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Short Communication

Monoketocholate can decrease transcellular permeation of methotrexate across Caco-2 cell monolayers and reduce its intestinal absorption in rat

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Abstract

Objectives Bile salts have been shown to decrease the absorption of methotrexate in the rat intestine by an unknown mechanism. We aimed to examine this effect.

Methods We assessed apical-to-basolateral (AP-BL) permeation of methotrexate (5 μM) across Caco-2 cell monolayers pretreated with various concentrations (0, 0.25, 0.5, 1, 3 and 5 mM) of sodium cholate or its semisynthetic analogue, sodium 12-monoketocholate. We also determined the effect of orally administered 12-monoketocholate on the intestinal absorption of methotrexate in rats to evaluate a possible in-vitro–in-vivo correlation.

Key findings It was found that sodium cholate and sodium 12-monoketocholate decreased the AP-BL permeation of methotrexate at low concentrations (maximal inhibition at 0.25 and 1 mM, respectively) and increased it at higher concentrations. Determination of [^{14}C] mannitol permeation and electrical resistance of monolayers during experiments showed that membrane integrity was not compromised at low concentrations of bile salts but was disrupted at higher concentrations. Subsequently, we examined the effect of the simultaneous oral administration of sodium 12-monoketocholate (4, 20, 40 and 80 mg/kg) on the intestinal absorption of methotrexate in rats after an oral dose (5 mg/kg). The pharmacokinetic study showed that 12-monoketocholate at 4 and 20 mg/kg did not change the methotrexate area under the serum concentration–time curve whereas sodium 12-monoketocholate at 40 and 80 mg/kg significantly reduced it.

Conclusions Sodium 12-monoketocholate appears to decrease the intestinal absorption of methotrexate in rats by inhibition of transcellular active transport.

Keywords Caco-2 cell permeation; methotrexate; 12-monoketocholate; pharmacokinetics; rat

Introduction

Bile acids are amphipathic derivatives of cholesterol that perform important physiological functions, including maintaining cholesterol homoeostasis, enhancing lipid absorption and facilitating the production of bile.[1] They also participate in the distribution and excretion of endogenous and exogenous toxins and undergo enterohepatic recycling, which depends on the coordinated activity of enterocyte and hepatocyte transporters.[2] It is well known that bile salts enhance drug absorption by increasing the dissolution rate of poorly soluble compounds[3] and perturbing cell membranes through the release of membrane phospholipids and protein.[4] However, they have also been shown to reduce the intestinal absorption of alanine[5] and the antifolate drug methotrexate by unknown mechanisms.[6]

Methotrexate is taken into cells by the influx transporters reduced folate carrier (RFC) and proton-coupled folate transporter/haem carrier protein (PCFT/HCP1). It is also a substrate of the efflux transporters breast cancer resistance protein (BCRP) and multidrug resistance-associated proteins 2 and 3 (MRP2 and MRP3) but not of P-glycoprotein (Pgp).[7] In the rat small intestine, RFC and PCFT/HCP1 are localised at the apical membrane of enterocytes and both have a higher affinity for methotrexate than for folate.[8,9] BCRP and MRP2 are localised on the apical brush border membrane of enterocytes with the highest expression found in the proximal jejunum.[10,11] MRP3 is found in the basolateral membrane with the highest expression in the distal ileum.[12] It has been shown that site-specific and membrane-specific expression of these methotrexate transporters in the intestine play an important role in methotrexate absorption in the rat.[13]

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Recently, the ability of bile salts to interfere with drug transporters has been suggested in several studies.[14,15] On this basis, we hypothesised that the reduction in intestinal absorption of methotrexate by bile salts is due to their ability to inhibit transcellular permeation of methotrexate at the level of transporters. Bile salts are possibly able to intercalate cell membranes and indirectly alter the activity of transporters in the same way that changes in cell membrane composition have been shown to affect the functions of Pgp and BCRP.[16,17] The aim of this study was to examine the influence of sodium cholate and its semisynthetic analogue, sodium 12-monoketocholate, on the permeation of methotrexate across Caco-2 cell monolayers. Sodium cholate and sodium 12-monoketocholate were chosen for this study because they have different facial amphilicity and lipophilicity, which considerably affect their ability to insert into phospholipid monolayers.[18,19] Caco-2 cell monolayers are a good in-vitro model for the study of methotrexate transporter-mediated intestinal absorption because they are known to retain differential expression and function of RFC, BCRP and MRP isoforms in their apical and basolateral membrane domains.[20–22] We also determined the effect of orally administered sodium 12-monoketocholate on the intestinal absorption of methotrexate in rats to evaluate a possible in-vitro–in-vivo correlation.

Materials and Methods

Materials

Methotrexate, sodium cholate, Hanks' balanced salt solution (HBSS), *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) and glucose were purchased from Sigma-Aldrich New Zealand Ltd (Auckland, New Zealand). [^{14}C] Mannitol (specific activity 61.0 mCi/mmol; radiochemical purity 98.6%) was obtained from Amersham Biosciences (Auckland, New Zealand). Caco-2 cells were obtained from the American Type Culture Collection (Rockville, USA). Methotrexate Injection BP (2.5 mg/ml) for use in the animal study was obtained from Mayne Pharma Pty Ltd (Mulgrave, Australia). Sodium 12-monoketocholate (sodium 3α,7α-dihydroxy-12-keto-5β-cholate, purity 98%) was kindly provided by the Department of Pharmacy, University of Novi Sad, Republic of Serbia. All other reagents were of analytical grade.

Drug solutions

For Caco-2 transport studies, a stock solution of methotrexate (1 mM, 454.5 μg/ml) was prepared in 0.1 M sodium hydroxide and diluted with transport buffer (HBSS containing 25 mM HEPES and 25 mM D-glucose, pH 7.4) to give solutions containing 0.5, 1, 2, 5, 10 and 20 μM. Stock solutions of sodium cholate and sodium 12-monoketocholate (10 mM, 4.30 and 4.29 mg/ml, respectively) were prepared and diluted with transport buffer to give solutions containing 0.25, 0.5, 1, 3 and 5 mM.

For the animal study, a methotrexate solution (1.25 mg/ml) was prepared by diluting methotrexate injection with saline. Solutions containing methotrexate and sodium 12-monoketocholate (1, 5, 10 and 20 mg/ml) were prepared by mixing appropriate volumes of methotrexate injection and sodium 12-monoketocholate stock solution (40 mg/ml in saline) and then making to volume with saline.

Transport studies in Caco-2 cell monolayers

Caco-2 cells at passage 27–36 were used to form monolayers according to a 21-day protocol.[23] Concentration dependence of methotrexate apical-to-basolateral (AP-BL) permeation was studied in Caco-2 cell monolayers at 37°C as follows. After rinsing monolayers twice with transport buffer, a prewarmed methotrexate solution (0.5 ml) was added to the AP side and prewarmed transport buffer (1.5 ml) added to the BL side. Samples (0.4 ml) were collected from the BL side after 0.5, 1, 1.5, 2, 2.5 and 3 h and replaced with equal volumes of prewarmed transport buffer. Transport studies were performed in triplicate. Samples were stored at −20°C until analysis.

To study the effect of bile salts, monolayers were preincubated with various concentrations of sodium cholate or sodium 12-monoketocholate on both sides at 37°C for 30 min. After pretreatment, solutions were aspirated from both sides and the AP-BL permeation of methotrexate (5 μM) was determined as described above. During experiments, the integrity of cell monolayers was assessed by measuring the transepithelial electrical resistance (TEER) and [^{14}C] mannitol flux at a concentration of 0.1 mM (1 μCi/ml). [^{14}C]Mannitol was analysed by liquid scintillation counting (Model LS 6500; Beckman Coulter, Fullerton, USA) of 0.2-ml samples diluted with 4 ml biodegradable scintillation fluid (Amersham Biosciences, Auckland, New Zealand) at a counting efficiency of 95.2%. To avoid the variability of transporter protein expression at different Caco-2 passage numbers,[24] permeation experiments on different days were conducted using monolayers prepared from the same batch of cells.

Pharmacokinetic study

Male Wistar rats, (age 10–12 weeks, weight 300 ± 50 g) were obtained from the Laboratory Animal Centre, University of Otago and housed under a 12-h light–dark cycle with free access to a rodent diet and water. Food was withdrawn 12 h before experiments. Approval for experimental procedures was obtained from the University of Otago Committee on Ethics in the Care and Use of Laboratory Animals (Approval No. 55/06).

Rats were randomised to four groups (n = 5 per group) to receive a single oral dose of methotrexate (5 mg/kg) alone (control) and in combination (treatment) with one of four doses of sodium 12-monoketocholate (4, 20, 40 and 80 mg/kg) by gavage at a volume of 4 ml/kg. In each group of rats, the period and sequence of administration of the control and treatment were randomised in a two-way crossover design with a washout period of at least 72 h. Blood samples (100 μl) were collected from the tail vein before the dose and at 10, 20, 30, 45, 60, 90, 120, 180, 240, 360 and 480 min after dosing. Blood samples were centrifuged at 15 000*g* for 10 min and serum stored at −20°C until analysis for methotrexate.

Assay of methotrexate

Methotrexate was determined by HPLC with post-column photochemically induced fluorescence detection as described

previously.[25] Serum (20 μl) and acetonitrile (50 μl) were thoroughly mixed and centrifuged (5 min, 15 000*g*). The supernatant (60 μl) was then mixed with de-ionised water (100 μl) and chloroform (200 μl) was added to remove acetonitrile.[26] After centrifugation (5 min, 15 000*g*), the aqueous supernatant (20 μl) was injected. Samples from transport studies were injected directly or after dilution with transport buffer. Standard curves were prepared in the range 5–1000 ng/ml for serum samples and 1–250 ng/ml for transport buffer samples. Standard curves were linear with acceptable accuracy (85–115% of true values) and precision (intra- and inter-day coefficients of variation <15%). The recovery of methotrexate from rat serum was 89 ± 4% at 100 ng/ml.

Data analysis

Data are presented as means ± standard deviation (SD) (n = 3). Transport of methotrexate through Caco-2 cell monolayers is expressed as apparent permeability coefficient (P_{app}, cm/s) calculated using Equation 1.

$$P_{app} = dQ/dt \times [1/(A \times C_0)] \quad (1)$$

where dQ/dt is the rate of methotrexate permeation (μmol/s), A is the surface area of the membrane (1.12 cm^2) and C_0 is the initial concentration in the donor solution (μM).

In the study of the concentration dependence of methotrexate permeation, kinetic parameters of the model shown in Equation 2 were estimated by nonlinear regression.

$$dQ/dt = \{V_{max}[S]/(K_m + [S])\} + k_d[S] \quad (2)$$

where V_{max} is the maximum active transport rate, K_m is the Michaelis constant, [S] is the initial methotrexate concentration and k_d[S] is the permeation rate by passive diffusion.

For each bile salt, the effect of concentration on methotrexate P_{app} was examined using one-way analysis of variance followed by Dunnett's post-hoc testing. Multiple t-tests with Bonferroni adjustment were used to compare the effects of the two bile salts at equal concentrations. Statistical analysis and nonlinear regression were carried out by Graphpad Prism 4 software.

For the pharmacokinetic study, peak plasma concentration (C_{max}) and time to C_{max} (T_{max}) were read directly from serum concentration–time profiles. Areas under the curve ($AUC_{0\rightarrow t}$ and $AUC_{0\rightarrow\infty}$), half-life ($t_{½}$) and mean residence times ($MRT_{0\rightarrow t}$ and $MRT_{0\rightarrow\infty}$) were calculated by WinNonlin 5.2. Statistical comparison was carried out by Minitab 15 software using a general linear model.[27] Differences for which $P < 0.05$ were considered statistically significant.

Results

Transport studies in Caco-2 cell monolayers

AP-BL permeation of methotrexate at various concentrations is shown in Figure 1. Calculated kinetic parameters were K_m = 5.86 μM, $V_{max} = 2.95 \times 10^{-9}$ μmol/s and $k_d = 0.243 \times 10^{-3}$ μl/s. Hence, when [S] = 5 μM, active transport and passive diffusion make an approximately equal contribution

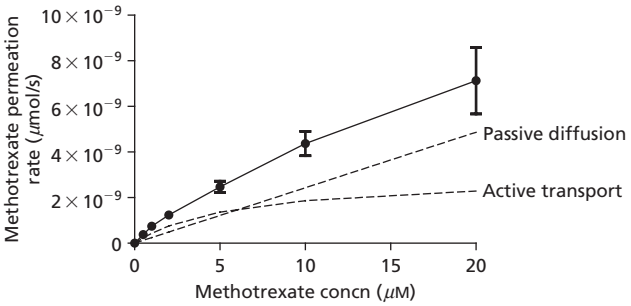

Figure 1 Concentration dependence of apical-to-basolateral permeation of methotrexate across Caco-2 cell monolayers. Data are means ± SD, n = 3. Dashed lines are the fits of nonlinear regression analysis according to Equation 2.

to methotrexate permeation (Equation 2). After pretreatment with sodium cholate or sodium 12-monoketocholate, the AP-BL permeability of methotrexate (5 μM) exhibited hyperbolic behaviour (Figure 2a). Significant reductions in methotrexate P_{app} were produced by sodium cholate at 0.25 and 0.5 mM and sodium 12-monoketocholate at 0.5 and 1 mM, whereas both bile salts significantly increased methotrexate P_{app} at 5 mM. Values of methotrexate P_{app} for sodium cholate were significantly higher than for sodium 12-monoketocholate at 1, 3 and 5 mM (Table1).

In terms of membrane integrity, sodium cholate at 0.25–1 mM and sodium 12-monoketocholate at 0.25–3 mM did not change the permeability of [^{14}C]mannitol (Figure 2b) or TEER values (Table 1). However, at 3 and 5 mM sodium cholate or 5 mM sodium 12-monoketocholate [^{14}C]mannitol permeability was significantly increased, and at 3 and 5 mM sodium cholate or sodium 12-monoketocholate the final TEER values were significantly reduced. [^{14}C]Mannitol permeability and TEER values for sodium cholate were significantly higher than for sodium 12-monoketocholate at 3 and 5 mM (Table 1).

Pharmacokinetic study

Serum concentration–time profiles of methotrexate administered with and without sodium 12-monoketocholate are shown in Figure 3 and corresponding pharmacokinetic parameters are summarised in Table 2. There was no sequence and period effect between control and treatment. For sodium 12-monoketocholate at 4 and 20 mg/kg, the AUC and C_{max} of methotrexate were not significantly different from values for methotrexate administered alone. For sodium 12-monoketocholate at 40 and 80 mg/kg, the AUC and C_{max} of methotrexate were significantly lower (approximately 40%) than corresponding values for methotrexate administered alone. There was no significant difference in any pharmacokinetic parameter between sodium 12-monoketocholate doses of 40 and 80 mg/kg and no significant change in T_{max}, $t_{½}$ and MRT of methotrexate for any sodium 12-monoketocholate dose.

Discussion

Bile salts have been extensively investigated as drug absorption enhancers in previous studies.[28,29] Recently,

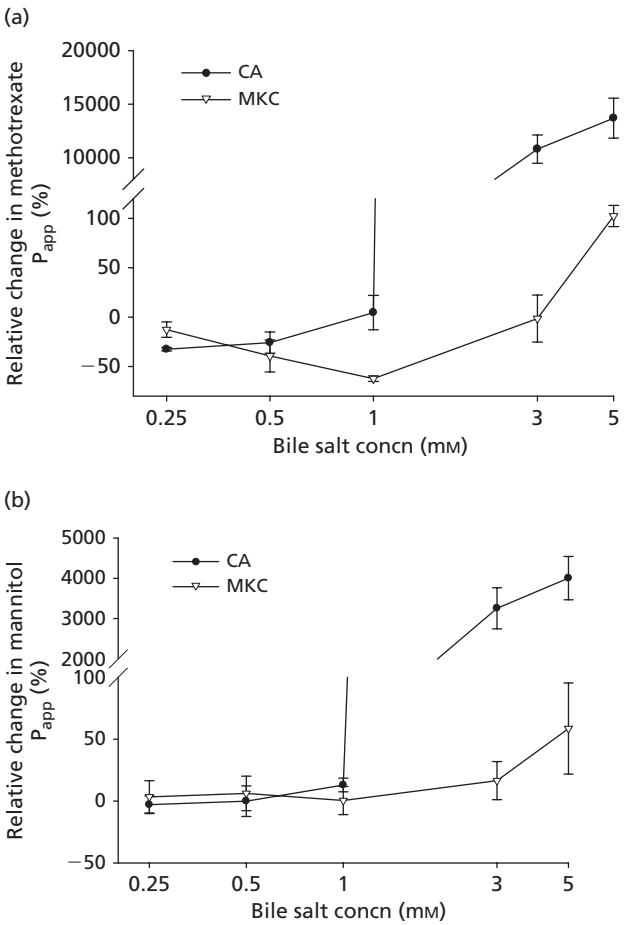

Figure 2 Relative change in the apical-to-basolateral permeation (P_{app}) of methotrexate (a) and mannitol (b) across Caco-2 cell monolayers after pretreatment with various concentrations of sodium cholate or sodium 12-monoketocholate on both apical and basolateral sides. CA, sodium cholate; MKC, sodium 12-monoketocholate. Relative change in P_{app} was calculated as ([treatment – control]/control) × 100%. Data are means ± SD, $n = 3$.

bile acid transporters have been considered as potential targets in the design of bile-acid conjugates of therapeutic agents as a means to improve their bioavailability.[30] Since some bile acid transporters, such as organic anion transporting proteins (OATPs) and multidrug resistance-associated proteins (MRPs), also carry a broad range of other organic anions and cations,[2,31] there is a need to better understand the potential interaction between bile acids and drugs at the level of their common transporters. Our Caco-2 transport study simultaneously examined methotrexate permeability, mannitol permeability and TEER to illuminate effects of bile salts on paracellular and transcellular permeation of methotrexate. Although a previous study has shown that natural bile salts (such as sodium cholate, deoxycholate and taurocholate) inhibit the permeation of [^{3}H]methotrexate in everted gut sacs,[32] our rat study shows for the first time that a bile salt can reduce the intestinal absorption of methotrexate *in vivo*.

Methotrexate is a hydrophilic compound that is unable to cross lipophilic membranes by transcellular passive diffusion.[33] Therefore, its permeation across Caco-2 cell monolayers probably involves a combination of paracellular passive diffusion and transcellular active transport. A model combining saturable and passive diffusion processes (Equation 2) was used to estimate K_m for the transcellular active transport of methotrexate across Caco-2 cell monolayers. The result of 5.85 μM was the basis for choosing a concentration of 5 μM methotrexate in the study of the effect of bile salts to ensure carrier-mediated transport would make a substantial contribution to methotrexate permeation.

As regards the effect of bile salts on methotrexate permeation, low concentrations (≤1 mM) of sodium cholate and sodium 12-monoketocholate inhibited AP-BL transport of methotrexate but did not change the paracellular flux of mannitol or the TEER. Since mannitol permeates cell monolayers exclusively by the paracellular pathway,[34] it appears that the effect of bile salts results from inhibition of transcellular active transport. This is also supported by the observation that the reduction in permeation caused by 1 mM sodium 12-monoketocholate (62 ± 3%) is close to the contribution made by active transport (approximately 50%) to the permeation of 5 μM methotrexate (estimated from Equation 2).

At 0.25 mM bile salts (Figure 2), where paracellular permeation as measured by mannitol P_{app} is unaffected, sodium cholate causes a greater reduction in methotrexate transcellular transport than sodium 12-monoketocholate in

Table 1 Apical-to-basolateral permeability of methotrexate and mannitol and changes in transepithelial electrical resistance of Caco-2 cell monolayers after pretreatment with various concentrations of sodium cholate or sodium 12-monoketocholate on both apical and basolateral sides

		Bile salt concentration (mM)					
	Bile salt	0	0.25	0.5	1	3	5
Methotrexate P_{app}	CA	0.14 ± 0.02	0.09 ± 0.01*	0.10 ± 0.01*	0.14 ± 0.01$^{\#}$	14.7 ± 1.09*,$^{\#}$	18.6 ± 1.08*,$^{\#}$
($\times 10^{-6}$ cm/s)	MKC	0.14 ± 0.02	0.12 ± 0.02	0.08 ± 0.01*	0.05 ± 0.01*,$^{\#}$	0.13 ± 0.01$^{\#}$	0.28 ± 0.05*,$^{\#}$
Mannitol P_{app}	CA	0.64 ± 0.01	0.62 ± 0.06	0.64 ± 0.08	0.72 ± 0.04	21.5 ± 3.7*,$^{\#}$	26.3 ± 3.9*,$^{\#}$
($\times 10^{-6}$ cm/s)	MKC	0.64 ± 0.01	0.66 ± 0.10	0.68 ± 0.10	0.64 ± 0.08	0.75 ± 0.11$^{\#}$	1.02 ± 0.25*,$^{\#}$
Change in TEER	CA	99.8 ± 8.7	87.9 ± 14.8	88.5 ± 11.6	77.4 ± 3.2	1.31 ± 0.85*,$^{\#}$	1.24 ± 1.32*,$^{\#}$
(% of initial)	MKC	99.8 ± 8.7	88.4 ± 13.1	85.5 ± 12.5	92.1 ± 16.8	59.3 ± 10.0*,$^{\#}$	27.2 ± 1.5*,$^{\#}$

CA, sodium cholate; MKC, sodium 12-monoketocholate; TEER, transepithelial electrical resistance. Data are means ± SD, $n = 3$. *$P < 0.05$ compared with control; $^{\#}P < 0.05$ sodium cholate compared with sodium 12-monoketocholate at equal concentrations.

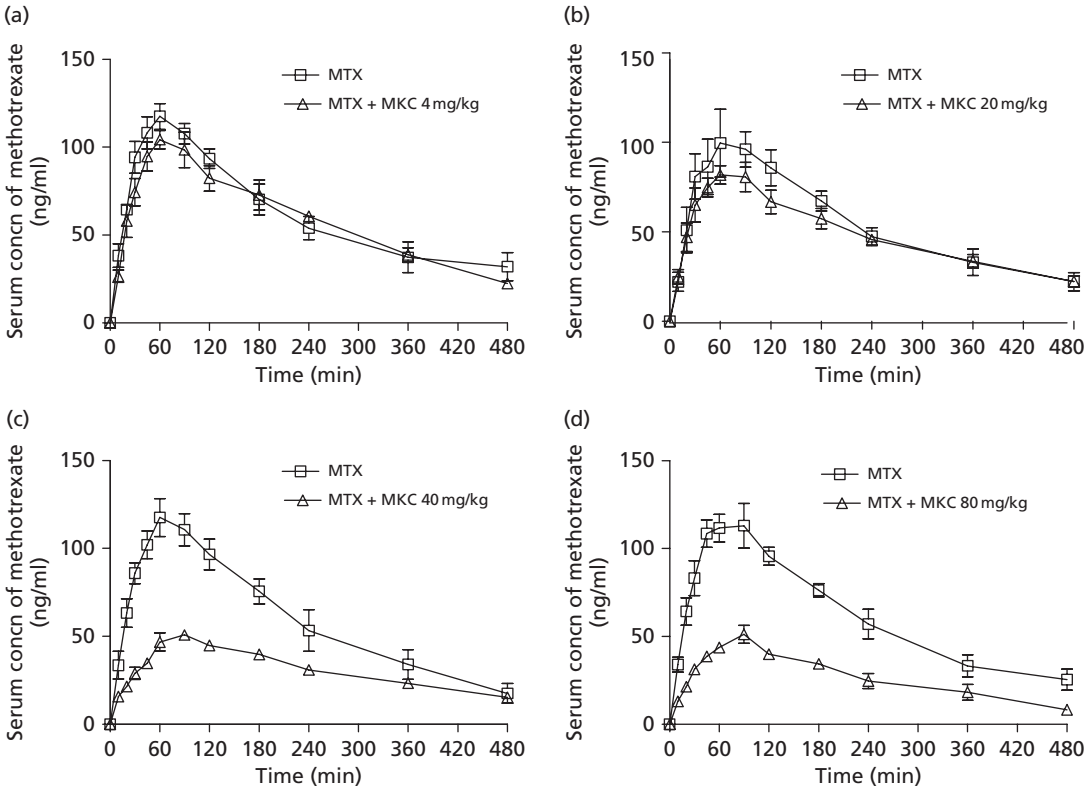

Figure 3 Serum concentration–time profiles of methotrexate in rat following a single oral dose of 5 mg/kg methotrexate and following a single oral dose of 5 mg/kg methotrexate plus single oral doses of 4 (a), 20 (b), 40 (c) or 80 mg/kg (d) sodium 12-monoketocholate. MTX, methotrexate; MKC, sodium 12-monoketocholate. Each pharmacokinetic study was conducted using a two-way randomised crossover design. Data are means ± SD, $n = 5$.

line with its greater lipophilicity (sodium cholate, log P = 3.29; sodium 12-monoketocholate, log P = 3.09).[18] At 1 mM bile salts, where again paracellular permeation is unaffected, sodium 12-monoketocholate causes a greater reduction in methotrexate transcellular transport than sodium cholate. These differences suggest that keto substitution of the hydroxy group on the steroid ring has a major effect on the ability of bile salts to intercalate phospholipid bilayers and indirectly to inhibit the function of transporters. A further study is underway to clarify structure–activity relationships for the effect of ketocholate derivatives on methotrexate permeation.

With regard to the mechanism of the reduction in methotrexate permeation by bile salts, one possible explanation is that bile salts competitively inhibit the basolateral transporter MRP3 for which they are known to be substrates.[35] Consistent with this is the fact that serosal methotrexate efflux in the everted sac of rat ileum preloaded with methotrexate is suppressed by pretreatment with the non-selective MRP inhibitor, probenecid.[13] An alternative

Table 2 Pharmacokinetic parameters for methotrexate in rat after a single oral dose of 5 mg/kg methotrexate alone and with single oral doses of 4, 20, 40 and 80 mg/kg sodium 12-monoketocholate

Treatment	Pharmacokinetic parameter						
	T_{max} (min)	C_{max} (ng/ml)	$AUC_{0\rightarrow t}$ (min μg/ml)	$AUC_{0\rightarrow\infty}$ (min μg/ml)	$MRT_{0\rightarrow t}$ (min)	$MRT_{0\rightarrow\infty}$ (min)	$t_{½}$ (min)
MTX	78.0 ± 16	121 ± 14	29.4 ± 5.5	43.4 ± 9.2	185 ± 26	278 ± 91	149 ± 25
MTX + 4 mg/kg MKC	72.0 ± 16	109 ± 16	28.0 ± 3.0	34.3 ± 2.9	192 ± 16	298 ± 69	165 ± 32
MTX	78.0 ± 16	107 ± 38	25.6 ± 7.0	33.5 ± 10	186 ± 18	338 ± 65	169 ± 43
MTX + 20 mg/kg MKC	66.0 ± 25	84.86 ± 14	22.8 ± 3.0	30.7 ± 5.6	196 ± 16	353 ± 82	194 ± 40
MTX	66.0 ± 13	120 ± 20	28.3 ± 5.6	32.3 ± 9.0	173 ± 33	228 ± 75	166 ± 34
MTX + 40 mg/kg MKC	84.0 ± 13	52.7 ± 8.2*	14.8 ± 1.7*	20.8 ± 5.1*	205 ± 13	288 ± 91	193 ± 22
MTX	72.0 ± 16	121 ± 26	29.1 ± 5.9	37.4 ± 12	181 ± 19	302 ± 114	154 ± 30
MTX + 80 mg/kg MKC	84.0 ± 13	51.4 ± 11*	12.6 ± 2.9*	15.0 ± 4.5*	185 ± 25	269 ± 79	163 ± 36

MTX, methotrexate; MKC, sodium 12-monoketocholate. Data are means ± SD, $n = 5$. *$P < 0.05$ versus methotrexate alone.

explanation is that bile salts, through their surfactant properties, nonspecifically disturb methotrexate transporters by changing cell membrane fluidity. Nonionic surfactants have been shown to do this for Pgp.[36] A third, less likely, explanation is that bile salts enhance intracellular polyglutamation of methotrexate since methotrexate has been shown to form methotrexate polyglutamates within tumour cells.[37]

At high concentrations of sodium cholate and sodium 12-monoketocholate (3 and 5 mM), methotrexate P_{app} increases in conjunction with increases in mannitol P_{app} and reductions in TEER. This suggests that the enhanced permeability of methotrexate is due to opening of tight junctions. This result is consistent with the finding of Lowes and Simmons (2001)[38] showing that 5 mM sodium cholate increased mannitol flux and reduced TEER of Caco-2 cell monolayers.

Due to the fact that sodium 12-monoketocholate produced greater inhibition of methotrexate P_{app} at low concentrations and less membrane disruption at high concentrations than sodium cholate, sodium 12-monoketocholate was chosen to study the effect of a bile salt on the absorption of orally administered methotrexate in rats. After the 5 mg/kg dose, the range of methotrexate serum concentrations (10–150 ng/ml) was similar to that previously reported.[39] The washout period of 72 h was sufficient as there was no significant sequence effect. This is to be expected from the elimination half-life of methotrexate of 3 h and the fact that 90% of an intravenous methotrexate dose is excreted unchanged by the kidneys.[40]

In terms of the sodium 12-monoketocholate dose range, previous studies (in our laboratory) have shown that a single oral dose of 100 mg/kg sodium 12-monoketocholate in rats causes neither hepatotoxicity nor nephrotoxicity. On this basis, a maximum dose of 80 mg/kg sodium 12-monoketocholate was chosen for the in-vivo study. At this dose, the concentration in the gastrointestinal tract (of a 300-g rat with 10-ml intestinal volume) was estimated to be approximately 5.7 mM. Exposure of Caco-2 cells to 5 mM sodium 12-monoketocholate increased the P_{app} of methotrexate whereas the in-vivo dose of 80 mg/kg reduced the methotrexate AUC. This contradiction between in-vitro and in-vivo results may arise because enterocytes in the gut wall are actually exposed to less sodium 12-monoketocholate than predicted due to the mucus layer or to the removal of sodium 12-monoketocholate by emulsification with dietary lipids in the gut lumen. This reduced exposure is supported by the fact that the C_{max} of methotrexate was reduced by the 80 mg/kg dose of sodium 12-monoketocholate to an extent ($58 \pm 7\%$) similar to the decrease ($62 \pm 3\%$) in methotrexate P_{app} in Caco-2 cell monolayers pretreated with 1 mM sodium 12-monoketocholate.

After the 80 mg/kg dose, the estimated concentration of sodium 12-monoketocholate in the gastrointestinal tract of 5.7 mM is well below its critical micelle concentration value (13 mM).[19] This indicates that the decrease in methotrexate absorption caused by sodium 12-monoketocholate is not due to methotrexate encapsulation by micelles. At doses of 40 and 80 mg/kg sodium 12-monoketocholate, the T_{max} of methotrexate appears to be later and the $t_{½}$ of methotrexate longer than methotrexate alone (Figure 3), although the differences are not significant. This may suggest that sodium 12-monoketocholate prolongs the intestinal absorption and total body elimination of methotrexate. A larger experiment will be required to substantiate this observation. The decrease in the rate of methotrexate absorption (as reflected in the C_{max}) by 40 and 80 mg/kg doses of sodium 12-monoketocholate may explain why, clinically, the simultaneous consumption of food (inducing bile secretion) can delay the absorption of methotrexate and reduce its concentration in the blood.[41]

Conclusions

The reduction in Caco-2 cell permeation of methotrexate caused by low concentrations of cholate and 12-monoketocholate is consistent with their ability to inhibit active transcellular permeation of methotrexate. The increase in permeation of methotrexate at higher concentrations of these bile salts is due to their ability to open tight junctions. Comparison of the reduction in intestinal absorption of methotrexate caused by 12-monoketocholate with its ability to inhibit Caco-2 cell permeation suggests that the decrease in intestinal absorption of methotrexate in rat is due to inhibition of active transcellular permeation.

Declarations

Conflict of interest

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

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