Local Inhibition of Liver Fibrosis by Specific Delivery of a Platelet-Derived Growth Factor Kinase Inhibitor to Hepatic Stellate Cells

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Received September 22, 2006; accepted March 13, 2007

ABSTRACT
Liver fibrosis is characterized by excessive proliferation and activation of hepatic stellate cells (HSC), a process in which platelet-derived growth factor (PDGF) plays an important role. Inhibition of liver fibrosis via specific delivery of a PDGF kinase inhibitor to HSC might therefore be an attractive strategy. The HSC-selective carrier mannose-6-phosphate modified human serum albumin (M6PHSA) was equipped with a tyrosine kinase inhibitor, 4-chloro-N-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-benzamide (PAP19) (an imatinib derivative), by means of the platinum-based universal linkage system (ULS). The antifibrotic activity of PAP19-M6PHSA was evaluated in culture-activated rat HSC and precision-cut liver slices from fibrotic rats. After 24-h incubation, both free inhibitor PAP19 and PAP19-M6PHSA showed potent activity, as determined by quantitative reverse transcription-polymerase chain reaction analysis of α-smooth muscle actin (αSMA) and procollagen 1α1. Next, we examined the organ distribution and antifibrotic activity of PAP19-M6PHSA in bile duct-ligated (BDL) rats. Male Wistar rats at day 10 after BDL were administered a single dose of PAP19-M6PHSA and sacrificed at 2 h, 1 day, or 2 days afterward. The accumulation of PAP19-M6PHSA in the liver was quantified by high-performance liquid chromatography analysis (30% of the injected dose at 2 h) and detected in the liver by staining of the carrier. Liver drug levels were sustained at 24 and 48 h after the single dose. Furthermore, PAP19-M6PHSA reduced collagen deposition (Sirius red staining) and αSMA staining of activated HSC at these time points in comparison with saline-treated rats. We therefore conclude that delivery of a PDGF-kinase inhibitor to HSC is a promising technology to attenuate liver fibrogenesis.

Liver fibrosis is a proliferative disease that may be initiated by a variety of factors, including chronic hepatitis, virus infections, alcohol drinking, and drug abuse. It has been extensively documented that activated hepatic stellate cells (HSC) play a fundamental role in the development of liver fibrosis (Friedman, 1999; Tsukada et al., 2006). During liver fibrosis, activated HSC proliferate and deposit extracellular matrix proteins, a process that is driven by an array of cytokines and growth factors. Among these, platelet-derived growth factor (PDGF) is one of the most potent mitogens for HSC (Pinzani, 2002). Activated HSC produce PDGF, and PDGFR-β receptors are highly up-regulated on the cell surface of hepatic stellate cells during fibrosis (Bachem et al., 1993; de Bleser et al., 1995; Weiner et al., 2000).

This study was supported by SenterNovem Grant TSGE1083 and NWO Science Netherlands Grant R 02-1719, 98-162.

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Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.106.114496.

ABBREVIATIONS: HSC, hepatic stellate cell(s); PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PAP19, phenyl-amino-pyrimidine derivative 19; M6PHSA, mannose 6-phosphate modified human serum albumin; PAP, phenyl-amino-pyrimidine; ULS, universal linkage system; DMF, dimethylformamide; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; BDL, bile duct ligated/ligation; PCR, polymerase chain reaction; αSMA, α-smooth muscle actin; TIMP, tissue inhibitor of metalloproteinase; HSA, human serum albumin, bp, base pairs.
Imatinib (STI-571, Gleevec) is used in the treatment of chronic myelogenous leukemia and gastrointestinal stromal tumors (von Mehren, 2005). It inhibits several tyrosine kinases that are mutated during cancer development. In addition, imatinib is a potent inhibitor of PDGF-B receptor kinase. Consequently, imatinib has been tested for its antifibrotic effect in cultured HSC (Kinnman et al., 2001; Yoshiji et al., 2005), and it has recently been evaluated in different animal models of liver fibrosis (Yoshiji et al., 2005; Neef et al., 2006). The fundamental role that PDGF signaling seems to play in liver fibrogenesis has made it an attractive therapeutic target for the treatment of liver fibrosis (Bataller and Brenner, 2005).

In the present study, we have investigated whether the antifibrotic effects of a PDGF tyrosine kinase inhibitor can be enhanced by local delivery to HSC. Drug targeting can improve the effect of a drug by increasing local concentrations at the target site and by providing slow local drug release. In addition, it can prevent side effects in other tissue or organs. PDGF tyrosine kinase activity plays a role in many physiological processes and its inhibition by imatinib may lead to side effects such as cardiotoxicity, as reported recently (Kerkela et al., 2006).

To effectuate local delivery within HSC in the liver, we have developed a new drug-targeting construct, in which a PDGF kinase inhibitor is coupled to the HSC-directed carrier protein mannose-6-phosphate-human serum albumin (M6PHSA). M6PHSA is a well established carrier that binds to the M6P/insulin-like growth factor-II receptor on HSC and accumulates rapidly and extensively in the liver of fibrotic rats (Beljaars et al., 1999). Figure 1 schematically depicts the structure of the PAP19-M6PHSA conjugate as well as the structures of imatinib and PAP19, the PDGF kinase inhibitor used in the present study. Zimmermann et al. (1997) have evaluated a series of phenyl-amino-pyrimidine (PAP) derivatives closely related to imatinib. Among the evaluated derivatives, PAP19 showed an inhibitory profile similar to imatinib with equivalent potency versus PDGF receptor kinase. We now have conjugated PAP19 to M6PHSA using a novel type of platinum linker chemistry called universal linkage system (ULS). ULS allows stable coupling of drug molecules to M6PHSA based on the formation of a platinum-ligand coordination bond (Gonzalo et al., 2006). Application of this novel linker technology was essential since it seems to be a straightforward and reliable method for linking PAP19 molecules to the carrier, allowing high synthesis yields in a relatively simple approach. In addition, the developed PAP19-M6PHSA conjugates display a unique behavior of slow release of drug molecules during a period of days, within the designated target cells.

In the present study, we describe the development of PAP19-M6PHSA and its impact on liver fibrogenesis in vitro and in vivo. Cultured HSC and fibrotic liver slices were used as in vitro systems to prove the antifibrotic effect of PAP19 and PAP19-M6PHSA. In addition, PAP19-M6PHSA was tested in a rat model of liver fibrosis to study its distribution to the liver and its effects on the development of liver fibrosis.

### Materials and Methods

#### Materials

The protein tyrosine kinase inhibitor PAP19 was kindly provided by György Keri (Vichem Chemie Research Ltd., Budapest, Hungary). M6PHSA was prepared as described previously (Gonzalo et al., 2006). The cis-[Pt(ethylenediamine)nitrate-chloride] (ULS) was prepared as described previously (Gonzalo et al., 2006).

![Fig. 1. Structures of the kinase inhibitors and of the drug targeting conjugate. PAP19-M6PHSA was synthesized by reacting PAP19 via its pyridyl aromatic nitrogen to the linker ULS, which was subsequently linked to M6PHSA. Typically, each PAP19-M6PHSA conjugate contained eight drug molecules and approximately 30 mannose-6-phosphate groups, which facilitate binding to activated hepatic stellate cells.](image-url)
Synthesis of PAP19-ULS-M6PHSA

PAP19-ULS was synthesized and purified by Kreacte Biotechnology (Amsterdam, The Netherlands). In brief, PAP19 (7.2 μmol; 3 mg; 10 mg/ml in dimethylformamide (DMF)) was mixed with an equimolar amount of ULS (7.2 μmol, 2.4 mg; 20 mM in dimethylformamide). The reaction mixture was heated at 37°C for 24 h after which consumption of the starting material was monitored by analytical HPLC. An additional amount of ULS was added (0.5 equivalent; 3.6 μmol), and the reaction was continued for 48 h at 37°C. The reaction mixture was concentrated under reduced pressure and dissolved in 600 μl of methanol. The crude product was purified by preparative HPLC, and the collected peaks of the main product were taken to dryness under reduced pressure. The resulting white solid was treated with water to remove inorganic salts and dried. Yield was 0.9 mg (20%). Mass spectrometry analysis confirmed the presence of the 1:1 PAP19-ULS species.

**H NMR of PAP19** (CD3OD). δH 2.33 (s, 3H, CH3), 7.26 (d, J = 8.28 Hz, 1H, CCH2CH), 7.37 (m, 2H, CHC), 7.57 (m, 3H, N(CH2)4CCH), 7.93 (d, J = 8.60 Hz, 2H, CHCCH), 8.22 (s, 1H, NCNCH), 8.47 (d, J = 5.23 Hz, 1H, CHCNCH), 8.64 (m, 2H, CH(CH3)C and CHCCHNC), 9.29 (s, 1H, NCH) ppm.


**HPLC Separations**. The mobile phase consisted of a binary solvent system of 100 mM triethylammonium acetate, pH 7.0 (solvent A) and 100 mM triethylammonium acetate, pH 5.0 (solvent B) (solvent B). The column was eluted at a flow rate of 1.1 ml/min. Compounds were eluted at a stepwise gradient (0% B from 0 to 4 min; 0−46% B from 4 to 17 min; 46−100% B from 17 to 19 min; 100% B from 19 to 25 min; 100−0% B from 25 to 27 min; and 0% B from 27 to 34 min). PAP19 eluted at 21.2 min (60.8% B) and PAP19-ULS eluted at 11.5 min (26.5% B).

PAP19-ULS was conjugated to M6PHSA according to a general protocol that has been described elsewhere for the synthesis of pentoxifylline-ULS-M6PHSA (Gonzalo et al., 2006). In brief, PAP19-ULS (143 nmol; 1.6 mg that was dissolved in DMF/H2O at 6.7 mg/ml) was added in 10-fold molar excess to M6PHSA (14.3 mg; 10 mg, dissolved in 1 ml of 20 mM Tricine/NaNO3 buffer, pH 8.3) and reacted overnight at 37°C. The final product was dialyzed against PBS at 4°C, sterilized by filtration via a 0.2-μm filter, and stored at −20°C. Protein content was assessed by the BCA protein assay (Pierce Chemical, Rockford, IL). PAP19-M6PHSA and M6PHSA were analyzed by size exclusion chromatography and anion exchange chromatography as described previously (Beljaars et al., 1999) to verify that coupling of PAP19-ULS did not alter the properties of the M6PHSA protein. The amount of PAP19 coupled to M6PHSA was analyzed by isocratic HPLC after competitive displacement of the drug by overnight incubation at 80°C with excess of potassium thio-yanate (KSCN; 0.5 M in PBS). Elutions were performed on a Waters system (Waters, Milford, MA) equipped with a 5-μm Hypersil BDS C8 column (250 × 4.6 mm; ThermoQuest, Runcorn, UK), a thermostated column oven operated at 40°C, and an UV-detector operated at 269 nm. The mobile phase consisted of acetonitrile/water/trifluoroacetic acid (40:60:0.1) at pH 2 and flow rate of 1.0 ml/min with a sensitivity of 0.01. Retention time for PAP19 was 7 min. Chromatograms were also monitored for PAP19-ULS (retention time 5 min) to confirm that drug release was complete.

**Cells**

Hepatic stellate cells were isolated and cultured as described previously (Gonzalo et al., 2006). Cells were split after 3 days, and they were cultured until day 10 to obtain the activated HSC phenotype. Culture-activated HSC were used for the experiments described below.

**Cell Viability Studies**

Activated HSC (10,000 cells/well seeded in 96-well plates; Corning Life Sciences, Acton, MA) were washed with serum-free medium and incubated for 24 h in medium supplemented with PAP19-M6PHSA (0.1 or 1 mg/ml, corresponding to 10 or 100 μM PAP19 or ULS linker) or with free PAP19, free ULS, or unmodified M6PHSA at equimolar concentrations. To evaluate the sensitivity of HSC to platinum toxicity, cells were incubated with cisplatin for 24 h. Cell viability was assayed by Alamar Blue assay (Serotec, Oxford, UK) according to the supplier’s instructions.

**Effects on Gene Expression**

The potential antifibrotic activity of PAP19-M6PHSA and PAP19 was evaluated in cultured HSC and in precision-cut liver slices prepared of fibrotic rat livers (BDL3 rats, i.e., 3 weeks after bile duct ligation). Activated HSC were incubated with indicated compounds for 24 h, after which they were processed for RNA analysis as described below. In total, four independent experiments were performed in HSC cultures from four different Wistar male rats.

Precision-cut liver slices were prepared as described previously (Olinga et al., 2001; van de Bovenkamp et al., 2005). After 2 h of preincubation in Williams’ medium E (Invitrogen, Paisley, Scotland, UK) supplemented with 25 mM D-glucose and 50 μg/ml gentamicin and saturated with 95% O2, 5% CO2 at 37°C, slices were transferred into fresh medium and incubated individually in six-well plates with the indicated compounds for 24 h, after which they were snap-frozen in liquid nitrogen and stored at −80°C until real-time PCR analysis.

Each measurement was performed on three liver slices from the same liver, and the experiment was repeated on livers from three different BDL3 rats.

**Animal Experiments**

All animal studies were approved by the local committee for care and use of laboratory animals at Groningen University, and they were performed according to strict governmental and international guidelines on animal experimentation. Animals had free access to tap water and standard laboratory chow, and they were housed under a 12-h light/dark cycle. All the animals included in these studies were monitored by analysis of body weight ratio and biochemical parameters reflecting liver functions, such as serum bilirubin levels, alanine aminotransferase, and alkaline phosphatase levels. These analyses were performed at the University Medical Center Groningen by standard biochemical procedures.

**BDL Model**

Liver fibrosis was induced in male Wistar rats (250 g; Harlan, Zeist, The Netherlands) by bile duct ligation as described previously (Beljaars et al., 1998). Animals were allowed to recover, and then they were carefully observed until final sacrifice at the end of the experiments.

**Experimental Protocol**

At day 10 after BDL, rats received a single intravenous injection of PAP19-M6PHSA (3.3 mg/kg, corresponding to 150 μg of PAP19 per kg). Control animals were injected with an equivalent volume of the vehicle (saline, 250 μl). Animals were sacrificed 2 h postinjection to determine initial accumulation of the conjugate within the liver, or they were sacrificed 24 or 48 h postinjection to determine the effects of the conjugate on liver fibrosis. Organs were harvested and processed for drug analysis, RNA isolation, and immunohistochemical analysis as described below.
Analysis of PAP19 Levels in the Liver

Drug levels in the liver were determined according to the HPLC method described above. In brief, approximately 400 µg of liver tissue was accurately weighed and homogenized in 2 ml of PBS using an Ultraturrax. One-fourth of the sample was incubated overnight at 80°C with 500 mM KSCN to release PAP19. The drug was extracted twice with diethyl ether, after which the organic layer was evaporated to dryness and reconstituted in 150 µl of mobile phase. Drug levels were expressed as percentage of the total amount of administered PAP19 that had accumulated in the liver.

RNA Isolation and Gene Expression Analysis

Total RNA isolation and synthesis of cDNA was performed according to standard procedures. Quantitative real-time reverse transcription-PCR was performed in duplicate on an ABI 7900HT system (Applied Biosystems, Foster City, CA) using SYBR Green primers. For each sample, 1 µl of cDNA was mixed with 0.4 µl of each gene-specific primer (50 µM), 0.8 µl of dimethyl sulfoxide, 8.4 µl of water, and 10 µl of SYBR Green PCR Master Mix. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used as a reference gene and for normalization of the other genes, whereas ultra-pure water was used as a negative control. Table 1 lists the primers that have been used to investigate antifibrotic responses. PCR reactions consisted of 40 cycles (denaturation, 15 s at 95°C; annealing, 15 s at 56°C; and extension, 40 s at 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 method and referred to control treatment.

Immunohistochemical Analyses

Cryostat sections (4 µm) of liver, heart, kidney, lung, and spleen were acetone-fixed and stained for the presence of the PAP19-M6PHSA conjugate with an antibody directed against HSA (Cappel ICN Biomedicals, Zoetermeer, The Netherlands) as described previously (Beljaars et al., 1998). Expression of PDGF receptor and its phosphorylation in BDL liver cryosections were investigated by staining with anti-PDGFR-β and anti-phospho-PDGFR-β (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

The degree of hepatic fibrosis was estimated by staining for collagen using the picrosiris red dye (Sigma, Gillingham, UK). Fibrogenic myofibroblasts were stained with anti-smooth muscle α actin (αSMA; Sigma). Microphotographs were taken with an optic microscope BX40 (Olympus, Tokyo, Japan) connected to a high-resolution camera (Camedia C-5050 zoom; Olympus) at an original magnification of 40X. Stainings were quantified by morphometric analysis of the sections using the ImageJ software package (National Institutes of Health, Bethesda, MD), and images were captured following automatic white balance and light intensity equilibration with a 40X magnification objective and digitized as RGB 24-bit. After shading correction and interactive thresholding, the selected positive pixels were measured. The positive area was the sum of the area of positive pixels per liver biopsy. Results were calculated as the average area of positive pixels per liver biopsy and divided into each group of animal treatment.

Statistical Analysis

Results are expressed as the mean of at least three independent experiments ± S.D., unless otherwise indicated. Statistical analysis was performed with an unpaired Student’s t test, and differences were considered significant at P < 0.05.

Results

Synthesis and Characterization of PAP19-M6PHSA Conjugate. When observing the structure of imatinib or the related kinase inhibitor PAP19 (Fig. 1), it is clear that their structures are mainly composed of aromatic rings. Typically, conjugation reactions for the preparation of a drug-linker adduct aim at carboxyl, hydroxyl, thiol, or primary amino groups. Because such functional groups are lacking in imatinib or PAP19, one would need to synthesize a derivatized molecule, which potentially may alter or destroy the pharmacological activity of the inhibitor. We therefore pursued a different strategy in which a novel linker was applied that binds the drug via a coordinative linkage at the pyridyl nitrogen of PAP19. HPLC analysis and mass spectrometry indicated complete derivatization of the drug into the drug-ULS 1:1 product. The PAP19-ULS adduct was subsequently conjugated to M6PHSA, and the final high-molecular-weight product was extensively purified by dialysis, which also removed free PAP19-ULS molecules not linked to the carrier (HPLC analysis; data not shown). An average of eight PAP19-ULS molecules was coupled per M6PHSA, as assessed by HPLC after release of the drug from the carrier. The overall yield of the final reaction step was 84%. Conjugation of PAP19 to M6PHSA did not change the charge or size features of M6PHSA, as determined by anion exchange chromatography and size-exclusion chromatography, respectively (Fig. 2).

Effects in Cultured Hepatic Stellate Cells and Fibrotic Liver Slices. The use of a platinum(II) compound as a linker may introduce platinum-associated effects in the drug-targeting preparation. We therefore evaluated HSC viability, although PAP19 at the highest concentration even increased the number of HSC.

To examine the potential antifibrotic effects of PAP19-M6PHSA conjugate and nonconjugated PAP19, we investi-
gated their effects on the expression of fibrosis-related genes in cultured HSC and fibrotic liver slices. Both PAP19-M6PHSA and free PAP19 down-regulated the expression of αSMA and collagen 1a1 in cultured HSC (Fig. 4A). In contrast, M6PHSA did not affect the expression of these genes in HSC. The other genes that were examined, PDGF receptor-β and TIMP-1, were not significantly reduced by either treatment.

Precision-cut liver slices were prepared from rats that had undergone bile duct ligation 3 weeks before, and thus they encompassed all liver cell types in the context of a fibrotic extracellular matrix. In agreement with the results in cultured HSC, PAP19-M6PHSA and PAP19 showed pronounced reductions in the expression of αSMA and collagen 1a1 (Fig. 4B). In addition, PDGF-β (PAP19-M6PHSA and PAP19) and TIMP-1 (PAP19) gene levels were inhibited significantly

![Fig. 2. Characteristics of PAP19-M6PHSA drug-targeting conjugate. A, Mono Q anion exchange chromatography confirmed that the charge of the protein was not affected after coupling of PAP19-ULS to the carrier. Both M6PHSA and PAP19-M6PHSA eluted at a later retention time than unmodified HSA due to the negatively charged mannose-6-phosphate groups. B, size exclusion chromatography showed the monomeric composition of PAP19-M6PHSA. Notice the similar elution profiles of M6PHSA and PAP19-M6PHSA.](image)

![Fig. 3. Activated HSC incubated with PAP19-M6PHSA. Effect of PAP19-M6PHSA and drug, linker and carrier on HSC cell viability, as determined by Alamar Blue assay. Cultured HSC were incubated for 24 h with the compounds. Indicated concentrations reflect the platinum content of the tested compounds or equivalent amounts of PAP19 or M6PHSA (*, P < 0.05).](image)

![Fig. 4. Effects of PAP19 and PAP19-M6PHSA on the expression of fibrotic genes. Activated HSC (A) and precision-cut slices (B) from BDL rats were incubated for 24 h with PAP19 or PAP19-M6PHSA (both at 10 μM drug). M6PHSA was tested in an equivalent concentration of carrier (0.1 mg/ml). Gene expression levels were normalized to the expression levels in control HSC or slices (*, P < 0.05).](image)
in slices, whereas those genes were not affected in cultured HSC. Furthermore, M6PHSA carrier showed inhibitory effects on some of the investigated genes, although TIMP-1 expression was enhanced by M6PHSA. Differences between PAP19-M6PHSA were significant for αSMA and collagen 1α1. These results encouraged us to evaluate PAP19-M6PHSA in an animal model of liver fibrosis.

**Animal Studies.** To demonstrate that PAP19-M6PHSA was capable of homing to the fibrotic liver and that the compound is capable of inhibiting early stages of fibrogenesis, BDL rats at day 10 after ligation of the bile were treated with a single dose of the product. In the rat BDL model, HSC activation associated with PDGF receptor expression and downstream signaling is prominent at this day (Neef et al., 2006). In addition, the expression of the M6P/insulin-like growth factor-II receptor, which recognizes M6PHSA, is also increased at day 10 after BDL (Grepunk et al., 2006a).

Table 2 shows the characteristics of the BDL rats included in the studies. The animals were treated with a relatively low dose of PAP19-M6PHSA (1 mg of conjugate/animal), which corresponds to 150 μg/kg conjugated drug. Administration of PAP19-M6PHSA did not affect the body weight of the rats, nor did it change the biochemical parameters that were analyzed.

**Distribution of PAP19-M6PHSA in Fibrotic Rats.** Previous studies in our laboratory have demonstrated that M6PHSA extensively binds to activated HSC in the fibrotic liver (Beljaars et al., 1999). Typically, about 60% of the injected carrier accumulated in the fibrotic liver already within 10 min after administration (e.g., Gonzalo et al., 2006). In the present study, PAP19-M6PHSA organ distribution was investigated at a later time point, 2 h after administration of the product, to allow for a more complete distribution of the conjugate (Fig. 5). PAP19-M6PHSA was detectable in the liver in a nonparenchymal staining pattern. Although double immunostaining to detect colocalization with HSC failed due to the faint staining (data not shown), these results suggest that PAP19-M6PHSA homed to the liver in a similar manner as other HSC-selective conjugates. PAP19-M6PHSA was undetectable in other organs, such as heart, kidney, lung, and spleen (Fig. 5, C–F).

We furthermore quantified the accumulation of PAP19-M6PHSA in the liver by assaying PAP19 levels by HPLC. As can be observed in Fig. 6, 30% of the injected dose had been accumulated in the liver after 2 h, and these drug levels persisted up to the last time point at 48 h.

**Effects of PAP19-M6PHSA in Fibrotic Rats.** Antifibrotic effects of PAP19-M6PHSA were studied at the latter two time points of the study, 24 and 48 h after administration of the conjugate. First, we investigated whether PAP19-M6PHSA affected fibrotic gene expression in a similar approach as described above for cultured HSC and precision-cut slices. We did not observe changes in the expression of fibrosis reporter genes (data not shown). Immunohistochemical staining of fibrotic markers, however, clearly indicated the establishment of fibrosis and the pharmacological activity of PAP19-M6PHSA. Sirius red staining for collagen showed a continuous increment in deposited extracellular matrix components from day 10 to 12 after bile duct ligation (Fig. 7A). Treatment with a single dose of PAP19-M6PHSA significantly attenuated collagen deposition at both 24 and 48 h.

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**Table 2**

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<td>Bilirubin (μmol/l)</td>
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after administration. Apart from reduced staining intensity, the antifibrotic effect of PAP19-M6PHSA was illustrated by a reduced portal-portal bridging in fibrotic areas (Fig. 7B). Likewise, treatment with PAP19-M6PHSA significantly attenuated the αSMA-stained area in the liver at both 24 and 48 h after single-dose treatment (Fig. 8).

**Discussion**

Liver fibrosis is in principle a reversible process in which the stellate cells have been identified as the key fibrogenic cells (Friedman, 2003). PDGF is the most potent mitogen for HSC in vitro, and it plays an important role in the transformation of HSC into myofibroblast-like cells in vivo (Pinzani et al., 1994). Targeting the PDGF signaling cascade therefore represents a promising antifibrotic approach. Several recent studies have described the antifibrogenic properties of the kinase inhibitor imatinib in different models of fibrotic disease (Lassila et al., 2005; Wang et al., 2005; Yoshiji et al., 2005). In contrast, concerns have been raised in relation to the effectiveness of imatinib as an antifibrogenic drug (Neef et al., 2006; Vittal et al., 2007), especially in later stages of liver fibrosis. In the present study, the new product PAP19-M6PHSA was designed to effectively and specifically deliver an imatinib-related kinase inhibitor to HSC, to improve its antifibrotic properties. We demonstrate the antifibrogenic effects of PAP19-M6PHSA in two different validated in vitro systems. Furthermore, we show the sustained delivery of the drug within the liver as well as its potential activity in rats with liver fibrosis.

PAP19 is a potent inhibitor of PDGR-kinase with an IC_{50} of 10 nM (Zimmermann et al., 1997). We confirmed the antifibrotic potential of PAP19 by incubating culture-activated HSC and BDL liver slices with the drug. Our results with PAP19 are in good agreement with previous studies in which imatinib was tested for its activity in HSC (Yoshiji et al., 2005) or liver slices (van de Bovenkamp et al., 2006). Imatinib dose-dependently inhibited fibrotic gene expression in slices, reaching 70 to 80% inhibition of collagen 1α1 and αSMA at 10 μM (van de Bovenkamp et al., 2006). No toxicity of imatinib was observed in fibrotic slices. In PDGF-stimulated HSC, 10 μM imatinib blunted the expression of αSMA and α2-(1)-procollagen in addition to an almost complete inhibition of PDGF-induced proliferation and migration (Yoshiji et al., 2005). We did not add external PDGF to our experiments, but it has been reported that the growth factor is synthesized in situ by activated HSC in culture or in slices (Pinzani, 2002). We also found that PAP19 reduced PDGFR-β and TIMP-1 expression in BDL slices. TIMP-1 expression is increased during liver fibrosis, and it plays an important role in liver fibrogenesis by modulating extracellular matrix remodeling (Iredale et al., 1998). Taken together, these data support our hypothesis that PAP19 is a proper candidate for HSC-directed drug delivery.

PAP19-M6PHSA potently inhibited fibrotic gene expression in both HSC and fibrotic liver slices. These results are remarkable taking into account that carrier-bound drug most probably should be released from the carrier to become active. First, the attachment of PAP19 to ULS and subsequently to the carrier will prevent the pharmacological activity of PAP19, because the active site of the drug is blocked by the linker. Intracellular release of the compound will revert PAP19-M6PHSA to the active kinase inhibitor. Sec-
ond, drug-M6PHSA conjugates cannot diffuse across cell membranes, but they will enter cells by receptor-mediated endocytosis, followed by lysosomal routing (Beljaars et al., 2001). Since the kinase domain of the PDGF receptor is located in the cytosol, the conjugate will not end up in the same intracellular compartment as the pharmacological target of PAP19. We postulate that the conjugate is degraded efficiently in the lysosomes of HSC and that during this process drug will be released from the carrier. A possible mechanism by which drug release can take place is competitive displacement by glutathione or other endogenous compounds that can coordinate to platinum (Gonzalo et al., 2006; Prakash et al., 2006; Temming et al., 2006a,b).

Rapid degradation of the conjugate within the lysosomal compartment of target cells also explains the faint staining of PAP19-M6PHSA in the liver after its administration to BDL rats. To allow for a more complete accumulation of the conjugate within the liver, we sacrificed the animals at a rather late time point (2 h) compared with other studies from our group in which staining was performed 10 to 20 min after administration (Gonzalo et al., 2006; Greupink et al., 2006b; Hagens et al., 2007). Most probably, this prolonged period allowed for the uptake and degradation of the carrier by target cells, whereas the majority of the compound was bound extracellularly or in the endosomes at the 10- to 20-min time point. Thus, anti-HSA staining resulted in poor immunodetection of the PAP19-M6PHSA conjugate. However, the analysis of the coupled drug by HPLC clearly showed the efficient accumulation of PAP19-M6PHSA in the liver. Furthermore, the persistence of high drug levels even at 2 days after its administration denotes that PAP19 is retained within the fibrotic liver. This result is in good agreement with the results of another drug-ULS construct aimed at the kidney, which could be detected up to 3 days after single dosing (Prakash et al., 2006). Whether the released PAP19 molecules will be retained within HSC or redistribute to other cell types within the liver is presently unknown. Although one may argue that redistribution of the delivered drug is unfavorable, it may also help in reaching profibrogenic liver cell types such as peribiliary fibroblasts. Highest drug levels can be expected, however, in target cells that have accumulated and internalized the conjugate. It is noteworthy that the drug delivery strategy greatly lowered the systemic distribution of imatinib, because a much lower dose was administered than commonly applied for liver fibrosis treatment, 150 \(\mu\)g/kg as a single dose versus 5 to 20 mg/kg daily, respectively. This lower dose was furthermore accumulated preferentially within the fibrotic liver. Thus, potential side effects of imatinib in nonfibrotic tissues will be avoided, which seems relevant in view of the reported cardiotoxicity of imatinib (Kerkela et al., 2006).

Eventually, released PAP19 should effectuate a sustained blockade of PDGF kinase activity within the fibrotic liver. Staining for phosphorylated PDGF receptor in untreated BDL rats at day 10 after ligation was, however, scattered, and it did not match the pattern of total PDGF receptor (data not shown). This may relate to a technical problem as the commercially available antibodies were not reported for immunostaining of fibrotic tissues. Yoshiji et al. (2005) have reported on the capability of imatinib to inhibit PDGF receptor phosphorylation in HSC, and the anti-PDGF kinase activity of PAP19 has been well documented (Zimmermann et al., 1997). Because pharmacological activity of PAP19-M6PHSA is observed in HSC and fibrotic slices, it is feasible that kinase inhibition has occurred in the cells that have accumulated PAP19-M6PHSA. Although this event could not be directly demonstrated, the pharmacological effects of the construct found in vivo are in accordance with this hypothesis.

After single administration, PAP19-M6PHSA markedly reduced collagen deposition together with a suppression of \(\alpha\)-SMA-positive cells, which reflects a reduction in the number of activated HSC (Olaso and Friedman, 1998). In contrast to the effects on protein expression of fibrotic markers, PAP19-M6PHSA administration did not affect the gene expression of such markers in BDL rats. This discrepancy either reflects different sensitivity of the assays, or it corresponds to a greater reduction of collagen and \(\alpha\)-SMA at the protein level than at gene expression level. This divergence has been described previously in experiments investigating the inhibition of hepatic fibrosis (Yata et al., 2002), and it might be due to post-transcriptional regulation of collagen expression in cultured HSC (Stefanovic et al., 1997).

Several other approaches for blockade of the PDGF sig-
naling cascade have been investigated as a strategy to block liver fibrosis. Some studies showed that monoclonal antibodies directed against the extracellular domain of the PDGF receptor can prevent binding of the PDGF ligand, thereby inhibiting mitogenic signaling (Heldin, 1997; Shulman et al., 1997). In a different strategy, Borkham-Kamphorst et al. (2004) produced a soluble PDGF receptor that was capable of reducing extracellular matrix deposition in the BDL model after daily administration for 14 days. Consistent with this, short-term treatment with imatinib in the BDL model caused a marked reduction in HSC proliferation (Kinnman et al., 2001). According to Neef and colleagues, the effect of imatinib is limited to the early phase of fibrogenesis, due to the lack of efficiency in advanced liver injury, i.e., 3 to 4 weeks after BDL (Neef et al., 2006). In contrast to this latter observation, another study reported a pronounced inhibition of SMA-positive fibroblast and expression of fibrosis markers after 8 weeks of imatinib administration to fibrotic rats (Yoshiji et al., 2005). These authors studied the effect of imatinib in the pig serum-induced model of liver fibrosis, which is characterized by a slow progression of fibrogenesis, resembling the human situation. Thus, it might be a relevant therapy in patients with early stages of liver fibrosis.

In conclusion, we have evaluated a new strategy for local delivery of a PDGF kinase inhibitor to the fibrotic liver. Our strategy differs completely from the aforementioned studies in which the free drug imatinib is used. We used a carrier molecule that is internalized by target HSC, after which the kinase inhibitor is released inside the cells. The delivered kinase inhibitor produced a significant effect on the fibrotic process 24 to 48 h after its administration to BDL rats. These results illustrate the potential of using PAP19-M6PHSA as a cell-specific drug that may effectively lead to reduced activation of HSC and consequently may reduce liver fibrosis.

Acknowledgments

We acknowledge Jan Visser and Jai Prakash (University of Groningen) for assistance in HPLC analysis and colleagues at Kreatech Biotechnology B.V. for critical reading of the manuscript.

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