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Effects of Monoglycerides on P-Glycoprotein: Modulation of the Activity and Expression in Caco-2 Cell Monolayers

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Abstract: The purpose of this study was to analyze the effects of two common monoglyceride components of lipid excipients, 1-monoolein and 1-monostearin, on the activity and expression of P-glycoprotein (P-gp) in Caco-2 cells. Non-cytotoxic concentrations of 1-monoolein and 1-monostearin were determined by assessing membrane permeability and mitochondrial activity in Caco-2 cells, a human colon adenocarcinoma cell line. Concentrations of 500 and 100 μM were used to evaluate P-gp activity through Rh123 accumulation and bifunctional transport studies. The P-gp protein expression levels were quantified through the use of immunoblots. The changes in cell membrane fluidity and nuclear membrane integrity upon the addition of monoglycerides were analyzed by fluorescence anisotropy using DPH and TMA-DPH as the fluorescent labels and by using increasing salt concentrations to release the nuclear contents, respectively. The absorptive flux (apical to basolateral) in the bifunctional transport studies was not found to be statistically significant for the non-cytotoxic concentrations of 1-monoolein and 1-monostearin. However, treatments of 500 and 100 μM of 1-monoolein or 1-monostearin displayed statistically lowered efflux (basolaterial to apical, $P < 0.05$) compared to the controls (7.9 ± 0.8, $12.9 \pm 2.6 \times 10^6$ cm/s for 1-monoolein or 11.1 ± 2.0, $11.4 \pm 2.3 \times 10^6$ cm/s for 1-monostearin, respectively, compared to the untreated control, $21.1 \pm 2.9 \times 10^6$ cm/s, $n = 5$). Rh123 accumulation was also found to be enhanced upon 24 h incubation with both concentrations of the monoglycerides; however, only concentrations of 500 μM of the monoglycerides were shown to significantly reduce the P-gp protein expression. The results from this study suggest that these two monoglycerides, common components in various lipid excipients, are inhibitors of P-gp.

Keywords: P-gp; monoglycerides; Rh123; verapamil; 1-monoolein; 1-monostearin; lipid excipients; DPH; TMA-DPH; Caco-2; bifunctional transport

Introduction

Oral drug delivery systems have many advantages over intravenous dosing including elimination of the need for frequent visits to outpatient clinics, easier chronic administration, and increased patient compliance.[1] However, oral delivery of 50% of the available drug compounds is hampered due to poor oral bioavailabilty.[2] Thus, a considerable amount of research is targeted toward modifying the physicochemical properties of these lipophilic drugs to increase their gastrointestinal absorption rate. Modifications, such as salt formation, particle size reduction, the use of cyclodextrins, nanoparticles, solid dispersions and permeation enhancers, are a few of the approaches being investigated to improve the dissolution rate of the drug.[1–3] In recent years,

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attention has also been focused on using lipid-based formulations to improve oral bioavailability.

Many of these lipid-based drug delivery systems have been shown to enhance the intestinal absorption of drugs that exhibit poor water solublility.[2,4,5] Specifically, self-emulsifying drug delivery systems (SEDDS), isotropic mixtures of oils and surfactants that form microemulsions or fine opaque emulsions upon dilution with water or GI fluids, have led to commercialized formulations of Neoral (cyclosporin A), Norvir (ritonavir), and Fortovase (saquinavir).[1,2] Although the exact mechanism of the absorption enhancement remains unclear, it is primarily accepted that the lipid excipients exert their effects through several complex mechanisms that lead to the alteration of the biopharmaceutical properties of the drug.[2,5] Some of these mechanisms include increasing the dissolution rate and solubility in intestinal fluids, protection of the drug from chemical and enzymatic degradation, increasing the gastrointestinal permeability by disrupting the structural organization of the lipid bilayer of the epithelial cell membrane, and/or stimulating the formation of lipoproteins inside the cell which promotes lymphatic transport of the water insoluble drugs (thereby avoiding first-pass hepatic metabolism).[3,5]

It has also been noted that a wide range of lipid-based drug delivery systems inhibit intestinal P-glycoprotein (P-gp) efflux.[1,4] P-glycoprotein, an ATP-dependent efflux pump encoded by the MDRI gene in humans, is a 170 kDa integral membrane protein.[6–9] Widely distributed in apical surfaces of epithelial tissues in the gut, kidney, placenta, testes, and the blood−brain barrier, P-gp plays an important role in the excretion of a variety of structurally and pharmacologically unrelated hydrophobic compounds. After binding to P-gp, the substrates are transported back to the apical surface of the tissue in an ATP-dependent manner, thereby preventing the accumulation of both exogenous and endogenous compounds. As a result, P-gp restricts the bioavailability of several xenobiotics.[6–9] Surfactants, including polyscorbate (Tween 80 and Tween 60), D-α-tocophoryl polyethylene glycol succinate 1000 (TPGS), Cremophore EL, and the block copolymers Pluronic P85 and L81, have been shown to affect P-gp in the enterocytes.[10–14] Other lipid excipients, such as Gelucire and Peceol, have also been shown to decrease the protein expression and functional activities of P-gp *in vitro* and *in vivo* (for Peceol).[15,16]

Nonetheless, the lack of mechanistic understanding has limited the widespread acceptance of these potentially useful drug delivery systems. One common oversight instrumental in understanding the effects of lipid excipients is the lack of knowledge on how each individual component of these solubilizing mixtures affects the enterocytes. Preliminary results by Konishi et al. suggested that monoglycerides, common components in lipid-based drug delivery systems, have a larger effect on the P-gp activity compared to tri- or diglycerides.[17,18] In order to follow up on these important findings, the effects of two common monoglycerides, the monounsaturated 1-monoolein and the saturated 1-monostearin, on P-gp were examined. Caco-2 cells, a well-established human carcinoma cell line that closely mimics the enterocytes of the small intestine, were used for all the *in vitro*

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studies.[19,20] By elucidating the mechanism of action of each lipid excipient component, one will not only be able to understand how lipid excipients affect the physicochemical properties of xenobiotics, but also be able to selectively choose the ideal vehicle excipient for each particular lipophilic drug.

Materials and Methods

Materials. Verapamil, rhodamine 123 (Rh123), sucrose, $MgCl_2$, phenylmethylsulfonylfluoride (PMSF), 1-monoolein, 1-monostearin, benzyl alcohol, cholesterol, Triton X-100, Tween-80, HEPES, protein inhibitor cocktail, sodium deoxycholate, Trizma hydrochloride, DPH, EDTA, OptiPrep Density Gradient Medium (60% (w/v) iodixanol solution) and NaCl were obtained from Sigma-Aldrich (St. Louis, MO). Peceol and Gelucire were purchased from Gattefossé Inc. (Montreal, QC, Canada). TMA-DPH and Quant-iT PicoGreen dsDNA Assay Kit were purchased from Invitrogen (Burlington, ON, Canada). All cell culture reagents were purchased from Gibco BRL (Grand Island, NY), while sterile 50 mL centrifuge tubes, and disposable 10 and 25 mL stereological pipettes were purchased from Starstedt (Montreal, PQ, Canada). Culture flasks, Transwell and multiwell plates were obtained from Corning-Costar (Cambridge, MA). CellTiter 96 Aqueous One Solution Assay and CytoTox 96 Nonradioactive Cytotoxicity Assay were from Promega Corporation (Madison, WI), and the BCA Protein Assay Kit was obtained from Pierce Biotechnology, Inc. (Rockford, IL). NP-40 was purchased from Roche Applied Science and Trans-Blot Transfer medium (nitrocellulose membrane 0.45 μM) from Bio-Rad (Hercules, CA).

Cell Culture. Caco-2 cells, human colon adenocarcinoma cells, were purchased from American type Culture Collection (Rockville, MD) and maintained between passages 36 and 45. Caco-2 cells were cultured in minimum essential medium (MEM) with Earls salts and L-glutamine (with phenol red), supplemented with 10% fetal bovine serum (FBS), 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 100 μg/mL penicillin and 100 μg/mL streptomycin at 37°C in humidified air containing 5% CO_2 in T75 flasks. The cells were detached with 0.25% trypsin and 0.02% EDTA and seeded in 6-, 12-, 48-, 96-well plates or 12-well Transwell plates with COL collagen-coated membrane inserts (0.4 μM pore size, 12 mm membrane diameter) depending on the type of experiment. The media were changed every other day, and plates were used for the experiments after 90% confluency was reached (in the case of the Transwell studies, cells were used when the transepithelial electrical resistance (TEER) values were above 400 $\Omega \cdot cm^2$, measured using a Millicell-ERS from Millipore).

Cytotoxicity Measurements. Two separate studies, the MTS assay that measures mitochondrial respiration and the LDH assay that assesses cell membrane integrity, were employed to determine non-cytotoxic concentrations of the monoglycerides. Caco-2 cells were seeded onto 96-well plates at a density of 40,000 cells/cm^2. On the day of the experiment, the cells were washed twice with PBS and the culture medium was exchanged for treatment solutions of 31.25, 62.5, 125, 250, 500, 750 and 1000 μM of 1-monoolein or 1-monostearin, 100 μM verapamil, and 1% Triton X-100 (as a positive control for cytotoxicity) in MEM complete media. After an incubation period of 24 h, 50 μL aliquots of the treatment solutions were removed from each well and transferred into a new 96-well plate. Using the protocol for the Cytotox96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI), 50 μL of the LDH Substrate Mix was added to each well of the new 96-well plate. The plate was covered with foil and incubated for 30 min at room temperature. A 50 μL aliquot of the stop solution was added to each well, and the absorbance was immediately read at 492 nm with a Multiskan Ascent Multiplate reader (Thermo Labsystems, Finland).

For the MTS assay, solutions were aspirated from the original 96-well plate and washed twice with PBS. 100 μL of HBSS was added to each well followed by the addition of 20 μL of CellTiter AQ_{ueous} One Solution Reagent from the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega, Maddison, WI). The plate was wrapped in aluminum foil and incubated for 1 h at 37 °C in a humidified, 5% CO_2:95% O_2 atmosphere. The absorbance was measured at room temperature at 492 nm with a Multiskan Ascent Multiplate reader. The percent cell viability for the MTS assay was calculated relative to the 100% control (media plus cells). Cytotoxicity for the LDH assay was calculated relative to the Triton X-100 group. The estimated standard deviations are based on at least 7 trials.

Uptake of Rhodamine 123. Caco-2 cells were seeded at a density of 40,000 cells/well in 48-well plates. On the day of the experiment, the culture media were removed and the cells were washed with PBS. Solutions of 100 μM and 500 μM of 1-monoolein or 1-monostearin, 100 μM of verapamil (positive control) in MEM complete media, or media were added to the cells and incubated for 24 h. The cells were washed 3 times with PBS, and a solution of fresh HBSS with 10 mM HEPES (pH 7.4) containing 5 μM Rh123 was added to each well. The uptake was halted after 3 h incubation by aspirating the Rh123/HBSS solution. The cells were washed 3 times with ice-cold PBS and lysed in 1% Triton X-100. The cellular debris and the membrane were pelleted by centrifugation. The fluorescence of the accumulated Rh123 in the cells was measured (excitation at 485 nm and emission measured at 530 nm) for each sample (50 μL aliquots removed from the supernatant and placed in a 96-well plate). The protein content of the aliquots was determined by the bicinchoninic acid protein assay (BCA

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Protein Assay Kit). The cellular accumulation of Rh123 was normalized with respect to the protein content in each well.

Bifunctional Transport Studies. The Caco-2 cells were seeded at a density of 300,000 cells/well onto 12 mm collagen-coated PTFE membrane inserts (pore size of 0.4 μm) prewet with 0.1 mL of media. After 24 h, the media was replaced with 0.5 or 1.5 mL of fresh media in the apical or basolateral chamber, respectively. The media was changed every second day until the TEER value of the cell monolayer, monitored using a Millicell-ERS (Millipore, Bedford, MA), exceeded 400 $\Omega \cdot cm^2$. Cells were washed once with PBS, and treatment solutions (100 μM and 500 μM of 1-monostearin or 1-monoolein, 100 μM verapamil in media, or media) were added to both the apical (0.5 mL) and basolateral sides (1.5 mL). After 24 h incubation, the cells were carefully washed twice with PBS. The donor side was loaded with a solution of 5 μM Rh123 in HBSS with 10 mM HEPES. In order to avoid hydrostatic pressure differences, the same surface levels in each chamber were maintained by adding 0.4 mL of the Rh123 solution in the apical chamber or 1.2 mL in the basolateral chamber.[21] The receiving side was filled with the appropriate volume of HBSS with 10 mM HEPES (either 0.4 or 1.2 mL for the apical or basolateral compartments, respectively). The amount of permeated Rh123 was monitored by removing 50 μL aliquots from the apical or basolateral side (B-to-A secretory flux or A-to-B absorptive flux, respectively) after 0, 15, 30, 60, 90 and 120 min and replaced with a fresh 50 μL aliquot of HBSS with 10 mM HEPES to maintain a constant volume. The plate was incubated at 37 °C in a humidified atmosphere of 5% CO_2:95% O_2 in between sample removal periods. The concentration of Rh123 in both the donor and receiving chambers was determined by measuring the fluorescence of each sample (λ_{ex} = 480 nm, λ_{em} = 537 nm, Fluoroskan Ascent from Thermo Electron Corporation, Finland) and comparing the readings to a standard curve of appropriate Rh123 concentrations. The integrity of the monolayer was assessed by monitoring the TEER values throughout the experiment.

The apparent permeability (P_{app}) coefficients were calculated using the following equation:

$$P_{app} = (dQ/dt)/(AC_0)$$

where dQ/dt is the drug permeation rate, A is the surface area of the membrane insert (1.13 cm^2), and C_0 is the initial concentration of Rh123 in the donor chamber at $t = 0$. The net efflux (P_{app} ratio) is expressed as the quotient of P_{app}(B-to-A) to P_{app}(A-to-B).

After the 120 min time point, the solutions where aspirated and each well was washed three times with PBS. The membrane was cut from each insert and placed in 1% Triton X-100 for 30 min to assess the cell accumulation of Rh123. The cellular debris and the membrane were pelleted by centrifugation. The concentration of Rh123 was assessed by measuring the fluorescence of each sample (50 μL aliquots removed from the supernatant). The intensity values were compared to the Rh123 calibration curve. The protein concentration for each well was determined using the BCA Protein Assay Kit.

P-gp Protein Expression. Caco-2 cells were seeded at a density of 300,000 cells/well in a 6-well plate. After reaching 90% confluency, the cells were washed with PBS and the media was replaced with media (control) or media plus treatments (100 μM and 500 μM of 1-monoolein or 1-monostearin, or 100 μM of verapamil). After a 24 h incubation period, the cells were washed 3 times with PBS and harvested with RIPA lysis buffer (50 mM HEPES, 150 mM NaCl, 2 mM EDTA, 0.5% Na-deoxycholate, 1% NP-40) containing PMSF and protease inhibitor cocktail (1:100 dilution). The protein content was determined using the BCA Protein Assay Kit. The proteins (30 μg/lane) were separated by electrophoresis through a 7.5% SDS−polyacrylamide gel at 80 V for 120 min. The gels were electroblotted at 0.07 mA/gel for 90 min onto 0.45 mM nitrocellulose membranes from Bio-Rad (Hercules, CA). A prestained protein standard was used to identify the P-gp band at 170 kDa and Actin at 42 kDa. The membranes were incubated for 2 h in blocking buffer (5% nonfat dried milk in TBS with 0.1% Tween-20) and probed overnight with a 1:300 dilution of mouse antihuman P-gp primary antibody (C219, Signet Pathology Systems). A 1:1000 dilution of goat antihuman actin (I-19, Santa Cruz Biotechnology) was used to probe actin, the internal control. The membranes were washed 3 times with TBS-0.1% Tween-20 (TBS-T) and incubated for 90 min in a 1:5000 dilution of rabbit antimouse IgG horseradish peroxidase (HRP)-conjugated antibody (Jackson Immuno-Research Laboratories) and a 1:3000 dilution of bovine antigoat IgG-HRP (Santa Cruz Biotechnology) for P-gp and actin, respectively. After washing the membrane 3 times with TBS-T, the bands were visualized using ECL Western Blotting Detection Reagents (GE Healthcare), exposed to an X-ray film (Kodak X-Omat film), and quantified with UVP-Labworks software.

Cell Fluidity Measurements. Caco-2 cells were seeded at a density of 300,000 cells/well in 6-well plates. Confluent monolayers were treated with either media or media plus compounds (100 μM and 500 μM 1-monoolein or 1-monostearin, 0.50% or 0.25% (v/v) Peceol, 0.02% or 0.01% (w/v) Gelucire, 50 μM cholesterol, 30 mM benzyl alcohol, or media). Treatments of 50 μM cholesterol and 30 mM benzyl alcohol were used as positive controls. Following a 24 h incubation, the treatments were removed and the cells were washed three times with PBS, trypsinized using 1 mL of typsin/well, and pelleted at 1000g for 5 min at 15 °C. The cells were resuspended in PBS at a concentration of 2 × 10^5 cells/mL and labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH) or 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) as described

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previously.[22] Briefly, 2.5 mL of the Caco-2 cell suspensions were labeled in the dark at room temperature with either 2.5 μL of 1 mM DPH in tetrahydrofuran for 30 min or 2.5 μL of 1 mM TMA-DPA in dimethyl formamide for 2 min. The fluorescence anisotropy of the DPH or TMA-DPH labeled Caco-2 cells was measured using a Varian Cary Eclipse fluorescence spectrophotometer with filters in the parallel and perpendicular orientations to the excitation light ($\lambda_{ex} = 360$ nm, $\lambda_{em} = 430$ nm). Fluorescence intensities were corrected for background influences and the fluorescence anisotropy (r) was calculated using the following equation:

$$r = I_{||} - I_{\perp}/(I_{||} + 2I_{\perp})$$

where $I_{||}$ and $I_{\perp}$ are the fluorescence intensities measured in the parallel and perpendicular directions to the excitation light, respectively. Cholesterol, shown to decrease the membrane fluidity (increase fluorescence anisotropy) with concentrations ≥ 20 μM, and benzyl alcohol, shown to increase the membrane fluidity (decrease fluorescence anisotropy) with concentrations ≥ 20 mM, were used as the positive controls.[23]

Nuclear Integrity Measurements. Caco-2 cells were seeded at a density of 300,000 cells/well in a 6-well plate and grown to confluency. Solutions of 100 and 500 μM 1-monoolein or 1-monostearin, 0.5 and 0.25% (v/v) Peceol, 0.02 and 0.01% (w/v) Gelucire, 30 mM benzyl alcohol (positive control) in MEM complete media, or media were added to the cells and incubated for 24 h. The treatment solutions were removed and the confluent monolayers were washed 3 times with PBS. The cells were removed from the wells by gentle scraping using a cell scraper and resuspended in 500 μL of nuclei extraction buffer (250 mM sucrose, 50 mM Tris (pH 7.4), 5 mM $MgCl_2$, 1% PMSF, 1% protein inhibitor cocktail, and 1% Triton X-100). The cell solutions were passed through a 25 gauge needle 10 times, and the nuclei were pelleted by centrifugation at 1000g for 10 min. The crude nuclei pellets were resuspended in 500 μL of 30% iodixanol. The nuclei suspension was carefully layered between 400 μL of 25% and 35% iodixanol (diluted with the nuclei extraction buffer without 1% Triton X-100). The solution was centrifuged at 10000g for 20 min, and the band of nuclei was collected at the 30−35% interface. The nuclei yield and integrity were confirmed by microscopic examination with trypan blue staining.

A modified procedure from Czebryt et al. was used to assess the nuclear membrane integrity.[24] STM buffer (250 mM sucrose, 50 mM Tris (pH 7.4), 5 mM $MgCl_2$, 1% PMSF and 1% protein inhibitor cocktail) was used for suspension and storage of nuclei. NaCl−STM solutions were made by adding NaCl to STM buffer in order to acquire the desired salt concentrations. All solutions were kept on ice throughout the study.

The protein concentrations of the extracted nuclei were determined using the BCA Protein Assay Kit. The same concentration of nuclei for each treatment was added to 10 separate 1.5 mL eppendorf tubes (equal to 10 μg of protein for each sample). Solutions of 0, 50, 100, 125, 150, 175, 200, 250, 300 and 1000 mM NaCl-STM were added to the nuclei for a final volume of 125 μL. The tubes were capped and inverted twice to mix all ingredients, and placed on ice for 30 min. After incubation, the solutions were centrifuged at 7500g for 10 min. Exactly 25 μL of the supernatant was transferred to a 96-well plate, and the DNA released from the nuclei was determined by adding 25 μL of Quant-iTPicoGreen dsDNA reagent (prepared following the procedure from Invitrogen). The solutions were incubated for 2 to 5 min at room temperature, protected from light and their fluorescence intensities were measured on a Fluoroskan Ascent multiplate spectrophotometer with excitation and emission wavelengths of 480 and 520 nm, respectively. NaCl-STM buffer solutions at all prepared concentrations were used as the blanks. The absorbance values were normalized to the maximum absorbance measured (usually at 1000 mM NaCl) and expressed as a percentage of maximum absorbance. The concentration of the salt needed to release 50% of the nuclear contents was interpolated from the curves.

Statistical Analysis. All data sets were analyzed by one way ANOVA with fixed effects and Newman-Keuls post-analysis using SigmaStat version 3.5 (Systat Inc.). Data is expressed as mean ± SD. Statistical significance was accepted at $P < 0.05$.

Results

Cytotoxicity of Monoglycerides. Both the cell membrane permeability and mitochondrial activity were assessed to determine non-cytotoxic concentrations of the monoglycerides on Caco-2 cells (Figures 1 and 2). No significant differences in cell viability (MTS assay, $n \geq 7$) and toxicity (LDH assay, $n \geq 7$) of both monoglycerides compared to the untreated control were observed with treatments from 31.25 to 1000 μM. Non-cytotoxic concentrations of 500 and 100 μM were used for all subsequent studies.

Rh123 Accumulation. The accumulation of Rh123 in Caco-2 cells was used to assess the efflux transport of P-gp (Figure 3). Concentrations of 500 μM of the monoglycerides resulted in similar Rh123 accumulations compared to verapamil, the positive control, whereas concentrations of 100 μM of either 1-monoolein or 1-monostearin resulted in lowered accumulations of Rh123 compared to verapamil. Nonetheless, both concentrations of 1-monoolein and 1-monostearin, and 100 μM of verapamil resulted in significant accumulation of Rh123 relative to the untreated controls ($n = 6$).

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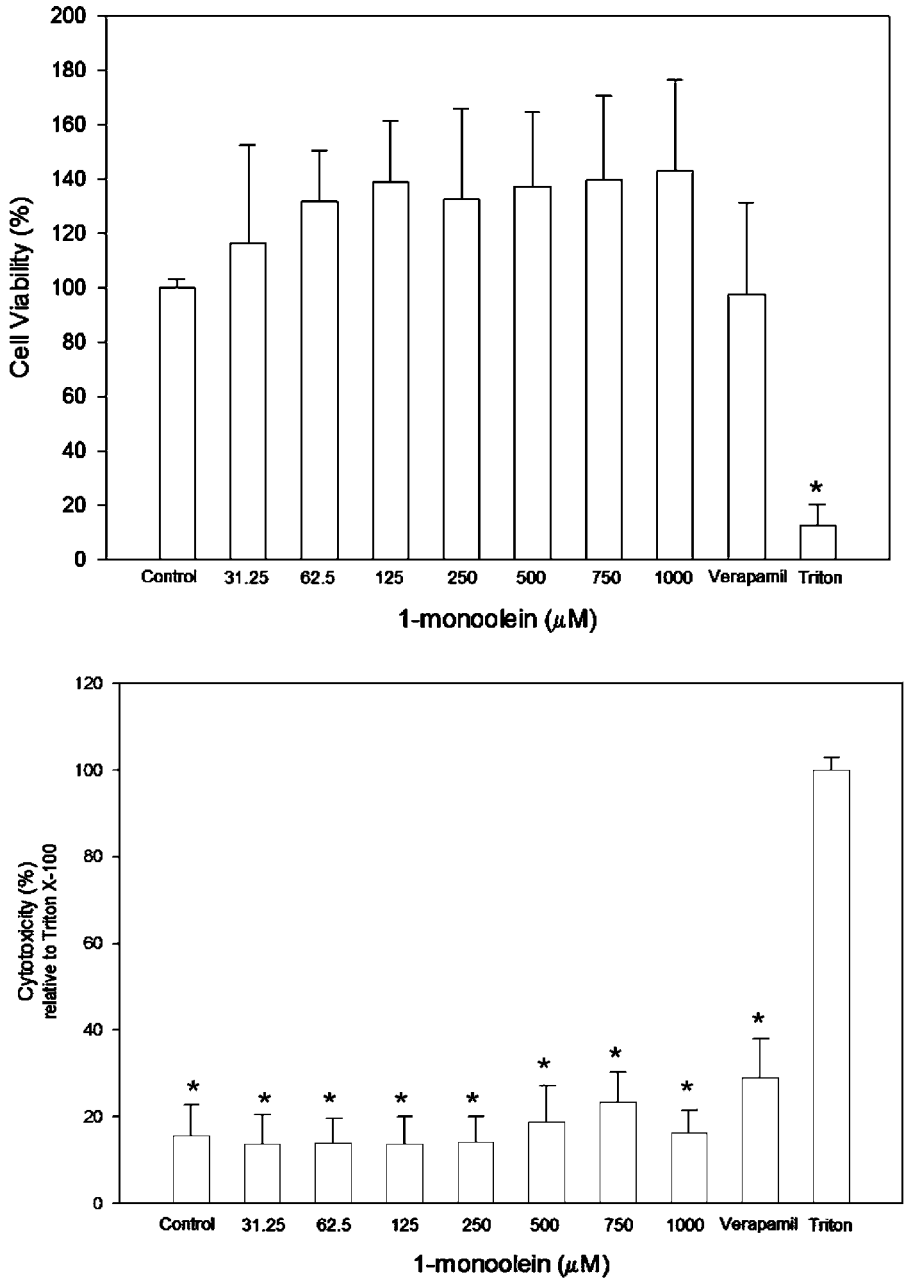

Figure 1. MTS (top) and LDH (bottom) assays of 1-monoolein. The results are expressed as the mean percent cell viability compared to the media control for the MTS assay and the mean percent cytotoxicity compared to Triton X-100 for the LDH assay ± standard deviation ($n \geq 7$). The cells were incubated for 24 h with treatments of 31.25−1000 μM 1-monoolein, 100 μM verapamil or 1% TritonX-100. * represents significant differences relative to the control ($P < 0.05$).

Bifunctional Transport of Rh123. The bifunctional flux of Rh123 across a Caco-2 cell monolayer was assessed after a 24 h treatment with 100 and 500 μM of 1-monoolein or 1-monostearin, or 100 μM verapamil ($n = 5$, Figure 4, Table 1). The absorptive flux (apical-to-basolateral, A-to-B) was not found to be statistically significant for any of the treatments compared to the media control. However, all treatments significantly reduced the secretory transport of Rh123 from the basolateral-to-apical transport (B-to-A). Specifically, treatments of 100 μM 1-monoolein, and 100 and 500 μM 1-monostearin (12.9 ± 2.6, 11.4 ± 2.3 or 11.1 ± 2.0 × 10^6 cm/s, respectively) showed similar depressed secretory transport compared to verapamil (11.0 ± 2.0 × 10^6 cm/s). Upon the addition of 500 μM 1-monoolein, the efflux of Rh123 in the B-to-A direction was reduced even further than the verapamil treated samples (7.9 ± 0.8 × 10^6 cm/s). TEER values were monitored prior to, during, and after the experiment (Table 1). No significant differences in the TEER values were noted throughout the experiment, indicating that the integrity of the Caco-2 cell monolayer was not compromised by the addition of the treatments.

P-gp Expression. The protein expression of P-gp was quantified in Caco-2 cells after being treated for 24 h with either 100 μM or 500 μM of 1-monoolein or 1-monostearin

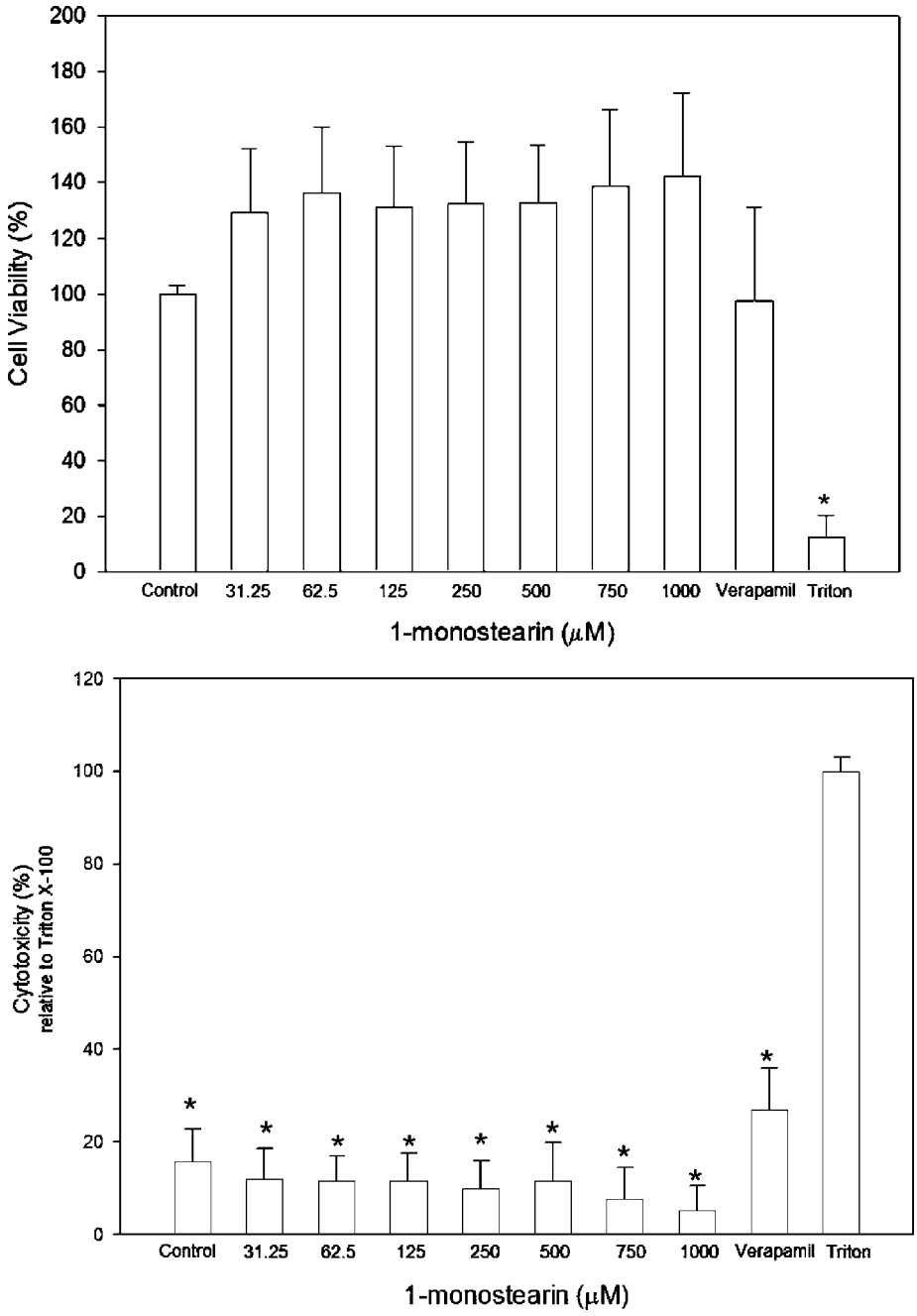

Figure 2. MTS (top) and LDH (bottom) assays of 1-monostearin. The results are expressed as the mean percent cell viability compared to the media control for the MTS assay and the mean percent cytotoxicity compared to Triton X-100 for the LDH assay ± standard deviation ($n \geq 7$). The cells were incubated for 24 h with treatments of 31.25−1000 μM 1-monostearin, 100 μM verapamil or 1% Triton X-100. * represents significant differences relative to the control ($P < 0.05$).

($n = 6$, Figure 5). The addition of both monoglycerides at concentrations of 500 μM significantly decreased the protein expression compared to the control (52.3 ± 1.1% or 39.1 ± 2.3% depressed, respectively). Alternatively, concentrations ≤100 μM of both monoglycerides did not appear to have any significant effects on the expression of P-gp.

Cell Membrane Fluidity. By taking the fluorescence anisotropy of two markers, DPH and TMA-DPH, changes in the fluidity of the cell membrane upon exposure to the monoglycerides was assessed (Figure 6, $n = 5$). DPH detects changes in the hydrophobic fatty acid side chains, whereas TMA-DPH is used to identify changes in the lipid/polar head interphase of the cell membrane.[22] Since fluorescence anisotropy is inversely related to the membrane fluidity, a decrease in DPH or TMA-DPH fluorescence anisotropy upon exposure to treatments concludes an increase in membrane fluidity.

Cholesterol, known to decrease membrane fluidity (increase in fluorescence anisotropy), and benzyl alcohol, a

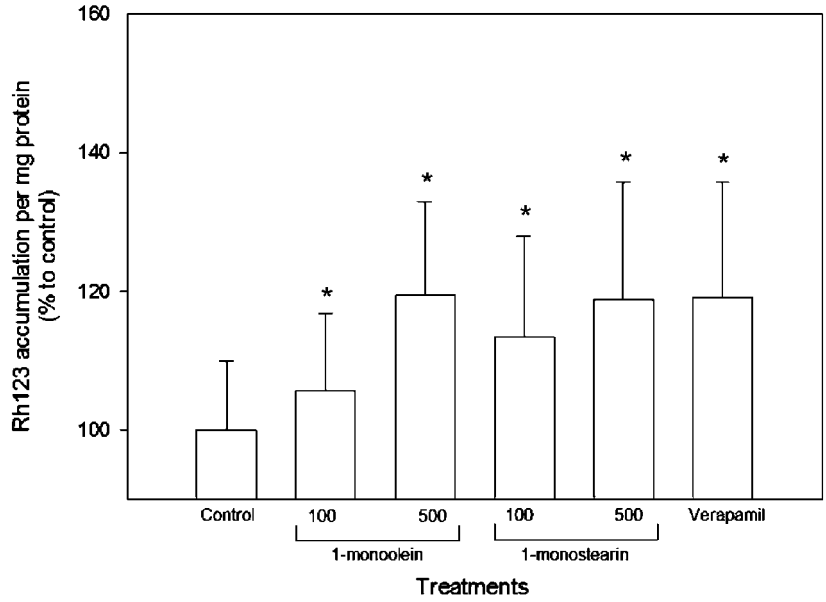

Figure 3. Rh123 accumulation in Caco-2 cells. The results are expressed as mean percent relative to the control after 24 h incubation with 100 or 500 μM of 1-monoolein or 1-monostearin, or 100 μM verapamil $\pm$ standard deviation (n = 6, *P < 0.05). All data have been normalized for protein concentrations.

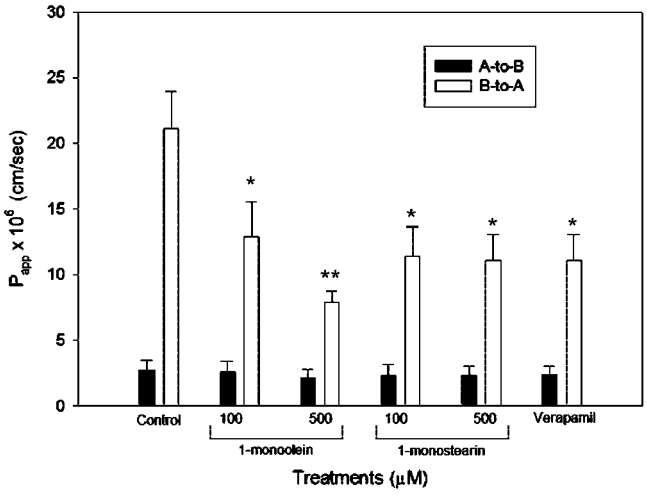

Figure 4. Apparent permeability coefficients for the absorptive and secretory flux of Rh123 across a Caco-2 cell monolayer. The results are expressed as the mean of either the apical to basolateral flux (P_{app}(A-to-B)) or the basolateral to apical flux (P_{app}(B-to-A)) after 24 h treatment with 100 or 500 μM 1-monoolein or 1-monostearin, or 100 μM verapamil $\pm$ standard deviation (n = 5, *P < 0.05 compared to the control, **P < 0.005 monoglycerides compared to verapamil).

compound documented to increase membrane fluidity (decrease fluorescence anisotropy), were used as positive controls. Upon 24 h exposure to 50 μM cholesterol, a 3% increase in the fluorescence anisotropy was observed for both the DPH and TMA-DPH fluorescent labeled Caco-2 cells. As expected, a decrease in the fluorescence anisotropy (3% for DPH and 10% of TMA-DPH studies) was observed upon incubation with 30 mM benzyl alcohol. Both results are similar to those found in the literature.[23]

Interestingly, the addition of 100 μM 1-monoolein, and 100 μM, 500 μM 1-monostearin only marginally perturbed the cell membrane fluidity. The addition of 500 μM 1-monoolein was the only monoglyceride treatment that showed any significant effect; however, it appears that only the fluidity of the fatty acid chains was affected by the higher concentration of 1-monoolein (13% increase in fluidity measured by the DPH fluorescence anisotropy). No significant change in the TMA-DPH fluorescence (measuring the fluidity of the polar headgroup region) was observed.

For comparison, the effects of two common lipid excipients, Peceol and Gelucire, on the cell membrane fluidity were also assessed. Both these lipid excipients are known to contain a reasonable percentage of monoglycerides in their formulation. Peceol, composed of 44% monoglycerides, increased the cell membrane fluidity of the fatty acid chains by 18%, and Gelucire, containing ~20% glycerides, increased the fluidity by 8%.

Nuclear Membrane Integrity. A modified assay was used to determine the effects of monoglycerides on the integrity of the nuclear membrane (Figure 7).[24] The assay measured the concentration of DNA released upon increasing concentrations of salt. In order to determine the noted effects, the concentration of salt at which 50% of the releasable material has exited the nucleus was interpolated from the data and compared to the various treatments. Thus, if the nuclear membrane integrity was destabilized, the salt concentration at which 50% of the DNA is released from the nucleus will also be decreased. The opposite is also true where a higher concentration of salt needed to destabilize the nuclear membrane suggests an increase in nuclear membrane integrity. Based on this study, no significant change in the salt concentrations needed to release 50% of the DNA for treatments of 100 and 500 μM 1-monoolein or 1-monostear-

Table 1. TEER Values and the Apparent Permeability Ratio (P_{app} Ratio) from the Bifunctional Transport Studies[a]

	TEER values ($\Omega \cdot cm^2$)				
	A-to-B		B-to-A		
treatments	before	after	before	after	P_{app} ratio
control	439.7 ± 19.6	436.6 ± 16.5	445.8 ± 22.7	435.2 ± 17.1	7.8
100 μM verapamil	431.6 ± 14.7	437.0 ± 11.8	437.9 ± 19.7	437.0 ± 22.4	4.7
500 μM 1-monoolein	439.8 ± 12.3	430.9 ± 18.6	442.1 ± 15.8	432.0 ± 13.2	3.8
100 μM 1-monoolein	433.5 ± 13.7	436.3 ± 20.4	438.1 ± 16.9	430.3 ± 18.4	5.3
500 μM 1-monostearin	442.7 ± 15.6	435.9 ± 15.7	434.9 ± 21.7	438.9 ± 10.8	4.8
100 μM 1-monostearin	429.6 ± 13.5	432.4 ± 19.5	440.6 ± 17.3	437.4 ± 21.9	4.9

[a] The TEER values of the Caco-2 cell monolayers were measured throughout the experiment; their values before the monoglyceride treatments and after the transport of Rh123 in the A-to-B and B-to-A directions are shown. Data is presented as mean ± standard deviation ($n = 5$). The P_{app} ratio is expressed as the mean quotient of P_{app}(B-to-A) to P_{app}(A-to-B).

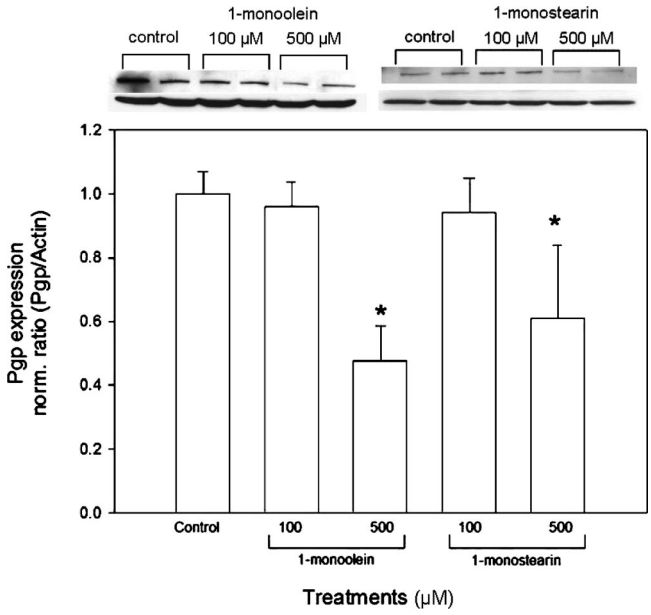

Figure 5. P-gp protein expression after 24 h exposure to 100 or 500 μM 1-monoolein or 1-monostearin. The data represent the mean ± standard deviation of P-gp protein normalized by the protein expression of actin ($n = 5$, $^*P < 0.001$ compared to the control).

in, 0.5 and 0.25% (v/v) Peceol, and 0.02 and 0.01% (w/v) Gelucire was observed when compared to the untreated control ($n \geq 5$).

Discussion

There has been an increased interest in designing and synthesizing novel P-gp inhibitors to overcome multidrug resistance. Thus, it is ironic that some of the agents which are being used to increase the solubility of lipophilic drugs paradoxically also inhibit P-gp. However, research into the actual components of lipid excipients that affect the activity of P-gp has not been widely investigated. As an attempt to methodically determine the compounds responsible for inhibiting the efflux mechanism in the enterocytes, we began analyzing two different monoglycerides used in many of the lipid excipients currently being investigated. Both 1-monoolein and 1-monostearin are monoacylglycerols with similar chemical structures, each 18 carbon units long. 1-Monoolein,

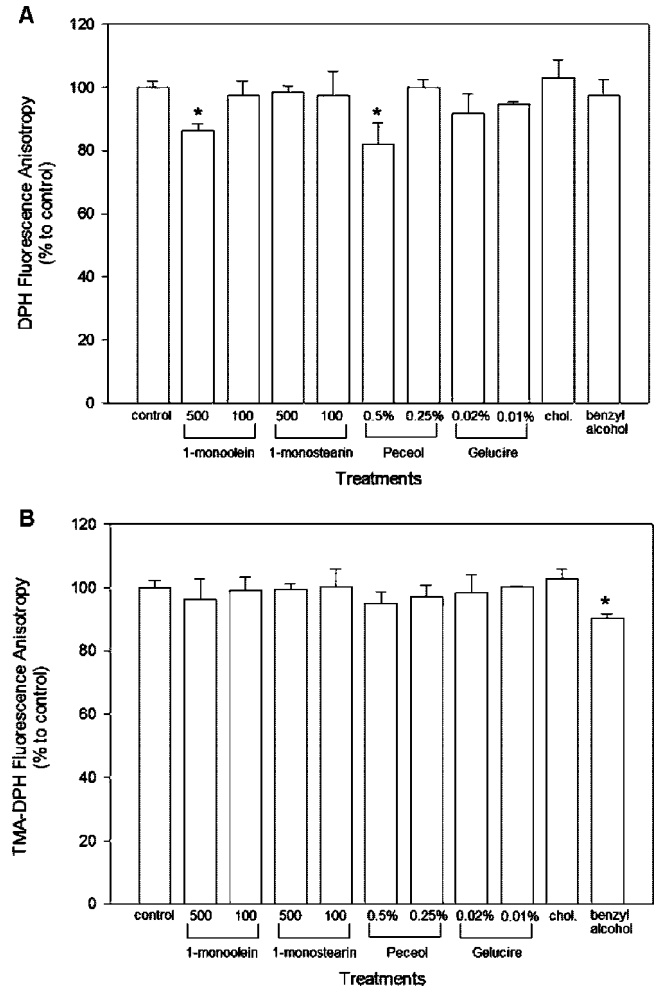

Figure 6. The effects of different concentrations of 1-monoolein, 1-monostearin, Peceol and Gelucire on the membrane fluidity (inverse of fluorescence anisotropy) of Caco-2 cell membranes. Cholesterol (50 μM) and benzyl alcohol (30 mM) were used as the positive controls. Units for the concentrations stated are μM for the monoglycerides, v/v for Peceol and w/v for Gelucire. In these studies, (A) DPH serves as the fluorescent probe for the fatty acid side chain region and (B) TMA-DPH is the fluorescent probe for the polar head groups of the cell membrane ($n = 5$, $^*P < 0.05$ compared to the control).

however, has one degree of unsaturation (9-*cis*-octadecanoic acid) compared to the completely saturated 1-monostearin.

Caco-2 cells, a well-accepted *in vitro* model of the intestinal mucosa, were chosen for this study to mimic the small-intestinal columnar epithelium.[20,25,26] These cells express an apically polarized efflux system that has previously been shown to limit the permeability of certain substrates similar to the observed P-gp mediated efflux observed *in vivo*.[11,27–30]

To reduce the nonspecific effects induced by toxicity-mediated reactions in Caco-2 cells during subsequent studies, the toxicities of both monoglycerides were completed using two separate cytoxicity assays. Although both monoglycerides showed minimal to no toxicity for the concentration range tested (31.25−1000 μM), the results of the MTS data should be evaluated with caution. The results for all concentrations tested using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium bromide) assay for both monoglycerides suggest an increase in cell viability compared to the media-treated control. One possible explanation for the increased cell viability is that the monoglycerides are stimulating the growth of the Caco-2 cells upon a 24 h exposure. Oleic acid, the fatty acid chain derivative of 1-monoolein, has been shown to stimulate the growth of Caco-2 cells at concentrations >150 μM.[31] However, we believe there may be another explanation for the increased MTS values.

Vellonen et al. also noted an apparent increase in cell viability using a similar toxicity assay, an MTT assay.[32] The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, like the MTS assay, detects the activity of the mitochondria by spectrophotometrically quantifying the reduction of tetrazolium salts to formazan crystals by NADPH or NADH. However, the MTT assay uses insoluble formazan crystals that must be first dissolved in DMSO before being analyzed. Nonetheless, Vellonen et al. noted an increased concentration of the reduced MTT reagent in the cytoplasm of the cell when using various known inhibitors of MRP and MDR1 in a MDCKII cell line.[32] They concluded that the inhibition of the efflux transporter proteins caused retention of the formazan products inside the cell, thereby leading to erroneously high absorption values. From these results, it suggests that the use of efflux inhibitors skews the results of assays that quantify cell toxicity by measuring the accumulation or formation of reduced compounds inside the cytoplasm including MTT and MTS assays.

The LDH assay, on the other hand, measures the release of lactate dehydrogenase (LDH) by damaged/nonviable cells into the media by monitoring the conversion of a tetrazolium salt into a red formazan product (quantified by its characteristic absorbance at 492 nm). In this assay, the results are independent of the activity of efflux transporters. Thus, the LDH assay is likely to be more reliable when measuring the cytotoxicity for inhibitor compounds, and was weighted more heavily when determining the toxicity of the monoglycerides.

Nonetheless, concentrations no greater than 500 and 100 μM of both monoglycerides were used for the remainder of the experiments. These concentrations are based on clinically relevant concentrations of monoglycerides administered when using non-cytotoxic amounts of either Peceol or Gelucire as the lipid excipients (44% of Peceol is composed of monoglycerides, whereas 20% of Gelucire are glycerides[33]).

Rh123, a low toxic fluorescent dye, is a well-known substrate for P-gp[34,35] and was used to assess the activity for both the accumulation and bidirectional flux studies. Inhibition of efflux transporters can be observed by an increase in the concentration of Rh123 trapped inside the Caco-2 cells, whereas an increase in the activity of P-gp is observed by detecting a decrease in the Rh123 accumulation compared to the control. Verapamil, a well-documented inhibitor of P-gp, was used as the positive control for both the accumulation and bidirectional flux studies.[36]

Both concentrations of monoglycerides in the accumulation studies (100 and 500 μM 1-monoolein and 1-monostearin) increased the intracellular Rh123 accumulation relative to

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the media control, with the higher concentration of monoglycerides showing similar Rh123 accumulation as the positive control, verapamil. Suppression of the Rh123 efflux was also observed in the bidirectional flux studies for both 1-monoolein and 1-monostearin. Although no significant changes in the A-to-B flux was observed for any of the treatments, there was a statistically significant reduction in the B-to-A transport. Comparisons of the P_{app} ratios for 1-monoolein and 1-monostearin with the P_{app} ratio for the media treated controls also confirmed the decrease in Rh123 secretions. Given that P-gp plays a key role in the efflux of Rh123, both the increased accumulation and the depressed P_{app} ratios upon treatment with 1-monoolein or 1-monostearin strongly suggest that monoglycerides inhibit the activity of P-gp. In addition, the unchanged TEER values and the total cellular protein content indicated that the reduced activity of P-gp was not caused by physical disruption to the monolayer, cell damage or cell death.

A possible explanation for the depressed activity of P-gp upon treatments of monoglycerides is the observed decrease in P-gp protein expression. Treatments of both 500 μM 1-monoolein and 1-monostearin resulted in a significant decrease of P-gp protein expression, with 1-monoolein decreasing the P-gp protein concentration by 48%. However, no significant changes in the P-gp expression were observed for concentrations $\leq$100 μM. Thus, it is believed that the decreased activity of P-gp may not be exclusively due to the depressed P-gp expression, but due to several other possible mechanisms.

This hypothesis is not based on an isolated observation. Sachs-Barrable et al. have also noted a decrease in the P-gp activity when treated for 24 h with Gelucire or Peceol;[15] similarly, the decrease in P-gp activity for the treated cells was greater than the decrease in P-gp expression.

A few groups have linked exicipient-induced changes in the cell membrane fluidity to inhibition of P-gp activity *in vivo*.[5,37,38] This change in P-gp activity would not translate to a decrease in protein expression providing a possible explanation for the discrepancy in our data. However, the only change in the bulk membrane fluidity for all treatments of the monoglycerides was isolated to the addition of 500 μM 1-monoolein.

It is not extremely surprising that 1-monoolein has an effect on the fluidity of the cell membrane. Degrees of unsaturation have been previously noted to alter the packing order of phospholipid by-layers.[39] For the rest of the monoglyceride teatments, however, it appears that the decreased activity of P-gp cannot be explained by changes in the bulk membrane fluidity. Yet, this hypothesis of affecting the activity of P-gp by inducing a change in the cellular environment should not be completely ruled out.

Unfortunately, fluorescence anisotropy is not a sensitive enough technique to detect changes in the proximal membrane components to the active transport domain of P-gp.[38] It has been previously shown that P-gp's performance is very dependent on the lipid milieu of the membrane environment.[40–43] Thus, even if a change in the bulk membrane fluidity upon treatments with monoglycerides was not detected, it is still possible that the environment around P-gp has been perturbed just enough to diminish the activity of the efflux protein.[37]

Other possible explanations for the decreased activity could also be attributed to either the depletion of intracellular levels of ATP or a decreased affinity of P-gp for ATP, similarly seen previously in both *in vivo* and *in vitro* studies.[3,44,45] P-gp, a member of the ATP-binding cassette (ABC) transporter protein superfamily, is dependent on ATP for its efflux activity.[6–9] Thus, any change in the affinity or concentration of ATP, possibly accounted for by the addition of monoglycerides, could cause a significant change in P-gp activity while not affecting the protein expression levels.

The discrepancy between the protein expression and P-gp activity results may also be due to the choice of Rh123 as the fluorescence marker. Although Rh123 is a high-affinity P-gp substrate (apparent K_m = 13.5 μM in membrane vesicles),[46] it is also known to interact with MRP2 and BCRP efflux transporters.[47,48] Thus, it is possible that the monoglycerides are also inhibiting other transporters besides P-gp resulting in an inflated accumulation of Rh123.

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Despite the numerous mechanisms that might be involved in decreasing the activity of P-gp, the decrease in protein expression is believed to occur at the transcriptional level. Risovic et al. has shown that concentrations of 0.5 and 0.25 (v/v) % Peceol downregulated the MDR1 gene.[16] However, the receptor pathway (specifically the transcriptional control of the ABC transporters) leading to the decrease in P-gp expression has not been identified. Many receptor proteins such as retinoic acid receptor (RAR), steroid-activated receptor (SXR; PXR in rodents), farnesoid receptor (FXR) and constitutive androstane receptor (CAR) have been implicated in the transcriptional control of transport proteins.[49–51] Many of these nuclear receptors have lipid ligands that provide interplay between the lipids (possibly including monoglycerides) and the transcriptional control of efflux transporters.[49,52–55]

To date, we have determined that the monoglycerides (100 and 500 μM 1-monoolein and 1-monostearin) do not affect the nuclear membrane integrity using a modified salt concentration assay.[24] This suggests that the disruption to the nuclear membrane, which potentially could affect the contents of the nucleus, is not a mechanism for the downregulation of MDR1. This finding is particularly significant since studies have shown that the nucleocytoplasmic barrier responds sensitively to changes in the cellular environment and are particularly sensitive to any changes in the lipid environment of the nuclear envelope.[56,57] To further examine the effects of the monoglycerides on the transcriptional/translational processes, we plan to study the mRNA levels of the MDR1 gene upon treatments of the monoglycerides, as well as identifying the receptor pathways related to the observed decrease of the P-gp expression.

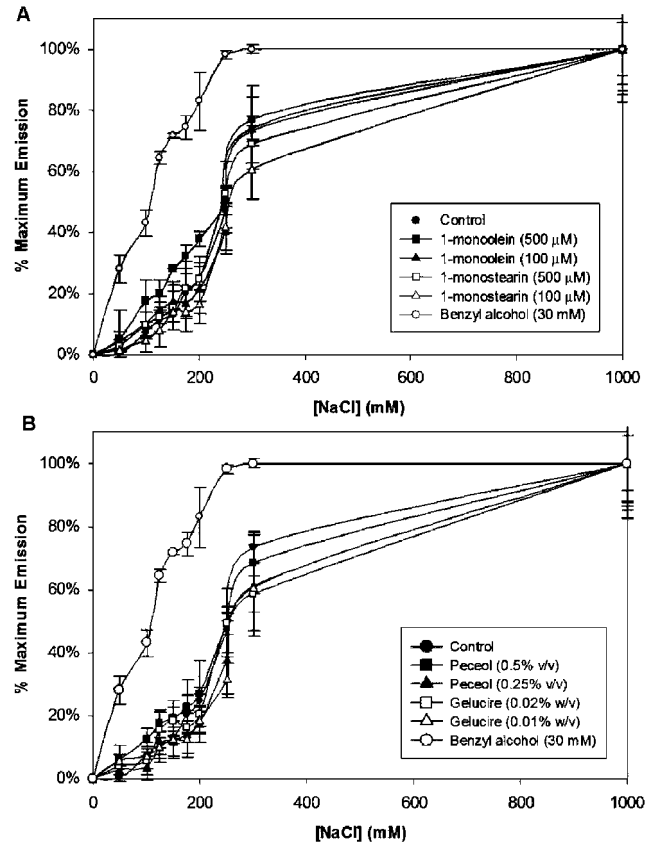

Figure 7. The effects of different salt concentrations on Caco-2 cell nuclei pretreated with (A) 1-monoolein and 1-monostearin and (B) Peceol and Gelucire. Benzyl alcohol (30 mM) was used as the positive control. The fluorescence signals are normalized to the largest emission observed for each of the samples (representing the highest concentration of DNA released). Treatments are compared at the value where 50% of the DNA has been released (50% maximum emission). Perturbations in the nuclear membrane integrity can be concluded based on an increase or decrease in the salt concentrations needed to release 50% of the DNA ($n \geq 5$).

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Conclusion

Both monoglycerides studied, the saturated 1-monostearin and the unsaturated 1-monolein, showed a decrease in the efflux activity of P-gp assessed by Rh123 accumulation and bifunctional transport studies. These findings suggest that monoglycerides, common components in various lipid excipients, are inhibitors of P-gp. Although the mechanism of the inhibition is not completely understood, the depressed activity of P-gp upon addition of 1-monoolein and 1-monostearin can be partially explained by a decrease in P-gp expression and an increase in cell membrane permeability (for treatments of 500 μM 1-monoolein). These results reinforce the need for an appreciation and understanding of the bioactive nature of individual components of the excipients in order to facilitate a logical choice of lipid-based drug delivery systems when designing multicomponent formulations.

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