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Effect of ketocholate derivatives on methotrexate uptake in Caco-2 cell monolayers

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ABSTRACT

Purpose: The bile salts (BS) cholate (C) and 12-monoketocholate (12-MKC) have been shown to inhibit the transcellular permeation of methotrexate (MTX) across Caco-2 cell monolayers. The aim of this study was to investigate the mechanism of this inhibition by comparing the effects of C, 7-MKC, 12-MKC, 3,7-diketocholate (DKC) and triketocholate (TKC) on MTX uptake by Caco-2 cells.
Methods: Critical micelle concentrations (CMCs) and cytotoxicities of BS and their effects on membrane fluidity Caco-2 cells were determined by standard methods. MTX uptake by Caco-2 cell monolayers was determined using LC–MS/MS.
Results: Replacing hydroxyl groups in C with keto groups and changing from 7-MKC to 12-MKC resulted in BS with lower cytotoxicity, higher CMC and decreased ability to inhibit the uptake of MTX. 7- and 12-MKC increased membrane fluidity of hydrophilic regions of Caco-2 cell membranes, DKC and TKC increased membrane fluidity of hydrophobic regions and C had little effect on membrane fluidity of either region.
Conclusion: Replacing hydroxyl groups in C with keto groups produces BS with different physicochemical properties and biological effects. Since ketocholates (but not C) decrease MTX uptake in parallel with increasing membrane fluidity, it is suggested that ketocholates inhibit MTX influx transporters indirectly through disturbing their lipid environment.

1. Introduction

Bile salts (BS), found predominantly in the bile of mammals, have been extensively investigated as absorption enhancers of orally administered drugs (Aungst, 2000; Sakai et al., 1999). The underlying mechanisms have been shown to include increasing the solubility of drugs by micelle formation (Mithani et al., 1996) and opening the tight junctions between enterocytes (Tsutsumi et al., 2008). In addition, taurocholate has been shown to inhibit the active efflux of P-glycoprotein (P-gp) substrates (Ingels et al., 2002, 2004) probably through an indirect effect on the lipid environment of the P-gp transporter (Romsicki and Sharom, 1999). This raises the possibility that BS in general can alter the activity of membrane embedded transporters by modulating their lipid environment. Cholesterol has been shown to modulate the activities of P-gp (Troost et al., 2004a,b) and breast cancer resistance protein (BCRP) (Mazzoni and Trave, 1993; Storch et al., 2007) in this way.

The use of unconjugated BS in pharmaceutical formulations has been limited by their tendency to cause mucosal irritation and cytotoxicity. Minor structural modifications of BS have been made in an attempt to reduce this cytotoxicity without compromising their ability to enhance membrane permeability (Michael et al., 2000). In this respect, it has been shown that replacing hydroxy groups in cholate (C) with keto groups produces BS with higher critical micelle concentration (CMC) (Poša et al., 2007) and lower cytotoxicity (Poša and Kuhajda, 2010). One of these ketocholates, 12-monoketocholate (12-MKC), has been shown to enhance nasal absorption of insulin (Kuhajda et al., 1997) and brain uptake of quinine in rat (Mikov et al., 2004).

The folate antagonist, methotrexate (MTX), is a hydrophilic compound [$\log P - 1.85$ (Leo et al., 1971)] with limited ability to cross lipophilic membranes by passive diffusion. However, because of its structural similarity to folate, it is actively transported into cells by the reduced folate carrier (RFC) (Dixon et al., 1994) and the proton-coupled folate transporter/haem carrier protein (PCFT/HCP1) (Inoue et al., 2008; Nakai et al., 2007; Yokooji et al., 2009). MTX is also a substrate for efflux transporters the main ones being apical BCRP (Chen et al., 2003; Volk and Schneider, 2003) and MRP2 as well as basolateral MRP3 (Yokooji et al., 2007; Vlaming

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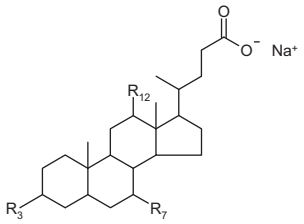

	C	7-MKC	12-MKC	DKC	TKC
R_3	-OH	-OH	-OH	=O	=O
R_7	-OH	=O	-OH	=O	=O
R_{12}	-OH	-OH	=O	-OH	=O

Fig. 1. Chemical structures of cholate and its ketocholate derivatives.

et al., 2009; Wang et al., 2011). We have recently shown that C and 12-MKC have concentration-dependent effects on the apical-to-basolateral permeation of MTX in Caco-2 cell monolayers (Chen et al., 2009a). At high concentrations they produce the predictable increase in permeation due to opening of tight junctions but at low concentrations they produce a reduction in transcellular permeation which may be the result of inhibiting influx transporters directly or by inhibiting them indirectly through disturbing their lipid environment. Such an indirect effect gains some support from the fact that modification of plasma membrane fatty acid composition affects the uptake of MTX in murine leukemia cells (Burns et al., 1979).

The aim of this study was to investigate the mechanism of the effect of cholate and ketocholates on MTX uptake through an examination of the effects of C, 7-MKC, 12-MKC, 3,7-diketocholate (DKC) and triketocholate (TKC) (Fig. 1) on Caco-2 cell membrane fluidity and MTX uptake. Caco-2 cell monolayers are a good model for such a study since they retain differential expression and function of RFC, PCFT/HCP1, BCRP and MRP isoforms in their apical membranes (Ashokkumar et al., 2007; Prime-Chapman et al., 2004; Subramanian et al., 2008; Xia et al., 2005). Cytotoxicities to Caco-2 cells and CMCs of the BS were also determined to ensure the results were not affected by cytotoxicity or micelle formation.

2. Materials and methods

2.1. Materials

MTX, phosphate buffered saline (PBS), Hank's balanced salt solution (HBSS) powder, glucose, C (sodium 3α,7α,12α-trihydroxy-5β-cholanate, purity ≥99%), diphenylhexatriene (DPH), trimethylammonium diphenylhexatriene (TMA-DPH) and 2-morpholinoethanesulfonic acid monohydrate (MES) were purchased from Sigma–Aldrich New Zealand Ltd. (Auckland, New Zealand). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), nonessential amino acids, penicillin and streptomycin were purchased from Invitrogen New Zealand Ltd. (Auckland, New Zealand). Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and used at passage 27–36. Acetonitrile, formic acid and 35% ammonia were AnalaR® grade purchased from BDH Chemicals Ltd. (Poole, UK). The cholic acid derivatives (purity ≥ 96.5%), 3α,12α-dihydroxy-7-keto-5β-cholanoic acid, 3α,7α-dihydroxy-12-keto-5β-cholanoic acid, 12α-hydroxy-3,7-diketo-5β-cholanoic acid and 3,7,12-triketo-5β-cholanoic acid were supplied by Professor Momir Mikov (University of Novi Sad, Republic of Serbia) and converted to their sodium salts by neutralization of alcoholic solutions with equivalent amounts of sodium hydroxide. Solid BS obtained by freeze-drying these aqueous solutions were stored in desiccators over dry silica gel when not in use.

2.2. CMC determination

CMC was determined by the surface tension method using a dynamic contact angle tensiometer (DCA-100, FTA Europe Ltd., Cambridge, UK) with a Wilhelmy plate. Surface tension of BS was measured in transport buffer (TB) (HBSS containing 5 mM MES and 25 mM D-glucose, pH 6.0) at 37 °C. Measurements were initiated with TB after which the concentration of BS was increased by titration of a stock solution. The plate was completely wetted by dipping before each measurement to ensure that the contact angle between the plate and buffer was zero. For each BS concentration, surface tension was measured in triplicate and all experiments were performed in duplicate on different occasions. The CMC was obtained from the intersection of the two best-fit least-squares lines (one in the descending part of the curve and the other through the plateau) of a plot of surface tension versus log[BS] (Fig. 2).

2.3. Cytotoxicity assay

Cytotoxicity of BS at selected concentrations was assessed using the lactate dehydrogenase (LDH) assay (CytoTox 96® kits purchased from Promega Corporation, Germany) in 96-well plates in accordance with the manufacturer's instructions. Briefly, Caco-2 cells (2×10^4 cells/well) were incubated with blank TB, lysis solution (0.9% Triton® X-100) or TB containing BS for 30 min. After centrifugation at 2000 × *g* for 5 min, supernatants were treated with equal volumes of substrate mix solution for 30 min. The reaction was stopped by the addition of 0.1 M NaOH and formazan concentration determined by measuring absorption at 490 nm. LDH release was expressed as percentage of the high control (lysis solution) calculated using Eq. (1). Cytotoxicity is defined as the minimum concentration producing LDH release ≥20% of the control.

$$\text{LDH release (\% of control)} = \frac{A_b - A_l}{A_h - A_l} \times 100\% \quad (1)$$

where A_b, A_l and A_h are absorbances of the wells containing BS, blank TB (low control) and lysis solution (high control), respectively. LDH release at each concentration of BS was measured in triplicate wells and all experiments were done in duplicate on different occasions.

2.4. Membrane fluidity

Membrane fluidity of Caco-2 cells was determined by steady state fluorescence polarization (FP) using DPH and TMA-DPH as probes (Audus et al., 1991). Conditions producing maximum incorporation of the fluorescent probe into the Caco-2 cell membrane and stable measurements over the experimental period were chosen based on a previous study (Hugger et al., 2002). Briefly, Caco-2 cells suspended in TB (2×10^5 cells/mL, 100 μl) were incubated with either DPH 2 μM for 30 min at 37 °C or TMA-DPH 2 μM for 2 min at 37 °C in the dark. TB (100 μl) containing various concentrations of BS was preheated to 37 °C and added to cell suspensions. After 30 min, FP was measured using a microplate reader (POLARstar Omega, BMG LABTECH, Germany) with excitation and emission wavelengths of 355 nm and 460 nm, respectively. Two identical 460 nm filters were fitted at 180° to each other to monitor the parallel (I_p) and perpendicular (I_s) emission intensities. FP was automatically calculated by the software as $1000 \times (I_p - I_s)/(I_p + I_s)$. Target FP was set at 0.2 for gain adjustment. To avoid cell aggregation, each measurement was performed after a 20 s shake. FP was measured in triplicate wells and all experiments were performed in duplicate.

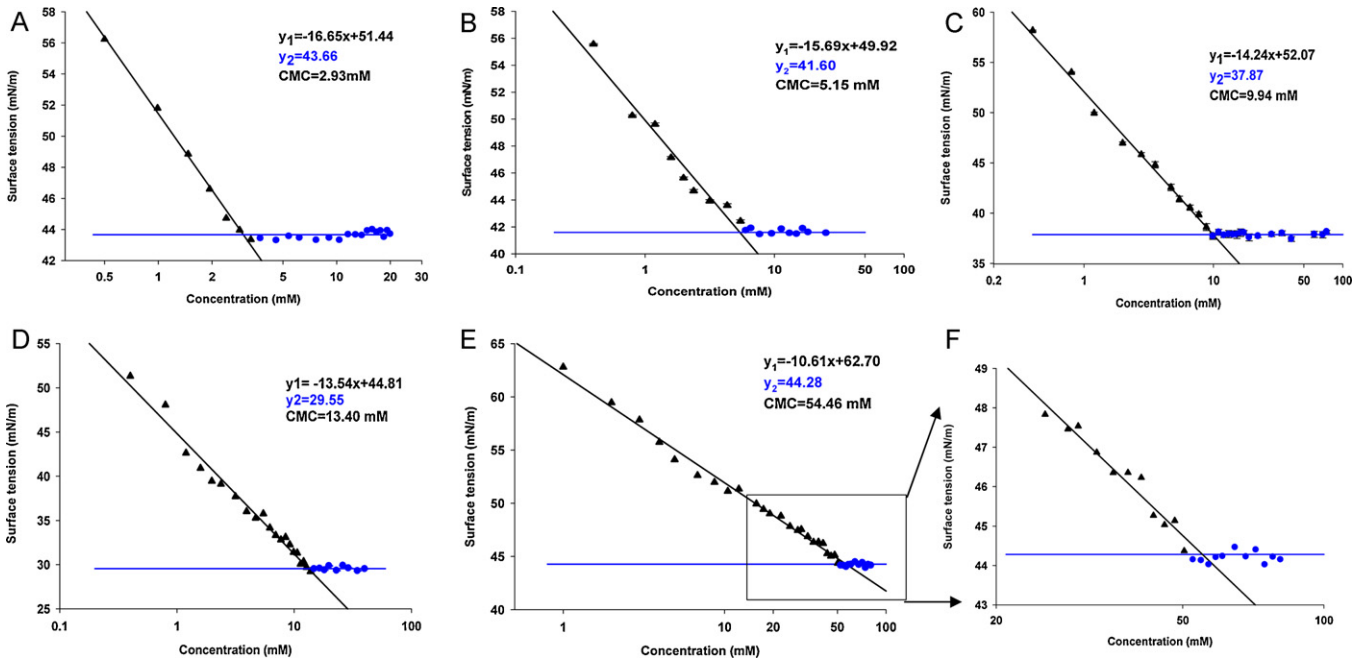

Fig. 2. CMC determination of (A) C, (B) 7-MKC, (C) 12-MKC, (D) DKC and (E and F) TKC by determination of surface tension (plot F is the enlarged interaction area of plot E). Data are means of 6 measurements.

2.5. MTX uptake by Caco-2 cells

The effect of BS on MTX uptake was studied in Caco-2 cell monolayers prepared by culturing cells in 24-well plates at a seeding density of 4×10^5 cells/well for 7 days. After rinsing twice with TB, cells were pre-incubated in triplicate with blank TB or TB containing BS at concentrations up to the minimum causing cytotoxicity at 37 °C for 30 min. Solutions were then removed, replaced with TB containing MTX (2 μM) and the same BS at the same concentration as in the pre-incubation period and incubated at 37 °C for a further 10 min. These conditions were chosen based on the results of previous studies showing MTX uptake into Caco-2 cells is linear for up to 15 min with a K_m of 1.31 μM (Narawa et al., 2005, 2007). After incubation, the MTX solution was aspirated, cells washed twice with ice-cold TB and then lysed with 400 μL 5% ammonia. MTX concentration in cell lysate (200 μL) was determined by a validated LC–MS/MS method (Chen et al., 2009b) with a lower limit of quantitation of 1 ng/mL and inter-day precision (as coefficient of variation) in the range of 8.3–12.9%. Cellular uptake of MTX is expressed as ng/mg cellular protein where protein concentration was determined using a Bradford assay kit (Bio-Rad Laboratories, Richmond, CA, USA) with bovine serum albumin as standard. IC_{50}, defined as the BS concentration producing 50% inhibition of MTX uptake, was estimated by nonlinear regression of sigmoidal dose–response curves with variable slope (Eq. (2)) using the Graphpad Prism 5 program.

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\log\ IC_{50} - X) \times \text{Hill slope}}} \quad (2)$$

where Y is the measured MTX uptake, top is the MTX uptake in the absence of BS, bottom is the predicted maximum inhibition of MTX uptake set as a global parameter for all BS, X is log[BS] and Hill slope is the slope at the steepest part of the curve. The program determines best fit values with associated standard errors (SEs) and 95% confidence intervals (CIs). Differences in IC_{50}s for the five BS were assessed by comparing the 95% CIs.

3. Results

3.1. CMC values

CMC values of BS are reported in Table 1. The CMC values of C and 12-MKC are lower than those previously obtained using the same method (4.09 and 13.35 respectively) (Yang et al., 2009) but follow the same order. The CMC values for all BS are also much lower than those determined by Poša et al. (2007) using a light scattering method (7.6, 58, 62.5, 93 and 132 mM for C, 7-MKC, 12-MKC, DKC and TKC respectively) but again follow the same order. Later work by Poša and Kuhajda (2010) showed the CMC value (with the exception of the two MKCs) is inversely proportional to the lipophilicity of the BS as measured by their relative retention on reversed phase HPLC.

3.2. Cytotoxicity

LDH release from Caco-2 cells as a function of BS concentration is shown in Fig. 3. The minimum concentrations causing cytotoxicity (≥20% of positive control) were 2, 2, 5, 10 and 15 mM for C, 7-MKC, 12-MKC, DKC and TKC respectively. This indicates that replacing hydroxyl groups in C with keto groups leads to a decrease in cytotoxicity of the BS.

Table 1
CMCs and best fit IC_{50} values (mean ± SE, $n = 3$) for inhibition of MTX uptake by Caco-2 cells of bile salts.

Bile salt	Number of keto groups	CMC (mM)	IC_{50} [a] (mM)
C	0	2.93	0.15 ± 0.02
7-MKC	1	5.15	0.41 ± 0.04
12-MKC	1	9.94	0.91 ± 0.04
DKC	2	13.4	1.56 ± 0.21
TKC	3	54.6	6.84 ± 0.46

[a] Determined by non-linear regression of dose–response curves (Eq. (2)) shown in Fig. 5.

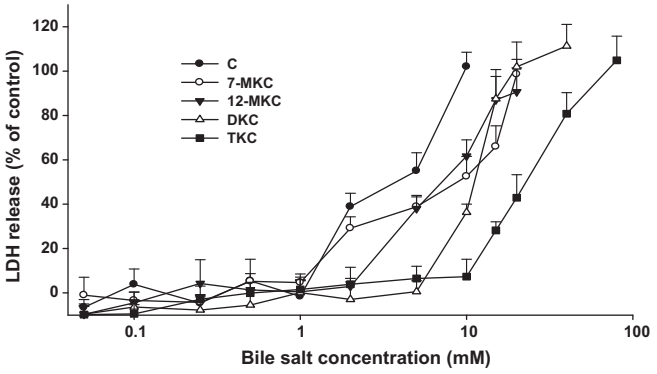

Fig. 3. Cytotoxicity of bile salts as measured by LDH release from Caco-2 cells exposed to increasing concentrations of BS for 30 min. Values of LDH release (means ± SD, $n = 6$) were calculated using Eq. (1).

3.3. Membrane fluidity

Dose–response curves for the effect of BS on FP of DPH and TMA-DPH in Caco-2 cells are shown in Fig. 4. The results show that FP decreases with increasing concentration of BS indicating that exposure to BS increases the fluidity of Caco-2 cell membranes. Using DPH, a fluorescent probe for hydrophobic regions of cell membranes, DKC and TKC cause a marked decrease in FP (increase in membrane fluidity) whereas 7-MKC and 12-MKC have little effect.

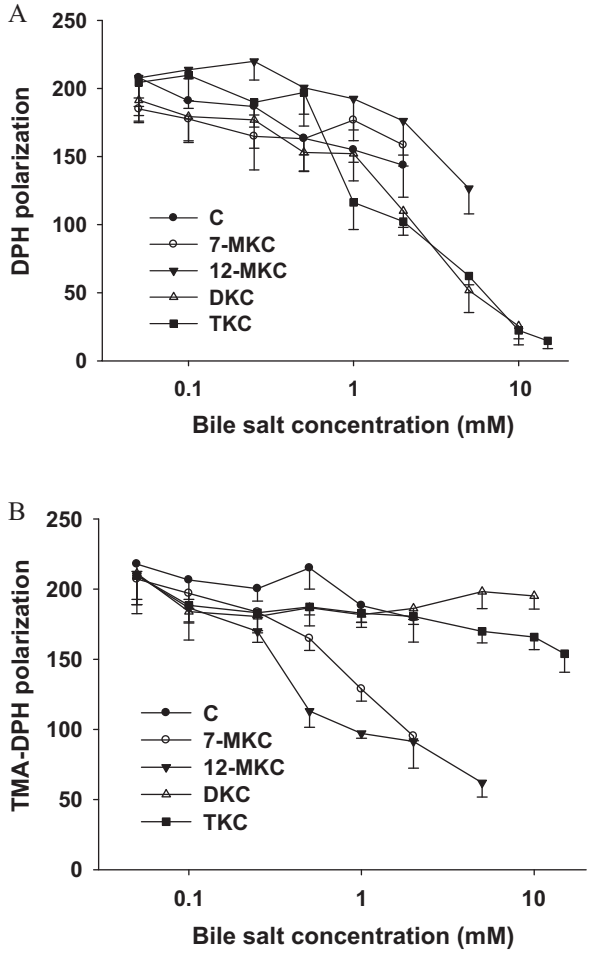

Fig. 4. Dose–response curves for the effect of bile salts on membrane fluidity of Caco-2 cells as measured by fluorescence polarization of (A) DPH and (B) TMA-DPH. Data are means ± SD ($n = 6$).

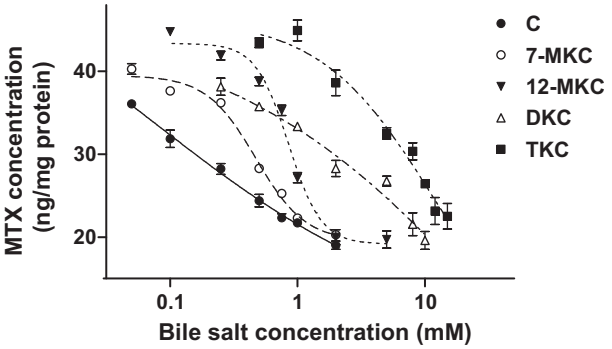

Fig. 5. Dose–response curves for the effect of BS on MTX uptake in Caco-2 cell monolayers. Cells in triplicate were pretreated with BS for 30 min and then incubated for 10 min at 37 °C. Data are means ± SD ($n = 3$).

Conversely, using TMA-DPH, a fluorescent probe for hydrophilic (head group) regions of cell membranes, 7-MKC and 12-MKC cause a notable decrease in FP whereas DKC and TKC have little effect. C causes little or no change in FP in both regions.

3.4. MTX uptake in Caco-2 cell monolayers

Dose–response curves for the effect of BS on MTX uptake are shown in Fig. 5 with corresponding best fit IC_{50} values shown in Table 1. All BS decreased MTX uptake with IC_{50} values increasing for BS with increasing numbers of keto groups (increasing CMC). The IC_{50} values are all significantly different from each other. The slopes of the dose–response curves are not parallel (Hill slopes ranging from 0.5 for DKC to 4.0 for 12-MKC) with that of 12-MKC being the steepest.

4. Discussion

It is known that BS are cytotoxic due to their ability to disrupt cell membranes. In the present study, all BS were cytotoxic to Caco-2 cells at higher concentrations. Cytotoxicity decreased with increasing CMC suggesting that lower cytotoxicity is the result of a reduced ability to penetrate cell membranes and sequester lipids from them.

BS were shown to increase membrane fluidity (decrease steady state FP) and decrease MTX uptake into Caco-2 cells in the same concentration range. Also the order of the ability of BS to decrease MTX uptake based on IC_{50} values (C > 7-MKC > 12-MKC > DKC > TKC) is, with the exception of C, the same as that for the ability of BS to increase the fluidity of hydrophilic regions of cell membranes. Since the current study of MTX uptake involves the apical membrane only, and since BS reduce MTX uptake rather than increase it (suggesting little or no effect on MTX apical efflux transporters), the results suggest that BS decrease MTX uptake by indirectly reducing the activity of MTX influx transporters through increasing the membrane fluidity of hydrophilic regions of cell membranes. However, the system is inherently very complex and our results do not rule out the possibility of a direct effect of BS on MTX influx transporters or some combination of direct and indirect interactions between BS and MTX influx and efflux transporters.

The fact that C has little effect on membrane fluidity of both hydrophobic and hydrophilic regions of Caco-2 cell membranes is surprising given that, of all the BS studied here, it has the highest lipophilicity (Poša and Kuhajda, 2010) and lowest CMC. However, a similar result was obtained by Jean-Louis et al. (2006) where C was found to have no effect on membrane fluidity of both hydrophobic and hydrophilic regions of cell membranes of the human colon adenocarcinoma cell line HCT116. In contrast, they found membrane fluidity of hydrophobic regions of cell membranes was decreased by

the more lipophilic deoxycholate (DC) and ascribed the difference to the demonstrated ability of DC but not C to increase membrane cholesterol content and redistribute it into macroscopic aggregates. Whether membrane perturbations of this sort are responsible for the changes in membrane fluidity observed with ketocholates in our study requires further research.

The ability of C to reduce MTX uptake without affecting membrane fluidity indicates some other mechanism is involved. One possibility is that C reduces the interaction of MTX with its influx transporters directly through competition for its binding sites.

5. Conclusion

An increasing number of keto groups in C and changing from 7- to 12-MKC are associated with a decrease in the ability of BS to inhibit MTX uptake by Caco-2 cells. 7- and 12-MKC but not C also increase membrane fluidity of hydrophilic regions of cell membranes suggesting they inhibit MTX influx transporters by disturbing their lipid environment. However, C inhibits MTX uptake without affecting membrane fluidity suggesting inhibition by BS involves other mechanisms.

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