RESEARCH ARTICLE

The FASEB Journal

Targeting thiol isomerase activity with zafirlukast to treat ovarian cancer from the bench to clinic

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Funding information
HHS | National Institutes of Health (NIH), Grant/Award Number: DEO29661; HHS | NIH | National Cancer Institute (NCI), Grant/Award Number: R21CA231000

Abstract

Thiol isomerases, including PDI, ERp57, ERp5, and ERp72, play important and distinct roles in cancer progression, cancer cell signaling, and metastasis. We recently discovered that zafirlukast, an FDA-approved medication for asthma, is a pan-thiol isomerase inhibitor. Zafirlukast inhibited the growth of multiple cancer cell lines with an IC_{50} in the low micromolar range, while also inhibiting cellular thiol isomerase activity, EGFR activation, and downstream phosphorylation of Gab1. Zafirlukast also blocked the procoagulant activity of OVCAR8 cells by inhibiting tissue factor-dependent Factor Xa generation. In an ovarian cancer xenograft model, statistically significant differences in tumor size between control vs treated groups were observed by Day 18. Zafirlukast also significantly reduced the number and size of metastatic tumors found within the lungs of the mock-treated controls. When added to a chemotherapeutic regimen, zafirlukast significantly reduced growth, by 38% compared with the mice receiving only the chemotherapeutic treatment, and by 83% over untreated controls. Finally, we conducted a pilot clinical trial in women with tumor marker-only (CA-125) relapsed ovarian

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cancer, where the rate of rise of CA-125 was significantly reduced following treatment with zafirlukast, while no severe adverse events were reported. Thiol isomerase inhibition with zafirlukast represents a novel, well-tolerated therapeutic in the treatment of ovarian cancer.

1 | INTRODUCTION

Thiol isomerases, including protein disulfide isomerase (PDI) and the endoplasmic reticulum resident proteins 5, 57, and 72 (ERp5, ERp57, and ERp72), are increasingly recognized for critical activities in regulating protein function external to the endoplasmic reticulum. Thiol isomerases play key roles in maintaining cellular homeostasis by catalyzing disulfide bond breakage, formation, and rearrangement.[1] Thiol isomerases are implicated in neurodegenerative diseases,[2,3] allergic responses,[4] thrombosis, and hemostasis,[5–7] as well as cancer.[8,9] These thiol isomerases are upregulated in many distinct cancer types including ovarian, prostate, lung, and colon.[1] Considering the ubiquitous roles of thiol isomerases in regulating cellular function, they have been linked to an array of oncologic events including oncogene activation, oncogenic transformation, apoptotic escape, mutation repair, cancer-induced thrombosis, and resistance to chemotherapeutic agents.[1,3,10–12] Importantly, these extracellular thiol isomerases are not redundant, each carrying out distinct roles in these processes, and seemingly they vary by cancer type.[1]

Selective inhibitors of PDI, such as PACMA-31 and CCF642, have been shown to inhibit cancer cell growth in vitro and prolong the life span of mice xenografted with multiple myeloma or ovarian cancer cell lines.[8,13] Additional selective inhibitors of PDI have also been identified and utilized in other disease states, such as thrombosis.[7,14] PDI, ERp5, ERp57, and ERp72 are all overexpressed in ovarian cancer and associated with shortened survival,[15] making a pan-thiol isomerase inhibitor a potentially attractive antineoplastic agent.

We previously performed a high-throughput screen of FDA-approved or known bioactive compounds for broad-spectrum inhibitors of these four thiol isomerases.[16] The asthma medications zafirlukast, and to a lesser extent, montelukast, which are leukotriene receptor 1 (LTR1) antagonists, were identified as broad-spectrum thiol isomerase inhibitors that inhibited platelet aggregation and thrombus formation in a thiol isomerase-dependent manner.[16] Whether zafirlukast has antitumor activity in ovarian cancer is not known, but this would be anticipated, considering that PACMA-31, another thiol isomerase inhibitor, does.[8] This is also supported by the finding that zafirlukast has demonstrated anticancer effects in hepatocellular carcinoma and glioblastoma, among other tumor types.[17,18] In this study, we assessed the antineoplastic activity of zafirlukast against ovarian cancer cells in culture, a xenograft model of ovarian cancer, and a pilot clinical study in women with relapsed ovarian cancer, marking the first instance when thiol isomerase inhibitors have been examined in patients for antineoplastic effects.

2 | MATERIALS AND METHODS

2.1 | Reagents

Recombinant PDI, ERp57, ERp72, and ERp5 were purchased from Abcam (Cambridge, MA). Zafirlukast was purchased from TCI America (Portland, OR), cisplatin, gemcitabine, and DI-E-GSSG were purchased from Cayman Chemicals (Ann Arbor, MI). Factor VII (FVII), Factor X (FX), and the fluorescent Factor Xa cleaving substrate were purchased from Prolytix (Essex Junction, VT). RL90 PDI inhibitory antibody was purchased from Thermo Fisher (Waltham, MA), while anti-PDI, anti-ERp57, anti-pEGFR-Y1068, anti-pGAB1-Tyr627, anti-beta-actin, and anti-mouse and anti-rabbit secondary antibodies, as well as EGF were purchased from Cell Signaling (Danvers, MA). Recombinant insulin (bovine), bacitracin, DTT, buffers, and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO); while 96-well and 384-well clear bottom plates were purchased from Corning (Corning, NY).

2.2 | Chemical synthesis

The zafirlukast analog was synthesized as described previously.[19]

2.3 | Cell culture

Human ovarian adenocarcinoma (OVCAR8) (NCI-DTP Cat# OVCAR-8, RRID:CVCL_1629), human prostate adenocarcinoma (PC3) (ATCC Cat# CRL-1435,

RRID:CVCL_0035), and human lung carcinoma cells (A549) (ATCC Cat# CCL-185, RRID:CVCL_0023) were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS), 1% w/v sodium pyruvate, 10 mM HEPES, 2 mM glutamine, and 100 I.U./mL penicillin/streptomycin. Human colorectal carcinoma cells (HCT116) (NCI-DTP Cat# HCT-116, RRID:CVCL_0291) were grown in McCoys medium supplemented with 2 mM glutamine, 10% FBS, and 100 I.U./mL penicillin/streptomycin. Human embryonic kidney cells (HEK293) (ATCC Cat# CRL-1573, RRID:CVCL_0045) were grown in DMEM high-glucose medium supplemented with 4 mM glutamine, 10% FBS, and 100 I.U./mL penicillin/streptomycin. For EGF stimulation, OVCAR8 cells were serum starved, treated with zafirlukast for 1 or 4 h then treated with EGF at a final concentration of 100 ng/mL for 10 min.

2.4 | Insulin-based turbidimetric assay

An insulin-based turbidimetric assay was utilized to determine the selectivity of zafirlukast and montelukast for thiol isomerases PDI, ERp57, ERp72, and ERp5. These drugs were diluted in a 6-point dose curve in a 384-well plate, and a final concentration of 10 μg/mL thiol isomerase (30 μg/mL for ERp5 only), 125 μM insulin, 2 mM EDTA, and 100 mM potassium phosphate buffer were added for a total volume of 30 μL per well. The turbidity of insulin aggregation was measured every minute for 75 min after initiating the reaction with 0.3 mM DTT using a SpectraMax M3 plate reader (Molecular Devices, Sunnyvale, CA).

2.5 | PrestoBlue assay

The indicated cell lines were plated at 5000 cells per well in a 96-well plate and allowed to grow for 24 h. The cells were then treated with either a drug or a vehicle control for an additional 2–24 h prior to the addition of PrestoBlue reagent (Invitrogen, Waltham, MA) for 10–20 min at 37°C. Cell viability was determined by measuring the fluorescent signal at an excitation wavelength of 560 nm and emission wavelength of 590 nm. The signal was normalized to a percentage of the control.

2.6 | Di-eosin-GSSG disulfide reductase assay

OVCAR8 cells were plated at 10000 cells per well, allowed to grow overnight, then treated with 0–100 μM of zafirlukast, montelukast, or the analog for 10 min. Samples were then subjected to 150 nM of the di-eosin-GSSG probe in the presence of 5 μM of DTT and potassium phosphate buffer (containing 100 mM potassium phosphate (pH 7.4) and 2 mM EDTA). Increase in fluorescence was monitored every 30 s for 30 min by excitation at 520 nm and emission at 550 nm. Generated data were then normalized to the control for each sample, with raw data representing relative fluorescent units (RFU)/minute.

With blood samples, the assay was performed as previously described,[20] with a modification to use plasma at a 1:1 dilution with potassium phosphate buffer (containing 100 mM potassium phosphate (pH 7.4) and 2 mM EDTA). Samples were then subjected to 150 nM of the di-eosin-GSSG probe in the presence of 5 μM DTT. Increase in fluorescence was monitored for 30 min by excitation at 520 nm and emission at 550 nm. Generated data from Day 28 were then normalized to Day 0 control for each sample, with raw data representing relative fluorescent units (RFU)/minute ($n = 3$ for each patient sample).

2.7 | Western blotting

Cells were seeded in a six-well plate, grown to confluence and incubated with zafirlukast for a determined amount of time depending on the experiment. Total cellular extracts were prepared in M-PER extraction buffer (Thermo Scientific, Waltham, MA) and total protein calculated using Pierce Coomassie Plus (Bradford) Assay Reagent (Thermo Scientific, Waltham, MA). Proteins were separated via SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked with 5% nonfat milk in Tris-Buffered Saline (TBS) and probed with the appropriate primary and HRP-coupled secondary antibodies in 3% bovine serum albumin (BSA) in TBS with 0.1% Tween 20.

Tumor lysates were prepared from 10 to 20 mg of flash-frozen tumor tissue in 1% Triton X-100 buffer, and total protein calculated using the Pierce Detergent Compatible Bradford Assay (Thermo Scientific, Waltham, MA). Samples were electrophoresed and transferred as described above. Membranes were probed with anti-PDI and anti-ERp57 followed by an HRP-coupled antirabbit secondary antibody.

2.8 | Factor Xa generation assay

Cells were plated in 12-well dishes at 100000 cells per well. After 24 h of growth, cells were treated with zafirlukast as indicated, washed twice with TBS, and read in a SpectraMax M3 plate reader with TBS containing 5 nmol/L FVIIa, 150 nmol/L of FX, and 5 mmol/L of $CaCl_2$. The reaction was read every minute for 45 min at a fluorescence excitation wavelength of 352 nm and emission wavelength

of 450 nm. The signal was normalized to a percentage of the mock-treated control cells.

2.9 | Animal studies

Four-week-old female NOG mice (Taconic Biosciences, Germantown NY) were subcutaneously (SC) implanted with OVCAR8 cells grown to confluence and diluted to a concentration of 250 000 cells/100 μL in serum-free medium then randomly assigned to a vehicle control ($n=13$) or test group ($n=13$). Four mice remained with no injection to serve as a baseline. Twice a week, mice were measured for weight and tumor growth then checked for health concerns. When tumors became apparent, measurements were taken twice a week with a caliper and size was estimated using LXWXW/2. When tumor size reached an average of 30 mm^3, animals were treated based on weight via i.p. injections, either with vehicle (1% DMSO, 20% PEG, 2% Tween 80) or with 30 mg/kg zafirlukast in vehicle. Injections were administered daily with freshly prepared drug. Health checks, weigh-ins, and tumor measurements continued twice a week for 32 days.

In a second xenograft study using the same experimental setup as above, all animals received a combined chemotherapy dose of 5 mg/kg cisplatin and 120 mg/kg gemcitabine once weekly in the presence or absence of a daily i.p. injection of zafirlukast (30 mg/kg). Injections, health checks, weigh-ins, and tumor measurements remained the same as the first study.

At the end of each study, lungs, kidneys, livers, spleens, and tumors were excised and sent for histology, and tumors were examined by Western blotting analysis. Organs and tumors were placed in 10% formalin and switched to 70% ethanol after 24 h. Organs were sectioned and stained with hematoxylin and eosin (H&E). Slides were viewed at 4× magnification via inverted microscopy and graded for metastasis. A 5-point scale of 0–4 was used for grading with a score of 0 equating to no observed metastasis; 1 being small, scattered tumors detected; 2 having scattered but larger tumors; 3 showing a high amount or regions of tumor growth; and 4 having large, dense tumor regions.

2.10 | Human studies

We conducted a single-arm pilot study in women with tumor marker-only relapsed ovarian cancer. The primary objective of the trial was to determine the potential efficacy of zafirlukast in terms of CA-125 response per Gynecologic Cancer Intergroup (GCIG) criteria.[21] The key secondary objective was to assess changes in CA-125 doubling time in the 3 months prior to enrollment and following the initiation of zafirlukast. Eligible patients were required to have histologically confirmed epithelial ovarian, fallopian tube, or primary peritoneal cancer and have completed at least first-line platinum-based chemotherapy and surgery with a response (Table 1). Eligible patients had tumor marker-only relapse, defined as CA-125 more than twice the upper limit of normal (35 U/mL) in the setting of normal baseline CA-125 levels or a CA-125 greater than twice the nadir count on two consecutive measurements for CA-125 values that remain above baseline without measurable radiographic disease. Participants were required to have adequate organ and marrow function defined as absolute neutrophil count above 1000/μL, platelets ≥90 000/μL, total bilirubin ≤1.3× institutional upper limit of normal (ULN), and creatinine ≤ULN. Participants were excluded if they received cytotoxic chemotherapy including bevacizumab or radiotherapy within 4 weeks prior to study entry, were receiving anticoagulant or antiplatelet therapy.

Zafirlukast was administered at 40 mg twice daily for 28-day cycles. The peak plasma concentration of zafirlukast 40 mg following oral ingestion is ~3 μM.[22] The 40 mg dose is double the FDA-approved dose for asthma, but is known to be well-tolerated.[23] The treatment continued until progression, study completion or taken off study for other reasons. CA-125 was assessed at baseline and every 28 days. Progression was defined as development of clinical symptoms deemed secondary to ovarian cancer and/or radiographically visible disease and/or doubling in the pretreatment CA-125 value, confirmed on successive measurements (1–3 weeks after initial measurement).[21]

TABLE 1 Patient characteristics.

Patient	1	2	3	4
Age	55	66	58	77
Histology	Serous carcinoma	Serous carcinoma	Serous carcinoma	Serous Carcinoma
Initial stage	IIB	IIIC	IIIB	IA
Grade	High grade	High grade	High grade	High grade
Previous treatment with carboplatin/paclitaxel?	Yes	Yes	Yes	Yes

2.11 | Statistics

The statistical analysis was performed using GraphPad Prism (Version 9.4.0, San Diego, CA). Data were presented as the mean $\pm$ SD. For di-eosin-GSSG assays and the factor Xa assay, a one-way ANOVA with a post hoc Dunnett's test was used for statistical analysis between test groups and the control. For the zafirlukast, RL90, and PACMA 31 time course study, a two-way ANOVA with a post hoc Sidak's test was used for statistical analysis between test groups and the control. For the Western blots, mouse studies, and human studies, a Student's t-test was used to evaluate the statistical significance between each test group and the control group. $*p < .05$, $**p < .01$, $***p < .001$, or $****p < .0001$ was considered to be statistically significant.

2.12 | Study approval

The animal work in this protocol was approved by the IACUC and IBC of UMass Chan Medical School-Baystate, the protocol for the human studies was approved by the institutional review board at Dana Farber Harvard Cancer Center and the trial was registered at clinicaltrials.gov (NCT04339140).

3 | RESULTS

3.1 | Zafirlukast is a pan thiol isomerase inhibitor

This study demonstrates a novel activity of zafirlukast and montelukast (Figure 1A,B) as thiol isomerase inhibitors. As shown in Figure 1C, zafirlukast inhibited PDI, ERp5, ERp57, and ERp72 in a concentration-dependent manner for all four enzymes at approximately the same IC_{50}, thereby demonstrating pan, nonpreferential, inhibition. Montelukast also inhibited all four thiol isomerases albeit with less potency (Figure 1D). It is interesting to note that montelukast is less potent than zafirlukast at inhibiting thiol isomerases, while being 2–3 times more potent than zafirlukast at inhibiting the LTR1 receptor.[24] To further confirm that these drugs are targeting thiol isomerases, we explored the effects of an analog of zafirlukast that lacks the cyclopentyl moiety (Figure 1E). Removal of the cyclopentyl is predicted to reduce the potency of this analog against the LTR1 receptor more than 100-fold.[25] Interestingly, this zafirlukast analog maintained a similar potency (~1.5-fold less) to that of zafirlukast at inhibiting thiol isomerases (Figure 1F).

3.2 | Zafirlukast inhibits cellular thiol isomerase activity

The thiol isomerase activity of OVCAR8 cells is also inhibited by zafirlukast treatment. The cleavage of the fluorescent di-eosin thiol isomerase substrate was inhibited after treatment with zafirlukast in a concentration-dependent manner. Treatment of OVCAR8 cells with 3, 10, or 30 μM of zafirlukast for 10 min significantly inhibited PDI activity, by 18%, 24%, and 45%, respectively (Figure 2A). To confirm that the addition of zafirlukast did not alter thiol isomerase expression, thiol isomerase levels were measured following 1-h treatment with 10 and 30 μM zafirlukast in OVCAR8 cells. No significant change in expression was observed (Figure 2B), confirming that the observed change in activity was due to enzymatic inhibition and not a decrease in enzyme levels. The cellular thiol isomerase activity of OVCAR8 cells also significantly decreased upon treatment with montelukast and the zafirlukast analog, with montelukast being somewhat less effective (reduction by 20%, 30%, and 40%, at 10, 30, and 100 μM, respectively) than zafirlukast (Figure 2C). In contrast, the zafirlukast analog was more potent than zafirlukast, significantly inhibiting activity by 25%, 39%, 29%, and 44% at 1, 3, 10, and 30 μM, respectively (Figure 2D).

3.3 | Zafirlukast has antineoplastic activity

As inhibitors of thiol isomerase activity have been shown to have antineoplastic activity,[1] the effect of these drugs on cancer cell viability was evaluated. Zafirlukast inhibited OVCAR8 cancer cell viability with an IC_{50} of 12 μM, while montelukast and the zafirlukast analog inhibited cell viability about five- and ~1.5-fold less potently (with IC_{50}'s of 60 and 20 μM, respectively) (Figure 3A). While differences in potency would not be surprising between a cell-free and a cellular activity assay as well as between a cellular activity assay and a viability assay, it is notable that relative potencies of zafirlukast, the zafirlukast analog, and montelukast remained consistent across the assays, with the analog having a similar potency to that of zafirlukast and montelukast being ~5-fold less potent in each assay (Figure 3B). Due to the potency differences along with the lack of availability of large quantities of the zafirlukast analog, only zafirlukast was selected for additional studies. Interestingly, zafirlukast inhibited the cell viability of three additional cancer cell lines, HCT116 colon cancer cells, A549 lung cancer cells, and PC3 prostate cancer cells with an IC_{50} value similar to (slightly more potent) that for OVCAR8
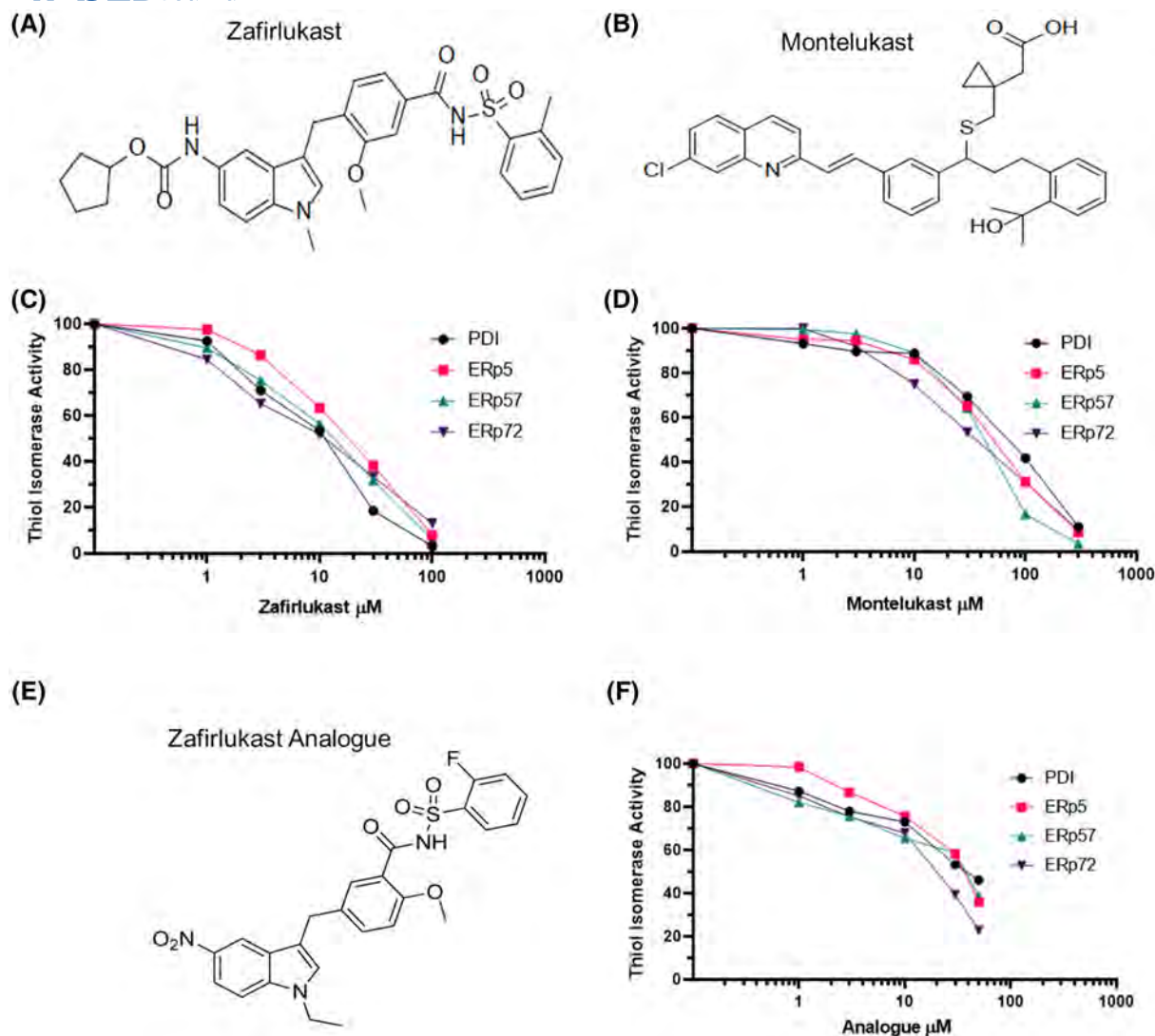
FIGURE 1 Zafirlukast and montelukast are broad-spectrum thiol isomerase inhibitors. The structures of zafirlukast (A) and montelukast (B). Both zafirlukast (C) and montelukast (D) inhibit the thiol isomerases PDI, ERp5, ERp57, and ERp72 in a concentration-dependent manner when examined via the insulin turbidity assay. (E) The structure of a zafirlukast analog missing the cyclopentyl moiety, decreasing or losing its affinity for the leukotriene receptor. (F) The analog inhibits PDI, ERp5, ERp57, and ERp72 similarly to zafirlukast.

cells; however, the IC_{50} for the non-neoplastic HEK293 cell line exceeded the concentration tested ($>100\,\mu M$) (Figure 3C).

3.4 | Zafirlukast and downstream measures of thiol isomerase inhibition in cancer cells

Thiol isomerases play important roles in cancer growth and thrombosis. For example, the activation of the EGFR receptor is dependent on ERp57 activity, as gene silencing of ERp57 leads to decreased phosphorylation of EGFR.[26] We evaluated whether incubation of cancer cells with zafirlukast altered EGFR activation and phosphorylation of GRB2-associated-binding-protein 1 (Gab1), an immediate downstream target of EGFR signaling, responsible for activation of the Akt cell survival pathway.[27] OVCAR8 cells were treated with zafirlukast for 1 or 4 h before activation with epidermal growth factor (EGF) for 5 min. After four hours of treatment with 10 or 30 μM zafirlukast, EGFR phosphorylation significantly decreased, by 39% and 52%, respectively. Phosphorylation also decreased after 1 h, by 18% and 27% after treatment with 10 or 30 μM zafirlukast, respectively (Figure 4A). To further confirm that the decreased activation of EGFR was due to thiol isomerase inhibition, the PDI and ERp57 function blocking antibody RL-90[28] and small molecule thiol isomerase inhibitor PACMA-31 were utilized to examine the effects of thiol isomerase inhibition on EGFR activation and also inhibited EGFR activation in a concentration-dependent
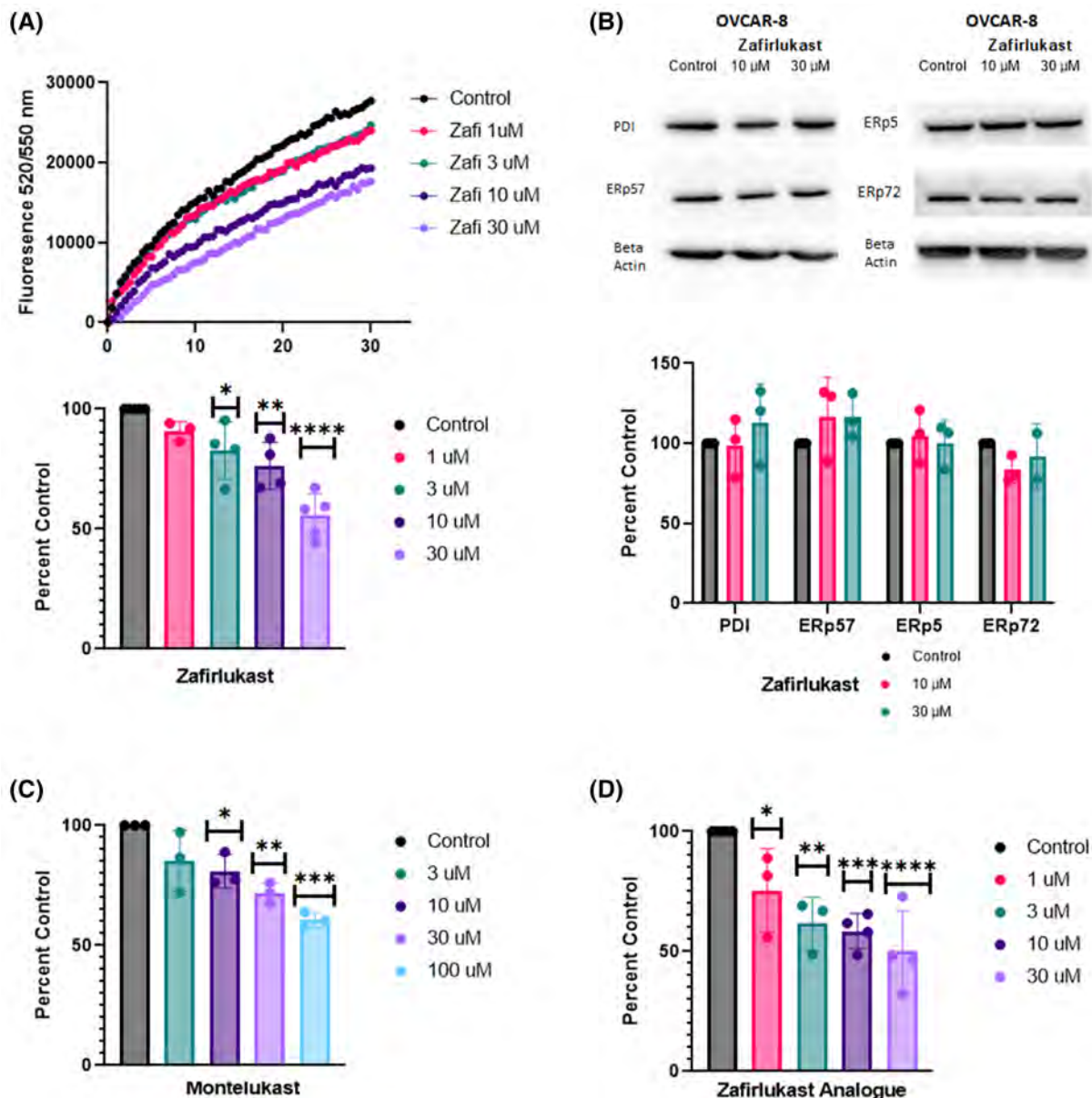
FIGURE 2 Cellular thiol isomerase activity is inhibited by zafirlukast. (A) Cellular thiol isomerase activity is inhibited by zafirlukast treatment in a concentration-dependent manner as measured by di-eosin-GSSG fluorescence ($n=4$) Data are presented as mean $\pm$ SD. One-way ANOVA and a post hoc Dunnett's test where *$p=.0217$ for 3 μM zafirlukast, **$p=.0020$ for 10 μM zafirlukast and ****$p<.0001$ for 30 μM zafirlukast compared with the control. (B) Levels of PDI, ERp5, ERp57, and ERp72 remain similar with increasing concentrations of zafirlukast in OVCAR8 cells ($n=3$). Data are presented as mean $\pm$ SD. A Student's t-test was used for comparison to control. No significant changes were present. (C,D) Cellular thiol isomerase activity is also inhibited by (C) montelukast ($n=3$) and (D) the zafirlukast analog ($n=4$). Data is presented as mean $\pm$ SD. One-way ANOVA and a post hoc Dunnett's test where *$p=.0219$ for 10 μM montelukast, **$p=.0018$ for 30 μM montelukast and ***$p=.0002$ for 100 μM montelukast compared with the control, while *$p=.0383$ for 1 μM zafirlukast analog, **$p=.0017$ for 3 μM zafirlukast analog, ***$p=.0004$ for 10 μM zafirlukast analog and ****$p<.0001$ for 30 μM zafirlukast analog compared with the control.

manner (Figure 4B). Notably, OVCAR8 cell viability was minimally altered by the concentrations and time points of zafirlukast, RL-90 and PACMA-31 used in Figure 4A,B, suggesting decreases in cell viability were not responsible for the observed decreases in EGFR activation (Figure 4C). Furthermore, phosphorylation of Gab1 was significantly decreased under all conditions

tested, by 37% following incubation with 10 μM zafirlukast at 1 h, and further decreased to 5% or less at all other time points and concentrations, demonstrating robust inhibition of the EGFR pathway after zafirlukast treatment (Figure 4D).

Second, PDI activity has been demonstrated to enhance the procoagulant activity of cancer cell lines.[29] To measure
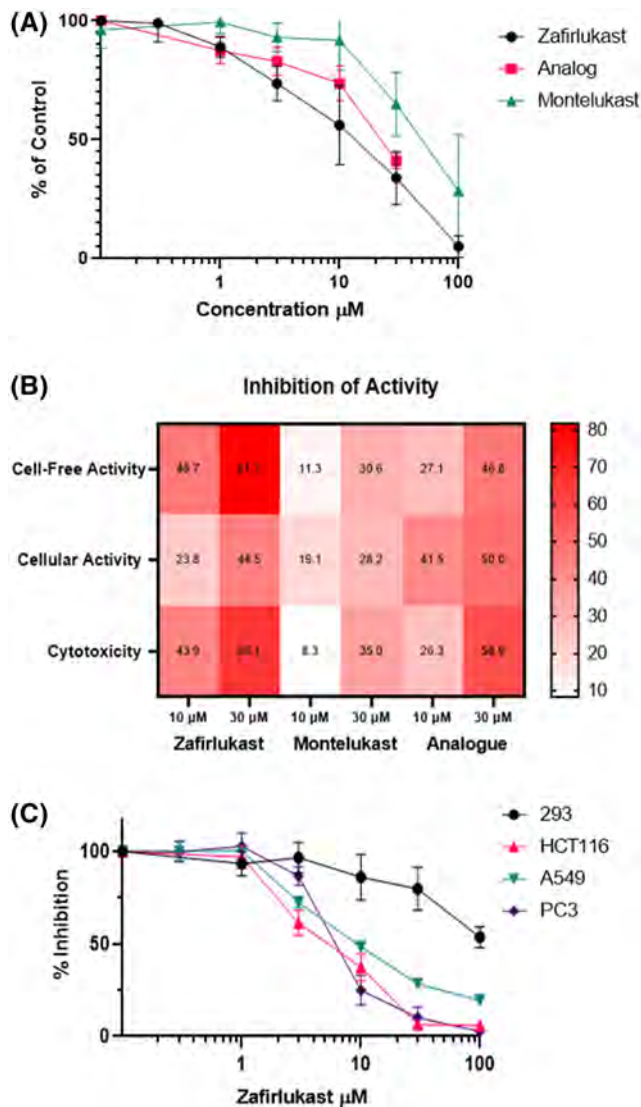
FIGURE 3 Zafirlukast and montelukast selectively cause cancer cell cytotoxicity. (A) Zafirlukast is about 5× more cytotoxic than montelukast and 1.5× more cytotoxic than the analogue ($n = 3$). (B) Comparison of the relative potency of each compound in the experiments from Figures 1 to 3A. (C) Zafirlukast also induced cytotoxicity to HCT116 colon tumor cells, PC3 prostate cancer cells, and A549 lung cancer cells selectively over the HEK293 non-cancerous cell line ($n = 3$).

the activity of tissue factor released by OVCAR8 cells, the generation of Factor Xa was monitored. Cells treated with 1, 3, 10, and 30 μM zafirlukast for 30 min demonstrated a dose-dependent decrease in Xa generation (Figure 4E), with significant differences observed at 10 and 30 μM.

3.5 | Zafirlukast inhibits tumor growth in ovarian cancer mouse xenografts

Following the observed antitumor activity of zafirlukast in vitro, we assessed whether zafirlukast inhibited tumor cell growth in a mouse xenograft of OVCAR8 cells. Mice were administered vehicle control or 30 mg/kg zafirlukast consecutively for 32 days via intraperitoneal injections. Tumor volumetric measurements were assessed twice weekly (Figure 5A). Zafirlukast treatment significantly decreased tumor size compared to controls with a mean decrease in volume of 42% at Day 32 ($p = .0005$). Examination of PDI and ERp57 levels in tumors by Western blot revealed similar levels of both thiol isomerases between the control and zafirlukast treated groups (Figure 5B). Organs (i.e., lung, kidney, liver, and spleen) were examined for metastatic deposits by histology, and representative animals from the control and zafirlukast group were stained with hematoxylin and eosin (H&E). Multiple metastases were detected in the lungs of the control group, while very few metastases were found in the lungs of the zafirlukast-treated group (Figure 5C). Using a rating scale of 0–4, where 0 represents no metastases detected and 4 represents large dense metastases, the zafirlukast-treated group (score of 0.25) had a significantly smaller amount and size of metastases compared with the control group (score of 2.5) ($p = .0004$) (Figure 5D). Examination of the livers of animals detected one control mouse that scored a 1, but no other metastases were detected in the livers, kidneys, or spleens of any animal.

Additionally, since multidrug therapy is commonplace in cancer treatment, zafirlukast was added to a common ovarian cancer regimen of cisplatin and gemcitabine in 5 mg/kg and 120 mg/kg, respectively, via weekly intraperitoneal injection. The addition of zafirlukast significantly reduced the tumor volume relative to cisplatin-gemcitabine alone with a mean decrease of 38% ($p = .011$) after 4 weeks (Figure 5E), and an 83% decrease in the tumor volume by the end of the study compared with the vehicle control (Figure 5F).

3.6 | A pilot clinical trial of zafirlukast in women with relapsed ovarian cancer

In order to assess potential antineoplastic activity of zafirlukast, we conducted a pilot clinical trial in women with tumor marker-only (CA-125) relapsed ovarian cancer. A total of four women were enrolled, and the CA-125 levels were measured at baseline and monthly intervals to assess response after initiation of zafirlukast 40 mg twice daily. None of the women demonstrated a decrease in CA-125 following the initiation of zafirlukast. However, in all four women, the rate of rise of CA-125 was reduced following treatment with zafirlukast (Figure 6A–C). Accordingly, the mean change in CA-125 was 0.036 U/mL per day prior to treatment compared with 0.015 U/mL per day following zafirlukast (paired t-test $p = .026$). This
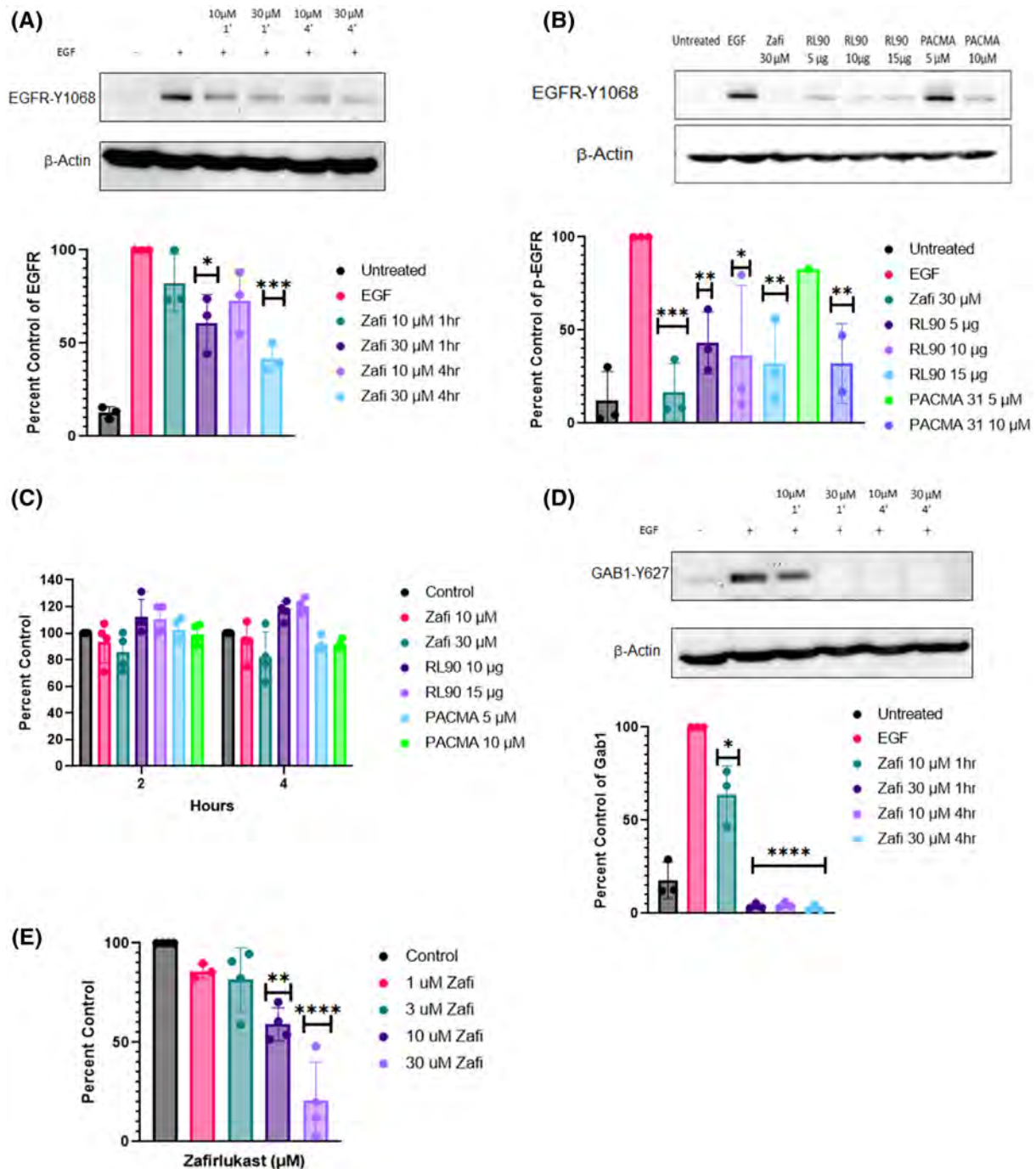
FIGURE 4 Zafirlukast effects downstream measures of thiol isomerase inhibition. (A) Zafirlukast treatment inhibits the activation of the EGFR receptor ($n = 3$). Data are presented as mean ± SD. A Student's t-test where *$p = .0112$ for 1 h 30 μM treated zafirlukast and ***$p = .0002$ for 4 h 30 μM treated zafirlukast compared with the EGF control. (B) The known ERp57 inhibitor RL90 and PACMA 31 also inhibit EGFR activation ($n = 3$). Data are presented as mean ± SD. A Student's t-test where ***$p = .0007$ for 30 μM zafirlukast, **$p = .004$ for 5 μg RL90, *$p = .0433$ for 10 μg RL90, **$p = .0059$ for 15 μg RL90 and **$p = .0092$ for 10 μM PACMA 31 compared with the EGF control. (C) A time-course study of zafirlukast RL-90, and PACMA 31's effect on OVCAR8 cell viability ($n = 4$). Data are presented as mean ± SD. A two-way ANOVA and a post hoc Sidak's test demonstrate no significant changes between the control and any of the test groups. (D) Phosphorylation of Gab1, an immediate downstream target of EGFR phosphorylation ($n = 3$) Data are presented as mean ± SD. A Student's t-test where *$p = .0153$ for 1 h 10 μM zafirlukast and ****$p < .0001$ for 4 h 10 μM and 1 and 4 h 30 μM zafirlukast compared with the EGF control. (E) Zafirlukast inhibits the tissue factor-dependent generation of Factor Xa ($n = 4$). Data are presented as mean ± SD. A one-way ANOVA and post hoc Dunnett's test where **$p = .0041$ for 10 μM zafirlukast and ****$p < .0001$ for 30 μM zafirlukast.
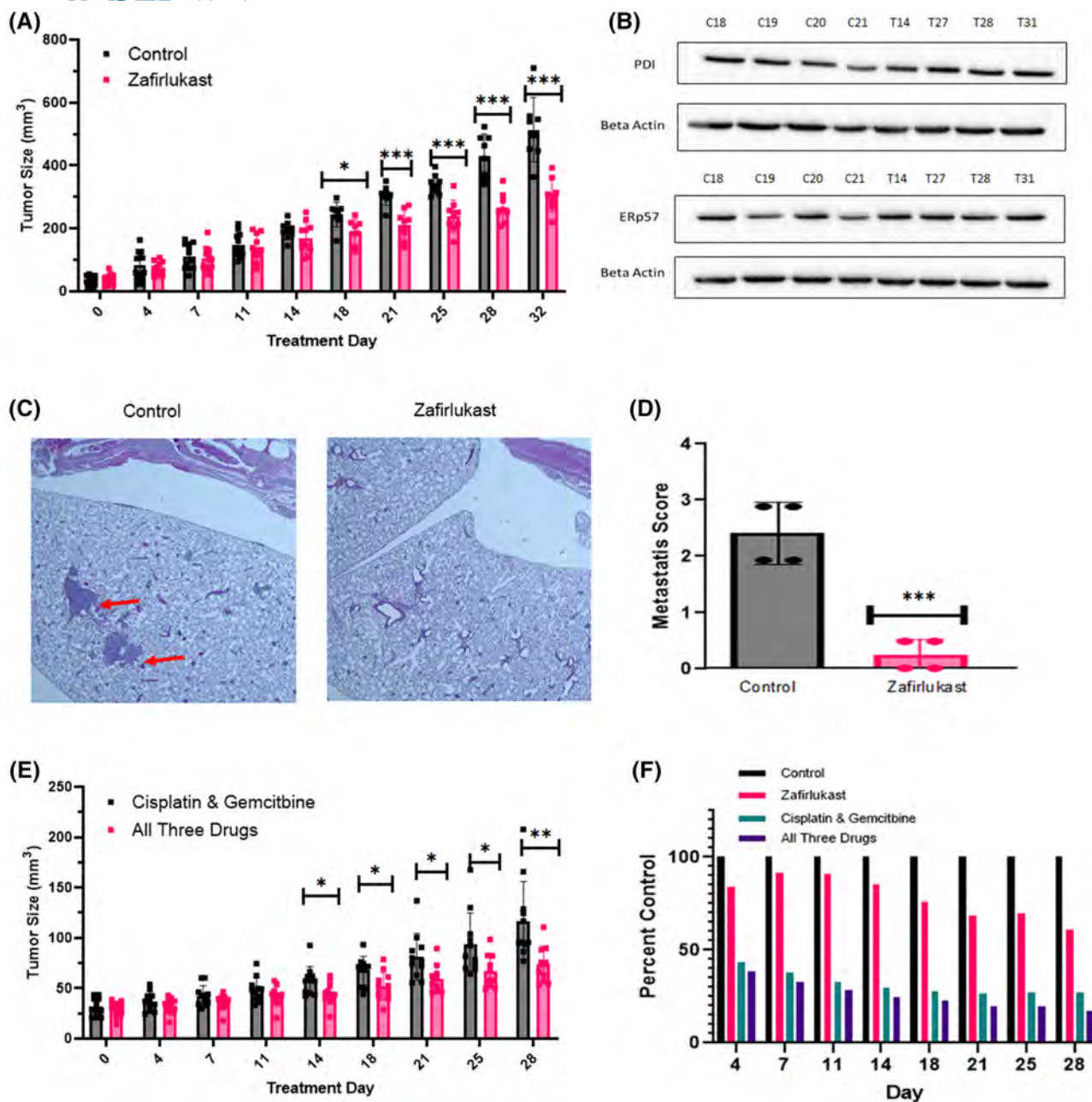
FIGURE 5 Zafirlukast inhibits the growth of OVCAR-8 tumors on xenograft mice. (A) NOG mice were SC injected with OVCAR8 cells at 4 weeks old ($n = 13$/group). Tumors were allowed to grow to an average size of 30 mm^3 before daily treatment with 30 mg/kg of zafirlukast (red bars) or vehicle control (black bars). Tumor size was measured twice weekly. Data are presented as mean $\pm$ SD. A Student's t-test where $*p = .0149$ at 18 days, $***p = .0002$ at 21 and 25 days and $***p = .0001$ at 28 days. (B) PDI and ERp57 levels in tumors were measured by immunoblotting after day 32 of treatment in control vs zafirlukast treated mice with minimal difference in expression. (C,D) Lung metastasis ($n = 4$/group) were graded on a scale of 0–4. A significant difference was seen between the control and zafirlukast treated groups. Data are presented as mean $\pm$ SD. A Student's t-test where $***p = .0004$ for the treated group compared with the control. (E) Similar to A, except mice were treated with 5 mg/kg cisplatin and 120 mg/kg gemcitabine once weekly in the presence (red bars) or absence (black bars) of 30 mg/kg zafirlukast daily ($n = 12$/group). Data are presented as mean $\pm$ SD. A Student's t-test where $*p = .023, .012, .018$ and $.027$ for Days 14, 18, 21 and 25, respectively, and $**p = .004$ for Day 28. (F) A summary of the three different treatment groups, normalized to the control group by percentage.

equated to a mean CA-125 doubling time of 29.8 days in the weeks prior to treatment compared with a mean doubling time of 85.69 days following treatment. Plasma thiol isomerase was evaluated prior (Day 0) and after 4 weeks of zafirlukast treatment (Day 28). In three of four patients, there was a reduction in plasma thiol isomerase activity (Figure 6D). The degree of thiol isomerase activity reduction correlated ($R = .91$) with the CA-125 doubling time
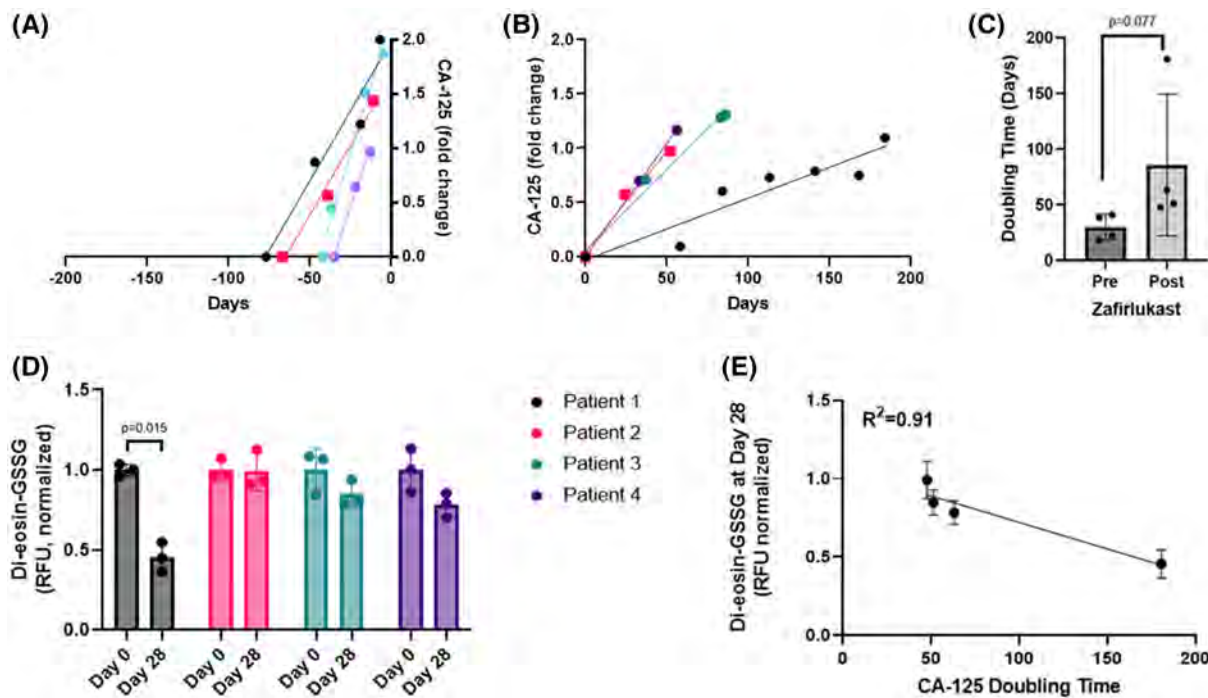
FIGURE 6 Zafirlukast inhibits CA-125 doubling time. The rate of CA-125 doubling time (A) prior to and (B) after zafirlukast treatment. The mean change in CA-125 was 0.036 U/mL per day prior to treatment compared with 0.015 U/mL per day following zafirlukast (paired *t*-test $p = .026$) (C) Comparison between the average pre- and posttreatment doubling times of CA-125. (D) The relative thiol isomerase activity of each patient 28 days after treatment, the reduction of which was well-correlated (E) to the decrease in CA-125 doubling time.

(Figure 6E). There were no cases of hemorrhage reported. The only instance of toxicity considered possibly related to zafirlukast was headache (Grade 1). Three patients reported abdominal pain (Grade 1) and one patient experienced Grade 2 hypertension and abdominal bloating. There were no Grade 3 or higher adverse events reported on study.

4 | DISCUSSION

Targeting thiol isomerase activity represents a promising mechanism of novel antitumor agents.[1,6,13,28,30] There are several compounds in therapeutic development including rutin, bepristat, isoquercetin,[5,6,12] PACMA-31, and CCF642, but none of these are pan-inhibitors.[8,13] The ability of zafirlukast to inhibit multiple thiol isomerases is potentially advantageous, since each thiol isomerase has distinct roles in cancer, and we observed that zafirlukast broadly inhibited thiol isomerases including PDI, ERp57, ERp5, and ERp72 almost equivalently (Figure 1). For example, PDI has a vital role in tumor growth and progression as well as protecting cells from apoptosis.[1,8,31] High levels of ERp5 expression correlate with preventing an efficient antitumor response in Hodgkin lymphomas[32,33] and are associated with prostate[34] and breast[35] cancer progression. ERp57 is involved in cancer metastasis,[36]

gene transcription through EFGR signaling,[26] STAT3,[37] and mTOR pathways[38] while also promoting resistance to treatment with paclitaxel in ovarian cancer.[39] While ERp72 is overexpressed in lung adenocarcinoma and plays a role in cisplatin resistance.[40] With the exception of bacitracin, which is not used systemically due to nephrotoxicity,[41] zafirlukast is the first known broad-spectrum thiol isomerase agent systematically evaluated as a therapeutic compound.

Considering the pleiotropic activity of zafirlukast, we cannot definitively conclude that the antitumor activity is exclusively related to thiol isomerase inhibition. Previous studies have determined that agents such as zafirlukast are associated with a decreased cancer risk that was assumed to be through interaction with the LTR1 receptor[42,43] and others have demonstrated the LTR2 receptor can promote invasiveness and metastasis of ovarian cancer cells.[44] However, we note that zafirlukast and montelukast demonstrate selectivity for the LTR1 receptor, with minimal effects on the LTR2 receptor.[24] Second, montelukast has an increased affinity for the LTR1 receptor in comparison with zafirlukast[24] yet was 3-5× weaker in our in vitro experiments (Figure 3B). Furthermore, using a zafirlukast analog that was modified to significantly decrease or abolish its affinity for the LTR1 receptor[25] while retaining its potency as a thiol isomerase inhibitor, we observed similar relative effects

when compared to zafirlukast and stronger effects than those observed with montelukast (Figure 3B). These observations strongly suggest that zafirlukast does not inhibit cancer cell viability by acting on the LTR1 receptor, but through thiol isomerase inhibition. Finally, our observed delay in CA-125 doubling in our clinical trial correlated with thiol isomerase inhibition (Figure 6D,E). Thus, we believe the available evidence supports that our observed effects are primarily due to thiol isomerase inhibition.

Zafirlukast significantly inhibited tumor growth in a xenograft model of ovarian cancer, which is consistent with previous studies of thiol isomerase inhibitors.[8] Interestingly, very few, small metastases were found in the lungs of treated animals in comparison with the control group (Figure 5), which, to our knowledge, is a novel finding. We have previously shown that zafirlukast can inhibit cell migration, which would be consistent with slowing metastasis.[16] Future studies should further examine the potential of thiol isomerase inhibitors in effecting metastasis.

Next to metastatic growth, cancer-induced thrombosis is the second leading cause of death due to cancer.[45,46] In our previous work, we demonstrated that zafirlukast also inhibits ex vivo human platelet aggregation and in vivo mouse thrombus formation induced via laser injury.[16] Thus, zafirlukast is the first reported thiol isomerase inhibitor that inhibits cancer cell growth as well as thrombus formation and, as such, should be further explored as a potential preventive agent for cancer-induced thrombosis. Our previous work has demonstrated that circulating PDI is associated with increased risks of cancer-induced thrombosis,[9] along with increased levels of tissue factor.[47] Interestingly, tissue factor is also expressed on the surface of some cancer cells[48] and in this study, we found that both cellular thiol isomerase activity (Figure 2A) and tissue factor-dependent Factor Xa generation (Figure 4E) was inhibited by zafirlukast in ovarian cancer cells, which would further support exploration of zafirlukast for cancer-induced thrombosis.

Based on the established safety profile of zafirlukast and its potential benefits as a broad-spectrum thiol isomerase inhibitor that is FDA-approved for the treatment of asthma, we proceeded to assess whether we could detect antineoplastic activity in humans. We elected to use 40 mg twice daily, which is a dose higher than typically used in the treatment of asthma but previously shown to be well-tolerated.[23] The CA-125 did not decline in any of the women treated with zafirlukast but in all instances, there was a slowing in the rate of rise of CA-125 with a greater than twofold increase in CA-125 doubling time. Additional studies are needed to assess whether higher doses of zafirlukast can effectively stabilize CA-125 levels.

The use of zafirlukast or eventually, its analog, in combination with other chemotherapeutics should be explored, which would be supported by the additive effect of zafirlukast with cisplatin and gemcitabine shown in our xenograft experiments.

An important message from this work is that zafirlukast could be repurposed as a cancer medication as it is already FDA-approved, being first used clinically in 1996 for the treatment of asthma via inhibition of the LTR1 receptor and is known to be well-tolerated.[49] In this study, we found that zafirlukast significantly inhibited tumor growth on its own and especially in tandem with a standard chemotherapeutic regimen in a xenograft model of ovarian cancer (Figure 5). We found that zafirlukast could reduce metastasis (Figure 5) and potentially cancer-induced thrombosis (Figure 4D), the top two leading causes of death due to cancer.[45,46] Thus, considering the potential thiol isomerase inhibitors have held as potential cancer therapies,[1,6–9,13,50,51] and our additional findings, it is notable that zafirlukast can be readily studied in the clinic due to its previous FDA approval and established safety profile.

In summary, zafirlukast is the first broad-spectrum thiol isomerase inhibitor that has been shown to inhibit cancer growth in both cells and mice. We demonstrated that cellular thiol isomerase activity was inhibited by zafirlukast and observed a clinical effect on CA-125 that appeared to correlate with thiol isomerase activity. Future clinical studies are needed to further assess the therapeutic benefit of targeting thiol isomerases in ovarian cancer.

DISCLOSURES
DRK is an inventor on a patent owned by Western New England University repurposing zafirlukast as a potential anticancer medication and receives research funding from Quercis Pharma. DRK and SGT are also inventors on a patent owned by Western New England University exploring zafirlukast analogues in thrombosis and cancer. JIZ: Prior research funding from Incyte and Quercegen; Consultancy for Sanofi, CSL Behring, Calyx; Advisory board participation with Pfizer/Bristol Myers Squibb (BMS), Portola, Janssen, and Daiichi.

DATA AVAILABILITY STATEMENT

The data generated in this study are not publicly available, as release of some information could compromise patient privacy or consent but are available upon reasonable request from the corresponding author.

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