Assessment of antiepileptic drugs as substrates for canine P-glycoprotein

Chadwick L. West, DVM, and Katrina L. Mealey, DVM, PhD

Objective—To determine whether antiepileptic drugs (AEDs) are substrates for canine P-glycoprotein (P-gp).

Sample Population—OS2.4/Doxo cells (canine osteosarcoma cells induced via exposure to doxorubicin to highly express P-gp).

Procedures—Competitive inhibition of rhodamine 123 efflux from OS2.4/Doxo cells was used to determine whether AEDs were substrates for canine P-gp. Flow cytometry was used to quantify mean fluorescence intensity of cells treated with rhodamine alone and in combination with each experimental drug.

Results—Known P-gp substrate drugs ivermectin and cyclosporin A altered rhodamine efflux by 90% and 95%, respectively. Experimental drugs altered rhodamine efflux weakly (diazepam, gabapentin, lamotrigine, levetiracetam, and phenobarbital) or not at all (carbamazepine, felbamate, phenytoin, topiramate, and zonisamide).

Conclusions and Clinical Relevance—At clinically relevant doses, it appeared that AEDs were weak substrates (diazepam, gabapentin, lamotrigine, levetiracetam, and phenobarbital) or were not substrates (carbamazepine, felbamate, phenytoin, topiramate, and zonisamide) for canine P-gp. Therefore, it seems unlikely that efficacy of these AEDs is affected by P-gp expression at the blood-brain barrier in dogs. (Am J Vet Res 2007;68:1106–1110)

Epilepsy is a common neurologic disorder in dogs estimated to affect from 1% to 5% of all dogs.1 The term idiopathic epilepsy is generally used to describe seizure disorders that occur in an animal without evidence of a clinically apparent metabolic or structural intracranial disorder. Because an underlying cause for the seizures is not identified, treatment of dogs with idiopathic epilepsy consists of pharmacologic treatment with AEDs. The goal of AED treatment is to decrease overall seizure frequency and severity. However, in approximately 25% to 30% of dogs with idiopathic epilepsy, seizure frequency or severity is unaffected despite adequate doses and serum concentrations of 1 or more AEDs.2–4 This is termed pharmacoresistant or medically refractory epilepsy. Many of these dogs have seizures that are resistant to multiple concurrent AEDs, even when these AEDs have diverse mechanisms of anticonvulsant activity. Consequently, affected dogs have severe, frequent seizures that adversely affect their quality of life or are ultimately euthanatized because of persistent seizure activity.

The biological basis for multidrug-resistant epilepsy in veterinary and human patients is poorly understood. It is likely that there are multiple causes of refractory epilepsy. One potential mechanism for multidrug-resistant epilepsy that has received a great deal of attention in the human literature is impaired drug penetration into the brain in general and into epileptic foci in particular. This is believed to be mediated by drug efflux pumps.3–6 The best characterized of these efflux pumps in dogs and humans is P-gp. This glycoprotein, which is encoded by the ABCB1 (formerly MDR1) gene, is a transmembrane protein that functions as an ATP-dependent drug efflux pump.7–9 The P-gp is expressed physiologically in a number of tissues with excretory function, such as the liver, kidneys, and intestines, and is involved in barrier functions, such as in the blood-brain barrier, the blood-testis barrier, and the placenta.10 Thus, P-gp is believed to exert a protective physiologic function by excreting potentially toxic xenobiotics. Substrates for P-gp are large (molecular weight, 400 to 1,200 kd), hydrophobic amphipathic molecules that usually include a ring structure.11 In abcb1 knockout mice (which lack functional P-gp), brain concentrations of P-gp substrates are 100 times as high as in abcb1 wild-type mice, which indicates that P-gp is effective as a drug efflux pump.10–14 Overexpression of P-gp may effectively limit entry of important therapeutic drugs to their site of action in the brain. If AEDs are substrates for canine P-gp, expression of P-gp at the blood-brain barrier would inhibit AED penetration to epileptic foci, resulting in severely compromised efficacy despite adequate plasma drug concentrations.

ABBREVIATIONS

<table>
<thead>
<tr>
<th>AED</th>
<th>Antiepileptic drug</th>
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<tr>
<td>P-gp</td>
<td>Antiepileptic drug</td>
</tr>
<tr>
<td>P-glycoprotein</td>
<td>Protein</td>
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Several lines of evidence support the assertion that P-gp–mediated AED efflux is responsible for pharmacoresistant epilepsy. Many AEDs used to treat human epileptic patients are substrates for human P-gp. Additional studies have revealed that P-gp expression in the brain is increased in rodents with epilepsy and in human patients with pharmacoresistant epilepsy. Interestingly, P-gp is expressed on brain capillary endothelial cells as well as in tissues surrounding epileptic foci, potentially leading to focal areas where AEDs cannot penetrate. If P-gp does contribute to pharmacoresistant epilepsy in dogs, additional treatments could use AEDs that are not substrates for P-gp or that abrogate P-gp function. The purpose of the study reported here was to determine whether AEDs used in dogs are substrates for canine P-gp.

**Materials and Methods**

**AEDs**—Experimental drugs consisted of antiepileptic agents (phenobarbital, gabapentin, diazepam, phenytoin, carbamazepine, felbamate, lamotrigine, zonisamide, levetiracetam, and topiramate). Positive control samples consisted of drugs known to be P-gp substrates (ivermectin and cyclosporin A), with a non–P-gp substrate (cisplatin) as the negative control sample. Concentrations of drugs used in the experiments were within therapeutic plasma concentrations, as determined by available data in canine or human patients, or extrapolated on the basis of recommended dose and volume of distribution. A human pharmacology textbook that lists effective plasma concentrations was used to determine the experimental concentrations for carbamazepine, diazepam, and phenytoin. A review article of therapeutic drug monitoring of newer AEDs for human patients was used to determine the experimental concentrations of felbamate, gabapentin, lamotrigine, levetiracetam, topiramate, and zonisamide. A veterinary clinical pharmacology textbook was used to determine the experimental concentrations for phenobarbital and cyclosporin A. For cisplatin and ivermectin, experimental concentrations were extrapolated by use of the following equation: Concentration = dose/volume of distribution, where doses were obtained from a veterinary formulary and volume of distribution was obtained from veterinary (ivermectin) or human (cisplatin) references.

**Sample population**—A canine osteosarcoma cell line, OS2.4/Doxo, which overexpresses P-gp, was used in the cell culture–flow cytometry experiments. Cells were grown in RPMI 1640 media supplemented with 10% fetal bovine serum in 5% CO₂, at 37°C and 100% relative humidity.

**Immunoblot analysis of P-gp expression**—The OS2.4/Doxo cell line has been characterized as expressing P-gp; however, we believed it was important to confirm P-gp expression before we used these cells for the rhodamine 123 retention studies. Cells from the experimental cell line (ie, OS2.4/Doxo) and MCF-7/ADR, a human cell line that highly expresses P-gp (positive control cells), were harvested by trypanosinization, pelleted, rinsed in PBS solution, and lysed with 1 mL of Laemmli sample buffer (62.5 mM Tris, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol [pH, 6.8]). Protein (50 μg/lane) was loaded on a polyacrylamide gel and electrophoresed. Proteins were transblotted onto a membrane. The primary antibody was a murine anti-human monoclonal antibody, followed by a peroxidase-conjugated rabbit anti-mouse IgG antibody. Protein bands were evaluated by use of chemiluminescence.

**Rhodamine 123 efflux studies**—The OS2.4/Doxo cells (2 × 10⁶) were grown in monolayer to approximately 80% confluency (overnight incubation) in 25-mm² flasks. Medium (RPMI 1640 with 10% fetal bovine serum) was aspirated from the cells, and the cells were washed with PBS solution. Phenol red–free RPMI 1640 media (4 mL) containing rhodamine 123 (200 ng/mL) alone or rhodamine 123 and an experimental drug were added to the flasks. Flasks were incubated for 2 hours by use of the described standard cell culture conditions. After incubation, medium was aspirated from the cells, and cells were trypsinized and washed with ice-cold PBS solution. Cells were centrifuged and the pellet resuspended in 150 μL of PBS solution. Cells were then subjected to fluorescence-activated cell sorting.

**Flow cytometry**—Cells were analyzed on a flow cytometer with an argon laser set at 488 nm. A minimum of 10,000 events was collected for each experimental drug. The fluorescence emission of rhodamine 123 was collected in the FL1 channel with a 530/30 bandpass filter. Duplicate flasks of each experimental drug were analyzed, and all experiments were repeated on separate days to ensure repeatability. Because rhodamine 123 is an intrinsically fluorescent molecule, the measured fluorescence intensity of a cell represents the amount of rhodamine 123 contained in that cell. As a substrate for P-gp, rhodamine 123 would be expected to be actively transported out of OS2.4/Doxo cells, yielding low mean fluorescence intensity. However, when a cell contains another substrate for P-gp, rhodamine 123 would be pumped out of the cell, resulting in greater mean fluorescence intensity. Conversely, when a drug that is not a substrate for P-gp (ie, cisplatin) is coincubated with rhodamine 123, P-gp function would remain intact, pumping rhodamine 123 out of the cell, with a low mean fluorescence intensity expected.

**Statistical analysis**—Data were converted to a linear scale (1 to 10,000) on the basis of the range of signal intensities. To determine the percentage of rhodamine 123 retained after coincubation with experimental drugs, the following calculation was used:

\[
\text{MF1} = \frac{\text{MFI}_{\text{exp drug + rhodamine}} - \text{MFI}_{\text{rhodamine alone}}}{\text{MFI}_{\text{exp drug + rhodamine}}} \times 100
\]

where MF1 is the median fluorescence intensity for the various treatments.

**Results**

**Immunoblot analysis of P-gp expression**—Immunoblot analysis was performed. Results revealed P-gp expression in OS2.4/Doxo cells (Figure 1).
Rhodamine 123 efflux experiments—Histograms of OS2.4/Doxo cells incubated with rhodamine 123 alone (A) or in conjunction with known P-gp substrates cyclosporine A (B) or ivermectin (C) or the negative control drug (non–P-gp substrate) cisplatin (D). FL1-H = Fluorescence emission collected in the FL1 channel.

Table 1—Percentage of rhodamine retention in OS2.4/Doxo cells (mean of at least 2 separate experiments) after treatment with various drugs at therapeutic concentrations.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (mg/mL)</th>
<th>Rhodamine retention (%)</th>
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<tbody>
<tr>
<td>Carbamazepine</td>
<td>0.008</td>
<td>1.1</td>
</tr>
<tr>
<td>Cisplatin†</td>
<td>0.005</td>
<td>7.5</td>
</tr>
<tr>
<td>Cyclosporin A†</td>
<td>0.0005</td>
<td>95.2</td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.0006</td>
<td>17.2</td>
</tr>
<tr>
<td>Felbamate</td>
<td>0.002</td>
<td>0.3</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>0.003</td>
<td>10.5</td>
</tr>
<tr>
<td>Ivermectin†</td>
<td>0.000125</td>
<td>90</td>
</tr>
<tr>
<td>Lamotrigine</td>
<td>0.005</td>
<td>18.1</td>
</tr>
<tr>
<td>Levetiracetam</td>
<td>0.01</td>
<td>17.3</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>0.025</td>
<td>15.6</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>0.015</td>
<td>–9.2</td>
</tr>
<tr>
<td>Topiramate</td>
<td>0.02</td>
<td>3.9</td>
</tr>
<tr>
<td>Zonisamide</td>
<td>0.01</td>
<td>–4.5</td>
</tr>
</tbody>
</table>

*Negative control treatment. †Positive control treatment.

Discussion

A number of studies in human epileptic patients and rodents with epilepsy have implicated P-gp as a major cause of pharmacoresistant epilepsy. The 2 major pieces of evidence that support this theory are increased P-gp expression in brain tissue surrounding epileptic focus11,15 and results indicating that AEDs are substrates for P-gp.13,15

In the study reported here, none of the AEDs commonly used to treat epilepsy in dogs were strong substrates for P-gp. It is possible that at higher concentrations, some of these drugs may have been substrates for P-gp. However, the primary reason for conducting this study was to determine whether P-gp may contribute to pharmacoresistant epilepsy in dogs by pumping AEDs away from epileptic foci. Therefore, drug concentrations were used that would approximate those in an epileptic patient. It is also possible that other drug transporters are involved in the efflux of AEDs from the CNS. However, because the OS2.4/Doxo cell line expresses P-gp at high concentrations, results of this study are likely to primarily reflect P-gp activity.

Because most of the drugs investigated in this study have also been used to treat human patients with epilepsy, data were available for comparison of our results with published reports for the same drugs studied in murine or human P-gp–expressing cell lines or in rodents with epilepsy. Phenobarbital is reported to be a substrate for P-gp2 on the basis of microdialysis studies in rodent. Studies in which investigators used P-gp–expressing human or rodent cell lines have not supported this result. In the study reported here, phenobarbital increased rhodamine retention by 1%, compared with baseline values, which indicated that at therapeutic doses, phenobarbital was at most a weak substrate for canine P-gp.
The most commonly studied AEDs, in terms of P-gp transport, are phenytoin, levetiracetam, and carbamazepine. In the study reported here, phenytoin did not increase rhodamine retention, thus indicating that it is not a substrate for canine P-gp. Although phenytoin is reportedly a substrate for P-gp in rodent cell lines, it is only a weak substrate for human P-gp.31,33 This species difference is not surprising because mice have 2 genes (abcb1a and abcb1b) that code for P-gp, whereas only a single gene (ABCB1) codes for P-gp in humans and dogs.34 Lefluracetam increased rhodamine retention by 17%, which indicated that levetiracetam may be a weak substrate for canine P-gp at therapeutic concentrations. Similar to phenytoin, levetiracetam appears to be a weak substrate for human P-gp.35 Studies conducted in human cell lines indicate that carbamazepine is a substrate for P-gp, but the concentrations used exceeded therapeutic plasma concentrations.36 At concentrations used in the study reported here, carbamazepine was not a substrate for canine P-gp.

Data regarding other AEDs are limited with regard to their status as P-gp substrates. Further confounding the issue is that various investigators have used different methods and techniques for identifying P-gp substrates, including microdialysis rodents, various rhodamine 123 transport models (human or rodent cell lines), substrate-binding assays, and ATP hydrolysis assays. Cell culture systems, such as the one used in our study, offer several advantages over substrate-binding or ATP hydrolysis assays. Substrate-binding assays do not measure drug transport; they simply measure drug binding. Noncompetitive inhibitors of P-gp can bind to P-gp and thus be deemed a P-gp substrate by use of the assay despite the fact they may not actually be transported by P-gp. By the same token, ATP hydrolysis is necessary for P-gp function, but it is not always associated with drug efflux.

It has been determined that P-gp substrate drugs have different binding affinities to P-gp.36 Thus, in cell culture transport models, such as the one used in the study reported here, weak P-gp substrates would have to be used at high concentrations to yield a positive result as a P-gp substrate. In our study, the goal was to determine whether AEDs at clinically relevant concentrations would serve as substrates for P-gp.

Species differences between humans and mice have been identified in P-gp–mediated transport of drugs. These species differences result from a qualitative difference in substrate recognition or a quantitative difference in the efficiency for P-gp transport. Although P-gp has a high degree of homology among species, there are differences in amino acids that could result in differing binding affinities or pumping efficiencies among species. Interestingly, human P-gp appears to behave more like canine P-gp than murine P-gp with respect to AED transport. This is not surprising because both human and canine P-gp are encoded by a single gene, whereas 2 genes encode murine P-gp.

The clinical implications of AEDs being weak P-gp substrates are unknown. Ivermectin is a P-gp substrate in dogs and mice and was determined to be a strong P-gp substrate in the study reported here. The concentration of ivermectin in the brains of abcblab double knockout mice is at least 100 times that of wild-type mice.37,38 There are obvious pharmacologic effects when 300 µg of ivermectin/kg is administered to a dog with the ABCB1–/– genotype (severe depression of the CNS), compared with a dog with a wild-type genotype (no detectable CNS abnormalities).39–40 The authors are not aware of any reports that ABCB1–/– dogs have sensitivity to any of the AEDs that were found to be weak substrates for P-gp in our study. Therefore, it seems unlikely that the efficacy of AEDs in this study (non–P-gp substrates or weak P-gp substrates) was affected by P-gp expression at the blood-brain barrier in dogs.

Reference