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MNT and Muta^{TM}Mouse studies to define the *in vivo* dose response relations of the genotoxicity of EMS and ENU

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ABSTRACT

Although there are a multitude of *in vitro* and *in vivo* studies on the genotoxic activity of EMS, no lifetime carcinogenicity studies, repeat dose mutation data or exposure analysis are available to serve as a solid basis for risk assessment for human exposure cases. The present studies were undertaken to investigate whether a threshold for mutagenic and clastogenic activity *in vivo* could be established, using the bone marrow micronucleus (MNT) and Muta^{TM}Mouse test systems, in the hope to provide reassurance to the patients that their accidental exposure to EMS at doses up to 0.055 mg/kg did not carry a toxicological risk. Dose levels ranging from 1.25 to 260 mg/kg/day were applied orally for up to 28 days. As a reference we included ENU at doses of 1.1–22 mg/kg/day. Our studies showed that daily doses of up to 25 mg/kg/day (bone marrow, GI tract) and 50 mg/kg/day (liver) did not induce mutations in the lacZ gene in the three organs tested. Doses up to 80 mg/kg/day (7-day dosing regime) did not induce micronuclei in mouse bone marrow. The genotoxic activity of EMS became apparent only at higher dose levels. Dose fractionation of EMS (28 times 12.5 mg/kg versus a single high dose 350 mg/kg) provided further evidence for the thresholded dose response of EMS and showed that no cumulation of gene mutations below a threshold was occurring. In contrast, for ENU no threshold was apparent and dose fractionation indicated full additivity of individual dose effects.

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1. Introduction

Over a period of three months in 2007 (March–May) several thousand HIV patients ingested Viracept (Nelfinavir mesylate) tablets containing the impurity ethylmethane sulfonate (EMS), a small alkylating agent with well known mutagenic, teratogenic and carcinogenic properties in animals (Müller and Singer, 2009).

In vitro and animal data did not permit a sufficiently convincing risk assessment for the levels at which HIV patients were exposed to EMS (maximal dose of 0.055 mg/kg/day). Therefore, the marketing authorization holder F. Hoffmann-La Roche conducted a series of preclinical investigations to better quantify the risk for adverse effects in the exposed individuals. For directly DNA damaging genotoxins, such as EMS, it has been generally assumed that dose response relations are linear due to their stochastic, single hit-single target, mode of action. Formation of tumours, heritable birth defects, and teratogenic effects, are also thought to follow a linear dose relation for these genotoxins as these effects are considered subsequent to the genotoxic action. Thus, our initial risk assessment was based on linear extrapolation of carcinogenicity data (Gocke et al., 2009a). However, a recent publication by Doak et al., 2007 provided strong evidence for a thresholded dose response relationship for *in vitro* induction of micronuclei and for gene mutations by EMS, in contrast to an apparent linear dose response for ENU.

Based on this publication we sought to obtain reassuring evidence for the absence of (geno)toxicological adverse effects in the Viracept patients exposed to the contaminant EMS. A series of general toxicity, genotoxicity and DMPK studies were conducted in discussion with the CHMP, the relevant regulatory authority in control of the marketing authorization of Viracept.

Here we present the results of the two *in vivo* genotoxicity studies we performed with EMS and ENU. Under the conditions of these tests no increase of clastogenic and mutagenic effects were observed below the dose of 25 mg/kg/day. Statistical analysis (Gocke and Wall, 2009) supported the visual impression of a thresholded dose response relationship. The full set of non-clinical data has been integrated into a comprehensive risk assessment evaluation (Müller et al., 2009).

2. Material and methods

The genotoxicity tests were performed under GLP at Covance, Harrogate, UK. Formulation analytics of EMS was done at F. Hoffmann-La Roche Ltd., Basel (for MNT test) and RCC Ltd., Itingen, Switzerland (for Muta^{TM}Mouse test). Bioanalytics

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(determination of ethylvaline adduct levels) was performed at Currenta GmbH & Co, Leverkusen, Germany.

2.1. Test items, formulations

EMS [CAS 62-50-0] and ENU [CAS 62-50-0] were purchased from Sigma–Aldrich, UK. Batches of EMS had purities of 99.4 and 100.4%, batches of ENU had purities of 89% (10% water, 1% acetic acid) and 50% (47% water, 3% acetic acid). EMS was stored under desiccant and nitrogen at room temperature in the dark, ENU was stored frozen at <−10 °C in the dark. Dosing preparations were freshly prepared at each dosing occasion in purified water (EMS) or PBS (ENU), protected from light and stored on wet ice prior to completion of dosing. For ENU the pH was adjusted to pH 5 for better stability. All formulations were used within 2 h of preparation. A dose volume of 10 mL/kg was used for all animals in all groups. Control animals were dosed with the vehicle alone using the same dosing regime and volumes as that for the test article treated animals. Remaining formulations were subsequently frozen at nominal −20 °C for subsequent analytical determinations.

2.2. Test system: micronucleus test

The protocol was designed to meet the known requirements of the OECD Guideline 474, 1997 and the ICH Tripartite Harmonised Guideline on Genotoxicity: Specific Aspects of Regulatory Tests, 1995. Out-bred, young adult Crl:CD-1 (ICR) mice were obtained from Charles River (UK) Ltd., Margate, UK and Harlan UK Ltd., Bicester, UK. The experiments with EMS and ENU were performed separately on consecutive weeks to keep the number of animals per experiment manageable. For EMS an initial dose range of up to 80 mg/kg/day was chosen which produced at most a marginal effect. Therefore an additional experiment with higher doses was added to obtain a full dose response curve. Each experiment had its appropriate control group. The animals were housed in a room air-conditioned to provide a minimum of 15 air changes/h. The temperature and relative humidity ranges were 19–25 °C and 40–70%, respectively. Fluorescent lighting was controlled automatically to give a cycle of 12 h light (06:00–18:00) and 12 h dark. Animals had access to diet and water ad libitum. They were acclimatised for at least 7 days and a veterinary inspection was performed before the start of dosing to ensure their suitability for study. Checks were made prior to dosing on the first day of treatment to ensure individual group weights (six animals/group) differed from the mean group weight by no more than 5%. All animals were observed daily for signs of ill health or overt toxicity. An individual record was maintained of the clinical condition of all animals dosed in the study. Individual body weights were recorded on each day of dosing and on the day of bone marrow sampling.

Animals were treated daily by oral gavage for 7 days (EMS: 0, 1.25, 2.5, 5, 20, 80, 140, 200, 260 mg/kg/day; ENU: 0, 1.11, 4.45, 17.8 mg/kg/day) and bone marrow smears prepared from animals sacrificed approximately 24 h post the last dose. A maximal dose of 20 mg/kg of ENU was planned, but since no allowance for the water/acetic acid content was made during weighing the achieved concentrations were 11% lower. At the time of bone marrow sampling, mice were anaesthesized with isoflurane gas, followed by cardiac puncture (for collecting bioanalytics samples), in the same sequence used for dosing. Mice were not allowed to recover and death was ensured by cervical dislocation. Both femurs from each animal were exposed, removed, cleaned of adherent tissue and the ends removed from the shanks. Using a syringe and needle, bone marrow was flushed from the marrow cavity with 1 mL foetal bovine serum into appropriately labelled centrifuge tubes. A further 1 mL of foetal bovine serum was added to the tubes, which were then centrifuged at $200 \times g$ for approximately 5 min; the serum was aspirated to leave one or two drops and the cell pellet. The pellet was mixed into this small volume of serum in each tube by using a Pasteur pipette, and from each tube one drop of suspension was placed on the end of each of two or three slides labeled with the appropriate study number, sampling time, sex, date of preparation and animal number. The latter served as the code so analysis could be conducted "blind". A smear was made from the drop by drawing the end of a clean slide along the labelled slide.

Slides were allowed to air-dry and then fixed for 5 min in absolute methanol before being stained according to the modification of Gollapudi and Kamra (1979). Spare slides were allowed to dry and stored at room temperature until required for staining. One slide from each set was taken (remaining slides were kept in reserve). After rinsing several times in distilled water, slides were stained for 10 min in filtered Giemsa stain diluted 1:5 (v/v) in distilled water. Slides were rinsed and allowed to dry before clearing in xylene for 3 min. When dry, slides were mounted with coverslips and stored at room temperature prior to analysis.

Initially the relative proportions of polychromatic erythrocytes (PCE), seen as pale blue or blue/grey enucleate cells, and normochromatic erythrocytes (NCE), seen as yellow/orange-stained enucleate cells, were determined until a total of at least 1000 cells where possible (PCE plus NCE) had been analysed. Counting continued until at least 4000 PCE per animal where possible had been examined. All PCE containing micronuclei observed during these two phases of counting were recorded. After completion of microscopic analysis and decoding of the data the following were calculated:

(1) % PCE for each animal and the mean for each group. The group mean % PCE values were examined to see if there was any decrease in groups of treated animals versus controls and historical levels that could be taken as evidence of bone marrow toxicity.
(2) % MN-PCE (frequency of micronucleated PCE in 4000 PCE) for each animal and the group mean % MN-PCE (± standard deviation).

The numbers of micronucleated PCE in vehicle control animals were compared with the laboratory's historical control data to determine whether the assay was acceptable.

2.3. Test system: lacZ gene mutation test

The study was designed to comply with the recommendations made at the IWGT meeting in 2002 (Thybaud et al., 2003) CD_2-lacZ80/HazfBR mice were obtained from Harlan, UK. The study consisted of two arms: a 28-day daily treatment scheme (sampling on day 31; EMS: 0, 1.56, 3.13, 6.25, 12.5, 25, 50, 100 mg/kg/day, ENU: 0, 1.39, 5.56, 22.25 mg/kg/day) and a single treatment scheme (sampling on days 7 or 31; EMS: 0, 350 mg/kg (= 28 × 12.5), ENU: 0, 15.58 mg/kg). ENU acute (single administration) treatments were intended to be dosed at 175 mg/kg, but due to a calculation error the treatment solution was formulated at 10% of the required concentration, and also no correction was made for the 89% purity of this batch, so the actual achieved dose level was 15.58 mg/kg. The group sizes were seven animals. Since the study comprised too many animals to perform all work in parallel the study was split in appropriate experiments each with separate controls (thus four independent control groups were available). Animals were housed as described for the MNT test. Individual body weights were recorded on the first seven days of dosing (where applicable) and subsequently at weekly intervals prior to necropsy, depending on the body weight changes in the first week. Necropsies were performed on all mutation experiment animals found dead or sacrificed in a moribund condition. For all animals bone marrow, liver and small intestine (duodenum/ileum) tissue were taken. Tissues were removed from the animals, rinsed (where appropriate) and flash-frozen in liquid nitrogen, prior to DNA extraction and determination of mutation frequency. Prior to digestion, the liver was homogenised using a Wheaton Dounce homogenizer in ice-cold lysis buffer. DNA was extracted using proprietary Stratagene RecoverEase™ (Liver and bone marrow) or Gentra® Puregen® (small intestine samples) DNA extraction kits.

Where possible, data were generated for at least 200,000 plaque-forming units (pfu) per tissue for all seven animals per group. Five animals of the high dose continuous ENU treatment group had to be terminated in extremis at day 26. Their DNA was sampled and data were included in the group value.

The proprietary strain of *E. coli* C *lac-galE-* Kanr (*galE*-Ampr) was developed and supplied by J Gossen and J Vijg, Ingeny BV, Leiden, The Netherlands. An overnight (approximately 16 h) culture of *E. coli* C *lac-galE-*, inoculated from a frozen stock, was grown in a shaking incubator at 37 ± 1 °C in 10 mL of Luria broth (LB) containing maltose (0.2%, w/v), Kanamycin (50 μg/mL) and Ampicillin (50 μg/mL). An aliquot (1 mL) of this overnight culture was inoculated into fresh LB medium (98 mL) containing maltose (1 mL of 20% w/v) and incubated, with shaking, at 37 ± 1 °C for approximately 4–5 h. The cells were centrifuged (approximately $2020 \times g$) for 10 min and resuspended in approximately 80 mL of LB medium containing 10 mM magnesium sulphate. The cell suspension was stored refrigerated (1–10 °C) or on ice until used for phage adsorption. Approximately 5 μL of DNA solution (ideally containing about 7.5 μg of DNA for maximum packaging efficiency) was mixed with an appropriate packaging extract according to the manufacturer's instructions. The assay involves the scoring of titration plates to determine the total number of plaque-forming units, and selection plates using the positive selection method to determine the numbers of mutants. For the titration plates, packaged DNA (10 μL) was diluted with SM buffer (190 μL) and 10 μL of this dilution adsorbed to 500 μL suspension of *E. coli* C *lac-galE-* Kanr (*galE*-Ampr) for approximately 30 min at room temperature. After adsorption, the phage/bacteria were suspended in 12 mL 1:3 LB:NaCl, 0.75% (w/v) agar (top agar) containing 10 mM magnesium sulphate and plated onto petri dishes (14 cm diameter) containing 12 mL 1:3 LB:NaCl, 1.5% (w/v) agar (bottom agar). Once the agar had gelled, the plates were inverted and incubated overnight at 37 ± 1 °C.

The remaining packaged DNA was divided into three tubes and incubated at room temperature with bacterial suspension (500 μL/tube) for approximately 20 min, suspended in top agar (as above) containing both magnesium sulphate (10 mM) and phenylgalactose (P-gal, 0.3%, w/v), poured onto plates containing bottom agar (as above) and incubated overnight at 37 ± 1 °C. After incubation, the clear plaques on each plate were counted.

Wherever possible, tissues were processed and analysed using a 'block' design, where DNA samples from the negative control group and each treatment group were processed together. A further sample of DNA, isolated from animals previously treated with a positive control chemical was processed in parallel with these samples. This DNA acted as an internal positive control, to confirm the success of each packaging and plating occasion. Data from a packaging occasion was only accepted if the concurrent positive control DNA packaging reaction yielded a characteristic elevated mutation frequency (MF) compared to historical negative control mutation frequencies.

Where possible, data were generated for at least 200,000 pfu per tissue for all seven animals per group, to yield a total of at least 1.4 million pfu per tissue per group. Acceptance of data where less than 200,000 pfu per tissue per animal were obtained was performed on a case-by-case basis. Data from packagings providing

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