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Nanoparticles in inflammatory bowel disease: Particle targeting versus pH-sensitive delivery

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Abstract

Tacrolimus proved its distinct mitigating potential in the treatment of inflammatory bowel disease (IBD). Due to the risk for severe adverse effects and to achieve increased efficiency and tolerability, a selective delivery to the site of inflammation is of interest. Tacrolimus nanoparticles (NP) were tested for their efficiency in local treatment of inflamed bowel tissue in IBD. Drug loaded NP were prepared from either biodegradable poly(lactide-co-glycolide) (PLGA) or pH-sensitive Eudragit P-4135F by using a simple oil/water emulsification method. Tests on the therapeutic effect were conducted using dextran sulfate model colitis in mice receiving tacrolimus formulations daily for 12 days. Clinical activity score and myeloperoxidase activity decreased while colon length increased significantly after administration of all tacrolimus containing formulations. Oral NP formulations were less efficient in mitigating the experimental colitis compared to subcutaneous drug solution (PLGA: 7.88 ± 0.83; P-4135F: 7.48 ± 0.42; subcutaneous: 5.27 ± 0.68 U/mg) but superior to drug solution given by oral route (oral: 8.75 ± 1.34; untreated colitis control: 9.95 ± 0.92, all U/mg tissue). Tacrolimus solution groups (oral/subcutaneous) exhibited increased levels of adverse effects, whereas both NP groups demonstrated their potential to reduce nephrotoxicity. Both strategies showed similar mitigating effects while nephrotoxic adverse effects were slightly less expressed with pH-sensitive NP.

Keywords: Nanoparticles; Drug delivery; Inflammation; Colon delivery; Inflammatory bowel disease

1. Introduction

Different groups of drugs are utilized therapeutically for inflammatory bowel disease (IBD) (Egan and Sandborn, 1998). Tacrolimus, initially developed as an immunosuppressant for the prevention of transplantat rejection, demonstrated to be a very potent drug in the treatment of severe cases of IBD in animal studies as well as in clinical trials where corticoid treatment failed (Fellermann et al., 1998; Matsuhashi et al., 2000; Aiko et al., 1997; Higa et al., 1993; Hoshino et al., 1995). The mechanism of its immunosuppresive effect is not selective to inflamed tissue, which is the cause for nephrotoxicity, the main adverse effect of tacrolimus (Finn, 1999). Long-term tacrolimus administration may induce renal dysfunction with functional nephrotoxicity consisting of dose-dependent reduction of renal blood flow and glomerular filtration rate. Consequently, local delivery of tacrolimus is of high interest as an approach to reduce systemic availability of the drug and, therefore, lowering its adverse effects.

Various drug delivery strategies have been commercialized for the treatment of IBD and many experimental therapeutic strategies have also been described (Rubinstein, 1995; Lamprecht et al., 2002). One major therapeutic strategy consists of oral drug delivery with selective release of entrapped active compounds to the colon. However, decreased efficiency is noted in many cases related to diarrhea, a major symptom of IBD that both accelerates carrier elimination (Hardy et al., 1988; Watts et al., 1992) and reduces possible drug release time. Since all macroscopic drug delivery systems are at risk for this type of therapy failure, alternative strategies are needed. Size reduction of drug carriers is one option to circumvent this problem. Microparticulate delivery systems have been successfully developed for experimental treatment of IBD (Nakase et al., 2000; Nakase et al., 2001; Lamprecht et al., 2005a). Nanoparticles (NP) have also shown potential for specific accumulation in areas with inflamed tissue increasing the selectivity of local drug

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delivery (Lamprecht et al., 2001a). This observation was based on two phenomena, uptake into immune related cells infiltrating inflamed tissue and adhesion to the mucus, which is highly excreted in areas of inflamed tissue.

Recent developments concerning the use of pH-sensitive microparticles for colonic drug delivery using the polymer Eudragit P-4135F appear to be an efficient approach to IBD therapy (Lamprecht et al., 2005a). The next step is to transfer this therapeutic strategy to a colloidal carrier. Drug release from proposed Eudragit P-4135F nanoparticles is based on the polymer's sensitivity to luminal pH during intestinal passage, which is similar to standard drug delivery systems in this disease.

Both therapeutic strategies, pH-sensitive, and polyester NP, were compared in terms of therapeutic and adverse effects. In vivo studies were conducted using dextran sulfate model colitis in mice in order to characterize the potential of a nanotechnological approach. While NP formulations were administered orally, control mice received tacrolimus as a solution either by oral or subcutaneous route.

2. Materials and methods

2.1. Materials

Eudragit P-4135F was provided as a gift from Röhm Pharma Polymers (Tokyo, Japan). Tacrolimus (FK506) was received as a kind gift from Fujisawa (Osaka, Japan). Poly(lactic-co-glycolic acid) 50/50 (Mw 20,000 Da) was purchased from Alkermes (Cambridge, USA). Polyvinyl alcohol and dextran sulfate sodium (DSS) were purchased from Sigma (Deisenhofen, Germany). All other chemicals were obtained from either VWR International (Fontenay sous Bois, France) or Fluka (Deisenhofen, Germany) and were of analytical grade.

2.2. Methods

2.2.1. Preparation and characterization of nanoparticles

The preparation of NP was based on an oil/water emulsification-solvent evaporation method. A fixed amount of polymer, either PLGA or Eudragit (200 mg), and drug (20 mg) was used. The matrix polymer was dissolved in 3 ml methylene chloride together with tacrolimus. This solution was poured into 75 ml of 1% (w/w) polyvinyl alcohol and an oil/water-emulsion was formed by ultrasonication Vibracell 72434; Bioblock, Illkirch, France, power level: 80%) for varying time intervals (PLGA: 30 s, P-4135F: 3 min) in order to obtain equivalent particle sizes for both NP types. The solvent was removed under reduced pressure. NP were then centrifuged at 40,000 × *g* for 15 min and redispersed in distilled water twice prior to lyophilization in a 5% sucrose solution (Lyolab 3000, Heto, Heto-Holten A/S, Allerød, Denmark).

The NP were analyzed for size distribution and zeta potential in 1:100 dilution with distilled water using a Zetasizer II® (Malvern Instruments, UK). The drug loading efficiency of NP was determined after extraction from the particles using high performance liquid chromatography (HPLC) as described elsewhere (Lamprecht et al., 2004). Lyophilized NP were dissolved in 600 μl of an acetone–ethanol mixture (2:1). Then, 6 ml of acetonitrile were added and the solution was vortexed for 5 min. The samples were centrifuged at 10,000 × *g* for 20 min and the supernatant was diluted 1:10 before injection into the HPLC system.

For in vitro drug release experiments, 10 mg of lyophilized drug-loaded NP were re-suspended in 20 ml phosphate buffer (pH 6.8 or 7.4) containing 0.1% polysorbate 80 and incubated at 37 °C under magnetic stirring. At appropriate intervals, 0.5 ml samples were withdrawn and filtrated through a 0.1 μm PTFE Millipore filter. The filtrate was assayed for drug release and replaced by 0.5 ml of fresh buffer. The amount of tacrolimus in the release medium was determined by the HPLC method described before.

2.2.2. Animal treatment

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 model colitis was induced in male Swiss mice (average weight 30 g; $n = 6$/group) by use of dextran sulfate sodium method: Mice received 5% of DSS with their drinking water over a period of 7 days. Starting on day 7, the different groups received tacrolimus as subcutaneous injection (FK506sc) or orally (FK506oral), either as solution or as NP formulation (PLGA or P-4135F). NP suspension or 0.1 ml of tacrolimus solution were administered once daily for 12 continuous days (tacrolimus solution: 1 mg/kg of body weight; NP suspensions: equivalent dose containing). Colitis controls received saline instead of free drug or drug-containing particles. Dosage was determined as 1 mg/kg body weight based on results of precedent studies. Similarly, the treatment period was adjusted to 12 days on preliminary results as well as earlier studies with tacrolimus (Hoshino et al., 1995; Lamprecht et al., 2005b). The animals were sacrificed 24 h after the last drug/particle administration. Their colons were resected as well as blood and urine samples were collected.

2.2.3. Weight loss, colon length, and myeloperoxidase activity

Weight loss of mice was determined by dividing actual daily weight by the initial values on day 1. Resected colon tissue samples were opened longitudinally and rinsed with iced phosphate buffer to remove luminal content before length measurements were taken.

A measurement of myeloperoxidase activity 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 the inflamed tissue. Activities were analyzed according to a standard method (Krawisz et al., 1984). Briefly, distal colon specimen was minced in 1 ml of hexadecyltrimethylammonium bromide buffer (0.5% in 50 mM phosphate buffer) on ice and homogenized. The homogenate was sonicated for 10 s, freeze-thawed three times, and centrifuged at 10,000 rpm for 3 min. Myeloperoxidase activity in the supernatant was measured spectrophotometrically. Supernatant (0.1 ml) was added

to 0.167 mg/ml of *o*-dianisidine hydrochloride and 0.0005% hydrogen peroxide, and the change in absorbance at 460 nm was measured. One unit of myeloperoxidase activity was defined as the amount that degrades 1 μmol of peroxidase per minute at 25 °C.

2.2.4. Creatinine and blood urea nitrogen levels

Plasma levels of creatinine and blood urea nitrogen (BUN) were determined as well as creatinine levels in the urine by use of standard colorimetric methods using the respective kits from Biomérieux (Marcy-l'Etoile, France) according to the supplier's instructions.

2.2.5. Statistical analysis

The results are expressed as mean values ± S.D. For analysis of statistical significance, the Kruskal–Wallis test was applied followed by Dunn's test (Tukey test where tests for normality and equal variance were passed) for all pair wise comparison. In all cases, $P < 0.05$ was considered to be significant.

3. Results and discussion

Both NP formulations were found to be spherical with particle diameter of approximately 450 nm. The two particle types had a zeta potential close to neutrality (Table 1). Encapsulation efficiency and drug load were slightly higher with P-4135F compared to PLGA NP. This may be primarily due to greater lipophilicity of P-4135F and its polymer/drug interaction with tacrolimus as reported in an earlier study (Lamprecht et al., 2004). Those findings also suggested using an emulsification method rather than a solvent diffusion method. With the latter, drug extraction towards the external aqueous phase had been observed, which subsequently lowers encapsulation efficiencies and drug loads, respectively.

Fig. 1 illustrates in vitro drug release profiles obtained from the different NP formulations in phosphate buffer systems at a pH of 6.8 or 7.4, respectively. PLGA NP exhibited a drug release that was mainly independent from the pH of tested release media. Drug release occurred in two phases, an initial burst release of approximately 50% of total drug load within the first 30 min followed by sustained release that was completed after 24 h. The initial burst release was described as an immediate dissolution of the adsorbed drug onto the particle surface (Sampath et al., 1992) which might occur similarly with PLGA NP. It was stated that some polyester NP are affected by polymer degradation after 50–100 days depending on the presence of anionic or nonionic surfactants (Coffin and McGinity, 1992). This led to the conclusion that in vitro drug release from PLGA NP results mainly from diffusion out of polymeric NP. However, variations are expected for in vivo behavior secondary to significant effects of enzymatic particle degradation leading to accelerated drug release (Lamprecht et al., 2001b).

Table 1
General characteristics of tacrolimus NP prepared by PLGA or P4135F ($n = 3$; data are shown as mean ± S.D.)

	PLGA NP	P-4135F NP
Diameter [nm]	455 ± 25	469 ± 4
Zeta potential [mV]	−1.8 ± 1.1	0.1 ± 2.1
Drug load [%]	4.7 ± 0.2	5.8 ± 0.1
Encapsulation efficiency [%]	52.2 ± 2.1	64.2 ± 2.0

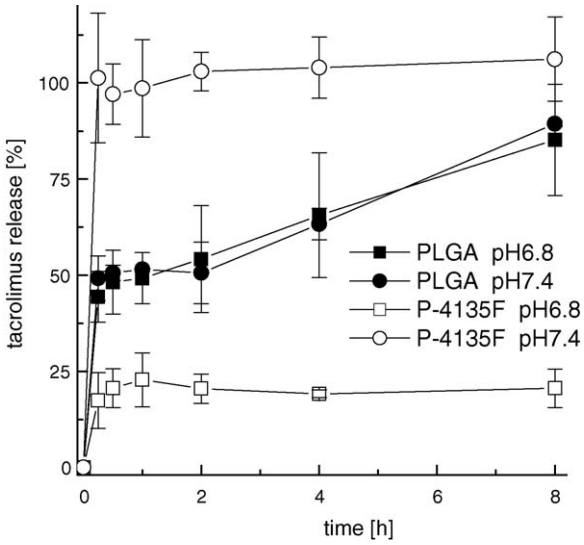

Fig. 1. Cumulated tacrolimus release vs. time of either PLGA or P-4581F NPs in phosphate buffer systems of pH 6.8 or 7.4, respectively ($n = 3$). Data are shown as mean ± S.D.

Release kinetics recorded for P-4135F NP showed substantial pH-sensitivity. Tacrolimus was retained efficiently inside the NP when tested in vitro at a pH of 6.8. Around 80% of the initial drug load was maintained inside the particle matrix during incubation for 8 h. A comparatively fast release was observed at a pH of 7.4 with delivery of nearly 100% of the incorporated drug within 30 min. These observations were in line with earlier findings where comparable release profiles were noted for microspheres (Lamprecht et al., 2004). The general mechanism of drug release from this carrier is based on dissolution of the pH-sensitive matrix polymer at a pH above 7.2. pH-sensitive NP are about 200-fold smaller than tacrolimus microspheres described earlier. The subsequent significantly larger surface area was observed to play a distinct role in higher drug leakage at a pH of 6.8 together with faster particle dissolution at a pH of 7.4.

In order to evaluate the therapeutic value of tacrolimus NP, the mitigating effect of the two different NP systems was studied on a preexisting colitis model in mice. After inducing experimental colitis, weight loss in response to intestinal inflammation became visible on day 5 and statistically significant after day 7 (Fig. 2). After day 14, tacrolimus formulations reduced the weight loss compared to the untreated colitis control group. This observation became statistically significant on day 18. Similar results were noted in response to administration of oral solution of tacrolimus and both NP formulations. A slightly more effective treatment was observed with tacrolimus when administered subcutaneously. However, the differences were statistically significant only in comparison to the untreated control group.

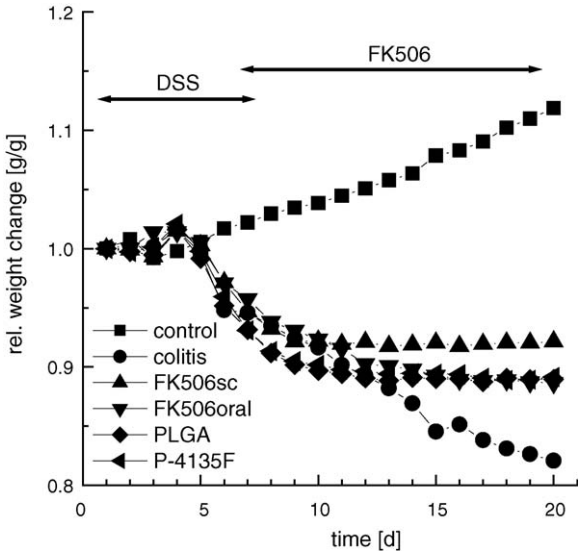

Fig. 2. Relative weight change during the whole experimental period always determined for $n = 6$ animals. $^{*}P < 0.05$ compared with colitis control mice given saline. Error bars were not shown for clarity reasons.

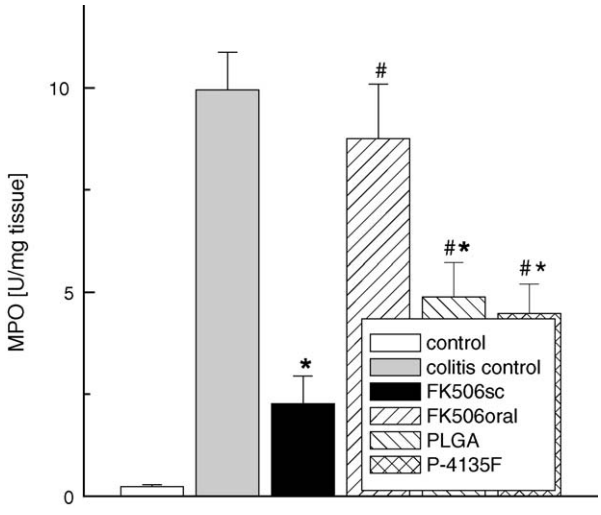

Fig. 4. Determination of myeloperoxidase activity (MPO) after final drug administration for $n = 6$ animals. $^{*}P < 0.05$ compared with colitis control mice given saline. $^{\#}P < 0.05$ compared with tacrolimus subcutaneous group. Data are shown as mean ± S.D.

Similar to observations made regarding the weight loss index, colon length levels after tacrolimus treatment were found to be significantly higher ($P < 0.05$) in comparison to the colitis control (Fig. 3). Again, oral administration of tacrolimus NP formulations proved slightly less efficient than the subcutaneous route while oral solution did not exhibit any therapeutic efficiency. Results from the two groups receiving NP formulations were comparable. Myeloperoxidase activity revealed similar therapeutic effects with PLGA NP and P-4135F NP significantly lowering myeloperoxidase activity compared to untreated control. This was not found to be the case after oral tacrolimus solution treatment (Fig. 4).

The clinical course of model colitis (characterized by weight loss, colon length, and myeloperoxidase activity) displays a distinct therapeutic effect of tacrolimus treatment as previously shown in other animal studies (Aiko et al., 1997; Higa et al., 1993). For both colon length and myeloperoxidase activity, tacrolimus NP formulations reduced the inflammatory activity below the level of oral tacrolimus solution but were less efficient than subcutaneously administered tacrolimus. Differences between the treatment groups were statistically significant in the case of myeloperoxidase activity but effects were less expressed in the colon length.

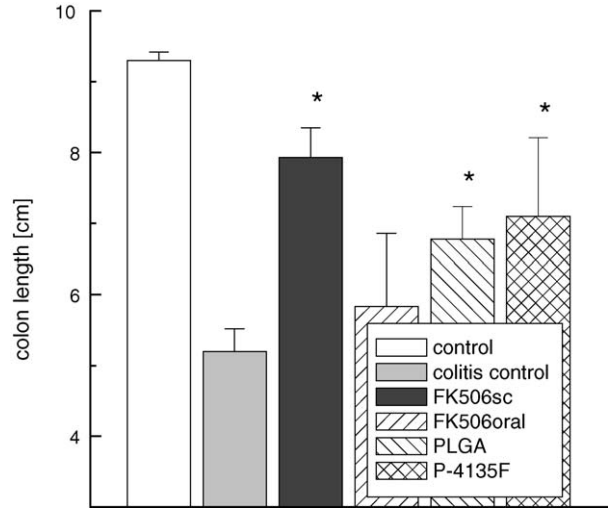

Fig. 3. Determination of colon length after final drug administration for $n = 6$ animals. $^{*}P < 0.05$ compared with colitis control mice given saline. $^{\#}P < 0.05$ compared with tacrolimus subcutaneous group. Data are shown as mean ± S.D.

Nephrotoxiciy, the main adverse effect of tacrolimus, was studied for the different formulations at the end of the treatment period. BUN and serum creatinine levels were increased after subcutaneous administration of tacrolimus (Fig. 5A and B). Tacrolimus NP treatment generally appeared to inhibit the increase of BUN and serum creatinine levels. Differences to the untreated control were not statistically significant while differences between the two particle types were minor. Endogenous creatinine clearance significantly decreased in FK506sc treated rats (Fig. 5C). Treatment with FK506oral also decreased creatinine clearance but not significantly. Both NP groups exhibited levels of creatinine clearance similar to the control group.

According to the results in Fig. 5, a successful reduction of adverse effects can be achieved by the use of NP formulations compared to FK506sc. Moreover, these measurements revealed that adverse effect levels after tacrolimus NP did not differ from that of untreated control animals. Although this was also observed for the FK506oral group, it must be kept in mind that, in terms of therapeutic efficiency the NP groups showed a statistically significant mitigating effect. Their effects were thus superior to the group receiving tacrolimus solution orally.

A general comparison of the treatment strategies elucidates the advantages and inconveniencies of the various therapeutic approaches. While subcutaneous injection of tacrolimus allows for full mitigating effect of the drug, increased adverse effects may be expected. When tacrolimus is administered orally as a solution, the drug is only partially systemically available due

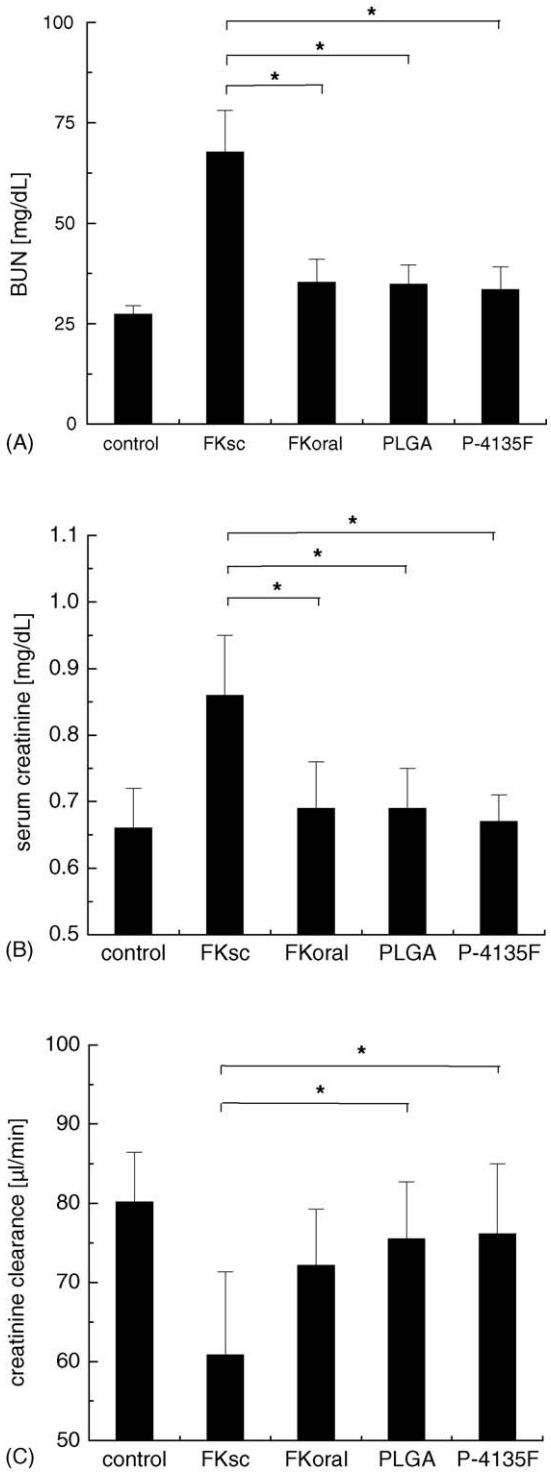

Fig. 5. (A–C) BUN, serum creatinine, and creatinine clearance after the treatment period of either tacrolimus solution (oral or subcutaneous) or the two tacrolimus NP formulations for $n=6$ animals. $^{*}P<0.05$. Data are shown as mean ± S.D.

to its low oral bioavailability, which is responsible for a lower therapeutic effect but decreased adverse effects as well.

P-4135F NP represent a standard drug delivery strategy for delivery of anti-inflammatory drugs in the treatment of colitis. This approach is similar to many commercialized macroscopic sustained release formulations. Although much smaller in size, the therapeutic principle is based on the selective drug release triggered by luminal pH. This can prevent premature drug absorption during passage through the small intestine until the lower ileal tissue is reached.

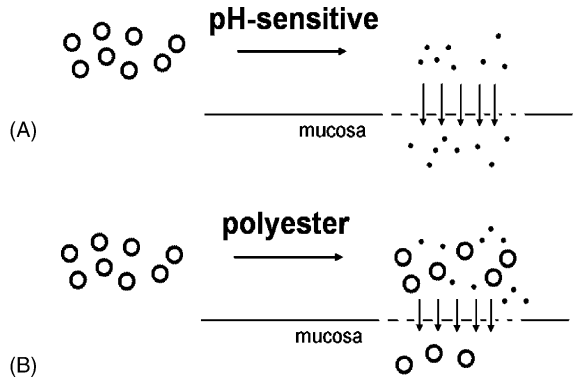

Fig. 6. Schematic presentation of the targeting strategies applied with the different NP in oral administration of anti-inflammatory drugs. The P-4135F NP representing the conventional pH-sensitive drug delivery is based on the change of luminal pH during transport towards the colon releasing the entrapped drug into the lumen near the inflammation site (A). PLGA NP allow targeting of the inflamed area directly. The strong presence of immune-related cells in the inflamed tissue which take up the drug carriers as well as the mucosal barrier breakdown at the ulceration sites allow a distinct accumulation of the drug delivery system inside the inflamed tissue (B).

However, initial drug leakage and complete drug release in the distal ileum make tacrolimus available to large healthy tissue areas surrounding the inflamed region. Surprisingly, this leads to lower adverse effects than would be expected, which may be related to some physiological particularities associated with the properties of tacrolimus. P-4135F NP disintegrate in the distal ileum where all drug is released. Usually, free drug has the potential to increase systemic drug concentration provoking adverse effects. In the case of tacrolimus, it leads rather to a significant loss of efficiency due to the drug's mucosal metabolism (Lampen et al., 1996). Tacrolimus is partially metabolized in the mucosa and partially expulsed towards the luminal side by P-glycoprotein efflux pumps. Mucosal metabolism is more intense in the upper parts of the intestine in comparison to colonic tissue. Besides, possible influences of the inflammation on metabolic activity of the mucosa are not completely understood. While the first factor decreases the drug amount to be delivered towards the inflammation side, the second is in favor of higher drug concentration in the colonic lumen resulting in higher drug availability at the inflammation site. An additional parameter is the observation that there is a generally lower absorptive activity for tacrolimus from colonic tissue (Kagayama et al., 1993).

PLGA NP follow a completely different therapeutic appoach which focuses on drug targeting (Fig. 6). This strategy is mainly based on two pathophysiological changes in the inflamed tissue allowing better adhesion of carriers in the inflamed tissue related to elevated levels of mucus production. Intense particle uptake inside the colitis tissue is also a result of an enhanced permeability and the presence of a highly increased number of immune related cells. This accumulation phenomenon was observed to be particle size dependent with effects increasing for a decrease

in size. Highest efficiency for NP was found to be at a diameter of approximately 100 nm (Lamprecht et al., 2001a).

Although the polyester NP provided a high selectivity in adhesion to the inflamed tissue areas, a major drawback seemed to be a considerably high initial drug loss based on the burst release during passage through the stomach and small intestine. As mentioned before, the effect of initially released drug will be limited in terms of systemic availability due to influences of P-glycoprotein efflux and mucosal metabolism.

Additionally, PLGA NP are impeded by Peyer's Patches uptake as well as enzymatic degradation of the carrier leading to a distinct drug loss despite the fact that the drug delivery system is more selective towards inflamed tissue. Thus, due to carrier degradation a loss of efficiency is probably based on a lower amount of NP reaching the inflamed tissue to exert its anti-inflammatory effect.

Orally administered tacrolimus loaded polyester NP were found to be of limited efficiency in a recent study (Lamprecht et al., 2005b) while the results here demonstrated a therapeutic success. One effect that has to be taken into account is the shorter and faster passage of the drug carriers throughout the intestinal tube in mice compared to rats, which were the basis of the animal model in that previous study.

In summary, both the targeting approach by PLGA NP and the standard strategy represented by P-4135F NP provided a significant mitigating effect in experimental colitis in mice. In terms of adverse effects, both particulate systems permitted a reduction of adverse effects similar to the level of untreated controls. Compared to PLGA NP, pH-sensitive NP exhibit a lack of specificity. However, their advantage is comparably lower drug leakage and apparently higher total amount of tacrolimus delivered to the colon. Oppositely, PLGA NP increase drug concentration specifically inside the inflamed tissue with a lower total amount of drug but more selective accumulation. Degradation of PLGA NP during passage in upper parts of the intestine resulted enhances the potential for adverse effects.

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