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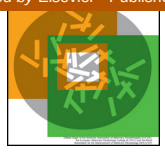

Discovery and mode of action of afoxolaner, a new isoxazoline parasiticide for dogs

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ABSTRACT

Afoxolaner is an isoxazoline compound characterized by a good safety profile and extended effectiveness against fleas and ticks on dogs following a single oral administration. *In vitro* membrane feeding assay data and *in vivo* pharmacokinetic studies in dogs established an afoxolaner blood concentration of 0.1–0.2 μg/ml to be effective against both fleas (*Ctenocephalides felis*) and ticks (*Dermacentor variabilis*). Pharmacokinetic profiles in dogs following a 2.5 mg/kg oral dosage demonstrated uniform and predictable afoxolaner plasma concentrations above threshold levels required for efficacy for more than one month. Dose ranging and a 5-month multi-dose experimental study in dogs, established that the 2.5 mg/kg oral dosage was highly effective against fleas and ticks, and produced predictable and reproducible pharmacokinetics following repeated dosing. Mode of action studies showed that afoxolaner blocked native and expressed insect GABA-gated chloride channels with nanomolar potency. Afoxolaner has comparable potency between wild type channels and channels possessing the A302S (resistance-to-dieldrin) mutation. Lack of cyclodiene cross-resistance for afoxolaner was confirmed in comparative *Drosophila* toxicity studies, and it is concluded that afoxolaner blocked GABA-gated chloride channels *via* a site distinct from the cyclodienes.

1. Introduction

To date, no therapeutic compound administered *per os* has had the attributes required to effectively kill both fleas and ticks on dogs safely for a month or more (Beugnet and Franc, 2012). Cythioate, an organophosphate, was the first oral agent to kill fleas but the short duration of its efficacy (~1 day; Gordon, 1995), variable efficacy against fleas (Arther et al., 1989), and poor safety profile limited its use. Nitenpyram, a closely related analog of the neonicotinoid, imidacloprid, has high levels of effectiveness against fleas only, and its half-life is such that its activity lasts but a single day (Cadiergues et al., 1999). Spinosad was the first oral agent to provide month long activity with good effectiveness but primarily against fleas (Snyder et al., 2007).

Herein, we reveal the structure of afoxolaner, a novel member of an isoxazoline class of compounds, and describe the initial preclinical program in dogs against fleas and ticks using oral administration. Isoxazoline insecticide represents a new class of potent ectoparasiticides, including afoxolaner, but also fluralaner, amongst others (Ozoe et al., 2010; Garcia-Reynaga et al., 2013; Gassel et al., 2014).

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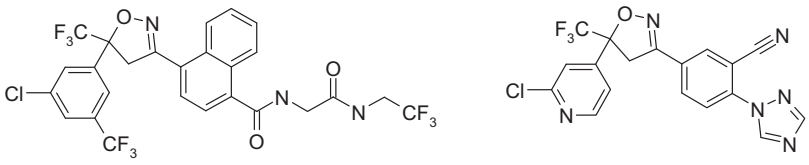

Fig. 1. Molecular structure of afoxolaner and the isoxazoline, CPD I.

The proof of concept studies presented here were not conducted with a final formulation to be registered as a veterinary medicine. Initial *in vitro* studies revealed remarkable activity of afoxolaner against fleas, and subsequent *in vivo* testing in dogs demonstrated outstanding effectiveness against fleas and ticks for an entire month following a single, low dose oral administration. Multi-month dosing tests further defined the effective oral dosage and safety of afoxolaner in dogs. Lastly, we outline the insecticidal/acaricidal mode of action of this compound and the class.

The history of insecticidal isoxazolines can be traced back to the discovery of the phthalic and the anthranilic diamides, and Lahm et al. (2013) have recently described the high levels of activity of a class of 4-azolylphenyl isoxazoline compounds for agricultural use. The isoxazoline compounds having both insecticidal and acaricidal activities have been proven to act on specific GABA/glutamate receptor inhibiting the chloride ion channels of arthropods (Ozoe et al., 2010; Garcia-Reynaga et al., 2013; Gassel et al., 2014).

The authors discovered a group of naphthalene isoxazoline compounds displaying remarkable activity against the cat flea (*Ctenocephalides felis*) and afoxolaner was selected as a representative compound for further testing and evaluation. The molecular structures of afoxolaner and of isoxazoline CPD1, a representative of the azolylphenyl isoxazoline class, are provided in Fig. 1.

2. Materials and methods

The efficacy of afoxolaner was assessed during different studies. A first *in vitro* study identified the potential activity of afoxolaner. It was followed by studies on dogs. A first simple assessment, then a one-month study, and finally a 5-months study. In parallel to the dog experiments, mode of action studies were conducted in insect models.

2.1. In vitro study

2.1.1. Study 1

Fleas are obligate haematophagous insects and therefore activity of insecticidal compounds that may be useful for their control can be evaluated by exposing them to any pharmaceutical compound added to the blood on which they feed (Zakson et al., 2001). This approach was used to determine the blood concentration of afoxolaner necessary to kill fleas.

The titration studies were performed in an *in vitro* membrane flea feeding system as generally described by Zakson et al. (2001). Compounds were formulated in 100% dimethyl sulphoxide (DMSO) to a concentration of 32.0 μg/ml and then through serial dilution in DMSO to 16, 8, 4, 2 and 1 μg/ml. Following the initial formulation and serial dilution, each drug sample was further diluted by addition of citrated bovine blood (99.2% blood, 0.8% sodium citrate) to create a final dose titration of 0.32, 0.16, 0.08, 0.04, 0.02 and 0.01 μg/ml. A vehicle treatment consisting of 1% DMSO and 99% citrated bovine blood was used for the control treatment. Each dilution of each drug was done in triplicate. One hundred *C. felis* fleas fed through the membrane on the blood containing the compound and counts of live and dead fleas were made after 24, 48 and 72 h.

2.2. In vivo studies on dogs

2.2.1. Studies 2, 3 and 4

Based on the *in vitro* activity against fleas (study 1), three studies were serially conducted to establish and define the *in vivo* activity of this compound against fleas and ticks on dogs. Initial studies involved single treatments, while subsequent studies evaluated the efficacy of different dosages and multiple treatments. These studies generally employed similar methodologies.

In these studies, afoxolaner was delivered in an oral solution. This was an experimental formulation, which provided good solubility of the compound and allowed for administration of accurate doses. Afoxolaner used in the studies was synthesized at DuPont Crop Protection. Each sample of afoxolaner used in the animal studies was weighed and then formulated in an experimental vehicle consisting of DMSO (2% of final volume) and propylene glycol/glycerol formal (3:2) (98% of final volume). The experimental vehicle was also used as the negative control in each experiment. A vortexer and sonicating bath were used, as needed, to achieve true solutions as determined by visual and, if necessary, microscopic inspection to confirm that no particles or crystals were present. Different dosages were used for the various studies. All dosages were administered orally using a calibrated syringe. Day 0 is defined in each study as the day of dog treatment.

All flea challenges were performed by placing 100 live, unfed adult cat fleas, *C. felis*, onto the dorsal midline of dogs at various times throughout the studies. Fleas were removed and counted by combing following 24 h of drug exposure. Comb counts were performed following the method described by Zakson et al. (1995).

All tick challenges were performed by placing 50 live, unfed adult American dog ticks, *Dermacentor variabilis*, onto the dorsal midline of dogs at various times

Table 1
Effect of afoxolaner in blood to exposed fleas (*Ctenocephalides felis*) in an artificial membrane feeding device.

Treatment	Evaluation time (h)	Mean % flea effectiveness[a,b]					
		0.32 μg/ml	0.16 μg/ml	0.08 μg/ml	0.04 μg/ml	0.02 μg/ml	0.01 μg/ml
Afoxolaner	24	100	100	62	33	6	0
	48	100	100	97	92	49	14
	72	100	100	100	100	96	46

[a] Vehicle control mortality was 1, 2.3 and 2.3% at 24, 48 and 72 h respectively.
[b] Each time and concentration was done in triplicate.

throughout the studies. Ticks were removed, categorized as dead or alive, and counted by hand and comb following 48 h of drug exposure.

The initial day of the flea or tick challenge is recorded in the specific table for each study (Tables 2–6) whereas the removal of fleas and ticks took place 24 and 48 h later, respectively. All flea and tick evaluations were blinded. Effectiveness was calculated at given time points, by the classic formula (1 – [geo mean flea/tick count treated dogs – geo mean flea/tick count control dogs]/[geo mean flea/tick count on control dogs]) × 100.

At a minimum, clinical observations for adverse reactions were performed post-treatment at 30 min, 2 and 4 h, and then daily for the duration of each study. Additional details were noted in Study 4 (see below).

Afoxolaner plasma levels were determined in each animal study. At various times during the studies, blood samples were collected from the dogs and placed in heparinized tubes. The blood samples were centrifuged and the plasma was separated and immediately frozen.

Plasma was thawed and afoxolaner was extracted from a 50-μL aliquot diluted with 450 μL of HPLC grade water in a 2-ml plastic centrifuge tube. The sample was further diluted with 500 μL of HPLC grade acetonitrile. Samples were centrifuged for 5 min at 14,000 rpm in a microcentrifuge tube after vigorous mixing at each step. A 10-μL aliquot was then analyzed for afoxolaner by LC/MS/MS. The LC/MS/MS system consisted of a Waters Quattro Micro™ Mass Spectrometer interfaced to a Waters Alliance HT 2795 HPLC and equipped with a 2.1 mm × 50 mm, 5 μm Zorbax SB C18 column. Afoxolaner was eluted from the column using a gradient of water/acetonitrile, each containing 0.1% formic acid.

Food and water were provided for all dogs following USDA guidelines and access to veterinary care was available throughout the studies. All testing was performed under the guidelines set forth by the Institutional Animal Care and Use Committee of DuPont (USDA, 2008).

2.2.2. Study 2: probe efficacy study in dogs

Afoxolaner was prepared for a dosage of 2.5 mg/kg to be administered once, orally, at the rate of 0.2 ml/kg. The vehicle was administered in the same manner. Two beagle dogs were used for the study with one randomly assigned to treatment and the other assigned to vehicle. Flea and tick challenges were made at periodic intervals over the course of 46 days and counts were performed approximately 24 h (fleas) or 48 h (ticks) after challenge. Blood samples were taken at least weekly for the duration of the study.

2.2.3. Study 3: preliminary dosage titration in dogs and assessment of post prandial effect

Afoxolaner was prepared for administration at dosages of 1.5, 2.5 or 3.5 mg/kg to be delivered once, orally at the rate of 0.2 ml/kg dog weight. The vehicle was administered in the same manner. Fifteen beagle dogs were allocated on restricted randomization based on weight and sex to form 5 equal groups of 3 dogs each. Those groups were allocated randomly to treatment. The treatment groups were: 0 mg/kg fed, 1.5 mg/kg fed, 2.5 mg/kg fed, 2.5 mg/kg fasted and 3.5 mg/kg fed. In the fed group, food was given prior to dosing whereas in the fasted group food was removed the night prior to dosing and not returned until 2 h post-dosing. Flea and tick challenges were conducted at periodic intervals over the course of one month. Blood samples were taken at least weekly for the duration of the study.

2.2.4. Study 4: assessment of 5 month repeat dosing on efficacy and safety in dogs

Afoxolaner was prepared for a dosage of 2.5 mg/kg to be administered five times orally at 30 days intervals at the rate of 0.2 ml/kg dog weight. The vehicle was administered in the same manner. Six beagles were allocated randomly to the 2.5 mg/kg treatment and six beagles were allocated to treatment with vehicle only. Flea and tick challenges were made every week over the course of five months, with counts conducted at appropriate intervals after each challenge. Blood samples were taken at least weekly for the duration of the study. Dog weights were measured on Day 1, and then on Days 7, 14, and 29 of each monthly dosing cycle. Final weights were collected on Day 31 after the fifth dose.

At each dosing, dogs were observed at 30 min, 2 and 4 h post dosing, and daily for the duration of the study (150 days). Each dog had a physical examination by a veterinarian at Day 1, and then on Days 1, 14 and 29 of each monthly dosing cycle.

2.2.5. Mode of action studies: elucidation of insecticidal/acaricidal mode of action by evaluation of toxicity and symptomology in insect models

Initial mode of action studies were conducted using the related isoxazoline, CPD I, with more detailed studies conducted using afoxolaner (Fig. 1).

2.2.6. Study 5 – toxicity and symptomology in model insects

Adult male American cockroaches (*Periplaneta americana*), were injected with 0.1–10 μg CPD I through the ventral intersegmental membrane of the abdomen with

Table 2
Flea (*Ctenocephalides felis*) counts and efficacy of afoxolaner following administration to a dog in an oral solution at 2.5 mg/kg.

Treatment	Dosage (mg/kg)	Flea counts						
		Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Vehicle	0	59	75	72	83	73	64	71
Afoxolaner	2.5	0	0	0	0	0	0	0
% Effectiveness		100	100	100	100	100	100	100

Table 3
Tick (*Dermacentor variabilis*) counts and efficacy of afoxolaner following administration to a dog in an oral solution at 2.5 mg/kg.

Treatment	Dosage (mg/kg)	Tick counts						
		Day 2	Day 9	Day 16	Day 23	Day 30	Day 37	Day 44
Vehicle	0	28	28	32	19	23	27	43
Afoxolaner	2.5	0	0	0	0	0	1	5
% Effectiveness		100	100	100	100	100	96	88

Table 4
Geometric means and efficacies of afoxolaner against multiple flea (*Ctenocephalides felis*) challenges following single administration to dogs in an oral solution.

Treatment	Dosage (mg/kg)	Fed/fasted	*n*	Geometric mean[a] (% efficacy[b])					
				Day 0	Day 7	Day 14	Day 21	Day 28	Day 32
Vehicle	0	Fed	3	81.0	73.1	63.5	67.6	67.5	68.8
Afoxolaner	1.5	Fed	3	0.4 (99.5%)	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)
	2.5	Fed	3	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.3 (99.6%)
	2.5	Fasted	3	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)
	3.5	Fed	3	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.8 (98.8%)

[a] Geometric mean is computed by subtracting 1 from the anti-logarithm of the mean of ln(count + 1).
[b] Percent efficacy = 100(1 − *T*/*C*), where *C* is GM of vehicle and *T* is GM of each treated group.

appropriate concentrations of CPD1 dissolved in 2 μL DMSO. Observations of insect toxicity and mortality were conducted over a 72 h period and a KD_{50} (50% knockdown concentration) was calculated.

2.2.7. Study 6 – cockroach electrophysiology studies

To aid in elucidating the target site of isoxazoline insecticides, activity of CPD I was investigated on an *in vitro* preparation. Cockroaches possess an escape reflex circuit (cercal reflex) in which mechanical stimulation of hairs of the cerci produce bursts of action potential spikes which travel through the ventral nerve cord in an anterior direction producing excitation of motor nerves (Fig. 4a). Nerve conduction for this reflex circuit involves the excitatory and inhibitory neurotransmitter receptors, acetylcholine and GABA, respectively, as well as voltage-gated sodium and potassium channels.

Extracellular recordings were conducted on nerve 5 (N5) of the metathoracic ganglion of American cockroaches.

2.2.8. Study 7 – oocyte injection and voltage clamp studies

The fruit fly (*Drosophila melanogaster*) *rdl* (resistance-to-dieldrin) gene encodes for a GABA-gated chloride channel subunit which can form a functional homomeric channel that exhibits pharmacology similar to native receptors (Gassel et al., 2014). To further investigate activity of afoxolaner, voltage clamp studies were conducted on *Xenopus laevis* oocytes expressing *Drosophila* Rdl receptors. Plasmids pNB40 and pALTER-Ex1 encoding for wild type (^{wt}Rdl) and dieldrin-resistant *R*dl (^{A302S}Rdl), respectively, were kindly provided by Prof. David Sattelle (University of Manchester). Constructs were transformed using One

Table 5
Geometric means and efficacies of afoxolaner against multiple tick (*Dermacentor variabilis*) challenges following single administration to dogs in an oral solution.

Treatment	Dosage (mg/kg)	Fed/fasted	*N*	Geometric mean[a] (% efficacy[b])				
				Day 2	Day 9	Day 16	Day 23	Day 30
Vehicle	0	Fed	3	26.8	23.4	22.9	23.3	23.5
Afoxolaner	1.5	Fed	3	0.4 (98.4%)	0.4 (98.1%)	0.3 (98.9%)	0.0 (100%)	0.3 (98.9%)
	2.5	Fed	3	0.3 (99.0%)	0.3 (98.9%)	0.0 (100%)	0.0 (100%)	0.0 (100%)
	2.5	Fasted	3	0.3 (99.0%)	0.3 (98.9%)	0.0 (100%)	0.3 (98.9%)	0.0 (100%)
	3.5	Fed	3	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.3 (98.9%)	0.8 (96.5%)

[a] Geometric mean is computed by subtracting 1 from the anti-logarithm of the mean of ln(count + 1).
[b] Percent efficacy = 100(1 − *T*/*C*), where *C* is GM of vehicle and *T* is GM of each treated group.

Table 6
Geometric means and efficacies of 5 repeated monthly dosages of afoxolaner, 2.5 mg/kg, administered to dogs in an oral solution against fleas (*Ctenocephalides felis*).

Treatment	Dosage (mg/kg)	*n*	Geometric mean[a] (% efficacy[b]) for Dose 1				
			Day 0	Day 7	Day 14	Day 21	Day 26
Vehicle	0	6	61.9	58.4	63.4	58.3	60.9
Afoxolaner	2.5	6	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.3 (99.6%)

Treatment	Dosage (mg/kg)	*n*	Geometric mean (% efficacy) for Dose 2				
			Day 0	Day 7	Day 14	Day 21	Day 26
Vehicle	0	6	62.8	66.3	65.0	58.5	73.9
Afoxolaner	2.5	6	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)

Treatment	Dosage (mg/kg)	*n*	Geometric mean (% efficacy) for Dose 3				
			Day 0	Day 7	Day 14	Day 21	Day 26
Vehicle	0	6	64.2	63.0	53.5	62.9	71.2
Afoxolaner	2.5	6	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.1 (99.8%)

Treatment	Dosage (mg/kg)	*n*	Geometric mean (% efficacy) for Dose 4				
			Day 0	Day 7	Day 14	Day 21	Day 26
Vehicle	0	6	72.9	51.2	70.1	79.0	77.1
Afoxolaner	2.5	6	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.4 (99.5%)

Treatment	Dosage (mg/kg)	*n*	Geometric mean (% efficacy) for Dose 5					
			Day 0	Day 7	Day 14	Day 21	Day 26	Day 30
Vehicle	0	6	73.8	73.6	73.4	72.5	69.0	69.9
Afoxolaner	2.5	6	0.0 (100%)	0.1 (99.8%)	0.0 (100%)	0.0 (100%)	0.2 (99.7%)	0.0 (100%)

[a] Geometric mean is computed by subtracting 1 from the anti-logarithm of the mean of ln(count + 1).
[b] Percent efficacy = $100(1 - T/C)$, where C is GM of vehicle and T is GM of treated group.

Shot® Top 10 competent *Escherichia coli* (Invitrogen) and cDNA purified using Plasmid Maxi Kit (Qiagen). ^{wt}Rdl cDNA was linearized with the restriction endonuclease, NotI and cRNA synthesized with SP6 RNA polymerase. ^{A302S}Rdl cRNA was synthesized with T7 RNA polymerase. The cDNA was not linearized as there is a T7 RNA polymerase termination sequence 3′ to the Rdl insert.

X. laevis oocytes were isolated from ovaries (purchased from Nasco) and defoliculated using 2 mg/ml collagenase (Type 1A, Sigma) in standard oocyte saline (SOS) having the following composition (mM): NaCl 100.0, KCl 2.0, $CaCl_2$ 1.8, $MgCl_2$ 1.0, HEPES 5.0, pH 7.6. Oocytes at growth stage V or VI were selected for injection with 20 ng of cRNA encoding for either ^{wt}Rdl or ^{A302S}Rdl using a micro-injector (Nanoject II; Drummond Scientific). Following injection, the oocytes were incubated at 18 °C in sterile SOS supplemented with 50 μg/ml gentamycin sulfate, 100 units/ml penicillin, 100 μg/ml streptomycin and 2.5 mM sodium pyruvate.

For electrophysiology studies, oocytes were secured in a Perspex chamber (RC-3Z Warner Instruments). Oocytes were impaled with KCl-filled (3 M) microelectrodes having resistance values of 0.5–1.5 MΩ (current passing) and 1–5 MΩ (recording). Membrane currents were recorded under two-electrode voltage-clamp mode with a holding potential of −60 mV using an Axoclamp 2B amplifier (Molecular Devices) with signal acquisition and processing using pClamp software (Molecular Devices). Solutions were bath perfused at a rate of 3–5 ml/min with GABA being applied at 2 min intervals. DMSO concentrations for test solutions did not exceed 0.1%.

2.2.9. Study 8 – cross-resistance studies

To evaluate whether there was potential for cross-resistance with cyclodienes, afoxolaner was evaluated in a contact toxicity study using wild type (Canton-S) and cyclodiene-resistant (Rdl) strains of *Drosophila* with dieldrin included for comparison. Both strains of *Drosophila* were obtained from Bloomington *Drosophila* Stock Center (Indiana University). Afoxolaner and dieldrin were dissolved in acetone and a 150 μl volume of test solution was dispensed into 12 ml glass vials. The vials were rotated on a carousel to evenly distribute afoxolaner and dieldrin while the acetone evaporated. Ten adult female *Drosophila* (less than 2 weeks post-emergence), were transferred into each test vial which was then sealed with a saturated cotton wick (10% sucrose). Mortality (moribund individuals were counted as dead) was measured at 72 h. A minimum of 3 replicates was obtained for each concentration along with an acetone control.

3. Results

3.1. In vitro study 1

A dosage and time titration effect was clearly identified for fleas ingesting afoxolaner with mean efficacies of >95% recorded for fleas fed blood containing the compound at

concentrations of 0.16, 0.08 and 0.02 μg/ml at the 24, 48 and 72 h observation points, respectively (Table 1). There was only 1%, 2.3% and 2.3% mean mortality in the vehicle-treated control at the 24, 48 and 72 h observation points, respectively. Therefore, afoxolaner was judged to be highly active against fleas following ingestion in blood.

3.2. Study 2: probe efficacy study in dogs

The percent reduction in flea counts in the afoxolaner-treated dog following 6 weekly flea challenges was 100% (Table 2). Percent reduction in tick counts in the afoxolaner-treated dog, following the first 5 tick challenges on Days 2, 7, 14, 21 and 28, was 100%. The effectiveness of the drug declined slightly to 96% on Day 37 and then to 88% on Day 44 (Table 3). No adverse events were noted during this experiment.

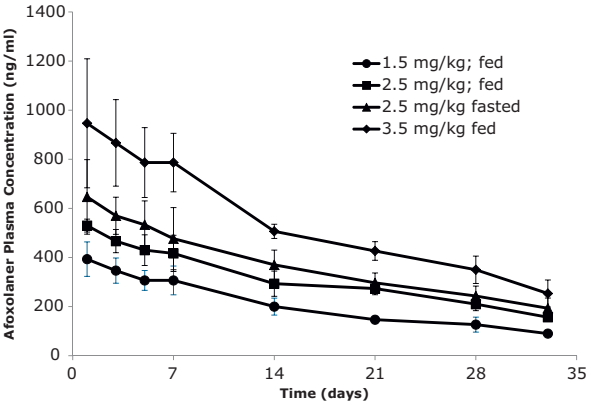

Fig. 2. Mean pharmacokinetic profiles of afoxolaner following administration to fed or fasted dogs in an oral solution (5 dogs/group).

3.3. Study 3: preliminary dosage titration in dogs and assessment of post prandial effect

Mean percent reduction in flea counts for the four afoxolaner treatment groups challenged throughout the study (flea infestations on Days 1, 7, 14, 21, and 28) ranged from 99% to 100% (Table 4). Mean percent reduction in flea counts on day 32 was 100, 99, 100, and 99% for the 1.5 mg/kg fed, 2.5 mg/kg fed, 2.5 mg/kg fasted and 3.5 mg/kg fed groups, respectively (Table 4). Mean percent reduction in tick counts for the four afoxolaner treatment groups challenged at intervals throughout the study (Days 2, 9, 16, 23 and 30) ranged from 97% to 100% (Table 5). Mean percent reduction in tick counts at Day 30 was 99, 100, 100 and 97% for the 1.5 mg/kg fed, 2.5 mg/kg fed, 2.5 mg/kg fasted and 3.5 mg/kg fed groups, respectively (Table 5).

Maximum afoxolaner plasma concentrations were observed at the first blood sampling time on Day 1 of the study (Fig. 2). Plasma concentrations of afoxolaner then decreased over the month but remained above 90 ng/ml on Day 33 for all dosage groups. Afoxolaner plasma concentrations showed dosage proportionally indicating linear

Table 7
Geometric means and efficacies of 5 repeated monthly dosages of afoxolaner, 2.5 mg/kg, administered to dogs in an oral solution against ticks (*Dermacentor variabilis*).

Treatment	Dosage (mg/kg)	*n*	Geometric mean[a] (% efficacy[b]) for Dose 1				
			Day 2	Day 9	Day 16	Day 23	Day 28
Vehicle	0	6	15.7	37.9	26.1	28.1	27.3
Afoxolaner	2.5	6	2.6 (83.5%)	0.1 (99.7%)	0.0 (100%)	0.0 (100%)	0.0 (100%)
Treatment	Dosage (mg/kg)	*n*	Geometric mean (% efficacy) for Dose 2				
			Day 2	Day 9	Day 16	Day 23	Day 28
Vehicle	0	6	29.7	22.6	25.2	24.7	18.4
Afoxolaner	2.5	6	0.0 (100%)	0.4 (98.2%)	0.6 (97.7%)	1.1 (95.5%)	0.2 (98.9%)
Treatment	Dosage (mg/kg)	*n*	Geometric mean (% efficacy) for Dose 3				
			Day 2	Day 9	Day 16	Day 23	Day 28
Vehicle	0	6	16.3	20.9	17.6	22.4	19.7
Afoxolaner	2.5	6	0.3 (98.4%)	0.5 (97.5%)	0.8 (95.6%)	5.2 (76.7%)	3.6 (81.5%)
Treatment	Dosage (mg/kg)	*n*	Geometric mean (% efficacy) for Dose 4				
			Day 2	Day 9	Day 16	Day 23	Day 28
Vehicle	0	6	19.1	21.9	26.0	26.4	19.1
Afoxolaner	2.5	6	0.1 (99.4%)	0.1 (99.4%)	0.0 (100%)	0.1 (99.5%)	0.1 (99.4%)
Treatment	Dosage (mg/kg)	*n*	Geometric mean (% efficacy) for Dose 5				
			Day 2	Day 9	Day 16	Day 23	Day 28
Vehicle	0	6	24.3	26.7	24.2	27.0	27.0
Afoxolaner	2.5	6	0.0 (100%)	0.1 (99.5%)	0.0 (100%)	0.0 (100%)	2.1 (92.3%)

[a] Geometric mean is computed by subtracting 1 from the anti-logarithm of the mean of ln(count + 1).
[b] Percent efficacy = 100(1 − *T*/*C*), where *C* is GM of vehicle and *T* is GM of treated group.

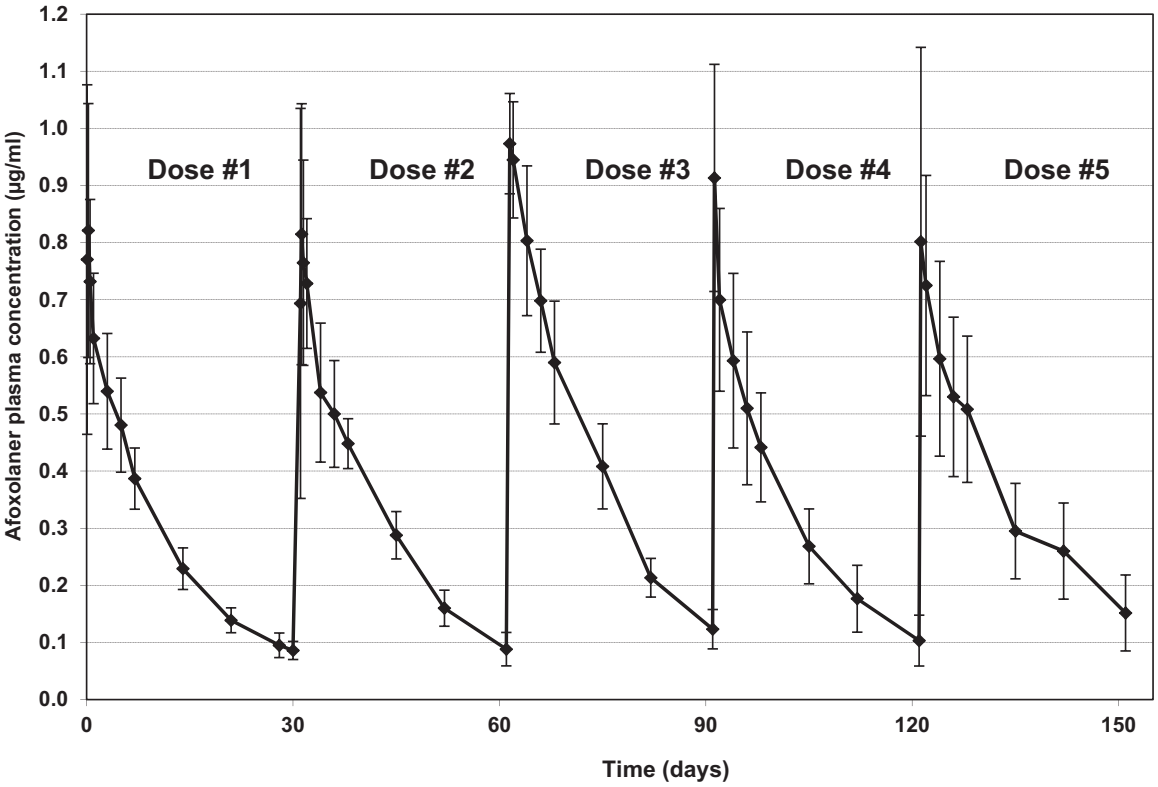

Fig. 3. Mean pharmacokinetic profile of afoxolaner2.5 mg/kg oral dosages in dogs following monthly dosing for 5 months.

kinetics over the range of 1.5–3.5 mg/kg (Fig. 2). There was no statistical difference in the maximum concentrations or overall exposure between dogs fed and fasted prior to treatment. No adverse reaction was noted during the study at any time point on any dog.

With efficacy established in fed as well as fasted dogs, and a strong indication of dosage proportionality, a fourth study was conducted to evaluate the effects of repeated dosing.

3.4. Study 4: assessment of 5 month repeat dosing on efficacy and safety in dogs

Over the five month period, mean effectiveness against fleas in the treated dogs was never less than 99% (Table 6). The first dose of afoxolaner in this test produced 83.5% mean effectiveness against ticks in the treated dogs at Day 2, and increased to 99% by the second week and then to 100% for the remaining two weeks of the first month (Table 7). For the following four months >98% efficacy was recorded against ticks for the first tick challenges in each month and efficacies of 100, 99, 82, 99 and 92% were recorded for the final challenge for months 1 through 5, respectively (Table 7).

For Dose 1 and Dose 2, early blood samples were taken at 2, 6 and 12 h after treatment, for the remaining doses the 2 and 12 h plasma collections were eliminated. The highest plasma concentration of 0.82 μg/ml was measured at the 6 h time point after dose 1 (Fig. 3). Pharmacokinetic profiles for afoxolaner were observed to be predictable and reproducible following multiple dosing (Fig. 3). Mean afoxolaner plasma concentrations at 6 h were 0.82, 0.81, 0.97, 0.91, and 0.80 μg/ml for Doses 1 through 5, respectively. There was no apparent difference in the trough concentrations as mean minimum afoxolaner plasma concentrations (C_{min}) collected at 30 days post-dose were 0.09, 0.09, 0.12, 0.10 and 0.15 μg/ml for Doses 1 through 5, respectively (Fig. 3). These data indicate that steady state had been reached by the 2nd dose. No adverse clinical signs were observed during the study.

3.5. Study 5 – toxicity and symptomology in model insects

A KD_{50} (50% knockdown concentration) value of 0.35 μg per cockroach was determined. At the higher injected dose, symptoms were observed within 10 min, initially appearing as brief periodic wing fluttering which progressed over time until the insects became uncoordinated and had difficulty remaining upright. Once prostrate, cockroaches displayed periodic volleys of leg tremors. The rapid onset and excitatory nature of toxicity suggested involvement of a neuronal target.

3.6. Study 6 – cockroach electrophysiology studies

By doing extracellular recordings on nerve 5 (N5) of the metathoracic ganglion of American cockroaches, under

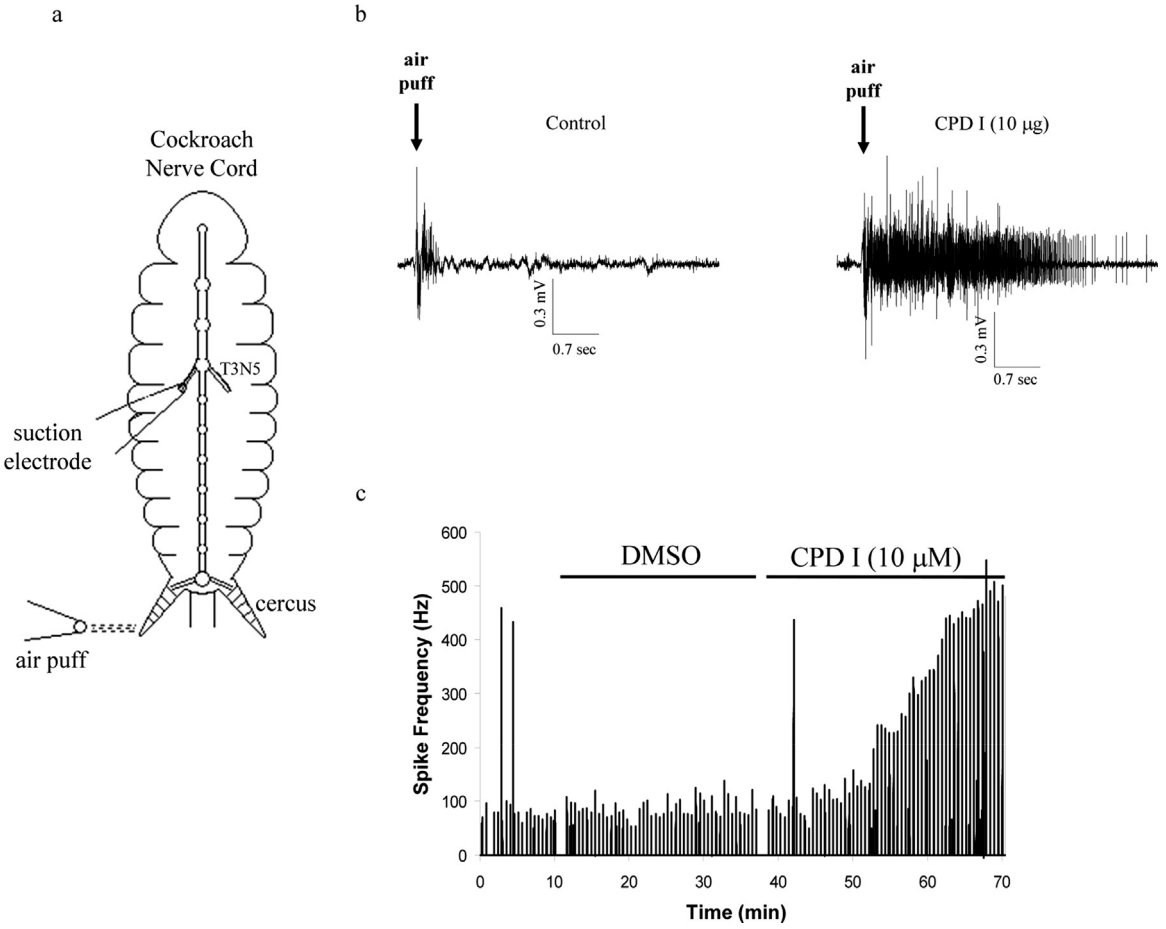

Fig. 4. Evaluation of the isoxazoline, CPD I, in the American cockroach, *P. americana*, cercal-reflex assay. (a) Diagram of the cerci and ventral nerve cord (VNC). Mechanical stimulation of hairs on the cerci produced action potentials which travel up the VNC. Integration of stimulatory and inhibitory input at the thoracic ganglia (T1–T3) determines the degree of nerve activity to the leg muscles. A suction electrode attached to motor nerve, N5, recorded the air puff-induced efferent signals. (b) T3N5 response to a 50 ms air puff before and after injection with CPD I. (c) In the presence of saline or DMSO, air puffs induced action potentials with a spike frequency below 150 Hz. Following injection of 1 μl CPD I (10 μM) a strong increase in the air-puff response is observed.

control conditions, a single air puff to the cerci produced a rapidly adapting volley of action potentials with a spike frequency between 75 and 175 Hz. Injection of CPD I (10 μg) into the body cavity produced no significant effect on spontaneous action potential frequency. However, adaptation of the air puff-induced N5 activity was inhibited by CPD I, resulting in a strong increase of spike frequency (Fig. 4b). Similarly, bath perfusion of CPD I (10 mM) induced a strong increase in the air puff-induced spike frequency indicating increased excitability due to blocking of inhibitory neuronal activity (Fig. 4c). The fact that the spontaneous action potential frequency remained unaffected suggested that action at the neurotransmitter receptors was a more likely target than action at voltage-gated ion channels.

As the neurotransmitters involved in the cercal reflex include both excitatory nicotinic acetylcholine receptors (nAChRs) and inhibitory GABA receptors (GABARs), action of CPD I was investigated on both neurotransmitter receptors. Although no effect was observed on nAChRs (data not shown), the compound potently inhibited GABA-induced currents in American cockroach thoracic neurons. CPD I inhibited GABA-induced currents with an IC_{50} value of 10.8 nM (Fig. 5) with prolonged saline rinse (>15 min) resulting in partial recovery of the GABA response.

3.7. Study 7 – oocyte injection and voltage clamp studies

Application of GABA (100 μM) induced a strong inward current in oocytes expressing the *Drosophila* RDL receptor. Perfusion of afoxolaner produced a dose-dependent inhibition in the GABA response with an IC_{50} value of 3.7 nM as shown in Fig. 6. This inhibition failed to reverse following extended saline washout. In *Drosophila*, resistance to cyclodiene insecticides is associated with a single amino acid substitution of serine for alanine at residue 302 of the *rdl* gene (Ffrench-Constant et al., 2000). *Xenopus* oocytes expressing ^{A302S}RDL were challenged with afoxolaner at 0.1, 1, and 10 nM to compare potency relative to that observed with ^{wt}RDL. As shown in Fig. 7, there was no statistically significant difference observed between ^{wt}RDl and ^{A302S}RDL at any of the concentrations, suggesting that

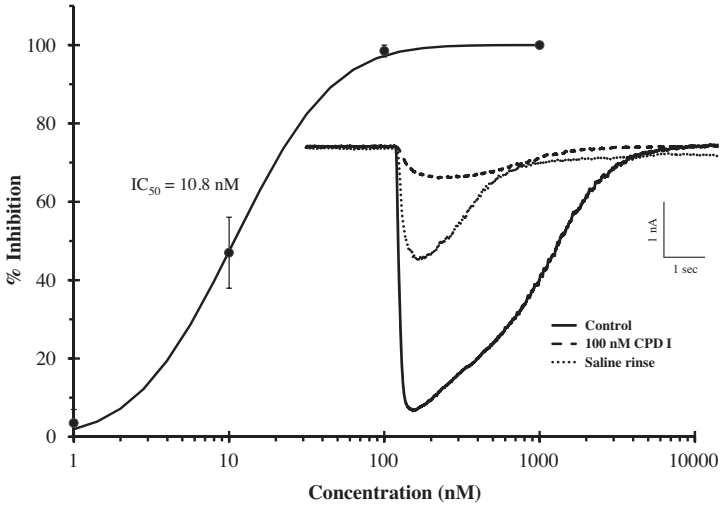

Fig. 5. CPDI inhibits GABA-gated currents in American cockroach, *P. americana*, neurons. Dissociated neurons were clamped at a holding potential of −60 mV and repeatedly stimulated with pulses of 100 μM GABA (inset, solid trace). Perfusion of CPD I inhibited the GABA response (inset, dashed trace) in a dose-dependent manner with an IC_{50} = 10.8 nM. Following prolonged saline rinse a partial recovery of the GABA response (inset, dotted trace) was observed.

no cross-resistance would be expected between isoxazolines and cyclodienes.

3.8. Study 8 – cross-resistance studies

As shown in Fig. 8, afoxolaner was highly potent on Canton-S flies with an LD_{50} value of 0.2 μg/vial (95% confidence interval = 0.1–0.4 μg/vial). Although this insecticide is an order of magnitude less potent against the susceptible strain than dieldrin (LD_{50} value of 0.02), excellent potency was observed against the RDL strain, as predicted by the receptor studies. RDL flies exhibited comparable sensitivity with a resistance ratio value (RR, expressed as RDL LD_{50}/Canton-S LD_{50}) of only two. In contrast, the RDL flies exhibited strong resistance to dieldrin (RR > 5000) consistent with earlier reports (Bloomquist, 1993). Based on the mode of action and differences in receptor interaction, it is unlikely that fleas and ticks carrying the *rdl* gene mutation and thereby resistant to dieldrin will demonstrate cross-resistance to afoxolaner.

4. Discussion

Data generated in these research studies provided evidence of the safety and month-long effectiveness of afoxolaner against fleas and ticks on dogs following oral

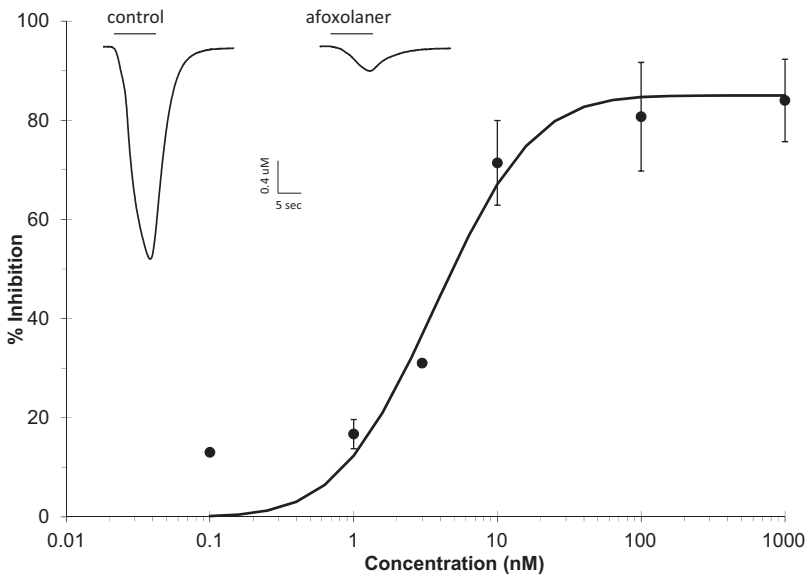

Fig. 6. Inhibitory effect of afoxolaner on expressed ^{wt}Rdl GABA-gated chloride channels. *Xenopus* oocytes expressing the ^{wt}Rdl receptor were clamped at a holding potential of −60 mV and repeatedly stimulated with pulses of 100 μM GABA to induce activation of GABA-gated Cl^- currents (Inset, control). Perfusion of afoxolaner inhibited the GABA response (inset, 100 nM afoxolaner) in a dose-dependent manner with an IC_{50} = 3.7 nM.

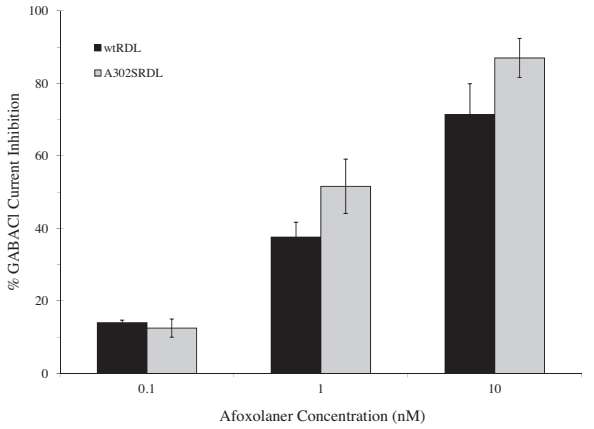

Fig. 7. Inhibitory effect of afoxolaner, on GABA-gated Cl^- currents recorded from *Xenopus* oocytes expressing either ^{wt}Rdl or ^{A302S}Rdl (resistant) receptors. Oocytes were recorded using TEVC with a holding potential of −60 mV.

administration at 2.5 mg/kg. The *in vitro* discovery results showed that afoxolaner was more potent than any other compound ever tested in this membrane feeding system, including the avermectins (Zakson et al., 2001). This *in vitro* assay was not only an important tool for estimation of compound potency in the discovery process, but established a preliminary *in vivo* target of 0.16 μg/ml as a blood level required in a dog for complete flea effectiveness for 30 days. The 0.16 μg/ml level was chosen because it provided 100% control at the 24 h *in vitro* observation and a 24 h *in vivo* challenge was to be used for fleas on dogs. In subsequent work conducted with formulated afoxolaner in dogs, the EC_{90} for fleas was determined to be 0.023 μg/ml (Letendre, 2014).

With strong evidence that blood containing afoxolaner could effectively control fleas, Study 2 was initiated and represented the first time afoxolaner was evaluated in a dog ($n = 1$). That study revealed effectiveness of afoxolaner against both fleas and ticks beyond a month following

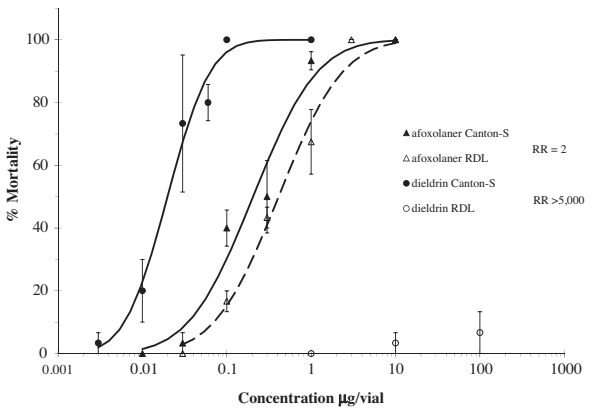

Fig. 8. Contact toxicity bioassay of wild type (Canton-S) and cyclodiene-resistant (Rdl) strains of *Drosophila*. Glass vials were treated with test compounds and mortality measurements taken 72 h after introduction of flies. The resistance ratio (RR) corresponds to the Rdl LD_{50}/Canton-S LD_{50}.

a single 2.5 mg/kg oral administration. In addition to attaining a blood concentration capable of providing flea control at the end of a month, it appeared that this blood level also was fully effective for ticks.

Study 3 provided a dosage titration of afoxolaner in dogs ($n = 3$) using oral administration. Results supported the previously observed flea and tick effectiveness for one month. Moreover, plasma level data and efficacy results indicated that there was no apparent prandial effect on the systemic absorption and effectiveness of afoxolaner. The compound was well absorbed with concentrations above those needed for effectiveness achieved at the first sampling time on the first day of the study and the plasma concentrations remained high enough to support effective flea and tick kill for the entire 33 days of the study. The pharmacokinetic profiles suggested dose proportionality over the range of doses tested, providing further indication of a good safety profile, and no dog showed an adverse event attributable to the drug at any time during this study.

Study 4 was conducted to explore the effects of repeated dosing and dogs ($n = 6$) received 5 monthly doses of afoxolaner in the experimental oral solution. Effectiveness results showed nothing less than 99% against fleas during the 26 challenges. Effectiveness against ticks at the end of each month (immediately before the next monthly dose) was also very good with values of 100, 99, 82, 99 and 92% for months 1 through 5, respectively. The pharmacokinetic profiles observed in this study were remarkably consistent with similar C_{max} and C_{min} for each monthly dose, and minimal accumulation of afoxolaner recorded over repeated dosages.

Veterinary clinical examinations conducted throughout the study showed no indication of adverse effects. The lack of accumulation following multiple dosing, the observed safety, and the sustained effectiveness confirmed the potential for afoxolaner to become a convenient and safe ectoparasiticide for use in dogs.

The mode of action studies demonstrated that afoxolaner and compounds of the isoxazoline class control insects by inhibition of GABA-gated chloride ion channels (Ozoe et al., 2010; Gassel et al., 2014). It can be expected that afoxolaner will act on fleas and ticks in a similar way. GABA-gated chloride ion channels are the target of ivermectin, fipronil, and cyclodienes; however ivermectin binds to a distinct site and activates rather than blocks GABA-gated chloride channels (Garcia-Reynaga et al., 2013; Gassel et al., 2014). It has been well established that replacement of alanine with serine at position 302 of the *rdl* gene confers strong resistance to cyclodienes and moderate resistance to fipronil in some arthropods (Ffrench-Constant et al., 2000; Bloomquist et al., 1992; Bloomquist, 1993). It is particularly noteworthy that no significant difference in afoxolaner sensitivity was observed in *Drosophila* toxicity or receptor studies for wild type *versus* A302S mutants. These findings indicate that afoxolaner binds to the target in a manner distinct from cyclodienes and phenylpyrazoles. Preliminary biochemical studies with ^{3}H-CPD I (unpublished but reported by Cordova et al., 2010) further support a distinct binding site for this chemistry. Consequently, one would expect afoxolaner to exhibit full potency on insects that bear the A302S mutation. Further,

it is unlikely that insects and acari which exhibit resistance to commonly used insecticides will show cross-resistance to afoxolaner given its unique mode of action.

In summary, the discovery studies reported here demonstrated the ability of afoxolaner to control fleas and ticks on dogs for more than a month when administered orally at the relatively low dose of 2.5 mg/kg. The predictable pharmacokinetic profiles of the compound, the absence of health abnormalities in treated dogs, together with the remarkable efficacy profile, and well-elucidated mode of action made afoxolaner the isoxazoline of choice for further development.

Conflict of interest statement

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