ELSEVIER

Archives of Medical Research ■ (2017) ■

Archives of Medical Research

PRELIMINARY REPORT

Combined Raloxifene and Letrozole for Breast Cancer Patients

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Received for publication August 2, 2017; accepted November 24, 2017 (ARCMED-D-17-00451).

Raloxifene, an anti-osteoporotic drug, is recently approved for prevention of breast cancer in postmenopausal women and thus the drug may be employed to combat the bony adverse effects of letrozole, another anticancer drug. However, the cytotoxic effect of their combination on human breast cancer (MCF-7) and human embryonic kidney (HEK) cell lines is not known. MCF-7 and HEK cell lines were treated with different graded doses of letrozole, raloxifene and their combination, then incubated for 24−48 h. MTT assay was performed to check the cytotoxicity of the drugs. The study indicates that the combination of letrozole and raloxifene possess additive effect in terms of cytotoxicity of cancer cell lines (MCF-7) and negligible effects in normal cell lines (HEK). Our study indicates that the addition of raloxifene doesn't interfere with anticancer efficacy of letrozole rather the combination acted additively for the treatment of breast cancer.

Key Words: Letrozole, Raloxifene, MCF-7, HEK, MTT.

Introduction

Several clinical studies have established the role of aromatase inhibitors as adjuvant treatment in postmenopausal women with endocrine responsive breast cancer in switching, sequential and upfront settings. The aromatase inhibitor letrozole has been shown to be superior to tamoxifen (TAM), a selective estrogen receptor modulator (SERM), in the first-line treatment of metastatic breast cancer (1). Another SERM, raloxifene, is approved for prevention and treatment of postmenopausal osteoporosis. Both SERMs have specific affinity to ERα and ERβ of estrogen receptors (ER) and show tissue specific ER agonistic or antagonist activity and thus have agonistic effects on bone and antagonistic effects in breast tissue. Raloxifene was approved by US Food and Drug Administration for the reduction of breast cancer risk in postmenopausal women at increased risk based on the results of the STAR (Study of Tamoxifen and Raloxifene) trial (2). The advantage of raloxifene over tamoxifen in breast cancer is that raloxifene use is not associated with endometrial carcinoma while tamoxifen is associated with the same due to its partial agonistic action (3). To increase the anti-cancer efficacy, combination therapy is most commonly used. Thus, in the present work, we aimed to explore the combination therapy of letrozole and raloxifene in MCF-7 cell lines, a model breast cancer cell-line often used to demonstrate the estrogen dependence of breast cancer growth, with a strategy to find an authentic and acceptable therapy to treat breast cancer with minimal osteoporotic effect. The present study is planned on the basis of our previous work on raloxifene exhibiting protection against letrozole-induced bone loss in a model of menopause in mice (4).

Materials and Methods

Chemicals and Reagents

DMEM (Dulbecco modified Eagles's Medium), FBS (Fetal Bovine Serum), Phosphate Buffer saline (PBS), Penicillin,

Conflict of Interest: The authors Divya Vohora, Abul Kalam, Ankita Leekha, Sushama Talegaonkar, Anita Kamra Verma declare no conflict of interest.

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0188-4409/$ - see front matter.
https://doi.org/10.1016/j.arcmed.2017.11.012

Streptomycin, Sodium bicarbonate, HEPES (Hydroxyl ethyl piperazine ethanesulphonic acid), Letrozole, Raloxifene and MTT reagent were purchased from Sigma-Aldrich (USA).

Cell Culture and Maintenance

HEK and MCF-7 cell lines were procured from National Cell Culture Science, Pune, India. The cell lines were maintained in DMEM +10% FBS supplemented with L-glutamine and Streptomycin/Penicillin, 25 mmol HEPES buffer with 5% CO2 at 37°C.

Methodology

The cytotoxicity of letrozole, raloxifene and their combination was examined according to a previously published protocol (5) with slight modifications. The cytotoxicity studies were conducted by evaluating the percent cytotoxicity by performing the MTT assay on MCF-7 cell-line (human breast cancer cell line) and HEK (human embryonic kidney cell-line). Briefly, cells were seeded at a density of $5x10^3$ cells/well in a 96 well microtiter plate, supplemented with 2.5% fetal calf serum followed by incubation at 37°C with an atmosphere of 90% N_2, 5% CO_2, 5% O_2 and allowed to grow for 24 h. The cells were incubated with the letrozole at concentrations between 500−0.9766 ng/mL and raloxifene at concentration between 160−1.25 µg/mL 24 h. Later, combination of letrozole and raloxifene at different doses was assessed. After the requisite time period, 20 µl of MTT solution (5 mg/mL in PBS pH 7.4) was added to each well. Formazan crystals were then dissolved in 100 µl of DMSO. After mixing with a mechanical plate mixer, the optical density was read at 540 nm on an ELISA microplate reader (Synergy HT, BioTek, USA). All measurements were in triplicates. IC_{50}values were calculated for each sample at the end of 24 h and the percent cytotoxicity was determined by the following formula (Equation 1):

$$\textit{Percent cytotoxicity} = \left\{([A]_{control} - [A]_{test})/[A]_{control}\right\} \times 100 \quad (1)$$

Where,

$[A]_{test}$ is absorbance of the test sample.
$[A]_{control}$ is the absorbance of the control sample.

Statistical Analysis

The results were expressed as mean ± SD. Comparison among groups were analyzed by one-way ANOVA and means were separated by Tukey's test using Prism (5.0) software (Prism software Inc. CA). Levels of significance were accepted at ≤0.05 level.

Results and Discussion

Aromatase inhibitors, during the last one decade, has opened a new prospective of successful treatment in estrogen receptor positive breast cancer. However, the major limiting factor of aromatase inhibitors is their negative impact on bone accrual due to depriving estrogen levels. Though several pre-clinical and clinical studies (6,7) have confirmed that aromatase inhibitors including letrozole adversely affects bone health, there are limited studies available on the efficacy of anti-osteoporotic agents in preventing or ameliorating such effects. Few studies demonstrate efficacy of bisphosphonates for management of aromatase-inhibitors associated bone loss in cancer patients (8,9). However, there were several concerns recently on the use of bisphosphonates. Apart from the gastrointestinal adverse effects including erosive esophagitis, there were long-term concerns regarding over-suppression of bone turnover (10), atypical fractures (11) and renal toxicity (12) in addition to some rare adverse effects including osteonecrosis of jaw (13) and esophageal cancer (14). These concerns highlight the importance of investigating other anti-osteoporotics for management of bone-loss caused by aromatase inhibitors. Recently, we demonstrated that raloxifene provided protection against letrozole-induced bone loss in a chemically-induced model of menopause in mice (4). Further, we demonstrated that such effects were found to be independent of a pharmacokinetic interaction between the two drugs. In the present work, we explored the effects of raloxifene alone and in combination with letrozole on human breast cancer cell lines (MCF-7) with the aim to evaluate whether the use of raloxifene for prevention/treatment of letrozole-induced bone-loss will interfere with its anticancer efficacy or not. We used MCF-7 cell lines as Zhou et al. reported that the aromatase gene is amplified in MCF-7 cells (15). Also, letrozole was found to be a potent *in vitro* inhibitor of cell proliferation and of type IV collagenases expressed by ER-positive MCF-7 cells, indicating that it may be of value for suppressing breast tumor growth and invasiveness (16).

The cytotoxicity of letrozole and raloxifene was evaluated in dose and time-dependent manner. Letrozole was evaluated at doses between 500−0.9766 ng/mL (Figure 1). The IC_{50} of Letrozole on MCF-7 cell line was found to be 10.89 ng/mL. Raloxifene was evaluated at dosage between 160−1.25 µg/mL (Figure 1). The IC_{50} of Letrozole and Raloxifene on MCF-7 cell line was found to be 29.07 µg/mL. An anti-proliferative effect of raloxifene in the ER-positive MCF-7 human breast cancer cell-line has been documented in model systems *in vitro* and *in vivo* (17,18). Further, MORE (Multiple Outcomes for Raloxifene Evaluation), a clinical study concluded that raloxifene diminishes the incidence of newly diagnosed breast cancer by 66% with a discernible effect on ER-positive tumors (reduced risk by 76%) and no effect on non-invasive