Acid ceramidase as a therapeutic target in metastatic prostate cancer

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Abstract Acid ceramidase (AC) catalyzes the hydrolysis of ceramide into sphingosine, in turn a substrate of sphingosine-1-phosphate. AC is expressed at high levels in several tumor types and has been proposed as a cancer therapeutic target. Using a model derived from PC-3 prostate cancer cells, the highly tumorigenic, metastatic, and chemoresistant clone PC-3/Mc expressed higher levels of the AC ASAH1 than the nonmetastatic clone PC-3/S. Stable knockdown of ASAH1 in PC-3/Mc cells caused an accumulation of ceramides, inhibition of clonogenic potential, increased requirement for growth factors, and inhibition of tumorigenesis and lung metastases. We developed de novo ASAH1 inhibitors, which also caused a dose-dependent accumulation of ceramides in PC-3/Mc cells and inhibited their growth and clonogenicity. Finally, immunohistochemical analysis of primary prostate cancer samples showed that higher levels of ASAH1 were associated with more advanced stages of this neoplasms. These observations confirm ASAH1 as a therapeutic target in advanced and chemoresistant forms of prostate cancer and suggest that our new potent and specific AC inhibitors could act by counteracting critical growth properties of these highly aggressive tumor cells.—Camacho, L., O. Meca-Cortés, J. L. Abad, S. García, N. Rubio, A. Díaz, T. Celiá-Terrassa, F. Cingolani, R. Bermudo, P. L. Fernández, J. Blanco, A. Delgado, J. Casas, G. Fabriàs, and T. M. Thomson. Acid ceramidase as a therapeutic target in metastatic prostate cancer. J. Lipid Res. 2013. 54: 1207–1220.

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Cancer cells develop a lipogenic phenotype that supports the energy and membrane synthesis requirements associated with the enhanced proliferation and survival under stress inherent to malignant progression (1, 2). The recognition of the importance of this phenotype in cancer has led to the use of enzymes of lipid metabolism as markers to monitor neoplastic progression and response to therapy, as well as to the development of drugs targeted at key lipogenic enzymes, such as fatty acid synthase (FASN) (2, 3). The excess fatty acid synthesis that results from the coordinated activation of lipogenic enzymes in many types of cancer leads to the accumulation of palmitate, which needs to be further processed by the cells due to the toxic effects of its accumulation. One pathway that...
many neoplastic cells activate to offset the toxic accumulation of palmitate is its peroxisome proliferator-activated receptor (PPAR)γ-dependent funneling to eventually form triglycerides, which can be further used as energy stores (4). A second pathway followed by palmitate is the condensation of palmitoyl CoA with L-serine, leading to the synthesis of ceramides (5). The accumulation of ceramides also poses a problem for cell survival because of its proapoptotic consequences (6–8). This can be counteracted by activities that convert ceramides to a variety of metabolites, including sphingosines through deacylation catalyzed by ceramidases. Sphingosines can be converted into the growth- and survival-promoting sphingosine-1-phosphate (S1P) through the action of sphingosine kinases (8). S1P can be either irreversibly degraded by S1P lyase or reutilized by sequential dephosphorylation and acylation for ceramide synthesis. The importance of ceramidases in the context of cancer is supported by the observation that their inhibition by drugs or RNAi severely compromises the growth and survival under stress of tumor cells (9).

De novo ceramide biosynthesis requires the coordinate action of serine palmitoyl transferase and ceramide synthase to generate ceramide. This process begins with the condensation of serine and palmitoyl-CoA to form 3-ketosphinganine (5), which is reduced to the sphingoid base sphinganine and acylated by ceramide synthase to generate dihydroceramide. This compound is oxidized to ceramide by introduction of a trans-4,5 double bond. This pathway can be stimulated by drugs and ionizing radiation and usually results in a prolonged ceramide accumulation (10).

Once generated, ceramide may amass or be converted into a variety of metabolites. Phosphorylation by ceramide kinase (11) generates ceramide 1-phosphate, while deacylation by alkaline, neutral or acid ceramidases (the products of different genes) (12) yields sphingosine, which may be phosphorylated by sphingosine kinase to S1P. Two distinct sphingosine kinases have been cloned. These two isoforms differ in temporal patterns of expression during development, are expressed in different tissues, and possess distinct kinetic properties (13), implying that they perform different cellular functions. Ceramide may also be converted back to SM by transfer of phosphorylcholine from phosphatidylcholine via SM synthases (14). Alternatively, it can be glycosylated by glucosylceramide synthase to form glucosylceramide, which may be further modified by various enzymes in the Golgi apparatus to form complex glycosphingolipids (15).

Many tumor types express high levels of acid ceramidase (AC). Specifically, the expression levels of AC in prostate cancer have been reported to be elevated relative to normal prostate tissue (16, 17). Prostate cancer (PC) is the most prevalent neoplasia in men in industrialized nations (18). Although PC is frequently initially sensitive to hormonal deprivation therapies and follows indolent clinical courses, a significant proportion of cases eventually become resistant to such therapeutic approaches, accompanied with aggressive growth, establishment of metastasis, and tumors that are highly resistant to conventional chemotherapeutic regimes (19, 20). Two major challenges in PC are to find predictive markers that identify those tumors most likely to follow a hormone-independent, aggressive clinical course as aids to decide early intervention and to identify molecular targets for improved therapies of castration-resistant cases that respond poorly to conventional chemotherapeutic regimes. Here, we provide new evidence to reinforce the notion that the acid ceramidase ASA1 is a valid therapeutic target in advanced prostate cancer, and we characterize new potent and specific inhibitors of AC.

**METHODS**

**Cells and reagents**

PC-3/Mc and PC-3/S cells (21) were grown in RPMI1640 medium supplemented with 10% fetal bovine serum, nonessential amino acids, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from PAA, Ontario, Canada). Fibroblasts from a Farber patient (FD, wild-type) and FD transformed to stably overexpress AC (FD10X) were grown in a humidified 5% CO₂ atmosphere at 37°C in DMEM medium supplemented as above.

**Transient transfection of fibroblasts**

Twenty-four hours before transfection, cells were plated at a density of 2.5 × 10⁵ cells in 35 mm diameter plates. Cells were then transfected with the specific constructs or empty vectors using lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, cells were either processed immediately or collected by trypsinization, washed twice with PBS, and centrifuged. Pellets were stored at −20°C until use. In transfections with neutral ceramidase (NC), the success of transfection was confirmed by activity assays with CerC12NBD in intact cells.

**Fluorogenic ceramidase assay**

Cells were collected by trypsinization, washed with PBS, resuspended in 0.25 M sucrose and lysed by ultrasonication in an ultrasonic bath. For the assay, 75 µl of reaction buffer (100 mM sodium acetate buffer, pH 4.5, for acid ceramidase activity), containing 40 µM RMR41C12 fluorogenic substrate (22) (with or without test compounds), was mixed with 25 µl of the cell lysates (20 µg of total protein content) and incubated at 37°C for 3 h. For time dependence of inhibition, incubations were carried out for 0.5, 1, 2, and 3 h with different amounts of protein. The reaction was stopped by addition of methanol followed by NaOEt (2.5 mg/ml in 200 mM glycine/NaOH buffer, pH 10.6). After 1 h at 37°C, 100 µl of 200 mM glycine/NaOH buffer were added and fluorescence detected at 355/460 nm excitation/emission wavelengths on a SpectraMax Microplate Reader (Molecular Devices, Sunnyvale, CA). To determine ceramidase activity in intact cells, 2 × 10⁴ cells/well were seeded in 96-well plates (Nunc, Roskilde, Denmark). The following day, medium was replaced by 100 µl fresh medium containing 40 µM RMM14C12 fluorogenic substrate incubated for 3 h at 37°C in a 5% CO₂ atmosphere, and the assay was continued as above.

**Papain activity**

Papain activity was determined in 96-well plates by a modification of the reported procedure (23). The reaction mixture contained 250 µl of 0.1 M phosphate buffer (pH 6.5) with 0.3 M KCl, 0.1 mM EDTA, and 3 mM DTT; 30 µl of substrate solution (L-pyroglutamyl-L-phenylalanyl-L-leucine-β-nitroanilide; 2.2 mM
in DMSO, 0.22 mM final concentration); 20 µl of enzyme solution (30 µg/ml in reaction buffer); and 5 µl of inhibitor solution or vehicle. Chymostatin at 1 µM and 10 µM was used as positive control of inhibition of papain activity. The reaction was stopped by the addition of 20 µl of 1 N HCl, and the OD was measured at 410 nm.

**Sphingolipid analysis by UPLC/MS**

The liquid chromatography-mass spectrometry equipment consisted of a Waters Acquity UPLC system connected to a Waters LCT Premier orthogonal accelerated time of flight mass spectrometer (Waters, Millford, MA), operated in positive electrospray ionization mode. Full scan spectra from 50 to 1,500 Da were acquired, and individual spectra were summed to produce data points every 0.2 s. Mass accuracy and reproducibility were maintained by using an independent reference spectrum by the LockSpray interference. The analytical column was a 100 mm × 2.1 mm id, 1.7 µm C8 Acquity UPLC BEH (Waters). The two mobile phases were phase A: MeOH/H2O/HCOOH (74:25:1 v/v/v); phase B: MeOH/HCOOH (99/1 v/v), both also containing 5 mM ammonium formate. A linear gradient was programmed as follows: 0.0 min: 80% B; 3 min: 90% B; 6 min: 90% B; 15 min: 99% B; 18 min: 99% B; 20 min: 80% B, at 0.3 mL/min flow rate. The column was held at 30°C. Quantification was carried out using the extracted ion chromatogram of each compound, using 50 mDa windows. Linear dynamic range was determined by injecting standard mixtures, and positive identification of compounds was based on accurate mass measurement (<5 ppm error) and LC retention time compared with that of a standard (±2%).

**HPLC/fluorescence detection**

These analyses were carried out in an Alliance Waters 2695 HPLC system coupled to a Waters 2475 Multi λ fluorescence detector (Waters, Milford USA) equipped with an Atlantis T3 C18 (50 mm × 4.6 mm) column (Waters). The mobile phase was composed of a mixture of acetonitrile/H2O (80:20), and the flow rate was set at 1 ml/min. All solvents contained 0.1% trifluoroacetic acid. Fluorescent compounds were monitored at 420/483 nm excitation/emission wavelengths. Peak quantification was carried out using the Empower Pro 2.0 software (Waters).

**Western blotting**

Cells were lysed (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na3PO4, 2 mM Na2VO4, 1% Triton-X 100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM PMSF, 1 mg/ml aprotinin, and leupeptin), proteins separated by SDS-polyacrylamide electrophoresis (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membrane blots (Bio-Rad). After blocking with 5% nonfat milk in PBS containing 0.1% Tween 20, membranes were incubated with primary antibody, washed, incubated with HRP-conjugated goat anti-rabbit IgG or anti-mouse IgG antibodies, and then washed again. Signals were detected by chemoluminescence (ECL Western Blotting Detection Kit, Amersham Biosciences, Barcelona, Spain). Actin signals were used as protein loading and transfer references.

**Real-time RT-PCR**

Total RNA was extracted with the RNeasy Kit (Qiagen, Venlo, Netherlands). Complementary DNAs were synthesized with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR assays were performed on a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) and analyzed with the LightCycler 480 Software release 1.5.0. The amplification levels of RN18S1 and HMBS were used as internal references to estimate the relative levels of specific transcripts, and relative quantification was determined by the ΔΔCp method. All determinations were done in triplicate.

**Cell cycle analysis**

Cells were seeded in 6-well Corning plates (Corning, NY), detached with Trypsin/EDTA/1% BSA, washed twice, resuspended in PBS, and fixed at 4°C for at least 2 h by dropwise addition of 70% ethanol. Subsequently, cells were washed with PBS/50 mM EDTA/1% BSA and incubated with 1 mg/ml RNase A (Sigma) at 37°C for 1 h and 0.1 mg/ml propidium iodide (Sigma, Alcobendas, Madrid, Spain). DNA content was determined in a Cytoomics EC500 instrument (Coulter, Hialeah, FL), and cell cycle distribution analyzed with Multicycle. All determinations were done in triplicate.

**Anchorage-independent growth**

For soft-agar colony formation assays, 0.5% agar in complete culture medium was placed at the bottom of 12-well plates, allowed to solidify, and overlayed with a suspension of 3 × 105 cells in 0.3% agar in complete medium. After solidification, wells were fed with complete medium twice a week. After three weeks, they were fixed with 0.5% glutaraldehyde, stained with 0.025% crystal violet, and visualized under a Leica magnifying glass (Wetzler, Germany) coupled to an Olympus digital camera (Olympus, Hamburg, Germany). Colonies > 0.2 mm diameter were scored with the ImageJ software (National Institutes of Health, MD). Each experimental condition was performed in triplicate.

**In vivo tumor formation**

For localized growth, 1 × 105 to 1 × 106 cells with stably integrated firefly luciferase were injected in a volume of 50 µl of RPMI 1640 (without FBS) intramuscularly in each hind limb of anesthetized six-week-old male NOD-SCID mice. Tumor growth was monitored by luminometry on an ORCA-2BT instrument (Hamamatsu Photonics, Hamamatsu, Japan), 5 min after intraperitoneal injection of luciferin (100 mg/kg in 150 µl of PBS). For lung colony formation, 5 × 104 cells in 150 µl RPMI1640 were injected through the dorsal caudal vein. Mice were imaged immediately after injection, and thereafter, tumor development was monitored by weekly imaging. For bioluminescence plots, photon flux was calculated relative to background values from luciferin-injected mice with no tumor cells and normalized to the value obtained immediately after xenografting. In lung colonization free survival analysis, lesions that had an increased photon flux value above day 0 were counted as events.

**Production and transduction of lentiviral particles**

Constructs based on the pLKOpuro vector and bearing ASAH1-targeting shRNAs or control sequences were purchased from Sigma-Aldrich. The lentivirus packaging cell line HEK293T was cotransfected with these DNAs, together with pCMVdeltaR8.91 and pVSV-G (Clontech, Mountain View, CA) for 12 h using Fugene HD (Roche). Supernatants were collected for the following 48 h and filtered through 0.45 µm methylcellulose filters (Millipore, Billerica, MA). Lentiviral particles were concentrated by ultracentrifugation at 27,000 rpm for 90 min on 20% sucrose density gradients. Viral particles were resuspended with medium and added to the cells together with 8 µg/ml polybrene (Sigma). Cells were infected for 24 h and allowed to recover in fresh medium for 24–48 h. Selection for cells with integrated sequences was carried out for three days in medium supplemented with 5 µg/ml puromycin (Biomol, Exeter, UK).

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Cell viability assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) was added to cultured cells at a final concentration of 0.5 mg/ml, incubated at 37°C for 3 h, and the resulting precipitates were solubilized with dimethyl sulfoxide. Absorbance was measured at 570 nm on a SpectraMax Microplate Reader (Molecular Devices, Sunnyvale, CA).

Synthesis of novel acid ceramidase inhibitors

A solution of 1-hydroxybenzotriazole (18 mg, 0.13 mmol), the corresponding carboxylic acid (0.1 mmol), and N-(3-di- methylaminopropyl)-N-ethylcarbodiimide hydrochloride (20 mg, 0.13 mmol) in CH₂Cl₂ (1 ml) was added to a mixture containing sphinganine (30 mg, 0.1 mmol), NEt₃ (30 µl, 0.2 mmol) in THF or CH₃CN (1 ml). The resulting solution was stirred for 1 h at room temperature and concentrated at reduced pressure. The residue was taken up in CH₂Cl₂ (2 ml), washed with saturated aqueous NaHCO₃ solution (3 x 0.5 ml), and then the solvent was evaporated to give a crude mixture that was purified by flash chromatography on silica gel using a gradient of 0–5% CH₃Cl/MeOH to afford the pure amide in 70–85% yield.

Spectroscopic data for the synthesized compounds: SABRAC : 3H-NMR (400 MHz, CDCl₃): δ 7.40 (1H, NH), 4.20 (2H), 4.05 (1H), 3.85 (1H), 3.83 (1H), 3.81 (1H), 1.55 (2H), 1.25–1.30 (26H), 0.90 (3H). 13C-NMR (101 MHz, CDCl₃): 167.4, 141.7, 138.2, 129.7, 120.2, 74.3, 62.7, 53.9, 34.7, 32.1, 29.8–29.3, 25.9, 22.7, 14.1. RBM1-15: (E)-1,3-dihydroxyoctadecan-2-yl-3-methyl-2-butenamide. 1H-NMR (400 MHz, CDCl₃): δ 6.75 (1H, NH), 5.72 (1H), 5.38 (1H), 4.05 (1H), 3.87 (1H), 3.81 (2H), 1.99 (3H), 1.42 (2H), 1.25–1.35 (26H), 0.88 (3H). 13C-NMR (101 MHz, CDCl₃): δ 166.4, 151.2, 118.5, 74.3, 62.7, 53.9, 34.7, 32.1, 29.8–29.5, 26.3, 22.8, 14.2. RBM1-16: (E)-[2(S,3R)-1,3-dihydroxyoctadecan-2-yl]-methyl-2-butenamide. 1H-NMR (400 MHz, CDCl₃): δ 6.26 (1H, NH), 5.64 (1H), 4.05 (1H), 3.85 (1H); 3.80 (1H), 2.17 (3H), 1.86 (3H), 1.54 (2H), 1.25 (26H), 0.88 (3H). 13C-NMR (101 MHz, CDCl₃): δ 167.4, 151.2, 118.5, 74.3, 62.7, 53.9, 34.7, 32.0, 29.8–29.5, 27.3, 26.1, 22.8, 19.9, 14.2. RBM1-17: (2E,4E)-N-[2(S,3R)-1,3-dihydroxyoctadecan-2-yl]-hexa-2,4-dienamide. 1H-NMR (400 MHz, CDCl₃): δ 7.11 (d, J = 14.8 Hz, 1H), 7.01 (1H, 1H), 6.90 (1H, NH), 6.05 (2H), 5.79 (d, J = 15.2 Hz, 1H), 3.95 (2H), 3.62 (1H), 1.72 (d, J = 6Hz, 3H), 1.42 (2H), 1.19 (2H), 0.82 (3H). 13C-NMR (101 MHz, CDCl₃, CD₃OD): δ 167.3, 141.7, 138.2, 129.7, 121.2, 73.9, 61.9, 54.3, 34.3, 31.9, 29.7–29.3, 26.0, 22.7, 18.5, 14.1. RBM1-18: (E)-N-[2(S,3R)-1,3-dihydroxyoctadecan-2-yl]-but-2-enamide. 1H-NMR (400 MHz, CDCl₃): δ 6.88 (1H, NH), 6.02 (1H), 5.87 (d, J = 14.3 Hz, 1H), 3.85 (1H), 3.71 (2H), 2.63 (1H), 1.87 (d, J = 6.6 Hz, 3H), 1.52 (2H), 1.25 (26H), 0.88 (3H). 13C-NMR (101 MHz, CDCl₃, CD₃OD): δ 166.5, 130.7, 126.4, 72.8, 60.9, 56.3, 35.0, 33.3, 30.9, 30.7–30.4, 26.9, 23.8, 18.0, 14.6. RBM1-19: N-[2(S,3R)-1,3-dihydroxyoctadecan-2-yl]acrylamide. 1H-NMR (400 MHz, CDCl₃): δ 6.35 (d, J = 17.0 Hz, 1H), 6.17 (d, J = 10.2 Hz, 1H), 5.69 (d, J = 11.1 Hz, 1H), 4.16–4.99 (1H), 3.96–3.71 (3H), 1.68–1.46 (4H), 1.25 (24H), 0.88 (3H). 13C-NMR (101 MHz, CDCl₃): δ 165.7, 131.0, 127.3, 74.5, 62.4, 54.0, 34.7, 32.1, 30.0, 29.7, 29.5, 26.3, 22.8, 14.2.

Immunohistochemistry

The procurement of human tissues complied with Spanish legislation regarding consent, privacy, and all legal requirements after approval by the Hospital Clinic Institutional Ethics Committee. Sections (2 µm thick) were obtained for immunohistochemistry either from formalin-fixed and paraffin-embedded tissue blocks or from tissue microarrays (TMA) built with a Manual Tissue Arrayer 1 (Beecher Instruments, Sun Prairie, WI). A total of 33 samples, containing normal, prostate intraepithelial neoplasia, and carcinomatous glands were analyzed. Tissue sections were mounted on xylan glass slides (Thermo Scientific, Braunschweig, Germany) and used for immunohistochemical staining using the Bond Polymer Refine Detection System (Leica Microsystems, Wetzlar, Germany). Samples were deparaffinized, antigen retrieval was performed at pH 6 for 20 min in citrate buffer, and primary antibody was incubated for 1 h at room temperature. Rabbit anti-ASAH1 (BD Transduction Laboratories, Franklin Lakes, NJ) was used at a dilution of 1/100. Staining was scored as the percentage of cells with clear positivity and the predominant staining intensity. Images were captured with an Olympus BX-51 microscope equipped with an Olympus DP70 camera.

Statistical analysis

Significance was determined by the two-tailed unpaired t-test using the Graph Pad Prism 4.0 software.

RESULTS

Increased expression of acid ceramidase in highly metastatic clones derived from PC-3 prostate cancer cells

The PC-3 prostate cancer cell line was used to generate two distinct clonal populations. PC-3/S cells were isolated in vitro by single-cell cloning from Luciferase-expressing PC-3 cells. A second single-cell progeny, hereafter designated PC-3/Mc, was isolated from Luciferase-expressing PC-3/M cells, a PC-3 subline that had been selected in vivo for its high metastatic potential (21). Intramuscular grafting in NOD-SCID mice of 2.5 x 10⁵ PC-3/Mc cells quickly produced large tumors (Fig. 1A) with the appearance of abdominal lymph node metastases by 19 days in 50% of mice (21). In vitro, PC-3/Mc cells grew much faster than PC-3/S cells (Fig. 1B). Moreover, PC-3/Mc cells were highly clonogenic, whereas PC-3/S cells showed limited anchorage-independent growth (Fig. 1C).

To investigate the sphingolipid profiles of both cell lines, cells were seeded (0.25 x 10⁶ cells/ml) and grown under standard conditions, and their sphingolipid composition determined after 48 h of culture. LC/MS analysis showed that total ceramide abundance in PC-3/S cells was 1.3-fold that of PC-3/Mc cells (Fig. 1D). Interestingly, this difference increased to 2.2 for the C14 and C16 N-acyl species (PC-3/S versus PC-3/Mc ratio: 2.2). Likewise, the cell content of SM and ceramide mono- hexosides (CMH, including both glucosylceramides and galactosylceramides) was 1.3–1.5 times higher in PC-3/S cells than in PC-3/Mc cells (Fig. 1E, F), and this difference was similar for all the differently Nacylated species. No significant differences between free bases and
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protein and activity levels were not paralleled by significant differences at the mRNA level (not shown), suggesting a posttranscriptional regulation of ASAH1 expression.

Requirement of ASAH1 for optimal growth and metastatic potential of PC-3/Mc cells

Given the above differential ceramidase activity and expression of ASAH1 protein between metastatic and nonmetastatic prostate cancer cell clonal populations and to determine its importance for the growth and metastatic properties of PC-3/Mc cells, we proceeded to stably knock down its transcript. Cells were transduced with five different lentiviral constructs expressing shRNAs targeting five distinct sequences on the ASAH1 mRNA. Three of these shRNAs were effective at specifically decreasing ASAH1 mRNA levels with variable efficacies, ranging from 90 to 60% (Fig. 2A). This gene knockdown was paralleled by a corresponding inhibition of the AC activity in these cells (Fig. 2A). The silencing efficacies
of shRNAs 399 and 402 were confirmed by Western blotting (Fig. 2B).

The sphingolipid content of ASAH1-knockdown PC-3/Mc cells was analyzed by UPLC-TOF. Both ASAH1-specific shRNAs caused the accumulation of ceramides, SM, and CMH compared with cells transduced with a control lentiviral vector (Fig. 2C–E), indicating an impairment of ceramide catabolism, which confirms the functionality of the knockdowns. Unexpectedly, sphingosine was increased in both knockdown clones (Fig. 2F), which suggests that other ceramidases are upregulated upon chronic knockdown of ASAH1 (24).

We next tested if this accumulation of ceramides provoked an impairment of the growth or viability of ASAH1-knockdown PC-3/Mc cells. Neither of the two knockdowns had any effect on the PC-3/Mc population, nor did they cause the accumulation of sub-G1 populations, suggesting that PC-3/Mc cells are resistant to the apoptotic and growth-inhibitory effects of ceramide accumulation under standard growth conditions. Consistently, the growth rate of ASAH1-knockdown PC-3/Mc cells in standard growth medium (10% FBS) did not differ significantly from that of control cells when seeded at initial densities of 1,000 cells/cm² (Fig. 3A). However, when seeded at a density of 500 cells/cm², ASAH1-knockdown cells showed a significantly reduced growth rate compared with control cells (Fig. 3B). This suggests that ASAH1 might be critically required to sense factors dependent on cell density, including paracrine factors or cell-cell interactions. In order to know whether ASAH1 knockdown sensitized PC-3/Mc cells to limiting concentrations of exogenous growth factors, their rate of proliferation was determined in medium containing 0.5% FBS. Under these conditions, ASAH1-knockdown PC-3/Mc cells grew at significantly slower rates.

![Fig. 2. ASAH1 knockdown causes an accumulation of sphingolipids in PC-3/Mc cells. A: Knockdown of ASAH1 mRNA by five distinct shRNAs, determined by qPCR, and of AC activity as determined with RBM14C12 as a substrate in intact cells. Negative controls were PC-3/Mc cells transduced with lentiviral particles carrying a LK0 vector expressing a nontargeting shRNA sequence. Results were normalized to 18S mRNA or amount of protein. Values are represented as the mean percentage over control of three replicates ± SD. B: Western blotting confirmatory of the specific and effective knockdown of ASAH1 mRNA by shRNAs 399 and 402. Actin signal was used as a control of protein loading and transfer. C–F: Sphingolipid content of PC-3/Mc cells knocked down for ASAH1 with shRNAs 399 and 402, showing the accumulation of ceramides (C), sphingomyelins (D), ceramide monohexosides (E), and sphingosine (F) compared with control PC-3/Mc-LK0 cells. Determinations were carried out by UPLC/TOF. Results are shown as the mean of three values ± SD.](image-url)
Acid ceramidase is required for tumor cell self-renewal and abrogates their anchor-independent colony-forming potential. A-D: Effect of ASAH1 knockdown by shRNA 399 or 402 on the 2D growth of PC-3/Mc cells. Cells were seeded at the specified initial densities on plastic dishes and grown in medium supplemented with either 10% FBS (A, B) or 0.5% FBS (C, D). Controls were PC-3/Mc cells transduced with lentiviral particles carrying a LK0 vector expressing a nontargeting shRNA sequence. The number of cells was determined with the MTT method at the indicated time points after seeding. Data correspond to the mean ± SD of triplicates. Statistical significance: *P < 0.05, **P < 0.005 (two-tailed unpaired t-test). E: Effect of ASAH1 knockdown on the cell cycle distribution of PC-3/Mc cells. Cells stably transduced with the indicated lentiviral particles or a control vector (LK0) were analyzed for DNA content by flow cytometry and analyzed for cell cycle distribution. Data are represented as the mean of triplicates ± SD. F: Effect of ASAH1 knockdown by shRNA 399 or 402 on anchorage-independent cell growth of PC-3/Mc cells. Cells were cultured on soft agar, and colonies were stained with crystal violet after three weeks. Data are represented as the mean of triplicates ± SD.

Fig. 3. ASAH1 knockdown inhibits the growth of PC-3/Mc cells under low-density and low-serum conditions and abrogates their anchor-independent colony-forming potential. A–D: Effect of ASAH1 knockdown by shRNA 399 or 402 on the 2D growth of PC-3/Mc cells. Cells were seeded at the specified initial densities on plastic dishes and grown in medium supplemented with either 10% FBS (A, B) or 0.5% FBS (C, D). Controls were PC-3/Mc cells transduced with lentiviral particles carrying a LK0 vector expressing a nontargeting shRNA sequence. The number of cells was determined with the MTT method at the indicated time points after seeding. Data correspond to the mean ± SD of triplicates. Statistical significance: *P < 0.05, **P < 0.005 (two-tailed unpaired t-test). E: Effect of ASAH1 knockdown on the cell cycle distribution of PC-3/Mc cells. Cells stably transduced with the indicated lentiviral particles or a control vector (LK0) were analyzed for DNA content by flow cytometry and analyzed for cell cycle distribution. Data are represented as the mean of triplicates ± SD. F: Effect of ASAH1 knockdown by shRNA 399 or 402 on anchorage-independent cell growth of PC-3/Mc cells. Cells were cultured on soft agar, and colonies were stained with crystal violet after three weeks. Data are represented as the mean of triplicates ± SD.

Development of novel, highly specific acid ceramidase inhibitors

The above results support the importance of ASAH1 for the self-renewal and metastatic phenotypes of PC-3 prostate cancer cells, and validate it as a potential therapeutic target.
target in hormone-insensitive, metastatic prostate cancer. Several AC inhibitors have been synthesized and tested with potencies in the low micromolar range. Considering that AC is a cysteine hydrolase, a small family of ceramide analogs modified at the amide linkage with thiol reactive functions was generated and tested. These compounds were inspired in reported cysteine protease inhibitors (26) and include two α-haloamides and several α,β-unsaturated amides as Michael acceptors. Their structures are shown in Fig. 5A.

The RBM1 series of compounds was synthesized by N-acylation of dihydrosphingosine following standard procedures. All compounds were tested in intact FD10X cells as well as in cell lysates at pH 4.5 following the reported fluorogenic assay (22, 27). The best inhibitors in intact cells were compounds RBM1-12, RBM1-13, RBM1-18, and SABRAC, with percentages of inhibition ranging from 50 to 70% (Fig. 5B). Whereas RBM1-12, RBM1-13, and SABRAC maintained their inhibitory activities in the in vitro assay, RBM1-18 had no inhibitory activity in cell lysates (Fig. 5B). Therefore, compounds RBM1-12, RBM1-13, and SABRAC were selected for further studies. To assess their specificity, the three compounds were tested for their effects on NC using the standard substrate (28) and on FD cells transiently transfected with the ASAH2 gene. None of the compounds inhibited NC (Fig. 5C), attesting to their specificity as inhibitors of acid ceramidase. In vitro dose-response determinations showed that SABRAC was the best inhibitor, with an IC₅₀ value of 52 nM, followed by RBM1-12 (IC₅₀ = 0.53 µM) and RBM1-13, which exhibited the lowest potency (IC₅₀ = 11.2 µM) (Fig. 5D). Furthermore, both in the presence of SABRAC or RBM1-12, the enzyme activity experienced an exponential decay versus incubation time at the two protein concentrations tested (supplementary Fig. I), indicating an irreversible type of inhibition.

Neither SABRAC nor RBM1-12 inhibited the activity of the cysteine protease papain, whereas chymostatin completely blocked papain activity at the standard concentrations (supplementary Fig. IIA). On the other hand, at conditions under which SABRAC inhibited AC, AC activity was unaffected by the cysteine protease inhibitor E64d (supplementary Fig. IIB).

Effects of AC inhibitors on the growth of PC-3/Mc cells

We next tested the effects of RBM1-12, RBM1-13, and SABRAC on the growth properties of PC-3/Mc cells. First, the inhibitory potency of these compounds on AC was determined by incubating the cells with increasing doses for 48 h, and then determining AC activity in cell lysates using a fluorogenic assay. Under these conditions, the inhibition of AC by RBM1-12 and SABRAC was dose-dependent (RBM1-12: 1 µM < IC₅₀ < 5 µM; SABRAC: IC₅₀ < 1 µM) (Fig. 6A). Surprisingly, RBM1-13, which had shown to be a good AC inhibitor in FD10X intact cells and cell lysates (Fig. 5B), did not display any AC inhibitory effect in PC-3/Mc cells. In fact, RBM1-13 tended to enhance the activity of AC in these cells (Fig. 6A). We do not know the reason for this striking difference in the effects of RBM1-13 between these two cell lines, although differential metabolism or uptake of the compounds in the prostate cancer cell lines tested may contribute to the discrepancy. The sphingolipid profiles of PC-3/Mc cells after treatment with these compounds reflected their AC inhibitory activities. Thus, both RBM1-12 and SABRAC induced an accumulation...
Acid ceramidase is required for tumor cell self-renewal facilitation. The short-term inhibition of AC by chemical inhibitors, such as in the above experiments, would not allow sufficient time to trigger such hypothetical long-term adaptive responses.

These compounds were assessed for their effects on the growth of PC-3/Mc cells. At a concentration of 5 µM and in medium containing 10% FBS, the growth of PC-3/Mc cells was significantly slowed by all the compounds, although RBM1-13 showed the least growth inhibitory activity at 1 µM (Fig. 6C). At 5 µM, the two AC inhibitors active on PC-3/Mc cells, RBM1-12 and SABRAC, completely abolished the ability of PC-3/Mc cells to form colonies in anchorage-independent conditions, while RBM1-13 did not show significant effects in these assays (Fig. 6D). None of the compounds had significant effects at a concentration of 1 µM, and similar to knockdown of ASAH1, none of ceramides (Fig. 6B), whereas, as expected for its lack of AC inhibitory activity in PC-3/Mc cells, RBM1-13 did not significantly alter the abundance of ceramides.

The abundance of complex sphingolipids such as SM or CMH was not significantly affected by any of the compounds, except for the levels of CMH in cells treated with RBM1-12 at 5 µM, which increased 50% over controls (Fig. 6B). This is in contrast with the effect of stably knocking down ASAH1 in PC-3/Mc cells, which caused the accumulation of these complex sphingolipids (Fig. 2D, E). In a second discrepancy between transcript knockdown and chemical inhibition of AC, levels of sphingosine did not change by treatment of PC-3/Mc cells with either RBM1-12 or SABRAC (data not shown), whereas they increased upon ASAH1 knockdown. We speculate that prolonged knockdown of ASAH1 may permit an adaptive response of cells by upregulating other ceramidases to facilitate the removal of the ceramides that accumulate as a consequence of ASAH1 silencing. The short-term inhibition of AC by chemical inhibitors, such as in the above experiments, would not allow sufficient time to trigger such hypothetical long-term adaptive responses.

These compounds were assessed for their effects on the growth of PC-3/Mc cells. At a concentration of 5 µM and in medium containing 10% FBS, the growth of PC-3/Mc cells was significantly slowed by all the compounds, although RBM1-13 showed the least growth inhibitory activity at 1 µM (Fig. 6C). At 5 µM, the two AC inhibitors active on PC-3/Mc cells, RBM1-12 and SABRAC, completely abolished the ability of PC-3/Mc cells to form colonies in anchorage-independent conditions, while RBM1-13 did not show significant effects in these assays (Fig. 6D). None of the compounds had significant effects at a concentration of 1 µM, and similar to knockdown of ASAH1, none
In summary, similar to ASAH1 knockdown, two of the newly developed AC inhibitors, RBM1-12 and SABRAC, showed strong inhibitory activities on AC activity and on the growth and clonogenic capacities of the highly metastatic PC-3/Mc cells. Interestingly, SABRAC displayed strong growth inhibitory effects on PC-3/Mc cells, while exhibiting very limited cytotoxicity. A third compound, RBM1-13, which had AC inhibitory activity in other cells, did not inhibit AC in PC-3/Mc cells, and although it exerted cytotoxic activity on these cells, it had less potent inhibitory activity on the growth of these cells on plastic and showed no effect on their capacity to grow in anchorage-independent conditions.

Expression of ASAH1 in nonmetastatic and metastatic prostate cancer

It has previously been reported that ASAH1 is expressed in a number of tumor types, including PC, at levels higher than in normal tissues (17). However, thus far its expression in PC has not been reported by immunohistochemistry, which permits to correlate staining intensities (as surrogates of expression levels) with morphological parameters. We used an ASAH1-specific antibody (Figs. 1H and 2B) to analyze by immunohistochemistry a total of 33 samples from prostate cancer patients that contained tumoral glands, glands with normal morphologies, and...
Acid ceramidase is required for tumor cell self-renewal with histological grade (Gleason score), stage, or the presence of lymph node metastasis. However, the observation that strong expression of ASAH1 was associated with the preneoplastic PIN lesions more than with normal glands and in turn with prostate adenocarcinoma more than with PIN lesions suggests that ASAH1 expression levels tend to increase during PC progression.

**DISCUSSION**

Advanced, hormone-independent, castration-resistant prostate cancer represents a devastating form of the disease that frequently develops from initially less aggressive tumors and shows resistance to conventional chemotherapeutic agents (18–20). In this evolutive process, the progressive dominance in the tumor of cancer stem cells, endowed with high survival and low drug sensitivity (25, 29), is emerging as crucial.

Here, we studied the clonal population PC-3/Mc, derived from prostate cancer cells and highly enriched in tumor-initiating cells (21), and found that its growth and clonogenic potential are extremely sensitive to the knockdown of ASAH1 or chemical inhibition of AC activity. In addition, knockdown of ASAH1 in these cells strongly inhibited their capacity to grow tumors upon local implantation or to colonize lungs after intravenous injection. Although the growth of PC-3/Mc cells under adherent conditions was also affected by ASAH1 knockdown, it was made most evident only upon deprivation of growth factors, suggesting that ASAH1 is required for optimal growth of PC-3/Mc cells. The expression of ASAH1 in PC-3/Mc cells was highly correlated with the growth and clonogenic potential of these cells, with a strong knockdown of ASAH1 leading to a significant decrease in the growth and clonogenic potential of PC-3/Mc cells.

Thus, strong ASAH1 immunostaining in human prostate tissues tended to be associated with prostate adenocarcinoma (Fig. 7D), with complement to previous reports in which immunohistochemical analysis was not performed, and thus the specific cell type, either epithelial or stromal, expressing ASAH1 was not determined (17). On the other hand, with the cases studied here, stronger ASAH1 staining did not show correlations with histological grade (Gleason score), stage, or the presence of lymph node metastasis. However, the observation that strong expression of ASAH1 was associated with the preneoplastic PIN lesions more than with normal glands and in turn with prostate adenocarcinoma more than with PIN lesions suggests that ASAH1 expression levels tend to increase during PC progression.

![Fig. 7. Immunohistochemical analysis of ASAH1 expression in prostate cancer. A–C: Staining for ASAH1 with a specific antibody of 33 cases of prostate cancer showed expression in normal glands (A), glands with prostate intraepithelial neoplasia or PIN (B), and adenocarcinomatous glands (C), with frequent (36.4% of cases) stronger staining in tumoral (T) than in morphologically normal (N) glands. D: Stronger staining (intensity 3) was observed more frequently in adenocarcinomatous glands (Tumor) than in PIN and morphologically normal glands. E: In samples in which tumoral and morphologically normal glands were analyzed simultaneously, the staining intensities were scored as equal in tumoral versus normal glands in 45.5%, as stronger in tumoral versus normal glands in 36.4%, and as weaker in tumoral versus normal glands in 18.2% of cases.](image-url)
growth of PC-3/Mc cells under limited growth factor supply but is dispensable for adherent growth when growth factors are present at high concentrations. These results, together with the strong phenotype that we observed in anchorage-independent growth assays, which correlates with self-renewal, tumorigenic, and metastatic potentials (21, 25) for both ASAH1 knockdown and AC inhibition, suggest that AC is required for self-renewal and growth factor signaling in drug-resistant and metastatic PC-3/Mc prostate cancer cells.

That ASAH1 knockdown and inhibition of AC produces a specific effect on a particular growth property of PC-3/Mc cells, namely, anchorage-independent spheroid formation, is further supported by the absence of significant cell death or the accumulation of sub-G1 cell populations, in spite of the expected accumulation of ceramides. Additionally, neither ASAH1 knockdown nor AC inhibition caused a significant sensitization of PC-3/Mc cells to drugs used in advanced prostate cancer therapy, including docetaxel, doxorubicin, and etoposide (supplementary Fig. IV), which confirms the chemoresistance of this highly aggressive subpopulation of prostate cancer cells enriched in tumor-initiating cells unaffected by the accumulation of ceramides. The failure of AC inhibition or ASAH1 knockdown to chemosensitize PC-3/Mc cells differs from the sensitization found in various other tumor cells (30–39). In PC-3/Mc cells, AC inhibition or ASAH1 knockdown may upregulate specific pathways to metabolize the resulting ceramide excess. Such pathways include phosphorylation, glycosylation, and conversion into SM. The possibility of phosphorylation as a tumor death escape route (40) does not seem plausible, as no ceramide-1-phosphate was ever detected in PC-3/Mc cells, regardless of any genetic manipulation or chemical treatment. In contrast, most SM and CMH species are significantly higher in ASAH1-knockdown cells than in mock cells. Although SM synthases have been seldom addressed as targets to overcome resistance (41, 42), the usefulness of inhibition of glucosyltransferases as a means to exploit ceramide as an antitumor agent has been extensively documented (43, 44). Furthermore, chemoresistance is not only the result of ceramide clearance by glycosylation but also the increased glycosylation products themselves have been reported to upregulate the expression of multidrug resistant protein 1 (MDR1) through c-Src kinase and β-catenin signaling (45). Whether UDP-glucose ceramide glucosyltransferase (UGCG) or MDR1 is upregulated upon ASAH1 silencing in PC-3/Mc cells has not been investigated. However, the UGCG transcript levels are higher in PC-3/S than in PC-3/Mc cells (unpublished observations), as opposed to their AC activity.

Nevertheless, the significant increase in SM and CMH in PC-3/Mc cells knocked down for AC deserves comment. Because AC is a lysosomal enzyme and ceramides cannot exit from the lysosome (46), a lack of AC activity leads to an intralysosomal accumulation of ceramide. However, sphingomyelin synthase 2 is localized in the plasma membrane, whereas sphingomyelin synthase 1 and glucosylceramide synthase reside in the Golgi apparatus (14, 47). Because of this different enzyme compartmentalization, the increase in SM and CMH is unlikely to arise from augmented synthesis from the lysosomal ceramide. A plausible explanation involves downregulation of lysosomal acid sphingomyelinase and glucocerebrosidase as a result of intralysosomal ceramide buildup. Interestingly, the sphingolipid profile of the PC-3/Mc cells knocked down for AC is similar to that seen in PC-3/S cells. This finding supports that the accumulation of sphingolipids inside the lysosome as a result of different AC activities in both clones is related to the different aggressiveness of the two phenotypes.

The growing recognition of AC as a potential therapeutic target in cancer has encouraged the development of AC inhibitors (12). AG belongs to the N-terminal nucleophile (Ntn) hydrolase family (48). This family of enzymes shares the common feature of having an N-terminal nucleophile, which is generated by autoproteolytic processing (49). The Cys143 nucleophilic thiol in AC is exposed at the N-terminus of the β-subunit after cleavage of the precursor protein. Therefore, AC belongs to the same subcategory of Ntn hydrolases as the cytochrome proteases. Based on this relationship, AC has been recently found to be inhibited by the cytochrome protease inhibitors, cystatins (50).

During the course of this study, we identified novel potent and specific inhibitors of AC within a series of small molecules inspired in reported irreversible cytochrome protease inhibitors (51–53). These compounds feature either an α-halocarboxyl unit or an α,β-double-bond Michael acceptor moiety. The first screening in cells overexpressing AC showed that, among the compounds prepared, the α-bromoamides RBM1-12 and SABRAC were the most potent inhibitors. Within the α,β-unsaturated amides, only the methacrylamide (RBM1-13) elicited AC inhibitory activities both in intact cells and cell lysates. None of the compounds was active on NC, consistent with their conception as thiol-targeting molecules. The low potencies of the other α,β-unsaturated amides relative to RBM1-13 suggest that substitution at the β-position hinders the attack of the enzyme nucleophilic thiolate. Surprisingly, acrylamide RBM1-19 had no activity as AC inhibitor. Although the reasons for this finding have not been investigated, it is possible that the high reactivity of unsubstituted acrylamides results in alternative reactions of RBM1-19 before reaching the AC target. The activity of the bromoamides is especially relevant. SABRAC and RBM1-12 are among the most potent AC inhibitors so far reported (54), with IC50 values of 52 nM and 530 nM, respectively, as assayed with lysates of cells overexpressing AC. Time dependence of inhibition supported that both SABRAC and RBM1-12 are irreversible inhibitors, which agrees with the expected mechanism considering their structure and the involvement of a nucleophilic cysteine residue in the catalytic site. Furthermore, they are inactive over both NC and papain, a cysteine protease, thus supporting their selectivity for AC.

Both SABRAC and RBM1-12 were also potent inhibitors of AC in intact PC-3/Mc cells. Surprisingly, RBM1-13, which inhibited AC in FD10X cells, failed to inhibit AC in
the PC-3/Mc cell line. This difference is likely due to the different incubation times (FD10X, 4 h; PC3Mc, 48 h) pointing to the metabolism of RBM1-13 after long-term incubations. In contrast, the boroamides maintained their inhibitory activity at long incubation times. In agreement with their AC inhibitory activity, both SABRAC and RBM1-12 induced a buildup of ceramides, which was significant compared with controls for all the different Nacyl species. However, levels of sphingosine remained unaffected. The latter results, although unexpected for AC inhibitors, are not unprecedented. Bielawska et al. (55) reported that the sphingolipid profiles of MCF-7 cells treated with different ceramidase inhibitors derived from B13 and D-e-MAPP followed different patterns depending on the chemical function substituted for the original amide. In the case of an Nalkyl analog of D-e-MAPP, namely, compound LCL284, ceramides accumulated, but long chain bases did not change significantly. A plausible explanation is the triggering of compensatory mechanisms to keep bioactive sphingolipids at nonlethal levels. One such mechanism could involve an increased activity of other ceramidas. A similar scenario might lead to the slight increase of sphingosine found in PC-3/Mc cells knocked down for AC. In support of this hypothesis, Hu et al. (24) demonstrated that knockdown of the alkaline ceramidase 3 upregulated the expression of the alkaline ceramidase 2 with increases of both sphingosine and its phosphate.

Our findings support the notion that ceramidase metabolism (56) and, more specifically, acid ceramidase activity, are critical regulators of the self-renewal, tumorigenic, and metastatic potentials of cancer stem cells, represented by the PC-3/Mc population (21), beyond their known regulation of signals that tilt the balance between cell survival and death. Furthermore, we argue that our cellular model, specifically selected for subpopulations with strong self-renewal and aggressive phenotypes, allows to better address therapeutic strategies against advanced cancer (45). The precise mechanism linking the elevated AC activity and the increased aggressiveness of PC-3/Mc cells compared with the PC-3/S clone is under investigation.

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