Study on liver targeting effect of vinegar-baked *Radix Bupleuri* on resveratrol in mice

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Abstract

**Background and purpose:** Site-direct delivery is a desirable and elusive goal. In Traditional Chinese Medicine, this goal is usually met by coadministration with a kind of meridian guide drug (MGD). Vinegar-baked *Radix Bupleuri* (VBRB) is usually used to focus other drugs pharmacological effect on liver in Traditional Chinese Medicine (TCM). However, the scientific data for this effect are not available. In this paper, the liver targeting enhancing effect of VBRB on resveratrol was investigated.

**Experimental approach:** Mice, 144, were divided into four groups by random, resveratrol group as control and resveratrol coadministered with three different doses of VBRB peroral. Concentrations of resveratrol in different tissues were determined by HPLC and the target efficiency was evaluated by relative uptake efficiency (RUE) and relative targeting efficiency (RTE).

**Key results:** Compared to the control group, medium dose VBRB enhanced the targeting efficiency of resveratrol significantly, and the RUE and RTE were 1.79 and 46.9%, respectively. Meanwhile, it considerably reduced the distribution of resveratrol in lung and blood, the RUE and RTE in blood were 1.1, -22.6%, and were 0.88, -55.0% in lung, respectively. VBRB reduced the Cmax of resveratrol in almost all the tissues except for liver, heart and kidney, with the extent in the range of 26–61%. In addition, VBRB prolonged the retention time of resveratrol in liver, and shortened the retention time in other tissues.

**Conclusions and implications:** VBRB could enhance the distribution of resveratrol in liver, and reduce the distribution in other tissues, implying that VBRB might be a potential drug for achieving target therapy.

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1. Introduction

It is pivotal to improve curative effect of anti-tumor drugs and reduce adverse reactions involving many organ systems. Many technologies were used for target delivery in recent years (Peek et al., 2008; Moses et al., 2003). However, site-directed pharmacodelivery is a desirable but elusive goal. Although there are great achievements in target delivery, the clinical results were beyond people’s expectation due to the difference between animals and human beings (Sams-Dodd, 2005; Rivera et al., 2002).

In Traditional Chinese Medicine (TCM), the above goal was achieved usually by coadministration with a kind of drug named meridian guide drug (MGD). For example, vinegar-baked *Radix Bupleuri* (VBRB), MGD of liver, is usually used to focus the effect of other drug on liver (Zhao and Liu, 2005).

*Radix Bupleuri*, the dry radix of *Bupleurum Chinense DC*, and *Bupleurum Scozoneri folium wide*, is an important Chinese Herb in the treatment of influenza, fever, malaria, hepatitis, jaundice, nephritis, dizziness, bitter taste in the mouth, lung disease, cancer, and menstrual disorders in China, Japan, and other Asian countries. The pharmacological effect and components in the drug had changed a little (Li, 2000; Nie et al., 2008) due to the vinegar-baked procedure. VBRB has a much stronger effect on acesodyne and bile secretion than that of *Radix Bupleuri* (Wu, 2008; Nie et al., 2002).

As the consequence, *Radix Bupleuri* is used more widely, but VBRB is mainly used in liver related diseases as liver MGD. The compatibility drugs include *Rhizoma polygonum cuspidatum*, Rhubarb, *Radix sophorae Flavescentis*, etc. (Ai, 2002; Yin and Liu, 1998).

Resveratrol (3,5,4′-trihydroxystilbene), a phytoalexin found in grapes, polygonum cuspidatum, mulberry and many other plants, was found to have cancer chemopreventive activity in different...
stages of carcinogenesis (Jang et al., 1997). Since this discovery, resveratrol has been the subject of a large number of preclinical and mechanism studies. It inhibits the proliferation of a variety of cancer cell lines (Gusman et al., 2001), a wide variety of biological effects germane to cancer chemoprevention, including the inhibition of cytochrome P450 enzyme expression activity (Chun et al., 1999; Guengerich et al., 2003), induction of apoptosis (Mahyar-Roemer et al., 2001), modulation of components of the cell cycle machinery (Schneider et al., 2000; Wolter et al., 2001), decrease in cyclooxygenase 1 (COX-1) activity and COX-2 expression (Subbarao et al., 1998), antioxidation (Li et al., 2002), inhibition of activities of protein kinase C and D (Sgambato et al., 2001; Haworth and Avkiran, 2001) and decrease in the activity of transcription factors NFκB and AP-1 (Surh et al., 2001; Banerjee et al., 2002). All the evidence showed resveratrol was a promising anticancer component, therefore, it was selected as the model drug to study the liver target enhancing effect of VBBR, and the method of target delivery system was used.

2. Materials and methods

2.1. Materials

Resveratrol was purchased from Shanxi Huike Phytochemistry Company (purity >98%), Xian, China. VBBR (B Chinese) was purchased from Kangmei Medical Company, Guangzhou China. The sample was authenticated and voucher specimens were deposited in the Pharmacognosy and Phytochemistry Department of Guangzhou University of Traditional Chinese Medicine. Sulfatase from Helix pomatia Type H-1 (S9626-10ku) and /H9252-glucuronidase from bovine liver Type B-1 (G0251-100ku) were purchased from Sigma (USA). Methanol was of HPLC grade, and other chemicals were of analytical grade.

2.2. Extraction of VBBR

VBBR (150 g) was soaked in 1500 mL of water for 0.5 h, and then heated to boil, keep boiling for 45 min, filtrate the extraction. The gruffs was extracted again with 1200 mL of water for a further 0.5 h, the filtrate was pooled, and condensed to 150 mL, and then stored at −20 °C until use. The extraction was divided into 4 parts according to the solubility, Parts 1 and 4 were polysaccharides which could be dissolved in water, Part 2 was high molecular polysaccharides, and Part 4 was low molecular polysaccharides, the contents of it in the extraction was 4.5%, 3.8%, respectively; the components in Part 2 were unclear which could be dissolved in ethanol but could not be dissolved in water, Part 3 was saponin, the contents of sum-saponin in the extraction was 1%. The components in Parts 2 and 3 were analyzed by TLC, the development for Part 2 was dichloromethane–acetoacetate–methanol–water (10:13:7:1), using saikoside a, c, d as the reference compounds. After the development, it was derivatized with 2% paradimethylaminobenzaldehyde in 10% sulfuric acid ethanol solution, the picture was taken at 365 nm and the results was shown in Fig. 1a. The extraction contained saikosaponin a, c, but not contained saikosaponin d.

2.3. Animals

Male Kunming mice (25 ± 2 g) were purchased from Animal Centre of Guangzhou University of Traditional Chinese Medicine (Guangzhou, China). Before experimentation they were kept in groups of six and fed with standard diet with water. Animals were kept under fasting overnight prior to experiment. All animal studies were reviewed and approved by the animal and ethics review committee of Guangzhou University of Traditional Chinese Medicine and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (Euthanasia and disposal of carcass was in accordance with the guidelines).

2.4. Tissue distribution studies

Tissue distribution studies were carried out in Kunming mice after a 7-day acclimatization period. Animals were divided into four groups by random. Resveratrol with different VBBR doses were given to the mice by intragastric administration. VBBR dosage and animal groups were as follows:

F-1 control group: resveratrol 200 mg/kg.
F-2 VBBR lower dosage group: resveratrol 200 mg/kg combined with 10 mg VBBR.

![Fig. 1.](image-url) (a) TLC analysis of component 2. (a) Spots 1, 3, and 5 are Radix Bupleuri, and spots 2, 4, and 6 are VBBR. (b) Spots 1, 2, 5, 6, 9, and 10 are Radix Bupleuri, spots 3, 4, 7, 8, 11, and 12 are VBBR in different batch drug and spots 13, 14, and 15 was saikoside a, c, d respectively.
2.5. Analytical method

2.5.1. Chromatography conditions
The chromatographic analysis was performed on an Agilent 1100 system, which consisted of a G1314A detector at 303 nm, a 10 μL filling loop, a column oven set at 25 °C, and a solvent degasser system, a chromatographic workstation. The column used was Diamonsil C18 analytical column (150 mm × 4.6 mm, 5 μm), with a security guard cartridg C18 (4.6 mm × 2.0 mm, 5 μm, by Phenomenex, USA), the mobile phase was methanol–water (48:52), at a flow rate of 1.0 mL min⁻¹.

2.5.2. Method validation
The specificity of the assay was checked by comparing the chromatograms of blank tissues with the corresponding spiked tissue samples. Each blank sample was tested to ensure that it had no interference on the elution of resveratrol. Calibration curves were constructed by plotting the peak area against the concentration of six different concentrated samples. The accuracy and precision were determined by replicate analysis (n=6) of QC samples at three concentration levels on three different validation days. The extraction recovery of resveratrol at three QC levels were evaluated by assaying the samples as described above and comparing peak areas with those obtained from direct injections of the compounds dissolved in the supernatant of the processed blank plasma.

2.6. Plasma and tissue sample analysis
100 μL plasma was pipetted into a 1.5 mL polypropylene eppendorf vial, then 20 μL sulfatase and β-glucuronidase mixture (1:1) of 1 mg/mL in 0.2% NaCl solution was added, subsequently the mixture was put into a water bath at 37 °C for 16 h. Thereafter, 130 μL of methanol was added, and the mixture was vortexed for 3 min, followed by centrifugation for 20 min at 4500 rpm. The supernatant was collected and 10 μL supernatant was used for HPLC analysis.

On the day of analysis, tissue samples were allowed to thaw, weighed accurately and homogenized with equal weight of 0.9% saline, then 100 μL tissue homogenate was taken and processed using the same procedures as described above for plasma samples and analyzed by HPLC.

2.7. Targeting efficiency evaluation
Drug concentration and retention time in tissues mainly determine its effect. Since the clinical application of VBRB in TCM is similar to the goal of the target delivery system, the targeting efficiency was used to study the distribution effect of VBRB on resveratrol in vivo.

In this paper, two methods were used to appraise the targeting efficiency (Gupta and Hung, 1989).

Relative targeting efficiency (RTE) = \( \frac{AUC_{\text{tissue}}}{AUC_{\text{sum}}} \),

where subscript “sample” represents tissues in groups co-administered with VBRB and “control” represents tissues in the control group of resveratrol:

AUCsum = AUCplasma + AUCheart + AUCliver + AUCkidney + AUCspleen + AUClung.

2.8. Data analysis
The pharmacokinetic parameters of the groups were evaluated using spss 9.0 for Windows, student test was used. Data are presented as means ± standard deviations of five experiments. The statistical significance was determined using one-way analysis of variance (ANOVA) followed by the Dunnett test. Probability P<0.05 were considered significant.

3. Results and discussion

3.1. Analytical method

The results of method validation are listed in Table 1. There was a good linear relationship between the drug concentration in different tissues and peak area, with a standard correlation coefficient >0.9996. The extraction method recoveries were not less than 81.27% in all the analyzed tissues. The inter-day and intra-day precisions described as RSD were below 11.54%.

Since resveratrol is glucuronidated and sulfated in intestinal cells and enters the blood circulation predominantly in its converted forms, further transport in the organism occurs mainly as resveratrol glucuronides and resveratrol sulfates (Wenzel and Somoza, 2005). The effect of resveratrol depends on its metabolites. Therefore, it is desired to determine the total contents of resveratrol including resveratrol and its metabolites.

Because resveratrol glucuronide or sulfate standard substances are currently commercially unavailable, the determination of the resveratrol glucuronides and sulfates in our study was performed by hydrolysis of the metabolites to resveratrol. Several hydrolysis methods such as acid and enzymatic hydrolysis were evaluated. Sulfuric acid, hydrochloric acid, phosphoric acid, β-glucuronidase, and aryl-sulfatase at different concentrations and reaction time on the hydrolysis of resveratrol metabolites were studied (data not shown). In the chromatogram of acid hydrolysis samples new peaks were observed with more noise, and sole enzyme hydrolysis could get a clear chromatogram but some metabolites were remained. When the two enzymes were used together, all the metabolites were transformed to resveratrol (Fig. 2). Therefore, both β-glucuronidase and aryl-sulfatase were used to hydrolyze resveratrol metabolites in this paper. The dose of hydrolysis enzymes and the proper reaction time were investigated and optimized, the optimized dose was 20 μL sulfatase and β-glucuronidase mixture (1:1) of 1 mg/mL in 0.2% NaCl solution, and the optimized reaction time was 16 h.

3.2. Pharmacokinetics of resveratrol in mice

As a control, drug levels in plasma, liver, heart, lung, spleen and kidney were measured after peroral administration of pure resveratrol. Resveratrol was absorbed rapidly, drug concentrations were measured 5 min after administration in all the tested tissues, and then it was eliminated rapidly. This is in accordance with the results
Table 1
Validation of the resveratrol analysis method.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Extraction recovery (%)</th>
<th>Intra-day precision (%)</th>
<th>Inter-day precision (%)</th>
<th>Linear range</th>
<th>Regression equation</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 μg/mL</td>
<td>5 μg/mL</td>
<td>10 μg/mL</td>
<td>0.5 μg/mL</td>
<td>5 μg/mL</td>
<td>10 μg/mL</td>
</tr>
<tr>
<td>Blood</td>
<td>84.93</td>
<td>81.27</td>
<td>88.3</td>
<td>8.21</td>
<td>3.71</td>
<td>2.24</td>
</tr>
<tr>
<td>Heart</td>
<td>86.51</td>
<td>85.58</td>
<td>88.10</td>
<td>1.33</td>
<td>7.04</td>
<td>1.78</td>
</tr>
<tr>
<td>Liver</td>
<td>95.85</td>
<td>94.13</td>
<td>93.13</td>
<td>1.79</td>
<td>9.32</td>
<td>7.34</td>
</tr>
<tr>
<td>Spleen</td>
<td>90.76</td>
<td>89.72</td>
<td>91.91</td>
<td>3.41</td>
<td>1.58</td>
<td>2.58</td>
</tr>
<tr>
<td>Lung</td>
<td>90.65</td>
<td>92.1</td>
<td>92.02</td>
<td>9.49</td>
<td>5.74</td>
<td>6.83</td>
</tr>
<tr>
<td>Kidney</td>
<td>95.68</td>
<td>99.24</td>
<td>93.28</td>
<td>10.61</td>
<td>9.23</td>
<td>11.54</td>
</tr>
</tbody>
</table>

Fig. 2. (a) Chromatogram of blank serum, (b) chromatogram of resveratrol in blank serum, (c) chromatograph of serum before hydrolysis after oral administered resveratrol, and (d) chromatograph of optimized enzyme hydrolysis after oral administered resveratrol.

of Yu et al. (2002). Based on our study, the distribution of resveratrol was mainly in kidney, and AUC values decreased in the order of kidney, liver, lung, plasma, heart and spleen (Fig. 3). In contrast, Sale et al. studied the distribution of resveratrol in mice with a 240 mg/kg dose, and the results showed that the AUC values decreased in the order of intestine, mucosa, liver, heart, lung, plasma and kidney. The reason for this difference is that they did not take the metabolites of resveratrol into account (Sale et al., 2004).

3.3. Target efficiency evaluation

3.3.1. RUE as the index

RUE is an index for targeting, and could reflect the effect of VBRB on absorption of resveratrol in tissues.

As shown in Fig. 4, VBRB could increase the RUE of resveratrol in liver significantly \((P < 0.05)\) when medium and low dose of VBRB were coadministered with resveratrol, and the RUE was 1.79 and 1.29, respectively, indicating that VBRB enforced the targeting of resveratrol in liver. However, when high dose of VBRB was coadministered with resveratrol, the RUE was 0.94 and no statistical difference with the control group was found. This study indicated that the target enhancing effect of VBRB is dose-dependent. In addition, medium dose of VBRB could decrease its RUE in lung \((P < 0.05)\), high dose of VBRB could increase the RUE of resveratrol in plasma \((P < 0.05)\) but decrease the RUE in other organs such as heart, spleen, lung and kidney, the RUEs was 0.68, 0.7, 0.7 and 0.7, respectively.

Fig. 3. Concentration–time profiles of resveratrol in different tissues after peroral administration (200 mg/kg) in mice.

Fig. 4. RUE in different tissues after coadministration of resveratrol (200 mg/kg) with three different doses of VPRB orally in mice.
3.3.2. **RTE as the evaluation criterion**

RTE is a drug distribution ratio in tissues compared to the control group. It implied the change of drug-tissue affinity when coadministered with VBRB. When RTE was over zero, it implied that compared with the control group, the distribution of the drug in the tissues was increased, and it is a targeting enhancing effect, on the contrary, when RTE was below zero, it indicated a targeting weaken effect.

As shown in Fig. 5, based on the RTE values, we can further conclude that medium dose VBRB enhanced the targeting effect of resveratrol in liver significantly, with the RTE values 46.9%. Meanwhile it remarkably decreased the RTE in lung and plasma, and the RTEs were −55% and −22.6%, respectively. Although low and high dose of VBRB exhibited only marginal liver targeting effect with RTE 5.6% and 13.4%, respectively, high dose of VBRB decreased the RTE in other tissues such as lung, kidney, heart, and spleen, the RTEs were between −14.8% and −18.2%. Low dose of VBRB decreased resveratrol distribution in lung and plasma with RTE −28.6% and −17.9%, respectively, and this is another reflection of liver targeting.

By using two different parameters, it was demonstrated that the target enforcing effect of VBRB was dose-dependent and medium dose VBRB could enhance the targeting effect of resveratrol in liver significantly. This is in good coincidence with its application in TCM. In TCM clinic, when VBRB is used as MGD to strengthen meridian, it could enhance the liver cell regeneration, therefore, it has the hepatoprotective effect (Matsumoto et al., 2008), and the results of this paper implied that VBRB could enhance the effect of anticancer drug, and decrease the side-effect of anticancer drug at same time, it should be useful for the therapy of liver cancer. So if there are not chemical interaction between the MGD and therapeutic agent, there should be a synergetic effect in therapy, therefore, a decreased dose for therapy should be considered.

Anyway, compared to the target delivery system, the method for site-directed delivery we presented here is simple, cheap, and easy to do. The results of this paper also indicated that meridian guide theory of TCM is credible and MGD may be a new way for target delivery.

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma</th>
<th>Heart</th>
<th>Liver</th>
<th>Spleen</th>
<th>Lung</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>0.018</td>
<td>0.024</td>
<td>0.034</td>
<td>0.028</td>
<td>0.013</td>
<td>0.044</td>
</tr>
<tr>
<td>Low dose group</td>
<td>0.020</td>
<td>0.009</td>
<td>0.016</td>
<td>0.018</td>
<td>0.016</td>
<td>0.012</td>
</tr>
<tr>
<td>Medium dose group</td>
<td>0.032</td>
<td>0.017</td>
<td>0.014</td>
<td>0.026</td>
<td>0.052</td>
<td>0.011</td>
</tr>
<tr>
<td>High dose group</td>
<td>0.032</td>
<td>0.018</td>
<td>0.018</td>
<td>0.020</td>
<td>0.014</td>
<td>0.019</td>
</tr>
</tbody>
</table>

3.4. **Effect of VBRB on the pharmacokinetics of resveratrol**

Pharmacokinetic parameters could reflect the absorption, distribution, metabolism and elimination of the drug, and comparison of the pharmacokinetic parameters might provide some evidence for the targeting effect.

3.4.1. **Effect of VBRB on the absorption and uptake of resveratrol**

Cmax is an absorption rate and extent parameter. Also, it is an index of targeting effect. As shown in Fig. 6, VBRB had marginal influence on the Cmax of resveratrol in liver. In contrast, compared to the control group, almost all the Cmax in other tissues decreased significantly except in spleen in medium dose, and in plasma in high and medium dose, this is beneficial to reduce the toxicity of drugs, and very important for targeting system design. In the Cmax point of view, liver targeting effect of VBRB was displayed by decreasing drug concentration in other tissues.

References


