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The intravenous and oral pharmacokinetics of afoxolaner used as a monthly chewable antiparasitic for dogs

Laura Letendre*, Rose Huang, Valerie Kvaternick, Jay Harriman, Marlene Drag, Mark Soll

Merial Limited, 3239 Satellite Blvd, Duluth, GA 30096, USA

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ABSTRACT

The pharmacokinetics of afoxolaner in dogs was evaluated following either intravenous or after oral administration of NEXGARD®, a soft chewable formulation. Afoxolaner is a member of one of the newest classes of antiparasitic agents, known as antiparasitic isoxazolines. The soft chewable formulation underwent rapid dissolution, and afoxolaner was absorbed quickly following oral administration of the minimum effective dose of 2.5 mg/kg, with maximum plasma concentrations (C_{max}) of 1655 ± 332 ng/mL observed 2–6 h (T_{max}) after treatment. The terminal plasma half-life was 15.5 ± 7.8 days, and oral bioavailability was 73.9%. Plasma concentration-versus-time curves fit a 2-compartment model and increased proportionally with dose over the oral dose range of 1.0–4.0 mg/kg, and over the oral dose range from 1.0 to 40 mg/kg. Following an intravenous dose of 1 mg/kg, the volume of distribution (V_d) was 2.68 ± 0.55 L/kg, and the systemic clearance was 4.95 ± 1.20 mL/h/kg. Afoxolaner plasma protein binding was >99.9% in dogs. One major metabolite, formed following hydroxylation of afoxolaner, was identified in dog plasma, urine and bile. When afoxolaner is administered orally, there is a strong correlation between afoxolaner plasma concentration and efficacy with EC_{90} values of 23 ng/mL for *Ctenocephalides felis* and ≥100 ng/mL for *Rhipicephalus sanguineus* sensu lato and *Dermacentor variabilis*. The pharmacokinetic properties of afoxolaner are suited for a monthly administration product because the fast absorption and long terminal half-life support a rapid onset of action while ensuring month-long efficacy.

1. Introduction

Afoxolaner is a member of one of the newest classes of antiparasitic agents known as antiparasitic isoxazolines (Fig. 1). Originally evaluated for use in crops, the isoxazolines are highly effective against flea and tick infestations in dogs (Ozoe et al., 2010; Woods et al., 2011). The antiparasitic mode of action is mediated primarily through interaction with the arthropod GABA receptor. Activity at the glutamate-gated chloride channel receptors also has been implicated (Garcia-Reynaga et al., 2013), with both channels functioning at the central nervous system and/or neuromuscular junction resulting in irreversible hyperexcitation in the targeted arthropods (Shoop et al., 2014). The specificity of a drug to insect and acari neuroreceptors, rather than mammalian neuroreceptors, is predictive of the margins of safety for the antiparasitic drug (Gupta, 2012; Ensley, 2012). As with the isoxazoline A1443 which is 2000-fold more potent for housefly GABA receptors than for those found in rat brain membranes (Ozoe et al., 2010), radioligand binding assays show that afoxolaner, at the doses used in dogs, does not bind to mammalian GABA or glutamate receptors (Chen and Lin, 2010). To support these in vitro results, the lack of effect on the mammalian nervous system

* Corresponding author.
E-mail address: laura.letendre@merial.com (L. Letendre).

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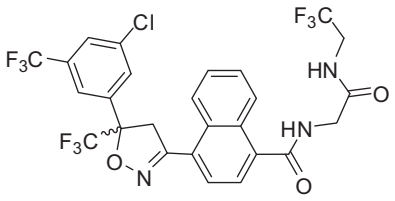

Fig. 1. Molecular structure of afoxolaner.

at clinically relevant doses was confirmed in numerous laboratory and target animal safety studies (Drag et al., 2014; Shoop et al., 2014).

Given both specificity and potency on the targeted parasite, the success of a systemically active antiparasitic agent largely depends on the pharmacokinetic properties in the targeted species. The speed and duration of action is driven by the absorption, distribution, metabolism and excretion of the antiparasitic agent in vivo (Beugnet and Franc, 2012). Two marketed active ingredients demonstrate this principle. Nitenpyram is a fast-acting oral flea treatment with a short oral plasma half-life of 2 h in dogs (Maddison et al., 2008), which makes the duration of its action too short for convenient and compliant use as an oral monthly product. Spinosad, on the other hand, has a long plasma half-life of 7–10 days (Holmstrom et al., 2012) therefore preventing and treating flea infestations for one month. In contrast to the currently available oral antiparasitic agents for dogs, the ideal pharmacokinetic and efficacy profile would demonstrate both a rapid onset of action and monthly duration of efficacy against fleas and multiple tick species. These attributes are achieved with an easy-to-administer oral soft chewable formulation for dogs, as demonstrated by the afoxolaner pharmacokinetic profile and PK/PD correlation discussed herein and supported by both efficacy and safety data.

2. Materials and methods

2.1. Protein binding

Protein binding was determined via equilibrium dialysis using a Dianorm Multi-Equilibrium DIALYZER at 200, 500, 1000, 2000 and 10,000 ng/mL of afoxolaner in dog plasma with buffer and plasma chambers separated by a semi-permeable cellulose membrane with a molecular weight cutoff of 10,000 Dalton. Incubations were performed for 2.5 h (37 °C), after which the afoxolaner concentrations were measured in the buffer and plasma compartments using LC–MS analysis (see below).

2.2. In vivo pharmacokinetic studies

For all in vivo studies, animals were treated with the final formulation of afoxolaner except when noted. All dogs were purpose-bred, ≥6 months of age, and weighed approximately 5–19 kg. The number of dogs in each study and other study details are described in Table 1 and in the text below. All animal procedures in these studies were reviewed and approved by Merial Institutional Animal Care and Use Committee (IACUC) and dogs were handled with due regard for their welfare (USDA, 2008, 9 CFR). Blood samples were taken prior to treatment, and periodically until the end of each study. All blood samples were processed into plasma and stored frozen until analyzed. Afoxolaner plasma concentrations were determined using a validated LC–MS method (Nguyen, unpublished data) and pharmacokinetic analysis via noncompartmental and/or compartmental analysis was performed. All study times are relative to Day 0, the day of dosing. Six pharmacokinetic studies were performed. The purpose and study design details are given in Table 1. Some details of PK Study 6 and two efficacy studies are given below.

2.3. Route of elimination studies

Six male Beagles were studied to determine the renal and biliary clearance of afoxolaner when administered as a soft chewable formulation once, orally, at 30–45 mg/kg (PK Study 6). Four of the dogs underwent bile duct cannulation prior to study start to allow bile collection throughout the study. Hematology and serum chemistry samples from all dogs were evaluated prior to treatment to assure normal liver functioning. Dogs were fasted prior to treatment. Plasma, urine and bile samples were collected and stored frozen (−20 °C) until analysis. Bile and urine were collected from the cannulated dogs over 3-h intervals for the first 12 h and then over 12 h intervals until 72 h post-dose. Blood was collected at time points between the bile and urine collection periods (e.g. at 1.5 h, the midpoint of the 0–3 h collection period). Afoxolaner urine and bile concentrations were determined quantitatively using an LC–MS method (Wenkui et al., 2013). Additionally, plasma, urine and bile samples were scanned for metabolites of afoxolaner. Biliary and renal clearances were calculated for each dosing interval (Rowland and Tozer, 1995) as in the following: biliary clearance = bile flow × concentration in bile/concentration in plasma and urinary clearance = urine flow × concentration in urine/concentration in plasma. The dosing intervals for each animal were averaged for each matrix. The bile and urine flow parameter for dogs were based on the data published by Davies and Morris (1993). To estimate the percentage of the total clearance attributed to biliary and renal clearance, the average total clearance (5.0 ± 1.2 mL/h/kg) from the IV Treatment Group in PK Study 2 was used.

Metabolite identification was performed on plasma samples taken 4 h and 1, 2, 4 and 6 days after administration of 25 and 100 mg/kg given orally as a solution in dog studies.

2.4. Preliminary efficacy studies

Sixteen male and female Beagle dogs (4 dogs/group; 3 oral dose groups/study and 1 control group/study) were studied to determine the effectiveness of afoxolaner when administered once as an oral soft chewable to dogs against induced infestations with 50 *D. variabilis* ticks and 100 *C. felis* fleas (Study 1) and 50 *R. sanguineus* sensu lato and 100 *C. felis* (Study 2). For each study, Treatment Group 1 included 4 untreated control dogs and Treatment Groups 2, 3 and 4 were treated with soft chewable formulations

Table 1
Study design details for pharmacokinetic studies.

Study name	PK Study 1	PK Study 2	PK Study 3	PK Study 4	PK Study 5	PK Study 6
Purpose	PK profile; dose proportionality assessment	PK profile; oral bioavailability	Multiple dose kinetics	Assess the effect of prandial state	Assess dose proportionality at high doses	Determine the route of elimination
Prandial state[a]	Fasted	Fasted	Fasted	1: Fed 2: Fasted	Fed	Fed
Breed	Mongrel	Beagle	Beagle	Beagle	Beagle	Beagle
Type	GLP	GLP	GLP	GSP	GSP	GSP
Dogs/group	8	6	6	5	5	6
Group: dose level	1: 1.0 mg/kg 2: 2.5 mg/kg 3: 4.0 mg/kg	1: 1.0 mg/kg 2: 2.5 mg/kg	1: 2.5 mg/kg	1: 2.5 mg/kg 2: 2.5 mg/kg	1: 20 mg/kg 2: 40 mg/kg	30–45 mg/kg
Treatment day	0	0	0, 28 and 56	0	0	0
Route of administration	Oral	1: IV 2: Oral	Oral gavage	Oral	Oral	Oral
Group: treatment	All groups: Nexgard®	1: IV solution[b] 2: Nexgard®	1: Solution[b]	1: Soft chew[c]	1: Soft chew[c]	1: Soft chew[c]
Blood sampling times[d]	Day 0: 2, 4, 6, 10 h Days 1, 7, 14, 21, 28, 35, 41, 48, 55	(Day 0: 15, 30 min IV Group only) Day 0: 1, 2, 4, 8, 12 h Days: 1, 3, 7, 18, 35, 42, 72	Day 0: 2, 4, 8 Day 1, 3, 7, 14, 21, 28 relative to each of the 3 doses	Day 0: 2, 4, 6, 8, 10 h Days 1, 2, 9, 16, 23, 30	Day 0: 4, 12 h Days 1, 2, 7, 14, 21, 30, 44, 58, 72, 80, 86 and 93 (Day 105 Group 1 only).	1.5, 4.5, 6, 8.5, 10.5, 12, 18, 30, 42, 54, 66, 72 h after treatment

GLP, good laboratory practices; GSP, good scientific practices. Validated bioanalytical methods were used for all studies.
[a] Fasted: food was removed the night before treatment and withheld until 4 h after treatment.
[b] Afoxolaner was formulated in a 8:2 (v/v) solution of PEG400:Ethanol.
[c] Chewable formulations contained afoxolaner and were similar to the Nexgard® formulation.
[d] Pretreatment samples taken for all groups. For PK Study 6, urine and bile collection intervals are discussed in the text.

administered orally at approximately 1.5, 2.5 and 3.5 mg/kg body weight, respectively. All dogs were infested with 100 ± 5 adult *C. felis* and 50 ± 5 adult *D. variabilis* or *R. sanguineus* sensu lato. Study 1 dogs were infested with *C. felis* on days −1, 8, 15, 22, 29, 35, 43 and 57. Study 1 dogs were infested with *D. variabilis* on days −1, 7, 14, 21, 28, 34 and 42. Study 2 dogs were infested with *C. felis* on days −1, 8, 15, 22, 29, 36, and 43. Study 2 dogs were also infested with *R. sanguineus* sensu lato on days −1, 7, 14, 21, 28, 35 and 42. All ticks and fleas were counted upon removal on Days 2, 9, 16, 23, 30, 37 and 44 for both studies, they were then categorized as live or dead (and for ticks also attached or free). Additionally, fleas were counted on Day 58 in Study 1. Blood samples were collected prior to treatment, on Day 0 at 4 and 12 h and on Days 1, 2, 9, 16, 23, 30, 36 (Day 37 for Study 2), and 44 in Study 1 and 2 and additionally on Days 51, 58, 64, 72, 79, 86 in Study 1. The later sampling times in study 2 were taken to determine the full pharmacokinetic profile of afoxolaner in dogs.

2.5. LC–MS analysis

Plasma samples from all studies were analyzed quantitatively to determine compound concentrations using a method based on 96-well solid phase extraction of afoxolaner from canine plasma and a proprietary internal standard. The extracted analytes were chromatographed by reverse-phase HPLC and quantified by a Waters Micromass® Quattro Ultima®, Waters Micromass® Micro or Sciex Qtrap® 3200 triple quadrupole mass spectrometer system using the electrospray interface. Drug and internal standard were detected in positive ionization mode using multiple reaction monitoring (MRM) of the precursor to product ions transition of *m*/*z* 626→470. Repeatability (precision), accuracy, assay specificity, stability in plasma, and all solutions and method robustness were verified and passed FDA guidelines for validated bioanalytical methods (http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf). The validated lower limits of quantitation were 5 ng/mL (lower) and 500 ng/mL (upper), based on calibration curves that ranged from 5 to 500 ng/mL afoxolaner in 0.250 mL plasma. The limit of detection was 1.0 ng/mL. Dilution quality control samples also passed all guideline criteria, and afoxolaner plasma concentrations above 500 ng/mL were diluted in plasma prior to sample extraction.

2.6. Metabolite identification

Selected dog plasma samples were scanned using LC–MS techniques for metabolites of afoxolaner. The plasma samples from control and treated dogs were prepared via solid phase extraction and protein precipitation. Structural elucidation was accomplished using accurate mass measurements and molecular formulae determinations. The molecular ion peak of the metabolite was confirmed by its characteristic $^{35}Cl/^{37}Cl$ isotope peak clusters representing each mass peak due to the natural relative abundance of each chlorine isotope in the mass spectrum. Elemental composition was determined by MassLynx® 4.1 software based on the accurate mass measurement using a Waters QTof mass spectrometer. The site of hydroxylation was determined based on the daughter ion, which was a result of loss of CF_3CHO group from the hydroxylated metabolite. HPLC-photodiode array (PDA) and total ion chromatograms were determined also. The UV (312 nm)

peak areas of both afoxolaner and the metabolite, for each animal and at each time point, were compared to estimate the relative percent of the metabolites formed. This gave a good estimate because the UV response (absorption maxima) for afoxolaner and the metabolites are approximately the same.

2.7. Pharmacokinetic analysis

Pharmacokinetic parameters were determined using the WinNonlin® software (Pharsight Corp, version 5.0.1). All plasma concentrations from samples taken prior to the first dose were below the LLOQ of the method. Concentrations < LLOQ were not used in PK calculations.

2.8. Noncompartmental analysis

All pharmacokinetic parameters were calculated using the individual plasma concentration data. The first order rate constant, λ_z, associated with the terminal log-linear portion of the curve was estimated via linear regression of the log plasma concentration versus time curve. The terminal plasma half-life ($T_{1/2}$) was calculated using the natural logarithm (2)/λ_z. The area under the plasma afoxolaner concentration versus time curve from the time of dosing to T_{last} ($AUC_{0\text{-}T_{last}}$) was calculated by a linear up log down trapezoidal method and extrapolated to infinity using the equation $AUC_{0\text{-}T_{last}} + C_{last}/\lambda_z$. The average steady state plasma concentration was calculated by dividing the AUC over one dosing interval by the time of the dosing interval.

2.9. PK/PD analysis for tick and flea efficacy

An E_{max} model (Eq. (1)) was used to describe the relationship between plasma concentration and percent efficacy (the effect). The flea or tick count taken 24 h (flea) or 48 h (tick) after infestation was compared to the flea or tick count at the same time on control dogs that were not treated, and a percent difference from control was calculated as follows: {1 − [count (X h post-infestation) for dog i]/[geometric mean count for the control dogs at X h post-infestation]} × 100, where count = the number of live fleas or ticks. The percent efficacy versus afoxolaner plasma concentration was input into the WinNonlin® software and fit to a Sigmoid E_{max} model (Eq. (1)). In the model the Effect is set to 0% when plasma concentrations are 0. The maximal effect, E_{max}, is a parameter determined by the model and expected to be close to 100% and is a measure of maximal efficacy. The following equation was used to fit the data:

$$\text{Effect}(t) = \frac{E_{max} \times C(t)^{Gamma}}{C(t)^{Gamma} + EC_{50}{}^{Gamma}} \quad E_{max}\,\text{Model} \qquad (1)$$

EC_{50} is the plasma concentration corresponding to $E_{max}/2$ and is a measure of potency. $C(t)$ is the measured afoxolaner plasma concentration at time t, and Gamma, a measure of the selectivity, is related to the steepness of the plasma concentration versus effect curve. The Nedler Mead algorithm was used without weighting to estimate the parameters of the model. The EC_{90}, the afoxolaner plasma concentration estimated to provide 90% efficacy, was then calculated using the following equation:

$$EC_{90} = EC_{50} * \left(\frac{90}{100 - 90}\right)^{1/Gamma}$$

2.10. Dose proportionality

Dose proportionality was assessed by calculating the strength of a linear relationship between AUC and dose or between C_{max} and dose using the power method (Hummel et al., 2009). Log dose versus log $AUC_{0\text{-}T_{last}}$, $AUC_{0\text{-Inf}}$ or C_{max} were fit using linear regression with reciprocal dose weighting. The upper and lower 95% confidence and prediction intervals also were determined, and the residuals were tested for normality. The parameters ($AUC_{0\text{-}T_{last}}$, $AUC_{0\text{-Inf}}$ or C_{max}) were considered to increase proportionally with dose if the slope of the Log dose versus Log parameter curve was completely within the 95% confidence interval of 0.8–1.25.

2.11. Linear pharmacokinetics

To confirm that the pharmacokinetic processes were linear, afoxolaner plasma concentration versus time curves for each dog following multiple monthly dosing were simulated using parameters from the single dose two-compartment analysis and assuming linear kinetics.

3. Results

3.1. Protein binding

The extent of plasma protein binding was greater than 99.9% in dog plasma over the range of afoxolaner plasma concentrations tested (200–10,000 ng/mL). Afoxolaner, therefore, is highly bound to plasma proteins of dog, and protein binding is independent of concentration over the range of 200–10,000 ng/mL.

3.2. Pharmacokinetic profile of afoxolaner in dogs

Following a 1 mg/kg IV dose, the afoxolaner plasma concentrations decreased bi-exponentially with a rapid distribution phase and a long elimination phase. The individual afoxolaner plasma concentration versus time curves fit well to a two compartment (bi-exponential) model (data not reported). The V_{dss} was 2.68 ± 0.55 L/kg, and the systemic clearance (Cl) was 4.95 ± 1.20 mL/h/kg. Afoxolaner IV and oral PK parameters are given in Table 2.

Following oral administration of Nexgard® to dogs, plasma concentrations peaked quickly, indicating rapid dissolution and absorption from the soft chewable formulation. Afoxolaner was well-absorbed with oral bioavailability in PK Study 2 of 73.9%. After T_{max}, the afoxolaner plasma concentrations declined bi-exponentially with a fast distribution phase occurring over the first day (Day 0). The oral plasma concentrations also fit well to a two compartment (bi-exponential) model (data not reported). The terminal plasma half-life was the same following IV and oral administration, indicating the terminal afoxolaner

Table 2
PK Study 1 and 2; mean ± standard deviation of pharmacokinetic parameters for afoxolaner following oral administration of afoxolaner in a soft chewable formulation or intravenous administration of a solution to Mongrel and Beagle dogs.

Parameter	PK Study 1 Mongrel dogs, $n=8$/group			PK Study 2 Beagle dogs, $n=6$/group	
	Group 2	Group 3	Group 4	Group 1 (IV)	Group 3
Route of administration	Oral chew	Oral chew	Oral chew	Intravenous solution	Oral chew
Dose (mg/kg)	1.0	2.5	4.0	1.0	2.5
$T_{1/2}$ (day)	14.4 ± 3.6	15.2 ± 5.1	15.6 ± 9.0	15.6 ± 7.3	15.5 ± 7.8
T_{max} (h)	2–6	2–4	2–6	0.25[a]	2–4
C_{max} (ng/mL)	538 ± 85	1384 ± 330	2147 ± 575	1107 ± 190[a]	1655 ± 332
T_{last}[b] (day)	55	55	55	42–72	14–72
C_{last} (ng/mL)	24.6 ± 13.6	72.6 ± 53.8	113 ± 90	21.9 ± 13.5	54.5 ± 31.4
$AUC_{0\text{-}T_{last}}$ (day μg/mL)	6.7 ± 1.3	17.2 ± 4.9	26.6 ± 5.6	8.18 ± 1.53	14.8 ± 8.2
$AUC_{0\text{-Inf}}$ (day μg/mL)	7.2 ± 1.6	19.1 ± 6.7	30.1 ± 7.3	8.78 ± 2.02	16.2 ± 9.4
AUC % Extrap	7.3 ± 4.4	8.7 ± 6.0	10.4 ± 10.3	6.0 ± 5.4	7.4 ± 3.8

[a] First time point.
[b] Last measured time point for PK Study 1 and last measured time point above the limit of quantitation for PK Study 2.

plasma concentrations represent a true elimination phase. This long terminal phase spanned from approximately Day 2 until the final time point for each study [see e.g. Fig. 2]. In PK Study 5, a single exponential decay accurately described the plasma concentration curve from Day 2 to Day 105.

The pharmacokinetic profile of afoxolaner following oral administration was determined in over 145 treated dogs and found to be predictable and comparable across all studies in the Nexgard® development program (some studies not reported in detail here). The pharmacokinetic parameters from PK Studies 1 and 2 are given in Table 2. The afoxolaner plasma concentration versus time curves on a semilog scale for the Nexgard® treatment groups reported in PK Study 1 are shown in Fig. 2.

A single major metabolite, hydroxylated afoxolaner, was observed in dog plasma as an oxidation product presumed to be formed via cytochrome P450 enzymes. Although the metabolite identification was performed qualitatively, the amount of metabolite present relative to parent afoxolaner was estimated using HPLC UV peak areas and found to be between approximately 2.5 and 17.8%.

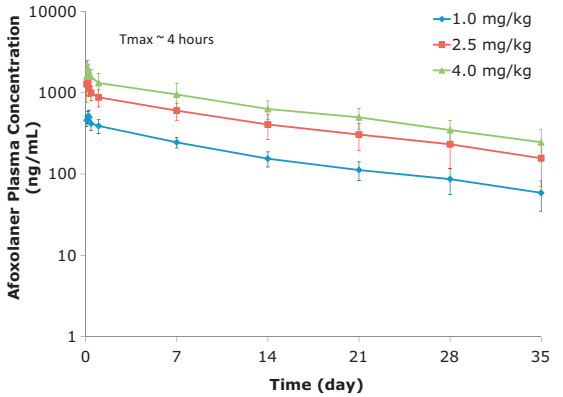

Fig. 2. Mean afoxolaner plasma concentration versus time curves following oral administration of 1.0, 2.5 and 4.0 mg/kg of Afoxolaner in Nexgard® chewable formulation to Mongrel dogs ($n=8$/treatment group) in PK Study 1.

Concentrations of afoxolaner in the bile ranged from 104 to 7900 ng/mL and the biliary clearance was on average 1.5 mL/h/kg. Afoxolaner urine concentrations were below the limit of quantitation of the bioanalytical method (<1.25 ng/mL), and the renal clearance of parent afoxolaner could therefore not be determined. Urine and bile samples also were analyzed for afoxolaner metabolites. The urine contained a hydroxylated afoxolaner and an afoxolaner acid metabolite. The bile samples contained the hydroxylated afoxolaner metabolite and afoxolaner. The acid of afoxolaner was not detected in the bile.

3.3. Multiple dose kinetics of afoxolaner to dogs

Following oral 2.5 mg/kg doses of a solution administered 3 times at 28 day intervals, the maximum afoxolaner plasma concentrations were 699 ± 315, 1150 ± 450, and 908 ± 147 ng/mL, observed approximately 3-h after the 1st, 2nd and 3rd dose, respectively. The average concentration at steady state was approximately 260 ng/mL and was similar after each dose.

Experimental data show low accumulation with a ratio of $AUC_{0\text{-Inf}}$ (Dose #3) to $AUC_{0\text{-Inf}}$ (Dose #1) of approximately 1.0. The ratio for C_{max} in this study was 1.3 (a 30% increase from Dose #1 to Dose #3). The half-life was comparable after each of the 3 monthly doses. The simulation assuming linear kinetics agrees with the measured plasma concentrations (Fig. 3). These results are consistent with those observed by Shoop et al. (2014) following 5 monthly doses of afoxolaner to dogs.

3.4. Impact of prandial state on afoxolaner exposure

Maximum afoxolaner plasma concentrations for the fed dogs averaged 1366 ± 276 ng/mL, and the time to maximum concentration was between 2 and 24 h for most dogs. Fasted dogs had maximum afoxolaner plasma concentrations of 1453 ± 374 ng/mL, and the time to maximum concentration was 2 h for all 5 dogs in this treatment group. The overall exposure (AUC_{Inf}) was not affected by the

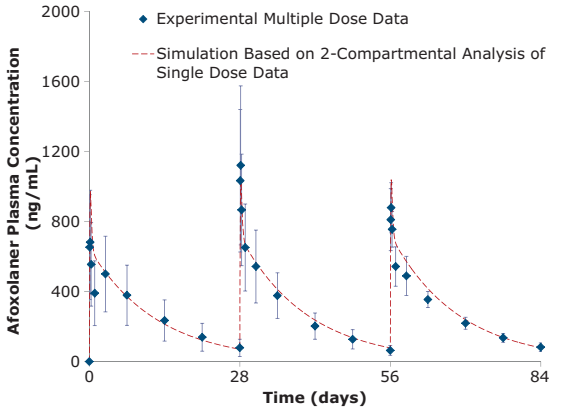

Fig. 3. Experimental (diamond) and simulated (dashed line) afoxolaner plasma concentration versus time curve following multiple 2.5 mg/kg doses to Beagle dogs ($n = 6$) on Days 0, 28 and 56.

prandial state of the dogs (13.0 ± 2.9 and 10.9 ± 2.6 μg day/mL for fed and fasted dogs, respectively).

3.5. Dose proportionality over the range of 1–40 mg/kg

C_{max}, $AUC_{0\text{-}T_{last}}$ and $AUC_{0\text{-}Inf}$ increased proportionally with dose, indicating linear pharmacokinetics over the range of 1.0–4.0 mg/kg when afoxolaner chews were administered orally in PK Study 1. Table 3 shows the parameters from the Power Model fit for PK Study 1.

Including the C_{max} and $AUC_{0\text{-}Inf}$ values from PK Study 5 reveals that afoxolaner exhibited close to linear kinetics for absorption, elimination and distribution processes (ADME) over the range of 1–40 mg/kg. The data were obtained from two different studies and therefore are not included in one statistical analysis; nonetheless, the log C_{max} versus Dose and Log $AUC_{0\text{-}Inf}$ versus Dose graphs covering the full range of doses tested are given in Fig. 4.

3.6. Effective afoxolaner plasma concentrations

Examples of the flea and tick efficacy as a function of plasma concentration are given in Fig. 5A and B, respectively. A direct relationship between plasma concentration and percent of effectiveness relative to control dogs was modeled using a Sigmoidal E_{max} model. The EC_{90} afoxolaner concentrations was estimated to be: 23 ng/mL (24 h post infestation) for *C. felis* and ≥100 ng/mL (48 h post infestation) for *R. sanguineus* and *D. variabilis*.

4. Discussion

The physicochemical properties of any drug affect its ability to cross cell membranes and therefore govern the absorption, tissue distribution, and elimination of the drug in vivo. The molecular size, solubility, degree of ionization (indicated by pK_a), and relative solubility in lipid and aqueous environments (indicated by the lipid:water partition coefficient, i.e., log P or log D) are therefore important parameters for understanding pharmacokinetic behavior (Jenkins and Cone, 1998). Afoxolaner has extremely weak acid/base properties and is essentially unionized over the

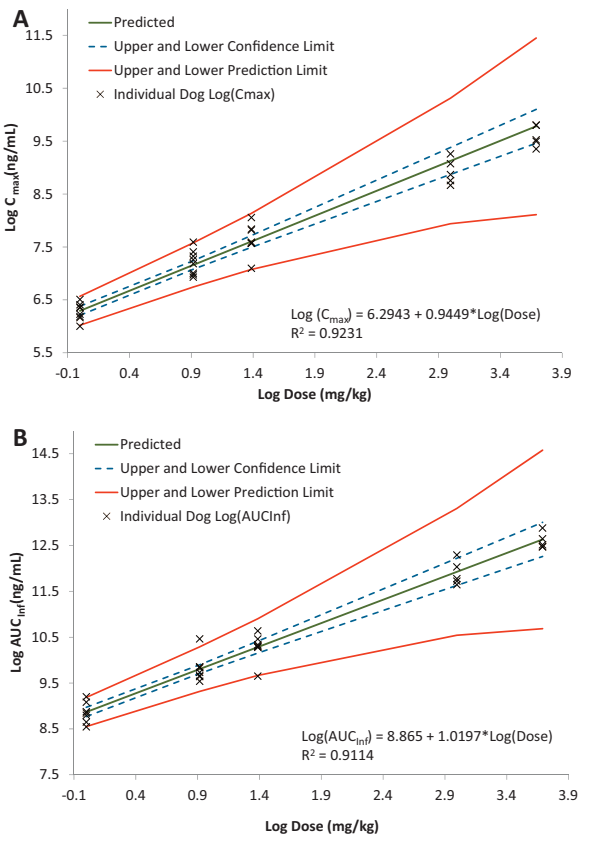

Fig. 4. Log afoxolaner C_{max} [A] and Auc_{Inf} [B] versus log dose following oral administration of afoxolaner in a soft chewable formulation to Mongrel and Beagle dogs over the range of 1–40 mg/kg from two studies: PK Study 1 (1, 2.5 and 4 mg/kg) and PK Study 2 (20 and 40 mg/kg). Fit using linear regression with reciprocal dose weighting is also shown along with the 95% confidence and prediction limits.

physiological pH range (pH 1–12). This small, lipophilic, unionized compound is therefore expected to cross cell membranes freely via passive diffusion driven by a concentration gradient.

Afoxolaner pharmacokinetic properties have been tested in a number of in vivo studies and follow the expectations for a Biopharmaceutics Classification System (BCS) Class II compound. For BCS Class II compounds, if dissolution is complete and the drug is in solution, high bioavailability is expected due to the high permeability. High permeability compounds readily access enzymes within the hepatocytes and therefore may be eliminated primarily by metabolism. These compounds also tend to distribute into tissues (Wu and Benet, 2005).

Afoxolaner distributes into tissues, V_d of 2.68 ± 0.55 L/kg, as expected for a lipophilic compound (Toutain and Bousquet-Melou, 2004). The single exponential decay of afoxolaner in plasma during the terminal phase from Day 2 to 3 months suggests that no special tissue depots are present in the dog. This conclusion is consistent with the physical chemical properties of afoxolaner, which favor passive diffusion into and out of tissues. Active transport, if occurring, was not saturated under the conditions/dose levels tested.

Table 3
Log parameter versus log dose fitted parameters, 95% confidence limits and dose proportionality assessment for PK parameters for range of 1–4 mg/kg [PK Study 1, $n = 8$ Mongrel dogs per treatment group].

Parameter	Intercept	Slope		Confidence limits		Dose
		Estimate	SE	Lower	Upper	Proportional
C_{max}	6.27812	0.99533	0.07296	0.84402	1.14663	Yes
$AUC_{0\text{-}T_{last}}$	8.79092	1.00333	0.07604	0.84563	1.16103	Yes
AUC_{Inf}	8.86672	1.02829	0.08754	0.84675	1.20983	Yes

Afoxolaner has a low systemic clearance of 4.95 ± 1.20 mL/h/kg, determined following IV administration. The low clearance is much less than the hepatic blood flow in dogs (1854 mL/h/kg), as reported in Davies and Morris (1993) and is responsible primarily for the long half-life of afoxolaner in dogs. Clearance may be closely dependent on either free drug concentrations, where significant protein binding (>99.9% for afoxolaner) limits the drug available for renal and hepatic elimination, or on the intrinsic ability of hepatocytes to metabolize the drug (Rowland and Tozer, 1995).

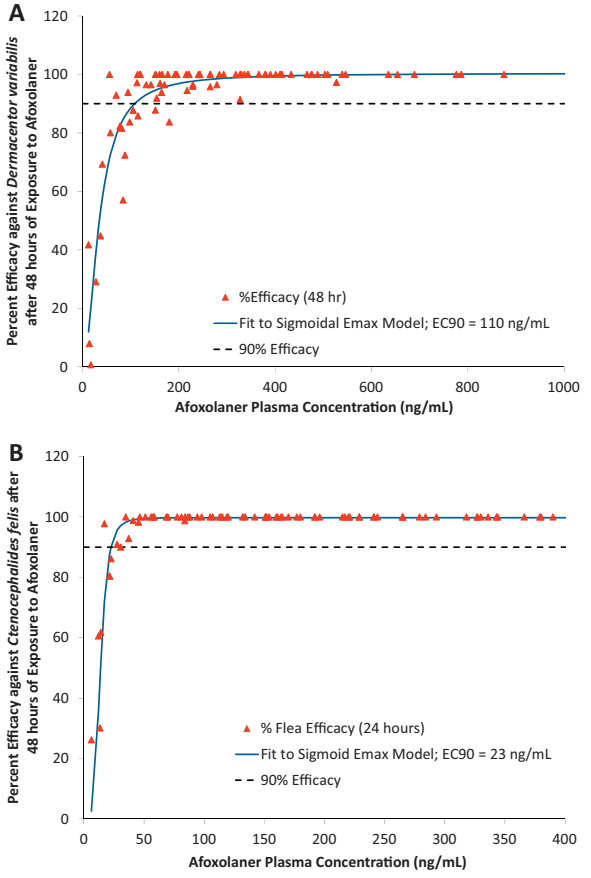

Fig. 5. Mean % efficacy against *Dermacentor variabilis* (Ticks) [A] and *Ctenocephalides felis* (Fleas) [B] versus afoxolaner plasma concentration following oral administration of afoxolaner in Nexgard® to Beagle dogs. The line represents the fit to a sigmoid E_{max} model. The triangles are the experimental values. In Fig. 5B, plasma concentrations > 400 ng/mL are not shown and efficacy for those levels was 100% in all cases.

Plasma, urine and bile were collected to establish the primary route for elimination. Afoxolaner concentrations in the bile were high, and the biliary clearance was on average 1.5 mL/h/kg. This clearance is about 30% of the total clearance measured in PK Study 2, with individual dogs ranging in biliary clearance from 10 to 44% of the total clearance. Afoxolaner reabsorption was experimentally hindered by the biliary collection in this study, therefore, 30% is considered an upper limit of the total afoxolaner biliary clearance from the body. Using the estimated urine afoxolaner values that were below the limit of quantitation (<1.25 ng/mL), renal clearance of the parent compound was calculated to be less than 0.01% of the total clearance.

The afoxolaner plasma concentrations from fed and fasted dogs are within the biological or inter-animal variability as shown by the standard deviations of the two groups. The differences were not therapeutically relevant or statistically significant ($\alpha = 0.05$).

As reported, the terminal plasma half-lives were 15.2 ± 5.1 and 15.5 ± 7.8 days in two studies investigating the minimum effective dose of 2.5 mg/kg. The mean half-life of afoxolaner administered orally at a dose of between 1 and 4 mg/kg, is 12.8 ± 5.6 days in 145 adult Beagle and Mongrel dogs from across the multiple studies. Given the observed half-lives, steady state plasma concentrations following a monthly dosing regimen will be well within the afoxolaner margin of safety in dogs.

Afoxolaner increased approximately proportionally with dose over a wide dose range of 1.0–40 mg/kg. Additionally the kinetics were unchanged upon multiple dosing. These parameters indicate that the clearance, distribution and absorption processes are neither saturated nor induced after monthly dosing and the kinetics are linear.

A strong relationship between afoxolaner concentration in plasma and efficacy against fleas and ticks was determined, thus confirming that afoxolaner acts systemically to kill fleas and ticks. The afoxolaner EC_{90} concentrations were 23 ng/mL for *C. felis* flea and ≥100 ng/mL for *R. sanguineus* sensu lato and *D. variabilis* ticks. Because afoxolaner is efficacious through most of the flea and tick sampling times, dogs had 100% efficacy at most time points. The variability was greater at lower plasma concentrations and especially so near the points on the curve that increase steeply, namely the slope (Gamma). The model was nonetheless judged to be useful because the CV of the parameters was low, the condition number was low, and there was a high correlation between predicted and observed values. In clinical studies, dogs administered a dose as close as possible to the minimum therapeutic dose of 2.5 mg/kg body weight had afoxolaner plasma concentrations above the EC_{90} for

fleas (*C. felis*), and ticks for at least one month. Efficacy studies have confirmed this result, with high levels of efficacy reported for fleas and these tick species for at least one month following treatment with Nexgard® oral chews (Dumont et al., 2014; Hunter et al., 2014; Mitchell et al., 2014).

5. Conclusion

Afoxolaner pharmacokinetic properties have been tested in a number of studies, and rapid absorption, high bioavailability, moderate distribution into tissues and low systemic clearance were the hallmarks of this novel, soft chewable oral formulation (Nexgard®). The prandial state of the dog does not affect the rate or extent of absorption. Afoxolaner plasma concentrations increase approximately proportionally with the dose from 1.0 to 40.0 mg/kg. The drug is highly bound to plasma proteins (>99% bound) in the dog, and protein binding is independent of concentration over the range of 200–10,000 ng/mL. Due to the terminal plasma half-life of approximately 2-weeks at a dose of 2.5 mg/kg, average afoxolaner plasma concentrations were consistently above the level needed for efficacy against fleas and ticks over one month.

Conflict of interest

The work reported herein was funded by Merial Limited, GA, USA. All authors are current employees or contractors of Merial.

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