The Involvement of Endoplasmic Reticulum Stress in the Suppression of Colorectal Tumorigenesis by Tolfenamic Acid

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Abstract

The nonsteroidal anti-inflammatory drug tolfenamic acid has been shown to suppress cancer cell growth and tumorigenesis in different cancer models. However, the underlying mechanism by which tolfenamic acid exerts its antitumorigenic effect remains unclear. Previous data from our group and others indicate that tolfenamic acid alters expression of apoptosis- and cell-cycle arrest–related genes in colorectal cancer cells. Here, we show that tolfenamic acid markedly reduced the number of polyps and tumor load in APCmin/+ mice, accompanied with cyclin D1 downregulation in vitro and in vivo. Mechanistically, tolfenamic acid promotes endoplasmic reticulum (ER) stress, resulting in activation of the unfolded protein response (UPR) signaling pathway, of which PERK-mediated phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) induces the repression of cyclin D1 translation. Moreover, the PERK-eIF2α-ATF4 branch of the UPR pathway plays a role in tolfenamic acid-induced apoptosis in colorectal cancer cells, as silencing ATF4 attenuates tolfenamic acid-induced apoptosis. Taken together, these results suggest ER stress is involved in tolfenamic acid-induced inhibition of colorectal cancer cell growth, which could contribute to antitumorigenesis in a mouse model. Cancer Prev Res; 6(12): 1–11. ©2013 AACR.

Introduction

Colorectal cancer is the third-leading cause of cancer-related death in the United States (1). Taking nonsteroidal anti-inflammatory drugs (NSAID) has been associated with a reduced risk of colorectal tumorigenesis in epidemiologic studies (2), and a large amount of evidence from cell culture and animal studies has also shown that NSAID treatments can inhibit growth of cancer cells and tumors (3). Many mechanisms have been proposed to elucidate the effect of NSAIDs in antitumorigenesis (4). One such proposed mechanism is through the inhibition of COX-2, which is overexpressed in human tumors and plays a role in carcinogenesis. However, a COX-independent function of NSAIDs also plays an important role in antitumorigenesis and has received much attention since the inhibition of COX-2 activity produces adverse effects (5).

Tolfenamic acid is a conventional NSAID that has been long used for treatment of migraines. Compared with the other NSAIDs, tolfenamic acid exhibits fewer upper gastrointestinal side effects (6). Increasing evidence has shown that tolfenamic acid also suppresses tumorigenesis in several cancer models (7–12), and that this suppression seems to be independent of COX inhibition. For example, suppression of specificity proteins Sp1, Sp3, and Sp4, and their target genes, has been considered to contribute to tolfenamic acid-induced anticancer activity (7). Previous results from our lab also indicate that tolfenamic acid inhibits cell proliferation and induces apoptosis in human colorectal cancer cells, which is in part mediated by the induction of the tumor suppressor proteins NAG-1 and ATF3 (13, 14). These data suggest that tolfenamic acid could exert anticancer activity through various molecular mechanisms, and prompted us to further investigate the additional molecular mechanisms by which tolfenamic acid induces anticancer activity.

Cyclin D1, an oncogenic protein, is often overexpressed in various cancer cells and tumor tissues. The activated cyclin D1-CDK4/6 complex phosphorylates Rb protein and subsequently induces the expression of E2F-target genes that are necessary for DNA synthesis (15). In a recent study, cyclin D1 was also shown to play a role in DNA repair through binding to DNA repair proteins, such as BRCA2 and RAD51 (16), indicating an extra role of cyclin D1. A variety of compounds, including NSAIDs, have been documented to mediate cyclin D1 degradation and cell growth
inhibition (17). It has been reported that tolfenamic acid also downregulated cyclin D1 expression in esophageal and breast cancer cells (8, 9); however, the detailed molecular mechanism(s) is not clear. Elucidation of the molecular mechanism(s) underlying cyclin D1 downregulation by tolfenamic acid would be beneficial to further understand the antitumorigenic activity of tolfenamic acid, and to develop novel derivatives, which could exert more pronounced anticancer effects.

Endoplasmic reticulum (ER) stress inhibits cyclin D1 translation and cell-cycle progression (18). Moreover, ER stress initiates apoptosis by activating transcription factor 4 (ATF4)-dependent C/EBP homologous transcription factor (CHOP), apoptosis signal-regulating kinase 1 (ASK1), and caspase-12 (19). Interestingly, two ER-stress-inducible genes (EGR1 and ATF3) have been previously identified as the anticancer targets of tolfenamic acid in colon cancer (13, 14). Thus, ER stress could be involved in tolfenamic acid-mediated cancer cell growth suppression. Indeed, some NSAIDs like indomethacin and celecoxib have been shown to trigger ER stress response (20, 21). In contrast, diclofenac blocked ER stress-induced apoptosis in SH-SY5Y cells (22), and pranoprofen also suppressed ER stress-induced glucose-regulated protein 78 (GRP78) and CHOP expression (23). Therefore, how ER stress is involved in tolfenamic acid’s effect on cell growth remains unclear and needs to be further elucidated.

In the current studies, we first evaluated the chemopreventive effect of tolfenamic acid in a mouse model of colorectal cancer. As a result, we found that tolfenamic acid inhibited polyp formation in APC<sub>min</sub>/C<sup>−/−</sup> mice, along with a dramatic decrease in cyclin D1 expression in tumors. Consistently, we have also shown that tolfenamic acid downregulates cyclin D1 expression in different cancer cells in vitro, which is associated with the increase in Rb activity. We further investigated the underlying mechanism, showing that tolfenamic acid repressed cyclin D1 translation through activation of the ER stress-mediated unfolded protein responses (UPR) pathway. In addition, ER stress played a role in tolfenamic acid-induced apoptosis in colorectal cancer cells. Therefore, our data strongly suggest that ER stress response could contribute to the antitumorigenic activity of tolfenamic acid.

Materials and Methods

Reagents and antibodies

Tolfenamic acid, SC-560, and lactacystin were purchased from Cayman Chemical, and 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone (DFU) was described previously (24). All other NSAIDs, cycloheximide, and propidium iodide (PI) were purchased from Sigma-Aldrich. Epoxomicin was obtained from Calbiochem. All other chemicals were purchased from Fisher Scientific. RNase A was purchased from 5PRIME. Antibodies for cyclin D1, ATF4, Bcl-2, and Actin were from Santa Cruz Biotechnology; antibodies for pRb (Ser780), total Rb, p-Cyclin D1 (Thr286), BiP, CHOP, p-eIF2α, total eIF2α, PERK, and PARP were from Cell Signaling Technology; and antibody for HA tag was from GenScript. Control siRNA (#6201) and siRNA for PERK (#9024) were obtained from Cell Signaling Technology. siATF4 (sc-35112) was purchased from Santa Cruz Biotechnology.

Cell culture

Human cancer cell lines (HCT-116, HT-29, SW480, LoVo, Caco-2, A549, PC-3, and AsPC-1) were purchased from American Type Culture Collection (ATCC). ATCC tests the authenticity of these cell lines using short tandem repeat analyses. HCT-116 and HT-29 cells were maintained in McCoy’s 5A medium (Bio Whittaker). SW480, A549, PC-3, and AsPC-1 cells were maintained in RPMI-1640 medium (Mediatech). Caco-2 and LoVo cells were kept in Eagle’s minimum essential medium and Ham’s F12 medium (HyClone), respectively. All cells were cultured in media supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO₂ atmosphere at 37°C.

Plasmid, mutagenesis, and transient transfection

The pRcCMV-cyclin D1-HA plasmid was generously provided by Dr. E. Dmitrovsky (Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH; ref. 25). The pCMV-cyclin D1 and ubiquitin (Ub)-HA were gifts from Dr. Richard G. Pestell (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA). Cyclin D1 transversion from threonine to alanine at the 286 amino acid position was generated using the QuickChange site-directed mutagenesis kit (Strategene) according to the manufacturer’s protocol. ATF4 expression construct was purchased from OriGene, and p5xATF6-GL3 luciferase reporter construct (plasmid #11976) was obtained from Addgene (26). Transient transfection was carried out using TransIT-2020 transfection reagent (Mirus Bio LLC) according to the manufacturer’s protocol. For luciferase assay, cells were seeded in 12-well plates at a density of 1.0 × 10<sup>5</sup> cells per well. After transfection with 1 µg p5xATF6-GL3 and pRL-null, cells were treated with dimethyl sulfoxide (DMSO) and tolfenamic acid for 24 hours. Cell lysates were harvested using 1XP passive lysis buffer (Promega), and then were subjected to luciferase activity analysis using DualGlo Luciferase Assay Kit (Promega).

Caspase-3/7 activity assay

Cells were seeded in a 96-well plate at a density of 1.0 × 10<sup>2</sup> cells per well. Empty vector and ATF-4 expression vector were transiently transfected using TransIT-2020 transfection reagent (Mirus Bio LLC) for 24 hours. Caspase-3/7 activity was determined by caspase-Glo 3/7 reagent (Promega). Briefly, 100 µL reagent was added to each well. After incubating the plate at room temperature for 1 hour in the dark, luminescence was measured using a FLX-800 microplate reader (Bio-Tek). Fold change compared with empty vector transfection is represented as the mean ± SD of three wells.
**Determination of sub-G₁ cells**

Cells were seeded in 6-well plates at a density of 3.0 × 10⁵ cells per well in three replicates, and cultured to 60% to 80% confluence. Then, cells were treated with vehicle and tolfenamic acid (50 μmol/L) for 24 hours, then harvested and fixed in 70% ethanol. After being stored at −20°C overnight, the fixed cells were washed with PBS and stained with propidium iodide (PI, 70 μmol/L) solution containing RNase A (1 mg/mL) for 15 minutes at room temperature. The sub-G₁ phase was determined by a Beckman Coulter Epixis XL flow cytometer equipped with ModFit LT software.

**RNA interference**

HCT-116 cells were seeded on 6-well plates at a density of 3.0 × 10⁵ cells per well overnight. Control siRNA, siATF4, or siPERK was transfected at a final concentration of 100 nmol/L using PepMute siRNA & DNA Transfection Reagent (SignaGen) according to the manufacturer’s instruction. After 24- (siATF4) or 48-hour (siPERK) transfection, cells were treated with vehicle and tolfenamic acid (50 μmol/L).

**RNA isolation, semi-quantitative reverse transcription PCR, and real-time PCR**

Total RNA of HCT-116 and SW480 cells treated by DMSO and tolfenamic acid were isolated by an E.Z.N.A Total RNA Kit (Omega Bio-Tek) according to the manufacturer’s protocol. Then, RNA (1 μg) was reverse transcribed using a Verso cDNA synthesis Kit (ThermoScientific). PCR was performed using GoTaq Green Master Mix PCR Reaction Mixture (Promega) with primers for human cyclin D1, EGR-1, or GAPDH as follows: cyclin D1, forward 5'-ATGGAACACCGCTCCGTGCTGC-3' and reverse 5'-TCAGATGTCCACGTCCCGCACGT-3'; EGR-1 forward 5'-CTCGGACATCTGTGGAAGAA-3' and reverse 5'-TGTCCTGGGAGAAAGGTGG-3'; ATF3: forward 5'-GTTTAGGGTGTCTGCAACTGAC-3' and reverse 5'-AGCTGCAATCTTATTTCTTTCTCGT-3'; GAPDH, forward 5'-CTGGATGAGCTTGTTGACGG-3' and reverse 5'-GCGGCTTTGTTATATAGTGG-3'; GAPDH, forward 5'-GGGCTTAAATGCTGT-3' and reverse 5'-TGGCAGGTTTGTCTAGACC-3'. Real-Time PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) with different primer sets for human cyclin D1, EGR-1, or GAPDH as follows: cyclin D1, forward 5'-GGGCGAGGAAACAAAGCCA-3' and reverse 5'-TGTAGGGCTTACAGTGACG-3'; EGR-1 forward 5'-CACTGACCCGAAGCTT-3' and reverse 5'-CTGCAACAGGTTGAAGGGG-3'; GAPDH, forward 5'-GGGACCGCAAAAGGTGACTCA-3' and reverse 5'-TATGCTGACCATGACTGTC-3'. Gene expression levels were calculated and GAPDH was used as a house-keeping gene, using MyiQ thermal cycler (Bio-Rad). The fold changes in mRNA levels were calculated using the ΔΔCt method.

**Western blot analysis**

Cells were washed with PBS and cell lysates harvested using radioimmunoprecipitation assay buffer (50 mmol/L Tris–HCl pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) supplemented with 1× protease inhibitor cocktail solution (Calbiochem) and phosphatase inhibitor (1 mmol/L Na₂VO₄, 1 mmol/L NaF), and centrifuged at 13,000 × g for 10 minutes at 4°C. Protein concentration was determined by the BCA protein assay (Pierce) using Bovine Serum Albumin as the standard. Protein (30 μg) was mixed with an equal amount of 2× SDS-PAGE sample loading buffer and boiled for 5 minutes. After separation by SDS-PAGE, the proteins were transferred to nitrocellulose membranes (Osmonics). The membranes were incubated with a specific primary antibody in TBS containing 0.05% Tween 20 (TSB-T) and 5% nonfat dry milk at 4°C overnight. After three washes with TBS-T, the blots were incubated with peroxide-conjugated IgG for 1 hour at room temperature, visualized using ECL (Amersham Biosciences), and quantified by Scion Image Software (Scion Corp.).

**Immunoprecipitation**

The cells were harvested using lysis buffer (0.025 mol/L Tris, 0.15 mol/L NaCl, 0.001 mol/L EDTA, 1% NP40, 5% Glycerol; pH 7.4) containing 1× protease inhibitor cocktail solution (Calbiochem) and phosphatase inhibitor (1 mmol/L Na₂VO₄, 1 mmol/L NaF), and then kept on ice for 30 minutes. After being spun down for 10 minutes, the suspension was precleared using protein A/G PLUS-agarose (Santa Cruz Biotechnology) for 30 minutes at 4°C. Protein concentration was determined as described above. Protein lysates (1,000 μg) were incubated with 5 μg primary antibody and IgG control for 1 hour at 4°C, followed by adding 50 μL resuspended protein A/G PLUS-agarose overnight. Immunoprecipitates were collected by centrifuging at 1000 × g for 5 minutes at 4°C. After washing five times with lysis buffer, the pellets were resuspended with 50 μL 2× SDS-PAGE sample loading buffer. The samples were boiled for 5 minutes, and 20 μL of samples were subjected to Western blot analysis.

**Animal study**

The APC<sub>min</sub> mice (C57BL/6background) were purchased from Jackson Laboratory. To investigate the long-term effect of tolfenamic acid on tumor formation, mice aged 6 to 8 weeks were randomly assigned to three groups, and 0, 25, or 50 mg/kg of tolfenamic acid was given by oral gavage with 0.5% methylcellulose (vehicle) every 2 days for 4 weeks. To investigate the short-term effect of tolfenamic acid on tumor formation and protein expression, mice aged 16 to 18 weeks were randomly divided into two groups, and 0 or 50 mg/kg body weight of tolfenamic acid was given by oral gavage with 0.5% methylcellulose once per day for 3 days. At the end of the experiment, the mice were euthanized by overdose of CO₂, and the entire intestinal tract was removed, flushed with cold saline, and opened longitudinally. The number and size (diameter) of polyps and tumors were scored blindly under a dissecting microscope as described previously (27, 28). Tumor load was calculated as number of tumors × average diameter. Both normal and
neoplastic tumors were kept under RNAlater solution for molecular analysis. All animal research procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accordance with NIH guidelines.

Statistical analysis

Statistical analysis was performed using the Student unpaired t test with statistical significance set at *, P < 0.5; **, P < 0.01; and ***, P < 0.001.

Results

Tolfenamic acid suppressed polyp formation in an APC\textsuperscript{min/+} mouse model

To evaluate the chemopreventive activity of tolfenamic acid in a mouse model, we examined the number of polyps and tumor load of APC\textsuperscript{min/+} mice treated with vehicle and tolfenamic acid. In the first experiment, we treated mice aged 6 to 8 weeks with tolfenamic acid for 4 weeks (total 14 times). The number of polyps and tumor load were calculated as described in Materials and Methods. Data indicate mean ± SD of all experiments. Results were considered statistically significant at ***, P < 0.001, compared to group 1. B, mice aged 16–18 weeks were randomly divided into two groups, and respectively treated with 0.5% methylcellulose or 50 mg/kg body weight tolfenamic acid once per day for 3 days (total three times). The number of polyps and tumor load were calculated, and the data are expressed as mean ± SD of all experiments. Results were considered statistically significant at **, P < 0.01, compared to group 1. C, proteins prepared from tissues of mice at age 16–18 weeks fed vehicle or 50 mg/kg body weight tolfenamic acid were subjected to Western blot analysis for COX-2, cyclin D1, and actin expression.

Figure 1. Tolfenamic acid suppresses colorectal tumorigenesis in a mouse model. A, mice aged 6–8 weeks were randomly divided into three groups, and respectively administered 0.5% methylcellulose, 25 mg/kg body weight, or 50 mg/kg body weight of tolfenamic acid (TA) every other day for 4 weeks (total 14 times). The number of polyps and tumor load were calculated as described in Materials and Methods. Data indicate mean ± SD of all experiments. Results were considered statistically significant at ***, P < 0.001, compared to group 1. B, mice aged 16–18 weeks were randomly divided into two groups, and respectively treated with 0.5% methylcellulose or 50 mg/kg body weight tolfenamic acid once per day for 3 days (total three times). The number of polyps and tumor load were calculated, and the data are expressed as mean ± SD of all experiments. Results were considered statistically significant at **, P < 0.01, compared to group 1. C, proteins prepared from tissues of mice at age 16–18 weeks fed vehicle or 50 mg/kg body weight tolfenamic acid were subjected to Western blot analysis for COX-2, cyclin D1, and actin expression.
report (29); however, only cyclin D1 was dramatically suppressed in tolfenamic acid-treated tumor samples.

**Tolfenamic acid downregulated cyclin D1 expression in cancer cells**

As cyclin D1 acts as a pro-oncogenic factor, it is not surprising that colorectal cancer cells harbor overexpressed cyclin D1 (Fig. 2A), and its downregulation may contribute to the antiproliferative effect of NSAIDs. As it has been documented that NSAIDs differ in their ability to suppress cyclin D1 expression or cell proliferation (30), we treated SW480 cells with the same dose (50 μmol/L) of different NSAIDs: conventional (diclofenac, ibuprofen, aspirin, naproxen, piroxicam, tolfenamic acid), COX-2 selective (SC-236, DFU), or COX-1 selective (SC-560). Tolfenamic acid and SC-560 completely decreased cyclin D1 expression, suggesting both of them were the most potent agents of those we tested with respect to cyclin D1 downregulation (Fig. 2B). As we have shown that tolfenamic acid can suppress cyclin D1 in an *in vivo* model (Fig. 1), we selected this compound for subsequent studies.

Tolfenamic acid caused rapid cyclin D1 downregulation in a dose- and time-dependent manner, and the decrease occurred within 1 hour (Fig. 2C). To further evaluate the effect of tolfenamic acid on other cancer cells, we treated colorectal cancer cells (HCT-116 and Caco-2), prostate (PC-3), pancreatic (AsPC-1), and lung (A549) cancer cells with tolfenamic acid. As shown in Fig. 2D, tolfenamic acid dose-dependently downregulated cyclin D1 expression in different cancer cells.

**Tolfenamic acid affected phosphorylation of Rb protein**

The cyclin D1-CDK4/6 complex phosphorylates and inactivates Rb, facilitating the G1→S phase transition. Specifically, cyclin D1 is required for phosphorylating the Ser780 amino acid position of Rb (31). We observed that tolfenamic acid decreased phosphorylated Rb at Ser780 in both SW480 and HCT-116 cells (Fig. 3A). To further investigate whether cyclin D1 plays a role in the loss of Rb phosphorylation by tolfenamic acid, we transiently transfected cyclin D1 expression vector containing an alanine for

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**Figure 2.** Tolfenamic acid downregulates cyclin D1 in cancer cells. A, endogenous cyclin D1 expression in different colorectal cancer cells. Western blot analysis was carried out for analyzing expression of cyclin D1 and actin in the indicated cell lines. B, SW480 cells were treated with various NSAIDs at 50 μmol/L for 24 hours. The cell lysates were harvested and subjected to Western blot analysis for cyclin D1 and actin. C, SW480 cells were treated with 0, 5, 10, 25, or 50 μmol/L tolfenamic acid for 24 hours (left) and 50 μmol/L tolfenamic acid for 0, 1, 3, 6, 12, or 24 hours (right). Cell lysates were analyzed by Western blot using cyclin D1 and actin antibodies. D, colorectal (HCT-116, Caco-2), prostate (PC-3), pancreatic (AsPC-1), and lung (A549) cancer cells were treated with 0, 5, 25, or 50 μmol/L of tolfenamic acid for 24 hours. Western blot analysis was carried out for analyzing cyclin D1 and actin.
Tolfenamic acid inhibited cyclin D1 at the translational regulation in colorectal cancer cells

To elucidate the molecular mechanism by which tolfenamic acid downregulated cyclin D1 expression in colorectal cancer cells, we treated HCT-116 and SW480 cells with tolfenamic acid for 1 hour and examined the expression of cyclin D1 transcripts. As shown in Fig. 4A, cyclin D1 transcript levels were not altered by tolfenamic acid within 1 hour, although there was a dramatic increase in transcript levels were not altered by tolfenamic acid within 1 hour, although there was a dramatic increase in transcript levels were not altered by tolfenamic acid within 1 hour, although there was a dramatic increase in transcript levels were not altered by tolfenamic acid within 1 hour, although there was a dramatic increase in EGR-1 and ATF3 mRNA expression as a control (13, 14). Given cyclin D1 is a short-lived protein, we examined whether tolfenamic acid triggered the cyclin D1 degradation. Results in Fig. 4B and C indicate that neither endogenous nor exogenous cyclin D1 stability was affected by tolfenamic acid. Furthermore, treatment of inhibitors responsible for cyclin D1 degradation did not restore cyclin D1 expression in the presence of tolfenamic acid (Fig. 4D and Supplementary Fig. S1). In addition, tolfenamic acid did not affect Thr286 phosphorylation nor ubiquitination of cyclin D1, which has been well documented to be associated with cyclin D1 degradation (Fig. 4E and Supplementary Fig. S2). Taking these results together, we found that tolfenamic acid did not affect transcription nor protein degradation pathway of cyclin D1; rather tolfenamic acid may inhibit cyclin D1 translation initiation.

Tolfenamic acid promoted ER stress response

To further understand the cellular signaling pathways involved in tolfenamic acid-mediated cyclin D1 translation, we investigated the effect of tolfenamic acid on the mTOR pathway, which is mainly responsible for protein translation. However, we did not observe that tolfenamic acid suppressed this pathway (data not shown). Another important protein translation control is mediated by eIF2α phosphorylation of which can lead to nearly global translation inhibition. As ER stress usually induces phosphorylated eIF2α, we therefore asked whether tolfenamic acid promoted ER stress in colorectal cancer cells. The ER stress-activated UPR pathway consists of a large variety of downstream responses, including ATF6-mediated transcriptional activation, splicing of x-box-binding protein-1 (XBP-1) mRNA, and induction of target gene expression, such as BiP, ATF4, and CHOP (33). As expected, treatment of HCT-116 and SW480 cells with tolfenamic acid resulted in the induction of ATF6 transcriptional activity, XBP-1 mRNA splicing, and expression of specific genes (BiP, ATF4, and CHOP) in a time-dependent manner (Fig. 5A–C), indicating tolfenamic acid could be a potential activator of ER stress.

Cyclin D1 suppression and ATF4 induction by tolfenamic acid are mediated by PERK-eIF2α pathway

Given that ER stress would activate the kinase PERK that resides in the ER lumen, resulting in the phosphorylation of eIF2α and subsequently the repression of cyclin D1 translation (18), we examined the relationship of PERK/eIF2α signaling and cyclin D1 expression in the presence of tolfenamic acid. As shown in Fig. 6A, tolfenamic acid caused rapid phosphorylation of eIF2α coupled with a dramatic decrease of cyclin D1 expression in a time-dependent manner. In addition, thapsigargin, a well-known activator of ER stress had an effect on cyclin D1 expression similar to that of tolfenamic acid (Fig. S3). Moreover, silence of PERK using specific siRNA slightly restored cyclin D1 expression in the presence of tolfenamic acid (Fig. 6B), suggesting the PERK-eIF2α pathway was involved in tolfenamic acid-mediated cyclin D1 downregulation. The UPR is a prosurvival

* Figure 3. Tolfenamic acid (TA) activates Rb via dephosphorylation. A, HCT-116 and SW480 cells were treated with 50 μmol/L tolfenamic acid for 0, 1, 3, 6, 12, or 24 hours. Cell lysates were harvested and analyzed by Western blot for phospho-Rb (Ser780) and total Rb. B, HCT-116 cells were transiently transfected with Mock and cyclin D1-HA (T286A) for 24 hours, followed by treatment with 50 μmol/L tolfenamic acid for 0, 6, 12, or 24 hours. Western blot analysis was performed for phospho-Rb (Ser780), total Rb, HA, and actin.
response to allow cells to remove unfolded proteins and reestablish homeostasis. However, if the primary stimuli causing unfolded proteins are persistent and the stress cannot be resolved, signaling switches from prosurvival to proapoptotic (34). Of the UPR signaling pathway branches, PERK-eIF2α pathway plays a crucial role in regulating BCL-2 downregulation, and attenuated apoptosis as measured by cleaved PARP (Fig. 6E). These data indicated tolfenamic acid caused cell apoptosis via, at least in part, the PERK-eIF2α-ATF4 pathway in colorectal cancer cells.

Discussion

We and others have previously reported that tolfenamic acid exhibits anticancer activity via different molecular targets, including Sps (7, 37), ATF2/ATF3 (14), NAG-1 (13), NF-κB (38), and Smads (39). In the present study, we further confirmed that tolfenamic acid is a powerful chemopreventive or therapeutic agent against colorectal cancer in vitro and in vivo, and that tolfenamic acid-mediated growth inhibition might be associated with ER stress response, especially the PERK-eIF2α axis, which resulted in downregulation of prosurvival proteins like cyclin D1, and upregulation of proapoptotic proteins like CHOP. In
addition, we found that tolfenamic acid markedly downregulated cyclin D1 in tumors and a large variety of cell lines, and that this downregulation partially contributed to the loss of phosphorylated Rb. Given the key role of cyclin D1 in G1-S phase transition, downregulation of cyclin D1 by tolfenamic acid could induce cell-cycle arrest. Indeed, there are several reports showing that treatment with tolfenamic acid could induce cell-cycle arrest. Indeed, there were previous reports in cells treated with the COX-2 selective inhibitor celecoxib and its analog (21, 50), were previously reported in cells treated with the COX-2 inhibitor, and tolacement of tumorigenesis. These results further indicate that tolfenamic acid-induced apoptosis in colorectal cancer cells. Tolfenamic acid triggered an ER stress response. A, HCT-116 and SW480 cells were transiently transfected with p53-ATF6-OL3 and pRL-null for 24 hours, followed by treatment with 0, 5, 25, or 50 μmol/L of tolfenamic acid for 24 hours. Luciferase activity was measured as described in Materials and Methods. RLU, relative luciferase unit. Results were considered statistically significant at *, P < 0.05; **, P < 0.01; and ***, P < 0.001, compared with DMSO. B, HCT-116 and SW480 cells were treated with 0, 5, 25, or 50 μmol/L of tolfenamic acid for 24 hours. Reverse transcription-PCR was carried out for analyzing XBP-1 and GAPDH mRNA expression using specific primers as described in Materials and Methods. C, HCT-116 and SW480 cells were treated with 50 μmol/L of tolfenamic acid for 0, 1, 3, 6, 12, or 24 hours. The cell lysates were subjected to Western blot analysis for analyzing BiP, ATF4, CHOP, and actin expression.

Figure 5. Tolfenamic acid promotes ER stress response. A, HCT-116 and SW480 cells were transiently transfected with p53-ATF6-OL3 and pRL-null for 24 hours, followed by treatment with 0, 5, 25, or 50 μmol/L of tolfenamic acid for 24 hours. Luciferase activity was measured as described in Materials and Methods. RLU, relative luciferase unit. Results were considered statistically significant at *, P < 0.05; **, P < 0.01; and ***, P < 0.001, compared with DMSO. B, HCT-116 and SW480 cells were treated with 0, 5, 25, or 50 μmol/L of tolfenamic acid for 24 hours. Reverse transcription-PCR was carried out for analyzing XBP-1 and GAPDH mRNA expression using specific primers as described in Materials and Methods. C, HCT-116 and SW480 cells were treated with 50 μmol/L of tolfenamic acid for 0, 1, 3, 6, 12, or 24 hours. The cell lysates were subjected to Western blot analysis for analyzing BiP, ATF4, CHOP, and actin expression.

It has been suggested that tolfenamic acid suppresses erbB2 and Sp transcription factors, which could result in the suppression of cyclin D1 expression (8, 9). However, our data clearly indicate that tolfenamic acid-induced cyclin D1 downregulation is an erbB2- and Sp-independent pathway; although tolfenamic acid did not affect the cyclin D1 transcription, it significantly decreased protein expression (Figs. 3 and 4A), and tolfenamic acid-induced cyclin D1 downregulation occurred much earlier than tolfenamic acid-mediated Sp downregulation (1 vs. 24 hours) (unpublished data). These results further indicate that tolfenamic acid-mediated cyclin D1 downregulation is independent of transcription factors such as Sp1 and erbB2, and even other transcription factors β-catenin or NF-kB.

Interestingly, we found that cyclin D1 degradation was not enhanced in the presence of tolfenamic acid (Fig. 4B–E). On the basis of our data, we suggest that tolfenamic acid could be able to suppress cyclin D1 translation, at least in colorectal cancer cells. Tolfenamic acid triggered an ER stress response, resulting in the phosphorylation of eIF2α, which leads to the repression of protein translation through limiting the delivery of initiator met-tRNA<sub>Met</sub> to translation machinery (42). After exposure of cells to ER stress, PERK is mainly responsible for phosphorylating eIF2α, which mediates cyclin D1 translation repression (43). Indeed, silencing PERK in HCT-116 cells partially restored cyclin D1 expression in the presence of tolfenamic acid. Because we could not completely block the expression of PERK (Fig. 6B), it is likely that remaining PERK activity contributed to the loss of cyclin D1 in HCT-116 cells treated with tolfenamic acid. In addition, it is possible that other kinases would be able to phosphorylate eIF2α in the absence of PERK, resulting in cyclin D1 repression (43). Therefore, ER stress-sensing pathways are very complicated, and the details need to be elucidated.

Accumulating evidence suggests that the activation of UPR exerts contradictory consequences to tumour development. There is some evidence that UPR signaling contributes to tumor growth through increasing tumor cell tolerance to hypoxia (44, 45). On the other hand, various compounds harboring anticancer activity have been shown to induce ER stress-mediated cell-cycle arrest and apoptosis (46–49). Our data indicate that tolfenamic acid exerts anticancer activity, in part dependent on the activation of the UPR signaling pathway. Similar findings were previously reported in cells treated with the COX-2 selective inhibitor celecoxib and its analog (21, 50), suggesting tolfenamic acid and celecoxib might exhibit comparable antineoplastic activity in a COX-independent manner.

We have identified EGR1 and ATF3 as mediators of tolfenamic acid-induced apoptosis in colorectal cancer cells (13, 14). Interestingly, both of them are inducible under ER stress (51, 52). Tolfenamic acid could also be able to suppress cyclin D1 translation, at least in colorectal cancer cells. Tolfenamic acid triggered an ER stress response, resulting in the phosphorylation of eIF2α, which leads to the repression of protein translation through limiting the delivery of initiator met-tRNA<sub>Met</sub> to translation machinery (42). After exposure of cells to ER stress, PERK is mainly responsible for phosphorylating eIF2α, which mediates cyclin D1 translation repression (43). Indeed, silencing PERK in HCT-116 cells partially restored cyclin D1 expression in the presence of tolfenamic acid. Because we could not completely block the expression of PERK (Fig. 6B), it is likely that remaining PERK activity contributed to the loss of cyclin D1 in HCT-116 cells treated with tolfenamic acid. In addition, it is possible that other kinases would be able to phosphorylate eIF2α in the absence of PERK, resulting in cyclin D1 repression (43). Therefore, ER stress-sensing pathways are very complicated, and the details need to be elucidated.

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We have identified EGR1 and ATF3 as mediators of tolfenamic acid-induced apoptosis in colorectal cancer cells (13, 14). Interestingly, both of them are inducible under ER stress (51, 52). Tolfenamic acid might transcriptionally upregulate expression of those two proapoptotic genes.
through regulation of ATF4 and CHOP, thereby causing apoptosis. In addition, tolfenamic acid also activated ER stress-associated kinases (JNK) in colorectal cancer cells, which would trigger apoptosis as well (14). Although Sp transcriptional factors played an important role in tolfenamic acid-mediated cell growth inhibition, that specific inhibition is likely to occur at a later stage. Here, we provided evidence that ER stress response was an early event of treatment with tolfenamic acid, whereas Sp down-regulation is later event affected by tolfenamic acid in antitumorigenesis.

We also reported here that tolfenamic acid dramatically reduced the intestinal tumor load and polyp number in an APCMin/+ mouse model of intestinal neoplasia. The potential relevance of our results to human familial and spontaneous colon cancer is provocative. Cyclin D1 overexpression has been demonstrated in adenomas from familial adenomatous polyposis patients (53), and more than 60% of spontaneous human colonic adenocarcinomas exhibit APC mutations (54). Our data are consistent with the recent report that tolfenamic acid exhibits anticancer activity, as assessed by nude mice experiments (37). Along with the fact that each NSAID exhibits distinctive action in anticancer activity, tolfenamic acid has great potential to be translated to the clinic for chemopreventive or therapeutic intervention of cancer. Moreover, our findings suggest that tolfenamic acid would be a very powerful and effective chemopreventive agent for colorectal cancer development, and inactivation of cyclin D1 would provide one mechanism to support tolfenamic acid effects on chemoprevention.
In conclusion, the current study provides further evidence for the antitumorigenic effect of tolfenamic acid on colorectal cancer and elucidates the underlying molecular mechanisms of its action. Together with previous reports, our results suggest tolfenamic acid would be a promising chemopreventive agent for cancer. Novel tolfenamic acid derivatives that show similar safety profiles and enhanced anticancer effects would be attractive in ongoing investigations.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed

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Conception and design: X. Zhang, S.-H. Lee, K.-W. Min, S.J. Baek
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References

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Acknowledgments
The authors thank Misty Bailey for her critical reading of this manuscript.

Grant Support
This work was supported by The University of Tennessee Center of Excellence in Livestock Diseases and Human Health grant (to S.J. Baek), NIH grant R01CA108975 (to S.J. Baek), American Cancer Society grant (RSG-11-133-01-CCE), to S.-H. Lee, and a grant from the Ministry of Agriculture of the People’s Republic of China (2011ZX08008-003; to Q. Li). Financial support for X. Zhang was provided by the Program in Organizational and Personal Cooperation with Foreign Counterparts (2010630161), China Scholarship Council, China.

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Received June 20, 2013; revised August 30, 2013; accepted September 17, 2013; published OnlineFirst October 8, 2013.

Published OnlineFirst October 8, 2013; DOI: 10.1158/1940-6207.CAPR-13-0220

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Tolfenamic Acid and ER Stress


The Involvement of Endoplasmic Reticulum Stress in the Suppression of Colorectal Tumorigenesis by Tolfenamic Acid

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Cancer Prev Res Published OnlineFirst October 8, 2013.

Updated version
Access the most recent version of this article at:
doi:10.1158/1940-6207.CAPR-13-0220

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