Effects of Cysteinyl Leukotriene Receptor-1 (CysLT-1) Antagonists in Metastatic Breast Cancer Cells

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ABSTRACT

Breast cancer metastasis is the major cause of mortality in breast cancer patients. There is no specific chemotherapies for triple negative breast cancer due to the absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression. This study aimed to determine the effects of cysteinyl leukotriene receptor-1 (CysLT-1) antagonists using MDA-MB 231 breast cancer cells. Cell viability, apoptosis and anti-migration effects were tested with MTT, Annexin V binding and wound healing assays. Montelukast at 10 µM reduced cell viability and increased apoptosis at 24 hours post-exposure. Non-toxic concentration of both montelukast and zafirlukast showed anti-migration effects. Therefore, CysLT-1 antagonists can be potential anti-cancer drugs.

Keywords: Cysteinyl leukotriene receptor-1 antagonists, Cytotoxicity, MDA-MB 231 cells

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Introduction

Breast cancer is a heterogeneous disease and each subtype response to treatment differently. The knowledge of biomarkers involved in breast tumorigenesis and metastasis is important for diagnosis and treatment. Although there are many chemotherapeutic drugs available in the market, triple negative breast cancer is not sensitive to either hormonal or monoclonal antibodies due to lack of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression.

Cysteinyl leukotrienes are potent pro-inflammatory mediators. Expression of their receptors and synthesis enzymes are highly expressed in several types of epithelial-related cancers such as gastric (Venerito, Kuester et al. 2011), colon (Ohd, Nielsen et al. 2003), prostate (Matsuyama, Hayama et al. 2007) and breast cancers (Magnusson, Liu et al. 2011). Together with LTC4 and LTE4, LTD4 binds CysLT-1 receptors with high affinity and activates CysLT-1 signaling, thereby producing various cellular processes such as cell proliferation, migration and survival (Savari, Vinnakota et al. 2014) (Massoumi and Sjolander 2007). CysLT-1 antagonists, montelukast, zafirlukast and pranlukast, are now widely used as anti-inflammatory drugs in bronchial asthma and allergic rhinitis.

A recent epidemiological study in 4,185 leukotriene receptor antagonist (LTRA) users and 20,925 non-leukotriene receptor antagonist (non-LTRA) users showed that the usage of LTRAs in asthma patients reduced the incidence rate of many types of cancers including breast cancer (Tsai, Wu et al. 2016).

In vitro studies showed that montelukast induced apoptosis in bladder transitional cell carcinoma (Matsuyama, Hayama et al. 2007) and neuroblastoma cell lines (Sveinbjornsson, Rasmuson et al. 2008). This drug also reduced LTD4-CysLT-1 receptor mediated migration of endothelial cell line EA.hy926 via the ERK1/2 pathway (Yuan, Fang et al. 2009). Moreover, a study in MCF-7 breast cancer cells reported that CysLT-1 antagonists had anti-migration and apoptotic effects together with the reduction of CysLT-1 expression. Combination treatment of LTRAs and tamoxifen led to high CysLT-2 expression, resulting in good prognosis (Magnusson, Liu et al. 2011). However, there is limited study of CysLT-1 antagonists' effects in highly metastatic MDA-MB 231 breast cancer cells.

Objective of this study

To evaluate the cytotoxic and anti-migration effects of cysteinyl leukotriene-1 antagonists on metastatic MDA-MB 231 breast cancer cells.

Materials and Methods

Cell culture

MDA-MB 231 is a human triple negative (no ER, PR and HER2 receptors expression) breast cancer cell line derived from metastatic site, categorized as basal-like subtype. Cells were cultured in Dulbecco's modified Eagle's

medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37^{O}C under 5% CO_2. Media were replaced every 2-3 days and cells were subcultured every 3-5 days.

MTT cell viability assay

MDA-MB 231 cells (12,000 cells/well in 96-well plates, 80% confluency) were treated with montelukast and zafirlukast at 1, 5 and 10 μM for 24 and 48 hours in serum-free medium. Dimethyl sulfoxide (DMSO) at 0.4% was used as vehicle control. MTT solution (final concentration 0.5 mg/ml) was added at 3 hours prior to the indicated time points. Cell viability was expressed as percentage and evaluated by measuring the concentration of purple formazan at 562 nm using a microplate reader. Experiments were repeated at least three times and data were shown as the mean ± SEM.

Wound healing assay

MDA-MB 231 cells (700,000 cells/60 mm dish) were cultured until 100% confluency. A scratch line was made in the middle of the dish on cell monolayer, then floating cells were removed by washing gently and incubated with DMSO (0.1%), montelukast and zafirlukast in serum free media. Four lines were drawn across the scratch line to mark analysis areas and captured at 0 and 24 hours using Nikon Eclipse TS1000 inverted microscope (40x magnification). The gap width between the cells was calculated using image J program. The data were expressed as the percentage of wound closure. The percentage of wound closure at 0 hour was assigned as 0%. Experiments were repeated at least three times and data were shown as mean ± SEM.

Apoptosis assay

MDA-MB 231 cells (400,000 cells/well) were plated in 6-well plates for 2 or 3 days until 80% confluency. Cells were treated with DMSO (0.2%), montelukast and zafirlukast 24 hours after incubation, supernatants and trypsinized cells were collected. Cells were then washed two times with annexin binding buffer. Cells were stained with annexin V antibody and incubated for 15 minutes. Then 7-aminoactinomycin D (7AAD) was added. Live and dead cells were analyzed by BD Accuri™ C6 Plus flow cytometer (Becton, Dickinson and Company, USA). Apoptosis and necrosis were analyzed from annexin V and 7AAD staining.

Western blot

MDA-MB 231 cells (700,000 cells/ dish) were seeded in 60-mm dishes until 80% confluency. Cell were treated with DMSO (0.1%), montelukast and zafirlukast for 30 minutes. Then, cells were lysed with lysis buffer plus protease inhibitors. Protein concentration was determined by Bradford assays. Crude protein lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blotted for phospho-ERK1/2 and β-actin followed by appropriate secondary antibodies linked to horseradish peroxidase enzymes. Levels of protein expression were visualized using enhanced chemiluminescence substrate (Bio-Rad, Cat. No. 170-5060) on film.

Statistical analysis

Data were expressed as mean ± SEM (n=3-4) and analyzed by a one-way analysis of variance (ANOVA) using Tukey's pairwise comparison. The level of significance is defined as *p*-value < 0.05 (* denotes P<0.05 and ** denotes P<0.01). Each value was obtained from at least triplicate samples. All statistical analyses was performed using Graphpad prism statistical analysis software.

Results

Cytotoxicity of CysLT-1 antagonists

Cell viability was determined using MTT assays. MDA-MB 231 cells were treated with DMSO (0.4%), montelukast and zafirlukast (1, 5 and 10 µM) for 24 and 48 hours. DMSO was used as a vehicle control. Montelukast was toxic at 10 µM at 24 and 48 hours while zafirlukast showed no toxicity at the same concentrations (Figure 1). At the same concentrations, montelukast was more toxic than zafirlukast.

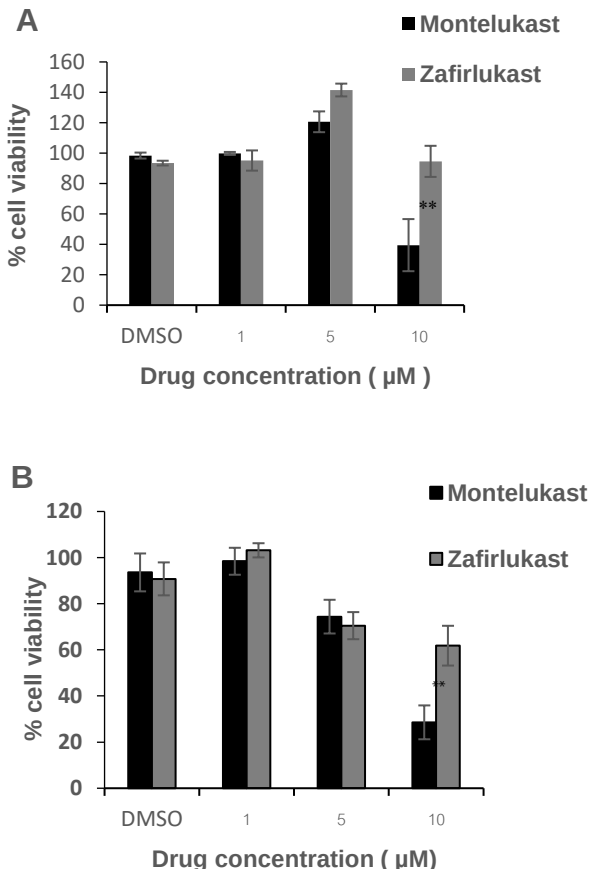
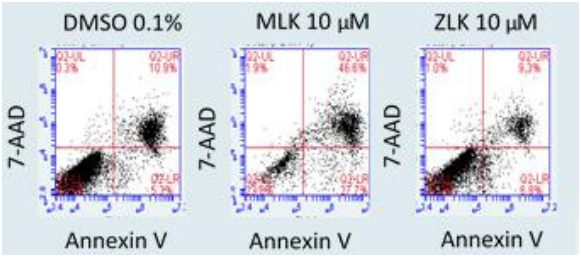


Figure 1. Cytotoxic effect of montelukast and zafirlukast on cell viability of MDA-MB 231 cells. MDA-MB 231 cells were exposed to 1, 5 and 10 µM of CysLT-1 antagonists for 24 hours (A) and 48 hours (B). The results were shown as the percentage of viable cells compared to DMSO-treated group.

In order to confirm cytotoxic effect of montelukast and zafirlukast, apoptosis and necrosis were determined by Annexin V and 7-Aminoactinomycin D (7AAD) staining using flow cytometry. Annexin V detects cells that express phosphatidylserine on the cell surface, showing apoptosis and other forms of cell death such as necrosis. 7AAD distinguishes viable cells from apoptotic cells and necrotic cells. MDA-MB 231 cells were treated with montelukast 10 µM, zafirlukast 10 µM and dimethyl sulfoxide 0.2% for 24 hours. Representative images of flow analysis were shown in Figure 2. The average percentage of apoptosis cells were showed in Table 1. Results showed that 10 µM of montelukast cause apoptosis of MDA-MB 231 cells while 10 µM of zafirlukast did not increase apoptosis.



Treatment	**Average of early apoptotic cells (%)** Annexin V-Positive, 7-AAD-Negative	**Average of late apoptotic cells (%)** Annexin V-Positive, 7-AAD-Positive	**Average of total apoptotic cells (%)**
DMSO (0.2%)	5.7 ± 0.4	9.5 ± 1.4	15.2 ± 1.8
MLK 10 µM	36.4 ± 7.63	35.1 ± 7.66	71.5 ± 15.29
ZLK 10 µM	9.1 ± 1.25	13.9 ± 5.32	23.0 ± 6.57

Figure 2. and **Table 1.** Apoptotic effect of montelukast (MLK) and zafirlukast (ZLK) in human MDA-MB 231 cells after 24 hours exposure.

Inhibition of MDA-MB-231 cell migration by CysLT-1 antagonists

Effects of CysLT-1 antagonists on cell migration were determined by wound healing assay. MDA-MB 231 cells were treated with montelukast (5 µM) and zafirlukast (5 and 10 µM). Images were captured at 0 and 24 hours to compare the migration rate of each treatment (Figure 3). The results showed that montelukast at 5 µM significantly inhibited cell migration compared to DMSO-treated group while zafirlukast at the same concentration did not slow down the migration. Anti-migration effect of zafirlukast were seen at 10 µM **(**Figure 3 and 4**).** Because montelukast at 10 µM were toxic to cells and caused morphology change, it is not possible to evaluate the anti-migration effect at this concentration.

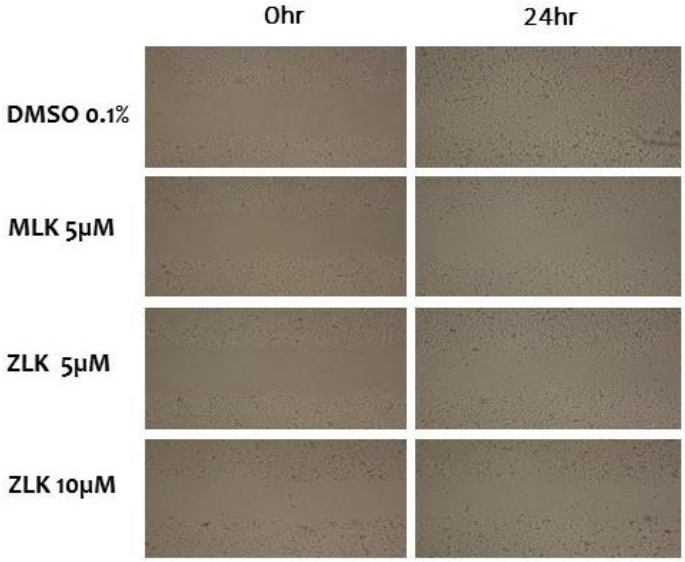


Figure 3. Representative images at 0 and 24 hours after exposure to montelukast **(**MLK**)** and zafirlukast **(**ZLK**)**

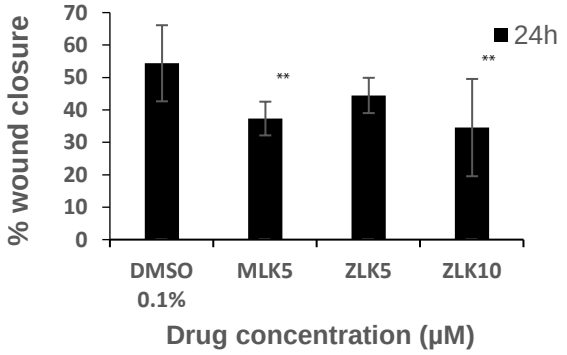


Figure 4. Effects of montelukast (MLK) and zafirlukast (ZLK) on cell migration of MDA-MB 231 cells.

Inhibition of ERK signaling by CysLT-1 antagonists

The effect of CysLT-1 antagonists on ERK signaling pathway was investigated by Western blot analysis. MDA-MB 231 cells were treated with montelukast and zafirlukast at 5 and 10 µM for 30 minutes. DMEM and DMSO-treated cells were used as a control. Results showed that both montelukast and zafirlukast at 10 µM (the fourth and sixth lanes) reduced protein expression of phosphor-ERK significantly whereas both antagonists at 5 µM (the third and the fifth lanes) showed no difference compared to mock-treated or DMSO-treated cells. Beta-actin was used as internal loading control.

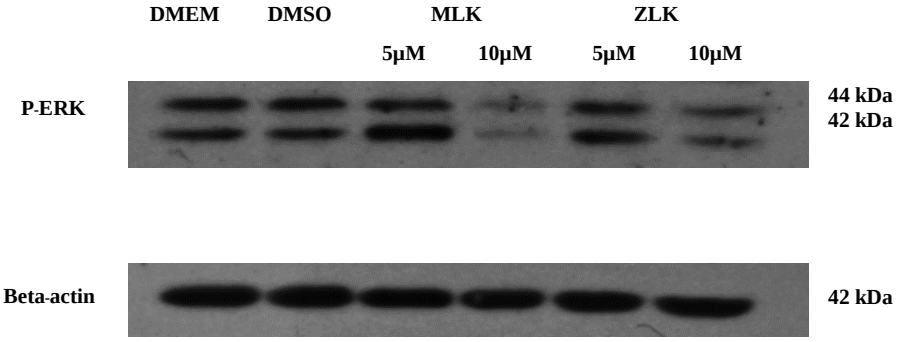


Figure 5. Effects of montelukast (MLK) and zafirlukast (ZLK) on the activation of ERK signaling pathway at 30 minutes post-exposure in human MDA-MB 231 cells.

Discussion

The present study demonstrates cytotoxic and anti-migration effects of CysLT-1 antagonists, showing the involvement of CysLT-1 signaling pathway in breast cancer progression.

Previous studies also demonstrated that CysLT-1 antagonists inhibit tumor growth by inducing apoptosis in renal cell carcinoma (Funao, Matsuyama et al. 2008), bladder (Matsuyama, Funao et al. 2009), prostate (Matsuyama M. et al, Oncol Rep 18,2007) and testicular cancers (Matsuyama, Funao et al. 2009). Here, we observed that montelukast at 10 µM, but not zafirlukast, reduce cell viability and induce early and late apoptosis of MDA-MB 231 after 24 hours exposure.

The earlier studies reported that CysLT-1 antagonists reduce LTD4-CysLT-1 receptor mediated migration of endothelial cell line EA.hy926 via the Erk1/2 pathway. In this present study, both antagonists showed anti-migration effects after 24 hours exposure.

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is noted that at the same concentration, montelukast is more potent than zafirlukast. Moreover, the cytotoxic and apoptotic effects of MDA-MB 231 breast cancer cells lines might be due to inhibition of Mitogen Activated Protein Kinase/Extracellular Regulated Kinase pathway, which is activated by both antagonists. Moreover, it is noted that at the same concentration of every experiment, montelukast is more potent than zafirlukast.

Conclusions

This study showed that LTRAs is toxic to human highly metastatic MDA-MB 231 cancer cells. Additionally, LTRAs also inhibit cell migration and activation of ERK pathway. These data indicated that LTARs can be potential target for basal type of breast cancer.

Acknowledgements

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Abbreviations

CysLT-1 = Cysteinyl leukotriene receptor-1, LTRA = Leukotriene receptor antagonist, MTT = 3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide, MLK = Montelukast, ZLK = Zafirlukast, ER = Estrogen receptor, PR = Progesterone receptor, HER2 = Human epidermal growth factor receptor 2, 7-AAD = 7-aminoactinomycin D

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