Antiproliferative effect of sulindac in colonic neoplasia prevention: role of COOH-terminal Src kinase

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Abstract

Although the nonsteroidal anti-inflammatory drugs (NSAID) protection against colorectal cancer is well established, the molecular mechanisms remain unclear. We show herein that induction of the tumor suppressor gene COOH-terminal Src kinase (Csk) by NSAID is important for their antiproliferative and hence chemopreventive effects. In the azoxymethane-treated rat model of experimental colon carcinogenesis, sulindac treatment markedly induced Csk with a corresponding increase in inhibitory phosphorylation of Src (Tyr^{527}). Sulindac-mediated Csk induction was replicated in the human colorectal cancer cell line HT-29, with a corresponding suppression of both Src kinase activity (63% of vehicle; $P < 0.05$) and E-cadherin tyrosine phosphorylation (an *in vivo* Src target). To determine the importance of Csk in NSAID antiproliferative activity, we stably transfected a Csk-specific short hairpin RNA (shRNA) vector into HT-29 cells, thereby blunting the sulindac-mediated Csk induction. These transfectants were significantly less responsive to the antiproliferative effect of sulindac sulfide (suppression of proliferating cell nuclear antigen was 21 $\pm$ 2.3% in transfectants versus 45 $\pm$ 4.23% in wild-type cells), with a corresponding mitigation of the sulindac-mediated G_1-S-phase arrest (S-phase cells 48 $\pm$ 3.6% versus 14 $\pm$ 2.8% of vehicle respectively). Importantly, the Csk shRNA cells had a marked decrease in the cyclin-dependent kinase inhibitor $p21^{cip/waf1}$, a critical regulator of G_1-S-phase progression (49% of wild-type cells). Moreover, although sulindac-mediated induction of $p21^{cip/waf1}$ was 113% in wild-type HT-29, this induction was alleviated in the Csk shRNA transfectants (65% induction; $P < 0.01$). Thus, this is the first demonstration that the antiproliferative activity of NSAID is modulated, at least partly, through the Csk/Src axis. [Mol Cancer Ther 2008;7(7):1797–806]

Introduction

Colorectal cancer is the second leading cause of cancer-related deaths in the United States. An estimated 153,760 new cases of colorectal cancer will be diagnosed in 2007, resulting in 52,180 deaths, underscoring the need for more effective preventive strategies (1). Epidemiologic, experimental, and randomized-controlled trials have unequivocally shown that nonsteroidal anti-inflammatory drugs (NSAID) afford significant protection against colorectal cancer through a myriad of complex, incompletely characterized mechanisms of action (2). However, the modest efficacy (~30-50% risk reduction), along with potential toxicity (gastrointestinal ulcerations, cardiac toxicity, etc.; ref. 3), has made clinical guidelines argue against the use of these agents for primary colorectal cancer prevention (4). Elucidating the molecular mechanisms through which NSAID prevent colon cancer is of paramount importance in rationally designing novel agents that can increase chemopreventive efficacy while reducing toxicity.

Among the numerous potential NSAID targets, cyclooxygenase-2 (COX-2) has received the most attention. However, several lines of evidence suggest that non-COX mechanisms may also be critical in colorectal cancer chemoprevention (5). For instance, NSAID derivatives without anti-COX activities have been reported to prevent colon carcinogenesis (6). Furthermore, NSAID were shown to exhibit antiproliferative activities in the COX-negative colon cancer cell lines (7). Also, the addition of prostaglandins (product of COX activity) was unable to rescue COX-producing colon cancer cells from sulindac-associated growth arrest (8). There have been numerous putative non-COX-related NSAID targets including β-catenin (9), Ras (10), nuclear factor-κB (11), cyclic GMP (12), peroxisome proliferator-activated receptor δ (13), NSAID-activated gene-1 (14), epidermal growth factor receptor (15), prostate apoptosis response gene-4 (16), etc. These targets share the ability to affect, on epithelial proliferation and/or apoptosis, processes believed to be critical for chemoprevention. However, it is unclear whether modulation of any of these putative molecular targets is actually responsible for the cellular changes seen during NSAID chemoprevention.

The nonreceptor tyrosine kinase Src is an oncogene that regulates a variety of cellular processes important in colorectal cancer development including proliferation, apoptosis, angiogenesis, and invasion. Src up-regulation is a ubiquitous event occurring in ~80% of colorectal

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cancers. Although Src may be activated through mutational events (17) or transcriptional overexpression (18), by far the most common mechanism of Src activation is through down-regulation of its negative regulator, COOH-terminal Src kinase (Csk). Csk inhibition is mediated by phosphorylation of Src at Tyr^{527} (19). The central role of Csk is highlighted by the observation that overexpression of Csk mitigates the neoplastic phenotype of colorectal cancer cells (20). We have noted previously that, in the azoxymethane-treated rat model of colon carcinogenesis, Csk was down-regulated with concomitant activation of Src in the histologically normal (predysplastic) mucosa (21). Importantly, this was associated with the diffuse colonic epithelial hyperproliferation, thereby providing a substrate for future tumorigenesis. Thus, the Csk/Src axis is a logical target for colorectal cancer chemoprevention. However, despite the central role of Src in colon carcinogenesis, to the best of our knowledge, there have been no previous studies that have explored the role of Csk/Src axis in colorectal cancer chemoprevention.

To explore this issue, we examined the ability of the well-established chemopreventive agent, sulindac, to modulate the Csk/Src axis and hyperproliferation in the azoxymethane-treated rat, a validated model of experimental colon carcinogenesis. We also compared the sulindac responsiveness of colorectal cancer cell line HT-29 and a stable construct that blunted the Csk induction through short hairpin RNA (shRNA). Finally, we determined the role of $p21^{cip/waf1}$ in the antiproliferative effect of NSAID as a result of Csk induction and Src inhibition. This provides the first evidence of the central role of Csk/Src axis in NSAID chemoprevention.

Materials and Methods

Animals

All animal studies were done in accordance with the Institutional Animal Care and Use Committee of Evanston Northwestern Healthcare. Male Fisher 344 rats (150-200 g) received two weekly i.p. injections of azoxymethane (15 mg/kg; Midwest Research Institute). Seven weeks after the second azoxymethane injection, the rats were randomized into two groups. The first (control) group was fed with AIN76A diet alone and the second group was fed with AIN76A diet supplemented with 320 ppm sulindac for 1 week after which the rats were euthanized 2 h after i.p. injections of bromodeoxyuridine (BrdUrd; 50 mg/kg) to measure the *in vivo* cell proliferation. Colons were removed and divided into proximal and distal segments. Mucosal scrapings from the distal segment were subjected to Western blotting analysis. Immunohistochemistry was used to detect levels of Csk, tyrosine phosphorylated Src (p-Src-Tyr^{527}), $p21^{cip/waf1}$, and BrdUrd incorporation.

Immunohistochemistry

Immunohistochemistry was done as described previously (21). Briefly, 5 μm tissue sections were deparaffinized, subjected to heat-based antigen retrieval, and probed with Csk (BD Transduction Labs), Src-Tyr^{527}, and $p21^{cip/waf1}$ (Cell Signaling). Slides were developed with a Vectastain ABC kit (Vector Laboratories). BrdUrd incorporation was detected by using a BrdUrd staining kit (Zymed Laboratories). The stained tissue sections were scored on a four-point scale by an observer blinded to treatment group.

Cell Culture

Colorectal cancer cell line HT-29 (American Type Culture Collection) was grown as described previously (22). To study the *in vitro* effect of NSAID on HT-29 cells and its shRNA stable construct, the cells were treated with either vehicle (DMSO) or sulindac sulfide (100-125 μmol/L) for 72 h. The proteins were subjected to immunoprecipitation and Western blotting analyses by standard methods.

Src Kinase Assay

HT-29 cells were treated with either vehicle or sulindac sulfide (100 μmol/L) for 72 h. Cells were lysed with the lysis buffer (Cell Signaling) and the lysates were precleared using anti-mouse IgG (5 μg). Total protein concentration was determined using the Bio-Rad RC/DC protein assay kit. Subsequently, 1 mg protein sample was immunoprecipitated with antibody against c-Src (Santa Cruz Biotechnology). The immunoprecipitates were washed three times with lysis buffer and resuspended in 25 μL of 1× tyrosine kinase buffer [20 mmol/L Tris (pH 7.2), 25 mmol/L $MgCl_2$, 5 mmol/L $MnCl_2$, 0.4 mmol/L EGTA, 50 mmol/L sodium orthovanadate, 0.4 mmol/L DTT]. The Src kinase activity from the immunoprecipitates was determined using a nonradioactive two-step tyrosine kinase activity assay kit (Chemicon International) following the manufacturer's instructions. Briefly, a biotinylated substrate peptide containing tandem repeats of poly(Glu_4-Tyr) was incubated with a tyrosine kinase enzyme sample in the presence of nonradioactive ATP and a Mn^{2+}-Mg^{2+}cofactor cocktail. The kinase assay was done with substrate peptide in a solution used to coat the surface of a streptavidin-coated 96-well plate. The second step involved detection of the phosphorylated substrate by direct ELISA with anti-phosphotyrosine horseradish peroxidase monoclonal antibody and a tetramethylbenzidine substrate. The quantity of phosphate incorporated into the tyrosine kinase substrate is determined using the phosphopeptide standard curve. One unit of Src kinase was defined as the amount of p60c-Src tyrosine kinase that will transfer 1 pmol phosphate/min to the biotinylated poly(Glu:Tyr) peptide at saturated substrate concentrations (pH 7.4) at 30°C.

E-Cadherin Tyrosine Phosphorylation

To study the phosphorylation status of E-cadherin, total cell proteins (750 μg protein) were immunoprecipitated with anti-phosphotyrosine antibody (Santa Cruz Biotechnology) as described above, subjected to Western blotting, and probed with anti-E-cadherin antibody (Santa Cruz Biotechnology).

Csk shRNA Constructs

To determine the role of Csk in NSAID-mediated antiproliferation, we stably transfected human colorectal cancer cell line HT-29 with the BD pSIREN-DNR-Csk shRNA vector (Clontech Laboratories) using Lipofect-AMINE 2000 (Invitrogen) according to the manufacturer's

instructions. After transfection, cells were incubated at 37°C in a humidified 5% CO_2 incubator for 24 h. Transfectants were then selected for hygromycin (600 μg/mL) resistance. The stable clones were isolated using cloning cylinders and were expanded and maintained in a medium containing hygromycin. Total RNA and protein was isolated from the clones using TRI-reagent (Sigma) and subjected to reverse transcription-PCR and Western blotting analyses respectively (21).

Western Blot Analysis

Each protein sample (40 μg) was separated by SDS-PAGE, transferred to polyvinylidene fluoride membranes, and probed with antibodies specific for p-Src-Tyr^{527} (1:300 dilution; Cell Signaling), proliferating cell nuclear antigen (PCNA; 1:500 dilution), and Csk (1:300 dilution; Santa Cruz Biotechnology) overnight at 4°C. Xerograms were developed with enhanced chemiluminescence (Santa Cruz Biotechnology). Images were acquired through UVP Bioimaging Systems (UVP) and analyzed using Labworks 4.6 software (UVP). Consistency in protein loading was assessed by probing membranes with anti-β-actin (1:500 dilution; Sigma).

Cell Cycle Analysis

HT-29 and the Csk shRNA stable cells were either incubated with 125 μmol/L sulindac sulfide or with vehicle (DMSO) at 37°C in a humidified 5% CO_2 incubator for 72 h. At the end the incubation, the cells were washed in PBS/bovine serum albumin (BSA), trypsinized, and recollected in PBS/BSA. Cells were then fixed with ice-cold 70% ethanol and incubated at −20°C for a minimum of 30 min before washing twice with PBS/BSA. The cells were stained with propidium iodide (40 μg/mL; Sigma) and RNase A (200 μg/mL; Sigma) in PBS/BSA and incubated at room temperature for 3 h before measuring their DNA content using flow cytometry (Becton Dickinson Labware). Raw data of the distribution of DNA content were expressed as a percentage of cells in G_0-G_1 through G_2-M populations. The CellQuest 3.1 software program was used to generate DNA content frequency histograms and to assist in data analysis.

p21$^{cip/waf1}$ Analysis by Flow Cytometry

HT-29 and the Csk knockdown cells treated with either vehicle or 125 μmol/L sulindac sulfide were fixed with ice-cold 90% methanol and incubated at −20°C for a minimum of 30 min and then washed twice with PBS/BSA. For p21 staining, cells were suspended in fluorescence-activated cell sorting buffer (PBS-2% FBS-0.2% BSA-saponin-0.02% azide). Cells were then incubated with anti-p21$^{cip/waf1}$ (1:200; Cell Signaling) antibody in fluorescence-activated cell sorting buffer for 1 h at room temperature. After washing three times with fluorescence-activated cell sorting buffer, the cells were incubated with secondary antibody (Alexa 488 Green FI1 labeled anti-mouse) for 40 min. Cells were again washed three times with fluorescence-activated cell sorting buffer and subjected to flow cytometric analysis (Becton Dickinson Labware). CellQuest 3.1 software was used in data analysis.

Statistical Analysis

A Student's t test was used for statistical analysis and the values were considered as significant with P values ≤ 0.05.

Results

Sulindac Reversed Csk Down-regulation in the Azoxymethane-Treated Rat Colon

We analyzed the expression of Csk and p-Src-Tyr^{527} in premalignant colonic mucosa of azoxymethane-treated rats treated with 320 ppm sulindac. Src tyrosine phosphorylation at residue 527 is inhibitory and pathognomonic for the Csk effect, thereby serving as a robust surrogate for Src activity in colon carcinogenesis. We examined the effect of sulindac on the premalignant mucosa 8 weeks after carcinogen injection in a model that requires ~20 and ~35 to 40 weeks to develop adenomas and carcinomas, respectively (23). We have reported previously that, in the histologically normal mucosa of the azoxymethane-treated rats (8 weeks after azoxymethane injection), Csk levels were ~50% lower with a concomitant increase in markers of Src activity compared with age-matched saline-treated animals (21). In the present study, we noted that treatment of azoxymethane-treated rats with sulindac for 1 week significantly increased the Csk protein levels (163.67% of control; $P < 0.05$) with a corresponding increase in the p-Src-Tyr^{527} (Fig. 1A and B). Furthermore, the sulindac treatment resulted in a marked decrease in cell proliferation as seen by decreased BrdUrd incorporation (36.3% of control; $P = 0.007$). Thus, sulindac reversed the down-regulation of Csk in azoxymethane-treated rats resulting in suppression of markers of Src activity.

Sulindac Induces Csk with Corresponding Src Inhibition in Colorectal Cancer Cell Line HT-29

HT-29 cells were treated with either sulindac sulfide or vehicle for 72 h. Treatment with sulindac sulfide failed to alter Src protein levels (data not shown). However, when Src activity was assayed by immunoprecipitation kinase assay (Chemicon International), we noted a significant reduction in Src activity with sulindac sulfide treatment (63% of the vehicle; $P < 0.05$; Fig. 2), which mirrored the inhibitory Src-Tyr^{527} phosphorylation (168% of vehicle; $P < 0.01$). This was accompanied by induction in Csk protein levels (163% of control; $P < 0.01$; Fig. 3A).

Role of Csk in Antiproliferative Activity of Sulindac Sulfide

To further elucidate the role of Csk in the NSAID antiproliferative effect, we engineered a stable Csk shRNA vector as a means of blunting the NSAID induction of Csk expression. We obtained a 54% knockdown in Csk gene expression in the Csk shRNA stable construct, which increased basal PCNA levels by 64% (data not shown). We then compared the NSAID responsiveness between the wild-type HT-29 cells (Fig. 3A) and the shRNA-transfected cells (Fig. 3B). Whereas sulindac sulfide significantly increased Csk and the inhibitory Src phosphorylation (163% and 168% of the vehicle, respectively) in the wild-type HT-29 cells, this NSAID caused only a moderate

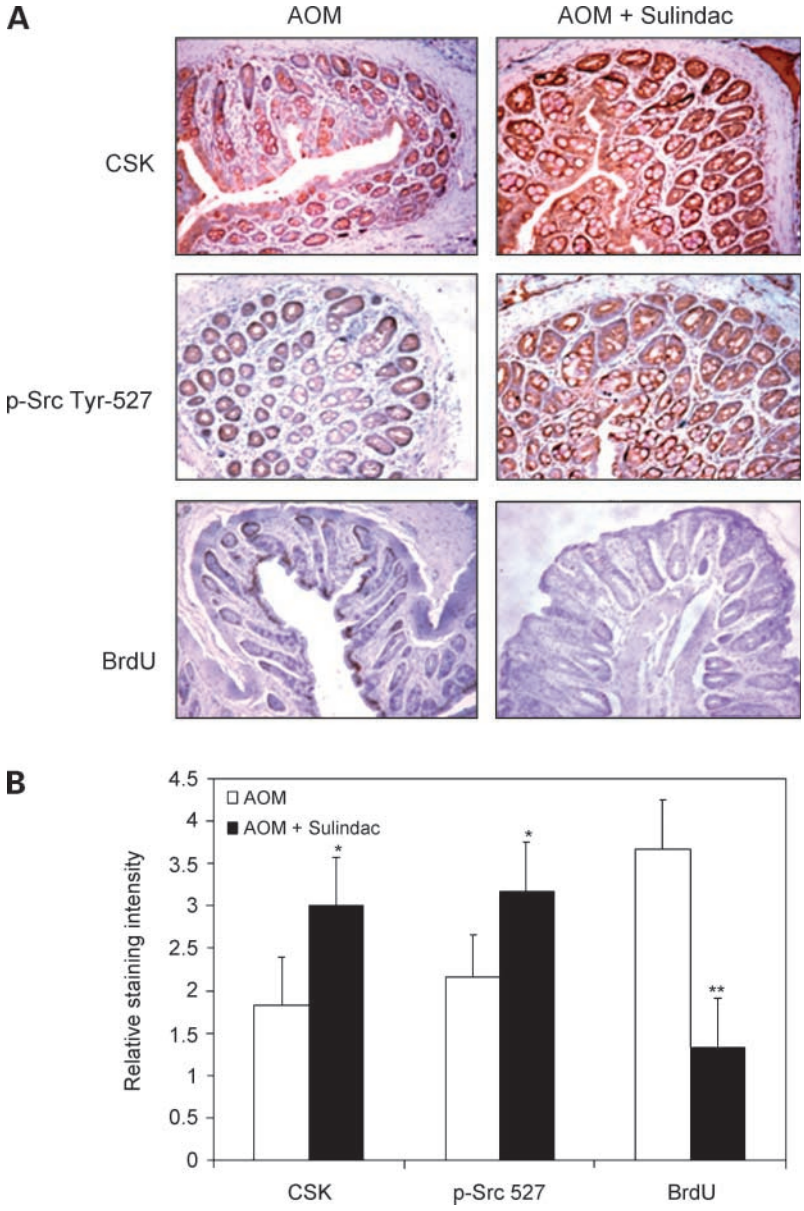

Figure 1. Effect of sulindac on expression of Csk and p-Src-Tyr^{527} and BrdUrd incorporation in preneoplastic colonic mucosa in azoxymethane-treated rats. Colonic tissue sections from azoxymethane-treated male Fisher 344 rats (randomized to AIN76a diet with or without supplementation with 320 ppm sulindac) were subjected to immunohistochemical analysis as described in Materials and Methods. Sulindac-treated rats showed significant Csk induction with corresponding increase in the inhibitory Src-Tyr^{527} phosphorylation. Sulindac markedly suppressed cell proliferation assessed by BrdUrd incorporation. Representative images are shown in **A**. The immunohistochemical staining was scored on a four-point scale by an observer blinded to treatment groups. In the sulindac-treated azoxymethane-treated rats, there was a significant increase in Csk and p-Src-Tyr^{527} with decrease in BrdUrd incorporation (marker of proliferation) compared with unsupplemented rats (**B**). *, $P < 0.05$; **, $P < 0.01$ ($n = 10$).

induction of Csk and Src-Tyr^{527} (129% and 110% of the vehicle; $P < 0.05$ and 0.07, respectively). To assess *in vivo* Src activity, we analyzed tyrosine phosphorylation status of E-cadherin, a well-established Src target in colorectal cancer (24, 25). We noted that sulindac sulfide caused a significant decrease in E-cadherin phosphorylation (35% of the vehicle; $P = 0.019$, supporting the decreased cellular Src activity); however, the Csk shRNA construct showed only a marginal decrease in E-cadherin tyrosine phosphorylation (84% of vehicle). Finally, from a functional, cellular perspective, the ability of sulindac sulfide to decrease cell proliferation was measured in terms of the decrease in the levels of the proliferation marker PCNA. In wild-type HT-29 cells, the sulindac sulfide caused significant reduction in PCNA levels (65% of vehicle; $P = 0.0086$), whereas in the transfectants the decrease in PCNA was only 79% of vehicle ($P = 0.038$). These experiments indicate that the Csk induction is not only associated but also actually necessary for the antiproliferative effects of NSAID in this system.

Loss of Csk Mitigates Sulindac-Mediated Cell Cycle Arrest

One common mechanism for the antiproliferative effect of NSAID is through retardation of cell cycle progression. In the wild-type HT-29 cells, we noted that sulindac sulfide caused an increase in the proportion of cells in G_0-G_1 while causing marked decrease (14% of vehicle; $P < 0.01$) in the proportion of cells in the S phase (Fig. 4), consonant with a G_1-S-phase cell cycle arrest. In the Csk shRNA construct, the decrease in S phase by sulindac sulfide was only 52% of vehicle, indicating that mitigating the Csk induction also

diminished the ability of NSAID to cause a G_1-S-phase cell cycle arrest.

Effect of Sulindac on $p21^{cip/waf1}$ in Azoxymethane-Treated Rat Model and in HT-29 and Csk Knockdown Construct

Because $p21^{cip/waf1}$ is an important modulator in the G_1-S-phase transition, we determined the levels of this cyclin-dependent kinase (CDK) inhibitor in both azoxymethane-treated rats treated with sulindac (using immunohistochemistry) and HT-29 cells (using flow cytometry). In the azoxymethane-treated rats, sulindac treatment resulted in a significantly higher $p21^{cip/waf1}$ (by immunohistochemical staining) in the colonic mucosa than the control rats (200% of control; $P = 0.0132$; Fig. 5A). These results were mirrored in the wild-type HT-29 cells wherein the flow cytometric analysis showed a marked induction of $p21^{cip/waf1}$ (213% of vehicle; $P < 0.05$) compared with significantly lesser induction (165% of vehicle; $P < 0.05$) in the Csk shRNA construct (Fig. 5B). These data thus corroborated with the differences in the sulindac sulfide–mediated cell cycle arrest in the HT-29 and the Csk shRNA construct.

Discussion

We show herein, for the first time, that the antiproliferative and hence chemopreventive response to the NSAID sulindac is mediated by, at least partly, through the Csk/Src axis. Treatment with sulindac markedly induced Csk in both colons of azoxymethane-treated rats and HT-29 cells. This was accompanied by decreased Src activity as shown *in vitro* by activity assays and *in vivo* via phosphorylation status of Src at Tyr^{527}. The functional consequence was indicated by a suppression of Src-mediated E-cadherin tyrosine phosphorylation with sulindac sulfide treatment of HT-29 cells. From a cellular point of view, our data implicate the Csk-mediated Src inhibition in the antiproliferative effects of NSAID through a $p21^{cip/waf1}$-related cell cycle arrest.

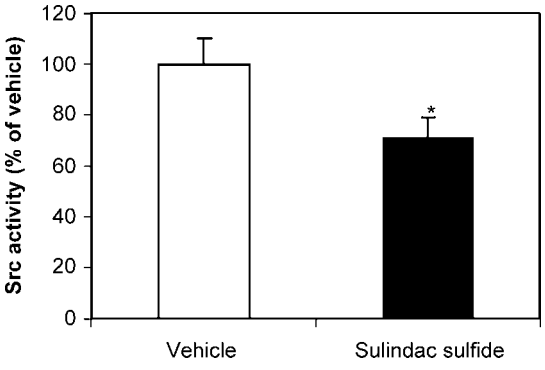

Figure 2. Sulindac sulfide decreases Src activity. HT-29 cells were treated with either vehicle (DMSO) or sulindac sulfide (100 μmol/L) for 72 h. Src activity was measured through an immunoprecipitation kinase assay as detailed in Material and Methods. Compared with vehicle (DMSO), the cells treated with sulindac sulfide showed significant decrease in Src kinase activity (67% of control; $P < 0.05$).

The azoxymethane-treated rat is a robust and well-established model of experimental colon carcinogenesis. This model is the ''gold standard'' in experimental colon carcinogenesis for both chemoprevention and cancer biology studies. In this model, the adenomas and carcinomas develop after 20 and 40 weeks, respectively. Thus, the time point selected in the present study (8 weeks after azoxymethane treatment) represents a preneoplastic stage.

Although there are numerous potential cellular mechanisms involved in NSAID chemoprevention (induction of apoptosis, suppression of motility, invasiveness, angiogenesis, etc.), several lines of evidence support the importance of suppression of epithelial proliferation. For instance, in early carcinogenesis, before any histologic abnormalities, there is a diffuse mucosal hyperproliferation noted in both azoxymethane-treated rat model and humans. Indeed, measurement of proliferation rates from the microscopically normal rectum has been shown to predict neoplasia throughout the colon (26). Modulation of proliferation has been an established intermediate biomarker in chemoprevention studies (27). Therefore, it appears to be a biologically relevant target for NSAID in colorectal cancer prevention.

Src is a logical molecular target for the antiproliferative activity of NSAID. This is supported by the observation that Src inhibition suppressed cell growth in colon cancer cell lines (28). In addition, emerging evidence from other systems indicate that the antiproliferative action of NSAID may be mediated through Src. For instance, Pai et al. noted decreased Src activity in gastric monolayers, a model of peptic ulcer disease (29). With regards to neoplasia, Bernardi et al. noted that the ability of indomethacin to suppress growth in glioma cells was abrogated by pretreatment with a Src inhibitor (30). Thus, there is biological precedence for Src to be modulated by NSAID, although, somewhat surprisingly, this has not been explored previously in chemoprevention of colon carcinogenesis.

For any candidate target of NSAID-mediated colorectal cancer chemoprevention, it would need to be dysregulated early event in neoplastic transformation. Although majority of reports on Src in colon carcinogenesis have focused on relatively late events (tumor progression/invasion), emerging evidence indicates that Src activation may occur much earlier. Src activity has been shown to be increased in the premalignant mucosa of both sporadic and colitis-related colon carcinogenesis (31). Our group has shown recently that, in the azoxymethane-treated rat model, Src activity is dysregulated in the histologically normal mucosa before occurrence of carcinomas or even adenomas (21). Furthermore, Src has been shown to be involved in the development of intestinal tumorigenesis in the MIN mouse, a genetic model of experimental colon carcinogenesis (32). Taken together, these reports support the role of Src in early colon carcinogenesis, thereby giving biological plausibility for targeting Src in chemoprevention.

The predominant means for Src activation in colon carcinogenesis is via suppression of its negative regulator,

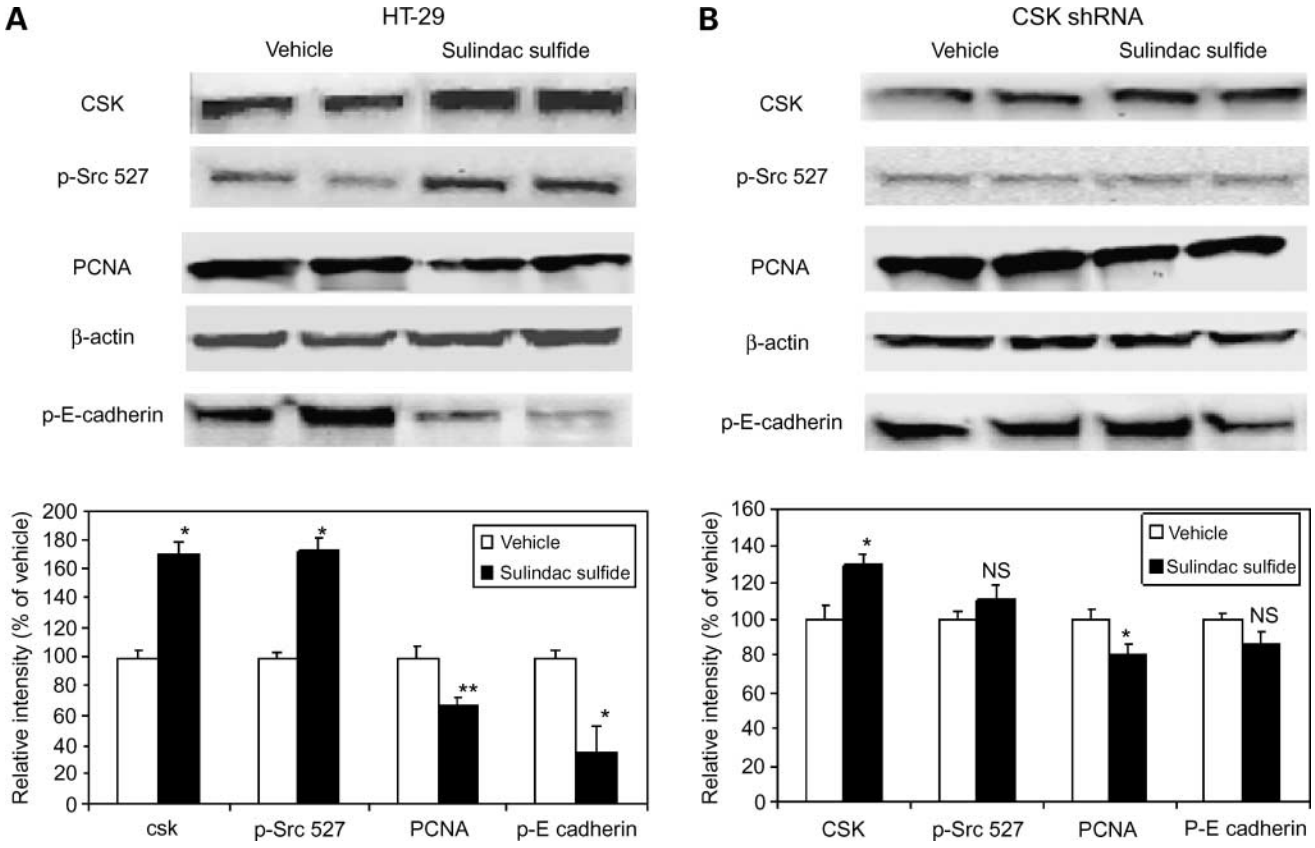

Figure 3. NSAID responsiveness of wild-type HT-29 cells and the Csk knockdown construct. Wild-type HT-29 cells and the Csk shRNA construct were treated with either vehicle (DMSO) or sulindac sulfide (100 μmol/L) for 72 h and proteins were subjected to standard Western blotting techniques. Levels of Csk, p-Src-Tyr^{527} and PCNA, phosphorylated E-cadherin, and densitometric analysis in wild-type HT-29 cells (**A**) and Csk shRNA construct (**B**). Whereas sulindac sulfide caused marked Csk induction with corresponding increase in p-Src-Tyr^{527} and a notable decrease in PCNA in the wild-type HT-29 cells, the Csk shRNA construct showed only a modest Csk induction with minimal alterations in p-Src-Tyr^{527} and lesser degree of PCNA reduction. *, $P < 0.05$; **, $P < 0.01$. *NS,* nonsignificant.

Csk. Indeed, there is a striking reciprocal relationship between Csk loss and Src activation in human colorectal cancer (33). Consistent with this is the observation that Csk overexpression in colorectal cancer cell lines resulted in decreased proliferation (20) and invasiveness (34). Csk appears to control Src activation in the premalignant mucosa as evident by the inverse relationship between Csk and Src activity in the predysplastic colonic mucosa of the azoxymethane-treated rat (21). Thus, Src activation, through Csk down-regulation, has been implicated in both colorectal cancer initiation and progression.

Our data indicate that Csk up-regulation plays a fundamental role in the antiproliferative mechanism of NSAID; however, the upstream events remain unclear. Indeed, it is still not understood how Csk is suppressed during colonic neoplastic transformation. One of the most likely possibilities is through epigenetic silencing (e.g., DNA methylation and histone acetylation) of Csk. Consistent with this is the observation that Csk promoter region has several CpG islands, thus making it a candidate for silencing via hypermethylation. Intriguingly, the NSAID celecoxib has been shown to reverse processes such as hypermethylation (35). A recent report suggests that the proto-oncogene phosphatase of regenerating liver 3 can suppress Csk in colon carcinogenesis (36). Thus, while the mechanisms of Csk induction by NSAID remain to be elucidated, reversal of Csk down-regulatory processes appears to be plausible.

With regards to downstream consequences of the modulation of the Csk/Src axis by NSAID, there are myriad of potential targets that could affect on proliferation and hence chemoprevention. The observation (confirming numerous previous reports) that sulindac sulfide caused a G_1-S-phase arrest was instrumental in narrowing our focus. The transition from G_1-S phase is complex and driven by CDK activity, which in turn is regulated by the interplay between pro-proliferative cyclins and antiproliferative CDK inhibitors (e.g., p21$^{cip/waf1}$, p27^{kip}, and p16). Several lines of evidence underscore the importance of p21$^{cip/waf1}$ loss in colon carcinogenesis (generally lost through epigenetic silencing). p21$^{cip/waf1}$ acts a critical conduit for the epidermal growth factor receptor pro-proliferative signal in intestinal cells (37). Additionally, in adenomatous polyposis coli and Muc-2 knockout-driven mouse models,

$p21^{cip/waf1}$ loss is noted to markedly augment tumorigenesis (32, 38). Celecoxib and sulindac among other NSAID have been shown to induce $p21^{cip/waf1}$ with a concomitant inhibition of E2F, the transcription factor central to G_1-S-phase progression (39). Clinically, $p21^{cip/waf1}$ induction has also seen in the uninvolved rectal mucosa of patients treated with sulindac (300 mg/d) for a month (40). Importantly, in adenomatous polyposis coli–driven models of intestinal tumorigenesis, loss of $p21^{cip/waf1}$ rendered the mice insensitive to the chemopreventive ability of sulindac (38). Thus, there are compelling data to believe that $p21^{cip/waf1}$ may be a critical mediator of the antiproliferative effects of NSAID.

There are numerous candidate regulators of $p21^{cip/waf1}$ including p53, K-ras, epidermal growth factor receptor, and c-Jun kinase. We observed that Csk knockdown with shRNA was accompanied by a ~50% increase in basal $p21^{cip/waf1}$, suggesting a role for Csk in p21 regulation. Similar to previous reports, we noted that sulindac sulfide treatment resulted in a 2-fold increase in $p21^{cip/waf1}$. Importantly, blunting sulindac-mediated Csk induction in the Csk shRNA construct resulted in mitigation of the $p21^{cip/waf1}$ up-regulation. Taken together, these experiments strongly implicate the NSAID modulation of Csk/Src axis in cell cycle arrest and hence chemoprevention. To our knowledge, this is the first report that Csk/Src axis regulates $p21^{cip/waf1}$ in colon carcinogenesis. There have been reports implicating Src to be involved in modulation of p21 by agents such as Adriamycin (in human breast cancer cells; ref. 41) or low-density lipoproteins (in endothelial cells; ref. 42). Thus, there is precedence in other systems, although our report is the first to link the Csk to the regulation of $p21^{cip/waf1}$ in both the basal state and during NSAID chemoprevention.

Although our data strongly suggest that Csk-Src modulation of $p21^{cip/waf1}$ expression is the most likely

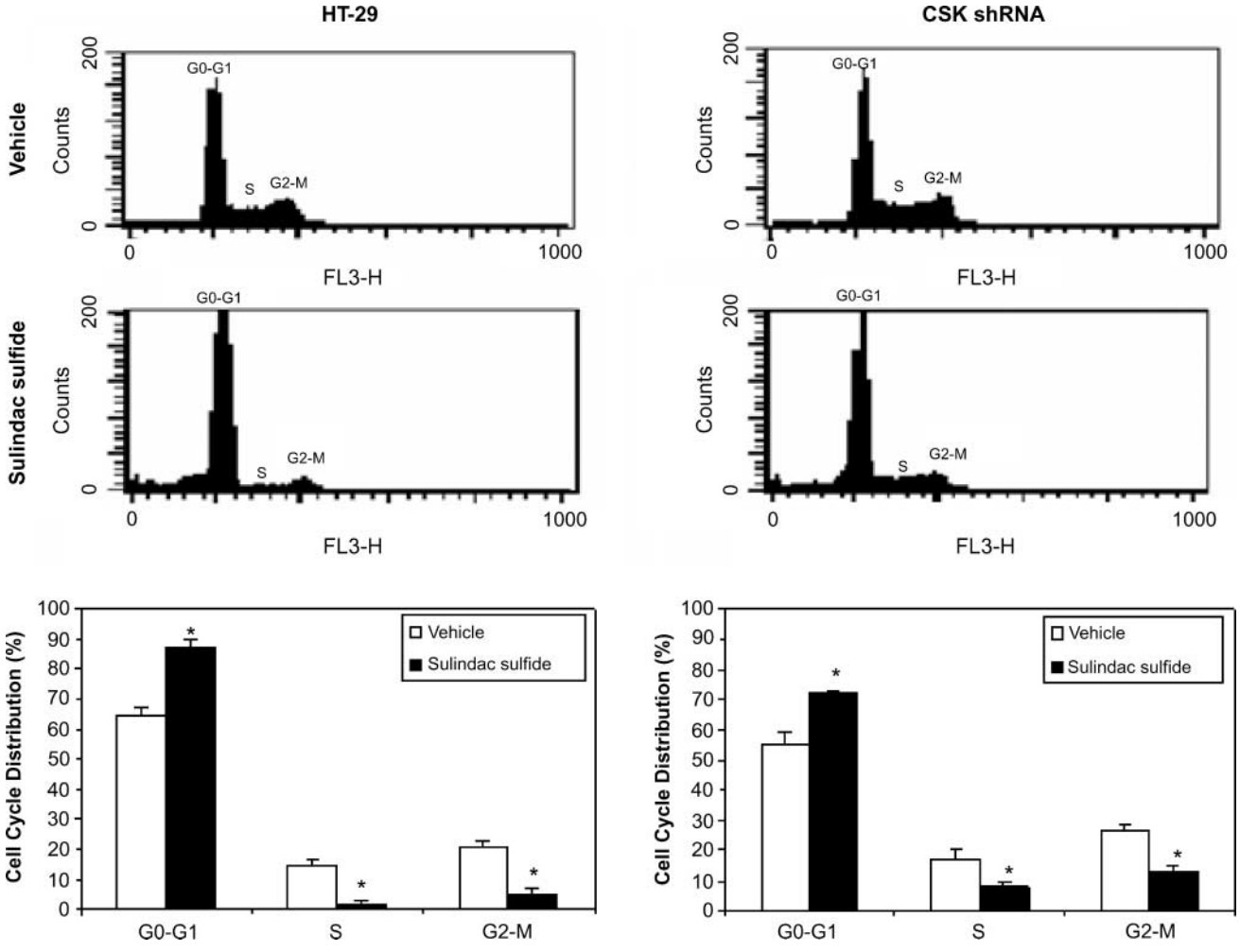

Figure 4. Effect of sulindac sulfide on cell cycle in HT-29 cells and its Csk knockdown construct. Wild-type HT-29 cells and the Csk shRNA construct were incubated with either vehicle (DMSO) or sulindac sulfide (125 μmol/L) for 72 h. Cells were fixed in 70% ethanol, stained with propidium iodide, and analyzed for cell cycle distribution by flow cytometry. In wild-type HT-29 cells, sulindac sulfide treatment caused a striking reduction in the percentage of cells in S phase (14% of vehicle; $P < 0.01$) compared with only 52% decrease in S-phase cells in the Csk shRNA construct. *, $P < 0.01$.

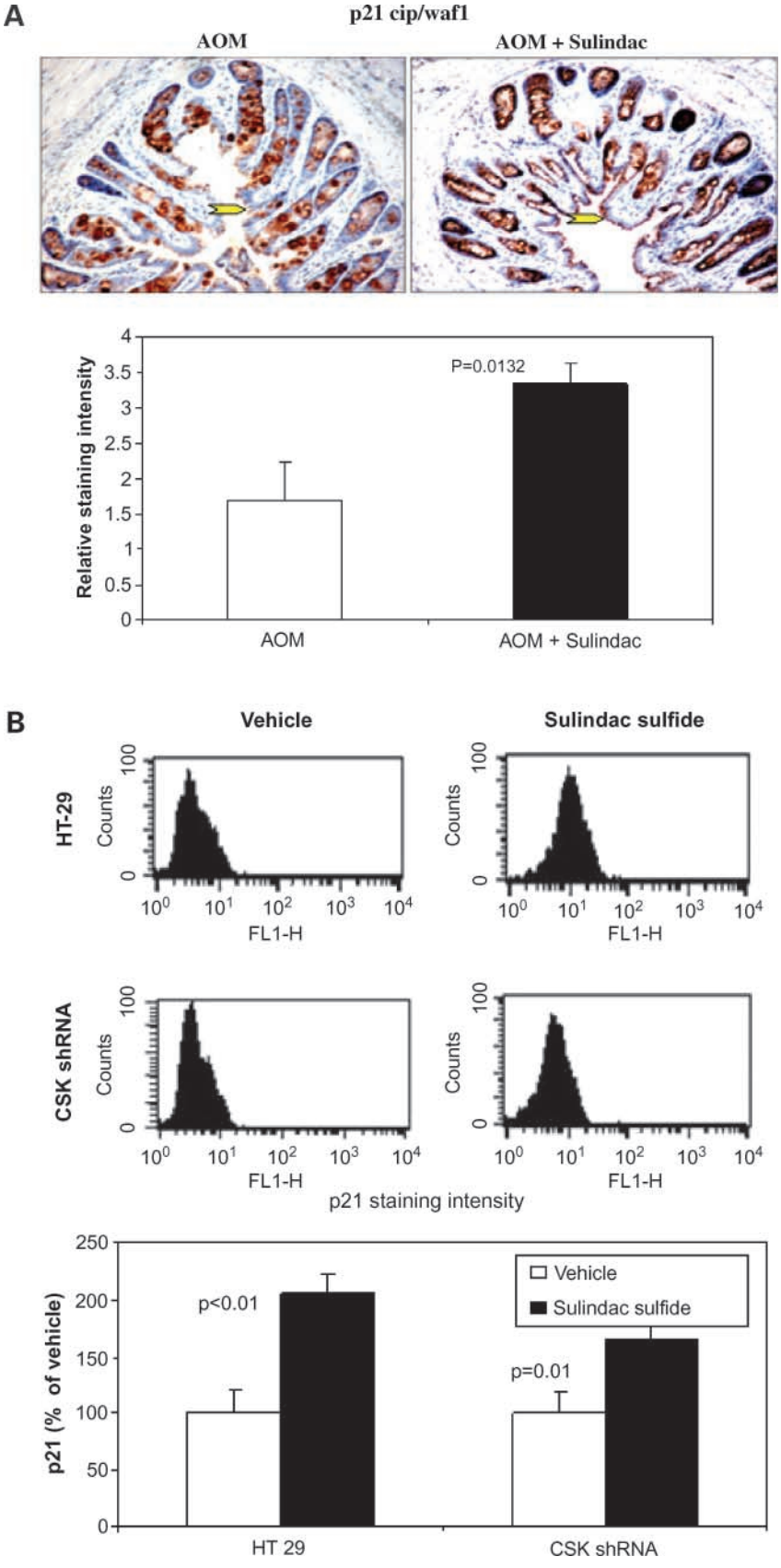

Figure 5. Effect of sulindac sulfide on p21 expression. **A,** immunohistochemical detection of $p21^{cip/waf1}$ in azoxymethane-treated rats. Sulindac treatment caused a significant induction of the CDK inhibitor $p21^{cip/waf1}$ in azoxymethane-treated rats (200% of control rats; $P = 0.0132$). **B,** flow cytometric detection of $p21^{cip/waf1}$ in the wild-type HT-29 cells and the Csk shRNA cells. The levels of $p21^{cip/waf1}$ were detected by flow cytometry as described in Materials and Methods. Whereas sulindac sulfide caused a significant induction (213% of vehicle; $P < 0.05$) on $p21^{cip/waf1}$ in wild-type HT-29 cells, the Csk knockdown showed a lesser degree of p21 activation (165% of vehicle; $P < 0.05$).

mechanism for NSAID-mediated G_1-S-phase arrest, there are alternative possibilities involving Src. For instance, Src has been shown to stabilize cyclin B1-CDK complex enhancing cell cycle progression (43). Src can also phosphorylate the CDK inhibitor, $p27^{kip}$, leading to down-regulation and hence increased proliferation (44). Moreover, the Csk/Src axis can also modulate several COX and non-COX-related putative targets implicated in NSAID-mediated suppression of proliferation. For instance, Src is necessary for prostaglandin E_2 (a product of COX)–induced epidermal growth factor receptor activation (45). Src can also affect the Wnt signaling (critical in early colonic hyperproliferation) through modulation of E-cadherin-mediated β-catenin regulation (46). In this regard, our data show that sulindac sulfide inhibition of E-cadherin tyrosine phosphorylation was mitigated in the Csk shRNA construct, suggesting that modulation of the Csk/Src axis may be causal to some of these downstream effects. Thus, it is clear that NSAID modulation of the Csk/Src axis may have implications for numerous pathways involved in colorectal cancer chemoprevention.

There are several unresolved issues in this report. Firstly, we present a linear paradigm from NSAID → ↑ Csk →↓ Src →↑ p21 →↓ proliferation (Fig. 6), which is obviously oversimplistic given the cross-talk among signaling cascades. Secondly, our assumption that Csk acts exclusively through Src remains to be proven given that there is evidence that Csk can act in a Src-independent manner. For instance, Csk can interact directly with c-Jun and lead to degradation (47) and others have noted that JNK can interact directly with p21 (48). Another issue is the translatability of these findings to other NSAID. Although sulindac is the prototypical NSAID used in cell culture, rodent models, and clinical trials, these results have been replicated with numerous other NSAID. Future studies will need to be done on other NSAID for the effect on Csk/Src/p21 axis. With regards to dosage, previous studies have used sulindac sulfide concentrations ranging from 5 to 200 μmol/L; however, there is a question about physiologic relevance. It is important to note that, in the azoxymethane-treated rat model, we observed a dose of sulindac that is equivalent to human dosing leads to Csk induction with a concomitant decrease in Src activity, arguing that our finding are biologically relevant for chemoprevention. Other issue is that Csk knockdown did not completely abrogate the antiproliferative effect of sulindac sulfide. This could be due to the fact that, in the Csk shRNA construct, sulindac sulfide *did* induce Csk (albeit only modestly), thus potentially accounting for its residual antiproliferative effects. It is also likely that the other potential targets contributed to the residual responsiveness to NSAID. Although, in this study, we show that Csk is important in targeting Src in NSAID-mediated chemoprevention, Csk may also inhibit other members of the Src family of tyrosine kinases (e.g., Fyn and Lyn; ref. 19). Thus, although our study provides a compelling evidence for the important role of the Src/Csk axis in NSAID-mediated chemoprevention, the possible role of non-Src family of tyrosine kinases remains unclear. Finally, we only assessed one aspect of chemoprevention, the inhibition of proliferation. It is probable that the Csk/Src axis can modulate many other facets of colonic neoplastic transformation, including apoptosis, angiogenesis, and motility/invasiveness. Indeed, our preliminary data indicate that Csk shRNA constructs also suppressed sulindac-induced apoptosis in HT-29 cells (49). It is intriguing to note that $p21^{cip/waf1}$ is also implicated in the control of apoptosis in colon cancer cells, suggesting that the paradigm specified in Fig. 6 may be relevant to other facets of chemoprevention (50).

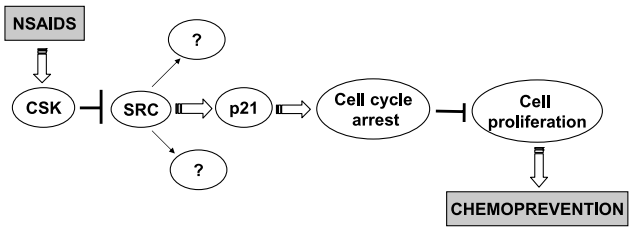

Figure 6. Proposed mechanism for NSAID-mediated colon cancer chemoprevention via Csk/Src axis.

In conclusion, we present evidence herein which indicates a novel mechanism through which NSAID suppress colonic epithelial proliferation. This provides the first compelling data that the Csk may be an important NSAID target in the chemoprevention of colorectal cancer. Moreover, it underscores the potentially significant role of Csk/Src axis in early colon carcinogenesis, especially in controlling cell cycle progression with $p21^{cip/waf1}$ representing a central downstream effector. Future studies are necessary to further elucidate the role of the Csk/Src axis in colon carcinogenesis and NSAID-chemoprevention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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