Arming drug carriers to disable the Hepatic Stellate Cell
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Gliotoxin non-selectively induces apoptosis
in fibrotic and normal livers

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Liver fibrosis is the common response to chronic liver injury, ultimately leading to cirrhosis. Several lines of evidence indicate that inducing apoptosis of Hepatic Stellate Cells (HSC) may lead to regression of liver fibrosis. Recently, it was shown that gliotoxin (GTX) induces apoptosis of HSC. However, the clinical use of GTX may be limited due to the lack of cell and tissue specificity, causing a high risk of potentially severe adverse effects. The aim of this study therefore, was to study the effect of GTX on different cells of the liver. We used normal and fibrotic precision-cut rat liver slices to study the effect of GTX on the various resident liver cell types. In these slices, the complex cell-cell interactions are preserved, which closely mimics the in vivo situation. GTX exhibited a potent apoptosis inducing activity in these slices. Both immunohistochemical stainings and real-time mRNA techniques showed that this apoptosis-inducing effect was seen in HSC. However, Kupffer cells and liver endothelial cells were also affected by GTX, whereas hepatocytes were only mildly affected. This study indicates that the apoptosis-inducing strategy to treat liver fibrosis has high potential, but it will be necessary to develop an HSC-specific therapy to avoid adverse effects.
INTRODUCTION

Chronic liver injury leading to liver fibrosis can occur in response to different forms of injuries, including alcohol abuse, drugs, viral hepatitis, and autoimmune diseases. During liver fibrosis, the hepatic stellate cell (HSC) plays a crucial role. After hepatocyte damage, this cell-type becomes activated and starts to proliferate. During this activation process, the HSC transforms from a resting vitamin A-storing cell into an activated myofibroblast-like cell and produces extensive amounts of scar tissue. Eventually, the end stage of fibrosis, cirrhosis, may be reached.

In experimental animal models for liver fibrosis, the fibrotic process seems quite reversible. Even in an advanced fibrotic state (i.e. after 4 weeks treatment with carbon-tetrachloride [CCl₄]) withdrawal of the fibrotic stimulus leads to restoration of the normal hepatic structure. This resolution of fibrosis was accompanied by the loss of activated HSC via spontaneous apoptosis. Recently, it has been shown that end stage liver cirrhosis (12 week CCl₄) can also undergo incomplete spontaneous recovery after terminating the fibrotic stimulus. Again, this was associated with induction of apoptosis in HSC.

The fungal metabolite Gliotoxin (GTX), a member of the epipolythiodioxopiperazine family, promotes apoptosis in human and rat HSC in vitro. In addition, it reduces hepatic fibrosis through HSC apoptosis while leaving the hepatocytes intact in the rat CCl₄ fibrosis model. However, GTX also displays immune suppressive effects and promotes apoptosis in several different cell types including thymocytes, macrophages, lymphocytes, enterocytes and several other cell lines of different origin, which may induce serious side-effects if GTX would be used as an anti-fibrotic drug.

To further investigate GTX as a potential anti-fibrogenic drug, it should be established whether other cells than activated HSC are affected by this drug. The effect of GTX on other non-parenchymal liver cells has not been examined in previous studies. Adverse effects of GTX on Kupffer cells and endothelial cells in the liver or on cells of the immune system could limit a successful application of this apoptosis-inducing strategy for the treatment of liver fibrosis in the future.

The aim of this study was to determine the specificity of effects of GTX within the liver. We therefore used precision-cut rat liver slices prepared from normal as well as fibrotic livers to study the specificity of GTX on various resident liver cells. In these slices, the complex cell-cell interactions are preserved, mimicking the in vivo situation. Moreover, this test system allows examination of the direct effects of GTX on fibrotic parameters without interference via its immunomodulatory properties.
MATERIALS AND METHODS

Animals and animal model

Specific pathogen-free male Wistar rats, purchased from Harlan (outbred strain, Horst, The Netherlands), were used in this study. The rats received a standard diet and were housed under standard laboratory conditions. To induce liver fibrosis, rats were subjected to bile duct ligation (BDL) as described by Kountouras et al. \(^{11}\) under anesthesia with 40% \(\text{O}_2\): 60% \(\text{N}_2\text{O}\) combined with 0.5% isoflurane (Abbot Laboratories Ltd, Queensborough, Kent, UK). Three weeks after the ligation (BDL3), rats were used for further experiments. Untreated, normal animals served as the control group. The study as presented was approved by the local committee for care and use of laboratory animals and was performed according to strict governmental and international guidelines on animal experimentation.

Liver slices

Precision-cut liver slices were prepared from normal (n=3) and BDL3 (n=3) rats \(^9\). After sacrificing the rats, the livers were taken out and stored in ice-cold University of Wisconsin preservation solution (UW) until preparation of the slices using the Krumdieck tissue slicer. After preparation, the slices were stored in UW on ice until use. Slices were pre-incubated for 2 hours in William’s Medium E (GIBCO, Life technologies LTD, Paisley, Scotland) supplemented with D-glucose (25 mM) and gentamycin (50 µg/ml) and saturated with 95% \(\text{O}_2\), 5% \(\text{CO}_2\) at 37 °C. Slices were transferred into fresh medium and incubated individually in 6-well plates with different concentrations (0.1, 1 and 10 µM) of Gliotoxin (GTX; Sigma-Aldrich, St. Louis, MO, USA, diluted in DMSO, 1mg/ml). Each measurement was performed on three slices. After 8 hours of incubation, the liver slices were snap-frozen in liquid N2 for the active caspase-3 assay and quantitative real-time PCR analysis, or frozen in isopentane (-80 °C) for immunohistochemical analysis.

Apoptosis

Three slices from each group were homogenized in caspase lysis buffer (according to the fluorimetric caspACE™ assay system, Promega, Madison, WI, USA) with a sonicator for 15 seconds. A protein assay was performed (BioRad Laboratories, Hercules, CA, USA) on these homogenates according to standard procedures. Active caspase-3 was measured in 75 µg (normal slices) or 25 µg (fibrotic slices) total protein with the fluorimetric caspACE™ assay system (Promega), incubated for 60 min at 37 °C according to the instructions described by the manufacturer.

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 manufacturers procedure (Roche Diagnostics, Indianapolis, IN, USA). The negative control for each slice consisted of adding the label solution only, omitting the terminal transferase. Additionally, all sections were stained with DAPI staining solution (Roche Diagnostics) to visualize all nuclei. Sections were analyzed with a fluorescence microscope (magnification 200X).

HSC isolation

Primary HSC were isolated from livers of male Wistar rats (450-550g) according to Geerts et al. \(^{12}\). The liver was digested with collagenase P, DNAse (both Roche Diagnostics) and pronase (Merck KGaA, Darmstadt, Germany). After separation of the HSC from other hepatic cells by density-
gradient centrifugation, the HSC were collected at the top of an 11% Nycodenz-solution. Subsequently, the cells were cultured in Dulbecco’s Modified Eagles Medium (DMEM, Gibco, Life technologies LTD) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and incubated in a 5% CO$_2$ humidified atmosphere at 37 °C. Cells cultured for 7 days were used for further experiments. During the growth period, the HSC transforms from a quiescent cell to an activated myofibroblast. The cells were daily observed and the presence of α-Smooth muscle actin (α-SMA) was confirmed by immunohistochemical staining cells.

**Viability assay**

HSC were seeded sub-confluent in 96 well culture plates and cultured for one day with serum (described above), then washed with serum-free medium and incubated for 24 hours in 200 µl medium without serum. Subsequently, GTX was added in various 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 8 hours (24 hours for HSC). The conversion of the Alamar blue REDOX indicator by metabolic activity of each cell reflects the viability of the cells present in each well and can be measured using a fluorimeter. Control incubations without cells did not show an affect of GTX on alamar blue.$^13$

**Immunohistochemical stainings**

Acetone-fixed cryostat sections (4 µm) of liver slices were stained using indirect immunoperoxidase methods according to standard procedures.$^{14}$ All HSC were detected with the simultaneous staining of two mouse monoclonal primary antibodies for the presence of desmin (II) (MP Biomedicals, Irvine, CA, USA) and glial fibrillary acidic protein (GFAP, Neomarkers, Fremont, CA, USA)$^{14}$. Detection of endothelial cells was performed with the mouse monoclonal antibody RECA-1 (Serotec). The mouse monoclonal antibody ED2 (Serotec) was used as a specific marker to detect the presence of Kupffer cells within the sections. The goat polyclonal antibody against rat collagen type III (Southern Biotech, Birmingham, USA) was used to stain the collagen type III in the liver. Apoptotic cells were demonstrated by the staining for active caspase-3 using a mouse monoclonal antibody (Cell Signaling Technology Inc, Beverly, MA, USA). After the primary antibody, sections were stained with a secondary peroxidase-conjugated rabbit anti-mouse (Desmin/GFAP, RECA-1, ED2) or rabbit anti-goat (collagen III) IgG (Dako, Glostrup, Denmark). The RECA-1 staining was further amplified using the peroxidase-conjugated goat anti-rabbit IgG (Dako). The peroxidase activity was subsequently visualized with 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich) for 20 min. Finally, sections were counterstained with hematoxylin (Fluka Biochemica, Buchs, Switzerland) and mounted in Kaisers glycerin-gelatin (Merck). The negative control was performed using the standard staining method$^{14}$ omitting the first antibody in the incubation step (PBS alone). Sections were evaluated at a magnification of 200X.

**Periodic acid Schiff (PAS)**

Cryostat sections (4 µm) of liver slices were fixed in 4% formalin / methanol, incubated with periodic acid and stained with Schiff’s reagent (Merck) according to standard methods. The staining of the sections was quantified with ImageJ software (National Institute of Health, Bethesda, Maryland, USA). On a digital photo, the total area stained positive for PAS was measured and related to the total area analyzed per liver slice at a magnification of 40X.
RNA isolation and reverse transcriptase PCR.

Total RNA was isolated from 3 slices using an RNA isolation kit (Qiagen GmbH, Hilden, Germany) according to the manufacturers procedure. The amount of RNA was estimated with a Ribogreen assay (Molecular Probes, Eugene, OR, USA) according to standard procedures. The reverse transcriptase reaction was performed (Promega) for 10 min at 25°C, 60 min at 45°C, 5 min at 95°C with the random primers.

Real-time PCR

Real-time quantitative PCR was performed with the ABI 7900HT apparatus (Applied Biosystems, Foster City, CA, USA). cDNA was added in each PCR reaction as well as the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the forward and reverse primers (50 pM). The housekeeping gene GAPDH was used as reference gene for normalization and H₂O was used as negative control. Desmin was chosen as a marker for the activated HSC, fucose-receptor was used as a marker for Kupffer cells, preproalbumin was used as a specific marker for hepatocytes and e-NOS was chosen as a marker for sinusoidal liver endothelial cells as well as endothelial cells from the vessel [12,15]. The primers used in the PCR reactions were: rat desmin, forward, 5'-AGGAACAGCAGGTCAGGTA-3', reverse, 5'-AGAGCATCAATCTCAGCAGGT-3' (product size 250 bp); rat eNOS forward, 5'-TGACCCACCCGCATACACA-3', reverse, 5'-TCTGCCTTCTGCTCA TTGT-3', (product size 211 bp); rat fucose receptor, forward, 5'-AGTCTGAGAGGCATTTGG-3', reverse, 5'-CATCTCTCATGGCCTGGATT-3', (product size 250 bp); rat preproalbumin, forward, 5'-GAAGCACACAAAGAGTGATCG-3', reverse, 5'-AGTTGTCAAGGATTTGGAAT-3', product size 250 bp); rat collagen type 1 α1 (collagen 1α1), forward, 5'-AGCCTGAGCAGCAGATTTGGAAT-3', reverse, 5'-CCAGGTTGCGCCCTGTGAT-3', (product size 145 bp); rat GAPDH, forward, 5'-CGCTGTGCTGAGATGTA-3', reverse, 5'-CTGTTGAGCAGCCTGCT-3', (product size 179 bp). The PCR reactions consisted of 40 cycles (denaturation 15 sec 95 °C, annealing 15 sec 56 °C, extension 40 sec 72 °C). The formation of single products was confirmed by analyzing the dissociation step at the end of each PCR reaction. Data were analyzed with the SDS 2.1 software program (Applied Biosystems). The relative amount of the designated PCR-product was calculated by the comparative threshold cycle (Cₜ) method.

Statistics

Results are expressed as mean ± SD. Statistical analysis was performed by two-tailed Student’s test. P values lower than 0.05 were considered statistically significant.

RESULTS

Gliotoxin induces apoptosis in precision-cut rat liver slices

To differentiate between the sensitivity of the various liver cells for GTX, we used precision-cut rat liver slices where all cell types (HSC, hepatocytes, endothelial cells and Kupffer cells) reside in the context of their natural environment and their complex cell-cell interactions are preserved [9].

We incubated normal and fibrotic rat liver slices with different concentrations (0.1, 1 and 10 µM) of GTX and measured induction of apoptosis by assessment of the caspase-3 activation. In liver slices from normal rat livers, GTX dose-dependently
increased apoptosis. The maximal fold induction of caspase-3 activation was $5.0 \pm 1.8$ after an 8 hour incubation with the highest concentration of GTX (10 µM) as compared to the normal slices incubated with vehicle (i.e. control incubation, Δ fluorescence units/hour at 37 °C/µg protein changed from $0.6 \pm 0.5$ [control incubation] to $3.0 \pm 0.9$ [10 µM GTX], Figure 1). The response to GTX in fibrotic slices was significantly higher as compared to normal liver slices; the maximal fold induction reached $10.2 \pm 1.0$ after an 8 hour incubation with the highest concentration of GTX (10 µM) as compared to control fibrotic slices (Δ fluorescence U/h at 37 °C/µg protein changed from $1.6 \pm 0.1$ [control incubation] to $21.4 \pm 3.5$ [10 µM GTX], Figure 1).

Figure 1: Induction of caspase-3 activity by GTX in normal (open bars) and fibrotic (closed bars) liver slices. Slices were incubated for 8 hours with different concentrations of GTX. The results are expressed as the mean ± SD (n=3 livers). * indicates a significant difference as compared to control liver slices (p < 0.05), # indicates a significant difference between normal and fibrotic liver slices incubated with the same concentration of GTX (p < 0.05).

To visualize induction of apoptosis after incubation with GTX in these slices, TUNEL and active caspase-3 stainings were performed. Both normal and fibrotic rat slices showed a strong increase in TUNEL-positive cells after incubation with GTX in a concentration-dependent manner starting at 0.1 µM GTX. Again, the number of apoptotic cells in the GTX-treated fibrotic liver slices was higher than in the normal liver slices (Figure 2). Immunohistochemical stainings of the liver slices with a specific active caspase-3 antibody confirmed the results of the TUNEL staining (data not shown). These results clearly show a concentration-dependent induction of apoptosis by GTX both in normal and fibrotic liver tissue. Furthermore, cells within fibrotic liver slices seem to be significantly more sensitive to GTX than cells within normal liver slices.
Figure 2: Representative microphotographs of the simultaneous detection of the nucleus (DAPI staining) and apoptotic cells (TUNEL staining) of normal and fibrotic liver slices after 8 hours incubation with different concentrations of GTX. Note the increase in TUNEL-positive cells in the fibrotic liver slices as compared to the same incubation of normal liver slices (Magnification 200X).

GTX reduces viability of activated primary isolated HSC in vitro

To investigate the response of activated primary isolated rat HSC incubated with GTX, alamar blue viability assays were performed. HSC are known to go into apoptosis after incubation with GTX \(^4\). Indeed, we found a dose-dependent decrease in viability in cultures of activated HSC. A significant decline in viability was already found at a GTX concentration of 0.1 \(\mu\)M (Figure 3).

Figure 3: Viability of isolated activated HSC 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. The results are expressed as the mean ± SD (n=3). * indicates \(p < 0.05\) compared to the control activated HSC.
Gliotoxin affects all cells in rat liver slices

To examine cell-specific responses of GTX within the liver slice, we performed immunohistochemical stainings of fibrotic livers with different markers for the resident cell types in the liver. A clear reduction in the staining of the HSC markers desmin and GFAP was visible after GTX incubation (Figure 4A). The stellate-shaped cells were nearly gone after incubation with 10 μM GTX as compared to the control fibrotic
slices. A profound reduction in the number of ED2-positive cells (Kupffer cells) was also noted in slices incubated with 10 µM GTX as compared to the control fibrotic slices (Figure 4B). Staining of the liver endothelial cells with the RECA-1-antibody revealed a slight reduction in the number of RECA-1 positive cells as compared to the control fibrotic slices (Figure 4C). Staining of collagen III clearly shows the fibrotic state of the rat liver slices (Figure 4D). Taken together, the staining for all non-parenchymal cell-markers was significantly reduced after incubation of fibrotic liver slices with GTX (Figure 4).

To test whether GTX might also affect the parenchymal cells in liver slices, hepatocytes were visualized by 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 area stained positive for PAS per liver section was quantified with imageJ software (Table 1). The data show a clear reduction in the staining for normal liver slices incubated with 10 µM GTX. The PAS staining in slices originating from fibrotic livers was significantly lower than in normal liver slices, but even this staining was further reduced by 10 µM GTX. Taken together, these data show that hepatocytes are also affected by GTX incubation, but only at the highest concentration used.

Table 1: Results of a morphometric analysis of normal and fibrotic liver slices incubated with different concentrations of GTX and stained with the PAS staining. The total area stained positive for PAS was measured and related to the total area analyzed per liver slice.

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<tr>
<th></th>
<th>Normal</th>
<th>Fibrotic</th>
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<tr>
<td>Control (%)</td>
<td>41 ± 6</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>0.1 µM GTX (%)</td>
<td>37 ± 3</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>1 µM GTX (%)</td>
<td>32 ± 8</td>
<td>5 ± 3</td>
</tr>
<tr>
<td>10 µM GTX (%)</td>
<td>4 ± 0.1</td>
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To examine the effects of GTX on the mRNA expression levels of cell-specific markers for the different hepatic cells within the livers, quantitative real-time PCR analysis on both normal and fibrotic liver slices was performed (Figure 5). The intrahepatic mRNA level for desmin was selected as a marker for the HSC, mRNA level for the fucose receptor was chosen as a Kupffer-cell selective marker, the mRNA level for eNOS reflected a endothelial cell-selective marker, and the mRNA level for preproalbumin was used as a marker for hepatocytes. All mRNA-levels of these markers were related to the expression of the house-keeping gene GAPDH.

Figure 5 shows that GTX affected the mRNA levels for all non-parenchymal cells in normal as well as fibrotic liver slices significantly (p<0.05). Compared to control incubations, mRNA levels for desmin were reduced by 46 ±11% in normal and
76 ± 14% in fibrotic liver slices by 10 µM GTX (Figure 5A). mRNA levels for the fucose receptor were reduced by 64 ± 8% in normal and 81 ± 11% in fibrotic liver slices (Figure 5B), and mRNA levels for eNOS were reduced by 25 ±15% in normal and 51 ± 10% in fibrotic liver slices (Figure 5C). The mRNA levels for the hepatocyte-specific marker preproalbumin were not significantly altered in normal (176 ± 51%) and fibrotic (152 ± 48 %) liver slices as compared to control incubations (Figure 5D). These real-time PCR results corroborate the results of the immunohistochemical stainings and clearly indicate that GTX not only significantly affects HSC, but also Kupffer and endothelial cells, and that hepatocytes are relatively unaffected.

**Figure 5:** Effect of different concentrations of GTX on several cell-specific intrahepatic mRNA levels in normal (open bars) and fibrotic (closed bars) liver slices; A: desmin mRNA levels reflecting the HSC; B: fucose receptor mRNA as a marker for Kupffer cells (KC); C: eNOS mRNA levels as a marker for liver endothelial cells (EC); D: preproalbumin mRNA levels as a marker for hepatocytes (PC). The results are expressed as the mean ± SD (n=3 livers). * indicates a significant difference as compared to control liver slices (p < 0.05), # indicates a significant difference between normal and fibrotic liver slices incubated with the same concentration of GTX (p < 0.05).

**Effect of Gliotoxin on the intrahepatic collagen 1a1 mRNA expression level**

A key parameter during liver fibrosis is the increased synthesis of matrix components. Fibrotic livers exhibit a significant increased collagen expression as compared to normal livers \(^1\). To investigate whether GTX could reduce the expression of collagen 1a1 mRNA, real-time PCR analysis on normal and fibrotic liver slices incubated with GTX was performed. Measurement of specific primers for collagen 1a1 mRNA showed that the Cycle time (Ct) value for the normal control was 23.6, the control fibrotic liver had a Ct of 18.8 with comparable GAPDH levels. This
clearly shows that the expression of collagen 1a1 is highly unregulated in fibrotic livers. The incubation of normal and fibrotic slices with GTX showed a significant reduction in the collagen 1a1 expression on the mRNA level. Compared to control incubations, mRNA levels for collagen 1a1 were reduced by 32 ±11% in normal and 59 ± 11% in fibrotic liver slices by the 10 µM GTX (p<0.05, Figure 6).

**Figure 6:** Effect of different concentrations of GTX on the intrahepatic mRNA levels of collagen 1a1 expression in normal (open bars) and fibrotic (closed bars) liver slices. The results are expressed as the mean ± SD (n=3 livers). * indicates p < 0.05 compared to control liver slices, # indicates p < 0.05 difference between normal and fibrotic liver slices incubated with the same concentration of GTX.

**DISCUSSION**

Several recent reports highlight the use of apoptosis-inducing agents as a potential therapeutic strategy to treat liver fibrosis. Some studies show that the compound Gliotoxin may be used for this purpose. It was shown that GTX promoted apoptosis in HSC *in vitro* and reduced hepatic fibrosis through HSC apoptosis in two rat models of liver fibrosis.

The aim of our studies was to examine cell-selectivity which is obviously a crucial element of apoptosis-inducing drugs. In addition, the effect of GTX on fibrogenesis could be examined in this manner without indirect effects on the immune system. The drug exhibited a potent apoptotic activity in our slice-system. Both immunohistochemical stainings and quantitative mRNA techniques elucidated that the HSC were strongly affected by GTX. However, also Kupffer cells and liver endothelial cells were affected by this compound. The hepatocytes were less sensitive compared to the other cell types although negative effects of GTX on hepatocytes were clearly visible with the PAS staining.

Induction of apoptosis in normal and fibrotic liver slices was shown with two techniques, the active caspase-3 assay and the TUNEL staining. Both results clearly point out that there is a concentration-dependent increase of the level of apoptosis.
Gliotoxin-induced apoptosis in liver slices

after GTX incubation. We also found that fibrotic slices significantly exhibited an increased sensitivity towards GTX as compared to normal liver slices which could be explained by the increased number of HSC in fibrotic livers and/or by an increased sensitivity of the individual cells.

This study elicited further investigations on the effect of GTX on other resident hepatic cells. In combination with the liver slice system, it is possible to study the effects of GTX on each cell type using semi quantitative mRNA real-time techniques combined with cell-specific markers. Real-time PCR techniques clearly demonstrated a decrease in expression levels of many specific cell markers relative to the housekeeping gene GAPDH. The decreases of the markers for HSC, Kupffer cells and endothelial cells were quite significant, whereas the hepatocyte marker was not significantly affected. Yet, the PAS staining in normal slices incubated with GTX showed a clear decrease in the glycogen content of the hepatocytes. This reduced glycogen content is an early response of hepatocytes to stress or injury due to an increased glycogenolysis. These data indicate that GTX may affect hepatocytes as well although they seem to be less sensitive towards GTX. This resistance of hepatocytes is related to the metabolism of GTX by glutathione conjugation by these cells. Hepatocytes metabolize GTX rapidly because these cells have high glutathione levels. This results in a prevention of apoptosis at low GTX concentrations.

Our data also show that GTX significantly attenuates the collagen 1a1 mRNA level in liver slices, which is another key process during liver fibrosis. Whether GTX affects the level of mRNA directly or reduces the amount of cells that produce collagen 1a1 (i.e. the HSC) remains to be established.

Recently, a growing number of publications show the ability of GTX to induce apoptosis in HSC. Not only in the tetrachloride model, but also in the thioacetamide model of fibrosis, significant in vivo effects of GTX were found. However, the effects of GTX on Kupffer and liver endothelial cells were not studied in any of these reports. In addition, the anti-fibrogenic effect found in all these experiments might have been secondary to the immunosuppressive effect of GTX found in other studies. Gliotoxin also effectively attenuated dextran sodium sulfate-induced colitis in rats. This was associated with a systemic immunosuppressive effect of GTX. In fact, this immunosuppressive effect appeared to seriously affect viability of the experimental animals in this model. For mice, the 50% lethal dose (LD50) of GTX was found to be 10 mg/kg. In this same study, it was demonstrated that intra-peritoneal injections of GTX also caused apoptosis in the thymus, spleen and mesenteric lymph nodes. This apoptotic effect was possibly a direct action of the toxin since low concentrations of GTX were measured in these organs. Based on the non-specific effect of GTX within the liver and the uptake in other organs, it
remains to be established whether the net effect of GTX as a chronic treatment for liver fibrosis is beneficial.

In general, the use of apoptosis-inducing drugs might be promising but full of risks. Cell-selectivity is a crucial element in this strategy. Apoptosis of hepatocytes for example might result in activation of the HSC by engulfment of apoptotic bodies. Apoptosis of the hepatocyte during liver fibrosis should therefore be prevented, while HSC apoptosis should be promoted. The specific delivery of the apoptotic stimulus of GTX to the HSC can in principle be accomplished using HSC-specific drug delivery systems. In recent years, these carrier systems have become available and active delivery of the potent GTX might be feasible.

In summary, we have demonstrated the ability of GTX to induce apoptosis in normal and fibrotic rat liver slices. We have shown that GTX also attenuates the synthesis of collagen 1a1 in rat liver slices, probably associated with the decrease in the number of HSC within the slice. Thus, direct effects on the key parameters of fibrosis (HSC proliferation and collagen synthesis) are demonstrated. However, we now also report that not only the HSC, but all resident cells in the liver are affected by GTX. We also conclude that fibrotic liver slices appear to be significantly more susceptible towards GTX than normal liver slices. This study shows the urge to develop an HSC-specific apoptosis-inducing drug for the treatment of liver fibrosis. Further research will explore the potency of this targeted approach.

REFERENCE LIST
