DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY®
Vol. 30, No. 7, pp. 767–774, 2004

Potential Mechanisms by Which Peceol® Increases the Gastrointestinal Absorption of Amphotericin B

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ABSTRACT

Purpose. The purpose of this study was to ascertain how the incorporation of AmpB into a glyceride-rich excipient Peceol® significantly increased Amphotericin B's (AmpB) gastrointestinal absorption in white male Sprague-Dawley rats. Based on preliminary studies, our working hypothesis was that incorporation of AmpB into mixed micelles composed of Peceol® would significantly enhance gastro-intestinal (GI) tract absorption by increasing lymphatic drug transport and decreasing P-glycoprotein (PGP)-mediated drug efflux. *Methods. I. Lymphatic Transport Studies:* Following an overnight fast (12–16 hr) and 48 hr postsurgery, rats were divided into two treatment groups and received a single-dose oral gavage (1 mL total volume) at 0700 h of either desoxycholate (DOC)–AmpB (5 mg AmpB/kg; n = 6 at each time point) or AmpB incorporated into 100% Peceol® (Peceol®–AmpB; 5 mg AmpB/kg; n = 6 at each time point). Mesenteric lymph samples were obtained prior to and at 0–4-hr, 4–6-hr, and 6–8-hr intervals post oral gavage. An equal volume of normal saline (1 mL) was administered intravenously to the animal following each blood draw to prevent fluid depletion throughout the duration of the study. Lymph was immediately harvested by centrifugation and analyzed for drug by high-performance liquid chromatography (HPLC). *II. Multidrug Resistance 1 (mdr-1) Studies:* Caco-2 cells were seeded at 10,000 cells/cm^2 in T-75 flasks. When the cells

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DOI: 10.1081/DDC-120039793
0363-9045 (Print); 1520-5762 (Online)

reached 80% confluency, they were treated for 1 day and 7 days with 0.1% to 1.0% (v/v) Peceol® or media alone (control). Following treatment, total RNA was isolated using TRIzol® reagent, followed by reverse transcription into single-stranded cDNA. Polymerase chain reactions (PCR) were performed with specific primers for *mdr-1*. The PGP protein was determined by Western Blot Analysis. *Results.* Mean weight of rats was not significantly different prior to and following drug administration. Similarly, kidney, liver, lung, spleen, and heart weights were not different between DOC–AmpB and Peceol®–AmpB treatment group. A significantly greater amount of AmpB was transported through the mesenteric lymph duct for all the time intervals used following the administration of Peceol®-AmpB treatment group compared to the administration of DOC–AmpB (suspension). A significant lower *mdr-1* mRNA and PGP protein expression within Caco-2 cells was observed following 1 and 7 days treatment with Peceol® 0.1% to 1.0% (v/v) compared to nontreated controls. *Conclusions*. Taken together, these findings suggest that Peceol® increases the gastrointestinal absorption of AmpB by increasing the amount of drug that is transported through the mesenteric lymph duct and by decreasing *mdr-1* mRNA and PGP protein expression, resulting in lower PGP-mediated AmpB efflux.

Key Words: Peceol®; Amphotericin B; Gastrointestinal absorption; P-glycoprotein.

INTRODUCTION

Amphotericin B (AmpB) formulated as a micellar suspension (Fungizone®) remains one of the most effective agents in the treatment of systemic fungal infections.[1,2] However, its use is often limited by the development of dose-dependent kidney toxicity manifested by renal vasoconstriction with a significant decrease in glomerular filtration rate and renal plasma flow and by renal potassium and magnesium wasting.[1–3] A number of studies have reported that AmpB, solubilized in methanol, is poorly absorbed from the gastrointestinal (GI) tract[4–6] and, therefore, is not commonly administered orally but intravenously, which can result in the aforementioned renal toxicity.

Improved GI absorption of poorly absorbable drugs can be achieved by increasing the dissolution rate of the drug in the presence of bile acids. Within the GI tract bile salts behave as biological detergents that form thermodynamically stable mixed micelles when mixed with phospholipids. Numerous studies have observed enhanced absorption of poorly absorbable drugs when administered as mixed micellar solutions.[7–9] When AmpB was incorporated into mixed micelles containing bile acids and phospholipids, it resulted in increased AmpB intestinal permeability and subsequent GI absorption using a rat intestinal perfusion methodology.[4] The limitation of this study was that various AmpB-mixed micelle formulations were perfused through a cannulated upper intestine of an anesthetized rat. This model does not account for the effect of anesthesia and was not done in a whole animal model. Furthermore, the toxicological consequences of improving GI absorption were not investigated in this study, specifically, AmpB's dose-dependent kidney toxicity, which limits the use of this compound.

Our laboratory has recently reported that plasma AmpB C_{max} and $AUC_{0\text{-}24\ h}$ were significantly elevated in rats administered triglyceride (TG)-rich AmpB formulations (i.e., Peceol®–AmpB) compared to desoxycholate (DOC) AmpB or AmpB solubilized in methanol.[10] Our preliminary data[10] suggest that the increase in AmpB GI absorption when formulated in Peceol® may be partly explained by increased lymphatic transport of the drug. However, the poor absorption of AmpB could also be explained secondary to P-glycoprotein™ (PGP) extrusion. Therefore, it may be possible that Peceol® may decrease PGP protein expression and activity,[11,12] resulting in less AmpB being excreted back into the intestine. This possibility may not be negligible if it is taken into account that AmpB can be transported by multidrug resistant systems of various fungal strains.[13–15]

The purpose of this study was to ascertain how the incorporation of AmpB into a glyceride-rich excipient [Peceol®; Glycerol Mono-oleate 40-EP; Gattefosse Canada Inc., Montreal, Quebec, Canada composed of mono- (32–52%), di- (30–50%), and triglycerides (5–20%)] significantly increased AmpB's gastrointestinal absorption in white male Sprague-Dawley rats. Based on preliminary studies, our working hypothesis was that incorporation of AmpB into mixed micelles composed of Peceol® would significantly enhance GI tract absorption by increasing lymphatic drug transport and decreasing PGP-mediated drug efflux.

METHODS AND MATERIALS

AmpB (purchased from Department of Pharmaceutical Services, Vancouver General Hospital, Vancouver, British Columbia, Canada) was administered as an oral gavage to rats, at a dose of 5 mg/kg. AmpB was preformulated as a micelle, which contains sodium desoxycholate with sodium phosphate as a buffer (DOC–AmpB; Fungizone; Bristol Myers Squibb, Nutley, NJ), and reconstituted in sterile water (5 mg/mL). In addition, AmpB was formulated as a lipid suspension using Peceol® (Peceol®–AmpB). Dispersion of lipid droplets into an emulsion of high surface area is an essential step in the efficient intestinal absorption of lipids. Peceol® is a readily dispersible, solubilizing agent comprised primarily of a mixture of mono- and diglycerides of oleic acid, which closely resembles the end products of intestinal lipid digestion.[7] Previous studies have demonstrated a significant increase in absorption of the hydrophobic drug, cyclosporine A, from ''predigested'' olive oil, when compared to a nondigested control.[16]

Peceol® purchased from Gattefosse Canada Inc. (Montreal, Quebec, Canada) was chosen for the Self-Emulsifying Drug Delivery System (SEDDS) formulation because of the ability of this combination to solubilize AmpB in high concentration while providing an oral delivery system with rapid self-emulsifying properties (unpublished results, Wasan et al.). The SEDDS formulations containing 10 mg/mL of AmpB were prepared by dissolving, with stirring and gentle heating, AmpB in a 100% Peceol®. AmpB has a triglyceride solubility of 10 to 30 mg AmpB per mg of triglyceride (Wasan et al., unpublished results).

Lymphatic Transport Studies in Rats

Adult male Sprague-Dawley rats (380–450 g) were used in this study. The rat is an appropriate animal model to investigate the GI absorption of AmpB following oral administration, due to similarities in intestinal characteristics (i.e., anatomical, metabolic, and biochemical characteristics)[17–20] between rats and humans. AmpB levels in mesenteric and thoracic lymph were analyzed by high-performance liquid chromatography (HPLC) as previously described,[3,21,22] using an LDC/Milton Roy pump with a Milton Roy variable wavelength detector set at a wavelength of 405 nm. Mobile phase (0.005 M EDTA: methanol 35:65 v/v) elutes on an LC-1 Supelco column (5 μm, 4.6×150 mm) at a flow rate of 2 mL/minute at ambient temperature. Retention time of AmpB was 3.5 minutes with a run time of 4 minutes. The assay was sensitive to 50 ng/mL with a linear range of 50–1000 ng/mL (%CV interday variability between 5–8%).[3,21,22] Prior to oral gavage, the mesenteric lymph duct was cannulated by methods previously developed by our laboratory[23] and others.[7]

All rats were cared for in accordance with the Canadian Council on Animal Care and the University of British Columbia (UBC) guidelines. Adult male Sprague-Dawley rats were obtained from UBC animal care unit (Vancouver, B.C., Canada). The rats were maintained under a 12-hr light (0700–1900)/dark cycle and supplied with a standard laboratory diet (PMI Feeds, Richmond, VA) and water ad libitum. Following an overnight fast (12–16 hr) and 48 hr post-surgery, rats were divided into two treatment groups and received a single-dose oral gavage (1 mL total volume) at 0700 h of either DOC–AmpB (5 mg AmpB/kg; $n=6$ at each time point) or AmpB incorporated into 100% Peceol® (Peceol®–AmpB; 5 mg AmpB/kg; $n=6$ at each time point). All animals had free access to the electrolyte solution throughout the course of the study. All animals were allowed recovery from the surgery and given postsurgical analgesics (with a short half-life) 24 h prior to the start of the study if necessary. Allowing for analgesics only 24 h before the study assured that the absorptive effects of AmpB observed from the different formulations was not due to any analgesic-induced disturbances in oral bioavailability.

Mesenteric lymph samples were collected prior to and from 0 to 4 hours, between 4 and 6 hours, and between 6 and 8 hours after the administration of the drug. An equal volume of normal saline (1 mL) was administered intravenously (IV) to the animal following each blood draw to prevent fluid depletion throughout the duration of the study. Lymph was immediately harvested by centrifugation and analyzed for drug by HPLC. The animals were permitted free access to food 4 h postdosing. After the 6–8-hr interval was completed, each rat was humanely sacrificed. A total lipid mass of 1.84 g/kg was administered with the lipid suspension. For the SEDDS formulations, a total lipid mass of 1.33 g/kg was administered. In all studies after each lymph collection interval, 1 mL of normal saline was administered to each rat to prevent dehydration.

AmpB concentrations recovered within the mesenteric lymph samples for each collection interval were compared between the different treatment groups by Mann-Whitney nonparametric test.[24] Critical differences were assessed by Newman-Keuls posthoc tests. A difference was considered significant if the probability of chance in explaining the results was reduced to less than 5% ($p<0.05$). All data were expressed as a mean ± standard deviation.

Multidrug Resistance 1 (*mdr-1*) and P-Glycoprotein (PGP) Studies in Caco-2 Cells

In a second series of studies, Caco-2 cells were seeded at 10,000 cells/cm^2 in Corning T-75 cell culture flasks. The growth media [Dulbecco's minimal essential medium (DMEM)] containing 10% heat-inactivated fetal bovine serum, 292 μg/mL glutamine, 0.1 mM nonessential amino acids, 100 U/mL penicillin, and 100 mg/mL glutamine was changed every other day. The cells were kept at 37°C in a humidified atmosphere of 5% CO_2. When the cells reached 80% confluency they were treated for 1 and 7 days with 0.10% to 1% (v/v) Peceol® or media alone (control). Media and treatment were changed every other day.

Total RNA was isolated using TRIzol® reagent (Invitrogen, Vancouver, B.C., Canada), followed by reverse transcription into single-stranded cDNA. The PCR reactions were performed with specific primers obtained from Oligonucleotide Synthesis Laboratory at UBC for *mdr-1* Forward: 5′-GTC-ATT-GTG-GAG-AAA-GGA-AAT-CAT-G-3′ and Reverse: 5′ATT-CCA-AGG-GCT-AGA-AAC-AAT-AGT-G-3′ and the internal standard gene GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) Forward: 5′-TGA-AGG-TCG-GAG-TCA-ACG-GAT-3′ and Reverse: 5′-TCG-CTC-CTG-GAA-GAT-GGT-GAT-3′. The PCR conditions were as follows: 94°C for 5 min, 94°C for 1 min, 55°C for 1 min, 72°C for 1 min 30 sec, repeat Step 2 29 times, 72°C for 10 min, 4°C. Primer drop at cycles 9 to 12 (Fig. 1). A sample from each PCR product was subjected to electrophoresis on a 1.5% agarose gel. The fluorescent bands were visualized under ultraviolet (UV) light (UV-Epi Chemi II) and quantified with UVP-labworks software.

After 1 or 7 days of treatment, cells were harvested in lysis buffer (Mem-PER® Eukaryotic Membrane Protein Extraction Kit from PIERCE Rockford, IL, USA) containing protease/phosphatase inhibitors. Total membrane protein (equivalent to 30 μg for each sample) was loaded onto 10% SDS-Page gel and then electroblotted onto a polyvinylidene difluoride (PVDF) membrane (0.45 micron) at 80 V for 2 hours

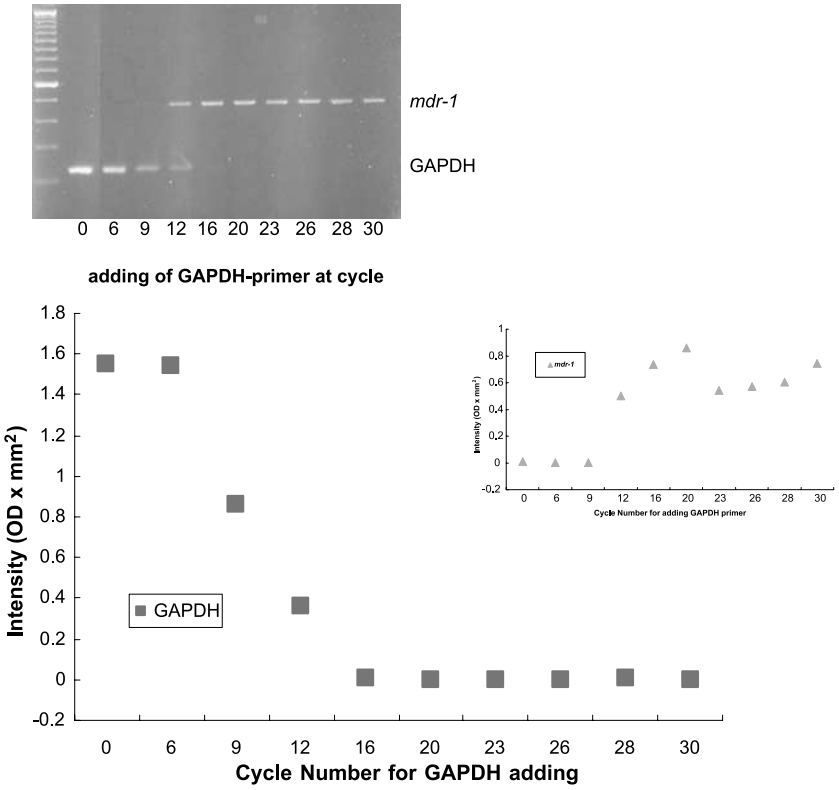

Figure 1. *mdr-1* gene expression in Caco-2 cells: Titration for primer drop of GAPDH. GAPDH was added at 0, 6, 9, 12, 16, 20, 23, 26, 28, and 30 cycle (0.5 μM GAPDH-Forward + 0.5 μM GAPDH-Reverse); Procedure: 50 ng cDNA (Caco-2 cells), 0.5 μM *mdr-1* Forward + 0.5 μM *mdr-1* Reverse; PCR program: 94°C for 5 min, 94°C for 1 min, 55°C for 1 min, 72°C for 1 min 30 sec, repeat from Step 2 for 29 more times, 72°C for 10 min, 4°C.

Table 1. Effect of Peceol® on the lymphatic transport through the mesenteric lymph duct of Amphotericin B in rats following a 5-mg/kg oral gavage dose of Amphotericin B.

	Amount (μg) of Amphotericin B transported lymphatically by time interval			Total lymph output (mL)
Formulation	0–4 h	4–6 h	6–8 h	0–8 h
Suspension (n=6)	25.4–6.4	14.0–2.3	5.2–0.9	30.5–5.6
Peceol® (n=6)	51.4–24.5	38.2–18.5[a]	64.4–27.5[a]	19.4–5.9

[a]$p<0.05$ vs. suspension formulation using normal scores ranks. Amphotericin B suspension is Fungizone and Amphotericin B in Peceol® is the drug dissolved in 100% Peceol. Data reported as mean +/− standard error of the mean.

and 50 V for 2 hours. The membrane was incubated overnight at 4°C in blocking buffer (1 × PBS, 1% nonfat, dried milk, 0.1% Tween-20). After a 2-hour incubation in primary monoclonal antibody (C219, Signet Pathology System Dedlam; dilution 1:300), the membrane was washed three times in blocking buffer, incubated for 1 hour in 1:2000 antimouse IgG rabbit HRP-conjugated antibody (from Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) and then followed by three washing steps with blocking buffer and one with 1 × PBS and 0.1% Tween-20. The membrane was exposed to X-ray film after incubation in Western Lightning Chemiluminescence Reagent for 1 minute (Perkin Elmer Life Sciences, Mississauga, Ontario, Canada).

The ratio of *mdr-1* mRNA and GAPDH was compared between the different treatment groups by the Mann-Whitney nonparametric test.[24] Critical differences were assessed by Newman-Keuls posthoc tests. A difference was considered significant if the probability of chance in explaining the results was reduced to less than 5% ($p<0.05$). All data were expressed as a mean ± standard deviation.

RESULTS AND DISCUSSION

The mean weight of rats was not significantly different prior to and following drug administration (data not shown). Similarly, kidney, liver, lung, spleen, and heart weights were not different between DOC–AmpB (suspension) and Peceol®–AmpB treatment groups (data not shown). A significantly greater amount of AmpB was transported through the mesenteric lymph duct for all the time intervals used following the administration of Peceol®–AmpB compared to the administration of DOC–AmpB (suspension) (Table 1). A significantly lower *mdr-1* mRNA (Figs. 2 and 3) and PGP protein expression (Fig. 4) within Caco-2 cells was observed following 1 [Fig. 2; at 0.25% Peceol® (v/v)] and 7 days [Fig. 3 at 0.25% Peceol® (v/v)] treatment with Peceol® compared to nontreated controls.

The administration of intravenous AmpB has been limited by its dose-dependent kidney toxicity that has not been predicted by monitoring plasma and/or serum drug concentration.[1–3] A number of studies have reported that AmpB, solubilized in methanol, is poorly absorbed from the gastrointestinal (GI) tract[4–6,25] and therefore is not commonly administered orally but intravenously, which can result in the aforementioned

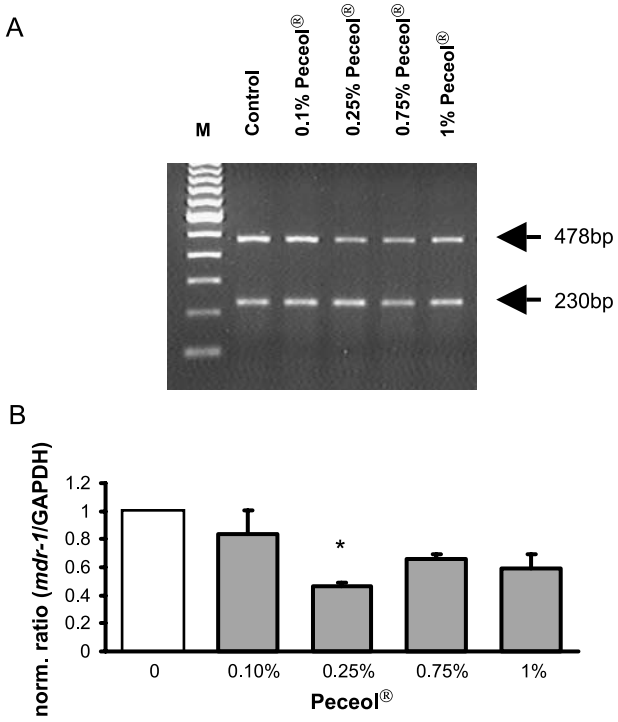

Figure 2. A) Expression profile of *mdr-1* (478bp) was examined as an effect of Peceol® (0.1% to 1% v/v) incubation for 24 hours. B) Bands were quantified with UVP-labworks software. Data represent the average ± SD of two experiments with two repeats *($P<0.05$) each.

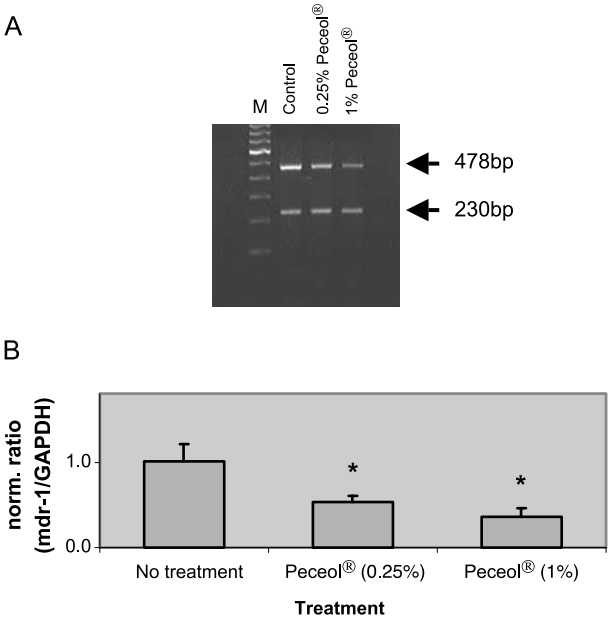

Figure 3. A) Expression profile of *mdr-1* (478bp) was examined as an effect of Peceol® (0.25% and 1% v/v) incubation for 1 week. B) Bands were quantified with UVP-labworks software. Data represent the mean ± standard deviation of three independent experiments with two repeats each ($p < 0.05$).

renal toxicity. However, to date, few studies investigating the development and evaluation of an oral AmpB formulation have been reported.

Our laboratory has recently reported considerable differences in the plasma and tissue concentration of AmpB following administration of Peceol®–AmpB compared to DOC–AmpB.[10] Plasma AmpB C_{max} and $AUC_{0-24\ h}$ were elevated in rats administered Peceol®–AmpB compared to DOC–AmpB.[10] In addition, we reported that AmpB incorporated into TG-based oral formulations (i.e., Intralipid and Peceol) are made less renal toxic than intravenous or orally administered DOC–AmpB by decreasing the concentration of AmpB recovered in the kidney and increasing the concentration of AmpB recovered in the liver.[10] Recently, others have reported that incorporation of AmpB into TG-rich parenteral formulations reduced AmpB-induced toxicity without altering its antifungal activity.[26–29] However, until now, few studies using oral TG-rich formulations to reduce AmpB-induced toxicity and explaining why Peceol® increases the GI absorption of AmpB have been published.

Results from our current investigation suggest that Peceol® may increase the GI absorption of AmpB by increasing the drug's transport through the mesenteric lymph duct (Table 1). This result may be explained by the fact that lymphatic transport of many water-insoluble drugs occurs concurrently with triglyceride absorption from the gastrointestinal tract,[7,8,30] and that Peceol® could provide an efficiently absorbed source of lipid for promoting lymphatic drug transport, thus increasing the oral absorption of AmpB. Previous studies have demonstrated a significant increase in absorption of the hydrophobic drug, cyclosporine A, from ''predigested'' olive oil, when compared to a nondigested control.[16]

Our results further suggest that lymphatic transport may only be a partial explanation for the increased absorption of AmpB when incorporated into Peceol®. We further hypothesized that lipid excipients, such as Peceol®, may increase AmpB GI absorption by inhibiting PGP-mediated efflux of AmpB back into the GI lumen. Results from our current investigation support this hypothesis by showing that Peceol® significantly decreased *mdr-1* mRNA (Figs. 2 and 3) and PGP protein expression (Fig. 4) following 1 and 7 days of treatment to Caco-2 cells. In the clinic AmpB is administered once daily over a 7–10-day time frame to treat fungal infections.[2] Therefore, demonstrating that Peceol® can down-regulate *mdr-1* mRNA and PGP protein expression continuously throughout the duration of AmpB therapy may further suggest that Peceol® could increase AmpB GI absorption for a sustained period of time. A limitation of this study was the use of an intestinal cell line and thus not being able to link these results to our prior lymphatic findings in rats. Future studies to determine if Peceol® decreases intestinal *mdr-1* mRNA expression and PGP efflux

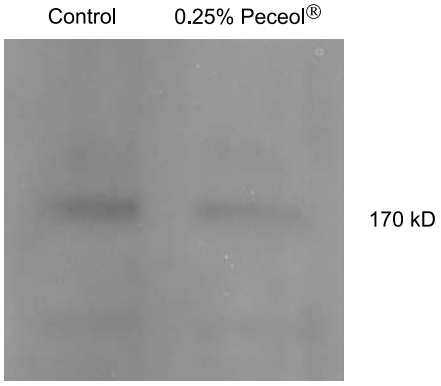

Figure 4. Expression of PGP in Caco-2 cells by Western Blotting. Control and 1-week treatment with 0.25% Peceol®. The Caco-2 cells, which were treated with 0.25% (v/v) Peceol®, show a decrease in PGP protein expression (representative figure).

activity in vivo are required. In addition, functional studies to confirm that this decrease in PGP protein expression results in decreased AmpB efflux from these cells are required.

In conclusion, these studies suggest that Peceol® increases the GI absorption of AmpB by increasing mesenteric lymphatic AmpB transport and decreasing *mdr-1* mRNA and PGP protein expression, resulting in lower PGP-mediated AmpB efflux.

ACKNOWLEDGMENTS

This study was supported with an operating grant from the Canadian Institutes of Health Research (Grant #MOP-49432 to KMW).

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