RESEARCH ARTICLE

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Leukotriene Receptor Antagonists Inhibit Mitogenic Activity in Triple Negative Breast Cancer Cells

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Abstract

Despite a discovery of hormonal pathways regulating breast cancer, a definitive cure for the disease requires further identification of alternative targets that provide a hormone-independent support. Apart from their role in inflammatory diseases, cysteinyl leukotriene (CysLT) receptor antagonists (LTRAs) decrease the risk of lung cancer in asthma patients and inhibit tumor progression in several malignancies. In the present study, we evaluate the effects of two chemically different, clinically relevant LTRAs (montelukast and zafirlukast) in a triple negative breast cancer cell line, MDA-MB-231. We found that these two LTRAs reduced breast cancer cell viability in a dose-dependent manner with the 50% inhibitory concentration (IC50) between 5-10 μM. Although both LTRAs have several pharmacological properties in common, we noticed that montelukast mainly induced apoptosis, while zafirlukast mainly exerted its action on cell cycle. However, the precise mechanisms responsible for such different effects remain unclear. In summary, our results suggest that CysLT plays a role in proliferation and survivability of breast cancer cells in the absence of hormonal stimuli.

Keywords: Montelukast- zafirlukast- proliferation- apoptosis- breast cancer- MDA−MB−231

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Introduction

Breast cancer is the most common malignancy developed in women worldwide (Torre et al., 2015) with approximately 12.4 percent of women will be diagnosed with breast cancer at some point during their lifetime (The Surveillance, Epidemiology, and End Results Program of the National Cancer Institute, USA). Advances in early detection and therapeutic have improved cancer survival resulting in a stable incidence rate; however, breast cancer remains the second most common cause of cancer death in women (Siegel et al., 2016). Current treatments for breast cancer include chemotherapy and antihormonal agents primarily targeting hormone dependent mechanism and a growth promoting protein, human epidermal growth factor 2 (HER2) (Senkus et al., 2015). Among several subtypes of breast cancer, triple-negative breast cancers (TNBC) are characterized by tumors that do not express estrogen receptor (ER), progesterone receptor (PR), and HER-2. These subtypes remain nowadays a clinical challenge due to their innate resistance to antihormonal therapy and other available targeted agents.

It is clear that the inflammatory response in tumor tissue associates with leukocytes and plays a major role in cancer development (reviewed in (Grivennikov et al., 2010)). An arachidonic acid (AA) pathway regulated by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes is well-known for its roles in pain and inflammation found in human diseases (Crofford et al., 1994; McAdam et al., 2000; Schonbeck et al., 1999). Both enzymes convert AA to an intermediate prostaglandin and eventually to several eicasanoids (i.e., prostaglandins, thromboxane A2, and leukotrienes). Evidence demonstrates that these metabolites can stimulate epithelial cell proliferation, inhibit apoptosis, and stimulate antigen suppression (Kawamori et al., 2003; Matsuyama et al., 2007; Mutoh et al., 2002; Sonoshita et al., 2001).

Cysteinyl leukotriene receptor antagonists (LTRAs) such as montelukast and zafirlukast have been recently reported to protect asthma patients from developing cancers, especially lung, breast, colorectal, and liver cancers, in a dose-dependent manner (Tsai et al., 2016). A follow-up study from the same group demonstrated that montelukast induces cell death and inhibits tumor growth in a lung cancer model contributing to its chemopreventive effect (Tsai et al., 2017). Furthermore, montelukast is able to prevent development of chemical-induced mammary carcinogenesis in an animal model (Jose et al., 2013). However, the direct effects of LTRAs on TNBC cells have not been studied yet. In the present study, we compared the effects of montelukast and zafirlukast, on cell viability, proliferation, apoptosis, and cell cycle in a triple negative breast cancer cell line, MDA-MB-231.

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Materials and Methods

Cell culture and chemicals

MDA-MB-231 cells were obtained from ATCC and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) with 1% penicillin/streptomycin (Merck) and 10% fetal bovine serum (FBS, Gibco). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO2. Montelukast and zafirlukast were purchased from Sigma. The maximal concentration of dimethyl sulfoxide (DMSO) in all experiments was 0.04%.

MTT cell viability assay

Cells were plated at 10,000 cells per well in 96-well plates. At 70-80% confluence, cells were treated with various concentrations (1-50 μM) of montelukast and zafirlukast for 24 and 48 h followed by 3 h incubation with MTT (final concentration 0.5 mg/ml) (Bio Basic, Canada). The purple formazan crystals were then solubilized with DMSO. The intensity was measured spectrophotometrically at 562 nm. The percentage of cell viability was normalized to mock-treated cells.

Apoptosis assay

Cells were plated in 6-well plates at 300,000 cells per well and then treated with 5, 10, or 20 μM montelukast and zafirlukast. At 24 h post-exposure, cells were detached with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA). Supernatant and detached cells were collected and centrifuged at 320 g for 4 min. Then, cells were washed twice in Annexin V binding buffer and labeled with Annexin V-FITC (Immunotools, Germany) followed by 7AAD staining (BD Bioscience). Early and late apoptosis were defined by Annexin V+7AAD- and Annexin V+7AAD+, respectively. All flow cytometric measurements were performed using BD Accuri 6 flow cytometer (BD Bioscience). A minimum of 5,000 events/sample was analyzed each time.

Proliferation assay

Cells were labeled with 1 μM of 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) (Sigma) in phosphate buffer saline (PBS). The excess CFSE was washed out by PBS containing 2% FBS. CFSE-labeled cells were seeded in media with 5% FBS at 100,000 cells/well in 6-well plates. Next day, cells were treated daily with montelukast and zafirlukast at 10 or 20 μM for 4 days. Cells were trypsinized and resuspended in PBS. Mean fluorescence intensity of cells was measured using BD Accuri C6 flow cytometer (BD Bioscience). A minimum of 5,000 events/sample was analyzed each time. Relative fluorescence intensity was compared to mean fluorescence intensity of mock-treated cells.

Cell cycle analysis

Cells were plated in 6-well plates at 100,000 cells per well in media with 5% FBS and then treated with 10 or 20 μM montelukast and zafirlukast. At 48 h post-exposure, cells were detached with 0.05% trypsin-EDTA and fixed with 70% ethanol on ice. Cells were then stained with propidium iodide (PI)/RNase Staining Buffer as recommended by manufacturer's protocol (BD Bioscience). Cells were gated to exclude aggregates on total fluorescence of P1 (FLA2-Area) vs maximum fluorescence of P1 (FLA2-Height). All flow cytometric measurements were performed using BD Accuri C6 flow cytometer (BD Bioscience). A minimum of 10,000 events of single cells/sample was analyzed each time.

Statistical analysis

Data were expressed as mean±SD (n=3-5) and analyzed by a one-way analysis of variance (ANOVA) using Dunnett's post-hoc test. The level of significance was defined as p-value<0.05.

Results

LTRAs decrease cell viability in MDA-MB-231 cell line by MTT cell viability assay

Since chemopreventive effects of cysteinyl leukotriene

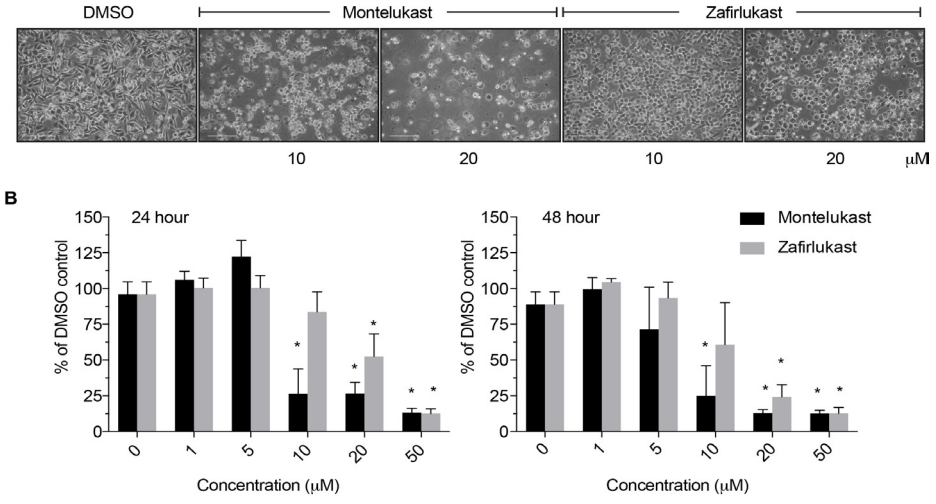

Figure 1. Effects of LTRAs on Breast Cancer Cell Viability. MDA-MB231 cells were treated with various concentrations of indicated LTRAs. A) Representative pictures of MDA-MB231 cells after 24 hours. B) MTT assays at 24 and 48 hours after treatment. Data represents mean±SD from 3-5 independent experiments. Scale bar = 200 μm. * indicates significance compared with DMSO ($p < 0.05$).

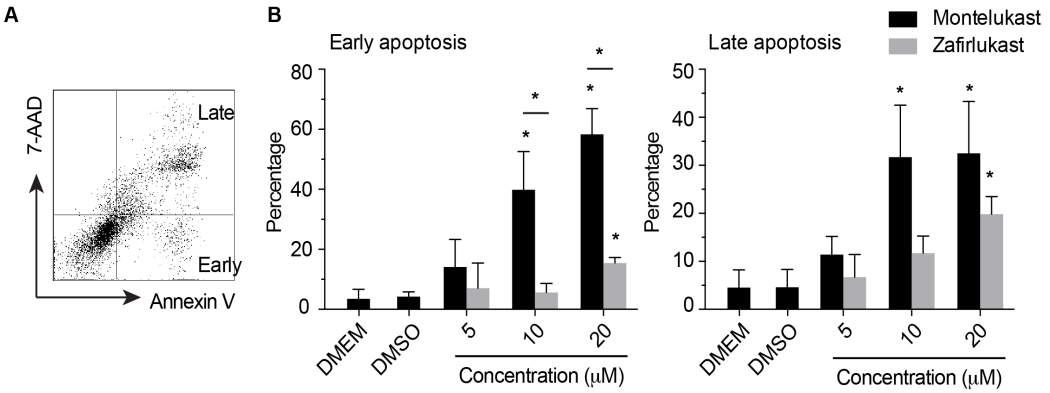

Figure 2. LTRAs Induced Apoptosis. A) Representative flow cytometric dot plot showing gating strategy to determine early and late apoptosis. B) Percentage of early (Annexin V+7AAD-) and late apoptosis (Annexin V+7AAD+) after 24-hour treatment. Data are mean±SD from 3-5 independent experiments. * indicates significance compared with DMSO ($p < 0.05$).

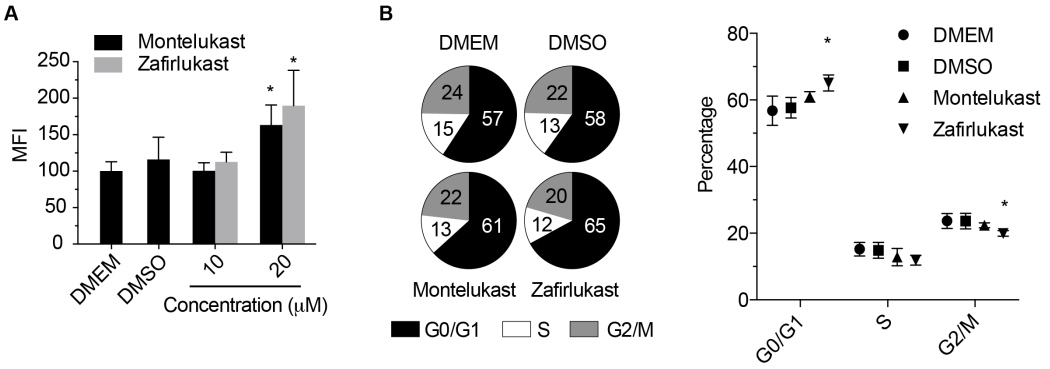

Figure 3. Zafirlukast Inhibited Cell Proliferation and Promoted Cell Cycle Arrest. A) CFSE-labeled cells were treated with indicated LTRAs for 4 days. Mean fluorescence intensity of cells was measured by flow cytometry. Results are mean±SD from 3-5 independent experiments. B) Pie charts represent average percentage of cell in each cell cycle phase. Dot plot to the right summarizes data from 3-5 independent experiments. Results are mean±SD. * indicates significance compared with DMSO ($p < 0.05$). MFI, mean fluorescence intensity.

receptor (CysLT) antagonists on lung cancer has been demonstrated in animal models (Gunning et al., 2002; Tsai et al., 2017) and human (Tsai et al., 2016), we promptly extended our investigation whether LTRAs affect breast cancer cells in a similar manner. We initially evaluated the effect of montelukast and zafirlukast on cell viability in a triple negative breast cancer cell line (MDA-MB-231) using MTT assays. We found that montelukast and zafirlukast effectively reduced cell viability in a dose-dependent fashion (Figure 1A and 1B, IC50 ~ 5-10 μM). We observed that cells treated with montelukast started to detach from the culture plate earlier than zafirlukast in a pairwise comparison (24 hours); however, similar maximal effect can be achieved by 48 hours.

LTRAs induced apoptosis and inhibited cell proliferation

To further examined whether the reduction in cell viability was caused by either apoptosis, proliferation inhibition, or both, we performed flow cytometric analysis using annexin V, CFSE dye, and cell cycle analysis. We found that both montelukast and zafirlukast triggered cell apoptosis (Figure 2). Consistent to the MTT data, montelukast induced apoptosis more rapid than zafirlukast (higher percentage of early apoptotic cells, $p < 0.05$). Our data demonstrated that both LTRAs inhibited breast cancer cell proliferation (Figure 3A), but only zafirlukast could induce cell cycle arrest (Figure 3B, higher percentage of cell stays in a dormant state (G0/G1, $p<0.05$) and less in a G2/M phase ($p<0.05$)).

Discussion

Among the most important prognostic factors in breast cancer, the expression of ER, PR, and HER2 are validated predictive factors determining the selection of patients for antihormonal therapies and anti-HER2 treatments (Blamey et al., 2007; Ravdin et al., 2001; Wishart et al., 2011). While several therapeutic options are available for many subtypes of breast cancer, a treatment for the triple-negative phenotype largely depends on destructive chemotherapy (reviewed in (Senkus et al., 2015)). Importantly, TNBCs are heterogeneous diseases with divergent profiles of chemosensitivity; however, overall prognosis is poor (Perou et al., 2000; Prat et al., 2010; Shah et al., 2012). In the present study we demonstrated that widely available and well-tolerated LTRAs used for the treatments of asthma and other allergy-related conditions are able to suppress proliferation and promote cell death in TNBC cells. We reported here that antihormone-resistant breast cancer cells are relatively sensitive to both

montelukast and zafirlukast (IC50 for the antiproliferative effect is between 5-10 μM) compared to lung cancer cells (Montelukast; IC50 for the same antiproliferative effect was between 50-75 μM (Tsai et al., 2017)). Although some experimental settings are different between our study and Tsai et al., it is worth to note that 5- to 15-fold difference in the IC50 likely represented differential sensitivity of the cells rather than the condition. Typically, the plasma concentrations of montelukast and zafirlukast after oral administration of therapeutic dose (10 and 20 mg, respectively) were 0.6 μM in healthy volunteers (Cheng et al., 1996; Dekhuijzen and Koopmans, 2002). Although we found that the concentration required for such effect was higher than a therapeutic range, a remarkable sensitivity to LTRAs (8 folds more sensitive than lung cancers (Tsai et al., 2017)) promises the therapeutic potential of their nonhormonal effects.

Interestingly, our data showed that montelukast primarily induced apoptosis while zafirlukast mainly inhibited proliferation and cell cycle. It is possibly due to the secondary anti-inflammatory activities, distinct from CysLTR1 antagonism such as cyclic nucleotide phosphodiesterases, 5'-lipoxygenase, and the nuclear factor kappa B (reviewed in (Theron et al., 2014)). Currently available LTRAs on the market share several major pharmacological properties (i.e., pharmacokinetic profile and selectivity on CysLT1 receptors) and demonstrate similar clinical outcome in asthmatic patients (Keam et al., 2003; Okubo and Baba, 2008; Riccioni et al., 2004); however, subtle differences in responses among LTRAs have been observed (Nozaki et al., 2010; Piromkraipak et al., 2017). Our group previously found that zafirlukast, but not montelukast, inhibited expression of matrix metalloproteinase 9 (MMP-9) in human glioblastoma cells (Piromkraipak et al., 2017). Pranlukast inhibited tumor cell migration through both the brain and peripheral capillaries, whereas montelukast inhibited tumor cell migration only in the peripheral capillaries (Nozaki et al., 2010).

Approximately 12 to 17% of women with breast cancer have a triple-negative subtype (Foulkes et al., 2010). Depending on the stage of its diagnosis, patients with TNBC have a relatively poor clinical outcome and cannot be treated with antihormonal therapy or HER2-targeted therapy. With a limited option of treatments, this subtype of breast cancers is clearly an important clinical challenge. As higher rates of triple-negative breast cancer have been observed in a younger age group (O'Brien et al., 2010), a discovery of other potential therapeutic targets are required to improve outcomes in these patients.

In summary, we found that cysteinyl leukotriene inhibition induced apoptosis and inhibited cell proliferation in hormone-independent breast cancer cells. These effects likely depended on the differences in secondary anti-inflammatory activities of LTRAs. Exploration of these alternative pathways modulating cell proliferation and survivability of breast cancer in the absence of hormones will provide insight and promise clinical applications of LTRAs for the treatment of TNBC.

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