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Toxicological evaluation of two flavors with modifying properties: 3-((4-amino-2,2-dioxido-1*H*-benzo[*c*][1,2,6]thiadiazin-5-yl)oxy)-2,2-dimethyl-*N*-propylpropanamide and (*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[*c*][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one

Amy J. Arthur [a,*], Donald S. Karanewsky [a], Mike Luksic [b], Geoff Goodfellow [b], Jon Daniels [b]

[a] *Senomyx, Inc., 4767 Nexus Centre Drive, San Diego, CA 92121, USA*
[b] *Intrinsik Health Sciences Inc., 6605 Hurontario Street, Suite 500, Mississauga, Ontario L5T 0A3, Canada*

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ABSTRACT

A toxicological evaluation of two structurally related flavors with modifying properties, 3-((4-amino-2,2-dioxido-1*H*- benzo[*c*][1,2,6]thiadiazin-5-yl)oxy)-2,2-dimethyl-*N*-propylpropanamide (S6973; CAS 1093200-92-0) and (*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[*c*][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one (S617; CAS 1469426-64-9), was completed for the purpose of assessing their safety for use in food and beverage applications. Both compounds exhibited minimal oxidative metabolism *in vitro*, and in rat pharmacokinetic studies, were poorly absorbed and rapidly eliminated. Neither compound exhibited genotoxic concerns. S6973 and S617 were not found to be mutagenic or clastogenic, and did not induce micronuclei *in vitro* or *in vivo*. In subchronic oral toxicity studies in rats, the no-observed-adverse-effect-levels (NOAELs) were 20 mg/kg/day and 100 mg/kg/day (highest doses tested) for S6973 and S617, respectively, when administered as a food ad-mix for 90 consecutive days. Furthermore, S617 demonstrated a lack of maternal toxicity, as well as adverse effects on fetal morphology at the highest dose tested, providing a NOAEL of 1000 mg/kg/day for both maternal toxicity and embryo/fetal development when administered orally during gestation to pregnant rats.

1. Introduction

Flavors with modifying properties (FMPs) is a term used by the flavor industry to describe ingredients that function as part of a flavor system, also known as compounded flavors (Hallagan and Hall, 2009), to modify or enhance the flavor profile of a variety of food and beverages. FMPs may not necessarily have a taste on their own (Marnett et al., 2013), but may work in concert with other flavor ingredients in a flavor system to change the flavor profile of a food product, such as by decreasing or increasing the intensity of specific flavor characteristics (Harman et al., 2013). One such class of FMPs are the recently discovered positive allosteric modulators (PAMs) of the human sweet receptor which, in addition to modifying certain aspects of the flavor profile, allow for a reduction of carbohydrate sweeteners in food and beverage products while maintaining the desired sweet taste of natural sugars (Servant et al., 2010, 2011; Zhang et al., 2010).

Two examples of this class of FMPs are 3-((4-amino-2,2-dioxido-1*H*-benzo[*c*][1,2,6]thiadiazin-5-yl)oxy)-2,2-dimethyl-*N*-propylpropanamide (S6973; CAS 1093200-92-0) and (*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[*c*][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one (S617; CAS 1469426-64-9). These FMPs have been shown to function in food and beverage products with reduced sweetener to restore sweetness as well as modify other aspects of the flavor profile, such as juicy, lemony, green notes, licorice, and clove notes. The structures of S6973 and S617 are shown in Fig. 1. S6973 and S617 differ only in the identity of the alkoxy side chain appended to the 5-position of a benzothiadiazine nucleus. The alkoxy side chain of both compounds contains an amide bond linkage in the linear backbone of the side chain. The alkoxy side chain of S6973 is derived from 3-hydroxy-2,2-dimethyl-*N*-propylpropanamide, while that of S617

Abbreviations: AUC, area under the curve; CL, plasma clearance; C_{max}, peak plasma concentration; EIC, extracted ion chromatogram; FDA, Food and Drug Administration; FEMA, Flavor and Extract Manufacturers Association of the United States; FL-no, FLAVIS number; FMP, flavor with modifying properties; GLP, Good Laboratory Practices; GMP, Good Manufacturing Practices; LC/MS, liquid chromatography with mass spectrometry; MC, methylcellulose; NOAEL, no-observed-adverse-effect-level; OECD, Organisation for Economic Co-operation and Development; PCE, polychromatic erythrocytes; PK, pharmacokinetics; RCG, Relative Cell Growth; RMI, Relative Mitotic Index; $t_{1/2}$, half-life; T_{max}, time to reach C_{max}; TK, toxicokinetics.

* Corresponding author. Senomyx, Inc., 4767 Nexus Centre Drive, San Diego, CA 92121, USA. Tel.: +1 858 646 8337; fax: +1 858 404 0750.
E-mail address: amy.arthur@senomyx.com (A.J. Arthur).

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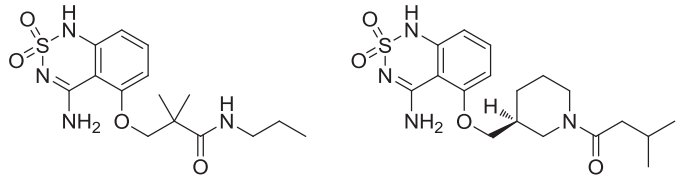

S6973
Chemical Formula: $C_{15}H_{22}N_4O_4S$
Molecular Weight: 354.4246

S617
Chemical Formula: $C_{18}H_{26}N_4O_4S$
Molecular Weight: 394.4884

Fig. 1. Structures of S6973 and S617.

is derived from (*S*)-1-(3-(hydroxymethyl)piperidin-1-yl)-3-methylbutan-1-one.

These substances were reviewed by the Expert Panel of the Flavor and Extract Manufacturers Association of the United States (FEMA) and determined to be generally recognized as safe (GRAS) under their conditions of intended use as flavor ingredients (FEMA, 2014; Smith et al., 2011; Hallagan and Hall, 2009) and therefore are available for use in human food in the United States as "FEMA GRAS" flavor ingredients. S6973 was assigned FEMA GRAS Number 4701 in 2009 (Smith et al., 2011). S6973 was also determined to be safe at the current levels of intake by the Joint FAO/WHO Expert Committee on Food Additives (Fields et al., 2012; assigned JECFA No. 2082) and the European Union (EFSA CEF Panel, 2013; assigned FL-no: 16.126). Other jurisdictions permit the use of S6973 including China, Korea, Indonesia, and Mexico (CO-24.01.2013). S617 was also determined to be FEMA GRAS in 2014 and was assigned FEMA GRAS Number 4802 (FEMA, 2014).

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, genotoxicity studies, and developmental toxicity studies conducted with S6973 and/or S617. Additional supporting data obtained in these studies with S6973 and S617 is included in a Supplementary Data section in the online publication.

2. Materials and methods

The batch of S6973 used for the *in vitro* metabolism, *in vivo* PK, and 28-day range-finding toxicity studies (Batch ID no. 31353842, purity >99.5%), was synthesized at Senomyx, San Diego, CA using the procedure described in US Patent No. 8,633,186 (Tachdjian et al., 2014a). The batch of S6973 used for the genotoxicity and 90-day subchronic toxicity studies (Batch ID no. 36669000, Lot no. P-1658, purity 99.9%) was synthesized at Norac Pharma, Azusa, CA using the same synthetic method but prepared in conformance with Good Manufacturing Practices (GMPs) as described in the ICH GMP Guidelines for APIs (ICH, 2000).

The batch of S617 used for the *in vitro* metabolism, *in vivo* PK, genotoxicity and 28-day range-finding toxicity studies (Batch ID no. 57926320, purity >99%), was synthesized at Senomyx using the procedure described in US Patent No. 8,877,922 B2 (Tachdjian et al., 2014b). The batch of S617 used for the 90-day subchronic and developmental toxicity studies (Batch ID no. 105809368, Lot no. CMLW-083/13-SS2, 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 (ICH, 2000).

With the exception of the abbreviated Ames assays, all genetic toxicology studies were conducted in compliance with the FDA Good Laboratory Practices (GLP) regulations 21 CFR Part 58 (FDA, 2014) and OECD guidelines (OECD, 1998). The experimental design for these studies followed the OECD Guidelines for the Testing of Chemicals – 471, 473, 474, and 487 (OECD, 1997a, 1997b, 1997c, 2010). The 28-day dose-range finding studies and 90-day toxicology studies in rats were conducted in compliance with the United States Food and Drug Administration (FDA) Guidelines (FDA, 2003a, 2003b) Toxicological Principles for the Safety of Food Ingredients; the 90-day subchronic toxicology study was also conducted in compliance with the FDA Good Laboratory Practice (GLP) Regulations, 21 CFR Part 58. The developmental toxicity range-finder and definitive studies were conducted in accordance with the OECD Guidelines for Testing of Chemicals Guideline 414, Prenatal Developmental Toxicity Study (OECD, 2001) and the United States FDA Redbook 2000: IV.C.9.b. Guidelines for Developmental Toxicity Studies (FDA, 2001); the definitive study was also conducted in compliance with the FDA GLP regulations 21 CFR Part 58 and OECD guidelines (OECD, 1998).

The microsomal metabolism studies on S6973 were conducted at QPS LLC, Newark, DE. Pharmacokinetic studies on S6973 in rats and monkeys were conducted at MPI Research, Mattawan, WI and Covance Laboratories, Madison, WI, respectively. Excretion studies on S6973, as well as microsomal metabolism and pharmacokinetic studies on S617 were conducted at Senomyx. Genotoxicity studies for both S6973 and S617 were conducted at Nucro-Technics, Scarborough, Ontario, Canada. The 28-day and 90-day subchronic toxicity studies for both S6973 and S617 were conducted at MPI Research. The developmental toxicity study on S617 was conducted at WIL Research, Ashland, OH. A detailed 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 files published online.

3. Absorption, distribution, metabolism, excretion

The *in vitro* metabolism of S6973 and S617 was studied using both rat and human liver microsomes. The *in vivo* metabolism and PK of S6973 and S617 were studied in rats; the PK of S6973 was also evaluated in monkeys.

3.1. In vitro *metabolism*

The potential of S6973 and S617 to undergo oxidative metabolism was investigated using Sprague-Dawley rat and human liver microsomes. Compounds were incubated with mixed gender, pooled liver microsomes from rat and human in the presence of NADPH for 60 or 120 minutes prior to quenching the samples with acetonitrile. After centrifugation, the supernatants were analyzed for the parent compound and its potential metabolites by tandem liquid chromatography/mass spectrometry (LC/MS). In the case of S617, metabolite identification was performed by liquid chromatography/accurate-mass quadrupole time-of-flight (Q-TOF) mass spectrometry.

S6973 was resistant to metabolism by both rat and human microsomes with ≥99% of the parent compound remaining intact after a 60 minute incubation period. The number of metabolites from each matrix was determined based on total ion chromatograms and extracted ion chromatograms (EICs) of molecular ions of possible Phase I metabolites. Two minor metabolites (M370A and M370B) of S6973 were observed in the 2 hour microsomal incubations with a total peak area of <1% of parent based on EIC peak intensities. Both M370A and M370B were produced by microsomes from both species and were identified as mono-hydroxylation products. The product ion spectra of the metabolites suggested that the hydroxylation occurred on benzothiadiazine ring, although the exact position of the hydroxylation could not be determined by MS/MS.

Likewise, S617 was also resistant to metabolism by either the rat or human microsomes with >97% of the parent compound remaining intact after the 60 minute incubation period. Accurate mass EICs were generated for the common Phase I transformations of M + 16 (mono-hydroxylation), M + 32 (di-hydroxylation), M-84 (amide bond hydrolysis), M-181 (O-dealkylation with loss of the *N*-(3-methylbutyryl)-3-methylpiperidinyl moiety), and M-165 (O-dealkylation with loss of the *N*-(3-methylbutyryl)-3-methylpiperidinyl moiety and mono-hydroxylation). Two M + 16 metabolites (M410A and M410B) were observed with a total peak area of <0.4% of the initial S617 peak area; no other metabolites were observed in the mass chromatograms. Based on the exact mass of M410A and M410B (m/z = 411.1697), both metabolites were identified as mono-hydroxylation products of S617, but the position of hydroxylation was not determined. The mass spectral fragmentation pattern suggests that the hydroxyl group of M410A is on the 3-methylbutanoyl group and the hydroxyl group of M410B is on the benzothiadiazine ring.