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Genotoxicity evaluation of benzene, di(2-ethylhexyl) phthalate, and trisodium ethylenediamine tetraacetic acid monohydrate using a combined rat comet/micronucleus assays

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ABSTRACT

As a part of the Japanese Center for the Validation of Alternative Methods (JaCVAM)-initiative international validation study of the *in vivo* alkaline comet assay (comet assay), we examined DNA damage in the liver, stomach, and bone marrow of rats dosed orally three times with up to 2000 mg/kg of benzene, di(2-ethylhexyl) phthalate, and trisodium ethylenediamine tetraacetic acid monohydrate. All three compounds gave negative results in the liver and stomach. In addition, a bone marrow comet and micronucleus analysis revealed that benzene, but not di(2-ethylhexyl) phthalate or trisodium ethylenediamine tetraacetic acid monohydrate induced a significant increase in the median % tail DNA and micronucleated polychromatic erythrocytes, compared with the respective concurrent vehicle control. These results were in good agreement with the previously reported genotoxicity findings for each compound. The present study has shown that combining the micronucleus test with the comet assay and carrying out these analyses simultaneously is effective in clarifying the mechanism of action of genotoxic compounds such as benzene.

1. Introduction

The *in vivo* rodent alkaline comet assay (comet assay) is used worldwide for detecting DNA damage induced by genotoxic effects. The assay is used for investigation of the genotoxic potential of test chemicals and currently is identified as a second *in vivo* genotoxicity assay in the ICH-S2[R1] guidance [1] along with the more usual micronucleus test in bone marrow or peripheral blood. The comet assay methodology has been discussed at meetings including the International Workshop on Genotoxicity Testing (IWGT) and the International Comet Assay Workshop (ICAW), and consensus statements have been published [2–4]. The assay, however, has not been validated formally using a standardized study protocol. Therefore, the Japanese Center for the Validation of Alternative Methods (JaCVAM) initiated 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), 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 or predict rodent carcinogenicity. The validation study results have been submitted to the Organization for Economic Co-operation and Development (OECD) for establishment of the OECD test guideline.

As part of the 2nd step in the 4th phase of the international validation study, we examined benzene (BZN), di(2-ethylhexyl) phthalate (DEHP), and trisodium ethylenediamine tetraacetic acid monohydrate (EDTA·3Na·H_2O) as coded test chemicals. These test chemicals were selected based on their genotoxicity and carcinogenicity properties [5]. Briefly, BZN is classified as a genotoxic carcinogen. Although BZN had no detectable effect in the bacterial mutation assay, several positive findings in genotoxicity and carcinogenicity studies in mice and rats have been reported [6–10]. DEHP is categorized as a non-genotoxic carcinogen based on its tumorigenicity and non-genotoxic potencies [11,12]. EDTA (trihydrate) is classified as a non-genotoxic non-carcinogen, while

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some reports showed positive findings in mouse micronucleus tests [13,14].

In this study, as a part of the 2nd step in the 4th phase of the international validation study, bone marrow samples were tested using the combination of a comet assay (for the analysis of DNA damage) and a bone marrow micronucleus assay (for the analysis of micronucleus induction), since positive findings were previously reported for BZN and EDTA in micronucleus studies *in vivo*.

2. Materials and methods

The study was conducted in accordance with the validation study protocol version 14.2 [15].

2.1. Test chemicals

The test substances were provided coded by JaCVAM. After completion of the validation Phase 4-2 study, JaCVAM decoded the test substances, which were benzene (BZN; Chemical Abstract Service Registry (CAS) No. 71-43-2), di(2-ethylhexyl) phthalate (DEHP; CAS No. 117-81-7), and trisodium ethylenediamine tetraacetic acid monohydrate (EDTA·3Na·H_2O; CAS No. 10378-22-0), all from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The positive control chemical ethyl methanesulfonate (EMS; CAS No. 62-50-0) was from Sigma–Aldrich (St. Louis, MO, USA), and vehicles corn oil (the vehicle for BZN and DEHP) and physiological saline (the vehicle for EDTA·3Na·H_2O and EMS) were from Nacalai Tesque Inc. (Kyoto, Japan) and Fuso Pharmaceutical Industries (Osaka, Japan), respectively.

Other chemicals were from the following suppliers: EDTA·2Na and Tris base, Nacalai Tesque Inc.; HCl and NaCl, Wako Pure Chemical Industries, Ltd.; NaOH, Kanto Chemical Co. (Tokyo, Japan); Triton X-100, ICN Biomedical (Irvine, CA, USA); low melting temperature agarose (NuSieve GTG, Lonza, Basel, Switzerland); dimethyl sulfoxide (DMSO), Dojindo Laboratories (Kumamoto, Japan); SYBR® gold and Hank's balanced salt solution, Invitrogen Corp. (Carlsbad, CA, USA).

2.2. Animals

Male Crl:CD(SD) rats bred and supplied by Charles River (Kanagawa, Japan) and weighing 264–322 g (8 weeks old) were used. Animals were allocated to plastic cages (W290 × D340 × H170 mm, Clea Japan Inc., Tokyo, Japan) with shavings (Whiteflake, Charles River) and fed laboratory animal chow (CRF-1, Oriental Yeast Co., Tokyo, Japan) and water *ad libitum*. Before treatment, the rats were quarantined for 1 week and acclimatized in an environmentally controlled room maintained at 22–26 °C and 40–70% relative humidity, with at least 10 air changes per hour and a 12-h light/12-h dark cycle. The animal care and use procedures in the current study conformed to institutional guidelines, which are in compliance with current Japanese laws and were approved by the Environmental Health Science Laboratory Institutional Animal Care and Use Committee of Sumitomo Chemical Co., Ltd.

2.3. Animal treatment

Based on available information on solubility obtained from JaCVAM and the results of solubility tests, corn oil for BZN, DEHP and physiological saline for EDTA·3Na·H_2O were selected as dosing vehicles, respectively.

The dosing mixtures were administered orally using a disposable syringe fitted with a disposable tube. The test substance and vehicle control were administered once daily at three time points (0, 24 and 45 h, *i.e.*, 24 and 21 h intervals). The positive control (EMS, 200 mg/kg) was administered once daily for 2 consecutive days, with doses separated by a 21-h interval. The dose volume for all groups was 10 mL/kg. Individual dose volumes were calculated based on body weight taken just before administration. Dosing mixtures of the test substance and the positive control were prepared just before use.

In order to determine the maximum tolerated dose (MTD; described as the highest dose that can give rise to the appearance of clear signs of toxicity without causing death), a preliminary toxicity test was conducted by treating one animal per dose (250, 500, 1000, or 2000 mg/kg) 3 times for each test chemical, examining clinical signs, and measuring body weight. Necropsy was also performed to assess pathological changes in the liver and stomach.

2.4. Comet assay

From the results of the preliminary toxicity test, three dose levels were chosen (*i.e.*, the MTD or the maximum recommended dose of 2000 mg/kg for non-toxic products), 50% of this dose, and 25% of this dose.

Six animals were treated and 5 animals per dose group were used for observation. All animals were humanely sacrificed with carbon dioxide 3 h after the final administration. The liver, stomach, and femurs were removed and the liver and stomach were examined.

Portions of the liver from the left lateral lobe were minced with a pair of scissors. The cells were suspended in cold homogenization buffer (20 mM EDTA·2Na and 10% DMSO in Hank's balanced salt solution [Ca^{2+}, Mg^{2+} free], pH 8.0), and the suspension was passed through a cell strainer. Portions of the glandular stomach were placed in cold homogenization buffer and the surface epithelium was scraped with a silicone rubber scraper, washed out with cold homogenization buffer, and the washings discarded. The lower layer of epithelium was scraped with a metallic spatula to release the cells, which were then suspended in cold homogenization buffer, and the suspension was passed through a cell strainer. Bone marrow cells were obtained by flushing the femur marrow with fetal bovine serum (Life Technologies, Carlsbad, CA, USA) containing 25 mM EDTA using a 1-mL syringe.

Cold homogenization buffer was used to dilute the liver and stomach cell suspensions and to suspend small portions of the bone marrow cells. Each cell suspension was mixed with 0.5% low-melting temperature agarose (sample:agarose ratio = 1:9), and the mixture was placed on a Matsunami adhesive silane (MAS) coated glass slide (Matsunami Glass Industries, Osaka, Japan) and allowed to solidify in an ice bath. Each slide was placed in lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, 1% Triton X-100, and 10% DMSO, pH 10) overnight under refrigeration and protected from light. Slides were placed randomly in a submarine-type electrophoresis chamber and completely immersed with cold electrophoresis buffer (0.3 M NaOH and 1 mM EDTA·2Na, pH > 13) for 20 min. After being subjected to electrophoresis (constant voltage of 0.7 V/cm [26 V], approximately 300 mA, below 5 °C) for 20 min, slides were immersed in neutralization buffer (400 mM Tris–HCl, pH 7.5), dehydrated with absolute ethanol and air dried. Prior to observation, each slide was stained with a few drops of a solution of SYBR® Gold diluted 10,000-fold and coverslipped. DNA migration was investigated by fluorescence microscopy using an Olympus fluorescence microscope with an IB excitation filter (BP460–495), auxiliary absorbing filters (BA510IF) and a 20× objective (total magnification 200×). The images were imported into a computer through a CCD camera attached to the microscope and analyzed by a comet assay analyzer (Comet Assay IV System, Perceptive Instruments, Suffolk, UK). The DNA in the tail expressed as a percentage of total comet DNA (% tail DNA) was used as a measure of DNA damage. One hundred cells per organ (50 cells per slide) were examined.