Cysteinyl Leukotriene Receptor Antagonists Induce Apoptosis and Inhibit Proliferation of Human Glioblastoma Cells by Down-regulating B-cell Lymphoma 2 and Inducing Cell Cycle Arrest

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Cysteinyl Leukotriene Receptor Antagonists Induce Apoptosis and Inhibit Proliferation of Human Glioblastoma Cells by Down-regulating B-cell Lymphoma 2 and Inducing Cell Cycle Arrest

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Running head: Effects of LTRAs on glioblastoma

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Abstract
Glioblastoma is the most aggressive type of brain cancer with the highest proliferation, invasion and migration. Montelukast and zafirlukast, two widely used leukotriene receptor antagonists (LTRAs) for asthma treatment, inhibited invasion and migration of glioblastoma cell lines. Montelukast induces apoptosis and inhibits cell proliferation of various cancer cells. Herein, apoptotic and antiproliferative effects of montelukast and zafirlukast were investigated in two glioblastoma cell lines, A172 and U-87 MG. Both LTRAs induced apoptosis and inhibited cell proliferation of glioblastoma cells in a concentration-dependent manner. Montelukast was more cytotoxic and induced higher levels of apoptosis than zafirlukast in A172 cells, but not in U-87 MG cells. Both drugs decreased expression of B-cell lymphoma 2 (Bcl-2) protein without affecting Bcl-2-associated X (Bax) levels. LTRAs also reduced the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2). In contrast, zafirlukast showed a greater antiproliferative effect than montelukast and induced G0/G1 cell cycle arrest by upregulating p53 and p21 expression. These results suggested that therapeutic potential of LTRAs in glioblastoma.

Key words: montelukast, zafirlukast, leukotriene, apoptosis, proliferation, glioblastoma, Bcl-2, cell cycle arrest
Introduction
Glioblastoma, one of the most common primary brain cancers, is notorious for its high invasion and metastasis (Kleihues et al. 1995). Surgical resection followed by chemotherapy and radiotherapy remains the gold standard therapeutic regimen for glioblastoma (Adamson et al. 2009). However, the recurrence of tumor is high and the survival rate is low, indicating a need for novel anticancer drugs.
The 5-lipoxygenase (5-LOX) pathway and cysteinylic leukotrienes (CysLTs) play a role in neuroinflammation and tumorigenesis in brain tumors. Tissue slices of human astrocytomas spontaneously release large amounts of CysLTs (Simmet et al. 1990). 5-LOX and cysteinylic leukotriene receptors (CysLTRs) were expressed in glioblastoma and meningioma (Ishii et al. 2009; Nathoo et al. 2004). In malignant astrocytoma patient, elevated urinary excretion of LTE4, a CysLT metabolite, was found. Interestingly, level of urinary LTE4 was greatly reduced after surgical resection (Simmet et al. 1990).
Montelukast and zafirlukast are cysteinylic leukotriene receptor antagonists (LTRAs) used for asthmatic prevention and control. A recent large epidemiological study showed asthmatic patients taking LTRAs have lower risk for several types of cancers compared to non-users (Tsai et al. 2016). In vitro studies reported montelukast-induced apoptosis in renal cell carcinoma, prostate cancer, testicular cancer, colon cancer, lung cancer and neuroblastoma (Funao et al. 2008; Matsuyama et al. 2009; Matsuyama et al. 2007; Savari et al. 2013; Sveinbjornsson et al. 2008; Tsai et al. 2017). Inhibition of 5-LOX reduces glioblastoma cell proliferation (09-Ishii). Rat astrocytes releases CysLTs in basal condition and after stimulation by various stimuli (Ciccarelli et al. 2004). Exogenous leukotriene D4 (LTD4) increases the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and induces rat astrocyte proliferation after oxygen-glucose deprivation (Ciccarelli et al. 2004; Huang et al. 2008). Furthermore, as previously shown, non-toxic concentrations of montelukast and zafirlukast inhibit migration, invasion and expression of matrix metalloproteinase-2 and matrix metalloproteinase-9 of glioblastoma cells (Piromkraipak et al. 2018).
In this study, we demonstrated that both LTRAs induced apoptosis and inhibited cell proliferation in human glioblastoma cell lines.

**Materials and Methods**

**Chemicals**

Montelukast and zafirlukast were purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO). The maximal concentration of DMSO in all experiments was 0.04%.

**Cell culture**

A172 and U-87 MG cells were obtained from ATCC and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) and Minimum Essential Medium (MEM, Gibco), respectively, at 37 °C in a humidified atmosphere of 5% CO₂. Culture media were supplemented with 1% penicillin/streptomycin (Merck) and 10% fetal bovine serum (FBS, Gibco) for A172 cells. In addition to antibiotics and FBS, MEM were supplement with 1% sodium pyruvate (Gibco) for U-87 MG cells.

**Apoptosis assay**

A172 and U-87 MG cells were plated in 6-well plates at 200,000 and 350,000 cells/well, respectively. At 70-80% confluence, cells were treated with 10 or 20 µM montelukast and zafirlukast for 24 h. Cell morphology was observed using an inverted light microscope (Eclipse TE2000-5, Nikon) with a 10x objective. The supernatant were collected and cells were detached with 0.05% trypsin-EDTA. The supernatant and detached cells were centrifuged at 320 g for 4 min. Then, cells were washed twice and resuspended in Annexin V binding buffer. Cells were labeled with Annexin V-FITC (BD Biosciences) in dark for 15 min followed by 7-Aminoactinomycin D (7-AAD) staining (BD Biosciences). All flow cytometric measurements were performed using a BD Accuri C6 flow cytometer (BD Biosciences). A minimum of 5,000 events/sample were analyzed each time.

**Western blotting**

A172 and U-87 MG cells were plated in 60-mm dishes at 500,000 and 800,000 cells/dish, respectively, for analysis of Bcl-2, Bax and ERK. Additionally, A172 and U-87 MG cells were plated in 60-mm dishes at 250,000 and 350,000
cells/dish, respectively, and then serum starved for 24 h prior to LTRA treatment for analysis of p53, p21Waf/Cip, and p27Kip. After the indicated time, cells were harvested with ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, and 0.5% NP-40) with protease inhibitor cocktail Set III (1:1000, Calbiochem) and centrifuged at 12,000 rpm for 15 minutes. Protein samples were separated on 12% SDS-PAGE gels and then transferred to nitrocellulose membranes. Protein levels were probed with anti-Bax (1:600, Cat. No. 5023, Cell Signaling), anti-Bcl-2 (1:600, Cat. No. 2872, Cell Signaling), anti-phospho-p44/42 (1:4000, Cat. No. 4370, Cell Signaling), anti-p44/42 (1:4000, Cat. No. 9102, Cell Signaling), anti-p21 (1:1000, Cat. No. 2947, Cell Signaling), anti-p27 (1:1000, Cat. No. 3686, Cell Signaling), anti-p53 (1:8000, Cat. No. 2527, Cell Signaling), anti-β-actin (1:5000, Cat. No. 4967, Cell Signaling), anti-β-tubulin (1:5000, Cat. No. 86298, Cell Signaling) or anti-GAPDH (1:10,000, Cat. No. 5174, Cell Signaling) followed by anti-rabbit or anti-mouse conjugated with horseradish peroxidase enzymes. The chemiluminescent signal was visualized on film. Band density was quantified by densitometry using Image J software (version 1.48). Protein expression was normalized with β-actin, β-tubulin or GAPDH. Protein expression of mock-treated cells was defined at 100%.

**Proliferation assay**

Cells were labeled with 1 µM 5(6)-carboxy-fluorescein diacetate N-succinimidyl ester (CFSE) (Sigma) in phosphate buffered saline (PBS). Excess CFSE was washed out with PBS containing 2% FBS. A172 and U-87 MG cells were seeded in media with 5% FBS at 120,000 and 240,000 cells/well in 6-well plates, respectively. Media with montelukast or zafirlukast at 10 or 20 µM were refreshed daily. Mean fluorescence intensity of cells was measured on day 4 post-treatment using a BD Accuri C6 flow cytometer (BD Biosciences). A minimum of 5,000 events/sample were analyzed each time. Relative fluorescence intensity was compared to mean fluorescence intensity of mock-treated cells.

**Cell cycle analysis**

A172 and U-87 MG cells were seeded in media with 10% FBS at 120,000 and 240,000 cells/well in 6-well plates, respectively. The next day, cells were washed
and incubated in media without serum. At 24 h after serum starvation, cells were treated with 20 µM montelukast and zafirlukast in fresh media containing 5% FBS. At 48 h post-exposure, cells were detached with 0.05% trypsin-EDTA and fixed with 70% ethanol on ice for 30 min. Cells were washed with PBS and then stained with PI/RNase Staining Buffer (BD Biosciences). Single cells were gated based on total fluorescence (FLA2-Area) vs maximum fluorescence (FLA2-Height) of propidium iodide. All flow cytometric measurements were performed using a BD Accuri C6 flow cytometer (BD Biosciences). A minimum of 10,000 events of single cells/sample were analyzed each time.

**Statistical analysis**

Data were expressed as mean±SEM (n=3-5) and analyzed by a one-way analysis of variance (ANOVA) using Tukey-Kramer post-hoc test. The difference of LTRA effects between A172 and UU87 MG cells was analyzed by a two-way ANOVA. The level of significance was defined as p-value<0.05. All statistical analyses were performed using GraphPad® Prism statistical analysis software (version 5.0).

**Results**

**Cysteinyi leukotriene receptor antagonists induced morphological changes and apoptosis of human glioblastoma cells**

A previous study in A172 cell line reported median toxic concentrations of montelukast and zafirlukast at 8.9 and 14.6 µM, respectively (Piromkraipak et al. 2018). Consistently, montelukast at 10 and 20 µM were toxic to A172 and U-87 MG cells, while zafirlukast at 20 µM reduced cell viability of both cell lines (Supp. Fig. 1). At 24 h post-exposure, DMSO-treated A172 and U-87 MG cells showed branching processes while cells treated with 10 µM montelukast became round and almost all of the cells were round after exposing to 20 µM montelukast. In contrast, zafirlukast showed less cytotoxicity in A172 cells (Fig. 1A and 1B). To test whether this finding resulted from apoptosis-inducing effect of LTRAs, cells were stained with annexin V and 7-AAD. The degree of early and late apoptosis was determined as the percentage of cells positive for annexin V-FITC without or
with 7-AAD staining, respectively. Flow cytometric dot plots of A172 and U-87 MG cells treated with both LTRAs showed increased percentages of annexin V positive cells in a concentration-dependent manner (Fig. 1A and 1B). Both LTRAs at 20 µM significantly increased early and late apoptotic cells compared to DMSO-treated cells (Fig. 1C and 1D). Montelukast at 10 µM also significantly promoted apoptosis of A172 cells (Fig. 1C). Moreover, montelukast at 20 µM induced a higher percentage of apoptotic cells in A172 than U-87 MG (p<0.05).

**Cysteinyl leukotriene receptor antagonists decreased protein expression of B-cell lymphoma 2 in human glioblastoma cells**

Previous studies indicate that LTD4 plays a role in anti-apoptotic Bcl-2 protein expression by decreasing ERK1/2 phosphorylation (Massoumi and Sjolander 2007; Tsai et al. 2017). To test whether LTRAs altered the expression of apoptosis-related proteins in glioblastoma cells, cells were treated with montelukast and zafirlukast for 24 h followed by analysis of Bcl2, Bax, phosphorylated ERK1/2 (p-ERK1/2) and ERK1/2 levels. Montelukast and zafirlukast at 20 µM reduced Bcl-2 expression in both A172 and U-87 MG glioblastoma cell lines (Fig. 2A and 2B) while both drugs did not alter the expression of proapoptotic Bax protein (Fig. 2C and 2D). Montelukast and zafirlukast decreased the levels of p-ERK1/2 in A172 and U-87 MG cell lines (Fig. 2E and 2F); moreover, both LTRAs reduced total expression of ERK1/2 in A172 cell line.

**Cysteinyl leukotriene receptor antagonists inhibited proliferation of human glioblastoma cells**

Montelukast inhibits LTD4-induced astrocytic proliferation (Huang et al. 2008). Zileuton, a 5-lipoxygenase inhibitor, inhibited cell proliferation of rat mammary glands (Chatterjee et al. 2013) and human colon carcinoma cells (Savari et al. 2013). We examined whether montelukast and zafirlukast inhibit cell proliferation in glioblastoma cell lines. Montelukast and zafirlukast were found to reduce cell density of A172 and U-87 MG cells (Fig. 3A and 3B). Cell proliferation was evaluated by CFSE-cell tracing assay using mean fluorescence intensity (MFI) of CFSE dye. After each cell division, the remaining CFSE dye in daughter cells is
reduced to half, resulting in the decrease of MFI. Montelukast and zafirlukast at 20 µM caused a rightward shift of fluorescence intensity at day 4 after the initial treatment in both glioblastoma cell lines (Fig. 3C and 3D). Both LTRAs increased MFI compared to DMSO-treated cells (Fig. 3E and 3F). In A172 cell line, zafirlukast demonstrated greater inhibition of cell proliferation than montelukast. Additionally, comparison of zafirlukast effects in two cell lines showed greater reduction of cell proliferation in A172 than in U-87 MG (p<0.05).

Zafirlukast caused G0/G1 cell cycle arrest in human glioblastoma cells by upregulating cell cycle inhibitor proteins

Montelukast induces G1 cell cycle arrest of human neuroblastoma cells (Sveinbjornsson et al. 2008) and human colon carcinoma cells (Savari et al. 2013). We investigated whether the inhibition of cell proliferation by LTRAs was due to cell cycle arrest. Both LTRAs at 20 µM were found to inhibit cell proliferation, but only zafirlukast significantly increased percentage of G0/G1 phase cells and decreased percentage of S phase cells in both A172 and U-87 MG cell lines at 48 h post-exposure (Fig. 4 and Table 1) Moreover, zafirlukast also significantly reduced percentage of G2/M phase cells in U-87 MG cells (Table 1). Next, to examine the effects of both LTRAs on proteins involved in cell cycle regulation, cells were treated with montelukast and zafirlukast at 20 µM for 48 h. In A172 cell line, zafirlukast, but not montelukast, upregulated the expression of p53 and p21\textsuperscript{Waf/Cip1} (Fig. 5A and 5C). In U-87 MG cell line, both montelukast and zafirlukast increased p53 expression (Fig. 5B), but only zafirlukast induced p21\textsuperscript{Waf/Cip1} expression (Fig. 5D). Interestingly, zafirlukast only increased p27\textsuperscript{Kip} expression in U-87 MG, but not A172 (Fig 5E and 5F).

Discussion

Montelukast is cytotoxic and induces apoptosis in several types of cancer cells. The present study reported apoptotic and anti-proliferative effects of montelukast and zafirlukast on two types of human glioblastoma cells. In A172 cells, montelukast showed higher apoptotic effects than zafirlukast, while zafirlukast exhibited a greater degree of cell cycle arrest than montelukast. On the other
hand, both LTRAs showed similar apoptotic and anti-proliferative effects in U-87 MG cells. Such subtle differences might be caused by different genetic alteration: A172 cells have missense mutation of RB1 and PTCH1 (http://cancer.sanger.ac.uk/cosmic) while U-87 MG cells contain the missense mutation of ATRX, NF1, and TERT genes (Patil et al. 2015).

Human glioblastoma cells are more sensitive to the apoptotic effect of LTRAs than several types of cancer cells. In the present study, montelukast at 10 μM and zafirlukast at 20 μM induced apoptosis of human glioblastoma cells while the apoptotic effect of montelukast on human renal, prostate and testicular cells is observed at 100 μM (Funao et al. 2008; Matsuyama et al. 2009; Matsuyama et al. 2007). In human colon carcinoma cells, montelukast at 25 μM, but not at 12.5 μM, triggers apoptosis (Savari et al. 2013) and apoptosis of human neuroblastoma is mediated by 10 μM montelukast (Sveinbjornsson et al. 2008).

Decreased Bcl-2 leads to apoptosis. Montelukast at 70 μM reduces expression of both anti-apoptotic Bcl-2 and pro-apoptotic Bax and Bad in A549 human lung cancer cells (Tsai et al. 2017). In the present study, both montelukast and zafirlukast downregulated Bcl-2 expression in A172 and U-87 MG human glioblastoma at much lower concentration than lung cancer cells, and did not change Bax expression in human glioblastoma cells. On the contrary, montelukast and zileuton reduce Bax expression and increase Bcl-2 expression in a mouse model of spinal cord injury (Genovese et al. 2008). Montelukast at 0.1 and 10 μM inhibit amyloid-β peptide1-42-mediated Bcl-2 downregulation in primary mouse neuronal cultures (Lai et al. 2014b). Similarly, oral montelukast increases Bcl-2 levels in hippocampus and cerebral cortex after brain injection of amyloid-β peptide1-42 and streptozotocin (Lai et al. 2014a; Zhang et al. 2016), indicating that montelukast may pass through the blood brain barrier and affect brain cells. Together, these studies suggest that LTRAs at low concentrations demonstrate an anti-apoptotic effect, while high concentrations induce apoptosis in several cancers including human glioblastoma.

LTD4 induces Bcl-2 expression by triggering ERK1/2, protein kinase C, cyclooxygenase-2 (COX-2), and β-catenin pathways (Massoumi and Sjolander
A recent study in human lung cancer cells also demonstrated that montelukast reduces the phosphorylation of ERK1/2 and several signaling proteins (Tsai et al. 2017). MK-886, a 5-lipoxygenase-activating protein inhibitor, reduces Bcl-2 expression and ERK1/2 phosphorylation in A172 and U-87 MG cell lines (Lim et al. 2010). Similarly, the present study found montelukast and zafirlukast inhibited ERK1/2 phosphorylation in both glioblastoma cell lines. Interestingly, LTRAs also reduced total expression of ERK1/2 in A172 cell line, which may explain montelukast’s greater apoptotic effects in A172 compared to U-87 MG cell line. In contrast to intestinal epithelial cells, montelukast increased COX-2 expression in human lung cancer cells (Tsai et al. 2017). Thus, further studies are required to elucidate the mechanisms underlying the down-regulation of Bcl-2 by LTRAs in glioblastoma cells. Several pieces of evidence indicate the importance of the leukotriene pathway in cancer cell proliferation. Montelukast at 12.5 to 50 µM inhibit proliferation of human colon carcinoma proliferation by inducing G1 arrest (Savari et al. 2013). In human neuroblastoma cells, montelukast at 10 µM, a 5-lipoxygenase inhibitor, and a 5-lipoxygenase-activating protein inhibitor cause G1 arrest (Sveinbjornsson et al. 2008). Pranlukast, another commercially available LTRA in Japan, inhibits Madine Darby canine kidney cell proliferation at 20 and 50 µM (Pathomthongtaweechai et al. 2014). Remarkably, montelukast at 20 µM inhibited cell proliferation of two glioblastoma cell lines without affecting cell cycle progression. Perhaps the higher dose of montelukast may lead to G0/G1 arrest but at doses higher than 20 µM, it is too toxic to investigate the effect on cell cycle. The present study showed that 20 µM zafirlukast induced G0/G1 arrest, leading to reduced cell proliferation in two glioblastoma cells. LTD4 induces rat astrocyte proliferation by activating the ERK1/2 pathway (Ciccarelli et al. 2004). The present study showed montelukast and zafirlukast reduced ERK1/2 phosphorylation, which could lead to the inhibition of astrocyte proliferation. Montelukast increases p53 expression in T lymphocytes (Spinozzi et al. 2004) and p21Waf/Cip1 expression in human colon carcinoma (Savari et al. 2013). In contrast, zafirlukast, not montelukast, upregulated p53 and p21Waf/Cip1 expression.
in A172 and U-87 MG cell lines. p21\textsuperscript{Waf/Cip1}, a cyclin-dependent kinase inhibitor, is upregulated by p53. Increased p21\textsuperscript{Waf/Cip1} blocks the transition between G1 and S phases, leading to G1 cell cycle arrest (Cmielova and Rezacova 2011). Increased p53 expression by zafirlukast could induce p21 synthesis, leading to G0/G1 cell cycle arrest. In addition to p21\textsuperscript{Waf/Cip1}, the decrease of p27\textsuperscript{Kip}, a cyclin dependent kinase inhibitor, is required for G0/G1 to S transition. Several types of cancer including glioblastoma showed reduced p27\textsuperscript{Kip} levels (Chu et al. 2008). Overexpression of a RING finger protein Znf179 induced G0/G1 arrest of primary glioblastoma cells by upregulating p53, p21\textsuperscript{Waf/Cip1}, and p27\textsuperscript{Kip} (Lee et al. 2017). Herein, zafirlukast also triggered the expression of p27\textsuperscript{Kip}, another cyclin-dependent kinase inhibitor, in U-87 MG cells; therefore, induction of p27\textsuperscript{Kip} expression could be beneficial in promoting G0/G1 arrest.

Montelukast, zafirlukast and pranlukast have similar affinity to CysLT1 receptors (Burke et al. 2016) and comparable clinical outcome in asthmatic patients (Keam et al. 2003; Riccioni et al. 2004); however, there are subtle differences between LTRAs. Zafirlukast inhibits cyclooxygenase-1 activity while montelukast and pranlukast have no effect (Kahnt et al. 2013). Pranlukast, but not montelukast and zafirlukast, inhibits P2Y(6) receptor-mediated signaling and ion transport in human bronchial epithelial cells (Lau et al. 2011). Pranlukast reduces the extravasation of rat colon carcinoma cells through both peripheral and brain capillaries, while montelukast only prevented the extravasation through peripheral capillaries (Nozaki et al. 2010). Zafirlukast and pranlukast also inhibit CysLT2 receptors (Burke et al. 2016), which when activated stimulate cell proliferation (Ito et al. 2008). The stronger anti-proliferative effect of zafirlukast compared to montelukast could be mediated by the inhibition of both CysLT receptors.

Peak plasma concentrations of a single oral administration of 10 mg montelukast and 20 mg zafirlukast were 0.63 and 0.58 \textmu M, respectively (Cheng et al. 1996; Dekhuijzen and Koopmans 2002). Both drugs are well-tolerated. Accidental overdose of montelukast at 135 mg in a 5-year-old asthmatic child did not produce any clinical symptom (Cobb et al. 2002). Patients receiving zafirlukast at
80 mg twice daily over 6 months did not report any serious side effect (Dekhuijzen and Koopmans 2002). One advancement in chemotherapy for glioblastoma multiforme treatment is the use of carmustine polymer wafers after tumor removal. This technique slightly improved patient survival but greatly increase complications due to carmustine toxicity (Bregy et al. 2013). Nevertheless, the direct delivery of drugs to tumor tissues creates the possibility to use drugs at concentrations higher than plasma levels and bypasses the problem of blood brain barrier penetration. Given LTRAs have an excellent safety profile, this direct delivery method could allow the use of toxic concentrations, which are higher than therapeutic range for asthma.

In summary, the present study showed inhibition of CysLT1 receptors resulted in apoptosis and reduced cell proliferation by reducing Bcl-2 expression and causing G0/G1 arrest via upregulation of p53 and p21Waf1/Cip1 expression in glioblastoma cell lines. Therefore, in vivo investigation of LTRAs’s effects in animal models of glioblastoma is warranted.

Acknowledgement

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Disclosure statement

All authors declare that there is no conflict of interest.

References:


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Figure 1. LTRA-induced apoptosis of human glioblastoma cells.
The morphology of A172 and U-87 MG cells was assessed under a light microscope. Montelukast (MLK) and zafirlukast (ZLK) caused morphological changes to A172 (A) and U-87 MG (B) cells. Cells were stained with FITC-Annexin V/7-AAD and analyzed by flow cytometry. The lower right quadrant represents early apoptosis (Annexin V-FITC-positive cells/7-AAD-negative cells). The upper right quadrant represents late apoptosis (Annexin V-FITC-positive cells/7-AAD-positive cells). Representative flow cytometric dot plots showed higher cell percentage in early and late apoptosis of A172 (A) and U-87 MG (B) cells treated with MLK and ZLK. An increased percentage of early and late apoptotic cells were found in A172 (C) and U-87 MG (D) cells treated with MLK and ZLK. Results were expressed as mean±SEM of at least four independent experiments. The scale bar was 20 µm. Statistical significance was denoted as * (p<0.05) and ** (p<0.01).

Figure 2. LTRA-induced reduction of an anti-apoptotic protein, Bcl-2, in human glioblastoma cells.
A172 and U-87 MG cells were treated with montelukast (MLK) and zafirlukast (ZLK) for 24 h. A172 (A) and U-87 MG (B) cells treated with MLK and ZLK at 20 µM showed lower expression of Bcl-2 proteins compared to DMSO-treated cells. In contrast, neither drug had an effect on Bax expression in A172 (C) or U-87 MG (D) cells. MLK and ZLK at 20 µM decreased the level of phospho-ERK1/2 (p-ERK1/2) in A172 (E) and U-87 MG (F) cells. Data were expressed as mean±SEM of three to five independent experiments. Statistical significance was denoted as * (p<0.05) and ** (p<0.01).

Figure 3. Inhibition of human glioblastoma proliferation by LTRAs.
A172 and U-87 MG cells were treated daily with montelukast (MLK) and zafirlukast (ZLK). At day 4 post-treatment, A172 (A) and U-87 MG (B) cells treated with MLK and ZLK at 20 µM showed less cell density than DMSO-treated
cells. In the representative histogram of CFSE fluorescence intensity, the black line, the gray line, and the dashed line represented cells treated with DMSO, 20 µM MLK and 20 µM ZLK, respectively. Both MLK and ZLK at 20 µM caused the rightward shift in CFSE histogram compared to DMSO in A172 (C) and U-87 MG (D) cells. Increased CFSE fluorescence intensity was observed in A172 (E) and U-87 MG (F) cells treated with MLK and ZLK at 20 µM. Additionally, 20 µM ZLK-treated A172 cells showed significantly higher fluorescence intensity than 20 µM MLK-treated cells. Results were expressed as mean±SEM of at least four independent experiments. The scale bar was 20 µm. Statistical significance was denoted as * (p<0.05) and ** (p<0.01) in comparison between DMSO-treated cells and LTRAtreated cells and ## (p<0.01) in comparison between 20 µM MLK and 20 µM ZLK.

**Figure 4.** Zafirlukast-induced G0/G1 cell cycle arrest.
Cell cycle progression was analyzed by flow cytometry. Representative histograms of cell cycle progression and bar graphs showed mean percentage of cells in sub-G1, G0/G1, S and G2/M phases of A172 (A) and U-87 MG (B) cells. Data were expressed as mean of five independent experiments.

**Figure 5.** LTRA-induced upregulation of cell cycle inhibitors in human glioblastoma cells.
A172 and U-87 MG cells were treated with montelukast (MLK) and zafirlukast (ZLK) at 20 µM for 48 h. ZLK upregulated expression of p53 and p21 compared to DMSO in A172 (A, C) and U-87 MG (B, D) cells. LTRAs did not affect p27 expression in A172 (E) while ZLK increased p27 expression in U-87 MG cells (F). Data were expressed as mean±SEM of three to five independent experiments. Statistical significance was denoted as * (p<0.05) and ** (p<0.01).
Table 1. Changes of percentage of cells in Sub-G1, G0/G1, S and G2/M phases by LTRAs.

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<td>U-87 MG</td>
<td>DMSO</td>
<td>3.42±0.35</td>
<td>67.67±2.40</td>
<td>5.63±0.84</td>
</tr>
<tr>
<td></td>
<td>MLK 20 µM</td>
<td>2.58±0.16</td>
<td>72.23±1.41</td>
<td>4.61±0.42</td>
</tr>
<tr>
<td></td>
<td>ZLK 20 µM</td>
<td>2.71±0.48</td>
<td>77.38±1.60**</td>
<td>3.24±0.28*</td>
</tr>
</tbody>
</table>

Data are mean±SEM (N=5).

Statistical significance was denoted as * (p≤0.05) and ** (p≤0.01).
Figure 1

279x361mm (300 x 300 DPI)
Figure 2

279x361mm (300 x 300 DPI)
Figure 3

279x361mm (300 x 300 DPI)
Figure 4
Figure 5

279x361mm (300 x 300 DPI)