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Intervening in β-Catenin Signaling by Sulindac Inhibits S100A4-Dependent Colon Cancer Metastasis[1,2]

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Abstract
Colon cancer metastasis is often associated with activation of the Wnt/β-catenin signaling pathway and high expression of the metastasis mediator S100A4. We previously demonstrated the transcriptional regulation of S100A4 by β-catenin and the importance of the interconnection of these cellular programs for metastasis. Here we probe the hypothesis that the nonsteroidal anti-inflammatory drug sulindac sulfide can inhibit colon cancer metastasis by intervening in β-catenin signaling and thereby interdicting S100A4. We treated colon cancer cell lines heterozygous for gain-of-function and wild-type β-catenin with sulindac. We analyzed sulindac's effects on β-catenin expression and subcellular localization, β-catenin binding to the T-cell factor (TCF)/S100A4 promoter complex, S100A4 promoter activity, S100A4 expression, cell motility, and proliferation. Mice intrasplenically transplanted with S100A4-overexpressing colon cancer cells were treated with sulindac. Tumor growth and metastasis, and their β-catenin and S100A4 expressions, were determined. We report the expression knockdown of β-catenin by sulindac, leading to its reduced nuclear accumulation. The binding of β-catenin to TCF was clearly lowered, resulting in reduced S100A4 promoter activity and expression. This correlated well with the inhibition of cell migration and invasion, which could be rescued by ectopic S100A4 expression. In mice, sulindac treatment resulted in reduced tumor growth in the spleen (P = .014) and decreased liver metastasis in a human colon cancer xenograft model (P = .025). Splenic tumors and liver metastases of sulindac-treated mice showed lowered β-catenin and S100A4 levels. These results suggest that modulators of β-catenin signaling such as sulindac offer potential as antimetastatic agents by interdicting S100A4 expression.

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Abbreviations: CAT-ELISA, chloramphenicol acetyltransferase–ELISA; COX, cyclooxygenase; EMSA, electrophoretic mobility shift assay; NSAID, nonsteroidal anti-inflammatory drug; TCF, T-cell factor

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Introduction

Colon cancer is the second most frequent malignancy in the Western world and represents one of the leading causes of cancer-related deaths. The 5-year survival rates are approximately 90% for early stage patients, dropping to 65% in patients with regional lymph node metastases and decreasing to less than 10% in patients with distant metastases [1]. The metastatic dissemination of primary tumors is directly linked to patient survival and accounts for approximately 90% of all colon cancer deaths [2]. Distant metastases develop in approximately 50% of all colon cancer patients, preferentially in the liver [3]. Half of these patients develop distant metastases after initial surgery of their primary tumor (metachronous metastasis). However, therapeutic options are limited, particularly for patients with metastases. Patients at high risk for metastasis, ideally identified well before the occurrence of metastasis, would benefit most from antimetastatic therapies [4].

The metastasis mediator, S100A4, a member of the S100 family of calcium-binding proteins, provides a marker for the early identification of patients at high risk for distant metastasis. S100A4 is overexpressed in many different types of cancer and has negative significance for prognosis and patient survival [5–7]. In colorectal cancer, S100A4 levels and nuclear localization increase during tumor progression [8–11]. High S100A4 levels in colorectal tumors are associated with aggressive growth, metastasis, poor prognosis, and shortened patient survival times [12–14]. We previously demonstrated a significant positive correlation of S100A4 levels in primary tumors with subsequent metastasis and patient survival [15].

More than 90% of colorectal cancers bear mutations that result in Wnt pathway activation [16,17]. These mutations generally affect β-catenin phosphorylation, hindering its degradation through the ubiquitin pathway. Nonphosphorylated β-catenin accumulates in the cytoplasm, enters the nucleus, and interacts with T-cell factor (TCF) transcription factors to control target genes. Nuclear β-catenin accumulation has been correlated with late stages of tumor progression and metastasis; the presence of mutated β-catenin is associated with aggressive tumor growth and poor prognosis [18,19]. We previously demonstrated the dependence of β-catenin–induced migration and invasiveness on S100A4. We identified S100A4 as a transcriptional β-catenin target gene. Transplantation of colon cancer cells with gain-of-function β-catenin and high S100A4 expression resulted in distant metastasis in mice [15].

Here we probed the functional significance of our findings using the nonsteroidal anti-inflammatory drug (NSAID) sulindac sulfide (sulindac), a known pharmacological inhibitor of β-catenin, for prevention of metastasis. Sulindac inhibits β-catenin expression in colorectal cancer cells and in patients with hereditary nonpolyposis colorectal cancer and familial adenomatous polyposis [20–22]. Sulindac induces proteasome-dependent degradation of β-catenin and suppresses tumorigenesis *in vivo* by downregulating β-catenin signaling [23,24]. It inhibits the nuclear accumulation of β-catenin in colorectal carcinoma cell lines and in adenomas of patients with familial adenomatous polyposis leading to reduced downstream signaling [25]. Consequently, β-catenin target genes like *Met*, *c-myc*, and *cyclin D1* are downregulated after sulindac treatment [22,25–28].

We studied the effects of sulindac on the expression and subcellular localization of β-catenin and on the expression and promoter activity of its transcriptional target gene *S100A4*. We evaluated the effect of sulindac on functional parameters such as cell migration, invasiveness, and proliferation of human colon cancer cells in the context of S100A4 expression inhibition. For preclinical evaluation, we identified sulindac concentrations that are nontoxic but effective for metastasis prevention in mice. Finally, we demonstrate that the antimetastatic potential of sulindac in mice is closely coupled to the intervention in β-catenin signaling and interdicting S100A4 expression.

Materials and Methods

Tumor Cell Lines, Transfections, and Treatments

HCT116 cells (heterozygous for β-catenin wild-type/Δ45 exon 3) and the β-catenin knockout sublines were kindly obtained from Todd Waldman, Georgetown University, Washington, DC [29]. HAB-68^{mut} cells express only the Δ45 mutant (mut) allele, and HAB-92^{wt} cells express only one wild-type (wt) allele of β-catenin. Sublines with reconstituted heterozygous genotype, HAB-68^{mut}/wt and HAB-92^{wt}/mut, were generated by stable transfection with wt β-catenin complementary DNA (cDNA) into HAB-68^{mut} and Δ45-gain-of-function β-catenin cDNA into HAB-92^{wt} cells. β-Catenin genotypes were confirmed by sequencing exon 3 and by reverse transcription–polymerase chain reaction (RT-PCR)–based restriction fragment length polymorphism [15]. Transfections of wt and Δ45-mutant β-catenin cDNA (kindly provided by Bert Vogelstein, Johns Hopkins University, Baltimore, MD [30]) of S100A4 cDNA (kindly provided by Claus Heizmann, University of Zurich, Switzerland [31]), of *c-myc* cDNA (cloned in our laboratory), and of cyclin D1 cDNA (kindly provided by Klaus Wethmar, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany) were performed using lipofectin (Invitrogen, Karlsruhe, Germany). For each transfection experiment, at least three independent transfected clones were analyzed; one representative clone thereof is shown, respectively. Cells were grown in RPMI 1640 (HCT116 cells and sublines, SW620) or Dulbecco modified Eagle medium (LS174T and DLD1) supplemented with 10% fetal calf serum. Sulindac treatment *in vitro* was performed with 100 μM sulindac sulfide (Sigma, Munich, Germany [25]) for 24 hours (RT-PCR, reporter expression, migration, and invasion), 48 hours (β-catenin immunocytochemistry and Western blot analysis), 4 days (wound healing), 5 days (proliferation), and 10 days (colony formation).

Quantitative Real-time RT-PCR

Quantitative real-time RT-PCR was performed in parallel and in duplicate per sample, as described previously [15]. For β-catenin and S100A4, amplicons of 152 and 124 bp were produced, respectively. The following primers and probes were used: β-catenin, forward primer 5′-gtgctatctgtctgctctagta-3′, reverse primer 5′-cttcctgtttagttgcagcatc-3′, FITC probe 5′-aggacttcacctgacagatccaagtca-3′, LCRed640 probe 5′-cgtcttgttcagaactgtctttggactctc-3′; S100A4, forward primer 5′-gagctgcccagcttcttg-3′, reverse primer 5′-tgcaggacaggaagacacag-3′, FITC probe 5′-tgatgagcaacttggacagcaaca-3′, LCRed640 probe 5′-gacaacgaggtggacttccaagagt-3′ (BioTeZ and TIB MolBiol, Berlin, Germany). Messenger RNA (mRNA) expressions of *c-myc* and *cyclin D1* were determined by SYBR green RT-PCR using the following primers: *c-myc*, forward primer 5′-acccttgccgcatccacgaaac-3′, reverse primer 5′-cgtagtcgaggtcatagttcctgttgg-3′; *cyclin D1*, forward primer 5′-ctgtttggcgtttcccagagtcatc, reverse primer 5′-agcctcctcctcacacctcctc-3′. For G6PDH, a 113-bp amplicon was generated (h-G6PDH Housekeeping Gene Set; Roche, Mannheim, Germany). The calibrator cDNA was derived from HCT116 cells and was used in serial dilutions simultaneously in each run.

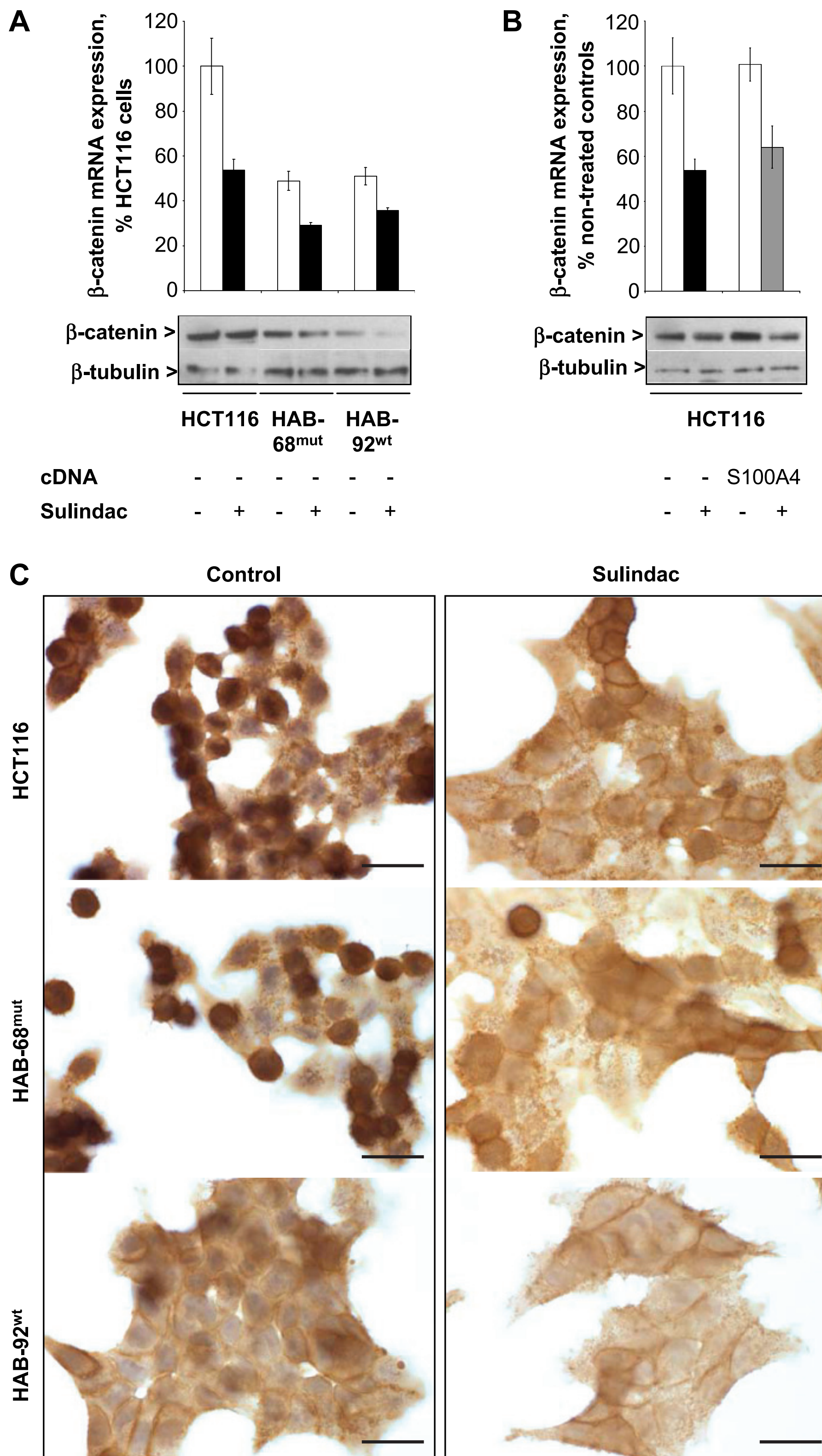
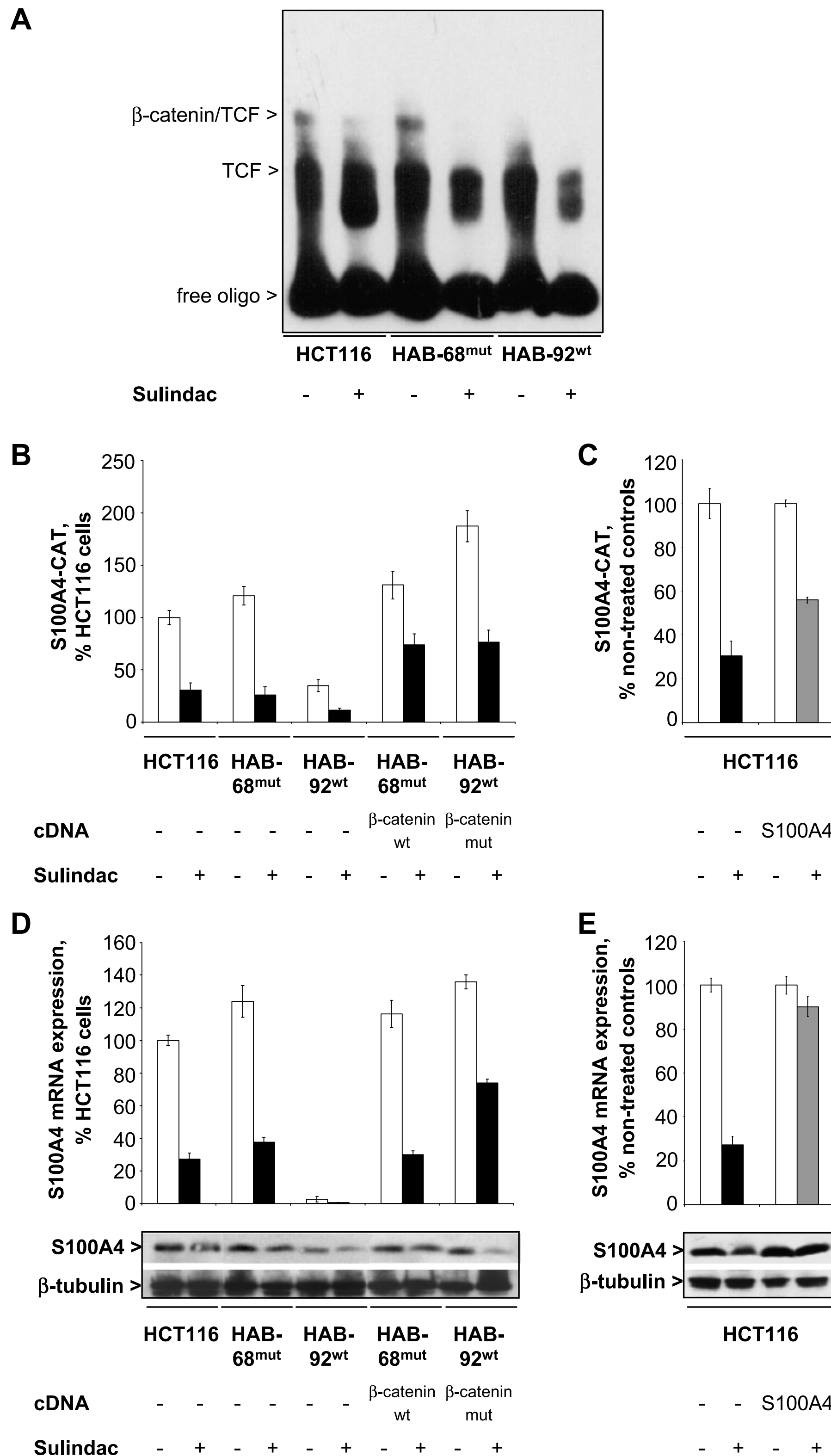


Figure 1. Sulindac inhibits expression and nuclear accumulation of β-catenin in human colon cancer cells. (A, B) Sulindac treatment led to down-regulation of β-catenin mRNA and protein expression in HCT116 cells and in the β-catenin knockout strains HAB-68^{mut} and HAB-92^{wt} *versus* their solvent-treated counterparts (A). Ectopic CMV promoter–driven overexpression of S100A4 in stably transfected HCT116/S100A4 cells did not prevent β-catenin down-regulation by sulindac (B). Expression levels of β-catenin mRNA were measured by quantitative real-time RT-PCR. Western blots for β-catenin were quantified by integrated density values by using the ChemiImager software (v5.5; Alpha Innotech Corporation). (C) Nuclear accumulation of β-catenin, mainly observed in HCT116 and HAB-68^{mut} cells, was decreased after sulindac treatment (*vs* solvent-treated cells). Bars, 20 μm.

Western Blot Analysis, Immunocytochemistry, and Immunohistochemistry

Western blot analysis was performed as described previously [15]. We used a polyclonal S100A4 antibody (Dako, Glostrup, Denmark), a monoclonal β-catenin, and a monoclonal β-tubulin antibody (both BD Biosciences, Heidelberg, Germany). Chemiluminescent reaction was done with an enhanced chemiluminescence solution. Quantification of the Western blots was performed by determining the integrated density values using the ChemiImager software (v5.5; Alpha Innotech Corporation, San Leandro, CA). For immunocytochemistry, cells were cultured on double-chamber slides (Nunc, Rochester, NY) and fixed, endogenous peroxidase was inactivated, and membranes were permeabilized. After blocking, cells were incubated with the β-catenin antibody. Detection was performed using the biotin-based ABC kit (Dako; antirabbit biotin antibody, antibiotin-streptavidin-HRP) and diaminobenzidine as substrate. For immunohistochemistry, incubations of the sections and



detection procedure were done as for immunocytochemistry. Sections were incubated with the β-catenin antibody or the S100A4 antibody.

S100A4 Gene Promoter Analysis

Electrophoretic mobility shift assay (EMSA) was performed as previously described using 3 μg of lysate, [15]. Briefly, we used biotin end-labeled double-stranded oligonucleotides for the human S100A4 promoter (sense 5′-ccgggcatggggatccccaccccagtttttgtttctgaatctttattttttaagagaca-3′, antisense 5′-ggcccgtacccctaggggtggggtcaaaaacaaagacttagaaataaaaaaattctctgt-5′; BioTeZ) harboring the wild-type TCF binding site. For chloramphenicol acetyltransferase (CAT)-ELISA, the S100A4 promoter fragment pCAT-1097-TCFwt (−1097 to +33; kindly provided by David Allard, University of Liverpool, Liverpool, UK; [32]) was used. The plasmid pCAT3-Basic and transfections without DNA served as controls. Transfer efficiency was controlled by transfection of pCAT-Control (Clontech, Mountain View, CA). Transfections and CAT-ELISA were carried out as described previously [15]. The amount of CAT protein was normalized to the protein content of the respective lysate and expressed as picograms of CAT per milligram of protein. Values are given as average of quadruplicates.

Cell Migration, Invasion, and Wound Healing

Cell migration was evaluated in transwell chambers, in which cells need to pass a 12-μm pore membrane (Corning, Schiphol-Rijk, the Netherlands). The number of migrated cells was counted after 24 hours. For the invasion assays, Matrigel (1:2; BD Biosciences) was added to the transwell membrane chambers 4 hours before seeding. Cells, which had invaded through the Matrigel, were counted after 24 hours. Independent migration and invasion assays were performed in triplicate [15]. For the scratch wound healing assay, wounds were created in confluent cells by cell scraping using a pipette tip and were washed with the medium to remove free-floating cells and debris. Wound healing within the scrape line was documented daily until day 4 and repeated twice, as described previously [33]. Scratch microphotographs were obtained with a Leica-Leitz DMIL microscope and a Kappa CF 15/4 MCC-MLUII Modul camera.

Colony Formation and Proliferation

For evaluation of cell growth in soft agar, cells were added to soft agar (Invitrogen) supplemented with medium and 10% fetal calf serum. Cells were grown in triplicate in soft agar cultures for 10 days, and colonies were counted in quadruplicate. For evaluation of *in vitro* growth, cell numbers were determined in quadruplicate until day 5 using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (Sigma, Munich, Germany) colorimetric assay.

In Vivo *Tumor Growth and Distant Metastasis after Intrasplenic Transplantation*

Intrasplenic transplantation of 5×10^6 HAB-92^{wt}/mut cells or HCT116/*S100A4* cells was performed using 6- to 8-week-old nonobese diabetic/severe combined immunodeficient mice (six mice per group) [15]. Sulindac sulfide was administered daily by gavage. Treatment was started at day 1 after cell transplantation and was finished the day before the animals were killed. Dose finding was performed with 6.25, 12.5, 18, 25, and 50 mg/kg per day of sulindac. Control group animals were treated with 10% Tween in saline (vehicle was used to solubilize sulindac). Experiments were performed in accordance with the UK Coordinating Committee on Cancer Research guidelines and approved by the responsible local authorities (State Office of Health and Social Affairs, Berlin, Germany). Mice were killed at day 19, and spleens (site of tumor injection) and livers (metastasis target organ) were shock-frozen in liquid nitrogen. To prove the effect of sulindac on β-catenin and S100A4 expression in spleen tumors and liver metastases, serial consecutive cryosections were made for immunohistochemistry and for microdissection and subsequent RNA isolation. RNA quality was proven (2100 Bioanalyzer; Agilent, Santa Clara, CA), and β-catenin and S100A4 mRNA levels were determined by quantitative real-time RT-PCR.

Statistical Analysis

Levels of statistical significance were evaluated by using the *t*-test or the nonparametric two-sided Mann-Whitney rank sum test depending on whether the data passed or failed a normal distribution test.

Results

Sulindac Inhibits Expression and Nuclear Accumulation of β-catenin

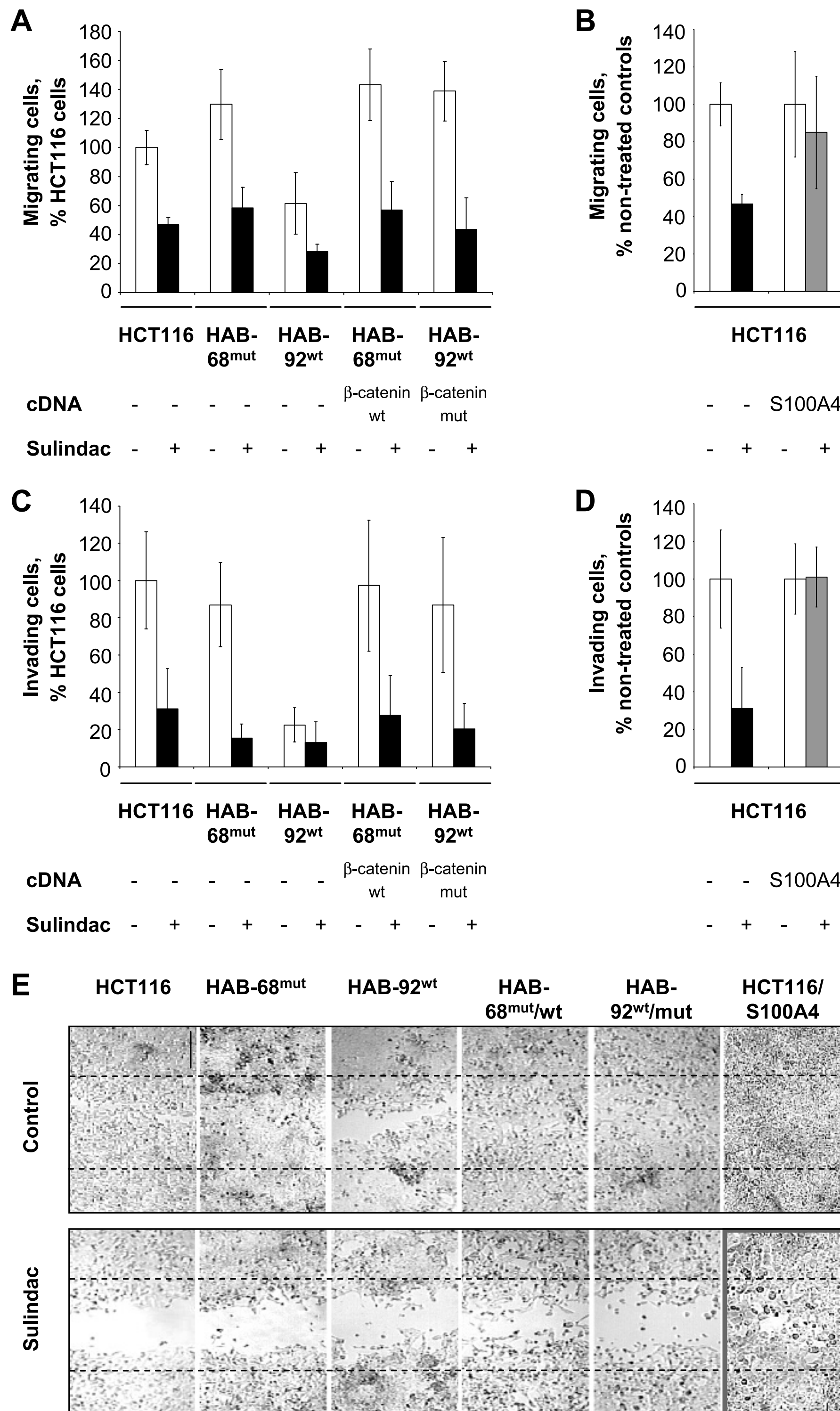
We first evaluated the effect of sulindac on β-catenin expression. We used a colon cancer model based on HCT116 cells, including the β-catenin knockout strains HAB-68^{mut} and HAB-92^{wt} [15]. Levels of β-catenin expression were dependent on the respective genotype, with higher levels in heterozygous cells. Sulindac treatment resulted in down-regulation of β-catenin mRNA and protein expression in all cell lines analyzed when compared with their solvent-treated counterparts (Figure 1*A*): in HCT116 cells, β-catenin mRNA expression was reduced

Figure 2. Sulindac inhibits transcriptional induction of S100A4 by β-catenin in human colon cancer cells. (A) Sulindac treatment led to reduced binding of β-catenin to the TCF/S100A4 promoter complex in HCT116 and HAB-68^{mut} cells, the cell lines harboring mutant β-catenin. In HAB-92^{wt} cells that express exclusively wt β-catenin, no β-catenin/TCF/S100A4 promoter oligonucleotide complex formation was detectable. EMSA was performed with 3 μg of lysate of solvent-treated or sulindac-treated cells and with biotin end-labeled double-stranded oligonucleotides for the human S100A4 promoter harboring the wild-type TCF binding site. (B, C) CAT-ELISA. The S100A4 promoter fragment pCAT-1097-TCFwt (−1097 to +33) was cloned to drive the reporter gene expression CAT. The plasmid pCAT3-Basic and transfections without DNA served as controls. The amount of CAT protein was normalized to the protein content of the respective lysate and expressed as picograms of CAT per milligram of protein. Values are average of quadruplicates and are given as percentage of CAT expression in HCT116 cells (set as 100%). Sulindac treatment led to down-regulation of S100A4 promoter–mediated reporter gene expression when compared with their solvent-treated counterparts (B). CMV promoter–driven ectopic S100A4 overexpression did not prevent down-regulation of S100A4 promoter–driven reporter gene expression by sulindac (C). (D, E) Sulindac treatment led to down-regulation of S100A4 mRNA and protein expression in HCT116 cells, in the β-catenin knockout strains HAB-68^{mut} and HAB-92^{wt}, and in the substrains with reconstituted heterozygous β-catenin genotype HAB-68^{mut}/wt and HAB-92^{wt}/mut (*vs* their solvent-treated counterparts) (D). Ectopic CMV promoter–driven overexpression of S100A4 was not responsive to sulindac (E). Expression levels of S100A4 mRNA were measured by quantitative real-time RT-PCR. Western blots for S100A4 were quantified by integrated density values by using the ChemiImager software (v5.5; Alpha Innotech Corporation).

to 53%, in the knockout strains HAB-68^{mut} and HAB-92^{wt} to 59% and 69%, respectively (*vs* solvent-treated counterpart). On the basis of measurements of the integrated density values, protein expression was diminished to 64%, 57%, and 43% in HCT116, HAB-68^{mut}, and HAB-92^{wt} cells, respectively (*vs* solvent-treated counterpart). Ectopic cytomegalovirus (CMV) promoter-driven six-fold overexpression of the β-catenin transcriptional target gene *S100A4* did not prevent β-catenin reduction by sulindac (Figure 1*B*). Nuclear accumulation of β-catenin was preferentially observed in HCT116 and HAB-68^{mut} cells harboring mutant β-catenin. This nuclear β-catenin accumulation was lost after treatment with sulindac (Figure 1*C*). These findings are consistent with previous reports [20–25].

Sulindac Inhibits Transcriptional Induction of S100A4 by β-Catenin

We previously identified the gene for the metastasis mediator S100A4 as a transcriptional target gene of β-catenin and demonstrated the direct dependence of S100A4 expression on β-catenin [15]. Because sulindac affected the expression and nuclear localization of β-catenin, we analyzed sulindac's effects on the binding of β-catenin to the



S100A4 promoter through the transcription factor TCF by EMSA. We observed clear binding of β-catenin to the TCF/S100A4 promoter complex in HCT116 and in HAB-68^{mut}, the cell lines harboring mutant β-catenin (Figure 2*A*). In HAB-92^{wt} cells, which express exclusively wild-type β-catenin, no β-catenin/TCF complex was detectable. Treatment with sulindac led to reduction of β-catenin binding to the TCF/S100A4 promoter complex in HCT116 and HAB-68^{mut} cells. Thus, we demonstrate that sulindac treatment results in the reduction of nuclear accumulation of β-catenin, which is associated with a reduction in β-catenin/TCF/S100A4 promoter oligonucleotide complex formation.

We then verified the effect of sulindac on S100A4 gene promoter activity in cells transfected with a S100A4 promoter–driven CAT reporter construct. We observed high S100A4 promoter activity in cells harboring mutant β-catenin, either heterozygously or in the knockout strain HAB-68^{mut}. Sulindac treatment reduced *S100A4* promoter–driven reporter gene expression to 30% in HCT116 cells, to 21% and 32% in HAB-68^{mut} and HAB-92^{wt} cells, and to 56% and 32% in HAB-68^{mut}/wt and HAB-92^{wt}/mut cells with the reconstituted heterozygeous β-catenin genotype, respectively (*vs* solvent-treated counterpart; Figure 2*B*). Concurrent expression of CMV promoter–driven S100A4 did not significantly affect the sulindac-induced down-regulation of the S100A4 promoter–driven reporter gene expression (Figure 2*C*).

Next, we directly analyzed the influence of sulindac on the expression of the β-catenin target gene S100A4. Sulindac caused knockdown of S100A4 mRNA expression, which ranged from 27% in HCT116 cells, 30% and 16% in the knockout strains HAB-68^{mut} and HAB-92^{wt}, to 25% and 54% in HAB-68^{mut}/wt, and HAB-92^{wt}/mut cells, respectively (*vs* solvent-treated counterpart; Figure 2*D*). This was confirmed by Western blots with reduced S100A4 protein in sulindac-treated cells, as determined by measurements of the integrated density values. Protein expression was diminished to 51%, to 52%, to 59%, to 51%, and to 37% in HCT116, HAB-68^{mut}, HAB-92^{wt}, HAB-68^{mut}/wt, and in HAB-92^{wt}/mut cells, respectively (*vs* solvent-treated counterpart). Ectopic overexpression of S100A4 was not inhibited by sulindac (Figure 2*E*).

Sulindac-Induced Inhibition of Cell Migration and Invasion by Interdicting S100A4

As we reported previously, migratory and invasive capabilities of these cells directly depend on the β-catenin genotype and thereby, on *S100A4* expression levels [15]. Here, we analyzed the effect of sulindac on migratory phenotypes and, correspondingly, on invasiveness. We observed an inhibition of migration by sulindac in all cell lines, with a decrease to 46% in HCT116 cells, to 45% and 46% in the knockout strains, and to 39% and 31% in HAB-68^{mut}/wt and HAB-92^{wt}/mut cells, respectively (*vs* solvent-treated counterpart; Figure 3*A*). Cell invasion was also decreased after sulindac treatment: to 31% in HCT116 cells, to 17% and 57% in the knockout strains, and to 28% and 23% in HAB-68^{mut}/wt and HAB-92^{wt}/mut cells, respectively (*vs* solvent-treated counterparts; Figure 3*C*). Ectopic CMV promoter–driven S100A4 overexpression was sufficient to rescue both inhibition of migration and invasion caused by sulindac (85% and 101% of sulindac-treated migrated and invaded cells compared with solvent-treated controls, respectively, as illustrated in Figure 3, *B* and *D*).

Sulindac's effects on cell motility were confirmed by a monolayer scratch assay, which tracks the migration of cells into a wound area. The situation at day 4 after injuring the cell monolayer is depicted (Figure 3*E*). Wound healing was dependent on β-catenin genotype and thus on S100A4 expression. Cells with mutant β-catenin, and therefore with high S100A4 levels, demonstrated accelerated wound healing compared with HAB-92^{wt} cells. However, sulindac treatment delayed wound healing in all cell lines analyzed. Again, ectopic overexpression of S100A4 largely prevented the sulindac-induced delay in directed cell motility. Although the wound was not completely closed at this time point, a difference of the distance of the gap is seen in sulindac-treated HCT116/*S100A4* cells with respect to the sulindac-treated cell lines that are not transfected with S100A4 (HCT116, HAB-68^{mut}, HAB-92^{wt}, HAB-68^{mut}/wt, and HAB-92^{wt}/mut). The delay of wound closure in the sulindac-treated HCT116/*S100A4* cells when compared with their solvent-treated counterparts may be partly explained by sulindac-induced proliferation inhibition.

To validate our findings, we also treated additional human colon cancer cell lines with sulindac: SW620 (harboring APC mutant, β- catenin wt), LS174T (harboring APC wt, β-catenin mutant), and DLD1 (harboring APC mutant, β-catenin wt), in addition to the HCT116 cells (harboring APC wt, β-catenin mutant). The expression of S100A4 was detectable in all cell lines analyzed (Figure W1*A*). We found sulindac-induced reduction of S100A4 mRNA expression to 37%, to 25%, and to 28% in SW620, LS174T, and DLD1 cells. We also tested the influence of sulindac treatment on cell migration (Figure W1*B*). Thereby, we observed a reduction of the number of migrating cells by treatment with sulindac of 23%, 22%, and 38% in SW620, LS174T, and DLD1 cells. Thus, the sulindac effect on S100A4 expression as well as on cell migration was verified in additional colon cancer cell lines.

We also analyzed the effect of sulindac on down-regulation of additional β-catenin transcriptional target genes including *c-myc* and

Figure 3. Sulindac inhibits cell migration and invasion in human colon cancer cells. (A, B) Cell migration assay. Cell migration was evaluated in transwell chambers with 12-μm pore membranes. The number of migrated cells was counted after 24 hours. Sulindac treatment led to inhibition of cell migration in HCT116 cells, in the β-catenin knockout strains HAB-68^{mut} and HAB-92^{wt}, and in the substrains with reconstituted heterozygous β-catenin genotype HAB-68^{mut}/wt and HAB-92^{wt}/mut (*vs* their solvent-treated counterparts) (A). Ectopic CMV promoter–driven S100A4 overexpression prevented migration inhibition by sulindac (B). (C, D) Cell invasion assay. Cell invasion was evaluated by adding Matrigel to the transwell chambers 4 hours before seeding. The number of invaded cells was counted after 24 hours. Sulindac treatment led to inhibition of cell invasion migration in HCT116 cells, in the β-catenin knockout strains HAB-68^{mut} and HAB-92^{wt}, and in the substrains with reconstituted heterozygous β-catenin genotype HAB-68^{mut}/wt and HAB-92^{wt}/mut (*vs* their solvent-treated counterparts) (C). Ectopic CMV promoter–driven S100A4 overexpression prevented invasion inhibition by sulindac (D). (E) Wound healing assay. Wounds were created in confluent cells by cell scraping using a pipette tip and were washed with medium to remove free-floating cells and debris. Wound healing within the scrape line was documented daily. Representative scrape lines are shown at day 4; dashed line indicates the margin of the scratch at day 1. Bar, 200 μm. Wound healing was dependent on β-catenin genotype and thus on S100A4 expression. Cells with mutant β-catenin, and therefore with high S100A4 levels, demonstrated accelerated wound healing compared with HAB-92^{wt} cells. Sulindac treatment led to inhibition of wound healing in all cell lines analyzed (*vs* their solvent-treated counterparts). Ectopic CMV promoter–driven S100A4 overexpression almost prevented the sulindac-induced delay in wound healing.

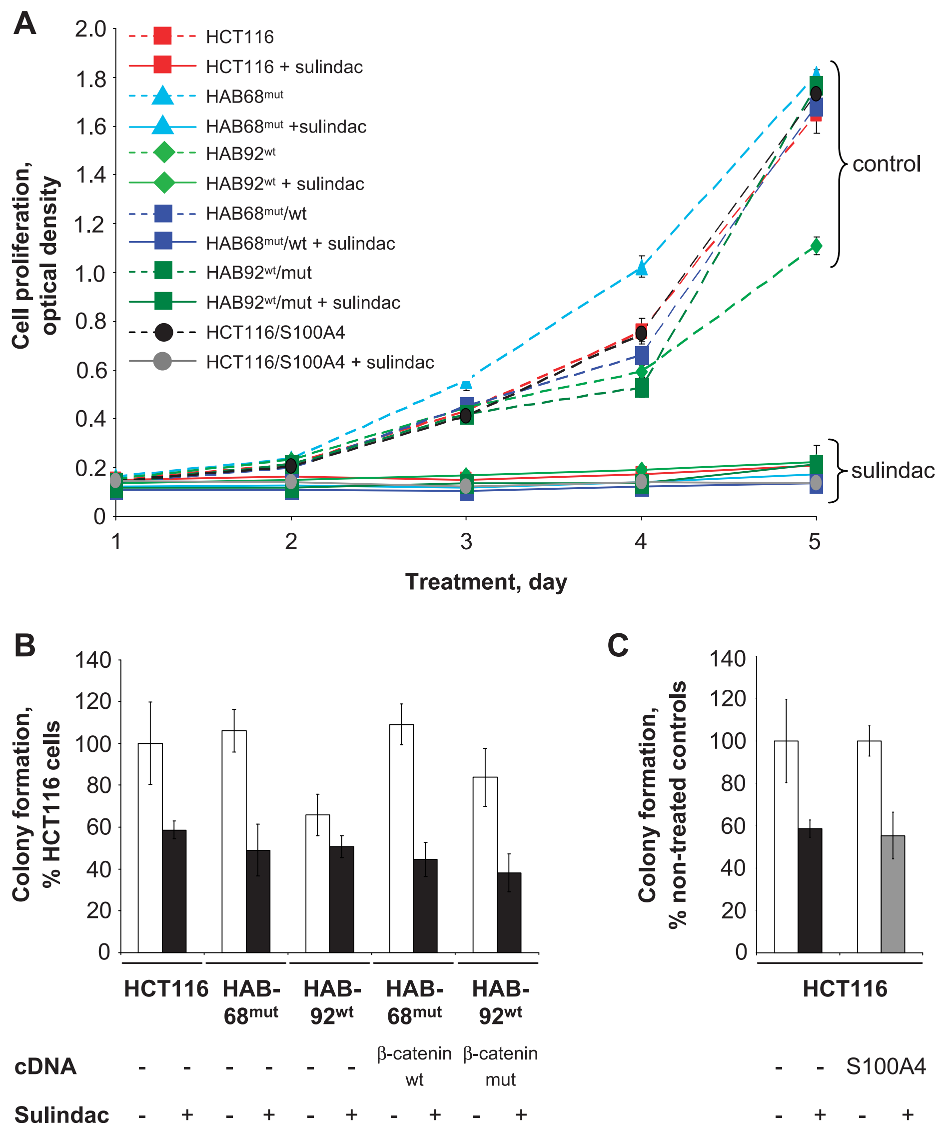


Figure 4. Sulindac inhibits colony formation and proliferation in human colon cancer cells. (A) Proliferation assay. *In vitro* growth was evaluated by determining the cell numbers using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide colorimetric assay until day 5. Adherent cell proliferation was comparable in all cell lines, except for a slightly reduced proliferation rate of HAB-92^{wt} cells. Sulindac treatment led to inhibition of cell proliferation in all cell lines analyzed (*vs* their solvent-treated counterparts). Ectopic CMV promoter–driven S100A4 overexpression did not prevent proliferation inhibition by sulindac. (B, C) Colony formation assay. For the evaluation of anchorage-independent growth, cells were grown in soft agar for 10 days, and colonies were counted. Colony formation was enhanced in cells harboring mutant β-catenin compared with HAB-92^{wt} cells. Sulindac treatment led to inhibition of colony formation in all cell lines analyzed (*vs* their solvent-treated counterparts) (B). Ectopic CMV promoter–driven S100A4 overexpression did not prevent colony formation inhibition by sulindac (C).

cyclin D1. We found that sulindac treatment resulted in mRNA down-regulation of *c-myc* and *cyclin D1*, as also observed for *S100A4* (Table W1). We then transfected the CMV promoter–driven cDNA of *c-myc* or *cyclin D1* into HCT116 cells, treated them with sulindac (*vs* solvent), and performed cell migration assays. We found that sulindac-induced reduction of cell migration was only prevented in HCT116 cells that ectopically overexpress S100A4. In HCT116/*c-myc* or HCT116/*cyclin D1* cells, no rescue of the sulindac-induced reduction in cell migration was observed (Table W2).

Sulindac-Induced Inhibition of Cell Proliferation and Colony Formation

Next, we evaluated the effect of sulindac on cell proliferation abilities. Adherent cell proliferation was measured for 5 days and was comparable in all cell lines, except for a somewhat reduced proliferation rate of HAB-92^{wt} cells. Colony formation was enhanced in cells harboring mutant β-catenin compared with HAB-92^{wt} cells. Sulindac treatment resulted in complete arrest of cell growth in all lines (Figure 4*A*). Treatment with sulindac also inhibited anchorage-independent proliferation in all cell lines: to 58% in HCT116 cells, to 46% and 76% in the knockout strains, and to 40% and 45% in HAB-68^{mut}/wt and HAB-92^{wt}/mut cells, respectively (*vs* solvent-treated counterpart; Figure 4*B*). This inhibition of colony formation was not prevented by ectopic S100A4 overexpression. In contrast to the rescue abilities of S100A4 overexpression on sulindac-induced inhibition of cell migration and invasion, ectopic S100A4 overexpression did not rescue sulindac-induced inhibition of adherent or anchorage-independent proliferation (Figure 4, *A* and *C*).

Antimetastatic Activity of Sulindac in Mice by Inhibition of β-Catenin and S100A4 Expression

Next, we evaluated the effect of sulindac on *in vivo* metastasis. We transplanted HAB-92^{wt}/mut cells intrasplenically leading to tumor growth in the spleen and to metastasis in the liver. Sulindac or solvent was administered orally every day, starting 1 day after tumor cell inoculation.

Dose finding experiments for sulindac were performed with 6.25, 12.5, 18, 25, and 50 mg/kg per day. We started these experiments with a sulindac concentration of 50 mg/kg per day. Because we found this concentration to be toxic, we lowered the concentration to 25 mg/kg per day, which was also not well tolerated. Although reduced metastases formation was observed in the animals treated with 50 and 25 mg/kg per day of sulindac (*vs* solvent-treated animals), these results were not considered owing to partial toxicity. On the other side, sulindac was very well tolerated but ineffective at concentrations of 12.5 and 6.25 mg/kg per day with respect to metastasis reduction.

However, sulindac at nontoxic concentrations, for example, 18 mg/kg per day, significantly reduced tumor growth as well as metastasis formation *in vivo*, compared with solvent-treated control animals (Figure 5*A*). This demonstrates the narrow concentration range where sulindac is nontoxic but effective with respect to metastasis inhibition. Tumor growth in the spleen was inhibited by sulindac to approximately 43%. Mean tumor weights with and without sulindac were 181 and 418 mg, respectively (P = .014; Figure 5*B*). We also investigated the effect of sulindac on the liver metastasis rate in mice. Metastases per animal were reduced by sulindac to approximately 37% of the solvent control. Mean numbers of metastases per animal with and without sulindac were 6.0 and 15.85, respectively (P = .025; Figure 5*C*). Body weights of the mice were unaffected by sulindac, but one of six mice treated with sulindac died of unknown reasons.

To assess the effect of sulindac treatment on molecular targets *in vivo*, we determined the levels of β-catenin and S100A4 in the spleen tumors and in the liver metastases of sulindac- and solvent-treated mice. We detected significantly reduced expressions of β-catenin (P = .0221) and S100A4 (P = .004) in the spleen tumors of sulindac-treated animals compared with solvent-treated animals at the mRNA level (Figure 6, *A* and *C*). However, the differences of the β-catenin and S100A4 mRNA expressions in the liver metastases of solvent- and of sulindac-treated mice did not reach statistical significance (Figure 6, *A* and *C*; β-catenin mRNA in liver metastases: median solvent-treated, 11.075; median sulindac-treated, 0.628; S100A4 mRNA: median solvent-treated, 63.49; median sulindac-treated, 7.945). At the protein level, diminished β-catenin as well as S100A4 protein expressions were

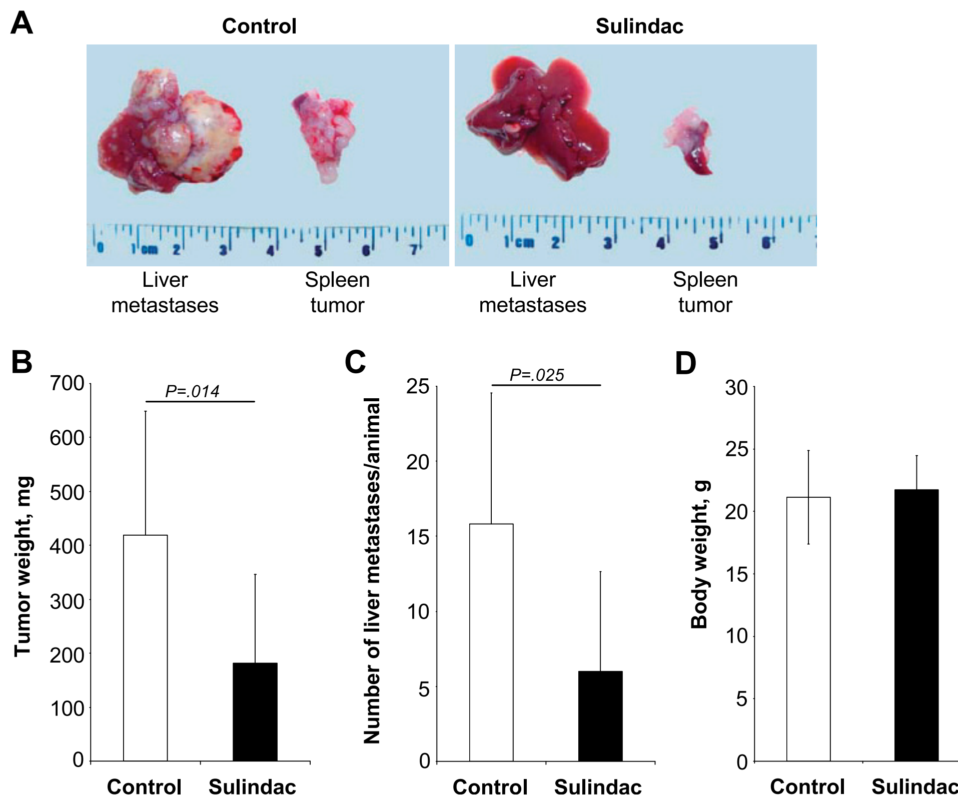


Figure 5. Sulindac inhibits tumor growth and metastasis in human colon cancer xenografts in mice. Intrasplenic transplantation of 5×10^6 HAB-92^{wt}/mut cells was performed using NOD/SCID mice. This led to tumor growth in the spleen and to liver metastases. Sulindac treatment was started at day 1 after intrasplenic transplantation, was administered daily by gavage (18 mg/kg per day), and was finished the day before the animals were killed. Mice were killed at day 19, and spleens (site of tumor injection) and livers (metastasis target organ) were shock-frozen in liquid nitrogen. Control group animals were treated with 10% Tween in saline (vehicle was used to solubilize sulindac). (A) Tumors in the spleens and metastases in the livers are shown (one representative mouse per group). Sulindac treatment resulted in the reduction of splenic tumor growth and of liver metastasis (*vs* the solvent-treated control mice). (B, C) Sulindac treatment resulted in significantly reduced tumor weight in the spleen (P = .014) (B) and in significantly reduced numbers of liver metastases (P = .025) (C) compared with solvent-treated control animals. (D) Body weight of mice was not influenced by sulindac.

observed by immunohistochemistry in the tumors as well as in the liver metastases of the sulindac-treated animals (Figure 6, *B* and *D*).

To evaluate the effect of ectopic overexpression of S100A4 on sulindac-induced reduction of tumor growth and liver metastasis, we transplanted HCT116/*S100A4* cells intrasplenically and administered sulindac daily (Figure 7*A*). Neither tumor growth in the spleen (P = .679) nor liver metastasis (P = .658) were reduced by sulindac when using cells ectopically overexpressing S100A4 (Figure 7, *B* and *C*). Body weight was also unaffected by treatment with sulindac (Figure 7*D*). We also analyzed the effect of sulindac on S100A4 protein levels by immunohistochemistry. In contrast to the sulindac-induced reduction of S100A4 protein in tumors and metastases originating

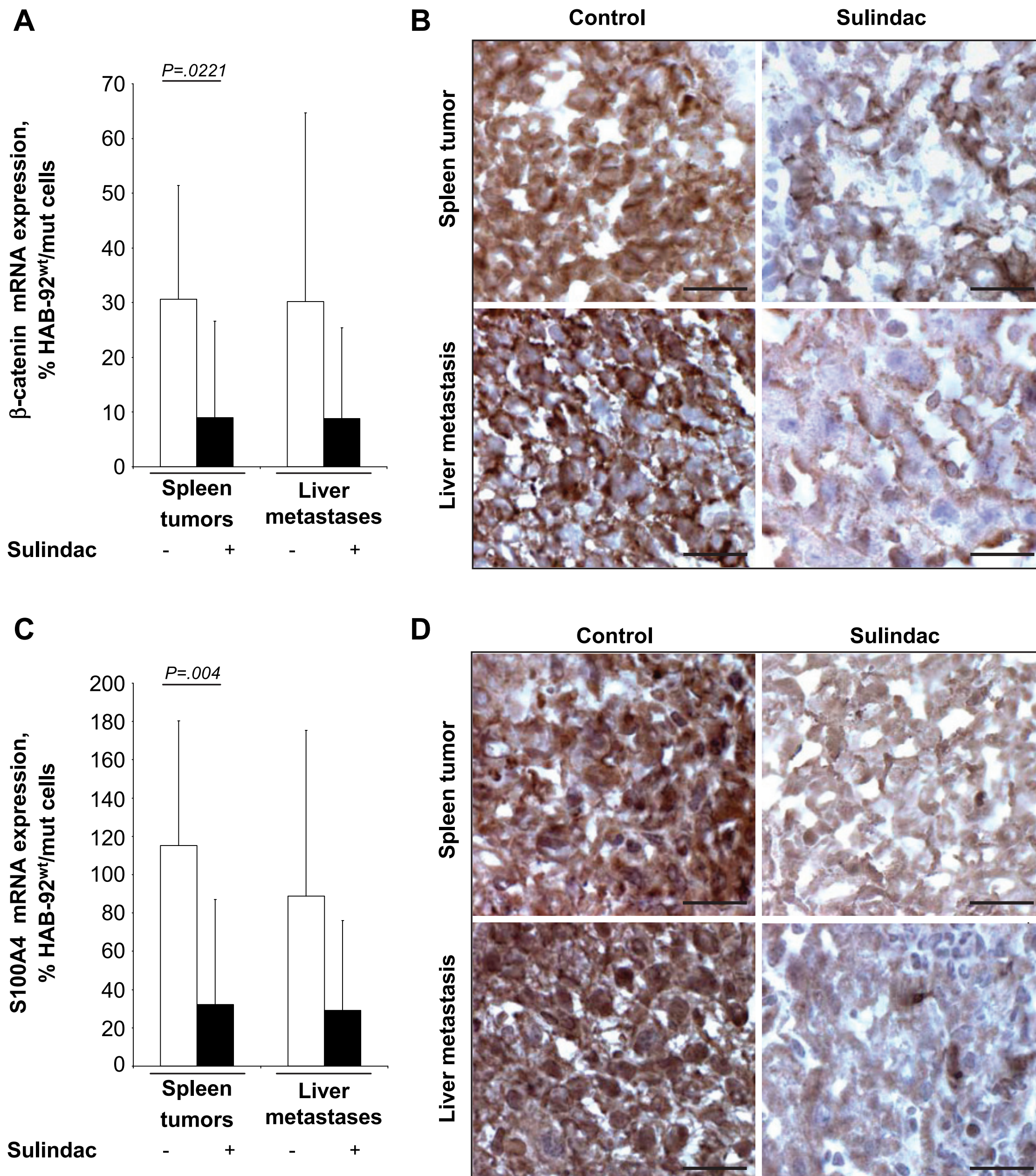


Figure 6. Sulindac inhibits β-catenin and *S100A4* gene expression in spleen tumors and liver metastases in mice. The effect of sulindac treatment (*vs* solvent treatment) on β-catenin and S100A4 mRNA expressions in the spleen tumors and liver metastases was evaluated by using serial consecutive cryosections for microdissection, subsequent RNA isolation, and quantitative real-time RT-PCR. The protein expression of β-catenin and S100A4 was determined by immunohistochemistry. (A, B) Sulindac treatment (18 mg/kg per day) resulted in down-regulation of β-catenin mRNA expression in spleen tumors (P = .021) and in liver metastases compared with solvent-treated control animals (A). Representative cryosections of spleen tumors and liver metastases also showed down-regulation of β-catenin protein expression after sulindac treatment (*vs* solvent-treated control animals) (B). Bars, 20 μm. (C, D) Sulindac treatment resulted in significant down- regulation of S100A4 mRNA expression in spleen tumors (P = .004) and in liver metastases compared with solvent-treated control animals (C). Representative cryosections of spleen tumors and liver metastases also showed down-regulation of S100A4 protein expression after sulindac treatment (*vs* solvent-treated control animals) (D). Bars, 20 μm.

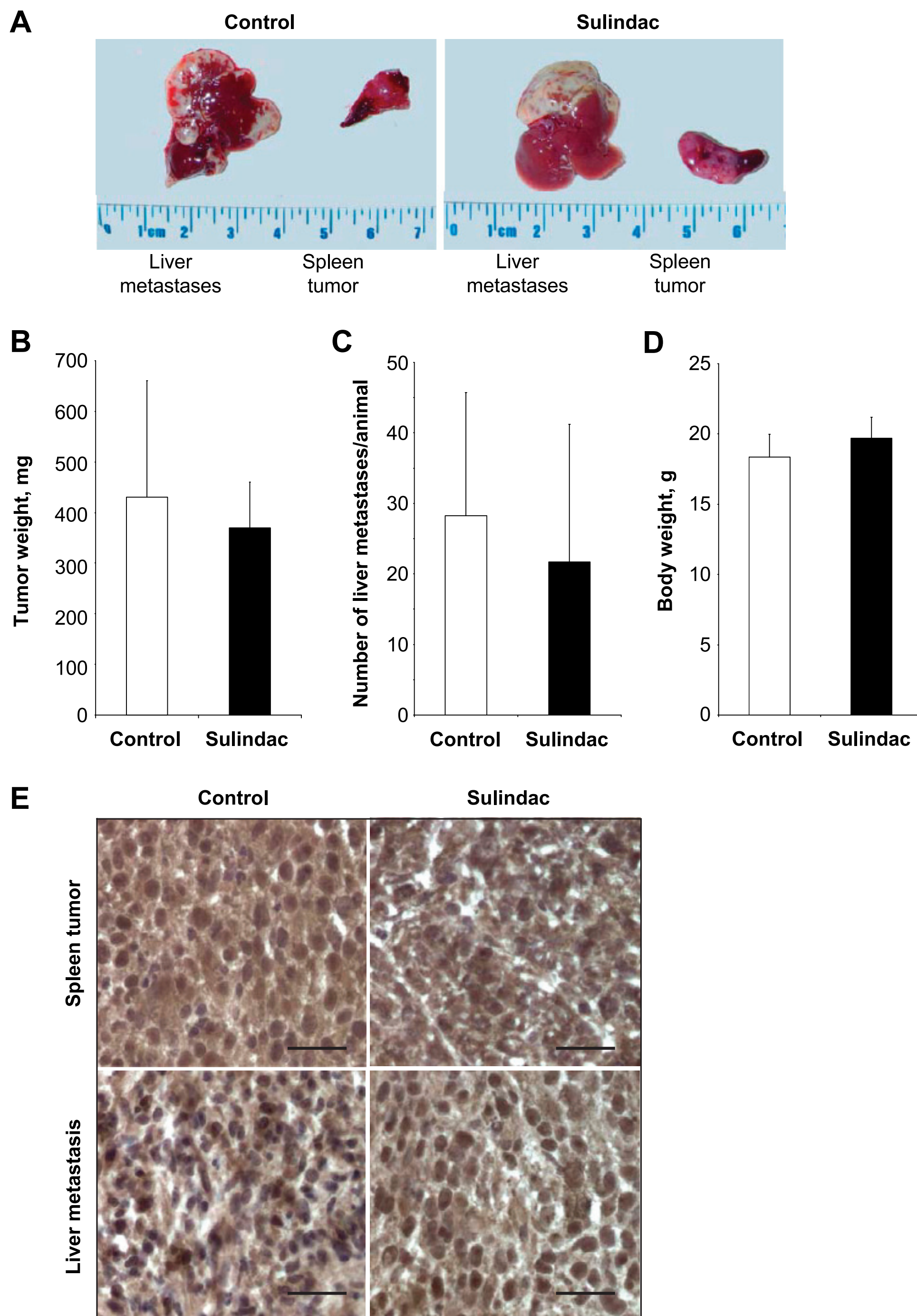


Figure 7. Ectopic CMV promoter–driven S100A4 overexpression prevents inhibition of tumor growth and metastasis in human colon cancer xenografts in mice by sulindac. To evaluate the effect of ectopic CMV promoter–driven overexpression of S100A4 on sulindac-induced reduction of tumor growth and liver metastasis, intrasplenic transplantation of 5×10^6 HCT116/S100A4 cells was performed using NOD/SCID mice. This led to tumor growth in the spleen as site of transplantation and to liver metastases. Sulindac treatment was started at day 1 after intrasplenic transplantation, was administered daily by gavage (18 mg/kg per day), and was finished the day before the animals were killed. Mice were killed at day 19, and spleens (site of tumor injection) and livers (metastasis target organ) were shock-frozen in liquid nitrogen. Control group animals were treated with 10% Tween in saline (vehicle was used to solubilize sulindac). (A) Tumors in the spleens and metastases in the livers are shown (one representative mouse per group). Sulindac treatment had no effect on tumor growth or liver metastasis. (B, C) Neither tumor growth (B) nor liver metastasis (C) was reduced by sulindac (*vs* solvent-treated control animals) when transplanting cells ectopically overexpressing CMV promoter–driven S100A4. Body weight of mice was not influenced by sulindac (D). (E) Sulindac treatment did not alter the S100A4 protein expression in ectopically S100A4-overexpressing HCT116/S100A4 cells (*vs* solvent-treated control animals). Representative cryosections of spleen tumors and liver metastases are shown. Bars, 20 μm.

from HAB-92^{wt}/mut cells, sulindac treatment did not alter the S100A4 protein expression in ectopically S100A4-overexpressing HCT116/*S100A4* cells (*vs* solvent-treated control animals; Figure 7*E*).

Discussion

Here we report the antimetastatic activity of sulindac. We demonstrate the mechanism of sulindac action, which involves intervening in β-catenin signaling and thus interdicting S100A4. Rescue of the metastatic phenotype by ectopic S100A4 provides a clear indication that this protein plays a key role in the process. Although this does not mean that other β-catenin–regulated genes do not also play important roles, the association of down-regulation of S100A4 in tumors treated *in vivo* with inhibition of metastasis and the lack of response of tumors expressing ectopic S100A4 support a critical role for this protein in Wnt/β-catenin–driven metastasis.

Sulindac has been well established as a colon cancer–chemopreventive agent [34,35]. It has pleiotropic activities as a cyclooxygenase (COX) inhibitor and as an inhibitor of polyamine biosynthesis. This latter activity provided the rationale for its combination with difluoromethylornithine in a large-scale trial for chemoprevention of sporadic colorectal adenomas [36,37]. This combination trial produced a striking preventative effect in the sulindac/difluoromethylornithine arm [38]. It is less well known that pharmacological manipulation with sulindac negatively regulates Wnt/β-catenin signaling, although NSAIDs have been repeatedly evaluated as potential Wnt/β-catenin pathway therapeutics [39]. Sulindac acts through inhibition of β-catenin expression and nuclear accumulation and by enhanced β-catenin degradation [21–25]. Traditional NSAIDs, including sulindac, inhibit COX resulting in reduced Wnt-signaling by induced β-catenin degradation, particularly in colon cancer [40].

We have previously shown that β-catenin regulates S100A4 expression transcriptionally and that mutant β-catenin acts in a dominant fashion. β-Catenin–induced cell migration and invasion was dependent on S100A4 and was abrogated by small interfering RNA acting on S100A4. These findings demonstrated that S100A4 is essential for increased cell motility initiated by β-catenin/TCF signaling [15].

The importance of S100A4 as a metastasis-inducing protein *in vivo* has been reported in several studies. Transgenic mouse models overexpressing S100A4 showed enhanced metastatic activity [41,42]. S100A4-deficient mice demonstrated suppression of tumor development and metastasis formation even when injected with highly metastatic mouse mammary carcinoma cells [43]. The *S100A4* gene functions as a pleiotropic mediator of metastasis with intracellular actions affecting motility, secretion of metalloproteases, and invasion. *S100A4* promotes the metastatic cascade by increasing cellular motility through interaction with cytoskeletal proteins. It coaggregates with actin filaments, binds to nonmuscle tropomyosin, and interacts with nonmuscle myosin II. S100A4 causes a migratory phenotype by modulating cell adhesion through binding liprin β_1 or p53, thereby affecting p53-mediated migration and invasion rates particularly in colon cancer cell lines. Beside its intracellular interactions, S100A4 is released to the extracellular space and promotes metastasis formation and angiogenesis *in vivo* (reviewed in references [5–7]). Metastatic phenotypes were reduced by experimental interventions that prevent S100A4 protein production, using S100A4 antisense oligonucleotides, anti–S100A4 ribozymes, or S100A4-RNAi [15,44–46]. Our approach aims at the pharmacologic inhibition of S100A4 expression as a strategy for metastasis prevention. On the basis of our signaling data and on sulindac actions on β-catenin, we hypothesized a reduction or prevention of metastasis by sulindac, mediated through β-catenin and its target gene *S100A4*. Indeed, we demonstrated sulindac-induced down-regulation of β-catenin *in vitro* and *in vivo* and reduced nuclear accumulation in cell culture. We had already shown that β-catenin binds to the *S100A4* gene promoter through a TCF site [15]. Here, we provide evidence that this binding of β-catenin to the S100A4 promoter through TCF is diminished by sulindac, leading not only to lowered reporter expression but also to reduced expression of the metastasis mediator S100A4 itself. These findings underline the importance of *S100A4* as transcriptional β-catenin target gene and, moreover, point to possible metastasis intervention strategies through β-catenin/TCF signaling.

Sulindac also knocked down functional parameters such as β-catenin–induced migratory and invasive capacities. In this context, we are aware of the complexity of sulindac actions and of the Wnt/β-catenin signaling pathway. For instance, the anti-invasive activity of sulindac was also regulated by ATF3 in colon cancer cells [47]. Therefore, we tested the effect of sulindac with respect to the inhibition of S100A4 expression and cell migration in additional colon cancer cell lines. Although these cell lines differ in their mutation status concerning APC and β-catenin, sulindac did reduce S100A4 expression as well as the migratory abilities of these lines. Furthermore, ectopic overexpression of *S100A4*, but not of *c-myc* or cyclin D1, rescued migration and invasion, making these functional sulindac effects dependent on the action of *S100A4* [48]. In addition to *S100A4*, other β-catenin target genes may contribute to cell motility inhibition by sulindac. However, the ectopic overexpression of *S100A4* was sufficient to completely overcome the anti-invasive effect of sulindac. Thus, we suggest a crucial role for *S100A4* in the context of sulindac-induced reduction of cell motility. Although ectopic overexpression of *S100A4* also accelerated directed migration in the wound healing assay, the closing of the wound was not complete in sulindac-treated HCT116/*S100A4* cells when compared with their solvent-treated counterpart. This might be due to the sulindac-induced reduction of cell proliferation that was not rescued by ectopic overexpression of *S100A4*. Other β-catenin target genes that were downregulated after sulindac treatment might contribute to proliferation inhibition. Furthermore, antiproliferative activity of sulindac in colon cancer cells was previously reported to be mediated through the Csk/Src axis [49].

Finally, we transplanted colon cancer cells with gain-of-function β-catenin and high S100A4 expression into mice and observed tumor growth and liver metastasis. Sulindac treatment resulted in significantly decreased splenic tumor growth and metastasis formation. Inhibition of tumorigenesis by sulindac has been well described [24,50]. In this report, the formation of experimental liver metastases in mice was significantly knocked down by sulindac, providing proof of principle for antimetastatic activity. Reduction in tumor growth and metastasis was accompanied by sulindac-induced knockdown of both β-catenin and S100A4 expression in these tissues, thereby confirming our initial hypothesis. Antimetastatic effects of sulindac were not observed, when using a cell line model with high ectopic overexpression of S100A4 for *in vivo* transplantation, illustrating the key importance of the S100A4 promoter in sulindac action.

Thus, we demonstrate here the activity of sulindac as an antimetastatic compound and show that the mechanism involves intervening in the β-catenin-S100A4 cellular program for colon cancer. Targeting S100A4 is a very promising approach for cancer therapy [51,52]. Other inhibitors targeting the Wnt/β-catenin pathway may also offer potential as antimetastatic agents by interdicting the expression of target genes such as *S100A4*. Small molecule inhibitors have been identified by

high-throughput screening that are able to disrupt the β-catenin/TCF interaction and block growth of colon cancer cell lines [53–55]. Other inhibitors like silibinin also decreased β-catenin expression resulting in down-regulation of its target genes *cyclin D1* and *c-myc* and inhibition of tumorigenesis [56]. It has also been taken into account that other signaling cascades besides the Wnt/β-catenin pathway might influence S100A4 expression; for instance, ErbB2 activates S100A4 expression in medulloblastoma cell lines during EMT through the Ras/Raf/Mek/Erk1/2 signaling pathways [32]. S100A4 expression induction by $\alpha_6\beta_4$ integrin through NFAT5 transcription factor was reported in breast cancer cell lines [57]. Recently, transcriptional regulation of S100A4 under hypoxic conditions was shown in a gastric cancer cell line [58]. Therefore, pathway-based intervention strategies should consider tissue-specific transcriptional regulation of S100A4.

In summary, we demonstrate here the antimetastatic activity of sulindac for restriction of colon cancer metastasis in mice. Sulindac acts through intervening in β-catenin signaling and interdicting expression of the metastasis mediator *S100A4*, a β-catenin target gene. Because ectopic S100A4 rescues motility abilities *in vitro* and the metastatic phenotype in mice, S100A4 plays a crucial role in the Wnt/β-catenin–driven metastatic process, despite other important β-catenin–regulated genes. This is also demonstrated by downregulated S100A4 expression in tumors and metastases of sulindac-treated mice that show inhibition of metastasis formation. Indeed, down-regulation of S100A4 expression in tumor tissue may provide a useful pharmacodynamic marker for potential clinical trials of sulindac or other Wnt/β-catenin inhibitors as antimetastatic agents. As mentioned above, S100A4 was previously shown to function as a prognostic indicator for the identification of patients at high risk for developing metastases metachronously and unfavorable survival prognosis. Thus, intervention strategies targeting key molecules of the Wnt/β-catenin pathway may represent promising approaches to inhibit metastasis formation especially when induced by S100A4. This new insight into the antimetastatic activity of sulindac could serve as the basis for chemoprevention of metastasis in individual colon cancer patients with high S100A4 expression and high risk for metastasis.

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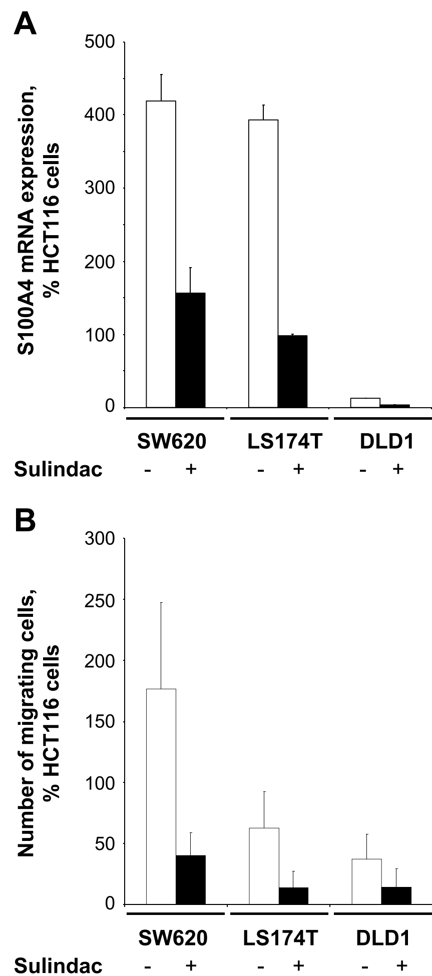

Figure W1. Sulindac inhibits S100A4 expression and cell migration in the human colon cancer cells SW620, LS174T, and DLD1. (A) Sulindac treatment led to down-regulation of S100A4 mRNA expression in SW620, LS174T, and DLD1 cells (*vs* their solvent-treated counterparts). Expression levels of S100A4 mRNA were measured by quantitative real-time RT-PCR. (B) Sulindac treatment led to inhibition of cell migration in SW620, LS174T, and DLD1 cells (*vs* their solvent- treated counterparts). Cell migration was evaluated in transwell chambers with 12-μm pore membranes. The number of migrated cells was counted after 24 hours.

Table W1. Sulindac-Induced Reduction of mRNA Expression of β-Catenin Target Genes *S100A4*, *c-myc*, and *cyclin D1* in HCT116 Cells, in the β-Catenin Knockout Strains HAB-68^{mut} and HAB-92^{wt}, and in the Sublines with Reconstituted Heterozygous β-Catenin Genotype HAB-68^{mut}/wt and HAB-92^{wt}/mut.

β-Catenin Target Gene	HCT116	HAB-68^{mut}	HAB-92^{wt}	HAB-68^{mut}/wt	HAB-92^{wt}/mut
	% Solvent-Treated Cells				
S100A4	28.01	30.12	16.89	25.91	54.94
c-myc	74.91	52.99	72.99	54.03	60.00
cyclin D1	52.46	44.17	69.93	55.44	74.19

Values are given as percentages of the mRNA expression of the respective β-catenin target gene in sulindac-treated cells *versus* their solvent-treated counterpart.

Table W2. Ectopic Overexpression of *S100A4*, But Not of Ectopic *c-myc* and *cyclin D1*, Prevents Sulindac-induced Reduction of Cell Migration of HCT116 Cells.

HCT116/β-Catenin Target Gene	No. Migrated Sulindac-Treated Cells, % Solvent-Treated Cells	
HCT116	46.78	<.001
HCT116/*S100A4*	85.11	NS (P = .212)
HCT116/*c-myc*	54.62	.002
HCT116/*cyclin D1*	37.85	<.001

Values are given as percentages of the number of migrated cells in sulindac-treated HCT116, HCT116/*S100A4*, HCT116/*c-myc*, and HCT116/*cyclin D1* cells *versus* their solvent-treated counterparts.
NS indicates not significant.