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Acute and 28-day repeated dose toxicity evaluations of 2-hydroxybenzylamine acetate in mice and rats

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ABSTRACT

2-hydroxybenzylamine (2-HOBA), a compound naturally found in buckwheat, has been shown to protect cells and tissues from the damaging effects of oxidative stress. The purpose of this report was to evaluate 2-HOBA in preclinical oral rodent toxicity studies. This report includes the results from three oral toxicity studies in rodents: a preliminary 28-day feeding study in mice, a 14-day acute oral toxicity study in rats, and a 28-day repeated dose oral toxicity study in rats. The preliminary mouse feeding study showed no adverse effects of 2-HOBA at concentrations up to 0.456% by weight in feed, but decreased food intake and weight loss were observed at 1.56% 2-HOBA in the diet, likely due to poor palatability. In the acute dosing study, 2000 mg/kg BW 2-HOBA resulted in mortality in one of the six tested female rats, indicating a median lethal dose of 2500 mg/kg BW. In the 28-day repeated oral dose study, small differences were observed between 2-HOBA treated and control group rats, but none of these differences were determined to be of toxicological significance. Together, these studies support the lack of toxicity of oral administration of 2-HOBA acetate at doses up to 1000 mg/kg BW d^{-1} in rodents.

1. Introduction

γ-Ketoaldehydes (also known as isolevuglandins or isoketals) are highly reactive lipid aldehydes that form via non-enzymatic rearrangement of intermediates created during either prostaglandin formation by cyclooxygenase (Iyer et al., 1989; Murthi et al., 1993) or free radical-catalyzed arachidonic acid oxidation (Brame et al., 1999; Morrow et al., 1990; Salomon and Miller, 1985). They rapidly react with proteins (Davies et al., 2002) and lipids (Bernoud-Hubac et al., 2009; Sullivan et al., 2010), forming adducts that alter protein function and induce cellular stress and inflammation. 2-Hydroxybenzylamine (2-HOBA, also known as salicylamine) is a potent γ-ketoaldehyde scavenger (Davies et al., 2002; Zagol-Ikapitte et al., 2010a) that is naturally found in buckwheat (Koyama et al., 1971). As 2-HOBA can prevent the formation of γ-ketoaldehyde-protein adducts associated with oxidative stress, it can protect cells from the detrimental effects of these adducts.

In vitro evidence for a protective effect of 2-HOBA was established in HepG2 cells; pre-treatment with 0.5 mM 2-HOBA protected these cells from H_2O_2-induced cytotoxicity (Davies et al., 2006). Beneficial effects of 2-HOBA in conditions associated with oxidative stress have also been identified *in vivo* in multiple organ systems and species. 2-HOBA administration dose-dependently decreased biomarkers of oxidant injury and increased lifespan in *C. elegans* (Nguyen et al., 2016). In mice, beneficial effects of 2-HOBA have been observed in multiple murine neuropathology models. Long-term treatment with 2-HOBA prevented the age-associated decline in spatial working memory in the ApoE4 model of Alzheimer's disease (Davies et al., 2011) and attenuated the neuronal loss and memory deficits in two mouse models of epilepsy (Pearson et al., 2017). There is also evidence for a protective effect of 2-HOBA against hypertension. 2-HOBA treatment attenuated angiotensin-II induced hypertension and renal damage in mice (Kirabo et al., 2014), prevented aortic stiffening and hypertension in mice with chronic vascular oxidative stress (Wu et al., 2016), and prevented pulmonary

Abbreviations: 2-HOBA, 2-hydroxybenzylamine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; BW, body weight; FDA, Food and Drug Administration; GLP, good laboratory practice; HED, human equivalent dosages; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance

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arterial hypertension in BMPR2 mutant mice (Egnatchik et al., 2017).

Though much of the preclinical efficacy data for 2-HOBA has been established in animal disease models, aging and other common conditions (e.g., obesity) are associated with excessive activation of oxidative processes (Marseglia et al., 2014; Finkel and Holbrook, 2000). In such conditions, 2-HOBA could be very effective as a nutritional supplement to help maintain good health and alleviate many of the co-morbidities associated with aging and/or obesity, such as cardiovascular events and development of many chronic diseases. Thus, these preclinical studies provide promising data supporting a potential benefit of 2-HOBA as a dietary supplement or functional food in humans. However, additional data on the safety of 2-HOBA are required. Although 2-HOBA has been studied in animal models, no specific toxicology data are currently available. Thus, we conducted a preliminary 28-day feeding study in mice to determine any acute toxicological properties of 2-HOBA. Based on the results from the preliminary feeding study, an acute oral dosing study and 28-day repeated dose oral toxicity study were conducted in rats to further assess the safety of 2-HOBA for future human clinical studies. Here we report the results of these three rodent toxicity studies.

2. Materials and methods

2-HOBA (as salicylamine acetate, CAS 1206675-01-5) was obtained from TSI (China) Co., Ltd. (Shanghai, China). For the initial mouse study, a pre-manufacturing batch run was used (Lot E2736-14-P1). This lot was verified as > 98% pure in our laboratory via high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectroscopy. For the dosing and 28-day rat studies, a commercial production lot was used (Lot 16120312). Our laboratory verified the purity of the commercial lot to be > 99% via HPLC and NMR spectroscopy. Additional information on the synthetic pathway and methods used to characterize the material are considered proprietary and thus, are not described in detail.

2.1. Preliminary 28-day oral toxicity study in mice

All procedures were approved by the Iowa State University Institutional Animal Care and Use Committee, (approval #8-14-7837-M). The study followed the guidelines outlined in Redbook (2000) IV.C.3. A Short-term toxicity studies with rodents (Office of Food Additive Safety U.S, 2003).

2.1.1. Feed preparation and analysis

The control feed was PMI LabDiet 5002 (Gateway Labsupply, St. Louis, MO), a certified rodent chow in meal form, with 3% by weight added corn oil. The test diets were mixed by adding 2-HOBA to the control feed at either 0.156, 0.469, or 1.56% 2-HOBA by weight. These percentages were designed to achieve target dose levels of 250–300, 750–900, and 2500–3000 mg/kg BW d^{-1}, respectively. All feed was mixed at the beginning of the study, and feed mixtures were stored at room temperature.

2-HOBA potency (percentage by weight) in all feed mixtures was assessed by HPLC at the beginning of the study. To determine stability of the feed mixtures, 2-HOBA potency was again assessed by HPLC after the study. Homogeneity of the feed mixture was confirmed in the 0.469% feed mixture by comparing the 2-HOBA potency in samples collected from the top, middle, and bottom of the feed mixture.

2.1.2. Animals and experimental protocol

All animals were housed at and all animal procedures were conducted at the Iowa State University Laboratory Animal Resources A Wing Unit. Necropsy and pathology services were provided by the Iowa State University Veterinary Pathology Laboratory. A total of 50 (25 male and 25 female) CD-1 IGS mice were obtained from Charles River Laboratories (Wilmington, MA) at approximately 5 weeks of age. Four treatment levels were used, with 5 male and 5 female mice randomized per treatment as described below.

All animals were individually caged throughout the study in approved InnoVive (San Diego, CA) ventilated solid bottom cages. All animals were provided with nestlets, paper rolls in the bedding, and short PVC tubes for psychological/environmental enrichment. Environmental conditions were controlled throughout the study. Specifically, light was provided for 12 h daily (0700–1900), room temperature was maintained at approximately 21 °C (range, 20.0–22.8 °C), relative humidity ranged from 16 to 49%, and room air was exchanged approximately 12 times per hour. Food and water were provided *ad libitum* throughout the pre-study and study periods. Marbles were placed in the feeders to minimize feed spillage.

Mice were acclimated to their cages and rooms for 7 days prior to the start of the treatments. On day −4, body weights were measured and used for the randomization. One male mouse was found to have a cataract in the right eye during this acclimation period and was therefore excluded from randomization and euthanized. Light and heavy outliers were identified, and the remaining mice (20 males and 20 females) were grouped by sex and weight into groups of 4 and assigned to one of the 4 treatments.

At Day 7 of the study, the 1.56% 2-HOBA-treated mice were consuming significantly less feed than the other treatment groups and were losing body weight. The study veterinarian recommended these mice be removed from the study and euthanized; accordingly, the 1.56% group mice were euthanized on day 8. The remaining animals were euthanized by timed CO_2 inhalation followed by cardiac puncture and bilateral thoracotomy on days 29 and 30 after the 28-day feeding protocol.

2.1.3. Observations

Brief veterinary examinations were performed during the pre-study period and again during the final week of the study. Twice daily observations were made throughout the study. These included general health/mortality, moribundity, alertness, coat condition, activity level, and physical condition (injury). Body weight and feed consumption were measured weekly (days 0, 7, 14, 21, and 28).

2.1.4. Hematology and clinical chemistry

Blood samples were collected via cardiac puncture immediately following euthanasia by timed CO_2 inhalation. Blood samples were immediately placed on ice. After clotting, the samples were centrifuged to obtain serum. Serum analyses were performed by the Vanderbilt University diagnostic laboratory. Due to the small amount of serum obtained from the mice, a limited clinical chemistry panel, including alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine, and glucose, was measured.

2.1.5. Pathology and histopathology

Brain, heart, liver, lung, and paired kidneys were collected and weighed. Organs were trimmed of fat and connective tissues before weighing, and the hearts were evacuated. Absolute and relative (relative to terminal body weight and brain weight) organ weights were calculated. The following tissues were collected for histopathology and preserved in 10% neutral buffered formalin: brain; heart; kidney; large intestine including cecum, colon, and rectum; liver; lung; pancreas; small intestine including duodenum, ileum, and jejunum; spleen; and stomach. A sample of hind limb muscle was also collected from control, 0.156%, and 0.469% 2-HOBA-treated animals. Tissues for histopathology were processed by The Comparative Pathology Core Service at the Iowa State University College of Veterinary Medicine (Ames, IA). Briefly, tissues were trimmed, embedded in paraffin, sectioned, mounted on glass slides, stained with hematoxylin and eosin, and evaluated by an experienced pathologist.

2.1.6. Statistical analysis

Statistical analyses were performed on body weights, body weight