

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

DRAFT DISCUSSION PAPER ON DATA SUBMITTED BY ROCHE IN SUPPORT OF A THRESHOLD FOR ETHYLMETHANE SULFONATE (EMS) MUTAGENICITY

INTRODUCTION

1. The COM considered a presentation from Dr Gareth Jenkins (University of Swansea) at its 26 February 2009 meeting. The presentation referred to studies undertaken by Roche to support a threshold for mutagenicity of ethylmethane sulfonate (EMS). The relevant studies have been forwarded to COM for consideration.

BACKGROUND

2. Over a period of three months in spring 2007, several thousand HIV patients had ingested Viracept (Nelfinavir mesylate) tablets, an HIV protease inhibitor, containing relatively high levels of the impurity EMS. The estimated maximal dose of EMS to which patients were exposed was 0.055 mg/kg bw/day. The available *in-vitro* mutagenicity and toxicity data on EMS did not allow a full risk assessment to be undertaken. Roche undertook *in-vivo* mutagenicity studies in mice (BM MN, *lacZ* gene mutation in bone marrow, liver and small intestine), employed a novel statistical approach to analysis of data, and undertook appropriate investigations to allow risk assessment based on kinetic data (Annex 1). All the documents provided by Roche (Annexes 1-5) are in various stages of peer-review and have been provided for Members use only. Brief overviews of the main points from documents in Annexes 2-5 have been provided below. Members are asked to consider the documents and the approach to risk assessment.

MICRONUCLEUS TEST (MNT) AND MUTA™MOUSE STUDIES (ANNEX 2)

MNT

3. Groups of 6 CD-1 mice were given seven daily oral doses of EMS 0, 1.25, 2.5, 5.0, 20, 80, 140, 200, 260 mg/kg bw/day EMS or 0, 1.11, 4.45, 17.8 mg/kg bw/day ENU and bone marrow smears were prepared 24 h after the last dose. Data reported included % PCE and % MN PCE from 4000 PCEs per animal. Ethylvaline adducts in globin were determined as a measure of exposure. The authors reported no increase in BM MN at doses up to 80 mg/kg bw/day EMS with evidence for a saturation of MN induction at cytotoxic doses of 260 mg/kg bw/day. The authors suggested the occurrence of a hormetic response up to 20 mg/kg bw/day as supported by a statistically

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significant negative slope of the dose response (Annex 4). When relating the clastogenic effect against the ethylvaline adducts, no evidence of a mutagenic response was reported at up to ethylvaline levels of 100 nmol/g globin. No evidence for a NOEL was reported in studies with ENU with a clear dose-response reported for MN induction.

Muta™Mouse Study

4. The study consisted of a 28-day and a single treatment scheme. Groups of 7 CD₂-lacZ80/HazfBR mice were treated daily for 28 days with oral doses of 0, 1.56, 3.13, 6.25, 12.5, 25, 50, 100 mg/kg bw/day EMS or 0, 1.39, 5.56, 22.25 mg/kg bw/ day ENU. Tissue sampling was undertaken on day 31. For the single dose scheme, animals were treated with oral doses of 350 mg/kg bw EMS and 15.56 mg/kg bw ENU with sampling at day 7 or day 31. The single dose ENU was approximately 10-fold lower than planned due to an error in treatment solution. Bone marrow, liver and small intestine were removed and DNA extracted. Packaged DNA was adsorbed to a suspension of *E.coli* C lac- *galE*- Kanr (*galE*-Ampr) for 30 mins at room temperature and the selection of lacZ mutants was undertaken using phenylgalactose. Where possible data were generated for at least 200,000 plaque forming units per tissue for all animals per group. Packagings with low PFU (mostly from high and toxic doses) showing evidence for excessive variability in plate counts or mutation frequency inconsistent with packagings from concurrent DNA samples were excluded.

5. The 28-day treatment resulted in ENU inducing significant increased MF in bone marrow, liver and small intestine, evident even at the lowest dose of 1.56 mg/kg/day. Effects for EMS were much smaller and clearly elevated MF values were only observed at the highest dose of EMS, 100 mg/kg. At this dose, relative increases in MF were reported for liver, intestine and bone marrow by factors less than 2, 3 and 4 respectively. Statistical analysis of the data indicated a linear dose response in the presence of ENU and threshold dose levels for EMS in bone marrow and liver. A NOEL of 25 mg/kg bw/day was reported for EMS in bone marrow and intestine and 50 mg/kg bw/day for liver. When assessment was done with respect to ethylvaline adduct levels no increase in MF was reported in animals dosed with EMS up to ethylvaline levels of 40 nmol/ g globin (bone marrow, intestine) and 100 nmol/g (liver).

6. The acute dosing regime produced similar positive results for mutagenicity with both EMS and ENU (roughly a 2- to 4-fold increase over controls), although the dose used for ENU was substantially lower. In detail, the mutant frequencies were found to vary with tissue and sampling time. A decreased tendency in MF bone marrow was observed at day 28 compared to day 7, and the MF for small intestine remained constant over the treatment period. For liver an effect was seen at day 28 only. The time course of MF in these tissues was attributed to differences in the relative sensitivities / repair capacities of the differentiated tissue cells compared to stem cells, or to the slower fixation of mutations in the liver with its slow cell turnover.

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7. The authors compared the mutagenic effects of tests substances following acute and repeated treatment schedules. Fractionation of the EMS dose of 350 mg/kg bw (which gave a mutagenic effect at 28 days post treatment in all tissues sampled) into 12.5 mg/kg bw/day for 28 days did not result in a mutagenic effect. By comparison, fractionation of ENU doses did not result in an appreciable reduction of mutagenic effect.

THRESHOLD FOR THE GENOTOXICITY OF EMS AND ENU (ANNEX 3)

8. This paper reports the studies presented in Annex 2 and gives some further information on the approach to risk assessment and threshold considerations. The statistical analysis of the EMS results of the 7-day treatment showed a linear dose relationship up to 80 mg/kg bw/day in bone marrow. Furthermore, the linear regression line for the dose region up to 20 mg/kg bw/day was negative and the authors considered that this indicated the possibility of a hormetic effect. Application of hockey stick software gave a threshold dose value for EMS in bone marrow of 89.8 mg/kg bw/day with a confidence interval of 56.7 – 118.2 mg/kg bw/day.

9. Fractionation of ENU doses did not result in the reduction of mutagenic effect and the authors concluded that either ENU demonstrated a linear dose response or that the threshold dose must be below the minimal daily dose of 1.39 mg/kg bw/day.

10. DMPK studies of patient EMS exposure verified that the doses of EMS ingested by the Viracept patients resulted in AUC and C_{max} values below the established threshold MF values.

11. EMS and ENU confer the same ethyl moiety to DNA but the ethylation profiles at the different sites within DNA strands differ substantially between the two compounds. Based on the measurement of ethylvaline adducts and the relative reactivity at DNA sites, the authors estimated that ENU induces around 60-fold more total oxygen adducts in DNA than EMS at the same dose. The authors determined that, if all oxygen adducts contribute equally, than the ENU threshold dose would be 0.4 mg/kg bw/day for bone marrow and GI tract, and 0.8 mg/kg bw/day for liver. The authors considered that their data for the low dose region of the ENU dose-response curve were compatible with these low threshold values.

12. The authors cite similar EMS dose-response levels of ethylvaline adduct formation in globin compared to DNA adducts as reported by other published studies.

STATISTICAL ASSESSMENT OF THE DOSE RESPONSE CURVES FOR EMS INDUCED EFFECTS (ANNEX 4)

13. The statistical assessment of the genotoxicity data, i.e. both MN and *lacZ* data, consisted of the following: comparison of the control groups

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from different arms of the studies by means of analysis of variance; assessment of the linearity of the entire dose range, and over the dose range up to the NOEL for each tissue; application of the threshold software developed by Lutz and Lutz (2009) to calculate the threshold values including confidence limits. In addition, as Viracept patients had been exposed accidentally to tablets contaminated with EMS at a maximal dose of 0.055 mg/kg/day, confidence limits were determined for the slope of the regression line below the NOEL and for genotoxic effects at 0.55 mg/kg as a percentage of the control.

14. There were no significant differences between the control groups, so they were grouped together in further analysis. Linear dose response relationships for MF data were rejected for the entire dose range in all three tissues. Linear dose response relationships were accepted below the NOEL values for the respective tissues. The thresholded dose responses for the three tissues closely correlated with the respective NOEL values. At the dose of interest, 0.055 mg/kg, the curves exhibited a negative slope at the low dose range in the MNT test and in the lacZ test for bone marrow and liver.

MODELLING OF PATIENT EMS EXPOSURE (ANNEX 5)

15. The effects observed in *in-vivo* genotoxicity studies with mice were correlated to PK parameters of EMS and these parameters were then extrapolated to predict the likely human exposure at the level of contamination of Viracept tablets.

16. The AUC and C_{max} values in mice at the threshold dose for mutagenicity of 25 mg/kg were calculated to be 350 µM*h and 315 µM respectively and predicted to be 13 µM*h and 0.85 µM for patients ingesting the most contaminated Viracept tablets.

17. The PK model was extended to include its interaction with haemoglobin to form ethylvaline haemoglobin adducts in mouse, rat and monkey to gain confidence in the model simulations of EMS exposures. The haemoglobin adduct levels in plasma were simulated in multiple dose experiments and based on the EMS plasma concentration versus time profiles obtained at the various doses. There was good correlation between EMS pharmacokinetics and haemoglobin adducts after single and multiple doses in mouse, rat and monkey.

18. The estimated safety factor, based on the threshold dose level in mice and the estimated maximal daily dose of EMS in patients, was calculated as 454 (i.e. 25 mg/kg/day / 0.055 mg/kg/day). A C_{max}-based safety factor for EMS in Viracept was calculated as 370, and a total exposure (AUC) based risk assessment for EMS in Viracept had a safety factor of 28. The lower AUC based safety factor was due to the predicted longer half-life of EMS in man versus mice.

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DISCUSSION

19. The Committee has not previously seen genotoxicity risk assessment conducted using margins of exposure based on NOEL or estimated C_{max} and AUC (at NOEL) compared to relevant indices of human exposure. What are Members' views?

HPA COM Secretariat

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ANNEXES

- Annex 1 Müller T, Gocke E, Lavé T, Pfister T. Ethyl methanesulfonate toxicity in Viracept – a comprehensive human risk assessment based on threshold data for genotoxicity. *Toxicol. Lett.* (accepted)
- Annex 2 Gocke E, Ballantyne M, Whitwell J, Müller L. MNT and MutaTM mouse studies to define the *in-vivo* dose response relations of the genotoxicity of EMS and ENU. *Toxicol. Lett.* accepted)
- Annex 3 Gocke E and Müller T. *In-vivo* studies in the mouse to define a threshold for the genotoxicity of EMS and ENU. *Mutation Research* (accepted)
- Annex 4 Gocke E and Wall E. *In-vivo* genotoxicity of EMS: statistical assessment of the dose response curves. *Toxicol. Lett.* (accepted)
- Annex 5 Lavé T, Paehler A, Grimm HP. Modelling of patient EMS exposure: translating pharmacokinetics of EMS *in-vitro* and in animals into patients. *Toxicol. Lett.* (under review)