Arming drug carriers to disable the Hepatic Stellate Cell
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Exacerbation of liver fibrosis after selective inhibition of cyclo-oxygenase-2 in Hepatic Stellate Cells

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Several cellular processes associated with fibrogenesis are controlled by cyclo-oxygenase-2 (COX-2) activity. Because opposite effects of COX-2 products are reported in different hepatic cell types, only a cell-selective intervention of COX-2 activity may lead to the unravelment of the role of this enzyme during fibrosis in vivo. To study COX-2 related effects in Hepatic Stellate Cells (HSC) in rats with liver fibrosis, we conjugated a COX-2 inhibitor, niflumic acid (NFA), to the HSC-selective drug carrier M6P-HSA (Human Serum Albumin modified with Mannose 6-Phosphate groups). HPLC analysis confirmed the covalent binding of NFA to M6P-HSA. To assess whether the conjugate NFA-M6P-HSA was able to exert a COX-2 related effect, we measured intracellular cAMP levels in human hepatic myofibroblast cultures. NFA-M6P-HSA reduced cAMP concentrations significantly in myofibroblasts. Subsequently, the in vivo distribution of NFA-M6P-HSA showed that after iv injection, this conjugate accumulated predominantly in the fibrotic rat livers, in particular in HSC. Finally, in vivo studies assessing the effects of this HSC-specific COX-2 inhibitor on the progression of liver fibrosis revealed that administration of NFA-M6P-HSA to bile duct ligated rats from day 3 to day 10 clearly enhanced the fibrotic process. The hepatic collagen deposition was significantly increased in these rats as compared to the control groups. In conclusion, coupling of NFA to M6P-HSA yielded a pharmacological active product. The effects of the HSC-specific COX-2 inhibitor in vivo appeared to be profibrotic. This study provides insight into the role of COX-2 in HSC during liver fibrosis in vivo and tools for a cell-specific manipulation of this enzyme activity.
INTRODUCTION

Prostaglandins (PG) are involved in the regulation of inflammation, cellular proliferation, apoptosis, and wound repair. These endogenous mediators are formed by enzymatic conversion of arachidonic acid by the enzyme cyclo-oxygenase (COX). Two isozymes are described, COX-1 and COX-2. COX-1 is constitutively expressed in most cell types, whereas COX-2 is mainly present in an inducible form. The induction of COX-2 occurs in particular in inflammatory conditions, and stimuli known to induce COX-2 are for instance LPS, IL-1, TNFα, PDGF and TGFβ. COX enzymes mediate the conversion of arachidonic acid into prostaglandin H₂ (PGH₂), and subsequently various prostaglandin synthases convert PGH₂ into other PG forms. In addition, PGH₂ can be converted in tromboxanes. Due to the fact that in different cell types different PG synthases are expressed and different receptors for the prostanoids are present, the biological effects of COX activity are cell-specific.

In the progression of liver fibrosis, induction of COX-2 activity is observed in both Kupffer cells (KC) and Hepatic Stellate Cells (HSC). In KC, COX-2 induction is associated with the production of PGE₂, PGD₂, and thromboxane A₂. Consequently, induction of COX-2 in KC leads to increased levels of TNFα and lipid peroxidation in KC, and triglyceride accumulation in hepatocytes. In HSC, COX-2 is reported to be constitutively expressed. However, this expression can be further upregulated by for instance endothelin-1 and TNFα via an activation of the NFκB pathway. Activation of COX-2 in HSC, and the formation of PGE₂, was associated with an inhibition of cellular proliferation. In addition, 15d-PGJ₂ can be formed by COX-2 in HSC, and this PG is a potent inducer of apoptosis in these cells. Administration of PGE₂ to rats with liver fibrosis was reported to result in a reduction of hepatic collagen production. So, COX-2 activity plays a significant role in the control of several, sometimes opposite, processes during fibrosis: whereas COX-2 products in one cell type (Kupffer cells) lead to profibrotic effects, COX-2 activity in other cells of the liver (HSC) lead to antifibrotic effects. Only a cell-selective intervention may lead to the unravelment of the role of COX-2 in a particular cell type in vivo, and possibly to an antifibrotic therapy.

To obtain cell-selective intervention in vivo, drug targeting can be applied. To be able to study the role of COX-2 or its products in HSC in vivo, we prepared a HSC-specific form of a COX-2 inhibitor by coupling the drug to the HSC-selective carrier M6P-HSA (Human Serum Albumin modified with Mannose 6-Phosphate groups). Many COX-2 inhibitors are described, of which the coxibs (celecoxib, rofecoxib, etc) are the most frequently used. We selected niflumic acid (NFA), because this particular COX-2 inhibitor possesses appropriate groups (carboxylic acid group) that allows chemical coupling to the albumin carrier. The IC₅₀ value of NFA for COX-2 was reported to be 20 nM, whereas the inhibition of COX-1 was less...
potent (IC$_{50}$ = 2 µM)$^{18,19}$. In the present series of experiments, we prepared NFA-M6P-HSA and studied the binding and uptake of this construct in vitro and in vivo. In vitro capability to inhibit COX-2 activity was measured by studying the effect of the construct on intracellular cAMP levels in HSC. Furthermore, this conjugate was administered to rats with liver fibrosis in order to study the effects of cell-selective COX-2 inhibition in HSC in vivo on the fibrogenic process.

**MATERIALS AND METHODS**

**Materials**

Human Serum Albumin (HSA) was purchased from the Central Laboratory of the Blood Transfusion Services (Sanquin Blood Supplies, Amsterdam, The Netherlands) and consisted of >95% monomeric protein. The COX-2 inhibitors used in this study were niflumic acid (NFA, obtained from ICN, Biomedicals Inc., Zoetermeer, The Netherlands) and NS398 (Biomol, Tebu, France). DMEM (Dulbecco’s modified Eagle’s medium) and Fetal Calf Serum (FCS) were obtained from Invitrogen (Carlsbad, CA, USA) and Biowhittaker Europe (Verviers, Belgium), respectively.

**Animals**

Male Wistar rats (Harlan, Horst, The Netherlands) were housed under standard laboratory conditions with free access to lab chow and water. 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.

**Rat model for liver fibrosis**

Liver fibrosis was induced in rats (≤ 250 g) by common bile duct ligation (BDL) under isoflurane/N$_2$O/O$_2$ anaesthesia$^{16}$.

**Synthesis**

M6P-HSA: HSA was modified with mannose 6-phosphate groups to obtain the carrier protein M6P-HSA, as described previously$^{16}$. The ratio used to couple thiophosgene-activated M6P to HSA was 300:1.

NFA-M6P-HSA: The carboxylic acid group of niflumic acid (NFA) (50 mg in 1 ml DMF) was activated with 74 mg N,N-dicyclohexylcarbodiimide (DCC, Sigma, St. Louis, MO, USA) dissolved in DMF 500 µl, and reacted for 1 hr. Then, the NFA/DCC solution was added to 30 mg M6P-HSA (dissolved in PBS in a concentration of 2 mg/ml) and the reaction was proceeded overnight. After this, the reaction mixture was extensively dialysed at 4°C against milliQ water to remove low molecular weight compounds, lyophilized, and stored at −20°C.

**Characterization**

M6P-HSA was characterized for protein, sugar and phosphate content as described$^{16,20}$. In the present series of experiments, we used the carrier that contained an average of 30 M6P groups coupled per albumin.

FPLC: M6P-HSA and NFA-M6P-HSA were analyzed with an FPLC (Fast Protein Liquid Chromatography) system (GE Healthcare, Uppsala, Sweden): the percentages of monomeric protein...
in the preparations were determined with a Sephadex200 column, and the net negative charge was analyzed with a MonoQ column. 

HPLC analysis was performed to establish the number of NFA drugs coupled to the protein and also to determine the amount of free drug in the preparation. The HPLC method used to detect NFA was based on the analysis as described by Avgerinos et al. Briefly, an HPLC (Waters 600 Controller 717 plus Autosampler) was equipped with a µBondapack C18 column (Waters) and a 486 Tunable Absorbance Detector (Waters). At 330 nm NFA was detectable, while other proteins (M6P-HSA components) and solvents did not disturb the NFA signal. To elute the compounds the eluent 0.1 M NaAc:ACN pH7 (60:40) was used at a flow rate of 1 ml/min.

To determine the amount of NFA molecules coupled to the carrier, the preparations (2 mg/ml) were dissolved in 6N HCl, incubated for 18 hrs at 110°C in order to completely hydrolyse the amide bond between NFA and the protein (acid hydrolysis). Subsequently, the mixture was diluted in eluent (30x), and a sample was injected on the HPLC system to determine the amount of total NFA. A calibration curve was prepared with various concentrations of NFA. NFA treated with 6N HCl overnight at 110°C showed the same calibration curve as a freshly prepared NFA standard curve. This indicated that the drug was stable during the hydrolysis procedure.

In order to assess the amount of uncoupled NFA in the preparations, the compound was freshly dissolved in milliQ in a high concentration (10 mg/ml), diluted in eluent (30x), and immediately injected on the HPLC system.

In vitro effects.

Cell isolation and culture: Human hepatic myofibroblasts (hMF) were obtained by outgrowth of normal liver explants as described previously. This procedure was performed in accordance with ethical regulations imposed by the French legislation. The cells were routinely characterized by positive immunostaining for α-smooth muscle actin. Experiments were performed with the cells between passages 3 and 8.

Cell Viability: hMF (10,000 cells/well in 96-well plates) were seeded and grown to confluency in DMEM containing 5% human serum and 5% fetal calf serum (DMEM 5/5), and serum-starved for 48 hours in Waymouth medium. Subsequently, cells were incubated with the various compounds (NFA, M6P-HSA, NFA-M6P-HSA) or their vehicles for 8 hours. The viability of the cells was determined after addition of CellTiter 96 AQueous One Solution reagent (Promega, Madison, WI, USA) to each well, and absorbance was recorded at 490 nm.

cAMP measurements. The in vitro effects of the conjugate were assessed with cAMP analysis as described. Briefly, human hMF were seeded in 24-well plates and grown to confluency in DMEM 5/5. Subsequently, the cells were serum-starved for 48 hours in Waymouth medium. At t=15 min, the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 1.2 mM) was added, and at t=0 min 1 µM sphingosine 1-phosphate (S1P, Biomol) was added for 10 min to induce cAMP. Cells were incubated for 3 hours with either NFA (1-5 µM), or the conjugated form NFA-M6P-HSA in various concentrations. To assess the potency of NFA in cultures of HSC, the COX-2 inhibitor NS-398 (5 µM) was used as a reference. NS-398 has been frequently applied as a COX-2 inhibitor in HSC. Control cells were incubated with vehicle, carrier M6P-HSA, or M6P-HSA with unbound NFA in a concentration that was maximally present in the preparations as determined with HPLC. Finally, cAMP was extracted into ethanol for 2 hours at room temperature and the cAMP levels were measured with a commercial radioimmunoassay (Sigma). The protein contents of the wells were determined (BioRad Laboratories, Hercules, CA, USA)
**In vivo distribution**

*In vivo* localization of NFA-M6P-HSA: A bolus dose of 2 mg/kg NFA-M6P-HSA or M6P-HSA was *iv* injected to rats 10 days after bile duct ligation (BDL). After 10 min, organs were removed and frozen in isopentane (-80°C). In addition, the distribution of NFA-M6P-HSA was studied after multiple dosing. Therefore, the protein (dose 5 mg/kg) was *iv* injected, under fluothane/N₂O/O₂ anaesthesia in the penile vein, every day starting at day 3 after the bile duct ligation until day 10. At 10 min or at 24 hours after the last injection, various organs (liver, spleen, kidney and lungs) were removed and snap-frozen in isopentane –80°C. Cryostat sections (4 µm) were immunohistochemically stained with polyclonal antibodies against HSA (MP Biomedicals, Irvine, CA, USA) as described \(^ {16}\). Double-immunostaining techniques were used to examine the cellular localization of NFA-M6P-HSA in the liver \(^ {16}\). The HSC were identified by a combination of two mouse monoclonal primary antibodies: desmin (MP Biomedicals) and glial fibrillary acidic protein (GFAP, Neomarkers, Fremont, CA, USA). The Kupffer cells and endothelial cells were identified, respectively, with the antibodies ED2 and HIS52 (both Serotec, Oxford, UK).

**Binding of NFA-M6P-HSA to HSC.**

The binding of the conjugates to HSC was immunohistochemically determined as described \(^ {25}\). Briefly, HSC were isolated from livers of male Wistar rats (>450 g) according to standard techniques \(^ {26}\). Rat livers were digested with collagenase P, DNAse (both Roche Diagnostics, Indianapolis, IN, USA) and pronase (Merck KGaA, Darmstadt, Germany). The HSC were separated from the other hepatic cells by density-gradient centrifugation and collected at the top of an 11% Nycodenz-solution. Subsequently, the cells were cultured at 37°C in DMEM containing 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. These culture conditions allow HSC to attach and proliferate as confirmed by light-microscopical evaluation. Cells cultured for 10 days after isolation displayed all signs of activated HSC, based on morphological observations and positive staining of the cells for α-smooth muscle actin (Sigma) and collagen type I and III (Southern Biotechnologies Associates, Birmingham, UK).

Activated HSC were trypsinized and seeded in 24-well-plates (30,000 cells/well), and cultured overnight in medium at 37°C. After a pre-incubation of 15 min with 1% bovine serum albumin in serum-free medium, 0.5 or 1 mg/ml M6P-HSA, NFA-M6P-HSA, HSA, or medium alone were incubated with these HSC for 2 hours at 37°C, and immunohistochemically stained for the presence of HSA with the polyclonal antibodies against HSA as described in the previous section.

**In vivo effects.**

Experimental setup: BDL-rats were treated with NFA-M6P-HSA (5 mg/kg; n=5), NFA (0.27 mg/kg; n=4), M6P-HSA (5 mg/kg; n=4) or PBS (n=4). These compounds were *iv* injected, every day starting at day 3 till day 10 after ligation. 24 hours after the last injection the rats were sacrificed. Heparinized blood was obtained to analyse the levels of various markers of (liver) injury and cholestasis: aspartate aminotransferase (AST), alanine aminotransferase (ALT) and bilirubin by routine clinical chemistry (Department of Clinical Chemistry, University Medical Centre Groningen, The Netherlands). The liver and spleen were weighed, and specimens of the livers were processed for immunohistochemical (frozen in isopentane –80°C) analysis.

Immunohistochemical analysis: Cryostat sections (4 µm) of the livers were fixed in acetone and stained with monoclonal antibodies directed against desmin/GFAP and α-smooth muscle actin (Sigma) to show the HSC in these livers, and with polyclonal antibodies directed against collagen type I and III (Southern Biotechnologies Associates) to demonstrate the fibrotic extracellular matrix. The
total area positively stained for collagen type I in the cryostat sections was quantified by Adobe Photoshop (original magnification 20x).

RESULTS

To couple NFA to the protein carrier, the carboxylic acid group of NFA was activated with DCC and subsequently attached to the εNH₂-lysine residues in M6P-HSA. HPLC analysis confirmed the covalent binding of the drug to the protein. After acid hydrolysis, a procedure to destruct the chemical amide bond between drug and albumin carrier, a peak emerged at the retention time of NFA. We assessed that the preparation of NFA-M6P-HSA used in the present series of experiments consisted of M6P₃₀-HSA substituted with a mean of 17 molecules of NFA (see Table 1). Acid hydrolysis of HSA or M6P-HSA did not result in HPLC-signals with retention time of NFA (at t=5.9 min). Furthermore, no peak was found in the chromatogram at the retention time of NFA when high concentrations of NFA-M6P-HSA were freshly dissolved in water, which indicated that the amount of free NFA in the preparation was below detection limits (i.e. less than 5 µmol NFA per mg NFA-M6P-HSA).

Table 1. Characteristics of NFA-M6P-HSA nd = not detectable

<table>
<thead>
<tr>
<th>Characteristics</th>
<th># M6P groups/HSA</th>
<th># NFA groups/HSA (HPLC)</th>
<th>% monomer (FPLC)</th>
<th>% di/polymers (FPLC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA</td>
<td>nd</td>
<td>nd</td>
<td>97</td>
<td>2</td>
</tr>
<tr>
<td>Carrier:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M6P-HSA</td>
<td>30</td>
<td>nd</td>
<td>80 ± 17</td>
<td>16 ± 11</td>
</tr>
<tr>
<td>Conjugate:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFA-M6P-HSA</td>
<td>30</td>
<td>17 ± 2</td>
<td>58 ± 2</td>
<td>41 ± 1</td>
</tr>
</tbody>
</table>

In addition, FPLC analysis was performed to characterize the products for the presence of mono-, di- and polymers (Table 1). Coupling of M6P or NFA groups to HSA resulted in a shift in retention time of the monomeric fraction from 34.5 min to 31.8 min indicating that the molecular weights of the carrier and conjugate were increased as compared to albumin. The prepared albumins were predominantly in the monomeric form, although for NFA-M6P-HSA an increased amount of polymers were detectable. This is relevant with regard to a possible uptake in Kupffer cells. In addition, FPLC-Sephadex analysis showed that low molecular weight compounds, such as NFA with a retention time of 44 min, were not detectable in the chromatograms of the conjugates, which confirmed our HPLC data.
We subsequently assessed whether NFA-M6P-HSA was able to exert a COX-2 related effect in vitro. Since cAMP levels are under the influence of COX-2 activity, intracellular cAMP measurements were performed in human hMF cultures to confirm the effects of the drug in the free and coupled form. Uncoupled NFA strongly reduced cAMP levels in myofibroblasts under basal and sphingosine 1-phosphate (S1P) stimulated conditions. For comparison, NS398, a frequently used COX-2 inhibitor, was used. This drug attenuated intracellular cAMP peaks in response to S1P in similar concentrations as NFA, i.e. 5 µM (Figure 1A). The reduction in cellular cAMP levels by NFA was dose-dependent with an IC₅₀ of 0.5 µM (Figure 1B). Similar to the free drug, the albumin-conjugated NFA also caused a significant reduction in the cAMP levels of S1P-stimulated myofibroblasts at concentrations higher than 1 µM (Figure 1C). M6P-HSA itself did not affect cAMP levels, while M6P-HSA mixed with a maximum amount of free NFA, that may be maximally present in the conjugate
preparations (as determined with HPLC analysis), affected the cAMP levels to some extent, but was less effective compared to NFA-M6P-HSA. In addition, we assessed that NFA was not cytotoxic for hMF up to concentrations of 30 µM. Also, neither the carrier M6P-HSA nor the conjugate NFA-M6P-HSA did affect the viability of hMF.

Subsequently, we studied the homing of the conjugate NFA-M6P-HSA to HSC. At first, we incubated cultured rat HSC with either the conjugate, the carrier, NFA, or medium alone. Immunohistochemical analysis showed that HSC were only positively stained for HSA, when NFA-M6P-HSA or M6P-HSA were incubated (data not shown), indicating that the carrier is able to bind to HSC.

Subsequently, the in vivo distribution of NFA-M6P-HSA was studied in rats 10 days after bile duct ligation. Ten min after intravenous injection of the proteins, high amounts of NFA-M6P-HSA were immunohistochemically detected in the fibrotic rat livers, in particular in the perisinusoidal areas (Figure 2). Extrahepatic localization of NFA-M6P-HSA was only observed in the spleen (red pulpa). However, the amount in the spleen was much less as compared to the liver. Other organs such as the kidneys and lungs were devoid of anti-HSA staining (Figure 2). The body distribution of NFA-M6P-HSA corresponded with the reported distribution of the drug carrier M6P-HSA\cite{16}. 24 hours after intravenous injection, all organs, including liver, of rats that received NFA-M6P-HSA or M6PHSA were negative after staining for the protein.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig2.png}
\caption{Organ distribution of NFA-M6P-HSA in rats with liver fibrosis (BDL 10 days) after iv injection. The localization of conjugated NFA was determined after staining for HSA. \textbf{A}: Spleen, \textbf{B}: Lungs, \textbf{C}: Kidneys, \textbf{D}: Liver (Magnification 100X).}
\end{figure}
Within the fibrotic livers, the staining patterns of NFA-M6P-HSA corresponded with a non-parenchymal cell distribution. Using double-immunohistochemical staining procedures, we found a co-localization with HSC markers (desmin/GFAP), and to some extent with the Kupffer cell marker ED2 (Figure 3). After counting the number of double-positive cells, we estimated that 32±11% of NFA-M6P-HSA was present in the HSC, while 23±3% was present in KC. Multiple dose injections of M6P-HSA or NFA-M6P-HSA yielded a similar distribution pattern: a predominant uptake in hepatic stellate cells was found with additional uptake in Kupffer cells.

Figure 3: Hepatic localization of NFA-M6P-HSA. Double staining of the livers for conjugate, with anti-HSA antibodies (in red), and cellular markers (in blue) for HSC (desmin/GFAP; A.) and KC (ED2; B). Figure A1 and B at magnification of 400. Figure A2 at magnification 1000.

Subsequently, we examined the effects of the targeted COX-2 inhibitor in rats with liver fibrosis. Therefore, NFA-M6P-HSA was administered to BDL rats during 7 consecutive days, starting at day 3. We used rats in an early stage of fibrosis to study the induction phase of the fibrotic process. Rats in all four groups (receiving NFA-M6P-HSA, PBS, carrier or drug alone) displayed a similar increase in body weight during treatment. Plasma bilirubin levels, which were elevated at day 10 as compared to normal rats, were similar in all 4 groups indicating that the bile duct obstruction was similar in all rats. Other plasma markers reflecting hepatocyte damage (AST, ALT) were also elevated as compared to normal rats, and were also similar in all 4 groups.
Figure 4: Representative photographs of the collagen type I staining in the parenchymal areas of the rat livers at 10 days after bile duct ligation. The rats received iv injections of NFA-M6P-HSA, NFA, M6P-HSA, or vehicle (PBS, control) for 7 consecutive days. Note the increased collagen fibers in the group of rats that received the HSC-specific COX-2 inhibitors.

Figure 5: Quantification of the immunohistochemical stainings presented in Figure 4. Analysis of collagen type I staining in the parenchymal areas revealed a significant increase in NFA-M6P-HSA treated rats (*p< 0.05), whereas rats that received the free drug NFA or M6P-HSA alone did not display such an increased matrix deposition.
After microscopic evaluation of the liver, we found that the intrahepatic staining for desmin, α-smooth muscle actin, collagen type I and III in the bile duct ligated rats were strongly enhanced as compared to normal livers. Ten days after ligation, the rats had a fibrous expansion of the portal areas (stage 2 Knodell scoring), and a few rats displayed portal to portal bridging (stage 3) \(^{28,29}\). In rats treated with NFA-M6P-HSA, collagen type I and III deposition was clearly enhanced compared to untreated BDL-rats (Figure 4). In particular in the perisinusoidal areas and enhanced staining for both collagen type I and III was seen. Quantitative analysis of this staining revealed a significant increase in NFA-M6P-HSA treated rats (p< 0.05), whereas rats that received the free drug NFA or M6P-HSA alone did not display such an increased matrix deposition (Figure 5). No significant differences in intrahepatic desmin and α-smooth muscle actin staining were found between the different experimental groups (data not shown).

**DISCUSSION**

Drug targeting strategies were used in this study to examine the effect of cell-selective COX-2 inhibition in HSC *in vivo*. Therefore, a COX-2 inhibitor (NFA) was coupled to the HSC-selective drug carrier M6P-HSA, and tested in a rat model of liver fibrosis (BDL). We selected niflumic acid (NFA) to attenuate COX-2 activity because this drug can be coupled to proteins and is equipotent to NS-398, a COX-2 inhibitor that has been frequently applied as an inhibitor of COX-2 activity in HSC *in vitro* \(^{22,24,30}\). The data show that the newly prepared construct accumulates in HSC and the COX-2 inhibitor is released from its carrier in a pharmacological active form within the target cells *in vitro*. The effects of NFA-M6P-HSA *in vivo* show that inhibition of COX-2 in HSC is associated with an exacerbation of the fibrotic process as reflected by increased collagen deposition.

NFA was coupled to lysine residues within the albumin carrier via the carboxylic acid group of the drug. The coupling procedure that was used (activation of -COOH by DCC) resulted in an amide bond between the drug and the amino acid lysine. When the drug-carrier is taken up by the target cells via receptor-mediated endocytosis, the protein is degraded but the amide bond between lysine and drug can not be cleaved by proteinases \(^{31}\). As a consequence, intracellular degradation of NFA-M6P-HSA will lead to the release of NFA-lysine. This modified NFA is still endowed with COX-inhibiting effects, as assessed with cAMP studies in human hepatic myofibroblasts (hMF). NFA-M6P-HSA attenuated c-AMP levels hMF to a similar level as free NFA and both compounds were equipotent *in vitro* in this respect. It has been reported that various non-steroidal anti-inflammatory drugs (NSAIDs) become more selective COX-2 inhibitors when the carboxylate-group is modified \(^{32}\);
the enzyme COX-2 has a larger active site as compared to the COX-1 enzyme. Attachment of lysine to the carboxylic groups of NFA apparently does allow binding to the active site of COX-2 resulting in pharmacological effects of carrier-bound NFA in our studies in vitro. Alterations of the carboxylic acid moiety in NFA however do inhibit the effects of this drug on chloride channels, but this was not examined in the present study.

A relation between COX-2 activity and various HSC activities has been shown in several studies, but most of these studies were performed in cell cultures in vitro. Several studies have shown that PGE2 can inhibit the proliferation of fibroblasts. Collagen production by HSC is attenuated by PGE2 and 15d-PGJ2. Hui et al showed in the human HSC cell line LX-1 and in isolated rat HSC that PGE2 inhibited collagen gene expression in these cells. Administration of PGE2 to rats with liver fibrosis caused a reduction in hepatic collagen production. In line with this, COX-2 deficiency led to enhanced fibrogenesis in lungs. Addition of a COX-2 inhibitor to HSC in vitro caused an increased collagen gene expression, an enhanced HSC proliferation, and an enhanced α-smooth muscle actin expression. All these in vitro studies implicate that inhibition of COX-2 activity in HSC or myofibroblasts promotes fibrogenesis.

However, several reports show antifibrotic effects of COX-2 inhibitors in vivo. In sinusoidal endothelial cells (SEC) and KC, COX-2 inhibition leads to down-regulation of the production of many pro-inflammatory mediators which may lead to antifibrogenic effects of these drugs. It is not clear which cells have the highest COX-2 activity during fibrosis. Hepatocytes express COX-2 activity and they constitute the majority of cells within the liver. KC respond to injury or stress by up-regulation of their COX-2 activity, and in several models, including BDL-induced liver injury, KC were shown to contribute predominantly to the total hepatic COX-2 activity. Inhibition of this KC-derived COX-2 activity may lead to reduced fibrogenesis.

However, most COX-2 inhibitors are cleared via/taken up by hepatocytes, and pharmacological effects of COX-2 inhibitors in this cell type should seriously be taken into account. Attenuation of prostaglandin production in this cell type generally lead to hepatoprotective effects, and hence antifibrogenic effects. Also, endothelial dysfunction during liver fibrosis may be corrected by the inhibition of thromboxane A2 production, although this appears to be primarily mediated by COX-1 rather than COX-2. On the whole, these studies suggest that COX-2 inhibitors within hepatocytes, KC or SEC exert anti-fibrogenic effects.

The opposite effects of COX-2 inhibitors in HSC and myofibroblasts versus hepatocytes, KC and SEC warrants further in vivo studies, and particular a cell-selective delivery appears to be essential for this kind of drugs. It will provide insight into the pathogenic role of COX-2 in different cell types and if uptake in particular
cells types can be prevented, counterbalancing effects will be circumvented and the effectivity of COX-2 inhibitors might be strongly enhanced.

The present study shows that such a cell-selective approach is feasible: accumulation in the liver of targeted NFA with predominant uptake in HSC was demonstrated. The minor uptake that was found in KC, still corrupts the targeting efficiency, yet the relative uptake of NFA in HSC is most likely much higher that in the untargeted situation since NFA is neither liver nor HSC-specific. Untargeted NFA is metabolized by hepatocytes and cleared by the kidneys.

COX-2 inhibitors may be prescribed by practitioners for various inflammatory diseases, including patients with chronic liver diseases. Care should be taken after administration of these drugs in patients with liver fibrosis/cirrhosis, because of the opposing effects in different cell types of the liver that lead to a complex situation.

In summary, we used the drug targeting approach to examine the effect of COX-2 inhibition in HSC in vivo. Our studies show that cell-selective delivery of pharmacologically active NFA in HSC is feasible and this leads to an enhanced collagen deposition in livers of bile duct ligated rats. This study provides insight into the role of COX-2 in HSC during liver fibrosis in vivo and provides also the tools for a cell-specific manipulation of this enzyme activity. This study implicates an important role for HSC-derived prostaglandins during fibrogenesis.

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REFERENCE LIST


