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

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Running Title: Tolfenamic acid and ER stress

Keywords: Tolfenamic acid; ER stress; cyclin D1; apoptosis; colorectal cancer

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

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Word count: 5703

Number of figures and tables: 6
Abstract

The nonsteroidal anti-inflammatory drug tolfenamic acid (TA) has been shown to suppress cancer cell growth and tumorigenesis in different cancer models. However, the underlying mechanism by which TA exerts its anti-tumorigenic effect remains unclear. Previous data from our group and others indicate that TA alters expression of apoptosis- and cell cycle arrest-related genes in colorectal cancer cells. Here, we show that TA markedly reduced the number of polyps and tumor load in \( AP\text{C}^{\text{min/+}} \) mice, accompanied with cyclin D1 down-regulation \textit{in vitro} and \textit{in vivo}. Mechanistically, TA 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 2\( \alpha \) (eIF2\( \alpha \)) induces the repression of cyclin D1 translation. Moreover, the PERK-eIF2\( \alpha \)-ATF4 branch of the UPR pathway plays a role in TA-induced apoptosis in colorectal cancer cells, as silencing ATF4 attenuates TA-induced apoptosis. Taken together, these results suggest ER stress is involved in TA-induced inhibition of colorectal cancer cell growth, which could contribute to anti-tumorigenesis in a mouse model.
Introduction

Colorectal cancer is the third-leading cause of cancer-related death in the United States (1). Taking nonsteroidal anti-inflammatory drugs (NSAIDs) has been associated with a reduced risk of colorectal tumorigenesis in epidemiological 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 anti-tumorigenesis (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 anti-tumorigenesis and has received much attention since the inhibition of COX-2 activity produces adverse effects (5).

Tolfenamic acid (TA) is a conventional NSAID that has been long used for treatment of migraines. Compared with the other NSAIDs, TA exhibits fewer upper gastrointestinal side effects (6). Increasing evidence has shown that TA 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 TA-induced anti-cancer activity (7). Previous results from our lab also indicate that TA 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 TA could exert anti-cancer activity through various molecular mechanisms, and prompted us to further investigate the additional molecular mechanisms by which TA induces anti-cancer activity.
Cyclin D1, an oncogenic protein, is often over-expressed 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 TA also down-regulated 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 down-regulation by TA would be beneficial to further understand the anti-tumorigenic activity of TA, and to develop novel derivatives, which could exert more pronounced anti-cancer 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 anti-cancer targets of TA in colon cancer (13, 14). Thus, ER stress could be involved in TA-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 TA’s effect on cell growth remains unclear and needs to be further elucidated.

In the current studies, we first evaluated the chemopreventive effect of TA in a mouse model of colorectal cancer. As a result, we found that TA inhibited polyp formation in APC<sup>min/+</sup> mice, along with a dramatic decrease in cyclin D1 expression in tumors. Consistently, we have also shown that TA down-regulates 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 TA repressed cyclin D1 translation through activation of the ER stress-mediated unfolded protein responses (UPR) pathway. Additionally, ER stress played a role in TA-induced apoptosis in colorectal cancer cells. Therefore, our data strongly suggest that ER stress response could contribute to the anti-tumorigenic activity of TA.
Materials and Methods

Reagents and antibodies

Tolfenamic acid, SC-560, and lactacystin were purchased from Cayman Chemical (Ann Arbor, MI), 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 (St. Louis, MO). Epoxomicin was obtained from Calbiochem (San Diego, CA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA). RNase A was purchased from 5PRIME (Gaithersburg, MD). Antibodies for cyclin D1, ATF4, Bcl-2, and Actin were from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies for pRb (Ser780), total Rb, p-Cyclin D1 (Thr286), BiP, CHOP, p-eIF2α, total eIF2α, PERK, and poly (ADP-ribose) polymerase (PARP) were from Cell Signaling Technology (Beverly, MA); and antibody for HA tag was from GenScript (Piscataway, NJ). 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, Manassas, VA). 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, Rockland, ME). SW480, A549, PC-3, and AsPC-1 cells were maintained in RPMI 1640 medium (Mediatech, Herndon, VA). Caco-2 and LoVo cells were kept in...
Eagle’s Minimum Essential Medium and Ham’s F12 medium (HyClone, Lagon, UT), respectively. All cells were cultured in media supplemented with 10% FBS, 100 units/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, USA) (25). The pCMV-cyclin D1 and ubiquitin (Ub)-HA were gifts from Dr. Richard G. Pestell (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, USA). Cyclin D1 transversion from threonine to alanine at the 286 amino acid position was generated using the QuickChange site-directed mutagenesis kit (Strategene, La Jolla, CA) according to the manufacturer’s protocol. ATF4 expression construct was purchased from OriGene (Rockville, MD), 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, Madison, WI) according to the manufacturer’s protocol. For luciferase assay, cells were seeded in 12-well plates at a density of 1.0×10⁵ cells/well. After transfection with 1 μg p5xATF6-GL3 and pRL-null, cells were treated with DMSO and TA for 24 h. Cell lysates were harvested using 1XPassive lysis buffer (Promega, Madison, WI), 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 × 10⁴ cells/well. Empty vector and ATF4 expression vector were transiently transfected using TransIT-2020
transfection reagent (Mirus Bio LLC) for 24 h. 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 h in the dark, luminescence was measured using a FLX-800 microplate reader (Bio-Tek, Winooski, VT). 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 × 10^5 cells/well in three replicates, and cultured to 60-80% confluence. Then cells were treated with vehicle and TA (50 μM) for 24 h, 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 μM) solution containing RNase A (1 mg/mL) for 15 min at room temperature. The sub G1 phase was determined by a Beckman Coulter Epixs 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 × 10^5 cells/well overnight. Control siRNA, siATF4, or siPERK was transfected at a final concentration of 100 nM using PepMute siRNA & DNA Transfection Reagent (SignaGen, Rockville, MD) according to the manufacturer’s instruction. After 24 h (siATF4) or 48 h (siPERK) transfection, cells were treated with vehicle and TA (50 μM).
RNA isolation, semi-quantitative reverse transcription PCR, and real-time PCR

Total RNA of HCT-116 and SW480 cells treated by DMSO and TA were isolated by an E.Z.N.A Total RNA Kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer’s protocol. Then RNA (1 µg) was reverse transcribed using a Verso cDNA synthesis Kit (ThermoScientific, Pittsburgh, PA). PCR was performed using GoTaq Green Master Mix PCR Reaction Mixture (Promega) with primers for human cyclin D1, EGR-1, ATF3, XBP1, and GAPDH as follows: cyclin D1, forward 5' - ATGGAAACACCAGCTCCTGTGCTGC-3' and reverse 5' - TCAGATGTCCACGTCCGCACGT-3'; EGR-1 forward 5' - CTGCGACATCTGGAAGAAG-3' and reverse 5'- TGTCCTGGGAGAAAAGTTG-3'; ATF3: forward 5'- GTTTGAGGATTGTTGAACCTGAC-3', and reverse 5' - AGCTGCAATCTTATTTCTTCTCGT-3'; XBP1: forward 5' - CTTGTAAGTTGAACAGG-3', and reverse 5'- GGGGCTTGGTATATATGTGG-3'; GAPDH, forward 5'- GGGCTT TTAACTCTGGT-3' and reverse 5' - TGTCAGGTTTTTCTAGACGG-3'. Real-Time PCR was performed using iTaq Universal SYBR Green Supermix (BioRad) with different primer sets for human cyclin D1, EGR-1, or GAPDH as follows: cyclin D1, forward 5'- GGCGGAGGAGAACAAACAGA-3' and reverse 5' - TGTGAGGCGGTAGTAGGACA-3'; EGR-1 forward 5' - CACCTGACCGCAGTCTTT-3' and reverse 5'- CTGACCAAGCTGAAGAGGG-3'; GAPDH, forward 5'- GGGAGCCAAAAGGGTCATCA-3' and reverse 5' - TGATGGCATGGACTGTGGTC-3'. Gene expression levels were calculated and GAPDH was used as a house-keeping gene, using MyiQ thermal cycler (BioRad). The fold changes in mRNA levels were calculated using the \( \Delta \Delta C_t \) method.
Western blot analysis

Cells were washed with PBS and cell lysates harvested using RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) supplemented with 1X protease inhibitor cocktail solution (Calbiochem) and phosphatase inhibitor (1 mM Na3VO4, 1 mM NaF), and centrifuged at 13,000 x g for 10 min at 4°C. Protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL) using BSA as the standard. Protein (30 µg) was mixed with an equal amount of 2XSDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer and boiled for 5 min. After separation by SDS-PAGE, the proteins were transferred to nitrocellulose membranes (Osmonics, Minnetonka, MN). 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 peroxidase-conjugated IgG for 1 h at room temperature, visualized using ECL (Amersham Biosciences, Piscataway, NJ), and quantified by Scion Image Software (Scion Corp., Frederick, MD).

Immunoprecipitation

The cells were harvested using lysis buffer (0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP40, 5% Glycerol, pH7.4) containing with 1× protease inhibitor cocktail solution (Calbiochem) and phosphatase inhibitor (1 mM Na3VO4, 1 mM NaF), and then kept on ice for 30 min. After being spun down for 10 min, the suspension was pre-cleared using protein A/G PLUS-agarose (Santa Cruz Biotechnology) for 30 min at 4°C.
Protein concentration was determined as described above. Protein lysates (1000 µg) were incubated with 5 µg primary antibody and IgG control for 1 h 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 min 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 5 min, and 20 µL of samples were subjected to Western blot analysis.

Animal study

The APCMin/+ mice (C57BL/6 background) were purchased from Jackson Laboratory (Bar Harbor, ME). In order to investigate the long-term effect of TA on tumor formation, mice aged 6-8 weeks were randomly assigned to three groups, and 0, 25, or 50 mg/kg of TA was given by oral gavage with 0.5% methylcellulose (vehicle) every 2 days for 4 weeks. To investigate the short-term effect of TA on tumor formation and protein expression, mice aged 16-18 weeks were randomly divided into two groups, and 0 or 50 mg/kg BW of TA 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 National Institutes of Health 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

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

To evaluate the chemopreventive activity of TA in a mouse model, we examined the number of polyps and tumor load of APC\textsuperscript{min/+} mice treated with vehicle and TA. In the first experiment, we treated mice aged 6-8 weeks with TA for 4 weeks. As shown in Fig. 1A, TA caused a striking reduction in the total number of polyps and tumor load in a dose-dependent manner. Next, we treated mice at 16-18 weeks of age with TA for a short period (3 days) in order to get a tumor sample and observe the short-term effect of TA treatment on tumorigenicity. As shown in Fig. 1B, TA still significantly reduced the number of polyps and tumor load, compared with the control group. Many genes, including β-catenin and Smad had altered expression in tissue samples (data not shown). However, the most interesting and consistent gene alteration was cyclin D1 (Fig. 1C). Both cyclin D1 and COX-2 were overexpressed in tumor tissues, which is in agreement with a previous report (29); however, only cyclin D1 was dramatically suppressed in TA-treated tumor samples.

TA down-regulated cyclin D1 expression in cancer cells

Since cyclin D1 acts as a pro-oncogenic factor, it is not surprising that colorectal cancer cells harbor overexpressed cyclin D1 (Fig. 2A), and its down-regulation may contribute to the anti-proliferative effect of NSAIDs. Since it has been documented that NSAIDs differ in their ability to suppress cyclin D1 expression or cell proliferation (30), we treated SW480 cells for 24 h with the same dose (50 µM) of different NSAIDs: conventional (diclofenac, ibuprofen, aspirin, naproxen, piroxicam, TA), COX-2 selective
(SC-236, DFU), or COX-1 selective (SC-560). TA 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 down-regulation (Fig. 2B). Since we have shown that TA can suppress cyclin D1 in an in vivo model (Fig. 1), we selected this compound for subsequent studies. TA caused rapid cyclin D1 down-regulation in a dose- and time-dependent manner, and the decrease occurred within 1 h (Fig. 2C). To further evaluate the effect of TA on other cancer cells, we treated colorectal cancer cells (HCT-116 and Caco-2), prostate cancer cells (PC-3), pancreatic cancer cells (AsPC-1), and lung cancer cells (A549) with TA. As shown in Fig. 2D, TA dose-dependently down-regulated cyclin D1 expression in different cancer cells.

TA 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 TA decreased phosphorylated Rb at Ser780 in both SW480 and HCT-116 cells (Fig. 3A). To further investigate if cyclin D1 plays a role in the loss of Rb phosphorylation by TA, we transiently transfected cyclin D1 expression vector containing an alanine for threonine substitution at codon 286 (cyclin D1-HA [T286A]), which is more stable than the wild type form (32). Overexpression of the stable form of cyclin D1 restored phosphorylated Rb at Ser780, suggesting that cyclin D1 down-regulation by TA, at least in part, facilitates dephosphorylation of Rb at Ser780 (Fig. 3B).
TA inhibited cyclin D1 at the translational regulation in colorectal cancer cells

To elucidate the molecular mechanism by which TA down-regulated cyclin D1 expression in colorectal cancer cells, we treated HCT-116 and SW480 cells with TA for 1 h and examined the expression of cyclin D1 transcripts. As shown in Fig. 4A, cyclin D1 transcript levels were not altered by TA within 1 h, 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 TA triggered the cyclin D1 degradation. Results in Fig. 4B and C indicate that neither endogenous nor exogenous cyclin D1 stability was affected by TA. Furthermore, treatment of inhibitors responsible for cyclin D1 degradation did not restore cyclin D1 expression in the presence of TA (Fig. 4D and S1). In addition, TA 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 S2). Taking these results together, we found that TA did not affect transcription nor protein degradation pathway of cyclin D1; rather TA may inhibit cyclin D1 translation initiation.

TA promoted ER stress response

To further understand the cellular signaling pathways involved in TA-mediated cyclin D1 translation, we investigated the effect of TA on the mammalian target of rapamycin (mTOR) pathway, which is mainly responsible for protein translation. However, we did not observe that TA 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. Since ER stress usually induces
phosphorylated eIF2α, we therefore asked if TA 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 TA 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 TA could be a potential activator of ER stress.

**Cyclin D1 suppression and ATF4 induction by TA 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 TA. As shown in Fig. 6A, TA 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 TA (Fig. S3). Moreover, silence of PERK using specific siRNA slightly restored cyclin D1 expression in the presence of TA (Fig. 6B), suggesting the PERK-eIF2α pathway was involved in TA-mediated cyclin D1 down-regulation. The UPR is a pro-survival response to allow cells to remove unfolded proteins and re-establish homeostasis. However, if the primary stimuli causing unfolded proteins are persistent and the stress cannot be resolved, signaling switches
from pro-survival to pro-apoptosis (34). Of the UPR signaling pathway branches, PERK-eIF2α-ATF4 up-regulates CHOP expression, which subsequently induces apoptosis in part through down-regulating BCL-2 gene expression (19). As shown in Fig. 6B, TA-induced CHOP expression is mediated by the PERK-eIF2α-ATF4 axis. Consistent with CHOP up-regulation, TA also down-regulated Bcl-2 expression accompanied with the induction of apoptosis as measured by cleaved PARP and the percentage of sub-G₁ fraction cells (Fig. 6C). In addition, overexpression of ATF4 increased caspase 3/7 activity, supporting the previously documented pro-apoptotic role of ATF4 in ER stress (35, 36) (Fig. 6D). Finally, knockout of ATF4 using specific siRNA blocked TA-induced CHOP expression and subsequent Bcl-2 down-regulation, and attenuated apoptosis as measured by cleaved PARP (Fig. 6E). These data indicated TA 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 TA exhibits anti-cancer activity via different molecular targets, including Sp1 (7, 37), ATF2/ATF3 (14), NAG-1 (13), NF-κB (38), and Smads (39). In the present study, we further confirmed that TA is a powerful chemopreventive or therapeutic agent against colorectal cancer in vitro and in vivo, and that TA-mediated growth inhibition might be associated with ER stress response, especially the PERK-eIF2α axis, which resulted in down-regulation of pro-survival proteins like cyclin D1, and up-regulation of pro-apoptotic proteins like CHOP. In addition, we found that TA markedly down-regulated cyclin D1 in tumors and a large variety of cell lines, and that this down-regulation partially contributed to the loss of phosphorylated Rb. Given the key role of cyclin D1 in G1-S phase transition, down-regulation of cyclin D1 by TA could induce cell cycle arrest. Indeed, there are several reports showing that treatment with TA results in G0/G1 cell cycle arrest (11, 40, 41). On the other hand, we also provided direct evidence that TA induced apoptosis via activation of the PERK-eIF2α-ATF4 pathway.

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

Interestingly, we found that cyclin D1 degradation was not enhanced in the presence of TA (Fig. 4B-E). Based on our data, we suggest that TA could be able to suppress cyclin D1 translation, at least in colorectal cancer cells. TA 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_i^{Met} 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 TA. 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 TA. 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 detail pathways need to be elucidated.

Accumulating evidence suggests that the activation of UPR exerts contradictory consequences to tumor 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 anti-cancer activity have been shown to induce ER stress-mediated cell cycle arrest and apoptosis (46-49). Our data indicate that TA exerts anti-cancer 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 analogue (21, 50), suggesting TA and celecoxib might exhibit comparable anti-neoplastic activity in a COX-independent manner.

We have identified EGR1 and ATF3 as mediators of TA-induced apoptosis in colorectal cancer cells (13, 14). Interestingly, both of them are inducible under ER stress (51, 52). TA might transcriptionally up-regulate expression of those two pro-apoptotic genes through regulation of ATF4 and CHOP, thereby causing apoptosis. In addition, TA 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 TA-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 TA, whereas Sp down-regulation is later event affected by TA in anti-tumorigenesis.

We also reported here that TA dramatically reduced the intestinal tumor load and polyp number in an APCMin/+ mouse model. Although we treated older mice with TA for only 3 days, the number of polyps and tumor load were still significantly decreased. The results described here extend the involvement of cyclin D1 to the development of benign lesions in the APCMin/+ 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 TA exhibits anti-cancer activity, as assessed by nude mice experiments (37).
Along with the fact that each NSAID exhibits distinctive action in anti-cancer activity, TA has great potential to be translated to the clinic for chemopreventive or therapeutic intervention of cancer. Moreover, our findings suggest that TA would be a very powerful and effective chemopreventive agent for colorectal cancer development, and inactivation of cyclin D1 would provide one mechanism to support TA effects on chemoprevention.

In conclusion, the current study provides further evidence for the anti-tumorigenic effect of TA on colorectal cancer and elucidates the underlying molecular mechanisms of its action. Together with previous reports, our results suggest TA would be a promising chemopreventive agent for cancer. Novel TA derivatives that show similar safety profiles and enhanced anti-cancer effects would be attractive in ongoing investigations.
Acknowledgements

We thank Misty Bailey for her critical reading of this manuscript.
Reference

Figure Legends

Figure 1. TA 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 BW, or 50 mg/kg BW of 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 BW TA 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 BW TA were subjected to Western blot analysis for COX-2, cyclin D1, and actin expression.

Figure 2. TA down-regulates cyclin D1 in cancer cells. (A) Endogenous cyclin D1 expression in different colorectal cancer cells. Western blot 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 µM for 24 h. 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 µM TA for 24 h (left panel) and 50 µM TA for 0, 1, 3, 6, 12, or 24 h (right panel). 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 µM of TA for 24 h. Western blot analysis was carried out for analyzing cyclin D1 and actin.

Figure 3. TA activates Rb via dephosphorylation. (A) HCT-116 and SW480 cells were treated with 50 µM TA for 0, 1, 3, 6, 12, or 24 h. 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 h, followed by treatment with 50 µM TA for 0, 6, 12, or 24 h. Western blot analysis was performed for phospho-Rb (Ser780), total Rb, HA, and actin.

Figure 4. TA suppresses cyclin D1 translation. (A) HCT-116 and SW480 cells were treated with 0, 5, 25, or 50 µM of TA for 1 h. Semi-quantitative RT-PCR was carried out for analyzing cyclin D1, EGR-1, ATF3, and GAPDH mRNA expression using specific primers as described in Materials and Methods. Realtime PCR was also performed as described in Material and Methods (right panel). (B) SW480 cells were treated with 0, 10, or 25 µM of TA for 1 h, followed by incubation with 10 µg/mL cycloheximide (CHX) for the indicated times. Cell lysates were subjected to Western blot using cyclin D1 and actin antibodies. (C) Cyclin D1-HA construct was transiently transfected into HCT-116 cells along with GFP expression vector serving as a transfection control for 24 h. Cells were treated with DMSO and 50 µM TA for 1 h, followed by incubation with 10 µg/mL cycloheximide (CHX) for the indicated times. Cell lysates were analyzed using HA (Cyclin D1) and GFP antibodies. (D) SW480 cells were treated with DMSO, lactacystin (10 µM), epoxomicin (1 µM), NH4Cl (20 mM), z-VAD-fmk (50 µM), calpeptin (50 µM), leupeptin (200 µM), KU55933 (10 µM), or staurosporine (2 µM) for 1 h,
followed by incubation with 50 µM of TA for 1 h, as indicated. Cell lysates were analyzed for cyclin D1 and actin using Western blot. (E) The pCMV-cyclin D1 and Ub-HA plasmids were co-transfected into HCT-116 cells for 24 h. Cell lysates were immunoprecipitated using either cyclin D1 antibody (IP: cyclin D1) or nonspecific IgG antibody (IP: IgG). Cyclin D1 ubiquitination, phosphorylation of cyclin D1, and total amount of cyclin D1 were determined with the indicated antibody.

Figure 5. TA promotes ER stress response. (A) HCT-116 and SW480 cells were transiently transfected with p5×ATF6-GL3 and pRL-null for 24 h, followed by treatment with 0, 5, 25, or 50 µM of TA for 24 h. Luciferase activity was measured as described in Materials and Methods. RLU, relative luciferase unit. Results were considered statistically significant at *P<0.5, **P<0.01 and ***P<0.001, compared to DMSO. (B) HCT-116 and SW480 cells were treated with 0, 5, 25, or 50 µM of TA for 24 h. RT-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 µM of TA for 0, 1, 3, 6, 12, or 24 h. The cell lysates were subjected to Western blot for analyzing BiP, ATF4, CHOP, and actin expression.

Figure 6. TA affects cyclin D1 and ATF4 expression via PERK-eIF2α axis. (A) HCT-116 cells were treated with 50 µM TA for the indicated times. Western blot was performed for analysis of phosphorylated eIF2α, total eIF2α, cyclin D1, and actin expression. (B) Knockout of PERK was performed as described in Materials and Methods in HCT-116 cells, followed by incubating with 50 µM TA for 24 h, as indicated. Cell lysates were analyzed for PERK, Cyclin D1, ATF4, CHOP, and actin expression. (C) HCT-116 cells
were treated with 50 µM TA for the indicated times. Western blot was carried out using Bcl-2, PARP, and actin antibodies. The proportion of the sub-G₁ phase of cells treated with 50 µM TA for 24 h was determined by flow cytometric analysis as described in Materials and Methods. The data are expressed as mean ± SD of three replicates. Results were considered statistically significant at **P<0.01, compared to DMSO. (D) The ATF4 construct was transfected into HCT-116 cells. Transfection efficiency was examined by Western blot using ATF4 antibody. Caspase 3/7 activity was measured as described in Materials and Methods, and the data are expressed as mean ± SD of three wells. Results were considered statistically significant at **P<0.01, compared to EV. (E) HCT-116 cells were transfected with siRNA against ATF4 for 24 h, followed by treatment with 50 µM TA for 24 h, as indicated. Cell lysates were subjected to Western blot analysis using ATF4, CHOP, Bcl-2, PARP, and actin antibodies.
Cancer Prevention Research

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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