Intestinal Transport of Pure Diester-type Alkaloids from an Aconite Extract across the Caco-2 Cell Monolayer Model

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Abstract

Aconitine (AC), mesaconitine (MA), and hypaconitine (HA) are the active alkaloids identified in aconite tuber, an important traditional Chinese medicine. The study is aimed to investigate their intestinal transport profiles and potential interaction during the intestinal absorption using the Caco-2 cell monolayer model. All three alkaloids had good permeability with \( P_{\text{app}} \) values greater than \( 1 \times 10^{-6} \text{ cm/s} \). However, AC, MA, and HA in a mixture and as an extract, in both cases with the same content of alkaloids, showed higher transport efficiency in the apical to basolateral, and lower transport efficiency in the basolateral to apical directions. Digoxin, as a P-glycoprotein (P-gp) substrate, was substantially effluxed in the basolateral to apical direction but inhibited by the three alkaloids. Furthermore, the backwards transport of MA and HA was inhibited by the P-gp inhibitor verapamil. These observations indicated that the three alkaloids may not only be P-gp inhibitors but also its substrates; they interact with each other and can potentially enhance their own bioavailability when taken concomitantly.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

>For centuries, Aconitum species (Ranunculaceae) have been widely used as a traditional medicine in East Asia [1,2]. Various alkaloids with properties such as analgesic, anti-inflammatory, anti-epileptic, local anesthetic, hypotensive, and spasmylotic activities have been made responsible for their pharmacological effects [3,4]. However, these alkaloids are compounds with cardiotoxic and neurotoxic activities and show a narrow therapeutic index [5,6]. Therefore, the improper use of Aconitum will result in a risk of severe intoxication [7,8]. Hence it is important to study their bioavailability after oral administration and the factors which may affect their absorption. Only a few references are available on pure aconite alkaloid absorption in vitro and in vivo [9–12]. However, Aconitum extract is usually instead of its pure alkaloids; therefore, the interaction between various alkaloids and the co-occurring components may affect their bioavailability.

Aconitine (AC), mesaconitine (MA), and hypaconitine (HA) are active ingredients in aconite tuber, all belonging to C19-diterpenoid diester-type alkaloids [13,14]. Their structures are shown in Fig 1. In this paper, the intestinal transport profiles of the three isolated alkaloids, a mixture with the same content of alkaloids as compared to the extract, and an aconite extract were studied using the Caco-2 monolayer model, which is the most popular in vitro method developed for permeability and absorption screening [15]. In addition, the P-gp mediated drug transport was also explored in order to estimate potential drug interactions [16]. Digoxin was used as a selective substrate and verapamil as a selective inhibitor of P-gp [17].

Materials and Methods

Chemicals and reagents

AC, MA, and HA (higher than 99% purity) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), verapamil hydrochloride, and digoxin were all obtained...
from Sigma-Aldrich with a purity > 98%. Water was prepared by a Milli-Q water purification system (Millipore), RPMI 1640 medium, Hank’s balanced salt solution (HBSS), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer were all from Gibco Laboratories (Life Technologies, Inc.). Fetal bovine serum, nonessential amino acids (NEAA), and penicillin/streptomycin were obtained from Hyclone. All other chemicals were of analytical grade and solvents used in HPLC were of HPLC grade.

HPLC analysis
The HPLC system (Waters 2695 series) consisted of a diode array UV-VIS detector, gradient pump, autosampler injection device, and Millennium station. Chromatographic separations were performed on an ODS C18 column (150 × 4.6 mm, 5 µm, Agilent Extent C18) at 30°C and a flow rate of 0.6 mL/min. The mobile phases were solvent A, acetic acid/ammonia water (25%)/water (1:10:200, v/v/v), and solvent B, methanol/acetonitrile (1:1, v/v). The gradient system was 50% solvent A at 0 min, 30% solvent A and 70% solvent B at 30 min, and 10% solvent A and 90% solvent B at 55 min. The injection volume was 25 µL, and detection at 235 nm. Retention times for MA, AC, and HA were 24.6 min, 34.1 min, and 41.5 min, respectively. For digoxin assays, the mobile phase was made of water and acetonitrile (65:35, v/v) at a flow rate of 0.6 mL/min at 30°C and detection at 220 nm with a retention time of 5.4 min.

Preparation of aconite extract and estimation of alkaloids
The aconite extract was prepared according to the procedures in a previous report [18], and the three alkaloids were analyzed using the HPLC mentioned above. AC, MA, and HA in the extract were calculated from their peak areas using the pre-established calibration curves; calibration equations were $y = 1807.435 x - 3146$ ($r = 0.9995$), $y = 2211.812 x - 8425$ ($r = 0.9999$), and $y = 4111.582 x - 7005$ ($r = 0.9999$) for AC, MA, and HA respectively. Validation data of the HPLC system are shown as Supporting Information (Table 1S, Table 2S, and Table 3S).

Cell culture
The Caco-2 cell line was purchased from Shanghai Institutes for Biological Science, Chinese Academy of Sciences. The Caco-2 cells were grown in RPMI 1640 medium containing 10 mM HEPES, 2.0 g/L NaHCO₃, supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 1% penicillin/streptomycin (10000 U/mL). Cells were maintained at 37°C in 5% CO₂ and 95% humidified air. For transport studies, the cells (passage 30 ~ 50) were seeded on Transwell polycarbonate insert filters (1.12 cm² surface, 0.4 µm pore size, 12 mm diameter; Corning Costar Corporation) in 12-well plates at a density of 1 × 10⁵ cells/cm². Cells were allowed to grow and differentiate to confluent monolayers for about 21 days post-seeding by changing the medium every two days. Then the filters with cell monolayers were used for transport studies. Integrity of cell monolayers was examined by measuring the transepithelial electrical resistance (TEER) with a Millicell-ERS-electrode (Millipore) before and after the transport experiment. The average TEER value of the monolayers used in the transport experiment was 550 ± 45 Ω·cm² ($n = 12$).

Transport studies and sample preparation
HBSS (pH 7.4) containing 25 mM glucose and 10 mM HEPES was used as the transport medium. Each alkaloid, their mixture with the same content as compared to the extract, and aconite extract dissolved in alcohol were diluted with HBSS to the appropriate concentrations (18 µM, 70 µM, and 100 µM for AC, MA, and HA, respectively) prior to the experiment. The growth medium was aspirated from both sides of the insert and replaced with 0.5 mL HBBS in the apical side and 1.5 mL HBBS in the basolateral side, then the plate was incubated at 37°C for 30 min. After this preincubation period, the experiment was initiated by replacing the transport medium on the donor side with medium containing the test compounds. Thereafter, 100 µL samples each were taken at time intervals of 20, 40, 60, 90, and 120 min from the acceptor compartment. Samples withdrawn from the acceptor side were immediately replaced with an equal volume of preheated HBBS. The transport experiment was monitored for 120 min, and it was conducted in a shaking incubator at 55 rpm. The samples were centrifuged at 14000 r·min⁻¹ for 15 min and then analyzed by HPLC. To check the mass balance, the recoveries of the three alkaloids were measured at both sides of the insert.

The transport experiments for digoxin were carried out as the protocol described above with slight modification. The effect of three alkaloids on the effective efflux of digoxin (35 µM) was determined in the basolateral to apical direction. Verapamil served as the positive control for P-gp inhibition. Verapamil or three alkaloids were added to the basolateral side at 100 µM were placed at both the apical side and the basolateral side of the monolayers.

To explore the influence of P-gp on the transport of the three alkaloids, their transport in the basolateral to apical direction was also performed in the presence of verapamil. In brief, the three alkaloids (35 µM) were added to the basolateral side, respectively, and verapamil (100 µM) was added to both sides of the monolayers. Then the experiment was performed following the method described above.

**Fig. 1** Chemical structures of the three alkaloids.
Data analysis
The apparent permeability coefficients ($P_{\text{app}}$) were calculated according to the following equation [19]:

$$P_{\text{app}} = \frac{\Delta Q}{\Delta t \cdot A \cdot C_0}$$

where $P_{\text{app}}$ is the apparent permeability coefficient (cm/s), $\Delta Q/\Delta t$ is the permeability rate (µmol/s), $A$ is the diffusion area of the monolayer (cm²), and $C_0$ is the initial concentration of the substance in the donor compartment (µmol/L). All the experiments were done in triplicate, and the data were expressed as mean ± SD. Statistical analysis was performed with one-way ANOVA followed by Dunnet's multiple comparison tests as post hoc analysis. The differences between means were considered to be statistically significant when the $p$ values were less than 0.05.

Supporting Information
Validation data of the HPLC system are shown in the Supporting Information.

Results and Discussion
The contents of AC, MA, and HA in aconite extract were 1.40%, 4.86%, and 6.34%, respectively. The mixture of pure AC, MA, and HA was composed of the same ratio. Accordingly, the test solutions for transport studies were prepared in a manner that the content of each alkaloid as a pure compound, in the mixture, and in the extract were the same. All concentrations and all samples were not toxic to the Caco-2 cells (data not shown). The HPLC of the aconite extract is shown in Fig. 2. Transport efficiency percentages of the three alkaloids at each time point were calcu-
AC was observed. The bilateral similar change trend of transport efficiency for MA and HA to that from the mixture. The difference of permeability from BL permeability from AP was the opposite. The results suggested that the three alkaloids promoted their own AP transport, and inhibited their BL transport, and inhibited their BL transport, and inhibited their AP transport to a lower extent compared to MA.

Pure compound

Pure MA, AC, and HA exhibited significantly different bidirectional P\textsubscript{app} values with efflux ratios of 8.44, 12.89, and 1.76, respectively, which suggested that the efflux transport of the three alkaloids was likely to be mediated by some efflux transporters. It is reported that some monoterpeneoids such as (R)-(−)-citronellal can be potential inhibitors of P-gp [20]. Thus, the three diterpenoid alkaloids may also interact with P-gp-mediated transport. Some studies indicated that a higher effect of P-gp inhibitors existed on the secretory pathway compared with the absorptive pathway [21,22]. Furthermore, the BL-AP transport was reported to have a much higher reproducibility compared with the AP-BL transport [23]. Therefore, we evaluated the effect of the three alkaloids on P-gp-mediated digoxin efflux, and the effect of verapamil on the BL-AP transport of the three alkaloids. First, the BL-AP transport of digoxin was evaluated in five experimental groups, namely, digoxin alone (normal control), digoxin in combination with AC, MA, HA, and verapamil, respectively. The two transport parameters for digoxin, transport efficiency and P\textsubscript{app} value, are shown in Table 1. The P\textsubscript{app} values of the three alkaloids are all more than 1 × 10\textsuperscript{6} cm\textsuperscript{2}s\textsuperscript{-1}, confirming that they had good permeability. Additionally, each alkaloid as a pure compound showed lower permeability from AP-BL compared to the mixture and extract, with the permeability from the extract being much higher than that from the mixture. The difference of permeability from BL-AP was the opposite. The results suggested that the three alkaloids promoted their own AP-BL transport, and inhibited their BL-AP transport as well, which was in particular obvious for the extract. Thus, a pharmacokinetic interaction exists between the three alkaloids, concerning their intestinal absorption, which influences their potential bioavailability. In addition, it seems that the presence of other substances (coeffectors) in the extract explains the increased absorption of the alkaloids. Because all three alkaloids had a good recovery rate > 95% in all experiments. Metabolism, cellular accumulation, and/or binding to the plate did not influence the data.

<table>
<thead>
<tr>
<th>Sample</th>
<th>C\textsubscript{0} (µmol/L)</th>
<th>P\textsubscript{app}(A → B) (cm/s × 10\textsuperscript{6})</th>
<th>P\textsubscript{app}(B → A) (cm/s × 10\textsuperscript{6})</th>
<th>Efflux ratio\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA Extract 1</td>
<td>70</td>
<td>16.07 ± 0.99*</td>
<td>20.10 ± 2.13*</td>
<td>1.25 ± 0.35*</td>
</tr>
<tr>
<td>Extract 2</td>
<td>35</td>
<td>14.63 ± 1.10*</td>
<td>18.90 ± 0.96*</td>
<td>1.29 ± 0.28*</td>
</tr>
<tr>
<td>Pure compound</td>
<td>70</td>
<td>3.90 ± 0.92</td>
<td>32.1 ± 0.39</td>
<td>8.44 ± 1.10</td>
</tr>
<tr>
<td>Mixture</td>
<td>70</td>
<td>9.59 ± 1.32*</td>
<td>25.66 ± 1.68</td>
<td>2.68 ± 0.92*</td>
</tr>
<tr>
<td>AC Extract 1</td>
<td>18</td>
<td>17.00 ± 0.85*</td>
<td>17.38 ± 2.11*</td>
<td>1.02 ± 0.44*</td>
</tr>
<tr>
<td>Extract 2</td>
<td>9</td>
<td>13.96 ± 1.24*</td>
<td>19.13 ± 1.74*</td>
<td>1.37 ± 0.52*</td>
</tr>
<tr>
<td>Pure compound</td>
<td>18</td>
<td>3.01 ± 0.72</td>
<td>38.73 ± 1.87</td>
<td>12.89 ± 1.98</td>
</tr>
<tr>
<td>Mixture</td>
<td>18</td>
<td>12.36 ± 1.52*</td>
<td>26.75 ± 1.39*</td>
<td>2.16 ± 0.60*</td>
</tr>
<tr>
<td>HA Extract 1</td>
<td>100</td>
<td>20.45 ± 2.01*</td>
<td>20.51 ± 1.44*</td>
<td>1.00 ± 0.55*</td>
</tr>
<tr>
<td>Extract 2</td>
<td>50</td>
<td>21.98 ± 1.30*</td>
<td>21.16 ± 1.63*</td>
<td>0.96 ± 0.48*</td>
</tr>
<tr>
<td>Pure compound</td>
<td>100</td>
<td>15.21 ± 0.90</td>
<td>26.81 ± 1.20</td>
<td>1.76 ± 0.73</td>
</tr>
<tr>
<td>Mixture</td>
<td>100</td>
<td>18.03 ± 1.45</td>
<td>29.04 ± 2.30</td>
<td>1.61 ± 0.90</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Results are expressed as mean ± SD (n = 3). Statistical analysis was performed with one-way ANOVA followed by Dunnet’s multiple comparison tests as post-hoc analysis. The values with asterisk are significantly different from the values of the corresponding pure compound (* p < 0.05)
This indicates that the three alkaloids (AC, MA, and HA) may not only be P-gp substrates but also its inhibitors, thus they enhanced absorptive transport and inhibited excretive transport for each other when coadministered. However, further investigations are necessary to determine the exact transport mechanism. In conclusion, the alkaloids from aconite show synergistic interaction during the course of intestinal absorption, with even a stronger interaction in the crude extract. This evidences that co-occurring components (coeffectors) from aconite also play an important role in the absorption of the three alkaloids, leading to significantly increased bioavailabilities. On the other side, it indicates that toxicity may be increased due to synergistic absorption of multicomponents in a generally toxic traditional medicine. Similar results have been reported in the literature, e.g., the presence of other compounds in the Rooibos plant was shown to assist in the transport of the flavonoid across the intestinal epithelial cells [24]; higher plasma concentrations of paeniflorin were achieved by using a more crude extract of the plant [25] or accompanying compounds from the Hypericum extract increased the bioavailability of hypericin [26].

Our study provides the basis for further clinical studies on the three alkaloids from aconite, investigating whether they influence the oral bioavailability of drugs which are either the substrates or inhibitors of P-gp. Coadministration of the three alkaloids with such drugs in clinical treatment may cause unwanted interactions by the inhibition of P-gp. Accordingly, Aconitum preparations should be appropriately labeled to alert consumers about their potential interactions when coadministered with other drugs.

Acknowledgements
This work was funded by the National Natural Science Foundation of China (No. 30873360) and National Basic Research Program of China (973 Program) (2011CB505300, 2011CB505305).
Conflict of Interest

There were no financial or commercial conflicts of interest concerning this work.

References

3 Ameri A. The effects of Aconitum alkaloids on the central nervous system. Prog Neurobiol 1998; 56: 211–235
15 Yang XX, Huang X, Mu LA, Wu Q, Xu W. The intestinal permeability of neoglignans from the seeds of Myristica fragrans in the Caco-2 cell monolayer model. Planta Med 2010; 76: 1587–1591