Inhibitor of P-glycoprotein–mediated drug transport by curcumin in broiler chickens

Mei Li, MS; Maynur Abdurexit, MS; Ziyong Xu, MS; Yanhong Gao, MS; Yujuan Zhang, PhD*

School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, China
*Corresponding author: Dr. Yujuan Zhang (zhangyujuan907@163.com)

OBJECTIVE
To investigate the role of curcumin in the regulation of P-glycoprotein (P-gp) and its influence on the pharmacokinetics of P-gp substrates.

SAMPLE
39 broiler chicken and chicken embryonic primary hepatocytes.

METHODS
Chicken embryonic primary hepatocytes were treated with curcumin, after which cell viability, P-gp expression, and transport were assessed. Broiler chickens were pretreated with curcumin, after which P-gp expression and the pharmacokinetic behavior of orally administered sulfadiazine (a substrate of P-gp) were measured.

RESULTS
The preliminary results showed that the viability of chicken embryonic primary hepatocytes was enhanced by pretreatment with 40, 60, and 100 μM curcumin. Curcumin inhibits the expression and transport of P-gp. In vivo experiments showed that curcumin decreased the expression of P-gp in the broiler chicken liver, kidney, and small intestine. Pretreatment with curcumin changed the pharmacokinetic behavior of orally administered sulfadiazine by increasing the area under the curve (47.36 vs 70.35 h·mg/L, \( P < .01 \)) and peak concentration (10.1 vs 14.53 μg/mL, \( P < .01 \)).

CLINICAL RELEVANCE
Curcumin inhibited the expression and efflux of chicken P-gp, thereby improving the oral bioavailability of P-gp substrate drugs. These findings provide a rationale for exploiting herbal-drug interactions in veterinary practice to improve the absorption of drugs.

Keywords: curcumin, P-gp, inhibitor, sulfadiazine, broiler chickens

© 2024 THE AUTHORS. Published by the American Veterinary Medical Association as an Open Access article under Creative Commons CCBY-NC license.
to improve absorption. High toxicity leads to clinical failure of first- and second-generation P-gp inhibitors. However, the third generation of P-gp inhibitors, such as tariquidar, still faces the shortcomings of insufficient efficacy and high toxicity. Owing to the poor success of these 3 generations of P-gp inhibitors, new strategies, such as “returning” to products of natural origin, have been used to find P-gp inhibitors. Curcumin is a drug extracted from the rhizomes of some plants of the ginger family and Araceae family. In animal husbandry, the addition of a certain proportion of curcumin can significantly increase the weight and daily gain of broilers and improve meat color. The addition of low (10 μM), medium (20 μM), or high (50 μM) concentrations of curcumin to oxaliplatin (L-OHP)-resistant colorectal cancer cell lines significantly decreases the expression of P-gp at both the mRNA and protein levels in a concentration-dependent manner, which reduces the efflux of P-gp substrate drugs in chemotherapy. He et al. showed that curcumin may reverse HCT-8/5-FU (5-fluorouracil-resistant cell line) resistance to 5-FU by downregulating P-gp expression. However, it has been noticed that there are species differences in the structure, substrate profile, and gene expression regulation of P-gp. For example, blast analysis revealed that the amino acid sequence of chicken P-gp had a 72%, 72%, 71%, and 68% identity to that of the homologs from human, pig, rat, and cattle, respectively. In addition, the change in one amino acid can cause differences in the substrate spectrum and regulatory mechanisms. The above research showed that curcumin has an inhibitory effect on human P-gp and can improve the accumulation of its substrate drugs, but the relationship between curcumin and chicken P-gp has not been reported.

Therefore, the purpose of this paper was to study the effect of curcumin on the expression and functional activity of broiler chicken P-gp. We hypothesized that curcumin would inhibit the expression and transport of P-gp, therefore increasing the oral bioavailability of the P-gp substrate sulfadiazine. The findings should be informative for guiding the rational use of curcumin in the poultry industry to improve the oral bioavailability of drugs.

Methods

Reagents and chemicals
Curcumin (CAS No. 458-37-7), sulfadiazine (CAS No. 68-35-9), and rhodamine 123 (Rho123; CAS No. 62669-70-9; Shanghai Yuanye Biotechnology Co, Ltd) were used. Transferrin (Beijing Solarbio Science & Technology Co, Ltd), DNA transfection reagents (Vazyme Biotech Co, Ltd), Advanced RNA Transfection Reagent (Zeta Life), and the Cell Counting Kit-8 (CCK-8; Sangon Biotech Co, Ltd) were used.

Chicken embryonic primary hepatocytes
We selected chicken embryonic primary hepatocytes as in vitro models to investigate the molecular mechanism of P-gp gene expression under the influence of exogenous curcumin. Chicken embryonic primary hepatocytes were isolated from livers taken from Arbor Acres broiler embryos after 14 days of incubation, unmodified as previously described. Isolated chicken embryonic primary hepatocytes were cultured in high-glucose DMEM supplemented with 5 μg/mL transferrin, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The cells were placed in a 37°C thermostatic cell incubator with 95% humidity and 5% CO2.

Cytotoxicity assays
To test the cytotoxic effect of curcumin on chicken embryonic primary hepatocytes, the CCK-8 method was used to evaluate the effect of curcumin on chicken embryonic primary hepatocyte activity. Briefly, after 100 μL of cell suspension (5,000 cells/well) was seeded in 96-well plates and grew to approximately 80% confluence, various concentrations of curcumin (20, 40, 60, 80, and 100 μM) were subsequently added in the cell culture. Chicken embryonic primary hepatocytes treated by PBS were added as a control. Then, the cells cultured in separated 96-well plates were allowed to incubate in a 37°C incubator for 36 or 60 hours. Thereafter, 10 μL of CCK-8 solution was added to cells and incubated at 37°C for 4 hours. Finally, the OD was detected by measuring absorbance at 450 nm using a microplate reader (Bio-TEK). Cell viability was calculated using the following equation: cell viability = (OD value of treated cells/OD value of control cells) X 100%.

RNA extraction and real-time PCR
Total cellular RNA was isolated from chicken embryonic primary hepatocytes by TRIzol reagent (Takara) and quantified via a microspectrophotometer. The RNA purity was checked by measuring the ratio of absorbance at 260 and 280 nm, which was between 1.8 and 2.0. The RNA was subsequently synthesized into cDNA using a reverse transcription kit (Takara). The chicken ABCB1 gene was quantitatively detected by NovoStart SYBR qPCR SuperMix (Novoprotein) and a real-time fluorescence quantitative PCR detection system, and the chicken B-actin housekeeping gene was used as an internal reference. The 2−ΔΔCt method was used for the analysis.

Functional detection of P-gp activity
Primary hepatocytes from chicken embryos were inoculated in 12-well plates and cultured to approximately 80% confluence. Untreated cells were used as controls, and the other cells were treated with 20, 60, or 100 μM curcumin for 24 hours. Then, the original medium was discarded, and the P-gp fluorescent substrate Rho123 was incubated with 5 μM for 1 hour. Trypsin was used to digest the cells into a single-cell suspension, after which the cells were washed with PBS 3 times. The fluorescence signal of the sample was recorded by a FACS Calibur with CellQuest Pro software using channels FL-2 for Rho123 (excitation wavelength, 485 nm; emission wavelength, 527 nm).
Experimental animals and sample collection

Arbor Acres broiler chickens were obtained from a local commercial poultry farm (Nanjing). The treatment method for the broiler chickens was approved by the Jiangsu Provincial Department of Science and Technology (approval No. 2017-0007) and followed the guidelines of the Jiangsu Provincial Department of Science and Technology and Nanjing Agricultural University. Fifteen 8-week-old broiler chickens with similar body weights (about 2 kg) were randomly divided into 3 groups with 5 broiler chickens per group. Group 1 was treated as a blank control group without any treatment. In group 2 and group 3, the broiler chickens were treated with curcumin orally; in group 2, the broiler chickens received a dose of 20 mg/kg orally once; and in group 3, the broiler chickens received a dose of 150 mg/kg curcumin orally once. The experimental diets and water were available ad libitum during the entire experimental period for all broiler chickens at the recommended humidity and temperature. Chickens were slaughtered by carbon dioxide asphyxiation machine after curcumin administration for 48 hours. Tissue samples from all broiler chickens including the liver, kidney, and small intestine were collected, rapidly frozen in liquid nitrogen, and stored at −70 °C for further analysis.

Pharmacokinetic studies of sulfadiazine in broiler chickens

Twenty-four 8-week-old broiler chickens were randomly divided into 4 groups. The first group was treated with sulfadiazine orally (20 mg/kg); the second group was given curcumin (150 mg/chicken) first and oral sulfadiazine (20 mg/kg) 24 hours later. The third group received a single dose of 20 mg/kg of sulfadiazine, IV, utilizing the left brachialis vein. The fourth group was first given curcumin (150 mg/chicken) orally, and sulfadiazine (20 mg/kg) was administered IV 24 hours later. At 0.17, 0.33, 0.5, 0.67, 0.83, 1, 2, 4, 6, 8, 10, and 12 hours after oral sulfadiazine administration, a blood sample was collected from the brachial vein of each broiler chicken and placed into heparin-containing tubes (33 IU/2 mL sodium heparin; Aladdin). All broiler chickens were treated following the protocol approved by Nanjing Agricultural University Animal Care and Use Committee (SYPK-Su 2021-0036). After centrifugation at 5,000 X g for 5 minutes, the plasma was rapidly collected and stored at −80 °C for further analysis.

The concentration of sulfadiazine in the plasma was determined via the Thermo Fisher U3000 HPLC system as previously reported with slight modification. In brief, frozen plasma (0.2 mL) was thawed at 4 °C, and the plasma sample was extracted with acetonitrile. The extraction solution was evaporated under nitrogen at 4 °C and dissolved in the mobile phase. The mobile phase consisted of a mixture of acetonitrile:water at a ratio of 18:82 (vol/vol). A C18 reversed-phase column (4.6 X 250 mm) was used for separation. The UV detector was set at 277 nm, and the flow rate of the mobile phase was 1 mL/min. The limit of detection and limit of quantification of sulfadiazine were 0.05 and 0.1 μg/mL, respectively. At concentrations of 0.1, 1, and 10 μg/mL, the recovery ranges of sulfadiazine were all greater than 92.1%, meeting the requirements of pharmacokinetic research for drug plasma recovery rates. The stability of sulfadiazine was measured by precision under different conditions as follows: short-term placement at room temperature (25 °C, 4 hours), repeated freezing and thawing 3 times, long-term placement (~20 °C, 2 weeks), and placement at room temperature before injection (25 °C, 13 hours). The average of inter- and intraday precision (relative SD) was < 11%. The calibration samples were prepared with 7 different concentrations of the respective drug using blank plasma. A linear relationship existed in the calibration curve from 0.1 to 100 μg/mL, which consistently yielded a correlation coefficient of > 0.999.

The pharmacokinetic parameters were calculated using a noncompartmental analysis and a computer program (WinNonlin 8.1; Phoenix Software).

Statistical analysis

The data are expressed as the mean ± SD. The statistical significance of the data was analyzed using Student t test (2 groups) or 1-way ANOVA followed by a least significant difference post hoc test with SPSS 20.0 (IBM Corp). P < .05 was considered to indicate statistical significance, and P < .01 was considered to indicate an extremely significant difference.

Results

Effect of curcumin on the viability of chicken embryonic primary hepatocytes

The cytotoxicity of curcumin to chicken embryonic primary hepatocytes was detected by a CCK-8 assay. As shown (Figure 1), there was no obvious
toxicity in chicken embryonic primary hepatocytes treated with 0 to 100 μM curcumin for 36 or 60 hours, and with increasing curcumin concentrations, cell viability was significantly greater than that in the control group. Therefore, chicken embryonic primary hepatocytes with exposure to up to 100 μM curcumin for up to 60 hours could be chosen as the appropriate experimental scheme, which resulted in a cell survival rate of more than 90%.

Concentration-dependent effects of curcumin on P-gp mRNA expression

Chicken embryonic primary hepatocytes were treated with different concentrations of curcumin to examine the effect of curcumin on cellular ABCB1 mRNA expression (Figure 2). With increasing curcumin concentrations from 10 to 100 μM, the expression level of ABCB1 mRNA in chicken embryonic primary hepatocytes significantly decreased in a concentration-dependent manner. Specifically, when the concentration of curcumin was 10, 20, 40, 60, or 100 μM, the mRNA expression levels of Abcb1 were 0.83-, 0.75-, 0.63-, 0.61-, and 0.54-fold of the control group, respectively.

Effect of curcumin on P-gp function in chicken embryonic primary hepatocytes

To further determine whether the effect of curcumin on P-gp expression modulates P-gp transport, we performed a fluorescent substrate drug accumulation assay in chicken embryonic primary hepatocytes (Figure 3), in which the fluorescence intensity of Rho123 (a selective fluorescent substrate for P-gp) was used to reflect P-gp transport activity. Compared with that in the control group, the fluorescence intensity of Rho123 in chicken embryonic primary hepatocytes treated with different concentrations (10 to 100 μM) of curcumin for 12 hours was significantly enhanced. Specifically, when the concentration of curcumin was 40, 60, or 100 μM, the fluorescence intensity of Rho123 in cells increased to 1.45-, 1.67-, and 2.4-fold that of the control group, respectively. Indicating a decrease in the efflux of Rho123 by P-gp in cells treated with curcumin.

Figure 2—Effect of different concentrations of curcumin on the expression of ABCB1 mRNA in chicken embryonic primary hepatocytes was detected via real-time fluorescence quantitative PCR. Chicken embryonic primary hepatocytes were exposed to different concentrations of curcumin (0, 10, 20, 40, 60, or 100 μM) for 48 hours. β-Actin was used as the reference gene for normalization (n = 5). *P < .05 and **P < .01, differences between control and curcumin-treated hepatocytes.

Figure 3—P-glycoprotein transport activity was assessed by Rho123 fluorescence in chicken embryonic primary hepatocytes untreated or treated with different concentrations of curcumin for 24 hours (A and B). Histograms showing fluorescence (y-axis) indicating Rho123 accumulation as a function of the curcumin concentration (x-axis). The bars show the means ± SDs of at least 3 independent experiments. *P < .05 and **P < .01.
Effect of curcumin on P-gp mRNA expression in broiler chickens

To further verify the regulatory effect of curcumin on ABCB1, 15 8-week-old chicks were randomly divided into 3 groups and given different doses (0, 20, or 150 mg/kg) of curcumin by gavage for 48 hours. Liver, kidney, and small intestine tissues were collected, and P-gp expression was measured (Figure 4). ABCB1 mRNA levels were decreased in a dose-dependent manner (0.2- and 0.23-fold, respectively, in liver tissue; 0.49- and 0.69-fold, respectively, in kidney tissue; and 0.46- and 0.54-fold, respectively, in small intestine tissue) compared with those in the control group, suggesting that curcumin has a suppressive effect on the ABCB1 gene.

Curcumin affects the pharmacokinetics of sulfadiazine in broiler chickens

To investigate whether the inhibition of P-gp by curcumin affects the absorption and metabolism of the drug in vivo, we conducted a pharmacokinetic analysis of the P-gp substrate sulfadiazine in 8-week-old broiler chickens (Figure 5). The relevant pharmacokinetic parameters are shown (Table 1). Compared with those in the control group, the peak concentration and area under the curve of sulfadiazine in the curcumin-treated group increased from 10.1 to 14.53 µg/ml (P < .01) and from 47.36 to 70.35 h·mg/L (P < .01), respectively. The peak concentration of the curcumin group was always greater than that of the control group within 10 hours after administration. At the same time, the apparent clearance and apparent volume of distribution per fraction of the dose absorbed by the curcumin treatment group were significantly different from those of the control group (0.28 vs 0.43 L/h/kg and 0.68 vs 1.48 L/kg, P < .01). However, the parameters and plasma concentration-time profiles of sulfadiazine after IV administration were not apparently changed by curcumin. The relevant pharmacokinetic parameters are shown (Table 2).
Table 1—Pharmacokinetic parameters of orally administered sulfadiazine in broiler chicken.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sulfadiazine</th>
<th>Sulfadiazine + curcumin</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (μg/mL)</td>
<td>10.10 ± 1.59</td>
<td>14.53 ± 0.95**</td>
<td>.000</td>
</tr>
<tr>
<td>$t_{\text{max}}$ (h)</td>
<td>1.86 ± 0.38</td>
<td>1.10 ± 0.42**</td>
<td>.004</td>
</tr>
<tr>
<td>$t_{1/2\alpha}$ (h)</td>
<td>2.35 ± 0.67</td>
<td>1.67 ± 0.35*</td>
<td>.034</td>
</tr>
<tr>
<td>$V_{z/F}$ (L/kg)</td>
<td>1.48 ± 0.58</td>
<td>0.68 ± 0.13**</td>
<td>.010</td>
</tr>
<tr>
<td>$C_{l/F}$ (L/h/kg)</td>
<td>0.43 ± 0.10</td>
<td>0.28 ± 0.03**</td>
<td>.007</td>
</tr>
<tr>
<td>$AUC_{0-t}$ (μg/mL)</td>
<td>47.36 ± 10.77</td>
<td>70.35 ± 7.79**</td>
<td>.001</td>
</tr>
<tr>
<td>F (%)</td>
<td>52.5</td>
<td>77.2</td>
<td></td>
</tr>
</tbody>
</table>

$AUC_{0-t}$ = Area under the plasma concentration-time curve. $Cl/F$ = Apparent clearance. $C_{\text{max}}$ = Peak concentration. $F$ = Bioavailability. $t_{1/2\alpha}$ = Elimination half-life time. $t_{\text{max}}$ = Peak time. $V_{z/F}$ = Apparent volume of distribution per fraction of the dose absorbed.

*P < .05; **P < .01.

Table 2—Pharmacokinetic parameters of IV administered sulfadiazine in broiler chicken.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sulfadiazine</th>
<th>Sulfadiazine + curcumin</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2\alpha}$ (h)</td>
<td>2.26 ± 0.77</td>
<td>2.24 ± 0.90</td>
<td>.976</td>
</tr>
<tr>
<td>$V_{z/F}$ (L/kg)</td>
<td>0.70 ± 0.22</td>
<td>0.68 ± 0.26</td>
<td>.939</td>
</tr>
<tr>
<td>$C_{l/F}$ (L/h/kg)</td>
<td>0.21 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>.791</td>
</tr>
<tr>
<td>$AUC_{0-t}$ (h-mg/L)</td>
<td>90.17 ± 2.44</td>
<td>91.11 ± 2.53</td>
<td>.669</td>
</tr>
</tbody>
</table>

$AUC_{0-t}$ = Area under the plasma concentration-time curve. $Cl/F$ = Apparent clearance. $t_{1/2\alpha}$ = Elimination half-life time. $V_{z/F}$ = Apparent volume of distribution per fraction of the dose absorbed.

Discussion

P-gp can transport a variety of substrate drugs, including anticancer drugs (topotecan, vincristine, and rhizocin), analgesics (morphine), antibiotics (erythromycin), and calcium channel blockers (verapamil). These substrate drugs often have low bioavailability when used therapeutically. Therefore, finding good inhibitors of P-gp is highly important for the absorption and utilization of these substrate drugs.

The absorption of oral drugs can be mediated by several efflux transporters, among which P-gp is the most important. Studies have shown that rifampicin upregulated the small intestinal level of P-gp, and therefore affected the pharmacokinetic behavior of orally administered enrofloxacin by limiting its absorption from the intestine in broiler chickens. Therefore, the presence of P-gp causes the substrate drug to be released from cells after oral administration, resulting in a reduction in the bioavailability of the drug and unguaranteed efficacy of the drug. Studies in humans and rodents have shown that when a P-gp substrate is administered in combination with an inhibitor or inducer, the exposure of P-gp substrates increases or decreases, respectively. For example, the use of epicatechin EC31, an inhibitor of P-gp, can restore the accumulation of P-gp substrate drugs in human breast cancer cell line LCC6MDR and human leukemia cell line K562/P-gp. Yang et al demonstrated that the oral bioavailability of (20S)-ginsenoside Rh2, the substrate drug of P-gp, was elevated when cyclosporine A, an inhibitor of P-gp, was used. Other studies have reported that the oral bioavailability of paclitaxel increases from 3.4% to 41.3% in rats after coadministration of paclitaxel with HM30181, a P-gp inhibitor. Several low-solubility drugs, such as irinotecan, a commonly used anticancer drug, can be beneficial for the treatment of colon cancer by increasing the permeability of cells under curcumin inhibition in rat models. Therefore, studying the function of P-gp and identifying its inhibitors are highly important. However, there are relatively few related studies in the field of veterinary medicine. Moreover, previous studies found that the use of synthetic inhibitors such as verapamil and quinidine often produced disappointing results in vivo because their low binding affinities necessitated the use of high doses, resulting in serious toxicity. Therefore, finding more potent and less toxic P-gp inhibitors of natural origin is a problem we need to solve at present.

Curcumin is an antitumor and anti-inflammatory natural active ingredient extracted from plant roots. Its use alone or in combination with other drugs is beneficial to the intestinal health of broilers. Studies have shown that curcumin has the effects of lowering serum triglycerides, lowering blood glucose, and promoting anticoagulation and antiplatelet aggregation. Our research results showed that curcumin inhibits the expression and function of P-gp in chicken embryonic primary hepatocytes. In vivo experiments revealed that the accumulation of sulfadiazine, the substrate drug of P-gp, occurs after curcumin is administered to broiler chickens, improving the oral bioavailability of sulfadiazine. With respect to IV administered sulfadiazine, curcumin did not significantly change the parameters of pharmacokinetics. Accordingly, the increased oral bioavailability of sulfadiazine by curcumin could be mainly due to the enhancement of intestinal absorption via P-gp downregulation by curcumin rather than reduced renal and/or hepatic elimination of sulfadiazine, despite the significantly decreased expression of P-gp in liver and kidney by curcumin. Liu et al discovered the same phenomenon. Consistent with our findings, curcumin has been demonstrated to inhibit P-gp expression and P-gp-mediated efflux in multidrug-resistant human cervical cancer cells. In addition, our research indicated that curcumin not only has cytotoxic effects on cells but also promotes the activity of chicken embryonic primary hepatocytes. Xu et al and Nabavi et al also showed that curcumin has hepatoprotective effects. Other research has proven that curcumin can promote neovascularization and wound healing in diabetic rats.

In summary, a series of in vivo and in vitro studies demonstrated that curcumin can inhibit P-gp expression at the gene and functional levels and improve
the oral absorption rate of sulfadiazine. These results indicate that curcumin can have an impact on drug efficacy and improve the oral bioavailability of P-gp substrate drugs. This study provides information for discovering and synthesizing new P-gp inhibitors with decreased toxicity and increased efficacy. However, the molecular mechanism through which curcumin inhibits P-gp expression in chickens is still unclear and is worthy of further study.

Acknowledgments

None reported.

Disclosures

The authors have nothing to disclose. No AI-assisted technologies were used in the generation of this manuscript.

Funding

This research was funded by the National Natural Science Foundation of China (grant No. 32002332) and the Postgraduate Research & Practice Innovation Program of Jiangsu Province (SJCX22_1978).

References


