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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