Glycosphingolipids and atherosclerosis
Lombardo, E.

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

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.
CHAPTER IV

Short term inhibition of glycosphingolipid synthesis does not promote atherosclerosis regression in LDLR(-/-) mice.

Elisa Lombardo¹; Cindy P.A.A. van Roomen¹; Roelof Ottenhoff¹; Herman S. Overkleeft²; Albert K. Groen³, Arthur J. Verhoeven¹; Johannes M. Aerts¹; Florence Bietrix¹.

¹Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands.
²Division of Biopharmaceutics, Leiden Institute of Chemistry, Leiden University, Leiden, the Netherlands.
³Department of Pediatrics, University Medical Center Groningen, Groningen, The Netherlands.
Abstract
The compound AMP-DNM (N-(5'-adamantane-1’yl-methoxy)-pentyl-1-deoxyoijirimycin) is a potent inhibitor of the glycosphingolipid synthesizing enzyme glucosyleramide synthase. In our previous study we have shown that administration of AMP-DNM prevented hyperlipidemia and atherosclerotic lesion development in APOE*3 Leiden and LDLR(-/-) mice. In the current study we determined whether treatment with AMP-DNM can promote regression and/or stabilization of already pre-existing plaques. For this purpose LDLR(-/-) mice were fed a western diet for 12 weeks to achieve lesion development. Subsequently, mice were treated with or without AMP-DNM for 6 weeks. The treatment ameliorated the hyperlipidemic status of the animals, but did not result in significant regression of atherosclerotic lesions. In conclusion, six weeks administration of the inhibitor AMP-DNM is not sufficient to positively affect atherosclerotic lesions.

Key words: atherosclerosis, cholesterol, glucosyleramide, regression
CHAPTER IV

Introduction

Atherosclerosis is a dynamic process involving different features such as endothelial dysfunction, lipid accumulation and chronic inflammation [1]. The evolution of a stable atherosclerotic lesion to a vulnerable stadium is crucial for the manifestation of acute coronary complications. Despite significant medical advances, atherosclerosis is still the leading cause of death in Western society. Treatment with statins, inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, is currently the therapy of choice to treat hypercholesterolemia associated with cardiovascular events. However two-thirds of the statin-treated patients still experience cardiovascular events [2]. Moreover atherosclerosis slowly develops over lifetime, but therapies that effectively stimulate the regression of atherosclerotic lesions are currently lacking and further research for new therapeutic agents are needed. Increasing evidence has accumulated demonstrating that glycosphinoglipids (GSL) play an important role in the atherogenetic process [3]. High GSL levels in atherosclerotic lesions and serum have been observed in atherogenic animal models as well as in humans [4–6]. Modulation of sphingolipid and GSL metabolism with the serine palmitoyltransferase inhibitor, myorcin, resulted in a decrease in the amount of atherosclerotic lesions in APOE -/- mice [7]. Our group recently developed a new compound that specifically targets glycosphingolipid synthesis, the iminosugar N-(5’-adamantane-1’y-l-methoxy)-pentlyl-1-deoxynojirimycin (AMP-DNM). This compound is an inhibitor of the enzyme glucosylceramide synthase [8]. In a previous study, we investigated the effect of GSL lowering in two mouse models for hyperlipidemia and atherosclerosis: APOE*3 Leiden and LDLR(-/-) mice. We were able to show that feeding the animals a western diet supplemented with AMP-DNM resulted in a strong inhibition of the development of atherosclerotic plaques in both models. This effect on the lesion progression was associated with low plasma levels of GSL but also of cholesterol and triglycerides. In that study the lipoprotein profile of treated mice was improved and the reverse cholesterol transport pathway was stimulated. In addition, mice receiving the treatment showed a decrease of inflammatory cytokines in plasma [3]. However, this study focused on lesions development but not on lesion regression.

In the current study the impact of AMP-DNM on established atherosclerotic lesions has been examined. To address this question, lesion size at the aortic sinus was monitored in LDLR(-/-) mice fed a western diet for 12 weeks and subsequently treated for 6 weeks with AMP-DNM[9] In a previous study, we showed that AMP-DNM treatment reduced plasma lipids and corrected non alcoholic steatohepatitis (the liver manifestation of the metabolic syndrome) induced by the high fat diet [9]. In the present study, we observed that despite the reduc-
tion of plasma lipids, no significant change in the extent of atherosclerosis in the aortic sinus of the animals was observed.

Materials And Methods

Materials
AMP-DNM was synthesized as previously described [10]. All solvents and reagents used were of analytical grade.

Animals study
Four groups of 10 female LDLR(-/-) mice (8-10 weeks-old) were fed for 12 weeks a western diet (0.25% w/w cholesterol, 15% w/w fatty acids; Arie Blok, Woerden, the Netherlands) to induce the development of atherosclerotic lesions. Then one group was sacrificed to establish the stage of the atherosclerotic plaques. The remaining three groups were fed for six additional weeks the western diet, supplemented with or without AMP-DNM at the doses of 50 and 100 mg/kg/day [9]. The study was approved by the local ethical committee for animal experiments.

Plasma and tissue sampling
Blood samples of non-fasted animals were collected during the study via the tail vein and by abdominal aorta puncture for terminal samples. Plasma samples were stored at -20°C. Hearts were flushed with PBS before fixation in formaldehyde 1% (formal-fixx, Thermo Electron Corporation, Pittsburgh, USA) for 24 h and stored at -80°C embedded in tissue medium (Tissue-Tek O.C.T, Sakura, Zoeterwoude, the Netherlands). Cryostat sections of 7 μM from the aortic sinus were prepared according to Paigen et al [11]. Atherosclerotic lesions were detected with Oil Red O staining. Collagen fibers were stained with 0.2% picro-sirius red. Anti-MOMA-2 (Abcam, Cambridge, UK diluted 1:50) was used for staining of macrophages and anti-alpha smooth muscle actin antibody (diluted 1:100) for staining of smooth muscle cells. Sections were then incubated with the corresponding secondary antibody and detected with DAB as chromogen. For all stainings, haematoxylin (Sigma-Aldrich, Zwijndrecht, the Netherlands) was used as counterstain. Primary antibodies were omitted in negative control samples. All quantifications were performed by using computer-assisted image quantification with Leica QWin software (Leica Microsystems, Wetzlar, Germany). Images were captured with a Leica DFC 420 video camera.
Analytical procedures
Cholesterol and triglycerides in plasma were determined using colorimetric enzymatic kits (Biolabo, Maizy, France). Ceramide and glucosylceramide were determined in liver and plasma samples after Folch extraction by high-performance liquid chromatography (HPLC) analysis of orthohtaldehyde-conjugated lipids according to a procedure described previously [12]. Plasma cholesterol concentrations in the main lipoprotein classes were determined in a pool of plasma of each group (10 mice) separated by high performance gel filtration chromatography (HPGC) as described before [13].

Statistical analysis
Values presented in figures represent mean ± SEM. Statistical significance between control group (CTRL) and the other groups was determined by ANOVA with Dunnet’s multiple comparison of means test. P-values < 0.05 were considered significant.

Results
AMP-DNM reduces GSLs in plasma
To evaluate the impact of treatment with AMP-DNM on atherosclerotic lesion regression, LDLR(-/-) mice fed 12 weeks with a western diet (baseline) and then fed 6 extra weeks with the western diet alone (ctrl) or supplemented with two different doses of AMP-DNM (50mg and 100mg) were used [9]. No differences in body weight and food intake before and after the treatment in the different groups were observed. At the end of the study, animals with AMP-DNM treatment showed a ~ 60% decrease of glucosylceramide levels in plasma for the 50 mg group and a~90% decrease with 100 mg, as compared with the baseline and control groups [9].

AMP-DNM corrects hyperlipidemia
8 weeks old LDLR(-/-) mice were fed a western diet for 12 weeks, in order to develop atherosclerotic lesion. After this period on western diet the animals of all groups had high levels of cholesterol and triglycerides in plasma. 6 weeks of treatment with AMP-DNM at the two doses resulted in correction of hyperlipidemia in the treated animals [9]. However, the lowering effect on plasma cholesterol did not occur directly after the switch of the diet. One week after starting the treatment the animals still showed high levels of cholesterol but low triglycerides in plasma (Fig 1A, B). The lipoprotein profile, analysed after 6 weeks of treatment, was also corrected in the treated animals, showing less cholesterol distributed in the VLDL and LDL particles (Fig 1C).
AMP-DNM is not able to promote regression of atherosclerotic lesions and does not induce the formation of stable plaques

We evaluated whether treatment with the iminosugar AMP-DNM could influence regression of established atherosclerotic plaques. Lesion severity was assessed at the aortic sinus for all the animals in the study. Lesion size was measured after lipid staining with Oil red O. Western diet feeding for 12 weeks induced the formation of severe atherosclerotic plaques. After 18 weeks, the control animals, as well as the treated ones, showed further development of the lesions with a 2-fold increase in lesion size compared with the group sacrificed after 12 weeks on western diet (Fig 2). AMP-DNM did not promote regression neither prevented the further progression. Lesions were therefore further characterized with
staining of collagen, macrophages and smooth muscle cells (SMC) (Fig 3). We observed no significant changes in the content of all these parameters relative to lesion area. Short-term treatment with AMP-DNM did not promote regression of the plaques neither arrest their progression, and did not improve any feature associated with lesion stabilization.

Figure 2: Effect of AMP-DNM treatment on atherosclerosis progression in LDLR(-/-) mice fed a western diet for 18 weeks, receiving in the last 6 weeks either 0, 50 or 100 mg AMP-DNM.

(A) Representative photomicrographs of Oil Red O-stained fatty streaks in the aortic root of the indicated mice (original magnification x 5) and (B) quantitative analysis of atherosclerosis lesion size.
Figure 3: 6 weeks treatment with AMP-DNM does not improve plaques quality.

(A) Representative photomicrographs of lesion collagen content stained with Sirius red; macrophages content staining with anti-MOMA-2 antibody (original magnification x 5) and smooth muscle cells (SMC) content staining with anti-α actin antibody (original magnification x 10).

(B) Quantitative analysis of staining positive areas relative to total lesion area.
Discussion

In a previous study we were able to show that treatment with the iminosugar AMP-DNM, inhibitor of glycosphingolipid synthesis, was able to prevent the formation of atherosclerotic lesions in mice fed with an atherogenic diet. In the present study we established whether AMP-DNM is able to regress existing atherosclerotic lesions.

For this purpose, we monitored of lesion size at the aortic sinus was in LDLR(-/-) mice fed a western diet for 12 weeks followed by 6 weeks treatment with AMP-DNM. The plasma data obtained at the end of the study, confirmed the strong effect of AMP-DNM on plasma cholesterol and triglyceride concentration previously observed [3,9]. In fact, despite the fact that AMP-DNM treated animals remained on western diet, they showed correction of the hyperlipidemia induced by the western diet alone. The improvement of the lipoprotein profile for cholesterol was associated with a reduction in VLDL and LDL content. It should be noted that the lowering of plasma lipids followed different patterns for cholesterol and triglycerides. The correction of triglyceride levels, in fact, occurred quickly after starting the treatment. Plasma levels after one week of AMP-DNM feeding were significantly lower compared to control mice (Fig 1B) and close to those before the western diet feeding, indicating an almost complete correction of the hyper-triglyceridemia in treated animals. The strong effect of AMP-DNM on plasma triglycerides might be related to the correction of liver steatosis observed earlier in these animals (chapter VIII). After one week of AMP-DNM treatment, the improvement of the cholesterolemia in treated animals was not yet observed. Moreover, the level of cholesterol achieved after 6 weeks treatment remained higher compared to control levels [3]. This delay in cholesterol lowering was new to us and the mechanism behind has not yet been investigated. With further investigation of the plaque size and composition, we could not observe any modification in either the size or the composition of the plaque that could indicate signs of regression. This finding was somehow disappointing, but may not be entirely unexpected. Plasma cholesterol plays beyond any doubt a key role in atherosclerosis development. However, a decrease of this parameter is not always associated with a regression of the atherosclerotic lesions. For example, in APOE*3 Leiden mice, Verschuren et al did not observe a regression of pre-existing atherosclerotic lesions despite a 70 % decrease of plasma cholesterol induced by a change of diet [14]. In ApoE(-/-) mice and LDLR(-/-) mice, regression or inhibition of progression of atherosclerotic lesions can be achieved only when restoring plasma cholesterol levels close to the wild type C57Bl/6 value [15,16]. Cholesterol plasma levels achieved in our study are far above the wild type ones. When Glaros et al. orally administered myoricin, the inhibitor of
serine-palmitoyl transferase, to ApoE(-/-) mice, they were only able to observe a slight effect in slowing lesion progression at the abdominal aorta whereas no effect was reported at the level of the aortic sinus [17]. Unfortunately, aortas of the animals were not collected in this study and, changes at this level can not be excluded. Moreover other parameters have to be taken into account, such as severity of the pre-existing lesions, the mouse strains used and the serum cholesterol exposure. Based on these findings, it seems possible that the noted absence of a beneficial effect of AMP-DNM treatment on existing atherosclerosis in this study results from the relatively short period of treatment and the limited reduction in plasma cholesterol. In addition, in our study, the animals had developed quite severe lesions in the aortic sinus prior to the intervention with AMP-DNM treatment, making an intervention treatment even more difficult. It appears that short term treatment with AMP-DNM on LDLR(-/-) mice presenting advanced lesions and severe hyperlipidemia is not sufficient to achieve regression of severe atherosclerotic lesions, but it is enough to ameliorate the hyperlipemic status of the animals.

**Acknowledgements**

We are grateful to Han Levels for helping with determination of the lipoprotein profile. We are grateful to the Netherlands Heart Foundation for supporting this work (grant 2007B030).
Reference List


