



# NOSH-sulindac (AVT-18A) is a novel nitric oxide- and hydrogen sulfide-releasing hybrid that is gastrointestinal safe and has potent anti-inflammatory, analgesic, antipyretic, anti-platelet, and anti-cancer properties

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## ABSTRACT

Sulindac is chemopreventive and has utility in patients with familial adenomatous polyposis; however, side effects preclude its long-term use. NOSH-sulindac (AVT-18A) releases nitric oxide and hydrogen sulfide, was designed to be a safer alternative. Here we compare the gastrointestinal safety, anti-inflammatory, analgesic, anti-pyretic, anti-platelet, and anti-cancer properties of sulindac and NOSH-sulindac administered orally to rats at equimolar doses. *Gastrointestinal safety*: 6 h post-administration, number/size of hemorrhagic lesions in stomachs were counted. Tissue samples were frozen for PGE<sub>2</sub>, SOD, and MDA determination. *Anti-inflammatory*: 1 h after drug administration, the volume of carrageenan-induced rat paw edemas was measured for 5 h. *Anti-pyretic*: fever was induced by LPS (*ip*) an hour before administration of the test drugs, core body temperature was measured hourly for 5 h. *Analgesic*: time-dependent analgesic effects were evaluated by carrageenan-induced hyperalgesia. *Anti-platelet*: anti-aggregatory effects were studied on collagen-induced platelet aggregation of human platelet-rich plasma. *Anti-cancer*: We examined the effects of NOSH-sulindac on the growth properties of 12 human cancer cell lines of six different tissue origins. Both agents reduced PGE<sub>2</sub> levels in stomach tissue; however, NOSH-sulindac did not cause any stomach ulcers, whereas sulindac caused significant bleeding. Lipid peroxidation induced by sulindac was higher than that from NOSH-sulindac. SOD activity was significantly lowered by sulindac but increased by NOSH-sulindac. Both agents showed similar anti-inflammatory, analgesic, anti-pyretic, and anti-platelet activities. Sulindac increased plasma TNF $\alpha$  whereas this rise was lower in the NOSH-sulindac-treated animals. NOSH-sulindac inhibited the growth of all cancer cell lines studied, with potencies of 1000- to 9000-fold greater than that of sulindac. NOSH-sulindac inhibited cell proliferation, induced apoptosis, and caused G<sub>2</sub>/M cell cycle block. These results demonstrate that NOSH-sulindac is gastrointestinal safe, and maintains the anti-inflammatory, analgesic, antipyretic, and antiplatelet properties of its parent compound sulindac, with anti-growth activity against a wide variety of human cancer cells.

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## 1. Introduction

The use of anti-inflammatory drugs (NSAIDs) in cancer

*Abbreviations*: NO, nitric oxide; H<sub>2</sub>S, hydrogen sulfide; NOSH, nitric oxide-hydrogen sulfide; NSAIDs, nonsteroidal anti-inflammatory drugs; SUL, sulindac; LPS, Lipopolysaccharide; PGE<sub>2</sub>, Prostaglandin E<sub>2</sub>; MDA, malondialdehyde; SOD, Super-oxide dismutase

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prevention is based on the recognition that inflammation is central to the carcinogenesis process [1]. There is considerable body of evidence suggesting that the long-term use of NSAIDs is associated with a significant reduction in many forms of cancers including, colon [2–5], breast [6–8], pancreas [9], bladder [10,11], head and neck [12], esophageal [13], ovarian [14,15], prostate [16], hepatocellular [17], and skin [18–20]. Of these, cellular and molecular mechanisms of colorectal cancer (CRC), which in many ways represent the prototypical case for cancer prevention, have been studied most extensively. From all accumulated data, what has become abundantly clear is that although NSAIDs are chemopreventive, they reduce the risk of, and mortality from, CRC by about

half [21]. Furthermore, long-term use of NSAIDs may lead to significant side effects, mainly gastrointestinal, cardiovascular, and renal which obviously limits their use [1].

Familial adenomatous polyposis (FAP) is an autosomal dominant disease caused by a mutation in the adenomatous polyposis coli (APC) gene [22] which is characterized by hundreds of colorectal adenomatous polyps that eventually progress to CRC. Almost all FAP patients will develop CRC if they are not identified and treated at an early stage. Generally, management of patients with FAP is with a total colectomy [23]. Therefore, chemoprevention in this setting is of paramount importance. Sulindac (SUL) belongs to the indene class of NSAIDs [24] that has extensively been studied and utilized as a chemopreventive agents in patients with FAP [25–28]. However, a limiting factor in the long term use of SUL is its toxicity, that can affect up to 20% of patients [24]. In our efforts on improving the safety profile of SUL, we developed NOSH-sulindac (NOSH-SUL), a hybrid molecule capable of releasing nitric oxide (NO) and hydrogen sulfide (H<sub>2</sub>S), two gasotransmitters of physiological significance [29]. Our rationale for developing NOSH-SUL was based on the observations that NO [30] and H<sub>2</sub>S [31] have some of the same properties as prostaglandins within the gastric mucosa, thus modulating some components of the mucosal defense systems. In the present study, we carried out a head-to-head comparison of the gastrointestinal safety, anti-inflammatory, analgesic, anti-pyretic, and anti-platelet, properties of SUL with those of NOSH-SUL. We also evaluated the effects of SUL and NOSH-SUL in 12 different cancer cell lines of 6 different tissue origins and on cell kinetics using a human colon cancer cell line.

## 2. Materials and methods

### 2.1. Chemicals

NOSH-SUL (AVT-18A), (Z)-4-(3-thioxo-3H-1,2-dithiol-5-yl) phenyl 5-(2-(5-fluoro-2-methyl-1-(4-(methylsulfinyl) benzylidene)-1H-inden-3-yl) acetoxy)-2-((4-(nitrooxy) butanoyl)oxy) benzoate was synthesized as described previously [29] and was a gift from Avicenna Pharmaceuticals Inc, (New York, NY). The structural components of the NOSH-SUL are shown in Fig. 1. Lipopolysaccharide (LPS) from *Escherichia coli*, SUL, and carrageenan were purchased from Sigma (St. Louis, MO, USA). Kits used for determination of PGE<sub>2</sub>, lipid peroxidation, and superoxide dismutase, were from Cayman Chemical (Ann Arbor, MI).

### 2.2. Experimental groups and treatments

In all the protocols described below, we used at least 5 male Wistar rats per group that weighed 180–200 g. The rats were obtained from Charles River Laboratories International (Wilmington,

MA) and were fed standard laboratory chow and water. All experimental procedures were approved by our institutional animal research committees and were performed in accordance with nationally approved guidelines for the treatment of laboratory animals.

### 2.3. Determination of ulcer index

Rats were fasted for 48 h with free access to drinking water. SUL and NOSH-SUL at equimolar concentrations, 200 mg/kg and 467 mg/kg respectively, were administered orally by gavage suspended in 1% carboxymethylcellulose (CMC) solution. Six hours post-administration, animals were euthanized in a CO<sub>2</sub> chamber; stomachs were then removed immediately, cut along the greatest curvature, and rinsed with ice-cold distilled water. The ulcer index (UI) was determined as described by Best et al. [32]. Briefly, the number and the length of ulcers observed in each stomach were determined using a magnifying lens. Using the following scoring module, the severity of each gastric lesion was measured along its greatest length with 1 mm = rating of 1, 1–2 mm = rating of 2, and > 2 mm = rating according to the measured length in mm. The “ulcer index” (UI) was then calculated by adding the total number of lengths (L, mm) in each stomach and then dividing the total by the total number of rats in each group (N=5): UI = (L1+L2+L3+L4+L5)/5.

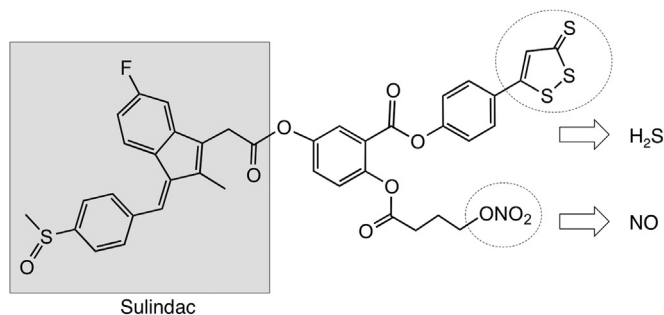
The excised tissues from each of the stomachs were processed for measurement of Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), malondialdehyde (MDA) and Superoxide dismutase (SOD) activity. Blood samples from each of the rats were taken by cardiac puncture into heparin-containing vials and used for determination of plasma TNF $\alpha$ .

### 2.4. Measurement of PGE<sub>2</sub> levels

One gram of tissue from each stomach was placed in a test tube containing 5 mL of 0.1 M phosphate buffer (pH7.4), 1 mM EDTA, and 10  $\mu$ M indomethacin. After homogenization, the homogenate was centrifuged at 10,000  $\times$  g for 10 min at 4  $^{\circ}$ C. PGE<sub>2</sub> content in the supernatant was determined in duplicate by an enzyme immunoassay kit following the protocol described by the manufacturer (Cayman Chemical, Ann Arbor, MI). Briefly, standard (50  $\mu$ L) or homogenate (50  $\mu$ L), enzymatic tracer (50  $\mu$ L) and specific antiserum (50  $\mu$ L) were mixed. After overnight incubation at 4  $^{\circ}$ C, the plates were washed with wash buffer and Ellman reagent (200  $\mu$ L) was added into each well. The absorbance at 412 nm was measured after 1 h incubation at room temperature. Results are expressed as pg of PGE<sub>2</sub> per mg of protein. Proteins were determined by Biorad assay.

### 2.5. Determination of Malondialdehyde (MDA) levels as index of lipid peroxidation

Snap frozen stomach tissue (25 mg) was sonicated for 15 s at 40 V over ice with 250  $\mu$ L of radioimmunoprecipitation (RIPA) buffer (25 mM TrisHCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with PMSF (phenylmethylsulphonyl fluoride). Homogenates were centrifuged for 10 min at 200  $\times$  g at 4  $^{\circ}$ C. Thiobarbituric acid reactant substances (TBARS) was measured in the supernatant using a kit from Cayman Chemical (Ann Arbor, MI) as described by the manufacturer. Briefly, reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) at high temperature (90–100  $^{\circ}$ C) in acidic conditions produced an adduct with a chromophore which absorbed visible light at 530–540 nm. Results are expressed as picomoles of malondialdehyde per gram protein.



**Fig. 1.** Structural components of NOSH-sulindac. The parent compound sulindac is shown in the shaded box. The parts of the molecule that releases NO and H<sub>2</sub>S are shown in the dotted ellipses.

## 2.6. Superoxide dismutase (SOD) activity

SOD activity in the gastric mucosa was assayed using a colorimetric kit from Cayman Chemical, (Ann Arbor, MI). Mucosal tissue (1 g) was homogenized with 5 mL of 20 mM HEPES buffer (pH 7.2) containing 1 mM EGTA and 300 mM of sucrose solution. Homogenates were centrifuged at  $200 \times g$  for 10 min at 4 °C. SOD activity of the supernatants were measured spectrophotometrically at 460 nm. As indicated in Cayman's SOD assay kit, "this procedure utilizes a tetazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine". SOD activity is expressed as the amount of the SOD standard showing activity equivalent to the determined activity. The results are expressed as units (U) of SOD activity/mg protein. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

## 2.7. Determination of plasma TNF- $\alpha$

Plasma TNF- $\alpha$  was measured using an enzyme immunoassay kit from R&D systems (Minneapolis, MN) following the protocol described by the manufacturer. Briefly, each sample (50  $\mu$ L) was incubated with antibodies specific for rat TNF- $\alpha$  and washed three times with assay buffer. An enzyme-linked polyclonal antibody specific for rat TNF- $\alpha$  conjugated to horseradish peroxidase was then added. Following washing of unbound antibody-enzyme reagent, substrate solution containing tetramethylbenzidine (TMB), plus hydrogen peroxide was then added. The enzyme reaction produced a blue product (oxidized TMB) that turned yellow when dilute hydrochloric acid (stop solution) was added. Color intensity was determined at 450 nm using a standard ELISA plate reader. Results are expressed as pg/mL.

## 2.8. Anti-pyretic activity

Fever was induced in animals as described previously [33]. Briefly, LPS (50  $\mu$ g/kg, Sigma, St. Louis, MO, USA) was administered intra-peritoneally to the animals an hour before the administration of SUL or NOSH-SUL at equimolar doses, 200 mg/kg and 467 mg/kg respectively, given orally by gavage suspended in 1% CMC. Rectal temperature was measured by inserting a lubricated thermistor probe (external diameter: 3 mm) 2.8 cm into the rectum of the animal. The probe was linked to a digital reader, which displayed the temperature at the tip of the probe ( $\pm 0.1$  °C). The values displayed were manually recorded. Rectal temperatures were taken every hour for 5 h.

## 2.9. Anti-inflammatory activity

Carrageenan (1%, 100  $\mu$ L, suspended in sterile saline solution) was subcutaneously injected into the plantar surface of the right hind paw in rat following the protocol described by Winter et al. [34]. Paw volume was measured using a water displacement plethysmometer (Model 520, IITC/Life Sciences Instruments, Woodland Hills, CA) before carrageenan injection and thereafter at 1 h intervals for 5 h. The paw volume measured just prior to carrageenan injection was used as the control volume. Data are expressed as the change in paw volume (mL) at each time point. At the end of the experiment, rats were euthanized by asphyxiation in a CO<sub>2</sub> chamber. After cutting each hind paw at the level of the calcaneus bone, exudates (oedema fluid) were collected and processed for measurement of PGE<sub>2</sub>, as described in Section 2.4.

## 2.10. Analgesic activity

Hindpaw inflammation was produced by intraplantar injection

of carrageenan (100  $\mu$ L of 1% carrageenan in sterile saline solution) into the right paw. SUL or NOSH-SUL at equimolar concentrations, 200 mg/kg and 467 mg/kg respectively, were administered orally by gavage suspended in 0.5% CMC 1 h after carrageenan injection, and the mechanical nociceptive threshold was determined 30 min after this and thereafter every 1 h for up to 5 h. The paw hyperalgesia was measured with an electronic pressure-meter. Each hindpaw was positioned in turn under a conical probe surface (tip radius approximately 1 mm) and gradually increasing pressure applied to the hindpaw surface until the animal vocalized at which point the measurement was terminated. Mechanical nociceptive threshold for both the injected and contralateral (i.e. non-injected) hindpaw were determined. The animals were tested before and after treatments and the results are expressed by the delta reaction force (g).

## 2.11. Inhibition of human platelet aggregation in vitro

Anti-aggregatory effects of SUL and NOSH-SUL were evaluated on collagen-induced platelet aggregation of human platelet-rich plasma (PRP). The collagen-induced aggregation occurs through a pathway dependent upon the arachidonic acid cascade [35]. Venous blood samples from healthy volunteers who had not taken any drugs for at least 2 weeks were used to prepare PRP by centrifugation of citrated blood at 200g for 20 min. Aliquots (500  $\mu$ L) of PRP were added into aggregometer cuvettes, and aggregation was recorded as increased light transmission under continuous stirring (1000 rpm) at 37 °C for 10 min after the addition of the stimulus. Collagen at submaximal concentrations (1.0  $\mu$ g/mL) was used as the platelet activator. Sulindac and NOSH-SUL at various concentrations were preincubated with PRP 10 min before the addition of collagen. Vehicle alone (0.5% DMSO) added to PRP did not affect platelet function in control samples. The anti-aggregatory activity of the two compounds was determined as percent inhibition of platelet aggregation compared to control samples. IC<sub>50</sub> values were calculated by nonlinear regression analysis.

## 2.12. Measurement of COX enzyme activity

NOSH-SUL was compared to SUL for its ability to inhibit COX-1 and COX-2 enzyme activities in vitro as described previously [36] using a colorimetric COX (ovine, o-COX) inhibitor screening kit from Cayman Chemicals (Ann Arbor, MI).

## 2.13. Cell culture and MTT assay

Human colon adenocarcinoma (HT-29, SW-480 and HCT-15), human breast cancer (MDA-MB 231, SK-BR-3 and MCF-7), human pancreatic cancer (MIA PaCa-2 and BxPC-3), human lung cancer (A549 and H383), human prostate cancer (LNCAP), and human leukemia (Jurkat T) cells were obtained from American Type Tissue Collection (Manassas, VA). All cells lines were grown as monolayers except for the Jurkat T cells which was grown as suspension culture. The pancreatic and breast cancer cells were grown in Dulbecco's modified Eagle's medium, the prostate, Jurkat, SW-480 and HCT-15 colon cells were grown in RPMI 1640 medium, the lung cells were grown in F-12 and the colon HT-29 cells were grown in McCoy 5A. All media were supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA) penicillin (50 U/mL), and streptomycin (50  $\mu$ g/mL) (Invitrogen, Carlsbad, CA). Cells were incubated at 37 °C in 5% CO<sub>2</sub> and 90% relative humidity. Single cell suspensions were obtained by trypsinization (0.05% trypsin/EDTA), and cells were counted using a hemacytometer. The final DMSO concentration was adjusted in all media to 1%. Viability was determined by the trypan blue dye exclusion method.

Cell growth inhibitory effect of SUL and NOSH-SUL was

measured using a colorimetric MTT assay kit (Roche, Indianapolis, IN). Cancer cells were plated in 96-well plates at a density of 25,000 cells/well. The cells were incubated for 24 h with different concentrations of SUL and NOSH-SUL after which 10  $\mu$ L of MTT dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, 5 mg/mL in phosphate buffered saline), was added to each well, and the plates were incubated for 2 h at 37 °C. Then, the media was aspirated, and 100  $\mu$ L of the solubilization solution (10% SDS in 0.01 M HCl) was added to each well. The absorbance was measured on a spectrophotometric plate reader at a wavelength of 570 nm.

#### 2.14. Cell proliferation

PCNA antigen expression was determined using an ELISA Kit (Calbiochem, La Jolla, CA), following the manufacturers protocol. HT-29 cells ( $1 \times 10^6$  cells/mL) were incubated with serum-free media for 24 h to remove the effect of endogenous growth factors; they were then treated for 24 h with various concentrations of NOSH-SUL or vehicle as previously reported [37].

#### 2.15. Cell cycle analysis

Cell cycle phase distributions of control and treated HT-29 cells were obtained using a Coulter Profile XL equipped with a single argon ion laser. For each subset, > 10,000 events were analyzed. All parameters were collected in list mode files. Data were analyzed on a Coulter XL Elite Work station using the Software programs Multigraph<sup>TM</sup> and Multicycle<sup>TM</sup>. HT-29 Cells ( $0.5 \times 10^6$ ) treated with various concentrations of NOSH-SUL or vehicle were fixed in 100% methanol for 10 min at  $-20$  °C, pelleted (5000 rpm  $\times$  10 min at 4 °C), resuspended and incubated in PBS containing 1% FBS/0.5% NP-40 on ice for 5 min. Cells were washed again in 500  $\mu$ L of PBS/1% FBS containing 40  $\mu$ g/mL propidium iodide (used to stain for DNA) and 200 mg/mL RNase type IIA, and analyzed within 30 min by flow cytometry. The percentage of cells in G<sub>0</sub>/G<sub>1</sub>, G<sub>2</sub>/M, and S phases was determined from DNA content histograms as reported previously [37].

#### 2.16. Assay for apoptosis

HT-29 cells ( $0.5 \times 10^6$  cells/mL) were treated for 24 h with various concentrations of NOSH-SUL or vehicle. Cells were washed with and resuspended in  $1 \times$  Binding Buffer (Annexin V binding

buffer, 0.1 M HEPES/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl<sub>2</sub>; BD BioSciences Pharmingen, San Diego, CA). Then 5 mL of Annexin V-FITC (final concentration 0.5 mg/mL) was added followed by propidium iodide as a counterstain (final concentration 20 mg/mL). The cells were then incubated at room temperature for 15 min in the dark. Finally, the cells were transferred to FACS tubes for analysis. Percentage of apoptotic cells were obtained using a Becton Dickinson LSR II equipped with a single argon ion laser. For each subset, about 10,000 events were analyzed. All parameters were collected in list mode files. Data was analyzed by Flow Jo software as reported previously [37].

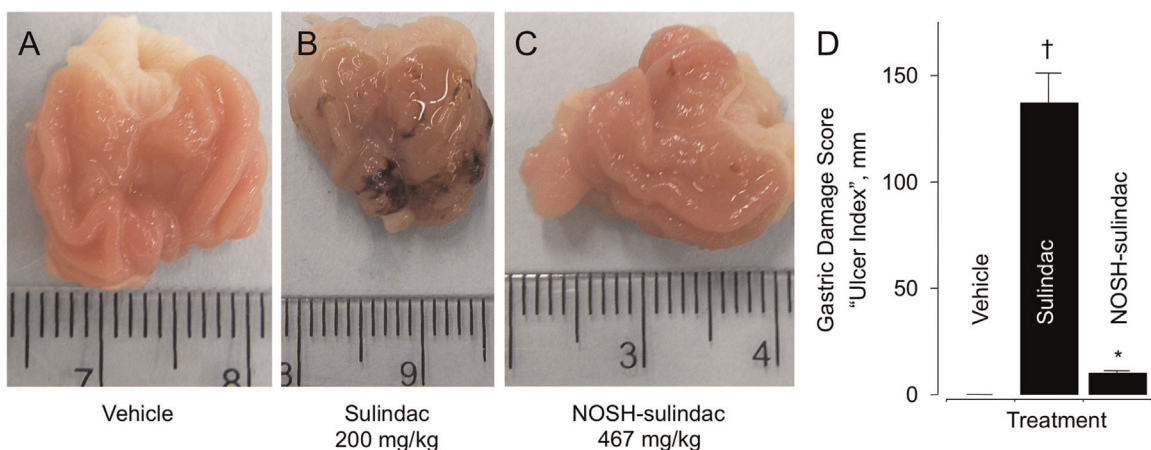
#### 2.17. Statistical analysis

In vivo treatment groups and number of animals in each group are indicated in the figure legend. In vitro data are presented as mean  $\pm$  SEM for at least three different sets of plates done in triplicate. Comparisons between groups were performed using a one-way analysis of variance followed by the Student-*t* test.  $P < 0.05$  was regarded as statistically significant.

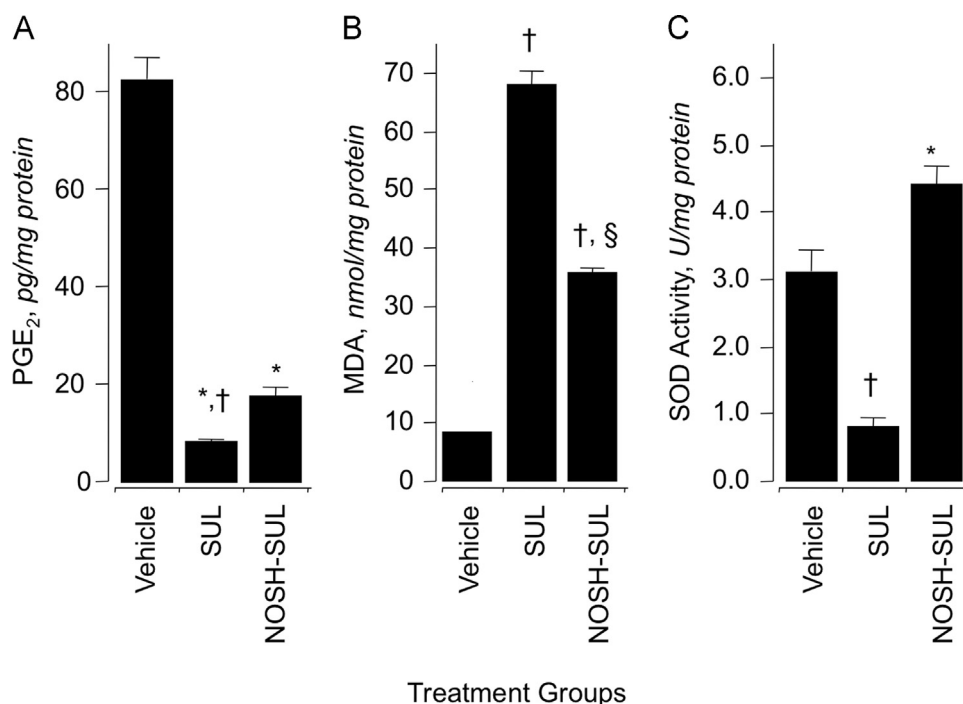
### 3. Results and discussion

#### 3.1. NOSH-SUL is gastrointestinal safe

The rats receiving the vehicle (0.5% CMC solution) had a normal glandular region on the surface of their stomach, and no ulcerative damage (Fig. 2A and D). For these rats, the gastric damage score (also described in the literature as “ulcer index”, or UI), was zero (UI=0). Administration of SUL (200 mg/kg) resulted in extensive mucosal injury, UI=130 (Fig. 2B and D). NOSH-SUL (476 mg/kg) did not produce significant ulcerative damage (Fig. 2C and D), UI=10 compared to SUL at equimolar doses, which represents a remarkable reduction ( $P < 0.01$ ) in gastrointestinal toxicity. Thus, this modified sulindac which has been shown to release NO and H<sub>2</sub>S [29] appears to be gastrointestinal safe. As alluded to in the introduction, SUL has extensively been utilized as a chemopreventive agent in patients with FAP [25–28]. However, a limiting factor in its long-term use is its GI toxicity. Based on the data presented here, NOSH-SUL would be an ideal drug candidate for development in such a setting.



**Fig. 2.** NOSH-sulindac is gastrointestinal safe. SUL and NOSH-SUL were administered orally at equimolar doses (0.56 mmol/kg; 200 mg/kg and 467 mg/kg for SUL and NOSH-SUL, respectively) and effects on the stomach were evaluated as indicated in Section 2.3. Panel A, shows the stomach of a vehicle-treated rat; Panel B, stomach of a SUL-treated rat showing ulceration and bleeding; Panel C, stomach of a NOSH-SUL-treated rat which is essentially devoid of ulcers. Panel D, gastric damage due to SUL, UI=135  $\pm$  15 mm ( $^{\dagger}P < 0.01$  compared to vehicle), NOSH-SUL was gastric damage-sparing, UI=10  $\pm$  1 mm ( $^*P < 0.01$  compared to SUL). Photographs in Panels A–C are representative from 5 rats in each group. Results in Panel D are mean  $\pm$  SEM of 5 rats in each group.



**Fig. 3.** Effects of sulindac and NOSH-sulindac on gastric PGE<sub>2</sub> levels, lipid peroxidation (MDA) and superoxide dismutase (SOD). At the end of the gastrointestinal safety evaluations as described in Section 2.3, tissues from the excised stomachs of each rat was snap frozen in liquid nitrogen and processed as described in Sections 2.4–2.6. SUL and NOSH-SUL caused a significant reduction in gastric mucosal PGE<sub>2</sub> levels (panel A). Results are mean  $\pm$  SEM of 5 rats in each group,  $^{\dagger}P < 0.05$  vs vehicle group,  $^{\ddagger}P < 0.05$  vs NOSH-SUL group. SUL caused an almost 9-fold increase in MDA levels, for NOSH-SUL-treated rats, MDA levels were about 2-fold higher (panel B). Results are mean  $\pm$  SEM for 5 rats in each group,  $^{\dagger}P < 0.01$  vs vehicle group,  $^{\S}P < 0.05$  vs SUL group. SUL caused a significant reduction in SOD activity, whereas NOSH-SUL did not have an effect (panel C). Results are mean  $\pm$  SEM of 5 rats in each group,  $^{\dagger}P < 0.05$  vs vehicle group,  $^{\ddagger}P < 0.01$  vs SUL group.

### 3.2. Gastric mucosal exudate prostaglandin E<sub>2</sub> content

We investigated the effect of SUL and NOSH-SUL on prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) content in gastric mucosa (Fig. 3A). Rats treated with SUL (200 mg/kg) produced about 88% less PGE<sub>2</sub> than rats in the control group. NOSH-SUL (467 mg/kg) also reduced PGE<sub>2</sub> levels but not to the same extent as SUL, the reduction being around 75% (Fig. 3A). Prostaglandins are the main product of cyclooxygenase-mediated arachidonic acid metabolism in gastric mucosa, therefore, comparison of PGE<sub>2</sub> content between control and drug-treated groups showed a clear and significant COX inhibition by both SUL and NOSH-SUL. In order to confirm that indeed COX enzyme activity was being inhibited, we evaluated the effects of these two compounds on ovine COX-1 and COX-2 enzymatic activity at their respective IC<sub>50</sub>s for cell growth inhibition, in HT-29 colon cancer cells (see Section 3.9). As shown in Table 1, NOSH-SUL at a concentration of 90 nM inhibited COX-1 enzymatic activity of more than that of COX-2, the respective values being 44  $\pm$  1% and 14  $\pm$  1%. SUL at 800  $\mu$ M inhibited COX-1 by 82  $\pm$  2% and COX-2 by 68  $\pm$  1%. Therefore, SUL at its IC<sub>50</sub>s for cell growth inhibition inhibits both COX-1 and COX-2 more than NOSH-SUL at IC<sub>50</sub>s for cell growth inhibition. Since NOSH-SUL is significantly

**Table 1**  
NOSH-SUL inhibits cyclooxygenase enzyme activity.

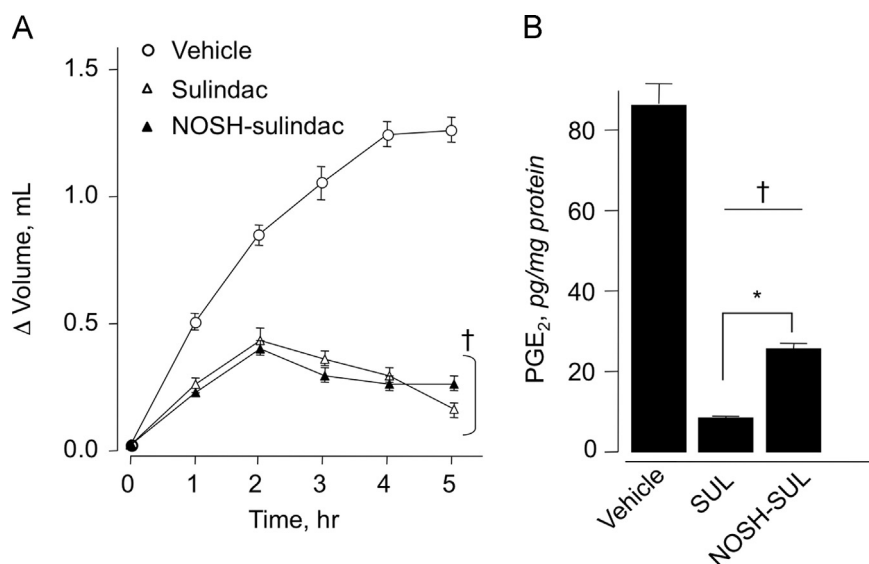
Treatment	COX-1 % Inhibition	COX-2 % Inhibition
SUL, 800 $\mu$ M	82 $\pm$ 2	68 $\pm$ 1
NOSH-SUL, 90 nM	44 $\pm$ 1	14 $\pm$ 1
Indomethacin, 1 $\mu$ M	74 $\pm$ 2	68 $\pm$ 1

Pure ovine COX enzymes were treated with SUL or NOSH-SUL at their respective IC<sub>50</sub>s for cell growth inhibition in HT-29 colon cancer cell line for 15 min at 4  $^{\circ}$ C after which *o*-COX-1 and *o*-COX-2 enzyme activity were determined. Results are mean  $\pm$  range of two independent studies performed in duplicate.

more potent than SUL, this strongly suggests that targets other than COX must be contributing to its mode of action. We also measured the degree of COX-1 and COX-2 inhibition by indomethacin (1  $\mu$ M) a nonselective COX inhibitor [38] as a reference compound in order to ensure that there were no anomalies with our assay system. Indomethacin inhibited COX-1 and COX-2 by 74  $\pm$  2% and 68  $\pm$  1%, respectively (Table 1).

### 3.3. Effect of NOSH-SUL on lipid peroxidation and superoxide dismutase activity

Measuring the concentration of MDA in intact mucosa 6 h post-administration of SUL and NOSH-SUL at 200 mg/kg and 476 mg/kg respectively was used to assess oxidative stress in gastric tissue. MDA levels were 8  $\pm$  1 nmol/mg protein in the vehicle treated rats (Fig. 3B), this was increased to 68  $\pm$  2 nmol/mg protein in the SUL treated rats but was significantly less in the NOSH-SUL treated animals, 32  $\pm$  1 nmol/mg protein, (Fig. 3B). Samples from the same gastric tissues were used to measure SOD activity. In the intact mucosa (control group) SOD activity was 3.2  $\pm$  0.3 U/mg protein. Following administration of SUL a significant decrease in SOD activity was observed (0.9  $\pm$  0.1 U/mg protein,  $^{\dagger}P < 0.05$  compared to vehicle). However, in the NOSH-SUL treated rats, SOD activity was significantly increased to 4.4  $\pm$  0.3 U/mg protein ( $^*P < 0.01$  compared to SUL, Fig. 3C). SOD is an antioxidative marker. Its activity was significantly lowered in the SUL-treated animals, this may explain the high levels of MDA and ulcerations observed in the stomachs. SOD activity was significantly higher in the NOSH-SUL-treated animals, which correlated with lower MDA levels and essentially no ulcerations to the stomachs. Thus, some if not all of the changes in the gastric mucosal tissue may be as the result of the antioxidative effects of NOSH-SUL.



**Fig. 4.** Anti-inflammatory properties of sulindac and NOSH-sulindac. Rat paw edema was induced by carrageenan injection as described in Section 2.9. SUL and NOSH-SUL were both equally effective in reducing paw volume at all time points (panel A). Results are mean  $\pm$  SEM of 5 rats in each group,  $^{\dagger}P < 0.05$  vs vehicle treated rats at all time points. SUL and NOSH-SUL also caused a significant reduction in PGE<sub>2</sub> levels in the paw exudate (panel B). Results are mean  $\pm$  SEM for 5 rats in each group,  $^{\dagger}P < 0.01$  vs vehicle,  $^*P < 0.05$  vs NOSH-SUL.

#### 3.4. Carrageenan-induced paw swelling

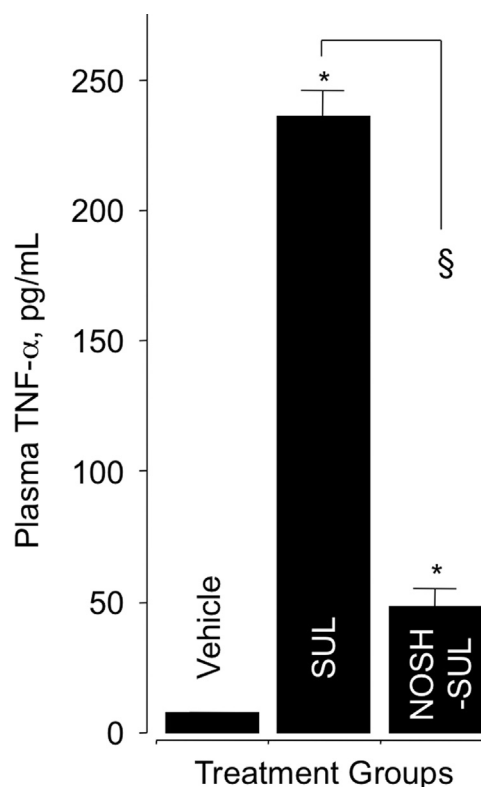
Sulindac is mainly used for treating inflammatory conditions. We therefore wanted to compare the COX-dependent anti-inflammatory activity of SUL to that of NOSH-SUL. For this, we used the carrageenan-induced edema model [34]. After inducing inflammation, animals receiving vehicle showed a fast time-dependent increase in paw volume ( $\Delta V = 0.5$  mL) within 1 h, and gradual increase to 1.3 mL over the course of the experiment (5 h) (Fig. 4A). In contrast, animals receiving SUL or NOSH-SUL showed a weak inflammatory response,  $\Delta V = 0.2$ – $0.3$  mL by 1 h, which peaked to  $\Delta V = 0.40$ – $0.45$  mL at 2 h and then decreased over the next 3 h (Fig. 4A). The anti-inflammatory effect registered in animals dosed with NOSH-SUL was comparable to those treated with SUL. Prostaglandins (PGE<sub>2</sub>) are the main products of cyclooxygenase-mediated arachidonic acid metabolism [1]. Comparison of PGE<sub>2</sub> content of paw exudates showed a clear and significant COX inhibition by SUL and NOSH-SUL (Fig. 4B). PGE<sub>2</sub> levels in control vehicle-treated rats were  $85 \pm 4$  pg mg<sup>-1</sup> and in the SUL and NOSH-SUL-treated rats it went down to  $9 \pm 1$  pg mg<sup>-1</sup> and  $24 \pm 2$  pg mg<sup>-1</sup>, respectively. This is equivalent to reduction of 89% and 72% by SUL and NOSH-SUL, respectively.

#### 3.5. Plasma TNF $\alpha$ levels

We determined the inhibitory effect of SUL and NOSH-SUL on the proinflammatory cytokine tumor necrosis factor- $\alpha$  in plasma obtained from control and drug-treated animals at the end of the gastrointestinal safety experiments, 6 h post-administration (Section 2.3). Administration of SUL (200 mg/kg, 0.56 mmol/kg) increased TNF $\alpha$  concentration by about 25-fold ( $9.5 \pm 0.3$  control and  $230 \pm 5$  pg/mL SUL). However, this rise was considerably lower in the NOSH-SUL-treated (476 mg/kg, 0.56 mmol/kg) animals,  $50 \pm 3$  pg/mL (Fig. 5).

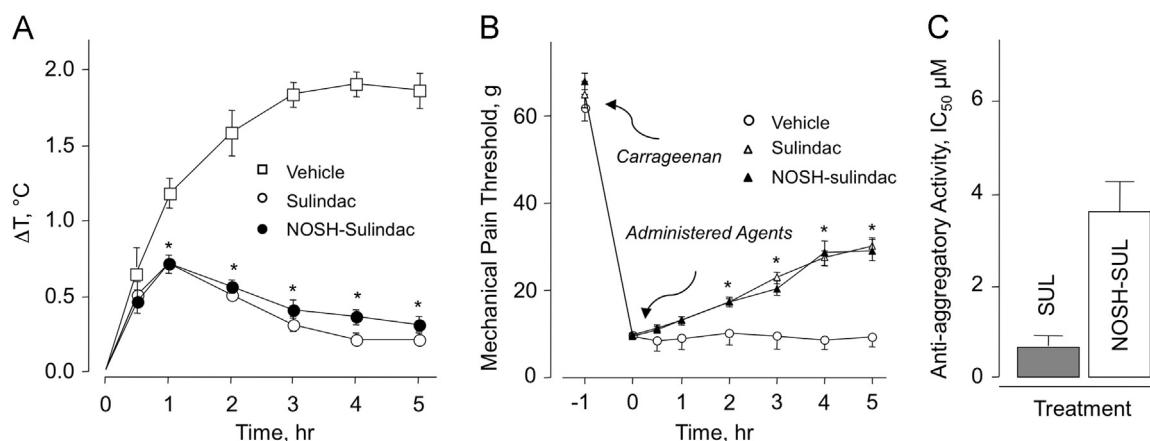
#### 3.6. Antipyretic activity

It is well known that NSAIDs exert a moderate antipyretic effect when administered orally; although SUL is seldom used for that purpose. Nevertheless for comparison considerations, we wanted to determine the decrease in body temperature induced by NOSH-



**Fig. 5.** Effect of sulindac and NOSH-sulindac on plasma TNF- $\alpha$ . At the end of the gastrointestinal safety evaluations as described in Section 2.3, blood was drawn and processed as described in Section 2.7 for determination of plasma TNF- $\alpha$ . SUL caused a significant rise in plasma TNF- $\alpha$ , however, this rise was significantly less in the NOSH-SUL-treated rats. Results are mean  $\pm$  SEM for 5 rats in each group,  $^*P < 0.001$  vs vehicle,  $^{\S}P < 0.01$  vs SUL.

SUL compared to that obtained with SUL. Experimental drugs, SUL and NOSH-SUL were administered orally at equimolar doses (0.56 mmol/kg; 200 mg/kg and 467 mg/kg for SUL and NOSH-SUL, respectively) 30 min before injecting the rats with LPS (50  $\mu$ g/kg ip). In this regard, control animals showed a time-dependent increase in body temperature which leveled off between 3 and 4 h



**Fig. 6.** Sulindac and NOSH-sulindac reduce LPS-induced fever, raise the threshold for hyperalgesia, and show anti-platelet activity. Panel A: LPS (50 μg/kg, ip) was administered to the rats one hour before administration of SUL or NOSH-SUL at equimolar doses (0.56 mmol/kg; 200 mg/kg and 467 mg/kg for SUL and NOSH-SUL, respectively). Core body temperature was recorded at 30 min and thereafter hourly for 5 h. Results are mean ± SEM for 5 rats in each group, \**P* < 0.01 vs vehicle for both SUL and NOSH-SUL from 1–5 h. Panel B: Mechanical pain threshold was increased in a time-dependent manner by SUL and NOSH-SUL. Results are mean ± SEM for 5 rats in each group. \**P* < 0.05 vs vehicle for SUL and NOSH-SUL from 2–5 h. Panel C: SUL and NOSH-SUL were equally effective in inhibiting human platelet aggregation. Results are mean ± range for two individuals.

with  $\Delta T = 1.8$  °C and this was maintained until the end of the screen (5 h). However, SUL and NOSH-SUL-treated animals showed only about a half-degree increase in body temperature at 30 min after LPS injection, this increased to  $\Delta T = 0.7$  °C by 1 h thereafter gradually decreased (Fig. 6A).

### 3.7. Carrageenan-induced mechanical hyperalgesia

This assay measures the ability of the test drugs to decreased threshold to a painful stimuli produced by injection of carrageenan onto the plantar surface of the right hind paw. The mechanical pain threshold was increased upon time by administering of SUL and NOSH-SUL (Fig. 6B). Pain threshold was markedly reduced from 65 g to about 10 g in animals receiving vehicle (control group), indicating a higher sensitivity to mechanical stimuli (non-painful at normal conditions). Hyperalgesia was decreased in animals receiving SUL and NOSH-SUL to the same extent, about 32 g or ~50% reduction compared to the initial response. Another NO- and H<sub>2</sub>S-releasing NSAID, NOSH-aspirin (NBS 11–20) was recently shown to have greater potency than aspirin in reducing inflammatory pain in several clinically relevant models [39]. The enhanced antinociceptive effect of NOSH-aspirin appeared to be due to its ability to reduce the production of pronociceptive cytokines such as IL-1β. NOSH-aspirin was also shown to reduce hyperalgesia, caused by a directly acting hyperalgesic mediator in a mechanism dependent on modulation of K<sub>ATP</sub> channels. The latter effect is presumably due to the released H<sub>2</sub>S as this gaso-transmitter in known to affect [1,40].

### 3.8. Platelet anti-aggregatory activity

Sulindac is not used as an anti-aggregatory agent whereas aspirin is frequently employed for this purpose. This is primarily because aspirin is an irreversible inhibitor of COX-1 whereas SUL is not [24]. Nevertheless, since SUL does inhibit COX-1 we wanted to compare the anti-aggregatory effects of NOSH-SUL to that of SUL for complete characterization of these two compounds. We used collagen-induced platelet aggregation of human platelet-rich plasma (PRP) for the comparison. The results expressed as IC<sub>50</sub>s are shown in Fig. 6C. Analysis of the data does not show any statistical differences between SUL and NOSH-SUL. It should be noted that NOSH-SUL releases NO and H<sub>2</sub>S [29] both of which can have independent anti-platelet properties [41–43].

### 3.9. NOSH-SUL inhibits the growth of various human cancer cell lines

We investigated the effects of SUL and NOSH-SUL on the growth properties of 12 different cancer cell lines of six different histological subtypes. The cell lines were that of colon (HT-29: COX-1 and COX-2 positive, HCT 15: COX null, and SW480: COX-1 positive, low levels of endogenous COX-2), breast (MCF7: [ER(+)], MDA MB-231 and SKBR3: [ER(-)]); pancreatic (BxPC3: both COX-1 and COX-2 positive, MIAPaCa-2: COX-null), lung (A549, H383), prostate (LNCaP), and T-cell leukemia (Jurkat). NOSH-SUL was extremely effective in inhibiting the growth of these cell lines (Table 2). The IC<sub>50</sub>s for cell growth inhibition at 24 h for NOSH-SUL ranged from 0.09 ± 0.01 to 0.32 ± 0.03 μM and that for SUL was 212 ± 37 to 935 ± 35 μM. The growth inhibition by NOSH-SUL versus SUL was very high in the panel of cancer cell lines studied. In a fold comparison study of the IC<sub>50</sub> values (SUL/NOSH-SUL), NOSH-SUL was at least 1000-fold to 9000-fold more potent than SUL in various cell lines (Table 2). Such fold increases imply that the NO and H<sub>2</sub>S-related structural modifications of the SUL molecule imparts a differential enhancement in potency. Furthermore, our data strongly suggests that this effect may be tissue-type independent since NOSH-SUL was effective against adenomatous, epithelial, and lymphocytic cancer cell lines. Here we studied 12 cell lines originating from six different tissues, therefore, it may be envisaged that our findings are part of a generalized effect. An interesting aspect of growth inhibition also emerges with respect to COX expression in the cell lines examined. NOSH-SUL showed similar effects on two colon cancer cell lines, HT-29 (expresses COX-1 and COX-2) and HCT 15 (no COX expression) [44] and on two pancreatic cancer cell lines, BxPC-3 (expresses COXs) and MIA PaCa-2 (no COX expression) [45] suggesting a COX-independent effect.

Currently we cannot explain the underlying mechanism(s) for the enhanced potency of NOSH-SUL observed in these studies. We do not yet know anything about the kinetics of NO and H<sub>2</sub>S release and their potential interactions. However, we do know that both contribute towards the potency of the intact molecule. This is based on our earlier observations where we showed that the biological activity of aspirin plus SNAP (S-Nitroso-N-acetylpenicillamine, which releases NO) plus ADT-OH (5-(4-hydroxyphenyl)-3H-1, 2-dithiole-3-thione, which releases H<sub>2</sub>S) was not the same as the biological activity of the intact NOSH-aspirin molecule [46].

**Table 2**  
IC<sub>50</sub> (μM) values at 24 h for cell growth inhibition in different cancer cell lines.

Agent	Colon		Breast		Pancreas		Lung		Prostate		Leukemia	
	HT-29	HTC 15	SW480	MDA MB 231	SKBR3	MCF-7	MIAPaCa2	BxPC3	A549	H383	LNCAP	Jurkat
SUL	835 ± 75	850 ± 55	710 ± 65	935 ± 35	845 ± 55	975 ± 80	792 ± 68	960 ± 58	212 ± 37	532 ± 38	810 ± 80	650 ± 45
NOSH-SUL	0.095 ± 0.01	0.11 ± 0.01	0.11 ± 0.009	0.098 ± 0.017	0.12 ± 0.01	0.10 ± 0.007	0.098 ± 0.018	0.12 ± 0.01	0.18 ± 0.015	0.088 ± 0.01	0.09 ± 0.01	0.32 ± 0.03
Enhanced Potency	~8700	~7700	~6400	~9500	~7000	~9700	~8000	~8000	~1100	~6000	~9000	~2000

Colon, breast, pancreas, lung, prostate, and leukemia cancer cell lines were treated with various concentrations of sulindac (SUL) and NOSH-sulindac (NOSH-SUL) as described in Section 2.13. Cell numbers were determined at 24 h from which IC<sub>50</sub> values were calculated. The ratios of SUL/NOSH-SUL represent fold-enhancement in potency of NOSH-SUL over SUL. Results are mean ± SEM of three independent determinations. In all cell lines,  $P < 0.001$  for NOSH-SUL vs SUL.

Thus the sum of parts did not equal the whole. The same was observed for NOSH-naproxen [29]. So, we suspect the same will hold true for NOSH-SUL. However, recent reports indicate that NO can react with H<sub>2</sub>S to produce HSNO which is a highly reactive intermediate [47,48]. Furthermore, NO and H<sub>2</sub>S signaling pathways appear to be intimately intertwined with mutual potentiation of biological responses [49].

### 3.10. Effect of NOSH-SUL on cell growth kinetics

The effects of NOSH-SUL on cell proliferation, apoptosis, and cell cycle transition, all of which affect cell growth were also examined in HT-29 colon cancer cells.

To determine the antiproliferative effects, HT-29 colon cancer cells were treated with different concentration of NOSH-SUL for 24 h, followed by PCNA quantification. The concentrations we used were,  $0.5 \times IC_{50}$  (50 nM),  $1 \times IC_{50}$  (100 nM), and  $2 \times IC_{50}$  (200 nM). NOSH-SUL reduced proliferation in a dose-dependent manner, as measured by the expression of PCNA, (Fig. 7A). Proliferation decreased to  $72 \pm 2\%$ , to  $55 \pm 3\%$  and to  $35 \pm 2\%$  at  $0.5 \times IC_{50}$ ,  $1 \times IC_{50}$ , and  $2 \times IC_{50}$ , respectively.

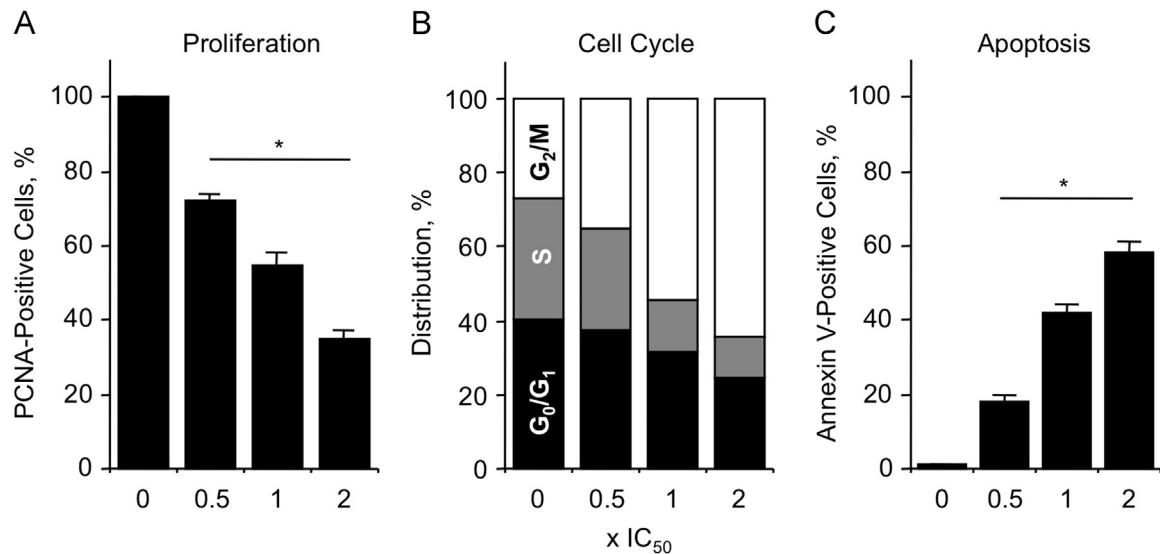
To determine whether cells were undergoing apoptosis in addition to inhibition of cell proliferation, apoptotic population was evaluated by Annexin V-FITC and propidium iodide staining, followed by flow cytometry. As shown in Fig. 7C, NOSH-SUL caused a significant increase in the number of cells undergoing apoptosis. The percentage of apoptotic cells increased from  $18 \pm 2\%$  at  $0.5 \times IC_{50}$  to  $42 \pm 2\%$  at  $1 \times IC_{50}$ , and  $58 \pm 3\%$  at  $2 \times IC_{50}$  compared to control.

We also determined the effect of the NOSH-SUL on the distribution of cells in G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M phases of the cell cycle. Cells were exposed to NOSH-SUL at concentrations of  $0.5 \times IC_{50}$ ,  $1 \times IC_{50}$ , and  $2 \times IC_{50}$  for 24 h, and analyzed for cell cycle phases by flow cytometry. DMSO-treated control cells proceeded through a normal cell cycle. Increasing concentrations of NOSH-SUL were associated with dose-dependent decreases in the percentage of cells in G<sub>0</sub>/G<sub>1</sub> and S phases, and a corresponding increase in the percentages of cells in G<sub>2</sub>/M phase (Fig. 7B), suggesting a G<sub>2</sub>/M phase cell cycle block. For example, at  $1 \times IC_{50}$  the population cells in G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle decreased from 40.6% to 31.4% and the S phase was reduced from 32.7% to 14%, while the cells in G<sub>2</sub>/M increased from 26.7% to 54.6%. This mode of cell cycle arrest has been reported for the parent drug sulindac in SW480 human colon cancer cells [50]. Thus, NOSH-SUL inhibits proliferation of HT-29 colon cancer cells by a combined induction of G<sub>2</sub>/M arrest and apoptosis.

### Summary and future directions

In the present study, proof-of-concept animal studies demonstrated that NOSH-sulindac is essentially devoid of any gastrointestinal side effects even though it reduces gastric tissue PGE<sub>2</sub> levels. The hybrid molecule retains all the positive pharmacological attributes of its parent NSAID, sulindac. That is, it is a potent anti-inflammatory and analgesic that has anti-pyretic and anti-platelet activity. In addition to its GI safety, NOSH-sulindac might also prove to have enhanced cardiovascular and renal safety profiles. This is because NO and H<sub>2</sub>S have protective roles in the cardiovascular and renal system [51–53]. NOSH-sulindac is also potentially useful as a chemopreventive agent against many types of cancer. In this regard, we are currently evaluating its utility in different animal models of cancer such as the APC<sup>Min/+</sup> mice. We are also deciphering its mechanism of action, focusing on molecular targets that are relevant to inflammation and cancer and to possible interactions between NO and H<sub>2</sub>S in producing a new signaling entity.





**Fig. 7.** Effect of NOSH-sulindac on colon cancer cell kinetics. NOSH-SUL inhibits proliferation by altering cell cycle progression and inducing apoptosis. HT-29 cells were treated with vehicle, 0.5x IC<sub>50</sub> (50 nM), 1 × IC<sub>50</sub> (100 nM) or 2 × IC<sub>50</sub> (200 nM) NOSH-SUL for 24 h and analyzed for (A) proliferation by PCNA antigen expression; (B) cell cycle phases by PI staining and flow cytometry; (C) apoptosis by Annexin V staining and flow cytometry. In (A) and (C), results are mean ± SEM for 3 different experiments performed in duplicate, \**P* < 0.05 compared to control. In (B), results are representative of two different experiments.

### Authorship contributions

Participated in research design: Kashfi, Chattopadhyay, Kodela.  
 Conducted experiments: Kodela, Chattopadhyay.  
 Performed data analysis: Kashfi, Chattopadhyay, Kodela.  
 Wrote or contributed to the writing of the manuscript: Kashfi, Chattopadhyay, Kodela.

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### Conflict of interest

The authors have nothing to disclose except for KK, who has an equity position in Avicenna Pharmaceuticals, Inc. the supplier of NOSH-sulindac used in these studies.

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