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Molecular Targeting of Acid Ceramidase: Implications to Cancer Therapy

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Abstract

Increasingly recognized as bioactive molecules, sphingolipids have been studied in a variety of disease models. The impact of sphingolipids on cancer research facilitated the entry of sphingolipid analogues and enzyme modulators into clinical trials. Owing to its ability to regulate two bioactive sphingolipids, ceramide and sphingosine-1-phosphate, acid ceramidase (AC) emerges as an attractive target for drug development within the sphingolipid metabolic pathway. Indeed, there is extensive evidence supporting a pivotal role for AC in lipid metabolism and cancer biology. In this article, we review the current knowledge of the biochemical properties of AC, its relevance to tumor promotion, and its molecular targeting approaches.

Keywords

Ceramidase; sphingolipids; ceramide; sphingosine; cancer therapy

A. Introduction

Sphingolipids, a class of sphingosine-based lipids, have received widespread importance in cancer research [1,2]. The acylated form of sphingosine, ceramide, has emerged as a tumor suppressor lipid owing to its role in cell cycle arrest, differentiation and cell death [3-5]. Ceramide plays key roles in mediating/regulating the cytotoxicity of several of the currently utilized cancer therapies including radiotherapy and chemotherapy. Other metabolites of ceramide have been also studied in the field of cancer biology. In contrast to ceramide, sphingosine-1-phosphate (S1P) is a tumor promoting lipid with well recognized functions in angiogenesis, cell survival, proliferation, migration and inflammation. S1P contributes to these processes mainly through engaging five extracellular G-protein-coupled receptors, S1P1R to S1P5R [6]. This wide variety of biological features has transformed our understanding of sphingolipids from inert structural components of membranes to critical bioactive lipids.

Sphingolipids are controlled by an intricate network of metabolic enzymes distributed over various compartments. Ceramide serves as a hub molecule where sphingolipid catabolism and anabolism intersect. *De novo* synthesis of ceramide is a multistep pathway resulting in ceramide formation on the cytosolic surface of the ER. Alternatively, ceramide can be generated through breakdown of complex sphingolipids mainly sphingomyelin. In turn, ceramide can be derivatised at its free hydroxyl group via phosphorylation to form

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ceramide-1-phosphate (C1P), transfer of phosphorylcholine to form sphingomyelin, or glycosylation to form glucosylceramide, the building block of gangliosides.

An intriguing step in the sphingolipid pathway, illustrated in Fig (1), is the ceramidase reaction which results in deacylation of ceramide to form sphingosine and free fatty acid. From a metabolic standpoint, the ability of ceramidase to directly regulate ceramide (substrate) and sphingosine (product) is critical in several cellular processes, including proliferation, cell cycle arrest, differentiation, death, and motility. On a larger scale, ceramidases indirectly regulate other bioactive lipids namely glucosylceramide, C1P and S1P. Here we shall review the biochemical properties of acid ceramidase, recent advances in understanding the role of AC in cancer biology and the potential for targeting AC in cancer therapy.

B. Acid Ceramidase from Gene to Protein

Acid ceramidase (*N*-acylsphingosine deacylase, EC 3.5.1.23; AC), is a lipid hydrolase that catalyzes the deamidation of ceramide into sphingosine and free fatty acids (reviewed in [7]). The capacity of AC to perform this cellular function is imparted at various levels, beginning with proper transcription and translation, followed by post-translational modifications, proteolytic processing and subcellular trafficking. Once a mature AC enzyme has been generated, there are a variety of other factors that influence its activity, including pH, ions, lipids and other proteins. Thus, following the life history of an AC molecule provides great insight, not only into the complexity of this maturation, but also into the multiple ways by which the cell can regulate AC protein levels, localization, and activity. With this knowledge, biomedical investigation into the physiologic and pathophysiologic function of AC and its ability of to regulate levels of its lipid substrate (ceramide) and product (sphingosine), both with distinct signaling properties, can proceed with greater mechanistic understanding of multiple levels of regulation of this complex and versatile enzyme.

Acid Ceramidase: the gene (ASAH1)

The human gene that encodes AC, *ASAH* (*N*-acylsphingosine acyl hydrolase), spans 30 kb, comprising 14 exons and 13 introns, and maps to the short arm of chromosome 8 (8p21.3-22) (cloned in 1999) [8]. AC expression, assessed only in a small number of tissues, was found to be highest in heart and kidney, with moderate expression in placenta, lung and skeletal muscle. Interestingly, besides the expected 2.4 kb mRNA, additional transcripts were detected in heart and skeletal muscle of sizes 1.7 and 1.2 kb, respectively. Whether or not these additional transcripts encode functional variants of AC is not known.

The full-length cDNA encoding AC is 1205 bp in length with an open reading frame (ORF) of 1185 bp corresponding to 395 amino acids, with a calculated molecular mass of this polypeptide was ~ 44 kDa [9]. The cDNA encoding murine AC has also been cloned, and mAC was found to have ~80% identity and ~90% homology with hAC [10]. This single DNA gives rise to a common glycoprotein precursor that is proteolytically processed into heterodimeric protein consisting of two subunits joined by disulfide bonds (processing is discussed in greater detail below).

The putative promoter region of human AC was characterized upon cloning of the full gene, as Li et al. [8] analyzed the activity of 475-bp of the putative promoter region of human *ASAH1*. Given that the promoter region contained consensus binding sequences for several transcription factors, including AP-2, SP-1 and NF- κ B Park et al. [11] set out to identify the minimal, essential region of the 1931-bp murine *ASAH1* promoter region in NIH3T3 fibroblasts using Luciferase reporter constructs. A 143-bp region from -297 to -154 was

found to be essential for promoter activity, and 34-bp GC-rich region of this portion of the murine AC promoter was found to bind KLF6, SP1 and AP2 transcription factors. Based on results of supershift assays using antibodies to these transcription factors, KLF6 was further investigated, and expression of KLF6 was shown to positively correlate with AC expression, as overexpression of KLF6 increased AC expression whereas KLF6 siRNA reduced AC expression [11].

Post-Translational Modification and Cellular Trafficking

Following translation from mRNA, the nascent AC polypeptide undergoes several modifications that are necessary for proper cellular targeting, enzyme stability, and activity. The maturation of the precursor polypeptide from the ER through the Golgi involves acquisition and processing of 5-6 N-linked glycosylations (roughly 2 kDa per Asn residue), increasing the M_r of the polypeptide from the expected 44 kDa to 53-55 kDa [12]. Further characterization of AC revealed that a single AC cDNA encodes both subunits of the fully functional mature AC protein. A single enzyme with a M_r of 53-55 kDa can be reduced to constituent α - and β -subunits, 13 kDa and 40 kDa in size, respectively, in the presence of sulfhydryl reducing agents. The 13 kDa α -subunit is not glycosylated [12,13], and the β -subunit possesses all N-glycosylations [12]. Site-directed mutagenesis confirmed the importance of the putative N-linked glycosylation sites, three of which were essential for proper proteolytic processing and full enzymatic activity [12]. Mass spectrometric analysis of recombinant hAC obtained from Sf9 cells using a baculovirus expression system indicated that indeed only five of the six predicted N-linked glycosylation sites were utilized [14].

Once in the acidic compartment, the immature AC glycoprotein undergoes proteolytic processing whereby the 53-55 kDa glycosylated precursor is cleaved to form an α -subunit (13 kDa) and a β -subunit (40 kDa) held together by disulfide linkages [12,13]. Under non-reducing conditions, the α - and β -subunits remain associated and co-migrate at 53-55 kDa, but can be dissociated in the presence of a sulfhydryl reducing agent. The pattern of disulfide linkages for recombinant AC purified from Sf9 insect cells was recently reported by Schulze et al. [14], only one of which (Cys10-Cys319) linked the α -subunit to the β -subunit. Of interest, the α -subunit is essential for AC activity as expression of the β -subunit alone did not enhance activity in COS-1 cells [8]. Providing additional evidence for the necessity of both subunits, Farber disease mutations are not isolated to the β -subunit, as several mutations in the α -subunit (Q22H, H23D, Y36C, V96E, Del96V, V97E, 128-152 del, E138V, E138x) have also been reported [7,8].

The mechanism of proteolytic maturation, including the specific compartment and the protease(s) responsible for this maturation, is currently not known. Interestingly, Konrad Sandhoff's group has suggested that this AC may itself catalyze this maturation [14]. Acidification of cell culture supernatant (pH 4.0, 37°C, 5 days) was sufficient to induce proteolysis of the precursor into α - and β -subunits. As discussed by the authors, it remains to be seen whether or not this process is autocatalytic, or involves a secreted protease that is activated by lowering the pH of the conditioned culture medium.

Additional evidence for maturation in endo-lysosomes comes from the qualitative analysis of (1) secretory AC and (2) intracellular AC in the context of impaired lysosomal targeting. The default fate of the portion of AC precursors that is not mannose-6-phosphorylated, and thus not trafficked to lysosomes, is the Golgi secretory pathway. Secretory AC never traverses the acidic compartment and retains its pro-form, as a monomeric 46-48 kDa glycoprotein [12,13], and subsequent shunting of AC precursor through the Golgi secretory pathway results. Disrupting lysosomal targeting, either artificially using lysosomotropic agents (e.g. NH_4Cl) or using I-cell fibroblasts with defective M6P-shuttling, enhances the

secretion of AC, but also prevents the maturation of intracellular AC, with accumulation of the 53-55 kDa precursor [12].

The initial characterization of secretory AC from human skin fibroblasts and COS-1 epithelial cells overexpressing AC cDNA, along with the purified AC from urine, described the protein as possessing qualitative differences from lysosomal AC. Specifically, secretory AC was initially shown to display a complex glycosylation pattern (partial EndoH-resistance) and was not reduced to α - and β -subunits, suggesting that this processing step was unique to the intracellular, lysosomal AC. However, analysis of purified AC obtained from the media of Chinese hamster ovary (CHO) cells stably overexpressing AC cDNA revealed several distinctions from these early reports: (1) AC obtained from conditioned medium could be reduced to constituent α - and β -subunits; (2) this “pseudo-secretory” AC displayed high-mannose type glycosylation pattern, typical of lysosomal enzymes; and (3) AC derived from CHO media co-immunoprecipitated with acid sphingomyelinase (ASMase) and β -galactosidase activity, suggesting a specific interaction between these three enzymes. Of note, AC immune complexes did possess activity for other lysosomal hydrolases, including β -glucosidase and α -iduronidase [15]. The nature and identity of secretory AC thus remains an unresolved issue.

Acid Ceramidase: Enzymology

The first report of ceramidase activity at an acidic pH came from Gatt in 1963 [16]. Since the study by Sugita et al. that linked AC deficiency to Farber disease, several groups have purified AC from various sources [17], although the most purified preparation described to date was published by Sandhoff's group in 1995, wherein AC was purified from urine and enriched > 4,000-fold [13]. The salient features of AC enzymology, including enzyme properties, enzyme kinetics, and reverse activity, will be discussed further.

The activity profile of AC demonstrates an optimal pH range between 4.0 and 5.0 [15], fitting with its presumed localization within acidic organelles. Also fitting with its presumed cellular localization, certain anionic phospholipids, especially bismonoacylglycerophosphate, known to be concentrated in endo-lysosomes, have been shown to not only enhance AC activity, but also increase binding of AC to lipid vesicles [18]. These lipids also increase binding of saposin D (SapD), an *in vivo* cofactor for AC [19] to these same vesicles, both alone and in the presence of AC. Maximum binding of SapD and AC occurred at pH 4.0. Of note, SapD was shown to lower the K_m of AC to substrate without affecting the V_{max} . Additional factors which have been shown to enhance AC activity include salt concentration and enhanced bilayer curvature (SUV>LUV) [18].

Kinetic analysis of the ceramidase reaction at acidic pH has been carried out with various enzyme sources and different substrates. For example, AC from human spleen was assessed using $C_{18:1}$ -Ceramide (N-oleoylsphingosine) by Al et al. [17] and V_{max} and K_m were determined to be 57 nmol/mg/hr and 220 μ M, respectively. In 1995 Bernardo et al. [13] showed that AC had one-quarter the activity towards $C_{18:1}$ -Ceramide (28 nmol/mg/hr) as it did towards C_{12} -Ceramide (N-lauroylsphingosine) [107 nmol/mg/hr], although in both cases double reciprocal plots demonstrated adherence to Michaelis-Menten kinetics. Kinetic analysis of AC secreted from CHO cells stably overexpressing AC cDNA showed further differences using ^{14}C - C_{12} -Cer as well as the fluorescent BODIPY- C_{12} -Cer. While the K_m 's for both substrates were similar (~400 μ M) the V_{max} was 10-fold less for the fluorescent BODIPY analog [15].

Another important enzymologic feature of AC is that in addition to functioning as a ceramide-degrading enzyme, it can also function as a ceramide-generating enzyme. This “reverse” activity, which results in the conversion of sphingosine and free fatty acids back to

ceramide by AC was initially reported soon after the identification of AC. Reverse ceramidase activity occurs at a pH optimum distinct from the forward reaction. While ceramide-cleaving activity is preferred from pH 4.0-5.0, "reverse activity" is maximal between pH 5.5-6.5 [15]. Okino et al. [20] further reported that reverse activity requires natural *D*-erythro-sphingosine and prefers medium chain fatty acids (C₁₂, C₁₄), is potentially activated by phosphatidic acid (PA) and phosphatidylserine (PS), and inhibited by sphingomyelin (SM). Most interesting about these studies was the finding that certain lipids that promoted reverse activity (e.g. PA, PS) inhibited the forward reaction whereas lipids that promoted the forward reaction (e.g. SM, PE, CL) inhibited the reverse reaction [20]. Taken together with the distinct pH profiles for the two activities, it is likely that subcellular or possibly extracellular localization of AC provides a unique mode of regulation of AC activity, in some regions promoting degradation of ceramide, and in others its synthesis. Other unique features of the reverse activity include potent inhibition by zinc (0.5 mM) and inhibition by nonionic detergents (Triton X-100, Igepal CA-630) at concentrations of 0.1% v/v [20].

C. Acid Ceramidase and Tumor Promotion

Increasingly compelling evidence points to important roles for AC and in the regulation of uncontrolled cellular growth and proliferation and in response to cancer therapy.

AC has been shown to be dysregulated in many cancers. A multivariate analysis of gene expression data from melanoma cells revealed that AC may be a useful biomarker for cancer detection and identification [21]. AC was found to be overexpressed in DUI45 (4.8 ×), LnCAP (7.5 ×) and PC3 (2.7 ×) human prostate cancer cell lines compared to a benign prostatic hyperplasia cell line [22]. These same researchers found AC overexpression in 15/36 (42%) prostate cancer tissue samples using competitive RT-PCR ($P < 0.05$), with at least 10 of the samples demonstrating increases higher than twice the SD of the normal tissue mean [22]. Additionally, increased AC expression was present in 60% of Gleason grade 7 tumors and 38% of grade 6 tumors, indicating a possible association between prostate cancer severity and elevated AC expression [22]. Another study used Western blotting to determine that AC was overexpressed in 67% of human prostate tumors as well as 70% of head and neck cancers when matched with normal controls [23].

Additional studies point to functional roles for the overexpressed AC in cancer. Blocking AC activity promoted accumulation of intracellular ceramide at cytotoxic levels. When SW403 human adenocarcinoma cells were treated with the AC inhibitor B13, cellular ceramide levels became significantly elevated ($P < 0.01$), and 90% cancer cell death was detected *in vitro*, implicating AC as a key regulator of ceramide levels in cancer [24]. On the other hand, increasing the activity of AC facilitated cellular proliferation *in vitro* and *in vivo*. Prostate cancer cell lines that have been transfected with AC exhibit a significantly higher growth rate than controls *in vitro* ($p < 0.0003$) and developed quicker into larger tumors when injected into nude mice ($p = 0.0067$) [25].

Based on these findings, AC contributes to cellular proliferation by [1] preventing the accumulation of pro-apoptotic ceramide and [2] generating pro-growth sphingolipid signaling molecules, especially S1P which helps to override normal cell arrest signals. This dual functionality qualifies AC as a prime target for the development of chemotherapeutic agents aimed at inhibiting its activity [26]. Some cancers, however, do not seem to conform to the paradigm of increased AC expression and resulting proliferation. In a limited analysis of thyroid cancers using semi-quantitative RT-PCR, decreased AC mRNA expression compared to normal thyroid tissues was demonstrated in 5/6 follicular adenomas, 2/2 adenomatous goiters, 3/6 papillary carcinomas, and 1/2 follicular carcinomas [27]. Likewise,

an earlier study [28] comparing enzymatic activities between 18 homogenates of colorectal cancer and normal tissue found no increase in ceramidase activity and has been cited as an exception to the paradigm [7]. It is unclear, however, whether the experimental conditions in this study adequately measure AC activity, as opposed to neutral or alkaline ceramidase activity. Overall, the variable expression of AC in different neoplasias likely reflects the diversity of carcinogenesis in specific tissues.

The section below provides a summary of growing evidence in the literature supporting a role for AC in tumor promotion. In particular, we focus the proposed role for AC in cell survival, resistance to cell death and metastasis.

Promotion of Survival

AC-overexpression, coupled with increased expression of sphingosine kinase, may lead to a cellular phenotype highly resistant to apoptosis and prone to proliferation [25]. Prostate cancer cells transfected with AC contained higher levels of sphingosine and S1P, demonstrating AC's enzymatic role in enhanced cellular proliferation signaling [25]. Treatment of the human hepatoma cell line HepG2 with daunorubicin, a chemotherapeutic drug shown to activate AC *in vitro*, resulted in higher levels of intracellular S1P, lower levels of ceramide, and increased cell survival compared to a control treated with the AC inhibitor, *N*-oleoylethanolamine (NOE) [29]. The enhanced cytotoxicity of the combined NOE and daunorubicin treatment was attenuated with the addition of exogenous S1P, increasing HepG2 survival by nearly 50% [29]. Thus, inhibition of AC activity affords a novel strategy for combination chemotherapy and chemosensitization.

Resistance to Death

As a consequence of its overexpression in cancer cells, enhanced AC activity has been linked with the resistance to cellular death. A radiation-resistant glioblastoma cell line revealed elevated levels of AC expression when exposed to γ -radiation [25]. Treatment with the AC inhibitor NOE, however, sensitized these glioblastoma cells to γ -radiation, resulting in ceramide levels 4.5 times greater than controls and increased apoptosis [25]. In addition, AC activity may play a major enzymatic role in cancer resistance to common chemotherapeutic drugs. Prostate cancers cell lines transfected with AC demonstrated greater resistance to pro-apoptotic agents, including doxorubicin, etoposide, cisplatin, gemcitabine, and C6-ceramide [30]. The resistance to doxorubicin in AC-overexpressing cells was significantly decreased with AC-siRNA treatment ($P = 0.0001$), confirming the relationship between AC activity and chemotherapy resistance [30]. Treatment of three human hepatoma cell lines with the AC inhibitor NOE along with vinblastine or doxorubicin resulted in significantly decreased cell survival compared to vinblastine or doxorubicin alone [31]. This sensitization to chemotherapeutic drugs was replicated *in vivo* using AC siRNA [31]. Nude mice with implanted intrahepatic tumors that were treated with AC siRNA and daunorubicin had lower levels of serum alpha-fetoprotein (a marker of hepatic cancer) compared to mice treated with daunorubicin and control siRNA [31]. Researchers have also identified a key role of AC in epidermal growth factor (EGF) inhibition of ceramide-induced apoptosis in cultured trophoblasts. In this study, the authors investigated role of sphingolipids in the EGF-induced survival of primary placental trophoblasts under stress conditions. [25]. Trophoblasts treated with the AC inhibitor NOE for 2 hours negated the anti-apoptotic effects of EGF and exhibited 50% higher levels of cellular ceramide than untreated controls [25]. Furthermore, cellular AC overexpression seems to confer resistance to TNF-induced apoptosis. An AC-transfected murine fibrosarcoma L929 cell line demonstrated markedly lower levels of TNF-induced apoptosis than non-transfected cells or cells transfected with a vector lacking the AC insert [25]. These L929 cells overexpressing AC were also found to have virtually unchanged levels of intracellular ceramide following

TNF treatment, unlike controls [24]. Resistance to TNF-induced apoptosis was overcome by treatment with exogenous C₆- and C₁₆-ceramide and with the AC inhibitor NOE, again illustrating the central role of AC and ceramide metabolism in resisting pro-apoptotic signals [24].

Role in Metastasis

In addition to promoting cell survival and resisting apoptosis, acid ceramidase may also contribute to cancer invasiveness. In a cell migration assay examining the effects of increased cellular AC expression, human prostate cancer cells transfected with AC migrated three times more rapidly than controls [24]. Pretreatment with the AC inhibitors LCL204 and B13 significantly decreased the enhanced migrated rate of AC-transfected cells [24]. It was also reported that cells overexpressing AC exhibited more adherence to collagen- and fibronectin-coated plates in comparison to controls [32]. This *in vitro* demonstration of pro-metastatic AC function verifies the results of an earlier *in vivo* experiment, which found that treatment with the AC inhibitor B13 prevented metastasis in nude mice [33]. Two colon cancer cell lines, SW403 and the aggressively metastatic Lovo, were injected into the portal veins of nude mice (Selzner 2001) [34,35]. After regular peritoneal injections of B13, 4 out of 7 mice in the Lovo injection group and 5 out of 7 mice in the SW403 group remained free of tumors [36]. Of the B13-treated mice that developed tumors, overall tumor mass was significantly less than in the control group [35].

D. Approaches for Targeting Acid Ceramidase

Given its important metabolic and biologic functions, AC emerges as a target enzyme that is attracting several experimental approaches. Pharmacologic inhibitors have been one of the earliest tools employed for studying AC and are still widely used. Various AC inhibitors have been designed with different specificities and potencies. Lipid biologists have utilized advances in RNA interference technology to target the AC message. More recently, genetic approaches were undertaken to generate an AC knock out mouse model. Collectively, these experimental venues have been indispensable for studying the role of AC in health and disease.

Early reports describing cellular ceramidase activity inspired efforts aimed at finding compounds that inhibit ceramide cleavage and cause ceramide accumulation. Sugita et al. identified the first AC inhibitor, N-oleoylethanolamine (NOE) [35,37]. The authors studied potential inhibitors of kidney ceramidase at pH 4.0 using radiolabeled ceramide as a substrate. In addition to the products sphingosine and oleic acid, the authors noted that complex sphingolipids as glucosylceramide and galactosylceramide can inhibit ceramidase. NOE was more potent than the above compounds; however it is a very low potency inhibitor as it inhibited 90% of ceramidase activity at a concentration of 3.8 mM (K_i 7 × 10⁻⁴ M). It is worth pointing that the specificity of NOE to AC versus other enzymes have not been studied yet. Also off note, NOE was found to decrease levels of complex sphingolipids through inhibition of ceramide glycosylation. More recently, Grijalvo et al. successfully generated novel NOE-related inhibitors which were N-acylated with oxoctanoyl or oleoyl moieties [38]. The newly synthesized compounds have promising potential future applications as they demonstrated improved potency and specificity towards AC inhibition.

A series of elegant studies pioneered by Bielawska et al. characterized a novel class of AC inhibitors based on N-acylated phenylammonopropanols of ceramide analogs [39]. A leading compound in this class is D-erythro-2-(N-Myristoylamino)-1-phenyl-1-propanol (D-e-MAPP). In vitro studies using HL-60 leukemia cells demonstrated that D-e-MAPP potently inhibited alkaline ceramidase (IC₅₀ 1-5 μM) and to a lesser extent acid ceramidase (IC₅₀ 500 μM). Importantly, D-e-MAPP demonstrated specificity to the ceramidase reaction as it

failed to inhibit other enzymes of sphingolipid metabolism. A more water soluble form of D-e-MAPP, B13, was shown to be more potent in elevating intracellular ceramide levels [40,41]. Interestingly, B13 was found to be more specific in inhibiting AC. The metabolic effects of B13 correlated with its biologic effects as it was shown to cause apoptosis human colon cancer cells in vivo and in vitro. In a recent follow up study [42], Bielawska and coworkers reported synthesis of novel analogs of D-e-MAPP and B13. Interestingly, some of the new compounds exhibited compartment specific effects on ceramide metabolism within lysosomes (LCL 204) and mitochondria (LCL 120, 85). For more information the reader is referred to the following references [43].

To circumvent specificity concerns of AC pharmacologic inhibitors, lipid biologists utilized recent advances in RNA interference (RNAi) technology. The first study to use this approach demonstrated that transfection of AC RNAi 21 bp duplexes resulted in significant knockdown of AC protein in L929 murine cell line. The study uncovered an intriguing role for AC in TNF-induced prostaglandin production. Another study by Morales et al. used RNAi to modulate AC expression in mouse models. Interestingly tail-vein injection of AC RNAi induced loss of AC mRNA in tumor liver graft cells sparing normal liver parenchyma. Clearly, RNA interference is a powerful tool for validity of results obtained with AC inhibitors.

Cloning of the murine AC gene prompted attempts aimed for generation of AC knockout mice. Interestingly, breeding experiments resulted only in AC^{+/-} offsprings but no viable AC^{-/-} mice. It was concluded that AC null mice are embryonically lethal. Of note, AC^{+/-} mice developed a ceramide storage phenotype. A follow up study revealed that AC^{-/-} embryos reached only the 2-cell stage before undergoing apoptosis. It is worth noting however that embryonic lethality is not part of Farber disease, a genetic disorder with incomplete loss of AC activity.

E. Summary and Conclusions

The above studies clearly demonstrate an important emerging role for AC in regulating sphingolipid metabolism, and especially the clearance of ceramide and the generation of S1P. Moreover, additional studies demonstrate dysregulated AC levels in many, but not all, cancer types with accumulating evidence suggesting a role for AC in mediating resistance of these cancers to radiation and chemotherapy and a role in imparting growth and metastasis advantages, as illustrated in Fig (2). Initial studies with first and second generation inhibitors point to the feasibility and effectiveness of inhibiting AC in experimental models of cancer.

While targeting AC is a promising approach in cancer therapy, several challenges have yet to be addressed. First, the essential role of AC in ceramide catabolism needs to be considered. Although mammalian cells are equipped with other ceramidases (neutral and alkaline ceramidases) only a defective AC results in ceramide-storage disease (Farber disease). Chronic inhibition of AC might compromise its basic metabolic function resulting in a lipid storage phenotype. However, if one targets the aberrant increase in AC in specific cancers, then this may prove to result in cancer-specific treatment. Second, the specificity of AC inhibitors is an important factor that reflects on their safety of application. For instance, in addition to AC inhibition, NOE was reported to block ceramide glucosylation and LCL 204 inhibited acid sphingomyelinase. This highlights the need for design, synthesis and validation of novel more specific and more potent AC inhibitors.

Genetic approaches provide another venue for targeting AC in malignancies. Recent breakthroughs in RNA interference, a Nobel prize-winning technology, allowed lipid investigators to specifically knockdown AC in cancer cells of animal and tissue culture

models. Targeted posttranscriptional gene silencing by RNAi is a rapidly developing field subject to clinical trials pertaining to different disease models. A leading example in cancer therapeutics is the VEGF RNAi, which has been shown to inhibit tumor growth and angiogenesis in multiple preclinical trials. With only one coding gene (ASAH1), AC becomes a favorable target for RNAi-based preclinical trials.

In conclusion, the biochemical characterization of AC catalyzed the current understanding of its intriguing role in tumor biology. Further identification of molecular mechanisms regulating the expression and activity of AC might afford additional methods to modulate its function. It is hope that the scientific findings reviewed in this article will stimulate further research aimed at better understanding of the biological role of AC and increase pharmaceutical recognition of AC as a current drug target.

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Figure 1.

Precursor polypeptide - 395 amino acids

28 kDa ~ 252 aa; 40 kDa including five N-linked glycosylation

Figure 2.

Figure 3.

Figure 4.

	Forward Reaction	Reverse Reaction
<i>pH</i>	4.0-5.0 [15]	5.5-6.5 [15]
<i>Lipids</i>	(↑↑↑) LBPA(BMP) [18]; (↑)SM, PE, CL [20] (↓↓↓) PA, PS [20]	(↑↑↑) PA, PS [20] (↓↓↓) SM, PE, CL [20]
<i>Cations</i>		(↑) Ca ²⁺ , Mg ²⁺ (↓↓↓) Zn ²⁺ [20]
<i>NaCl</i>	N/A	>2-fold activation above 150 mM [20]
<i>proteins</i>	Saposin D [18]	
<i>Substrate</i>	D- <i>e</i> -C ₁₂ -Cer [13]	D- <i>e</i> -sphingosine + C ₁₂ -/C ₁₄ -CoA [20]
<i>Misc</i>	Deceased membrane curvature (SUV>LUV) [18]	