Bioorganic Chemistry of Ceramide

Susanne Brodesser,[a] Peter Sawatzki,[a] and Thomas Kolter*[a]

Keywords: Bioorganic chemistry / Ceramide / Cell surface engineering / Glycolipids / Sphingolipids

Ceramide is the hydrophobic membrane anchor of glycosphingolipids, a vital component of the human skin, and a novel signaling substance. We give an overview of ceramide analogues that have been prepared to address biochemical questions related to the various functions of this lipid. We also discuss possible therapeutic applications.

1. Introduction

Bioorganic chemistry can be understood as a discipline that addresses relevant biochemical problems with synthetic organic chemistry as the key technique.[1] In this microreview, we report on the application of bioorganic concepts to a naturally occurring lipid, ceramide (**1**). We give a short overview of ceramide-mediated biology, and the current problems related to it that cause the demand for structurally modified ceramide analogues. We include selected potential applications of such analogues.

[a] Kekulé-Institut für Organische Chemie und Biochemie der Universität,
Gerhard-Domagk-Str. 1, 53121 Bonn, Germany
Fax: (internat.) + 49-(0)228/737-778
E-mail: tkolter@uni-bonn.de

2. Ceramide: Structure and Function

Ceramide (**1**) consists of a long-chain amino alcohol, D-*erythro*-sphingosine, which is acylated with a fatty acid (Scheme 1). Ceramides of different alkyl chain lengths and degrees of unsaturation and hydroxylation occur in low concentrations in living cells and, in higher concentrations, in the epidermis of human skin. In addition, ceramides are found as the core structure of neutral and of negatively charged glycosphingolipids, and also of a phospholipid, sphingomyelin (**2**, Scheme 1).[2,3] In sphingomyelin, phosphorylcholine is linked to the 1-position of ceramide, whereas the hydrophilic head group in glycosphingolipids is represented by various oligosaccharide residues. Glycosphingolipids, which contain negatively charged sialic acids, are called gangliosides. An example is ganglioside GM1, the receptor for cholera toxin on intestinal cells (**3**, Scheme 1). Like other lipids, glycosphingolipids are integrated into

Susanne Brodesser, born 1975, studied chemistry at the University of Bonn. In 2002, she received her diploma with a work on the synthesis of potential suicide inhibitors of dihydroceramide desaturase. In her Ph.D. thesis, she is currently working on the isolation and characterisation of dihydroceramide desaturase and human epidermal sphingolipid-4-hydroxylase.

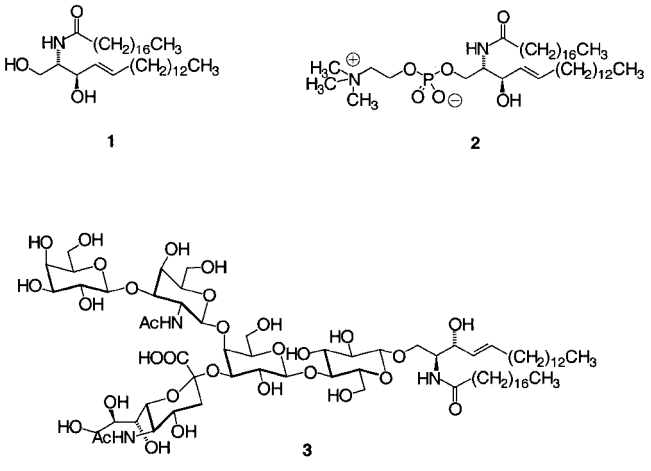
Peter Sawatzki, born in Stolp (now: Slupsk), Poland, in 1968, started to study Chemistry at the Rheinische Friedrich-Wilhelms-University of Bonn in 1990. In 1997 he obtained his diploma degree in the group of Prof. Dr. K. Sandhoff for his studies on the total synthesis of ganglioside GM2. He finished his Ph.D. degree in February 2003. His main research interests are the design and synthesis of biologically active sphingolipid derivatives and their application to cultured cells.

Thomas Kolter was born in 1963 and studied Chemistry at the University of Bonn from 1984 to 1990. He completed his Ph.D. under the supervision of Prof. Dr. Athanassios Giannis on the development of peptidomimetics in 1993. From 1993 to 1998 he joined the group of Prof. Dr. Konrad Sandhoff as a postdoctoral fellow. In 2002, he finished his Habilitation for Organic Chemistry and Biochemistry at the Kekulé-Institut. His research interests are in the area of lipid and glycolipid chemistry and biochemistry. His particular interests are the application of Organic Chemistry to investigate and to influence glycolipid metabolism.

MICROREVIEWS: This feature introduces the readers to the authors' research through a concise overview of the selected topic. Reference to important work from others in the field is included.

 DOI: 10.1002/ejoc.200200518

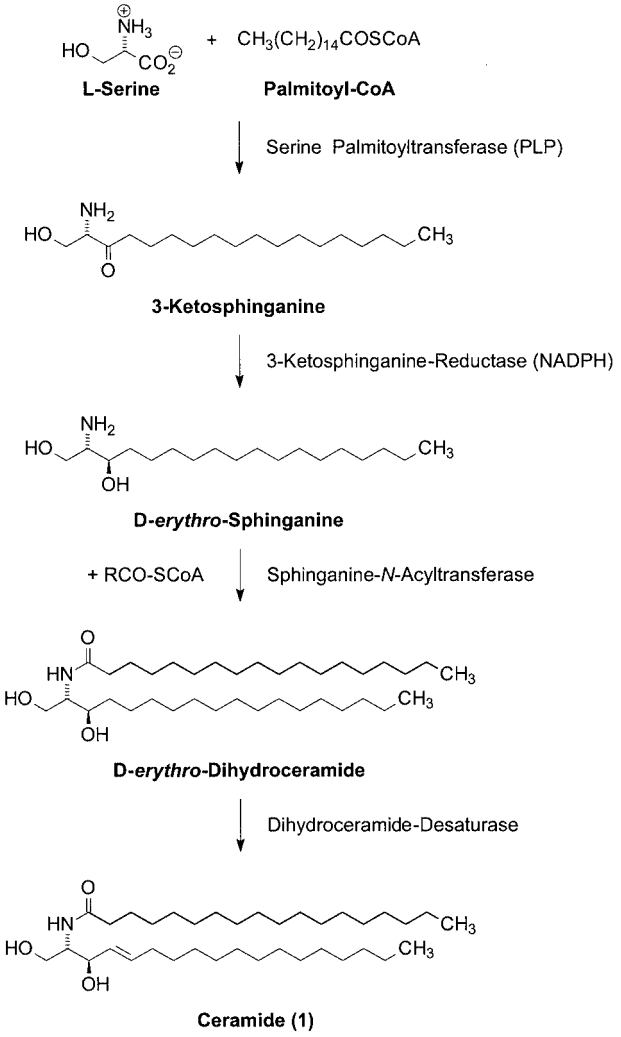
membranes. They show an extracytosolic orientation of their hydrophilic carbohydrate head groups and are primarily localised in the outer leaflet of the plasma membrane. They are, however, also part of intracellular membranes: during biosynthesis, they are embedded in the membranes of the endoplasmic reticulum and the Golgi apparatus. During their transport, they are part of exocytotic and endocytotic vesicles, and during their degradation they become part of lysosomal membranes. In contrast to many phospholipids, glycosphingolipids are expressed in a cell-type-specific manner and are often only slowly metabolized. Variations in type, number, and linkage of carbohydrate residues within the oligosaccharide chains give rise to complex glycosphingolipid patterns, which change during biological processes such as cell growth, differentiation, viral and oncogenic transformation. The function of these complex glycolipid patterns on cellular surfaces is largely unclear. It has been demonstrated for gangliosides of the nervous system that they are biosynthetically formed in a combinatorial manner.[4] As far as we know, the basic functions of glycosphingolipids are twofold: they can bind to soluble or cell-bound proteins outside the cell, or can interact with substances within the same membrane. As ligands of lectins outside the cell, they mediate recognition events, e.g. as blood-group substances, or as binding sites for bacteria, viruses, and toxins. Within cellular membranes they form microdomains (“rafts”; “detergent-insoluble glycolipid/cholesterol-rich domains”, “DIGS”), which are the physiological surroundings of many membrane proteins.[5,6] As individual molecules, or as components of microdomains, they can regulate the activity of receptors in the plasma membrane, and thus signal transduction. In addition, they protect biological membranes from uncontrolled fusion and serve as precursors for bioactive metabolites such as ceramide and sphingosine-1-phosphate. α-Galactosylceramides, initially isolated from a marine sponge, are potent stimulators of the mammalian immune system.[9] In mammals, sphingolipids are essential for embryogenesis and for the permeability barrier of the skin. Investigation of their function is often complicated: while even complex systems such as cultured cells can survive without sphingolipids, they are required for the development of multicellular organisms.[7] Owing to their various biological functions, sphingolipids and glycosphingolipids have been discussed as therapeutic agents and therapeutic targets.[8,9]



Scheme 1. Structures of ceramide (**1**), sphingomyelin (**2**) and ganglioside GM1 (**3**), the receptor for cholera toxin on intestinal cells

2.1. Metabolism

The formation of ceramide is catalysed by membrane-bound enzymes on the cytosolic leaflet of the endoplasmic reticulum (ER).[10] Starting from the amino acid L-serine and two molecules of the palmitoyl-coenzyme A, dihydroceramide is formed in three steps (Scheme 2). This *N*-acyl-2-aminoalkyl-1,3-diol (*N*-acylsphinganine) is dehydrogenated to ceramide with a 4,5-*trans*-double bond by a dihydroceramide desaturase. At the membranes of the Golgi apparatus, hydrophilic head groups are attached to ceramide leading to sphingomyelin, galactosylceramide, glucosylceramide, and higher glycosphingolipids, which are synthesised by the stepwise addition of monosaccharides to glucosylceramide. Their biosynthesis is coupled to exocytotic vesicle flow to the plasma membrane.



Scheme 2

The constitutive degradation of sphingolipids occurs in both the endosomes and the lysosomes.[2,11] Parts of the plasma membrane are endocytosed and transported via the endosomal to the lysosomal compartment. Hydrolytic enzymes cleave the carbohydrate residues of glycolipids sequentially. Many glycosphingolipids, and also ceramide, require the additional presence of activator proteins and negatively charged lysosomal lipids for degradation.[12] In humans, inherited defects of glycosphingolipid and sphingolipid catabolism give rise to lysosomal storage diseases, the sphingolipidoses.[13]

2.2. Signal Transduction

Ceramide has been recognized as a signaling substance, which can be released from sphingomyelin in response to extracellular (e.g. tumour necrosis factor α, platelet-derived growth factor) and intracellular stimuli.[14] Ceramide modulates multiple downstream targets and causes antimitogenic effects such as cell-cycle arrest, cell differentiation, and apoptosis in most cell types. There is some evidence that ceramide-binding proteins are involved in this signaling pathway, but their identity and physiological relevance has not always been unequivocally demonstrated to date. According to one hypothesis, ceramide signaling is mediated by interaction of its head group with cysteine-rich domains of protein kinases.[15] Several other models, however, also exist, which operate in part without the contribution of binding proteins.[16] It is possible that some of the observed effects are mediated by ceramide-rich domains within the plasma membrane and not by single lipid molecules.[17] In this case, sphingomyelin hydrolysis and concomitant formation of ceramide would lead to an alteration of membrane properties and in this way induce one or more signal processes. The physiological role of these alternatives is not easily addressed experimentally. Furthermore, the metabolic coupling of ceramide to other signalling substances such as sphingosine-1-phosphate or diacylglycerol prevents clear conclusions from cell-culture studies on the molecular details of this pathway.[18] For example, ceramide can be hydrolysed to sphingosine, which in turn can be phosphorylated at the 1-position to sphingosine-1-phosphate. In contrast to ceramide, sphingosine-1-phosphate behaves as a mitogenic regulator in most cell types. Extracellular receptors for sphingosine-1-phosphate, of the EDG family, have been characterised, but it is currently unclear how cytosolically formed sphingosine-1-phosphate is transported to the outside of the cell and how its intracellular effects are mediated.[19] The ratio of the ceramide and sphingosine-1-phosphate concentrations can be viewed as a rheostat that directs the cell cycle towards differentiation or cell division.[19] Ceramide can also be transformed to ceramide-1-phosphate and thus be converted from a differentiation signal into a mitogen, which can stimulate cell division and DNA synthesis.[20]

3. Bioorganic Concepts

Several biochemical problems have been addressed by chemical modification of the ceramide structure. Our knowledge of sphingolipid metabolism and function is much less well advanced than it is for other metabolic pathways. This is in part due to the amphiphilic properties of these lipids, which can cause severe complications and artifacts. Especially in this field, chemical approaches can be valuable extensions of the powerful techniques of molecular and cell biology. Some key questions that can be addressed by this approach are: What is the identity of the binding proteins and enzymes in ceramide signalling and metabolism? What reaction mechanisms of ceramide metabolism are catalysed by enzymes? What structural elements are required for the molecular recognition of ceramides by binding proteins? What does the protein-bound conformation of these hydrophobic substances look like? Can the affinity and selectivity of binding be enhanced? Can derivatives that show sufficient metabolic stability be prepared? Can ceramide function be mimicked or antagonized by substances with nonlipid structures (lipid mimetics)? In addition to the analysis of the molecular details of this pathway, bioorganic chemistry is able to provide ideas and substances for conceptual new therapeutics that are summarised in Section 9. Although we focus on structural modifications of ceramide, the structural modification of other sphingolipids such as sphingosine,[21] sphingosine-1-phosphate,[22−24] ceramide-1-phosphate,[25] glucosylceramide,[26] or sphingomyelin,[27−29] as well as the development of enzyme inhibitors, [30−32] are closely related to this field.

4. Structural Elements in Signal Transduction

4.1. Alkyl Chain Length and Stereochemistry

Many, but not all, effects of extracellular agents that lead to the formation of endogenous ceramide can be imitated by the exogenous addition of ceramide derivatives. Because of their hydrophobicity, ceramides of native structure are not easily delivered to cell cultures or enzyme assays.[33] Current methods for the application of native ceramides and their long-chain analogues to cells involve the addition to the cell culture medium of either a complex of bovine serum albumin and ceramide or liposomes containing the lipid.[34] Also a solvent mixture of ethanol/dodecane, 98:2 (v/v) is able to disperse long-chain ceramides into aqueous solution and deliver it to cells.[35] In order to increase their water solubility, ceramides of native structure are often replaced by synthetic derivatives with short-chain fatty acids like *N*-acetyl- or *N*-hexanoylsphingosine. The ceramide analogues designed and investigated by us in cell culture experiments (see Section 8) show octanol/water partition coefficients[36] similar to that of acetylsphingosine, log P = 4.65.[37] They are applied as a 10 mM stock solution in methanol with a final concentration of 1−100 μM in the culture medium. In most cases, where nonlabelled ceramide analogues are applied, their mode of delivery, their intracellular concentration, e.g. in terms of analogue per mol phospholipid, their subcellular distribution, and their metabolic fate are not known.

Besides those with modified alkyl chain length, synthetic derivatives with different stereochemistry and 4-hydroxylation are also valuable in the molecular characterisation of ceramide-recognition events. For example, the activation of protein phosphatases-1 and -2A by ceramides was demonstrated to be stereospecific.[38] Derivatives bearing radioisotopes at suitable positions within the ceramide structure are of considerable importance.[39−41]

4.2. Significance of the 4,5-trans-Double Bond

In contrast to *N*-acetylsphingosine, its saturated derivative *N*-acetylsphinganine is much less able to induce the signaling effects of endogenous ceramide.[42] Therefore, the requirement of the 4,5-*trans*-double bond for the signaling properties of ceramide has been demonstrated with the aid of synthetic analogues of the native lipid. The reader should, however, be aware, that administration of short-chain ceramides can lead to enhanced levels of long-chain, endogenous ceramides.[43] Also, inhibition of sphingomyelin biosynthesis by short-chain ceramides has been considered as an artificial cause of apoptosis.[44] As an additional example, ceramide-mediated fusion of the Semliki Forest virus with target membranes requires the presence of the 4,5-*trans*-double bond. This has been demonstrated with the aid of another synthetic derivative, an enantiomerically pure D-*erythro*-ceramide analogue with a C-5−C-6 *trans*-double bond.[45]

For the analysis of structural elements required for ceramide-mediated signal transduction, a ceramide library might be useful as a source of biologically active ceramide analogues. A combinatorial ceramide library has been prepared and investigated by cell-based screens of apoptosis and signal transduction.[46] Under optimised conditions, sphingosine-like core structures were acylated with a solid-phase reagent in THF at room temperature. A nitrophenol ester on polystyrene turned out to be the most suitable acyl donor. In this way, a library of 528 compounds using 16 sphingosine analogues and 33 acyl groups has been generated. The biological activities of members of this library were tested in two cell-based assays. The agonistic effects on NF-κB signalling were investigated in an NF-κB reporter gene (luciferase) assay using C6 glioma cells. An activation of NF-κB was only observed for a group of ceramides with a β-galactose head group, when 10-μM concentrations of the compound were added to the cells. None of the natural ceramides or their membrane-permeable derivatives showed any effect in this assay. An apoptosis screen was carried out with U937 leukemic cells. In this assay, the most active members of the library had IC_{50} values in the micromolar range and an optimum sum of carbon chain length of 18. Although the stereochemistry of the head group turned out not to be critical, the C-4−C-5 double bond in the ceramide core structure seems to be important.

4.3. Other Structural Elements in Signal Transduction

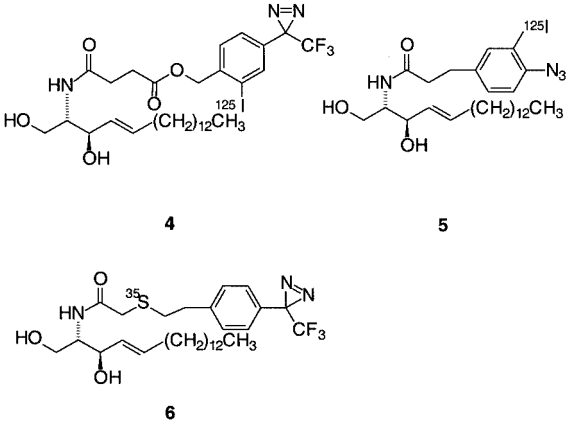
The analysis of structural elements usually occurs in whole cells by the detection of an apoptotic response, or changes in growth behaviour. Therefore, the results are often not satisfactory from the molecular point of view. Nevertheless, with advanced understanding of the molecular details of the pathway, the structural modifications will become important for the characterisation of the downstream targets in this pathway. Replacement of the amide group of ceramide by an amine [47] or a thioacetyl group,[48,49] as well as of the 1-OH group by alkyl residues [48,49] indicate that the antiproliferative and proapoptotic properties are retained in those derivatives, at least in the analysed systems, U937, HaCaT, and Molt4 cells. Even acylated serinol derivatives were reported to induce apoptosis in neuroblastoma cells.[50] Ceramide analogues with a fluoro substituent in the 3-position of the sphingosine moiety have been prepared and analysed as inhibitors of protein kinase C.[51] Compounds of this type showed apoptogenic properties in three different cell lines.[52] Different short-chain ceramides with an additional 4-OH group in the D-*ribo* configuration of naturally occurring phytoceramides induce apoptosis with higher potency than ceramides. Again, substitution of the carbonyl oxygen atom by sulfur did not influence cytotoxicity.[53]

5. Identification of Ceramide-Binding Proteins

Ceramide can be bound by receptors involved in signal transduction and by enzymes that metabolize the lipid. The identities of the receptors and of the enzyme introducing the 4,5-double bond into the sphingosine moiety have not been unambiguously demonstrated to date. These problems can be addressed by chemically modified analogues. Unknown receptors for a distinct ligand can be identified by labelling techniques, if the ligand bears a suitable label, e.g. a radioisotope or a fluorophore. Also, in cases where the receptors are already known and available in pure or enriched form, the binding site for the ligand can be analysed in this way on the molecular level. Labelling techniques replace reversible interactions between two components by a stable covalent bond.[54] Crosslinking techniques are also based on this principle. The formation of a covalent bond can be achieved by chemical reactivity inherent to the ligand (affinity labelling), by photochemical (photoaffinity labelling[55]) or enzymatic (suicide inhibition[56]) activation. Photoaffinity labelling requires the modification of the natural ligand by the incorporation of a chemically inert but photochemically labile functionality. On irradiation, the photolabile group is converted into a species of very high chemical reactivity such as a nitrene, a carbene, or a carbenium ion, which reacts with adjacent residues of the receptor with concomitant formation of a covalent bond. This approach has been applied to the identification of ceramide-binding proteins.

To identify downstream signaling targets of ceramide, ceramide analogue **4** (Scheme 3), with a (trifluoromethyl)diazirine as photoactivatable carbene precursor, has been synthesised. The photoaffinity ligand was radioiodinated by halogen/metal exchange.[57] This approach has identified

protein kinase c-Raf as a specific molecular target for interleukin 1b stimulated ceramide formation.[58] Photoactivatable derivatives of ceramide, glucosylceramide, and sphingomyelin have been synthesised in an attempt to identify compartment-specific proteins involved in sphingolipid sorting or metabolism.[59] In this case, an aryl azide moiety serves as a photoactivatable nitrene precursor. In HT29 and BHK cells, the ceramide analogue **5** was efficiently internalized at low temperature (4 °C) and predominantly metabolized to the corresponding sphingomyelin analogue, but small amounts of glucosylceramide and galactosylceramide were also formed. Incubation of cells with the photoactivatable analogues at 4 °C, followed by irradiation, led to the association of the corresponding sphingolipid analogues with a specific subset of proteins. The protein-labelling pattern of ceramide differed from that of glucosylceramide. Moreover, most of the proteins labeled by photoreactive sphingomyelin seemed to be detergent-insoluble, which indicates a location in sphingolipid-rich microdomains at the plasma membrane. These results emphasise the role of metabolism in labelling of cellular proteins in vivo and the need for suitable sphingolipid analogues of enhanced metabolic stability. Ceramide analogue **6** with a (trifluormethyl)-diazirine moiety and ^{35}S as radioisotope has been prepared using thiourea as the ^{35}S source.[60] **6** has been used to identify binding proteins after affinity chromatography of the proteins of SH-SY5Y neuroblastoma cells. Cytoskeletal and stress proteins with hydrophobic domains were labeled by this derivative. A serious problem inherent in the application of this labelling approach to the ceramide field is that hydrophobic interactions can outweigh the specific interactions between ceramide ligand and receptor. Proteins denature with time so that even water-soluble proteins expose hydrophobic surfaces. These might bind nonspecifically to the ligand and be detected by the extremely sensitive radioisotope detection.[60] This is especially likely when the labelling reaction is performed outside the cell, where partially denatured proteins are not continuously removed by the cellular degradation machinery. Although independent methods are applied to confirm results from photoaffinity labelling, this hydrophobicity problem limits the success of this approach in the ceramide field.



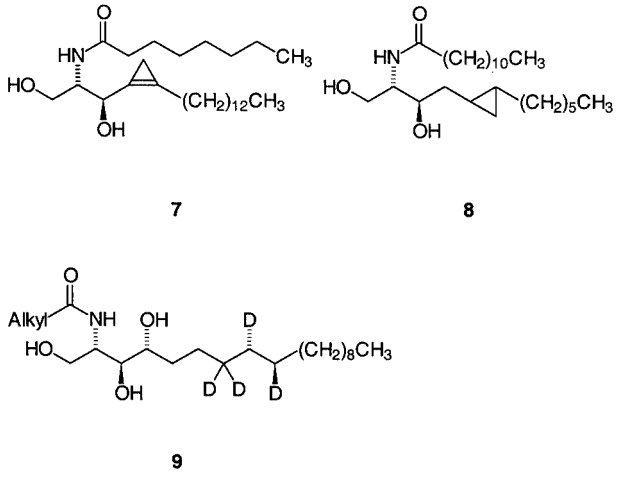
Scheme 3

5.1. Dihydroceramide Desaturase

At least in mammals, a dihydroceramide desaturase catalyzes the introduction of the 4,5-*trans*-double bond into the sphinganine moiety of dihydroceramide.[61] To date, it has not been possible to isolate this protein or to clone its cDNA. Very recently, a new family of sphinganine Δ^4-desaturases and hydroxylases from animals, plants, and fungi has been identified and characterised by expression in *Saccharomyces cerevisiae*.[62]

Based on experiments with fatty acyl desaturases, it can be assumed that the first and rate-determining step of the dihydroceramide desaturase-catalysed reaction is the homolytic cleavage of a C−H bond by a non-heme oxo diiron species.[63,64] Dihydroceramide derivatives, which were regiospecifically dideuterated at C-4 or C-5 of the sphinganine backbone, have been synthesised. Determination of the kinetic isotope effects clearly indicates that initial oxidative attack of the desaturase occurs at C-4 and not at C-5 of dihydroceramide.[65] Since the 4,5-*trans*-double bond is necessary for the signaling properties of ceramide, inhibitors of dihydroceramide desaturase are valuable for investigating ceramide-mediated signal transduction. Two dihydroceramide analogues with different alkyl chain lengths have been prepared, in which the 3-hydroxy group has been replaced by a fluorine atom. They were investigated as potential inhibitors of the desaturase, but showed only a slight inhibition of enzyme activity.[66] Irreversible inhibitors would allow the identification of the protein and the determination of the active site of the enzyme.

The concept of affinity labelling has been applied to this enzyme by a ligand that eventually operates by covalent modification: substrate analogue **7** (Scheme 4) with a cyclopropene ring caused a dose-dependent inhibition with half-maximal effect at concentrations around 25 μM.[67] Since thiol groups can be alkylated by addition to the double bond of cyclopropenes, the inhibition is eventually caused by reaction of the cyclopropene ring with a cysteine residue of the enzyme.[68]



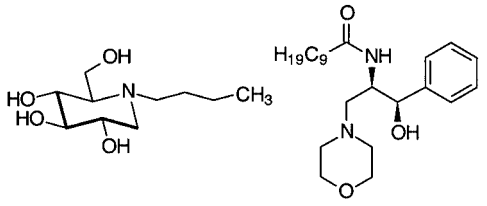
Scheme 4

As putative suicide inhibitors of the dihydroceramide desaturase, we prepared several substrate analogues, among them **8** with a cyclopropane ring at C-5 and C-6 of the sphinganine backbone. After enzymatic generation of a radical centre or a positive charge in position 4 of the sphinganine moiety, the reactive intermediate should be able to bind covalently to an amino acid side chain of the desaturase, inhibit the enzyme irreversibly, and lead to the covalent modification of the protein. **8** was investigated at a concentration of 750 μM in an in vitro assay for the desaturase from rat liver microsomes. As expected for irreversible inhibitors, **8** showed a time-dependent increasing inhibition of the enzyme activity and is currently prepared in radiolabeled form to demonstrate the covalent modification of the enzyme.[69]

A Δ^8-sphingolipid desaturase of higher plants is one of the desaturases that yield a mixture of (*E*)- and (*Z*)-olefins by *syn* elimination of two vicinal hydrogen atoms. Stereospecifically dideuterated palmitic acids have been synthesised[70] and biosynthetically incorporated into sphingolipids of a transgenic yeast strain, whose fatty acid biosynthesis was inhibited by cerulenin.[71] Ceramide **9** has been used to obtain mechanistic information on the reaction catalysed by this desaturase. The kinetic isotope effects of the hydrogen abstraction at C-8 and C-9 indicate that the initial C−H bond cleavage leading to the (*E*) isomer occurs at C-8 whereas the (*Z*) isomer is generated by abstraction of a primary hydrogen atom at C-9. Since each C−H bond cleavage, leading to either the (*E*) or the (*Z*) isomer, occurs at different methylene groups, a common intermediate (radical) can be excluded. It seems that the Δ^8-sphingolipid desaturase is able to convert two different substrate conformations, *anti* and *gauche,* independently from each other into the (*E*)- or (*Z*)-olefin.[71]

6. Enzyme Inhibition

Several enzymes utilize ceramide as a substrate, for example glucosylceramide-synthase (GlcCer-synthase), galactosylceramide-synthase, sphingomyelin-synthase, ceramide-1-phosphate-synthase, different ceramidases, and inositol-phosphorylceramide-synthase of lower eukaryotes. Dihydroceramide desaturase and putative hydroxylases of mammalian skin accept dihydroceramide as substrates. Some of these enzymes serve as therapeutic targets.[2] Inhibitors of these enzymes might be generated by modification of the ceramide structure, but most of them have been derived from the pool of natural compounds.[2] Most advanced is the inhibition of GlcCer-synthase as a target for substrate deprivation therapy of sphingolipidoses, and also of bacterial infections (see Sections 9.2 and 9.3). To the best of our knowledge, this is the only example in advanced clinical study of a glycosyltransferase for which an inhibitor (**10**, Scheme 5) has been developed. Two classes of compounds are the most actively investigated as inhibitors of GlcCer-synthase: imino sugar derivatives and derivatives of D-*threo*-(1*R*,2*R*)-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP, **11,** Scheme 5).[72] *N*-Butyldeoxynojirimycin (**10**), and also the derivative with D-*galacto* configuration inhibits the ceramide-specific glucosyltransferase in vitro and in vivo. Enzyme kinetics demonstrated noncompetitive inhibition with respect to the glucosyl donor, and, surprisingly, competitive inhibition with respect to ceramide. This indicates that, in this case, **10** acts not as a transition-state analogue of glycosyl transfer, but as a ceramide mimic. Attempts have been made to rationalize these data for transferase inhibition: energy-minimised molecular models of **10** and ceramide **1** reveal structural homology between the three chiral centres and the *N*-alkyl chain of **10**, and both the *trans*-alkenyl and the *N*-acyl chain of ceramide.[73] The enzyme kinetics of inhibition are in contrast to the inhibition of ceramide glucosyltransferase by analogues like PDMP (**11**), which act by mixed competition against ceramide (K_i = 0.7 μm) and are noncompetitive for the nucleotide sugar donor.[74]



10 **11**

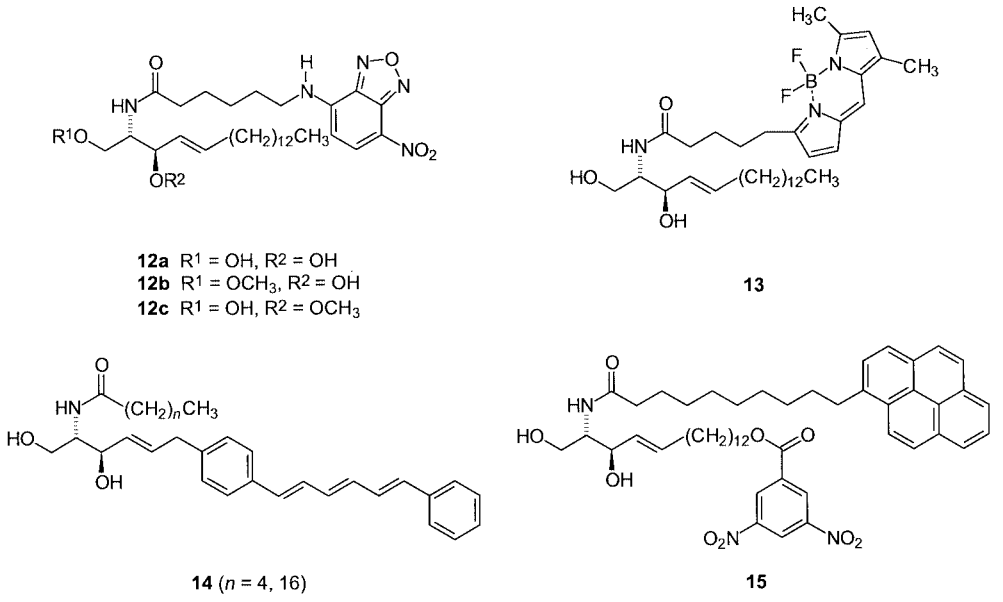
Scheme 5

The structural requirement for inhibition of another enzyme, mitochondrial ceramidase, was investigated with the aid of synthetic derivatives like all stereoisomers of ceramide, a urea-modified ceramide, *cis*-D-*erythro*-ceramide, *N*-methylceramide, 1-*O*-, and 3-*O*-methylceramide, 3-oxoceramide, ceramine, and other derivatives. Both OH groups, the amide nitrogen atom, and the 4,5-*trans*-double bond turned out to be required for inhibition.[75] For a more detailed discussion of other inhibitors of sphingolipid metabolism, compare ref.[2]

7. Other Structural Modifications of Ceramide

7.1. Intracellular Membrane Traffic

Fluorescent ceramide analogues bearing a short-chain fluorescent fatty acid have been used to investigate the membrane lipid traffic and concomitant metabolism in animal cells.[76] In contrast to native ceramides, they are integrated into cellular membranes by spontaneous lipid transfer from an exogenous source, and their intracellular distribution can then be observed in living cells by high-resolution fluorescence microscopy. Many investigations relied on C_6-NBD-ceramide **12a** (NBD = 4-nitro-2,1,3-benzoxadiazole, Scheme 6).[34] Synthesis of the anabolically stable derivative **12b** and of **12c** allowed the conclusion that staining of the Golgi apparatus with **12a** and **12c** requires metabolism.[77] An analogue with the BODIPY ("borondipyrromethene difluoride") fluorophore **13** shows improved



Scheme 6

properties in terms of higher fluorescence yield, higher photostability, and better membrane anchoring due to a lower polarity than **12a**.[78] In addition, lipids labeled with this fluorophore acid exhibit a shift in their fluorescence emission maximum from green to red with increasing concentration in membranes, allowing the estimation of their concentration in a particular organelle.[79]

The conversion of dihydroceramides modified with the NBD fluorophore to higher glycolipids and their respective subcellular localization are dependent on the stereochemistry of the sphingosine backbone.[80] Also synthesis and transport properties of **14** bearing a diphenylhexatrienyl fluorophore with *cis* and *trans* configuration of the 4,5-double bond have been described.[81]

Although fluorescent sphingolipid analogues have been widely used in cell biology, we should mention that their transport properties can be entirely different from that of native or radioactively labelled sphingolipids. For example, natural ceramide is unable to escape from the lysosome, in contrast to a fluorescent analogue.[82] A nonfluorescent ceramide analogue, **15**, which becomes fluorescent on hydrolysis of the amide bond, has been prepared and might become valuable in the screening of ceramidase inhibitors.[83]

7.2. Metabolism

Ceramide analogues are metabolized to neosphingomyelin, neoglycolipids, and also to degradation products after cleavage of the amide bond. As summarised in Sections 7.1 and 8, alteration in the structure of the lipid can lead to differences in metabolism compared to native ceramide as demonstrated by radiolabeled metabolic precursors such as L-serine and sphinganine. It appears that neoglucosylceramides and neosphingomyelins are more readily formed from ceramide analogues than higher glycolipids. This might be due to the lower specificity of GlcCer- and sphingomyelin-synthase for the lipid portion, but also to the different subcellular localization of their active sites: ceramides should easily reach glucosylceramide synthase on the cytosolic leaflet and sphingomyelin synthase on the luminal leaflet of the Golgi membrane. Access to the active sites of the sequentially acting "higher" glycosyltransferases (Scheme 10)[2,4] on the luminal site of the Golgi apparatus requires transversal membrane translocation ("flip flop") of neoglucosylceramide through the Golgi membrane. This transport is thought to be protein-mediated and might become rate-determining.

7.3. Additional Modifications

After aryl-fused sphingosine derivatives with anti-inflammatory properties had been prepared and investigated as inhibitors of protein kinase C,[84,85] ceramide analogues have also been prepared that have the C-4−C-5 bond of the long-chain base as part of an aromatic or heteroaromatic system.[86] 1-(Methylthio)dihydroceramide reduces de novo ceramide biosynthesis in concentrations of 10 μM, presumably by activation of sphingosine kinase and subsequently enhanced sphingolipid degradation.[87] Short-chain ceramides with aromatic rings were synthesised and their effect on the growth of hippocampal neurons was investigated.[88,89] In these cells, the biosynthesis of glucosylceramide appears to be required for axonal growth. Homoceramides bearing an additional methylene group at the 1-position of sphingosine were prepared with different alkyl chain lengths and also with a phenyl ring within the sphingosine moiety. The effects of target compounds on the morphology of cultured hippocampal neurons have also been investigated. In contrast to short-chain ceramide analogues, no significant effect was observed and no metabolic transformations of the homoceramides were detected in this system.[90]

The molecular details of ceramide function in the permeability barrier of the human skin are largely unknown. Modified ceramides bearing an additional ω-carboxyl group within the fatty acid moiety have been prepared for the immobilization of ceramide analogues on silica-gel particles to predict skin permeability of chemicals by HPLC.[91]

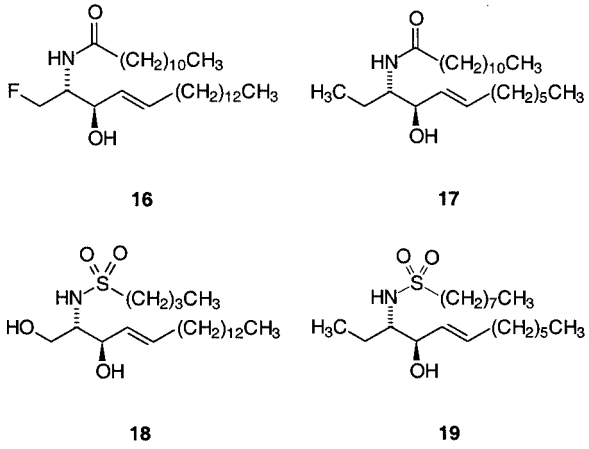
8. Application of Ceramide Analogues to Complex Systems

An important question in bioorganic chemistry is that of the appropriate assay system.[1] We designed and investigated compounds for their influence on sphingolipid metabolism in cultured cells, especially in primary cultured neurons. These cells are rich in complex gangliosides. In contrast to the current paradigm in medicinal chemistry, we applied few substances of defined structure to complex cell culture systems. Primary cell cultures are artificial compared to multicellular organisms,[7] but are sufficiently complex compared to cell lines or enzyme assays. As will become clear, some of the observed results would not have been obtained in enzyme assays.

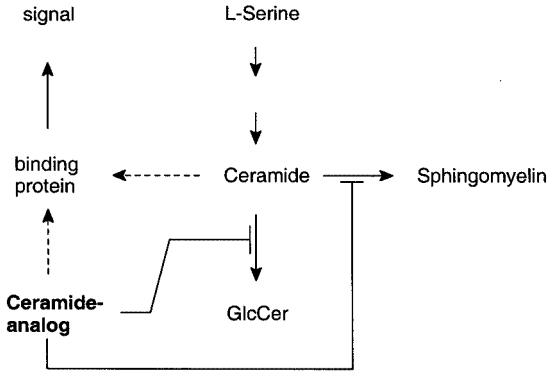
8.1. Metabolic Stability

Ceramide derivatives, when added to cultured cells, can be metabolized to a variety of products[59,60,92] by biosynthetic enzymes, leading to analogues of glycosphingolipids and sphingomyelin, by catabolic enzymes, leading to analogues of sphingosine or sphingosine-1-phosphate, or by enzymes that are not involved in metabolism of endogenous sphingolipids. This additional metabolic coupling to other signaling substances has complicated the functional analysis of ceramide. Although inhibitors are available for ceramide-metabolizing enzymes,[2] a valuable contribution of bioorganic chemistry would be to provide metabolically stable ceramide analogues as analytical, and, eventually, pharmacological tools. It has been demonstrated before that ceramides with nonnatural L-*erythro* configuration are not metabolized to the corresponding glucosylceramide and sphingomyelin species, while the L-*threo*-configured lipid is metabolized to sphingomyelin, but not to glucosylceramide.[93] Our strategy in the preparation of ceramide analogues of enhanced metabolic stability was the bioisosteric replacement[94] at positions within the ceramide structure, where the major metabolic reactions occur. If the signaling properties of ceramide analogues modified in this way are preserved, analysis of the downstream events of ceramide signaling should be facilitated.

We decided to use the replacement of the 1-OH group by fluorine (see ref.[95] for a review of this replacement) and by a methyl group, leading to **16** and **17** as target compounds (Scheme 7). In both cases, the nucleophilic attack of the 1-OH group on electrophilic head-group donors such as UDP-glucose or phosphatidylcholine, is no longer possible. The compounds have been prepared from D-galactose and L-serine, respectively.[96,98] According to Scheme 8, exogenously added ceramide analogues have to be expected to influence levels of endogenous ceramide.[43] It is not a priori clear whether the signaling effects eventually observed are due to the synthetic derivative or to (locally) enhanced concentrations of the natural compound. Therefore, we investigated the effect of synthetic derivatives on sphingolipid metabolism. **16** inhibited sphingomyelin and glucosylceramide formation in cultured murine neurons, but only at high concentrations (100 μM) in the culture medium,[98] and showed no proapoptotic properties in HaCaT cells, although about 30% of **16** was taken up by the cells (S. Barz, C. Geilen, personal communication).



Scheme 7



Scheme 8

Since it is not clear how phytoceramides (D-*ribo*-2-*N*-acylamino-1,3,4-alkanetriols) are biosynthetically formed in the human skin, we prepared **17** in radiolabeled form with a ^{14}C isotope at C-1 of the fatty acid moiety. Investigation in cultured human keratinocytes indicates that **17** is nearly completely metabolized with concomitant release of the radiolabeled fatty acid within 24 h of cell culture (O. Macheleidt, personal communication). At present, we have to conclude that effects observed after administration of compounds like **16** or **17** to cultured cells might be the sum of the effects of the exogenously applied analogue and the corresponding sphingosine derivative formed by catabolism.

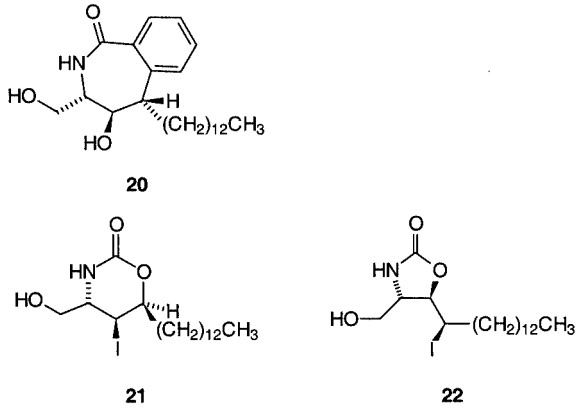
Ceramide can be degraded to sphingosine by ceramidases of different subcellular localization. The replacement of an amide moiety by a sulfonamide group is an approach to

enhance the metabolic stability of the parent compound.[97] Therefore, we have prepared catabolically stable compound **18**. Inhibition of glucosylceramide and sphingomyelin formation in cultured murine cerebellar neurons occurs in a concentration-dependent manner, but, as with **16**, high concentrations of about 100 μM in the culture medium are required for efficient inhibition.[98] Since **18** inhibited recombinant human acid ceramidase only slightly at 50 μM concentration in a micellar assay (H. Schulze, personal communication), it appears that the sulfonamide moiety is not a suitable mimic of the amide group in the transition state of the amide-cleavage reaction catalysed by acid ceramidase. The limited metabolic stability of **17** led us to combine the structural elements in **17** and **18**. Accordingly, we prepared **19** in D-*erythro* and L-*threo* configuration with and without the 4,5-*trans*-double bond. Addition of low concentrations (less than 10 μM) to the medium of cultured neurons led to a drastic decrease of sphingomyelin and glycosphingolipid formation.[98] We conclude that **19** either inhibits GlcCer- and sphingomyelin-synthase or activates an undefined control element that leads to the down-regulation of one or both proteins. Apparently, **19** cannot be removed by the cells by the major metabolic routes.

8.2. Conformational Restriction: Inhibition of Glycosyltransferases

The restriction of conformationally flexible ligands is an appropriate strategy in drug development, which has been particularly successful in the development of peptidomimetics.[99] In some cases, this strategy has also been applied to lipids.[100] We assumed that major questions within ceramide metabolism and function can be addressed by this approach, which aims at the development of analogues with enhanced affinity, selectivity, and metabolic stability.[100] To date, few compounds with remote structural similarity to **1** have been prepared as conformationally rigid ceramide analogues. Isoquinoline derivatives have been reported to slightly modulate protein phosphatase 2A,[101] and uracil derivatives show moderate effects in terms of toxicity and anti-tumour activity in vitro and in vivo.[102]

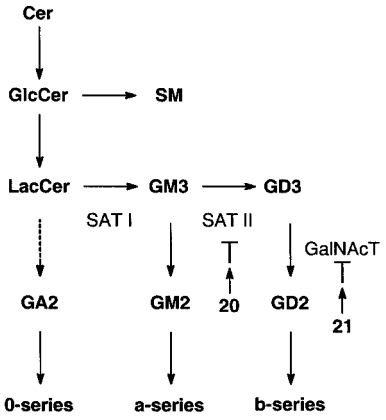
Ceramide-metabolizing enzymes have also to recognize and to modify the head group of this lipid, so we were especially interested in the conformational restriction of the head group. Since the target compounds should be investigated in primary cultured cells derived from the brain of mice, combinatorial strategies were not considered. Instead of this, we went back to the concept of series design[103] and designed ceramide analogues that represent remote points within the conformational space available to the head group of this lipid. Our group has synthesised several derivatives according to this strategy. Three members of the series are shown in Scheme 9. Since they lack the 4,5-*trans*-double bond usually required for signal transduction, interference with this pathway is not necessarily to be expected. We investigated the influence of the target compounds on the incorporation of biosynthetic precursors into sphingolipids of primary cultured murine cerebellar granule cells, which are rich in complex gangliosides.[104–106] Labelling with L-[3-^{14}C]serine reflects de novo biosynthesis, while labelling with [^{14}C]galactose additionally reflects the contribution of the salvage pathway – which predominates in neurons – and, eventually, also metabolism of the exogenously added ceramide analogue.



Scheme 9

L-[3-^{14}C]Serine labelling of sphingolipids in the presence of different concentrations (10–100 μm) of **20** (Scheme 9) led to a concentration-dependent decrease of sphingomyelin and glucosylceramide levels while labelling of ceramide increases. Unexpectedly, [^{14}C]galactose labelling indicated inhibition of glycosyltransferases that do not accept ceramide as substrate: **20** led to the inhibition of sialyltransferase II (SAT II, GD3-synthase) at low concentrations (10 μM) in the culture medium and, at higher concentrations, also of GalNAc-transferase (GM2-synthase, Scheme 10). Even more exciting results were obtained with **21**. As determined by [^{14}C]galactose labelling, 10-μM concentrations in the culture medium caused drastic elevations, up to 40-fold, in the levels of LacCer, GM3, and GD3, which indicate inhibition of GM2-synthase. These findings were confirmed by metabolic labelling with L-[3-^{14}C]serine and [4,5-^{3}H$_2$]sphinganine. The compound with opposite stereochemistry at the carbon atom bearing the alkyl chain (C-6) had nearly no effect, indicating the specificity of the molecular interaction. We investigated **21** for its influence on murine GM2-synthase recombinantly expressed in insect cells.[107] Since no inhibition was detected, the effect of **21** has to be indirect and to be mediated by the cellular surroundings. Because different ceramide analogues target different transferases, we exclude interference of **21** with membrane transport as caused by the drug brefeldin A,[108] which blocks the activation cycle of ARF.[109] The most convincing explanation of our results is the assumption of metabolism of **21**, but also of other ceramide analogues, to neoglycolipids that, as substrate analogues, lead to inhibition of higher glycosyltransferases. Chemical replacement of one of the two carbon-bound hydrogen atoms of the hydroxymethyl group of **21** by tritium enabled investigation of the metabolic fate of **21**, which is indeed metabolized to higher gang-

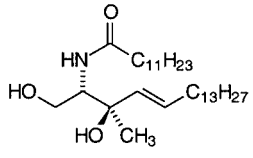
liosides.[98] Final proof of this assumption requires the synthesis of neoglycolipids with heterocyclic backbones and subsequent kinetic analysis of glycosyltransferase inhibition.



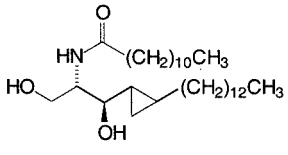
Scheme 10. Early steps in combinatorial ganglioside biosynthesis;[4] the sites of (indirect) inhibition by **20** and **21** are indicated; the next a-series ganglioside would be ganglioside GM1 (Scheme 1)

Other derivatives that we prepared and investigated caused comparable effects. For example, **22** leads to qualitatively similar glycolipid patterns like **21**.

Careful analysis of the effects of other ceramide analogues indicated properties similar to the conformationally rigid analogues **20**−**22**. We prepared a series of 3-methylceramides with different stereochemistry at C-3 with 4,5-single bond, *cis*- and *trans*-double bond, and triple bond. Besides structure-dependent inhibition of glucosylceramide and sphingomyelin formation, addition of **23** (Scheme 11; 25 μM in the medium), as well as the derivative with a triple bond, leads to inhibition of sialyltransferase II, while the *threo*-configured compound with a *cis*-double bond led to inhibition of GalNAc-transferase.[98] 10-μM concentrations of cyclopropyl derivative **24** led to a 1.5-fold increase in the levels of lactosylceramide, despite reduced formation of glucosylceramide.[98] These data suggest that ceramide analogues can be used as cell-permeable prodrugs of inhibitors of glycosyltransferases at nontoxic concentrations.



23



24

Scheme 11

9. Potential Applications

Bioorganic chemistry can also provide approaches for conceptionally new therapeutics.[110] We present a brief review on the interference of synthetic ceramide analogues with ceramide metabolism. The reader will become aware that the identical pharmacological strategy should be beneficial for entirely different diseases. This indicates the demand for ceramide analogues with higher selectivity, that show some, but not all, of the properties of this lipid.

9.1. Cardiovascular Diseases

Cardiovascular diseases might be influenced by synthetic ceramide analogues. A short-chain ceramide derivative is already in use for coating balloon catheters of carotid arteries.[111] Short-chain ceramides have been reported to reduce infarct size after brain ischemia in spontaneously hypertensive rats,[112] to induce,[113] but also to attenuate, vasoconstriction.[114] On the other hand, cell-permeable short-chain ceramides can trigger apoptosis in cardiac myocytes.[115] Although we have currently no clear picture of the molecular details, the different effects of short-chain ceramides might be selectively induced by synthetic derivatives of enhanced selectivity. Also synthetic derivatives of enhanced metabolic stability should be useful, since termination of the vasodilating effects of short-chain ceramides in methoxamine-contracted rat mesenteric microvessels was in part attributed to its metabolism to an inactive intermediate.[116]

9.2. Sphingolipidoses

Sphingolipidoses are rare, but mostly very severe human diseases, which result from inherited defects in the genes encoding proteins that are required for lysosomal sphingolipid degradation.[2,13] For most of these diseases, no causal therapy is available. Substrate deprivation[2,117] as a therapeutic strategy for GSL storage disorders was suggested by N. S. Radin[118] and aims to reduce the biosynthesis of sphingolipids, whose degradation is impaired. Inhibition of glucosylceramide biosynthesis by ceramide analogues like those discussed in Section 6 is currently being investigated for Gaucher's disease and others.[119,120] Inhibition of higher glycosyltransferases in the Golgi apparatus appears to be a promising extension of the current strategies. Suitable cell-permeable substances are not available to date, but our strategy summarised in Section 8.2 should provide such substances for this approach.

Another new therapeutic strategy is the application of "chemical chaperones",[121] as demonstrated in cells of patients with Fabry disease [122] and Gaucher's disease.[123] In these cases, lysosomal enzymes that are unstable due to inherited defects in the corresponding gene are protected from premature degradation by binding to inhibitors of the respective enzyme. Currently, we are investigating the metabolically resistant ceramide analogues mentioned in Section 8.1 as chemical chaperones for human acid ceramidase, the enzyme that is defective in Farber's disease.

9.3. Infections

Glycolipids are known to serve as cellular receptors for toxins, viruses, and bacteria. Pharmacological reduction of glycolipid-based cell surface receptors is a novel strategy in antimicrobial therapy in cultured cells[124] and in mice.[125] To date, it relies on inhibition of glucosylceramide-synthase by compounds like **10** and **11** discussed in Section 6. This strategy might be extended by the prodrug approach discussed in Section 8.2.

Therapeutic treatments of infectious diseases of humans, animals, and plants caused by lower eukaryotes such as yeasts and fungi are essentially based on interference with the biosynthesis and function of membrane sterols.[126] Inositol phosphorylceramide (IPC) synthase is an enzyme that accepts ceramides as substrates and is a new target in the development of fungicides.[2,127−129] The concept of conformational restriction might lead to the development of lipid mimetics for the inhibition of IPC synthase based on heterocyclic structures. Glycolipids have been identified in nematodes, where they are assumed to be responsible for a variety of immunological effects.[130] The biosynthesis of these pathogen-specific compounds might become a new target for the development of anthelmintic drugs.

9.4. Cancer

Pharmacological intervention in sphingolipid metabolism for cancer chemotherapy was proposed and discussed in detail by N. S. Radin.[131] The approach is based on the fact that ceramide induces apoptosis. Increase of ceramide concentrations and triggering of apoptosis can be induced by ceramidase inhibition as demonstrated for metastatic colon cancer cells.[132] Inhibition of glucosylceramide formation is achieved not only by the substances discussed in Section 6 but also by drugs like the breast cancer drug tamoxifen,[133,134] the progesterone receptor antagonist mifepristone (RU486),[135,136] combinations of both,[137] or the vitamin-A derivatives all-*trans*-retinoic acid and *N*-(4-hydroxyphenyl)retinamide.[138]

Ceramide analogues with increased biological half-lives might induce apoptosis directly or by inhibition of endogenous ceramide turnover. Phenylethyl isothiocyanate adducts of sphingosine and sphinganine show an inhibitory effect on the growth of human leukemia 60 (HL60) cells in vitro.[139]

9.5. Transmissible Spongiform Encephalopathies

In the Golgi apparatus, membrane lipids already segregate into membrane areas that are rich in cholesterol, sphingolipids, and glycosylphosphatidylinositol-(GPI-)anchored proteins.[140] The prion protein (PrP) is one of these GPI-anchored proteins. It has been suggested that the pathological (scrapie) form of the prion protein (PrPSc) is formed within rafts by change of the prion protein conformation of the host (PrPC) into the scrapie isoform (PrPSc).[141] Inhibition of glycosphingolipid biosynthesis in persistent scrapie-infected neuroblastoma N2a cells by PDMP (**11**) led to a dose-dependent reduction of PrPSc. When the sphingomyelin concentration was reduced at the same time, PrPSc formation increased.[142] This study demonstrated for the first time that glycolipid-rich membrane domains can be involved in a pathological process. This is confirmed by the isolation of sphingolipids from prion aggregates. The infectiveness of material is inversely correlated with the content of sphingomyelin and galactosylceramide.[143] Recently, a common sphingolipid-binding domain was identified in prion, Alzheimer, and HIV-1 proteins.[144] Therefore, cell surface engineering as discussed below should be considered as a strategy for pharmacological prevention of the new variants of Creutzfeldt−Jakob disease.

9.6. Cell Surface Engineering by Ceramide Derivatives

The surfaces of cultured cells can be modified by interference with carbohydrate metabolism. As demonstrated by W. Reutter, replacement of the acetyl group in *N*-acetyl-D-mannosamine by artificial residues leads to the biosynthesis of modified sialic acid derivatives and thus to modified glycoconjugates of the cell surface.[145] Depending on the cell type, 10−85% of the endogenous sialic acids can be replaced. Since pathogens adhere to the sialic acid moiety of glycoconjugates, the infectivity of living cells can also be modified by this approach.[146,147] Our work with chemically modified ceramide derivatives offers the possibility of modifying cellular surfaces by a different strategy: we demonstrated in Section 8.2 that different glycosyltransferases bound to the membrane of the Golgi apparatus can be inhibited by treatment of cells with cell-permeable ceramide analogues at nontoxic concentrations. While GlcCer-synthase can be directly inhibited by substances of the types **10** and **11** discussed in Section 6, the approach introduced in Section 8.2 requires a functional cellular environment, operates indirectly, and allows selective inhibition of glycosyltransferases that accept substrates of complex structure. We avoid substances of poor pharmacokinetic properties such as nucleotide sugars or carbohydrate derivatives with presumed violation of drug-likeliness algorithms like Pfizer's "rule of five".[148] Glycosyltransferase inhibition leads to modified glycolipid patterns on cell surfaces and is the pharmacological equivalent to mice with the corresponding genetically engineered defects.[4]

Anabolism of ceramide analogues to neoglycolipids, as demonstrated for **21**, might not only lead to modulation of glycosyltransferase activity in the Golgi apparatus, but might also alter the activity of the receptors of the plasma membrane. It is known that glycosphingolipids can drastically modify the activity of pharmacologically relevant receptors such as those for insulin,[149] epidermal growth factor,[150] or nerve growth factor.[151] We postulate that in addition to glycosyltransferase inhibition, a direct modulation of such membrane proteins by endogenously formed neoglycolipids should also be possible by this approach.

Acknowledgments

Work in the laboratory of the authors was supported by the Deutsche Forschungsgemeinschaft (SFB, 284) and the Fonds der Chemischen Industrie.

[1] H. Waldmann, M. Famulok, *ChemBioChem* **2001**, *2*, 3−6.
[2] T. Kolter, K. Sandhoff, *Angew. Chem.* **1999**, *111*, 1633−1670, *Angew. Chem. Int. Ed.* **1999**, *38*, 1532−1568.
[3] A. H. Merrill, Jr., K. Sandhoff, in *Biochemistry of Lipids, Lipoproteins and Membranes* (Eds.: D. E. Vance, J. E. Vance), 4th ed., Elsevier Science, B. V., chapter 14, in press.
[4] T. Kolter, R. L. Proia, K. Sandhoff, *J. Biol. Chem.* **2002**, *277*, 25859−25862.
[5] D. A. Brown, E. London, *J. Biol. Chem.* **2000**, *275*, 17221−17224.
[6] G. van Meer, Q. Lisman, *J. Biol. Chem.* **2002**, *277*, 25855−25858.
[7] T. Kolter, T. Magin, K. Sandhoff, *Traffic* **2000**, *1*, 803−804.
[8] M. Gagnon, H. U. Saragovi, *Expert Opin. Ther. Patents* **2002**, *12*, 1215−1223.
[9] T. Sakai, Y. Koezuka, *Expert Opin. Ther. Patents* **1998**, *8*, 1673−1682.
[10] A. H. Merrill, Jr., *J. Biol. Chem.* **2002**, *277*, 25843−25846.
[11] K. Sandhoff, T. Kolter, *Trends Cell Biol.* **1996**, *6*, 98−103.
[12] K. Sandhoff, T. Kolter, *Phil. Trans.*, in press.
[13] T. Kolter, K. Sandhoff, *Brain Pathology* **1998**, *8*, 79−100.
[14] Y. A. Hannun, L. M. Obeid, *J. Biol. Chem.* **2002**, *277*, 25847−25850.
[15] W. J. van Blitterswijk, *Biochem. J.* **1998**, *331*, 679−680.
[16] L. J. Siskind, M. Colombini, *J. Biol. Chem.* **2000**, *275*, 38640−38644.
[17] J. M. Holopainen, M. Subramanian, P. K. J. Kinnunen, *Biochemistry* **1998**, *37*, 17562−17570.
[18] A. Huwiler, T. Kolter, J. Pfeilschifter, K. Sandhoff, *Biochim. Biophys. Acta* **2000**, *1485*, 63−99.
[19] S. Spiegel, S. Milstien, *J. Biol. Chem.* **2002**, *277*, 25851−25854.
[20] A. Gomez-Munoz, L. M. Frago, L. Alvarez, I. Valero-Nieto, *Biochem. J.* **1997**, *325*, 435−440.
[21] K.-H. Jung, R. R. Schmidt, *Methods Enzymol.* **1999**, *311*, 441−457.
[22] A. Boumendjel, S. P. F. Miller, *J. Labelled Compd. Radiopharm.* **1995**, *36*, 377−383.
[23] A. Berger, R. Bittman, R. R. Schmidt, S. Spiegel, *Mol. Pharmacol.* **1996**, *50*, 451−457.
[24] A. Tarnowski, T. Bär, R. R. Schmidt, *Bioorg. Med. Chem. Lett.* **1997**, *7*, 573−576.
[25] A. S. Bushnev, V. I. Shvets, H. S. Hendrickson, *Chem. Phys. Lipids* **1996**, *82*, 85−88.
[26] M. Plewe, K. Sandhoff, R. R. Schmidt, *Carbohydr. Res.* **1992**, *235*, 151−161.
[27] C. Kan, Z. Ruan, R. Bittman, *Biochemistry* **1991**, *30*, 7759−7766.
[28] L. Grönberg, Z. Ruan, R. Bittman, J. P. Slotte, *Biochemistry* **1991**, *30*, 10746−10754.
[29] M. D. Lister, Z. S. Ruan, R. Bittman, *Biochim. Biophys. Acta* **1995**, *1256*, 25−30.
[30] C. Arenz, A. Giannis, *Angew. Chem. Int. Ed.* **2000**, *39*, 1440−1442.
[31] C. Arenz, M. Thutewohl, O. Block, H. Waldmann, H. J. Altenbach, A. Giannis, *ChemBioChem* **2001**, *2*, 141−143.
[32] C. Arenz, M. Gartner, V. Waschowski, A. Giannis, *Bioorg. Med. Chem.* **2001**, *9*, 2901−2904.
[33] R. Ghidoni, G. Sala, A. Giuliani, *Biochim. Biophys. Acta* **1999**, *1439*, 17−39.
[34] R. E. Pagano, R. G. Sleight, *Science* **1985**, *229*, 1051−1057.
[35] J. Li, G. Zhang, S. Uematsu, Y. Akahori, Y. Hirabayashi, *FEBS Lett.* **1995**, *358*, 211−214.
[36] A. Leo, C. Hansch, D. Elkins, *Chem. Rev.* **1971**, *71*, 525−616.
[37] Calculated with ChemPlus, Hypercube, **1993−1995**.
[38] C. A. Chalfant, K. Kishikawa, M. C. Mumby, C. Kamibayashi, A. Bielawska, Y. A. Hannun, *J. Biol. Chem.* **1999**, *274*, 20313−20317.
[39] G. Schwarzmann, *Biochim. Biophys. Acta* **1978**, *529*, 106−114.
[40] J. K. Anand, K. K. Sadozai, S. Hakomori, *Lipids* **1996**, *31*, 995−998.
[41] A. Bielawska, Y. A. Hannun, *Methods Enzymol.* **1999**, *311*, 499−518.
[42] A. Bielawska, H. M. Crane, D. Liotta, L. M. Obeid, Y. A. Hannun, *J. Biol. Chem.* **1993**, *268*, 26226−26232.
[43] B. Ogretmen, B. J. Pettus, M. J. Rossi, R. Wood, J. Usta, Z. Szulc, A. Bielawska, L. M. Obeid, Y. Y. Hannun, *J. Biol. Chem.* **2002**, *277*, 12960−12969.
[44] D. Allan, *Biochem. J.* **2000**, *345*, 603−610.
[45] L. He, H.-S. Byun, J. Smit, J. Wilschut, R. Bittman, *J. Am. Chem. Soc.* **1999**, *121*, 3897−3903.
[46] Y.-T. Chang, J. Choi, S. Ding, E. E. Prieschl, T. Baumruker, J.-M. Lee, S.-K. Chung, P. G. Schultz, *J. Am. Chem. Soc.* **2002**, *124*, 1856−1857.
[47] N. Karasavvas, R. K. Erukulla, R. Bittman, R. Lockshin, Z. Zakeri, *Eur. J. Biochem.* **1996**, *236*, 729−737.
[48] T. Wieder, C. C. Geilen, T. Kolter, F. Sadeghlar, K. Sandhoff, R. Brossmer, P. Ihrig, D. Perry, C. E. Orfanos, Y. A. Hannun, *FEBS Lett.* **1997**, *411*, 260−264.
[49] M. Bektas, Y. Dullin, T. Wieder, T. Kolter, K. Sandhoff, R. Brossmer, P. Ihrig, C. E. Orfanos, C. C. Geilen, *Exp. Dermatol.* **1998**, *7*, 342−349.
[50] E. Bieberich, T. Kawaguchi, R. K. Yu, *J. Biol. Chem.* **2000**, *275*, 177−181.
[51] S. De Jonghe, I. van Overmeire, S. Poulton, C. Hendrix, R. Busson, S. van Calenbergh, D. De Keukeleire, S. Spiegel, P. Herdewijn, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1375−3180.
[52] S. De Jonghe, I. van Overmeire, J. Gunst, A. De Bruyn, C. Hendrix, S. van Calenbergh, R. Busson, D. De Keukeleire, J. Philippé, P. Herdewijn, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3159−3164.
[53] O. Hwang, G. Kim, Y. J. Jang, S. W. Kim, G. Choi, H. J. Choi, S. Y. Jeon, D. G. Lee, J. D. Lee, *Mol. Pharmacol.* **2001**, *59*, 1249−1255.
[54] F. K. Knoll, T. Kolter, K. Sandhoff, *Methods Enzymol.* **1999**, *311*, 568−600.
[55] G. Dorman, G. D. Prestwich, *Trends Biotechnol.* **2000**, *18*, 64−77.
[56] C. Walsh, *Tetrahedron* **1982**, *38*, 871−909.
[57] T. Weber, J. Brunner, *J. Am. Chem. Soc.* **1995**, *117*, 3084−3095.
[58] A. Huwiler, J. Brunner, R. Hummel, M. Vervoordeldonk, S. Stabel, H. van den Bosch, J. Pfeilschifter, *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93*, 6959−6963.
[59] M. M. P. Zegers, J. W. Kok, D. Hoekstra, *Biochem. J.* **1997**, *328*, 489−498.
[60] L. Elsen, R. Betz, G. Schwarzmann, K. Sandhoff, G. van Echten-Deckert, *Neurochem. Res.* **2002**, *27*, 717−727.
[61] C. Michel, G. van Echten-Deckert, J. Rother, K. Sandhoff, E. Wang, A. H. Merrill, *J. Bio. Chem.* **1997**, *272*, 22432−22437.
[62] P. Ternes, S. Franke, U. Zähringer, P. Sperling, E. Heinz, *J. Biol. Chem.* **2002**, *277*, 25512−25518.
[63] B. J. Wallar, J. D. Lipscomb, *Chem. Rev.* **1996**, *96*, 2625−2657.
[64] P. H. Buist, B. Behrouzian, *J. Am. Chem. Soc.* **1996**, *118*, 6295−6296.
[65] C. K. Savile, G. Fabriàs, P. H. Buist, *J. Am. Chem. Soc.* **2001**, *123*, 4382−4385.
[66] S. De Jonghe, I. Van Overmeire, S. Van Calenbergh, C. Hendrix, R. Busson, D. De Keukeleire, P. Herdewijn, *Eur. J. Org. Chem.* **2000**, 3177−3183.
[67] G. Triola, G. Fabriàs, A. Llebaria, *Angew. Chem.* **2001**, *113*, 2014−2016; *Angew. Chem. Int. Ed.* **2001**, *10*, 1960−1962.
[68] J. Quintana, M. Barrot, G. Fabriàs, F. Camps, *Tetrahedron* **1998**, *54*, 10187−10198.
[69] S. Brodesser, Diploma Thesis, University of Bonn, **2002**.
[70] O. Thum, C. Hertweck, H. Simon, W. Boland, *Synthesis* **1999**, 2145−2150.
[71] C. Beckmann, J. Rattke, N. J. Oldham, P. Sperling, E. Heinz, W. Boland, *Angew. Chem.* **2002**, *114*, 2394−2397.
[72] F. M. Platt, T. D. Butters, *Expert Rev. Mol. Med.* **2000**, 1−17.
[73] T. D. Butters, L. A. G. M. van den Broek, G. W. J. Fleet, T. M. Krulle, M. R. Wormald, R. A. Dwek, F. M. Platt, *Tetrahedron: Asymmetry* **2000**, *11*, 113−124.

[74] J. Inokuchi, N. S. Radin, *J. Lipid Res.* **1987**, *28*, 565−571.
[75] J. Usta, S. El Bawab, P. Roddy, Z. M. Szulc, Y. A. Hannun, A. Bielawska, *Biochemistry* **2001**, *40*, 9657−9668.
[76] G. van Meer, J. C. M. Holthuis, *Biochim. Biophys. Acta* **2000**, *1486*, 145−170.
[77] U. Pütz, G. Schwarzmann, *Eur. J. Cell Biol.* **1995**, *68*, 113−121.
[78] R. E. Pagano, O. C. Martin, H. C. Kang, R. P. Haugland, *J. Cell Biol.* **1991**, *113*, 1267−1279.
[79] J. Bai, R. E. Pagano, *Biochemistry* **1997**, *36*, 8840−8848.
[80] J. W. Kok, M. Nikolova-Karakashian, K. Klappe, C. Alexander, A. H. Merrill, Jr., *J. Biol. Chem.* **1997**, *272*, 21128−21136.
[81] P. Antes, G. Schwarzmann, K. Sandhoff, *Eur. J. Cell Biol.* **1992**, *59*, 27−36.
[82] M. Chatelut, M. Leruth, K. Harzer, A. Dagan, S. Marchesini, S. Gatt, R. Salvayre, P. Courtoy, T. Levade, *FEBS Lett.* **1998**, *426*, 102−106.
[83] W. F. Nieuwenhuizen, S. van Leeuwen, F. Gotz, M. R. Egmond, *Chem. Phys. Lipids* **2002**, *114*, 181−191.
[84] J. J. Tegeler, B. S. Rauckman, R. R. L. Hamer, B. S. Freed, G. H. Merriman, L. Heller, M. Orteganános, S. C. Bailey, E. S. Kurtz, *Bioorg. Biomed. Chem. Lett.* **1995**, *5*, 2477−2482.
[85] G. Merriman, J. J. Tegeler, R. R. L. Hamer, B. S. Rauckman, B. S. Freed, E. S. Kurtz, S. C. Bailey, M. Orteganános, P. A. Przekop, L. Hellyer, *Bioorg. Biomed. Chem. Lett.* **1995**, *5*, 2483−2488.
[86] J. Chun, L. He, H.-S. Byun, R. Bittman, *J. Org. Chem.* **2000**, *65*, 7634−7640.
[87] G. van Echten-Deckert, A. Giannis, A. Schwarz, A. H. Futerman, K. Sandhoff, *J. Biol. Chem.* **1998**, *273*, 1184−1191.
[88] I. van Overmeire, S. A. Boldin, F. Dumont, S. Van Calenbergh, G. Slegers, D. De Keukeleire, A. H. Futerman, P. Herdewijn, *J. Med. Chem.* **1999**, *42*, 2697−2705.
[89] I. van Overmeire, S. A. Boldin, K. Vankataraman, R. Zisling, S. de Jonghe, S. van Calenbergh, D. de Keukeleire, A. H. Futerman, P. Herdewijn, *J. Med. Chem.* **2000**, *43*, 4189−4199.
[90] S. De Jonghe, I. Lamote, K. Vankataraman, S. A. Boldin, U. Hillaert, J. Roziniski, C. Hendrix, R. Busson, D. de Keukeleire, S. van Calenbergh, A. H. Futerman, P. Herdewijn, *J. Org. Chem.* **2002**, *67*, 988−996.
[91] J. Yin, H. Liu, C. Pidgeon, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 179−182.
[92] G. van Echten-Deckert, A. Giannis, A. Schwarz, A. H. Futerman, K. Sandhoff, *J. Biol. Chem.* **1998**, *273*, 1184−1191.
[93] K. Venkataraman, A. H. Futerman, *Biochim. Biophys. Acta* **2001**, *1530*, 219−226.
[94] G. A. Patani, E. J. LaVoie, *Chem. Rev.* **1996**, *96*, 3147−3176.
[95] D. O′Hagan, H. S. Rzepa, *Chem. Commun.* **1997**, 645−652.
[96] P. Sawatzki, M. Skowron, G. van Echten-Deckert, T. Kolter, unpublished results.
[97] D. B. A. de Bont, K. M. Sliedregt-Bol, L. J. F. Hofmeyer, R. M. J. Liskamp, *Bioorg. Med. Chem.* **1999**, *7*, 1043−1047.
[98] P. Sawatzki, Doctoral Thesis, University of Bonn, in preparation.
[99] A. Giannis, T. Kolter, *Angew. Chem.* **1993**, *105*, 1303−1326; *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 1244−1267.
[100] T. Kolter "Conformational restriction of sphingolipids" in: C. Schmuck, H. Wennemers (Eds.) *Bioorganic Chemistry II*, Wiley-VCH, Weinheim, in press.
[101] L. M. Leoni, H. C. Shih, L. Deng, C. Tuey, G. Walter, D. A. Carson, H. B. Cottam, *Biochem. Pharmacol.* **1998**, *55*, 1105−1111.
[102] M. Macchia, S. Barontini, S. Bertini, V. Di Bussolo, S. Fogli, E. Giovannetti, E. Grossi, F. Minutolo, R. Danesi, *J. Med. Chem.* **2001**, *44*, 3994−4000.
[103] V. Austel, in *Chemometric Methods in Molecular Design* (Ed.: H. van der Waterbeemd), Wiley-VCH, Weinheim, **1995**, p. 49−62.
[104] In brief, primary neurons were prepared from the cerebellum of 6-day-old mice and treated with different concentrations of ceramide analogue for 24 h. 3-[^{14}C]serine or [^{14}C]galactose was added to the culture medium and newly synthesised sphingolipids were analysed after 24 h of labelling. Lipids were extracted, separated by TLC, and visualized with a phosphoimager. Radioactivity found in the selected lipids is expressed in relation to untreated cells.
[105] G. van Echten-Deckert, A. Zschoche, T. Bär, R. R. Schmidt, A. Raths, T. Heinemann, K. Sandhoff, *J. Biol. Chem.* **1997**, *272*, 15825−33.
[106] G. van Echten, K. Sandhoff, *J. Neurochem.* **1989**, *52*, 207−14.
[107] M. Wendeler, H. Reilaender, J. Hoernschemeyer, G. Schwarzmann, T Kolter, K. Sandhoff, "Recognition of Carbohydrates in Biological Systems", in *Methods in Enzymology* (Eds.: Y. C. Lee, R. T. Lee), Academic Press, San Diego, in press.
[108] G. van Echten, H. Iber, H. Stotz, A. Takatsuki, K. Sandhoff, *Eur. J. Cell Biol.* **1990**, *51*, 135−139.
[109] C. L. Jackson, J. E. Casanova, *Trends Cell Biol.* **2000**, *10*, 60−67.
[110] R. Breslow, *J. Chem. Educ.* **1998**, *75*, 705−718.
[111] R. Charles, L. Sandirasegarane, J. Yun, N. Bourbon, R. Wilson, R. P. Rothstein, S. W. Levison, M. Kester, *Circ. Res.* **2000**, *87*, 282−288.
[112] K. Furuya, I. Ginis, H. Takeda, Y. Chen, J. M. Hallenbeck, *J. Cereb. Blood Flow Metab.* **2001**, *21*, 226−232.
[113] D. G. Johns, J.-S. Jin, D. W. Wilde, R. C. Webb, *Gen. Pharmacol.* **1999**, *33*, 415−421.
[114] T. Zheng, W. Li, J. Wang, B. T. Altura, B. M. Altura, *Lipids* **1999**, *34*, 689−695.
[115] N. Andrieu-Abadie, J.-P. Jaffrézou, S. Hatem, G. Laurent, T. Levade, J.-J. Mercadier, *FASEB J.* **1999**, *13*, 1501−1510.
[116] P. Czyborra, M. Saxe, C. Fetcher, D. Meyer zu Heringdorf, S. Herzig, K. H. Jakobs, M. C. Michel, A. Bischoff, *Br. J. Pharmacol.* **2002**, *135*, 417−426.
[117] T. Kolter, *Angew. Chem.* **1997**, *109*, 2044−2048; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 1955−1959.
[118] R. R. Vunnam, N. S. Radin, *Chem. Phys. Lipids* **1980**, *26*, 265−278.
[119] N. S. Radin, *J. Inherit. Metab. Dis.* **2000**, *23*, 767−777.
[120] T. D. Butters, R. A. Dwek, F. M. Platt, *Chem. Rev.* **2000**, *100*, 4683−4696.
[121] T. Kolter, M. Wendeler, *ChemBioChem*, in press.
[122] J. Fan, S. Ishii, N. Asano, Y. Suzuki, *Nature Med.* **1999**, *5*, 112−115.
[123] A. R. Sawkar, W. C. Cheng, E. Beutler, C. H. Wong, W. E. Balch, J. W. Kelly, *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 15428−15433.
[124] M. Svensson, R. Lindstedt, N. S. Radin, C. Svanborg, *Infect. Immun.* **1994**, *62*, 4404−4410.
[125] M. Svensson, B. Frendeus, T. Butters, F. Platt, D. Dwek, C. Svanborg, *Mol. Microbiol.* **2003**, *47*, 453−461.
[126] J. M. Balkovec, *Annu. Rep. Med. Chem.* **1998**, *33*, 173−182.
[127] T. Kolter, K. Sandhoff, *Chem. Soc. Rev.* **1996**, *25*, 371−381.
[128] M. M. Nagiec, E. E. Nagiec, J. A. Baltisberger, G. B. Wells, R. L. Lester, R. C. Dickson, *J. Biol. Chem.* **1997**, *272*, 9809−9817.
[129] J. M. Fostel, P. A. Lartey, *Drug Discovery Today* **2000**, *5*, 25−32.
[130] G. Lochnit, R. D. Dennis, R. Geyer, *Biol. Chem.* **2000**, *381*, 839−847.
[131] N. S. Radin, *Eur. J. Biochem.* **2001**, *268*, 193−204.
[132] M. Selzner, A. Bielawska, M. A. Morse, H. A. Rüdiger, D. Sindram, Y. A. Hannun, P.-A. Clavien, *Cancer Res.* **2001**, *61*, 1233−1240.
[133] M. C. Cabot, A. E. Giuliano, A. Volner, T. Y. Han, *FEBS Lett.* **1996**, *394*, 129−131.
[134] Y. Lavie, H. Cao, A. Volner, A. Lucci, T.-Y. Han, V. Geffen, A. E. Giuliano, M. C. Cabot, *J. Biol. Chem.* **1997**, *272*, 1682−1687.
[135] S. S. Koide, *J. Reprod. Med.* **1998**, *43*, 551−560.
[136] P. Xia, J. R. Gamble, L. J. Wang, S. M. Pitson, P. A. B. Moretti, B. W. Wattenberg, R. J: D′Andrea, M. A. Vadas, *Curr. Biol.* **2000**, *10*, 1527−1530.

[137] M. F. El Etreby, Y. Liang, *Breast Cancer Res. Treat.* **1998**, *49*, 109−117.
[138] B. J. Maurer, L. S. Metelitsa, R. C. Seeger, M. C. Cabot, C. P. Reynolds, *J. Natl. Cancer Inst.* **1999**, *91*, 1138−1146.
[139] K. Xu, P. J. Thornalley, *Bioorg. Med. Chem. Lett.* **2000**, *10*, 53−54.
[140] K. Simons, E. Ikonen, *Nature* **1997**, *387*, 569−572.
[141] N. Naslavsky, N. Stein, R. Yanai, G. Friedlander, A. Taraboulos, *J. Biol. Chem.* **1997**, *272*, 6324−6331.
[142] N. Naslavsky, H. Shmeeda, G. Friedlander, A. Yanai, A. H. Futerman, Y. Barenholz, A. Taraboulos, *J. Biol. Chem.* **1999**, *274*, 20763−20771.
[143] T. R. Klein, D. Kirsch, R. Kaufmann, D. Riesner, *Biol. Chem.* **1998**, *379*, 655−666.
[144] R. Mahfoud, N. Garmy, M. Maresca, N. Yahi, A. Puigserver, J. Fantini, *J. Biol. Chem.* **2002**, *277*, 11292−11296.
[145] H. Kayser, R. Zeitler, C. Kannicht, D. Grunow, R. Nuck, W. Reutter, *J. Biol. Chem.* **1992**, *267*, 16934−16938.
[146] O. T. Keppler, R. Horstkorte, M. Pawlita, C. Schmidt, W. Reutter, *Glycobiology* **2001**, *11*, 11R−18R.
[147] C. R. Bertozzi, *Science* **2001**, *291*, 2357−2364.
[148] C. A. Lipinski, F. Lombardo, B. W. Dominy, P. J. Feeney, *Adv. Drug Deliv. Rev.* **1997**, *23*, 3−25.
[149] S. Tagami, J. Inokuchi, K. Kabayama, H. Yoshimura, F. Kitamura, S. Uemura, C. Ogawa, A. Ishii, M. Saito, Y. Ohtsuka, S. Sakaue, Y. Igarashi, *J. Biol. Chem.* **2002**, *277*, 3085−3092.
[150] G. Zhou, S. Hakomori, K. Kitamura, Y. Igarashi, *J. Biol. Chem.* **1994**, *269*, 1959−1965.
[151] T. Mutoh, A. Toluda, T. Miyadai, M. Hamaguchi, N. Fujiki, *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 5087−5091.

Received September 17, 2002

