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Toxicological evaluation of a novel umami flavour compound: 2-(((3-(2,3-Dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio)methyl)pyridine

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ABSTRACT

A toxicological evaluation of a umami flavour compound, 2-(((3-(2,3-dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio)methyl)pyridine (S3643; CAS 902136-79-2), was completed for the purpose of assessing its safety for use in food and beverage applications. S3643 undergoes extensive oxidative metabolism *in vitro* with rat microsomes producing the S3643-sulfoxide and 4′-hydroxy-S3643 as the major metabolites. In incubations with human microsomes, the *O*-demethyl-S3643 and S3643-sulfoxide were produced as the major metabolites. In pharmacokinetic studies in rats, the S3643-sulfoxide represents the dominant biotransformation product. S3643 was not found to be mutagenic or clastogenic *in vitro*, and did not induce micronuclei in CHO-WB_L cells. In subchronic oral toxicity studies in rats, the no-observed-adverse-effect-level (NOAEL) for S3643 was 100 mg/kg bw/day (highest dose tested) when administered in the diet for 90 consecutive days.

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1. Introduction

Umami, the savory taste of the amino acid L-glutamate, is one of the five basic taste qualities detected by humans. Monosodium glutamate (MSG) is the prototypical umami substance commonly added to many food and beverage compositions, often at concentrations of 0.1–0.8% (1000–8000 ppm) by weight, to improve their overall fullness and savory flavour. In addition, it is known that naturally occurring purine ribonucleotides such as inosine-5′-monophosphate and guanosine-5′-monophosphate which elicit no umami taste on their own, can synergistically potentiate the umami taste of glutamate, thereby requiring less MSG for a given flavouring application. While these purine ribonucleotides can be present along with glutamate in certain food ingredients such as autolyzed yeast extracts, they are expensive to either isolate from natural sources or to synthesize. Until recently, little progress has been made in identifying high potency artificial substitutes for MSG or potentiators of the effectiveness of naturally occurring glutamate already present in food products.

Umami 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 hTAS1R family of class C G-protein-coupled receptors (GPCRs), which are distantly related to calcium sensing receptor, V2R pheromone receptors, and metabotropic glutamate receptors [13,14,31]. Co-expression of both hTAS1R1 and hTAS1R3 in heterologous cells results in a functional, heteromeric receptor which is highly selective for umami stimuli, responding only to glutamate, aspartate, and L-2-amino-4-phosphonobutrate. Most importantly, the glutamate-induced activity of the hTAS1R1/hTAS1R3 heterodimer is also strongly potentiated by inosine-5′-monophosphate and guanosine-5′-monophosphate, thereby proving further support for its identity as the human receptor for umami taste. This functional assay for hTAS1R1/hTAS1R3 has been adapted for high-throughput screening of natural extract and synthetic libraries leading to the discovery of several classes of novel umami agonists including a series of highly potent oxalamide compounds [24–26]. Several of these oxalamide umami agonists have been evaluated for potential genotoxicity and *in vivo*

Abbreviations: amu, atomic mass unit; 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; FL-no, FLAVIS number; GLP, Good Laboratory Practices; GMP, Good Manufacturing Practices; GPCR, G-protein-coupled receptor; GRAS, generally recognized as safe; HPBL, human peripheral blood lymphocytes; LC/MS, liquid chromatography with mass spectrometry; MC, methylcellulose; MSG, monosodium glutamate; NOAEL, no-observed-adverse-effect-level; NOEL, no-observed-effect-level; OECD, Organization for Economic Cooperation and Development; PK, pharmacokinetics; RCG, Relative Cell Growth; RMI, Relative Mitotic Index; $t_{1/2}$, half-life; T_{max}, time to reach C_{max}; TK, toxicokinetics; V_{ss}, volume of distribution at steady-state.

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toxicity in rodents and have received regulatory approval as flavouring agents including N^1-(2,4-dimethoxybenzyl)-N^2-(2-(pyridin-2-yl)ethyl)oxalamide (S336, CAS 745047-53-4, FEMA 4233, FL-no. 16.099, Savorymyx® UM33). S336 has worldwide regulatory approval for use as a flavour compound and has been used to reduce or replace MSG in a variety of products including sauces, frozen foods, cooking aids and snack foods.

More recently, researchers at Senomyx, Inc. have reported a series of novel 3-aryl-5-alkylthio-1,2,4-triazoles, including 2-(((3-(2,3-dimethoxyphenyl)-1*H*-1,2,4-triazol-5-yl)thio)methyl)pyridine (S3643; CAS 902136-79-2), which are also potent agonists of the human umami receptor [27,28]. Like several of the aforementioned oxalamides, S3643 can provide an umami flavour effect in product applications equivalent to that of MSG at a 1000-fold lower concentration. The structure of S3643 along with representative analogs from the oxalamide series is shown in Fig. 1.

S3643 was 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 [1,8,17] and therefore is available for use in human food in the United States as a "FEMA GRAS" flavour ingredient. S3643 was assigned FEMA GRAS Number 4798 in 2014 [1].

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, and genotoxicity studies conducted with S3643. Additional supporting data obtained in these studies with S3643 is included in a Supplementary Data section in the online publication.

2. Materials and methods

The batch of S3643 used for the *in vitro*/*in vivo* metabolism, *in vivo* pharmacokinetic, and 28-day range-finding toxicity studies (Lot no. BP110707, purity >99%, mp 114.7–115.5 °C), was synthesized at Ajinomoto Co., Inc., Kawasaki-Shi, Japan using the procedure described in US Patent No. 8,784,782 B2 and 8,968,708 B2 [27,28]. The batch of S3643 used for the *in vitro* genotoxicity and 90-day subchronic toxicity studies (Lot no. 60287-12-001-R, purity >98.5%, mp 114.7–115.3 °C) was synthesized at Ricerca Biosciences, LLC, Concord, OH using a slight modification of the same synthetic method but also prepared in conformance with Good Manufacturing Practices (GMPs) as described in the ICH GMP Guidelines for APIs [10]. Both batches of S3643 used for these studies gave identical ^{1}H NMR (400 MHz, d$_6$-DMSO), ^{13}C NMR (100 MHz, d$_6$-DMSO), FT-IR (KBr pellet), and mass spectral data.

All genetic toxicology studies were conducted in compliance with the United States Food and Drug Administration (FDA) Good Laboratory Practices (GLP) regulations 21 CFR Part 58 [6] and OECD guidelines [22]. The experimental design for these studies followed the OECD Guidelines for the Testing of Chemicals – 471, 473, and 487 [20,21,23]. The 28-day dose-range finding studies and 90-day toxicology studies in rats were conducted in compliance with FDA guidelines [7] Toxicological Principles for the Safety of Food Ingredients; the 90-day subchronic toxicology study was also conducted in compliance with GLP regulations, 21 CFR Part 58.

The receptor panel profiling and cytochrome P450 (CYP) inhibition assays on S3643 were conducted at MDS Pharma Services-Taiwan Ltd, Taipei, Taiwan. The *in vitro* microsomal metabolism studies on S3643 were carried out by PharmOptima, Portage, MI. The microsomal metabolism studies utilized male and female rat liver microsomes (Lot no. 1010122 and 0710104, respectively) and mixed gender human microsomes (Lot no. 087K1440) obtained from XenoTech, Lenexa, KS. The hERG channel inhibition assay on S3643 was carried out by Aviva Biosciences, San Diego, CA. Additional *in vitro* microsomal metabolism studies, as well as pharmacokinetic (PK) and *in vivo* metabolism studies on S3643 in rats were conducted at Senomyx, San Diego, CA. The analytical methods used for the *in vitro* metabolism, PK and *in vivo* metabolism studies can be found in the Supplementary Data section published online.

The *in vitro* genotoxicity studies for S3643 were conducted at Nucro-Technics, Scarborough, Ontario, Canada. The strains of *S. typhimurium* and *E. coli*, as well as rat liver S9 (9000 × *g* supernatant fraction of liver homogenate from Sprague-Dawley rats treated with Aroclor™ 1254) used in the reverse bacterial mutation assay were obtained from Molecular Toxicology Inc., Boone, NC. Cultures of human lymphocytes (Lot no. A2544) used for the chromosome aberration test were obtained from StemCell Technologies (Vancouver, BC, Canada) and were prepared from blood collected aseptically from a 20 year old, healthy, non-smoking donor. Chinese hamster ovary cell line WB$_L$ (CHO-WB$_L$) used for the *in vitro* micronucleus test was obtained from Dr. M.D. Baker, The Department of Pathobiology, University of Guelph (Guelph, ON, Canada). Rat liver S9 (9000 × *g* supernatant fraction of liver homogenate from Sprague-Dawley rats treated with phenobarbital and 5,6-benzoflavone) used in the chromosome aberration and micronucleus tests was obtained from Molecular Toxicology Inc., Boone, NC. The 28-day and 90-day subchronic toxicity studies for S3643 were conducted at MPI Research, Mattawan, WI. A description of the study designs is included in the individual study sections below. Detailed data tables for the genotoxicity, 28-day range-finder, and 90-day subchronic toxicity studies can be found in the Supplementary Data section published online.

3. Results and study designs

3.1. *In vitro* receptor and cytochrome P450 profiling of S3643

In vitro tests were conducted with S3643 to assess whether the compound interacts with any enzymes or receptors that might cause adverse or unexpected effects or affect drug metabolism. Preliminary *in vitro* screening for potential off-target activity of S3643 included tests for CYP inhibition, a receptor lead profiling panel (consisting of 68 receptor binding assays for GPCRs, ion channels, nuclear receptors, transporters), and a hERG inhibition assay. The preliminary tests for CYP inhibition were performed using recombinant human enzymes expressed in insect Sf9 cells using spectrofluorimetric substrates [2,18]. All assays were performed at a concentration of 10 μM of S3643. No significant responses (≥50% inhibition or stimulation) were found with S3643 in the lead profiling receptor screen. S3643 did not significantly inhibit the hERG ion channel current (<10%) in an *in vitro* hERG electrophysiology (patch clamp) assay [29]. However, S3643 did show significant inhibition of CYP1A2 and 3A4 (72% and 68% inhibition, respectively) at 10 μM in the spectrofluorimetric assay using recombinant human enzymes. As a follow up to the results obtained using spectrofluorimetic substrates, S3643 retested on the same panel of CYP enzymes utilizing human liver microsomes and CYP-specific substrates with detection of the CYP-specific metabolites by LC–MS/MS [15,30]. None of the CYP isoforms (including CYP1A2 and 3A4) were inhibited by >43% in the presence of 10 μM S3643 in this more definitive assay format (*i.e.*, all IC$_{50}$'s >10 μM). The results from the CYP inhibition studies are summarized in Table 1.

3.2. Absorption, distribution, metabolism, excretion

The *in vitro* metabolism of S3643 was studied using rat and human liver microsomes. A study of the PK and *in vivo* metabolism of S3643 was carried out in male and female Sprague-Dawley rats.

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