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Medicinal Chemistry Aspects of Drug Targets in Sphingolipid Metabolism

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Introduction

Sphingolipids are characterised by a long-chain base (sphingoid) backbone, commonly comprising 18 to 20 carbon atoms, substituted by two to three hydroxy groups (at least at positions 1 and 3) and in most cases a *trans* double bond in position 4. In human cells the common backbone of the majority of sphingolipids is (2*S*,3*R*,4*E*)-2-amino-4-octadecene-1,3-diol, that is, D-erythro-sphingosine, whereas the saturated analogue dihydrosphingosine is present as a minor component (Figure 1). Ceramides are derivatives acylated at the amino

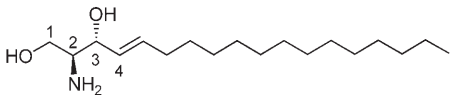

Figure 1. D-erythro-sphingosine, the common backbone of human sphingolipids.

group with fatty acids, and more polar and complex sphingolipids feature a further modification at position 1. Lysosphingolipids are characterised by a free amino group but with polar head groups in position 1. In total, sphingolipids are a class of more than 1000 different, naturally occurring compounds. The unique component of this structural class was isolated and identified by Thudichum in 1884 when investigating brain extracts. As the substance posed so many mysteries and puzzles to him he called it "sphingosine" after the Egyptian Sphinx. For more than 100 years, it was believed that sphingolipids were merely structural components of higher eukaryotic cells. In the meantime, it is well established that sphingolipids can also serve as extra- and intracellular mediators in cell signalling and that specific receptors exist. Consequently, sphingolipid research has become a very active field, and a number of hypotheses for therapeutic opportunities have emerged. With the invention of FTY720, which is currently in Phase III clinical trials for multiple sclerosis, medicinal chemistry proved that sphingolipid-like structures can be successfully developed into drugs.[1] This has further stimulated interest in drug discovery in this field but overall medicinal chemistry aspects are still rather unexplored.

In general, potential drug targets for therapeutic intervention with low-molecular weight compounds in sphingolipid signalling include 1) receptors for the respective sphingolipid mediators (extracellular function) and 2) enzymes involved in their biosynthesis and metabolism modulating endogenous levels of the signalling molecules (intracellular function). This review deals only with the latter approach and does not cover sphingolipid receptors. Moreover, the focus is on medicinal chemistry aspects such as drugability, available tool compounds, and target validation, complementing (selected) recent reviews on inhibitors of sphingolipid metabolism enzymes,[2] chemical tools to investigate sphingolipid metabolism and functions,[3] chemistry and biology of sphingolipids,[4] pharmacological modulation of sphingolipids and their role in disease,[5] function of sphingolipids in cell signalling,[6] sphingolipid metabolism on the cell surface and extracellular space,[7] translational aspects of sphingolipid metabolism,[8] and sphingolipid metabolism diseases.[9]

Sphingolipid Biosynthesis: Potential Intervention and the Rheostat Principle

The first step in the biosynthesis of sphingolipids (Figure 2) is the condensation of L-serine and palmitoyl-CoA catalysed by the enzyme serine palmitoyl transferase (SPT). The keto function is then stereoselectively reduced by 3-ketosphinganine reductase to generate sphinganine, which is the saturated analogue of sphingosine. Interestingly, free sphingosine itself—which is the core of most of the sphingolipids—is only generated in the degradation path and not produced from sphinganine which is acylated with fatty acids by sphinganine N-acyl transferases to give dihydroceramides. The *trans* double bond present in most sphingolipids is regioselectively introduced at this stage catalysed by dihydroceramide desaturase. The resulting ceramides (such as the dihydroceramides) are a mixture of analogues mainly varying in the length of the N-acyl side chain with C16 (palmitoyl) as the major component in humans. There is some evidence that ceramides with different fatty acid side chains may have different biological functions, but generally throughout the literature the singular term ceramide is being used for simplicity. Ceramide is the central molecule of sphingolipid biosynthesis and degradation. It is the branching point for the formation of sphingomyelin and of complex sphingolipids which feature polar substituents at the hydroxy

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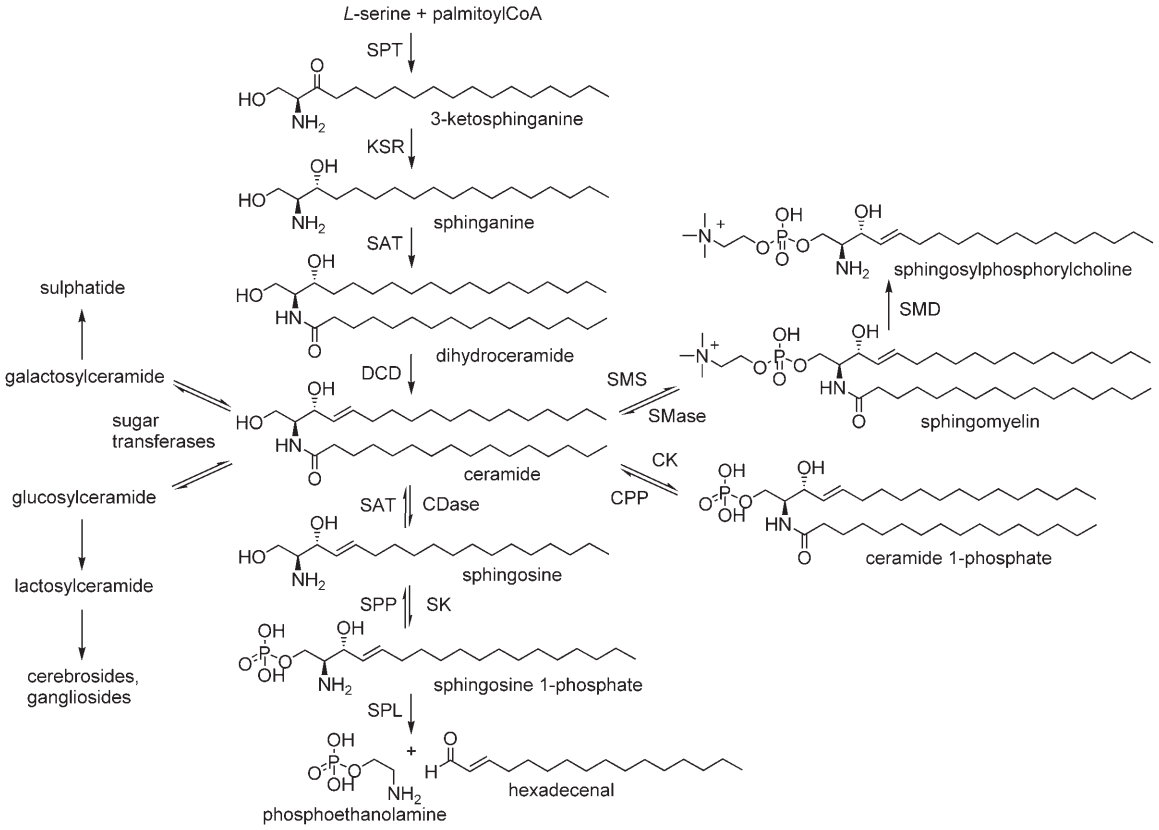


Figure 2. Biosynthesis and catabolism of sphingolipids; SPT = serine palmitoyl transferase, KSR = 3-ketosphinganine reductase, SAT = sphinganine/sphingosine N-acyl transferase, DCD = dihydroceramide desaturase, CDase = ceramidase, SPP = sphingosine 1-phosphate phosphatase, SK = sphingosine kinase, SPL = sphingosine 1-phosphate lyase, SMS = sphingomyelin synthase, SMase = sphingomyelinase, SMD = sphingomyelin deacylase, CK = ceramide kinase, CPP = ceramide 1-phosphate phosphatase.

group at position 1. Furthermore, ceramide is the first molecule in the biosynthesis with an attributed signalling function leading to induction of apoptosis and having implications in inflammation. Therefore, intervention with the biosynthesis of ceramide might be considered as a pharmaceutical target. On the other hand, stimulation of ceramide production to induce apoptosis might be of interest for cancer indications supported by the finding that ceramide is a key factor in radiation- and chemotherapy-induced cell death.

However, it has become evident that one particular sphingolipid does not have an assigned function per se but that its balance with respect to other sphingolipids determines effects like cell survival versus cell death.[6,10] This rheostat concept has been established for the ceramide/sphingosine 1-phosphate pair for T-cell apoptosis and also for the ceramide/ceramide 1-phosphate pair for mast cell phagocytosis. This already implies the importance of the catabolism path for targeting sphingolipid signalling by modulation of their endogenous levels. Moreover, it has to be considered that inhibition of enzymes involved in the biosynthesis of ceramide (serine palmitoyl transferase, 3-ketosphinganine reductase, sphinganine N-acyl transferases, dihydroceramide desaturase) to reduce levels of the signalling molecule and consequently alleviate its negative function will also affect the biosynthesis of all other sphingolipids, including cerebrosides and gangliosides.

This simultaneous disturbance of many functionally and structurally important molecules poses a high risk for side effects. Therefore, therapeutic intervention in the early part of sphingolipid synthesis is not very attractive, with the possible exception of life-threatening diseases with very high medical need lacking any alternative treatment option.

In summary, based on these facts and considerations, my recommendation is to modulate ceramide levels not by interfering along the general sphingolipid biosynthesis path, but rather by targeting those enzymes involved in either the generation of ceramide from pools of sphingomyelin or complex sphingolipids, or in the conversion of ceramide to more complex derivatives.

In the latter context, some glucosylceramide synthase inhibitors were shown to raise ceramide levels in addition to glucosylceramide depletion,[5] but for therapeutic use in glycosphingolipid storage diseases the dissociation of the two effects is required. By transferring the phosphocholine moiety from phosphatidylcholine to ceramide generating sphingomyelin (SM), sphingomyelin synthase (SMS) also regulates ceramide levels in cells and, hence, potentially controls cellular processes. Some SMS inhibitors and their ability to induce apoptosis have been reported but a clear link of SMS inhibition to a therapeutic potential still has to be established.

Sphingolipid Catabolism and Potential Drug Targets

As in the biosynthesis, ceramide is also the central molecule in the catabolism of sphingolipids (Figure 2). Sphingomyelin (SM), the most abundant sphingolipid in mammalian cells, is metabolised to ceramide by the family of sphingomyelinases (SMases), which significantly contributes to (local) concentrations of ceramide. SM can also be deacylated by sphingomyelin deacylases to generate sphingosyl phosphorylcholine which has signalling properties affecting cell growth and cell migration.[11] Ceramide can presumably be generated by degradation of ceramide 1-phosphate (C1P) catalysed by a postulated, as yet uncharacterised C1P phosphatase,[12,13] but is also the substrate for the reversed reaction, that is, formation of C1P by ceramide kinase (CK). The breakdown of ceramide is then initiated by ceramidases to generate sphingosine which itself can be converted to sphingosine 1-phosphate (S1P) by sphingosine kinases (SK). The next step catalysed by S1P lyase is the final, irreversible degradation of the sphingosine backbone yielding phosphoethanolamine and (*E*)-hexadec-3-enal. The first two degradation steps from ceramide to S1P are reversible because ceramide synthase and S1P phosphatases can recycle sphingosine and S1P, respectively, but the catabolism cascade of reactions is of general importance for the housekeeping function to eliminate sphingolipids. S1P is an important signalling molecule and, particularly, its balance versus ceramide is considered to be critical for cell death (stimulated by ceramide) versus cell survival (induced by S1P). In addition to cell survival, S1P effects are associated with cell migration, mast cell function, endothelial cell activation, angiogenesis, and inflammation.[14]

Several approaches can be envisaged to modulate the balance of ceramide versus S1P levels, depending on the indication: inhibition of SMases to downregulate ceramide effects, inhibition of ceramidases to augment ceramide effects, inhibition of SKs to reduce the S1P effects, inhibition of S1P lyase and S1P phosphatases to amplify the S1P effects. These points of intervention are now discussed in more detail.

Sphingomyelinases (SMases) as potential drug targets

SMases are a family of phospholipases that cleave sphingomyelin (SM) to ceramide and phosphocholine. The difference in the pH optimum for the enzymatic activity is the basis for their classification into alkaline, neutral, and acidic SMases. The alkaline SMase is mainly expressed in the intestine and may function in nutritional sphingomyelin degradation. As this can affect cholesterol uptake, alkaline SMase might have a potential implication in the development of atherosclerosis. Furthermore, an association of downregulated alkaline SMase with ulcerative colitis and with adenocarcinoma has been suggested recently.[15] Three neutral SMases (nSMase 1–3) have been cloned so far and all are associated with membranes. Inhibition of nSMases has been proposed as a potential target for anti-inflammatory and anticancer therapy. Several general but no specific nSMase inhibitors have been reported, and for some anti-inflammatory activity in vivo was also demonstrated.[16,17] Enzyme inhibitory activity was always measured in cell lysates only and not on isolated enzymes. The most investigated compound, the natural product scyphostatin (Figure 3), inhibits nSMase in the low μM range with about 50-fold selectivity over aSMase.[16]

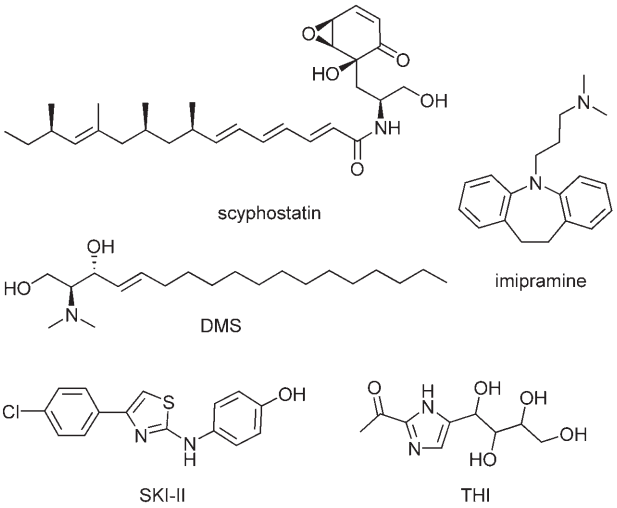


Figure 3. Selected inhibitors of nSMase (scyphostatin), aSMase (imipramine), sphingosine kinase (DMS, SKI-II), and sphingosine 1-phosphate lyase activity (THI).

There is one human gene encoding for acid SMase (aSMase) which generates two glycosylated precursor forms of 75 and 72 kDa, respectively.[18] A minor amount is secreted and the major fraction is transported to the lysosomes where it is processed to the mature form of about 52 kDa. As a housekeeping function, aSMase is essential in the SM turnover. Inborn deficiency in lysosomal aSMase leads to SM storage and causes Niemann-Pick disease. When there is complete loss of aSMase (Niemann-Pick type A) the disease is fatal in early childhood, whereas 5–10% residual aSMase (Niemann-Pick type B) results in non-neurological visceral progression.

Secreted aSMase differs from cellular aSMase by additional post-translational processing (proteolytic cleavage, glycosylation); it requires exogenous addition of zinc to get measurable enzyme activity, whereas the lysosomal form of aSMase has zinc very tightly bound to the active site. The physiological role of the secreted isoform is unknown, however a potential role in the pathogenesis of atherosclerosis was postulated.[19] Moreover, its elevation in septic shock patients and in LPS-treated mice was observed.[20]

aSMase is believed to play a critical role in signalling through the generation of ceramide. One model postulates that the enzyme needs to be translocated from the lysosomal compartment to the outer leaflet where it produces ceramide, which is critical for formation of lipid platforms.[21] In these ceramide-rich membrane domains receptors, such as CD40 and Fas/CD95, are concentrated ("receptor clustering") as a prerequisite for efficient signalling and/or internalisation. This model implies that aSMase inhibitors do not have to enter cells but that it should be sufficient to exert the inhibitory effect at the

cell surface and/or membrane. With respect to inhibitor design it is still uncertain whether the compounds have to incorporate into the membrane or not.

For aSMase there is quite substantial target validation available. Several cationic amphiphilic drugs (tricyclic antidepressants) have been used as tool compounds. They induce premature degradation of aSMase by dissociation from the inner lysosomal membrane followed by proteolytic degradation of the enzyme.[22,23] By this mechanism the catalytic enzyme activity is abolished, and inhibition of the signalling component can be studied. Thus, blocking of LPS-induced TNF-α secretion was observed in vivo after administration of imipramine (Figure 3).[24] The essential role of aSMase in apoptosis was demonstrated independently with human aSMase deficient cells and with cells from aSMase knock-out mice, but also with inhibitors. This was further corroborated by recent results in our labs, showing that Jurkat cells incubated with anti-Fas Ab were protected from apoptosis by aSMase siRNA, by a "maturation" inhibitor, and by an active-site enzyme inhibitor we had recently discovered (unpublished results).

Recently, aSMase deficient mice were reported to be protected from TNF-induced liver damage suggesting the enzyme as a target for apoptosis-induced liver diseases.[25] In addition, there is supporting evidence for a broad range of potential indications for aSMase inhibitors including COPD,[26] stroke,[27] and atherosclerosis.[28]

Ceramidases (CDase) as potential drug targets

According to their activity pH optimum, ceramidases are classified as acid (aCDase), neutral, and alkaline. Ceramidases in one subgroup do not share any sequence similarity to those in the other subgroups although all ceramidases catalyse the same reaction. Interestingly, aCDase not only hydrolyses ceramide to sphingosine, but also can catalyse the reverse reaction generating ceramide which occurs at a distinct pH from the hydrolysis.[29,30] aCDase which is localised in the lysosomes is the most investigated type and deficiencies cause Farber's disease or lipogranulomatosis, a rare lipid storage disease.[9] As inhibition of ceramidase can result in elevated levels of proapoptotic ceramide, the enzyme is considered a potential drug target, particularly for cancer therapy. Antitumour activity has been demonstrated with a lipidic prototype inhibitor (for information on inhibitors see references [2] and [5]) in several tumour cell lines and also in an in vivo model as well as with siRNA targeting aCDase.[30–32] Furthermore, human aCDase was found to be overexpressed in some cancers. These data together suggest a role of aCDase in various cancers, however the therapeutic potential still has to be proven. Aberrant aCDase activity has been also described in Alzheimer's disease suggesting that aCDase might be involved in the molecular mechanisms leading to this disease.[33]

Sphingosine kinases (SK) as potential drug targets

Two isoforms of sphingosine kinases (SK1 and SK2) are known, but there is some indication that other variants may exist. The regulation of SK and its role in disease was recently reviewed.[34] All cells have low basal levels of SK activity and low levels of S1P which constitutes the housekeeping function in sphingolipid degradation. The catalytic efficiency of SK1 and SK2 is of the same order of magnitude, but there are some differences in substrate specificity with SK2 accepting a much broader range of structural diversity.[35]

In immune cells, the activity of SK1 rises upon cell activation generating peak levels of S1P which acts both intra- and extracellularly. The results are effects on cell migration and also NFκB activation and induction of inflammatory mediators (COX-2 and PGE_2). In some cancer cells, SK1 is constitutively overexpressed contributing to tumourigenic growth. Target validation for SK1 inhibition is still incomplete and has been hampered by the lack of a good tool compound. Many claims on functional roles of SKs are based on results with *N*,*N*-dimethylsphingosine (DMS, Figure 3), a weak (IC_{50} in the 10 μM range), lipidic inhibitor with questionable specificity. Various natural products and substrate-based analogues were published as SK1 inhibitors with the most potent having IC_{50} values in the low μM range, but no candidate for drug development has emerged so far. Most promising appears to be a series of SK1 inhibitors first published in 2003.[36] The compound called SKI-II (Figure 3) is more and more often replacing DMS as the standard inhibitor in current literature. Recently, it was shown by inhibition with DMS and also with siRNA that SK1 is essential for survival of the A549 tumour cell line which is dependent on constitutive NFκB expression.[37] SK1 is upregulated in colon carcinogenesis and downregulation of SK1 in HT-29 human colon cancer cells by siRNA was shown to decrease COX-2 expression and PGE_2 production.[38] In addition, significant inhibition of tumour growth in mice after oral administration of SKI-II was reported,[39] confirming the potential of SK inhibition in antitumour therapy.

In support of anti-inflammatory indications, it was demonstrated that cytokine-induced COX-2 expression is suppressed by siRNA and dominant-negative mutants of SK1 indicating that SK1 activation is necessary for full stimulation of COX-2 transcription.[37] SK1 deficient mice have been established, and it was shown that renal fibroblasts of these animals are deficient in COX-2 expression and PGE_2 production after stimulation with TNF-α or IL-1β (A. Billich, personal communication). However, we and others found that SK1 deficient mice are not protected in COX-2 dependent models of arthritis,[40] which challenges the relevance of the target for anti-inflammatory therapy.

To study the role of SKs in allergy, mast cells of SK1 and SK2 deficient mice were used. Whereas SK2 activity was found to be required for normal mast cell function, susceptibility to in vivo anaphylaxis is associated with circulating S1P generated by SK1 predominantly from nonmast cell sources. Thus, deficiency in SK1 results in resistance to anaphylaxis even though the mast cells from these mice contain normal intracellular amounts of S1P and were unaffected in their responses.[41]

There is also growing evidence that SK1 plays an important role in cellular proliferation and angiogenesis. Along this line, the effects of SK1 inhibitors such as SKI-II were studied on the

responses of retinal endothelial cells to VEGF and TNF-α in vitro and in vivo. The results indicate that SK1 inhibition may have potential in the treatment of diabetic retinopathy.[42]

For SK2, no clear implication in disease pathology has been identified so far.

S1P lyase (SPL) as potential drug target

SPL was recently suggested as a novel immunosuppressant drug target when it was discovered that the long-known, immunosuppressive food colorant 2-acetyl-4-(tetrahydroxybutyl)imidazole (THI, Figure 3) inhibits SPL activity and induces lymphopenia.[43] From these studies it was concluded that lymphocyte egress from lymph nodes is mediated by S1P gradients controlled by SPL. Similar effects were seen with the vitamin B6 antagonist 4′-deoxypyridoxine and with siRNA against SPL and could be restored by adding an excess of vitamin B6, the co-enzyme of SPL. It appears that THI is neither an active-site inhibitor of SPL nor acts by inhibiting pyridoxal phosphate incorporation into the enzyme; thus the compound appears to have an indirect effect on S1P lyase activity, for example, by interfering with pyridoxal phosphate metabolism (A. Billich, personal communication). Investigations with cells of SPL deficient mice indicate that the enzyme might have a role in the regulation of angiogenesis and other developmental processes.[44] In a screen for genes with increased expression, the gene encoding human SPL was identified in skin samples of atopic dermatitis.[45] However, there is also evidence that loss of SPL expression or activity might contribute to tumourigenesis.[44] Interestingly, phosphoethanolamine, one product of SPL activity, is used as substrate in phospholipid biosynthesis and, thus, connects the two lipid pathways.

S1P phosphatases (SPP) as potential drug targets

S1P can be recycled to sphingosine by specific phosphatases SPP1 and SPP2, but also to some extent by unspecific lipid phosphate phosphatases (LPP1-3). Very recently, it was reported that SPP2 is specifically and transiently upregulated by inflammatory stimuli in neutrophils and endothelial cells.[46] Silencing of SPP2 expression led to full inhibition of IL-1β expression and partial inhibition of IL-8 expression after TNF induction. These findings suggest a proinflammatory role of SPP2 and its inhibition might have potential for anti-inflammatory therapy. Interestingly, SPP2 expression was found to be upregulated in psoriasis which is an inflammatory skin disease.

Ceramide kinase (CK) as potential drug target

C1P, produced from ceramide by action of CK, is another important signalling mediator with proinflammatory properties. C1P is postulated to directly interact with cytosolic phospholipase A2 alpha ($cPLA_2$) resulting in activation and, hence, increased release of arachidonic acid and downstream inflammatory mediators.[47] Inhibition of CK is expected to be therapeutically valuable in situations where high levels of C1P may contribute to $cPLA_2$-mediated inflammation but also in cancer.[48] C1P is also emerging as an important mediator in mast cell degranulation.[35] A CK inhibitor derived from a known, natural product-based SK inhibitor was found to suppress mast cell degranulation, suggesting that CK inhibition could be a potential therapeutic option for allergic diseases.[49] However, the therapeutic potential in vivo still needs to be demonstrated.

Issues Associated with Drug Targets in Sphingolipid Metabolism

From a medicinal chemistry point of view, sphingolipid metabolism and signalling is an exciting but still rather unexplored field. Target validation is progressing, especially with the generation of knock-out mice and with siRNA experiments, but relevance in diseases still has to be proven and good tool compounds are missing. In principle, the enzymes involved in sphingolipid metabolism have high drugability potential by definition, however several issues and challenges are associated with these targets. Because of the complex network of sphingolipid metabolism and signalling as well as the lack of any precedence, only enzyme inhibition and not stimulation is considered in the discussion.

To start with, protein expression is a common difficulty in this field, mainly because of the membrane association of many of these enzymes leading to low solubility. In addition, in many cases eukaryotic expression systems are needed to produce functional protein. Enzyme crystallisation is a so far an insurmountable hurdle, and the lack of structural information hampers rational design and progress in understanding of the catalytic mechanisms of the enzymes. The latter aspect is important as the catabolic enzymes appear to form separate families within classes of enzymes with similar functions, for example, SMases are distinct from other phosphodiesterases, SKs are a separate family even within the class of lipid kinases and very different from protein kinases. Therefore, it is not possible to follow approaches successfully applied in other areas for inhibitor design. As for the catalytic mechanism, substrate specificity, and structural requirements for the enzymatic reactions, not much is known yet about regulation of the enzymes.

Another important issue is the physicochemical profile of the sphingolipids, including the enzyme substrates and signalling molecules. Many sphingolipids are sparingly soluble in physiologically acceptable solvents, sometimes even in common organic solvents, and have a tenside-like behaviour. They usually tend to form micelles but very little is known about their critical concentration for micelle formation, how this depends on additives and solvent mixtures, and how this affects biological studies and their read-outs. It is a challenge to apply such compounds to biological systems and the degree of bioavailable fraction is not known. Therefore, in many biological investigations high concentrations (10 μM and higher is quite common) of sphingolipids and additives are used to facilitate dissolution and cellular targeting. Results might have to be interpreted with some caution in both cases. On the one hand some sphingolipid mediators exert additional nonspecific effects at high concentrations, on the other hand additives might not be inert. As an example, dodecane has

been used in mixtures with alcohols to deliver C1P to cells but, recently, it has been discovered that some biological effects previously attributed to C1P are in fact dependent on the presence of dodecane.[50] To study the effects of ceramides in cells, C2-ceramide is frequently used because this short-chain analogue readily enters cells (in contrast to the natural long-chain ceramides). However, there is increasing evidence that ceramides with different N-acyl residues can have different biological function. In addition, there are active transport mechanisms for some sphingolipids which have to be taken into account and investigated further. Addressing issues associated with physicochemical properties of sphingolipids, Szulc et al. investigated a series of cationic pyridinium ceramides designed to act as organelle-targeted analogues.[51] They showed significantly improved water solubility, fast cellular uptake, and higher cellular anticancer activities compared to the uncharged parent ceramides. Thus, such derivatives may have therapeutic potential.

Many of the aspects mentioned above are also important for establishing and running enzymatic assays. Frequently, these assays rely on radiolabelled substrates and radioactive detection methods. More and more fluorescently labelled substrates are emerging (see section on tool compounds) enabling convenient readouts and throughput. Validated, sensitive, and accurate detection methods for quantitative determination of sphingolipid substrates and products from cells and tissues are just emerging (sphingolipidomics) and liquid chromatography tandem mass spectrometry (LC MS/MS) has been successfully introduced for this purpose. Exact quantification is even more complex because natural ceramide (and consequently substituted derivatives) consists of several molecules with different fatty acid chains. Although the LC MS/MS method works well regarding separation and quantification, tissue workup, extraction, and sample preparation are still a challenge, mainly because of poor and different solubility of the sphingolipids, and because of high matrix contamination by glycerolipids. Several workup procedures, sometimes optimised for particular analytes, have been published.[52–56] Results from methods involving treatment with a strong base to hydrolyse and eliminate the glycerolipids have to be applied with caution. We investigated the effect of workup procedures on several pure, single sphingolipids and found substantial degradation of sphingomyelin to C1P. As sphingomyelin is abundant in cells, C1P levels are highly overestimated when strong bases such as potassium or sodium hydroxide are used during work-up. Ceramides were also found to partially undergo N-acyl migration under acidic conditions and upon storage, especially in chloroform, which calls for appropriate storage of ceramides and derivatives.[57]

The currently available inhibitors often are only low-potency, nondruglike compounds with limited selectivity for target subtypes. In addition to close substrate analogues, only singular natural products identified by screening without much follow-up have been reported. It is expected that the increasing interest of the pharmaceutical industry will soon change this picture. Rational inhibitor design is extremely difficult due to the lack of structural and mechanistic information. The situation is even more complicated by findings indicating that the whole cell or tissue concentrations of sphingolipid mediators are not important for biological (mal)function but distinct local peak concentrations generated at particular (sub)locations from particular precursors/sources. This sounds plausible when taking into account that some of the sphingolipid signalling molecules are quite abundant in the human body. To give one example, it is believed that ceramide-induced apoptosis in T-cells is caused upon stress stimulus by activation of sphingomyelinase resulting in locally increased ceramide concentrations.

Tool Compounds and Probes

By designing and synthesising tool compounds, medicinal chemistry is substantially contributing to gaining more insight in new fields and solving biological problems. With regard to sphingolipids, Robert Bittman's address to the "2003 ASMB-Avanti Award in Lipids" gives an excellent overview of advances in this direction.[58] Such tools and probes comprise selective inhibitors, selective assay substrates, labelled and tagged derivatives for detection and visualisation, and analytical standards. The currently available inhibitors of sphingolipid metabolising enzymes have been recently reviewed[2] and the most useful examples are mentioned in the section above.

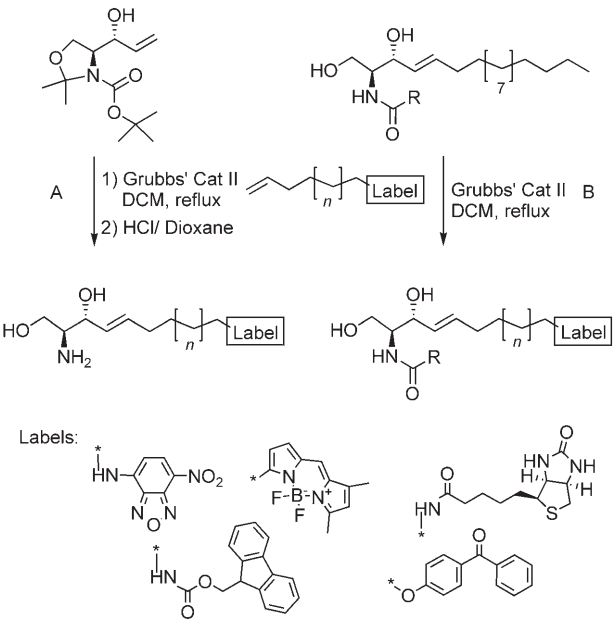
Bioactive labelled derivatives are required for localisation, binding, and metabolism studies and as assay substrates. Labels such as dyes, biotin, and photoactivatable groups can be introduced by modification of either the N-acyl fatty acid side chain in ceramides and analogues thereof or the sphingosine backbone. In sphingolipids with substituents at position 1, labelling in the polar head group is also an option. For example, a sphingomyelin derivative with dansyl in the phosphocholine moiety was synthesised and proven to be cleaved by aSMase with good efficiency,[59] thus, representing a potential assay substrate for aSMase. The label should be lipophilic to minimise an effect on biological activity. There is some information available on the influence of the nature, position, and linkage of a label in sphingosine derivatives using pyrene, dansyl, and NBD as dyes.[60] "Biocompatibility" was assessed by measuring the phosphorylation efficiency by SK1 and SK2. Compounds with a polar linker (connecting the sphingosine head group to the label) in the middle of the alkyl chain were not efficiently phosphorylated by the SKs. However, when the linker and label were moved to the terminus, the pyrene and NBD-labelled derivatives were well accepted as substrates and converted to the corresponding phosphates. Less polar linkers such as an ester group appeared to be favoured much more than polar amide or sulfonamide linkers, supporting the idea that the structural modification should be as apolar as possible.

Quite a number of ceramide derivatives with labels in the N-acyl side chain have become commercially available. Synthesis is easily accomplished by reacting lyso-sphingolipids with commercially available labelling reagents, such as activated esters of NBD-, BODIPY-, or biotin-substituted alkanoic acids, and was successfully applied even to complex gangliosides.[61] The alkyl spacer between the label and the sphingosine amino group is most commonly five to six atoms long, that is, pentanoic or

hexanoic acid. There are reports of some derivatives with longer or shorter spacers, but apparently no systematic study has been conducted on the influence and optimisation of the chain length. It also remains to be investigated whether lipophilic dyes such as pyrene, NBD, and BODIPY already mimic parts of the linear fatty acid chains to some extent. When radiolabelled and fluorescently N-acyl side chain labelled sphingolipids were compared in cell studies, different transport properties were observed, for example, for a pair of nondegradable glucosylceramide analogues.[62] Moreover, even NBD- and BODPY-labelled ceramides can give different results in cellular studies, most likely due to the much faster rate of spontaneous transfer between artificial membranes observed for NBD-ceramide.[63–65]

Labelling of the sphingoid backbone is more challenging as a terminal functionality has to be introduced first before a label can be attached. Until recently, derivatives have been only accessible by lengthy total syntheses, also requiring the stereoselective construction of the sphingosine head group for which several precursors of the chiral pool were used. Starting from commercially available Garner's aldehyde two sphingosine phosphate derivatives with photoactivatable moieties linked by an ether function were synthesised.[66] Biological evaluation revealed that the S1P type-1 receptor interacted with the benzophenone-containing derivative in a specific manner. This interaction was in the same potency range as with the natural ligand S1P, whereas no specific interaction with the diazirine-containing analogue was detected. Applications in photoaffinity labelling experiments has not yet been reported for those compounds. Three independent total syntheses of protected ω-amino-sphingosines (with different chain lengths) were reported.[60,67,68] Kozikowski et al. used the terminal amino functionality for immobilisation onto activated agarose. The other groups synthesised NBD-labelled sphingosines which were found to be readily phosphorylated by SKs and metabolised by cells to labelled sphingomyelin, ceramide, and sphingosine phosphate in a similar manner as ^{3}H-labelled sphingosine. Hakogi et al. also reported on the functional activity of the NBD-labelled S1P derivative by detection of MAPK activation as seen with natural S1P.[68] Based on comparable results obtained for the natural products and their NBD-labelled analogues, it can be concluded that the NBD-label is tolerated in the studied biological systems and that the labelled derivatives are useful tools in sphingolipid biology, although their alkyl chains are shorter by three and four methylene groups, respectively. It can be speculated that the rather lipophilic NBD-label partly mimics the missing alkyl residue. An NBD-labelled sphingosine derivative was also successfully applied in the first nonradioactive SK assay.[69]

Boosted by the needs for such derivatives and for analogues with a dye having improved fluorescent properties, we developed two efficient and flexible synthetic approaches using olefin cross-metathesis as the key reaction for the introduction of various functionalities such as fluorophores, photoaffinity tags, and biotin (Scheme 1). In parallel to our efforts, Katsumura and co-workers also reported efficient introduction of the fluorescent dye NBD and a photoaffinity label into the



Scheme 1. Two new, efficient methods (A and B) for the synthesis of backbone-labelled sphingolipids; a variant of method A using a different head group precursor is described in reference [70].

sphingosine backbone by cross-metathesis using practically identical conditions.[70] In addition, several syntheses of unlabelled sphingosine and ceramide derivatives by cross-metathesis have been described starting from different head group precursors.[71] Our approaches start either from commercially available Garner's aldehyde[72] which allows labelling of both natural and unnatural sphingolipids or from the natural parent sphingolipids themselves avoiding lengthy, individual total synthesis of each compound.[73] Both methods provide the opportunity to vary the length of the alkyl spacer between the sphingolipid head group and the label. In addition, we pioneered the compatibility of the BODIPY dye with the conditions of the cross-metathesis reaction and prepared sphingolipids with the BODIPY dye incorporated into the sphingoid backbone. Beyond exhibiting the excellent fluorescent properties of the dye, these new derivatives offer another big advantage, that is, the label is attached by a carbon–carbon bond avoiding the usual introduction of a polar linker for connecting the bioactive molecules and the label, thus, minimising the effect of the structural modification to the biological properties. BODIPY-labelled sphingosines are phosphorylated by SKs with reasonable efficiency, although for some derivatives preferential phosphorylation by SK1 was observed. We have successfully used fluorescently labelled derivatives to assess effects of enzyme overexpression, knockdown, and inhibition enzyme in cells and tissues.

Following an alternative approach, a novel ceramide analogue and its use in a high-throughput fluorogenic assay for ceramidases was published very recently.[74] This molecule features the umbelliferone moiety linked to the ceramide head group by an ether bond replacing the double bond common in sphingolipids. After the enzymatic deacylation, a chemical oxidation followed by an elimination reaction is required to

generate the fluorescent umbelliferone which is used as the read out.

As a general remark of caution: when using labelled analogues, one always has to consider that the structural modification might not only have an impact on potency, but also on the biological profile, in particular cell penetration, membrane incorporation, distribution in cellular compartments, and metabolism.

Outlook

Enzymes involved in the production of sphingolipid mediators represent novel drug targets with many potential therapeutic applications that are best studied for cancer and inflammation. Further validation of these targets is required and, in particular, clinical validation of the concepts is missing. The keyword is "shifting the balance" in a disease state back to normal, as strong interference to fight one condition might favour another disorder (for example, SPL inhibition is postulated to have a beneficial effect in autoimmune diseases, but might contribute to tumourigenesis). Therefore, results with knock-out mice might not be predictive because full depletion shifts the balance to the extreme end. However, knock-out animals are useful to investigate potential compensatory mechanisms. It is likely that there are still enzymes and additional interconnections in the complex sphingolipid signalling network to be discovered, for example, enzymes catalysing the potential acylation of S1P to C1P and the hypothesised phospholipase D-like phosphoester cleavage of sphingomyelin to C1P, respectively. The tough challenge for medicinal chemistry is to discover and invent specific, druglike inhibitors of sphingolipid metabolising enzymes to further validate or to dismiss therapeutic concepts.

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