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ORIGINAL ARTICLE

The effects of anastrozole on the proliferation of FM3A cells

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Summary

Purpose: *In this study, the antiproliferative effects of the aromatase inhibitor anastrozole (arimidex®) was evaluated on estrogen receptor (ER) positive FM3A cell line originated from C3H mouse mammary carcinoma.*

Methods: *For this purpose cell kinetic parameters including viability analysis, mitotic index and labelling index were used. Three different doses of anostrozole (D_1= 0.01 μM, D_2= 0.1μM, D_3= 1μM) were applied to cells for 24 h to determine the most effective dose. A dose of 1μM dose was determined as the most effective and this was used in all subsequent applications for 0-72 h.*

Results: *The results showed that there was a significant decrease in viability, mitotic index and labelling index for all experimental groups. The differences between control and all experimental groups were statistically significant ($p<0.01$) for all applications.*

Conclusions: *Anastrozole offers a promising treatment modality in estrogen sensitive breast cancer.*

Key words: *breast cancer, cell viability, FM3A, labelling index, mitotic index*

Introduction

Breast cancer is the most common type of cancer among women worldwide [1]. Long-term exposure of breast tissue to estrogen plays a major role in breast carcinogenesis. Consequently, reproductive factors such as total numbers of pregnancies, age at first pregnancy, breastfeeding, age at first menstruation, age at menopause and hormone replacement therapy, which affect a woman's lifetime exposure to estrogen, have been shown to be strongly associated with breast cancer risk [2].

Breast cancer is a heterogeneous disease with different morphologies, molecular profiles, clinical behavior and response to therapy. It is divided into molecular subtypes according to its expression levels of ER, progesterone receptor (PR) and epidermal growth factor receptor (Her2/neu) [3,4]. ER and PR are the most widely studied markers in breast tissue. When compared with hormone receptor–negative tumors, hormone receptor–positive breast cancers exhibit stronger clinical responses to hormonal treatment, better differentiated morphologic appearance, and incidence rates that rise continuously with aging rather than slowing after menopause [5].

Hormonal therapy plays an important role among other treatment modalities of breast cancer. Hormonal therapy is a very effective treatment for hormone receptor-positive breast cancers. This therapy blocks estrogens, thus preventing the proliferation of breast cancer cells. Aromatase inhibitors are used in hormonal therapy of breast cancer and block the conversion of androgens (androstenedione) to estrone in peripheral tissues including fat, liver, muscle and breast without detectable effects on adrenal synthesis of corticosteroids or aldosterone, so they have become an attractive therapeutic modality for steroid sensitive breast cancer [6].

Anastrozole (Arimidex; AstraZeneca, Wilmington, DE, and Macclesfield, United Kingdom) is a new generation, selective nonsteroidal aromatase inhibitor administered orally as a once-daily tab-

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let and has been available since 1995. Anastrozole has been shown to provide potent aromatase inhibition, resulting in near maximal estrogen suppression, both in the blood stream and within the tumor itself. Its use has so far been restricted to the treatment of advanced breast cancer in postmenopausal women whose disease has recurred or progressed despite treatment with tamoxifen [7].

In this study, we investigated the antiproliferative effects of anostrozole, on C3H mouse mammary carcinoma-derived FM3A cell line, which was used as a model of ER positive breast cancer. Therefore cell kinetic parameters including cell viability, mitotic index and labelling index were evaluated.

Methods

Cell culture

ER positive C3H mouse mammary carcinoma-derived FM3A cell line was used. The FM3A cell line was obtained from European Cell Culture Collection (CCL). Cells were cultured in RPMI 1640 (Gibco Lab) containing 10% fetal bovine serum (FBS, Gibco Lab), 100 µg/ml streptomycin (Streptomycin sulphate, I.E.Ulugay), 100 IU/ml penicilin (Pronapen,Pfizer), amphotericin B (Sigma,USA) and 2 mM glutamine at 37 °C in humidified atmosphere of 5% CO_2 in air. The pH of the medium was adjusted to 7.2 with $NaHCO_3$.

Estrogen receptor assay

ER levels were studied by the methods of Lippman and Huff and Raynaud et al. [8,9] with minor modifications. ER activity as demonstrated by the dextran-coated charcoal technique is closely correlated with the clinical effectiveness of anastrozole to inhibit tumor growth.

Drug doses

Anastrozole concentrations that were used in the this study were determined based on previous *in vitro* and clinical studies. At first, 1 mM stock solution was prepared with RPMI 1640 supplemented with 10% FBS. Three different doses were prepared by dilution of the stock solution and were determined as dose 1 (D_1) = 0.01 µM, dose 2 (D_2) = 0.1 µM and dose 3 (D_3) = 1 µM.

Cell viability analysis

For analyzing cell viability after exposure to anastrozole, MTT assay (Thiazolyl Blue Tetrazolium Bromide, Sigma), which effects mitochondrial dehydrogenase of the cells and reduces tetrazolium salts to colored formazan compounds, was used. For this application FM3A cells were seeded into 96-well plates at a density of $2x10^4$ cell per well. At the end of the experimental period, the medium in each well was removed and 40 µl fresh MTT solution (5 mg/ml in PBS) were added into each well and cells were incubated at 37 °C for 4 h. Then, DMSO (Dimethyl Sulfoxide, Sigma) was added into each well and cells were shaked thoroughly for 1 h on a shaker. Then, the absorbance of the samples was measured against a background control as a blank using an Elisa reader (µQuant, Bio-Tek Instruments Inc) at 450-690 nm.

Determination of optimal dose with cell viability analysis

To determine the most effective dose of anastrozole on FM3A cells, three different doses (D_1, D_2, D_3; see above) were applied to cell culture for 24 h. The cytotoxic effects of the different doses were evaluated with MTT assay. At the end of this application, the most effective dose determined according to the absorbance values of different drug concentrations was used for the following parameters:

Mitotic index analysis

Mitotic index was evaluated by the Feulgen method. Before the cells were treated with Feulgen, they were treated with 1 N HCl at room temperature for 1 min and then hydrolyzed with 1 N HCl for 10.5 min at 60 °C. After slides were treated with Feulgen, they were rinsed for a few minutes in distilled water and stained with 10% Giemsa stain solution (pH 6.8) for 3 min and washed twice in phosphate buffer. After staining, the slides were rinsed in distilled water and then were air-dried. Mitotic index was calculated by counting metaphases, anaphases and telophases for each tested drug concentration and control. At least 3,000 cells were examined from each slide for mitotic index evaluation [10].

^{3}H-thymidine labelling index

At the end of the drug administration, cells were treated with medium containing 1 µCi/mL ^{3}H-thymidine for 20 min to evaluate the labelling index.

Autoradiography

After labelling, the cells were fixed with Carnoys fixative [ethanol: glacial acetic acid (3:1)] and the remaining radioactive material was washed twice with 2% perchloric acid at 4 °C for 30 min. Slides were prepared and were coated with K.2 gel emulsion (Ilford, England) prepared with distilled water at 40 °C to determine thymidine labelling index. After 3 days exposure at 4 °C, autoradiograms were washed with D-19 b developer (Kodak) and fixed with Fixaj B (Kodak). The slides were evaluated after being stained with Giemsa for 3 min. The labelled cells were counted on each slide

with 100x12.5 magnification in 100 areas. The same person evaluated all the slides by counting at least 3,000 cells from each slide [11].

Statistics

Viability, mitotic index and labeling index were evaluated relative to controls and to each other. For this reason, values obtained from all experimental groups were analyzed using one-way ANOVA test. The significance between control and experimental groups was determined by Dunnett's test and the significance between experimental groups was determined by Student's t-test. All analyzed data were extracted from 3 independent experiments. A p value < 0.05 was accepted as statistically significant.

Results

Determination of optimal dose with cell viability analysis

The absorbance values for 24 h of each dose were 372.268x10^{-3} for controls, 336.365x10^{-3} for D_1, 246.312x10^{-3} for D_2 and 136.732x10^{-3} for D_3 (Figure 1). The results indicated that 24 h after the administration of anastrozole to FM3A cells, viability values were 90.36 % for D_1, 66.17 % for D_2 and 36.73 % for D_3 compared to control group which was considered as 100% (Figure 2). The differences between control and all experimental groups were statistically significant (p<0.01).

Determination of cytotoxic activity with cell viability analysis

After administration of D_3 dose of anastrozole to FM3A cells for 0-72 h, it was observed that there was a significant decrease in cell viability values. While the absorbance values of the control groups were 367.234x10^{-3}, 398.126x10^{-3}, 427.543x10^{-3}, 445.712x10^{-3}and486.106x10^{-3}for0,12,24,48and72 h respectively, the absorbance values of the experimental groups were 364.678x10^{-3}, 253.236x10^{-3}, 182.784x10^{-3}, 131.368x10^{-3} and 87.625x10^{-3} respectively for 0, 12, 24, 48 and 72 h respectively as shown in Figure 3. After administration of D_3 dose of anastrozole to FM3A cells, viability values were 99.304 % for 0 h, 63.607 % for 12 h, 42.75 % for 24 h, 29.47 % for 48 h and 18.03 % for 72 h compared to control groups which was considered as 100% (Figure 4). The differences between the control and all experimental groups were significant (p<0.01).

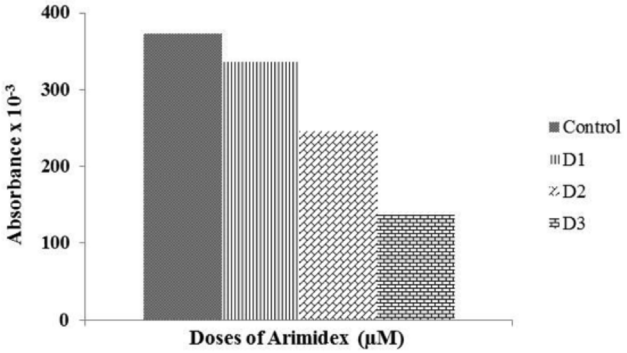

Figure 1. Absorbance values of mitochondrial dehydrogenase activity of FM3A cells treated with three different doses of Anastrozole (D_1= 0.01 µM, D_2= 0.1µM, D_3= 1µM) for 72 h (450-690 nm) (p<0.01).

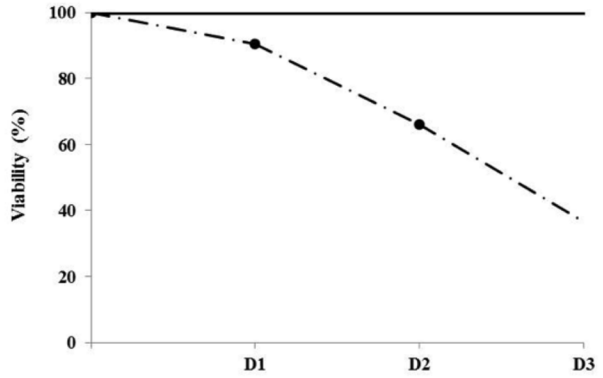

Figure 2. Percent viability values of FM3Acells treated with three different doses of Anastrozole (D_1= 0.01 µM, D_2= 0.1µM, D_3= 1µM) for 24 h (p<0.01).

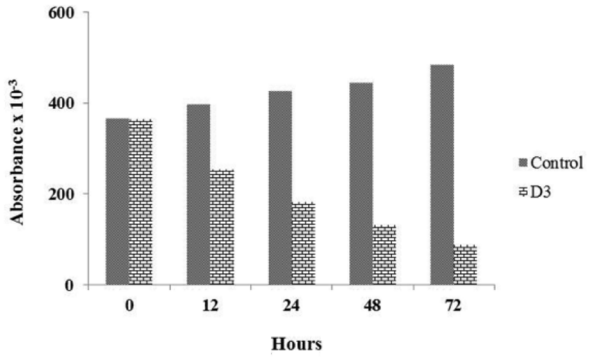

Figure 3. Absorbance values of mitochondrial dehydrogenase activity of FM3A cells treated with D_3 dose of Anastrozole (D_3= 1µM) for 0-72 h (450-690 nm) (p<0.01).

Mitotic index

After administration of D_3 dose of anastrozole for 0-72 h, the mitotic index values of FM3A cells decreased significantly. The differences between the control and all experimental groups were significant (p<0.01). As seen in Figure 5, D_3 dose of anastrozole which was the most effective dose among all doses decreased the mitotic index from

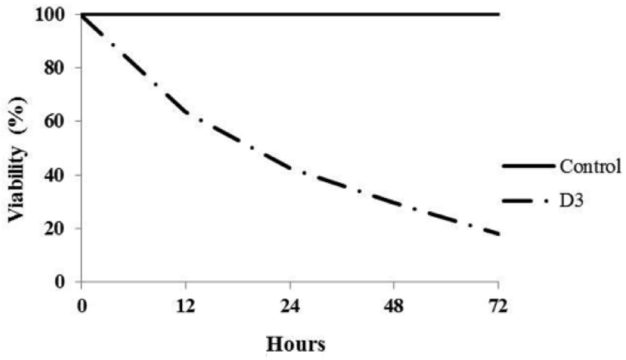

Figure 4. Percent viability values of FM3A cells treated with D_3 dose of Anastrozole (D_3= 1μM) for 0-72 h (450-690 nm) (p<0.01).

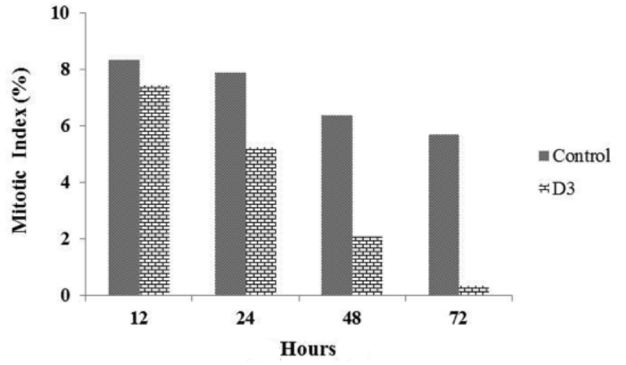

Figure 5. Percent mitotic index values of FM3A cells treated with D_3 dose of Anastrozole (D_3= 1μM) for 0-72 h (p<0.01).

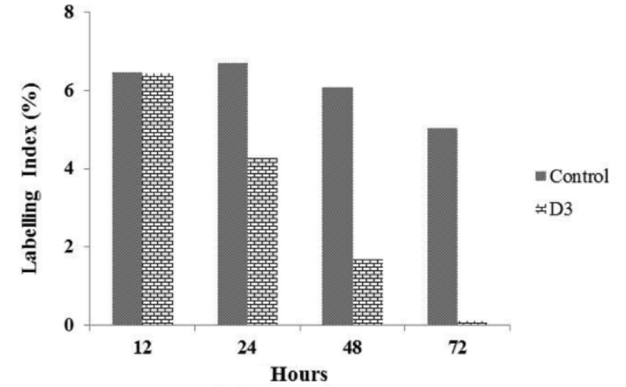

Figure 6. Percent labelling index values of FM3A cells treated with D_3 dose of Anastrozole (D_3= 1μM) for 0-72 h (p<0.01).

8.32 to 7.42 % at 12 h; from 7.87 to 5.23 % at 24 h; from 6.34 to 2.08 % at 48 h and from 5.68 to 0.34 % at 72 h.

Labelling index

After administration of D_3 dose of anastrozole for 0-72 h, the labelling index values of FM3A cells decreased significantly. The differences between the control and all experimental groups were significant (p<0.01). D_3 dose of anastrozole which was the most effective dose among all doses decreased DNA synthesis from 6.45 to 6.42% at 12 h; from 6.68 to 4.26 % at 24 h; from 6.07 to 1.64 % at 48 h and from 5.01 to 0.08 % at 72 h (Figure 6).

Discussion

In the present study we aimed to evaluate the effects of anastrozole on FM3A cell proliferation. In this context, cell kinetics parameters used in this study showed significant decrease in cell viability, mitotic index and labelling index.

After treatment of breast cancer with anastrozole, tamoxifen alone or combination of the two (ATAC), Ki-67 levels were measured and found significantly decreased 2 and 12 weeks after anastrozole treatment [12].

In an *in vitro* study using MCF-7 cell line as ER positive breast cancer, the effects of anastrozole on cell viability and mRNA expression levels were evaluated. A statistically significant growth inhibition was observed with 10^9 mol/L anastrozole and higher concentrations compared with tamoxifen. The PR A, PR B and cyclin D1 mRNA levels in anastrozole-treated cells were significantly below the level of tamoxifen-treated cells (p< 0.05) [13].

An *in vitro* comparison between anastrozole, tamoxifen and the combination of the two drugs was carried out by Xanthopoulos et al. In this study, 4T1 cells were used and cell viability was measured using WST-1 solution. This study demonstrated that there was a decrease in 4T1 cell viability following 24 h anastrozole and combination drug exposures when compared to untreated cells [14].

In a study by Miller et al., after anastrozole administration, aromatase activity and oestrogen levels in the blood stream and oestrogen receptor-rich breast tissue were investigated in postmenopausal patients. Following 12-week anastrozole treatment (1 and 10 mg) there was a profound decrease in aromatase activity in all patients, irrespective of dose. After 3 months of anastrozole therapy, there was statistically significant difference (p=0.0009) between pre-treatment and post-treatment values and the median inhibition value was 89% [15].

Now, 117 years after Beatson's pioneering work with oophorectomy, endocrine therapy has been shown to have a major role in the adjuvant setting. Hormonal therapy may eventually prove

to have its greatest impact in breast cancer prevention [16].

The results of our study seem to be concordant with the above mentioned studies suggesting that anastrozole has antiproliferative effects on breast cancer cells.

In conclusion, the results of this study indicated that there were significant decreases in cell viability, mitotic index and labelling index after treatment of FM3A cells with aromatase inhibitor anastrozole. These explain the mechanism of antiproliferative effects of anastrozole.

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