

The permeability and efflux of Vinca alkaloids in a Caco-2 cell model

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ABSTRACT

Vinca alkaloids are used extensively in the treatment of various diseases and despite their usefulness, drug resistance, attributed to a number of mechanisms associated with the multidrug-resistance phenotype including overexpression of P-glycoprotein (P-gp), remains a serious clinical problem. To identify the possible role of P-gp on the intestinal permeability of anti-arrhythmic crude alkaloids (Vingerbine) from Vinca herbaceae, Caco-2 cells were used in this study. The four Vingerbine constituent alkaloids were analyzed by high performance liquid chromatography (HPLC). Transport parameters, permeability coefficients and percent transports were calculated. Vingerbine constituent alkaloids displayed the identical tendency but with dissimilar degree of modulation in absorptive transport direction. Vincarine and Herbadine demonstrated higher-level intestinal transcellular efflux; the co-presence of verapamil, the absorptive transport of alkaloids increased, while the secretory decreased. No asymmetric permeation was observed for Herbamine and Vincamajine. The study suggests the involvement for multidrug resistance-associated proteins (MRPs) in the intestinal transcellular efflux of Vincarine and Herbadine. The further studies will be focused upon the screening appropriate nanomedicine-based strategies to combat MDR and thus improve intestinal absorption of anti-arrhythmic alkaloids.

KEYWORDS: Vinca alkaloids, Indoline alkaloids, Vingerbine, Caco-2, P-glycoprotein (P-gp).

Introduction

Vinca alkaloids (periwinkle plant, *Catharanthus roseus*) are an important group of natural products widely used as medicinal agents. Despite of various therapeutical efficacies such as antiarrhythmic, hypotensive, antitumor, these compounds are facing problems with less oral bioavailability and interpatient variability (1).

It was found out that the responsible for a low oral bioavailability of alkaloids are the ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp, MDR1), by pumping a variety of drugs out cells at the expense of ATP hydrolysis (2). In comparison to most other transport proteins that recognize specific chemical substrates, P-gp is unusual since it pumps out a variety of lipophilic and cationic compounds including vinca alkaloids (3). The molecular mechanisms underlying broad substrate specificity of this transporter are generally unknown. Different attempts have been made to find a common set of structural and functional features required for a substrate to interact with P-glycoprotein. It has thus been suggested that common property of P-glycoprotein substrates is their relative hydrophobic nature and the minimum set of structural features includes a basic nitrogen atom and planar aromatic domains (4-5).

Vinca herbacea Waldst et Kit, the least investigated among the *Vinca* species, is widely distributed in Georgia. The detailed chemical investigation of this plant has been performed in the laboratory of alkaloids of I. Kutateladze Institute of Pharmacochemistry TSMU and novel natural anti-arrhythmic component – Vingerbine, has been isolated from its aerial part (6-8). Vingerbine is composed by four indoline alkaloids of ajmaline series; the pharmacological studies confirmed that its therapeutic efficacy is generated by specific activity of each compounds (9-12). Thus, high intracellular accumulation of these alkaloids will provide maximal therapeutic effect for Vingerbine.

Since *Vinca* alkaloids are known to be pumped out by P-gp, the transport of Vingerbine constituent alkaloids was investigated in this study through the Caco-2 cells, which can imitate the transport in vitro. P-gp is known to be located in the apical (brush-border) membrane of Caco-2 cells and presents a major barrier to the oral delivery of many structurally diverse drugs and drugs candidates (13-14).

Materials and Methods

Materials. DMSO (Dimethyl sulfoxide) was purchased from Acros organics (USA). Sodium chloride (NaCl), Potassium chloride (KCl) and Glucose were purchased from Fluka chemical and all other reagents and solvents were obtained from Sigma (St. Louis, MO, Austria). The chemicals were of analytical grade and solvents used in high-performance liquid chromatography (HPLC) were of HPLC grade. Vingerbine (Fig. 1), sum of indoline alkaloids was obtained according to the procedures in a previous report (10, 15).

Vingerbine is represented by 4 indoline alkaloids of ajmaline series:

Vincarine (1) [$C_{21}H_{24}N_2O_3$ (353.3), m.p. 264 - 265°C, $[\alpha]_D + 13.75 \pm 0.80$ (ethanol)];

Vincamajine (3) [$C_{22}H_{26}N_2O_3$ (366), m.p. 226- 227°C, $[\alpha]_D - 21 \pm 0.1^0$ (chloroform)];

Herbadine (2) [$C_{21}H_{24}N_2O_4$ (368), m.p. 203- 206°C, (acetone)];

Herbamine (4) [$C_{22}H_{26}N_2O_4$ (382), m.p. 174-176°C, (acetone)] (16).

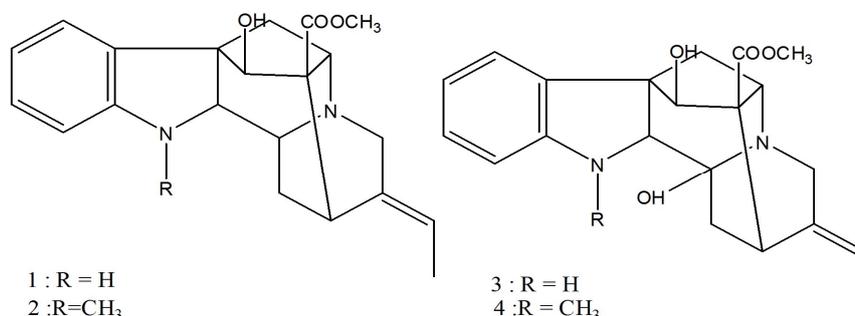


Fig.1. Structures of Vingerbine constituent alkaloids alkaloids.

In-vitro transport studies across Caco-2 cell monolayers. Caco-2 cell monolayer (passage number 85) was grown onto 12 well Transwell polycarbonate membranes (Transwell®, Costar, 0.4 μm pore size, 12 mm diameter) according to the protocol as described by Sattler S. et al. (1997). The cells were cultured in Minimum Essential Medium Eagle (MEM) medium supplemented with 20% fetal calf serum (FCS). The culture medium was exchanged every other day and the cells were stored in a 5% CO_2 -incubator at 37°C. To evaluate the integrity of Caco-2 cell monolayers, transepithelial electrical resistance (TEER) was measured with EVOM instrument (World Precision Instrument, Sarasota, FL). Cell monolayers with TEER values in the range of 500 to 600 $\Omega \text{ cm}^2$ were included in the in-vitro transport studies only.

On the day of experiment, the cell monolayers were rinsed with phosphate buffer saline (pH 6.8). Afterwards 1 mL of HEPES buffer was added to the apical and 1.5 mL to the basal sides of the Transwell insert and after a 30 min equilibration period in 5% CO_2 incubator, TEER was measured to ensure integrity of the Caco-2 cell monolayers. Vingerbine stock solution in a final concentration of 0.02 % (w/v) was added to the apical side for absorptive (AP to BL) transport. Respectively the same concentration of DMSO was made in basolateral side. 200 μL samples were withdrawn from the acceptor sides every 60 min over a time period of 3 h and immediately replaced by 2 % DMSO solution in incubation medium equilibrated at 37°C. The final concentration of DMSO was less than 2% in all experiments, which did not have any detectable effect on the cell monolayers.

Inhibition of transport. Inhibition of Vinca alkaloids transport was tested with known P-gp inhibitor Verapamil (100 μM), which was freshly prepared by dissolving in incubation medium and applied in either the apical or basolateral side. Unidirectional and net flux of Vingerbine was measured in the presence and absence of Verapamil. After 30 min equilibration the drug stock solution was added giving a final concentration of 0.02 % (w/v) to the apical side for absorptive (AP

to BL) / or basolateral for secretory (BL to AP) transport. Then the experiment was performed as described above. TEER was measured every hour. The amount of transported Vinca alkaloids were analyzed as described below.

After completion of the permeation studies, transport medium was removed carefully and Caco-2 cell monolayers were rinsed with phosphate buffer saline (pH 6.8) and the culture medium was applied on the monolayers. The Caco-2 cell monolayers were allowed to regenerate for 24 h in the CO₂-incubator.

Chromatographic conditions. The quantitative analysis of samples that permeated through the monolayers was measured by reversed-phase HPLC (Merck Hitachi ELITE LaChrom, Autosampler L-2200, Pump L-2130). To determine the corresponding alkaloids, the samples were centrifuged at 15000 g for 5 min at room temperature and 50 µL of supernatant was subjected to HPLC. The amount of permeated Vinca alkaloids were measured by HPLC in accordance with the previously reported analytical method (15). The column used was an Agilent Zorbax Eclipse XDB-C8 reversed-phase column (150 x 4.6mm i.d.; 5µm) operated at 25°C and eluted with 0.1% triethylamine (solvent A) and methanol (solvent B) according to the following protocol: 0–40 min, linear gradient from 50:50 (A:B) to 30:70; 40–50 min, isocratic elution with 10:90; and 50–60 min, isocratic elution with 50:50 for column equilibration. The flow rate was 0.8 mL/min and absorbance of the alkaloids was detected at 280 nm. No interference from any Caco-2 monolayers substances was observed during the elution. Compounds were identified according to peak retention times and MS (Fig. 2) (18).

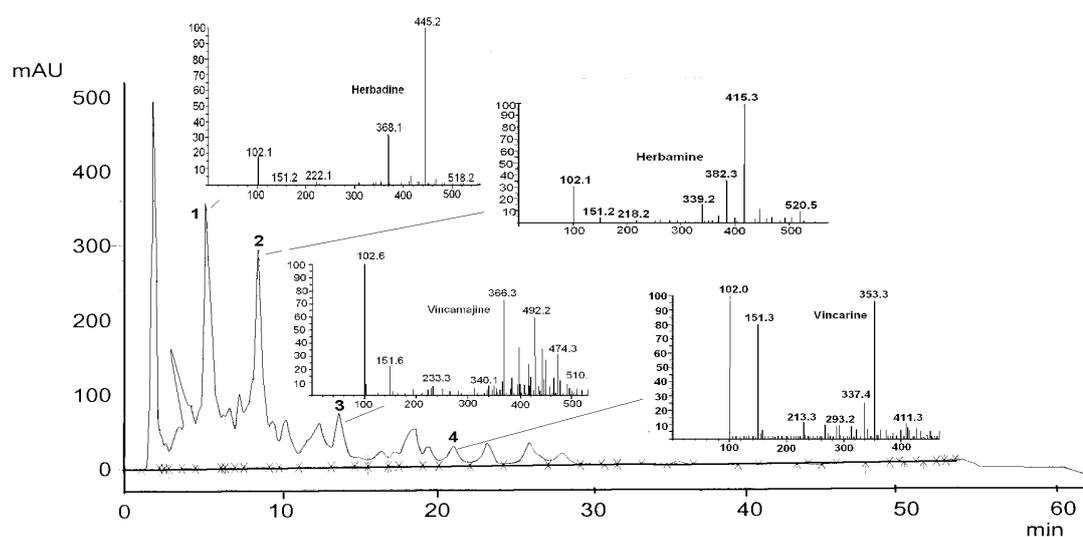


Fig 2. Retention time and identification of Vingerbine alkaloids: Herbadine (1), Herbamine (2), Vincamajine (3), Vincarine (4)

Data analysis. The Permeability coefficient was calculated from:

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{A}$$

Where dQ/dt is the permeability rate ($\mu\text{g/s}$), C_0 is the initial concentration in the donor chamber ($\mu\text{g/mL}$), and A is the membrane surface area (cm^2).

Efflux ratios (P_{ratio}) were calculated from Papp values by $P_{\text{efflux}} = \text{secretory Papp}/\text{absorptive Papp}$.

Percent transport (%T) was calculated as the ratio of permeated alkaloids in the receiver side to the initial concentration in the donor side $\times 100$. Transport was monitored for a period of 3 h.

Statistical analysis. All the experiments were conducted in triplicate and the data are expressed as mean \pm SEM. The statistical significance of difference was analyzed using student's t-test. Values of $P < 0.05$ were considered

Results and discussion

The present study was undertaken to determine the bioavailability and intestinal transport of the Vingerbine compounding vinca alkaloids: Vincarine, Herbadine, Vincamajine and Herbamine (Fig. 1) using Caco-2 cell monolayer. The two transport parameters, Permeability coefficient (Papp) and percent transport (%T) for these compounds were determined in the apical to basolateral (absorptive), as well as in the basolateral to apical (secretive) direction of the monolayer. Transport was monitored for a period of 3 h. The cumulative amount transported with respect to time is shown in Fig. 3; the percent of transported compounds and compared degree of AP-to-BL and BL-to-AP fluxes are listed in Table.

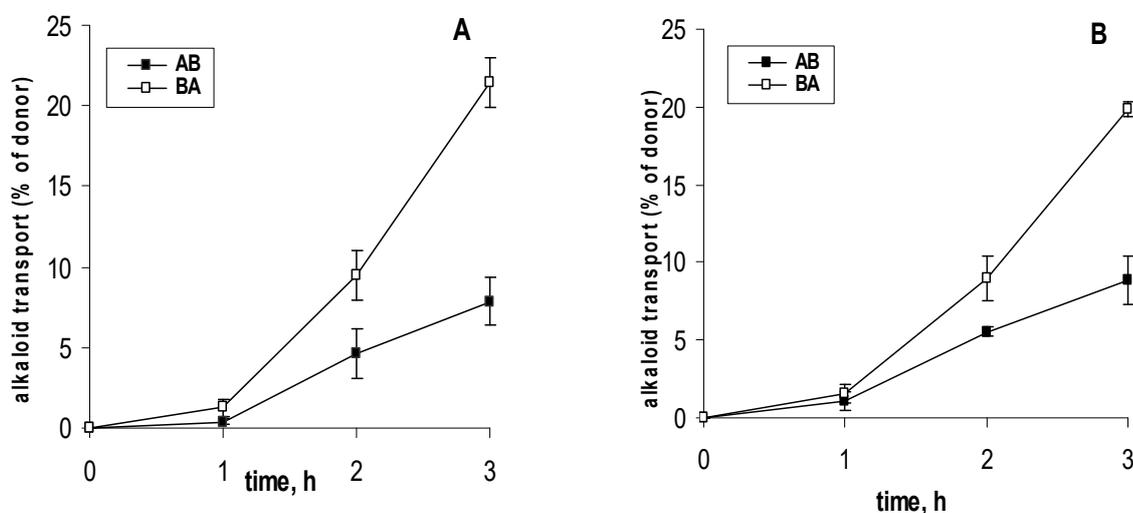


Fig. 3. Transepithelial transport across Caco-2 cells of Vincarine (A) and Herbadine (B). Each value represents the mean \pm SD of at least 3 trials.

Table Transport of Vingerbine Alkaloids across Caco-2 Monolayer (means \pm SD; n = 3-5)

compounds	Absorptive (AP to BL)		Secretory (BL to AP)		P _{ratio}
	P _{app} (cm/s) x 10 ⁻⁵	%T	P _{app} (cm/s) x 10 ⁻⁵	%T	
Vincarine	0.65 \pm 0.12	7.82 \pm 1.48	1.78 \pm 0.13	21.48 \pm 1.54	2.74
Herbadine	0.73 \pm 0.12	8.85 \pm 1,50	1.64 \pm 0.04	19.83 \pm 0.49	2.27

As illustrated in Fig. 3, two Vingerbine alkaloids demonstrated bidirectional transport; BL-to-AP flux for Vincarine and Herbadine was found to be significantly higher than AP-to-BL; the mean apparent permeability coefficients (P_{app}) of Vincarine and Herbadine in the absorptive direction were accordingly 2.74 and 2.27- fold higher than those in the secretory direction (Table). P-gp efflux resulted in higher BL to AP transport than AP to BL transport (21). This assay, where P ratio was compared with a value of 1, was regarded as the standard for identified P-gp substrates. Involvement of a p-gp mediated efflux mechanisms was indicated if the P_{ratio} > 2. The %T for these alkaloids in the mixture vary from 7.8 - 8.85% for absorptive and 19.83 - 21.5% for secretory directions. The results suggest that specific efflux systems such as P-gp may be involved in the permeation of these alkaloids.

This directional difference in transport was not observed with Vincamajine and Herbamine; for them fluxes were approximately equal in both directions. After one hour exposure, the rapid raise of transport was observed in both directions. This pronounced rise of influx and efflux cannot be attributed to drug-induced membrane damage, as the used concentrations of alkaloids and DMSO do not change normal mucosal cell morphology. The efflux ratio value around of 1 (0.84 \pm 0.35 and 0.72 \pm 0.14 for Vincamajine and Herbamine correspondingly) means that the permeation of these compounds in the AP-to-BL and BL-to-AP directions is identical, and is consistent with permeation by simple passive diffusion.

The functional involvement of P-gp in this process was verified by the addition of verapamil, often used at 100 μ M to selectively inhibit the P-gp-based efflux transport. Studies have suggested that verapamil competes with other substrates for binding to P-gp-associated ATPase (19). The influence of inhibitor on the Efflux ratios of Vingerbine constituent alkaloids across Caco-2 cell monolayers is shown on Fig 4. The co-presence of P-gp inhibitor substantially reduced the basolateral to apical efflux of Vincarine and Herbadine and significantly increased the AB-BL flux, thus provided supportive evidence for the involvement of an ATP-dependent active mechanism for intestinal transport of these compounds; on the other hand, the insignificant effect of verapamil on Herbamine and Vicamajine transport indicated a minor role of P-gp in the oral absorption of these alkaloids and excluded them as a substrate for P-gp. These results are compatible with the data obtained earlier from rat intestine model for Vingerbine constituent alkaloids (15).

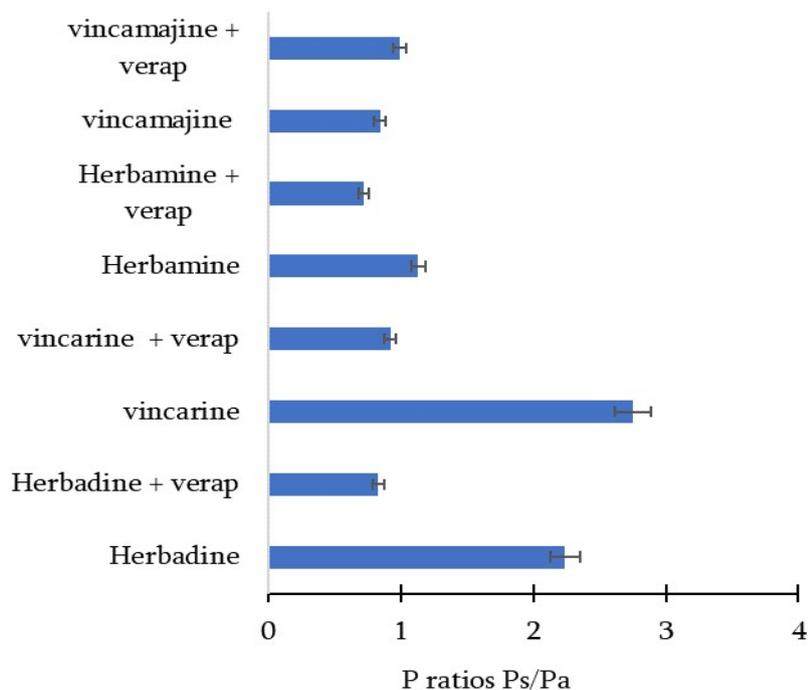


Fig. 4. Paracellular permeability dependence of Vingerbine constituent alkaloids transport across Caco-2 cell monolayers in the presence and absence of Verapamil

In conclusion, Vingerbine constituent alkaloids, having in common certain chemical and physical features, such as hydrophobicity, cationic charge, planar aromatic domain and nitrogen atom, demonstrated different permeability properties across Caco-2 cells. Vincarine and Herbadine, bearing hydrogen atom at first position (N-H), have typical P-gp-involved transport properties including preferential transport in the basolateral to apical direction; meanwhile, in the transport of Herbamine and Vincamajine, possessing methyl group (N-CH₃), PgP/MDR1 played a minor role. Strong relationship has been observed between the lipid solubility of compound and its affinity for P-gp; more hydrophobic Vincarine expressed more effectiveness for interaction with P-gp.

Due to the involvement of P-gp in Vingerbine transport, the further studies will be focused upon the screening appropriate nanomedicine-based strategies to combat MDR and thus improve intestinal absorption of anti-arrhythmic alkaloids.

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ვინკას ალკალოიდების Caco-2 უჯრედებში შეღწევადობა და ემანაცია

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აბსტრაქტი

ვინკას ალკალოიდები ფართოდ გამოიყენება სხვადასხვა დაავადებების სამკურნალოდ; მიუხედავად მათი თერაპიული ეფექტურობისა და მაღალი ფარმაცოლოგიური აქტიურობისა, P-გლიკოპროტეინის (P-gp) ჭარბ ექსპრესიასთან დაკავშირებული ფარმაცორეზისტენტობა რჩება სერიოზულ კლინიკურ პრობლემად. ანტიარითმული ალკალოიდების ჯამის (ვინგერბინი) ნაწლავში ბიოშეღწევადობაზე P-gp-ის შესაძლო როლის დასადგენად კვლევები ჩატარდა ადენოკარცინომულ ურედებზე (Caco-2), როგორც ნაწლავის ბარიერის მოდელზე. ვინგერბინი გამოყოფილია საქართველოში მოზარდი გველის სუროდან (*Vinca herbaceae*) და აიმალინის წარმოებული ოთხი ალკალოიდის (ვინკარინი, ჰერბადინი, ჰერბამინი, ვინკამაინი) ჯამური პრეპარატს წარმოადგენს. ალკალოიდების რაოდენობრივი ანალიზი ჩატარდა მაღალეფექტური სითხური ქრომატოგრაფიით (HPLC). განისაზღვრა ტრანსპორტირების პარამეტრები, ბიოშეღწევადობის კოეფიციენტები და ტრანსპორტირების პროცენტი. ვინკარინი და ჰერბადინი ტრანსცელულარული გამოდევნის მაღალი მაჩვენებლებით გამოირჩეოდნენ; ვერაპამილის თანაარსებობისას, ალკალოიდების აპიკალური ტრანსპორტი გაიზარდა, ხოლო ბაზოლატერული შემცირდა. ჰერბამინისა და ვინკამაინის შემთხვევაში მემბრანაში ასიმეტრიული გამტარობა არ დაფიქსირებულა. მიღებული შედეგებით ვინკარინისა და ჰერბადინისთვის დამახასიათებელია P-gp -ით განპირობებული ტიპური ტრანსპორტირების თვისებები, მათ შორის პრეფერენციული ტრანსპორტი ბაზოლატერულიდან აპიკალური მიმართულებით. ვინგერბინის ტრანსპორტირებაში P-gp-ის ჩართულობის გამო ანტიარითმული ალკალოიდების ნაწლავური შეღწევადობის გასაუმჯობესებლად შემდგომი კვლევები ფოკუსირდება ნანომედიცინაზე დაფუძნებული შესაბამისი სტრატეგიების სკრინინგზე.

საკვანძო სიტყვები: ვინკას ალკალოიდები, ვინგერბინი, Caco-2, P-გლიკოპროტეინი (P-gp).