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Toxicological evaluation of two novel bitter modifying flavour compounds: 3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)imidazolidine-2,4-dione and 3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)-5,5-dimethylimidazolidine-2,4-dione

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ABSTRACT

A toxicological evaluation of two novel bitter modifying flavour compounds, 3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)imidazolidine-2,4-dione (S6821, CAS 1119831-25-2) and 3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)-5,5-dimethylimidazolidine-2,4-dione (S7958, CAS 1217341-48-4), were completed for the purpose of assessing their safety for use in food and beverage applications. S6821 undergoes oxidative metabolism *in vitro*, and in rat pharmacokinetic studies both S6821 and S7958 are rapidly converted to the corresponding O-sulfate and O-glucuronide conjugates. S6821 was not found to be mutagenic or clastogenic *in vitro*, and did not induce micronuclei in bone marrow polychromatic erythrocytes *in vivo*. S7958, a close structural analog of S6821, was also found to be non-mutagenic *in vitro*. In short term and subchronic oral toxicity studies in rats, the no-observed-adverse-effect-level (NOAEL) for both S7958 and S6821 was 100 mg/kg bw/day (highest dose tested) when administered as a food ad-mix for either 28 or 90 consecutive days, respectively. Furthermore, S6821 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

Bitter taste is generally considered to be an undesirable taste attribute in most food products and is believed to trigger an innate stereotypical response to prevent mammals from intoxication by avoiding ingestion of potentially harmful food constituents. Although a clear correlation between bitterness and toxicity has not been established, many naturally occurring toxic compounds induce a bitter-taste response [30,47]. However, many potentially beneficial constituents in certain fruits and vegetables such as plant-based phenols and polyphenols, flavonoids, isoflavones, terpenoids, and glucosinolates, have been described as having a bitter taste quality [31,11]. One potential approach to improve the palatability of foods with beneficial phytonutrients is to attenuate their bitter off-tastes with bitterness-masking compounds.

Bitter substances are detected by a specific subset of taste receptor cells localized in the taste bud and characterized by the expression of members of the hTAS2R family of G protein-coupled

Abbreviations: AUC, area under the curve; CL, plasma clearance; C_{max}, peak plasma concentration; CYP, cytochrome P450; FDA, Food and Drug Administration; FEMA, Flavour and Extract Manufacturers Association of the United States; GLP, Good Laboratory Practices; GMP, good manufacturing practices; GPCR, G protein-coupled receptors; HPBL, human peripheral blood lymphocytes; JECFA, Joint FAO/WHO Expert Committee on Food Additives; LC/MS, liquid chromatography with mass spectrometry; MC, methylcellulose; mnPCE, micronucleated bone marrow polychromatic erythrocytes; MRM, multiple-reaction monitoring; MSDI, maximized survey-derived intake; MTD, maximum tolerated dose; NOAEL, no-observed-adverse-effect-level; NOEL, no-observed-effect-level; OECD, Organization for Economic Cooperation and Development; PCE, polychromatic erythrocytes; PK, pharmacokinetics; SPET, single portion exposure technique; $t_{1/2}$, half-life; T_{max}, time to reach C_{max}; TE, total erythrocytes; TK, toxicokinetics; V_{ss}, volume of distribution at steady-state.

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receptors (GPCRs) [1,7,34,37,2]. Humans have at least 25 full-length hTAS2Rs, clustered on 3 human chromosomes, which are highly divergent in sequence, sharing only 30–70% amino acid homology [27]. Additionally, there are more than 80 single nucleotide polymorphisms among individual hTAS2R genes [8,26], several of which are responsible for the variation in the intensity of human bitter taste perception to various bitter tastants [6,25,46,49]. Unlike most GPCRs, a single hTAS2R can recognize a diverse variety of chemical entities and most bitter tastants can activate multiple hTAS2Rs.

The discovery of the hTAS2Rs and recent development of high-throughput screening methods for hTAS2R antagonists have enabled the development of potential bitterness-blocking agents [3–5]. Despite the characterization of the hTAS2Rs, which are activated by various bitter tastants, and the growing commercial interest in developing bitter blockers to mask the bitter taste of drugs and certain foods, relatively few synthetic inhibitors against this class of GPCRs has been reported to date [48,18,28].

Researchers at Senomyx, Inc. have recently reported a series of novel substituted 3-(pyrazol-4-yl) imidazolidine-2,4-diones, including 3-(1-((3,5-dimethylisoxazol-4-yl) methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)imidazolidine-2,4-dione (S6821, CAS 1119831-25-2) and 3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1*H*-pyrazol-4-yl)-1-(3-hydroxybenzyl)-5,5-dimethylimidazolidine-2,4-dione (S7958, CAS 1217341-48-4), which are selective antagonists of the human bitter receptor hTAS2R8 (IC_{50}'s = 0.035 and 0.073 μM, respectively) [23]. Both S6821 and S7958 have demonstrated the ability to significantly attenuate the bitter taste of a variety of bitter tastants present in consumer products including caffeine, rebaudioside A, whey protein, and hydrolyzed soy protein. The structures of S6821 and its 5,5-dimethyl analog S7958 are shown in Fig. 1.

Both S6821 and S7958 have been 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 conditions of intended use as a flavour ingredient [33,19] and therefore are available for use in human food in the United States as "FEMA GRAS" flavour ingredients. S6821 and S7958 were assigned FEMA GRAS Numbers 4725 and 4726, respectively, in 2010 [33]. S6821 and S7958 have also determined to be safe at the current levels of intake by the Joint FAO/WHO Expert Committee on Food Additives [22] (assigned JECFA No. 2161 and 2162, respectively) and S6821 has recently been submitted to the European Union for review. Other jurisdictions permit the use of S6821 including Japan, Korea, and Mexico (794 DO 5.9.2013).

The purpose of this publication is to summarize the results obtained from *in vitro* metabolism and *in vivo* pharmacokinetic (PK) studies, general toxicology studies in rodents, developmental toxicity studies, and genotoxicity studies conducted with S6821 and S7958. Additional supporting data obtained in these studies with S6821 and S7958 is included in a Supplementary Data section in the online publication.

2. Materials and methods

The batches of S6821 used for the *in vivo* metabolism and 28-day range-finding toxicity studies (Batch ID nos. 36881437 and 37284090, respectively; purity >99%), were synthesized at Senomyx, San Diego, CA using the procedure described in US Patent 8,076,491 [23]. The batch of S6821 used for the *in vitro* metabolism, *in vivo* PK, *in vitro* and *in vivo* genotoxicity, and 90-day subchronic toxicity studies (Batch ID no. 100968056, Lot no. CMLW-585/09-EX2, purity 99.9%), was synthesized at Cambridge Major Laboratories, Germantown, WI using the same synthetic method but prepared in conformance with GMPs as described in the ICH GMP Guidelines for APIs [20]. The batch of S6821 used for the range-finding and definitive developmental toxicity studies (Batch ID no. 110280840, Lot no. 140491, purity 99.7%) was synthesized at Labochim, Milan, Italy, also according to GMP, using a slight modification of the same synthetic method.

The batch of the S7958 used for the *in vivo* metabolism, *in vitro* genotoxicity and 28-day subchronic toxicity studies (Batch ID no. 44500878, purity >99%), was synthesized at Senomyx, San Diego, CA using the procedure described in US Patent 8,076,491 [23].

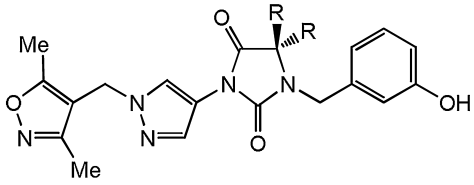
The experimental design for genetic toxicology studies followed the OECD Guidelines for the Testing of Chemicals—471, 473, and 474 [39–41]. The 28- and 90-day toxicology studies in rats were conducted in accordance with United States FDA Redbook 2000 [13]: IV.C.3.a. Short Term Toxicity Studies with Rodents [14], United States FDA Redbook 2000: IV.C.4.a. Subchronic Toxicity Studies with Rodents [15], and OECD Guidelines for Testing of Chemicals Guidelines 407 and 408, Repeated Dose 28- or 90-Day Oral Toxicity Study in Rodents [42,45]. All of the genetic toxicology and rodent toxicity studies were also conducted in compliance with the UK Good Laboratory Practice (GLP) regulations [35] and OECD guidelines [43]. 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 [44] and the United States FDA Redbook 2000: IV.C.9.b Guidelines for Developmental Toxicity Studies [13]; the definitive study was also conducted in compliance with the FDA GLP regulations 21CFR Part 58 and OECD guidelines [43].

The receptor panel profiling and preliminary cytochrome P450 (CYP) inhibition assays were conducted at MDS Pharma Services, Taipei, Taiwan; the follow-up CYP inhibition assays were carried out by Ricerca Biosciences, Bothell, WA using pooled human liver microsomes prepared by XenoTech, Lenexa, KS. The hERG channel inhibition assay was carried out by Aviva Biosciences, San Diego, CA. The *in vitro* microsomal metabolism and pharmacokinetic (PK) studies were carried out by Huntington Life Sciences (HLS), Cambridgeshire, UK. The microsomal metabolism studies utilized rat liver microsomes prepared in-house at HLS; human microsomes were from a pool of 50 donors and were obtained from BD Biosciences (Cat. No. 452156, lot 88114). The bioanalysis for the S6821 PK study was carried out by Nuvisan Pharma Services GmbH, Neu-Ulm, Germany. The *in vivo* metabolism studies on S6821 and S7958 were conducted at Senomyx, San Diego, CA. The analytical methods used for the PK and *in vivo* metabolism studies can be found in the Supplementary Data section published online.

All genotoxicity and rodent toxicology studies for both S6821 and S7958 were conducted at HLS, Suffolk and Cambridgeshire, UK. The strains of *Salmonella typhimurium* used in the reverse bacterial mutation assay were obtained from the National Collection of Type Cultures, London, England; the strain of *Escherichia coli* was obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland. Cultures of human lymphocytes for the chromosome aberration test were prepared from pooled blood collected aseptically from two, healthy, non-smoking donors. The developmental toxicity study on S6821 was conducted at WIL Research, Ashland, OH. A description of the study designs is included in the individual study sections below. Detailed data tables for the genotoxicity, subchronic and developmental toxicity studies can be found in the Supplementary Data section published online.

3. *In vitro* receptor and cytochrome P450 profiling of S6821 and S7958

In vitro tests were conducted with S6821 and S7958 to assess whether the compounds interact with any enzymes or receptors that might cause adverse or unexpected effects or affect drug



S6821 (R = H)
Chemical Formula: $C_{19}H_{19}N_5O_4$
Molecular Weight: 381.39

S7958 (R = Me)
Chemical Formula: $C_{21}H_{23}N_5O_4$
Molecular Weight: 409.44

Fig. 1. Structures of S6821 and S7958.

Table 1
Cytochrome P450 Inhibition of S6821 and S7958.

Cmpd	CYP	Spectrofluorimetric assay, human recombinant enzymes, Sf9 cells		LC–MS/MS assay in human liver microsomes	
		Probe Substrate	% Inhibition (10 μM)	Probe Substrate	% Inhibition (10 μM)
S6821	1A2	3-cyano-7-ethoxycoumarin	3%	phenacetin	18%
	2C9	3-cyano-7-ethoxycoumarin	34%	tolbutamide	−3%
	2C19	3-cyano-7-ethoxycoumarin	26%	S-mephenytoin	8%
	2D6	3-cyano-7-ethoxycoumarin	8%	bufuralol	−2%
	3A4	7-benzyloxy-4- (trifluoromethyl)-coumarin	1%	midazolam	4%
S7958	1A2	3-cyano-7-ethoxycoumarin	1%	ethoxyresorufin	16%
	2C9	3-cyano-7-ethoxycoumarin	80%	tolbutamide	62%
	2C19	3-cyano-7-ethoxycoumarin	11%	S-mephenytoin	17%
	2D6	3-cyano-7-ethoxycoumarin	3%	bufuralol	17%
	3A4	7-benzyloxy-4- (trifluoromethyl)-coumarin	26%	midazolam	12%

metabolism. Preliminary *in vitro* screening for potential off-target activity of S6821 and S7958 included tests for CYP inhibition, a lead profiling receptor screen (a panel of 68 receptor binding assays for GPCRs, ion channels, nuclear receptors, transporters), and a hERG inhibition assay. The preliminary tests for CYP inhibition were performed using recombinant human enzymes expressed in insect Sf9 cells using spectrofluorimetric substrates [9,36]. All assays were performed at a concentration of 10 μM of either S6821 or S7958. No significant responses (≥50% inhibition or stimulation) were found with either compound in the lead profiling receptor screen. Neither S6821 nor S7958 significantly inhibited the hERG ion channel current (<10%) in an *in vitro* hERG electrophysiology (patch clamp) assay [50]. The results from the CYP inhibition studies are summarized in Table 1.

As a follow up to the results obtained using spectrofluorimetic substrates, S6821 and S7958 were retested on the same panel of CYP enzymes utilizing pooled human liver microsomes and CYP-specific substrates with detection of the CYP-specific metabolites by LC–MS/MS [29,51]. S6821 did not significantly inhibit any of the CYP isozymes in either assay format. In contrast, 10 μM of S7958 demonstrated significant inhibition of CYP2C9 in both the spectrofluorimetric and LC–MS/MS assays (see Table 1). In a separate study, S7958 was also evaluated for evidence of time-and metabolism-dependent inhibition of CYP2C9 again using pooled human liver microsomes and diclofenac as a CYP2C9 substrate. To evaluate time- and metabolism-dependent inhibition, S7958 was preincubated with human liver microsomes in the presence and absence of a NADPH-generating system for 30 min prior to incubation with the marker substrate. In this assay format, S7958 was a direct inhibitor of CYP2C9 with an IC_{50} value of 8.8 μM. In addition, there was no evidence of either time- or metabolism-dependent inhibition of CYP2C9 by S7958.

4. Absorption, distribution, metabolism, excretion

The *in vitro* metabolism of S6821 was studied using rat and human liver microsomes. The *in vivo* metabolism of both S6821 and S7958 was studied in rats. A complete PK study of S6821 and its major metabolites was carried out in male Sprague-Dawley rats.

4.1. In vitro *metabolism of S6821*

The potential of S6821 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. Reference standards were synthesized for the three potential oxidative metabolites that could be produced by mono-hydroxylation of the 3-hydroxybenzyl moiety (see Fig. 2). S6821 (1, 10, and 50 μM) was incubated with mixed gender, pooled rat and human liver microsomes (0.5 mg/mL) in the presence of NADPH at 37 °C for 0, 10, 30 or 120 min prior to quenching the samples with acetonitrile. Control incubations in the absence of microsomes were conducted in parallel, for 120 min only, at all substrate concentrations. [^{14}C]Testosterone (53 mCi/mmol) was tested in parallel with S6821 to confirm the functionality of the microsomes (10 min incubation). Samples were centrifuged to separate the precipitated microsomes from the supernatant containing the parent compound and it metabolites. The supernatants from the S6821 incubations were analyzed by liquid chromatography-mass spectrometry (LC–MS) using a Waters Symmetry C18 column (150 × 3.9 mm) with 0.1% formic acid and acetonitrile gradient system, and a TSQ7000 mass spectrometer operated in positive ionization mode. The samples were scanned in the positive ionization mode for metabolites with +16 mass units greater than parent (mono-hydroxy metabolites), +18 mass units greater than parent (hydantoin ring opened metabolites), +32 mass units greater than parent (dihydroxy metabolites), and masses of 276 (loss of hydroxybenzyl), 207 (loss of dimethylisoxazol-pyrazole group), and 273 (loss of dimethylisoxazole group). Positive control incubations with [^{14}C]testosterone were analyzed using an Agilent 1200 series HPLC, monitoring for UV absorbance at 254 nm using an Agilent 1200 series variable wavelength detector, and for ^{14}C-radioactivity using a β-Ram Radioisotope System. There was greater than 30% turnover of testosterone in parallel incubations with microsomes of both species. Details of the experimental and analytical methods can be found in the Supplementary Data section.

The extent of metabolism (loss of parent S6821) was generally greater by rat than by human liver microsomes, but more detectable mono-hydroxylated metabolites were seen in incubations with

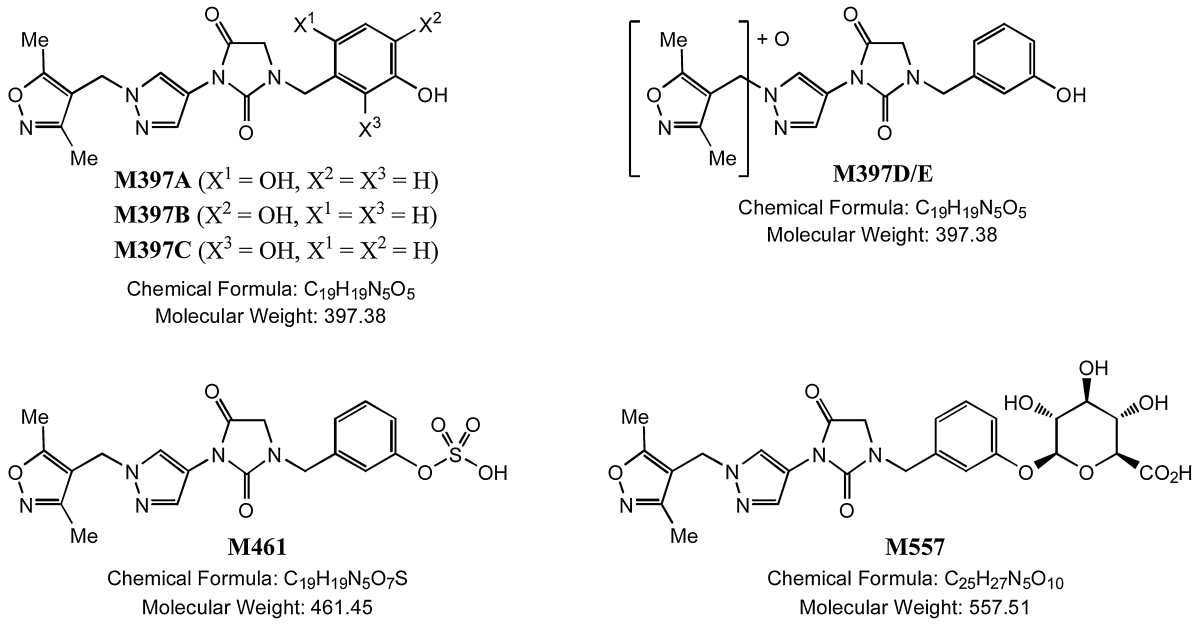


Fig. 2. Structures of S6821 Major Microsomal and *In Vivo* Metabolites.

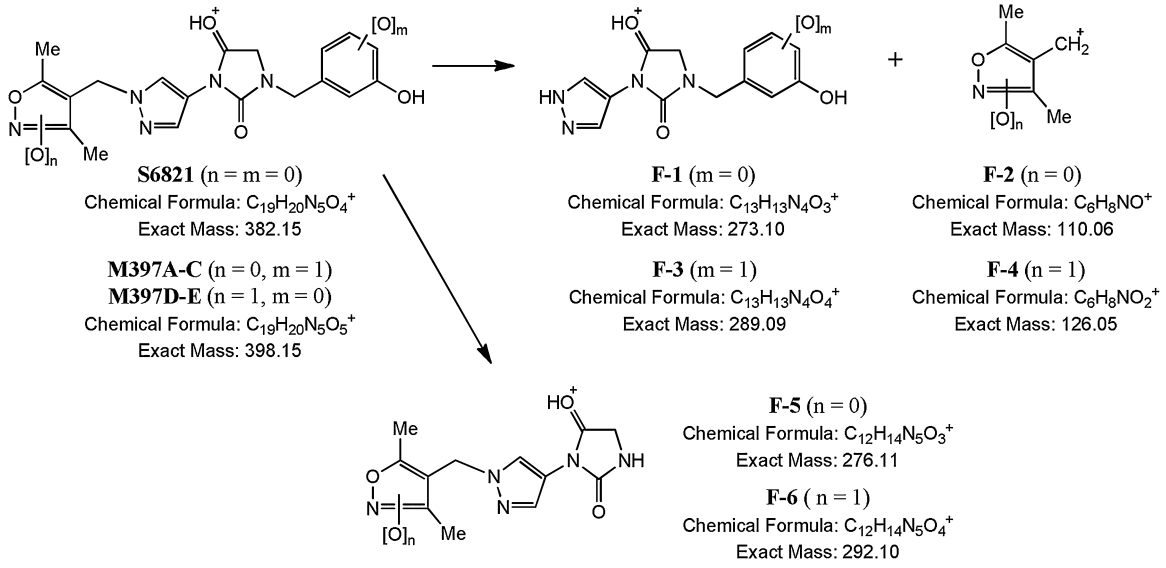


Fig. 3. Mass Spectral Fragmentation Pathway for S6821 and M397A-E.

the later. However, dihydroxylated and other degradation products were not detected in either the rat or human liver microsome incubation samples. At the 120 min time point in the human microsome incubations with 50 μM of S6821, 75% of the initial S6821 was remaining based on MS peak areas. Human liver microsomes formed at least four identifiable mono-hydroxylated metabolites which in total represented 23% of the initial S6821 peak area. Two are hydroxylated on the phenyl ring, M397A and M397B. The structures were confirmed by LC/MS comparison to reference standards (see Fig. 2). The corresponding 2,3-dihydroxybenzyl analog M397C was not observed in the human microsomal incubations but was subsequently identified as an *in vivo* metabolite in rats (*vide infra*). The other two metabolites, M397D and M397E, were mono-hydroxylated at undetermined positions on the dimethylisoxazole ring as confirmed by product ion mass spectra. The mass spectral fragmentation patterns for S6821 and its hydroxylyated metabolites M397A-E are shown in Fig. 3. At the 120 min time point in the rat microsome incubations with 50 μM of S6821, 56% of the initial S6821 was remaining based on mass spec peak areas. LC–MS results indicate that rat liver microsomes formed primarily one mono-hydroxylated metabolite of S6821 identified as M397D, one of the metabolites observed in the human microsomal incubations which is hydroxylated on the dimethylisoxazole ring. At the 120 minute time point in the incubations with 50 μM S6821, M397D only represented 0.5% of the initial S6821 peak area.

4.2. In vivo *metabolism of S6821 and S7958 in rats*

The *in vivo* metabolism of S6821 and S7958 was evaluated following a single oral administration in male Sprague-Dawley rats. A group of 4 male Sprague-Dawley [Crl:CD®(SD)] rats (Charles River Laboratories, Hollister, CA) was administered either 100 mg/kg bw of S6821 or 30 mg/kg bw of S7958 in 1% methylcellulose by oral gavage. Blood samples were taken at approximately 30 min, 2, and

4 h post-dose for animals dosed with S6821, and at 30 min, 2, 4, 8, and 24 h post-dose for animals treated with S7958. Plasma samples were analyzed for the parent compounds and metabolites by LC–MS/MS using a HALO RP-amide column (150 × 3.0 mm, 2.7 um) with a 0.1% formic acid and methanol gradient system, and an ABI API 3200 mass spectrometer operated in positive ionization mode. S7958 served as an internal standard for the plasma samples from animals dosed with S6821, and S6821 served as an internal standard for animals dosed with S7958. Details of the analytical methods can be found in the Supplementary Data section.

In the case of the plasma samples from animals treated with S6821, the parent compound and the internal standard (S7958) were detected using a source which was configured with turboionspray ionization in the positive mode using multiple-reaction monitoring (MRM) of mass transition pairs at *m*/*z* of 382.2/110.1 and 410.2/110.1 amu. Peak area ratios of S6821 to the internal standard (S7958) were used to quantify the samples. The maximum concentration of S6821 in rat plasma was observed at the 0.5 h time point in all the test animals (mean concentration: 105.4 ± 68.2 ng/mL). In addition to the expected S6821 peak in the extracted ion chromatogram (EIC) in the plasma samples monitored by the 382 → 110 MRM transition, two additional major metabolite peaks were observed. The spectra from enhanced product ion (EPI) scans and enhanced MS (EMS) scans are consistent with the corresponding glucuronide M557 (M + H: *m*/*z* 558; M + Na: *m*/*z* 580) and sulfate M461 (M + H: *m*/*z* 462; M + Na: *m*/*z* 484) conjugates of S6821. The structures of these Phase II metabolites were confirmed by direct comparison to synthetic standards (see Fig. 2). Both secondary metabolites are present at significantly higher concentrations than the parent S6821 throughout the 4 h post-dosing period. During the observation period, the mean concentrations of M557 and M461 in the plasma samples ranged from 32 to 166 ng/mL and 334–555 ng/mL, respectively.

The mono-hydroxylated metabolites of S6821 were detected using a source which was configured with turboionspray ionization in the positive mode using MRM of mass transition pair at *m*/*z* of 398.2/110.1 amu. Of the three possible benzyl mono-hydroxylation products of S6821, only M397C was confirmed to be present in rat plasma. The mean concentrations of M397C in the plasma samples were in the range of 12.6–18.9 ng/mL throughout the observation period. This mono-hydroxylation product was not observed in the rat microsomal incubations. Two additional peaks were present in the 398.2/110.1 MRM chromatogram that did not co-elute with the other possible benzyl mono-hydroxylation products M397A and M397B. They were also present in the MRM chromatograms monitored for the 478 → 110 transition suggesting that they may be the sulfate derivatives of the benzyl mono-hydroxylated metabolites M477A/B. The position of the hydroxylation and that of the sulfate moiety are unknown at this time.

In addition to the benzyl mono-hydroxylated metabolites of S6821 described above, three others were detected using MRM of mass transition pair at *m*/*z* of 398.2/126.2 amu. The 398 → 126 transition is consistent with metabolites in which the isoxazole ring of S6821 has undergone hydroxylation. All three of these peaks are also present in the MRM chromatogram when monitored for the 398 → 273 transition confirming the site of hydroxylation. Two of these peaks are likely the same isoxazole-hydroxylated metabolites M397D and M397E observed in the rat and human microsomal incubations. One of the three peaks was also present in the MRM chromatogram when monitored for the 478 → 126 transition suggesting that it is a sulfate derivative of an isoxazole-hydroxylated metabolite M477C. The position of the hydroxylation and that of the sulfate moiety are unknown at this time. Based on EIC peak areas, one of the two sulfated mono-hydroxylated metabolites M477A/B, is present at 3–4 fold higher concentration than M397C; all of the other metabolites (M397D/E and M477C) appear to be in significantly lower amounts than M397C. As stated above, the mono-hydroxylated compounds M397A and M397B were not observed in rat plasma, but may be the biosynthetic precursors of sulfates M477A/B. The metabolic pathway of S6821 in rats is shown in Fig. 4.

The plasma samples from the animals treated with S7958 were analyzed by LC–MS/MS by methods analogous to those used for the plasma samples from animals treated with S6821. The maximum concentration of S7958 in rat plasma throughout the observation period was only 5.2 ± 7.1 ng/mL. Due to the limited number of animals tested and the large fluctuation of the S7958 concentration between the animals, the T_{max} for S7958 could only be estimated to occur between 2 and 8 h. Mass ion transition pairs at *m*/*z* of 426.2/110.1, 490.2/110.1, and 586.3/110.1 amu were used to detect the mono-hydroxylated, O-sulfated, and O-glucuronidated metabolites of S7958, respectively. Ions consistent with the presence of S7958 glucuronide and sulfate metabolites as well as a sulfate derivative of a mono-hydroxylated metabolite were observed in the extracted ion chromatograms of the plasma samples. Although plasma concentrations of the parent compound S7958 are significantly less than those seen with S6821, S7958 demonstrates an overall metabolism profile similar to that of its close analogue S6821.

4.3. Pharmacokinetics of S6821 in rats

The PK parameters and oral bioavailability of S6821 in plasma was evaluated following either a single intravenous or oral administration in male Sprague-Dawley [Crl:CD®(SD)] rats (Charles River UK Ltd, Margate, Kent, England). Plasma samples were also analyzed for the presence of the sulfate M461 and glucuronide M557 conjugates observed in the preliminary *in vivo* metabolism study in rats. For intravenous administration, 6 male Sprague-Dawley rats were bolus injected with S6821 at 1 mg/kg bw in PEG 300:purified water (1:1). For oral administration, 6 male Sprague-Dawley rats per group were administered S6821 at either 10, 30, or 100 mg/kg bw in PEG 300:purified water (1:1) by oral gavage. Blood samples were collected by venipuncture of a caudal vein at approximately 15, 30 min, 1, 2, 4, 8, 12, 24, and 48 h post-dose at alternating time points from two cohorts of 3 animals/dose group with an additional time point (5 min) for the intravenously treated animals. Plasma samples spiked with S7958 as an internal standard were analyzed for S6821 and metabolites by LC–MS/MS using a HALO RP-amide column (150 × 3.0 mm, 2.7 um) with a 0.1% formic acid and methanol gradient system and a Sciex API 3000 mass spectrometer operated in positive ionization mode. The parent compound, internal standard, and metabolites were detected using a source which was configured with turboionspray ionization in the positive mode using MRM of mass transition pairs at *m*/*z* of 382.2/110.1 (S6821, M461, M557) and 410.2/110.1 (S7958) amu. Details of the analytical methods can be found in the Supplementary Data section. Test article formulations prepared for this study were analyzed for concentration by LC–MS/MS. The PK parameters were analysed by non-compartmental methods using WinNonlin Pro version 5.0 software (Pharsight Corporation, USA). The pharmacokinetic parameters for S6821 and its sulfate M461 and glucuronide M557 conjugates are shown in Table 2.

Both S6821 and its glucuronide conjugate M557 were rapidly eliminated after intravenous administration with terminal half-lives ($t_{1/2}$) of 0.1 and 0.2 h, respectively. The terminal $t_{1/2}$ of the sulfate conjugate M461 however, was longer than that of S6821 (1.9 h) indicating that the rate of formation of M461 was not the rate-limiting step in its elimination. Mean maximum plasma concentrations of 3380 and 315 ng/mL for M461 and M557 respectively, occurred at 5 min post dose. Systemic exposure (AUC_{last}) was found to be highest for metabolite M461 while

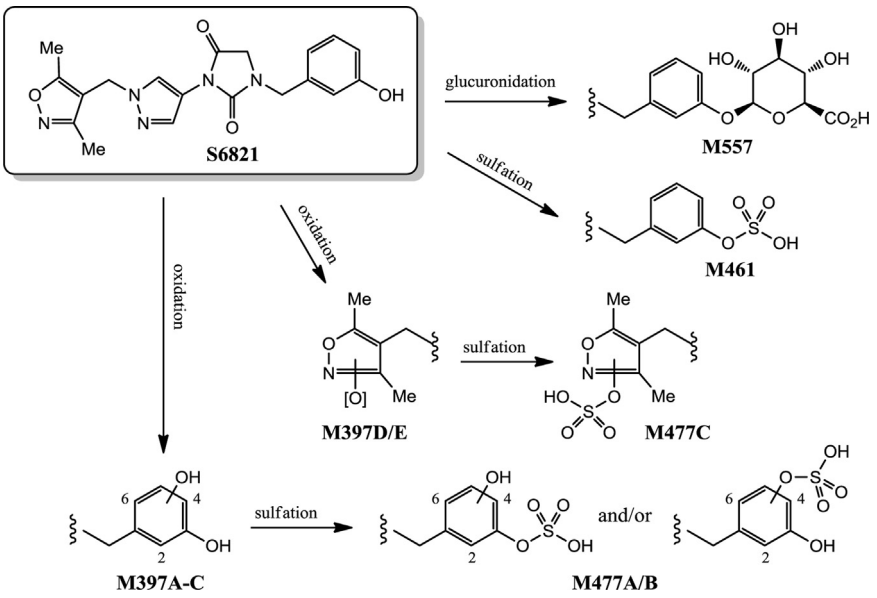


Fig. 4. Metabolic Pathway of S6821 in Rat.

Table 2
Pharmacokinetics of S6821, M461, and M557 in Male Sprague-Dawley Rats Treated with S6821.

Route	Dose (mg/kg bw)	Analyte	C_{max} (ng/mL)	T_{max} (h)	$t_{1/2}$ (h)	$AUC_{0\text{-last}}$ (ng·h/mL)	$AUC_{0\text{-last}}$/dose (ng·h/mL/mg/kg)	%F
iv	1.0	S6821	619	0.08	0.1	161	161	–
		M461	3380	0.08	1.9	4500	4500	–
		M557	315	0.08	0.2	104	104	–
oral gavage	10	S6821	12.3	0.5	2.0	28.0	2.80	1.74%
		M461	2600	0.5	2.9	18200	1820	–
		M557	2540	1.0	1.4	6050	605	–
	30	S6821	38.5	0.5	7.1	201	6.70	4.16%
		M461	4620	0.5	2.5	32000	1070	–
		M557	8050	0.5	1.7	22900	763	–
	100	S6821	68.1	0.5	4.3	410	4.10	2.55%
		M461	6110	4.0	4.7	72500	725	–
		M557	9090	0.5	4.2	44700	447	–

S6821: CL = 105 mL/min/kg; V_{ss} = 563 mL/kg CL = clearance; V_{ss} = steady-state volume of distribution; %F = bioavailability.

the exposure to S6821 and glucuronide M557 were similar. The mean plasma clearance (CL) of S6821 after an intravenous dose was 190% (105 mL/min/kg) of the hepatic blood flow in the rat (55.2 mL/min/kg [10]), and the volume of distribution at steady state (V_{ss}) was 0.84 times (563 mL/kg) the total body water volume in the rat (668 mL/kg [10]). A plot of the mean plasma concentrations of S6821, M461 and M557 verses time after intravenous administration of S6821 is shown in Fig. 5.

Following oral gavage administration of S6821 to male rats at 10 mg/kg bw, absorption was rapid and the mean maximum S6821 plasma concentration (C_{max}) of 12.3 ng/mL occurred 0.5 h post dose. Following administration of S6821 at 30 and 100 mg/kg bw, the mean maximum plasma concentrations of S6821 were 38.5 and 68.1 ng/mL respectively, occurring at 0.5 h post dose. S6821 plasma concentrations increased with increasing oral doses from 10 to 100 mg/kg bw and the rate and extent of systemic exposure, as reflected by the parameters C_{max} and AUC_{last} respectively, increased with increasing dose. However, these increases were not proportionate to the dose increase. At the highest dose level (100 mg/kg bw), C_{max} was 45% lower than that predicted by a linear relationship. At this dose, the increase in systemic exposure (AUC_{last}) was more than proportionate to the dose increase and was almost 1.5-fold higher than that predicted by a linear relationship. The systemic bioavailability of S6821 following oral administration was low, with percentage values (%F) of 1.74%, 4.16% and 2.55% following oral doses of 10, 30 and 100 mg/kg bw respectively.

Metabolism of S6821 to sulfate M461 was rapid with C_{max} values of 2600 and 4620 ng/mL occurring at 0.5 h post dose following administration of S6821 at 10 and 30 mg/kg bw respectively. Following S6821 administration at 100 mg/kg bw the mean maximum plasma M461 concentration of 6110 ng/mL occurred at 4 h post dose. The terminal $t_{1/2}$ of metabolite M461 following oral doses of S6821 at 10, 30 and 100 mg/kg bw, was 2.9, 2.5 and 4.7 h respectively, compared to a terminal $t_{1/2}$ of 1.9 h after intravenous bolus dose administration of S6821. The systemic exposure of M461 ranged from 160- to 650-fold higher than that of S6821. Similarly, metabolism of S6821 to M557 was also rapid at all dose levels with C_{max} occurring between 0.5 and 1 h post dose. Following administration of S6821 at 10, 30 and 100 mg/kg bw, the mean maximum plasma concentrations of M557 were 2540, 8050 and 9090 ng/mL respectively. The terminal $t_{1/2}$ of metabolite M557 was 1.4, 1.7 and 4.2 h following oral doses of 10, 30 and 100 mg/kg bw S6821, compared to a terminal $t_{1/2}$ of 0.2 h after intravenous bolus dose administration of S6821. The systemic exposure of M557 ranged from 109- to 216-fold higher than that of S6821. A plot of the mean

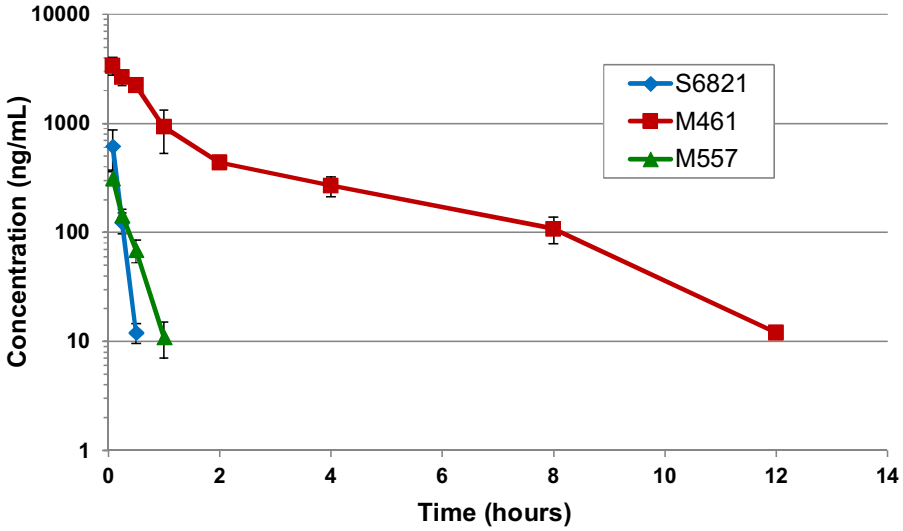


Fig. 5. Mean plasma concentrations of S6821, M461, and M557 after intravenous administration of S6821 (1.0 mg/kg bw) to male S-D rats.

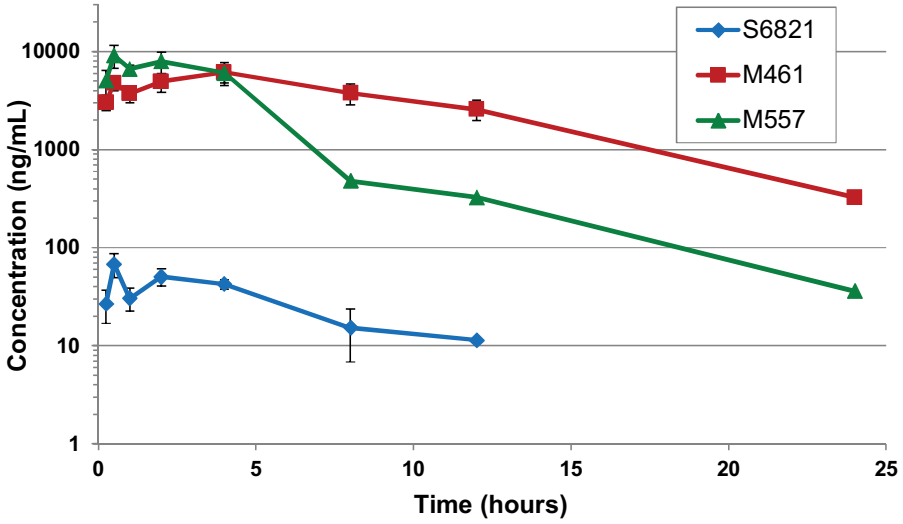


Fig. 6. Mean plasma concentrations of S6821, M461, and M557 after oral administration of S6821 (100 mg/kg bw) to male S-D rats.

plasma concentrations of S6821, M461 and M557 verses time after oral administration of S6821 (100 mg/kg bw) is shown in Fig. 6.

Both C_{max} and AUC_{last} for M461 and M557 increased with increasing dose of S6821 over the range 10–100 mg/kg bw S6821. However, these increases were less than the proportionate dose increase. At the highest dose level, the C_{max} and AUC_{last} values for M461 were approximately 76% and 60% lower than the values predicted by a linear relationship, respectively. Similarly, C_{max} and AUC_{last} values for M557 at this dose level were approximately 64% and 26% lower than the value predicted by a linear relationship, respectively.

5. Genotoxicity and mutagenicity studies

S6821 was evaluated for its genotoxic potential through a standard (5-strain) Ames, chromosome aberration, and micronucleus tests (see Table 3). A reverse bacterial mutation assay was also conducted on S7958. All genetic toxicology studies were conducted in compliance with the United States Food and Drug Administration (FDA) Good Laboratory Practices (GLP) regulations 21CFR Part 58 [17] and OECD guidelines [43]. The data tables for the genotoxicity studies can be found in the Supplemental Material.

5.1. Bacterial reverse mutation test (5-Strain ames)

S6821 and S7958 were evaluated for the potential to induce point 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 rats induced with phenobarbital and 5,6-benzoflavone. The assay was designed to meet the current OECD Guideline for Testing of Chemicals No. 471, Bacterial Reverse Mutation Test [39].

The concentrations of S6821 and S7958 investigated for both the plate incorporation and pre-incubation tests ranged from 50 to 5000 μg per plate. In both the plate incorporation and pre-incubation assays, toxicity was not observed at any concentration of either compound as evident by a normal background lawn and colony counts similar to the concurrent negative controls. No precipitates were observed in any of the plates containing S6821. Precipitate was observed on all plates containing S7958 at 5000 μg/plate in both the absence and presence of S9 mix, and at 1500 μg/plate in the absence of S9 mix, in both tests. Neither S6821

Table 3
Summary of genotoxicity studies conducted on S6821 and S7958.

End-Point	Test System	Cmpd No.	Concentration/Dose	Result
Reverse mutation (*in vitro*)	*S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* strain WP2 *uvrA*	**S6821**	50–5000 μg/plate, plate incorporation and pre-incubation, ±S9[a]	Negative
		S7958	50–5000 μg/plate, plate incorporation and pre-incubation, ±S9[a]	Negative
Chromosome aberration (*in vitro*)	Primary human lymphocytes	**S6821**	297–824 μg/mL, 3 hr exposure −S9 297–824 μg/mL, 3 hr exposure +S9[a] (2%) 1100–1300 μg/mL, 3 hr exposure +S9[a] (5%) 200–600 μg/mL, 21 hr exposure −S9	Negative
Micronucleus formation (*in vivo*)	Male Swiss albino mice (CD-1), bone marrow PCEs	**S6821**	500, 1000, 2000 mg/kg bw/day (oral), 2 days	Negative

[a] S9 from rat liver homogenate from male Sprague-Dawley rats treated with phenobarbital/5,6-benzoflavone.

nor S7958 increased the number of revertant colonies in either the plate incorporation or pre-incubation assays with any of the tester strains both in the presence and absence of metabolic activation with rat liver S9. The concurrent positive controls demonstrated the sensitivity of the assay and the metabolizing activity of the liver preparations. Thus, it was concluded that both S6821 and S7958 were not mutagenic to *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* strain, WP2 *uvrA* in the absence and presence of metabolic activation under the test conditions employed.

5.2. In vitro *chromosome aberration test*

S6821 was investigated for its potential to induce structural and numerical chromosome aberrations in human peripheral blood lymphocytes (HPBL), both in the presence and absence of a supplemental rat liver fraction (S9) from rats induced with phenobarbital and 5,6-benzoflavone. Solvent and positive control (mitomycin C, −S9; cyclophosphamide, +S9) cultures were also included. The experimental design followed the OECD Guideline for the Testing of Chemicals No. 473, *In Vitro* Mammalian Chromosome Aberration Test [40]. In order to determine the toxicity of S6821 to cultured HPBL, the mitotic index was assessed for all cultures treated with the test substance in the presence and absence of S9, as well as that of the solvent control, dimethyl sulfoxide (DMSO).

On the basis of these data, the doses chosen for the chromosome aberration assay ranged from 297 to 824 μg/mL for the non-activated and S9-activated (2% v/v) 3-hour exposure groups, from 1100 to 1300 μg/mL for the S9-activated (5% v/v) 3-hour exposure group, and from 200 to 600 μg/mL for the non-activated 21-hour exposure group. All conditions were tested at the limit of test article toxicity as assessed by the Relative Mitotic Index (RMI ≥47% under all conditions).

Under these test conditions, no structural or numerical chromosome aberrations were observed in the S6821 treated cultures beyond those seen in the concurrent solvent controls. All concurrent positive controls induced significant numbers ($p < 0.001$) of cells with chromosome aberrations. It was concluded that exposure to S6821 did not induce chromosome aberrations in the *in vitro* mammalian chromosome aberration test using HPBL in both the absence and presence of rat liver S9, when tested in accordance with regulatory guidelines.

5.3. In vivo *micronucleus assay in mice*

S6821 was evaluated for potential *in vivo* clastogenic activity and/or disruption of the mitotic apparatus, as measured by its ability to increase the incidence of micronucleated polychromatic erythrocytes (mnPCEs) in the bone marrow of CD-1 mice. The study was designed to meet the current OECD Guideline for the Testing of Chemicals No. 474, Mammalian Erythrocyte Micronucleus Test [41]. A dose-range finding study was performed to assess test articles toxicity and determine the maximum tolerated dose (MTD) or maximum feasible dose (MFD) for the definitive assay.

For the dose range finding phase of the study, male and female (2 animals/sex/group) Crl:CD-1(ICR) mice (Charles River UK Ltd, Margate, Kent, England) were treated with 2000 mg/kg bw of S6821 suspended in vehicle (1% methylcellulose (MC) in purified water) at a volume of 10 mL/kg body weight for two consecutive days, approximately 24 h apart, by oral gavage. Since there was no toxicity noted up to 2000 mg/kg bw, dose levels of 500, 1000, and 2000 mg/kg bw (6 animals/group), were used for the definitive study with S6821. Since no substantial differences in toxicity were observed between the sexes, the main test was performed using male animals only. In the definitive phase of the study, 1% MC was used as the vehicle (negative) control and mitomycin C, at a dose of 12 mg/kg bw (dose volume 20 mL/kg), was used as the positive control article. Animals were observed for signs of toxicity during the course of these studies.

In the definitive assay, all animals from each of the test article and vehicle treated groups were euthanized 24 h after administration of the second dose by carbon dioxide inhalation; animals in the positive control group were sacrificed 24 h after a single dose. Immediately following euthanasia, femoral bone marrow was collected from each animal. Bone marrow slides were prepared and polychromatic erythrocytes (PCEs, 2000/animal) were examined microscopically for the presence of micronuclei (mnPCEs). The ratio of PCEs to total erythrocytes (TE) in the test article groups relative to the vehicle control groups was also evaluated to reflect the test article's cytotoxicity.

No clinical signs of toxicity were noted for the vehicle control, positive control and S6821 group animals over the duration of the test. Some small incidences of bodyweight loss were observed in all study groups. No statistically significant reductions in the PCE/TE ratio in the S6821 treated groups compared to the vehicle control group were observed indicating that the test article did not induce cytotoxicity. No statistically significant increase in the incidence of mnPCEs in the S6821 treated groups was observed relative to the negative control group. The positive control (mitomycin C) induced statistically significant increases in the incidence of mnPCEs when compared to both the negative control groups and the test article treated groups at all three dose levels ($p < 0.01$). It was concluded that oral administration of S6821 did not induce micronuclei or bone marrow toxicity in male CD-1 mice when tested in accordance with regulatory guidelines.

6. *In vivo* toxicological studies

S6821 was evaluated in 28-day dose-range finding and 90-day subchronic toxicology studies in rats in compliance with the FDA Guidelines [16] Toxicological Principles for the Safety of Food Ingredients. S7958 was evaluated in a 28-day subacute toxicology study in rats. S6821 was also evaluated for potential embryo/fetal tox-

icity 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 (see Table 4). Summary data tables for the 28-day toxicology study for S7958, and for the 90-day toxicology and the definitive developmental toxicity studies for S6821 can be found in the Supplemental Material.

6.1. Rodent toxicology studies

6.1.1. 28-Day dose-range finding toxicity study on S6821

The purpose of this study was to evaluate the potential systemic toxicity of S6821 in rats after dietary administration for 28 days in order to select doses for 90-day subchronic toxicity study in rats. Three treatment groups of male and female CD® [Crl:CD®(SD)] rats (n = 5/sex/group, Charles River UK Ltd, Margate, Kent, England) were administered S6821 in the diet at dose levels of 10, 30, or 100 mg/kg bw/day. One additional group of five animals/sex served as the control and received the vehicle diet. The test substance was administered continuously via the diet throughout the 28-day treatment period. Dietary concentrations (ppm) of S6821 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, animals were sacrificed by carbon dioxide asphyxiation before subsequent exsanguination. Survival, clinical observations, body weight, food consumption, hematology, clinical chemistry, organ weights, and macroscopic evaluations of all animals were used to assess potential toxicity. The livers were examined microscopically for animals in the 0 and 100 mg/kg bw/day dose groups.

Administration of S6821 in the diet for 28 days was well tolerated in rats at dose levels up to 100 mg/kg bw/day, the highest dose tested. There was no test article-related mortality observed and all animals survived until scheduled euthanasia. There were no test article-related clinical signs or changes in mean body weight, body weight gain, food consumption, hematology parameters, coagulation parameters, red blood cell morphology, or clinical chemistry parameters during this study. In the high dose females, there was a slight reduction of hematocrit, hemoglobin concentration and erythrocyte count, compared with the controls. Plasma calcium concentrations were low at all doses in males ($p < 0.01$) and at 100 mg/kg bw/day in females ($p < 0.05$), and phosphate concentrations were also low in females receiving 100 mg/kg bw/day ($p < 0.05$). However, these differences were minor and the majority of the individual values were within the laboratory's historical control values and were therefore attributed to normal biological variation.

There were no test article-related gross observations, changes in absolute or relative organ weights, or microscopic findings observed in the livers of the high dose animals examined at study termination. Based on these results, MTD was considered to be >100 mg/kg bw/day (highest dose tested).

6.1.2. 90-Day subchronic toxicity study on S6821

The purpose of this study was to evaluate the potential subchronic toxicity and toxicokinetic (TK) profile of S6821, in rats after administration for 90 consecutive days. Test article was administered in the diet to four groups of twenty male and twenty female Sprague-Dawley [Crl:CD®(SD)] rats (Charles River UK Ltd, Margate, Kent, England) at dose levels of 0 (control), 10, 30, or 100 mg/kg bw/day for 90 consecutive days. The test substance was administered continuously via the diet throughout the treatment period. Dietary concentrations (ppm) of S6821 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. For the purpose of the collection of the data for several of the study parameters, the animals were divided into two cohorts of 10 animals/sex/dose group (Cohorts A and B). At the conclusion of the study, 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 were performed to assess potential toxicity. A sensory reactivity and motor activity assessment (including, but not limited to, evaluation of motor activity, arousal, auditory startle response, tail pinch response, and grip strength) was conducted during 12th week of test article administration for all animals in Cohort A. Opthalmoscopic examinations were conducted pre-dose and during 12th week of test article administration for animals in the control and high dose groups in Cohort A. Samples for hematology, coagulation, and clinical chemistry evaluations were collected from all animals in Cohort A on Day 14, and during Weeks 7 and 13. Samples for urinalysis evaluations were collected from all animals during Week 12. Blood for TK analysis was collected on Day 7 and during Weeks 6 and 13 at six time points (each separated by 2 h) at alternating time points from three groups of 3 animals/sex/dose group from the animals in Cohort B. Microscopic examination of fixed hematoxylin and eosin-stained paraffin sections were performed on sections of tissues from the control and high-dose (100 mg/kg bw/day) groups from both Cohorts A and B. For organ weight data, analysis of covariance was performed using terminal bodyweight as covariate. The treatment comparisons were made on adjusted group means in order to allow for differences in bodyweight which might influence the organ weights.

The blood samples taken on Day 7 and during Week 6 and Week 13 of the 90-day toxicity study were used to assess the systemic exposure of male and female rats to S6821 administered in diet. Plasma concentrations of S6821 in samples taken at 6 time points over a 24-hour period were measured by a validated LC–MS/MS method. Maximum mean plasma concentrations (C_{max}) of S6821 and areas under the mean plasma S6821 concentration-time curves estimated over a 24-hour interval ($AUC_{0\text{-}24h}$) on Day 7 and during Week 6 and Week 13 are summarized in Table 5. Consistent with the results of the single dose PK study in rats where S6821 was administered by oral gavage, systemic exposure to S6821 was very low. For example, at the 100 mg/kg bw/day dose, C_{max} of S6821 ranged from 95.6 to 107 ng/mL (0.251–0.281 μM) in females, and from 41.4 to 54.7 ng/mL (0.109–0.143 μM) in males, throughout the duration of the study.

The rate (C_{max}) and extent ($AUC_{0\text{-}24h}$) of systemic exposure of rats to S6821 on Day 7 and during Week 6 and Week 13 increased with increasing dose over the nominal dose range 10–100 mg/kg bw/day. In males, however, these increases were less than the proportionate dose increment and at the highest nominal dose level (100 mg/kg bw/day) the C_{max} and $AUC_{0\text{-}24h}$ values were *ca* 32% lower than those values predicted from a linear relationship. In contrast, in females the increases were greater than the proportionate dose increment, and at the highest nominal dose level the C_{max} and $AUC_{0\text{-}24h}$ values were *ca* 1.3-fold higher. The dose-adjusted C_{max} of the female rats to S6821 was generally slightly higher than that in males. The dose-adjusted $AUC_{0\text{-}24h}$ of the female rats to S6821 was generally similar to that in males at the two lower dose levels (10 and 30 mg/kg bw/day), but was significantly higher for the females at the highest dose level (100 mg/kg bw/day). The dose-adjusted $AUC_{0\text{-}24h}$ of the male and female rats to S6821 during Week 6 and Week 13 was similar to that on Day 7 and there was no statistically significant evidence for any time (day of sampling) related differences in systemic exposure.

There were no test article-related deaths during the study. Two males and five females died or were sacrificed prematurely due to

Table 4
Summary of in vivo toxicity studies conducted on S6821 and S7958.

Study	Cmpd No.	Species/Gender (N value)	Dose	Findings
28-Day Dose Range Finding Toxicity Study	**S6821**	Male & Female Sprague-Dawley Rats 5 animals/sex/group	10, 30, 100 mg/kg bw/day (food ad-mix)	No test-article related findings; MTD > 100 mg/kg bw/day
90-Day Sub-Chronic Toxicity Study	**S6821**	Male & Female Sprague-Dawley Rats 20 animals/sex/group	10, 30, 100 mg/kg bw/day (food ad-mix)	No test-article related findings; NOAEL = 100 mg/kg bw/day
28-Day Sub-Acute Toxicity Study	**S7958**	Male & Female Sprague-Dawley Rats 10 animals/sex/group	10, 30, 100 mg/kg bw/day (food ad-mix)	No test-article related findings; NOAEL = 100 mg/kg bw/day
Dose Range Finding Developmental Toxicity Study	**S6821**	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	**S6821**	Bred Female Sprague-Dawley Rats 25 animals/group	125, 300, 1000 mg/kg bw/day (oral gavage)	NOAEL for both maternal toxicity and embryo/fetal development = 1000 mg/kg bw/day

Table 5
Toxicokinetics of S6821 in male and female Sprague-Dawley rats (food ad-mix).

Time Point	Dose (mg/kg bw)	Sex	C_{max} (ng/mL)	$AUC_{0\text{-}24h}$ (ng·h/mL)	$AUC_{0\text{-}24h}$/dose (ng·h/mL/mg/kg)	C_{max} Ratio[a]	$AUC_{0\text{-}24h}$ Ratio[b]	ACUM Ratio[c]
Day 7	10	M	6.05	118	11.8	1	1	–
		F	7.22	122	12.2	1	1	–
	30	M	16.7	295	9.83	2.8	2.5	–
		F	27.7	436	14.5	3.8	3.5	–
	100	M	41.4	844	8.44	6.8	7.2	–
		F	99.0	1500	15.0	13.7	12.3	–
Week 6	10	M	10.7	126	12.6	1	1	1.07
		F	6.34	113	11.3	1	1	0.93
	30	M	24.6	293	9.77	2.3	2.3	0.99
		F	17.5	331	11.0	2.8	2.9	0.76
	100	M	43.7	758	7.58	4.1	6.0	0.90
		F	95.6	1300	13.0	15.1	11.5	0.87
Week 13	10	M	5.99	123	12.3	1	1	1.04
		F	8.32	125	12.5	1	1	1.02
	30	M	18.3	305	10.2	3.1	2.5	1.03
		F	19.8	344	11.5	2.4	2.8	0.79
	100	M	54.7	925	9.25	9.1	7.5	1.10
		F	107	1670	16.7	12.9	13.4	1.11

[a] C_{max} Ratio = C_{max}/C_{max} at 10 mg/kg bw dose.
[b] $AUC_{0\text{-}24hr}$ Ratio = $AUC_{0\text{-}24hr}$/$AUC_{0\text{-}24hr}$ at 10 mg/kg bw dose.
[c] ACUM Ratio = $AUC_{0\text{-}24hr}$ at Week 6 or 13/$AUC_{0\text{-}24hr}$ at Day 7.

accidental causes associated with routine blood sampling. Body-weight gain and food consumption were unaffected by treatment (see Figs. 7 and 8). There were no test article-related clinical signs observed during the study and there were no treatment-related ophthalmic lesions. The appearance and behaviour of the animals, sensory reactivity and grip strength were unaffected by treatment. Motor activity scores for males receiving 100 mg/kg bw/day were slightly higher than those of controls ($p < 0.05$) and historical control scores during the second half of the 1-hour recording period, with scores for high beams and low beams (rearing and cage floor activity, respectively) similarly increased. The pattern of activity indicated that males receiving 100 mg/kg bw/day failed to habituate to the test cage environment to the same extent as control males. As there were otherwise no changes that were indicative of an adverse effect of treatment, the increase in motor activity was considered not adverse. Scores for females receiving 100 mg/kg bw/day and for males and females receiving 10 or 30 mg/kg bw/day were unaffected.

There were no test article-related effects among hematology parameters, coagulation times, clinical chemistry analytes, or urinalysis parameters in either sex at any dose level. Any observed differences were minor and as the majority of individual values were within the background range, were not considered toxicologically significant. There were no test article-related organ weight, macroscopic or microscopic changes noted at any dose level. All inter-group differences from controls were minor, seen in one sex only and were therefore attributed to normal biological variation. These included the slightly lower adjusted mean liver weights in males ($p < 0.05$) and the slightly higher adjusted mean spleen weights for females which received 100 mg/kg bw/day ($p < 0.05$).

In conclusion, once daily oral administration of S6821 for 90 days was well tolerated in rats at dose levels up to 100 mg/kg bw/day, with the only effect occurring in the high dose males where there was a slight increase of motor activity. No test article-related mortality or evidence of any systemic toxicity was observed and no target organs were identified. Based on the findings in this study the no-observed-effect-level (NOEL) was considered to be 30 mg/kg bw/day for males and 100 mg/kg bw/day for females and the no-observed-adverse-effect level (NOAEL) was 100 mg/kg/day in both sexes. See Supplementary Data for summary of the 90-day study data for S6821.

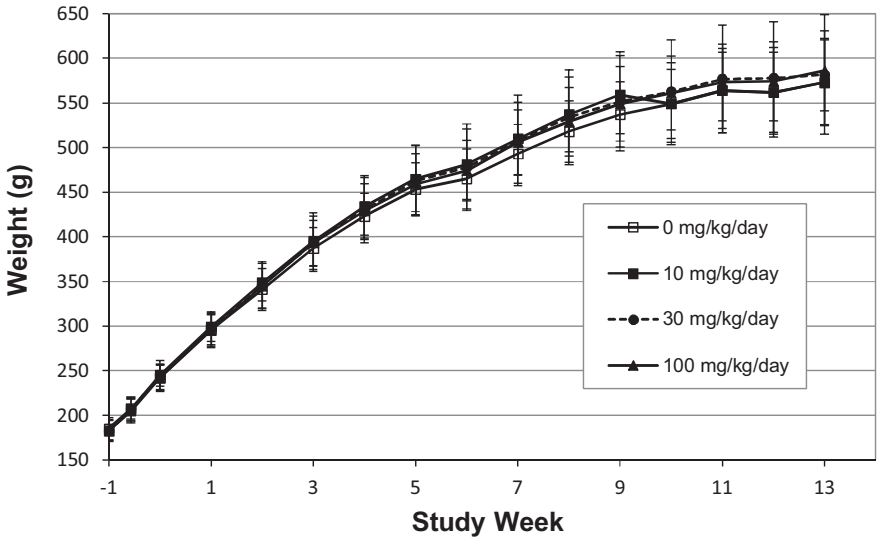


Fig. 7. Mean body weights of male Sprague-Dawley rats receiving S6821 for 13 weeks.

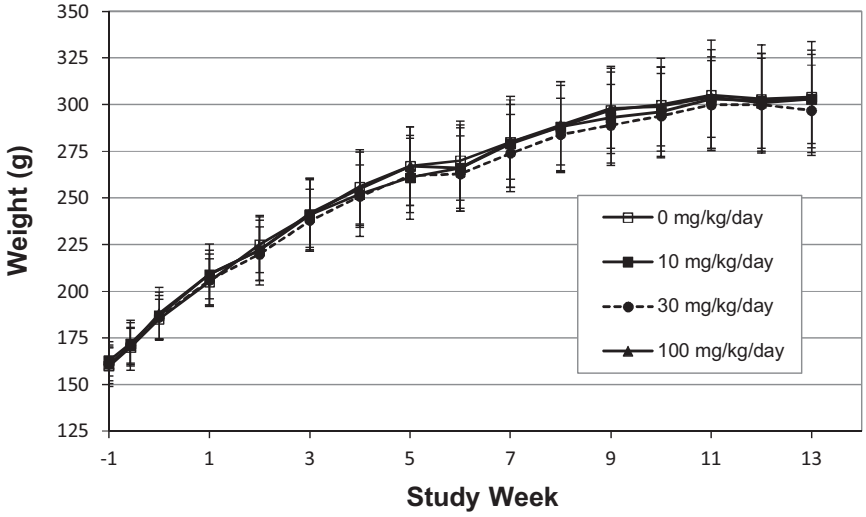


Fig. 8. Mean body weights of female Sprague-Dawley rats receiving S6821 for 13 weeks.

6.1.3. 28-Day subacute toxicity study on S7958

The purpose of these studies was to evaluate the potential toxicity of S7958 in rats after administration in diet for 28 consecutive days. Three treatment groups of male and female CD® [Crl:CD®(SD)] rats (n = 10/sex/group, Charles River UK Ltd, Margate, Kent, England) were administered S7958 in the diet at dose levels of 10, 30, or 100 mg/kg bw/day. One additional group of ten animals/sex served as the control and received the vehicle diet. The test substance was administered continuously via the diet throughout the treatment period. Dietary concentrations were adjusted at the same frequency as bodyweight and food consumption measurement to achieve constant doses in terms of mg/kg bw/day. At the conclusion of the study, 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 were performed to assess potential toxicity. A sensory reactivity and motor activity assessment (including, but not limited to, evaluation of motor activity, arousal, auditory startle response, tail pinch response, and grip strength) was conducted during the 4th week of test article administration for all animals. Opthalmoscopic examinations were conducted pre-dose and during the 4th week of test article administration for animals in the control and high dose groups. Samples for hematology, coagulation, and clinical chemistry evaluations were collected from all animals during Week 4. Samples for urinalysis evaluations were collected from all animals during Week 4. Blood samples for proof of exposure were obtained from the retro-orbital sinus on Day 29 at a single time point (06:00 GMT) from five male and five female animals in each dose group. Samples were also taken from the same animals at necropsy from the hepatic portal vein. Microscopic examination of fixed hematoxylin and eosin-stained paraffin sections were performed on sections of tissues all animals in the control and high-dose (100 mg/kg bw/day) groups.

Mean plasma concentrations of S7958 in samples obtained from the peripheral blood at 06:00 h GMT on Day 29, the mean plasma concentrations of S7958 in samples obtained from the hepatic portal vein at necropsy, and the relationship between the mean plasma concentrations are summarised in Table 6.

The mean concentrations of S7958 in the peripheral blood on Day 29 increased with increasing dose over the nominal range

Table 6
Mean Concentrations of S7958 in the Peripheral and Portal Vein Blood.

Dose	Sex	Peripheral Blood		Hepatic Portal Vein Blood	
		Mean Conc. (ng/mL)	Mean Conc. Ratio[a]	Mean Conc. (ng/mL)	Mean Conc. Ratio[a]
10 mg/kg bw/day	M	0.441	1	24.4	1
	F	18.4	1	19.4	1
30 mg/kg bw/day	M	1.79	4.1	68.2	2.8
	F	66.7	3.6	85.0	4.4
100 mg/kg bw/day	M	18.3	41.5	345	14.1
	F	136	7.4	334	17.2

[a] Mean Concentration Ratio = mean concentration/mean concentration at 10 mg/kg bw/day.

10–100 mg/kg bw/day but these increases were greater than the proportionate dose increment in males and lower than the dose increment in females. The mean concentrations of S7958 in the portal blood at necropsy also increased with increasing dose, however, these increases were greater than the proportionate dose increment in both males and females. Plasma concentrations of S7958 in the samples taken from the hepatic portal vein were generally higher than those taken from the peripheral blood, which may indicate a marked first-pass effect. These differences were particularly marked in males. The plasma concentrations of S7958 in all the samples taken from the control animals were below the limit of quantification (<0.500 ng/mL) demonstrating that there was no quantifiable contamination in these animals.

There were no test article-related deaths during the study and all animals survived until scheduled euthanasia. There were no ophthalmic lesions in Week 4 that were considered to be associated with treatment. There were no test article-related clinical signs observed during the study. The appearance, behaviour, sensory reactivity findings, grip strength values and motor activity scores of the animals were unaffected by treatment. Motor activity scores for males and females showed some inter-group variation but the overall pattern of activity during the 1-hour recording period showed no differences considered to be associated with treatment.

Bodyweight gain was unaffected by treatment (see Figs. 9 and 10). The overall bodyweight gain of treated males was slightly lower than that of the controls. There was no dose relationship and these small inter-group differences were not considered associated with treatment. The overall bodyweight gain of females receiving 100 mg/kg bw/day was also slightly low when compared to the controls, however weekly gains were variable, particularly in Week 3 which accounted for the majority of the difference from controls.

The hematological investigation in Week 4 did not identify any toxicologically significant findings. The biochemical examination of the blood plasma in Week 4 revealed slightly low phosphorus concentrations in males and females receiving 10, 30 or 100 mg/kg bw/day, though statistical significance was only achieved at 30 and 100 mg/kg bw/day ($p < 0.05$), and high chloride levels in females receiving 10, 30 or 100 mg/kg bw/day ($p < 0.01$). A number of other inter-group differences from controls occurred but these tended to be minor, confined to one sex or lacked dosage-relationship and were therefore likely to represent normal biological variation. The appearance and composition of urine was not affected by treatment.

There were no test article-related organ weight, macroscopic or microscopic changes noted at any dose level of S7958. All macroscopic and microscopic observations were considered incidental/spontaneous, of the nature commonly observed in this strain and age of rats, and/or were of similar incidence and severity in control and treated animals.

In conclusion, once daily oral administration of S7958 for 28 days was well tolerated in rats at dose levels up to 100 mg/kg bw/day. No test article-related mortality or evidence of any systemic toxicity was observed and no target organs were identified. Based on these results, the NOAEL was considered to be 100 mg/kg bw/day. See Supplementary Data for summary of the 28-day study data for S7958.

6.2. Developmental toxicity studies

6.2.1. Dose range-finding developmental toxicity study

The objective of the study was to determine dosage levels of S6821 to be evaluated in a definitive developmental toxicity study conducted in rats. The test article, S6821, in the vehicle (1% MC [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). A concurrent control group composed of 8 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 twice daily for mortality and moribundity. Clinical observations, body weights, and food consumption were recorded at appropriate intervals. On gestation Day 21, all animals were euthanized by carbon dioxide inhalation and 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, their sex determined, and examined for external malformations and developmental variations.

All females survived to the scheduled necropsy on gestation Day 21; 2 females in the 1000 mg/kg bw/day dose group delivered on this day (the expected day of delivery for this species). One female in the 500 mg/kg bw/day group was determined to be nongravid. Clinical findings noted at the daily examinations, including hair loss or scabbing on various body surfaces, occurred infrequently, at similar frequencies in the control group, and/or in a manner that was not dose-related. No test article-related clinical or macroscopic findings were noted at any dosage level. Mean maternal body weights, body weight gains, net body weights, net body weight gains, gravid uterine weights, and food consumption were unaffected by test article administration up to 1000 mg/kg bw/day.

Intrauterine growth and survival were not affected by test article administration at any dosage level. Parameters evaluated included post-implantation loss, live litter size, mean fetal body weights, and fetal sex ratios. Mean numbers of *corpora lutea* and implantation sites and the mean litter proportions of pre-implantation loss were

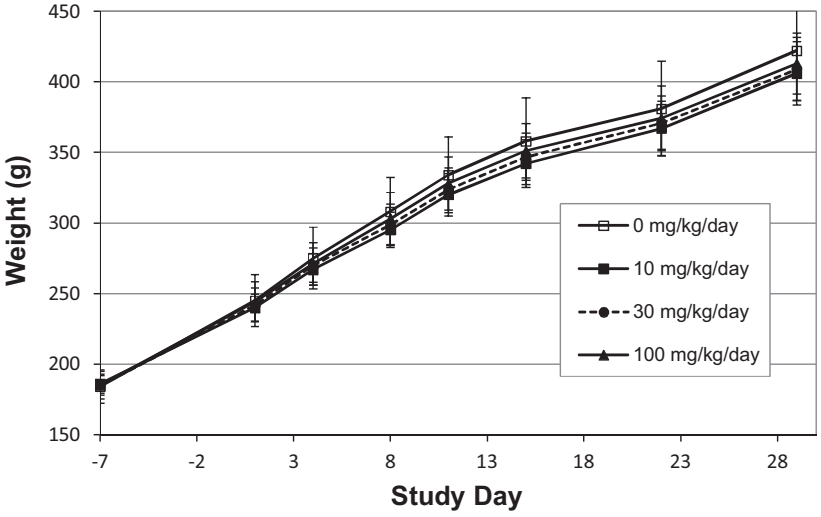


Fig. 9. Mean body weights of male Sprague-Dawley rats receiving S7958 for 4 weeks.

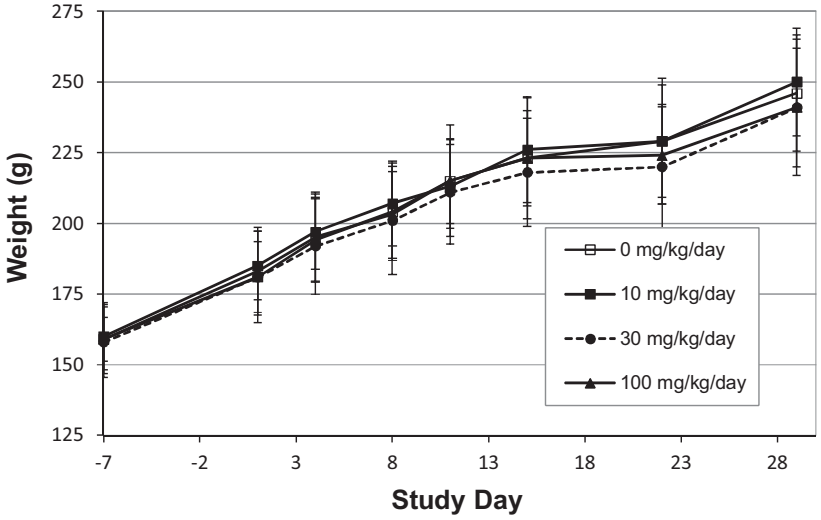


Fig. 10. Mean body weights of female Sprague-Dawley rats receiving S7958 for 4 weeks.

similar across all groups. Differences from the control group were slight and not statistically significant. The numbers of fetuses (litters) available for morphological evaluation were 109(8), 114(8), 119(8), 101(7), and 118(8) in the control, 125, 250, 500, and 1000 mg/kg bw/day groups, respectively. Cyclopia (proboscis-like nose, microstomia, and bilateral anophthalmia) was noted in one fetus in the 250 mg/kg bw/day group. This finding was not considered test article-related because it occurred in a single fetus and was not observed in the higher dosage groups. No other external malformations and no external developmental variations were noted in fetuses in this study.

No evidence of maternal toxicity or embryo-fetal toxicity was noted in this study at dosage levels up to 1000 mg/kg bw/day. Therefore, dosage levels of 125, 500, and 1000 mg/kg bw/day were selected for the definitive embryo/fetal development study of S6821.

6.2.2. Definitive developmental toxicity study

The objective of the study was to determine the potential of S6821 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. The test article, S6821, in the vehicle (1% MC [400 cps]) was administered orally by gavage to 3 groups of 25 bred female Crl:CD(SD) rats (Charles River Laboratories, Kingston, NY) once daily from gestation days 6 through 20, at dosage levels 125, 500, and 1000 mg/kg bw/day (dose volume 10 mL/kg). A concurrent control group composed of 25 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, all animals were euthanized by carbon dioxide inhalation and 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, their sex determined, and examined for external, visceral, and skeletal malformations and developmental variations.

All females survived to the scheduled necropsy on gestation Day 21. Two females in the control group (0 mg/kg bw/day), and three in the 1000 mg/kg bw/day dose group were determined to be non-gravid. No significant article-related clinical findings were noted at the daily examinations or 1 h following dose administration at any dosage level.

Mean body weight, body weight gains and food consumption were comparable across all groups (See Fig. 11). Mean food consumption was significantly ($p < 0.01$) higher on gestational Days 8–9 in the 1000 mg/kg bw/day dosage group compared to the control; however, this was transient and did not affect the overall food consumption or body weight gain for the interval. Therefore, the higher food consumption for this interval was not considered test article-related. There were no significant maternal necropsy findings in any of the dose groups.

At the scheduled necropsy on gestation Day 21, no test article related internal findings were observed at any dosage levels. 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 any dosage level. 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. There appeared to be a small, though dose-dependent, increase in early resorptions and post-implantation loss; however, the increase did not reach statistical significance and the high dose values were relatively similar to those reported in the historical control data (Table 7). A significantly ($p < 0.01$) higher mean female fetal weight was noted in the 125 mg/kg bw/day group. The higher mean fetal weight in this group did not occur in a dose-dependent manner, and therefore was not considered test article-related. Any other differences from the control group were slight and/or did not occur in a dose-related manner.

The numbers of fetuses (litters) available for morphological evaluation were 320(23), 347(25), 364(25), and 327(22) in the control, 125, 500, and 1000 mg/kg bw/day groups, respectively. Malformations were observed in 4(4), 3(2), 2(2), and 4(3) fetuses (litters) in these same respective dose groups and were considered spontaneous in origin.

One fetus in the 1000 mg/kg bw/day group had localized fetal edema (neck and thorax). One fetus in the control group and one in the 500 mg/kg bw/day group had meningoencephalocele; both of these findings was confirmed skeletally, consisting of misshapen parietal and interparietal bones in the control group fetus and misshapen frontal and nasal bones in the fetus in the 500 mg/kg bw/day group. One fetus in the 125 mg/kg bw/day group had unilateral microphthalmia. These external malformations occurred infrequently in single fetuses, at similar frequencies in the concurrent control group, and/or in a manner that was not dose-related. In addition, the mean litter proportions of these findings were not statistically significantly different from the control group and/or were within the ranges of values in the historical control data. Therefore, these findings were not attributed to test article administration.

External variations were limited to twinning, noted for two fetuses in the 125 mg/kg bw/day group; however, there was only a single occurrence of this finding and it did not occur in a dose-related manner. In addition, the mean litter proportion of this finding was not statistically significantly different from the concurrent control group. Therefore, this finding was not attributed to test article administration.

The aforementioned fetus with localized edema in the 1000 mg/kg bw/day group and one of the fetuses in the 125 mg/kg bw/day group had a total of 5 and 4 visceral malformations, respectively. These visceral malformations included situs inversus (trachea, esophagus, heart, great and major vessels, liver, stomach, pancreas, spleen, kidneys, adrenals, and/or intestine laterally transposed), lobular dysgenesis of the lungs (1 lobe present, bilateral), small atrio-ventricular valve (bilateral), and interventricular septal defect for both fetuses, and a malpositioned vena cava (coursed between the right adrenal and kidney) for the fetus in the 1000 mg/kg bw/day group. In addition, the fetus in the 125 mg/kg bw/day group had a small left ventricle (visceral developmental variation). Lobular dysgensis of the lungs (1 lobe present, bilateral) was also noted for one fetus in the concurrent control group, and situs inversus (trachea, esophagus, heart, great and major vessels, lungs, liver, stomach, pancreas, spleen, kidneys, adrenals, and/or intestine laterally transposed) was also noted for two fetuses from the same litter in the concurrent control group. Two fetuses from the same litter in the 1000 mg/kg bw/day group were noted with a persistant truncus arteriosus which consisted of pulmonary arteries that arose from the ascending aorta with an interventricular septal defect. Right-sided aortic arch (aortic arch and descending aorta coursed to the right of the vertebral column; right carotid and right subclavian arteries arose independently from the aortic arch [no brachiocephalic trunk]; left subclavian coursed retroesophageal) was seen in the fetus in the 125 mg/kg bw/day group noted above which had unilateral microphthalmia. These visceral malformations occurred infrequently in single fetuses, at similar frequencies in the concurrent control group, within single litters, and/or in a manner that was not dose-related. In addition, the mean litter proportions of these findings were not statistically significantly different from the control group and/or were within the ranges of values in the historical control data. Therefore, these findings were not attributed to test article administration.

Visceral developmental variations, including renal papilla(e) not fully developed and/or distended ureter, small heart, hemorrhagic ring around the iris, accessory lobules of the liver, and pale spleen, occurred infrequently, at similar frequencies in the concurrent control group, and/or in a manner that was not dose-related. In addition, the mean litter proportions of these findings were not statistically significantly different from the control group and/or were within the ranges of values in the laboratories historical control data. Therefore, these findings were not attributed to test article administration.

There were no test article-related skeletal malformations noted for fetuses at any dosage level. The fetus in the 1000 mg/kg bw/day group noted above with persistent truncus arteriosus also had sternoschisis (sternal bands not joined). Another fetus from a different litter in the 1000 mg/kg bw/day group had bent limb bones (femur and humerus, bilateral). One fetus in the 500 mg/kg bw/day group had fused sternebrae. Severely malaligned sternebrae were noted for 2(2) fetuses (litters) in the 125 mg/kg bw/day group. Vertebral anomaly with associated rib anomaly (absent, misshapen, and fused arches and fused ribs) was also noted in one of these fetuses. This is the same fetus described above that also had unilateral microphthalmia and a right-sided aortic arch. A vertebra anomaly (malpositioned and missized arches) was also noted for one fetus in the concurrent control group. These skeletal malformations occurred infrequently in single fetuses, at similar frequencies in the concurrent control group, and/or in a manner that was not dose-related. In addition, the mean litter proportions of these findings were not statistically significantly different from the control group and/or were within the ranges of values in the historical control data. Therefore, these findings were not attributed to test article administration.

Skeletal variations observed in the test article-treated groups occurred infrequently, at similar frequencies in the concurrent control group, and/or in a manner that was not dose related. A slight increase in 27 presacral rib(s) was noted in the 1000 mg/kg

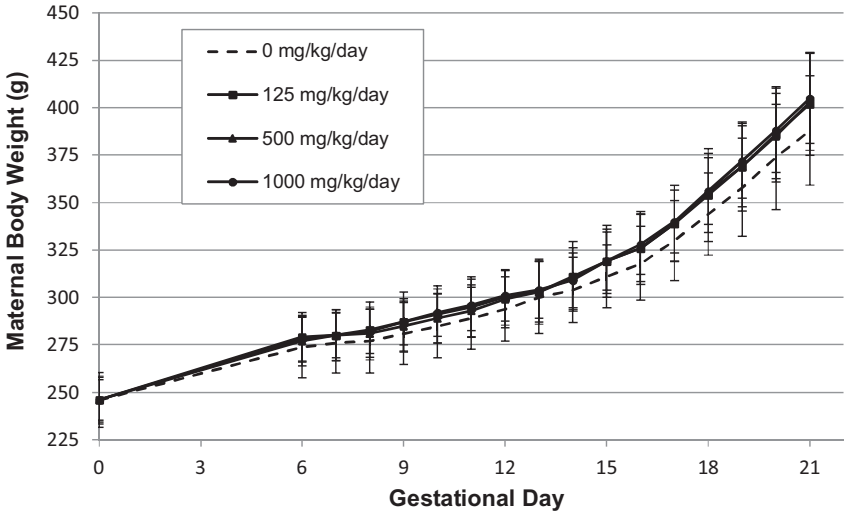


Fig. 11. Oral (Gavage) Developmental Toxicity Study of S6821 in Rats: Mean maternal body weights during gestation (0 mg/kg bw/d: n = 23; 125, and 500 mg/kg bw/d: n = 25/group; 1000 mg/kg bw/d: n = 22/group).

Table 7
Developmental Toxicity Study of S6821 in Rats: Summary of Fetal Data.

Dose Group (mg/kg bw/day)	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	165	155	320	0	9	0	9	329	388	59	NA	23
	Mean	7.2	6.7	13.9	0.0	0.4	0.0	0.4	14.4	16.9	2.6	5.5	
	S.D.	2.37	3.32	3.87	0.00	0.72	0.00	0.72	3.77	3.88	6.04	0.43	
	S.E.	0.49	0.69	0.81	0.00	0.15	0.00	0.15	0.79	0.81	1.26	0.09	
125	Total	177	170	347	0	17	0	17	364	484	20	NA	25
	Mean	7.1	6.8	13.9	0.0	0.7	0.0	0.7	14.6	15.4	0.8	5.7	
	S.D.	2.02	1.50	1.86	0.00	0.75	0.00	0.75	1.87	2.10	0.96	0.25	
	S.E.	0.40	0.30	0.37	0.00	0.15	0.00	0.15	0.37	0.42	0.19	0.05	
500	Total	179	185	364	0	19	1	20	384	415	31	NA	25
	Mean	7.2	7.4	14.6	0.0	0.8	0.0	0.8	15.4	16.6	1.2	5.6	
	S.D.	1.65	1.80	1.89	0.00	0.88	0.20	0.91	1.70	2.24	1.42	0.24	
	S.E.	0.33	0.36	0.38	0.00	0.18	0.04	0.18	0.34	0.45	0.28	0.05	
1000	Total	165	162	327	0	20	0	20	347	372	25	NA	22
	Mean	7.5	7.4	14.9	0.0	0.9	0.0	0.9	15.8	16.9	1.1	5.6	
	S.D.	2.13	2.74	2.64	0.00	1.54	0.00	1.54	2.05	1.90	1.28	0.33	
	S.E.	0.46	0.56	0.56	0.00	0.33	0.00	0.33	0.44	0.41	0.27	0.07	

NA = not applicable.

bw/day group compared to the control group (8 from 2 litters in the 1000 mg/kg bw/day group compared to 1 from 1 litter in the control group). This increase was primarily due to a single litter that consisted of 7 fetuses with 27 presacral rib(s) in association with a 14th rudimentary rib. Due to the majority of this variation stemming from a single litter, and that this finding did not reach statistical significance, the higher incidence of 27 presacral rib(s) is not considered test article-related. In addition, the mean litter proportions of these findings were not statistically significantly different from the control group and/or were within the ranges of values in the historical control data. Therefore, these findings were not attributed to test article administration.

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 S6821 was administered orally by gavage to bred Crl:CD(SD) rats.

7. Discussion

S6821 and S7958 are members of a novel series of antagonists of the human bitter receptor hTAS2R8 which have demonstrated the ability to significantly attenuate the bitter taste of a variety of bitter tastants present in consumer products including caffeine, rebaudioside A, whey protein and hydrolyzed soy protein. In CYP inhibition assays using both spectrofluorimetric substrates with recombinant enzymes and CYP-specific substrates with pooled human liver microsomes, S7958 exhibited significant inhibition of CYP2C9 (IC_{50} = 8.8 μM, using pooled human liver microsomes). However, given the low use levels of S7958 and its low systemic bioavailability after oral administration, this is not expected to interfere with normal metabolic processes. In contrast, S6821 did not significantly inhibit any of the CYP isozymes in either assay format. No other significant off-target activities were seen with either S6821 or S7958 in any of the receptor profiling assays.

Based on *in vitro* studies with rat and human liver microsomes, S6821 is metabolized by CYP oxidative enzymes to produce several mono-hydroxylated metabolites. Subsequent *in vivo* metabolism

studies in the rat indicated that the major metabolic pathway for elimination of S6821 involved reaction of the phenolic moiety of S6821 with Phase II enzymes to produce the corresponding sulfate (M461) and glucuronide (M557) conjugates. *In vivo*, the rat produces all of the mono-hydroxylated metabolites seen in the human microsomal incubations along with the conjugated forms (*e.g.*, sulfate, glucuronide) of the parent compound and its mono-hydroxylated metabolites. Taken together, these findings support the rat is a suitable species for assessing the potential toxicity of S6821. Similarly, in the case of S7958, ions consistent with the presence of S7958 glucuronide and sulfate metabolites as well as a sulfate derivative of a mono-hydroxylated metabolite were observed in the extracted ion chromatograms of the plasma samples. Although plasma concentrations of the parent compound S7958 are significantly less than those seen with S6821, S7958 demonstrates an overall metabolism profile similar to that of its close analogue S6821.

Following intravenous administration of S6821 to male SD rats elimination of both the parent and the glucuronide metabolite M557 was found to be rapid ($t_{1/2}$ of 0.1 and 0.2 h respectively), while elimination of the sulfate metabolite M461 was significantly slower ($t_{1/2}$ = 1.9 h), indicating that the rate of formation of the metabolite was not the rate-limiting step in its elimination. Systemic exposure (AUC_{last}) was shown to be highest for metabolite M461 while the exposure to S6821 and metabolite M557 were similar. The total plasma clearance of S6821 was higher than the reported hepatic blood flow in rats and the volume of distribution at steady state was less than the reported total body water in rats.

Following oral administration of S6821 to male SD rats, S6821 absorption was rapid with a terminal $t_{1/2}$ of 4.3 h (at the highest dose level). The terminal $t_{1/2}$ of metabolites M461 and M557 ranged between 2.5–4.7 and 1.4–4.2 h respectively, with the longest $t_{1/2}$ being exhibited at the highest dosage. The rate (C_{max}) and extent (AUC_{last}) of systemic exposure to the parent and metabolites was shown to increase with increasing dosage, but tended not to be proportional to dose as predicted from a linear relationship. Most significantly, the systemic exposure of metabolites M461 and M557 ranged from 160- to 650- and from 109- to 216-fold higher than that of S6821, respectively. Given the significantly lower systemic exposure of S6821 relative to metabolites M461 and M557, any *in vivo* findings in the rodent toxicology studies might more likely be attributed to these metabolites rather the parent compound S6821.

S6821 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 a chromosome aberration test in HPBL. S6821 was found to be neither mutagenic or clastogenic in these *in vitro* genotoxicity assays. Its close analog S7958, also showed no evidence of mutagenicity in the bacterial reverse mutation assays either in the presence or absence of metabolic activation.

S6821 also evaluated in an *in vivo* mouse micronucleus assay. Oral administration of S6821 at doses of 500, 1000, and 2000 mg/kg bw to male and female CD-1 mice for two consecutive days were well tolerated and did not induce clastogenicity nor indicate interactions with the mitotic spindle in bone marrow erythrocytes. No appreciable reductions in the PCE/TE ratio in the test article group compared to the vehicle control group were observed indicating the S6821 also did not induce cytotoxicity. No significant changes in body weight or apparent signs of toxicity were observed in mice administered S6821 during the study period.

The doses of S6821 and S7958 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 margin of safety of 1000-fold 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]; [12].

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) employed both the maximized survey-derived intake (MSDI) method and single portion exposure technique (SPET) as measures of dietary exposure to flavouring agents for use in their safety evaluation of these compounds [21]. The MSDI is based on the reported amount of a flavouring agent introduced into the food supply per year in specific regions of the world and provides a *per capita* estimate of the exposure to the flavouring agent, assuming that 10% of the relevant population would consume foods containing the flavouring agent. However, in many cases the MSDI is believed to underestimate the dietary exposure to some flavouring agents. The SPET was developed to account for specific consumer patterns of behaviour with respect to food consumption and possible uneven distribution of dietary exposure for consumers of foods containing flavouring agents [21]. The SPET provides an estimate of the dietary exposure for an individual who consumes a specific food product containing the flavouring agent every day and combines an average added use level with the standard portion size for a particular food category. The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate. Based on the SPET value obtained from non-alcoholic beverages, JECFA estimated the dietary exposure of both S6821 and S7958 (JECFA Nos. 2161 and 2162) to be 4000 μg/day or 67 μg/kg bw/day [22]. Therefore, based on this calculation, a NOAEL of 100 mg/kg bw/day in the 28- and 90-day toxicology studies would be 1500 times the estimated dietary exposure to either S6821 or S7958 when used as a flavouring agent.

Dietary administration of S6821 rats for either 28 or 90 days at doses of up to 100 mg/kg bw/day was generally well tolerated. There was no effect of treatment on clinical signs, bodyweight, food consumption, ophthalmic function, hematological parameters, blood chemistry parameters, urine, macropathology or histopathology.

The TK investigations in the 90-day study indicated that the rate and extent of systemic exposure of rats to S6821 was characterised by nonlinear (dose-dependent) kinetics over the nominal dose range 10–100 mg/kg bw/day. Increasing the dose above 30 mg/kg bw/day resulted in a lower systemic exposure in males, but a higher systemic exposure in females, than would be predicted from a linear relationship. In general, there were no differences in the systemic exposure of male and female rats to S6821 at the lower doses of 10 or 30 mg/kg bw/day. However, at the highest dose (100 mg/kg bw/day) the systemic exposure of females was significantly higher than that of males. There were no time-related differences in systemic exposure in either sex. The less than dose-proportional increase in exposure in males suggests that the higher doses of S6821 may be inducing the further metabolism of S6821 on repeat dosing in males, but not in female rats. This is further supported by the observation that in the single dose PK study in male rats, exposure (AUC) at the 100 mg/kg bw/day dose was somewhat greater than dose proportional. In female rats, the greater than dose-proportional exposure at 100 mg/kg bw/day may be due to saturation of a clearance pathway. *In vivo* metabolism studies in rats have shown that S6821 is not only rapidly metabolized to the corresponding sulfate M461 and glucuronide M557 conjugates, but can also be hydroxylated and subsequently sulfated. Therefore, it is possible that these metabolic pathways are further induced at high doses in males, but become saturated in females at the same dose. Gender-dependent metabolism of xenobiotics and sexual dimorphisms in response to inducing agents are well known phenomena

in rats that has been attributed to differences in the profile of CYP isozymes found in male and female rat liver [24,38].

In the 90-day toxicity study of S6821, motor activity (both high and low beam scores; observation period of 60 min) was slightly increased for males receiving 100 mg/kg bw/day when compared to that of controls ($p < 0.05$) and historical control data. Females receiving 100 mg/kg bw/day were unaffected (see motor activity scores in the Supplemental Material for details). As noted above, the TK study indicated that the exposure to S6821 was significantly lower in males than females at 100 mg/kg bw/day dose and therefore the increased motor activity in males may be more likely due to higher levels of a S6821 metabolite. The pattern of findings at the motor activity assessment indicated that the high dose males failed to habituate to the test cage environment to the same extent as control males. No increase in activity was noted during clinical signs recorded throughout the treatment period. As there were otherwise no changes that were indicative of an adverse effect of treatment, the increase in motor activity was not considered adverse. Based on the findings in this study the NOEL for S6821 was considered to be 30 mg/kg bw/day for males and 100 mg/kg bw/day for females, and the NOAEL was 100 mg/kg bw/day in both sexes.

Dietary administration of S7958 rats for 4 weeks at doses of up to 100 mg/kg bw/day was also generally well tolerated. There was no effect of treatment on clinical signs, behavioural tests, bodyweight, food consumption, ophthalmic function, hematological parameters, urine, macropathology or histopathology demonstrating that the test substance was of low toxicity.

The mean concentrations of S7958 in the peripheral and portal vein blood increased with increasing dose over the dose range. These increases were greater than the proportionate dose increment in the peripheral blood of males and in the portal vein blood of both males and females. In contrast, the increase in the mean concentrations of S7958 in the peripheral blood of the females was lower than the dose increment. Plasma concentrations of S7958 in the samples taken from the hepatic portal vein were generally higher than those taken from the peripheral blood, which may indicate a marked first-pass effect. These differences were particularly marked in males.

Minor electrolyte changes were noted for all treated groups but as there was no histopathological correlate in the kidneys, these changes were not considered to be adverse. Based on the findings in this study the NOAEL for S7958 was 100 mg/kg bw/day in both sexes.

In the developmental toxicity study of S6821, there were no test article-related clinical or macroscopic findings were noted at any dosage level. Mean maternal body weights, body weight gains, gravid uterine weights, and food consumption were unaffected by test article administration at all dosage levels. No test article-related findings were noted on intrauterine growth and survival and fetal morphology at any dosage level. When the total malformations and developmental variations were evaluated on a proportional basis, no statistically significant differences from the control group were noted. Fetal malformations and developmental variations, when observed in the test article treated groups, occurred infrequently or at a frequency similar to that in the control group, did not occur in a dose-related manner, and/or were within the laboratories historical control data ranges. Based on these data, no fetal malformations or developmental variations were attributed to the test article at any dosage level.

Based on the lack of 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 S6821 for both maternal toxicity and embryo/fetal development.

8. Conclusions

S6821 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 S6821 at doses up 100 mg/kg bw. The corresponding 5,5-dimethyl analog S7958 also demonstrated a lack of mutagenicity in a reverse bacterial mutation assay. S6821 had a NOAEL of 1000 mg/kg bw/day for both maternal toxicity and embryo/fetal development in rats. The results of a 90-day subchronic toxicity study with S6821 and a 28-day short term toxicity study with S7958 established NOAELs for both compounds of 100 mg/kg bw/day (the highest dose evaluated), for both male and female rats. Assuming that the systemic exposure of these compounds 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 both compounds under the conditions of intended use.

Conflict of interest

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxrep.2016.02.007.

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