

ABSTRACT

Title of Thesis:

ANTICANCER MECHANISM OF TOLFENAMIC ACID IN COLORECTAL CANCER

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Colorectal cancer (CRC) is the third leading cause of cancer-related death in the United States. Chemopreventive therapies could be effective way to treat CRC. Tolfenamic acid, one of the NSAIDs, shows anti-cancer activities in several types of cancer. Aberrant Wnt/ β -catenin regulation pathway is a major mechanism of colon tumorigenesis. Here, we sought to better define the mechanism by which tolfenamic acid suppresses colorectal tumorigenesis focusing on regulation of β -catenin pathway. Treatment of tolfenamic acid led to a down-regulation of β -catenin expression in dose dependent manner in human colon cancer cell lines without changing mRNA. MG132 inhibited tolfenamic acid-induced downregulation of β -catenin and exogenously overexpression β -catenin was stabilized in the presence of tolfenamic acid. Tolfenamic acid induced an ubiquitin-mediated proteasomal degradation of β -catenin. In addition, tolfenamic acid treatment decreased transcriptional activity of β -catenin and expression of Smad2 and Smad3 while overexpression of Smad 2 inhibited tolfenamic acid-stimulated transcriptional activity of β -catenin. Moreover, tolfenamic acid decreased β -catenin target gene such as vascular endothelial growth factor (VEGF) and cyclin D1. In summary, tolfenamic acid is a promising therapeutic drug targeting Smad 2-mediated downregulation of β -catenin in CRC.

ANTICANCER MECHANISM OF TOLFENAMIC ACID IN COLORECTAL
CANCER

By

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Chapter 1: Background

1.1 Significance of chemoprevention and chemotherapy in colorectal cancer (CRC)

Cancer is one of the major causes of death throughout the world and second leading cause of death in US. Based on the annual report that American Cancer Society estimated, 1,685,210 new cancer cases will be diagnosed and 595,690 cancer deaths will occur in the United States in 2016 (1). Among them, colorectal cancer (CRC) occupy third places in prevalence and cancer-related death in both men and women (1). Around 75% incidences of colorectal cancer are sporadic and remaining 25% is hereditary. Numerous epidemiological studies showed that CRC can be preventable by changing eating pattern and lifestyle and avoiding environmental risk factors. They include smoking, drinking, obesity, physical inactivity, low calcium intake, low intake of fruit and vegetable and high intake of red or processed meat (2). For the past decades, there was a decent decreasing cancer mortality in the United States due to the advances in early detection and screening technology (3). However, projected numbers of CRC-related deaths in US is 49,190 in 2016 (1) because advanced stage of CRC remain untreatable. Therefore, it is urgent to screen and find effective anticancer compounds for chemoprevention. Chemopreventive strategy using nature products or synthetic drugs could prevent or delays the onset and reverse the process of several types of cancers (4). More specifically, long term use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been showed to prevent CRC in different stage of tumorigenesis in basic studies, epidemiological studies and clinical trials (5-7).

1.2 CRC and nonsteroidal anti-inflammatory drugs (NSAIDs)

NSAIDs are a class of drugs that contain analgesic, antipyretic, and anti-inflammatory properties. Most well-known mechanism of NSAIDs is to inhibit prostaglandin (PG) synthesis via deactivating activity of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), first limiting enzymes of prostaglandin biosynthesis (8). COX enzyme converts arachidonic acid to prostaglandin H₂ (PGH₂). Then PGH₂ is converted into diverse PG including PGE₂, PGI₂, PGJ₂ and thromboxane and carries out a diverse biological activity including oncogenesis and thrombogenesis. Another function is a cytoprotective activity in the gastric mucosa and renal epithelial cells (9). Thus, long-term use of COX inhibitors may cause gastrointestinal tract bleeding, ulcer or kidney failure, which mainly due to inhibition of COX-1 (10). While COX-1 is constitutive function, COX-2 is an inducible enzyme and responsible for the chemopreventive effect of NSAIDs. As COX-2 expression and PG synthesis is elevated in CRC (11), COX-2 inhibitors such as celecoxib are regarded as the promising CRC preventive agents. The responsible anticancer mechanisms of NSAIDs include increased apoptosis, cell-cycle arrest and inhibition of angiogenesis (12, 13). However, unexpected side effect and toxicity led to withdrawal of COX-2 selective inhibitors from market. However, inhibition of COX is not the only pathway of chemopreventive activities of NSAIDs, but COX-independent pathways exist (14, 15). Thus, many research group study to find effective and safe anticancer drugs targeting other cancer pathway as well as COX.

1.2.1. Aspirins

Aspirin has been the most commonly used to treat inflammation, fever, and pain in clinical setting since Felix Hoffmann at Bayer in Germany found that extracts from willow bark possessed anti-inflammatory and anti-pyretic activity in 1898 (16). It is typical traditional NSAID inhibiting both COX-1 and COX-2 irreversibly by acetylation of serine residues in the active and catalytic binding domain for arachidonic acid (11, 13). Low-dose of aspirin intake (around 70–100 mg/day) is widely used for health benefit including prevention of cardiovascular disease and CRC. Kune et al. reported an inverse association between use of aspirin and the risk of CRC through human clinical trials for the first time (17). In addition, recent other cohort and case-control studies indicated that aspirin may be effective at preventing several types of cancer particularly in colorectal cancer (18-20). As a COX inhibitor, aspirin inhibits synthesis of prostaglandin E2 (PGE2) which is well known oncogenic pathway in tumor development and progression. However, aspirin effect is also observed in COX-null colorectal cancer cells, proposing COX-independent anticancer pathway (21). Pathi et al pointed that aspirin inhibits specificity protein (Sp), Sp1, Sp3 and Sp4 transcription factors through caspase-dependent proteolysis in colon cancer cells (22).

1.2.2. Sulindac sulfide (SS)

Sulindac has been shown to inhibit tumorigenesis activities including angiogenesis and tumor cell invasion in preclinical setting (23, 24). For example, sulindac treatment decreases polyp formation in FAP patients (15, 25). Sulindac

sulfide is a metabolite of sulindac and effectively suppresses colon cancer as an inhibitor of COX-1 and COX-2 whereas it results in severe side effect including gastrointestinal ulceration and bleeding (30). Sulindac, lacks COX inhibitory activities, can undergo reversible reduction to pharmacologically active sulindac sulfide. And sulindac and sulindac sulfide will also irreversibly oxidize to sulindac sulfone metabolites with hepatotoxicity (26).

Sulindac sulfone has been shown that anti-tumorigenesis undergoes COX independent pathway in animal model in different cancer models (27-30). Some mechanistic studies showed that sulindac sulfone can suppress oncogenic β -catenin signaling by inhibiting cyclic guanosine monophosphate phosphodiesterase (cGMP PDE) (31). Sulindac sulfide also induces the expression of tumor suppressor NSAIDs activated gene 1 (NAG-1) (32). However, sulindac do not completely protect all individuals from developing cancer. Keller et al pointed out that treatment of sulindac may produce resistant adenomas and breakthrough carcinomas, which limit chemopreventive activity of NSAIDs (33).

1.2.3. Celecoxib

Celecoxib is most common selective COX-2 inhibitor. Many side effects including gastrointestinal ulceration and kidney failure are mainly attributed to inhibition of COX-1. As the result, selective COX-2 inhibitor can avoid side effects of non-selective NSAIDs because they are not targeting COX-1 (10). Prolonged treatment of celecoxib may also be associated with increased risk of cardiovascular

side effects, such as an increase in blood pressure, myocardial infarction, and stroke (10, 34, 35).

Celecoxib can directly inhibit the DNA-binding activity of Sp1 transcription factor which is a crucial driver of VEGF overexpression in cancer cells (36). In addition, celecoxib has been shown to inhibit invasion through downregulation of matrix metalloproteins (MMP) 2 and 9, the enzymes to help disassociate with cell junctions and eventually lead to metastasis (37, 38). All of these evidences suggest NSAIDs can go through COX-independent pathway to exhibits anti-cancer effects.

1.3 Tolfenamic acid

Tolfenamic acid ((N-(2-methyl-3-chlorophenyl)-anthranilic acid) is a derivative of anthranilic acid (39) and inhibits inflammation through inhibition of leukotriene B4 (LTB4)-induced chemotaxis (40) and has been broadly used for the treatment of migraines. The optimal dose of migraine treatment is 200 mg when the first symptoms appear (41). Recently we reported an anti-inflammatory activity of tolfenamic acid in human CRC (42).

Like other NSAIDs, tolfenamic acid exhibits anti-cancer activities in different cancer models, such as lung, esophageal, breast, prostate, ovary and pancreatic cancer cells (43-49). Since tolfenamic acid show little side effect (41, 50), our lab chose tolfenamic acid as a study compound and investigated detailed anti-cancer mechanisms.

We observed that tolfenamic acid induced growth arrest and apoptosis in colon cancer cells through NF-kappa B activation (51), reactive oxygen species (ROS)

generation (52), ESE-1/EGR-1 activation (53) and mitogen-activated protein kinases (MAPK) pathway (54). Recently, we also found tolfenamic acid suppressed formation of tumor in $Apc^{Min/+}$ mice *in vivo* (55). In terms of metastasis, Abdelrahim et al have been showed tolfenamic acid was associated with suppression of vascular endothelial growth factor (VEGF) and its receptor (VEGFR1) in pancreatic cancer (56, 57). Taken together, there are several proposed molecular mechanisms of anti-tumorigenic activity by tolfenamic acid.

1.4 Introduction of colon cancer tumorigenesis

1.4.1. Significant of β -catenin in CRC

Truncated deletion of APC gene is identified in familial adenomatous polyposis (FAP) syndrome, which is characterized by early onset colorectal cancer (58) and ovarian tumors (59). Wild-type APC make a complex with axin, GSK3b, casein kinase 1 and β -catenin and facilitates the proteasomal degradation of β -catenin through ubiquitination. As a result, mutation in APC gene leads to accumulation of β -catenin (60). In addition, mutation of axin, the scaffolding protein in complex formation, or deletion of β -catenin at N-terminal Ser/Thr destruction motif also escapes β -catenin degradation. Surplus β -catenin is translocated into nuclear and binds to TCF4. The β -catenin/TCF4 complex directly binds to TCF-binding site and regulates expression of target genes such as VEGF, cyclin D1 and c-myc. Thus, β -catenin is promising preventive and therapeutic target for colorectal cancer (61, 62).

β -catenin is a downstream target of Wnt pathway (63). It was first classified as a protein which binds to E-cadherin in adherent junctions that are required to maintain

the architecture of epithelia. With down-regulation of E-cadherin, β -catenin can be released from cadherin complexes. The level of β -catenin in cells is tightly controlled through interactions with other proteins, such as APC, GSK-3 β , and axin (64, 65). With the absence of Wnt, cytoplasmic β -catenin forms a complex with APC, glycogen synthase kinase 3 (GSK3), axin and casein kinase 1 (CK1). β -catenin gets phosphorylated and recognized by ubiquitin ligase, and lead to proteasomal degradation at 26S proteasome (68) through interaction with β -TrCP (66). However, with the presence of Wnt ligand, Axin-mediated phosphorylation and degradation of β -catenin was disrupted by receptor complex formed between Frizzled (Fz) and low density lipoprotein receptor-related protein 5/6 (LRP5/6). Most identified mutations results in removing binding sites of beta-catenin and axin, and results in β -catenin elevated. Therefore, β -catenin accumulated and moved into nucleus to function as a coactivator for TCF to activate Wnt-response downstream gene by interacting with N terminus of DNA-binding proteins lymphoid enhancer-binding factor 1/T cell-specific transcription factor (LEF/TCF) proteins family (60, 67).

Nuclear β -catenin accumulation can be detected in more than 80% of CRC tumors (68). Moreover, an epidemiological study conducted by Baldus et al pointed that high levels of nuclear β -catenin have been associated with a poor prognosis in CRC patients (69) and this idea was also supported by other groups (70-72). Oncogenic target genes such as VEGF and cyclin D1 can also be transactivated by β -catenin translocation and binding to LEF/TCF (63, 73-75). Thus β -catenin downregulation and the β -catenin/TCF complex interruption could be promising strategies for prevention and treatment of colon cancer.

1.4.2. Upstream regulators of β –catenin: Wnt, TGF- β , PI3K

Wnt. The Wnt signaling plays important roles in regulation of tumorigenesis. Dysregulation of Wnt signaling is involved in the development of multiple cancers, especially in colorectal cancer (76). There are two different pathways of Wnt signaling, one is canonical Wnt pathway for cell growth and apoptosis, and another is non-canonical Wnt pathway (the planar cell polarity, PCP pathway; and the WNT–Ca²⁺ pathway) for control of tissue polarity and cell movement (77). Different ligands' activation can be used to differentiate canonical (Wnt1, 2, 3, 3A, 8A, 8B, 10A and 10B) and non-canonical (Wnt4, 5A, 5B, 6, 7A, 7B) pathways(78). Numbers of studies have been shown that dysregulation of canonical Wnt can lead to cancer development and progression (79).

TGF- β . Transforming growth factor beta (TGF- β) represents a major growth-inhibitory signal that normal cells, such as epithelial cells, evade in order to become cancer cells (80). Smad transcriptional factors serve as major mediators in TGF-beta signaling of the Smad-dependent pathway (81). TGF- β ligands bind to type two TGF- β receptor and then recruit type one TGF- β receptor. These allow Smad2 and Smad3 phosphorylation. Activated Smad proteins bind with Smad4 and translocate to nucleus to mediate gene activities (82, 83). In cancer development, TGF- β may have dual functions: a tumor suppressor or tumor promoter, depending on the stage of tumorigenesis. TGF- β could inhibit cell growth in normal or pre-neoplastic epithelial cells. However, TGF- β would promote tumor growth and metastasis in late stage of cancer (84).

Recent studies demonstrated TGF- β /Smad and Wnt/ β -catenin signaling cross-talk to each other (85, 86). TGF- β and Wnt ligands can work together to regulate cell differentiation and apoptosis by controlling gene expression. Taketo et al developed Smad4/APC heterozygotes mice, and observed more malignant tumors in these mice compare to those in APC heterozygotes mice (87). Another *in vivo* study performed by Hamamoto et al, Smad2/APC heterozygotes mice was observed that disruption of Smad2 mediated malignant progression of intestinal tumors in the APC mice, but the difference was not severe (88). More work need to be done in order to understand TGF- β and Wnt interactions.

PI3K. Phosphoinositide 3-kinase (PI3K) is a family of enzymes that important for cell cycle, proliferation, survival and protein synthesis (89). Dysregulation of oncogenic PI3K pathway would drive to cancers. In cancer cells, receptor tyrosine kinase (RTK) constitutively activated and leads to PI3K activation (90). Since PI3K is a critical mediator in cancer development, making it an important target in the treatment of cancer. Recent study also indicated that PI3K would have some association between β -catenin. Liu et al suggested level of β -catenin could alter PI3K effect on NF-kappa B activity in CRC cells (91). The detailed β -catenin and PI3K pathway interactive still remain unclear.

In the present study, we examined whether treatment of tolfenamic acid could alter β -catenin expression and found β -catenin decreased in dose dependent manner in human colon cancer cell lines. We further found β -catenin downregulation by tolfenamic acid treatment undergone ubiquitin-mediated proteasomal degradation.

We also found there is a cross-talk between Wnt and TGF- β signaling in our designed study. Tolfenamic acid treatment would downregulate Smad2 and Smad3 via overexpression of Smad 2, but not Smad3. Moreover, tolfenamic acid also decreased β -catenin target gene. Thus, tolfenamic acid could be a potential cancer therapeutic drug colorectal cancer.

Chapter 2: Materials and Methods

2.1 Materials

Tolfenamic acid was purchased from Cayman Chemicals (#70480) (Ann Arbor, Michigan, and dissolved in dimethyl sulfoxide (DMSO). MG-132 was purchased from Calbiochem (San Diego, CA). Antibodies for β -catenin (#9562), Smad2 (#5339) and Smad3 (#9513) were purchased from Cell Signaling (Beverly, MA). Antibodies for cyclin D1 (sc-718), green fluorescent protein (GFP) (sc-8334), and actin (sc-1615) were purchased from Santa Cruz (Santa Cruz, CA). The GFP-tagged β -catenin expression vector was described previously (92, 93). Media for cell culture were purchased from Invitrogen (Carlsbad, CA). All chemicals were purchased from Fisher Scientific or VWR, unless otherwise specified.

2.2 Cell culture

Human colon adenocarcinoma cells (SW480, HCT116, LoVo and CaCO₂) were purchased from American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle medium (DMEM/F-12) media supplemented with 10% fetal bovine serum (FBS), a mixture of penicillin (100 U/mL) and streptomycin (100 μ g/mL) under a humidified atmosphere of 5% CO₂ at 37°C.

2.3 Isolation of RNA and semi-quantitative RTPCR

Total RNA was prepared using RNeasy Mini kit (Qiagen). Total RNA (1 μ g) was reverse-transcribed with the Verso cDNA kit (Thermo Scientific) according to the

manufacturer's instruction. PCR was carried out using ReadyMix Taq polymerase (Sigma) with primers for human β -catenin and GAPDH.

β -catenin (CTNNB1):

Forward 5'-CCCACTAATGTCCAGCGTTT-3'

Reverse 5'-AATCCACTGGTGAACCAAGC-3'

GAPDH:

Forward 5'-GGGCTGCTTTTAACTCTGGT-3'

Reverse 5'-TGGCAGGTTTTTCTAGACGG-3'.

2.4 DAPI staining

The cells were washed with PBS three times and fixed with 3.7% formaldehyde.

After three times washed with PBS, 0.2% Triton X-100 was used to permeabilize the cells for 5 mins. The cells washed again, and 4', 6-diamidino-2-phenylindole (DAPI stock diluted 1:5000 in PBS) labeling solution was added at room temperature for 5 mins. The Nuclear DNA images were taken by fluorescence microscope.

2.5 Transient transfection and Luciferase assay

The expression vectors (for GFP-tagged β -catenin, Smad2 and Smad3) and luciferase reporters (TOP/FOP FLASH) were transiently transfected using PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD, USA) according to the manufacturer's instruction. For luciferase assay, the cells were transfected with plasmid containing β -catenin/TCF-LEF-responsive (TOP-FLASH) and mutant (FOP-FLASH) promoters for 24 hours. The transfected cells were treated in the compounds

for 24h. The cells were harvested in 1× luciferase lysis buffer. The luciferase activity was measured and normalized to the pRL-null luciferase activity using a dual-luciferase assay kit (Promega).

2.6 SDS-PAGE and Western blot:

Cells were washed with 1 × phosphate-buffered saline (PBS), sat on ice for 15 minutes in radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA) supplemented with protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and phosphatase inhibitor cocktail (Sigma Aldrich) and then harvested. The cell lysate was centrifuged at $12,000 \times g$ for 15 min at 4°C. Protein content was measured by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). The proteins were separated on SDS-PAGE, transferred to nitrocellulose membranes (Osmonics, Minnetonka, MN) and blocked in 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1h at room temperature. Membranes were probed with specific primary antibodies in 3% Bovine Serum Albumin (Santa Cruz) at 4 °C overnight and then with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 h at room temperature. Chemiluminescence was detected with Pierce ECL Western blotting substrate (Thermo Scientific) and visualized by ChemiDoc MP Imaging system (Bio-Rad, Hercules, CA).

2.7 Statistical analysis

Statistical analysis was performed with ImageJ (NIH) and SPSS. Data was analyzed by Student's t-test. Data represent mean \pm SD from three replicates.

Chapter 3: Tolfenamic acid downregulates β -catenin at post-transcriptional level in human colon cancer cells

3.1 Effects of NSAIDs and dietary phytochemicals on cell growth arrest in SW480 cell

According to recent studies, several types of NSAIDs and dietary phytochemicals possess strong anticancer activity in CRC (4). Since aberrant regulation of Wnt/ β -catenin is common during colon tumorigenesis (94-96), we investigated if several NSAIDs and dietary phytochemicals may affect β -catenin expression in human colon cancer cells. Selected NSAIDs (50 μ M sulindac sulfide, 50 μ M tolfenamic acid, 50 μ M aspirin, 50 μ M diclofenac, 50 μ M SC-560 and 50 μ M celecoxib) and phytochemicals (50 μ M epigallocatechin-3-gallate, 50 μ M resveratrol and 50 μ M genistein) are broadly used as potential anticancer drugs to treat many types of cancer. Sulindac sulfide (a metabolite of sulindac), tolfenamic acid, aspirin and diclofenac are non-selective NSAIDs. SC-560 and celecoxib is COX-1 and COX-2 selective inhibitor, respectively. For screening the compounds, we used SW480 human colorectal adenocarcinoma cells. This cell line has APC mutation and active β -catenin signaling. The results indicated that sulindac sulfide and tolfenamic acid significantly decreased expression of β -catenin and celecoxib and epigallocatechin-3-gallate treatment tend to decreased β -catenin expression (Figure 3.1A). These data suggest that decreased β -catenin could be a potential mechanism of anticancer activity of sulindac sulfide and tolfenamic acid. We selected tolfenamic acid for further studies due to its novelty and potential of promising anticancer drugs and less toxicity in human studies (50).

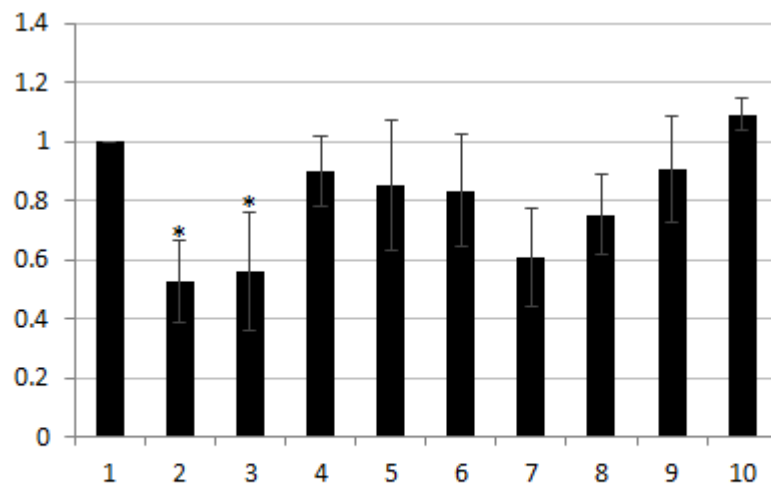
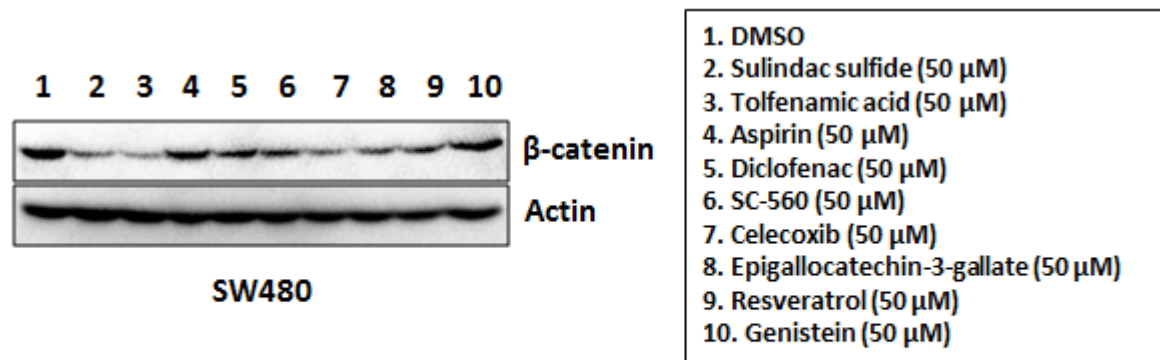


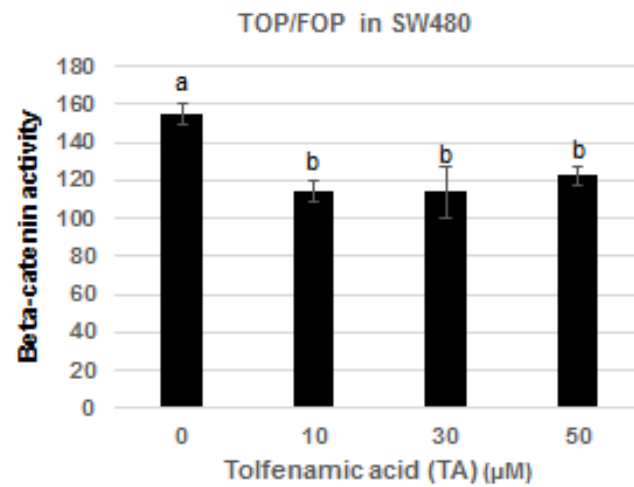
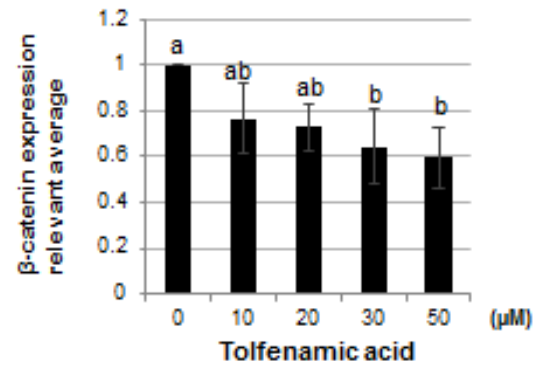
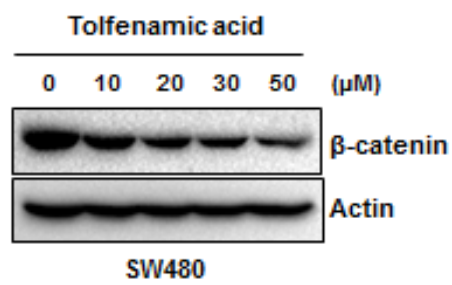
Figure 3.1. Several NSAIDs and dietary compounds decrease β -catenin expression level. SW480 cells were treated for 24 hours with 50 μ M of different compounds (DMSO, 6 NSAIDs and 3 dietary phytochemicals) listed above. Lysates were harvested and western blot was performed to analyze the β -catenin expression level in SW480. Actin is the housekeeping marker.

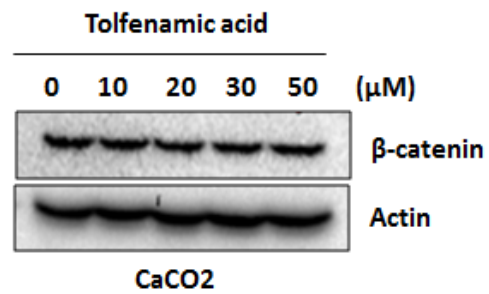
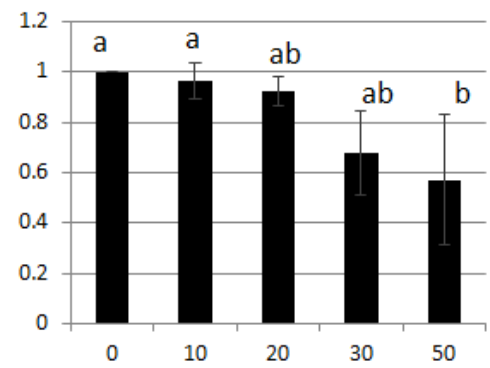
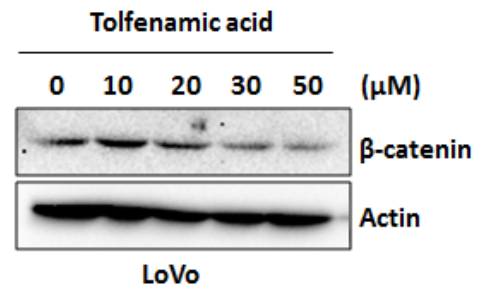
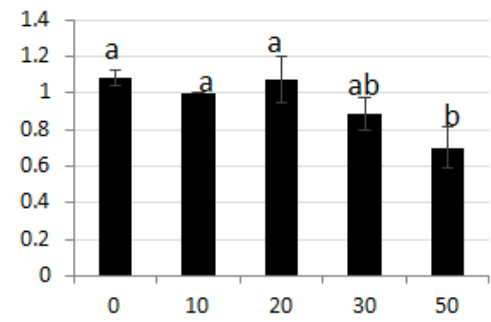
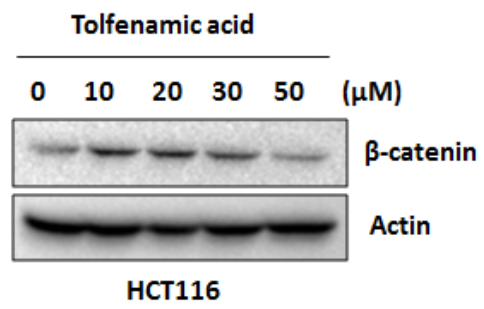
3.2 Tolfenamic acid decreased β -catenin expression level in human colon cancer cells

We tested if tolfenamic acid downregulates expression of β -catenin in dose dependent manner. The cells were treated with different dose of tolfenamic acid (0, 10, 20 30 or 50 μ M) for 24 hours and western blot was performed against β -catenin and actin antibody. As shown in Fig. 3.2A, tolfenamic acid decreased β -catenin expression in dose-dependent manner in SW480. TOP/FOPflash data also confirmed β -catenin declining with the tolfenamic acid treatment (Figure 3.2A, bottom) because the downstream target gene luciferase expression decreased . We also tested if tolfenamic acid affect expression of β -catenin in other human colorectal cancer cells with different genetic background. HCT116 and LoVo cells decreased β -catenin expression in response to tolfenamic acid, but Caco-2 cells did not change the β -catenin expression (Figure 3.2B).

Since β -catenin downregulation is associated with increase apoptosis, we stained tolfenamic acid-treated cells with DAPI to look at DNA fragmentation and chromatin condensation. As a result, we found an increase of DNA fragmentation in the SW480 cells treated with 30 and 50 μ M of tolfenamic acid (Figure. 3.2C).

A



B

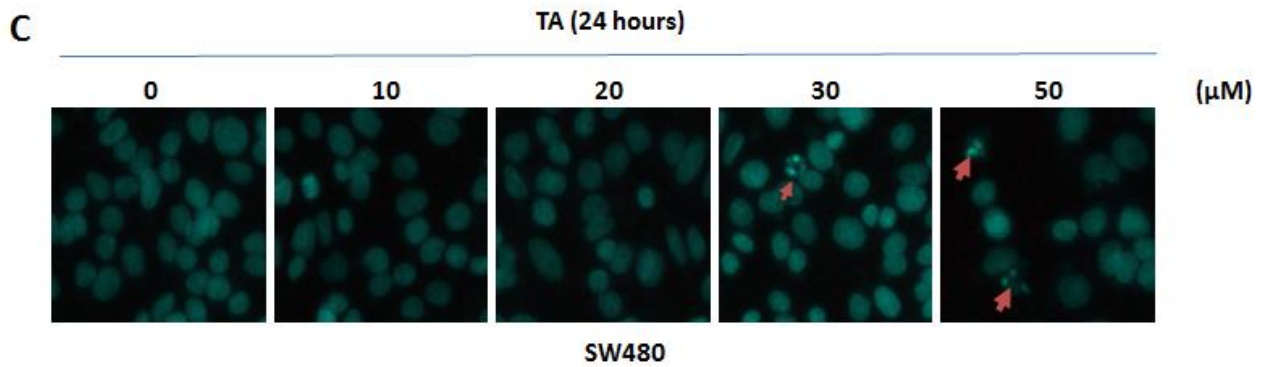
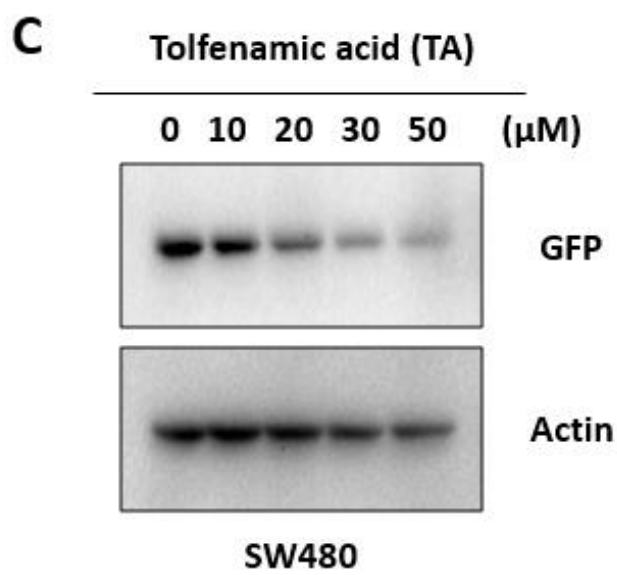
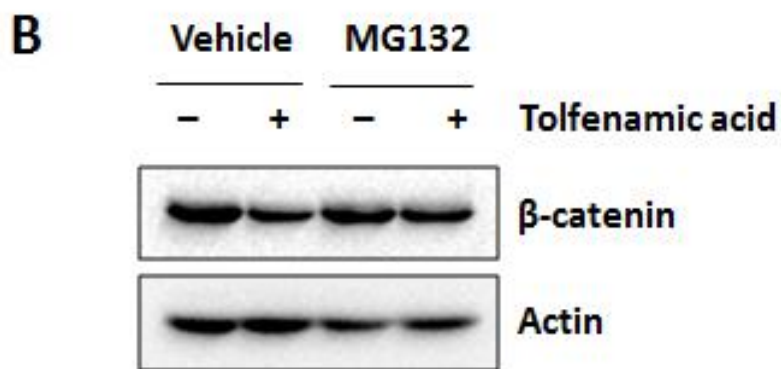
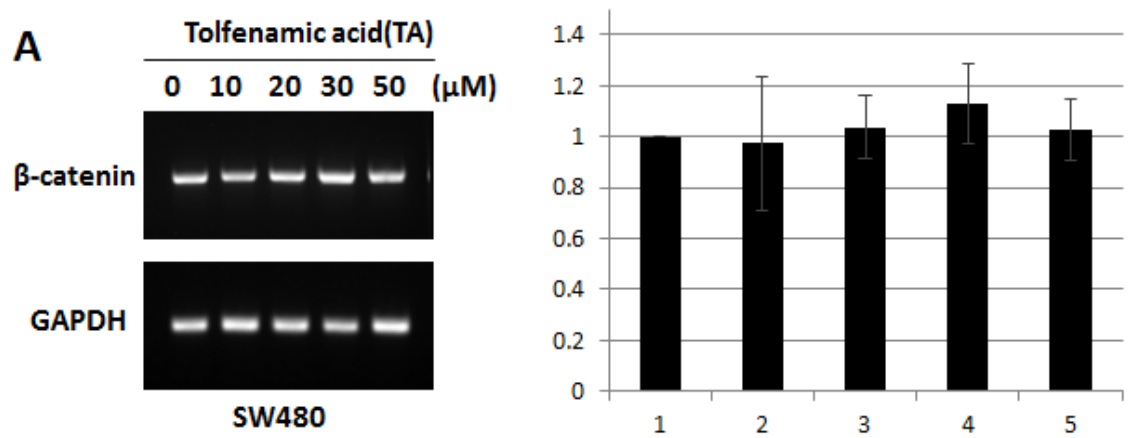


Figure 3.2. Tolfenamic acid decreased expression of β -catenin protein in dose-dependent manner in human colorectal cancer cells. A top and B) SW480, HCT116, LoVo, and CaCO2 were treated with tolfenamic acid as labeled for 24 hours. Lysates were harvested and western blot was performed to analyze the β -catenin expression level. Actin is the housekeeping marker; A bottom) SW480 were transfected with reporter plasmids (TOP/FOP, pRL-null). Cells were subsequently treated with tolfenamic acid as labeled for 24 hours. Luciferase activity was measured using a dual luciferase assay kit (Promega). Data represent mean \pm SD from three replicates. C) SW480 cells were treated with tolfenamic acid, 0, 10, 20, 30, 50 μ M, respectively for 24 hours. . The Nuclear DNA was stained with 4', 6-diamidino-2-phenylindole (DAPI) and pictures were taken by fluorescence microscope.

3.3 Tolfenamic acid induced ubiquitination and degradation of β -catenin

In order to test if tolfenamic acid decreases β -catenin expression through transcriptional downregulation of β -catenin gene, RT-PCR was performed to measure mRNA. As a result, the β -catenin mRNA level in SW480 cell was not changed with tolfenamic acid treatment (Figure 3.3A), indicating that downregulation of β -catenin by tolfenamic acid is not related to transcriptional regulation, instead at posttranscriptional level in colon cancer cells. Since some literatures showed β -catenin is directly targeted by ubiquitination and subsequent degradation (64, 97), we tested if proteasomal degradation mediates tolfenamic acid-induced β -catenin down-regulation. The cells were co-treated with MG-132 (inhibitor of proteasome) and tolfenamic acid and then western blot was performed. As shown in Figure 3.3B, tolfenamic acid-induced β -catenin down-regulation was blocked by the treatment of MG-132. Moreover, overexpressed exogenously GFP-tagged β -catenin was decreased by tolfenamic acid (Figure 3.3C). These data suggest that tolfenamic acid induces β -catenin degradation through activating ubiquitin-proteasome system. We also compared protein stability with the treatment of tolfenamic acid by performing cycloheximide (CHX) assay. CHX is an inhibitor of protein biosynthesis due to its prevention in translational elongation. Interestingly, tolfenamic acid decreased the stability of β -catenin protein in 12 hours but slightly increased the stability at 24 hours after treatment of cycloheximide (Figure 3.3D).



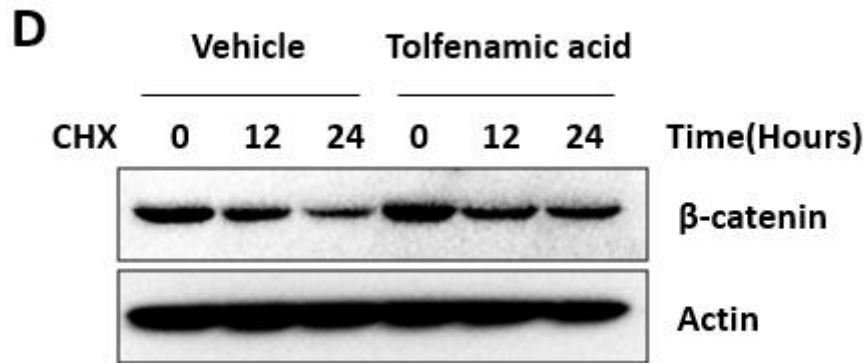
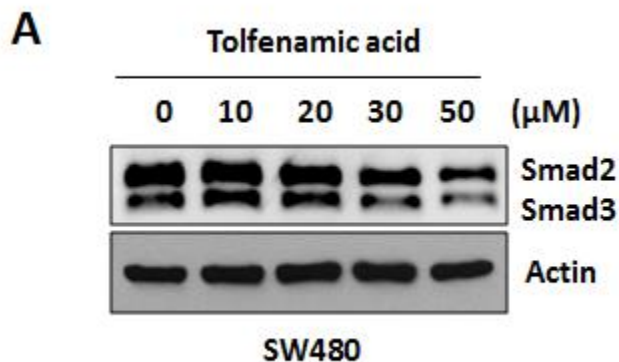


Figure 3.3. Tolfenamic acid induced ubiquitination and degradation of β -catenin. A) SW480 cells were treated with tolfeamic acid using indicated amount for 24 hours. Semi-quantitative reverse transcriptase (RT)-PCR was performed for β -catenin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. B) SW480 cells were pretreated with MG-132 for 30 mins and co-treated with tolfeamic acid for 24 hours. Whole cell lysates were harvested and western blot was performed to analyze the β -catenin and actin expression level. C) SW480 cells were transfected with GFP-tagged β -catenin expression vector and then treated with tolfeamic acid at different concentration. Western blot was performed to detect exogenously expressed β -catenin using GFP antibody. D) SW480 cells pretreated with DMSO or 50 μ M tolfeamic acid for 2 hours at 90% confluent and then co-treat with 10 μ g/ml CHX for indicated time period. Western blot was performed to detect β -catenin and actin.

3.4 Tolfenamic acid-induced crosstalk between TGF- β /Smad and Wnt/ β -catenin signaling

There are growing evidences that transforming growth factor- β (TGF- β) /Smad and Wnt/ β -catenin signaling cross-talk to each other (85, 86). In TGF- β signaling, Smad2 and Smad3 are major mediators(81). Therefore, we hypothesized that Smad2 and Smad3 are involved in tolfenamic acid-induced β -catenin degradation. Expression level of Smad2/Smad3 decreased in dose dependent manner with tolfenamic acid treatment in SW480 cells (Figure 3.4A). LoVo cells showed the same pattern as well (Figure 3.4B).

Since TGF- β and Wnt are major activator of β -catenin-mediated oncogenic signaling pathway, we tested if tolfenamic acid influences TGF- β or Wnt-stimulated stability of β -catenin protein. The SW480 cells were co-treated with tolfenamic acid and TGF- β or Wnt3A for 24 hours. As shown in Figure 3.4C, tolfenamic acid did not affect the responsiveness of β -catenin expression to external TGF- β 1 and Wnt3A.



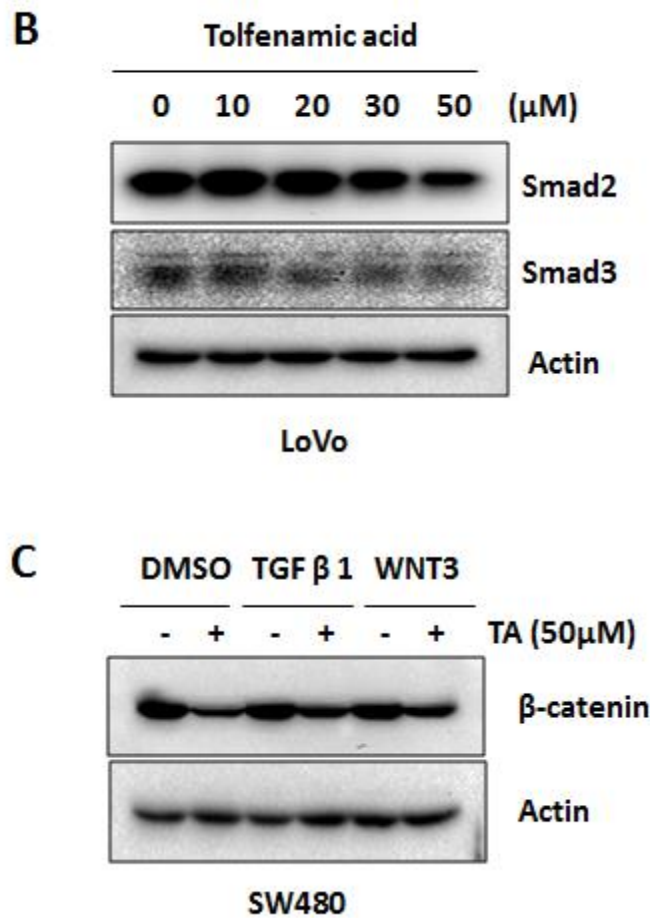


Figure 3.4. Tolfenamic acid decreases expression of Smad2 and Smad3 via TGF β and Wnt3 independent pathway A) SW480 cells were treated with indicated concentrations of tolfenamic acid for 24 hours. Cells were harvested with RIPA buffer and subjected to western blot assay using Smad2/Smad3 antibody and actin. B) LoVo cells were treated with indicated concentrations of tolfenamic acid for 24 hours. Cells were harvested with RIPA buffer and subjected to western blot assay using Smad2, Smad3 and actin. C) SW480 cells were co-treated with 50 μ M tolfenamic acid and DMSO (control) or TGF β 1 (2.5ng/ml) or WNT3 (150ng/ml) as labeled for 24 hours. Lysates were harvested and western blot was performed to analyze the β -catenin expression level. Actin was also measured.

3.5 Tolfenamic acid suppressed Smad2-stimulated transcriptional activity of β -catenin

Since Smad2 and Smad3 are downregulated by tolfenamic acid treatment, we hypothesized that Smad2 and Smad3 may be involved in tolfenamic acid-induced β -catenin degradation. Smad2 and Smad3 were overexpressed in the presence of SB216763 (selective inhibitor of GSK-3 β) to block β -catenin degradation and then transcriptional activity of β -catenin were measured using TOP/FOP Flash luciferase system. Overexpression of Smad2 dramatically increased transcriptional activity of β -catenin whereas Smad3 minimally affected β -catenin activity. Tolfenamic acid decreased Smad2-stimulated transcriptional activity of β -catenin. (Figure. 3.5). These data indicated that Smad2, but not Smad3, mediates tolfenamic acid-induced suppression of β -catenin activity.

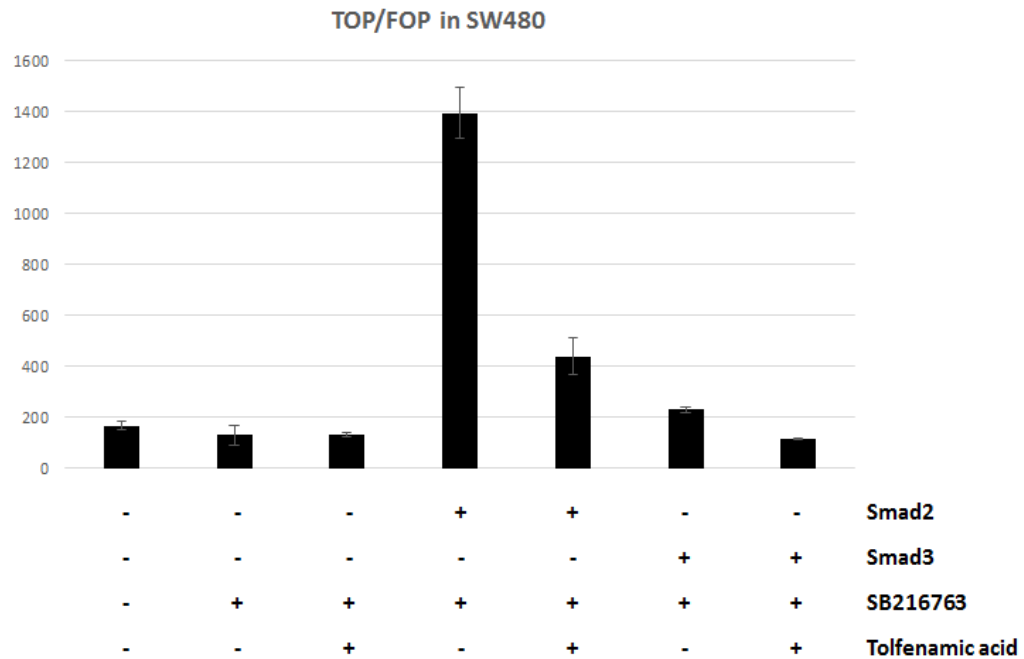


Figure 3.5. Tolfenamic acid suppressed Smad2-stimulated transcriptional activity of β -catenin A) SW480 cells were transfected with empty vector, Smad2 or Smad3 expression vector and then treated with tolfenamic acid for 24 hours. Cells were harvested with lysis buffer and luciferase activity was measured using a dual luciferase assay kit (Promega). Data represent mean \pm SD from three replicates.

3.6 Tolfenamic acid reduced expression of β -catenin target genes.

VEGF and cyclin D1 are downstream transcriptional activation oncogenic targets of β -catenin (63, 73-75). We observed whether tolfenamic acid could alter expression of VEGF and cyclin D1. Our result showed the expression level of VEGF and cyclin D1 also decreased in dose dependent manner in SW480 cells treated with tolfenamic acid.

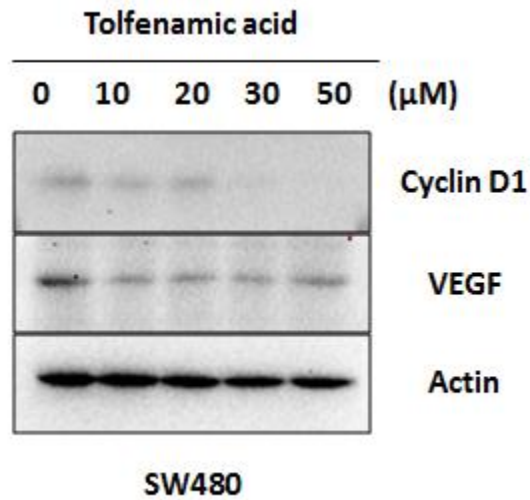


Figure 3.6. Tolfenamic acid reduced expression of β -catenin target genes. SW480 were treated with tolfenamic acid as labeled for 24 hours. Lysates were harvested and western blot was performed to analyze the VEGF and cyclin D1 expression level. Actin is the housekeeping marker.

Chapter 4: Discussion, Conclusion and Future Perspective

4.1 Discussion

NSAIDs are used traditionally to treat pain, fever and inflammation. Tolfenamic acid is the one used to treat acute migraine in the UK. Like many other NSAIDs that exhibited anti-cancer activities, tolfenamic acid has been reported to inhibit the proliferation of lung, esophageal, breast, prostate, ovary and pancreatic cancer cell and tumor growth *in vivo* (43-49). Recently we found that tolfenamic acid suppressed formation of tumor in Apc^{Min/+} mice (55) and proposed several molecular mechanisms of anti-tumorigenic activity by tolfenamic acid. They include activation of tumor suppressive EGR-1, NAG-1 and ATF3 (52, 53). Other groups also indicated that anti-cancer activities of tolfenamic acid may involve in degradation of specificity protein (Sp) and suppression of TGF- β (98, 99).

Aberrant Wnt/ β -catenin regulation pathway is a major mechanism of colon tumorigenesis (20-22). In most colon cancer tissues, the β -catenin/TCF4-mediated oncogenic activity is constitutively active due to defective APC or β -catenin genes. Thus they could be a promising therapeutic target for colon cancer. Since Apc mutation is found in 90% of colon cancer patients and leads to constitutive activation of β -catenin signaling, we hypothesized that anticancer activities of tolfenamic acid might be associated with suppression of β -catenin pathway. Six different NSAIDs (sulindac sulfide, tolfenamic acid, aspirin, diclofenac, SC-560, celecoxib) and three dietary compounds (epigallocatechin-3-gallate, resveratrol and genistein) were screened based on suppression of β -catenin expression in SW480 cells. The result indicated that sulindac sulfide, tolfenamic acid, celecoxib and epigallocatechin-3-

gallate treatment apparently decreased β -catenin expression. Sulindac sulfide is a metabolite of sulindac and effectively suppresses colon cancer as an inhibitor of COX-1 and COX-2 whereas it results in severe side effect including gastrointestinal ulceration and bleeding (100). Celecoxib (selective inhibitor of COX2) has been accepted as effective NSAIDs to inhibit inflammation but it also is associated with increased risk of cardiovascular side effects (10). However, it is generally accepted that tolafenamic acid show a little side effect compared to other NSAIDs (50). Epigallocatechin-3-gallate, resveratrol and genistein are very strong dietary antioxidants and their anticancer activities were already widely studied. Therefore, current study focused on tolafenamic acid as a potential effective anti-cancer drug and reliable anti-cancer mechanisms.

Tolafenamic acid suppressed β -catenin expression in SW480, HCT116 and LoVo cells. Since both SW480 and LoVo cells are APC mutant cells whereas HCT116 is wild type Apc gene (61), effects of tolafenamic acid on down-regulation of β -catenin is likely go through Apc-independent pathways. In later studies, we chose SW480 cells as our model cells. Our data indicated the β -catenin mRNA level in SW480 cell was not changed with tolafenamic acid treatment, indicating that tolafenamic acid-stimulated β -catenin downregulation is not at transcriptional level. We further showed tolafenamic acid induced β -catenin down-regulation was blocked by the treatment of MG-132. Moreover, exogenously GFP-tagged β -catenin was also decreased by tolafenamic acid. (Figure 3.3) Results strongly support that tolafenamic acid induces β -catenin degradation through activating ubiquitin-proteasome system. However, mechanism of β -catenin stability is still unclear.

Recent studies showed transforming growth factor- β (TGF- β) /Smad and Wnt/ β -catenin signaling cross-talk to each other (85, 86). In TGF- β signaling, Smad2 and Smad3 are major mediators (81). In our study, we found that Smad2 and Smad3 are downregulated by tolferamic acid treatment. Therefore, we purposed that Smad2 and Smad3 are involved in tolferamic acid-induced β -catenin degradation. We found that tolferamic acid suppressed Smad2-stimulated transcriptional activity of β -catenin (Fig. 3.5). Moreover, according to Dr. Ha's data in our lab, overexpression of Smad2, but not Smad3, blocked tolferamic acid-stimulated downregulation of β -catenin, which suggest that Smad2 might specifically regulate β -catenin in SW480 cells (data now shown). Taken together, Smad2 is upstream stimulator of β -catenin and tolferamic acid suppresses expression of both Smad2 and β -catenin in human colon cancer cells.

Furthermore, Cyclin D1 and VEGF are downstream targets of β -catenin (63, 73-75). Cyclin D1 involved in G1 to S transition and activates cell cycles (73). VEGF is a key player in the process of angiogenesis. Tolferamic acid decreased cyclin D1 and VEGF expression in dose dependent manner. Data were implied that decreasing of cyclin D1 and VEGF under tolferamic acid treatment could be a consequence of protein degradation.

4.2 Conclusion

Tolfenamic acid downregulated β -catenin through activating ubiquitination and subsequent proteasomal degradation of β -catenin. Decreased β -catenin activity led to a decreased expression of target gene such as VEGF and cyclin D1. Smad2 mediated tolfenamic acid-induced suppression of β -catenin transcriptional activity. All taken together, tolfenamic acid might be a potential cancer therapeutic drug in colon cancer.

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