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The 14-day repeated dose liver micronucleus test with methapyrilene hydrochloride using young adult rats

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ABSTRACT

The repeated dose liver micronucleus (RDLMN) assay using young adult rats has the potential to detect genotoxic hepatocarcinogens that can be integrated into a general toxicity study. The assay methods were thoroughly validated by 19 Japanese facilities. Methapyrilene hydrochloride (MP), known to be a non-genotoxic hepatocarcinogen, was examined in the present study. MP was dosed orally at 10, 30 and 100 mg/kg/day to 6-week-old male Crl:CD (SD) rats daily for 14 days. Treatment with MP resulted in an increase in micronucleated hepatocytes (MNHEPs) with a dosage of only 100 mg/kg/day. At this dose level, cytotoxicity followed by regenerative cell growth was noted in the liver. These findings suggest that MP may induce clastogenic effects indirectly on the liver or hepatotoxicity of MP followed by regeneration may cause increase in spontaneous incidence of MNHEPs.

1. Introduction

A collaborative study by the Mammalian Mutagenicity Study (MMS) Group, which is a subgroup of the Japanese Environmental Mutagen Society (JEMS), was conducted to evaluate the suitability of the repeated dose liver and gastrointestinal tract micronucleus assays using young adult rats. This study, using methapyrilene hydrochloride (MP), was performed as part of a collaborative study at Maruho Co., Ltd.

MP is an antihistaminic compound that was once used as a popular over-the-counter sleep-aid component and was also used in cold and allergy medications. However, MP was found to induce hepatocellular carcinomas and cholangiocarcinomas in rats [1], and it was subsequently withdrawn from the market. MP gave positive [2] and negative [3] results under metabolism-activated conditions for the *in vitro* mouse lymphoma assay (MLA), but MP gave negative results for the Ames assay [4], *in vivo* Comet assay [5], UDS assay [6] and DNA adduct assay [7–9]. These data are consistent with the hypothesis that MP is carcinogenic in rats *via* non-genotoxic mechanisms [6,10].

In this study, MP was used as a non-genotoxic hepatocarcinogen to examine the selectivity of the liver MN assay. To assess the possibility that the liver MN assay could be incorporated into a general toxicity study, 6-week-old rats, which are usually used in these types of studies, were used. At the same time, we attempted to assess whether the dose levels in the 4-week, repeated-dose toxicity study would be compatible with the dosage levels required for an acceptable liver MN assay.

2. Materials and methods

2.1. Animals

Male Crl:CD (SD) rats were purchased from Charles River Japan Inc. and were aged 6 weeks with weights between 180 and 200 g at the beginning of the experiments. The animals were housed one per cage in an air-conditioned room with a 12 h light/dark cycle and free access to food and drinking water. The animal experiments were approved by the Institutional Animal Care and Use Committee prior to conducting the experiments.

2.2. Chemicals

Methapyrilene hydrochloride (MP; CAS No. 135-23-9, 99.6% purity) was purchased from Sigma–Aldrich Co., LLC (Tokyo, Japan). MP was dissolved in water (Otsuka Pharmaceutical Factory, Inc., Japan) at concentrations of 1, 3 and 10 mg/mL before use. A positive control, diethylnitrosamine (DEN; CAS No. 55-18-5, 99.9% purity), was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). DEN was dissolved in water at a concentration of 1.25 mg/mL before use.

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2.3. Doses levels and treatment

The maximum dose for repeated dose studies was defined as the dose inducing clinical signs without causing lethality. The high dose for MP (100 mg/kg/day) was determined according to published literature information [5]. Three dose levels were set, and the mid- and low-dose levels were each approximately 1/3 of the next higher dose. DEN given at 12.5 mg/kg/day served as a positive control for the liver MN assay. Animals were divided into groups of 5 and received the test chemicals or the vehicle alone by oral gavage (10 mL/kg of body weight volume) once a day for 14 consecutive days. Clinical signs and mortality were observed every day, and the body weights were measured twice a week. At 24 h following the last administration, fasted rats were euthanised under isoflurane anaesthesia. Blood samples were used for blood chemistry examinations. Livers were subjected to the liver MN assay and histopathological examination after weighting. Femurs were used for the bone marrow MN (BM MN) assay.

2.4. MN assays

Both hepatocytes (HEPs) and bone marrow immature erythrocytes (IMEs) were analysed.

2.4.1. Liver MN assay

The HEPs were prepared by the method of Narumi et al. [11], except for the slice method, the concentrations of collagenase and acridine orange and the conditions for centrifugation. The livers were excised, and an approximately 1 g portion of the left lateral lobe was sliced into several strips, each approximately 1-mm in width, using a razor blade. These strips were rinsed with Hanks' balanced salt solution (HBSS; Sigma–Aldrich Co., LCC, Tokyo, Japan) and treated with HBSS containing 100 U/mL of collagenase (Collagenase Yakult-S, Yakult Pharmaceutical Industry, Co., Ltd., Tokyo, Japan) in a screw-capped flask with shaking (approximately 50 rpm) at 37 °C for 1 h. During this time, they were shaken at the maximum speed (approximately 180 rpm) for 1 min after 30 min have passed. The resulting material was repeatedly pipetted vigorously to break apart cell clumps, and then was forced through a cell strainer (Becton Dickinson and Co., Franklin Lakes, NJ, USA). The resulting cell suspension was then rinsed with 10% neutral buffered formalin and centrifuged at 50 × *g* for 5 min. Finally, the HEP pellet was resuspended in 10% neutral buffered formalin and kept at room temperature until further analysis was performed. Immediately before observation, 10 μL of the HEP suspension was mixed with an equal volume of an acridine orange (AO; 250 μg/mL) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 10 μg/mL) staining solution, dropped onto a glass slide, and covered with a coverslip (24 mm × 32 mm). The specimens were observed under a fluorescent microscope at 400× magnification with U-excitation (ultraviolet rays excitation, wave length: 330–385 nm), and the number of micronucleated HEPs (MNHEPs) per 2000 HEPs, including mono-, bi-, and multi-nucleate cells, was counted for each animal. MNHEPs were defined as HEPs with round or distinct micronuclei stained with the same colour as the main nuclei, and with diameters of 1/4 or less than those of the main nuclei. MNHEPs were confirmed by focusing up and down while taking into account the thickness of the HEPs. The number of metaphase (M-phase) cells among the 2000 HEPs was also counted to determine the mitotic index (MI). M-phase cells were identified as cells in a division stage from prophase to telophase which had identifiable chromosomes, or a poorly defined nuclear envelope, or where the appearance of the single nucleus looked like two unevenly shaped nuclei but fluoresced strongly.

2.4.2. BM MN assay

After the removal of the livers as described in the liver MN assay, the femurs were removed from the same animals. BM cells were collected from the femurs according to a method reported by Hayashi et al. [12], and the resultant BM cell suspensions were stored at room temperature until used for microscopic observation. Immediately prior to microscopic observation, 20 μL of each BM sample were placed onto an AO-coated slide and covered with a 24 mm × 32 mm cover slip. The specimens were observed under a fluorescent microscope at 800× magnification with B-excitation (blue light excitation, wave length: 460–495 nm), and the number of micronucleated immature erythrocytes (MNIMEs) per 2000 erythrocytes was counted for each animal. As a parameter of hematopoietic function in BM, the proportion of immature erythrocytes (IMEs) among 500 erythrocytes was also calculated for each animal.

2.5. Blood chemistry examination

Blood chemistry was examined using blood samples collected from the posterior vena cava of all animals in each group. Blood chemistry parameters included total protein (TP), albumin (Alb), albumin/globulin ratio (A/G), total bilirubin (T-Bil), direct bilirubin (D-Bil), gamma glutamyl transpeptidase (γ-GTP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total cholesterol (T-Cho), triglycerides (TG), glucose (Glu), blood urea nitrogen (UN), creatinine, phospholipids (PL), inorganic phosphorus (IP), calcium (Ca), sodium (Na), potassium (K) and chlorine (Cl).

2.6. Histopathological examination

Upon euthanasia, the residual liver tissue of the left lateral lobe after the isolation of HEPs was fixed with 10% phosphate buffered formalin, embedded in paraffin, and then stained with haematoxylin and eosin (H.E.) according to the standard method. The histopathological examination was performed under a light microscope.

2.7. Statistical analysis

Differences in the incidences of MNHEPs and MNIMEs between the test and the vehicle control groups were analysed using the conditional binomial test, reported by Kastenbaum and Bowman [13], at an upper-tailed significance level of 5%. Additional quantitative data were analysed for their statistical significance by the multiple comparison test for the MP and the vehicle control groups at a significance level of 5%. Namely, the homogeneity of variance was examined by Bartlett's test. When a homogeneous variance was demonstrated, one-way analysis of variance was applied; however, when a heterogeneous variance was observed, the Kruskal–Wallis test was applied. When a statistical significance was demonstrated between the groups, the difference was assessed by Dunnett's test or the Dunnett-type multiple comparison test. In addition, the homogeneity of variance was examined by the *F*-test between the positive and vehicle control groups at a significance level of 5%. When a homogeneous variance was demonstrated, Student's *t*-test was applied, whereas the Aspin–Welch *t*-test was applied when a heterogeneous variance was observed.

3. Results

3.1. Liver MN assay

The results of the liver MN assay are shown in Table 1. The MNHEP frequency significantly increased at 100 mg/kg/day of MP treatment. The mean value in the 100 mg/kg/day group was 0.23%, and the mean value in the vehicle control group was 0.03%. The mean MI value in the HEPs tended to be higher in the MP treated groups when compared with the vehicle control group, although there was no statistically significant difference between the MP treated groups and the vehicle control group, and this seemed comparable to the magnitude of MI values among the three MP treated groups. In the positive control group, the MNHEP frequency and the MI significantly increased.

3.2. BM MN assay

The results of the BM MN assay are given in Table 1. The MNIME frequencies and the IME ratios in the MP treated groups were similar to those in the vehicle control group. There was no statistically significant difference in the MNIME frequency among the samples in the positive control group. The IME ratio significantly decreased at 10 mg/kg/day, but it was considered incidental because there was no dose-dependency.

3.3. Clinical signs, body weight and liver weight

The results pertaining to the clinical signs, body weight and liver weight are shown in Table 2. Decreased body weight gain was noted at 100 mg/kg/day. The relative liver weight significantly increased at 100 mg/kg/day. In regards to any clinical signs, no MP-related abnormalities were noted in any of the animals during the administration period. In the positive control group, decreased body weight gain and a low absolute and relative liver weight were noted.

3.4. Blood chemistry examination

The results of the blood chemistry examination are shown in Table 3. Na was significantly low in the 30 and 100 mg/kg/day groups and K was significantly high in the same groups. UN was significantly high or tended to be high in the 30 and 100 mg/kg/day groups. There were significantly high T-Bil, D-Bil, γ-GTP, AST, ALT and ALP levels and significantly low TP and Alb

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