STUDIES ON THE TARGETED DELIVERY OF THE ANTIFIBROGENIC COMPOUND MYCOPHENOLIC ACID TO THE HEPATIC STELLATE CELL

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Hepatic stellate cell (HSC) activation and proliferation are key events in the pathology of liver fibrosis. Inhibiting these parameters therefore is a relevant option to treat liver fibrosis pharmacologically. The immunosuppressive drug mycophenolic acid (MPA) has been shown to inhibit proliferation and activation of various types of fibroblasts. In an effort to circumvent the immunosuppression and at the same time enhance this antifibrotic effect, we coupled MPA to the HSC-selective drug carrier mannose-6-phosphate modified human serum albumin and evaluated this conjugate for its specificity and antifibrotic activity. We found that MPA inhibited proliferation of HSC in vitro. The drug coupled to the drug carrier bound specifically to HSC and reduced HSC proliferation in vitro. In vivo studies in bile duct-ligated rats demonstrated that our conjugate accumulated selectively in the liver with significant uptake in HSC apart from Kupffer and endothelial cells, whereas primary and secondary lymphoid tissues were avoided. Treatment of bile duct-ligated rats with this conjugate reduced hepatic inflammation and hepatic α-β-crystallin mRNA expression, a marker for HSC activation. In conclusion, this study shows that targeted delivery of MPA to HSC results in a decrease in HSC activation, making it the first drug that is successfully delivered to this cell type.
INTRODUCTION

During liver fibrosis, hepatic stellate cells (HSC) change from a quiescent cell type into activated cells with a myofibroblastic phenotype that extensively proliferate and produce an excess of extracellular matrix proteins. This gradually causes an impairment of liver function (1, 2). Inhibiting HSC functioning therefore is an interesting strategy to develop an effective pharmacological treatment for this disease (3-7).

The immunosuppressive drug mycophenolic acid (MPA) is a potential inhibitor of HSC proliferation and activation. In the treatment of the fibrotic process in the kidney, MPA has been successfully used to ameliorate fibrosis in two animal models (8-10). In vitro studies showed that MPA has a direct antiproliferative effect and inhibits cell activation in cultures of mesangial cells, the fibrogenic cells within glomeruli (11, 12). Other studies report that MPA is able to inhibit the proliferation of tenon fibroblasts and vascular smooth muscle cells, which are main players in fibrotic processes in the eye and during atherosclerosis, respectively (13, 14). This may imply that MPA can also act on HSC.

The ideal antifibrotic drug for liver fibrosis should be organ-specific and, if possible, even HSC-specific. Inhibition of fibroblast proliferation other than HSC, in principle, can result in impaired wound healing elsewhere in the body, which is not desired. In addition to that, systemically administered MPA would lead to suppression of T and B lymphocyte functioning, which may seriously affect the host-defence system of patients.

In order to avoid immunosuppression and at the same time to deliver MPA specifically to the HSC, we coupled the drug to the HSC-selective drug carrier mannose-6-phosphate-modified human serum albumin (M6PHSA). M6PHSA has been shown to accumulate selectively in activated HSC in vivo, through binding to Mannose-6-Phosphate/Insuline-like Growth Factor-II receptors (M6P/IGF-II receptors) (15). These receptors are abundantly present on activated HSC (16, 17).
In this study we explore the potential of MPA conjugated to M6PHSA to achieve specific delivery to the HSC in order to treat HSC activation and proliferation, making this the first drug to be targeted to the HSC.

MATERIALS AND METHODS

Preparation of drug targeting construct

Synthesis of M6PHSA-MPA via ester bond

M6PHSA was synthesized as described (15). MPA (Sigma, Gillingham, UK) was coupled to M6PHSA via an ester bond by activation with 2-iodoethanol. Briefly, MPA (20 mg) was reacted with 20 µl of SOCl₂ (Sigma) and 0.4 µl of dimethylformamide (Merck, Darmstadt, Germany) in dichloromethane (Merck) for 5 hours. The reaction mixture was evaporated to dryness. The remaining product was reacted with 200 µl of 2-iodoethanol (Sigma) in dichloromethane for 90 minutes on ice, protected from light. The resulting MPA-2-iodoethanol ester was coupled to the sulphydryl groups of M6PHSA which were incorporated via S-acetylthioglycolic acid N-hydroxysuccinimide ester (Sigma) derivatization (18). The final product was purified by filtration followed by dialysis against water and was then lyophilized. The protein content was assessed by the Biorad protein assay. Drug content was assessed by HPLC after hydrolysis of the ester bond between MPA and M6PHSA at pH=12. Drug loading was calculated from the molar ratio between MPA and protein. The percentage of monomeric protein in the preparation was assessed by size exclusion chromatography as described previously (15).

Synthesis of M6PHSA-MPA via amide bond

To enhance drug loading to the carrier, MPA was also coupled to M6PHSA via an amide bond. Briefly, MPA (8 mg) was reacted in PBS with M6PHSA (10 mg) and 5 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma). Purification was performed as described above. The amount of conjugated MPA was
determined as described above, after degradation of the conjugate overnight at 120 °C in 6 N HCl.

**Experimental Animals**

For HSC isolation, male Wistar rats of 400-500g (Harlan, Horst, The Netherlands) were housed under a 12-hour dark/light cycle, at constant humidity and temperature. Animals had free access to tap water and standard lab chow (Harlan). For *in vivo* experiments male Wistar rats of 220-240g were used. All experiments were approved by the local committee for care and use of laboratory animals and were performed according to strict governmental and international guidelines for the use of experimental animals.

**In vitro experiments**

**HSC isolation**

HSC were isolated and cultured according to the method of Geerts et al. (19). After 9 days of culture, when cells had an activated phenotype, the cells were plated in 24-well plates at a density of 30,000 cells/well and were allowed to attach to the plates overnight before performing experiments.

**Effects on cell proliferation**

To assess the effect of test substances on cell proliferation, HSC were cultured with MPA, M6PHSA (1 mg/ml), M6PHSA-MPA (1 mg/ml) or vehicle for 48 hours. Immunohistochemical staining was performed to assess BrdU-incorporation. The number of BrdU-positive cells was counted and expressed relative to the total number of cells counted. HSC were cultured in the presence of 10 µM BrdU with 50 ng/ml human recombinant PDGF-BB (Peprotech, Rocky Hill, NJ, USA) and 10% FCS to stimulate proliferation.

To verify whether MPA acts by decreasing intracellular guanosine synthesis, MPA-treated HSC (1.5 µM) were co-incubated with 1 mM guanosine (Sigma) for 48 hours and cell proliferation was measured by assessment of BrdU incorporation.
Binding of M6PHSA-MPA to HSC in vitro

To authenticate specific binding of the conjugate to HSC, binding assays were performed with $^{125}$I-labeled M6PHSA-MPA. Cells (10 days after isolation) were pre-incubated with 1% BSA in DMEM to block non-specific binding. HSC were then incubated at 37 °C with 100,000 cpm of $^{125}$I-labeled M6PHSA-MPA in the absence of a competitor for receptor binding, in the presence of 1 mg/ml HSA or with 1 mg/ml M6PHSA. After 2 hours, cells were washed and the cell-associated radioactivity was measured on a $\gamma$-counter (Riastar, Packard instruments, Palo Alto, USA).

Inosine monophosphate dehydrogenase (IMPDH) mRNA expression in HSC

RNA was isolated from primary isolated rat HSC with the Absolutely RNA microprep kit (Stratagene, La Jolla, CA, USA) after culture-activation of the cells on plastic for 3, 7 or 10 days. cDNA was synthesized according to standard techniques and real time PCR was performed on an ABI PRISM 7900HT Sequence Detection System to assess the expression of IMPDH type 2 mRNA relative to GAPDH. SYBR Green (Applied Biosystems, Warmington, UK) was used for fluorescent detection of the amplified product. Appropriate primers were used in a concentration of 50 µM.

In vivo experiments

Organ distribution

Organ distribution experiments were performed in bile duct-ligated rats (BDL) as described previously (15). Three weeks after BDL, animals were injected i.v. with a tracer dose of $^{125}$I-labeled M6PHSA-MPA under $O_2/N_2O$/Isoflurane anaesthesia. Ten minutes after injection, the animals were sacrificed by heart puncture. The organs were removed and washed with saline, before measuring radioactivity with a $\gamma$-counter.
Intra-hepatic distribution

Three BDL rats received i.v. injections with PBS from day 3 until day 10 after BDL and a final injection of M6PHSA-MPA (4 mg/kg) 10 minutes before sacrifice. Another three BDL rats were injected daily from day 3 until day 10 after BDL with conjugate before their final injection, 10 minutes prior to sacrifice. This allowed us to study the difference in distribution after single and multiple dosing. Immunohistochemical double-stainings were performed for the conjugate and markers for HSC (desmin/GFAP), Kupffer cells (ED2) and endothelial cells (RECA-1) on 4 µm acetone-fixed cryostat sections of liver, as described earlier (15). The number of double-positive cells per microscopic field was counted at a magnification of 200x. In each microscopic field this number of double-positive cells was related to the total number of HSA-positive cells. This yielded a relative accumulation of M6PHSA-MPA by each cell type. At least 5 microscopic fields per section were analyzed.

In vivo effect study

BDL rats were i.v. injected daily with PBS, M6PHSA-MPA (4 mg/kg/day), M6PHSA (4 mg/kg/day) or M6PHSA (4 mg/kg/day) in combination with 8 µg/kg/day of uncoupled MPA which is equivalent to the amount of MPA present in the conjugate. Animals were treated from day 3 after BDL until sacrifice at day 10 after BDL. Staining for oxygen-free radical-producing cells, using diaminobenzidine (DAB) was performed on 4 µm cryostat sections of liver as described (20). The staining was quantified by counting the number of DAB-positive cells in 6 microscopic fields per section (magnification 10 x 10). The extent of fibrosis and the number of desmin/GFAP-positive cells were assessed by picrosirius red staining and immunohistochemical staining for desmin/GFAP, respectively, followed by morphometric analysis with Image J software (NIH, Bethesda, USA).
Real-time RT-PCR for hepatic α-β-crystallin mRNA, a marker for HSC activation was performed as described above (21-23).

Statistical analysis

Results were expressed as the mean +/- SD. Data were subjected to a one-way ANOVA followed by the LSD post-hoc test. The differences were considered statistically significant at P < 0.05.

Table 1: Characteristics of the synthesized M6PHSA-MPA constructs

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RESULTS

Characterization of M6PHSA-MPA conjugates

Synthesis of M6PHSA-MPA via an ester bond

HPLC-analysis revealed that the maximum drug to protein ratio that could be achieved was 1.2:1 (Table 1). Bulk synthesis yielded lower coupling ratios and the construct used in the in vitro studies, therefore, had a drug to protein ratio of 0.4:1. The conjugate that was used for in vivo studies had a near equal drug to protein ratio of 0.5:1. Size exclusion chromatography showed that more than 70% of the conjugate consisted of monomeric protein.
Synthesis of M6PHSA-MPA via an amide bond

Analysis of the construct for the coupling ratio of MPA to M6PHSA showed that a total of 22 drug molecules were coupled per carrier molecule (Table 1).

In vitro experiments with MPA

When HSC were incubated with increasing concentrations of MPA, BrdU-incorporation decreased strongly in a dose-dependent manner (Fig. 1), whereas cell viability was not affected (data not shown).

Since it is possible that MPA acts by decreasing intracellular guanosine synthesis through inhibition of IMPDH, we investigated whether HSC express mRNA for IMPDH. In Fig. 2A it can be seen that IMPDH type 2 mRNA is expressed after 3, 7 and 10 days of culture activation. In addition, Fig. 2B shows that the effect of MPA is completely blocked when exogenous guanosine is added to the culture medium.

Fig. 1. Representative microphotographs of the immunohistochemical detection of BrdU-incorporation (red staining) in nuclei of primary isolated rat HSC, 11 days in culture. Figures show cultures treated for 48 hours with vehicle (A), or treated with 0.75 (B), 1.5 (C) and 3 µM (D) MPA. Magnification 200x. E: Percentage of BrdU-positive cells expressed as a percentage of control. Data represent the average of 3 independent experiments. * indicates p < 0.05.
Fig. 2. A: Expression of IMPDH type 2 mRNA in primary isolated rat HSC after culture activation for 3, 7 and 10 days. B: The effect of co-incubation of HSC with guanosine (1 mM) and MPA (1.5 µM) on cell proliferation. Note that the response of HSC to MPA is attenuated when exogenous guanosine is supplemented to the culture medium. * indicates P < 0.05 compared to control. Data represent the average +/- SD of 3 experiments, from 3 different HSC isolations.

In vitro activity of M6PHSA-MPA conjugates

In experiments with 3T3-fibroblasts, the conjugate in which MPA was linked to M6PHSA via an ester bond significantly inhibited BrdU-incorporation, whereas MPA that was conjugated via an amide bond to the drug carrier, displayed no pharmacological activity at all, in spite of the much higher amount of MPA that was coupled to the protein (Table 1). In subsequent experiments we therefore only tested the pharmacologically active M6PHSA-MPA conjugate in cultures of HSC, the ultimate target cells. Incubation of HSC with this conjugate, strongly reduced BrdU-incorporation, whereas M6PHSA alone was completely inactive (Fig. 3).
**Fig. 3.** The effect of M6PHSA (1 mg/ml) and M6PHSA-MPA (conjugation via ester bond, 1 mg/ml) on BrdU incorporation in cultures of primary isolated HSC (10 days in culture). Data represent the average of 3 independent experiments, from 3 different HSC isolations. * indicates P < 0.05 compared to control.

**Fig. 4.** The influence of competitors on the binding and uptake of $^{125}$I-labeled M6PHSA-MPA by primary isolated HSC (10 days in culture). Note that M6PHSA, a ligand for the M6P/IGF-II receptor reduces binding of the conjugate by $87 \pm 2\%$, whereas the control protein HSA, only reduces binding by $30 \pm 7\%$. * indicates P < 0.05. Data are the average of 3 independent experiments from 3 different HSC isolations.
Binding of M6PHSA-MPA to HSC in vitro

To verify that M6PHSA-MPA binds specifically to receptors on HSC, we performed binding studies on culture activated HSC with $^{125}$I-labeled M6PHSA-MPA. Binding to HSC was strongly inhibited by M6PHSA, an M6P/IGF-II receptor ligand, whereas the control protein HSA only had a minor effect (Fig 4).

In vivo distribution of M6PHSA-MPA

All in vivo experiments were performed with the conjugate in which MPA was coupled to M6PHSA via an ester bond.

Organ distribution

Ten minutes after i.v. administration of a tracer dose of $^{125}$I-labeled M6PHSA-MPA to animals with liver fibrosis, the conjugate distributed only to the liver. Thymus and spleen, which are organs with resident T and B lymphocytes, did only take up minor amounts of conjugate. Accumulation in other major organs like heart, kidney and lung was also very low (Fig. 5). In bone marrow no significant radioactivity was found (data not shown).

Fig. 5. Organ distribution of $^{125}$I-labeled M6PHSA-MPA, 10 minutes after i.v. injection (penal vein) in rats, 3 weeks after BDL (n=2).
**Intra-hepatic distribution**

Immunohistochemical staining for HSA within the liver showed a distribution to the non-parenchymal cells, 10 minutes after injection. Double-staining indicated co-localization of HSA with the HSC markers desmin/GFAP (Fig. 6A). However, double-staining for endothelial cells (EC) and HSA, and double staining for Kupffer cells (KC) and HSA also showed double-positive cells (Fig. 6B and 6C). Quantification of the particular staining patterns revealed an equal distribution to HSC, EC and KC (Fig. 6D). The intra-hepatic distribution in the multiple-dose and single-dose groups was similar.

*Fig. 6.* Co-localisation of M6PHSA-MPA with HSC (A), EC (B) and KC (C) in rat livers, demonstrated by immunohistochemical double-staining for the conjugate (α-HSA, red staining) and non-parenchymal cell types (KC, EC and HSC were identified with the antibodies ED2, RECA-1 and anti-desmin/GFAP respectively; blue staining) in 4 µm cryostat sections. Magnification 1000x. D: Quantification of the intra-hepatic cellular distribution of M6PHSA-MPA in the livers of fibrotic rats after a single dose and after multiple injections (n=3). HSC: hepatic stellate cell, EC: endothelial cell, KC: Kupffer cell and Hep: hepatocyte.
In vivo effects

To assess the effects of test compounds on the liver, we treated BDL animals for 7 days. We started dosing at day 3 after BDL because M6P/IGF-II receptor expression is being up regulated from this time point on (data not shown) and we stopped at day 10 when signs of fibrosis are evident but not excessive yet.

Fig. 7. Representative microphotographs of a DAB-staining for the amount of oxygen-free radical-producing cells in livers of bile duct ligated rats (n=5 per group) after daily i.v. injections with PBS (A), M6PHSA 4 mg/kg (B), M6PHSA 4 mg/kg combined with an equimolar amount of uncoupled MPA as present in bound form in the conjugate (C), and conjugate 4 mg/kg (D). Animals were injected from BDL day 3 until BDL day 10. Magnification 200x. E: Quantification of DAB staining by counting the number of DAB-positive cells per microscopic field (magnification 10 x 10). Per rat 6 fields were counted, each group consisted of 5 animals (average +/- SD). * indicates P < 0.05 compared to the PBS-treated group, # indicates p < 0.05 compared to both the M6PHSA-treated animals and the M6PHSA + uncoupled MPA-treated animals.
Fig. 8. A: Results of a morphometric analysis of liver sections stained for desmin/GFAP. The area of desmin/GFAP positive cells is expressed as a percentage of the total area. B: Results of the morphometric analysis of liver sections stained for collagen with picrosirius red. Data present the average of 5 animals per group +/- SD. For both stainings no significant differences were observed between the groups.

Fig. 9. Effect of drugs and control substances on the hepatic mRNA levels of α-β-Crystallin expression after daily i.v. injections (penal vein) with PBS, conjugate (4 mg/kg), M6PHSA (4 mg/kg) and M6PHSA (4 mg/kg) that was combined with an equimolar amount of uncoupled MPA compared to the amount present in the conjugate. Animals were treated from day 3 to day 10 after bile duct ligation (n=5 per group). As a reference α-β-Crystallin mRNA expression in healthy, non-fibrotic rats is showed. * indicates P < 0.05 compared to both the M6PHSA-treated animals and the M6PHSA + MPA-treated group.
Oxygen-free radical-producing cells

Treatment with carrier alone or with M6PHSA + unconjugated MPA elevated the number of DAB-positive cells in the liver, reflecting an increase in the number of oxygen-free radical-producing neutrophils and monocytes (Fig. 7). In contrast, the M6PHSA-MPA conjugate significantly lowered the amount of DAB-positive cells compared to these two groups. The dose of unconjugated MPA was equimolar to the amount of coupled MPA in conjugate-treated animals.

Extent of hepatic collagen deposition and amount of desmin/GFAP-positive cells

The extent of collagen deposition and the amount of desmin/GFAP-positive cells did not differ between the groups (Fig. 8). Because these late markers for the extent of liver fibrosis were not affected by either targeted or untargeted MPA, we assessed the influence of the various test substances on an early marker for hepatic stellate cell activation, i.e. α-β-Crystallin (21-23).

α-β-Crystallin mRNA levels

Fig. 9 shows the effect of treatment on α-β-Crystallin mRNA expression. When the conjugate-treated group was compared to the animals treated with M6PHSA + unconjugated MPA or the M6PHSA-treated animals, a significant decrease in expression of α-β-Crystallin mRNA levels could be seen. This indicates that this early marker for HSC activation is lowered by the targeted form of MPA only.

DISCUSSION

In the present study we investigated the antifibrogenic potential of mycophenolic acid and of a conjugate of mycophenolic acid coupled to the HSC-selective drug carrier M6PHSA. We report here, for the first time, that MPA also has antiproliferative effects in culture-activated rat HSC.

Since long-term administration of immunosuppressive drugs will cause problems in patients with liver fibrosis, we addressed the issue of avoiding adverse
effects by exploring the antifibrotic effects of MPA after coupling it to an HSC-selective drug carrier. We showed that, for effective targeting of MPA, a construct in which the drug is coupled via an ester bond to the carrier backbone has to be used, since a conjugate in which MPA was linked via a non-peptide-like amide bond exerted no effect \textit{in vitro}. Most likely this is due to the inability to degrade non-peptide like amide bonds within lysosomes of cells (24, 25).

Distribution studies \textit{in vivo} showed uptake of the conjugate selectively in the liver, whereas lymphocyte-containing organs were avoided. Within the liver, the conjugate accumulated in HSC, although uptake of the construct by EC and KC was also evident. Quantitative estimations of drug concentrations within target cells can not be easily done based on these immunohistochemical data, but it is highly likely that the drug concentration in HSC after administration of uncoupled drug, which distributes throughout the body, is only a fraction compared to the targeted situation, in which the drug accumulates within the liver, in only a limited part of the cells.

A possible reason for the uptake of conjugate in KC and EC may be the presence of protein polymers in the construct which can be taken up by KC. Another possibility is that the negatively charged protein backbone of the construct is recognized by scavenger receptors on KC and EC (26, 27). Yet, the delivery of MPA to these cell types is not expected to produce profibrotic or pro-inflammatory effects. In fact, MPA may exert anti-inflammatory and antifibrotic effects in these cell types. In human umbilical chord vein endothelial cells (HUVEC), MPA attenuated the expression of adhesion molecules, and incubation of monocytes and macrophages with MPA caused a reduced cell adhesion and a reduced cytokine and nitric oxide production by these cells (28, 29). Based on these findings, MPA delivery to EC and KC in the liver may even attenuate hepatic inflammation.

\textit{In vivo}, the targeted delivery of MPA clearly reduced inflammation compared to the group that was treated with untargeted MPA mixed with carrier. This effect may be explained by the actions of MPA in EC and KC as outlined above. Yet, an
active role of HSC in hepatic inflammation has also been suggested and the
delivery of MPA to this cell type may also contribute to the observed effects (30,
31). Although inflammation in rats treated with M6PHSA-MPA was reduced
compared to the group injected with carrier alone or the group that received carrier +
untargeted MPA, the number of activated neutrophils was still higher compared
to PBS-treated controls. This may be due to a pro-inflammatory effect of the carrier
counteracting the anti-inflammatory effect of targeted MPA. Possibly this is
effected by KC that take up a portion of the injected carrier protein (15). Both
lowering the amount of protein aggregates in the preparation and limiting the
overall negative charge of the conjugate may circumvent this KC uptake.

When studying the antifibrotic effects of targeted MPA we could not find a
significant effect on the amount of desmin/GFAP positive cells in the liver, which
was anticipated because of the antiproliferative properties of MPA.

Because recently it was reported that MPA reduced the activation of cultured
mesangial cells (12) we also evaluated our liver samples for the expression of an
early, sensitive and specific marker for HSC activation, α-β-Crystallin (21-23).
Data show that in contrast to untargeted MPA, an equimolar concentration of
targeted MPA reduced the expression of α-β-Crystallin in fibrotic animals,
reflecting reduced HSC activation.

In conclusion: MPA appears to be a potent inhibitor of HSC proliferation *in vitro*
and therefore may be a promising antifibrotic agent in liver fibrosis. We
showed that the targeted delivery of MPA to the HSC in the liver is possible, and
results in an anti-inflammatory effect and in signs of reduced HSC activation *in vivo*. Simultaneously, the uptake in extrahepatic tissues in which side effects of
MPA may occur is avoided. The mild pharmacological effect of targeted MPA
within the liver however stresses the need for additional structure-activity and
dose-dependency studies on this targeting construct. This includes investigations
on the targeting of even more potent drugs. Yet, the construct presented in this
HSC-selective targeting of mycophenolic acid

report may be the first of a generation of antifibrotic drug targeting preparations that accumulates in HSC.

ACKNOWLEDGEMENTS

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REFERENCE LIST


