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Evaluation of *p*-phenylenediamine, *o*-phenylphenol sodium salt, and 2,4-diaminotoluene in the rat comet assay as part of the Japanese Center for the Validation of Alternative Methods (JaCVAM)-initiated international validation study of *in vivo* rat alkaline comet assay

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ABSTRACT

As part of the Japanese Center for the Validation of Alternative Methods (JaCVAM)-initiated international validation study of *in vivo* rat alkaline comet assay (comet assay), *p*-phenylenediamine dihydrochloride (PPD), *o*-phenylphenol sodium salt (OPP), and 2,4-diaminotoluene (2,4-DAT), were analyzed in this laboratory as coded test chemicals. Male Sprague–Dawley rats (7–9 weeks of age) were given three oral doses of the test compounds, 24 and 21 h apart and liver and stomach were sampled 3 h after the final dose administration. Under the conditions of the test, no increases in DNA damage were observed in liver and stomach with PPD and OPP up to 100 and 1000 mg/kg/day, respectively. 2,4-DAT, a known genotoxic carcinogen, induced a weak but reproducible, dose-related and statistically significant increase in DNA damage in liver cells while no increases were observed in stomach cells.

1. Introduction

The *in vivo* rodent alkaline comet assay (comet assay) is used worldwide for detecting DNA damage induced by genotoxic agents. The assay is applied for investigating the genotoxic potential of chemicals, and can be used as a second *in vivo* genotoxicity assay in the ICH-S2(R1) guidance [1] in addition to the *in vivo* micronucleus assay. The comet assay methods were often discussed at the meetings of the International Workshop on Genotoxicity Testing (IWGT) and the International Comet Assay Workshop (ICAW), and consensus articles have been published [2–4]. The assay, however, has not been validated formally with a standardised study protocol. Therefore, the Japanese Center for the Validation of Alternative Methods (JaCVAM) organised an international validation study of the *in vivo* comet assay, in cooperation with the U.S. National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the European Centre for the Validation of Alternative Methods (ECVAM), and the Mammalian Mutagenicity Study Group (MMS)/Japanese Environmental Mutagen Society (JEMS). The purpose of this validation study was to evaluate the ability of the *in vivo* comet assay to identify genotoxic chemicals as a potential predictor of rodent carcinogenicity by evaluation of their DNA damaging potential in liver and stomach cells. The validation study results have been submitted to the Organization for Economic Co-operation and Development (OECD) as the basis for a test guideline.

As part of the 2nd step of 4th phase international validation study [5], *p*-phenylenediamine dihydrochloride (PPD), *o*-phenylphenol sodium salt (OPP), and 2,4-diaminotoluene (2,4-DAT) were evaluated in this laboratory as coded test chemicals. PPD was described in the literature to be genotoxic *in vitro* but not *in vivo* and there was no convincing evidence for carcinogenicity in rodents. OPP induces bladder tumors in male rats for which it is unclear if genotoxicity plays a role. 2,4-DAT is a genotoxic carcinogen inducing hepatocellular carcinomas after oral administration. Under the conditions of the test, no increases in DNA damage were observed in liver and stomach with PPD and OPP up to 100 and 1000 mg/kg/day, respectively. 2,4-DAT, a known genotoxic carcinogen, induced a weak but reproducible, dose-related and statistically significant increase in DNA damage in liver cells while no increases were observed in stomach cells.

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2. Materials and methods

The study was conducted in accordance with the validation study protocol version 14.2 [5]. The following are detailed testing conditions of this study.

2.1. Test chemicals, selection of dose levels and formulation preparation

The three test chemicals were received and evaluated coded. PPD dihydrochloride (Chemical Abstracts Service [CAS] 624-18-0) was supplied by Wako Pure Chemical Industries Ltd. (Japan). Based on information provided by the validation management team (VMT), PPD was water soluble. Hence, 0.9% physiological saline (Merck, Germany) was chosen as vehicle. The solubility was checked and PPD was found to be completely soluble up to 20 mg/mL. PPD was dissolved in 0.9% physiological saline at 2.5, 5.0, and 10.0 mg/mL. The corresponding dose levels of 25, 50, and 100 mg/kg/day were selected based on a dose range finding study in which PPD was administered once daily by the oral route at 25, 50, 100, and 150 mg/kg/day to male rats (3 animals per group) for 3 consecutive days. The VMT had indicated that the oral LD_{50} for PPD in rats is 147 mg/kg. Mortality occurred in the 150 mg/kg/day dose group; one male died around 1 h after the second administration and another one was found dead at 24 h after the third administration. The third male rat of the 150 mg/kg/day dosage group survived the experimental period. No adverse clinical effects were observed in the animals of the lower dose groups. Based on these results, the highest dose of PPD selected for the comet assay was 100 mg/kg/day. The lower doses were 25 and 50 mg/kg/day.

OPP sodium salt tetrahydrate (CAS 132-27-4) was supplied by Wako Pure Chemical Industries Ltd. (Japan). Based on information provided by the VMT, OPP was insoluble in water. Following the instructions of the validation study protocol, demineralised water containing 0.5% w/v sodium carboxymethylcellulose was chosen as vehicle. The ability to formulate OPP at 200 mg/mL was checked and a homogeneous milky suspension was obtained. Dose levels of 250, 500 and 1000 mg/kg/day were selected based on a dose range finding study in which OPP was administered once daily by the oral route at 500, 1000, and 2000 mg/kg/day to male rats (3 animals per group) for 3 consecutive days. During the conduct of the range finding study, the test item formulations (milky suspensions) appeared to be unstable and OPP precipitates were formed. No mortality occurred and no clinical signs were noted as animals probably were not substantially exposed. Hence, it was decided to select another vehicle for the main study. OPP could be formulated as a clear suspension in corn oil up to 100 mg/mL. According to information provided by the VMT, the oral LD_{50} of OPP in rats is 2000 mg/kg. The comet assay was therefore conducted with 1000 mg/kg/day as highest dose (*i.e.*, 50% of the LD_{50}); the lower doses selected were 250 and 500 mg/kg/day.

2,4-DAT (CAS 95-80-7) was supplied by Wako Pure Chemical Industries Ltd. (Japan). Based on information provided by the VMT, 2,4-DAT was water soluble up to 7.74 mg/mL. Hence, physiological saline was chosen as vehicle. The solubility was checked and 60 mg/mL was completely soluble after 2 h of incubation at 37 °C. 2,4-DAT was dissolved in 0.9% physiological saline at 3.75, 7.5 and 15.0 mg/mL in the first study and at 10.0, 15.0 and 20.0 mg/mL in the second study. Dose levels of 37.5, 75 and 150 mg/kg/day for the first study were selected based on a dose range finding study in which 2,4-DAT was administered once daily by the oral route at 100, 200, 400 and 600 mg/kg/day to male rats (3 animals per group) for 3 consecutive days. The VMT had indicated that the oral LD_{50} for 2,4-DAT is 590 mg/kg. Mortality occurred from the 200 mg/kg/day dose onwards; one male rat of the 200 mg/kg/day dosage group died around 24 h after the third administration and one male rat of the 400 mg/kg/day dosage group died within 1 h after the third administration. All males of the 600 mg/kg/day dosage group and the remaining males of the 400 mg/kg/day dosage group were found dead around 24 h after the third administration. Clinical abnormalities were noted primarily in animals that died during the course of the study. No adverse clinical effects were observed at 100 mg/kg/day. Based on these results, the highest dose of 2,4-DAT selected for the comet assay was 150 mg/kg/day. The lower doses were 37.5 and 75 mg/kg/day. No clinical abnormalities were observed in the 2,4-DAT-dosed rats, except for one rat at 75 mg/kg/day showing excessive salivation at 3 h after the first administration. To clarify the obtained results, a second study with 2,4-DAT was performed in which animals were dosed with 100, 150 and 200 mg/kg/day.

Formulations for each test chemical were prepared freshly on each day of dosing at room temperature, protected from light. The positive control, ethyl methanesulphonate (EMS; CAS 62-50-0) supplied by Sigma–Aldrich (Belgium) was dissolved in 0.9% physiological saline at 20 mg/mL.

2.2. Animals

Male Sprague–Dawley rats (Crl:CD® (SD) IGS), obtained from Charles River, (Germany) were approximately 7–9 weeks old at dosing and were housed five per cage in polysulphone cages with a wire-mesh roof in an air-conditioned room under routine test conditions of temperature (20–23 °C), relative humidity (40–70%), ventilation and illumination, with a 12-h light/dark cycle. All animals were handled in accordance with the approved ethical protocol.

The rats were given free and continuous access to water and the diet, consisting of pelleted rat food, was fed *ad libitum* throughout the studies.

2.3. Animal treatment

The rats were not starved prior to dosing. Groups of five male rats were given three oral doses of test article or vehicle, 24 and 21 h apart, or two oral doses of positive control (200 mg/mL EMS in physiological saline; 24 and 3 h before terminal kill), in a dose volume of 10 mL/kg by use of a stomach tube. Approximately 3 h after the last dose administration, the rats were anesthetized with an isoflurane (Isoba® Vet)/oxygen mixture by inhalation and killed by exsanguination *via* the carotid artery, and liver and stomach were sampled.

2.4. Comet assay

2.4.1. Preparation of single cell suspensions and comet assay procedure

Mincing buffer (pH 7.4) was prepared with Hank's balanced salt solution (Gibco, Life Technologies, Paisley, UK) containing 20 mM ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2; Sigma–Aldrich, Diegem, Belgium) and kept on ice during tissue preparation. Immediately prior to necropsy, 10% dimethylsulphoxide (DMSO; Merck, Germany) was added to the tissue mincing buffer. Single cell preparation was done within one hour after animal sacrifice. Immediately following terminal kill, the stomach and a portion of the liver were removed from the animals. The liver and the stomach were processed under dim yellow light and on a cold surface in order to prevent induction of additional damage.

For the liver, a portion of the left lateral lobe was excised and washed in cold mincing buffer to remove excess of blood. The size of the portion was standardized. The portion was minced with a pair of fine scissors to release the cells. The cell suspension was strained through a cell strainer to remove clumps and the remaining