Effects of Pluronic Block Copolymers on Drug Absorption in Caco-2 Cell Monolayers

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Received December 1, 1997; accepted February 23, 1998

Purpose. The present work characterizes the effects of Pluronic copolymers on the transport of a P-gp-dependent probe, rhodamine 123 (R123) in Caco-2 cell monolayers.

Methods. The accumulation and efflux studies were performed on the confluent Caco-2 monolayers using fluorescent probes with and without Pluronic copolymers.

Results. At concentrations below the critical micelle concentration single chains ("unimers") of Pluronic P85 enhanced the accumulation and inhibited the efflux of R123 in Caco-2 monolayers. The transport of the P-gp-independent probe, rhodamine 110 was not altered under these conditions. In contrast the micelles increased R123 accumulation to a much lower extent when compared to the unimers and enhanced R123 efflux in Caco-2 monolayers.

Conclusions. Pluronic P85 unimers increase accumulation of a P-gp-dependent drug in Caco-2 monolayers through inhibition of the P-gp efflux system. The mechanism of the micelle effect is not known, however, it is very similar to the micelle effects in BBMEC. This has been previously shown to involve vesicular transport of the micelle-incorporated drug. The study suggests that Pluronic copolymers can be useful in increasing oral absorption of select drugs.

KEY WORDS: block copolymer; intestinal delivery; drug; micelles.

INTRODUCTION

Amphiphilic block copolymers, such as Pluronic copolymers with the basic poly(ethylene oxide)-*block*-poly(propylene oxide)-*block*-poly(ethylene oxide) structure ($EO_{m/2}$-PO_n-$EO_{m/2}$), are of interest as potential drug delivery and drug targeting systems due to their ability to form self-assembling micelles (1–6). In particular the studies using micelles as "microcontainers" for drug delivery have demonstrated the enhanced delivery of haloperidol to the brain, resulting in a significant increase in neuroleptic activity of the drug (2,7). Using the bovine brain microvessel endothelial cells (BBMEC) we have recently demonstrated that Pluronic block copolymers affect drug transport in cells through multiple mechanisms involving inhibition of the glycoprotein P (P-gp) efflux system and redirection of the vesicular transport (8).

Similarly to BBMEC the intestinal epithelium cells, Caco-2 have increased expression of P-gp (9) and display a number of other carrier-mediated and vesicular transport systems found in the intestine *in vivo* (10). The present work characterizes the effects of Pluronic copolymers on the transport of a P-gp-dependent probe, rhodamine 123 (R123) and its non-P-gp-dependent analog rhodamine 110 (R110) in Caco-2 monolayers. This study suggests existence of multiple transport mechanisms with Pluronic copolymers in Caco-2 monolayers.

MATERIALS AND METHODS

Cell Culture

Caco-2 cells, originating from a human colorectal carcinoma (11) were kindly provided by R.T. Borchardt (The University of Kansas, Lawrence, KS). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids, benzylpenicilin (100 U/ml) and streptomycin (10 μg/ml), in an atmosphere of 5% CO_2 as as described elsewhere (12). All tissue culture media were obtained from Gibco Life Technologies, Inc. (Grand Island, NY). The cells were grown on uncoated 24-well plates from Costar Corp. (Bedford, MA). The 50,000 cells were added to each well and grown for 14 days with feeding every second day before the monolayers were used in drug accumulation experiments. Only the cells of passage number 32–45 were used (the cells of high passages (50–60) have shown less resistance to R123 due to decreased P-gp levels).

Preparation of Block Copolymer Solutions

The present study used Pluronic block copolymers (Table 1): Pluronic F68 (F68) (lot #WPOP-590B), Pluronic P85 (P85) (lot #WPOP-587A) and Pluronic L81 (L81) (lot #WSOO-83457 25087) that were provided by BASF Corp. (Parispany, NJ). The polyoxyethylated castor oil, Cremophor EL (lot #106H0615) and polyoxyethylene sorbitan ester, Tween 60 (lot #46H0348) were purchased from Sigma Chemical Corp. (St. Louis, MO). The solutions of Pluronic block copolymers and polyoxyethylated detergents were prepared in assay buffer containing 122 mM sodium chloride, 25 mM sodium bicarbonate, 10 mM glucose, 10 mM HEPES, 3 mM potassium chloride, 1.2 mM magnesium sulfate, calcium chloride (1.4 mM) and potassium phosphate dibasic (0.4 mM). The R123 and R110 were added to the copolymer solutions and incubated at 37°C for at least one hour prior to their use in the experiments.

Cellular Accumulation of R123 and R110

The cellular accumulation of the fluorescent dyes (R123 and R110) was used to examine the effects of the Pluronic block copolymers and polyoxythylated detergents on the cell monolayers. Previous studies with R123 (8,13) indicate that this fluorescent marker is a good probe for examining P-gp. Furthermore, compared to R123, the structural analog R110 has little interaction with P-gp (14). Cell monolayers were preincubated for 30 min. at 37°C with assay buffer. After this the assay buffer was removed and the cells were exposed to 3.2 μM R123 (or R110) in either assay buffer or solutions of Pluronic block copolymers, Cremophore EL or Tween 60. The

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0724-8741/98/0600-0850$15.00/0

cells were incubated with dye solutions for up to 90 min. at 37°C. After that the dye solutions were removed and cell monolayers were washed three times with ice-cold PBS. The cells were then solubilized in 1.0% Triton X-100 and aliquots (25 μl) were removed for determination of cellular dyes using a Shimadzu RF5000 fluorescent spectrophotometer: λ_{ex} = 505 nm, λ_{em} = 540 nm. Samples were taken for protein assay using the Pierce BCA method.

Efflux of R123

For the R123 efflux studies Caco-2 monolayers were grown on 24-well plates as described above. The cell monolayers were preincubated for 30 min. at 37°C with assay buffer and loaded with R123 using three different treatment protocols (A, B, C) for 60 min at 37°C. The A-protocol used 3.2 μM R123 in assay buffer, the B-protocol used 3.2 μM R123 in 22 μM P85, and the C-protocol used 3.2 μM R123 in 11 mM P85. Then all cell monolayers were washed three times with ice-cold PBS. Four monolayers in each treatment protocol were left for determining total R123 accumulation in Caco-2 monolayers. The remaining monolayers were incubated at 37°C in R123-free assay buffer (A-protocol), 22 μM P85 (B-protocol) or 11 mM P85 (C-protocol) respectively. The efflux of R123 in each treatment group was determined by sampling buffer solutions at 0.5, 7, 15 and 22 min. time intervals and measuring the fluorescence in each sample using fluorescent spectroscopy as described above. At the conclusion of the efflux study the monolayers were solubilized in 1% Triton X-100 for protein determination using the Pierce BCA method. The efflux was expressed for each treatment protocol as a percentage of R123 in the media (nmol/mg protein) to R123 accumulated in Caco-2 cell monolayers during the loading period (nmol/mg protein).

Fluorescence Microscopy

For examining R123 cellular accumulation using fluorescence microscopy Caco-2 cell monolayers were grown on uncoated chamber slides (Fisher, St. Louis, MO). The Caco-2 cells were then exposed to R123 in assay buffer, R123 in 22 μM P85 and R123 in 11 mM P85 for 60 min. at 37°C. After this incubation period the R123 solutions were removed, the Caco-2 cell monolayers were washed a total of three times with ice-cold PBS and examined immediately using a Leitz fluorescent microscope.

Table 1. Molecular Characteristics and CMC for Pluronic Block Copolymers ($EO_{m/2}$-PO_n-$EO_{m/2}$)

Copolymer	Molecular mass	Number of EO units, m	Number of PO units, n	HLB[a]	CMC, μM
L81	2500	5.7	39	1–7	24
P85	4500	51	39	12–18	67
F68	8800	159	30	>24	1140

[a] HLB as determined using gel permeation chromatography (BASF Performance Chemicals, Specialty Products, BASF Co. 1991). High HLB values indicate hydrophilic compounds, while low HLB values indicate hydrophobic compounds.

CMC Determination

The CMC of L81, P85, F68, Cremophore EL and Tween 60 were determined at 37°C, pH7.2 using a fluorescent probe (pyrene) technique as previously described (15).

RESULTS

Effects of P85 Unimers on R123 Accumulation in Caco-2 Monolayers

To characterize the effects of the single chains of P85 ("unimers") the cellular accumulation of R123 in Caco-2 monolayers was examined in the presence of 22 μM P85 (0.01% w/v). This concentration of P85 was below its critical micelle concentration (CMC), which is ca. 67 μM (0.03% w/v). As shown in Figure 1A the cellular levels of R123 were increased significantly in the presence of 22 μM P85 compared to those in the assay buffer. The effects of 22 μM P85 on R123 accumulation were observed by 5 min. with the greatest increases in R123 accumulation in Caco-2 monolayers treated with 22 μM P85 occurring at the later time points (30-90 min.). At these time points the cellular levels of R123 were increased approximately 12-fold compared to the control (Figure 1A).

Studies comparing the cellular accumulation of the non-P-gp substrate, R110 (14) were also performed in the presence and absence of 22 μM P85 in Caco-2 monolayers. Unlike the studies with R123, the amount of cell-associated R110 in Caco-2 monolayers was low and unaffected by P85 unimers (Figure 1B).

Effects of P85 Micelles on R123 Accumulation in Caco-2 Monolayers

The effects of P85 were also evaluated above the CMC to characterize the interactions of the P85 micelles with the Caco-2 monolayers (Figure 1A). The treatment of Caco-2 cells with 11 mM P85 (5% w/v) caused an initial increase in R123 accumulation compared to the control groups. At 15 min. the levels of R123 in Caco-2 monolayers treated with 11 mM P85 were approximately 4-times greater than those observed in the control group. However, at time points past 15 min. the cellular accumulation of R123 in the presence of micelles leveled off,

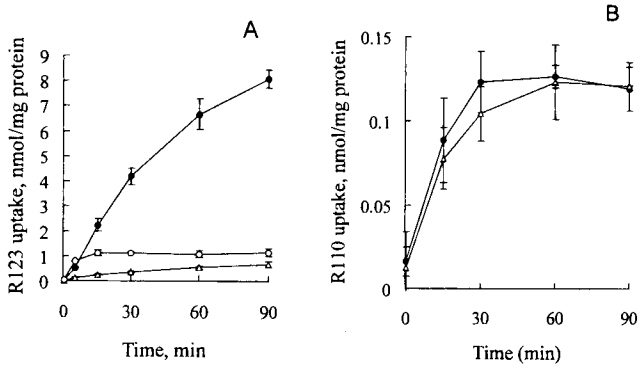

Fig. 1. Effect of P85 on R123 (A) and R110 (B) accumulation in Caco-2 monolayers. Accumulation of the probes was examined using assay buffer (open triangles), 22 μM P85 (filled circles) and 11 mM P85 (open circles).

while cellular R123 in control monolayers continued to increase during the 90 min. of observation (Figure 1A).

Fluorescent Microscopy Studies in Caco-2 Monolayers

Fluorescent microscopy studies confirm the effect of P85 unimers and micelles on R123 accumulation in Caco-2 monolayers (Figure 2). Following a 60 min. exposure to R123 there is a very significant increase in cellular fluorescence in the P85 unimer treatment groups, compared to control groups receiving R123 in assay buffer (Figure 2A,B). While the cellular fluorescence in the micelle treatment group is also increased compared to the control groups (Figure 2A,C). However, this effect was much less compared to the unimer treatment group (Figure 2B,C). The increased accumulation of R123 with the P85 unimer and micelle treatment groups appears as an increased fluorescence in the cytosol of the cell.

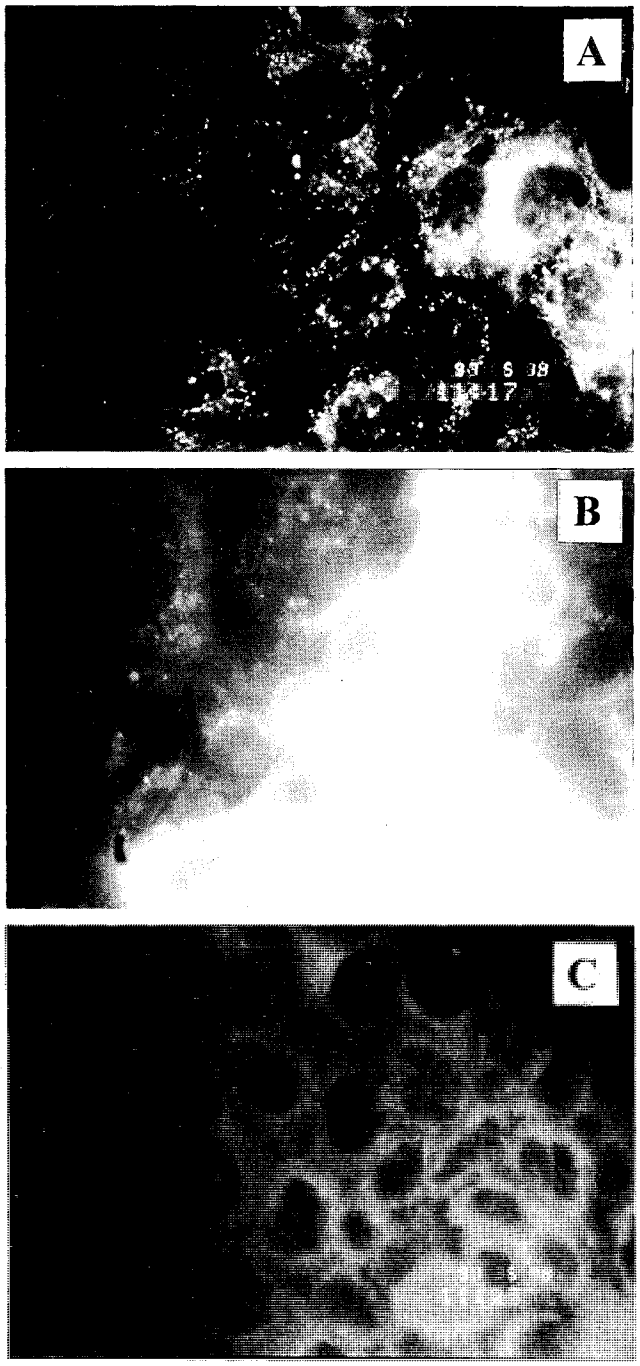

Fig. 2. Fluorescent microphotography of R123 accumulation in Caco-2 monolayers following 60 min. exposure to (A) R123 in assay buffer, (B) R123 in 22 μM P85 and (C) R123 in 11 mM P85.

Effects of P85 Unimers and Micelles on R123 Efflux in Caco-2 Monolayers

The effects of P85 unimers and micelles on efflux of R123 in the Caco-2 monolayers were examined following 60 min. loading of these cells with the drug. In the control group treated with the assay buffer approximately 55% of R123 contained in the cells at time zero effluxes in the external medium during the 20-min. efflux period (Figure 3). Almost complete inhibition of the R123 efflux was observed at 22 μM P85: the amounts of R123 released in the media during 20 min. were less than 10% of the initial levels of R123 in the cells (Figure 3). In contrast, in the presence of 11 mM P85 the efflux was significantly increased when compared to the control group (Figure 3). In this case over 90% of cellular R123 effluxes in the external media during 20 min. incubation. Therefore the P85 micelles appear to increase R123 efflux while the unimers inhibit it.

Effects of Pluronic Copolymer Structure on R123 Accumulation

The effects of Pluronic block copolymers with different lengths of hydrophilic (EO) and hydrophobic (PO) segments on R123 accumulation in Caco-2 monolayers were examined using L81, P85 and F68. The results are presented in Figure 4 as dependencies of R123 accumulation at 60 min on copolymer concentration (M). One common pattern observed with these block copolymers was that the accumulation of R123 reached maximal levels in the proximity of the respective copolymer CMC (Figure 4). Below the CMC there was an initial increase in the R123 accumulation with increasing copolymer concentration. Above the CMC the accumulation first leveled off and

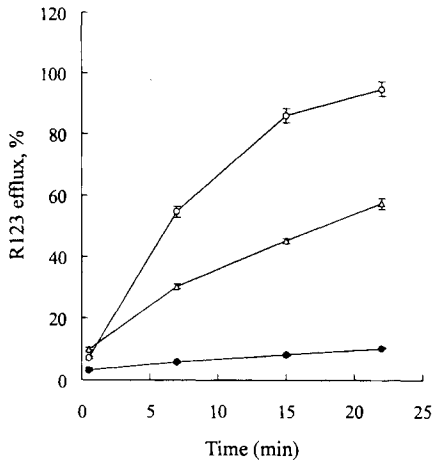

Fig. 3. Effect of P85 on R123 efflux in Caco-2 monolayers. Efflux was examined using assay buffer (open triangles), 22 μM P85 (filled circles) and 11 mM P85 (open circles).

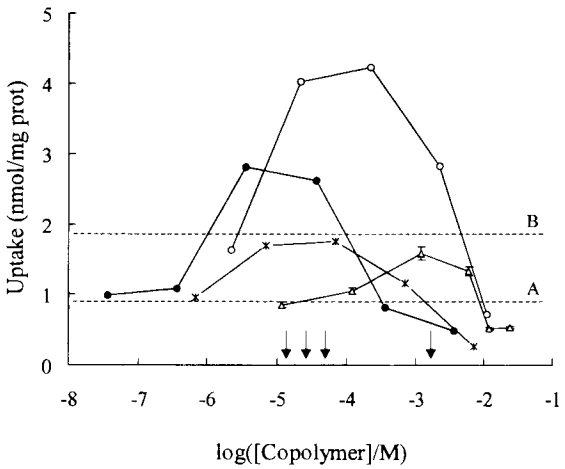

Fig. 4. Effect of L81 (filled circles), P85 (open circles), F68 (triangles) and Cremophor EL (crosses) on R123 accumulation in Caco-2 monolayers following 60-min. exposure to the probe. The dashed lines show R123 accumulation in (A) assay buffer and (B) 4 μg/ml CSA. The vertical arrows from left to right show the CMC of L81, Cremophor EL, P85, and F68.

then decreased, reaching levels below those observed in the control groups. The cellular levels of R123 varied significantly for the different Pluronic block copolymers. The greatest R123 accumulations were observed with P85 at 22 to 220 μM (Figure 4). In these cases the increases in cellular R123 surpassed significantly those observed following treatment with the P-gp inhibitor, CSA (Figure 4). Approximately 3.5-fold increases in R123 levels compared to the control were also observed with 4 to 40 μM of the most hydrophobic copolymer, L81 (Figure 4). These increases were less compared to the P85 effects but they were observed at approximately 5-fold lower concentrations of the copolymer. In contrast, the most hydrophilic copolymer, F68 was the least active among the Pluronic block copolymers studied. The R123 accumulation in Caco-2 cells was increased with F68 only 25% compared to the control group. The effects were observed near the CMC of F68 at ca. 1.1 mM, which was a much higher concentration compared to both P85 and L81.

Comparison with Nonionic Detergents

The effects of Pluronic block copolymers in Caco-2 cell monolayers were compared with those of the Cremophor EL and Tween 60 that were previously described as P-gp modifying agents (16,17). The concentration dependencies for R123 accumulation with the Cremophor EL (Figure 4) and Tween 60 (not shown in figure) were generally similar to those observed with the Pluronic block copolymers. Maximal levels of cellular R123 were observed at respective CMC values: 40 μM for Cremophor EL and 42 μM for Tween 60. The R123 levels observed with Cremophor EL were slightly higher than these observed with Tween 60 (not shown in figure). But they were much less than the maximal effects of P85 and L81 and did not exceed the increases observed with the P-gp modifying agent, CSA. Similarly to Pluronic block copolymers, at concentrations of Cremophor EL and Tween 60 above the CMC, a substantial decrease in the R123 accumulation was observed.

DISCUSSION

Effects of the Unimers

This study suggests that at concentrations below the CMC Pluronic block copolymers enhanced accumulation and inhibited efflux of R123 in Caco-2 monolayers. The block copolymer unimers did not alter the transport of the P-gp-independent probe, R110 under these conditions. This suggests that the unimers are blocking P-gp efflux system rather than altering the membrane permeability in a nonspecific way. Furthermore, inhibition of R123 efflux with P85 unimers supports their effects on the P-gp efflux pump in Caco-2 monolayers. The fluorescence microscopy studies revealed that P85-induced changes in cell associated R123 resulted from an increased fluorescence in the cytosol of the cell. This conclusion is consistent with the previous work describing inhibitory effects of Pluronic block copolymers on P-gp efflux systems in multiple drug resistant cancer cell lines (18,19) and BBMEC (8).

The effects of nonionic detergents, such as Cremophor EL and Tween 60 on P-gp efflux system in Caco-2 cells were previously reported (16,20). This paper compares the effects of Pluronic block copolymers on R123 accumulation in Caco-2 monolayers with the effects of Cremophor EL and Tween 60. The results suggest that the most effective Pluronic block copolymers, P85 and L81 induce significantly higher levels of cellular R123 than these detergents. Therefore, Pluronic copolymers can be useful in increasing oral absorption of P-gp substrates.

This paper suggests that the effects of Pluronic block copolymers on R123 transport in Caco-2 monolayers strongly vary for copolymers having different segment lengths. To summarize these effects the activity of unimers increases with elevation in the hydrophobicity of block copolymers. As a result more hydrophobic copolymers, having longer PO segments or shorter EO segments (21), cause higher R123 accumulations at lower concentrations, i.e. are more "potent" P-gp inhibitors. However, copolymers with intermediate hydrophobicity cause higher R123 accumulations and are more efficient at intermediate concentrations. This pattern observed in Caco-2 monolayers has been also found with other P-gp expressing cells, such as multiple drug resistant KBv cells and BBMEC (in preparation).

To explain the differences in the potency and efficacy of Pluronic copolymers one should take into account the differences in their CMCs. The more hydrophobic the block copolymers are the lower the CMC they have (21). Since CMC is the cut-off point at which the concentration of the unimers levels off (15) the P-gp inhibition effects mediated by the unimers are also saturated at the CMC. Therefore upon concentration increase the amount of the unimers and effects of the more hydrophobic (more potent) copolymers level off at their (lower) CMC. At the same time the amount of the unimers of less hydrophobic (less potent) copolymers continue to elevate until their (higher) CMC is reached. This results in a situation when the block copolymer with an intermediate hydrophobicity, such as P85, is the most efficient since higher concentration of its unimers can be achieved at the CMC compared to more hydrophobic L81. With very hydrophilic block copolymers, such as F68, the net efficacy decreases due to much lower potency in P-gp inhibition, even though higher concentrations of the unimers can be achieved.

Effects of the Micelles

The effects of the micelles on R123 accumulation in cells were very different from those of the P85 unimers. In the case of the micelles there was an initial rapid increase in R123 accumulation during the first 15-min, with cellular R123 leveling off at incubation times more than 15 min. Conversely, the free R123 or R123 and P85 unimer mixtures revealed steady increase in R123 levels during the entire incubation period. Furthermore, in contrast to P85 unimers the P85 micelles increased the efflux of R123 in Caco-2 monolayers, rather than inhibited it. A similar difference in transport mechanisms with the P85 unimers and micelles were reported recently for several cells, including BBMEC, KB, KBv and human umbilical vein endothelial cell (HUVEC) monolayers (8).

At physiological conditions P85 micelles represent aggregates of ca. 60 block copolymer molecules with a hydrodynamic diameter of ca. 15 nm. (15). The hydrophilic shell of these micelles is made of EO chain segments and their core consists of tightly packed hydrophobic PO chain segments. Above the CMC the hydrophobic and amphiphilic probes such as R123 incorporate into the micelle core thus becoming masked from the external medium. The fraction of the micellar R123 increases and the fraction of the free R123 decreases with elevating the micelle concentration. That is at 11 mM P85 (5% w/v) used for characterization of the micelle effects in this study about 95% of R123 is incorporated into the P85 micelles and only 5% of the probe are still in the free form (8). Under these conditions interactions of the micellar form of R123 with cells become very significant. Indeed, the effects of the P85 micelles have been previously shown to involve vesicular transport of the micelle-incorporated probe, which is different from the free probe entering cell through the transmembrane diffusion (8).

The recent studies by Nerurkar et al. (16,20) suggested that the micelles of nonionic detergents, such as Cremophor EL and Polysorbate 80 decrease the transport of peptides through Caco-2 cells. This effect was attributed to the association of the peptides to the micelles resulting in the decrease of the concentration of the peptide above the detergent CMC. Similar consideration is valid also in the case of the Pluronic block copolymers. It explains the bi-modal (bell-shaped) dependencies of R123 transport reported earlier for BBMEC (8) and observed in this paper with Caco-2 monolayers (Figure 4). That is although above CMC there are unimers present in the system, there is also much less free R123 available for transport. However, in contrast to Nerurkar et al. (16,20) we believe that the micelles carrying the drug might be transported into the cells and then recirculated back at the cell surface through vesicular transport mechanisms (8). Further studies shall validate this hypothesis with Caco-2 monolayers as well as other cells.

ACKNOWLEDGMENTS

We would like to acknowledge the support of this work by the National Institutes of Health (R01 NS36229-01A1) and Nebraska Research Initiative. We would like to thank Dr. R.T. Borchardt for providing Caco-2 cells, and BASF Co. for the gift of Pluronic block copolymers.

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