Mutation Research xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Evaluation of methyl methanesulfonate, 2,6-diaminotoluene and 5-fluorouracil: Part of the Japanese center for the validation of alternative methods (JaCVAM) international validation study of the *in vivo* rat alkaline comet assay

Ulla Plappert-Helbig*, Ursula Junker-Walker, Hans-Joerg Martus

Novartis Institutes for BioMedical Research, Novartis Pharma AG, Switzerland

ARTICLE INFO

Article history:
Received 15 April 2015
Accepted 15 April 2015
Available online xxx

Keywords:
In vivo rat comet assay
In vivo mammalian alkaline comet assay
JaCVAM validation study
Methyl methanesulfonate
2,6-Diaminotoluene
5-Fluorouracil

ABSTRACT

As a part of the Japanese Center for the Validation of Alternative Methods (JaCVAM)-initiative international validation study of the *in vivo* rat alkaline comet assay (comet assay), we examined methyl methanesulfonate, 2,6-diaminotoluene, and 5-fluorouracil under coded test conditions. Rats were treated orally with the maximum tolerated dose (MTD) and two additional descending doses of the respective compounds. In the MMS treated groups liver and stomach showed significantly elevated DNA damage at each dose level and a significant dose–response relationship. 2,6-diaminotoluene induced significantly elevated DNA damage in the liver at each dose and a statistically significant dose–response relationship whereas no DNA damage was obtained in the stomach. 5-fluorouracil did not induce DNA damage in either liver or stomach.

1. Introduction

The *in vivo* rodent alkaline comet assay (comet assay) has increasingly been used for regulatory genotoxicity testing in recent years. The assay is applied for the investigation of the genotoxic potential of chemicals, and recommended as second *in vivo* genotoxicity assay in the ICH-S2(R1) guidance [1] in addition to the *in vivo* micronucleus assay with bone marrow and/or peripheral blood.

The methods of the comet assay were often discussed in the meetings of the International Workshop on Genotoxicity Testing (IWGT) and the International Comet Assay Workshop (ICAW), and consensus recommendations for conduct and interpretation have been published [2–6]. The assay, however, has not been validated formally with a standardized study protocol. Therefore, the Japanese Center for the Validation of Alternative Methods (JaCVAM) organized 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 validation study results were submitted to the Organization for Economic Co-operation and Development (OECD) for establishment of the OECD test guideline.

As a part of the 2nd step of 4-phase international validation study, we examined methyl methanesulfonate, 2,6-diaminotoluene, and 5-fluorouracil under coded test chemical conditions in the *in vivo* rat comet assay.

2. Materials and methods

The study was conducted in accordance with the validation study protocol version 14.2 [7]. Chemicals, solutions and buffers as described in the protocol were used.

2.1. Test chemicals

Three chemicals, methyl methanesulfonate (MMS; Chemical Abstracts Service Number (CAS no.: 66-27-3); 2,6-diaminotoluene (2-6-DAT; CAS no.: 823-40-5); and 5-fluorouracil (5-FU; CAS no.: 51-21-8) were selected by the validation management team (VMT) and sent as coded test chemicals. Solubility was determined for all chemicals. Physiological saline was used as the vehicle for MMS and

* Corresponding author at: Preclinical Safety, Novartis Institutes of BioMedical Research, 4057 Basel, Switzerland. Tel.: +41 61 3241309; fax: +41 61 3248001.
E-mail address: ulla.plappert_helbig@novartis.com (U. Plappert-Helbig).

http://dx.doi.org/10.1016/j.mrgentox.2015.04.009
1383-5718/

5-FU, and corn oil was used as the vehicle for 2,6-DAT. The positive control item ethyl methanesulfonate (EMS, CAS No. 62-50-0) was also dissolved in physiological saline.

2.2. Animals

Male rats Crl:CD (SD) were purchased from Charles River Laboratories, Inc. At the beginning of the experiment, the animals were approximately 8 weeks old and weighed

150–320 g. Five animals were used for each group: vehicle group, treatment group, or positive control group. Housing and feeding of animals was in conformity with the Swiss Animal Welfare Law (Tierschutzgesetz 2005, 2008) and in accordance with the in-house SOP and guidelines for care and use of laboratory animals. Commercial pelleted standard rodent diet (PROVIMI-KLIBA, no. 3893.PX S25 pelleted standard diet from Provimi, Kliba AG, Kaiseraugst, Switzerland) and tap water from the domestic supply were available *ad libitum*. The animals were kept in an air conditioned animal room under periodic bacteriological control, at $22^{\circ} \pm 2\,^{\circ}C$ with monitored 40–80% humidity, a 12 h light/dark cycle, and background radio coordinated with the light hours.

2.3. Animal treatment

Dose selections of the compounds were based on dose-range finding experiments and the toxicological information such as the rat oral LD_{50}, provided by VMT. The maximum dose was defined as the dose inducing clinical signs and/or histological findings (*i.e.*, maximum tolerated dose or MTD). The mid- and low dose levels were 1/2 and 1/4, respectively, of the high dose. Negative control animals received the vehicle alone. The animals were administered orally by gavage after they had been weighed. The control and treatment groups were dosed three times with an interval of 24 h between the first two administrations and 21 h between the second and third application. The animals were sacrificed 3 h after the final administration. The positive control item ethyl methanesulfonate (EMS) was dosed at 200 mg/kg on two occasions with an interval of 21 h between the first and second administrations (*i.e.* at 24 and 3 h before sacrifice and dissection).

2.4. Comet assay

The comet assay was performed according to our laboratory Standard Operating Procedures (SOPs) and the JaCVAM test protocol [7].

2.4.1. Tissue sampling

Animals were humanely sacrificed and organs were immediately removed. A piece of the left liver lobe and one half of the stomach was rinsed sufficiently with the cold mincing buffer (HBSS; 20 mM EDTA; 10% DMSO; pH 7.5) to remove residual blood, placed into ice-cold mincing buffer and stored ($\leq$1 h) on ice until processed. The remaining tissues were fixed in neutral phosphate-buffered formalin (Baker, USA) for histopathological evaluation.

2.4.2. Preparation of single cell suspensions and microscope slides

A small piece of the liver was taken and minced in mincing buffer with tweezers into fine pieces to obtain a single cell suspension. The cell suspension was stored on ice for 15–30 sec to allow large clumps to settle then immediately mixed with liquid 0.5 % low-melting point agarose (Lonza, NuSieve GTG Agarose; end concentration >0.45%) and transferred onto each of the two slides prepared for each animal. The stomach was opened longitudinal the forestomach was removed and discarded and only the glandular stomach was used for analysis. The surface of the mucosa was rinsed with cold mincing buffer and the superficial mucosal layer was gently scraped off with a scalpel blade and discarded. The gastric mucosa was moistened with cold mincing buffer and carefully scraped with a scalpel blade and minced with tweezers to generate a single cell suspension. The cell suspension was strained through a cell strainer (on ice) to remove lumps. The resulting cell suspensions were immediately mixed with liquid 0.5 % low-melting point agarose (Lonza, NuSieve GTG Agarose; end concentration >0.45%) and transferred onto each of the two slides prepared for each animal. The agarose was allowed to solidify on ice for 5–10 min; afterwards the slides the were immediately submersed into chilled lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10; 4 °C) and stored overnight. After this incubation period, the slides were rinsed in purified water and neutralization solution. For unwinding, the slides were placed into the electrophoresis unit and submersed in alkaline buffer (300 mM NaOH, 1 mM EDTA, pH > 13; 4 °C) for 20 min, and thereafter subjected to an electric field of 0.7 V/cm (approximately 300 mA). A balanced design was used to run the electrophoresis, *i.e.*, in each run slides of treated, positive and vehicle control were included. Following electrophoresis the gels were neutralized in 0.4 M Tris (pH 7.5), rinsed with water, dehydrated in absolute ethanol for at least 5 min and allowed to dry at room temperature.

2.4.3. Staining

Coded slides were stained with the fluorescent DNA stain SYBR Gold (Invitrogen-Molecular Probes), prepared and used according to the manufacturer's specifications.

Analysis: For the analysis of the samples a semi-automatic image analysis system (Comet Assay III, version 3.0, Perceptive Instruments, UK) was used. DNA damage was measured as increased DNA migration determined as % tail DNA (% tail intensity) and Olive tail moment [8]. For each sample (animal/tissue) two slides were prepared and fifty comets per slide were analyzed. Heavily damaged cells (hedgehogs) were not included in the measurement but their number was determined per sample, based on the visual scoring of 100 cells.

2.5. Histopathology

From all animals of the negative control group and the low, mid, and high dose groups, the remaining left liver lobe, and half of the stomach were sampled at necropsy, fixed in neutral phosphate-buffered formalin (Baker, USA), embedded in paraffin wax, sectioned at 2–5 microns, stained with hematoxylin and eosin (Sigma, USA) and examined by light microscopy. Histopathological findings were graded as grade 1 (minimal/very few/very small), grade 2 (slight/few/small), grade 3 (moderate /moderate number/moderate size), grade 4 (marked, many, large), or grade 5 (severe, extensive, very large).

2.6. Statistics

2.6.1. Statistical analysis

The result of the statistical analysis was evaluated according to the following criteria, a test item is classified as positive if the mean % Tail DNA of the treated group is statistically significant compared to the mean % Tail DNA of the vehicle control group.

1. Log-transformation ($\log_e$) of the values at cell level.
2. Median of the log-transformed values at the slide level.
3. Arithmetic mean of all slides per animal.
4. 1-Way ANOVA with treatment groups as fixed factor (negative control and all treated groups, without positive control).
5. Linear trend test: a linear regression analysis (negative control and all treated groups, without positive control) was used for the assessment of a linear dose–response relationship. Only one sex

Please cite this article in press as: U. Plappert-Helbig, et al., Evaluation of methyl methanesulfonate, 2,6-diaminotoluene and 5-fluorouracil: Part of the Japanese center for the validation of alternative methods (JaCVAM) international validation study of the *in vivo* rat alkaline comet assay, Mutat. Res.: Genet. Toxicol. Environ. Mutagen. (2015), http://dx.doi.org/10.1016/j.mrgentox.2015.04.009