Aiming at the cirrhotic liver

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Summary, conclusions and perspectives

In this thesis, we have explored the concept of drug targeting for the treatment of liver cirrhosis. This fatal disease is currently among the top ten causes of death in the Western World and can not be treated properly with drugs. The only effective treatment to date is a liver transplantation. The lack of donor organs and the complexity of the surgical procedures, however, prevent widespread use of this treatment. Pharmacological intervention has failed up until now because of insufficient uptake of drugs in the target cells or extrahepatic side-effects, or a combination of both. Organ or cell-specific targeting of drugs may circumvent these problems.

Liver cirrhosis is a disorder that is characterized by the deposition of large amounts of extracellular matrix components like collagens (1). The fibrotic process leading to liver cirrhosis is induced by the concerted action of many cell types and is regulated by many mediators. Inciting stimuli like viruses (hepatitis B, C), toxins (alcohol, drugs), bile obstruction or metabolic disorders may damage hepatocytes and cause the activation of other resident hepatic cells like Kupffer cells, sinusoidal endothelial cells, and hepatic stellate cells, or infiltrating inflammatory cells. Chronic activation of this inflammatory process leads to an irreversible accumulation of extracellular matrix. In particular, the sinusoidal endothelial and Kupffer cells play an important role in continuing the inflammatory process by releasing a cascade of

inflammatory mediators: prostaglandins, leukotrienes, cytokines, chemokines, and oxygen radicals.

Pharmacological intervention with several anti-inflammatory drugs has only met with limited success. Some of the drugs (corticosteroids) were able to slow down the disease progress in some cases (autoimmune hepatitis, primary biliary cirrhosis), but their use was hampered by the severe side-effects (2,3). By targeting anti-inflammatory drugs to endothelial and Kupffer cells of the liver the efficacy of these drugs may be increased while avoiding the side-effects elsewhere in the body.

For the drug targeting studies described in this thesis we used of two kinds of anti-inflammatory drugs, naproxen and dexamethasone. Naproxen is a nonsteroidal anti-inflammatory drug, related to aspirin. It inhibits the enzyme cyclo-oxygenase which is involved in the synthesis of prostaglandins. The side-effects of naproxen are usually mild, but during cirrhosis it can cause serious problems in the kidney. Its pharmacological potential, however, is also limited, because it only inhibits the production of prostaglandins. Dexamethasone is a steroidal anti-inflammatory drug derived from the hormone cortisol. This corticosteroid is a very potent anti-inflammatory drug because it inhibits the production of many inflammatory mediators. These include cytokines, chemokines, prostaglandins, leukotrienes, and oxygen radicals. This effectiveness, however, coincides with serious side-effects, especially during long-term use. Dexamethasone, for instance, can cause osteoporosis, an increased susceptibility to infections, and diabetes. To avoid these problems we aimed at the targeting of naproxen and dexamethasone to the relevant cell types.

In the studies presented in this thesis, we succeeded in delivering naproxen and dexamethasone to endothelial and Kupffer cells using human serum albumin (HSA) and its mannosylated derivative. Endothelial and Kupffer cells have scavenger receptors that remove negatively charged proteins and proteins with increased hydrophobicity from the circulation. For naproxen it had already been shown that coupling of naproxen to HSA changes HSA into a substrate for these scavenger receptors (4). The coupling of naproxen to positively charged amino groups in the HSA-molecule increases the relative negative charge. In addition, the naproxen groups add extra hydrophobicity to the molecule. Likewise, we anticipated that a similar coupling of dexamethasone to HSA would cause uptake by the same scavenger receptors. We therefore also synthesised a conjugate of dexamethasone coupled to HSA.

To study the effects of targeting of dexamethasone specifically to Kupffer cells we developed another drug targeting preparation using mannosylated HSA. Previous studies have shown that Man_{10}-HSA (10 molecules of the sugar mannose coupled to

1 molecule of HSA) is specifically taken up by the mannose receptors on Kupffer cells (5). Dexamethasone was therefore coupled to this neoglycoprotein to achieve specific targeting to Kupffer cells.

As mentioned before, previous studies showed that naproxen coupled to HSA (Nap_{23}-HSA) is taken up specifically by endothelial and Kupffer cells. After cellular degradation, active naproxen was shown to be released. However, this active naproxen was not the original naproxen, but naproxen-lysine. This degradation product fortunately had the same activity profile as naproxen. **Chapter 3** describes a follow-up study with Nap_{23}-HSA. This study was conducted to gain insight in the pharmacokinetic behaviour of coupled naproxen. This information is needed for the practical application of drug targeting preparations. Coupling of naproxen to HSA distinctly altered the kinetic properties of naproxen. It was removed from the blood stream at a much higher rate than uncoupled naproxen ($t_{½}$: 60±11 min vs. 152±44 min). Liver targeting of Nap_{23}-HSA was clearly demonstrated: 180 min after injection, the naproxen content in the liver was 30 times higher for a dose Nap_{23}-HSA as compared to an equimolar dose of free naproxen. The elimination of naproxen from the body had also changed dramatically after coupling to HSA. Free naproxen is mainly excreted by the kidneys (95%). HSA-coupled naproxen, however, was predominantly excreted in bile as naproxen-lysine. The biliary appearance of Nap-lysine, observed after injection of the HSA-conjugate, had a considerable lag time (20 min). Injected naproxen-lysine, on the other hand, appeared almost immediately in bile. This difference of 20 min probably reflects the time necessary for endocytosis, proteolytic degradation and transportation of naproxen-lysine through the hepatocytes to the bile. The concentration of active naproxen in the target cells is not exclusively dependent on the rate of uptake in the cells. This concentration is also largely dependent on the degradation rate of the conjugate, the liberation of naproxen-lysine, and its rate of elimination.

In vivo studies, in collaboration with the group of Prof. J. Reichen in Bern, showed a clear protective effect of Nap_{23}-HSA during acute intrahepatic inflammation (6). However, the chronic hepatic inflammation underlying liver fibrosis, would probably benefit more from potent anti-inflammatory drugs like dexamethasone, with a long biological half life. We therefore decided to concentrate our efforts on the targeting of dexamethasone. This drug was coupled to HSA and Man_{10}-HSA with a succinate spacer linking dexamethasone to the protein. The spacer ensured the release of native dexamethasone after degradation of the protein. We developed two conjugates of dexamethasone that we used in further studies: $Dexa_{10}$-HSA (**chapters 4, 5, 7 and 8**) and $Dexa_5$-Man_{10}-HSA (**chapter 6**).

In **chapters 4 and 6** the tissue and intrahepatic distributions of both conjugates

were studied in healthy rats and rats with liver cirrhosis. Cirrhosis was induced by ligation of the bile duct (BDL), which causes a quick and reproducible type of fibrosis of the liver. Ten minutes after injection of either dexamethasone conjugate, about 70% of the dose had been taken up by the liver. No differences were found between healthy rats and BDL-rats. $Dexa_{10}$-HSA distributed to both endothelial and Kupffer cells, whereas $Dexa_5$-Man_{10}-HSA had mostly been taken up by Kupffer cells. Uncoupled dexamethasone was also taken up by the liver. Most of this uptake, however, was found in the hepatocytes, the cells responsible for the elimination of dexamethasone from the body.

The release of *active* dexamethasone from the proteins was tested in an *in vitro* system with precision-cut liver slices. In liver slices the cells are unaffected by isolation and culture procedures and still have their normal cell-cell contacts (7). The effect of anti-inflammatory preparations can be studied by the inhibition of mediator release after activation of resident cells with lipopolysaccharide (LPS). Both conjugates inhibited the release of the inflammatory mediator tumor necrosis factor alpha (TNFα) after LPS-incubation more effectively than free dexamethasone. This indicates that active dexamethasone is indeed released within the target cells.

The release of active dexamethasone was confirmed with another type of experiment in **chapter 5**. Kupffer cells from untreated rats or rats that had received $Dexa_{10}$-HSA were isolated and subsequently incubated with LPS. The Kupffer cells of the untreated rats released TNFα after LPS stimulation, whereas the Kupffer cells of the $Dexa_{10}$-HSA-treated rats did not respond to LPS-stimulation.

In addition to these *in vitro* studies, we also demonstrated the release of active dexamethasone in BDL-rats *in vivo* (**chapter 4**). These rats were found to be very sensitive to LPS. A small dose of this toxin caused the release of lethal amounts of inflammatory mediators. BDL-rats treated with 10 $mg.kg^{-1}$ $Dexa_{10}$-HSA, however, survived the dose of LPS up to the end of the experiment. This, again, indicates the release of active dexamethasone.

A study investigating the kinetic properties of $Dexa_{10}$-HSA is described in **chapter 5**. At a dose below receptor saturation (3 $mg.kg^{-1}$) a rapid removal of the conjugate from the blood was found in both healthy and cirrhotic rats ($t_{½}$: 6.1±1.6 min vs. 6.6±1.5 min respectively). None of the determined kinetic parameters ($t_{½}$, clearance, volume of distribution) were significantly different between healthy and cirrhotic rats. The involvement of the scavenger receptors in the uptake of $Dexa_{10}$-HSA was demonstrated by its reduced uptake in rats pretreated with polyinosinic acid, an inhibitor of scavenger receptors. The kinetic profile of $Dexa_5$-Man_{10}-HSA and the receptor type involved in its uptake have not been studied yet, due to the technical problems encountered in attempts to analyse this compound in blood.

Since we found uptake of our dexamethasone conjugates and release of active dexamethasone in the target cells, we continued with testing these preparations in rats with liver fibrosis. The biological activity of dexamethasone has a very long half life (36-72 hours), we therefore decided to treat BDL rats three times a week with 10 $mg.kg^{-1}$ $Dexa_{10}$-HSA (**chapter 5**). This dose had previously been shown to protect BDL-rats against LPS-induced toxicity. During BDL-induced liver fibrosis, however, 10 $mg.kg^{-1}$ $Dexa_{10}$-HSA induced weight loss and caused the animals to die within a week. Therefore the dose was reduced hundred-fold to 0.1 $mg.kg^{-1}$ $Dexa_{10}$-HSA and the experiment was repeated. This time we also treated rats with an equimolar amount of the $Dexa_5$-Man_{10}-HSA conjugate, which amounted to 0.2 $mg.kg^{-1}$ (**chapter 6**).

In these experiments we did not find signs of overdosing in the rats treated with either preparation. $Dexa_{10}$-HSA and $Dexa_5$-Man_{10}-HSA did clearly show effects on the extent of intrahepatic inflammation. Intrahepatic inflammation was determined as the number of cells producing reactive oxygen species (ROS). Activated Kupffer cells produce ROS and release chemokines to attract neutrophils that also produce ROS. Dexamethasone has been shown by others to inhibit both ROS-and chemokine production. The livers of BDL-rats treated with the conjugates contained less ROS-producing cells as compared to livers of untreated BDL-rats or BDL-rats treated with uncoupled dexamethasone. However, the reduction of the hepatic inflammation did not have the desired effect on the fibrotic process in these livers. The livers of the rats treated with $Dexa_{10}$-HSA and $Dexa_5$-Man_{10}-HSA were found to contain even *more* collagen when compared to the livers of untreated rats. For $Dexa_5$-Man_{10}-HSA we tried to establish the mechanism behind this increase (**chapter 6**). The collagen-increasing effect of dexamethasone was not likely to be related to an increased production of transforming growth factor beta (TGFβ), the major profibrotic growth factor. Nor was it likely to be related to an decreased production of interleukin-10, a very important anti-inflammatory cytokine. Dexamethasone, however, was found to stimulate the mRNA expression of tissue inhibitor of metalloproteinase-1 (TIMP-1). This factor, also produced by Kupffer cells, inhibits the enzyme collagenase, which degrades collagens. During the fibrotic process the production of collagen is already favoured over the degradation. The increase in TIMP-1 seen after treatment with targeted dexamethasone can shift the balance towards excessive collagen production even more.

A further study in which BDL-rats were treated with even lower amounts of $Dexa_{10}$-HSA (0.02 and 0.01 $mg.kg^{-1}$) is described in **chapter 5**. These doses did not have a demonstrable effect on either hepatic inflammation or collagen deposition. The amount of dexamethasone in the target cells apparently did not reach levels for

significant biological activity in these cases.
An unexpected finding was the fact that the carrier Man_{10}-HSA (without dexamethasone coupled to it) also affected hepatic inflammation and fibrosis. The livers of BDL-rats treated with this carrier showed a dramatic increase in hepatic inflammation and collagen deposition. To our knowledge, the intrinsic effect of this neoglycoprotein has not been reported before. In **chapter 6** we speculate that this observation may be related to the disease model we have been using. Only Kupffer cells in *diseased* livers express the receptor for LPS, the CD14 receptor (8). Upon ingestion of mannose-containing macromolecules, the mannose receptor has been reported to activate cells in conjunction with the CD14 receptor (9). This may explain the increase in ROS-production found for Man_{10}-HSA and may be indirectly responsible for the increased collagen deposition. Studies examining the use of this type of drug targeting preparation in nondiseased livers would therefore not reveal this effect.

A potential problem with the use of protein-based carriers may be their antigenicity. Especially in the case of targeting to sinusoidal endothelial and Kupffer cells that have been reported to be antigen-presenting cells (10), thus increasing the risk of immunogenic responses. Rats treated once a week for three months with $Dexa_{10}$-HSA (**chapter 5**) or $Dexa_5$-Man_{10}-HSA (unpublished observation) indeed exhibited high antibody titers after one month. Treatment for more than a month, however, caused a secondary decrease in antibody titers suggesting tolerance induction.

Since treating cirrhosis in humans is the ultimate goal of our efforts, we also investigated distribution and efficacy of $Dexa_{10}$-HSA in human liver tissue (**chapters 7 and 8**). Studies in experimental animals may not adequately predict the behaviour of drugs in humans. Testing in humans is therefore essential, but unfortunately such experiments are often not possible. We used healthy as well as cirrhotic human liver tissue to study the uptake of $Dexa_{10}$-HSA in the human condition. As found in the rat studies, scavenger receptors were responsible for the uptake of $Dexa_{10}$-HSA in human endothelial and Kupffer cells. In healthy human liver tissue both cell types were found to have taken up the conjugate. In cirrhotic human liver tissue, however, only Kupffer cells were responsible for the uptake. This difference with the situation in rat livers awaits further clarification yet. The release of active dexamethasone in the target cells was demonstrated in slices of human livers incubated with LPS and $Dexa_{10}$-HSA. Slices incubated with only LPS upregulated the mRNA expression of inducible NO synthetase (iNOS), the enzyme responsible for the production of NO radicals. Coincubation with $Dexa_{10}$-HSA completely abrogated the upregulation of iNOS. Dexamethasone has been reported to inhibit iNOS expression, indicating that

active dexamethasone is released within the slice. These results all suggest that the handling of $Dexa_{10}$-HSA in human livers is probably not very different from the handling in rat livers.

Conclusions

The studies described in this thesis represent the entire process of developing drug targeting preparations: from the synthesis of such preparations to their testing in diseased animals and human tissue (both healthy and diseased). Drug targeting has often been proposed as the solution to problems of low efficacy of drugs and side-effects caused elsewhere in the body. However, these aspects are seldom tested in appropriate disease-models. In the present studies, we therefore tested the concept of drug targeting as a possible treatment for liver cirrhosis. Fibrosis of the liver, leading to liver cirrhosis, is a multifactorial process, in which both endothelial and Kupffer cells have been identified as key players in the inflammation underlying liver fibrosis. To target anti-inflammatory drugs to these cell types, we prepared conjugates of naproxen and dexamethasone with albumin. Using these preparations we were able to selectively deliver *active* naproxen and dexamethasone to endothelial and Kupffer cells in rats. For dexamethasone we could confirm this in human liver tissue as well.

Treatment of rats with bile duct ligation-induced liver fibrosis with the dexamethasone conjugates was studied in further detail. Both $Dexa_{10}$-HSA and $Dexa_5$-Man_{10}-HSA were able to inhibit intrahepatic inflammation associated with liver fibrosis. They failed, however, to reduce the collagen deposition within the liver. We even found an increase in rats treated with the dexamethasone conjugates most likely caused by stimulation of TIMP-1 expression by dexamethasone. The selective cellular delivery of this corticosteroid with its broad activity profile may therefore not provide sufficient pharmacological selectivity to shift the overall cytokine/autocoid levels to an antifibrotic state.

These results, however, do reveal one other interesting aspect of drug targeting: specific cellular intervention strategies in a disease like liver fibrosis can shed light on the role of the particular cell types in the pathogenesis. Our results indicate that in liver fibrosis the endothelial and Kupffer cells may be responsible for continuing hepatic inflammation, but they also appear to have an antifbrotic role.

The immunogenicity of these drug targeting preparations does not appear to be a major problem. Multiple injections over a prolonged period of time only led to a transient rise in antibody titers, suggesting tolerance induction.