Evidence for Modulation of P-glycoprotein–Mediated Efflux by Methoxypolyethylene Glycol-*block*-Polycaprolactone Amphiphilic Diblock Copolymers

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Purpose. The purpose of this study was to investigate the pathways involved in rhodamine 123 (R-123) accumulation enhancement in Caco-2 cells with a low molecular-weight methoxypolyethylene glycol-*block*-polycaprolactone (MePEG-*b*-PCL) diblock copolymer.

Methods. R-123 accumulation by Caco-2 cells with $MePEG_{17}$-*b*-PCL_5 was measured in the presence of endocytosis inhibitors or under ATP depletion conditions. Directional flux studies were conducted with cell monolayers on Transwell plates.

Results. Endocytosis inhibitors had no effect on reducing R-123 accumulation with $MePEG_{17}$-*b*-PCL_5. The apical to basolateral (AP→BL) flux of R-123 with $MePEG_{17}$-*b*-PCL_5 or verapamil was similar to R-123 alone. However the BL→AP flux was significantly decreased with $MePEG_{17}$-*b*-PCL_5 and verapamil. The efflux ratio for R-123 flux was 3.2 and was reduced to 1.06 with $MePEG_{17}$-*b*-PCL_5 confirming the inhibition of P-glycoprotein (P-gp)-mediated efflux. R-123 accumulation at the conclusion of each of the flux experiments was similar for $MePEG_{17}$-*b*-PCL_5 and verapamil in the BL→AP direction. The AP→BL direction demonstrated a 2-fold increase for verapamil and a 5-fold increase with $MePEG_{17}$-*b*-PCL_5. This difference in R-123 accumulation was possibly due to the diblock enhancing passive membrane diffusion of R-123.

Conclusions. $MePEG_{17}$-*b*-PCL_5 diblock reduced R-123 efflux through inhibition of P-gp efflux, and unimers may interact with cell membranes, increasing permeability and enhancing R-123 influx through increased transmembrane diffusion.

KEY WORDS: amphiphilic diblock copolymers; efflux ratio; P-glycoprotein; polymeric micelles; rhodamine 123.

INTRODUCTION

The expression of the ATP-binding cassette transporter, P-glycoprotein (P-gp), on the apical membrane of enterocytes in the gastrointestinal tract can effectively limit oral drug availability for many drugs (1,2). The overexpression of P-gp is also associated with the development of multidrug resistance (MDR) in tumor cells and presents a major problem in terms of reduced clinical responsiveness to chemotherapy of various human malignancies (3). The structure of P-gp consists of two homologous halves each composed of six transmembrane spanning domains (TMD) and a cytoplasmic nucleotide binding domain (NBD) (4). Upon substrate binding, ATP binds to one of the NBD's inducing a conformational change that translocates the substrate to a lower affinity binding region or exposes the substrate binding domain to the extracellular milieu (5,6). An alternating catalytic cycle of ATP hydrolysis occurs where the first ATP bound to one NBD is hydrolyzed followed by release of ADP and Pi, which promotes the release of bound substrate. Binding and hydrolysis of ATP to the second NBD then results in the reconfiguration of P-gp for additional substrate binding (5–7).

It has been suggested that the function of P-gp may involve substrate transport from the lipid bilayers of the plasma membrane to the external medium ("vacuum cleaner" hypothesis) (8). Within this hypothesis, P-gp could function as a "flippase," catalyzing the translocation of hydrophobic substrates present in the cell membrane from the inner to the outer leaflet (9). P-gp substrates are molecules that typically possess a degree of hydrophobicity. It has been proposed that substrates partition into the membrane as a rate-limiting step and then interact with the P-gp binding domain within the lipid membrane (10). Thus, the ability of the substrate to enter the membrane and the rate at which it can diffuse through, or the length of time it resides within the membrane, are important parameters dictating the effectiveness of P-gp efflux. The transbilayer movement rates across multilamellar lipid vesicles or liposomes for numerous P-gp substrates and P-gp modulators demonstrated that substrates have a tendency to reside longer in the membrane allowing efficient P-gp efflux, while modulators can enter and move across the lipid bilayer at a faster rate and overcome the outward efflux (11). Eytan *et al.* evaluated a series of P-gp substrates, rhodamine dyes, for P-gp–mediated exclusion from rodent and human tumor MDR lines, their ability to stimulate ATPase activity, and transmembrane movement rate in P-gp–reconstituted liposomes (12). The transmembrane movement rate was the major factor determining the efficiency of P-gp in excluding rhodamine dyes from MDR cells (12).

There are numerous studies demonstrating that P-gp–mediated efflux of drugs may be inhibited by the use of non-ionic surfactants. Amphiphilic compounds such as polysorbates, vitamin E TPGS (d-alpha-tocopheryl polyethylene glycol-1000 succinate), Pluronic block copolymers, and Cremophor EL have demonstrated enhanced cellular accumulation and improved transepithelial flux when co-administered with compounds susceptible to P-gp–mediated efflux (13). The mechanism for surfactants inhibiting P-gp–mediated efflux appears to involve interactions with the lipid bilayer. Sinicrope *et al.* showed that alterations in membrane lipid fluidity of canalicular membrane vesicles modulated the P-gp–mediated accumulation of MDR drugs (14). Membrane fluidization by various agents including surfactants was shown to modulate drug efflux from MDR cells and substantially reduce P-gp ATPase activity (15). Amphiphilic Pluronic triblock copolymers have been described to inhibit P-gp via a dual mechanism involving a reduction in membrane microviscosity and cellular depletion of ATP levels (16).

Several surfactants that have demonstrated enhanced cellular accumulation of P-gp substrates have also been evaluated as permeation enhancers for poorly permeable drugs. The non-ionic surfactant polysorbate 80 has been shown to increase permeability of transport markers in Caco-2 cells as well as improving the permeability of proteins susceptible to P-gp–mediated efflux (17,18). Yamazaki *et al.* demonstrated that polysorbate 80 enhanced the cellular accumulation of an

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epipodophyllotoxin derivative susceptible to P-gp–mediated efflux, and this was attributed to an increased influx of drug and not to an inhibition of P-gp–mediated efflux (19). Pluronic triblock copolymers have also been shown to enhance the diffusion of compounds such as doxorubicin across model lipid bilayers (20). These investigations suggest that surfactant interactions with membranes leading to increased membrane permeability may play an important role in enhancing the transmembrane diffusion of a P-gp substrate and cell accumulation, independent of the inhibitory effects of these surfactants on P-gp.

Recently, we have demonstrated that a novel series of low molecular-weight methoxypolyethylene glycol-*block*-polycaprolactone (MePEG-*b*-PCL) amphiphilic diblock copolymers can enhance the Caco-2 cellular accumulation of the P-gp substrate, rhodamine 123 (R-123) (21). Diblock copolymers with optimal activity were composed of MePEG of molecular weight 750 with either 2 or 5 repeat units of caprolactone with larger blocks of MePEG or PCL all showing less activity. R-123 cellular accumulation increased with increasing concentration of MePEG-*b*-PCL copolymers up to a maximum, after which accumulation decreased. The decrease in accumulation was attributed to R-123 partitioning into micelles reducing the free R-123 fraction available for cellular uptake. The MePEG-*b*-PCL copolymers maximally enhanced R-123 accumulation at concentrations above the critical micelle concentration (CMC) with little or no activity below the CMC, contrary to other surfactants that have demonstrated activity only at concentrations at or below the CMC (18,22). Based on these observations, we proposed three possible pathways for MePEG-*b*-PCL diblock copolymer mediated accumulation enhancement: 1) inhibition of P-gp–mediated efflux; 2) increase in substrate membrane permeability or transmembrane diffusion; 3) vesicular transport of micellized substrate.

In this work, we have selected $MePEG_{17}$-*b*-PCL_5 diblock copolymer for further study because it showed maximum R-123 accumulation enhancement activity (21). The human colon adenocarcinoma cell line, Caco-2, was used as an intestinal epithelial cell model. Caco-2 cells undergo spontaneous enterocytic differentiation displaying a well-organized cellular polarity, including microvilli and functional transporters including P-glycoprotein (23). The aim of this study was to determine the contributions of passive transmembrane diffusion, vesicular transport, and inhibition of P-gp efflux, to enhanced R-123 cellular accumulation in the presence of $MePEG_{17}$-*b*-PCL_5 diblock copolymer. Specific objectives were to investigate the effects of endocytosis inhibitors and ATP depletion conditions on cellular R-123 accumulation and to compare the directional transepithelial fluxes of R-123 in the presence of $MePEG_{17}$-b-PCL_5 diblock copolymer.

MATERIALS AND METHODS

Materials

Sucrose and ammonium chloride (NH_4Cl) were from Fisher Scientific (Nepean, ON, Canada). Stannous 2-ethylhexanoate ($Sn(Oct)_2$), Potassium cyanide (KCN), lucifer yellow (LY), verapamil, cyclosporin A (CSA), rhodamine 123 (R-123), brefeldin A (Br-A), and deoxyglucose (DOG) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methoxypolyethylene glycol (MePEG) with molecular weight of 750 and 6-caprolactone monomer were purchased from Fluka (Oakville, ON, Canada). All tissue culture reagents were from Invitrogen/Life Technologies (Grand Island, NY, USA).

Synthesis and Characterization of MePEG-*b*-PCL Diblock Copolymer

The diblock copolymer $MePEG_{17}$-*b*-PCL_5 was synthesized and characterized using methods previously described (21). Diblock notation describes the number of repeat units in subscript, therefore $MePEG_{17}$-*b*-PCL_5 is a diblock copolymer composed of MePEG with 17 repeat units or a molecular weight of 750 with 5 repeat units of caprolactone attached. Briefly, a 60:40 feed weight ratio of MePEG 750:caprolactone was polymerized at 140°C for 24 h with 0.3% w/w $Sn(Oct)_2$ as catalyst. The resulting diblock was characterized for molecular weight and polydispersity using gel permeation chromatography (GPC) and for degree of polymerization by nuclear magnetic resonance.

Although degradation of MePEG-*b*-PCL diblock copolymer most likely occurs via hydrolytic chain scission between MePEG and PCL and by cleavage of the ester bonds between caprolactone units, limited work has been reported to characterize the extent of this degradation with low-molecular-weight copolymers. Indirect evidence suggests that up to 200 min there was little to no degradation of polyethylene-*b*-polycaprolactone micelle nanoparticles by low concentrations of lipase enzymes in water (24). Furthermore, in previous investigations we reported that MePEG 750 oligomers up to 3% w/v did not enhance R-123 accumulation by Caco-2 cells (21). Therefore over the time course of these studies, degradation was considered to be negligible and not contributing to the results of these studies.

Cell Culture

The human colon adenocarcinoma cell line Caco-2 was obtained from ATCC (Rockville, MD, USA). Cells were grown in a humidified atmosphere of 5% CO_2 at 37°C. Caco-2 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% HI-FBS (heat-inactivated fetal bovine serum), 1% nonessential amino acids (NEAA), 1% L-glutamine, and 100 units/ml penicillin and 100 μg/ml streptomycin. Stock cultures were grown in T-175 cm^2 flasks (BD-Falcon, BD Biosciences, Bedford, MA, USA) at a seed density of 5000 cells/cm^2. Upon 80–90% confluency, cells were split using 0.25% trypsin containing 1.0 mM EDTA. Cells were seeded into 48-well flat-bottom plates (Corning Costar, Cambridge, MA, USA) at a density of 40,000 cells/cm^2 and grown in plates for a minimum of 14 days before being used for experimentation. Media were changed in plates and T-175 flasks every second day.

For directional flux experiments, Caco-2 cells were seeded at a density of 60,000 cells/cm^2 onto collagen-coated PTFE (polytetrafluoroethylene) membrane (Transwell-COL, Corning Costar) with a pore size of 0.4 μm and insert diameter of 12 mm. Media were changed every second day, with 0.5 ml placed on the apical (AP) side and 1.5 ml in the basolateral (BL) side. Cell monolayers were used for experiments between 21 to 28 days post seeding to allow for differentiation and tight junction formation. Transepithelial electrical resis-

tance (TEER) was monitored routinely using a Millicell-ERS (Millipore, Bedford, MA, USA).

R-123 Cellular Accumulation Studies

Cellular accumulation of R-123 by Caco-2 was carried out as previously described (21). Briefly, cells grown in 48-well plates were exposed to 5.0 μM R-123 in either assay buffer (HBSS + 10 mM Hepes, pH = 7.4) alone or in solutions of $MePEG_{17}$-*b*-PCL_5 diblock copolymer. The standard P-glycoprotein (P-gp) inhibitor, verapamil (50 μM), was used as a positive control. Cells were incubated for 90 min at 37°C. The cellular fluorescent intensity of R-123 was measured using a fluorescent microplate reader (CytoFluor 4000, PerSeptive Biosystems, Framingham, MA, USA) with λ_{EX} = 485 nm and λ_{EM} = 530 nm (with filter bandwidths of 20 and 25 nm, respectively). R-123 cellular accumulation was normalized with respect to total protein content in each well using the BCA protein assay method (Pierce, Rockford, IL, USA).

ATP Depletion

Caco-2 cells grown in 48-well plates were washed 2 times with glucose free HBSS + 10 mM Hepes (glucose free assay buffer) and preincubated in glucose-free assay buffer at 37°C for 15 min. The buffer was removed and the cells pretreated with 1.5 mM potassium cyanide (KCN) + 25 mM deoxyglucose (DOG) in glucose-free assay buffer for 30 min at 37°C. The pretreatment solution was removed, and 0.5 ml of 5.0 μM R-123 containing 1.5 mM KCN + 25 mM DOG in glucose-free assay buffer, with or without various concentrations of $MePEG_{17}$-*b*-PCL_5 diblock copolymer, were then added to each well. Cells were incubated at 37°C for 90 min, after which the solutions were removed and the plate placed on ice. Cells in each well were collected and R-123 concentrations determined as described above.

Endocytosis Inhibition

Caco-2 cells grown in 48-well plates were washed 2 times with assay buffer and preincubated in assay buffer at 37°C for 15 min. The buffer was removed and the cells pretreated with either 75 mM NH_4Cl or 20 μM brefeldin A in assay buffer for 60 min or 0.45 M sucrose for 30 min at 37°C. The pretreatment solution was removed and 0.5 ml of 5.0 μM R-123 containing the corresponding endocytosis inhibitor in assay buffer, with or without various concentrations of $MePEG_{17}$-*b*-PCL_5 diblock copolymer, were added to each well. Cells were incubated at 37°C for 90 min, after which the solutions were removed and the plate placed on ice. Cells in each well were collected and R-123 concentrations determined as described above.

As a control for endocytosis inhibition, cellular accumulation with the fluid phase endocytosis marker, lucifer yellow (LY), was performed. Cells were treated with inhibitors as described above and LY was used at a concentration of 0.5 mM. LY fluorescence was determined using a fluorescent microplate reader (CytoFluor 4000) with λ_{EX} = 450 nm and λ_{EM} = 530 nm (with filter bandwidths of 50 and 25 nm, respectively) and normalized to total cellular protein.

R-123 Directional Flux

The flux of R-123 across Caco-2 monolayers was determined in the apical to basolateral (AP→BL) and the BL→AP directions. Caco-2 cells grown on Transwell inserts as described above were washed with assay buffer and allowed to equilibrate with assay buffer at 37°C for 15 min. For AP→BL flux, 0.5 ml of either 5.0 μM R-123 with assay buffer, 50 μM verapamil, 0.01% w/v $MePEG_{17}$-*b*-PCL_5, or 0.25% w/v $MePEG_{17}$-*b*-PCL_5 were placed on the AP side and 1.5 ml of assay buffer placed on the BL side. The plate was placed on an orbital shaker (Microshaker, Bellco Biotechnology, Vineland, NJ, USA) set to 50–75 rpm at 37°C. At specified time points, the Transwell inserts were lifted out and placed in a new well containing 1.5 ml of assay buffer. The amount of R-123 in the basolateral solution was determined as described above.

For BL→AP flux, 1.5 ml of 5.0 μM R-123 were placed on the BL side and 0.5 ml of assay buffer, 50 μM verapamil, 0.01% w/v $MePEG_{17}$-*b*-PCL_5, or 0.25% w/v $MePEG_{17}$-*b*-PCL_5 were placed on the AP side. Aliquots of 50 μl were taken from the AP side at each time point for R-123 quantitation, and 50 μl of fresh solution was replaced.

As a control for Caco-2 cell monolayer integrity, TEER was monitored before and after experimentation. Lucifer yellow flux (1 mM) was also measured in the presence of assay buffer and with 0.25% w/v $MePEG_{17}$-*b*-PCL_5.

The apparent permeability coefficient (P_{app}) was calculated using the following equation:

$$P_{app} = \frac{\Delta Q}{\Delta t \cdot A \cdot C_o} \quad (1)$$

Where ΔQ is the amount of R-123 transported during the time interval Δt, C_o is the concentration of R-123 applied to the donor side, and A is the monolayer surface area.

The amounts of R-123 accumulated by Caco-2 cells at the conclusion of the directional transport experiments were measured. Cell monolayers were rinsed with ice-cold PBS after which the membrane support was excised using a scalpel. The membrane was then placed in 1% Triton x-100 to solubilize the cells. The cellular debris and membrane were pelleted by centifugation at 13,000 rpm for 5 min and aliquots of the supernatant measured for R-123 fluorescence and protein content as described above.

Confocal Fluorescence Microscopy

Caco-2 cells were grown on 8-well Lab-tek chamber glass slides (Nalge Nunc International, Naperville, IL, USA) for a minimum of 14 days under the same culture conditions as described above. Cells were then washed with HBSS + 10 mM Hepes and exposed to 5.0 μM R-123 in assay buffer or with 0.25% $MePEG_{17}$-*b*-PCL_5 diblock copolymer for 90 min at 37°C. After incubation, the cells were washed with ice-cold PBS and immediately viewed using an Axiophat confocal fluorescence microscope using a standard rhodamine filter set and 40x objective (Carl Zeiss, Don Mills, ON, Canada).

RESULTS

Role of Endocytosis in Enhanced R-123 Accumulation by $MePEG_{17}$-*b*-PCL_5

Caco-2 cells were treated with endocytosis inhibitors such as hyperosmotic sucrose (0.45 M), 75 mM ammonium chloride, or 20 μM brefeldin A. Control studies using the fluid phase marker lucifer yellow (LY) demonstrated a 40–50%

reduction in LY cellular uptake when treated with the endocytosis inhibitors, suggesting that the endocytosis pathway was inhibited under these experimental conditions (Fig. 1). Using varying concentrations of $MePEG_{17}$-*b*-PCL_5, accumulation of 5.0 μM R-123 was determined in the presence of these endocytosis inhibitors. Figure 2 shows that the levels of R-123 accumulation were similar when treated with endocytosis inhibitors compared to untreated control group (no inhibitors). Figure 2 also demonstrates that R-123 accumulation increased up to a critical concentration of 0.25% $MePEG_{17}$-*b*-PCL_5 and then decreased, consistent with our earlier reported data (21).

Directional Flux of R-123

The directional flux of R-123 was conducted using Caco-2 cell monolayers grown on collagen coated Transwell 0.4-μm pore size membrane supports that provide discrete apical and basolateral surfaces. No increases in lucifer yellow flux in the presence of 0.25% $MePEG_{17}$-*b*-PCL_5 and no changes in TEER at the conclusion of the flux experiments were observed (Table I).

The AP→BL absorptive flux of R-123 was determined by exposing the monolayers to either R-123 with assay buffer, or to R-123 in combination with 50 μM verapamil or $MePEG_{17}$-*b*-PCL_5 at concentrations above (0.25%) or below (0.01%) the CMC on the apical surface and monitoring the amount of R-123 entering the basolateral side. For each time point the Transwell insert was placed into fresh buffer to maintain sink conditions. Figure 3A demonstrates that compared to R-123 alone, verapamil or 0.01% $MePEG_{17}$-*b*-PCL_5 did not increase the amount transported across Caco-2 cells. However, 0.25% diblock showed a small, but not statistically significant increase in the R-123 flux. Apparent permeability coefficients for AP→BL ($P_{app_{AP\rightarrow BL}}$) compared in Table II show no significant differences between verapamil and diblock treatment compared to R-123 alone.

The BL→AP secretory flux of R-123 was measured by placing R-123 on the basolateral side and either assay buffer, 50 μM verapamil or either 0.01% and 0.25% $MePEG_{17}$-*b*-PCL_5 on the apical surface and monitoring the amount of R-123 entering the apical side. Sample aliquots were withdrawn from the apical solution and replaced with fresh solution to avoid repeated removal and replacement of the entire apical buffer contents from disrupting the Caco-2 monolayer integrity. Figure 3B demonstrates a linear relationship for flux across the Caco-2 monolayer for all treatment groups, suggesting that sink conditions were still maintained using this sampling method. Verapamil and 0.25% diblock lowered the BL→AP flux of R-123 to the same extent and 0.01% diblock also lowered the flux but not down to the same level (Fig. 3B). The corresponding $P_{app_{BL\rightarrow AP}}$ values were significantly reduced with exposure to verapamil or 0.25% diblock (Table II).

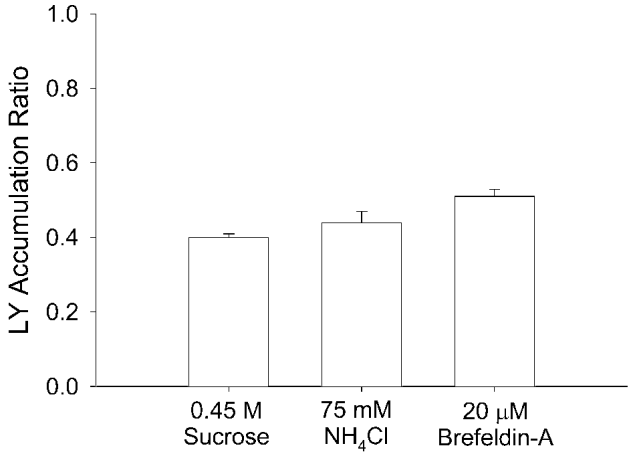

Fig. 1. Lucifer yellow (LY) accumulation ratio of endocytosis inhibitors. LY accumulation by caco-2 with or without endocytosis inhibitors was measured at 37°C for 90 min. Accumulation ratio is the amount of LY accumulated in the presence of inhibitors to the amount of LY accumulated without inhibitors. Data expressed as mean ± SD with n = 3.

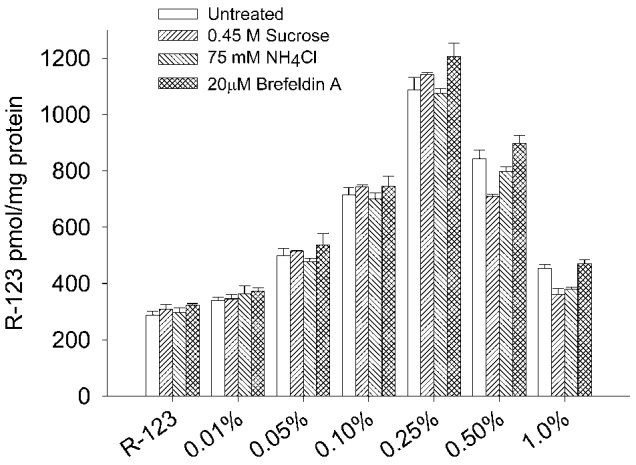

Fig. 2. R-123 accumulation by Caco-2 cells comparing untreated to treatment with endocytosis inhibitors. Cells were treated with 5.0 μM R-123 containing either 0.45 M sucrose, 75 mM NH_4Cl, or 20 μM brefeldin A with varying concentrations of $MePEG_{17}$-*b*-PCL_5 (0.01% to 1.0% w/v) for 90 min at 37°C. R-123 with inhibitors but without diblock was used as control. Data expressed as mean ± SEM with n = 3.

R-123 accumulation by Caco-2 monolayers at the conclusion of the directional flux studies is shown in Figs. 4A and 4B. For AP→BL, 0.25% diblock increased the accumulation approximately 5-fold compared to verapamil, which increased cellular accumulation approximately 2-fold (Fig. 4A). The amount of R-123 accumulated by Caco-2 cells in the BL→AP direction was similar for verapamil and 0.25% diblock, with both demonstrating approximately a 2-fold increase (Fig. 4B). In either direction, 0.01% diblock showed little or no increase in R-123 accumulation compared to R-123 with assay buffer (Figs. 4A and 4B). The overall extent of R-123 cellular accumulation for all treatment groups in the BL→AP direction was substantially higher than the extent of R-123 cellular accumulation in the AP→BL direction (Figs. 4A and 4B). For example, 0.25% diblock produced R-123 accumulation values of approximately 500 pmol/mg protein and 1200 pmol/mg protein for AP→BL and BL→AP directions, respectively.

The intracellular localization of R-123 was determined by confocal fluorescence microscopy (CFM). Figure 5A displays a punctated pattern of R-123 fluorescence localized within the cells after exposure to R-123 in assay buffer. In the presence of 0.25% $MePEG_{17}$-*b*-PCL_5, a punctated fluorescence pattern was difficult to ascertain because there was an intense and diffuse fluorescence observed throughout the cytosol (Fig. 5B).

Table I. TEER Ratio and Percent LY Transported in Assay Buffer and in the Presence of Diblock Copolymer for Caco-2 Monolayers Grown on Transwell Membrane Inserts

Treatment group	TEER ratio*	% LY transport†
Assay buffer	1.06 ± 0.01	0.42 ± 0.06
0.25% $MePEG_{17}$-*b*-PCL_5	1.07 ± 0.03 (ns)	0.37 ± 0.03 (ns)

TEER measurements conducted before and after directional flux experimentation and LY transport represents the cumulative amount of LY transported in the AP→BL direction over 120 min at 37°C. Two-tailed two-sample *t* test used to compare treatment group to assay buffer group. (ns) = not statistical significant $p > 0.05$. LY, Lucifer Yellow; TEER, transepithelial electrical resistance.
* TEER ratio = $TEER_{after\ expt}/TEER_{before\ expt}$.
† % LY transport = (Amt LY transported/Amt LY applied)100.

R-123 Accumulation Under ATP Depletion

Caco-2 cells were pretreated for 30 min with an inhibitor of oxidative phosphorylation, KCN, and an inhibitor of glycolysis, DOG. All R-123 samples contained 1.5 mM KCN and 25 mM DOG during the accumulation time period. Figure 6 shows the R-123 accumulation by ATP depleted Caco-2 cells with various concentrations of $MePEG_{17}$-*b*-PCL_5 diblock copolymer. Accumulation of R-123 under ATP depletion conditions was significantly increased approximately 3-fold compared to R-123 accumulation by untreated cells. R-123 accumulation was significantly increased with the addition of 0.05, 0.1, or 0.25% $MePEG_{17}$-*b*-PCL_5 compared to the R-123 accumulation without diblock under ATP depleted conditions.

DISCUSSION

In recent work, we showed that a series of MePEG-*b*-PCL amphiphilic diblock copolymers varying in MePEG and PCL length were able to enhance the accumulation of R-123 within Caco-2 cells (21). The diblock copolymer with the greatest activity was found to be composed of MePEG with 17 repeat units (MePEG of molecular weight 750) and with 5 repeat units of PCL, denoted as $MePEG_{17}$-*b*-PCL_5. R-123 accumulation was enhanced 3.8-fold using 0.25% of $MePEG_{17}$-*b*-PCL_5, which was substantially higher than 1.5- and 1.7-fold for the P-gp inhibitors verapamil and cyclosporin A, respectively (21). The concentration of $MePEG_{17}$-*b*-PCL_5 at maximum R-123 accumulation was approximately 10-fold higher than the critical micelle concentration (CMC = 0.03% w/v) with minimal enhancement of accumulation found at concentrations below the CMC (21). At concentrations greater then 0.25% $MePEG_{17}$-*b*-PCL_5, R-123 accumulation was observed to decrease substantially (21). Our data differ from those of other investigators who showed that other surfactants, including the triblock copolymers in the Pluronic series, enhanced cell accumulation or flux of P-gp substrates, but did so only at concentrations below the CMC (18,22). Substrate accumulation or flux was maximal close to the CMC of the amphiphiles and free surfactant unimers were suggested as the species primarily responsible for the inhibition of P-gp (18,22,25). The decrease in accumulation at concentrations above the CMC for these surfactants has been suggested to be due to substantial micellization of the P-gp substrate which reduces the free fraction of substrate available for entry into the cell (18).

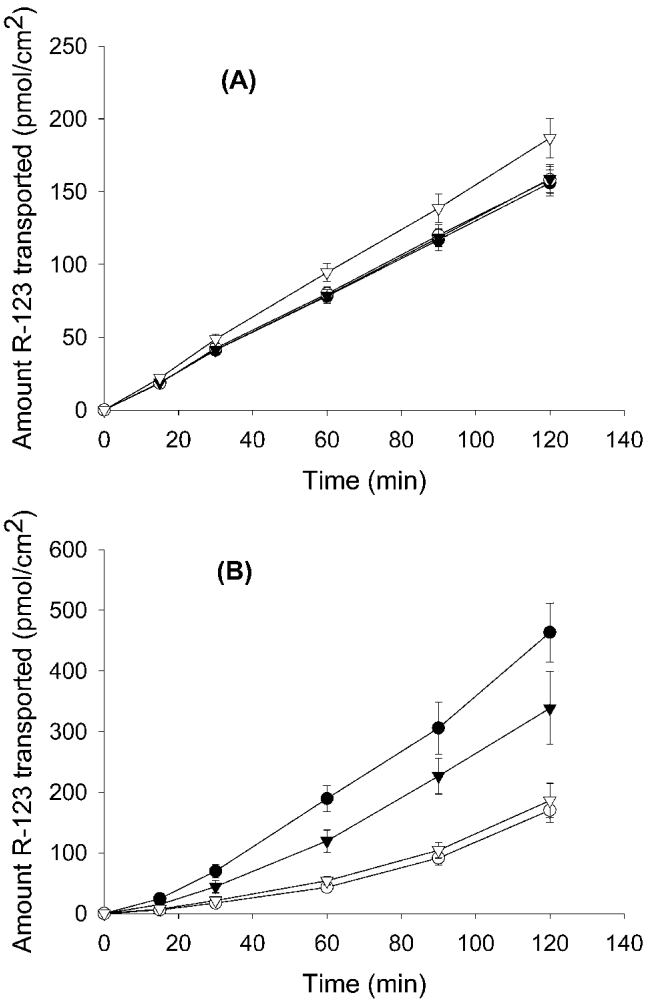

Fig. 3. Directional flux of 5.0 μM R-123 across Caco-2 monolayers over 120 min at 37°C. R-123 AP→BL flux (A) measured the amount of R-123 in the BL solution when R-123 was placed on the AP side in the presence of (●) HBSS + 10mM Hepes, (○) 50 μM verapamil, (▼) 0.01% $MePEG_{17}$-*b*-PCL_5, or (∇) 0.25% $MePEG_{17}$-*b*-PCL_5. BL→AP flux of R-123 (B) was determined by placing R-123 on the BL side and monitoring the amount that entered the AP side, which was exposed to (●) HBSS + 10mM Hepes, (○) 50 μM verapamil, (▼) 0.01% $MePEG_{17}$-*b*-PCL_5, or (∇) 0.25% $MePEG_{17}$-*b*-PCL_5. Data expressed as the cumulative amount of R-123 transported across a unit area of Caco-2 monolayers and represented as the mean ± SEM with n = 3.

The association of R-123 with $MePEG_{17}$-*b*-PCL_5 micelles showed that approximately 25% of a 5.0 μM R-123 solution was bound within micelles at 0.25% $MePEG_{17}$-*b*-PCL_5 diblock concentration. This increased to 50% binding at 1.0% $MePEG_{17}$-*b*-PCL_5 (21). This suggested that although the extent of R-123 micellization is relatively low at the maximal accumulation concentration of 0.25% $MePEG_{17}$-*b*-PCL_5, enhanced accumulation of R-123 with $MePEG_{17}$-*b*-PCL_5 may have occurred in whole or in part by an endocytotic pathway involving uptake of micelle-bound R-123. Endocytosis of micellized compounds has been previously demonstrated for chemically similar diblock copolymers composed of polyethylene glycol-*block*-polycaprolactone in PC12 and P19 cell lines (26,27). Therefore, to determine if R-123 loaded $MePEG_{17}$-*b*-PCL_5 micelles contributed to enhanced R-123

Table II. Apparent Permeability Coefficients (P_{app}) for R-123 Flux Across Caco-2 Monolayers in the AP→BL and BL→AP Direction

Treatment group	AP→BL $P_{app} \times 10^{-6}$ (cm/s)	BL→AP $P_{app} \times 10^{-6}$ (cm/s)	Efflux ratio*
Assay buffer	4.31 ± 0.26	13.8 ± 1.62	3.20
50 μM verapamil	4.40 ± 0.27 (ns)	5.05 ± 0.61 (✶)	1.15
0.01% $MePEG_{17}$-*b*-PCL_5	4.40 ± 0.28 (ns)	10.3 ± 1.68 (ns)	2.34
0.25% $MePEG_{17}$-*b*-PCL_5	5.17 ± 0.38 (ns)	5.52 ± 0.82 (✶)	1.06

P_{app} calculated using Eq. 1 from the directional flux of 5.0 μM R-123 with the indicated treatment groups. Data expressed as mean ± SEM with n = 3. Two-tailed two-sample *t* test used to compare treatment group to assay buffer group. (ns) = not statistical significant $p > 0.05$ and (✶) = statistically significant $p < 0.05$.

* Efflux ratio = $P_{app_{BL\rightarrow AP}}/P_{app_{AP\rightarrow BL}}$.

accumulation via an endocytotic pathway, R-123 accumulation was evaluated in the presence of endocytosis inhibitors.

Preliminary studies evaluated common endocytosis inhibitors such as monensin and cytochalasin B. However, it was shown that these compounds increased R-123 accumulation that may be facilitated by P-gp modulation by these endocytosis inhibitors (data not shown). The endocytosis inhibitors chosen have been reported to affect various stages of the endocytotic process without modulating the activity of P-gp. Hyperosmotic sucrose inhibits fluid phase and receptor mediated endocytosis, ammonium chloride is used to increase the pH of the endosomes and thus prevent the acidification of the endocytotic pathway, and the macrocyclic antibiotic, brefeldin A (Br-A), can cause morphological changes in the

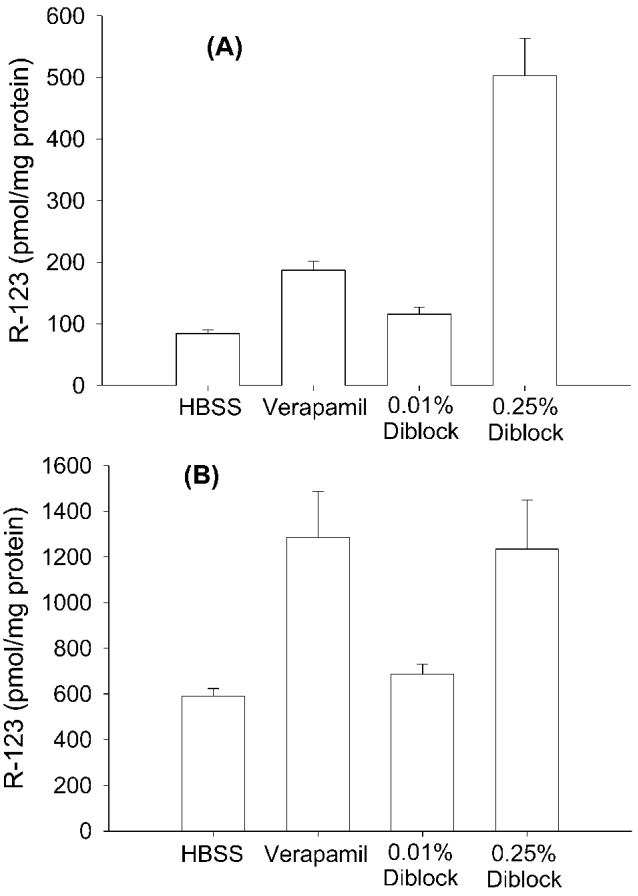

Fig. 4. R-123 accumulation by Caco-2 monolayers at the conclusion of the directional flux studies. Transwell membranes were excised and cells lysed to quantitate the cellular fluorescence. (A) Cellular accumulation in the AP→BL direction and (B) BL→AP direction. Data expressed as mean ± SEM with n = 3.

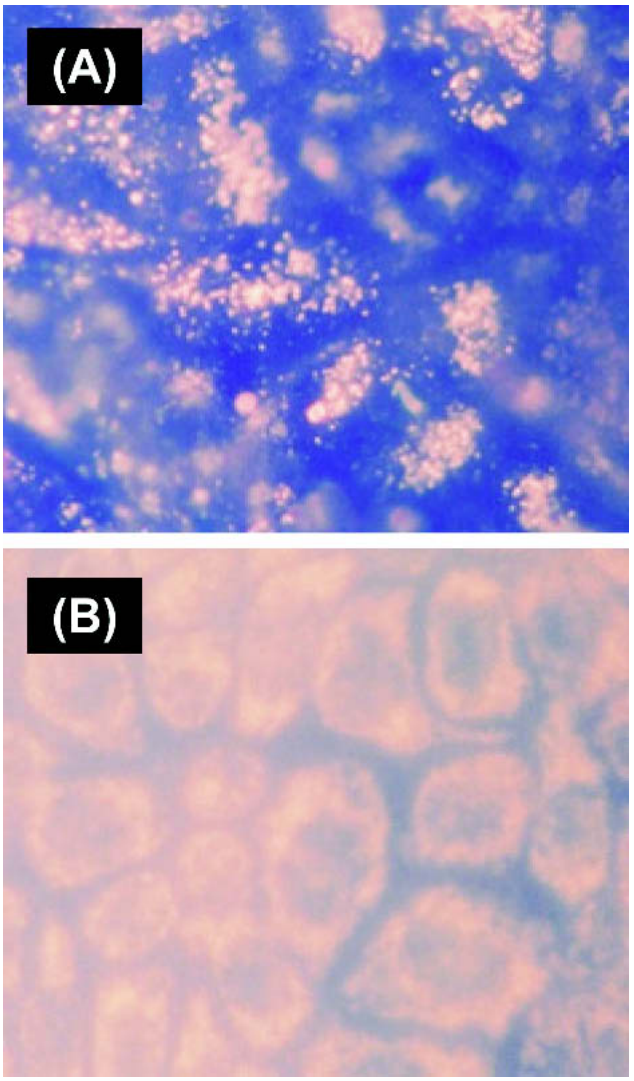

Fig. 5. Confocal fluorescence microscopy photographs of R-123 accumulation by Caco-2 cells after exposure to either (A) 5.0 μM R-123 in assay buffer or (B) 5.0 μM R-123 with 0.25% $MePEG_{17}$-*b*-PCL_5 for 90 min at 37°C. Photographs were taken using a 40× objective.

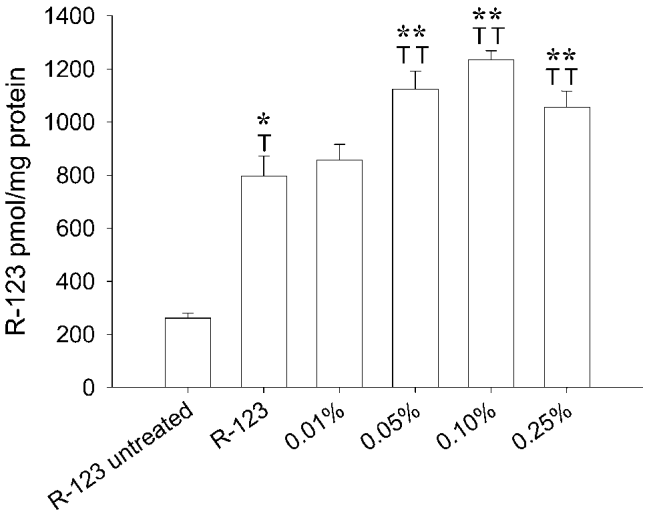

Fig. 6. R-123 accumulation by Caco-2 cells under ATP depleted conditions. Caco-2 cells were exposed to 5.0 μM R-123 in glucose-free assay buffer containing 1.5 mM KCN + 25 mM DOG and varying concentrations of $MePEG_{17}$-*b*-PCL_5 for 90 min at 37°C. 5.0 μM R-123 accumulation by ATP depleted and untreated cells without diblock copolymer was used as control. Data expressed as mean ± SEM with n = 3. Two-tailed two sample *t* test with $p < 0.05$ was used to compare R-123 accumulation groups. (*) Statistically significant comparison of R-123 accumulation of untreated cells to ATP depleted cells. (**) Statistically significant accumulation of R-123 with diblock copolymer compared to R-123 alone under ATP depletion.

endosomes and inhibit recycling between the endoplasmic reticulum and the cis-golgi complex (28–30).

In control experiments with the fluid phase marker lucifer yellow (LY), all three inhibitors reduced cellular LY accumulation to approximately 40–50% in our Caco-2 model, demonstrating that the endocytotic pathway was inhibited under the experimental conditions (Fig. 1). Using varying concentrations of $MePEG_{17}$-*b*-PCL_5, accumulation of 5.0 μM R-123 was determined in the presence of these endocytosis inhibitors. Figure 2 showed that R-123 accumulation with $MePEG_{17}$-*b*-PCL_5 at concentrations above the CMC in the presence of endocytosis inhibitors was similar to the untreated control group. Both treatment and control groups demonstrated an increase in R-123 accumulation up to 0.25% diblock then a drop as the concentration of diblock was further increased as described previously (21). The lack of any reduction in R-123 accumulation with endocytosis inhibitors over the concentrations tested suggested that endocytosis of micellized R-123 was not a pathway contributing to the R-123 accumulation enhancement.

We speculated that $MePEG_{17}$-*b*-PCL_5 micelles could be functioning as a "depot" for free unimers that partitioned into the membrane and either reached a threshold concentration for P-gp inhibition, or promoted influx through enhanced R-123 membrane permeability. Therefore, studies evaluating the effect of $MePEG_{17}$-*b*-PCL_5 diblock on the directional flux of R-123 across Caco-2 monolayers were carried out to assess inhibitory effects on P-gp–mediated efflux activity. Concentrations of $MePEG_{17}$-*b*-PCL_5 used in these studies were either below the CMC (0.01%) or above the CMC at the concentration of peak R-123 accumulation (0.25%) (21). Efflux ratios ($P_{app_{BL\rightarrow AP}}$ / $P_{app_{AP\rightarrow BL}}$) were determined to assess the possible role of P-gp modulation by $MePEG_{17}$-*b*-PCL_5 diblock. In general, it has been established that efflux ratios of greater than 2.0 are indicative of the involvement of an efflux mechanism and in the presence of an inhibitor, efflux ratios are typically reduced to approximately 1.0 (31). The efflux ratio of R-123 with assay buffer was 3.2 confirming its polarized efflux by P-gp (Table II). Efflux ratios for verapamil and 0.25% $MePEG_{17}$-*b*-PCL_5 were 1.15 and 1.06, respectively, indicative of inhibitory effects on P-gp–mediated efflux of R-123.

During the flux experiments the TEER was monitored before and after the experiment and the flux of LY was monitored in the presence or absence of 0.25% $MePEG_{17}$-*b*-PCL_5 to determine any change in tight junction integrity over the course of the experiment. A large increase in LY flux or a decrease in the TEER would be indicative of a decrease in monolayer integrity and also might indicate that the diblock could enhance the R-123 flux via effects on the paracellular transport pathway. No significant difference in the TEER and LY transport with $MePEG_{17}$-*b*-PCL_5 diblock was observed compared to assay buffer group suggesting that the integrity of the tight junctions was maintained during treatment (Table I).

Interestingly, the $P_{app_{AP\rightarrow BL}}$ values for verapamil and both concentrations of $MePEG_{17}$-*b*-PCL_5 were not statistically different from the $P_{app_{AP\rightarrow BL}}$ for R-123 in assay buffer (Table II). The $P_{app_{BL\rightarrow AP}}$ was significantly reduced with verapamil and 0.25% $MePEG_{17}$-*b*-PCL_5 compared to R-123 with assay buffer (Table II). This indicates that verapamil and $MePEG_{17}$-*b*-PCL_5 are able to inhibit the secretory R-123 efflux through Caco-2 monolayers but do not enhance the absorptive AP→BL influx. However, cellular accumulation studies with $MePEG_{17}$-*b*-PCL_5 have consistently shown an approximately 4-fold increase in R-123 accumulation compared to R-123 alone both in this work (Fig. 2, untreated group) and in previous studies (21). Therefore, although $MePEG_{17}$-*b*-PCL_5 enhanced R-123 accumulation, no increase in the AP→BL flux was observed. A similar effect on R-123 influx has been described by Altenberg *et al.,* who showed that verapamil did not enhance R-123 influx despite abolishing R-123 efflux in P-gp overexpressing Chinese hamster lung fibroblasts (32).

Recent work by Troutman and Thakker demonstrated a similar lack of R-123 flux enhancement in the AP→BL direction when Caco-2 cells were treated with a P-gp inhibitor (33). The inability of P-gp inhibitors to increase AP→BL flux of R-123 was thought to be the result of poor membrane penetration of R-123 owing to its cationic charge and low octanol/water partition coefficient (11,12,33,34). Furthermore, a large BL→AP flux of R-123 was shown, which was significantly decreased using a P-gp inhibitor, similar to our observations (Fig. 3B) (33). Because R-123 cannot penetrate the lipid membrane effectively, kinetic evidence suggested that the large BL→AP flux of R-123 was the result of an unknown influx transporter in the BL membrane that actively transported R-123 into the cell and was then transported by P-gp out of the cell (33). Apically applied P-gp inhibitors would then be able to effectively decrease the BL→AP flux through modulation of P-gp. This asymmetry in the flux for R-123 was further found for doxorubicin, another hydrophilic compound with poor membrane permeability (33).

At the conclusion of the AP→BL and BL→AP direc-

tional flux studies the Caco-2 monolayers were excised to determine the extent of R-123 accumulation occurring through either the AP membrane or the BL membrane. Assessment of R-123 accumulation after the AP→BL flux study was considered to be analogous to R-123 accumulation studies previously determined in flat-bottom plates and should describe effects of verapamil and $MePEG_{17}$-*b*-PCL_5 on the influx of R-123 across the AP membrane. Approximately 5-fold and 2-fold increases in R-123 cellular accumulation were observed after the AP→BL flux experiment with 0.25% $MePEG_{17}$-*b*-PCL_5 and verapamil, respectively, suggesting that $MePEG_{17}$-*b*-PCL_5 might have inhibited P-gp to a greater extent than verapamil (Fig. 4A). On the other hand, Table II and Fig. 4B show that the $P_{app_{BL\rightarrow AP}}$ and the extent of R-123 accumulation was similar for verapamil and 0.25% $MePEG_{17}$-*b*-PCL_5, demonstrating that they are equally effective at reducing the apical efflux of R-123. Hence, it seems unlikely that the differences we observed in R-123 accumulation after AP→BL flux studies for verapamil and diblock can be explained solely by inhibition of P-gp efflux. We suggest that there may be a combination of mechanisms including both P-gp efflux inhibition and changes in R-123 membrane permeability caused by diblock interactions with the AP membrane. Furthermore, the significantly larger R-123 cell accumulation following BL→AP flux experiments compared to AP→BL flux experiments, in the presence of either verapamil or 0.25% $MePEG_{17}$-*b*-PCL_5 diblock, indicates that R-123 crossed the BL membrane relatively easily and produced higher intracellular concentrations (Fig. 4A and 4B). This observation may provide indirect support for the concept proposed by Troutman and Thakker that the BL membrane of Caco-2 cells may possess an active transporter pumping R-123 into the cell (33). Hence, the apparent high BL membrane permeability may in fact be due to this proposed active transport pump and not a result of intrinsic membrane permeability of R-123.

If the diblock caused an increase in R-123 membrane permeability leading to an increase in R-123 influx through the AP membrane, why this did not translate to an increase in the $P_{app_{AP\rightarrow BL}}$ is not clear. For AP→BL flux to occur for R-123, once inside the cell R-123 must cross the BL membrane. Because the intracellular localization of R-123 primarily resides within the mitochondria, this deposition may act as an intracellular sink reducing the concentration gradient across the BL membrane and effectively reducing the AP→BL flux. Figures 5A and 5B display confocal fluorescence microscopy photographs of the R-123 fluorescence localization within Caco-2 cells when exposed to either R-123 in assay buffer or with 0.25% $MePEG_{17}$-*b*-PCL_5. A lower level of R-123 fluorescence and a punctated pattern was observed throughout the cells with assay buffer (Fig. 5A). This pattern is indicative of the cellular localization of R-123 within the mitochondria (12). With 0.25% $MePEG_{17}$-b-PCL_5 an intense and diffuse fluorescence is observed throughout the cytosol with no discernable punctation due to the fluorescence intensity (Fig. 5B). From the micrograph in Fig. 5B, it appears that there is extensive cytosolic deposition of R-123, which should be available for diffusion across the BL membrane. The low partition coefficient and charged nature of R-123 may have limited the BL membrane permeability of R-123 once inside the cell and reduced AP→BL flux. Although a greater influx through the AP membrane occurred with $MePEG_{17}$-*b*-PCL_5, this does not significantly increase the overall $P_{app_{AP\rightarrow BL}}$ for R-123, possibly due to the lack of $MePEG_{17}$-*b*-PCL_5 effects on the BL membrane since exposure was on the apical surface.

In an attempt to determine the possible contributions of R-123 permeability changes producing an increased influx of R-123 by $MePEG_{17}$-*b*-PCL_5 diblock, R-123 accumulation studies were performed under ATP depletion conditions. We hypothesized that if Caco-2 cells were depleted of ATP, the ability of P-gp to function as an efflux transporter would be abolished and any observed increase in R-123 accumulation by $MePEG_{17}$-*b*-PCL_5 would be the result of increased transmembrane diffusion caused by R-123 membrane permeability changes. Several groups have demonstrated an increase in P-gp substrate accumulation under ATP depletion (35,36). Using similar conditions, the accumulation of R-123 in assay buffer could be increased by depleting Caco-2 cells of ATP (Fig. 6). There was a statistically significant increase in the R-123 accumulation in the presence of $MePEG_{17}$-*b*-PCL_5 diblock compared to the accumulation of R-123 in assay buffer when Caco-2 cells were treated with metabolic inhibitors (Fig. 6). This increase may be attributed to the diblock increasing the transmembrane passive diffusion of R-123. However the contributions of diblock inhibition of residual P-gp activity that may not have been abolished from our treatment cannot be ruled out.

In conclusion, we believe the ability of $MePEG_{17}$-*b*-PCL_5 diblock to enhance the cellular accumulation of the P-gp substrate, R-123, may involve a combination of mechanisms. In the secretory direction, $MePEG_{17}$-*b*-PCL_5 was equally as effective as verapamil in reducing P-gp–mediated efflux of R-123. In the absorptive direction, $MePEG_{17}$-*b*-PCL_5 enhanced the cellular internalization of R-123 at high concentrations of diblock above the CMC that did not appear to involve endocytosis of micellized R-123. This suggests that $MePEG_{17}$-*b*-PCL_5 micelles may provide a "depot" for free unimer to interact with the cell membrane and contribute either to enhanced passive membrane diffusion of R-123 through membrane permeability changes or inhibition of P-gp–mediated efflux or both, resulting in enhanced accumulation across the apical membrane. Once inside the cell, although R-123 is distributed throughout the cytosol and is available for diffusion across the basolateral membrane, we speculate that the basolateral membrane permeability of R-123 may limit the AP→BL flux. Further work is underway to determine the effects of MePEG-*b*-PCL diblock copolymers on membrane microviscosity and elucidate the effects these changes may have on the transmembrane diffusion of P-gp substrates.

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