RESEARCH ARTICLE

Effects of Femara and Tamoxifen on Proliferation of FM3A Cells in Culture

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Abstract

In this study, antiproliferative effects of the selective estrogen receptor modulator Tamoxifen and the aromatase inhibitor letrozole (Femara) were evaluated and compared using the FM3A cell line, originating from a C3H mouse mammary carcinoma and positive in terms of estrogen receptor (ER) expression. Cell kinetic parameters including labelling index, mitotic index and labelling index were assessed after exposure of the. FM3A cell line to 0.001μg/ml of Tamoxifen and 0.25μg/ml of Femara for 4, 8, 16 and 32 h for all parameters. The results showed that cell growth was inhibited by both agents. There was a significant decrease in labelling index and mitotic index and significant increase in apoptotic index for all experimental groups. The differences between control and all experimental groups were statistically significant (p<0.001) for all applications.

Keywords: FM3A - Tamoxifen - femara - mitotic index - labelling index - apoptotic index

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Introduction

Estrogens are important regulators of growth and differentiation in the normal mammary gland and are also important in the development and progression of breast carcinoma (Gruvberger et al., 2001) Breast cancers can be divided into subtypes including hormone-dependent and hormone-independent subtype depending on their hormon receptor status (Santen and Harvey, 1999).

Since estrogens are known to play a role in the growth and development of many breast cancers, a logical approach for the treatment of estrogen-sensitive breast cancer is the use of anti-estrogens that inhibit the estrogen function in breast cancer cells. The first "classic anti- estrogen" is Tamoxifen (Bai and Gus, 2009). For more than 35 years, tamoxifen has been the gold standard for the endocrine treatment of all stages of estrogen-receptor-positive breast cancer (Jordan, 2003) Tamoxifen is selective estrogen receptor modulator and its antagonist effect is particularly prominent with respect to breast cancer. Among women with ER-positive breast cancer, tamoxifen reduces the risk of recurrence and death when given as adjuvant therapy for early stage disease and can provide palliation in those with metastatic disease (Osborne, 1998; Davies et al., 2011).

Aromatase inhibitors (AIs) have become the first choice endocrine drugs for postmenopausal breast cancer patients since they are associated with superior activity and better general tolerability when compared with tamoxifen both in the adjuvant and metastatic settings (Ponzone et al., 2008).

Aromatase inhibitors lower the estrogen level in postmenopausal women by inhibition of the P450 cytochrome enzyme aromatase, which catalyzes the conversion of androgens to estrogens. These agents are only effective in patients in whom ovarian function has been effectively suppressed either naturally or therapeutically (Buzdar, 2003). One such group of agents is the aromatase inhibitors specifically the new generation triazole aromatase inhibitors, such as anastrozole and letrozole, which have both shown tolerability and efficacy advantages over standard treatments in postmenopausal women with advanced breast cancer (Baum, 1999).

Third- generation aromatase inhibitors have become the gold standard in second-line therapy and are being considered as an alternative to tamoxifen for first-line endocrine therapy based on results from randomized trials in postmenopausal patients with metastatic, receptor-positive, or receptor-unknown breast cancer (Mouridsen et al., 2003)

In the present study, to evaluate and compare the antiproliferative effects of Tamoxifen and Femara, estrogen receptor positive breast cancer cell line FM3A was used and for this purpose labelling index, mitotic index and apoptotic index were evaluated as cell kinetic parameters.

Materials and Methods

Cell line

FM3A cell line originated from C3H mouse mammary carcinoma used in this study was obtained from İstanbul University, İstanul Medical School and grown in our cell and tissue laboratory since 1995.

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Cell culture

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 $NaHCO_3$. Cells were seeded 15.000 cells/well in 24 well plates. After these cells incubate at 37°C for 24 hours, experiments were done.

Determination of estrogen receptor

ER levels were studied by the methods of Lippman and Huff and Raynaud et al. (Lippman and Huff, 1976; Raynaud et al., 1978) with minor modifications. ER activity as demonstrated by the dextran-coated charcoal technique is closely correlated with the clinical ability of arimidex to inhibit tumour growth. Cell suspension was mixed with buffer. This mixture was homogenized with a Teflon-glass homogenizer for 5-6 seconds 800 rev/min. The homogenate was centrifuged at 100.000 g for 60 min and then 0.1 ml cytosol sample was incubated 1 nM [3H] estradiol with 60 nM diethylstilbestrol and without diethylstilbestrol at +4°C for one night. After incubation, non-bound estradiol was destroyed by treatment with dextran-coated charcoal and the radioactivity of the supernatant was caunted in a liquid scintillation counter. Specific binding was determined from tritium composition differences between incubated samples and diethylstilbestrol. Data were analyzed according to Scatchard (Klotz, 1982) and estrogen content was found as 12.1 fmol/mg protein. Such a level is a characteristic of estrogen responsive tumors (McGuire, 1990).

Preparing drug concentrations

Arimidex concentrations that were used in the this study were determined based on previous in vitro and clinical studies. 10 mg Tamoxifen (Nolvadex®, Zeneca Pharmaceuticals, A.B.D.) and 2.5 mg Femara (Femara®, Novartis, İsviçre) was dissolved in sterile BSS (Balanced Salt Solution, Gibco lab.) as a 100 μg/ml stock solution. The required final concentrations (0.001μg/ml for Tamoxifen and 0.25 μg/ml for Femara) of the drugs were obtained by diluting of the stock solution in cell culture medium (RPMI-1640) supplemented with 10% FBS.

Preparing 3H-thymidine

9 ml deionized water was added to a vial containing 1 mCi/ml 3H-thymidine (TRA-120, Amersham, England) and stock solution was prepared. Then 1 mCi/ml solution was diluated to 1 μCi/ml with cell culture medium. The cells will be labelled with this solution.

Application of drug and 3H-thymidine

The drug concentrations applied to FM3A cells were determined according to previous in vitro and clinical studies. Cell were exposed to 0.001μg/ml dose of Tamoxifen and 0.25μg/ml dose of Femara for 4, 8, 16 and 32 h for all the cell kinetic parameters. At the end of these periods, to investigate the labeling index parameter cells were incubated in medium containing 1 μCi/ml 3H-thymidine for 20 min. and cells were labelled. Then fixation was carried out.

Labelling index (LI)

Autoradiograms were stained with Giemsa stain at 16°C for 3 min. For each drug concentration and time period of each application 3000 cells were counted under light microscope and percentage of cells labelling was calculated.

Mitotic Index (MI)

Mitotic index was determined by Feulgen method. Feulgen stain is a staining technique used to identify chromosomal material or DNA in cell specimens. It depends on acid hydrolysis of DNA, therefore fixating agents using strong acids should be avoided (Bedi and Goldstein, 1976; Kjellstrand, 1980). Before the cells were treated with Feulgen, they were treated with 1 N HCl at room temperature for 1 min and then hydrolized 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 the slides were air dried. At last 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 MI.

Apoptotic index (AI)

The apoptotic index (the percentage of cells undergoing apoptosis) was studied using fluorescence microscope . For the determination of the AI, cells were fixed with methanol and stained with 4'-6 diamidine-2 phenylindol (DAPI). Following extensive washing in phosphate-buffered saline (PBS), slides were scored under fluorescence microscope. For evaluation of the AI, at least 100 cells were counted for control and each experimental groups.

Statistics analysis

Values of MI, LI 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.

Results

Labelling index

After administration of Tamoxifen and Femara to FM3A cell line for 0, 4, 8, 16 and 32 h, labelling index values of the cells were decreased significantly. The differences between the control and all experimental groups were significant ($p<0.001$). As seen in the Figure 1, while labelling index decreased from 6.64 % to 6.62 % at 0 h; 6.59% to 6.54% at 4 h; 6.28% to 5.86% at 8 h; 5.92% to 4.29% at 16 h and 5.37% to 2,77% at 32 h for 0.001μg/ml dose of Tamoxifen, while decreasing from

6.64 % to 6.63% at 0 h; 6.59% to 6.13% at 4 h; 6.28% to 5.08% at 8 h; 5.92% to 2.36% at 16 h and 5.37% to 1.03% at 32 h for 0.25μg/ml dose of Femara.

Mitotic index

After administration of Tamoxifen and Femara to FM3A cell line for 0, 4, 8, 16 and 32 h, mitotic index values of the cells were decreased significantly. The differences between the control and all experimental groups were significant ($p<0.001$). As seen in the Figure 2, while mitotic index decreased from 10.21 % to 10.19 % at 0 h; 9.87% to 9.63% at 4 h; 9.42% to 8.17% at 8 h; f 9.06% to 7.91% at 16 h and 8.86% to 5.14% at 32 h for 0.001μg/ml dose of Tamoxifen, mitotic index decreased from 10.21 % to 10.19 % at 0 h; 9.87% to 8.84% at 4 h; 9.42% to 7.28% at 8 h; 9.06% to 4.71% at 16 h and 8.86% to 1.17% at 32 h for 0.25μg/ml dose of Femara.

Apoptotic index

After administration of Tamoxifen and Femara to FM3A cell line for 0, 4, 8, 16 and 32 h, apoptotic index values of the cells were increased significantly. The differences between the control and all experimental groups were significant ($p<0.001$). As seen in the Figure 3, while apoptosis increased from 2.26 % to 2.24 % at 0 h; from 2.21% to 2.56% at 4 h; from 2.14% to 2.82% at 8 h; from 1.98% to 4.46% at 16 h and 1.83% to 6.03% at 32 h for 0.001μg/ml dose of Tamoxifen, apoptosis increased from 2.26 % to 2.24 % at 0 h; 2.21% to 2.59% at 4 h; 2.14% to 3.27% at 8 h; 1.98% to 5.42% at 16 h and 1.83% to 9.72% at 32 h for 0.25μg/ml dose of Femara.

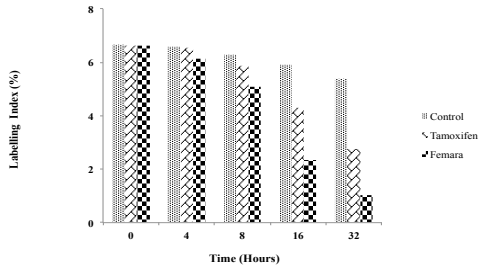

Figure 1. Labelling Index (%) Values of FM3A Cells Treated with 0.001μg/ml Tamoxifen and 0.25 μg/ml Femara 0-32 h

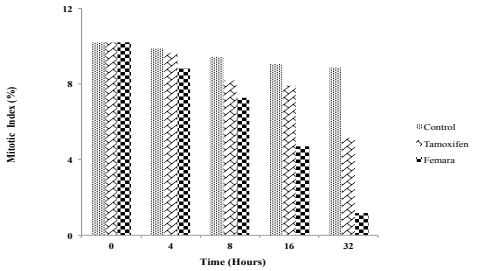

Figure 2. Mitotic Index (%) Values of FM3A Cells Treated with 0.001μg/ml Tamoxifen and 0.25 μg/ml Femara 0-32 h

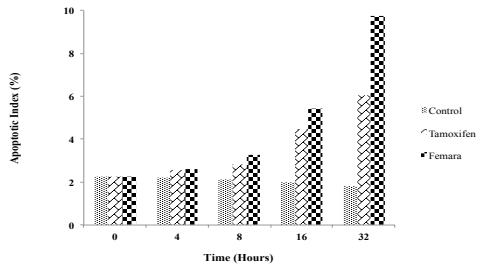

Figure 3. Apoptotic Index (%) Values of FM3A Cells Treated with 0.001μg/ml Tamoxifen and 0.25 μg/ml Femara 0-32 h

Discussion

The aim of this study was to evaluate and compare the antiproliferative effects of antiestrogen Tamoxifen and aromatase inhibitor letrozole (Femara) on FM3A cell line which was originated from estrogen receptor positive breast cancer. For this purpose various cell kinetic parameters including labelling index, mitotic index and apoptotic index were used.

In the breast tissue, tamoxifen principally acts as a competitive antagonist of estrogen receptors. In contrast, nonsteroidal aromatase inhibitors function by interrupting the biosynthesis of estradiol from androgen precursors through competitive inhibition of aromatase.

Reduction in incidence and eventually eradication of breast cancer will recquire development of more effective methods for prevention. Rational prevention strategies for breast cancer require understanding of the molecular mechanisms of carcinogenesis.

Treatment of the aromatase gene expressing MCF7-aro cells with letrozole, doxorubicin and docetaxel in vitro showed that administration of letrozole with either doxorubicin or docetaxel resulted in increased levels of cytotoxicity under all treatment schedules (O'Neill et al., 2012).

Experiments by Azria et al. demonstrated that compared with radiation alone, the combination of radiation and letrozole produced a significant decrease in radiation-induced G2 phase arrest and a decrease of cells in the S phase, with cell redistribution in the G1 phase. These radiobiological results may form the basis for concurrent use of letrozole and radiation as postsurgical adjuvant therapy for breast cancer (Azria et al., 2005)

The translational potential on a well-designed clinical trial considering factors that are typically controlled in preclinical settings.

In comparative studies with tamoxifen, each of the third generation aromatase inhibitors demonstrated clinical efficacy in postmenopausal women with advanced breast cancer. Letrozole was compared with tamoxifen in one study, in which it demonstrated statistically significant superiority in OR rate, clinical benefit and TTF (Campos, 2004).

In a study by Long et al. (2004) they found that the combination of tamoxifen plus letrozole was equivalent to tamoxifen alone. Their result parallels the recently published findings of the Anastrozole, Tamoxifen, Alone or in Combination (ATAC) trial (Baum et al., 2002).

Letrozole is the only aromatase inhibitor to demonstrate consistent superiority over tamoxifen in first-line treatment. It associated with an OS advantage for the first- line setting indication at 1-year and 2-year follow-up (Mouridsen, 2007).

Previous neo-adjuvant trials using oestrogen antagonists have demonstrated no close relationship between clinical response as measured by changes in

apoptosis and proliferation (Dowsett et al., 2005). In contrast, our results of the study indicated that whereas labelling index and mitotic index values which reflect proliferation rate decreased significantly, apoptotic index values increased significantly.

Our findings showed that Femara is more effective than Tamoxifen on estrogen receptor positive FM3A cell line.

In recent years treatment of breast cancer have gained a new direction with the discovery of cancer stem cells. If stem cell differentiation potential becomes impaired and their proliferative capacity becomes uncontrolled, these mutated, potentially tumorigenic, self-renewable stem cells have the potential to cause cancer and are called cancer stem cells (CSCs). CSCs are responsible for tumor relapse and metastasis (Cetin and Topcul, 2012). Ovariectomy markedly diminished mammary stem cells and mammary stem cell activity is increased by treatment with estrogen and progesterone. Intriguingly, treatment with aromatase inhibitor letrozole is for three weeks was sufficient to reduce mammary stem cell pool. Simpliarly aromatase knockout animals showed reduced number of mammary stem cells (Asselin-Labat et al., 2010).

Our results indicated that aromatase inhibitors are superior to anti-estrogens for breast cancer prevention. However, further clinical studies are required to directly compare tamoxifen with aromatase inhibitors in women at high risk of developing breast cancer.

Thus, the results of our study seem to be concordant with the above mentioned studies, suggesting that femara is better than tamoxifen for early-stage. But, in ER-positive and ER-negative breast cancer cell lines, the growth, cytotoxic, and cell-cycle effects must be examined and compared. Also investigating the effects of femara combination with different kinds of anticancer treatments would be useful.

Unique to our study, we demonstrated that the effects of Femara and Tamoxifen on the proliferation of FM3A cells has shown that labelling index and mitotic index values decreased while apoptotic index values decreased effectively by Femara and Tamoxifen treatment. These results suggest the use of Femara fort he treatment of ER positive breast cancer to be an alternative of Tamoxifen treatment. But available evidence suggests that switching strategies are rational and effective for most patients and that they are the only ones able to provide a modest, though not trivial, survival advantage as well.

In conclusion, persistance with all endocrine treatments in women with hormone receptor positive breast cancer is low and needs to be significantly increased to improved outcome in clinical practice. Further research is recquired to understand this complex issue.

Acknowledgements

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