Alendronate, Raloxifene and Tibolone Inhibit the Proliferation-Stimulating Activity of 17β-Estradiol in MCF-7 Cells

N. Sookvanichsilp* and C. Boonleang

Department of Pharmacology, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand.

Abstract

Estrogen replacement therapy has been considered for many decades as the gold standard in the management of osteoporosis, but the risk of developing breast cancer outweighs its benefits. It is not known whether addition of another antiresorptive agent to estrogen replacement therapy will provide any beneficial effects against such risk. This in vitro study aimed to investigate the inhibitory effects of alendronate, raloxifene and tibolone on the proliferation-stimulating activity of 17β-estradiol in MCF-7 cells. The effects of individual drugs, alendronate, raloxifene and tibolone, alone at concentrations of 10^-10 - 10^-4 M and their combinations with 10^-8 M 17β-estradiol on MCF-7 cell proliferation were determined on day 2 and day 6 of incubation using the standard tetrazolium MTT (cell viability) method. The cytotoxic effect of the drugs either alone or in combination with 17β-estradiol was determined by visualizing morphological features of the MCF-7 cells and measuring the release of lactate dehydrogenase (LDH) from the cells. The results indicated that the three drugs inhibited the proliferation of MCF-7 cells in a concentration-related manner on both days of investigation. At all indicated concentrations, they could significantly inhibit (p < 0.001) the proliferation-stimulating activity of 10^-8 M 17β-estradiol. Among the three drugs, tibolone exhibited the most potent inhibitory effect. Morphological detection and LDH determination revealed a strong cytotoxic activity of these three drugs. Extrapolations from these in vitro data suggest that the three drugs, alendronate, raloxifene and tibolone, do not increase the risk of cancer mediated via estrogen receptors and probably reduce the risk of developing breast cancer in estrogen users.

INTRODUCTION

Postmenopausal women usually suffer from osteoporosis. Available therapies include estrogens, estrogens with progestins, vitamin D, bisphosphonates, selective estrogen receptor modulators (SERMs), calcitonin, tibolone and new compounds such as menatetrenone, teriparatide and strontium.1-5 In addition to being used each drug individually, a combination therapy such as estrogen (alone or plus a progestin) and the other antiresorptive drugs, particularly a bisphosphonate, has been introduced.3,6-8 Although it is well documented that longer-term use of estrogens alone as well as estrogens plus the synthetic progestins increases the risk of breast cancer,9,10 few data are available for other antosteoporotic drugs. Alendronate and raloxifene are among the commonly used antosteoporotic drugs. The use of alendronate, a nitrogen-containing bisphosphonate, and other bisphosphonates has increased dramatically during the last few years. Long-term use of some bisphosphonates is also useful in patients with metastatic bone disease from breast cancer.11,12 Raloxifene, a selective estrogen receptor modulator, produces both estrogen-agonistic effects on

*Corresponding author: Faculty of Pharmacy, Mahidol University, 447 Sri-Ayudhaya Road, Bangkok 10400, Thailand. Fax: (662) 354-4326. Email: pynsw@mahidol.ac.th
bone and lipid metabolism and estrogen-antagonistic effects on uterine endometrium and breast tissue. Raloxifene treatment, at a median 48-month follow-up, resulted in a 72% reduction in breast cancer incidence without association with an increased risk of uterine endometrial cancer. Tibolone, a 19-nortestosterone derivative, structurally related to norethynodrel, possesses weak estrogenic, progestagenic and androgenic properties. It has a wide range of postmenopausal therapy options and rarely causes endometrial proliferation. Prevention of bone loss with tibolone is comparable to that seen with estrogen therapy. With regard to breast cancer, several studies show that the risk with tibolone is low. In a study on breast cell proliferation, tibolone had less impact on the breast than conventional hormone therapy. Moreover, a previous prospective randomized study showed that mammographic density decreased after 12 months of tibolone use. However, in the Million Women Study, a slight increase in breast cancer risk was also reported in tibolone users.

Estrogen replacement therapy has been considered for many decades as the gold standard in the management of osteoporosis, but the risk of developing breast cancer outweighs its benefits. Whether addition of another antiresorptive agent to estrogen replacement therapy would provide any beneficial effects against such risk is a question. Therefore, we performed the in vitro study to investigate the effects of the three drugs, alendronate, raloxifene and tibolone on the proliferation-stimulating activity of 17β-estradiol in MCF-7 human breast cancer cells.

**METHODS**

**Preparation of Drug Solutions**

The working solution of 17β-estradiol (ICN Biomedicals, USA) at $10^{-9}$ M concentration (chosen from our preliminary studies) was prepared by diluting the stock solution ($10^{-1}$ M in ethanol) with the treated cell culture medium (see “MCF-7 Cell Proliferation Assay”). The concentrations of ethanol in the assay media were limited at 0.1%. The stock solution of each drug, alendronate (Fosamax), raloxifene (Celvista) or tibolone (Livial), was prepared in the appropriate solvent that could dissolve only the target drug but not or very few the inactive ingredients. For this reason, water was used to prepare alendronate stock solution and ethanol was used to prepare tibolone and raloxifene stock solutions. Final appropriate working solutions of individual drugs that would provide the drug concentrations of $10^{-10}$, $10^{-8}$, $10^{-6}$ and $10^{-4}$ M in the assay media were prepared by diluting the stock solutions with the treated cell culture medium and the concentrations of ethanol in assay media were also limited at 0.1%.

The blank of each drug consisting of the inactive ingredient(s) that could be dissolved in the solvent for each drug, except those which have been reported to exhibit no proliferative activity on MCF-7 cells at the range of concentration up to $10^{-3}$ M or 1000 μg/ml, was prepared at the maximum concentration(s) of the inactive ingredient(s) as being in the highest concentration ($10^{-4}$ M) of the final working solution. Lactose, propylene glycol, macrogol 400, polysorbate 80, povidone and shellac, did not demonstrate any significant effects on MCF-7 cell proliferation in this study.

**Cell Cultures**

MCF-7 human breast cancer cells were grown in T-25 flasks (25 cm²) in culture medium consisting of Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, USA) with phenol red, supplemented with 5% fetal bovine serum (FBS; Gibco BRL, USA), 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (Gibco BRL, USA) in a humidified atmosphere containing 5% CO₂ at 37°C. The cells were passaged twice a week.

**MCF-7 Cell Proliferation Assay**

During the proliferation assay, the “treated cell culture medium” consisting of phenol red-free DMEM added with 5% charcoal-dextran-treated FBS (charcoal-dextran-treated FBS being used instead of normal FBS in order to remove endogenous steroids), 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate was used.
Exponentially growing cells were trypsinized, counted with a hemocytometer, and seeded into 96-well plates at 5,000 cells/well in 100 μl volume. After a 24-hour period of attachment, the culture medium in each well was replaced with 100 μl treated medium containing different concentrations (10^{-10}, 10^{-8}, 10^{-6} and 10^{-4} M) of the test compound (alendronate, raloxifene or tibolone), 10^{-8} M 17β-estradiol, 0.1% ethanol, and the blank solutions. For 6-day incubation, the drug-containing media were replaced on day 3 of incubation. The effects of the drugs and 17β-estradiol on MCF-7 cell proliferation were assessed on day 2 and day 6 of incubation using the standard tetrazolium MTT (cell viability) assay. Briefly, the media were removed and after washing the cells with phosphate buffered saline (PBS; Invitrogen, USA), 200 μl of 3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma–St. Louis, MO) reagent at a concentration of 0.5 mg/ml in the treated medium were added to each well. The plates were incubated at 37°C for additional 4 hours. At the end of incubation, the media were removed and the formazan crystals formed as a result of MTT metabolism by viable cells were solubilized by the addition of 100 μl dimethyl sulfoxide (DMSO) to each well. At 10 minutes after adding DMSO, the absorbance of the solubilized product was then measured at 560 nm with background subtraction at 650 nm on a microplate reader (Molecular Devices Thermomax Absorbance Reader, USA).

To determine the effects of the three drugs on MCF-7 cell proliferation in the presence of 10^{-8} M 17β-estradiol, the drugs at different concentrations were added 15 minutes before adding 17β-estradiol. The evaluation was also performed on day 2 and day 6 of incubation as described above.

**Determination of Median Inhibitory Concentrations**

The absorbance values obtained per treatment from the MTT assay were converted to cell viability, expressed as cell number (calculated in percent of the seeding amount). Regression analysis was performed on the cell viability data and the resulted equation was used to determine the estimated median inhibitory concentration (IC_{50}).

**Determination of Morphological Alterations**

Exponentially growing cells were seeded into 24-well plates at 50,000 cells/well in 500 μl of the culture medium. After a 24-hour period of attachment, the culture medium in each well was replaced with 500 μl treated medium containing each test compound (alendronate, raloxifene or tibolone) alone at the concentration of 10^{-6} M (only one concentration tested), 10^{-8} M 17β-estradiol alone, the test drug in combination with 17β-estradiol, 0.1% ethanol, or the blanks. The living cells were examined in their natural state without being killed by an inverted microscope (Zeiss, Germany) at 400× magnification at 24 hours as well as on day 2 and day 6 of incubation before submitting to benzimidazole Ho33342 staining.

Ho33342 staining was performed on day 2 (at 48 hours) and day 6 (at 144 hours) of incubation (the same incubation periods as indicated in the MTT assay). In brief, the media were removed and cells in wells were washed once with PBS. A volume of 200 μl of Ho33342 (Sigma–St. Louis, MO) at 1 μg/ml in Hank’s balance salt solution (Gibco BRL, USA) was added to each well and the plates were further incubated at 37°C for 30 minutes in the dark. Normal and apoptotic cells were visualized through a blue filter of the fluorescence inverted microscope (Zeiss, Germany) at 400× magnification.

**Cytotoxic Assay by LDH Method**

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme which is present in most cells. It is released into the cell culture supernatant upon damage of the cytoplasmic membrane (an indication of cell death) in the early stage of necrosis, and in the late stage of apoptosis.

The LDH activity was measured by colorimetric method using 2,4-dinitrophenylhydrazine (DNPH) as a coloring agent. Briefly, the MCF-7 cells were incubated with the drugs in the same ways as mentioned in the MTT
proliferation assay. Triton X-100 (Fisher Scientific, UK) at 1% v/v in PBS was used to produce a total cell lysis. Measuring LDH leakage was performed on day 2 of incubation, the same day as doing the MTT assay. Due to routine culture medium changing every 3 days, we did not perform the study of LDH release on day 6 of incubation. On the assay day, the substrate solution (containing 0.187 mM pyruvate and 0.5 mg/ml NADH) at a volume of 100 μl was placed into each well of new 96-well plates. The enzyme supernatant from all experimental wells (after 2-day incubation), including wells containing cells treated with Triton X-100, at a 20 μl volume was then added to the substrate solution and thoroughly mixed. The plates were incubated for exactly 30 minutes at 37°C. DNPH (Fluka, Switzerland) solution (0.2% in 1M HCl) at a volume of 20 μl was added to each well and the plates were further incubated at room temperature for 20 minutes. To develop the final color, 50 μl of 4M NaOH were added to each well, mixed and allowed to stand for 5 minutes. At the end of the color developing period, the absorbance was then measured at 450 nm on the microplate reader.

The percentage of LDH release produced by the test compounds was calculated based on their absorbance, relative to absorbance values for medium background and values resulting from total cell lysis (100% cell kill) by 1% Triton X-100.

Data Analysis

Data were expressed as mean ± S.D., calculated from four separated quadruplicate experiments for the MTT assay and three separated quadruplicate experiments for the LDH assay, and then analyzed by Student’s two tailed, unpaired t-test using the Microsoft Excel 97. Statistical significance was set at p value of less than 0.05. In morphological detection, data were expressed as mean ranges of apoptotic cells from two separated triplicate experiments and the statistical evaluation was not done.

RESULTS

Antiproliferative Effect

Alendronate, raloxifene and tibolone inhibited the proliferation of MCF-7 cells in a concentration-related manner (from 10⁻¹⁰ M to 10⁻⁴ M) either evaluated on day 2 or day 6 of incubation with significant differences (p < 0.001) from the control at all indicated concentrations, except on day 2 for alendronate at concentrations of 10⁻¹⁰ M (nonsignificant) and 10⁻⁸ M (p < 0.05) (Figure 1). The order of antiproliferative potency either evaluated on day 2 or day 6 of incubation was tibolone > raloxifene > alendronate. The estimated IC₅₀ (obtained from regression equations) on day 2 were 1.9 x 10⁻⁸ M, 1.6 x 10⁻⁶ M and 5.0 x 10⁻² M, respectively, and on day 6 were 7.0 x 10⁻¹² M, 7.4 x 10⁻¹⁰ M and 9.9 x 10⁻¹⁰ M, respectively.

Inhibition of the Proliferation-Stimulating Activity of 17β-Estradiol

The three drugs at all indicated concentrations could significantly inhibit (p < 0.001) the proliferation-stimulating activity of 10⁻⁸ M 17β-estradiol in a concentration-related manner on both day 2 (Figure 2a) and day 6 (Figure 2b) of incubation. Tibolone exhibited the most potent inhibitory effect against such activity of 17β-estradiol on both days of investigations. The estimated IC₅₀ on day 2 were 6.1 x 10⁻⁸ M, 2.9 x 10⁻¹¹ M and 4.7 x 10⁻¹³ M, and on day 6 were 1.3 x 10⁻¹⁰ M, 1.5 x 10⁻⁸ M and 2.0 x 10⁻¹⁵ M for alendronate, raloxifene and tibolone, respectively.

Cytotoxic Effect – Morphological Alterations

Several unhealthy cells with granularity around the nucleus and cytoplasmic vacuolization
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**Figure 1.** Inhibition of MCF-7 cell proliferation by alendronate, raloxifene and tibolone at concentrations of 10⁻⁶-10⁻⁴ M on day 2 and day 6 of incubation. Values are means (± S.D.) of four separated quadruplicate experiments. Ethanol at 0.1% and blanks of the three drugs exhibited no significant differences from control on both days of incubation, data not shown. * p < 0.05, ** p < 0.001 vs. control on day 2 of incubation; * p < 0.001 vs. control on day 6 of incubation.

Cytotoxic Effect − LDH release

The release of the cellular LDH into the media was used as an index of cytotoxicity. On day 2 of incubation, a minimal cell lysis could occur in the control cells (2.8%) and cells treated with 0.1% ethanol (3.0%), the drug blanks (2.9-3.3%) and 10⁻⁸ M 17β-estradiol (4.1%). The amounts of LDH release from MCF-7 cells caused by the three drugs either alone or in combination with 17β-estradiol were concentration-related (Figure 4) and were all significantly different from the control (p < 0.05 to < 0.001), except alendronate alone at the lowest concentration. The results of LDH release were correlated with the results of antiproliferative effect from the viability MTT assay (Figure 2a) – that was the
Figure 2. Effect on MCF-7 cell proliferation of alendronate, raloxifene or tibolone at concentrations of $10^{-10}$-$10^{-4}$ M in combination with $10^{-8}$ M $17\beta$-estradiol on day 2 (a) and day 6 (b) of incubation. Values are means ($\pm$ S.D.) of four separated quadruplicate experiments. Data on cell proliferation in the presence of the three drugs alone are also shown (significant figures being indicated in Figure 1). * $p < 0.01$, ** $p < 0.001$ vs. control; * $p < 0.001$ vs. $17\beta$-estradiol alone.

DISCUSSION

The results of inhibitory action on MCF-7 cell proliferation of alendronate, raloxifene and tibolone in the present study were in accordance with many previous studies which have demonstrated the antiproliferative effect of the three drugs on various cell types, including MCF-7 cells. In addition, the observation that the three drugs could also...
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Figure 3. Morphological alterations of MCF-7 cells, cultured in phenol red-free DMEM added with 5% charcoal-dextran-treated FBS in 24-well plates, following 48-hour exposure to 10⁻⁸ M alendronate alone or in combination with 10⁻⁸ M 17β-estradiol and being observed under an inverted microscope at 400x magnification; a: normal MCF-7 cells, b: cells treated 17β-estradiol, c: cells treated with alendronate blank, d: cells treated with alendronate—the cells rounding up and detaching from the wells, e: cells treated with alendronate plus 17β-estradiol, and f: cells treated with alendronate after benzimidazole Ho33342 staining—the characteristic morphological features of apoptosis, e.g. chromatin condensation and formation of apoptotic bodies being observed. 1→: apoptotic cells, 2→: normal MCF-7 cells.

effectively inhibit the proliferation-stimulating activity of 10⁻⁸ M 17β-estradiol in MCF-7 cells in the present study coincided with some previous findings which revealed that raloxifene²⁶,²⁹,³⁰ and tibolone³¹ antagonized the stimulating effect of estradiol in MCF-7 cells. To date, no data have been reported about the effect of alendronate on the proliferation-promoting activity of the estrogen in MCF-7 cells. Among the three drugs, tibolone showed the most potent inhibitory effect on MCF-7 cell proliferation in the present study either investigated on day 2 or day 6 of incubation and both in the absence or presence of 10⁻⁸ M 17β-estradiol.

To the best of our knowledge, no comparative studies on the antiproliferative activity among the three drugs either individually or in combination with the estrogen have been reported.

Several mechanisms of action of these three drugs in inhibiting cell growth have been studied and found that they could produce cell apoptosis (cell elimination due to cell death).²¹,²⁷,²⁸,³¹-³⁴ The present results also indicated, by morphological detection, that the three drugs could produce cell apoptosis. Although in the present study significant differences in the ability to induce cell apoptosis
Figure 4. LDH release from MCF-7 cells following 48-hour exposure to alendronate, raloxifene or tibolone alone at concentrations of 10^{-10}-10^{-4} M and these three drugs in combination with 10^{-8} M 17β-estradiol. Data are expressed as the percentage of LDH release produced by the test compounds in relative to the total cell lysis (100% cell kill) produced by 1% Triton X-100. Values are means (± S.D.) of three separated quadruplicate experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. control.

among the three drugs either alone or in combination with 17β-estradiol were unable to demonstrate by morphological detection, the amount of LDH release from the cells into the surrounding medium (an indication of cell death) caused by these three drugs on day 2 of incubation coincided with the loss of viability in the MTT assay on the same day, and again tibolone exhibited the most potent effect. A slight but significant increase in LDH release was observed when the drugs were added in combination with 10^{-8} M 17β-estradiol, suggesting that 17β-estradiol stimulates cell proliferation and the cytotoxic effect of the three drugs depend on not only the drug concentration but also the amount of cells being exposed to the drugs. Since LDH leakage is a sign of cell necrosis and cultured cells that are undergoing apoptosis in vitro eventually undergo secondary necrosis and LDH leakage, cytotoxicity as indicated by LDH release on day 2 of incubation in the present study could exclude neither the possibility of cell necrosis nor cell apoptosis.

The direct cytotoxic agent usually causes LDH release from cultured cells within 4 hours after exposure while the agent that causes cell death by apoptosis as the primary mechanism usually causes LDH release later.

There are many other studies which have provided evidences for the antiproliferative activity of the three drugs such as decrease in the activity of telomerase (a cellular ribonucleoprotein reverse transcriptase responsible for elongation of the telomere and its expression being increased in many cancers)\cite{28} and inhibition of the mevalonate pathway by inhibition of farnesyl diphosphate (FPP) synthase (resulted in induction of apoptosis)\cite{32,35} caused by alendronate or other nitrogen-containing bisphosphonates; inhibition of collagen biosynthesis and prolylase activity,\cite{26} inhibition of IGF-I action\cite{36} and binding to inhibitory androgen receptor in the cells\cite{37} caused by raloxifene; and inhibition of estrone sulfatase activity (resulted in strongly inhibited transformation of estrone sulfate to
17β-estradiol in the cells),\(^{38-40}\) binding to progesterone receptors and/or androgen receptors\(^{33}\) (binding to the androgen receptor\(^ {41}\) and the progesterone receptor\(^ {42}\) resulted in decrease of proliferation of MCF-7 cells) and decrease in the expression of the anti-apoptotic proteins Bcl-2 and Bcl-x\(_L\)^{43} caused by tibolone and/or its active metabolites. In the present study, incubating the MCF-7 cells with the three drugs for 15 minutes prior to adding 17β-estradiol produced a rapid and pronounced inhibition of the proliferation-stimulating activity of 17β-estradiol as evidenced by the results on day 2 of incubation from the MTT assay. Moreover, the results from morphological detection at 24 hours and 48 hours of incubation showed unhealthy cells detaching from the wells. Taken together, these findings suggest that the inhibitory effect of the three drugs could be partly due to a rapid alteration in cell (physical and/or biochemical) properties before the binding of 17β-estradiol to its receptors, and therefore resulted in no fully stimulated cell proliferation.

In conclusion, this study have demonstrated the antiproliferative effect of alendronate, raloxifene and tibolone with their concentration-related cytotoxic effect in MCF-7 human breast cancer cells. The three drugs could effectively inhibit the proliferation-stimulating activity of 17β-estradiol. Among the three drugs, tibolone exhibited the most potent activity. This study is the first report on the effect of alendronate in combination with 17β-estradiol on the MCF-7 cell proliferation and has compared the inhibitory effect of three drugs, alendronate, raloxifene and tibolone, either alone or in combination with 17β-estradiol, on the proliferation of MCF-7 cells. Extrapolations from the present in vitro data suggest that the three drugs do not increase the risk of cancer mediated via estrogen receptors and probably reduce the risk of developing breast cancer in estrogen users.

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