The glucosylceramide synthase inhibitor AMP-DNM induces SREBP regulated gene expression and cholesterol synthesis in HepG2 cells.

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Non-standard Abbreviations: AMP-DNM, N-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin; CER, ceramide; CTH, ceramide trihexoside; DRM, detergent resistant membrane; EGF, epidermal growth factor; GCS, glucosylceramide synthase; GlcCer, glucosylceramide; GM3, sialocyllactosylceramide; GSL, glycosphingolipids; LacCer, lactocylceramide; SM, sphingomyelin; SREBP, sterol regulatory element-binding protein;

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Abstract

Recent findings have implicated glycosphingolipids as modulators of insulin receptor activity. Studies with C57BL/6J ob/ob mice have shown that insulin sensitivity is enhanced by the synthetic hydrophobic iminosugar AMP-DNM (N-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin) that inhibits glucosylceramide synthase. Here we treated the liver hepatoma cell line HepG2 with AMP-DNM, resulting in a 70% reduction of glycosphingolipids, and analyzed the effect on gene expression. Using Agilent whole human genome 44K oligo arrays, we identified 89 genes that were significantly (p<0.01) up- or down regulated by AMP-DNM treated treatment. Of the 56 up-regulated genes, 17 were direct target genes for transcription factors sterol regulatory element-binding protein 1 (SREBP1) or SREBP2, which activate genes in the sterol biosynthesis pathway. An increase in cholesterol production rate confirmed that the induction of SREBP target genes seen at the mRNA level resulted in activation of the cholesterol biosynthesis pathway. Interestingly, the cholesterol content of the cells did not increase. Importantly, no effects were found on expression of genes related to cell receptor signalling pathways, neither on toxicity or cell growth. Our findings indicate that inhibition of glucosylceramide synthase with AMP-DNM leads to activation of SREBP target genes and synthesis of cholesterol in HepG2 cells.
Introduction

It has become increasingly clear that the lipid composition of cell membranes and especially of lipid rafts play a major role in cell signaling processes. Lipid rafts or detergent resistant membranes (DRMs) are specialized domains of cell membranes enriched in cholesterol, sphingomyelin (SM), and glycosphingolipids (GSLs) (Lahiri and Futerman, 2007). The signaling capacity of various receptors such as the insulin receptor and the epidermal growth factor (EGF) receptor that reside in these lipid rafts can be influenced by changing the glycosphingolipid composition of these membrane domains (Inokuchi, 2006; Kabayama, et al., 2005; Rebbaa, et al., 1996). This process was first described by Bremer and colleagues (Bremer, et al., 1986) who showed that EGF-mediated signaling is inhibited by the ganglioside sialocyllactosylceramide (GM3). Furthermore, addition of GM3 to adipocytes in culture suppresses phosphorylation of the insulin receptor resulting in reduced glucose uptake (Tagami, et al., 2002). This could have important physiological consequences since glycosphingolipids have indeed been shown to accumulate in tissues from insulin-resistant rodents and humans (Summers and Nelson, 2005). Moreover, GM3 synthase deficient mice are protected from high fat diet-induced insulin resistance (Yamashita, et al., 2003). Thus either blocking GM3 synthase or inhibiting the synthesis of excess glycosphingolipids could improve insulin signaling in vivo.

We have previously described AMP-DNM, (N-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin), an inhibitor of glucosylceramide synthase that specifically lowers glycosphingolipid levels without affecting ceramide levels in various cell models (Aerts, et al., 2003; Overkleeft, et al., 1998; Wennekes, et al., 2007). AMP-DNM was
found to reverse insulin resistance and normalize blood glucose levels in animal models of diabetes and obesity (Zucker fa/fa rats, diet-induced obese mice and ob/ob mice). The compound also ameliorated lipotoxicity in kidney and pancreas (Aerts, et al., 2007).

Although these effects can be ascribed to improved insulin receptor function via modulation of glycosphingolipid levels by AMP-DNM, the question remains if this is the only effect of AMP-DNM in vivo. Glycosphingolipids are associated with a wide range of functions, ranging from mediation of cell adhesion to modulation of signal transduction (Lahiri and Futerman, 2007). To be able to begin to understand the effects of AMP-DNM in vivo, where various hormones and organs are involved in maintaining glucose homeostasis, we set out to investigate the effects of established low glycosphingolipids at the cellular level in vitro. The liver is one of the major players in the physiology of diabetes and obesity, due to its gluconeogenic capacity and its role in lipoprotein metabolism. We monitored the effect of AMP-DNM on total gene expression in human hepatoma HepG2 cells using genome wide microarray analysis. We found that treatment of HepG2 cells with AMP-DNM results in 70% reduction of glycosphingolipid content without an effect on expression of genes related to cell receptor signaling pathways, toxicity or cell growth. However, we did observe a specific activation of sterol regulatory element-binding protein (SREBP) target genes, associated with the synthesis of lipids and cholesterol.
Methods

**HepG2 cell culture**

The human hepatic cell line HepG2 was obtained from the ATCC (American Type Culture Collection, Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium/HAM’s Nutrient Mixture F-12 (DMEM/HAMF-12) (Invitrogen, Carlsbad, CA), supplemented with 10% foetal calf serum at 10% CO₂. For each experiment, cells were seeded in either 75 cm² or 25 cm² tissue-culture flasks or tissue culture plates as indicated and grown to 60-70% confluency. Cells were incubated in DMEM/HAMF-12 with 10µM AMP-DNM in 0.01% DMSO, or vehicle only. Incubation of control and AMP-DNM-treated cells was continued for 0, 24, 48 or 72 hours. AMP-DNM was synthesized as described (Overkleeft, et al., 1998).

**Lipid measurements**

Ceramide and neutral lipids levels were determined exactly as previously described (Groener, et al., 2007). In short, cells were washed 3 times in PBS, lysed in 500 µl water and further disrupted by sonication (3 strokes) on ice. Lipids were extracted with 2 ml chloroform/ methanol (1:1, v/v) according to Folch (FOLCH, et al., 1957) followed by deacylation in 500 µl 0.1 mol/L NaOH in methanol using a microwave oven (CEM microwave Solids/Moisture System SAM-155). The deacylated lipids were derivatised for 30 minutes with the addition of 25 µl O-phtaldehyde reagent to 50µl lipid mixture and separated with a high performance liquid chromatography (HPLC) method. Gangliosides were detected as recently described (Ghauharali-van der Vlugt, et al., 2007) by analysis.
of the acidic glycolipid fraction obtained by the Folch extraction. In short, the upper-phase was desalted on a C18 Sep-Pak (Bakerbond) column and the eluted gangliosides were digested with ceramide glycanase. The released oligosaccharides were labelled at their reducing end with the fluorescent compound anthranilic acid (2-aminobenzoic acid), prior to analysis using normal-phase HPLC. For total cholesterol and cholesterol-ester determination, cells were washed in PBS, lysed in 500µl 1% Triton X-100 (v/v) and further disrupted by sonication. Cholesterol content in these samples was measured using the cholesterol oxidase reaction coupled to fluorimetric determination of hydrogen peroxide as described (Elferink, et al., 1998).

Microarray expression profiling and pathway analysis

Cells were incubated in 75 cm² tissue-culture flasks for 48 hours in either normal medium (n=6 for reference RNA on the arrays), medium with vehicle (n=3 control samples), or medium with 10µM AMP-DNM (n=3 treated samples). Subsequently, RNA was extracted using the TRIzol RNA isolation method (Invitrogen), followed by a purification step using RNA II Nucleospin columns including DNAse treatment (Machery Nagel, Düren, Germany). RNA concentration and integrity was determined with a Nanodrop and Agilent 2100 Bioanalyzer respectively. The individual samples from the control and treated cells were used for probe synthesis and hybridized on separate arrays against a probe made from a pool of reference RNA. Probe synthesis and microarray hybridizations were performed by Service XS (Leiden, The Netherlands) using a direct labeling reaction and human whole genome 44 k arrays (Agilent Technologies, Santa Clara, CA). Processed images were imported into the Rosetta Resolver database and
analysis software (Rosetta Biosoftware). Statistical analysis was performed using false discovery rate (FDR)-corrected p-values, involving a recalculation of the P values using a Benjamini-Hochberg correction for multiple testing. Genes with a significantly (p<0.05) altered expression profile were imported into pathway analysis software (MAPPfinder version 2.0) and PubMed-mining software (Ingenuity Pathway Analysis version 2; Ingenuity Systems) for further analysis.

Expression-level analysis

Quantitative gene analysis was used to confirm differential gene expression of candidate transcripts found by microarray analysis. Total RNA (2µg) was used for first-strand cDNA synthesis with SuperScript II Reverse Transcriptase and oligo-dT primer (Invitrogen, Carlsbad, CA). The PCR primers were designed using Primer 3 (Rozen and Skaletsky, 2000) and specificity was verified by conventional PCR. Quantitative PCR was performed with the MyIQ real-time detection system (Bio-Rad Laboratories Inc., Hercules, CA) using the SYBR Green I reaction mix (Bio-Rad). PCRs were performed in duplicate and normalized to cyclophilin and acidic ribosomal phosphoprotein P0 (36B4).

Preparation of nuclear extracts and western blot analysis

HepG2 cells were incubated in DMEM/HAMF-12 with 10µM AMP-DNM in 0.01% DMSO, or vehicle only for 48 hrs in 10 cm² tissue-culture dishes. Subsequently, cells were washed with ice-cold PBS. To prevent degradation of proteins a cocktail of protease inhibitors was added to the lysis buffer (Complete, Roche Molecular Biochemicals). Nuclear and cytosolic extracts were prepared using a nuclear extract kit (NE-PER, Pierce,
Rockford, IL) according to the manufacturer’s protocol. The protein concentration was measured with the BCA protein assay kit (Pierce, Rockford, IL). Nuclear extracts containing samples were desalted using protein desalting spin columns (Pierce, Rockford, IL). Equal amounts of proteins (20µg) were subjected to electrophoresis on 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Millipore) using an electroblotting apparatus (Bio-Rad Laboratories, Hercules, CA). The blots were blocked in Starting Block buffer (Pierce) and incubated with a polyclonal antibody against SREBP1 (Santa Cruz Biotechnology) diluted (1:500) in block buffer containing 0.01% (v/v) Tween-20, overnight at 4ºC. Blots were washed for 30 minutes in TBS (10 mM Tris-HCl (pH 8.0), 150 mM NaCl) containing 0.01% (v/v) Tween-20. After washing, the membranes were incubated with secondary antibody (anti-rabbit IgG peroxidase conjugate; Dako) diluted 1:3000 in block buffer containing 0.01% Tween-20, for 1 hour at room temperature. Proteins were detected with the enhanced chemiluminescence system (Amersham) and x-ray film. The densitometric analysis of bands was done with Image Quant software. To confirm equal protein loading and blotting, total protein on the blots was detected with a MemCode reversible protein stain kit (Pierce, Rockford, IL).

**Cholesterol synthesis measurements**

HepG2 cells were pre-incubated with DMEM/HAMF-12 with 10µM AMP-DNM in 0.01% DMSO, or vehicle only for 40 hrs in 25 cm² tissue culture flasks. Subsequently 1µCi [1-14C]-acetate was added to the culture medium and flasks were sealed with a rubber cap. After 4 hours 200µl 2M NaOH was injected through the rubber cap into a
small cup inside the culture flask and the produced (radioactive) CO₂ was trapped for 30 minutes. Cells were washed 3 times with 0.2% (w/v) BSA in TBS, 3 times with TBS only and then lysed in 0.2 M NaOH. Radioactivity in the homogenates and trapped CO₂ was measured by liquid scintillation counting and total protein content by BCA staining (Pierce, Rockford, IL).

Lipids in the homogenates were hydrolysed in 50% (w/v) NaOH and extracted 3 times with hexane. Extracts were evaporated under nitrogen and separated by silica thin layer chromatography with diethylether/petroleumbenzin/acetic acid (50:50:1) as the mobile phase. Cholesterol was quantified by phosphorimaging (Fuji FLA-3000) with the aid of Aida software package.

**Cholesterol efflux**

Cholesterol efflux in HepG2 cells was measured with or without AMP-DNM incubation. HepG2 cells were incubated with DMEM/HAMF-12 with 10µM AMP-DNM in 0.01% DMSO, or vehicle only for 24 hours. Cells were then loaded for a following 24 hours with 0.5 µCi/ml [³H] labelled cholesterol in fresh culture medium with 10µM AMP-DNM in 0.01% DMSO, or vehicle only. After the 48 hour incubation period cells were washed 4 times with 0.2% (w/v) free fatty acid-free BSA in HBSS. Acceptor medium (DMEM/HAMF-12 with 0.2% free fatty acid free BSA with 10µM AMP-DNM in 0.01% DMSO, or vehicle only) supplemented with various cholesterol acceptors (10% FCS, 10µg/ml ApoA1 or 100µg/ml HDL) was added followed by 24 hours incubation. Subsequently the medium was collected, cells were lysed in isopropanol and [³H] cholesterol content in the medium and lysates was quantified by liquid scintillation
counting. Cholesterol efflux to the various acceptors was calculated by dividing the output of $[^3\text{H}]$ cholesterol to the medium by the input (total counts in both medium and isopropanol extracted lipids). The influence of possible cholesterol exchange between medium and cells on net flux was evaluated by measuring cholesterol mass transfer. To this end, efflux experiments as described above were performed with the omission of $[^3\text{H}]$ cholesterol. Free cholesterol and cholesterol-ester were measured in input media, cells and efflux media. Net cholesterol mass transfer was calculated by subtracting the free cholesterol in the input media from the amount of free cholesterol in the acceptor media after the efflux period.

**Statistics**

All results are presented as means ± SD. Statistical significance of differences was evaluated by Student’s $t$-test. Significance was set at $P$ values <0.05.
Results

Modulation of glycosphingolipid levels by AMP-DNM in HepG2 cells.

HepG2 cells were incubated for up to 72 hours with 10µM of AMP-DNM, a concentration that was previously shown to block GCS activity in cultured cells completely (IC50 1.5µM, (Aerts, et al., 2007)). After the indicated time intervals, lipids were extracted and ceramide, GlcCer, LacCer, CTH and GM3 contents were quantified (Fig. 1). In accordance with previous results ceramide levels remained constant upon exposure to AMP-DNM. In contrast, GlcCer and LacCer levels decreased to 58% and 67% respectively within 24 hours, declining even further upon prolonged treatment (to less than 35% after 72 hours). The GM3 content showed an even more rapid decrease in the first 8 hours resulting in a 49% reduction after 24 hours and declining still further to a level of 17% after 72 hours. We could not detect changes in total cell number, protein or RNA concentration between treated and control cell culture flasks at any of the indicated time intervals (data not shown).

Identification of AMP-DNM responsive genes.

Inhibition of glycosphingolipid synthesis occurs almost instantly after addition of AMP-DNM to the culture medium and a reduction in glycosphingolipids is therefore visible at an early time point. In this study we monitored the adaptations on the level of gene expression due to established low levels of glycosphingolipids as can be found in the in vivo situation where liver sphingolipid levels are permanently reduced by about 60% after AMP-DNM treatment. A similar reduction in glycosphingolipid levels was reached after
48 hrs of AMP-DNM treatment in vitro and we thus used this time point to monitor gene expression. Using Agilent whole human genome 44K oligo arrays, we identified 89 genes that were significantly (p<0.01) up- or down regulated after treating HepG2 cells for 48 hours with 10µM AMP-DNM. Literature data mining and pathway analysis, using the Ingenuity pathway Analysis software on this set of 89 AMP-DNM responsive genes revealed a significant induction of genes classified to sterol metabolic processes (Table 1).

Transcription of genes in the sterol synthesis pathway is controlled by a family of transcription factors, the sterol regulatory-element binding proteins (SREBPs). Three isoforms exist, SREBP-1a, SREBP-1c and SREBP-2. SREBP-1a is a potent activator of all SREBP-responsive genes, and the dominant isoform in most cultured cell lines including HepG2 (memiya-Kudo, et al., 2002; Shimano, et al., 1997; Shimomura, et al., 1997), precluding the possibility to differentiate between SREBP1 and SREBP2 mediated effects.

Further analysis of the microarray data indicated that of the 51 published SREBP1 and 2 target genes that were present on the array (Horton, et al., 2003), 27 were significantly up-regulated (17 with $P<0.01$ and 10 with $0.01<P<0.05$ indicated with * in Table 1) upon AMP-DNM treatment of HepG2 cells. A total of 21 of these genes were classified to the sterol metabolic processes, including the rate-limiting enzyme in the cholesterol synthesis pathway 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR). Up-regulation of these genes was modest (up to 1.4 fold change in expression level), but none of the SREBP responsive genes present on the array were down-regulated.
In addition to the 21 genes encoding proteins involved in the sterol biosynthesis pathway, a large group of genes involved in lipid metabolic processes was significantly induced by AMP-DNM treatment of HepG2 (Table 1, lipid metabolic processes). Six of these, e.g. the fatty acid desaturases, are part of the SREBP1a activated pathway for monounsaturated and polyunsaturated fatty acid synthesis. Other genes found are also involved in lipid metabolic processes, though they are not directly regulated by SREBPs. For example, the acyl-CoA-binding proteins bind medium- and long-chain acyl-CoA esters with very high affinity and may function as intracellular carriers of acyl-CoA esters. Fatty acid binding protein 1 also binds long-chain fatty acids and is able to bind bile salts as well. It is an abundant constituent of the cytoplasm and has a major role in bile acid and biliary cholesterol metabolism (Martin, et al., 2005).

The third group detected, consists of genes classified as involved in general metabolic processes. However, some of these are also involved in cholesterol and lipid homeostasis such as lysosomal acid lipase (LIPA), otherwise known as acid cholesteryl ester hydrolase. LIPA is essential for the intracellular hydrolysis of cholesteryl esters and triglycerides that have been internalised via receptor-mediated endocytosis of lipoprotein particles.

Remaining classification groups (responsive to chemical stimuli, miscellaneous and unclassified) are provided as supplementary data.

**AMP-DNM treatment gives higher levels of nuclear SREBP and induces cholesterol synthesis.**
The concerted induction of various key genes in the sterol biosynthesis pathway suggests an effect of AMP-DNM on their mutual transcription factor SREBP. Indeed, real-time RT-PCR detected an almost twofold induction of SREBP1a mRNA by AMP-DNM incubation (Fig. 2A). SREBPs are located as inactive precursors in the ER. Only when cells become cholesterol depleted, SREBP cleavage activating protein (SCAP) escorts the SREBP from the ER to the Golgi apparatus for processing and activation, followed by transportation to the nucleus (Horton, 2002). Western blot analysis of total cell lysates and isolated nuclei indeed showed that AMP-DNM treatment specifically increased the amount of nuclear SREBP (Fig. 2B). We next investigated the physiological consequences of enhanced SREBP target gene expression. Total cholesterol production was measured using [14C]-acetate in HepG2 cells incubated for 48 with or without 10µmol/l AMP-DNM. An increase of 84% in total cholesterol synthesis was observed in AMP-DNM treated cells as compared to control cells (Fig. 3), in line with the increase in mRNA expression of the sterol biosynthesis genes that was observed under the same conditions. Stimulated cholesterol production was not due to an increase in overall metabolic activity, since the CO2 production was equal in control and treated cells (Fig. 3).

AMP-DNM treatment induces cholesterol efflux.

Following the observation that cholesterol production was increased, we analysed the efflux of cholesterol upon glycosphingolipid (GSL) reduction in AMP-DNM treated cells to various possible acceptors (HDL, ApoA-I or normal culture medium). We detected a small but significant increase in efflux rates of cholesterol in AMP-DNM treated cells.
with normal culture medium (containing 10% FCS) as the acceptor. Although there seemed to be a small additive efflux in AMP-DNM treated cells when ApoA-1 and HDL were provided as acceptors these results were not significant (Fig. 4). Similar efflux results were obtained when we measured total cholesterol mass transfer confirming that the efflux was due to an increase in cholesterol output. We did not find an effect of AMP-DNM treatment on bile acid secretion (data not shown).

The increase in cholesterol production and the small increase in efflux influenced the cellular cholesterol pool size. After 24 hours no change in cholesterol pool size was observed. After 48 hours of treatment the cholesterol ester content and total levels of cholesterol were below control levels (Fig. 5).
Discussion

The most striking result in this study is that treatment of the liver hepatoma cell line HepG2 with the iminosugar AMP-DNM had only a minor effect on gene expression despite a strong reduction of GlcCer, LacCer and GM3 content of these cells to 33%, 35% and 27% respectively (Fig. 1). Using a 44k Agilent chip-based approach we identified only 89 genes that showed reproducible up- or down-regulation in response to AMP-DNM treatment (Table 1). This effect was not observed in earlier time-points (data not shown), indicating that it was a result of glycosphingolipid lowering rather than a direct effect of AMP-DNM on gene expression. Interestingly, of the 56 up-regulated genes, 17 (p<0.01) were direct target genes for transcription factors SREBP1 or SREBP2 that activate genes in the sterol and lipid biosynthesis pathways. Indeed, we found an increased amount of SREBP in the nucleus (Fig. 2 B). An 84 % increase in cholesterol production rate confirmed that the presence of nuclear SREBP resulted in activation of the cholesterol biosynthesis pathway (Fig. 3). The increase in cholesterol production did not lead to increased cholesterol pools. On the contrary, cholesterol pool size slightly decreased after 48 hours of AMP-DNM treatment, probably reflecting an increase in cholesterol efflux.

Cholesterol efflux from cells is a complex process mediated primarily by the ABC transporters ABCA1 and ABCG1, but also the HDL-receptor SR-BI and passive aqueous diffusion have been implicated. Expression levels of these transporters were not significantly altered by AMP-DNM. Consequently, the lack of significant increase in efflux to apolipoprotein A-I or HDL came not as a surprise. In hepatocytes in vivo cholesterol can also be effluxed by the cholesterol transporter heterodimer
ABCG5/ABCG8 which is presented at the canalicular membrane in polarized cells. HepG2 cells express ABCG5 and ABCG8. However, these cells are not polarized and ABCG5 and ABCG8 are probably only expressed in intracellular vacuoles. Furthermore, it has been shown recently that efflux mediated by ABCG5 and ABCG8 occurs only in the presence of micellar concentrations of bile salt (Vrins, et al., 2007). We did not add bile salts in our experiments so even if the proteins are partly present on the plasma membrane no ABCG5/ABCG8 mediated efflux can occur.

Previous studies in fibroblasts have shown that depletion of GSL following incubation with PDMP, a GCS inhibitor, promoted the apoA-1 dependent cholesterol efflux (Glaros, et al., 2005). Apparently, liver cells behave differently. We did detect a small but significant increase in efflux rate of cholesterol in AMP-DNM treated cells to normal culture medium. The culture medium contains 10% FCS which apparently does contain as yet uncharacterized cholesterol acceptors explaining the slight decrease in intracellular cholesterol observed after 48 hours of incubation. No increase in bile acids was observed, the primary metabolite of cholesterol in liver cells. We can, however not exclude that some cholesterol has been converted to cholesterol-glucuronide, sulfate or glucoside.

Events leading to SREBP activation upon reduction in sphingolipid levels, rather than by classical activation by cholesterol depletion are somewhat enigmatic. However, experiments by Scheek et al. (Scheek, et al., 1997) suggest that the balance between cholesterol and other components of the ER membrane such as sphingomyelin, is more important for SREBP activation than cholesterol levels as such. Although we could not detect changes in total sphingomyelin levels in these cells (data not shown), it is possible
that AMP-DNM treatment causes a disturbed balance in cholesterol and (glyco)sphingolipids in the ER membrane, (in-) directly leading to SREBP activation. More recent studies have also shown that synthesis of sphingolipids in itself activates SREBP processing, independently of cellular cholesterol concentration (Worgall, et al., 2002; Worgall, et al., 2004). Cholesterol-independent SREBP regulation is also found in Drosophila melanogaster, where SREBP levels are regulated by phospholipids instead of sterols (Rawson, 2003).

Despite the substantial effect of AMP-DNM treatment on GSL levels in HepG2 cells, the effects on gene expression patterns were small scale, with only 89 genes significantly up- or down-regulated. Although AMP-DNM was shown to have an effect on insulin sensitivity (Aerts, et al., 2007), we did not find genes involved in any of the known receptor signalling pathways to be significantly regulated. The microarray data results show that AMP-DNM modulates SREBP processing, possibly by altering the glycosphingolipid content of the cells. It is not clear how the in vitro induction of SREBP target genes in liver upon AMP-DNM treatment relates to the beneficial effects on blood glucose levels and insulin resistance in animal studies. Additional in vivo and in vitro studies will be performed to elucidate the effects of glycosphingolipid manipulation in metabolically important tissues such as liver, gut, fat and muscle cells. Studying the effects of AMP-DNM in various tissues will bring us a better understanding of how glycosphingolipids are involved in various features of the Metabolic Syndrome.
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References


Footnotes

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Legend for figures

Fig. 1: Effect of AMP-DNM on ceramide and glycosphingolipid levels in HepG2 cells.
HepG2 cells were treated with control or AMP-DNM (10μmol/l) medium for 48 hours. Sphingolipid content was determined by HPLC-based procedures as described in methods. Values represent means of 3 wells of AMP-DNM treated cells related to means of 3 wells of control treated cells ± SD. Data are representative of three independent experiments.

Fig. 2: AMP-DNM treatment induces nuclear SREBP.
HepG2 cells were treated with control or AMP-DNM (10μmol/l) medium for 48 hours.
A. RNA levels for SREBP1 were analysed by quantitative PCR (n=3). Fold induction by AMP-DNM treatment is indicated. **P<0.01. B. Western blot analysis performed with antibody against SREBP1 on the cytosolic fraction and isolated nuclei of control or AMP-DNM-treated HepG2 cells. P and M indicate the precursor (125 kd) and mature (68 kd) forms of SREBP1. Bars indicate means (n=3) of quantified SREBP1 levels ± SD corrected for total protein levels. *P<0.05

Fig.3: AMP-DNM treatment induces cholesterol synthesis in HepG2 cells.
HepG2 cells were treated with 10μM AMP-DNM in 0.01% DMSO, or vehicle only for 40 hrs. Subsequently cells were loaded with [14C]-acetate and after 4 hours CO₂ was
captured and quantified with LSC as described in materials and methods. Cholesterol was extracted and spotted on TLC. Data are means of 3 separate flasks ± SD. *P<0.05

**Fig. 4: Effect of AMP-DNM on cholesterol efflux in HepG2 cells.**

HepG2 cells were treated with 10µM AMP-DNM in 0.01% DMSO, or vehicle only for 24 hours. Cells were then loaded for a following 24 hours with [3H] labelled cholesterol in fresh culture medium with 10µM AMP-DNM in 0.01% DMSO, or vehicle only. After this incubation, the efflux of [3H]-cholesterol to various acceptors was measured as described in methods. Data are means of 3 separate wells ± SD. Similar results were obtained in 3 independent experiments. *P<0.05

**Fig. 5: Effect of AMP-DNM on cholesterol pools in HepG2 cells.**

HepG2 cells were treated with 10µM AMP-DNM in 0.01% DMSO, or vehicle only for either 24 or 48 hours. Total cholesterol was extracted and quantified as described in materials and methods. Data are means of 3 independent experiments ± SD. *P<0.05
Table 1: Genes whose transcripts in HepG2 cells change with 10µM AMP-DNM treatment. Classification groups: sterol-, lipid-, and general metabolic processes.

HepG2 cells were treated with control or AMP-DNM (10µmol/l) medium for 48 hours. The mRNA expression of each gene in AMP-DNM treated cells compared to vehicle treated cells is shown. Genes with a relative expression exceeding 1.2 (absolute fold change>1.2) and (p<0.01) are grouped according to function and are listed in alphabetical order under each heading.

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### Lipid metabolic processes

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Fold Change</th>
<th>Accession</th>
<th>#</th>
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<tbody>
<tr>
<td>Sterol-CS-desaturase-like</td>
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</table>

### General metabolic processes

| Gene Name | Fold Change | Accession | 
|-----------|-------------|-----------|---|
| 2-oxoglutarate 4-dioxygenase | 1.45 | P4HA2 | NM_004199 |
| Cadherin 1 | 1.38 | CDH1 | NM_004360 |
| Calreticulin | 1.55 | CALR | NM_004343 |
| CD4 molecule | 1.25 | CD4 | NM_000616 |
| Lipase A | 1.46 | LIPA | NM_000235 |
| Meprin A, alpha | 1.33 | MEP1A | NM_005588 |
| NADP+ specific isocitrate dehydrogenase | 1.24 | ENST00000305820 | ENST00000305820 |
| Phosphatidylinositol binding clathrin assembly protein | 1.66 | PICALM | BF335836 |
| Protein with high similarity to klotho (mouse Kl) | 1.32 | I_1109100 | I_1109100 |
| Serpin peptidase inhibitor, clade E, member 2 | -1.24 | SERPINE2 | NM_006216 |
| Solute carrier family 2 (facilitated glucose transporter) | -1.38 | SLC2A3 | NM_006931 |
JPET #139394

| Transthyretin | 1.32 | TTR | NM_000371 |

*: **SREBP** regulated genes are added if they fulfilled the criteria: absolute fold change > 1.2 AND p < 0.05

#: **SREBP1a** (#1), **SREBP2** (#2) or both (#1/2) regulated as described (*Horton, et al.,* 2003)
Figure 1

The graph shows the relative lipid content (%) over time (hours) for different lipids: cer, glccer, laccer, CTH, GM3. Each bar represents the lipid content at specific time points: 0, 8, 24, 48, and 72 hours. Significant differences are indicated by asterisks (*).
Figure 4

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Figure 5

![Graph showing total cholesterol levels over 24 and 48 hours for control and AMP-DNM groups.](image-url)