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Toxicological evaluation of a novel cooling compound: 2-(4-methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(2-thienylmethyl)acetamide

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ABSTRACT

A toxicological evaluation of a novel cooling agent, 2-(4-methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(2-thienylmethyl) acetamide (S2227; CAS 1374760-95-8), was completed for the purpose of assessing its safety for use in food and beverage applications. S2227 undergoes rapid oxidative metabolism *in vitro*, and in rat and dog pharmacokinetic studies is rapidly converted to its component carboxylic acid and secondary amine. S2227 was not found to be mutagenic or clastogenic *in vitro*, and did not induce micronuclei in polychromatic erythrocytes *in vivo*. The secondary amine hydrolysis product, *N*-(2-thienylmethyl)-1*H*-pyrazol-3-amine (M179), was also evaluated for genotoxicity. In subchronic oral toxicity studies in rats, the no-observed-adverse-effect-level (NOAEL) for S2227 was 100 mg/kg/day (highest dose tested) when administered by oral gavage for 90 consecutive days. Furthermore, S2227 demonstrated a lack of maternal toxicity, as well as adverse effects on fetal morphology at the highest dose tested, providing a NOAEL of 1000 mg/kg/day for both maternal toxicity and embryo/fetal development when administered orally during gestation to pregnant rats.

1. Introduction

Menthol, a monocyclic terpene derived from mint oil, has been used in a wide variety of consumer products such as toothpaste, mouthwashes, chewing gum, and candy mints to elicit a cooling sensation that is associated with freshness and cleanliness. The cooling sensation elicited by menthol has been attributed to its ability to activate a member of the transient receptor potential (TRP) ion channel family, TRPM8, which is also activated by cold temperatures (<30 °C). Many synthetic derivatives of menthol have been developed for use in oral care products that produce the same cooling sensation without the nasal and oral irritant effects seen with higher concentrations of menthol due to its volatile nature [2]. Most notable of these are the non-volatile *p*-menthane carboxamides originally developed by Wilkinson Sword, Ltd. in the 1970's [31]. Several of these *p*-menthane carboxamides have been evaluated for potential genotoxicity and *in vivo* toxicity in rodents [7–9] and have been commercialized including the widely used *N*-ethyl-*p*-menthane-3-carboxamide (WS-3, FEMA 3455). A subset of these compounds, notably FEMA 4496 (Evercool™ 180), FEMA 4549 (Evercool™ 190), and FEMA 4681 (WS-12), are significantly more potent than menthol (>10-fold) and are known to produce a long-lasting cooling sensation [1,13,14].

More recently, researchers at Senomyx, Inc. have reported a series of novel phenoxyacetylamides, including 2-(4-methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(2-thienylmethyl) acetamide (S2227, CAS 1374760-95-8), which are potent activators of TRPM8 [29]. Like several of the aforementioned *p*-menthane carboxamides, S2227 demonstrates potent, long-lasting cooling effects in sensory testing in a variety of prototype product applications. The structure of S2227 along with representative analogs from the *p*-menthane carboxamide series is shown in Fig. 1.

S2227 was reviewed by the Expert Panel of the Flavor and Extract Manufacturers Association of the United States (FEMA) and determined to be generally recognized as safe (GRAS) under conditions of intended use as a flavor ingredient [3,20,15] and therefore

Abbreviations: AUC, area under the curve; CL, plasma clearance; C_{max}, peak plasma concentration; CYP450, cytochrome P450; FDA, Food and Drug Administration; FEMA, Flavour and Extract Manufacturers Association of the United States; GMP, Good Manufacturing Practices; HPBL, human peripheral blood lymphocytes; JV, jugular vein; LC/MS, liquid chromatography with mass spectrometry; MC, methylcellulose; mnPCE, micronucleated bone marrow polychromatic erythrocytes; NOAEL, no-observed-adverse-effect-level; NOEL, no-observed-effect-level; OECD, Organization for Economic Cooperation and Development; PCE, polychromatic erythrocytes; PK, pharmacokinetics; PV, portal vein; RCG, Relative Cell Growth; RMI, Relative Mitotic Index; $t_{1/2}$, half-life; T_{max}, time to reach C_{max}; TE, total erythrocytes; TK, toxicokinetics; TRPM8, transient receptor potential melastatin 8; V_{ss}, volume of distribution at steady-state.

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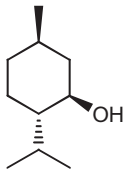

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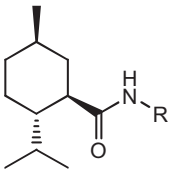
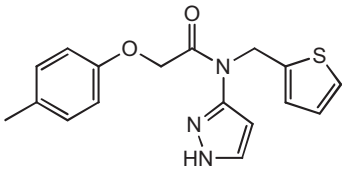


p-menthane carboxamides

FEMA no.	FL-no.	R
3455	16.013	Et
4309	16.111	CH_2CO_2Et
4549	16.118	$(CH_2)_2$(2-pyridinyl)
4496	16.117	(4-$NCCH_2$)Ph
4681	16.123	(4-MeO)Ph
4684	16.125	(4-H_2NCOCH_2)Ph



S2227
Chemical Formula: $C_{17}H_{17}N_3O_2S$
Molecular Weight: 327.40

Fig. 1. Structures of S2227 and currently marketed cooling compounds.

is available for use in human food in the United States as a "FEMA GRAS" flavor ingredient. S2227 was assigned FEMA GRAS Number 4809 in 2014 [3].

The purpose of this publication is to summarize the results obtained from *in vitro* metabolism and *in vivo* pharmacokinetic (PK) studies, general toxicology studies in rodents, developmental toxicity studies, and genotoxicity studies conducted with S2227 and its secondary amine hydrolysis product *N*-(2-thienylmethyl)-1*H*-pyrazol-3-amine (M179). Additional supporting data obtained in these studies with S2227 and M179 is included in a Supplementary data section in the online publication.

2. Materials and methods

The batch of S2227 used for the *in vitro* metabolism, *in vivo* PK, *in vitro* and *in vivo* genotoxicity, 28-day range-finding, and range-finding developmental toxicity studies (Batch ID no. 58705651, purity 99.1%), was synthesized at Senomyx, San Diego, CA using the procedure described in US Patent Application No. 2013/0324557 A1 [29]. The batch of S2227 used for the 90-day subchronic, and definitive developmental toxicity studies (Batch ID no. 10640134, Lot no. GXS-2013-052-1, purity 99.3%) was synthesized at Firmenich, Geneva, Switzerland using a slight modification of the same synthetic method.

The batches of the M179 hydrochloride salt used for the *in vitro* genotoxicity studies (Batch ID no. 58711139, purity >97%; Batch ID no. 59094944, purity >98%), were synthesized at Senomyx, San Diego, CA using the procedure described in US Patent Application No. 2013/0324557 A1 [29]. Likewise, the M179 hemisulfate salt used for the *in vivo* genotoxicity studies (Batch ID no. 60063722, purity >98%) was also prepared at Senomyx, San Diego, CA by an analogous method.

All genetic toxicology studies were conducted in compliance with the FDA Good Laboratory Practices (GLP) regulations 21CFR Part 58 [11] and OECD guidelines [26]. The experimental design for these studies followed the OECD Guidelines for the testing of chemicals - 471, 473, 474 and 489 [23–25]. The 28-day dose-range finding studies and 90-day toxicology studies in rats were conducted in compliance with the United States Food and Drug Administration (FDA) Guidelines [12] Toxicological Principles for the Safety of Food Ingredients; the 90-day subchronic toxicology study was also conducted in compliance with the FDA Good Laboratory Practice (GLP) Regulations, 21CFR Part 58. The developmental toxicity range-finder and definitive studies were conducted in accordance with the OECD Guidelines for Testing of Chemicals Guideline 414, Prenatal Developmental Toxicity Study [27] and the United States FDA Redbook 2000: IV.C.9.b Guidelines for Developmental Toxicity Studies [10]; the definitive study was also conducted in compliance with the FDA GLP regulations 21CFR Part 58 and OECD guidelines [26].

The receptor panel profiling and preliminary CYP450 inhibition assays were conducted at Ricerca Biosciences, Taipei, Taiwan; the follow up CYP450 inhibition assays were carried out by Absorption Systems, Exton, PA. The hERG channel inhibition assay was carried out by Aviva Biosciences, San Diego, CA. The plasma stability, *in vitro* microsomal metabolism, pharmacokinetic, and *in vivo* metabolism studies on S2227 and M179 were conducted at Senomyx, San Diego, CA. The in-life portion of the pharmacokinetic study in dogs was conducted at Charles River Laboratories, Wilmington, MA. The analytical methods used for the pharmacokinetic and *in vivo* metabolism studies can be found in the Supplementary data section published online.

The *in vitro* genotoxicity studies for S2227 were conducted at Nucro-Technics, Scarborough, Ontario, Canada; the *in vivo* mouse micronucleus study was conducted at WIL Research, Ashland, OH. All genotoxicity studies for M179 (hydrochloride and hemisulfate salts) were conducted at BioReliance Corporation, Rockville, MD. The 28-day and 90-day subchronic toxicity studies for S2227 were conducted at MPI Research, Mattawan, WI. The developmental toxicity study on S2227 was conducted at WIL Research, Ashland, OH. A description of the study designs is included in the individual study sections below. Detailed data tables for the genotoxicity, subchronic and developmental toxicity studies can be found in the Supplementary Data section published online.

3. In vitro receptor and cytochrome P450 profiling of S2227

In vitro tests were conducted with S2227 to assess whether the compound interacts with any enzymes or receptors that might cause adverse or unexpected effects or affect drug metabolism. Preliminary *in vitro* screening for potential off-target activity of S2227 included tests for cytochrome P450 (CYP450) inhibition, a receptor lead profiling panel (67 receptor binding assays for GPCRs, ion channels, nuclear receptors, transporters), and an hERG inhibition assay. The preliminary tests for CYP450 inhibition were performed using recombinant human enzymes expressed in insect Sf9 cells using spectrofluorimetric substrates [4,21]. All assays were performed at a concentration of 10 μM of S2227. No significant responses (≥50% inhibition or stimulation) were found in the lead profiling receptor screen. S2227 also did not significantly inhibit the hERG ion channel current (<10%) in an *in vitro* hERG electrophysiology (patch clamp) assay [30]. The results from the CYP450 inhibition studies are summarized in Table 1.

Table 1
Cytochrome P450 inhibition of S2227.

CYP	Spectrofluorimetric assay, human recombinant enzymes, Sf9 cells		LC–MS/MS assay in human liver microsomes	
	Probe substrate	% Inhibition (10 μM)	Probe substrate	% Inhibition (10 μM)
1A2	3-Cyano-7-ethoxycoumarin	59%	Phenacetin	41%
2C9	3-Cyano-7-ethoxycoumarin	39%	Diclofenac	12%
2C19	3-Cyano-7-ethoxycoumarin	95%	*S*-mephenytoin	89%
2D6	3-Cyano-7-ethoxycoumarin	18%	Bufuralol	15%
3A4	7-Benzyloxy-4-(trifluoromethyl)-coumarin	88%	Testosterone	35%
			Midazolam	57%

As a follow up to the results obtained using spectrofluorimetic substrates, S2227 was retested on the same panel of CYP enzymes utilizing pooled human liver microsomes (Absorption Systems, Exton, PA) and CYP-specific substrates with detection of the CYP-specific metabolites by LC-MS/MS [18,32]. In this more definitive assay format, 10 μM of S2227 demonstrated significant inhibition CYP2C19 and CYP3A4 (midazolam substrate) (see Table 1). In a separate study, S2227 was also evaluated for evidence of time-dependent inhibition of CYP2C19 and CYP3A4 again using human liver microsomes and CYP-specific substrates. In this assay format, the IC_{50}'s of S2227 on CYP2C19 (*S*-mephenytoin substrate) and CYP3A4 (midazolam substrate) were 1.3 μM and 15 μM, respectively. While no evidence of time-dependent CYP inhibition was seen on CYP2C19, there was a suggestion of time-dependent inhibition on CYP3A4 as indicated by a significant increase in inhibition when the microsomes were pre-incubated with S2227 in the presence of NADPH.

4. Absorption, distribution, metabolism, excretion

The *in vitro* metabolism of S2227 was studied using rat, dog, rabbit, Gottingen pig, and human liver microsomes. The *ex-vivo* plasma stability of S2227 was evaluated in rat, dog, rabbit, monkey, Gottingen pig, and human plasma. The *in vivo* metabolism and PK of S2227 was studied in both rats and dogs. The PK of both S2227 and its secondary amine hydrolysis product M179 was also determined in mice.

4.1. In vitro metabolism

The potential of S2227 to undergo oxidative metabolism was investigated using Sprague-Dawley rat, dog, rabbit, Gottingen pig, and human liver microsomes in order to determine the similarity of the metabolic profile across species and to assess the suitability of the rat as a species for toxicology studies. S2227 (10 μM) was incubated with mixed gender, pooled liver microsomes (0.5 mg/mL) from human, rat, dog, and rabbit, or with pooled liver microsomes from male Gottingen pigs (XenoTech, Lenexa, KS) in the presence of NADPH at 37 °C for 10, 20, or 60 min prior to quenching the samples with acetonitrile. Control samples included time zero and 60 min incubates without NADPH. Buspirone and loperamide were tested in parallel with S2227 to confirm the functionality of the microsomes. Samples were centrifuged to separate the precipitated microsomes from the supernatant containing the parent compound and it metabolites. The supernatants were analysed on an Agilent 6550 Accurate Mass Q-TOF LC/MS system in order to evaluate the metabolism of S2227. Details of the experimental and analytical methods can be found in the Supplementary data section.

S2227 was more rapidly metabolized by rat and pig than by the dog or human microsomes during the 60 min incubation period. At the end of the 60 min incubation, roughly 7.67%, 3.15%, 1.00%, and 0.89% of S2227 was remaining for the dog, human, rat, and pig, respectively. The amide bond hydrolysis product, *N*-(2-thienylmethyl)-1*H*-pyrazol-3-amine (M179), was observed in microsomal incubations with and without NADPH (*i.e.*, non-CYP450 dependent pathway). In the case of the rabbit microsomes, hydrolysis was rapid; less than 15% of S2227 remained at $T = 0$ min (*i.e.*, time of NADPH addition). In all other species, greater than 98% of S2227 remained at $T = 0$ min. Since relatively little S2227 remained at the time of NADPH addition in the rabbit microsomes, the metabolite profile in rabbit microsomes was not included in this analysis. A graph of the S2227 % remaining (100% for $T = 0$ min) verses time in the microsomal incubations is shown in Fig. 2.

A total of 27 metabolites were observed across all species. Because S2227 was metabolized at different rates across species, metabolite profiles in human, rat, dog, and pig microsomes were compared at a time-point where 3.15–9.01% of S2227 remained. The relative extracted ion chromatogram (EIC) peak areas (%) compared to the S2227 peak area at $T = 0$ min for the major metabolites (relative peak area >1% for at least one species) of S2227 is shown in Table 2 (positive ionization mode only). The structures assigned to the major metabolites are shown in Fig. 3. The structures of M179, M231, and M343A were confirmed by direct comparison to synthesized materials. The remaining structures are based on exact masses and mass spectral fragmentation patterns and should be considered tentative. The mass spectral fragmentation pathway for the parent compound S2227 is shown in Fig. 4. The ions corresponding to *m*/*z* 244.1081, 220.0539, and 97.0103 were the major fragments seen in the electrospray ionization (ESI) product ion spectra of S2227 and served as diagnostic fragments for determining the position of the oxidative modification of the parent structure in the microsomal metabolites. For example, metabolites in which the thiophene ring has been oxidized (*e.g.*, M341A, M343D, M359E, M361) lack the signal for the thienylmethyl cation (*m*/*z* 97.0103). Likewise, while this fragment ion is seen in the spectra of metabolites M343B/C, the fragment corresponding to *m*/*z* 244.1081 now appears at *m*/*z* 260.1030 indicating that the pyrazole moiety has undergone an oxidative modification. Other fragment ions seen in the mass spectra of these metabolites demonstrate that the (4-methylphenoxy) acetic acid moiety remains intact and lend further support to the proposed positions of oxidative metabolism.

The amide bond hydrolysis product M179 was produced by all species in greater amounts than in human microsomes. The major Phase I metabolites produced in human microsomes result from the oxidative cleavage of the thienylmethyl group (M231), hydroxylation of the 4-methylphenoxy moiety (M343A), and from oxidation of the thiophene ring (M343D and M361). The metabolite profile in dog microsomes was nearly identical to that of the human microsomes. The rat microsomes did not produce significant quantities of the M361 metabolite, and also produced large amounts of a M341A (a further oxidation product of M343D) which is produced only in trace quantities in human microsomes.

4.2. Ex-vivo plasma stability of S2227 in human, rat, dog, rabbit, monkey, and Gottingen pig plasma

The relative stability of S2227 in human plasma as compared to other species was investigated in order to assess the suitabil-

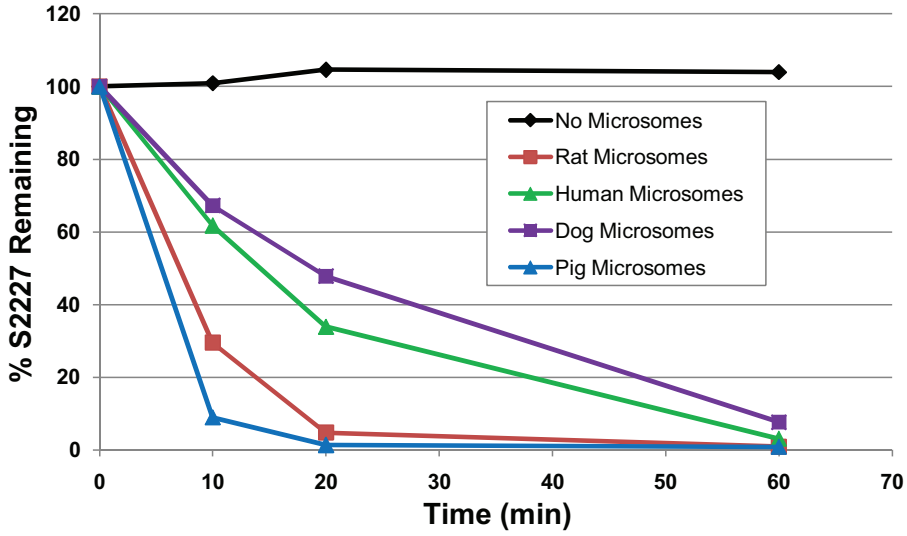


Fig. 2. Rate of metabolism of S2227 by rat, pig, dog and human liver microsomes.

Table 2
Major metabolites of S2227 in rat, dog, pig, and human microsomal incubations.

Metabolite	*m/z* (M + H)	Formula	Rat (20 min)	Pig (10 min)	Dog (60 min)	Human (60 min)
S2227	328.1114	$C_{17}H_{18}N_3O_2S^+$	4.83	9.01	7.67	3.15
M179	180.0590	$C_8H_{10}N_3S^+$	6.47	37.29	10.26	0.91
M231	232.1081	$C_{12}H_{14}N_3O_2^+$	1.89	3.68	5.14	8.68
M341A	342.0907	$C_{17}H_{16}N_3O_3S^+$	2.65	0.40	0.41	0.78
M343A	344.1063	$C_{17}H_{18}N_3O_3S^+$	16.78	7.92	13.67	6.31
M343B	344.1063	$C_{17}H_{18}N_3O_3S^+$	0.17	0.75	1.48	0.92
M343C	344.1063	$C_{17}H_{18}N_3O_3S^+$	1.91	0.21	0.43	0.26
M343D	344.1063	$C_{17}H_{18}N_3O_3S^+$	5.00	3.52	3.75	4.00
M359E	360.1013	$C_{17}H_{18}N_3O_4S^+$	0.48	2.64	0.19	0.78
M361	362.1169	$C_{17}H_{20}N_3O_4S^+$	0.42	0.78	1.99	2.52

Numbers represent relative EIC peak areas (%) compared to S2227 peak area at $T = 0$ min. All statements of scale (quantitative) assume that the relative mass spectrometry response factors for all metabolites and S2227 are equivalent.

ity of the rat as a species for toxicology studies. S2227 (750 ng/mL) was incubated with human, rat, dog, rabbit, monkey, or Gottingen pig plasma (Bioreclamation LLC, Westbury, NY) at 37 °C for 24 h. Aliquots (50 μL) were taken at 0, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, and 24 h and diluted into acetonitrile (150 μL) containing an internal standard (IS). The samples were centrifuged to separate the precipitated proteins from the supernatant. The supernatant was analysed by LC–MS/MS using an API 3200 QTrap equipped with an Agilent 1100HPLC system in order to determine the percent of S2227 remaining at the various time points. All data were normalized by ratio of peak area (S2227/IS) at the 0 h time point. Details of the experimental and analytical methods can be found in the Supplementary data section.

S2227 was rapidly hydrolyzed in rat plasma with only 3.8% remaining at the 15 min time point. In contrast, S2227 was very stable in human, dog, and pig plasma with 90.0%, 110.4%, and 91.7%, respectively, remaining at the 24 h time point. S2227 was less stable in either monkey or rabbit plasma with 36.7% and 20.1%, respectively, remaining at the 24-h time point (see Fig. 5).

4.3. *Pharmacokinetics and in vivo metabolism of S2227 in rats and dogs*

The PK parameters and oral bioavailability of S2227 in plasma was evaluated following either a single intravenous or oral administration in male/female Sprague-Dawley rats and male beagle dogs. Plasma samples were also analyzed for the presence of the metabolites observed in incubations of S2227 with rat and dog liver microsomes. For intravenous administration, 4 male and 4 female Sprague-Dawley [Crl:CD®(SD)] rats (Charles River Laboratories, Hollister, CA), or 2 male dogs were bolus injected with S2227 at 1 mg/kg in PEG 400. Blood samples were collected at approximately 0, 2, 5, 10, 30 min, 1, 2, 4, and 8 h post-dose. For oral administration, 4 male and 4 female Sprague-Dawley rats, or 3 male dogs per group were administered S2227 at either 10, 30, or 100 mg/kg in 1% methylcellulose by oral gavage (rats) or in gelatin capsule (dogs). Blood samples were taken at approximately 0, 15, 30 min, 1, 2, 4, 8, and 24 h post-dose. Plasma samples were analyzed for S2227 and metabolites by LC–MS/MS using an Agilent 6550 iFunnel Q-TOF equipped with a 1290 UHPLC system. Details of the analytical methods can be found in the Supplementary data section. Samples analyzed by mass spectrometry (API 3200 QTRAP) included internal standards for the parent compound S2227, as well as the carboxylic acid (M166) and secondary amine (M179) that result from hydrolysis of the amide bond. The PK parameters were analysed by non-compartmental methods using Phoenix WinNonlin software (Pharsight, A Certara Company, Princeton, NJ). Plasma samples from rats and dogs dosed orally at 100 mg/kg were analysed by Q-TOF for the presence of the metabolites that were observed in the rat and dog *in vitro* microsomal incubations. Test article formulations prepared for this study were analysed for concentration before and after dosing by HPLC-UV.

S2227 was rapidly eliminated after intravenous administration in both rats ($t_{1/2}$ = 0.25-0.30 h) and dogs ($t_{1/2}$ = 0.56 h) (see Table 3 and Fig. 6). Mean plasma clearance (CL) in rats averaged 79.0 mL/min/kg for males (144% of hepatic blood flow, [5] and

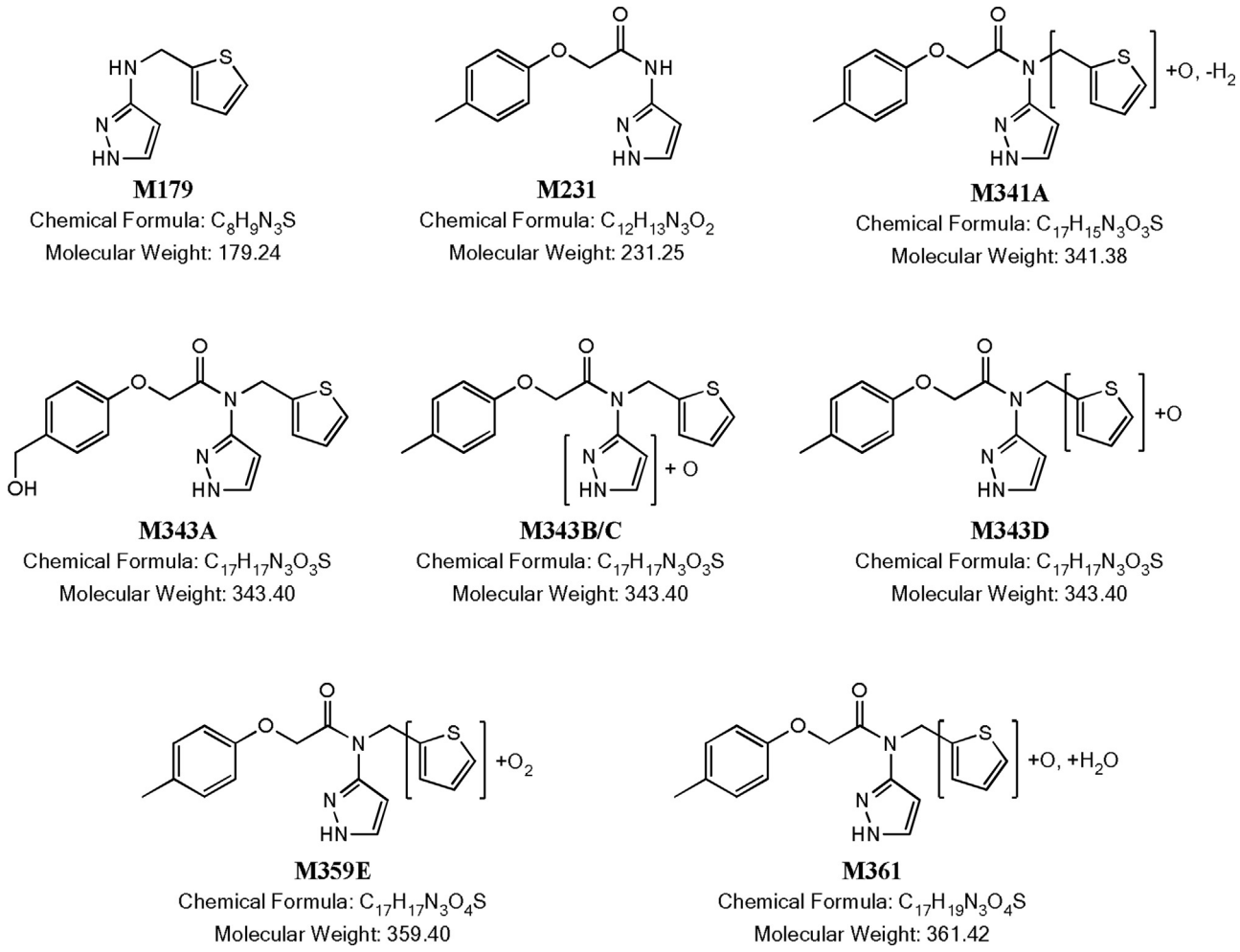


Fig. 3. Tentative structures of S2227 major microsomal metabolites.

Table 3
Pharmacokinetics of S2227 in male/female S-D rats and male beagle dogs.

Route	Dose (mg/kg)	Species (sex)	C_{max} (ng/mL)	T_{max} (h)	$t_{1/2}$ (h)	$AUC_{0\text{-last}}$ (ng·h/mL)	$AUC_{0\text{-last}}$/dose (ng·h/mL/mg/kg)	%F
iv	1.0	Rat (M)	900 ± 196	0.03	0.25	222 ± 65.8	222	–
		Rat (F)	1332 ± 431	0.03	0.30	299 ± 59.6	299	–
		Dog (M)	1329 ± 94.0	0.13	0.56	942 ± 274	942	–
oral gavage	10	Rat (M)	2.62 ± 0.33	0.75	0.60	1.64 ± 0.98	0.164	0.07%
		Rat (F)	8.51 ± 3.60	0.25	1.46	7.86 ± 3.60	0.786	0.26%
		Dog (M)	33.7 ± 27.5	1.50	1.92	119 ± 102	11.9	1.26%
	30	Rat (M)	6.22 ± 4.58	0.31	0.89	6.22 ± 3.93	0.208	0.09%
		Rat (F)	33.1 ± 32.7	0.31	2.15	29.1 ± 16.0	0.971	0.32%
		Dog (M)	41.9 ± 25.5	1.67	4.47	223 ± 29.8	7.42	0.79%
	100	Rat (M)	18.7 ± 4.58	1.00	3.02	50.1 ± 6.22	0.501	0.23%
		Rat (F)	47.8 ± 26.9	0.50	2.91	157 ± 71.5	1.57	0.53%
		Dog (M)	121 ± 29.5	1.67	5.25	704 ± 404	7.04	0.75%

Male rat: CL = 79.0 mL/min/kg; V_{ss} = 1810 mL/kg; Female rat: CL = 56.4 mL/min/kg; V_{ss} = 2440 mL/kg; Male dog: CL = 18.4 mL/min/kg; V_{ss} = 593 mL/kg; CL = clearance; V_{ss} = steady-state volume of distribution; %F = bioavailability.

56.4 mL/min/kg for females (103% of hepatic blood flow), and the volume of distribution at steady-state (V_{ss}) averaged 1810 and 2440 mL/kg for males and females, respectively. In dogs, CL averaged 18.4 mL/min/kg (59% of hepatic blood flow) and V_{ss} averaged 593 mL/kg. Despite the significantly greater stability of S2227 in dog *vs* rat plasma *ex-vivo*, S2227 was rapidly converted to its component carboxylic acid M166 and secondary amine M179 on intravenous administration in both rat and dog (see Tables 4 and 5, and Figs. 6 and 7). After oral administration, systemic exposure to S2227 was also relatively low in both species, but tended to be somewhat higher in dogs (see Table 3, Fig. 7). For example, at 100 mg/kg, the mean C_{max} for the male rats was 18.7 ng/mL (0.057 μM) and 121 ng/mL (0.386 μM) in male dogs; the mean AUC_{last} was 50.1 ng·h/mL and 704 ng·h/mL in rats and dogs, respectively (see Table 3). In contrast, systemic exposure to the acid M166 hydrolysis product was significantly greater in both species (see Table 4). At the 100 mg/kg oral dose, the C_{max} for acid M166 was 9619 ng/mL (57.9 μM) in male rats and 8342 ng/mL (50.2 μM) in male dogs; AUC_{last} was 47520 and 92580 ng·h/mL for male rats and dogs, respectively. However, the amine hydrolysis product M179,

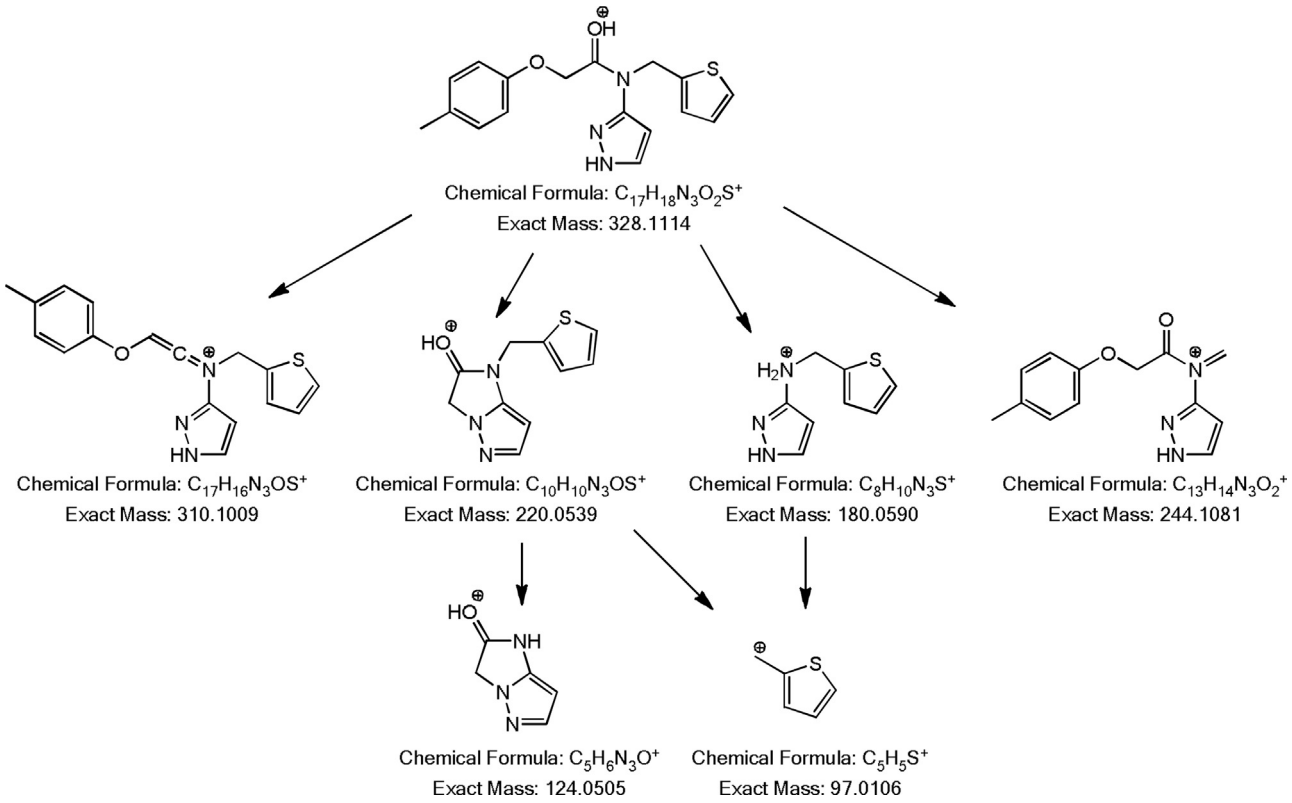


Fig. 4. Proposed mass spectral fragmentation pathway for S2227.

appears to be more rapidly excreted and/or metabolized in dogs than in rats (see Table 5). At 100 mg/kg, the C_{max} for amine M179 was 1011 ng/mL (5.64 μM, male rat) and 23.1 ng/mL (0.129 μM, male dogs); AUC_{last} was 3500 and 171 ng·h/mL for male rats and dogs, respectively. The exposure (AUC_{last}) to S2227 and its hydrolysis products M166 and M179 was somewhat higher in female rats than in males, and increased in a dose proportional manner with increasing oral dose. In dogs, exposure to S2227 and its hydrolysis products also increased with increasing oral dose, but tended to be less than dose proportional. There was also evidence of enterohepatic recirculation of the carboxylic acid hydrolysis product M166 in the dog. For example, the mean plasma concentration of M166 in dogs at the 100 mg/kg oral dose was 7130 ng/mL (42.9 μM) at the 24 h time point verses 1790 (10.8 μM) at the 8 h time point. This phenomenon was not observed in the rat.

In addition to the carboxylic acid M166 and secondary amine M179 amide bond hydrolysis products, seven Phase I and two Phase II metabolites of S2227 were observed in the rat plasma

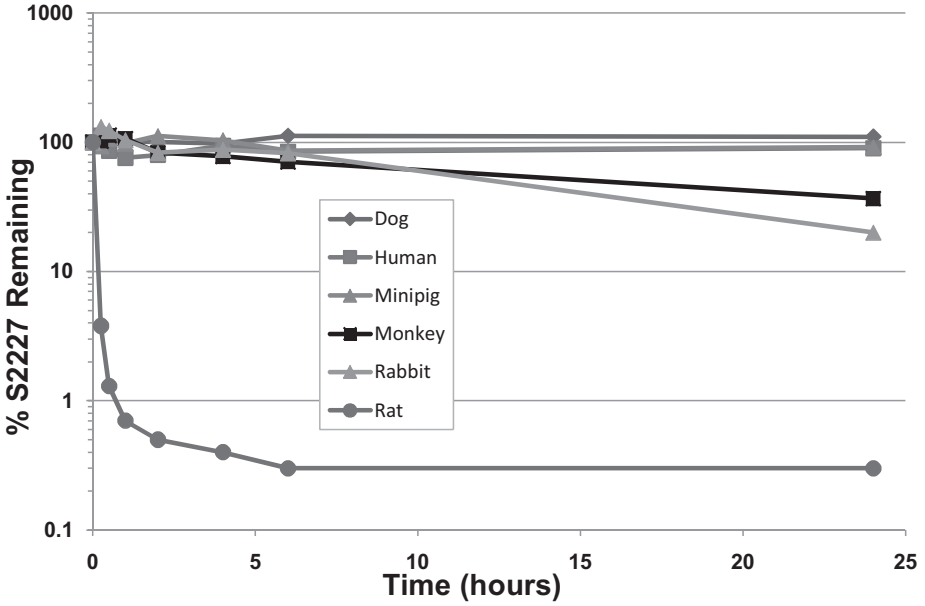


Fig. 5. Time course of S2227 plasma stability in various species at 37 °C.

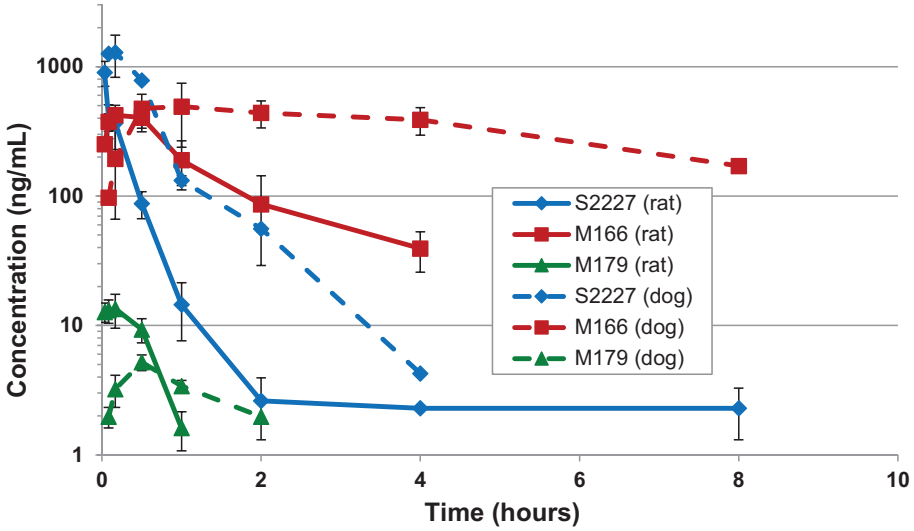


Fig. 6. Mean plasma concentrations of S2227, M166, and M179 after intravenous administration of S2227 (1.0 mg/kg) to male S-D rats and male beagle dogs.

Table 4
Pharmacokinetics of carboxylic acid metabolite M166 in male/female S-D rats and male beagle dogs.

Route	Dose (mg/kg)	Species (sex)	C_{max} (ng/mL)	T_{max} (h)	$t_{1/2}$ (h)	$AUC_{0\text{-last}}$ (ng·h/mL)	$AUC_{0\text{-last}}$/dose (ng·h/mL/mg/kg)	$AUC_{0\text{-last}}$ ratio M166/S2227
iv	1.0	Rat (M)	448 ± 25.1	0.33	0.86	554 ± 219	554	2.50
		Rat (F)	756 ± 184	0.33	1.01	1010 ± 501	1010	3.38
		Dog (M)	506 ± 159	1.50	4.17	2790 ± 1031	2790	2.96
oral gavage	10	Rat (M)	2558 ± 1240	0.63	1.34	5710 ± 2120	571	3480
		Rat (F)	3470 ± 1120	0.63	1.23	7700 ± 1440	770	979
		Dog (M)	1507 ± 552	4.67	4.77	15130 ± 6170	1513	127
	30	Rat (M)	4843 ± 1680	1.38	1.91	14845 ± 3210	495	2380
		Rat (F)	9732 ± 4240	0.81	1.70	25920 ± 6260	864	890
		Dog (M)	1783 ± 471	9.33	4.42	25930 ± 5840	864	117
	100	Rat (M)	9619 ± 4650	1.50	3.29	47520 ± 19000	475.2	949
		Rat (F)	12304 ± 5360	2.00	2.85	74230 ± 30700	742.3	473
		Dog (M)	8342 ± 5560	17.3	6.07	92580 ± 62600	925.8	132

Table 5
Pharmacokinetics of secondary amine metabolite M179 in male/female S-D rats and male beagle dogs.

Route	Dose (mg/kg)	Species (sex)	C_{max} (ng/mL)	T_{max} (h)	$t_{1/2}$ (h)	$AUC_{0\text{-last}}$ (ng·h/mL)	$AUC_{0\text{-last}}$/dose (ng·h/mL/mg/kg)	$AUC_{0\text{-last}}$ ratio M179/S2227
iv	1.0	Rat (M)	15.1 ± 3.23	0.11	0.28	8.78 ± 1.97	8.78	0.040
		Rat (F)	14.0 ± 4.84	0.13	0.82	12.6 ± 5.02	12.6	0.042
		Dog (M)	5.20 ± 0.90	0.50	1.10	6.63 ± 0.90	6.63	0.007
oral gavage	10	Rat (M)	189 ± 94.5	0.50	1.12	254 ± 102	25.4	155
		Rat (F)	407 ± 289	0.44	1.02	384 ± 267	38.4	48.9
		Dog (M)	10.2 ± 4.84	4.67	4.77	22.2 ± 8.78	2.22	0.187
	30	Rat (M)	290 ± 115	0.75	1.29	614 ± 201	20.5	98.6
		Rat (F)	1538 ± 1050	0.75	1.06	2511 ± 1700	83.7	86.2
		Dog (M)	6.81 ± 2.15	1.00	2.27	31.0 ± 15.4	1.03	0.139
	100	Rat (M)	1011 ± 367	1.13	1.83	3500 ± 813	35.0	69.9
		Rat (F)	1774 ± 457	0.83	1.84	5980 ± 745	59.8	38.1
		Dog (M)	23.1 ± 7.71	10.0	4.69	171 ± 144	1.71	0.243

samples. The Phase I metabolic biotransformation of S2227 in the rat involved oxidative cleavage of the thienylmethyl group to M231, hydroxylation of the pyrazole moiety to M343B and M343C, hydroxylation and oxidation of the 4-methylphenoxy moiety to the corresponding alcohol M343A and carboxylic acid M357D, hydrolysis of M343A to the carboxylic acid/alcohol M182, and hydrolysis of M357D to the dicarboxylic acid M196. Phase II metabolites consisted of the acyl glucuronide M533A derived from carboxylic acid M357D and M519B derived from glucuronidation of M343C. Based on EIC peak areas, metabolites M166, M179, M357D, M343C, and M533A were present at higher concentrations than the parent compound S2227 throughout the entire 24 h collection period. Alcohol M343A appears to be rapidly converted to acid M357D, which in turn, is converted to acyl glucuronide M533A in the rat.

With the exception of oxidative metabolites M343C, M182, and dicarboxylic acid M196, the same Phase I metabolites were also observed in dog plasma. In addition to the acyl glucuronide metabolite M533A, a third Phase II metabolite M519A derived from glucuronidation of alcohol M343A, was also observed in the dog. Based on MS peak areas, carboxylic acids M166 and M357D, and glucuronides M519A and M533A, were the major metabolites observed in dog and were present at higher concentrations than the parent compound S2227 throughout the entire 24 h collection period. As was observed in the case of the rat, alcohol M343A appears to be a transient intermediate which is rapidly converted

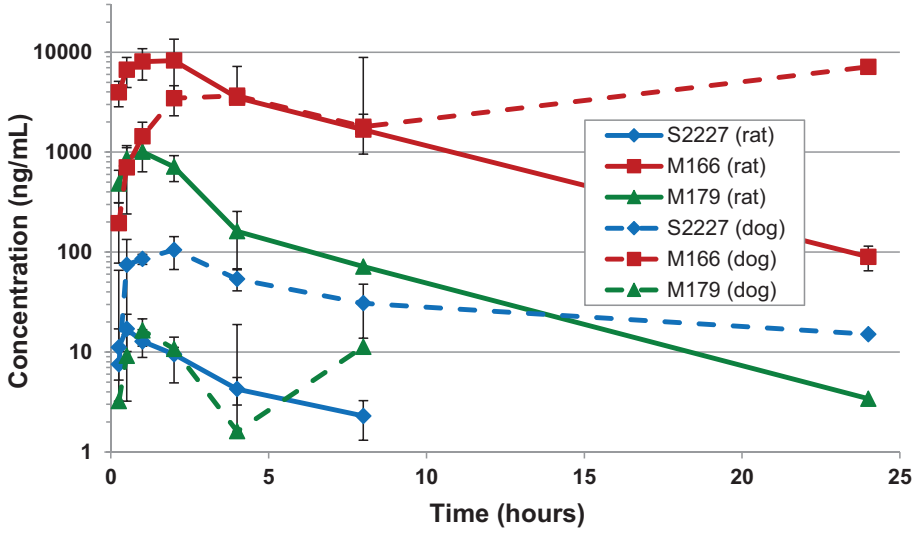


Fig. 7. Mean plasma concentrations of S2227, M166, and M179 after oral administration of S2227 (100 mg/kg) to male S-D rats and male beagle dogs.

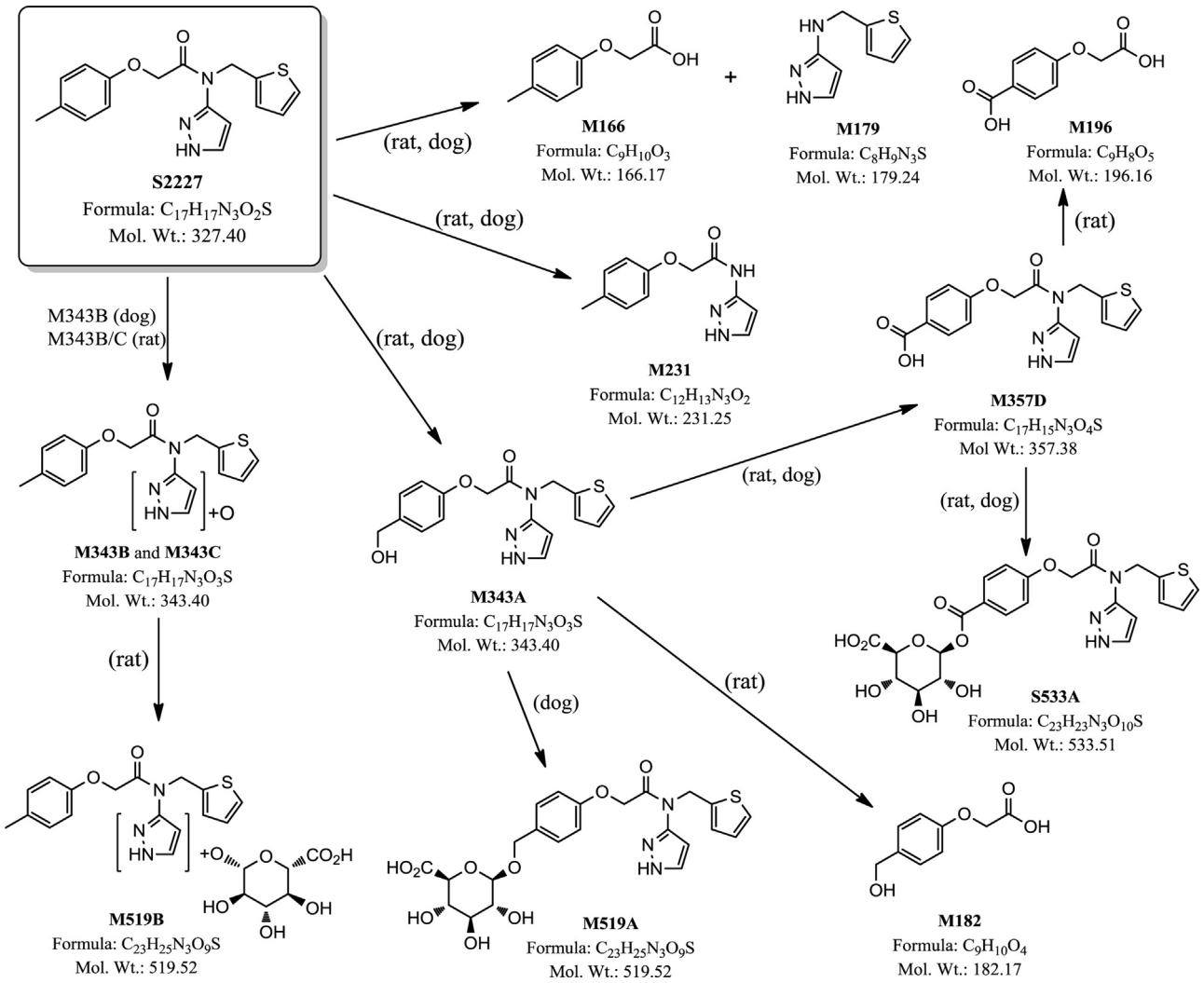


Fig. 8. Metabolic Pathway of S2227 in Rat and Dog.

Table 6
S2227 and its metabolites oberved in rat and dog plasma at 1 h post-dose (100 mg/kg, po).

			Rat		Dog	
Metabolite	*m/z* (positive)	Formula	Peak area	% Peak area	Peak area	% Peak area
S2227	328.1114	$C_{17}H_{18}N_3O_2S^+$	11800	0.52	11400	10.45
M179	180.0590	$C_8H_{10}N_3S^+$	1760000	78.05	7090	6.50
M231	232.1081	$C_{12}H_{14}N_3O_2^+$	7270	0.32	–	–
M343B	344.1063	$C_{17}H_{18}N_3O_3S^+$	–	–	3560	3.26
M343C	344.1063	$C_{17}H_{18}N_3O_3S^+$	76000	3.37	–	–
M357D	358.0856	$C_{17}H_{16}N_3O_4S^+$	369000	16.36	18900	17.33
M519A	520.1384	$C_{23}H_{26}N_3O_9S^+$	–	–	45100	41.36
M519B	520.1384	$C_{23}H_{26}N_3O_9S^+$	5230	0.23	–	–
M533A	534.1177	$C_{23}H_{23}N_3O_{10}S^+$	25700	1.14	23000	21.09
Metabolite	*m/z* (negative)	Formula	Peak area	% Peak area	Peak area	% Peak area
M166	165.0557	$C_9H_9O_3^-$	4420000	95.43	478000	100.00
M182	181.0506	$C_9H_9O_4^-$	23400	0.51	–	–
M196	195.0299	$C_9H_7O_5^-$	188000	4.06	–	–

to metabolites M357D, M519A, and M533A in the dog. None of the microsomal metabolites involving oxidation of the thiophene ring (M341A, M343D, M359E, and M361) were seen in either the rat or dog plasma samples at any time point. With the exception of M343B/C and M519B, the identities of the remaining nine of rat/dog *in vivo* metabolites were confirmed by direct comparison to synthetic standards. The metabolic pathway for S2227 in rat and dog is shown in Fig. 8. The plasma concentrations of S2227 and its metabolites at the 1-h time point can be found in Table 6. The time course data for S2227 and its metabolites over the entire 24 h collection period can be found in the Supplementary data section.

4.4. Pharmacokinetics of S2227 and S179 in mice

The plasma concentrations of the secondary amine hydrolysis product *N*-(2-thienylmethyl)-1*H*-pyrazol-3-amine (M179) were determined following oral administration of equimolar levels of either S2227 or M179 hydrochloride salt to male Crl:CD-1(ICR) mice (Charles River Laboratories, Hollister, CA). Two groups of 28 male CD-1 mice/group were dosed by oral gavage with a suspension of either S2227 (30 mg/kg) or M179 hydrochloride salt (20 mg/kg) in 1% methyl cellulose (MC). Blood samples were taken at approximately 15, 30 min, 1, 2, 4, 8, and 24 h post dose from 4 animals/group/ time point by cardiac puncture. Plasma samples from animals dosed with S2227 were analysed for S2227 and metabolites M179 and M166 (carboxylic acid hydrolysis product) using internal standards by LC–MS/MS on an AB Sciex 3200 QTRAP system equipped with an Agilent 1100 binary pump and a CTC PAL injector. Plasma samples from animals dosed with M179 hydrochloride salt were analyzed only for M179 utilizing the same system. The PK parameters were analysed by non-compartmental methods using Phoenix WinNonlin software. Test article formulations prepared for this study were analyzed for concentration before and after dosing by HPLC-UV. Details of the experimental and analytical methods can be found in the Supplementary data section.

As was observed previously in both rats and dogs, S2227 was rapidly hydrolysed to the corresponding carboxylic acid M166 and secondary amine M179 after oral administration to mice. As a result, the systemic exposure to the parent amide S2227 was very low; C_{max} = 125 ng/mL (0.382 μM), AUC_{last} = 128 ng·h/mL. After an oral dose of S2227, the exposure to the secondary amine metabolite M179 was also very low; C_{max} = 27.8 ng/mL (0.155 μM): AUC_{last} = 27.6 ng·h/mL. In contrast, exposure to the carboxylic acid metabolite M166 was nearly 100-fold greater [C_{max} = 11290 ng/mL (67.95 uM), AUC_{last} = 61730 ng·h/mL] than either S2227 or M179. These data are consistent with results obtained in the PK study of S2227 in dogs, and differ from the results of the PK study in rats, in which much higher levels of amine M166 were seen after oral administration of S2227.

M179 hydrochloride salt was rapidly absorbed (T_{max} = 0.25 h) and rapidly eliminated ($t_{1/2}$ = 0.46 h) on oral administration to mice, but still resulted in much higher levels of M179 in plasma [C_{max} = 9790 ng/mL (54.62 μM), AUC_{last} = 4370 ng·h/mL] than was seen with an equimolar dose of S2227. Taken together, the data suggest that the majority of the amide bond hydrolysis of S2227 occurs post-absorption and not within the intestinal lumen.

5. Genotoxicity and mutagenicity studies

Both S2227 and M179 were evaluated for their genotoxic potential through a standard (5-strain) Ames, chromosome aberration, and micronucleus tests (see Table 7). An alkaline comet assay was also conducted on the livers of the mice treated with M179. All genetic toxicology studies were conducted in compliance with the FDA GLP regulations 21CFR Part 58 (2006) and OECD guidelines (1998). The data tables for the genotoxicity studies can be found in the Supplemental material.

5.1. Bacterial reverse mutation test (5-strain Ames)

S2227 and M179 hydrochloride salt were evaluated for the potential to induce point mutations in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* strain WP2 *uvrA* (Molecular Toxicology Inc., Boone, NC) in the presence and absence of metabolic activation with rat liver S9 from rats induced with Aroclor™ 1254. The assay was designed to meet the current OECD Guideline for Testing of Chemicals No. 471, Bacterial Reverse Mutation Test [23].

The concentrations of S2227 investigated for both the plate incorporation and pre-incubation tests ranged from 63 to 1000 μg per plate. In the plate incorporation assay, toxicity was not observed at any concentration evident by a normal background lawn and colony counts similar to the concurrent negative controls; a slight precipitate was visible at 250 and 1000 μg/plate. In the pre-incubation assay, precipitate was visible only at 1000 μg/plate and the colony counts for TA1537 without S9 were slightly reduced at the highest concentration of 1000 μg/plate. The background lawns for TA1537 were also slightly reduced at the highest concentration of 1000 μg/plate with and without S9. Despite this apparent toxicity, all 5 concentrations were analyzable for mutagenicity. S2227 did not increase the number of revertant colonies in either the plate incorporation or pre-incubation assays with any of the tester strains both in the presence and absence of metabolic activation with rat liver S9.

Table 7
Summary of genotoxicity studies conducted on S2227 and M179.

End-Point	Test system	Cmpd No.	Concentration/dose	Result
Reverse mutation (in vitro)	S. typhimurium strains TA98, TA100, TA1535, TA1537 and E. coli strain WP2 uvrA	S2227	63–1000 μg/plate, plate incorporation and pre-incubation, ±S9[a]	Negative
		M179	50–5000 μg/plate, initial and confirmatory plate incorporation, ±S9[a]	Negative
Chromosome aberration (in vitro)	Primary human lymphocytes	S2227	35–160 μg/mL, 3 h exposure −S9, 1.3–5.0 μg/mL, 3 h exposure +S9[b], 23–65 μg/mL, 20 h exposure −S9	Negative
		M179	280–2160 μg/mL, 4 h exposure −S9 100–1840 μg/mL, 4 h exposure +S9[a] 25–280 μg/mL, 20 h exposure −S9	Positive (4 h,−S9)
Micronucleus formation (in vivo)	Male & female Swiss albino mice (CD-1), bone marrow PCEs	S2227	Males and females: 500, 1000, 2000 mg/kg bw (oral)	Negative
		M179	Males: 125, 250, 500 mg/kg bw (oral) Females: 250, 500, 1000 mg/kg bw (oral)	Negative
DNA damage (in vivo)	Male & female Swiss albino mice (CD-1), liver Comet assay	M179	Males: 125, 250, 500 mg/kg bw (oral) Females: 250, 500, 1000 mg/kg bw (oral)	Negative

[a] S9 from rat liver homogenate (9000 × g fraction) from male Sprague-Dawley rats treated with Aroclor-1254
[b] S9 from rat liver homogenate (9000 ×g fraction) from male Sprague-Dawley rats treated with phenobarbital/5,6-benzoflavone

The assay for M179 hydrochloride salt was conducted in two phases (initial toxicity-mutation and confirmatory mutation assays), using the plate incorporation method at nominal concentrations ranging from 50 to 5000 μg/plate. Neither precipitate nor background lawn toxicity was observed in either test at M179 concentrations up to 5000 μg/plate. M179 did not increase the number of revertant colonies in either the initial toxicity-mutation or the confirmatory mutation assays with any of the tester strains both in the presence and absence of metabolic activation with rat liver S9.

In all tests, the positive and vehicle controls yielded the expected results. Thus, it was concluded that both S2227 and M179 were not mutagenic to *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* strain, WP2 *uvrA* at concentrations up to 1000 μg/plate for S2227 and 5000 μg/plate for M179, in the absence and presence of metabolic activation.

5.2. In vitro chromosome aberration test

S2227 and M179 hydrochloride salt were investigated for their potential to induce structural and numerical chromosome aberrations in mammalian cells, both in the presence and absence of a supplemental rat liver fraction (S9). The experimental design followed the OECD guideline for the testing of chemicals No. 473, In vitro mammalian chromosome aberration test [24]. For S2227, a preliminary toxicity test was performed to establish the dose range for testing in the cytogenetic test.

In the case of S2227, cultures of human peripheral blood lymphocytes (HPBL, StemCell Technologies, Vancouver, BC, Canada) were treated for 3 and 20 h in the non-activated test system, and for 3 h in the presence of S9 from rats induced with phenobarbital and 5,6-benzoflavone. Solvent and positive control (mitomycin C, -S9; cyclophosphamide, +S9) cultures were also included. Test article precipitate was only observed in the test system for 3 h exposure at a concentration of 260 μg/mL. In the preliminary toxicity assay, substantial toxicity (at least 50% reduction in mitotic index relative to the vehicle control) was observed at 260 μg/mL in the non-activated 3-h exposure groups, at 5.0 μg/mL in the S9-activated 3-h exposure group, and at dose levels ≥110 μg/mL in the non-activated 20-h exposure group. Based on these findings, the doses chosen for the chromosome aberration assay ranged from 35 to 160 μg/mL for the non-activated 3-h exposure group, from 1.3 to 5.0 μg/mL for the S9-activated 4-h exposure group, and from 23 to 65 μg/mL for the non-activated 20-h exposure group. All conditions were tested at the limit of test article toxicity evaluated by relative cell growth (RCG) and relative mitotic index (RMI) levels.

Under these test conditions, no structural or numerical chromosome aberrations were observed in the S2227 treated cultures beyond those seen in the concurrent solvent controls. All concurrent positive controls induced significant numbers ($p < 0.01$) of cells with chromosome aberrations. It was concluded that exposure to S2227 did not induce chromosome aberrations in the *in vitro* mammalian chromosome aberration test using HPBL in both the absence and presence of rat liver S9, when tested in accordance with regulatory guidelines.

In the case of M179 hydrochloride salt, cultures of HPBL from a healthy, non-smoking adult female were treated for 4 and 20 h in the non-activated test system, and for 4 h in the presence of S9 from rats treated with treated with Aroclor™ 1254. Solvent and positive control (mitomycin C, -S9; cyclophosphamide, +S9) cultures were also included. In the preliminary toxicity assay, substantial toxicity (at least 50% reduction in mitotic index relative to the vehicle control) was observed at 2160 μg/mL in the non-activated and S9-activated 4 h exposure groups, and at dose levels ≥216 μg/mL in the non-activated 20 h exposure group. Based on these findings, the doses chosen for the chromosome aberration assay ranged from 280 to 2160 μg/mL for the non-activated 4 h exposure group, from 100 to 1840 μg/mL for the S9-activated 4 h exposure group, and from 25 to 280 μg/mL for the non-activated 20 h exposure group.

No significant or dose-dependent increases in structural aberrations were observed in the S9-activated 4 h and the non-activated 20 h exposure groups ($p > 0.05$). In the non-activated 4 h exposure group, a statistically significant and dose-dependent increase in structural aberrations (5.5–6.0%, $p \leq 0.01$) was observed at dose levels 2000 and 2160 μg/mL. No significant or dose-dependent increases in numerical aberrations were observed in any of the treatment conditions ($p > 0.05$). All vehicle control values were within historical ranges, and the positive controls induced significant increases in the percent of aberrant metaphases ($p \leq 0.01$). M179 was concluded to be positive in the non-activated test system, but negative in S9-activated test system, for the induction of structural aberrations in the *in vitro* chromosome aberration assay in HPBL. M179 was negative for the induction of numerical aberrations in both the non-activated and S9-activated test systems under the conditions of this assay.

5.3. In vivo micronucleus assay in mice

S2227 and M179 hemisulfate salt were evaluated for potential *in vivo* clastogenic activity and/or disruption of the mitotic apparatus, as measured by their ability to increase the incidence of micronucleated polychromatic erythrocytes (mnPCEs) in the bone marrow of CD-1 mice. The study was designed to meet the current OECD Guideline for the Testing of Chemicals No. 474, Mammalian Erythrocyte Micronucleus Test [25].

Dose-range finding studies were performed to assess test articles toxicity and determine the maximum tolerated dose (MTD) or maximum feasible dose (MFD) for the definitive assay. For both the dose range finding and definitive phases of the study, male and female Crl:CD-1(ICR) mice (Charles River Laboratories, Portage, MI) were treated with S2227 suspended in vehicle (1% methylcellulose (MC) in purified water) at a volume of 10 mL/kg body weight for three consecutive days by oral gavage. In the definitive phase of the study, 1% MC was used as the vehicle (negative) control and cyclophosphamide monohydrate (CP), at a dose of 60 mg/kg, was used as the positive control article. For the study with M179 hemisulfate salt, male and female Hsd:ICR(CD-1) mice (Harlan Laboratories, Frederick, MD) were treated with M179 hemisulfate salt suspended in 1% MC at a volume of 20 mL/kg body weight for three consecutive days by oral gavage. In the definitive phase of the study, 1% MC was used as the vehicle (negative) control and methyl methanesufonate (MMS), at a dose of 40 mg/kg/day, was used as the positive control article. Animals were observed for signs of toxicity during the course of these studies.

In the preliminary dose range finding study with S2227, mice (3 animals/sex/group) were dosed with S2227 at 500, 1000, 1500 and 2000 mg/kg for three consecutive days. Since there was no toxicity noted up to 2000 mg/kg, dose levels of 500, 1000, and 2000 mg/kg (6 animals/sex/group), were used for the definitive study with S2227. In the dose ranging study with M179, mice (3 animals/sex/group) received three consecutive daily doses of M179 hydrochloride salt at 125, 250, 500, 1000 and 2000 mg/kg/day. Mortality occurred during the dose range finding assay in 3/3 males and 3/3 females at 2000 mg/kg and 2/3 males at 1000 mg/kg/day. Clinical signs included piloerection observed at all dose levels; lethargy observed at the 500 and 1000 mg/kg/day dose levels and prostration and irregular breathing observed at the 1000 and 2000 mg/kg/day dose levels. All other mice appeared normal throughout the observation period. Due to the mortality and observations seen during the dose range finding assay, the dose levels of 125, 250, and 500 mg/kg in male mice, and 250, 500 and 1000 mg/kg in female mice, were used for the definitive study with M179 (5 animals/sex/group).

In the definitive assay, all animals from each of the test article treated, vehicle, and positive control groups were euthanized either 18–24 h (S2227 study) or 3–4 h (M179 study) after the last dose by CO_2 inhalation. Immediately following euthanasia by carbon dioxide inhalation, femoral bone marrow was collected from each animal. Bone marrow slides were prepared and polychromatic erythrocytes (PCEs, 2000/animal) were examined microscopically for the presence of micronuclei (mnPCEs). The ratio of PCEs to total erythrocytes (TE) in the test article groups relative to the vehicle control groups was also evaluated to reflect the test article's cytotoxicity. In the case of the M179 treated animals, all animals were also dissected and the liver removed and collected for a liver alkaline comet assay (*vide infra*).

All animals dosed with S2227 appeared normal throughout the conduct of the study. There were no test article-related clinical observations or effects on body weights or food consumption. For M179, no mortality occurred at any dose level during the course of the definitive assay; however, clinical signs included piloerection observed at all M179 dose levels; lethargy observed at 500 and 1000 mg/kg/day; prostration, irregular breathing and crusty eyes observed at 1000 mg/kg/day in female mice. All other mice appeared normal throughout the observation period.

No appreciable reductions in the PCE/TE ratio in either the S2227 or M179 treated groups compared to the vehicle control group were observed indicating that the test articles did not induce cytotoxicity. No statistically significant increase in the incidence of mnPCEs in either the S2227 or M179 treated groups was observed relative to the negative control group. The positive controls (CP or MMS) induced statistically significant increases in the incidence of mnPCEs when compared to both the negative control groups and the test article treated groups at all three dose levels. It was concluded that oral administration of either S2227 or M179 at did not induce micronuclei in male and female CD-1 mice when tested in accordance with regulatory guidelines.

5.4. In vivo alkaline comet assay in mice

In conjunction with the *in vivo* micronucleus study of M179 hemisulfate salt described in Section 5.3, M179 was also assessed for its potential to induce DNA damage in the liver cells by single cell gel electrophoresis (*i.e.*, the alkaline comet assay) in the same animals evaluated for micronuclei induction in PCEs in bone marrow. The comet assay design was based on JaCVAM protocol version 14.2 [16] and is in accord with the recently adapted OECD Guideline for the Testing of Chemicals No. 489, *In Vivo* Mammalian Alkaline Comet Assay [28].

In the same animals assessed for micronuclei induction, M179 was negative (non-DNA damaging) in the liver based on the results of the comet assays. There were no statistically significant differences in the % tail DNA in the liver cells of the male and female M179 treated mice (all three dose groups) and the vehicle control mice. The number of "clouds" (*aka* "hedgehogs") in the test article dosed animals were similar to that the vehicle control. The % tail DNA for the positive control (MMS) was significantly ($p < 0.05$) above that of the vehicle control. Based on the results of the comet assay, it was concluded that M179 hemisulfate salt, at doses up to and including 500 mg/kg/day in male mice and up to and including 1000 mg/kg/day in female mice, was non-DNA damaging to the liver, under the conditions of this test.

6. In vivo toxicological studies

S2227 was evaluated in 28-day dose-range finding and 90-day toxicology studies in rats in compliance with the United States Food and Drug Administration (FDA) Guidelines [12] Toxicological Principles for the Safety of Food Ingredients. S2227 was also evaluated for potential embryo/fetal toxicity in a gestational developmental toxicity study in rats. The developmental toxicity study consisted of two phases, a range-finding study and a definitive study in which the test animals were evaluated for both maternal toxicity and effects on embryo/fetal development. Summary data tables for 90-day toxicology and the definitive developmental toxicity studies for S2227 can be found in the Supplemental material (see Table 8).

6.1. Subchronic toxicology studies

6.1.1. 28-Day dose-range finding toxicity study

The purpose of these studies was to evaluate the potential systemic toxicity of S2227 in rats after dietary administration for 28 days in order to select doses for 90-day subchronic toxicity studies in rats. Three treatment groups of male and female CD® [Crl:CD®(SD)] rats ($n = 8$/sex/group, Charles River Laboratories, Portage, MI) were administered S2227 as a suspension in 1% MC by oral gavage at dose levels of 10, 30, or 100 mg/kg/day (dose volume of 10 mL/kg). One additional group of eight animals/sex served as the control and received only vehicle.

Table 8
Summary of subchronic and developmental toxicity studies conducted on S2227.

Study	Species/gender (*N* value)	Dose	Findings
28-Day dose range finding toxicity study	Male & female Sprague-Dawley rats 8 animals/sex/group	10, 30, 100 mg/kg/day (oral gavage)	No test-article related findings; NOEL = 100 mg/kg/day
90-Day subchronic toxicity study	Male & female Sprague-Dawley rats Main study: 20 animals/sex/group TK satellite group: 6 animals/sex/group	10, 30, 100 mg/kg/day (oral gavage)	No test-article related findings; NOEL = 100 mg/kg/day
Dose range finding developmental toxicity study	Bred female Sprague-Dawley rats 8 animals/group	125, 250, 500, 1000 mg/kg/day (oral gavage)	No maternal toxicity up to 1000 mg/kg/day; slight effect on intrauterine growth (fetal weight) at 1000 mg/kg/day
Definitive developmental toxicity study	Bred female Sprague-Dawley rats 25 animals/group	125, 300, 1000 mg/kg/day (oral gavage)	NOAEL for both maternal toxicity and embryo/fetal development = 1000 mg/kg/day

Survival, clinical observations, body weight, food consumption, clinical chemistry, ophthalmic examinations, organ weights, and macroscopic evaluations of all animals were used to assess potential toxicity. The livers and gastrointestinal tissues (stomach, duodenum, jejunum, and ileum) were examined microscopically for animals in the 0 and 100 mg/kg/day dose groups.

Once daily oral administration of S2227 for 28 days was well tolerated in rats at dose levels up to 100 mg/kg/day, the highest dose tested. There was no test article-related mortality observed and all animals survived until scheduled euthanasia. There were no test article-related clinical signs or changes in mean body weight, body weight gain, food consumption, ophthalmic examinations, hematology parameters, coagulation parameters, red blood cell morphology, clinical chemistry parameters, macroscopic or microscopic urinalysis data, or urine chemistry parameters during this study. There were no test article-related gross observations, changes in absolute or relative organ weights, or microscopic findings observed in the limited protocol-required tissues examined at study termination. Based on these results, the no-observed-effect level (NOEL) was considered to be ≥100 mg/kg/day.

6.1.2. 90-Day subchronic toxicity study

The purpose of these studies was to evaluate the potential subchronic toxicity and toxicokinetic (TK) profile of S2227, in rats after administration by oral gavage for 90 consecutive days. A suspension of S2227 in 1% MC was administered by oral gavage to four groups of twenty male and twenty female Sprague-Dawley [Crl:CD®(SD)] rats (Charles River Laboratories, Portage, MI) at dose levels of 0 (control), 10, 30, or 100 mg/kg/day for 90 consecutive days. Additionally, one control group of three animals/sex and three treated groups of six animals/sex/group served as TK animals and received the vehicle or test article in the same manner as the main study groups at respective dose levels of 0 (control), 10, 30, and 100 mg/kg/day.

Survival, clinical observations, body weight gain, food consumption, hematology, clinical chemistry, urinalysis, organ weights, macroscopic examination, and histopathologic evaluation of at least 54 tissues (control and high dose animals only; see Supplementary data for list of tissues examined histopathologically) were performed to assess potential toxicity. A functional observational battery (including, but not limited to, evaluation of activity, arousal, autonomic and physical function, neuromuscular function, salvation, and respiration) and opthalmoscopic examinations were conducted pretest and again during 13th week of test article administration for all main study animals. Samples for hematology and clinical chemistry evaluations were collected from all main study animals during Week 1 and Week 6, and again prior to termination. Urinalysis and samples for coagulation evaluations were collected prior to termination only. Blood for TK analysis was collected on days 1, 44 and 90 at pre-dose, 1, 3, 6, 12, and 24 h at alternating time points from two cohorts of 3 animals/sex/group. Microscopic examination of fixed hematoxylin and eosin-stained paraffin sections were performed on sections of tissues from the control and high-dose (100 mg/kg/day) groups.

Previous PK studies on the test article S2227 in rats have shown that the amide bond of S2227 rapidly hydrolyzes *in vivo* to the corresponding carboxylic acid M166 and secondary amine M179. As a result, the plasma levels of S2227 are very low. In contrast, plasma levels of the carboxylic acid hydrolysis product M166 were quite high and increased in proportion to dose. Therefore, for the TK analysis associated with this study, M166 was used as a surrogate for demonstrating proof of exposure to S2227 using a validated analytical procedure. All animals in the 10, 30, and 100 mg/kg dose groups were exposed to M166 on Days 1, 44, and 90 and were generally quantifiable until at least 12 h post-dose (see Table 9). For the 100 mg/kg males, M166 was quantifiable over the whole sampling interval (24 h). Time to maximum plasma concentration (T_{max}) was reached at 1 or 3 h post-dose across occasions. At the 100 mg/kg/day dose, C_{max} of M166 ranged from 6790 to 7730 ng/mL (40.9–46.6 μM) in females, and from 5040 to 9290 ng/mL (30.4–56.0 μM) in males, throughout the duration of the study. Maximum plasma concentration was followed by a mono-exponential decline of M166 with half-lives ($t_{1/2}$) ranging between 1.43 and 4.41 h. Overall, AUC_{last} increased in a less than dose-proportional manner between 10 and 30 mg/kg/day, and between 30 and 100 mg/kg/day. The exposure to M166 on Day 90 when compared to Day 1 was similar for all female animals of all dose levels with accumulation ratios (R_{AUC}) ranging from 1.07 to 1.19. For male animals, M166 exposure on Day 90 decreased by approximately half when compared to Day 1 and accumulation ratios ranged from 0.413 to 0.701. A gender difference was observed for AUC_{last} values on Days 44 and 90 where higher systemic exposure in females compared to males was observed, whereas on Day 1, exposure was similar.

There were no test article-related deaths during the study. All animals survived until scheduled euthanasia on Days 91–93. There were no test article-related clinical signs observed during the study. Labored breathing and abnormal breathing sounds were observed only once (in a single animal) during the study (Day 63) and were most likely related to inadvertent aspiration during the gavage procedure; therefore, these findings were considered incidental. No test article-related changes were observed in functional observational battery or ophthalmic assessments in test article-

Table 9
Toxicokinetics of M166 in male and female Sprague-Dawley rats treated with S2227 by oral gavage for 90 days.

Day	Dose (mg/kg)	Sex	C_{max} (ng/mL)	T_{max} (h)	$t_{1/2}$ (h)	AUC_{0-24h} (ng·h/mL)	AUC_{0-24h}/dose (ng·h/mL/mg/kg)	R_{AUC}[a]
1	10	M	1820 ± 494	3.0	1.63	9840 ± 1630	984	–
		F	1740 ± 163	1.0	1.43	8040 ± 542	804	–
	30	M	4400 ± 921	3.0	2.26	25200 ± 4630	841	–
		F	4070 ± 922	3.0	2.47	22500 ± 3200	751	–
	100	M	9290 ± 618	3.0	2.75	55000 ± 4490	550	–
		F	7730 ± 2500	3.0	2.28	54500 ± 11 400	545	–
44	10	M	1250 ± 94.4	3.0	1.98	8220 ± 876	822	0.835
		F	1910 ± 484	3.0	2.03	10200 ± 1600	1020	1.27
	30	M	2970 ± 382	3.0	4.41	16200 ± 1270	540	0.642
		F	4500 ± 845	3.0	2.23	24800 ± 4390	826	1.10
	100	M	5040 ± 557	3.0	2.96	39300 ± 2490	393	0.716
		F	6790 ± 1170	3.0	2.78	56100 ± 12 100	561	1.03
90	10	M	1180 ± 275	3.0	4.21	6900 ± 1010	690	0.701
		F	1670 ± 226	1.0	2.79	8760 ± 929	876	1.09
	30	M	2450 ± 274	1.0	3.80	10400 ± 1200	347	0.413
		F	3890 ± 1350	3.0	2.43	26800 ± 5200	894	1.19
	100	M	5140 ± 1260	3.0	3.71	31600 ± 5850	316	0.575
		F	7210 ± 1820	3.0	3.32	58100 ± 9030	581	1.07

[a] R_{AUC} = Day 44 or 90 AUC_{0-24h}/Day 1 AUC_{0-24h}

treated animals compared to the controls or pretest values in this study.

There were no test article-related changes observed in mean absolute body weights or body weight gain (see Figs. 9 and 10) in the S2227-treated animals compared to the controls in this study. There were no test article-related effects among hematology parameters, coagulation times, clinical chemistry analytes, or urinalysis parameters in either sex at any dose level.

There were no test article-related organ weight, macroscopic or microscopic changes noted at any dose level. All macroscopic and microscopic observations were considered incidental/spontaneous, of the nature commonly observed in this strain and age of rats, and/or were of similar incidence and severity in control and treated animals.

In conclusion, once daily oral administration of S2227 for 90 days was well tolerated in rats at dose levels up to 100 mg/kg/day. No test article-related mortality or evidence of any systemic toxicity was observed and no target organs were identified. Based on these results, the NOEL was considered to be ≥100 mg/kg/day. See Supplementary data for summary of the 90-day subchronic toxicity study data for S2227.

6.2. Developmental toxicity studies

6.2.1. Dose range-finding developmental toxicity study

The objective of the study was to determine dosage levels of S2227 to be evaluated in a definitive developmental toxicity study conducted in rats. The test article, S2227, in the vehicle (1% MC) was administered orally by gavage to 4 groups of 8 bred female Crl:CD(SD) rats (Charles River Laboratories, Raleigh, NC) once daily from gestation Days 6 through 20, with dosage levels of 125, 250, 500, or 1000 mg/kg/day (dose volume 10 mL/kg). A concurrent control group composed of 8 bred females received the vehicle on a comparable regimen. The females were approximately 13 weeks of age when paired for breeding. Positive evidence of mating was confirmed by the presence of a vaginal copulatory plug or the presence of sperm in a vaginal lavage. The day on which evidence of mating was identified was termed gestation Day 0. All animals were observed for mortality, moribundity, clinical observations, body weights, and food consumption. On gestation Day 21, a laparohysterectomy was performed on each female. The uteri, placentae, and ovaries were examined, and the numbers of fetuses, early and late resorptions, total implantations, and *corpora lutea* were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes were calculated. The fetuses were weighed, sexed, and examined for external malformations and developmental variations.

All females survived to the scheduled necropsy on gestation Day 21. Limited occurrences of clear and/or red material around the mouth were observed at 125, 250, 500, and 1000 mg/kg/day at approximately 1 h following dose administration during the latter portion of gestation (Days 17–20). In addition, increased occurrences of hair loss on various body surfaces were noted in the 500 and 1000 mg/kg/day groups at the daily examinations throughout the treatment period.

Following the initiation of dose administration on gestation Day 6–7, lower mean body weight gains and corresponding reduced mean food consumption were noted in the 500 and 1000 mg/kg/day groups during gestation Days 6–9. Mean body weight gains and food consumption in these groups were similar to the control group during the remainder of gestation. The initial mean body weight loss noted in the 500 and 1000 mg/kg/day groups resulted in slightly lower mean body weight gains when the entire treatment period (gestation Days 6–21) was evaluated, but was not of sufficient magnitude to affect mean body weights. Mean net body weights, net body weight gains, and gravid uterine weights in the 125, 250, 500, and 1000 mg/kg/day groups were similar to that in the control group. At the scheduled necropsy on gestation Day 21, no remarkable internal findings were observed at dosage levels of 125, 250, 500, and 1000 mg/kg/day and all females were determined to be gravid.

Mean combined fetal weight in the 1000 mg/kg/day group (5.1 g) was 8.9% lower than the concurrent control group value (5.6 g). Intrauterine growth in the 125, 250, and 500 mg/kg/day groups and survival and external fetal morphology in the 125, 250, 500, and 1000 mg/kg/day groups were unaffected by test article administration. Based on these results, dosage levels of 125, 300, and 1000 mg/kg/day were selected for a definitive embryo/fetal development study of S2227 administered orally by gavage to bred Crl:CD(SD) rats.

6.2.2. Definitive developmental toxicity study

The objective of the study was to determine the potential of S2227 to induce developmental toxicity after maternal exposure from implantation to one day prior to expected parturition, to characterize maternal toxicity at the exposure levels tested, and to determine a NOAEL for maternal and developmental toxicity. S2227 was administered orally by gavage (in vehicle 1% MC) to 3 groups of

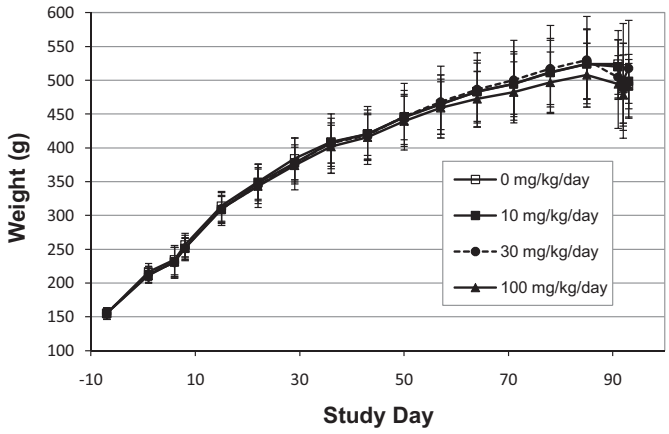


Fig. 9. Mean body weights of male Sprague-Dawley rats receiving S2227 for 13 weeks (Days -7 to 85, $n = 20$/group; Days 91–92, $n = 7$/group; Day 93, $n = 6$/group).

25 bred female Crl:CD(SD) rats (Charles River Laboratories, Portage, MI) once daily from gestation Days 6 through 20, at dosage levels 125, 300, or 1000 mg/kg/day. A concurrent control group composed of 25 bred females received the vehicle on a comparable regimen. The females were approximately 11 weeks of age when paired for breeding. Positive evidence of mating was confirmed by the presence of a vaginal copulatory plug or the presence of sperm in a vaginal lavage. The day on which evidence of mating was identified was termed gestation Day 0. All animals were observed for mortality, moribundity, clinical observations, body weights, and food consumption. On gestation Day 21, a laparohysterectomy was performed on each female. The uteri, placentae, and ovaries were examined, and the numbers of fetuses, early and late resorptions, total implantations, and *corpora lutea* were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes were calculated. The fetuses were weighed, sexed, and examined for external, visceral, and skeletal malformations and developmental variations.

All females survived to the scheduled necropsy on gestation Day 21. Non-adverse test article-related clinical findings in the 300 and 1000 mg/kg/day groups included clear and/or red material around the mouth and nose at approximately 1 h following dose administration in 11 and 15 females, respectively. These findings began as early as gestation Day 12 with the majority occurring between gestation Days 17 and 20. These material findings were considered test article-related, but non-adverse because they generally did not persist to the daily examinations the following day. No test article-related findings were noted at 1 h following dose administration in the 125 mg/kg/day group. No test article-related clinical findings were noted at the daily examinations for any of the test article-treated groups.

Mean body weight loss and lower food consumption were noted following the first day of dose administration (gestation Day 6–7) in the 1000 mg/kg/day group compared to the control group. This weight loss was followed by a significant mean body weight gain on gestation Day 7–8 compared to the control group. Mean body weight gains in the 1000 mg/kg/day group were similar to the control group during the remainder of the treatment period (gestation Days 8–21). The effects on body weight change and/or food consumption in the 1000 mg/kg/day group was transient and not of sufficient magnitude to affect mean body weights for the overall treatment period, and therefore, were not considered adverse. Mean net body weights (Fig. 11), net body weight changes, and gravid uterine weights in the 125, 300 and 1000 mg/kg/day groups were unaffected by test article administration.

At the scheduled necropsy on gestation Day 21, no test article-related internal findings were observed at dosage levels of 125, 300, and 1000 mg/kg/day. Macroscopic findings observed in the test article-treated groups occurred infrequently, at similar frequencies in the control group, and/or in a manner that was not dose-related. One female in the 1000 mg/kg/day group was non-gravid.

Intrauterine growth and survival were unaffected by test article administration at any dosage level. Parameters evaluated included post-implantation loss, live litter size, mean fetal body weights, and fetal sex ratios (see Table 10). Mean numbers of *corpora lutea* and implantation sites and the mean litter proportions of pre-implantation loss were similar across all groups. Differences from the control group were slight and not statistically significant.

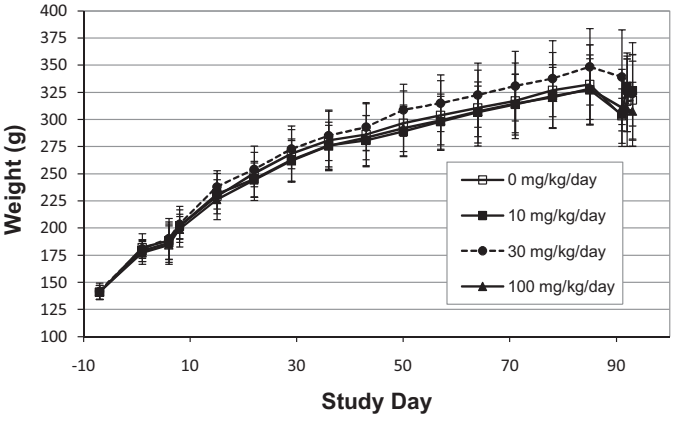


Fig. 10. Mean body weights of female Sprague-Dawley rats receiving S2227 for 13 weeks (Days -7 to 85, $n = 20$/group; Days 91–92, $n = 7$/group; Day 93, $n = 6$/group).

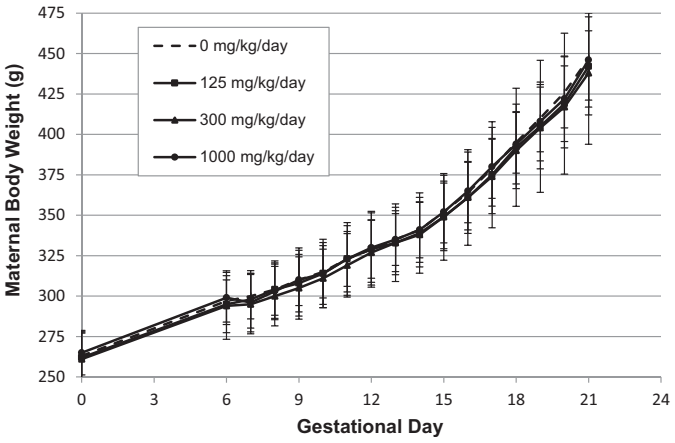


Fig. 11. Oral (gavage) developmental toxicity study of S2227 in rats: Mean maternal body weights during gestation (0, 125, and 300 mg/kg/d: $n = 25$/group; 1000 mg/kg/d: $n = 24$/group).

Table 10
Developmental toxicity study of S2227 in rats: summary of fetal data.

Dose group (mg/kg/d)	Fetuses	Sex		Viable fetuses	Dead fetuses	Resorptions		Post-implant. Loss	Implant. sites	*Corpora Lutea*	Pre-Implant. loss	Fetal Wt.(g)	No. of gravid females
		M	F			Early	Late						
0	Total	183	197	380	0	17	0	17	397	415	18	NA	25
	Mean	7.3	7.9	15.2	0.0	0.7	0.0	0.7	15.9	16.6	0.7	5.8	
	S.D.	2.27	2.09	2.20	0.00	0.80	0.00	0.80	2.07	2.40	0.94	0.27	
	S.E.	0.45	0.42	0.44	0.00	0.16	0.00	0.16	0.41	0.48	0.19	0.05	
125	Total	192	168	360	0	20	0	20	380	433	53	NA	25
	Mean	7.7	6.7	14.4	0.0	0.8	0.0	0.8	15.2	17.3	2.1	5.7	
	S.D.	2.98	2.72	4.36	0.00	1.00	0.00	1.00	3.84	3.76	3.05	0.25	
	S.E.	0.60	0.54	0.87	0.00	0.20	0.00	0.20	0.77	0.75	0.61	0.05	
300	Total	176	185	361	0	24	2	26	387	422	35	NA	25
	Mean	7.0	7.4	14.4	0.0	1.0	0.1	1.0	15.5	16.9	1.4	5.6	
	S.D.	2.11	2.53	2.53	0.00	1.02	0.28	1.06	2.47	2.54	1.53	0.29	
	S.E.	0.42	0.51	0.51	0.00	0.20	0.06	0.21	0.49	0.51	0.31	0.06	
1000	Total	188	185	373	0	14	0	14	387	419	32	NA	24
	Mean	7.8	7.7	15.5	0.0	0.6	0.0	0.6	16.1	17.5	1.3	5.7	
	S.D.	2.78	2.65	1.74	0.00	0.83	0.00	0.83	1.48	2.41	1.83	0.21	
	S.E.	0.57	0.54	0.36	0.00	0.17	0.00	0.17	0.30	0.49	0.37	0.04	

NA = not applicable

The numbers of fetuses (litters) available for morphological evaluation were 380(25), 360(24), 361(25), and 373(24) in the control, 125, 300, and 1000 mg/kg/day groups, respectively. Malformations were observed in 0(0), 2(2), 3(2), and 0(0) fetuses(litters) in these same respective dose groups and were considered spontaneous in origin. In the 300 mg/kg/day group, a malformation of anophthalmia (bilateral) was observed in one fetus. There was no apparent skeletal origin for this finding. In the 125 mg/kg/day group, a malformation of cleft palate was observed in one fetus. The aforementioned findings at 125 and 300 mg/kg/day were not considered test article-related because they occurred in single fetuses in a manner that was not dose-related, and mean litter proportions were within the laboratories historical control data ranges. No other external malformations were observed at any dosage level. No external developmental variations were noted in this study.

There were no test article-related soft tissue malformations or variations noted for fetuses at any dosage level. In the 300 mg/kg/day group, lobular dysgenesis of the lungs (one lobe present, bilateral) and right-sided aortic arch were observed in one fetus. A retroesophageal aortic arch was noted for another fetus in this same litter. No other soft tissue malformations were observed in the test article-treated groups. The soft tissue developmental variation of renal papilla(e) not developed and/or distended ureters was noted in 13(3), 13(4), 4(2), and 26(5) fetuses(litters) in the control, 125, 300, and 1000 mg/kg/day groups respectively. The 125 and 1000 mg/kg/day mean litter proportion (3.7% and 6.7% per litter, respectively) exceeded the maximum mean value in the laboratory's historical control data (3.5% per litter). However, this common variation was not considered test article-related because it was not statistically significantly different from the concurrent control group, and only occurred in a limited number of litters. Other soft tissue developmental variations observed in the test article-treated groups consisted of a major blood vessel variation (right carotid and right subclavian arteries arose independently from the aortic arch) and hemorrhagic ring around the iris.

There were no test article-related skeletal malformations or variations noted for fetuses at any dosage level. A vertebral anomaly with or without associated rib anomaly was noted for one fetus in the 125 mg/kg/day group and one fetus in the 300 mg/kg/day group. No skeletal malformations were noted in the 1000 mg/kg/day group. Skeletal variations were observed with similar frequency in all groups, including the control group, and consisted mainly of 14th rudimentary rib(s) and 7th cervical rib(s). The aforementioned visceral and skeletal malformations and variations were not

considered test article-related because they were noted in single fetuses, not in a dose-related manner, and/or the mean litter proportions were within the laboratory's historical control data ranges. A detailed summary of all of the external, visceral, and skeletal malformations and variations seen in this study can be found in the Supplemental data section.

Based on the lack of adverse maternal toxicity or effects on intrauterine growth and survival and fetal morphology at any dosage level, a dosage level of 1000 mg/kg/day (the highest dosage level evaluated) was considered to be the NOAEL for maternal toxicity and embryo/fetal development when S2227 was administered orally by gavage to bred Crl:CD(SD) rats.

7. Discussion

S2227 is a member of a novel series of substituted phenoxyacetylamide TRPM8 activators which differ from the currently marketed *p*-menthane carboxamide cooling agents mainly by the replacement of the *p*-menthane-3-carboxylic acid moiety with a phenoxyacetic acid derivative. In CYP450 inhibition assays using both spectrofluorimetric substrates with recombinant enzymes and CYP-specific substrates with pooled human liver microsomes, S2227 exhibited significant inhibition of CYP2C19 and CYP3A4 (IC_{50}'s = 1.3 μM and 15 μM, respectively using pooled human liver microsomes). However, given the low anticipated use levels of S2227 [3] and its low systemic bioavailability after oral administration, this is not expected to interfere with normal metabolic processes. Other cooling compounds such as FEMA 3455 and 4496 have also shown significant inhibition of CYP2C19 when profiled against the same panel of CYP450 enzymes (74% and 84% inhibition, respectively at 10 μM using spectrofluorimetric substrates; Arthur, unpublished results). No other significant off-target activities were seen in any of the receptor profiling assays with 10 μM of S2227.

S2227 was rapidly converted to its component carboxylic acid M166 and secondary amine M179 on both intravenous and oral administration in mouse, rat, and dog. In contrast, the amide bond of the *p*-menthane carboxamides FEMA 4309 and 4496 has been shown to be resistant to hydrolysis in artificial pancreatic juice, liver homogenate, and rat and human liver microsomes [7,8]. On oral administration of S2227 to either mouse, rat, or dog, the exposure (AUC) to the carboxylic acid hydrolysis product M166 was significantly higher than to either the parent amide S2227 or to the secondary amine hydrolysis product M179. This appears to be particularly true in the dog and in the mouse, where only very low levels of M179 were seen in plasma. The observation that amine M179 is not produced in proportion to carboxylic acid M166 suggests that either M179 is undergoing further metabolism, or that the amine moiety of S2227 is being metabolized prior to cleavage of the amide bond. However, the plasma half-life of M179 in the dog is not significantly different from that of M166 suggesting that the latter may be the case. This is further supported by the observation that oral administration of M179 hydrochloride salt to mice results in significantly higher exposure of M179 in plasma (158-fold) than an equimolar dose of S2227, and also indicates that hydrolysis of S2227 is not occurring to a significant extent in the intestinal lumen.

In a PK study on a closely related compound, 2-(4-methylphenoxy)-*N*-ethyl-*N*-(2-thienylmethyl) acetamide (S5031), in rats equipped with jugular and hepatic vein catheters, there was far more intact S5031 relative to the hydrolysis products in the portal blood verses the jugular blood [AUC_{0-8h} ratio (amide/acid): PV = 0.52; JV = 0.013; Chi, unpublished results]. This indicates that, although some hydrolysis of the amide occurred on absorption, the majority of the amide bond cleavage of S5031 occurred on first-pass through the liver. As also seen in the case of S2227, the secondary amine hydrolysis product was not present in proportion to the acid in either the portal or jugular veins [S5031 AUC_{0-8h} ratio (acid/amine): PV = 13.3; JV = 23.0]. Taken together with the results of the *in vitro* microsomal metabolism studies, these findings indicate that the amine moiety of S2227 is, at least to some extent, undergoing oxidative metabolism in the liver (and possibly the intestine) prior to cleavage of the amide bond on first-pass through the liver.

In addition to the carboxylic acid M166 and secondary amine M179 amide bond hydrolysis products, seven Phase I and two Phase II metabolites of S2227 were observed in the rat plasma samples. A third Phase II metabolite, glucuronide M519A, was only seen in dog plasma. None of the microsomal metabolites involving oxidation of the thiophene ring were seen in either the rat or dog plasma samples at any time point. Most of the remaining human microsomal Phase I metabolites were present in both rat and dog plasma and tended to be present in higher concentrations in the rat than in the dog. Based both the pharmacokinetic and *in vivo* metabolism data in both rat and dog, the rat was viewed as a suitable species for the evaluation of the *in vivo* toxicology of S2227.

Both S2227 and its secondary amine hydrolysis product M179 were evaluated for their genotoxic potential through a standard battery of *in vitro* genotoxicity assays which included a bacterial reverse mutation assay (*S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* strain WP2 *uvrA*), and a chromosome aberration test in HPBL. S2227 was found to be neither mutagenic or clastogenic in these *in vitro* genotoxicity assays. Although secondary amine M179 showed no evidence of mutagenicity in the bacterial reverse mutation assays or clastogenicity in the chromosomal aberration assay in presence of metabolic activation, human lymphocytes exposed to M179 in the absence of S9 showed a significant increase in cells with structural (but not numerical) chromosomal aberrations (5.5–6.0%, $p \leq 0.01$). This was only seen in the 4 h non-activated cultures and was not seen in the 20 h cultures in the absence of S9. *p*-Menthane carboxamides FEMA 4496, 4549, and 4681 have also been shown to be non-mutagenic in a bacterial reverse mutation assay using the same tester strains at concentrations up to 5000 μg/plate with and without metabolic activation [7,8]. FEMA 4309 was found to be weakly positive to TA1535 in the absence of metabolic activation, but was non-mutagenic in the other tester strains and also in a L5178Y $TK^{+/-}$ mouse lymphoma assay at concentrations up to 672.5 μg/mL (-S9) and 1008.75 μg/mL (+S9) [7]. FEMA 4496 and 4549 were also found to be non-clastogenic in a chromosomal aberration test in HPBL in the presence and absence of metabolic activation at concentrations up to 2984 μg/mL and 300 μg/mL, respectively [8].

Both S2227 and M179 hemisulfate salt were also evaluated in an *in vivo* mouse micronucleus assay. Oral administration of S2227 (males and females: 2000 mg/kg) or M179 (males: 500 mg/kg; females: 1000 mg/kg) to CD-1 mice for three consecutive days was well tolerated and did not induce clastogenicity nor indicate interactions with the mitotic spindle in bone marrow erythrocytes. No appreciable reductions in the PCE/TE ratio in the test article groups compared to the vehicle control group were observed indicating neither compound induced bone marrow toxicity. FEMA 4549, the only *p*-menthane carboxamide evaluated in the *in vivo* mouse bone marrow micronucleus assay, also was negative for clastogenicity at oral doses (gavage) up to 2000 mg/kg [8].

Given the apparent positive results for M179 in the chromosomal aberration assay, the livers of the mice treated with M179 were also examined for evidence of DNA damage by single cell gel electrophoresis (*i.e.*, the alkaline comet assay) in the same animals evaluated for induction of mnPCEs in bone marrow. Although M179 is produced *in vivo* from an oral dose of S2227 in mice, the exposure to M179 after an oral dose its hydrochloride salt was significantly higher than seen with a molar equivalent dose of S2227 (158-

fold). Therefore, it was felt that an independent evaluation of the *in vivo* genotoxicity potential of M179 was necessary. In the same animals assessed for micronuclei induction, M179 was negative (non-DNA damaging) in the liver based on the results of the comet assays. Overall, the results of the genotoxicity studies conducted on both S2227 and the *p*-menthane carboxamide cooling agents indicate no safety concern for these substances with respect to genotoxicity.

The doses of S2227 selected for the 28 and 90 day toxicology studies were designed to provide a high margin of safety rather than define a maximum tolerated dose (MTD) in rats. Applying a 1000-fold margin of exposure in extrapolating animal data to humans to account for species differences in susceptibility, numerical differences in population ranges between the test animals and the human population, the greater variety of complicating disease processes in the human population, and the possibility of synergistic action among food additives, is believed to be an adequate margin of safety for most substances proposed for use in food [19,6]. Based on the anticipated annual volume of use (2000 kg), the *per capita* intake ("eaters only") of S2227 for use as a flavor ingredient was calculated to be 290 μg/person/day (5 μg/kg bw/day, Cohen et al., 2015). Therefore, based on the low anticipated use level of S2227, a no-observed-effect-level (NOEL) of 100 mg/kg/day in a sub-chronic toxicology study would provide over a 20,000-fold margin of safety.

For the TK analysis associated with the 90-day subchronic toxicology study of S2227, carboxylic acid M166 was used as a surrogate for demonstrating proof of exposure to S2227. While the exposure to M166 on Day 90 when compared to Day 1 was similar for all female animals at all dose levels, M166 exposure for the male animals decreased by approximately half when compared to Day 1. A gender difference was observed for AUC_{last} values on Days 44 and 90 where higher systemic exposure in females compared to males was observed, whereas on Day 1, exposure was similar. Taken together, these results suggest that repeat dosing of S2227 may be inducing the further metabolism of M166 in males, but not in female rats. *In vivo* metabolism studies in rats have shown that M166 can be further metabolized to alcohol/acid M182 and dicarboxylic acid M196. Gender-dependent metabolism of xenobiotics and sexual dimorphisms in response to inducing agents are well known phenomena in rats that has been attributed to differences in the profile of cytochrome P450 isozymes found in male and female rat liver [17,22].

In the 90-day subchronic toxicology study with S2227, there were no test article-related effects among clinical signs, body weights, ophthalmic examinations, hematology parameters, coagulation times, clinical chemistry parameters, or urinalysis parameters, or in the functional observation battery in either sex at any dose level. There were no test article-related organ weight, macroscopic or microscopic changes in the tissues examined noted at any dose level. The 90-day subchronic toxicity study established a NOEL for S2227 of 100 mg/kg/day (the highest dose evaluated), for both male and female Sprague-Dawley rats.

The *in vivo* toxicology of several of the *p*-menthane carboxamides has also been studied in rats. In both 28-day and 22-week toxicology studies with FEMA 3455, administered by oral gavage at doses up to 1000 mg/kg/day to Sprague-Dawley rats, mild liver and kidney toxicity was observed at doses of 40 mg/kg/day and above. Likewise, in a 28-day toxicology study in beagle dogs at 100, 300, and 1000 mg/kg/day, administered by gelatin capsule, mild liver toxicity was seen at all doses. Based on these studies, the NOEL for FEMA 3455 was considered to be 8 mg/kg/day [9]. In a 90-day study with FEMA 4309 in Crl:CD (SD) rats (males and females) at doses of 25, 75, 225, and 675 mg/kg/day (oral gavage), hematological changes (increase in monocytes, neutrophils, and white blood cells; decrease in hematocrit, hemoglobin, and red blood cell count) seen at 225 and 675 mg/kg/day were dose-related and considered adverse. At 675 mg/kg/day, increases in serum creatinine, urea nitrogen, triglycerides, and total urine volume were observed in both genders. Test-article related microscopic findings where noted in the kidney (tubular degeneration and dilation, interstitial fibrosis, and tubular epithelium vacuolation) and in the liver (periportal hepatocellular vacuolation and centrilobular hepatocellular hypertrophy) in both males and females, and in the hearts of females (cardiomyopathy) at 675 mg/kg/day. The NOAEL for FEMA 4309 was considered to be 75 mg/kg/day [7]. In a similar 90-day study in rats with FEMA 4496 at doses of 100, 300, and 1000 mg/kg/day administered in diet, a test-article related increase in methemoglobin was observed in the females in the 1000 mg/kg/day group, and increased cholesterol and potassium in males dosed at 300 and 1000 mg/kg/day. Significantly increased liver weight to body weight was observed in both males and females dosed at 300 and 1000 mg/kg/day. There were no histopathological substance-related changes in any tissue. The NOAEL for this study was considered to be 100 mg/kg/day [8].

In a 28-day toxicology study in Sprague-Dawley rats with FEMA 4549 at doses of 10, 50, and 300 mg/kg/day administered in diet (8 animals/sex/group), increases in albumin, globin, triglycerides, cholesterol, T3, absolute and liver/body weight ratio were seen in animals in the 300 mg/kg/day group. Follicular cell hypertrophy of the thyroid gland was observed in 7 males and 4 females in the 300 mg/kg/day group, 2 males in the 50 mg/kg/day group, and 1 male each in the 10 and 0 mg/kg/day groups. The NOAEL was considered to be 10 mg/kg/day. In a follow up 90-day study with FEMA 4549 at 5, 20, and 50 mg/kg/day administered in diet (10 animals/sex/group), an increase in liver weights was observed in the high dose males with no histopathological correlate. Mild hepatocellular hypertrophy in females dosed at 20 and 50 mg/kg/day was not considered to be adverse since there was no clinical chemistry correlate. Dose-related increases in TSH, T4, and T3 were observed in animals receiving 50 mg/kg/day. A dose-related follicular cell hypertrophy was observed in the thyroid gland of males (all doses) and females (5 and 50 mg/kg/day) treated with FEMA 4549. Given that thyroid follicular hypertrophy can progress to neoplasia in rats, the effects seen in the 20 and 50 mg/kg groups was considered to be adverse. Thyroid gland effects were considered to be secondary to hepatic microsomal enzyme induction. Induction of UDP glucocuronyltranferanse that conjugates T4 in liver would give rise to compensatory increase in TSH in rat. In humans, T4 is metabolized by sulfation, and therefore the observed effect in rat may not be relevant to humans. The NOAEL for this study was considered to be 5 mg/kg/day [8]. With the exception of FEMA 4496, the NOAEL for these 28- and 90-day toxicology studies of the *p*-menthane carboxamides in rats was below the 100 mg/kg/day NOEL observed in the case of the 90-day study of phenoxyacetamide S2227 in Sprague-Dawley rats. In the case of the *p*-menthane carboxamides, target organs of toxicity included the liver, kidney, and thyroid gland. There were no signs of toxicity in any of these organs in the 90-day study of S2227 in rats at doses up to 100 mg/kg/day (highest dose tested). According to the maximized survey-derived daily intake (MSDI) approach, the margin of safety for the *p*-menthane carboxamide based on the estimated daily *per capita* intake of these substances in Europe was viewed to be adequate and they are not anticipated to pose a safety concern when used as flavoring substances at the estimated levels of intake [8]. Although an estimate of daily *per capita* intake of S2227 is not currently available, based on its approved use levels in various products [3] and its higher NOEL in the 90-day toxicology study in rats, S2227 is also believed not to pose a safety hazard when used as a flavoring substance.

In the developmental toxicity study of S2227, there were no test article-related clinical or macroscopic findings were noted at

any dosage level. Mean maternal body weights, body weight gains, gravid uterine weights, and food consumption were unaffected by test article administration at all dosage levels. No test article-related findings were noted on intrauterine growth and survival and fetal morphology at any dosage level. Non-adverse test article-related clinical findings in the 300 and 1000 mg/kg/day groups included clear and/or red material around the mouth and nose at approximately 1 h following dose administration. These findings were considered test article-related, but non-adverse because they generally did not persist to the daily examinations the following day.

There were no test article-related external malformations, soft tissue, or skeletal malformations noted for fetuses at any dosage level. In the 300 mg/kg/day group, a malformation of anophthalmia was observed in one fetus and in the 125 mg/kg/day group, a malformation of cleft palate was observed in one fetus. All of the malformations observed in the study were considered to be spontaneous in origin and not test article-related. Based on the lack of adverse maternal toxicity or effects on intrauterine growth and survival and fetal morphology at any dosage level, a dosage level of 1000 mg/kg/day (the highest dosage level evaluated) was considered to be the NOAEL for S2227 for both maternal toxicity and embryo/fetal development. No developmental or reproductive toxicity studies have been reported for cooling compounds in the *p*-menthane carboxamide series.

8. Conclusions

S2227 demonstrated a lack of genotoxicity with or without metabolic activation *in vitro* at concentrations that greatly exceed those observed in rat plasma following oral administration of S2227 at doses up 100 mg/kg. Although the corresponding secondary amine hydrolysis product M179 did show evidence of clastogenicity in an *in vitro* chromosome aberration assay, both S2227 and M179 demonstrated a lack of genotoxicity in mice at oral doses of 2000 mg/kg (male and female) and 500 (male)/1000 (female) mg/kg, respectively. S2227 had a NOAEL of 1000 mg/kg/day for both maternal toxicity and embryo/fetal development in rats. The results of a 90-day subchronic toxicity study established a NOEL for S2227 of 100 mg/kg/day (the highest dose evaluated), for male and female rats. Assuming that the systemic exposure of S2227 after oral administration to humans is comparable to that observed at an equivalent dose in the rat, these NOEL/NOAELs are several orders of magnitude higher than the anticipated human exposure for S2227 under the conditions of intended use [3].

Conflict of interest

All the studies described herein were funded by Senomyx, Inc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxrep.2015.09.001.

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