



Discovery of sarolaner: A novel, orally administered, broad-spectrum, isoxazoline ectoparasiticide for dogs



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ABSTRACT

The novel isoxazoline ectoparasiticide, sarolaner, was identified during a lead optimization program for an orally-active compound with efficacy against fleas and ticks on dogs. The aim of the discovery program was to identify a novel isoxazoline specifically for use in companion animals, beginning with *de novo* synthesis in the Zoetis research laboratories. The sarolaner molecule has unique structural features important for its potency and pharmacokinetic (PK) properties, including spiroazetidone and sulfone moieties. The flea and tick activity resides in the chirally pure *S*-enantiomer, which was purified to alleviate potential off-target effects from the inactive enantiomer. The mechanism of action was established in electrophysiology assays using CHO-K1 cell lines stably expressing cat flea (*Ctenocephalides felis*) RDL (resistance-to-dieldrin) genes for assessment of GABA-gated chloride channel (GABACs) pharmacology. As expected, sarolaner inhibited GABA-elicited currents at both susceptible (CfRDL-A285) and resistant (CfRDL-S285) flea GABACs with similar potency. Initial whole organism screening was conducted *in vitro* using a blood feeding assay against *C. felis*. Compounds which demonstrated robust activity in the flea feed assay were subsequently tested in an *in vitro* ingestion assay against the soft tick, *Ornithodoros turicata*. Efficacious compounds which were confirmed safe in rodents at doses up to 30 mg/kg were progressed to safety, PK and efficacy studies in dogs. *In vitro* sarolaner demonstrated an LC₅₀ of 0.3 µg/mL against *C. felis* and an LC₁₀₀ of 0.003 µg/mL against *O. turicata*. In a head-to-head comparative *in vitro* assay with both afoxolaner and fluralaner, sarolaner demonstrated superior flea and tick potency. In exploratory safety studies in dogs, sarolaner demonstrated safety in dogs ≥ 8 weeks of age upon repeated monthly dosing at up to 20 mg/kg. Sarolaner was rapidly and well absorbed following oral dosing. Time to maximum plasma concentration occurred within the first day post-dose. Bioavailability for sarolaner was calculated at >85% and the compound was highly protein bound (>99%). The half-life for sarolaner was calculated at 11–12 days. Sarolaner plasma concentrations indicated dose proportionality over the range 1.25–5 mg/kg, and these same doses provided robust efficacy (>99%) for ≥35 days against both fleas (*C. felis*) and multiple species of ticks (*Rhipicephalus sanguineus*, *Ixodes ricinus* and *Dermacentor reticulatus*) after oral administration to dogs. As a result of these exploratory investigations, sarolaner was progressed for development as an oral monthly dose for treatment and control of fleas and ticks on dogs.

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1. Introduction

In spite of an abundance of parasiticides available to protect dogs and cats from ectoparasites, the market for novel, more effective and more convenient products continue to grow. Isoxazolines are a potent new class of ectoparasiticide for companion animals.

These compounds have demonstrated remarkable activity against the most common parasites of dogs, fleas and ticks, via the oral route of administration. This has been a major breakthrough in parasite control, particularly for ticks, as the best previous acaricides have been topically administered products. This new isoxazoline class of parasiticide also has advantages over the first generation of oral parasiticides available to veterinarians, which had limited ability to effectively kill both fleas and ticks on dogs (Beugnet and Franc, 2012). Fast and consistent efficacy for the full dosing period against fleas and multiple species of ticks is an important attribute

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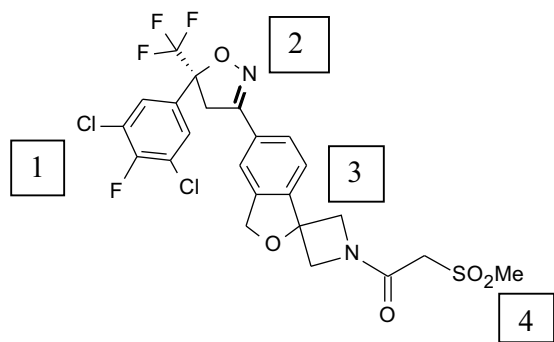


Fig. 1. Structure of sarolaner: 1) phenyl head group, 2) isoxazoline core, 3) spiroazetidibenzofuran moiety and 4) methylsulfonyl ethanone tail.

of effective ectoparasiticides, both as a means to improve basic parasite control and to reduce the potential for pathogen transmission. Products with a faster onset of action will help achieve both these aims.

The mechanism of action (MOA) of the isoxazoline class of compounds is well documented. Previous reports from the literature have demonstrated that the isoxazolines exhibit antiparasitic activity through specific blockade of insect GABA- and glutamate-gated chloride channels (Garcia-Reynaga et al., 2013; Gassel et al., 2014; Ozoe et al., 2010). The *in vivo* efficacy of several of these compounds (afoxolaner and fluralaner) have been previously described in numerous publications (Hunter et al., 2014; Rohdich et al., 2014; Shoop et al., 2014; Wengenmayer et al., 2014).

This paper describes the compound attributes, *in vitro* screening strategy, initial *in vivo* ectoparasiticidal activity, pharmacology and mechanism of action (MOA) and basic pharmacokinetic (PK) profile of the novel isoxazoline, sarolaner. Sarolaner was designed in the research laboratories at Zoetis and is the product of an intensive effort to discover and develop a novel isoxazoline specifically for use in companion animals.

2. Materials and methods

2.1. Compound background and details

Sarolaner (1-(5'-((5S)-5-(3,5-dichloro-4-fluorophenyl)-5-(trifluoromethyl)-4,5-dihydroisoxazol-3-yl)-3'-H-spiro(azetidine-3,1'-(2) benzofuran)-1-yl)-2-(methylsulfonyl) ethanone) was discovered during a 2-year lead optimization program. Over 3000 isoxazoline compounds were prepared and tested to build an understanding of the structure activity relationships of the isoxazoline class that led to the selection of sarolaner as the best molecule for advancement as a candidate for product development.

The molecule's structure can be described as four connected sub-units; a substituted phenyl ring head group, the isoxazoline core, a spiroazetidibenzofuran moiety and a methylsulfonyl ethanone tail (Fig. 1). The molecule was optimized for broad-spectrum ectoparasiticidal potency, pharmacokinetics and safety in the dog as follows. The addition of a 4-substituted fluorine to a 3,5-dichlorophenyl head unit provided superior tick potency compared to any 4-hydroxy-3,5-substituted patterns. The isoxazoline was prepared as a single *S*-enantiomer due to all of the activity residing in this chiral conformation; the *R*-enantiomer provided no potency against fleas or ticks. Mindful of enhancing safety in canine patients, selecting the chirally pure form minimized potential off-target effects that could result from the incorporation of the inactive enantiomer. The spiroazetidibenzofuran moiety is a unique structure, previously undescribed in the parasitology literature, providing rigidity, potency and novelty to the molecule. The final piece of optimization, yielded the methylsulfonyl ethanone tail, increasing the polar surface area of the molecule and maximizing the pharmacokinetic exposure to target the rapid kill of fleas and ticks.

Afoxolaner and fluralaner, which were originally both discovered in the agrochemical field, are presented below for reference (Fig. 2).

2.2. Evaluation strategy

Preliminary efficacy screening was conducted *in vitro* against the cat flea, *Ctenocephalides felis felis*, with active hits subsequently screened against the soft tick, *Ornithodoros turicata*. Once initial efficacy had been profiled *in vitro*, the safety of selected compounds was assessed in a mouse symptomatology model and those compounds with an acceptable rodent safety profile were progressed to target animal toleration, pharmacokinetics and efficacy studies in the dog.

For all studies involving animals reported in this paper, animals were handled with due regard to their welfare and all protocols and procedures were reviewed by appropriate welfare authorities and applicable procedures were conducted according to state and national/international regulations.

2.3. *In vitro* screening—fleas (*Ctenocephalides felis*)

Fleas were reared using an artificial dog apparatus to provide regular supplies of an established laboratory strain of fleas (*C. felis felis*) not more than 48 h post-emergence. Approximately 30 adult fleas (mixed sex) were collected and aspirated into the feeding chambers (modified 50 mL centrifuge tube with a 300 mm mesh top) and held at 25° C and 75% relative humidity (RH) until use. Test compounds were made up as stock solutions in 0.5% dimethyl

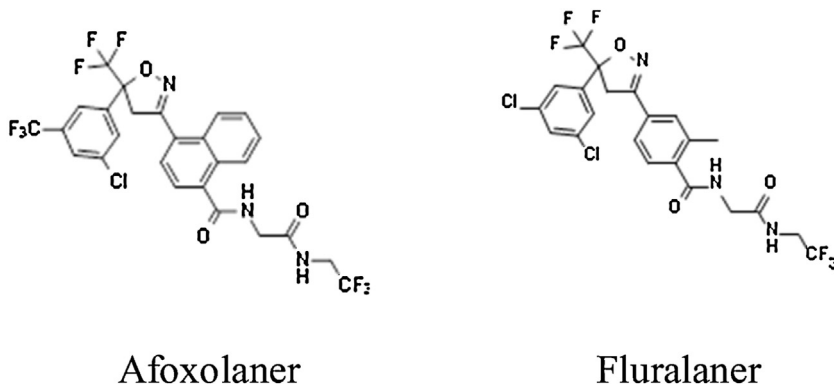


Fig. 2. Structures of other isoxazolines.

sulphoxide (DMSO). Membrane feeding was carried out using a flat-bed incubator apparatus maintained at 37°C. Test solutions were added to 5 mL of citrated bovine blood in a 50 mm diameter petri dish and sealed with a thin Nesco/Parafilm membrane. Control fleas were fed on blood containing 0.5% (v/v) DMSO. Tubes with fleas were placed in contact with membranes to allow fleas to feed through the mesh top. Fleas were allowed to feed for 2 h. On removal from the feeding apparatus, fleas were maintained at 25°C and 75% RH.

Efficacy recordings (a subjective visual assessment of flea viability based on motility) were made at 2 and 24 h after initiation of feeding. Screening assays were conducted in duplicate and comparative assays in triplicate. Each compound was tested at half-log intervals, and endpoint data was recorded as LC₈₀ (the lowest concentration to cause ≥ 80% mortality) in µg/mL.

2.4. In vitro screening—soft ticks (*O. turicata*)

Compounds demonstrating activity in the primary *in vitro* flea screens were then evaluated in an *in vitro* soft tick screen that used a system similar to that described above for testing compounds against fleas. Five individual nymphal (N3–N4) *O. turicata* were placed on top of the membrane covering a 50 mm petri dish containing blood with test compound, and ticks were allowed to feed to repletion (~5–15 min). Assays were conducted in duplicate for initial screening and in triplicate for comparative assays. Ticks were removed and placed in a separate container with sand and incubated for 72 h at 25°C and 75% RH. Ticks were observed at 24 and 72 h for paralysis/mortality. Endpoint results for subjective visual assessment of organism viability were recorded as EC₅₀ (the lowest concentration to cause ≥ 50% morbidity/mortality) and LC₁₀₀ (the lowest concentration to cause 100% mortality) in µg/mL.

2.5. Safety screening

2.5.1. Mouse symptomatology

Compounds that met *in vitro* efficacy potency targets were selected for initial toxicity screening in mice. Symptomatology was evaluated in mice (n = 3 per compound), administering compounds by oral gavage using aqueous micelle formulations. Visual observations for adverse effects were made at 0, 0.25, 0.5, 1, 2, 4 and 6 h post-dose. Observations included, but were not limited to, body posture, hyper/hypo activity, grip strength, convulsions and ptosis. Compounds were dosed up to 30 mg/kg body weight. Compounds that passed safety screening in mice were progressed to initial safety screening in the dog.

2.5.2. Target animal safety testing

Study 1: sarolaner was tested in adult dogs in an exploratory margin of safety (MOS) repeat-dose study. Twenty-four adult Beagle dogs (12 males and 12 females) were allocated randomly to four treatments of six dogs each: placebo or sarolaner at 2, 6 or 10 mg/kg. Treatments were blocked by pen location and day of study completion. This study had a split-plot design, with sex as the whole plot factor and treatment as the sub-plot factor. The compound was formulated in capsules with excipients (microcrystalline cellulose, sodium starch glycolate, sodium lauryl sulfate and magnesium stearate) and delivered orally. Each treatment group was dosed three times at 28-day intervals. Dogs were fed an appropriate ration of a commercial dry canine feed for the duration of the study. Water was available *ad libitum*.

Observations included body weight, serum chemistry (including serum thyroxine, T4), hematology, coagulation, urinalysis, and a qualitative estimate of food intake. Directed clinical and neurologic examinations were conducted by a veterinarian. Blood samples were collected and analyzed for pharmacokinetic assessment. A

full post-mortem examination was conducted one week after the third dose. This included macroscopic examination and collection of selected organs. Microscopic evaluation was conducted by a board-certified veterinary pathologist.

Study 2: an exploratory repeat dose tolerance study with sarolaner in young dogs was conducted. Thirty-two 8-week-old intact male and female beagle dogs were allocated randomly to four treatments of 8 dogs each: placebo or sarolaner at 4, 12 or 20 mg/kg. Treatments were blocked by pen and day of study completion. Dogs were pair housed because of their young age. This study had a split-plot design with sex as the whole plot factor and treatment as the sub-plot factor. Each dog was dosed twice at a 28-day interval, followed by a complete post-mortem examination one week after the second dose. The compound was formulated and delivered as in Study 1. Similar end point observations to those in Study 1 were made for the dogs in Study 2. Dogs were fed an appropriate ration of a commercial dry canine feed for the duration of the study. Water was available *ad libitum*.

2.6. Pharmacokinetics

The pharmacokinetics of sarolaner were assessed in a parallel design bioavailability study.

2.6.1. Animals

Purpose-bred Beagle dogs (male and female), at least 6 months of age and weighing at least 10 kg were used in the study. Each animal was uniquely identified and was in good health at treatment. Dogs were housed individually in accordance with accepted laboratory animal care and use guidelines, and dogs were allowed regular opportunity for exercise and social interaction. Dogs were fed an appropriate ration of a commercial dry canine feed for the duration of the study. Water was available *ad libitum*.

2.6.2. Experimental design and methods

Dogs were acclimated to the study conditions for at least 15 days prior to treatment. The dogs were observed for general health twice daily throughout the study. A physical exam was performed on each dog by a veterinarian to determine health and suitability prior to inclusion in the trial. Animals were fasted overnight prior to dose administration, and fed at 4 h post dose. Dogs were randomly allocated to 2 treatments of 12 dogs each (6 male/6 female). On Study Day 0, dogs were administered study treatments: sarolaner was dosed either as an intravenous solution, or as a compressed tablet for oral administration. Intravenous doses were administered at 2 mg/kg and oral doses were administered at 20 mg/dog. Pharmacokinetic data was dose normalized to 2 mg/kg, based on the Day 0 body weights. Blood samples for pharmacokinetic analysis were collected from all animals prior to dosing, and at multiple time points post dose, through Day 56.

2.7. Pharmacology and mechanism of action (MOA)

Chinese Hamster Ovary (CHO-K1) cells stably expressing flea (*C. felis*) RDL (resistance-to-dieldrin) genes were generated to assess GABA-gated chloride (GABA_A) pharmacology of sarolaner. RDL subunits of interest (including the alanine-to-serine dieldrin resistance mutation) were codon optimized for CHO-K1 expression by GeneART-Invitrogen (Carlsbad, CA) and subcloned into the mammalian expression vector pcDNA3.1(+) using introduced BamHI and NotI restriction sites. The resulting constructs (CfRDL-A285, CfRDL-S285) were introduced into CHO-K1 cells using FuGene HD transfection reagent (Promega). CHO cells were cultured in Dulbecco's modified Eagle's medium + Glutamax (Invitrogen) supplemented with 10% dialyzed fetal bovine serum (dFBS), 10 mM

HEPES, 1X Anti–Anti (Invitrogen) at 37 °C and 5% CO₂. Transfected cell lines were selected by the addition of 500 µg/mL G418. Resistant colonies were apparent within 2 weeks of selection and picked to individual wells of 24-well plates for assessment of function. Growth media was HAMS-F12 supplemented with GlutaMAX, 10% Fetal Bovine Serum, 1% Non-Essential Amino Acids, 1% Penicillin/Streptomycin along with G418 (800 µg/mL) and hygromycin B (300 µg/mL) selection. Cells were maintained in a humidified incubator (37 °C/5% CO₂) in T225 flasks and expanded to 80–90% confluence prior to the experiment. For electrophysiology experiments, a cell suspension of 3–4 × 10⁶ cells/mL was prepared immediately prior to the studies.

Electrophysiology studies were conducted using the IonWorks platform. Basic principles and operation of the IonWorks platform have been described by Schroeder et al. (2003). The external solution used in the experiments was a HEPES-buffered saline solution. The composition of extracellular solution was (in mM): 137 NaCl, 4 KCl, 1.8CaCl₂, 1.0 MgCl₂, 10HEPES, pH adjusted to 7.4 with NaOH. The internal solution used for the experiment was (in mM): 100 KGlucuronate, 40 KCl, 3.2 MgCl₂, 3.2 EGTA and 5HEPES; pH adjusted to 7.25–7.30 with KOH. The antibiotic perforating agent used in this experiment was amphotericin B added to the internal solution at a concentration of 120 µg/mL. All salts and antibiotic perforating agents were obtained from Sigma Aldrich, except KGlucuronate that was purchased from VWR. All recordings were made at room temperature.

γ-Aminobutyric acid (GABA, A2129 Sigma Aldrich) was dissolved in water at a concentration of 100 mM and diluted in external solution to a concentration of 1 mM for agonist concentration-response experiments and 300 µM for antagonist experiments. An eight point, 3-fold serial dilution series was prepared from the 1 mM stock solution to a final low assay concentration of 450 nM to evaluate agonist potency. An eight-point concentration series was constructed for each compound (n = 8 wells/test concentration) with a 3-fold dilution scheme. External solution (99.5 µL) was added to each well of the plate resulting in an eight-point concentration-response series with a top final assay concentration of 10 µM and a low final assay concentration of 4.6 nM. In antagonist evaluations, test compounds were also included in the second addition along with EC₈₀ of GABA.

After achieving voltage-clamp control, all cells were held at a potential of –80 mV for the duration of the experiment and sampled at a frequency of 2–10 kHz. Fluidic additions were made simultaneously in all 384 wells using the integrated 384 well pipettor on the IonWorks Barracuda. Each concentration of test compound was added to 4–8 wells in each experiment for subsequent analysis. For agonist effects, test compounds were added at a volume of 15 µL at 10 µL/s. Recordings were sampled for a period of 1 s prior to compound addition and 15 s following compound addition. For antagonist effects, test compounds were added and voltage clamp recordings were sampled as described above. Five minutes after test compound addition, agonist was added at approximately EC₈₀ concentration for evaluation of inhibitory activity of the test compound. Recordings were sampled for a period of 1 s prior to agonist addition and 10 s following agonist addition.

Voltage-clamp recordings from each of the 384 wells of the PatchPlate were evaluated for seal resistance, current amplitude and stability. Data from wells with seal resistances less than 20 MΩ during the course of the experiment or with unstable baseline currents prior to or following fluidic addition (assessed by visual inspection of voltage-clamp traces) were eliminated from the data analysis.

GABA produced concentration-dependent inward Cl[–] currents under the assay conditions. For wells passing the criterion above, peak inward current was determined by subtracting baseline current (average currents for a 50 ms window 100 ms before

compound addition) from maximum inward currents from a window for 4 s following compound addition. Concentration-response data for peak inward current were fit using non-linear regression analysis to determine EC₅₀ and EC₈₀ concentrations using a sigmoidal dose-response equation (GraphPad version 5). The approximate EC₈₀ concentration of agonist was used in subsequent experiments to evaluate test compound antagonist activity.

Percent inhibition of GABA-induced inward current was calculated for each concentration of test compound by comparing with in-plate agonist controls by the following equation:

$$(\text{AgonistEC}_{80}\text{average} - \text{TestCompound}) / (\text{AgonistEC}_{80}\text{average}) \times 100$$

Eight-point concentration-response curves were constructed from pooled data and were fit using non-linear regression analysis (GraphPad version 5) to determine IC₅₀ concentrations using a sigmoidal concentration-response relationship. For most curve fits, maximum block was constrained to 100% and minimum constrained to 0%.

2.8. Efficacy evaluation in dogs

The *in vivo* efficacy against fleas and ticks was profiled in two studies in dogs. The studies were conducted in accordance with the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for evaluating the efficacy of parasiticides for the treatment, prevention and control of flea and tick infestation on dogs and cats (Marchiondo et al., 2013). Masking was assured through the separation of functions. All personnel conducting observations or animal care, or performing flea infestations and counts were masked to treatment allocation.

2.8.1. Animals

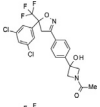
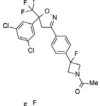
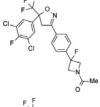
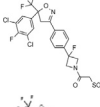
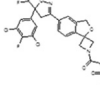
Adult (male and female), purpose-bred Beagles at least 8 months of age and weighing greater than 20 kg were used in both studies. Each dog was individually identified, had not been treated with an ectoparasiticide for at least 60 days and was in good health at treatment. Dogs were housed individually in indoor runs. Dogs were fed an appropriate maintenance ration of a commercial dry canine feed for the duration of the study. Water was available *ad libitum*.

2.8.2. Experimental design and methods

General methods. Day 0 for each study was the day dogs were administered the study treatment. Dogs were acclimated to the study conditions for at least 14 days prior to treatment. The dogs were observed for general health at least once daily throughout the studies. A physical exam was performed on each dog by a veterinarian to determine health and suitability prior to inclusion in the trial. For infestations in Study 1, approximately 100 cat fleas (*C. felis felis*, 1:1 sex ratio) or approximately 50 ticks (~1:1 sex ratio) of each species to be assessed were applied directly to a site proximal or adjacent to the shoulder blades and allowed to crawl into the dog's coat. In Study 2, dogs were sedated prior to tick infestation, to reduce animal movement and enhance tick attachment. Forty-eight hours after treatment and after each tick infestation and/or 24 h after each flea infestation, the dogs were examined and combed to remove and count fleas and ticks. All dogs were first examined visually, and any ticks detected were removed using forceps. Ticks were examined to determine their viability. Any tick able to move in a coordinated manner was considered live. The dogs were then thoroughly flea combed to count and remove fleas and any remaining ticks. Commercial fine-toothed flea combs were used. Dogs were systematically combed initially while standing starting from the head, then proceeding caudally along the dorsum; then the sides and ventral aspects were combed. Dogs were repeatedly combed until no fleas were recovered for about 5 min. Each animal was examined for a minimum of 10 min. Fleas able to stand upright

Table 1

Efficacy of a representative sample of novel isoxazolines in *in vitro* screening assays with compounds fed in blood to fleas (*Ctenocephalides felis felis*) and soft ticks (*Ornithodoros turicata*) (n = 2).

Compound ID	Chemistry	Structure	Flea Feed (<i>C. felis</i>) LC ₈₀ (µg/mL)	Soft Tick Feed (<i>O. turicata</i>) LC ₁₀₀ (µg/mL)
PF-06279149	Racemic		>3	N.T.
PF-06272420	Racemic		3	0.1
PF-06279093	Racemic		1	0.1
PF-06377581	Racemic		1	0.1
PF-06450567 (Sarolaner)	S-enantiomer		0.3	0.003

N.T. = not tested.

Table 2

Comparative *in vitro* efficacy of isoxazolines in *in vitro* assays with compounds fed in blood to fleas (*Ctenocephalides felis felis*) and soft ticks (*Ornithodoros turicata*) (n = 3).

Compound ID	Chemistry	Flea Feed (<i>C. felis</i>) LC ₈₀ (µg/mL)	Soft Tick Feed (<i>O. turicata</i>) LC ₁₀₀ (µg/mL)
Afoxolaner	Racemic	1	0.1
Fluralaner	Racemic	1	0.1
Sarolaner	S-Enantiomer	0.1	0.03

and/or move in a coordinated manner were considered live. Protective gloves and clothing were changed between dogs. Separate flea combs were used for each animal.

Study 1. On Day-7, each dog was infested with *Rhipicephalus sanguineus*. On Day-5, the ticks on each dog were counted, dogs were ranked by descending tick count into blocks, and randomly allocated within blocks to two treatment groups of eight dogs each. On Day-2, the dogs were weighed and infested with *R. sanguineus* and *Dermacentor reticulatus* ticks. On Day-1, the dogs were infested with fleas. On Day 0, each animal was treated via oral gavage at 0.5 mL/kg body weight with either placebo control or sarolaner solution (5 mg/mL) to provide a dose of 2.5 mg/kg. A tetraglycol/solutol solution base was used to formulate the sarolaner. Dogs were observed for general health and any reaction to treatment approximately 1, 3 and 6 h after treatment on Day 0, then once daily for the remainder of the study. On Day 2, each dog was examined and combed to count and remove fleas and ticks. Subsequently, all animals were infested with *R. sanguineus* and *D. reticulatus* on Days 5, 12, 19, 25 and 33, and with fleas on Days 6, 13, 20, 26 and 34. All dogs were examined, combed and parasite counted on Days 7, 14, 21, 27 and 35.

Study 2. On Day-9, each dog was infested with *Ixodes ricinus*. On Day-7, the ticks on each dog were counted, the dogs ranked by descending tick count into eight blocks of four, and randomly allocated within blocks to four treatment groups of eight dogs each. On Day-2, the dogs were weighed and infested with *I. ricinus* ticks. On Day 0, each animal was treated via oral gavage at 0.5 mL/kg body weight with either placebo control, sarolaner suspension (10 mg/mL) to provide a dose of 5 mg/kg, sarolaner suspension (5 mg/mL) to provide a dose of 2.5 mg/kg or sarolaner suspension (2.5 mg/mL) to provide a dose of 1.25 mg/kg. The suspensions were formulated in a carboxymethylcellulose/Tween-80 base with water. Dogs were observed for general health and any reaction to

treatment approximately 1, 3 and 6 h after treatment on Day 0, then once daily for the remainder of the study. On Day 2, each dog was examined and combed to remove and count ticks (as described above). Subsequently, all animals were infested with *I. ricinus* on Days 5, 12, 19, 26, 33 and 55. All dogs were examined and combed and live ticks were counted on Days 7, 14, 21, 28 and 57. Blood samples for pharmacokinetic assessment were collected at least weekly for the duration of the study.

2.8.3. Data analysis

The individual dog was the experimental unit and the primary endpoint was live flea and/or tick count. Flea and tick counts were transformed by the $\log_e(\text{count} + 1)$ transformation prior to analysis in order to stabilize the variance and normalize the data. Using the PROC MIXED procedure (SAS 8.2, Cary NC), transformed counts were analyzed in a mixed linear model for repeated measures. The model included the fixed effect of treatment, day of study and the interaction between treatment and day of study. The random effects included room, block within room, the interaction between block and treatment within room (animal term) and error. In Studies 1 and 2, *a priori* contrasts were used to compare treatment means to the control at the one-sided significance level $\alpha=0.05$. Testing was two-sided at the significance level $\alpha=0.05$. Percent efficacy, relative to the control group and based on arithmetic (and geometric) means, was calculated as follows:

$$\% \text{Efficacy} = \frac{(\text{Mean Control} - \text{Mean Treated})}{\text{Mean Control}} \times 100$$

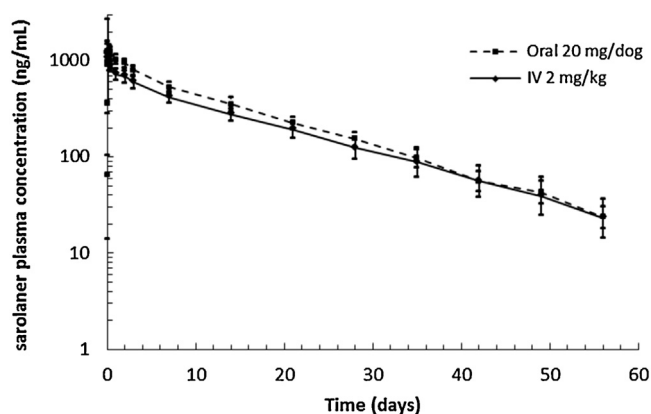


Fig. 3. Least squares (LS) mean plasma concentrations (ng/mL) with 95% confidence intervals, following intravenous or oral administration to dogs.

3. Results

3.1. In vitro screening for flea and ticks

Sarolaner (PF-06450567) was identified as the most potent of the isoxazolines tested in the *in vitro* screening system with a LC_{80} of 0.3 $\mu\text{g/mL}$ for fleas and LC_{100} of 0.003 $\mu\text{g/mL}$ for *O. turicata* soft ticks (Table 1). In addition sarolaner was profiled against other isoxazolines, afoxolaner and fluralaner, in a head-to-head comparison and was shown to be the most potent of the three compounds in these *in vitro* assays with a LC_{80} of 0.1 $\mu\text{g/mL}$ against fleas and a LC_{100} of 0.03 $\mu\text{g/mL}$ against *O. turicata* ticks (Table 2). The minor differences observed between sarolaner potency in these assays are within the biological variability often observed with these assays. The assays were conducted at different times and this underscores the importance of conducting head-to-head assays when comparing relative potencies of compounds *in vitro*.

3.2. Safety screening

3.2.1. Mouse symptomatology

Twenty isoxazoline compounds were assessed in mice as the primary *in vivo* safety screen. Sarolaner did not cause any adverse reactions in mice at screening doses of 10 and 30 mg/kg.

3.2.2. Target animal safety testing

Oral administration of 2, 6 and 10 mg/kg of sarolaner 3 times at 28-day intervals to adult dogs and oral administration of 4, 12 and 20 mg/kg of sarolaner 2 times, at 28-day intervals to 8-week-old dogs was well-tolerated at all dose levels. No adverse reactions associated with treatment were observed for any of the sarolaner-treated dogs. Toxicokinetic evaluation indicated dose proportional systemic exposure for both adult and young dogs. Test article-related changes for clinical pathology, gross pathology, organ weight and histopathology parameters were minimal and not considered adverse or clinically relevant.

3.3. Pharmacokinetics

Following administration of the intravenous dose at 2 mg/kg, the least square (LS)-mean value for volume of distribution (V_{dss}) was 2.81 L/kg bodyweight, and the LS-mean value for clearance (CL) was 0.12 mL/min/kg bod weight. Sarolaner was rapidly and well absorbed following oral dosing at 20 mg/dog (dose range 2.17–3.79 mg/kg) (Fig. 3). Oral pharmacokinetic parameters C_{max} and $AUC_{0-\infty}$ were dose normalized according to the following equation: (PK parameter)/(2 mg/kg)/Actual dose admin-

Table 3

GABACl channel pharmacology: GABA EC_{50} values and antagonist IC_{50} values.

Compound ID	CfRDL-A285	CfRDL-S285
GABA (EC_{50} , μM)	68 \pm 7.8	43 \pm 2.9
Sarolaner (IC_{50} , nM)	135 \pm 33	136 \pm 16
Afoxolaner (IC_{50} , nM)	539 \pm 74	412 \pm 79
Dieldrin (IC_{50} , nM)	6920 \pm 992	>33,000+

Concentration–response curves represent mean \pm standard error (S.E.) of 3–8 experiments.

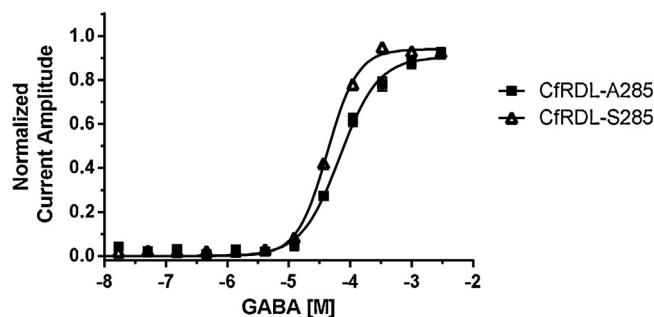


Fig. 4. GABA concentration-responses at homomeric *C. felis* (CfRDL-A285 and CfRDL-S285) GABACls. Concentration-dependent activation of GABACls was measured by voltage-clamp electrophysiology. Concentration–response curves represent mean \pm S.E. (bars) of 3–8 independent experiments.

istered. Bioavailability was determined by the ratio of $AUC_{0-\infty}/\text{Oral dose}$: $AUC_{0-\infty}/\text{IV dose}$. LS mean t_{max} following oral administration to fasted dogs occurred within the first day post dose, with dose-normalized LS mean C_{max} at 1100 ng/mL, and bioavailability calculated at >85%. $T_{1/2}$ for sarolaner was calculated at 11–12 days.

3.4. Mechanism of action (MOA) and pharmacology

In voltage clamp electrophysiology studies with recombinant CHO-K1 cell lines stably expressing RDL subunits from *C. felis*, GABA induced strong inward currents and exhibited agonist responses with EC_{50} of 68 μM and 43 μM at CfRDL-A285 and CfRDL-S285, respectively (Table 3 and Fig. 4).

In functional assays with invertebrate GABACls, sarolaner failed to activate the CfRDL-A285 or CfRDL-S285 as measured by voltage-clamp electrophysiology (data not shown). However, consistent with the published mechanism of action for this chemical class (Gassel et al., 2014; Ozoe et al., 2010; Shoop et al., 2014), sarolaner potently blocked GABA-induced currents with IC_{50} of 135 nM and 136 nM at CfRDL-A285 and CfRDL-S285 receptors, respectively (Table 3 and Fig. 5A and B). For comparative purposes, afoxolaner was evaluated for inhibitory activity and demonstrated 3–4 fold weaker blockade of GABA-induced currents with IC_{50} of 539 nM and 412 nM at CfRDL-A285 and CfRDL-S285 receptors, respectively (Table 3 and Fig. 5A and B).

To confirm the dieldrin resistance phenotype in CfRDL-S285, dieldrin concentration–response curves were examined. Dieldrin blocked GABA-induced currents in CfRDL-A285 with an IC_{50} of 6920 nM, while demonstrating a significant rightward shift in potency at CfRDL-S285 with an IC_{50} of >33,000 nM. (Table 3 and Fig. 5C). These data are consistent with previous literature reports demonstrating dieldrin functionality being sensitive to a single amino acid substitution of alanine to serine at position 285 of the CfRDL (Gassel et al., 2014), and therefore confirmed the resistance phenotype.

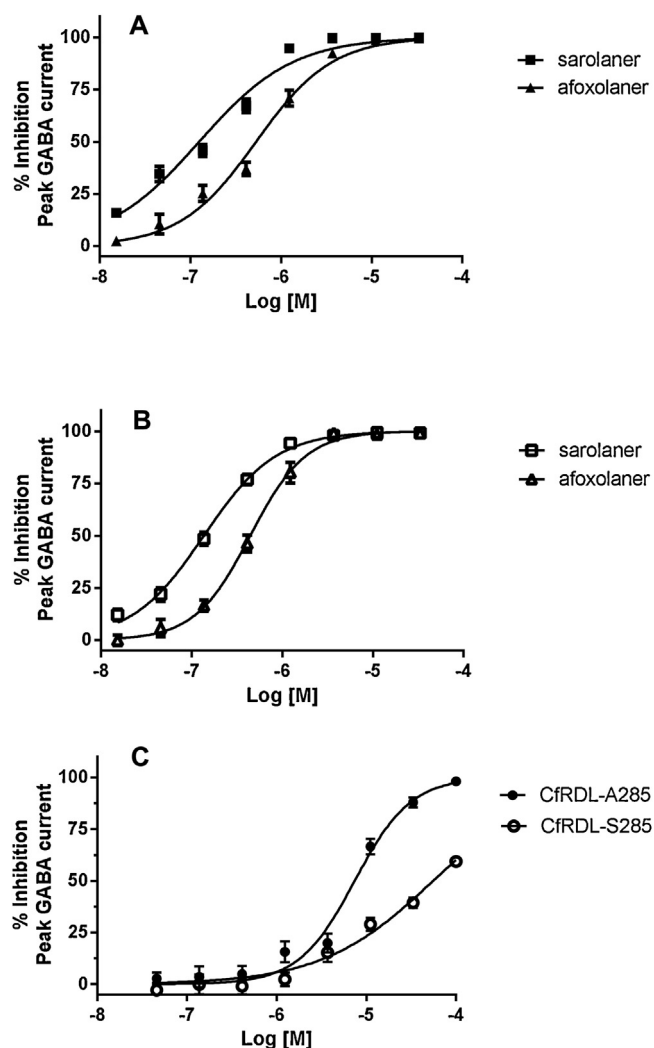


Fig. 5. Inhibition of GABA-induced currents at homomeric *C. felis* (CfrDL-A285 and CfrDL-S285) GABACs. Concentration-dependent inhibition of a fixed concentration (approximate EC₈₀ concentration) of GABA was measured by voltage-clamp electrophysiology. (A) Sarolaner (closed squares) and afoxolaner (closed triangles) inhibition of GABA-induced currents in CfrDL-A285 GABACs. (B) Sarolaner (open squares) and afoxolaner (open triangles) inhibition of GABA-induced currents in CfrDL-S285 GABACs. (C) Dieldrin inhibition of GABA-induced currents in CfrDL-A285 (closed circles) and CfrDL-S285 (open circles) GABACs. Concentration–response curves represent mean \pm S.E. (bars) of 3–8 independent experiments.

3.5. Dog efficacy

Study 1. Placebo-treated animals maintained good flea (mean range, 59.8–80.1) and tick infestations (mean range: *R. sanguineus*, 12.7–26.0; *D. reticulatus*: 24.3–33.1) throughout the study (Tables 4–6). Live flea and tick counts for all sarolaner groups were significantly lower than the placebo group ($P \leq 0.05$) on all post-treatment count days. Percentage reductions are reported based on arithmetic mean calculations.

Sarolaner, administered in a solution formulation at 2.5 mg/kg, provided 100% reduction in live flea counts 48 h after treatment of the existing infestation, and $\geq 99.9\%$ reduction in live flea counts when evaluated 24 h after each weekly re-infestation for 35 days (Table 4).

Against *R. sanguineus*, sarolaner (2.5 mg/kg, oral solution) was 100% efficacious at 48 h after treatment and against subsequent infestations through Day 35 (Table 5). Similar efficacy was seen

Table 4

Arithmetic (geometric) mean flea (*C. felis*) counts for placebo control and sarolaner-treated dogs and percent efficacy relative to controls.

Count Day	Placebo Control	Sarolaner Solution 2.5 mg/kg (<i>C. felis</i>)
2	62.5 (59.8)	0.0* [100]
7	72.4 (71.2)	0.0* [100]
14	78.9 (77.8)	0.0* [100]
21	82.0 (80.1)	0.0* [100]
28	76.5 (75.3)	0.1* [99.9]
35	70.9 (69.4)	0.0* [100]

* Mean counts are significantly different to control; $P < 0.05$. Percent efficacy, based on arithmetic means, is given in brackets []. 48-h post-treatment counts on Day 2 and 24 h post-infestation counts on all other days.

Table 5

Arithmetic (geometric) mean tick (*Rhipicephalus sanguineus* and *Dermacentor reticulatus*) counts for placebo control and Sarolaner-treated dogs and percent efficacy relative to controls.

Count Day	Placebo Control		Sarolaner Solution (2.5 mg/kg)	
	<i>R. sanguineus</i>	<i>D. reticulatus</i>	<i>R. sanguineus</i>	<i>D. reticulatus</i>
2	20.0 (19.3)	25.9 (24.3)	0.0* [100]	0.0* [100]
7	27.0 (26.0)	33.8 (33.1)	0.0* [100]	0.1* [99.6]
14	13.5 (12.7)	32.4 (31.9)	0.0* [100]	0.0* [100]
21	23.3 (22.3)	31.4 (31.0)	0.0* [100]	0.4 (0.3)* [98.8]
28	18.0 (17.1)	31.9 (31.2)	0.0* [100]	0.6* [98.0]
35	26.3 (25.4)	26.4 (25.7)	0.0* [100]	0.0* [100]

* Mean counts are significantly different to control; $P < 0.05$. Percent efficacy, based on arithmetic means, is given in brackets []. 48-h counts on all days.

Table 6

Arithmetic (geometric) mean *Ixodes ricinus* counts for placebo control and sarolaner-treated dogs and percent efficacy relative to controls.

Count Day	Placebo Control	Sarolaner (mg/kg, suspension)		
		1.25	2.5	5.0
2	22.9 (22.5)	0.0* [100]	0.0* [100]	0.0* [100]
7	18.0 (17.6)	0.0* [100]	0.0* [100]	0.0* [100]
14	20.6 (20.5)	0.1* [99.4]	0.0* [100]	0.0* [100]
21	19.3 (18.8)	0.0* [100]	0.0* [100]	0.0* [100]
28	18.5 (18.3)	0.0* [100]	0.1* [99.3]	0.0* [100]
35	17.4 (17.0)	0.1* [99.3]	0.0* [100]	0.0* [100]
57	22.0 (21.8)	4.4* [80.1]	0.1* [99.4]	0.0* [100]

* Mean counts are significantly different to control; $P < 0.05$. Percent efficacy, based on arithmetic means, is given in brackets []. 48-h counts on all days.

against *D. reticulatus*, with $\geq 98.0\%$ efficacy achieved for the entire study period (Table 5).

Study 2. Placebo-treated animals maintained adequate tick infestations of *I. ricinus* for the entire study period (Days–2–57), with a geometric mean of 18–23 ticks per dog (Table 6). Live tick counts for all of the sarolaner groups were significantly lower than those for the placebo group ($P \leq 0.05$) on all post-treatment count days. Percentage reductions are reported based on arithmetic mean calculations.

Against an existing infestation of *I. ricinus*, sarolaner at all doses provided 100% reduction in live tick counts at 48 h after treatment (Table 6). The two highest doses, 5.0 and 2.5 mg/kg, provided $>99.3\%$ reduction against subsequent re-infestations through Day 57. Persistent efficacy for the 1.25 mg/kg dose was $\geq 99.3\%$ for 35 days, dropping to 80.1% on Day 57.

Maximum sarolaner plasma concentrations were most often observed within the first day post-dose (Fig. 6). Following C_{max}, plasma concentrations decreased over the two-month study duration. Sarolaner plasma concentrations indicated dose proportionality over the range 1.25–5 mg/kg.

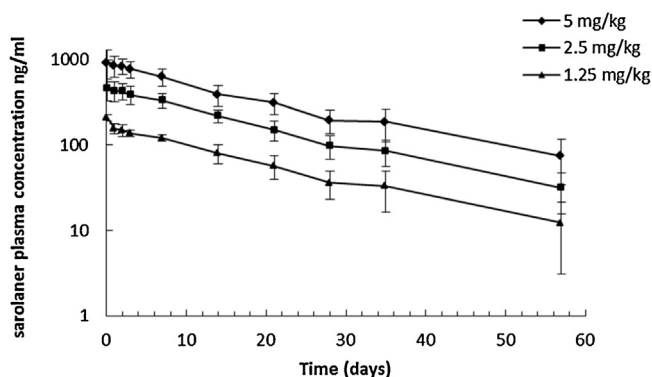


Fig. 6. Mean sarolaner plasma profiles, with standard deviations, following administration to dogs in an oral suspension.

3.6. Health observations

There were no adverse health events noted for any dogs in Studies 1 and 2.

4. Discussion

The discovery of the novel isoxazoline, sarolaner, was the result of an intensive collaborative and multi-disciplinary research program involving medicinal chemistry, *in vitro* and *in vivo* parasitology, pharmacology and metabolism and safety research scientists at Zoetis. Sarolaner is a chirally pure molecule optimized for potency, safety and pharmacokinetics in the dog.

Sarolaner emerged as the most potent of more than 3000 compounds tested and was more potent than the currently marketed isoxazolines, afoxolaner and fluralaner, in our *in vitro* parasite screening systems. Similar *in vitro* parasite screening systems have been reported previously for fleas (Zakson-Aiken et al., 2001) and have been used to evaluate and screen other isoxazoline compounds (Shoop et al., 2014). In our *in vitro* screening system, the flea assay was used as a primary screening assay and the tick assay to discriminate between the potencies of previously identified flea active molecules.

Sarolaner demonstrated oral safety in initial *in vivo* studies in rodents up to 30 mg/kg, the highest dose evaluated, and was progressed to safety studies in dogs, where no adverse effects were observed in dogs \geq 8 weeks of age with repeated monthly doses up to 20 mg/kg.

Pharmacokinetics was used as one of the pillars in the screening program to select sarolaner as the lead molecule for further progression. Sarolaner was rapidly and well absorbed following oral dosing at 20 mg/dog. Following oral administration to fasted dogs, C_{max} occurred within the first day post dose, with dose-normalized (2 mg/kg) LS mean C_{max} at 1100 ng/mL, and bioavailability calculated at $>85\%$. $T_{1/2}$ for sarolaner was calculated at 11–12 days. Doses across the range of 1.25–5 mg/kg in an aqueous suspension appeared proportional, indicating linearity. These PK characteristics made sarolaner an acceptable candidate molecule for oral monthly dosing.

Previous literature has shown that the primary mechanism of action for the isoxazoline class is through inhibition of invertebrate ligand-gated chloride channels, specifically GABA_ACl_s (Ozoe et al., 2010). Sarolaner was evaluated for functional activity using recombinant CHO-K1 cell lines stably expressing RDL subunits from *C. felis* (CfRDL-A285 and CfRDL-S285). Voltage-clamp electrophysiology confirmed that sarolaner potently blocked GABA-induced currents with IC_{50} values of 135 nM and 136 nM at CfRDL-A285 and CfRDL-S285 receptors, respectively. Of particular interest, was that sarolaner exhibited similar potency in blocking GABA-induced

currents in the resistant flea allele containing an alanine-to-serine mutation at position 285, which has been well established to confer cyclodiene resistance (ffrench-Constant et al., 2004; Bloomquist et al., 1992; Gassel et al., 2014). By contrast, dieldrin showed potent blockade of the susceptible CfRDL-A285 channel (IC_{50} of 6920 nM), while demonstrating poor antagonism at the resistant CfRDL-S285 channel (IC_{50} $>$ 3300 nM), thus confirming the resistance phenotype (Gassel et al., 2014). These data suggest that sarolaner binds at GABA_ACl_s in a differential manner to cyclodienes and is not negatively impacted by the dieldrin resistance mutation.

Direct comparisons of the receptor pharmacology of sarolaner to other isoxazolines are made difficult by the different receptor expression systems and pharmacological methods employed in the literature. For instance, while fluralaner inhibitory activity at GABA_ACl_s was demonstrated using similar recombinant cell lines as the current study (Gassel et al., 2014), IC_{50} s ($<$ 10 nM) were generated using a membrane potential fluorescence assay which is an indirect measure of channel depolarization compared with voltage-clamp electrophysiology. Moreover, afoxolaner was evaluated against the *Drosophila* RDL (IC_{50} = 3.7 nM), using oocyte expression systems rather than recombinant cell lines expressing flea and tick channels (Shoop et al., 2014). For a direct comparison, afoxolaner inhibitory potency was investigated using the currently described methodologies in a side-by-side voltage-clamp experiment with sarolaner. Afoxolaner yielded IC_{50} values of 539 nM and 412 nM at CfRDL-A285 and CfRDL-S285 receptors, respectively, which were significantly weaker than the reported inhibition of *Drosophila* RDL in the literature (Shoop et al., 2014). Moreover, sarolaner produced IC_{50} values that were significantly more potent ($P < 0.001$) than afoxolaner at both CfRDL-A285 and CfRDL-S285 receptors, thus highlighting the importance of comparing compounds using the same receptor expression systems and pharmacological methods.

In an initial exploratory *in vivo* study in dogs, sarolaner demonstrated robust efficacy ($\geq 99.8\%$) for 35 days against both fleas (*C. felis*) and adult ticks (*R. sanguineus/D. reticulatus*) when delivered in an oral solution at 2.5 mg/kg. In a subsequent dose finding study in dogs against adult *I. ricinus*, sarolaner demonstrated very high efficacy ($>99.5\%$) at the lowest dose tested (1.25 mg/kg) using an oral suspension formulation. No adverse events associated with treatment were observed for any animal in either of the efficacy studies in dogs.

Taken collectively these data indicate that sarolaner has the potential to provide month-long efficacy against fleas and multiple species of tick via the oral route of administration.

5. Conclusions

Sarolaner, the product of a comprehensive research program to identify a novel isoxazoline parasiticide specifically for use in companion animals, demonstrated the *in vitro* whole organism potency, the pharmacological and pharmacokinetic attributes, and the target animal safety and *in vivo* efficacy profiles to warrant progression as a development candidate for a monthly oral parasiticide product for dogs.

Conflict of interest

The work reported herein was funded by and conducted under the direction of Zoetis. All authors are current employees of Zoetis.

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