Toxicology Reports 4 (2017) 507–520

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Full Length Article

Toxicological evaluation of the flavour ingredient *N*-(1-((4-amino-2,2-dioxido-1*H*-benzo[*c*][1,2,6]thiadiazin-5-yl)oxy)-2-methylpropan-2-yl)-2,6-dimethylisonicotinamide (S2218)

Donald S. Karanewsky*, Guy Servant, Hanghui Liu, Bert Chi, Lily Ida, Michael Saganich, Sara Werner, Joseph R. Fotsing, Andrew Patron, Catherine Tachdjian, Amy Arthur[1]

Senomyx, Inc., 4767 Nexus Centre Drive, San Diego, CA 92121, United States

ARTICLE INFO

Keywords:
Flavours with modifying properties
S2218
FEMA GRAS
Subchronic toxicological evaluation
Genetic toxicological evaluation
Developmental toxicity evaluation

ABSTRACT

A toxicological evaluation of *N*-(1-((4-amino-2,2-dioxido-1*H*-benzo[*c*][1,2,6]thiadiazin-5-yl)oxy)-2-methylpropan-2-yl)-2,6-dimethylisonicotinamide (S2218; CAS 1622458-34-7), a flavour with modifying properties, was completed for the purpose of assessing its safety for use in food and beverage applications. S2218 exhibited minimal oxidative metabolism *in vitro*, and in rat pharmacokinetic studies, the compound was poorly orally bioavailable and rapidly eliminated. S2218 was not found to be mutagenic in an *in vitro* bacterial reverse mutation assay, and was found to be neither clastogenic nor aneugenic in an *in vitro* mammalian cell micronucleus assay. In subchronic oral toxicity studies in male and female rats, the NOAEL was 140 mg/kg bw/day (highest dose tested) for S2218 sulfate salt (S8069) when administered as a food ad-mix for 13 consecutive weeks. Furthermore, S2218 sulfate salt 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 bw/day for both maternal toxicity and embryo/fetal development when administered orally during gestation to pregnant rats.

1. Introduction

The dramatic increase in the consumption of sugary soft drinks during the last 40 years has been cited as a major contributor of the obesity epidemic in the United States which can lead to the development of early onset type II diabetes [1,2]. As a result, food and beverage companies have utilized a number of synthetic and naturally occurring non-caloric sweeteners in an effort to reduce dietary sugar intake. Unfortunately, all of the existing non-caloric sweeteners fail to mimic the taste of real sugar. These alternative sweeteners can exhibit objectionable off-tastes (bitter, metallic, liquorish, cooling), inadequate temporal properties (slow onset and/or lingering of sweet taste), or even a limited sweetness intensity at higher concentrations [3,4].

The recent discovery of the human sweet receptor, hTAS1R2/hTAS1R3 [5], and its application in the high-throughput screening of natural extract and synthetic libraries, has led to the discovery of positive allosteric modulators (PAMs) of the human sweet receptor as an alternative approach to reducing the caloric content of food and beverage products currently sweetened with sucrose or high fructose corn syrup [6–8]. By enhancing the affinity of the carbohydrate sweetener to hTAS1R2/hTAS1TR3 heterodimer, these PAMs allow for a reduction of carbohydrate sweeteners in food and beverage products while maintaining the desired sweet taste of natural sugars. These compounds fall into a class of flavour compounds known as flavours with modifying properties (FMPs) which is a term used by the flavour industry to describe ingredients that function as part of a flavour system [9] to modify or enhance the flavour profile of a variety of food and beverages. FMPs may not necessarily have a taste on their own [10], but may work in concert with other flavour ingredients in a flavour system to change the flavour profile of a food product, such as by decreasing or increasing the intensity of specific flavour characteristics [11].

Abbreviations: amu, atomic mass units; AUC, area under the curve; CBPI, cytokinesis-blocked proliferation index; CL, plasma clearance; C_{max}, peak plasma concentration; CYP, cytochrome P450; EIC, extracted ion chromatogram; FDA, Food and Drug Administration; FEMA, Flavour and Extract Manufacturers Association of the United States; FL-no, FLAVIS number; FMP, flavour with modifying properties; GLP, Good Laboratory Practices; GMP, Good Manufacturing Practices; HPBL, human peripheral blood lymphocytes; LC/MS, liquid chromatography with mass spectrometry; MC, methylcellulose; NOAEL, no-observed-adverse-effect-level; OECD, Organization for Economic Cooperation and Development; PK, pharmacokinetics; $t_{1/2}$, half-life; T_{max}, time to reach C_{max}; TK, toxicokinetics; V_{ss}, volume of distribution at steady-state

* Corresponding author.
E-mail address: don.karanewsky@gmail.com (D.S. Karanewsky).
[1] Current address: Vextex Pharmaceuticals, 11010 Torreyana Road, San Diego, CA 92121, United States.

http://dx.doi.org/10.1016/j.toxrep.2017.09.004
Received 8 August 2017; Received in revised form 6 September 2017; Accepted 13 September 2017
Available online 14 September 2017
2214-7500/ © 2017 The Authors. Published by Elsevier Ireland Ltd. This is an open access article under the CC BY-NC-ND license
(http://creativecommons.org/licenses/BY-NC-ND/4.0/).

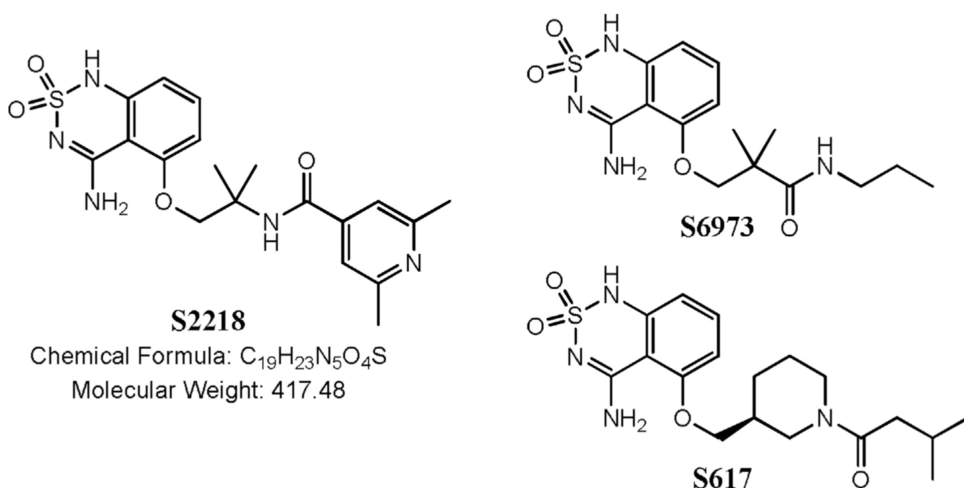

Fig. 1. Structures of S2218 and Related Sweet Modifiers.

Researchers at Senomyx have previously reported on the toxicological evaluation of two representatives of a series of 5-alkoxy substituted benzothiadiazine analogs, 3-((4-amino-2,2-dioxido-1*H*-benzo[*c*][1,2,6]thiadiazin-5-yl)oxy)-2,2-dimethyl-*N*-propylpropanamide (S6973; FEMA 4701, CAS 1093200-92-0) and (*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[*c*][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one (S617; FEMA 4802, CAS 1469426-64-9), which were identified as PAMs of the human sweet receptor [12]. The structures of S6973 and S617 are shown in Fig. 1. These substances were reviewed by the Expert Panel of the Flavour and Extract Manufacturers Association of the United States (FEMA) and determined to be generally recognized as safe (GRAS) under their conditions of intended use as flavour ingredients [13,14,9] and therefore are available for use in human food in the United States as "FEMA GRAS" flavour ingredients. S6973 was also determined to be safe at the current levels of intake by the Joint FAO/WHO Expert Committee on Food Additives ([15]; assigned JECFA No. 2082) and the European Union ([16]; assigned FL-no: 16.126).

The purpose of this publication is to summarize the results obtained from *in vitro/in vivo* metabolism and *in vivo* pharmacokinetic (PK) studies, general toxicology studies in rodents, genotoxicity studies, and developmental toxicity studies conducted on a third member of this class of PAMs of the human sweet receptor, *N*-(1-((4-amino-2,2-dioxido-1*H*-benzo[*c*][1,2,6]thiadiazin-5-yl)oxy)-2-methylpropan-2-yl)-2,6-dimethylisonicotinamide (S2218, CAS 1622458-34-7). This compound differs from S6973 and S617 only in the structure of the alkoxy side chain appended to the 5-position of a benzothiadiazine nucleus (see Fig. 1). The presence of the 2,6-dimethylisonicotinamide moiety was found to improve the physical properties of S2218 over S6973 and S617, including significantly improved photostability to UV light and improved water solubility at low pH [17]. Additional supporting data obtained in these studies with S2218 is included in a Supplementary data section in the online publication.

2. Materials and methods

The batch of S2218 used for the *in vitro* profiling assays, *in vitro/in vivo* metabolism, *in vivo* pharmacokinetic, *in vitro* genotoxicity, and 28-day range-finding toxicity studies (Batch ID 112593519, purity 99.23%, mp 235–240 °C, decomp), and the batch of S2218 sulfate salt (S8069, CAS 2079034-28-7) used for the *in vivo* pharmacokinetic studies (Batch ID 113502673, purity > 98%) was synthesized at Senomyx, San Diego, CA using the procedure described in US Patent No. 9,000,151 B2, 9,371,317 B2, and 9,475,803 B2 [17–19]. The batches of S2218 sulfate salt (S8069) used for the 90-day subchronic toxicity (Batch ID 113825463, purity 98.60%) and for the developmental toxicity studies (Batch ID 113765640, purity 97.26%, mp 229–230 °C, decomp.) were synthesized at Labochim, Milan, Italy, using a slight modification of the same synthetic method but also prepared in conformance with Good Manufacturing Practices (GMPs) as described in the ICH GMP Guidelines for APIs [20]. The batches of S2218 and S2218 sulfate salt (S8069) used for these studies gave ^{1}H NMR (400 MHz, d_6-DMSO), ^{13}C NMR (100 MHz, d_6-DMSO), FT-IR/ATR (ZnSe crystal), mass spectra, and elemental analysis which were consistent with the proposed structure and purity.

All genetic toxicology studies were conducted in compliance with the United States Food and Drug Administration (FDA) Good Laboratory Practices (GLP) regulations 21 CFR Part 58 [21] and OECD guidelines [22]. The experimental design for these studies followed the OECD Guidelines for the Testing of Chemicals – 471 and 487 [23,24]. The 28-day dose-range finding studies and 90-day toxicology studies in rats were conducted in compliance with FDA guidelines [25] Toxicological Principles for the Safety of Food Ingredients; the 90-day subchronic toxicology study was also conducted in compliance with GLP regulations, 21 CFR Part 58 [21]. 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 [26] and the United States FDA Redbook 2000: IV.C.9.b Guidelines for Developmental Toxicity Studies [27]; the definitive study was also conducted in compliance with the FDA GLP regulations 21 CFR Part 58 and OECD guidelines [22].

The receptor panel profiling and cytochrome P450 (CYP) inhibition assays on S2218 were conducted at Eurofins PanlabsTaiwan Ltd., Taipei, Taiwan. The hERG channel inhibition assay on S2218 was carried out by Aviva Biosciences, San Diego, CA. The *in vitro* microsomal metabolism studies, as well as pharmacokinetic (PK) and *in vivo* metabolism studies on S2218 and S2218 sulfate salt in rats were conducted at Senomyx, San Diego, CA. The microsomal metabolism studies utilized male and female rat liver microsomes (Lot no. 1310030 and 0310205, respectively) and mixed gender human microsomes (Lot no. 1410013) obtained from XenoTech, Lenexa, KS. The analytical methods used for the *in vitro* metabolism, PK and *in vivo* metabolism studies can be found in the Supplementary data section published online.

The *in vitro* genotoxicity studies for S2218 were conducted at BioReliance Corporation, Rockville, MD. The *S. typhimurium* tester strains were from Dr. Bruce Ames' Master cultures, and the *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland. Tester strains TA100, TA1535 and TA1537 were obtained from Molecular Toxicology Inc., Boone, NC, using cultures derived from the above sources. The rat liver S9 (9000 × *g* supernatant fraction of liver homogenate from Sprague-Dawley rats treated with Aroclor™ 1254) used in the reverse bacterial mutation assay (Lot No. 3586) was obtained from Molecular Toxicology Inc., Boone, NC. Peripheral blood lymphocytes used for the *in vitro*

micronucleus test were collected aseptically from a 29 year old, healthy, non-smoking female donor. The donor had no recent history of radiotherapy, viral infection or the administration of drugs. Rat liver S9 (9000 × *g* supernatant fraction of liver homogenate from Sprague-Dawley rats treated with Aroclor™ 1254) used in the micronucleus test (Lot No. 3563) was obtained from Molecular Toxicology Inc., Boone, NC. The 28-day and 90-day subchronic toxicity studies for S2218 and S2218 sulfate salt (S8069) were conducted at MPI Research, Mattawan, WI. The developmental toxicity study on S2218 sulfate salt (S8069) was conducted at Charles River, Ashland, OH. A description of the study designs is included in the individual study sections below. Detailed data tables for the genotoxicity, 28-day range-finder, 90-day subchronic toxicity, and developmental toxicity studies can be found in the Supplementary data section published online.

3. Results and study designs

3.1. In vitro receptor and cytochrome P450 profiling of S2218

In vitro tests were conducted with S2218 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 S2218 included tests for CYP inhibition, a receptor lead profiling panel (consisting of 68 receptor binding assays for GPCRs, ion channels, nuclear receptors, transporters), and a hERG inhibition assay. The tests for CYP inhibition were performed using recombinant human enzymes expressed in insect Sf9 cells using spectrofluorimetric substrates [28,29]. All assays were performed at a concentration of 10 μM of S2218. No significant responses (≥50% inhibition or stimulation) were found with S2218 in the lead profiling receptor screen. S2218 did not significantly inhibit the hERG ion channel current (< 10%) in an *in vitro* hERG electrophysiology (patch clamp) assay [30]. None of the five CYP isoforms tested (CYP's 1A2, 2C19, 2C9, 2D6 and 3A4) were inhibited by > 7% in the presence of 10 μM S2218 in the spectrofluorimetric assay.

3.2. Absorption, distribution, metabolism, excretion

The *in vitro* metabolism of S2218 was investigated using rat and human liver microsomes. A study of the pharmacokinetics and bioavailability of both S2218 and its sulfate salt S8069 was carried out in male and female Sprague-Dawley rats. Finally, the metabolic profile and excretion of S2218 was studied following a single oral dose of its sulfate salt to male and female Sprague-Dawley rats.

3.2.1. In vitro metabolism of S2218

The potential of S2218 to undergo oxidative metabolism was investigated using Sprague-Dawley rat and human liver microsomes in order to determine the similarity of the metabolic profile across these species. Solutions of S2218 (10 μM) were incubated with mixed gender, pooled liver microsomes (0.5 mg/mL) from both rat and human (XenoTech, Lenexa, KS) in the presence of NADPH (0.91 mM) at 37 °C for 20, 60, or 120 min prior to quenching the samples with acetonitrile. Control samples included time zero and 120 min incubates without NADPH. Buspirone (10 μM) and loperamide (10 μM) were tested in parallel with the test compounds to confirm the functionality of the microsomes. Samples were centrifuged to separate the precipitated microsomes from the supernatant containing the parent compound and its metabolites. The supernatants were analyzed by LC-QTOF/MS using a Waters Acquity UPLC HSS T3 column (50 × 2.1 mm, 1.8 μm) with a 0.1% formic acid/water (v/v) and acetonitrile gradient system, and an Agilent iFunnel 6550 UHD Accurate Mass QTOF with iFunnel Technology operating in positive ionization mode equipped with an Agilent 1290 Infinity Binary pump and an Agilent 1290 Infinity autosampler. Accurate mass extracted ion chromatograms (EICs) were generated for the following possible Phase I transformations for S2218: M + 16 (mono-hydroxylation), M + 32 (di-hydroxylation), M + 14 (mono-hydroxylation and dehydrogenation), M − 133 and M − 266 (amide hydrolysis), M − 197 and M − 204 (*O*-dealkylation), M − 226 (*O*-dealkylation and mono-hydroxylation), and M − 195 (*O*-dealkylation and carbonyl reduction). EICs were examined in detail and recorded to support the presence or absence of each possible metabolite at levels at or above roughly 0.1% of the parent compound peak area in the time 0 samples. MS/MS experiments were performed to support or refute potential metabolites observed in a respective species based on EIC data. Details of the experimental and analytical methods can be found in the Supplementary data section.

The control samples verified the microsome functionality with 2% (human) and 21% (rat) of buspirone, and 30% (human) and 47% (rat) of loperamide remaining at the 120 min time point. Based on detailed analysis of the full scan accurate LC/MS of study samples, S2218 was resistant to oxidative metabolism by either the rat or human microsomes with 98.4 ± 0.4% (human) and 96.5 ± 0.4% (rat) of the parent remaining at the end of the microsomal incubation period. Mono-hydroxylation metabolites M433A and M433B (m/z = 434.1493) were observed for both species, with a combined EIC peak area at the 120 min time point of 0.6% (human) and 3.3% (rat) of the initial S2218 peak area. The mass spectral fragmentation pattern suggests that the hydroxyl group is on the benzothiadiazine ring of the 4-amino-2,2-dioxide-1*H*-benzo [*c*][1,2,6]thiadiazine scaffold (see Fig. 2). A primary alcohol resulting from *O*-dealkylation followed by carbonyl reduction (M222, m/z = 223.1441), was also observed for both species, with relative EIC peak areas at the 120 min time point of 0.3% (human) and 2.0% (rat) of the initial S2218 peak area. An aldehyde resulting from *O*-dealkylation, M220 (m/z = 221.1285), was detected for rat (0.2% at the 120 min time point), but not for human likely due to its low abundance. Note: All statements of scale (quantitative) assume that the relative response factor for all metabolites is equivalent for the mass spectrometry data. Taken together with the minimal loss of the parent compound during the microsomal incubations, the results suggest that the Phase I metabolic turnover rate be rather slow. No other potential metabolites were observed based on the EICs for the possible Phase I transformations.

3.2.2. Pharmacokinetics and oral bioavailability of S2218 and its sulfate salt (S8069) in Sprague-Dawley rats

The objective of this study was to estimate the pharmacokinetic (PK) parameters of S2218 in plasma following single oral dose and single intravenous administration in rats. This study also evaluated the oral bioavailability of S2218 and its sulfate salt (S8069) in 1% methyl cellulose formulations. For single intravenous administration, 4 male and 4 female Sprague-Dawley rats per group (Charles River, Hollister, CA) were bolus injected with S2218 at 1 mg/kg bw in 10% DMSO in 50 mM potassium phosphate buffer (pH = 7.4). Blood samples were collected from a jugular catheter at approximately 2, 5, 10, 30 min, 1, 2, 4, and 8 h post-dose. For oral administration, 4 male and 4 female Sprague-Dawley rats per group were given a single dose of either S2218 at 10, 30, or 100 mg/kg bw, or S8069 at 10 or 100 mg/kg bw, in 1% methylcellulose (MC) by oral gavage. S8069 is very soluble in water and completely dissolved in 1% MC at a concentration of 10 mg/mL. In contrast, S2218 formed a suspension in 1% MC at all dose levels. Blood samples were taken from a jugular catheter at approximately 15, 30 min, 1, 2, 4, 8, and 24 h post-dose. Plasma samples were prepared by centrifugation and frozen.

Proteins from plasma samples (25 μL) were precipitated by addition of acetonitrile (75 μL) containing an internal standard [*N*-(1-((4-amino-2,2-dioxido-1*H*-benzo[*c*][1,2,6]thiadiazin-5-yl)oxy)-2-methylpropan-2-yl)-2-(1*H*-imidazol-1-yl)isonicotinamide; S9284, 10 ng/mL], centrifuged, and 50 μL of the resulting supernatant was mixed with deionized water (100 μL). The resulting solutions were analyzed for S2218 by LC–MS/MS using a Waters XSELECT™ CSH 130 C18 column (50 × 2.1 mm, 3.5 μm) with a 0.1% formic acid/water and 0.1% formic acid/acetonitrile gradient system, and a API 3200 Q-Trap mass

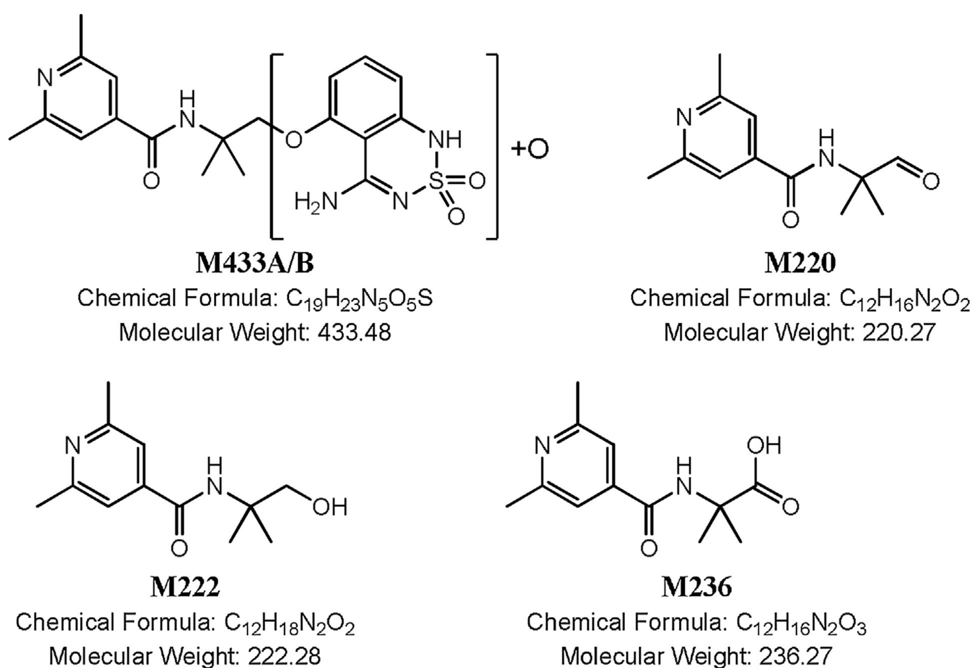

Fig. 2. Structures of S2218 *In Vitro* and *In Vivo* Metabolites.

spectrometer equipped with an Agilent 1100 binary pump with a CTC PAL injector. The parent compound and internal standard (IS) were detected using a source that was configured with turboionspray ionization in the positive mode using multiple-reaction monitoring (MRM) of mass transition pairs at *m/z* of 418.1/205.1 (S2218) and 456.1/243.2 (IS, S9284) amu. The PK parameters were analyzed by non-compartmental methods using Phoenix WinNonlin version 1.2 software (Pharsight/Certara company). Test article formulations prepared for this study were analyzed for concentration before and after dosing by HPLC-UV on an Agilent 1200 LC-DAD (240 nm). The test articles in suspension formulations for rats were within the acceptance criteria of ± 20% of their nominal concentrations. Details of the analytical methods can be found in the Supplementary data section.

S2218 was rapidly eliminated after intravenous administration in both male and female rats with mean terminal half-lives ($t_{1/2}$) of 0.185 and 0.173 h, respectively. Mean plasma clearance (CL) in rats averaged 33.5 mL/min/kg for males (60.9% of hepatic blood flow, [31]) and 38.3 mL/min/kg for females (69.6% of hepatic blood flow), and the volume of distribution at steady-state (V_{ss}) averaged 536 and 575 mL/kg (80.2% and 86.1% of total body water volume) for males and females, respectively (see Table 1).

Both test articles, S2218 and S8069, were poorly orally bioavailable in both male and female rats (%F = 0.14–0.77%). After and oral dose of either S2218 or its sulfate salt S8069, the mean values for the $t_{1/2}$ of S2218 in plasma ranged from 0.67 to 0.86 h in male rats and 0.75 to 1.25 h in female rats. No significant difference in half-life mean values between S2218 and S8069 were observed. For both S2218 and S8069, mean AUC_{last} and mean C_{max} increased with increasing oral dose, but the increase was not dose proportional in either male or female rats.

The ratio of female/male exposure to S2218 (C_{max} and AUC_{last}) in plasma was used for comparison of gender differences. For intravenous administration, the female/male ratios of mean AUC_{last} and C_{max} were 0.88 and 1.06, respectively. For oral administration, the female/male ratios of mean AUC_{last} ranged from 0.68 to 1.14 for S2218 and from 1.12 to 1.78 for S8069. Likewise, the ratios of mean female/male C_{max} ranged from 0.51 to 1.32 for S2218 and ranged from 1.14 to 1.75 for S8069. The relative bioavailability of S8069 to S2218 ranged from 0.74 to 1.92. Considering the variability in plasma C_{max} and AUC_{last} between individual animals, gender differences between male and female rats were not significant for either S2218 or S8069. The data from this study, as well as results from a separate excretion study discussed below, suggest that the absorption of S2218 or S8069 in rats was limited by permeability of S2218 in the small intestine.

Table 1
Pharmacokinetic Parameters for S2218 and S8069 in Sprague-Dawley Rats.

Cmpd	Route	S2218 Equiv. Dose (mg/kg bw)	Sex	C_{max} (ng/mL)	AUC_{last} (ng*h/mL)	T_{max} (h)	$t_{1/2}$ (h)	%F
S2218	iv	1	M	2980 ± 291	500 ± 47.9	0.033 ± 0.0	0.185 ± 0.026	–
			F	3150 ± 399	440 ± 57.1	0.033 ± 0.0	0.173 ± 0.004	–
	oral gavage	10	M	16.8 ± 6.03	18.1 ± 4.84	0.313 ± 0.125	0.763 ± 0.073	0.36%
			F	22.1 ± 8.55	19.1 ± 10.5	0.313 ± 0.125	0.749 ± 0.202	0.43%
		30	M	31.2 ± 14.6	33.2 ± 20.7	0.375 ± 0.144	0.669 ± 0.092	0.22%
			F	36.8 ± 14.8	37.7 ± 14.0	0.313 ± 0.125	0.774 ± 0.184	0.29%
		100	M	76.3 ± 28.8	96.6 ± 31.4	0.333 ± 0.144	0.842 ± 0.153	0.19%
			F	38.7 ± 20.4	65.9 ± 34.6	0.250 ± 0.0	1.25 ± 0.600	0.15%
S8069	oral gavage	7.83	M	21.4 ± 1.54	23.6 ± 5.26	0.313 ± 0.125	0.786 ± 0.110	0.60%
			F	24.4 ± 10.1	26.4 ± 14.3	0.250 ± 0.0	1.15 ± 0.353	0.77%
		78.3	M	60.5 ± 16.9	55.6 ± 17.8	0.250 ± 0.0	0.860 ± 0.340	0.14%
			F	106 ± 39.7	99.1 ± 23.2	0.250 ± 0.0	0.969 ± 0.315	0.29%

Male rat: CL = 33.5 mL/min/kg, V_{ss} = 536 mL/kg; Female rat: CL = 38.3 mL/min/kg, V_{ss} = 575 mL/kg; CL = clearance; V_{ss} = steady-state volume of distribution; %F = bioavailability.

3.2.3. In vivo metabolism of S2218 in rats

The *in vivo* metabolism of S2218 was evaluated following a single oral administration of its sulfate salt S8069 in male and female Sprague-Dawley rats. A group of 4 male and 4 female Sprague-Dawley rats (Charles River, Hollister, CA) was administered 100 mg/kg bw of S8069 (S2218 sulfate salt) in 1% MC (10 mL/kg bw) by oral gavage. Blood samples were collected from a jugular catheter at approximately 15, 30 min, 1, 2, 4, 8, and 24 h post-dose. Plasma samples (10 µL) from each animal were pooled together by each time point. Proteins from the combined plasma samples (80 µL) were precipitated by addition of acetonitrile (240 µL), centrifuged, and 260 µL of the resulting supernatant was concentrated to a volume of ~40 µL. The resulting solutions were analyzed for the parent compound and metabolites by LC-QTOF/MS using a Waters Acquity UPLC HSS T3 C18 column (50 × 2.1 mm, 1.8 µm) with a 0.1% formic acid/water and acetonitrile gradient system, and an Agilent iFunnel 6550A MS QTOF operating in positive ionization mode equipped with an Agilent 1290 Infinity Binary pump and an Agilent 1290 Infinity autosampler. Test article formulation prepared for this study was analyzed for concentration before and after dosing by HPLC-UV on an Agilent 1200 LC-DAD (240 nm). The test article concentration in the formulation was within the acceptance criteria of ± 20% of the nominal concentration of 10 mg/mL. Details of the analytical methods can be found in the Supplementary data section.

Three Phase I metabolites of S2218 were observed in rat plasma. The parent compound S2218, represented 95.98% of the relative +ESI Extracted Ion Chromatogram (EIC) peak area at the 0.25 h time point. The *O*-dealkylated metabolite of S2218 (M222) was the major metabolite which represented 2.61% of the relative +ESI EIC peak area at the 0.25 h time point. M222 was also observed in rat liver microsome incubations of S2218. A carboxylic acid M236, derived from alcohol M222, represented 1.05% of the relative +ESI EIC peak area at the 0.25 h time point. Only one of the two metabolites M433A/B resulting from mono-hydroxylation of the 4-amino-2,2-dioxido-1*H*-benzo[*c*][1,2,6]thiadiazine scaffold that were seen in the rat microsomal incubations was observed in rat plasma (*i.e.*, M433B). M433B represented 0.36% of the relative +ESI EIC peak area at the 0.25 h time point and was only seen at the 0.25 and 0.5 h time points. No Phase II metabolites were observed in the rat plasma samples (see Table 2 and Figs. 2 and 3).

3.2.4. Excretion of S2218 in rats

The purpose of this study was to evaluate the route of excretion of S2218 following a single oral administration of S8069 (S2218 sulfate salt) in 1% MC formulation to Sprague-Dawley rats. Four male and four female Sprague-Dawley rats (Charles River, Hollister, CA) were administered a single oral dose of S8069 at 10 mg/kg bw in 1% MC by oral gavage. Urine and fecal samples were collected from metabolic cages at room temperature over 0–24, 24–48, and 48–72 h post-dose. Samples were stored at the test facility at −20 °C prior to analysis. Calibration standards were prepared by spiking solutions of S8069 into blank feces and urine samples. Fecal samples were extracted with 0.2% formic acid/methanol containing an internal standard (S9284) using a tissue homogenizer (OMNI TH_Q) and an orbital shaker. The resulting fecal homogenate was centrifuged and supernatant diluted with 40% acetonitrile/water. Urine samples were extracted by the addition of 0.2% formic acid/water containing an internal standard (S9284), centrifuged, and supernatant diluted with 0.1% formic acid/water. The extracted fecal and urine samples were analyzed for S2218 by liquid chromatography-tandem mass spectrometry (LC–MS/MS) along with the internal standard S9284. Details of the analytical methods can be found in the Supplementary data section.

Table 2
S2218 and its Metabolites Oberved in Rat Plasma at 0.25 h Post Dose.

Compound	*m/z* (positive)	Formula	RT (min)	Peak Area ($\times 10^6$)	% Peak Area
S2218	418.1544	$C_{19}H_{24}N_5O_4S^+$	4.96	6.940	95.98
M222	223.1441	$C_{12}H_{19}N_2O_2^+$	1.83	0.189	2.61
M236	237.1234	$C_{12}H_{17}N_2O_3^+$	1.70	0.0761	1.05
M433B	434.1493	$C_{19}H_{24}N_5O_5S^+$	4.17	0.0262	0.36

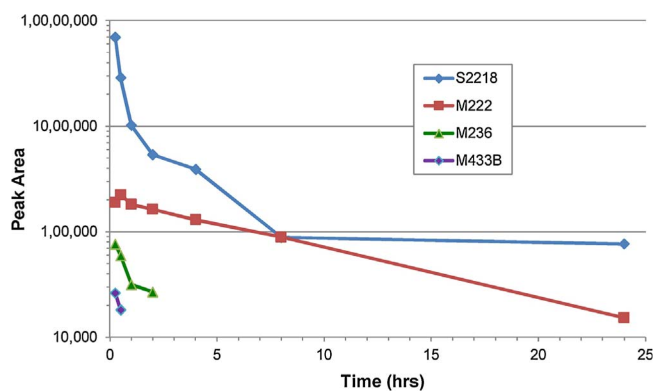

Fig. 3. Mean plasma concentrations of S2218, M222, M236 and M433B after oral administration of S2218 sulfate salt (S8069, 100 mg/kg) to Sprague-Dawley rats (male and female combined, n = 8).

The main route of excretion of S2218 after an oral dose of S8069 (S2218 sulfate salt) was through the feces with a relatively small percentage being excreted in the urine. The recovery of S2218 in feces within the first 24 h was 76.68% for males and 82.19% for females. Over the course of 72 h, approximately 85.94% of S2218 (82.08% in males and 89.79% in females) was excreted in the feces, and 0.137% of S2218 (0.044% in males and 0.230% in females) was excreted in the urine. On average, the total recovery of the orally administered dose was 86.07% (82.1% in males and 90.0% in females, see Table 3). These results suggest that S2218 does not undergo significant metabolism in the gut and is largely excreted unchanged. Taken together with the compound's rather low systemic bioavailability after oral administration, the rat excretion data suggests that S2218 is poorly absorbed by the intestinal tract and rapidly eliminated predominately in the feces.

3.3. Genotoxicity and mutagenicity studies

S2218 was evaluated for its genotoxic potential *in vitro* through a standard bacterial reverse mutation (5-strain Ames) and mammalian cell micronucleus test (see Table 4). All genetic toxicology studies were conducted in compliance with the FDA GLP regulations 21 CFR Part 58 [21] and OECD guidelines [22]. The data tables for the genotoxicity studies can be found in the Supplemental material.

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

S2218 was evaluated for the potential to induce point or frame shift mutations in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* strain WP2 *uvrA* in the presence and absence of metabolic activation with rat liver S9 from male rats induced with Aroclor™ 1254. The assay was performed in two phases, using the plate incorporation method. The first phase, the initial toxicity-mutation assay, was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. The second phase, the confirmatory mutagenicity assay, was used to evaluate and confirm the mutagenic potential of the test article. Both negative and positive controls were included in the study. The assay was designed to meet the current OECD Guideline for Testing of Chemicals No. 471, Bacterial Reverse Mutation Test [23].

The concentrations of S2218 tested in the initial toxicity-mutation assay were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate. Precipitate was observed at 5000 µg per plate. No cytotoxicity (*i.e.*, reduction in the background lawn and/or mean number of revertant colonies) was observed in any of the tester strains in either the presence

Table 3
Percent Recovery of S2218 in Feces and Urine Over 72 h Post-Dose.

Matrix	Gender	% Recovery of S2218			
		0–24 h	24–48 h	48–72 h	0–72 h
Feces	Male	76.68 ± 13.82	5.247 ± 4.432	0.298 ± 0.225	82.08 ± 15.35
	Female	82.19 ± 10.89	7.504 ± 6.517	0.152 ± 0.062	89.79 ± 7.63
Urine	Male	0.036 ± 0.025	0.007 ± 0.004	0.001 ± 0.001	0.044 ± 0.022
	Female	0.202 ± 0.216	0.020 ± 0.027	0.008 ± 0.010	0.230 ± 0.252
Feces + Urine	Male	–	–	–	82.1 ± 15.4
	Female	–	–	–	90.0 ± 7.5

or absence of S9 activation. In the confirmatory mutagenicity assay, the dose levels tested were 100, 333, 1000, 3333 and 5000 μg per plate. No toxicity was observed. Precipitate was observed beginning at 3333 μg per plate. In both the initial toxicity-mutation and confirmatory mutagenicity assays, no positive mutagenic responses were observed with any of the tester strains in the presence or absence of S9 activation. The negative controls for each tester strain were all within the historical negative control and/or spontaneous reversion ranges. All concurrent positive controls induced significant increase ($p < 0.01$, *t*-test) in colony counts (at least 4.7-fold) when compared to the corresponding negative controls and were at levels similar to the historical positive control data. It was concluded that S2218 was not mutagenic to *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* strain, WP2 *uvrA* at concentrations up to 5000 μg/plate, in the absence and presence of metabolic activation.

3.3.2. In vitro micronucleus test

The purpose of this study was to evaluate the genotoxic (clastogenic/aneugenic) potential of S2218 as measured by its ability to induce micronuclei in human peripheral blood lymphocytes (HPBL) in both the absence and presence of liver preparations (S9 mix) from male rats treated with Aroclor™ 1254. The experimental design followed the OECD Guideline for the Testing of Chemicals – 487, *In Vitro* Mammalian Cell Micronucleus Test [24].

A preliminary toxicity test was performed to establish the dose range for testing in the micronucleus test. In the micronucleus assay, human lymphocytes were exposed to varying concentrations of S2218 under three different conditions: 1) a 4 h exposure in the absence of S9 metabolic activation; 2) a 4 h exposure in the presence of S9; and 3) a 24 h exposure in the absence of S9 activation. Solvent (DMSO) and positive control (vinblastine, −S9; cyclophosphamide, +S9) cultures were also included. After the 4 h treatment in the non-activated and the S9-activated studies, the cells were centrifuged, the treatment medium was aspirated, the cells were washed with calcium and magnesium free phosphate buffered saline, re-fed with complete medium containing cytochalasin B (6.0 μg/mL) and returned to the incubator under standard conditions. For the 24 h treatment in the non-activated study, cytochalasin B (6.0 μg/mL) was added at the beginning of the treatment. Cells were collected after being exposed to cytochalasin B for 24 h (± 30 min), 1.5–2 normal cell cycles, to ensure identification and selective analysis of micronucleus frequency in cells that have completed one mitosis evidenced by binucleated cells. The cytochalasin B exposure time for the 4 h treatment in the non-activated and the S9-activated studies was 20 h (± 30 min). For the preliminary toxicity test, at least 500 cells were evaluated to determine the cytokinesis-blocked proliferation index (CBPI) at each dose level and the control. For the micronucleus assay, at least 1000 cells (500 cells from each duplicate culture), were evaluated to determine the CBPI at each dose level and the control. A minimum of 2000 binucleated cells from each concentration (1000 binucleated cells from each duplicate culture) were examined and scored for the presence of micronuclei.

In the preliminary toxicity assay, the doses tested ranged from 0.2 to 2000 μg/mL, which was the limit dose for this assay. Cytotoxicity (55 ± 5% CBPI relative to the vehicle control) was not observed at any dose in any of the three treatment conditions. At the conclusion of the treatment period, visible precipitate was observed at 2000 μg/mL in all three treatment conditions. Based upon these results, the doses chosen for the micronucleus assay ranged from 100 to 2000 μg/mL for all three treatment conditions.

In the micronucleus assay, cytotoxicity (55 ± 5% CBPI relative to the vehicle control), was not observed at any dose in any of the three treatment conditions. At the conclusion of the treatment period, visible precipitate was observed at 2000 μg/mL in the non-activated and S9-activated 4 h exposure groups, and at doses ≥ 1000 μg/mL in the non-activated 24 h exposure group. The doses selected for microscopic evaluation were 500, 1000, and 2000 μg/mL for the non-activated and S9-activated 4 h exposure groups, and 250, 500, and 1000 μg/mL in the non-activated 24 h exposure group.

No significant or dose-dependent increases in micronuclei induction were observed in treatment groups with or without S9 ($p > 0.05$; Fisher's Exact and Cochran-Armitage tests). Positive controls showed a significant increase in the percent micronucleated binucleated cells per dose ($p \leq 0.01$, Fisher's exact test, relative to the solvent control). These results indicate S2218 was negative for the induction of micronuclei in HPBL in the presence and absence of the exogenous metabolic activation system.

3.4. In vivo toxicological studies

S2218 was evaluated in 28-day dose-range finding and 13-week subchronic toxicology studies in rats in compliance with the FDA guidelines [25] Toxicological Principles for the Safety of Food Ingredients. S2218 was also evaluated for potential embryo/fetal toxicity

Table 4
Summary of Genotoxicity Studies Conducted on S2218.

End-Point	Test System	Concentration/Dose	Result
Reverse mutation (*in vitro*)	*S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* strain WP2 *uvrA*	*S. typhimurium* strains: 100–5000 μg/plate, plate incorporation, ± S9[a] *E. coli* strain WP2 *uvrA*: 100–5000 μg/plate, plate incorporation, ± S9[a]	Negative
Micronucleus formation (*in vitro*)	Human peripheral blood lymphocytes (HPBL)	500–2000 μg/mL, 4 h exposure, ± S9[a] 250–1000 μg/mL, 24 h exposure, −S9	Negative

[a] S9 from rat liver homogenate from male Sprague-Dawley rats treated with Aroclor-1254.

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 28-day and 13-week toxicology studies, as well as the range-finder and definitive developmental toxicity studies for S2218 can be found in the Supplemental material (see Table 5).

3.4.1. 28-Day dose-range finding toxicity study

The purpose of this study was to evaluate the potential systemic toxicity of S2218 in rats after dietary administration for 28 days in order to select doses for the 13-week subchronic toxicity study in rats. Three treatment groups of eight male and eight female CD® [Crl:CD®(SD)] rats (Charles River Laboratories, Raleigh, NC), were administered the test article in the diet at nominal dose levels of 10, 30, and 100 mg/kg bw/day. One additional group of eight animals/sex served as the control and received untreated (vehicle) diet. The test substance was administered continuously via the diet throughout the 28 day treatment period. Dietary concentrations (ppm) of S2218 for each group were adjusted each week based on bodyweight and food consumption data, in order to achieve constant doses in terms of mg/kg bw/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. Ophthalmoscopic examinations were conducted pre-test and prior to the terminal necropsy. Blood and urine samples for clinical pathology evaluations were collected from all animals prior to the terminal necropsy. At study termination, animals were sacrificed by carbon dioxide asphyxiation before subsequent exsanguination. Necropsy examinations were performed, organ weights were recorded, and only the liver was microscopically examined for animals treated at 0 and 100 mg/kg bw/day.

All animals survived to the scheduled necropsy. There were no test article-related clinical findings in detailed clinical observations at any dose level. When compared to control values, mean body weight was higher in males at 10 and 30 mg/kg bw/day with concurrent and statistically significant increases in food consumption ($p < 0.05$) during the dosing period. The higher mean body weight corresponded to a higher mean body weight gain (+7 to 9%) for these animals during the dosing period. The changes observed were not dose dependent and were only present in males. These changes may have been related to the administration of S2218 as corresponding increases in food consumption were also present; however, the changes were considered non-adverse. There were no changes in body weight noted in males at 100 mg/kg bw/day or in the females at any dose level.

There were no test article-related ophthalmoscopic findings. At the terminal interval, one control male was observed with retinal detachment; one female at 10 mg/kg bw/day was observed with chorioretinal hypoplasia; and one female at 30 mg/kg bw/day was observed with a retinal hemorrhage. These findings were not considered to be test article-related effects. The observations noted were representative of pathology that would be expected for this group of animals considering age, sex, and strain.

There were no test article-related effects among hematology parameters, coagulation times, or clinical chemistry analytes in either sex at any dose level. All mean and individual values were considered within an acceptable range for biologic and/or procedure-related variation despite occasional mean values that reached statistical significance. There were no test article-related alterations observed among urinalysis parameters in either sex at any dose level. There were occasional differences found in urine volume and specific gravity that were not considered toxicologically meaningful due to their sporadic nature and the inherent variability of these endpoints.

There were no test article-related organ weight, macroscopic or microscopic changes noted at any dose level. Increase in mean absolute weights and organ-to-body and -brain weight ratios of the liver in males at ≥10 mg/kg bw/day were considered incidental because the magnitude of increase was small without statistical significance, lack of microscopic correlates, and, in general, a lack of dose response in either sex. All microscopic findings were considered incidental due to minimal magnitude, similar incidence in control and treated animals, lack of similar findings in higher dose animals, lack of similar findings in opposite sex, and/or known as background findings in this species.

There were no adverse effects noted for any parameter examined. As a result, the no-observed-adverse-effect-level (NOAEL) following 28 days of dietary administration of S2218 was 100 mg/kg bw/day, the highest dose level tested. Average daily compound consumption for animals given 10 mg/kg bw/day was 12.5 and 11.5 mg/kg bw/day; for 30 mg/kg bw/day, 36.2 and 31.9 mg/kg bw/day, and 100 mg/kg bw/day, 119.8 and 111.8 mg/kg bw/day, for males and females, respectively.

3.4.2. 13-Week dietary toxicity study in rats

The purpose of this study was to evaluate the potential subchronic toxicity and toxicokinetic (TK) profile of the test article, S8069 (S2218 sulfate salt), in rats after dietary administration for 13 weeks. This study is based on the United States Food and Drug Administration (FDA) Toxicological Principles for the Safety of Food Ingredients [25] and conducted in accordance with the FDA Good Laboratory Practice (GLP) Regulations, 21 CFR Part 58 [21]. Compound was administered in the diet to four groups of twenty male and twenty female CD® [Crl:CD®(SD)] rats (Charles River Laboratories, Raleigh, NC) at nominal dose levels of 0 (control), 30, 70, and 140 mg/kg bw/day. Additionally, one control group of four animals/sex and three treated groups of eight animals/sex/group served as toxicokinetic (TK) animals and received the vehicle or test article diet in the same manner as the main study groups at respective dose levels of 0, 30, 70, and 140 mg/kg bw/day. The vehicle or test article diet was available *ad libitum* for 13 weeks.

Table 5
Summary of Subchronic and Developmental Toxicity Studies Conducted on S2218 or its Sulfate Salt S8069.

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 bw/day (food ad-mix)	Slight increase in food consumption and mean body weight in males at 10 and 30 mg/kg bw/day. No other test-article related findings; NOAEL = 100 mg/kg bw/day
13-Week Sub-Chronic Toxicity Study	Male & Female Sprague-Dawley Rats Main study: - 20 animals/sex/group TK satellite group: - 8 animals/sex/group	30, 70, 140 mg/kg bw/day (food ad-mix)	No test-article related findings; NOAEL = 140 mg/kg bw/day
Dose Range Finding Developmental Toxicity Study	Bred Female Sprague-Dawley Rats - 8 animals/group	125, 250, 500, 1000 mg/kg bw/day (oral gavage)	No maternal toxicity or effect on intrauterine growth up to 1000 mg/kg bw/day
Definitive Developmental Toxicity Study	Bred Female Sprague-Dawley Rats - 25 animals/group	250, 500, 1000 mg/kg bw/day (oral gavage)	NOAEL for both maternal toxicity and embryo/fetal development = 1000 mg/kg bw/day

Dietary concentrations (ppm) of S8069 for each group were adjusted each week based on bodyweight and food consumption data, in order to achieve constant doses in terms of mg/kg bw/day. At the conclusion of the study (Day 92), animals were sacrificed by carbon dioxide asphyxiation before subsequent exsanguination.

Survival, clinical observations, body weight gain, food consumption, hematology, clinical chemistry, urinalysis, organ weights, macroscopic examination, and histopathologic evaluation of at least 51 tissues were performed to assess potential toxicity (control and high-dose animals only; see Supplementary data for list of tissues examined histopathologically). Observations for morbidity, mortality, injury, and the availability of food and water were conducted twice daily for all animals. Cageside clinical observations were conducted once daily for main study animals. A functional observational battery (including, but not limited to, evaluation of activity, arousal, autonomic and physical function, neuromuscular function, salvation, and respiration), motor activity assessment, 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 the main study animals on Days 14 and 46, and again prior to termination. Urinalysis and samples for coagulation evaluations were collected prior to termination only. Blood for TK analysis was collected from one cohort of 3 animals/sex (control animals) at 1 h post the start of the dark cycle on Days 7 and 91. Samples were collected at alternating time points from 2 cohorts of 3 animals/sex (treated animals) at pre-dose, 1, 3, 6, 12, and 24 h post the start of the dark cycle on Days 7 and 91. At study termination, necropsy examinations were performed and organ weights were recorded for all main study animals and appropriate organ weight ratios were calculated (relative to body and brain weights). Microscopic examination of fixed hematoxylin and eosin-stained paraffin sections were performed on sections of tissues from the control and high-dose (140 mg/kg bw/day) groups.

Average daily compound consumption for animals given 30 mg/kg bw/day was 30.1 and 30.6 mg/kg bw/day; for 70 mg/kg bw/day, 69.4 and 69.3 mg/kg bw/day; and for 140 mg/kg bw/day, 139.4 and 140.9 mg/kg bw/day, for males and females, respectively. Formulation analysis demonstrated that the formulation preparation method produced homogeneous preparations (RSD ≤ 15%).

Blood sample collections were based on the start of the dark cycle. S2218 was quantifiable up to 24 h post the start of the dark cycle on both Days 7 and 91. Systemic exposure to S2218 was highly variable and appeared to be independent of sex following dietary administration of S2218 sulfate salt (S8069) to male and female rats (see Table 6). Individual and mean plasma concentration data and female to male dose normalized AUC ratios were not indicative of any consistent gender difference (female to male exposure ratio ranged from 0.248 to 1.75). Therefore, the following discussion is based on TK parameters calculated the combined mean plasma concentrations from both the male and female animals.

Peak S2218 mean plasma concentrations were achieved by 3 or 6 h post the start of the dark cycle for the low-dose group, 3 or 12 h post the start of the dark cycle for the mid-dose group, and 6 or 12 h post the start of the dark cycle for the high-dose group on Days 7 and 91. Consistent with results from the single dose PK studies in rats, systemic exposure to S2218 was relatively low. For example, at 70 mg/kg bw/day, the combined mean C_{max} on Day 91 was 40.0 ng/mL (77.6 nM), and the combined mean $AUC_{0\text{-}24h}$ was 627 ng*h/mL. Systemic exposure ($AUC_{0\text{-}24h}$) and C_{max} values of S2218 generally increased with increasing dose (determined average achieved daily doses). On Day 7, a 1: 2.4: 4.9-fold increase in achieved daily dose resulted in an approximate 1: 3.2: 6.9-fold increase in $AUC_{0\text{-}24h}$ values and an approximate 1: 3.1: 7.8-fold increase in C_{max} values. On Day 91, a 1: 2.3: 4.1-fold increase in achieved daily dose resulted in an approximate 1: 1.6: 7.1-fold increase in $AUC_{0\text{-}24h}$ values and an approximate 1: 0.85: 4.7-fold increase in C_{max} values. The combined dose normalized systemic exposure ($AUC_{0\text{-}24h}$/Dose) did not display a consistent increase from Day 7 to Day 91. The combined dose normalized comparison ratios of Day 91/Day 7 were 1.88, 0.954, and 2.30 at nominal doses of 30, 70, and 140 mg/kg, respectively. However, the dose normalized comparison ratios of Day 91/Day 7 were generally higher in the male animals than in the females. For example, the dose normalized comparison ratios of Day 91/Day 7 at the 140 mg/kg bw/day nominal dose for the male animals was 4.55 and that of the female animals was 0.768.

There were no test article-related unscheduled deaths. Mortality occurred in a high-dose main study male that was euthanized *in extremis* on Day 69. Clinical observations noted for this animal prior to euthanasia included a malocclusion and associated clinical signs of black material around the eyes/nose, red material in the bedding, and unkempt appearance. These clinical signs were noted beginning on Day 42 with a general persistence though Day 68. A reduction in movement during standard open-field testing was observed for this animal

Table 6
Toxicokinetics of S8069 in Male and Female Sprague-Dawley Rats Administered in Diet for 13-Weeks.

Time Point	Nominal Dose (mg/kg bw)	Actual Dose (mg/kg bw)	Sex	C_{max} (ng/mL)	C_{max}/Dose (ng/mL/mg/kg)	T_{max} (h)	$AUC_{0\text{-}24h}$ (ng*h/mL)	$AUC_{0\text{-}24h}$/Dose (ng*h/mL/mg/kg)	ACUM Ratio[a]	F:M Ratio[b]
Day 7	30	25.8	M	14.0	0.543	1	134	5.19	NA	NA
		27.8	F	20.1	0.723	3	253	9.10	NA	1.75
		26.8	M + F	11.1	0.414	3	194	7.24	NA	NA
	70	62.8	M	32.8	0.522	6	641	10.2	NA	NA
		65.4	F	44.4	0.679	0	610	9.33	NA	0.914
		64.1	M + F	34.3	0.535	12	626	9.77	NA	NA
	140	125	M	57.0	0.456	6	1020	8.16	NA	NA
		137	F	137	1.00	24	1640	12.0	NA	1.48
		131	M + F	86.5	0.660	6	1330	10.15	NA	NA
Day 91	30	28.7	M	35.8	1.25	6	427	14.9	2.86	NA
		30.5	F	58.8	1.93	6	376	12.3	1.35	0.828
		29.6	M + F	47.3	1.60	6	402	13.6	1.88	NA
	70	68.6	M	60.4	0.880	3	748	10.9	1.07	NA
		66.1	F	27.8	0.421	0	507	7.67	0.821	0.703
		67.3	M + F	40.0	0.594	3	627	9.32	0.954	NA
	140	123	M	358	2.91	12	4550	37.0	4.55	NA
		122	F	85.0	0.697	12	1120	9.18	0.768	0.248
		122	M + F	222	1.82	12	2840	23.3	2.30	NA

NA = Not applicable.
[a] ACUM Ratio = ($AUC_{0\text{-}24h}$ at Day 91/Dose Day 91)/($AUC_{0\text{-}24h}$ at Day 7/Dose Day 7).
[b] F:M = ($AUC_{0\text{-}24h}$/Dose female)/($AUC_{0\text{-}24h}$/Dose male).

beginning during Week 1 when compared to individual counts from other animals designated to this dose level/group. A gradual decrease in food consumption and body weight loss was noted in this animal beginning on Day 42. Clinical pathology changes included evidence of an inflammatory stimulus (increases in neutrophil counts and fibrinogen), which was not considered test article-related due to the lack of similar findings in animals that survived to scheduled termination. Other values for clinical pathology endpoints in this animal were generally within expected values for biologic and procedure-related variation. Macroscopic findings consistent with a malocclusion were observed for this animal following gross evaluation. Microscopic findings included inflammation/ulceration of the nose. The cause of moribundity was considered to be incidental and not related to S8069 due to the absence of similar clinical observations in other animals at this dose level.

Mortality occurred in a mid-dose toxicokinetic female at 70 mg/kg bw/day that was euthanized *in extremis* on Day 70. Clinical observations noted for this animal prior to euthanasia included brown hair discoloration of the anogenital region, hunched posture, loss of skin elasticity, and thin body condition. These clinical signs were noted beginning on Day 67 and persisted to euthanasia. Body weight loss was also observed for this animal beginning on Day 56 and continued to be noted through Week 10. The cause of the death of this animal could not be determined following macroscopic examination (*e.g.*, all tissues within normal limits). However, the death was considered incidental and not related to S8069 due to the absence of similar clinical observations or declination of body weight in other animals at this dose level or at the next higher dose level.

There were no test article-related clinical findings. A clonic/tonic convulsion was observed for one mid-dose main study male at 70 mg/kg bw/day on Day 90. The convulsion may have been a result/reaction to handling. This was a single occurrence and was transient in nature. Similar observations were not noted in any other animals at this dose level or in animals receiving S8069 at higher doses. There were also no S8069-related effects noted on FOB or neurobehavioral examinations/endpoints at this dose or at the highest dose level; therefore, this observation was considered incidental and not related to S8069. There were no clear test article-related effects on locomotor activity at any dose level over the course of the study. There were no test article-related ophthalmic effects at any dose level. One control male and two males at 30 mg/kg bw/day were observed with chorioretinal hypoplasia at the terminal interval, however, these findings were not considered to be a test article related effect. There were no test article-related body weight effects or effects on food consumption (see Figs. 4 and 5).

There were no test article-related effects among hematology parameters, coagulation times, or clinical chemistry analytes in either sex at any dose level. There were a few sporadic animals across all groups, including controls, with greater than expected aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) activities, which were not considered test article-related due to the sporadic nature and similar occurrence in control and treated animals. All other statistically significant or apparent differences among clinical chemistry endpoints were not considered test article-related due to their negligible magnitude, sporadic nature, and/or relation to expected values for biologic and procedure-related variation. There were no test article-related alterations observed among urinalysis parameters in either sex at any dose level. There were occasional differences found in urine volume and specific gravity that were not considered toxicologically meaningful due to their sporadic nature and the inherent variability of these endpoints.

There were no S8069-related macroscopic observations, organ weight changes, or microscopic findings noted in either sex. Macroscopic observations were few and were of the type typically seen in rats of this strain and age. The only statistically significant changes in the 140 mg/kg bw/day group occurred in females. There was a decrease in both liver

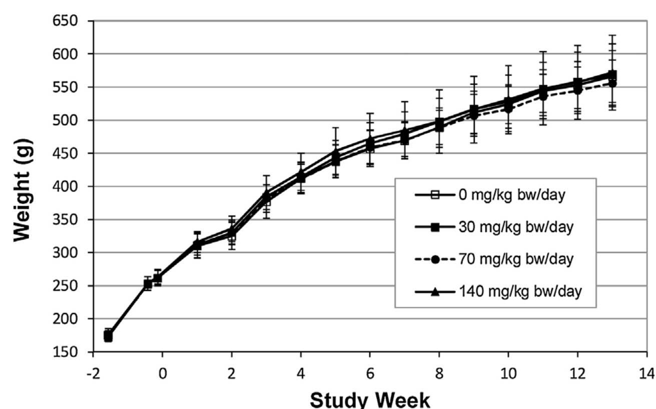

Fig. 4. Mean body weights of male Sprague-Dawley rats receiving S8069 for 13 weeks (0, 30, 70 mg/kg bw/d, n = 20; 140 mg/kg bw/d, n = 20 thru Week 9, n = 19 Weeks 10–13).

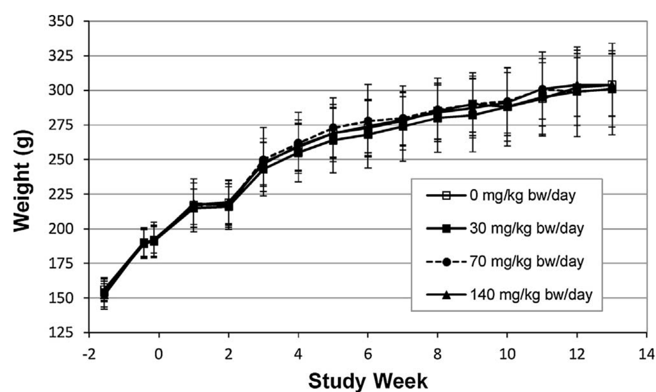

Fig. 5. Mean body weights of female Sprague-Dawley rats receiving S8069 for 13 weeks (all doses, all time points, n = 20).

and kidney relative to brain weight (liver/brain weight: 9.8% decrease, $p < 0.05$; kidney/brain weight: 8.1% decrease, $p < 0.05$) as a result of a slightly heavier brain weight (3.4% increase relative to control, $p > 0.05$) in this group. Also, the pituitary gland weight in the 140 mg/kg bw/day females was decreased as compared to controls by mean absolute weight (15.1% decrease, $p < 0.05$) and relative to both body and brain weight (pituitary/body weight: 14.6% decrease, $p < 0.05$; pituitary/brain: 17.7% decrease, $p < 0.01$). None of these differences had microscopic correlates and all were considered spurious and not related to administration of S8069. Microscopic findings noted in these animals were of the type typically seen in rats of this strain and age and none were attributed to administration of S8069.

Overall, no adverse test article-related effects were noted in any parameter evaluated. As a result, the No-Observed-Adverse-Effect-Level (NOAEL) following 13-weeks of dietary administration of S2218 sulfate salt (S8069) was 140 mg/kg bw/day, the highest dose level tested, in male and female rats. Following 13-weeks of dietary administration, the NOAEL AUC_{0-24} for S2218 was 2840 ng*h/mL, and C_{max} was 222 ng/mL, for males and females combined.

3.4.3. Dose range-finding developmental toxicity in rats

The objective of the study was to determine dosage levels of S8069 (S2218 sulfate salt) to be evaluated in a definitive developmental toxicity study conducted in accordance with the OECD Guidelines for Testing of Chemicals Guideline 414, Prenatal Developmental Toxicity Study, January 2001 [26] and the United States FDA Redbook 2000: IV.C.9.b Guidelines for Developmental Toxicity Studies, January 2001 [27] in rats.

The test article, S8069, in the vehicle (1% methyl cellulose [400

cps]) 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, at dosage levels of 125, 250, 500, and 1000 mg/kg bw/day (dose volume 10 mL/kg bw). 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. Clinical observations noted at the daily examinations or approximately 1 h following dose administration, including hair loss on various body surfaces, occurred infrequently, at similar frequencies in the control group, and/or in a manner that was not dose-related. Mean maternal body weights, body weight gains, net body weights, net body weight gains, and gravid uterine weights in the 125, 250, 500, and 1000 mg/kg bw/day groups were similar to that in the control group. Differences from the control group were slight and not statistically significant. Mean maternal food consumption, evaluated as g/animal/day and g/kg bw/day, in the 125, 250, 500, and 1000 mg/kg bw/day groups was comparable to that in the control group. Differences from the control group were slight and not statistically significant, with the exception of significantly ($p < 0.05$) higher mean food consumption for the 250 mg/kg bw/day group on Gestation Day 20–21 compared to the control group. In the absence of higher food consumption in the 500 and 1000 mg/kg bw/day groups for this same interval, the higher food consumption in the 250 mg/kg bw/day group during Gestation Day 20–21 was not considered to be test article-related.

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 bw/day and all females were determined to be gravid. 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.

Intrauterine growth and survival were unaffected by test article administration at dosage levels of 125, 250, 500, and 1000 mg/kg bw/day. Parameters evaluated included post-implantation loss, live litter size, mean fetal body weights, and fetal sex ratios. Mean numbers of *corpora lutea* and implantation sites and the mean litter proportions of pre-implantation loss were comparable across all groups. Differences from the control group were slight and not statistically significant. The numbers of fetuses (litters) available for morphological evaluation were 115(8), 117(8), 108(8), 112(8), and 114(8) in the control, 125, 250, 500, and 1000 mg/kg bw/day groups, respectively. No external developmental malformations or variations were observed in fetuses in this study.

Based on lack of effects on survival, body weight, food consumption, maternal necropsy observations, intrauterine growth and survival, and fetal external morphology, dosage levels of 250, 500, and 1000 mg/kg bw/day were selected for a definitive embryo/fetal development study of S8069 (S2218 sulfate salt) administered orally by gavage to bred Crl:CD(SD) rats.

3.4.4. Developmental toxicity study in rats

The objective of the study was to determine the potential of S8069 (S2218 sulfate salt) 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. This study was conducted in general accordance with the United States Food and Drug Administration (FDA) Redbook 2000: IV.C.9.b Guidelines for Developmental Toxicity Studies, January 2001 [27] and the Organization of Economic Co-operation and Development Guidelines (OECD) for Testing of Chemicals Guideline 414 [26].

The test article, S8069, in the vehicle (1% methyl cellulose [400 cps]) was administered orally by gavage to 3 groups of 25 bred female Crl:CD(SD) rats (Charles River Laboratories, Raleigh, NC) once daily from Gestation Days 6 through 20, at dosage levels 250, 500, and 1000 mg/kg bw/day (dose volume 10 mL/kg bw). A concurrent control group composed of 25 bred females received the vehicle on a comparable regimen. The females were approximately 12 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.

One female from the 1000 mg/kg bw/day dose group was found dead on Gestation Day 17. This animal was noted with 14 dead fetuses and one early resorption during the macroscopic exam. No other macroscopic findings, clinical observations, post-dose observations, or significant effects on body weight or food consumption were noted. Therefore, this death was likely not test article-related. One female from the 500 mg/kg bw/day dose group was euthanized *in extremis* on Gestation Day 16 due to reduced food consumption, resulting in decreased defecation and reduced body weight gain/body weight losses, beginning Gestation Day 11 and continuing through euthanasia. During the macroscopic exam, this female was noted with adhesions on the lung, a mass on the heart, and an esophageal perforation, indicating a dosing error. Therefore, the moribund condition of this female was considered accidental and not attributed to the test article. A separate female from the 500 mg/kg bw/day group delivered on Gestation Day 21, and was noted with no remarkable findings during necropsy. One female each from the 250, 500, and 1000 mg/kg bw/day dose groups were determined to be non-gravid. All remaining females were determined to gravid at the scheduled euthanasia on Gestation Day 21.

No test article-related clinical observations were noted during the daily exams at any dosage level. Observations such as hair loss, dried or wet red/clear material around the nose, mouth or urogenital area, scabbing, and rales were noted sporadically, in single females, and/or in a non-dose-related manner. At the 1000 mg/kg bw/day dosage level, rales was observed for 6 females 1 h following dose administration between Gestation Day 8 and 17, and salivation was noted for 4 females between Gestation Day 12 and 16 at the time of dose. These observations were considered test article-related but not adverse, since they were generally noted as single occurrences for each of the animals, and did not persist at the daily clinical exams. Other post-dose findings were observed in single animals in a non-dose-related manner.

Food consumption was comparable across all groups throughout the study. Females at the 1000 mg/kg bw/day dosage level had a mean body weight loss (2 g) during Gestation Day 8–9, resulting in a significantly lower mean body weight gain ($p < 0.01$) during Gestation Day 6–9 compared to controls. Slightly lower mean body weight gain was also noted in females at the 500 mg/kg bw/day dose level during Gestation Day 6–9 (see Fig. 6). However, in both dose groups, this was transient and did not affect the overall body weight gain for the overall study interval (Gestation Day 6–21). Mean body weight gains and mean

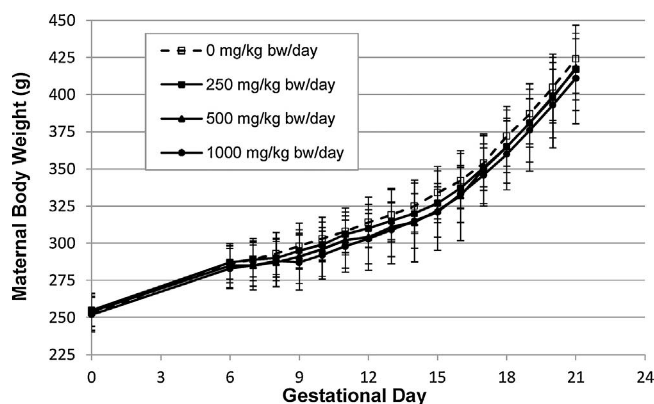

Fig. 6. Oral (Gavage) Developmental Toxicity Study of S8069 in Rats: Mean maternal body weights during gestation.
0 mg/kg bw/d: n = 25/group (GD 0–21);
250 mg/kg bw/d: n = 24/group (GD 0–21);
500 mg/kg bw/d: n = 24/group (GD 0–16), n = 23/group (GD 17–20), n = 22/group (GD 21);
1000 mg/kg bw/d: n = 24/group (GD 0–17), n = 23/group (GD 18–21).

body weights at the 250 mg/kg bw/day dosage level were unaffected by treatment. The initial lower body weight gain in the 1000 and 500 mg/kg bw dose groups was considered test article-related but not adverse, as mean absolute body weights were generally unaffected. There were no significant test article-related effects on gravid uterine weights, net body weights, or net body weight changes at any dosage level. There were no significant maternal necropsy macroscopic findings at any dosage level. At the scheduled necropsy on Gestation Day 21, there were no significant maternal macroscopic findings at any dosage level.

Intrauterine growth and survival were unaffected by test article administration at dosage levels of 250, 500, and 1000 mg/kg bw/day. Parameters evaluated included post-implantation loss, live litter size, mean fetal body weights, and fetal sex ratios. Mean numbers of *corpora lutea* and implantation sites and the mean litter proportions of pre-implantation loss were similar across all groups (Table 7).

The numbers of fetuses (litters) available for morphological evaluation were 355(25), 331(24), 339(23), and 328(23) in the control, 250, 500, and 1000 mg/kg bw/day groups, respectively. Malformations were observed in 3(3), 3(2), 4(4), and 1(1) fetuses (litters) in these same respective dose groups and were considered spontaneous in origin.

There were no external malformations observed in the test article-treated groups. In the control group, one fetus had omphalocele (several loops of intestine protruded through an opening in the umbilicus) and a second fetus had anophthalmia (bilateral).

Visceral malformations were noted in 0(0), 3(2), 3(3), and 1(1) fetuses (litters) in the control, 250, 500, and 1000 mg/kg bw/day groups, respectively. Two fetuses from one litter in the 250 mg/kg bw/day group and one fetus in each of the 500 and 1000 mg/kg bw/day groups had an interventricular septal defect (an opening in the anterior portion of the septum). A single fetus in each of the 250 and 500 mg/kg bw/day groups had a retroesophageal aortic arch. A single fetus in the 500 mg/kg bw/day group had an absent left kidney, ureter, and uterine horn. The mean litter proportions of these malformations were not statistically significantly different from the concurrent control group. The visceral malformations noted in the test article-treated groups were noted infrequently and in a manner that was not dose-related and therefore, were not attributed to the test article.

Skeletal malformations were noted in one fetus in the control group and 3(3) fetuses (litters) in the 500 mg/kg bw/day group. A single fetus in the control group had sternoschisis (sternal bands not joined, entire length). In the 500 mg/kg bw/day group, one fetus had severely malaligned sternebrae, a second fetus had fused sternebrae (this same fetus also had a retroesophageal aortic arch noted above) and a third fetus had a vertebral anomaly with an associated rib anomaly. The vertebral anomaly consisted of fused arches, ribs, and costal cartilages, a thick rib, a short rib with no associated costal cartilage, malpositioned costal cartilages, and a severely malaligned sternebra. These malformations in the 500 mg/kg bw/day group were not attributed to the test article because they occurred in single fetuses and no skeletal malformations were observed at 1000 mg/kg bw/day (highest dosage level tested).

No test article-related external, visceral, or skeletal developmental variations were noted. Findings observed in the test article-treated groups were noted infrequently, similarly in the control group, were not observed in a dose-related manner, the differences in the mean litter proportions were not statistically significant compared to the concurrent control group, and/or the values were within the ranges of the testing laboratories historical control data.

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 bw/day (the highest dosage level evaluated) was considered to be the NOAEL for maternal toxicity and embryo/fetal development when S8069 was administered orally by gavage to bred Crl:CD(SD) rats.

Table 7
Developmental Toxicity Study of S8069 (S2218 sulfate salt) in Rats: Summary of Fetal Data.

Dose Group (mg/kg bw/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	178	177	355	0	21	0	21	376	403	27	NA	25
	Mean	7.1	7.1	14.2	0.0	0.8	0.0	0.8	15.0	16.1	1.1	5.8	
	S.D.	2.28	2.02	2.08	0.00	1.31	0.00	1.31	2.37	2.37	1.91	0.36	
	S.E.	0.46	0.40	0.42	0.00	0.26	0.00	0.26	0.47	0.47	0.38	0.07	
250	Total	158	173	331	0	23	0	23	354	363	9	NA	24
	Mean	6.6	7.2	13.8	0.0	1.0	0.0	1.0	14.8	15.1	0.4	5.9	
	S.D.	1.44	1.41	1.96	0.00	1.08	0.00	1.08	1.54	1.51	0.58	0.31	
	S.E.	0.29	0.29	0.40	0.00	0.22	0.00	0.22	0.31	0.31	0.12	0.06	
500	Total	151	188	339	0	16	0	16	355	370	15	NA	23
	Mean	6.6	8.2	14.7	0.0	0.7	0.0	0.7	15.4	16.1	0.7	5.6	
	S.D.	2.11	1.77	2.30	0.00	0.82	0.00	0.82	2.04	1.90	0.93	0.39	
	S.E.	0.44	0.37	0.48	0.00	0.17	0.00	0.17	0.43	0.40	0.19	0.08	
1000	Total	168	160	328	0	7	0	7	335	348	13	NA	23
	Mean	7.3	7.0	14.3	0.0	0.3	0.0	0.3	14.6	15.1	0.6	5.6	
	S.D.	2.57	2.34	2.34	0.00	0.63	0.00	0.63	2.09	2.42	1.16	0.37	
	S.E.	0.54	0.49	0.49	0.00	0.13	0.00	0.13	0.43	0.50	0.24	0.08	

NA = not applicable.

4. Discussion and conclusions

S2218 is a member of a novel series of 5-alkoxy substituted benzothiadiazine analogs which function as positive allosteric modulators (PAMs) of the human sweet receptor heterodimer hTAS1R2/hTAS1R3. S2218 differs from the previously reported PAMs S6973 and S617 only in the structure of the alkoxy side chain appended to the 5-position of a benzothiadiazine nucleus [12]. Like the previously reported benzothiadiazine analogs, S2218 is highly selective for activity on hTAS1R2/hTAS1R3 and does not exhibit significant cross-reactivity with other GPCRs, ion channels, nuclear receptors, or transporters. In addition, none of the aforementioned benzothiadiazine analogs significantly inhibit the major CYP enzymes indicating that these compounds should not affect the metabolism of drug substances. *In vitro* studies using both rat and human liver microsomes have shown that these benzothiadiazine derivatives themselves are resistant to oxidative metabolism by CYP enzymes with > 97% of the parent compounds remaining after a 60 min incubation with microsomes of either species [12].

The PK profile of S2218 in male and female Sprague-Dawley rats is very similar to that of the previous reported 5-alkoxy benzothiadiazine analogs S6973 and S617 ([12]; see Tables 1 and 8). All three compounds were rapidly eliminated after intravenous administration in both male and female rats with mean terminal $t_{1/2}$'s ranging from 0.173 and 0.78 h. After oral dosing, the plasma $t_{1/2}$ values for S2218 tended to be significantly shorter for S2218 relative to comparable oral doses of S617 and S6973. For example, the $t_{1/2}$ for a 10 mg/kg bw oral dose of S2218 in males rats was 0.76 h, compared to $t_{1/2}$'s of 3.85 and 2.98 h for S617 and S6973, respectively, at the same oral dose.

For all three compounds, AUC_{last} increased in a less than dose proportional manner. The oral bioavailability (%F) for all three compounds was rather poor and tended to decrease with increasing dose. For S2218 administered as a suspension in 1% MC, oral bioavailability ranged from 0.15% to 0.43% while the oral bioavailability of an aqueous solution of S2218 sulfate salt (S8069) ranged from 0.14% to 0.77%, suggesting that the absorption of the compound was not limited by its rate of dissolution. The dose normalized S8069 $AUC_{0\text{-}24h}$ values from the TK animals in the 13-week dietary toxicology study were somewhat higher than those of the single dose PK study. Using the $AUC_{0\text{-}24h}$ values from the intravenous PK study of S2218, and correcting the dose normalized oral S8069 $AUC_{0\text{-}24h}$ values for the S2218 equivalent dose, the bioavailability of S8069 in the 13-week toxicology study ranged from 1.28–3.36% on Day 7, and from 2.15–9.14% on Day 91 in male and female rats. Given that S8069 was administered by oral gavage as a solution in 1% MC in the PK study, and as a food ad-mix in the TK study, this difference is likely to be due to a food effect on oral absorption.

For S617 administered as a suspension in 1% MC, oral bioavailability ranged from 0.20% to 1.73%, while that of an aqueous solution of S617 sodium salt (30 mg/kg bw) was significantly higher ranging from 5.29% to 10.0% in male and female rats, indicating that its oral absorption may be limited by its rate of dissolution (Liu and Chi, unpublished results). Consistent with this finding, the bioavailability of S617 seen in the TK studies associated with the 90-day subchronic toxicity study where S617 was administered in the diet as a food admix, was similar to that seen with the aqueous solution of S617 sodium salt and ranged from 8.97% to 13.9% on Day 90 of the study [12]. The oral bioavailability of a suspension of S6973 in male rats decreased from 9.56% at the 5.0 mg/kg bw dose to 3.93% at the 20 mg/kg bw dose. In contrast, the bioavailability of S6973 administered in the diet as a food ad-mix was 8.04% at the 5.0 mg/kg bw dose versus 7.73% at the 20 mg/kg bw dose on Day 1 of the 90-day subchronic toxicity study [12]. This suggests that the decrease in bioavailability seen with increasing dose of S6973 in the PK study may also be associated with its rate of dissolution, although not to the extent seen with S617.

In order to determine whether pre-systemic metabolism could be responsible for the rather poor oral bioavailability of these compounds, their mode of elimination was studied in rats. After a single oral dose of either S2218 sulfate salt (S8069), S617 sodium salt, or S6973 to rats, 86.1% to 93.5% of the administered dose of all three compounds was recovered in the urine and feces over 72 h post-dose, with the vast majority (79.4–90.8%) being excreted in the feces within the first 24 h. These results indicate that all three compounds do not undergo significant metabolism in the gut and are largely excreted unchanged. Taken together with their low systemic bioavailability after oral administration, the rat excretion data suggests that all three benzothiadiazine analogs are poorly absorbed by the intestinal tract and rapidly eliminated in the feces [12].

S2218 was evaluated for its 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 an *in vitro* micronucleus test in human peripheral blood lymphocytes (HPBL). S2218 was found to be neither mutagenic, clastogenic, nor aneugenic in these *in vitro* genotoxicity assays. Benzothiadiazines S6973 and S617 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, and non-clastogenic in a chromosome aberration test in HPBLs [12]. S617 was also found to be neither clastogenic nor aneugenic in an *in vitro* micronucleus test in $CHO\text{-}WB_L$ cells in the presence and absence of metabolic activation at concentrations up to 2500 μg/mL [12]. Likewise, S6973 was found to be neither clastogenic nor aneugenic in an *in vivo* mouse micronucleus assay in male Swiss albino (CD-1) mice at a dose of 2000 mg/kg bw [12]. Overall, the results of the genotoxicity studies conducted on S2218 and the other benzothiadiazine analogs S6973 and S617 indicate no safety concern for these substances with respect to genotoxicity.

The doses of S2218 and its sulfate salt S8069 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 [32,33]. One of the methods used for determining exposure to flavouring substances in the US is the estimated possible average daily intake (PADI). The PADI is determined by multiplying the usual use levels of a flavour ingredient in each of 33 food categories by the average amount of that food category consumed

Table 8
Pharmacokinetics of S6973 and S617 in Sprague-Dawley Rats.

Cmpd	Route	Dose (mg/kg)	Sex	C_{max} (ng/mL)	AUC_{last} (ng*h/mL)	T_{max} (h)	$t_{1/2}$ (h)	%F
S6973	iv	2.5	M	3997	1760	0.083	0.78	–
	oral gavage	5.0	M	74.8	337	1.33	3.67	9.56%
		10	M	103	453	1.00	2.98	6.43%
		20	M	111	553	1.33	4.53	3.93%
S617	iv	1.0	M	1663	263	0.03	0.23	–
			F	1308	212	0.03	0.29	–
	oral gavage	10	M	3.30	17.5	0.44	3.85	0.67%
			F	4.80	36.7	0.88	4.25	1.73%
		30	M	7.10	42.5	1.25	3.03	0.54%
			F	9.40	56.3	1.38	3.57	0.89%
		100	M	10.1	53.2	1.38	3.48	0.20%
			F	18.3	92.6	0.50	1.45	0.44%

S6973 male rat: CL = 24.1 mL/min/kg; V_{ss} = 856 mL/kg.
S617 male rat: CL = 65.2 mL/min/kg; V_{ss} = 910 mL/kg.
S617 female rat: CL = 82.1 mL/min/kg; V_{ss} = 2040 mL/kg.
CL = clearance, V_{ss} = steady-state volume of distribution, %F = bioavailability.

daily and summing the intake over all 33 categories [34]. However, because the PADI calculation assumes that all foods in a food category always contain that substance and that the food category is consumed daily, the PADI is a gross exaggeration of the average daily intake. That being said, based on the anticipated use levels and proposed product categories of S8069 for the use as a flavour ingredient, the PADI for this flavouring agent was calculated to be 2.84 mg/person/day (47 μg/kg bw/day). Therefore, based on the low anticipated use level of S8069, a NOAEL of 140 mg/kg bw/day in a subchronic toxicology study would provide over a 2975-fold margin of safety.

In the 13-week subchronic toxicology study with S2218 sulfate salt (S8069), there were no test article-related unscheduled deaths or adverse test article-related effects noted for any parameter evaluated establishing a NOAEL for S8069 of 140 mg/kg bw/day (the highest dose evaluated), for both male and female Sprague-Dawley rats. The potential for *in vivo* toxicity of two of the structurally related 5-alkoxy benzothiadiazine analogs has also been evaluated in rats [12]. As seen in the case of S2218 sulfate salt (S8069), there were no test article-related unscheduled deaths or adverse test article-related effects noted for any parameter evaluated for either compound. The 90-day subchronic toxicity studies established NOAELs for S6973 and S617 of 20 and 100 mg/kg bw/day, respectively (the highest doses evaluated for each compound), for male and female Sprague-Dawley rats. Overall, the results of the subchronic toxicity studies conducted on S2218 and the other benzothiadiazine analogs S6973 and S617 indicate no safety concern for these substances at exposures that are orders of magnitude higher than their expected human exposures when used as flavouring substances in food applications.

Both S2218 sulfate salt (S8069) and S617 [12] have also been evaluated for their potential to induce developmental toxicity when administered orally to bred female rats from Gestation Days 6 through 20, at dosage levels 250, 500, or 1000 mg/kg bw/day. The high dose chosen for these studies was the recommended limit dose according to OECD guidelines for developmental toxicity studies [26]. Based on the lack of adverse maternal toxicity or effects on intrauterine growth and survival and fetal morphology at any dosage level for either compound, a dosage level of 1000 mg/kg bw/day (the highest dosage level evaluated) was considered to be the NOAEL for both S2218 sulfate salt (S8069) and S617 for both maternal toxicity and embryo/fetal development.

In conclusion, S2218 has demonstrated a toxicity profile comparable to that of the previously reported 5-alkoxy benzothiadiazine analogs S6973 and S617 currently in use as flavours with modifying properties. S2218 has 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 doses up to 100 mg/kg bw. In a developmental toxicity study in rats, S2218 sulfate salt (S8069) had a NOAEL of 1000 mg/kg bw/day for both maternal toxicity and embryo/fetal development. The results of a 13-week subchronic toxicity study established a NOAEL for S2218 sulfate salt (S8069) of 140 mg/kg bw/day (the highest dose evaluated), for male and female rats. Assuming that the systemic exposure of this compound after oral administration to humans is comparable to that observed at an equivalent dose in the rat, these NOAELs are orders of magnitude higher than the expected human exposure for this compound under the conditions of intended use.

Conflict of interest

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

Acknowledgement

The authors wish to thank Justin Kerr of Senomyx, Inc., for carrying out the in-life phase of the pharmacokinetic studies on S2218 and S8069.

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.2017.09.004.

References

[1] World Health Organization, Diet, nutrition, and prevention of chronic diseases, World Health Organ. Tech. Rep. Ser. 916 (2003) 1–149.
[2] B.A. Swinburn, I. Caterson, J.C. Seidell, W.P. James, Diet, nutrition, and the prevention of excess weight gain and obesity, Public Health Nutr. 7 (1A) (2004) 123–146.
[3] S.S. Sciffman, C.A. Gatlin, Sweeteners: state of knowledge review, Neursci. Biobehav. Rev. 17 (1993) 313–345.
[4] S.S. Schiffman, B.J. Booth, M.L. Losee, S.D. Pecore, Z.S. Warwich, Bitterness of sweeteners as a function of concentration, Brain Res. Bull. 36 (1995) 505–513.
[5] X. Li, L. Staszewski, H. Xu, K. Durick, M. Zoller, E. Adler, Human receptors for sweet and umami taste, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 4692–4696.
[6] G. Servant, C. Tachdjian, X.-Q. Tang, S. Werner, F. Zhang, X. Li, P. Kamdar, G. Petrovic, T. Ditschun, A. Java, P. Brust, N. Brune, G.E. DuBois, M. Zoller, D.S. Karanewsky, Positive allosteric modulators of the human sweet taste receptor enhance sweet taste, Proc. Natl. Acad. Sci. U. S. A. 107 (10) (2010) 4746–4751.
[7] G. Servant, C. Tachdjian, X. Li, D.S. Karanewsky, The sweet taste of true synergy: positive allosteric modulation of the human sweet taste receptor, Trends Pharmacol. Sci. 32 (11) (2011) 631–636.
[8] F. Zhang, B. Klebansky, R.M. Fine, H. Liu, H. Xu, G. Servant, M. Zoller, C. Tachdjian, X. Li, Molecular mechanism of the sweet taste enhancers, Proc. Natl. Acad. Sci. U. S. A. 107 (10) (2010) 4752–4757.
[9] J.B. Hallagan, R.L. Hall, Under the conditions of intended use – new developments in the FEMA GRAS program and the safety assessment of flavour ingredients, Food Chem. Toxicol. 47 (2009) 267–278.
[10] L.J. Marnett, S.M. Cohen, S. Fukushima, N.J. Gooderham, S.S. Hecht, I.M.C.M. Rietjens, R.L. Smith, T.B. Adams, J.B. Hallagan, C. Harman, M.M. McGowen, S.V. Taylor, GRAS Flavouring Substances 26: the 26th publication by the Expert Panel of the Flavour and Extract Manufacturers Association provides an update on recent progress in the consideration of flavouring ingredients generally recognized as safe under the Food Additive Amendment, Food Technol. 67 (8) (2013) 38–56.
[11] C.L. Harman, J.B. Hallagan, The FEMA Science Committee Sensory Data Task Force, Sensory testing for flavouring with modifying properties, Food Technol. 67 (11) (2013) 44–47.
[12] A.J. Arthur, D.S. Karanewsky, M. Luksic, G. Goodfellow, J. Daniels, Toxicological evaluation of two flavors with modifying properties: 3-((4-amino-2,2-dioxido-1*H*-benzo[*c*][1,2,6]thiadiazin-5-yl)oxy)-2,2-dimethyl-*N*-propylpropanamide and (*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[*c*][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one, Food Chem. Toxicol. 76 (2015) 33–45.
[13] S.M. Cohen, S. Fukushima, N.J. Gooderham, S.S. Hecht, L.J. Marnett, I.M.C.M. Rietjens, R.L. Smith, GRAS Flavouring Substances 27: the 27th publication by the Expert Panel of the Flavour and Extract Manufacturers Association provides an update on recent progress in the consideration of flavouring ingredients generally recognized as safe under the Food Additive Amendment, Food Technol. 69 (8) (2015) 40–59 Key findings of the FEMA Expert Panel GRAS determinations for each substance on the GRAS 27 list are available online: www.femaflavor.org.
[14] R.L. Smith, W.J. Waddell, S.M. Cohen, S. Fukushima, N.J. Gooderham, S.S. Hecht, L.J. Marnett, P.S. Portoghese, I.M.C.M. Rietjens, T.B. Adams, C.L. Gavin, M.M. McGowen, S.V. Taylor, GRAS Flavouring Substances 25: the 25th publication by the Expert Panel of the Flavour and Extract Manufacturers Association provides an update on recent progress in the consideration of flavouring ingredients generally recognized as safe under the Food Additive Amendment, Food Technol. 65 (7) (2011) 44–75.
[15] B. Fields, M. DiNovi, J.-C. Leblanc, U. Mueller, A. Renwick, Who Food Additives Series: 67 – Safety Evaluation of Certain Food Additives, Aliphatic and Aromatic Amines and Amides, (2012), pp. 69–93.
[16] EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), Scientific opinion on flavouring group evaluation 312 (FGE.312): 3-[(4-amino-2,2-dioxido-1*H*-2,1,3-benzothiadiazin-5-yl)oxy]-2,2-dimethyl-*N*-propylpropanamide, EFSA J. 11 (10) (2013) 3404 1–35.
[17] S.L. Adamski-Werner, V. Darmohusodo, C. Tachdjian, D.S. Karanewsky (2015). Sweet Flavour Modifier. United States Patent 9,000,151 B2, issue date April 7, 2015.
[18] S.L. Adamski-Werner, V. Darmohusodo, C. Tachdjian, D.S. Karanewsky (2016). Sweet Flavour Modifier. United States Patent 9,371,317 B2, issue date June 21, 2016.
[19] S.L. Adamski-Werner, V. Darmohusodo, C. Tachdjian, D.S. Karanewsky, G. Petrovic, R. Kimmich, J. Fotsing (2016). Sweet Flavour Modifier. United States Patent 9,475,803 B2, issue date October 25, 2016.
[20] ICH Harmonised Tripartite Guideline: Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients-Q7, (2000).
[21] Good Laboratory Practice for Nonclinical Laboratory Studies, 21 CFR Part 58, National Archives and Records Administration, 2006.
[22] Principles on Good Laboratory Practice (as revised in 1997), OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, Number 1, OECD Environmental Health and Safety Publications, Environment Directorate, Organisation for Economic Co-Operation and Development, Paris, 1998.

[23] Guideline test no. 471: bacterial reverse mutation test, OECD Guidelines for the Testing of Chemicals, Organisation for Economic Co-Operation and Development, Paris, 1997.
[24] Guideline test no. 487: in vitro mammalian cell micronucleus test, OECD Guidelines for the Testing of Chemicals, Organisation for Economic Co-Operation and Development, Paris, 2014.
[25] Guidance for Industry and Other Stakeholders. Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, 2010.
[26] Guideline test no. 414: prenatal developmental toxicity study, OECD Guidelines for the Testing of Chemicals, Organisation for Economic Co-Operation and Development, Paris, 2001.
[27] Chapter IV.C.9.b guidelines for developmental toxicity studies, Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, 2001.
[28] C.C. Crespi, C.L. Miller, B.W. Penman, Microtiter plate assays for inhibition of human, drug-metabolizing cytochromes P450, Anal. Biochem. 248 (1997) 188–190.
[29] V.P. Miller, D.M. Stresser, A.P. Blanchard, S. Turner, C.L. Crespi, Fluorometric high-throughput screening for inhibitors of cytochrome P450, Ann. N. Y. Acad. Sci. 919 (2000) 26–32.
[30] H. Tao, D. Santa Ana, A. Guia, M. Huang, J. Ligutti, G. Walker, K. Sithiphong, F. Chan, T. Guoliang, Z. Zozulya, S. Saya, R. Phimmachack, C. Sie, J. Yuan, L. Wu, J. Xu, A. Ghetti, Automated tight seal electrophysiology for assessing the potential hERG liability of pharmaceutical compounds, Assay Drug Dev. Technol. 2 (5) (2004) 497–506.
[31] B. Davies, T. Morris, Physiological parameters in animals and humans, Pharm. Res. 10 (1993) 1093–1095.
[32] B. Magnuson, I. Munro, P. Abbot, N. Baldwin, R. Lopez-Garcia, K. Ly, L. McGirr, A. Roberts, S. Socolovsky, Review of the regulation and safety assessment of food substances in various countries and jurisdictions, Food Addit. Contam. Part A 30 (7) (2013) 1147–1220.
[33] EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids. 2010. Draft Guidance on the data required for the risk assessment of flavourings, EFSA J. 8 (6) (2010) 1623. Available online: www.efsa.europa.eu.
[34] R.L. Smith, J. Doull, V.J. Feron, J.I. Goodman, I.C. Munro, P.M. Newberne, P.S. Portoghese, W.J. Waddell, B.M. Wagner, T.B. Adams, M.M. McGowen, GRAS Flavouring Substances 20: the 20th publication by the FEMA Expert Panel on recent progress in the consideration of flavoring ingredeients generally recognized as safe under the Food Additives Amendment, Food Technol. 55 (12) (2001) 34–55.