The role of glycosphingolipids in insulin signaling and lipid metabolism
Bijl, N.

Citation for published version (APA):
Bijl, N. (2009). The role of glycosphingolipids in insulin signaling and lipid metabolism

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Improved glycemic control in obese rodents by combined pharmacological reduction of visceral glycosphingolipids and buffering of carbohydrate assimilation

Tom Wennekes¹, Alfred J. Meijer², Albert K. Groen², Rolf G. Boot², Johanna E. Groener², Marco van Eijk², Roelof Ottenhoff², Nora Bijl², Karen Ghauharali², Hang Song¹, Tom J. O’Shea³, Hanlan Liu³, Nelson Yew², Diane Copeland³, Richard J. van den Berg¹, Gijs A. van der Marel¹, Hermen S. Overkleeft¹, Johannes M. Aerts²*

¹Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, The Netherlands. ²Department of Medical Biochemistry, Academic Medical Center, Amsterdam, The Netherlands. ³Genzyme, Drug and Biomaterial R&D, Waltham, MA, USA

Submitted
Abstract

Objectives and background
The iminosugar N-(5-adamantane-1-ylmethoxy)-pentyl-L-deoxynojirimycin (AMP-DNM) potently controls hyperglycemia in rodent models of insulin resistance. The reduction of visceral glycosphingolipids by AMP-DNM is thought to underlie its beneficial action. It can however not be excluded that concomitant inhibition of intestinal glycosidases and associated buffering of carbohydrate assimilation adds to the effects of AMP-DNM. To establish firmly the mode of action of AMP-DNM, we developed a panel of hydrophobic iminosugars varying in configuration at C-4/C-5 and N-substitution of the iminosugar.

Results
From these we identified N-(5-adamantane-1-ylmethoxy)-pentyl-L-ido-L-deoxynojirimycin (L-ido-AMP-DNM) as a selective inhibitor of glycosphingolipid synthesis. L-ido-AMP-DNM lowered visceral glycosphingolipids in ob/ob mice and ZDF rats on a par with AMP-DNM. In contrast to AMP-DNM, L-ido-AMP-DNM did not inhibit sucrase activity in vitro, and did not reduce sucrose assimilation in vivo. L-ido-AMP-DNM treatment was significantly less effective in lowering blood glucose and reducing HbA1C.

Conclusions
We conclude that combined reduction of glycosphingolipids in tissue and buffering of carbohydrate assimilation produces a superior glucose homeostasis. Therefore AMP-DNM seems intrinsically more suited for controlling type 2 diabetes associated hyperglycemia, whilst L-ido-AMP-DNM is more attractive for treatment of diseases where exclusive reduction of glycosphingolipids is required such as the hereditary lysosomal glycosphingolipidoses.
Introduction

Coinciding with obesity, type 2 diabetes has reached epidemic proportions worldwide. Insulin resistance is one of the earliest detectable abnormalities during the development of type 2 diabetes. The precise cause for the rapidly increasing occurrence of insulin resistance has not been firmly established, but there is growing evidence that obesity and associated lipotoxicity play a crucial role (1). Recent literature links insulin resistance in tissues to the presence of excessive amounts of a particular group of lipids, the so-called glycosphingolipids. These lipids are found in specific (detergent resistant) membrane microdomains in close physical proximity to the insulin receptor (2). A regulatory role for glycosphingolipids, in particular the ganglioside GM3, in insulin sensitivity is substantiated by a rapidly growing body of experimental evidence (3). Interaction of gangliosides and the insulin receptor was originally described by Nojiri et al. (4), demonstrating the ganglioside-mediated inhibition of insulin-dependent cell growth of leukemic cell lines. Tagami and coworkers were the first to demonstrate that addition of GM3 to cultured adipocytes suppresses phosphorylation of the insulin receptor and its down-stream substrate IRS-1, resulting in reduced glucose uptake (5). Inokuchi and coworkers reported that exposure of cultured adipocytes to TNF-α increases GM3 and inhibits IR and IRS-1 phosphorylation. This was found to be counteracted by 1-phenyl-2-decanoylamino-3-morpholinopropanol (PDMP), an inhibitor of glycosphingolipid biosynthesis (6). Mutant mice lacking GM3 have been reported to show an enhanced phosphorylation of the skeletal muscle insulin receptor after ligand binding and to be protected from high-fat diet induced insulin resistance (7). Consistent with this is the recent report on improved insulin sensitivity and glucose tolerance in mice with increased expression of the GM3 degrading sialidase Neu3 (8). Conversely, GM3 levels are elevated in the muscle of certain obese, insulin resistant mouse and rat models (5). Altered sphingolipid metabolism, reflected by increased glycosphingolipid levels, has recently also been documented in relation to neuronal pathology in diabetic retinopathy (9). Very recently Kabayami et al provided evidence that the interaction of GM3 with the insulin receptor is mediated by a specific lysine residue located just above the transmembrane domain of the receptor, and that excess levels of GM3 promote dissociation of the insulin receptor from caveolae, a location which is essential for insulin signal transduction (10).

The value of pharmacological lowering of excessive glycosphingolipid levels to improve insulin sensitivity has recently been demonstrated by others and us (3, 11-13). Holland and coworkers reported that inhibition of the synthesis of ceramide, the precursor of glycosphingolipids, markedly improves glucose tolerance and
prevents the onset of overt diabetes in obese rodents (11). Zhao et al demonstrated that inhibition of the first step in the biosynthesis of glycosphingolipids, catalyzed by glucosylceramide synthase (GCS), exerts beneficial effects. The GCS inhibitor (1R,2R)-nonanoic acid[2-(2',3'-dihydro-benzo[1,4]dioxin-6'-yl)-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide-L-tartaric acid salt (Genz-123346) lowered blood glucose and HbA1C levels and improved glucose tolerance in insulin resistant rodents (12). Finally, we showed that treatment of various rodent models of insulin resistance with the hydrophobic iminosugar N-(5-adamantane-1-yl-methoxy)-pentyl-1-deoxynojirimycin (AMP-DNM), a well tolerated and potent inhibitor of GCS, very markedly lowered circulating glucose levels, improved oral glucose tolerance, reduced HbA1C, and improved insulin sensitivity in muscle and liver (13). In addition, AMP-DNM treatment was found to cause a marked improvement in insulin sensitivity of adipocytes and to reduce inflammation in adipose tissue of obese mice (14).

The marked beneficial effect of AMP-DNM on glycemic control in obese mice might not only be exerted by reduction of glycosphingolipids in tissues. The same compound was found to inhibit in vitro the enzymatic activities of some glycosidases like sucrase and maltase (15, 16). The latter effect is similar to the mode of action of registered anti-diabetics, including the iminosugar Miglitol (N-(1-hydroxyethyl)-1-deoxynojirimycin) (17). To establish whether concomitant reduction of carbohydrate assimilation by AMP-DNM contributes to its beneficial effect on glycemic control, we looked for an analogue of AMP-DNM that inhibited GCS more specifically. Therefore we developed a range of structural analogues of AMP-DNM varying in configuration at C-4 and C-5 (glycopyranose numbering) of the piperidine ring and in the type of substitution on the endocyclic nitrogen.

We here demonstrate that both the D-galacto (C-4 epimer) and L-ido (C-5 epimer) analogues of AMP-DNM, but not the L-altro (C4 and C5 epimer) analogue, are still potent inhibitors of GCS. Of these, N-(5-adamantane-1-yl-methoxy)-pentyl-L-ido-1-deoxynojirimycin (L-ido-AMP-DNM) is the most specific and potent iminosugar-based GCS inhibitor reported to date. L-ido-AMP-DNM, was head-to-head compared with AMP-DNM in ob/ob mouse and ZDF rat models of insulin resistance and type 2 diabetes. The ability of these compounds to improve insulin sensitivity and buffer sucrose assimilation was examined. Their effects on glycemic control were also compared with those of two registered deoxynojirimycin-type drugs Miglustat ((N-butyl-1-deoxynojirimycin) and Miglitol. The outcome of these investigations is here reported, indicating that the prominent beneficial effect of AMP-DNM on glycemic control results from dual inhibition of both carbohydrate assimilation and visceral glycosphingolipid synthesis.
Experimental Procedures

Animals
Experimental procedures were all approved by the appropriate Ethics Committee for Animal Experiments. C57Bl/6J and ob/ob mice (C57Bl/6J background) were obtained from Harlan (Horst, The Netherlands) and ZDF (ZDF/GMi-fa/fa) rats and lean littermates from Charles River Laboratories (Wilmington, USA). Animals were housed in a light- and temperature controlled facility. Animals were fed a commercially available lab chow (RMH-B, Hope Farms BV, Woerden, The Netherlands) containing about 6% fat and ~0.01% cholesterol (w/w). Iminosugars were mixed in the food. In the case of experiments with ZDF rats compound was administered by oral gavage two times daily.

 Plasma and tissue sampling
Blood samples were collected by either tail vein or retro-orbital plexus puncture. Animals were sacrificed under isoflurane anaesthesia. A large blood sample was collected by cardiac puncture. Tissues were quickly removed and frozen for further analysis.

Iminosugars
AMP-DNM (N-(5-adamantane-1-yl-methoxy)-pentyl-1-deoxynojirimycin) was synthesized as described previously (16, 18). The synthesis, characterization and purity of L-ido-AMP-DNM and all other reported iminosugars is described in SI. Plasma levels of AMP-DNM and L-ido-AMP-DNM were determined by mass spectrometry following high pressure liquid chromatography (Xendo, Groningen, The Netherlands). N-butyl-DNM was obtained from Sigma (St Louis, USA).

Analysis of lipids and measurement of enzyme activities
Lipids were extracted according to Folch et al. (19). Ceramide and glucosylceramide collected from the chloroform phase were determined by HPLC analysis of o-phthalaldehyde-conjugated lipids according to a procedure described previously (20). The chloroform layer was thoroughly dried and deacylation of lipids was performed in 0.5 ml 0.1 M NaOH in methanol in a microwave oven (CEM microwave Solids/Moisture System SAM-155). After deacylation 0.5 ml methanol and 2 ml chloroform were added and phase separation was performed. The chloroform layer was dried under N2 and the deacylated lipids were taken up in 250 μl methanol. Deacylated glycolipids were derivatised on line for 30 min with o-phthalaldehyde. Analysis was performed using an HPLC system (Waters Associates, Milford, MA) and a Hypersil
BDS C18 3 μm, 150 * 4.6 mm reverse phase column (Alltech). Chromatographic profiles were analyzed using Waters Millennium software. All samples were run in duplicate and in every run a reference sample was included. Ganglioside composition was determined by analysis of the acidic glycolipid fraction obtained after Folch extraction using chloroform/methanol/water (65:25:4) as solvent. Gangliosides were quantified following HPLC separation of oligosaccharides released from glycolipids by ceramide glycanase digestion (21). IC50 values of AMP-DNM for various enzyme activities were determined by exposing cells or enzyme preparations to an appropriate range of iminosugar concentrations. IC50 values for glucosylceramide synthase activity were measured using living cells with NBD-ceramide as substrate (22). IC50 values for the lysosomal glucocerebrosidase (GBA1) were measured using 4-methylumbelliferyl-beta-D-glucoside as substrate (23). IC50 values for the non-lysosomal glucocerebrosidase (GBA2) were measured with the same substrate as earlier described (24, 25). Lactase, maltase and sucrase were determined with homogenates of freshly isolated rat intestine using assay conditions described earlier (16). Debranching enzyme (α-1, 6-glucosidase) was measured with an erythrocyte preparation as enzyme source as described previously (16).

### Analysis of insulin signalling in liver

Livers were quickly collected and lysed in modified RIPA buffer as described earlier (13). Equal amounts of lysates were separated by SDS-PAGE and immunoblots performed in parallel using anti-pTyr1446 IR-β, anti-pSer473 AKT, anti-pSer2448 mTOR and anti-p-70S8K (Cell Signalling Technology Inc., US).

**Glucose tolerance test and analysis of sucrose assimilation**

The tolerance test was performed in fasted animals (>6 h) with oral gavage of glucose (0.5 or 1 g of glucose per kg of body weight). Blood glucose values were measured immediately before and 10, 20, 30, 60, 90 and 120 min after glucose injection. AUCs (areas under the curve; arbitrary units per minute) were determined for individual animals. For analysis of sucrose assimilation, fasted animals (>6 h) received oral gavage of sucrose (1 g per kg of body weight), and subsequently blood glucose values were measured as described above.

**Blood glucose and HbA1C analysis**

The concentrations of glucose, insulin and HbA1C levels in blood samples were determined as exactly described previously (13).
Statistical testing
Values presented in figures represent mean ± SEM. Statistical analysis of two groups was assessed by Student’s t-test (two-tailed) or ANOVA for repeated measurement (clamp experiment). Level of significance was set at p < 0.05.

Pharmacokinetic Evaluation
Four ZDF/Crl-leprfa male rats were administered a single 3 mg/kg intravenous dose of either AMP-DNM or L-ido-AMP-DNM in normal saline. In another group four ZDF/Crl-leprfa male rats were administered a single 10 mg/kg oral dose of either AMP-DNM or L-ido-AMP-DNM in normal saline. Each animal was dosed following an overnight fast. Following dose administration, whole blood samples were collected via the jugular vein catheter from each animal for up to 12 hours. Blood samples were processed to plasma and analyzed by liquid chromatography with tandem mass spectrometry. Pharmacokinetic parameters were determined using standard non-compartmental methods by WinNolinTM version 5.1.

Results
Effects of various existing iminosugars on glycemic control in ob/ob mice
Three existing 1-deoxynojirimycin-based iminosugars, AMP-DNM, N-butyl-DNM (registered as Miglustat (26)), N-(1-hydroxyethyl)-DNM (registered as Miglitol) and 1-deoxynojirimycin (DNM) itself were prepared and comparatively investigated with respect to their ability to inhibit three intestinal glycosidases and GCS. Their structures are depicted in Table 1. As expected, the anti-diabetic Miglitol is a potent inhibitor of the intestinal glycosidases maltase and sucrase. DNM, Miglustat and AMP-DNM also inhibit these enzymes at micromolar concentrations. AMP-DNM in particular is a potent inhibitor of glucosylceramide synthase (IC$_{50}$ 150 nM), Miglustat a weaker inhibitor (IC$_{50}$ 50000 nM), whilst DNM and Miglitol do not inhibit GCS at all.
Table 1. Apparent IC<sub>50</sub> values in μM for DNM-based iminosugars

<table>
<thead>
<tr>
<th>Iminosugar inhibitor structure / abbreviation</th>
<th>GCS &lt;i&gt;in vitro&lt;/i&gt;</th>
<th>Sucrase &lt;i&gt;in vitro&lt;/i&gt;</th>
<th>Maltase &lt;i&gt;in vitro&lt;/i&gt;</th>
<th>Lactase &lt;i&gt;in vitro&lt;/i&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNM</td>
<td>&gt;100</td>
<td>2</td>
<td>2</td>
<td>62</td>
</tr>
<tr>
<td>Miglitol</td>
<td>&gt;1000</td>
<td>0.5</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>Miglustat</td>
<td>50</td>
<td>0.5</td>
<td>9</td>
<td>&gt;100</td>
</tr>
<tr>
<td>AMP-DNM</td>
<td>0.15</td>
<td>0.5</td>
<td>4</td>
<td>35</td>
</tr>
</tbody>
</table>

The effects of Miglitol, Miglustat and AMP-DNM on glucose homeostasis in obese, insulin resistant ob/ob mice were studied. For this purpose, 7-week old, C57Bl/6j (control group) and ob/ob mice were treated for 4 weeks with 100 mg/kg/d of AMP-DNM, Miglustat or Miglitol. Only in the case of AMP-DNM a significant lowering of plasma and liver glycosphingolipids was observed, without concomitant changes in ceramide content (Figure 1A, B). AMP-DNM treated mice showed a significantly lower circulating blood glucose and insulin, improved HOMA and oral glucose tolerance, and reduced HbA1C (Figure 1C-F). Miglustat treatment had no significant positive effects on these parameters. Miglitol treatment resulted only in a significant, but minor, reduction of blood glucose, HOMA and HbA1C.

**L-ido-AMP-DNM, a potent and more specific inhibitor of glucosylceramide synthase**

1-Deoxynojirimycin and the piperidine ring of Miglitol, Miglustat and AMP-DNM possess D-gluco stereochemistry. It is a well established fact that structural mimicry is one of the main causes for inhibition of intestinal glycosidases by this type of iminosugar. Therefore, changing the iminosugar stereochemistry could be a means to developing more specific GCS inhibitors. Platt, Butters and co-workers have demonstrated previously that N-butyl-D-galacto-1-eoxynojirimycin (D-galacto-butyl-DNM), a C-4 epimer of Miglustat, still inhibits GCS (27).
Chapter 2

The same has also been reported for N-pentyl-L-ido-1-deoxynojirimycin, a N-pentyl substituted C-5 epimer of Miglustat (28). To obtain more specific iminosugar-based inhibitors of GCS, we developed a panel of nine structural and stereochemical analogues of AMP-DNM and analyzed their inhibitory capacity towards the three intestinal glycosidases and GCS. We prepared all three C-4/C-5 epimers of 1-deoxynojirimycin (D-galacto, L-altro and L-ido) and either left the endocyclic nitrogen unsubstituted, or substituted it with a butyl for Miglustat analogues or a 5-(adamantan-1-yl-methoxy)-pentyl (AMP) group for AMP-DNM analogues. The synthesis of all nine iminosugars started from either 2,3,4,6-tetra-O-benzylated D-glucose or D-galactose and followed adapted routes previously reported by us and others (see SI for details on the synthesis of the iminosugars). The structures of the panel of iminosugars and their inhibitory capacity towards the three intestinal glycosidases and GCS are depicted in Table 2.

Figure 1. Effects of AMP-DNM, Miglustat and Miglitol treatment on GSLs and glycemic control in ob/ob mice and comparative values in untreated normal mice. Animals were treated for 4 weeks daily with 100 mg compound per kg bodyweight. Panel A: plasma content (nmol/ml) of GSLs: ceramide (black); glucosylceramide (grey); total gangliosides (white). Panel B: liver content (nmol/g) of GSLs: (left to right) ceramide/10; glucosylceramide; lactosylceramide; GM3; GM2; GM2-glycol/10; GD1a. Panel C: HbA1c. Panel D: Blood glucose (black) and insulin (white). Panel E: HOMA-IR index. Panel F: Oral glucose tolerance (OGT; area under the curve).
Of the nine compounds only the three D-galacto-iminosugars (C-4 epimer; entries 1-3) still substantially inhibit intestinal glycosidases. All three L-altro-iminosugars (C-4 and C-5 epimer; entries 4-6) show very weak to no inhibition of GCS. In line with literature reports, the D-galacto- and L-ido-iminosugars (C-5 epimer, entries 7-9) do inhibit GCS. The unsubstituted D-galacto- and L-ido-iminosugars (entries 1 and 7) show the lowest to no inhibition of GCS. The D-galacto- and L-ido-N-butyl analogues (entries 2 and 8) are inhibitors of GCS in the micromolar range. Analogous to Miglustat versus AMP-DNM, a great increase in inhibitory potency for GCS is observed in switching from N-butyl substitution to N-AMP substitution of the D-galacto- and L-ido-iminosugars. Compared to AMP-DNM, the AMP-substituted D-galacto-iminosugar (entry 3) is an only slightly less potent inhibitor of GCS, but as mentioned above still inhibits the intestinal glycosidases. However, of particular interest was L-ido-AMP-DNM (entry 9), which did exhibit the required profile for a potent GCS-selective inhibitor. L-ido-AMP-DNM is slightly better than AMP-DNM with regard to inhibition of GCS (IC50 < 150 nM), but sharply contrasts from this compound in its much reduced capacity to inhibit intestinal glycosidases. Exposure of various types of cultured cells to AMP-DNM and L-ido-AMP-DNM resulted in comparable lowering of glycosphingolipids without concomitant increases in ceramide (not shown). The pharmacokinetic properties of L-ido-AMP-DNM and AMP-DNM were also found to be very similar (for more detailed information, see SI).
Figure 2. Effects of AMP-DNM (2) and L-ido-AMP-DNM (4) treatment on (G)SLs and glycemic control in ob/ob mice and comparative values in untreated normal mice. Animals were treated for 4 weeks daily with 100 mg compound per kg bodyweight. Panel A: Plasma content (nmol/ml) of ceramide and GSLs: ceramide (black); glucosylceramide (grey); total gangliosides (white). Panel B: Liver content (nmol/g) of ceramide and GSLs: (left to right) ceramide/10; glucosylceramide; GM2; GM2-glycol/10. Panel C: HbA1c. Panel D: Blood glucose (black) and insulin (white). Panel E: HOMA1-IR index. Panel F: Oral glucose tolerance (OGT; area under the curve).
Comparison of effects of AMP-DNM and L-ido-AMP-DNM in ob/ob mice and ZDF rats

The effect of L-ido-AMP-DNM and AMP-DNM on ob/ob mice was comparatively investigated. For this purpose, 7-week old animals were treated for 4 weeks with 100 mg/kg/d compound. Treatment with AMP-DNM and L-ido-AMP-DNM resulted in significant reductions in glycosphingolipids in plasma and liver without affecting ceramide levels (Figure 2A, B). Although clear improvements in blood glucose concentration, insulin levels, HOMA and HbA1C were observed in L-ido-AMP-DNM treated animals, these were significantly smaller than those detected in AMP-DNM treated animals (Figure 2C-E). Oral glucose tolerance was comparably improved in animals treated with L-ido-AMP-DNM and AMP-DNM (Figure 2F).

Next, ZDF rats were treated with AMP-DNM and L-ido-AMP-DNM. In addition, the effect of Genz-123346 (12, 29, 30), a ceramide analogue that specifically inhibits glucosylceramide synthase and not intestinal glycosidases, was studied. For 4 weeks treatment with all these compounds resulted in significant reductions of plasma and liver glycosphingolipids without changes in ceramide content (Figure 3A, B). Similar to the findings with ob/ob mice, treatment of ZDF rats with AMP-DNM resulted in more prominent improvements in blood glucose concentration and HbA1C compared to treatment with L-ido-AMP-DNM or GENZ-123346 (Figure 3 C, D).

**Different effects of AMP-DNM and L-ido-AMP-DNM on sucrose assimilation**

Next, we investigated the effect of AMP-DNM and L-ido-AMP-DNM on the uptake of glucose from orally administered sucrose. For this purpose, 7-week old ob/ob mice were treated for 4 weeks with 100 mg/kg/d compound or no addition. Sucrose (1g/kg) was orally administered, and blood glucose was monitored. The increase of blood glucose and its clearance from the circulation was slower in AMP-DNM

![Figure 3](image-url). Effects of AMP-DNM, ido-AMP-DNM treatment on GSLs and glycemic control in ZDF rats and comparative values in untreated normal mice. Animals were treated for 4 weeks daily with indicated amount of compound per kg bodyweight. Panel A: plasma content (nmol/ml) of GSLs: ceramide (black); glucosylceramide (grey); total gangliosides (white). Panel B: liver content (nmol/g) of GSLs: (left to right) ceramide/10; glucosylceramide; lactosylceramide; GM3; GM2; GM2-glycol/10; GD1a. Panel C: HbA1c. Panel D: Blood glucose.
treated mice than in L-ido-AMP-DNM treated animals (Figure 4A). The AUC for the first hour after sucrose administration of AMP-DNM and L-ido-AMP-DNM treated mice differed significantly (975 ± 75, 840 ± 65 mM.min, \(p<0.05\)). After one week, the same animals received orally glucose (0.5 g/kg). No differences were noted in the kinetics of glucose appearance and disappearance from the blood when AMP-DNM treated mice were compared with L-ido-AMP-DNM treated animals (Figure 4B). Untreated ob/ob mice showed higher blood glucose concentrations and impaired glucose tolerance upon administration of sucrose or glucose due to their insulin resistance.

**Discussion**

In our earlier study, we have demonstrated that the GCS inhibitor, AMP-DNM, has dramatic beneficial effects on the insulin resistance and hyperglycemia seen in ZDF rats, ob/ob mice and high-fat diet-induced glucose-intolerant mice via a mechanism that does not require a reduction in food intake or loss of bodyweight (13). In the ZDF type 2 diabetes model, protection of the pancreas by AMP-DNM was also observed. Given the ability of AMP-DNM to also inhibit the activity of intestinal glycosidases like sucrase in vitro, we considered the possibility that the compound might not only reduce visceral glycosphingolipids but also buffer intestinal carbohydrate assimilation in treated animals. Indeed, the assimilation of sucrose in ob/ob mice was...
found to be buffered in AMP-DNM treated animals. This dual action of AMP-DNM is not surprising since the structurally related Miglitol (N-(1-hydroxyethyl)-1-deoxyxojirimycin) positively affects glucose homeostasis via its specific inhibition of intestinal glycosidases. Based on this mechanism of action Miglitol is registered as anti-diabetic drug (17). Miglitol itself does not inhibit glucosylceramide synthase as measured with cultured cells (13), and our present investigation rendered no indication that some metabolite is formed that causes visceral glycosphingolipid reduction. The development of Miglitol as anti-diabetic drug was stimulated by the ancient use in the Far East of iminosugar-rich mulberry leaves to control hyperglycemia (31). Very recently, it has indeed been demonstrated that, compared with a placebo, co-ingestion of mulberry extract with 75 g sucrose reduced the increase in blood glucose observed over the initial two hours of testing in control and type 2 diabetic subjects (32).

The realization of dual effects exerted by AMP-DNM raised the question of the relative importance of buffering of carbohydrate assimilation on the one hand, and visceral glycosphingolipid lowering on the other hand, for the improved glycemic control in AMP-DNM treated animals. To dissect the two actions of AMP-DNM, we looked into the possibility to generate more specific analogues. Evaluation of known iminosugar-based inhibitors combined with the design and preparation of novel analogues has shown that the C-4/ C-5 configuration of the iminosugar and the type of substitution on the endocyclic nitrogen are critical for potent inhibition of GCS. In general, iminosugars with D-gluco-, D-galacto and L-ido-stereochemistry in combination with a hydrophobic substituent on the endocyclic nitrogen inhibited GCS. Substitution with 5-adamantane-1-yl-methoxy)-pentyl (AMP) provided the most potent inhibitors of GCS. Epimerization of the C-5 position of AMP-DNM greatly reduced inhibition of intestinal glycosidases and slightly increased the inhibitory potency for GCS, making L-ido-AMP-DNM the most potent iminosugar-based GCS-selective inhibitor reported to date. In order to further evaluate the effects of configurational manipulation and endocyclic nitrogen substitution on the inhibitory capacity and specificity we tested the iminosugars reported in Table 1A and 1B on four additional enzymes known to be inhibited by AMP-DNM. These enzymes are the glycogen debranching enzyme and lysosomal acid α-glucosidase, and the glucosylceramide metabolism related lysosomal glucocerebrosidase (GBA1) and non-lysosomal glucosylceramidase (GBA2) (13). The individual IC50 values of the iminosugars for these enzymes are shown in SI. Of note, compared to AMP-DNM, L-ido-AMP-DNM is a much poorer inhibitor of glucocerebrosidase (IC50 1.0 μM versus 0.2 μM), acid α-glucosidase (IC50 > 1 mM versus 0.4 μM), and debranching enzyme (IC50 > 1 mM versus 10 μM), which
further emphasizes its specificity in GCS inhibition. The generation of L-ido-AMP-DNM allowed us to dissect the two actions of AMP-DNM. We established that L-ido-AMP-DNM also in obese mice inhibits GCS comparably to AMP-DNM, and and does not effectively buffer sucrose assimilation by its lack of sucrase inhibition. Treatment of ob/ob mice and ZDF rats with L-ido-AMP-DNM demonstrated that sole reduction of visceral glycosphingolipids is sufficient to induce major improvements in blood glucose, HbA1C, oral glucose tolerance and insulin signaling in the liver. These findings are consistent with the observation that Genz-123346, that does not affect intestinal glycosidases and carbohydrate assimilation, helps to control hyperglycemia (12). Importantly, our study also points out that the concomitant inhibition of intestinal carbohydrate assimilation by AMP-DNM adds to its prominent beneficial effect on glycemic control (see scheme).

**Scheme 1. Proposed model for improved glycemic control by dual action (A/B) of AMP-DNM in type 2 diabetes.**
A considerable drawback of compounds that buffer carbohydrate assimilation by virtue of inhibition of intestinal glycosidases are the associated intestinal complaints that lower drug compliance. L-ido-AMP-DNM does not affect intestinal glycosidases. In this respect, the compound is an appealing drug, particularly for conditions in which the exclusive lowering of visceral glycosphingolipid levels is desirable without the need for buffering of carbohydrate assimilation. Examples in this respect are the inherited glycosphingolipidoses, such as Gaucher disease, Sandhoff disease, Tay-Sachs disease and Fabry disease (15). In all these disorders, a particular glycosphingolipid accumulates in the lysosomes due to an inherited deficiency in a catabolic lysosomal glycosidase. Reduction of glycosphingolipid biosynthesis by inhibition of glucosylceramide synthase is envisioned to be beneficial in all these conditions (15, 33, 34). Miglustat (N-butyl-1-deoxynojirimycin) has been registered as orphan drug for the treatment of mild to moderate type 1 Gaucher disease, and has proven to be efficacious (26, 35). Given the significantly improved features of L-ido-AMP-DNM as compared to Miglustat, such as better bioavailability, specificity and potency of inhibition of glucosylceramide synthase, it seems warranted to investigate its potential as therapeutic agent for inherited glycosphingolipidoses.

In summary, AMP-DNM exerts beneficial effects on glycemic control by virtue of its dual lowering of visceral glycosphingolipids and buffering of carbohydrate assimilation. This dual action is desirable for control of hyperglycemia, a hallmark of type 2 diabetes. The analogue of AMP-DNM we developed, L-ido-AMP-DNM, specifically inhibits glycosphingolipid biosynthesis and may be of interest to intervene in inherited glycosphingolipidoses. The outcome of this study also indicates that tailored iminosugars can be developed for specific therapeutic indications by designing them to exclusively act on glycosphingolipid metabolism or on other related glycoprocessing pathways.

Acknowledgements.
The authors wish to thank Stephen O’Rahilly for useful and stimulating discussions. The described research was funded by the Academic Medical Center and a governmental TOP grant from NWO-CW.
Reference List