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RESEARCH ARTICLE

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Acute and subacute inhalation toxicity assessment of WS-23 in Sprague–Dawley rats

Ze-Hong Wu[1] | Yue-Song Liu[2] | Xin-Duo Li[1] | Te Xu[1] | Jing Xu[1] | Xue-Ming Yang[1] | Ren-Qiang Ma[2] | Xing-Tao Jiang[1]

[1]RELX Lab, Shenzhen RELX Tech. Co,. Ltd., Shenzhen, China

[2]Drug Evaluation Center, Guangzhou Boji Pharmaceutical Biotechnology Co., Ltd., Guangzhou, China

Correspondence
Xing-Tao Jiang, RELX Lab, Shenzhen RELX Tech. Co,. Ltd., Shenzhen, China.
Email: xingtao.jiang@relxtech.com

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Abstract

2-isopropyl-N,2,3-trimethylbutyramide (WS-23) is a well-known artificial synthesis cooling agent widely used in foods, medicines, and tobaccos. As a commonly cooling agent in e-cigarette liquids, WS-23 has led to concerns about the inhalation toxicity with the prosperous of e-cigarettes in recent years. Thus, the aim of this study is to assess the acute and subacute inhalation toxicity of WS-23 in Sprague–Dawley (SD) rats according to the Organization for Economic Cooperation and Development (OECD) guidelines. In the acute toxicity study, there was no mortality and behavioral signs of toxicity at the limit test dose level (340.0 mg/m^3) in the exposure period and the following 14-day observation period. In the subacute inhalation toxicity study, there was no significant difference observed in the body weights, feed consumption, and relative organ weights. Haematological, serum biochemical, urine, and bronchoalveolar lavage fluid (BALF) analysis revealed the non-adverse effects after 28-day repeated WS-23 inhalation (342.85 mg/m^3), accompanied by slight changes in few parameters which returned to normal during the 28-day recovery period. The histopathologic examination also did not show any differences in vital organs. In conclusion, the maximum tolerated dose for WS-23 acute inhalation is not less than 340.0 mg/m^3, and the No Observed Adverse Effect Level (NOAEL) of WS-23 subacute inhalation was determined to be over 342.85 mg/m^3.

KEYWORDS
acute inhalation toxicity, NOAEL, subacute inhalation toxicity, WS-23

1 | INTRODUCTION

Cooling agent, which used to provide a cooling sensation, has penetrated into everyone's daily life. Such as menthol, the most familiar cold taste agent with the advantages of fresh cool taste, low price, and easy availability is widely used in daily chemicals, tobacco, medicine, and personal hygiene products. However, with the continuous improvement of people's requirements for cool taste experience, the deficiencies of menthol such as effumability, the short and intense instantaneous cool irritation accompanied by bitter are also exposed (Wu et al., 2008). Therefore, varieties of new cooling agents have been developed by chemists based on the structure of menthol.

2-isopropyl-N,2,3-trimethylbutyramide (WS-23, CAS No. 51115-67-4; FEMA No. 3804) is one nearly odorless white crystalline powder widely used as a coolant in medicinal preparations, oral care products, foodstuffs, cosmetics, and confection-eye products (Li et al., 2019). It is an artificial synthesis cooling agent and could be synthesized by the Ritter reaction of 2,2-diisopropylpropionitrile (DIPPN) with methanol in the presence of acid (Chavakula et al., 2014; Li & Zhang, 2007). Comparing with menthol, WS-23 is characterized by a continuous and long-lasting cooling and refreshing effect with no side effects of burning or stinging sensations (Chavakula et al., 2014). As an important additive in menthol cigarettes, WS-23 can cover up the bitter and astringent taste of cigarettes, make the cigarette feel fresh, improve

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the coordination and softness of cigarette smoke, and reduce the thorn in the mouth and throat (Wu et al., 2008). WS-23 is also compatible with other flavors and cooling agents. Thus, it is widely used in cigarette industry enterprises including e-cigarettes.

The in vitro and vivo studies showed that WS-23 is unlikely to be a mutagen and present a genotoxic hazard. The potential of WS-23 to induce bacterial mutagenicity was investigated in vitro using the Ames assays equivalent (ECHA, Online). The study showed that WS-23 did not induce mutations in the genomes of the organisms used under the conditions of the assay. Three in vivo studies of genotoxicity showed that WS-23 did not induce chromosome aberrations in rat bone marrow cells or comet effects in female rat kidney cells, whereas it was weakly genotoxic in the comet assay in male rat kidney cells which was postulated that this effect was male specific (Eighty-Second Report of the Joint FAO/WHO Expert Committee on Food Additives, 2016). According to the reports from ECHA, the LD_{50} of oral consumption of WS-23 was 533 and 490 mg/kg in male and female rats, respectively (ECHA, Online).

As a commonly cooling agent in e-cigarette, the probability and dose of inhalation exposure to WS-23 is significantly increased in consumers with the prosperous of e-cigarettes in recent years. Therefore, the inhalation toxicity study of WS-23 is becoming increasingly urgent for the absence of its inhalation toxicology information. Thus, the aim of this study is to assess the acute and subacute (28-day repeated) inhalation toxicity in Sprague–Dawley (SD) rats.

2 | MATERIALS AND METHODS

2.1 | Chemicals

WS-23 (CAS No. 51115-67-4) was purchased from Kunshan Asia Aroma spice Co., LTD (China) with the purity ≧ 99.9%, the vapor pressure of 0.0573 mmHg at 25°C, the melting point of 58–61°C, and the boiling point of 233°C at 760 mmHg. The medicinal grade carrier solvent of propylene glycol (PG) and vegetable glycerin (VG) were purchased from Dow and P&G, respectively.

2.2 | Animals

Specific-pathogen-free (SPF) SD rats (6 weeks old, male and female per half) were purchased from Hunan STA Lab. Animal Co., LTD (China). The temperature of the animal rooms was set to 23 ± 3°C and 50% ± 20% relative humidity. The rats used for the experiment were with the mean initial body weight of 196.0 g (male) and 182.8 g (female), respectively. A light–dark cycle of 12 hr was in operation, and the exposure was carried out in the light phase of the cycle. Rats were housed in pairs in cages with polypropylene material and environmental enrichment. A gamma-irradiated pellet diet (Medicience, China) and sterilized drinking water were provided ad libitum except during exposure. Following an acclimatization period of 1 week, the rats were randomly divided into two groups (10 rats/group) for acute toxicity study and four groups (20 rats/group) for subacute toxicity study. The changes in body weight were recorded once per week from the beginning of the study, and the clinical signs and symptoms were observed during the exposure and recovery periods. All animal experiments were conducted in accordance with the guidelines of the OECD 403 (OECD Test Guideline No. 403, 2009) and OECD 412 (OECD Test Guideline No. 412, 2018) with some modification for taking into account the practical application of WS-23 in the e-cigarette industry.

2.3 | Inhalation exposure assembly

The aerosols of WS-23 solutions were generated by synchronously using homemade smoking robots and the commercially available e-cigarette devices (RELX 0 with 6.5 watts fixed operating power) (RELX, China) with a clean diluted air flow of 8 L/min. The vaping method was according to the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) Recommended Method No. 81 vaping regime, which specifies a square-wave puff profile, 55-ml puff volume, 3-s duration and a 30-s interval (CORESTA, 2015). A nose-only exposure system HRH-MNE3026 (Huironghe Technology, China) was used to achieve the inhalation exposure of the diluted WS-23 aerosols, and also synchronously monitor the parameters of temperature, humidity, air flow, and air pressure in the exposure system. The concentration of WS-23 and its particle size determination were detected by the filter film adsorption method (Gautam et al., 2018), which firstly collected by Cambridge filter (WHATMAN, UK) attached to an aerosol mass concentration detector CEL-712 (Sampling flow: 1 L/min, t = 30 min) (CASELLA, UK) or an aerodynamic particle size analyzer HRH APS-1 (Sampling flow: 28.3 L/min, *t* = 30 min) (Huironghe Technology, China) and then analyzed by a Shimadzu gas chromatography (GC), model Nexis GC-2030 (SHIMADZU, Japan).

2.4 | Acute study

The acute inhalation toxicity study of WS-23 was conducted according to the OECD Guideline 403 method (OECD Test Guideline No. 403, 2009). Rats were exposed to the aerosol of WS-23 solvent (Quality ratio, WS-23:PG:VG = 10:40:50) with the target concentration of 300 mg/m^3, taking the blank solvent (PG:VG = 1:1) as the control group. Ten percent is pretty close to the upper limit solubility of WS-23 in PG and VG (1:1) at room temperature. Half of the rats were sacrificed immediately after 4 hr exposure, and the remaining rats were monitored for 14 days and then euthanized.

2.5 | Subacute study

The subacute inhalation toxicity study was carried out according to OECD Guidelines 413 for repeated dose 28-day inhalation toxicity