Transport Characteristics of Tryptanthrin and its Inhibitory Effect on P-gp and MRP2 in Caco-2 Cells

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ABSTRACT - Purpose. Tryptanthrin, an indole quinazoline alkaloid with multiple medical activities, has been recently under preclinical development as an anti-tuberculosis and anti-tumor drug. The aims of this study are to characterize the intestinal transport of tryptanthrin in Caco-2 cells, to determine whether P-glycoprotein (P-gp) and multidrug resistance-associated protein 2 (MRP2) are involved in this issue, and to evaluate the potential influence of tryptanthrin on the function of P-gp and MRP2. **Methods**. Transport assays of tryptanthrin were performed in Caco-2 monolayers with or without the supplement of P-gp and MRP2 inhibitors. Transport assays of P-gp and MRP2 substrates were also performed in the presence of tryptanthrin. The effect of tryptanthrin on the expression of P-gp and MRP2 was analyzed by reverse transcriptase-PCR. **Results**. Both absorption and secretion of tryptanthrin were concentration-independent at a low concentration range of 0.8–20 μM. The apparent permeability (P_{app}) for the apical (AP) to basolateral (BL) was $6.138 \pm 0.291 \times 10^{-5}$. The ratio of $P_{app\ (BL\rightarrow AP)}$ to $P_{app\ (AP\rightarrow BL)}$ was 0.77, suggesting greater permeability in the absorptive direction. Both the P-gp inhibitor, verapamil, and the MRP2 inhibitor, glibenclamide, didn't affect the efflux transport of tryptanthrin. The efflux transport of the P-gp substrate, digoxin, and the MRP2 substrate, pravastatin sodium, decreased when tryptanthrin was present. However, tryptanthrin didn't change the expression of P-gp and MRP2. **Conclusions.** Tryptanthrin was well absorbed across the Caco-2 monolayers, and its transepithelial transports were dominated by passive diffusion. Tryptanthrin was not a substrate, but a potential inhibitor of P-gp and MRP2.

INTRODUCTION

Tryptanthrin (indolo[2,1-b]quinazolin-6,12-dione, Figure 1) is a natural product of the Chinese medicinal plant, *Strobilanthes cusia*. It has generated interest as a potential therapeutic agent because of its simple structure convenient to synthesis and broad spectrum of biological activities. Some of these biological properties of tryptanthrin include antifungal activity against various *Trichophyton, Microsporum,* and *Epidermophyron* species (1), anti-growth of *Leishmania donovani*, *Trypanosoma brucei*, and *Plasmodium falciparum* (2-4), and anti-inflammation via inhibiting cyclooxygenase-2 activity or down-regulating the expression of nitric oxide synthase (5-7). Particularly, the in vitro antitumor activity of tryptanthrin has been observed in a number of cancer cell lines, including leukemia U937, breast MCF-7, glioma U251, colon SW620, and lung H522 (8-9), and the *in vivo* antitumor activity of tryptanthrin has been observed as well (10). Above all, the most attractive and important biological activity of tryptanthrin is its anti-tuberculosis property. It has been demonstrated that tryptanthrin is more potent against *Mycobacterium tuberculosis* [minimum inhibitory concentration (MIC) = 1 mg/L], in Bactec studies, than against *M. smegmatis* (4 mg/L), in agar-dilution work (11). Furthermore, the previous study with a panel of multiple drug-resistant (MDR) tuberculosis strains showed that tryptanthrin retained its potency (0.5–1.0 mg/L); while isoniazid decreased its activity from 4.0 to 16.0 mg/L (11). Taken together, these reports reveal that tryptanthrin is a potential agent against tuberculosis, particular MDR-tuberculosis.

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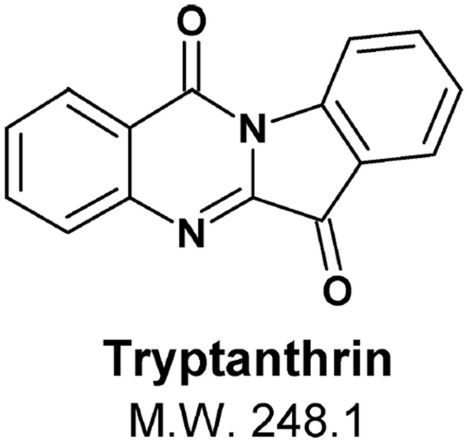

Figure 1. Structure of tryptanthrin (indolo - [2,1-b] – quinazoline - 6, 12 - dione)

As a potential drug candidate with multiple medical activities, the pharmacological actions of tryptanthrin have been widely studied; but its pharmacokinetic properties have rarely been reported. The preclinical trials are currently investigating the efficacy of tryptanthrin as an anti-tuberculosis and anti-tumor drug (12-13). Understanding the absorption and transport of drug candidates is a critical step in the process of drug development. Therefore, the detailed information regarding intestinal absorption and secretion of tryptanthrin are of specific importance and should be investigated. In this study, Caco-2 cells (human colorectal adenocarcinoma cell line), a widely accepted in vitro model for drug absorption and metabolism studies (14), were used to characterize the intestinal transport of tryptanthrin.

It is well known that ATP-driven efflux pumps, such as P-glycoprotein (P-gp encoded by MDR1) and multidrug resistance-associated protein 2 (MRP2), play significant roles in drug absorption, distribution, metabolism and excretion processes. In the oral absorption level, these transporters are present on the apical brush border membrane of gut enterocytes and actively cause efflux of drugs from the gut epithelial cells back into the intestinal lumen. Up to now, it is still unclear whether P-gp and MRP2 participate in the transport of tryptanthrin in Caco-2 cells. On the other hand, it has been reported that tryptanthrin downregulates the expression of the MDR1 gene in MCF-7 cells (human breast cancer cell line) and thus enhances the cytotoxicity of doxorubicin against MCF-7 cells due to the uptake of doxorubicin increased in the presence of tryptanthrin (15). Consequently, determining whether tryptanthrin directly affects the function of P-gp or MRP2 also is of particular importance.

In this study, using Caco-2 cells, the intestinal transport profile of tryptanthrin was studied in a low concentration range and the type of tryptanthrin transport was identified. In addition, the possible participation of P-gp and MRP2 in tryptanthrin transport was examined with the supplement of transporter inhibitors. Furthermore, the effect of tryptanthrin on the function of P-gp and MRP2 was also evaluated.

METHODS

Materials

Tryptanthrin was generously given by Prof. Jianli Liu from The College of Life Sciences, Northwest University, Xi'an, China. Digoxin and verapamil were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pravastatin sodium was purchased from Beta Pharma Inc. Co., Ltd. (Shanghai, China). Glibenclamide was purchased from Dongfang Pharma Technology Inc. (Shanghai, China). Hank's balanced salt solution (HBSS), trypsin-EDTA, Dulbecco's modified Eagle's medium (DMEM), non-essential amino acid solution, and penicillin-streptomycin solution were purchased from Gibco Laboratories (Invitrogen Co, Grand Island, NY, USA). Heat-inactivated fetal bovine serum (FBS) was purchased from Shanghai Al-Amin Bio-tech Co., Ltd. (Shanghai, China). TRIzol Reagent was purchased from the Invitrogen Corporation (Carlsbad, CA, USA). Acetonitrile and methanol (HPLC grade) were from Fisher Scientific Co., Ltd. (Fair Lawn, New Jersey, USA). All other chemicals used in the study were at least of analytical grade.

Cell Culture

The Caco-2 cell line was obtained from the American Type Culture Collection (Rockbille, MD, USA). Cells were routinely grown in 75 cm^2 plastic culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA) in DMEM supplemented with 10% (v/v) FBS, 1% (v/v) non-essential amino acid solution, and 1% (v/v) penicillin-streptomycin solution at 37 °C under a 5% CO_2 atmosphere with 90% relative humidity. The medium was replaced every 2–3 days after incubation. Cells were passaged every 5 days approximately between 70%–80% confluence at a split ratio of 1:5, using 0.25%

trypsin and 0.02% EDTA. For the transport experiments, the cells of passages between 32 and 40 were seeded at a density of 5×10^4 cells/cm^2 onto permeable polycarbonate inserts (0.6 cm^2, 0.45 μm pore size, Millipore, USA) in 6-well tissue culture plates (NUNC, Roskilde, Denmark). Media in the culture plates were changed every two days for the first week post seeding and was then replaced daily. Testing cell monolayer integrity on Transwell inserts was done by measuring transepithelial electrical resistance (TEER) across the cell monolayer or by measuring the amount of a nontransportable fluorescent compound fluorescein that leaks from the apical chamber between the cells down into the basolateral chamber. When the cell monolayers displayed that the TEER values were above 600 Ω•cm^2 and the permeability of fluorescein was less than 0.5×10^{-6} cm/s, the monolayers were considered not leaky. In our culture condition, Caco-2 monolayers, post seeding between 21 and 24 days, usually were available for the experiments. To ensure the integrity of the monolayers, the TEER value was measured again right before the transport assay.

Transport Studies in Caco-2 Cell Monolayers

Transport of tryptanthrin across the Caco-2 cell monolayers was studied using monolayers 21–24 days post seeding. Before the experiments, the monolayers were washed twice with HBSS (pH 7.4). After washing, the monolayers were preincubated at 37 °C for 20 min, and TEER was measured. HBSS solution on both sides of the cell monolayers was then removed by aspiration.

For measurement of the apical (AP) to basolateral (BL) transport, 400 μL of HBSS containing tryptanthrin (0.8–20 μM) was added to the AP side and 600 μL of HBSS without the drug was added to the BL side. For measurement of the BL to AP transport, 600 μL of HBSS containing tryptanthrin (0.8–20 μM) was added to the BL side and 400 μL of HBSS without the drug was added to the AP side. Tryptanthrin solutions were freshly prepared dissolving it in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the HBSS was below 0.1%. The monolayers were incubated at 37 °C, and then placed in a shaker at 50 rpm during the transport process to minimize the influence of the aqueous boundary layer. Samples were taken from the receptor chamber every 20 min for 2 hours, followed by an immediate replacement of the same volume of prewarmed fresh HBSS.

Tryptanthrin was evaluated for its potential as a substrate of P-gp or MRP2 in Caco-2 cell monolayers. The transport assay of tryptanthrin was performed with the addition of the P-gp inhibitor, verapamil, and the MRP2 inhibitor, glibenclamide. The absorption and efflux transport apparent permeability (P_{app}) of tryptanthrin were determined at a fixed substrate concentration (4 μM) and inhibitor concentration (100 μM).

To evaluate the potential effect of tryptanthrin on P-gp and MRP2 function, the efflux transport assays of digoxin (5μM) and pravastatin sodium (0.5 mM), selective substrates of P-gp and MRP2, respectively, were performed in the presence of tryptanthrin (4 μM). The efflux P_{app} of both digoxin and pravastatin sodium were determined.

Reverse Transcription–polymerase Chain Reaction (RT-PCR) Analysis of MDR1 and MRP2 Gene Expression

Caco-2 monolayers ready for the transport assay were treated with tryptanthrin (4 μM) or DMSO (0.1%, v/v) as vehicle control. Untreated Caco-2 monolayers were used as the control. After exposure to tryptanthrin for 2h or 4h, Caco-2 cells were washed with ice-cold HBSS (pH 7.4) and harvested.

Total mRNA was extracted using TRIzol reagent according to the manufacturer's protocol and quantified by UV-visible Recording spectrophotometer (Shimadzu, Kyoto, Japan). The cDNA were synthesized using M-MuLV reverse transcriptase with Oligo $(dT)_{18}$ random primer from 1 μg total mRNA. The primers (Invitrogen, Carlsbad, CA, USA) for MDR1 were MDR1-F (5'-TGA CTA CCA GGC TCG CCA ATG AT-3') and MDR1-R, (5'-TGT GCC ACC AAG TAG GCT CCA AA-3'). The primers for MRP2 were MRP2-F (5'-AGG TTT GCC AGT TAT CCG TG-3') and MRP2-R, (5'-AAC AAA GCC AAC AGT GTC CC-3'). The primers for GAPDH were as follows: GAPDH-F (5'-ATG GTG AAG GTC GG-3') and GAPDH-R (5'-TTA CTC CTG GAG GCC ATG T-3'). GAPDH was used as the internal control. PCR was performed in a S1000 Thermal Cycler system (Bio-Rad, Richmond, CA, USA). Samples were amplified with a precycling hold at 96 °C for 5 min, followed by 31 cycles of denaturation at 96 °C for 1 min, 56 °C annealing for 1 min, and extension at 72 °C for 1 min. PCR products were separated on 1.5% agarose gel and photographed with a gel analysis system.

High-performance Liquid Chromatography (HPLC) Analysis of the Samples

HPLC analysis was carried out using a LC-10AVP HPLC system equipped with a LC-10AT pump and a SPD-10A UV detector (Shimadzu, Kyoto, Japan). The detection wavelength was 254 nm. HPLC analysis of the samples was performed using a YMC ODS C_{18} column (150 ± 4.2 mm i.d., 5μm, Japan) preceded by a C_{18} guard column (Dikma, China). The column temperature was maintained at 35 °C at a flow rate of 1 mL/min. The mobile phase was a mixture of acetonitrile/2% ethanoic acid (40:60, v/v). The retention time of tryptanthrin was 5.5 min. Determination of digoxin and pravastatin sodium was performed with HPLC as reported (16-17).

The samples were analyzed by the modified reversed-phase HPLC described previously. After centrifugation at 12,000 rpm for 5 min, 20 μL of the supernatant of the samples obtained from the Caco-2 cells transport assays was directly analyzed with HPLC. The standard curve of tryptanthrin was linear within the range 0.04–20 μM (r^2 = 0.9999). The standard curve of digoxin was linear within the range 0.5–50 μM (r^2 = 0.9997). The standard curve of pravastatin sodium was linear within the range 12–1200 μM (r^2 = 0.9999).

Calculation and Data Analysis

The P_{app} coefficients were calculated for the directional flux studies according to Equation 1:

$$P_{app} = \frac{(dQ/dt)}{(A \times C_0)} \qquad \text{Equation 1.}$$

where dQ/dt (μg/min) is the drug permeation rate, A is the cross-sectional area (0.6 cm^2), and C_0 (μg/mL) is the initial drug concentration in the donor compartment at t = 0 min.

The net efflux of a test compound is assessed by calculating the ratio of P_{app} from BL to AP versus P_{app} from AP to BL ($P_{app\ (BL\rightarrow AP)}$/ $P_{app\ (AP\rightarrow BL)}$). A ratio substantially greater than 1.0 indicates a net efflux of the drug; otherwise, the absorptive transport is preponderant.

Statistical Analysis

Results are given as mean ± S.D. Statistical significance was tested by two-tailed Student's t-test. Statistical significance was set to $P < 0.05$.

RESULTS

Absorptive and Efflux Transport of Tryptanthrin Across Caco-2 Cells

Due to the poor solubility of tryptanthrin in HBSS, the absorptive and efflux transport of tryptanthrin were examined over a low concentration range (0.8–20 μM). As indicated in Table 1, both the absorptive $P_{app\ (AP\rightarrow BL)}$ and the secretory $P_{app\ (BL\rightarrow AP)}$ of tryptanthrin had no significant change at three different concentrations (0.8, 4, 20 μM). These results suggested that the transport of tryptanthrin was concentration-independent in this concentration range. The efflux ratios, calculated from the permeability of BL to AP versus the permeability of AP to BL, were all below 1 in the concentration range of 0.8–20 μM, suggesting that the absorptive transport of tryptanthrin was preponderant.

Effects of P-gp and MRP2 Inhibitors on the Permeation of Tryptanthrin Across Caco-2 Cell Monolayers

To determine the effect of P-gp and MRP2 inhibitors on tryptanthrin, the transport assays of tryptanthrin (4 μM), tryptanthrin (4 μM) + verapamil (100 μM) and tryptanthrin (4 μM) + glibenclamide (100 μM) were carried out respectively.

The P_{app} coefficients of tryptanthrin across Caco-2 cell monolayers in the AP→BL and BL→AP directions, in the presence vs. absence of verapamil or glibenclamide, are presented in Table 2. In the efflux direction (BL→AP), the P_{app} of tryptanthrin remained almost constant when verapamil (100 μM) or glibenclamide (100 μM) was added to both sides of Caco-2 cell monolayers. These results suggested that the efflux transport of tryptanthrin was not affected by both P-gp and MRP2 inhibitors, in the other word, neither P-gp nor MRP2 participated in the efflux transport of tryptanthrin. In the uptake direction (AP→BL), the P_{app} of tryptanthrin was hardly influenced by verapamil, which means that the P-gp did not affect the uptake transport of tryptanthrin. But the $P_{app\ (AP\rightarrow BL)}$ of tryptanthrin was significantly decreased when glibenclamide was added ($P < 0.01$). Nevertheless, this result cannot lead to the conclusion that the MRP2 was involved in the uptake of tryptanthrin because glibenclamide is not a specific inhibitor of the MRP2 and may inhibit the function of other uptake transporters rather than that of the MRP2 (18).

To better understand the performance of tryptanthrin in the process of transport assays with or without presence of transporter inhibitors, the samples were taken at each desired time point over the period of 2h. Figure 2 showed the results of these time course assays. The transport of tryptanthrin in both uptake (Fig 2A) and efflux directions (Fig 2B) was linearly increased over the time regardless in the presence or absence of the inhibitors. At each desired time point, the efflux permeation of tryptanthrin (Fig 2B) was not significantly affected by either verapamil or glibenclamide. However, the uptake permeation of tryptanthrin (Fig 2A) was substantially decreased in the presence of glibenclamide (P<0.05), but not of verapamil, at the late assay period (80-120 min). These results are consistent with the end point of transport assays shown in Table 2.

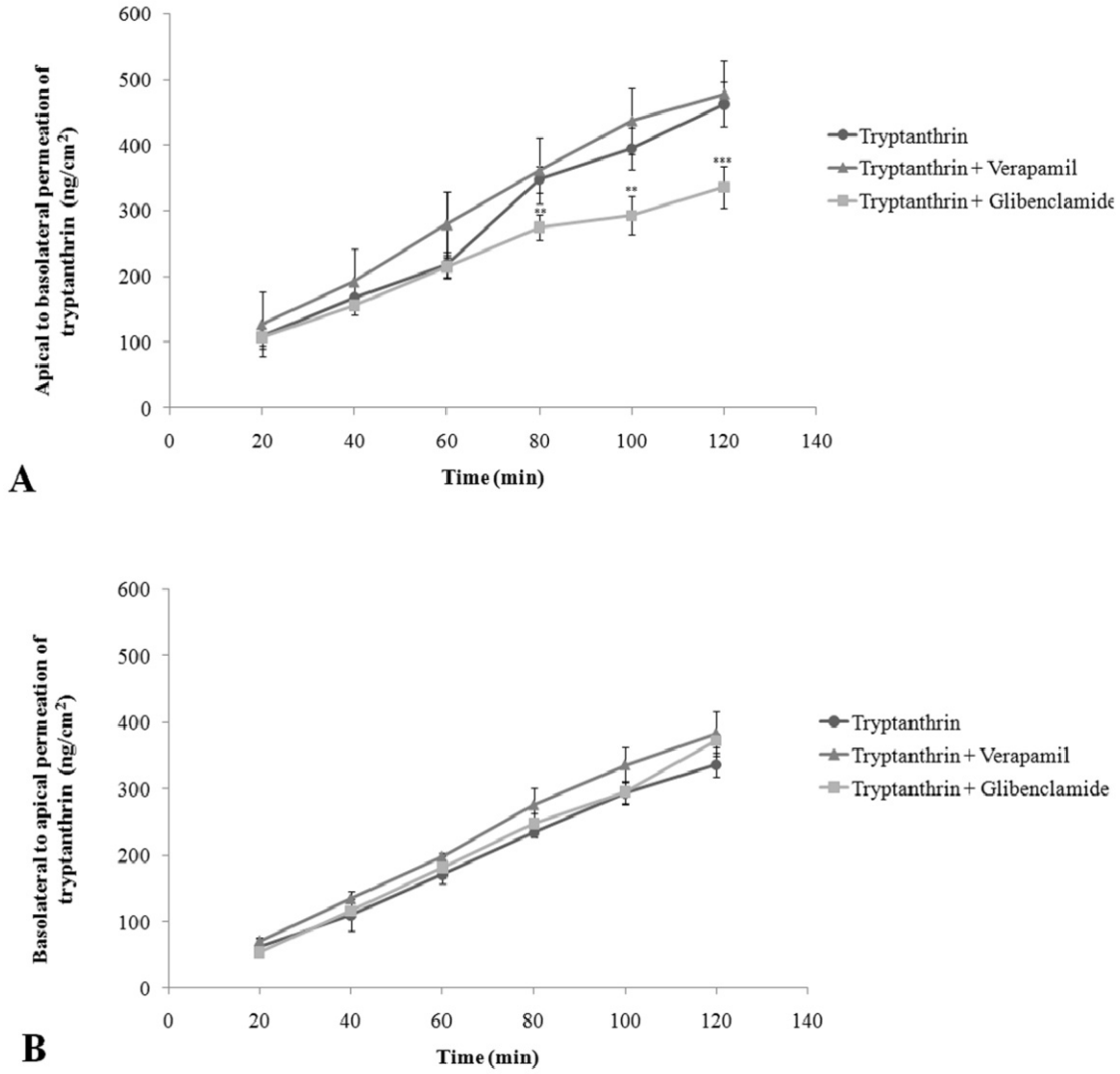

Figure 2. Effects of P-gp and MRP2 inhibitors on tryptanthrin permeation across the Caco-2 cell monolayers. Apical to basolateral (A) and basolateral to apical (B) permeation of tryptanthrin (4 μM) was investigated in the presence and absence of verapamil (100 μM) or glibenclamide (100 μM). Each point represents the mean ± S.D. for at least three monolayers.
** $P < 0.05$, *** $P < 0.01$

Table 1. Permeability of tryptanthrin in Caco-2 cell monolayers.

Tryptanthrin (μM)	$P_{app(AP→BL)}$ (cm/s×10^{-6}) (Mean±SD)	$P_{app(BL→AP)}$ (cm/s×10^{-6}) (Mean±SD)	$P_{app(BL→AP)}$/ $P_{app(AP→BL)}$ Ratio
0.8	69.46±14.27	45.32±2.99	0.65
4	61.38±2.91	47.31±1.01	0.77
20	57.39±2.17	49.08±6.38	0.86

Caco-2 cells were incubated at 37 °C for 2h with tryptanthrin added either to the apical or basolateral side of the cell monolayers. Data represents the mean ± S.D. of three monolayers (n=3).

Table 2. Effect of P-gp and MRP2 inhibitors on transport of tryptanthrin across Caco-2 cell monolayers

	$P_{app(AP→BL)}$(cm/s×10^{-6}) (Mean±SD)	$P_{app(BL→AP)}$ (cm/s×10^{-6}) (Mean±SD)	$P_{app(BL→AP)}$/ $P_{app(AP→BL)}$ Ratio
Tryptanthrin (4 μM)	61.38±2.91	47.31±1.01	0.77
Tryptanthrin (4 μM) + Verapamil (100 μM)	61.19±3.75	53.42±6.57	0.87
Tryptanthrin (4 μM) + Glibenclamide (100 μM)	38.39±5.30***	52.34±2.86	1.36

Caco-2 cells were incubated at 37 °C for 2h with tryptanthrin added either to the apical or basolateral side of the cell monolayers. Verapamil or glibenclamide was added to both the apical and basolateral sides. Data represents the mean ± S.D. of three monolayers (n=3).
*** Significantly different from control with $P < 0.01$

Effects of Tryptanthrin on the P-gp–mediated Transport of Digoxin and the MRP2-mediated Transport of Pravastatin Sodium in Caco-2 Cell Monolayers

To evaluate the effect of tryptanthrin on the function of P-gp or MRP2, an efflux transport assay of digoxin or pravastatin sodium was performed in the presence of tryptanthrin. The concentrations of 5μM digoxin and 0.5 mM pravastatin sodium in the transport assays were chosen according to the literatures (19-20). Efflux P_{app} coefficients of digoxin and pravastatin sodium were obtained in the presence vs. absence of tryptanthrin. The P_{app} of each group was calculated and listed in Table 3. Compared to those from the assays in the absence of tryptanthrin, the efflux $P_{app\ (BL→AP)}$ of both digoxin and pravastatin sodium was significantly decreased to more than 30% in the presence of tryptanthrin ($P < 0.01$).

In addition, the time courses of pravastatin transport assays in the presence and absence of tryptanthrin were performed and their results were shown in Figure 3. Compared to those in the absence of the tryptanthrin (solid circles) the efflux permeation of pravastatin sodium in the presence of tryptanthrin (solid squares) were gradually reduced over the time and reached more than 30% reduction at 2h. The similar results of the P-gp–mediated digoxin efflux inhibited by tryptanthrin were presented in Figure 4. These results indicated that tryptanthrin was an inhibitor of the MRP2 and the P-gp through reducing the efflux transport of their substrates.

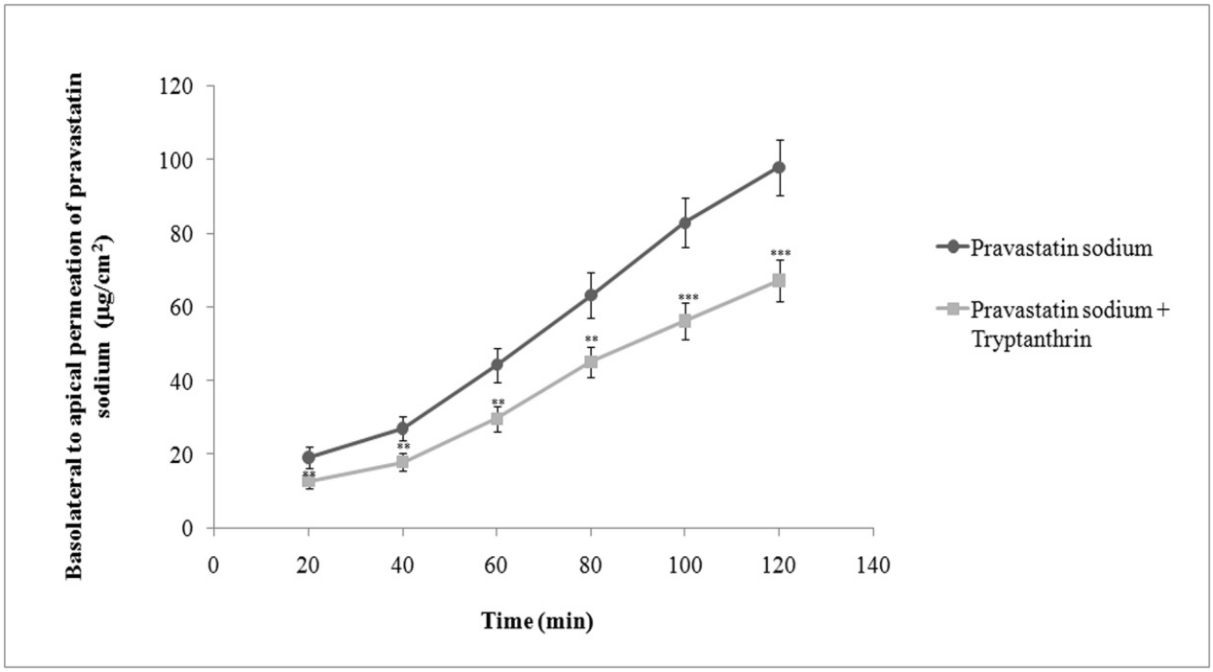

Figure 3. Inhibition of MRP2-mediated pravastatin sodium efflux by tryptanthrin across the Caco-2 cell monolayers. Basolateral to apical permeation of pravastatin sodium (0.5 mM) was investigated in the presence and absence of tryptanthrin (4 µM). Each point represents the mean ± S.D. for at least three monolayers. ** $P < 0.05$, *** $P < 0.01$

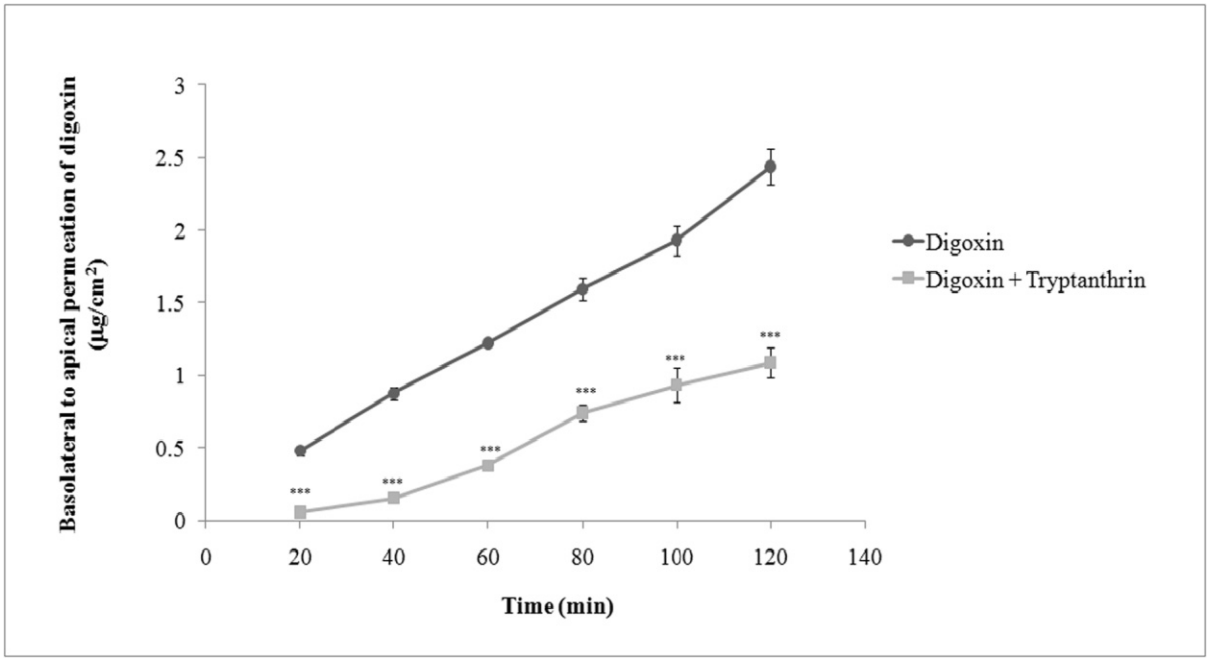

Figure 4. Inhibition of P-gp–mediated digoxin efflux by tryptanthrin across the Caco-2 cell monolayers. Basolateral to apical permeation of digoxin (5µM) was investigated in the presence and absence of tryptanthrin (4 µM). Each point represents the mean ± S.D. of at least three monolayers. *** $P < 0.01$

Effect of Tryptanthrin on MDR1 and MRP2 Expression

To address whether tryptanthrin affects the P-gp–mediated transport of digoxin and MRP2-mediated transport of pravastatin sodium via regulating the expression of these transporters, the mRNA expression of MDR1 and MRP2 genes in the Caco-2 cells, cultivated in the presence of tryptanthrin, was evaluated by RT-PCR. The Caco-2 cells were treated with 4 μM tryptanthrin for up to 4h, and then the mRNA of MDR1 and MRP2 was isolated for RT-PCR analysis. As shown in Figure 5, the expression levels of MDR1 or MRP2 genes from the cells treated with tryptanthrin were comparable to those from the untreated cells or vehicle control. These results suggested that the expression of MDR1 and MRP2 remained the same during the transport assays.

DISCUSSION

Although tryptanthrin has been extensively studied for its multiple medical activities since 1990s, there is lack of comprehending information regarding its pharmacokinetic properties. This study represents the first investigation of the intestinal absorption and transport characteristics of tryptanthrin using Caco-2 cell monolayers, a well-established in vitro model.

Our results showed that both the influx and efflux transport of tryptanthrin were concentration-independent (Table 1), suggesting that passive diffusion is the dominant transport process for tryptanthrin in Caco-2 cell monolayers. The absorptive P_{app} of tryptanthrin was calculated to be $(61.38 \pm 2.91) \times 10^{-6}$ cm/s, whereas the secretory P_{app} was $(47.31 \pm 1.01) \times 10^{-6}$ cm/s. These values are nearly fivefold higher than the threshold value (1×10^{-5} cm/s) suggested for poorly absorbed compounds (21). The absorptive P_{app} was 1.3 times higher than the efflux P_{app}, showing that absorptive transport was preponderant. These results indicate that tryptanthrin is well absorbed and the main efflux transporters probably do not participate in its transport across Caco-2 cell monolayers. These results were further confirmed by the observation that the efflux transporter P-gp and MRP2 inhibitors, verapamil and glibenclamide, had no inhibitory effect on the efflux of tryptanthrin transport (Table 2, Figure 2B).

However, the uptake transport of tryptanthrin was significantly inhibited by glibenclamide (Table 2, Figure 2A), which is the most commonly used inhibitor (22-23). Given the fact that glibenclamide is not a specific inhibitor of the MRP2, the observed inhibitory effect of glibenclamide might result from its inhibition of other uptake transporters. In future studies, a specific MRP1 inhibitor should be applied. It was reported that the Caco-2 cell basolateral transporters MRP1, MRP3, and MRP5 are responsible for drug uptake,

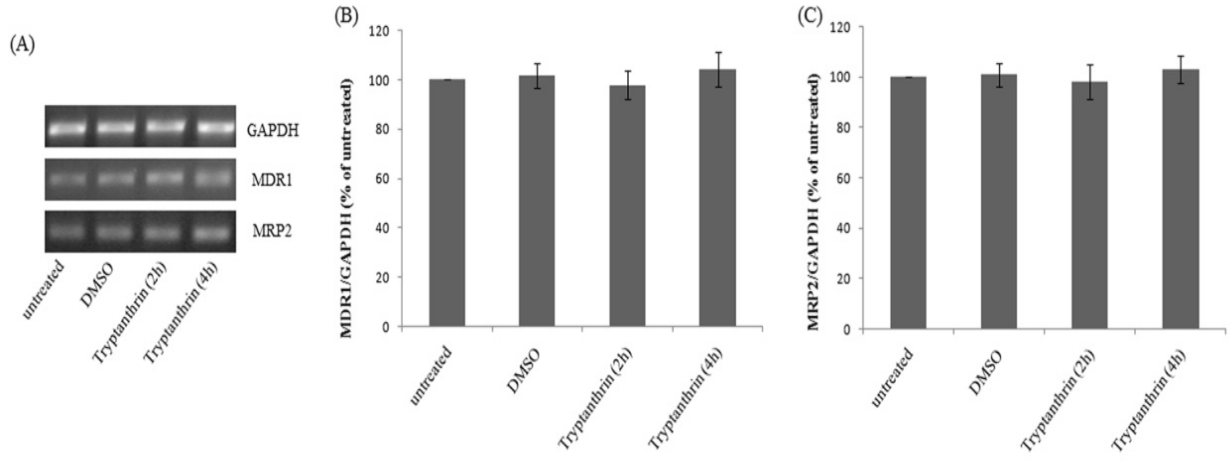

Figure 5. MDR1 and MRP2 mRNA levels in the Caco-2 cells exposed to tryptanthrin (4 μM) for 2h, 4h, DMSO (0.1%, v/v, vehicle) or culture medium (untreated, control) (A). GAPDH was used as the internal standard. MDR1 (B) and MRP2 (C) mRNA levels were quantified by densitometry in relation to GAPDH. Data were expressed as mean ± SD from three independent experiments.

whereas the apical transporters P-gp and MRP2 regulate drug efflux (24). Previous literature has also shown that glibenclamide could inhibit the activity of both MRP2 and MRP1 in GLC4/Sb30 cells (18). Our study showed that in the efflux direction (BL→AP), the P_{app} of tryptanthrin remained almost constant when glibenclamide (100 μM) was added, suggesting that MRP2 did not participate in the efflux transport of tryptanthrin. If the MRP1 played a key role in the uptake of tryptanthrin in Caco-2 cells, presumably, glibenclamide decreased tryptanthrin uptake by inhibiting the activity of MRP1.

The evaluation of the potential effect of a compound on the function of an efflux transporter usually only investigates the change in efflux transport of substrate. For instance，to find the effect of pravastation on MRP2 mediated cyclosporin A transport in Caco-2 monolayer，only efflux transport assay of cyclosporin A in the presence of pravastation was performed (19). Herein, to address whether tryptanthrin is a potential inhibitor of P-gp and MRP2 in Caco-2 cell monolayers, efflux transport assays of P-gp substrate, digoxin, and MRP2 substrate, pravastatin sodium were performed in the presence of tryptanthrin. As shown in Table 3 both the efflux $P_{app\ (BL\rightarrow AP)}$ values of digoxin and pravastatin sodium were decreased in the presence of tryptanthrin. However, the presence of tryptanthrin did not downregulate the expression of P-gp or MRP2 genes over either 2h or 4h period of the transport assays. Therefore, our results suggested that tryptanthrin directly inhibited the functional activities of these two transporters. Recently, it was reported that tryptanthrin could reverse doxorubicin resistance in MCF-7 cells by suppressing MDR1 gene expression and P-gp production (15). Similarly, our findings showed that tryptanthrin could directly inhibit the activity of P-gp and MRP2.

It is worth to notice that in the previous study tryptanthrin downregulated the MDR1 gene in breast cancer MCF-7 cells (15), but in the present study tryptanthrin did not show the downregulation of the MDR1 gene expression over 2-4hrs. This discrepancy is due to the different experimental designs between the two studies. In Yu ST's report, the MCF-7/adr cells were seeded into six-well plates at a density of 2×10^5/well in the presence of tryptanthrin at 10^{-6} M for 5 days. On the fifth day, the expression MDR1 gene and protein in the cells was decreased. In addition, they found that tryptanthrin down-regulated MDR1 expression by interfering the binding of CAAT motif to nuclear transcription factors (15). In the present study, because the tryptanthrin treatment was only lasted for 2 hours, tryptanthrin did not show any downregulation of the MDR1 gene expression.

It was reported that the minimum inhibitory concentration (MIC, 2-4 μM) of tryptanthrin was efficient against multiple drug-resistant (MDR) tuberculosis strains (11), and within the concentration range of 0.95-5μM tryptanthrin showed effective in vitro antitumor activity against numerous of cancer cell lines, including renal A498, ovarian SKOV3, melanoma M14, colon SW620, and lung H522 (8). Therefore, the concentration of tryptanthrin (0.8-20 μM) was chosen for the present transport study.

Table 3. Effect of tryptanthrin on efflux transport of P-gp or MRP2 substrate across Caco-2 cell monolayers

	$P_{app(BL\rightarrow AP)}$ (cm/s×10^{-6}) (Mean±SD)
Digoxin (5μM)	62.80±3.80
Digoxin (5μM) + Tryptanthrin (4 μM)	43.59±2.93***
Pravastatin sodium (0.5 mM)	79.33±4.80
Pravastatin sodium (0.5 mM) + Tryptanthrin (4 μM)	46.69±4.61***

Caco-2 cells were incubated at 37 °C for 2h with digoxin or pravastatin sodium added to the basolateral side of the cell monolayers. Tryptanthrin was added to both the apical and basolateral sides. Data represents the mean ± S.D. of three monolayers (n=3).

*** Significantly different from control with $P < 0.01$

For further investigation, a series of concentrations of tryptanthrin would be set up for transport assays in Caco-2 cell monolayers to determine whether the tryptanthrin effect on P-gp and MRP2 is concentration dependent. It is known that the overexpression of P-gp and MRP2 in tumor cells can lead to drug resistance and chemotherapeutic failure (25-26). Thus, it seems that tryptanthrin might be a potential adjuvant agent for chemotherapy targeting these particular tumor cells.

In conclusion, tryptanthrin was well absorbed across the Caco-2 monolayers, and its absorptive and efflux transport were both dominated by passive diffusion. Tryptanthrin was not a substrate of P-gp or MRP2, but a potential inhibitor of P-gp and MRP2. This study provides useful information for characterizing the pharmacokinetic properties of tryptanthrin.

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