Effects of BCRP and P-gp Modulators on the Penetration of Aflatoxin B₁ into the Mouse Brain

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Abstract
This study was conducted to determine whether the plasma and brain concentrations of AFB₁ are affected by the modulation of P-gp and BCRP using zosuquidar (ZQR) and prazosin (PRZ), respectively. In this study, a total of 40 healthy adult male BALB/c mice (32±3.7 g) were used. The animals were randomly divided into 5 groups, with 8 animals per group. Group 1 was used for method validation. Group 2 (AF) received intraperitoneal AFB₁ at a dose of 20 mg/kg of body weight. Groups 3 (AF+PRZ), 4 (AF+ZQR), and 5 (AF+PRZ+ZQR) received 20 mg/kg of AFB₁ intraperitoneally 30 min after the intraperitoneal administration of prazosin (0.3 mg/kg), zosuquidar (25 mg/kg), and prazosin+zosuquidar (0.3 mg/kg prazosin + 25 mg/kg zosuquidar), respectively. Six hours after the administration of AFB₁, blood and brain samples were collected from the animals in Groups 2 to 5. AFB₁ concentrations were determined using an HPLC system with fluorescence detection. Individual and simultaneous administration of prazosin and zosuquidar significantly reduced the brain concentrations of AFB₁ in comparison to a single administration of AFB₁ (P<0.05). The brain/plasma ratio of the AF group was higher than that of the other groups (AF+PRZ, AF+ZQR, and AF+PRZ+ZQR) (P<0.05). Inducers of transmembrane proteins, especially BCRP, can be life saving during acute AFB₁ poisoning.

Keywords: Aflatoxin B₁, Brain, Drug transporter proteins, Modulation, Mice

INTRODUCTION
Aflatoxin B₁ (AFB₁), which is an environmental dietary carcinogen, is one of the most toxic mycotoxins and causes significant losses of livestock [1,2]. While the clinical signs of acute aflatoxicosis include epistaxis, blood stained faeces, and convulsions, sudden death is observed as the clinical sign of severe acute aflatoxicosis [3,4]. The elimination half-life of AFB₁ was determined to be 77 min after intraperitoneal administration in mice [5]. There is a...
high interaction potential to occur of AFB, with food/feed because AFB is a common food/feed contaminant. Also, the ingredients found in the food/feed compositions can cause to change the efficiency and disposition of drug and toxic substances through enzyme-and transporter [7-9].

Aflatoxin B₁ is a substrate of BCRP, and BCRP function may affect systemic exposure to this mycotoxin [10,11]. Additionally, AFB₁, and its metabolite aflatoxin B₁-epoksit-glutathione are substrates of multidrug resistance protein 1 (MRP1), although the affinity of MRP1 for AFB₁ is low [12-14]. BCRP substrates tend to overlap with P-gp substrates [13,15], but no information is available concerning whether AFB₁ is a substrate of P-gp. Van Herwaarden et al. [10] noted that BCRP plays an important role in the renal excretion of AFB₁. If AFB₁ is a substrate of both BCRP and P-gp, the tissue penetration of AFB₁ may be changed by the modulation of these transmembrane proteins.

The blood-brain barrier (BBB) protects the brain from a variety of endogenous and exogenous substances. The BBB not only limits substance flow from the blood to the brain tissue via the paracellular and transcellular routes but also permits the efflux of substances via several transmembrane proteins, such as P-glycoprotein (P-gp) and the breast cancer resistance protein (BCRP). P-gp (ABCB1) and BCRP (ABCG2) are members of the ATP-binding cassette transporter superfamily. Both P-gp and BCRP are expressed in mammalian capillary endothelial cells at BBB sites, and these transporters work in tandem to limit the accumulation of substances in tissues [11,16-18]. P-gp and BCRP are inducible and inhibitable in vivo and in vitro by various substances. The efflux activity of these transmembrane proteins has been described as saturable [19]. Because P-gp and BCRP have a broad substrate specificity and tissue distribution, the modulation of these transmembrane proteins may result in significant alterations in the pharmacokinetics, pharmacodynamics, and toxicity of their substrates. For example, Van Herwaarden et al. [10] found that the brain concentration of [³³C] 2-amino-3-methylimidazo[4,5-f] quinoline, which is a BCRP substrate, was higher in bcrp-/- mice than wild-type mice.

The aim of this study was 1) to determine the passage of AFB₁ into the brain and 2) to evaluate whether the plasma and brain concentrations of AFB₁ are affected by the modulation of P-gp and BCRP using prazosin and zosuquidar, respectively. This study is the first in vivo experimental study intended to evaluate the modulation of AFB₁ passage into the brain.

**MATERIAL and METHODS**

**Chemicals and Reagents**

All reagents were of recognised analytical grade. AFB₁ was obtained from Biopure Chemical Co. (Romer Labs Inc., 1301 Stylemaster Drive Union) and dissolved in corn oil for injection. Zosuquidar hydrochloride (99%) was obtained from MedKoo Biosciences (Canada, USA) and dissolved in a solution that included glycine (15 mg) and mannitol (200 mg) in 1 mL of distilled water for injection. Prazosin hydrochloride (≥99%) was purchased from Sigma Chemical Co. (Saint Louis, MO, USA) and dissolved in 1 mL of distilled water for injection. Immunoaffinity columns (Aflatest® WB) were purchased from Vicam (Watertown, MA, USA). Monobasic potassium phosphate (KH₂PO₄), sodium phosphate dibasic (Na₂HPO₄), sodium chloride (NaCl), potassium bromide (KBr), nitric acid (4 mol/L), and HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). All water used in this study was deionised and distilled. For the high performance liquid chromatography (HPLC), water was passed through a water purification system (aqua Max-Ultra System, Younglin Instrument Co. Ltd., South Korea). Phosphate buffer solution (PBS) was prepared by dissolving 0.2 g of KCl, 0.2 g of KH₂PO₄, 1.16 g of anhydrous Na₂HPO₄, and 8 g of NaCl in 1,000 mL of water, and the pH of the solution was adjusted to 7.4 with 0.1 N NaOH. A Tween-20 (Amresco, USA) solution was prepared by adding 15 mL of Tween to 85 mL of PBS and mixing.

**Experimental Design**

In this study, a total of 40 healthy adult male BALB/c mice (32±3.7 g) were used. Mice were supplied from Experimental Animal Production and Research Laboratory, Faculty of Veterinary Medicine, University of Mehmet Akif Ersoy, Burdur, Turkey. All experimental administrations on animals were carried out in here. The animals were housed individually in plastic cages with grated stainless steel floors in a controlled environment (temperature 25±1°C, relative humidity 60±10%, and artificial lighting sequenced to generate a 12-h light/dark cycle). The animals had ad libitum access to water and a commercial diet (Optima Feeds, Kırklareli, Turkey) that included the following: 89% dry matter, 21% crude protein, 2850 kcal/kg metabolic energy, 5% crude fibre, 0.75% methionine and cysteine, 1.0-2.0% calcium, 0.5-1.0% phosphorus, and 0.5% sodium.

All animal protocols in this study were approved by the Ethical Committee of the Faculty of Veterinary Medicine, University of Mehmet Akif Ersoy (No: 2014-71).

The animals were randomly divided into 5 groups of 8 animals each. Group 1 was used for method validation without any treatment of the animals. AFB₁ was administered intraperitoneally at a dose of 20 mg/kg of body weight to mice in Group 2-5. In the present study, the dose of AFB₁ was determined by taking into consideration concerns about AFB₁; analysis, the natural poisoning state and the dose previously reported Bastaki et al. [20] because no experimental research is available concerning the passage of AFB₁ into the brain. Group 2 (AF) received intraperitoneal (IP) AFB₁ at a dose of 20 mg/
kg of body weight 30 min after the IP administration of 1 mL of the glycine/mannitol solution. Groups 3 (AF+PRZ), 4 (AF+ZQR), and 5 (AF+PRZ+ZQR) were administered IP AFB1 at a dose of 20 mg/kg of body weight 30 min after the IP administration of prazosin (0.3 mg/kg of body weight) plus the glycine/mannitol solution (1 mL), zosuquidar (25 mg/kg of body weight), and prazosin (0.3 mg/kg of body weight) plus zosuquidar (25 mg/kg of body weight), respectively. The animals were observed for general behavioural changes, signs of toxicity, and mortality continuously for 6 h following the administration of AFB1. Six hours after the administration of AFB1 in Groups 2 to 5, blood samples from the hearts of animals under ether anaesthesia were collected into tubes containing heparin. All animals were euthanized using the cervical dislocation method. Plasma and brain tissues were collected from the animals and carried to Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Selçuk, Konya, Turkey for HPLC analysis. All samples were stored at -70°C until the time of analysis.

**HPLC and Chromatographic Conditions**

HPLC analyses were performed using an Agilent 1100 series HPLC system, which consisted of a G1379A degasser, a G1310A isocratic pump, a G1313A autosampler, a G1316A column oven, and a fluorescence detector (model G1321A, Agilent Technologies, Palo Alto, California, USA). Data acquisition was performed using the Chemstation 3D software (Agilent Technologies, Palo Alto, California, USA). For HPLC analysis, a reverse-phase ACE C18 analytical column (5 mm particle size, 4.6 x 250 mm) was employed. The column temperature was maintained at 30°C. Postcolumn derivatisation resulting in enhanced fluorescence was achieved with electrochemically-generated bromine in a Cobra cell (Coring System Diagnostics GmbH, Gernsheim, Germany), using a reaction tube that consisted of a 0.5 mm id x 34 cm length of polyether ketone tubing. The LC mobile phase solvent used with electrochemically-generated bromine was water/methanol/acetonitrile (60:20:20, v/v/v). To each litre of mobile phase, 120 mg of potassium bromide and 350 mL of 4 M nitric acid were added. The flow rate was 1 mL/min. The fluorescence detector was set to excitation and emission wavelengths of 360 and 430 nm, respectively. The injection volume for the standards and samples was 100 µL.

**Standard Preparation**

A stock solution was prepared by dissolving 5 mg of AFB1 in 5 mL of methanol and stored at -70°C. The stock solution was further diluted with water/methanol/acetonitrile (60:20:20, v/v/v) to generate a series of working standard solutions. Calibration standards of AFB1 (0.5, 1, 5, 10, 25, 50, 100, 200 ng/mL) were prepared by adding 20 µL of the working standard solution to 180 µL of plasma or 180 mg of brain tissue. Quality control samples (QC) at low (5 ng/mL), medium (25 ng/mL) and high (200 ng/mL) concentrations were used for the method validation program. These calibration standards and QC samples were processed according to the described method of sample preparation prior to HPLC analysis.

**Sample Preparation**

Plasma and brain tissue samples were extracted and cleaned up according to the procedures described by Association of Official Analytical Chemist [20] and Chiavarro et al.[21], with some modifications. Briefly, 200 µL of plasma or 200 mg of tissue sample were blended with 200 mg of NaCl in 1.2 mL of methanol-water (80:20) using a tissue homogeniser (Heidolph Silent Crusher M, Germany) at 9,000 rpm for 1 min. The extract was filtered through Whatman No. 4 filter paper. A 1-mL filtrate sample was diluted to 7.2 mL with PBS containing 0.1% Tween-20. The final filtrate was passed through an immunoaffinity column at a flow rate of 1 drop/s. The column was then washed twice with 4 mL of ultrapure water. AFB1 was eluted with 0.5 mL of methanol followed by 0.5 mL of water in a glass vial. Finally, 100 µL of the sample was injected into the HPLC system.

**Method Validation**

The specificity, linearity, sensitivity, recovery, precision, and accuracy of the employed method were determined to evaluate the performance of the analytical method. The specificity of the method was evaluated to identify interference from plasma and brain tissue. The calibration curve of AFB1 was constructed using seven calibration standards over a calibration range of 0.5-200 ng/mL. The limits of detection (LOD) and quantification (LOQ) were determined via signal-to-noise ratio evaluations of samples spiked from 0.1-10 ng/mL. The LOD was defined at a signal-to-noise (S/N) ratio of 3:1. The LOQ was defined as the lowest quantifiable concentration of analyte with an accuracy within 20% and a precision <20%. The recovery of AFB1 was evaluated at concentrations corresponding to the three QC values (5, 25, and 200 ng/mL), with six replicates. This parameter was determined by comparing the peak areas of the extracted QC samples with those of the working standards. The intra- and inter-day precision and accuracy were assessed by extracting and analysing five replicates of each QC sample. The intra- and inter-day precision and accuracy of the assay were determined based on the percent coefficient of variation (CV) and percent relative error (RE) values, respectively.

**Statistical Analysis**

All data were expressed as the mean ± SD. Data obtained from plasma and brain concentrations were analysed using one-way ANOVA, followed by the Duncan test. Statistical significance was assigned at P<0.05. The SPSS (Version 16.0 for Windows, Chicago, USA) statistical software was used for the statistical analyses.
Effects of BCRP and P-gp ...

RESULTS

No interfering peaks were observed in the blank plasma and brain tissue samples after the extraction. The assay was linear from 0.5 to 200 ng/mL, and the concentrations of the calibration standards were back-calculated using the peak area ratios of AFB1. The data were analysed using linear regression analysis, and the correlation coefficients for the calibration curves prepared from plasma and brain tissue were ≥0.9996 and ≥0.9994, respectively. The LOD values were determined to be 0.2 and 0.5 ng/mL in plasma and brain tissue, respectively. The LOQ values were determined to be 0.5 and 1 ng/mL in plasma and brain tissue, respectively. The LOD values were back-calculated using the peak area ratios of AFB1. The data were analysed using linear regression analysis, and the correlation coefficients for the calibration curves prepared from plasma and brain tissue were ≥0.9996 and ≥0.9994, respectively. The LOD values were determined to be 0.2 and 0.5 ng/mL in plasma and brain tissue, respectively. The LOQ values were determined to be 0.5 and 1 ng/mL in plasma and brain tissue, respectively. The LOQ values were determined to be 0.2 and 0.5 ng/mL in plasma and brain tissue, respectively. The LOD values were determined to be 0.2 and 0.5 ng/mL in plasma and brain tissue, respectively. The LOQ values were determined to be 0.2 and 0.5 ng/mL in plasma and brain tissue, respectively.

The effects of prazosin and zosuquidar on murine plasma and brain concentrations of AFB1, after the IP administration of AFB1, at a dose of 20 mg/kg of body weight are presented in Table 1. Single and simultaneous administrations of prazosin and zosuquidar significantly reduced brain concentrations of AFB1, in comparison to a single administration of AFB1. (P<0.05). The ratios of the brain concentrations of AFB1, to the plasma concentrations of AFB1, in the mice are shown in Fig 1. The brain/plasma ratio in the AF group was higher than those of the groups (AF+PRZ, AF+ZQR and AF+PRZ+ZQR) that received prazosin and zosuquidar (Fig. 1, P<0.05).

No mortality was observed in the AFB1-treated groups during the study; however, signs of toxicity, including oedema of the lower extremities, lethargy, muscular weakness, and convulsions, were observed in the AF group.

DISCUSSION

Although AFB1 was found at levels of 1-5 ppb in human brain biopsy samples, as reported by Hooper et al. [22] no experimental study investigated the ability of AFB1, to cross the BBB. However, AFB1 was reported to be unable to pass into the rat brain [23]. In this study, AFB1 crossed the BBB after the administration of 20 mg/kg AFB1, and the brain/plasma ratio of AFB1 was 7.85 six hours after administration. Our results suggest that AFB1, passed more intense to the brain and cannot be removed immediately. We concluded that AFB1 accumulates in tissues, such as the brain, and cannot be removed immediately due to the greater concentration of AFB1, in the brain than that in the plasma at the 6-hour blood collection time.

Contrary to expectation in this study, prazosin administration caused a statistically significant decline in the brain concentration of AFB1, (P<0.05); however, the decline in the plasma concentration of AFB1, was not statistically significant in the AF group (P>0.05). The brain/plasma ratio of AFB1, in prazosin-treated mice was 0.45 (Fig. 1). Our results indicate that prazosin is an inducer of BCRP, although some authors reported that prazosin is a BCRP inhibitor and substrate [24-27]. The reason for this contradiction may be differences in the materials used in the studies and in the employed doses of BCRP inhibitors and substrates. The drug affinity and the expression rate of BCRP differ among species, breeds, and organs [28,29]. Studies of inhibitors and inducers are typically conducted in vitro. The majority of in vivo studies concerning the passage of BCRP and P-gp substrates into the brain were conducted using transgenic experimental animals (bcrp/ p-gp -/-), such as mice and rats. Our study was conducted in vivo using wild-type mice. The type of modulation (i.e., induction and/or inhibition) and the effectiveness of a substance on transporter proteins are reported to depend on the dose, the type of tissue, the substrate examined, and the time of administration [30,31]. Additionally, the
difficulties of predicting in vivo relationships based on in vitro transport assays were discussed by Enokizono et al.[32]. Alternatively, the causes of the decreased AFB, concentration in the brain that is caused by prazosin may be associated with the roles of AFB, as a P-gp substrate and prazosin as a P-gp inducer. The inhibition of BCRP by prazosin may cause the activation of P-gp in the brain capillaries of mice. A compensatory relationship has been reported to exist between P-gp and BCRP, and P-gp has also been reported to be more prominent and inducible than BCRP at the BBB[33,34]. Prazosin has been indicated to be only an inducer of P-gp [23-27]. In addition, many BCRP and P-gp substrates and modulators overlap [13,27,33].

In the present study similar to the results of prazosin, zosuquidar, which is a potent and selective P-gp inhibitor in vitro, caused a non-statistically significant increase in the plasma concentration of AFB, but a statistically significant decrease in the brain concentration of AFB, (P<0.05). Cripe et al.[34] reported that the in vivo inhibition of P-gp by zosuquidar might lead to the activation of non-P-gp transmembrane proteins, such as BCRP. In addition, BCRP and P-gp presumably work in synergy or in a compensatory manner to restrict the passage of their concerted substrates into the CNS in mice, and P-gp can compensate for the deletion of BCRP, as suggested by Zhou et al.[33]. In addition, zosuquidar can be considered to be an inducer of P-gp or a dual P-gp/BCRP inducer in brain capillaries. Zosuquidar is described as a specific P-gp inhibitor [13,63,65], and a number of BCRP and P-gp substrates and modulators overlap [3,27,33].

Although the co-administration of prazosin and zosuquidar reduced the level of AFB, in the brain, this reduction was not statistically significant in comparison to the other two treatment groups. Thus, prazosin and zosuquidar do not show the combined effect to prevent the passage of AFB, into the brain. This may be associated with that prazosin or zosuquidar in combined use alter the response to the modulation of other transmembrane protein. Also, it has been reported that BCRP and P-gp work in synergy or in a compensatory manner for the efflux of their substrates and the modulation of a transmembrane protein by the inducer/inhibitor alters the efflux effect of other transmembrane protein [31,34].

The administration of prazosin, zosuquidar and prazosin+zosuquidar did not cause a statistically significant reduction of the plasma concentrations of AFB, in comparison to a single administration of AFB; (AF group). Similar to our findings, some previous studies reported that transmembrane protein modulators caused no changes in the plasma concentrations or pharmacokinetics of the substrates of transmembrane proteins but did cause important changes in tissue concentrations [34,36,37]. Based on the results of studies in this field, we believe that this difference may occur because the transmembrane proteins in each tissue respond differently to inducers and because drugs/substances are transported by more than one transmembrane protein. Demeule et al.[38] found that dexamethasone, which is a P-gp inducer, increased P-gp expression in the liver and the lung but reduced the expression of this molecule in the kidney. Transmembrane proteins in each tissue respond differently to inducers, as stated by Drescher et al.[39].

AFB, is activated via the conversion into AFB, 8-9 epoxide by cytochrome 450 enzymes, especially CYP1A2 and CYP3A4, and glutathione S-transferases are the most important enzymes for detoxifying AFB, in all species, including mice [40-42]. The plasma concentration of AFB, in the ZQR-AF group was found to be significantly higher than those of the PRZ-AF and PRZ+ZQR-AF groups. The causes of this difference may be the inhibition of AFB, metabolism by zosuquidar and/or the induction of BCRP in excretion organs by prazosin. Van Herwaarden et al.[43] posited that BCRP plays an important role in the renal excretion of AFB. P-gp inhibitors have also been reported to inhibit CYP3A activity, which plays an important role in AFB, metabolism.

In summary, both prazosin and zosuquidar significantly reduced the brain concentration of AFB, but not the plasma concentration of this molecule. Thus, prazosin is a better inducer than zosuquidar for both BCRP and P-gp in brain capillaries. In addition, AFB, may be a substrate of both BCRP and P-gp. Inducers of transmembrane proteins, such as prazosin, can be life-saving during acute poisoning with AFB, based on the overall health status and brain concentrations of AFB, in mice. The results of in vitro studies in this issue should be confirmed with in vivo studies of wild-type animals.

REFERENCES


