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Research paper

In vivo evaluation of thiolated poly(acrylic acid) as a drug absorption modulator for MRP2 efflux pump substrates

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ABSTRACT

Recently, several polymers have been reported to modulate drug absorption by inhibition of intestinal efflux pumps such as multidrug resistance proteins (MRPs) and P-glycoprotein (P-gp). The aim of the present study was to evaluate the efficiency of thiolated poly(acrylic acid) (PAA-Cys) to act as a drug absorption modulator for MRP2 efflux pump substrates in vivo, using sulforhodamine 101 as representative MRP2 substrate. In vitro, the permeation-enhancing effect of unmodified PAA and PAA_{250}-Cys, displaying 580 μmol free thiol groups per gram polymer, was evaluated by using freshly excised rat intestinal mucosa mounted in Ussing-type chambers. In comparison to that of the buffer control, the sulforhodamine 101 transport in the presence of 0.5% unmodified PAA_{250} and 0.5% (w/v) PAA_{250}-Cys was 1.3- and 4.0-fold improved, respectively. In vivo, sulforhodamine 101 solutions containing 4% (w/v) unmodified PAA_{250} or 4% (w/v) thiolated PAA_{250} were orally given to rats. The PAA_{250}-Cys solution increased the area under the plasma concentration–time curve (AUC_{0-12}) of sulforhodamine 101 3.8-fold in comparison to control and 2.2-fold in comparison to unmodified PAA_{250}. This in vivo study revealed that PAA_{250}-Cys significantly increased the oral bioavailability of MRP2 substrate sulforhodamine 101.

1. Introduction

Oral delivery of poorly taken up drugs represents one of the major challenges in pharmaceutical technology since it provides more convenient dosing for patients. Many orally administered drugs, however, must overcome several barriers before reaching their target site. After passing the low pH of the stomach and the possible enzymatic degradation of enzymes of stomach and intestine, the next obstacle is to cross the intestinal epithelium. Intestinal enterocytes form a selective barrier to drugs and xenobiotics. Drugs that cross the apical membrane may be substrates for transmembrane efflux transporters, which extrude compounds back into the lumen [1–3]. These apically polarized efflux transporters are ABC proteins such as multidrug resistance protein (MRP) and P-glycoprotein (P-gp), which are ideally situated to act as the first line of defence by limiting the absorption and accumulation of potentially toxic substances. MRP2 was identified as a 1545-amino acid, 190 kDa protein efflux pump that contains two ATP-binding regions. A schematic presentation of MRP2 is depicted in Fig. 1 [4].

It has been reported previously that inhibition of efflux pumps by various compounds can lead to enhanced transport of drugs across intestinal membranes [5]. MRP2, which preferably transports relatively hydrophilic and anionic compounds [6] and which is expressed mainly in intestine, liver and kidney tubules [7], can be inhibited by a range of substances such as pravastatin and cyclosporine [4,8]. However, most of these inhibitors do not only inhibit efflux pumps as requested but are also absorbed by the gut which leads to systemic toxic undesired adverse effects, caused by high concentrations necessary for sufficient gastrointestinal inhibition of MRP2.

One alternative promising class of efflux pump inhibitors is represented by high molecular mass polymers and copolymers, which offer the advantage of not being absorbed from the gastrointestinal tract, whereby systemic toxic adverse effects can be excluded [9]. Over the last few years thiolated polymers (thiomers) have been widely investigated as promising permeation modulators, featuring improved mucoadhesive, controlled release, permeation-enhancing and enzyme inhibitory properties [10,11]. Besides these properties thiolated polymers have recently been reported to exert an inhibitory effect on efflux pumps. Föger et al., for example, could show that the thiolated polymer chitosan-4-thiobutylamidine (Ch-TBA) could be a useful tool for oral delivery of P-gp substrates [12]. Additionally, delivery systems based on Pluronic P85, Myrj 52 and Ch-TBA were tested in vivo in rats, showing the greatest promise for Ch-TBA [13].

The aim of this study was to further examine the potential of thiolated PAA_{250} in vivo to act as an MRP2 inhibitor, thus improving the oral bioavailability of MRP2 substrates. Sulforhodamine 101 was chosen as the representative substrate for MRP2 [14].

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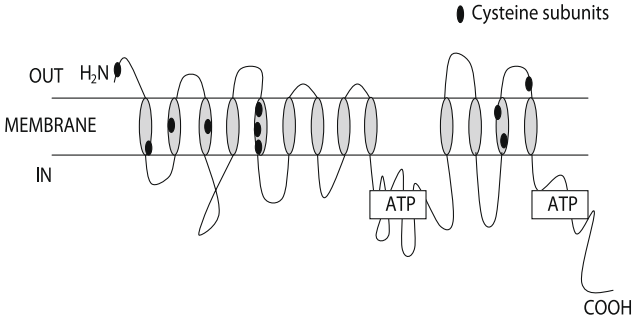

Fig. 1. Schematic presentation of MRP2.

2. Materials and methods

2.1. Materials

All compounds and reagents were purchased from Sigma, Austria. All chemicals were of analytical grade.

2.2. Polymer synthesis

Poly(acrylic) acid–cysteine conjugate of 250 kDa (PAA_{250}-Cys) was synthesized according to a method described previously by our research group [15]. In brief, one gram of PAA was first hydrated in 100 mL demineralised water and the pH value of the PAA solution was adjusted to 6 by the addition of 5 M NaOH. Then, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) in a final concentration of 100 mM was added in order to activate the carboxylic acid moieties of the hydrated polymers. After 15-min incubation under stirring at room temperature, one gram of L-cysteine was added and the pH was readjusted to 6. The reaction mixture was incubated for 3 h at room temperature under stirring. The resulting conjugate was dialysed twice against 0.2 mM HCl, then twice against 0.2 mM HCl containing 1% NaCl and finally again twice against 0.2 mM HCl. After dialysis, the pH of the sample was readjusted to 6. Thereafter, the polymer was freeze-dried at −30 °C and at 0.01 mbar (Benchtop 2K, VirTis, NY, USA) and was stored at 4 °C until further use.

2.3. Determination of thiol/disulfide groups

The amount of thiol groups on the PAA_{250}-Cys conjugate was determined via Ellman's reagent [DTNB, 5,5′-dithiobis(2-nitrobenzoic acid)] as described previously [10].

2.4. In vitro permeation studies across freshly excised rat intestinal mucosa

For permeation studies non-fasting male Sprague Dawley rats weighting between 240 and 250 g were used. After sacrificing the rats, the first 20 cm of the small intestine was immediately removed. The excised intestine was cut into strips of 1.5 cm, rinsed free of luminal contents and mounted in Ussing-type chambers (0.64 cm^2 surface area) without stripping off the underlying muscle layer. The preheated transport medium, containing 250 mM NaCl, 2.6 mM $MgSO_4$, 10 mM KCl, 40 mM glucose and 50 mM $NaHCO_3$ buffered with 50 mM Hepes (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) pH 6.8, was added to the apical and basolateral sites. In order to ensure oxygenation and agitation, a mixture of 95% O_2 and 5% CO_2 was bubbled through each compartment. The Ussing-type chambers were then placed in a water bath at 37 °C. After a 20-min equilibration period, sulforhodamine 101 in a final concentration of 0.001% (w/v) was added to the apical chamber for absorptive (AP to BL) transport. The transport of sulforhodamine 101 was investigated in the absence and presence of the test compounds 0.5% (w/v) unmodified PAA_{250} or 0.5% (w/v) PAA_{250}-Cys. After 0, 30, 60, 90, 120, 150 and 180 min, 100 μL samples were taken out from the acceptor chambers and were replaced by the same amount of fresh transport medium. The amount of permeated sulforhodamine 101 was determined by fluorimetric detection (Fluostar Galaxy) at 485 nm (extinction) and 590 nm (emission) (BMG Labtechnologies) and was calculated by interpolation from an according standard curve. Cumulative corrections were made for previously removed samples. Apparent permeability coefficients (P_{app}) for sulforhodamine 101 were calculated as follows: $P_{app} = Q/Act$, where Q is the total amount permeated throughout the incubation time (μg), A is the diffusion area of the Ussing-type chambers (0.64 cm^2), c is the initial concentration of sulforhodamine 101 in the donor chamber (μg/cm^3), and t is the time of the permeation study (s).

2.5. Preparation of sulforhodamine 101 test solutions

The composition of test solution A, of test solution B, of control A solution (oral) and control B solution (intravenous) is given in Table 1.

2.5.1. Preparation of test solution A and test solution B

Test solution A and test solution B were prepared by dissolving 1.5 mg of sulforhodamine 101 in 15 μL of DMSO and 485 μL of a 0.1% (w/v) aqueous ascorbic acid solution. Thereafter, 20 mg of PAA_{250}-Cys and of PAA_{250}, respectively, were added and the solutions were vortexed for 1 min until the polymers were completely dissolved.

2.5.2. Preparation of control A solution

Control A solution for oral administration was prepared by dissolving 1.5 mg of sulforhodamine 101 in 15 μL of DMSO and 285 μL of a 0.1% (w/v) aqueous ascorbic acid solution.

2.5.3. Preparation of control B solution

Control B solution for intravenous injection was prepared by dissolving 50 μg of sulforhodamine 101 in 5 μL of DMSO and 45 μL of sterile 0.9% (w/v) NaCl solution.

2.6. In vivo evaluation of the test solutions

The protocol for the in vivo studies on animals was approved by the Animal Ethical Committee of Vienna, Austria and it adheres to the Principles of Laboratory Animal Care. For the in vivo studies male Sprague Dawley rats (240–250 g body weight), fasted over night, were used. The rats were divided into four cohorts and were treated separately with the different dosage forms. Two cohorts received 500 μL of test solution A or test solution B, respectively, and one cohort received 500 μL control A solution orally. To determine the oral bioavailability versus i.v. injection, 50 μL of a sulforhodamine 101 solution was intravenously injected into the tail vein. Dosed rats were fasted for the duration of the experiment but

Table 1
Composition of the dosage forms used for in vivo studies in rats.

	Test solution A (500 μL)	Test solution B (500 μL)	Control A (300 μL solution)	Control B (50 μL solution)
Sulforhodamine	1.5 mg	1.5 mg	1.5 mg	50 μg
PAA_{250}	–	20 mg	–	–
PAA_{250}-Cys	20 mg	–	–	–

had free access to water. Blood samples of 120 μL were taken from the tail vein after 40, 80, 120, 160, 200, 240, 360 and 720 min after oral administration of the solutions and after 1, 10, 25, 60, 120, 180 and 240 min after intravenous injection of control B solution. Blood samples were centrifuged (1500 g, 6 min, sigma 3–16 K) and plasma samples were collected and stored at −80 °C until analysis. Prior to analysis, an equal volume of methanol was added to 60 μL aliquots of each plasma sample for deproteinization. The mixture was vortexed for 10 s and centrifuged at 12,000*g* for 10 min (sigma 3–16K). Thereafter, 100 μL of the supernatant was transferred to the wells of 96-well microplates and sulforhodamine 101 concentrations in the samples were measured as already described.

2.7. Statistical and pharmacokinetic data analysis

Pharmacokinetic parameters of sulforhodamine 101 after intravenous and oral administration were calculated by applying a non-compartmental pharmacokinetic analysis to the plasma concentration–time data using the computer software OriginPro 7G SR4 version 7.0552. The area under the concentration versus time curve up to the ultimate measured time point ($AUC_{0\text{-ultimate}}$) was calculated by using the linear trapezoidal rule, using kinetic data collected from individual values. The absolute bioavailability was calculated from the dose-corrected areas under the curves for oral versus intravenous administration.

Statistical data analyses were performed using student's *t* test with $p < 0.05$ as the minimal level of significance. All values are expressed as the means ± SD.

3. Results and discussion

3.1. Characterization of the PAA_{250}-cysteine conjugate

Detailed studies concerning the properties of PAA_{250}-Cys, including an evaluation of the swelling behaviour and mucoadhesive as well as cohesive properties, have been performed already [16]. The isolated polymer–cysteine conjugate was of fibrous structure, white and odourless, and displayed 580 ± 15 μmol free thiol groups per gram polymer ($n = 3$, mean ± SD).

3.2. In vitro permeation studies across freshly excised rat intestinal mucosa

The effect of unmodified as well as thiolated PAA_{250} on the permeation of sulforhodamine 101 across freshly excised rat intestinal mucosa was evaluated in Ussing-type chambers. Sulforhodamine 101, a hydrophilic anionic molecule, was chosen as the representative MRP2 substrate as its efficiency to act as a suitable test compound for MRP2-mediated transport studies had been demonstrated in previous studies [14,17], but Masereeuw et al. also state that several mechanisms are involved in the cellular uptake and secretion of organic anions [18]. As rat jejunum exhibited a higher apical MRP2 expression than ileum [19] and also Cao et al. found much lower MRP2 expression level in colon than in duodenum [20], the first 20 cm of the intestine was used because of the highest MRP2 expression there. A classical indication of efflux pump-mediated involvement in transport kinetics is the difference in permeation rates of compounds in the apical to basolateral and basolateral to apical direction [5]. Additional studies could show that the secretory P_{app} ($7.7 \pm 0.5 \times 10^{-6}$ cm/s) of sulforhodamine 101 was significantly higher than the absorptive P_{app} ($2.8 \pm 0.7 \times 10^{-6}$ cm/s) with an efflux ratio of 2.8, indicating the presence of apically polarized efflux proteins, for which sulforhodamine 101 can act as substrate. PAA_{250}-cys was added in the studies only to the apical site and was not added to the basolateral site, as there are other types of efflux pumps present (e.g. MRP1, MRP3, MRP5 and MRP6) [21]. As PAA_{250}-cys is not a selective inhibitor of MRP2, these pumps will likely be inhibited as well resulting in almost un-interpretable data. In addition, because of the considerable high molecular mass, it is very unlikely that PAA_{250}-Cys will be taken up from the GI tract reaching also the basolateral site of the membrane.

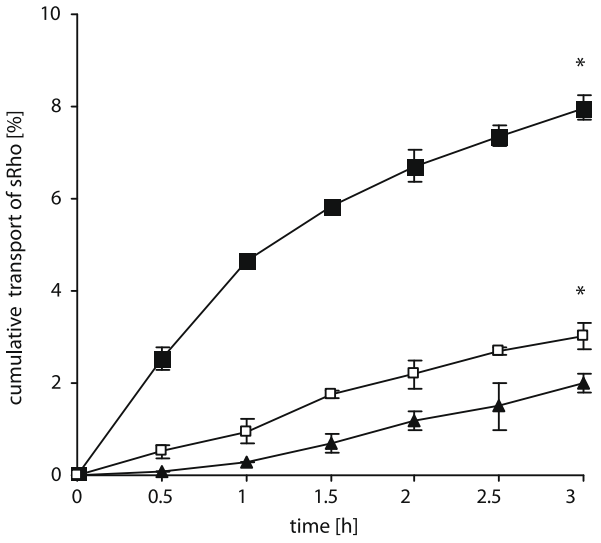

Fig. 2. Permeation studies on freshly excised rat intestinal mucosa mounted in Ussing-type chambers. Effect of 0.5% (w/v) PAA_{250} (white squares) and 0.5% (w/v) PAA_{250}-Cys (black squares) in comparison to that of sulforhodamine 101 (sRho) control (black triangles). Indicated values are means (±SD) of at least four experiments. *Differs from buffer control, $p < 0.001$.

Results of the in vitro permeation studies are shown in Fig. 2 and listed out in Table 2. Due to the addition of 0.5% (w/v) unmodified PAA_{250} to the buffer, sulforhodamine 101 transport was 1.3-fold improved, in comparison to that of buffer control. After addition of 0.5% (w/v) PAA_{250}-Cys, corresponding to 2.9 μM thiol groups/mL, a 4.0-fold higher uptake of sulforhodamine 101 was achieved in comparison to that of buffer control. These results indicated a significantly improved permeation-enhancing effect of thiolated PAA_{250} than of unmodified PAA_{250}, whereas the permeation enhancement of PAA_{250} is in good correlation with that found for other low molecular mass inhibitors in the literature. Naruhashi et al., for example, found that 1 mM probenecid, a low molecular mass MRP2 inhibitor, enhanced the transport of grepafloxacin 1.5-fold in transport experiments by the Ussing-type chamber method in vitro [22]. Also Legen and Kristl reported 1.5-fold increased P_{app} values of fluorescein sodium across rat jejunum when 50 and 100 μM of the MRP inhibitor benzbromarone were added [23].

Permeation-enhancing effects of thiolated polymers, such as PAA-Cys, have already been demonstrated in several studies for various hydrophilic macromolecular drugs [24]. Until recently, thiolated polymers were supposed to enhance intestinal absorption only via paracellular route. However, results of the studies per-

Table 2
Comparison of the in vitro apparent permeability coefficients (*P*app) of sulforhodamine and improvement ratios in the presence of indicated test compounds (means ± SD, $n = 4$).

Test compounds	*P*app (cm/s) × 10^{-6}	Improvement ratio
Buffer	2.9 ± 0.3	–
PAA_{250} (0.5%)	3.9 ± 0.4	1.3
PAA_{250}-Cys (0.5%)	11.5 ± 0.4	4.0

formed within our research group showed that chitosan-4-thiobuthylamidine can act as an inhibitor of P-gp, thus increasing absorption of P-gp substrate rhodamine 123 across rat small intestine in vitro [25] and in vivo [12]. The mechanism responsible for efflux pump inhibition by non-absorbable sulfhydryl compounds such as thiolated polymers seems not to be based on substrate competition or ATP-depletion, as reported for other multidrug resistance reversing agents [26], since thiomers are not taken up from cells due to their high molecular mass. It seems more likely, that thiomers act via: (1) changes in the membrane structure, which affect the conformations of the efflux transport proteins, and/or (2) the incorporation of the bulky polymer may lead to a steric hindrance which limits active efflux. For example, Demina et al. [27] suggested that the ability of polyglycerol-based copolymers to affect the P-gp activity is mediated by their effect on the membrane structure, since P-gp activity is supposed to highly depend on the membrane microviscosity. According to it, the hydroxyl groups of polyglycerols form hydrogen bonds with the head groups of cell membrane lipids, resulting in the incorporation of the polymer into the polar region of the membrane. The same mechanism could offer an explanation for the efflux pump inhibitory effect of thiolated polymers considering the similarity of physico-chemical properties of hydroxyl and sulfhydryl groups.

In addition, PAA_{250}-Cys might also affect the MRP2 activity through a covalent interaction between thiol groups on the polymer and cysteine residues of MRP2 [28]. As illustrated in Fig. 1, MRP2 exhibits two extracellular located cysteine subunits, one cysteine subunit in each of the transmembrane regions 1, 2 and 3, two cysteine subunits in the transmembrane region 12 and three cysteine subunits in the transmembrane region 5 [29,30]. Thiomer sulfhydryl residues may react directly with one cysteine moiety or more than one of these 10 cysteine moieties of MRP2, thus leading to a blockade of the efflux pump. This hypothesis is supported by the observation that the corresponding unthiolated polymers show significantly lower efflux pump inhibitory properties and an increase of the paracellular transport because PAA_{250}-Cys can be excluded as Kast and Bernkop-Schnürch demonstrated that PAA-cys with a molecular mass smaller than 450 kDa does not enhance the paracellular transport at all [31].

Furthermore, strongly improved mucoadhesive properties of thiomers, due to the immobilization of thiol groups on the polymers, could be demonstrated [32]. These strongly improved mucoadhesive properties are based on the formation of disulfide bonds between the thiolated polymer and cysteine residues of mucus glycoproteins [33]. Therefore thiolated polymers may be considered as potentially interesting candidates for efflux pump inhibition.

3.3. In vivo study

The different oral solutions tested in the present study are listed out in Table 1. Plasma concentration–time profiles of sulforhodamine 101 administered intravenously into the tail vein are shown in Fig. 3. Sulforhodamine 101 was eliminated after 4 h, following intravenous injection. Plasma concentration–time curves of sulforhodamine 101 given orally as solution without or with unmodified and thiolated PAA_{250}, respectively, are presented in Fig. 4. After administration of an oral solution the MRP2 substrate sulforhodamine 101 was poorly absorbed with an absolute bioavailabilty of 0.09% and an $AUC_{0\text{-}12}$ of 38 ± 3 ng/mL. Administration of an oral solution containing sulforhodamine 101 and unthiolated PAA_{250} resulted in a significant increase in the plasma concentration of sulforhodamine 101. The $AUC_{0\text{-}12}$ was improved by 68% in comparison to that of the control solution. These results correlate with the findings within a report by Carreno-Gomez and Duncan [34], describing polysaccharides, surfactants, and dendrimers as

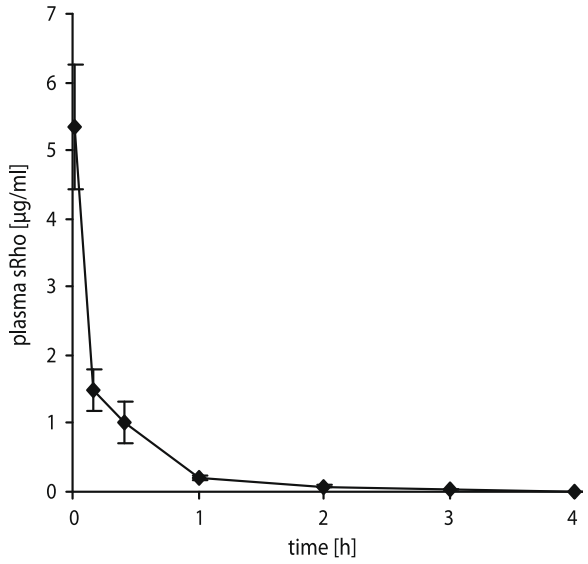

Fig. 3. Plasma concentration of sulforhodamine 101 (sRho) (µg/mL) after intravenous administration to rats. Indicated values are the means (±SD) of four experiments. The intravenous dose of sulforhodamine was 200 µg/kg. Blood samples were taken after 1, 10, 25, 60, 120, 180, 240 min.

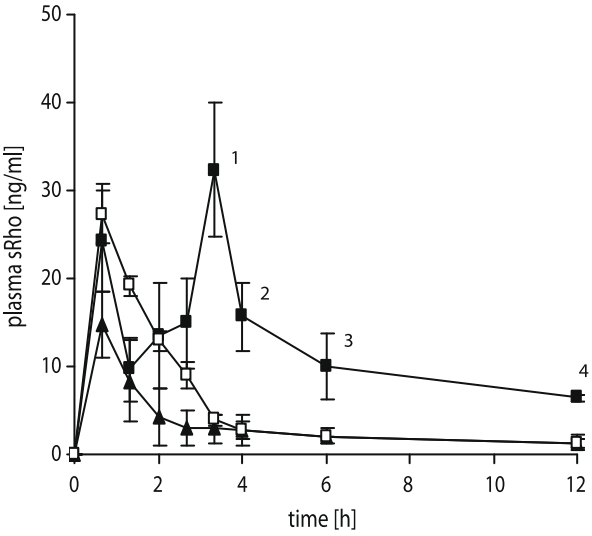

Fig. 4. Plasma curves of sulforhodamine 101 (sRho) (ng/mL) after oral administration of 1.5 mg sulforhodamine in solution (black triangles), in solution with PAA_{250} (white squares) or in solution with PAA_{250}-Cys (black squares). Indicated values are means (±SD) of four experiments. ^{1,2,3,4}Differ from oral solution with PAA_{250}, $p < 0.003$; $p < 0.005$; $p < 0.025$; $p < 0.001$, respectively.

bioavailability enhancers for oral pharmaceutical compositions which exert an inhibitory action on gastrointestinal efflux pumps.

Sulforhodamine 101 plasma concentration was significantly further increased after administration of an oral solution containing sulforhodamine 101 and thiolated PAA_{250}. The $AUC_{0\text{-}12}$ was improved by 276% in comparison to that of the control solution and by 123% in comparison to that of the solution containing unmodified PAA_{250}. The absolute bioavailability of the sulforhodamine 101/PAA_{250}-Cys solution was 0.34% (Table 3). This result is comparable to that of another study in which coadministration of MK571, another MRP2 inhibitor, increased saquinavir absorption 2- to 3-fold without improving the variation in AUCs [35].

Regarding the plasma curves of the different oral solutions, it can be pointed out that the highest plasma concentration through-

Table 3
Main pharmacokinetic parameters calculated after oral administration of the test and control solutions and intravenous administration of sulforhodamine to rats (means ± SD, $n = 4$).

	Test solution A (PAA_{250}-Cys)	Test solution B (PAA_{250})	Control A (oral solution)	Control (i.v. solution)
AUC_{0-12} (ng/mLh)	143 ± 10	64 ± 5	38 ± 3	1386 ± 176
C_{max} (ng/mL)	32	27	15	–
t_{max} (h)	3.3	0.6	0.6	–
Absolute bioavailability (%)	0.34%	0.15%	0.09%	–

out the test period could be reached after 40 min after administration of the oral sulforhodamine 101 solution and the solution containing unthiolated PAA_{250}. Thereafter the plasma level of sulforhodamine 101 decreased continuously, suggesting that sulforhodamine 101 is absorbed in the stomach and/or in the upper intestine. In comparison to these findings, two sulforhodamine 101 plasma level peaks were obtained after oral administration of a sulforhodamine 101/PAA_{250}-Cys solution in all rats (Fig. 5), the lower peak again after 40 min and the higher one after 200 min. Accordingly, sulforhodamine 101 seems to be absorbed not only from the stomach but also from the gut when being administered in combination with a thiolated polymer. Due to the mucoadhesive properties of the thiomer the retention time is prolonged and t_{max} occurs later than in solutions without PAA_{250}-Cys.

Regarding the results from the in vivo study, as MRP2 expression was reported to be highest in the duodenum and subsequently decreases in direction to the terminal ileum and colon where it is only minimal [36], thiolated polyacrylic acid seems to provide the opportunity to enhance absorption of the MRP2 substrate sulforhodamine 101 from MRP2-rich intestinal segments.

4. Conclusion

Within the present study the inhibitory potential of thiolated poly(acrylic acid) on MRP2 efflux protein pumps was investigated in vitro and in vivo. Thereby, PAA_{250}-Cys has shown to provide absorption of a MRP2 substrate from the gut, whereas the same substrate was only absorbed from the stomach when being administered in combination with unmodified PAA_{250}. In comparison to low molecular mass inhibitors, thiolated polymers offer additional advantages such as mucoadhesive properties facilitating an intimate contact with the area of drug absorption. Furthermore, thiomers are not absorbed from the intestine due to their high molecular mass, whereby systemic toxic adverse effects can be excluded. All these features should therefore challenge the development of new drug delivery systems for MRP2 substrates based on thiolated poly(acrylic acid).

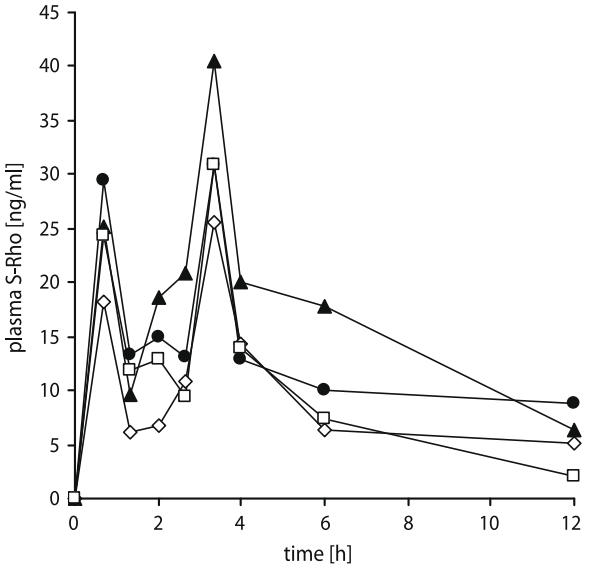

Fig. 5. Plasma curves of sulforhodamine 101 (sRho) (ng/mL) of all four rats after oral administration of 1.5 mg sRho in solution with PAA250-Cys.

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