Published in final edited form as:

Lung Cancer. 2010 May ; 68(2): . doi:10.1016/j.lungcan.2009.06.012.

Dithiolethione modified valproate and diclofenac increase E-cadherin expression and decrease proliferation of non-small cell lung cancer cells

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Abstract

The effects of dithiolethione-modified valproate, diclofenac and sulindac on non-small cell lung cancer (NSCLC) cells were investigated. Sulfur(S)-valproate and S-diclofenac at 1 µg/ml concentrations significantly reduced prostaglandin (PG)E₂ levels in NSCLC cell lines A549 and NCI-H1299 as did the COX-2 inhibitor DuP-697. In vitro, S-valproate, S-diclofenac and S-sulindac half-maximally inhibited the clonal growth of NCI-H1299 cells at 6, 6 and 15 µg/ml, respectively. Using the MTT assay, 10 µg/ml S-valproate, NO-aspirin and Cay10404, a selective COX-2 inhibitor, but not SC-560, a selective COX-1 inhibitor, inhibited the growth of A549 cells. In vivo, 18 mg/kg i.p. of S-valproate and S-diclofenac, but not S-sulindac, significantly inhibited A549 or NCI-H1299 xenograft proliferation in nude mice, but had no effect on the nude mouse body weight. The mechanism by which S-valproate and S-diclofenac inhibited the growth of NSCLC cells was investigated. Nitric oxide-aspirin but not S-valproate caused apoptosis of NSCLC cells. By Western blot, S-valproate and S-diclofenac increased E-cadherin but reduced vimentin and ZEB1 (a transcriptional suppressor of E-cadherin) protein expression in NSCLC cells. Because S-valproate and S-diclofenac inhibit the growth of NSCLC cells and reduce PGE₂ levels, they may prove beneficial in the chemoprevention and/or therapy of NSCLC,

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Conflicts of Interest: Drs. Piero Del Soldato and Anna Sparatore are shareholders of Sulfidris, Milan, Italy. This company has patent rights of the reagents used in this study.

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Keywords

S-valproate; S-diclofenac; lung cancer; PGE₂; E-cadherin

1. Introduction

Lung cancer is a serious public health problem which kills over 160,000 U.S. citizens annually [1]. Traditionally small cell lung cancer (SCLC) is treated with chemotherapy, and radiotherapy is added in patients with early disease, whereas non-SCLC (NSCLC) is treated with surgery at an early stage, while combination chemotherapy is standard in advanced disease. Unfortunately, the 5 year survival rate in NSCLC patients diagnosed with lung cancer is less than 15%. A subset of NSCLC patients which are resistant to chemotherapy, respond to the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors gefitinib and erlotinib [2]. These patients usually have EGFR mutations at or near the tyrosine kinase domain [3]. The mutated EGFR may have excessive tyrosine kinase activity, which is inhibited by gefitinib or erlotinib.

EGF addition to NSCLC cells causes increased cyclooxygenase (COX) expression [4]. Aspirin (Asa), which inhibits both COX-1 and COX-2 enzymes, reduces prostaglandin (PG)E₂ levels in NSCLC cells and inhibits their proliferation [5]. Because PGE₂ causes inflammation, transactivation of the EGFR and epithelial to mesenchymal transitions [6], reduction of PGE₂ levels in NSCLC may be important. Unfortunately, Asa caused toxic side-effects in some patients, such as stomach ulcers. The better-tolerated selective COX-2 inhibitor celecoxib inhibits the growth of NSCLC cells [7]. In addition, celecoxib may increase the sensitivity of NSCLC patients to radiation [8] or chemotherapy [9]. The use of rofecoxib or celecoxib in combination with EGFR tyrosine kinase inhibitors is currently being investigated in lung cancer patients [10]. In a phase I trial, celecoxib and erlotinib caused a partial response in some NSCLC patients. In patients which had a partial response, soluble E-cadherin, MMP-9, TIMP-1 and the chemokine CCL15 significantly decreased [11]. Also, progression-free survival was increased in NSCLC patients which had high COX-2 and were treated with celecoxib and the EGFR tyrosine kinase inhibitor erlotinib [12]. These results indicate the COX-2 in addition to the EGFR is a molecular target for NSCLC.

Recently non-steroidal anti-inflammatory drugs (NSAID) analogs were derivitized with moieties that can release NO. Nitric oxide-aspirin (NO-Asa) inhibited COX-2 and was 1000-fold more potent than Asa at inhibiting the proliferation of colon cancer cells [13]. In Min mice, intestinal tumors were reduced by 55% after treatment with NO-Asa for 3 weeks [14]. NO-Asa caused apoptosis of colon cancer cells as a result of increased levels of peroxides and superoxide [15]. Previously, we reported that S-valproate, S-diclofenac and S-sulindac decrease angiogenesis [16]. S-Valproate, S-diclofenac and S-sulindac inhibited endothelial cell proliferation and induced Ser78 phosphorylation of hsp27, a known anti-angiogenic signal. Also, S-valproate, S-diclofenac and S-sulindac inhibited angiogenic responses in muscle and HT29 colon cancer tumor explants. In this communication the effects of S-valproate, S-diclofenac and S-sulindac were investigated on NSCLC cells. The S-valproate and S-diclofenac significantly reduced PGE₂ levels and inhibited the proliferation of NSCLC cells *in vitro*. S-valproate and S-diclofenac, but not S-sulindac, inhibited significantly NSCLC xenograft proliferation in nude mice *in vivo*. NO-Asa but not S-valproate caused apoptosis of NSCLC cells. S-Valproate increased expression of E-cadherin but decreased the expression of ZEB1 in NSCLC cells. The results suggest that S-valproate may decrease expression of the transcriptional suppressor of E-cadherin, ZEB1, leading to increased E-cadherin expression in NSCLC cells.

2. Materials and Methods

2.1 Cell culture

NCI-H1299 and A549 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen; Grand Island, NY). The cells were split weekly 1/20 with trypsin-ethylene diamino tetraacetic acid (EDTA). The cells were mycoplasma free and were used when they were in exponential growth phase after incubation at 37°C in 5% CO₂/95% air.

2.2 PGE₂ assay

The ability of the S-valproate, S-diclofenac and S-sulindac to alter PGE₂ was investigated by enzyme ELISA (Cayman Chemical, Ann Arbor, MI). S-valproate, S-diclofenac and S-sulindac were synthesized as described previously [16] whereas NO-Asa was purchased from Cayman Chemical (Ann Arbor, MI). S-valproate was synthesized by refluxing 2-propylpentanoyl chloride with dithiolethione-OH and N,N-diisopropylethylamine in anhydrous tetrahydrofuran. After evaporation of the solvent, the product was purified by chromatography and crystallized with ethyl ether; purity > 98%. S-valproate, S-diclofenac, S-sulindac and NO-Asa were dissolved in DMSO at a concentration of 10 mg/ml for the *in vitro* experiments. A549 or NCI-H1299 cells in 24 well plates were washed twice with 250 µl of SIT medium (RPMI-1640 containing 3×10^{-8} M sodium selenite, 5 µg/ml bovine insulin and 10 µg/ml transferrin (Sigma-Aldrich, St. Louis, MO)). S-Valproate, S-diclofenac, S-sulindac, DuP697 (Sigma-Aldrich, St. Louis, MO), CAY10404, SC-560 or NO-Asa (Cayman Chemicals, Ann Arbor, MI) were added for 30 min, then arachidonic acid (20 µg/ml) was added at 37°C for 5 min. The samples were frozen at -80°C, until assayed for PGE₂. The supernatant (100 µl) was added to EIA buffer (900 µl). Routinely 25 µl of the diluted supernatant was added to a 96 well plate. The samples were incubated overnight at 4°C and after washing the plate 5 times, 200 µl of Edman's reagent was added. The absorbance at 450 nm was determined in an ELISA reader.

2.3 Western Blot

The ability of the S-valproate and S-diclofenac to alter E-cadherin expression was investigated. S-Valproate or S-diclofenac was added to A549 or NCI-H1299 cells and incubated for 16 hr at 37°C. Cells were washed twice in SIT medium and lysed with 50 mM Tris.HCl (pH 7.5), 150 mM sodium chloride, 1% Triton X-100, 1% deoxycholate, 1% sodium azide, 1 mM ethyleneglycoltetraacetic acid, 0.4 M EDTA, 1.5 µg/ml aprotinin, 1.5 µg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride and 0.2 mM sodium vanadate (Sigma-Aldrich, St. Louis, MO). The lysate was sonicated for 5 s at 4°C and centrifuged at $10,000 \times g$ for 15 min. Protein concentration was measured by BCA protein reagent (Pierce Chemical Co., Rockford, IL), and 20 µg of protein was analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis using 4-20% acrylamide gels (Novex) and Western blotting. Proteins were transferred to nitrocellulose membranes and the membranes were blocked overnight at 4°C using blotto (5% non-fat dried milk in solution containing 50 mM Tris/HCl (pH 8.0), 2 mM CaCl₂, 80 mM sodium chloride, 0.05% Tween 20 and 0.02% sodium azide) and incubated for 2 h at 25°C with 1 µg/ml anti-E-cadherin antibody (BD Bioscience, San Jose, CA) followed by anti-mouse immunoglobulin G-horseradish peroxidase conjugate (Upstate Biotechnologies, Lake Placid, NY). The membrane was washed for 10 min with blotto and twice for 10 min with washing solution (50 mM Tris/HCl (pH 8.0), 2 mM CaCl₂, 80 mM sodium chloride, 0.05% Tween 20 and 0.02% sodium azide). The blot was incubated with enhanced chemiluminescence detection reagent for 5 min and exposed to XAR film. The density of bands was determined using a densitometer. As a loading control, 20 µg of cellular extract was analyzed using anti-tubulin antibody (Cell Signalling Technologies; Danvers, MA). Also, the ability of S-valproate or S-diclofenac to

alter vimentin, ZEB1, VEGF, ERK, EGFR or COX-2 was investigated using antibodies from Cell Signalling Technologies; Danvers, MA and Santa Cruz Biotechnology Inc.; Santa Cruz, CA.

2.4 Proliferation and apoptosis assays

Growth studies *in vitro* were conducted using the a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium (MTT) bromide colorimetric and clonogenic assays. NCI-H1299 or A549 cells (10^4 /well) were placed in SIT medium and various concentrations of S-valproate, S-diclofenac or S-sulindac added. After 4 days, 15 μ l of 1 mg/ml MTT was added. After 4 h, 150 μ l dimethylsulfoxide was added. After 16 h, the optical density at 570 nm was determined. In the clonogenic assay, the effects of S-valproate, S-diclofenac or S-sulindac on the growth of NCI-H1299 or A549 cells were investigated. The bottom layer contained 0.5% agarose in SIT medium containing 5% FBS in 6 well plates. The top layer consisted of 3 ml of SIT medium in 0.3% agarose, S-NSAIDs and 5×10^4 NCI-H1299 cells. Triplicate wells were plated and after 2 weeks, 1 ml of 0.1% piodonitrotetrazolium violet was added and after 16 hours at 37°C, the plates were screened for colony formation; the number of colonies larger than 50 μ m in diameter were counted using an Omnicon image analysis system.

For detection of early apoptotic events, floating and adherent cells were collected. NCI-H1299 or A549 cells were treated with 10 μ g/ml NO-Asa or S-valproate for 24 hr at 37°C. Cells (10^6) were double stained with FITC-conjugated Annexin V and propidium iodide using the Vybrant Apoptosis Kit (Molecular Probes) and were analyzed by cytofluorometric analysis.

Growth studies *in vivo* were performed using female athymic nude mice (20 g) which were purchased from NCI-Frederick. The mice were injected s.c. with NCI-H1299 or A549 cells (10^7). After 1 week, xenografts formed, and the animals were injected (i.p.) with PBS or 18 mg/kg S-valproate, S-diclofenac or S-sulindac in 100 μ l of PEG-400, twice weekly. The nude mouse weight and tumor volume using calipers was determined 2 times a week. When the tumor volume was greater than 2000 mm³, the mice were euthanized with CO₂ vapors in accord with the protocol approved by the NCI Animal Care and Use Committee. The xenografts were weighed and frozen at -80°C, for Western blot analysis.

3. Results

3.1 S-valproate and S-diclofenac decrease cellular PGE₂

The ability of the S-valproate, S-diclofenac or S-sulindac to alter PGE₂ levels were investigated using lung cancer cells. Table I shows that addition of 1 μ g/ml NO-Asa, S-valproate, S-diclofenac or DuP-697, a selective COX-2 inhibitor, significantly reduced PGE₂ levels by over 70%. S-sulindac (1 μ g/ml) reduced PGE₂ concentrations by 36% and 25% in cell lines A549 and H1299 respectively. Preliminary data (T. Moody, unpublished) indicate that the selective COX-1 inhibitor SC-560 [17] has little effect on NSCLC cellular PGE₂ levels. These results suggest that the S-valproate and S-diclofenac inhibit COX-2 enzymatic activity in NSCLC cells.

3.2 S-valproate and S-diclofenac inhibit NSCLC proliferation

The ability of the S-valproate, S-diclofenac or S-sulindac to alter lung cancer proliferation was investigated. Figure 1A shows the S-valproate had little effect on NCI-H1299 colony growth at 1 μ g/ml but strongly inhibited large colony formation at 30 μ g/ml. The IC₅₀ values for S-valproate, S-diclofenac and S-sulindac were 6, 6 and 15 μ g/ml, respectively.

Similar results were obtained using A549 cells (data not shown). The results indicate that S-valproate, S-diclofenac and S-sulindac inhibit the growth of NSCLC cells *in vitro*.

Using the MTT assay, S-diclofenac inhibited A549 proliferation in a concentration dependent manner. Figure 1B shows that there was little growth inhibition using 1 $\mu\text{g/ml}$, but substantial growth inhibition using 100 $\mu\text{g/ml}$ S-valproate or NO-Asa. Similarly, the selective COX-2 inhibitor CAY10404 [18] but not the selective COX-1 inhibitor SC-560, strongly inhibited the proliferation of A549 cells. Similar results were obtained using NCI-H1299 cells. Preliminary data (T. Moody, unpublished) indicate that S-valproate and S-diclofenac preferentially inhibit COX-2 relative to COX-1.

The mechanism by which S-valproate inhibits lung cancer proliferation was investigated. NO-Asa is cytotoxic for cancer cells [15]. Figure 1C shows that, only 3.0% of the NCI-H1299 cells treated with S-valproate were apoptotic, which was similar to untreated cells. In contrast, 32.3% of the NCI-H1299 cells treated with NO-Asa for 24 hours were apoptotic (Fig. 1D). These results indicate that NO-Asa, but not S-valproate, causes apoptosis for lung cancer cells.

Figure 2 shows that A549 xenografts formed 1 week after injection of cells (s.c.) into nude mice. In animals injected with PBS, the tumors grew exponentially and after 3.5 weeks, the tumor volume was 472 mm^3 . In contrast, animals injected with S-diclofenac or S-valproate but not S-sulindac, had significantly slower tumor growth and the tumor volume after 3.5 weeks was 248, 198 and 373 mm^3 , respectively. These results indicate that S-diclofenac or S-valproate but not S-sulindac significantly inhibited A549 growth *in vivo*.

Table II shows that after 4.5 weeks, the NCI-H1299 xenograft volume was 1941 and 2134 mm^3 in animals injected with PBS or the vehicle control (PEG400). The xenograft volume was 1962, 398 and 1154 mm^3 in nude mice injected with S-valproate (3.6 mg/kg), S-valproate (18 mg/kg) or S-sulindac (18 mg/kg), respectively. These results indicate that S-valproate, in a dose-dependent manner, but not S-sulindac, inhibited significantly lung cancer proliferation *in vivo*. Animals injected with PBS had an average weight of 24.7 g (Table II). Because animals injected with S-valproate, S-sulindac or the vehicle control had a similar weight, these results suggest that the drugs have little systemic toxicity.

3.3 S-valproate and S-diclofenac increase E-cadherin

The ability of S-valproate to alter E-cadherin expression was investigated by Western blot. Figure 3A shows that addition of 0.003 μM S-valproate to A549 cells increased E-cadherin expression 5-fold, and this increase was maximal using 0.28 μM S-valproate, which increased E-cadherin expression 8-fold (Fig. 3B). As a loading control, equal amounts of tubulin were present in the cellular extracts.

Tumors were analyzed for E-cadherin expression (Fig. 3C). In nude mice treated with 18 mg/kg S-valproate, the A549 xenografts expressed approximately 40% more E-cadherin than did xenografts from control mice treated with PBS (Fig. 3D). These results indicate that S-valproate causes significant increases in E-cadherin expression *in vitro* and *in vivo*.

Similarly S-diclofenac treatment of A549 cells caused increased E-cadherin expression in a dose-dependent manner (Fig. 4). As a control tubulin was not altered by S-diclofenac treatment of A549 cells. Similarly, diclofenac treatment of A549 cells had no effect on COX-2, EGFR, or ERK but vimentin and ZEB1 were decreased. Previously it was shown that addition of PGE_2 to A549 cells increased expression of ZEB1, a transcriptional suppressor of E-cadherin [19]. Conversely, PGE_2 addition to A549 cells decreased E-

cadherin expression [19]. The results indicate that S-valproate and S-diclofenac cause increased E-cadherin expression in NSCLC cells.

Discussion

The metastatic process of malignancy has many similarities to the epithelial to mesenchymal transitions (EMT) that occur in normal development including resistance to anoikis, enhanced survival, genomic instability and resistance to chemotherapeutic drugs [20]. EMT is associated with the loss of cell-cell adhesion, cellular elongation, and invasion of the underlying extracellular matrix [6]. Proteins involved in cell junctions such as E-cadherin are reduced whereas vimentin is increased [21]. A number of factors which transcriptionally repress E-cadherin driven EMT during development and cancer include TGF β , Wnt, Notch and receptor tyrosine kinase pathways activating zinc finger Snail homologs and basic helix-loop-helix factors such as Twist, ZEB1 and TCF3/E47/E12 [22]. PGE₂ addition to A549 cells reduced E-cadherin as a result of increased expression of Snail and ZEB1 [19]. Further, E-cadherin expression was reduced if A549 cells were transfected with COX-2, and E-cadherin expression was increased if NSCLC cells were treated with COX-2 antisense [19]. In this communication, S-valproate and S-diclofenac caused increased expression of E-cadherin but decreased expression of ZEB1 and vimentin in A549 cells. Also, A549 tumors from nude mice treated with S-valproate had significantly greater E-cadherin expression than did A549 tumors from control mice. It remains to be determined if S-valproate causes mesenchymal to epithelial transitions in NSCLC cells.

S-Valproate increases the sensitivity of A549 cells to gefitinib (T. Moody, unpublished). The growth of NSCLC cells, which have high E-cadherin expression, such as NCI-H3225 [23], were strongly inhibited by the EGFR tyrosine kinase inhibitor gefitinib (IC₅₀ = 0.15 μ M). Cell line A549 had moderate levels of E-cadherin and moderate sensitivity to gefitinib (IC₅₀ = 8.2 μ M). A549 has wild type EGFR whereas cell line NCI-H3225 has the EGFR L858R point mutation [23]. E-cadherin expression and EGFR mutation status are predictive of the response of NSCLC patients treated with gefitinib [24].

S-valproate, S-diclofenac and S-sulindac inhibited the growth of NSCLC cells in a concentration-dependent manner. In nude mice bearing A549 or NCI-H1299 xenografts, S-valproate or S-diclofenac but not S-sulindac administration, slowed significantly the proliferation of NSCLC tumors. Because the tumors rapidly regrew after the drug injection was stopped, S-valproate or S-diclofenac may be a cytostatic and not cytotoxic agents. E-cadherin regulation is facilitated by interaction with the transcriptional corepressor, CtBP [25], which recruits histone deacetylases (HDAC). E-cadherin expression is increased by HDAC inhibitors such as trichostatin A [26]. Also the HDAC inhibitor MS-275 increased gefitinib sensitivity in NSCLC cells [27]. Recently, S-valproate was found to be a potent HDAC inhibitor [28]. S-valproate was a more potent inhibitor of HDAC activity than valproate. Thus one mechanism by which S-valproate decreases NSCLC proliferation may be inhibition of HDAC [29].

Activation of the EGFR by transforming growth factor (TGF β) increased COX-2 resulting in elevated levels of PGE₂ [30]. COX-2 levels are elevated in numerous epithelial tumors including colon and lung [31,32]. COX-2 metabolizes arachidonic acid to PGG₂ which is subsequently metabolized by PG endoperoxide synthase and PGE synthase to PGE₂. PGE₂ is pro-inflammatory mediating the progression of cancer [33]. PGE₂ is increased in malignant tissue [34] compared with normal tissue and PGE₂ is the predominant PG in colorectal cancer. NSAIDs decrease small intestinal adenomas in a murine model of colon cancer (APC^{min} mice), whereas treatment of APC^{min} mice with PGE₂ accelerates the growth of intestinal adenomas [35]. S-valproate and S-diclofenac reduced strongly PGE₂ in NSCLC

cells. The effects were dose-dependent in that 1 $\mu\text{g/ml}$, but not 0.1 $\mu\text{g/ml}$ S-valproate, S-diclofenac, DuP-697 or NO-Asa reduced PGE_2 concentrations in NSCLC cells (T. Moody, unpublished). Further the S-diclofenac had little effect on COX-2 protein levels in A549 and NCI-H1299 cells. These results suggest that S-valproate and S-diclofenac may inhibit COX-2 enzymatic activity in NSCLC cells.

NSAIDs inhibit the proliferation of NSCLC cells. Indomethacin or Asa, 300 μM , inhibited the growth of NSCLC *in vitro* [4]. Similarly, 6, 6 and 15 $\mu\text{g/ml}$ of S-valproate, S-diclofenac and S-sulindac half-maximally reduced NSCLC colony formation. Because S-valproate, S-diclofenac and S-sulindac have molecular weights of 353, 526 and 587 Daltons, respectively, the IC_{50} values to inhibit clonal growth are 17.2, 11.4 and 25.5 μM respectively. Thus the S-valproate is over 1-order of magnitude more potent than Asa at inhibiting NSCLC clonal growth. *In vivo*, 80 mg/kg of Asa inhibited the growth of NSCLC xenografts in nude mice [4]. S-valproate or S-diclofenac, but not S-sulindac (18 mg/kg) inhibited the growth of A549 or NCI-H1299 xenografts in nude mice. Thus S-valproate or S-diclofenac are over 5-fold more potent than is Asa *in vivo*. Relatively high doses of Asa or indomethacin, but not S-valproate or S-diclofenac, however, caused toxic side effects e.g. gastrointestinal ulcers (T. Moody, unpublished).

Previously, it was hypothesized that S-diclofenac was metabolized by rat liver esterases to form diclofenac along with ADT-OH from which hydrogen sulfide (H_2S) is liberated [36]. H_2S , similar to NO, may be a vasodilator in the microvasculature. While S-diclofenac and diclofenac were both found to reduce rat liver PGE_2 levels, S-diclofenac caused less gastric or intestinal mucosal damage than did diclofenac. S-diclofenac was anti-inflammatory and did not affect blood pressure or heart rate of the anesthetized rat. Currently we are investigating if S-valproate is more potent or has less side effects than valproate in the mouse.

Genetic studies indicate that colorectal polyp growth and vascular density are significantly attenuated on $\text{Cox-2}^{-/-}/\text{APC}^{\text{min}}$ mice [37, 38]. Overexpression of COX-2 in colorectal cancer cells stimulates endothelial cell migration and tube formation resulting in increased levels of PGE_2 and VEGF [39]. Previously, we found that PGE_2 increased the expression of VEGF in NSCLC cells [40]. S-valproate and S-diclofenac inhibited endothelial cell proliferation and HT29 tumor explant angiogenesis, but had little effect on zebrafish angiogenesis [16]. These results indicate that S-valproate is more potent at inhibiting tumor angiogenesis than embryonic angiogenesis.

Previously, we found that PGE_2 elevated the cAMP in NSCLC cells by activating EP2 receptors [41]. Specific $^3\text{H-PGE}_2$ binding to NSCLC cells was inhibited by AH6809, an EP2 receptor antagonist. Addition of PGE_2 to NSCLC cells caused elevated cAMP, an effect which was antagonized by AH6809. Also, AH6809 inhibited the clonal growth of NSCLC cells. EP2 null mice had significantly less lung adenomas than did normal mice treated with an initiation-promotion carcinogenesis protocol [42]. These results suggest that EP2 receptors are important in the proliferation of lung cancer cells.

PGE_2 causes transactivation of the EGFR in NSCLC cells. Addition of PGE_2 to NCI-H157 squamous carcinoma cells causes tyrosine1068 phosphorylation of the EGFR [43]. Similarly, addition of PGE_2 to A549 adenocarcinoma cells causes EGFR tyrosine phosphorylation (T. Moody, unpublished). Addition of PGE_2 to NSCLC cells leads to activation of ERK, leading to the proliferation of NSCLC cells. Addition of gefitinib to cell line NCI-H3255, which has EGFR mutations, caused apoptosis of the NSCLC cells [10]. Addition of celecoxib and gefitinib, strongly inhibited the proliferation of NCI-H3255 cells

and caused down regulation of COX-2, EGFR, pEGFR, Akt, pAkt and PGE₂. The results indicate that COX-2 and the EGFR are important molecular targets in NSCLC.

Conclusions

S-valproate and S-diclofenac represent new agents which inhibit COX-2 activity and increase E-cadherin expression in NSCLC cells. Because they inhibit the growth of NSCLC cells and reduce PGE₂ levels, they may prove beneficial in the chemoprevention and/or therapy of NSCLC.

Acknowledgments

This research was partially supported by the intramural research funds of the NIDDK and NCI, of NIH.

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Figure 1.

S-valproate, S-diclofenac and S-sulindac inhibit NSCLC proliferation. (A) The effects of varying doses of S-valproate (●), S-diclofenac (◻) and S-sulindac (○) were determined on NCI-H1299 colony formation. The mean value $\pm$ S.D. of 3 determinations is indicated. This experiment is representative of 3 others. (B) The effect of varying doses of S-valproate (○), NO-Asa (◻), CAY10404 (●) and SC-560 (◻) on A549 cells were determined using the MTT assay. The mean value $\pm$ S.D. of 8 determinations is indicated. This experiment is representative of 5 others. (D) NO-Asa but not (C) S-valproate causes apoptosis of NSCLC cells. NCI-H1299 cells were treated with 10 μ g/ml NO-Asa or S-valproate for 24 hrs at 37°C. This experiment is representative of 2 others.

Figure 2. S-valproate and S-diclofenac slow nude mouse xenograft proliferation. The mean tumor volume $\pm$ S.E. of 7 determinations is determined as a function of time after injection of A549 cells. Animals were injection twice weekly i.p. with PBS (o), 18 mg/kg S-valproate (•), S-sulindac () or S-diclofenac (); $p < 0.05$, *; $p < 0.01$, ** using Student's t-test. This experiment is representative of 1 other.

Figure 3.

Western blot and S-valproate. (A) E-cadherin and tubulin were determined 16 hr after addition of varying doses of S-valproate to A549 cells. The densitometry analysis is shown (B) and this experiment is representative of 4 others; $p < 0.05$, *; $p < 0.01$, **. (C) E-cadherin and tubulin were determined in A549 xenografts derived from nude mice treated with PBS or S-valproate. (D) The densitometry analysis is shown and this experiment is representative of 2 others; $p < 0.05$, *.

Figure 4. Western blot and S-diclofenac. A549 cells were treated with varying doses of S-diclofenac for 16 hr and cellular extracts analyzed for E-cadherin, tubulin, vimentin, ZEB1, EGFR, ERK and COX-2. This experiment is representative of 2 others.

Table I

S-valproate, S-diclofenac and S-sulindac cause reduced PGE₂ in NSCLC cells.

Addition	Relative PGE ₂ (%)	
	A549	NCI-H1299
None	100 ± 13	100 ± 8
NO-Asa, 1 µg/ml	18 ± 3 ^{**}	23 ± 4 ^{**}
S-valproate, 1 µg/ml	26 ± 8 ^{**}	33 ± 8 ^{**}
S-diclofenac, 1 µg/ml	18 ± 6 ^{**}	25 ± 4 ^{**}
S-sulindac, 1 µg/ml	64 ± 9 [*]	75 ± 10
DuP-697, 1 µg/ml	30 ± 7 ^{**}	21 ± 4 ^{**}

The mean value ± S.E. of 4 determinations is indicated using A549 or NCI-H1299 cells using Student's t-test). This experiment is representative of 4 others.

*
(p < 0.05

**
p < 0.01

Table II

S-valproate inhibits NCI-H1299 lung cancer xenograft proliferation.

Addition	Tumor volume, mm ³	Nude mouse weight, g
PBS	1941 ± 322	24.7 ± 0.7
S-valproate, 3.6 mg/kg	1962 ± 510	24.1 ± 1.1
S-valproate, 18 mg/kg	398 ± 64 **	24.7 ± 0.7
PEG400	2134 ± 399	23.9 ± 0.4
S-sulindac, 18 mg/kg	1154 ± 294	23.9 ± 1.0

S-valproate or S-sulindac was freshly made in 100 µl weekly i.p. The mean tumor volume ± S.E. of 8 determinations is indicated 4.5 weeks after injection of tumor cells

This experiment is representative of 2 others.

**
p < 0.01 using Student's t-test.