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Sphingolipids and acid ceramidase as therapeutic targets in cancer therapy

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ABSTRACT

Background: Sphingolipids have been shown to play a key part in cancer cell growth and death and have increasingly become the subject of novel anti-cancer therapies. Acid ceramidase, a sphingolipid enzyme, has an important role in the regulation of apoptosis. In this review we aim to assess the current evidence supporting the role of sphingolipids in cancer and the potential role that acid ceramidase may play in cancer treatment.
Methods: A literature search was performed for published full text articles using the PubMed, Cochrane and Scopus databases using the search criteria string "acid ceramidase", "sphingolipid", "cancer". Additional papers were detected by scanning the references of relevant papers. A summary of the evidence for each cancer subgroup was then formed. Given the nature of the data extracted, no meta-analysis was performed.
Results: Over expression of acid ceramidase has been demonstrated in a number of human cancers. *In vitro* data demonstrate that manipulation of acid ceramidase may present a useful therapeutic target. In the clinical setting, a number of drugs have been investigated with the ability to target acid ceramidase, with the most promising of those being small molecular inhibitors, such as LCL521.
Conclusion: The role of the sphingolipid pathway in cancer is becoming very clearly established by promoting ceramide accumulation in response to cancer or cellular stress. Acid ceramidase is over expressed in a variety of cancers and has a role as a potential target for inhibition by novel specific inhibitors or off-target effects of traditional anti-cancer agents. Further work is required to develop acid ceramidase inhibitors safe for progression to clinical trials.

1. Introduction

Sphingolipids have been shown to play a key role in the regulation of cell growth and proliferation in cancer (Ponnusamy et al., 2010). Sphingolipids are the structural components of biological membranes, maintaining barrier function and fluidity (Hannun and Obeid, 2018). First described by Thudichum in the 19th Century, it took over a century (until Herbert E Carter in the 1920s) to first describe the structure of various sphingolipids including sphingosine and ceramidase (Yu RK, 2009). The term "sphingosine" is Greek in origin and means "to bind tight" and shares its name with the mythical Sphinx, so named due to its initially elusive structure. More recently the role of sphingolipids in the regulation of cancer cell growth and death has been investigated such that they are increasingly becoming the subject of novel anti-cancer therapies (Ogretmen, 2018).

Ceramide is the central molecule of sphingolipid metabolism. Composed of a sphingosine base and amide-linked acyl chains varying in length from C14 to C26, ceramide serves as the structural and metabolic precursor of more complex sphingolipids such as sphingomyelin and ceramide-1-phosphate. Ceramide synthesis and metabolism occur in the endoplasmic reticulum and Golgi apparatus, and thus transport of ceramide (by both vesicular and non-vesicular mechanisms) are of critical importance in the sphingolipid pathway (Perry and Ridgway, 2005). There are two major pathways for ceramide biosynthesis (Hannun et al., 2001; Mathias et al., 1998); sphingomyelinase-dependent hydrolysis of sphingomyelin (in the outer plasma membrane) by specialised enzymes known as SMases (Levade et al., 1999), or *de novo* synthesis in the endoplasmic reticulum through condensation of serine and palmitoyl CoA by serine-palmitoyl CoA transferase (SPT) leading to the synthesis of dihydroceramide by dihydroceramide synthases (CerS). Ceramide is metabolised to ceramide-1-phosphate by ceramide kinase, or to glucosylceramide by glucosylceramide synthase. Ceramide can also be degraded by ceramidases to form sphingosine (SPH), which can then be phosphorylated to sphingosine-1-phosphate (S1P) by sphingosine kinase (SK). SPH can be produced from S1P under the action of phosphatases, and ceramide

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generated from SPH under the action of ceramide synthase. SPH, ceramide and S1P are the main bioactive molecules and are generated in response to cellular stresses such as chemotherapy, radiotherapy and/or oxidative stress where they mediate cell death, senescence or cell cycle arrest (Cuvillier et al., 1996; Lee et al., 1998).

The major enzymes involved in sphingolipid metabolism have been identified and cloned, providing data revealing that the abundance of sphingolipids is highly regulated by these metabolic enzymes. The altered expression or activity of these enzymatic pathways may play a key role in the regulation of cancer signalling and/or treatment (Ogretmen, 2006). At least five human ceramidases (encoded by five distinct genes) exist, each defined by their unique pH optima, subcellular locations, substrate specificities towards different ceramides, and/or effects on downstream biological pathways (Mao and Obeid, 2008). One of these ceramidases, acid ceramidase (AC) was first discovered in rat brain homogenates (Gatt, 1963) and was further characterised and purified from human urine in 1995 (Bernardo et al., 1995). Through regulation of sphingolipid metabolism, AC has been implicated in a number of diseases, but more recently in cancer where it plays a particular role in the regulation of apoptosis and has recently been the target of cancer therapy. The aim of this review is to describe the roles of AC and other sphingolipid enzymes in cancer, and also to summarise recent attempts to manipulate its expression as a potential cancer therapy.

2. Ceramide and cancer cell regulation

The synthesis and accumulation of ceramide in response to cellular stress is known to mediate cancer cell death though various mechanisms including apoptosis and autophagy. In contrast, many tumours inherently exhibit increased ceramide metabolism through the actions of acid ceramidase and other enzymes such as ceramide kinase and sphingomyelin synthase, which increases the production of pro-survival sphingolipids.

Induction of apoptosis by ceramide was first described by Obeid et al. (1993) using human leukaemic cells treated with exogenous ceramide. The exact mechanisms by which ceramide induces apoptosis are yet to be fully elucidated but it appears to be regulated by the SpT and/or CerS enzymes (Bose et al., 1995). There is growing evidence linking individual CerS enzymes and their preferentially generated ceramide chain with both pro- and anti-tumour effects. For example, in the head and neck squamous cancer xenograft CerS1/C18-ceramide suppress tumour growth whereas CerS6/C16-ceramide induces tumour proliferation in SCID mice (Senkal et al., 2010). Similarly, Mesicek et al. reported opposing actions for the same ceramide molecules but generated by different CerS enzymes (Mesicek et al., 2010). Taken together these studies suggest that ceramides with distinct fatty chain lengths generated by different CerS enzymes play distinct roles in the regulation of cell death in cancer.

The reported pathways that are involved in mediating apoptosis are numerous and diverse but are generally viewed as involving direct or indirect targets of ceramide. Ceramide accumulation in the mitochondria induces the pro-apoptotic protein Bax to be recruited to the mitochondria, resulting in caspase activation and apoptosis (Siskind et al., 2008; Chang et al., 2015; Chipuk et al., 2012). Other direct mechanisms include regulation of ceramide transport from late endosomal organelles, leading to ceramide mediated caspase-3 activation and apoptosis (Blom et al., 2015). Ceramide also acts as a second messenger in regulation of the apoptotic cascade via CD95, and induction of caspase-8 (Grassmé et al., 2001). Other factors such as tumour necrosis factor-related apoptosis inducing ligand (TRAIL) and nitric oxide synthase may also be induced by ceramide (Dumitru and Gulbins, 2006; Goldkorn et al., 1998). Nitric oxide levels have also been suggested to play a role in ceramide levels and cell survival, with high levels contributing to cell death in leukaemia cells (Takeda et al., 1999). Ceramide has also been associated with reduced telomerase activity and with the acceleration of telomere shortening, thus increasing the likelihood of cell senescence or apoptosis once the telomere reaches a critical length (Ogretmen et al., 2001).

Table 1
Historical landmarks in the study of acid ceramidase.

1963	Enzyme first discovered and partially purified by Gatt
1963	“Reverse” ceramidase reaction first identified
1972	Deficiency of acid ceramidase first found in Farber disease patients
1995	First substantial purification of acid ceramidase from human urine
1996	First cloning of the human acid ceramidase cDNA
1996	First mutation identified in a Farber disease patient
1999	First cloning of the human acid ceramidase gene
2002	First production of recombinant, human acid ceramidase
2002	First acid ceramidase “knock-out” mouse model constructed

It is proposed that many cancer cells are able to resist apoptosis as a consequence of limiting ceramide generation, or by its rapid removal (Truman et al., 2014). One such mechanism is via the de-acetylation of ceramide by AC to SPH, which is then modified by a SK to S1P. S1P has been demonstrated to initiate the pro-survival PI3K/AKT signalling pathway, thus opposing the effect of ceramide, with the balance between ceramide and S1P determining cell fate (Bonnaud et al., 2010). A number of studies have reported increased expression of SK/S1P in cancer tumour tissue, with *in vitro* studies demonstrating inhibition of apoptosis if over expressed or decreased tumour growth when down regulated (Patmanathan et al., 2017)· Targeting both SK and S1P with small molecular inhibitors has shown promise recently as potential cancer treatment strategies (Zheng et al., 2019).

After its discovery in 1963 (FARBER, 1952), inherited acid ceramidase deficiency was found to be responsible for Farber disease, study of which ultimately led to further work identifying the genetic mutations and development of “knock out” AC mouse models (see Table 1 for AC timeline). AC has an optimal pH of 4.5 and the enzyme catalyses the hydrolysis of ceramide into sphingosine and a fatty acid with a preference for unsaturated ceramides with 6–16-carbon acyl chains (Okino et al., 2003). Similar to the other sphingolipid enzymes, AC catalyzes the reverse reaction as well as the forward synthetic reaction32, such as C12 fatty acid and sphingosine combining to produce ceramide, although this reaction is at a higher pH than the forward reaction (6 *vs.* 4.5). AC is located in the lysosome, is ubiquitously expressed and has been found to be highly expressed in the heart and kidney, with lower expression in placenta, lung and skeletal muscle (Li et al., 1999). Following on from its initial discovery and implication in Farber disease, (which is incredibly rare with only 80 cases worldwide described since its discovery), further work began to implicate AC in human cancer via two main observations:

1 Identification of its over expression in human cancer and/or relationship to stage or prognosis.
2 Observation that its inhibition and consequent rise in ceramide levels led to apoptotic cell death.

3. Acid ceramidase in malignancy

Numerous studies have highlighted the significance of acid ceramidase in the initiation and propagation of a number of human cancers (*See* Table 2*)*.

3.1. Prostate cancer

Our depth of understanding of the role of AC in tumour proliferation and resistance to treatment is greatest in relation to prostate cancer. Importantly, the study from Mahdy *et al.*[35] has convincingly demonstrated that downregulation of the corresponding gene for AC with siRNA in a prostate cancer cell line confers radio-sensitivity. This *in vitro* study in PPC-1 cells assessed radiation response by clonogenic and cytotoxic assays, demonstrating that upregulation of acid ceramidase

Table 2
The role of AC in cancer and the corresponding studies highlighting the significance of acid ceramidase.

Reference	Cancer	Focus	The role of AC in cancer
(Mahdy et al., 2009; Cheng et al., 2013; Camacho et al., 2013a; Holman et al., 2008; Gouazé-Andersson et al., 2011; Turner et al., 2011)	Prostate	Inhibition, Over expression	siRNA and SMI inhibition of AC conferred radiosensitivity *in vitro*. Upregulation of AC in response to radiation with surviving cells being radio-resistant. IHC data conferred higher levels of AC in patient tissue with prior radiation failure compared to radiation naïve patients. Higher AC expression found on IHC associated with more advanced disease stage in cell models. Significant cytotoxic effects on hormone refractory cell lines when treated with ceramide generating chemotherapy and AC inhibition. PPC1 and DU 45 cell lines over express AC and found to have higher expression of lysosomal stabilising proteins thought to explain ability to tolerate increased ceramide post irradiation.
(Roh et al., 2016; Korbelik et al., 2016a; Separovic et al., 2013; Elojeimy et al., 2007)	Head and Neck	Inhibition, Over expression	AC overexpression in SCC's, pharmacological and biological AC inhibition increased chemosensitivity. AC inhibition with small molecular inhibitors (SMI's)on SCC's reduced survival after photodynamic therapy. Overexpression of AC increased resistance to apoptosis and pharmacological AC inhibition sensitised cells to apoptosis in vivo and in vitro.
(Realini et al., 2016; Bedia et al., 2011; Lai et al., 2017)	Melanoma	Inhibition, Over expression	Melanocytes and proliferative melanoma over-express AC relative to other skin cells and pharmacological inhibition increased cytotoxicity to 5-FU. Over and down regulation of AC in melanoma cells *in vitro* conferred resistance and sensitivity respectively.
(Tan et al., 2016; Hu et al., 2011)	Myeloid Leukaemia	Inhibition	Primary AML cells highly express AC leading to the production of anti-apoptotic proteins. Pharmacological inhibition of AC increased survival of mouse model of AML. AC found to influence IRF8 which is a key transcription factor lost in myeloid leukaemia.
(Ramírez de Molina et al., 2012)	Non-Small Cell Lung	Inhibition	Cells with acquired resistance to pro-apoptotic choline kinase α (ChoKα) inhibitors have raised AC expression compared to non resistant cells. AC inhibition combined with (ChoKα) inhibition showed improved antiproliferative effects.
(Sänger et al., 2015; Flowers et al., 2012)	Breast	Baseline expression	High expression of ASAH1 gene correlates with better prognosis in invasive breast cancer. AC expression is related to HER2 status, suggesting a prognostic relevance to ceramide-targeted therapy.
(Hanker et al., 2013)	Ovarian	Baseline expression	Immunohistochemical analysis of >100 patient samples showed low AC expression correlated with poorer cancer survival, this goes against findings in other cancers.
(Morales et al., 2007) (Giovannetti et al., 2010)	Hepatobilary	Inhibition Ceramide Analogues	siRNA inhibition of AC in vivo enhanced daunorubicin therapy efficacy and reduced tumour growth in liver murine xenografts. The novel ceramide analogue (AL6) dose-dependently inhibited cell growth, induced apoptosis and synergistically enhanced the cytotoxic activity of gemcitabine in pancreatic cancer cell lines.
(Klobučar et al., 2018; Bowden et al., 2018)	Colon	Baseline expression	High AC expression linked with poorer neoadjuvant chemoradiotherapy response in patients with locally advanced rectal cancer confirmed using proteomic and IHC analysis from patient tissue. AC expressed higher in colonic adenocarcinoma tissue compared to normal colonic tissue and AC inhibition sensitises HCT 116 cells to oxaliplatin.
(Young and Van Brocklyn, 2007; Abuhusain et al., 2013; Doan et al., 2017a; Zeppernick et al., 2008; Zorniak et al., 2012; Qiu et al., 2014; Doan et al., 2017b)	Glioblastoma	Baseline expression	S1P from AC metabolism of ceramide promotes GBM invasiveness. S1P levels higher in GBM tissue compared to normal brain tissue. AC expression highly correlates with poorer survival in GBM patients based on >600 biomarkers that were screened. CD133 is a marker of GBM like stem cells associated with radioresistance and tumour repopulation, AC found to be expressed highly populations expressing high CD133.

decreased sensitivity to radiation and created cross-resistance to chemotherapy, with the small molecule acid ceramidase inhibitor LCL385 also sufficient to sensitize PPC-1 cells to radiation.

In addition, the cell line is observed to preferentially upregulate AC in response to irradiation, with over-expression of AC in clones that survived a course of irradiation and that were further associated with increased radio-resistance and proliferation (Cheng et al., 2013). This was validated with immunohistochemical (IHC) analysis of human prostate cancer tissues, where higher levels of AC were observed after radiotherapy failure than in irradiation-naïve cancer, intra-epithelial neoplasia or benign tissue. In addition to AC inhibition in an animal xenograft model producing radio-sensitisation, it also prevented relapse.

Higher IHC expression of AC in primary prostate cancers is associated with more advanced disease stage, and in a model derived from PC-3 prostate cancer cells, the highly tumourigenic, metastatic and chemo-resistant PC-3/Mc clone expressed higher levels of AC than the non-metastatic PC-3/S clone, with stable knockdown of ASAH1 in PC-3/Mc cells resulting in an accumulation of ceramide, reduced clonogenic potential and inhibition of tumourigenesis and lung metastases (Camacho et al., 2013a). The DU 145 prostate cancer cell line has also been demonstrated to be sensitive to the effect of AC inhibition following treatment with LCL204, with raised ceramide and decreased sphingosine levels, caspase activation and ultimately apoptosis observed (Holman et al., 2008). In this study, the degradation of AC by LCL204 was cathepsin-dependent.

The PC-3 and DU 145 prostate cancer cell lines are considered to be hormone-refractory. In a study by Gouazé-Andersson et al. (2011), combination therapy in these cell lines with the ceramide-generating chemotherapeutic fenretinide and the synthetic AC inhibitor DM102 significantly decreased cell viability in a synergistic manner, with single-agent treatment being only weakly cytotoxic. An alternative known AC inhibitor N-oleoylethanolamine did not produce a synergistic effect with fenretinide in the cells and blocking ceramide generation (with either vitamin E or myriocin) did not prevent cytotoxicity from combined fenretinide/DM102 treatment, suggesting alternative metabolic pathways in this context.

Turner et al. (2011). demonstrated that the PPC1 and DU 145 prostate cancer cell lines over-expressing AC relative to controls have increased lysosomal density, high expression of the lysosomal stabilising protein KIF5B and increased levels of autophagy, complimenting the enhanced stress-resistance inherent to the cells, and to potentially explain the ability of the prostate cancer cells to respond to ceramide accumulation following irradiation.

3.2. Head and neck cancer

AC over-expression has been observed in squamous cell head and neck cancer (HNC) in four out of six primary tumours and six out of nine HNC cell lines in a study by Roh *et al.* (Roh et al., 2016) and was correlated with resistance to cisplatin chemotherapy in the cell lines. Pharmacological (with N-oleoyl-ethanolamine) and genetic (with short hairpin RNA) inhibition of AC in the cell lines significantly increased their sensitivity to cisplatin, with increased ceramide production and activation of pro-apoptotic proteins. In another study of squamous cell cancer cells, the inhibition of AC with the small molecule inhibitor LCL521 significantly reduced the survivability of SCCVII cells on the basis of clonogenic assay after photodynamic therapy (Korbelik et al., 2016a). AC activity inhibition and increases in ceramide levels have also been observed after photodynamic therapy in isolation (Separovic et al., 2013). Over-expression of AC in the SCC-1 cancer cell line increased resistance to Fas-induced apoptosis, with down-regulation inducing sensitivity. The AC inhibitor LCL204 was also demonstrated in this study to sensitise HNC cell lines to Fas-induced apoptosis in both *in vitro and* in a xenograft model *in vivo* (Elojeimy et al., 2007).

3.3. Melanoma

Melanocytes and proliferative melanoma cell lines have been demonstrated to over-express AC relative to other skin cells and non-melanoma skin cancer cells (Realini et al., 2016). Application of the AC inhibitor ARN14988 acted synergistically with 5-FU to increase cytotoxicity in the proliferative melanoma cell line in this study, with increased ceramide levels and reduced S1P levels noted. This may have positive implications for 5-FU based chemoradiotherapy (CRT) in rectal cancer. In the context of the clinical management of metastatic melanoma, where dacarbazine is a chemotherapeutic option, targeting of AC may improve sensitivity to therapy. This was demonstrated by over-expression and down-regulation of AC in human A375 melanoma cells *in vitro* conferring resistance and sensitivity respectively to dacarbazine (Bedia et al., 2011). More recently CrispR-Cas9 gene editing has been used to delete the ASAH-1 gene in the A375 cell line, as well as resulting in ceramide accumulation, ASAH-1 null cells lost the ability to form cancer initiating cells and undergo self-renewal (Lai et al., 2017).

3.4. Myeloid leukaemia

A comprehensive experimental assessment of the role of AC in acute myeloid leukaemia (AML) was undertaken by Tan et al. (2016). Primary AML cells were observed to highly express AC, with AC over-expression increasing the expression of the anti-apoptotic Mcl-1, and reduced Mcl-1 expression observed with the synthetic AC inhibitor LCL204. LCL204 treatment significantly increased the overall survival of C57BL/6 mice engrafted with leukaemic C1498 cells and significantly decreased disease burden in NSG mice engrafted with primary human AML cells, implicating AC as an independent therapeutic target. IFN regulatory factor 8 (IRF8) is a key transcription factor for myeloid cell differentiation, with expression frequently lost in haematopoeitic cells in myeloid leukaemia. Hu *et al.* identified AC as a general transcription target of IRF8, with expression of IRF8 regulated by promoter DNA methylation (Hu et al., 2011). In myeloid cells, restoration of IRF8 expression supressed AC and resulted in ceramide accumulation and increased sensitivity to FasL-induced apoptosis. In cells derived from IRF8-deficient mice AC was dramatically increased, with AC inhibition or the application of exogenous ceramide sensitising cells to FasL-induced apoptosis, suggesting a mechanism for a pathway of resistance to Fas-mediated apoptosis and disease progression.

3.5. Non-small cell lung cancer

In non-small cell lung cancer cells with acquired resistance to the pro-apoptotic effect of choline kinase α (ChoKα) inhibitors, raised levels of AC have been demonstrated compared to non-resistant cells. The anti-proliferative effect of ChoKα therapy is enhanced with the synergistic inhibition of AC in primary cell culture, suggesting a model for combination therapy (Ramírez de Molina et al., 2012).

3.6. Breast cancer

High genetic expression of ASAH1 has also been observed to correlate with a better prognosis in invasive breast cancer, and with a reduced incidence of recurrence in pre-invasive DCIS (Sänger et al., 2015) although how this correlates with a functional post-translational expression of AC is not identified.

In three breast cancer cell lines, Flowers et al. assessed the effect of AC inhibition (with DM102) on the apoptosis inducing effects of C6-ceramide (Flowers et al., 2012). As single agents, C6-ceramide and DM102 were only moderately cytotoxic but co-administration produced a reduction in viability in all cell lines. This was considered to be synergistic in MDA-MB-231 and MCF-7 cells but antagonistic in BT-474 cells. Correlation of AC expression and sensitivity to C6-ceramide/DM102 were independent of oestrogen receptor status or molecular subtypes but AC expression was related to HER2 status. This potentially suggests a prognostic relevance of AC tumour expression and the sensitivity of certain breast cancer subtypes to ceramide-targeted therapy and warrants further investigation. More recently, the AC inhibitor ceranib-2 has been shown to mediate apoptosis in breast cancer cell lines through activation of the stress activated protein kinase/c-Jun N-terminal kinase and mitogen-activated protein kinase pathways (Vethakanraj et al., 2018). Ceranib-2 has shown similar effects in prostate cancer cell lines also (Kus et al., 2015).

3.7. Ovarian cancer

On IHC analysis of 112 ovarian cancer tumour samples, low AC expression correlated with a poorer cancer specific survival (Hanker et al., 2013), although this is contradictory to the centrally described outcomes of sphingolipid metabolism as discussed. It may be that AC expression and function is not ubiquitous across all tumours, that its expression is implicated in response to selected therapies, or that it is involved in alternative pathways in certain tumours, and further investigation in these cancers is required.

3.8. Hepatobiliary cancers

AC is activated by the chemotherapeutic daunorubicin in human (HepG2) and mouse (Hepa1c17) hepatoma cell lines as well as in primary cells from murine liver tumours but not in cultured mouse hepatocytes. Inhibition of AC by siRNA or pharmacological means sensitised the cell lines to daunorubicin-induced cell death, preceded by structural mitochondrial changes, stimulation of reactive oxygen species generation and cytochrome c and caspase-3 activation. *in vivo* AC inhibition with siRNA also reduced growth in liver tumour xenografts of HepG2 cells and also enhanced daunorubicin therapy, offering a potential therapeutic target in the management of liver cancers (Morales et al., 2007).

It is known that in pancreatic cancer that the key chemotherapeutic agent ; gemcitabine, displays different efficacy due to polymorphism in the expression of enzymes that regulate its metabolism. Further *in vitro* evidence has shown that in pancreatic cancer cell lines (MIA PaCa-2 and PANC-1) the novel ceramide analogue (AL6) dose-dependently inhibited cell growth, induced apoptosis and synergistically enhanced the cytotoxic activity of gemcitabine. Further mechanistic work revealed that AL6 favourably modulated gene expression of gemcitabine metabolic enzymes therefore increasing its efficacy as a syngergistic agent (Giovannetti et al., 2010). Therefore it could support a role for AC inhibition as a target in combination chemotherapeutics in pancreatic cancer.

3.9. Colon cancer

In vitro experiments have demonstrated that AC expression is higher in colon cancer cells and this was also confirmed by IHC when comparing cancer to normal tissue (Klobučar et al., 2018). *In vitro* experiments of AC inhibition by this group and others (Baspinar et al., 2017) have demonstrated increased apoptosis mediated via increased ASAH mRNA levels and p53 activity. A proteomic analysis of patients undergoing neoadjuvant chemoradiotherapy identified over expression AC in patients whom undergo a poor response or disease progression compared to those that have a good response (Bowden et al., 2018). Whether AC represents a biomarker of disease response or a therapeutic target remains to be seen.

3.10. Glioblastoma

AC expression and glioblastoma (GBM) is another important area of research given the poor prognosis of this disease stimulating efforts to molecularly target AC. The sphingolipid pathway has been shown to be involved in GBM at multiple points. Sphingosine-1-phosphate (S1P) promotes GBM invasiveness *in vitro* via the upregulation of the urokinase plasminogen activator, its receptor, and the pro-invasive molecule *CCN1* (cysteine-rich angiogenic protein 61) (Ogretmen and Hannun, 2004; Young and Van Brocklyn, 2007). S1P levels were found to be higher in GBM tissue compared to normal cerebral tissue (Abuhusain et al., 2013). A recent study by Doan *et al.* (Doan et al., 2017a), examined tumour tissue from 10 GBM patients with known survival data and screened for 601 biomarkers. ASAH1 (AC) had the highest R_2 value of 0.53 correlating high AC expression with poorer survival and lower expression correlating with higher survival, it was also shown that AC was expressed in GBM post irradiation. This suggests a role for AC expression as an inducible survival factor post irradiation, findings which have also been seen in prostate cancer. Subsequent immunohistochemical analysis of patient samples from 9 newly diagnosed GBM patients also showed the same results as the proteomic analysis with higher AC expression correlating with poorer survival. It is known that, whilst killing tumour cells, chemotherapy can leave cancer stem cells behind which can allow for a repopulation of tumour and another mechanisms of resistance. In GBM it has been shown that CD1D33 are biomarkers of glioma like stem cells and are associated with poorer prognosis (Zeppernick et al., 2008). From glioma stem cell (GSC) populations it was noted that those expressing higher CD133 also expressed higher AC levels (Zorniak et al., 2012) suggesting a role for AC in promoting survival and proliferation of CD133+ cells that are known to be chemo and radioresistant (Qiu et al., 2014).

These findings are important for GBM as there is no drug currently available that induces cell death. Temozolomide is the only FDA approved oral chemotherapy for GBM and inhibits cell growth. Doan et al. 2017 successfully demonstrated *in vitro* that by pharmacological inhibition of AC U87MG GBM cells and GSCs (Doan et al., 2017a) (which are known to be more resistant to chemo and radiotherapy) were successfully killed via apoptosis, in contrast with conventional treatment with Temozolomide. Carmofur has already been identified as a potential AC inhibitor that is already commercially available for possible therapeutic use in both adult and paediatric brain tumours[68] with planned trials to potentially repurpose carmofur as a therapy in GBM. With GBM, the challenge has been to find a predictable and reliable method of the AC inhibitor crossing the blood brain barrier and several groups are working on this issue at present (Realini et al., 2013) including murine studies involving nanogel vectors to assist in penetrance of brain tissue (Miyazaki and Tabata, 2009). Immunotherapy to AC and S1P has also been shown to reduce the proliferation *in vitro* of GBM cells (Doan et al., 2017c).

4. AC as therapeutic target in cancer therapy

The mounting *in vitro* data clearly demonstrates the critical role the sphingolipid pathway plays in a wide spectrum of human cancers, suggesting it is of fundamental importance and therefore a key molecular target. A number of agents currently exists that target the sphingolipid pathway, some of which have made it to clinical testing. By comparison, most AC inhibitors have only been tested in the *in vitro* phase, suggesting that although promising, more translational work is urgently needed in this area. However, a number of specific inhibitors have been approved for clinical use, and a number of other agents with off target effects affecting AC have also been observed. See Table 3.

The first AC inhibitor reported was N-Oleoylethanolamide (NOE) (Sugita et al., 1975), producing anti-tumour effects in several *in vitro* studies however its pharmacokinetics meant that it was not suitable in clinical therapy. First generation AC inhibitors were developed from the ceramide analogues by Bielawska et al. in the 1990s (Bielawska et al., 1996). Second generation AC inhibitors were then developed by Draper et al. in 2011 that did not utilize the ceramide scaffold (Draper et al., 2011). An important second-generation AC inhibitor is Carmofur (1-hexylcarbamoyl-5-fluorouracil), a derivative of 5-FU that is an oral prodrug which becomes converted intracellularly to release 5-FU and inhibit thymidylate synthetase thus inhibiting tumour proliferation (Kubota et al., 1991). It is also a potent AC inhibitor that has been approved for clinical use in Japan since 1981 for adjuvant treatment of colon and breast cancer. A recent meta-analysis of its role in the adjuvant setting has shown that overall survival and disease free survival were improved when compared to patients who underwent curative surgery alone (Sakamoto et al., 2005). Carmofur however has still not been licenced for FDA approval due to the associated higher rates of leukoencephalopathy as reported in a trial on hepatocellular carcinoma that was ended prematurely (Yamamoto et al., 1996). In addition to the encouraging *in vitro* results, clinical studies in breast and colon cancer have demonstrated some benefits (Morimoto and Koh, 2003). Treatment of GBM is perhaps the area of greatest interest, where its ability to at least partially cross the blood-brain barrier is of substantial importance, however this may partially explain its risk of inducing leukoencephalopathy.

More recently a number of small molecular AC inhibitors have been developed and used in the pre-clinical setting. Whilst none of these have yet to make it to the clinical setting, the development of an AC enzyme activity assay will facilitate the development of these inhibitors. The LCL series of small molecular inhibitors are perhaps the most studied. They are composed of N-dimethylglycine (DMG)-B13 prodrugs that are metabolised to B13, a known AC inhibitor, and target AC in the lysosome. As discussed above, LCL521 improved the efficacy of photodynamic therapy in head and neck cancer and has also been

Table 3
Summary of AC inhibitors (OS – overall survival, DFS- disease free survival, CS – cumulative survival, RFS – recurrence free survival).

Reference	AC Inhibitor	Pre-clinical / Clinical	Findings
(Realini et al., 2013)	N-Oleoylethanolamide (NOE)	In vitro	Pharmacokenetics deemed not suitable for clinical therapy
(Vethakanraj et al., 2018; Kus et al., 2015; Baspinar et al., 2017; Draper et al., 2011) (Yildiz-Ozer et al., 2018)	Ceranib-2	In vitro Mice models In vitro	More potent AC inhibitors than NOE. Inhibit cell proliferation and induce cell death in ovarian cancer cell line. Reduced tumour growth with no overt toxicity in mice models. Anti-cancer effects on breast and prostate cancer cell lines and synergestic effects with oxaliplatin in colon cancer cell lines. Synergistic effects when used with carboplatin on non-small cell lung cancer cells, reduced toxicity to normal cells
(Kubota et al., 1991; Sakamoto et al., 2005; Yamamoto et al., 1996; Morimoto and Koh, 2003)	Carmofur	Meta-analysis (3 RCTs; 2152 patients) Clinical Clinical	Resection alone v resection + carmofur in colon cancer – increasing efficacy of carmofur for Dukes' B and C ((OS hazard ratios 0.73 (p = 0.086), 0.83 (p = 0.11), DFS 0.64 (p = 0.008), 0.80 (p = 0.032)) Adjuvant carmofur in hepatocellular cancer – no difference in CS, RFS between groups, discontinued due to toxicity Adjuvant carmofur v carboquone in breast cancer – No difference in
(Korbelik et al., 2016b)	LCL521	In vitro Mice models	In combination with photodynamic therapy (PDT) in head and neck cancer – enhanced effects of PDT with LCL521 use in vitro. In mice significant improvement in response to PDT, only in immunocompetent mice
(Realini et al., 2016)	ARN14899	In vitro	Reduces AC activity and increases ceramide levels in stage II melanoma. Effects more pronounced with combination with other chemotherapeutic agents. Inhibits AC with nanomolar potency.
(Camacho et al., 2013b)	RBM 1-12 SABRAC	In vitro	Caused strong effect on AC inhibition and a dose-dependent accumulation of ceramides in PC-3 prostate cancer cell lines and inhibited their growth and clonogenicity. Interestingly, SABRAC displayed strong growth inhibitory effects on PC-3/Mc cells, while exhibiting very limited cytotoxicity.

shown to prevent relapse of prostate cancer in mice (Korbelik et al., 2016b). Another inhibitor, ARN14899, has been shown to reduce AC activity and increase ceramide levels in stage II melanoma. Importantly these effects were more pronounced when the inhibitor was combined with chemotherapeutic agents (Realini et al., 2016). Similar synergistic findings have been observed in colon cancer cells where carmofur has been shown to increase sensitivity to oxaliplatin (Klobučar et al., 2018).

Whilst specific AC inhibitors are yet to make it to the clinical setting, there are a number of studies showing the possibility of utilising existing agents that have off target AC inhibitory effects. The most commonly used of these agents is tamoxifen. Both tamoxifen and its metabolite N-desmethyltamoxifen block the conversion of ceramide to glucosylceramide and inhibit AC independent of their anti-oestrogen mechanisms. AML cells treated with tamoxifen and exogenous ceramide or ceramide inducing drugs induced synergistic apoptosis in AML cells (Morad and Cabot, 2015) Interestingly, the combination of tamoxifen in combination with the specific inhibitor LCL521 has been shown to also result in synergistic effects on tumour cell proliferation and death in breast cancer cells (Bai et al., 2017).

Traditional chemotherapeutic agents may also work via the ceramide pathway, with *in vitro* work demonstrating chemotherapeutic induced increased ceramide levels in some cancer cells (Lucci et al., 1999). Clinical data in head and neck cancer has also revealed that elevated ceramide levels may be associated with improved response to chemotherapy (Saddoughi et al., 2011). AC inhibition with the agent ceranib-2 has been shown to have synergistic effects when used with carboplatin on non-small cell lung cancer cells (Yildiz-Ozer et al., 2018). Additionally, ceranib-2 appeared to be less toxic to normal cells than carboplatin, suggesting that such combination therapies of AC inhibitors and reduced chemotherapy schedules would be very attractive as a cancer therapy.

If AC inhibition is to progress from *in vitro* studies to larger scale studies in patients, developments in the measurement of AC activity will also be required. Traditional assays have relied on time consuming and expensive radioactive substrates, however novel fluorogenic high throughput enzymatic assays have now been developed (Bedia et al., 2007). In addition to being significantly more cost-effective, such assays also have the promising ability to measure AC activity directly from serum or plasma samples (Mühle and Kornhuber, 2017).

5. Conclusion

The role of the sphingolipid pathway, and in particular ceramide accumulation in cancer is becoming very clearly established. The subcellular localisations and targets of sphingolipids are of critical importance as they appear to determine their anti-cancer or pro-carcinogenic properties. The ceramidases appear to play a critical role in these actions by promoting ceramide accumulation in response to cancer or cellular stress. Acid ceramidase in particular has been shown to be elevated in a wide spectrum of cancers, and furthermore its direct inhibition by novel specific inhibitors or from off-target effects of traditional cancer agents has demonstrated its potential either alone or in combination with other agents. Further work is required to further develop safe and accurate assays of AC activity that could be used in routine clinical practice and to progress pre-clinical studies of AC inhibition into phase II clinical trials.

Conflict of interest

None to declare

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