Inhibitors of Glycosphingolipid Biosynthesis

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Dedicated to Prof. Hans Paulsen on the occasion of his 75th birthday.

1 Structure and Function of Glycosphingolipids

Glycosphingolipids (GSLs) are characteristic components of the outer leaflet of the plasma membrane of eukaryotic cells.[1] Each GSL contains a hydrophobic ceramide moiety that acts as membrane anchor and a hydrophilic, extracellular oligosaccharide chain. Ceramide itself consists of a long chain amino alcohol, D-*erythro*-sphingosine, which is acylated with a fatty acid. It is also a structural component of a plasma membrane phospholipid, sphingomyelin. GSLs are heterogeneous with respect to both their carbohydrate and ceramide portion. Sphingolipids with unusual ceramide structures are found in the skin where they contribute to the epidermal water permeability barrier. Variations in the type, number and linkage of sugar residues within the oligosaccharide chain give rise to the wide range of naturally occurring GSLs. More than 300 different structures have been characterised from natural sources. GSL structures depend on the species and can be classified into series which are characteristic for a group of evolutionary related organisms. Beside the species dependence, GSLs form cell-type specific patterns on the cell surface. In particular sialic acid containing GSLs of the ganglio-series, the gangliosides, are abundant on neuronal cells. Moreover, these patterns change with cell growth, differentiation, viral transformation, ontogenesis and oncogenesis. Together with glycoproteins and glycosaminoglycans the GSLs contribute to the glycocalix which covers the cell surface with a carbohydrate wall.

At the cell surface, GSLs can interact with toxins, viruses and bacteria.[2] These pathogens take advantage of the close spatial neighbourhood between specific carbohydrate recognition sites and the plasma membrane. *E.g.* the cholera toxin B subunit has to bind to ganglioside GM1 before the A subunit of the toxin can enter the cell. GSLs can also interact with membrane bound receptors and enzymes and are involved in cell type specific adhesion processes. Various physiological events can be influenced by GSLs, *e.g.* embryogenesis, neuronal and leukocyte differentiation, cell adhesion and signal transduction.[3] Lipophilic products of GSL metabolism such as sphingosine, ceramide and their phosphorylated derivatives play a role in signal transduction events.[4,5] Finally, GSLs form a protective layer on biological membranes protecting them from inappropriate degradation and uncontrolled membrane fusion. Limited knowledge about the precise *in vivo* function of GSLs is available today. A variety of observations indicate that they can participate in different biological events, but in most cases definite proofs for their importance are missing. In general, the conservation of the overall GSL structure during evolution and the absence of inherited diseases affecting GSL biosynthesis indicate their functional importance for the living organism.

2 Sphingolipid Biosynthesis

GSLs and sphingomyelin occur predominantly on the plasma membrane of eukaryotic cells. Since their biosynthesis and degradation proceed within cellular organelles, GSLs and their precursors are also found on intracellular membranes communicating with the plasma membrane by processes of membrane fusion and fission. The enzymes involved in sphingolipid biosynthesis are membrane bound proteins. Little is known about their structure, catalytic mechanism, biosynthesis and regulation. *De novo* biosynthesis of GSLs[6,7] takes place in the same intracellular compartments as glycoprotein biosynthesis and is coupled to intracellular vesicular transport of the growing molecules through the cisternae of the Golgi apparatus and to the plasma membrane. It starts with the formation of ceramide at the membranes of the endoplasmic reticulum (ER). The condensation of the amino acid L-serine with a fatty acyl coenzyme A, usually palmitoyl coenzyme A, to 3-ketosphinganine is catalysed by the enzyme serine palmitoyl transferase (SPT). The SPT is a pyridoxalphosphate dependent enzyme. Two mechanistic pathways for the enzyme-catalysed reaction can be considered, which are distinguished by the order of the loss of carbon dioxide and the acylation.[8] The finding that tritium labelling of the 2-position in serine is retained during sphingolipid biosynthesis in rats supports the first mechanism (Figure 2, pathway A). Another study using [2,3,3-$^2H_3$]serine as substrate and *H. ciferrii* as enzyme source led to the observation that the deuterium at the 2-position of serine is eliminated during condensation. This finding supports the second mechanism (Figure 2, pathway B).[8] A related reaction which occurs in haem biosynthesis, the condensation of succinyl coenzyme A and glycine, is catalysed by the enzyme aminolevulinate synthase according to the second mechanism. Sequence homologies on the cDNA level between this two enzymes suggest a common three-dimensional structure and mechanism.

The SPT has a lower activity than the other enzymes of ceramide biosynthesis and catalyses the rate-limiting step of this pathway. It preferentially utilises fatty acid CoA esters with a chain length of

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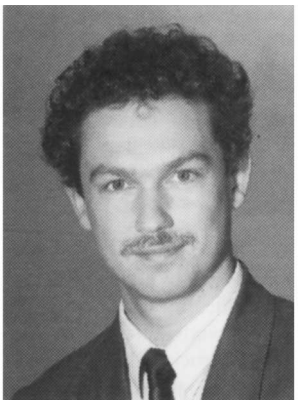

Konrad Sandhoff was born in 1939 and studied chemistry at the University of Munich. He received his Ph.D. with Horst Jatzkewitz and Feodor Lynen in 1965. He joined the Max-Planck-Institut für Psychiatrie, München, the Johns Hopkins University, Baltimore, and the Weizmann Institute, Rehovot. In 1979 he became full professor for biochemistry and director of the Institute for Organic Chemistry and Biochemistry in Bonn. Among the honours he received is the use of the eponym 'Sandhoff disease' for a certain inherited disorder and the award of the Richard-Kuhn-medal from the Gesellschaft Deutscher Chemiker. His research interests include the analysis of lysosomal storage diseases and the biochemistry of glycolipid metabolism.

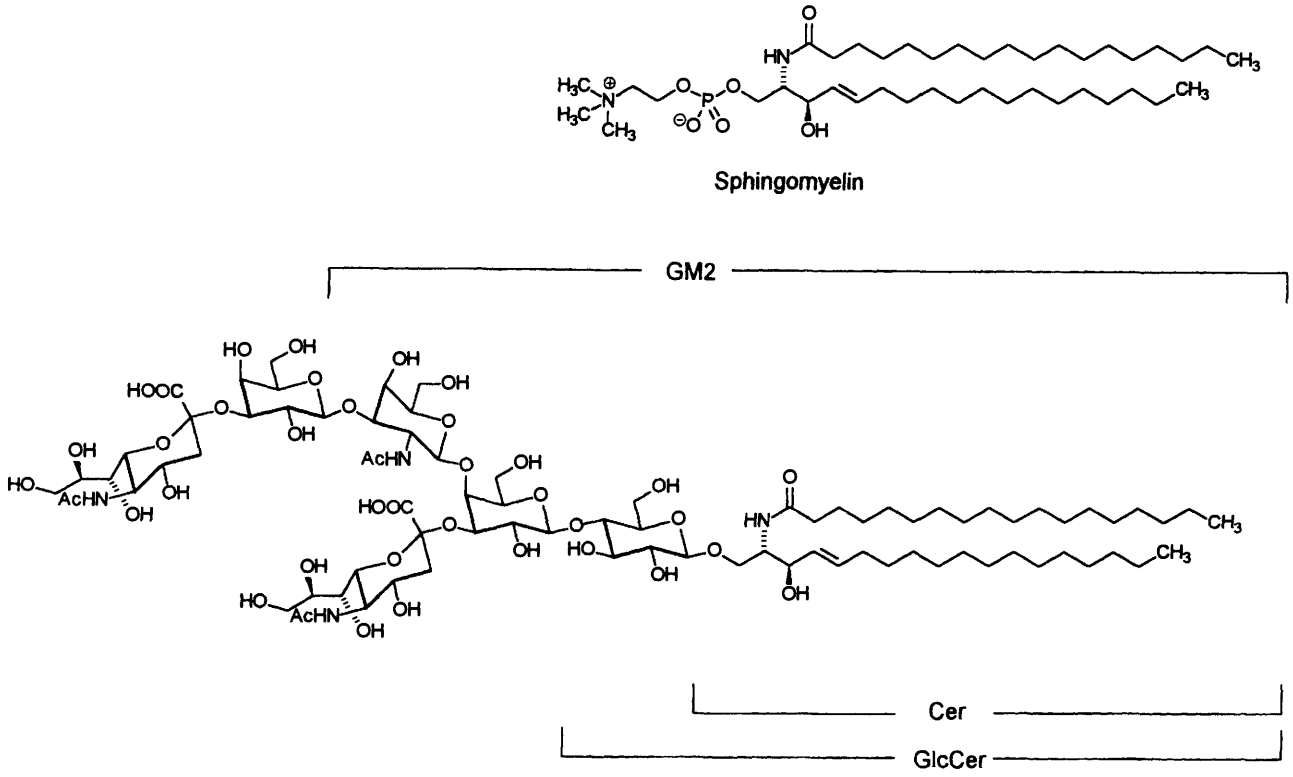

Figure 1 Structures of sphingomyelin and ganglioside GD1a, the most abundant glycosphingolipid (GSL) in adult human brain Abbreviations refer to partial structures Cer – ceramide, GlcCer – glucosylceramide, GM2 – ganglioside GM2

16 carbon atoms resulting in long chain bases with a C_{18} chain In the following NADPH-dependent reaction, 3-ketosphinganine is reduced to D-*erythro*-sphinganine by the enzyme 3-ketosphinganine reductase Sphinganine is acylated to dihydroceramide by the enzyme sphinganine N-acyltransferase Dihydroceramide is subsequently desaturated to ceramide in the dihydroceramide desaturase reaction The order of introduction of the double bond and aclyation was controversial for some time, but it can be regarded as accepted that dihydroceramide is desaturated and not sphinganine [6 7] Therefore, sphingosine, the parent compound of the sphingolipids, is not an intermediate in sphingolipid biosynthesis Instead of this it is formed during sphingolipid degradation Besides sphinganine and sphingosine, another long chain base, phytosphingosine [C_{18} homologue (2*S*,3*S*,4*R*)-2-amino-1,3,4-octadecanetriol] is the structural constituent of many plant, yeast and mammalian epidermis sphingolipids

Ceramide is the common precursor of GSLs and sphingomyelin In the case of GSLs in vertebrates, a glucose or a galactose moiety is β-glycosidically linked to the 1-position of ceramide through the action of glycosyltransferases The transferases utilise nucleotide activated sugars Galactosylation of ceramide takes place predominantly in oligodendrocyte cells of the brain and in the kidneys Galactosylceramide (GalCer) and sulfatide (GalCer-3-sulfate) occur in high concentrations in the multilamellar layer of the myelin sheet, which covers the axons of neuronal cells On the other hand, the biosynthesis of most GSLs of vertebrates requires the glucosylation of ceramide The GlcCer synthase transfers a glucose residue from UDP-glucose to ceramide LacCer, the common precursor for the five GSL series found in vertebrates, is formed by the addition of a galactose moiety from UDP-Gal to GlcCer catalysed by galactosyltransferase I

Ceramide is also a precursor for sphingomyelin, a structural component of the plasma membrane It is a 1-ceramide phosphocholine and occurs largely on the extracellular leaflet of the plasma membrane [1] The sphingomyelin content of plasma membranes depends on the cell type and can reach 25% Sphingomyelin is functionally distinguished from glycerophospholipids like phosphatidyl choline by the higher melting temperature of sphingomyelin bilayers due to the prevalence of saturated alkyl chains and the occurrence of intermolecular H-bonds between the 3-OH group and the amide-NH Sphingomyelin biosynthesis requires the transfer of phosphorylcholine headgroup from phosphatidyl choline to ceramide Diacylglycerol is liberated in this step which suggests a tight coupling between sphingolipid and glycerolipid metabolism Indeed an inverse correlation between the amounts of sphingomyelin and phosphatidyl choline is observed in many membranes

2.1 Topology

The first three steps of sphingolipid biosynthesis leading to dihydroceramide are catalysed by membrane-bound enzymes at the cytosolic face of the endoplasmic reticulum (ER) [6] Since formation of glucosyl ceramide occurs on the cytosolic face of the Golgi apparatus or a pre-Golgi compartment, dihydroceramide has to be transported from the ER to the Golgi apparatus, either by vesicle flow or by a protein-mediated process Introduction of the next sugar residue leading to lactosyl ceramide appears to be restricted to the lumenal site of the Golgi apparatus This implicates a membrane translocation of glucosylceramide which is thought to be facilitated by a protein, a yet uncharacterised flippase Biosynthesis of higher GSL proceeds on the lumenal site of the Golgi apparatus Therefore, the oligosaccharide chain of the membrane-bound GSLs is orientated anticytosolic This orientation is topologically equivalent to the situation in the plasma membrane, where the GSLs face the extracellular space

Sphingomyelin synthesis takes place on the lumenal site of an early Golgi or pre-Golgi compartment This requires an additional membrane translocation on the stage of ceramide It is not clear whether this process is facilitated by a protein

2.2 Biosynthesis of complex glycosphingolipids

Since the majority of cellular functions of complex GSLs can be attributed to sialic acid containing gangliosides, their biosynthesis is briefly discussed here [6] Most GSL found in vertebrates share lactosylceramide as a common precursor and structural element The sequential addition of further sugar residues including sialic acid requires the action of membrane-bound glycosyltransferases in the

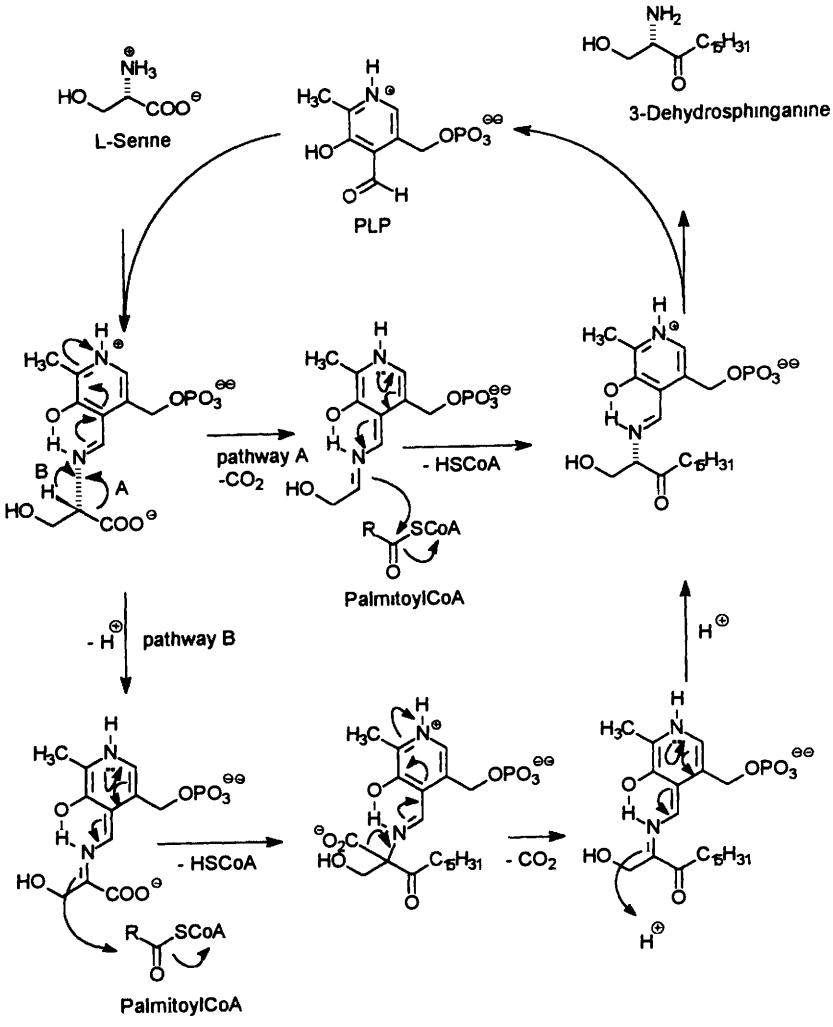

Figure 2 Mechanism of serine palmitoyltransferase (SPT) See text for details

lumen of the Golgi apparatus LacCer and its sialylated derivatives GM3, GD3 and GT3 serve as precursors for more complex gangliosides of the O, a, b and c series C series gangliosides have been found only in trace amounts in human tissues Sequential glycosylation of these precursors is performed by less specific glycosyltransferases, which transfer a respective sugar residue to glycosyl acceptors which differ only in the number of sialic acids bound to the inner galactose *In vitro* data indicate that the sialyltransferases I and II are much more specific for their glycolipid substrates than sialyltransferases IV and V as well as the Gal and GalNAc transferase The distribution of glycosyltransferases within the stacks of the Golgi apparatus has been investigated with the aid of inhibitors of vesicular membrane flow Monensin, a cationic ionophore, impedes vesicular membrane flow between proximal and distal Golgi cisternae and causes an increased biosynthetic galactose labelling of GlcCer, LacCer, GM3, GD3 and GM2 while labelling of more complex gangliosides was reduced Brefeldin A, which causes fusion between the ER and largely *cis*- and medial Golgi, causes label reduction in gangliosides GM1a, GD1a, GD1b, GT1b, GQ1b and, to some extent, sphingomyelin in neuronal cells Although glycosyltransferase activities are not exclusively found in one Golgi subcompartment, these data suggest that GM3 and GD3 are formed in early Golgi compartments whereas more complex GSL like GM1a are formed in a late compartment Besides *de novo* biosynthesis GSLs can also be formed in salvage pathways utilizing monosaccharides, sphingosine and possibly also ceramide released in glycoconjugate catabolism (part 4)

2.3 Regulation

The maintenance of balanced GSL patterns on individual cell surfaces requires a stringent control of GSL biosynthesis, degradation and intracellular traffic The regulation of GSL metabolism and transport is not well understood and only a few indications about it are available [6] SPT seems to be the first control point for sphingolipid formation The enzyme activity correlates with the relative amounts of sphingolipids found in different tissues Sphingosine reduces the SPT activity in cultured neurons and removal of lipids from the skin leads to increased SPT activity

During ontogenesis and cell transformation a correlation between GSL expression and the activity of the glycosyltransferases leading to its synthesis has been observed Therefore, transcriptional control of glycosyltransferases seems to be a major point of regulation Since most glycosyltransferases have been cloned within the last few years, information required for the understanding of transcriptional control is expected to be available in the near future Besides regulation on the genomic level some findings hint on epigenetic regulation mechanisms [6] Feedback control of several glycosyltransferases either by its respective reaction product or final products within the corresponding series has been observed *in vitro* Also the phosphorylation status of the glycosyltransferases can influence their activity Lowering the pH of murine cerebellar cell culture media from 7 4 to 6 2 resulted in a reversible shift of ganglioside biosynthesis from the a- to the b- series This observation can be explained by the complementary pH profiles of the key regulatory glycosyltransferases, sialyltransferase II and GalNAc transferase

3 Glycosphingolipid Degradation[9]

The final degradation of GSLs occurs in the lysosomes of the cells The plasma membrane containing GSLs destined for degradation are endocytosed and traffic through the endosomal compartments to reach the lysosome A detailed model of the topology of this process

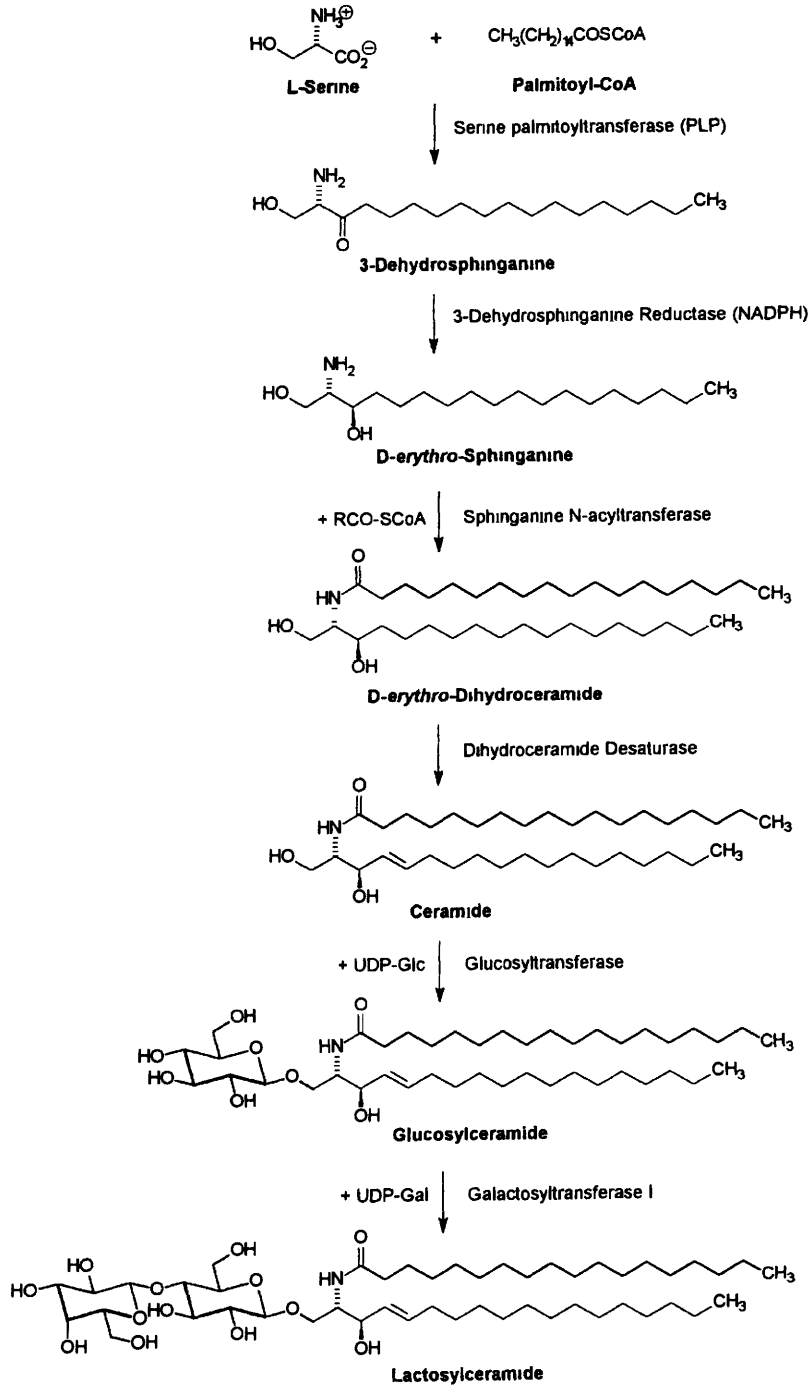

Figure 3 Biosynthesis of lactosylceramide [6] Heterogeneity within the lipid portion is not indicated

has been suggested [9] Within the lysosome, hydrolysing enzymes sequentially cleave off the sugar residues to produce ceramide which is deacylated to sphingosine This can leave the lysosome, reenter the biosynthetic pathway or be further degraded More than ten different exohydrolases are involved in GSL degradation If any of these enzymes is deficient, the corresponding lipid substrate accumulates and is stored in the lysosomal compartment This leads to inherited lipid storage diseases with broad clinical and biochemical heterogeneity For GSLs with long carbohydrate chains of more than four sugar residues the presence of an enzymatically active exohydrolase is sufficient for degradation *in vivo* However, degradation of mem brane bound GSLs with short oligosaccharide chains requires the cooperation of an exohydrolase and a protein cofactor, a so-called sphingolipid activator protein Several sphingolipid activator proteins are now known including the GM2 activator and the saposins SAP-A, -B, -C and -D Inherited deficiencies of either lysosomal hydrolases or activator proteins give rise to GSL storage diseases [9]

Sphingolipid degradation is not necessarily restricted to occur in the lysosome Sphingomyelin and ceramide can be cleaved by sphingomyelinases and ceramidases of various subcellular localisation Prior to degradation, sphingosines with the natural *erythro* configuration are phosphorylated by a sphingosine kinase with cytosolic localisation The sphingosine-1-phosphate generated in this reaction can be cleaved by an enzyme localised on the cytosolic face of the endoplasmic reticulum, the sphingosine-1-phosphate lyase The enzyme is PLP-dependent and the reaction corresponds to a retro aldol cleavage The products are ethanolamine phosphate and an unsaturated aldehyde (Fig 5)

4 Sphingolipids in Signal Transduction

To clarify the role of cell surface GSLs is not the only motivation to modulate sphingolipid metabolism Lipophilic intermediates of GSL catabolism have been identified as putative signalling mole-

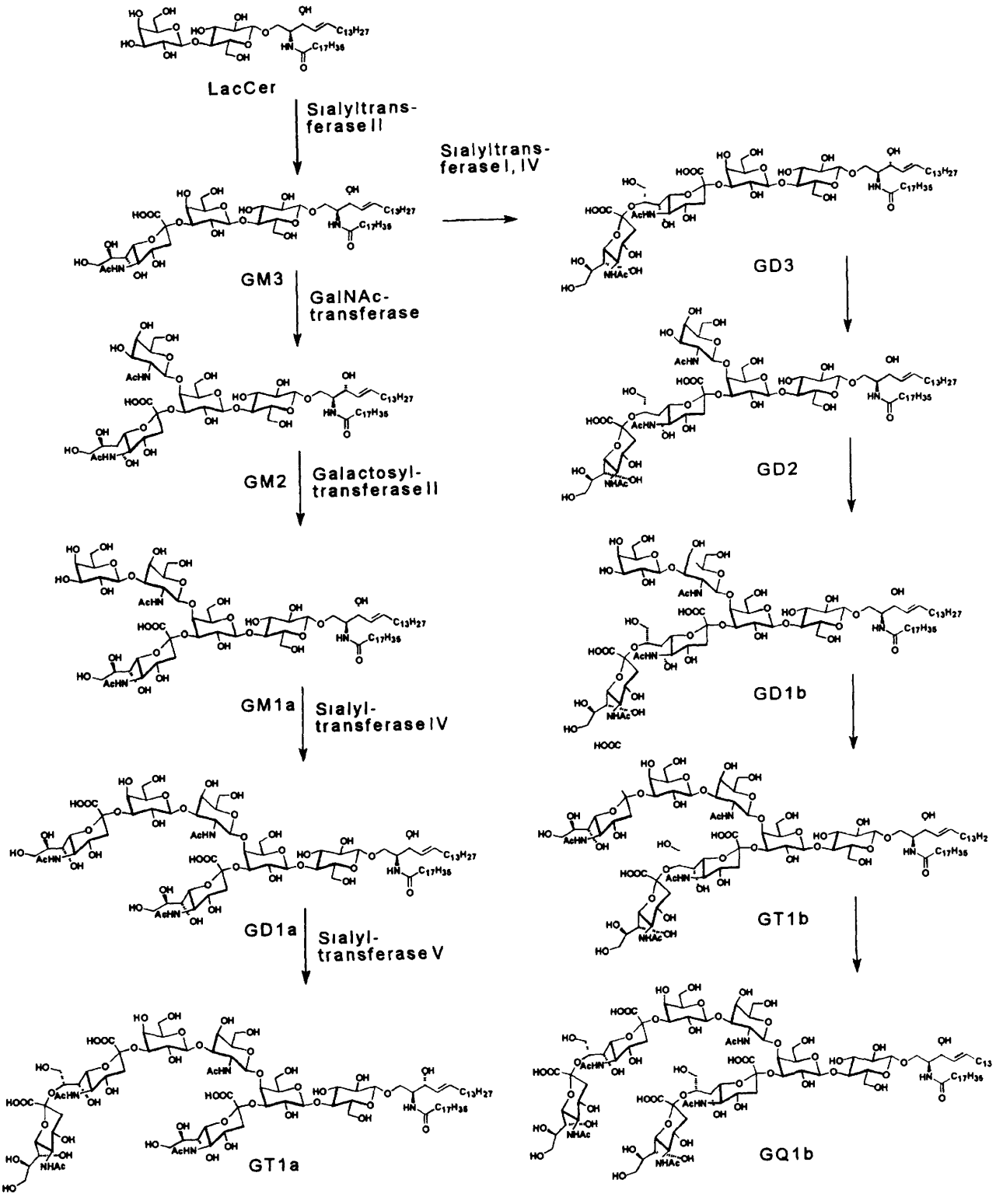

Figure 4 Biosynthesis of a and b series gangliosides, the predominant GSLs in adult human brain The biosynthesis of O series and c series gangliosides[6] is not shown Biosynthesis of O series gangliosides starts from LacCer by the action of GalNAc transferase to yield GA2 that of c series gangliosides from GD3 by the action of an α2,8 sialyltransferase to yield GT3 Heterogeneity within the lipid portion is not indicated

cules involved in the transmission of extracellular signals to intracellular regulatory systems [4,5] In the case of the structurally related glycerolipids it has been well established for several years that extracellular agents are able to cause the formation or the release of lipid-derived second messengers like diacylglycerol, inositol-1,4,5-trisphosphate and others Therefore, the function of phospholipids is not restricted to being structural constituents of the lipid bilayer of biological membranes Also sphingosine, ceramide and their 1-phosphorylated derivatives are currently discussed as signalling molecules

Increasing evidence suggests that ceramide plays a role comparable to its structural and functional glycerolipid counterpart, diacylglycerol (DAG) DAG, together with inositol- 1,4,5 trisphosphate, is released from phosphatidylinositol-4,5-bisphosphate by phospholipase C in response to an extracellular signal The observation that sphingomyelin hydrolysis can also be induced by extracellular agents in various cell types, like lymphocytes, myelocytes or fibroblasts, led to the discovery of the so called sphingomyelin cycle Tumour necrosis factor, α,γ-interferon or interleukin-1, which act on receptors in the plasma membrane, but also calcitriol,

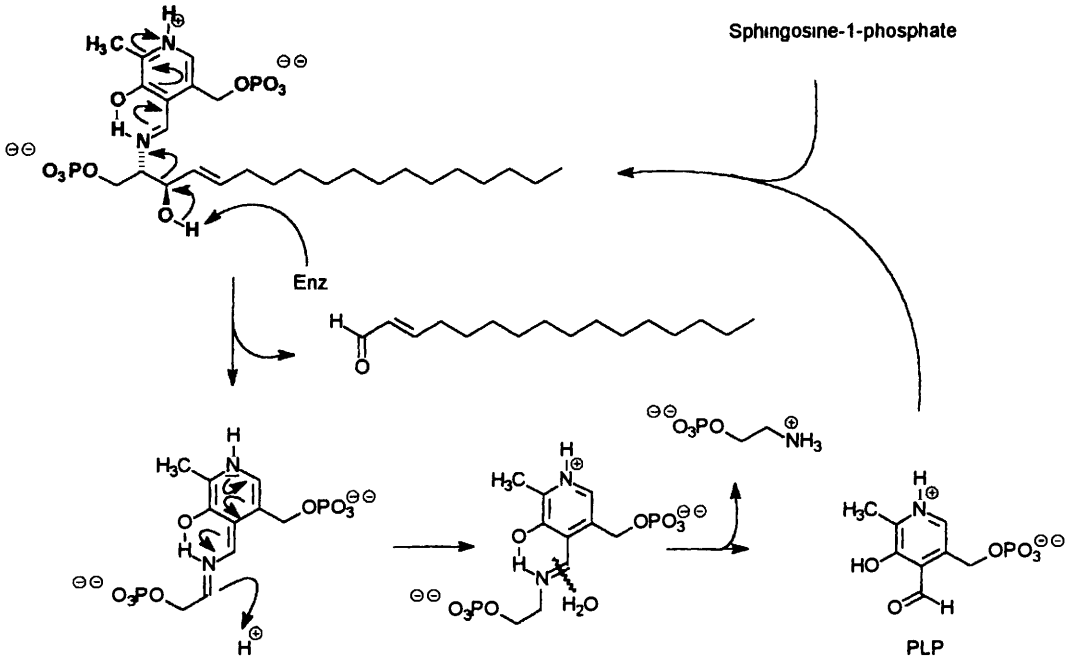

Figure 5 Sphingosine 1 phosphate lyase reaction

which acts on intracellular receptors cause the formation of ceramide The cellular and molecular effects of these extracellular agents, inhibition of cell growth, induction of differentiation, modulation of protein phosphorylation or regulation of gene transcription, are mimicked by application of a membrane permeable ceramide derivative, C2-ceramide, which is the abbreviation used for *N*-acetyl-sphingosine Importantly, the effects of C2-ceramide are generally not observed with the corresponding saturated derivative, C2-dihydroceramide This finding suggests a specific interaction between ceramide and an intracellular target molecule The identity of the cellular targets of ceramide and other molecules downstream within the signal flow is not known unambiguously A ceramide-dependent kinase, a phosphatase and a protein kinase C subtype are currently under investigation

In general, ceramide appears to mediate antimitogenic effects like cell differentiation, cell cycle arrest and cell senescence The most spectacular among the various cellular roles of ceramide is that of a physiological mediator of programmed cell death Programmed cell death, or apoptosis, is a well-defined process regulated by biochemical pathways, which are only partially clarified to date It is controlled by receptor-mediated mechanisms which in turn activate intracellular signal cascades which influence the phosphorylation status of target proteins, and finally gene expression Beside the sphingomyelin cycle, several signal transduction pathways to apoptosis seem to be involved in this process, which is necessary for normal development of organs, tissues and the immune system

A large number of events are reported to be influenced by sphingosine or sphingosine-1-phosphate In these cases a clear coupling between extracellular receptor activation, intracellular elevation of sphingosine or sphingosine-1-phosphate and corresponding cellular responses is less evident compared to ceramide

Other effects of sphingolipids on signal transduction[5] are the inhibition of protein kinase C by sphingosine and lysoGSLs lacking the amide-bonded fatty acid Sphingosine-1-phosphate mediates mitogenic effects, in contrast to ceramide It induces proliferation of Swiss 3T3 cells and stimulates the liberation of calcium ions from internal sources Since ceramide, sphingosine and sphingosine-1-phosphate are metabolically coupled, it is not clear which of these molecules is responsible for a distinct effect and why this pathway is mitogenic in some cells and antiproliferative in others To date it awaits elucidation how the cell avoids confusion between the function of these molecules either as metabolic intermediates or second messengers In other words, how the cell regulates normal metabolism as opposed to signal dependent events

5 Inhibition of Sphingolipid Biosynthesis

The precise role of cell surface GSLs as well as their metabolic intermediates for cell function is not defined A strategy to clarify their cellular and molecular roles is the interruption of their biosynthetic pathway at a definite step This can be achieved either by inhibitors or by the generation of mutant cells (or animals), which are deficient in a distinct biosynthetic enzyme (see ref 7 for review) The effects of these approaches are twofold the cell is depleted of metabolites downstream of the inhibited or mutated enzyme On the other hand metabolites upstream of the blocked step can accumulate allowing the investigation of their biological function This is of particular importance for sphingolipid biosynthesis since ceramide and the catabolic metabolite sphingosine as well as their phosphorylated derivatives are currently discussed as signalling molecules

As we are focusing on low molecular mass inhibitors of GSL biosynthesis, mutant cells as well as inhibitors of sphingolipid degradation are outside the scope of this review However, important contributions to the understanding of GSL metabolism and function may arise with the aid of these valuable tools

Several inhibitors of GSL biosynthesis have been described They have been isolated from natural sources or have been generated by design and chemical synthesis Most of them act on early steps of the synthetic pathway and have a lipid-like structure The compounds share the advantage of a higher membrane-permeability compared to carbohydrate-based inhibitors Suitable inhibitors of glycosyltransferases are only available for the steps associated with the addition of the first two sugars leading to glucosyl- and lactosyl-ceramide

5.1 Inhibition of Serine Palmitoyltransferase (SPT)

5 1 1 Cycloserine, Fluoroalanine, Chloroalanine

The SPT is inhibited by suicide inhibitors of PLP-dependent enzymes which are directed against the serine binding site L-Cycloserine[10] leads to a reduction of GalCer levels in mouse brain but has little effect on gangliosides and sphingomyelin SPT is also irreversibly inhibited by β-chloro- and β-fluoro-alanine with IC_{50}

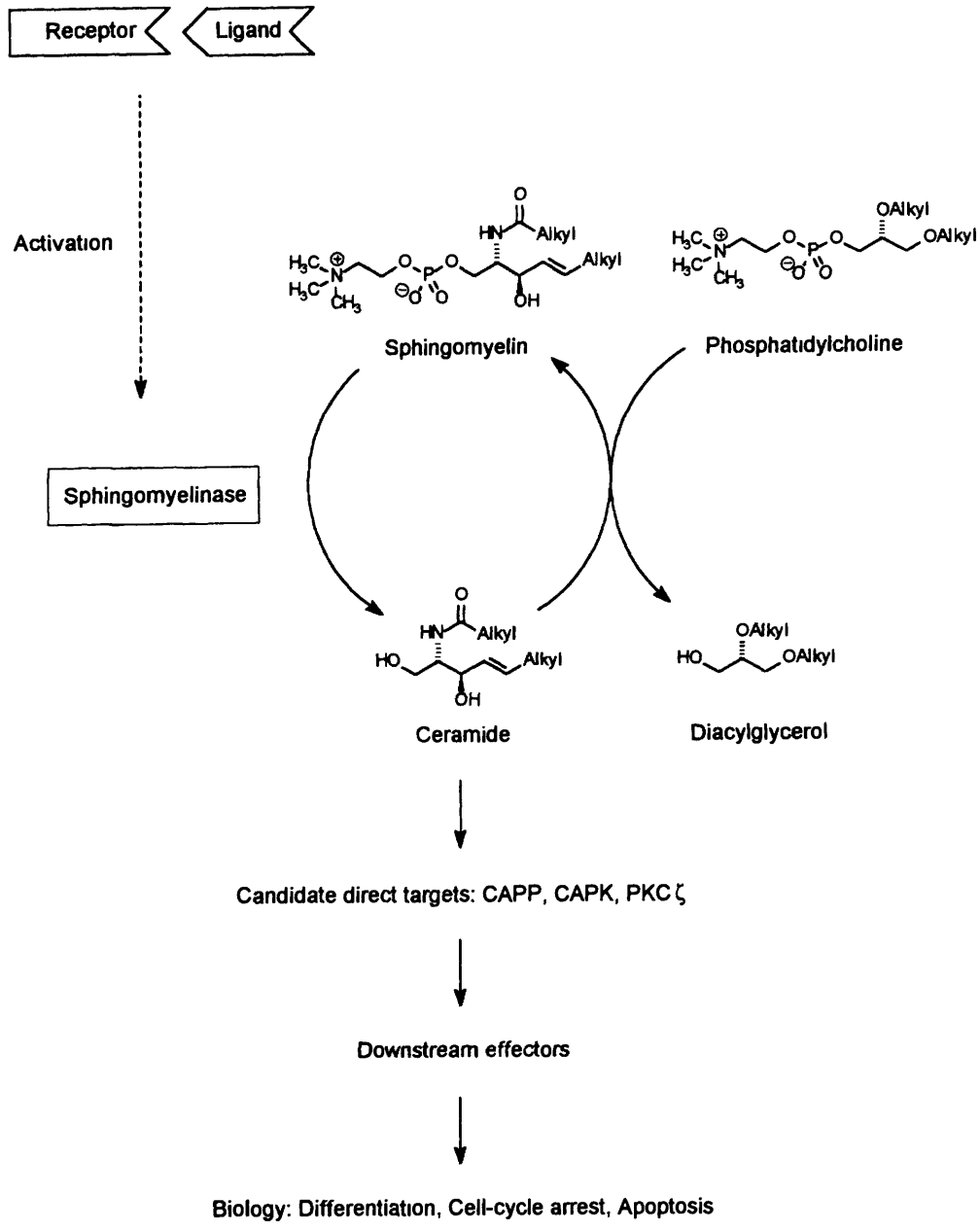

Figure 6 Spingomyelin cycle (modified from ref 4) See text for details [38]

values of about 50 μM [11] Inhibition can be blocked by high serine concentrations These compounds seem to be suicide inhibitors of several PLP-dependent enzymes and therefore of limited use in clarifying sphingolipid function Especially high SPT activity is found in human keratinocytes, which is inhibited by L-cycloserine and β-chloroalanine with IC_{50} values of 3 0 and 25 μM, respectively [12]

5 1 2 Sphingofungin

Two compounds with structural relationship to sphingolipids, sphingofungin B and C showed a broad spectrum antifungal but no antibacterial activity They have been isolated from a culture of *Aspergillus fumigatus* and have been investigated towards inhibition of sphingolipid biosynthesis in yeast (*Saccharomyces cerevisiae*) Sphingofungin B caused an inhibition of *de novo* sphingolipid synthesis (IC_{50} = 8 nM) measured by incorporation of [^{3}H]inositol into yeast sphingolipids [13] Yeast SPT is inhibited by sphingofungin B (IC_{50} = 20 nM) *in vitro* and also by the 5-*O*-acetyl derivative, sphingofungin C (IC_{50} = 20 nM) Inhibition is accompanied by growth inhibition and cell death was observed in growing cultures The effect of sphingofungin can be reversed by addition of phytosphingosine, but not sphingosine This could be expected since yeast sphingolipids contain phytosphingosine instead of sphingosine as major long chain base The sphingofungins act competitively with respect to serine for yeast and mammalian SPT [13]

5 1 3 Myriocin (ISP-1)

Myriocin[14] is a structural analogue of the sphingoid backbone and inhibits biosynthesis of ceramide and the two major GSLs in yeast, inositolphosphorylceramide (IPC) and mannosyl-IPC (MIPC) It causes a reduction in the rate of transport of GPI-anchored proteins to the Golgi apparatus and the remodelling of the GPI-anchor to ceramide-containing structures

ISP-1 is a very potent immunosuppressant of fungal origin It turned out to be identical with the antibiotics myriocin and thermozymocidin In contrast to the widely used immunosuppressants cyclosporine and FK-506, ISP-1 does not interfere with interleukin-2 production but suppressed the IL 2 dependent growth of a cytotoxic murine T-lymphocyte cell line, CTLL-2 SPT of these cells is inhibited *in vitro* in a noncompetitive manner with an apparent

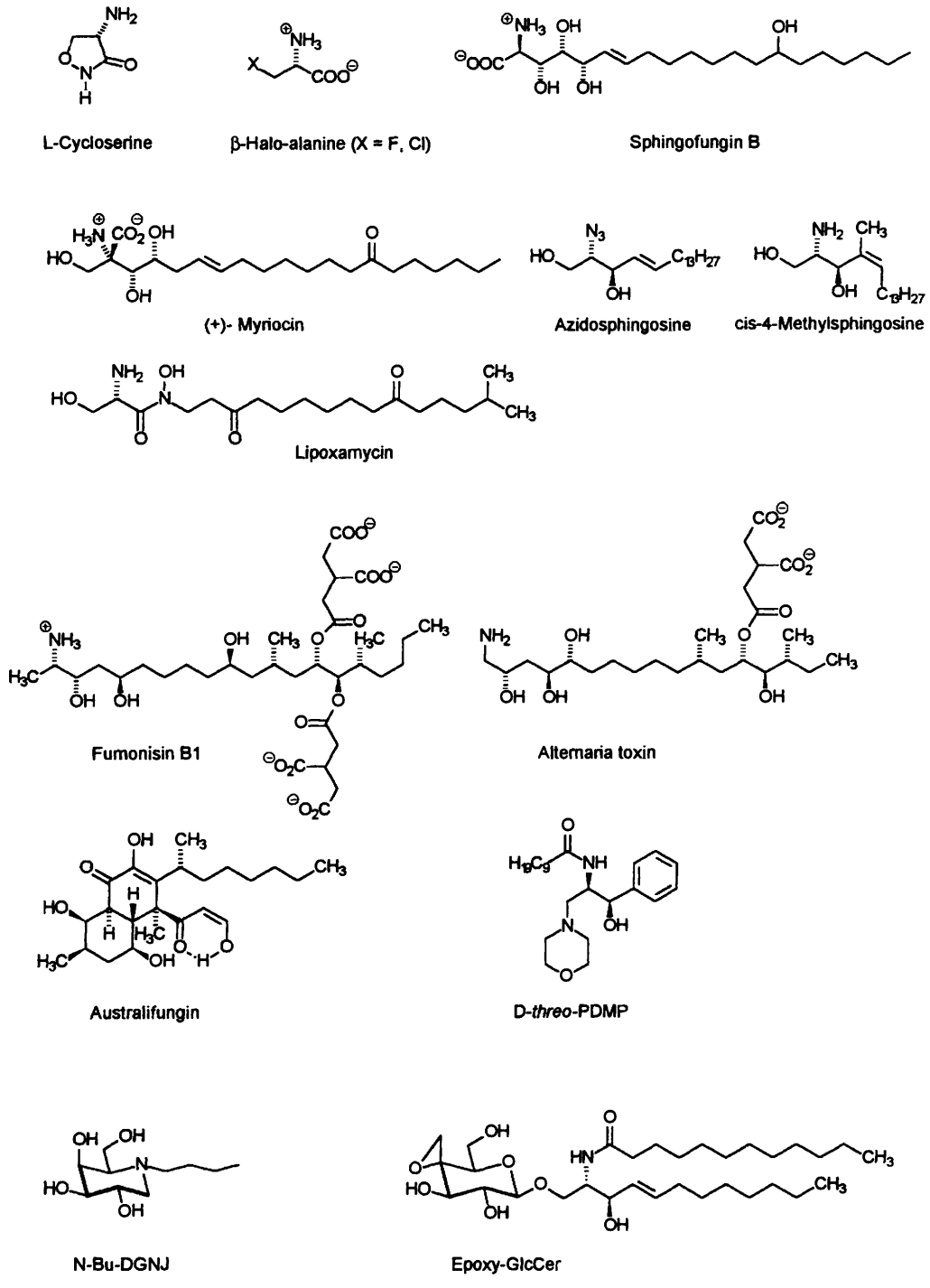

Figure 7 Inhibitors of sphingolipid biosynthesis. The absolute configuration of the tricarballylic acid side chains in fumonisin B1 and alternaria toxin are not indicated.

inhibition constant of 0.28 nM.[15] SPT inhibition was accompanied by suppression of T cell growth which could be restored by C2 ceramide, sphinganine or sphingosine-1-phosphate, but not by sphingomyelin, GlcCer, GalCer or GM3. Later it was assumed that growth suppression of CTTL-2 cells by ISP-1 was due to induction of apoptosis in these cells.[16]

5.1.4 Lipoxamycin

Lipoxamycin has been reported to inhibit SPT from *Saccharomyces cerevisiae* (IC_{50} = 21 nM) *in vitro*.[17] Also the corresponding 13-hydroxy-derivative inhibits (IC_{50} = 88 nM). Ten-fold lower IC_{50}-values were obtained against SPT from cultured HeLa cells. The high toxicity of the compound when applied to mice subcutaneously or topically is remarkable. Also, antifungal activity against several human pathogens was found which could be reversed by sphinganine or phytosphingosine.

5.1.5 SPT downregulation

Other agents have been reported to reduce SPT activity without inhibiting the enzyme directly. The fact that D-*erythro*-sphingosines of various chain length can down-regulate SPT activity suggests an autoregulatory mechanism in sphingolipid biosynthesis. The mechanism of SPT downregulation is not known, but interaction of sphingoid bases with a transcriptional factor followed by reduction of SPT biosynthesis would be a possible mechanism. In this way, the cell might prevent overproduction of these cytotoxic molecules.

A similar effect is exerted by synthetic analogues of sphingosine: D-*erythro*-azidosphingosine either with *trans* or *cis* double bond downregulates SPT activity in primary cultured neurons.[18] These compounds are metabolically stable due to the fact that acylation to ceramide is not possible. In contrast to sphingosine, *de novo* sphingolipid biosynthesis is strongly inhibited by this compound in concentrations lower than 50 μM.

Also the synthetic 4-methyl derivative of *cis*-sphingosine downregulates SPT activity (IC_{50} = 10 μM), causes a transient increase of the intracellular concentration of calcium ions and behaves as a potent mitogen in quiescent Swiss 3T3 fibroblasts. Thymidine incorporation into DNA is stimulated tenfold at 10 μM concentration. Moreover the compound initiates drastic morphological alterations of the cells and initiates cell death.[19] Both the *cis*-double bond and the 4-methyl group are necessary for the observed effects since the *trans*- and 5-methyl derivatives are ineffective. Work is in progress to discriminate between the effects – basically biosynthesis inhibition, mitogenic effect and morphological alterations – with the aid of synthetic analogues of the compound.

5.2 Inhibitors of Sphinganine *N*-Acyltransferase

5.2.1 Fumonisin

Fusarium moniliforme is a mould frequently found on corn and grains. Consumption of contaminated agricultural products leads to diseases in animals and correlates with oesophageal cancer in humans. Mycotoxins from *F. moniliforme*, the fumonisins have been shown to cause the diseases associated with *F. moniliforme* uptake. Fumonisins B1 and B2 (lacking the 10-hydroxy group of FB1), have been identified as inhibitors of sphinganine-*N*-acyltransferase with IC_{50}-values of about 0.1 μM.[20,21] They are structural analogues of sphingoid bases with the 1-OH function missing in fumonisin. This may contribute to persistence of inhibition since long chain bases are cleaved only after phosphorylation in the 1-position. The tricarballylic acid moiety and the 5-OH group of fumonisins are important but not critical for inhibition: Fumonisin B3 lacking the 5-OH group is still active, albeit at 1 μM concentration. A similar value is obtained for fumonisin B1H prepared by mild base cleavage of the tricarballylic acid. Inhibition of ceramide formation is accompanied by accumulation of its biosynthetic precursor sphinganine. On the one hand this accounts for some effects of these toxins since long chain bases are known to be toxic and mitogenic at low concentrations. On the other hand, the ratio of sphinganine to sphingosine in the serum of animals is a sensitive means to detect fumonisin consumption.

5.2.2 Alternaria toxin

Alternaria toxin is a phytotoxin with structural similarity to the sphingolipid backbone. It inhibits sphingolipid biosynthesis (IC_{50} = 1 μM) on the stage of ceramide formation[21] but is of limited use due to cytotoxicity in mammalian cell culture and less potency compared to fumonisins.

5.2.3 Australifungin

Recently, the fungal metabolite australifungin was reported as an inhibitor of sphinganine *N*-acyltransferase *in vitro*. The IC_{50} value is less or equivalent to fumonisin B1, dependent on the cell type.[22] Australifungin is a potent antifungal agent and shows no structural similarity to sphingoid bases.

5.3 Inhibitors of GlcCer Synthase

Inhibition of ceramide biosynthesis results in depletion of cell surface GSLs and of sphingomyelin. This is a principle disadvantage of such inhibitors as far as they should provide insight into the function of cell surface carbohydrates. The observed phenomena might be obscured by effects due to the inhibition of membrane biosynthesis due to depletion of sphingomyelin. Therefore, the availability

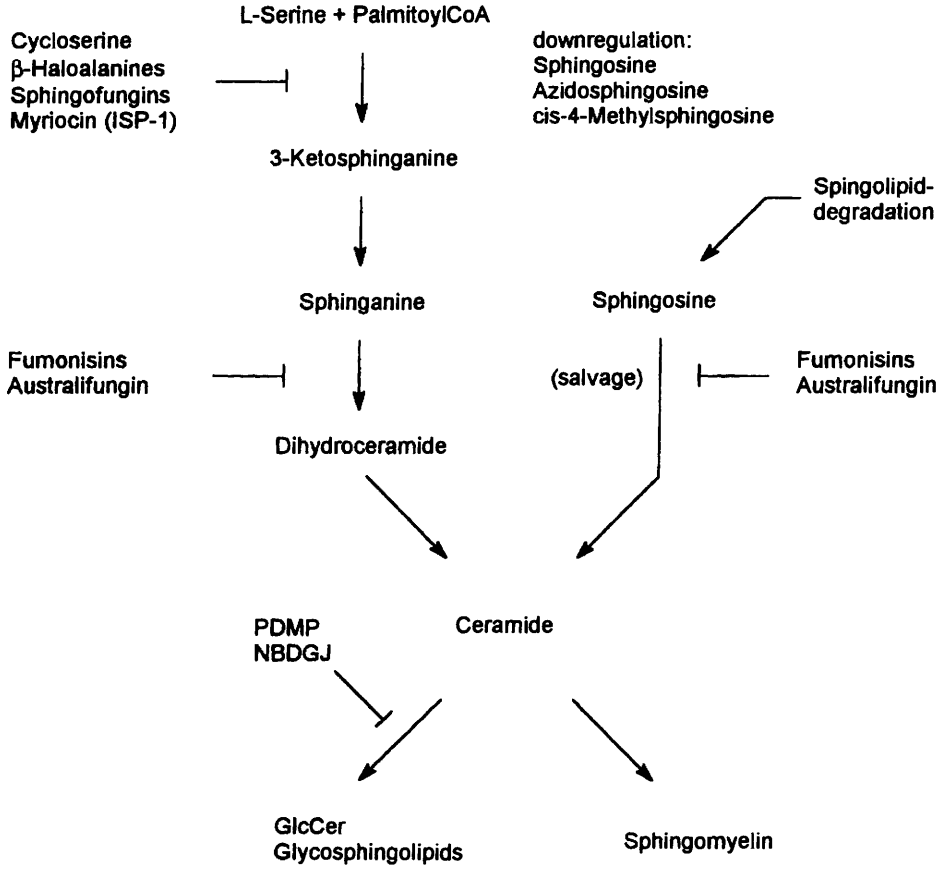

Figure 8 Flow scheme of sphingolipid biosynthesis.

of potent and specific inhibitors of glucosylceramide biosynthesis would be highly desirable Two classes of synthetic compounds are reported to date as inhibitors of GlcCer synthase [23]

5 3 1 D-*threo*-PDMP

D-*threo*-PDMP [D-*threo*-(1*R*,2*R*)-1-phenyl-2-decanoylamino-3-morpholinopropan-1-ol] is the most thoroughly investigated member of a series of ceramide analogous inhibitors of GlcCer synthase [24] D-*threo*-PDMP inhibits formation of glucosylceramide in concentrations of 2 5 to 10 μM and has already been used in functional studies [25] D-*threo*-PDMP shows a mixed type inhibition mode with respect to ceramide and is uncompetitive for the glucosyl donor The apparent K_i is 0 7 μM In concentrations of more than 25 μM also sphingomyelin biosynthesis and protein transport along the secretory pathway are inhibited [26] PDMP stereoisomers and analogues have been synthesised and investigated, among them D-*threo*-1-morpholino-1-deoxyceramide (73% inhibition of GlcCer synthase in Madin–Darby canine kidney (MDCK) cells at 5 μM concentration compared with 20% inhibition through D-*threo*-PDMP) [27] Concentrations of more than 100 μM D-*threo*-PDMP or 10 μM of its palmitoyl derivative PPMP are toxic for HL-60 cells D-*threo*-PDMP exhibits multiple cellular effects like cell growth inhibition, eventually mediated by ceramide accumulation or inhibition of sphingomyelin synthesis, and is metabolised by cytochrome P450 Various other effects of PDMP, its isomers and analogues are summarized in Ref 25

5 3 2 DGNJ

Recently it has been shown that a synthetic derivative of the naturally occurring glycosidase inhibitor deoxynojirimycin, *N*-butyldeoxynojirimycin (NB-DNJ), inhibits GlcCer formation with an IC_{50} value of 20 μM NB-DNJ was known to inhibit HIV replication *in vitro*, obviously *via* inhibition of viral glycoprotein processing Butyldeoxygalactonojirimycin (*N*-Bu-DGNJ) is a related compound with improved selectivity (IC_{50} = 40 μM) [28] Glycosidases such as β-gluco- and β-galacto cerebrosidase, α-glucosidase I and II are either not or weakly inhibited by this compound Structure–activity relationships revealed that the alkyl chain length requires three carbons for inhibition with C_4 and C_6 being optimal Longer chain length leads to improved inhibition *in vitro* but also to cytotoxicity *in vivo* The corresponding derivatives of mannose, fucose and GlcNAc are inactive A major advantage is their metabolic stability and their low toxicity, up to 2–5 mM are tolerated The mechanism of inhibition by alkylated iminosugars is not known but it might be argued that they mimic the transition state of the transferase reaction This is of particular importance since with rare exceptions, attempts for the development of potent and selective glycosyl transferase inhibitors have not been successful to date

5.4 Inhibition of LacCer Synthase

A synthetic truncated derivative of glucosylceramide bearing an additional epoxide function in the 4-position of the glucose residue caused an irreversible and concentration dependent decrease of the specific activity of LacCer synthase [29] In primary cultured neurons of chick embryos the biosynthetic GSL patterns changed in such a way that labelling of GSL downstream from GlcCer was reduced and label accumulated in GlcCer The gluco derivative was active while the derivative with galacto configuration showed no effect Inhibition of LacCer synthase *in vitro* by epoxy-GlcCer was much less pronounced 250 μM concentration was required to cause only 30% inhibition of enzyme activity Therefore, it cannot be excluded that the observed effect *in vivo* is due to inhibition of a GlcCer translocator or a transcriptional factor

5.5 Miscellaneous

A recent study[30] shows that azidothymidine (AZT), which is used as chemotherapeutic agent in the treatment of HIV infection, inhibits cellular glycosylation of glycolipids and glycoproteins in clinical relevant concentrations of 1–5 μM The primary intracellular metabolite of AZT, the monophosphate, possibly inhibits the uptake of nucleotide sugars by the Golgi apparatus, thereby reducing the content of complex acidic GSLs Toxic side effects of AZT especially on maturation of blood stem cells seems to be due to modified glycosylation patterns on these cells and not on inhibition of DNA replication

Antisense oligodeoxynucleotides to GM2 synthase and GD3 synthase led to downregulation of more complex GSLs downstream of GM3 in the biosynthetic pathway (Figure 4) The human leukaemia cell line HL-60 treated with these antisense-DNAs underwent monocytic differentiation under these conditions and accumulated GM3 [31] Since suitable low molecular mass inhibitors for glycosyl transferases are not available today, this approach constitutes a promising tool for the investigation of GSL function

6 Perspective: Therapeutic Potential of GSL Biosynthesis Inhibitors

There are several potential fields for the application of inhibitors of GSL biosynthesis Only three of them are briefly mentioned here

6.1 Chemotherapy of Parasite Infections

Various observations indicate that inhibition of sphingolipid biosynthesis can become helpful in the treatment of diseases caused by lower eukaryotes, *e g* fungal and protozoan infections The great number of infections (one million children die of malaria each year) and the occurrence of drug resistance led to an urgent requirement for new drugs in this field

Recently it has been shown that D,L-*threo*-PDMP, which is usually used as an inhibitor of glucosylceramide formation, and a chain homologue of it effectively inhibited sphingomyelin formation in the human malaria parasite, *Plasmodium falciparum* Inhibition was achieved with concentrations of less than 1 μM and accompanied by inhibition of parasite proliferation in culture [32] Plasmodial sphingomyelin synthase appears to be a rational target for the development of antimalarial drugs

Many observations indicate that inhibition of GSL biosynthesis might become advantageous in the treatment of parasitic or fungal infections In contrast to vertebrates, lower eukaryotes like yeast (*Saccharomyces cerevisiae*) have only a simple set of GSLs which are also predominantly found in the plasma membrane Studies with mutant cells (reviewed in ref 7) indicate that sphingolipids appear to be essential for the viability of yeasts Also inhibition studies with the fungal metabolite myriocin showed that ceramide stores are rapidly depleted in these fast proliferating cells if ceramide is not regenerated by biosynthesis [14] Often natural compounds with sphingolipid-like structure of fungal or marine origin[33] have antifungal properties Both in MDCK cells and yeast, the intracellular transport of glyosyl-phosphatidylinositol-anchored proteins and of sphingolipids seems to be tightly coupled and commonly regulated [34] Since the content of GPI-anchored proteins of the cell surface is particularly high in lower eukaryotes, these organisms should be sensitive towards inhibition of this process Furthermore, the H^+-ATPase of such organisms is dependent on inositolphosphoryl ceramide which is not found in higher eukaryotes [35]

6.2 Antiproliferative Agents

Signal transduction is an attractive target for the discovery of antiproliferative agents A pharmacological approach of this type has the potential advantage that the action of a drug is not necessarily accompanied by toxic side effects associated with the action of traditional chemotherapy based on the inhibition of DNA synthesis Efforts in this direction are rare within this very new area of research

The generic synthesis of aryl-fused sphingosine derivatives designed as agents for the topical treatment of inflammatory skin disorders like psoriasis has been reported [36] Compounds of this type inhibit protein kinase C in micromolar concentrations *in vitro*

6.3 Treatment of Sphingolipidoses

Sphingolipidoses are a group of inherited disorders due to impaired proteins responsible for sphingolipid catabolism within the lysosomes of the cell With rare exceptions a treatment of these often lethal diseases is not possible to date Several factors influence the pathogenesis of the sphingolipidoses [9] Accumulation of lipids occurs mainly in those cell types and organs in which the lipids are predominantly synthesized or taken up by endocytosis In Tay–Sachs disease, for example, β-hexosaminidase A is deficient This causes accumulation of the ganglioside GM2 in neuronal cells, the main site for synthesis of gangliosides (sialic acid-containing GSLs) According to a kinetic model,[37] severity and onset of these diseases depend on the residual enzyme activities Their decrease beyond a critical threshold value leads to the accumulation of the substrate of the deficient enzyme since substrate influx into the lysosomes exceeds the degradation rate Substrate influx into the lysosome due to biosynthesis can be reduced by inhibiting this process [23 24] From kinetic considerations it should be possible to influence the severity as well as the onset of these diseases with the aid of synthetic inhibitors

7 Outlook

Many questions about the details of sphingolipid biosynthesis and function remain open and might in part be answered with the aid of enzyme inhibitors or receptor ligands Inhibitors of dihydroceramide desaturase are not available They would permit purification of the enzyme and eventually dissect structural and signalling sphingolipid pools Also no suitable inhibitors of sphingomyelin synthase are known They would be interesting candidates for the treatment of malaria infections as discussed above More potent and selective inhibitors are required for the treatment of sphingolipidoses

Ligands of sphingolipid binding proteins might be helpful in confirming the current hypotheses about details of sphingolipid signalling function The development of antiproliferative drugs based on this approach would yield pharmacological application

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