Experimental Article

Letrozole and zoledronic acid changed signalling pathways involved in the apoptosis of breast cancer cells

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Abstract

Objectives: Oestrogen plays a key role in the development of breast malignancies. Therefore, aromatase inhibitors (e.g. letrozole [LTZ]) are widely used in the treatment of breast cancer. On the other hand, oestrogen is important to the integrity of bone mass. Research has shown that zoledronic acid (ZLA) may prevent osteoporosis. Therefore, the present research aims to investigate the effect of a combination of LTZ and ZLA in the treatment of breast cancer and in reducing osteoporosis in patients with breast cancer.

Methods: We used immunocytochemistry and Western immunoblotting techniques in this study.

Results: We observed that LTZ inhibited cellular growth of Michigan Cancer Foundation-7 (MCF-7) and T-47D at IC50 70 nM and 140 nM, respectively, whereas ZLA inhibited cellular growth at IC50 50 μM and 150 μM for MCF-7 and T-47D cell lines, respectively. Interestingly, the LTZ and ZLA combination increased the apoptotic rate of breast cancer cells.

Conclusions: The combination of LTZ and ZLA showed a synergistic effect on the treatment of breast cancer and in reducing osteoporosis in patients with breast cancer.
Introduction

Breast cancer is the most common malignancy and the second cause of cancer-related death among women worldwide. Increased exposure to circulating oestrogen is considered a major risk factor of breast cancer. Inhibition of the effects of oestrogen can be achieved either by using anti-oestrogens to inhibit the binding of oestrogen to its receptors or by using aromatase inhibitors (AIs) to block its synthesis. AIs have replaced tamoxifen as initial adjuvant therapy for the treatment of postmenopausal patients with hormone-sensitive breast cancer. The third generation of AIs includes letrozole (LTZ), which shows the highest potency and inhibition of aromatase enzyme activity, is the third generation of AIs. This inhibition consequently inhibits the conversion of androgens to oestrogen which is mediated by the aromatase enzyme.

On the other hand, bisphosphonates are effective in preventing fragility fractures and are currently used in the treatment of patients with numerous myeloma and bone metastasis. In an approach that seeks to minimise bone loss caused by AIs, bisphosphonates such as ZLA can be co-administrated with LTZ to bypass the occurrence of osteoporosis in breast cancer patients. ZLA also has a great affinity for bone mineralisation and binding to hydroxyapatite of the bone matrix. Additionally, several studies have reported that the major enzymatic target of ZLA is farnesyl pyrophosphate synthase (FPP), considered to be one of the enzymes involved in lipid biosynthesis. This enzyme is proven to have both direct and indirect anti-tumour effects on breast cancer progression. Furthermore, in vitro studies have shown that ZLA has direct anti-tumour effects via the induction of apoptosis and inhibition of cancer cell proliferation.

Breast cancer type 1 susceptibility protein (BRCA1) is a tumour-suppressor protein that plays a key role in maintaining proper genomic repair and cell division by preserving genomic integrity throughout the regulation of DNA replication, repair, and transcription. Indeed, inherited or sporadic mutations in the BRCA1 gene may disrupt these processes, resulting in chromosome instability and defective checkpoints which speed up cellular transformation in breast or ovarian cancers. On the other hand, it is reported that increased expression of BRCA1 leads to inhibition of oestrogen receptor-mediated transcription. Consequently, there is a great demand to develop and improve molecularly-based therapies through a better understanding of biochemical pathways and genetic alterations involved in different subtypes of human breast cancer, with special emphasis on the pathogenic significance of these events related to the progression of the disease.

In another avenue, it is well known that signal transducer and activator of transcription 3 (STAT3) belongs to the STAT family of proteins, both signal transducers and transcription factors activated in response to many cytokines and growth factors. This family of proteins is involved in cell proliferation, differentiation, and apoptosis. STAT3 mainly acts as an anti-apoptotic factor and has been found to be overexpressed in many malignancies. Under normal circumstances, STAT activation is temporary, whereas, in resting cells, STATs remain inactive and located in the cytoplasm. Upon their activation by many cytokines and growth factors, they start to dimerise through phosphorylation, leading to their translocation from the cytoplasm into the nucleus, followed by their binding to DNA. As a result, several studies have reported that the targeting inhibition of the STAT3 signalling pathway could arrest the growth of tumour cells in vitro and lead to tumour regression in vivo. In addition to its enhanced effect on bone density maintenance, ZLA was found to inhibit the phosphorylation of serine residues in the aromatase enzyme, leading to inhibition of its enzymatic activity. Therefore, LTZ alone and/or in conjunction with ZLA was used to treat both MCF-7 and T-47D human breast cancer cells. The present research also demonstrated the mechanism of cell apoptosis in both cell lines by observing the protein expression of BRCA1 and STAT3 under the influence of this combination.

Materials and Methods

Cell culture and reagents

Human breast cancer cell lines MCF-7 and T-47D were purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA). Cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640) and...
Dulbecco’s Modified Eagle’s Medium (DMEM), respectively, supplied with 10% bovine serum (FBS). Fifty IU/ml penicillin and 50 IU/ml streptomycin. LTZ and ZLA were purchased from Sigma–Aldrich (St. Louis, USA). Anti-β-Actin IgG, anti-STAT3 (F-2) IgG1, and anti-BRCA1 IgG were purchased from Santa Cruz Biotechnologies, Inc., CA, USA.

Cell viability by MTT assay

Cellular viability was estimated using the Vybrant® MTT Cell Proliferation Assay Kit (Invitrogen Corporation, CA, USA). Both MCF-7 and T-47D cell lines were seeded (10^4 cells/well) in complete DMEM and RPMI-1640 medium, respectively, and left until reaching the desired densities within 48 h. Then, the cells were treated for 24 h with different LTZ concentrations (1–140 nM, in the case of the MCF-7 cell line, and 1–200 nM, in the case of the T-47D cell line). ZLA was used in concentrations of 1–100 μM, in the case of the MFC-7 cell line, and 1–200 μM, in the case of the T-47D cell line. Mock cells were treated with 0.1% Dimethyl sulfoxide (DMSO). The medium was removed and replaced with a fresh culture medium (100 μL), followed by the addition of 10 μL of MTT stock solution in each well. Afterwards, the treated cells were incubated at 37 °C for 4 h under light protection in the 96-well plate, then 100 μL of the prepared SDS–HCl solution were added to each well and mixed thoroughly, followed by incubation at 37 °C for another 18 h in the CO2 incubator. Finally, absorbance at 545 nm was read using a micro-plate ELISA reader (enzyme-linked immunosorbent assay reader, Stat Fax 2100, USA). The inhibitory concentration (IC50) values of LTZ, ZAL, and the LTZ/ZAL combination were determined by constructing a sigmoidal curve for each treatment.

Morphological examinations

Changes in the morphology of both types of human breast cancer cell lines (MCF-7 and T-47D) were examined before and after treatment with LTZ, ZAL, and LTZ/ZAL combination. Equal numbers of cells/well were seeded in 12 well plates in DMEM or RPMI medium for MCF-7 and T-47D cell lines, respectively, with different concentrations of each drug alone or in combination and incubated for 72 h. Afterwards, cells were washed with Phosphate buffer solution (PBS), fixed with 10% formalin buffer, and visualised using an inverted optical microscope with 200X magnification (Inverted Microscope, Optika, Italy). Digital images were then captured using a Kodak microscopic digital camera.

Western immunoblotting

Cells were seeded in a 25 cm² flask and incubated at 37 °C and 5% CO₂ in a CO₂ incubator for 48 h, followed by treatment with appropriate concentrations determined according to the obtained IC50 of LTZ and ZAL alone or in combination. Radioimmunoprecipitation assay (RIPA) buffer was used for whole cells and for membrane-bound protein extracts. The samples were kept on ice, and proteinase inhibitors and Phenylmethylsulfonyl fluoride (PMSF) were freshly added to the lysis buffer. The cells were rinsed with an ice-cold PBS buffer after treatment and then easily harvested by cell scraping using a cold plastic cell scraper after ice-cold lysis (RIPA buffer) was applied at a concentration of 1 ml per 107 cells/25 cm² flask. Cell lysates were gently transferred to pre-cooled microfuge tubes and kept for 60 min at 4 °C with agitation. Afterwards, cells were centrifuged at 1500 rpm for 15 min, then the supernatant was carefully aspirated and kept on ice. Protein lysates of both MCF-7 and T-47D cell lines were separated using denaturing polyacrylamide gel electrophoresis, followed by their transfer to a 0.45-mm nitrocellulose membrane (Bio-Rad Laboratories). Membranes were blocked using 5% skim milk in TTBS (Tris-buffered saline +0.1% Tween-20) for 1 h at 4 °C with constant agitation to prevent non-specific binding. Specific primary antibodies against STAT3 and BRCA1 were diluted in 5% skim milk in TTBS and incubated with the membranes at room temperature for 4 h. After washing the membranes with TTBS, they were incubated with their corresponding secondary antibodies. Transferred protein bands were detected using 3, 3, 5, 5 tetramethylbenzidine (TMB) (Sigma–Aldrich Corp. St. Louis, MO, USA) in a dark room for 15 min. Finally, the membranes were stripped using the blot stripping buffer, Thermo Scientific Restore™ (Thermo Scientific, IL, USA), and reprobed with anti-β-actin as a control to ensure loading of equal amounts of protein. Densitometry of the obtained bands was estimated using Quantity One Analysis Software (Bio-Rad).

Immunocytochemistry

The MCF-7 and T-47D cells were cultured to be sub-confluent while incubated at 37 °C, followed by their treatment with LTZ (100 nM) or ZAL (50 μM) for 24 h in a culture medium containing foetal bovine serum. Cells were then collected and placed on microscopic glass slides. Afterwards, they were deparaffinized and rehydrated with xylene, ethyl alcohol (70, 95, and 100%), and, finally, with distilled water for 5 min per each step. The slides were then immersed in pre-warmed sodium citrate (10%) in the microwave for 20 min, then left to cool. The fixed cells were incubated with anti-BRCA1 antibody (1:100) or anti-STAT3 mouse polyclonal antibody (1:50) overnight at 4 °C, then detection was conducted using the Pierce Peroxidase IHC Detection Kit (Thermo Scientific, IL, USA). Finally, the stained cells were mounted in DPX (Biostatin Ready Reagents Ltd, Manchester, UK) and visualised under a light microscope at 1000x magnification.

Statistical analysis

All data were represented as mean ± SE, and the results were analysed using a one-way analysis of variance
(ANOVA) and Tukey’s Multiple Comparison test using Graphpad Prism 6 software (San Diego, CA). $P < 0.05$ was considered statistically significant. All experiments were run in triplicate.

**Results**

*Letrozole and zoledronic acid inhibit the viability of breast cancer cells*

In an attempt to study the inhibitory effect of both drugs on breast cancer cells, MCF-7 and T-47D human breast cancer cell lines were treated for 24 h with different concentrations of LTZ (1−140 nM, in the case of MCF-7 cells, or 1−200 nM, in the case of T-47D cells. Concentrations of 1−100 μM or 1−200 μM of ZLA were used for the MCF-7 cells and T-47D cells, respectively. Upon measuring cellular viability using MTT assay as previously described, it was observed that both drugs inhibited the cellular viability of both types of breast cancer cells in a dose-dependent manner, as shown in Figure 1. IC$_{50}$ values were calculated for both drugs. The MCF-7 cells displayed higher inhibition with LTZ (70 ± 0.001 nM), compared to ZLA (50 ± 0.005 μM). Similarly, T-47D cells exhibited higher inhibition with LTZ (140 ± 0.004 nM) when compared to ZLA (50 ± 0.005 μM). Interestingly, the combinational treatment with both drugs for 24 h showed a significant inhibition of the growth of MCF-7 and T-47D cells at concentrations of 70 nM/25 μM and 90 nM/50 μM of LTZ/ZLA, respectively.

*Letrozole and zoledronic acid induced apoptosis in breast cancer cells*

For further confirmation of the cell viability data, the effects of LTZ and ZLA on the morphology of MCF-7 human breast cancer cells were examined. The cells were cultured in 12-well plates and treated with different concentrations of LTZ (30−90 nM) and ZLA (10−50 μM). Two different combinational ratios were used, including 30 nM/10 μM and 50 nM/25 μM of LTZ/ZLA, respectively. Following treatment of MCF-7 cells with each drug individually or in combination, the results revealed a significant increase in the proportion of cells undergoing apoptosis ($P < 0.05$). In addition, the treated cells were shown to have the characteristic apoptotic nuclear morphology, including nuclear fragmentation, chromatin condensation, and the formation of dense, rounded apoptotic bodies, as shown in Figure 2.

**BRCA1 protein is a key regulator protein in the preservation of genomic integrity through the DNA repair process.**

To estimate the effects of LTZ, ZLA, and LTZ/ZLA combination on the BRCA1 and STAT3 protein expression levels, MCF-7 and T-47D cells were treated for 24 h with LTZ (100 nM), ZLA (50 μM), and a combination of LTZ/ZLA (50 nM/25 μM). BRCA1 protein expression was then determined using Western immunoblotting. In the case of MCF-7 cells, LTZ individually or in combination with ZLA significantly increased the expression of BRCA1 protein in a dose-dependent manner, compared to the mock cells, whereas cells treated with ZLA alone did not exhibit any change in the expression of BRCA1 protein (Figure 3A and B). In addition, substantial increases in the expression of BRCA1 protein in T-47D cells were observed, whereas the level of STAT3 protein in MCF-7 cells decreased significantly in a dose-dependent manner following treatment with LTZ, ZLA and their combination. By contrast, the T-47D cells displayed a reduced expression of STAT3 protein after treatment of cells with LTZ alone or in combination with ZLA compared to the mock cells. However, treating cells with ZLA did not change the expression of STAT3 protein (Figure 3C and D).

**Letrozole and zoledronic acid induced BRCA1 and inhibited STAT3 nuclear localisation in T-47D breast cancer cells**

Nuclear localisation of BRCA1 and STAT3 were examined in T-47D cells using immunocytochemistry, as previously described in the materials and methods section. After treating the cells for 24 h with LTZ (100 nM) or ZLA (50 μM), there was a notable enhancement of the nuclear localisation of BRCA1 protein compared to the control cells in the nuclei of T-47D cells (Figure 4). In contrast, there was a significant ($P < 0.05$) reduction in the nuclear localisation

**Figure 1:** LTZ and ZLA inhibit the growth of breast cancer. MCF-7 cells and T-47D were treated with different concentrations of LTZ (10, 30, 50, 70, 90, 120, 140, 200 nM) for 24 h (A). MCF-7 cells and T-47D were treated with different concentrations of ZLA (1, 5, 10, 15, 25, 50, 80, 100 μM) for 24 h (B). Percentage of cell viability was calculated compared to mock. The calculated anti-log concentration represent LTZ IC$_{50}$ of 70 nM (anti-log 1.8) for MCF-7 cells and IC$_{50}$ of 140 nM (anti-log 2.14) for T-47D. The calculated anti-log concentration represent ZLA IC$_{50}$ of 50 μM (anti-log 1.6) for MCF-7 cells and IC$_{50}$ of 150 μM (anti-log 2.17) for T-47D cells.
Figure 2: LTZ and ZLA induce morphological changes in breast cancer cells. MCF-7 cells were treated with different concentrations of LTZ and ZLA individually or both drugs in combination for 72 h. Cells were washed with PBS and fixed with 10% formalin, then examined at 100x using an inverted light microscope. All treatments were conducted in triplicate.
of STAT3 protein associated with its transfer to the cytoplasm, suggesting that STAT3 could act as an attractive target for inhibition of Estragon Receptor (ER)-positive breast cancer proliferation and progression.

Discussion

Oestrogen, with its proliferative effect, is well known as the key hormone involved in breast cancer etiology. Therefore, its synthetic pathway is considered to be an attractive target pathway for treatment of hormone-sensitive breast cancer. However, oestrogen is highly important for maintaining bone integrity in women, and inhibition of its synthesis pathway elevates the rate of bone turnover and loss in LTZ-treated breast cancer patients. Therefore, post-menopausal breast cancer women could have a higher rate of bone turnover than normal postmenopausal women. Supporting this suggestion, it has been found that AI-treated breast cancer women showed an increased risk of osteoporosis and bone fractures. Moreover, nitrogen-containing bisphosphonates, e.g. ZLA, were found to enhance bone mineral density in normal postmenopausal women via inhibition of osteoclast-mediated bone resorption. Therefore, in the present study, LTZ has been used in addition to ZLA to possibly prevent osteoporosis and treat postmenopausal women with breast cancer.

BRCA1 plays an important role in regulating aromatase activity in breast cancer cell lines via its direct interaction with a DNA sequence in the proximal promoters I.3 and II regions of aromatase, suggesting the inhibitory effect of BRCA1 might be due to its ability to suppress oestrogen production in breast tissue. In the present study, the protein expression of BRCA1 was up-regulated in both cell lines after their treatment with the combination of LTZ and ZLA for 24 h. The mechanism of up-regulation of BRCA1 might be due to inhibition of ER transactivation via suppression of P300, which is a transcriptional activator for ER. In accordance with the present study, it has been found that raloxifene, an anti-osteoporotic drug, does not interfere with anticancer efficacy of LTZ; rather, the combination acted additively for the treatment of breast cancer. In addition, LTZ/ZLA treatment resulted in inhibition of growth and apoptosis in both cell lines. The results of the present study are inconsistent with the previously reported studies because the anti-apoptotic and anti-proliferative effects of LTZ on MCF-7 human breast cancer cells were time-dependent. In parallel, ZLA also effectively inhibited the growth of both breast cancer cell lines in a dose-dependent manner after treatment for 24 h. Moreover, ZLA use has been associated with a decreased risk of skeletal-related events among patients with bone metastases from breast cancer or prostate cancer and/or multiple myeloma.

Treatment of MCF-7 with LTZ/ZLA (30 nM/10 μM) or (50 nM/25 μM) treatment of MCF-7 for 72 h caused considerable growth inhibition because morphological changes occurred in both cell lines, including cell shrinkage and cytoplasmic condensation with a higher tendency to float on the growth media surface. Supporting our finding, the growth rate inhibition of breast cancer cells increased if LTZ was added first, for a certain time, prior to the addition of ZLA. In another study, the administration of LZT in combination with ZLA was found to be effective in patients with a low frequency of γδ T cells, which are a unique subtype of T cells having both γ and δ T cell receptor (TCR) chains with multiple immune responses during cancer progression. Therefore, selecting a panel of molecular markers that elucidate the mechanism of

Figure 3: LTZ individually or in combination with ZLA up-regulates BRCA1 and down-regulates STAT3 protein expression levels in breast cancer cells. Western blot analysis of BRCA1 and STAT3 expression levels in MCF-7 cells (A); quantification of their expression levels normalised by the levels of β-actin (B). Western blot analysis of BRCA1 and STAT3 expression levels in T-47D cells (C); quantification of their expression levels normalised by the levels of β-actin (D). Data are represented as means of three independent experiments.
resistance and predicting the protocol for breast cancer treatment is highly recommended.

STAT3 is reported to be an overexpressed transcription factor which participates in cell proliferation in many malignancies. Therefore, the strategy of STAT3 signalling pathway inhibition could aid in arresting the growth of tumour cells through the inhibition of cell division. On the other hand, the protein expression of STAT3 was downregulated in the MCF-7 cell line when treated with LTZ or ZLA alone or in combination compared to the mock cells. Previous studies have shown that ER-α plays a critical role in regulation of the STAT3 signalling pathway through inhibition of IL-6, leading directly to the suppression of STAT3 activity through activation of protein tyrosine phosphatase receptor type O (PTPRO). Elevated levels of PTPRO were found to be correlated with the down-regulation of STAT3 in tumour tissue through binding of ER-α with oestrogen response elements (EREs) in the DNA sequence on the promoter region of the PTPRO gene, which, in turn, increased its expression. The level of PTPRO expression was significantly decreased in MCF-7 and MDA-MB-231 human breast cancer cell lines, primarily because of promoter methylation relative to its expression in normal human mammary epithelial cells (48R and 184). Therefore, the mechanism of down-regulation of STAT3 in the present study might be due to suppression of PTPRO. Additionally, it has been reported that cytokines secreted by breast cancer cells can bind to cytokine receptors on the stromal cells causing phosphorylation of STAT3 via activation of the tyrosine kinases. Then, STAT3 will be dimerised and down-regulated.

**Conclusion**

It is concluded that an LTZ/ZLA combination could be used in the treatment of ER-positive breast cancers. It can also be stated that there is strong cross talk between different signalling molecules involved in the onset of breast cancer, including BRCA1, STAT3, and ER. Moreover, treatment of patients having ER-positive breast cancer with combined LTZ and ZLA is important as a new strategy for minimising and/or reducing bone turnover in those patients.
Recommendation

A combination of LTZ/ZLA is strongly recommended for the treatment of patients with breast cancer and for the prevention of osteoporosis in those patients.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

This work was approved by the Ethical Committee of the University of Alexandria, the Master’s Degree Protocol for Mrs Rania Ammar under Accession number 654/4/2014 in April 2014.

Authors’ contributions

SAS, and AS designed the study, conducted research, and provided research materials and wrote the article. RA did the experimental work. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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