Effects of nonionic surfactants on membrane transporters in Caco-2 cell monolayers

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Abstract

The objectives of this study were (1) to investigate the transporter inhibition activity of three nonionic surfactants on P-glycoprotein, the human intestinal peptide transporter, and the monocarboxylic acid transporter in Caco-2 cell monolayers, and (2) to evaluate the role of membrane fluidity and protein kinase C in surfactant-induced transporter inhibition. All three surfactants inhibited P-glycoprotein (P-gp). Over a range from 0 to 1 mM, Tween 80 and Cremophor EL increased apical-to-basolateral permeability (AP-BL) and decreased basolateral-to-apical (BL-AP) permeability of the P-gp substrate rhodamine 123. Vitamin E TPGS’s effect was equally large, but essentially only reduced the BL-AP permeability of rhodamine 123, and did so at a vitamin E TPGS concentration of only 0.025 mM. These P-gp inhibition effects would appear to be related to these excipients’ modulation of membrane fluidity, where Tween 80 and Cremophor EL fluidized cell lipid bilayers, while vitamin E TPGS rigidized lipid bilayers. However, among the three surfactants, only Tween 80 inhibited the peptide transporter, as measured by glycyl sarcosine permeability. Likewise, only Cremophor EL inhibited the monocarboxylic acid transporter, as measured by benzoic acid permeability. Nevertheless, at least one of these three surfactants inhibited each P-gp, the human intestinal peptide transporter, and the monocarboxylic acid transporter. A common functional feature of these three surfactants was their ability to modulate fluidity, although results indicate that even strong membrane fluidity modulation alone was not sufficient to reduce transporter activity. N-octyl glucoside, a nonionic surfactant that did not modulate membrane fluidity, did not affect transporter functioning. Protein kinase C inhibitors failed to affect rhodamine 123 and glycyl sarcosine permeability, suggesting protein kinase C inhibition was not the mechanism of transporter inhibition. These results suggest that surfactants can inhibit multiple transporters but that changes in membrane fluidity may not be a generalized mechanism to reduce transporter activity.

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1. Introduction

Surfactants are extensively used in pharmaceutical formulations as wetting agents to improve dissolution and absorption of poorly soluble drugs. Low molecular weight ionic surfactants like sodium lauryl sulfate, in concentrations that are not toxic to the intestinal mucosa, are probably the most commonly used surfactants for this purpose. Nonionic surfactants have been shown to be even less toxic than ionic surfactants to biological membranes (Davis et al., 1970). Additionally, being more hydrophobic than ionic surfactants, nonionic surfactants also possess greater capacity to dissolve poorly soluble drugs, are very efficient emulsifiers, and can be used in self-emulsifying drug delivery systems.

Several nonionic surfactants have been shown to inhibit transporters (Rege et al., 2001; Koga et al., 2000; Miller et al., 1999; Nerurkar et al., 1996; Woodcock et al., 1992). Most reports of surfactant-induced inhibition of membrane transporters have focused on P-glycoprotein (P-gp). P-gp is expressed extensively in the GIT and potentially contributes to reduced oral absorption of drugs. Many nonionic surfactants effectively inhibit P-gp, although the mechanism of inhibition remains unclear. These nonionic surfactants have potential to increase oral absorption of P-gp substrates. Examples of nonionic surfactant with activity on various efflux pumps include Tweens, Spans, Crem-
phors (EL and RH40), Pluronic block copolymers, and vitamin E TPGS.

Numerous drugs are substrates for active influx transport systems, which facilitate absorption. Examples include the human apical sodium-dependent bile acid transporter (hASBT), the human intestinal peptide transporter (hPepT-1), and the monocarboxylic acid transporter (MCT). Inhibition of the peptide transporter by nonionic surfactants has been observed (Koga et al., 1998, 1999a,b, 2000). This effect of influx transporters would make surfactants potentially undesirable formulation additives, as these surfactants may reduce drug absorption.

It has been suggested that surfactants can alter membrane fluidity, thereby changing the conformation of membrane bound transporters (Dudeja et al., 1995; Woodcock et al., 1992). This conformation change is associated with inhibition of P-gp’s ATPase activity. It has also been suggested that such surfactants inhibit protein kinase C (PKC), which is involved in the functioning of many transporters like P-gp (Zhao et al., 1989). PKC phosphorylates several membrane transporters, such that transporter function can be modulated by PKC inhibition or activation. Some of these surfactants have also been shown to inhibit metabolizing enzymes like cytochrome P450 (Mountfield et al., 2000). It has also been reported that only surfactant monomers active in transporter inhibition, such that inhibitory activity essentially plateaus above the critical micelle concentration (cmc) (Nerurkar et al., 1997).

The first objective of this study was to evaluate the influence of three nonionic surfactants on three membrane transporters, namely P-gp, PepT-1, and MCT. The three evaluated surfactants were Tween 80, Cremophor EL, and vitamin E TPGS (α-tocopheryl polyethylene glycol 800 succinate), each of which contains poly(ethylene oxide) groups. N-octyl glucoside, a nonionic surfactant without poly(ethylene oxide) groups, was also evaluated. Additionally, the concentration dependence of surfactant-induced transporter inhibition was determined. Specifically, we evaluated whether micelles were active species, like the monomers, and aimed to compare the transporter inhibition potency of the three surfactants. The second objective was to evaluate the role of membrane fluidity and PKC in the surfactant-induced inhibition of these membrane transporters.

2. Materials and methods

2.1. Materials

Transwell® cell culture chambers with polycarbonate filters (3.0 μm) were purchased from Corning Costar Corporation (Cambridge, MA). 14C-Mannitol (specific activity of 51 mCi/mmol) was obtained from DuPont NEN (Boston, MA). 3H-Glycyl sarcosine or gly-sar (specific activity of 4 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Caco-2 cells were obtained from ATCC (Manassas, VA). Dulbecco’s modified Eagle’s medium (DMEM), Hank’s balanced salt solution (HBSS), phosphate-buffered saline (PBS), bovine serum albumin (BSA), rhodamine 123 (R123), benzoic acid, leucyl methionine (leu-met), sodium azide, staurosporine, chelerythrine chloride and cholesterol were obtained from Sigma Chemical Co. (St. Louis, MO). GF 120918 was a gift from GlaxoSmithKline (Research Triangle Park, NC). HEPES buffer (1 M, pH 7.0) and non-essential amino acids (NEAA) were purchased from Biofluids (Rockville, MD). Fetal bovine serum (FBS) and antibiotic–antimycotic solution were purchased from Life Technologies, Inc. (Rockville, MD). 1,6-Diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMA-DPH) were purchased from Molecular Probes, Inc. (Eugene, OR). Polyvinyl pyrrolidone (PVP, molecular weight 40 000) was obtained from ISP Technologies, Inc. (Wayne, NJ). All organic solvents were of HPLC grade. All other chemicals were reagent grade.

2.2. Cell culture

Caco-2 cells were grown in T-75 flasks to 80–90% confluency in DMEM supplemented with 10% FBS, 1% antibiotics (80 units/ml penicillin, 50 μg/ml streptomycin and 0.125 μg/ml amphotericin B. Cells at passage numbers 25–45 were seeded onto polycarbonate Transwell® inserts (3 μm pore size, 4.71 cm² area) at a density of 63 000 cells/cm². Culture medium was changed every 48 h. Cells were cultured for at least 21 days at 37 °C, 90% relative humidity and 5% CO₂, then used for transport studies between 21 and 25 days.

2.3. Fluorescence polarization

To measure membrane fluidity, fluorescence polarization studies were carried out at 37 °C using a spectrofluorimeter (model CM1T10L, SPEX Industries, Edison, NJ) fitted with polarizer attachments and a temperature regulated stir cell. Caco-2 were labeled with either DPH solution (1 mM stock solution in tetrahydrofuran) for 30 min in darkness or TMA-DPH solution (0.1 mM stock solution in dimethyl formamide) for 2 min by adding 2.5 μl of stock dye solution to 2.5 ml of Caco-2 cell suspension cells (2×10⁵ cells/ml in PBS). Steady state anisotropy was measured in the absence and presence of selected concentrations of Tween 80, Cremophor EL, Vitamin E TPGS, N-octyl glucoside, and the known membrane fluidizer benzyl alcohol (final concentration 30 mM). In addition, cells were pre-incubated with cholesterol to study the influence of the known membrane rigidizer cholesterol (final concentration 0.2 mM), and anisotropy studies were conducted as described. The wavelengths of excitation and emission were 355 and 430 nm, respectively.
In comparing the effect of excipient on membrane fluidity, the steady state anisotropy in the presence of excipient is compared to the steady state anisotropy in the absence of excipient (i.e. control anisotropy), on a percentage basis. Control anisotropy values were measured each day (i.e. for each excipient experiment). This approach toward anisotropy evaluation is typically performed, since absolute control anisotropy values vary, depending on cell passage number, days in culture, and specific culture conditions. For example, control anisotropy values for DPH ranged from 0.144 to 0.171. Control anisotropy values for TMA-DPH varied from only 0.262–0.263.

2.4. Permeability studies

Control permeability studies of R123 [a P-gp substrate] (Yumoto et al., 1999) without surfactants were performed in HBSS containing 10 mM HEPES buffer at pH 6.8. Control permeability studies of gly-sar [a hPepT-1 substrate] (Thwaites et al., 1993a) and benzoic acid [a MCT substrate] (Tsuji et al., 1994) were conducted without surfactants in the presence of a pH gradient with apical pH of 6.0 and basolateral pH of 7.4. Additionally, control permeability studies of gly-sar and benzoic acid were performed in HBSS containing 10 mM HEPES buffer at pH 6.8 in both chambers, to study the effect of lack of pH gradient on their transport. R123 was evaluated in both AP-BL and BL-AP directions, whereas gly-sar and benzoic acid were evaluated in AP-BL direction only. To demonstrate the expression of P-gp, hPepT-1, and MCT in Caco-2 cells, inhibition studies were performed in presence of the P-gp inhibitor GF 120918 (500 nM) for R123, the competitive inhibitor leu-met (10 mM) for gly-sar, and the metabolic inhibitor sodium azide (1 mM) for benzoic acid.

To evaluate surfactant effect on membrane transporters, treatment studies entailed the permeability measurements of R123, gly-sar, and benzoic acid in the presence of Tween 80, Cremophor EL, Vitamin E TPGS, and N-octyl glucoside over a range of surfactant concentrations. The Table 1 shows the influence of two membrane fluidity modulators benzyl alcohol (final concentration 30 mM) and cholesterol. Benzyl alcohol fluidizes membranes. Cholesterol is the chief lipid rigidifier in cell membranes. For the cholesterol study, Caco-2 monolayers were enriched with cholesterol using a BSA–PVP–cholesterol complex developed by Lebo and Gupta (1995). The complex was prepared in HBSS by sonication and Vitamin E TPGS (0.025 mM) 128.7 mg/100 ml cholesterol. The BSA–PVP–cholesterol complex was added on the apical side of Caco-2 cell monolayers and incubated for 2 h. After 2 h, the complex was removed, the apical side was washed with transport medium and the permeability studies were conducted.

The involvement of PKC in the surfactant-induced inhibition of transporters was evaluated by performing permeability studies in the presence of the specific protein kinase inhibitors staurosporine (5 nM) and chelerythrine chloride (50 μM).

All permeability studies were carried out at 37 °C and 50 oscillations/min. Monolayer integrity was monitored in each study by 14C-mannitol permeability. In all cases for all monolayers, mannitol permeability was less than 1×10−6 cm/s, indicating monolayer exhibited suitable integrity. R123 was quantified using Wallac Victor 1420 fluorescence plate reader operating at excitation wavelength of 485 nm and emission wavelength of 535 nm. Gly-sar was quantified on a Beckmann liquid scintillation counter. Benzoic acid was quantified by HPLC.

2.5. Statistical analysis

The effects of different concentrations of nonionic surfactants on the transport of different substrates were analyzed using Spearman’s ranked correlation analysis at α=0.05. Spearman’s ρ (r) measures the positive or negative rank order relationship between the two variables (i.e. concentration of surfactant and substrate permeability). Other data were analyzed by Student’s t-test at α = 0.05.

3. Results

3.1. Membrane fluidity

Table 1 shows the influence of two membrane fluidity modulators (cholesterol and benzyl alcohol) and four surfactants (Tween 80, Cremophor EL, Vitamin E TPGS, and N-octyl glucoside) on changes in steady state anisotropy.

<table>
<thead>
<tr>
<th>Fluidity modulators/nonionic surfactants</th>
<th>Steady state anisotropy as % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPH</td>
</tr>
<tr>
<td>Cholesterol (0.2 mM)</td>
<td>186.6±2.4</td>
</tr>
<tr>
<td>Benzy alcohol (30 mM)</td>
<td>90.4±1.1</td>
</tr>
<tr>
<td>Tween 80 (0.025 mM)</td>
<td>65.2±1.8</td>
</tr>
<tr>
<td>Tween 80 (1 mM)</td>
<td>50.9±2.6</td>
</tr>
<tr>
<td>Cremophor EL (0.025 mM)</td>
<td>64.0±0.9</td>
</tr>
<tr>
<td>Cremophor EL (1 mM)</td>
<td>63.1±0.9</td>
</tr>
<tr>
<td>Vitamin E TPGS (0.025 mM)</td>
<td>105.8±0.6</td>
</tr>
<tr>
<td>Vitamin E TPGS (1 mM)</td>
<td>128.7±3.5</td>
</tr>
<tr>
<td>N-octyl glucoside (0.034 mM)</td>
<td>97.0±1.3</td>
</tr>
</tbody>
</table>

Table 1 Influence of excipients and known fluidity modulators on anisotropy of DPH<sup>a</sup> and TMA-DPH<sup>b</sup>

<sup>a</sup> Control anisotropy values for DPH were between 0.144 and 0.171.
<sup>b</sup> Control anisotropy values for TMA-DPH were between 0.262 and 0.263.
tropy of DPH and TMA-DPH. DPH is a fluorescent probe for the fluidity of the hydrophobic core of the lipid bilayer, whereas TMA-DPH probes the fluidity of polar headgroup region of the bilayer. As expected, cholesterol caused a sharp decrease in membrane fluidity (i.e. increase in anisotropy) of the hydrophobic core of the bilayer. Cholesterol did not change the anisotropy of TMA-DPH, suggesting that it did not affect polar head group fluidity. Benzyl alcohol decreased anisotropy of both DPH and TMA-DPH, suggesting fluidization of the entire lipid bilayer.

TWEEN 80 and Cremophor EL caused a significant decrease in the anisotropy of DPH, but they did not affect the anisotropy of TMA-DPH. In contrast, vitamin E TPGS significantly increased the anisotropy of DPH, suggesting rigidization of the hydrophobic portion of bilayer. Like TWEEN 80 and Cremophor EL, vitamin E TPGS did not affect the anisotropy of TMA-DPH. N-octyl glucoside, the only nonionic surfactant of the four without poly(ethylene oxide) groups, did not change fluorescence anisotropy of either DPH or TMA-DPH, suggesting it had no effect on Caco-2 cell membranes fluidity.

3.2. Inhibition of P-gp

Table 2 gives the AP-BL and BL-AP permeability coefficients of R123, in the presence of the P-gp inhibitor GW 120918, the two fluidity modulating controls, and the PKC inhibitors staurosporine and chelerythrine chloride. GW 120918 reduced the BL-AP versus AP-BL ratio (i.e. B/A ratio) of R123 transport from 6.48 to 1.21. The presence of the fluidity modulating controls cholesterol and benzyl alcohol did not affect B/A transport ratio of R123. Each PKC inhibitor failed to reduce the efflux of R123.

Table 3 gives the AP-BL and BL-AP permeability coefficients of R123, in the presence of different concentrations of the fluidity modulating nonionic surfactants TWEEN 80, Cremophor EL, and vitamin E TPGS, as well as N-octyl glucoside, which did not modulate membrane fluidity. The efflux ratio for R123 ranged from 6.24 to 8.99 in control studies. TWEEN 80 (cme~50 µM) and Cremophor EL (cme~30 µM) inhibited P-gp in a concentration dependent manner, by both increasing AP-BL permeability and decreasing BL-AP permeability. Enhanced inhibition was evident even above the cmc of each surfactant. Vitamin E TPGS (cmc~132 µM) inhibited P-gp in a markedly different manner. It decreased BL-AP permeability significantly at all concentrations to the same extent, but AP-BL permeability did not increase. N-octyl

### Table 2

<table>
<thead>
<tr>
<th>Excipient (mM)</th>
<th>P迎 (±S.E.M.)×10⁶ [cm/s]</th>
<th>Ratio B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AP-BL</td>
<td>BL-AP</td>
</tr>
<tr>
<td>Benzy l alcohol (30 mM)</td>
<td>0.548±0.026**</td>
<td>2.81±0.13**</td>
</tr>
<tr>
<td>Cholesterol pretreatment</td>
<td>0.518±0.081*</td>
<td>2.38±0.24</td>
</tr>
<tr>
<td>Chelerythrine chloride (50 µM)</td>
<td>0.375±0.031</td>
<td>1.88±0.28</td>
</tr>
<tr>
<td>Staurosporine (5 nM)</td>
<td>0.472±0.043</td>
<td>3.00±0.30</td>
</tr>
<tr>
<td>GW 120918 (500 nM)</td>
<td>0.601±0.029*</td>
<td>0.672±0.035***</td>
</tr>
</tbody>
</table>

* P<0.05; ** P<0.01.

a Control P迎×10⁶ (cm/s): AP-BL 0.311±0.027, BL-AP 1.91±0.05, B/A 6.24±0.56.

b Control P迎×10⁶ (cm/s): AP-BL 0.506±0.010, BL-AP 3.28±0.10, B/A 6.48±0.23.
glucoside, the only surfactant without poly(ethylene oxide) groups, did not inhibit P-gp.

3.3. Inhibition of peptide transporter

Fig. 1 plots AP-BL and BL-AP permeability coefficients and A/B permeability ratios of gly-sar in the presence and absence of a proton gradient. Table 4 lists the AP-BL permeability coefficients of gly-sar, in the presence of a competitive inhibitor leu-met, the PKC inhibitor staurosporine, and the two fluidity modulating controls. Tables 5 lists the AP-BL permeability coefficients of gly-sar, in the presence of different concentrations of Tween 80, Cremophor EL, vitamin E TPGS, and N-octyl glucoside with proton gradient.

As shown in Fig. 1, the transport of gly-sar across Caco-2 monolayers was 2–4-fold higher in the presence of a proton gradient (apical pH 6.0; basolateral pH 7.4), than in the absence of a gradient (apical and basolateral pH 7.4). This suggests that a proton dependent peptide transport system may be involved in gly-sar transport. Table 4 shows that leu-met (10 mM) decreased the transport of gly-sar by almost 90% in the presence of the proton gradient. This observation confirms the expression of the peptide transporter system in Caco-2 cells. Only Tween 80 was able to inhibit gly-sar transport in the presence of the proton gradient (Table 5). Cremophor EL, vitamin E TPGS, and N-octyl glucoside had no influence on gly-sar transport.

3.4. Inhibition of monocarboxylic acid transporter

Tables 6 and 7 give the permeability coefficients of benzoic acid in the presence and absence of proton gradient, in the presence of sodium azide (1 mM), and in different concentrations of nonionic surfactants. With no proton gradient (apical and basolateral pH 6.8), benzoic acid permeability was reduced almost 50%, than when apical pH was 6.0 and basolateral pH was 7.0. Similarly, sodium azide, a metabolic inhibitor of active transport, reduced benzoic acid permeability by almost 50%, indicating benzoic acid was transported by an active transport system. Most concentrations of each surfactants had no effect on benzoic acid permeability. However, higher concentrations of Cremophor EL (cmc~30 μM) decreased benzoic acid permeability.

4. Discussion

4.1. Membrane bound transport proteins

Along with passive diffusion and facilitated diffusion, which are energy independent processes of drug transport, membrane bound active transport systems play an important role in the transport of drugs across biological barriers. These active transport systems directly or indirectly utilize the free energy released by the hydrolysis of ‘high energy’ compounds, such as ATP, to pump solutes across membranes. The multidrug resistance (mdr) protein, also known as P-glycoprotein (P-gp), is a widely studied example of such an active transport mechanism. It is
Table 5
Effect of nonionic surfactant on AP-BL gly-sar permeability in the presence of proton gradient

<table>
<thead>
<tr>
<th>Excipient (mM)</th>
<th>( P_{app} ) (±S.E.M.) ( \times 10^5 ) [cm/s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80 (^a)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10.1±0.44</td>
</tr>
<tr>
<td>0.01</td>
<td>6.57±0.67</td>
</tr>
<tr>
<td>0.025</td>
<td>6.17±0.17</td>
</tr>
<tr>
<td>0.05</td>
<td>7.32±0.73</td>
</tr>
<tr>
<td>0.25</td>
<td>4.37±0.20</td>
</tr>
<tr>
<td>1.00</td>
<td>2.48±0.29</td>
</tr>
<tr>
<td>Cremophor EL (^b)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>13.0±0.56</td>
</tr>
<tr>
<td>0.01</td>
<td>13.9±0.42</td>
</tr>
<tr>
<td>0.02</td>
<td>13.5±0.82</td>
</tr>
<tr>
<td>0.03</td>
<td>14.6±1.01</td>
</tr>
<tr>
<td>0.25</td>
<td>12.1±0.38</td>
</tr>
<tr>
<td>1.00</td>
<td>11.2±0.89</td>
</tr>
<tr>
<td>Vitamin E TPGS (^c)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7.81±0.60</td>
</tr>
<tr>
<td>0.025</td>
<td>8.52±0.24</td>
</tr>
<tr>
<td>0.075</td>
<td>7.88±0.59</td>
</tr>
<tr>
<td>0.132</td>
<td>7.69±0.41</td>
</tr>
<tr>
<td>0.25</td>
<td>8.57±0.35</td>
</tr>
<tr>
<td>0.75</td>
<td>9.19±0.38</td>
</tr>
<tr>
<td>N-octyl glucoside</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>14.3±0.75</td>
</tr>
<tr>
<td>0.034</td>
<td>15.3±1.00</td>
</tr>
</tbody>
</table>

\(^a\) \( r_s = -0.83, P=0.04. \)
\(^b\) \( r_s = -0.49, P=0.33. \)
\(^c\) \( r_s = -0.49, P=0.33. \)

expressed in many membrane barriers relevant to drug absorption and disposition. The drugs that are substrates for P-gp are actively effluxed by the direct utilization of energy released by ATP hydrolysis by P-gp. Alternatively, the energy required for active transport can also be derived from the transmembrane gradient of other species like sodium (\( \text{Na}^+ \)) or protons (\( \text{H}^+ \)). Examples of transport systems utilizing inward proton gradient include the human intestinal peptide transporter (hPepT-1), the organic cation transporter expressed in kidneys, and the monocarboxylic acid transporter (MCT) (Nabekura et al., 1996; Thwaites et al., 1993a; Tsuji et al., 1994). As most of transport proteins are membrane bound, the physical state of the cell membrane is probably important in the regulation of transporter function. Several studies have investigated the influence of cholesterol content and cell membrane fluidity on P-gp function (Dudeja et al., 1995; Saeki et al., 1992; Sinicrope et al., 1992). Fluidization usually resulted in decreased P-gp activity. Studies of the effect of rigidizing the cell membrane on P-gp activity have yielded contradictory results, with some reports suggesting an increase in P-gp activity, while others suggest a decrease (Dudeja et al., 1995; Saeki et al., 1992).

Several nonionic surfactants with membrane fluidity modulating activity have been reported to inhibit P-gp. Most of these active surfactants contained poly(ethylene oxide) groups, with no similarities in their hydrophobic components (Woodcock et al., 1992). Cremophor EL is a complex mixture of hydrophobic and hydrophilic components, with the main components being glycerol polyethylene glycol ricinoleate and glycerol ethoxylates, respectively. The average molecular weight is approximately 2510. Membrane fluidity has also been shown to modulate the activity of sodium or proton coupled membrane transporters (Lee et al., 1999; Nabekura et al., 1996). The effects of nonionic surfactants have been demonstrated for the peptide transporter system (Koga et al., 1998, 1999a,b, 2000).

Protein kinase C (PKC), a phospholipid/Ca\(^{2+}\)-depended-

Table 6
Permeability (AP-BL) of benzoic acid in the presence of sodium azide and in the absence of proton gradient

<table>
<thead>
<tr>
<th>Excipient (mM)</th>
<th>( P_{app} ) (±S.E.M.) ( \times 10^5 ) [cm/s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (proton gradient)</td>
<td>131±5.07</td>
</tr>
<tr>
<td>Control (no proton gradient)</td>
<td>71.2±3.28*</td>
</tr>
<tr>
<td>Sodium azide (1 mM) with proton gradient</td>
<td>68.8±1.40*</td>
</tr>
</tbody>
</table>

\(^*\) \( P < 0.01. \)
dent protein kinase, is known to phosphorylate several proteins for cellular functions, in response to extracellular stimuli. PKC activity has been linked to various transporters, such as P-gp and the peptide transporter (Brandsh et al., 1994; Castro et al., 1999). For example, P-gp is phosphorylated by PKC. Although P-gp function may be modulated by phosphorylation, it has been shown that phosphorylation is not essential to confer multidrug resistance (Germann et al., 1995). In addition to its membrane fluidizing activity as shown here, Cremophor EL has also been shown to inhibit PKC purified from human leukemia ML-1 cells (Zhao et al., 1989). Hence, the basis of Cremophor EL’s P-gp inhibition may involve the excipient’s general influence on the membrane, or inhibition of PKC. However, since neither PKC inhibitor affected R123 permeability across Caco-2 monolayers (Table 2), Cremophor’s in vitro inhibition of PKC would appear to be attenuated in a cellular system, such as the present one.

Our first objective was to investigate the effects of three commonly used nonionic surfactants on the activities of P-gp, hPepT-1 and MCT. Additionally, we wanted to evaluate the concentration dependence of surfactant action, both below and above the cmc. As these surfactants are primarily used for solubilization of hydrophobic drugs, it would be advantageous if they were active above the cmc, since they would then have dual action of solubilizing hydrophobic substrates of efflux systems and inhibiting efflux. In contrast, if they are also active against influx transport systems, then they might actually hinder the absorption of substrate drugs. Our second objective was to determine whether surfactant-induced inhibition of the membrane transporter systems is associated with membrane fluidity changes or with PKC inhibition.

4.2. Effects of nonionic surfactants on cell membrane fluidity

As shown in Table 1, pretreatment of Caco-2 cells with cholesterol caused a strong increase in fluorescence anisotropy of DPH, without affecting the anisotropy of TMA-DPH. These results suggest that cholesterol becomes embedded in, and rigidizes, the hydrophobic core of the bilayer, leaving the polar region largely unaffected. Benzyl alcohol decreased anisotropy of both DPH and TMA-DPH, suggesting an increase in the fluidity of both the hydrophobic region and polar headgroup region of the bilayer.

Tween 80 (cmc~50 μM) and Cremophor EL (cmc~30 μM) caused a significant decrease in DPH anisotropy at concentrations below and above their respective cmc values. Below their respective cmcs, Tween 80 and Cremophor EL were equipotent in fluidizing lipid bilayers. Above their respective cmcs, however, Tween 80 was more effective in fluidizing the membrane than Cremophor EL. In contrast, Vitamin E TPGS (cmc~132 μM) significantly increased DPH anisotropy, indicating rigidification of the cell membrane, and did so below and above its cmc. None of these three surfactants affected TMA-DPH anisotropy. N-octyl glucoside did not affect cell membrane fluidity, perhaps due to its lacking a poly(ethylene oxide) group.

4.3. Effects of nonionic surfactants, fluidity modulators and PKC inhibitors on R123 transport

R123 was used as a substrate for P-gp to study the effects of nonionic surfactants. As shown in Tables 2 and 3, BL-AP permeability of R123 was almost six to nine times higher than its AP-BL permeability. The P-gp inhibitor GW 120918 almost completely abolished the efflux of R123 (Table 1) by reducing BL-AP permeability significantly (P < 0.01), thus demonstrating that R123 is a substrate for P-gp. GW 120918 also increased AP-BL permeability modestly (P<0.05). This effect of P-gp on R123, where P-gp mostly affects R123 BL-AP permeability, has been previously observed (Troutman and Thakker, 2001). The membrane rigidizer cholesterol and the membrane fluidizer benzyl alcohol each resulted in a modest increase in AP-BL permeability of R123 (P<0.05). Neither affected BL-AP permeability.

The transport of R123 was not affected by staurosporine and chelerythrine chloride, which are potent and specific PKC inhibitors. P-gp is known to be a PKC substrate. This observation suggests that these surfactants probably do not inhibit P-gp by blocking PKC phosphorylation. This result is in agreement with a previously published observation, in which staurosporine was reported to be ineffective in inhibiting P-gp in murine neuroblastoma cells (Chervinsky et al., 1993).

As shown in the Table 3, all three nonionic surfactants Tween 80, Cremophor EL and vitamin E TPGS inhibited P-gp. However, vitamin E TPGS showed a markedly different phenomenon of P-gp inhibition than Tween 80 and Cremophor EL. Tween 80 and Cremophor EL each progressively increased AP-BL permeability (significant positive correlation, P=0.005), decreased BL-AP permeability (significant negative correlation, P < 0.05) and reduced B/A permeability ratio (significant negative correlation, P < 0.05) with increase in surfactant concentration. This phenomenon was observed at all concentrations tested, even above their respective cmc values (~50 μM for Tween 80 and ~30 μM for Cremophor EL).

While it is not clear why the pattern of P-gp inhibition was not dependent on the surfactant concentration being above or below the cmc, one possible explanation is that the cited cmc’s do not reflect experimental conditions here, where cells are present. The cited cmc values reflect cell-free conditions, that may under-estimate a cmc in the presence of cells, since the presence of cells may increase the apparent cmc. For example, in the case of Tween 80, Tween 80 may partition into the cells, such that a Tween 80 system concentration above 50 μM may result in a Tween 80 concentration less than 50 μM in the aqueous...
media. Hence, with the cmc not attained, further Tween 80 above 50 μM could potentially result in greater P-gp inhibition, as was the case.

In contrast to the pattern of P-gp inhibition by Tween 80 and Cremophor EL, vitamin E TPGS drastically reduced BL-AP permeability even at a very low concentration of 25 μM, and reduced BL-AP ratio from 7.14 to 1.73. There was no significant correlation between the studied concentrations of surfactant and these two variables \((P \gg 0.05)\), with the lowest concentration achieving a full effect. The BL-AP permeability, and the B/A permeability ratio of R123 in the presence of subsequent higher concentrations of vitamin E TPGS, remained virtually unchanged. In fact, the AP-BL permeability of R123 showed a continuous decline \((P < 0.05)\).

It is noteworthy that vitamin E TPGS was the lone membrane rigidizing surfactant, and that its P-gp inhibition properties were different than the properties of the two membrane fluidizing surfactants (i.e. Tween 80 and Cremophor EL). Vitamin E TPGS's effect was essentially one of reducing BL-AP permeability, which was already maximally achieved at low concentration. It should be noted that an alternative explanation of these effects of vitamin E TPGS on R123 permeability is the possible binding of R123 molecules with surfactant monomers and micelles, to result in the large decrease in BL-AP permeability at even low concentrations.

\(N\)-octyl glucoside, which did not alter the cell membrane fluidity, had no effect on R123 transport. Although there appears to be some correspondence between fluidity modulation of the hydrophobic core of the lipid bilayer and P-gp inhibition, this association is not shown with certainty.

4.4. Effects of nonionic surfactants, fluidity modulators, and PKC inhibitors on gly-sar transport

Gly-sar was used as a substrate for peptide transporter to study the effects of nonionic surfactants. This transporter is active in the presence of an inward proton gradient (Thwaite et al., 1993a,b). From Fig. 1, in the presence of an inward directed proton gradient (i.e. pH 6.0 in the apical chamber and pH 7.4 in the basolateral chamber), the AP-BL permeability of gly-sar was almost 4-fold higher than its BL-AP permeability. The AP-BL and BL-AP permeabilities of gly-sar were the same in the absence of proton gradient (i.e. pH 6.8 in both apical and basolateral chambers). In Table 4, the competitive substrate leu-met reduced gly-sar transport by almost 90% \((P < 0.01)\), confirming that gly-sar transport is mediated by a dipeptide transporter. Both fluidity modulating treatments (cholesterol and benzyl alcohol) as well as \(N\)-octyl glucoside failed to affect gly-sar transport. The PKC inhibitor staurosporine also did not affect gly-sar transport, although staurosporine has been previously reported to affect the peptide transporter in the Caco-2 cells (Hu et al., 1995).

As shown in Table 5, Tween 80 reduced the permeability of gly-sar in a concentration dependent manner \((P = 0.04)\). At the highest concentration of Tween 80 (1 mM), the transport of gly-sar was reduced almost 5-fold. Although Cremophor EL has similar fluidizing effects as Tween 80, it did not affect the transport of gly-sar \((P = 0.33)\). Vitamin E TPGS did not inhibit gly-sar transport, suggesting that it had no activity on the peptide transporter \((P = 0.33)\).

4.5. Effects of nonionic surfactants on benzoic acid transport

Benzoic acid was used as a substrate for MCT to study the effects on nonionic surfactants. A detailed study has shown that benzoic acid is a substrate for a pH dependent MCT in Caco-2 monolayers (Tsuji et al., 1994). This transporter is activated by a proton gradient and the \(K_m\) value for benzoic acid was reported as 4.98 mM. This reported \(K_m\) value for benzoic acid suggests a low affinity transporter. Table 6 shows that benzoic acid exhibited a very high permeability of \(131 \times 10^{-6}\) cm/s in the presence of a proton gradient. In the absence of proton gradient, the permeability of benzoic acid was reduced two-fold to \(71.2 \times 10^{-6}\) cm/s, which reflects a high benzoic acid passive permeability. The metabolic inhibitor sodium azide reduced benzoic acid transport almost two-fold, to the same level as was obtained in the absence of the proton gradient. Cremophor EL inhibited the MCT transporter, as evident from the strong negative correlation between surfactant concentration and benzoic acid transport \((P = 0.005)\). However, Tween 80 and vitamin E TPGS did not show an effect on benzoic acid transport. It should be noted that benzoic acid’s high passive permeability has the potential to mask the excitant’s inhibitory effect on MCT-mediated benzoic acid transport (Lentz et al., 2000).

4.6. In vivo implications of surfactant-induced inhibition of membrane transporters

The nonionic surfactants Tween 80, Cremophor EL, and vitamin E TPGS are effective inhibitors of efflux systems in vitro. It has been reported that the bioavailability and the tissue distribution of P-gp substrates is enhanced in the presence of P-gp inhibitors (Sparreboom et al., 1997; Polli et al., 1999), including Cremophor EL and vitamin E TPGS (Badary et al., 1998; Sokol et al., 1991). However, it is not clear if this improvement is due to micellar solubilization or efflux inhibition, or a combination of both (Yu et al., 1999; van Heeswijk et al., 2001). Tween 80 and Cremophor EL were both effective in increasing R123 permeability. They could potentially be useful in increasing the bioavailability and tissue distribution of substrates through efflux inhibition (Chang et al., 1996) and micellar solubilization.

Surfactant-induced transporter inhibition may be im-
portant when a drug is a substrate for multiple transporters. For example, the HMG-CoA reductase inhibitor atorvastatin has been shown to be a substrate for both P-gp and MCT (Wu et al., 2000). In such cases, nonionic surfactants like Tween 80 may be useful, since Tween 80 effectively inhibits P-gp efflux, while leaving MCT influx unaffected. The inhibition of intestinal P-gp will potentially reduce P-gp mediated efflux, while influx mediated by MCT will remain unaffected, resulting in a possible increased absorption of the drug.

In summary, all three nonionic surfactants containing poly(ethylene oxide) were effective in inhibiting P-gp, although vitamin E TPGS showed a markedly different pattern of P-gp inhibition than Tween 80 and Cremophor EL. Tween 80 and Cremophor EL progressively increased AP-BL permeability of rhodamine 123 over a concentration range of 0–1 mM. Vitamin E TPGS’s effect was equally large, mainly due to reduced BL-AP permeability of rhodamine 123. Moreover, its effect was already maximal at 0.025 mM. These P-gp inhibition effects would appear to be related to these excipients’ ability to modulate membrane fluidity. Tween 80 and Cremophor EL fluidized lipid bilayers, while vitamin E TPGS rigidized lipid bilayers. Additionally, these three surfactants include poly(ethylene oxide) in their chemical composition. However, among the three surfactants, only Tween 80 inhibited the peptide transporter. Likewise, only Cremophor EL inhibited the monocarboxylic acid transporter. Nevertheless, P-gp, the human intestinal peptide transporter, and the monocarboxylic acid transporter were each inhibited by at least one of these surfactants. A common functional feature of these surfactants was their ability to modulate fluidity, although the present studies indicate that strong membrane fluidity modulation by itself was not sufficient to reduce transporter activity. The PKC inhibitors failed to inhibit transporter activity, suggesting inhibition of PKC was not the mechanism of surfactant-induced transporter inhibition. In total, these results suggest that surfactants can inhibit multiple transporters, but that changes in membrane fluidity may not be a general mechanism underlying reduced transporter activity.

References


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