Differential effects of montelukast and zafirlukast on MDA-MB-231 triple-negative breast cancer cells: Cell cycle regulation, apoptosis, autophagy, DNA damage and endoplasmic reticulum stress

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Abstract. Montelukast and zafirlukast, cysteinyl leukotriene receptor antagonists (LTRAs), trigger apoptosis and inhibit cell proliferation of triple-negative breast cancer MDA-MB-231 cells. By contrast, only zafirlukast induces G_0/G_1 cell cycle arrest. The present study compared the effects of these drugs on proteins regulating cell proliferation, apoptosis, autophagy, and endoplasmic reticulum (ER) and oxidative stress using reverse transcription-quantitative PCR, western blotting and flow cytometry. The expression of proliferating markers, Ki-67 and proliferating cell nuclear antigen, was decreased by both drugs. Zafirlukast, but not montelukast, decreased the expression of cyclin D1 and CDK4, disrupting progression from G_1 to S phase. Zafirlukast also increased the expression of p27, a cell cycle inhibitor. Both drugs decreased the expression of anti-apoptotic protein Bcl-2 and ERK1/2 phosphorylation, and increased levels of the autophagy marker LC3-II and DNA damage markers, including cleaved PARP-1, phosphorylated (p)-ATM and p-histone H2AX. The number of caspase 3/7-positive cells was greater in montelukast-treated cells compared with zafirlukast-treated cells. Montelukast induced higher levels of the ER stress marker CHOP compared with zafirlukast. Montelukast activated PERK, activating transcription factor 6 (ATF6) and inositol-requiring enzyme type 1 (IRE1) pathways, while zafirlukast only stimulated ATF6 and IRE1 pathways. GSK2606414, a PERK inhibitor, decreased apoptosis mediated by montelukast, but did not affect zafirlukast-induced cell death. The knockdown of CHOP by small interfering RNA reduced apoptosis triggered by montelukast and zafirlukast. In conclusion, the effects on cell cycle regulator proteins may contribute to cell cycle arrest caused by zafirlukast. The greater apoptotic effects of montelukast may be caused by the higher levels of activated caspase enzymes and the activation of three pathways of ER stress: PERK, ATF6, and IRE1.

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Introduction

Cysteinyl leukotriene receptor antagonists (LTRAs), currently used for the treatment of asthma and allergic rhinitis, demonstrate anticancer effects in *in vitro* and *in vivo* models (1,2). Overexpression of 5-lipoxygenase, a leukotriene synthesis enzyme, and leukotriene receptors are found in numerous types of cancer, including breast cancer (BC) (3). A large population study in Taiwanese patients with asthma using LTRAs identified a decreased risk of breast, lung, colorectal and liver cancer compared with non-LTRA users (4). In this study, a hazard ratio of LTRA users was 0.31 and the increase of cumulative dose progressively decreased cancer risk. Two subsequent large retrospective cohort studies also reported a lower risk of cancers in asthmatic patients taking LTRAs. Korean patients showed significant lower risk of cancers after taking high dose of LTRAs (adjusted HR=0.56) or 3 years or more of LTRA use (adjusted HR=0.68 after 3 years and 0.33 after 5 years) (5). In Japanese patients, the high accumulative dose of LTRAs was associated with a decrease in risk of cancer (adjusted HR=0.57) (6). Cysteinyl leukotriene receptor 1 (CysLT1R), a receptor of leukotriene D4, is highly expressed in breast tumor tissues compared with in normal breast tissues, and its expression is greater in higher grade tumors (3). MDA-MB-231, a triple-negative BC (TNBC) cell line, expresses CysLT1R but not CysLT2R (3). Our previous study reported the apoptotic and antiproliferative effects of two LTRAs, montelukast and zafirlukast, on MDA-MB-231 cells (7). In addition, LTRAs

have been reported to induce the apoptosis of glioblastoma and lung cancer cells by reducing ERK1/2 phosphorylation and Bcl-2 levels (8,9). Zafirlukast has also been shown to induce G_0/G_1 cell cycle arrest by upregulating p53 and p21 expression in both glioblastoma A172 and U-87 MG cells, and p27 expression in U-87 MG cells (10). Similarly, only zafirlukast can induce G_0/G_1 cell cycle arrest in MDA-MB-231 cells (7). However, the mechanisms underlying the cytotoxic and anti-proliferative effects of LTRAs on MDA-MB-231 cells remain unknown. The present study aimed to compare the effects of montelukast and zafirlukast on the molecular mechanisms regulating proliferation and death in MDA-MB-231 cells.

Materials and methods

Cell line and culture. MDA-MB-231 cells (cat. no. HTB-26) were obtained from American Type Culture Collection and cultured in DMEM (cat. no. 12800-017) supplemented with 1% penicillin/streptomycin (cat. no. 15140) and 10% fetal bovine serum (cat. no. 10270) (all Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2.

Chemicals. Montelukast (cat. no. SML0101) and zafirlukast (cat. no. Z4152) were purchased from Sigma-Aldrich (Merck KGaA), and were dissolved in DMSO (cat. no. D4540; Sigma-Aldrich; Merck KGaA). GSK2606414 (cat. no. HY-18072) was purchased from MedChemExpress. The final concentration of DMSO was 0.04% (v/v) in culture media, as previously described (7).

Cell treatment. Cells were treated with DMSO, montelukast at 20 μM, or zafirlukast at 20 μM at 37˚*C* for 3 h in reverse transcription-quantitative PCR analysis, 6, 12 or 48 h in western blotting, and 6 or 12 h in flow cytometric analysis and apoptosis assay. GSK2606414 was treated 1 h prior to the addition of montelukast or zafirlukast.

Small interfering RNA (siRNA) transfection. Cells were transfected with 50 nM ON-TARGETplus siRNA Human DNA damage-inducible transcript 3 (DDIT3, also known as C/EBP homologous protein or CHOP) SMARTpool (cat. no. L-004819-00-0005) or ON-TARGETplus Non-targeting Control Pool (cat. no. D-001810-10-05; both GE Healthcare Dharmacon, Inc.; Table I). All four siRNAs were mixed in the same reaction. DharmaFECT 4 Transfection Reagent (cat. no. T-2004-02; GE Healthcare Dharmacon, Inc.) was used according to the manufacturer's instructions. Cells were transfected at 37˚C for 48 h for gene expression studies and for 96 h before determining protein expression. Cells were immediately treat with DMSO, montelukast at 20 μM, or zafirlukast at 20 μM for 12 h.

Western blotting. Proteins were harvested from cells and detected as previously described (10). Protein concentrations were determined by Bradford Protein Assay (cat. no. 5000006; Bio-Rad) and 25 μg protein were loaded into each lane. Nitrocellulose membranes were blocked with 5% non-fat dry milk (cat. no. 1706404; Bio-Rad) in 0.5% TBS-T at 4˚C overnight and probed with primary antibodies against phosphorylated (p)-ERK1/2 (rabbit; 1:4,000; cat. no. 4370), ERK1/2 (rabbit; 1:2,000; cat. no. 4695), Bcl-2 (rabbit; 1:600; cat. no. 4223), PARP-1 (rabbit; 1:2,000; cat. no. 9542), LC3-A/B (rabbit; 1:1,000; cat. no. 12741), activating transcription factor 6 (ATF6; rabbit; 1:1,000; cat. no. 65880), CHOP (mouse; 1:1,000; cat. no. 2895), p21 (rabbit; 1:1,000; cat. no. 2947), p27 (rabbit; 1:1,000; cat. no. 3686), CDK4 (rabbit; 1:1,000; cat. no. 12790), cyclin D1 (rabbit; 1:1,000; cat. no. 2978) (all Cell Signaling Technology, Inc.) and cyclin E (mouse; 1:1,000; cat. no. 05-363; Merck KGaA) for 2 h at room temperature. β-actin (rabbit; 1:10,000; cat. no. 4970) and β-tubulin (mouse; 1:5,000; cat. no. 86298) (both Cell Signaling Technology, Inc.) were incubated for 45 min at room temperature. After that, membranes were incubated with anti-rabbit or anti-mouse conjugated with horseradish peroxidase enzymes for 1 h at room temperature (goat; 1:4,000; cat. no. 111-035-003 or 115-035-003; Jackson ImmunoResearch Laboratories). Bands were visualized using Clarity Western ECL substrate (cat. no. 1705061; Bio-Rad) and quantified using ImageJ software (version 1.54; National Institutes of Health and the Laboratory for Optical and Computational Instrumentation). β-actin or β-tubulin was used as loading controls depending on the molecular weight of proteins of interest. The band intensity was normalized to the expression of the loading control.

Flow cytometric analysis. Cells were detached with 0.05% trypsin-EDTA (cat. no. 25300054; Gibco; Thermo Fisher Scientific, Inc.) at 37˚*C* for 2 min and centrifuged at 320 x g for 10 min at room temperature. The levels of monomeric JC-1, caspase 3/7 activity, autophagic LC3, p-ATM and p-histone H2AX (H2AX) were measured using Guava Mitochondrial Depolarization Kit, Guava® Caspase 3/7 FAM Kit (cat. no. 4500-0540; Merck Millipore; Merck KGaA), FlowCellect Histone H2AX Phosphorylation Assay Kit (cat. no. FCCS100182; Merck Millipore; Merck KGaA), phycoerythrin-conjugated anti-p-ATM (Ser1981) (cat. no. FCMAB110P; Merck Millipore; Merck KGaA) and FlowCellect™ Autophagy LC3 Antibody-based Assay Kit (cat. no. FCCH100171; Merck Millipore; Merck KGaA), respectively. All tests were performed according to the manufacturer's protocol. The percentage of dead cells was determined by 7-aminoactinomycin D (7-AAD) staining when assessing mitochondrial depolarization. Cells were assessed using the Guava® easyCyte 5HT benchtop flow cytometer with InCyte 3.1 (both Cytek Biosciences). A minimum of 2,000 events was analyzed for each sample.

Reverse transcription-quantitative (RT-q)PCR. Total RNA from cells was extracted using the Total RNA Mini kit (cat. no. RB300; Geneaid Biotech Ltd.) according to the manufacturer's protocol. cDNA synthesis and qPCR were performed using superscript III reverse transcriptase (cat. no. 18080-044; Invitrogen; Thermo Fisher Scientific, Inc.) and SensiFAST SYBR Lo-ROX (cat. no. BIO-94005; Bioline; Meridian Bioscience) on an Applied Biosystems Real-time PCR 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) as previously described (11). All primers are listed in Table II. The thermocycling conditions were as follows: Initial denaturation at 50˚C for 2 min and

Table I. siRNA target sequences.

siRNA	Target sequence, 5'-3'
ON-TARGET plus Non-targeting Pool (control)	UGGUUUACAUGUCGACUAA
	UGGUUUACAUGUUGUGUGA
	UGGUUUACAUGUUUUCUGA
	UGGUUUACAUGUUUUCCUA
ON-TARGET plus Human DDIT3	GGUAUGAGGACCUGCAAGA
	CACCAAGCAUGAACAAUUG
	GGAAACAGAGUGGUCAUUC
	CAGCUGAGUCAUUGCCUUU

DDIT3, DNA damage-inducible transcript 3; siRNA, small interfering RNA.

Table II. Reverse transcription-quantitative PCR primers.

Gene	Forward primer, 5'-3'	Reverse primer, 5'-3'
GAPDH	AGCCTTCTCCATGGTGGTGAAGAC	CGGAGTCAACGGATTTGGTCG
Ki-67	CTTTGGGTGCGACTTGACG	GTCGACCCCGCTCCTTTT
PCNA	GGCCGAAGATAACGCGGATAC	GGCATATACGTGCAAATTCACCA
ATF4	CTTCCTGAGCAGCGAGGTG	TCTCCAACATCCAATCTGTCC
ATF6	TCGGTCAGTGGACTCTTATT	CCAGTGACAGGCTTATCTTC
CHOP	AAGGCACTGAGCGTATCATGT	TGAAGATACACTTCCTTCTTGAACA
GADD34	GAGGAGGCTGAAGACAGTGG	AATTGACTTCCCTGCCCTCT
PERK	GTCCGGAACCAGACGATGAG	GGCTGGATGACACCAAGGAA
XBP-1s	TCTGCTGAGTCCGCAGCAG	GAAAAGGGAGGCTGGTAAGGAAC
CAT	CCATTATAAGACTGACCAGGGC	AGTCCAGGAGGGGTACTTTCC
GST	GGATCTGCTGGAACTGCTTATCAT	TGTCCGTGACCCCTTAAAATCTT
GR	AACATCCCAACTGTGGTCTTCAGC	TTGGTAACTGCGTGATACATCGGG
GPx1	TTCCCGTGCAACCAGTTTG	TTCACCTCGCACTTCTCGAA
SOD	CTGAAGGCCTGCATGGATTC	CCAAGTCTCCAACATGCCTCTC

PCNA, proliferating cell nuclear antigen; ATF, activating transcription factor; XBP-1s, X-box binding protein 1 splice variant; GADD34, growth arrest and DNA damage-inducible gene 34; CAT, catalase; SOD, superoxide dismutase; GR, glutathione reductase; GST, glutathione S-transferase; GPx1, glutathione peroxidase 1.

95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 58˚C for 1 min, with a melting curve stage of 95˚C for 10 sec and 60˚C for 1 min. The Cq value of each target gene was normalized to the expression of GAPDH. Relative fold-change was calculated against untreated cells based on the $2^{-\Delta\Delta Cq}$ method (12).

Apoptosis assay. Floating cells and cells detached with 0.05% trypsin-EDTA were collected and centrifuged at 320 x g for 10 min at room temperature. Pellets were washed twice in Annexin V binding buffer, and were then with Annexin V-FITC (cat. no. 556419) and 7-AAD (cat. no. 51-68981E) (both BD Biosciences). The percentage of Annexin V positive cells representing early and late apoptotic cells was determined using a BD Accuri C6 Plus with BD Accuri C6 Plus Software version 1.0.1 (both BD Biosciences). A minimum of 5,000 events/sample were collected.

Dichlorofluorescein (DCFH) assay. Cells were seeded in 96-well clear bottom black plates and incubated with 20 μM DCFH diacetate (cat. no. D6883; Sigma-Aldrich; Merck KGaA) in phenol red-free MEM (cat. no. 12800-017; Sigma-Aldrich; Merck KGaA) in the dark before being treated with DMSO, montelukast at 20 μM, or zafirlukast at 20 μM for 3 h. The DCF fluorescence intensity was detected using a Varioskan™ Flash spectral scanning Multimode Reader (Thermo Fisher Scientific, Inc.) with 490/535 nm excitation/emission wavelengths. The DCF fluorescence intensity of each treatment was normalized to fluorescence intensity of untreated cells.

Statistical analysis. Data are presented as the mean ± SEM (n≥3). Data were analyzed by one- or two-way ANOVA followed by Tukey's post hoc test using GraphPad Prism version 5.0 (Dotmatics). The level of significance was defined as $P<0.05$.

Results

Zafirlukast induces p27 expression and decreases cyclin D1 expression. Although both montelukast and zafirlukast at 20 μM have been reported to inhibit MDA-MB-231 cell proliferation, only 20 μM zafirlukast can induce G_0/G_1 cell cycle arrest at 48 h post-exposure, according to a previous study (7). Therefore, the effects of both drugs on the expression of two proliferating markers, Ki-67 and proliferating cell nuclear antigen (PCNA), were assessed. Montelukast and zafirlukast at 20 μM decreased Ki-67 and PCNA at 6 h post-exposure (Fig. 1A and B). Cells were treated with 20 μM drugs for 48 h and the expression levels of proteins regulating cell cycle progression were determined using western blotting. Cyclin D1 and CDK4 levels were decreased in cells that were treated with zafirlukast (Fig. 1C and D), while both drugs had no effect on cyclin E levels (Fig. S1). Zafirlukast significantly decreased p21 expression, whereas the increase of p21 by montelukast was not significant (Fig. 1E). Increased p27 expression was found only in cells treated with zafirlukast (Fig. 1F).

LTRAs activate the intrinsic apoptosis pathway in MDA-MB-231 TNBC cells. Montelukast at ≥10 μM and zafirlukast at 20 μM were previously reported to induce the apoptosis of MDA-MB-231 cells at 24 h post-exposure (7); therefore, 20 μM was used to assess cell death in the present study. The expression levels of p-ERK1/2 and Bcl-2 were detected using western blotting. Montelukast and zafirlukast decreased the expression levels of p-ERK1/2 at 6 h (Fig. 2A and B) and the anti-apoptotic protein Bcl-2 at 12 h post-exposure (Fig. 2A and C). Both LTRAs did not alter levels of ERK1/2 (Fig. 2A and B). Next, cells were stained with JC-1 and 7-AAD to assess mitochondrial membrane potential and cell death, respectively. Both LTRAs increased the levels of cells with green fluorescent signal, indicative of monomeric JC-1, at 12 h post-exposure (Fig. 2D and E). The levels of 7-AAD-positive cells after being exposed to montelukast and zafirlukast were 48.98±7.22 and 31.61±2.04%, respectively, compared with 3.97±0.50% in DMSO-treated cells at 12 h (data not shown). Caspase 3/7 activity was also measured by flow cytometry. Both drugs increased caspase 3/7 activity at 12 h post-treatment, with a greater effect observed in montelukast-treated cells (Fig. 2F and G)

LTRAs trigger autophagy in MDA-MB-231 TNBC cells. To test whether autophagy was caused by LTRAs, LC3 levels were measured by western blotting and flow cytometry. Montelukast and zafirlukast did not alter LC3-I levels at 6 h post-exposure, while zafirlukast decreased LC3-I levels at 12 h post-exposure (Fig. 3A and B). Both drugs elevated LC3-II levels at 6 and 12 h post-treatment (Fig. 3A and C). The expression of lipidated LC3 sequestered into autophagosomes was determined by using flow cytometry. Both drugs caused a rightward shift of autophagic LC3 fluorescence intensity and increased the mean fluorescent intensity of autophagic LC3 at 12 h post-exposure (Fig. 3D and E).

LTRAs induce DNA damage and endoplasmic reticulum (ER) and oxidative stress in MDA-MB-231 TNBC cells. Apoptosis and autophagy are triggered in response to DNA damage (13). The levels of PARP-1, a key regulator of the DNA repair process, were measured by western blotting in the present study. Montelukast and zafirlukast increased levels of 89-kDa fragments of PARP-1 at 6 and 12 h after treatment (Fig. 4A and B). Cells with positive staining of p-ATM and p-H2AX, two DNA damage markers, were counted by flow cytometry at 12 h post-exposure. Montelukast and zafirlukast increased p-ATM and p-H2AX levels (Fig. 4C-E). Montelukast showed significantly higher p-ATM levels than zafirlukast.

Activation of ER stress can lead to either autophagy or apoptotic cell death (14,15). In the present study, the expression of ER membrane-associated sensors and signaling molecules was evaluated using RT-qPCR at 6 h post-treatment. Montelukast, but not zafirlukast, upregulated the expression levels of PERK and ATF4 (Fig. 5A and B). Both montelukast and zafirlukast increased the mRNA expression levels of X-box binding protein 1 splice variant (XBP-1s), CHOP and growth arrest and DNA damage-inducible gene 34 (GADD34), but had no effect on ATF6 mRNA levels (Fig. 5C-F). The protein expression levels of CHOP in montelukast-treated cells were significantly higher than those in zafirlukast-treated cells (Fig. 5G and H). The activation of ER stress triggers cleavage of ATF6 (16). Montelukast and zafirlukast decreased the expression levels of total ATF6 protein (Fig. 5G), and the cleaved ATF6/ATF6 level in zafirlukast-treated cells was greater than that in montelukast-treated cells (Fig. 5I). The cleaved ATF6/ATF6 levels at 12 h post-zafirlukast treatment were higher than those at 6 h.

To determine the contribution of ER stress to LTRA-induced apoptotic cell death, GSK2606414, a PERK inhibitor, and siRNA targeting CHOP) were used. The percentage of cells with Annexin V staining was measured at 12 h post-exposure to LTRAs. Montelukast and zafirlukast increased the percentage of Annexin V-positive cells; however, pre-treatment with GSK2606414 only decreased the percentage of montelukast-induced Annexin V-positive cells (Fig. 6). CHOP siRNA decreased the CHOP mRNA levels to 14% compared to non-targeting control siRNA (Fig. 7A). The mRNA expression levels of CHOP triggered by montelukast were decreased to 23% at 48 h post-transfection with CHOP siRNA compared with cells with non-targeting control siRNA (Fig. 7A). By contrast, the downregulation of Ki-67 and PCNA by montelukast was not reversed by CHOP siRNA (Fig. 7B and C). Transfection with CHOP siRNA resulted in lower levels of Annexin V-positive cells than in the group transfected with the non-targeting control siRNA following exposure to montelukast or zafirlukast (Fig. 7D and E).

To examine whether LTRAs induced oxidative stress, intracellular reactive oxygen species and the expression of antioxidant enzymes were measured using DCF assays and RT-qPCR, respectively. Montelukast and zafirlukast at 20 μM did not increase DCF fluorescent intensity compared with DMSO at 1 and 3 h post-treatment (Fig. 8A). Both drugs decreased the expression of catalase, glutathione reductase and glutathione S-transferase at 6 h post-exposure to montelukast and zafirlukast; however, neither drug affected the expression of superoxide dismutase and glutathione peroxidase (Fig. 8B-F).

Discussion

Our previous findings showed that LTRAs, including montelukast and zafirlukast, have the potential for BC treatment

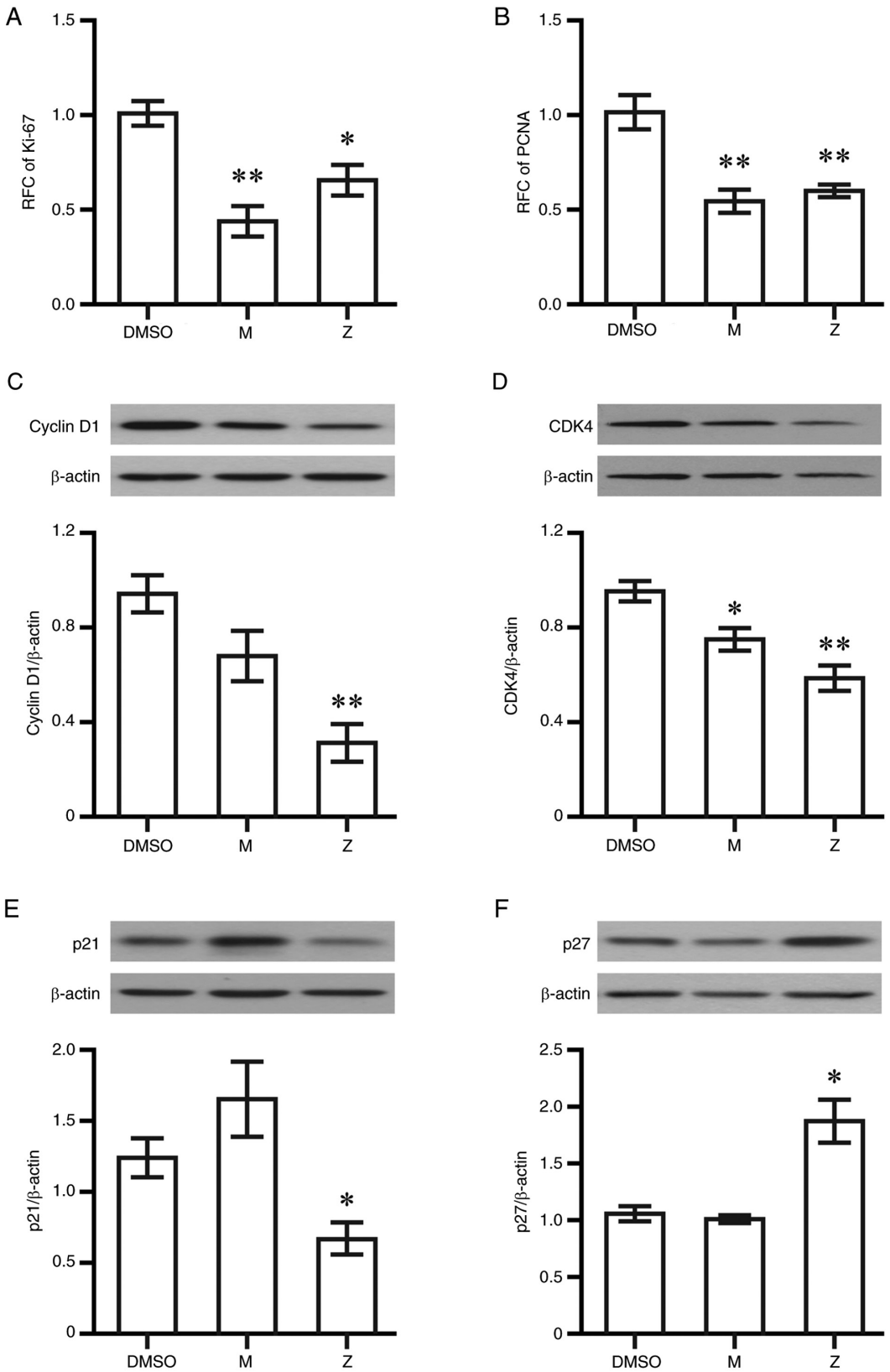

Figure 1. Effects of M and Z on proliferation markers and cell cycle regulatory proteins. mRNA expression of (A) Ki-67 and (B) PCNA were decreased in cells treated with M and Z. Levels of cell cycle regulatory proteins for G_1 phase (C) cyclin D1 and (D) CDK4 were reduced in Z-treated cells. Z (E) decreased the expression of p21 and (F) increased the expression of p27. Data are expressed as the mean ± SEM of four independent experiments. $^{*}P<0.05$ and $^{**}P<0.01$ vs. DMSO. M, montelukast; Z, zafirlukast; PCNA, proliferating cell nuclear antigen; RFC, relative fold change.

by inhibiting cancer progression and inducing apoptosis in the absence of hormonal stimuli (7). The apoptotic effects of montelukast are more potent than zafirlukast in TNBC MDA-MB-231 cells. Zafirlukast induces cell cycle arrest at G_0/G_1 phase, while montelukast treatment does not induce cell cycle arrest at a specific phase and preferentially induced cell

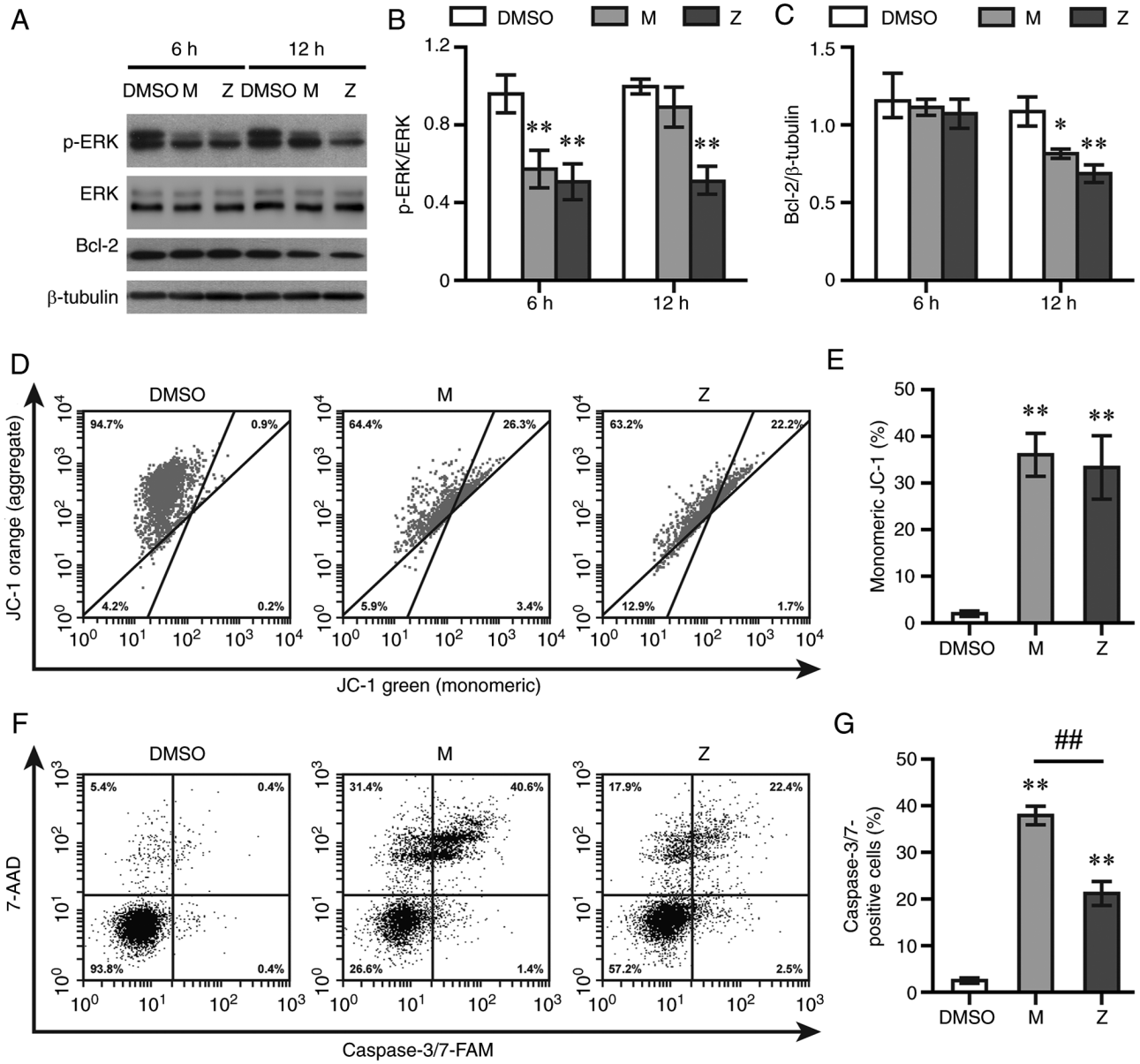

Figure 2. Activation of intrinsic apoptotic pathways by LTRAs. (A) Cells treated with M and Z showed decreased expression of (B) p-ERK1/2 and (C) Bcl-2 compared with DMSO-treated cells. (D) Representative dot plots of JC-1 staining showed the shift of JC-1 orange to green in LTRA-treated cells. (E) Both LTRAs increased the levels of monomeric JC-1. (F) Caspase 3/7 levels in M-treated cells were higher than in Z-treated cells. (G) Caspase 3/7-positive cells. Data are expressed as the mean ± SEM of three or four independent experiments. *P<0.05 and **P<0.01 vs. DMSO. $^{\#\#}$P<0.01 vs. zafirlukast. LTRA, leukotriene receptor antagonist; M, montelukast; Z, zafirlukast; p-, phosphorylated; 7-AAD, 7-aminoactinomycin D.

death (7). The present study demonstrated that montelukast and zafirlukast differentially mediated CysLT1R signaling in MDA-MB-231 cells. Montelukast and zafirlukast activates two ER stress sensors: ATF6 and IRE1; but only montelukast triggers PERK. The accumulation of zafirlukast-treated cells in G_0/G_1 phase as previously report (7) is mediated by the upregulation of p27, and the decrease of CDK4 and cyclin D1. Montelukast exhibited a greater effect than zafirlukast on the upregulation of caspase 3/7, DNA damage and CHOP. Montelukast activated all three pathways of ER stress: PERK, ATF6, and IRE1; while zafirlukast only triggered the cleavage of ATF6 and XBP-1. Both LTRAs also triggered autophagic response and decreased the expression of antioxidant enzymes.

A key feature of cancer is the loss of cell cycle regulation. Cell cycle arrest is the primary mechanism controlling the proliferation of cancer cells and cell death (17). Ki-67 is a prognostic biomarker of BC and was used in the recent American Joint Committee on Cancer Staging System for BC (18,19). In the present study, both LTRAs significantly decreased the expression of two cell proliferating markers, Ki-67 and PCNA, in MDA-MB-231 cells. The present results are consistent with a previous study that showed inhibition of proliferation of non-tumor and tumor-derived intestinal epithelial cells by LTRAs (20).

The upregulation of cyclin D1 promotes progression of cells in the G_1 phase while cyclin E is a key regulator for cells to enter S phase. Cyclin D1 forms complexes with CDK4 or CDK6 to trigger phosphorylation of retinoblastoma protein (Rb), leading to the release of Rb from transcription factor E2F. E2F then induces the synthesis of cyclin E and cyclin

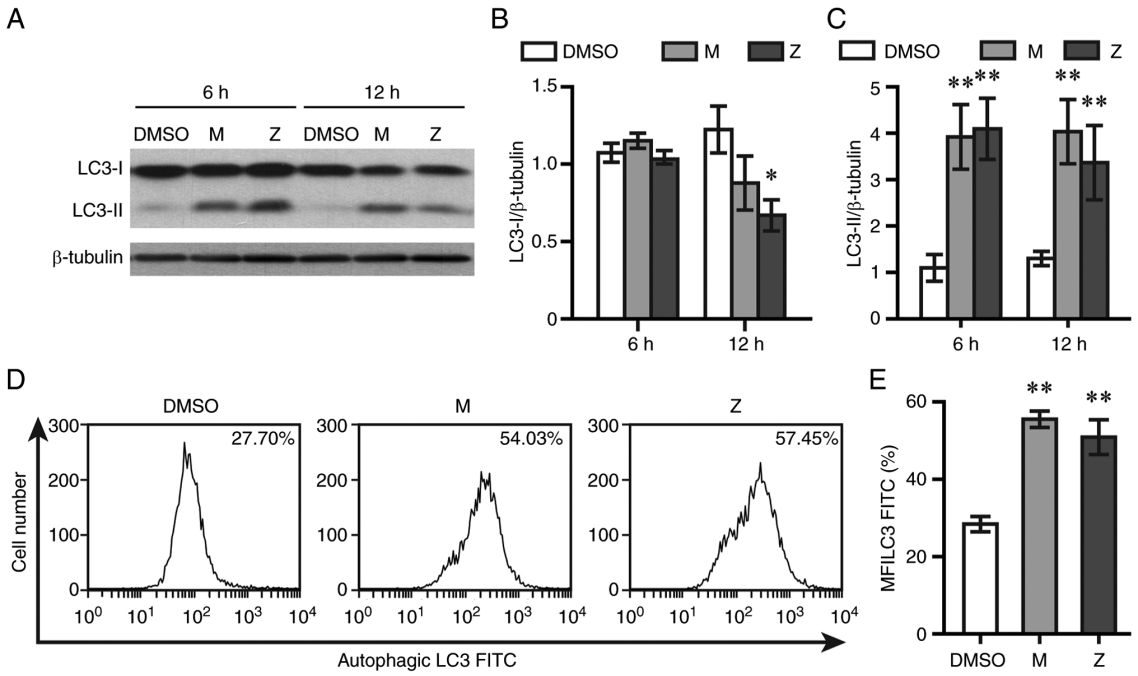

Figure 3. LTRAs induce autophagy. (A) Cells treated with M and Z showed increased LC3-II levels. Expression of (B) LC3-I and (C) LC3-II. (D) Representative LC3-FITC fluorescence intensity showed a rightward shift in LTRA-treated cells. (E) MFI of LTRA-treated cells was higher than that in DMSO-treated cells. Data are expressed as the mean ± SEM of three or four independent experiments. *P<0.05 and **P<0.01 vs. DMSO. LTRA, leukotriene receptor antagonist; M, montelukast; Z, zafirlukast; MFI, mean fluorescence intensity.

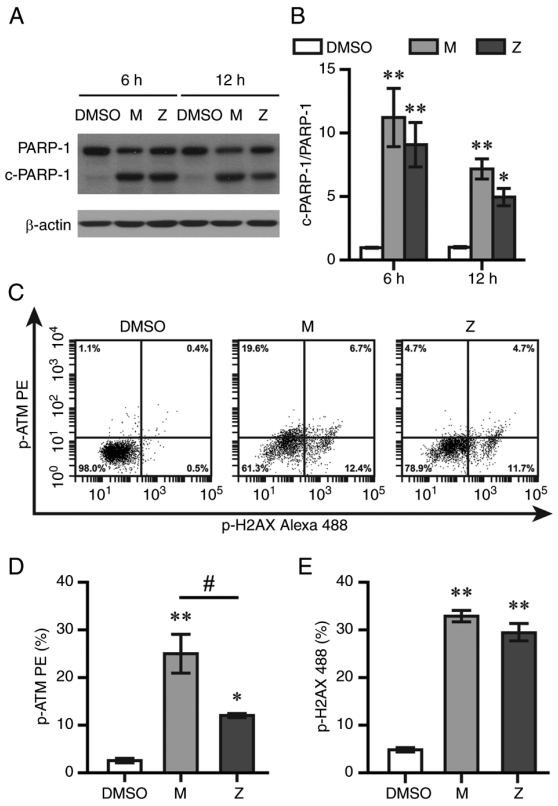

Figure 4. LTRAs upregulate DNA damage markers. (A) Cells treated with M and Z showed decreased PARP-1 and increased c-PARP-1. (B) Expression of c-PARP-1 normalized to PARP-1. (C) Representative dot plots showed the increased number of cells expressing (D) p-ATM and (E) p-H2AX following exposure to LTRAs. Data are expressed as the mean ± SEM of three or four independent experiments. *P<0.05 and **P<0.01 vs. DMSO. $^{\#}$P<0.05 vs. zafirlukast. LTRA, leukotriene receptor antagonist; M, montelukast; Z, zafirlukast; c-, cleaved; PR, phycoerythrin; p-, phosphorylated; H2AX, histone H2AX.

A, which regulate the S phase (21). In the present study, zafirlukast decreased the expression of cyclin D1 and CDK4 but showed no effect on cyclin E expression. This suggested that the potential G_0/G_1 cell cycle arrest was mediated by the reduction of cyclin D1/CDK4 complexes. In addition to cyclin D1, length of the G_1 phase is regulated by the CDK inhibitor p27. The inhibitory effect of p27 is key for entry into S phase because p27 blocks the activity of cyclin E/CDK2, an initiator of DNA synthesis. The inhibition of p27 expression decreases the length of the G_1 phase, and cyclin D1 inhibits the activity of p27 (22). Therefore, the G_0/G_1 cell cycle arrest induced by zafirlukast in MDA-MB-231 cells in our previous study (7) may be mediated by the combination of cyclin D1 downregulation and p27 upregulation. On the other hand, decreased CDK4 expression without altered expression of cyclin D1, cyclin E and p27 could explain the decrease of cell proliferation without cell cycle arrest at specific phases induced by montelukast. Our previous study showed that zafirlukast can increase the levels of p53, p21 and p27 in U-87 MG glioblastoma cells (10). In the present study, zafirlukast decreased p21 expression. U-87 MG expresses wild-type p53, while p53 in MDA-MB-231 is mutated (23). Although the most well-known function of p21 is as a cell cycle inhibitor protein, there are reports of p21 having oncogenic effects (24,25).

To date, three CDK 4/6 inhibitor agents (palbociclib, ribociclib and abemaciclib) have been approved by the Food and Drug Administration of United States and are available for patients with BC. The major side effect of these CDK 4/6 inhibitors is bone marrow suppression, resulting in anemia, neutropenia and thrombocytopenia (26). LTRAs are generally considered safe and well-tolerated drugs because of their mild side effects (1,27). With the growing evidence of anticancer effects and the inhibition of CDK4/6, LTRAs could

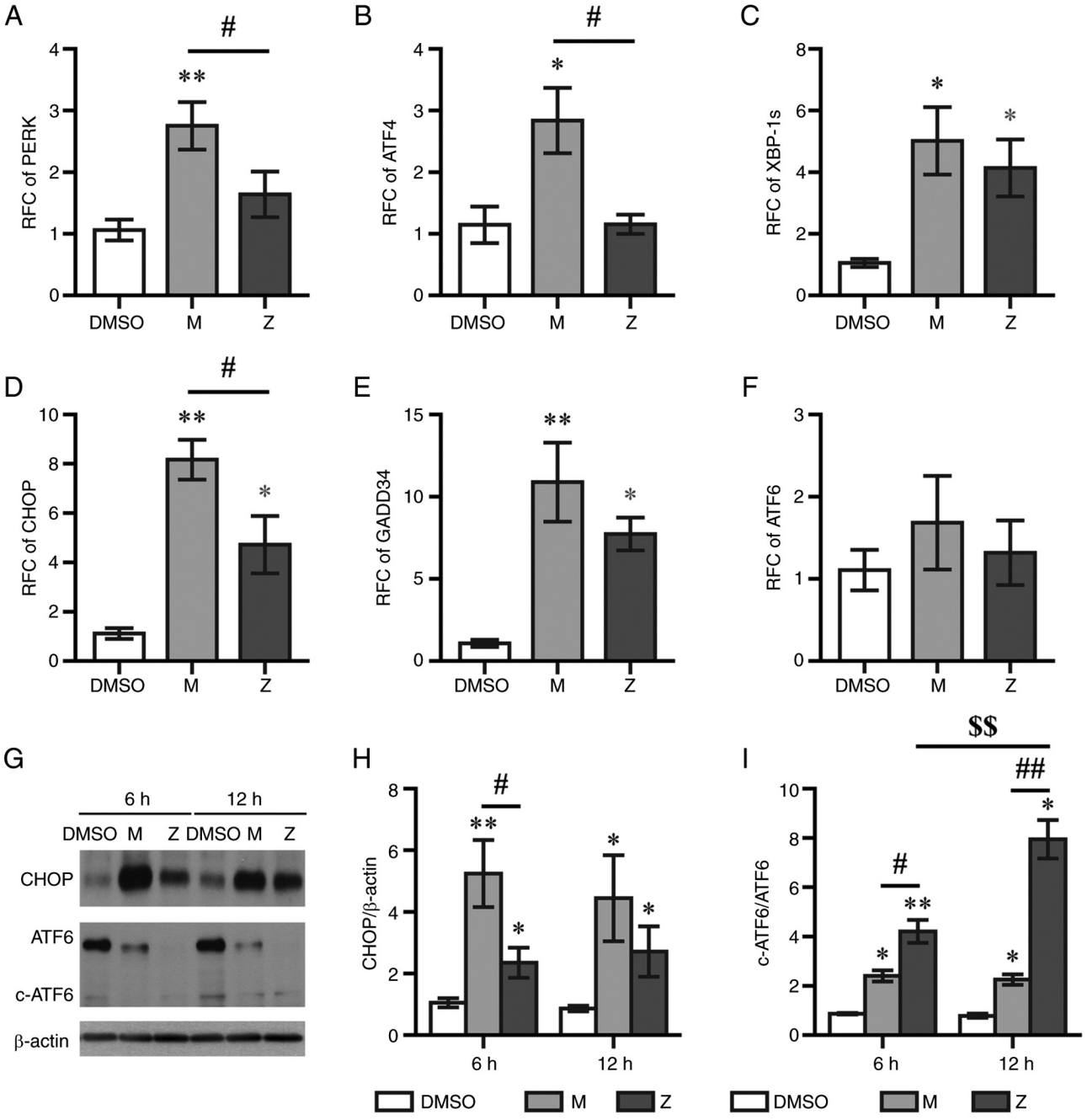

Figure 5. LTRAs upregulate endoplasmic reticulum stress markers. Cells treated with M exhibited increased mRNA expression levels of (A) PERK, (B) ATF4, (C) XBP-1s, (D) CHOP and (E) GADD34. Z triggered only the expression of XBP-1s, CHOP and GADD34. (F) Both LTRAs did not alter mRNA expression of ATF6. (G) Western blotting showed the increase of CHOP and the decrease of ATF6 expression. (H) M significantly increased the levels of CHOP protein compared with Z. (I) Z significantly increased the levels of c-ATF6/ATF6 compared with M. Data are expressed as the mean ± SEM of 3-5 independent experiments. *P<0.05 and **P<0.01 vs. DMSO. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. zafirlukast. $^{\$\$}$P<0.01 vs. zafirlukast at 12 h. LTRA, leukotriene receptor antagonist; M, montelukast; Z, zafirlukast; ATF, activating transcription factor; XBP-1s, X-box binding protein 1 splice variant; GADD34, growth arrest and DNA damage-inducible gene 34; c-, cleaved; RFC, relative fold change.

be repurposed as a chemopreventive agent or adjuvant in BC therapy. In the present study, zafirlukast reduced the expression of both cyclin D1 and CDK4; therefore, this drug may be a better candidate than montelukast as a cyclin-CDK inhibitor. However, studies have reported neuropsychiatric effects, such as depression, hallucination and suicidal ideation, related to the use of montelukast and zafirlukast (28,29).

The regulation of Bcl-2 by decreasing ERK1/2 activation following LTRA treatment has been reported in glioblastoma (10), lung cancer (9), leukemia (30) and colorectal cancer (31). In the present study, decreased expression of p-ERK1/2 in the cells treated with LTRAs was observed before a time-dependent reduction of Bcl-2 expression. Therefore, LTRA-induced Bcl-2 downregulation in MDA-MB-231 cells is potentially mediated by the inhibition of ERK1/2 phosphorylation. The activation of the ERK1/2 pathway triggers the degradation of p27 (22). Therefore, the upregulation of p27 observed in the present study could be mediated by inhibition of ERK1/2 phosphorylation. Bcl-2 expression maintains the integrity of the mitochondrial outer membrane and prevents the

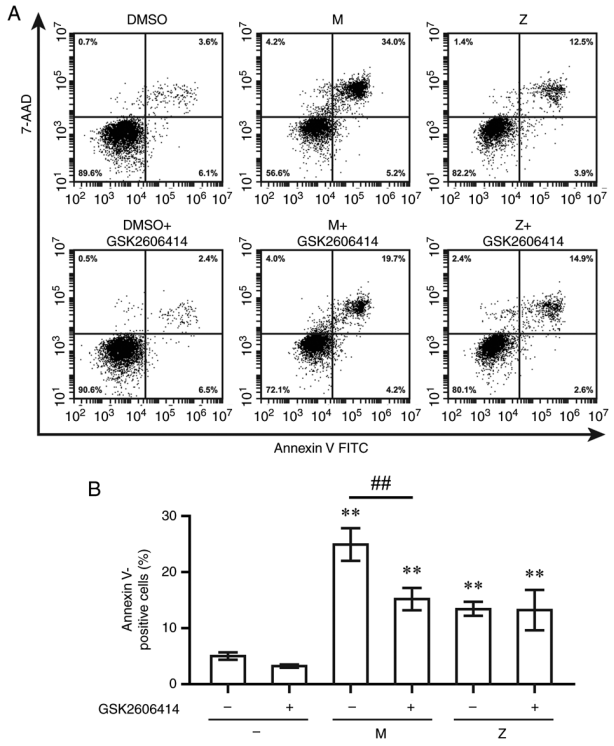

Figure 6. Inhibition of the PERK-dependent pathway decreases M-induced apoptosis. (A) Representative flow cytometric dot plots showed higher percentage of Annexin V-positive cells in M and Z groups. (B) Pretreatment with GSK2606414 decreased the percentage of Annexin V-positive cells induced by M. Data are expressed as the mean ± SEM of four independent experiments. **P<0.01 vs. DMSO. $^{\#\#}$P<0.01 vs. zafirlukast. M, montelukast; Z, zafirlukast; 7-AAD, 7-aminoactinomycin D.

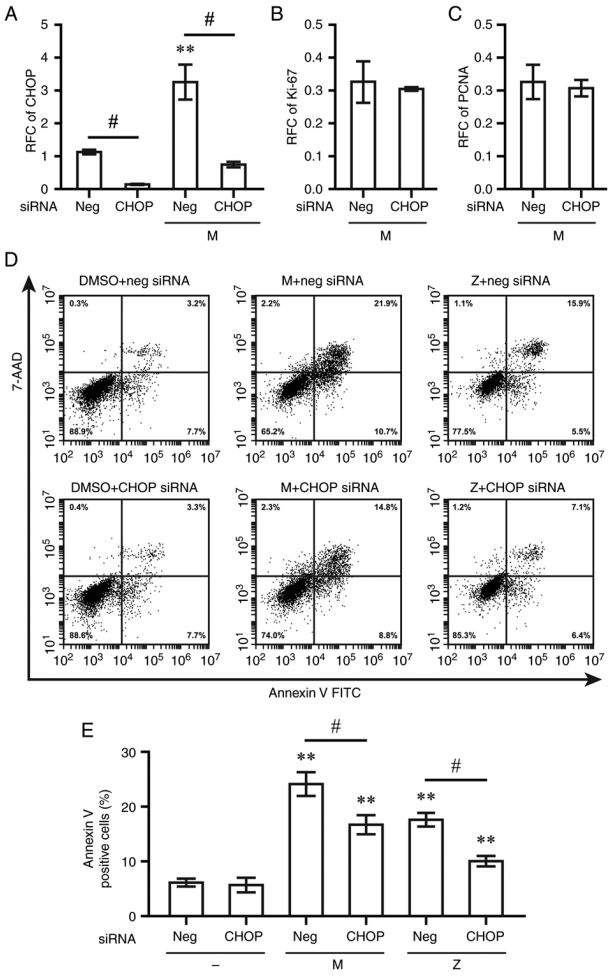

Figure 7. Knockdown of CHOP decreases leukotriene receptor antagonist-induced apoptosis. Cells were transfected with neg or CHOP siRNA to knockdown CHOP. (A) RFC of CHOP mRNA expression was reduced in cells transfected with CHOP siRNA. RFC of (B) Ki-67 and (C) PCNA mRNA expression reduced by M was similar between cells transfected with CHOP and neg siRNA. (D) Representative flow cytometric dot plots showed a higher percentage of Annexin V-positive cells in M and Z groups. (E) CHOP siRNA group showed a lower percentage of Annexin V-positive cells following M and Z treatment. Data are expressed as mean ± SEM of four independent experiments. *P<0.05 and **P<0.01 vs. DMSO. $^{\#}$P<0.05 vs. CHOP siRNA. M, montelukast; Z, zafirlukast; CHOP, C/EBP homologous protein; siRNA, small interfering RNA; RFC, relative fold change; PCNA, proliferating cell nuclear antigen; neg, negative; 7-AAD, 7-aminoactinomycin D.

release of cytochrome *c* and caspase activation (32). In healthy cells, JC-1 dye enters and forms aggregates with orange fluorescent signals. The decrease of JC-1 aggregation by LTRAs indicated a loss of mitochondrial membrane potential. Both LTRAs also increased levels of caspase 3/7-positive cells, indicating that LTRAs enhanced cell death via the intrinsic mitochondrial apoptotic pathway in MDA-MB-231 cells. The increase of caspase-3 activation by montelukast is consistent with previous studies in colon cancer (33) and leukemia (30).

Caspase-mediated apoptotic cell death is accomplished via cleavage of several key proteins, including PARP-1. Cleavage of PARP-1 by caspases, a hallmark of apoptosis, decreases DNA-binding capacity, which disrupts the routine repair of DNA damage. Bcl-2 is an upstream regulator of PARP-1 activation (34). Decreased Bcl-2 levels and the upregulation of caspase 3/7 may lead to the increase of cleaved-PARP-1 by LTRAs in MDA-MB-231 cells. This is consistent with previous findings in chronic myeloid leukemia, which revealed that montelukast triggers the mitochondrial apoptosis pathway by increasing the activities of cytochrome *c*, caspase-3 and cleavage of PARP-1 (20,30). The inhibition of PARP-1 can increase ATM activation and promote H2AX phosphorylation (13). The elevation of cleaved-PARP-1, p-ATM and p-H2AX by LTRAs in the present study indicated DNA damage in MDA-MB-231 cells.

Autophagy serves a protective role in cancer cells during chemotherapy, allowing cancer cells to alleviate cellular stress, causing drug resistance. However, a number of cytotoxic drugs promote cancer cell death via autophagy (35). The present study showed the increased cleavage of LC3 (LC3-II), a marker of autophagy, and decreased LC3-I in MDA-MB cells following LTRA treatment, suggesting that the inhibition of leukotriene signaling could induce autophagy. This observation aligns with the increase in LC3-II induced by LTRAs in retinal pigment epithelial cells and a rat model of hemorrhagic cystitis (36,37).

ER stress serves a dual role in cell survival and death via apoptosis or autophagy in BC cells (14,15). In the present study, the induction of key effectors involved in ER stress and/or unfolded protein response (UPR) signaling by both LTRAs was evidenced by an increase in ER stress markers, including CHOP and XBP-1s, as well as the reduction of ATF6 due to proteolytic cleavage. CHOP is a key link between the UPR and pro-apoptotic activation (38). The PERK/eukaryotic translation

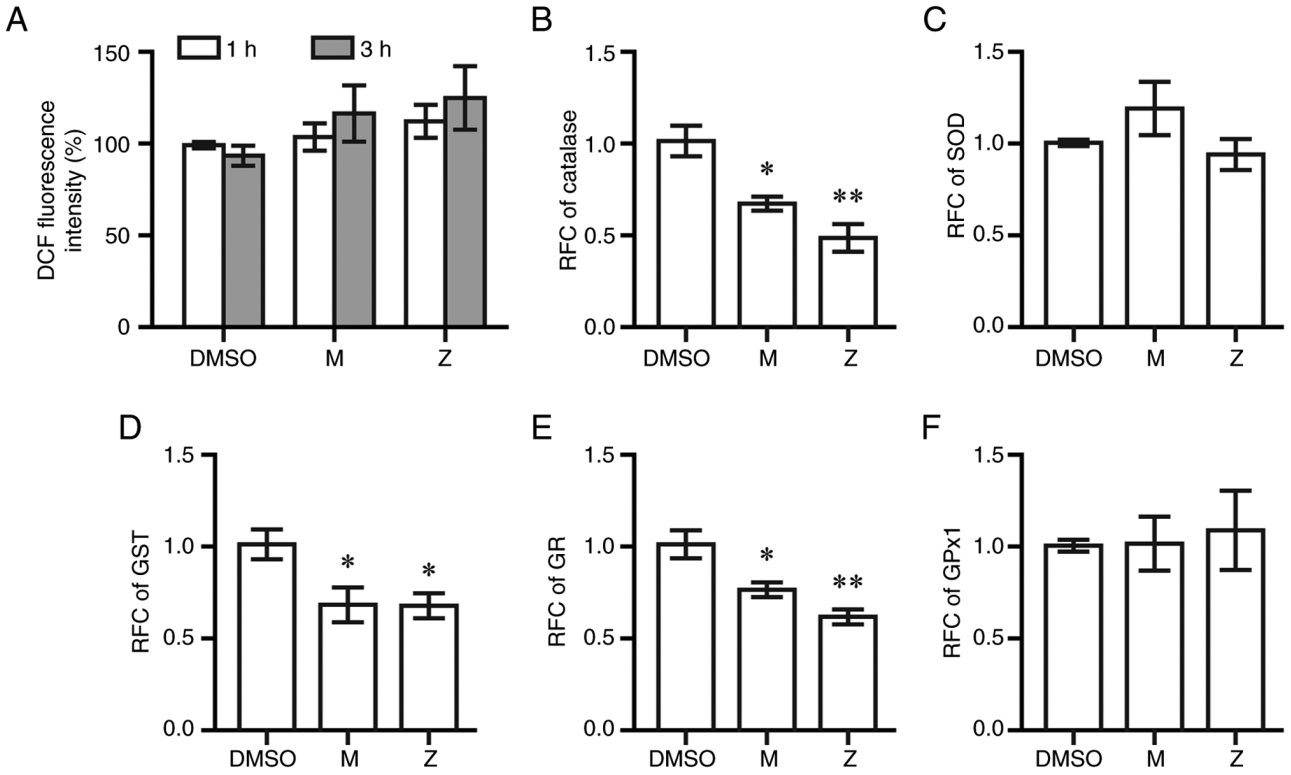

Figure 8. LTRAs downregulate antioxidant enzymes. (A) M and Z did not increase the fluorescence intensity of DCF. (B) Both LTRAs reduced the mRNA expression of catalase. (C) Neither drug altered expression of SOD. Both drugs decreased expression of (D) GST and (E) GR. (F) Neither drug altered expression of GPx. Data are expressed as the mean ± SEM of four independent experiments. *P<0.05 and **P<0.01 vs. DMSO. LTRA, leukotriene receptor antagonist; M, montelukast; Z, zafirlukast; SOD, superoxide dismutase; GR, glutathione reductase; GST, glutathione S-transferase; GPx, glutathione peroxidase; RFC, relative fold change.

initiation factor 2α/ATF4 pathway plays an important role in the upregulation of CHOP in cancer cells (39). Activation of IRE1 induces the splicing of XBP-1 mRNA. XBP-1s and cleaved-ATF6 also trigger CHOP expression (38). In the present study, the higher levels of CHOP induced by montelukast compared with zafirlukast are likely caused by the activation of all three ER stress sensors: PERK, ATF6, and IRE1.In contrast, zafirlukast did not upregulate the PERK/ATF4 pathway. GADD34 is one of the downstream transcriptional targets of CHOP. This protein acts as a part of negative feedback loop for the response essential for cell survival by prolonged CHOP expression (40). In the present study, both LTRAs triggered the expression of CHOP and GADD34.

CHOP is a key protein responsible for ER stress-induced cell death by downregulating Bcl-2 (41). CHOP activates caspase-3 and PARP-1 (42), and induces the expression of autophagy-related genes (38). In BC, the increased expression of CHOP indicates ER stress-induced autophagic cell death (14,15). In the present study, the downregulation of CHOP by siRNA decreased LTRA-induced apoptosis. Montelukast upregulated PERK and the inhibition of PERK using its specific inhibitor reduced montelukast-induced apoptosis. These results suggested that the upregulation of ER stress contributed to LTRA-induced cell death. The expression of CHOP has been shown to be associated with a lower risk of recurrence and prolonged disease-free survival in patients with BC (43). Therefore, the increase of CHOP expression by LTRAs could be an important mechanism for BC treatment. Upregulation of CHOP could lead to cell cycle arrest. Although the downregulation of CHOP via siRNA did not alter the decrease the expression levels of the proliferating markers Ki-67 and PCNA in MDA-MB-231 cells, further studies on CHOP knockdown and cell proliferation are of interest in other types of cancers to investigate the role of ER stress in the inhibition of cell proliferation.

Excessive oxidative stress leads to cell death. Antioxidants and olaparib, a PARP-1 inhibitor, have been shown to decrease zafirlukast-induced cell death in renal cell carcinoma (44). In the present study, montelukast and zafirlukast decreased the expression of antioxidant enzymes including catalase, glutathione S-transferase and glutathione reductase. Montelukast exhibits protective effects against oxidative and ER stress in non-cancerous models (45-48). Montelukast can ameliorate acetaminophen-induced liver injury and methotrexate-induced kidney damage by decreasing oxidative stress (45,46). Montelukast has also been shown to decrease the expression of ER stress markers, including CHOP, GADD34, ATF4 and IRE1a, in models of hepatotoxicity and diabetes (47,48). These differential effects require further studies in other cancerous cells.

The toxic concentrations of LTRAs required for anticancer effects are greater than plasma concentrations used in asthma treatment. Pharmacokinetic studies of a single dose of 10 mg montelukast and 20 mg zafirlukast detected 0.63 μM of montelukast and 0.58 μM of zafirlukast in human plasma (49,50). Novel localized drug delivery using polymeric wafers, nanofibrous scaffolds and hydrogels could increase drug concentrations at cancer sites (51). It is also possible to

use nanocarriers to deliver LTRAs via intraductal delivery (52). Graphine oxide nanoparticles of montelukast have been shown to increase apoptosis of inflammatory cells in a mouse asthma model (53). The volume of distribution of montelukast and zafirlukast is ~10 and 70 l, respectively (49,54). The greater the volume of distribution, the greater the tissue accumulation. Zafirlukast, with a higher volume of distribution and effects on both cell cycle arrest and apoptosis, could be a better candidate than montelukast. The anti-inflammatory effects of LTRAs for capsular contracture after breast augmentation have suggested a potential role for these drugs in BC treatment (55,56). Zafirlukast has been shown to prevent the metastasis of MDA-MB-231 cells to the bone and lung by blocking CysLT1R on platelets, subsequently inhibiting platelet aggregation and adhesion on cancer cells (57). In addition, Montelukast may suppress the epithelial-mesenchymal transition in MDA-MB-231 cells (58). The knockdown of CysLT1R using siRNA or CRISPR may confirm the role of this receptor in cell death pathways.

In conclusion, LTRAs effectively inhibited MDA-MB-231 cells via multiple downstream signaling pathways, including induction of cell cycle arrest, apoptosis, autophagy, DNA damage and ER stress. The use of one cell line is a limitation of this study. The different effects on cell death and proliferation induced by montelukast and zafirlukast should be further investigated in other BC cells and animal models.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

PV, NS and PW conceived the study. PV, TS and SP analyzed data and wrote the manuscript. PV, KK and TS collected data. PV and TS confirm the authenticity of all the raw data. PV and TS edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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