Epithelial heparin delivery via microspheres mitigates experimental colitis in mice

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IBD inflammatory bowel disease, MS microspheres, TNBS 2,4,6-trinitrobenzenesulfonic acid, OXA oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one), MPO myeloperoxidase, PVA polyvinyl alcohol, LMWH low molecular weight heparin
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Abstract

Low molecular weight heparins (LMWH) have been shown to be efficient in the treatment of inflammatory bowel disease (IBD). Parenteral heparin therapy, however, may cause hemorrhagic adverse effects. In order to reduce this risk, epithelial LMWH delivery in combination with a system ensuring selective drug release to the inflamed tissue was tested here. Enoxaparin loaded microspheres (MS) were administered orally to male Balb mice suffering from a preexisting experimental colitis while control groups received subcutaneous or rectal LMWH solution. Colon weight/length index, alkaline phosphatase, and myeloperoxidase activity were assessed to determine the inflammation. Tissue penetration experiments elucidated the processes involved in the proposed new therapeutic approach. Oral LMWH-MS proved to be equally efficient in mitigating experimental colitis as rectally administered LMWH solution when quantified by myeloperoxidase activity (MS: 10.2±1.5U/mg tissue; rectal: 9.2±1.6U/mg) and to be superior to subcutaneous LMWH (sc: 21.6±5.6U/mg; untreated colitis control: 30.0±3.8U/mg). Pharmacokinetic studies found a notably low systemic availability of oral LMWH delivered from MS (<5%) indicating a low potential for adverse effects. The tissue permeability was selectively enhanced in the inflamed regions where a 9-fold higher LMWH penetration was found compared to healthy tissue. Epithelial LMWH delivery has been found a promising anti-inflammatory therapeutic approach. The use of LMWH-MS in this context offers a promising tool for IBD therapy by enhancing specifically drug availability at inflamed tissue sites while reducing the risk for systemic adverse effects to a negligibly low level.
Introduction

The general principle of pharmacological treatment for inflammatory bowel disease (IBD) is to induce remission of outbreaks and to prevent outbreaks during remission. With this goal in mind, a wide range of anti-inflammatory pharmaceutical products have been commercialized mainly for oral administration with modified delivery profiles in the gastrointestinal tract (Lamprecht et al., 2002; Hanauer and Present, 2003; Podolsky, 2002). In many cases, pharmacotherapy for IBD consists of life-long administration of one or more of aforementioned drugs. Therefore, quality and severity of adverse effects of these therapeutic regimens is an essential issue to address. Consequently, innovative drug delivery strategies have been designed for more selective delivery of drug to sites of inflamed tissue meanwhile reducing the risk for systemic adverse effects (Lamprecht et al., 2002). State of the art drug release strategies such as enzymatically degradable carriers rely on enzymatic activity of colonic bacteria similar to the mechanism of prodrugs. Some drug delivery systems perform time-dependent drug release. Others, among them most of currently commercialized systems, are based on the change of luminal pH during gastrointestinal passage (Lamprecht et al., 2002). Since these systems are known to exhibit a lack of specificity in terms of drug release, newer strategies were developed in order to increase selectivity of drug deposition towards inflamed colon tissue. Microcarriers were proposed to minimize drug loss related to accelerated carrier elimination by diarrhea associated with IBD (Nakase et al., 2000; Lamprecht et al., 2005a). Systems that are even smaller, e.g. liposomes and nanoparticles have shown significant improvements (Lamprecht et al., 2001; Lamprecht et al., 2005b; Kesisoglou et al., 2005). The limiting factor in most of abovementioned strategies is premature loss of encapsulated drug during passage of upper parts of the intestine. This compromises smaller carrier systems’ advantage based on their ability to mitigate the effect of diarrhea on drug loss (Hardy et al., 1988; Watts et al., 1992). Since this partial drug loss is an unavoidable phenomenon related to the principal physicochemical properties of such microsystems, a closer look at the drug’s distinct properties appears warranted also in order to find ways to limit undesired early drug absorption and subsequent systemic drug availability. This was partially achieved by use of several budesonide...
formulations reducing systemic availability by drug’s mucosal metabolism (Klotz and Schwab, 2005). However, the number of such drugs is rather limited.

Besides its anticoagulant property, heparin was recently found to possess anti-inflammatory properties and to be effective in the treatment of ulcerative colitis after subcutaneous administration (Törkvist et al., 1999; Dotan et al., 2001). Although mechanisms of action are not completely clear, its clinical application in IBD appears to be interesting. On the other hand, long term administration of heparin increases the risk for hemorrhagic events (Papa et al., 2000). This issue has prevented heparin from becoming a standard therapeutic adjunct in the pharmacotherapy of IBD. Surprisingly, the therapeutic efficiency of heparins delivered locally to areas of inflammation for epithelial uptake is completely unknown. Thus, a local and defined epithelial delivery of heparin appears to be of high interest in order to reduce systemic drug availability and, hence, lower the risk for adverse effects. This is especially of interest since heparins show a minimal tendency to cross the intact intestinal mucosa which would further reduce undesired side effects.

In this study, the therapeutic efficiency of low molecular weight heparin (LMWH) in IBD treatment delivered by epithelial route was analyzed. Therefore, LMWH loaded pH-sensitive microcarriers were developed in order to ensure a selective delivery of the drug towards areas of inflammation in the colon. The mitigating potential of epithelial LMWH in IBD was evaluated in two different colitis models in mice. This study focused specifically on the comparative analysis of the new microsphere (MS) carrier with control groups receiving LMWH as a solution either by rectal or subcutaneous route.

**Materials and methods**

**Materials**

Eudragit P-4135F was a kind gift from Röhm Pharma Polymers (Tokyo, Japan; for details see Lehmann and Höss, 2001). For LMWH, enoxaparin sodium (Lovenox® 10.000 UI anti-Xa / 1ml) was purchased from Sanofi-Aventis (Paris, France). Polyvinyl alcohol (PVA), sorbitan monostearate
(Span® 60), 2,4,6-trinitrobenzene sulphonic acid (TNBS), and oxazolone (OXA) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals were of analytical grade.

Methods

Microparticle preparation and characterization

MS were prepared by a water-in-oil-in-water-emulsion (w/o/w) technique. Briefly, 200 mg of Eudragit P-4135F were dissolved in 3 ml dichlormethane containing 28 mg sorbitan monostearate. Subsequently, 300 µl aqueous LMWH (=3000IU) were emulsified in the polymer solution by ultrasonication for 15 seconds. This primary water-in-oil (w/o)-emulsion was then poured into 75 ml aqueous PVA-solution (0.5%) to form a water-in-oil-in water (w/o/w)-emulsion. This emulsion was stirred for an hour with a three-blade propeller at 500 rounds per minute (rpm) at room temperature until the organic solvent of the internal phase was entirely removed inducing polymer precipitation creating solid MS with encapsulated LMWH. After the formulation, MS were filtrated (Millipore®, HA, 0.45 µm), washed extensively with deionized water and dried at room temperature.

MS were analyzed for their size distribution by laser light diffraction (Mastersizer®, Malvern Instruments, UK). For scanning electron microscopy (SEM), the particles were fixed on supports with carbon-glue, coated with gold using a gold sputter module in a high-vacuum evaporator, and then observed with the scanning electron microscope (JEOL JSM-T330A scanning microscope, Tokyo, Japan) at 15 or 20kV. The drug content was determined by nephelometry measuring directly the amount of LMWH entrapped in MS (Meissner et al., 2006). The encapsulation rate was expressed as the percentage of LMWH encapsulated with respect to the theoretical value.

In-vitro drug release was initiated in a buffer system at pH 1.2. After 2 and 4 hours pHs were adapted to values of 6.8 and 7.4, respectively. Drug loaded MS were suspended under magnetic stirring in 20ml phosphate buffer of pH 1.2 at 37°C. Aliquots of the dissolution medium (1ml) were withdrawn at predetermined time intervals and analyzed by nephelometry. All experiments were performed in triplicate.

Animal experiments
All animal experiments were carried out in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, US). The animals were kept under standard laboratory conditions with drinking water and food provided ad libitum if not specified otherwise.

**Bioavailability studies**

Low molecular weight heparin loaded MS were administered to male New Zealand rabbits to study bioavailability of LMWH (since bioavailability studies on mice are hardly feasible under these conditions). The rabbits with a mean body weight of 3020 ± 136 g were fasted overnight but had free access to water. MS were filled into gelatin hard capsules and administered orally (600 IU/kg). Experiments started at 8am. Solutions of LMWH administered subcutaneously or orally were used for control. In each case, blood samples (1.5 ml) were withdrawn from the marginal ear vein at predetermined times (2, 4, 6, 8, 10, 12 hours after administration) and gently mixed with sodium citrate (70 µl) before centrifugation for 10 min at 4000 rpm. The obtained plasma samples were stored at -20°C before they were assayed for the anti-Xa activity with a standard kit (Stachrom heparin, Diagnostica Stago, France).

**Colitis models**

The TNBS and OXA mice models were chosen as well recognized experimental models (Neurath et al., 1995; Heller et al., 2002) that allow induction of colitis at an exact location. This permitted rectal administration of LMWH in form of an exclusively local delivery to the inflamed tissue. Male BALB/c mice (average weight 25g, n=6/group) were used for the inflammation models. In the first model colitis, inflammation was induced by TNBS after the following procedure: animals were catheterized 4 cm intrarectally after light narcotizing with ether. One hundred µl of TNBS in ethanol were applied in a dose of 160 mg/kg body weight of TNBS in ethanol (50% solution). The mice were housed for a day without treatment to attain a complete IBD model. The OXA model was set up as follows: The mice were immunized with an ethanol solution containing 3% OXA topically applied to the skin. The challenge was performed a week later by rectal administration of 10 mg of haptenating
agent; 3% OXA solution was administered per rectum in a total volume of 100 µl of an ethanol/water mixture.

During the treatment period, all animals received either 0.1 ml of LMWH solution or LMWH-MS suspension once daily for six consecutive days at a LMWH dose of 600 and 2000 IU/kg body weight. Administrations were performed daily at 11am. Doses were selected according to precedent studies (Dotan et al., 2001). While aqueous LMWH solution was administered by subcutaneous, oral or rectal route (all 600IU/kg), MS suspended in carboxymethylcellulose (0.5 %) were administered orally. Oral administration was performed by gavage while rectal administration consisted of 4cm intrarectal catheterization delivering LMWH directly to the site of inflammation. The control groups received saline only (colitis control) or blank MS. The mice were treated once daily for six consecutive days. The animals were sacrificed 24 h after the last drug/particle administration and their colons were resected.

Pathophysiological parameters

Colitis activity was quantified with a clinical score assessing weight loss, stool consistency, and rectal bleeding as previously described elsewhere (Hartmann et al., 2000).

Resected colon tissue samples were opened longitudinally and rinsed with iced phosphate buffer to remove luminal content. Then tissue wet weight and colon length were determined and expressed as colon weight/length quotient. Histological assessment was carried out by light microscopy of colon tissue samples. The degree of inflammation was graded using the criteria described previously (Lamprecht et al., 2005a). The score represented the sum of eight individual variables graded 0-3 depending upon the severity of the changes (0 no=change; 1=mild; 2=moderate; 3=severe). The variables evaluated were: erosion, ulceration, necrosis, hemorrhage, edema, and inflammatory cell infiltration.

For the determination of alkaline phosphatase (AP) activity, tissue samples were weighed and homogenized in cold saline solution. After centrifugation, the homogenates were assayed for protein content. AP activity was determined spectrophotometrically using disodium p-nitrophenylphosphate as
substrate (Bessey et al., 1946). One unit of AP activity was defined as that degrading 1 µmol/min of substrate at 25°C. The results are expressed as AP units per mg of protein.

The measurement of myeloperoxidase activity (MPO) was performed to quantify the severity of the colitis. It is a reliable index of severity of inflammation caused by infiltration of activated neutrophils into inflamed tissue. Enzymatic activity was analyzed according to a standard method (Krawisz et al., 1984).

Tissue penetration

Control tissue samples were taken from the healthy group. Inflamed or non-inflamed tissue samples were taken from the colitis group where non-inflamed tissue was resected from areas without macroscopic damages with a distance of approximately 3cm from sites of major inflammation. The resected mouse tissue samples were washed with ice-cold phosphate buffer (pH7.4) and full thickness specimens were mounted in modified Ussing chambers. Both chambers were filled with Dulbecco’s modified eagle medium and kept under carbogen bubbles at 37° by water jackets. Tissues were preincubated for 15 min before the samples were added into the apical compartment and incubated for 30 min (at final carboxyfluorescein and LMWH-fluoresceinamine concentrations of 1µM). The LMWH labeling protocol was adapted to a method described earlier (Lamprecht et al., 2006).

Statistical analysis

The results were expressed as mean values ± S.D. For the analysis of statistical significance ANOVA on ranks was applied followed by Dunn’s test for all pair wise comparison. In all cases, P < 0.05 was considered to be significant.

Results

in-vitro characteristics of microspheres
LMWH-MS were spherical with a particle diameter below 150µm and a relatively rough surface (Fig. 1). The polymer matrix trapped an internal aqueous phase containing the LMWH with the surrounding polymeric matrix material resulting in a sponge-like structure. Further MS characteristics such as particle size, LMWH encapsulation efficiency, and drug load are shown in Table 1. In general, in-vitro drug release occurred with strong dependency on the pH of the respective buffer system, in which the MS were suspended (Fig. 2). LMWH was retained efficiently inside MS when tested at pH 1.2 and 6.8 where at least 80% of the initial drug load was still present inside the MS after 4h of incubation. On the contrary, a comparatively fast release was observed at pH 7.4, which delivered nearly 100% of the incorporated drug within 30 min.

**Therapeutic efficiency**

For purposes of testing the therapeutic concept in a preexisting experimental colitis in rats, LMWH formulations were administered subcutaneously as comparative standard, orally as the new MS formulation or rectally as solution in order to deliver a maximum concentration of LMWH to the site of inflammation. LMWH-MS were administrated in two different concentrations (600 and 2000 IU/kg) to detect potential dose-dependent effects.

In the TNBS model, only LMWH-MS lowered clinical activity after a lag time of 24 to 48 h and maintained this effect during the whole treatment period while the two solution receiving groups exhibited a continuous high level (Fig. 3A). Control experiments with blank MS or oral LMWH solution were not significantly different from untreated colitis control (data not shown). The difference between LMWH-MS (600IU/kg) and colitis controls became significant on day 5 while for the other treated groups statistically significant differences were not observed. Principally, in line with observations from clinical activity, LMWH treated groups showed decreased values in the colon weight/length ratio in comparison to the untreated colitis control group (Fig. 4A). Although rectal LMWH administration showed a higher mitigating effect than in clinical activity scores, only levels after the LMWH-MS treatment were found to be significantly lower than the colitis control. Histological sections of the colon demonstrated significant influences by the various treatments (Fig. 5). With LMWH-MS treatment, mucosal and submucosal tissue was found partially intact, whereas in
colitis control a complete disintegration of the mucosa occurred. Also, swelling of the submucosa was reduced in LMWH-treated groups although not reaching the level of healthy control. Histological damage score again revealed higher treatment efficiency for rectal LMWH and LMWH-MS compared to subcutaneous LMWH where results were not statistically different from untreated colitis controls (Fig. 4C). Aside from that, MPO activity in samples from inflamed colonic tissue demonstrated similar therapeutic effects for LMWH-MS and rectal LMWH treated groups but a significantly lower efficiency of subcutaneous LMWH solution (Fig. 6A). The tissue concentrations of alkaline phosphatase were in line with MPO activity exhibiting significant mitigating effects for oral and rectal LMWH formulations (Fig. 7A). TNF-alpha values were determined inside the tissue samples of both colitis models, however, changes were not statistically significant (data not shown).

Slightly different tendencies for clinical activity score were found in the OXA model (Fig. 3B). Here, rectal administration of LMWH provided the strongest reduction of clinical activity. It must be noted that differences between all non-parenteral groups were not statistically significant. Generally, the administration of LMWH was noted to be more efficient for the OXA model compared to the TNBS model with marginally better results for LMWH-MS. Although the observed effect after oral and rectal administration of LMWH was similar to the TNBS model, a faster response to treatment was found in the OXA model (different from untreated control, rectal: day 4, LMWH-MS 2000IU/kg: day 5; P < 0.05). Colon weight/length ratios as well as histological damage scores were decreased in all LMWH groups. However, differences were only significant for LMWH-MS as well as for histological damage score after rectal LMWH administration (Fig. 4B+D). Oppositely, after rectal or subcutaneous administration of LMWH, significant differences were observed in terms of MPO activity (Fig. 6B). Similarly to observations made regarding clinical activity, rectal administration of LMWH solution mitigated colitis to a greater extent reaching levels of LMWH-MS at values near complete remission. Again, consistent with results of MPO activity, alkaline phosphatase activity was reduced for all LMWH treatments (Fig. 7B).

Systemic LMWH availability after administration of MS formulations was compared to that of subcutaneously administered LMWH solution, which was regarded as 100% value of bioavailability. Relative bioavailability of LMWH-MS at 600IU/kg was overall less than 3% in healthy animals and
less than 5% in animals suffering from TNBS colitis (Table 2). Oral delivery of LMWH solutions led to non-detectable drug absorption with consequent lack of bioavailability (data not shown). Ussing chamber studies allowed an insight into drug penetration for the different tissue samples and subsequent changes depending on the disease state. In this context, carboxyfluorescein was selected as a model compound for low molecular weight drugs. LMWH was fluorescently labeled prior to all experiments. Tissue penetration of carboxyfluorescein in colitis tissue was significantly greater than for healthy controls (Fig. 8). LMWH tissue penetration was less than that of carboxyfluorescein. Similarly to observations made for carboxyfluorescein, drug penetration into inflamed tissue was significantly greater compared to tissue from healthy control animals. It was also slightly increased in non-inflamed tissue surrounding inflamed regions. In comparison of healthy versus colitis tissue in terms of penetration, differences were immensely increased for LMWH (a 9-fold increase). This finding underlines the notion of LMWH tendency for selective penetration into inflamed tissue.

Discussion

LMWH demonstrated to be a potent approach in the treatment of IBD in animal studies as well as in clinical trials (Törkvist et al., 1999; Dotan et al., 2001). However, long-term routine administration of LMWH increases the risk for hemorrhagic events (Papa et al., 2000) requiring a modification of this early approach to treatment. A selective and local delivery of LMWH could reduce systemic availability of the drug potentially lowering the risk for adverse effects. Thus, the oral administration pathway might be suggested, however, demanding a significant technological progress in drug formulation science in order to avoid LMWH loss during its passage through the upper intestinal tract. Still nowadays, standard drug delivery systems release anti-inflammatory drug non-specifically to the colonic epithelium, irregardless from healthy or inflamed state. Therefore, with regards to drug delivery, the optimized strategy would be to combine a higher degree of specificity of drug tissue penetration with an increased selectivity of drug release towards inflamed tissue. In this context, heparin and its derivatives are especially interesting considering their minimal tendency to cross the
intact intestinal barrier related to their macromolecular structure. This in turn leads to a very low oral bioavailability in healthy subjects (Hoffart et al., 2006; Lamprecht et al., 2006).

The study determining therapeutic efficiency of LMWH was performed in the TNBS and OXA colitis models in mice in order to analyze therapeutic efficiency on models resembling Crohn’s disease (TNBS) or ulcerative colitis (OXA) in humans, since therapeutic efficiency can vary significantly in both diseases. Despite the fact that the relationship of the TNBS model to human disease is imperfect (Fiocchi, 1998; Neurath et al., 1995), it displays several Crohn’s disease resembling features. Most notably comparable is full-thickness transmural mononuclear inflammation driven by Th1- stimulated secretion of IL-2, IL-4 and TNF-alpha. OXA colitis is a mucosal model of colitis as an IL-4 driven Th2 inflammation with histologic similarities to ulcerative colitis (Boirivant et al., 1998). It remains relatively superficial at the microscopic level affecting mainly the lamina propria of gastrointestinal lumen tissue.

The results of the two models exhibit comparable tendencies with regards to therapeutic effect. Some considerable exceptions were noted, however. One essential difference seems to be efficiency of subcutaneous LMWH. A significant therapeutic effect was noted for the OXA colitis model but it was found to be distinctly less efficient in the TNBS model. The reasons for this observation are not clear and explanations may range from less mucosal and submucosal swelling with OXA (and subsequent less LMWH penetration hindrance) to other disease specific mechanisms. Colon weight/length index, MPO, and AP activity results underlined the improved therapeutic efficiency found with LMWH-MS where colitis activity was reduced compared to values from subcutaneous LMWH solution. Rectal administration of LMWH solution reflects local deposition of the drug avoiding early loss or degradation of the drug during its transport along the intestinal tract. It therefore represents a kind of “best effect value” for epithelial LMWH. It is thus a very promising finding that LMWH-MS attained an equivalent level in mitigating efficiency.

An in-depth comparison of the different treatments elucidates the advantages of the developed MS system. After parenteral administration of LMWH, targeted areas of inflamed colonic tissue were not reached by a sufficiently high drug dose. This administration pathway appears particularly
inappropriate as suboptimal drug doses may be utilized in order to decrease the risk for adverse effects. When LMWH is administered orally, all drug undergoes intraluminal enzymatic inactivation in the upper parts of the gastrointestinal tract. Rectally administered LMWH, however, showed significant improvements of inflammation in both tested animal models. Similarly, LMWH-MS can protect the drug from early degradation during passage through the upper intestine and may allow its intact passage until reaching colonic tissue near sites of inflammation.

In terms of efficiency, this microparticulate system might be compared in the context of other studies applying LMWH. However, relevant information derived from preceding studies is limited since those dealt exclusively with parenteral LMWH administration (Fries et al., 1998; Xia et al., 2004). Improvements of colonic inflammation were observed after a 14 days treatment with dalteparin and suggested time- and dose-dependent effects of LMWH accompanied by severe intestinal bleeding (Xia et al., 2004). This highlights advancements that may be obtained through epithelial LMWH delivery.

The very low oral bioavailability demonstrates that, in terms of adverse effects, epithelial LMWH may allow significant progress compared to existing oral delivery approaches. Although oral bioavailability was mentioned to be altered in active state of IBD (Fries et al., 1999; Schurmann et al., 1999), no significant impact was determined in this study with LMWH-MS. This was determined to be due to efficient retention inside the particle matrix until delivery to the colon.

Apparantly, LMWH effect is mainly local since its systemic concentration is negligible after administration of LMWH-MS and therapeutic effect is limited after subcutaneous administration. Aside from possible effects on microcirculation (Vrij et al., 2001), several other mechanisms of action may explain the therapeutic action of heparins. They were found to interact with a wide variety of biological proteins such as proinflammatory chemokines, leukocyte proteases, growth factors, and extracellular matrix proteins (Tyrrell et al., 1995). Inhibition of IL-1 production (Jones and Geczy, 1990) may be considered as a specific mechanism as well as the non-specific plain physicochemical interaction between heparins and a variety of interleukins, namely IL-2, IL-6, IL-10, and IL-12 (Hasan et al., 1999; Salek-Ardakani et al., 2000). On the other hand, the inhibition of TNF-alpha production
by macrophages (Cahalon et al., 1997) appears improbable, since TNF-alpha levels remained unchanged with LMWH treatment, which is a similar finding to results from other groups (Wan et al., 2002). Also an interaction with P- and L-selectin, the adhesion molecules responsible for the leucocyte recruitment, from the endothelial side (Nelson et al., 1993; Koenig et al., 1998) is also not a likely explanation due to the rather long diffusional transport distance for the drug. These aspects may require further in-depth studies to elucidate whether the inhibitory effect of epithelial LMWH is the result of a non-specific binding of LMWH to one or more cytokines or involves a selective inactivation mechanism.

Selective epithelial LMWH delivery appears to be a promising approach in the therapy of IBD. LMWH-MS allow the desired drug to be released with a high degree of selectivity in areas of inflamed tissue ensuring a therapeutic concentration of the entrapped drug near the site of action. Furthermore, the entrapped LMWH enhanced the phenomenon of specificity by its very own minimal tendency to cross intact intestinal barrier resulting in extremely low oral bioavailability and a specific tissue penetration at the inflammation site. This approach proposes the clinical use of LMWH for the oral treatment of IBD with enhanced therapeutic efficiency of LMWH by selective drug delivery combined with negligible systemic adverse effects due to particularly low systemic drug absorption.
References


Legends for Figures:

**Figure 1:** Scanning electron microscopic image of pH-sensitive LMWH loaded microspheres.

**Figure 2:** Cumulated LMWH release versus time of LMWH trapping microspheres in phosphate buffer systems of pH 1.2, 6.8, and 7.4 replaced after 2 hours, respectively (n=3). Data are shown as mean ± SD.

**Figure 3:** Clinical activity score in TNBS or OXA model during the whole experimental period after either oral or rectal drug administration (■=colitis control; △=FK506 solution oral; ○=FK506 NP oral; ▲=FK506 solution rectal; ●=FK506 NP rectal; NP controls being similar to the colitis control as well as error bars are not shown for clarity reasons; n = 6) * P < 0.05 compared with colitis control rats given saline.

**Figure 4:** Colon weight/length ratio and histological damage score on day 8 in TNBS or OXA colitis model after administration of LMWH and LMWH-MP, respectively. Data are shown as mean ± S.D for n = 6 animals. * P < 0.05 compared with colitis control rats given saline.

**Figure 5:** Examples for histologic colon sections of healthy (A), untreated TNBS colitis (B), and LMWH treated tissue (C: LMWH sc; D: LMWH rectal; E: LMWH-MS 600IU/kg; F: LMWH-MS 2000IU/kg) in mice after the treatment period (×30 magnification).

**Figure 6:** MPO activity on day 8 in TNBS or OXA colitis model after administration of LMWH and LMWH-MP, respectively. Data are shown as mean ± S.D for n = 6 animals. * P < 0.05 compared with colitis control rats given saline. ** P < 0.05 compared with rats given LMWH solution subcutaneously.
Figure 7: alkaline phosphatase (AP) activity on day 8 in TNBS or OXA colitis model after administration of LMWH and LMWH-MP, respectively. Data are shown as mean ± S.D for n = 6 animals. * $P < 0.05$ compared with colitis control rats given saline, ** $P < 0.05$ compared with rats given LMWH solution subcutaneously.

Figure 8: penetrative behavior of carboxyfluorescein or fluorescently labeled LMWH (LMWH-FA) into healthy, inflamed, or non-inflamed tissue samples was analyzed (n=6). * $P < 0.05$ for differences observed between healthy and inflamed group tissue samples. Data are shown as mean ± S.D.
Table 1: Characteristics of LMWH loaded MS after solvent evaporation

<table>
<thead>
<tr>
<th>MS batches</th>
<th>bioavailability</th>
<th>colitis (600 IU/kg)</th>
<th>colitis (2000 IU/kg)</th>
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<tr>
<td>diameter (µm)</td>
<td>126.5±6.6</td>
<td>133.8±8.4</td>
<td>120.1±13.1</td>
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<tr>
<td>process yield (%)</td>
<td>94.0±2.1</td>
<td>95.9±1.2</td>
<td>96.6±8.0</td>
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<tr>
<td>encaps. rate (%)</td>
<td>75.8±3.3</td>
<td>73.9±2.0</td>
<td>70.7±1.7</td>
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</table>
Table 2: Main pharmacokinetic parameters after oral administration of LMWH loaded MS versus subcutaneous LMWH solution in rabbits

<table>
<thead>
<tr>
<th></th>
<th>sc</th>
<th>MS healthy</th>
<th>MS colitis</th>
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</thead>
<tbody>
<tr>
<td>Dose (IU)</td>
<td>827.0±113.7</td>
<td>2090.0±75.5</td>
<td>1980.0±36.8</td>
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<tr>
<td>AUC (mg/ml)</td>
<td>6.01±0.72</td>
<td>0.55±0.51</td>
<td>0.98±0.69</td>
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<tr>
<td>Weight (kg)</td>
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<td>AUC/kg</td>
<td>2.19±0.14</td>
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<td>F rel (%)</td>
<td>100</td>
<td>2.3±2.5</td>
<td>5.5±2.1</td>
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Figure 2

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Figure 2: Graph showing the LMWH release percentage over time at different pH levels (pH 1.2, pH 6.8, pH 7.4).
Figure 3

**TNBS**

- colitis contr
- LMWH sc 300IU
- LMWH rectal 600IU
- LMWH MP 600IU
- LMWH MP 2000IU

**OXA**

- colitis contr
- LMWH sc 300IU
- LMWH rectal 600IU
- LMWH MP 600IU
- LMWH MP 2000IU

Clinical activity score over time for TNBS and OXA with different dosages of LMWH.
Figure 4
Figure 6

**TNBS**

- healthy contr
- colitis contr
- LMWH sc 300IU
- LMWH rect 600IU
- LMWH MP 600IU
- LMWH MP 2000IU

**OXA**

- healthy contr
- colitis contr
- LMWH sc 300IU
- LMWH rect 600IU
- LMWH MP 600IU
- LMWH MP 2000IU
Figure 7

![Bar chart for TNBS and OXA](chart.png)

**TNBS**
- Healthy control
- Colitis control
- LMWH sc 300IU
- LMWH rect 600IU
- LMWH MP 600IU
- LMWH MP 2000IU

**OXA**
- Healthy control
- Colitis control
- LMWH sc 300IU
- LMWH rect 600IU
- LMWH MP 600IU
- LMWH MP 2000IU
Figure 8

![Bar graph showing LMWH [%] for healthy, inflamed, and non-inflamed conditions. The graph compares fluorescein and LMWH-FA treatments.]

- Healthy: Gray bars
- Inflamed: Dark gray bars
- Non-inflamed: Black bars

* indicates significant difference.