Monensin Inhibits Canonical Wnt Signaling in Human Colorectal Cancer Cells and Suppresses Tumor Growth in Multiple Intestinal Neoplasia Mice

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Abstract
The Wnt signaling pathway is required during embryonic development and for the maintenance of homeostasis in adult tissues. However, aberrant activation of the pathway is implicated in a number of human disorders, including cancer of the gastrointestinal tract, breast, liver, melanoma, and hematologic malignancies. In this study, we identified monensin, a polyether ionophore antibiotic, as a potent inhibitor of Wnt signaling. The inhibitory effect of monensin on the Wnt/β-catenin signaling cascade was observed in mammalian cells stimulated with Wnt ligands, glycogen synthase kinase-3 inhibitors, and in cells transfected with β-catenin expression constructs. Furthermore, monensin suppressed the Wnt-dependent tail fin regeneration in zebrafish and Wnt- or β-catenin–induced formation of secondary body axis in Xenopus embryos. In Wnt3a-activated HEK293 cells, monensin blocked the phosphorylation of Wnt coreceptor low-density lipoprotein receptor related protein 6 and promoted its degradation. In human colorectal carcinoma cells displaying deregulated Wnt signaling, monensin reduced the intracellular levels of β-catenin. The reduction attenuated the expression of Wnt signaling target genes such as cyclin D1 and SP5 and decreased the cell proliferation rate. In multiple intestinal neoplasia (Min) mice, daily administration of monensin suppressed progression of the intestinal tumors without any sign of toxicity on normal mucosa. Our data suggest monensin as a prospective anticancer drug for therapy of neoplasia with deregulated Wnt signaling, Mol Cancer Ther; 13(4); 812–22. ©2014 AACR.

Introduction
The Wnt pathway is an evolutionarily conserved signaling mechanism that evolved in metazoans. During embryonic development, the pathway is essential for cell proliferation, differentiation, and migration. In adult organisms, Wnt signaling is involved in somatic tissue homeostasis and tissue regeneration upon injury (reviewed in ref. 1); moreover, deregulation of Wnt signaling is a hallmark of various types of cancer (reviewed in ref. 2). The key component of the canonical Wnt signaling pathway is β-catenin (reviewed in ref. 3). In unstimulated cells, β-catenin is phosphorylated at the N-terminus by the cytoplasmic "destruction complex" that includes axis inhibition protein (Axin), adenomatous polyposis coli (APC) and serine/threonine kinases casein kinase-1ε (CK1ε), and glycogen synthase kinase-3 (GSK-3). The phosphorylation promotes ubiquitination and subsequent degradation of the protein keeping the cellular level of the free pool of β-catenin low. Binding of the secreted Wnt ligands to the Frizzled (Fz) receptor and Wnt coreceptor lipoprotein receptor related protein (LRP)-5/6 initiates the CK1ε-dependent phosphorylation of multidomain cytoplasmic transducer Dishevelled (Dvl). In addition, LRP is phosphorylated at its intracellular part by CK1γ and GSK3. The latter event leads to the formation of the LRP–Axin complex and dephosphorylation of Axin. Dephosphorylated Axin constitutes inactive conformation that is unable to interact with LRP and β-catenin. Without the Axin scaffold β-catenin phosphorylation is inhibited and, consequently, the protein accumulates in the cell cytoplasm and nucleus. In the nucleus, β-catenin forms complexes with DNA-binding factors of the lymphoid enhancer factor/T-cell factor (LEF/TCF) family (further referred to as TCFs). The complexes act as...
bipartite transcriptional activators of specific Wnt signaling target genes.

Cancer affecting colon and rectum constitutes one of the most commonly diagnosed neoplasia in developed countries (4). Intriguingly, pathogenesis of the colorectal carcinoma is connected with the aberrant activity of the Wnt/β-catenin signaling cascade. Germlinal mutations of the APC gene underlie the hereditary familial adenomatous polyposis (FAP) syndrome (5). Similarly, about 50% of sporadic colorectal tumors arise upon biallelic loss of APC (6). Hyperactive Wnt signaling might also result upon activation mutations in the β-catenin (also designated as CTNNB1) gene (7). In either case, stabilized β-catenin mediates inappropriate transcriptional activation of TCF/β-catenin target genes, thus driving pathologic transformation of the gut epithelium (8).

In the present study, we performed a reporter gene-based high-throughput screen (HTS) to identify inhibitors of the Wnt signaling pathway. We identified monensin, an antibiotic isolated from Streptomyces cinnamonensis bacteria (9), as a potent blocker of the Wnt-induced transcription in cells stimulated with Wnt ligands or GSK3 inhibitors. The suppressive effect of monensin on Wnt signaling was also observed in the tail fin regeneration assay in zebrafish and Xenopus body axis duplication experiment. In human colorectal carcinoma cells harboring mutations in APC or β-catenin, the monensin-mediated block of the TCF/β-catenin transcription activity led to slowdown in cell-cycle progression. Finally, monensin treatment reduced the size of the Apc-deficient tumors in the mouse model of intestinal cancer.

Materials and Methods

Cell lines and generation of Wnt1-producing cells

SuperTOPFLASH HEK293 (STF) cells (10) harboring the genome-integrated Wnt-responsive luciferase reporter SuperTOPFLASH were a gift of Q. Xu and J. Nathans (Johns Hopkins University, Baltimore, MD). COLO320, HCT116, HEK293, L, LS174T, RKO, and SW480 cell lines were purchased from the American Type Culture Collection. All cell lines were obtained in 2006 and maintained in Dulbecco’s modified Eagle medium (Sigma) supplemented with 10% FBS (Gibco), penicillin, streptomycin, and gentamicin (Invitrogen). Upon receipt, cells were expand- ed and aliquots of cells at passage number <10 were stored frozen in liquid nitrogen. Cells from one aliquot were kept in culture for less than 2 months after resuscitation. The cell identity was not authenticated by the authors. Mouse Wnt1 cDNA (11) was cloned into the lentiviral vector pCDH1 (System Biosciences). Lentiviruses were prepared using the Trans-Lentiviral Packaging System (Open Biosystems). Transduced STF cells were selected without subcloning using puromycin (Alexis; 5 μg/mL).

Compounds, luciferase reporters and assays, transfections, and biochemistry

The small compound collections included the Library of Pharmacologically Active Compounds (LOPAC1280; Sigma-Aldrich), Prestwick Chemical Library (Illkirch, France), and NIH Clinical Trial Collection. Monensin sodium salt, (2′Z,3′E)-6-Bromoindirubin-3′-oxime (BIO) and bafilomycin A1 were purchased from Sigma, and CHIR99021 from Selleckchem. Luciferase reporter constructs, NF-κB-Luc and pRL-TK, were purchased from Promega. The TCF/β-catenin–dependent reporter TOPFLASH and negative control reporter FOPFLASH were described previously (8). The luciferase assays were performed as described previously (11) using the ONE-Glo Luciferase Assay System (Promega) for HTS and Dual-Glo Luciferase Assay System (Promega) for subsequent analysis. Mouse Wnt3a ligand was isolated from the culture medium of Wnt3a-producing L cells as described previously (12). Human recombinant lymphotoxin-α (LTα) was purchased from R&D Systems. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen). RNA purification, quantitative reverse transcription PCR (qRT-PCR), coimmunoprecipitations, and immunoblotting were performed as described previously (11). The primers for qRT-PCR are listed in Supplementary Table S1.

Cell viability, apoptosis, and cell proliferation assays

Cell viability and apoptosis were determined after overnight incubation with respective compounds using the Cell Titer-Blue Cell Viability Assay Kit and Caspase-Glo 3/7 Assay Kit, respectively (Promega). In control experiments, cells were incubated with recombinant TRAIL (kindly provided by L. Andera, Institute of Molecular Genetics, Prague, Czech Republic). Apoptotic cells in the mouse intestine were detected using TumorTACS Detection Kit (RD Systems). Metabolic incorporation of [3H]-thymidine (MP Radiochemicals; final concentration 0.1 mCi/μL) was measured using MicroBeta2 Microplate Counter (PerkinElmer) after overnight incubation at 37°C.

Monitoring cell proliferation and attachment in "real time"

xCELLigence Real-Time Cell Analysis System (Roche Applied Science) was used according to the instructions of the manufacturer. Cells were seeded at the density of 1,500 cells per well. The electronic impedance of sensor electrodes was monitored every 15 minutes. Eighteen hours after seeding, monensin or dimethyl sulfoxide (DMSO) was added to the wells and the measurement continued for additional 24 hours. Cell index was quantified as described previously (13).

Immunocytochemistry and immunohistochemistry

The techniques were performed as described previously (14, 15).

Zebrafish tailfin regeneration assay

Zebrafish (less than 6 months of age) were kept in E3 medium (5 mmol/L NaCl, 0.17 mmol/L KCl, 0.33 mmol/L CaCl2, and 0.33 mmol/L MgSO4 in distilled H2O) at 28°C. Fishes that were approximately 2.5 cm long were anesthetized with tricaine (Sigma) and tips of their tail fin were
carefully amputated using scissors. The animals were randomly distributed into aquaria (4–5 fish per tank) containing E3 medium with 2 μmol/L (prepared from stock solution of 20 mmol/L monensin in ethanol) or equivalent volume of ethanol alone. One week later, fishes were anesthetized and photographed. The regenerated area (recognizable by lack of pigmentation) was scored in three independent experiments using ImageJ software.

**Xenopus double axis formation assay**

The assay was performed as described previously (16). A marginal zone of the ventral blastomers of 4-cell stage *Xenopus laevis* embryos was injected (4 nL) using 20 or 800 pg of XWnt8 or β-CATENIN mRNA, respectively. Messenger RNA was injected together with monensin (0.04 pmol) or vehicle (DMSO). The developing embryos were kept at 20°C; the duplication of the body axis was scored 36 hours after injection.

**Tumor treatment in mice**

Multiple intestinal neoplasia (Min) mice (further referred to as the Apc<sup>−/−Min</sup> strain) were purchased from the Jackson Laboratory. Animals were housed and handled in accordance with the approved guidelines. Four-week-old pups were weaned, genotyped, and randomized. The animals were divided into two groups and treated with monensin (10 mg/kg) or vehicle (DMSO). Daily oral applications continued for 6 weeks. In addition, six pairs of Apc<sup>−/−Min</sup> mice ages 7, 10, 13, 16, 19, and 22 weeks were treated with monensin or vehicle for 5 weeks. The mice were sacrificed and the intestines were dissected, washed in PBS, and fixed in 4% formaldehyde (v/v) in PBS for 3 days. Fixed intestines were embedded in paraffin, sectioned and stained. The number and size of the neoplastic lesions were quantified using Ellipse software (ViDiTo).

**Statistical analysis**

Fisher exact test was used to analyze the statistical significance of the results of the double axis formation assay. Data obtained in the gene reporter and qRT-PCR analyses were evaluated by Student t test.

Additional materials and methods, including details of HTS, plasmid constructs, and antibodies, are given in Supplementary Materials and Methods.

**Results**

**HTS for inhibitors of the Wnt signaling pathway**

Luciferase reporter gene assay in STF cells was used to search for novel inhibitors of Wnt/β-catenin signaling. The screen included 2,448 compounds from three commercially available collections. STF cells were stimulated with recombinant Wnt3a ligand and, simultaneously, the tested compounds were added to culture medium to 1 μmol/L concentration. The luciferase activity was quantified 18 hours later using bioluminescent signal detection. The primary screen identified seven "small molecules" displaying a profound inhibitory effect on the SuperTOP-FLASH activity. These molecules included the previously identified Wnt pathway inhibitors indometacin (17), thapsigargin (18), and harmine (19). In addition, four compounds without any (published) relation to Wnt signaling were discovered. The putative novel Wnt pathway modulators were examined for their effective concentration range, cell toxicity, and direct repressive effect on the luciferase reaction. Moreover, because the zebrafish has the ability to regrow damaged tissues in the process that depends on active Wnt/β-catenin signaling (20), we used the tail fish regeneration assay to validate the action of the identified Wnt pathway inhibitors in vivo. Polyether antibiotic monensin, which suppressed the activity of the TCF/β-catenin reporter SuperTOPFLASH at concentrations 0.2 to 5 μmol/L and decreased the tail fish regeneration to 50% (compared with control vehicle-treated animals), was selected for subsequent studies (Fig. 1).

**Monensin inhibits the Wnt signaling cascade at multiple levels**

To confirm the specificity of the monensin action, reporter gene assays in HEK293 cells were performed using the Wnt-responsive reporter TOPFLASH (8), negative-control reporter FOPFLASH, and the NF-κB pathway luciferase reporter plasmid NF-κB-Luc. Cells transiently transfected with the reporters were stimulated with recombinant Wnt3a or LTR to activate Wnt or NF-κB signaling, respectively. In agreement with the results obtained in STF cells, 1 and 5 μmol/L monensin reduced the TOPFLASH activity to 34% and 32%, respectively (Fig. 2A). Conversely, monensin had no effect on the transcription from the NF-κB-Luc and FOPFLASH reporters (Fig. 2A; data not shown). In addition, we performed qRT-PCR analysis of HEK293 cells. Monensin treatment resulted in downregulation of the previously described Wnt target genes AXIN2, CYCLIN D1, LGR5, NKD1, and SP5 (Fig. 2B). To verify these results in a different cell type, the β-catenin stabilization was visualized in L cells (15). In these mouse fibroblasts, monensin reduced Wnt3a-mediated accumulation of β-catenin in the cytoplasm and nucleus (Fig. 2C). Recently, Morrall and colleagues have reported that some inhibitors of the Wnt pathway antagonize recombinant Wnt3a protein but are ineffective against ectopically expressed Wnt ligands (21). To exclude this possibility, the results of luciferase and qRT-PCR assays were confirmed using Wnt1-transduced STF cells (not shown). In addition, Western blot analysis of Wnt1-producing STF cells showed that monensin treatment decreased the cellular levels of β-catenin, including the presumably transcriptionally active forms of the protein either nonphosphorylated at the N-terminus (non-P-β-CATENIN) or containing the phosphorylated serine residue at position 675 (P-S675-β-CATENIN; ref. 22). Reduction in the production of the AXIN2 protein was also observed, indicating that monensin did not inhibit tankyrase (14). In contrast, monensin had no effect on the cellular levels of nuclear Wnt signaling effector TCP4 (Fig. 2D).

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To identify the molecular mechanism of the monensin action in more detail, we used GSK3 inhibitors BIO (23) and CHIR99021 (24) to trigger Wnt signaling. Monensin suppressed the activity of both compounds in STF and L cells (Fig. 3A and B; data not shown). In HEK293 cells, BIO increased the cellular levels of total and "active" non-P-β-CATENIN. Combined cell treatment with BIO and monensin caused a moderate decrease in all tested β-catenin forms (Fig. 3C and Supplementary Fig. S1). We noted that monensin antagonized GSK3 inhibitors less efficiently than the signaling initiated by Wnt ligands, indicating that monensin "hits" the Wnt pathway upstream and at the level (or downstream) of the β-catenin destruction complex. This conclusion was confirmed in STF cells displaying aberrant Wnt signaling after disruption of the APC gene induced by transcription activator-like effectors (Fig. 3C and Supplementary Fig. S1). We next tested whether monensin inhibits Wnt-induced dorsalization of Xenopus embryos. Injection of XWnt8 with vehicle alone resulted in 65.9% embryos with a duplicated body axis. In contrast, coinjection of XWnt8 with monensin caused a significant (*P = 0.002) decrease in the proportion of dorsalized animals to 30.7%. Interestingly, monensin also reduced the secondary body axis formation initiated by β-catenin mRNA (Fig. 3E). In agreement with the results obtained in Xenopus embryos, in STF cells monensin suppressed the transcription activity of wild-type (wt) β-catenin. Strikingly, monensin also inhibited stable forms of β-catenin mutated at the N-terminal regulatory residues but had no effect on transcription induced by the Lef1-VP16 fusion protein (Fig. 3F).

Disruption of intracellular pH homeostasis suppresses paracrine Wnt signaling; moreover, inhibition of vacuolar acidification interferes with Wnt secretion (26). Because monensin acts as a ionophore blocking cellular acidification, we compared its effect on Wnt signaling with bafilomycin A, a compound disrupting vacuolar acidification through pharmacologic inhibition of V-ATPase (27). In contrast with monensin, bafilomycin inhibited the signaling activity of the recombinant and ectopically expressed Wnt ligand, but the inhibitor was not effective against signaling stimulated by β-catenin or constitutively active LRP6 (ΔN-LRP6; Supplementary Fig. S4). Taken together, these results provided evidence that monensin...
specifically antagonized the Wnt signaling pathway at the LRP and β-catenin levels.

**Monensin attenuates aberrant Wnt signaling in human colorectal carcinoma cells**

The effect of monensin was investigated in four colorectal carcinoma-derived cell lines. SW480 and COLO320 cells are APC deficient, whereas LS174T and HCT116 cells contain intact APC but produce stabilized S45A and ΔS45 β-catenin proteins, respectively (28). Interestingly, monensin decreased the activity of the TOPFLASH reporter and the expression of Wnt signaling target genes in SW480, COLO320, and LS174T cells but not in HCT116 cells (Fig. 4A and B; Supplementary Table S3; Supplementary Fig. S5A). Microscopy supported this finding, showing reduced anti-β-catenin staining in monensin-treated SW480, COLO320, and LS174T cells. No reduction in the anti-β-catenin signal was observed in HCT116 cells, although the treated cells gained a spindle-like shape (Fig. 4C and Supplementary Fig. S5B). Using immunoblotting, we detected a decrease in all forms of β-catenin in SW480 cells. In the other colorectal carcinoma-derived cells, the decrease in the stability of different β-catenin forms was moderate (COLO32O and HCT116 cells) or negligible (LS174T cells; Fig. 4D and Supplementary Fig. S5C). We noted a discrepancy between clear reduction of the β-catenin signal as documented by microscopy and rather moderate changes in the β-catenin levels obtained by immunoblotting. The phenomenon was not associated with the specificity of the monoclonal antibody because another two anti-β-catenin monoclonal antibodies displayed a similar variation in the β-catenin detection (data not shown). The observed discrepancy possibly reflects differences in the robustness of these two readouts, and monensin to some extent promoted β-catenin degradation in three (SW480, COLO320, and LS174T) out of four colorectal carcinoma cells tested.
Figure 3. Monensin suppresses Wnt signaling initiated by GSK3 inhibitors and ectopic β-catenin. A, luciferase reporter assays in STF cells stimulated with recombinant Wnt3a or GSK3 inhibitors BIO (1 μmol/L) and CHIR99021 (3 μmol/L); error bars, SDs. B, fluorescent microscopy images of L cells treated overnight with BIO in combination with vehicle or monensin. C, immunoblotting of lysates obtained from STF cells stimulated overnight with BIO in combination with vehicle or monensin. Densitometric analysis of the Western blot analyses is given in Supplementary Fig. S1B. D, monensin reduces the protein level and phosphorylation of the Wnt coreceptor LRP6 in HEK293 cells; P-LRP6, LRP6 phosphorylated at S1490. E, monensin reduces double axis formation in Xenopus embryos; representative images of embryos are shown at the right. n, total number of injected embryos; error bars, SEM. F, results of the luciferase reporter assays performed in STF cells transfected with the indicated β-catenin–expressing constructs. S33Y, S37A, S45A, stable β-catenin with the amino acid changes at the serine residue in positions 33, 37, and 45; ΔS45, S45 deleted; ΔN, the N-terminally truncated variant of β-catenin; Lef1-VP16, Lef1 lacking the N-terminal β-catenin–interaction domain fused to the transcription transactivation domain of herpes simplex virus protein VP16; *, P < 0.05; **, P < 0.01.
Cell proliferation assays in SW480 and COLO320 cells revealed more than 50% decrease in [3H]-thymidine incorporation in cultures with monensin when compared with controls treated with DMSO alone. The proliferation rate of LS174T cells was affected by monensin to a lesser extent; nevertheless, the antibiotic caused 25% decline in [3H]-thymidine counts. No substantial changes in proliferation of HCT116 and Wnt “signaling inactive” HEK293 and HeLa cells were recorded (Fig. 4E and Supplementary Fig. S5D). Subsequently, cell-cycle analysis showed that monensin reduced the fraction of SW480 cells in S and G2-M phases. Conversely, the cell fraction in G1 phase was increased (Supplementary Table S4). In addition, we used the xCELLigence system to gain continuous information about the cell growth, death, and morphological changes of SW480 and HCT116 cells. As shown in Fig. 4F, monensin significantly influenced the cell index (this parameter depends on the number and dimensional changes of attached cells) of SW480 cells. In HCT116, the cell index fluctuations were possibly attributed to the
Monensin reduces tumor size in the APC$^{+/Min}$ mouse model of intestinal cancer

The possible antitumor activity of monensin was analyzed in Apc$^{+/Min}$ mice. The mice harbor a truncation mutation in one allele of the Apc gene, and all adult animals eventually develop large amounts of intestinal polyps and die of cancer (35). The initial daily oral applications of monensin (dose 10 mg/kg) started at the weaning age. Animals in the control group received vehicle (DMSO) only. No effect on body weight was noticed throughout the experiment and individuals in both groups were steadily gaining weight as they were reaching sexual maturity (not shown). After six weeks, the mice were sacrificed and the dissected intestines were embedded in paraffin and sectioned. Immunohistochemical staining showed elevated production of β-catenin in neoplastic lesions found mainly in the small intestine (Fig. 5A). Stained tumors contrasted with the healthy mucosa enabled quantitative analysis of the tumor size and numbers using the image analysis software Ellipse (14). Although the numbers of tumors did not change substantially, a significant ($P = 0.0144$) reduction in the average size of lesions was observed in monensin-treated Apc$^{+/Min}$ mice when compared with control animals (mean $0.199 \text{ mm}^2 \pm 0.299 \text{ mm}^2$). Consequently, the total tumor area estimated in one animal was decreased in individuals receiving monensin (mean $10.16 \text{ mm}^2 \pm 16.46 \text{ mm}^2$; $P = 0.0125$; Fig. 5B). The inhibitory effect of monensin on the tumor growth was also observed in the second experiment, in which the compound (or vehicle) was administered to paired adult animals at various ages (Fig. 5C). Interestingly, the proportion of proliferating cells positive for the Ki-67 or proliferating cell nuclear antigen marker did not change in adenomas exposed to monensin (Fig. 5D, c and d' and Supplementary Fig. S9). However, monensin treatment increased the numbers of apoptotic cells and cells expressing the p21 cell-cycle inhibitor at the surface area of the neoplastic outgrowths (Fig. 5D, e–h'). This indicated that the smaller size of lesions in monensin-treated APC$^{+/Min}$ mice was related to the cell-cycle arrest and/or cell death at the tumor periphery. Importantly, no changes in the cell proliferation, differentiation, and tissue architecture in the healthy parts of mucosa were noted after exposure to monensin (Fig. 5E, i–l).

Discussion

Monensin belongs to the group of natural carboxylic polyether ionophores (36). The ionophoric antibiotics, the group currently includes more than 100 compounds, are studied mainly for their antibacterial, antifungal, and antiparasitic biologic activity (9). Monensin was approved by the U.S. Food and Drug Administration for use in veterinary practice as a coccidiostat in poultry and growth-promoting agent in cattle (New Animal Drug Application No. 95-735). The antibiotic increased dairy cattle milk production with no negative side effects on the animal health or reproduction (37, 38). Stimulation of growth in ruminants is associated with changes in intestinal microflora that lead to increased amounts of digestible proteins (39). Monensin antibacterial and antiparasitic activity is related to the intracellular changes in pH and sodium–potassium balance that can result in cell death (40). Several recent studies have demonstrated that monensin inhibits growth and induces apoptosis of cells derived from renal, prostate, and colon carcinoma (29, 41, 42). In addition, monensin induced cell-cycle arrest of acute myelogenous leukemia and lymphoma cells (43, 44). In glioma cells, monensin provoke endoplasmic reticulum stress and sensitized these cells to TRAIL-induced apoptosis (45). According to our results, monensin antagonized the Wnt signaling cascade at multiple levels involving LRP6 and β-catenin. Interestingly, a similar “mode of action” was described for other potassium ionophores salinomycin and nigericin in chronic lymphocytic leukemia.

Changes in cellular shape because monensin did not induce apoptosis in any of the colorectal carcinoma cells tested (Fig. 4G). In prostate cancer cells, monensin treatment reduced the amount of androgen receptor mRNA and elevated oxidative stress (29). However, monensin did not increase the levels of reactive oxygen species (ROS) in HEK293 and SW480 cells (Supplementary Fig. S6); thus, we could exclude that its effect on Wnt signaling was indirectly mediated by changes in the intracellular ROS concentration.

The insensitivity of HCT116 cells to monensin was peculiar as LS174T cells, which also express mutant β-catenin, were sensitive to the antibiotic. Interestingly, HCT116 cells show considerably less TOPFLASH activity than other colorectal carcinoma cells carrying APC truncations or β-catenin mutations (Fig. 4A; ref. 28). Moreover, in HCT116 cells, β-catenin is mainly associated with the cellular membrane (Supplementary Fig. S7). Recently, several studies documented that β-catenin localization or intracellular concentration can be regulated by several kinases, including RAF1, c-Jun NH2-terminal kinase 2, AKT, protein kinase A (PKA), and F21-activated kinase 1 (22, 30–33). We therefore evaluated the effect of monensin on the phosphorylation of β-catenin. The endogenous protein was precipitated from control and monensin-treated SW480 and HCT116 cells, and phosphorylated peptides were determined using liquid chromatography/tandem mass spectrometry (LC/MS–MS). The analysis revealed that β-catenin is indeed phosphorylated at S191 (HCT116 cells only), S552, and S675; however, the observed modifications did not change in monensin-sensitive SW480 cells upon the treatment (Supplementary Table S5). Because acetylation influences β-catenin stability and transcriptional activity (34), the impact of monensin on β-catenin acetylation was also determined. Nevertheless, the extent of β-catenin acetylation did not change in the cells cultured with monensin (Supplementary Fig. S8).
cells (46). However, the detailed inhibitory mechanism of these antibiotics on the Wnt signaling pathway has not been identified.

Protein phosphorylation is the key modification in virtually all signaling cascades. Therefore, we performed kinase selectivity profiling, but no conclusive result was obtained. This would imply that (i) the monensin action is highly specific and monensin-sensitive kinase was not included in the test, (ii) the selected concentrations of the antibiotic were too low to elicit any effect in this particular type of experiment, and (iii) the monensin action is not related to the inhibition of any protein kinase. The last possibility seems to be most likely, at least in relation to β-catenin, because LC/MS-MS analysis did not identify any phosphorylation sites that changed in monensin-sensitive SW480 cells.

Figure 5. Monensin treatment decreases the size of adenomas in APC^Min^ mice. A, hematoxylin and anti-β-catenin–stained sections of the jejunum of APC^Min^ mice. Tumors are indicated by black arrowheads. B and C, quantification of the tumor size and count in the ileum of vehicle- and monensin-treated APC^Min^ mice; the treatment started either in 4-week-old animals just after weaning (B) or in adult animals 7 to 22 weeks of age (C). The total tumor area determined in each individual is indicated in the boxplots. The boxed areas correspond to the second and third quartiles; the spread of the values is given by "whiskers" above and below each box. Median (transverse line) and mean (cross) is marked inside each box; statistical significance was determined using Student (B) or paired (C) t test; *, P < 0.05; error bars, SD. D, detection of proliferation marker Ki-67 (c and d), cell-cycle inhibitor p21 (e and f), and apoptotic cells (TACS; g and h) in the small intestinal tumors. Detailed images are shown in panels c', d', e', f', g', and h'. E, phenotype of healthy parts of the small intestine is not affected by monensin. The specimens were stained with Ki-67 (i and k) and cytokeratin 20 (Krt20; j and l) to label proliferating cells in the crypts and terminally differentiated epithelial cells on the villi, respectively. Bar, 0.5 mm (a and b), 0.15 mm (a', b', c, d, e, f, g, and h), 0.08 mm (i, j, k, and l), and 0.05 mm (c', d', e', f', g', and h').
In APC−/−Min mice, monensin suppressed tumor growth without any noticeable negative impact on healthy mucosa. In the treated animals, the size of neoplastic lesions was decreased, but the average number of tumors remained unchanged. This result indicated that monensin inhibited tumor progression rather than the tumor initiation process. The conclusion was confirmed by immunohistochemical staining that showed markers of cell-cycle arrest and apoptosis at the surface of neoplastic outgrowths. In colorectal carcinoma cells, this proapoptotic activity of monensin was not observed, presumably due to the genetic alterations impairing cell death-inducing mechanisms (47).

In HCT116 cells, β-catenin displayed clear membrane localization; however, in LS174T cells, the protein was detected not only at the cellular membrane, but also in the cytoplasm and nucleus. As reported previously, the level of aberrant TCF/β-catenin–driven transcription depends on mutations in the genome of colorectal carcinoma cells. Cells expressing “short” APC mutants lacking the nuclear export signals exhibit high activity of the TOPFLASH reporter when compared with cells with wt APC but mutant β-catenin (28). HCT116 cells harbor one wt and one mutant (ΔS45) allele of β-catenin, whereas LS174T are homozygous for missense mutations (S45A) in the same triplet of the gene. This implies that subcellular distribution of β-catenin is related to the partially retained ability of HCT116 cells to regulate Wnt/β-catenin signaling. Nevertheless, because both mutant alleles (i.e., ΔS45 and S45A) are inhibited by monensin in reporter gene assays, the reason why HCT116 and LS174T cells display differential sensitivity to monensin is unclear. We suggest that particular cellular “wiring” of various signaling pathways or networks can contribute to the response of the respective cell to monensin.

Despite some prevailing uncertainties about the detailed inhibitory mechanism of monensin, our data imply that the antibiotic might be used as an anticancer drug, especially in neoplasia displaying aberrant Wnt signaling.

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

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