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Research Paper

Susceptibility of glucocorticoids to colonic metabolism and pharmacologic intervention in the metabolism: implication for therapeutic activity of colon-specific glucocorticoid 21-sulfate sodium at the target site

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Abstract

Objectives The systemic side effects of glucocorticoids have prevented their long-term use for treatment of inflammatory bowel disease. Colon-specific delivery of glucocorticoids has been adopted as a strategy to circumvent the toxicological trouble. Glucocorticoids delivered to the large intestine might undergo metabolisms by colonic microflora, which should affect therapeutic availability at the target site. It was investigated whether the susceptibility of glucocorticoids to the colonic metabolisms and pharmacologic intervention in the metabolism could modulate the therapeutic availability of colon-targeted glucocorticoids.

Methods Various glucocorticoids and their derivatives, glucocorticoid 21-sulfate sodium compounds, were incubated in the cecal contents in the presence or absence of reduction inhibitors and the change in the levels of the drugs was monitored.

Key findings The accumulation profiles of the corresponding glucocorticoids liberated from glucocorticoid 21-sulfate sodium compounds vary, depending on the metabolic susceptibility of glucocorticoids. Reduction inhibitors prevented the cecal metabolisms of glucocorticoids, which was most prominent for prednisolone (PD) and methylprednisolone (MP). Moreover, reduction inhibitors increased the accumulated amount of MP and PD released from PD- and MP-21-sulfate sodium in the cecal contents.

Conclusions Our data provide information useful for selection of a glucocorticoid and a pharmacologic strategy for the design of an efficient colon-specific glucocorticoid prodrug.

Introduction

Glucocorticoids, which have been used frequently for the treatment of inflammatory bowel diseases,[1,2] are well absorbed in the upper intestine and only a limited fraction of the orally administered dose is delivered to the inflammatory site in the distal ileum or colon. Long-term administration causes serious side effects, such as osteoporosis, hypertension, edema, diabetes or decreased immunity by the systematically absorbed drugs.[3,4] For this reason, they are used for intensive therapy in large doses and are not for long-term maintenance therapy for the prevention of relapse. To reduce the serious side effects caused by systemic absorption and increase the accessibility of the drug to the site of action, development of colon-specific delivery of a glucocorticoid is highly desirable.[5]

Prodrugs aiming at the delivery of glucocorticoids to the colon have been introduced which use polymers such as dextran or highly hydrophilic small molecules such as glucuronic acid, monosaccharides and sulfuric acid as a colon-specific pro-moiety.[6–9] Limited absorption of highly polar molecules and polymers allows the delivery of such prodrugs to the colon. Various microbial enzymes, including glycosidases and sulfatases, which originate from microbes in the large intestine, are usually responsible for the hydrolysis of the linkages between the promoieties and glucocorticoids to

produce the active drug in the large intestine.[5] Recently, it was reported that the sulfate ester derivatives of methylprednisolone (MP) and dexamethasone elicit colon targetability, leading to therapeutic advantages for treatment of experimental colitis.[10,11]

The 4,5 double bond and the 3-keto group on A ring of the glucocorticoid, which are essential for an anti-inflammatory effect,[12] seem to be susceptible to reductive metabolism, which is a highly probable metabolic reaction in the colon. Therefore, in designing a potential colon-specific prodrug of a glucocorticoid, reductive susceptibility of glucocorticoids should be taken into consideration. In addition to the adoption of a metabolically stable glucocorticoid, the co-administration of an inhibitor against reductive metabolism has been suggested as a strategy to overcome the hurdle of reductive inactivation in the development of an efficient colon-specific prodrug of a glucocorticoid.[5] In this study, the metabolic tendencies of various glucocorticoids were investigated in the cecal contents of rats, which was related to the accumulation profiles of the active drugs released from colon-specific prodrugs of the glucocorticoids. Moreover, it was investigated whether the reduction inhibitors from synthetic and natural sources could intervene in the cecal metabolism of glucocorticoids and subsequently modulate the accumulation profiles of metabolism-sensitive glucocorticoids liberated from their colon-specific prodrugs. Based on our investigation, it was suggested that co-administration with some reduction inhibitors such as glycyrrhizin or carbenoxolone, which prevented the reductive metabolism of glucocorticoids, could increase the therapeutic availability of glucocorticoids susceptible to large intestinal metabolism. Furthermore, we discussed the relationship between structure and cecal metabolism of glucocorticoids, which should be useful information for selecting a glucocorticoid for colon-specific delivery.

Materials and Methods

Materials and instruments

Glucocorticoids and reduction inhibitors including glycyrrhizin, carbenoxolone and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were reagent-grade, commercially available products. Buffer solutions were prepared as described in USP XXIII. Male Sprague–Dawley (SD) rats (200–270 g, 7–8 weeks old) were purchased from Daehan Biotec Co. Ltd (Daegu, Republic of Korea). The animal protocol used in this study has been reviewed and approved by the Pusan National University–Institutional Animal Care and Use Committee (PNU-IACUC) according to their ethical procedures and scientific care (approval number PNU-2009–0029). IR spectra were recorded on a Bomem MB 100 FT-IR spectrophotometer (Bomem, Quebec, Canada). ^{1}H-NMR spectra were taken on a Varian AS 500 spectrometer (Varian, Palo Alto, CA, USA). Elemental analysis was carried out by a VarioEL III (ELEMENTAR Analysensysteme GmbH, Hanau, Germany). The HPLC system consisted of model 305 and 306 pumps, a 117 variable UV detector, a model 234 autoinjector, a model 805 manometric module and a model 811C dynamic mixer from Gilson (Middleton, WI, USA).

Preparation of various glucocorticoid 21-sulfate sodium compounds

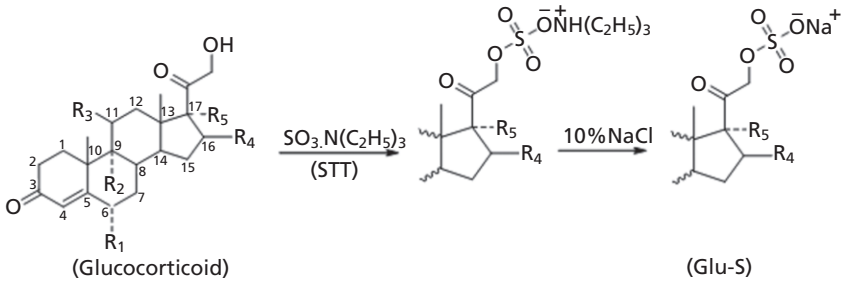
To the solution of prednisolone (PD; 5.0 mmol) in 8 ml of anhydrous pyridine was added sulfurtrioxide triethylammine complex (1.72 g, 9.5 mmol) in portions with stirring at 56–60°C. After the addition was completed, the reaction mixture was evaporated under reduced pressure to remove the solvent. The residue, PD 21-sulfate triethylammonium, was suspended in a solution of 10–25% NaCl with mechanical stirring for 1 h. The same reaction was carried out using various glucocorticoids. The resulting precipitate, the respective glucocorticoid 21-sulfate sodium (sulfate-conjugated glucocorticoid, Glu-S), was collected by suction filtration and recrystallized from absolute ethanol. The synthetic scheme and the structures of the reactants and products are shown in Figure 1.

HPLC analysis

The concentration of glucocorticoids and various Glu-S compounds was measured by a reversed-phase HPLC. The standard or blank solution (1 ml) was mixed on a vortex mixer for 2 min, centrifuged at 14 000 rpm for 5 min and filtered through a membrane filter (0.45 μm). A portion of the filtrate (20 μl) was injected on a μBondapak C_{18} (Waters, Milford, MA, USA; 3.9 × 300 mm, 10 μm) column and eluted with mobile phase at a flow rate of 1.0 ml/min. The mobile phase consisted of a solution of acetonitrile in 0.067 M phosphate buffer, pH 4.5, which was filtered through a 0.45 μm membrane filter before use. The eluate was monitored by measuring the absorption at 248 nm at a sensitivity of AUFS 0.001. The Gilson 712 HPLC software was used for data analysis. The retention time and the constituents of the mobile phase for various glucocorticoids and the respective Glu-S compounds are listed in Table S1. Calibration curves for the glucocorticoids and Glu-S compounds in the various biological media were constructed from the concentration versus the peak area on HPLC ($n = 6$ for each concentration).

Analysis of the glucocorticoid and Glu-S in various biological specimens

A non-fasting male SD rat (200–270 g, 7–8 weeks old) fed with pellet feed (Samtako, Kyung-gi-do, Republic of Korea) was anesthetized by diethyl ether and a midline incision



(Glucocorticoid)	C1,C2	R_1	R_2	R_3	R_4	R_5
Cortisone	single	H	H	=O	H	OH
Hydrocortisone	single	H	H	OH	H	OH
Fludrocortisone	single	H	F	OH	H	OH
Prednisolone	double	H	H	OH	H	OH
Methylprednisolone	double	CH_3	H	OH	H	OH
Triamcinolone	double	H	F	OH	β-OH	OH
Betamethasone	double	H	F	OH	β-CH_3	OH
Flucinolone acetonide	double	F	F	OH	acetonide	

Figure 1 Reaction scheme and structures of reactants and products.

was made and gastrointestinal tract segments were obtained. The tissue and contents of the stomach, proximal small intestine and distal small intestine were homogenized separately and diluted with pH 4.5 isotonic acetate buffer (for the stomach) and pH 6.8 isotonic phosphate buffer (for the small intestine) to 20% (w/v). The contents of the cecum were diluted with pH 6.8 isotonic phosphate buffer to 20% (w/v). The pH values for the stomach and intestinal tract were adopted as 4.5 and 6.8, respectively, considering the reported pH values for the rat gastrointestinal tract.[8] To each 2 ml portion of the above suspensions was added 2 ml of each stock solution of glucocorticoids or Glu-S compounds (20, 40, 100 or 200 μg/ml), which was dissolved in an appropriate buffer solution. A 200 μl portion of the mixture was removed and centrifuged at 14 000 rpm for 3 min. To a 100 μl portion of the supernatants was added 900 μl of methanol to precipitate protein in the samples. This solution was vortexed for 2 min and centrifuged for 5 min at 14 000 rpm. The supernatants provided the standard solutions of the glucocorticoids or the Glu-S compounds at concentrations of 1, 2, 5 or 10 μg/ml, respectively, in various biological specimens. Fifty microlitres of standard or sample solutions filtered through a membrane filter (0.45 μm) was used for the HPLC analysis.

Incubation of various glucocorticoids with the cecal contents of rats

The cecal contents were diluted with pH 6.8 phosphate buffer solution to a concentration of 20% (w/v) and filtered through a piece of medical gauze to remove the fibrous mass. Solutions of the glucocorticoids were prepared by dissolving each compound in ethanol/pH 6.8 isotonic phosphate buffer solution (1/9) at a concentration of 0.3 mM. A 2 ml portion of each glucocorticoid solution was mixed with 2 ml of 20% cecal contents suspension in pH 6.8 phosphate buffer solution. The mixture was incubated at 37°C in a shaking incubator. The whole process was carried out in a glove box, which was previously displaced by nitrogen. At an appropriate time interval, the concentration of each glucocorticoid in the sample was determined by HPLC as described previously.

Incubation of glucocorticoids with the cecal contents of rats in the presence of various reduction inhibitors

Solutions of either the glucocorticoids or natural and synthetic reduction inhibitors (glycyrrhizin, carbenoxolone, morin, rutin, n-octyl gallate) were prepared by dissolving each compound in ethanol/pH 6.8 isotonic phosphate buffer (1/9) at a concentration of 0.6 mM. A 1 ml portion of each glucocorticoid solution was mixed with 1 ml of each inhibitor solution followed by addition of 2 ml of 20% cecal contents in pH 6.8 isotonic phosphate buffer solution. The samples were incubated at 37°C in a shaking incubator under nitrogen. Drugs in samples were analysed by HPLC.

Incubation of Glu-S compounds with the contents of rat gastrointestinal tract segments

Homogenates of tissue and the contents of the stomach or small intestine and suspension of the cecal contents were prepared as described previously. A 2.5 ml portion of each gastrointestinal tract contents suspension was mixed with 2.5 ml of each Glu-S solution in pH 6.8 isotonic phosphate buffer (0.6 mM). The mixture was incubated at 37°C in a shaking incubator. The whole process was carried out in a glove box, which was previously displaced by nitrogen (except for an incubation study using the homogenates of tissue and contents of the stomach or small intestine). At an appropriate time interval, the concentration of each glucocorticoid in the samples was determined by HPLC.

Incubation of Glu-S compounds with the cecal contents of rats in the presence of various reduction inhibitors

Solutions of either Glu-S compounds or reduction inhibitors were prepared by dissolving each compound in pH 6.8 isotonic phosphate buffer at a concentration of 0.6 mM. A 1 ml portion of each Glu-S solution was mixed with 1 ml of each inhibitor solution followed by addition of 2 ml of 20% cecal contents in pH 6.8 isotonic phosphate buffer solution. The samples were incubated at 37°C in a shaking incubator under nitrogen. Drugs in samples were analysed by HPLC.

Statistical analysis

The results are expressed as mean ± SE. At least six replicate experiments were carried out for *in vitro* hydrolysis studies. The two-way ANOVA or one-way ANOVA followed by post-hoc test was used to assess the statistical significance ($P < 0.05$) of results. XLSTAT® Software (Addinsoft, Inc, New York, USA) was used for the statistical analysis.

Results

Preparation of Glu-S compounds

21-Sulfate ester triethylammonium derivatives of triamcinolone, betamethasone, fludrocortisone, fluocinolone acetonide, PD and MP were prepared by reacting each glucocorticoid with sulfurtrioxide triethylamine complex (STT) using anhydrous pyridine as a solvent. The reaction process was monitored by TLC to adjust the reaction time and the ratio of the reactants, which were critical to the suppression of by-product formation. The reaction proceeded rapidly and was terminated when a trace of by-product was monitored on TLC. The reaction temperature was maintained at 55–60°C. The optimum ratio for STT/glucocorticoid was 1.0, 1.2, 2.0, 3.0, 1.7 and 1.9 for triamcinolone, betamethasone, fludrocortisone, fluocinolone acetonide, PD and MP, respectively. Conversion of 21-sulfate ester triethylammonium derivatives of glucocorticoids to their corresponding sodium salts proceeded readily by reacting them with concentrated NaCl solution (10–25%). The overall yield was 60–80%. The structures of Glu-S compounds were confirmed from the data of IR and ^{1}H-NMR spectra. Data from the elemental analysis were in good agreement with the calculated values for C, H and S (within 0.5% from the calculated values). The physical data of the synthesized Glu-S compounds are listed in Table 1. Infrared spectra showed two strong absorption bands originating from –S = O asymmetric and symmetric stretching of sulfate ester double bond around 1250–1280 and 1022–1034 cm^{-1}, respectively. The most characteristic change in ^{1}H-NMR spectra was the downfield shift of the two protons on C-21 to 0.5 ppm by the introduction of sulfate ester group. NMR and IR data of parent glucocorticoids are shown in Table S1.

The cecal metabolisms of various glucocorticoids

The glucocorticoids were incubated with 10% cecal contents of rats at 37°C. At an appropriate time interval, the amount of each glucocorticoid remaining in the medium was analysed by HPLC. The results are shown in Figure 2a. It is seen that the amount of glucocortioids decreased to a varied degree as the incubation time extended. The concentration of fluocinolone acetonide did not change at all during the whole 24 h incubation period. Cortisone decreased most drastically and was zero 4 h after the incubation. The susceptibility toward the cecal metabolisms was in the order cortisone > hydrocortisone > PD > MP, fludrocortisone > triamcinolone > betametasone > fluocinolone acetonide.

The cecal conversion of various Glu-S to the respective glucocorticoids

After administration of a colon-specific prodrug, the therapeutic concentration of its parent drug at the target

Table 1 Physical data of various glucocorticoid 21-sulfate sodium compounds

Glu-S	Melting point (°C)	IR (nujol, cm^{-1})	^{1}H-NMR (D_2O, ppm)	Elemental analysis
PDS	120 (D)	3450, 1710, 1692, 1259, 1034, 920	0.78(s,3H), 1.30(s,3H), 4.89(ABq,2H), 5.94(s,1H), 6.21(d,1H), 7.42(d,1H)	C21H27NaO8S Calcd: C, 54.54; H, 5.88; S, 6.93 Found: C, 53.43; H, 5.32; S, 7.12
MPS	156 (D)	3377, 1735, 1648, 1598, 1281, 1056	0.87(s,3H), 1.40(s,3H), 4.95(ABq,2H), 6.07(s,1H), 6.34(d,1H), 7.56(d,1H)	C22H29NaO8S Calcd: C, 55.45; H, 6.13; S, 6.73 Found: C, 54.66; H, 5.94; S, 6.98
BTS	185 (D)	1719, 1662, 1611, 1455, 1259, 1034	1.00(s,3H), 1.55(s,3H), 4.9~5.2(ABq,2H), 6.23(s,1H), 6.43(d,1H), 7.54(d,1H)	C22H28FNaO8S Calcd: C, 53.43; H, 5.71; S, 6.48 Found: C, 54.23; H, 5.97; S, 6.40
TRS	171	3365, 1719, 1666, 1604, 1255, 1032	1.00(s,3H), 1.55(s,3H), 4.9~5.2(ABq,2H), 7.3(m,10H)	C21H26FNaO9S Calcd: C, 50.80; H, 5.28; S, 5.98 Found: C, 51.54; H, 5.58; S, 6.01
FAS	147	3370, 1729, 1666, 1612, 1255, 1048	0.88(s,3H), 1.20(s,3H), 1.41(s,3H), 1.51(s, 3H) 4.37~4.78(ABq,2H), 6.21(d,1H), 7.43(d,1H)	C24H29F2NaO9S Calcd: C, 51.98; H, 5.27; S, 5.78 Found: C, 52.64; H, 5.57; S, 5.91
FCS	125	3520, 3245, 1722, 1643, 1250, 1022	0.78(s,3H), 1.30(s,3H), 1.41(s,3H) 4.81~5.00(ABq,2H), 5.74(s,1H)	C21H26FNaO8S Calcd: C, 52.49; H, 5.45; S, 6.67 Found: C, 51.99; H, 5.28; S, 6.59

PDS, PD 21-sulfate sodium; MPS, MP 21-sulfate sodium; BTS, betamethasone 21-sulfate sodium; TRS, triamcinolone 21-sulfate sodium; FAS, fluocinolone acetonide 21-sulfate sodium; FCS, fludrocortisone 21-sulfate sodium; D, decomposed.

site (large intestine) could be determined by rates of colonic metabolisms of the prodrug and the parent drug.[5] To examine this notion for Glu-S compounds, colon-specific prodrugs of glucocorticoids, various Glu-S compounds were incubated with 10% cecal contents. At appropriate time intervals, the concentration of remaining Glu-S compounds and the released corresponding glucocorticoids was analysed by HPLC. The results are shown in Figures 2b and c. Fluocinolone acetonide 21-sulfate sodium was not hydrolysed during the 24 h incubation period. Naturally fluocinolone acetonide was not detected from the incubation medium. Triamcinolone 21-sulfate sodium hydrolysed most rapidly among the tested compounds and produced triamcinolone. All of the Glu-S compounds except fluocinolone acetonide 21-sulfate ester sodium hydrolysed to produce the respective glucocorticoids. The amount of glucocorticoids produced was less than that of the hydrolysed prodrugs to a varied degree and this correlated to the metabolic susceptibility of the glucocorticoids. Overall, the more susceptible a glucocorticoid is, the less glucocorticoid is produced from the Glu-S.

Effect of reduction inhibitors on the cecal metabolism of various glucocorticoids

Our data above demonstrate that the achievable maximal concentrations and the accumulated amount of glucocorticoids decreased as metabolic susceptibility increased. To examine whether the cecal metabolisms could be modulated pharmacologically, various glucocorticoids were incubated with 10% cecal contents in the presence of equimolar reductive inhibitors, glycyrrhizin or carbenoxolone, which might have an inhibitory activity against the cecal reductive metabolisms of glucocorticoids.[13,14] As shown in Figures 3a and b, the reduction inhibitors exhibited inhibitory effects in early session of the incubation period (Figure 3a). As the incubation period extended to 12 h, cortisone, which was most susceptible to the cecal metabolism (Figure 2a), was barely detectable in the medium (Figure 3b). Triamcinolone and betamethasone, which were resistant to the reduction, were not affected significantly by the reduction inhibitors. The inhibitory effect was most prominent with PD and MP, which showed moderate metabolic susceptibility. For MP and PD, pharmacologic intervention in the reduction metabolism was further examined using other reduction inhibitors such as n-octyl gallate, rutin and morin.[15] The structures of the reduction inhibitors, including glycyrrhizin or carbenoxolone, are shown in Figure S1. Figure 4a shows the results when MP was incubated with 10% cecal contents in the presence of a variety of equimolar reduction inhibitors. When n-octyl gallate was added in the incubation medium, MP did not change at all throughout the incubation period. The inhibitory activity was in the order of n-octyl gallate > rutin > carbenoxolone > glycyrrhizin > morin. Figure 4b shows the results when PD was incubated with 10% cecal contents in the presence of a

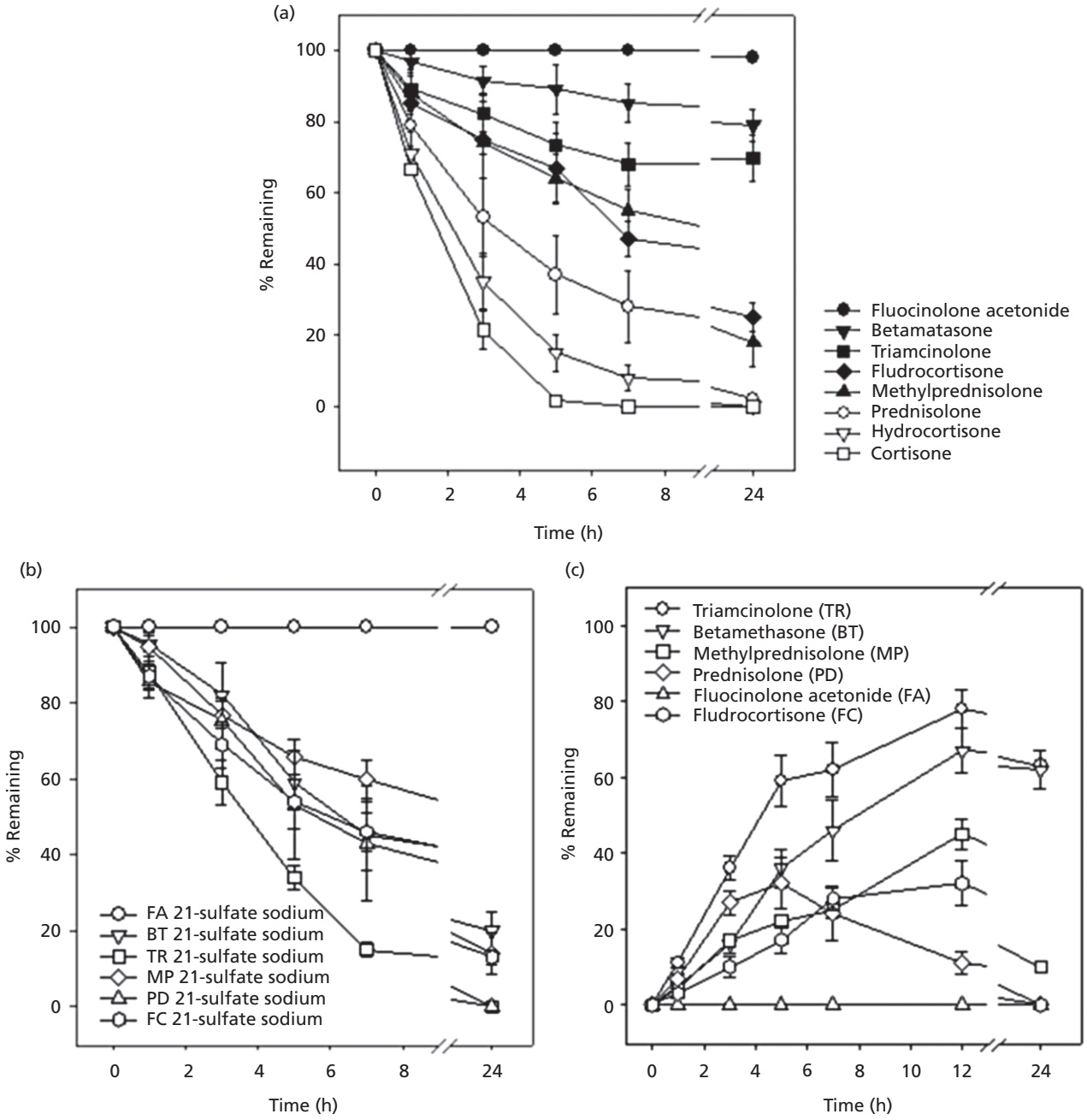


Figure 2 The metabolic susceptibility of various glucocorticoids and conversion of various Glu-S compounds to the respective glucocorticoids in the cecal contents. (a) Various glucocorticoids (0.3 mM) were incubated with 10% cecal. In an appropriate time interval, the concentration of each glucocorticoid in the samples was determined by HPLC. (b) and (c) Various Glu-S compounds (0.3 mM) were incubated with 10% cecal contents. At an appropriate time interval, the concentration of each (b) glucocorticoid and (c) Glu-S in the samples was determined by HPLC.

variety of equimolar reduction inhibitors. Again, n-octyl gallate completely inhibited the reductive metabolism of PD during the 24 h incubation period. The inhibitory activity was in the order of n-octyl gallate > carbenoxolone > rutin > glycyrrhizin > morin. Carbenoxolone exhibited relatively greater inhibitory effect toward PD than MP.

Effect of reduction inhibitors on the accumulation profiles of the corresponding glucocorticoids liberated from Glu-S in the cecal contents

Since reduction inhibitors intervened in the cecal metabolisms of glucocorticoids, we examined whether the inhibitors

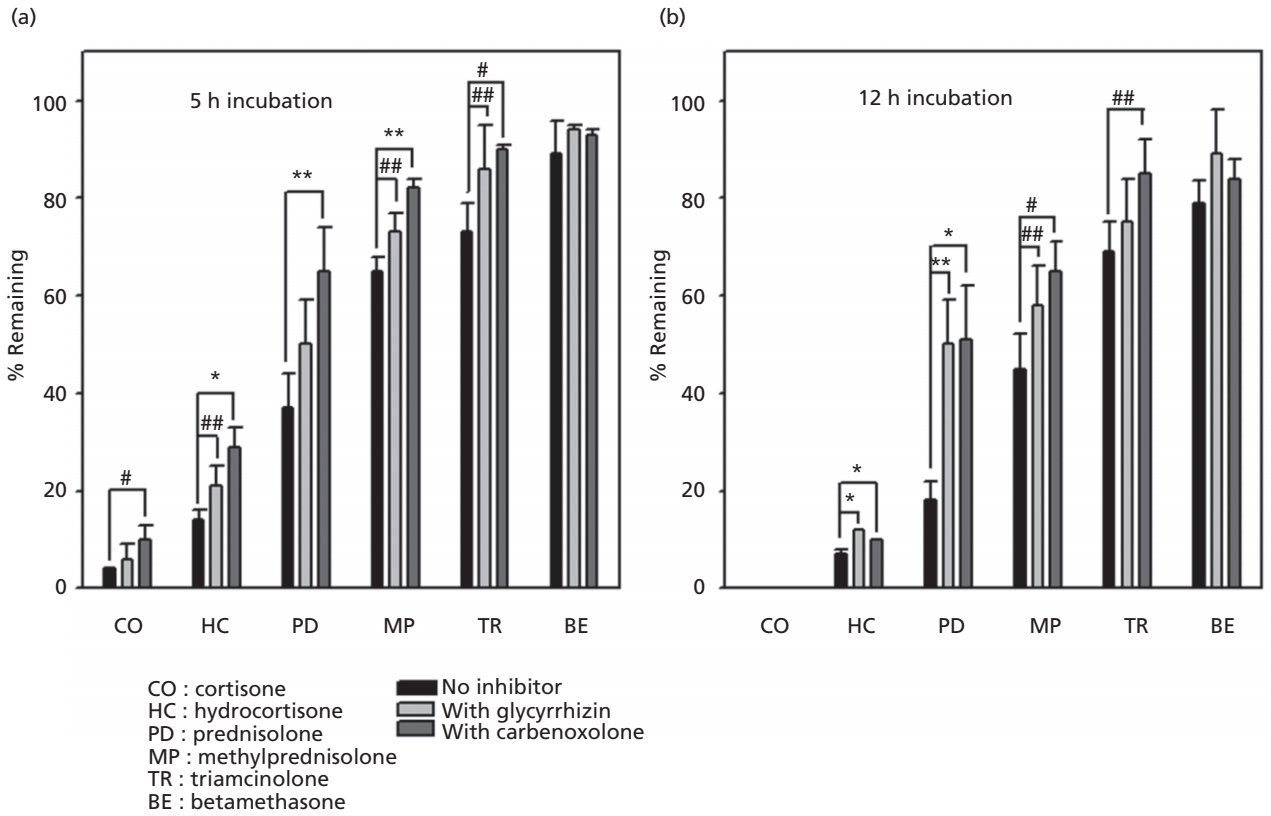


Figure 3 Effect of reduction inhibitors, glycyrrhizine and carbenoxolone, on the cecal metabolism of various glucocorticoids. Various glucocorticoids (0.3 mM) were incubated with 10% cecal contents in the presence of an equimolar reductase inhibitor, glycyrrhizin or carbenoxolone. At (a) 5 h and (b) 12 h after incubation, the concentration of each glucocorticoid in the samples was determined by HPLC. The two-way ANOVA followed by the post-hoc test (Fisher's least significant difference method) was used to assess the statistical significance ($P < 0.05$) of results. *$P < 0.0001$ vs. no inhibitor, **$P < 0.001$ vs. no inhibitor, #$P < 0.01$ vs. no inhibitor, ##$P < 0.05$ vs. no inhibitor.

affected the accumulation profiles of MP and PD when co-incubated with MP 21-sulfate sodium (MPS) or PD 21-sulfate sodium (PDS) in the cecal contents. MP and PD were chosen because the cecal metabolisms of them were modulated most by reduction inhibitors. Before carrying out this experiment, the effect of the inhibitors on the bioactivation (hydrolysis, desulfation) of the sulfate conjugated prodrugs, MPS and PDS, was tested. The reduction inhibitors n-octyl gallate, carbenoxolone, rutin and glycyrrhizin that exhibited a significant inhibitory effect were incubated with the prodrugs in the cecal contents and the disappearance of them was monitored. As shown in Figures 5a and b, while n-octyl gallate and rutin significantly deterred the disappearance of MPS and PDS, the influence of the other inhibitors, carbenoxolone and glycyrrhizin, was not great. Therefore, either PDS or MPS was incubated with the cecal contents in the presence of either carbenoxolone or glycyrrhizin and the accumulation profiles of MP and PD were examined. As shown in Figures 6a and b, on incubation without the inhibitors the levels of PD and MP increased then began to decrease, therefore two phases are presented in the accumulation profiles while the breakpoint in the profile for PD is shown much earlier. The inhibitors caused a steady increase in the level of PD, which exceeded the level of PD (obtained from incubation without the inhibitors) at around 7 h. For MP, the level of MP was kept higher through the experimental period than MP obtained from incubation without the inhibitors. These results suggest that reduction inhibitors could positively modulate the therapeutic availability of glucocorticoids susceptible to reductive metabolisms in the large intestine.

Discussion

In this study our data demonstrate that the susceptibility of glucocoticoids to the cecal metabolisms affected the accumulation profiles of the glucocoticoids on incubation of their colon-specific prodrugs, sulfate-conjugated glucocorticoids, in the cecal contents and reduction inhibitors could positively modulate the cecal availability of

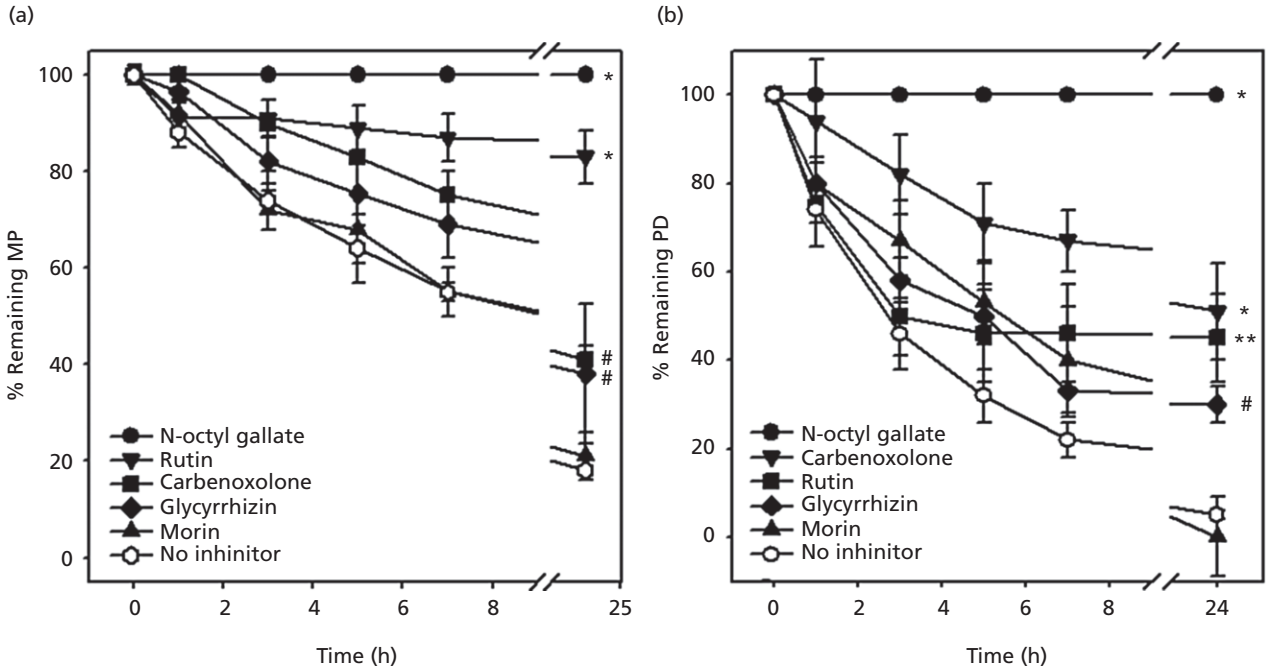


Figure 4 Effect of various reduction inhibitors on the cecal metabolism of MP and PD. Either MP (0.3 mM) or PD (0.3 mM) was incubated with 10% cecal contents in the presence of a variety of equimolar reductase inhibitors. At an appropriate time interval, the concentration of (a) MP and (b) PD in the samples was determined by HPLC. The one-way ANOVA followed by the post-hoc test (Fisher's LSD method) was used to assess the statistical significance ($P < 0.05$) of results. *$P < 0.0001$ vs. no inhibitor, **$P < 0.001$ vs. no inhibitor, #$P > 0.05$ vs. no inhibitor.

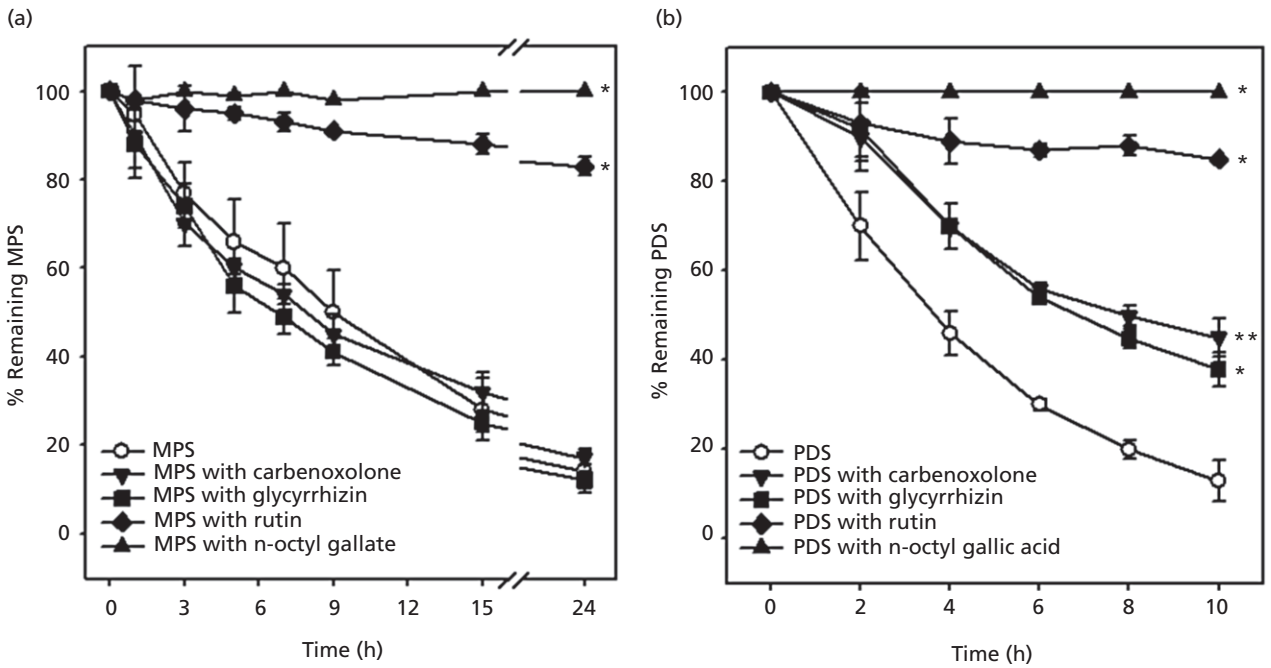


Figure 5 Effect of various reduction inhibitors on the cecal metabolism of MP 21-sulfate sodium and PD 21-sulfate sodium. Either MPS (0.3 mM) or PDS (0.3 mM) was incubated with 10% cecal contents in the presence a variety of equimolar reductase inhibitors. At an appropriate time interval, the concentration of (a) MPS and (b) PDS in the samples was determined by HPLC. The one-way ANOVA followed by the post-hoc test (Fisher's LSD method) was used to assess the statistical significance ($P < 0.05$) of results. *$P < 0.0001$ vs. (a) MPS or (b) PDS, **$P < 0.001$ vs. (a) MPS or (b) PDS.

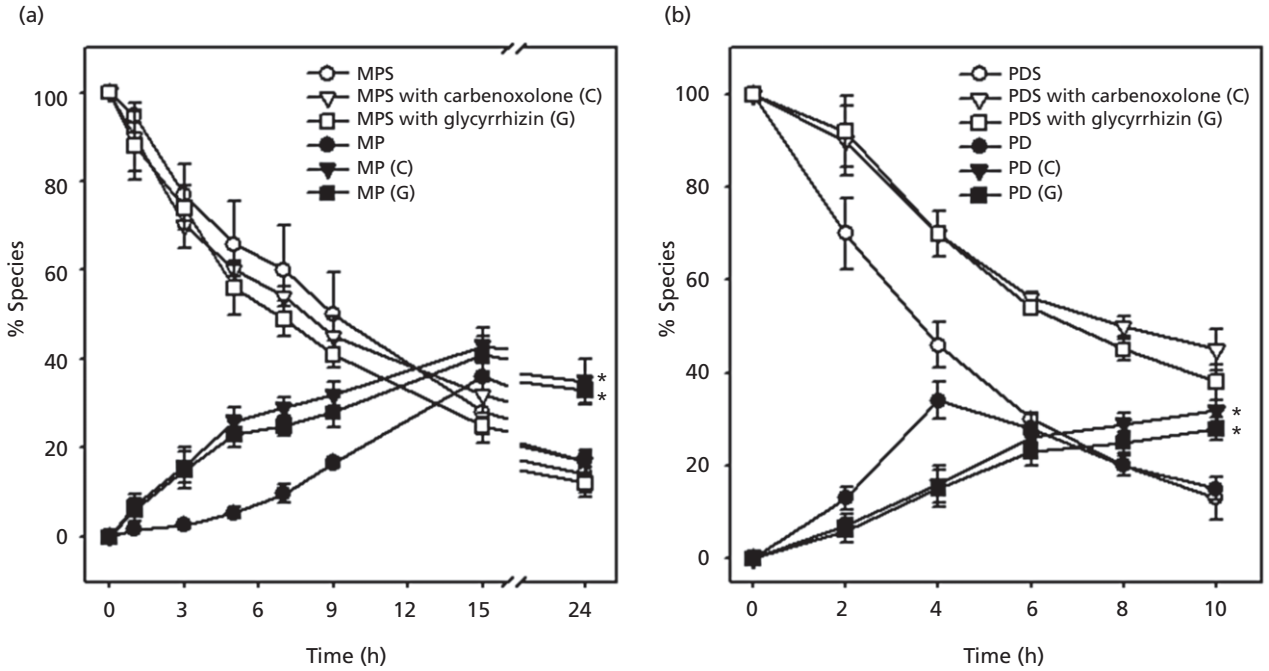


Figure 6 Effect of reduction inhibitors, glycyrrhizine and carbenoxolone, on the accumulation profiles of the corresponding glucocorticoids liberated from MPS and PDS in the cecal contents. Either MPS (0.3 mM) or PDS (0.3 mM) was incubated with 10% cecal contents in the presence an equimolar reductase inhibitor, glycyrrhizine or carbenoxolone. At an appropriate time interval, the concentration of (a) MP and MPS and (b) PDS and PD in the samples was determined by HPLC. The one-way ANOVA followed by the post-hoc test (Fisher's LSD method) was used to assess the statistical significance ($P < 0.05$) of results. *$P < 0.05$ vs. (a) MP or (b) PD.

glucocorticoids sensitive to the metabolism by intervening in the cecal metabolisms.

As reported, synthesis of Glu-S compounds was achieved in good yield by the reaction of glucocorticoids with sulfatrioxide-triethylamine complex in dry pyridine, followed by treating it with concentrated NaCl solution. The ratio of the reactants was critical to the suppression of by-product formation and the optimum ratio for STT/glucocorticoid varies depending on glucocorticoids.[6,16]

Since reductive metabolism(s) of the 4,5 double bond and the 3-keto group on ring A of glucocorticoids essential for an anti-inflammatory effect,[12] should result in a loss of biological activity, the metabolic susceptibility should be considered in the design of a prodrug of glucocorticoids for specific delivery to the large intestine, where reductive metabolisms prevail. In this regard, the metabolic tendencies of a variety of glucocorticoids in the cecal contents were investigated. The metabolic stability of glucocorticoids seems to be dependent on their structures. It was recognized that fluocinolone, which was completely free from the cecal metabolism, has a 1,2 double bond and substituents (fluoro) on the 6 and 9 positions, while cortisone and hydrocortisone, which were most susceptible to the cecal metabolism, have a 1,2 single bond with no substituent on either the 6 or the 9 position. Betamethasone and triamcinolone, which were stable to the metabolism, have a 1,2 double bond with one substituent (fluoro) on the 9 position. Fludrocortisone, which was much more stable than hydrocortisone, is structurally the same as hydrocortisone except that it has a substituent (fluoro) on the 9 position. PD, which was more stable than hydrocortisone, is structurally the same as hydrocortisone except that it has a 1,2 double bond. MP, which was more stable than PD, has a substituent (methyl) on the 6 position. Summarizing these results, certain structural characteristics are derived: (i) glucocorticoids with a 1,2 single bond, such as cortisone and hydrocortisone, are very susceptible to the cecal metabolisms and (ii) glucocorticoids with substituents on the 6 and/or 9 positions are more resistant to cecal metabolisms. The susceptibilities toward the cecal metabolisms are in the order cortisone > hydrocortisone > PD > MP, fludrocortisone > triamcinolone > betametasone > fluocinolone acetonide, which is in parallel order with the doses of the glucocorticoids.[12] Such relationship between the doses and metabolic tendencies of the glucocorticoids might be quite understandable, considering previous findings demonstrating that the susceptibility of glucocorticoids to colonic metabolisms is in line with that to hepatic metabolisms.[10] In addition, our data showing that the cecal metabolisms of glucocorticoids were significantly prevented by reduction inhibitors suggest that the metabolisms taking place during

incubation with cecal contents should be reductive ones, which are directly related to the loss of biological activity.

All of the Glu-S compounds except fluocinolone acetonide 21-sulfate sodium hydrolysed to produce the respective glucocorticoids, which is consistent with previous papers demonstrating colon-specific hydrolysis of sulfate conjugated PD, dexamethasone and MP.[6,10,16] Overall, the amount of glucocorticoids produced was less than that of the hydrolysed prodrugs, which was probably because of the metabolisms of the glucocorticoids liberated from their prodrugs in the cecal contents. This argument is supported by showing that the metabolic tendencies of the glucocorticoids were well-correlated with the cecal accumulation profiles of the glucocorticoids liberated from the respective Glu-S. The more susceptible to the cecal metabolisms a glucocorticoid is, the lower the cecal accumulation of it. Thus, these results suggest that the metabolic susceptibility of glucocorticoids should be taken into consideration in the design of an efficient colon-specific glucocorticoid prodrug.

Application of a metabolic inhibitor is suggested to be a strategy to improve the colonic therapeutic availability of glucocorticoids susceptible to colonic metabolisms.[5] We suggest that reduction inhibitors can be used for this purpose. This argument is supported by our data showing that the reduction inhibitors glycyrrhizin and carbenoxolone substantially prevented the cecal metabolisms of glucocorticoids moderately susceptible to the metabolisms, like PD and MP. However, it does not seem that the reduction inhibitor is applicable to all glucocorticoids as the protective effect of the reduction inhibitors was not significant for glucocorticoids highly susceptible or resistant to cecal metabolism, such as hydrocortisone and cortisone (highly susceptible) and triamcinolone and betamethasone (resistant).

For PD and MP, polyphenols with a catechol ring, such as rutin and n-octyl gallate, exhibited greater inhibitory effects than morin, a polyphenol with a resorcinol ring. The inhibitory activity of glycyrrhizin and carbenoxolone, which are α,β-unsaturated ketones, was lower than that of n-octyl gallate and greater than that of morin. Although rutin and n-octyl gallate elicited greater inhibitory activity, glycyrrhizin and carbenoxolone are likely to be more appropriate for co-administration with reduction-sensitive glucocorticoids for treatment of inflammatory bowel disease (IBD). This is based on the observation that polyphenolic compounds prevented the hydrolysis of the sulfate-conjugated glucocorticoids MPS and PDS in the cecal contents, which should decrease the availability of the parent drugs, MP and PD, at the target site. Moreover, it is reported that glycyrrhizin and carbenoxolone *per se* possess an anti-inflammatory activity,[17–19] probably leading to an additive or synergic beneficial effect on IBD upon co-treatment with PDS or MPS. It is under investigation whether co-administration of a reduction-sensitive glucocorticoid and glycyrrhizin can elicit such a beneficial effect in TNBS-induced rat colitis.

Conclusions

Our data suggest that glucocorticoids delivered to the large intestine could have variable therapeutic availability depending on the colonic metabolic susceptibility of the glucocorticoids and reduction inhibitors such as carbenoxolone and glycyrrhizin may positively modulate the therapeutic availability of the drugs by intervening in their colonic metabolisms. Thus, our data provide information useful for selection of an appropriate glucocorticoid and a pharmacologic strategy for the design of an efficient colon-specific glucocorticoid prodrug.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Structures of reduction inhibitors.
Table S1 HPLC data and physicochemical properties of various glucocorticoids and their sulfate conjugates.

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