Regulatory Toxicology and Pharmacology 73 (2015) 501–508

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

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

Evaluation of the mode of action of mouse lung tumors induced by 4-methylimidazole

George Cruzan [a, *], Jack R. Harkema [b], Hiromi Hosako [c], Jennifer M. Wasil [c], F. Jay Murray [d]

[a] ToxWorks, 1153 Roadstone Rd., Bridgeton, NJ 08302, USA
[b] Michigan State University, 1129 Farm Ln., Rm. 212, East Lansing, MI 48824, USA
[c] WIL Research Laboratories LLC, 1407 George Rd., Ashland, OH 44805, USA
[d] Murray & Associates, 5529 Perugia Cir., San Jose, CA 95138, USA

ARTICLE INFO

Article history:
Received 24 August 2015
Received in revised form
25 September 2015
Accepted 5 October 2015
Available online 9 October 2015

Keywords:
4-Methylimidazole
Styrene
Toxicity
Mouse
Lung
Mechanism
CYP2F2

ABSTRACT

4-Methylimidazole (4-MEI) occurs in certain foods and beverages as a product of browning reactions. An increased incidence of lung tumors was reported in mice, but not rats, exposed to levels of 4-MEI in their diet that far exceed human dietary intake. This investigation evaluated the hypothesis that 4-MEI induces mouse lung tumors by the same mode of action (MOA) as styrene: CYP2F2 metabolic activation and increased BrdU labeling. Using styrene (200 mg/kg/day by gavage) as a positive control, histopathology and DNA synthesis (measured by BrdU incorporation) in the bronchiolar region were evaluated in: (1) a 5-day comparative toxicity study in C57BL/6 "wild type" and CYP2F2 "knock out" (KO) mice given 4-MEI at the same dietary concentrations used in the NTP cancer bioassay, and (2) a 13-week comparative toxicity study of C57BL/6 and B6C3F1 mice receiving 0, 1250 or 2500 ppm of 4-MEI in the diet for 6, 15, 34 and 91 days. In contrast to styrene, 4-MEI had no consistent effect on BrdU labeling or histopathology in the lungs of mice in the dose range that had been shown to produce lung tumors in another study. The results of these studies do not support the hypothesis that 4-MEI and styrene induce lung tumors by the same MOA.

1. Introduction

4-Methylimidazole (4-MEI) is used in the manufacture of pharmaceuticals, photographic chemicals, dyes and pigments, cleaning and agricultural chemicals, and rubber. In addition, it may be present or subject to unavoidable formation during cooking in a wide array of food products, including – among many other things that contain certain caramel colors or give rise to caramelization – carbonated beverages, coffee, beer, wine, soy sauce, Worcestershire sauce, molasses, and certain baked goods (Chan et al., 2008). It has also been identified in milk from cows fed ammoniated forage (Morgan and Edwards, 1986; Perdok and Leng, 1987).

The National Toxicology Program (NTP) conducted a number of studies including two-year feeding cancer bioassays of 4-MEI in B6C3F1 mice and F/344 rats (NTP, 2007; Chan et al., 2008). 4-MEI demonstrated a unique pattern of tumor results that has not been observed in any other NTP cancer bioassay. NTP reported "clear evidence of carcinogenic activity" of 4-MEI in male and female mice based on increased incidences of combined alveolar/bronchiolar adenomas and carcinomas. 4-MEI had no significant effect on any other type of tumor in mice.

In contrast, no increase in lung tumors was observed in male or female rats exposed to 4-MEI. NTP reported "equivocal evidence of carcinogenic activity" in female rats based on increased incidences of mononuclear cell leukemia and "no evidence of carcinogenic activity" in male rats. Of note, dose-related, statistically significant decreases in multiple tumors were observed in both male and female rats exposed to 4-MEI in the NTP bioassay; in most cases, reduced body weight did not appear to be the primary cause of the decreased tumor incidences observed in rats exposed to 4-MEI (Murray, 2011).

The mechanism by which 4-MEI induces lung tumors in mice has not been elucidated. The NTP reported that 4-MEI was neither mutagenic in the *Salmonella typhimurium* mutation assay, with and without metabolic activiation, nor genotoxic in micronucleus assays in rats and mice (Chan et al., 2008). The observation of

* Corresponding author.
E-mail addresses: toxworks@aol.com (G. Cruzan), harkemaj@msu.edu (J.R. Harkema), hiromi.hosako@wilresearch.com (H. Hosako), jmurray2@sbcglobal.net (F.J. Murray).

http://dx.doi.org/10.1016/j.yrtph.2015.10.006
0273-2300/

increased tumors in a single organ in a single species suggests a mode of action (MOA) related to specific factors in that organ and species. The NTP reported no evidence of histological changes in the lungs of male or female B6C3F1 mice in an NTP 14-week toxicity study in which 4-MEI was given in the diets at concentrations of 0, 625, 1,250, 2,500, 5,000, or 10,000 ppm (NTP, 2004). One of 10 males and seven of 10 females from the 10,000 ppm groups died early. Body weight gains of mice exposed to 1250 ppm or greater were significantly reduced compared to the controls.

Similarly, in a 15-day toxicity study, NTP observed no evidence of pulmonary toxicity among B6C3F1 mice administered diets containing up to 2500 ppm of 4-MEI (Chan, 2004; Chan et al., 2006; NTP, 2004). All animals survived to the end of the studies, and there were no significant differences in mean body weights, clinical findings, organ weights, or gross or microscopic lesions between exposed and control groups.

Other substances, such as styrene, naphthalene, ethylbenzene, alpha-methylstyrene, cumene, divinylbenzene, benzofuran, and fluensulfone have been demonstrated to cause lung tumors in mice, but not in rats (Cruzan et al., 2009; Strupp et al., 2012). For example, increased incidences of alveolar/bronchiolar tumors were observed in mice exposed to 40–160 ppm of styrene by inhalation, but not in rats exposed to concentrations of styrene up to 1000 ppm (Cruzan et al., 1998, 2001). A mode of action (MOA) for styrene, naphthalene, ethylbenzene, isoniazid, fluensulfone, and other chemicals has been proposed involving mouse-specific metabolic activation by CYP2F2 followed by lung cell proliferation. In the mouse, CYP2F2 is present in high concentrations in Club cells (formerly known as Clara cells) in the terminal bronchioles (Carlson, 2008; Cruzan et al., 2009); in contrast, rats have much lower levels of the rat CYP2F isoform (CYP2F4), and rats do not develop lung toxicity or increased lung tumors from these compounds. A CYP2F2-knockout (KO) mouse line was developed from the C57BL/6 mouse with an inactive gene that does not produce the CYP2F2 protein (Li et al., 2011). Short-term exposures to styrene in mice with normal levels of CYP2F2 (Wild-type, WT) by inhalation, oral gavage or ip administration result in early exfoliation of cells in the terminal bronchioles followed by increased cell proliferation, as measured by BrdU-incorporation (Cruzan et al., 2012). In comparison, no lung toxicity was observed in CYP2F2 KO mice similarly exposed to styrene (Cruzan et al., 2012).

The purpose of the current study was to evaluate the hypothesis that 4-MEI induces lung tumors in mice by the same MOA as styrene and similar compounds mediated through CYP2F2 metabolic activation and/or induction of cell proliferation. Two studies were conducted in mice exposed to 4-MEI in order to assess BrdU incorporation in the alveolar/bronchiolar region of the lungs, as well as the histopathology of the lungs. The studies included (1) a 5-day toxicity study in C57BL/6 "wild type" (WT) mice and CYP2F2 KO mice given 4-MEI in the diet at the same concentrations used in the NTP 2-year study (0, 125, 312, 650, 1250 ppm), and (2) a 13-week comparative toxicity study of dietary exposure to 4-MEI in C57BL/6 and B6C3F1 mice (1250 and 2500 ppm), with assessments at 6, 15, 34 and 91 days. Styrene was employed as the positive control in both of these studies.

2. Methods and materials

Test substance: 4-Methylimidazole (CAS # 822-36-6) was obtained from Sigma–Aldrich (Lot no. 17097PJV and 09505BJ, ≥99.1% purity). Styrene (CAS # 100-42-5), purchased from Sigma–Aldrich (Lot no. MKBH1232V and MKBC9108V, 99.9% purity), was used as a positive control agent.

Animals and husbandry: For Study One, 35 male and 35 female C57BL/6 (WT) and 30 male and 30 female CYP2F2 KO mice were obtained from Taconic (Germantown, NY) at 8–11 weeks of age and acclimated before testing. For Study Two, 97 male C57BL/6 mice were obtained from the same supplier and 97 male B6C3F1 mice were obtained from Charles River Laboratories, Inc. (Raleigh, NC) at approximately 10 weeks of age and acclimated before testing. The animals judged suitable for assignment to the study were selected for use in a computerized randomization procedure based on body weight stratification in a block design. Animals were identified by metal eartag. Animal rooms were set to maintain 71° ± 5 °F and relative humidity of 50 ± 20%. Fluorescent lighting was provided with a 12 h on-off schedule. Ventilation was set to provide 10 changes of fresh air per hour. The mice were housed in solid bottom cages containing nesting material. Reverse osmosis-treated water was available *ad libitum*. PMI Nutrition International, LLC Certified Rodent LabDiet® 5002 (meal) was offered *ad libitum* during acclimation to all mice, and it was used in preparation of control and test diets. The animal care procedures and facilities were fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

These studies were conducted at WILResearch Laboratories LLC (Ashland, OH) in compliance with United States Environmental Protection Agency (EPA) Good Laboratory Practice (GLP) Standards (40 CFR Part 792) with two exceptions: first, the tissue harvesting (conducted at WIL), tissue preparation, and microscopic examinations were conducted by Dr. Harkema and colleagues of Michigan State University (MSU) according to the standard operating procedures in place at MSU, and second, 4-MEI and styrene were characterized (Certificates of Analysis) by the supplier according to their standards.

Study One Experimental Design: The experimental design for Study One is summarized in Table 1. Groups of five male and five female WT mice and five male and five female KO mice were administered diets containing 0, 312, 625 or 1250 ppm of 4-MEI for five consecutive days. These dietary concentrations were chosen because 312, 625, and 1250 ppm of 4-MEI were the low, middle and high dose groups, respectively, in the NTP 2-year cancer bioassay of 4-MEI in mice. An additional group of five male and five female WT mice were administered a diet containing 125 ppm of 4-MEI for five consecutive days to determine whether any effect could be determined at a dose lower than that used for the NTP cancer bioassay. For each dietary concentration, 4-MEI was finely ground and dissolved in acetone; this mixture was blended with PMI Nutrition International, LLC, Certified Rodent LabDiet® 5002 (meal); diet for the control group received the same amount of acetone only. As a positive control, groups of 5 male and five female WT and KO mice were given 200 mg/kg/day of styrene by gavage in corn oil at a dose volume of 5 mL/kg bw for five consecutive days. The stability and homogeneity of the diets and the positive control dosing solutions were evaluated in a preliminary study. During these studies the concentrations of 4-MEI in the diet and styrene in the dosing solutions were measured. The first day of test substance administration was considered study day 0.

All animals were observed twice daily, once in the morning and once in the afternoon, for mortality and moribundity. Clinical examinations were performed on the day of surgery at approximately 2–3 h following implantation and once daily during the treatment period. Detailed physical examinations were conducted once prior to randomization, at the time of randomization, prior to BrdU pump implantation, on study days 0 and 4, and on the day of the scheduled euthanasia. Individual body weights were recorded once prior to randomization, at the time of randomization, prior to and following implantation on the day of surgery, on study days 0 and 4, and on the day of the scheduled euthanasia (study day 5). Individual food consumption was recorded once prior to randomization and on study days 0 and 5. Food intake was calculated as g/animal/day for the corresponding body weight intervals.