Cellular Targeting of the Apoptosis-Inducing Compound Gliotoxin to Fibrotic Rat Livers


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ABSTRACT

Liver fibrosis is associated with proliferation of hepatic stellate cells (HSCs) and their transformation into myofibroblastic cells that synthesize scar tissue. Several studies indicate that induction of apoptosis in myofibroblastic cells may prevent fibrogenesis. Gliotoxin (GTX) was found to induce apoptosis of hepatic cells and caused regression of liver fibrosis. However, the use of apoptosis-inducing drugs may be limited due to lack of cell specificity, with a risk of severe adverse effects. In previous studies, we found that mannose-6-phosphate-modified 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 HSCs and reduced their viability. Apoptosis was induced in cultures of human hepatic myofibroblasts (hMFs) and in liver slices obtained from rats with liver fibrosis. In vivo treatment with GTX or GTX-M6P-HSA in bile duct ligated rats revealed a significant decrease in α-smooth muscle actin mRNA levels and a reduced staining for this HSC marker in fibrotic livers. In addition, although GTX also affected hepatocytes, GTX-M6P-HSA did not significantly affect other liver cells. In conclusion, we developed an HSC-specific compound that induced apoptosis in human hMFs, rat HSCs, and in fibrotic liver slices. In vivo, both GTX and GTX-M6P-HSA attenuated the number of activated HSCs, but GTX also affected hepatocytes. This study shows that cell-selective delivery of the apoptosis-inducing agent GTX is feasible in fibrotic livers.

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 (MF)-like cell. Activated HSCs produce excessive amounts of extracellular matrix compounds and inhibitors of matrix degradation; therefore, this cell type is a key player in the fibrogenic response. Eventually this process may lead to cirrhosis, the end stage of fibrosis (Friedman, 2000; Lotersztajn et al., 2005).

Animal studies revealed that the fibrotic process can be reversible. Livers can undergo regression of fibrosis after withdrawal of the damaging stimulus (Iredale et al., 1998), even in an advanced stage of cirrhosis (Iasa et al., 2004). This regression was found to be accompanied by the disappearance of activated HSCs via apoptosis. This suggests that induction of apoptosis in HSCs during fibrogenesis could attenuate or reverse the fibrogenic process (Iredale et al., 1998; Canbay et al., 2004b; Elsharkawy et al., 2005; Julien et al., 2005; Teixeira-Clerc et al., 2006). However, selective apoptosis of HSCs is difficult to achieve.

The fungal metabolite gliotoxin (GTX), a member of the epipolythiodioxopiperazine family, has been shown to induce apoptosis in human and rat HSCs in vitro (Wright et al., 2001; Hagens et al., 2006). Induction of apoptosis in HSCs by GTX led to attenuation of hepatic fibrosis in vivo. However,
the applied dosages did not affect the viability of hepatocytes in vivo in the rat CCl₄ fibrosis model (Wright et al., 2001), although GTX induced oxidative stress in hepatocytes in vitro (Orr et al., 2004). Other studies showed that GTX displayed immune-suppressive effects and promoted apoptosis in several cell types including thymocytes, macrophages, lymphocytes, enterocytes, and other cell lines of various origins (Müllbacher et al., 1987; Waring et al., 1998; Pahl et al., 1996; Waring and Beaver, 1996). 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 HSCs, but also other nonparenchymal cell types within this organ (Hagens et al., 2006).

Specific delivery of the apoptotic agent GTX to the HSCs may therefore be quite relevant. Such cell-specific uptake of GTX might be achieved 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 HSCs of fibrotic rats (Beljaars et al., 1999, 2001). Therefore, the aim of this study was to synthesize the compound GTX-M₆P-HSA. The binding of this targeted compound to HSCs, and the pharmacological effects of this conjugate were explored in vitro and in vivo in rats with liver fibrosis.

Materials 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-Aldrich (St. Louis, MO). All other chemicals used were of analytical grade.

Animals

Male Wistar rats (outbred strain; Harlan, Zeist, 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 M₆P₂₅-HSA (Beljaars et al., 1999). 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) were dissolved in 2 ml of dry acetone. Fifteen milligrams 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 (Soyez et al., 1996; Melgert et al., 2001). The product was purified by phase separation (dichloromethane-water), and formation of GTX-hemisuccinate was confirmed by thin-layer chromatography and mass spectrometer analysis.

As a result, GTX-hemisuccinate was dissolved in dimethylformamide (300 μl). Isobutylchlorocarbonate (30 μl; Accros) and Tributylamin (42 μl; Fluka) were added, and this mixture was stirred for 30 min. Meanwhile, M₆P-HSA (70 mg) was dissolved in phosphate-buffered saline (PBS), pH 7.2, to a final concentration of 5 mg/ml. GTX-hemisuccinate mixture was added to the M₆P-HSA mixture, and the reaction was stirred for 4 h in which GTX-hemisuccinate was coupled to the primary amine groups (ε-NH₂) 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 (GE Healthcare, Uppsala, Sweden). The purified conjugate was dialyzed against milliliter-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 after hydrolysis of the ester bond between GTX and M₆P-HSA by 0.1 M citric acid/0.2 M phosphate buffer, pH 2.5. Samples were injected on a C₁₈ reversed-phase column (Thermo-Hypersil Keystone, Bellefonte, PA) preceded by C₁₈ Guardpak precolumn (Waters Inc., Milford, MA). The mobile phase consisted of acetonitrile/H₂O/PBS (35:64:1, v/v) at a flow rate of 1.5 ml/min and detection at 254 nm.

Human Hepatic Myofibroblast Isolation

Human hepatic myofibroblasts (hMFs) were obtained by outgrowth of explants from normal human livers (Win et al., 1993; Julien et al., 2005). This procedure was performed in accordance with ethical regulations imposed by French legislation. Cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (FCS) and 5% human serum and used between the third and seventh passages. The cell cultures were >99% viable (trypan blue exclusion), and the myofibroblastic nature of the cells was routinely evaluated by microscopy and checked for the presence of α-smooth muscle actin (SMA). All cells were positive for αSMA.

Rat Hepatic Stellate Cell Isolation

Primary HSCs were isolated from livers of male Wistar rats (>500 g; Harlan) (Geerts et al., 1998). The HSCs were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% FCS and incubated in a 5% CO₂ humidified atmosphere at 37°C. The isolated HSCs were >95% pure (as determined microscopically based on the presence of lipid vitamin A droplets in these cells) and had >99% viability (trypan blue exclusion test). Cells cultured for 10 days were used for further experiments. At this time point, all cells have the phenotype of activated HSCs as assessed by routine immunohistochemical methods.

Biological Effects on HSCs

Binding Assays. HSC binding assays (70,000 cells/six wells) were performed with 125I-labeled GTX-M₆P-HSA (100,000 cpm; tracer amount containing approximately 0.5 ng of protein) according to standard methods as described previously (Beljaars et al., 2001).

Viability Assays. The effect of GTX-M₆P-HSA on the viability of the HSCs was assessed. HSCs (5000 cells/well) were incubated for 24 h in 200 μl of medium with or without 10% FCS. GTX, GTX-M₆P-HSA, or M₆P-HSA was subsequently added in increasing concentrations, and the cells were incubated for another 18 h. Alamar Blue (20 μl; Serotec, Oxford, UK) was added, and the cells were incubated for an additional 24 h. The conversion of Alamar Blue redox indicator by metabolic activity of cells reflects the number of viable cells present in each well. The Alamar Blue conversion was measured using a fluorometer (O’Brien et al., 2000).

Apoptosis Assays

Myofibroblasts. Caspase-3-like activity was assayed on cell lysates according to standard methods as described previously (Li et al., 2001). In brief, after incubation of hMFs (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-DEVDAPC (Biomol, Tebu, France) as fluorogenic substrate.

Cellular Targeting of Gliotoxin 903
Apoptosis was also assessed by nuclear staining with DAPI (Roche Diagnostics, Indianapolis, IN) in cultures of nonconfluent HMFs (10,000/cm²) incubated with GTX, carrier, or conjugate as described previously (Li et al., 2001). The cells were viewed by fluorescence microscopy (Carl Zeiss GmbH, Jena, Germany) using the blue filter at a magnification of 680×.

**Liver Slices.** Slices were homogenized in caspase lysis buffer (Fluorometric Caspase Assay Kit; Promega, Madison, WI) with a sonicator. The total protein concentration of these homogenates was determined according to standard procedures (Bio-Rad Laboratories, Hercules, CA). The caspase-3 activity was measured in 25 µg of total protein homogenate and incubated for 60 min at 37°C according to the instructions of the manufacturer (Promega).

**Induction of Fibrosis.** Rats (220–240 g; Harlan) were subjected to bile duct ligation (BDL) and used for slice experiments, 3 weeks after BDL, or for in vivo effect studies, 10 days after BDL.

**Liver Slices.** Precision-cut liver slices were prepared from 3-week BDL fibrotic rats (n = 3) according to standard techniques (Olinga et al., 2001). Slices were incubated individually in six-well plates with vehicle, GTX-M6P-HSA, or M6P-HSA. After 24 h of incubation, the liver slices were snap-frozen in liquid N₂ for the active caspase-3 assay (see above) or frozen in isopentane (−80°C) for terminal deoxynucleotidyl transferase dUTP nick-end labeling analysis.

**In Vivo Studies**

**Distribution Studies.** The organ and cellular distribution of GTX-M6P-HSA was determined in rats with BDL-induced liver fibrosis as described previously (Beljaars et al., 1999).

**Effect Studies.** To assess the effects of the compounds on the number of HSCs within fibrotic livers, we treated BDL fibrotic rats at days 6 to 9 after ligation and examined the rats 24 h later at day 10 (n = 5 per group). Rats received i.v. injections 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.

Serum bilirubin levels were measured on the day of sacrifice to confirm the occurrence of cholestasis in each rat. Serum bilirubin levels were similar in each group (PBS-treated BDL rats, 201.5 ± 23.6 µM; GTX-M6P-HSA-treated BDL rats, 210.2 ± 10.4 µM; GTX-treated BDL rats, 192.6 ± 10.2 µM; and M6P-HSA-treated BDL rats, 213.8 ± 9.6 µM).

**Immunohistochemical Staining.** Acetone-fixed cryostat sections (4 µm) of liver sections were stained with indirect immunoperoxidase methods (Beljaars et al., 1999). The HSCs in the livers were detected with the monoclonal antibodies against desmin (Sigma-Aldrich) and αSMA (Sigma-Aldrich). Endothelial cells and Kupffer cells were detected with the monoclonal antibodies RECA-1 and ED2 (both obtained from Serotec). Goat polyclonal antibodies against rat collagen types I and III (both obtained from Southern Biotechnology Associates, Birmingham, AL) 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 (Sigma-Aldrich). The level of fibrosis was scored for each collagen-stained liver according to the Ishak Knodell score (Ishak et al., 1995). αSMA staining was also analyzed with ImageJ software (National Institutes of Health, Bethesda, MD). On 10 digital graphs per liver, the total area stained positive for αSMA was measured and related to the total area analyzed at a magnification of 100×.

**Periodic Acid-Schiff Staining.** Paraffin-embedded sections (5 µm) of livers were fixed in 4% formalin/methanol, incubated with periodic acid, and stained with the Schiff reagent (Merck, Darmstadt, Germany) (Hagens et al., 2006). The periodic acid-Schiff (PAS)-stained areas were quantified using ImageJ software (National Institutes 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 200×.

**Real-Time PCR.** Total RNA was isolated from rat livers using the RNaseasy kit (QIAGEN, Hilden, Germany), and the amount of RNA was measured with the NanoDrop ND1000 (Nanodrop Technologies, Wilmington, NC). The reverse transcriptase reaction (Promega) was performed with random primers.

The transcription levels of rat collagen type Iα1 (forward, 5'-AAGCTGACCCCCAGGTTGACGCTGGTTA-3'); reverse, 5'-CCAGGTGTGGACGCTGGTTA-3') and αSMA (forward, 5'-GAGCCAGGGAATGGATGTT-3'; reverse, 5'-GTTAGCAAGGGTGAGCTGC8-3') were detected by quantitative real-time PCR methods with SYBR Green on an ABI 7900HT apparatus (Applied Biosystems, Foster City, CA). Formative 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 (with GAPDH as housekeeping gene), and the gene expression levels in livers from either untreated BDL or normal rats were set at baseline.

**Liver Function.** Heparinized blood was collected by heart-puncture and plasma levels for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin were measured according to routine clinical chemical methods (Department of Clinical Chemistry, University Medical Centre Groningen, The Netherlands).

**Statistics.** Results were expressed as mean ± S.E.M. 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 one-way ANOVA followed by the least significant difference post-hoc test. P Values lower than 0.05 were considered statistically significant.

**Results**

**Characterization of the GTX-M6P-HSA Conjugate.** M6P-HSA was prepared as described previously (Beljaars et al., 1999), and GTX was coupled to this neo-glycoprotein by coupling the drug to carboxylic groups in HSA via an ester bond. Because di- or polymeric protein fractions are rapidly endocyotoded by the reticuloendothelial system in vivo, 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 the present study. High-performance liquid chromatography analysis revealed that the coupled GTX/M6P-HSA ratio was 0.4:1.

**Distribution Studies with GTX-M6P-HSA.** We previously assessed that M6P-HSA accumulated in fibrotic rat livers for 59 ± 9%, 10 min after i.v. administration of this carrier (Beljaars et al., 1999). We now showed that M6P-HSA conjugated with GTX is also predominantly taken up in the fibrotic liver. The liver accumulation of GTX-M6P-HSA at t = 30 min was 38 ± 10% (Fig. 1A), indicating that the liver accumulation of the drug carrier remains high in time. Moreover, we previously proved that M6P-HSA was taken up in HSCs of the rat liver (Beljaars et al., 1999). Using the same technique, we now also scored a predominant uptake of GTX-M6P-HSA in the HSCs of the fibrotic rat liver (Fig. 1B).

Studies with radiolabeled proteins in cultures of primary isolated rat HSCs showed significant binding of 125I-labeled GTX-M6P-HSA to these cells (Fig. 1C). This binding was reduced by 89 ± 3% (*, p < 0.05) after coincubation with M6P-HSA, which is an M6P/IGF-II receptor ligand, the designated target receptor on activated HSCs (Beljaars et al., 2001). This near-complete reduction in binding induced by M6P-HSA indicates that GTX-M6P-HSA binds to specific receptors on HSCs that recognize M6P-HSA.
In Vitro Studies of GTX-M6P-HSA on Rat HSCs and Human MFs. To test whether covalent attachment of GTX to M6P-HSA influences the biological effects of this compound, we incubated primary isolated rat HSCs with GTX and GTX-M6P-HSA. Figure 2A shows that uncoupled GTX reduced cell viability of HSCs, as reflected by a dose-dependent decrease in viability of activated HSCs that were treated for 24 h with GTX. A significant decline in viability was found at a GTX concentration of 0.1 µM (Fig. 2A), which is in accordance with previous reports on GTX (Wright et al., 2001; Hagens et al., 2006). In addition, GTX-M6P-HSA dose-dependently decreased the viability of HSCs (Fig. 2B). This decrease was significant at a concentration of 25 µg/ml GTX-M6P-HSA corresponding with a drug concentration of 0.13 µM GTX. Incubation of HSCs with the carrier alone did not have any effect on the viability of HSCs (Fig. 2B).

To assess whether GTX-M6P-HSA also affects human cells, we performed an active caspase-3 assay on hMFs. Both GTX and GTX-M6P-HSA were able to increase the level of active caspase-3 compared with the control (PBS)-incubated hMFs (Fig. 3A). In addition, DAPI staining showed the presence of condensed nuclei in GTX- and GTX-M6P-HSA-treated hMF cultures, confirming the results found with the active caspase-3 assay (Fig. 3B). The carrier alone (M6P-HSA) did not induce apoptosis of hMFs, even at high concentrations (500 µg/ml). These in vitro data show that GTX-M6P-HSA is pharmacologically active and in vitro equipotent to unmodified GTX.

GTX-M6P-HSA Induces Apoptosis in Fibrotic Livers. We previously showed that GTX induced an apoptotic response in rat liver slices (Hagens et al., 2006). Such liver slices contain all resident hepatic cells in their normal context and hence represent an excellent tool to study the effects of cell-selective compounds in vitro. In the present study, we incubated freshly prepared fibrotic rat liver slices with control substance (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 ± 0.3 after 24-h incubation with 500 µg/ml GTX-M6P-HSA compared with slices incubated with vehicle (Fig. 4A), whereas M6P-HSA did induce caspase-3 activity. In previous studies, apoptosis in fibrotic slices occurred at a
Staining of fibrotic liver slices with markers for HSCs (αSMA and desmin), Kupffer cells (ED2), or endothelial cells (RECA-1) revealed that GTX-M6P-HSA reduced the staining for desmin strongly. Staining for αSMA was difficult to evaluate because this staining was mostly present in portal areas. In cryostat sections of ultra thin liver slices, only a very few portal areas are present (zero to two). αSMA staining per section was therefore greatly determined by the number of portal areas within the section, which hampered quantitative evaluation of the fibrotic process. Staining for ED2 and RECA-1 was still present throughout the slice in GTX-M6P-HSA-treated slices (Fig. 4B). This indicates that hepatic stellate cells were the main target cells for GTX-M6P-HSA. No reduction in collagen I and III staining was seen in any of the slices incubated with GTX-M6P-HSA or M6P-HSA compared with control slices (Fig. 4A). This indicates that hepatic stellate cells were not affected by carrier-bound or M6P-HSA, GTX, and M6P-HSA did not affect the markers for parenchymal cells within the liver, mRNA levels for a Kupffer cell-specific marker (i.e., the fucose receptor) and an endothelial cell-specific marker (i.e., ENOS) were determined (Hagens et al., 2006) (Table 1). Quantitative evaluation of mRNA expression levels of these markers showed that GTX-M6P-HSA, GTX, and M6P-HSA did not affect the markers for these cells, indicating that at the applied dosages, other nonparenchymal cells were not affected by carrier-bound or unbound GTX.

In Vivo Effects of GTX-M6P-HSA. We next progressed to in vivo studies to determine the ability of targeted GTX to affect myofibroblast-like cells in fibrotic livers. α-Smooth muscle actin was used as a marker for these cells. Measurement of serum bilirubin levels showed that the levels were similar in all groups (data; see Materials and Methods), confirming that the surgical procedure led to a similar level of cholestasis in all experimental groups. To assess apoptosis, we measured caspase-3 activity in homogenized liver tissue samples. In PBS-treated fibrotic rats, caspase-3 activity was 9.89 ± 2.1 ΔFU/h, in GTX-M6P-HSA-treated BDL rats it was 21.0 ± 22.4 ΔFU/h, in M6P-HSA-treated BDL rats it was 11.9 ± 3.8 ΔFU/h, and in GTX-treated BDL rats caspase 3 activity was 12.8 ± 3.5 ΔFU/h. Differences between groups were not statistically significant. Because terminal deoxynucleotidyl transferase dUTP nick-end labeling staining indicated apoptosis in many different cell types in PBS-treated fibrotic livers (data not shown), cell-specific events in nonparenchymal cells induced by the treatment might not be detectable in tissue homogenates. Therefore, cell-specific markers were studied. αSMA mRNA levels were examined in livers of fibrotic rats after treatment with GTX, M6P-HSA, GTX-M6P-HSA, or PBS. Treatment of rats with GTX or with GTX-M6P-HSA resulted in a significant reduction in αSMA mRNA levels compared with the PBS-treated groups (Fig. 5; p < 0.05 compared with control). The carrier alone (M6P-HSA) had no effect on the αSMA mRNA expression levels. To assess the effects of the different compounds on other nonparenchymal cells within the liver, mRNA levels for a Kupffer cell-specific marker (i.e., the fucose receptor) and an endothelial cell-specific marker (i.e., ENOS) were determined (Hagens et al., 2006) (Table 1). Quantitative evaluation of mRNA expression levels of these markers showed that GTX-M6P-HSA, GTX, and M6P-HSA did not affect the markers for these cells, indicating that at the applied dosages, other nonparenchymal cells were not affected by carrier-bound or unbound GTX.

Also at the protein level, αSMA expression was affected. Liver sections of rats treated with GTX or GTX-M6P-HSA displayed a clearly reduced immunohistochemical staining for αSMA compared with control treatment (Fig. 6A). More...
phometric analysis of this staining revealed that this reduction was significant for carrier-bound GTX as well as for unbound GTX (ANOVA, $p < 0.05$; Fig. 6B). In addition, we studied the expression of a Kupffer cell marker (ED2) and endothelial cell marker (RECA-1) at the protein level. No changes in the immunohistochemical staining for these markers were noted in any of the rats treated with GTX, GTX-M6P-HSA, M6P-HSA, or vehicle, again indicating that the number of Kupffer cells or sinusoidal endothelial cells was not affected by the GTX or the GTX-M6P-HSA treatment at the applied dosages. So, in contrast to our in vitro studies (Hagens et al., 2006), GTX did not induce detectable effects in Kupffer cells (KCs) and endothelial cells (ECs).

Liver sections were also stained for the presence of collagen and the degree of fibrosis was scored according to Ishak-Knodell (Ishak et al., 1995). No significant effects were seen on the degree of fibrosis or on the deposition of collagens in the different groups receiving different treatments (Table 1).

PAS staining was performed to visualize the glycogen content of the liver as a marker for hepatocytes. A clear decrease in PAS staining was observed only in animals treated with GTX (Fig. 6A). Quantification of this PAS staining by using ImageJ software demonstrated a significant reduction in livers of GTX-treated rats (Fig. 6B), whereas the PAS staining in GTX-M6P-HSA-treated animals was similar to control animals. Serum levels for ALT and AST were measured to assess hepatocyte damage in different groups. No significant differences in serum levels for ALT were found between the different treated groups, but AST levels were higher in the GTX-treated rats compared with any other group ($p < 0.05$, ANOVA; Table 1).

**Discussion**

In the past few years, several reports have shown beneficial effects of the apoptosis-inducing agent GTX in experimental models of fibrosis (Wright et al., 2001; Dekel et al., 2003; Kweon et al., 2003; Orr et al., 2004). The induction of apoptosis in HSCs may be a pivotal step in the resolution of fibrosis (Iredale et al., 1998). However, the activities of GTX are not specific for HSCs alone (Hagens et al., 2006). GTX was discovered as an antibiotic drug made by the fungus *Gliocladium fimbriatum*, and later, it was found that GTX also suppressed the immune system (Sutton et al., 1994; Waring and Beaver, 1996), and related to this, GTX effectively attenuated dextran sodium sulfate-induced colitis in rats. This GTX-induced immunosuppressive effect seemed to seriously affect viability of the experimental animals (Fitzpatrick et al., 2000). For mice, the 50% lethal dose of GTX was found to be 10 mg/kg (Sutton et al., 1994), and it was demonstrated that i.p. injections of GTX also caused apoptosis in the thymus, spleen, and mesenteric lymph nodes (Sutton et al., 1994). In view of the multiple effects of GTX in all cells of the liver (Hagens et al., 2006) and the uptake in other organs, we designed a more cell-selective approach for this apoptosis-inducing drug in fibrotic livers to enhance its antifibrotic effects in HSCs.

In recent years, HSC-specific drug carrier systems have been developed (Beljaars et al., 1999, 2000). The modification of HSA with mannose-6-phosphate groups resulted in a carrier that accumulated in HSCs by binding to the M6P-IGFII receptors found on activated HSCs (de Bleser et al., 1995; Beljaars et al., 1999). After binding of M6P-HSA to this receptor, receptor-mediated endocytosis of the ligand-receptor complex in endosomes occurred, and within this cellular compartment, lysosomal enzymes and the low pH caused degradation of the endocytosed proteins (Dahms and Hancok, 2002). We now coupled GTX via an ester bond to the protein backbone of the carrier. The use of this ester bond ensures the release of native GTX after internalization and lysosomal degradation of the protein backbone within the target cell (Melgert et al., 2001).

Binding of GTX-M6P-HSA to the target cells was studied in cell cultures, and the pharmacological effects of this com-

**TABLE 1**

<table>
<thead>
<tr>
<th>Effect on treatment on markers for ECs, KCs, hepatocytes, and collagen deposition</th>
<th>PBS</th>
<th>GTX-M6P-HSA</th>
<th>GTX</th>
<th>M6P-HSA</th>
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<td><strong>EC marker</strong></td>
<td><strong>eNOS mRNA expression (-fold over basal)</strong></td>
<td>1.15 ± 0.74</td>
<td>0.97 ± 0.21</td>
<td>1.02 ± 0.32</td>
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<td><strong>KC marker</strong></td>
<td><strong>Fucose receptor mRNA expression (-fold over basal)</strong></td>
<td>1.02 ± 0.25</td>
<td>1.46 ± 0.64</td>
<td>1.34 ± 0.49</td>
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<td><strong>Hepatocyte markers</strong></td>
<td><strong>-ALT (U/l)</strong></td>
<td>90.5 ± 8.8</td>
<td>107.8 ± 9.7</td>
<td>116.8 ± 10.5</td>
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<td></td>
<td><strong>-AST (U/l)</strong></td>
<td>209.9 ± 45.8</td>
<td>294.4 ± 41.0</td>
<td>314.9 ± 33.1*</td>
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<tr>
<td></td>
<td><strong>Collagen deposition</strong></td>
<td><strong>Ishak-Knodell score</strong></td>
<td>3.3 ± 0.5</td>
<td>2.8 ± 0.4</td>
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* $p < 0.05$, ANOVA.
pound were examined in vitro and in vivo. Our studies showed specific binding of the conjugate to HSCs, which was inhibitable by M6P-HSA, indicating receptor-mediated uptake by a receptor that recognizes M6P-ligands. In addition, significant apoptosis-inducing activity of GTX-M6P-HSA was found, as reflected by increased caspase-3 activity and the

Fig. 6. Effects of treatment with GTX-M6P-HSA, GTX, and M6P-HSA on fibrotic rat livers. A, left, representative microphotographs of immunohistochemical staining for activated HSCs, detected with a monoclonal antibody against αSMA. Note the decrease in αSMA staining after treatment of rats with GTX or GTX-M6P-HSA. Right, representative microphotographs of PAS staining for glycogen content of hepatocytes (magnification, 100×). Note the reduced PAS staining in livers of GTX-treated rats. B, morphometric analysis of the area positively stained for αSMA (left) and for PAS (right) in livers of BDL rats receiving different treatments. Analysis was performed using ImageJ software by measuring the total area stained as a percentage of the total area per digital photo. n = 5 per group. *, p < 0.05 compared with control (PBS).
presence of apoptotic bodies in both rat HSCs and human MFs. This shows that binding of the drug carrier to the receptors on target cell is intact, and active drug is released from the carrier. In vitro, the effectiveness of a receptor-targeted drug can be hampered by receptor binding, internalization, and lysosomal degradation, which all may be rate-limiting steps, whereas the beneficial effects of cell-specific targeting are absent in vitro. Uncoupled drug will not be hampered by all these steps. However, our experiments indicated that the potency of GTX and GTX-M6P-HSA to induce apoptosis in HSCs in vitro was near equal.

We previously reported on the activity of GTX in rat liver slices (Hagens et al., 2006). The normal tissue architecture and complex cell-cell interactions are preserved in liver slices, which mimic the in vivo situation (Olinga et al., 2001). Incubation of fibrotic slices with GTX-M6P-HSA resulted in a strong induction of apoptosis as detected by the caspase-3 assay, and this was paralleled by a reduced staining for desmin in GTX-M6P-HSA-treated liver slices, whereas other nonparenchymal cell types were not affected. Our studies indicate that the HSC is the main target cell for GTX-M6P-HSA in the liver. These data show that selective removal of fibrogenic cells in the liver by an apoptosis-inducing agent is feasible.

We started treatment of fibrotic rats at day 6 because expression of the target receptor, i.e., the M6P-insulin-like growth factor II receptor, is known to be enhanced on activated HSCs (Greupink et al., 2006). Twenty-four hours after the final injection, the rats were sacrificed, and the presence of activated HSCs was examined. A significant decrease in the number of activated HSCs, as reflected by a decreased αSMA staining, was induced by GTX-M6P-HSA as well as by untargeted GTX. Both the targeted and the nontargeted GTX were administered in a dose of 10 µg/kg, which is in case of GTX-M6P-HSA equaled 5 mg protein/kg b.wt. (GTX/protein ratio was 0.4:1). Also in other studies (Wright et al., 2001), a GTX-induced reduction in the number of activated HSCs during CCl4-induced liver fibrosis was found, but much higher doses of GTX were used in these studies. The effect of low-dose GTX found in this experimental set-up was surprising and indicates the potency of this compound. From these experiments, we concluded that coupling of GTX to M6P-HSA yields a pharmacologically active compound.

Previous in vitro studies from our laboratory have shown that GTX can also affect KCs and ECs, but in the present in vivo studies, no deleterious effects were found on KCs and ECs, as assessed by ED2 and RECA1 immunostaining and by measurement of cell-specific markers at the mRNA level (i.e., the fucose receptor and eNOS as markers for KCs and ECs, respectively). It is apparent that the dosage of GTX (10 µg/kg) applied in the current animal study was too low to induce unwanted effects in these cells in vivo. However, we found significant effects of GTX on hepatocytes in vivo. Earlier studies detected no significant effects of GTX on hepatocytes in vivo (Wright et al., 2001), although in vitro studies showed an increased oxidative stress in hepatocytes induced by GTX (Orr et al., 2004). Hepatocytes rapidly respond to stress or injury by activation of glycogenolysis, leading to a reduced glycogen content that can be demonstrated by PAS staining (Hagens et al., 2006). GTX clearly affected hepatocytes as reflected by the PAS staining (Fig. 6, A and B) and displayed a small but significant effect on serum AST levels (Table 1), a serum marker for hepatocyte damage (Moss and Henderson, 1999). These data indicate that GTX not only affects the HSCs, but hepatocytes as well, whereas GTX-M6P-HSA only had an effect on HSC markers. The oxidative stress induced by GTX in hepatocytes (Orr et al., 2004) can explain the intrahepatic glycogen degradation found in our studies.

During liver fibrosis, hepatocyte damage and loss of liver function parallel the proliferation of HSCs. As much as it may be important to induce apoptosis in HSCs, it is important to prevent apoptosis in hepatocytes to prevent further loss of liver function during progression of disease. The success of recent studies with the caspase inhibitor IDN-6556 that prevents apoptosis in hepatocytes (Canbay et al., 2004a; Pockros et al., 2007) supports this notion. Uptake of apoptosis-inducing agents like GTX in hepatocytes therefore needs to be prevented, whereas uptake in HSCs needs to be promoted. The use of HSC-selective drug carriers may provide an opportunity to achieve this.

Although we found an induction of apoptosis in vitro on cells and in fibrotic liver slices, we were not able to find direct evidence of apoptosis in vivo. An explanation for this may be that apoptosis can occur very fast, ranging from minutes to a few hours after induction (Rust and Gores, 2000), which prevents its detection after 24 h. Moreover, it has been shown that apoptotic cells are rapidly and efficiently removed by macrophages (Maderna and Godson, 2003), resulting in a short half-life and a narrow window for detection of the apoptotic bodies (Cummings et al., 2004). We were 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) or the length of the treatment, which lasted for 4 days and is probably too short to affect collagen deposition. It is apparent that the most potent and direct effect of GTX is on the number of activated HSCs, which fits with its action as an apoptosis-inducing drug. Further long-term studies examining the effects of chronic treatment, the immunogenicity and toxicity are required to assess the benefits of such a targeted therapy, but the present study shows proof-of-concept for the cell-specific targeting of an apoptosis-inducing compound.

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

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References

albumin modified with cyclic peptides that recognize the collagen type VI receptor.


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