Biochemical Pharmacology 83 (2012) 715–722

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Hydrogen sulfide-releasing NSAIDs inhibit the growth of human cancer cells: A general property and evidence of a tissue type-independent effect

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ARTICLE INFO

Article history:
Received 14 September 2011
Accepted 15 December 2011
Available online 26 December 2011

Keywords:
Hydrogen sulfide
NSAIDs
Cancer prevention
COX-independent

ABSTRACT

Hydrogen sulfide-releasing non-steroidal anti-inflammatory drugs (HS-NSAIDs) are an emerging novel class of compounds with significant anti-inflammatory properties. They consist of a traditional NSAID to which an H_2S-releasing moiety is covalently attached. We examined the effects of four different HS-NSAIDs on the growth properties of eleven different human cancer cell lines of six different tissue origins. Human colon, breast, pancreatic, prostate, lung, and leukemia cancer cell lines were treated with HS-aspirin, -sulindac, -iburofen, -naproxen, and their traditional counterparts. HS-NSAIDs inhibited the growth of all cancer cell lines studied, with potencies of 28- to >3000-fold greater than that of their traditional counterparts. HS-aspirin (HS-ASA) was consistently the most potent. HS-NSAIDs inhibited cell proliferation, induced apoptosis, and caused G_0/G_1 cell cycle block. Metabolism of HS-ASA by colon cells showed that the acetyl group of ASA was hydrolyzed rapidly, followed by hydrolysis of the ester bond linking the salicylate anion to the H_2S releasing moiety, producing salicylic acid and ADT-OH from which H_2S is released. In reconstitution studies, ASA and ADT-OH were individually less active than the intact HS-ASA towards cell growth inhibition. Additionally, the combination of these two components representing a fairly close approximation to the intact HS-ASA, was 95-fold less active than the intact HS-ASA for growth inhibition. Taken together, these results demonstrate that HS-NSAIDs have potential anti-growth activity against a wide variety of human cancer cells.

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1. Introduction

Hydrogen sulfide (H_2S), along with nitric oxide (NO) and carbon monoxide (CO), forms part of a group of biologically active gases that are termed gasotransmitters or gasomediators [1]. These gases have attracted attention for their roles in several intracellular signaling processes. At appropriate concentrations, they play a fundamental role in the regulation of a variety of cellular and physiological functions. For example, they act on the gastrointestinal smooth muscle inhibiting contraction [2,3], and on the cardiovascular system causing vasodilatation, promoting angiogenesis and exerting a key role in cardioprotection [1]. In addition, they are also involved in the modulation of sepsis by mediating protective effects through reduction in inflammation [4]. Of these three endogenous gases, NO has been undoubtedly the most widely investigated and characterized. H_2S is emerging as an important endogenous modulator, which exhibits the same beneficial effects as NO on the cardiovascular system [5–7], and has beneficial effects as an anti-inflammatory and antiperoxidative agent in inflammation, oxidative stress and angiogenesis [8].

Novel H_2S-releasing drugs have been developed in order to conjugate the beneficial effect of H_2S with other pharmaceuticals. For example, hydrogen sulfide-releasing NSAIDs (HS-NSAIDs) have been designed through the conjugation of the parent NSAID with a dithiolethione moiety [5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione] (ADT-OH) which releases H_2S [9,10]. In animal models, HS-NSAIDs release the parent compound, which act according to their own pharmacological properties while the H_2S released has marked activity against inflammatory cells including a cytoprotective effect in non-inflamed tissue [10]. More specifically, stronger reduction in hindpaw swelling by H_2S-releasing hybrid of diclofenac compared to diclofenac has been reported [11]. This hybrid has also shown to have a safer gastrointestinal profile [12] and be as effective as diclofenac in inhibiting both COX-1 and

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0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.12.018

COX-2 enzymatic activities [13]. Similarly, HS-indomethacin and HS-naproxen are more GI sparing than their traditional counterparts [12,14]. HS-mesalamin exhibits a marked increase in anti-inflammatory activity and potency in a murine model of colitis, compared to mesalamine [15,16], while GYY4137, a slow-releasing H_2S donor, has anti-inflammatory properties in a mouse model of endotoxic shock [17].

Research in the field of HS-NSAIDs is in its infancy. To our knowledge there have been no reports describing the effects of HS-NSAIDs on growth inhibition of any human cancer cell lines or in any in vivo animal models of cancer. In the present study we evaluated the effects of four different HS-NSAIDs on the growth properties of eleven different human cancer cell lines of six different tissue origins. These cell lines are of adenomatous (colon, pancreatic, lung, prostate), epithelial (breast), and lymphocytic (leukemia) origin. In the accompanying two manuscripts, we examined various molecular targets of H_2S-releasing aspirin, its effects on NF-κB, which is central to the inflammatory process and on drug metabolizing enzymes responsible for metabolism and elimination of xenobiotics. Our results establish the remarkable potency of these compounds on a wide variety of tissue molecular targets and suggest a strong clinical potential for these compounds.

2. Materials and methods

2.1. Reagents

HS-aspirin (HS-ASA), [4-(5-thioxo-5H-1,2-dithiol-3-yl)-phenyl 2-acetoxybenzoate], HS-naproxen (HS-NAP), [2-(6-methoxy-naphthalen-2-yl)-propionic acid 4-(5-thioxo-5H-[1,2]dithiol-3-yl)-phenyl ester], HS-Sulindac (HS-SUL), [(Z)-5-fluoro-2-methyl-1-[[4-(methylsulfinyl) phenyl]-methylene]-1H-indene-3-acetic acid 4-(5-thioxo-5H-1,2-dithiol-3-yl)-phenyl ester] and HS-ibuprofen (HS-IBU), [4-(5-thioxo-5H-1,2-dithiol-3-yl)-phenyl 2-(4-isobutyphenyl)propanoate] were synthesized, purified and verified by ^{1}H NMR. Stock (100 mM) solutions of HS-NSAIDs and their corresponding NSAIDs were prepared in DMSO (Fisher Scientific, Fair Lawn, NJ). Traditional NSAIDs and fine chemicals were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Synthesis of HS-NSAIDs (HS-ASA, HS-NAP, HS-SUL, and HS-IBU)

The 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione (ADTOH) was prepared from anethole and sulfur heated at 200 °C for 6 h to obtain anethole dithione, subsequently undergo demethylation in the presence of pyridine hydrochloride to yield the 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione (ADTOH) [10]. All HS-NSAIDs were prepared by adding their NSAID counterparts with ADT-OH, dicyclohexylcarbodiimide (DCC) and a catalytic amount of 4-dimethylaminopyridine (DMAP) in dichloromethane [10]. All the compounds were purified by column chromatography and characterized by ^{1}H NMR spectroscopy, Fig. 1 shows the HS-NSAIDs synthesized.

(1) HS-ASA [4-(5-thioxo-5H-1,2-dithiol-3-yl)-phenyl 2-acetoxybenzoate]: ^{1}H NMR ($CDCl_3$, 500 MHz): 8.23 (d, J = 7.8 Hz, 1H), 7.74 (d, J = 8.3 Hz, 2H), 7.68 (t, J = 7.8 Hz, 1H), 7.43 (t, J = 7.8 Hz, 2H), 7.34 (d, J = 8.8 Hz, 2H), 7.20 (d, J = 7.8 Hz, 1H) 2.32 (s, 3H).
(2) HS-NAP [2-(6-methoxy-naphthalen-2-yl)-propionic acid 4-(5-thioxo-5H-[1,2] dithiol-3-yl)-phenyl ester]: ^{1}H NMR ($CDCl_3$, 500 MHz): 7.80 (d, J = 7.8 Hz, 2H), 7.77 (d, J = 8.8 Hz, 1H), 7.65 (d, J = 8.8 Hz, 2H), 7.51 (dd, J = 8.8 Hz, 1.5 Hz, 1H), 7.39 (s, 1H), 7.20 (dd, J = 8.8 Hz, 2.4 Hz, 1H), 7.17 (d, J = 2.4 Hz, 1H), 7.14 (d, J = 8.8 Hz, 2H), 4.15 (q, J = 7.3 Hz, 1H), 3.94(s, 3H), 1.73 (d, J = 7.32 Hz, 3H).
(3) HS-SUL [(Z)-5-fluoro-2-methyl-1-[[4-(methylsulfinyl) phenyl]-methylene]-1H-indene-3-acetic acid 4-(5-thioxo-5H-1,2-dithiol-3-yl)-phenyl ester]: ^{1}H NMR ($CDCl_3$, 500 MHz): 7.74 (d, J = 7.8 Hz, 2H), 7.69 (t, J = 8.8 Hz, 3H), 7.38 (s, 1H), 7.23 (d, J = 8.8 Hz, 3H), 7.19 (dd, J = 8.8 Hz, 1.5 Hz, 2H), 6.98 (dd, J = 7.8 Hz, 1.5 Hz, 1H), 6.60 (t, J = 7.8 Hz, 1H), 3.83 (s, 2H), 2.81 (s, 3H), 2.30 (s, 3H).
(4) HS-IBU [4-(5-thioxo-5H-1,2-dithiol-3-yl)-phenyl 2-(4-isobutyphenyl) propanoate]: ^{1}H NMR ($CDCl_3$, 500 MHz): 7.64 (d,

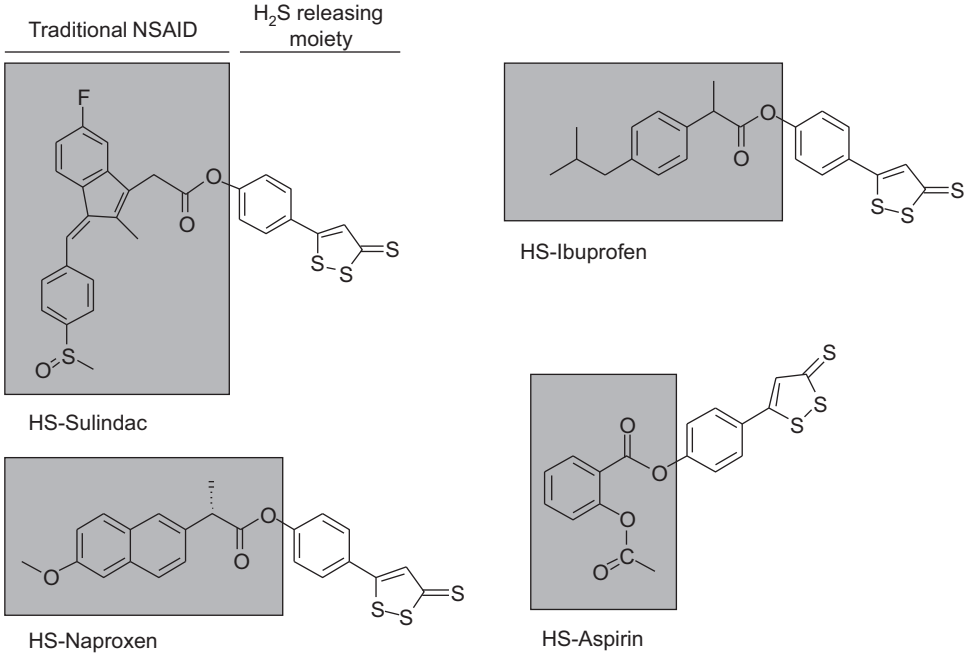

Fig. 1. The chemical structures of HS-NSAIDs. The structural components of HS-aspirin (salicylic acid derivative), HS-sulindac (indole derivative), and HS-ibuprofen, HS-naproxen (arylpropionic acid derivative) are indicated; the traditional NSAID is shown in the shaded box; this is covalently attached to a dithiolethione moiety [5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione] which can release hydrogen sulfide.

J = 8.3 Hz, 2H), 7.37 (s, 1H), 7.28 (d, J = 7.8 Hz, 2H), 7.14 (d, J = 7.8 Hz, 4H), 3.96 (q, J = 7.3 Hz, 1H), 2.48 (d, J = 7.32 Hz, 2H). 1.87 (m, 1H), 1.62 (d, J = 7.32 Hz, 3H), 0.91 (d, J = 7.32 Hz, 3H).

2.3. Cell culture

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, MDA-MB-231 and SK-BR-3 human breast cancer and Jurkat T cell human leukemia cell lines 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 μg/mL) (Invitrogen, Carlsbad, CA). Cells were seeded on culture dishes at a density of 25×10^3 cells/cm^2 and incubated at 37 °C in 5% CO_2 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.

2.4. Growth inhibition

Cell growth inhibitory effect of all HS-NSAIDs was measured using a colorimetric MTT assay kit (Roche, Indianapolis, IN). Cancer cells were plated in 96-well plates at a density of 30,000–50,000 cells/well depending on cell type. The cells were incubated for 24 h with different concentrations of HS-NSAIDs. After the indicated time, 10 μL 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 μL of the solubilization solution (10% SDS in 0.01 M HCl) was added to each well to solubilize the formant crystals. The absorbance of the plates was measured on a spectrophotometric plate reader at a wavelength of 570 nm. Each experiment was performed in triplicate, and the entire experiment was repeated three times.

2.5. Cell proliferation

Proliferating cell nuclear antigen (PCNA) was determined using an ELISA Kit (Calbiochem, La Jolla, CA), in accordance with the manufacturers protocol. HT-29 cells were incubated with serum-free media for 24 h to remove the effect of endogenous growth factors. The cells (1×10^6 cells/mL) were then treated for 24 h with various concentrations of the four HS-NSAIDs. In brief, we made a suspension of 1×10^6 HT-29 cells/mL in suspension buffer (5 mM EDTA, 0.2 mM PMSF, 1 μg/mL pepstatin, 0.5 μg/mL leupeptin, and 50 mM Tris–HCl, pH 8.0). Samples of the suspension were pipetted into the wells of the plate enclosed with the kit, where rabbit polyclonal antibody, specific for the human PCNA protein was immobilized. The mouse monoclonal antibody clone PC10 was then added to each well as a detector antibody and the mixture was incubated for 2 h at room temperature. After the wells had been washed, horseradish peroxidase streptavidin was added and the plates were incubated for 30 min at room temperature. The chromogenic substrate tetramethylbenzidine was then added, and the plates were again incubated for a further 30 min. Finally, the stop solution was added and the absorbance of the solutions in the wells was measured at 450 nm with a spectrophotometric plate reader.

2.6. 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 MultigraphTM and MulticycleTM. HT-29 Cells (0.5×10^6) treated with various concentrations of HS-NSAIDS were fixed in 100% methanol for 10 min at −20 °C, pelleted (5000 rpm × 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 μL of PBS/1% FBS containing 40 μg/mL propidium iodide (used to stain for DNA) and 200 μg/mL RNase type IIA, and analyzed within 30 min by flow cytometry. The percentage of cells in G_0/G_1, G_2/M, and S phases was determined from DNA content histograms.

2.7. Assay for apoptosis

HT-29 cells (0.5×10^6 cells/mL) were treated for 24 h with various concentrations of the four HS-NSAIDs. Cells were washed with and resuspended in 1× Binding Buffer (Annexin V binding buffer, 0.1 M HEPES/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$; BD BioSciences Pharmingen, San Diego, CA). Then, 5 μL of Annexin V-FITC (final concentration: 0.5 mg/mL) was added followed by 5 μL propidium iodide as a counterstain. 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 were analyzed by Flow Jo software.

2.8. HPLC analyses of HS-ASA metabolites

HS-ASA, its de-acetylated derivative and ADT-OH were prepared as described above. Salicylic acid, HPLC grade solvents and standard organic compounds were obtained from Fisher Chemicals (Bridgewater, NJ). HT-29 cells in McCoy's 5 A medium were treated with HS-ASA for 1 min, 5 min, 10 min, 15 min, 30 min, 60 min, 2 h, 3 h, 5 h, 6 h and 24 h, after which media was taken out, filtered and immediately subjected HPLC analysis. We used a Shimadzu HPLC system which consisted of two pumps LC-6AD with an automated gradient solvent delivery controller, UV–vis detector SPD-20A/SPD-20AV prominence and LiChrospher C18 reverse-phase column (250 mm × 4.6 mm; particle size, 5 mm; Varian, Lakefarest, CA) with a manual sample injector. Binary phase system, buffer A consisted of 0.05% (v/v) trifluoroacetic acid (TFA); buffer B consisted of acetonitrile. The flow rate was 1 mL/min. We applied gradient elution from 100% buffer A to 70% buffer B from 0 to 30 min; it was maintained at 70% buffer B for 45 min. Analyses were recorded at 435 nm wavelength.

2.9. Statistical analysis

In vitro data are presented as mean ± SEM for at least three different sets performed in triplicate. In vivo treatment groups and number of animals in each group are indicated in the figure legend. Comparison between treatment groups was done using Student's t tests, p-values <0.05 were considered significant.

Table 1
IC_{50} (μM) values for cell growth inhibition by different HS-NSAIDs in different cancer cell lines.

Agent	Colon			Breast			Pancreas		Lung	Prostate	Leukemia
	HT-29	HCT15	SW480	MDA MB231	SKBR3	MCF7	MIAPaCa2	BxPC3	A549	LNCAP	Jurkat
ASA	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	4560 ± 210	>5000
HS-ASA	3.7 ± 0.9	3.2 ± 1.1	1.8 ± 0.6	3.6 ± 0.6	3.3 ± 0.9	4.2 ± 1.1	2.1 ± 0.9	1.9 ± 0.7	1.6 ± 0.7	2.3 ± 0.8	2.3 ± 0.8
Ratio	>1300	>1500	>2700	>1300	>1500	>1100	>2300	>2600	>3100	1983	>2100
NAP	2730 ± 121	3000 ± 189	2980 ± 151	3260 ± 125	3110 ± 129	2550 ± 121	3300 ± 150	2160 ± 128	2770 ± 133	3170 ± 165	2650 ± 161
HS-NAP	72 ± 2	66 ± 2	71 ± 3	51 ± 2	58 ± 2	63 ± 3	81 ± 3	76 ± 2	69 ± 2	74 ± 2	59 ± 2
Ratio	38	45	42	64	54	40	41	28	40	43	45
SUL	740 ± 32	836 ± 43	678 ± 46	987 ± 37	865 ± 41	1045 ± 62	834 ± 29	985 ± 30	147 ± 22	871 ± 36	736 ± 33
HS-SUL	6 ± 1.6	7 ± 0.9	8 ± 2	10 ± 2	9 ± 1	10 ± 1	7 ± 1	5 ± 1	7 ± 2	7 ± 1	5 ± 0.9
Ratio	123	119	85	99	96	105	119	197	21	124	147
IBU	1090 ± 77	1120 ± 120	1135 ± 121	789 ± 47	763 ± 53	843 ± 61	567 ± 40	621 ± 51	968 ± 40	1075 ± 92	897 ± 27
HS-IBU	2.8 ± 0.9	3.5 ± 0.7	3.1 ± 0.8	5.4 ± 0.7	6.2 ± 0.9	4.1 ± 0.9	2.9 ± 0.8	3.7 ± 0.9	1.9 ± 0.5	2.8 ± 0.9	4.3 ± 0.7
Ratio	389	320	366	146	123	206	196	168	509	384	209

Colon, breast, pancreas, lung, prostate, and leukemia cancer cell lines were treated with various concentrations of HS-ASA, HS-NAP, HS-SUL, HS-IBU and their traditional counterparts as described in Section 2. Cell numbers were determined at 24 h from which IC50 values were calculated. The ratios of NSAID/HS-NSAID represent fold-enhancement in potency of the HS-NSAID over the parent compound. Results are mean ± SEM of at least four different experiments performed in triplicates. $P < 0.001$ for all HS-NSAIDs compared to their respective NSAID in all cell lines

3. Results

3.1. HS-NSAIDs inhibit the growth of various human cancer cell lines

We investigated the effects of HS-ASA, HS-SUL, HS-NAP, and HS-IBU and their respective parent compounds on the growth properties of eleven 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 SKBR-3: [ER(−)]); T-cell leukemia (Jurkat), pancreatic (BxPC3: both COX-1 and COX-2 positive, MIAPaCa-2: COX-null), prostate (LNCaP), and lung (A549). All four HS-NSAIDs were extremely effective in inhibiting the growth of these cell lines (Table 1). It is noteworthy that ASA was the least potent of the four NSAIDs in inhibiting the growth of all the various cell lines, yet HS-ASA was the most potent HS-NSAID. The IC_{50}s for cell growth inhibition for HS-ASA ranged from 1.6 ± 0.7 to 4.2 ± 1.1 μM. The corresponding IC_{50} values for HS-NAP, HS-SUL and HS-IBU were 51–81, 4.6–10.2 and 1.9–6.2 μM, respectively. The potency of HS-IBU, HS-SUL and HS-NAP were 2, 3 and 20-fold lower than that of HS-ASA. Therefore, HS-ASA was more potent than other HS-NSAIDs followed by HS-IBU, HS-SUL and then HS-NAP.

The growth inhibition by HS-NSAIDs versus their traditional NSAID counterparts was very high in the cell lines studied. In a fold comparison study of the IC_{50} values (Traditional/HS-NSAIDs), HS-ASA was at least 3000-fold more potent than ASA in A549 lung cancer cells. In all the cell lines examined, the IC_{50} ratios (fold potency increase) for HS-SUL and HS-NAP were 90 and 60, respectively, compared to their parent compounds. In A549 lung cells, HS-SUL was 20-fold more potent than the parent compound. Compared to Ibuprofen, HS-IBU was more potent by 200-fold in colon, prostate and lung cancer cells, 80-fold more potent in breast cancer cells, and 120-fold in pancreatic cancer cells. Such fold increases imply that the H_2S-related structural modification of the parent molecules imparts a differential enhancement in potency. Among the HS-NSAIDS, HS-ASA was determined to be the most potent H_2S releasing drug.

3.2. Effect of HS-NSAIDs on cell growth kinetics

In view of the growth-inhibitory effects of all the HS-NSAIDs in different cancer cell lines, we further examined their effects on cell proliferation, apoptosis, and cell cycle transition, all of which affect cell growth. For these studies using HT-29 cells, we chose concentrations of HS-ASA at its IC_{50} (4 μM, Table 1), half its IC_{50} (2 μM) and twice its IC_{50} (8 μM) and likewise for HS-NAP, HS-SUL and HS-IBU we used their respective IC_{50}, 0.5× IC_{50}, and 2× IC_{50} (Table 1).

3.2.1. Cell proliferation

To determine the antiproliferative effects of these four HS-NSAIDs, HT-29 cells were treated with different concentration of the drugs for 24 h, followed by PCNA quantification. HS-ASA, HS-NAP, HS-SUL and HS-IBU reduced proliferation in a dose-dependent manner, as measured by the expression of PCNA (Fig. 2A). For HS-ASA, the proliferation decreased to 53.7 ± 3%, and 39.6 ± 4% at IC_{50} (4 μM) and 2× IC_{50} (8 μM), respectively, compared to the untreated control. Antiproliferative effect of HS-NAP was the highest among the four compounds; PCNA expression was reduced to 49.1 ± 3% and 28 ± 2% at its IC_{50} (72 μM) and 2× IC_{50} (144 μM), respectively, compared to control. HS-IBU decreased cell proliferation to 32.1 ± 2% when treated with 2× IC50 (6 μM). HS-SUL was the least effective among the four compounds; PCNA expression was reduced to 46.1 ± 3% at 2× IC_{50} (12 μM).

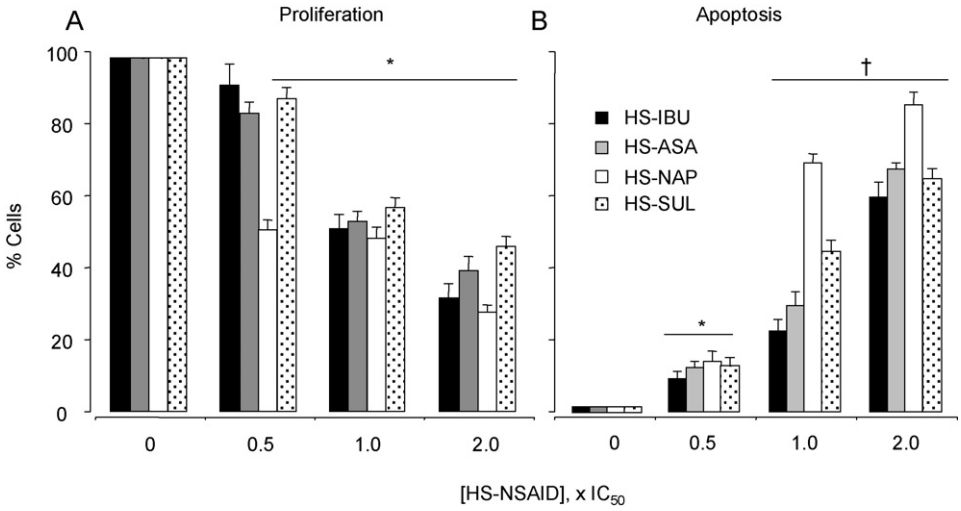

Fig. 2. Effect of HS-NSAIDs on HT-29 colon cancer cell kinetics. Cells were treated with the respective HS-NSAIDs at the concentration indicated for 24 h. Apoptosis (panel A) and PCNA (panel B) were determined as described in Section 2. Results are mean ± SEM of three different experiments. $^{*}P < 0.05$; $^{\dagger}P < 0.01$ compared with untreated cells. IC_{50}s for HS-ASA, HS-NAP, HS-SUL, and HS-IBU were 3.7 ± 0.9, 72 ± 2, 6 ± 1.6, and 2.8 ± 0.9 μM, respectively.

3.2.2. Apoptosis

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. Actively dividing HT-29 colon cancer cells were treated with HS-NSAIDs for 6 h. A significant increase in the number of cells undergoing apoptosis was observed (Fig. 2B). For HS-ASA, the percentage of apoptotic HT-29 cells increased from 30 ± 4% at 1× IC_{50} to 68 ± 2% at 2× IC_{50}, compared to control. Induction of apoptosis by HS-NAP ranged from 64 ± 3% 1× IC_{50} to 85 ± 4% at 2× IC_{50}, while for HS-SUL, the apoptotic population ranged from 44 ± 3% at 1× IC_{50} to 64 ± 3% 2× IC_{50}. HS-IBU increased the apoptotic population to 60 ± 3% at 2× IC_{50} compared to untreated control. Among the HS-NSAIDs studied, HS-NAP was the strongest contributor to apoptosis, whereas HS-IBU was the least (Fig. 2B).

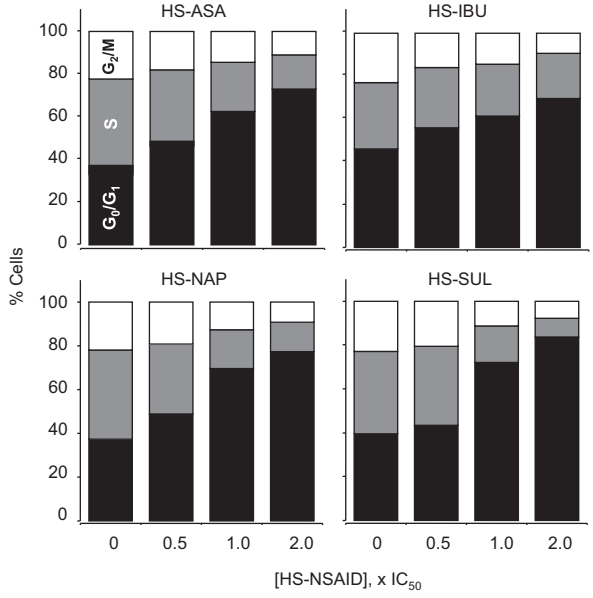

Fig. 3. Effect of HS-NSAIDs on cell cycle. Colon HT-29 cells were treated with various HS-NSAIDs and harvested at 24 h. The percentage of cells in each phase was determined by flow cytometry, as described in Section 2. Results are representative of two different experiments. This study was repeated twice generating results within 10% of those presented here.

3.2.3. Cell cycle

We determined the effect of the HS-NSAIDS on the distribution of cells in G_0/G_1, S, and G_2/M phases of the cell cycle. HT-29 cells were exposed to the HS-NSAIDs at concentrations of 0.5× IC_{50}, 1× IC_{50}, and 2× 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 HS-NSAIDs were associated with dose-dependent increase in the percentage of cells in G_0/G_1 phase, and this accumulation was accompanied by a corresponding reduction in the percentages of cells in S and G_2/M phases (Fig. 3). Since more pronounced effects were observed at 2× IC_{50} for HS-ASA, HS-NAP, and HS-IBU, here we highlight the changes observed for these HS-NSAIDs at these respective concentrations. Specifically, 8 μM (2× IC_{50}) of HS-ASA increased the population of cells in G_0/G_1 phase from 32.7% to 73.1%, with simultaneous decreases of cell populations in S phase from 45.1% to 16.0%, and G_2/M phase from 22.2% to 10.6%, compared to control. Similarly, 144 μM (2× IC_{50}) of HS-NAP increased the population of cells in G_0/G_1 phase from 39.7% to 79.6%, while it decreased the percentage of cells in S phase from 37.4% to 8.6%, and G_2/M from 22.9% to 7.8%, respectively, compared to control. For HS-IBU at 6 μM (2× IC_{50}), a prominent increase in G_0/G_1 cells (from 48.3% to 69.4%) was also observed. HS-SUL caused accumulation of cells in G_0/G_1; 6 μM (1× IC_{50}) increased the population of cells in G_0/G_1 phase from 38.2% to 77.3%, and decreased the percentage of cells in S phase from 40.7% to 13.3%, and G_2/M from 21.9% to 11.2%, respectively.

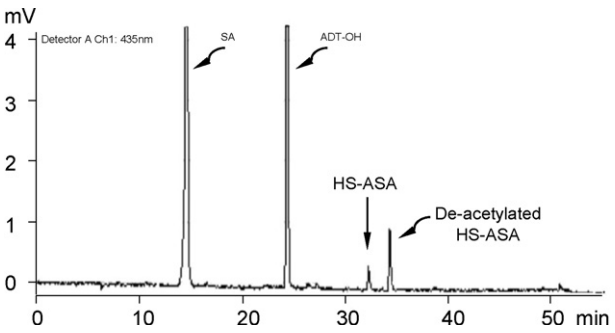

Fig. 4. Metabolism of HS-ASA by HT-29 colon cancer cells. HS-ASA, 50 μM, was incubated for 6 h with HT-29 human colon cancer cells, metabolites were extracted from the culture medium and fractionated by HPLC as described in Section 2.

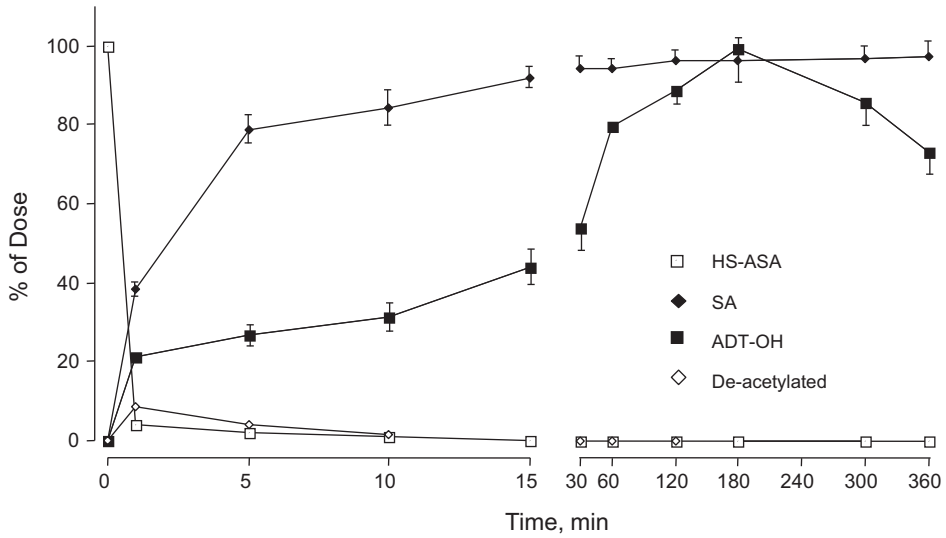

Fig. 5. Metabolism of HS-ASA by HT-29 colon cancer cells. HS-ASA, 50 μM, was incubated for up to 360 min with HT-29 human colon cancer cells, extracted, and fractionated by HPLC as described in Section 2. Values were calculated based on area under the peak determinations, and data were expressed as percentage of input HS-ASA. Results are mean ± range of two different experiments done in duplicate.

3.3. Metabolism of HS-ASA by HT-29 colon cancer cells

Since HS-ASA was characterized with a very low IC_{50} for cell growth inhibition, we focused on this compound for metabolism studies in HT-29 colon cancer cells. HT-29 cells were treated with a high concentration of 50 μM HS-ASA for 6 h and metabolites determined from the culture medium HPLC fractionation as described in Section 2. Several metabolites of HS-ASA were detected and identified (Fig. 4). HS-ASA and its deacetylated metabolite were detected in low amounts. The major metabolites were ADT-OH, which is the H_2S releasing moiety, and salicylic acid. In a time course study of 6 h (Fig. 5), HS-ASA was rapidly hydrolyzed in 30 min yielding salicylic acid and ADT-OH. The deacetylated derivative of HS-ASA was produced within 2 min which was also rapidly hydrolyzed, as expected, to ADT-OH and salicylic acid. The appearance of the metabolite ADT-OH paralleled the kinetics of deacetylated HS-ASA disappearance in the first 15 min, and was fairly stable throughout the remaining time evaluated, reaching its maximal level at 3 h, while still maintaining high levels by 6 h. On the other hand, salicylic acid formed was maximal at 15 min followed by a plateau effect. Since deacetylated-HS-ASA and concomitant ADT-OH production occurred very rapidly in less than 5 min, it is possible that the metabolites are not secreted by the cells but are generated before entry into the cells.

3.4. SAR and reconstitution studies

A structure–activity and reconstitution study of the individual components of HS-ASA; ASA and ADT-OH, was performed in HT-29 cells in order to determine equivalency of HS-ASA to the sum of its parts. We examined cell growth inhibitory function of intact HS-ASA molecule and the combination of ASA and ADT-OH. For the combination, various concentrations of ASA were combined with different fixed concentrations of ADT-OH. Such simulation of intact HS-ASA using ASA plus ADT-OH represents a fairly close approximation to the intact HS-ASA.

The growth inhibition curves of HT-29 cells were analyzed with these combinations, the respective IC_{50}s of ASA in these were evaluated for a possible shift. Table 2 shows that combined ASA and ADT-OH had a synergistic effect in terms of cell growth inhibition, but the respective IC_{50}s of ASA in the combinations were far higher than those of HS-ASA. In other words, the combination of these two molecules did not reconstitute the potency of HS-ASA. These findings indicate that neither ASA, ADT-OH nor the combined molecular components can completely account for the biological activity of intact HS-ASA and that these constituents may only, in part, contribute to its activity.

Table 2
IC_{50} values for cell growth inhibition in HT-29 cells.

Treatment (μM)	IC_{50} (μM)
ASA	>1000
HS-ASA	4.0 ± 0.8[†]
ADT-OH	27.5 ± 1.8
ASA + ADT-OH	380 ± 45

Cells were treated with various concentrations of test agents shown above as described in Section 2. Cell numbers were determined at 24 h from which IC_{50} values were calculated. Results are mean ± SEM of four different experiments performed in triplicates.

[†] $P < 0.001$ compared to all other treatment groups.

4. Discussion

In the present study, we demonstrated that four different H_2S-releasing NSAIDs inhibit the growth of several cancer cell lines arising from a variety of tissue types such as colon, breast, pancreas, lung prostrate and T cell leukemia. These HS-NSAIDs were more potent than their traditional counterparts, with enhanced potency ranging from 21 to greater than 3000-fold. Of the four HS-NSAIDs evaluated here, HS-ASA was consistently the most potent in all cell lines tested, and in some cases this enhancement was in excess of 140-fold over the other HS-NSAIDs. It is noteworthy that the potency of an NSAID in inhibiting cell growth does not predict the potency of the corresponding HS-NSAID. For example, ASA was the weakest of the four traditional NSAIDs in inhibiting the growth of any of the cell lines, yet HS-ASA was the most potent. Since the H_2S-releasing moiety in the four HS-NSAIDs was the same and ASA was the weakest in terms of its potency in cell growth inhibition, it may be inferred that enhanced effects may be imparted predominantly by the H_2S contributing moiety. Further, our data indicate that this effect may be tissue-type independent since the HS-NSAIDs were effective against adenomatous, epithelial, and lymphocytic cancer cell lines and all were susceptible to different HS-NSAIDs. Here we studied eleven

cell lines originating from six different tissues, therefore, it may be envisaged that our findings are part of a generalized effect, especially since all cell types responded, although in a differential manner. However, more studies are needed to further substantiate the generalization of this property.

An interesting aspect of growth inhibition also emerges with respect to COX expression in the cell lines examined. All HS-NSAIDs showed similar effects on two colon cancer cell lines, HT-29 (expresses COX-1 and COX-2) and HCT 15 (no COX expression) [18], and on two pancreatic cancer cell lines, BxPC-3 (expresses COXs) and MIA PaCa-2 (no COX expression) [19], suggesting a COX-independent effect.

This study also demonstrates that HS-NSAIDs achieve their growth inhibitory effect in cultured colon cancer cells through a complex effect on cell kinetics, which include inhibiting cell proliferation, inducing apoptosis and altering the cell cycle. After 24 h, the HS-NSAIDs, at concentrations close to their IC_{50}s inhibited PCNA expression by roughly about 45%. Another contributor to their growth inhibitory effect may be the block in the cell cycle phase transitions induced by these compounds, such as the G_0/G_1 block that we noted in colon cancer cells. Traditional NSAIDs are known to profoundly affect cell cycle transitions through changes in proteins that control them [20,21], these effects may be enhanced by H_2S, as yet to be determined.

In a reconstitution study using HT-29 cells, two major constituents of HS-ASA, namely ASA and the H2S-releasing moiety, ADT-OH, were individually less active than the intact HS-ASA for cell growth inhibition. ASA was poorly effective as expected while ADT-OH was about 7 times less active (IC_{50} = 27 μM, Table 2). Additionally, the combination of these two constituents representing a fairly close approximation to HS-ASA, was 120-fold less active than intact HS-ASA. Since the effect of the parts did not equal the effect of the whole molecule, it suggests that ADT-OH may be contributing partially to the overall cell growth inhibition, but more importantly, that the intact HS-ASA molecule may be the predominant molecule important for its growth inhibitory effect. We also speculate that with the intact molecule, H_2S may be released over a long period of time. We are currently evaluating this. Regarding ASA as a component of HS-ASA, it is known to be a poor inhibitor of cancer cell growth [22]. It is also well established that ASA does not undergo metabolism that would improve its biological activity. The major transformation product from the ASA moiety of HS-ASA is salicylic acid, shown here and in agreement with previous studies [23]. Based on our results from HT-29 cells, future goals will attempt to define the metabolic steps of biotransformation and its kinetics by the liver, which has a complete drug metabolizing enzyme system.

The role of H_2S as a gasotransmitter is believed to be rather diverse, depending in part, on its concentration, and additionally may be ascribed to the difference in cell types, culture conditions, and durations of H_2S administration. For example, H_2S was cytoprotective and pro-proliferative on various types of normal cells such as cardiomyocytes [24], neutrophils [25] and endothelial cells [26]. A recent study showed that an H_2S donor, NaHS increased human colon cancer cell proliferation at 200 mmol/L [27] On the other hand, higher level of NaHS (1000 mmol/L) had no effect on HCT 116 cell proliferation and even inhibited SW480 cell proliferation [27]. Antiproliferative and pro-apoptotic effects of H_2S were demonstrated in smooth muscle cells [28], lung fibroblast cells [11] and HEK-293 kidney cells [29]. This diversity is similar to nitric oxide (NO) which is another important gaseous transmitter, that exerts a wide variety of biological effects. It has been well documented that lower NO concentrations promote cell survival and proliferation, whereas higher levels favor cell cycle arrest, apoptosis, and senescence (reviewed in ref. [30]). For example, NO has been shown to inhibit cell growth and induce apoptosis in colon cancer cells [31,32], whereas its tumor-promoting effects have also been reported [30,33], and may be ascribed mainly to levels of NO. It may be envisaged that such dichotomy also exists for H_2S which produce a specific cellular response.

In our study taken together, the induction of cell death appears to be a more prominent effect of HS-NSAIDs on colon cancer cell line, along with inhibition of proliferation, and may represent a common property targeted by these agents. On the basis of these results we speculate that H_2S liberated by these compounds activates or enhances cell death. Further work is directed to address these complex issues.

Acknowledgment

Supported in part by the National Cancer Institute through a subcontract from ThermoFisher, contract # FBS-43312-26.

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