Vestibular schwannomas (VSs) are the most common tumors of the cerebellopontine angle. Significant clinical need exists for pharmacotherapies against VSs. Motivated by previous findings that immunohistochemical expression of cyclooxygenase 2 (COX-2) correlates with VS growth rate, we investigated the role of COX-2 in VSs and tested COX-2 inhibiting salicylates against VSs. COX-2 was found to be aberrantly expressed in human VS and primary human VS cells in comparison with control human nerve specimens and primary Schwann cells (SCs), respectively. Furthermore, levels of prostaglandin E2, the downstream enzymatic product of COX-2, were correlated with primary VS culture proliferation rate. Because COX-2 inhibiting salicylates such as aspirin are well tolerated and frequently clinically used, we assessed their repurposing for VS. Changes in proliferation, cell death, and cell viability were analyzed in primary VS cultures treated with aspirin, sodium salicylate, or 5-aminosalicylic acid. These drugs neither increased VS cell death nor affected healthy SCs. The cytostatic effect of aspirin in vitro was in concurrence with our previous clinical finding that patients with VS taking aspirin demonstrate reduced tumor growth. Overall, this work suggests that COX-2 is a key modulator in VS cell proliferation and survival and highlights salicylates as promising pharmacotherapies against VS. (Translational Research 2015;166:1–11)

Abbreviations: 5-ASA = 5-aminosalicylic acid; BrDU = 5-bromo-2-deoxyuridine; GAN = great auricular nerve; COX-2 = cyclooxygenase 2; IκB = I kappa B kinase; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NaSal = sodium salicylate; NF-κB = nuclear factor kappa-light-chain-enhancer of activated B; PBS = phosphate-buffered saline; PBST = phosphate-buffered saline with Tween 20; PTG = prostaglandin; PTGS2 = gene encoding COX-2 protein; RIPA = Radioimmunoprecipitation Assay; S100 = Schwann cell/schwannoma cell marker; SC = Schwann cell; SD = standard deviation; SEM = standard error of the mean; VS = vestibular schwannoma
INTRODUCTION

Vestibular schwannomas (VSs) are the most common tumors of the cerebellopontine angle and the fourth most common intracranial tumors. Although VSs are histologically nonmalignant, they can lead to substantial morbidity, including sensorineural hearing loss, vestibular dysfunction, and facial nerve paralysis, because of their location within the internal auditory canal and the cerebellopontine angle. Large VSs can cause additional paralysis of other cranial nerves, brainstem compression, and death. Currently, patients with symptomatic or growing VSs can undergo surgical resection or radiotherapy. Both these procedures can result in serious complications. Surgical resection entails full or partial removal of the tumor via craniotomy and carries substantial risks, including sensorineural hearing loss, vestibular dysfunction, facial nerve paralysis, cerebrospinal fluid leaks, and meningitis. Stereotactic radiotherapy entails delivering a radiation dose to the tumor and can be associated with severe adverse effects such as further exacerbation of the sensorineural hearing loss, vestibular dysfunction, and potential malignant transformation of the tumor. Patients with nongrowing or asymptomatic VSs can undergo conservative management and follow tumor progression through serial magnetic resonance imaging, but because of the lack of biomarkers for VS growth and associated symptoms, conservative monitoring can be a risky approach. Effective drug therapies that can limit VS growth would greatly advance health care for patients with VS.

Cyclooxygenase 2 (COX-2), a major inflammatory mediator, has been implicated in VS. Previous studies demonstrate that the expression level of COX-2 in VSs is correlated with tumor proliferation rates, as judged by the intensity of COX-2 immunostaining in VS specimens. The COX enzymes catalyze the biosynthesis of prostaglandins (PGs), hormone-like lipid compounds that can trigger the inflammatory response. In contrast to COX-1, which is expressed constitutively as a homeostatic enzyme in several cell types such as platelets and gastrointestinal mucosal cells, COX-2 is expressed at sites of inflammation and neoplasia. Specifically, COX-2 has been described to modulate cell proliferation and apoptosis in many solid tumors, such as colorectal, breast, and prostate cancers. Salicylates, a class of nonsteroidal anti-inflammatory drugs (NSAIDs) defined by their chemical structure, are attractive therapeutics because they are clinically relevant, well-tolerated, effective COX-2 inhibitors, commonly used against pathologies such as pain and arthritis. Furthermore, in some cases, chronic intake of salicylates has led to a significant reduction in the incidence and burden of various tumors, such as colorectal cancer. In our study, we assessed the efficacy of 3 different salicylates, aspirin, sodium salicylate (NaSal), and 5-aminosalicylic acid (5-ASA), against VS because they are clinically used and well tolerated. Specifically, aspirin has been confirmed to provide chemoprevention for multiple human malignancies, including colon, gastric, breast, and prostate cancer—reviewed in Thorat and Cuzick. NaSal is a sodium salt of salicylic acid. It is used clinically as an analgesic and antipyretic and as an alternative to aspirin for people sensitive to aspirin. NaSal has shown effectiveness against myeloid leukemia cell lines. 5-ASA is commonly used to treat inflammatory bowel disease including ulcerative colitis and Crohn’s disease, and it can prevent colorectal cancer. In addition to its anti-inflammatory properties, 5-ASA is thought to be an antioxidant that traps free radicals. These 3 salicylates, although acting through similar mechanisms to inhibit COX activity, have nuances that can lead to differential therapeutic and toxic profiles. We explored the expression of COX-2 in human VS and the therapeutic efficacy of salicylate-mediated COX-2 inhibition in primary VS cells. All salicylates tested were effective in selectively reducing proliferation and viability of cultured VS cells, accompanied by reduced secreted PG levels. Our work suggests
promising potential of commonly used salicylates against VS.

MATERIALS AND METHODS

Specimen collection. Human great auricular nerves (GANs) were used as healthy control nerves and as the source for healthy human Schwann cells (SCs), as these nerves are routinely sacrificed for access during parotidectomies and neck dissections. Immediately after GAN resection, nerve specimens measuring 1 cm (from parotidectomies) to 5 cm (from neck dissections) were placed in sterile saline on ice and transported to the laboratory. Human VS tumor specimens were also collected from independent surgical resections via indicated craniotomies. Specimens were handled according to the institutional review board’s study protocol approved by the Human Studies Committee of Massachusetts General Hospital and Massachusetts Eye and Ear Infirmary.

Reverse transcription–quantitative polymerase chain reaction. Gene expression of COX-2 (PTGS2 gene) was measured using real-time quantitative polymerase chain reaction (qPCR). Specifically, human VS or GAN tissue was placed in RNA later (Qiagen, Valencia, California) and stored at −20°C until RNA extraction. Total RNA was extracted using RNeasy Mini-Kit (Qiagen) according to the manufacturer’s protocol. Quantification and quality assessment of the RNA were performed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California) or Nanodrop (ThermoScientific, Wilmington, Delaware). All samples yielded undegraded RNA as shown by electropherograms or through 260/280 nm absorbance ratios. Isolated RNA was stored at −80°C. The RNA was reverse-transcribed to complementary DNA with TaqMan Reverse Transcription Reagent kit (Applied Biosystems, Foster City, California) following the manufacturer’s protocol. The complementary DNA was stored at either 4°C for short-term use or −20°C for long-term storage. qPCR was performed with TaqMan primers and 6-carboxyfluorescein (6-FAM) linked fluorescent probes (Applied Biosystems) for PTGS2 (Hs00153133_m1) with reference gene ribosomal RNA 18s (Hs9999901_s1). The PCR measurements were performed using Applied Biosystems 7700 Sequence Detection System.

Immunohistochemistry of GAN and VS specimens. Human VS and GAN specimens were fixed in 4% paraformaldehyde for 2 hours at room temperature (RT) on shaker. The specimens were transferred to phosphate-buffered saline (PBS) and kept on shaker at −4°C until being embedded into paraffin. Paraffin-embedded tissue on slides was deparaffinized and antigen retrieval (#S1700; Dako, Glostrup, Denmark) was performed using manufacturer’s instructions. Tissue sections on slides were placed in 5% normal horse serum with 0.4% Triton X-100 (#X-100; Sigma-Aldrich, St. Louis, Missouri) for blocking, incubated with primary antibodies against S100 (#Z031129; Dako), an SC marker, or COX-2 (#ab15191; Abcam, Cambridge, UK) at 4°C overnight, and then incubated for 2 hours at RT in secondary antibodies (Jackson-Immuno Research, West Grove, Pennsylvania). Nuclei were labeled using Hoechst 33342 stain ( Invitrogen, Carlsbad, California). The sections were washed with PBS and a cover slip was mounted with VectaShield (Vector Laboratories, Burlingame, California). The tissue was visualized and imaged using a Carl Zeiss 2000 upright microscope (Carl Zeiss, Jena, Germany).

Schwann and schwannoma cell isolation and culture. Details of the simplified culture method are provided in Dilwali et al.17 Briefly, for SC cultures, GAN samples were washed with sterile PBS thrice to remove accompanying blood or scar tissue and transferred to an equal mixture of supplemented Dulbecco’s Modified Eagle’s Medium (DMEM) and F12 medium, consisting of 44% DMEM (Life Technologies, Grand Island, New York), 44% F12 nutrient mixture (ThermoScientific, Waltham, Massachusetts), 10% fetal bovine serum (Life Technologies), 1% of a mixture of penicillin and streptomycin (#15140-122; ThermoScientific), and 1% GlutaMAX (Life Technologies). Nerve segments were incubated in an enzymatic mixture containing 250 U/mL hyaluronidase type I-S (Sigma-Aldrich) and 160 U/mL collagenase type I (Sigma-Aldrich) in DMEM/F12 medium for 24 hours at 37°C with 5% CO2 levels. No further growth factors were added. After the enzymatic incubation, the cell culture−containing medium was triturated using an 18-gauge needle (BD Biosciences, San Jose, California). Cells were recovered by centrifugation and the pellet was resuspended in supplemented DMEM/F12 medium and plated on Poly-L-lysine and Laminin precoated cover slips (BD Biosciences). Culture medium was replaced with fresh medium after 24 hours and then every 3 days after the initial exchange.

The same protocol was followed for VS cell cultures with the only major difference being 18 hours enzymatic incubation rather than the 24 hours used here for healthy SCs.17

Protein extraction and Western blot. Protein levels of COX-2 were investigated semiquantitatively through Western blot analysis. Protein was extracted from VS specimens and cultures using Radioimmunoprecipitation Assay (RIPA) buffer fortified with phosphatase and protease inhibitor tablets (Roche Applied Science, Penzberg, Germany).
After quantifying the protein concentration in the tissue lysate using spectrophotometry, protein was loaded at a total protein concentration of 7.5 μg per lane, separated on a 4%–20% Tris-glycine gel (Invitrogen), and transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, Massachusetts). The membrane was blocked for an hour with 5% of bovine serum albumin diluted in phosphate-buffered saline with Tween 20 (PBST) (wt/vol) solution and probed with Abcam antibody against COX-2 (#ab15191; Abcam), followed by corresponding secondary antibodies (Jackson-Immuno Research). Antibody against glyceraldehyde 3-phosphate dehydrogenase (#2118; Cell Signaling, Danvers, Massachusetts) served as a total protein loading control. Membranes were visualized with an enhanced chemiluminescence detection system: ChemiDoc XRS+ (Bio-Rad Laboratories, Hercules, California). Band densities were quantified using ImageJ and were normalized to glyceraldehyde 3-phosphate dehydrogenase for a given lane.

**PTG E2 assay.** PTG E2 was assayed in the media of VS cultures and in tumor lysates using the PTG E2 Parameter Assay Kit (#KGE004B; R&D Systems, Minneapolis, Minnesota). Tumor lysates were collected by extracting total protein in PBS fortified with protease and phosphatase inhibitors. A total of 21 μg tumor lysate protein was loaded per well. The media were treated with 2 mM of aspirin, NaSal, and 5-ASA, or 10 μM COX-2 inhibitor II (EMD Millipore). Cultured VS cells and GAN cells were kept in the dark after the addition of BrdU.

**Drug preparation and treatment.** Primary VS and SC cultures were treated with aspirin (#sc-202471), NaSal (#sc-3520), and 5-ASA (#sc-202890) purchased from Santa Cruz Biotechnology (Dallas, Texas). One and 5 mM aspirin, 1, 5, and 10 mM NaSal, and 1 and 5 mM 5-ASA were prepared by mixing the appropriate amount of drug (powder form) into prewarmed culture media. The drug concentrations we used are based on the reported half maximal inhibitory concentration (IC₅₀) values of 2.5–5 mM for aspirin-induced growth inhibition and around 5 mM for NaSal-induced growth inhibition. The cultures were incubated with the drugs for 48 hours. To label proliferating cells, 5-bromo-2′-deoxyuridine (BrdU) was added 20 hours before fixation. pH was measured in the media after drug addition to ensure no significant deviations. Salicylate levels in the media pretreatment were measured by high-performance liquid chromatography using a photodiode array detector at the Massachusetts General Hospital Clinical Laboratory.

**Proliferation assay.** Proliferation was assessed in cultured cells as described in Dilwali et al. Briefly, BrdU was added to the cells at a concentration of 10 μg/mL 20 hours before the cells were fixed. The cells were kept in the dark after the addition of BrdU. Cell and nuclear membranes were permeabilized by incubation in 1% Triton X-100 (#X-100; Sigma-Aldrich) for 10 minutes and by incubation in 2N hydrochloric acid for 20 minutes, respectively, after fixation. Primary antibodies against BrdU (#OBT0030G; AbD Serotec, Oxford, UK) and S100 (#Z031129; Dako) followed by fluorescent anti-rat and anti-rabbit immunoglobulin Gs (Life Technologies) were used. BrdU- and Hoechst-stained nuclei were counted in 3–5 fields and the ratio of BrdU-positive to Hoechst-positive nuclei was used to determine the proliferation rate in vitro. Manual counts were performed by S.D., who was blinded to treatment conditions.

**Apoptosis assay.** Apoptosis was assessed in cultured cells as described in Dilwali et al using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Roche Applied Science) following manufacturer’s instructions. The cells were permeabilized and then incubated in TUNEL mix for 1 hour at 37°C, then for 30 minutes at RT on shaker. Nuclei were labeled with Hoechst stain. The cover slips were mounted onto slides for imaging. TUNEL- and Hoechst-stained nuclei were counted in ≥3 fields and the ratio of TUNEL-positive to Hoechst-positive nuclei was used to determine apoptosis rate in vitro. A positive control of a 10-minute DNase (Roche Applied Science) treatment before TUNEL labeling was used. Manual counts were performed by S.D., who was blinded to treatment conditions.

**MTT assays.** Cell viability was assessed using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (#M-6494; Life Technologies). Cultured VS cells and GAN cells were treated with 2 mM of aspirin, NaSal, and 5-ASA, or 10 μM COX-2 inhibitor II (EMD Millipore). Each treatment was performed in 5–6 random wells from 3 different patients. After 48 hours, 10 μL of the 12 mM MTT was added in each well, and cells were cultured for additional 4 hours. The crystals were dissolved in 500 μL of dimethyl sulfoxide in each well. The optical density (OD) at 540 nm of each well was detected using a photometer. The average OD value of the GAN cells exposed to vehicles (NT) was set as 100% and used to normalize OD values of the GAN cells treated with drugs. Similarly, the average OD value of the VS cells treated with vehicles (NT) was set as 100% and used.
to normalize OD values of the VS cells treated with drugs. The viability in VS cells was compared with that in GAN cells and reported as percent change.

**Statistical analyses.** A 2-tailed *t* test was used to compare differences in qPCR, Western blot analyses, and PTG levels. Spearman’s correlation was used to assess the relationship between PTG levels and culture growth. A paired 2-tailed *t* test was used to compare differences in proliferation and cell death after treatment with salicylates. *P* values for multiple comparisons for the different treatments were adjusted using the Benjamini-Hochberg adjustment for false discovery rate. *P* < 0.05 was considered significant for all analyses.

**RESULTS**

**COX-2 is aberrantly expressed and active in VS and its derived cultures.** COX-2, an enzyme responsible for PTG synthesis, is encoded by the *PTGS2* gene. *PTGS2* was found to be 7.4-fold higher (range of 3.7–15.1, *P* = 0.02) in human VS (n = 9) in comparison with healthy nerves (GAN, n = 8), as measured through qPCR on extracted RNA from fresh human VS and GAN tissue (Fig 1, A). Furthermore, through immunohistochemistry, COX-2 was minimally detectable in 2 of 5 healthy GAN specimens: although the SCs were S100-positive as they wrapped around nerve fibers of GANs, only a few (approximately 4–5 cells per frame) COX-2–positive cells could be identified (Fig 1, B (a)). COX-2 staining was noted in most of the cytoplasmic and perinuclear regions of VS cells in 4 of 6 specimens, with 2 having a smaller COX-2–positive cell population (Fig 1, B (b)). PTGs were also detected in different tumor lysates (n = 5) with an average and standard deviation (SD) of 818.9 ± 273.4 pg/100 µg (Fig 1, C). Although PTGs were also detected in healthy nerves (n = 3) with an average and SD of 289.4 ± 85.2 pg/100 µg, the minimal values in tumors (488.8 pg/100 µg) were higher than the maximal levels in healthy nerves (382.2 pg/100 µg) (Fig 1, C). PTG levels in VS lysates were significantly higher than in nerve lysates (*P* = 0.019). Measuring PTGs in buffer only yielded a concentration of 153.8 ± 22.5 pg/100 µg.

At the protein level, COX-2 was present at substantially higher levels in cultured VS cells compared with SCs. Expressed as the mean ± SD, COX-2 expression was 3.6 ± 2.7-fold higher in cultured human VS (n = 6) than SCs derived from GAN (1.0 ± 0.8, n = 6) as quantified through Western blot analysis (*P* = 0.06; Fig 1, D). Similar results were also observed in RIPA-extracted freshly isolated VS tumor tissues (n = 3) and GAN (n = 3) tissues. The COX-2 protein expression level in the VS tumors was 2.05 ± 0.82-fold higher than that in the GAN tissue (*P* = 0.04; Fig 1, E). To understand the role of COX-2 in VS, we examined the correlation of PTG levels in culture media with VS cultures’ growth rates, as quantified by the percentage of BrdU-positive cells in the culture. VS cultures secreted PTGs at varied levels, with an average of 1351 pg/mL and a range of 12–4880 pg/mL, and the PTG concentrations in media strongly correlated (*R* = 0.93, *P* = 0.007) with VS proliferation rate in vitro (n = 6; Fig 1, F).

**Salicylates reduce proliferation and viability of cultured VS cells.** To assess the therapeutic efficacy of COX-2 inhibition, we used 3 clinically relevant and well-tolerated salicylates: aspirin, NaSal, and 5-ASA. These drugs were tested on primary VS cultures established from different tumors, with n representing the number of different primary VS cultures used. We found that these inhibitors, used at physiologically relevant concentrations, selectively reduce VS-cultured cell proliferation. Representative images of NT cells, 5 mM aspirin-treated cells, and 1 mM NaSal-treated cells are shown in Fig 2, A (a–c), respectively. Data are summarized as average ± standard error of the mean (SEM). Benjamini-Hochberg adjusted *P* values are provided. Proliferation is normalized to the NT cells for each culture. After 1 and 5 mM aspirin treatment, proliferation changed in VS cells to 129.6 ± 26.2% (n = 4, *P* = 0.34) and 19.3 ± 5.5% (n = 5, *P* = 0.00008), respectively, of the NT cells (having an SEM of 42.3%) (Fig 2, B). After 1, 5, and 10 mM NaSal treatment, VS cell proliferation changed to 18.9 ± 5.0% (n = 3, *P* = 0.004), 25.4 ± 11.1% (n = 7, *P* = 0.0002), and 20.6 ± 11.2% (n = 6, *P* = 0.0008), respectively, of the NT cells (having an SEM of 33.4%) (Fig 2, B). After 5 and 10 mM 5-ASA treatment, VS proliferation changed to 66.0 ± 15.1% (n = 6, *P* = 0.10) and 54.8 ± 16.5% (n = 6, *P* = 0.03), respectively, of the NT cells (having an SEM of 36.3%) (Fig 2, B). Going from most effective to least effective based on dosage, NaSal, aspirin, and 5-ASA were all effective in reducing proliferation in VS cells.

Salicylates at these concentrations did not induce significant cell death in VS cells as measured by TUNEL staining (Fig 2, C (a–c)). After treatment with 1 and 5 mM aspirin, the cell death rate did not change, going from 0.8 ± 0.4% in the NT cells to 0.6 ± 0.3% (n = 6, *P* = 0.31) and 2.8 ± 2.2% (n = 5, *P* = 0.42), respectively (Fig 2, D). Similarly, the cell death rate was not significantly affected for 5 mM NaSal and 5-ASA treated cells, going from 1.0 ± 0.5% in the NT-cultured VS cells to 3.3 ± 2.3% (P = 0.19) and 5.6 ± 3.6% (P = 0.19), respectively (n = 5; Fig 2, D).
Fig 1. COX-2 is aberrantly upregulated in VS and derived primary cultures. (A) PTGS2 gene expression in human VSs (n = 9 different tumors) vs GANs (n = 8 different nerves) as measured through qPCR. Error bars represent range. (B) Representative images of COX-2 expression (green) as visualized through immunohistochemistry in (a) GAN (n = 5 different nerves) and (b) VS (n = 6 different tumors). Schwann or schwannoma cells are labeled with S100 (red) and nuclei are labeled with Hoechst (blue). (C) PTG levels in tissue lysates of VS (n = 5 different tumors) and GAN (n = 3 different nerves). Error bars represent standard error of the mean. (D) COX-2 expression in cultured human VS (n = 6 different tumors) normalized to the expression in SC cultures (n = 6 different nerves) as quantified through Western blot analysis. Error bars represent standard deviation. (E) COX-2 expression in tissue specimens of VS (n = 3 different tumors) and GAN (n = 3 different nerves) assessed by Western blot analysis. Error bars represent standard deviation. (F) Correlation of PTG concentrations secreted in VS culture media with VS proliferation rate (% BrdU-positive cells) in vitro. R represents Spearman’s correlation coefficient (n = 6).
Our results suggest that these salicylates are selectively cytostatic against VS cells.

Because the hypothesized mechanism of antiproliferative effect of salicylates is COX-2 inhibition, we tested a specific COX-2 inhibitor (COX-2 inhibitor II) in 3 different tumor samples. Using a substantially smaller concentration of COX-2 inhibitor II than of salicylates, we found that 10 μM COX-2 inhibitor II reduced the proliferation in VS cells to 48.76 ± 11.93% (P = 0.0007), as reflected in BrdU labeling (Fig 3, A).

To further characterize the cytostatic effect of salicylates, we used the MTT assay. VS cells and GAN cells were treated with 2 mM of aspirin, NaSal, 5-ASA, or 10 μM COX-2 inhibitor II for 48 hours. Compared with that in GAN cells, treatment with aspirin, NaSal, or 5-ASA reduced the viability of VS cells to 70.65 ± 6.82% (n = 5, P = 0.0007), 72.23 ± 6.68% (n = 6, P = 0.0002), or 69.35 ± 9.27% (n = 5, P = 0.002), respectively, whereas COX-2 inhibitor II treatment reduced VS cell viability to 62.58 ± 4.95% (n = 6, P = 0.00001) (Fig 3, B). Taken together, these data suggest that the cytostatic effect of salicylates may depend on the inhibition of COX-2.

Additionally, we measured levels of PTGs in VS to assess COX-2 inhibition. Treatment with 1 and 5 mM aspirin, and 5 mM NaSal reduced secreted PTG levels to 3.1% (n = 4, P = 0.000002), 3.8% (n = 4, P = 0.000005), and 32.2% (n = 3, P = 0.07) of NT cells, respectively (Fig 3, C). Our results suggest that COX-2 was inhibited after salicylate treatment.

Salicylate levels measured in culture media with 1 mM aspirin, 5 mM aspirin, 1 mM NaSal, and 5 mM NaSal, shown as the mean ± SD were 0.88 ± 0.28, 3.33 ± 1.33, 17.44 ± 0.15, and 68.24 ± 2.61 mg/dL, respectively. No salicylate was detected in plain media or media with 5 mM 5-ASA.

**Salicylates do not reduce proliferation of SCs.** The cytostatic effect of salicylates against VS cells seemed to be specific to the neoplastic cells because treating healthy SCs with the same concentrations of the drugs did not lead to a decrease in cell proliferation. These drugs were tested on primary SC cultures established from different GANs, with n representing the number of different primary SC cultures used. After aspirin treatment, proliferation did not change in SCs, going to 124.4 ± 72.9% (P = 0.48) and 198.1 ± 141.3% (P = 0.47) of the NT cells with 1 and 5 mM aspirin, respectively (n = 3; Fig 3, D). After NaSal treatment, proliferation was not affected until the highest dose of 10 mM NaSal. Proliferation rate was 104.4 ± 13.2% (n = 3, P = 0.45) and 64.9 ± 18.9% (n = 4, P = 0.03) of the NT cells with 5 and 10 mM NaSal, respectively (Fig 3, D). After 5-ASA treatment, proliferation did not change in SCs at 107.8 ± 22.4% (P = 0.51) and 109.7 ± 26.7% (P = 0.54) of the NT cells with 5 and 10 mM 5-ASA, respectively (n = 3; Fig 3, D). These results suggest the promising utility of salicylates to specifically target VS cells.

**DISCUSSION**

We have shown that well-tolerated and clinically common salicylates led to selective decrease in proliferation and in secreted PTG levels in primary VS cultures. Our in vitro results parallel our findings of a clinical study in which we correlated the growth rates of human VSs, calculated by measuring changes in tumor size on serial magnetic resonance imaging scans, with the patient’s intake of aspirin (for unrelated medical diagnoses to VS). In that retrospective study, based on a review of the medical records over the past 32 years at our clinical center, we found that the probability of VS growth in patients who took aspirin was approximately half of that in patients with VS who did not take aspirin. Medical records that specified aspirin dose reported oral intake of either 81 or 325 mg daily, with most (38) patients taking 81 mg for comorbidities such as cardiovascular disease. Although a low-dose aspirin (81 mg daily) may not reach the concentration in sera that we found therapeutic in our present in vitro work (1–5 mM), the acidic properties of salicylates allow them to have a high affinity toward sites of inflammation, potentially explaining their efficacy at low dosages. Other clinical studies have shown a protective and therapeutic effect of a low dose aspirin against different types of cancers. Although it has been known for decades that blood levels after intake of these drugs vary in humans, the therapeutic serum concentrations of the active metabolite (salicylate) that are considered adequate to treat inflammatory conditions range from 1.1 to 2.2 mM, comparable with dosages we found efficacious in vitro. The salicylate levels measured in media with dosages that led to significant reduction in VS proliferation in vitro, being 17.4 mg/dL at 1 mM NaSal and 3.3 mg/dL at 5 mM aspirin, would be detected in serum with a dose of around 200 and 800 mg of the respective drugs. This dose is less than the range of salicylate toxicity, with milder symptoms such as tinnitus being noted at different cultures). *P < 0.05. BrdU, 5-bromo-2'-deoxyuridine; COX-2, cyclooxygenase 2; GANs, great auricular nerves; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PTG, prostaglandin; qPCR, quantitative polymerase chain reaction; re = in comparison with; SCs, Schwann cells; VSs, vestibular schwannomas.
approximately 25–35 mg/dL serum salicylate levels. Because of the simplified nature of a culture model, it is difficult to directly translate the concentration effective on cultured tumor cells with the concentration required in vivo to be efficacious when administered systematically. To gain some insight into whether these concentrations would be feasible in vivo, we applied salicylates onto healthy SCs. We did not find a decrease in SC proliferation with salicylates, suggesting the dosages to be tolerable to SCs. Additionally, salicylates readily cross the blood-brain barrier and can reach up to 50% of the concentration present in the blood, an appealing aspect that makes translation of salicylates against VS even more promising. Regardless, salicylate concentrations in tumor tissue are likely to be similar to those in serum because the blood-brain barrier is compromised in intracranial tumors. Nonetheless, because NSAID concentrations effective against VSs in vivo have not been established, it would be important to define drug dosage curves for NSAIDs in vivo through the use of mouse models or phase 0 trials in humans. Further, the use of a specific COX-2 inhibitor, COX-2 inhibitor II, at a concentration of 10 μM also led to decreased proliferation of cultured VS cells, suggesting that clinically relevant specific COX-2 inhibitors, such as celecoxib, could be effective at even lower dosages and may be more so well tolerated than NSAIDs at the ≥1 mM dosages efficacious in our VS culture work.

Fig 2. Salicylates decrease proliferation of VS cells. (A) Representative VS culture proliferation images are shown after treatment for (a) no treatment control (NT), (b) 5 mM aspirin, and (c) 1 mM NaSal. S100 marks schwannoma cells, BrdU in nuclei marks proliferating cells. Nuclei are labeled with Hoechst. Scale bar = 100 μm for all images. (B) Quantification of proliferation changes after treatment with aspirin, NaSal, and 5-ASA in primary VS cells normalized to proliferation in NT cells (n = 3–7 different cultures). Error bars represent standard error of the mean. (C) Representative VS culture cell death images are shown after treatment for (a) NT, (b) 5 mM aspirin, and (c) 5 mM NaSal. TUNEL (green) marks dying cells. Nuclei are labeled with Hoechst. Scale bar = 100 μm for all images. (D) Quantification of cell death rate changes after treatment with aspirin, NaSal, and 5-ASA in primary VS cells (n = 5–6 different cultures for each). Error bars represent standard error of the mean. *P < 0.05; **P < 0.01; ***P < 0.001. 5-ASA, 5-aminosalicylic acid; BrdU, 5-bromo-2′-deoxyuridine; NaSal, sodium salicylate; re = in comparison with; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; VSs, vestibular schwannomas.
and cytotoxic in neoplastic cells, most studies implicate salicylate-mediated cytotoxic effect to mechanisms other than COX-2 inhibition. In our case, salicylates may have a different therapeutic window for cytotoxic than for cytostatic effects in VS cells; we did not test higher salicylate concentrations because they would be greater than the range considered safe in vivo.

Interestingly, salicylate was not detected in media with 5-ASA, suggesting that 5-ASA may be acting through an alternative active metabolite to inhibit VS proliferation. Further, as we have only shown a correlated decrease in PTG levels with salicylate application, it is feasible that the salicylates could be acting through other molecular pathways along with COX-2 inhibition to lead to VS cytostaticity as salicylates do have multiple targets. For instance, although COX-2 is a preferential target for salicylates compared with COX-1, it is possible that COX-1 is also inhibited in VS cells as COX-1 expression and activity was not assessed in this study. Additionally, aspirin and NaSal can also inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) directly, through blockade of I kappa B kinase (IκB), especially at higher dosages (≥5 mM). Aspirin may operate through this mechanism in our work, as we do not note decreased proliferation at 1 mM aspirin, although PTG secretion may have a different therapeutic window for cytotoxic effects in VS cells; we did not test higher salicylate concentrations because they would be greater than the range considered safe in vivo.

It has also been shown that celecoxib, a COX-2-specific inhibitor, could induce apoptosis in colon cancer lines by inhibiting the 3-phosphoinositide-dependent kinase 1 (PKD-1) activity. PKD-1 is an upstream molecule of AKT; it can phosphorylate AKT and induce AKT activities. PKD-1 is also involved in NF-κB activation. These results in colon cancer cells indicated that PKD-1 and AKT are both involved in the proliferation of tumor cells. By analogy, similar pathways may be regulating the growth of VS. Indeed, it has been shown that the promotion of VS invasion by epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) was modulated by AKT.

Although COX-2 inhibition does not seem to lead to significant adverse effects, COX-1 inhibition can interfere with homeostatic functions, which may cause increasing incidence of gastrointestinal hemorrhage and ulceration with chronic intake. Among the salicylates tested, aspirin is a more potent drug. It leads to an
irreversible inhibition of COX enzymes by acetylating their binding sites, whereas NaSal and 5-ASA inhibit COX enzymes through reversible competitive binding.\textsuperscript{9,10} We tested NaSal and 5-ASA because they can serve as alternatives to aspirin for people with hypersensitivity to aspirin. Our results also motivate trials of COX-2-selective inhibitors such as celecoxib against VS as these compounds further curb the adverse effects of general COX inhibitors.\textsuperscript{9}

Our preclinical data motivate future work studying the mechanisms behind the therapeutic efficacy of salicylates against VS cells and clinical translation of these drugs against VS. We have established the aberrance of COX-2 in VS and VS cultures. The secreted levels of its enzymatic product, PTGS, correlated with VS culture proliferation rates. We found clinically well-tolerated COX-2 inhibitors, namely aspirin, NaSal, and 5-ASA, to minimize proliferation of VS cells, without affecting healthy SCs. Our in vitro findings corroborate our retrospective clinical observation that the probability of VS growth decreased to approximately half in patients taking aspirin.\textsuperscript{20} To the best of our knowledge, salicylates would be the most promising treatments against sporadic VS as they are commonly used for a variety of pathologies, including other tumors such as colon cancer, with minimal adverse effects when used within the clinically well-established therapeutic range. For the histologically nonmalignant VSs, the cytostatic effect alone, without the cytotoxic effect, would be therapeutic.

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REFERENCES

13. Kruis W, Schreiber S, Theuer D, et al. Low dose balsalazide (1.5 g twice daily) and mesalazine (0.5 g three times daily) maintained remission of ulcerative colitis but high dose balsalazide (3.0 g twice daily) was superior in preventing relapses. Gut 2001;49:783–9.