Toxicology Reports 4 (2017) 507–520

Contents lists available at ScienceDirect

Toxicology Reports

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

Full Length Article

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

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ARTICLE INFO

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

ABSTRACT

A toxicological evaluation of *N*-(1-((4-amino-2,2-dioxido-1*H*-benzo[*c*][1,2,6]thiadiazin-5-yl)oxy)-2-methylpropan-2-yl)-2,6-dimethylisonicotinamide (S2218; CAS 1622458-34-7), a flavour with modifying properties, was completed for the purpose of assessing its safety for use in food and beverage applications. S2218 exhibited minimal oxidative metabolism *in vitro*, and in rat pharmacokinetic studies, the compound was poorly orally bioavailable and rapidly eliminated. S2218 was not found to be mutagenic in an *in vitro* bacterial reverse mutation assay, and was found to be neither clastogenic nor aneugenic in an *in vitro* mammalian cell micronucleus assay. In subchronic oral toxicity studies in male and female rats, the NOAEL was 140 mg/kg bw/day (highest dose tested) for S2218 sulfate salt (S8069) when administered as a food ad-mix for 13 consecutive weeks. Furthermore, S2218 sulfate salt demonstrated a lack of maternal toxicity, as well as adverse effects on fetal morphology at the highest dose tested, providing a NOAEL of 1000 mg/kg bw/day for both maternal toxicity and embryo/fetal development when administered orally during gestation to pregnant rats.

1. Introduction

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

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

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

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http://dx.doi.org/10.1016/j.toxrep.2017.09.004
Received 8 August 2017; Received in revised form 6 September 2017; Accepted 13 September 2017
Available online 14 September 2017

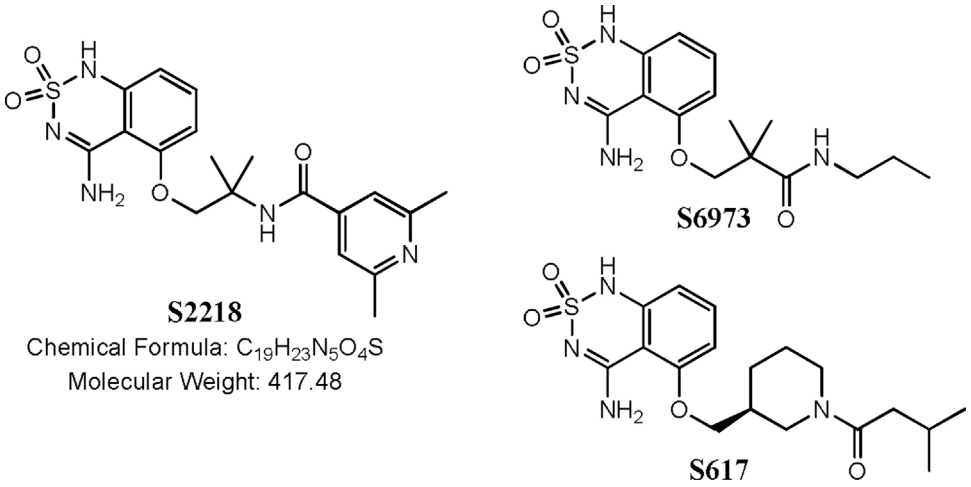

Fig. 1. Structures of S2218 and Related Sweet Modifiers.

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

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

2. Materials and methods

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

All genetic toxicology studies were conducted in compliance with the United States Food and Drug Administration (FDA) Good Laboratory Practices (GLP) regulations 21 CFR Part 58 [21] and OECD guidelines [22]. The experimental design for these studies followed the OECD Guidelines for the Testing of Chemicals – 471 and 487 [23,24]. The 28-day dose-range finding studies and 90-day toxicology studies in rats were conducted in compliance with FDA guidelines [25] Toxicological Principles for the Safety of Food Ingredients; the 90-day subchronic toxicology study was also conducted in compliance with GLP regulations, 21 CFR Part 58 [21]. The developmental toxicity range-finder and definitive studies were conducted in accordance with the OECD Guidelines for Testing of Chemicals Guideline 414, Prenatal Developmental Toxicity Study [26] and the United States FDA Redbook 2000: IV.C.9.b Guidelines for Developmental Toxicity Studies [27]; the definitive study was also conducted in compliance with the FDA GLP regulations 21 CFR Part 58 and OECD guidelines [22].

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

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