Pompe disease
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2010

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 5

Mimicking Pompe phenotypes for future therapeutic studies: Biodistribution of non-modified siRNA

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ABSTRACT
Pompe disease, an autosomal recessive lysosomal disorder, is caused by a genetic mutation of acid alpha glucosidase (GAA). The current therapy, enzyme replacement therapy (ERT), compensates for the deficient enzyme by administering recombinant enzyme. As ERT does not cure the genetic background, we focus on designing targeted nucleases to induce homologous recombination for gene correction. To evaluate gene correction strategies, animal models with low GAA plasma levels resembling clinical situation would be preferential. In this study, we explored the ability of the cationic amphiphilic lipid SAINT as a delivery agent for GAA secreting cells in vivo. For this purpose, we used small interfering RNA and demonstrated lowered GAA plasma levels after intravenous injection. Detailed investigation to demonstrate affected cells did not result in identifying such cells. However, biodistribution studies using radioactive labeled SAINT showed that SAINT mainly localized in the liver. The data presented here demonstrates that SAINT is able to deliver siRNA and proves a promising delivery agent to study future therapeutic approaches for Pompe disease in an in vivo setting.
INTRODUCTION

Pompe disease is a rare autosomal recessive lysosomal storage disease caused by the deficiency of acid-alpha-glucosidase (GAA). This disease is characterized by glycogen accumulation in the lysosome leading to generalized muscle weakness combined with cardiomyopathy ultimately leading to death of the patient. The only therapy clinically available for Pompe disease is Enzyme Replacement Therapy (ERT), which aims to compensate for the lack of enzyme by continuous supply of recombinant produced enzyme. Although the therapy is extending lives, diminishing severeness, some drawbacks hampers the success dramatically. Life-long treatment is necessary to continue effectiveness of ERT, sufficient amounts of the recombinant enzyme needs to be produced which is associated with high costs. Moreover, the low uptake of the exogenously administered enzyme leads to a variability of response. Finally, the treatment often leads to an immune response. To address these limitations of ERT, we previously investigated the uptake of recombinant human GAA (rhGAA) in vitro using the cationic lipid compound SAINT as a shielding and potentially targeting delivery device. We demonstrated efficient uptake of the recombinant produced enzyme in patient derived skin fibroblasts when complexed with SAINT (chapter 3, submitted).

However, even though reducing the amount of enzyme needed for ERT would decrease the costs, the genetic defect underlying the disease will not be cured. To overcome this, gene therapy using integration-competent viruses can be envisioned. As several disadvantages are associated with such approaches, we and others explore strategies for true gene correction therapy for Pompe disease using targeted nucleases. Gene correction, which is based on specific induction of a double strand break (DSB) in the DNA followed by homologous recombination (HR), could be used to correct the genetic defect. Lu et al. previously demonstrated gene correction of the C1935→A mutation found in Pompe patients using single stranded oligonucleotides both in vitro and in vivo. We want to investigate the role of targeted nucleases, DNA binding domains (either Triple helix Forming Oligonucleotides (TFO) or Zinc Finger Proteins (ZFP)) fused to a DNA damaging agent, to induce gene correction in Pompe disease (chapter 7). The cellular effects of such therapeutic approaches needs to be evaluated in animal models with low GAA plasma levels resembling the clinical situation.

In this study, we investigate whether siRNA in complex with SAINT can target GAA secreting cells. If SAINT delivers siRNA in vivo, which leads to GAA downregulation in plasma and organs, then this animal model allows us and others to test future therapeutic approaches for Pompe disease in an in vivo setting.

MATERIAL AND METHODS

Reagents

Predesigned gene-specific siRNAs for GAA (mouse siRNA 1, target sequence AACGAACCGTCCAACTTCGT; mouse-human siRNA 2, target sequence AACAGCAATGCCATGGATGT; and human siRNA 3, target sequence AACTTCATCAGAGGCTCTGAG) were obtained from Qiagen.
Benelux (Venlo, the Netherlands). Amniochrome was obtained from Lonza (Verviers, Belgium), 4 methylumbelliferyl-α-d-glucopyranoside was obtained from Sigma. SAINT-RED was purchased from Synvolux Therapeutics B.V. (Groningen, The Netherlands).

**Cell Culture**

Human skin fibroblasts (from healthy controls) were cultured in F10HAM medium (Invitrogen, Breda, the Netherlands) supplemented with 10% FCS (BioWhittaker, Walkersville, USA) and 2% Penicillin-Streptomycin (Invitrogen) and trypsinized with 1% trypsin (Invitrogen). Transfection experiments (in human skin fibroblasts) were carried out using POM medium which consists of F10HAM medium supplemented with 5% heat-inactivated FBS (BioWhittaker), 2% Penicillin-Streptomycin (Invitrogen) and 3mM Pipes (dissolved in 8ml H₂O and 2ml 1M NaOH).

Mouse 3T3 fibroblasts (obtained from ATCC, Manassas, VA, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 50 μg/ml gentamycine sulfate, 2 mM L-glutamine, 10% FBS (BioWhittaker) and trypsinized with trypsin.

**Transfection assay**

SAINT siRNA delivery system (product name SAINT-RED, Synvolux Therapeutics B.V.) was used and consisted of a 1:1 mixture of the cationic SAINT lipid and the neutral helper lipid DOPE (dioleoylphosphatidylethanolamine). For transfection cells were seeded 0.75 x10⁶ / well (6-well plate) using normal culture medium (for mouse 3T3 fibroblasts) or 0.01mg protein/ ml cell suspension for a 6 well using Amniochrome (for human skin fibroblasts). SiRNA delivery was performed when cells were 50-80% confluent (for human skin fibroblasts: cells were seeded three days before transfection). Cells were transfected with 1µg of siRNA (=71 pmol) in complex with SAINT-RED according to the manufacturer’s protocol. For one 6-well: 1µg of siRNA in 100 μl HBS was complexed with 20 μl SAINT-RED in an equal volume of HBS. The SAINT-RED:siRNA complex was incubated for 5 min at RT, 800 μl serum-free DMEM (mouse 3T3 fibroblasts) or 800 μl POM medium (human skin fibroblasts) was added and the complete mixture was applied directly onto the cells. Three to four hours later, 2ml serum-containing DMEM (mouse 3T3 fibroblasts) or POM medium (human skin fibroblasts) was added to each well. The cells were incubated for 48 hrs (mouse 3T3 fibroblasts) or 10 days (human skin fibroblasts) at 37ºC in humidified 5% CO₂.

**Measuring acid-α-glucosidase activity using the synthetic fluorogenic substrate 4-methylumbelliferyl-α-d-glucopyranoside (4-MUG assay)**

Fibroblasts cells were trypsinized for 1 minute at room temperature, after removal of trypsin cells were incubated for 5 minutes at 37°C. Cells were collected in 1.5ml eppendorf cups with 500µl POM medium and centrifuged for 1 minute at 13000 rpm and 4°C. Supernatant was discarded and 1ml of ice-cold 0.9% NaCl was added to the pellets and resuspended. After a second centrifugation step (1 minute at 13000 rpm at 4°C) the cell pellets without supernatant were stored at -80°C until further
After sonification of the cell pellets, the protein concentration of the homogenate was measured by BCA protein assay reagent (Pierce, Etten-Leur, the Netherlands). GAA activity was measured according to Okimuya et al\textsuperscript{10} using a fluorescence spectrophotometer (Zenyth 3100, excitation at 355 nm and emission at 460 nm). For the \textit{in vivo} experiments, GAA activities measured in plasma of mice treated with siRNA:SAINT-RED were normalized against GAA activities measured in plasma of HBS treated mice.

\textbf{\textit{In vivo} delivery of siRNA}

C57/BL6 mice, male, (Harlan, the Netherlands) were injected each day through the tail vein with HBS, 50µg siRNA alone (= 3.54 nmol) or 50µg siRNA in complex with SAINT-RED (ratio 1:0.5). Every other day (as ordered by the animal ethical commission), blood samples were taken from the mice to analyze GAA activity. At day 14, all mice were sacrificed and blood was drawn for analysis of GAA activity. Muscle, liver, and brain tissues were harvested for mRNA expression analysis. Experimental protocols were approved by the Institutional Animal Care Committee of the Groningen University, project number D4488D. All animals received human care in compliance with the Dutch Law on Experimental Animal Care.

\textbf{ASAT and ALAT measurements}

To investigate possible liver damage due to daily injections of siRNA1 in complex with SAINT-RED, the liver enzymes aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) were analyzed in plasma. The measurements were performed by a Roche Modular P analyzer.

\textbf{Lasermicrodissection of liver endothelial cells}

Serial 9 μm frozen sections were taken from liver samples from untreated mice or mice treated with SAINT + siRNA and placed on MembraneSlide 1.0 PEN (Carl Zeiss Microlmaging GmbH, Munich, Germany). The slides were fixed in aceton and cells were counterstained using hematoxilin. Laser dissection was performed using the Leica LMD6000 system and the Leica LMD Software. Captured areas were collected on the cap of a microcentrifuge tube and homogenized in RNA lysis buffer (RNeasy micro-kit, Qiagen). The laser cut areas were pooled to ensure sufficient numbers of cells were obtained for RNA analysis.

\textbf{RT-PCR}

Total RNA was extracted from cell cultures, from mouse liver, muscle and brain tissues using the RNeasy plus mini kit. RNA from selected sinusoid al endothelial cells was extracted using the QIAGEN RNeasy Micro kit. (Qiagen Benelux) according to the manufacturer’s recommendation. RNA was reverse transcribed using SuperscriptIII reverse transcriptase (Invitrogen) and random hexamer primers (Promega, Leiden, The Netherlands). Quantitative PCR amplifications were performed in
triplicate for each sample according to manufacturer’s protocol on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Relative gene expression of mouse GAA taqman primer/probe (Mm00484581_m1) and human CD31 primer/probe (Hs00169777_m1) was normalized for the expression of the house keeping gene GAPDH (taqman mouse primer Mm99999915_g1 and taqman human primer Hs99999905_m1) according to the comparative Ct method ($\Delta$Ct = number of amplification cycles needed to achieve a fixed threshold value; $\Delta$Ct = Ct$_{\text{gene of interest}}$ – Ct$_{\text{housekeeping gene}}$). Comparison of gene expressions in different samples was performed based on the differences in $\Delta$Ct of individual samples ($\Delta$$\Delta$Ct).

**Biodistribution studies in vivo**

Mice (4 per treatment group) were intravenously injected with 15, 50 and 150 nmol SANT-RED mixed with trace amount (0.03 nmol) of [3H] radiolabelled SAINT-18 (specific activity 57 Ci/mmol (RC Tritec), in the presence or absence of AllStar Negative control siRNA (Qiagen). The solution was vortexed for 1 min just prior to injection and when appropriate 60 pmol siRNA were added. Blood was sampled at time points of 2, 5, 10, 30, 60 and 90 minutes. Blood was centrifuged, plasma was separated, and 3H-radioactivity was measured as described by Asgeirsdottir et al.$^{11}$ The mice were sacrificed at 90 minutes, the urine was collected from the bladder, and the organs were removed and processed for measurement of radioactivity, as described previously.$^{12}$

**Stabilization studies of SAINT lipoplexes**

Trace amount (0.03 nmol) of [3H] radiolabelled SAINT-18 was mixed with 50 nmol SAINT-RED as described above, vortexed vigorously for 1 minute and incubated for various periods of time (1, 6 and 24 h, respectively) at room temperature in either water, HBS (20 mM HEPES, 150 mM NaCl, pH = 7.4), PBS (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na$_2$HPO$_4$, 1.49 mM KH$_2$PO$_4$, pH = 7.4), 0.9% Saline (150 mM NaCl) or 5% (w/v) glucose. Just prior to removing aliquots (n = 3 per experimental condition) for measuring the radioactivity, the solution was vortexed for 1 minute.

**Statistics**

The data were expressed as means ± SD. The in vitro data were analyzed by univariate ANOVA. Data obtained from the in vivo experiment were analyzed by repeated measures ANOVA. Significance level was set at P < 0.01 (***) and P < 0.001 (****).

**RESULTS**

**Efficient delivery of siRNA leads to downregulation of acid-α-glucosidase activity in vitro.**

In order to investigate whether siRNA can be applied to efficiently decrease GAA activity in vitro, three siRNAs were generated. Initial transfection experiments were performed in mouse 3T3 fibroblasts using increasing concentrations (0.1 – 1.5 µg) of siRNA1 or siRNA2, to analyze the minimal amount of siRNA needed to cause efficient downregulation of GAA. Figure 1A demonstrates optimal
Mimicking Pompe phenotypes for future therapeutic studies: Biodistribution of non-modified siRNA
downregulation of GAA activity at a concentration of 1µg as detected by the 4-MUG assay.

Downregulation of GAA was assessed in mouse 3T3 fibroblasts using 1µg siRNA 1 or siRNA 2 (Figure 1B). In 3T3 mouse fibroblasts, siRNA1 and siRNA2 decreased GAA activity with 45% and 65%, respectively, while siRNA 3 did not affect the GAA activity (Figure 1B). RT-PCR confirmed downregulation of GAA at mRNA level in mouse 3T3 cells transfected with siRNA (data not shown).

Subsequently, we evaluated the efficient downregulation of GAA in human skin fibroblasts. siRNA2 and siRNA3 were delivered in human skin fibroblasts and 4-MUG assays revealed optimal downregulation of GAA activity with ± 75% at day 12 after transfection, demonstrating that non-modified siRNA enwrapped in SAINT-RED is stable for at least 12 days after one single transfection experiment (Figure 2).

Figure 1. Downregulation of GAA in mouse 3T3 fibroblasts and human skin fibroblasts.

A. To determine the optimal concentration of siRNA for transfection experiments, mouse 3T3 fibroblasts were transfected with increasing amounts of siRNA 1 (grey bars) or siRNA 2 (white bars) for 48 h (n=2). B. Mouse 3T3 fibroblasts were transfected with 1µg of siRNA 1, 2 or 3 and incubated at 37°C for 48 h (n=3). After the indicated time points, the cells were collected and GAA activity was analyzed using the 4-MUG assay as described in "Material and Methods". ** represents $P < 0.01$, and *** represents $P < 0.001$ compared to control.

Figure 2. Efficient downregulation of GAA in human skin fibroblasts.

1µg of siRNA 2 (white bars) and siRNA 3 (grey bars) were transfected in human skin fibroblasts and incubated at 37°C. Cells were collected at day 3, 5, 10, 12 and 14 and analyzed for GAA activity using the 4-MUG assay as described in "Material and Methods".
siRNA delivered by SAINT-RED inhibits acid-/α-glucosidase activity in vivo.

To evaluate the effect of siRNA in vivo, C57/Bl6 mice were injected each day with 50µg of siRNA1 complexed with SAINT-RED in a ratio of 1:0.5. Every other day, blood samples were taken from the mice and analyzed for GAA activity using the 4-MUG assay and acarbose as inhibitor of maltase-glycoamylase. Plasma analysis revealed that siRNA1 was able to decrease GAA expression with approximately 45% after 14 days (Figure 3). Furthermore, no toxicity was observed due to daily injections of siRNA enwrapped in SAINT-RED as the liver enzymes ASAT and ALAT were not increased in plasma in mice treated with siRNA in complex with SAINT-RED compared to mice treated with HBS (Table 1).

Because we observed lowering of GAA plasma levels in SAINT:siRNA treated mice, we next analyzed the brain, liver and muscle for their GAA mRNA levels by RT-PCR as in these organs the highest GAA mRNA expression were found. Results demonstrated no reduction of GAA mRNA in SAINT:siRNA treated mice compared to HBS treated mice.

Figure 3. Downregulation of GAA in plasma of mice after daily injections with siRNA in complex with SAINT-RED.

BL6 mice (male) were injected daily with 50µg of siRNA alone, or in complex with SAINT-RED (ratio 1:0.5) and sacrificed 14 days after the first injection (n=2 for control group and n=4 for siRNA-SAINT-RED treated group). Every other day, blood samples were collected and analyzed for downregulation of GAA using the 4-MUG assay and acarbose as inhibitor of maltase-glycoamylase as described in “Material and Methods”. Statistical analysis revealed a significant effect (P < 0.05) of siRNA treatment over time in the siRNA:SAINT-RED (ratio 1:0.5) treated mice.

Table 1 Effect of siRNA treatment on the biochemical parameters ASAT and ALAT

Blood samples taken from mice either untreated (HBS) or treated with SAINT, siRNA, or SAINT + siRNA were analyzed for the serum enzymes for alanine aminotransaminase (ALAT) and aspartate aminotransaminase (ASAT).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ASAT (U/I) average ± stdev</th>
<th>ALAT (U/I) average ± stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBS</td>
<td>101,5 ± 23,3</td>
<td>24,5 ± 4,9</td>
</tr>
<tr>
<td>SAINT</td>
<td>62,3 ± 16,3</td>
<td>21,7 ± 3,2</td>
</tr>
<tr>
<td>siRNA</td>
<td>79,5 ± 2,1</td>
<td>27,5 ± 3,5</td>
</tr>
<tr>
<td>Saint + siRNA (1:0,5)</td>
<td>53,3 ± 19,3</td>
<td>19,5 ± 2,3</td>
</tr>
</tbody>
</table>
Table 2 Determination of GAA mRNA downregulation in liver, muscle and brain
Total RNA was extracted from liver, muscle and brain tissues from mice treated with HBS, SAINT, siRNA alone or SAINT:siRNA. Using reverse transcriptase, cDNA was synthesized and used for RT-PCR reactions containing GAA primers or GAPDH primers. The data in the table are represented per group.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Treatment</th>
<th>GAA $C_T$ (average)</th>
<th>GAPDH $C_T$ (average)</th>
<th>$\Delta C_T$ (Avg. GAA $C_T$ - Avg. GAPDH $C_T$)</th>
<th>$\Delta \Delta C_T$ (Avg. $\Delta C_T$ - Avg. $\Delta C_T$, liver)</th>
<th>Normalized GAA amount relative to liver $2^{\Delta \Delta C_T}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>HBS</td>
<td>25,33 ± 0,11</td>
<td>19,60 ± 0,06</td>
<td>5,73 ± 0,13</td>
<td>0,000 ± 0,13</td>
<td>1,00</td>
</tr>
<tr>
<td></td>
<td>SAINT</td>
<td>24,99 ± 0,18</td>
<td>19,19 ± 0,04</td>
<td>5,81 ± 0,19</td>
<td>0,072 ± 0,19</td>
<td>0,95</td>
</tr>
<tr>
<td></td>
<td>siRNA</td>
<td>24,87 ± 0,08</td>
<td>19,04 ± 0,06</td>
<td>5,84 ± 0,10</td>
<td>0,104 ± 0,10</td>
<td>0,93</td>
</tr>
<tr>
<td></td>
<td>SAINT + siRNA (1:0,5)</td>
<td>25,14 ± 0,11</td>
<td>19,48 ± 0,13</td>
<td>5,66 ± 0,17</td>
<td>0,074 ± 0,17</td>
<td>1,05</td>
</tr>
<tr>
<td>Muscle</td>
<td>HBS</td>
<td>25,92 ± 0,04</td>
<td>20,05 ± 0,06</td>
<td>5,87 ± 0,07</td>
<td>0,000 ± 0,07</td>
<td>1,00</td>
</tr>
<tr>
<td></td>
<td>SAINT</td>
<td>24,87 ± 0,04</td>
<td>18,79 ± 0,09</td>
<td>6,09 ± 0,10</td>
<td>0,218 ± 0,10</td>
<td>0,86</td>
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<tr>
<td></td>
<td>siRNA</td>
<td>25,16 ± 0,06</td>
<td>19,53 ± 0,08</td>
<td>5,63 ± 0,10</td>
<td>-0,239 ± 0,10</td>
<td>1,18</td>
</tr>
<tr>
<td></td>
<td>SAINT + siRNA (1:0,5)</td>
<td>25,90 ± 0,27</td>
<td>20,20 ± 0,08</td>
<td>5,70 ± 0,28</td>
<td>-0,173 ± 0,28</td>
<td>1,13</td>
</tr>
<tr>
<td>Brain</td>
<td>HBS</td>
<td>23,71 ± 0,04</td>
<td>19,61 ± 0,11</td>
<td>4,09 ± 0,11</td>
<td>0,000 ± 0,11</td>
<td>1,00</td>
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<tr>
<td></td>
<td>SAINT</td>
<td>23,80 ± 0,20</td>
<td>19,78 ± 0,07</td>
<td>4,02 ± 0,21</td>
<td>-0,074 ± 0,21</td>
<td>1,05</td>
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<tr>
<td></td>
<td>siRNA</td>
<td>24,06 ± 0,14</td>
<td>19,82 ± 0,10</td>
<td>4,24 ± 0,17</td>
<td>0,144 ± 0,17</td>
<td>0,91</td>
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<tr>
<td></td>
<td>SAINT + siRNA (1:0,5)</td>
<td>23,70 ± 0,28</td>
<td>19,74 ± 0,08</td>
<td>3,96 ± 0,29</td>
<td>-0,139 ± 0,29</td>
<td>1,10</td>
</tr>
</tbody>
</table>

Table 3 Determination of GAA mRNA downregulation in isolated sinusoidal endothelial cells from liver
Laser dissection was performed on sections from liver samples of untreated (HBS) or treated (SAINT:siRNA) mice in order to isolate sinusoidal endothelial cells. RT-PCR reactions containing GAA primers or GAPDH primers were carried out to analyze the GAA mRNA levels in SECs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tissue</th>
<th>GAA $C_T$ (average)</th>
<th>GAPDH $C_T$ (average)</th>
<th>$\Delta C_T$ (Avg. GAA $C_T$ - Avg. GAPDH $C_T$)</th>
<th>$\Delta \Delta C_T$ (Avg. $\Delta C_T$ - Avg. $\Delta C_T$, SEC untr.)</th>
<th>Normalized GAA $2^{\Delta \Delta C_T}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>SEC</td>
<td>32,15 ± 0,29</td>
<td>27,22 ± 0,11</td>
<td>4,93 ± 0,32</td>
<td>-0,50 ± 0,32</td>
<td>1,00</td>
</tr>
<tr>
<td>siRNA treated</td>
<td>SEC</td>
<td>31,28 ± 0,17</td>
<td>26,27 ± 0,11</td>
<td>5,02 ± 0,20</td>
<td>-0,22 ± 0,20</td>
<td>0,94</td>
</tr>
</tbody>
</table>
Because particles entering the bloodstream first have to pass the endothelial cells, and because, in general, the liver is the main secreting organ, we reasoned that sinusoid endothelial cells (SECs) in the liver would have lowered GAA levels and hence are the main source of GAA production. As these cells only contribute to a small part of the liver, possible downregulation of GAA due to siRNA delivery in these cells could therefore not be detected when whole liver is examined. Laser dissection experiments were performed to isolate and investigate these cells. The data are shown in Table 3 and revealed no significant decrease in GAA mRNA levels in the mice treated with SAINT:siRNA compared to HBS treated mice.

**Biodistribution studies using radioactive labelled SAINT**

As no decrease in mRNA levels was found in the brain, muscle, and liver including the SECs dissected from the liver, we set out new experiments to investigate the biodistribution of SAINT. Radioactive

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**Figure 4. Evaluation of 3H-SAINT uptake with or without siRNA in vivo.**

Mice were injected with different concentrations of 3H-SAINT with or without siRNA. Blood samples were taken after different time points and analyzed for radio-activity (A) and for the recovery of the injected dose (B). For biodistribution studies, mice were injected with different doses of 3H-SAINT with or without siRNA. Ninety minutes after the injections, mice were sacrificed and organs were collected followed by analysis of radioactivity (C). The total amount of 3H-SAINT for each mouse is set at 100%.
SAINT (³H-SAINT) was injected i.v. in mice in three different concentrations (15 nmol, 50 nmol and 150 nmol) with or without siRNA to study its uptake. Blood samples were taken after 2, 5, 10, 30, 60 and 90 min to analyze the clearance of ³H-SAINT +/- siRNA from the blood. Ninety minutes after the injection, all mice were sacrificed, their organs were collected and analyzed for ³H-SAINT +/- siRNA uptake. Figure 4A shows the rapid clearance of radioactive SAINT from the blood. The recovery of radioactivity after injection differs per concentration of SAINT used. Moreover, addition of siRNA led to a lower overall recovery of the radioactivity after injection compared to ³H-SAINT alone (Figure 4B). Figure 4C demonstrates that most of the radioactive SAINT +/- siRNA recovered from the mice was found in the liver.

We next set out to investigate the low (and inconsistent) recovery of the ³H-SAINT. After excluding physical aspects like SAINT sticking either to the glass vials or to the syringe, we investigated solubility of the SAINT in buffer. Stability experiments using HBS (the buffer used in the in vivo experiments) showed that radioactivity was “lost” after overnight storage. This loss of activity was recovered by the addition of EtOH. Timeline experiments (5, 15, 30 and 60 minutes) demonstrated a substantial decrease in recovery after 15 minutes. Subsequently, we tested different buffers and analyzed the recovery after 1, 6 and 24 h: PBS (10% recovery) < HBS ≤ saline ≤ saline + glycerol < 5% glucose (100% recovery). PBS showed the lowest percentage of recovery, and 5% glucose has a 100% recovery (Figure 5).

![Graph showing stabilization of SAINT lipoplexes](image)

**DISCUSSION**

In this paper, we showed that SAINT is a promising candidate to deliver therapeutic agents. As we envision future gene correction approaches to be mediated via non-viral delivery, this finding provides a solid basis for animal studies. To assess whether SAINT delivers molecules to GAA secreting cells, we used siRNA directed against GAA as a cargo and measured GAA secretion by the targeted cells in the plasma.

The use of siRNA has become a powerful tool to induce downregulation of genes to study diseases and to develop new therapeutic approaches. Several successful delivery methods have
already been described for \textit{in vivo} applications, but most of these methods require modification of siRNA. Liposomal carrier systems appear the most promising approach for \textit{in vivo} studies. Moreover, non-viral siRNA delivery is already in a phase I clinical trial (www.clinicaltrials.gov).

In this study, we showed siRNA induced downregulation of GAA plasma levels, after delivery using the lipid compound SAINT. Our findings thus indicate that SAINT delivers siRNA to GAA secreting cells. What exactly the GAA secreting cells are, is to our knowledge mainly unknown. We previously established that most lipoplexes are found in the lung and liver after \textit{i.v.} administration. Because the liver is the organ responsible for metabolism and secretion, it is very likely that this organ excretes GAA to the plasma which will be then be taken up by other cells/organs.

Ponce \textit{et al.} investigated the GAA expression in different organs and demonstrated that the highest levels of GAA were found in brain, followed by intermediate levels in skeletal muscle, liver and heart, and the lowest levels were found in lung and kidney. With this in mind, we analyzed brain, liver and muscle for their GAA expression after siRNA:SAINT treatment. Analysis of mRNA GAA levels in these organs did not reveal downregulation of GAA. As Santel et al. demonstrated that liposomal formulated siRNAs are able to inhibit gene expression specifically in endothelial cells in liver and tumor and based on the fact that endothelial cells (ECs) produce GAA mRNA levels (personal communication with B. St.Croix) and are accessible after \textit{i.v.} injection, we reasoned that in our case the sinusoidal ECs in the liver might be responsible for the reduction of GAA plasma levels. Laser dissection was performed to isolate SECs and RT-PCR demonstrated that SECs isolated from the liver did not show downregulation of GAA mRNA levels.

As no decrease was found on mRNA level in brain, muscle and whole liver nor in SECs, we set out to determine the biodistribution of SAINT using radioactivity after \textit{i.v.} injection. Our initial data showed delivery of \textsuperscript{3}H-SAINT mainly to the liver, as expected. Based on the evaluation of buffers in stabilizing the SAINT mix, we hypothesize that further improvements of siRNA mediated downregulation will be observed when SAINT:siRNA solutions are prepared in 5% glucose.

For Pompe disease, five mouse models have been developed by targeted disruption of the GAA gene. All models demonstrate absence of GAA activity in the organs. However, high GAA plasma levels have been observed in our laboratory for the exon 13 KO model, which is in contrast with activity of GAA in plasma from Pompe patients. The animal model described here can be an ideal model to investigate future therapeutic approaches (gene correction). Due to the low GAA plasma levels, non-invasive methods can be used to monitor the effects of these approaches, as such non-invasive methods are also used in the clinic.

In conclusion, the data described here demonstrate the feasibility of SAINT to deliver siRNA \textit{in vivo}. Future therapeutic approaches for Pompe disease can be studied in an \textit{in vivo} setting with SAINT as delivery device.
Acknowledgements

The authors thank Professor Dr. F. Kuipers for support; Professor Dr. G.P.A. Smit for skin fibroblasts and F. van der Sluijs, I. van Veen, H. van de Molen, P. Zwiers and A. Arendzen (UMCG) for excellent technical assistance. The authors thank Professor Dr. G. Molema for her expert advice and the possibility to use the Laser Dissection Microscope and E. Talman for production of 3H-SAINT.

Grant support: European Union FP6 program, activity NEST (contract number 015509)
Chapter 5

Reference List


