Effects of Monoglycerides on Rhodamine 123 Accumulation, Estradiol 17 β-D-Glucuronide Bidirectional Transport and MRP2 Protein Expression within Caco-2 cells

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ABSTRACT – Purpose. Oral drug development had been hindered by the bioavailability issue despite vast market popularity. Lipid excipients had shown to enhance bioavailability of a number of reformulated hydrophobic oral drugs, yet the underlying mechanisms of action by lipids are still unclear. One proposed mechanism is that lipid excipients could facilitate drug uptake by altering the activities of apical membrane intestinal efflux transporters. Thus, this study aimed to investigate the effects of 1-monopalmitin, 1-monoolein and 1-monostearin on the efflux activity and protein expression of multidrug resistance-associated protein 2 (MRP2) *in vitro*. **Methods.** The 24-hour non-cytotoxic ranges of these monoglycerides were first determined using MTS and LDH assays in Caco-2 cells. Then, both accumulation and bidirectional transport studies were conducted using 10 µM rhodamine 123 (Rh123) and 10 nM estradiol 17 β-D-glucuronide ($E_2$17βG), respectively, to assess the functional activities of MRP2. 50 µM MK-571, a specific MRP1 and MRP2 inhibitor, was used as the positive control in both studies. Western blotting was followed to determine the effect of these monoglycerides on MRP2 protein expression. **Results.** Caco-2 cells were viable when treated with 1-monopalmitin, 1-monostearin and 1-monoolein at concentrations equal or less than 1000 µM, 1000 µM and 500 µM, respectively. Cells treated with 1-monoplamitin, 1-monostearin, 1-monoolein and MK571 resulted in significant increases in Rh123 accumulation and decreases in $E_2$17βG efflux ratio compared to the control (medium treated only). MRP2 protein expressions in 1-monopalmitin and 1-monoolein treated cells were decreased by 19% and 35% compared to the control; however, there was no change of MRP2 protein expression in 1-monostearin treated cells. **Conclusions.** These

findings suggested that 1-monoolein, 1-monostearin and 1-monopalmitin could attenuate the activity of MRP2 and possibly other efflux transporters in Caco-2 cells. The reduction of efflux activity of MRP2 by 1-monoolein treatment could be partially accounted by the non-specific down-regulation of MRP2 protein expression.

INTRODUCTION

A large number of drugs on the market are formulated for oral intake. The main advantages of oral administration are that it is usually the most convenient and economical route (1). However, one major constraint in oral drug development is the bioavailability issue, especially for hydrophobic drugs. Approximately 40% of new drug candidates suffer from low aqueous solubility (2). Bioavailability is defined as a measurement for the extent of a therapeutically active drug that reaches the systemic circulation (1). Some of the barriers effecting oral drug bioavailability include drug degradation prior to absorption at the gastrointestinal (GI) tract, poor absorption along the GI tract and elimination through hepatic first-pass metabolism (3).

For many years, clinical observations had indicated that lipids could enhance the bioavailability of many hydrophobic oral drugs (4). However, lipid-based drug delivery systems have only generated considerable interest in recent years after the commercial successes of a number of hydrophobic oral drugs reformulated with lipids, such as Neoral (Cyclosporine A), Fortovase (Saquinavir) and Norvir (Ritonavir) (5).

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Nonetheless, the underlying mechanism for enhanced bioavailability with lipid-based oral formulation is still under intensive investigation.

So far, there are several proposed mechanisms aiming to explain the possible clinical benefits of lipid-based drug delivery systems in oral drug applications (3). The three primary theories that attracted the most attention on lipid-enhanced bioavailability are the following ones: alteration of the composition and character of the intestinal environment (6); bypass of the first-pass hepatic metabolism via the lymphatic system (7, 8); interaction with enterocyte-based transporters (9). The effect of lipids on intestinal efflux transporters will be the focus of this study.

The small intestine is a major site of absorption for both nutrients and orally administered drugs due to its large surface area. The two routes which drugs could permeate across the intestinal membranes are the paracellular and transcellular pathways. Small hydrophilic and ionized drugs can get across the enterocytes via the paracelluar route; whereas the majority of the drugs have to permeate the enterocytes via the transcellular route (10).

After a drug has penetrated into the cytosol of an enterocyte, it is subject to either efflux by apical membrane ATP-binding cassette (ABC) efflux transporters, for instance, P-glycoprotein (Pgp), multidrug resistance-associated protein 2 (MRP2) and breast cancer resistance protein (BCRP); or degradation by intracellular metabolizing enzymes, for example, the cytochrome P450 (CYP), the uridine diphosphate–glucuronosyltransferase (UGT) families and the glutathione S-transferase (GST). Enterocytes regulate the absorption of drugs mainly through these two processes to effectively remove xenobiotics from the body (10, 11). Hence, modulating the activity of apical membrane ABC efflux transporters has became a popular pursuit by pharmaceutical companies to enhance the bioavailability of orally administered drugs.

ABC transporter superfamily encompasses 49 members, and they distribute widely throughout the body. ABC transporters can pump a wide range of substances out of the cells using energy generated by hydrolysis of ATP (12). Many studies had shown that lipid-based drug delivery systems could attenuate the functional activities of the intestinal ABC efflux transporters, which in turn,

increase the drug bioavailability when co-administered orally with the drug of interest. Most of these studies were focused on Pgp, an apical-membrane ABC efflux transporter. Several *in vivo* animal studies had shown that when highly non-soluble drugs like ontazolast and Amphotericin B (AmpB) were incorporated into Peceol (a lipid excipient primarily composed of mono- and di-glycerides), there were significant increases in the bioavailability of these orally formulated drugs (8, 13). In addition, a number of studies using natural lipids like castor oil and monoglycerides had shown to inhibit Pgp-mediated drug efflux *in vitro* (10, 14). In contrast to Pgp, the interaction of lipids with other major intestinal apical-membrane ABC efflux transporters, for instance MRP2, has received less attention.

MRP2 shares many similarities with Pgp in terms of tissue distribution, function and localization on polarized cells (15). MRP2 is an ABC transporter encoded by the ABCC2 gene, and it has a molecular weight around 200kDa. MRP2 is mainly located in the gut, liver, kidney, blood-brain barrier and placenta which overlaps with the tissue distribution of Pgp (16). Furthermore, MRP2 is anchored on the apical side of polarized cells acting as efflux pumps in a similar fashion as Pgp. In addition, MRP2 transports a wide-spectrum of substrates like Pgp, in particularly substances such as glutathione and glucuronide conjugates, steroid, bile salt conjugates and hydrophobic and anionic xenobiotics (17, 18). Despite the shared similarities between MRP2 and Pgp, there were only a limited number of studies have been conducted which looked at the lipid-effect on MRP2.

In the past, studies of lipid effects on transporters were mostly focused on the more complicated lipid excipients which often composed of a mixture of long- or medium-chain fatty acids, mono-, di- and tri-glycerides, various types of surfactants and hydrophilic solvents. Yet, the effects of the specific components in those lipid excipients on ABC efflux transporters have not been adequately assessed. Therefore, the first objective of this study was to investigate the effect of monoglycerides on efflux transporters' functional activity, in particularly MRP2, using rhodamine 123 (Rh123) and estradiol 17 β-D-glucuronide (E$_2$17βG). The monoglycerides used in this study were monopalmitin, monostarin and monoolein,

which are the three predominant lipid-digestion products in plants and animals (19).

Although lipid excipients had shown to inhibit the functional activities of ABC efflux transporters, particularly on Pgp, the mechanism of action exerted by these lipids excipients is still unclear. Some suggested theories include disrupting the cell membrane integrity, depleting available intracellular ATP, blocking binding sites on the transporters, inhibiting the transporters' ATPase activity, reducing the protein expression of the transporters (9, 20-21). An *in vitro* study by Sachs-Barrable *et al.* had shown that Peceol could inhibit Pgp protein expression (9); however, there has been no other study done to address the issue of whether the lipid-effect on the protein expression of ABC efflux transporters is transporter-specific or not. Hence, the second objective of this study was to investigate the effect of monoglycerides on MRP2 protein expression using Caco-2 cells.

Caco-2 cell line is a well-established and most studied human small intestinal *in vitro* model for the study of absorption in the intestinal mucosa (22). Caco-2 cells were obtained from human colon adenocarcinoma cells; they could differentiate into monolayers that both structurally and functionally resemble the small intestinal epithelium (22). The transcript level of MRP2 in Caco-2 was comparable to its level in human jejunum of the small intestine, which is the major site for oral drug absorption (23). In addition, MRP2 transcript level in both Caco-2 cells and jejunum is much higher than MDR1 and other MRP transporters (24). Thus, Caco-2 cell line is a suitable cell model serving the purpose of this study.

MATERIALS AND METHODS

Materials

The 1-monopalmitin, 1-monostearin, 1-monoolein (purity >99%), rhodamine 123 (Rh123), Triton X-100, Tween-80, protease inhibitors cocktail, Na-deoxycholate, EDTA and NaCl were obtained from Sigma-Aldrich (St. Louis, MO, USA). MK-571 was purchased from Alexis Biochemical (San Diego, CA, USA). All tissue culture reagents, such as fetal bovine serum (FBS), MEM Non-essential amino acids solution, HEPES buffer solution, sodium pyruvate solution, penicillin and streptomycin, Phosphate-buffered saline (PBS), Hanks' Balanced Salt Solution (HBSS), Minimum Essential Medium (MEM) with Earle's salts and L-glutamine (with and without phenol-red), were purchased from Invitrogen Technologies (Grand Island, NY, USA). T-75 flasks and tissue culture treated plates were from Corning Incorporated (NY, USA). CytoTox96 Non-Radioactive Cytotoxicity Assay and MTS CellTiter 96 AQueous One Solution Cell proliferation Assay were purchased from Promega Corporation (Madison, WI, USA). Tritium radio-labeled estradiol 17 β-D-glucuronide (E$_2$17βG) was purchased from PerkinElmer, Inc. (Boston, MA, USA). The E$_2$17βG was labeled at the C6 and C7 positions of the estradiol. The specific activity of [^{3}H] E$_2$17βG was 47.1 Ci/mmol packed in 1.0 mCi/mL ethanol:water (9:1) solution. BCA Protein Assay Kit was obtained from Pierce Biotechnology Inc. (Rockford, IL, USA). NP-40 was purchased from Roche Applied Science (Laval, QC, CA). Nitrocellulose membrane was purchased from Bio-Rad (Hercules, CA, USA). High range markers, anti-MRP2 (M$_2$III-6), anti-β actin (I-19) primary antibodies and peroxidase-conjugated bovine anti-goat IgG for Western blotting were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Peroxidase-conjugated AffiniPure goat anti-mouse IgG (H+L) was purchased from Jackson ImmunoResearch Laboratory, Inc. (West Grove, PA, USA).

Cell culture

Caco-2 cells from a human colon adenocarcinoma cell line were purchased from American Type Culture Collection (Rockville, MD, USA) and maintained between passages 18-43. The passages used were from 26 to 43. Caco-2 cells were cultured in MEM with Earl's salts and L-glutamine (with phenol red) supplemented with 10% fetal bovine serum 0.1 mM MEM Non-essential amino acids solution, 1mM HEPES buffer solution, 1mM sodium pyruvate solution, 100 U/mL penicillin and 100 μg/mL streptomycin at 37˚C in humidified air containing 5% CO$_2$. Stock cultures were grown in T-75 flasks, 48-well, 96-well (Sargent-Welch, NY, USA) or 12-well plates with COL collagen-coated membrane inserts (pore size 0.4 μm, membrane diameter 12mm) (Corning, Sigma-Aldrich, USA), depending on the type of experiment. The medium was changed every other day and the plates were to

be used for experiments when the seeded cells in each well reached 90% confluency.

Cytotoxicity study

The cytotoxicity study was conducted using the CytoTox 96 non-radioactive cytotoxicity assay, which was often referred to as the LDH assay. LDH assay is a colorimetric enzymatic assay alternative to ^{51}Cr release cytotoxicity assay. It quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis, thus it is considered an indirect measure of cell membrane integrity. To assess the toxicity profile of each monoglyceride in Caco-2 cells, 20,000 Caco-2 cells were seeded per well in 96-well plates and medium was changed every other day until the cells reached 90% confluency. Each monoglyceride treatment was prepared with the fully supplemented MEM medium without phenol red by sonication at 37°C for 30 minutes. On the day of the experiment, 100 μL of each monoglyceride (1-monoolein, 1-monostearin, 1-monopalmitin) was added per well, and the cells were incubated in a humidified chamber at 37°C, 5% CO_2 for 24h. The cytotoxicity profiles of 1-monostearin and 1-monopalmitin were evaluated at 0, 100, 250, 500, 750, 1000, 1250, 1500, 1750, 2000 and 5000 μM; the cytotoxicity profile of 1-monoolein was evaluated at 0, 100, 250, 500, 750 and 1000 μM. For each monoglyceride experiment, a positive control group with 0.1% Triton X-100 was added. Triton X-100 is a potent non-ionic detergent that disrupts cell membrane which leads to cell death. For each experiment, there were three to six replicates for each concentration of the monoglyceride. After the cells incubated with the monoglyceride treatment for 24 hours, 25 μL of the medium from each well was transferred to a new 96-well flat-bottomed plate. Subsequently, 25 μL of reconstituted substrate mix from the CytoTox96 Non-Radioactive Cytotoxicity Assay Kit was transferred to each well of the new 96-well plate containing the aliquots. The plate was covered with aluminum foil and incubated at room temperature for 15 minutes. Stop solution was immediately added after the incubation time. Before any reading was taken, bubbles in the wells were popped using a syringe needle. The absorbance was recorded at 492nm within 1 hour after the addition of stop solution using Ascent Multiskan, a 96-well plate reader. Cytotoxicity was determined in the following fashion: (sample – background) / Triton X-100 treated.

Cell viability study

The cell viability in response to each monoglyceride treatment was assessed using the CellTiter 96 AQueous One Solution cell proliferation assay (also known as the MTS assay). The MTS tetrazolium compound can be bio-reduced by metabolically active cells into a colored formazan product that is soluble in tissue culture medium. This conversion is accomplished by NADPH or NADH produced by dehydrogenases from mitochondria. Hence, MTS assay is an indirect measure of mitochondrial respiration. Caco-2 cells were seeded and treated with different monoglycerides (1-monoolein, 1-monostearin, 1-monopalmitin) at various concentrations in 96-well plates as described in the cytotoxicity study. After 24-hour treatment, wells were washed with 100 μL PBS (pH 7.4) once. Then, 100 μL of CellTiter 96 AQueous One Solution Reagent diluted with HBSS (1:5) was added to each well. Cover the plate with aluminum foil and incubated it at 37°C, 5% CO_2 atmosphere for 1 hour. Any large bubbles in the wells were popped using a syringe needle, and record the absorbance at 492nm using Ascent Multiskan, a 96-well plate reader. 0.1% Triton X-100 was used as a negative control. Percentage cell viability was determined from readings in the following fashion: (sample – background) / no treatment group

Rh123 accumulation study

Forty thousand Caco-2 cells were seeded per well in 48-well plates. When the cells reached 90% confluency, 200 μL of appropriate treatment was added per well (Control: completed MEM; 1-monostearin: 500 and 1000 μM; 1-monopalmitin: 500 and 1000 μM; 1-monoolein: 500 μM; MK-571: 50 μM). After 24 hours of the monoglyceride treatment, cells were washed once with PBS and 200 μL of 10 μM Rh123 diluted in HBSS with 10 mM HEPES (pH 7.4) was added to each well. After 2-hour incubation, the uptake was stopped by aspirating the Rh123/HBSS solution and washing the cells 3 times with ice-cold PBS. Subsequently, cells were lysed with 200 μL 0.1% Triton X-100 for 30 minutes at room temperature

and 100 μL aliquots were used to measure the fluorescence using CytoFluor 4000, Perspective Biosystems (Framingham, MA, USA) at excitation and emission wavelengths of 485 nm and 530 nm, respectively. A Rh123 standard curve was generated to quantify the total amount of Rh123 accumulated in each sample. To determine the total protein content in each sample, 50 μL of the original cell lysate from each sample was used in BCA assay. Cellular accumulation of Rh123 was then normalized with the total protein content as determined by the BCA assay.

E$_2$17βG bidirectional flux study

The protocol used was a modification of the one from Hubatsch *et al.*'s group (25). 300,000 Caco-2 cells were seeded onto collagen-coated PTFE membrane insert with a pore size of 0.4 μm and 1.2 cm diameter (Transwell-COL from Corning Costar) in 12-well plates. The transepithelial electrical resistance (TEER) was monitored using a Millicell-Electrical Resistance System (Millipore, Bedford, MA, USA). Once the TEER value exceeded 350 Ω·cm^2 (around 21 days post-seeding), the cell monolayers were ready to be used for bidirectional flux experiments. Cells were washed once with PBS before adding treatment solutions (Control: completed MEM; 1-monostearin: 1000 μM; 1-monopalmitin: 1000 μM; 1-monoolein: 500 μM; MK-571: 50 μM) were loaded on both the apical side (0.5 mL) and the basolateral side (1.5 mL). After 24-hour incubation, the cells were washed twice with HBSS (with 10 mM HEPES) and loaded with 10 nM radioactive E$_2$17βG on the appropriate side. A standard curve was prepared for radioactively labeled MRP2 specific substrate E$_2$17βG at 0.3125, 0.625, 1.25, 2.5, 5 and 10 nM. For the absorptive flux (A->B), 750 μL of E$_2$17βG was sampled and replaced with buffered HBSS at 0, 30, 60 and 120 min; for secretory flux (B->A), 250 μL of E$_2$17βG was sampled and replaced with buffered HBSS at 0, 30, 60 and 120 min. The transferred E$_2$17βG concentration was determined by using standard curves of E$_2$17βG in the appropriate treatment solutions. At the end of the experiment the TEER values were measured to assure the integrity of the monolayer. For each treatment, duplicates were used. The apparent permeability coefficient (P$_{app}$) were calculated by the equation P$_{app}$ = $dQ/dt \cdot [1/(A \cdot C_0)]$, where dQ/dt

is the steady-state flux (in disintegrations per minute per second or micromolar per second), A is the surface area of the filter (in square centimeters), and C_0 is the initial concentration in the donor chamber at each time interval (in disintegrations per minute per liter or micromolar). The net efflux (Papp ratio) is expressed as the quotient of Papp (B-to-A) to Papp (A-to-B).

MRP2 protein expression study

There were 500,000 Caco-2 cells seeded per well in 6-well plates. After the cells reached 90% confluency (approximately 1 week), they were washed with 2 mL/well of PBS/HBSS. Caco-2 cells were treated for 24 hours with culture medium (control) or culture medium containing 1-monostearin (500 μM and 1000 μM), 1-monopalmitin (500 μM and 1000 μM), 1-monoolein (500 μM). 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 protease inhibitor cocktail (1:100 dilution) and 1 mM phenylmethanesulphonylfluoride (PMSF). Samples were then centrifuged for 30 minutes at 12,850 x *g* at 4ºC. Whole cell protein content in each sample was determined using BCA^{TM} assay and 60μg of protein from each sample were separated by electrophoresis with a 7 % SDS-polyacrylamide gel (SDS-PAGE) gel at 100 V constant voltage for 120 minutes. The gel was then transferred to a nitrocellulose membrane at 0.07A constant current for 90 minutes. A pre-stained high range marker was used to identify the MRP2 at 200 kD and β-actin at 42 kD. The membrane was blocked with 5% non-fat milk (in 1x TBS-T) for 2 hours. Mouse anti-human MRP2 (M$_2$III-6) primary antibody was added in 1:200 dilution with 5% non-fat milk to detect MRP2; goat anti-human β-actin (I-19) was added in 1:500 dilution with 5% non-fat milk to detect β-actin as an internal control. The membranes were washed 4 times with TBS-Tween (1% Tween-20). The membrane was incubated for 90 minutes in a 1:3000 dilution of goat anti-mouse IgG-HRP and bovine anti-goat IgG-HRP secondary antibodies for MRP2 and β-actin, respectively, followed by four washes with TBS-T. Bands were visualized using ECL Western blotting detection reagent from Amersham Biosciences (Piscataway,

NJ, USA), exposed to an X-Omat film from Kodak and quantified with UVP-Labworks software.

Statistical analysis

One Way Analysis of Variance (ANOVA) was used to analyze the data from cell viability, toxicity, Rh123 accumulation, $E_2 17\beta G$ bidirectional transport and MRP2 protein expression (monostearin and monopalmitin) studies. Student's t-test was used to analyze MRP2 protein expression for cells treated with monoolein. Statistically significant differences of multiple treatment groups versus control group were assessed using Tukey post-test method with a pre-determined alpha value of 0.05. All statistical analysis was performed using SigmaStat version 3.5 from Systat Inc.

RESULTS

Effects of monoglycerides on toxicity and viability of Caco-2 cells

To assess the non-cytotoxic ranges of these monoglycerides (mono-palmitin, -stearin, -olein) in Caco-2 cells, two different enzymatic assays were performed to ascertain the cytotoxicity data obtained.

In Figure 1, it had shown that there was no increased toxicity associated with 24-hour 1-monoolein treatment compared to the control when its concentration was equal or less than 500 μM. For cells treated with 1-monostearin and 1-monopalmitin as shown in Figure 2 and 3, their non-cytotoxic concentrations fell within 1000 μM of each type of lipid. In summary, the cytotoxic profiles generated by LDH and MTS assays for each monoglyceride were in agreement.

## Effects of monoglycerides on Rh123 accumulationin in Caco-2 cells

To assess the effect of these lipid treatments on MRP2 activity, a non-MRP2 specific fluorescent substance Rh123 was first used. The positive control used in the accumulation study was 50 μM MK-571, a specific MRP inhibitor. In Table 1, cells treated with mono-olein, -palmitin and -stearin had shown 22%, 19% and 23% increases, respectively, while 50 μM MK-571 had shown 81% in the accumulation of Rh123 compared to the control.

Effects of monoglycerides on $E_2 17\beta G$ bidirectional flux acorss Caco-2 cell monolayers

To monitor the transport of $E_2 17\beta G$ across Caco-2 cell monolayer, it was crucial to ensure that the tight junction of the monolayer remained intact throughout the experiment. One way to estimate the formation of tight junction was using the TEER value. As shown in Table 2, all TEER values were higher than 350 $\Omega*cm^2$, and there was no change in TEER values before and after the experiment for all treatment groups (control, monopalmitin, monostearin, monoolein and MK-571) in either apical to basolateral or basolateral to apical directions. These data suggested that the Caco-2 cell monolayer had maintained its integrity during the experiment. In another word, the transport of $E_2 17\beta G$ across Caco-2 cell monolayer was restricted to the transcellular route.

In Figure 4, the $P_{app,ab}$ (from apical to basolateral side) for the $E_2 17\beta G$ transport of 500 μM monoolein, 1000 μM monostearin and 1000 μM monopalmitin treated groups were (3.8 ± 2.1), (5.0 ± 1.8) and (6.1 ± 1.4) x10^{-6} cm/s, respectively; while the $P_{app, ab}$ for the control and 50 μM MK-571 (a specific MRP1 and MRP2 inhibitor) treated groups were (3.2 ± 1.7) and (3.6 ± 1.4) x10^{-6} cm/s, respectively. The apparent permeabilities in the absorptive direction for 500 μM monoolein, 1000 μM monostearin and 50 μM MK-571 treated groups had shown no statistically significant different to the control group (treated with medium only), with the exception of 1000 μM monopalmitin treated group which had its apparent permeability in the absorptive direction almost doubled comparing to the control. For the basolateral to apical $E_2 17\beta G$ transport, 500 μM monoolein [$P_{app,ba}$: (13.2 ± 4.5) x10^{-6} cm/s], 1000 μM monostearin [$P_{app,ba}$: (11.5 ± 3.6) x10^{-6} cm/s] and 50 μM MK-571 [$P_{app,ba}$: (10.3 ± 3.3)] x10^{-6} cm/s] treated groups had shown statistically significant decreases in their apparent permeability coefficients compared to the control group [$P_{app,ba}$: (31.1 ± 13.9) x10^{-6} cm/s], with the exception of 1000 μM monopalmitin treated group [$P_{app,ba}$: (31.5 ± 14.9) x10^{-6} cm/s] which had shown no change in $P_{app,ba}$ compared to the control. unchanged compared to the control.
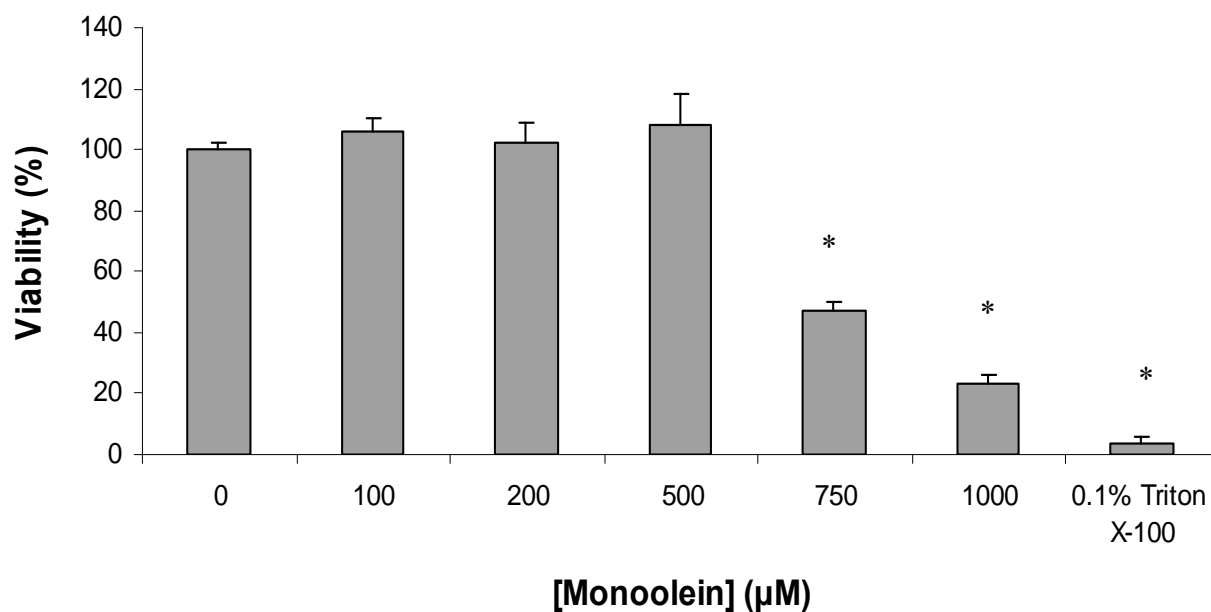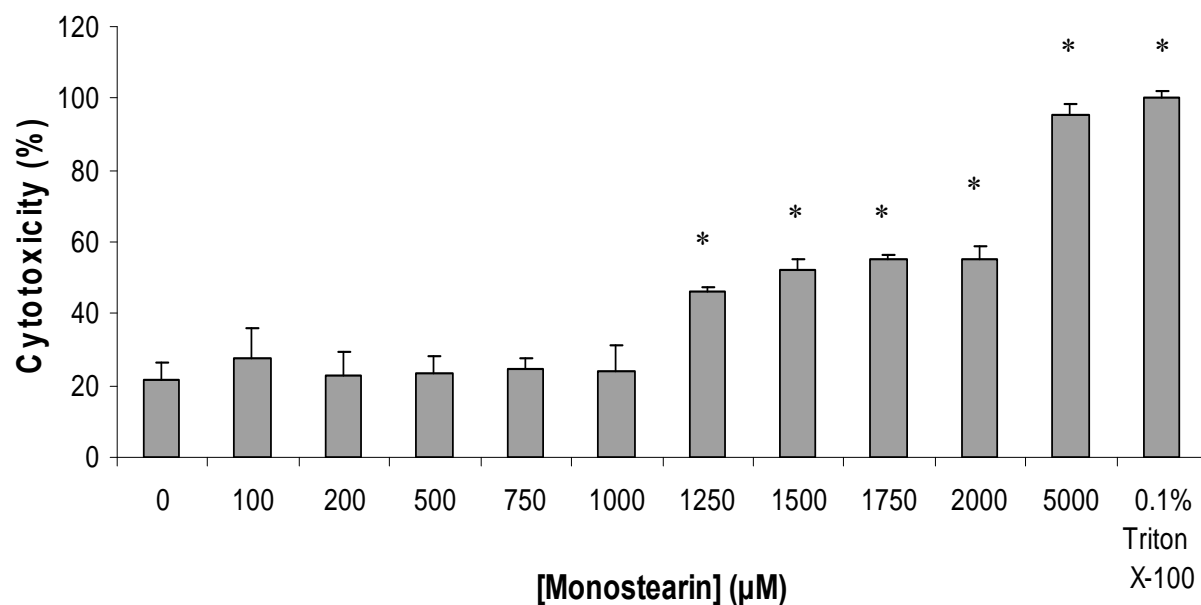
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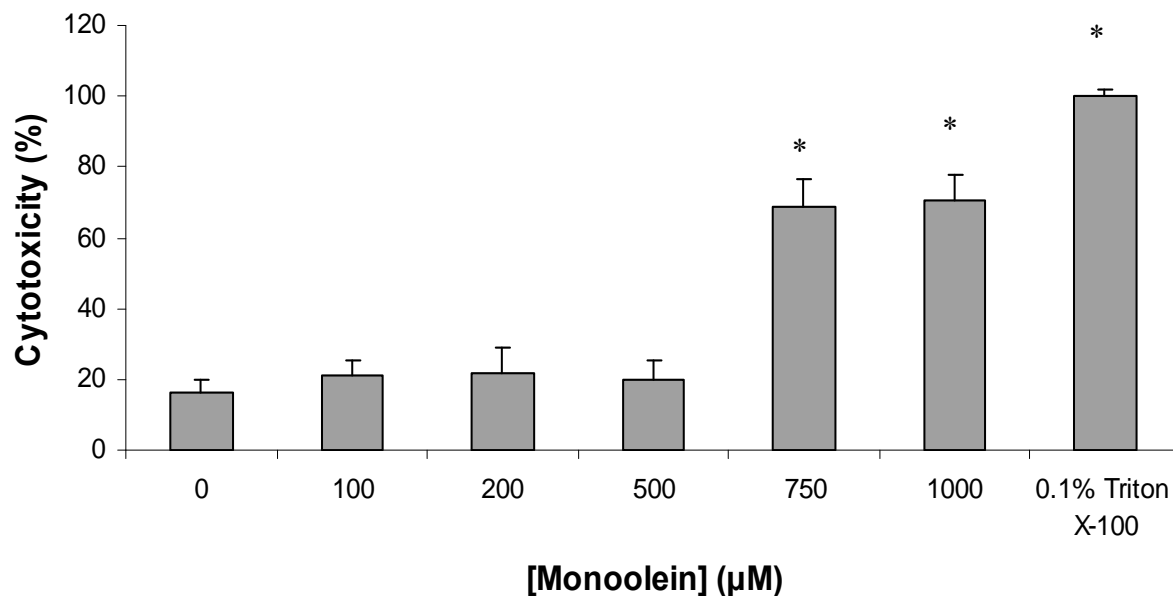
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Figure 1. Effects of Monoolein on Cell Viability and Cytotoxicity in Caco-2 cells. (A) The 24-hour toxicity profile of Caco-2 cells treated with 1-monoolein measured by LDH assay. The positive control group was the one treated with 0.1% Triton X-100. (n=6; *p<0.05, 1-monoolein treated groups vs. Triton X-100 treated group). Six replicates were used for per treatment group in each independent experiment, and the values were expressed as mean ± SD. (B) The 24-hour viability profile of Caco-2 cells treated with 1-monoolein measured by MTS assay. The control group was the one treated with media alone (n=6; *p<0.05, treatment group vs. the negative control group without treatment). Six replicates were used for per treatment group in each independent experiment, and the values were expressed as mean ± SD.
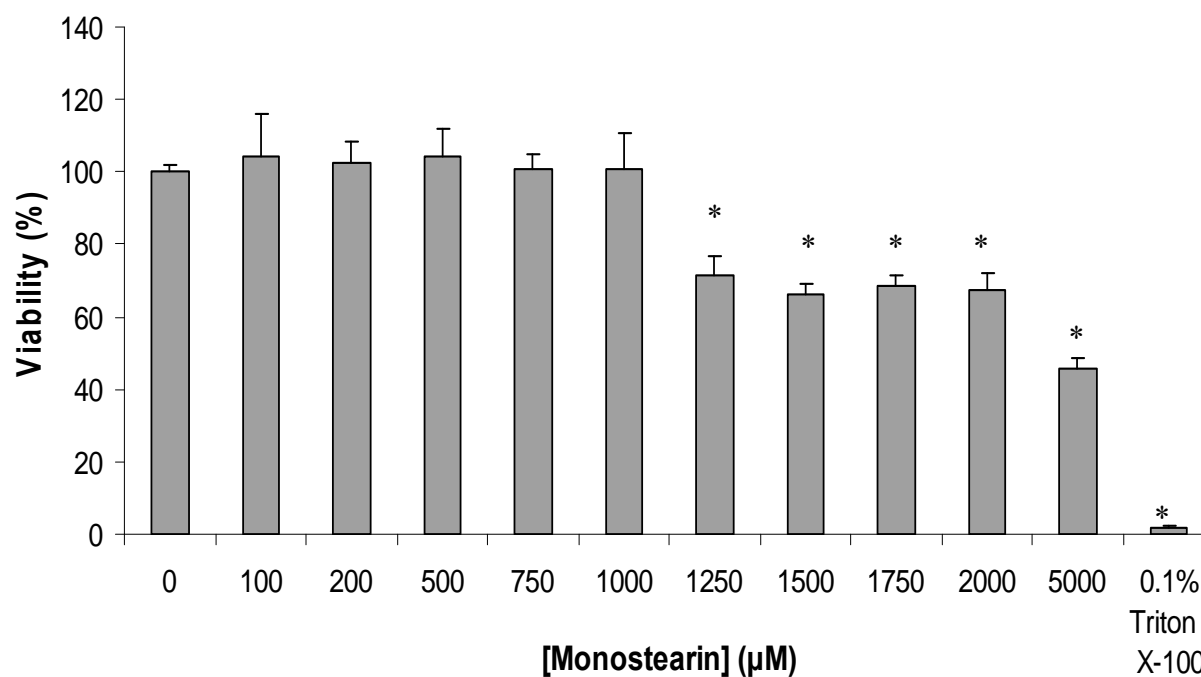
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Figure 2. Effects of Monostearin on Cell Viability and Cytotoxicity in Caco-2 cells. (A) The 24-hour toxicity profile of Caco-2 cells treated with 1-monostearin measured by LDH assay. The positive control group was the one treated with 0.1% Triton X-100. (n=6; *p<0.05, 1-monostearin treated groups vs. Triton X-100 treated group). Six replicates were used per treatment group in each independent experiment, and the values were expressed as mean ± SD. B) The 24-hour viability profile of Caco-2 cells treated with 1-monostearin measured by MTS assay. The control group was the one treated with media alone (n=6; *p<0.05, treatment group vs. the negative control group without treatment). Six replicates were used per treatment group in each independent experiment, and the values were expressed as mean ± SD.
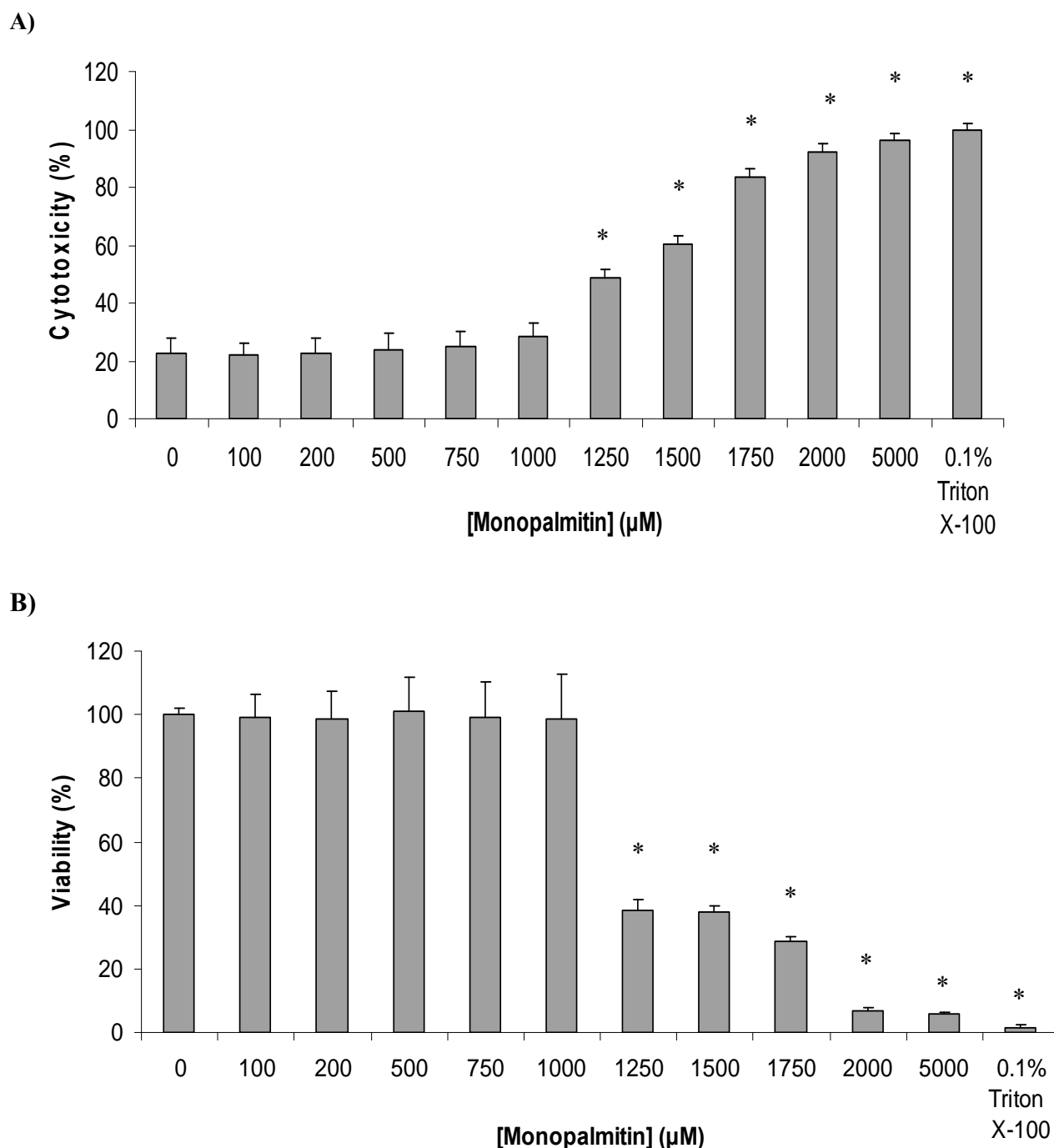
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Figure 3. Effects of Monopalmitin on Cell Viability and Cytotoxicity in Caco-2 cells. (A) The 24-hour toxicity profile of Caco-2 cells treated with 1-monopalmitin measured by LDH assay. The positive control group was the one treated with 0.1% Triton X-100. (n=6; *p<0.05, 1-monopalmitin treated groups vs. Triton X-100 treated group). Six replicates were used per treatment group in each independent experiment, and the values were expressed as mean ± SD. B) The 24-hour viability profile of Caco-2 cells treated with 1-monopalmitin measured by MTS assay. The control group was the one treated with media alone (n=6; *p<0.05, treatment group vs. the negative control group without treatment). Six replicates were used per treatment group in each independent experiment, and the values were expressed as mean ± SD.

Table 1. Accumulation of Rh123 in 24-hour Monoglycerides and MK-571 Treated Caco-2 Cells

Treatment Group	Relative Rh123 Accumulation (%)
Control	100 ± 9
500 µM 1-monoolein	122 ± 6 *
1000 µM 1-monopalmitin	119 ± 2 *
1000 µM 1-monostearin	123 ± 6 *
50 µM MK-571	181 ± 3 *

The accumulation was measured after 2-hour incubation of the cells with the substrate, Rh123. The absolute accumulation of Rh123 in each well was first normalized by using protein content divided by the amount of Rh123 in pmole/µg. The relative Rh123 accumulation with each treatment was then compared in % with the control group. The negative control group was treated with medium alone. MK-571, a specific inhibitor of MRP, was used as a positive control. Data expressed as mean ± standard deviation (n=5, *P<0.05, each monoglyceride group vs. the control; n=3, *P<0.05, MK-571 vs. the control). There were three replicates per treatment in each independent experiment.

Table 2. The effect of monoglycerides (mono-olein, -stearin, -palmitin) and MK571 on the Transepithelial Electrical Resistance (TEER).

Treatment Group	TEER A→B Before	($\Omega \cdot cm^2$) A→B After	B→A Before	B→A After
Control	386 ± 12	384 ± 10	390 ± 10	382 ± 14
500 µM monoolein	386 ± 21	380 ± 24	381 ± 14	374 ± 9
1000 µM monopalmitin	383 ± 10	378 ± 17	381 ± 23	379 ± 21
1000 µM monostearin	381 ± 16	382 ± 16	384 ± 15	382 ± 14
50 µM MK-571	380 ± 23	376 ± 22	384 ± 8	379 ± 8

TEER values of Caco-2 cells monolayer were measured before and after the bi-directional transport of $E_2 17\beta G$ in the A→B (apical-to-basolateral) and B→A (basolateral-to-apical) directions in the presence of medium only, 500 µM monoolein, 1000 µM monopalmitin, 1000 µM monostearin and 50 µM MK-571.

Thus, the apparent permeability of transporting $E_2 17\beta G$ in the effluxing direction (basolateral to apical) was greatly reduced in cells treated with either 500 µM monoolein, 1000 µM monostearin or 50 µM MK-571; in the meantime, the apparent permeability in the absorptive direction (apical to basolateral) had not changed for neither of these three treatments. The only exception observed for the transport of $E_2 17\beta G$ was the 1000 µM monopalmitin treated group which had shown an increased apparent permeability from the apical to basolateral direction, while the apparent permeability in the reverse direction remained.

In Table 3, the efflux ratios for each treatment were tabulated, and all treatment groups (500 µM monoolein, 1000 µM monostearin, 1000 µM monopalmitin and 50 µM MK-571) had shown statistically significant reductions in the efflux of $E_2 17\beta G$, a typical substrate for MRP2, compared to the control group. The rank order from the most

significant to the least reduction in efflux ratios was listed as following: 1000 µM monostearin > 500 µM monoolein > 1000 µM monopalmitin.

Effects of monoglycerides on MRP2 protein expression

The MRP2 protein expression in response to each monoglyceride treatment was evaluated by Western blotting technique. The relative MRP2 protein content was normalized using β-actin as the housekeeping protein. As shown in Figure 5 and 6, there were 35% and 19% down-regulation of the relative MRP2 protein expression in cells treated with 500 µM monoolein and 1000 µM monopalmitin, respectively, as compared to the control group (medium treated only). However, there had been no change in the relative MRP2 protein expression of cells treated with 1000 µM monostearin compared to the control group (Figure 7).
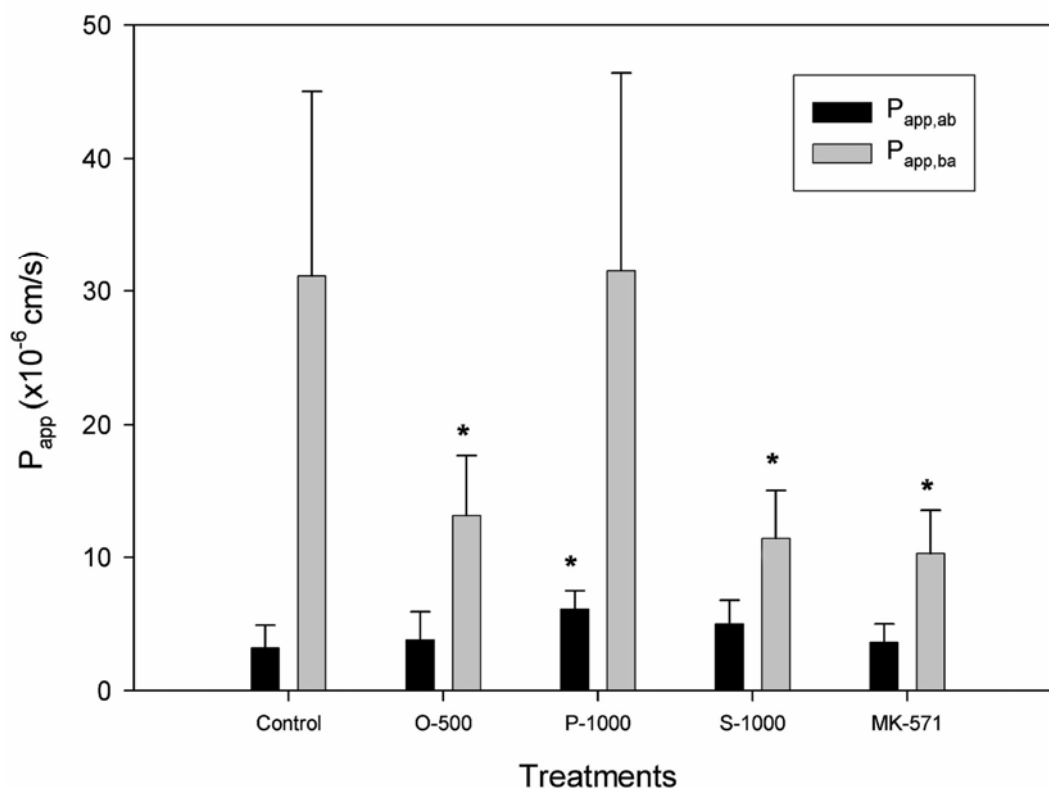
Figure 4. Apparent Permeability Coefficients (P_{app}) for Bi-directional transfer of $E_2 17\beta G$ across Monoglycerides Treated Caco-2 Cell Monolayers. The apparent permeability coefficient (Papp) was calculated with the following equation: Papp = $dQ/dt \cdot [1/(A \cdot C_0)]$, where C_0 is the initial $E_2 17\beta G$ concentration in the donor compartment at t=0, A is the surface area of the monolayer (in cm2), dQ/dt is the drug permeation rate. The control group was treated with medium alone which used as the negative control. MK-571 (50μM), a specific MRP inhibitor, was used as a positive control. O-500, P-1000, S-1000 were the three monoglyceride treatments which were 500μM monoolein, 1000μM monopalmitin and 1000μM monostearin, respectively. Data were expressed as mean ± standard deviation (n=6, *P<0.05, $P_{app, ab}$ for each lipid treatment vs. $P_{app, ab}$ for the control; $P_{app, ba}$ for each lipid treatment vs. $P_{app,ba}$ for the control).

Table 3. The Efflux Ratio of Caco-2 Cell Monolayer Treated with Monoglycerides and MK-571 Treated Caco-2 Cell Monolayer

Treatment Group	Efflux Ratio
Control	10.1 ± 1.0
500 μM monoolein	3.8 ± 1.0 ***
1000 μM monopalmitin	5.6 ± 3.2 ***
1000 μM monostearin	2.4 ± 0.5 ***
50 μM MK-571	3.4 ± 0.8 ***

The calculated efflux ratio was the quotient of $P_{app,ba}$ over $P_{app, ab}$, where $P_{app,ba}$ was apparent permeability coefficient from basolateral to apical side, $P_{app, ab}$ was of the apparent permeability coefficient from the apical to basolateral side. The control group was treated with medium alone which was used as the negative control. MK-571, a specific MRP inhibitor, was used as a positive control. Data were expressed as mean ± standard deviation (n=6, ***P<0.001, each lipid treatment group vs. the control).
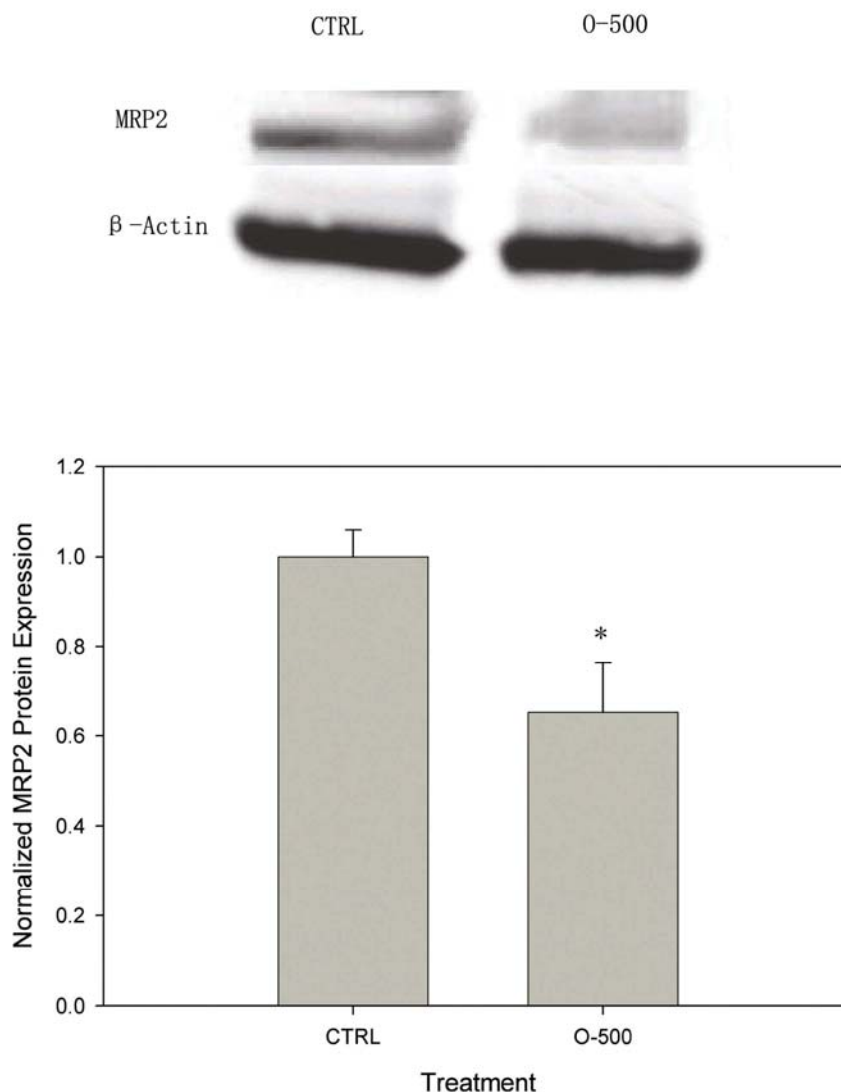
Figure 5. The 24-hour Effect of 1-Monoolein on the MRP2 protein expression in Caco-2 cells by Western blotting. The primary antibodies used to probe against MRP2 and β-actin were mouse anti-human MRP2 (M$_2$III-6) and goat anti-human β-actin (I-19), respectively. The molecular weight of MRP2 is around 200kDa; whereas the molecular weight of β-actin is around 42kDa. The insert shown was an image of a representative blot. Each bar value was presented as mean ± standard deviation of MRP2 protein expression normalized by β-actin expression. MRP2 protein expression in treatment groups were compared with the untreated control group. (n=5, *P<0.05).

DISCUSSION

In this study, the effects of monoglycerides (monopalmitin, monoolein and monostearin) on Rh123 accumulation, E$_2$17βG bi-directional transport and MRP2 protein expression were investigated *in vitro*.

For each monoglyceride treatment, the non-cytotoxic concentration range was first determined before proceeding to any transporter activity or protein expression studies to eliminate factors, such as cell necrosis and apoptosis, which could alter Caco-2 cells' normal physiology (26, 27).
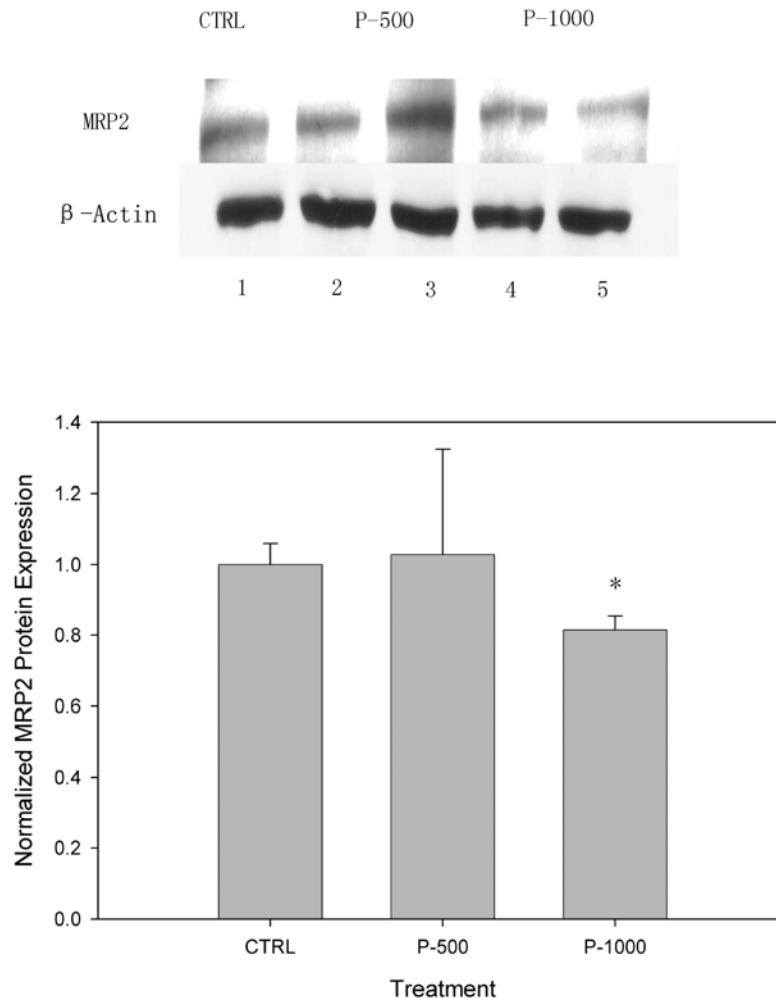
Figure 6. The 24-hour Effect of 1-Monopalmitin on the MRP2 protein expression in Caco-2 cells by Western blotting. The primary antibodies used to probe against MRP2 and β-actin were mouse anti-human MRP2 (M₂III-6) and goat anti-human β-actin (I-19), respectively. The molecular weight of MRP2 is around 200kDa; whereas the molecular weight of β-actin is around 42kDa. The insert shown was an image of a representative blot. Lane 1 was the control group; lane 2 and 3 were the cells treated 500μM of monopalmitin; lane 4 and 5 were the cells treated with 1000μM of monopalmitin. Each bar value was presented as mean ± standard deviation of MRP2 protein expression normalized by β-actin expressionMRP2 protein expression in treatment groups were compared with the untreated control group. (n=5, *P<0.05).

The non-cytotoxic ranges obtained from both MTS and LDH studies for each type of monoglyceride were in agreement with each other which ensured the accuracy of the results, and the 24-hour non-cytotoxic ranges of 1-monoolein, 1-monostearin and 1-monopalmitin in Caco-2 cells were equal or less than 500 μM, 1000 μM and 1000 μM, respectively (Figure 1-3). The toxicity of MK-571 at 50 μM was also assessed using MTS and LDH assays (data not shown), and there was no increased toxicity associated with this treatment compared to the control group after 24 hours.
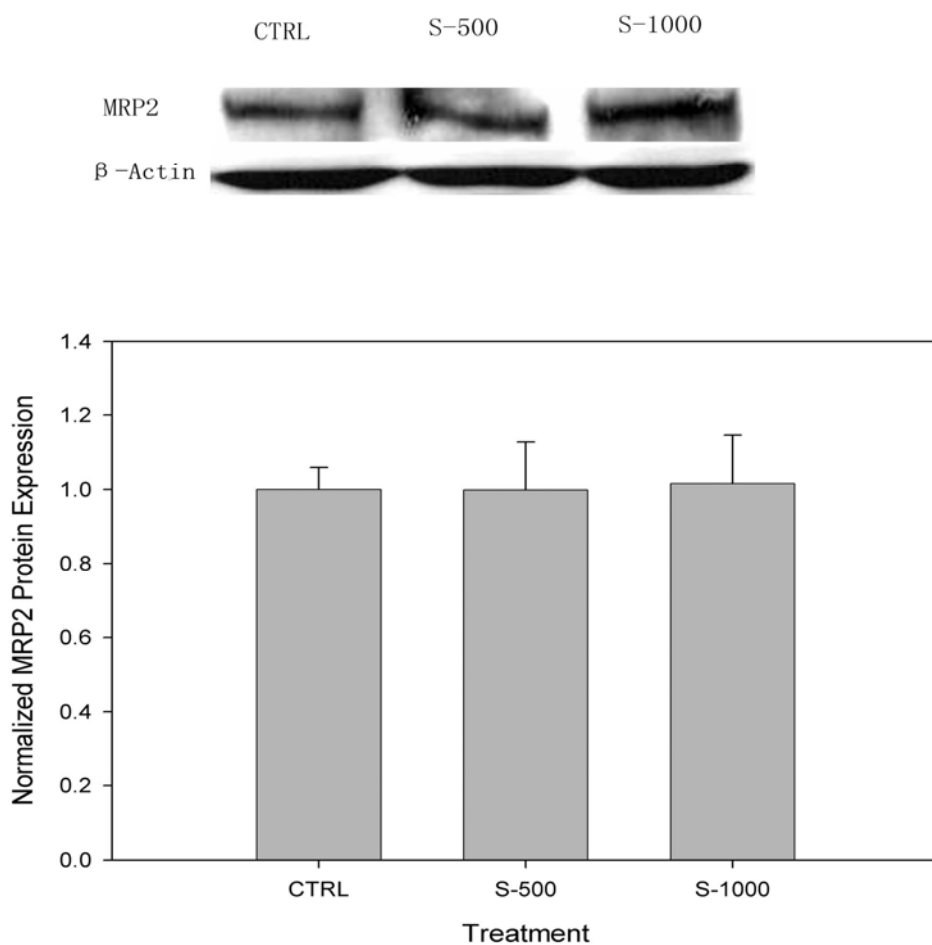
Figure 7. The 24-hour Effect of 1-Monostearin on the MRP2 protein expression in Caco-2 cells by Western blotting. The primary antibodies used to probe against MRP2 and β-actin were mouse anti-human MRP2 (M₂III-6) and goat anti-human β-actin (I-19), respectively. The molecular weight of MRP2 is around 200 kDa; whereas the molecular weight of β-actin is around 42 kDa. The insert shown was an image of a representative blot. Each bar value was presented as mean ± standard deviation of MRP2 protein expression normalized by β-actin expression. Lane 1 was the control group; lane 2 was the cells treated 500μM of monostearin; lane 3 was the cells treated with 1000μM of monostearin. MRP2 protein expression in treatment groups were compared with the untreated control group. (n=5).

Both accumulation and transport studies were conducted to assess the activity or vectorial transport of ABC efflux transporters, in particularly MRP2, in respond to the monoglyceride treatments in Caco-2 cells. For the accumulation study, Rh123 was used to monitor the changes of the functional activities of Pgp and MRPs (28-31). Rh123 is a cationic fluorescent dye that accumulates selectively in the mitochondria of eukaryotic cells, and it could be transported by Pgp and MRPs but not BCRP (32, 33). Although studies had shown that both Pgp and MRP could transport Rh123 via direct bindings (34, 35), a recent study had indicated that MRPs contributed to a lesser degree on Rh123 efflux compared to Pgp (37). MK-571, a MRP1 and MRP2 inhibitor was used in this study as a positive control. There was about 20% increase in Rh123 accumulation for each monoglyceride

treatment (Table 1) which implied that efflux activities of Pgp and MRPs were affected.

Vectorial transport is an asymmetrical transport across a monolayer of polarized cells, and it plays a major role in the intestinal absorption of drugs from the viewpoint of drug absorption and disposition (1). After screened the effects of these three monoglycerides on the functional activities of Pgp and MRPs using Rh123, we further conducted the bi-directional transport studies using $E_2 17\beta G$ as the substrate to assess the transport activity of MRP2. $E_2 17\beta G$ is a well known substrate for the study of MRPs (such as MRP1, MRP2, MRP3, MRP4 and MRP7); in recent years, studies had shown that it could be transported by Pgp, BCRP and OATP as well. Regardless, $E_2 17\beta G$ has a significantly higher affinity toward MRP1 and MRP2 compared to other transporters (38-41). Despite the high affinity of $E_2 17\beta G$ with MRP1, there is a considerably lower expression level of MRP1 compared to MRP2 in Caco-2 cells. Hence, $E_2 17\beta G$ could be considered as a relatively specific MRP2 substrate (42, 43) in this study. One major advantage of the bi-directional transport study over the accumulation study was the polarization and tight junction formation of these Caco-2 cells, which provided the best possible *in vitro* simulation of intestinal epithelial cells. The unchanged TEER values before and after each experiment (Table 2) ensured that the observed reduced transport activity was not caused by cell damage, death or monolayer leakage.

In the $E_2 17\beta G$ bi-directional transport study, it had shown that all three monoglycerides treatments lowered their efflux ratios compared to the control (Table 3). However, efflux ratio could not indicate which side of the cell membrane was affected by the treatments. In Figure 4, Caco-2 cells treated with monopalmitin increased the absorptive (the apical to basolateral) transport while maintained its efflux transport (basolateral to apical) compared to the control; on the contrary, cells treated with monoolein and monostearin decreased their efflux transport without changing their absorptive transport (apical to basolateral) compared to the control. Therefore, these suggested that there may not be a generalized mechanism applying to all monoglycerides on how they affecting transporters' activities. The unchanged basolateral to apical flux and enhanced apical to basolateral flux of $E_2 17\beta G$ in monopalmitin treated

cells suggested that monopalmitin may not affect the functional activities of efflux transporter like MRP2 on the apical side of the monolayer, instead it may affect apical to basolateral transport of $E_2 17\beta G$ by either enhancing efflux activity of some efflux transporters on the basolateral side (like MRP1) or stimulating uptake activity of absorptive transporters on the apical side (like OATP) in Caco-2 cells. For monostearin and monoolein treated cells, the basolateral to apical transports of $E_2 17\beta G$ were reduced while apical to basolateral transports remained unchanged compared to control. Thus, the decrease in efflux transport of $E_2 17\beta G$ in cells treated with monostearin and monoolein might be partially accounted by the attenuation of the functional activity of MRP2. Furthermore, it was noticed that monopalmitin has a 16-carbon saturated acyl chain attached to the glycerol backbone; whereas monostearin and monoolein have one saturated and another unsaturated 18-carbon acyl chains, respectively, attached to the glycerol backbone. It was speculated that the chain length of the monoglyceride might play a role in selectively affecting the functional activities of enterocyte based efflux/uptake transporters.

We further investigated the effect of monoglycerides on alteration of MRP2 protein expression. Our data had indicated monopalmitin and monoolein treated cells had shown decreased MRP2 protein expression while monostearin had shown no change in MRP2 protein expression in Caco-2 cells (Figure 5-7). Considering monostearin had shown the greatest reduction in transport activity yet demonstrate no alteration in MRP2 protein expression level, it was speculated that monostearin might accumulate in the cytosolic compartment of Caco-2 cells which directly inhibit the MRP2-mediated transport or it might disrupt membrane fluidity or suppress ATPase activity of MRP2 (Figure 7). Monopalmitin treated cells had shown a lesser degree of MRP2 down-regulation at a much higher concentration compared to the monoolein treated cells (Figure 6). Considering it mostly affected the apical to basolateral transport, monopalmitin may not have a significant impact on MRP2 activity (Figure 4). For monoolein treated cells, there was a significant reduction in MRP2 protein expression compared to the control (Figure 5). Thus, the down-regulation of MRP2 protein expression by monoolein treatment might be partially accounted for the accumulation of Rh123

and reduction of $E_2 17\beta G$ efflux. This was the first study to our knowledge had shown a specific monoglyceride could reduce the activity of a major enterocyte efflux transporter, namely MRP2, by down-regulating its protein expression in Caco-2 cells.

In conclusion, these findings suggested that monoolein, monostearin and monopalmitin could attenuate the efflux activities partially by modulation of enterocyte-based efflux transporters, such as MRP2, in Caco-2 cells. Furthermore, the reduction of MRP2 efflux activity in monoolein treated cells could be partially accounted by the down-regulation of MRP2 protein expression. Some preliminary results from our lab had pointed out that monoolein could also down-regulate Pgp protein expression (data now shown). Thus, it was speculated that monoolein might have some indirect interplays with nuclear receptors at the transcriptional level to exert a non-specific downregulation on ABC efflux transporters. Recently, a number of nuclear receptors, such as PXR, had shown to exert global transcriptional control over MRP2 and Pgp in response to rifampin treatment (44, 45). For future studies, mRNA levels of MRP2 and other major efflux transporters as well as some nuclear receptors could be determined to assess the effects of monoglycerides at the transcriptional level.

Treatment groups used in this study were incubated with these three monoglyceride for 24 hours. Since many lipid-formulated oral drugs were administered on a relatively long-term basis, the effect of longer exposure of these monoglycerides in Caco-2 cells could be explored in the future. Some preliminary studies of cells treated with monoglycerides for 48-hour and 72-hour had already shown a greater percentage of Rh123 accumulation than they were when treated for 24-hour in Caco-2 cells (data not shown). Moreover, the small intestine is a very dynamic environment; in contrast, Caco-2 cell model is a relatively static model consisting of one single cell type with a thicker unstirred water layer than *in vivo* situation (46). Thus, the exact concentrations of monoglycerides that the intestinal cells might be exposed to in an *in vivo* situation were difficult to assess and correlate with the concentrations used in the cell studies. Ultimately, the effects of these monoglycerides on ABC efflux transporters in enterocytes need to be determined in a more physiologically relevant system, for instance, an animal model. If lipids or lipid excipients indeed influence efflux transporters' expressions, pharmaceutical industry and the regulatory agencies need to address the concerns in drug formulation development.

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