with positive reactions to proliferative antigens.

As shown by Mangiarotti et al., all-trans retinoic acid isomers inhibit the growth of MCF-7 cells by two mechanisms: by blocking cell proliferation mainly in the G1 phase of the cell cycle, and by apoptosis induction [27] (the critical role of retinoic acid receptors β, RAR β) [28].

13-cis retinoic acid also induced the pathway of apoptosis. In our own studies (unpublished data), isotretinoin, like tamoxifen, acted mainly by stimulating apoptosis (36.0% ± 4.96 of cells compared to the control: 1.8% ± 0.8, p<0.0001). Moreover, it statistically significantly reduced the percentage of MCF-7 live cells of breast cancer (56.25% ± 2.21 of cells compared to the control, p<0.0001), (100%=live (%) + apoptotic (%) + apoptotic/necrotic (%) + necrotic (%)) (Fig. 8). Isotretinoin induced apoptosis actively, while tretinoin only in 16% of cells. It seems that apoptosis induction in MCF-7 cells is connected with blocking bcl-2 protein expression. Following cell exposure to 3x10^{-5} M isotretinoin, a statistically significant decrease was found in the percentage of
Fig. 8. Micrographs of Wright-Giemsa-stained MCF-7 cells (a) Control MCF-7 cell culture, magnification 1000x, (b) cells exposed to isotretinoin $3 \times 10^{-5}$ M; note the presence of the morphological features of apoptosis, magnification 1000x.
bcl-2 antigen-positive cells (6.25% ±1.5 compared to 25.00% ±1.29 in the control group (p<0.0004) and 67.80% ±21.99 (p<0.0001) in the 17β estradiol (5μM) group [unpublished data].

The retinoid-activated processes inhibit proliferation and induce apoptosis, which may suggest the possibility of retinoid application in breast cancer therapy.

REFERENCES


