Tetrandrine Potentiates the Hypoglycemic Efficacy of Berberine by Inhibiting P-Glycoprotein Function

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This study was designed to improve the absorption and hypoglycemic efficacy of berberine (BBR), which is a substrate of P-glycoprotein (P-gp), by combination with a P-gp inhibitor tetrandrine (Tet). Flow cytometry and LC-MS/MS were used to determine the cellular efflux or retention of chemicals. Pharmacokinetic study was performed in ICR mice following oral administration of the study compounds. The hypoglycemic efficacies of the compounds were evaluated in diabetic KK-Ay mice. In the *in vitro* experiments, Tet significantly inhibited the efflux and increased the uptake of P-gp substrates rhodamine-123 as well as BBR in MCF7/DOX cells and Caco-2 intestinal cells. Meanwhile, Tet greatly reduced the expression of P-gp in Caco-2 cells. The inhibition of BBR efflux by Tet was translated into improved pharmacokinetics *in vivo*. When co-administered, Tet dose-dependently increased the average maximum concentration ($C_{\max}$) and area under concentration–time curve (AUC_{0-24}) of BBR in mice. Tet itself had no impact on glucose metabolism. However, it greatly potentiated the hypoglycemic efficacy of BBR in diabetic KK-Ay mice. In addition, we found that Tet had moderate inhibitory effect on the catalytic activity of CYP3A4, which played a role in the bio-transformation of BBR, and this may also take part in the improvement of the pharmacokinetics of BBR. In summary, combination with P-gp inhibitors such as Tet can improve the pharmacokinetics and hypoglycemic efficacy of BBR greatly; this implicates a feasible strategy for exploring the therapeutic effects of BBR and other pharmaceuticals which are substrates of P-gp.

Key words tetrandrine; P-glycoprotein; hypoglycemic effect; berberine; pharmacokinetics

Treatment of chronic metabolic disorders such as diabetes mellitus usually requires long-term pharmaceutical therapy. Using chemically synthetic drugs in such cases may cause unpredictable adverse effects. For example, over the past years, several reports suggested that rosiglitazone, which was used as a first-line pharmaceutical for type 2 diabetes mellitus, may be linked to an increased risk of heart problems.^{1–3} So, it is of great significance to develop new hypoglycemic drugs with reliable safety, for example, from natural origin.

Berberine (BBR, Fig. 1A) is a natural isoquinoline alkaloid isolated from such plants as *Coptis chinensis* and *Hydrastis canadensis*. BBR has been used in China as a nonprescription medicine for diarrhea since 1950s⁴) and has good safety records in the clinic. We found that BBR can improve hyperlipidemia by increasing the expression of low-density-lipoprotein receptor (LDLR).^{5–7}) The hypoglycemic effect of BBR was firstly reported in 1986⁸) and was proved to be associated with AMP-activated protein kinase (AMPK) activation^{9–11}) as well as insulin receptor (InsR) up-regulation.^{12–14}) Exploration of the therapeutic effects of BBR will help discovering new hypoglycemic drugs with reliable safety.

However, the oral bioavailability of BBR is less than 5%¹⁵); one of the reasons is that cellular efflux transporter P-glycoprotein (P-gp) recognizes BBR as a substrate and directly pumps it out of the cell, which limits the intestinal absorption of BBR.^{16–18}) P-gp is an ATP-dependent efflux transporter on cell membrane which can pump out many chemicals from the cell.^{19–21}) It is extensively distributed and expressed in

intestinal epithelium, hepatocytes, kidney tubules and capillary endothelial cells comprising the blood–brain barrier.²²) Highly-expressed P-gp in tumor cells will result in multi-drug resistance to chemotherapy.²³)

Overcoming P-gp efflux is a strategy to improve the absorption and pharmacokinetics of BBR. In our previous work,²⁴) we have identified a BBR analogue with decreased affinity to P-gp and had improved hypoglycemic efficacy in diabetic mice. In this study, we used another strategy targeting P-gp to improve the intestinal absorption and hypoglycemic efficacy of BBR. In traditional Chinese medicine, a formula always consists of principal components and adjuvant ones which assist the effects or facilitate the delivery of principal components.²⁵) Therefore we tried to use a P-gp inhibitor as an adjuvant component to potentiate the hypoglycemic efficacy of BBR.

Fig. 1. Chemical Structures of Berberine (BBR, A) and Tetrandrine (Tet, B)

The authors declare no conflict of interest.

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Natural botanic product tetrandrine (Tet, Fig. 1B) was selected to compose a combined formula with BBR. Tet is a natural bis-benzylisoquinoline alkaloid isolated from *Stephania tetrandra*. It was found that Tet can reverse multidrug resistance of cancers by inhibiting P-gp on the membrane of cancer cells.^{26–28} In what presented below, we showed that Tet could improve the intestinal absorption and potentiate the hypoglycemic efficacy of BBR by inhibiting P-gp mediated drug efflux and decreasing P-gp expression.

MATERIALS AND METHODS

Cell Culture HepG2 human hepatocellular carcinoma cells, Caco-2 human colorectal adenocarcinoma cells, MCF7 human breast cancer cells and MCF7/DOX human breast cancer cells with resistance to doxorubicin were all obtained from Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences (Shanghai, China). HepG2 cells were cultured in minimum essential medium (MEM) plus 10% fetal bovine serum (FBS), Caco-2 cells were cultured in MEM plus 20% FBS and 1% nonessential amino acids, MCF7 and MCF7/DOX cells were cultured in MEM plus 10% FBS and 0.01 mg/mL human insulin (Sigma-Aldrich Co., St. Louis, MO, U.S.A.).

Chemicals and Reagents BBR (with a purity $\geq 98\%$), Tet (with a purity of 98%) and rhodamine-123 (Rho) were purchased from Sigma-Aldrich Co. Antibodies against P-gp and β -actin (ACTB) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Protein purification kit, Pierce BCA Protein Assay kit and Halt Protease and Phosphatases Inhibitor Cocktail were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.). Cell culture media, FBS and other cell culture related reagents were obtained from Life Technologies Corporation (Grand Island, NY, U.S.A.). Glucose determination reagents were obtained from Beijing Strong Biotechnologies, Inc. (Beijing, China). CYP1A2/CEC, CYP2D6/AMMC and CYP3A4/BFC High Throughput Inhibitor Screening Kits were obtained from BD Biosciences (San Jose, CA, U.S.A.).

Flow Cytometry Analysis Rho (5 $\mu\text{g/mL}$) was added to MCF7 or MCF7/DOX cells with or without Tet (2 μM) for 1 h as indicated. Cells were detached and re-suspended in 1 mL of phosphate buffered saline (PBS) after washed twice with PBS. The fluorescence intensities were analyzed with 488 nm excitation laser and 533 nm emission detection by Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA, U.S.A.).

Cellular Uptake and Efflux Assays Caco-2 cells between passages 30 to 45²⁹ were seeded onto 60-mm culture dishes at a density of $5 \times 10^4/\text{cm}^2$ and were let grown for 14 d with fresh media replaced every other day. Then, confluent cell monolayers were used for chemical uptake and efflux assays.

BBR (20 μM) was added to Caco-2 cells with or without Tet (2 μM) and incubated for 3 h. Then some of the cells were harvested for determination of the uptake of BBR. For the rest of cells, chemical-containing media were discarded; fresh media without chemicals were added to cells initially incubated without Tet, fresh media with 2 μM of Tet were added to cells initially incubated with Tet. Cells were incubated for another 3-hour and harvested for determination of the efflux of BBR.

In another set of experiment, three groups of Caco-2 cells (3 dishes per group) were treated with BBR at 20 μM for 3 h

(without Tet). Then, one group of cells was harvested for determination of the uptake of BBR. For the other 2 groups of cells, drug containing media were discarded, fresh media with or without Tet (2 μM) were added and incubated for another 3-h for determination of the efflux of BBR.

After treatment as indicated, cells were washed twice with cold PBS and harvested by scraping. Harvested cells were then re-suspended in 100 μL of PBS; 50 μL of which was used for protein extraction and quantification, another 50 μL was mixed with 150 μL of acetonitrile (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) for extraction and assay of BBR concentration, which was determined by LC-MS/MS as described previously.^{24,30} The intracellular BBR concentrations were normalized to protein contents and presented as $\mu\text{mol/g}$ protein.

Immunoblot After treatment, cells were harvested and total proteins were extracted. Samples were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation, and then transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore Corporation, Billerica, MA, U.S.A.). For detection of P-gp protein expression, membranes were probed with a specific mouse monoclonal antibody with ACTB as internal control. After incubation with appropriate peroxidase-conjugated secondary antibodies, protein bands were visualized using Chemiluminescence Kit (EMD Millipore Corporation). Signal intensities of the blots were determined with Image Lab Software (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.). P-gp levels were normalized to that of ACTB and plotted as percent of solvent control, which was designated as 100.

Pharmacokinetic Study Male ICR mice (25 $\pm$ 1 g, 8-weeks old) were obtained from Vital River Laboratories (Beijing, China). After fasting overnight, the mice (6 each group) were administered with 100 mg/kg of BBR alone, or 100 mg/kg of BBR plus 10 mg/kg of Tet, or 100 mg/kg of BBR plus 20 mg/kg of Tet by gavage, respectively. Blood samples were collected by retro-orbital puncture and anti-coagulated with heparin at 0, 0.167, 0.5, 1, 2, 4, 8, 12 and 24 h after chemical administration. Plasma concentrations of BBR were determined by LC-MS/MS as described previously.^{24,30}

Glucose Consumption Assay HepG2 cells were treated with BBR (with or without Tet) or Tet as indicated. After incubation for 24 h, cultured media were collected and centrifuged at 1200 rpm for 5 min. Glucose levels in the supernatant were assayed with commercially available kits and standard enzymatic method. Glucose consumption was calculated as follows: glucose level of the fresh medium minus glucose level of the cultured medium.

Hypoglycemic Study in Diabetic Mice Male diabetic KK-Ay mice at 10 to 11 weeks of age were obtained from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (Beijing, China). KK-Ay mice were fed with a high-fat diet containing 34.5% fat, 48.03% carbohydrate and 17.47% protein. Animals were left untreated or orally administered with BBR at 100 mg/(kg d) or 200 mg/(kg d), or BBR at 100 mg/(kg d) plus Tet at 10 mg/(kg d), or Tet at 10 mg/(kg d), respectively. After 3 weeks of treatment, animals were sacrificed; fasting blood glucose (FBG) levels were measured.

Together with the pharmacokinetic study, the animal experiments were reviewed and approved by the Research Committee of the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences; animals were cared for ac-

Fig. 2. Inhibition of P-gp Mediated Efflux of Rhodamine-123 (Rho) by Tet in MCF7/DOX Cells

(A) Protein expression level of P-gp in different cell lines. Total protein contents were extracted from different cell lines as indicated and subjected to SDS-PAGE as described in Materials and Methods. The protein expression level of P-gp was determined with Western blot by using a mouse monoclonal antibody with β -actin (ACTB) as internal control. (B) Flow cytometry analysis. MCF7 and MCF7/DOX cells were treated with solvent or $5 \mu\text{g/mL}$ of Rho (with or without $2 \mu\text{M}$ of Tet) for 1 h as indicated. After wash, cellular fluorescence intensities were assayed by flow cytometry as described in Materials and Methods. The experiments were repeated for at least 3 times, presented are representative images.

cording to the institutional guidelines of Chinese Academy of Medical Sciences.

Cytochrome P450 (CYP450) Catalytic Activity Assay
Effects of chemicals on the catalytic activities of CYP450 isoenzymes were investigated by Gentest P450 High Throughput Inhibitor Screening Kits (BD Biosciences). By following the instruction manuals provided by the vender, the catalytic activities of CYP1A2, CYP2D6 and CYP3A4 were measured by incubation with specific substrates 3-cyano-7-ethoxycoumarin (CEC), 3-[2-(*N,N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC) and 7-benzoyloxy-trifluoromethylcoumarin (BFC), respectively, in the presence of serially diluted chemicals. CEC, AMMC and BFC can be transformed into fluorescent metabolites by CYP1A2, CYP2D6 and CYP3A4, respectively. The fluorescence intensities of metabolites were detected by an Enspire 2300 multilabel reader (PerkinElmer, Inc., Waltham, MA, U.S.A.). The catalytic activities of

CYP450s were plotted against chemical concentrations and IC_{50} values were calculated by SPSS predictive analytics software (IBM Corporation, Armonk, NY, U.S.A.). Furaflavine, quinidine and ketoconazole were used as selective positive control inhibitors for CYP1A2, CYP2D6 and CYP3A4, respectively. IC_{50} value $>50 \mu\text{M}$ indicates no inhibitory effect on CYP450s, at $10\text{--}50 \mu\text{M}$ indicates weak, at $1\text{--}10 \mu\text{M}$ indicates moderate, and IC_{50} value $<1 \mu\text{M}$ indicates strong inhibitory effect on CYP450s, respectively.

Statistical Analysis After validation of the test for homogeneity of variance, differences between or among study groups were examined by one-way analysis of variance followed by the Newman-Keuls test for multiple comparisons. A $p < 0.05$ was considered to be statistically significant.

RESULTS

Tet Overcomes P-gp Efflux and Decreases P-gp Expression in Cultured Cells The inhibitory effect of Tet on P-gp function was verified by using Rho, a classic substrate of P-gp.³¹⁾ Breast cancer cells MCF7 which has no P-gp expression (Fig. 2A) and MCF7/DOX which has high level of P-gp expression (Fig. 2A) were incubated with Rho, fluorescence intensities were determined by flow cytometry. As shown in Fig. 2B, MCF7/DOX cells exhibited obviously decreased fluorescence intensity than MCF7 cells did, which is due to P-gp mediated efflux of Rho. However, when Tet was added to MCF7/DOX cells together with Rho, its fluorescence intensity

increased greatly and reached the level of MCF7 (Fig. 2B). This means that Tet can inhibit P-gp efflux and enable P-gp substrates to retain in cells.

As a substrate of P-gp, BBR was poorly absorbed in the intestinal epithelium, which has high level of P-gp expression.^{16–18)} Our previous study proved that Tet could inhibit the efflux of BBR greatly in Caco-2 cells.²⁴⁾ To learn if Tet had any influence on the uptake of BBR, Caco-2 cells were treated with BBR (with or without Tet) for 3 h, intracellular concentration of BBR was determined with LC-MS/MS. As shown in Fig. 3 (efflux time 0), when co-administered with Tet, Caco-2 intestinal cells will uptake 71.4% more BBR ($p < 0.01$ vs. BBR alone). After removal of chemicals, Caco-2 cells treated with Tet pumped 46.5% of BBR out of cells in 3 h, which was greatly lower than that of BBR alone (74.6%, $p < 0.05$) and was agree with our previous report.²⁴⁾ As a result, in the presence of Tet, the remained BBR in Caco-2 cells after 3 h' efflux increased about 3.6 times compared to that without Tet ($p < 0.001$). These results implied that Tet may improve the intestinal absorption of BBR.

As Caco-2 cell will uptake more BBR in the presence of Tet, to exclude the possibility that low percent of BBR efflux in the presence of Tet is due to saturation of P-gp, we performed another set of experiment, in which Tet was added after removal of BBR. As shown in Fig. 3B, even added after BBR removal, Tet still could reduce its efflux significantly, resulting in retention of more BBR in cells ($p < 0.01$ vs. that of efflux time 3 h without Tet).

Tet not only inhibited P-gp efflux, but also decreased its expression. As shown in Fig. 4, the protein expression level of P-gp reduced dose-dependently after Tet treatment in Caco-2 cells. 1.25 μM to 20 μM of Tet decreased P-gp protein level by 25.8 to 68.3% ($p < 0.05$ or $p < 0.001$ vs. solvent) after exposure for 48 h. Theoretically, this would improve the absorption of BBR further more in long-term combined treatment.

Fig. 3. Tet Increased the Uptake and Inhibited the Efflux of BBR in Caco-2 Cells

(A) Caco-2 intestinal cells were treated with 20 μM of BBR (with or without 2 μM of Tet) for 3 h, cells were harvested for determination of the uptake of BBR (efflux time 0 h). For determination of BBR efflux, chemical-containing media were discarded; fresh media without chemicals were added to cells initially incubated without Tet, fresh media with 2 μM of Tet were added to cells initially incubated with Tet. Cells were then incubated for another 3 h (efflux time 3 h) and harvested. Quantities of BBR in the cells were determined by LC-MS/MS, normalized to protein contents and presented as $\mu\text{mol/g}$ protein. Values were mean $\pm$ S.D. of 5 separate assays. ** $p < 0.01$, *** $p < 0.001$ vs. that of efflux time 0 h without Tet; †† $p < 0.01$ vs. that of efflux time 0 h with Tet. The down arrows indicated the percents of BBR pumped out of cells (efflux rates), ‡ $p < 0.05$ between the two efflux rates. §§§ $p < 0.001$ vs. that of efflux time 3 h without Tet. (B) Three groups of Caco-2 cells (3 dishes per group) were treated with BBR at 20 μM for 3 h (without Tet). Then, one group of cells was harvested for determination of the uptake of BBR (efflux time 0 h). For the other 2 groups of cells, drug containing media were discarded, fresh media with or without Tet (2 μM) were added and incubated for another 3 h for determination of the efflux of BBR (efflux time 3 h). Quantities of BBR in the cells were determined by LC-MS/MS, normalized to protein contents and presented as $\mu\text{mol/g}$ protein. Values were mean $\pm$ S.D. of 3 separate assays. ** $p < 0.01$, *** $p < 0.001$ vs. that of efflux time 0 h without Tet; §§ $p < 0.01$ vs. that of efflux time 3 h without Tet. The down arrows indicated the percents of BBR pumped out of cells (efflux rates), ‡ $p < 0.05$ between the two efflux rates.

Fig. 4. Tet Down-Regulated the Expression of P-gp in Caco-2 Cells

Caco-2 intestinal cells were treated with various concentrations of Tet for 48 h; total protein contents were extracted and subjected to SDS-PAGE as described in Materials and Methods. The protein expression level of P-gp was determined with Western blot by using a mouse monoclonal antibody with ACTB as internal control. Representative blots were presented in the upper panel. Blots were scanned and quantified; the level of P-gp was normalized to that of ACTB and plotted as percent of DMSO (lower panel), which was designated as 100. The quantitative data in histogram were mean $\pm$ S.D. of 3 separate experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. that of DMSO.

Fig. 5. Pharmacokinetic Study

Male ICR mice were orally administered with BBR alone at 100mg/kg, or 100mg/kg of BBR plus 10mg/kg or 20mg/kg of Tet, respectively. Blood samples were taken at 0, 0.167, 0.5, 1, 2, 4, 8, 12, and 24h after administration. Plasma concentrations of BBR were determined by LC-MS/MS and plotted against time. Values were mean $\pm$ S.D. of 6 animals in each group. * p <0.05, ** p <0.01 vs. that of the same time point.

Tet Improves the Pharmacokinetics of BBR in Mice To determine whether increased uptake and reduced cellular efflux by Tet could improve the intestinal absorption and pharmacokinetic profiles of BBR, ICR mice were orally administered with BBR alone (100mg/kg) or BBR plus 10mg/kg or 20mg/kg of Tet; then plasma concentrations of BBR were assayed at different time intervals. As shown in Fig. 5, the average maximum concentrations (C_{max}) of BBR when co-administered with 10 or 20mg/kg of Tet were 15.4 and 18.3ng/mL, respectively. They were about 1.36-fold (p <0.05) and 1.62-fold (p <0.01) of that of BBR alone (11.3ng/mL), respectively. The average area under concentration–time curve (AUC_{0-24}) of BBR when co-administered with 10mg/kg of Tet was 128.7ng/mL, about 1.33-fold (p <0.05) of that of BBR alone (96.8ng/mL); and that of BBR plus 20mg/kg of Tet was 155.8ng/mL, about 1.61-fold (p <0.01) of that of BBR alone. These results suggested that co-administration of Tet could improve the intestinal absorption and bioavailability of BBR in mice.

Tet Has No Effect on Glucose Metabolism *in Vitro* Before investigating whether improved intestinal absorption of BBR by Tet could be translated into potentiated hypoglycemic effect *in vivo*, we firstly tested the effect of Tet on glucose metabolism *in vitro*. As shown in Fig. 6, in HepG2 liver cells, Tet could not stimulate glucose consumption itself and had no impact on the glucose consumption stimulating effect of BBR. Also, we found that Tet had no activity on the AMPK pathway or InsR expression in cultured cells, either treated alone or combined with BBR (data not shown).

Tet Notably Potentiates the Hypoglycemic Effect of BBR in Diabetic Mice Next we investigated the hypoglycemic effect of the formula BBR combined with Tet in diabetic mice. Male KK-Ay diabetic mice were left untreated or orally treated with BBR with or without Tet. After treatment for 3 weeks, mice were sacrificed; fasting blood glucose (FBG) levels were measured. As shown in Fig. 7, 100mg/(kg d) and 200mg/(kg d) of BBR lowered FBG by 20.6% (p <0.05 vs. untreated control) and 39.5% (p <0.01 vs. untreated control), respectively; 10mg/(kg d) of Tet alone could not lower FBG. One hundred milligrams/(kg d) of BBR plus 10mg/(kg d) of Tet lowered

Fig. 6. Glucose Consumption

Hep G2 cells were treated with DMSO, BBR (with or without Tet), or different concentrations of Tet alone as indicated for 24h. Glucose levels in the media were assayed; glucose consumption was calculated as described in Materials and Methods and plotted as fold of DMSO, which was designated as 1. Values are mean $\pm$ S.D. of 5 separate experiments, ** p <0.01 vs. that of DMSO.

FBG by 55.2% (p <0.001 vs. untreated control), more effective than the 20.6% reduction seen in the 100mg/(kg d) of BBR treated group (p <0.01), and even more effective than the 39.5% reduction seen in the 200mg/(kg d) of BBR treated group (p <0.05).

Tet Has Moderate Inhibitory Effect on the Catalytic Activity of CYP3A4 In our previous work, we found that BBR is metabolized mainly to thalifendine (M2) and demethylenoberberine (M3) through transformation by CYP450 isoenzymes CYP1A2, CYP2D6 and CYP3A4.³⁰⁾ To determine whether Tet had any influence on the metabolism of BBR, we investigated the catalytic activities of CYP1A2, CYP2D6 and CYP3A4 in the presence of Tet. As shown in Fig. 8, furafylline, which is a selective positive control inhibitor for CYP1A2, had weak inhibitory effect on it with IC_{50} value of 16.4 μ M; Tet had no inhibitory effect on CYP1A2 with IC_{50} value >100 μ M (Fig. 8A). Quinidine, which is a selective positive control inhibitor for CYP2D6, had moderate inhibitory effect on it with IC_{50} value of 2 μ M; Tet had no inhibitory

Fig. 7. Tet Potentiated the Hypoglycemic Effect of BBR in Diabetic Mice

Male KK-Ay diabetic mice were left untreated or orally administered with BBR at 100 mg/(kg d) or 200 mg/(kg d), or BBR at 100 mg/(kg d) plus Tet at 10 mg/(kg d), or Tet at 10 mg/(kg d), respectively. After treatment for 3 weeks, mice were sacrificed; fasting blood glucose (FBG) levels were measured. Values were mean $\pm$ S.D. of 8 mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. that of untreated control; †† $p < 0.01$ vs. that of BBR 100; ‡ $p < 0.05$ vs. that of BBR 200.

effect on CYP2D6 with IC_{50} value of $58.3 \mu M$ (Fig. 8B). Ketoconazole, which is a selective positive control inhibitor for CYP3A4, had moderate inhibitory effect on it with IC_{50} value of $1.62 \mu M$; Tet also had moderate effect on CYP3A4 with IC_{50} value of $3.9 \mu M$ (Fig. 8C).

DISCUSSION

P-gp is a member of the super family of ATP-binding cassette (ABC) transporters and mediates the clearance of xenotoxins at the expense of ATP hydrolysis.^{19–21} P-gp could prevent the uptake of drugs from the gut and may interfere with the delivery of drugs to target tissues. Here in the present study, we make a new progress that the hypoglycemic efficacy of BBR, which is a substrate of P-gp, is successfully potentiated by combination with Tet.

As a natural product, Tet is a non-competitive inhibitor and not a substrate of P-gp.^{26–28} Tet could reverse the resistance to paclitaxel, a well known P-gp substrate, both *in vitro* in P-gp highly expressed cancer cells and in nude mice bearing corresponding xenografts.³² There were reports that $2.5 \mu M$ of Tet with no cytotoxicity could fully restore the sensitivity of multidrug resistant cancer cells to paclitaxel and vincristine.^{26,32} And as low as $0.5 \mu M$ of Tet still could partially restore the sensitivity.²⁸ Our results were in agree with those previous reports; as shown in Fig. 2, Tet at $2 \mu M$ could inhibit P-gp mediated efflux of Rho in MCF7/DOX multidrug resistant cells.

The mechanisms of action of Tet on P-gp are not fully elucidated now. However, they involved direct interaction with P-gp²⁶ and were different from that of verapamil and cyclosporine A (CsA), which were substrates and also competitive inhibitors of P-gp.^{26–28} In multidrug resistant cancer cells, Tet exhibited stronger and significantly prolonged resistance reversal activity than CsA.²⁸

Besides anti-cancer drugs, Tet had influence on other P-gp substrates, too. For example, there was a report that when co-administered with Tet, the pharmacokinetic parameters of CsA were greatly improved in rabbits.³³ Together with our results, they suggested that Tet had substantial influence on the pharmacokinetics and pharmacological effects of P-gp substrates.

Our results showed that as a substrate of P-gp, the efflux

Fig. 8. Catalytic Activities of CYP450 Isoenzymes Responsible for the Bio-Transformation of BBR

Serially diluted Tet was incubated with recombinant human CYP450 isoenzymes CYP1A2 (A), CYP2D6 (B) and CYP3A4 (C) in the presence of specific substrates, with furafylline, quinidine and ketoconazole as selective positive control inhibitors, respectively. The fluorescence intensities of metabolites were read on an Enspire 2300 multilabel reader with an excitation and emission wavelength suitable to detect the fluorescent metabolites. Catalytic activities of CYP450 isoenzymes were plotted against chemical concentrations; IC_{50} values were calculated and classified as described in Materials and Methods. The experiments were repeated for 3 times, presented were representative results.

of BBR was blocked by Tet in P-gp highly expressed Caco-2 intestinal cells; and the inhibitory effect of Tet was obvious even when it was added after BBR removal from the culture media. In addition, we found that the uptake of BBR was increased greatly in the presence of Tet, which resulted in accumulation of BBR in Caco-2 cells. These beneficial effects of Tet were translated into improved pharmacokinetics of BBR in animals. Tet dose-dependently improved the pharmacokinetic parameters of BBR when co-administered.

Besides Tet, other P-gp inhibitors such as verapamil and CsA also increased the absorption of BBR, both in Caco-2 cells, and *in vivo* in a rat model.^{16,17,34} And also, a monoclonal antibody against P-gp could improve the uptake of BBR in Caco-2 cells.¹⁶ Taken together, these data suggested that P-gp did play an important role in the pharmacokinetics of BBR.

In the *in vitro* experiments, we found that Tet did not stimulate glucose utility in HepG2 cells when treated alone, as expected. Our results also showed that Tet could not enhance the glucose consumption stimulating efficacy of BBR in HepG2 cells. This could be explained by the limited contribution of P-gp to the efflux of BBR in liver cells.²⁴ As shown in our

previous report,²⁴⁾ P-gp was expressed at a low level in liver cells, and Tet only could slightly inhibit the efflux of BBR in liver cells with no statistical significance. Here in the present study, we found that Tet did not increase the uptake of BBR in HepG2 cells (data not shown). There were reports that multidrug resistance-associated proteins (MRPs) were expressed in the liver and could pump BBR out of cells.^{18,35)} Currently, there is no report whether Tet could influence the functions of MRPs. However, based on our results, we infer that Tet could not inhibit the transporters responsible for BBR efflux other than P-gp in liver cells.

Also, we noticed that Caco-2 cells treated with Tet still could pump out a part of BBR after 3 h of efflux time (Fig. 3). This could be due to the existence of MRPs in the intestine,³⁶⁾ too. However, based on other reports and our results,^{16,17,34)} P-gp was a key factor responsible for the efflux of BBR in the intestine and Caco-2 cells.

Although Tet had no effect on glucose metabolism itself, it notably potentiated the hypoglycemic efficacy of BBR in diabetic mice, through facilitating the absorption of BBR. The hypoglycemic effect of 100 mg/(kg d) of BBR plus 10 mg/(kg d) of Tet was more effective than that of BBR alone, either at 100 mg/(kg d) or at 200 mg/(kg d). This means more efficient and more economical for clinic use. Such components of a combined formula are classified as Guide Drugs in traditional Chinese medicine,³⁷⁾ which has been used as a rule for more than 2500 years (Yellow Emperor's Inner Canon). Traditional medicines of natural origin may be a beneficial choice for pharmaceutical R&D, especially for chronic metabolic disorders.

In our results, the hypoglycemic effect of BBR when co-administered with Tet was even more outstanding than double-dosed. This could be explained by the poor oral bioavailability of BBR. It is possible that the absorption of BBR was increased only by a limited extent when double-dosed, and less than that of BBR/Tet combination. We are now designing experiments to prove this point.

Besides cellular efflux, metabolism/degradation is another important factor affecting pharmacokinetics of drugs. In this study, we found that Tet had moderate inhibitory effect on the catalytic activity of CYP3A4, which play a role in the degradation of BBR. This means that inhibition of CYP3A4 transformation may take part in the improvement of pharmacokinetics of BBR by Tet. Furthermore, it had been shown that there was some kind of functional relationship existed between CYP3A4 and P-gp, for their similar distribution and substrate profile.^{38,39)} It is possible that Tet inhibits CYP3A4 and P-gp in coordination to facilitate the absorption of BBR. More experimental data are required to support this hypothesis.

In this study, we provided another strategy targeting P-gp to improve the efficacy of existing drugs. Though it has been found that CsA and verapamil may increase the intestinal absorption of BBR,¹⁶⁾ they were not practical for clinical use considering the obvious side effects of those chemicals. At present time, new kinds or third-generation of P-gp inhibitors which were low toxic and high selective are under development, and some of them have already entered clinical trials for cancer therapy.⁴⁰⁾

Tet is a potent and specific inhibitor of P-gp,³²⁾ it has been used to treat silicosis and mild hypertension in China since

1960s, clinical data showed that it was safe for long-term use.⁴¹⁾ Our data suggest that formula of BBR combined with Tet may be suitable for the treatment of chronic metabolic disorders in the future. In addition, Tet can not only reduce P-gp efflux but also decrease P-gp expression in Caco-2 intestinal cells (Fig. 4). This would be likely to counteract the up-regulatory effect of BBR on P-gp expression²⁴⁾ and be beneficial for long-term therapy with BBR.

In summary, combination with P-gp inhibitors such as Tet can improve the intestinal absorption and *in vivo* hypoglycemic efficacy of BBR. This implicates a feasible strategy for exploring therapeutic effects of BBR and other pharmaceuticals which are substrates of P-gp.

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