Influence of tamoxifen on cisplatin-sensitivity and estrogen receptors expression in ovarian carcinoma cell lines

Wpływ tamoksifenu na platynowrażliwość i ekspresję receptorów estrogenowych linii komórkowych raka jajnika

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Abstract

Background: Tamoxifen, used in breast cancer treatment, competitively inhibits estrogen receptor (ER) and also demonstrates direct antiproliferative effect on cancer cells even in ER lacking cancer tissue. However, its molecular mechanism of action is still unclear.

Material and methods: We exposed on tamoxifen 11 ovarian cancer cell lines, including well-documented platinum-sensitive and platinum-resistant ones, and studied tamoxifen-, cisplatin-sensitivity and expression of ERα and β.

Results: We observed: no correlation between TAM-sensitivity and ERα and ERß expressions, no correlation between TAM influence on cisplatin-sensitivity and ERα and ERß expressions, increase of ERß expression after TAM-exposure in 3 cell lines; decrease in the 1 line, no TAM-exposure influence on ERα expression and increase of IC50 for cisplatin after TAM-exposure in 5 (45%) cell lines. These results show ovarian cancer cells being affected by TAM have different platinum sensitivity.

Conclusions: Our data suggests that ovarian cancer cells platinum-sensitivity are not linked with ER expressions. We claim the necessity of seeking some TAM predicting factors, using DNA microarrays.

Key words: ovarian carcinoma / cisplatin / tamoxifen / estrogen receptor alpha / estrogen receptor beta /
Introduction

Epithelial ovarian cancer (EOC) is the leading cause of death among gynecological malignancies in Western countries. About 190,000 new cases and 114,000 deaths are estimated to occur annually due to ovarian cancer with the highest rates reported in Scandinavia, Eastern Europe, the USA and Canada [1].

Only about 35% of women will survive for 5 years without any symptoms of the disease. Treatment results have not improved significantly in the course of the last 30 years in spite of new chemotherapeutical agents and surgery protocol development. The unsatisfactory outcome is related to the ovarian tumor localization (deep in the pelvis) and, consequently, late clinical symptoms appearance, which does not facilitate earlier diagnosis. Over 75% of cases are diagnosed in stage FIGO III and IV, with only 20% surviving the period of 5 years [2].

Surgery is the first step of the medical procedures and the optimal cytoreduction remains the most predictive value of the treatment results and patient outcome [3]. Chemotherapy, administered in the vast majority of cases, constitutes the necessary next step. Non-radical surgical performance, together with the phenomenon of chemoresistance (MDR – Multidrug Resistance), are mostly responsible for ovarian cancer treatment failure and that is why ovarian cancer appears to be a persistent disease for most women. Thus, search for new solutions increasing chemosensitivity, remains an essential research area. Recently, certain authors have redirected their attention to tamoxifen (TAM), selective estrogen receptor modulator, and its ability to inhibit the ovarian cancer tissue growth. What is more, it has been proven that, contrary to breast cancer, this mechanism is independent of ERα expression [4, 5].

Moreover, TAM may enhance the cytostatic chemosensitivity [6]. In case of patients with advanced ovarian cancer, clinical TAM administration results, together with platinum-based agents, have not been homogenous. Markman et al. have not managed to prove the positive influence of TAM on chemotherapy efficacy, whereas Benedetti-Panici et al. have indeed demonstrated that patients, both resistant and sensitive to platinum-based chemotherapy, treated with platinum and TAM (80mg/day or 40mg/day) have shown the same treatment response and similar overall survival rate [7, 8].

Due to lack of coherent data, we have made an attempt to check the influence of TAM on various ovarian cancer cell lines, what may help to determine its usefulness in clinical practice.

The aim of our work was to investigate the influence of TAM exposure on cisplatin sensitivity in 11 cell lines, including well-documented platinum-sensitive and platinum–resistant ones. We have also assessed the connection between the TAM effect and expressions of ERα and ERβ on investigated cell-lines.

Material and methods

Cell culture

Human carcinoma cells were grown in Leibovitz L-15 medium (Biowhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (FCS) (GIBCO/BRL, Grand Island, NY), 1 mM L-glutamine, 6.25 mg/l fetauin, 80 IE/1 insulin, 2.5 mg/ml transferrin, 0.5 g/l glucose, 1.1 g/l NaHCO3, 1% minimal essential vitamins and 20,000 kIE/l trasylol in a humidified atmosphere of 5% CO2 at 37°C as described previously [9, 10, 11, 12]. The cisplatin-resistant cell line, A2780RCIS, was derived from the ovarian carcinoma cell line, A2780 [9].

The human ovarian carcinoma cell lines CAOV-3, EFO 21, EFO 27, ES-2, Mdah 2774, OAW 42, OVCAR-3, PA-1, and SKOV-3 were kindly provided by Dr. Carsten Denkert (Institute of Pathology, Charité’, Berlin, Germany). In order to ensure maintenance of cisplatin-resistant phenotype of A2780RCIS cells, the medium was supplemented with 10 Ag/ml of cisplatin (33.3μmol/L; GRY-Pharm, Kirchzarten, Germany).

Cell proliferation assay

Chemoresistance was tested with the use of the proliferation assay, based on sulphorhodamine B (SRB) staining as described previously [13]. Briefly, 800 cells per well were seeded in 96-well plates in triplicates. After 24-hour attachment, cisplatin or TAM (GRY-Pharm, Kirchzarten, Germany) were added in dilution series for a 5-day incubation, before SRB staining was performed. Incubation with studied substances was terminated by replacing the medium with 10% trichloroacetic acid, followed by incubation at 4°C for 1h. Subsequently, the plates were washed five times with water and stained by adding 100 μl 0.4% SRB (Sigma, St. Louis, MO, USA) in 1% acetic acid for 10 min at room temperature. Washing the plates five times with 1% acetic acid eliminated unbound dye. After air-drying and re-solubilization of the proteinbound dye in 10 mM Tris-HCl (pH=8.0), absorbance was read at 562 nm in an Elisa-Reader (EL 340 Microplate Bio Kinetics Reader, BIO-TEK Instruments, Winooski, VT, USA). The measurements were performed in triplicates in three independent experiments. IC50-values were calculated from three independent experiments for each cell line.

Exposure on TAM treatment

4-OH TAM was purchased from Sigma (Deisenhofen, Germany). In our first step we have determined TAM IC50 of the investigated cell lines. Next, the cell lines were cultured for two weeks with addition of 0.0 μM (control), and 0.5 μM, and 1 μM of TAM. The choice of such, TAM concentrations, nontoxic for ovarian cancer cells, was imposed to avoid direct TAM toxicity on the results of the assessment platinum sensitivity. Similar, non toxic for culture cells, TAM concentrations were described by Zhou et al [14]. Scambia et al. have proven that TAM concentrations starting from 0.1 μM increase the ovarian cancer cell platinum sensitivity [15].
**Immunocytochemistry**

In order to detect estrogen receptors α and β proteins by immunocytochemistry, cells were seeded on slides, fixed 10 min in a methanol: acetone (1:1) mixture at -20°C, and air-dried. Afterwards, the slides were incubated 5 min in 3% H₂O₂ to block endogenous peroxidase. Immunocytochemical reactions were performed using of following antibodies: mouse mAb 1D5 (DakoCytomation, Glostrup, Denmark) directed against ERα (optimally prediluted, 1 h at 20°C) and mouse mAb PPG5/10 (DakoCytomation) directed against ERβ (dilution 1:50 in Antibody Diluent, Background Reducing (DakoCytomation), 1 h at 20°C). Each reaction was accompanied by a negative control using Primary Mouse Negative Control (DakoCytomation). The antigens were visualized using biotinylated antibodies (15 min at room temperature), streptavidin–peroxidase complex (15 min at room temperature), LSAB+, HRP (DakoCytomation), and DAB (10 min at room temperature) (DakoCytomation). Preparations were counterstained with Mayer’s hematoxylin (DakoCytomation), dehydrated, and mounted as described previously [11, 12].

**Evaluation of reaction intensity**

In case of immunostained cancer cell lines, specific staining reactions were localized by two experienced histologists. Immunostaining reactions were evaluated using a simplified scale, providing merely a score for the intensities of the reactions (0, total absence of staining; 1, only faint staining; 2, moderate staining; and 3, strong, intense staining) as described previously [12].

**Statistical analysis**

Statistical analyses of the results have been performed with the help of Statistica 98 PL software (Statsoft, Krakow, Poland). \( P < 0.05 \) indicated a significant relationship.

**Results**

**TAM-sensitivity of investigated cells**

The TAM IC₅₀ values of the investigated cells are enclosed in table I.

**TAM influence on ERα and ERβ expressions in investigated cell lines**

In individual cell lines of the ovarian cancer, the performed immunocytochemical reactions showed nuclear or cytoplasmic localization and a variable intensity. Only cells A2780P have shown intense nuclear expression of ERα (score 3). (Figure 1).

Investigated cell lines exposure to TAM did not alter ERα expression. The results of immunocytochemical reactions of ERβ expression are shown in Table II.

In cells not subjected to TAM the nuclear expression of ERβ has only been observed in EFO 21 cell line (Figure 2A1). In these cells the down-regulation of the ERβ expression was noted (Figure 2A2-3). Cell lines CAOV-3, ES-2 and PA-1 after two-week incubation with 0.5 µM TAM have caused up-regulation of nuclear expressions ERβ. (Figure 2B). Nuclear expressions of ERβ have not been observed after two-week incubation with 1µM of TAM. Cell lines A2780P, A2780RCIS, EFO27, Mdah 2774, OAW 42, OVCAR-3 and SKOV-3 have not demonstrated the nuclear expression of ERβ. (Figure 2C). In some cases cytoplasmatic expression of ERβ were traced. (Table II, Figure 2A2).
The IC_{50} results in the investigated cell lines, both exposed to 0.5 μM and 1 μM of TAM for two weeks and not exposed at all (the control group) are presented in Table III and in Figure 3.

In the most platinum resistant cell line, A2780RCIS, platinum IC_{50} was independent of TAM exposure (ANOVA rank test of Kruskal-Wallis, P >0.05). Cell lines CAOV-3, EFO21, EFO27 and PA-1 incubations with 0.5 μM TAM have increased their IC_{50} for platinum (ANOVA rank test of Kruskal-Wallis, P <0.05). Similarly, cell lines CAOV-3, EFO21 and OVCAR-3 incubations with 1 μM of TAM have increased their platinum IC_{50} (ANOVA rank test of Kruskal-Wallis, P <0.05). In the remaining cell lines the ovarian cancer cells have demonstrated a certain sensibilization to platinum (ANOVA rank test of Kruskal-Wallis, P <0.05).

ERs expressions and TAM sensitivity

The ANOVA rank test of Kruskal-Wallis has not demonstrated correlations between ERα and ERβ expressions and TAM IC_{50} of the investigated cells (P >0.05).

**Discussion**

The underlining principle of TAM action is the competitive inhibition of ERα activity. At present, TAM is routinely used only as hormonal drug in breast cancer treatment, where it is administered to women showing ERα expression in cancer tissue [4].

TAM toxicity is generally low and adverse events are noticed rarely [16]. Both types of ER (α and β) are found in normal ovarian epithelium, benign as well as border-line tumors and in ovarian cancer (61% to 79%), especially in its serous and endometrial histological subtypes [17]. However, the ratio of ERα to ERβ is different in cancer tissue comparing to normal ovarian epithelium [17, 18, 19]. ERα expression does not change but ERβ expression is considerably lower in cancer cells, probably due to its selective down-regulation in carcinogenesis process, consequently promoting the mitogenic activity of ERα [20].

Two notable phenomena are directly responsible for TAM testing in ovarian cancer. The above mentioned expressions of ERα and ERβ might be observed in most cases of ovarian cancers, therefore, theoretically, application of selective estrogen modulators should modify EOC development, growth and spread. However, clinical trials have shown only 7 to 18% clinical responses to treatment with TAM in case of women suffering from EOC [21, 22].

Currently, insufficient data prevents us from interpreting those results correctly. Other observations from clinical trials suggest that TAM, irrespective of its direct involvement in ER inhibitions, may increase platinum susceptibility in recurrent ovarian cancer, especially in platinum sensitive diseases [8, 23]. The nature of this platinum – TAM action has not been fully comprehended.
The expected advantages might result from their synergistic effect in cancer cells and the possibility of increasing ovarian cancer cells platinum sensitivity. In ovarian cancer management we attempt to use TAM in partial platinum-sensitive tumor in order to prolong the platinum free interval, what may be effective in tumor cells platinum sensitization; however the molecular mechanism of this action remains unclear [24, 25]. Clinical trials involving tamoxifen in case of ovarian cancer patients have not given unambiguous results so far, and in vitro investigations have been conducted in small groups of cells. We have shown, for the first time, the TAM influence on platinum sensitivity in a large group of cell lines (11 cell lines).

The present results demonstrate different platinum sensitivity of the investigated cell lines being affected by TAM. We have shown that TAM might influence cancer cells platinum sensitivity in different ways. In the cell line CAOV-3, EFO21, EFO27 and PA-1 well-defined decreasing platinum sensitivity was observed upon 0.5 μM of TAM but in the cell lines OVCAR-3 the effect was noted upon 1 μM of TAM. In the cisplatin-resistant A2780RCS cell line, TAM has not affected platinum sensitivity. Similarly to other authors, we observed that inhibition of proliferations in cancer cells and their platinum sensitivity does not depend on ERα or ERβ expression. Mabuchi et al. have proven that TAM inhibits ovarian cancer cells proliferation by means of mitogen-activated protein kinase cascades and this mechanism is mediated independently of ER [5]. Tavassoli et al. have reported the dual effect of proliferation prohibition by TAM action, irrespective of ER. TAM has induced p53-independent cell-cycles arrest at the G1 phase but has caused up-regulation of cyclin dependent kinase inhibitors p15/INK4b, p27/Kip-1, p21/WAF-1 activity and RB hypophosphorylation. P53 independent apoptosis induction might, potentially, be an important anticancer mechanism since it is cisplatin which induces p53 independent apoptosis processes [26]. Zhou et al. have reported ERβ expression down-regulation on the BG-1 (ATCC) cell line in the course of TAM exposure [14].

The present results demonstrated that cancer cell incubation with TAM changes ERβ expression. Our data shows that upon exposure to the 0.5 mM of TAM in cell line EFO 21 ERβ down-regulation was observed and in the cell line CAOV-3, ES-2 and PA-1 its up-regulation were noticed. We have not observed ERα expression in the course of TAM exposure in the investigated cell lines. Bearing in mind considerable TAM differences in IC50 and related to them various cisplatin sensitivity, not to mention, the absence of correlation between ERα and ERβ expression after cell lines TAM exposure results, we claim the necessity of seeking some TAM predicting factors, using DNA microarrays. It might allow us to distinguish the group of ovarian cancer patients who may benefit from TAM therapy.

References


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