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Physicochemical and Biological Characterization of Monoketocholic Acid, a Novel Permeability Enhancer

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Abstract: Bile salts are endogenous surfactants which have been widely used in drug formulation and drug delivery systems to increase drug permeation. When given by subcutaneous injection to rats, the novel bile salt, monoketocholate (MKC) has been shown to increase brain uptake of several drugs. This study aimed to characterize the physicochemical and some biological properties of MKC as a basis for understanding the mechanism by which it enhances membrane permeability. Comparison was made with three natural bile salts, cholate, deoxycholate and taurocholate. Critical micelle concentrations (CMC) were measured by the surface tension method and partition coefficients in *n*-octanol/buffer were measured by liquid−liquid extraction. The effects of bile salts on three different biological membrane models were investigated. Penetration studies in Langmuir monolayers indicated that MKC has only a weak ability to insert into phospholipid monolayers, but it can increase their elasticity once incorporated. In the erythrocyte model, MKC did not cause hemolysis at concentrations up to 10 mM, but changed the deformability of erythrocytes. Studies of the permeability of mannitol and transepithelial electrical resistance (TEER) across Caco-2 cell monolayers showed MKC did not cause significant increases in mannitol permeability or decreases in TEER values. In conclusion, MKC does not display strong membrane-solubilizing properties, but does change the mechanical properties of biological membranes. This effect might influence both passive and active transcellular permeation.

Keywords: Monoketocholate; bile salts; permeability; biological membrane; hemolysis; Langmuir monolayer; Caco-2; BBB

Introduction

Bile salts are amphiphilic steroids which have been extensively studied as permeability enhancers of various biological membranes such as the gastrointestinal wall,[1] buccal epithelium,[2] skin,[3] cornea,[4] nasal mucosa[5] and the blood brain barrier (BBB).[6] Bile salts not only increase the permeability of membranes to low molecular weight compounds, but also to macromolecules such as insulin.[7] Certain physicochemical properties of bile salts such as their solubil-

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ity, ionization, hydrophobicity and micelle formation vary due to structural differences in the type of conjugation and the number, position and orientation of hydroxyl groups. Other properties, particularly their detergent activity and hydrophobicity, are especially relevant to the ability to enhance permeability. At submicellar concentrations, it is believed that bile salt monomers intercalate phospholipid bilayers and change their lipid packing, thereby affecting the membrane permeability.[8] At higher concentrations, mixed micelles of bile salts and the components of the plasma membrane are formed, leading to a loss of integrity of the membrane structure[9] and opening of tight junctions.[10]

All bile salts have a four ring steroid structure and a five or eight carbon side chain terminating in a carboxylic acid group which may be conjugated. They differ in the number and orientation of substituents (e.g., hydroxyl groups), and they are facially amphiphilic with a convex hydrophobic upper (β) side and a concave hydrophilic lower (α) side. In monoketocholate (MKC, 3α,7α-dihydroxy-12-oxo-5β-cholanate) the OH at position 12 in cholate is replaced by a keto group,[11] thus the α-side of this semisynthetic bile salt with a keto group at position 12 is less hydrophilic than that of cholate. MKC has been studied as a permeability enhancer for drug transport across the BBB[6] where it was found to promote quinine uptake, enhance the analgesic effect of morphine and prolong the sleeping time induced by pentobarbital. However details of the efficacy, toxicity and mechanism of MKC as a permeability enhancer are unknown.

The aim of the work reported here was to study some physicochemical and biological properties of MKC in order to compare it with three well-known bile salts: cholate (C), deoxycholate (DC) and taurocholate (TC). Erythrocytes, Langmuir monolayers and Caco-2 cell monolayers were employed to study the effects of MKC on biological membranes.

Materials and Methods

Materials. Sodium cholate, sodium deoxycholate and sodium taurocholate (purities: 99%, 97% and 97% respectively) were purchased from Sigma-Aldrich (New Zealand). Sodium monoketocholate, purity 96.5% with 3.1% cholate as the major impurity, was a gift from Professor Ksenija Kuhajda (University of Novi Sad, Serbia). Dipalmitoyl phosphatidylcholine (DPPC), Ringer's buffer (10 mM D-glucose; 0.23 mM $MgCl_2$; 0.45 mM KCl; 120 mM NaCl; 0.70 mM Na_2HPO_4; 1.5 mM NaH_2PO_4), HEPES, calcium chloride, formic acid and sodium bicarbonate were purchased from Sigma-Aldrich. Pop-Top and Hemafil polycarbonate track-etched membranes (13 mm diameter) were obtained from Biolab (Auckland, New Zealand). The Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM), Hanks balanced salt solution (HBSS), fetal bovine serum (FBS), trypsin-EDTA, nonessential amino acids, penicillin−streptomycin and glutamine were all from Invitrogen (Auckland, New Zealand). Milli-Q water was used throughout all studies and other reagents were of analytical grade.

Apparent Octanol/Buffer Partition Coefficients. *n*-Octanol/water partition coefficients were determined in triplicate by liquid−liquid extraction using a Mixxor phase transfer device.[12,13] Bile salt solutions (1−20 μg/mL) were prepared in *n*-octanol-saturated Ringer's-HEPES buffer. *n*-Octanol was presaturated with Ringer's-HEPES buffer. Organic and aqueous phases of appropriate volume ratios were mixed 20 times over 3 min. Preliminary experiments showed that equilibrium was reached using this procedure. The mixture was then centrifuged at 2000*g* for 10 min and an aliquot of the aqueous layer taken and diluted with blank aqueous solution for analysis by a validated LC−MS/MS assay. Apparent partition coefficients (*P*) were calculated as

$$P = \frac{V_W(C_{initial} - C_{end})}{V_O C_{end}} \quad (1)$$

where V_W is the volume of aqueous phase, V_O is the volume of octanol phase, and C_{inital} and C_{end} are the concentrations of bile salt in the aqueous phase initially and at equilibrium respectively.

Critical Micelle Concentrations. A Du Nouy ring tensiometer (Torsion balance, U.K.) was used to measure surface tension of bile salts in Ringer's buffer containing 10 mM HEPES and 1.2 mM calcium chloride at room temperature.[14] The ring was raised slowly to minimize the nonequilibrium problem inherent with this method. Triplicate surface tension measurements were initiated with bile salt-free Ringer's-HEPES buffer after which a bile salt stock solution was added incrementally and surface tension measured after each

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increment until equilibrium was reached. The CMC was obtained from the intersection of the two best-fit least-squares lines of a plot of surface tension versus the logarithm (ln) of bile salt concentration.

Langmuir Monolayer Studies. Studies of bile salts with phospholipid monolayers were conducted using a Langmuir−Blodgett trough (NIMA, U.K.) with an area (A) of 100 cm^2 and a volume of 50 mL at ambient temperature. A Wilhelmy paper plate (Whatman's No.1 chromatography paper) was connected with the pressure sensor to measure the surface pressure (π). Before each experiment, the Teflon trough and the barriers were thoroughly cleaned with chloroform and Milli-Q water. The subphase consisted of isotonic Ringer's-HEPES buffer pH 7.2 with or without bile salt. A 10−20 μL volume of a 1 mM DPPC solution in chloroform was spread on the subphase using a Hamilton syringe. After waiting 10 min for solvent evaporation, compression was started at a rate of 10 cm^2/min and the $\pi-A$ isotherms were recorded by the instrument software (Nima516).

For penetration studies, a stock solution (1 mM or 4 mM) of a bile salt in Ringer's-HEPES buffer was prepared. The DPPC monolayer on bile salt-free buffer was compressed into the liquid-condensed phase at 15, 20 or 25 mN/m at a rate of 10 cm^2/min. After the target surface pressure was reached, the surface area was kept constant. Then the bile salt solution (1 mL) was injected into the subphase through the injection port without breaking through the monolayer. A magnetic stirrer was used to ensure uniform distribution of bile salt in the subphase. The change in the surface pressure with time was recorded.

For the equilibrium study, the isotherms of DPPC monolayers on subphases containing different bile salts at different concentrations were recorded.

***In Vitro* Hemolytic Activity.** Rat blood was centrifuged and the red blood cells (RBC) were washed three times in Ringer's-HEPES buffer. A calibration curve for percentage intact RBC was constructed by mixing intact RBC suspensions in Ringer's-HEPES buffer with the supernatant of lysed RBC. The total amount of hemoglobin, which includes hemoglobin from lysed and intact RBC, was the same in all suspensions, but the percentage of intact RBC in the suspensions varied from zero to 100%. The absorbance of each suspension was measured at 700 nm using a microplate reader. Bile salts and the supernatant of lysed RBC solutions did not absorb at 700 nm. The calibration curve of absorbance verses percentage intact RBC was linear ($r = 0.996$), indicating the absorbance at 700 nm was a good indicator of intact RBC and hence of hemolysis.

To study the hemolytic activity of bile salts, aliquots (100 μL) of bile salt solutions of various concentrations in Ringer's-HEPES buffer were added into 96-microplate wells, and aliquots (100 μL) of RBC suspension were then added. Bile salt solutions, erythrocyte suspensions and the plate chamber of the microplate reader were prewarmed to 37 °C. After shaking for 10 s, the absorbance (700 nm) of each well was monitored at one minute intervals for two hours. The percentage of intact RBC remaining after incubation with bile salts was calculated from the calibration curve.

Erythrocyte Deformability. The deformability of RBC was evaluated by the filtration method[15] using gravity as the driving force. A RBC suspension (0.5 mL) with 10% hematocrit was mixed with various concentrations of bile salt solution (0.5 mL) with and without 1.2 mM Ca^{2+} and incubated at 37 °C for 60 min. The assembled membrane (Hemafil) holder was filled with Ringer's-HEPES buffer avoiding any trapped air, and the outlet of the filter holder was vertically connected to a Teflon tap. A 1.0 mL plastic syringe without its plunger was connected to the inlet of the filter holder and then filled with a RBC suspension. After the first drop of RBC suspension passed through the membrane, the tap was closed and the syringe filled to 1 mL with RBC suspension. The filtration time of 1 mL of RBC suspension was recorded from when the tap was opened. In this study, a 3 μm pore-sized filter was used as it is more sensitive to the viscoelastic properties of the cell membrane than a 5 μm filter which is more sensitive to the cytoplasm viscosity.[16] All deformability studies were conducted in pentuplicate at room temperature. The relative cell transit time (RCTT) was calculated as the filtration time of the erythrocyte suspension divided by the filtration time of the same volume of buffer.

Caco-2 Cell Monolayer Permeability. Caco-2 cells at passage 15 were cultured in complete DMEM containing 10% FBS, 1% penicillin−streptomycin−glutamine and 1% nonessential amino acids under an atmosphere of 5% CO_2/95% air at 37 °C and 95% humidity. Caco-2 cells at 80−90% confluence were split with 0.05% trypsin-EDTA and seeded into Transwell inserts at a density of 2×10^5 cells/insert. The medium was replaced every two days until day 21. Transepithelial electrical resistance (TEER) ($\Omega \cdot cm^2$) of the monolayers was measured at room temperature using a Millicell-ERS apparatus (Millipore Corp., Bedford, MA).

Monolayers were washed twice with HBSS at 37 °C before experiments. Transport buffer (0.5 mL of HBSS with 25 mM HEPES and 25 mM glucose at pH 7.4) containing cholate or MKC (0−20 mM) was added to the apical side and 1.5 mL transport buffer to the basolateral side. The integrity of the cell monolayer was checked by measuring the TEER at 0, 60, 120, 180 min and by measuring the permeability of [^{14}C]mannitol. After 180 min, fresh medium (without bile salts) was added and recovery of TEER monitored over 21 h.

The apparent permeability coefficient (P_{app}) for each bile salt was calculated using

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$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A} \times \frac{1}{C_0} \quad (2)$$

where P_{app} is the apparent permeability coefficient (cm/s), $(\Delta Q)/(\Delta t)$ the steady state flux (μmol/s), A the surface area of the membrane (cm^2), and C_0 the initial concentration in the donor chamber (mM).

LC−MS/MS Assay. Bile salts were analyzed by an LC−MS/MS system consisting of an Agilent 1200 HPLC system and an Applied Biosystems API3200 mass spectrometer. Separation was achieved using a Zorbax 5 μm SB-C18 column (2.1 × 50 mm, Agilent) protected with a guard column C18 4 × 3.0 mm (Phenomenex, NZ). The column oven was set at 40 °C. The flow rate was 0.3 mL/min, and the mobile phase was a gradient of 20−80% acetonitrile containing aqueous 0.1% (v/v) formic acid. MS/MS analysis was performed in the multiple reaction monitoring (MRM) mode. For unconjugated bile salts, the precursor ions themselves were selected as product ions as no characteristic product ions could be identified. The MS/MS transitions were *m/z* 407.2 → 407.2 for C, *m/z* 391.2 → 391.2 for DC, *m/z* 405.2 → 405.2 for MKC and *m/z* 514.2 → 79.9 for TC All the samples were mixed with acetonitrile (1:1 v/v) before injection. The calibration was linear over the range 20 ng/mL to 500 ng/mL. Intraday and interday precisions were in the range of 0.5−9.4% for all compounds at 20, 100 and 400 ng/mL.

Statistical Analysis. Data for the four bile salts were analyzed by one-way ANOVA. Linear regression was used to determine the cutoff value for the Langmuir monolayer penetration study.

Results and Discussion

Lipophilicity. The lipophilicity of bile salts is frequently linked with their biological activities, such as permeability enhancement,[5] interaction with biological membranes[17] and induction of apoptosis.[18] In this study, *n*-octanol−physiological buffer partition coefficients (log *P*) were measured at bile salt concentrations below their CMC. It has been suggested that side chain amidation, number of hydroxyl groups and position of hydroxyl groups all influence lipophilicity. The unconjugated bile salts are more hydrophobic than conjugated bile salts, and dihydroxyl bile salts are more hydrophobic than those with three hydroxyl groups.[19] The results (Table 1) show that the order of the three well-known bile salts, C, DC and TC, complies with this relationship between log *P* and structure; however, MKC, an unconjugated dihydroxy bile salt, is less hydrophobic than the unconjugated trihydroxy bile salts (Table 1).

Table 1. Partition Coefficients, Critical Micelle Concentrations and Area per Molecule of Bile Salts

bile salt	partition coefficient log *P*	CMC (mM)	area/molecule (Å²)
cholate	0.01	4.09	87
monoketocholate	−0.41	13.35	86
deoxycholate	1.46	1.69	77
taurocholate	−1.50	3.56	129

Critical Micelle Concentration and Area per Bile Salt Molecule. The critical micelle concentration (CMC) is an important physicochemical property which provides information about the physical status of bile salt molecules in an aqueous environment. This is important since the monomers and the micelles have different physicochemical properties and biological behaviors.[20] It is well-known that the CMC is dependent on the experimental method, the temperature, pH value and the composition of the dispersion medium.[21] In this study, the surface tension method was used to determine the CMC of the four different bile salts in Ringer's-HEPES buffer. CMC values were sufficiently sharp to readily allow their estimation from the intersection of the linear segments of the surface tension versus log concentration profiles. Compared with the CMC of MKC, those of C, DC and TC were relatively low (<5 mM) (Table 1) consistent with values previously obtained in physiological saline using the same surface tension method.[22] It has been suggested that the CMC is strongly influenced by the number and position of hydroxyl groups with the trihydroxy bile salts having higher CMC than the dihydroxy bile salts.[23] In this study, C and TC, with three hydroxyl groups, have higher CMC values than DC, with two hydroxyl groups. However, MKC, with only two hydroxyl groups, shows a much higher CMC. This suggests that the keto group at position 12 has a significant effect on the properties of monoketocholate.

The area per bile salt molecule (A_m) was calculated as

$$A_m = \frac{-RT}{N_a\left(\frac{\partial \gamma}{\partial \ln C}\right)} \quad (3)$$

where N_a is the Avogadro number, γ is the surface tension and C is the bile salt concentration. $(\partial\gamma)/(\partial \ln C)$ was obtained

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from the linear segment of the plot of surface tension versus the logarithm of the bile salt concentration below the CMC. The results (Table 1) suggest that all four bile salts are horizontally packed at the air/aqueous interface. TC has the largest surface area (129 Å^2) due to the taurine group. C and MKC have very similar chemical structures and molecular areas (87 and 86 Å^2 respectively). DC has the smallest area (77 Å^2) because it is an unconjugated bile salt without any moiety at position 7. The hydroxyl groups of bile salts anchor them at the air/water interface[24] and the evenly distributed hydroxyl groups of trihydroxy bile salts cause them to lie flat. The absence of a 7-OH group allows DC to tilt thereby reducing the area per molecule.

Langmuir Trough Study. The Langmuir trough technique is frequently used to prepare a phospholipid monolayer and has been adopted as a simple biological membrane model. The monolayer has been used to study the interaction between membranes and substances such as drugs,[25,26] surfactants,[27] polymers[28] and proteins.[28,29] In this study, the interaction between water soluble bile salts and DPPC was studied by observing the surface pressure changes.

1. Penetration Study. The penetration study is a technique to investigate if a substance can insert into a biomembrane. The DPPC monolayer was compressed to an initial surface pressure at which the DPPC is in the liquid condensed state where it has similar structural characteristics with DPPC/water bilayers in the gel state.[30] After the DPPC monolayer was compressed to an initial surface pressure (π_i), the bile salts were added to the subphase and the increment of surface pressure was recorded as a function of time. Various initial surface pressures were studied. When the surface pressure showed no further increase, the monolayer was assumed to be in equilibrium with bile salt in the subphase.

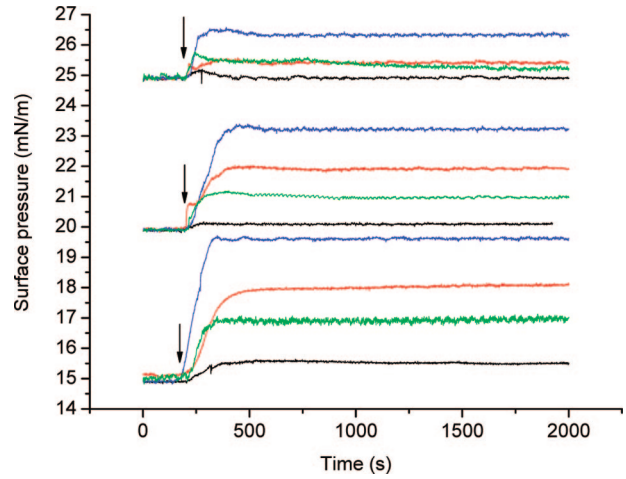

Figure 1. Penetration of C, DC, MKC and TC into DPPC monolayers with different initial surface pressure (green, C; blue, DC; black, MKC; red, TC). The arrows indicate when bile salts were injected into the subphase.

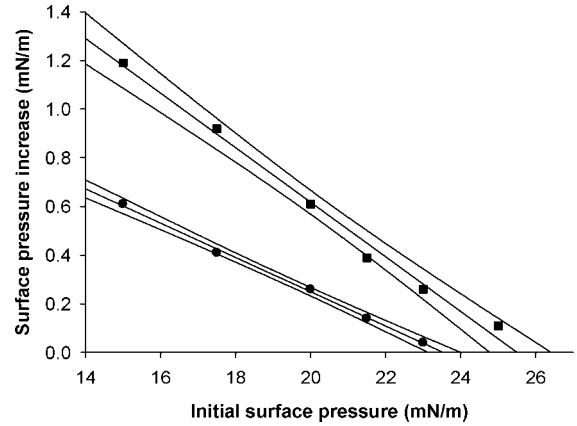

Figure 2. Effect of concentration of MKC on its penetration into DPPC monolayers (● 13.3 μM and ■ 53.2 μM). Curves are 95% confident limits.

The final concentration of all four bile salts was 13.3 μM, which is well below the CMCs. Different bile salts showed different effects on pressure. The penetration of DC caused the greatest increase in the surface pressure. TC and C showed modest increases, and MKC had the smallest effect on surface pressure. Given the similarity in molar volumes (315.3, 318.3, 314.8 and 378.2 cm^3 for C, DC, MKC and TC respectively, calculated by ACDLABS 11.0) and cross section areas of the bile salts, this suggests that the difference in pressure increase for the four bile salts is due to the difference in the number of bile salt molecules penetrating the monolayer. Therefore MKC has the least penetration into the DPPC monolayer. The rank order of penetration of the four bile salts correlates with their CMC values rather than *n*-octanol/water partition coefficients. This suggests that the penetration of a substance into a phospholipid monolayer is dependent not only on the hydrophobicity of the substance but also on its amphiphilicity.

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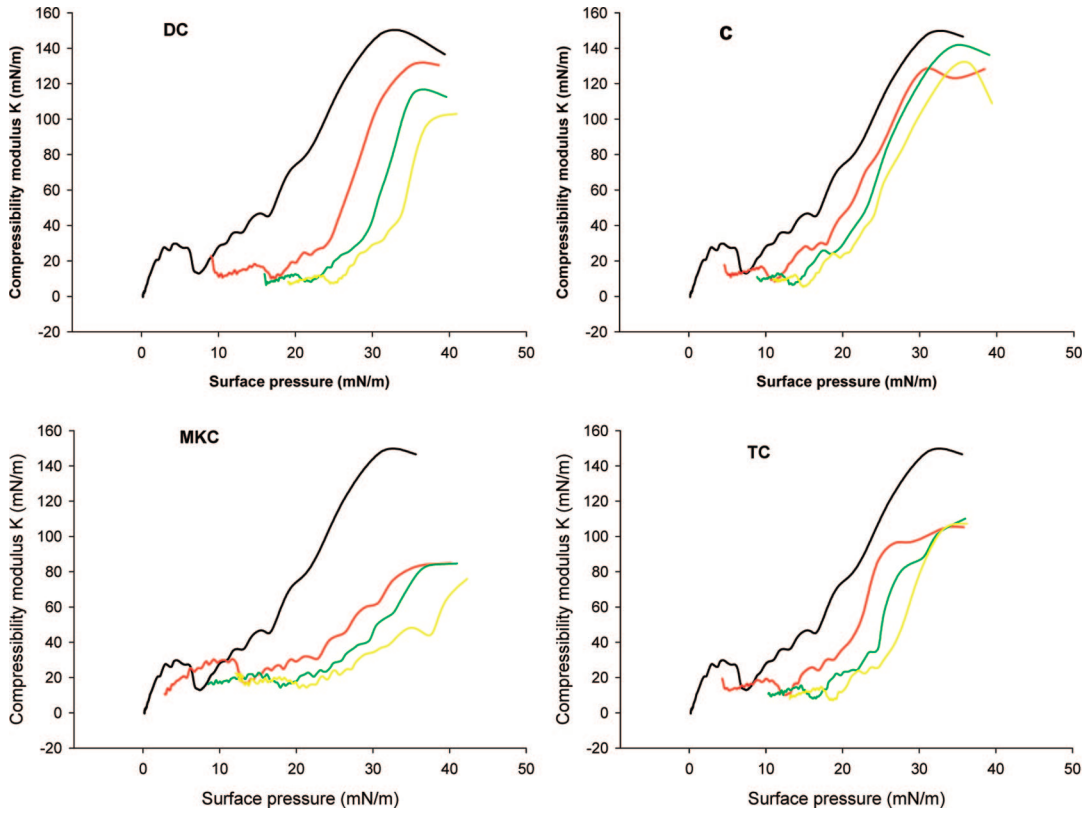

Figure 3. Effect of different bile salts in the subphase on the compressibility of DPPC monolayers (black, no bile salt; red, 10 μM; green, 50 μM; yellow, 100 μM).

The penetration of bile salts into a DPPC monolayer was dependent on the initial surface pressure, penetration being less at higher π_i values because it is more difficult for bile salts to insert into tightly packed monolayers (Figure 1). The surface pressure increase ($\Delta\pi$) calculated as $\Delta\pi = \pi_s - \pi_i$, where π_s is the surface pressure at saturation and π_i is the initial surface pressure, was regressed against π_i to obtain the critical surface pressure (π_{cr}) from the intercept on the *x*-axis. The critical surface pressures of 29.7 mN/m, 27.7 mN/m, 27.3 and 25.2 mN/m for DC, TC, C and MKC respectively at a concentration of 13.3 μM suggest that these bile salts would not penetrate biological membranes which are reported to have π_i of 30 mN/m, a surface pressure higher than the π_{cr}.[31,32] MKC, with the lowest π_{cr}, is the least likely to penetrate membranes. However, the critical surface pressure is dependent on the concentration of bile salt in the subphase (Figure 2), whereby the π_{cr} for MKC is significantly ($p < 0.05$) increased from 23.5 to 25.4 mN/m on increasing its concentration from 13.3 to 52.5 μM. This would suggest that, at high concentrations, MKC could penetrate biological membranes.

2. Compressibility. The bile salt/phospholipid mixed monolayers can also be characterized by the elastic modulus of area compressibility (K) calculated using

$$K = -A_\pi\left(\frac{\partial\pi}{\partial A}\right)_\pi \qquad (4)$$

where A_π is the area per DPPC molecule at the indicated surface pressure π. The lower the K value, the higher the monolayer elasticity and bile salts increase elasticity (Figure 3). The liquid-expanded to lipid-condensed phase transition, as shown by the peak in the range $\pi = 0-10$ mN/m (Figure 3), also becomes less significant and tends to occur at higher surface pressure at higher concentrations of bile salt. The increase in elasticity has also been shown with liposomes, a lipid bilayer model.[33] This increase in elasticity should facilitate insertion of foreign molecules into the bilayer thereby enhancing the transport across the membrane.[34]

Hemolytic Activity. The hemolysis assay is a simple *in vitro* model to evaluate the damage to biological membrane caused by bile salts. The hemolytic activities of the three

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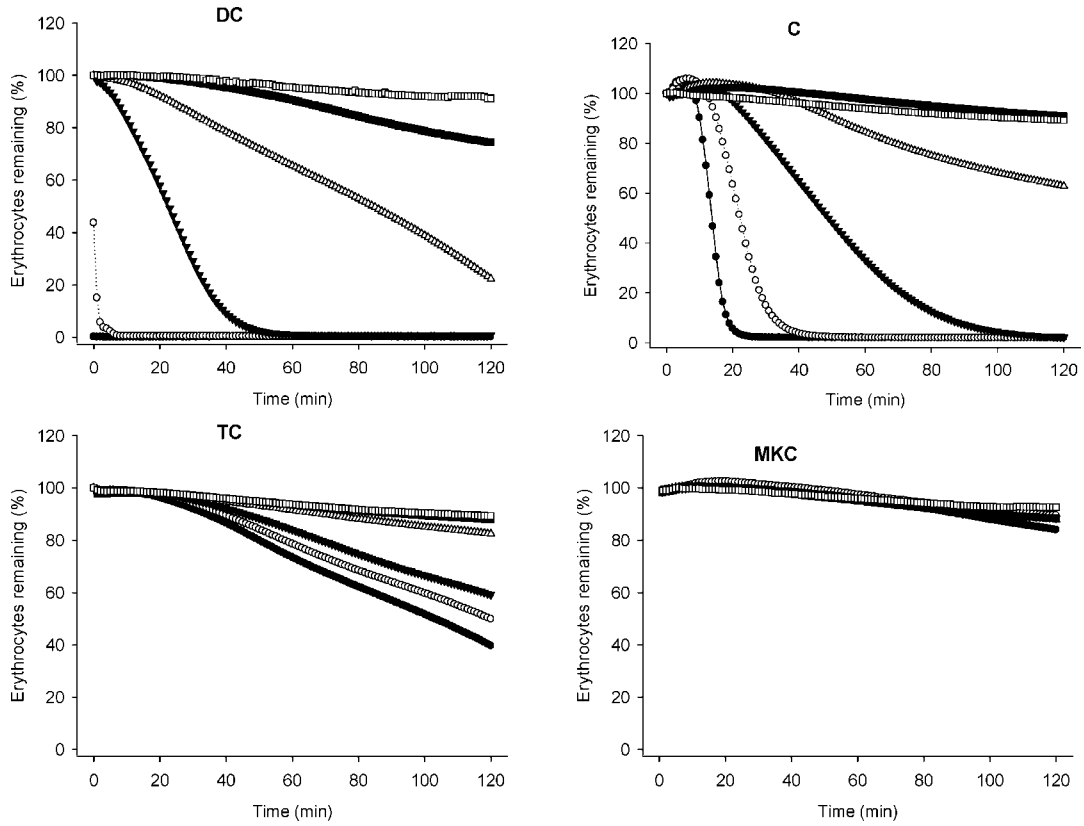

Figure 4. Effect of bile salts on hemolysis of erythrocyte at 37 °C: C, TC, MKC (● 10 mM; ○ 8 mM; ▼6 mM; Δ 4 mM; ■ 2 mM; □ 0 mM) and DC (● 2.0 mM; ○ 1.5 mM; ▼1.0 mM; Δ 0.75 mM; ■ 0.5 mM; □ 0 mM).

natural bile salts were concentration-dependent (Figure 4). The most hydrophobic bile salt, DC, showed the greatest hemolytic activity, followed by C and TC; however, the semisynthetic MKC did not show any hemolytic activity in the 1−10 mM range. It has been suggested that hemolytic potency increases with partitioning into the plasma membrane and hydrophobicity.[35] Although the order of hemolytic activities of the three natural bile salts followed their hydrophobicities (*n*-octanol/water partition coefficient), this was not the case for MKC.

C and TC showed hemolytic activity at concentrations (4 mM) just above their CMC, but the most hydrophobic DC induced hemolysis at a concentration of 0.5 mM, that is below its CMC (1.69 mM). There is a negative correlation between CMC and hemolytic activity of bile salts, which has been reported previously.[36] Some studies suggest hemolytic activity of bile salts occurs at concentrations above the CMC, but other studies have demonstrated the possibility of hemolysis induced by bile salts when present as monomers.[37] It has been observed that more hydrophobic bile salts cause hemolysis below their CMC and more hydrophilic ones above their CMC.[38] The hydrophilic bile salt, MKC, is supposed to only induce hemolysis at a concentration higher than its CMC. However the hemolytic activity of MKC at concentrations above its CMC (13.35 mM) could not be studied because of its limited solubility.

The rank order of hemolytic activities of the four bile salts correlates with their penetration capabilities into DPPC monolayers. This suggests that, although hydrophobicity (octanol/water partition coefficient) is an important property, it does not adequately describe the complexities of the bile salt/membrane interaction. Bile salts are negatively charged amphiphilic compounds under physiological conditions; therefore, the electrostatic interactions can be important for membrane partitioning. The isotropic octanol/water model may not be a suitable model of the biological membrane. It has been shown that the hydrophilic−hydrophobic balance of bile salts has a significant impact on membrane partition-

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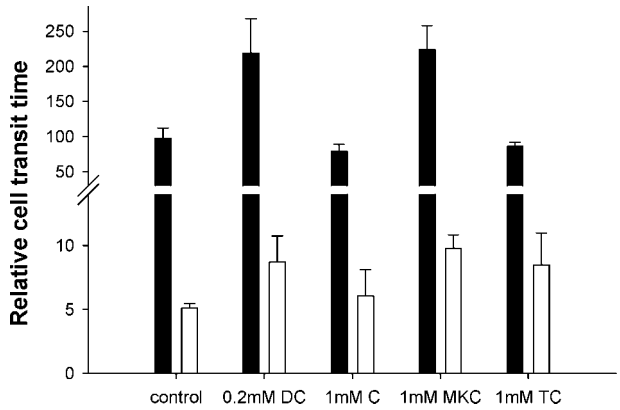

Figure 5. Relative cell transit time (RCTT) of erythrocytes after incubation with bile salts (■ with 1.2 mM Ca^{2+}; □ without Ca^{2+}).

ing[39] and the membrane/water partition coefficients of detergents are correlated with critical micelle concentration.[40]

Erythrocyte Deformability. It is reported that some substances affect the deformability of endothelial cells[41] and epithelial cells,[42] thereby affecting paracellular permeability of membranes. In this paper, we used erythrocytes as a readily available model of endothelial cells to investigate if bile salts can change the mechanical properties of a plasma membrane. The effect of bile salts on erythrocyte deformability was studied by a filtration method at final concentrations of bile salts below those which cause hemolysis. The erythrocyte deformability is determined by three factors: cell geometry; viscosity of cytoplasm; and intrinsic viscoelastic properties of the erythrocyte membrane.[43] It has been reported that once bile salts intercalate the cell membrane, they lower the membrane tension so as to transform erythrocytes from biconcave to spherical cells with impaired deformability.[44] Since the stability of a phospholipid bilayer and many intracellular biochemical events are controlled by calcium, the role of calcium in bile salt induced deformability was also studied.

The effect of extracellular calcium on erythrocyte deformability was very significant (Figure 5). All erythrocytes in buffer containing 1.2 mM Ca^{2+} had much longer RCTT compared with erythrocytes in buffer without Ca^{2+} in agreement with previous work which showed deformability decreases on elevation of intracellular Ca^{2+}.[41] Bile salts also showed effects on the deformability of erythrocytes. In the group without extracellular Ca^{2+}, only 1 mM MKC was necessary to decrease the deformability significantly ($p = 0.05$). However, when Ca^{2+} was added, both 0.2 mM DC and 1 mM MKC impaired the deformability significantly ($p = 0.01$). This deformability study suggests that bile salts can change the mechanical properties of a plasma membrane which may cause changes in its permeability. In addition, some chemicals can change the paracellular permeability by modulating cell deformability.[41,42] It is believed that inter-

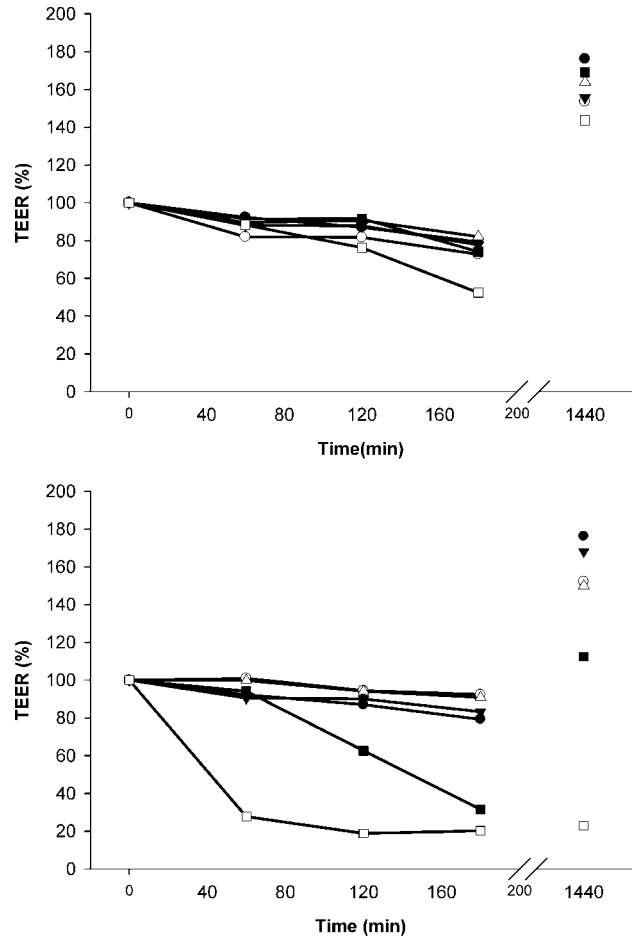

Figure 6. Effect of MKC and C on TEER of Caco-2 monolayers (● control; ○ 0.05 mM; ▼ 0.1 mM; Δ 1 mM; ■ 10 mM; □ 20 mM).

Table 2. Effect of Concentration of C and MKC on the Permeability of Mannitol ($n = 2$)

	mannitol P_{app} (10^{-6} cm/s)	
bile salt concn (mM)	C	MKC
control	1.27	1.27
0.05	1.47	1.55
0.1	1.04	0.73
1	0.84	0.57
10	1.57,[a] 11.66[b]	0.52
20	75.6	0.42

[a] Permeability of first one hour. [b] Permeability of last one hour.

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cellular contact (tight junction) is modified when the mechanical properties or morphology is changed.

Caco-2 Permeability. TEER was monitored during transport experiments as an indicator of paracellular permeability which is controlled by tight junctions and for a further 12 h in bile salt free medium to monitor recovery (Figure 6). C caused a marked decrease in TEER when its concentration was above its CMC (10−20 mM), and the reduction in TEER was concentration dependent. The Caco-2 monolayer lost its integrity very soon after incubation with 20 mM C, and the TEER value did not recover, suggesting that 20 mM C caused permanent damage to the cell monolayer. In contrast, MKC showed much less effect on paracellular permeability than C. The TEER only decreased after three hours incubation with 20 mM MKC and rose to 140% of the initial value after 21 h.

The permeability of [^{14}C] mannitol, a paracellular marker, was also investigated. Although C enhanced paracellular permeability at 10 and 20 mM, the trend for MKC was toward decreased permeability, suggesting MKC does not compromise the integrity of Caco-2 monolayers (Table 2).

Conclusion

MKC, a novel semisynthetic bile salt, showed very different physicochemical and biological properties compared with natural bile salts. MKC did not show strong "membrane-solubilizing" properties, but it did modulate the mechanical properties of phospholipid monolayers and erythrocytes. Based on hemolysis of erythrocytes and Caco-2 permeability studies, it is suggested that MKC is less toxic than the other bile salts, neither solubilizing plasma membranes nor opening tight junctions. However, once MKC intercalates the cell membrane, it affects membrane fluidity which we postulate may enhance passive diffusion and indirectly affect transporters. These postulates are now being studied in our laboratory.

Abbreviations Used

C, cholate; DC, deoxycholate; MKC, monoketocholate; TC, taurocholate; CMC, critical micelle concentration; DPPC, dipalmitoyl phosphatidylcholine; RCTT, relative cell transit time; TEER, transepithelial electrical resistance.

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Supporting Information Available: Figures depicting the chemical structures of C, DC, MKC and TC, the penetration of C, DC, MKC and TC into DPPC monolayers at a bile salt concentration of 13.3 μM, and the determination of CMC of bile salts from surface tension versus log concentration. This material is available free of charge via the Internet at http://pubs.acs.org.

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