Note
An Inhibitor of Glucosylceramide Synthase Inhibits the Human Enzyme, but Not Enzymes from Other Organisms

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Specific inhibitors of glucosylceramide biosynthesis are used as drugs for the treatment of some human diseases correlated to glycosphingolipid metabolism. The target of the presently available inhibitors is the human glucosylceramide synthase (GCS), but effects on enzymes from other organisms have not been studied. We expressed cDNAs encoding GCS enzymes from lower animals, plants, fungi, and bacteria in the yeast P. pastoris. In vitro GCS assays with the GCS inhibitor D-threo-1-(3’4’-ethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol showed that this inhibitor did not affect non-human GCS enzymes.

Key words: glucosylceramide synthase (GCS); glycosphingolipids; D-threo-1-(3’4’-ethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (ethylenedioxy-P4)

A membrane-bound glucosylceramide synthase (GCS, EC 2.4.1.80) transfers glucose to ceramide, resulting in the formation of glucosylceramides (GlcCer) which serve as precursors for the biosynthesis of a multitude of higher glycosylated sphingolipids.1) Thus the regulation or external manipulation of GCS gene expression or its catalytic activity interferes with both the function of ceramide as a second messenger2) and all functions of GlcCer and higher glycosphingolipids.3–5) These functions include two phenomena of medical importance. First, turnover of higher glycosphingolipids requires their continuous but matching degradation. Functions of GlcCer and higher glycosphingolipids. 3–5) Thus the regulation or external manipulation of GCS gene expression or its catalytic activity interferes with both the function of ceramide as a second messenger2) and all functions of GlcCer and higher glycosphingolipids.3–5) These functions include two phenomena of medical importance. First, turnover of higher glycosphingolipids requires their continuous but matching degradation. Accumulation of these lipids at pathological levels due to degradation deficiencies results in glycosphingolipidoses, among which Gaucher’s disease is the most common lysosomal storage disorder. Inhibition of GlcCer biosynthesis by drugs can reduce the accumulation of higher sphingolipids.5) Secondly, the development of cancer cells towards apoptosis or proliferation and their level of multi-drug resistance depend on the ratio of ceramide to glycosphingolipids.7,8)

In view of these phenomena, both for basic research and for therapeutic use, efforts have been directed towards the development of specific inhibitors of GCS activity. The iminosugar deoxynojirimycin9) and D-threo-1-phenyl-2-decanoyl-amino-3-morpholinol-1-propanol (PDMP,10) were the parent compounds of two series of structurally related homologs.11) But despite their successful use in mammalian systems, GCS inhibitors have been used only in a few non-mammalian organisms such as insects, Plasmodium falciparum, and fungi.12–14) In previous experiments with Aspergillus fumigatus and Aspergillus nidulans, the PDMP derivative D-threo-1-(3’4’-ethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (ethylendioxy-P4)11) strongly inhibited GlcCer synthesis, germination, and hyphal growth,13) although the actual target was not identified. These results were not confirmed by studies on inhibition of the GCS enzyme by ethylenedioxy-P4, since an in vitro assay for fungal GCS was lacking.

To continue these investigations, we overexpressed GCS cDNAs from different organisms in the yeast P. pastoris to prepare cell-free membrane fractions suitable for in vitro determination of GCS activity. The GCS sequences used for these experiments were cloned from Homo sapiens, the plant Gossypium arboreum, the nematode Caenorhabditis elegans, the fungi Ustilago maydis, Aspergillus nidulans, Candida albicans, and Pichia pastoris, and the bacterium Agrobacterium tumefaciens. DNA fragments corresponding to the ORFs of the GCS sequences from U. maydis (GenBank accession no. XM_402111), A. nidulans (XM_412943), and A. tumefaciens (NP_354792; locus tag: AGR_C_3323) were amplified by PCR from genomic DNA and cloned into the plasmid pPIC3.5 (Invitrogen, Carlsbad, CA, U.S.A.). This vector makes possible the expression of heterologous sequences under the control of the strong AOX1 promoter. The GCS sequence from C. elegans was transferred from pYeCe215) into pPIC3.5. Cloning of the GCS sequences from H. sapiens, G. arboreum, C. albicans, and P. pastoris into pPIC3.5 was performed previously.15) For expression of the different GCS clones, a mutant of P. pastoris was transformed that does not contain glucosylceramide and sterolglucoside due to the inacti-
vation of the two corresponding glucosyltransferase genes. Expression cultures of the transformed cells were grown as described previously. After harvesting, the cells were used for lipid analysis and *in vitro* enzyme assays.

The expression of the GCS sequences from all organisms resulted in the accumulation of new glycolipids in the transformed yeast cells (Fig. 1). As observed previously, the expression of the human GCS resulted in the biosynthesis of several different GlcCer species and of monoglucosyldiacylglycerol (MGlcD). The structure of the ceramides in the various GlcCer species differed with regard to amide-linked fatty acids and sphingobases (details not discussed here). In confirmation of previous results, the expression of the GCS from *G. arboreum* resulted in the accumulation of GlcCer species and in the production of sterolglucoside. The expression of the GCS from *C. elegans*, *U. maydis*, *A. nidulans*, *C. albicans*, *P. pastoris*, and *A. tumefaciens* resulted in the synthesis of GlcCer species and several other glycolipids, as detailed elsewhere.

In summary, the expression of all the sequences annotated as GCS confirmed that the ORFs indeed encode the assigned enzymatic activity.

The *P. pastoris* cultures subjected to lipid analysis were also used for the determination of *in vitro* Gcs activity, as described previously. The assay was based on the incorporation of radioactivity from UDP-[14C]glucose into GlcCer. As enzyme source, a membrane fraction from transgenic yeast cultures was used, which at the same time provided internal ceramide as acceptor. The assay products were extracted, separated by TLC, and detected by radioscanning. In parallel assays the concentration of the GCS inhibitor ethylenedioxy-P4 was increased from 0 to 10, 20, and 50 μM. In the absence of inhibitor all GCS preparations showed activity, as was evident from the formation of radiolabelled GlcCer. In confirmation of previous data, the activity of the human GCS was inhibited in the presence of ethylenedioxy-P4 (Fig. 2A). On the other hand, none of the other GCS activities of animal, plant, fungal, or bacterial origin examined in the present investigation was inhibited by ethylenedioxy-P4 (Fig. 2B–H), as was evident from the insensitivity of GlcCer labelling towards all concentrations of the inhibitor tested. This result indicates that the inhibitor ethylenedioxy-P4 is highly specific for the human GCS enzyme.

This result might be important in view of the assumption that GlcCer and their derivatives are of vital, though partially unknown, importance for all organisms expressing GCS. The demonstration of significant differences in the inhibitor sensitivity of GCS enzymes from different organisms would suggest that it might be possible to develop an inhibitor specific to fungal GCS activity, but not affecting the human enzyme. This would revert the characteristics of the presently studied inhibitor and provide the chance for specific interference with functions vital for pathogenic fungi.

In this context, the strongly inhibitory effects of ethylenedioxy-P4 on GlcCer synthesis and the germination of conidia and hyphal growth of *A. nidulans* are interesting and puzzling. In view of our results here, the reduced GlcCer content of the cells might in fact not result from a direct inhibition of the GCS, since *in vitro*, fungal GCS was insensitive towards concentrations of ethylenedioxy-P4 that showed inhibition with intact...
cells of *A. nidulans*. The decrease in GlcCer might rather be caused by impairment of other enzymes of sphingolipid metabolism leading to declined GlcCer synthesis. Hence, the actual target of the inhibitor in *A. nidulans* remains to be determined.

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**References**


