Pluronic L81 enhances triacylglycerol accumulation in the cytosol and inhibits chylomicron secretion

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Abstract **Pluronic L81 (PL81) inhibits fat absorption, and other Pluronic copolymers help overcome drug resistance in cancer cells. To understand how PL81 acts, we synthesized a radiolabeled analog, [^{14}C]PL81, and showed that it was structurally similar to PL81 based on ^{1}H NMR as well as mass spectrometric analysis. [^{14}C]PL81 inhibited the secretion of chylomicrons (CMs), lipoproteins essential for fat absorption, by differentiated Caco-2 cells similar to PL81. Moreover, PL81 competed with the cellular uptake of [^{14}C]PL81. Thus, [^{14}C]PL81 and PL81 behave similarly in these physiologic assays. Uptake of [^{14}C]PL81 by Caco-2 cells was concentration-, time-, and temperature-dependent and occurred mainly from the apical side. Intracellularly, it was assimilated in the cytosol. Cells excreted PL81 toward the apical side via a pathway partially sensitive to verapamil. Small amounts were secreted toward the basolateral side unassociated with CM, and this secretion was unaffected by the inhibition of CM assembly. Nonetheless, PL81 significantly inhibited the secretion of triacylglycerols (TGs) and phospholipids as part of CM. PL81-treated cells showed decreased activity of microsomal triglyceride transfer protein and accumulated more TGs, but not phospholipids, in their cytosol. We propose that Pluronic copolymers act by interfering with the export of molecules from the cytosol. They inhibit fat absorption by decreasing TG transport to the endoplasmic reticulum and increase drug efficacy against cancer cells by competing for their excretion.** —Fatma, S., R. Yakubov, K. Anwar, and M. M. Hussain. **Pluronic L81 enhances triacylglycerol accumulation in the cytosol and inhibits chylomicron secretion. *J. Lipid Res.* 2006.** 47: **2422–2432.**

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Pluronic® (also known as poloxamers) surfactants are synthetic copolymers of ethylene oxide and propylene oxide. They are synthesized by the controlled addition of propylene oxide to the two hydroxyl groups of propylene glycol (1). These and other nonionic surfactants are widely used as defoaming and antifoaming agents in dishwashing, antifreeze, cutting, and grinding fluids, water treatment, fermentation, paper processing, etc. They are now being evaluated as vehicles for drug delivery (2), for overcoming drug resistance in cancer cells (3), and to increase the transport of drugs across the blood-brain barrier (4).

Pluronic L81 (PL81) contains 10% hydrophilic and 90% hydrophobic residues. The observations that PL81 decreased plasma cholesterol and triacylglycerols (TGs), liver cholesterol, and body weight in rats fed a high-fat and high-cholesterol diet generated significant interest in this compound (5). Subsequently, chronic PL81 feeding was shown to decrease the secretion of TG and cholesterol into the lymph without affecting the digestion, absorption, and reesterification of TG and cholesterol during fat infusion (6). PL81 infusion has been shown to increase cellular lipid droplets (7, 8). Negative staining of the secreted particles showed that the lymph obtained from normal and PL81-infused rats contained chylomicron (CM)- and very low density lipoprotein-sized particles, respectively. Based on these studies, it was proposed that intestinal cells have two separate pathways for the assembly of these lipoproteins and that PL81 is a specific inhibitor for the assembly and secretion of CM (9). We showed that PL81 inhibited CM secretion but did not affect the secretion of smaller lipoproteins by Caco-2 cells (10). Moreover, it inhibited the secretion of retinyl esters that are transported mainly on CM (11). We proposed that PL81 might interfere with the formation of lipid droplets, which are believed to coalesce with primordial lipoproteins, and thereby inhibit the biosynthesis of larger lipoproteins without affecting the assembly and secretion of smaller particles (12–14).

Recent studies to understand the mode of inhibition of CM assembly and secretion by PL81 have focused on the air/water interfacial properties of the surfactant. Weinberg et al. (15) observed that PL81 binds to hydrophobic surfaces very rapidly and reduces interfacial tension, and

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Abbreviations: apoB, apolipoprotein B; CM, chylomicron; ER, endoplasmic reticulum; MTP, microsomal triglyceride transfer protein; OA, oleic acid; PL81, Pluronic L81; TC, taurocholate; TG, triacylglycerol.

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they proposed that PL81 may disrupt CM assembly by destabilizing the surface and promoting the formation of larger intracellular droplets. In fact, Morita et al. (16) showed that PL81 binds to triglyceride/phospholipid emulsions and to low density lipoproteins and induces particle aggregation/fusion as well as a conformational change in apolipoprotein B (apoB). They proposed that PL81 might inhibit CM secretion by causing excess core expansion, leading to poor secretion of these larger particles. Using [^{14}C]PL81, we studied its uptake, intracellular assimilation, secretion, and mode of action. Our studies indicate that PL81 might specifically inhibit the transport of triglycerides from the cytosol to the endoplasmic reticulum (ER) and interfere with CM assembly.

EXPERIMENTAL PROCEDURES

Materials

Oleic acid (OA), taurocholate (TC), and other chemicals were from Sigma. The microsomal triglyceride transfer protein (MTP) inhibitor BMS197636 was a gift from Dr. David Gordon of Bristol-Myers Squibb Co. To prepare OA/TC (20 × 1.6:0.5 mM) stocks, 97.4 mg of OA was added to 10 ml of 10 mM TC solution, mixed by gentle swirling, and incubated at 37°C until a clear solution was achieved.

Synthesis of radiolabeled PL81

PL81 was from BASF Corp. [^{14}C]PL81 (31.48 mCi/g, 0.283 mCi/ml) was synthesized by NEN Life Science Products by the controlled addition of [^{14}C]ethylene oxide to unlabeled PL81. PL81 was ionized using sodium hydride and reacted with [^{14}C]ethylene oxide (two equivalents by weight), and the reaction was stopped by the addition of diluted acetic acid. Volatile compounds were removed by vacuum. Because PL81 is insoluble in water, it was diluted in ethanol to obtain a concentration of 100 μg/ml. The amount of ethanol added to cells did not exceed 0.01%.

Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in DMEM containing high glucose, 10% FBS, and a 1% antibiotic-antimycotic mixture. From a 70–80% confluent 75 mm^2 flask, cells were plated on 24 mm Transwells (3 μM pore size; Corning Costar Corp.) and allowed to differentiate into enterocyte-like cells (10, 11, 17, 18).

Uptake and secretion of PL81 by Caco-2 cells

For uptake studies, differentiated Caco-2 cells were washed twice with serum-free DMEM and incubated in the same medium with 1.6 mM OA, 0.5 mM TC, and [^{14}C]PL81 for the indicated time periods. Serum-free DMEM containing 0.1% BSA was added to the basal side. After incubation, the apical and basal conditioned media were collected and used to measure apoB and [^{14}C]PL81. To extract lipids, cells were washed twice with chilled PBS and incubated overnight with 2 ml of isopropanol at 4°C. Isopropanol was collected from each well and evaporated, and radioactivity was measured in a liquid scintillation counter. For excretion studies, Caco-2 cells were incubated with [^{14}C]PL81 for 18 h, washed three times with PBS at room temperature, and supplemented with medium containing 10% serum or 10% serum and OA+TC. At different times, [^{14}C]PL81 was quantified in both the apical and the basolateral media. For secretion studies, cells were labeled with [^{14}C]PL81 for 8 h, washed, and incubated with medium containing TC or OA+TC for 18 h.

Subcellular fractionation

Differentiated Caco-2 cells were washed twice with PBS at room temperature, and 0.5 ml of homogenizing medium (0.25 M sucrose, 1 mM EDTA, and 20 mM HEPES-KOH, pH 7.4) was added to each well and incubated for 1 h at 4°C. The cells were scraped off and ruptured by passing at least 10 times through a 25 gauge needle attached to a 5 ml syringe. Cellular homogenates were subjected to differential centrifugation to obtain various subcellular fractions as described previously (19). Briefly, the cell lysate was centrifuged (500 *g*, 10 min, 4°C) to collect the nuclear pellet. The supernatant was centrifuged (3,000 *g*, 10 min, 4°C), and the mitochondrial pellet was obtained. The resulting supernatant was centrifuged (100,000 *g*, 1 h, 4°C) again to harvest microsomes. This supernatant consisted of the cytosolic fraction. Various pellets were resuspended in 2.5 ml of the homogenizing medium by passing through a 25 gauge needle 15 times.

Density gradient ultracentrifugation

The conditioned media obtained from the basolateral side were subjected to density gradient ultracentrifugation to separate various lipoprotein fractions, as described previously (10, 11, 18, 19). Briefly, to the conditioned media (4 ml) was added KBr (0.57 g) to obtain a density of 1.10 g/ml. The media were then overlaid with 3 ml each of 1.063 and 1.019 g/ml, and 2 ml of 1.006 g/ml, density solutions using the Auto Density flow (Buchler Instrument) and subjected to sequential ultracentrifugation. To obtain large CMs (CM_L; $S_f > 400$), samples were ultracentrifuged (SW 41 rotor, 33 min, 40,000 rpm, 15°C) and the top 1 ml was collected. The samples in the ultracentrifuge were then overlaid with 1 ml of d = 1.006 g/ml solution. CM_S (S_f = 60–400; 1 ml) was obtained from the top after a second ultracentrifugation (3 h and 28 min, 40,000 rpm, 15°C). The top 1 ml was again replenished with d = 1.006 g/ml solution and ultracentrifuged for 17 h. The first 1 ml fraction collected from the top represented VLDL (CM_{VLDL}, d < 1.006 g/ml, S_f = 20–60). The rest of the gradient was fractionated into seven additional 1.5 ml fractions. Fractions 2–4 and 5–7 were considered intermediate/low density lipoprotein (CM_{IDL}, CM_{LDL}; d = 1.02–1.063 g/ml) and high density lipoprotein (I-HDL; 1.063–1.1 g/ml), respectively.

Determination of MTP activity in cells

Differentiated Caco-2 cells were washed with PBS and then incubated at room temperature for 2 min in a hypotonic buffer (1 mM Tris-Cl, pH 7.4, 1 mM $MgCl_2$, and 1 mM EGTA) (20, 21). The buffer was aspirated, cells were scraped in 750 μl of ice-cold hypotonic buffer containing protease inhibitors and homogenized (20 passages through a 21 gauge needle), lysates were centrifuged (50,000 rpm, 4°C, 1 h; SW55Ti rotor), and supernatants were used for lipid transfer assays (20, 21) as well as protein determination (22).

RESULTS

Structural similarities between unlabeled and radiolabeled PL81

The radiolabeled polymer had one [^{14}C]ethylene oxide moiety at both termini (compare **Fig. 1A, B**). The ^{1}H NMR

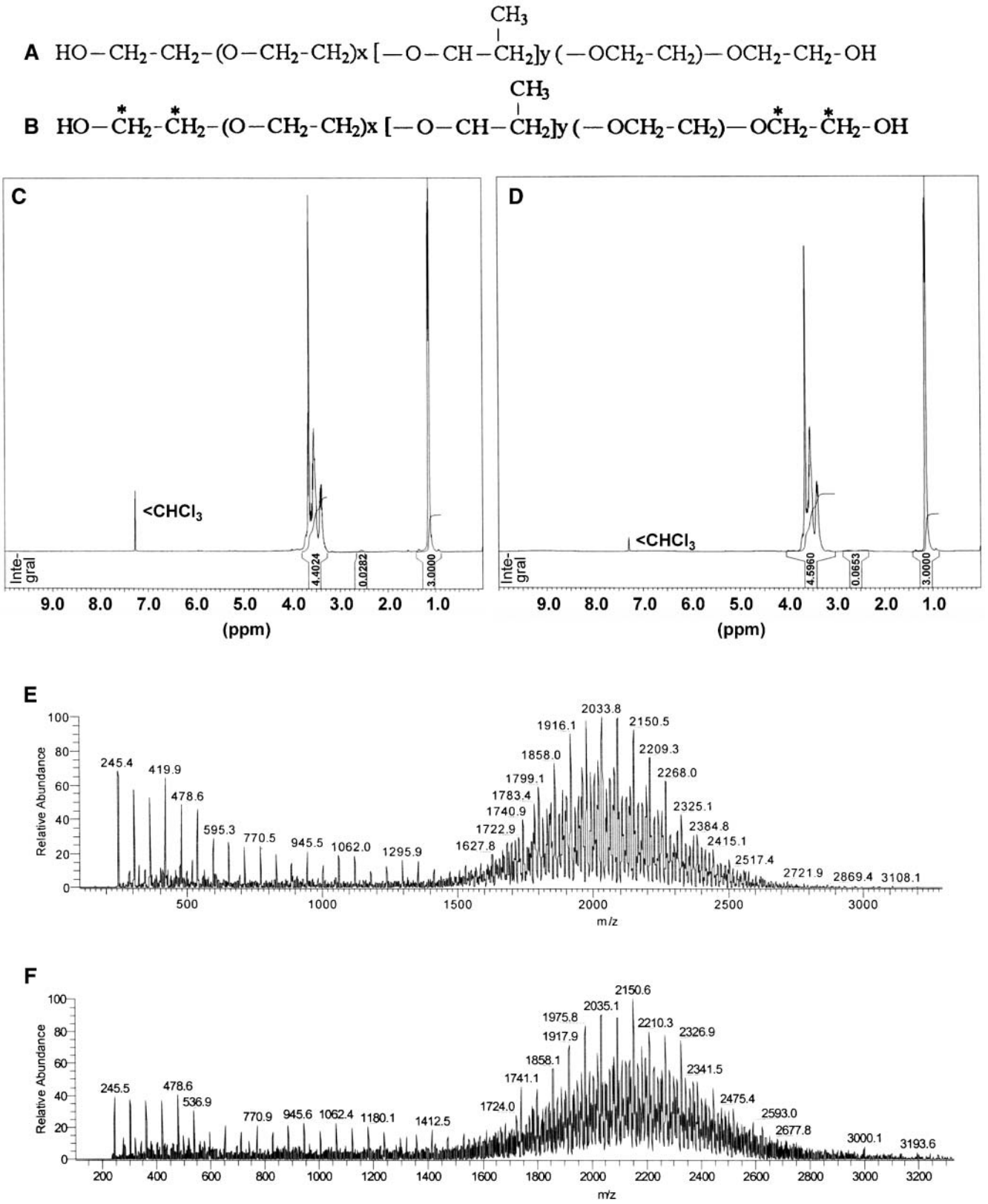

Fig. 1. Physicochemical characterization of unlabeled and radiolabeled Pluronic L81 (PL81). A, B: Chemical structure of unlabeled and radiolabeled PL81, respectively. *C denotes the position of a ^{14}C-labeled carbon atom. C, D: ^{1}H NMR spectra of unlabeled and labeled PL81, respectively. E, F: Mass spectrometric analyses of unlabeled and labeled compounds, respectively, after positive atmospheric pressure chemical ionization.

spectra of both PL81 and [^{14}C]PL81 showed two peaks representing two types of equivalent hydrogen (Fig. 1C, D). The peaks at 1.0–1.3 and 3.4–3.6 most likely represent hydrogen atoms in methyl groups and methylene groups attached to oxygen, respectively. Mass spectroscopic analysis of [^{14}C]PL81 and PL81 showed a similar distribution of ions (Fig. 1E, F). These studies indicate that [^{14}C]PL81 is structurally similar to unlabeled PL81.

Similar physiologic behavior of [^{14}C]PL81 and PL81

We determined whether [^{14}C]PL81 behaves similar to PL81 using two physiologic assays: competition for cellular

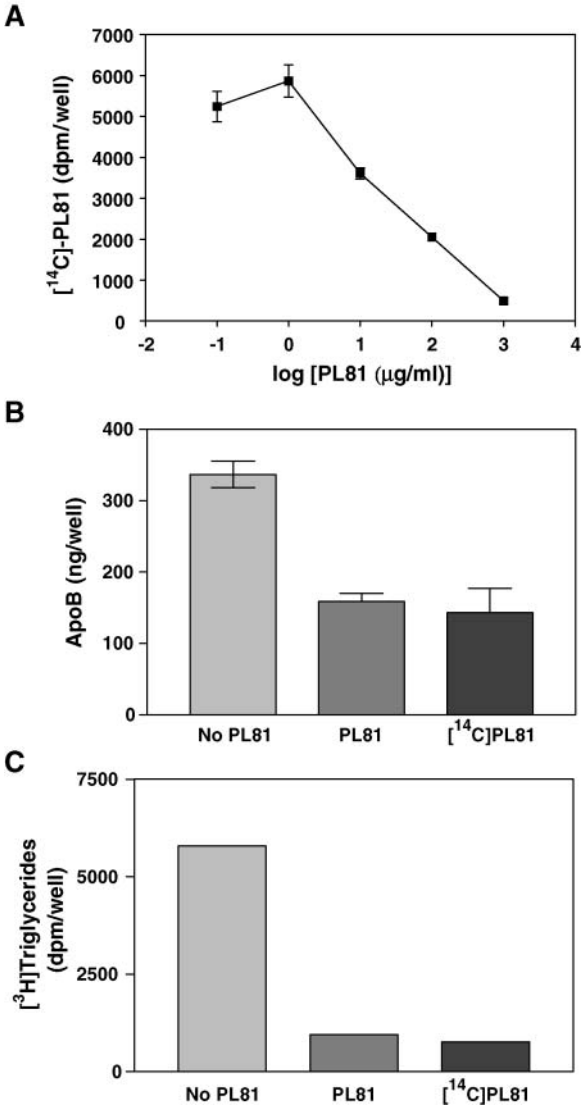

Fig. 2. [^{14}C]PL81 behaves similar to PL81 in physiological assays. A: Competition between unlabeled and labeled PL81. Differentiated Caco-2 cells were incubated with 2 μg/ml [^{14}C]PL81 along with increasing concentrations of unlabeled PL81 (0.1–1,000 μg/ml). After 8 h, cells were washed and radioactivity in cells was determined. B, C: Inhibition of chylomicron (CM) secretion by radiolabeled and unlabeled PL81. Differentiated Caco-2 cells received media, toward the apical side, containing oleic acid plus taurocholate (OA+TC) and [^{3}H]glycerol. In addition, these cells got either no PL81 or 6 μg/ml of either PL81 or [^{14}C]PL81. The basolateral side received serum-free medium containing 0.1% BSA. After 18 h, basolateral media were subjected to density gradient ultracentrifugation as described in Experimental Procedures. The top two fractions (CM_L and CM_S) were combined and used to quantify apolipoprotein B (apoB; B) by ELISA and to measure triacylglycerol (TG; C). The data are presented as means ± SD (n = 3).

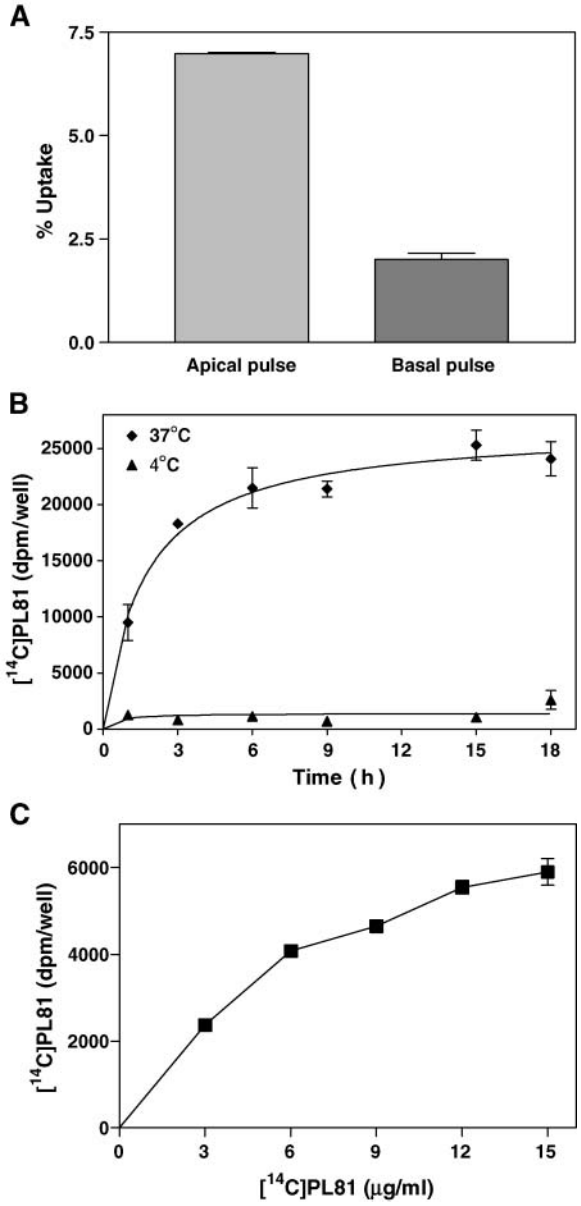

Fig. 3. Uptake of [^{14}C]PL81 by Caco-2 cells. A: Uptake from the apical and basolateral sides. Caco-2 cells were supplemented with [^{14}C]PL81 (6 μg/ml) toward the apical or basolateral side. After 4 h, amounts of radioactivity in cells were measured and the percentage of the radioactivity taken up by cells was calculated. B: Effect of time and temperature on the uptake of PL81 by differentiated Caco-2 cells. Cells were incubated in triplicate with 9 μg/ml [^{14}C]PL81 at 37°C or 4°C for the indicated times as described in Experimental Procedures, and the amount of radioactivity taken up by cells was measured. C: Concentration-dependent PL81 uptake. Cells were incubated in triplicate with increasing concentrations of [^{14}C]PL81 in the presence of OA and TC at 37°C for 4 h. Radioactivity present in the cells was quantified. The data are presented as means ± SD (n = 3).

uptake and inhibition of CM secretion. For competition studies, Caco-2 cells received a fixed concentration of [^{14}C]PL81 along with increasing concentrations of PL81 (**Fig. 2A**). Increasing concentrations of PL81 inhibited the cellular uptake of [^{14}C]PL81 in a dose-dependent manner. A 50% inhibition was achieved at 19 μg/ml PL81, indicating effective competition between these compounds.

The signature activity of PL81 is its ability to inhibit CM secretion. To determine whether [^{14}C]PL81 would inhibit CM secretion, differentiated Caco-2 cells were incubated with either PL81 or [^{14}C]PL81 in the presence of OA/TC to induce CM assembly and secretion (10, 11, 18, 19). The effect of PL81 was studied by measuring the amounts of apoB and TG secreted as CM. ApoB secretion by Caco-2 cells was reduced by 57% and 53% when incubated with PL81 and [^{14}C]PL81, respectively (Fig. 2B). Both PL81 and [^{14}C]PL81 inhibited TG secretion by ~85% (Fig. 2C). These studies indicate that [^{14}C]PL81 and PL81 behave similarly in these physiologic assays.

Uptake of [^{14}C]PL81 by Caco-2 cells

Differentiated Caco-2 cells received equal amounts of [^{14}C]PL81 from either the apical or the basolateral side (**Fig. 3A**). The amount of [^{14}C]PL81 taken up by cells was ~3-fold higher when supplemented from the apical side compared with cells that received it from the basolateral side. Next, the effect of time and temperature on PL81 uptake was studied. There was an initial time-dependent increase in the uptake of PL81 that tended to saturate at later time points (Fig. 3B, 37°C). On the other hand, incubation of cells at 4°C resulted in no cellular uptake. Subsequently, concentration-dependent uptake was studied (Fig. 3C). At low concentrations, PL81 uptake was linear and started to saturate at higher concentrations. These studies show that PL81 uptake is a time-, temperature-, and concentration-dependent process.

Intracellular distribution of PL81

Cells were incubated with [^{14}C]PL81 under two conditions that support (**Fig. 4A**, OA+TC) or do not support (Fig. 4A, TC) CM assembly for 18 h, and various subcellular organelles were isolated by differential ultracentrifugation. Under both conditions, the majority of [^{14}C]PL81 was in the cytosol (Fig. 4A) instead of the expected microsomes. Because this was unexpected, we considered the possibility that cytosol was contaminated with microsomes and examined the presence of calnexin, a microsomal marker, in various subcellular fractions (Fig. 4B). As expected, calnexin was present mainly in homogenates and microsomes. However, small amounts of calnexin were present in the nucleus (11%), mitochondria (14%), and cytosol (15%), indicating a small contamination of microsomes in other subcellular fractions. This small contamination is unlikely to explain the amounts of PL81 found in the cytosol (Fig. 4A). We also studied the separation of cytosol from other organelles by studying the distribution of GAPDH (Fig. 4B). GAPDH was present mainly in the cytosol. It should be noted that similar studies with vitamin E showed that α-tocopherol accumulates mainly in microsomes (19). Thus, we conclude that PL81 accumulates primarily in the cytosol.

Excretion of PL81 by Caco-2 cells

Differentiated Caco-2 cells were incubated overnight with [^{14}C]PL81, washed, and supplemented with fresh media on both the apical and basolateral sides (**Fig. 5A, B**). The amounts of PL81 excreted toward the apical side were significantly higher (~3- to 4-fold) than those secreted toward the basolateral cells (Fig. 5A). Consideration was given to the possibility that higher excretion toward the apical side was because cells received [^{14}C]PL81 from the apical side. To test this possibility, cells were supplemented

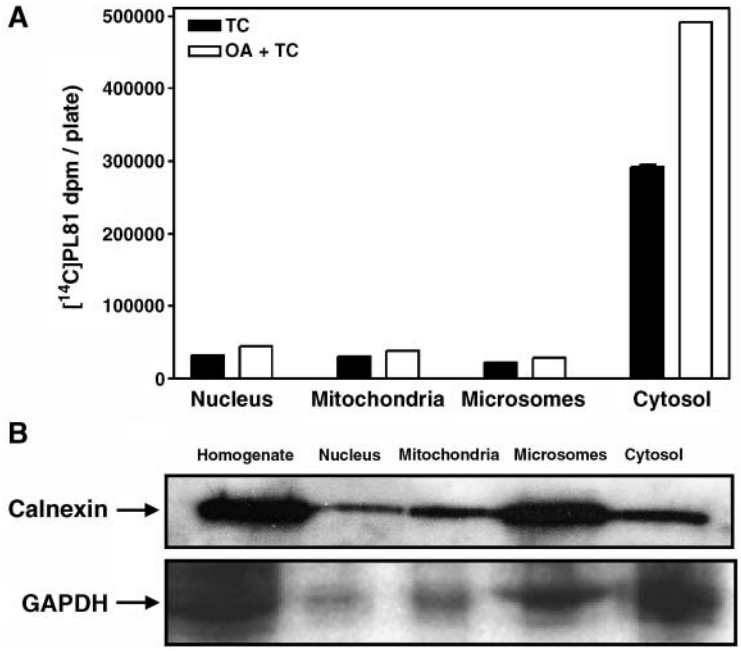

Fig. 4. Intracellular distribution of PL81. A: Differentiated Caco-2 cells were incubated with OA+TC or TC as well as [^{14}C]PL81 (6 μg/ml) for 18 h and homogenized, and various subcellular organelles were isolated by differential ultracentrifugation as described in Experimental Procedures and counted. B: Western blot analysis of calnexin and GAPDH in various purified subcellular fractions. Proteins (10 μl) from each fraction were used for SDS-PAGE. Western blotting was performed using rabbit anti-calnexin antibody (1:3,000 dilution; Stressgen) or rabbit anti-GAPDH (1:2,000 dilution; Novus Biologicals) followed by secondary goat anti-rabbit antibody (1:2,000 dilution; Zymed). Bands were then quantified using NIH Image software.

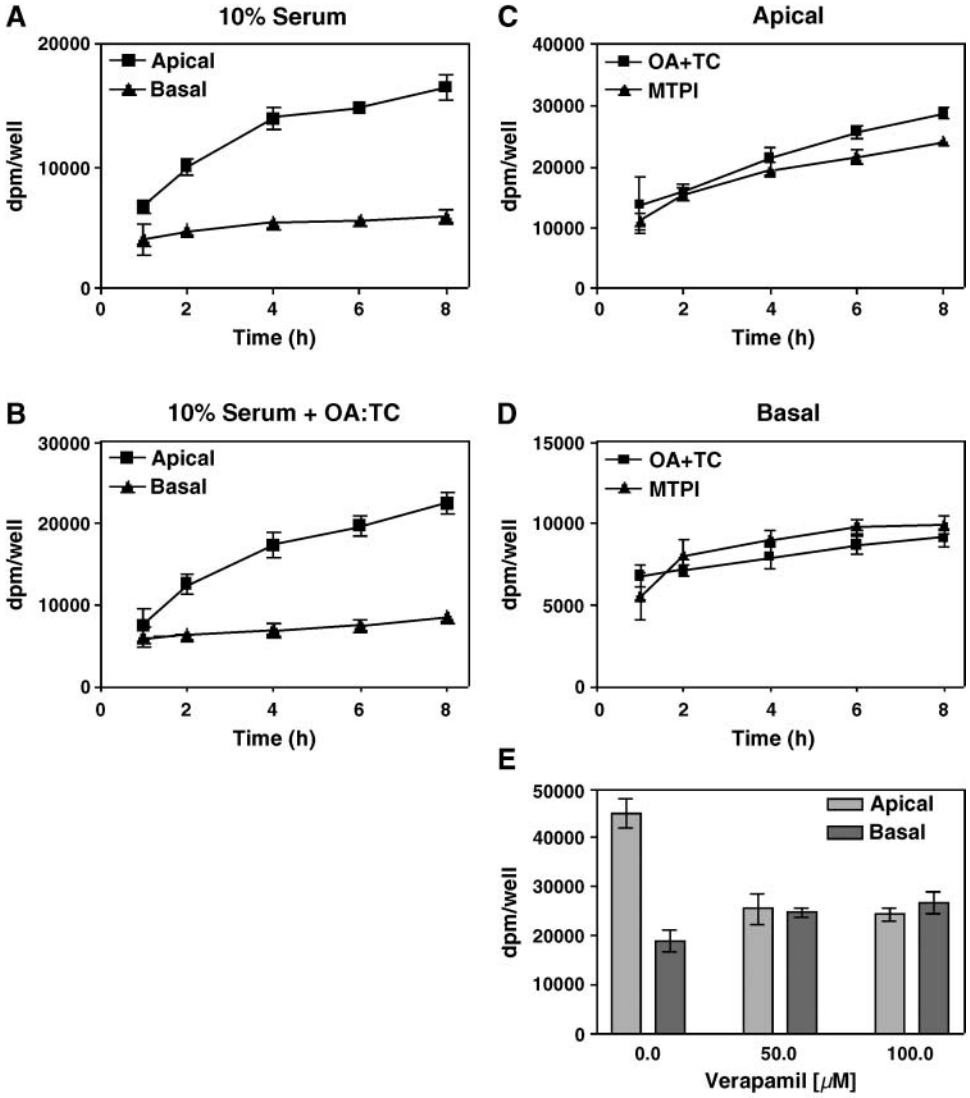

Fig. 5. Excretion of PL81 by Caco-2 cells. A, B: Differentiated Caco-2 cells were incubated with [^{14}C]PL81 on the apical side. After 18 h, cells were washed and supplemented with fresh medium containing 10% serum (A) or 10% serum and OA+TC (B). Media from both the apical and basolateral sides were collected at the indicated times and counted. C, D: To study the effect of the microsomal triglyceride transfer protein (MTP) inhibitor BMS197636 (MTPi; 1 μM) on the secretion of PL81, cells were incubated with [^{14}C]PL81 (6 μg/ml) for 18 h. Cells then received medium containing serum and OA+TC or OA+TC+MTPi on the apical side. The basolateral side received medium supplemented with 0.1% BSA. Conditioned media from the apical and basolateral sides were collected to measure radioactivity. E: Effect of verapamil on [^{14}C]PL81 excretion. Caco-2 cells were allowed to take up [^{14}C]PL81 for 18 h, washed, and incubated with different concentrations of verapamil for 6 h. Conditioned media obtained from both the apical and basolateral sides were counted. The data are presented as means ± SD (n = 3).

with [^{14}C]PL81 from the basolateral side. PL81 taken up from the basolateral side was also excreted mainly toward the apical side (data not shown). Next, we studied the effect of the induction of lipoprotein assembly on PL81 excretion (Fig. 5B). Cells were allowed to take up [^{14}C]PL81, washed, and supplemented with media containing OA+TC. Again, PL81 was excreted mostly toward the apical side. These data indicate that Caco-2 cells actively excrete PL81 from the apical side. We also studied the effect of the inhibition of lipoprotein assembly by providing cells with the MTP inhibitor BMS197636. This MTP antagonist affected neither the excretion of PL81 toward the apical side (Fig. 5C) nor the secretion toward the basolateral side (Fig. 5D). These studies indicate that PL81 excretion and secretion are not perturbed by CM production or its inhibition.

Intestinal cells actively excrete organic compounds by ATP-dependent transporters such as P-glycoprotein (3, 23–25). To check for their involvement, we used verapamil, a known inhibitor of P-glycoprotein-mediated efflux.

Verapamil (50 μM) inhibited the apical excretion of PL81 by ~45% and increased (30%; $P < 0.01$) basolateral secretion (Fig. 5E). These data indicate that verapamil-sensitive (P-glycoprotein or its family members) and -insensitive pathways are involved in the excretion of PL81.

PL81 is secreted independent of lipoproteins

Even though majority of the PL81 was excreted back to the apical side, some was secreted toward the basolateral side (Fig. 5). To study the secretion of PL81, we first studied the floatation properties of PL81 and its ability to associate with lipoproteins extracellularly (**Fig. 6A**). When PL81 was mixed with nonconditioned media and subjected to density gradient ultracentrifugation, PL81 was present mainly in the bottom fractions (Fig. 6A, nonconditioned media). PL81 was then incubated with conditioned media obtained from Caco-2 cells induced to assemble and secrete CM and centrifuged. Again, all of the PL81 was in the bottom fractions, indicating that there is no significant association of PL81 with secreted CM (Fig. 6A, conditioned media). Thus, PL81 has a density that is very different from CM and does not associate with these lipoproteins extracellularly.

To determine whether PL81 is secreted associated with lipoproteins, cells were incubated with [^{14}C]PL81 and subjected to three different experimental manipulations (Fig. 6B). When cells were incubated with TC only, conditions that do not support CM assembly and secretion, the secreted PL81 was in the bottom fractions. Treatment of cells with OA+TC to induce CM assembly and secretion had no significant effect on PL81 secretion (Fig. 6B). Similarly, treatment of cells with MTP inhibitor had no effect on the secretion of PL81. These data indicate that PL81 is not secreted with CM and that the assembly of these lipoproteins does not affect PL81 secretion.

Inhibition of CM secretion by PL81

To study the effect of PL81 on CM secretion, cells were labeled with glycerol and the secretion of TG and phospholipids was studied. No detectable TG was present in lipoprotein fractions when cells were incubated with TC (**Fig. 7A**, TC). However, incubation of differentiated Caco-2 cells with OA+TC resulted in significant secretion of TG as part of CM_L, CM_S, and CM_{VLDL} (Fig. 7A, OA+TC), consistent with previous studies (10, 11, 18, 19). The presence of PL81 significantly inhibited the secretion of TG

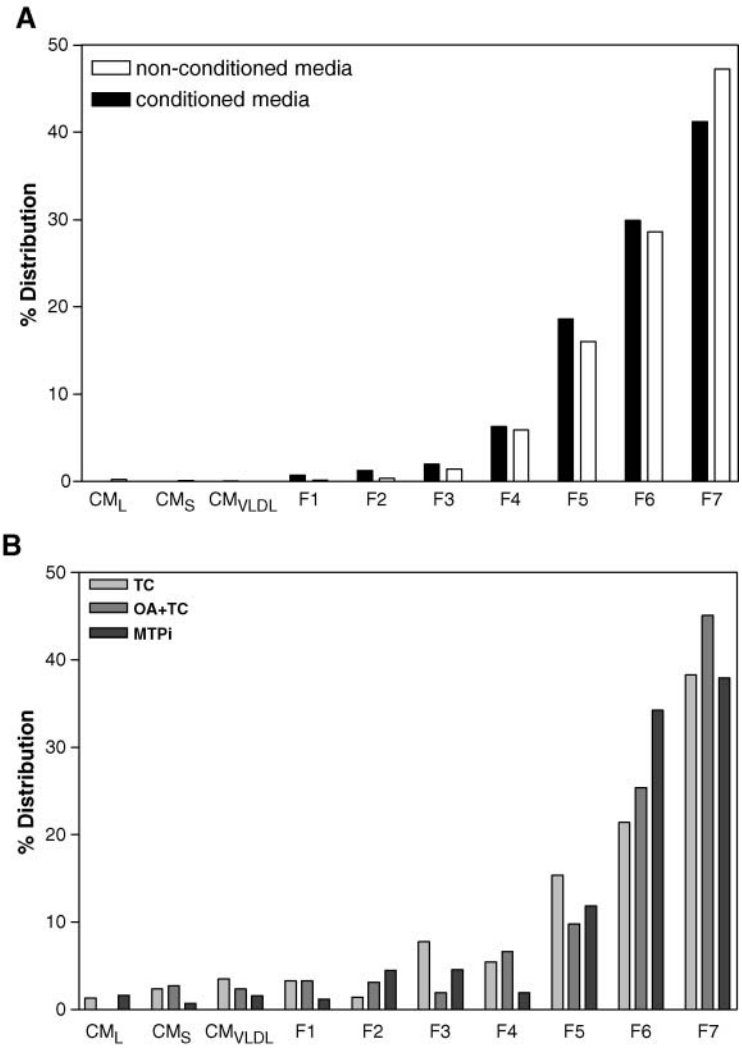

Fig. 6. PL81 does not associate with secreted lipoproteins. A: Floatation properties of PL81. Differentiated Caco-2 cells were incubated with OA+TC for 18 h to obtain conditioned media. Conditioned media and nonconditioned media were incubated with [^{14}C]PL81 for 3 h at 37°C and subjected to density gradient ultracentrifugation, and counts were measured in individual fractions. B: Secretion of PL81. Caco-2 cells were incubated with [^{14}C]PL81 for 8 h, washed, and incubated with medium containing TC, OA+TC, or OA+TC+MTP inhibitor (MTPi; BMS197636; 1 μM) for 18 h. Basolateral conditioned media were subjected to ultracentrifugation, and radioactivity in each fraction was quantified.

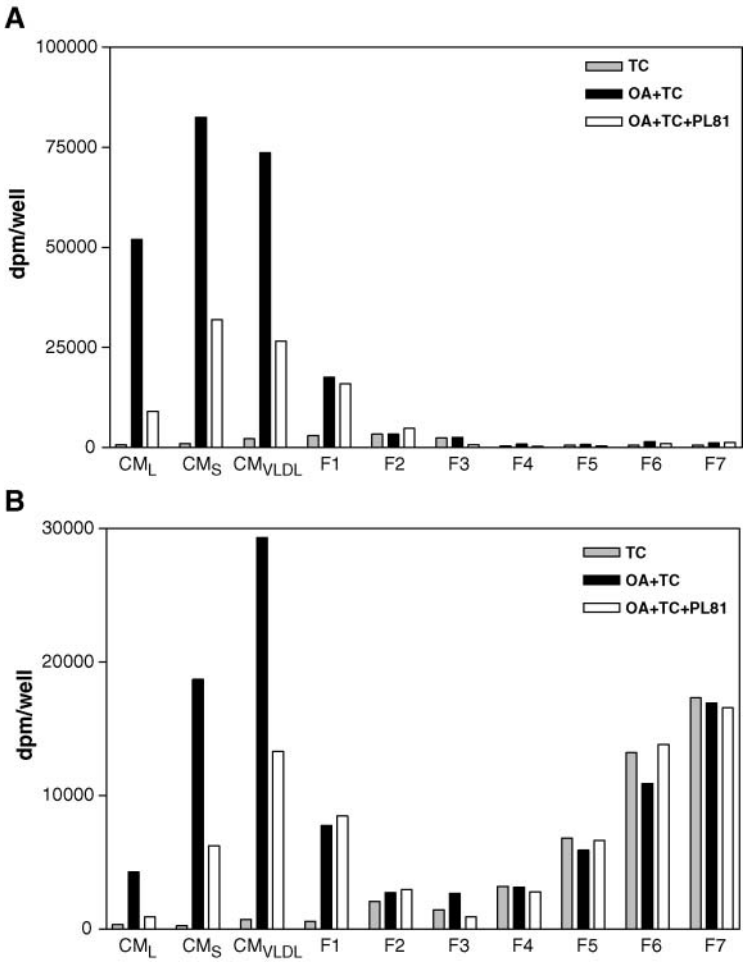

Fig. 7. Inhibition of lipoprotein secretion by PL81. Caco-2 cells received [^{3}H]glycerol (8 μCi/ml) and serum-containing medium supplemented with TC (0.5 mM), OA+TC (1.6 + 0.5 mM), or OA+TC+PL81 (6 μg/ml) for 18 h. Basolateral media were subjected to density gradient ultracentrifugation. Lipids were extracted from each fraction and separated by thin-layer chromatography, and bands corresponding to TG (A) and phospholipids (B) were counted.

with these lipoproteins (Fig. 7A, OA+TC+PL81). In TC-treated cells, phospholipids were present mainly in the bottom fractions (Fig. 7B, fractions 5–7) known to contain I-HDL (18, 26). Incubation of Caco-2 cells with OA+TC resulted in a significant increase of phospholipids in CM fractions (Fig. 7B, OA+TC), and this increase was effectively inhibited in the presence of PL81 (Fig. 7B, OA+TC+PL81). Treatment of cells with OA+TC or OA+TC+PL81 had no effect on the secretion of phospholipids with I-HDL. These studies showed that PL81, even though it is secreted independent of CM, inhibits the secretion of these lipoproteins.

Effect of PL81 on MTP activity

Because MTP is critical for CM assembly, we studied the effect of PL81 on cellular MTP activity. MTP activity was decreased by ~25% in cells treated with PL81 compared with untreated cells (**Fig. 8A**). To understand how PL81 decreases MTP activity, we studied the effect of PL81 on cellular (Fig. 8B) and purified (Fig. 8C) MTP. Increasing amounts of PL81 (0–10 μg/ml) had no inhibitory effect on cellular TG transfer activity (Fig. 8B). Incubation of purified MTP with PL81 slightly increased, rather than decreased, the transfer of TG by MTP. These data suggest that PL81 does not affect MTP activity directly.

PL81 increases the accumulation of TG in the cytosol

TGs are the major constituents of CM (Fig. 7A), constituting 90–95% of the mass (12, 14, 27). We had observed that PL81 significantly inhibits TG secretion (Figs. 2C, 7A). Therefore, the effect of PL81 on the intracellular distribution of TGs and phospholipids was studied (**Fig. 9**). When cells were labeled with [^{3}H]glycerol, very small amounts of TGs were present in the nucleus, mitochondria, and microsomes, and their distribution was not affected by the presence or absence of PL81 (Fig. 9A). In contrast, there were significant amounts of TG in the cytosol of control cells, and these amounts were further increased (207%) in cells treated with PL81 (Fig. 9A). This increase was specific to TG, as phospholipid distribution in different organelles was not affected by PL81 (Fig. 9B). When cells were labeled with OA, TGs again were present mainly in the cytosol (Fig. 9C), and these levels were increased (177%) in cells treated with PL81. Analysis of phospholipids revealed that the majority of OA was in microsomal phospholipids (Fig. 9D), most likely reflecting the preferential use of OA for the biosynthesis of microsomal phospholipids. Again, PL81 treatment had no significant effect on the amount of phospholipid in any of the organelles. These studies indicate that PL81-treated cells specifically accumulate more TG in their cytosol.

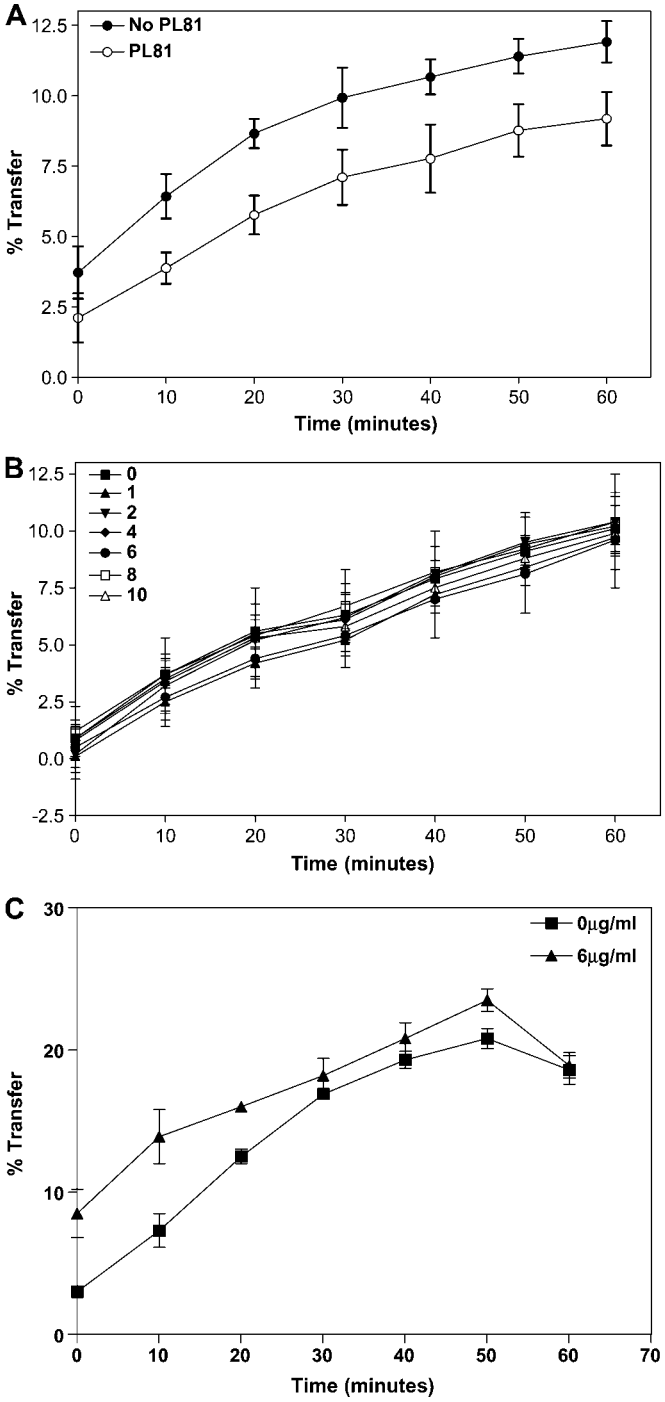

Fig. 8. Effect of PL81 on MTP activity. A: Cells were incubated with or without PL81 (6 μg/ml) for 18 h in the presence of OA+TC, washed, treated with hypotonic buffer (20, 21), and centrifuged, and supernatants were used (50 μg protein/assay) to measure MTP activity using a fluorescence assay (20, 21). B: Differentiated Caco-2 cells were incubated with OA+TC and treated with hypotonic buffer, and lumenal contents were prepared as described for A. Lumenal proteins (50 μg) were incubated with increasing concentrations (0–10 μg/ml) of PL81 in the presence of donor and acceptor vesicles. Transfer of fluorescent TG was recorded at the indicated times. C: Purified rat liver MTP was incubated with or without PL81 (6 μg/ml) and acceptor and donor vesicles at 37°C. Increases in fluorescence attributable to the transfer of fluorescent TG were recorded at the indicated times. The data are presented as means ± SD (n = 3).

DISCUSSION

Mode of action of PL81

The major observations made here are that PL81 accumulates in the cytosol and that PL81-treated Caco-2 cells assimilate more TG in the cytosol as well as exhibit diminished MTP activity. Based on these observations, we propose that the major site of action of PL81 is the cytosol and that PL81 most likely inhibits CM assembly and secretion by interfering with the transport of TG from the cytosol to the ER. By limiting this transport, PL81 could differentially inhibit the assembly of larger CM particles without affecting the secretion of smaller lipoprotein particles.

It has been appreciated for some time that the transport of TG from the cytosol to the ER lumen is crucial for the assembly and secretion of apoB-lipoprotein assembly. First, it was proposed that the hydrolysis and reesterification of TG might be involved in the transfer of TG from the cytosol to the ER lumen (28). Microsomal enzymes involved in the hydrolysis of TGs (29) and their synthesis (30) have been characterized. Second, evidence has been presented in Huh-7 cells for the presence of a mechanism that transports TG across the ER membrane and is partially sensitive to verapamil (31). Potentially, PL81 could interfere with both of these mechanisms. A third possibility is that PL81 might interact with cytosolic lipid droplets and cause their aggregation/fusion (15, 16) and inhibit their accretion into the ER lumen. Further studies are required to understand how PL81 affects any of these mechanisms and causes increased TG assimilation in the cytosol.

Recently, Pluronic block copolymers have been used to sensitize drug-resistant tumor cells (3). Tumor cells acquire drug resistance by increasing the expression of efflux proteins, such as P-glycoprotein, and by sequestering drugs in cytosolic vesicles (3). We observed that PL81 is excreted out of the cells, most likely via P-glycoprotein or a related family member. Its excretion could competitively inhibit the excretion of cancer drugs and increase their cellular residence time. Furthermore, our observation that PL81 accumulates in the cytosol suggests that PL81 might destabilize cytosolic vesicles that sequester and excrete drugs. Thus, Pluronic copolymers may increase the efficacy of cancer drugs and help overcome drug resistance by competitively inhibiting drug excretion and the destabilization of cytosolic vesicles.

PL81 inhibits MTP activity

PL81-treated cells showed a small (~20–25%) but significant decrease in MTP activity (Fig. 8A), although it is unclear how PL81 affects MTP activity. PL81 had no direct effect on the TG transfer activity of the purified (Fig. 8C) or cellular (Fig. 8B) MTP. Furthermore, very small amounts of [^{14}C]PL81 were found in microsomes, and PL81 does not show any structural similarity with known MTP antagonists. It is possible that a metabolite of PL81 might inhibit MTP.

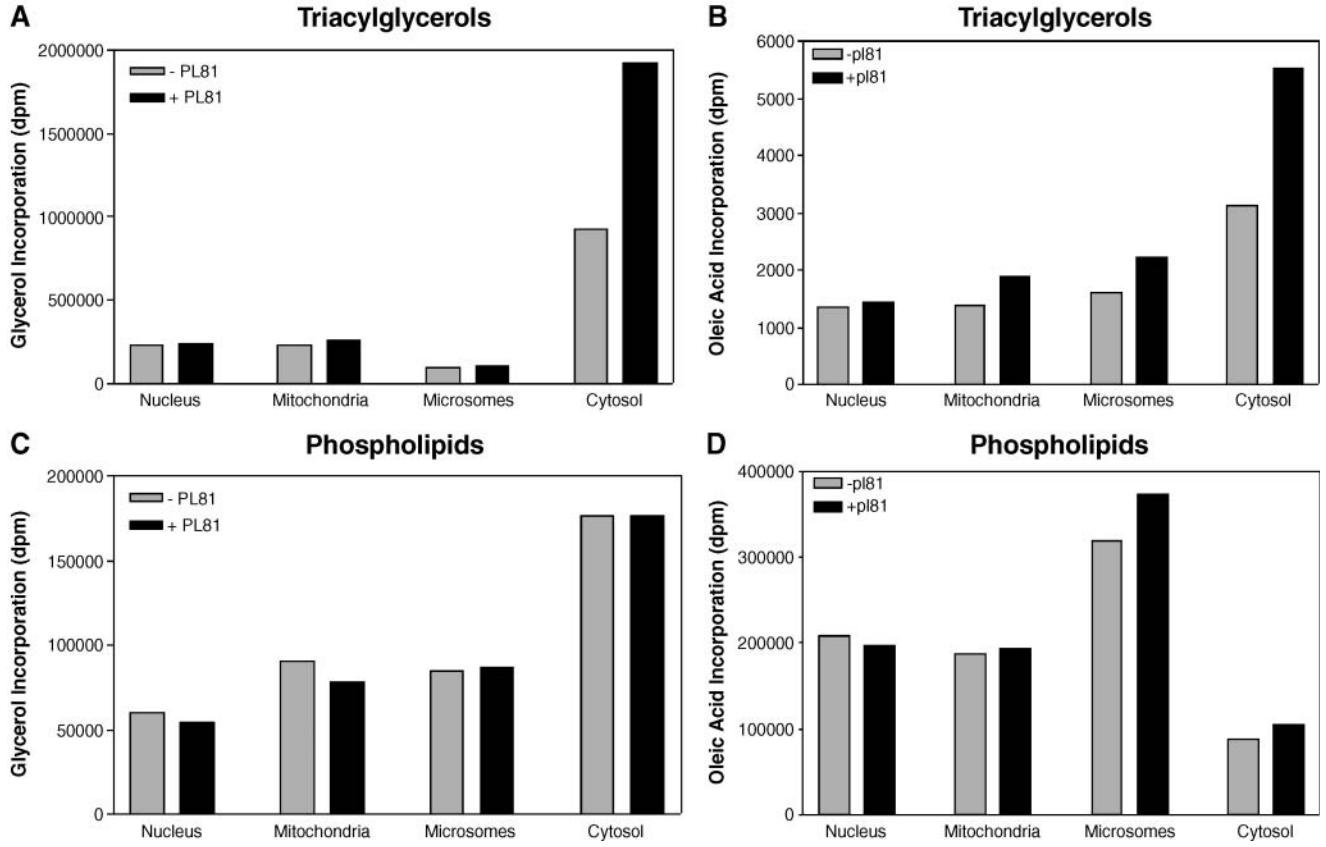

Fig. 9. PL81 increases the accumulation of TGs in the cytosol. Caco-2 cells were incubated with [^{3}H]glycerol (8 μCi/ml; A, B) or [^{14}C]OA (0.45 μCi/ml; C, D) with OA+TC in the presence (+ PL81) or absence (− PL81) of PL81 (6 μg/ml) for 18 h. Cells were washed with PBS, scraped, homogenized by passing through a 15 gauge needle, and processed for subcellular fractionation as described in Experimental Procedures. Total lipids were extracted using chloroform-methanol (3:1, v/v) from the subcellular fractions and separated by thin-layer chromatography. Lipid standards were chromatographed simultaneously, and the bands corresponding to TG and phospholipids were cut and processed to determine radioactivity.

Inhibition of MTP could contribute to the accumulation of TG in the cytosol. MTP has been shown to play a role in the accretion of TG from the cytosol to the ER lumen. For example, MTP inhibition (32, 33) and its gene deletion (34) lead to the accumulation of TG in the cytosol. It remains to be determined whether there is a precursor-product relationship between MTP inhibition by PL81 and the accumulation of TG in the cytosol.

Cellular metabolism of PL81

Very little is known about the cellular metabolism of surfactants such as PL81. Rodgers, Friday, and Bochenek (35) showed that rats absorb ~50% of the ingested [^{14}C] poloxalene and excrete it in the bile and urine. Here, we show that PL81 uptake by differentiated Caco-2 cells is a time-, temperature-, and concentration-dependent (Fig. 3) indicating a protein-facilitated active process. After uptake, cells excrete PL81 toward the apical side. The excretion is at least partially dependent on P-glycoprotein or a related protein, as shown by its partial inhibition by verapamil.

Even though the majority of the PL81 was excreted from the apical side, a small fraction was secreted at the basolateral side. Surprisingly, PL81 was secreted unassociated with lipoproteins. We also observed that PL81 does not associate to any significant extent with CM after secretion in the extracellular milieu. Moreover, PL81 secretion and excretion were not affected by the induction or inhibition of lipoprotein assembly and secretion. Thus, PL81 is neither secreted with nor associated with CM. Yet, it profoundly affects the assembly and secretion of these lipoproteins.

In short, our studies show that PL81 accumulates in the cytosol, causes TG assimilation in this compartment, and inhibits MTP activity. All of these mechanisms could interfere with the transfer of TG from the cytosol to the ER and inhibit the assembly and secretion of CM by the intestinal cells. The observations that PL81 is predominantly excreted apically and stored in the cytosol suggest that Pluronic copolymers could increase the efficacy of cancer drugs by inhibiting their excretion and destabilizing cytosolic vesicles in drug-resistant tumor cells.

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REFERENCES

1. Schmolka, I. R. 1977. A review of block polymer surfactants. *J. Am. Oil Chem. Soc.* **54:** 110–116.
2. O'Driscoll, C. M. 2002. Lipid-based formulations for intestinal lymphatic delivery. *Eur. J. Pharm. Sci.* **15:** 405–415.
3. Kabanov, A. V., E. V. Batrakova, and V. Y. Alakhov. 2002. Pluronic block copolymers for overcoming drug resistance in cancer. *Adv. Drug Deliv. Rev.* **54:** 759–779.
4. Kabanov, A. V., E. V. Batrakova, and D. W. Miller. 2003. Pluronic block copolymers as modulators of drug efflux transporter activity in the blood-brain barrier. *Adv. Drug Deliv. Rev.* **55:** 151–164.
5. Bochenek, W. J., and J. B. Rodgers. 1977. Effect of polyol detergents on cholesterol and triglyceride absorption. Hypolipidemic action of chronic administration of hydrophobic detergent. *Biochim. Biophys. Acta.* **489:** 503–506.
6. Tso, P., J. A. Balint, and J. B. Rodgers. 1980. Effect of hydrophobic surfactant (Pluronic L-81) on lymphatic lipid transport in the rat. *Am. J. Physiol.* **239:** G348–G353.
7. Reger, J. F., S. Frase, and P. Tso. 1989. Fine structure observations on rat jejunal epithelial cells during fat processing and resorption following L-81 exposure and reversal. *J. Submicrosc. Cytol. Pathol.* **21:** 399–408.
8. Tso, P., J. A. Balint, M. B. Bishop, and J. B. Rodgers. 1981. Acute inhibition of intestinal lipid transport by Pluronic L-81 in the rat. *Am. J. Physiol.* **241:** G487–G497.
9. Tso, P., D. S. Drake, D. D. Black, and S. M. Sabesin. 1984. Evidence for separate pathways of chylomicron and very low-density lipoprotein assembly and transport by rat small intestine. *Am. J. Physiol.* **247:** G599–G610.
10. Luchoomun, J., and M. M. Hussain. 1999. Assembly and secretion of chylomicrons by differentiated Caco-2 cells: nascent triglycerides and preformed phospholipids are preferentially used for lipoprotein assembly. *J. Biol. Chem.* **274:** 19565–19572.
11. Nayak, N., E. H. Harrison, and M. M. Hussain. 2001. Retinyl ester secretion by the intestinal cells is a specific and regulated process that is dependent on the assembly and secretion of chylomicrons. *J. Lipid Res.* **42:** 272–280.
12. Hussain, M. M. 2000. A proposed model for the assembly of chylomicrons. *Atherosclerosis.* **148:** 1–15.
13. Hussain, M. M., M. H. Kedees, K. Singh, H. Athar, and N. Z. Jamali. 2001. Signposts in the assembly of chylomicrons. *Front. Biosci.* **6:** D320–D331.
14. Hussain, M. M., R. K. Kancha, Z. Zhou, J. Luchoomun, H. Zu, and A. Bakillah. 1996. Chylomicron assembly and catabolism: role of apolipoproteins and receptors. *Biochim. Biophys. Acta.* **1300:** 151–170.
15. Weinberg, R. B., V. R. Cook, J. A. DeLozier, and G. S. Shelness. 2000. Dynamic interfacial properties of human apolipoproteins A-IV and B-17 at the air/water and oil/water interface. *J. Lipid Res.* **41:** 1419–1427.
16. Morita, S. Y., M. Kawabe, M. Nakano, and T. Handa. 2003. Pluronic L81 affects the lipid particle sizes and apolipoprotein B conformation. *Chem. Phys. Lipids.* **126:** 39–48.
17. Singh, K., O. A. Batuman, H. O. Akman, M. H. Kedees, V. Vakil, and M. M. Hussain. 2002. Differential, tissue-specific, transcriptional regulation of apolipoprotein B secretion by transforming growth factor beta. *J. Biol. Chem.* **277:** 39515–39524.
18. Iqbal, J., K. Anwar, and M. M. Hussain. 2003. Multiple, independently regulated pathways of cholesterol transport across the intestinal epithelial cells. *J. Biol. Chem.* **278:** 31610–31620.
19. Anwar, K., H. J. Kayden, and M. M. Hussain. 2006. Transport of vitamin E by differentiated Caco-2 cells. *J. Lipid Res.* **47:** 1261–1273.
20. Athar, H., J. Iqbal, X. C. Jiang, and M. M. Hussain. 2004. A simple, rapid, and sensitive fluorescence assay for microsomal triglyceride transfer protein. *J. Lipid Res.* **45:** 764–772.
21. Rava, P., H. Athar, C. Johnson, and M. M. Hussain. 2005. Transfer of cholesteryl esters and phospholipids as well as net deposition by microsomal triglyceride transfer protein. *J. Lipid Res.* **46:** 1779–1785.
22. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
23. Seeballuck, F., M. B. Ashford, and C. M. O'Driscoll. 2003. The effects of Pluronics block copolymers and Cremophor EL on intestinal lipoprotein processing and the potential link with P-glycoprotein in Caco-2 cells. *Pharm. Res.* **20:** 1085–1092.
24. Nerurkar, M. M., P. S. Burton, and R. T. Borchardt. 1996. The use of surfactants to enhance the permeability of peptides through Caco-2 cells by inhibition of an apically polarized efflux system. *Pharm. Res.* **13:** 528–534.
25. Batrakova, E. V., H. Y. Han, V. Y. Alakhov, D. W. Miller, and A. V. Kabanov. 1998. Effects of Pluronic block copolymers on drug absorption in Caco-2 cell monolayers. *Pharm. Res.* **15:** 850–855.
26. Iqbal, J., and M. M. Hussain. 2005. Evidence for multiple complementary pathways for efficient cholesterol absorption in mice. *J. Lipid Res.* **46:** 1491–1501.
27. Hussain, M. M., R. W. Mahley, J. K. Boyles, M. Fainaru, W. J. Brecht, and P. A. Lindquist. 1989. Chylomicron-chylomicron remnant clearance by liver and bone marrow in rabbits: factors that modify tissue-specific uptake. *J. Biol. Chem.* **264:** 9571–9582.
28. Wiggins, D., and G. F. Gibbons. 1992. The lipolysis/esterification cycle of hepatic triacylglycerol. Its role in the secretion of very-low-density lipoprotein and its response to hormones and sulphonylureas. *Biochem. J.* **284:** 457–462.
29. Dolinsky, V. W., D. Gilham, M. Alam, D. E. Vance, and R. Lehner. 2004. Triacylglycerol hydrolase: role in intracellular lipid metabolism. *Cell. Mol. Life Sci.* **61:** 1633–1651.
30. Buhman, K. K., H. C. Chen, and R. V. Farese, Jr. 2001. The enzymes of neutral lipid synthesis. *J. Biol. Chem.* **276:** 40369–40372.
31. Higashi, Y., H. Itabe, H. Fukase, M. Mori, Y. Fujimoto, and T. Takano. 2003. Transmembrane lipid transfer is crucial for providing neutral lipids during very low density lipoprotein assembly in endoplasmic reticulum. *J. Biol. Chem.* **278:** 21450–21458.
32. Wang, Y., K. Tran, and Z. Yao. 1999. The activity of microsomal triglyceride transfer protein is essential for accumulation of triglyceride within microsomes in McA-RH7777 cells. A unified model for the assembly of very low density lipoproteins. *J. Biol. Chem.* **274:** 27793–27800.
33. Kulinski, A., S. Rustaeus, and J. E. Vance. 2002. Microsomal triacylglycerol transfer protein is required for lumenal accretion of triacylglycerol not associated with apo B, as well as for apo B lipidation. *J. Biol. Chem.* **277:** 31516–31525.
34. Raabe, M., M. M. Véniant, M. A. Sullivan, C. H. Zlot, J. Björkegren, L. B. Nielsen, J. S. Wong, R. L. Hamilton, and S. G. Young. 1999. Analysis of the role of microsomal triglyceride transfer protein in the liver of tissue-specific knockout mice. *J. Clin. Invest.* **103:** 1287–1298.
35. Rodgers, J. B., S. Friday, and W. J. Bochenek. 1984. Absorption and excretion of the hydrophobic surfactant, 14C-poloxalene 2930, in the rat. *Drug Metab. Dispos.* **12:** 631–634.