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Research paper

Colonic delivery of carboxyfluorescein by pH-sensitive microspheres in experimental colitis

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ABSTRACT

The colonic drug delivery in inflammatory bowel disease (IBD) by microcarriers has been suggested over the past decade; however, pharmacokinetic and biopharmaceutical details are hardly known. A model colitis was induced to male Wistar rats by trinitrobenzenesulfonic acid. Carboxyfluorescein (CF) was entrapped into microspheres (MS) prepared with the pH-sensitive polymer Eudragit® S100, in order to simulate drug delivery to the colon. Pharmacokinetic behaviour of CF–MS was compared to oral or rectal administration of CF as solution in healthy or colitis group. Colitis lowered the oral bioavailability of CF solution, compared to healthy controls (healthy: 8.4 ± 1.5; colitis: 3.0 ± 0.9; all μg/ml h), and similar results were obtained after rectal administration of CF solution (healthy: 5.6 ± 2.1; colitis: 1.8 ± 0.8). Surprisingly, CF–MS showed only minor differences between colitis and healthy controls (healthy: 1.9 ± 0.8; colitis: 2.3 ± 0.4). In contrary, the intra-tissue concentrations of CF of the various formulations in colitis showed lower levels than the comparable healthy group after oral drug administration. Pharmacokinetic outcome was largely disease-dependent, while CF–MS confirmed their ability to local drug delivery.

1. Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) usually affect different areas of the gut. Mucosal inflammation is mainly limited to the distal regions of the colon in UC. Transmural chronic inflammation may involve all intestinal segments in CD with a usual main focus in the distal parts of the small intestine [1]. The natural course of both variants consists of quiescent phases that are interrupted by relapses [2].

The general principle of a pharmacological treatment in inflammatory bowel disease [IBD] is to induce remission of outbreaks and to prevent outbreaks during remission. A large diversity of drugs is of therapeutic interest, such as 5-aminosalicylic acid and glucocorticoids but also immune suppressive drugs in severer cases [3–6]. For such highly potent drugs, a specific and locally limited delivery is most desirable in order to limit drug availability towards non-inflamed tissue, which lowers the efficiency and risk distinct adverse effects. Therefore, several strategies to deliver drugs to the large intestine after oral administration have been developed over the past decades. For instance, enzymatically degradable carriers rely on the enzymatic activity of colonic bacteria similar to the mechanism of prodrugs. However, disease related variability in colonic flora mainly observed in CD can distinctly impede efficient drug delivery [7,8]. Others, among them most of the commercialised systems, are based on the change of the luminal pH during the gastrointestinal passage [9–12].

The pH-sensitive approaches have been mainly reported for coated solid dosage forms using a variety of different polymers, such as methacrylates and substituted polyvinyl acetate and cellulose derivates [13]. Methacrylate/methacryl acid polymers Eudragit® S, L, and FS dissolve in aqueous media in the range of pH 6–7, respectively, which may be equivalent to a drug release to the distal ileum and seemingly most appropriate for IBD therapy.

Gastrointestinal transit in IBD can vary from that in healthy state due to mucosal inflammation, and the changes in the normal mechanism of transit. The inflamed colon presents abnormalities in fluid and electrolyte absorption and secretion [14]. An additional common pathophysiological attribute of ulcerative colitis patients is diarrhoea influencing the transit of the drug delivery system.

Solid dosage forms, especially drug carrier systems with a size larger than 200 μm are strongly subjected to the diarrhoea symptoms. This results in a decreased gastrointestinal transit time and leading therefore to a distinct risk of inefficiency. Thus, the efficiency of drug delivery systems can be decreased due to both accelerated carrier elimination and reduced drug release time and drug availability from the delivery system [15,16].

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A size reduction of the drug carrier system might be an option in order to circumvent those problems, as it was proposed by using microspheres (MS) [17,18].

Due to the smaller size of the delivery system, not only streaming becomes less dramatic but also other factors such non-specific mucoadhesion play a more important role than it is the case of standard oral dosage forms. This can have a significant impact when MS designed for colonic delivery also show mucoadhesive properties [19].

However, there are surprisingly few details in the literature on the pharmacokinetics of these experimental MS, especially in the context of IBD. Similarly, only little is known about how drug absorption takes place across the inflamed barrier at the inflammation site and tissue permeability is potentially modified. Also, the actual tissue concentrations after different delivery routes are rarely addressed.

In order to elucidate the role of the drug delivery system in healthy or diseased state and to estimate the efficiency of the local drug tissue accumulation, healthy rats and those suffering from an experimental colitis underwent pharmacokinetic studies using various administration routes and formulations. Besides, the intra-tissue concentration following the different administration pathways was recorded.

2. Methods

2.1. Materials

Eudragit® S100 was a kind gift from Degussa/Roehm Pharma Polymers (Darmstadt, Germany). Carboxyfluorescein (CF) and trinitrobenzenesulfonic acid (TNBS) were purchased from Cooper, Melun, France and Sigma–Aldrich Chemie GmbH (Steinheim, Germany), respectively. All other chemicals were of analytical grade.

2.2. Preparation of microspheres

Two hundred milligrams Eudragit® S and 25 mg CF were dissolved in 8 ml acetone/ethanol mixture 3:2 containing 10 μl of 1 N HCl. This solution was poured into 40 ml of liquid paraffin containing 4% w/w Span 80, and an oil/oil emulsion was formed by stirring with a three-blade propeller at 800 rpm for 2 h. The emulsion was stirred under vacuum until solvents were removed. MS were collected by filtration, and washing steps were performed with 150 ml *n*-hexane before drying at atmospheric pressure. All preparation steps were performed under subdued light.

2.3. In vitro characterisation of microspheres

Particle size analyses of all MS batches were carried out by laser light diffraction (Mastersizer® X, Malvern Instruments, UK). MS were dispersed in 2 ml of an aqueous solution of Tween 80 (0.2%). Particle morphology was analysed by SEM. MS were fixed on supports with carbon–glue and coated with gold using a gold sputter module in a high-vacuum evaporator. Samples were then observed with the scanning electron microscope (JEOL JSM-5600 Scanning Electron Microscope, Tokyo, Japan) at 24 kV.

Drug loading was determined by fluorescence spectrophotometry after dissolution of MS in phosphate buffer at pH 7.4 as described elsewhere [20]. The in vitro drug release was analysed as follows: drug loaded MS were suspended in 100 ml phosphate buffer systems of pH 1.2, 4.5, and 7.4, respectively. The dissolution medium was kept under stirring at 100 rpm. All the experiments were carried out at 37 °C for 4 h. Aliquots of the dissolution medium (1 ml) were withdrawn at predetermined time intervals and replaced by fresh buffer. Drug concentrations in the supernatant were directly analysed by fluorescence spectrophotometry. CF was determined with an F-3010 Fluorescence spectrophotometer (Hitachi, Tokyo, Japan) without any additional processing.

2.4. 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 TNBS rat model was chosen as well recognised experimental model [21] that allow induction of colitis at an exact location. This permitted administration of rectal drug in the form of an exclusively local delivery to the inflamed tissue.

Male Wistar rats (10 weeks; $n = 4$/group) were treated by the following procedure in order to induce the TNBS model colitis: after light narcotizing with ether, the rats were catheterised 8 cm intrarectal, and 500 μl of TNBS (140 mg/kg) in an ethanol/water mixture was applied. Control groups received a rectal installation of an ethanol/saline mixture without TNBS instead. For 48 h, the rats were housed without treatment to maintain the development of a full inflammatory bowel disease model.

In order to ensure pharmacokinetic studies under reproducible conditions, colitis activity was quantified with a clinical score assessing weight loss, stool consistency, and rectal bleeding as previously described elsewhere [22]. No weight loss was counted as 0 point, 1–5% as 1 point, 5–10% as 2 points, 10–20% as 3 points and >20% as 4 points. For stool consistency, 0 point was given for well-formed pellets, 2 points for pasty and semiformed stools that did not stick to the anus, and 4 points were given for liquid stools that stick to the anus. Bleeding was scored as 0 point for no blood, 2 points for positive finding, and 4 points for gross bleeding. The mean of these scores was forming the clinical score ranging from 0 (healthy) to 4 (maximal activity of colitis).

The determination of the myeloperoxidase activity (MPO) in resected colonic tissue samples allowed quantifying the severity of the colitis as a reliable index for the infiltration of activated neutrophils into the inflamed tissue. Activities were analysed according to a standard method [23]. Briefly, distal colon specimen was minced in 1 ml of hexadecyltrimethylammonium bromide buffer (0.5% in 50 mM phosphate buffer) on ice and homogenised. The homogenate was sonicated for 10 s, freeze–thawed three times, and centrifuged at 10,000 rpm for 3 min. 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.

All rats were anaesthetised with isoflurane during intravenous injection, oral administration, and blood sampling. In the experiments of oral CF administration, rats received either MS formulations (dispersed in 0.5% carboxymethyl cellulose) or CF solution at a dose of 1 mg/kg. For rats receiving a rectal administration of CF, the dye solution was administered in analogue to the TNBS induction procedure described earlier.

Blood specimens of 0.5 ml were collected from the jugular vein sampling at different predetermined time points in tubes containing 20 μl of heparin (2500 I.U/ml) as anticoagulant and centrifuged at 10,000*g* for 5 min. The plasma samples were diluted with phosphate buffer (pH 7.4) and analysed for their CF content by fluorescence spectrophotometry as described earlier [24].

2.5. Tissue penetration

Experimental setup was adapted from a method described elsewhere [25]. Control tissue samples were taken from the healthy group. Inflamed or non-inflamed tissue samples were taken from the treated healthy or colitis group, where inflamed tissues were

resected from areas with macroscopic damages or equivalent tissues from healthy controls. The resected rat tissue samples were washed with ice-cold phosphate buffer (pH 7.4) and minced with an ultraturrax in a polysorbate solution (0.1%) for 2 min at 10,000 rpm and centrifuged. CF concentrations were quantified in the supernatant by fluorescence spectrophotometry as described earlier against untreated intestinal tissue as blanks.

2.6. Statistical analysis

The results were expressed as mean values ± SD. 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.

3. Results

3.1. In vitro characteristics of microspheres

CF–MS were spherical with a particle diameter below 150 μm and a relatively smooth surface exhibiting a distinct number of pores (Fig. 1). Further, MS characteristics such as particle size, CF 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). CF was retained inside MS within certain limits when tested at pH 1.2 and 4.5, where around 80% of the initial drug load was still present inside the MS after 2 h of incubation. On the contrary, a comparatively fast release was observed at pH 7.4, which delivered nearly 100% of the incorporated drug within 15 min.

3.2. Pharmacokinetic studies

The presence and stability of a reliable experimental colitis was confirmed by the MPO activity in all groups. The average MPO activity in the areas with macroscopic damages was 36.9 ± 6.6 units/g tissue, while in the healthy control the activity was as low as 0.8 ± 0.1 units/g tissue. These results were comparable to values reported from earlier work [18]. Besides, no significant influence by CF on the colitis model was observed (MPO activity of 39.2 ± 8.5 units/g tissue 24 h after CF administration). Additionally, it was observed that the colon wet weight/body ratio increased by a factor of 4.1 compared with the healthy control group, which has been reported as an indication for inflammation as well [18].

The plasma concentrations after oral administration of CF increased to their highest values after 2 h for healthy animals, while in the colitis group C_{max} was observed after 30 min (Fig. 3A). After 8 h, 95% of CF was found to be cleared from the plasma in both cases.

Table 1
General characteristics of the CF–MS ($n = 3$; data are shown as mean ± SD).

Diameter (μm)	145.2 ± 10.9
Drug load (%)	10.8 ± 1.1
Encaps. rate (%)	52.8 ± 4.9

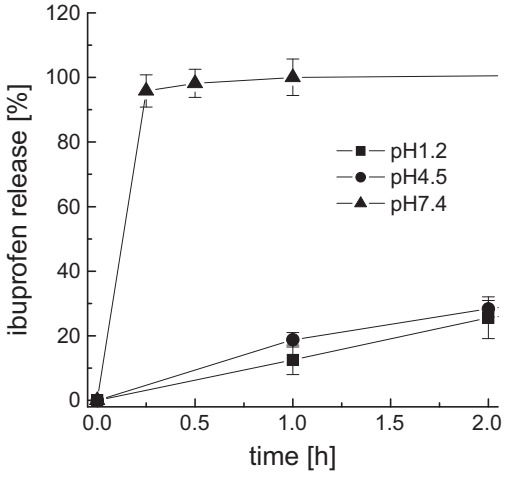

Fig. 2. In vitro drug release vs. time of CF loaded Eudragit® S100 MS in buffer systems of pH 1.2, 4.5, and 7.4.

After rectal administration, C_{max} was twice as high as after oral administration in healthy animals (Fig. 3B). In the colitis group, t_{max} was delayed to 2 h, and C_{max} was only one-tenth of that observed in healthy animals. In the healthy controls, a prolonged presence of CF in the plasma was found after oral CF administration, where plasma concentrations after 2 and 4 h were significantly higher compared to the rectal administration of CF solution.

The most significant finding, however, was that in both cases, oral or rectal administration, CF bioavailability was around factor three lower when animals suffered from colitis.

Using CF loaded MS highly limited the bioavailability of the entrapped CF (Fig. 4). C_{max} was reached after 2 h in the healthy and colitis group. While bioavailability in the colitis group was comparable to results obtained with the oral solution, MS lowered bioavailability significantly in healthy animals.

In healthy animals, rectal CF administration led to a decrease to around 67% relative bioavailability (Table 2). Besides, the use of MS reduced the systemic availability of CF down to values of around 23%. After colitis induction, systemic CF availability varied between 20% and 35% regardless the formulation or administration route. Statistical analyses revealed no significant differences between all groups.

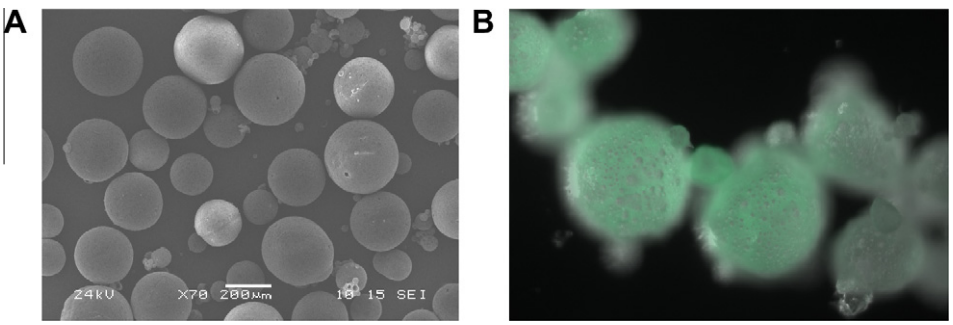

Fig. 1. SEM images of CF containing Eudragit® S100 MS showing the general appearance (A), where the spherical shape as well as a smooth particle surface can be easily seen. Internal structure analysis by fluorescence microscopy confirmed the homogeneous CF distribution throughout the particle matrix (B).

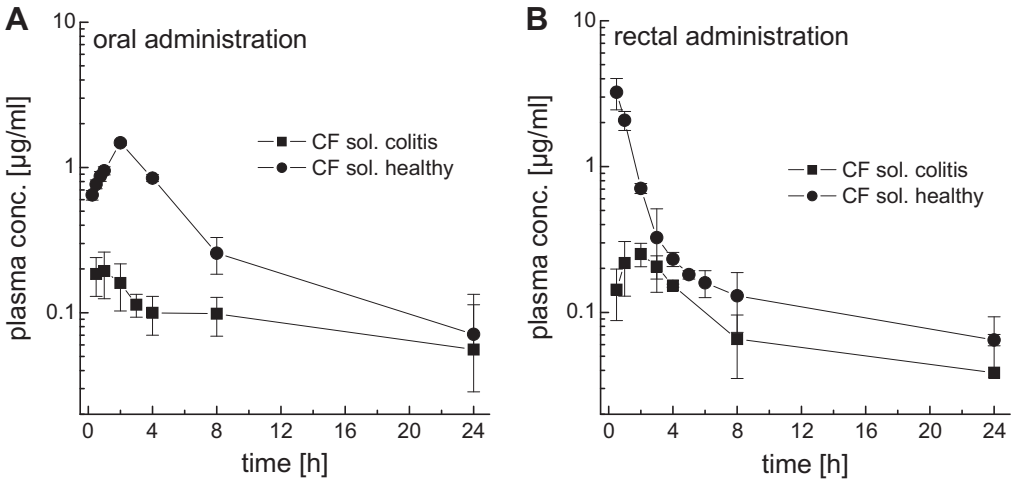

Fig. 3. Pharmacokinetic study of CF after oral (A) or rectal (B) administration comparing healthy rats with a TNBS experimental colitis group ($n = 4$). Data are shown as mean ± SD.

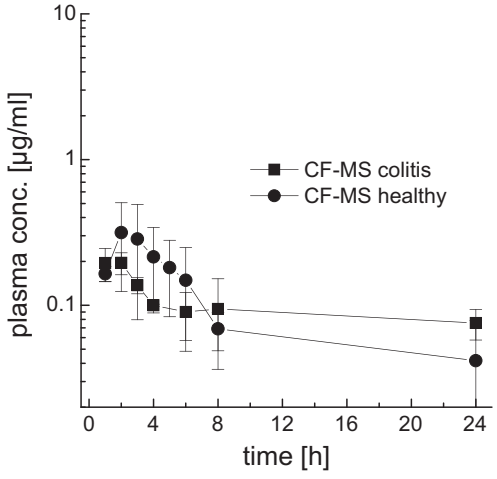

Fig. 4. Pharmacokinetic study of CF loaded MS after oral administration comparing healthy rats with a TNBS experimental colitis group ($n = 4$). Data are shown as mean ± SD.

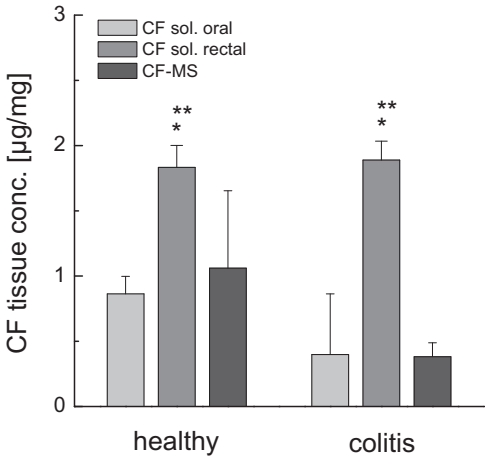

Fig. 5. CF accumulation in colonic tissue after the various administration routes in healthy or colitis group after single dose administration ($n = 4$). $^{*}P < 0.05$ for differences observed between oral CF solution and rectal CF solution, $^{**}P < 0.05$ for differences observed between oral CF solution and CF–MS. Data are shown as mean ± SD.

Table 2
Main pharmacokinetic parameters after oral or rectal administration of CF solution versus oral CF–MS in rats.

	C_{max} (µg/ml)	t_{max} (h)	AUC (µg/ml h)	F_{rel} (%)
Healthy oral	1.63 ± 0.56	2.19 ± 1.34	8.39 ± 1.50	100 ± 17.9
Healthy rectal	2.26 ± 1.62	0.83 ± 0.29	5.62 ± 2.12	67.0 ± 25.3
Healthy MS	0.36 ± 0.21	2.00 ± 0.82	1.90 ± 0.84	22.7 ± 10.0
Colitis oral	0.23 ± 0.05	0.83 ± 0.29	2.98 ± 0.85	35.5 ± 10.1
Colitis rectal	0.25 ± 0.05	2.00 ± 0.00	1.83 ± 0.84	21.8 ± 10.0
Colitis MS	0.20 ± 0.04	1.33 ± 0.58	2.27 ± 0.43	27.1 ± 5.1

3.3. Tissue penetration

CF intra-tissue concentration in colonic tissue samples allowed an insight into drug penetration for the different tissue samples and subsequent changes depending on the disease state. CF amounts accumulated in the colonic epithelial tissue were similar in healthy groups receiving CF orally, independently from the formulation (Fig. 5). Rectal administration, however, led to a higher local drug concentration in the colonic tissue.

Similarly, in the colitis group tissue concentration of CF was not significantly changed when CF was administrated by oral route as solution or MS. In these two cases, drug tissue concentrations were lower than in the healthy control groups for the oral route, but differences were not statistically significant. Again, CF tissue concentrations were increased after rectal solution in colitis versus healthy control group.

4. Discussion

Since gastrointestinal transit in IBD can vary from that in healthy state due to mucosal inflammation and the changes in the normal mechanism of absorption and transit, drug delivery systems require to further adapt to common pathophysiological attributes of UC and CD. Major changes are the abnormalities in fluid and electrolyte absorption and secretion in the inflamed colon [14] and the diarrhoea influencing the transit of the drug delivery system.

Micrometre size drug delivery became interesting at the time when "streaming" was discovered to impede the efficiency of macroscopic drug delivery systems. Principally, two systems are currently proposed to limit influences by "streaming", pellets and MS.

The therapeutic interest is to limit the drug availability mainly to the inflammation site with the goal of high drug concentrations in the inflamed tissue and rather low systemic drug availability. Various drug delivery strategies have been proposed, such as enzyme triggered, pH-sensitive, or time-controlled release. Especially, pH-sensitive MS by oral route have been proposed in this context considered as the most robust system with the aim of local delivery of the entrapped drug towards the inflamed colon.

The pH-dependent drug release from MS is based on diverse formulation approaches described in the literature, while the release mechanisms remained principally the same. Most of them are based on the use of pH-sensitive polymethacrylates, namely Eudragit® L, S, or P-4135F [26–30]. Mainly, all studies reported a more selective delivery to the site of inflammation based on the pharmacologic effects that could further improve the therapeutic efficiency and tolerability. Surprisingly, only few data beyond simple in vitro dissolution and very preliminary in vivo data are available to date. Moreover, pharmacokinetics for all these MS never has been studied in detail.

When CF is administered orally as a solution, high amounts of CF are systemically available due to an early absorption during the passage of the upper parts of the gastrointestinal tract, where CF was found to be mainly absorbed in the duodenum, jejunum, and ileum [31]. This also can explain the lower systemic availability of CF after rectal administration being potentially based on the lower permeability of the colonic tissue for this compound. Similar observations have been reported for other highly water-soluble compounds [32,33].

pH-sensitive MS are known for their ability to retain the drug from early absorption during the passage in the upper intestine and may allow an intact passage until the distal ileal and colonic tissue. Priority was given to the choice of Eudragit® S in order to retain drug release towards distal parts of the colon coming as close as possible to the site of inflammation. Although it is clear that drug release occurs before reaching the inflamed tissue, other pH-sensitive polymers were considered as even less specific since CF release will start earlier.

The relative early onset of CF availability from MS in this study is potentially related to the high water solubility. This characteristic increases the leakage tendency of the drug from the intact particle matrix leading to a premature CF release similar to that found in vitro. Nevertheless, MS delay the CF plasma appearance and ensure a low systemic bioavailability. In healthy groups, one can easily see the potential of pH-sensitive MS to limit systemic availability, which is only one-fifth of the ordinary oral CF solution. Due to the observation that the relative bioavailability from CF–MS is barely differing between healthy and colitis group, it appears rather improbable that the mucoadhesion of the MS could influence the oral bioavailability of CF. This is potentially related to the fact that CF is highly water-soluble and is immediately dissociating from the MS as soon as matrix dissolution takes place.

Remarkably, systemic CF availability is generally reduced in colitis groups regardless the administration route. One very simple reason for the lower CF availability in the case of colitis might be the high amount of liquid on the luminal side due to the diarrhoea. Consequently, the CF elimination is accelerated, and the diffusion gradient towards the basolateral tissue is decreased. The lower systemic availability in the case of colitis also correlates well with the reduced colonic tissue concentrations. This suggests a general mechanism influencing both pharmacokinetic parameters in parallel, such as a flux balance.

Beside the hypothesis that general permeability of the barrier has changed in the inflammation state, it is also probable that the expression of transporter proteins has changed. From recent studies, it is known that absorption can be modified in the state of in inflammation, mainly by changes in the expression of efflux pumps and mucosal metabolism [33]. However, potential changes in other transporters, such as MCT 1–4 transporter, OATP, or MRP1 (involved in the efflux of organic anions), could be a reason for the observed results as well, especially since CF is known to be subjected to OATP transport pathway [31].

The local delivery after rectal CF leads to the highest tissue concentrations in healthy as well as in inflamed tissue. This is in line with other observations [25] underlining the importance of a local drug delivery strategy. Surprisingly, no significant influence on the colonic tissue concentration was observed by the use of MS compared to free CF solution. pH-sensitive MS do not show any increased local drug level at the inflammation site and suggest that the ability to provide local delivery is limited. Following the obtained results, the question arises whether local delivery is essential when a drug is not exhibiting adverse effects since local drug concentration is not increased.

However, this observation may be altered when the therapeutic compound is substrate of certain transport or metabolic systems, e.g. *P*-glycoprotein or cytochrome P450. On the other hand, colonic tissue concentration after rectal administration is observed to be distinctly higher, which may be simply related to the higher local amount directly delivered to the inflammation site and consequently favour rectal administration in any possible case.

To our knowledge, there is no clinical study until now with pH-sensitive MS in humans, and data were mainly obtained from disease models in mice and rats. It should be kept in mind that the applied animal model may differ in terms of changes of efflux systems, transporter expression and other potentially modified disease-specific factors.

After these initial observations, it will be necessary to analyse the multifactorial interplay between the properties of the dosage form, i.e. drug release and mucoadhesive properties, and the biopharmaceutical peculiarities of the inflamed tissue, such as transporter protein expression and all over tissue permeability. This appears very complex, since moreover therapeutic requirements in terms of a local delivery change from one drug to the other.

5. Conclusion

Oral and rectal administration of the model compound CF differed significantly in terms of systemic as well as local availability. This was confirmed in the presence or absence of an experimental colitis, respectively. The entrapment of CF into MS did exhibit only minor influences on the systemic availability of CF at inflammation state. It was generally observed that the systemic availability of drugs correlate with the local concentration in healthy or inflamed tissue without any influence from a potential formulation.

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