

RESEARCH PAPER

Modulation of angiogenesis by dithiolethione-modified NSAIDs and valproic acid

JS Isenberg¹, Y Jia¹, L Field¹, LA Ridnour², A Sparatore³, P Del Soldato⁴, AL Sowers², GC Yeh⁵, TW Moody⁶, DA Wink², R Ramchandran¹ and DD Roberts¹

¹Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA; ²Radiation Biology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA; ³Istituto di Chimica Farmaceutica, University of Milan, Milan, Italy; ⁴CTG Pharma, Milan, Italy; ⁵Laboratory of Metabolism, NCI-Frederick, Frederick, MD, USA and ⁶Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

Background and purpose: Angiogenesis involves multiple signaling pathways that must be considered when developing agents to modulate pathological angiogenesis. Because both cyclooxygenase inhibitors and dithioles have demonstrated anti-angiogenic properties, we investigated the activities of a new class of anti-inflammatory drugs containing dithiolethione moieties (S-NSAIDs) and S-valproate.

Experimental approach: Anti-angiogenic activities of S-NSAIDs, S-valproate, and the respective parent compounds were assessed using umbilical vein endothelial cells, muscle and tumor tissue explant angiogenesis assays, and developmental angiogenesis in Fli:EGFP transgenic zebrafish embryos.

Key results: Dithiolethione derivatives of diclofenac, valproate, and sulindac inhibited endothelial cell proliferation and induced Ser⁷⁸ phosphorylation of hsp27, a known molecular target of anti-angiogenic signaling. The parent drugs lacked this activity, but dithiolethiones were active at comparable concentrations. Although dithiolethiones can potentially release hydrogen sulphide, NaSH did not reproduce some activities of the S-NSAIDs, indicating that the dithioles regulate angiogenesis through mechanisms other than release of H₂S. In contrast to the parent drugs, S-NSAIDs, S-valproate, NaSH, and dithiolethiones were potent inhibitors of angiogenic responses in muscle and HT29 tumor explants assessed by 3-dimensional collagen matrix assays. Dithiolethiones and valproic acid were also potent inhibitors of developmental angiogenesis in zebrafish embryos, but the S-NSAIDs, remarkably, lacked this activity.

Conclusions and implication: S-NSAIDs and S-valproate have potent anti-angiogenic activities mediated by their dithiole moieties. The novel properties of S-NSAIDs and S-valproate to inhibit pathological versus developmental angiogenesis suggest that these agents may have a role in cancer treatment.

British Journal of Pharmacology (2007) **151**, 142–151. doi:10.1038/sj.bjp.0707198; published online 12 March 2007

Keywords: angiogenesis inhibitors; hsp27 phosphorylation; dithioles; nonsteroidal anti-inflammatory drugs

Abbreviations: ADT, 5-[p-methoxyphenyl]-3H-1,2-dithiole-3-thione; D3T, 1,2-dithiole 3-thione; hpf, hours postfertilization; EGFP, enhanced green fluorescent protein; FCS, fetal calf serum; Hsp27, heat-shock protein 27; HUVEC, human umbilical vein endothelial cells; NSAID, nonsteroidal anti-inflammatory drug; Fli, Friend leukemia inhibitor

Introduction

Angiogenesis is the process of new vessel formation from an established vascular plexus (Folkman, 2006). Under nonpathological conditions, it is a tightly regulated process representing a balance between stimulating and inhibiting factors (Ferrara and Kerbel, 2005). A number of diseases

involve dysregulation of angiogenesis (Carmeliet, 2005). Lack of an angiogenic response in the face of tissue ischemia underlies myocardial infarction and peripheral vascular disease. Inappropriate angiogenesis is required for tumor growth and metastasis (Cao, 2005). Thus, selective control of angiogenic responses would have clinical utility in several major diseases (Verheul and Pinedo, 2005).

Dithiolethiones are sulfur-based compounds widely distributed in the human diet with a range of known activities, including inhibition of lipid peroxidation, protective properties against radiation injury and redox cytotoxicity, and anticancer effects (Kensler *et al.*, 2000). Some dithioles have

Correspondence: Dr DD Roberts, Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Building 10, Room 2A33, 10 Center Drive, Bethesda, MD 20892-1500, USA.

E-mail: droberts@helix.nih.gov

Received 30 August 2006; revised 29 November 2006; accepted 24 January 2007; published online 12 March 2007

known effects on endothelial cells or antiangiogenic activities (Ben-Mahdi *et al.*, 2000; Ruggeri *et al.*, 2002). These compounds also protect against tetrachloride and acetaminophen hepatotoxicity, possibly owing to their antioxidant properties modulating cellular glutathione, which provides cytoprotection against oxidative stress (Kensler *et al.*, 1992; Kwak *et al.*, 2001).

A number of molecular pathways are induced by dithiolethiones. Dithiolethiones induce redox coactivator Ref-1, which enhances AP-1-dependent transcription of *c-fos* and *c-jun* (Yao and O'Dwyer, 2003). Dithiolethiones also induce antioxidant-response element (ARE)-regulated genes via dissociation of nuclear transcription factor erythroid 2p45 (NF-E2)-related factor 2 (Nrf2) from Kelch-like ECH-associated protein 1 (Keap1), which sequesters Nrf2 in the cytoplasm (Lee and Surh, 2005). The activation of these pathways is dependent on oxidation of key thiols in the molecular targets.

The above observations led us to investigate the effects of a novel class of dithiolethione-modified anti-inflammatory drugs (Li *et al.*, 2006) on angiogenic responses in vascular cells. Unlike the unmodified drugs, we found that dithiolethione-modified S-nonsteroidal anti-inflammatory drug (NSAIDs) and S-valproate demonstrated significant antiangiogenic activities, inhibiting endothelial cell proliferation and vascular cell outgrowth and invasion of extracellular matrix under wound healing as well as tumor-driven conditions. Likewise, simple dithiolethiones potently inhibited these angiogenic responses, suggesting that the antiangiogenic properties of the S-NSAIDs and S-valproate reside in their dithiolethione moiety.

Methods

Animals

C57B16 mice and Cr:(NCr)-athymic nu fBR mice (NCI, Frederick, MD, USA) were housed in a pathogen-free environment and allowed *ad libitum* access to food and

water. Handling and care of animals was in compliance with the guidelines established by the Animal Care and Use Committees of the National Cancer Institute and the National Institutes of Health. Zebrafish were grown and maintained at 28.5°C under the National Cancer Institute guidelines.

Cells

Human umbilical vein endothelial cells (HUVEC; Cambrex, Walkersville, MD, USA) were maintained in endothelial cell growth medium (Cambrex) and 2% fetal calf serum (FCS) in 5% CO₂ at 37°C. Cells were utilized between passages 4 and 8. Purity of cultures was monitored by immunochemical staining with monoclonal human anti-CD31 antibody (Sigma, St Louis, MO, USA).

Synthesis of dithiolethione derivatives S-diclofenac, S-sulindac and S-valproate

The 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione (ADT-OH) was prepared by demethylation of ADT as described previously (Bottcher *et al.*, 1951). The compounds S-diclofenac and S-sulindac were prepared adding to the respective free acids, ADT-OH, dicyclohexylcarbodiimide and a catalytic amount of 4-dimethylaminopyridine in dichloromethane (Figure 1) (Li *et al.*, 2006). After removal of dicyclohexylurea, the compounds were purified by column chromatography. S-valproate was prepared by refluxing 2-propylpentanoyl chloride with ADT-OH and *N,N*-diisopropylethylamine in anhydrous tetrahydrofuran. After evaporation of the solvent, the residue was purified by chromatography and crystallized with ethyl ether. All the compounds had chromatographic purity >98%. The compounds were characterized by melting point (m.p.) (Buchi apparatus), ¹H-NMR spectra (Varian Mercuri 300 VX spectrometer in CDCl₃ or DMSO-*d*₆), high-resolution mass spectra (APEX II ICR-FTMS Bruker Daltonics mass

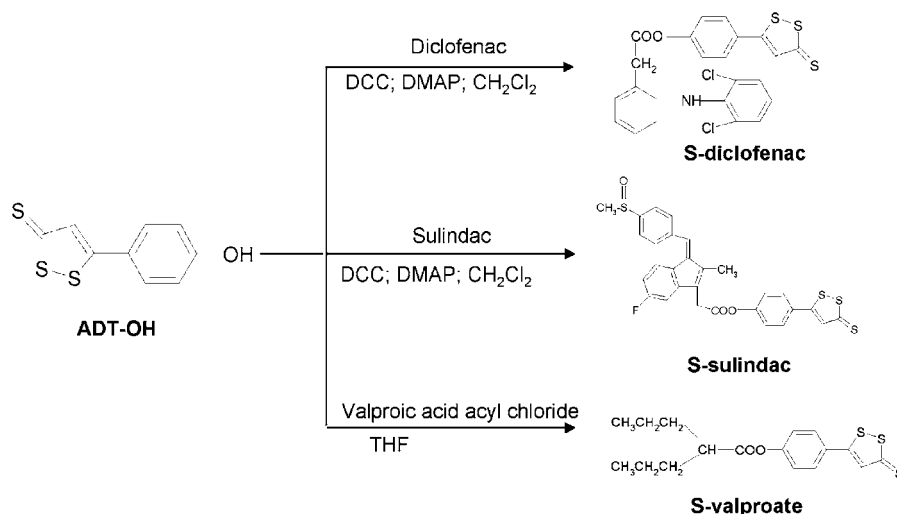


Figure 1 Synthesis and chemical structures of S-NSAIDs and S-valproate. Synthetic pathways and chemical structures of the ADT-OH derivatives of diclofenac, sulindac and valproate.

spectrometer) and by elemental analysis (Carlo Erba EA-1100CHNS-O instrument).

- (1) 2-[(2,6-dichlorophenyl)amino]benzeneacetic 4-(5-thioxo-5H-1,2-dithiol-3-yl)phenyl ester (S-diclofenac): m.p. 151–153°C; ^1H NMR (CDCl_3): δ 7.75–7.60 (m, 2H), 7.45–7.15 (m, 7H), 7.10–6.90 (m, 2H), 6.65–6.55 (m, 2H), 4.10 (s, 2H); HRMS (ESI) calculated for $\text{C}_{23}\text{H}_{15}\text{Cl}_2\text{NO}_2\text{S}_3\text{Na}$ ($\text{M} + \text{Na}$) $^+$: 525.95342; found: 525.95438. Elemental analysis calculated for $\text{C}_{23}\text{H}_{15}\text{Cl}_2\text{NO}_2\text{S}_3$: C 54.76, H 3.00, N 2.78, S 19.07; found: C 54.64, H 3.27, N 2.64, S 19.18.
- (2) (Z)-5-Fluoro-2-methyl-1-[[4-(methylsulfinyl)phenyl]-methylene]-1H-indene-3-acetic acid 4-(thioxo-5H-[1,2]dithiol-3-yl)-phenyl ester. (S-sulindac): m.p. 155–156°C; ^1H NMR ($\text{DMSO}-d_6$): δ 8.00 (d, 2H), 7.65–7.80 (m, 5H), 7.40 (s, 1H), 7.30 (d, 2H), 7.10–7.25 (m, 2H), 6.70 (t, 1H), 4.00 (s, 2H), 2.80 (s, 3H), 2.20 (s, 3H). HRMS (ESI) calculated for $\text{C}_{29}\text{H}_{21}\text{FO}_3\text{S}_4\text{Na}$ ($\text{M} + \text{Na}$) $^+$: 587.02498; found: 587.02397.
- (3) 2-Propylpentanoic acid 4-(5-thioxo-5H-1,2-dithiol-3-yl)-phenyl ester (S-valproate): mp 69.5–70.5°C; ^1H NMR (DMSO): δ 7.95 (d, 2H), 7.80 (s, 1H), 7.25 (d, 2H), 2.70–2.55 (m, 1H), 1.42–1.70 (m, 4H), 1.25–1.40 (m, 4H), 0.85–1.00 (m, 6H); HRMS (ESI) calculated for $\text{C}_{17}\text{H}_{21}\text{O}_2\text{S}_3$ ($\text{M} + \text{H}$) $^+$: 353.06982; found: 353.06956. Elemental analysis calculated for $\text{C}_{17}\text{H}_{20}\text{O}_2\text{S}_3$: C 57.92; H 5.72, S 27.29; found: C 58.05, H 5.69, S 27.34.

Explant invasion assay

Biopsies (1 mm^3) from the pectoralis major muscle of 8-week-old wild-type C57B16 mice were harvested and explanted into type I collagen gel in 96-well tissue culture plates as described by Isenberg *et al.* (2005). Following gelation, explants were incubated in endothelial growth medium (EGM) + 2% FCS in the presence or absence of a dose range of the indicated treatment agents or the comparable concentration of vehicle at 37°C and 5% CO_2 . The selection of treatment agent concentrations used in explant and other *in vitro* cell assays was based upon data obtained from *in vivo* experiments (Li *et al.*, 2006). Maximum cell migration through the matrix was measured following 7 days of incubation. In other experiments, C3H athymic nude mice were injected subcutaneously with 10^6 HT29 adenocarcinoma tumor cells to the lateral thigh. Animals were killed when tumors reached 1 cm. Tumor biopsies of 1 mm^3 were harvested immediately following death and explanted into type I collagen matrix as described above and incubated in EGM with 1% FCS. Following 7 days of incubation in the presence of treatment agents, maximum vascular cell outgrowth was measured. The treatment doses employed *in vitro* and *in vivo* were based on previously published data utilizing these agents (Li *et al.*, 2006). Vascular cell outgrowth of collagen matrices was quantified as the distance of farthest cell invasion from the explant border in each of the four quadrants. Results represent the mean $\pm$ s.e. of at least three separate experiments.

Cell proliferation

Proliferation of HUVEC was measured using a nonradioactive colorimetric assay (CellTiter 96, Promega, Madison, WI,

USA). Briefly, 5×10^3 cells suspended in $100\text{ }\mu\text{l}$ of culture medium were added to each well of a 96-well culture plate (Nunc, Denmark) and incubated for 72 h. Appropriate zero-time controls were run for all assays and the optical density readings obtained then subtracted from those obtained at 72 h. Results represent the mean $\pm$ s.e. of at least three separate experiments.

Explant cytospin analysis

Type I collagen matrix from explant constructs was digested with type 2 collagenase in growth medium and collected by centrifugation. The cell pellet was resuspended in growth medium at a concentration of 0.5×10^6 cells ml^{-1} . Standard cytospin cuvettes were loaded with $200\text{ }\mu\text{l}$ of cell suspension and spun at 800 r.p.m. for 3 min and allowed to air dry. Slides were then fixed with acetone, stained with anti-CD31 (clone WM-59; Sigma) and anti- α smooth muscle actin (clone 1A4, Sigma), developed with the Vectastain Elite ABC kit (Vector Labs, Burlington, CA, USA) following the manufacturer's instructions and the cell counts obtained.

Cyclooxygenase assay

HT-29 cells were incubated with the S-NSAID compounds at $1\text{ }\mu\text{M}$ for 30 min, and then $20\text{ }\mu\text{M}$ arachidonic acid was added for 5 min. The supernatant was then removed and assayed for prostaglandin E_2 (PGE_2) using an enzyme-linked immunoassay.

Western blot analysis

HUVEC at 60% confluence were starved overnight in endothelial basal medium + 1% FCS and then treated with the indicated dosages of compounds for 60 min. Cells were collected into $1 \times$ sodium dodecyl sulfate sample buffer containing $1 \times$ protease inhibitors. Cell lysates were separated by electrophoresis and blotted onto polyvinylidene fluoride membranes. Membranes were blocked with 5% nonfat dry milk at RT for 1 h, and incubated with anti-Hsp27 Ser 78 phosphorylation Ab (Stressgen, Ann Arbor, MI, USA) overnight at 4°C, then with goat-anti-mouse-horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA) at RT for 1 h. After three washes with phosphate-buffered saline, protein was determined by enhanced chemiluminescence. Membranes were reprobed with antiactin antibody for sample loading control.

Embryonic Zebrafish toxicology studies

Zebrafish were maintained as described in The Zebrafish Book (Westerfield, 2000). Transgenic $Tg(fli1:EGFP)^{y10}$ embryos were dechorinated at 10 h postfertilization (hpf) with 2 mg ml^{-1} pronase (Sigma, Cat. No. P-8811) stock in 2–3 ml Blue Water (E3 solution: 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl_2 , 0.33 mM MgSO_4 , $10^{-5}\%$ Methylene Blue). Embryos were then added to 2 ml of Blue Water containing desired concentration of drug compound or dimethylsulfoxide (DMSO) control at either 10 hpf or in some experiments at 18 hpf. All drug compounds were dissolved in DMSO to 1 mM

before being diluted in Blue Water. Embryos were incubated at 28°C until 48 hpf and images were captured with a Zeiss AxioCam HRC and Carl Zeiss SV11 Epi-Fluorescence stereo microscope (Thornwood, NY, USA). The number of animals or embryos employed ranged from 30 to 100/drug concentration per experiment.

Statistics

Results represent the mean $\pm$ s.e. of at least three separate experiments. Significance was determined with the Student's *t*-test, or one- or two-way analysis of variance with Tukey's *post hoc* analysis and using a $P < 0.05$. A standard software package (Origin version 7, OriginLab Corp., Northampton, MA, USA) was utilized throughout.

Materials

3H-1,2-dithiole-3-thione (D3T) was obtained from LKT Laboratories (St Paul, MN, USA). Sodium hydrosulfide and valproic acid were purchased from Sigma (St Louis, MO, USA). Agents with limited solubility in water were initially prepared as stock solutions in DMSO and serial dilutions in the indicated cell-culture medium performed with final concentrations of DMSO never exceeding 0.1%.

Results

Chemical characterization and solubility of S-NSAIDs

The ADT-OH derivatives of diclofenac, sulindac and valproate were synthesized as outlined in Figure 1 and according to Li *et al.* (2006). Nuclear magnetic resonance, mass spectrometry and elemental analysis were all consistent with the predicted structures. The compounds S-diclofenac, S-sulindac and S-valproate were found to have lower solubility as compared with the parent compound and were all practically insoluble in water (water solubility $< 0.01\%$) and very lipophilic. The calculated log *P* (ACD/Labs software V9.02, Toronto, Canada) at 25°C, for example, of compound S-diclofenac is 7.18 vs a calculated log *P* of the parent compound of 4.06, for compound S-valproate, the calculated log *P* is 5.88 and where as that of the parent compound is 2.72.

COX inhibition by S-NSAIDs

To confirm that the S-NSAIDs retain their COX inhibitory activities, they were assessed for inhibiting conversion of arachidonic acid to PGE₂ in HT-29 cells. Compared to basal levels of 90 ± 21 pg ml⁻¹, S-diclofenac and S-sulindac at 1 μ M reduced PGE₂ production to 16 ± 9 and 58 ± 13 , respectively (mean $\pm$ s.e., $n = 4$).

S-NSAIDs and S-valproate inhibit wound-driven angiogenesis

Muscle biopsies from C57Bl6 mice were explanted into 3D type I collagen gels in a 96-well plate format and incubated in the presence of EGM and the indicated concentrations of S-NSAIDs, S-valproate or H₂S (Figure 2a). The S-NSAIDs, S-valproate and H₂S were dose-dependent inhibitors of

vascular cell outgrowth. The dithiolethiones ADT and D3T also significantly inhibited wound-driven vascular cell outgrowth (Figure 2b). In contrast, the corresponding unmodified valproate, sulindac and diclofenac had either no effect on wound-driven angiogenesis or tended to stimulate the response (data not shown), indicating that the dithiole moieties of the S-NSAIDs and S-valproate mediated their antiangiogenic activities. Vehicle alone did not inhibit the muscle explant vascular cell response (data not shown).

S-NSAIDs and S-valproate inhibit tumor-driven vascular outgrowth in 3D collagen explants

Utilizing a novel *ex vivo* model of tumor-driven angiogenesis, we found that HT-29 tumor-driven angiogenic responses were also very sensitive to the inhibitory effects of S-NSAIDs and S-valproate (Figure 2c and d). Cytospin analysis of cell outgrowth from tumor explants demonstrated a preponderance of vascular cells composed of endothelial cells ($35 \pm 5\%$), vascular smooth muscle cells ($33 \pm 1\%$) and other unidentified cells ($33 \pm 7\%$) (Table 1). S-NSAID and S-valproate treatment of HT-29 explants resulted in a decrease in vascular migration through extracellular matrix (Figure 2c) as and in a decrease in the number of cells in the outgrowth (Figure 2d). As in the muscle explants, nonmodified parent compounds were much less active and did not significantly inhibit tumor-driven angiogenesis except at $> 100 \mu$ M (Figure 2e). Both ADT and D3T potently inhibited tumor-driven angiogenesis in the explant assay (Figure 2f). Vehicle alone did not inhibit the tumor explant vascular cell response (data not shown).

S-NSAIDs and S-valproate inhibit endothelial cell proliferation

HUVEC proliferation was inhibited in a dose-dependent manner by S-NSAIDs and S-valproate (Figure 3a). Consistent with the explant data, the nonmodified parent compounds had little or no effect on cell proliferation at equivalent dosages (Figure 3b). As in vascular outgrowth, ADT inhibited HUVEC proliferation. D3T demonstrated a slight, but not statistically significant inhibition on proliferation (Figure 3c). In contrast, H₂S stimulated endothelial cell proliferation in a dose-dependent manner (Figure 3d). Vehicle alone did not inhibit the vascular cell response (data not shown).

Dithioles but not S-NSAIDs disrupt embryonic angiogenesis

To investigate the effect of the drugs on developmental angiogenesis, we utilized the zebrafish angiogenesis model system. In zebrafish, the dorsal aorta and posterior cardinal vein, which traverse along the axis of the embryo, represent vasculogenic vessels, whereas sprouts emerging from the axial vessels represent the angiogenic intersomitic vessels. The effect of drugs were studied in transgenic fish *Tg(fli1:EGFP)* that carry a vascular specific 15 kb fli (Friend leukemia inhibitor) promoter driving enhanced green fluorescent protein (Lawson and Weinstein, 2002) in angioblasts and mature vasculature. S-NSAIDs and S-valproate demonstrated minimal toxicity and had little effect upon inter-

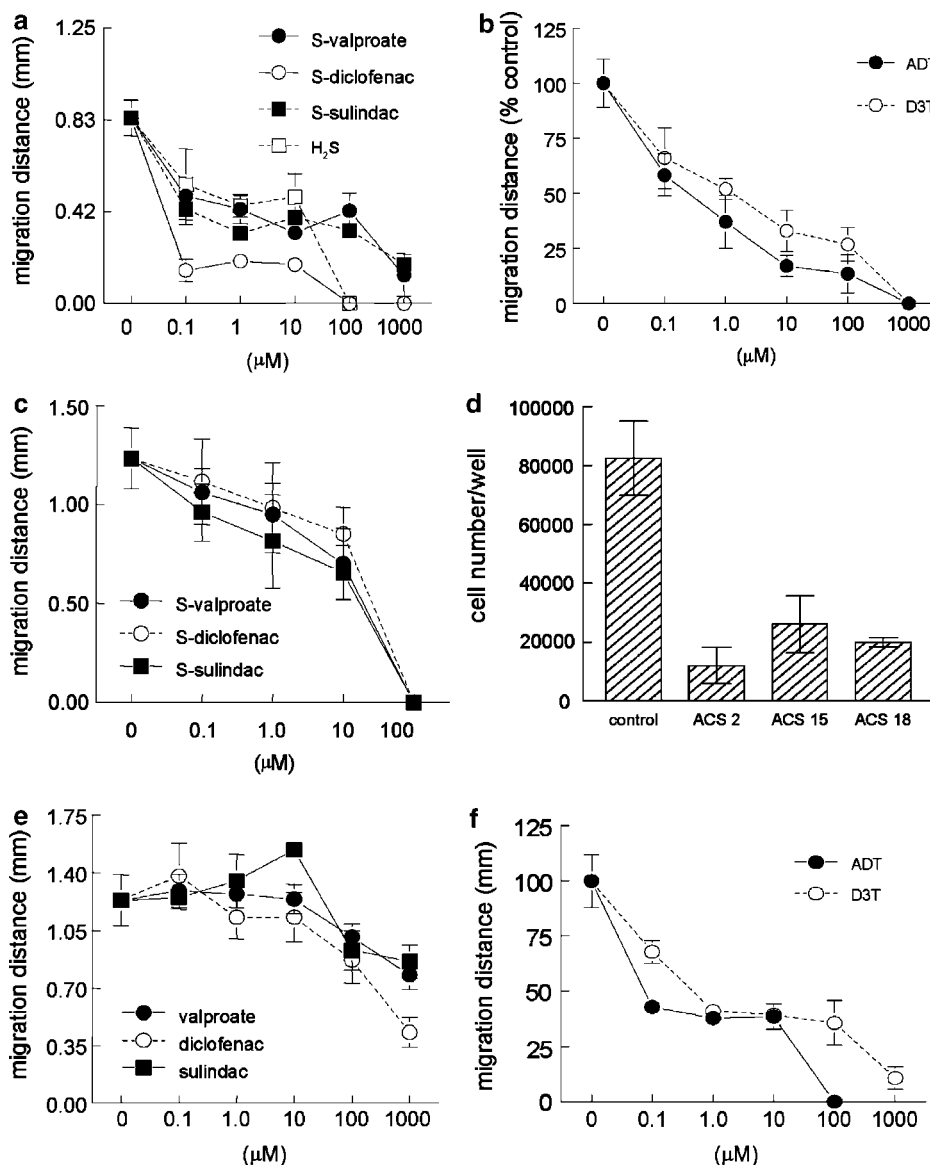


Figure 2 S-NSAIDs, S-valproate and dithiole thiones inhibited vascular outgrowth from muscle and tumor explants. Pectoralis major muscle (a and b) or HT29 tumour biopsies (c, e and f) were explanted in type I collagen matrices and incubated in growth medium with the indicated treatments for 7 days and vascular outgrowth quantified. Control and treated-HT29 adenocarcinoma explants were incubated under similar conditions and, following matrix digestion, total cell counts determined using cytopsin preparations (d). In (d), S-valproate, S-diclofenac and S-sulindac are shown as ACS2, ACS15 and ACS18, respectively. S-NSAIDs and S-valproate caused significant inhibition ($P < 0.05$) at all concentrations (a, b, d and f) and from 10 to 100 μM (c), compared with control. Parent compounds were effective ($P < 0.05$) at 100–1000 μM (e) compared with control.

Table 1 Immunohistochemical analysis of HT29 xenograft explants

Cell type	%
Endothelial cells	34.6 $\pm$ 5 ^a
Vascular smooth muscle cells	32.7 $\pm$ 1
Other cell types	32.7 $\pm$ 7

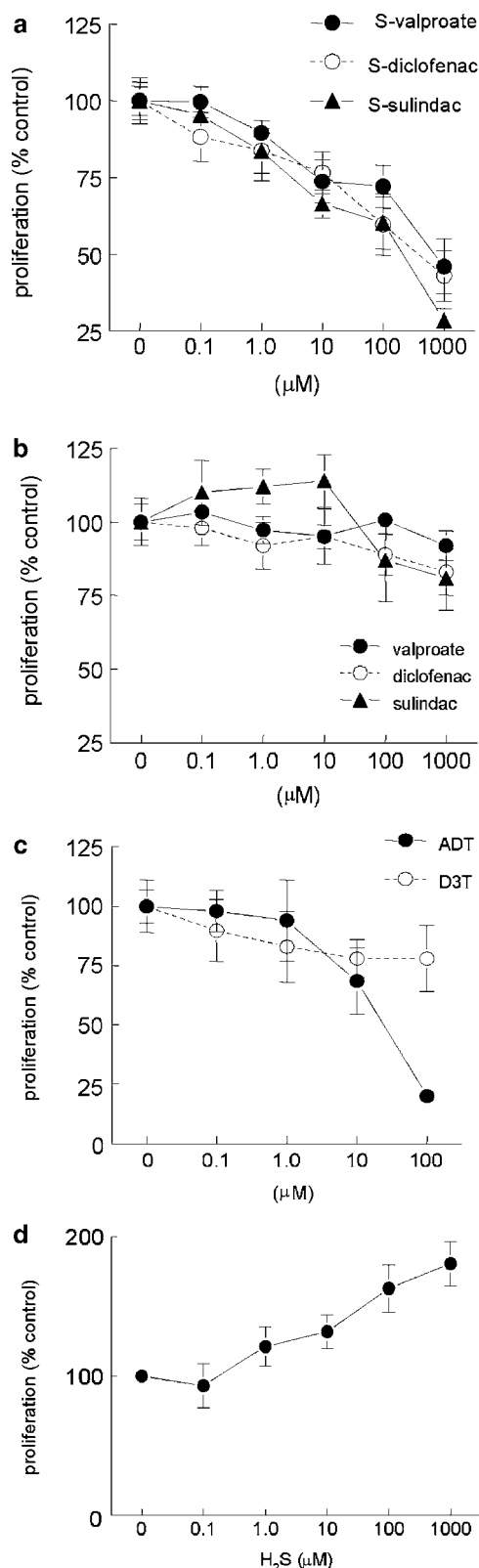
HT29 adenocarcinoma xenograft explants were cultured in 3D matrices for 7 days. Vascular outgrowths were collected after digesting the collagen gels using collagenase type 2. Slides were prepared by cytopsin and stained using CD31 (PECAM-1) or α -smooth muscle actin antibodies. CD31-positive endothelial cells and α -smooth muscle actin-positive perivascular cells were counted in 10 randomly selected fields. Results are the means of at least three independent experiments.

^aValues are the means $\pm$ s.e.m.; $n = 3$.

somatic vessels formation at the dose ranges employed in zebrafish embryos (Figure 4a). However, there were moderate numbers of gross developmental abnormalities noted among embryos treated with these agents (Figure 4a). Importantly, solubility limits for these compounds were encountered at 25 μM , above which precipitation occurred, probably owing to the reactivity with Blue Water components. In contrast, treatment of embryos with valproate (Figure 4b–b'') and higher doses of sulindac (Figure 4c–c''), the parent compounds of S-valproate and S-sulindac, respectively, were associated with a significant increase in intersomitic vessel defects. However, structural defects were also observed in 25 μM sulindac-treated embryos. D3T and ADT treatment

resulted in both vessel defects and gross developmental defects (Figure 5a and d). In the case of D3T, we noticed defects in the notochord at concentrations as low as $1\text{ }\mu\text{M}$ that progressed in severity as the concentration increased to

$5\text{ }\mu\text{M}$ (Figure 5c, asterisk) and beyond. To investigate whether vessel defects were indeed primary, we added the drug at 18 hpf postnotochord development (Figures 5b, e and f). We noticed intersomitic vessel defects in these embryos, although at much higher concentrations of $25\text{ }\mu\text{M}$ (Figure 5e). Interestingly, ADT treatment at 10 hpf was associated with significant delay in gastrulation (Figure 5d), and the drug uptake was evident by the yellow color seen in the yolk photographs (Figure 5d). When ADT was added to embryos at 18 hpf, the vessel defects were apparent (Figure 5f), although the concentrations required to observe this effect was higher ($25\text{ }\mu\text{M}$) than when treated at 10 hpf ($7.5\text{ }\mu\text{M}$). In most of the drug-treated embryos, we did not notice any defect in vasculogenesis, as both the dorsal aorta and posterior cardinal vein remained unaffected.



S-NSAIDs, *S*-valproate, H_2S and dithioles induce Hsp27 phosphorylation

Phosphorylation of heat shock protein 27 (Hsp27) is a convergent target of signaling in endothelial cells exposed to the angiogenesis inhibitors thrombospondin-1, fumagillin, endostatin and TNP-470 (Keezer *et al.*, 2003). Similar regulation of Hsp27 phosphorylation was subsequently reported for endorepellin, an antiangiogenic fragment of the heparan sulfate proteoglycan perlecan (Bix *et al.*, 2004). To determine whether Hsp27 phosphorylation is also regulated by *S*-NSAIDs, endothelial cells were exposed to various concentrations of *S*-NSAIDs, H_2S and D3T (Figures 6 and 7). After 60 min, *S*-sulindac stimulated Hsp27 Ser⁷⁸ phosphorylation at 10–100 μM (Figure 6a), but sulindac lacked activity at the same concentrations (Figure 6b). The dithiolethione D3T showed an inverse dose response in the same range, with strongest induction of Hsp27 phosphorylation at 1 μM (Figure 6a). Further dilution of the D3T showed maximal phosphorylation at 0.3 μM (Figure 5c). ADT was much less active and stimulated only weak Hsp27 phosphorylation at 10 μM (Figure 6b). H_2S was also much less active, inducing maximal Hsp27 phosphorylation at 100 μM (Figure 6a).

H_2S in some cases parallels NO in cellular responses, and NO, via activation of cGMP-dependent protein kinase, is known to induce phosphorylation of Hsp27 at Thr¹⁴³ (Butt *et al.*, 2001). Interestingly, spermine-NONOate also increased Hsp27 Ser⁷⁸ phosphorylation in endothelial cells (Figure 6a). The maximal response at 100 μM suggests that this is mediated by reactive nitrogen intermediates that form at

Figure 3 *S*-NSAIDs and *S*-valproate inhibited endothelial cell proliferation. HUVEC (5×10^3 cells/well⁻¹) were plated on 96-well plates and incubated for 72 h in EGM + 2% FCS with the indicated concentrations of *S*-NSAIDs and *S*-valproate (a), parent compounds (b), dithioles alone (c) and hydrogen sulfide (d). Cell proliferation was assayed via the colorimetric change obtained after incubation with MTS [3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] reagent using a microplate reader at 490 nm and is presented as percent of untreated control wells and corrected for the initial cell signal. Treatments were effective ($P < 0.05$) at concentrations from 10 to 1000 μM (a), 0.1–100 μM (c) and 1–1000 μM (d) compared with control.

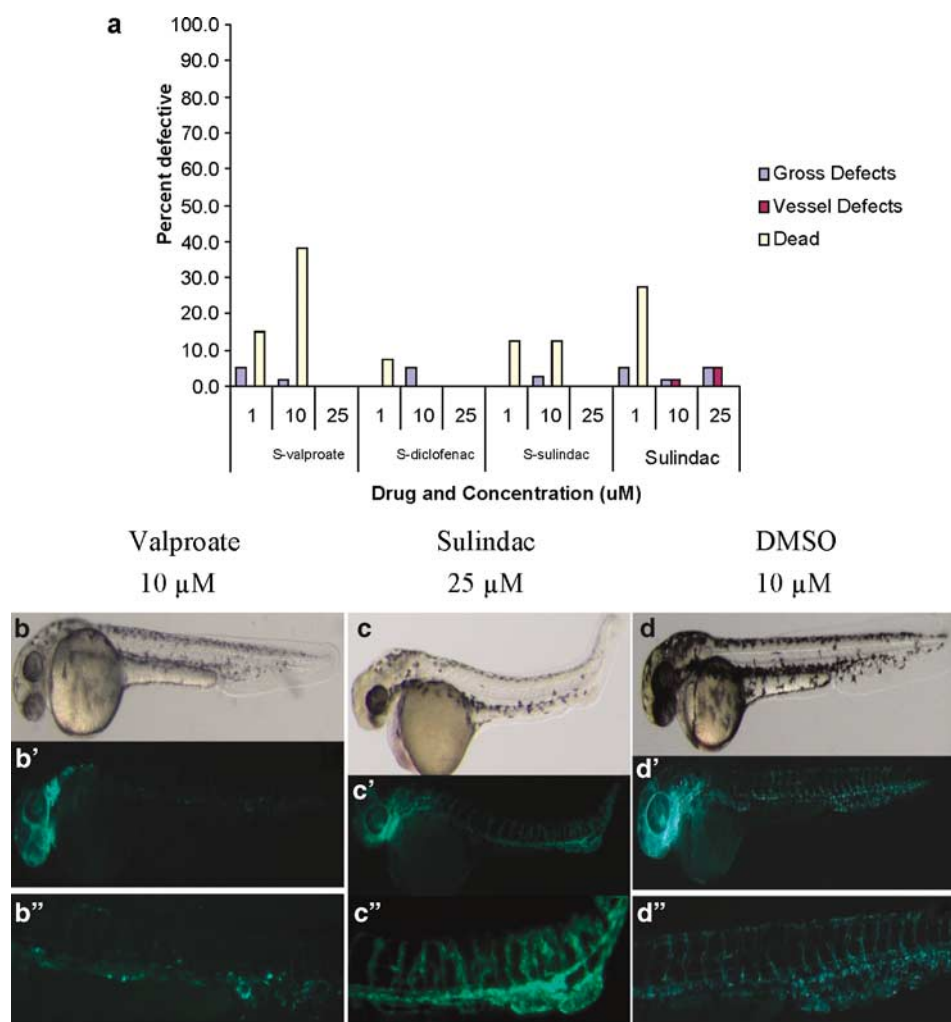


Figure 4 S-NSAIDs and S-valproate did not cause significant defects in vessels in developing zebrafish embryos. Dechorinated zebrafish embryos were incubated in the presence of the indicated concentrations of S-NSAIDs and S-valproate or nonderivatized parent compound of S-sulindac. Vascular defects in intersegmental vessels, gross defects or embryo death were quantified (a) ($n = 40$ – 60 embryos/experimental point). Representative embryos treated as indicated were photographed 48 hpf with light (b–d) or fluorescent illumination (b'–d'). Enlargements of the fluorescent images (b''–d'') show trunk intersegmental vessels branching from the dorsal aorta. Data are expressed as % defective.

stress levels of NO rather than by NO/cGMP signaling that is typically observed at $<1 \mu\text{M}$ spermine-NONOate (Thomas *et al.*, 2004). Oxidizing intermediates formed from reactive oxygen species induce Hsp27 phosphorylation in endothelial cells through the p38/MAPKAP2 pathway (Nguyen *et al.*, 2004). Using $100 \mu\text{M}$ H_2O_2 , we observed moderate phosphorylation of Ser⁷⁸ (Figure 6b) but much less than in response to D3T, suggesting that the latter induces Hsp27 phosphorylation through a different mechanism.

Treatment with S-diclofenac and S-valproate also induced Hsp27 phosphorylation, with maximal responses at $100 \mu\text{M}$ and similar to S-sulindac (Figure 7a). However, the corresponding parent compounds diclofenac and valproate also moderately increased Hsp27 phosphorylation (Figure 7a). These responses were maximal at 10 and/or $100 \mu\text{M}$. The Hsp27 response to valproate is consistent with its reported antiangiogenic activity (Michaelis *et al.*, 2004) and the intersomitic vessel defects in valproate-treated zebrafish embryos.

To examine further the potential role of p38/MAPKAP2 signaling in dithiolethione-induced Hsp27 phosphorylation, we examined the effects of these on mitogen-activated protein (MAP) kinase activation (Figure 7b). Neither p38, JNK nor Erk showed increased phosphorylation after treatment for 60 min with ADT, D3T or S-valproate. S-diclofenac, S-sulindac and sulindac modestly increased p38 phosphorylation. Therefore, increased p38 activation does not account for the dithiolethione-induced phosphorylation of Hsp27.

Discussion and conclusions

NSAIDs such as diclofenac are well studied for their anti-inflammatory properties via COX inhibition (Ulrich *et al.*, 2006). Here, we show that S-NSAIDs and S-valproate significantly inhibit pro-angiogenic responses of vascular cells under both wound healing and tumor growth condi-

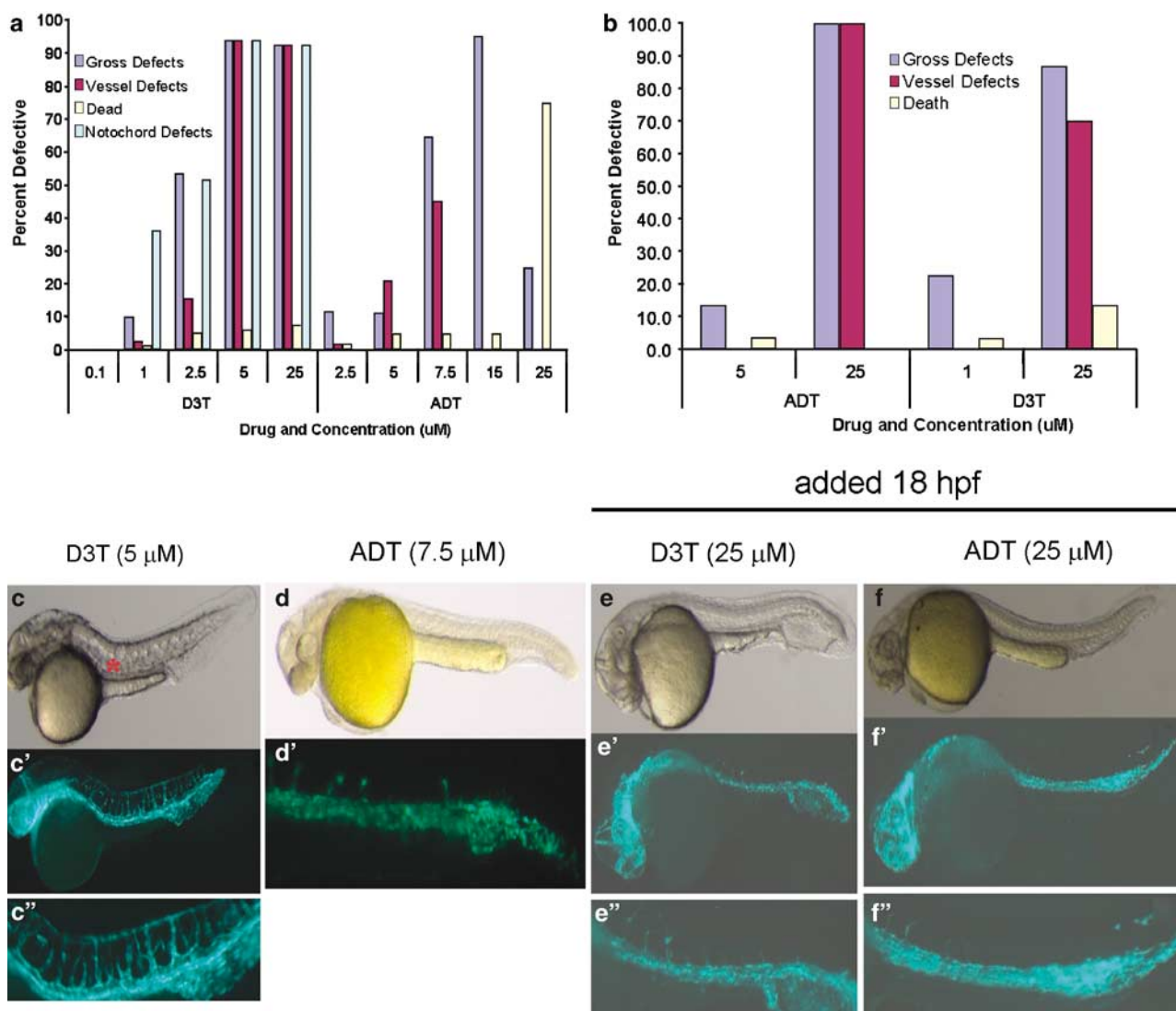


Figure 5 Dithiole moieties caused vascular and developmental defects in zebrafish embryos. Zebrafish embryos were incubated in the presence of the indicated concentrations of dithiole moieties and counted for developmental and vascular abnormalities (**a**) ($n=40-100$ embryos/experimental point) or added at 18 hpf (**b, e, f**) ($n=30$ embryos/experimental point). Representative embryos were photographed 48 hpf with light (**c-f**) or fluorescent illumination (**c'-f'**). Data are expressed as % defective. High magnification images showing intersomatic vessels are shown in **c'', d'', e''** and **f''**.

tions. Vascular cell migration through extracellular matrix was significantly blocked by S-NSAIDs and S-valproate. Additionally, total cell numbers obtained from digested extracellular matrix in tumor explants were dramatically decreased by S-NSAIDs, S-valproate and dithioles, consistent with the activities of these agents in inhibiting endothelial cell proliferation. In contrast, the parent drugs had no inhibitory effects on vascular cell outgrowth in either muscle or tumor explants. Treatment with the dithioles ADT and D3T mimicked the inhibitory effects of the S-NSAIDs and S-valproate in *ex vivo* wound and tumor-driven explants, suggesting that the antiangiogenic activity of the S-NSAIDs and S-valproate resides in their dithiole moiety. Although the two S-NSAIDs tested are COX inhibitors (Li *et al.*, 2006), their antiangiogenic activities appear to be independent of COX inhibition.

Angiogenesis requires a number of specific responses in both endothelial and vascular smooth muscle cells including

increased cell proliferation and migration. Consistent with their activity for blocking angiogenic responses under wound healing and tumor-driven conditions, we found that S-NSAIDs, S-valproate and dithioles inhibited endothelial cell proliferation *in vitro*. In contrast, hydrogen sulfide stimulated endothelial cell proliferation in a dose-dependent manner. In contrast to some other activities of S-NSAIDs and dithioles, their antiangiogenic activity is probably independent of H_2S release (Distrutti *et al.*, 2006; Li *et al.*, 2006).

Because cyclooxygenases are also known to play a role in angiogenesis (Gately and Li, 2004), the lack of effects of diclofenac and sulindac on endothelial cell proliferation and their weak inhibition of explant angiogenesis were unexpected. The most active metabolite of sulindac for cyclooxygenase inhibition is the sulfide, but previous studies have indicated that other sulindac metabolites can also influence angiogenic responses independent of cyclooxygenase inhibition (Gourzoulidou *et al.*, 2005). Sulindac sulfide induces

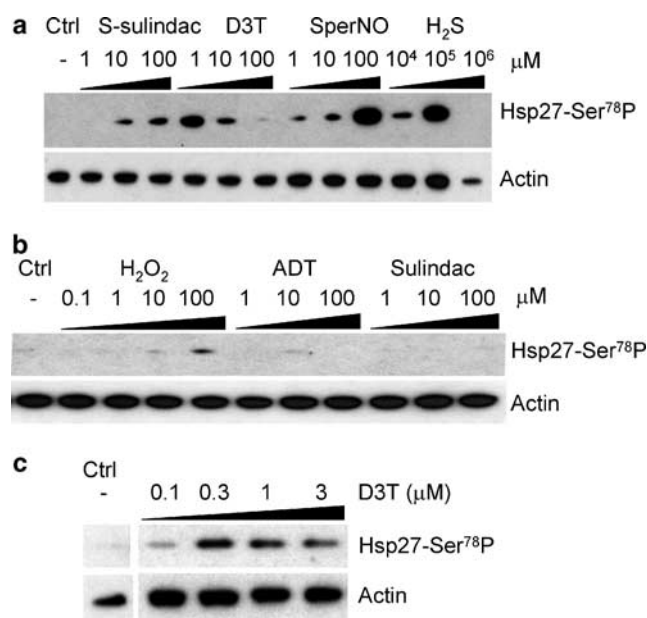


Figure 6 S-sulindac and D3T but not sulindac increased phosphorylation of Hsp27. HUVEC were serum starved overnight and then incubated for 60 min in the presence of S-sulindac, D3T, spermine-NO (1–100 μ M) or hydrogen sulfide (10–1000 mM) (a) and with H₂O₂, ADT or sulindac (1–100 μ M) (b), cells lysed and Western blot assays performed using an antibody specific for Hsp27 phosphorylated at Ser⁷⁸. In other experiments (c), an expanded concentration range of D3T was employed.

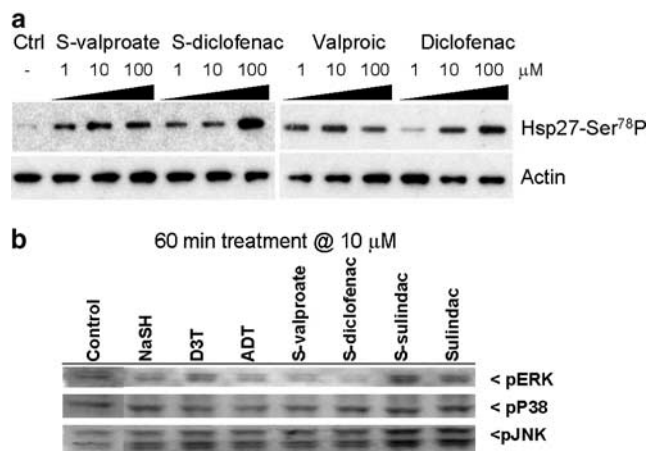


Figure 7 S-valproate and S-diclofenac and their parent drugs altered Hsp27 phosphorylation. HUVEC were treated with S-valproate, S-diclofenac and their representative parent compounds at the indicated concentrations for 60 min, and Western blot analysis performed for Hsp27 (Ser⁷⁸P) (a). Cell lysates from HUVEC treated for 60 min with the indicated agents were analyzed for phospho-p38, phospho-JNK or phospho-ERK (b).

endothelial cell apoptosis via caspase-3 activation (Flis *et al.*, 2006). However, earlier studies showed that sulindac sulf-oxide, the sulfone and sulfide all inhibit angiogenesis in chick chorioallantoic membrane angiogenesis assays, and the sulfone was in fact 10-fold more active than the sulfide, which in turn was twice as active as sulindac itself (Sharma *et al.*, 2001; Elwich-Flis *et al.*, 2003). Our data that sulindac is

antangiogenic in zebrafish embryos but not for endothelial cell proliferation suggests that this activity of sulindac is context-dependent.

As a heat-shock protein, Hsp27 supports cell survival and recovery from thermal and oxidative stress (Arrigo *et al.*, 2005). However, it also plays important roles in normal cell signaling. Mediated by MAPKAP-2 downstream from p38, phosphorylation of Hsp27 stabilizes the actin cytoskeleton and decreases cell migration (Landry and Huot, 1999; Keezer *et al.*, 2003). Consistent with the effects of other angiogenesis inhibitors to stimulate Hsp27 phosphorylation (Keezer *et al.*, 2003; Bix *et al.*, 2004) we found that phosphorylation of Hsp27 was induced by S-NSAIDs, S-valproate, D3T and H₂S, suggesting that these agents exert some of their antiangiogenic effects by regulating signaling pathways downstream of Hsp27. It is known that activation of the p38 pathway stimulates Hsp27 phosphorylation at all three serine residues (S15, S78 and S82). However, S-NSAID-induced Hsp27 phosphorylation is mainly at S78. In addition, the phosphorylation of Hsp27-S78 is not sensitive to the p38-specific inhibitor SB203580 (data not shown). Together, these results suggest these compounds might induce Hsp27-S78 phosphorylation through other signaling pathways.

The potent effects of S-NSAIDs, S-valproate and simple dithiols on wound healing and tumor-driven angiogenesis in an explant model correlated with their inhibitory effects on endothelial cell proliferation *in vitro*. Although direct toxicity studies were not carried out in murine models, others have employed H₂S-releasing derivatives of diclofenac *in vivo* in rat models with success, suggesting that these agents may be useful in therapeutic situations (Bhatia *et al.*, 2005; Li *et al.*, 2006; Ong *et al.*, 2006). However, in a zebrafish model the S-NSAIDs and S-valproate demonstrated relatively modest effects upon intersomitic vessel formation. This selective activity of S-NSAIDs and S-valproate may reflect the known mechanistic differences between developmental angiogenesis and pathological angiogenesis in the adult (Carmeliet, 2004). From this standpoint, our zebrafish screening approach could be useful to differentiate drugs that inhibit embryonic angiogenesis as opposed to tumor-associated angiogenesis in the adult. Alternatively or concomitantly, hydrolytic cleavage of the S-NSAIDs and S-valproate maybe required to release the antiangiogenic dithiole moiety. It is possible that hydrolysis does not occur in the zebrafish developmental angiogenesis model owing to the absence of a secreted esterase.

Activity of valproate in the zebrafish model is consistent with earlier published reports of its inhibition of vessel development in the chicken chorioallantoic membrane and in mice (Michaelis *et al.*, 2004; Zgouras *et al.*, 2004), and thereby validates the zebrafish model as a screening tool for angiogenesis inhibitors. The endothelial inhibition results and the tumor explant studies argue that both S-NSAIDs and S-valproate are angiogenesis inhibitors, with the primary antiangiogenic effects most likely residing in the dithiolthione moieties. These effects are separate from any antiangiogenic or anti-inflammatory effects of the parent compounds. S-NSAIDs and S-valproate, by combining the favorable activities of conventional anti-inflammatory drugs

and dithioles, and the decreased gastrointestinal side effects in the case of S-diclofenac (Li *et al.*, 2006), may increase their effectiveness as drugs. The antiangiogenic activities of S-NSAIDs may be useful in cancer therapy.

Acknowledgements

This research was supported in part by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research.

Conflict of interest

Dr Piero Del Soldato is a shareholder of CTG Pharma, Milan, Italy. This company has patents on reagents used in this study. Dr Anna Sparatore received a grant from CTG Pharma.

References

- Arrigo AP, Virot S, Chaufour S, Firdaus W, Kretz-Remy C, Diaz-Latoud C (2005). Hsp27 consolidates intracellular redox homeostasis by upholding glutathione in its reduced form and by decreasing iron intracellular levels. *Antioxid Redox Signal* 7: 414–422.
- Ben-Mahdi MH, Gozin A, Driss F, Andrieu V, Christen MO, Pasquier C (2000). Anethole dithiolethione regulates oxidant-induced tyrosine kinase activation in endothelial cells. *Antioxid Redox Signal* 2: 789–799.
- Bhatia M, Sidhapuriwala JAS, Moore PK (2005). Treatment with H2S-releasing derivative of diclofenac reduces inflammation in carrageenan-induced hindpaw oedema. *Inflammation Res* (Suppl. a): S185.
- Bix G, Fu J, Gonzalez EM, Macro L, Barker A, Campbell S *et al.* (2004). Endorepellin causes endothelial cell disassembly of actin cytoskeleton and focal adhesions through α 2 β 1 integrin. *J Cell Biol* 166: 97–109.
- Bottcher B, Bauer F, Trithionones V (1951). Some new trithionones. *Chemische Berichte* 84: 458–463.
- Butt E, Immler D, Meyer HE, Kotlyarov A, Laass K, Gaestel M (2001). Heat shock protein 27 is a substrate of cGMP-dependent protein kinase in intact human platelets: phosphorylation-induced actin polymerization caused by HSP27 mutants. *J Biol Chem* 276: 7108–7113.
- Cao Y (2005). Tumor angiogenesis and therapy. *Biomed Pharmacother* 59: S340–S343.
- Carmeliet P (2004). Manipulating angiogenesis in medicine. *J Intern Med* 255: 538–561.
- Carmeliet P (2005). Angiogenesis in life, disease and medicine. *Nature* 438: 932–936.
- Distrutti E, Sediari L, Mencarelli A, Renga B, Orlandi S, Russo G *et al.* (2006). 5-Amino-2-hydroxybenzoic acid 4-(5-thioxo-5H-[1,2]dithiol-3-yl)-phenyl ester (ATB-429), a hydrogen sulfide-releasing derivative of mesalazine, exerts antinociceptive effects in a model of post-inflammatory hypersensitivity. *J Pharmacol Exp Ther* 319: 447–458.
- Elwich-Flis S, Soltysiak-Pawluczuk D, Splawinski J (2003). Anti-angiogenic and apoptotic effects of metabolites of sulindac on chick embryo chorioallantoic membrane. *Hybrid Hybridomics* 22: 55–60.
- Ferrara N, Kerbel RS (2005). Angiogenesis as a therapeutic target. *Nature* 438: 967–974.
- Flis S, Soltysiak-Pawluczuk D, Jedrych A, Jastrzebski Z, Remiszewska M, Splawinski J (2006). Antiangiogenic effect of sulindac sulfide could be secondary to induction of apoptosis and cell cycle arrest. *Anticancer Res* 26: 3033–3041.
- Folkman J (2006). Angiogenesis. *Annu Rev Med* 57: 1–18.
- Gately S, Li WW (2004). Multiple roles of COX-2 in tumor angiogenesis: a target for antiangiogenic therapy. *Semin Oncol* 31: 2–11.
- Gourzoulidou E, Carpintero M, Baumhof P, Giannis A, Waldmann H (2005). Inhibition of angiogenesis-relevant receptor tyrosine kinases by sulindac analogues. *Chembiochem* 6: 527–531.
- Isenberg JS, Ridnour LA, Perruccio EM, Espey MG, Wink DA, Roberts DD (2005). Thrombospondin-1 inhibits endothelial cell responses to nitric oxide in a cGMP-dependent manner. *Proc Natl Acad Sci USA* 102: 13141–13146.
- Keezer SM, Ivie SE, Kruttsch HC, Tandle A, Libutti SK, Roberts DD (2003). Angiogenesis inhibitors target the endothelial cell cytoskeleton through altered regulation of heat shock protein 27 and cofilin. *Cancer Res* 63: 6405–6412.
- Kensler T, Styczynski P, Groopman J, Helzlsouer K, Curphey T, Maxuitenko Y *et al.* (1992). Mechanisms of chemoprotection by oltipraz. *J Cell Biochem Suppl* 161: 167–172.
- Kensler TW, Curphey TJ, Maxiutenko Y, Roebuck BD (2000). Chemoprotection by organosulfur inducers of phase 2 enzymes: dithiolethiones and dithiols. *Drug Metabol Drug Interact* 17: 3–22.
- Kwak MK, Egner PA, Dolan PM, Ramos-Gomez M, Groopman JD, Itoh K *et al.* (2001). Role of phase 2 enzyme induction in chemoprotection by dithiolethiones. *Mutat Res* 480–481: 305–315.
- Landry J, Huot J (1999). Regulation of actin dynamics by stress-activated protein kinase 2 (SAPK2)-dependent phosphorylation of heat-shock protein of 27 kDa (Hsp27). *Biochem Soc Symp* 64: 79–89.
- Lawson ND, Weinstein BM (2002). *In vivo* imaging of embryonic vascular development using transgenic zebrafish. *Dev Bio* 248: 307–318.
- Lee JS, Surh YJ (2005). Nrf2 as a novel molecular target for chemoprevention. *Cancer Lett* 224: 171–184.
- Li L, Rossoni G, Sparatore A, Lee L, Del Soldato P, Moore P (2006). Anti-inflammatory and gastrointestinal sparing activity of a novel H2S-releasing diclofenac agent: new insights into the biological roles of H2S. *Free Radic Biol Med* 42: 706–719.
- Michaelis M, Michaelis UR, Fleming I, Suhan T, Cinatl J, Blaheta RA *et al.* (2004). Valproic acid inhibits angiogenesis *in vitro* and *in vivo*. *Mol Pharmacol* 65: 520–527.
- Nguyen A, Chen P, Cai H (2004). Role of CaMKII in hydrogen peroxide activation of ERK1/2, p38 MAPK, HSP27 and actin reorganization in endothelial cells. *FEBS Lett* 572: 307–313.
- Ong MM, Sparatore A, del Soldato P, Moore PK, Treinen-Moslen M, Boelsterli UA (2006). Differential covalent adduct biodistribution of diclofenac and a diclofenac sulfured ester in rat liver and small intestine. *The Toxicologist* S2136: 438.
- Ruggeri BA, Robinson C, Angeles T, Wilkinson J, Clapper ML (2002). The chemopreventive agent oltipraz possesses potent antiangiogenic activity *in vitro*, *ex vivo*, and *in vivo* and inhibits tumor xenograft growth. *Clin Cancer Res* 8: 267–274.
- Sharma S, Ghoddoussi M, Gao P, Kelloff GJ, Steele VE, Kopelovich L (2001). A quantitative angiogenesis model for efficacy testing of chemopreventive agents. *Anticancer Res* 21: 3829–3837.
- Thomas DD, Espey MG, Ridnour LA, Hofseth LJ, Mancardi D, Harris CC *et al.* (2004). Hypoxic inducible factor 1 α , extracellular signal-regulated kinase, and p53 are regulated by distinct threshold concentrations of nitric oxide. *Proc Natl Acad Sci USA* 101: 8894–8899.
- Ulrich CM, Bigler J, Potter JD (2006). Non-steroidal anti-inflammatory drugs for cancer prevention: promise, perils and pharmacogenetics. *Nat Rev Cancer* 6: 130–140.
- Verheul HM, Pinedo HM (2005). Angiogenesis inhibitors: what is the clinical future? *Prog Drug Res* 63: 67–91.
- Westerfield M (2000). *The Zebrafish Book*. University of Oregon Press, Eugene, Oregon, p363.
- Yao KS, O'Dwyer PJ (2003). Role of the AP-1 element and redox factor-1 (Ref-1) in mediating transcriptional induction of DT-diaphorase gene expression by oltipraz: a target for chemoprevention. *Biochem Pharmacol* 66: 15–23.
- Zgouras D, Becker U, Loitsch S, Stein J (2004). Modulation of angiogenesis-related protein synthesis by valproic acid. *Biochem Biophys Res Commun* 316: 693–697.