Effect of five novel 5-substituted tetrandrine derivatives on P-glycoprotein-mediated inhibition and transport in Caco-2 cells

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Abstract. Tetrandrine (Tet) is a potent inhibitor that reverses P-glycoprotein-mediated multidrug resistance (MDR). A number of novel 5-substituted tetrandrine derivatives were synthesized by the authors. The present study aimed at identifying potential P-gp inhibitor candidates, and intracellular uptake and efflux experiments and Caco-2 cell-based Transwell transport studies were performed. It was demonstrated that all five test compounds were able to inhibit efflux and increase intracellular uptake of the P-gp substrate, rhodamine-123 (Rho-123); the test compounds were P-gp inhibitors. The transepithelial transport experiment indicated that the selectivity (basolateral-to-apical) of Rho-123 decreased, the absorption (apical-to-basolateral) increased and the transport efflux ratio (ER) reduced in the presence of the five compounds. Among the compounds, fluobenzene-Tet (TF) exhibited similar inhibitory effect as Tet. Although the other four test compounds exhibited weaker inhibitory effects than Tet and TF, the compounds exhibited stronger inhibitory effects compared with the reference compound verapamil. The study demonstrated that the five novel 5-substituted tetrandrine derivatives are able to act as inhibitors of P-gp to overcome P-gp-mediated drug resistance.

Introduction

Tetrandrine (Tet), a bisbenyloquinoline alkaloid isolated from the dried root of Stephenia tetrandra S. Moore, exhibits broad pharmacological actions. Tet has potential either as a tumoricidal agent or as an adjunct to chemotherapy to serve as a model of paracellular movement of compounds across the monolayer. The test compounds are added to either the apical or basolateral sides of the monolayer. Following incubation for various lengths of time, aliquots of the buffer in opposite chambers are removed in order to determine the concentration of test compounds. The rates of permeability for

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It has been indicated that the naturally occurring compound may be used as a chemosensitizer for treating P-glycoprotein (P-gp)-mediated cancer with multidrug resistance (MDR) (2). Selective alkylation substitution of Tet at the 5-position was systematically investigated, and series of novel 5-substituted derivatives were prepared in the present study.

P-gp belongs to a family of ATP-binding cassette (ABC) transporters, and it is encoded by the MDR1 gene. The overexpression of MDR1 has been associated with chemotherapy failure in a number of types of cancer, including kidney, colon and liver cancer, as well as leukemia and lymphoma (3). The P-gp, membrane transporter protein, has a crucial role in the modulation of absorption, distribution, metabolism and excretion of drugs. P-gp is also known to function as a barrier protein to extrude toxins and xenobiotics from cells (4,5). P-gp is able to efflux various anticancer drugs out of the cells in order to decrease the intracellular accumulation of cytostatic drugs, including doxorubicin and paclitaxel (5). Therefore, the development of P-gp inhibitors, which are able to efficiently overcome MDR, is necessary (6). The P-gp inhibitors can either downregulate the expression of transporter proteins or have a synergistic effect with chemotherapeutic agents by inhibiting the efflux function of ABC transporters (7).

P-glycoprotein is overexpressed in the Caco-2 cell line. The Caco-2 cell line has a number of biophysical and biochemical characteristics, which are similar to the features of a normal intestinal absorptive cell. The Caco-2 cell line has become the most common and extensively characterized cell-based model in predicting the absorption and transport potential of compounds (8). Caco-2 cells also have been employed in several other studies that have investigated the mechanisms of transport, metabolism and the effect of P-glycoprotein on the efflux of compounds (8-12).

Caco-2 cell monolayers are usually cultured on semi-permeable plastic supports that may be fitted into the wells of multi-well culture plates. When cultured as a monolayer, Caco-2 cells differentiate to form tight junctions between cells to serve as a model of paracellular movement of compounds across the monolayer. The test compounds are added to either the apical or basolateral sides of the monolayer. Following incubation for various lengths of time, aliquots of the buffer in opposite chambers are removed in order to determine the concentration of test compounds. The rates of permeability for
each compound (apparent permeability coefficients) are then determined (13). The apparent permeability coefficient values obtained using Caco-2 cell monolayers are correlated with the in vivo absorption capability of the molecule (14-16).

The expression of P-gp may reduce the intracellular accumulation of Rhodamine-123 (Rho-123). Measuring the uptake or efflux of Rho-123 allows the characterization of cells with a MDR phenotype and P-gp overexpression even with low levels of resistance (17,18). Rho-123 is a substrate for P-glycoprotein (P-gp) and therefore can be used as a molecular probe for the investigation of the multidrug resistance (MDR) phenotype (19).

In the present study, the inhibition of P-gp and transepithelial transport properties in the presence of five novel 5-substituted tetrandrine derivatives in Caco-2 cells were studied. The Rho-123 uptake and efflux assay indicated that the five novel 5-substituted tetrandrine derivatives tested were able to inhibit efflux and increase the intracellular accumulation of Rho-123 in Caco-2 cells. Additionally, the 5-substituted tetrandrine derivatives were P-gp inhibitors. The analysis of transepithelial transport across Caco-2 cell monolayers indicated that the 5-substituted tetrandrine derivatives were able to increase the absorption and decrease the secretory transport of Rho-123 via the inhibition of P-gp-mediated drug efflux.

Materials and methods

Materials. The Caco-2 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) at passage 17. Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids (NEAA), fetal bovine serum (FBS), 0.25% trypsin, 10,000 U/ml penicillin, 10,000g/ml streptomycin were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Dimethyl sulfoxide (DMSO), Rhodamine-123 (Rho-123), verapamil (VER) and MTT were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Tet was a gift from Dr Jiekai Cheng (Qinghai Ecion Pharmaceutical Co., Ltd., Xining, China), and novel 5-substituted Tet derivatives (purity, >98%) were synthesized by the lab of the authors of the present study. The 5-substituted tetrandrine derivatives were evaluated by chromatography, high-resolution electrospray ionization mass spectrometry (Table I and Fig. 1) and nuclear magnetic resonance spectroscopy. Chromatographic separation was performed on a waters Acquity UPLC HSS T3 column (2.1x100 mm, 1.7 µm) by using the Agilent 1290 Series UHPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA).

Cell culture. The Caco-2 cells were cultured in DMEM medium, which was supplemented with 10% FBS, 1% glutamine, 1% penicillin and streptomycin. In addition, 1% sodium pyruvate and 1% NEAA were added to the cell medium. In order to maintain a high P-glycoprotein 1 (P-gp) level, the cells were cultivated for 24 h in fresh culture medium (DMEM) with 2 µg/ml doxorubicin (Selleck Chemicals, Houston, TX, USA). All cells were cultured at 37°C with 5% CO₂ and 95% humidified atmosphere. The experiments were performed with cells in the logarithmic growth phase. The cells used for all the experiments were taken between passage number 30 and 50.

Cytotoxicity of Caco-2 cells. The viability of the Caco-2 cells was analyzed using the MTT assay as previously described (20). The cells (5x10⁴ cells/well) were seeded in 96-well plates overnight. A series of concentrations of Tet (100, 200, 300, 400, 500, 600, 700, 800, 900 and 1,000 µg/ml) were added and incubated for 24 h at 37°C. Subsequently, 100 µl MTT (0.5 mg/ml) was added to each well following the removal of the culture medium and incubated for an additional 4 h at 37°C. Following the removal of the culture medium, the formed formazan was dissolved in 150 µl DMSO. The plates were placed on a shaker for 5 min at room temperature, and optical density was measured using a microplate reader (M1000; Tecan Trading AG, Männedorf, Switzerland) at a wavelength of 570 nm. The IC₅₀ value was calculated by using Graphpad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA).

Determination of concentration and inhibition time of Rho-123 and Tet. In order to determine the concentration of Rho-123, Caco-2 cells were incubated with a series of Rho-123 concentrations (0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 µg/ml) at 37°C for 1 h. The cells were analyzed with a flow cytometer (BD FACSArray; BD Biosciences, Franklin Lakes, NJ, USA) following digestion with trypsin. For the determination of the concentration of compounds, Caco-2 cells were incubated with a series of Tet concentrations (0, 1, 2 and 5 µg/ml) at 37°C for 1 h, and then Rho-123 was added and the cells were incubated at 37°C for 1 h. The cells were subsequently analyzed by flow cytometry following trypsinization. For the determination of the duration of inhibition, Caco-2 cells were incubated with Rho-123 with different durations (0, 0.25, 0.5, 1, 2, 4 and 6 h). The cells were analyzed with a flow cytometer following digestion with trypsin. All experiments were analyzed independently for 3 times. The relative fluorescence intensity for each type of treatment was calculated as follows: % Inhibitory efficiency=(fluorescence intensity of test compound/VER) x100.

Rho-123 uptake and efflux assay. For the Rho-123 uptake assay, Caco-2 cells were seeded in 24-well plates. When confluent monolayers were formed, the cells were incubated with 5 µg/ml Tet and its novel 5-substituted derivatives [tetrandrine-aromatic (TA), tetrandrine-toluene (TT), tetrandrine-pyridine (TP), tetrandrine-fluorobenzene (TF) and tetrandrine-trifluorobenzene (TTF); 5 µg/ml; for 1 h. Subsequently, 5 µM Rho-123 was added, and the cells were incubated at 37°C for 1 h following digestion with trypsin. The cells were analyzed by flow cytometry. For the Rho-123 efflux assay, Caco-2 cells were incubated with 5 µM Rho-123 in the presence or absence of 5 µg/ml test compounds at 37°C for 1 h. The cells were analyzed by flow cytometry after digestion with trypsin. In each independent experiment, 50 µM VER (a known P-gp inhibitor) was used as a reference compound.

Transepithelial transport across Caco-2 cell monolayers. The transport of Rho-123 across Caco-2 cell monolayers in the presence or absence of Tet and novel 5-substituted...
Table I. High-Resolution Mass Spectrometry data of tetrandrine and its novel 5-substituted derivatives (ESI-POS).

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Formula</th>
<th>Expected (m/z)</th>
<th>Found at (m/z)</th>
<th>Isotope difference (%)</th>
<th>Error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tet</td>
<td>C_{18}H_{24}N_{3}O_{6}</td>
<td>623.3116</td>
<td>623.3113</td>
<td>4.4</td>
<td>-0.4</td>
</tr>
<tr>
<td>TA</td>
<td>C_{18}H_{24}N_{3}O_{6}</td>
<td>699.3429</td>
<td>699.3423</td>
<td>13.7</td>
<td>-0.8</td>
</tr>
<tr>
<td>TT</td>
<td>C_{18}H_{24}N_{3}O_{6}</td>
<td>713.3585</td>
<td>713.3579</td>
<td>5.5</td>
<td>-0.9</td>
</tr>
<tr>
<td>TP</td>
<td>C_{18}H_{24}N_{3}O_{6}</td>
<td>700.3381</td>
<td>700.3385</td>
<td>3.6</td>
<td>0.6</td>
</tr>
<tr>
<td>TF</td>
<td>C_{18}H_{24}F_{2}N_{3}O_{6}</td>
<td>717.3334</td>
<td>717.3341</td>
<td>1.9</td>
<td>0.9</td>
</tr>
<tr>
<td>TTF</td>
<td>C_{18}H_{24}F_{2}N_{3}O_{6}</td>
<td>767.3302</td>
<td>767.3308</td>
<td>1.9</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Tet, tetrandrine; TA, tetrandrine-aromatic group; TT, tetrandrine-toluene; TP, tetrandrine-pyridine; TF, tetrandrine-fluobenzene; TTF, tetrandrine-trifluobenzene.

Figure 1. Molecular structure of tetrandrine and its novel 5-substituted derivatives. Tet, tetrandrine; TA, tetrandrine-aromatic group; TT, tetrandrine-toluene; TP, tetrandrine-pyridine; TF, tetrandrine-fluobenzene; TTF, tetrandrine-trifluobenzene.

The apparent permeability coefficient (Papp) is Papp in the absorptive (A→B) direction. All results are presented as the mean ± standard deviation (n=3).

HPLC analysis. The samples for Rho-123 transport study were determined using the Waters 2695 HPLC system as previously described (21), which was equipped with a fluorescence detector. HPLC was performed at 488 nm (λexc, 488 nm; λem, 575 nm). The system was controlled using Empower 2 software (Waters Corporation, Milford, MA, USA). Chromatographic separation was performed on the XBridgeTM C18 column (4.6x250 mm, 5 µm column). The column was kept at 25°C with a flow rate of 1 ml/min. The mobile phase consisted of methanol and water (50:50, v/v). The injection volume of the test sample was 20 µl.

Statistics analysis. All data sets were analyzed with GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The results were expressed as the mean ± standard deviation. The comparisons were performed using Student's t-test (two-tailed) and one-way analysis of variance with post-hoc Dunnett's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxicity of Caco-2 cells. As indicated in Fig. 2, the survival rate of Caco-2 cells was 100% at <100 µg/ml Tet. The IC_{50} value was calculated using the GraphPad Prism 5.0 software. The IC_{50} value was 240 µg/ml, which indicated that Tet exhibited no toxicity on Caco-2 cells at 0-100 µg/ml. Therefore, the concentrations for subsequent experiments were selected from 0-100 µg/ml.

Determination of the concentration and duration of inhibition of Tet and Rho-123. Prior to investigating the P-gp inhibitory activity and transepithelial transport properties, the concentration of Tet and Rho-123 and the inhibition time were determined. As indicated in Figs. 3-5, Caco-2 cells were incubated with a series of different concentrations of Tet and Rho-123 or for different periods of time (37°C). The cells were analyzed by flow cytometry. VER (50 µM) was used as a reference compound. All experiments were analyzed independently for 3 times. Based on the results of the assay, 5 µg/ml of each test compound and 2 µg/ml (5 µM) Rho-123 [the value...
of Rho-123 used was the same as that used in a previous study (22)] were used in following experiments. Additionally, 1 h was used as the duration of inhibition.

**Rho-123 uptake and efflux assays.** The intracellular uptake and efflux of Rho-123 were evaluated in Caco-2 cells by flow cytometry. For the Rho-123 uptake assay, Caco-2 cells were incubated with 5 µg/ml test compounds, and then the cells were incubated with 5 µM Rho-123 for 1 h. The results indicated that the amount of Rho-123 in cells that were treated with the test compounds was significantly higher compared with the untreated control (Fig. 6A). Additionally, the findings indicated that the test compounds may increase the uptake of Rho-123. For the Rho-123 efflux assay, Caco-2 cells were incubated with 5 µM Rho-123 in the presence or absence of 5 µg/ml compounds for 1 h (Fig. 6B). The test compounds were able to inhibit the efflux of Rho-123.

**Transepithelial transport of Rho-123 in Caco-2 cells that were treated in the presence or absence of test compounds.** The results are presented in Table II. The P_{app} values of Rho-123 across Caco-2 cell monolayers were calculated (4). The value of P_{app} B→A (secretory) was 7.32±0.82 and P_{app} A→B (absorptive) was 2.65±0.33. The value was well in the reported P_{app} value of 1x10⁻⁶ cm/s in Caco-2 cells, which is necessary for a compound to exhibit efficient absorption through the gastrointestinal epithelium (15).

**Basolateral to apical (B→A) transport.** The transport of Rho-123 in the B→A direction (secretory) across the monolayer was decreased in the presence of 5 µg/ml TA, TT, TP, TF or TTF compared with cells that were not treated with the compounds. The value of P_{app} B→A was decreased from 7.32±0.82 to 3.94±0.44, 4.18±0.39, 2.97±0.27, 2.34±0.21 and 2.83±0.25, respectively (Table II). The value of TF (2.34±0.21) was lower compared with the value for Tet (2.38±0.24). In the experiment, VER, the gold standard P-gp inhibitor (23), was used as a reference compound. The treatment of cells with VER led to a decrease in the secretion of Rho-123 from 7.32±0.82 to 2.37±0.18. The value for VER was approaching
the value for Tet (2.38±0.24). The results indicated that 50 µM VER had the same inhibitory effect as 8 µM (5 µg/ml) Tet. In addition, TF had almost the same inhibitory effect as Tet. Although the other four test compounds had weaker inhibitory effects compared with Tet and TF, the effects of the four test compounds were stronger than VER. These findings demonstrated that these compounds (TA, TT, TP, TF and TFF) were P-gp inhibitors and exerted a strong inhibitory effect on the transport of Rho-123 across Caco-2 cells.

**Apical to basolateral (A → B) transport.** The transport of Rho-123 in the A → B direction (absorptive) across the monolayer was increased in the presence of 5 µg/ml TA, TT, TF or TTF compared with transport in the absence of these compounds.

The value of $P_{\text{app}}$ A → B was increased from 2.65±0.33 to 3.38±0.48, 2.73±0.18, 2.78±0.18, 3.04±0.31 and 2.80±0.20, respectively (Table II). The increase was 1.28-, 1.03-, 1.05-, 1.15- and 1.06-fold, respectively. The maximum increase (TA) in $P_{\text{app}}$ for the test compounds (1.28-fold) was higher compared with the increase with Tet (1.20-fold) and VER (1.20-fold). It demonstrated that the absorption of Rho-123 was improved in the presence of the compounds.

**ER.** ER was determined by the equations: ER=$P_{\text{app}}$ (B → A)/$P_{\text{app}}$ (A → B), where the $P_{\text{app}}$ (B → A) is the $P_{\text{app}}$ in the secretory (B → A) direction, $P_{\text{app}}$ (A → B) is $P_{\text{app}}$ in the absorptive (A → B) direction. The presence of the 5 compounds did affect the transport ER of Rho-123; they all reduced the efflux of Rho-123. The value was significantly reduced from 2.79±0.39 to 1.18±0.16, 1.54±0.15, 0.76±0.11, 1.07±0.09 and 1.01±0.11 in the presence of TA, TT, TF, VER and TTF, respectively. The value of TF (0.78±0.10) was approaching the value of Tet (0.76±0.11; Table II).

**Discussion**

Tet, a benzylisoquinoline alkaloid, is a potent inhibitor in reversal of P-gp-mediated MDR (24). There is an urgent requirement for the development of strong inhibitors to overcome MDR. Consequently, five novel 5-substituted tetrandrine derivatives were synthesized in the present study.

In the present study, the inhibitory activity on P-gp and transepithelial transport experiments in the presence of the five compounds were performed by using P-gp-overexpressed Caco-2 cells. The results indicated that the five test compounds were P-gp inhibitors. The test compounds were not only able to increase the absorption but also decrease the secretory transport of Rho-123 via the inhibition of P-gp-mediated drug efflux. Among the test compounds, TF exhibited a similar extent of inhibitory effect as Tet.

P-gp was the first ABC transporter to be identified (25). The Caco-2 cell line with an overexpression of P-gp can be used to screen P-gp inhibitors and predict the absorption and transport potential of the test compounds. Rho-123 was selected as a representative P-gp substrate as its efficiency to combine with inhibitors for P-gp-mediated transport had been demonstrated in previous studies (26).

In the present study, the uptake and efflux of Rho-123 was quantified by flow cytometry in the presence or absence of the test compounds. By comparing the fluorescent intensity of Rho-123 in Caco-2 cells, the inhibitory effect of P-gp-mediated drug efflux was determined. The results showed that the five test compounds increased the intracellular uptake and decreased the Rho-123 efflux in Caco-2 cells.

In the transepithelial transport study, the Caco-2 cell monolayer was used for the assessment of absorption of drugs via the intestinal membrane enterocytes, which have been described in previous studies (9,14,27,28). A fully differentiated and tight monolayer was required for the permeability experiments. Differentiation also included the formation of tight junction between the cells. However, a monolayer that is too tight may underestimate the absorption of hydrophilic compounds that are not substrates for any absorptive transporters (29).

In the present study, the $P_{\text{app}}$ value was calculated for Rho-123, which was well in the reported value of 1x10$^{-6}$ cm/s in Caco-2 cells, which is necessary for a compound to exhibit efficient absorption through the gastrointestinal
epithelium (15). The transport of Rho-123 showed the ratio between two fluxes; from the basolateral to apical compartments (B→A, secretory, representative of passive diffusion) and from the apical to basolateral compartments (A→B, absorptive, representative of active transport). The transport of Rho-123 of B→A was significantly higher compared with A→B in the absence of these compounds. The B→A transport of Rho-123 was reduced and the A→B transport was increased in the presence of these compounds. These findings indicated that the compounds were not only able to increase absorption but also decrease the secretory transport of Rho-123.

These results confirmed that the combination of the test compounds with the P-gp substrate Rho-123 was able to increase uptake and absorption in the uptake assay and transport experiments. Furthermore, reductions in efflux and secretion were demonstrated with the test compounds, which supported the role of P-gp transporters in mediating the inhibitory effects of Rho-123.

In the present study, VER was used as a reference compound. VER is the gold standard P-gp inhibitor, and it had been demonstrated to inhibit P-gp (23). However, VER was indicated to be a relatively weak P-gp inhibitor compared with other P-gp inhibitors including cyclosporine A and Valspodaar, and data from clinical trials regarding its efficacy had been disappointing (3,25). In the present study, 5 µM Rho-123 was used. Tet and its novel 5-substituted derivatives were used. Tet and data from clinical trials regarding its efficacy had been supported the role of P-gp transporters in mediating the inhibitory effects (14-16). The use of the Caco-2 cell line model should enable the elucidation of P-gp-mediated transport and the overall membrane permeability of a P-gp substrate (30).

The findings in the present study demonstrated that these test compounds were P-gp inhibitors and exhibited P-gp-mediated inhibitory effects on the transport of Rho-123 across Caco-2 cells. Furthermore, the apparent permeability coefficient values obtained using Caco-2 cell monolayers are consistent with the in vivo absorption capability of the molecule (14-16). The use of the Caco-2 cell line model should enable the elucidation of P-gp-mediated transport and the overall membrane permeability of a P-gp substrate (30).

The results of the study indicated that the efflux function of P-gp was inhibited by the test compounds; the compounds were able to act as inhibitors of P-gp to decrease the secretion and increase the absorption of the P-gp substrate, Rho-123, across Caco-2 cell monolayers.

Although the five novel 5-substituted tetrandrine derivatives have been demonstrated to be able to act as inhibitors of P-gp to increase the absorption of drug, further studies are required to optimize the structure of the compounds to improve the inhibitory activity on P-gp. It was inferred that the P-gp inhibitory activity might be improved by using specific steric substituent, electro-donating and electro-withdrawing groups. Fluoride is a strong electro-withdrawing group (31), while the triple fluorine results in the weakness of electro-withdrawing group due to the steric hindrance and symmetry. Therefore, the 5-substituted tetrandrine derivatives, TF, exhibited stronger inhibitory effect on P-gp compared with TTF and TF exhibited similar inhibitory efflux effect as Tet. The lone-pair electrons nitrogenous compound, TF, had an electro-donating group, and TP had potential applications in improving the effect of P-gp to decrease the secretion and increase the absorption of drugs. Furthermore, several substituted groups with high lipid solubility might be utilized so that low-weight molecules can move more easily into the lipid bilayer and improve the inhibitory activity of the compounds on P-gp.

In summary, the molecular structural variations of Tet at 5-position provided good predictive information and would

### Table II. P\textsubscript{app} in A→B and B→A direction and efflux ratio (ER) of Rho-123 across Caco-2 cell monolayers treated in the presence or absence of tetrandrine and its novel 5-substituted derivatives.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>P\textsubscript{app} 10\textsuperscript{-6} (cm/s)</th>
<th>Efflux ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B→A (secretory)</td>
<td>A→B (absorptive)</td>
</tr>
<tr>
<td>5 µM Rho-123</td>
<td>7.32±0.82\textsuperscript{f}</td>
<td>2.65±0.33\textsuperscript{c}</td>
</tr>
<tr>
<td>5 µM Rho-123 + 50 µM VER</td>
<td>2.37±0.18\textsuperscript{a}</td>
<td>3.18±0.40\textsuperscript{b}</td>
</tr>
<tr>
<td>5 µM Rho-123 + 5 µg/ml Tet</td>
<td>2.38±0.24\textsuperscript{a}</td>
<td>3.19±0.37\textsuperscript{a}</td>
</tr>
<tr>
<td>5 µM Rho-123 + 5 µg/ml TA</td>
<td>3.94±0.44\textsuperscript{a}</td>
<td>3.38±0.48\textsuperscript{c}</td>
</tr>
<tr>
<td>5 µM Rho-123 + 5 µg/ml TT</td>
<td>4.8±0.39\textsuperscript{a}</td>
<td>2.73±0.18\textsuperscript{d}</td>
</tr>
<tr>
<td>5 µM Rho-123 + 5 µg/ml TP</td>
<td>2.97±0.27\textsuperscript{a}</td>
<td>2.78±0.18\textsuperscript{a}</td>
</tr>
<tr>
<td>5 µM Rho-123 + 5 µg/ml TF</td>
<td>2.34±0.21\textsuperscript{a}</td>
<td>3.04±0.31\textsuperscript{a}</td>
</tr>
<tr>
<td>5 µM Rho-123 + 5 µg/ml TTF</td>
<td>2.83±0.25\textsuperscript{e}</td>
<td>2.80±0.20\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\*P<0.05, \*P<0.01, \*P<0.001, vs. Rho-123; \*P<0.05, \*P<0.01, \*P<0.001, vs. Rho-123 + 5 µg/ml Tet. Data are expressed as the mean ± standard deviation of three independent experiments. Rho-123 was used at a concentration of 5 µM, Tet and its novel 5-substituted derivatives were used at a concentration of 5 µg/ml (equivalent to 8.0, 7.2, 7.0, 7.1, 7.0 and 6.5 µM, respectively). VER was used at a concentration of 50 µM. Tet, tetrandrine; TA, tetrandrine-aromatic group; TT, tetrandrine-toluene; TP, tetrandrine-pyridine; TF, tetrandrine-fluobenzene; TTF, tetrandrine-trifluobenzene; Rho-123, Rhodamine-123; VER, verapamil; P\textsubscript{app}, apparent permeability coefficient.
be beneficial to the design of potent P-gp inhibitors. These findings would aid in understanding the structure design of tetrandrine derivatives and therefore support further structure optimization to develop novel P-gp inhibitors.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZC and DL conducted the experiments. PY and LL conceived and designed the experiments. ZC analyzed the data. ZC wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References