Sulindac Induces Apoptosis and Inhibits Tumor Growth *In Vivo* in Head and Neck Squamous Cell Carcinoma[1]

Mark A. Scheper, Nikolaos G. Nikitakis‡, Risa Chaisuparat*, Silvia Montaner*,† and John J. Sauk*,†*

*Department of Diagnostic Sciences and Pathology, University of Maryland, Baltimore, MD, USA; †Greenebaum Cancer Center, University of Maryland, Baltimore, MD, USA; ‡Department of Oral Pathology and Oral Medicine, National and Kapodistrian University of Athens, Greece

Abstract

Sulindac has antineoplastic effects on various cancer cell lines; consequently, we assessed sulindac's effects on laryngeal squamous cell carcinoma (SCC) cells *in vitro* and *in vivo*. *In vitro*, SCC (HEP-2) cells treated with various cyclooxygenase inhibitors or transfected with constitutively active signal transducer and activator of transcription 3 (Stat3) or survivin vectors were analyzed using Western blot analysis, annexin V assay, and cell proliferation assay. In parallel, nude mice injected subcutaneously with HEP-2 cells were either treated intraperitoneally with sulindac or left untreated, and analyzed for tumor weight, survivin expression, and tyrosine-phosphorylated Stat3 expression. *In vitro* studies confirmed the selective antiproliferative and proapoptotic effects of sulindac, which also downregulated Stat3 and survivin protein expression. Stat3 or survivin forced expression partially rescued the antiproliferative effects of sulindac. *In vivo* studies showed significant repression of HEP-2 xenograft growth in sulindac-treated mice *versus* controls, with near-complete resolution at 10 days. Additionally, tumor specimens treated with sulindac showed downregulation of phosphorylated tyrosine-705 Stat3 and survivin expression. Taken together, our data suggest, for the first time, a specific inhibitory effect of sulindac on tumor growth and survivin expression in laryngeal cancer, both *in vitro* and *in vivo*, in a Stat3-dependent manner, suggesting a novel therapeutic approach to head and neck cancer.

Neoplasia (2007) **9,** 192–199

Keywords: Sulindac, signal transducer and activator of transcription 3 (Stat3), survivin, head and neck cancer, squamous cell carcinoma.

Introduction

Head and neck squamous cell carcinoma (SCC) is a common cancer, with 40,000 new cases diagnosed annually in the United States and a collective worldwide incidence of 500,000 new cases yearly, making it the sixth most common cancer in the United States and the third most common cancer worldwide [1,2]. However, over the past 50 years, the prognosis for this cancer has not improved over the 50% 5-year survival rate [3].

The *in vitro* antineoplastic effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on various types of cancer, including oral SCC cells, have been recognized for a number of years [4–6]. In recent studies, the use of the NSAID sulindac has shown an inhibitory effect on tumor growth in gastric, lung, and colorectal cancers in nude mice, with a concomitant decrease in cell growth and an increase in apoptosis [7–12]. Furthermore, studies using sulindac in combination with other anticancer drugs (cisplatin, paclitaxel, and docetaxel), epidermal growth factor receptor inhibitors, tumor necrosis factor-α, mitomycin, or lactacystin (a proteasome inhibitor) have shown a synergistic effect [12–19]. Although it is well known that sulindac and other cyclooxygenase (COX) inhibitors exert analgesic, antipyretic, and anti-inflammatory effects through the inhibition of prostaglandins, the exact mechanism of their ability to prevent cancer is still unknown [20,21].

The constitutive activation of signal transducer and activator of transcription 3 (Stat3) is known to be associated with various human cancers, including head and neck SCC, in which abnormal upstream tyrosine kinase signaling has been implicated as the predicted culprit [22–27]. Oncogenic Stat3 signaling results in activation of target genes, including *cyclin D1*, *Bcl-2*, and *Bcl-xL*, affecting cell proliferation, cell cycle, tumor formation, and prevention of apoptosis [23–26]. We have previously shown that sulindac treatment of oral SCC cell lines SCC4, SCC9, SCC15, and SCC25 causes a downregulation of activated Stat3, with a concomitant inhibition of cell growth and an increase in apoptosis [28]. Recent *in vivo* studies using silencer siRNA for Stat3 have shown an inhibition of transplanted laryngeal tumor growth in mice, with a concomitant increase in apoptosis [29].

Survivin, acting as an inhibitor of apoptosis, is normally expressed in developing tissues, the thymus, basal colonic

Address all correspondence to: Mark A. Scheper, DDS, Department of Diagnostic Sciences and Pathology, Dental School, University of Maryland at Baltimore, 7 North, 650 West Baltimore Street, Baltimore, MD 21201. E-mail: mscheper@umaryland.edu

[1]This work was supported by grants from the National Institutes of Health (DE13118 and DE12606 to J.S.) and the University of Maryland Greenebaum Cancer Center Pilot Grant Program (to N.N.).
Received 1 December 2006; Revised 18 January 2007; Accepted 19 January 2007.

DOI 10.1593/neo.06781

tissues, endothelial tissues, and neural stem cells, but not in normally differentiated tissues [30]. It has been reported to be overexpressed in lung, breast, colon, gastric, esophageal, pancreatic, liver, bladder, uterine, ovarian, and brain cancers, as well as in melanomas, lymphomas, leukemias, neuroblastomas, sarcomas, and skin cancers, providing a defect in the normal apoptotic pathway [30–32]. Furthermore, its expression has been detected in preneoplastic lesions, suggesting a possible participation in the induction of malignant transformation [30]. Current *in vivo* studies in mice, using antisense oligodeoxynucleotides, dominant-negative mutants combined with recombinant adenovirus, or siRNA against survivin, have shown inhibition of transplanted tumor growth and induction of apoptosis in laryngeal, liver, and hepatocellular carcinoma xenografts [30]. Recent investigations have focused on the potential function of survivin as a downstream target of Stat3 signaling [33–35]. Our recent studies have suggested that in oral cancer cell lines SCC9 and SCC25, survivin may be a target of sulindac, which mediates its antineoplastic effects [21].

Currently, no studies have explored the *in vivo* effects of sulindac on cancer growth and the Stat3/survivin signaling pathway in primary head and neck SCC in mouse models. Here, we show for the first time the antiproliferative and proapoptotic effects of sulindac using laryngeal SCC (HEP-2) xenografts in nude mice, suggesting that sulindac may be a potential therapeutic alternative for patients with SCC. In addition, we demonstrate that the antiproliferative effects of sulindac on head and neck SCC may be mediated through the downregulation of activated Stat3 and survivin *in vivo*.

Materials and Methods

Cell Lines and Cell Culture

All experiments were performed using the established primary laryngeal SCC cell line HEP-2 (donated by Dr. Silvio Gutkind; National Institutes of Health). Cells were cultured in a 1:1 mix of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 U of penicillin, 100 μg/ml streptomycin, and 0.4 g/ml hydrocortisone (Sigma Chemical Co., St. Louis, MO). The cells were cultured at 37°C in a 5% CO_2 air atmosphere. Cells were subcultured using a disaggregation assay with trypsin (0.1%) and ethylenediaminetetraacetic acid (EDTA; 0.01%) in phosphate-buffered saline (PBS), pH 7.5.

Cells were grown in 6-well or 24-well plates at 5×10^4 cells/well and grown to 80% confluence. The cells were then either left untreated or treated with dimethyl sulfoxide (DMSO) only or drug dissolved in DMSO. When DMSO was used, its final concentration did not exceed 0.1%.

Drug Treatments

The following COX-2 inhibitors were used for *in vitro* experiments:

Nonselective: 150 μM sulindac (Sigma Chemical Co.) and 150 μM indomethacin (Sigma Chemical Co.)

Selective: 150 μM nimesulide (Sigma Chemical Co.) and 150 μM celecoxib (Pfizer, New York, NY).

Transfection with Constitutively Active Stat3 Mutant or Survivin Forced Expression Vectors

Vectors for constitutively active Stat3 mutant (*c-Stat3*) and survivin forced expression, and corresponding control vectors (clone name pCDNA 3.1 + Hygro constitutively active C-terminus–tagged Stat3 and pcDNAIII myc-tagged survivin, respectively) were generously donated by Dr. Silvio Gutkind of the National Institutes of Health. These vectors were created with the following primers: 5′ *Bam*HIII and 3′ *Hin*dIII (for Stat3) or 3′ *Eco*RI (for survivin), with resistance to ampicillin. DNA were bacterially transformed using Bacto agar (Difco, Sparks, MD), LB medium (Fisher, Hampton, NH), and ampicillin (Sigma Chemical Co.), using GC5-competent cells (Gene Choice, Frederick, MD). Plasmid DNA purification was accomplished using HiSpeed Plasmid Purification Maxi-Prep Kit (Qiagen, Valencia, CA). To determine the existence of a construct within purified DNA, digestion with *Bam* and *Eco* enzymes (New England Biolaboratories, Ipswich, MA) was preformed. Cells were plated in six-well plates using a density of 5×10^4 cells/well, allowed to grow to 80% confluence, and treated with serum-free medium for 24 hours. Expression or control mock vectors were added at 0.4 μg/ml to 25 μl of Optimem media (Invitrogen, Carlsbad, CA) and 4 μl of Plus Regent (Invitrogen) and allowed to incubate for 15 minutes, whereas 1 μl of Lipofectamine 2000 reagent (Invitrogen) was added to 25 μl of Optimem media and incubated for 15 minutes. The vectors were combined with the Lipofectamine 2000 reagent and incubated for an additional 15 minutes at room temperature. The combined mix was then added with 0.2 ml of serum-free medium to the cells, which were incubated at 37°C at 5% CO_2 for 3 hours, followed by the addition of a normal medium or various treatments for prescribed times. Transfection efficiency was evaluated by immunofluorescence analysis. Further analysis included Western blot analysis and cell proliferation assay.

Cell Proliferation

Cells were plated in 24-well plates using a density of 5×10^4 cells/well, allowed to grow to 80% confluence, and then treated with serum-free medium for 24 hours. Subsequently, a normal medium with DMSO (at a maximum concentration of 0.1%); sulindac sulfide, indomethacin, nimesulide; or celecoxib (all at 150 μM, dissolved in DMSO) was added to a normal growth medium and incubated for 72 hours. Alternatively, cells were transfected with vectors for *c-Stat3*, survivin forced expression, or control mock vector for 24 hours alone or followed by sulindac treatment for 72 hours. The cells were removed enzymatically and counted using a Coulter counter (Model ZI; Coulter, Miami, FL). The percentage of cell growth was determined by setting as 100% the growth of cells treated only with the vehicle (0.1% DMSO). All analyses were performed in triplicate.

Flow Cytometry and Annexin V Studies

Apoptosis was evaluated using annexin V–fluorescein isothiocyanate methods. Cells were treated with either the vehicle alone (DMSO at a maximum concentration of 0.1%) or sulindac at 150 μM for 72 hours and washed with Hank's balanced salt solution, followed by lysis using trypsin (0.1%) and EDTA (0.01%) in PBS at pH 7.5. The cells were washed with normal medium and cold PBS, and resuspended in 1× binding buffer (BD-Pharmingen Biosciences, San Diego, CA) Five microliters of annexin and 5 μl of propidium iodide were added to the cells, vortexed, and incubated for 15 minutes in the dark. Finally, 400 μl of 1× binding buffer was added, and samples were evaluated by flow cytometry.

Western Blot Analysis

Cell were treated with either normal medium with DMSO (at a maximum concentration of 0.1%); 150 μM sulindac sulfide for 72 hours; or *c-Stat3* mutant, survivin forced expression vector, or control mock vector for 24 hours, followed by sulindac treatment for 72 hours. The cells were washed twice with ice-cold PBS, followed by lysis with radioimmunoprecipation assay buffer (50 μM Tris pH 7.4, 150 μM NaCl, 1% Triton X-100, 1% deoxycholic acid, sodium salt, 0.1% sodium dodecyl sulfate, 100 μg/ml phenylmethysulfonyl fluoride, 1 μg/ml aprotinin, 1 mM dichlorodiphenyltrichloroethane, and 1 mM sodium orthovanadate) for 10 minutes at 4°C. The wells were scraped, and recovered cell products were centrifuged at 40,000*g* for 15 minutes at 4°C. Recovered proteins were measured and equalized using Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA) per manufacturer's instructions. Tumor tissue samples were placed in lysis buffer on ice for 10 minutes, crushed and sonicated, and finally centrifuged to obtain the protein supernatant. Western blot analysis was then performed using a survivin polyclonal antibody (Abcam, Cambridge, UK), or phosphorylated tyrosine-705 (p-tyr) Stat3 or total Stat3 monoclonal antibodies (Cell Signaling, Beverly, MA).

Establishment and Treatment of SCC Tumor Xenografts in Athymic nu/nu *Mice*

The HEP-2 cell line was used to induce xenografts in 6-week-old athymic (*nu/nu*) nude female mice. The animals received food and water *ad libitum* and were housed in the Association for Assessment and Accreditation for Animal Care–approved Animal Facility of the University of Maryland at Baltimore under the care and management of full-time veterinarians and veterinary staff. All procedures involving animals were approved by the Institutional Animal Care and Use Committee. Exponentially growing cells were harvested, washed with PBS, and resuspended in DMEM, and 1×10^6 viable cells were transplanted subcutaneously into the right flank of mice. For drug treatment, tumor-bearing animals were randomly grouped (control, $n = 5$; test, $n = 5$) and treated with sulindac sulfide (60 mg/kg). Treatment schedule comprised a single injection per animal given intraperitoneally every other day (six injections in total). For analysis, tumor weight was determined by converting tumor volume ($LW^2/2$, where L and W represents the longest length and the shortest width of the tumor, respectively) to weight. At the end of the study period, animals were euthanized for tissue retrieval, which was fixed for immunohistochemical analysis (4% paraformaldehyde overnight before processing for paraffin embedding).

Immunohistochemistry

Paraffin-embedded tissue sections of tumor samples (treated with sulindac or untreated) were deparaffinized, immersed in ethanol 100% and 95%, and heated for antigen retrieval in 0.01 M citrate buffer for 25 minutes in a pressure cooker inside a microwave oven. After dehydration in hydrogen peroxide, the sections were incubated with primary antibodies at room temperature for 1 hour. The applied antibody was a monoclonal p-tyr Stat3 antibody (Cell Signaling) diluted at 1:500, monoclonal survivin antibody (Abcam) diluted at 1:100, or Ki-67 antibody (Dako, Carpinteria, CA) diluted at 1:150. Standard streptavidin–biotin–peroxidase complex method was employed to bind to the primary antibody along with multilink concentrated biotinylated anti-IgG as secondary antibody. Reaction products were visualized by counterstaining with the 3,3′-diaminobenzidine reagent set (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Sections were counterstained with hematoxylin. As a negative control, sections were treated with PBS, with the omission of the primary antibody. Additionally, tumors were stained with Harris' hematoxylin (Harleco, Kansas City, MO) and eosin (Sigma Chemical Co.) for microscopic evaluation.

Immunostains were reviewed by two independent evaluators (M.S. and N.N.). Immunohistochemical reactivity for p-tyr Stat3, survivin, or Ki-67 was graded according to the percentage of positive tumor cells (0, *0%*; 1, *< 20%*; 2, *20–50%*; 3, *> 50%*) and intensity of staining (–, *no staining*; w, *weak*; m, *moderate*; s, *strong*) compared to negative control tissues.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP-Biotin End Labeling (TUNEL) Staining

Paraffin-embedded tissue sections of tumor samples (treated with sulindac and untreated) were deparaffinized, immersed in ethanol 100% and 95%, and heated for antigen retrieval in 0.01 M citrate buffer for 10 minutes in a pressure cooker inside a microwave oven. The tissues were rinsed in PBS and labeled using 50 μl of a 9:1 solution of Label and Enzyme solutions from the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Applied Sciences, Mannheim, Germany), with appropriate controls labeled only with the Label solution. The tissues were incubated for 1 hour at 37° in a humidified atmosphere in the dark. The tissues were rinsed in PBS and analyzed directly under a fluorescence microscope with a detection range of 515 to 565 nm.

Statistical Analysis

For all measurements, as needed, a Student's *t* test or *t*-test was employed to assess the statistical significance of treated groups *versus* control groups. A statistically significant difference was considered to be present at $P \leq .05$.

Results

Treatment with Sulindac, But Not Other COX Inhibitors, Inhibits Cell Proliferation and Induces Apoptosis in HEP-2 Cells

We first sought to study the *in vitro* antiproliferative effects of sulindac on laryngeal SCC (HEP-2) cells and to examine whether these effects are independent of COX-2 inhibition. Treatment of HEP-2 cells with sulindac sulfide, the active metabolite of sulindac, caused significant decreases in cell proliferation ($P \leq .05$; *versus* control cells); in contrast, treatment with selective or nonselective COX inhibitors (indomethacin, nimesulide, or celecoxib) did not significantly affect HEP-2 cell proliferation (Figure 1*A*). The inhibitory effects of sulindac sulfide on cell growth were accompanied by a 3.3-fold increase in apoptosis (Figure 1*B*), which was statistically significant ($P \leq .05$).

In summary, treatment with sulindac, but not other COX inhibitors, exerts *in vitro* cell growth–inhibitory and proapoptotic effects on HEP-2 head and neck SCC cells.

Downregulation of Stat3 and Survivin Expression By Sulindac Mediates Its Antiproliferative Effects on HEP-2 Cells

Previous evidence has shown that constitutive activation of Stat3 in cancer cell lines, including head and neck cell lines, leads to cell proliferation, survival, and sustained growth, through induction of downstream molecules such as survivin [23–26,33–35]. We then set out to determine whether the *in vitro* effect of sulindac's antineoplastic activity on HEP-2 cells is also directly related to its inhibitory effect on Stat3 signaling and subsequent downregulation of survivin expression.

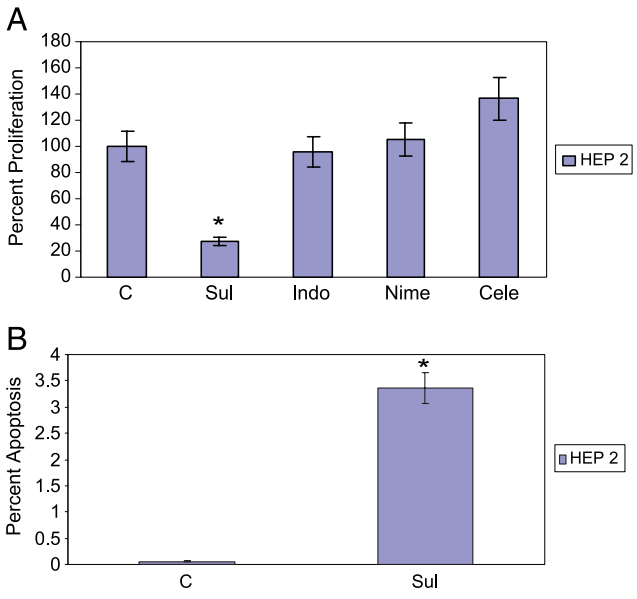

Figure 1. In vitro *COX-independent effects of sulindac on HEP-2 tumor cell proliferation and apoptosis. (A) Assessment of HEP-2 cell proliferation following 72 hours of treatment with the vehicle alone (0.1% DMSO; C) or with COX inhibitor 150 μM sulindac (Sul), 150 μM indomethacin (Indo), 150 μM nimesulide (Nime), or 150 μM celecoxib (Cele). The growth of control cells (C) has been set to 100%. *Statistically significant ($P \leq .05$) differences compared to control cells. (B) Annexin V assay in HEP-2 cells for a comparison of apoptosis following 72 hours of treatment with the vehicle alone (0.1% DMSO; C) or with 150 μM sulindac (Sul). *Statistically significant ($P \leq .05$) differences compared to control cells.*

First, the effects of sulindac treatment on p-tyr Stat3, Stat3, and survivin expression of HEP-2 cells were determined by Western blot analysis. We observed that in HEP-2 cells, sulindac downregulates p-tyr and total Stat3, and survivin protein levels (Figure 2*A*). HEP-2 cells were then transfected with either a flag-tagged constitutively active Stat3 vector (*c-Stat3*), a myc-tagged survivin vector, or a control vector for 24 hours. Western blot analysis showed that sulindac treatment caused significant decreases in the protein levels of p-tyr Stat3, Stat3, and survivin in cells treated with the mock vector (Figure 2*B*). However, survivin forced expression abrogated the effects of sulindac treatment on the downregulation of the levels of this protein (Figure 2*B*). Moreover, transfection with *c-Stat3* reversed the effects of sulindac on p-tyr and total Stat3 levels, as well as on survivin protein expression (Figure 2*B*). In addition, the forced expression of survivin or transfection with *c-Stat3* mutant partially rescued the cell proliferation of HEP-2 cells despite sulindac treatment. Specifically, following sulindac treatment, the cell proliferation rate of survivin-transfected cells (67.4%) and active Stat3-transfected cells (82.1%) was significantly higher than that of similarly treated nontransfected (40.7%) or mock vector–transfected (46.8%) cells (Figure 2*C*).

Therefore, transfection with *c-Stat3* or survivin forced expression is able to decrease the antineoplastic effects of sulindac treatment, suggesting that the ability of sulindac to repress survivin and Stat3 mediates, at least partially, its effects on HEP-2 cancer cell growth.

Sulindac Reduces Xenograft Tumor Burden and Weight

Previous results suggesting an antiproliferative activity of sulindac in head and neck cancer cells prompted us to examine the potential clinical benefit of such drug for the treatment of head and neck cancer patients. For that purpose, athymic (*nu/nu*) nude female mice were injected subcutaneously with HEP-2 cells in the right flank, allowing for the development of subcutaneous tumors. Following tumor formation, five mice were treated every other day (six doses in total) with 60 mg/kg sulindac sulfide, whereas five control mice were left untreated for the same period of time. Drug toxicity, as assessed by animal weight loss, was minimal in the sulindac-treated group (reduction < 5%) during the duration of the study period (results not shown). Sulindac-treated tumors showed a considerable reduction in tumor burden over 11 days of observation, compared with nontreated tumors (Figure 3*A*). Tumor regression in treated animals was observed for 19 days after the beginning of the treatment, and inhibition of tumor growth was sustained for the duration of the experiment (Figure 3, *A–C*). At the end of the study (day 19), we observed that although the average weight of vehicle-treated tumors was 1024 mg (an almost five-fold increase), the sulindac-treated group demonstrated minimal growth over the same period, with an average tumor weight

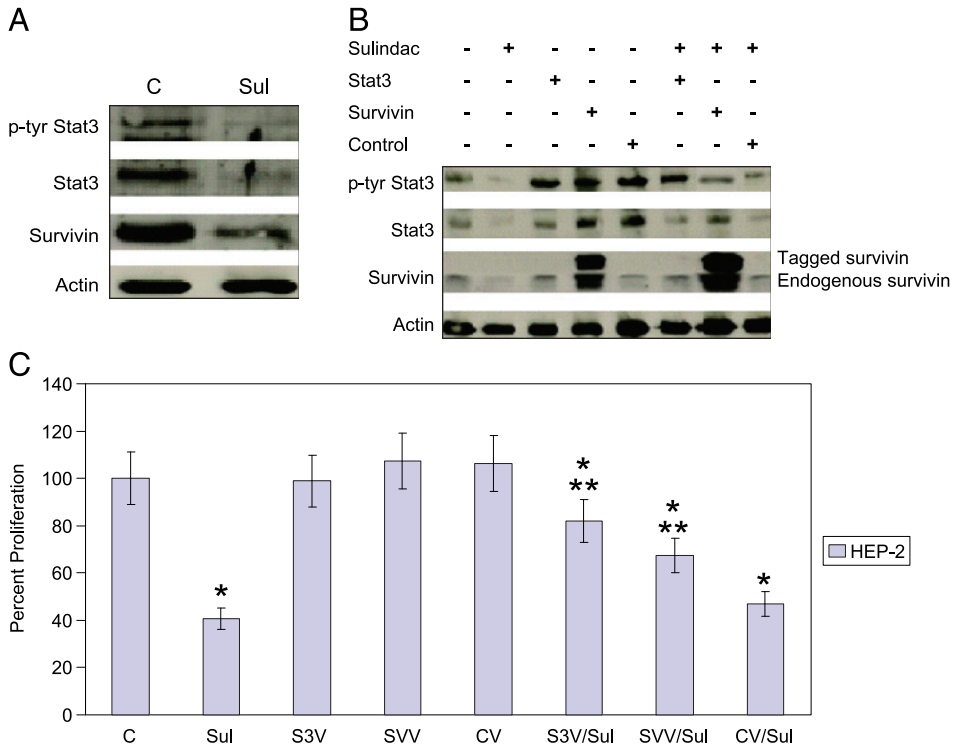

Figure 2. *The role of Stat3 and survivin in sulindac's effects on HEP-2 cells. (A)* In vitro *effects of sulindac treatment on Stat3 and survivin expression. Western blot analysis of p-tyr Stat3, total Stat3, or survivin expression in HEP-2 cells following treatment for 72 hours with the vehicle alone (0.1% DMSO) (lane 1; C) or with 150 μM sulindac (lane 2; Sul). Actin was used as loading control. (B) Effects of transfection with constitutively active Stat3* (c-Stat3) *or survivin forced expression on sulindac's ability to modulate Stat3 and survivin protein expression. Western blot analysis of p-tyr Stat3, total Stat3, or survivin protein expression in HEP-2 cells, following treatment with the vehicle alone (0.1% DMSO) (lane 1); sulindac (150 μM) for 72 hours (lane 2); C-terminus Flag epitope–tagged* c-Stat3 *vector for 24 hours only (lane 3); myc-tagged survivin vector for 24 hours only (lane 4); control mock vector for 24 hours only (lane 5); C-terminus Flag epitope–tagged* c-Stat3 *vector for 24 hours followed by sulindac (150 μM) for 72 hours (lane 6); myc-tagged survivin vector for 24 hours followed by sulindac (150 μM) for 72 hours (lane 7); or control mock vector for 24 hours followed by sulindac (150 μM) for 72 hours (lane 8). Actin was used as loading control. (C) Effects of transfection with* c-Stat3 *or survivin forced expression on sulindac's antiproliferative ability. Assessment of HEP-2 cell proliferation following treatment with the vehicle alone (0.1% DMSO; C); sulindac (150 μM) over 72 hours (Sul); expression vector for* c-Stat3 *(S3V) for 24 hours; expression vector for survivin (SVV) for 24 hours; control vector (CV) for 24 hours; combined treatment of* c-Stat3 *vector for 24 hours followed by sulindac (150 μM) for 72 hours (S3V-Sul); combined treatment of survivin vector for 24 hours followed by sulindac (150 μM) for 72 hours (SVV-Sul); or combined treatment of control vector for 24 hours followed by sulindac (150 μM) for 72 hours (CV-Sul). The cell proliferation of control cells treated with 0.1% DMSO has been set to 100%. *Statistically significant (P ≤ .05) differences compared to control cells. **Statistically significant (P ≤ .05) differences compared to nontransfected or control vector–transfected cells treated with sulindac.*

of 8 mg on day 19 (Figure 3*C*), consistent with continued tumor regression over the same treatment period.

p-tyr Stat3 and Survivin Protein Levels Decrease in Sulindac-Treated Xenografts

Excised tumors were further examined to determine histologic characteristics and degree of differentiation. All tumors demonstrated moderately differentiated SCC characteristics; however, the tumors resected from sulindac-treated animals showed decreased cellularity and mitotic activity, and increased necrosis and number of apoptotic bodies on hematoxylin and eosin (H&E) analysis, compared to untreated control mice (Figure 4*A*, *a* and *b*). To confirm the H&E findings, both apoptosis and proliferation assays were preformed. A TUNEL assay confirmed an increase of apoptosis in sulindac-treated animals compared to untreated control mice (Figure 4*A*, *c* and *d*), whereas Ki-67 staining confirmed an increase in cell proliferation in untreated control mice tumors *versus* sulindac-treated animals (Figure 4*A*, *e* and *f*).

Next, an immunohistochemical expression analysis of survivin and p-tyr Stat3 was evaluated in sulindac-treated *versus* untreated mouse tumors. The untreated tumors showed a strong expression (3S) of survivin (Figure 4*B*, *a*) and a moderate expression (2M) of p-tyr Stat3 (Figure 4*B*, *c*), whereas sulindac-treated tumors showed a diminished expression (1W) of survivin (Figure 4*B*, *b*) and failed to express p-tyr Stat3 (Figure 4*B*, *d*).

To further evaluate whether sulindac treatment reduces the protein expression of p-tyr Stat3 and survivin *in vivo*, protein was extracted from tumors resected from nude mice. Western blot analysis of tumor protein levels showed a downregulation of p-tyr Stat3 and survivin protein levels in sulindac-treated tumors *versus* untreated control mice (Figure 4*C*).

Taken together, these results support the ability of sulindac to downregulate Stat3 signaling and survivin expression *in vivo*, which may provide a molecular explanation for its potent *in vivo* anticancer properties.

Discussion

Aberrant constitutive activation of Stat3 signaling has been well established in various cancers, including head and neck cancer [23–27,33,36], where it is implicated in early tumor

formation and progression and correlates with adverse prognosis [24,36]. Recent studies in gastric carcinomas and primary effusion lymphomas have shown that Stat3 inhibition results in the downregulation of survivin expression and its proproliferative and antiapoptotic effects, implicating survivin as a significant downstream target of Stat3 signaling in cancer [33,34]. This notion is corroborated by the recent demonstration of the *in vivo* downstream effects of aberrant Stat3 activation on survivin expression in hepatoma cells, endothelial cells, and laryngeal tumors [37–39]. Therefore, novel therapeutic approaches with the ability to target aberrant Stat3/survivin signaling in cancer hold great promise.

In this regard, we have previously observed that sulindac, a nonselective COX inhibitor with antiproliferative and proapoptotic effects, causes downregulation of Stat3 tyrosine phosphorylation and protein expression, also inhibiting survivin expression in oral cancer cell lines [21,28]. Other investigators have established that sulindac and other COX inhibitors decrease survivin expression in colorectal carcinoma, breast cancer, non–small cell lung cancer, and lymphoma [35,40–42]. However, the *in vivo* ability of sulindac to inhibit tumor growth in head and neck cancer has not been evaluated. This, combined with the lack of effective treatment strategies for head and neck SCC, prompted us to examine the role of sulindac in head and neck SCC, with the aim of assessing the potential *in vivo* efficacy of this drug and to elucidate its molecular mechanisms of function.

Using the highly tumorigenic HEP-2 cell line [29], we demonstrated that laryngeal SCC cells were sensitive to *in vitro* treatment with sulindac (but not other COX inhibitors), exhibiting significant reduction of cell proliferation and survival. These effects were accompanied by downregulation of the protein levels of active and total Stat3 and survivin, which appeared to be, at least partially, responsible for sulindac's *in vitro* anticancer properties. When HEP-2 cells were transfected with constitutively active Stat3 or subjected to survivin forced expression, there was a rescue effect on Stat3/survivin signaling, cancer cell proliferation, and survival despite sulindac treatment. These *in vitro* observations support the specific antineoplastic effects of sulindac on laryngeal SCC cells, highlighting Stat3 and survivin as significant targets for this drug. Interestingly, this effect of Stat3 downregulation on survivin expression is a supposed direct effect, as there is evidence that the promoter area of survivin contains a Stat3-binding element that directly regulates its expression [43].

We also determined the *in vivo* effectiveness of sulindac against laryngeal SCC cells by showing that sulindac was able to significantly reduce tumor burden and weight in mice bearing xenograft HEP-2 tumors. Moreover, sulindac eliminated the protein expression of activated Stat3 and severely decreased the protein levels of survivin in tumor xenografts, suggesting that downregulation of Stat3/survivin signaling may account for the *in vivo* effects of sulindac on laryngeal SCC xenograft tumors. Therefore, both *in vitro* and *in vivo*, sulindac-induced Stat3 and survivin downregulation may be directly linked to the inhibition of cancer cell proliferation and tumor growth.

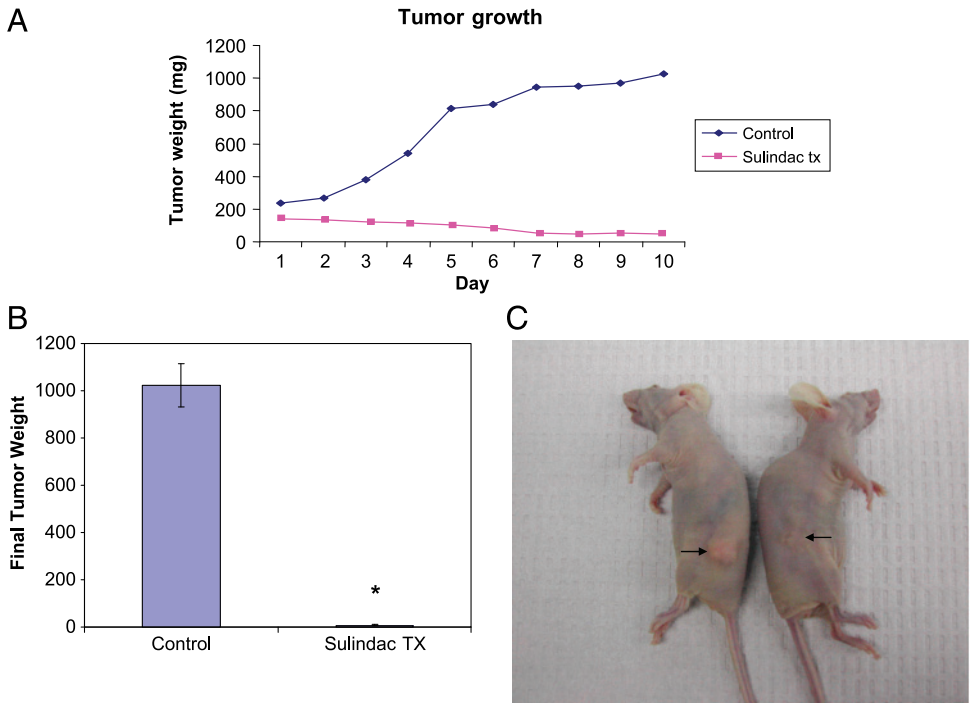

Figure 3. *Effects of sulindac on HEP-2 xenografts tumor volume and weight. (A and B) Growth inhibition (as assessed by tumor volume and weight) of subcutaneously transplanted HEP-2 xenografts treated with sulindac (60 mg/kg) every other day over a period of 11 consecutive days followed by 8 days of follow-up evaluation* versus *untreated control animals. The difference in tumor volume and weight between sulindac-treated and untreated tumors was statistically significant ($P \leq .05$) on day 3 and on all subsequent time points for tumor volume and weight. (C) Representative mice showing untreated (left) and sulindac (60 mg/kg)–treated (right) tumors on day 19.*

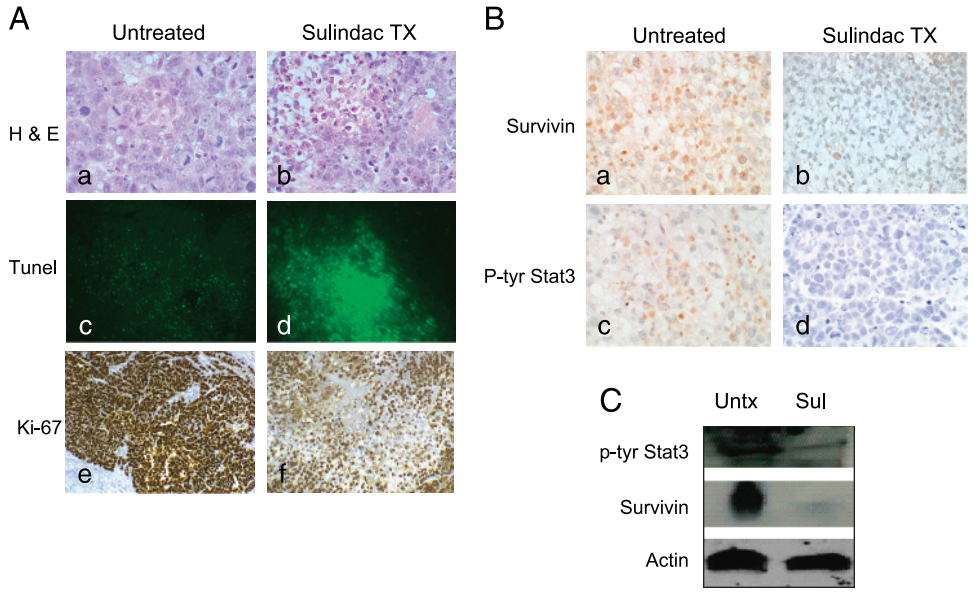

Figure 4. *Tumor protein expression of Stat3 and survivin, and histologic and immunohistochemical analyses of resected tumors. Effects of sulindac treatment on apoptosis and proliferation* in vivo. *(A) (a and b) H&E staining of untreated* versus *sulindac-treated tumor tissues resected from nude mice, showing decreased cellularity and mitotic activity, and increased necrosis and number of apoptotic bodies in sulindac-treated tissues (original magnification. 400×). (c and d) TUNEL staining of untreated* versus *sulindac-treated tumor tissues resected from nude mice, showing increased [apoptosis in sulindac-treated] tissues (original magnification, 200×). (e and f) Ki-67 staining of untreated* versus *sulindac-treated tumor tissues showing increased cell proliferation in untreated tumor tissues (original magnification, 200×). (B) Effects of sulindac treatment on p-tyr Stat3 and survivin expression* in vivo. *Immunohistochemical expression of survivin and p-tyr Stat3 in representative resected HEP-2 xenografts showing (a) a strong expression of survivin in > 50% of tumor cells of untreated resected tumors; (b) a weak expression of survivin in < 20% of tumor cells of sulindac-treated tumors; (c) a moderate expression of p-tyr Stat3 in 20% to 50% of tumor cells of untreated resected tumors; and (d) a lack of expression of p-tyr Stat3 in tumor cells of sulindac-treated tumors (a–d: original magnification, 400×). (C) Western blot analysis of p-tyr Stat3 or survivin expression in mouse tumor tissues resected on day 19; untreated tumors (lane 1; Untx) or sulindac-treated tumors (lane 2; Sul). Actin was used as loading control.*

Demonstration of the immunohistochemical expression of the tyrosine-phosphorylated (active) form of Stat3 and survivin in the tumor cells of laryngeal SCC xenografts correlates well with previous studies showing the overexpression of activated Stat3 and survivin in head and neck SCC tumors [21,26,32,44,45]. It is possible that assessment of the pretreatment levels of Stat3 and/or survivin expression in tumors may facilitate the selection of the most effective treatment. To this end, future studies should attempt to correlate the protein levels of these molecules with the efficacy of sulindac and other Stat3-targeting and/or survivin-targeting therapeutic regimens. Moreover, further animal studies are needed to determine the possible additive or synergistic effects of sulindac, along with other anticancer modalities, in controlling tumor formation and the progression of head and neck SCC. In addition, the effects of sulindac treatment on angiogenesis and downstream effectors of survivin need to be elucidated [46–50].

References

[1] Massano J, Regaterio FS, Januario G, and Ferreira A (2006). Oral squamous cell carcinoma: review of prognostic and predictive factors. *Oral Surg Oral Med Oral Pathol Oral Radiol Endo* **102**, 67–76.

[2] Patel V, Leethanakul C, and Gutkind JS (2001). New approaches to the understanding of the molecular basis of oral cancer. *Crit Rev Oral Biol Med* **12**, 55–63.

[3] Jemal A, Tiwari RC, Murray T, Ghafoor A, Samels A, Ward E, Feuer EJ, and Thun MJ (2004). Cancer statistics 2004. *CA Cancer J Clin* **54**, 8–29.

[4] Mohan S and Epstein JB (2003). Carcinogenesis and cyclooxygenase: the potential role of COX-2 inhibition in upper aerodigestive tract cancer. *Oral Oncol* **39**, 537–546.

[5] Lin DT, Subbaramaiah K, Shah JP, Dannenberg AJ, and Boyle JO (2002). Cyclooxygenase-2: a novel molecular target for the prevention and treatment of head and neck cancer. *Head Neck* **24**, 792–799.

[6] Subbaramaiah K and Dannenberg AJ (2003). Cyclooxygenase 2: a molecular target for cancer prevention and treatment. *Trends Pharmacol Sci* **24**, 96–102.

[7] Fu S-L, Wu Y-L, Zhang Y-P, Qiao M-M, and Chen Y (2004). Anticancer effects of COX-2 inhibitors and their correlation with angiogenesis and invasion in gastric cancer. *World J Gastroenterol* **10**, 1971–1974.

[8] Thompson HJ, Jiang C, Lu J, Mehta RG, Piazza GA, Paranka NS, Pamukcu R, and Ahnen DJ (1997). Sulfone metabolite of sulindac inhibits mammary carcinogenesis. *Cancer Res* **57**, 267–271.

[9] Castonguay A and Rioux N (1997). Inhibition of lung tumourigenesis by sulindac: comparison of two experimental protocols. *Carcinogenesis* **18**, 491–496.

[10] Oida Y, Gopalan B, Miyahara R, Inoue S, Branch CD, Mhashilkar AM, Lin E, Bekele BN, Roth JA, Chada S, et al. (2005). Sulindac enhances adenoviral vector expressing mda-7/IL-24–mediated apoptosis in human lung cancer. *Mol Cancer Ther* **4**, 291–304.

[11] Dvory-Sobol H, Kazanov D, Liberman E, Birkenfeld S, Bulvik B, Luk P, Leshno M, and Arber N (2006). MF tricyclic and sulindac retard tumor formation in an animal model. *Int J Cancer* **118**, 11–16.

[12] Sun BC, Zhao XL, Zhang SW, Liu YX, Wang L, and Wang X (2005). Sulindac induces apoptosis and protects against colon carcinoma in mice. *World J Gastroenterol* **11**, 2822–2826.

[13] Teicher BA, Korbut TT, Menon K, Holden SA, and Ara G (1994). Cyclooxygenase and lipoxygenase inhibitors as modulators of cancer therapies. *Cancer Chemother Pharmacol* **33**, 515–522.

[14] Soriano AF, Helfrich B, Chan DC, Heasley LE, Bunn PA Jr, and Chou TC (1999). Synergistic effects of new chemopreventive agents and conventional cytotoxic agents against human lung cancer cell lines. *Cancer Res* **59**, 6178–6184.

[15] Torrance CJ, Jackson PE, Montgomery E, Kinzler KW, Vogelstein B,

Wissner A, Nunes M, Frost P, and Discafani CM (2000). Combinatorial chemoprevention of intestinal neoplasia. *Nat Med* **6**, 1024–1028.

[16] Yasui H, Adachi M, and Imai K (2003). Combination of tumor necrosis factor-alpha with sulindac in human carcinoma cells *in vivo*. *Ann NY Acad Sci* **1010**, 273–277.

[17] Yasui H, Adachi M, and Imai K (2003). Combination of tumor necrosis factor-alpha with sulindac augments its apoptotic potential and suppresses tumor growth of human carcinoma cells in nude mice. *Cancer* **97**, 1412–1420.

[18] Ma L, Xie YL, Yu Y, and Zhang QN (2005). Apoptosis of human gastric cancer SGC-7901 cells induced by mitomycin combined with sulindac. *World J Gastroenterol* **11**, 1829–1832.

[19] Choi HJ, Kim HH, Lee HS, Huh GY, Seo SY, Jeong JH, Kim JM, and Yoo YH (2003). Lactacystin augments the sulindac-induced apoptosis in HT-29 cells. *Apoptosis* **8**, 301–305.

[20] Meric JB, Rottey S, Olaussen K, Soria JC, Khayat D, Rixe O, and Spano JP (2006). Cyclooxygenase-2 as a target for anticancer drug development. *Crit Rev Oncol Hematol* **59**, 51–64.

[21] Scheper MA, Sauk JJ, and Nikitakis NG (2006). COX-independent antineoplastic effects of sulindac in oral cancer are mediated by survivin downregulation. *Anticancer Res* **26**, 4103–4113.

[22] Levy DE, Darnell JE Jr (2002). Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* **3**, 651–662.

[23] Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE Jr (1999). Stat3 as an oncogene. *Cell* **98**, 295–303.

[24] Kijima T, Niwa H, Steinman RA, Drenning SD, Gooding WE, Wentzel AL, Xi S, and Grandis JR (2002). STAT3 activation abrogates growth factor dependence and contributes to head and neck squamous cell carcinoma tumor growth *in vivo*. *Cell Growth Differ* **13**, 355–362.

[25] Grandis JR, Drenning SD, Zeng Q, Watkins SC, Melhem MF, Endo S, Johnson DE, Huang L, He Y, and Kim JD (2000). Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis *in vivo*. *Proc Natl Acad Sci USA* **97**, 4227–4232.

[26] Song JI and Grandis JR (2000). STAT signaling in head and neck cancer. *Oncogene* **19**, 2489–2495.

[27] Buettner R, Mora LB, and Jove R (2002). Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. *Clin Cancer Res* **8**, 945–954.

[28] Nikitakis NG, Hamburger AW, and Sauk JJ (2002). The nonsteroidal anti-inflammatory drug sulindac causes down-regulation of signal transducer and activator of transcription 3 in human oral squamous cell carcinoma cells. *Cancer Res* **62**, 1004–1007.

[29] Gao LF, Xu DQ, Wen LJ, Zhang XY, Shao YT, and Zhao XJ (2005). Inhibition of STAT3 expression by siRNA suppresses growth and induces apoptosis in laryngeal cancer cells. *Acta Pharmacol Sin* **26**, 377–383.

[30] Zaffaroni N, Pennati M, and Daidone MG (2005). Survivin as a target for new anticancer interventions. *J Cell Mol Med* **9**, 360–372.

[31] Li F (2003). Survivin study: what is the next wave? *J Cell Physiol* **197**, 8–29.

[32] Lo Muzio L, Pannone G, Staibano S, Mignogna MD, Rubini C, Mariggio MA, Procaccini M, Ferrari F, De Rosa G, and Altieri DC (2003). Survivin expression in oral squamous cell carcinoma. *Br J Cancer* **89**, 2244–2248.

[33] Kanda N, Seno H, Konda Y, Marusawa H, Kanai M, Nakajima T, Kawashima T, Nanakin A, Sawabu T, Uenoyama Y, et al. (2004). STAT3 is constitutively activated and supports cell survival in association with survivin expression in gastric cancer cells. *Oncogene* **23**, 4921–4929.

[34] Aoki Y, Feldman GM, and Tosato G (2003). Inhibition of STAT3 signaling induces apoptosis and decreases survivin expression in primary effusion lymphoma. *Blood* **101**, 1535–1542.

[35] Zhang T, Fields JZ, Ehrlich SM, and Boman BM (2004). The chemopreventive agent sulindac attenuates expression of the antiapoptotic protein survivin in colorectal carcinoma cells. *J Pharmacol Exp Ther* **308**, 434–437.

[36] Nikitakis NG, Siavash H, and Sauk JJ (2004). Targeting the STAT pathway in head and neck cancer: recent advances and future prospects. *Curr Cancer Drug Targets* **4**, 637–651.

[37] Selvendiran K, Koga H, Ueno T, Yoshida T, Maeyama M, Torimura T, Yano H, Kojiro M, and Sata M (2006). Luteolin promotes degradation in signal transducer and activator of transcription 3 in human hepatoma cells: an implication for the antitumor potential of flavonoids. *Cancer Res* **66**, 4826–4834.

[38] Mahboubi K, Li F, Plescia J, Kirkiles-Smith NC, Mesri M, Du Y, Carroll JM, Elias JA, Altieri DC, and Pober JS (2001). Interleukin-11 up-regulates survivin expression in endothelial cells through a signal transducer and activator of transcription-3 pathway. *Lab Invest* **81**, 327–334.

[39] Gao LF, Wen LJ, Yu H, Zhang L, Meng Y, Shao YT, Xu DQ, and Zhao XJ (2006). Knockdown of Stat3 expression using RNAi inhibits growth of laryngeal tumors *in vivo*. *Acta Pharmacol Sin* **27**, 347–352.

[40] Teh SH, Hill AK, Foley DA, McDermott EW, O'Higgins NJ, and Young LS (2004). COX inhibitors modulate bFGF-induced cell survival in MCF-7 breast cancer cells. *J Cell Biochem* **91**, 796–807.

[41] Krysan K, Merchant FH, Zhu L, Dohadwala M, Luo J, Lin Y, Heuze-Vourc'h N, Pold M, Seligson D, Chia D, et al. (2004). COX-2–dependent stabilization of survivin in non–small cell lung cancer. *FASEB J* **18**, 206–208.

[42] Kobayashi M, Nakamura S, Shibata K, Sahara N, Shigeno K, Shinjo K, Naito K, and Ohnishi K (2005). Etodolac inhibits EBER expression and induces *Bcl-2*–regulated apoptosis in Burkitt's lymphoma cells. *Eur J Haematol* **75**, 212–220.

[43] Gritsko T, Williams A, Turkson J, Kaneko S, Bowman T, Huang M, Nam S, Eweis I, Diaz N, Sullivan D, et al. (2006). Persistent activation of stat3 signaling induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells. *Clin Cancer Res* **12**, 11–19.

[44] Dong Y, Sui L, Watanabe Y, Sugimoto K, and Tokuda M (2002). Survivin expression in laryngeal squamous cell carcinomas and its prognostic implications. *Anticancer Res* **22**, 2377–2383.

[45] Lo Muzio L, Pannone G, Leonardi R, Staibano S, Mignogna MD, De Rosa G, Kudo Y, Takata T, and Altieri DC (2003). Survivin, a potential early predictor of tumor progression in the oral mucosa. *J Dental Res* **82**, 923–928.

[46] Kohli M, Yu J, Seaman C, Bardelli A, Kinzler KW, Vogelstein B, Lengauer C, and Zhang L (2004). SMAC/Diablo–dependent apoptosis induced by nonsteroidal antiinflammatory drugs (NSAIDs) in colon cancer cells. *Proc Natl Acad Sci USA* **101**, 16897–16902.

[47] Reed JC (2001). The survivin saga goes *in-vivo*. *J Clin Invest* **108**, 965–969.

[48] Flis S, Soltysiak-Pawluczuk D, Jedrych A, Jastrzebski Z, Remiszewska M, and Splawinski J (2006). Antiangiogenic effect of sulindac sulfide could be secondary to induction of apoptosis and cell cycle arrest. *Anticancer Res* **26**, 3033–3041.

[49] Wu YL, Fu SL, Zhang YP, Qiao MM, and Chen Y (2005). Cyclooxygenase-2 inhibitors suppress angiogenesis and growth of gastric cancer xenografts. *Biomed Pharmacother* **59**, S289–S292.

[50] Skopinska-Rozewska E, Piazza GA, Sommer E, Pamukcu R, Barcz E, Filewska M, Kupis W, Caban R, Rudzinski P, Bogdan J, et al. (1998). Inhibition of angiogenesis by sulindac and its sulfone metabolite (FGN-1): a potential mechanism for their antineoplastic properties. *Int J Tissue React* **20**, 85–89.