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Acid ceramidase inhibition: a novel target for cancer therapy

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1. ABSTRACT

During the last decade, sphingolipid deregulation, namely the balance between the pro-apoptotic molecule ceramide and the anti-apoptotic sphingolipid sphingosine-1-phosphate, has emerged as an important factor in cancer pathology and resistance to therapy. Thus, our research has been focused on developing drugs that are able to restore normal sphingolipid balance, precisely through increasing the levels of ceramide and decreasing sphingosine-1-phosphate. Particularly, inhibition of the ceramide metabolizing enzyme acid ceramidase, whose over-expression in cancer cells has been implicated in resistance to treatment, is proving to be an efficient and promising strategy. In this review, we consider our recent work with acid ceramidase inhibitors, in combination with radiation or gene therapy as a sensitizer that enhance cancer therapy.

2. INTRODUCTION

Increasingly, evidence suggests that sphingolipids, of which ceramide is the most widely characterized, play an important role in signal transduction for a wide variety of cancer therapeutics including chemo, radiation, and gene therapy. Important factors that determine the end result of ceramide action include cell type, the absolute level and duration of ceramide synthesis, the lipid chain lengths of ceramide produced and last but not least the specific intracellular location of ceramide generation. In regard to the latter, mRNA and protein levels of the lysosomal localized ceramide metabolizing enzyme, acid ceramidase (AC), have been identified as over-expressed in a wide variety of cancers including prostate cancer, head and neck cancer, and melanoma (1-3). Not only were the levels of AC expression found to be elevated

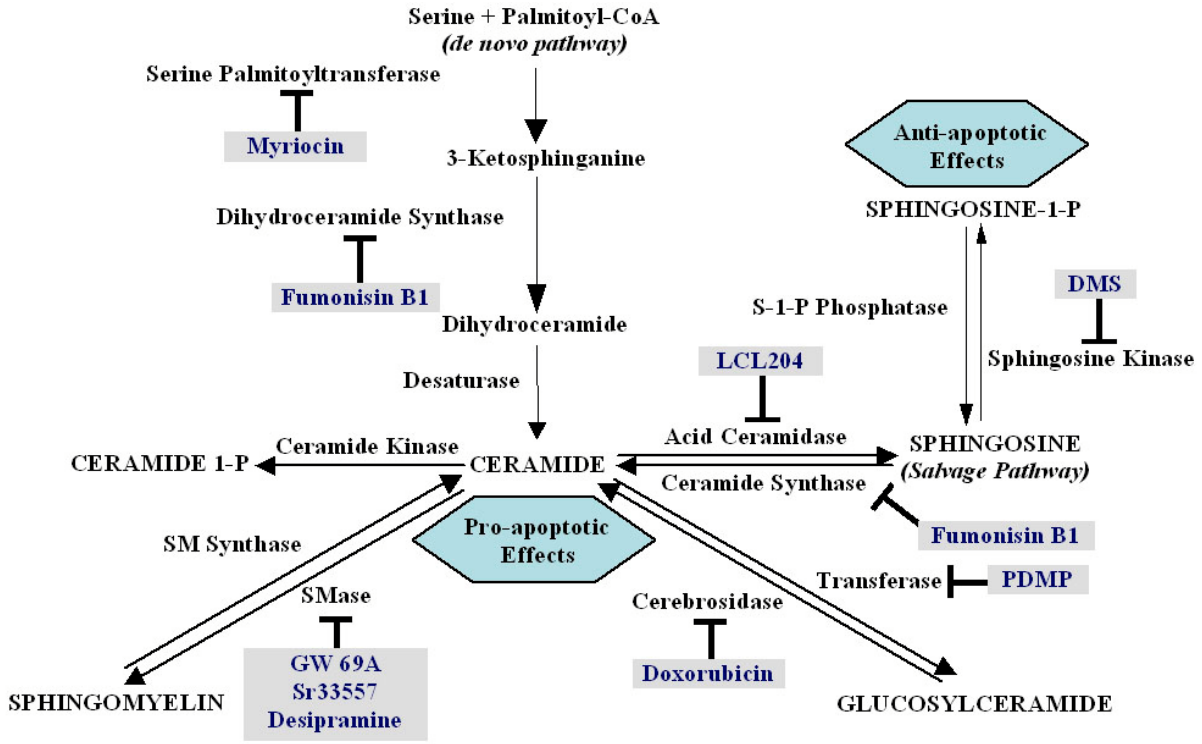

Figure 1. Major synthetic and metabolic pathways for ceramide.

in tumor tissues compared with adjacent "normal" tissue, but AC expression was also found to correlate with the malignant stage of the disease (3).

What are the causes of AC over-expression in cancer? Although the mechanism of over-expression of AC is not yet understood, preliminary data from our laboratory reveals that it may be related to the level and species of ceramide, that are observed to be increased in cancer tissues (4). Likely, systemic stress placed on the tumor signals by hypoxia, nutrient deprivation, and the immune system increases production of ceramide (5). Therefore, to escape ceramide-induced apoptosis, cancer cells upregulate enzymes that degrade ceramide. Specifically, in the case of AC over-expression, cancer cells metabolize ceramide by deacylation. This forms sphingosine which can be further metabolized to sphingosine-1-phosphate (S1P), another well-characterized sphingolipid with anti-apoptotic and angiogenic properties that functions to counter-act the effects of ceramide-mediated pro-apoptotic signaling (Figure 1). This pathway can be further aggravated in cancer cells that have become resistant to cancer therapeutics that act to kill cancer, in part, by inducing ceramide up-regulation mainly by stress mediated mechanisms. The end result is that cancer cells are able to survive in the presence of increased ceramide as long as ceramide is efficiently metabolized and balanced by S1P levels. This balance between ceramide and S1P has been well studied in the literature and is referred to as the Cer/S1P rheostat (6) (Figure 1). Ongoing studies in our group have identified another possible balance between two major ceramides with different lipid chain lengths (Cer-16 and Cer-24) that also play an important role in determining tumor cell survival or apoptosis. Our data suggests AC is functioning to control the relative balance of these two important ceramides where Cer-16 is pro-apoptotic and the Cer-24 is anti-apoptotic (7).

The over-expression of AC in different types of tumors predicted its involvement in cancer pathogenesis making it a "druggable" target for treatment of cancer with the goal of enhancing therapeutic responses during cancer therapy. In this review, we will highlight our rationale for using AC inhibitors and our latest achievements in combining AC inhibitors with different treatment modalities including radiation therapy, FasL and Apoptin cancer gene therapy, all of which are currently in preclinical development in our laboratory.

3. AC INHIBITION AND CANCER THERAPY

3.1. AC inhibitors: How do they work?

In order to illustrate the role of sphingolipids and their role in cancer therapy, we will compare them to the widely known Bcl-2 family of proteins insofar as both families share many similarities in their mode of action. Like Bcl-2 family members which consist of pro-apoptotic (Bak, Bax) and anti-apoptotic molecules (Bcl-2, Bcl-xl), the sphingolipid family also includes pro-apoptotic (mainly long chain ceramides) and anti-apoptotic (very long chain ceramides and S1P) molecules. Like the Bcl-2 family, where the relative absolute values of pro- and anti-apoptotic proteins determine apoptosis induction, a balance between the pro-apoptotic ceramide and anti-apoptotic S1P

also controls apoptosis during cellular stress, i.e. the Ceramide/ S1P rheostat (6). However, there are differences between the two pathways which are important to delineate. First, the Bcl-2 family are proteins that seem to be largely involved in controlling the intrinsic apoptotic pathway, while the ceramide/S1P lipid pathway controls multiple cell functions including the intrinsic pathway, differentiation, and inflammation (8). Second, unlike the Bcl-2 family, the pro-apoptotic and anti-apoptotic sphingolipids are somewhat inter-convertible making this pathway even more intriguing. An illustration of this is that ceramide can be metabolized to sphingosine which can be phosphorylated by sphingosine kinase to generate S1P. Consequently, when it comes to therapeutic applications, one must be cautious when administering ceramide analogues. This is a concern because, although ceramide administration may be helpful in mediating apoptosis in cells that have low levels of enzymes that metabolize ceramide, cancer cells frequently have elevated ceramide catabolism, such as occurs in cells with elevated AC. In this case AC may rapidly metabolize ceramide to sphingosine, the substrate used by sphingosine kinase to form S-1-P. This would result in minimal pro-apoptotic effects with the paradoxical result of elevating the anti-apoptotic and angiogenic sphingolipid S-1-P which would tend to enhance tumor survival as opposed to the action of ceramide induction of apoptosis. As a result, inhibition of ceramide metabolizing enzymes such as AC, has emerged as a more attractive therapeutic option than administration of exogenous ceramides.

One of the earliest compounds to show efficacy in cancer treatment was the AC inhibitor B13. Interestingly, B13 reduced viability of colon cancer cells by 90% (9). This effect was partially reversed with simultaneous inhibition of Caspase 3 indicating that cell death was mainly through apoptosis. B13 has also been shown to inhibit AC in the immortalized human keratinocyte line HaCaT as well as in melanoma cells (10), and induces apoptosis and suppression of proliferation. Similarly, in prostate cancer cell lines, Samsel, *et al.*, reported varied responsiveness to B13 enantiomers with a maximum of 90% cell death in LNCaP cells in response to the R enantiomer (11). LNCaP cells have significantly upregulated AC and would be predicted to be sensitive to AC inhibition. In this paper, it was also shown that radiation or radiation plus B13 resulted in a reduction in PC3 tumor volume in nude mice over a 28 day period compared to control values or B13 alone. Since B13 is a weak non-lysosomally targeted AC inhibitor we predicted that the design of newer drugs which target the lysosome (9, 12) will be significantly more effective. The data presented below sustains that belief.

Earlier studies of the B13 analog LCL204 (AD2646), demonstrated that this lysosomal targeted drug was able to induce apoptosis in two leukemic cell lines (13, 14). Following treatment of prostate cancer (PCa) cells with LCL204, we observed that LCL204 was concentrated in the heavy membrane fraction compared to cytosol or nuclear fractions. It induced dose- and time-dependent cell death with a significant improvement in cytotoxicity over B13 (12). By analysis of intracellular lipid levels, we observed up-regulation of ceramide followed by down-regulation of sphingosine indicating inhibition of AC. Activated Caspases were detected in the treated cells and cytotoxicity could be partially reversed by pre-treatment with the Caspase inhibitor zVAD-fmk. Following LCL204 treatment, we also detected increased cathepsin B levels in the cytosol of treated cells. These events were followed by mitochondrial membrane permeabilization, release of cytochrome c into the cytosol, and activation of executioner Caspases. Combined, these results suggest that treatment of PCa cells with LCL204 activate multiple pathways leading to apoptosis.

3.2. AC inhibition and apoptin gene therapy

Our laboratory has demonstrated that introducing pro-apoptotic genes into cancer cells significantly increases induction of apoptosis and in many cases exhibits bystander activity (2). Apoptin, a 121 amino acid protein which constitutes the third open reading frame of chicken anemia virus is able to induce apoptosis in both cancerous and transformed cell lines while being much less toxic to normal cells (15). Apoptin's mechanism of action does not require upstream Caspases, but does involve activation of Caspase 3 to induce apoptosis (16). Apoptin is equally active in tumor cells with wild type or mutant p53 and still functions if cells over-express Bcl-2 or BCR-ABL (17). Thus, Apoptin's mechanism of action likely involves multiple pathways in tumor cells and suggests widespread ability to induce apoptosis in diverse cancer cell types. Recently an adenovirus that expressed Apoptin has shown efficacy in systemic treatment of human hepatoma xenograft tumors in nude mice with no observed toxicity to the animal (18). Non-viral systemic delivery vehicles containing the Apoptin gene linked to asialoglycoprotein have also been shown to target the asialoglycoprotein receptor (ASGPR) present only on the surface of hepatocytes (19). These studies demonstrated specific and efficient distribution of Apoptin in both hepatocellular carcinoma cells and normal hepatocytes after tail vein administration. However, only the *in situ* hepatomas cancer cells showed significant signs of regression, whereas the surrounding normal hepatocytes did not. Because of its tumor targeting specificity, Apoptin may have future utility as a systemic therapy.

Recently, our laboratory has described a new mechanism of action for Apoptin, namely up-regulation of ceramide which may explain, in part, how Apoptin induces apoptosis based on the known apoptotic pathways induced by ceramide (5, 20). Our data demonstrated that Apoptin expression from an adenoviral base vector (20) induced an increase in long chain ceramides (C14-C18) via sphingomyelin hydrolysis in three different prostate cancer cell lines during Apoptin induced cell death (21). Evidence for activation of the ceramide-sphingomyelin pathway included the translocation of acid sphingomyelinase from the lysosome to the plasma membrane, increased enzyme activity and upregulated enzyme mRNA expression. This same study showed that C6-ceramide (which mimics natural ceramide) also promotes Apoptin-induced apoptosis (21).

Table 1. Sensitivity of prostate cancer cells to radiation therapy.

#	Cell line	Radiation dose Gy	Time after Radiation days	%Cell Apoptosis	Ref
1	PC3	10-30	3	Insignificant	39-42
2	Du145	10-12	3	10-15%	39, 43
3	PC3	20	3	40%	44
4	LNCaP	20	3	35%	44
5	PPC1	40	2	25%	32

As mentioned previously, when we examined AC protein levels in primary prostate cancer tissues, we foundthat in 35 pairs of PCa versus "normal" adjacent tissue 60% of the samples demonstrated AC up-regulation. Since the ceramide pathway was involved in Apoptin induced cell killing, a new therapeutic strategy for treating prostate cancer by combining Apoptin with AC down-regulation was considered. This approach was studied by first observing a decreased sensitivity to Apoptin in AC over-expressing cells and more importantly by finding enhanced cell killing in cells when AC was down-regulated by siRNA. Furthermore, as we predicted in our model, the AC inhibitor LCL204, enhanced Apoptin killing *in vitro* and *in vivo* (22)**,** suggesting a new clinical approach for treatment of prostate cancer.

3.3. AC inhibition and FasL gene therapy

Despite emerging advances in both surgical and chemo-radiation therapy for the treatment of head and neck cancer, five year survival rates hover around the 50% mark indicating a need for development of new therapeutic approaches. FasL gene therapy has shown to be effective for treating many tumor cell models including glioblastoma, bladder, renal, and prostate cancers (2, 21-30). Recently we have published both *in vitro* and *in vivo* results which supported the efficacy of intratumoral administration of AdGFPFasL gene therapy as a new therapeutic modality for head and neck cancer (24). In order to further enhance the efficacy of FasL gene therapy in head and neck cancer AC was down regulated using AC siRNA. Our results demonstrate a significant sensitization of head and neck cancer cells to FasL gene therapy in combination with AC knockdown, further illustrating the importance of targeting this enzyme. Current work in our lab is focusing on the combination of FasL gene therapy and the AC inhibitor LCL204 for the treatment of head and neck cancer, and we are obtaining promising data both *in vitro* and *in vivo* in mouse models suggesting that the combination of FasL gene therapy and AC inhibitors may soon become a new efficient therapeutic modality for the treatment of head and neck cancer.

3.4. AC inhibition and radiation

The application of ionizing radiation for treating cancer is well established with >750,000 patients treated per year (31). Tumor responsiveness to radiation therapy is highly variable. Factors as diverse as lack of acid sphingomyelinase (ASMase) to problems in DNA repair mechanisms are attributed as causes of resistance. Model systems for studying cancer also exhibit radiation resistance (Table 1). In our experiments we found that PPC1 cells showed a maximum of 25% cell killing 48 hours following treatment with up to 40 Gy (32) making them very radio-resistant.

Improved understanding of the different mechanisms for insensitivity to radiation-induced apoptosis has provided clues to rational development of radio-sensitizing agents that interdict specific mechanisms of resistance. We and others have evidence which suggests that the tumor suppressor lipid, ceramide, is a critical component of ionizing radiation-induced apoptosis (33-36). This apoptotic pathway is initiated by hydrolysis of sphingomyelin by sphingomyelinases (SMases) to generate ceramide (34, 36, and 37). It is believed that acid sphingomyelinase is most likely responsible for radiation-induced ceramide based on studies on patients with Niemann-Pick disease and on ASMase knockout mice, both exhibiting high levels of resistance to ionizing radiation. An alternative mechanism for generation of ceramide in response to radiation is a pathway that involves *de novo* synthesis of ceramide catalyzed by the enzymes serine palmitoyl CoA synthase and ceramide synthase. A recent study demonstrated that radiation activates both ceramide generation pathways concurrently (38).

The generation of ceramide seems to be important in the response of cells after radiation exposure. Defects in the ceramide generation pathway contribute to radio-resistance. Our lab recently determined that increasing the rate of ceramide degradation in irradiated-cells also results in radio-insensitivity. In these unpublished studies, we demonstrated a role for AC in regulating ceramide levels in response to radiation. While carrying out radiation studies on PPC1 prostate cancer cells, we were surprised to observe that AC was up-regulated in response to radiation exposure with concomitant minor changes in ceramide. However, importantly we observed significantly higher levels of sphingosine and sphingosine-1-phosphate. This suggests radiation exposure induces a situation where the cell becomes less susceptible to radio-therapy. To test this we down-regulated AC using ACsiRNA, and noticed a substantial sensitization of the cells to radiation-induced cell death, by both clonogenic and cytotoxicity assays. Conversely AC over-expression reverses the sensitivity of PPC1 to radiation. Further, we observed using the AC inhibitor LCL385 that PPC1 tumor cells become more sensitive to radiation treatment by inhibition of AC enzymatic activity, resulting in accumulation of ceramide. The combination of AC inhibition with radiation significantly reduced tumor growth in PPC1 xenografts grown in nude mice, suggesting a new combination modality for improvement of prostate radiation therapy.

4. SUMMARY

In conclusion, this review highlights the critical central role that ceramide metabolism plays in the application of either genetic or radiation therapy for treating prostate and head and neck cancers. Our data suggests that development of small molecules that interdict the action of AC will play a crucial role in future therapies that involve the combination of these molecules with application of gene therapy, either systemically or topically

and/or with application of radiation in prostate and head and neck cancers. In the future we hope to provide details of clinical trials on all of these fronts.

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Abbreviations: AC: acid ceramidase; PCa: prostate cancer; ASMase: acid sphingomyelinase; SMases: sphingomyelinases

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