Glycosphingolipids and atherosclerosis

Lombardo, E.

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

Glycosphingolipid synthesis inhibitor AMP-DNM lowers plasma cholesterol levels by promoting faecal cholesterol excretion without inhibiting cholesterol absorption

Carlos L.J. Vrins¹, Florence Bietrix¹, Elisa Lombardo¹, Cindy P.P.A van Roomen¹, Roelof Ottenhoff¹, Herman S. Overkleeft², and Johannes M. Aerts¹

¹ Department of Medical Biochemistry, Academic Medical Center, Amsterdam, The Netherlands
² Leiden Institute of Chemistry, Leiden University, Leiden, The Netherlands

Clinical Lipidology (2012) 7(2)
Abstract

Inhibition of GSL synthesis with iminosugar N-(5’- adamantane-1’-yl-methoxy)-pentyl-1-deoxynojirimycin (AMP-DNM) increases faecal neutral sterol (FNS) output in mice. To investigate which pathways were involved in this increase, we treated C57Bl/6J mice with AMP-DNM and/or ezetimibe. FNS output was three-fold increased with combined AMP-DNM/ezetimibe treatment compared to the approximately two-fold increases with single treatments. Bile canulations and intestine perfusions showed that biliary cholesterol secretion and trans-intestinal cholesterol efflux (TICE) are increased in mice receiving AMP-DNM treatment. Our study indicates that AMP-DNM treatment of mice increases FNS by promoting biliary and intestinal cholesterol secretion without inhibiting cholesterol absorption.

Keywords: glycosphingolipids, reverse cholesterol transport, faecal neutral sterol, intestine, glucosylceramide synthase, hypercholesterolemia
**Introduction**

Dyslipidemia is a risk factor for cardiovascular diseases (CVD) [1]. In this connection, attention has largely been focussed on triglyceride and cholesterol levels. A role for sphingolipids in CVD is considered due to the growing awareness of the relationship between sphingolipids and cholesterol [2-6]. Since plasma sphingolipids are mainly associated with very low density lipoprotein (VLDL) and low density lipoproteins (LDL), their correlation with CVD could be a mere consequence of the already established atherogenic nature of these lipoproteins [7-9]. Additional roles for sphingolipids in CVD might exist. For example, our group has shown that specific pharmacological lowering of glycosphingolipids (GSL’s) with glycosylceramide synthase inhibitor N-(5’-adamantane-1’-yl-methoxy)-pentyl-1-deoxynojirimycin (AMP-DNM), reduces plasma cholesterol levels by increasing biliary cholesterol secretion, and increasing faecal neutral sterol (FNS) excretion in mice [10] and consistently drug treatment prevents atherosclerosis in hyperlipidemic mouse models [11].

AMP-DNM does not only improve cholesterol homeostasis. In fact, AMP-DNM treatment was firstly studied in genetically modified ob/ob mice and found to improve glycemic control resulting in reduced blood glucose levels, reduced glycated hemoglobin (HBA1c) levels and improved insulin sensitivity as measured in muscle and liver by euglycemic clamps [12]. In liver and adipose tissue, improvement of insulin-stimulated insulin receptor autophosphorylation was also observed [13;14]. As a consequence fat storages are clearly reduced in liver of AMP-DNM treated animals, and expression of sterol responsive element binding protein 1-c (SREBP1c) target genes involved in fatty acid synthesis are normalized [13]. In the adipose tissue, adipogenesis is restored and inflammation is reduced by AMP-DNM treatment [14].

How the drug exactly promotes increased faecal excretion of cholesterol in the various mouse models used is still not precisely known. It may be that AMP-DNM by changing sphingolipid levels via inhibition of glucosylceramide synthase activity stimulates the reverse cholesterol transport (RCT) pathway [15]. This may result in increased FNS by increased biliary cholesterol secretion and/or transintestinal cholesterol excretion (TICE), the recently described alternative direct secretion of cholesterol via the intestine [16]. In addition to these pathways, a direct inhibitory effect of AMP-DNM on intestinal cholesterol absorption cannot be excluded. Intestinal cholesterol absorption is mediated by the Niemann-Pick C1 Like 1 (NPC1L1) protein [17;18]. Ezetimibe, and particularly its glycosylated metabolite, is a potent inhibitor of the NPC1L1 mediated cholesterol absorption [19]. AMP-DNM, being a compound consisting of an iminosugar moiety and a large hydrophobic group, resembles to some extent ezetimibe in these
structural features. In figure 1, the biological active metabolite of ezetimibe, i.e. ezetimibe glucuronide is shown, as well as AMP-DNM. There is indeed a similarity between the anti-atherogenic effect of AMP-DNM in ApoE3*Leiden mice and that reported for ezetimibe [11;20]. However, in an earlier study we did not observe a changed uptake of radiolabeled cholesterol in AMP-DNM treated ApoE3* Leiden mice [11].

To definitely exclude a direct effect of AMP-DNM on cholesterol absorption and to elucidate pathways involved in the increased FNS, we treated mice with AMP-DNM in the presence or absence of ezetimibe and determined biliary cholesterol secretion and TICE.

Figure 1 Structure formula’s of AMP-DNM and ezetimibe-glucuronide, the active metabolite of ezetimibe. The two compounds present similar structural proprieties. They both present a sugar moiety and a large hydrophobic group.
Materials And Methods

Animals and diet
Male C57Bl/6J mice (4-5 months; n=5) were housed in the animal facility (2-3 mice per cage) on a 12 h light- 12 h dark cycle, and food and water were supplied ad libitum. For two weeks, one group received a reference diet (AM-II chow, Arie Blok BV, The Netherlands), two groups received either a reference diet containing AMP-DNM (100 mg/kg bw/day) or a diet containing ezetimibe (Ezetrol, Schering-Plough, Utrecht, The Netherlands; 10 mg/kg bw/day), the last group received ezetimibe (10 mg/kg bw/day) and AMP-DNM (100 mg/kg bw/day) in combination. All experiments were performed with the approval of the local Ethical Committee for Animal Experiments.

Cholesterol intake and output measurements
After 10 days of treatment, daily food intake and faecal neutral sterol excretion per cage were measured for a period of 2 days. During this period, the mice and remaining diet were weighed and faeces were collected per cage each day. FNS was determined as described below.

Intestine perfusion procedures
After 2 weeks of diet, mice were anaesthetized (7 ml fluanisone (17.5 mg), fentanyl citrate (0.55 mg), and midazolam (8.75 mg) per kg bw) with an intraperitoneal injection and placed on a heat pad to maintain body temperature. The bile duct was cannulated via the gallbladder to divert biliary cholesterol. The first 15 min fraction was used to measure biliary cholesterol secretion. Intestine perfusions were performed as described previously [16]. Perfusate fractions were stored at -20 °C until further analysis. At the end of the perfusion period, blood was collected by cardiac puncture to obtain plasma. Plasma was stored at -80 °C.

Determination of mRNA levels
Total RNA was isolated using Trizol reagent according to the manufacturer’s protocol (Invitrogen, Breda, The Netherlands). Purified RNA was treated with RQ1 RNase-free DNase (1 units/ 2 µg of total RNA; Promega, Leiden, The Netherlands) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen, Breda, The Netherlands) according to protocols supplied by the manufacturers. Gene expression analysis was performed by use of SYBR green. Quantitative gene expression analysis was performed on a Bio-Rad MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Veenendaal, The Netherlands). Hypoxanthine-guanine phosphoribosyl transferase (HPRT), cyclophilin, and acidic ribosomal phosphoprotein P0 (36B4) were used as standard housekeeping genes.
Details of primers used for gene expression analysis are available upon request.

**Analytical procedures**

Cholesterol in bile and in intestinal perfusate was measured by a fluorescent method as described previously [21]. Total cholesterol concentrations of plasma samples were determined using the cholesterol RTU kit from Biomerieux (Marcy-l’Etoile, France). Triglycerides in plasma were determined using the Triglycerides Ecoline S+ kit (Diagnostic Systems GmbH, Holzheim, Germany). Total cholesterol content of the main lipoprotein classes (very low density lipoprotein (VLDL), low density lipoprotein (LDL) and HDL) was determined in pooled plasma using high performance gel-filtration chromatography (HPGC) as described previously [22]. For FNS analysis, 1-day faecal samples were collected, lyophilized and grinded. Samples were prepared for analysis by gas chromatography as described previously [23]. Glucosylceramide levels in plasma were determined after lipid extraction according to Folch [24] by high-performance liquid chromatography analysis according to procedure as described previously [12].

**Statistics**

All results are presented as mean ± SD. Group means for TICE, as depicted in the figures, were calculated by averaging the outcomes of all mice that got the same treatment. Statistical differences between groups were determined by ANOVA. Outcomes of p < 0.05 were considered to be significant.

**Results**

**AMP-DNM stimulates additional cholesterol excretion in ezetimibe treated mice**

C57Bl/6J mice were divided in two groups by feeding them a reference diet with or without ezetimibe. To assess the effect of AMP-DNM on cholesterol excretion, we supplemented the diets of these mice with AMP-DNM. Treatments did not affect the body weights of the mice even if the group that received ezetimibe and AMP-DNM in combination showed a slight reduction in the food intake (Table1). FNS output was measured after 10 days of treatment. In mice that did not receive ezetimibe, AMP-DNM treatment resulted in an about 2-fold increase in FNS excretion compared to the group that received the reference diet (Fig. 2). A quite similar increase was observed for animals treated with ezetimibe only. Combined treatment with AMP-DNM and ezetimibe increased more markedly FNS by approximately 3-fold compared to the control group.
AMP-DNM stimulates cholesterol secretion via bile and TICE

To determine the pathways involved in the increase of FNS in treated animals, we determined biliary cholesterol secretion after gallbladder cannulations and TICE after intestine perfusions [16]. As reported previously, AMP-DNM treatment stimulated secretion of cholesterol via bile [10]. Ezetimibe treatment alone did not affect this pathway (Fig. 3A). Interestingly animals that received AMP-DNM alone or in combination with ezetimibe showed a significant increase of TICE (Fig. 3B), whereas ezetimibe alone did not affect this pathway as shown in a previous study [25].

Major cholesterol transport genes are not affected

To determine if the changes in cholesterol secretion could be attributed to altered expression of genes related to cholesterol transport, we analysed mRNA levels in both liver and intestine out from animals (Table 2). We did not observe significant changes in the expression of most analyzed genes upon AMP-DNM treatment. Scarb1 and Scarb2 were slightly but significantly modulated in livers of treated animals.

Table 1. Effect of AMP-DNM and ezetimibe on body weight and food intake

<table>
<thead>
<tr>
<th></th>
<th>Ref. Diet</th>
<th>AMP-DNM</th>
<th>Ezetimibe</th>
<th>Ezetimibe + AMP-DNM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bodyweight at start treatment</td>
<td>31.1 ± 1.8</td>
<td>30.9 ± 2.2</td>
<td>31.8 ± 2.9</td>
<td>32.4 ± 0.4</td>
</tr>
<tr>
<td>Bodyweight at end treatment</td>
<td>31.3 ± 2.0</td>
<td>29.6 ± 1.4</td>
<td>31.2 ± 1.7</td>
<td>29.9 ± 1.5</td>
</tr>
<tr>
<td>Food intake (g/day.100g bw)</td>
<td>15.1 ± 1.0</td>
<td>14.8 ± 0.7</td>
<td>13.9 ± 1.7</td>
<td>11.8 ± 0.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Figure 2. AMP-DNM and ezetimibe have an additive effect on FNS excretion.
After 10 days of treatment, faeces were collected for 2 days from two cages (2-3 mice per cage) per group and the sterol content was analysed.
Figure 3. AMP-DNM treatment stimulates biliary cholesterol secretion and TICE.

After 2 weeks of treatment, gallbladder cannulations and intestine perfusions were performed. Cholesterol in collected bile and perfusate was measured to determine biliary cholesterol secretion (A) and TICE (B).

Table 2. Hepatic and intestinal relative gene expression in mice after treatment

Values are expressed as means ± SD. * Indicates a significant difference between the AMP-DNM treated animals and their corresponding controls. † Indicates a significant difference between treated groups and animals that received reference diet only.

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abca1</td>
<td>1.00±</td>
<td>0.78±</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>0.42</td>
</tr>
<tr>
<td>Abcg5</td>
<td>1.00±</td>
<td>0.82±</td>
</tr>
<tr>
<td></td>
<td>0.34</td>
<td>0.41</td>
</tr>
<tr>
<td>Abcg8</td>
<td>1.00±</td>
<td>0.67±</td>
</tr>
<tr>
<td></td>
<td>0.37</td>
<td>0.41</td>
</tr>
<tr>
<td>Npc1l1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Scarb1</td>
<td>1.00±</td>
<td>0.62±</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.13</td>
</tr>
<tr>
<td>HMG-CoAR</td>
<td>1.00±</td>
<td>1.86±</td>
</tr>
<tr>
<td></td>
<td>0.22</td>
<td>1.01</td>
</tr>
<tr>
<td>Rab9</td>
<td>1.00±</td>
<td>1.06±</td>
</tr>
<tr>
<td></td>
<td>0.23</td>
<td>0.20</td>
</tr>
<tr>
<td>Scarb2</td>
<td>1.00±</td>
<td>1.04±</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.11</td>
</tr>
</tbody>
</table>
AMP-DNM lowers plasma cholesterol
AMP-DNM treatment resulted in a reduction of plasma levels of glucosylceramide and cholesterol as earlier observed [10;11]. Ezetimibe treatment, on the contrary, either with or without AMP-DNM treatment, did not induce any changes in plasma cholesterol (Fig. 4A). The reduction of cholesterol observed with AMP-DNM (+ or – ezetimibe) was not restricted to one specific fraction in the lipoprotein profile (Fig. 4B, Table 3). Given the fact that the cholesterol lowering observed in the group treated with both drugs is due to AMP-DNM action only, we excluded that the modulation of lipid was linked to the observed reduction in food intake.

Figure 4. AMP-DNM treatment reduces plasma cholesterol levels.
Plasma levels of glucosylceramide (GluCer), total plasma cholesterol, and triglyceride levels were measured (A). Lipoproteins from pooled plasma samples were separated by HPGC combined with subsequent in-line total cholesterol determination (B).
Discussion

Previously, we have shown in mice that AMP-DNM treatment stimulates FNS excretion which can be partly explained by induced biliary cholesterol secretion [10;11]. In this study we determined which other pathways are additionally involved in the FNS increase: cholesterol absorption and TICE (intestinal excretion of cholesterol). To this end, we firstly compared the effect of AMP-DNM on cholesterol homeostasis in mice that were either simultaneously treated with ezetimibe or not. NPC1L1 mediates intestinal cholesterol absorption and its inhibitor ezetimibe is known to reduce cholesterol absorption to levels similar to what is observed in Npc1l1 deficient mice [18]. Our study shows that single treatment with either AMP-DNM or ezetimibe results in quite similar output of FNS. Interestingly, the addition of AMP-DNM in the ezetimibe treated group, increased additionally FNS excretion, indicating that AMP-DNM induces cholesterol excretion without affecting cholesterol uptake in the intestine. Indeed, we have earlier been unable to demonstrate that absorption of labelled cholesterol is impaired by AMP-DNM treatment [11].

Part of the sterols excreted via the faeces originates from cholesterol secreted in bile and from TICE that involves direct secretion of circulating cholesterol by the intestine [16;26]. We therefore examined by intestine perfusions the rate of TICE in animals treated with or without AMP-DNM. Iminosugar treatment was found to significantly increase TICE, in animals treated with or without ezetimibe. Thus, AMP-DNM seems to effect cholesterol homeostasis by promoting both biliary cholesterol secretion and TICE, but not by inhibiting cholesterol absorption. Both cholesterol secretion routes were found to be unaffected by ezetimibe treatment alone as described earlier [25].

Although glucosylceramide synthase inhibition induces FNS excretion, the expression of established genes involved in cholesterol transport in the liver or the intestine were found to be mostly unaffected. Treatment with ezetimibe alone did increase hepatic Hmg-CoA reductase compared to reference diet fed group, which indicates de novo cholesterol synthesis in response to reduced cholesterol
uptake \[27;28\]. Previous studies performed on TICE so far also revealed no direct relationship between intestinal expression of genes related to lipid homeostasis and activity of TICE \[25;29;30\]. Such studies suggested that expression of two genes, Scarb2 (also referred as lysosomal integral membrane protein type (LIMP 2)) and Rab9, may be associated with TICE \[25\]. Scarb2 is linked to glycosphingolipid metabolism by its recently demonstrated role in intracellular transport of the enzyme glucocerobrosidase \[31\]. Of interest, we observed a significant increased expression of Scarb2 mRNA in liver of mice treated with AMP-DNM and ezetimibe. This was however not seen in the intestine of the same animals. Scarb1 is the gene encoding for the scavenger receptor class B, type 1 (SR-B1), the receptor for HDL. HDL binds to SRB1 localized in the hepatocyte basolateral plasma membrane. Part of the HDL free-cholesterol is converted to bile acids and transported to the bile canaliculus for cosecretion with HDL-derived free cholesterol into bile for elimination\[32\]. Gene expression of Scarb1 was unexpectedly down regulated with AMP-DNM and this is in contrast with previous study where upregulation of SR-B1 mRNA was observed with AMP-DNM, in line with its effect on cholesterol lowering and bile production\[10; Bietrix et al unpublished\]. This gene was instead unregulated when mice were treated with both drugs, explaining the reduction in HDL and the effect on biliary cholesterol. Scarb1 mRNA was upregulated also in the intestine of animals treated with ezetimibe only. This could be a compensation mechanism to inhibition of cholesterol absorption and since it was shown that in vitro ezetimibe is able to bind SR-B1 and to block cholesterol uptake via this receptor \[33\].

A cholesterol lowering effect of AMP-DNM has now been demonstrated in both normo- and hyperlipidemic mouse models (this study and \[10;11\]). This beneficial effect of AMP-DNM is exerted by stimulation of RCT in a pleiotropic manner involving increased biliary cholesterol secretion and increased TICE. However, further research is needed to elucidate in which manner the inhibition of glycosphingolipid synthesis is regulating cholesterol homeostasis. In conclusion, our study shows that inhibition of glycosphingolipid synthesis by AMP-DNM increases FNS in mice by both stimulating biliary cholesterol secretion and TICE, two pathways of the RCT without affecting intestinal cholesterol absorption.

Coronary heart disease forms a major public health concern worldwide, with dyslipidemia as major risk factor. Current therapeutic strategies aim to reduce plasma cholesterol by inhibiting its hepatic synthesis \[34\] Statin drugs are recommended by several guidelines for both primary and secondary prevention \[35\]. They reduce the incidence of cardiovascular events by 25\% \[36\]. To achieve a further substantial drop in cardiovascular morbidity and mortality, new therapeutic modalities are desired. Pathways involved in removal of arterial cholesterol and elimination of cholesterol in excess from the body are therefore under
intense investigation. Targeting the synthesis of glycosphingolipids appears an interesting avenue in this respect. We have recently shown that the AMP-DNM, an iminosugar inhibitor of the glucosylceramide synthase catalyzing the rate controlling step in the biosynthesis of glycosphingolipids [12], effectively lowers plasma cholesterol in mice and improves excretion of cholesterol from the body [10;11]. AMP-DNM promotes reverse cholesterol transport by stimulating both secretion of cholesterol in bile and trans-intestinal cholesterol excretion (TICE), the more recently described pathway involving secretion of cholesterol from blood through the intestine [16]. In this manner AMP-DNM is able to prevent atherosclerosis development in two models of hypercholesterolemic mice [11]. The anti-atherogenic action of AMP-DNM warrants further investigations with respect to underlying mechanisms and possible application as drug for prevention of coronary heart disease in man.
Reference List


25. Vrins CL, van der Velde V, van den Oever K et al: PPARd activation leads to increased trans intestinal cholesterol efflux. J. Lipid Res. 50(10), 2046-2054 (2009).


