Pharmacokinetic Interaction of the Antiparasitic Agents Ivermectin and Spinosad in Dogs

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ABSTRACT:

Neurological side effects consistent with ivermectin toxicity have been observed in dogs when high doses of the common heartworm prevention agent ivermectin are coadministered with spinosad, an oral flea prevention agent. Based on numerous reports implicating the role of the ATP-binding cassette drug transporter P-glycoprotein (P-gp) in ivermectin efflux in dogs, an in vivo study was conducted to determine whether ivermectin toxicity results from a pharmacokinetic interaction with spinosad. Beagle dogs were randomized to three groups treated orally in parallel: Treatment group 1 (T01) received ivermectin (60 μg/kg), treatment group 2 (T02) received spinosad (30 mg/kg), and treatment group 3 (T03) received both ivermectin and spinosad. Whereas spinosad pharmacokinetics were unchanged in the presence of ivermectin, ivermectin plasma pharmacokinetics revealed a statistically significant increase in the area under the curve (3.6-fold over the control) when ivermectin was coadministered with spinosad. The majority of the interaction is proposed to result from inhibition of intestinal and/or hepatic P-gp-mediated secretory pathways of ivermectin. Furthermore, in vitro Transwell experiments with a human multidrug resistance 1-transfected Madin-Darby canine kidney II cell line showed polarized efflux at concentrations ≤2 μM, indicating that spinosad is a high-affinity substrate of P-gp. In addition, spinosad was a strong inhibitor of the P-gp transport of digoxin, calcein acetoxymethyl ester (IC50 = 3.2 μM), and ivermectin (IC50 = 2.3 μM). The findings suggest that spinosad, acting as a P-gp inhibitor, increases the risk of ivermectin neurotoxicity by inhibiting secretion of ivermectin to increase systemic drug levels and by inhibiting P-gp at the blood-brain barrier.

Introduction

Ivermectin is one of the most effective and widely used antiparasitic agents ever discovered because of its broad-spectrum activity against numerous endo- and ectoparasites, especially nematodes and arthropods (Geary, 2005; Omura, 2008). Chemically, ivermectin is a high-molecular-weight natural product macrocyclic lactone produced by the actinomycete, Streptomyces avermitilis (Campbell et al., 1983). Originally developed for veterinary use, ivermectin is commonly used to eliminate gastrointestinal nematodes in livestock and heartworms in companion animals. In several instances, ivermectin has also proven to be an effective treatment for worm infections, as well as for mites, lice, and scabies in human medicine (Omura, 2008). A number of companies currently market ivermectin and have adapted a range of formulations for dosing by the oral, parenteral, and topical routes.

Much of the success of ivermectin could be attributed to its high therapeutic index. For instance, in dogs, the effective heartworm prevention dose is 6 μg/kg once monthly, but the more difficult-to-treat dermatological Demodex infections are often treated daily with extra-label ivermectin doses in excess of 50-fold of the heartworm prevention dose (Mueller, 2004). The primary factors contributing to the therapeutic index of ivermectin appear to be 1) the high affinity of ivermectin for its primary pharmacological targets in parasites, the glutamate-gated chloride ion channels (Yates et al., 2003; Wolstenholme and Rogers, 2005); 2) the absence of the glutamate-gated chloride (anion) channels in mammalian hosts (Raymond and Sattelle, 2002); and 3) “protection” from ivermectin binding to its secondary target, GABA-gated chloride channels, because the expression of these GABA channels in mammals is mostly limited to the central nervous system (CNS) (Campbell et al., 1983). When the limits of the safety margin are exceeded in the treatment of difficult-to-treat infections, the toxicological syndromes appear to be consistent with GABA receptor modulation caused by ivermectin-stimulated neuronal GABA release (Lovell, 1990).

ABBREVIATIONS: AUC, area under the curve; CNS, central nervous system; P-gp, P-glycoprotein; BBB, blood-brain barrier; MEM, minimal essential medium; HBSS, Hanks’ balanced salt solution; calcein AM, calcein acetoxymethyl ester; MDCK, Madin-Darby canine kidney; MDR, multidrug resistance; MDCK-MDR1, human MDR1 gene-transfected MDCK II; A, apical; B, basolateral; ER, efflux ratio; CP100356, N-(3,4-dimethoxyphenethyl)-4-((6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)-6,7-dimethoxyquinazolin-2-amine.
Despite the well deserved superdrug status held by ivermectin, drug interaction concerns have recently arisen in dogs for use of extra-label doses in conjunction with the oral flea preventative, spinosad. According to a warning statement issued by the U.S. Food and Drug Administration, increased incidence of ivermectin toxicoses have been observed during coadministration of the two antiparasitic agents in dogs (http://www.fda.gov/AnimalVeterinary/NewsEvents/CVMUpdates/ucm047942.htm). We are not aware of any study detailing the rate of occurrence of adverse events with extra-label ivermectin doses. When spinosad was administered with ivermectin at its heartworm prevention dose, its safety has not been questioned on the basis of field experience. In a recent study, the safety of extra-label doses of the anthelmintic milbemycin oxime with concurrent spinosad treatment has also been confirmed (Sherman et al., 2010).

Of interest, a clue to the potential mechanism behind the ivermectin-spinosad drug interaction in dogs comes from a well characterized P-glycoprotein (P-gp) mutation in dogs found predominantly in the collie breed but also in other breeds (Mealey et al., 2001; Mealey and Meurers, 2008). Ivermectin is a substrate and inhibitor of the transmembrane drug transporter P-gp (Didier and Loor, 1996). A 4-base pair deletion in the ABCB1 gene encoding P-gp results in a truncated, nonfunctional protein (Mealey et al., 2001). This P-gp variant is associated with increased sensitivity to ivermectin because of the enhancement of BBB drug penetration (Pulliam et al., 1985; Mealey et al., 2001). It is clear that clinician knowledge of the mutation is relevant during ivermectin treatment, which is associated with neurotoxicity at high doses (≥100 µg/kg) typically tolerated by wild-type allele carriers (Hopkins et al., 1990; Mealey, 2004; Fecht and Distl, 2008). Because the safe use of high-dose ivermectin therapies is related to P-gp function, it has been conjectured that spinosad may inhibit ivermectin efflux by P-gp in dogs with wild-type P-gp alleles. In such an instance, ivermectin would be predicted to have greater CNS penetration and GABA-related pharmacology. Because ivermectin is a P-gp substrate that is primarily eliminated in feces and bile and not extensively metabolized, it is also reasonable to envision how P-gp inhibition could increase circulating ivermectin via decreased hepatic or intestinal secretion. The goal of this work was to test whether the ivermectin-spinosad interaction in dogs is related to P-gp inhibition via pharmacokinetic studies and in vitro P-gp efflux and inhibition experiments.

Materials and Methods

Chemicals and Reagents. The dosing material was commercially available ivermectin solution [Ivermectin (1%) in a sterile solution of 40% glycerol formal and propylene glycol, in a sufficient quantity to make 100%]. Spinosad tablets (Comfortis) were obtained from commercial retail resources for research use. Ivermectin was purchased from Sigma-Aldrich (St. Louis, MO). An internal standard was added to treatments and runs by weight according to a randomized block design with a one-way treatment structure. Blocking was based on run location within the study room. The experimental unit for treatment was the animal. Nine dogs were assigned to one of three groups for oral dosing with either ivermectin (T01), spinosad (T02), or concurrent doses of spinosad and ivermectin (T03).

Dosing and Blood Draws. Ivermectin (69 µg/kg average) and spinosad (>30 mg/kg) were administered orally via gavage and by chewable tablet, respectively. The dose of ivermectin was 10-fold higher than that used for routine heartworm prevention to enable pharmacokinetic analysis. Spinosad was administered at the recommended dose using the dose banding guidelines provided. Each dog was fed 30 min before dosing to stimulate digestive processes. After dosing, all subjects were administered 15 ml of water by syringe to ensure delivery of the dose. An intensive blood sampling regimen followed the dose with samples taken at predose and at 1, 2, 3, 4, 6, 10, 24, 48, and 72 h and 5, 7, 10, and 14 days. Two-milliliter blood samples were collected via jugular venipuncture with Vacutainer tubes containing K3-EDTA anticoagulant. Plasma was prepared by centrifugation and stored at −20°C until analysis. Clinical signs were recorded at 2, 4, and 10 h and once daily for the remainder of the study. No adverse events were observed in any study subjects.

Plasma Analysis. Ivermectin in plasma was assayed by liquid chromatography with fluorescence detection after solid-phase extraction and chemical derivatization. Standards and quality control samples (prepared from separate weighings) were prepared by the addition of appropriate volumes of standard solutions of ivermectin to aliquots of control dog plasma. Internal standard solution (75:25, water-acetonitrile) was added to each standard and sample, mixed, and then centrifuged. An ISOLUTE extraction plate was primed with 2 ml of methanol/water and allowed to drain under gravity. Wells were then washed with 2 ml of water. Plasma standards and samples were applied to the extraction plate, which was then washed with 2 ml of water, followed by 1 ml of water-methanol (75:25) and dried under vacuum. The extraction plate was then eluted with 2 ml of methanol. The eluate was collected in a 96-well plate (2-ml capacity) and evaporated to dryness under a stream of nitrogen gas at 60°C using a sample concentrator (TurboVap 96; Caliper Life Sciences, Hopkinton, MA). N-Methylimidazole (60 µl of 50% v/v in acetonitrile) was added to each well in the plate, followed by trifluoroacetic anhydride (100 µl of 50% v/v in acetonitrile) to derivatize the samples. Detection was accomplished using an Acuity ultraperformance liquid chromatography system and fluorescence detector (Waters, Milford, MA) with an excitation wavelength of 365 nm and an emission wavelength of 475 nm. Chromatographic separations were performed using an Acuity BEH C18 column (1.7 µm, 2.1 × 100 mm) and gradient elution. The flow rate was 500 µl/min, with a column temperature of 45°C and an injection volume of 10 µl. Solvent A was water and solvent B was 0.2% acetic acid in water, methanol, and acetonitrile (4:32:64%, v/v/v). Gradient conditions began at 50% B, increasing to 99% B over 3.5 min, holding at 99% B for 3.5 min, and then returning to the initial conditions over 2.5 min. Ivermectin eluted at 6.08 min, and internal standard eluted at 5.45 min. Standard curves were shown to be linear in dog plasma over the concentration range covering the unknown samples, and no weighting was applied to the fit (r = 0.9967).

Spinosyn A was assayed by liquid chromatography separation and tandem mass spectrometry methods. Standards, quality controls, and samples were prepared using the Microlab STAR (Hamilton Robotics, Inc., Reno, NV). Appropriate volumes of standard solutions of spinosyn A were added to aliquots of control dog plasma in a 96-well format. Likewise, appropriate volumes of control solution were added to sample plasma, to maintain a common matrix. Internal standard was then added to each standard and sample. The plate was thoroughly vortex-mixed and centrifuged, and aliquots of supernatant were transferred to an injection plate. Mass spectrometry detection
of test compounds was accomplished with an API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDSc Sciex, Foster City, CA). Chromatographic separations were performed using a Zorbax Eclipse XDB C8 column (3.5 μm, 2.1 × 50 mm), and gradient elution. The flow rate was 600 μl/min with an injection volume of 10 μl. Solvent A was 0.1% formic acid and solvent B was methanol. Gradient conditions began at 10% B, increasing to 90% B over 1 min, holding at 90% B for 1.4 min, and then returning to the initial conditions over 0.5 min. Spinosad eluted at 1.89 min and internal standard at 2.06 min. Analysis was performed in the positive ion mode at unit resolution for the parent ion and forosamine transition of 732.5 → 142.3. Calibration curves were shown to be quadratic up to 1000 ng/ml in dog plasma, and 1/X weighting was used to provide the fit (r = 0.9982).

Pharmacokinetic Analysis. Pharmacokinetic calculations were performed using the noncompartmental approach (linear trapezoidal rule for AUC calculation) with the aid of Watson (version 7.2; Inphapace, Inc., Wayne, PA). The ivermectin and spinosad plasma concentrations used to calculate the terminal elimination rate constant and half-life were selected from the 72- to 336-h time points.

Statistical Analysis. Plasma concentrations for ivermectin and spinosad from the nine study animals were analyzed using linear mixed models for repeated measures. Separate repeated-measures models were used for each drug. The models contained fixed effects for treatment, time, and the treatment by time interaction and random effects for block and residual. Plasma concentrations were log transformed before statistical analysis. If the treatment main effect or treatment by time interaction was significant, pairwise comparisons between treatment groups were performed. Treatment comparisons (two-tailed) within time points were conducted at the 10% level of significance to compensate for the small group sizes. Treatment comparisons between treatment groups were performed. Treatment comparisons (two-tailed) within time points were conducted at the 10% level of significance. All statistical analyses were performed by using SAS 9.2 (SAS Institute, Cary, NC).

Cell Culture. A MDCK II single-cell isolation was double-sorted by flow cytometry for low P-gp efflux function against calcein AM accumulation, then expanded in culture for use in trans-monolayer assays (L. Di, manuscript in preparation). This subpopulation is referred to as MDCK low efflux (MDCKLE) and was used to define a null-P-gp transcellular flux. A ratio of ratios (ERMDCK-MDR1/ERMDCK-LE) greater than 1.7 has been established as the cutoff to identify P-gp substrates (Feng et al., 2008).

Ivermectin Efflux Inhibition. Ivermectin (1 μM) was added to Transwell MDCK-MDR1 plates and the P_app was determined for the B → A direction as described above. Experiments were performed in the presence of spinosad (0.1–30 μM) with each concentration being tested in triplicate. The inhibition of P_app for ivermectin was normalized to the absence of inhibitor, and the resulting data were fit to a one-site relative IC50 inhibition model using GraphPad Prism software (version 5.02; GraphPad Software Inc., La Jolla, CA):

\[
P_{\text{app, observed}} = P_{\text{app, min}} + \frac{(P_{\text{app, max}} - P_{\text{app, min}})}{1 + 10^{[\log IC_{50} - \text{test concentration}]}}
\]

Caliene AM Accumulation Assay. MDCK-MDR1 cells were seeded into 96-well clear-bottom black-wall sterile culture plates (BD Biosciences) at 5.3 × 104 cells/well and grown for 3 days before testing. Cells were washed twice in Dulbecco’s phosphate-buffered saline and then overlaid with test compounds in HBSS at the indicated concentrations in duplicate, along with a positive control inhibitor and blank HBSS wells, and incubated at 37°C for 30 min. Buffer was aspirated off cells and replaced with identical drug concentrations containing 0.1 μM calcein AM. Fluorescence was immediately scanned (t = 0 min) using XFluor4 version 4.40 software on a Saffire fluorescence plate reader (Tecan, Durham, NC) set at an excitation wavelength of 488 nm and emission wavelength of 535 nm. The plate was incubated at 37°C for 40 min, followed by a second scan (t = 40 min). Percentage inhibition was calculated from the difference between the two scans, using positive control data as the maximum inhibition (100%), and an IC50 was generated for each test compound using nonlinear regression with GraphPad Prism software.

Results

Pharmacokinetics. The pharmacokinetics of all treatment groups are summarized in Table 1. During the in-life phase of the study no clinical signs of changes in health or behavior were observed in any subject or treatment group. Ivermectin (T01) was readily absorbed in plasma spinosad concentrations, measured as the primary spinosyn (T03) were significantly higher than the control T01 from 6 h to the last half-life of ivermectin (106 h) was determined to be somewhat longer than that reported previously, which ranged from ~37 to 80 h (Lo et al., 1985; Kojima et al., 1987; Gokbulut et al., 2006). The time after dosing that ivermectin remained detectable in plasma seemed to have some influence on the calculated terminal t1/2 in these previous studies because of the multicompartmental kinetic profile.

Plasma spinosad concentrations, measured as the primary spinosyn A constituent, indicated rapid absorption from its chewable formulations (Table 1). Like ivermectin, spinosad demonstrated a long terminal half-life (Fig. 1).
P-gp-mediated Efflux. P-gp mediated efflux of spinosad was measured in MDCK-MDR1 cells over a range of concentrations, along with the well characterized P-gp substrate digoxin at 2 μM. Both compounds were also tested in MDCKLE cells, a subpopulation of MDCK II wild-type cells with low P-gp efflux function. As shown in Table 2, transport was not polarized in MDCKLE cells for either compound, with efflux ratios at unity. Digoxin showed strongly polarized transport in MDCK-MDR1 cells; this was completely eliminated by ivermectin or by spinosad, each at a 10 μM concentration. Polarized efflux was seen for spinosad at low concentrations (less than 2 μM) in MDCK-MDR1 cells, with an efflux ratio of approximately 3.5. However, as concentrations increased, apparent saturation of the efflux pump occurred, preventing P-gp function and resulting in decreasing efflux ratios. Spinosad efflux at 0.5 μM was also blocked by ivermectin and cyclosporine, a known P-gp inhibitor.

The potent inhibition of P-gp-mediated digoxin efflux by spinosad and the saturation of spinosad transport by P-gp at low concentrations suggested that spinosad was a high-affinity P-gp ligand that could mediate P-gp drug interactions. Spinosad inhibition of P-gp was further examined in the calcine AM accumulation assay, in parallel with ivermectin. Ivermectin was previously shown to inhibit P-gp (Didier and Loor, 1996). Calcine AM is freely taken up by passive permeability across cell membranes but is rapidly effluxed from the membrane as a substrate of P-gp. Once internalized, however, calcine AM is irreversibly cleaved by nonspecific endogenous esterases to produce the fluorescent free acid calcine. Inhibition of the P-gp transporter results in the accumulation of calcine within the cell and a corresponding increase in cellular fluorescence (Tiberghien and Loor, 1996). A proprietary Pfizer compound, N-(3,4-dimethoxyphenethyl)-4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2[1H]-yl)-6,7-dimethoxyquinazolin-2-amine (CP100356), has been validated as a potent P-gp inhibitor with maximal inhibition at 5 μM (Kalugutkar et al., 2009). The inhibition of P-gp with 5 μM CP100356 was used as a 100% inhibitory control, which was then used to compare calcine AM inhibition by ivermectin and spinosad. Figure 2A shows that ivermectin and spinosad are similar in their human P-gp calcine AM inhibition potency, with IC_{50} values of 1.19 and 3.23 μM (1041 and 2364 ng/ml), respectively. In addition, spinosad was shown to inhibit human P-gp basolateral to apical transport of ivermectin (1 μM) with an IC_{50} of 2.3 μM or 2013 ng/ml (Fig. 2B).

**TABLE 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>T01: Ivermectin</th>
<th>T02: Spinosad</th>
<th>T03</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>0.0815 ± 0.00319</td>
<td>38.0 ± 7.0</td>
<td>0.0562 ± 0.0211*</td>
</tr>
<tr>
<td>C_{max,obs} (ng/ml)</td>
<td>24.0 ± 6.6</td>
<td>1550 ± 356</td>
<td>60.1 ± 20.9</td>
</tr>
<tr>
<td>T_{max,obs} (h)</td>
<td>5.3 ± 4.0</td>
<td>3.3 ± 2.3</td>
<td>5.0 ± 4.4</td>
</tr>
<tr>
<td>t_{1/2} (h)</td>
<td>106 ± 33</td>
<td>271 ± 30</td>
<td>140 ± 11</td>
</tr>
<tr>
<td>AUC_{0-12} (ng × h/ml)</td>
<td>934 ± 241</td>
<td>55,900 ± 23,900</td>
<td>3400 ± 538</td>
</tr>
<tr>
<td>CL/F (ml/h · kg⁻¹)</td>
<td>67.3 ± 15.1</td>
<td>571 ± 183</td>
<td>17.7 ± 2.5</td>
</tr>
</tbody>
</table>

* Ivermectin doses were calculated using the density of the most abundant formulation excipient, propylene glycol (density = 1.036 g/ml).

Materials and Methods

The transmembrane ATP-binding cassette transporter P-gp is known to influence drug absorption in the intestinal tract, distribution to the central nervous system, tumors, and other tissues, and drug elimination by the liver and intestinal tract (for review, see Ho and Kim, 2005). As a result, P-gp may influence the efficacy and safety of a drug or cause an interaction with other coadministered drugs. P-gp-sensitive drug pharmacokinetics and drug interactions have been studied in dogs for only a few drugs, although inferences have been drawn from other species (Martinez et al., 2008). Probably the most well known examples of P-gp-sensitive pharmacokinetics in
TABLE 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc.</th>
<th>Inhibitor</th>
<th>Cell Line</th>
<th>Efflux Ratio (BA/AB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digoxin</td>
<td>2 μM</td>
<td>MDCK-L</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>MDCK-MDR1</td>
<td>13.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>MDCK-MDR1</td>
<td>1.64</td>
<td></td>
</tr>
<tr>
<td>Ivermectin*</td>
<td>2 μM</td>
<td>MDCK-MDR1</td>
<td>1.34</td>
<td></td>
</tr>
<tr>
<td>Spinosad</td>
<td>2 μM</td>
<td>MDCK-MDR1</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 μM</td>
<td>MDCK-MDR1</td>
<td>3.58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5 μM</td>
<td>MDCK-MDR1</td>
<td>3.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 μM</td>
<td>MDCK-MDR1</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 μM</td>
<td>MDCK-MDR1</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 μM</td>
<td>MDCK-MDR1</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>Cyclosporine*</td>
<td>0.5 μM</td>
<td>MDCK-MDR1</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>Ivermectin*</td>
<td>0.5 μM</td>
<td>MDCK-MDR1</td>
<td>0.82</td>
<td></td>
</tr>
</tbody>
</table>

* Included in apical and basolateral chambers at 10 μM.

** Included in apical and basolateral chambers at 20 μM.

dogs have been associated with a coding sequence 4-base pair deletion beginning at nucleotide 294 of the dog P-gp gene (Mealey et al., 2001). In dogs and other mammals, ivermectin toxicity is caused in part by activation of GABA receptors, but at low doses P-gp prevents CNS accumulation of ivermectin. Characterization of brain tissue ivermectin concentrations in ivermectin-sensitive collies (Pulliam et al., 1985) supports a link between P-gp function and the CNS distribution of ivermectin. The CNS disposition of ivermectin and related macrocyclic lactones has also been linked to P-gp function in rodents (Schinkel et al., 1994; Kwei et al., 1999; Kiki-Mvouaka et al., 2010).

The impetus for the current study was a U.S. Food and Drug Administration warning on coadministration of spinosad with extra-label high doses of ivermectin, which resulted in ivermectin-like toxicities in dogs. Of interest, the U.S. Food and Drug Administration findings did not mention any breed effects (i.e., collies) that might implicate variants of P-gp. However, if spinosad were to be confirmed as a P-gp inhibitor, then the increased incidence of ivermectin toxicity observed with concurrent spinosad treatment could be explained by a P-gp drug-drug interaction. For instance, spinosad inhibition of ivermectin efflux at the BBB could result in increased CNS penetration of ivermectin and GABA-associated adverse events in P-gp wild-type dogs.

After we conducted several in vitro cellular assays commonly used to assess P-gp transport and inhibition, spinosad was confirmed to be a substrate and inhibitor of human P-gp using established substrates and ivermectin. However, the question of species differences in P-gp ligand recognition and kinetics must be considered even though the human P-gp amino acid sequence is calculated to be 90.3% identical to dog P-gp. Yokoi and colleagues (Katoh et al., 2006; Suzuyama et al., 2007) have demonstrated several cases in which P-gp ligands have different affinities between species, although human P-gp substrates and inhibitors were also dog P-gp substrates and inhibitors. Further complicating the question of species differences are the current limitations to characterizing dog-specific P-gp activity and inhibition. Cell-based systems for dog P-gp are not commercially available, although some contract research companies offer a dog P-gp screening service. Beagle P-gp membranes are commercially available but are only useful for showing inhibition of ATPase activity and are not well suited to establishing transport or interactions with substrates that do not produce high ATPase activity.

Although suggestive of an ivermectin-spinosad interaction, the use of human P-gp in in vitro screening was not considered definitive. Therefore, an in vivo study was next conducted to further probe the potential for drug interaction. A 10 times heartworm prevention dose of ivermectin was administered as a liquid formulation rather than a chewable formulation to achieve a dose that would enable detection of plasma drug concentrations. The liquid formulation is further postulated to mimic demodicosis treatment in the clinic for which the high doses of ivermectin needed are not easily achieved with chewable formulations. Statistical analysis demonstrated significant increases in the AUC and C_{max, obs} values for ivermectin upon dosing with spinosad (Fig. 1A; Table 1). A lack of ivermectin toxicity in T03 is attributed to the low, single dose of ivermectin used (~60 μg/kg). Even with a spinosad interaction, it can be estimated that the ivermectin plasma exposures in our study would not reach levels achieved during repeated demodicosis treatments (~300 μg/kg/day).

Several laboratories have used various mice strains or knockouts to demonstrate at least a partial role of P-gp inhibition in intestinal drug interactions involving ivermectin (for review, see Chen et al., 2003; Murakami and Takano, 2008; del Amo et al., 2009). Ivermectin is eliminated mostly unchanged in bile and feces (González Canga et al., 2009) and so, as a P-gp substrate, it is a candidate for secretion by the liver or intestine. Thus, the possibility that systemic ivermectin concentrations increase as a result of spinosad inhibition of secretion should be considered, especially because ivermectin is known to undergo P-gp-mediated exsorption in rodents (Sparreboom et al., 1997; Lafont et al., 2002; Ballent et al., 2006). As an alternative, the increase in the dog ivermectin C_{max} and lack of change in the terminal half-life could suggest that spinosad increased the oral fraction of ivermectin absorbed. This could occur either by inhibition of ivermectin metabolism or inhibition of its intestinal efflux. Unfortunately, oral ivermectin bioavailability data are scarce and a primary reference was not available for dog bioavailability. In addition, published intravenous data do not include AUC values from which to estimate F_{oral}. One reference does cite the oral bioavailability of ivermectin in dogs as being high (95%) (Plumb, 2005), so if this value accurate, the inhibition of intestinal efflux would not greatly increase the fraction absorbed, and the first-pass metabolism must be minimal. In fact, it was shown previously that only low levels of the 3-O-desmethyl...
metabolite are found in dogs and that the potent P-gp inhibitor ketoconazole did not alter metabolism of ivermectin in dogs despite increasing its AUC and C\textsubscript{max} (Hugnet et al., 2007). These findings lend support to the secretion inhibition model. However, in this scenario, pharmacokinetic theory would predict C\textsubscript{max}, T\textsubscript{max}, and half-life to increase if inhibition of the systemic elimination were responsible for the drug interaction. Spinosad did not increase the oral ivermectin T\textsubscript{max} or half-life (Table 1), but the pharmacokinetics do not necessarily rule out inhibition of ivermectin secretion. For instance, inhibition of hepatic canalicular P-gp secretion may have relatively modest effects on the plasma T\textsubscript{max} or half-life for drugs excreted in bile (i.e., ivermectin) because inhibition of biliary elimination is predicted to affect liver concentrations to a greater degree than systemic drug concentrations (Watanabe et al., 2009). However, until more intravenous data are available, the possibility that spynosad increases the fraction of oral ivermectin absorbed by inhibiting intestinal efflux cannot be discounted.

Two final discussion points deserve comment. First, ivermectin did not alter spinosad pharmacokinetics even though it might be expected because spinosad is a P-gp substrate and, like ivermectin, does not appear to be extensively metabolized in rats or livestock (Environmental Protection Agency, 1999; Rutherford et al., 2000). It is proposed that the large difference in spinosad and ivermectin doses and exposure probably prevented the observation of a spinosad interaction. Therefore, plasma protein binding of the two drugs could also be relevant. Second, the ability of the spinosad formulation to enhance ivermectin absorption cannot yet be ruled out. A formulation mimicking the one used in the commercial product could not be reproduced to serve as a control for these studies. However, formulation effects are considered to be less likely than a drug-drug interaction based on the P-gp activity and inhibition profiles of spynosad.

In summary, the results of these studies demonstrate that spinosad causes a pharmacokinetic interaction with ivermectin in dogs and that the interaction is probably due to inhibition of P-gp as assessed in vitro using human P-gp. Based on these findings, it is the P-gp inhibition by spinosad that is hypothesized to increase the risk of ivermectin toxicoses during high-dose ivermectin treatments, although the extent of BBB P-gp involvement remains in question. On one extreme, increased systemic ivermectin exposures may simply translate to higher CNS drug exposures even if inhibitory concentrations of spinosad are not present in the BBB. In contrast, it may be that the local BBB inhibition of P-gp drives CNS penetration of ivermectin. Further complicating the issue is the possibility that multidrug resistance-associated proteins, organic anion-transporting polypeptides, and other transporters that may have ivermectin and spinosad activities contributing to the drug interaction are co-localized in cell lines.


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