PRP Pharmacology Research & Perspectives
Open Access

ORIGINAL ARTICLE

Gastrointestinal safety, chemotherapeutic potential, and classic pharmacological profile of NOSH-naproxen (AVT-219) a dual NO- and H_2S-releasing hybrid

Mitali Chattopadhyay[1], Ravinder Kodela[1], Pascale L. Duvalsaint[1] & Khosrow Kashfi[1,2]

[1]Department of Physiology, Pharmacology and Neuroscience, Sophie Davis School of Biomedical Education, City University of New York School of Medicine, New York, NY 10031
[2]Avicenna Pharmaceuticals Inc., New York, NY 10019

Keywords
AVT-219, chemotherapeutic, cyclooxygenase, gastrointestinal, hydrogen sulfide, inflammation, naproxen, nitric oxide, NOSH-naproxen, pain, platelet, ulcer

Correspondence
Khosrow Kashfi, Department of Physiology, Pharmacology and Neuroscience, City University of New York School of Medicine, 138th Street and Convent Avenue, New York, NY 10031. Tel: (212) 650-6641; Fax: (212) 650-7692; E-mail: kashfi@med.cuny.edu

Funding Information
This work was supported in part by the National Institutes of Health (grant R24 DA018055). The funding agency had no role in the study design, collection, analysis, and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

Received: 28 December 2015; Revised: 27 January 2016; Accepted: 29 January 2016

Pharma Res Per, 4(2), 2016, e00224, doi: 10.1002/prp2.224

doi: 10.1002/prp2.224

Abstract

Naproxen (NAP) is a potent nonsteroidal anti-inflammatory drug (NSAID) with a favorable cardiovascular profile. However, its long-term use may lead to serious gastrointestinal and renal side effects. NOSH- (nitric oxide and hydrogen sulfide) releasing naproxen (NOSH-NAP, AVT-219) belongs to a new class of anti-inflammatory agents designed to overcome these limitations. We compared the gastrointestinal safety, anti-inflammatory, analgesic, antipyretic, and antiplatelet properties of AVT-219 to that of NAP in preclinical animal models. We also evaluated its anticancer effects in 11 human cancer cell (HCC) lines of six different tissue origins and in a chemotherapeutic xenograft mouse model of colon cancer. AVT-219: (1) was orders of magnitude more potent than NAP in inhibiting the growth of cultured HCC; (2) was safe to the stomach, whereas NAP caused significant ulceration; (3) showed strong anti-inflammatory, analgesic, antipyretic, and antiplatelet properties comparable to NAP; and (4) NAP caused a significant rise in plasma tumor necrosis factor-alpha (TNFα), whereas in the AVT-219-treated rats this rise was significantly less. Mechanistically, AVT-219 was a strong antioxidant, inhibited cyclooxygenase (COX)-1 and -2, thus reducing prostaglandin (PG) E_2. In xenografts, AVT-219 significantly reduced tumor growth and tumor mass with no sign of GI toxicity, whereas NAP-treated mice died due to GI bleeding. AVT-219 displayed considerable safety and potency in inhibiting HCC growth; was an effective analgesic, antipyretic, antiplatelet, and anti-inflammatory; and was significantly more efficacious than NAP in reducing the growth of established tumors in a xenograft mouse model.

Abbreviations

ADT-OH, 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione; H_2S, hydrogen sulfide; LPS, lipopolysaccharide; MDA, malondialdehyde; NAP, naproxen; NO, nitric oxide; NOSH, nitric oxide and hydrogen sulfide; NSAIDs, nonsteroidal anti-inflammatory drugs; PGE_2, prostaglandin E_2; SNAP, *S*-Nitroso-*N*-acetyl-penicillamine; SOD, superoxide dismutase.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the mainstay in the treatment of pain, fever, and inflammation. Unfortunately, their long-term use may lead to life-threatening side effects, mainly gastrointestinal, cardiovascular, and renal. The gastric damage is as a result of direct epithelial damage due to their acidic properties and also through the breakdown of mucosal defense mechanisms (leukocyte adherence, decreases in blood flow, and bicarbonate and mucus secretions) due to a reduction in mucosal prostaglandin (PG) biosynthesis as

a result of cyclooxygenase-1 (COX-1) inhibition (Wallace 1993, 2008). Among this class of compounds, naproxen is one of the most cardiovascular-safe drugs because of its sustained suppression of platelet thromboxane synthesis (Kearney et al. 2006) and is the one most often recommended for the treatment of osteoarthritis. However, it is notorious for causing gastrointestinal ulceration and bleeding.

Regular use of NSAIDs has been associated with lower incidence of cancer in general, the most compelling data available to date being that for colon cancer (Kune et al. 1988; Baron et al. 2003; Sandler et al. 2003; Rothwell et al. 2012). Unfortunately again, potentially life-threatening side effects alluded to above preclude their widespread use as chemopreventive agents (Wolfe et al. 1999), reviewed in Kashfi (2009). In our search for a "better NSAID", we developed NOSH-NSAIDs (Kodela et al. 2012, 2013). These are regular NSAIDs, such as aspirin, naproxen, sulindac, and others, to which nitric oxide- (NO) and hydrogen sulfide- (H_2S) releasing moieties have been covalently attached. Both NO and H_2S are gaseous signaling molecules of biological importance (Li et al. 2006; Predmore et al. 2012; King et al. 2014). The rationale for incorporating these gaseous mediators into NSAID molecules was based on the observations that NO (Brown et al. 1993; Wallace et al. 1994; Wallace and Miller 2000) and H_2S (Fiorucci et al. 2005; Wallace et al. 2007) have some of the same properties as PGs within the gastric mucosa, thus modulating some components of the local mucosal defense systems which should lead to reduced gastrointestinal toxicity.

In this study, we carried out a head-to-head comparison of the gastrointestinal safety, anti-inflammatory, analgesic, antipyretic, and antiplatelet properties of naproxen with those of AVT-219. We also evaluated the efficacy of naproxen and AVT-219 on established tumors in a xenograft mouse model of colon cancer.

Materials and Methods

Reagents

NOSH-naproxen (AVT-219) [6-(1-oxo-1-(4-(3-thioxo-3*H*-1,2-dithiol-5-yl) phenoxy) propan-2-yl) naphthalen-2-yl 4-(nitrooxy) butanoate] was synthesized as previously described (Kodela et al. 2013) and was a gift from Avicenna Pharmaceuticals Inc. (New York, NY). Its structural components are shown in Figure 1. Lipopolysaccharide (LPS) from *Escherichia coli*, naproxen, and carrageenan was purchased from Sigma (St. Louis, MO). The kits used for determination of PGE_2, lipid peroxidation, and superoxide dismutase (SOD) were purchased from Cayman Chemical (Ann Arbor, MI).

Experimental groups and treatments

In all the protocols described below, we used at least five 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 ad libitum. The animals were housed in suspended, wire-bottom cages in a room maintained at 22 ± 2°C, humidity 65–70%, and 12-h light/12-h dark cycle. 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. It should be noted that in the anti-inflammatory and antihyperalgesia studies, the experimenter was blinded to the treatment protocols; whereas in all the other protocols the experimenter was not blinded.

Ulcer index

Rats were fasted for 48 h with free access to drinking water. Naproxen and AVT-219 at equimolar concentrations, 80 and 188 $mg \cdot kg^{-1}$, respectively, were administered orally by gavage suspended in vehicle (0.5% carboxymethylcellulose, CMC). Six hours post administration, animals were euthanized in a CO_2 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. (1984) and reported by us previously (Chattopadhyay et al. 2010; Kodela et al. 2013). Excised tissues from each stomach were flash frozen in liquid nitrogen for assessing levels prostaglandin E_2 (PGE_2), malondialdehyde (MDA), and SOD activity. Blood samples from each rat were taken by cardiac puncture into heparin-containing vials and used for determination of plasma tumor necrosis factor-alpha (TNF-α).

PGE_2 levels

About 1 g of stomach tissue from each rat was homogenized in 5 mL of 0.1 mol/L phosphate buffer (pH 7.4)

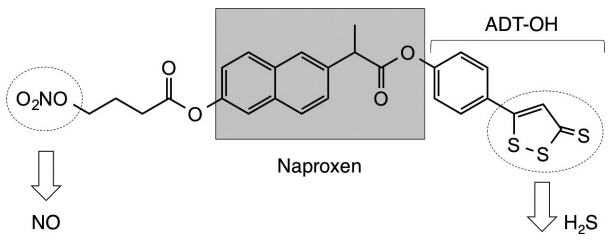

Figure 1. Structural components of AVT-219. The parent compound naproxen is shown in the shaded box. The parts of the molecule that releases NO and H_2S are shown in the dotted ellipses.

containing 1-mmol/L EDTA and 10-μmol/L indomethacin. The homogenate was then centrifuged for 10 min at 12,000*g* at 4°C. PGE_2 content in the supernatant was determined in duplicate using an enzyme immunoassay kit from Cayman Chemical, following the manufacturer's protocol. Briefly, 50 μL each of standard or homogenate, enzymatic tracer, and specific antiserum were mixed. After overnight incubation at 4°C, the plates were washed with wash buffer, and Ellman's reagent (200 μL) was added to each well. After 1 h of incubation at room temperature, the absorbance was measured at 412 nm. PGE_2 levels are expressed as pg mg^{-1} of protein. Protein concentration was determined by Biorad assay.

Lipid peroxidation

Levels of MDA were measured as an index of lipid peroxidation using a colorimetric kit from Cayman Chemical, as detailed by the manufacturer and reported by us (Chattopadhyay et al. 2010). Snap frozen stomach tissue (25 mg) was sonicated for 15 sec at 40 V over ice with 250 μL of radioimmunoprecipitation (RIPA) buffer (25 mmol/L TrisHCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) with phenylmethylsulphonyl fluoride (PMSF) as protease inhibitor. Homogenates were centrifuged for 10 min at 200*g* at 4°C. Thiobarbituric acid reactant substances (TBARS) content was then measured in the supernatant and expressed as nmoles of MDA·g^{-1} of protein.

SOD activity

Using a colorimetric kit from Cayman Chemical, we measured SOD activity in the gastric mucosa by homogenizing 1 g of tissue in 5 mL of a buffer system containing 20 mmol/L HEPES (pH 7.2) with 1 mmol/L EGTA and 300 mmol/L of sucrose solution as reported previously (Chattopadhyay et al. 2010). SOD activity was measured spectrophotometrically at 460 nm and expressed as units (U) activity·mg^{-1} protein. One unit of activity is defined as the amount of enzyme needed to exhibit 50% dismutaion of the superoxide radical.

Plasma TNF-α level

An enzyme immunoassay kit from R&D systems (Minneapolis, MN) was used to measure plasma TNF-α levels as reported by us (Chattopadhyay et al. 2010; Kodela et al. 2013). Briefly, fresh blood (50 μL) was incubated with antibodies specific for rat TNF-α and washed three times with assay buffer. Following washing of unbound antibody-enzyme reagent, substrate solution (tetramethylbenzidine, TMB, plus hydrogen peroxide) was then added. The reaction was stopped by adding dilute hydrochloride acid and its intensity measured at 450 nm. Sensitivity of this assay was determined by adding 2 standard deviations to the mean optical density value of 20 × zero standard replicates and calculating the corresponding concentration; sensitivity is estimated to be 1.6 pg·mL^{-1}.

Antipyretic activity

Rats received LPS (50 $\mu g \cdot kg^{-1}$) intraperitoneally to induce fever 30 min after equimolar administration of naproxen (80 mg·kg^{-1}) or AVT-219 (188 mg·kg^{-1}) as described by Pinto et al. (1998). 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 (±0.1°C). Rectal temperatures were recorded hourly for 5 h.

Determination of anti-inflammatory response

Rats ($n = 5$ in each group) were administered vehicle (1%, CMC in 1 mL), naproxen (80 mg·kg^{-1}), or AVT-219 (188 mg·kg^{-1}) 30 min before inducing inflammation which was done by subcutaneously injecting carrageenan (1%, 100 μL, suspended in sterile saline solution) into the plantar surface of the right hind paw as described by Winter et al. (1962) and reported by us (Chattopadhyay et al. 2010; Kashfi et al. 2015). The change in paw volume before and after carrageenan injection was measured using a water displacement plethysmometer (Model 520; IITC/Life Sciences Instruments, Woodland Hills, CA) for 5 h. At the end of the experiment, rats were euthanized by asphyxiation in a CO_2 chamber. After cutting each hindpaw at the level of the calcaneus bone, exudates (oedema fluid) were collected and processed for measurement of PGE_2, as described above.

Hyperalgesia

Hindpaw inflammation was produced by intraplantar injection of carrageenan (100 μL of 1% carrageenan in sterile saline solution) into either hindpaw chosen at random. Suspensions of naproxen (80 mg·kg^{-1}), AVT-219 (188 mg·kg^{-1}), or vehicle (CMC, 0.5% w.v^{-1}) were administered orally 1 h after carrageenan injection, and the mechanical nociceptive threshold determined 30 min after this and thereafter every hour for 5 h. The paw hyperalgesia was measured with an electronic pressure meter as reported earlier (Chattopadhyay et al. 2010; Kodela et al. 2013; Kashfi et al. 2015). The animals were

tested before and after treatments, the results are expressed by the delta reaction force (g).

Antiplatelet activity

Collagen-induced platelet aggregation of human platelet-rich plasma (PRP) occurs through a pathway dependent upon the arachidonic acid cascade (Nieswandt and Watson 2003). We therefore compared the antiaggregatory properties of AVT-219 to that of naproxen using this system as reported previously (Chattopadhyay et al. 2010; Kashfi et al. 2015). The antiaggregatory activity of the two compounds was determined as percent inhibition of platelet aggregation compared to control samples. IC_{50} values were calculated by nonlinear regression analysis.

COX enzyme activity

The ability of AVT-219 to inhibit COX-1 and COX-2 enzymatic activity was compared to that of naproxen using a colorimetric COX (ovine, *o*-COX) inhibitor screening kit from Cayman Chemicals as described previously (Kulmacz and Lands 1983).

Cell culture and MTT

HT-29, SW-480, and HCT-15 human colon adenocarcinoma, MIA PaCa-2 and BxPC-3 human pancreatic cancer, LNCAP human prostate cancer, A549 human lung cancer, MCF-7 (estrogen receptor positive), MDA-MB 231 and SK-BR-3 (estrogen receptor negative) human breast cancer, and Jurkat T (human leukemia) cell lines were obtained from American Type Tissue Collection (Manassas, VA). All cells lines were grown as previously described (Kodela et al. 2012). Single-cell suspensions were obtained by trypsinization (0.05% trypsin/EDTA), and cells were counted using a hemocytometer. Viability was determined by the trypan blue dye exclusion method. Cell growth inhibitory effect of naproxen and AVT-219 was measured using a colorimetric MTT assay kit (Roche, Indianapolis, IN).

Therapeutic evaluation of NOSH-naproxen in a mouse xenograft model

Male athymic nude (NU/NU) mice ($n = 15$), age 5 weeks, were purchased from Charles River Laboratories, Inc. After 1 week of acclimation, the mice were inoculated subcutaneously in the right flanks with SW480 human colon cancer cells (3×10^6) suspended in Matrigel (50% $v{\cdot}v^{-1}$, BD Biosciences, San Jose, CA) using a 1-mL syringe and 22-gauge needles. When the tumors reached an average sizes of ~100 mm^3 (10 days post inoculation), the mice were randomly divided into three groups and gavaged daily with either vehicle (0.5% CMC) or equimolar (0.18 $mmol{\cdot}kg^{-1}$) concentrations of AVT-219 (100 $mg{\cdot}kg^{-1}$ body weight) or naproxen (45 $mg{\cdot}kg^{-1}$ body weight). Tumor size was measured every other day using electronic calipers, and the tumor volumes were calculated using the following formula: length $\times$ width^2/2. After 30 days of treatment, the mice were sacrificed, the tumors collected, weighed, and photographed. Blood was also collected for determination of serum NO and H_2S levels. The tumors were stored in formalin for immunohistochemistry studies.

Immunohistochemistry

All specimens were fixed in formalin and paraffin enclosed for examination. Five-micrometer-thick tissue sections were prepared to conduct immunohistochemistry using standard techniques as reported by us (Chattopadhyay et al. 2012). For proliferation we used primary mouse proliferating cell nuclear antigen (PCNA) at the appropriate dilution (1:100 dilution), and for detection of apoptotic cell death in tumor tissues, we used a TUNEL system kit from Promega (Madison, WI).

Determination of plasma NO and H_2S levels

Plasma concentration of NO was quantified indirectly as the concentration of nitrate (NO_3^-) and nitrite (NO_2^-) by the Griess method using an assay kit from Cayman Chemical and following the protocol described by the manufacturer, as reported by us previously (Chattopadhyay et al. 2012; Kodela et al. 2012, 2013). Plasma was filtered using a 10-KD molecular weight cut-off filter from Millipore (Bedford, MA) before each analysis, to reduced background absorbance due to the presence of hemoglobin. After centrifugation for 10 min at 3000 rpm, samples (40 μL/well) were mixed with 10 μL nitrate reductase mixture and incubated for 3 h after which Griess reagents 1 and 2 (50 μL each) were added. Absorbance was read after 10 min at 540 nm using a plate reader. The concentration of nitrate/nitrite was calculated graphically from a calibration curve prepared from $NaNO_2$ standard solution.

H_2S levels were measured as previously described using the standard methylene blue method (Li et al. 2007; Huang et al. 2010), and reported by us (Chattopadhyay et al. 2012; Kodela et al. 2012, 2013). Plasma (100 μL) was mixed with distilled water (100 μL), Zinc acetate (1% w/v, 250 μL), trichloroacetic acid (10% w/v, 250 μL), and *N*, *N*-dimethyl-*p*-phenylenediamine sulfate (133 μL,

20 μmol/L) in 7.2 mol/L HCl and $FeCl_3$ (133 μL, 30 μmol/L) in 7.2 mol/L HCl. The absorbance of the resulting mixture was determined after 15 min using a 96-well microplate reader at 670 nm. H_2S levels were calculated against a calibration curve of NaHS (1–250 μmol/L). Because of the strong acidic conditions, this method overestimates H_2S levels as it measures free H_2S, HS^- (hydrosulfide anion), S^{2-} (sulfide), acid-labile sulfide, and other, as yet unidentified, sulfides and can only provide a rough estimate of H_2S production (Olson 2009). Therefore, our results presented here indicate the sum total of these species.

Data analysis

All data are presented as the mean ± SEM, with sample sizes of at least 5 rats/mice in each group. Comparisons among groups were performed using a one-way analysis of variance followed by the Student *t*-test.

Results

Naproxen, but not AVT-219, induced GI damage in rats

Visual inspection showed that there were no gastric mucosal lesions in vehicle-treated rats (control group, Fig. 2A and D), for these rats, the gastric damage score (also described in the literature as "UI") was zero (UI = 0). However, all rats treated with naproxen (80 mg·kg^{-1}) developed pronounced gastric ulcers and erosions, UI = 80 (Fig. 2B and D). In contrast, AVT-219 (188 mg·kg^{-1}) did not induce significant gastric damage UI = 2 (Fig. 2C and D). This is consistent with previously published data, where NO (Brown et al. 1993; Wallace et al. 1994; Wallace and Miller 2000) and H_2S (Fiorucci et al. 2005; Wallace et al. 2007) were shown to act as mediators in mucosal defense.

Gastric PGE_2 content

The main products of cyclooxygenase-mediated arachidonic acid metabolism in the gastric mucosa are the PGs. In that respect, PGE_2 levels in the normal mucosal tissue of the vehicle-treated rats, the naproxen-treated rats, and the AVT-219-treated rats were 72.3 ± 4.2, 10.2 ± 1.6, and 22.1 ± 2.6 pg·mg^{-1} protein, respectively (Fig. 3A). That is, a rat treated with naproxen (80 mg·kg^{-1}) produced about 86% less PGE_2 than rats in the control group. AVT-219 at an equimolar concentration (188 mg·kg^{-1}) reduced PGE_2 by about 70%. These comparative PGE_2 levels showed a clear and significant COX inhibition by naproxen and AVT-219.

Effects of AVT-219 on suppression of COX activity

When metabolized, AVT-219 should produce naproxen, H_2S, and NO. Previously, we had shown that NO and H_2S are released from AVT-219 (Kodela et al. 2013). In order to show the effects of the naproxen component, we compared the effects of AVT-219 to that of naproxen on pure ovine COX-1 and COX-2 enzymatic activity. As shown in Table 1, AVT-219 dose dependently and selectively inhibited the enzymatic activity of COX-1 more than that of COX-2. Naproxen at 3000 and 6000 μmol/L concentrations, inhibited COX-1 by 80 ± 4% and 92 ± 3%; and COX-2 by 65 ± 3% and 75 ± 2%. AVT-219 at 100 and 200 nmol/L inhibited COX-1 by 45 ± 3% and 54 ± 2%; and COX-2 by 16 ± 2% and 27 ± 3%. The concentrations of naproxen and AVT-219 chosen in these studies were based on their respective IC_{50}s for cell growth inhibition in SW480 colon cancer cell line (see below). We used indomethacin (1 μmol/L), a nonselective COX inhibitor (Riendeau et al. 1997), as a reference compound in these studies, which inhibited COX-1 by 69 ± 3% and COX-2 by 70 ± 2% (Table 1).

Effect of AVT-219 on lipid peroxidation and SOD activity

MDA levels representing oxidative stress in the gastric tissue were 6.5 ± 0.6 nmol·mg^{-1} protein for the vehicle-treated group, this increased to 59 ± 3 nmol·mg^{-1} in the naproxen-treated group, but was only 26 ± 2 nmol·mg^{-1} protein in the AVT-219-treated group (Fig. 3B). On the other hand, SOD activity in the vehicle-treated group was 3.4 ± 0.5 U·mg^{-1} protein, this was decreased to 1.4 ± 0.3 U·mg^{-1} protein in the naproxen-treated group, and was significantly increased (compared to the naproxen group) to 3.6 ± 0.2 U·mg^{-1} protein in the AVT-219-treated group (Fig. 3C).

AVT-219 effectively reduces carrageenan-induced paw swelling

Naproxen is very effective in treating inflammation and inflammatory pain. Here, we compared the COX-dependent anti-inflammatory activity of naproxen to that of AVT-219 using the carrageenan-induced rat paw edema model (Winter et al. 1962). After inducing inflammation, animals receiving vehicle showed a fast time-dependent increase in paw volume, which appeared to be plateauing toward the end of the experiment after 5 h (ΔV = 1.4 mL, Fig. 4A). In contrast, animals receiving naproxen and AVT-219 showed a weak inflammatory response (ΔV = 0.3–0.4 mL by 2 h), which decreased

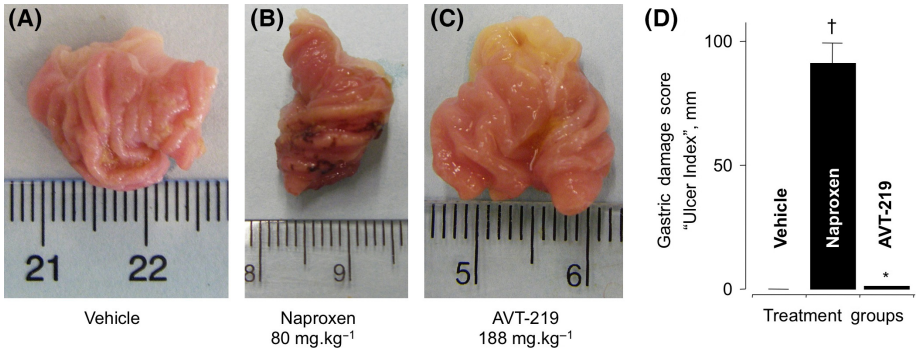

Figure 2. AVT-219 does not cause gastric damage. Naproxen (80 mg·kg^{-1}) and AVT-219 (188 mg·kg^{-1}) were administered orally at equimolar concentrations (0.35 mmol·kg^{-1}) and effects on the stomach were evaluated as indicated in "Ulcer index". (A) Stomach of a vehicle-treated rat; (B) stomach of a naproxen-treated rat showing ulceration and bleeding; (C) stomach of a AVT-219-treated rat which is essentially devoid of ulcers; and (D) gastric damage due to naproxen, UI = 80 ± 5 mm ($^{\dagger}P < 0.01$ compared to vehicle), AVT-219, UI = 2 ± 0.4 mm ($^{*}P < 0.01$ compared to naproxen).

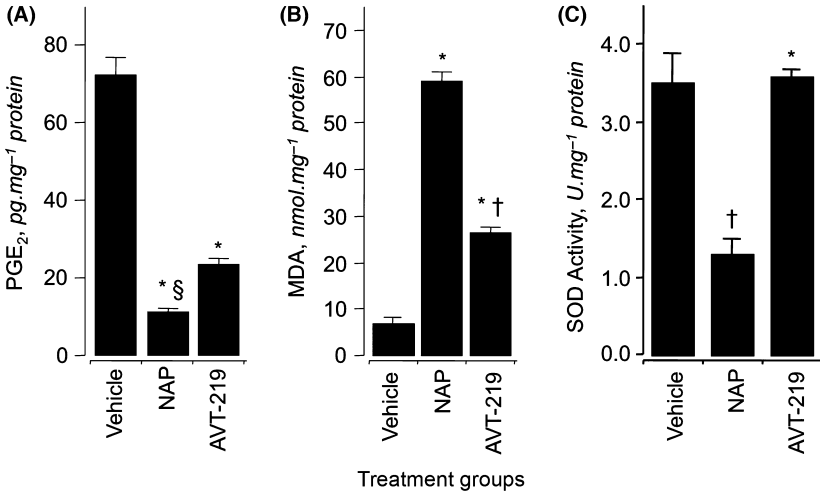

Figure 3. Effects of AVT-219 and naproxen on gastric PGE_2 level, lipid peroxidation (MDA), and superoxide dismutase (SOD). Three groups of rats were treated with vehicle, equimolar concentrations of AVT-219, and naproxen and their stomachs were removed and processed as described in "PGE_2 levels". AVT-219 and naproxen caused significant reductions in gastric mucosal PGE_2 levels (A). Results are mean ± SEM of five rats in each group, $^{*}P < 0.05$ versus vehicle group, $^{\S}P < 0.05$ versus AVT-219 group. Naproxen caused an almost nine fold increase in MDA levels, for AVT-219-treated rats, MDA levels were about 2.3-fold higher (B). Results are mean ± SEM for five rats in each group, $^{*}P < 0.01$ versus vehicle group, $^{\dagger}P < 0.01$ versus naproxen group. Naproxen caused a 2.4-fold reduction in SOD activity, whereas AVT-219 had no effect (C). Results are mean ± SEM of five rats, $^{\dagger}P < 0.05$ versus vehicle group, $^{*}P < 0.01$ versus naproxen group.

over the next 3 h (Fig. 4A). From hours 3–5, AVT-219 was significantly ($P < 0.05$) better than naproxen in its anti-inflammatory action (Fig. 4A). PGE_2 is one of the main products of cyclooxygenase-mediated arachidonic acid metabolism and is a key mediator of the inflammatory process (Kashfi 2009). Comparison of PGE_2 content of paw exudates showed a clear and significant COX inhibition by naproxen and AVT-219 (Fig. 4B). PGE_2 level in control vehicle-treated rats was 82 ± 3 pg·mg^{-1} protein, and in the naproxen- and AVT-219-treated rats were 9.3 ± 0.3 pg·mg^{-1} protein and 32 ± 2 pg·mg^{-1} protein, respectively. This is equivalent to reduction of 89% and 61% by naproxen and AVT-219, respectively.

Plasma TNF-α levels are lower in AVT-219-treated rats

The proinflammatory cytokine TNF-α in plasma of naproxen-treated rats was increased by about 14-fold. Plasma TNF-α levels were 11 ± 0.3 pg·mL^{-1} in vehicle-treated rats and 150 ± 2 pg·mL^{-1} in the naproxen-treated rats (Fig. 5). The corresponding value in

Table 1. AVT-219 inhibits cyclooxygenase enzyme activity.

Treatment	COX-1 % inhibition	COX-2 % inhibition
NAP, 3000 μmol/L	80 ± 4	65 ± 3
NAP, 6000 μmol/L	92 ± 3	75 ± 2
AVT-219, 100 nmol/L	45 ± 3	16 ± 2
AVT-219, 200 nmol/L	54 ± 2	27 ± 3
Indomethacin, 1 μmol/L	69 ± 3	70 ± 2

Pure ovine COX enzymes were treated with NAP or AVT-219 at their respective 1 × IC_{50} and 2 × IC_{50} for cell growth inhibition in SW480 colon cancer cell line for 15 min at 4°C after which *o*-COX-1 and *o*-COX-2 enzyme activity was determined. Results are mean ± range of two independent studies performed in duplicate.

AVT-219-treated rats was 50 ± 3 pg·mL^{-1}, this represents a three fold reduction compared to the native compound.

Antipyretic activity of AVT-219

NSAIDs in general have a moderate antipyretic effect when administered orally. Here, we compared at equimolar doses (0.34 mmol·kg^{-1}) the effects of AVT-219 (188 mg·kg^{-1}) to that of naproxen (80 mg·kg^{-1}) on LPS-induced fever in rats. Both agents were administered (*po*) 30 min before injecting LPS (50 μg/kg *ip*). Vehicle-treated rats showed a time-dependent increase in body temperature (ΔT = 1.9 ± 0.1°C) which peaked at 3 h and then slowly declined by about 0.2°C over the next 2 h (Fig. 6A). Naproxen- and AVT-219-treated rats showed only about 0.3–0.4°C increase in body temperature within the first 30 min which fell down to 0.15–0.25°C by 1 h after LPS injection and continuously declined for the next 4 h, in fact at the end of the experiment the AVT-219-treated rats showed no detectable fever (Fig. 6A).

AVT-219 is an effective analgesic

This assay measures the ability of a test drug to reverse hyperalgesia (decreased threshold to a painful stimuli) produced by injection of carrageenan. The mechanical pain threshold was increased in a time-dependent manner after administering naproxen or AVT-219 (Fig. 6B). Pain threshold before administration of naproxen or AVT-219 ranged from 62 to 70 g. This was reduced to about 10 g after carrageenan injection confirming a higher sensitivity to mechanical stimuli (nonpainful at normal conditions). The mechanical threshold for pain increased to about 32 g in both naproxen- and AVT-219-treated groups.

Antiplatelet properties of AVT-219

Antiaggregatory effects of naproxen and AVT-219 were studied on collagen-induced platelet aggregation of human PRP. The results expressed as IC_{50} are shown in Figure 6C. Analysis of the data, which represents the mean ± range for two different individuals, does not lend

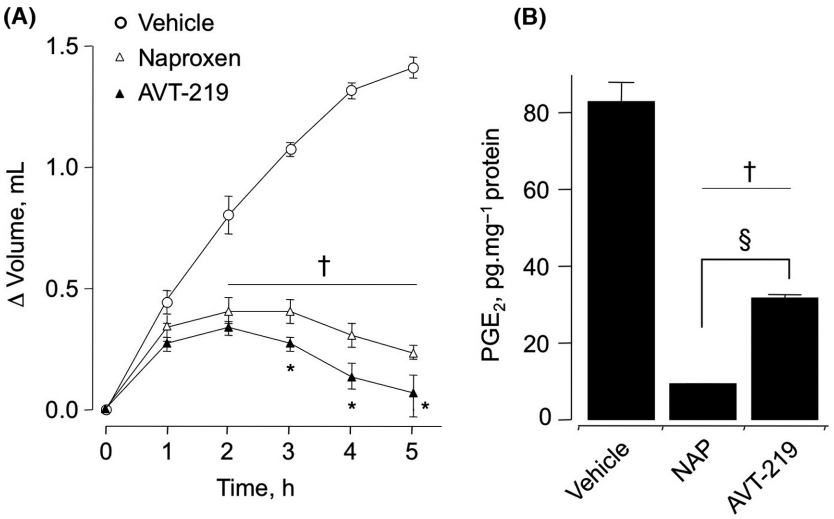

Figure 4. AVT-219 has effective anti-inflammatory properties. Rat paw edema was induced by carrageenan injection as described in "Determination of anti-inflammatory response". AVT-219 caused a significant reduction in paw volume at all time points, whereas naproxen's anti-inflammatory action became significant after 2 h; further AVT-219 from 3 to 5 h was significantly better than naproxen in its anti-inflammatory action (A). Results are mean ± SEM of five rats in each group, $^{\dagger}P < 0.05$ versus vehicle-treated rats; $^{*}P < 0.05$ versus naproxen 3–5 h. In the paw exudate, naproxen reduced PGE_2 levels by about 88% and AVT-219 by about 60% (B). Results are mean ± SEM for five rats in each group, $^{\dagger}P < 0.01$ versus vehicle, $^{\S}P < 0.05$ versus AVT-219.

itself to any statistical analysis; however, based on the ranges, there does not appear to be any differences between the two groups.

AVT-219 inhibits the growth of various human cancer cell lines

We evaluated the effects of naproxen and AVT-219 on the growth properties of eleven different human 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), prostate (LNCaP), and T-cell leukemia (Jurkat). AVT-219 was very effective in inhibiting the growth of these cell lines (Table 2). The IC_{50}s for cell growth inhibition at 24 h for AVT-219 ranged from 0.09 ± 0.01 to 0.14 ± 0.02 μmol/L and that for naproxen was 2100 ± 200 to 3110 ± 185 μmol/L. In a fold comparison of the IC_{50} values (naproxen/AVT-219), AVT-219 was at least 18,000-fold to 33,000-fold more potent than naproxen in various cell lies (Table 2). It should be noted that the data presented for HT-29, MCF-7, BxPC3, and Jurkat cells are replication of our previously published work (Kodela et al. 2013).

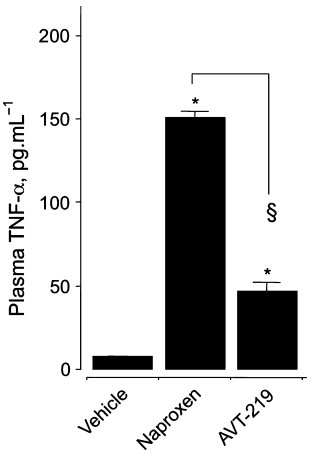

Figure 5. Plasma TNF-α levels are lower in AVT-219-treated rats. Rats were treated with equimolar concentrations (0.35 mmol·kg^{-1}) of naproxen and AVT-219 and plasma TNF-α were measured as described in "Plasma TNF-α level". Naproxen increased plasma TNF-α by about 14-fold, whereas this rise was about 3-fold in AVT-219-treated rats. Results are mean ± SEM for five rats in each group, *$P < 0.001$ versus vehicle, §$P < 0.01$ versus naproxen.

Efficacy of AVT-219 in established tumor xenografts in nude mice

Male athymic nude (NU/NU) mice were injected subcutaneously with SW480 colon cancer cells in the right flank, allowing for the development of subcutaneous tumors with an average volume of about 100 mm^3 after 10 days. Following tumor formation, the mice were divided into three groups. The control group was treated with the vehicle (0.5% CMC); the other two groups were treated orally with equimolar (0.18 mmol·kg^{-1}) concentrations of naproxen (43 mg·kg^{-1}) and AVT-219 (100 mg·kg^{-1}). All

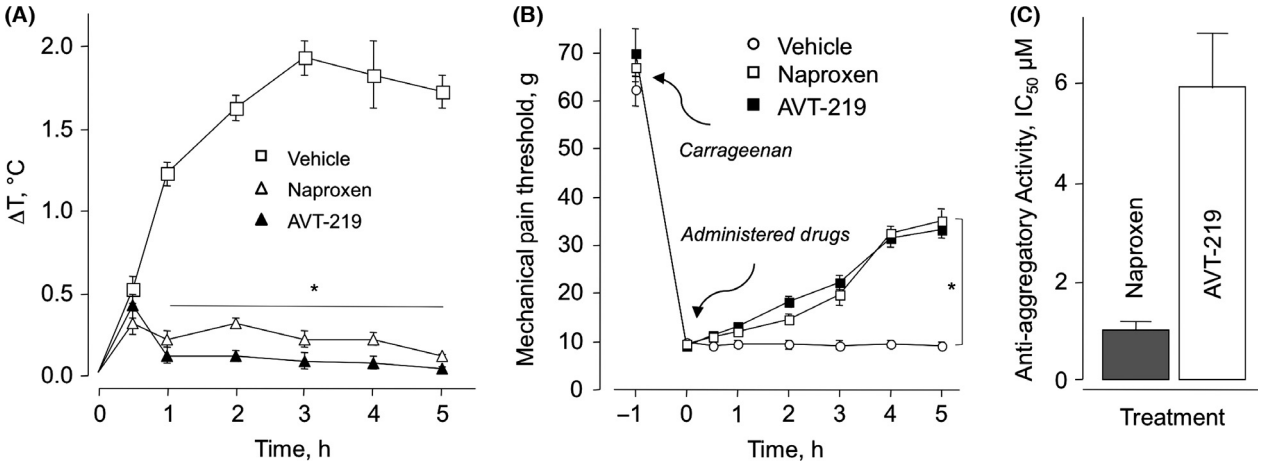

Figure 6. AVT-219 reduces LPS-induced fever, increases the threshold for pain, and has antiplatelet activity. (A) LPS (50 μg·kg^{-1}, *i.p.*) was administered to the rats 30 min after naproxen and AVT-219 administration. Core body temperature was recorded at 30 min and thereafter hourly for 5 h. Results are mean ± SEM for five rats in each group, *$P < 0.01$ versus vehicle for both naproxen and AVT-219 from 1 to 5 h. (B) Mechanical pain threshold was increased as a function of time by AVT-219 and naproxen. Results are mean ± SEM for five rats in each group. *$P < 0.05$ versus vehicle for AVT-219 and naproxen 2–5 h. (C) AVT-219 and naproxen were equally effective in inhibiting human platelet aggregation. Results are mean ± range for two individuals.

Table 2. IC50 (mol/L) values at 24 h for cell growth inhibition in different cancer cell lines.

Agent	Colon			Breast			Pancreas		Lung	Prostate	Leukemia
	HT-29	HCT 15	SW480	MDA MB231	SKBR3	MCF-7	MIAPaCa2	BxPC3	A549	LNCAP	Jurkat
NAP	2800 ± 165	2950 ± 215	3110 ± 185	2900 ± 225	2890 ± 147	2100 ± 200	3200 ± 195	2600 ± 85	2650 ± 110	2990 ± 175	2385 ± 177
AVT-219	0.09 ± 0.01	0.1 ± 0.008	0.098 ± 0.007	0.11 ± 0.01	0.10 ± 0.007	0.11 ± 0.008	0.095 ± 0.009	0.14 ± 0.02	0.10 ± 0.01	0.13 ± 0.02	0.11 ± 0.01
Enhanced potency	~31,000	~29,000	~28,000	~26,000	~28,000	~19,000	~33,000	~18,000	~26,000	~23,000	~21,000

Colon, breast, pancreas, lung, prostate, and leukemia cancer cell lines were treated with various concentrations of naproxen and AVT-219 as described in Methods. Cell numbers were determined at 24 h from which IC50 values were calculated. The ratios of NAP/AVT-219 represent fold enhancement in potency of AVT-219 over NAP. Results are mean ± SEM of three independent determinations. In all cell lines, $P < 0.001$ for AVT-219 compared to NAP.

the mice treated with naproxen died within 2 weeks from what appeared to be sever GI bleeding and damage to the spleen and liver. However, the AVT-219-treated mice did not show any overt signs of toxicity, their average group weight was comparable to that of the controls. After 30 days of treatment (protocol shown in Fig. 7A), the AVT-219-treated mice (Figs. 7C and 8A) showed a considerable reduction in tumor growth compared with untreated mice (Figs. 7B and 8A). The stomachs of the AVT-219-treated mice were completely devoid of any GI erosions or bleeding (Fig. 7D). Compared with the control group with a mean tumor volume of 2100 ± 380 mm^3, AVT-219-treated mice had a tumor volume of 370 ± 50 mm^3, equivalent to a mean reduction of 82% ($P < 0.05$). Compared to the control group with average tumor mass 1.4 ± 0.5 g, AVT-219 reduced the tumor mass to 0.63 ± 0.11 g, equivalent to a reduction of 55% ($P < 0.05$) which was relatively consistent with continued regression of tumor growth over the same treatment period. Immunohistochemical analysis of AVT-219-treated and untreated mouse tumor sections was evaluated by PCNA, which is a biomarker for proliferation, and by TUNEL to determine the number of cells undergoing apoptosis. The vehicle-treated tumors showed a strong expression index for PCNA ($80 \pm 3\%$, Fig. 9A and C), whereas AVT-219-treated tumors showed a diminished expression of PCNA ($20 \pm 0.5\%$, Fig. 9B and C). TUNEL-positive cells in the vehicle-treated tumors were $1.5 \pm 0.4\%$ (Fig. 9D and F); AVT-219 increased apoptosis in the tumors ($83 \pm 3\%$) as measured by TUNEL staining for multiple tissue sections (Fig. 9E and F). Therefore, AVT-219 suppressed tumor growth by a combination of increased apoptosis and reduced cell proliferation.

AVT-219 releases both NO and H_2S in vivo

AVT-219 was designed to release both NO and H_2S. To that end, using in vitro and in vivo protocols, we had previously shown that indeed that was the case (Kodela et al. 2013). Here, using yet another in vivo model, that is, in the mice bearing the colon cancer xenografts, we have confirmed our previous finding showing that both NO and H_2S were significantly higher in the AVT-219-treated animals (Figure 10). It should be noted that the serum concentrations of H_2S as determined by the methylene blue method described in "Determination of plasma NO and H_2S levels" are not accurate, as this method is associated with considerable artifact (Olson 2009). What our data show are not absolute H_2S levels in the serum, but there appears to be an increase in some form of sulfide species ($H_2S + HS^- +$ and $S^{2-} +$ others) that must clearly be due to the administered AVT-219.

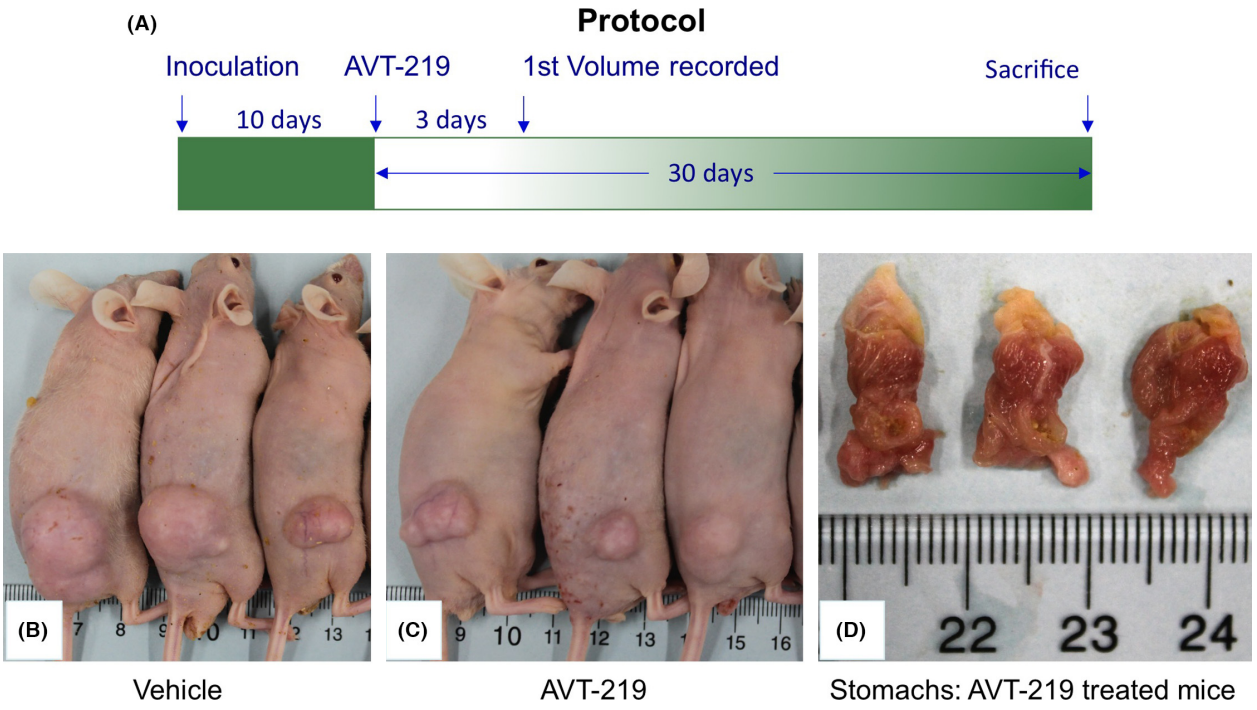

Figure 7. Efficacy of AVT-219 in established tumor xenografts. Study protocol: (A) Male athymic nude mice were injected subcutaneously with SW480 colon cancer cells in the right flank, after 10 days average volume was about 100 mm^3. Following tumor formation, the mice were divided into three groups and treated daily by gavage with the vehicle (0.5% CMC, B), or equimolar concentrations of AVT-219 (100 $mg{\cdot}kg^{-1}$, C) or naproxen (43 $mgkg^{-1}$). Naproxen-treated mice died within 2 weeks. (D) Stomachs of the AVT-219-treated mice showing no signs of GI erosions or bleeding.

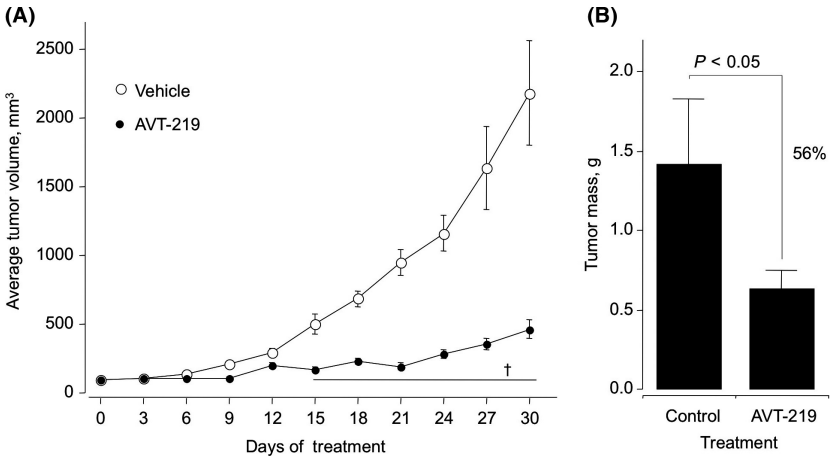

Figure 8. AVT-219 reduced tumor growth and tumor mass. Animals were treated as depicted in Figure 7. Average tumor volume as function of time and tumor mass at sacrifice are shown in A and B, respectively. AVT-219 significantly reduced tumor growth from day 15 of treatment to sacrifice, $*P < 0.01$. Tumor mass was also significantly reduced by AVT-219 treatment, $*P < 0.01$. Naproxen-treated mice died within 2 weeks of treatment.

Discussion

In this study, we evaluated the effects of a novel NO- and H_2S-releasing derivative of one of the most commonly used NSAIDs, naproxen. AVT-219 exhibited all the classic pharmacological profiles of its native compound, the so-called four "A"s that is analgesic, antipyretic, anti-inflammatory, and antiplatelet aggregation. One of the most

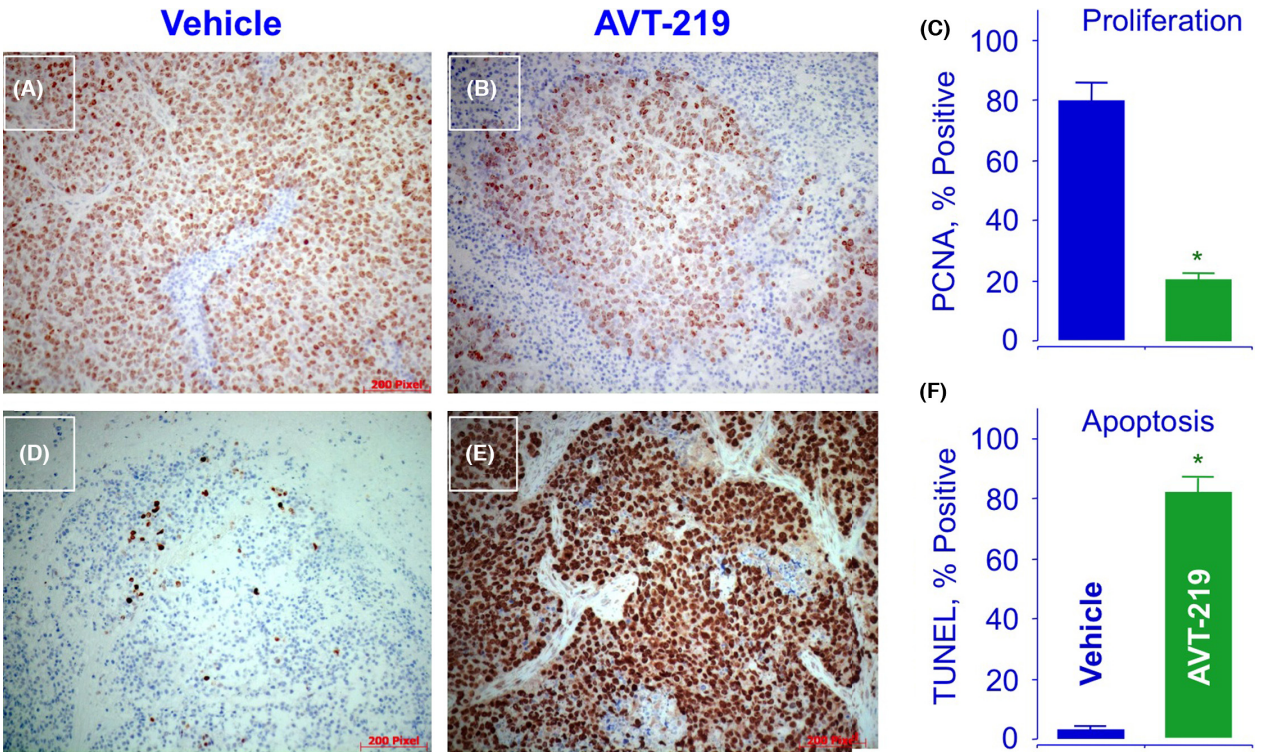

Figure 9. AVT-219 inhibits proliferation and induces apoptosis in vivo. Stored tumors were sectioned, probed, and scored as described in "Immunohistochemistry". Average mitotic index at sacrifice was determined by PCNA staining ($P < 0.05$) and TUNEL staining ($P < 0.02$). Representative fields used for quantification of the staining are shown. The scale bar represents 200 μm.

significant side effects associated with naproxen is its GI toxicity, which limits its use. AVT-219 was designed to overcome this side effect and be a safer naproxen by releasing NO and H_2S, two gasotransmitters of physiological relevance that have been shown to be important mediators of gastric mucosal protection (Brown et al. 1993; Wallace et al. 1994, 2007; Wallace and Miller 2000; Fiorucci et al. 2005). In our comparative study at equimolar concentrations, AVT-219 did not produce any significant GI injury despite inhibiting mucosal PGE_2 synthesis, whereas naproxen caused a tremendous amount of GI bleeding. This GI injury is as a result of COX-1 inhibition. We further confirmed that both compounds dose dependently inhibited ovine COX-1 and COX-2 enzymatic activity in vitro. Our data clearly indicated that AVT-219 selectively inhibited COX-1 versus COX-2, whereas naproxen essentially inhibited both COX isoforms quite effectively. Another COX-1-mediated effect that we evaluated was the antiaggregatory effects of naproxen and AVT-219, which showed that both drugs had similar antiplatelet effects. On the other hand AVT-219 was just as effective as naproxen if not better in reducing LPS-induced fever, and exhibited potent anti-inflammatory properties using the carrageenan paw edema model, both of which are COX-2-mediated effects. Therefore, although in vitro AVT-219 selectively inhibited COX-1, its effects in vivo are consistent with actions that are mediated by both COX isoforms. In this regard there are two important observations to be made: one, it is important to exercise caution when applying in vitro data to any in vivo settings; two, as AVT-219 is more potent than naproxen in many of its actions, released NO and H_2S must contribute to its overall pharmacological properties.

Analysis of gastric tissue showed that naproxen reduced SOD activity by about three fold, whereas AVT-219 essentially had no effect on the activity of this antioxidative enzyme. This may explain in part the high levels MDA, an index of lipid peroxidation, seen in the naproxen-treated groups. We also observed that naproxen caused increases in the circulating levels of the proinflammatory mediator TNF-α, this may release oxygen-derived free radicals and proteases by triggering the adherence and activation of leukocytes, thus producing epithelial injury (Perini et al. 2004). It has been reported that leukocyte adherence is an early key event in the pathogenesis of mucosal injury associated with NSAIDs (Wallace 1993). Both NO (Wallace and Miller 2000) and H_2S (Zanardo

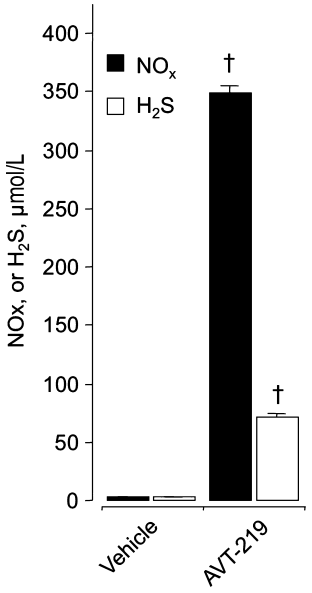

Figure 10. NO and H_2S levels are increased in vivo after administration of AVT-219. Plasma concentration of NO_x and H_2S prepared at the end of the xenograft studies was quantified as described in "Determination of plasma NO and H_2S levels". Results are mean ± SEM for five mice in each group. $^{\dagger}P < 0.001$ versus vehicle.

et al. 2006), two components of AVT-219, are potent inhibitors of leukocyte adherence induced by NSAIDs, thus contributing to its GI safety profile.

Naproxen is often used to treat inflammatory conditions. We therefore compared the anti-inflammatory profile of AVT-219 to that of naproxen using the in vivo carrageenan-induced rat paw edema model. AVT-219 exhibited significantly better anti-inflammatory activity compared to naproxen. Of note is the observation that naproxen reduced PGE_2 levels to a greater extent in the paw exudate compared to AVT-219, but that mice treated with equimolar concentrations of AVT-219 had better anti-inflammtory response. These observations suggest a strong role by NO and H_2S, which we know are released by AVT-219 (Kodela et al. 2013), in modulating the anti-inflammatory response.

Naproxen is also employed in treatment of pain. We therefore compared the analgesic effect of AVT-219 to that of naproxen in an in vivo model of hyperalgesia (Chattopadhyay et al. 2010). Both drugs were equally effective in increasing the threshold response to a painful stimulus. These results are in line with our recent reports showing both NOSH-aspirin (Kodela et al. 2015) and NOSH-sulindac (Kashfi et al. 2015) to have equal analgesic efficacy to that of their respective native NSAID. The enhanced antinociceptive effect of AVT-219 may be due to its ability to reduce the production of proinflammatory cytokines, such as IL-1β, and restore neuronal sensitization caused by PGE_2 through upregulation of K_{ATP} channels, as was observed with NOSH-aspirin but not with aspirin (Fonesca et al. 2015).

In United States, colorectal cancer (CRC) is one of the top three most common cancers in both men and women, resulting in an estimated 50,000 deaths this year alone (American-Cancer-Society, 2015). Epidemiological data show NSAIDs as the prototypical class of drugs that prevent CRC (Harris et al. 2005). While aspirin is the NSAID that has been investigated the most (Baron et al. 2003; Sandler et al. 2003; Rothwell et al. 2012), other NSAIDs including naproxen are also effective anticancer agents (Harris et al. 2008). In this study we first showed that AVT-219 was orders of magnitude more potent than naproxen in inhibiting the growth of adenomatous, epithelial, and lymphocytic cancer cell lines; that is, eleven cell lines originating from six different tissues. Thus, demonstrating a generalized property that was tissue-type independent. Recently we reported that this growth inhibition was as a result of reductions in cell proliferation, G_0/G_1 cell-cycle arrest, leading to increased apoptosis (Kodela et al. 2013). The underlying mechanism(s) for the enhanced potency of AVT-219 observed in these studies is not known, but must be due to contributions from both NO and H_2S. While we do not know anything about the kinetics of NO and H_2S release and their potential interactions, we do know that in vitro both contribute toward the potency of the intact molecule. This is based on our recent report where we showed that the biological activity of naproxen plus SNAP (releases NO) plus ADT-OH (releases H_2S) was not as same as the biological activity of the intact NOSH-naproxen (AVT-219) molecule (Kodela et al. 2013), thus the sum of parts did not equal the whole. NO can also react with H_2S to produce HSNO, which is a highly reactive intermediate (Filipovic et al. 2012; Cortese-Krott et al. 2015). Furthermore, NO and H_2S signaling pathways appear to be intimately intertwined with mutual potentiation of biological responses (Cortese-Krott et al. 2015).

In this study, we also compared the efficacy of AVT-219 to that of naproxen at equimolar concentrations on established tumors using an in vivo xenograft mouse model of colon cancer. AVT-219 treatment resulted in large reductions in tumor volume and mass. Decrease in tumor size and mass was associated with inhibition of cell proliferation and induction of apoptosis. The dose of AVT-219 used in this mouse model was well tolerated with no apparent harmful side effects or overall gross toxicity; the mice in both control and AVT-219-treated groups had similar average body weights at necropsy and visual inspection of the organs did not show anything untoward. However, all the naproxen-treated mice died

by the end of the second week of treatment from what appeared to be GI bleeding and damage to other organs such as the spleen and the liver. In this regard, further work is necessary to establish the safe limit for long-term administration of naproxen in such an animal model and then compare its efficacy with equimolar concentrations of AVT-219.

In summary, AVT-219 retains all the classic therapeutic profile of native naproxen, such as the ability to inhibit inflammatory response, nociception, platelet aggregation, and fever, but spares the GI tract. It has all the attributes that make it an ideal promising alternative to existing therapies for the treatment of inflammation and pain. AVT-219 may also prove useful either alone or in combination with other classical chemotherapeutic agents in treatment of colon and possibly other cancers. Current work is directed toward unraveling the molecular targets of AVT-219 in different in vivo models of colon cancer and further evaluating its safety profile.

Author Contributions

Participated in research design: Kashfi, Chattopadhyay, and Kodela.

Conducted experiments: Kodela, Chattopadhyay, and Duvalsaint.

Performed data analysis: Kashfi, Chattopadhyay, Kodela, and Duvalsaint.

Wrote or contributed to the writing of the manuscript: Kashfi, Chattopadhyay, Kodela, and Duvalsaint.

All authors have read the final version of the manuscript and have approved its content for publication.

Disclosures

The authors have nothing to disclose except for K. K., who has an equity position in Avicenna Pharmaceuticals, Inc. to which NOSH-naproxen (AVT-219) has been licensed.

References

American-Cancer-Society. 2015. What are the key statistics about colorectal cancer?. Available at http://www.cancer.org/cancer/colonandrectumcancer/detailedguide/colorectal-cancer-key-statistics (accessed in November 2015).

Baron JA, Cole BF, Sandler RS, Haile RW, Ahnen D, Bresalier R, et al. (2003). A randomized trial of aspirin to prevent colorectal adenomas. N Engl J Med 348: 891–899.

Best R, Lewis DA, Nasser N (1984). The anti-ulcerogenic activity of the unripe plantain banana (Musa species). Br J Pharmacol 82: 107–116.

Brown JF, Keates AC, Hanson PJ, Whittle BJ (1993). Nitric oxide generators and cGMP stimulate mucus secretion by rat gastric mucosal cells. Am J Physiol 265: G418–G422.

Chattopadhyay M, Velazquez CA, Pruski A, Nia KV, Abdellatif KR, Keefer LK, et al. (2010). Comparison between 3-nitrooxyphenyl acetylsalicylate (NO-ASA) and O2-(acetylsalicyloxymethyl)-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (NONO-ASA) as safe anti-inflammatory, analgesic, antipyretic, antioxidant prodrugs. J Pharmacol Exp Ther 335: 443–450.

Chattopadhyay M, Kodela R, Nath N, Barsegian A, Boring D, Kashfi K (2012a). Hydrogen sulfide-releasing aspirin suppresses NF-kappaB signaling in estrogen receptor negative breast cancer cells in vitro and in vivo. Biochem Pharmacol 83: 723–732.

Chattopadhyay M, Kodela R, Olson KR, Kashfi K (2012b). NOSH-aspirin (NBS-1120), a novel nitric oxide- and hydrogen sulfide-releasing hybrid is a potent inhibitor of colon cancer cell growth in vitro and in a xenograft mouse model. Biochem Biophys Res Commun 419: 523–528.

Cortese-Krott MM, Fernandez BO, Kelm M, Butler AR, Feelisch M (2015a). On the chemical biology of the nitrite/sulfide interaction. Nitric Oxide 46: 14–24.

Cortese-Krott MM, Kuhnle GG, Dyson A, Fernandez BO, Grman M, DuMond JF, et al. (2015b). Key bioactive reaction products of the NO/H2S interaction are S/N-hybrid species, polysulfides, and nitroxyl. Proc Natl Acad Sci USA 112: E4651–E4660.

Filipovic MR, Miljkovic J, Nauser T, Royzen M, Klos K, Shubina T, et al. (2012). Chemical characterization of the smallest S-nitrosothiol, HSNO; cellular cross-talk of H2S and S-nitrosothiols. J Am Chem Soc 134: 12016–12027.

Fiorucci S, Antonelli E, Distrutti E, Rizzo G, Mencarelli A, Orlandi S, et al. (2005). Inhibition of hydrogen sulfide generation contributes to gastric injury caused by anti-inflammatory nonsteroidal drugs. Gastroenterology 129: 1210–1224.

Fonesca MD, Cunha FQ, Kashfi K, Cunha TM (2015). NOSH-aspirin (NBS-1120), a dual nitric oxide and hydrogen sulfide-releasing hybrid, reduces inflammatory pain. Pharmacol Res Perspect 3: 1–12.

Harris RE, Beebe-Donk J, Doss H, Burr Doss D (2005). Aspirin, ibuprofen, and other non-steroidal anti-inflammatory drugs in cancer prevention: a critical review of non-selective COX-2 blockade (review). Oncol Rep 13: 559–583.

Harris RE, Beebe-Donk J, Alshafie GA (2008). Similar reductions in the risk of human colon cancer by selective and nonselective cyclooxygenase-2 (COX-2) inhibitors. BMC Cancer 8: 237.

Huang S, Chua JH, Yew WS, Sivaraman J, Moore PK, Tan CH, et al. (2010). Site-directed mutagenesis on human

cystathionine-gamma-lyase reveals insights into the modulation of H2S production. J Mol Biol 396: 708–718.

Kashfi K. Anti-inflammatory agents as cancer therapeutics. Adv Pharmacol 2009;57:31–89.

Kashfi K, Chattopadhyay M, Kodela R (2015). 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. Redox Biol 6: 287–296.

Kearney PM, Baigent C, Godwin J, Halls H, Emberson JR, Patrono C (2006). Do selective cyclo-oxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory drugs increase the risk of atherothrombosis? Meta-analysis of randomised trials. BMJ 332: 1302–1308.

King AL, Polhemus DJ, Bhushan S, Otsuka H, Kondo K, Nicholson CK, et al. (2014). Hydrogen sulfide cytoprotective signaling is endothelial nitric oxide synthase-nitric oxide dependent. Proc Natl Acad Sci USA 111: 3182–3187.

Kodela R, Chattopadhyay M, Kashfi K (2012). NOSH-aspirin: a novel nitric oxide-hydrogen sulfide-releasing hybrid: a new class of anti-inflammatory pharmaceuticals. ACS Med Chem Lett 3: 257–262.

Kodela R, Chattopadhyay M, Kashfi K (2013a). Synthesis and biological activity of NOSH-naproxen (AVT-219) and NOSH-sulindac (AVT-18A) as potent anti-inflammatory agents with chemotherapeutic potential. Med Chem Commun 4: 1472–1481.

Kodela R, Chattopadhyay M, Goswami S, Gan ZY, Rao PP, Nia KV, et al. (2013b). Positional isomers of aspirin are equally potent in inhibiting colon cancer cell growth: differences in mode of cyclooxygenase inhibition. J Pharmacol Exp Ther 345: 85–94.

Kodela R, Chattopadhyay M, Velazquez-Martinez CA, Kashfi K (2015). NOSH-aspirin (NBS-1120), a novel nitric oxide- and hydrogen sulfide-releasing hybrid has enhanced chemo-preventive properties compared to aspirin, is gastrointestinal safe with all the classic therapeutic indications. Biochem Pharmacol 98: 564–572.

Kulmacz RJ, Lands WE (1983). Requirements for hydroperoxide by the cyclooxygenase and peroxidase activities of prostaglandin H synthase. Prostaglandins 25: 531–540.

Kune G, Kune S, Watson L (1988). Colorectal cancer risk, chronic illnesses, operations, and medications: case-control results from the Melbourne Colorectal Cancer Study. Cancer Res 48: 4399–4404.

Li L, Bhatia M, Moore PK (2006). Hydrogen sulphide–a novel mediator of inflammation? Curr Opin Pharmacol 6: 125–129.

Li L, Rossoni G, Sparatore A, Lee LC, Del Soldato P, Moore PK (2007). Anti-inflammatory and gastrointestinal effects of a novel diclofenac derivative. Free Radic Biol Med 42: 706–719.

Nieswandt B, Watson SP (2003). Platelet-collagen interaction: is GPVI the central receptor? Blood 102: 449–461.

Olson KR (2009). Is hydrogen sulfide a circulating "gasotransmitter" in vertebrate blood? Biochim Biophys Acta 1787: 856–863.

Perini R, Fiorucci S, Wallace JL (2004). Mechanisms of nonsteroidal anti-inflammatory drug-induced gastrointestinal injury and repair: a window of opportunity for cyclooxygenase-inhibiting nitric oxide donors. Can J Gastroenterol 18: 229–236.

Pinto L, Borreli F, Bomberdelli E, Cristonic A, Capasso F (1998). Antiinflammatory, analgesis and antipyretic effects of glaucine in rats and mice. Pharm Pharmacol Communication 4: 502–505.

Predmore BL, Lefer DJ, Gojon G (2012). Hydrogen sulfide in biochemistry and medicine. Antioxid Redox Signal 17: 119–140.

Riendeau D, Charleson S, Cromlish W, Mancini JA, Wong E, Guay J (1997). Comparison of the cyclooxygenase-1 inhibitory properties of nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors, using sensitive microsomal and platelet assays. Can J Physiol Pharmacol 75: 1088–1095.

Rothwell PM, Price JF, Fowkes FG, Zanchetti A, Roncaglioni MC, Tognoni G, et al. (2012). Short-term effects of daily aspirin on cancer incidence, mortality, and non-vascular death: analysis of the time course of risks and benefits in 51 randomised controlled trials. Lancet 379: 1602–1612.

Sandler RS, Halabi S, Baron JA, Budinger S, Paskett E, Keresztes R, et al. (2003). A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. N Engl J Med 348: 883–890.

Wallace JL (1993). Gastric ulceration: critical events at the neutrophil–endothelium interface. Can J Physiol Pharmacol 71: 98–102.

Wallace JL (2008). Prostaglandins, NSAIDs, and gastric mucosal protection: why doesn't the stomach digest itself? Physiol Rev 88: 1547–1565.

Wallace JL, Miller MJ (2000). Nitric oxide in mucosal defense: a little goes a long way. Gastroenterology 119: 512–520.

Wallace JL, Reuter B, Cicala C, McKnight W, Grisham MB, Cirino G (1994). Novel nonsteroidal anti-inflammatory drug derivatives with markedly reduced ulcerogenic properties in the rat. Gastroenterology 107: 173–179.

Wallace JL, Dicay M, McKnight W, Martin GR (2007). Hydrogen sulfide enhances ulcer healing in rats. FASEB J 21: 4070–4076.

Winter CA, Risley EA, Nuss GW (1962). Carrageenin-induced Edema in hind Paw of the rat as an assay for antiiflammatory drugs. Proc Soc Exp Biol Med 111: 544–547.

Wolfe MM, Lichtenstein DR, Singh G (1999). Gastrointestinal toxicity of nonsteroidal antiinflammatory drugs. N Engl J Med 340: 1888–1899.

Zanardo RC, Brancaleone V, Distrutti E, Fiorucci S, Cirino G, Wallace JL (2006). Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. FASEB J 20: 2118–2120.