Glycosphingolipids and atherosclerosis
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Citation for published version (APA):
Lombardo, E. (2013). Glycosphingolipids and atherosclerosis

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CHAPTER III

The beneficial effect of hydrophobic iminosugars on cholesterol excretion is dependent on concomitant inhibition of glucosylceramide synthase and glucocerebrosidase activity.

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Submitted
Abstract

The administration to rodents of the lipophilic iminosugar AMP-DNM, \( \text{(N-}(5'\text{-adamantane-1'yl-methoxy)-pentyl-1-deoxyojirimycin)} \), reduces glycosphingolipids in various tissues as a result of inhibition of glucosylceramide synthase. In both APOE*3 Leiden and LDLR (-/-) mice fed a western type diet, AMP-DNM treatment strikingly lowers plasma cholesterol and prevents development of atherosclerosis. The underlying mechanism for the beneficial effect is unknown. AMP-DNM inhibits not only glucosylceramide synthase, but also the lysosomal and non-lysosomal glucocerebrosidases, GBA1 and GBA2. The idose-analogue of AMP-DNM, L-ido-AMP-DNM, similarly to AMP-DNM inhibits glucosylceramide synthase and the non-lysosomal glucocerebrosidase GBA2. However, the idose-analogue hardly inhibits the lysosomal glucocerebrosidase GBA1 in contrast to AMP-DNM. In this study we compared the capacity of AMP-DNM and L-ido-AMP-DNM to prevent the development of atherosclerosis in LDLR (-/-) mice receiving an atherogenic diet for 12 weeks. L-ido-AMP-DNM and AMP-DNM treatment nicely lowered tissue glucosylceramide and gangliosides, but only AMP-DNM prevented markedly lesion development (-16% with L-ido-AMP-DNM vs -60% with AMP-DNM). In contrast to AMP-DNM, L-ido-AMP-DNM hardly caused increased biliary and fecal cholesterol excretion. Our results suggest that the potent anti-atherogenic effect of AMP-DNM requires concomitant inhibition of GBA1.

**Keywords:** iminosugars, atherosclerosis, lipids, glucosylceramide synthase, glycosphingolipids, glucocerebrosidase
CHAPTER III

Introduction

Cardiovascular diseases constitute a high societal burden. Abnormalities in lipid metabolism contribute to the development of atherosclerosis. A high plasma cholesterol level is a well-established risk factor in this respect, especially a high sterol concentration in the low density lipoprotein (LDL-C) [1]. Ample evidence exists from population-based studies and clinical trials for the notion that LDL-C reduction is an effective strategy to prevent atherosclerosis [2]. Statins are powerful LDL-C lowering agents that represent the therapy of choice for treatment of hyperlipidemia associated with cardiovascular events [3]. Unfortunately, recent surveys have shown that certain categories of patients with a high cardiovascular risk fail often to achieve their therapeutic goal [4–6]. Therefore, novel classes of sterol-lowering drugs acting by different mechanisms are considered to be needed [7]. Such a new class of drugs might be hydrophobic iminosugars that modulate the glycosphingolipid metabolism [8]. Glycosphingolipids have been earlier implicated in atherosclerosis [8], and their cellular function as components of lipid rafts is related to that of cholesterol [9]. Cholesterol and glycosphingolipids are also both components of lipoprotein particles [10,11]. In both humans and rodents, high levels of glycosphingolipids have been observed in lesions and plasma [12,13]. Moreover, lactosylceramide (LacCer) has been suggested to stimulate proliferation of aortic smooth muscle cells and high concentrations of gangliosides seem to increase LDL uptake by mouse peritoneal macrophages [14]. Of interest in this connection, oxidized LDL has been reported to stimulate the synthesis of LacCer [15]. Deoxynojirimycins with hydrophobic N-substituents, a subclass of iminosugars, are inhibitors of several enzymes involved in glycosphingolipid synthesis. A member of this class, the lipophilic iminosugar AMP-DNM (N-(5’-adamantane-1’-yl-methoxy)-pentyl-1-deoxynojirimycin) is a potent inhibitor of the enzyme glucosylceramide synthase (GSC) [16]. In a previous study [8] we fed mice prone to develop atherosclerosis (APOE3*Leiden and LDLR (-/-) mice) an atherogenic diet supplemented with AMP-DNM, and we observed a strong inhibition of atherosclerotic plaques development. The inhibition of atherosclerosis progression was accompanied by a correction of the hyperlipidemic status of the mice and by a strong stimulation of bile and fecal sterol output, a possible indicator of increased reverse cholesterol transport [8]. AMP-DNM is not a specific inhibitor of glucosylceramide synthase. It is able to inhibit other enzymes, such as the lysosomal glucocerebrosidase GBA1 and the non-lysosomal glucocerebrosidase GBA2 as well as intestinal sucrase [17]. One may therefore wonder whether the impressive effect of AMP-DNM on lesion development and lipid metabolism can be explained solely by reduction of glycosphingolipid levels. Recently, our group developed several
derivatives of AMP-DNM aiming to improve selectivity and potency towards GCS inhibition [17]. Among the different derivatives, L-ido-AMP-DNM, a C-5 epimer of AMP-DNM (figure 1) proved to be a more selective inhibitor [17]. The newly synthesized L-ido analogue inhibits GCS on a par with AMP-DNM (IC$_{50}$ of 150 and 180 nM, respectively). The non-lysosomal GBA2 is potently inhibited by both AMP-DNM and L-ido-AMP-DNM (IC$_{50}$ of 1 and 30 nM, respectively). The intestinal sucrase is not inhibited by L-ido-AMP-DNM in contrast to AMP-DNM [17]. The major difference between the idose analogue and AMP-DNM is in the much poorer inhibition of GBA1 by L-ido-AMP-DNM compared to AMP-DNM (IC$_{50}$ of >2000 and 200 nM, respectively). Importantly, the pharmacokinetic properties of both iminosugars have been found to be very similar [17]. A comparison of AMP-DNM and L-ido-AMP-DNM treatment of LDLR (-/-) mice with respect to cholesterol homeostasis and development of atherosclerotic lesions was undertaken by us. The comparison allows an assessment of the importance of GBA1 inhibition for the noted beneficial effect of AMP-DNM on atherosclerosis. The outcome of the investigation suggests that the concomitant inhibition lysosomal glucocerebrosidase GBA1 is necessary to promote a marked correction in cholesterol homeostasis and the prevention of atherosclerosis.
Figure 1. Chemical structures of the two iminosugar and respective IC\textsubscript{50} values in nM.
(A) AMP-DNM and (B) L-ido-AMP-DNM. The two compounds are C-5 epimers and present similar pharmacokinetic properties. Based on the IC\textsubscript{50} values the main difference between the two iminosugars is laid in their inhibition of GBA1.
Materials And Methods

Materials
AMP-DNM and L-ido-AMP-DNM were synthesized as previously described [17]. All solvents and reagents used were of analytical grade.

Mice and diets
For the study we used female LDLR (-/-) mice 8 to 10 weeks old. The animals were fed a western type diet (0.25 % w/w cholesterol; 15% w/w fat, Arie Blok, Woerden, the Netherlands) for 12 weeks supplemented or not with a calculated dose of 50 mg/kg bw/day AMP-DNM or L-ido-AMP-DNM. LDLR (-/-) mice were housed at the Institute Animal Core Facility in a temperature- and humidity-controlled-room with a 12-h light/dark cycle. All experiments were approved by the institutional review board for animal experiments (Dier Ethische Commissie (DEC)) at the Academic Medical Center (Amsterdam, The Netherlands).

Plasma and tissue sampling
Blood samples were collected via the tail vein during the study. At the end of the experiments, large blood samples were collected by abdominal aorta puncture and plasma samples were stored at -20°C. Hearts were flushed with PBS to clean up excess of blood before fixation in formaldehyde 1% (Thermo Electron Corporation, Pittsburgh, USA) for 24 hours and storage at -80°C embedded in tissue medium (Tissue-Tek O.C.T, Sakura, Zoeterwoude, the Netherlands). Other tissues were snap frozen in liquid N2 and stored at -80°C.

Characterization of Atherosclerotic Lesions
Frozen sections from the aortic sinus were prepared according to Paigen et al. [18]. Surface lesion area was measured after Oil Red O staining by computer-assisted image quantification with Leica QWin software (Leica Microsystems, Wetzlar, Germany). Images were captured with a Leica DFC 420 (Leica Microsystems, Wetzlar, Germany) video camera.

Bile and feces sampling
Twenty-four hours fecal collection for each cage (2-5 animals per cage) was performed at the end of the study. Samples were freeze-dried, weighed and powdered before storage at -20°C. Cannulation of the gallbladder and bile collection was performed as described previously [8]. After cannulation, bile was collected for 15 minutes and bile samples were immediately frozen and stored at -80°C. Bile flow was determined gravimetrically assuming a density of 1 g/ml.


**Analytical procedures**

(Glyco)sphingolipids were determined in plasma and liver samples after lipid extraction according to Folch [19]. Ceramide (Cer), glucosylceramide (GlcCer) and sphingomyelin (SM) were analyzed by high-performance liquid chromatography (HPLC) analysis according to a procedure described previously [20]. Gangliosides, largely GM2-glycolyl, were separated by normal-phase HPLC and quantified as previously described [21], using the gangliosides Gt1b as an internal standard. Fecal neutral sterols were extracted from the feces and measured by gas chromatography [22]. Biliary parameters were measured by fluorescent methods as described previously [8]. Cholesterol (CH) and triglycerides in plasma and liver were determined using commercial colorimetric enzymatic kits (Biolabo, Maizy, France). Plasma cholesterol distribution in the main lipoprotein classes were determined in a pool of plasma of each group (8 to 10 mice) separated by high performance gel filtration chromatography (HPGC) as described before [23]. AMP-DNM and L-ido-AMP-DNM in plasma were measured after extraction using the method of Bligh and Dyer. Samples were then analyzed by LC-ESI-MS/MS as previously described [24].

**Statistical analysis**

Values presented in figures represent mean ± SEM. Statistical significance between control (ctrl) and treated groups was determined by ANOVA with Dunnett’s multiple comparison. P-values < 0.05 were considered significant.
Results

Effect of iminosugars on glycosphingolipids, cholesterol and triglycerides

Female LDLR (-/-) mice were fed with a lipid-rich western diet for 12 weeks. AMP-DNM and L-ido-AMP-DNM (structure formula’s shown in figure 1) were added to the diet to obtain an intake of 50 mg iminosugar/kg bw/day. The steady-state plasma concentrations of the iminosugars were 300 and 200 nM for AMP-DNM and L-ido-AMP-DNM treated animals respectively. Control animals received the same food without iminosugars. No significant differences in body weight or food intake in the different experimental groups were observed.

As earlier reported, LDLR (-/-) mice receiving a western diet for 12 weeks, showed marked abnormalities in plasma and hepatic glycosphingolipids, cholesterol and triglycerides [8]. The administration of both iminosugars resulted in improved lipid concentrations in the liver (Table 1). The hepatic glucosylceramide concentration was lowest in L-ido-AMP-DNM treated animals. In the plasma of iminosugar treated LDLR (-/-) mice, hyperlipidemia was less prominent after 12 weeks of western diet (see figure 2). Intriguingly, cholesterol and triglycerides levels were significantly lower with AMP-DNM treatment than the ones achieved with L-ido-AMP-DNM treatment. Analysis of the cholesterol distribution over the different lipoproteins revealed that, the iminosugar-induced reduction of cholesterol took largely place at the level of VLDL and IDL/LDL particles (figure 2E). Plasma lipid ratios (table 2) were unchanged in the three experimental groups except for ratios of lipids to glucosylceramide, due the direct inhibition of glucosylceramide synthesis responsible for its synthesis.

Table 1. Lipid levels in liver of AMP-DNM and L-ido-AMP-DNM treated LDLR (-/-) mice.
Concentrations are expressed as nmol/g liver. Abbrevations GlcCer: glucosylceramide; Cer: ceramide; GM2-gl: ganglioside GM2-glycolyl ; SM: sphingomyelin. Values are expressed as mean ± SEM *p<0.05; **p<0.01; ***p<0.001; n=5 to 10

<table>
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<th></th>
<th>GlCer</th>
<th>Cer</th>
<th>GM2-gl</th>
<th>SM</th>
<th>Cholesterol</th>
<th>Triglyceride</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL</td>
<td>47±5.4</td>
<td>127±7.6</td>
<td>282±38</td>
<td>470±76</td>
<td>14.8±0.9</td>
<td>9.3±1</td>
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<tr>
<td>AMP-DNM</td>
<td>34.8±5*</td>
<td>179±14*</td>
<td>285±30</td>
<td>615±68</td>
<td>13.5±1.3</td>
<td>7±0.5*</td>
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<tr>
<td>Ldo-AMP-DNM</td>
<td>17.5±1.7***</td>
<td>153±8.3</td>
<td>129±12**</td>
<td>516±51</td>
<td>10.5±0.3**</td>
<td>10±0.8</td>
</tr>
</tbody>
</table>
Table 2. Lipid ratios in plasma of untreated and iminosugar treated LDLR (-/-) mice.
Abbreviations: CH: cholesterol; GlcCer: glucosylceramide; SM: sphingomyelin; Cer: ceramide. Values are expressed as mean ± SEM *p<0.05; **p<0.01; ***p<0.001; n=5 to 10

<table>
<thead>
<tr>
<th></th>
<th>CH/Cer</th>
<th>CH/GlcCer</th>
<th>CH/SM</th>
<th>Cer/GlcCer</th>
<th>Cer/SM</th>
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</thead>
<tbody>
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<td>CTRL</td>
<td>0.99±0.06</td>
<td>0.5±0.05</td>
<td>0.05±0.003</td>
<td>0.5±0.03</td>
<td>0.05±0.006</td>
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<tr>
<td>AMP-DNM</td>
<td>1.1±0.08</td>
<td>1.1±0.1***</td>
<td>0.04±0.002</td>
<td>0.9±0.1**</td>
<td>0.03±0.003</td>
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<tr>
<td>Ido-AMP-DNM</td>
<td>0.9±0.05</td>
<td>1.5±0.08***</td>
<td>0.05±0.005</td>
<td>1.7±0.09***</td>
<td>0.05±0.006</td>
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</table>

Figure 2. Treatment with the two iminosugars decreased GSL and ameliorates hyperlipidemia in LDLR (-/-) mice.
(A) Glucosylceramide; (B) Ceramide and (C) Sphingomyelin levels in plasma. Chow group represents mice on a standard chow diet (n=5). (D) Plasma cholesterol and (E) triglycerides, before (PD) and at the end of the study. Values are expressed as mean ± SEM *p<0.05; ***p<0.001. (F) Lipoprotein profile showing distribution of cholesterol determined in pooled plasma (n=8 to 10) for each group.
**Effect of iminosugars on lesion development**

When fed the atherogenic western diet, LDLR (-/-) mice develop severe atherosclerotic lesions, especially at the aortic sinus. In our study, control animals after 12 weeks of western diet, showed advanced and extended plaques (figure 3). AMP-DNM treated mice showed, consistent with our previous study [8], a 60% inhibition of the development of lesions. In contrast, L-ido-AMP-DNM treatment did hardly (-16%) inhibit the progression of the atherosclerotic plaques (figure 3).

**Effect of iminosugar treatment on cholesterol excretion**

In our previous study we showed that AMP-DNM stimulates biliary and fecal cholesterol excretion from the animal body [8]. We investigated the effect of L-ido-AMP-DNM on these pathways. Bile of 5 animals from each group was collected and analyzed for cholesterol, bile salts and phospholipid content. Neutral sterol content was analyzed in the animal fecal samples. In the present study the animals treated with AMP-DNM again showed increased cholesterol excretion in both bile and feces. In contrast to the findings with the parent compound AMP-DNM, we did not observe an increase of biliary secretion with L-ido-AMP-DNM.
treatment when compared to control animals (figure 4). Fecal sterol excretion was neither increased (figure 5).

**Figure 4.**
AMP-DNM but not L-ido-AMP-DNM treatment stimulates biliary lipid secretion. 
(A) Bile flow; 
(B) Biliary cholesterol; 
(C) Bile salts; 
(D) Phospholipids. Lipids were measured in bile collected for 15 minutes as described in Material and Methods. Values are expressed as mean ± SEM; n= 5 *p<0.05; **p<0.01.

**Figure 5.**
AMP-DNM but not L-ido-AMP-DNM treatment increased fecal neutral sterol output.
During 24 hours feces was collected from the different cages (n= 2 to 5 animals per cage). LDLR (-/-) mice were treated with 0 or 50 mg/kg bw/day AMP-DNM or L-ido-AMP-DNM mixed in the diet for 12 weeks.
Discussion

In this study we compared two structurally closely related iminosugars, AMP-DNM and L-ido-AMP-DNM, with respect to their potency to ameliorate atherosclerotic lesion formation. Surprisingly, a major difference in ability of L-ido-AMP-DNM and AMP-DNM in prevention of atherosclerosis was noted. A prominent beneficial anti-atherogenic effect was only demonstrable for AMP-DNM. Concomitantly only this compound resulted in pronounced cholesterol lowering and stimulation of cholesterol output. The distinct effect of the two iminosugars, both inhibitors of glucosylceramide synthase, on cholesterol homeostasis is of interest. Inhibition of glucosylceramide synthase alone seems to be not sufficient to maximally prevent atherosclerosis. Clearly, AMP-DNM, but not L-ido-AMP-DNM, contains an additional feature that is essential for its full beneficial effect on cholesterol homeostasis. A closer inspection of AMP-DNM and its idose analogue is therefore of interest. The two compounds, showing similar pharmacokinetics and dynamics [17], are similar inhibitors of the enzyme glucosylceramide synthase and both are very potent (low nanomolar) inhibitors of the non-lysosomal glucocerebrosidase GBA2. L-ido-AMP-DNM and AMP-DNM do however clearly differ in ability to inhibit the lysosomal glucocerebrosidase GBA1.

At the measured plasma steady state iminosugar concentrations in AMP-DNM and L-ido-AMP-DNM treated mice (concentrations 300 nM and 200 nM respectively), and considering the IC$_{50}$ values for GBA1 (listed in figure 1), it is likely that GBA1 is inhibited in AMP-DNM but not with L-ido-AMP-DNM treated animals. In our previous study we used a higher dose of AMP-DNM (resulting in even stronger GBA1 inhibition) and observed an even more complete protective effect on lesion development [8]. We observed that L-ido-AMP-DNM treatment resulted in a more effective reduction of glucosylceramide in liver, presumably due to the fact that the degradation of this lipid by GBA1 is not impaired in contrast to situation in AMP-DNM treated animals. We also noted that iminosugar treatment reduces excessive plasma lipids and corrects the abnormal lipoprotein profile of these hyperlipidemic mice. Again, the extent of this correction is dependent on the iminosugar used, with AMP-DNM being more potent.

AMP-DNM and its idose-analogue exerted a different effect on bile production and excretion. Only AMP-DNM treatment increased bile production and excretion. Increasing bile acids and bile cholesterol excretion is a well-known method to improve lipid metabolism, to decrease LDL-C and to improve atherosclerosis [25,26]. Studies on patients with CAD have revealed a decreased bile acid production and excretion [27]. Also in our study, bile acid excretion negatively correlated with lesion area ($r^2=0.67; P= 0.007; n=5$).
Another indication that the beneficial action of AMP-DNM may be dependent on inhibition of GBA1 comes from clinical observations on Gaucher disease patients. GBA1 is deficient in this inherited lysosomal storage disorder [28,29]. Of interest, Gaucher disease is associated with the occurrence of gallstones, even in young man. This phenomenon is ascribed to increased biliary cholesterol excretion [30]. In mice treated with AMP-DNM increased bile flow together with increased biliary cholesterol and bile salts excretion is generally observed [8,31]. Similar to the lipoprotein lowering effect of AMP-DNM, Gaucher patients present low levels of both LDL and HDL cholesterol [32,33]. Despite low levels of HDL-C, Gaucher patients are not at risk for cardiovascular disease [34]. Very recently it has been reported by Harzer and colleagues that the lysosomal glucocerebrosidase GBA1 also hydrolyzes bile acid 3-O-beta-glucoside [35]. It is at present unclear whether abnormalities in this bile acid induced by AMP-DNM treatment underlie the potent iminosugars cholesterol-lowering effect. This possibility warrants further investigation.

Based on our results it seems that AMP-DNM positively modulates lipid metabolism and has anti-atherogenic features by its ability to inhibit glucosylceramide synthase as well as partially GBA1. A role in atherogenesis for the enzyme GBA2, the non-lysosomal glucosylceramidase that is also inhibited by AMP-DNM and L-ido-AMP-DNM, seems to be unlikely. Firstly, at the concentration of around 200 nM L-ido-AMP-DNM, just like AMP-DNM, completely inhibits GBA2 without exerting the beneficial anti-atherogenic effect. In addition to this, a study performed with GBA2 -/- mice by Yildiz and co-workers showed that cholesterol and bile metabolism where not altered [36].

Given the striking beneficial effects of AMP-DNM on atherogenesis, further investigations are needed to identify the precise mode of action and in particular the need for partial GBA1 inhibition. A better understanding of the mode of action would be of great value in developing an iminosugar-type drug for prevention of coronary heart disease in man.

Acknowledgements

We are grateful to Han Levels for helping with the lipoprotein profile and Nick Dekker for measuring AMP-DNM and L-ido-AMP-DNM in plasma. We are grateful to the Netherlands Heart Foundation for supporting this work (grant 2007B030).
Reference List


