Arming drug carriers to disable the Hepatic Stellate Cell
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Selective targeting of Gliotoxin to Hepatic Stellate Cells maintains it’s pro-apoptotic characteristics in both *in vitro* and *in vivo* models of liver fibrosis

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Liver fibrosis is the common response to chronic liver injury. The fibrogenic process is consecutive to intense proliferation and accumulation of myofibroblastic cells that synthesize scar tissue. Several lines of evidence indicate that inducing apoptosis of myofibroblastic cells may prevent fibrogenesis. In this respect, it was shown that Gliotoxin (GTX), a natural compound derived from a fungus, induced apoptosis of hepatic fibrogenic cells and regression of liver fibrosis. However, this antifibrotic approach may find limitations due to the lack of cell specificity, with a risk of potentially adverse effects. In previous studies, we found that Mannose-6-phosphate coupled to Human Serum Albumin (M6P-HSA) selectively accumulated in liver fibrogenic cells. The aim of this study therefore was to couple GTX to M6P-HSA and test its pharmacological effects in vitro and in rats with liver fibrosis. The conjugate GTX-M6P-HSA bound specifically to HSC and reduced their viability. Apoptosis was induced in cultures of human hepatic myofibroblasts (hMF) and in liver slices obtained from rats with liver fibrosis. In vivo treatment with either GTX or GTX-M6P-HSA in bile duct ligated rats revealed a significant decrease in αSMA mRNA levels and reduced staining for this HSC-marker in fibrotic livers. In addition, GTX affected hepatocyte functioning as reflected by a significant reduced PAS staining and increased serum AST levels, whereas GTX-M6P-HSA did not affect these parameters. In conclusion, we developed a HSC-specific GTX construct which showed clear biological effects in human hMF, rat HSC and in fibrotic liver slices. Both GTX and GTX-M6P-HSA attenuated the number of activated HSC, but GTX affected also hepatocytes. This study shows that cell-selective delivery of GTX is feasible in fibrotic livers.
INTRODUCTION

The hepatic stellate cell (HSC) plays a crucial role during liver fibrosis. After hepatocyte damage, this cell-type becomes activated and starts to proliferate, during which it transforms from a resting vitamin A-storing cell into an activated myofibroblast-like cell. This activated HSC produces excessive amounts of extracellular matrix compounds and inhibitors of matrix degradation, thereby strongly affecting liver homeostasis eventually leading to cirrhosis, the end stage of fibrosis \(^1,2\).

Animal model studies revealed that this fibrotic process is reversible. Livers can undergo regression of fibrosis after withdrawal of the damaging stimulus \(^3\), even in an advanced stage of cirrhosis \(^4\). This regression was found to be accompanied by the disappearance of activated HSC via apoptosis. This suggests that inducing or accelerating HSC apoptosis during fibrogenesis might attenuate or reverse the fibrogenic process \(^3,5,6\). However, selective apoptosis of HSC is difficult to achieve.

The fungal metabolite Gliotoxin (GTX), a member of the epipolythiodioxo-piperazine family, has shown to induce apoptosis in human and rat HSC \textit{in vitro}. In addition, it reduced hepatic fibrosis \textit{in vivo} by induction of HSC apoptosis while leaving the hepatocytes intact in the rat CCl\(_4\) fibrosis model \(^7\). However, other studies showed also that GTX displayed immune-suppressive effects and promoted apoptosis in different cell types including thymocytes, macrophages, lymphocytes, enterocytes and several other cell lines of various origins \(^8-11\). This indicates that GTX can induce serious side effects outside the liver after systemic administration. Also within the liver, GTX might induce adverse effects because we previously reported that GTX affected not only the HSC, but also the other hepatic cell types \(^12\).

Specific delivery of the apoptotic agent GTX to the HSC might avoid these adverse effects. This cell-specific uptake of GTX might be accomplished by conjugation of GTX to HSC-specific drug delivery systems. In recent years, these carrier systems have become available. We previously reported that mannose-6-phosphate modified human-serum-albumin (M6P-HSA) predominantly accumulated in the HSC of fibrotic rats \(^13,14\). Therefore, the aim of this study was to synthesize the conjugate GTX-M6P-HSA. The binding of this construct to HSC and the pharmacological effects of this conjugate were explored \textit{in vitro} and in rats with liver fibrosis.
MATERIAL AND METHODS

Materials

Human Serum Albumin was purchased from the Central Laboratory of Blood Transfusion Services (Sanquin Blood Supplies, Amsterdam, The Netherlands). Gliotoxin was purchased from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Animals

Male Wistar rats (Harlan, outbred strain, Horst, The Netherlands) were housed under standard laboratory conditions and had free access to food and water. This study was performed in accordance with ethical regulations imposed by Dutch legislation.

Synthesis of GTX-M6P-HSA

HSA was modified with mannose 6-phosphate groups as described previously to obtain the drug carrier M6P<sub>60</sub>-HSA<sup>13,14</sup>. The products of the synthesis were purified and characterized according to standard procedures.

The subsequent conjugation of GTX to M6P-HSA is a two step reaction. First, 8 mg of 4-dimethylaminopyridine (Fluka, Buchs, Germany) and 140 mg of succinic anhydride (Acros, Morris Plains, NJ, USA) were dissolved in 2 ml of dry acetone. 15 mg of GTX was dissolved in 1.8 ml of the acetone solution and reacted for 4 days. The succinic spacer will form an ester bond with the hydroxyl-group of GTX. This ester bond can be cleaved by esterases, present within the lysosomes, releasing the native GTX drug. The product was purified by phase separation (dichloromethane-water) and formation of GTX-hemisuccinate was confirmed by thin layer chromatography and mass-analysis.

Subsequently, GTX-hemisuccinate was dissolved in dimethylformamid (300 µl). Isobutyl-chlorocarbonate (30 µl, Acros,) and Tri-Butylamin (42 µl, Fluka) were added and this mixture was stirred for 30 minutes. Meanwhile, M6P-HSA (70 mg) was dissolved in PBS (5 mg/ml). GTX-hemisuccinate mixture was added to the M6P-HSA mixture and the reaction was stirred for 4 hours in which GTX-hemisuccinate was coupled to the primary amine groups (εNH<sub>2</sub>) of the lysines within HSA. The formed product (GTX-M6P-HSA) was extensively dialyzed against PBS to remove all low molecular weight compounds. The monomeric form of GTX-M6P-HSA was separated from the dimeric and polymeric form using sephadex preparative fast performance liquid chromatography (FPLC, GE Healthcare, Uppsala, Sweden). The purified conjugate was dialyzed against milliQ-water to remove salts, lyophilized and stored at -20 °C.

The degree of drug loading of the conjugate was determined by high performance liquid chromatography (HPLC) after hydrolysis of the ester bond between GTX and M6P-HSA by 0.1M citric acid / 0.2M phosphate buffer (pH 2.5). Samples were injected on a C<sub>8</sub> reversed-phase column (Thermo-Hypersil Keystone, Bellefonte, PA, USA) preceded by C<sub>18</sub> Guardpak precolumn (Waters Inc, Milford, MA, USA). The mobile phase consisted of acetonitril/H2O/PBS (35/64/1, v/v/v) at a flow rate of 1.5 ml/min and detection at 254 nm.

Human hepatic myofibroblast isolation

Human hepatic myofibroblasts (hMF) were obtained by outgrowth of explants from normal human livers<sup>15</sup>. This procedure was performed in accordance with ethical regulations imposed by French legislation. Cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 5% Fetal Calf Serum (FCS) and 5% Human Serum (HS) and used between the third and seventh passage. The myofibroblastic nature of the cells was routinely evaluated by microscopy and checked for the presence of α-Smooth Muscle Actin (α-SMA).
Rat hepatic stellate cell isolation

Primary HSC were isolated from livers of male Wistar rats (> 500 g, Harlan)\textsuperscript{16}. The HSC were cultured in DMEM (Invitrogen) supplemented with 10% FCS, and incubated in a 5% CO\textsubscript{2} humidified atmosphere at 37 °C. Cells cultured for 10 days were used for further experiments. At this time point, all cells have the phenotype of activated HSC as assessed by routine immunohistochemical methods.

Biological effects on HSC

HSC binding assays were performed with\textsuperscript{125}I-labelled GTX-M6P-HSA\textsuperscript{17} according to standard methods\textsuperscript{14}. Furthermore, the effect of GTX-M6P-HSA on the viability of the HSC was assessed. HSC (5,000 cells/96-well) were incubated for 24 hours in 200 µl medium without serum. Subsequently, GTX, GTX-M6P-HSA or M6P-HSA was added in increasing concentrations and the cells were incubated for another 18 hours. Alamar blue (20 µl, Serotec, Oxford, UK) was added and the cells were incubated for an additional 24 hours. The conversion of alamar blue redox indicator by metabolic activity of cells reflects the number of cells (i.e. net result of proliferation and apoptosis) present in each well. The alamar blue conversion was measured using a fluorimeter\textsuperscript{18}.

Apoptosis assays on hMF

Caspase-3-like activity was assayed on cell lysates according to standard methods\textsuperscript{19}. Briefly, after incubation of hMF (300,000 cells in 60-mm dishes) with GTX, GTX-M6P-HSA or M6P-HSA, cells were lysed and DEVDase activity was measured in 200 µl of assay buffer, containing 50 µg of total protein and 20 µM AC-DEVDAFC (Biomol, Tebu, France) as fluorogenic substrate. Apoptosis was also assessed by nuclear staining with DAPI (Roche Diagnostics, Indianapolis, IN, USA) in cultures of non-confluent hMF incubated with either GTX, carrier or conjugate according standard methods\textsuperscript{19}.

Induction of fibrosis

Rats (220-240 g, Harlan) were subjected to bile duct ligation (BDL)\textsuperscript{20} and used for slice experiments, 3 weeks after BDL, or for in vivo effect studies, 10 day after BDL (BDL-10d).

Liver slices

Precision-cut liver slices were prepared from 3 week BDL fibrotic rats (n=3) according standard techniques\textsuperscript{21}. Slices were incubated individually in 6-well plates with vehicle, GTX-M6P-HSA or M6P-HSA. After 24 hours of incubation, the liver slices were snap-frozen in liquid N\textsubscript{2} for the active caspase-3 assay or frozen in isopentane (-80 °C) for TUNEL analysis.

Apoptosis assay on liver slices

Slices were homogenized in caspase lysis buffer (fluorimetric caspase assay kit, Promega, Madison, WI, USA) with a sonicator. The total protein concentration of these homogenates was determined according to standard procedures (BioRad Laboratories, Hercules, CA, USA). The caspase-3 activity was measured in 25 µg total protein homogenate and incubated for 60 min at 37 °C according to the instructions of the manufacturer (Promega).

TUNEL assay

Cryostat sections (4 µm) of the liver slices were fixed in 4% paraformaldehyde and apoptotic cells were stained with a TUNEL staining kit according to the procedure provided by the manufacturer (Roche Diagnostics). In addition, sections were stained with the DAPI staining solution (Roche Diagnostics) to visualize all nuclei. Subsequently, sections were analyzed with a fluorescence microscope (magnification 200X).
**In vivo effect studies**

To assess the effects of the compounds on the number of HSC within fibrotic livers, we treated BDL fibrotic rats at day 6, 7, 8 and 9 after the ligation and examined the rats 24 hours later at day 10 (n=5 per group). Rats were i.v. injected with vehicle (PBS), M6P-HSA (5 mg/kg), GTX-M6P-HSA (5 mg/kg) or GTX (10 µg/kg, equivalent to 0.4 molecules of GTX coupled to one M6P-HSA molecule), all dissolved in PBS.

**Immunohistochemical stainings**

Acetone-fixed cryostat sections (4 µm) of liver sections were stained with indirect immunoperoxidase methods. The activated HSC in the livers were detected with the monoclonal antibody against αSMA (Sigma). Goat polyclonal antibodies against rat collagen type I and III (both Southern Biotech, Birmingham, USA) were used simultaneously to stain the most important interstitial collagens in the livers. Staining was visualized with peroxidase-conjugated rabbit anti-mouse or rabbit anti-goat IgG (DAKO, Glostrup, Denmark) and with 3-amino-9-ethylcarbazole (AEC; Sigma). The level of fibrosis was scored for each collagen-stained liver according to Ishak Knodell score. SMA staining was also analyzed with ImageJ software (National Institute of Health, Bethesda, Maryland, USA). On ten digital photos per liver, the total area stained positive for αSMA was measured and related to the total area analyzed at a magnification of 100X.

**Real-time PCR**

Total RNA was isolated from rat livers using the RNasy kit (Qiagen, Hilden, Germany) and the amount of RNA was measured with the NanoDrop ND1000 (Nanodrop Technologies, Wilmington, USA). The reverse transcriptase reaction (Promega) was performed with random primers.

The transcription levels of rat collagen type 1α1 (forward, 5’-AGCCTGAGCCAGCAGATTGA-3’, reverse, 5’-CCAGGTTGCGAGCCTTGTGTA-3’) and αSMA (forward, 5’-GACACCAGGGAGTGGTT-3’, reverse, 5’-GTTAGCAAGGTCCGATGCTC-3’) were detected by quantitative real time PCR methods with SYBR Green on a ABI 7900HT apparatus (both Applied Biosystems, Foster City, CA, USA). Formation of single products was confirmed by analyzing the dissociation step at the end of each PCR reaction. The data were quantified via comparative ΔΔCt calculation (GAPDH was used as housekeeping gene), and the gene expression levels in livers from normal rats were set at baseline.

**Liver function**

Periodic acid Schiff (PAS) histochemistry: Paraffin imbedded sections (5 µm) of livers were fixed in 4% formalin / methanol, incubated with periodic acid and stained with Schiff’s reagent (Merck). The PAS-stained areas were quantified using ImageJ software (National Institute of Health). On digital photos, the total area stained positive for PAS was measured and related to the total area analyzed per liver section at a magnification of 200X.

Blood analysis: Heparinized blood was collected by heart-puncture and plasma levels for Alanine Aminotransferase (ALT), Asparate Aminotransferase (AST) and total bilirubin were routinely measured (Department of Clinical Chemistry, University Medical Centre Groningen, The Netherlands).

**Statistics**

Results were expressed as mean ± SEM. Statistical analysis of the in vitro experiments was performed by a two-tailed Student’s t-test. The results of the in vivo effect studies were analyzed by a one-way ANOVA followed by the LSD post-hoc test. P values lower than 0.05 were considered statistically significant.
RESULTS

Characterization of the GTX-M6P-HSA conjugate

The monomeric protein fraction of the GTX-M6P-HSA conjugate was separated from the total protein fraction by preparative size exclusion chromatography. This monomeric fraction was used in this study. HPLC-analysis revealed that the coupled GTX to M6P-HSA ratio was 0.4:1.

*In vitro* studies of GTX-M6P-HSA on rat HSC

We investigated the pharmacological effects of GTX-M6P-HSA on primary isolated rat HSC by binding and viability studies. The binding studies showed selective binding of $^{125}$I-labeled GTX-M6P-HSA to receptors on HSC (Figure 1A). Binding of the conjugate to HSC was reduced by $89 \pm 3\%$ ($P<0.05$) after co-incubation with M6P-HSA, which is an M6P/IGF-II receptor ligand $^{14}$.

![Figure 1: Effect of GTX-M6P-HSA on rat HSC. A: Binding of $^{125}$I-GTX-M6P-HSA to activated rat HSC. Note that preincubation with M6P-HSA, a ligand for the M6P/IGF-II receptor, reduces binding of the conjugate by $89 \pm 3\%$. B: Viability of isolated activated HSCs incubated with different concentrations of GTX. The control cells (incubated with vehicle) are set at 100% viability, whereas control wells without cells are set as 0% viability. C: Viability of isolated activated HSCs incubated with different concentrations of GTX-M6P-HSA (closed bars) and M6P-HSA (open bars) Protein concentration and the corresponding GTX load is depicted for GTX-M6P-HSA (* indicates $P<0.05$ compared to control).
As previously described, GTX dramatically induced apoptosis of HSC resulting in a dose-dependent decrease in viability of cultures of activated HSC, treated for 24 hours with GTX. A significant decline in viability was found at 0.1 µM concentration of GTX (Figure 1B). GTX-M6P-HSA also dose-dependently decreased the viability of HSC after incubation. This decrease was significant at a concentration of 25 µg/ml corresponding with a drug concentration of 0.13 µM GTX. Incubation of HSC with the carrier alone did not affect the viability of HSC at all (Figure 1C).

**GTX-M6P-HSA induces apoptosis in human hepatic myofibroblasts**

To assess whether GTX-M6P-HSA would also affect human cells, we conducted an active caspase-3 assay on human hepatic myofibroblasts (hMF). Both GTX and GTX-M6P-HSA were able to increase the level of active caspase-3 compared with the control (vehicle) incubated hMF (Figure 2A). The carrier alone (M6P-HSA) did not induce apoptosis of hMF, even at high concentrations (500 µg/ml, Figure 2B). Condensated nuclei were detected with DAPI staining in GTX and GTX-M6P-HSA treated cultures, confirming the results found with the active caspase-3 assay (Figure 2B).

![Figure 2](https://via.placeholder.com/150)

**Figure 2:** Apoptotic effects of GTX and GTX-M6P-HSA in cultures of human hMF. **A:** The effect of GTX and GTX-M6P-HSA on caspase-3-activity (* indicates p < 0.05 compared to control incubations). **B:** DAPI staining of the nuclei (x680). Serum deprived cells were incubated for 20 h with vehicle or in the presence of 0.25 µM GTX, 100 µg/ml GTX-M6P-HSA or 500 µg/ml M6P-HSA.
GTX-M6P-HSA induces apoptosis in fibrotic liver slices

Previously, we showed a GTX-induced apoptotic response in rat liver slices. In the present study, we incubated fibrotic rat liver slices with control (PBS), GTX-M6P-HSA or M6P-HSA and measured induction of apoptosis by assessment of the caspase-3 activation. The maximal fold induction of caspase-3 activation was $4.2 \pm 0.3$ after 24 hours incubation with 500 µg/ml GTX-M6P-HSA as compared with slices incubated with vehicle (Figure 3). Incubation of slices with M6P-HSA did not result in a significant induction of caspase-3 activity. Previous experiments showed that apoptosis in fibrotic slices occurred at a concentration of 1 µM GTX, which is equimolar to the present conjugate concentration $^{12}$.

![Figure 3](image)

**Figure 3**: Induction of caspase-3 activity by GTX-M6P-HSA in fibrotic liver slices. Slices were incubated for 24 hours with vehicle, GTX-M6P-HSA or M6P-HSA (* indicates p < 0.05 compared to control incubations).

TUNEL staining was performed on fibrotic liver slices to visualize the induction of apoptosis. These slices showed a strong increase in TUNEL-positive cells after incubation with 500 µg/ml GTX-M6P-HSA. Again, incubation of slices with vehicle or with M6P-HSA did not result in an increase in TUNEL-positive cells (Figure 4).

**In vivo effects of GTX-M6P-HSA**

We next progressed to in vivo studies in order to determine the ability of targeted GTX to modulate the fibrogenic response in the BDL model. αSMA mRNA levels were examined in livers of fibrotic rats following reatment with GTX, M6P-HSA, GTX-M6P-HSA or PBS (Figure 5). Treatment of rats with GTX and GTX-M6P-HSA resulted in a significant reduction in αSMA mRNA levels compared with the PBS-treated groups ($p < 0.05$). The carrier M6P-HSA had no effect on the αSMA mRNA expression levels. No significant differences in the mRNA expression levels of collagen 1α1 were found between livers of the different experimental groups (data not shown).
**Figure 4:** Representative microphotographs of the simultaneous detection of the nucleus (DAPI staining) and apoptotic cells (TUNEL staining) in fibrotic liver slices after 24 hours incubation with vehicle, GTX-M6P-HSA or M6P-HSA (both 500 µg/ml, magnification 200X).

**Figure 5:** Real-time PCR analysis of mRNA levels for αSMA in livers after treatment of BDL fibrotic rats with PBS, GTX-M6P-HSA, M6P-HSA or GTX. The αSMA expression was significantly reduced when rats were treated with GTX-M6P-HSA and GTX compared to PBS and M6P-HSA-treated rats. As a reference, αSMA expression levels of the treated groups were compared with the expression in healthy, non-fibrotic rats. GAPDH was used as a reference gene. Data represent the average of 5 animals per group ± SEM, * p < 0.05, ANOVA.
Liver sections of rats treated with GTX or GTX-M6P-HSA displayed a clearly reduced immunohistochemical staining for αSMA compared with control treatment. Morphometric analysis of these sections revealed a significant reduction in the number of αSMA positive cells in rats treated with GTX and GTX-M6P-HSA as compared with rats treated with M6P-HSA or PBS (ANOVA, p<0.05, Figure 6A and 6C). Liver sections were also stained for the presence of collagens and the degree of fibrosis was scored according to Ishak-Knodell.22,23 No significant effects were seen on the degree of fibrosis or the deposition of collagen between the different treated groups (Table 1).

Table 1: Effects of the different treatments in fibrotic rats. The interstitial collagen deposition was scored according Ishak-Knodell. Serum levels for ALT, AST and total bilirubin were analyzed. ALT and AST are expressed as unit per liter (U/L), whereas total bilirubin levels are expressed as µM (n=5 rats per group, * p < 0.05, ANOVA).

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<th>PBS</th>
<th>GTX-M6P-HSA</th>
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<td><strong>Ishak-Knodell</strong></td>
<td>3.3 ± 0.5</td>
<td>2.8 ± 0.4</td>
<td>2.8 ± 0.2</td>
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<td><strong>Serum levels for</strong></td>
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<tr>
<td>ALT (U/L)</td>
<td>90.5 ± 8.8</td>
<td>107.8 ± 9.7</td>
<td>116.8 ± 10.5</td>
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<td>AST (U/L)</td>
<td>200.8 ± 45.8</td>
<td>294.4 ± 41.0</td>
<td>341.0 ± 33.1*</td>
<td>226.6 ± 34.9</td>
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<td>Bilirubin (µM)</td>
<td>201.5 ± 23.6</td>
<td>201.2 ± 10.4</td>
<td>192.6 ± 10.2</td>
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To assess the effects of the different treatments on markers for other hepatic cells within the liver, mRNA levels for Kupffer cells (fucose receptor mRNA) and Endothelial cells (eNOS mRNA) were determined.12 In addition, sections were stained for ED2 and RECA1 as protein markers for Kupffer cells and Endothelial cells respectively. These experiments revealed no significant effect on Kupffer cells and Endothelial cells in mRNA expression levels and immunohistochemical staining, indicating that the number of Kupffer cells or sinusoidal endothelial cells were not affected by GTX, GTX-M6P-HSA, M6P-HSA or vehicle treatment (data not shown).

PAS staining was performed to visualize the glycogen content of the liver, and clear changes were found. Quantification of this PAS staining using ImageJ demonstrated a significant reduction in livers of GTX-treated rats whereas the PAS staining in all other groups were similar to control animals (Figure 6B and 6C).

Measurement of serum parameters showed that bilirubin levels were similar in all groups, confirming that the surgical procedure led to a similar level of cholestasis in all experimental groups. No significant difference in serum levels for ALT were found between the different treated groups, but AST levels were higher in the GTX-treated rats (p< 0.05, ANOVA, Table 1).
Figure 6: Representative microphotographs of A: Immunohistochemical staining of HSC, detected with a monoclonal antibody specific for αSMA. Note the decrease in αSMA staining after treatment of rats with GTX or GTX-M6P-HSA. B: Periodic Acid Schiff (PAS) staining for glycogen content of hepatocytes. (Magnification 100X) C: Morphometric analysis of the area positive or αSMA or PAS staining using ImageJ software by measuring the total area stained as a percentage of the total area per digital photo (For αSMA, *p<0.05, ANOVA, GTX-M6P-HSA and GTX compared to PBS and M6P-HSA; for PAS, *p<0.05, ANOVA, GTX compared to GTX-M6P-HSA, PBS and M6P-HSA).
DISCUSSION

In the past few years, several reports have shown beneficial effects of GTX in experimental models of fibrosis\textsuperscript{7,24-26}. The ability of GTX to induce apoptosis in HSC may be a pivotal step in the resolution of fibrosis\textsuperscript{3}. However, the activities of GTX are not specific for HSC alone\textsuperscript{12}. GTX was discovered as an antibiotic drug made by the fungus \textit{Gliocladium fimbriatum}\textsuperscript{27}. Later, it was found that GTX also suppressed the immune system\textsuperscript{10,28}, and related to this, GTX effectively attenuated dextran sodium sulfate-induced colitis in rats. This GTX-induced immunosuppressive effect appeared to seriously affect viability of the experimental animals in this model\textsuperscript{29}. For mice, the 50\% lethal dose (LD\textsubscript{50}) of GTX was found to be 10 mg/kg\textsuperscript{28} and it was demonstrated that intra-peritoneal injections of GTX also caused apoptosis in the thymus, spleen and mesenteric lymph nodes\textsuperscript{28}. In view of the non-specific effects of GTX within the liver\textsuperscript{12} and the uptake in other organs, we designed a more selective approach for this apoptosis-inducing drug in fibrotic livers.

In recent years, HSC-specific drug carrier systems have been developed\textsuperscript{13,30}. The modification of HSA with Mannose-6-phosphate groups resulted in a carrier that accumulated in HSC by binding to the M6P-IGFII receptors found on activated HSC\textsuperscript{13,31}. After binding of M6P-HSA to this receptor, internalization of the ligand-receptor complex in endosomes occurred and within this cellular compartment, lysosomal enzymes degrade the proteins\textsuperscript{32}. We now coupled GTX via an ester bond to the protein backbone of the carrier. After internalisation, lysosomal degradation of the protein or destruction of this bond by esterases will result in the release of native GTX within HSC.

Binding of GTX-M6P-HSA to the target cells was studied in cell cultures and the pharmacological effects of released GTX were examined \textit{in vitro}. These studies showed specific binding of the conjugate to HSC. In addition, a decrease in cell viability and an increase in caspase-3 activity were found after incubation of rat HSC or human hMF with GTX-M6P-HSA. This shows that active GTX is released from the carrier and receptor interaction of the drug-carrier construct is intact. These experiments also indicated that the potency of GTX and GTX-M6P-HSA \textit{in vitro} was near equal.

We previously reported on the activity of GTX in rat liver slices\textsuperscript{12}. The normal tissue architecture and complex cell-cell interactions are preserved in these liver slices, mimicking the \textit{in vivo} situation\textsuperscript{21}. Incubation of fibrotic slices with GTX-M6P-HSA resulted in a strong induction of apoptosis as detected by the caspase-3 assay and by increased TUNEL staining in these slices. These results prompted us to start the \textit{in vivo} experiments.
We injected BDL fibrotic rats on day 6, 7, 8 and 9 with PBS (vehicle), GTX, M6P-HSA or GTX-M6P-HSA. Twenty-four hours after the last injection, the rats were sacrificed and the presence of activated HSC was examined. Since activated HSC express αSMA, we stained the livers for the presence of αSMA and found a decreased αSMA staining in the rats that were treated with GTX or GTX-M6P-HSA compared with M6P-HSA or vehicle treatment. Quantitative measurements of αSMA mRNA levels confirmed the reduction in αSMA found by immunohistochemical techniques. No effects of GTX and GTX-M6P-HSA were found on other non-parenchymal cells, as demonstrated with ED2 and RECA1 staining and measurement of cell-specific mRNA levels (fucose receptor and eNOS). In addition, we examined the effect of GTX on hepatocytes by serum-analysis and PAS staining. Hepatocytes rapidly respond to stress or injury by activation of glycogenolysis leading to a reduced glycogen content which can be demonstrated by PAS staining. The PAS staining of livers was significantly reduced in the hepatocytes after treatment with GTX as compared with vehicle, M6P-HSA or GTX-M6P-HSA treated rats. A small but significant effect of GTX was also noted on AST levels. AST is associated with hepatocytes and is raised as a result of acute liver damage. These data indicate that GTX not only affects the HSC, but hepatocytes as well, whereas GTX-M6P-HSA did not display these side-effects on hepatocytes at all.

Although we found an induction of apoptosis in vitro on cells and in fibrotic liver slices, we were not able to find a significant apoptotic response in vivo. An explanation for this may be that apoptosis can occur very fast, ranging from minutes to a few hours after treatment, which prevents its detection after 24 hours. Moreover, it has been shown that apoptotic cells are rapidly and efficiently removed by macrophages resulting in a short half-life and a narrow window for detection of the apoptotic bodies.

We were also not able to detect an effect of GTX or targeted GTX on collagen deposition. Neither Ishak-Knodell scores nor mRNA levels for collagen were affected by the treatment. This may be due to the very low doses of GTX that were applied in the present study (10 µg/kg rat). Apparently, the most potent effect of GTX is on the number of activated HSC, which fits with its action as an apoptosis inducing drug in vitro.

In conclusion, we developed a HSC-specific gliotoxin construct which showed clear biological effects in rat HSC and in fibrotic liver slices as well as in human hMF. Both GTX and GTX-M6P-HSA attenuated the synthesis of αSMA in vivo. However, adverse effects on liver toxicity after treatment with untargeted GTX were detected, whereas treatment with targeted GTX did not reveal any adverse effects. This demonstrates that a targeted approach with GTX for the treatment of liver fibrosis is feasible.
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