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# Pro-apoptotic actions of exisulind and CP461 in SW480 colon tumor cells involve β-catenin and cyclin D1 down-regulation

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**Abstract**

Exisulind and its analogues are inhibitors of cyclic GMP phosphodiesterases (PDEs) that have been shown to activate and induce protein kinase G, resulting in the induction of apoptosis in colon cancer cells. These drugs also reduce β-catenin protein levels and decrease cyclin D1 mRNA levels in SW480 cells. Herein we report on studies pertaining to exisulind regulation of β-catenin levels and activity in colon tumor cells. Exisulind and its higher-affinity PDE analogues, (*Z*)-5-fluoro-2-methyl-(4-pyridylidene)-3-(*N*-benzyl)-indenylacetamide hydrochloride (CP461) and (*Z*)-1*H*-indene-3-acetamide, 5-fluoro-2-methyl-*N*-(phenylmethyl)-1-[(3,4,5-trimethoxyphenyl)methylene] (CP248), reduced β-catenin, including the nuclear β-catenin in SW480 cells ($EC_{50} \approx 200$ μM, 1 μM, and <1 μM, respectively). The 50% reduction of β-catenin was seen in 8–14 hr. There was no change in β-catenin mRNA. Exisulind-induced β-catenin reduction was blocked by the proteasomal inhibitor MG132 (Z-leu–Leu–Leu-CHO), indicating that the effect of exisulind involved ubiquitin–proteasomal degradation. A consequence of reduced β-catenin in SW480 cells was that exisulind, CP461, and CP248 caused a concentration- and time-dependent decrease in cyclin D1 levels ($EC_{50} \approx 300$ μM, 1 μM, and <1 μM, respectively) in 4 hr. The effect was via decreased cyclin D1 mRNA levels. Exisulind-induced degradation of β-catenin was not blocked by the inhibition of caspase-3 activity and/or apoptosis, and some SW480 cells showed a reduction in β-catenin levels before the appearance of early apoptosis indicators. Expression of the N-terminal 170 amino acid fragment of β-catenin reduced the effects of β-catenin degradation, cyclin D1 reduction, and the apoptosis response to exisulind. These results indicate that exisulind-induced β-catenin degradation precedes the induction of apoptosis and that the down-regulation of inappropriate β-catenin-activated genes accounts in part for the pro-apoptotic effects of exisulind and CP461 in colon tumor cells.


*Keywords:* Familial adenomatous polyposis; Colon cancer; Cyclin D1; Sulindac sulfone; cGMP; Apoptosis

## 1. Introduction

β-Catenin, a 97 kDa phosphoprotein with important cell adhesion functions, is also critical to both embryogenesis and oncogenesis through the Wnt signaling pathways in combination with Lef/Tcf modulated transcription [1,2]. In addition to integrin and α-catenin, β-catenin binds to the APC protein in association with axin/axil, PP2A, and GSK3β. The structural integrity of the complex leads to the phosphorylation of β-catenin that is required for its ubiquitination and degradation by proteasomes [3]. Phosphorylation of β-catenin involving, in part, four serines and one threonine in or near the UTS ($Ser^{29}$ to $Ser^{45}$) is required for recognition by E3 ligase components [4]. The tumor-suppressing APC protein is also phosphorylated and gains an increased affinity for β-catenin [5]. Mutations in APC or β-catenin may lead to sporadic and familial adenomatous polyps [6,7].

Exisulind (sulindac sulfone, Aptosyn™) inhibits growth and induces apoptosis in a wide range of human tumor cell lines [8–10]. Exisulind-treated SW480, HT29, T84, and HCT116 colon tumor cells show activated PKG secondary to increased cellular cGMP, resulting from cGMP PDE inhibition [11,12]. However, none of the known selective substrates for PKG, e.g. VASP, Hsp27, and IRAG, are

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*Abbreviations:* APC, adenomatous polyposis coli; cGMP, cyclic GMP; DAPI, 4′,6-diamidino-2-phenylindole; G6PDH, glucose-6-phosphate dehydrogenase; GSK3β, glycogen synthase kinase 3 beta; Lef/Tcf, lymphocyte enhancer binding factor/T cell factor; PDE, phosphodiesterase; PKG, cGMP-dependent protein kinase; PP2A, protein phosphatase 2A; RT-PCR, reverse transcriptase-polymerase chain reaction; SAANDs, selective, apoptotic anti-neoplastic drugs; TCA, trichloroacetic acid; UTS, ubiquitin targeting sequence.

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growth regulators [13–15], but we have shown previously that β-catenin is a substrate for PKG *in vitro* [12]. β-Catenin may serve as one of the downstream targets in exisulind-regulated pro-apoptotic signaling.

Several lines of evidence have shown that mutations in APC cause an accumulation of β-catenin to transduce an oncogenic signal. The middle portion of APC contains three successive 15 amino acid repeats followed by seven 20 amino acid repeats. Both types of repeats bind independently to β-catenin and are sufficient for its degradation [16]. Truncated or missing APCs are found in patients with familial adenomatous polyposis (FAP) and most colorectal cancers do not form fully functional APC/β-catenin/GSK3β/axin complexes [17,18]. Recent studies also show β-catenin accumulation in human colon polyps not displaying APC mutations [6,19], further emphasizing the importance of β-catenin regulation.

Mutations in or near the β-catenin UTS, such as $Ser^{33} \rightarrow Tyr^{33}$ in SW48 cells, or $Ser^{45}$ deletions in HCT116 cells, also disrupt β-catenin phosphorylation and result in its accumulation [20]. Frequent somatic *CTNNB1* (β-catenin gene) mutations around the UTS, i.e. $Ser^{45} \rightarrow Phe^{45}$, $Thr^{44} \rightarrow Ala^{44}$, $Ser^{29} \rightarrow Phe^{29}$, $Asp^{32} \rightarrow Gly^{32}$, have been found in colorectal and gastric cancers, and β-catenin mutations also occur in breast, ovarian, skin, bone, and soft tissue [20–26].

β-Catenin accumulation transactivates Lef/Tcf transcription of oncogenes including *cyclin D1* [27,28]. Over-expression of cyclin D1 has been reported in a variety of tumors, including esophagus, prostate, and adenomatous polyps of the colon [29–33]. Suppression of cyclin D1 expression inhibited the growth and tumorigenicity of colon cancer cells [34]. More recently, studies using antisense oligonucleotides to cyclin D1 have shown that tumor cell apoptosis increases when β-catenin-driven over-expressed cyclin D1 levels are reduced [35].

SW480 colon adenocarcinoma cells are appropriate models to study β-catenin accumulation. These cells show truncated APC proteins. Introduction of a wild-type *APC* gene into SW480 cells significantly reduces the accumulation of β-catenin and induces apoptosis and cell growth inhibition [16,21]. We and others have reported that exisulind (sulindac sulfone) and sulindac sulfide decrease β-catenin in *APC* mutant cell lines [12,36–38]. The studies reported here test the hypothesis that exisulind circumvents defective regulation of β-catenin accumulation, resulting in reduced β-catenin and cyclin D1 leading to increased apoptosis and neoplastic cell death.

## 2. Materials and methods

### 2.1. Materials

MG132 (Z-leu–Leu–Leu-CHO) was purchased from Biomol. Caspase-3 inhibitor II (Ac-DEVD-CMK) was obtained from Calbiochem. Exisulind [(*Z*)-5-fluoro-2-methyl-(*p*-methylsulfonylbenzylidene)-3-indenylacetic acid] and CP461 [(*Z*)-5-fluoro-2-methyl-(4-pyridylidene)-3-(*N*-benzyl)-indenylacetamide hydrochloride] were synthesized at Cell Pathways, Inc. $LiCl_2$, other chemicals, and fetal bovine serum (FBS) were purchased from the Sigma Chemical Co, if not otherwise mentioned in the text. Anti-PKG 1β, anti-β-catenin, anti-cyclin D1, and anti-G6PDH antibodies were purchased from the StressGen Biotechnologies Corp., Upstate Biotechnology, NeoMarkers, Inc., and Sigma, respectively. RPMI culture medium and other cell culture reagents were from Life Technologies.

### 2.2. Methods

#### 2.2.1. Cell growth

SW480 cells (ATCC No. CCL-228) were grown in RPMI 1640 medium, 2 mM glutamine, 1% penicillin/streptomycin/amphotericin B solution, 5% FBS in 5% $CO_2$ at 37°. Cells at 70–100% confluence were treated with drugs dissolved in 100% DMSO and diluted to 0.1% in medium.

#### 2.2.2. Western blot analysis

Drug-treated cells were rinsed twice with ice-cold PBS and lysed in the culture dishes using modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM $Na_3VO_4$) containing protease inhibitors (Complete Protease Inhibitor Tablets; Boehringer Mannheim). The lysate was centrifuged for 15 min at 20,800 *g* in the cold room. Fifty micrograms of protein, determined using Bio-Rad DC protein assays, from control and treated dishes was loaded on 10% BT NuPAGE gels (Novex, Inc.), electrophoresed under reducing conditions, and transferred onto nitrocellulose membranes (0.45 μm) (Bio-Rad Laboratories). Blots were probed with primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies and developed with BM Blue POD substrate (Roche). The results were analyzed using an AlphaImager and software system (Alpha Innotech).

#### 2.2.3. Relative quantitative RT-PCR

Cells in complete RPMI 1640 medium were treated with drugs for 48 hr and rinsed with cold PBS (two times). Total RNA was extracted using QIAGEN's RNeasy mini kit, and cDNA was prepared using Superscript II RNase $H^-$ Reverse Transcriptase (Life Technologies) with random hexamers. Relative quantitative PCR was performed using Quantum RNA 18S internal standard Competimer™ techniques (Ambion). In PCR reactions, 18S cDNA was used as an internal control to normalize inter-sample variability. The specific primers used for β-catenin were 5′-ATTTGATGGAGTTGGACATGGC-3′/5′-CCAGCTACTTGTTCTTGAGTGAAGG-3′ and for cyclin D1 were 5′-GGATGCTGGAGGTCTGCGAGGAAC-3′/5′-GAGAGGAAGCGT-

GTGAGGCGGTAG-3′. PCR was conducted for 30 cycles and primers were annealed to template at 60° and extended at 72°. The PCR products were run on 1% agarose gels in Tris–borate–EDTA (TBE) and analyzed after ethidium bromide (EtBr) staining, using an AlphaImager and software system.

*2.2.4. Apoptosis and cell growth inhibition*

DNA fragmentation in SW480 cells was measured using a double-antibody ELISA kit (Roche) that requires both histone protein and fragmented DNA detection for a signal. Cells were seeded in 96-well plates at 10,000/well and after 24 hr, were dosed and grown for an additional 48 hr. Cytoplasmic fractions of control and treated cells were transferred into streptavidin-coated 96-well plates and incubated with biotinylated mouse anti-histone antibody and peroxidase-conjugated mouse anti-DNA antibody at room temperature for 2 hr. After the removal of unbound antibodies by washing, the amount of apoptosis-generated nucleosomes was quantified as the peroxidase retained in the immuno-complex using ABTS (2,2′-azino-di[3-ethylbenzthiazolin-sulfonate]) as the substrate. Absorbance was determined at 405–90 nm using a Spectra Max340 microplate reader (Molecular Devices).

Growth inhibition was determined using cells seeded at 1000/well in 96-well plates. After 24 hr in culture, cells were dosed with drug and incubated for 6 days. Cells were fixed with 10% TCA at 4° for 1 hr, rinsed five times with deionized $H_2O$, and incubated for 10 min with 0.4% sulforhodamine B in 1% acetic acid. Plates were rinsed four times with 1% acetic acid, dried for 30 min, and solubilized in 10 mM Tris. Absorbance was determined at 540 nm using a Spectra Max340 plate reader.

*2.2.5. Immunofluorescence*

SW480 cells were seeded on coverslips 24 hr before being treated with 600 μM exisulind for 4–48 hr in RPMI medium with 5% FBS. The cells were fixed in −20° methanol for 30 min, air-dried, and rehydrated in Dulbecco's phosphate-buffered saline (D-PBS). The samples were incubated for 1 hr in a humid chamber with the appropriate primary antibody. β-Catenin was detected using rabbit anti-β-catenin IgG (Upstate Biotechnology) (1:1000) that recognizes the consensus GSK site and monoclonal anti-β-catenin IgG (Alexis Biochemicals) that stains nuclear β-catenin but not cytoplasmic or membrane-bound β-catenin. Apoptotic cells were detected using the M30 antibody (Roche) at a dilution of 1:50. M30 antibody binds only to the neoantigen, caspase cleaved cytokeratin 18. All primary antibodies were recognized with the appropriate fluorescein isothiocyanate (FITC)- or Cy3-conjugated secondary antibodies (Jackson Laboratories). Samples were washed for 15 min in D-PBS between each antibody incubation, counterstained with DAPI, mounted in Vectasheild (Vector Laboratories, Inc.), and observed using an Olympus IX70 fluorescence microscope. Digital images were collected using a spot 2 camera (Diagnostic Instruments) and labeled with PhotoShop 5.0 (Adobe).

*2.2.6. Plasmid construction and transfection*

Total RNA was extracted from SW480 cells using an RNeasy mini kit (QIAGEN), and cDNA was prepared using Superscript II RNase $H^-$ Reverse Transcriptase (Life Technologies) with random hexamers. The 2.3 kb β-catenin cDNA was PCR amplified using PCR Supermix High Fidelity (Invitrogen) with β-catenin primers (5′-AGCGTGGACAATGGCTACTCAAGC-3′/5′-TCAGTATCAAACCAGGCCAGCTGATTG-3′) and ligated into pCR-Blunt TOPO II vector (Invitrogen). A 505 bp DNA fragment of β-catenin N-terminal residues 1–170 was amplified by PCR with PCR SuperMix High Fidelity and primers (5′-CGGGATCCCTACTCAAGCTGATTTGATGG-3′/5′-ATAGTTTAGCGGCCGCTGGTCCTCGTCATTTAGCAG-3′) containing *Bam*HI and *Not*I sites, respectively. The DNA product was digested with *Bam*HI and *Not*I (Invitrogen), cleaned, and ligated into pcDNA4/His-myc A. The resulting plasmid, pcDNA4/His-myc-NT-β-cat and empty vector alone were transfected into SW480 cells using Lipofectin (Invitrogen) following the protocols of the manufacturer. After selection with 200 μg/mL of zeocin, colonies were isolated and expanded. SW480 NT-β-cat was identified after screening 16 colonies for expression of a 22 kDa NT-β-cat fusion protein (see Fig. 8) by Western blot analysis.

## 3. Results

### *3.1. Exisulind-induced decrease in β-catenin in APC-defective colon tumor cells*

Subconfluent SW480 cells treated with 200–600 μM exisulind for 48 hr showed a concentration-dependent decrease in the level of total cellular β-catenin of up to

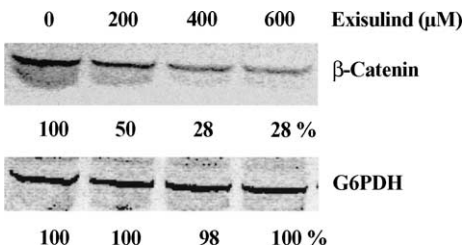


Fig. 1. Concentration-dependent exisulind reduction of β-catenin. SW480 cells were grown to 70% confluence and treated for 48 hr with exisulind at the concentrations indicated. Fifty micrograms of whole cell lysate from attached cells was loaded to each lane, electrophoresed, and transferred. β-Catenin (molecular mass 93 kDa) and G6PDH (molecular mass 117 kDa) proteins were probed with anti-β-catenin and anti-G6PDH and HRP-conjugated secondary antibody and detected by BM Blue POD substrate as described in "Materials and methods." Band intensities were compared to DMSO-treated cells using an AlphaImager system. These data are representative of six experiments.

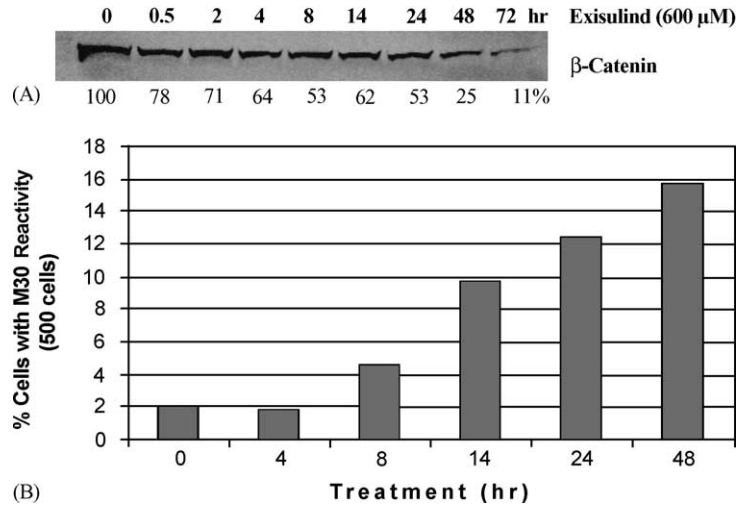


Fig. 2. Correlation of exisulind-induced apoptosis and total cellular β-catenin reduction. SW480 cells were treated with 600 μM exisulind for the times indicated and harvested, and 50 μg of whole cell lysates was analyzed by Western blot as described in ''Materials and methods'' (panel A). For M30 analysis of exisulind-induced apoptosis, both attached and floating cells were collected at each endpoint and stained with DAPI and M30 antibody (Roche) as described in ''Materials and methods.'' Consecutive cells from each culture (500) were scored for M30 reactivity, indicating caspase-3 activity (panel B).

70% (Fig. 1). G6PDH did not change with the drug treatments. Densitometric scans of Western blots of cells treated with 600 μM exisulind and harvested from 0.5 to 72 hr showed that some small decreases even in total cellular β-catenin levels could be detected as early as 30 min after cells were treated (Fig. 2A). This indicated that β-catenin degradation begins early in exisulind-induced apoptosis and growth inhibition. Approximately one-half of the total β-catenin was processed in response to the drug at 8–24 hr. When the number of apoptotic cells was determined over a time course of 4–8 hr, the apoptosis index increased in parallel with the drug-dependent reduction of β-catenin (Fig. 2B).

### 3.2. Lack of caspase-3 inhibition effect on exisulind-induced β-catenin decreases

Since caspase-3 has been reported to cleave β-catenin during apoptosis [39], we tested whether the exisulind-induced decrease in total β-catenin was a consequence of apoptosis, rather than an event to initiate apoptosis. SW480 cells were treated with exisulind and a caspase-3 inhibitor (caspase inhibitor II) simultaneously to block potential caspase-3 cleavage of β-catenin. Caspase-3 inhibitor II is a peptide derivative that binds irreversibly to caspase-3 to inhibit activity. At 100 μM, caspase-3 inhibitor II completely blocked exisulind-induced apoptosis (Fig. 3B), but

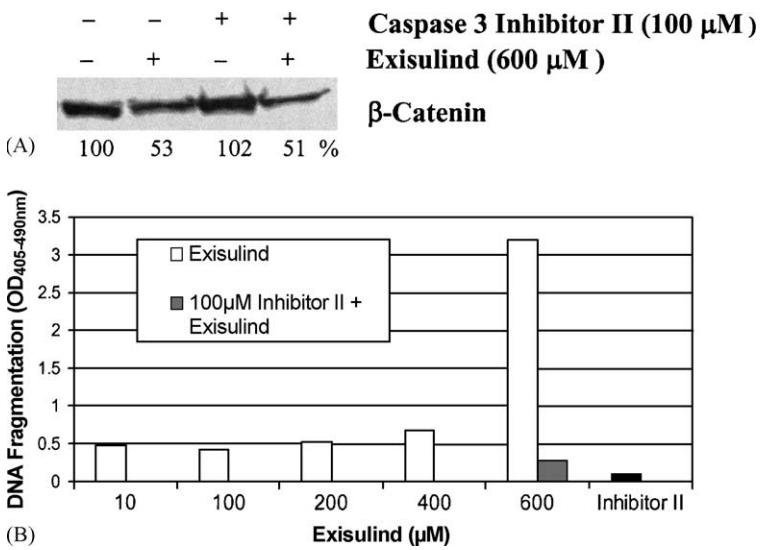


Fig. 3. Caspase-3 independence of exisulind reduction of β-catenin. SW480 cells were treated with 600 μM exisulind and 100 μM caspase-3 inhibitor II for 48 hr. (A) Western blots of β-catenin were as described in the legend of Fig. 1 and in ''Materials and methods.'' The blot is representative of two similar experiments. Control cells were treated with 0.1% DMSO. (B) DNA fragmentation in SW480 cells at 10,000 cells/well in 96-well plates was measured using a double-antibody ELISA kit as described in ''Materials and methods.''

did not affect exisulind-induced decreases in β-catenin (Fig. 3A). Therefore, the reduction of total cellular β-catenin levels by exisulind is not a general consequence of apoptosis, but rather a direct effect of the drug on β-catenin regulation.

### 3.3. β-Catenin distribution in exisulind-treated SW480 cells

Because the functions of β-catenin occur in specific subcellular locales, we studied the distribution of β-catenin within individual cells in response to exisulind (Fig. 4). Indirect immunofluorescence of apoptotic cells employed an anti-cytokeratin monoclonal antibody M30 [40] that recognizes a neo-epitope of cytokeratin 18 made accessible by caspase proteolysis [41]. Thus, the distribution patterns of caspase-cleaved cytokeratin 18 indicate whether a cell is in an early or a late stage of apoptosis. Cells in the early stages of apoptosis exhibit a filamentous staining pattern as in Fig. 4B, while cells in the later stages of apoptosis display the granular staining pattern seen in Fig. 4E. SW480 cells undergoing apoptosis in response to exisulind displayed decreased β-catenin levels (Fig. 4C and F, arrows). Overall, no selective locales were affected

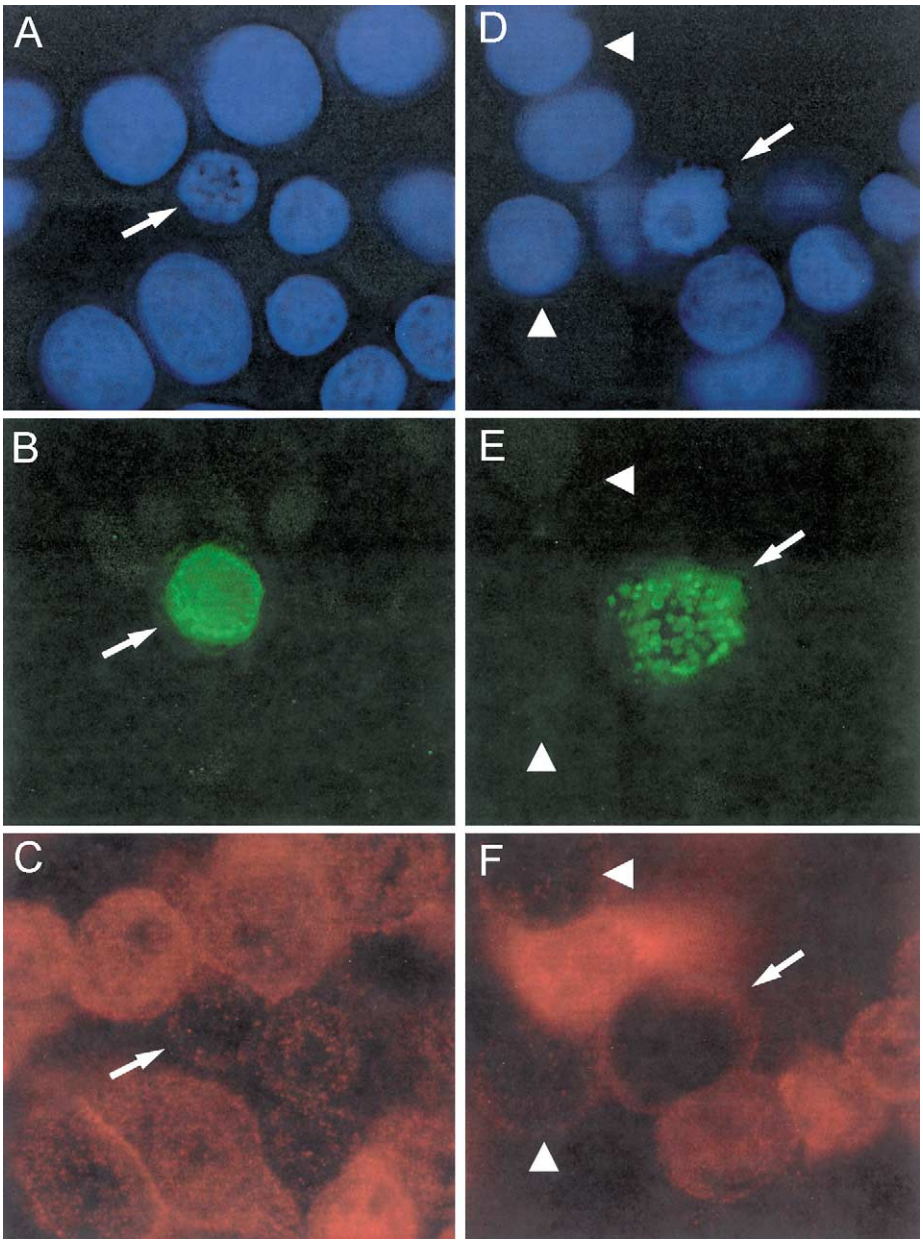


Fig. 4. β-Catenin distribution and apoptosis in exisulind-treated SW480 cells. SW480 cells were stained with DAPI (A and D), M30 (B and E), and β-catenin (C and F). Two separate fields of view (A–C and D–F) are shown. Early- (B) and late-stage (E) apoptotic cells (denoted by arrows) were identified by both nuclear morphology (DAPI) and M30 reactivity. Early-stage apoptotic cells show filamentous M30 reactivity, while late-stage apoptotic cells have a granular M30 pattern. Apoptotic cells exhibited decreased levels of nuclear and cytoplasmic β-catenin staining (C and F, arrows). Some exisulind-treated cells with no M30 reactivity also displayed decreased β-catenin levels as illustrated (F, arrowheads).

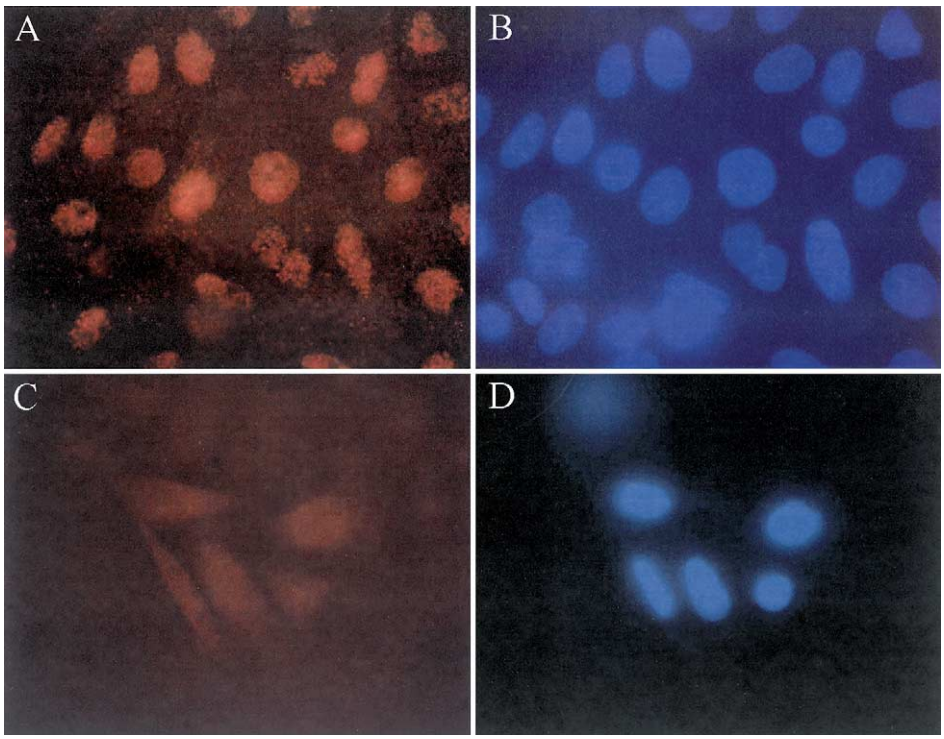


Fig. 5. Decreased nuclear β-catenin in exisulind-treated SW480 cells. Levels of nuclear β-catenin were compared between control and exisulind-treated SW480 cells by indirect immunofluorescence, utilizing a monoclonal antibody that recognizes a dephosphorylated-peptide (a.a. 27–37) of β-catenin. This antibody specifically labels only the nuclear β-catenin. Control SW480 cells exhibited strong nuclear β-catenin labeling (A and B). Cells treated with 600 μM exisulind for 48 hr exhibited a dramatic decrease of nuclear β-catenin (C and D). β-Catenin (A and C), DAPI counterstain (B and D).

because both cytoplasmic and nuclear β-catenin were reduced significantly. We also found that there were populations of exisulind-treated cells that displayed decreased β-catenin staining with no corresponding M30 reactivity or morphology changes (Fig. 4D–F, arrowheads). The reduction of nuclear β-catenin was confirmed further using a monoclonal antibody that specifically labels β-catenin in the nucleus. In contrast to the heterogeneous β-catenin staining in control cell nuclei, there was no nuclear β-catenin staining in the SW480 cells treated with 600 μM exisulind for 48 hr (Fig. 5). These data indicate that decreased β-catenin levels in response to exisulind treatment may precede the induction of apoptosis.

### 3.4. Post-translational regulation of exisulind-reduced β-catenin in SW480 cells

β-Catenin mRNA levels were measured by relative quantitative RT-PCR to determine if exisulind decreased β-catenin by degradation rather than down-regulation of transcription and protein synthesis. SW480 cells were treated for 48 hr with 200–600 μM exisulind, and total

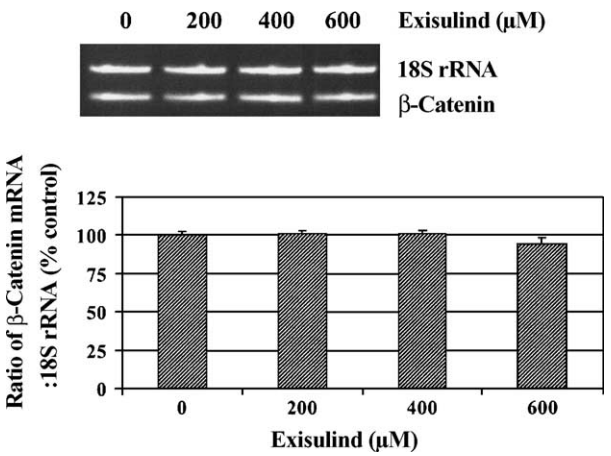


Fig. 6. Unchanged β-catenin mRNA level by exisulind. Total RNAs were extracted from SW480 cells treated with exisulind for 48 hr as described in "Materials and methods" and amplified by relative quantitative RT-PCR as described in "Materials and methods." The ratio of the β-catenin bands over the 18S rRNA cDNA bands was analyzed. No changes in β-catenin mRNA levels were found with exisulind treatment.

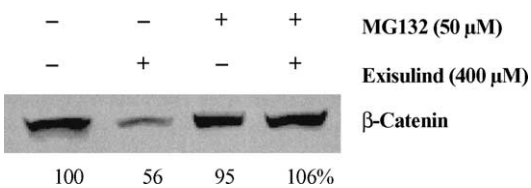


Fig. 7. Exisulind reduction of β-catenin depending on the ubiquitin–proteasomal system. SW480 cells were treated for 48 hr with 0.1% DMSO, 400 μM exisulind, 50 μM MG132 (ubiquitin–proteasome inhibitor), and 400 μM exisulind plus 50 μM MG132, respectively. Western blot analysis was performed as described in "Materials and methods" and the legend of Fig. 1. These data are representative of four experiments.

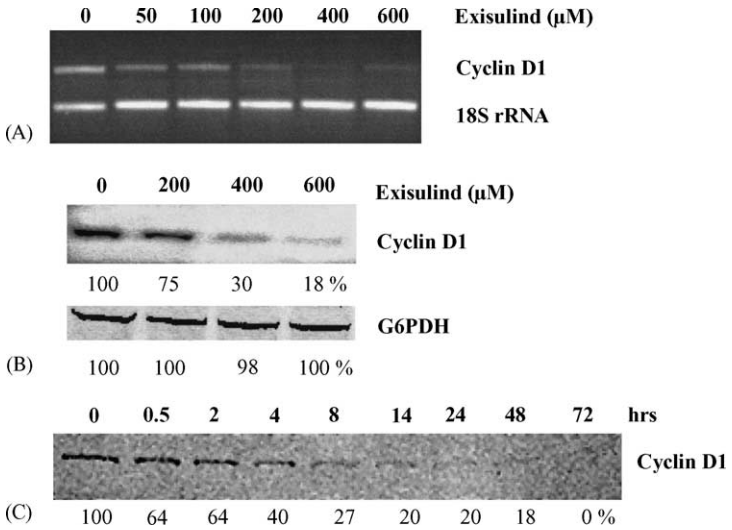


Fig. 8. Exisulind-decreased cyclin D1 mRNA levels. (A) Relative quantitative RT-PCR was used to analyze the effects of exisulind on cyclin D1 mRNA. Total RNA was extracted as described in "Materials and methods." Cyclin D1 cDNA was amplified using human cyclin D1 primers, and 18S rRNA was co-amplified as an internal standard using a pre-judged ratio of 18S primer:18S competimer of 4:6 as described in "Materials and methods." Both cyclin D1 cDNA and 18S rRNA bands were stained with EtBr and analyzed using an AlphaImager system. The experiment was repeated two times. Cyclin D1 protein was analyzed by Western blotting as described in "Materials and methods" (B and C). SW480 cells were treated for 48 hr with up to 600 μM exisulind (B) or with 600 μM exisulind for 0.5–72 hr (C). G6PDH was analyzed as a loading control (B). These data are representative of six experiments.

RNA was extracted. The ratio of the specific β-catenin cDNA products amplified by PCR over the cDNA of 18S rRNA did not change in response to exisulind (Fig. 6). Therefore, decreased β-catenin due to exisulind treatment is not caused by an effect at the level of mRNA.

### 3.5. Dependence of exisulind-reduced β-catenin on ubiquitin–proteasomal degradation

MG132, a ubiquitin–proteasome inhibitor, was used to determine if the exisulind-induced decreases in β-catenin

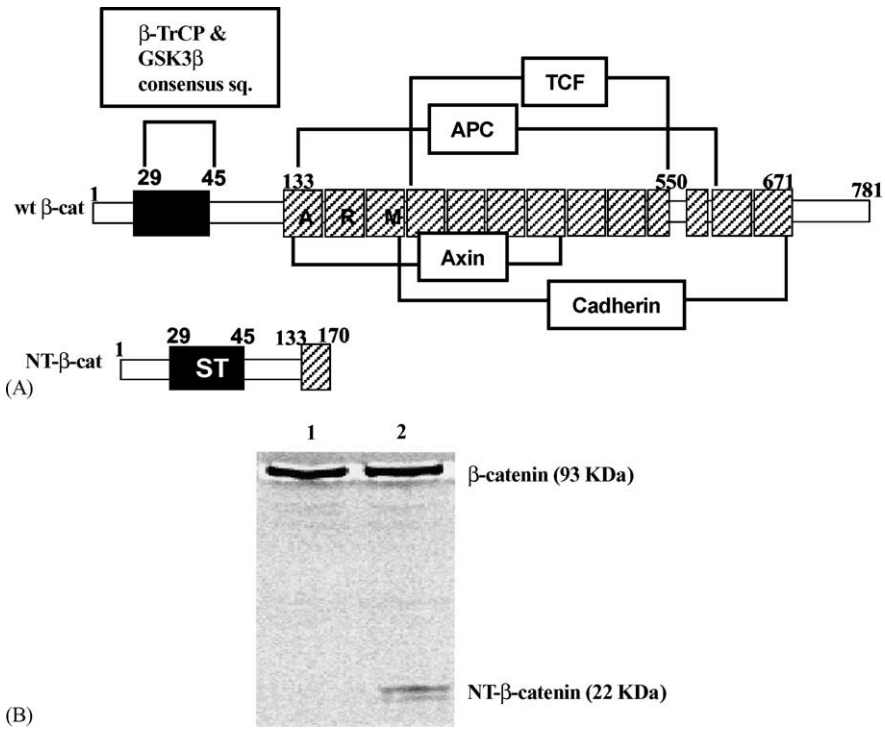


Fig. 9. Expression of NT-β-catenin in SW480 cells. (A) Schematic representations of wild-type human β-catenin and NT-β-catenin. The black boxes indicate the GSK3β consensus sequence and the crosshatched boxes are the armadillo repeats with amino acid numbers of human β-catenin. (B) Western blotting showing the expression of NT-β-catenin in SW480 cells stably transfected with pcDNA4/His-myc-NT-β-cat. Fifty micrograms of whole cell lysates from empty vector and pcDNA4/His-myc-NT-β-cat transfected SW480 cells were loaded in lanes 1 and 2, respectively. After electrophoresis and transfer as described in "Materials and methods," the blot was probed using polyclonal antibody against residues 29–49 of human β-catenin (GSK3β consensus sequence).

could be blocked at the proteasomal level. MG132 (50 μM) was added to cultured SW480 cells with either 0.1% DMSO or 400 μM exisulind for 48 hr. Total cellular β-catenin protein was analyzed using Western blot immunoreactivity. MG132 inhibited the exisulind-induced reduction of β-catenin and the inhibitor alone did not change the level of β-catenin, indicating that the protein is processed by the ubiquitin–proteasome system (Fig. 7).

### *3.6. Down-regulation of cyclin D1 mRNA levels in SW480 cells by exisulind*

The accumulation of β-catenin serves to initiate transcription of cyclin D1 [27,28]. Therefore, we studied whether exisulind influenced cyclin D1 mRNA levels secondary to reduced β-catenin reduction. Comparison of the amount of cyclin D1 DNA and 18S rRNA fragments amplified by relative quantitative RT-PCR using specific cyclin D1 and 18S rRNA primers showed that exisulind decreased cyclin D1 mRNA (Fig. 8A). In addition, Western blots using mouse monoclonal anti-cyclin D1 antibody detected a single 36 kDa band corresponding to cyclin D1 protein. Cyclin D1 protein levels were reduced approximately 80% by 600 μM exisulind after 48 hr (Fig. 8B), while the G6PDH protein remained unchanged. A time course with 600 μM exisulind showed that cyclin D1 protein decreased 36% at 30 min and 80% after a 24-hr drug treatment. Cyclin D1 immunoreactivity was near zero after 72 hr of drug treatment in the cells remaining on the plates (Fig. 8C).

### *3.7. Reduced apoptosis by exisulind in N-terminal β-catenin expressing SW480 cells*

To further address the role of β-catenin and cyclin D1 in the regulation of SW480 apoptosis, we expressed the N-terminal β-catenin (a.a. 1–170) that included the GSK3β consensus sequence and ubiquitination site in SW480 cells (Fig. 9). In comparison to the empty vector transfected cells, SW480 cells containing NT-β-cat demonstrated elevated β-catenin levels. Exisulind-induced decreases of β-catenin and cyclin D1 were diminished compared with vector-only cells (Fig. 10A). Apoptosis measured by DNA fragmentation showed that in the NT-β-cat expressing SW480 cells, the concentration–response curve to exisulind was shifted to the right (Fig. 10B). The attenuation of apoptosis by NT-β-cat in response to exisulind was confirmed by the M30 antibody labeling experiments (Fig. 10C). The apoptosis indicator, M30 reactivity, was lower in exisulind-treated SW480 NT-β-cat cells than in SW480 V2 cells.

### *3.8. Effects of high-affinity SAANDs on β-catenin/cyclin D1 signal transduction*

CP461 and (*Z*)-1*H*-indene-3-acetamide, 5-fluoro-2-methyl-*N*-(phenylmethyl)-1-[(3,4,5-trimethoxyphenyl)-

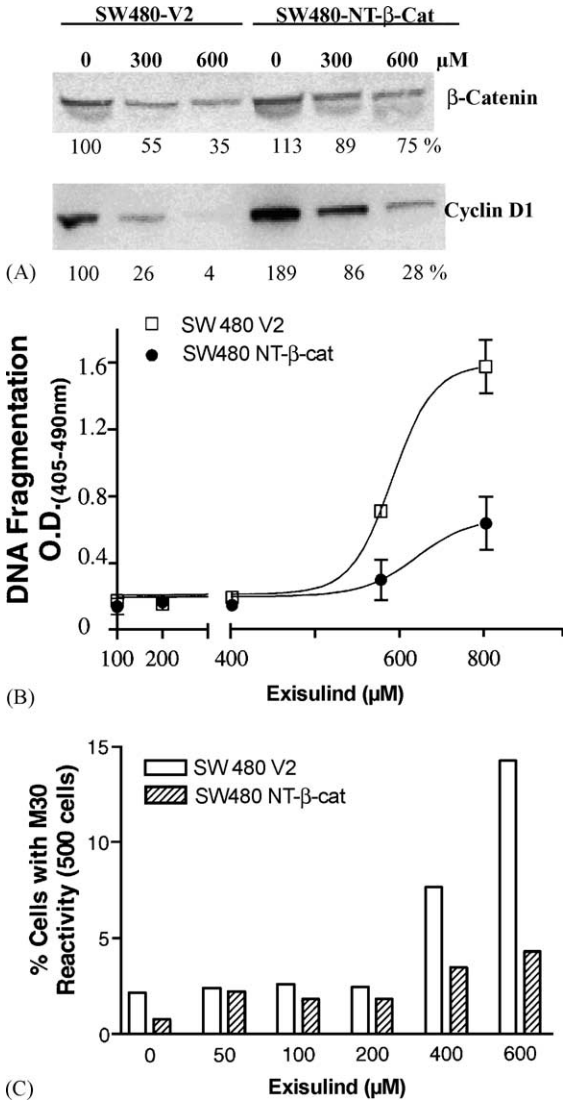


Fig. 10. Reduced exisulind-induced apoptosis in NT-β-catenin expressing SW480 cells. SW480 cells either stably transfected with empty vector or expressing NT-β-cat were treated with exisulind for 48 hr. Western blot analysis showed attenuated reduction of β-catenin and cyclin D1 in NT-β-cat expressing SW480 cells after drug treatment (A). Fifty micrograms of cell lysate per sample was analyzed as described in "Materials and methods." NT-β-cat expression attenuated exisulind-induced apoptosis in SW480 cells (B and C). DNA fragmentation (B) in SW480 cells seeded at 10,000/well in 96-well plates was measured using a double-antibody ELISA as described in "Materials and methods" after treatment for 48 hr using the indicated concentrations of exisulind. M30 reactivity was measured as described in "Materials and methods" (C). M30 antibody labeling was counted in 500 cells after 48 hr of drug treatment under each exisulind concentration as indicated.

methylene] (CP248) are exisulind analogues with approximately 40- and 400-fold higher affinity for PDE5 and PDE2 inhibition (e.g. 3.6 and 0.3 vs. 128 μM for PDE5) and for apoptosis induction (2 and 0.6 vs. 557 μM) in colon, prostate, and breast tumor cells [10,12,42]. β-Catenin and cyclin D1 changes after CP461 or CP248 treatment were analyzed using Western blots. SW480 cells treated for 48 hr with 5 μM CP461 and 1 μM CP248 reduced β-catenin levels by 70%. Cyclin D1 levels were reduced

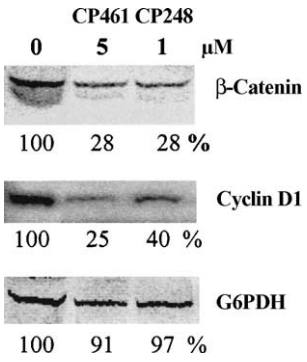


Fig. 11. Reduction of β-catenin and cyclin D1 by high-affinity SAANDs. SW480 cells were treated for 48 hr with 5 μM CP461 and 1 μM CP248. Fifty micrograms of cell lysate per sample was analyzed by Western blot as described in the legend of Fig. 1 and in "Materials and methods." G6PDH was analyzed as the loading control. The results were quantified using an AlphaImager system. These data are representative of three experiments.

75% by CP461 and 60% by CP248 (Fig. 11). G6PDH was analyzed as a loading control.

## 4. Discussion

This study reports that exisulind, CP461, and CP248 reduced β-catenin levels and defines the mechanism of exisulind- and analog-induced β-catenin reduction in the colon tumor cell line SW480. Exisulind and its analogues reduced β-catenin via proteasomal degradation independent of caspase activity. The reduction of β-catenin resulted in cyclin D1 down-regulation and increased SW480 cell apoptosis.

β-Catenin accumulation occurs with mutations in APC and is thought to play a critical role during tumorigenesis of colon cancer [7,43,44]. The hypothesis is that mutations in APC prevent formation of the APC/axin/GSK3β/PP2A complex, which in turn blocks phosphorylation of β-catenin by GSK3β, a prerequisite for degradation by the ubiquitin–proteasome pathway. This hypothesis is supported by the findings that the introduction of wild-type *APC* into *APC*-defective colon cancer cells restored β-catenin degradation and induced apoptosis [16,21,45]. However, recent evidence suggesting that β-catenin accumulates in colon polyps without APC truncations raises additional complications [6,19]. Accumulated β-catenin, along with Tcf/Lef, facilitates the expression of multiple genes involved in proliferation and apoptosis [46]. The expression of many oncogenes including *cyclin D1*, c-*myc* and *PPARδ*, is under the control of β-catenin/Tcf/Lef [27,43,47]. Exisulind, CP461, and CP248 provide a pharmacological approach for circumventing the *APC* mutational defect.

The reduction in β-catenin induced by exisulind, CP461, and CP248 suggests that the drug either activated a proteolysis pathway or inhibited transcription. RT-PCR data and Northern blots (data not shown) showed that exisulind did not lower β-catenin mRNA levels even after 48 hr of treatment at several concentrations, indicating a proteolysis regulatory mechanism. Although proteolysis of β-catenin occurs via the ubiquitin–proteasomal pathway, previous reports have also shown that β-catenin can be cleaved by caspases [39,48,49]. Thus, we determined whether exisulind and its analogues reduced β-catenin as the result of pre-apoptotic digestion by the ubiquitin–proteasomal pathway or as the result of post-apoptotic caspase activity. Inhibition of caspase-3 did not block the degradation of β-catenin in exisulind-treated cells at concentrations of caspase-3 inhibitor II that blocked apoptosis. Conversely, β-catenin degradation in response to exisulind was blocked when the ubiquitin–proteasome pathway was inhibited by MG132. Additionally, time courses comparing an early marker of apoptosis with β-catenin Western blots indicated that, even with analysis using total cellular β-catenin, changes were seen in a similar time range. Immunofluorescence analysis of individual cells showed reduced β-catenin labeling before caspase activation (more substantial in the nucleus), confirming the Western blot data. The early reduction of β-catenin after exisulind treatment affected mRNA levels because nearly 50% of cyclin D1 was reduced after only 2 hr of drug exposure. These results indicate that the exisulind-induced decrease in β-catenin is the direct result of drug-induced post-translational regulation.

Exisulind decreased nuclear β-catenin and both cyclin D1 mRNA and protein levels, indicating that reduced β-catenin levels could result in the down-regulation of Tcf/Lef-mediated promoter transcription. The reduction of cyclin D1 levels may have a direct role in the increased apoptosis observed for exisulind- and analog-treated cells. This hypothesis is supported by a recent report that the reduction of cyclin D1 by antisense oligonucleotide methods induced apoptosis in squamous carcinoma cells [35].

Our results also showed a β-catenin/cyclin D1 linkage to the regulation of apoptosis. When transfected into SW480 cells, the NT-β-cat fusion protein containing the GSK3β consensus sequence and ubiquitination sites competed with endogenous β-catenin for its degradation machinery and resulted in the elevation of endogenous β-catenin. The effect of NT-β-cat on apoptosis was an attenuated exisulind response along with diminished β-catenin and cyclin D1 reduction. Thus, exisulind, by reducing the level of Tcf/Lef-dependent cyclin D1 transcription and expression via β-catenin, also increases apoptosis in SW480 cells.

Cyclin D1 protein can be degraded through a ubiquitin–proteasomal pathway triggered by GSK3β phosphorylation [14,50]. We excluded the possible involvement of such a mechanism in exisulind- and analog-induced cyclin D1 reduction by showing that GSK3β was not activated by these drugs (data not show) and that MG132, a ubiquitin–proteasomal inhibitor, did not block exisulind-induced cyclin D1 protein decrease (data not shown). In addition to post-transcriptional regulation, the cyclin D1 promoter

is also under the control of AP1, Lef-1, and CRE elements [51,52]. c-Jun, ERK1, β-catenin, CREB, and GSK-β are all reported to be involved in regulating cyclin D1 transcription [51,53]. Therefore, overall regulation of the cyclin D1 level is complex. On the other hand, *cyclin D1* has been considered to be the most tightly β-catenin-controlled gene among the Tcf/Lef-driven genes. We have observed that cyclin D1 mRNA levels decreased parallel to exisulind- and analog-induced β-catenin reduction, suggesting that these drugs reduce cyclin D1 levels, at least in part, through β-catenin-regulated Tcf/Lef transcription.

CP461 and CP248 share the mechanism of action described here for exisulind with respect to restored β-catenin/cyclin D1 down-regulation in APC defective cell lines. Exisulind, CP461, and CP248 show the same rank orders of potency for PDE5 and PDE2 inhibition, apoptosis, and growth inhibition constants [12]. Since β-catenin and cyclin D1 regulation by these drugs occurred in similar concentration ranges, these effects may be downstream to PKG activation, secondary to PDE inhibition. YC-1, a soluble guanylyl cyclase activator, showed the effects of reducing β-catenin and cyclin D1 in SW480 cells (data not shown). Moreover, PKG efficiently phosphorylates recombinant β-catenin *in vitro*, and exisulind treatment of colon cancer cells increases the phosphorylation of β-catenin [11]. Studies are ongoing to determine whether PKG may directly phosphorylate β-catenin to cause its degradation, or whether some other kinase, e.g. JNK, also activated by PKG and involved in exisulind- and analog-induced apoptosis [54], mediates the process. Presumably, the expression of additional oncogenes (c-*myc*, *PPARδ*) [43,47], whose expression is regulated by Tcf/Lef, may also be lowered by the exisulind- and analog-induced degradation of β-catenin.

In addition to APC mutations causing accumulation of β-catenin in cancer cells, mutations in axin or in β-catenin can have the same result [6,55–57]. This is because cells with mutations in either APC, β-catenin, or axin may interrupt the process of β-catenin phosphorylation. We reported that exisulind reduced β-catenin levels in HCT116 cells [58], colon tumor cells expressing wild-type APC. β-Catenin in HCT116 cells shows mutations near the UTS [20] and, therefore, affects processing. Thus, the pathway activated by exisulind and its analogues does not require either the formation of an APC–protein complex as a prerequisite for β-catenin phosphorylation or the integrity of the GSK consensus sequence. These data are consistent with a kinase such as PKG substituting for GSK3β to initiate proteolysis of β-catenin. The PKG phosphorylation site(s) on β-catenin is currently being determined and will be reported separately.

While the nonsteroidal anti-inflammatory drug sulindac has been shown to reduce β-catenin *in vivo* [38], exisulind and its analogues are the first drugs lacking anti-inflammatory activity shown to have this pharmacological effect. Since these drugs do not inhibit cyclooxygenases, reduction in β-catenin is cyclooxygenase-independent. Many other cancers have defects in the APC/β-catenin pathway [6,7,18,22–26,55–57]. Exisulind and its derivatives have shown positive results in other animal models of tumorigenesis including prostate, bladder, mammary, and lung, suggesting a wide occurrence of β-catenin regulation [8,10,59–61]. Exisulind and its analogues provide a pharmacological mechanism for reducing β-catenin and cyclin D1 levels to increase apoptosis. This process may also reduce the aberrant transcription of multiple oncogenes and allow cells to undergo apoptosis. Exisulind does not affect normal colonic crypt cells [62], perhaps, in part, because of normal APC/β-catenin processing. Thus, exisulind and its analogues are considered SAANDs. The development of SAANDs provides a previously unexplored approach to treating cancer.

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